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Determining \( xE2-xE3 \) Pair of RNF38 by Phage Display for Orthogonal Ubiquitin Transfer

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ABSTRACT

Ubiquitin (UB) cascades in vivo are complex to study with cross-reactivity of E1-E2 pairs, E2-E3 pairs, and E3-substrate pairs. RNF38, RING finger protein 38, is a RING-type E3 ligase in the UB transfer cascade in the cell, and the role of RNF38 in cell regulation is unknown, but one study identifies p53 ubiquitination. Previously, the orthogonal UB transfer (OUT) cascade with xUB~xE1 and xE1~xE2 pairs is used to determine xE2-xE3 pair. The current project focuses on generating RNF38 Ring domain libraries with the randomization of four key residues in the E2-binding site then selecting out specific xE2-xRNF38 RING pairs allowing exclusive transfer of xUB to substrate proteins of RNF38. RNF38 RING library is displayed on the surface of M13 phage for the selection of RING mutants binding with xE2. After identifying functional xE2-xRNF38 pairs, xRNF38 is used to assemble the OUT cascade to identify potential substrates by proteomics.

INDEX WORDS: RING E3 Ligases, Substrate Ubiquitination, Multi-mutant Libraries, Phage Display, Selection, Autoubiquitination
DETERMINING XE2-XE3 PAIR OF RNF38 BY PHAGE DISPLAY FOR ORTHOGONAL UBIQUITIN TRANSFER

by

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I appreciate my professor, Jun Yin, for guiding me through the theory and believing in my ability to conduct research with ubiquitin cascades and phage display. He is encouraging and helps clarify any issues I am unsure on, especially on protein expression. Dr. Yin’s background in ubiquitin cascades has deeply caught my interest and has allowed me to perform both organic synthesis of Thz-Lys UAA and other compounds as well as my main project for identifying engineered RNF38 to work in the OUT cascade previously designed by Karan Bhuripanyo. I also thank Dr. Bhuripanyo for providing many references and aid in my understanding of OUT system and M13 phage display from his dissertation thesis.

My mentor, Li Zhou, helps to polish many of my skills and teaching library construction, model selection, phage display, and library selection. He helps mold my ambition and techniques to become successful in the lab and we have worked closely together to tackle many issues. He has shaped my personality in the lab to work together and work on communicating effectively to ensure successful assay development and results. Li trusts me to work on his experiments as well since my efficiency has grown quite a bit since my first time entering the lab.

Dr. Alfons Baumstark provides one of the main driving forces to critically thinking in terms of an organic chemist in addition to a biologist and biochemist. Thus, it pleases me to even daresay I follow into a new field of chemical biology which is crucial to tackling new challenges present within biological processes. I am grateful to being open to new ideas and orient my presentations towards different fields which may very well be a key step towards field cohesion. Dr. Kathy Grant’s inorganic class helps understand how important
metals are in structure and function within some proteins, especially in RNF family of E3 ligases.

I want to take some time to recognize many colleagues within the lab. Dr. Geng Chen helps clarify many technical issues, and her knowledge on wt-PARKIN when I did an extra project on phosphorylation assays with PINK1. The phosphorylation assays are not included in this thesis, but phosphorylation work with PINK1 and FL-PARKIN with HA-UB will be included in Dr. Chen’s article submission for Parkin activity. Ruochuan Liu has helped with expression of xE2, xUB, xUba1, UbcH5B-myc, and Uba1 for the multiple assays with wt-RNF38 RING, library selection assays, and assays with OUT cascade. Han Zhou helped with organic synthesis of Thz-Lys UAA and the other compounds along with HPLC set up, NMR analysis, and MALDI-TOF MS analysis for UAA compounds. I want to thank Alicia Freeman the previous work on pComb-wt-RNF38 RING, though only sequence analysis for pComb-wt-RNF38 RING with TEV site is used for sequence alignment. I thank all the undergraduates I am currently teaching: Nikolas Halloway, Autumn Tobin, and Victory Nwankwo for their help within the lab.
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LIST OF ABBREVIATIONS

UB – Ubiquitin
E1 – Ubiquitin activating enzyme
E2 – Ubiquitin conjugating enzyme
E3 – Ubiquitin ligase
HECT - homologous to E6AP carboxyl terminus
RING – Really Interesting New Gene
RBR – Ring between RING
Ab – antibody
SILAC – stable isotope labeling of amino acids
DUB – deubiquitinating enzyme
UBL – ubiquitin-like protein
RNF38 – RING finger protein 38
OUT – orthogonal ubiquitin transfer
xUB – engineered UB
xE1 – engineered E1 / xUba1
xE2 – engineered E2 / xUbcH5B
AD – adenylation domain
UFD – ubiquitin fold domain
fE1 – forward mutation in AD on E1
bE1 – back mutation in UFD on E1
+ ssDNA – positive sense single stranded DNA
LacZ – LacZ operon
S.D. – shine-delgano sequence
pComb – pComb3H vector
UB-Bio – biotinylated UB
ELISA – enzyme-linked immunosorbent assay
1° Ab – primary antibody
HRP – horseradish peroxidase enzyme
TMB – 3, 3’, 5,5’-tetramethylbenzidine
xRNF38 RING – engineered RNF38 RING domain
FL-RNF38 – full-length RNF38
FLAG – DYKDDDD sequence tag
RE – restriction enzyme
IB – immunoblot
pfu – phage forming unit
PCR – polymerase chain reaction
cfu – colony forming unit
4th library -- 4th RNF38 RING 4-mutant library
INTRODUCTION

1.1 Background Information

1.1.1 Ubiquitin Signaling Pathway

The ubiquitin cascade is a highly regulated process within cells targeting proteins for post-translational modification with ubiquitin (UB).\textsuperscript{1} Proteins modified with UB are thought to signal for degradation, but non-degradation pathways can occur after ubiquitin modification.\textsuperscript{2-3} Ubiquitinated protein substrates can respond differently within the cell depending on the number and where UB modifications occur on the substrate.\textsuperscript{4-10} UB signaling is still under study, but new advances will help elucidate pieces of the signal pathway.

The UB cascade is transfers uses three enzymes to transfer UB through thioester linkages before forming an amide linkage with specific substrates (Figure 1.1). UB activating enzymes (E1) activate UB with adenosine triphosphate (ATP) through nucleophilic attack of the oxyanion from C-terminal carboxyl group to the α-phosphate in a two-step manner to release a pyrophosphate and form UB-AMP conjugate allowing for nucleophilic attack from a reactive cysteine thiol on E1 onto the C-terminal carboxyl group of ubiquitin and release AMP.\textsuperscript{11} The UB~E1 conjugate interacts with the UB conjugating enzymes (E2) within the ubiquitin fold domain (UFD) of E1 where nucleophilic attack of E2’s reactive cysteine thiol onto the C-terminal carboxyl group of UB form a thioester intermediate.\textsuperscript{12} The UB~E2 conjugate interacts with the E2 binding domain of ubiquitin ligase (E3) before the substrate associates with the substrate binding domain of E3 to undergo UB modification. E3s are categorized into three major class types, HECT-type, RING-type, and RBR-type, via their interaction with E2 and the substrate. HECT E3s directly have UB transferred by nucleophilic attack from a reactive cysteine residue, then interact with the substrate for the final transfer of ubiquitin (Figure 1.1 arrow a).\textsuperscript{13}
RBR E3s, such as Parkin and HHARI, have the two RING domains with a linker in between, but RING2 has a catalytic cysteine residue which will behave similarly to HECT-type E3s.\textsuperscript{14} RING E3s act as scaffolds to transfer UB to the substrate from the E2-UB conjugate when the substrate is close in proximity (Figure 1.1 arrow b). In most cases, UB is transferred to the $\varepsilon$-amino group of lysine residue(s) of the substrate, but in rare cases the $\alpha$-amino group can be ubiquitinated.\textsuperscript{15-16} In the absence of the substrate, E3s will undergo autoubiquitination, where multiple UBs conjugate onto the E3 lysine residue and signal E3s for degradation in a regulatory event to control UB cascade.\textsuperscript{17}

![Figure 1.1 UB cascade with RING/ U-box and HECT E3 ligases](image)

Ubiqutin (UB) associates with the adenylation domain (AD) of E1 and ATP associates with ATP-binding site. UB is activated through nucleophilic attack of C-terminal carboxyl group on ATP allowing nucleophilic attack of catalytic cysteine residue on E1. E2 associates with ubiquitin fold domain (UFD) of E1 and uses catalytic cysteine to attack UB at C-terminal. Two paths are presented between HECT (path a) and RING/ U-box (path b) E3s based on whether the ubiquitination on the substrate occurs directly or indirectly from the type of E3. Substrate will be polyubiquitinated and signaled for protein degradation by 26S proteasome.
1.1.2 Challenges with Studying UB Substrates

Studying the ubiquitin cascade has its challenges identifying E2-E3 and E3-UB pairs (Figure 1.2). The main issue is studying each interaction separately to understand and identify potential substrates. Currently only a few E1s, dozens of E2s, and several hundred E3s have been identified. Multiple cross-reactivities occur between E1-E2, E2-E3, and E3-substrate pairs, therefore, complicating identification. Another difficulty in understanding the UB cascade is the multiple lysine residues on ubiquitin which can alter the chain linkages (Figure 1.3). Ubiquitin itself has multiple lysine residues (K6, K11, K27, K29, K33, K48, and K63) which can react with C-terminal carboxyl groups on ubiquitin to form linear chains and branched linkages. Ubiquitin chains can be homotypic, the chains and linkages are formed via the same lysine residue, and heterotypic, the chains and linkages are formed with different lysine residues.

Figure 1.2 Cross reactivity within the ubiquitin cascade

The complexity in the ubiquitin cascade makes it difficult to identify substrates of ubiquitin ligases. Enzymes within UB signaling are promiscuous where a substrate can be ubiquitinated by multiple E3s and there are multiple E2-E3 pairs for a specific E3.
The different chains and linkages with UB lysine residues are reported to serve as signals for different functions within the cell.\textsuperscript{4-6} Linear chains of K48 are commonly seen to signal for the degradation by the 26S proteasome, but linear K11 and K29 chains have also been reported to signal for degradation.\textsuperscript{30-31} Recently, there are cases of ubiquitination on histones to serve as epigenetic markers.\textsuperscript{32}

\textit{Figure 1.3 Modes of ubiquitination and chain linkages}

Ubiquitin protein has seven lysine residues used as markers for different signaling pathways. The substrate can be monoubiquitinated (single ubiquitin), polyubiquitinated (ubiquitin chain at a single site on substrate), or multiubiquitinated (ubiquitin at multiple sites on substrate). Polyubiquitination can be further categorized into homotypic or heterotypic linkages. Homotypic linkages use the same Lys residue (K48 or K63) or C-terminus of one UB and N-terminus of another UB (linear M-1) to form the chain. Heterotypic linkages use different Lys residues to form a chain or form a branched linkage forming two differing chains.
Several techniques have been used to determine potential substrates of E3s as well as classification of E3s. One technique takes advantage of ubiquitinated protein substrates by using antibodies (Abs) targeting the diGly fragment after the substrates undergo trypsin digestion. Additionally, the use of stable isotope labeling of amino acids in cell culture (SILAC) with diGly antibodies allows for identifying ubiquitinated substrates as well as which lysine residues get modified.\textsuperscript{36} Another method uses the diGly fragments for ubiquitylation mapping with mass spectrometry after using proteasome inhibitor or deubiquitinating enzymes (DUBs).\textsuperscript{37-39} Such techniques have been employed to identify culin-RING E3s, the CRL family, and ubiquitin-like (UBL) protein, NEDD8.\textsuperscript{40} Substrates can be determined via their interaction with the E3 and categorizing the interacting domain help elucidate a substrate’s E3 counterpart.\textsuperscript{41} Microarrays also have been used to identify potential substrates and E3s \textit{in vitro}.\textsuperscript{42} Electrochemiluminescence

