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Development of *in vitro* Sensitive FRET Technology to Dissect Reaction Dynamics and Intermediates in NEDD8 Conjugation Cascade

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by

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March 2014

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ABSTRACT OF THE DISSERTATION

Development of *in vitro* Sensitive FRET Technology to Dissect Reaction Dynamics and Intermediates in NEDD8 Conjugation Cascade

by

Harbani Kaur Malik Chaudhry

Doctor of Philosophy, Graduate Program in Bioengineering
University of California, Riverside, March 2014
Dr. Jiayu Liao, Chairperson

Reversible post-translational modifications are widely used to dynamically regulate protein activities *in vivo*. The most well known example of polypeptide modifiers is ubiquitin and ubiquitin like proteins (Ubl). Ubiquitin and Ubiquitin like proteins (Ubls), such as NEDD8 and SUMO, play critical roles in various physiological and pathological processes, ranging from signal transduction, cell cycle to tumorgenesis. NEDD8 specifically is known regulate many critical targets, which play crucial role in cellular processes such as, DNA transcription and repair, signal transduction and cell cycle regulation. And misregulation of NEDD8 cascade is observed in various cancers and neurodegenerative disorders.

The Ubls are conjugated to their respective substrates by similar but distinct multi enzyme cascades that involve sequential actions of the E1 activating enzymes, E2 conjugating enzymes and E3 ligases, requiring multiple protein-protein interactions between NEDD8 and NEDD8's E1, E2, E3 enzymes and NEDD8 substrates. Förster resonance energy transfer (FRET) technology has been widely used in biological and biomedical research and is a valuable tool for elucidating molecular interactions *in vitro*

and *in vivo*. Quantitative FRET analysis is a powerful method for determining biochemical parameters and molecular distances at nanometer levels. The goal of my research is to develop FRET based protein assays to dissect the mechanisms of NEDDylation cascade and develop a HTS platform to identify bioactive chemical compounds, which can inhibit the activity of NEDD8 pathway. Using peptide linker-engineering strategy, I developed a high sensitive fluorescent-tagged NEDD8 for a trans-FRET assay, which allowed us to follow the covalent conjugation of NEDD8 to its E2 ligase in the presence of E1 and ATP, which was difficult to determine without linker. The understanding of the covalent attachment of NEDD8 to target proteins and its kinetics of conjugation cascade will provide greater insights into this process in normal and pathological conditions, such as cancers. To understand the system of NEDDylation cascade, I develop a series of novel and quantitative FRET-based assays to dissect reaction intermediates and dynamics of NEDD8 to its E1 activating enzyme and its E2 conjugating enzyme. We were able to follow the thioester intermediates of NEDD8 with its E1 and E2 for the first time.

The Ubls are conjugated to their respective substrates by similar but distinct multi enzyme cascades that involve sequential actions of the E1 activating enzymes, E2 conjugating enzymes and E3 ligases. Although all the Ubls are conjugated to their substrates through a similar enzymatic cascade, the requirement for either single or heterodimer of E1 activating enzymes in various Ubl conjugation cascade is still a mystery. Here, by employing our high sensitive engineered trans-FRET assay we report a significant discovery of the requirements of each subunit of E1 heterodimer for NEDD8 activation. We, for the first time, systematically dissect the NEDD8 activation

step in real time by employing our new quantitative FRET technology. These studies show that APPBP1 (a subunit of NEDD8 E1 heterodimer) is not absolutely required for the NEDD8 activation. These findings provide not only detail molecular mechanisms of Ubl activation, but also potential evolution process at molecular level. I further employed by linker-engineering strategy for engineering Cullin (NEDD8's well studied target protein) tagged with YPet, to aid in development of FRET based HTS platform for drug discovery. I was able to successfully engineer YPet-Cullin1 with linker, to drastically improve fluorescence emission. In future this engineered YPet-Cullin1 will be employed for development of FRET based HTS platform for drug discovery for NEDD8 conjugation cascade.

Table of Contents

List of Figures

corresponding to the sequence and structure of MoeB. The E1 of Ubiquitin and ISG15 are single polypeptides. In case of heterodimeric E1s (SUMO and NEDD8 cascade), one subunit corresponds to N terminal half of the single-chain E1s and other subunit of the heterodimer corresponds to C-terminal of half. The N and C-terminal halves of the E1s for ubiquitin, SUMO, NEDD8 and ISG15 are partially homologous to each other, and the region of sequence homology between the two halves is also the region of sequence homology to MoeB and ThiF.