\textbf{Figure 1.4 RNF38 RING Crystal Structure}

(A) Cartoon representation of RNF38 RING (blue) with two loop regions interacting with Zn\textsuperscript{2+} ions (grey). The two loops chelating the Zn\textsuperscript{2+} ions are characteristic of RING-type E3s similar to zinc-finger motifs. (B) Interface of RNF38 RING domain with UbcH5B and UB. Cartoon representation of RNF38 RING domain (blue), ubiquitin (yellow), and UbcH5b (green) with two spherical representations of Zn\textsuperscript{2+} ions. Crystal structure, PDB: 4V3L, for this complex was identified by Buetow, L. \textit{et al.} 2015.\textsuperscript{63}
helps elucidate E3s after undergoing polyubiquitination from specific E2s on multi-array assays.\textsuperscript{43}

Current techniques to identify E3s and substrates have drawbacks which may hinder the study of UB signaling. Overexpression of E3s can ultimately decrease overall activity while \textit{in vivo} during the process of collecting the cells, thus no change in ubiquitination of the substrate may be observed. Techniques involving diGly Abs are useful for substrate identification and multi- or poly ubiquitination chains, but the only the substrates can be identified. Therefore, the E3s in the pathway are not known leaving a mystery as to which E3 ubiquitimates the substrate. The technique for using the type of interactions to identify substrates with E3s is extremely labor intensive as well as some E3s within large families have conserved domains, but the substrates could be very different or similar. Screening methods which rely on affinity towards E3s do not result in actual biological activity within the UB cascade. Lastly, \textit{in vitro} experiments occur under highly controlled conditions, but do not represent how the enzymes may function \textit{in vivo}. New strategies to help solve these complications are still being pursued to determine the substrates of E3s and which interactions are present throughout a single ubiquitin cascade from UB transferred to substrate.

\textbf{1.1.3 Rationale in Studying RING E3s}

The RING family make up a majority of the identified E3 enzymes which are regulated in several biological signaling pathways.\textsuperscript{44-50} Identification of the E3s responsible in substrate ubiquitination will aid in determining potential causes for a variety of diseases. A few of the RING E3s identified are known to be involved in the development or suppression in a variety of different diseases.\textsuperscript{51-56} RING E3s are classified based on the highly conserved RING domain involved in activity as an E3 ligase.\textsuperscript{57}
RING finger proteins dominate the class of E3s, and as previously stated, they lack a catalytic cysteine to directly transfer UB to the substrate. RING finger proteins have a unique zinc-binding motif with either cysteine or histidine in the first few residues of the RING.\textsuperscript{58} The zinc ions help maintain the structural integrity and activity for the RING domain. The zinc coordination pattern is distinct between the classes of E3s RING-type, and RING-type E3s act as a scaffold for the E2 to transfer the UB to the substrate.

1.2 RNF38

The E3 of interest is RING finger protein 38 (RNF38), a highly understudied E3 ligase. RNF38 is reported to function as a nuclear E3 ligase which ubiquitinates tumor suppressor protein, p53, and evidence supports the role of nuclear localization for p53 after being targeted for ubiquitination.\textsuperscript{59} Both structural and type of ubiquitination of RNF38 still remain elusive, thus

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.5.png}
\caption{E2-E3-UB interaction}
\end{figure}

(A) Close-up of RNF38 RING domain-UbcH5B interaction with color scheme of side reside chains represented as sticks as follows: O atoms (red), S atoms (yellow), and N atoms (blue). Key residues are labeled. (B) Close-up of RNF38 RING domain-UbcH5B-UB interaction with similar color scheme as (A). Hydrogen bonds are red dashed lines with labeled distances and grey spheres are Zn\textsuperscript{2+}. Labeling scheme is as follows: RNF38 RING (blue), UbcH5B (green), and UB (yellow).
complicating how this E3 acts in vivo. Similar RNF E3s have been identified, but their role is quite varied within several signal pathways.60-62

A partial protein structure for RNF38 RING domain elucidates the protein interaction with E2, UbcH5B, and UB (Figure 1.4).63 RNF38 RING domain is composed of three β-strands in antiparallel configuration, two pairs of Cys residues interacting with two Zn$^{2+}$ (not shown), an alpha helix, and several loop regions.63 One of the loops interacting with Zn$^{2+}$ has an interaction with the n-terminal of the helix interaction with UbcH5B. The E2-binding domain from RNF38 RING has hydrogen bond formation from Cys418 with K8 from UbcH5B (Figure 1.5 A) and potentially an electrostatic interaction of Asp419 from RNF38 RING and K8 from UbcH5B.

*Figure 1.6 OUT cascade*
xE1 interacts with xUB-HA via mutated AD while wt-UB cannot associate due to charge reversal in AD. xE2 associates with the mutated UFD of xE1 to transfer xUB-HA to the catalytic cysteine. wt-E2 cannot associate to UFD due to the charge reversal within the UFD. RING-type xE3 acts as a scaffold to allow transfer of xUB-HA to the substrate from xE2. wt-E2 will not associate with mutated E2-binding domain of xE3 based on the charge reversal while the substrate binding domain for xE3 remains unchanged to allow the substrate to associate.
RNF38 RING domain interacts with UB and UbcH5b (Figure 1.5 B) with R454. R454 from the RING domain interacts with Q40 and R72 from UB as well as Q92 from UbcH5b.

1.3 Orthogonal Ubiquitin Transfer Pathway

Previous research has developed bio-orthogonal cascade of engineered enzyme mutants known as orthogonal ubiquitin transfer (OUT).\textsuperscript{64} OUT clarifies a single pathway between the transfer of ubiquitin to the substrate by mutating key residues between the interactions of class of enzymes in the cascade (Figure 1.6) The engineered enzymes will not interact with their wild-type counterparts; therefore, making this system highly useful to identify substrates for

\textbf{Figure 1.7 Reversal of charge in the AD and/ or UFD of E1}

(A) wt-E1 has negative charges from aspartate or glutamate in both adenylation domain (AD) and ubiquitin fold domain (UFD), and UB associates with AD via positive charge by two arginine residues. (B) AD domain is mutated to reverse the charge by mutation of Asp or Glu to Arg or Lys rejecting wt-UB, therefore; mutant UB with charges reversed will associate to AD. AD mutated E1 is known as “forward E1” (fE1) while wt-E2 can associate with UFD. (C) Mutated UFD on E1 allows for wt-UB to associate to AD, but only mutant E2 (xE2) can associate to UFD. Mutated UFD E1 is known as “back E1” (bE1). (D) Both AD and UFD are mutated to have a complete charge reversal for E1 known as xE1. xUB and xE2 will associate to AD and UFD, respectively, on xE1 but wt-UB and wt-E2 cannot associate.
engineered E3s. The OUT approach utilizes pairs based on their interface interactions, UB-E1, E1-E2, and E2-E3. Understanding these interface interactions allows for developing an interaction solely between engineered species, thus wild-type enzyme counterparts would not interfere.

Two orthogonal pairs, engineered UB (xUB) with engineered E1 (xE1) and xE1 with engineered E2 (xE2), have already been identified. Studying the interaction between wild-type E1, Uba1, and UB the AD of E1 was a primary target where an electrostatic interaction between the positive charges of R42 and R72 on UB and Q576, S589, and D519 from the AD domain of Uba1. Therefore, through site-directed mutagenesis both sets of enzymes had a reversal of charges to generate an orthogonal pair. Uba1 also has a UFD necessary to interact with E2, and three negative charge residues, E1004, D1014, and E1016 are located within this domain. Similarly a charge reversal for both the n-terminal helix on E2 and UFD on E1 would create the orthogonal pair. If only the UFD domain on E1 is mutated while the AD domain remains unchanged, then a partially mutated E1 exist, known as bE1 (Figure 1.7). If the AD domain is mutated while the UFD remains unchanged then a mutant E1 (fE1) is produced. When both AD and UFD domains are mutated then a fully engineered E1, xE1, will exist allowing for a complete the both xUB and engineered E2, xE2, to bind. The last factor for the orthogonal pathway is the generate a reactive engineered E3, xE3, to interact with xE2. Potential E3 mutants must be screened against xE2 to find reactive xE3s using a display system. Some common display systems use bacterial surface display, yeast surface display, and phage display, but phage display is used almost exclusively for determination of potential xE3s by previous research.
1.4 Phage Display and Library Selection

1.4.1 M-13 Phage Display

The bacteriophage, M-13, is the display vector used for infection of F factor containing \textit{E. coli} strains (XL1-blue) because M-13 phage is a member of the Ff class within the genus \textit{Inovirus} bacteriophage uses \textit{E. coli}’s F conjugative pilus for attachment.\textsuperscript{71} M-13 bacteriophage physiology is rod shaped with six capsid proteins surrounding a circular (+) single-stranded DNA (ssDNA) genome encoding for 11 proteins, pI-pXI (Figure 1.8 A).\textsuperscript{72-76} The lysogenic

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{m13_virion}
\caption{M-13 phage structure and display vector}
\begin{itemize}
\item A Cartoon representation for the structure of M-13 phage. Circular (+) ssDNA (red) M-13 phage with rod-like capsid. Capsid proteins for structural and attachment: pIII (purple), pVI (orange), pVII (brown), pVIII (grey), and pIX (black).
\item B Partial representation for the different types of pIII phagemid vectors. The notations are as follows: LacZ (LacZ operon), S.D. (shine-delgano), OmpA (signal peptide), pIII protein.
\end{itemize}
\end{figure}
lifecycle of M-13 virion is beneficial for phage-displaying proteins as the viral genome is integrated into the host genome and plasmid DNA. Display methods for M-13 use phagemid vectors incorporating pIII are pComb3, pComb3X, pComb3H, and pJuFo where the pComb3H (pComb) vector is used in current research (Figure 1.8 B). pIII and pVIII are commonly used for display of proteins on the bacteriophage surface. pIII is the viral protein of interest for display with proteins fused to N-terminal end of pIII, but this results in slight reduction of infectivity. The use of a “helper phage” or wild-type M-13 phage helps kick start the phagemid, modified bacterial plasmid with pIII and gene fused to N-terminal.

1.4.2 Library Selection

Phage-displayed E3 will be selected out with a technique known as biopanning. Biopanning is an affinity-based approach to select specific sequences from a large pool, like a library. Purified antigens which have been immobilized are used to select out antibody libraries displayed on phage. New advances to selection techniques have advanced since then allowing for antibodies to target antigens presented on the cell surface, known as cell panning. Another way is to post-translationally modify proteins by biotin-labelled reactants, an approach employed by previous research for ubiquitin signaling using biotin-labeled ubiquitin undergoing ubiquitin pathway onto E3-displayed phage to be pulled down by streptavidin-coated plates. Recently chemically modifying molecules with biotin has been shown to be very effective in selecting out sequences by using streptavidin coated plates.
1.5 Purpose of the Study

Here determining the xE2-xE3 pair with RNF38 for the OUT cascade is crucial before identifying potential substrates. First, the activity of wt-RNF38 RING with a GST tag is observed with autoubiquitination assay then p53 ubiquitination assay before insertion of the RNF38 RING domain within the pComb phagemid. Activity of the phage-displayed RNF38 RING is observed to rule out RNF38-pIII fusion does not interfere with the autoubiquitination of RNF38 RING using the UB cascade. Phage-displayed E3 assays using both enzyme-linked immunosorbent assay (ELISA) with a direct immunoassay approach with primary antibody (1° Ab) targeting the pVIII protein of M13 phage (Figure 1.9) and targeting against the N-terminal tag of RNF38 RING with an immunoblot (IB). After confirming phage-displayed activity, the efficiency of the RNF38 RING-phage is compared with a control phage in a process known as

Figure 1.9 ELISA for wt-E3-phage
Cartoon representation of an ELISA with a direct immunoassay approach to determine E3-phage display activity. Streptavidin-coated plates will bind biotin-tagged ubiquitin conjugated to E3-phage. Anti-M13 primary Ab conjugated with horseradish peroxidase (HRP) binds to pVIII protein of M13 phage. The oxidation reaction of TMB with H₂O₂ catalyzed by HRP will allow for color-inducing intermediate resulting in a positive result.
model selection (Figure 1.10). Model selection uses variable ratios of wt-E3-phage and control-phage using colony PCR to show efficiency of the E3-phage. Then

Construction of a 4-mutant library for possible RNF38 RING domain sequences will be a two-step process: first an alanine screen on the four mutants: L412, M417, C418, and D419, and second will be the randomization of these four residues to generate all possible sequence variations. The generation of the DNA construct will occur through overlap PCR and inserted into the pComb phagemid, then to be transformed into XL1-blue cells (Figure 1.11). The library will be displayed on M-13 phage similarly as was previously stated in the model selection process. The library selection process will undergo a pseudo-orthogonal pathway with bE1, xE2, and UB-Bio. As previously stated bE1 is the mutated UFD domain of E1 where UB interacts

\[ wt\ E3 + \text{Fab} \xrightarrow{1:1, 1:10, 1:100} \text{wt-E3-phage: Fab-phage} \]

\[ \text{UB-Bio} \xrightarrow{+ E1, E2} \]

\[ \text{Pull down with Streptavidin plate} \]

\[ \text{Elute and infect cells on selective media} \]

\[ \text{Colonies PCR} \]

\[ \text{Figure 1.10 E3-phage Model Selection} \]

wt-E3 and Fab (control) are displayed on pIII protein of M-13 phage. wt-E3-phage will undergo the ubiquitin pathway \textit{in vitro} and set against varying ratios of control phage, then be pulled down by streptavidin coated wells. Bound E3-phage will be eluted and infect XL1-blue cells then a colony PCR for varying ratios will determine the efficiency of E3-phage.
with the AD domain and N-terminal helix of xE2 interacts with UFD domain. Sequences from the RNF38 RING domain library will be selected out based on the interaction of the xE2 and E3 library interface. Sequences are displayed on pIII and activity is tested in the OUT cascade with xUba1 (xE1), xUbcH5b (xE2), and HA-xUB. Sequences which are autoubiquitinated with HA-xUB are termed engineered RNF38 RING (xRNF38 RING). xRNF38 RING sequences will be inserted into full-length RNF38 (FL-RNF38) for protein expression in HEK293 cells. Selected FL-RNF38 proteins will undergo autoubiquitination in the OUT pathway using xE1, xE2,

Figure 1.11 Library construction and phage display

Construction of the library for all possible sequences from four mutation sites is performed via overlap PCR. Initially up and down fragments are amplified which have a section of identical DNA, therefore both fragments can anneal to one another to generate a full-length insert. The insert is ligated into pComb phagemid and transformed via electroporation into Xl1-blue cells. The cells are grown on selective media specific for the resistance within the phagemid then pDNA of the library are purified from isolate colonies. The E3 library is displayed on the pIII viral protein of M-13 phage.
xRNF38 proteins with HA-xUB. Reactive xRNF38 will ubiquitinate the substrate, p53, in the OUT pathway in vitro to provide evidence for FL-xRNF38 targeting p53.\textsuperscript{59}
2 Model Selection for wt-RNF38

2.1 Introduction

Here wt-E3, RNF38 RING, is studied to confirm activity within the UB cascade \textit{in vitro} before displaying on M-13 phage. First wt-RNF38 is inserted into pGex vector for expression in BL21 cells where RNF38 RING undergoes autoubiquitination \textit{in vitro} then ubiquitination of the substrate, p53 \textit{in vitro} to confirm activity of the RING domain. wt-RNF38 is inserted into the pComb phagemid where wt-RNF38 RING will be displayed onto the N-terminal end of pIII protein from M-13 phage. ELISA and autoubiquitination assay confirm activity of phage-displayed RNF38 (refer to Figure 1.9). Model selection with 1:1, 1:10, and 1:100 wt-RNF38 RING-phage: 7G12 control-phage confirm efficiency of the phage-displayed E3 (refer to Figure 1.10).

2.2 pGex vector and wt-RNF38 RING

![pGex vector with wt-RNF38 RING insert, H6 and GST tags, TEV recognition, ampicillin resistance gene, and BamHI and EcoRI restriction sites.](image)

\textit{Figure 2.12pGex vector}

pGex vector with wt-RNF38 RING insert, H6 and GST tags, TEV recognition, ampicillin resistance gene, and BamHI and EcoRI restriction sites.
2.2.1 pGex vector

(A) Full length wt-RNF38 sequence overlapped together from pGex-FL1-RNF38 and pGex-FL2-RNF38 with key features of TEV recognition, proline-rich region and RING domain. (B) wt-RNF38 RING inserted into the pGex vector. Key features include TEV site, H6 tag and GST tags, and RNF38 RING.

Figure 2.2 Sequence analysis of pGex-wt-RNF38 and RNF38 RING
pGex vector is used as a protein expression vector for insertion of the FL-wt-RNF38 and wt-RNF38 RING (only RNF38 RING is included in study). pGex vector includes H6 (HHHHHH) and GST tags, resistance gene, and TEV recognition site (Figure 2.1). pGex vector can be used for plasmid expression in XL1-blue cells, but protein expression for FL-RNF38 is reported to yield active protein with HEK293 cell line. However, only the RING domain will

![Figure 2.3 Activity of GST-RNF38 RING protein](image)

(A) Scheme for autoubiquitination of E3 RING domain with N-terminal GST tag. UB transfers to a lysine residue on E3 RING in the absence of a substrate. Polyubiquitin chain formation occurs in vitro without degradation of the E3 RING domain. (B) Coomassie staining for the expression of GST-RNF38 RING comparing the protein lysate with the GST elution showing a ~35 kDa band verifying GST-RNF38 RING. (C) Autoubiquitination of GST-RNF38 RING in vitro with Uba1, UbcH5B, GST-RNF38 RING, and HA-UB. Lanes from left to right: +, -Uba1, -UbcH5B, and -HA-UB. GST-RNF38 RING is identified at ~35 kDa in IB: GST at 1 min exposure. Polyubiquitin chain is shown on GST-RNF38 RING in the “+” reaction only when compared to three negative controls.
be fused with GST and expressed in BL21 cell line for overexpression. RNF38 RING insertion into the vector occurs with BamHI and EcoRI restriction enzymes (REs).

2.2.2 wt-RNF38 RING

Genomic studies for FL-wt-RNF38 in humans have determined the coding sequence (1545 bp) from the translated sequence (515 aa). However the crystal structure and function for FL-RNF38 remains elusive, but only RING domain has been shown to have function and the sequence has two featured regions: proline-rich region and RING domain (Figure 2.2A). RNF38 RING will be studied for activity with protein assay, model selection, and library selection. wt-RNF38 RING is inserted into the pGex vector and sequence analysis confirms ligation of RING domain (Figure 2.2B). The GST protein is fused with the N-terminal protein of RNF38 RING for protein purification and using GST tag as a marker to study whether RING domain only is active in UB cascade. pGex-RNF38 RING expression in BL21 cells will be tested for activity within UB cascade.

Figure 2.4 Substrate ubiquitination by RING domain
(A) Scheme for substrate ubiquitination with E3 RING domain with N-terminal GST tag. UB transfers to a lysine residue on the substrate. The interaction of E2 with substrate will determine whether mono- or polyubiquitin chain formation occurs in vitro. (B) Substrate ubiquitination with p53 in vitro with Uba1, UbcH5B, GST-RNF38 RING, and HA-UB. Lanes from left to right: +, -Uba1, -UbcH5B, -GST-RNF38 RING, and -HA-UB. p53 is identified at ~53 kDa in IB: p53 at 1 min exposure. Polyubiquitin chain is shown on p53 in the “+” reaction only when compared to four negative controls.
2.3 wt-RNF38 RING Activity

FL-RNF38 does not express well nor showed significant activity in BL21 or HEK293T cells, but GST-RNF38 RING domain is expressed well in BL21 cells.59,63 Expression of wt-RNF38 RING in BL21 cells is purified with fusion of GST tag resulting a protein size of ~35 kDa shown in the figure below (Figure 2.3 A). GST-RNF38 RING fusion protein yields ~71.2 mg / mL with minimal impurities. A scheme for the autoubiquitination of E3 RINGs with N-

![Diagram A](image1.png)

![Diagram B](image2.png)

![Diagram C](image3.png)

Figure 2.5  pComb phagemid and pComb-wt-RNF38 RING sequence
(A) Phagemid of pComb with Fab, 7G12, insert, ampicillin resistance gene, pIII gene, SacI, SpeI, and ClaI restriction sites, and TEV recognition. (B) Phagemid of pComb with wt-RNF38 RING insert and addition of FLAG tag, SacII site. (C) wt-RNF38 RING inserted into the pComb vector. Key features include TEV site, FLAG tag, and pIII protein, and RNF38 RING.
terminal GST tag occurs in the absence of substrate (Figure 2.3 B). Autoubiquitination of GST-RNF38 RING in IB: GST shows polyubiquitination in only the “+” reaction while no ubiquitin bands occurred in the three negative controls: -Uba1, -UbcH5B, and -HA-UB (Figure 2.3 C). Interestingly there is the appearance of a band ~70 kDa which may show GST-RNF38 RING exist as a dimer, but further evidence is required to prove dimeric activity. A scheme for substrate ubiquitination from E3 RING with GST tag occurs when E2~UB is proximal to the substrate after both substrate and E2 associate with E3 RING domain (Figure 2.4 A). GST-RNF38 RING targets p53 after showing activity in p53 ubiquitination providing evidence that

Figure 2.6 wt-RNF38 RING-phage activity
(A) wt-RNF38 phage display in anti-FLAG immunoblot. Lanes (left to right): Previous wt-RNF38-phage (10 days old) and freshly prepared wt-RNF38-phage with 60 sec. exposure time. (B) Scheme for autoubiquitination of E3 RING domain conjugated to N-terminus of pIII on M-13 phage. (C) Autoubiquitination assay for wt-RNF38 RING-phage with Uba1, UbcH5B, wt-RNF38 RING-phage, and Bio-UB in IB: FLAG with exposure time at 30 sec. Reactions left to right: “+”, -Uba1, -UbcH5B, and -Bio-UB are incubated overnight at 37°C. Polyubiquitin chain may interfere with the FLAG tag, thus preventing the 1° Ab against FLAG tag as the three negative controls show FLAG tag ~30 kDa. (D) ELISA assay for activity of wt-RNF38 RING-phage activity using α-M-13 phage 1° Ab-HRP conjugate. Positive reaction occurs in “+” reaction while no reaction occurred for three negative controls in 10⁰ reactions, but not reactions are observed for 10⁻¹ and 10⁻² “+” reactions.
the RING domain associates with both UbcH5B and p53 (Figure 2.4 B). p53 ubiquitination occurs only in the “+” reaction when compared to four negative controls: -Uba1, -UbcH5B, -GST-RNF38 RING, and -HA-UB. Surprisingly, p53 is shown to act as a dimer as a band ~100 kDa occurs after a four-hour reaction (data not shown). The other bands present lower than p53 may be a result of the 1° Ab binding to fragments of recombinant p53 which is shown on both commercial and expressed p53 (data not shown).