Thermodynamic cycle for calculation of electrostatic free energy of association and solvation. The iso-potential contours illustrated are the spatial distribution of electrostatic potential. Blue and red surfaces represent iso-values of $\pm 1 k_B T/e$, respectively, where k_B is the Boltzmann constant, T is temperature, and e is the unit charge of an electron. The proteins A, B, and AB in the figure are APPBP1, UBA3, and APPBP1-UBA3 complex respectively. The top process models the reference state (dielectric coefficient of 20 for both proteins in interior and exterior and no counter ions) and the bottom process models the association step in a solvated state with dielectric coefficient of 20 for protein interior, 78.54 for protein exterior, and 150mM concentration of counter ions. The three vertical processes show solvation of each free protein and complex.

A)Alanine scan electrostatic clustering and free energies of association of APPBP1 and UBA3. Clustering dendrogram of the alanine scan mutants of APPBP1 using the average

weighted difference ESD. Free energy of mutants ordered according to average weighted difference clustering. B)Alanine scan electrostatic clustering and free energies of association of UBA3 and NEDD8. Clustering dendrogram of the alanine scan mutants of UBA3 using the average weighted difference ESD. Free energy of mutants ordered according to average weighted difference clustering.

Figure 29. p.112

NEDD8 modification is required to activate Cullin-RING ubiquitin ligases (CRLs). Protein degradation by the UPS is a highly regulated process. Proteasomes degrade proteins that are tagged with poly-ubiquitin chains. The formation of the poly-ubiquitin chain on a protein is catalyzed by a pyramidal cascade of enzymes; a single E1-activating enzyme activates Ub (UAE), transfers it via a transthiolation reaction to one of dozens of E2 enzymes that in turn collaborate with specific E3 ligases to catalyze the formation of a poly-ubiquitin chain on a substrate protein recruited by that E3. E3 ligases can be subdivided into three broad subclasses; HECT, U-box, and RING-finger domain E3s with CRLs representing the largest subfamily of RING-finger E3s. A key feature of CRLs is that the Cullin subunit must be modified on a conserved lysine by the ubiquitin-like protein NEDD8 to activate holoenzyme activity. NEDD8 activation and conjugation to Cullin proteins is catalyzed via an enzymatic cascade that is homologous to ubiquitination involving NEDD8's E1 (NAE) and E2 (Ubc12). Removal of NEDD8 from Cullin is catalyzed by the COP9 signalosome. Deneddylation facilitates dissociation of CRL components. Binding to CAND1 sequesters the Cullin-RING core in an inactive state until it is recruited to form a new CRL.

List of Tables

List of Abbreviations

PTM: Post-translational modifications

Ub: Ubiquitin

Ubl: Ubiquitin-like modifiers

NEDD8: Neural precursor cell Expressed Developmentally Down-regulated 8

SUMO: Small Ubiquitin-like modifier

ISG15: IFN-stimulated gene 15

FAT10: F-Antigen Associated Transcript 10

CRL: Cullin-ring ligases

RING: Really interesting new gene

FRET: Förster resonance energy transfer

HTS: High- throughput screening

SPR: Surface plasmon resonance

ITC: Isothermal titration calorimetry

SD: Standard deviation

Chapter 1: Introduction

1.1 Post-translational modification

Inside the cell, information is transcribed from DNA to RNA and then further translated into proteins. A major mechanism for regulating the protein and its function is through posttranslational modifications. Post-translational modifications (PTM) are covalent modifications, including small molecule, such as phosphorylation, acetylation, methylation, carboxylation, etc. and protein modification such as Ubiquitin and Ubiquitin like modifiers (Table.1). Cells respond very rapidly to their internal and external environment. Post-translational modification of proteins is one of the sensitive, rapid and reversible mechanisms to respond to environmental stimuli or internal change in cell state (Figure.1). Cells uses PTMs as means to modulate the function of proteins by regulating their activity, sub cellular localization, stability and interaction with other proteins and as it is a reversible mechanism that aids target protein's participation in multiple rounds of functional pathways. The diversity and functionality of cellular proteome can be greatly expanded by covalent post-translational modifications.