2.4 wt-RNF38 RING-phage Activity

2.4.1 pComb vector

pComb vector, as previously stated, is a phagemid with resistance gene, pIII protein from M-13 phage, and LacZ operon (Figure 2.5 A). A TEV recognition site: ENLYFQG is included right after the SpeI recognition sequence. TEV site will be further explained in chapter 4 when used in selection of RNF38 RING 4-mutant library. The 7G12 Fab insert (1410 bp) will be used as a control in the model selection with wt-RNF38 RING domain (Figure 2.5 B). pComb vectors will be optimal for male E. coli cells with F conjugative pilus for the helper phage to initiate infection, hence XL1-blue E. coli will be used to uptake phage. pComb phagemid includes an ampicillin resistance allowing XL1-blue cells to be selected out from natural resistance to tetracycline, but XL1-blue with pComb phagemid will grow in tetracycline/ampicillin media. The RING domain is inserted into the pComb vector for phage display on pIII of M-13 phage. The template for the phagemid used in model selection, library construction, and library selection is shown below (Figure 2.5 C). The phagemid construct includes FLAG tag (DYKDDDD), SacI, SacII, SpeI, and Clal REs, TEV recognition site, and pIII.
2.4.2 wt-E3-Phage Activity

Native RNF38 RING domain is ensured to be displayed by M-13 phage before testing the efficiency of wt-RNF38 against 7G12 control. Phage-displayed wt-RNF38 is verified with IB: FLAG showing a ~30 kDa band (Figure 2.6 A). An indirect approach for IB ensures the efficacy of the 2° Ab binding to the 1° Ab, thus providing stronger and more accurate signal. wt-RNF38 RING-pIII conjugate is ~30 kDa in size, but a slightly smaller band appearing less than 25 kDa was seen which a cleaved fragment or truncation of wt-RNF38 RING-pIII occurs in vivo during capsid protein expression and assembly for the M-13 virion. 7G12-phage does not appear in IB: FLAG due to the absence for the FLAG-tag in the sequence. Autoubiquitination of FLAG-RNF38-RING-phage occurs similarly as autoubiquitination with GST-RNF38 RING in the scheme above to demonstrate phage-displayed proteins will not have have activity interference from the phage protein (Figure 2.6 B). Autoubiquitination of FLAG-RNF38 RING-phage shows evidence for activity of E3 RING displayed on pIII capsid protein (Figure 2.6 C). Polyubiquitination of FLAG-RNF38 RING-phage in the “+” reaction may affect the binding of α-FLAG 1° Ab by shielding the tag, but the three negative controls show only one band ~30 kDa indicating no autoubiquitination. An ELISA assay helps determine the sensitivity of phage-displayed activity for FLAG-RNF38 RING-phage (Figure 2.6 D). A positive result occurs in the “+” reaction indicating E3 RING-phage has Bio-UB conjugated to a lysine residue, thus α-M-13 phage 1° Ab-HRP conjugate is able to bind and oxidize TMB substrate in the presence of H₂O₂. There is no reaction observed for the three negative controls. Unfortunately, only 10⁰ reaction produces a positive result while 10⁻¹ and 10⁻² reactions do not show activity.
### 2.5 wt-E3-phage Model Selection

The titer for 7G12- and wt-RNF38 RING-phage, respectively, are $5.4 \times 10^7$ pfus and $5 \times 10^9$ pfu resulting a in 100-fold decrease and would make the model selection parameters more difficult to obtain. 7G12-phage is prepared once more to provide a better phage titer to be near wt-RNF38 RING-phage yielding a titer of $1.3 \times 10^9$ cfus (data not shown). Reactions for the ELISA and model selection have a total wt-RNF38-phage concentration of $9 \times 10^{10}$ pfu while 7G12-phage is varied for 1:1, 1:10, and 1:100 wt-RNF38-phage: 7G12-phage. Colony PCR with primers, Jun13 and Jun14 (refer to Table 3.1), tests the efficiency of RNF38 RING-phage: 7G12-phage 1:1, 1:10, and 1:100 ratios (Figure 2.7). All eight isolates in 1:1 reactions correspond to the RNF38 RING with ~500 bp band. In 1:10, isolates 1-3 and 5-7 (75% efficiency) are wt-RNF38 RING compared to 7G12, but isolates 1 and 8 have slight competition.

![Figure 2.7 Model Selection of wt-RNF38 RING.](image)

*Figure 2.7 Model Selection of wt-RNF38 RING.*

Colony PCR of isolate colonies with phagemid primers, Jun13 and Jun14, in ratios (1:1, 1:10, and 1:100) of wt-RNF38-phage: 7G12-phage. RNF38 RING domain is 500 bp band while 7G12 is 1500 bp band. wt-RNF38 RING-phage is effective in phage activity appearing in 6 of 8 isolates in 1:10 reaction and 1 of 8 within the 1:100 reaction.
with 7G12. In 1:100, isolates 1-8 are 7G12 while isolates 1-7 have slight competition of wt-RNF38 RING.

2.6 Discussion

RNF38 RING Sequence analysis for FL-RNF38 was spliced together from two-part sequence with an overlap within the proline rich domain (refer to Figure 2.2). Autoubiquitination for GST-RNF38 RING provides evidence of the RING domain only and does not provide data on how FL-RNF38 will undergo autoubiquitination. The ~70 kDa band present for autoubiquitination may elucidate GST-RNF38 RING dimeric activity only, but further assays are required to explore whether FL-RNF38 may exhibit dimeric activity. Ubiquitination of p53 shows polyubiquitination in vitro as there is no regulatory signal for monoubiquitinating p53. When introducing RNF38 RING into the pComb phagemid, OmpA leader sequence is attached to the N-terminus of RING domain and directs the FLAG-tagged wt-RNF38 RING-pIII conjugate to the Sec pathway in E. coli to translocate the protein from the cytosol to the periplasm for assembly of the M13 virion. RNF38 RING-phage activity is not affected by being displayed on the N-terminus of pIII capsid protein suggesting little interference of the virion with the E3.

2.7 Experimental Procedure

2.7.1 Cloning of pGex-FL-RNF38 and RNF38 RING

wt-FL-RNF38 and wt-RNF38 RING (sequenced and prepared by previous lab member, Li Zhou) is inserted into pGex vector (previous construct provided by previous lab member, Li Zhou) with H6 and GST tag. pGex vector was purchased from Addgene.
2.7.2 Expression and purification of GST-RNF38 RING

Transformation with 2 μL of pGex-RNF38 RING plasmid into 50μL BL21 cells via electroporation is treated immediately with 1 mL SOC media and incubated for 1 hour in 37°C shaker. 50 μL of the solution is streaked onto 2% glucose ampicillin plates and incubated overnight in 37°C incubator. The remaining 950 μL is transferred to 200 mL 2XYT media with 100 μg/mL ampicillin incubating overnight in a 37°C shaker. The cells are measured at O.D.₆₀₀ to be ~0.7-0.8 before transferring to 800 μL 2XYT media with 50 μM ZnCl₂ and 100 μg/mL ampicillin and incubated in 37°C shaker until O.D.₆₀₀ measures ~1.2. The cells are cooled in 20°C shaker for 20 min before inducing cells with 1 mL of 1M IPTG overnight in 20°C shaker. Cells are transferred to large centrifuge tubes and centrifuged at 5500 rpm for 15 minutes at 4°C. The supernatant is discarded while the pellet is resuspended in 40 mL of GST lysis buffer (pH 8.8, 20 mM Tris, 500 mM NaCl, 0.1% Triton X-100, 10% glycerol, 10 mM DTT, 50 μM ZnCl₂, 1 mM PMSF, and 1 mM benzamidine). Lysozyme (1 mg/mL) and 1 protease inhibitor tablet are added to resuspended pellet, mixed by inversion, and incubated on ice for 1 hour. Lysis mixture is sonicated at 19% for 30 mins with 10 s on / 10 s off. Lysate is transferred to ultracentrifuge tubes and centrifuged at 12,000 rpm for 30 min at 4°C. Glutathione-agarose beads were washed with 50 mL GST wash buffer (pH 8.8, 20 mM Tris, 500 mM NaCl, 10% glycerol, 10 mM DTT, 1 mM PMSF, and 1 mM benzamidine) then centrifuged at 2,200 rpm for 5 min at 4°C while the supernatant is discarded. Glutathione-agarose beads were resuspended in protein lysate before incubating at 4°C on orbital shaker overnight. Lysate is run through protein column and collected in 50 mL tube. Beads are washed with 15 mL GST lysis buffer and collected in 15 mL tube. Beads are washed twice with 15 mL GST wash buffer and collected separately in 15 mL tubes. Beads are eluted with 6 mL GST elution buffer (pH 8.8, 30 mM reduced glutathione, 50 mM
Tris, and 150 mM NaCl) and dialyzed in GST dialysis buffer (pH 8.8, 50 mM Tris, 150 mM NaCl, 50 μM ZnCl₂, 10% glycerol, and 10 mM DTT) overnight. The solution is concentrated in 30 kDa protein centrifuge column and stored in -80°C freezer. An aliquot of 25 μL was collected from protein lysate and GST-elution fractions and mixed with 2X Lamelli with BME loading dye, boiled for 5 minutes, then spun down before electrophoresis at 200V for 30 min. in precast 4-15% SDS-PAGE gel. GST-RNF38 RING is verified following Coomassie stain protocol.

Bradford Protein Assay protocol was followed to determine protein concentration in 1 cm cuvette with Genesys20 spectrophotometer. Concentration was determined using equation (determined by previous lab member following Bradford Protein Assay protocol): (A₅₉₅ + 0.0245)/ 0.03945 * 0.8 μL sample (1:1 dilution factor) = mg/ mL). GST-RNF38 RING concentration (mg/ mL) is multiplied by following equation to determine μM concentration: 1 / 35 kDa GST-RNF38 RING * 1000 μM / 1 mM * (concentrated mL / 1 mL) = μM.

2.7.3 Ubiquitination Assays with wt-RNF38 RING and p53

Auto-ubiquitination for wt-RNF38 RING was conducted in 50 μL reactions with E1, Uba1 (1 μM), E2, UbcH5B (10 μM), E3, wt-RNF38 RING (5 μM), HA-UB (40 μM), ATP pH 7.6 (5 μM), 1 X Tris HCl / MgCl₂, 0.1 mM DTT, and remaining volume with diH₂O. Three controls: -E1, -E2, and -UB are also conducted to serve as negative controls for auto-ubiquitination. Reactions were incubated at 2 h in 37°C shaker then half of the reactions were stopped in 2X Lamelli with BME loading dye while the other half continued for 2 additional hours. The reactions for 4 h were stopped in 2X Lamelli with BME loading dye. Reactions were run following similar conditions as the western blot for wt-RNF38 phage with 1: 1500 α-GST 1° Ab and 1: 10000 α-mouse-HRP conjugate 2° Ab.
Substrate ubiquitination for p53 was conducted in 60 μL reactions with E1: Uba1 (1 μM), E2: UbcH5B (10 μM), E3: wt-RNF38 RING (5 μM), HA-UB (40 μM), p53 (Thermo Fischer) (5 μM), ATP, pH 7.6 (5 μM), 1 X Tris HCl / MgCl₂, DTT (0.1 mM), and remaining volume with diH₂O. Three controls: -E1, -E2, and -UB are also conducted to serve as negative controls for self-ubiquitination. Reactions were incubated at 2 h in 37°C shaker before 25 μL of the reactions were stopped in 2X Lamelli BME loading dye. The remaining 25 μL incubated for 4 h before being stopped with 2X Lamelli BME loading dye. Reactions were run following similar conditions as the western blot for wt-RNF38 phage with 1: 1500 α-GST and 1: 1500 α-p53 1° Ab and 1: 10000 α-mouse-HPR conjugate 2° Ab.

2.7.4 Cloning pComb-RNF38 RING and pComb-7G12

pComb vector was purchased from Addgene, but the pComb-wt-RNF38 RING construct was provided by previous lab member. FAB, 7G12, was inserted with SacI and SpeI REs into pComb vector. 7G12 Fab Ab was designed and purified by Dr. Andrea Cochran.

2.7.5 wt-RNF38 RING phage display

Previously wt-RNF38 was purified and ligated to pComb vector to be used for selecting the efficiency of wt-RNF38-phage compared to 7G12- phage. pComb-wtRNF38 plasmid was transformed into commercial XL1-Blue cells via electroporation method, and streak plated 50 μL of inoculum onto 2% glucose, 100 μg/ mL ampicillin agar plates then incubated overnight in 37°C incubator. Isolate colony was incubated in 10 μg/ mL tetracycline, 2XYT media for 12-15 hours at 37°C. 2 mL of cell culture was pipetted to 20 mL solution containing 100 μg/ mL ampicillin, 2% glucose, and 2XYT media then incubated in 37°C shaker for 4-6 hours until O.D. was 0.7-0.8 via spectrophotometer. Cells were infected with 20 μL VCSM13 helper phage for 1 hour in 37°C shaker then cells well centrifuged at 3700 rpm at 4°C for 12 min. The supernatant
was decanted while the cell pellet was resuspended in 200 mL 2XYT media with 70 µg/mL kanamycin and 100 µg/mL ampicillin and incubated in 30°C shaker overnight. Cells were transferred to large sterile centrifuge tubes to be centrifuged at 5000 rpm for 12 min. at 4°C. The supernatant was transferred to another larger centrifuge tube with 38 mL of 5X PEG, mixed, and solution was incubated on ice for 1 hour. The solution was centrifuged at 5000 rpm for 1.5 hours at 4°C, decanted the supernatant, and resuspended the pellet in 3 mL 1X TBS pH 7.6 buffer. Resuspension was centrifuged at 13000 rpm for 10 min at 4°C, supernatant was transferred to chilled 1.5 mL tubes and stored in 4°C.

2.7.6 Western Blot and Phage Titer

wtRNF38-phage was denatured in 20 µL 2X Lamelli w/ BME loading dye and boiled for 5 minutes, then spun down before adding 9 µL of each reaction to precast 4-15% SDS-PAGE gel running at 200 V for 30 minutes. The nitrocellulose membrane was pre-soaked in methanol before transferring to 1X Transfer Blot Buffer. The gel was soaked in Transfer Buffer along with the filter papers, and then stacked in a cast for transferring the gel to membrane running for 10 minutes at 25 V. The membrane was incubated for 1 hour in 5% milk in 1X TBS buffer. The membrane was sealed in pouch with 2000-fold dilution of anti-FLAG Ab (purchased from Sigma Aldrich) in 5% milk solution and incubated overnight on rotary shaker in 4°C. The membrane was washed thrice at 10-minute intervals with 1X TBS buffer, then was added to 20 mL of 5% milk with 5000-fold dilution anti-mouse-HRP conjugate Ab (purchased from Santa Cruz Biotechnology) and incubated at 25°C for 1-2 hours before being washed thrice at 10-minute intervals with 1X TBS buffer, and finally was subjected to 1 mL of Super Signal Pico Solution and 1 mL Super Signal Peroxide Solution and incubated for 5 minutes at 25°C. Film was developed for 1, 3, 5-min in a dark room.
wt-RNF38-phage and 7G12-phage conjugates were diluted to $10^{-6}$ by 1000-fold dilution in 1 mL of 1X PBS Buffer, pH 7.6, then 1000-fold dilution into 1 mL XL1-blue cell culture with O.D. ~0.7 in 10 μg/ mL tetracycline 2XYT solution. The cells infected by RNF38 RING-phage were incubated for 1 hour before serial dilution in 2XYT media. 5 μL of the serial dilutions are pipetted onto 2% glucose, 100 μg/ mL ampicillin agar plates in triplicate and incubated in 37°C overnight. Isolate colonies were counted to provide accurate titers.

2.7.7 Autoubiquitination of RNF38 RING-phage and ELISA

Autoubiquitination for wt-RNF38 RING-phage was prepared similarly to autoubiquitination of GST-RNF38 RING except with $1\times10^{10}$ pfus for phage concentration.

The ELISA assay was prepared in 100 μL reactions with Uba1 (1μM), UbcH5B (10 μM), wt-RNF38 RING-phage (1 $\times$ 10$^{10}$ pfu), wt-UB-Biotin (Boston Biochem) (0.5 μM), ATP pH 7.5 (5 μM), 1X Tris pH 7.5 HCl / MgCl$_2$ Solution, 0.1 mM DTT, remaining volume with diH$_2$O. Three negative controls with -E1, -E2, and -UB-Bio were prepared similarly. Reactions were incubated for 2 h in the 37°C shaker, then reactions were bound to the streptavidin-coated wells with 1:1 reaction: 3% BSA in 1X TBS-T incubating for 1 h on orbital shaker. Wells were washed 20 times with TBS-T buffer and incubated for 1 h at room temperature with 100 μL BSA with 20,000-fold dilution anti-M13 phage antibody (Fischer Scientific). Each well was washed 20 times with 1 X PBS buffer, and mixed with 1:1 peroxide solution: TMB substrate for 5 minutes at room temperature.

2.7.8 RNF38 RING model phage selection

wt-RNF38 RING-phage: 7G12-phage reactions were incubated in 1 mL of XL1 blue cell cultures (O.D. ~0.7) for 1 hour in 37°C shaker. Cell cultures were streaked onto 2% glucose, 100 μg/ mL ampicillin plates incubating overnight in 37°C incubator. Colony PCR for eight isolate
colonies from 1:1, 1:10, and 1:100 plates was set up in 20 µL reactions with 0.5 nM Jun13 (5'-ACTTTATGCTTCCGGCTCGTATGT-3') (primers from IDT) and 0.5 nM Jun14 (5'-AATCAAAATCACCGGAACCAGAGC-3'), 2X DreamTaq Green PCR Master Mix (Thermo Fischer), and remaining volume with diH2O for RNF38 RING-phage: 7G12-phage with thermocycler conditions: 95°C for 2:00, (95°C for 30 s, annealing at 52°C for 30 s, extending at 72°C for 45 s for 35 cycles), 72°C for 4:00 min, and holding @ 4°C. The PCR products were verified in 1% agarose gel running for 35 min at 150 V with 1 µg of 1.0 kb DNA ladder and 20 µL of 1:1, 1:10, 1:00 reactions were loaded into the corresponding wells.
3 RNF38 RING Library Construction

3.1 Introduction

Characterization of conserved residues among RING E3s elucidate key residues within the RING domain responsible for interacting with E2. A library will be generated to cover all possible sequences to later be selected out when using xUbcH5B where K4E and K8E mutations are present on the N-terminal helix. An alanine screen is used to test potential primers to generate two fragments, upstream fragment and downstream fragment, for overlap polymerase chain reaction (PCR) (refer to Figure 1.1). RNF38 RING AAAA-mutant overlap insert for alanine screen is inserted into pComb vector after digestion, transformed into XL1-blue cells, and sequences are analyzed. RNF38 RING AAAA-mutant is used as a template for the 4-mutant library by randomizing the four alanine sites.

3.2 Library Design

The interface of UbcH5B and wt-RNF38 RING shows hydrogen bonding between carbonyl on the backbone, located on an N-terminal loop containing Cys413 and Cys416 chelating Zn$^{2+}$, of RNF38 RING with K8 located on a N-terminal helix of UbcH5B (Figure 3.1 A). Residues: L412, M417, C418, and D419 are targeted as these positions flank Cys413 and Cys416. The four residues are close to the N-terminal helix from UbcH5B which may interact intra- or intermolecular to stabilize the binding interface. The tight loop seems to be necessary to allow for hydrogen bond formation. This crystal structure provides only a small piece of evidence since a great deal of flexibility would achieve a potential electrostatic interaction between D419 of RNF38 RING and K8 of UbcH5B as well as a two hydrogen bonds form by
Figure 3.19E2-E3 Interface and E3 RING domain alignment of RING E3s

(A) Interface of E2-binding domain of wt-RNF38 RING with N-terminal helix of UbcH5B. Residues for structural integrity and E2-E3 binding interface are labeled with hydrogen bond distance (Å). Blue labels represent basic residues while red labels are acidic residues. Mutations for xUbcH5B are included to determine new interface for xE2-xE3 at the E2 binding domain. (B) Alignment of translated RING E3s based on RING domain truncated to analyze conserved residues (shaded in black) to elucidate similarities for E2 binding interface. RING E3s have CXXC domain crucial for Zn$^{2+}$ binding and residues near this motif interact with E2s.
both carbonyl groups of Val414 and Val415 from RNF38 RING with Arg5 from UbcH5B. Several RNF E3s RING domains are aligned to show conserved residues within the RING domain which may interact with their respective E2s (Figure 3.1 B). The CXXC moiety is conserved among several RING-type E3s and residues neighboring will interact with N-terminal helix from E2. Randomization of four neighboring residues: L412, M417, C418, and D419 from RNF38 RING domain around the CVVC moiety will allow for interaction with xUbcH5B.