Table1. Types of Post-translational Modifications (PTM).

Peptide	Small Molecules
Ubiquitin	Phosphorylation
NEDD8	Glycosylation
SUMO	Acetylation
ISG15	Carboxylation
FAT10	Alkylation

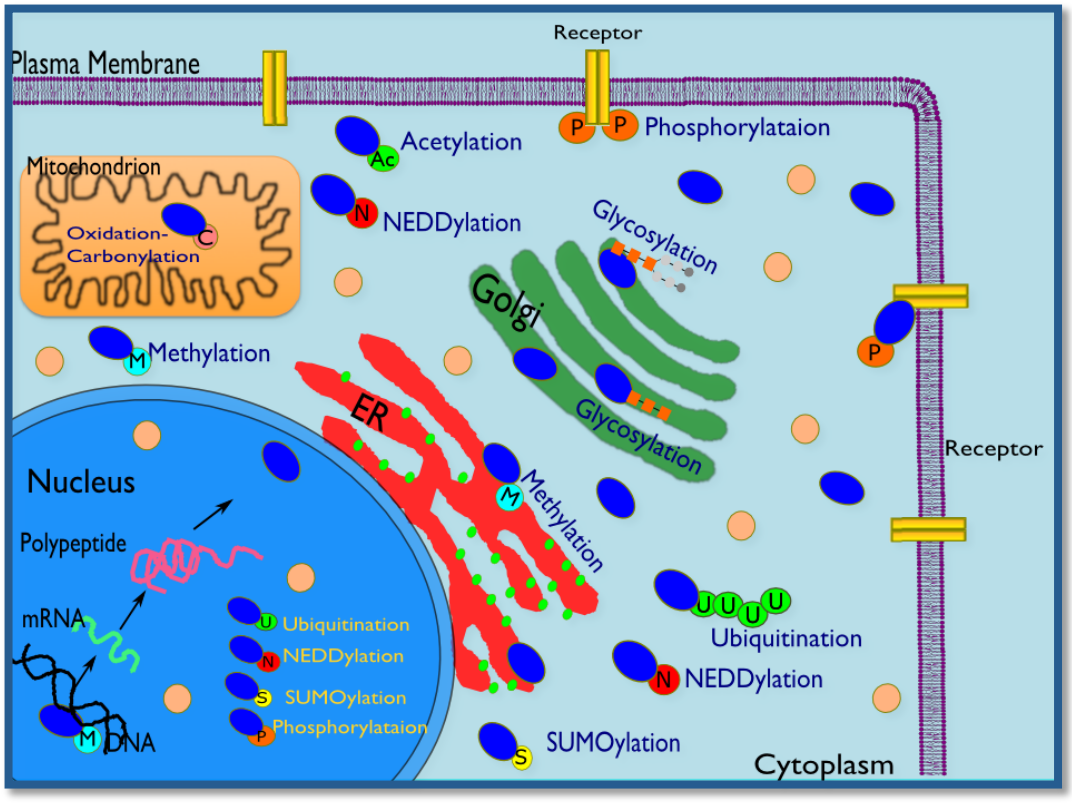


Figure 1. Various Post-translational Modifications in different cellular compartments.

1.2 Ubiquitin and Ubiquitin-like modifiers

The 2004 Nobel Prize in Chemistry was awarded to Avram Hershko, Aaron Ciechanover and Irwin Rose for the discovery of the functions of a small (76-residue), ubiquitous, eukaryotic cellular protein that was given the name ubiquitin[9]. The first protein characterized as a PTM modifier, was a 76 amino acid protein, Ubiquitin (Ub)[4,10]. As compared to the other small molecule modifiers such phosphoryl and methyl groups, Ubiquitin and Ubiquitin like modifiers provide much larger and more chemically varied surface. Thus these covalent modifiers can act as flexible adaptor modules for altering substrate's conformation or protein-protein interactions. Ubiquitination process attaches Ubiquitin molecules to proteins usually to signal proteosomal degradation. Targeting of various proteins by different Ubls can lead to different protein fate, cellular localization, degradation, stabilization, conformation change, change in enzymatic activity or protein-protein interaction. Ubiquitin and Ubiquitin like protein modification are involved in DNA replication and repair, transcription, signal transduction, endocytosis and sorting, cell cycle regulation and development, metabolism regulation, protein quality control (Figure.2). Posttranslational modification by ubiquitin and ubiquitin like protein family has surfaced as an important cellular mechanism to regulate various cellular processes.