Figure 3.20pComb-RNF38 RING alanine mutants
(A) PCR of up and down fragments from RNF38 RING AAAA mutant. Lanes from left to right: 1.0 kb DNA ladder, RNF38 RING AAAA mutant upstream fragment, and RNF38 RING AAAA mutant downstream fragment. (B) Overlap PCR of RNF38-AAAA mutant show ~250 bp band consistent with the overall size of the RING domain. (C) pComb-wtRNF38 digestion with SacII and SpeI REs resulting in 3.0 kb band and ~250 bp band, respectively. (D) RNF38 RING-AAAA mutant digestion with SacII and SpeI with ~250 bp band. (E) Ligation of pComb-RNF38 RING AAAA mutant in 1:3 ligation of SacII/ SpeI digested pComb phagemid: SacII/ SpeI RNF38 AAAA mutant insert in comparison with vector-only control. Effective ligation is determined by at least 10-fold difference in colony forming units (cfus) between control and ligation reaction.
3.3 RNF38 RING AAAA mutant construction

pComb-RNF38 RING (refer to Figure 2.5 C) is used as a template while primers included L412A, M417A, C418A, and D419A mutations. As previously stated with overlap PCR, an upstream fragment including a SacII restriction site and a downstream fragment consisting of four alanine mutations and SpeI restriction site are amplified. There is an area on the fragments, ~28 bp overlap near the 3’ end on the upstream fragment and the 5’ end of the downstream fragment, where the upstream fragment and downstream fragment will anneal, and extension of the fragments will produce a ~250 bp fragment (Figure 3.2 A & B). Digestion for

![Image of gel electrophoresis](image)

**Figure 3.3 pComb-RNF38 RING 4-mutant library**

(A) PCR of up and down fragments from RNF38 RING 4-mutant library. Lanes from left to right: RNF38 RING AAAA mutant upstream fragment, 1.0 kb DNA ladder, and RNF38 RING AAAA mutant downstream fragment. (B) Overlap PCR of RNF38-AAAA mutant shows ~520 bp band consistent with the overall size of the RING domain as well as ~350 bp band in higher with more intensity. (C) pComb-7G12 digestion with SacI and ClaI REs result in 3.0 kb band and ~1500 bp band, respectively. (D) RNF38 RING 4-mutant library digestion with SacI and ClaI with ~520 bp band as well as the ~350 bp band. (E) Ligation of pComb-RNF38 RING 4-mutant library in 1:10 ligation of SacI/ ClaI digested pComb phagemid to SacI/ ClaI RNF38 RING 4-mutant library insert in comparison with vector-only control. Effective ligation is determined by at least 10-fold difference in colony forming units (cfus) between control and ligation reaction.
pComb-RNF38 RING and RNF38 RING AAAA mutant occurs with restriction enzymes (REs), SacII and SpeI (Figure 3.2 C & D). The pComb-wt-RNF38 RING did not have complete digestion as verified by the low intensity of the ~250 bp band which may affect ligation of RNF38 RING AAAA mutant into pComb phagemid. Though the digestion did not provide complete digestion, ligation of pComb-RNF38 RING AAAA mutant yields many colony forming units (cfus) when compared to digested pComb phage only (Figure 3.2 E). Sequence analysis from four of six isolate colonies confirm L412A, M417A, C418A, and D419A mutations.

3.4 RNF38 RING 4-mutant library construction

Construction of RNF38 RING 4-mutant library is generated from RNF38 RING AAAA-mutant template similar to alanine mutant construction except that the four mutation sites use NNK sequence to represent twenty amino acid combinations. The upstream fragment includes SacI restriction site with FLAG tag while the downstream fragment includes four NNK sequences, ClaI restriction site, pIII protein and TEV recognition site (Figure 3.3 A). The overlap of upstream and downstream fragments occurs in the similar region as the alanine mutant insert but yields a larger band ~520 bp (Figure 3.3 B). The 4-mutant library insert is repeated multiple times to achieve at least 100-150 µg before digestion. pComb-7G12 and RNF38 RING 4-mutant library are digested with SacI and ClaI (Figure 3.3 C & D). pComb-7G12 is almost completely digested as the band intensities are similar from a qualitative perspective where 7G12 is verified by ~1500 bp band. The 4-mutant library after digestion yields intense bands ~520 bp and ~350 bp suggesting that many of the fragments could not have undergone overlap PCR as effectively as alanine mutant insert (Figure 3.3 D). Ligation of pComb-RNF38 RING 4-mutant library yields a 10-fold increase of SacI/ ClaI digested pComb with SacI/ ClaI digested 4-mutant
library in a 1:10 ratio, respectively, when compared to digested pComb-only (Figure 3.3 E). The relatively low amount of cfus present in the 1:10 ligation could be the result of the downstream fragment being amplified in excess while less intensity is observed for the ~520 bp band.

The ligation for pComb-RNF38 RING 4-mutant library is verified through colony PCR for twelve isolate colonies with primers, Jun13 and Jun14 (refer to Table 3.1). The primers

![Image](image.png)

**Figure 3.4 RNF38 RING 4-mutant library**

(A) Colony PCR with primers, Jun13 and Jun14, on twelve isolate colonies of pComb-RNF38 RING 4-mutant library. A ~500 bp band includes OmpA leader sequence, RNF38 RING domain sequence, and pIII sequence. (B) Library titer comparing all possible sequences for a 4-mutant library with the number of potential cfus generated in the RNF38 RING 4-mutant library. (C) Sequencing analysis and alignment of translated DNA sequences of 7 isolates colonies. Sequences are aligned with translated wt-RNF38 RING domain with residues: L412, M417, C418, and D419 randomized.
anneal upstream of the OmpA leader sequence and downstream of the ClaI restriction site verifying ~520 bp fragment in the twelve isolate colonies (Figure 3.4 A). After large-scale ligation and plasmid purification, a library size for a 4-mutant library consists of $1.6 \times 10^5$ is required to have all possible sequences. RNF38 RING 4-mutant library is titered showing a potential library size to be $\sim 7.2 \times 10^6$ cfus when using commercial XL1-blue cells (Figure 3.4 B). Sequence analysis sent from 10 isolate colonies reveal 7 of 10 randomized residues at L412, M417, C418, and D419 within the RING domain (Figure 3.5 C). Isolates 2 and 6 had a deletion causing a frame shift while isolate 10 was not sequenced (data not shown). Isolates 1, 8 and 9 have missense mutations from either sequencing errors or replication of the plasmid within XL1-blue cells.

3.5 Discussion

RNF38 RING sequence is similar to many other RING-type E3s following the CXXC moiety in the N-terminal loop coordinating with Zn$^{2+}$, but a majority of E3s use two nonpolar residues in between two flanking cysteine residues with the exception of RING domains of Table 3.1 Primer for overlap and colony PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>1. Jun13</td>
<td>5’-ACCTTTATCCTCCGGCTCGTATG-3’</td>
</tr>
<tr>
<td>2. Jun14</td>
<td>5’-AACCCGGAATCCAGCAGATTT-3’</td>
</tr>
<tr>
<td>3. pComb-TEV-RNF38 up</td>
<td>5’-TCCGCCGCGGACTAAAGCAGATTT-3’</td>
</tr>
<tr>
<td>4. pComb-TEV-RNF38 overlap down</td>
<td>5’-AGCTGTTCCTCGACTGTTGTTAGGAAATTT-3’</td>
</tr>
<tr>
<td>5. pComb-TEV-RNF38 overlap up</td>
<td>5’-CAGTCGAAACAGACTGATATGATATTCGCGAGGACGATTTGAGTCAAGGAGCAGCTAC-3’</td>
</tr>
<tr>
<td>6. pComb-TEV-RNF38 down</td>
<td>5’-CGTGAATTTCCTGAATCCCGATG-3’</td>
</tr>
<tr>
<td>7. SseI up primer</td>
<td>5’-GCCTACGAGCTCAGAAGACGATATGAACTGACACC-3’</td>
</tr>
<tr>
<td>8. pComb-RNF38 TEV overlap NNK Re</td>
<td>5’-GCTGACGCTGAGGTGTTTT-3’</td>
</tr>
<tr>
<td>9. pComb-RNF38-TEV overlap NNK Fw</td>
<td>5’-CAGTCGAAACAGACTGATATGATATTCGCGAGGACGATTTGAGTCAAGGAGCAGCTAC-3’</td>
</tr>
<tr>
<td>10. pComb-RNF38-TEV ClaI down</td>
<td>5’-GGAAGTATCGATTCCCTAATCTCCCGATGACTCTCCTG-3’</td>
</tr>
</tbody>
</table>
BARD1, MDMX, Rbx1, cIAP2, Birc7, and Cbl-b from the E3s aligned. Similarly, many RING E3s have nonpolar residues such as M or L immediately before and after the CXXC moiety. Mutations of C418 and D419 would be thought to be exclusive in charge reversal as C418 interacts directly with K4 on N-terminal helix of UbcH5B (refer to Figure 1.5 A). RNF38 RING AAAA-mutant ligation to pComb vector and transformation into XL1-blue cells could have been more efficient if the digestion of pComb-wt-RNF38 RING would have yielded more cut pComb vector for the ligation. But the total transformation of colonies for pComb-RNF38 RING AAAA-mutant could barely be counted as the cells were beginning to grow over one another. Therefore, more than 10-fold difference could be speculated between control and 1:3 ligations. Colony PCR for the library mutants yield only the ~500 bp fragment similar to the wt-RNF38 RING colony PCR. Library titer and sequence analysis for RNF38 RING 4-mutant library do provide evidence sequence coverage and all four sites well randomized before starting on library selection.

3.6 Experimental Procedures

3.6.1 Alanine mutant screen

RNF38 RING AAAA mutant was amplified from pComb-wt-RNF38 RING in two fragments. The “up fragment” was prepared in 50 μL reaction with 100 ng pComb-wt-RNF38 RING, 0.5 μM pComb-TEV-RNF38 up primer (5’-TCCTGCCCGGACTAAAGCAGATATT-3’), 0.5 μM pComb-TEV-RNF38 overlap down primer (5’-AGTCTGTTCGACTGTTGTTAGGATTG-3’), 2X DreamTaq Green PCR Master Mix, and remainder with diH2O. “Up fragment” reaction was amplified in thermocycler under following conditions: 95°C for 2:00, (95°C for 30 s, annealing at 52°C for 30 s, extending at 72°C for 45 s for 35 cycles), 72°C for 4:00 min, and holding @ 4°C. The “down fragment” was prepared in a 50 μL reaction with 0.5 μM pComb-TEV-RNF38 overlap up AAAA primer (5’-
pComb-TEV-RNF38 down primer (5'-CGTGACTAGTTTCTGAATCCCGATG-3’) and amplified under similar conditions as the up fragment. Fragments were run in 1% agarose gel and DNA was purified following Qiagen DNA Extraction protocol.

Overlap PCR was prepared in a 100 μL reaction with 300 ng “up fragment”, 300 ng “down fragment”, 0.5 μM pComb-TEV-RNF38 up primer, 0.5 μM pComb-TEV-RNF38 down primer, 2X DreamTaq Green PCR Master Mix, and remainder with diH₂O. RNF38 RING AAAA mutant overlap PCR was amplified, verified, purified, and quantified under similar conditions previously stated.

Digestion of pComb-wt-RNF38 RING was prepared in 50 μL reaction with 200 ng pComb-wt-RNF38 RING, 1 unit of SacII, 1 of unit SpeI, and 1X Cutsmart Buffer and remaining volume with diH₂O. Digestion of RNF38 RING AAAA mutant was prepared in 200 μL reaction with 1 μg RNF38 RING AAAA-mutant, 1 unit of RE SacII, 1 unit of RE SpeI, 1X Cutsmart Buffer, and remaining volume with diH₂O. Digestion reactions for pComb-wt-RNF38 RING and RNF38 RING AAAA-mutant are incubated in 37°C incubator for 5 hours and overnight, respectively. Digestions are verified in 1% agarose gel, excised and purified following Qiagen DNA excision protocols.

Ligation of pComb vector: RNF38 RING AAAA-mutant in 20 μL reactions with 50 ng SacII/ SpeI digested pComb vector, 150 ng SacII/ SpeI digested RNF38 RING AAAA-mutant, 1X T4 ligase buffer, 1 unit of T4 ligase, and remaining volume with diH₂O. Vector-only control was set up similarly without insert. Reactions were incubated overnight at 25°C. Transformation via electroporation occurred with 1 μL ligation reaction pComb-RNF38 RING AAAA-mutant into 50μL XL1-blue cells, then cells were immediately treated with 1 mL SOC media incubating
in 37°C shaker for 1 hour. 300 μL of transformed cells are plated onto 2% glucose and 100 μg/mL ampicillin plates incubating overnight in 37°C incubator. Transformation of ligation control with SacII/ SpeI digested pComb was prepared similarly. Plasmid purification for each isolate colony followed QIAGEN Miniprep Purification protocol. Each purified pDNA was quantified with Nanodrop spectrophotometer before diluted to 40-50 ng/μL with 0.5 μM primer, Jun13 or Jun14 and sent to Genewiz for Sanger Sequence analysis.

3.6.2 4-mutant library construction

The 4-mutant library “up fragment” was prepared in 50 μL reactions with 100 ng pComb-RNF38 RING AAAA mutant as a template, 0.5 nM pComb-RNF38 SacI up primer (5’-GCCTACGAGCTCATAAAGCAGATGAAACAAC-3’), 0.5 nM pComb-RNF38-TEV-overlap NNK Re primer (5’-GTCTGTGACTGTTGGTGGTTAGG-3’), 2X DreamTaq Green PCR Master Mix (Thermo Fischer), and remainder with diH2O. Reactions for “down fragment” was prepared similarly, but with 0.5 μM pComb-RNF38-TEV overlap NNK Fw primer (5’-CAGTCAGAACAGACTNNKTGTGTAGTATGCGNNKNNKNTTTGAGTCAAGGCACG-3’), 0.5 μM pComb-RNF38-TEV ClaI down primer (5’-GGAAGTATCGATTTCTGAAATCCGATGCACCTCTG-3’). Both amplified under similar conditions as the up fragment.

Overlap PCR was prepared in a 100 μL reaction with 300 ng “up fragment”, 300 ng “down fragment”, 0.5 nM pComb-TEV-RNF38 SacI up primer, 0.5 nM pComb-TEV-RNF38 ClaI down primer, 2X DreamTaq Green PCR Master Mix, and remainder with diH2O. RNF38 RING 4-mutant library overlap PCR was amplified, verified, purified, and quantified under similar conditions previously stated.
Digestion of pComb-7G12 was prepared in a 200 μL reaction with 26 μg pComb-7G12, 1 unit of SacI, 1 of unit ClaI, and 1X Cutsmart Buffer and remaining volume with diH₂O. Digestion of RNF38 RING 4-mutant library was prepared in 200 μL reaction with 60 μg RNF38 RING 4-mutant library, 3 unit of SacI, 3 unit of ClaI, 1X Cutsmart Buffer, and remaining volume with diH₂O. Digestion reactions for pComb-7G12 and RNF38 RING 4-mutant library are incubated in 37°C incubator for 4 hours and overnight, respectively. Digestions are verified in 1% agarose gel, excised and purified following Qiagen DNA excision protocols.

Ligation of 1: 10 pComb vector: RNF38 RING 4-mutant library in 10 μL reactions with 50 ng SacI/ ClaI digested pComb vector, 500 ng SacI/ ClaI digested RNF38 RING 4-mutant library, 1X T4 ligase buffer, 1 unit of T4 ligase, and remaining volume with diH₂O. Vector-only control was set up similarly without insert. Reactions were incubated overnight at 25°C. Transformation via electroporation occurred with 1 μL ligation reaction pComb-RNF38 RING 4-mutant library into 50μL XL1-blue cells, then cells were immediately treated with 1 mL SOC media incubating in 37°C shaker for 1 hour. 300 μL of transformed cells are plated onto 2% glucose and 100 μg/ mL ampicillin plates incubating overnight in 37°C incubator.

Transformation of ligation control with SacI/ ClaI digested pComb was prepared similarly. Isolate colonies were selected for plasmid purification following QIAGEN miniprep purification protocol.

Large-scale ligation was set up in 1 mL reaction with 2.8 μg SacI/ ClaI digested pComb vector, 22 μg SacI/ ClaI digested RNF38 RING 4-mutant library, 1X T4 ligase Buffer, 20 units of T4 ligase, and remaining volume with diH₂O. Ligation reaction was incubated for 15 hours at 25°C. Ligation is concentrated to 20 μL following the phenol-chloroform ethanol precipitation protocol. Transformation for large-scale ligation was prepared similarly using commercial XL1-
blue cells. Serial dilution in 10-fold decrease from 50 μL from SOC treated XL1-blue cells. Dilutions were plated onto 2% glucose, 100 μg/mL ampicillin plates with 5 μL serial dilutions. Plasmid purification for each isolate colony followed QIAGEN Miniprep Purification protocol. Each purified pDNA was quantified with Nanodrop spectrophotometer before diluted to 40 nM with 0.5 nM Jun13 or Jun14. Colony PCRs were prepared in 20 μL reactions under similar conditions as RNF38 RING model phage selection protocol.
4 Phage Display and Selection of RNF38 RING 4-mutant Library

4.1 Introduction

The library will be displayed onto the N-terminal end of pIII protein from M-13 phage. Selection of the library using a pseudo-OUT pathway to enrich potential sequences after multiple rounds. E3-phage library interacts with charge reversed N-terminal helix (K4E and K8E) of xE2, xUbch5B, to rule out unreactive sequences for RING domain. bE1 will be used for interaction with wt-UB-Bio for efficient selections of the current library. Sequences which have undergone convergence will then be displayed onto pIII proteins of M13 phage to be tested for activity within the OUT pathway. Active sequences termed xRNF38 RING will be inserted into FL-

![Figure 4.1 Library selection scheme and phage display](image)

(A) Immunoblot of 1\textsuperscript{st} (left) and 2\textsuperscript{nd} (right) RNF38 RING 4-mutant library with anti-FLAG 1\textsuperscript{o} Ab exposed for 30 sec. Phage displayed RNF38 RING 4-mutant library is fused to pIII protein to yield ~30 kDa band. (B) Library selection scheme for autoubiquitination of phage displayed E3 RING domain library with N-terminal FLAG tag. bE1 (UFD domain is mutated) interacts with wt-Bio-UB similar to wt-E1 but interacts with xE2 only. xE2 will then transfer Bio-UB to its catalytic Cys residue before interacting with active sequences from phage-displayed E3 RING domain library. Active phage-displayed E3 RINGs will undergo autoubiquitination to a lysine residue within the E3 RING domain. Bio-UB will be pulled down onto streptavidin-coated plates then washed after binding to remove unbound phage. TEV cleavage by TEV protease occurs to free bound phage to infect XL1-blue cells for to generate the selected sequences for the new library.
RNF38 to be expressed in HEK293 cell line to test activity of FL-xRNF38 within the OUT pathway.

**Figure 4.2 Library selection with phage titers.**

(A) Input phage for each round of library selection. Final phage concentration for each selection is $1 \times 10^{11}$ pfus for effective phage-displayed E3 RING library to interact with xE2. (B) Library selection titers represented in a bar graph. The “+” reactions are usually at least 10-fold higher compared to three negative controls. (C) Library selection titers represented in numerical table. The positive “+” show almost ~100-fold increase in pfu compared to three negative controls. The 4th library shows a 10-fold increase in activity compared to the other libraries which may lead to decrease overall E3 RING-phage selection.
4.2 Selection of 4-mutant library

Native RNF38 RING 4-mutant library displays on pIII protein of M-13 phage before selecting out active sequences through a pseudo OUT cascade (Figure 4.1 A). Each library-phage is displayed to verify successful fusion of E3 RING library to pIII before selection of the library can occur. The pseudo OUT cascade is a hybrid system of wt-UB cascade and OUT cascade to select out xE2-xE3 pair from a library of E3 RING domain mutants (Figure 4.1 B). The selection scheme uses bE1 to interact with wt-Bio-UB with wt-AD and xE2 with mutant UFD (refer to Figure 1.7). wt-Bio-UB is transferred to catalytic Cys from xE2 then through the xE2 binding interface to select for active phage-displayed E3 RING sequences are polyubiquitinated. Polyubiquitinated E3 RING sequences are pulled down with streptavidin and washed to remove unbound E3-phage. Bound E3-phage undergo TEV cleavage to release the phage to infect XL1-blue cells before selection titers and purifying the new selected library.

Each library-phage is quantified by a phage titer before selection of the library for keeping the phage input of 1 x 10^{11} pfus for every selection to ensure many E3 RING library is displayed on the phage for streptavidin pull-down (Figure 4.2 A). After XL1-blue cells are infected, selection titers are monitored for each library selection and compared with three negative controls (Figure 4.2 B & C). The selection titers representation as a bar graph helps visually monitor the efficiency where the “+” reaction is roughly 10-100-fold higher in pfu compared to the three negative controls: “-bE1”, “-xE2”, and “-UB”. Selections leading to the 4th library show a slight 9-fold increase in pfus while monitoring “-bE1” control. Each library after selection undergoes double digestion with REs, SacII and ClaI, on pComb-RNF38 RING 4-mutant library from purified isolates before sequence analysis (Figure 4.3 A). Digestion results leading up to the 4th library show significant decrease in the amount of RNF38 RING 4-mutant
library. Therefore, the 4th library undergoes a technique known as sub-cloning to clean up the 4th library before continuing in the selection process.

![Diagram of library selection process]

**Figure 4.3 Selection library verification**

(A) Scheme for double digestion of purified pComb-RNF38 RING 4-mutant library isolates after library selection. XL1-blue cells infected with the selected library are grown on selective media to monitor selection titers. Isolate colonies from the positive reaction grown in selective media have pDNA purified then digested with two restriction enzymes (REs), SacII and ClaI. Library quality is measured from the number of digested fragments and isolates with the correct size fragment are sent for sequence analysis. (B) Double digestion with REs, SacII and ClaI, on 10 isolates from the 4th library selection showing 4 of 10 isolates with the RNF38 RING 4-mutant library-pIII conjugate with ~500 bp band (top). A contaminant with similar RE sites is observed by the ~1700 bp band and the digested pComb vector with a ~3000 bp band. The 4th library undergoes sub cloning to improve library quality for the next selection round. Double digestion for 10 isolates from the 5th library selection shows 9 of 10 isolates with ~500 bp band consistent with RNF38 RING 4-mutant library (bottom). Two isolates have the contaminant with a ~1700 bp band and all isolates have the digested pComb vector at ~3000 bp band.
4.3 4th library sub cloning

The 4\textsuperscript{th} RNF38 RING 4-mutant library (4\textsuperscript{th} library) shows decreasing quality of the library after verifying ten isolates with double digestion (Figure 4.3 B). A sub cloning for the 4\textsuperscript{th} library is used to improve the library by double digestion with REs, SacI and ClaI, and ligated into SacI/ ClaI digested pComb vector. A ligation with 1:5 ratio of digested pComb vector to 4\textsuperscript{th} library and vector only control yield ~660 cfu and 1 cfu, respectively (data not shown). Double digestion with REs SacII/ ClaI confirm successful ligation of the 4\textsuperscript{th} library as all ten isolates show ~3.0 kb band consistent with digested pComb vector and ~500 bp band consistent with 4\textsuperscript{th}

Table 4.1 Sequence convergence for the 5th library.
Sequence analysis from several isolates of the 5\textsuperscript{th} library shows convergence of sequences of the four mutation sites. Several isolates from the 5\textsuperscript{th} library selection are compared to wt-RNF38 RING at positions: 412, 417, 418, and 419. Letters represent amino acid residues where converged positions are highlighted in the following: non-polar (gray), basic (blue), small chain and polar (yellow), and long chain and polar (green). Met417 is converged to Leu residues, Cys418 has converged to Thr/ Ser or Arg/Lys residues, and Asp419 shows some convergence with Arg/His/Lys or Asn/Gln residues.
library-pIII conjugate (data not shown). The sub cloned 4th library then undergoes selection for the 5th library yielding very little pfus from negative control (Figure 4.3 A). Double digestion with REs, SacII and ClaI, for ten isolates results result in 9 of 10 isolates with ~500 bp band for 5th library-pIII conjugate (Figure 4.3 B).

4.4 Sequence Analysis for the 5th library

Selection for the 5th library shows ~100-fold difference between “-bE1” control and almost ~1000-fold difference between “-xE2” and “-UB” controls with relatively pure library from several isolates digested with REs, SacII and ClaI. Sequences analysis for digested isolates

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<th>4S-5-21 (LLTR)</th>
<th>4S-5-28 (KLSQ)</th>
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Figure 4.4 Loop stability affects activity in pseudo-OUT cascade

Autoubiquitination of three xRNF38 RING mutants with differing residues for L412 mutation but other three residues are relatively similar. FLAG-xRNF38 RING-pIII is ~35 kDa band in IB: FLAG at 1 min exposure with lanes left to right: +, -bE1, -xE2, and -HA-UB. Polyubiquitin chain is shown for “+” reaction only when compared to negative controls. L412G from isolate 4S-4-3 shows some activity, L412 from isolate 4S-5-21 show highest activity, and L412K from isolate 4S-5-28 does not show activity.
reveal a convergence within three positions from RNF38 RING domain: M417, C418, and D419 (Table 4.1). Met417 has converged to Leu residue to resemble characteristics of methionine. Interestingly, Cys418 has converged to similar residues such as threonine and serine, but also arginine and lysine with a longer chain. Lys/Arg residues can electrostatically interact with K4E or K8E on N-terminal helix of xUbcH5B. Asp419 did not show as much of a convergence, but Arg/Lys and Asn/Gln seem to reoccur. Theoretically, D419R/K would present an electrostatic interaction with K8E; however, the amide group of D419N/Q would potentially form a hydrogen bond with K8E. Isolates are selected based on retaining stability for the Zn$^{2+}$ interacting loop, potential electrostatic or hydrogen bond formation with mutations on N-terminal helix of xE2.

4.5 Activity of xRNF38 RING in OUT cascade

Isolates 4S-4-3 (GLTK), 4S-5-21 (LLTR), and 4S-5-28 (KLSQ) are selected to determine if loop stability is necessary to form an interaction with xE2 within a pseudo-OUT cascade (Figure 4.4). The three isolates have similar residue mutations for M417L, C418S/T, and D419K/R/Q, which can help determine Isolate 4S-4-3 uses L412G mutation to introduce a flexible residue to allow for loop flexibility, but according to the data xRNF38 only has slight autoubiquitination after interacting with xUbcH5B. Isolate 4S-5-21 retains L412 and shows highest activity through polyubiquitin chain formation. Isolate 4S-5-28 has L412K mutation changing the polarity which may affect activity as no ubiquitin chains formed. These three isolates are tested in vitro in the OUT cascade to obtain further evidence if loop stability affect xE2-xRNF38 RING interaction.

4.6 Discussion

bE1, bUba1, is used for selection to allow wt-UB-Bio to be transferred to RNF38 RING 4-mutant library-phage as the sequences interact with xE2. Autoubiquitination of RNF38 RING
4-mutant library-phage with UB-Bio will be pulled out with streptavidin plates, and to ensure selections are efficient the “+” reaction is compared to three negative controls. “-bE1” reaction is important to compare the “+” reaction because wt-UB-Bio cannot be transferred if there is no bE1 to activate UB with ATP to allow for reactive thiol from bUba1 to transfer UB-Bio to xE2.

There is a strong possibility that RNF38 RING AAAA-mutant-phage could have contaminated the 2nd and 3rd libraries due to the abundance of the sequences with this genotype. The contaminant with ~1700 bp band is not known since non-RNF38 RING sequences did not have a sequence analysis with anything over 1200 bp, therefore, the source is still unknown. Nevertheless, sub cloning the 4th library is necessary to continue the selection of the library, and the sub cloning is very successful since the ligation ratio 1:5 control: 4th library is more than 99% 4th library. The digestion for the sub cloned pComb-4th library-phage result in 100% 4th library fragment further proving minimal contamination. Sequence analysis for the sub cloned 4th library-phage also shows no sequences with AAAA-mutant because pComb-RNF38 RING AAAA-mutant is digested with SacII/ ClaI and cannot be fully digested since SacI RE site is not present within the sequence.

Selection after sub cloning has proven very effective only after fifth trial for 5th library as the selection titer is ~17-fold higher compared to the selection titer for the 4th library. The fourth trial did not yield any significance between any of the reactions due to lack of growth from old ampicillin plates and the same plates are tested with the 5th library-phage from fourth trial selection producing similar results for lack of growth. Hence a fifth trial for the selection of the 5th library provides the best results, but isolate colonies from the fourth trial provide some evidence for convergence of sequences.
4.7 Experimental Procedures

4.7.1 Phage display of RNF38 RING 4-mutant library

Phage display for library mutants was similar prepared similarly to wt-RNF38 RING phage display except for the use of commercial XL1-blue cells in transformation. Phage titers and western blots for library phage display was prepared similarly.

4.7.2 RNF38 RING library selection

Selection assay for RNF38 RING libraries was conducted in 250 μL reactions with bE1, bUba1 (1 μM), xUbcH5B (10 μM), RNF38 RING 4-mutant-library-phage (1 x 10¹¹ pfus), UB-Bio (0.2 μM), ATP pH 7.6 (5 μM), 1 X Tris HCl / MgCl₂, 0.1 mM DTT, and remaining volume with diH₂O. Three controls: -E1, -E2, and -UB are also conducted to serve as negative controls in 50 μL reactions. Reactions were incubated for 2 hr in 37°C shaker then transferred to streptavidin-coated wells in 1:1 reaction: 3% BSA in 1X PBS. Wells were incubated for 1 hr on orbital shaker at room temperature before being washed 20X with 1X PBS Buffer. Wells were incubated for 45- 60 min in 30°C with 100 μL TEV solutions of 1X ProTEV Buffer (1M HEPES pH 7.0 and 10 mM EDTA), DTT (0.1 mM), 1 u/μL ProTEV Protease (50 mM Tris HCl (pH 7.5), 500 mM NaCl, 5 mM DTT, 1 mM EDTA, 50% glycerol, and 0.1% Triton X-100), and remaining volume with diH₂O. Cleaved phage were incubated for 1 hr in 37°C shaker in XL1-blue cells (OD₆₀₀ ~0.7). Serial dilution for each reaction was prepared similarly as phage titer. Isolate colonies were prepared similarly to RNF38 RING AAAA-mutant. Digestion of the isolates were prepared in 20 μL reactions with 10 μL digestion master mix (2 u/μL SacII, 1 u/ μL ClaI, 1X Cutsmart Buffer, and remaining with diH₂O) and 10 μL purified pDNA incubating for 1 hr in 37°C incubator. Reactions were verified under similar conditions as digestion of RNF38 RING 4-mutant library with SacI/ ClaI.
4.7.3 4th Library Digestion, Ligation, and Transformation

Digestion, ligation, and transformation of the 4th RNF38 RING 4-mutant library was prepared similarly to RNF38 RING 4-mutant library, but with a 1:5 ligation of vector: insert. Large scale ligation was prepared similarly to RNF38 RING 4-mutant library.

4.7.4 Selection conditions for 6th library

Selection assay for the 6th library was prepared similarly except for the decrease in total 5th RNF38 RING 4-mutant library-phage concentration was decreased to 5 x 10⁹ pfus and increase of DTT to 0.2 mM.

4.7.5 Ubiquitination Assay in OUT cascade

Autoubiquitination for xRNF38 RING mutants within the pseudo-OUT cascade was prepared in 25 μL reactions with bE1, bUba1 (1 μM), xE2, xUbcH5B (10 μM), xE3-phage, xRNF38 RING mutant-phage (1x10¹¹ pfus), HA-UB (40 μM), ATP pH 7.6 (5 μM), 1 X HEPES / MgCl₂, 0.1 mM DTT, and remaining volume with diH₂O. Three controls: -bE1, -xE2, and -UB are also conducted to serve as negative controls for auto-ubiquitination. Reactions were incubated for 20 h in 37°C shaker and stopped with 2X Lamelli with BME loading dye. Reactions were run following similar conditions as the western blot for wt-RNF38 phage with 1:1500 α-GST 1° Ab and 1:10000 α-mouse-HRP conjugate 2° Ab.

Autoubiquitination for xRNF38 RING mutants within the OUT cascade was prepared similarly as the pseudo-OUT cascade except with xE1, xUba1 (1 μM) and xUB-HA (20 μM). The western blot was also prepared similarly as wt-RNF38 phage.
5 CONCLUSION and FUTURE WORK

wt-RNF38 RING domain shows activity in autoubiquitination by interacting with UbcH5B through the E2-binding domain, and shows substrate, p53, ubiquitination in vitro through the recognition of p53 in the substrate binding domain. Phage-displayed RNF38 RING shows autoubiquitination activity without interference from pIII protein on M-13 phage. RNF38 RING 4-mutant library is successfully generated with 10-fold higher cfus to cover all possible sequences and ligated to the pComb phagemid. Sequence convergence for 3 out of 4 key residues, M417L, C418S/T or C418R/K, and D419R/K are achieved for the xE2-xE3 pair after four rounds of selection. The Zn$^{2+}$ interacting loop on xRNF38 RING mutants, the xE2-xE3 interface, is important for xUbcH5B recognition before autoubiquitination of xE3 occurs.

Substrate ubiquitination with p53 xRNF38 RING mutants needs to be confirmed before testing activity of FL-xRNF38. xRNF38 RING needs to be inserted into FL-RNF38 to determine activity in vivo in HEK293T cells. Ubiquitination of substrates in vivo in HEK293T cells after tandem purification using HBT-UB (Hexahistidine-Biotin-UB) using both Ni-NTA column purification followed by streptavidin column purification will then undergo trypsin digestion. Proteomics for ubiquitinated substrates will by LC/MS determines potential substrates for xRNF38 to help understand the function of this RING-type E3 in the cell.
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