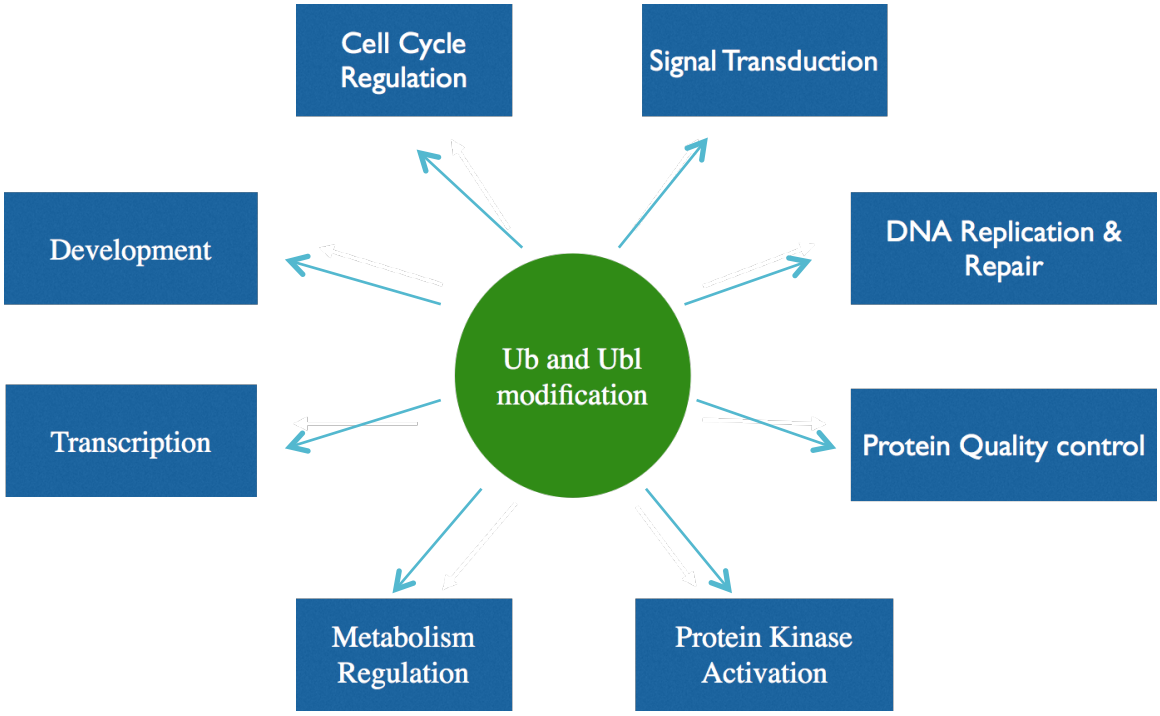


Figure 2. Importance of Ubiquitin and Ubiquitin like modifiers conjugation[3,4] Ubiquitin and Ubiquitin-like modification play crucial roles in various cellular processes.

There are 17 known ubiquitin-like modifiers (UBLs), NEDD8, SUMO, ISG15, FUB1, FAT10, Atg8, Atg12, Urm1, and UFM1 that have been identified to conjugate to its target proteins in a similar fashion to ubiquitin[4,9,10]. Ubls adopt similar structures consisting of two regions: (a) a globular domain comprising a four-stranded mixed β-sheet and an α-helix, and (b) a flexible C-terminal tail terminating with a glycine[11] (Figure.3). Ubiquitin and Ubiquitin like modifiers conjugate to various target proteins through a conserved enzymatic pathway with their own specific enzymes. As there are different and critical consequences of target proteins after their Ubl conjugation, thus Ubl conjugation to their specific targets through the coordinating enzymatic cascade is very important. It is critical to understand how different Ubl's, shuffling through their

respective conserved enzymatic pathway, conjugate to the targets. Different Ubls are conjugated to their respective targets by a conserved mechanism but distinct cascades of enzymes, which sequentially involve, activating enzyme: E1, conjugation enzyme: E2 and ligases: E3[10].

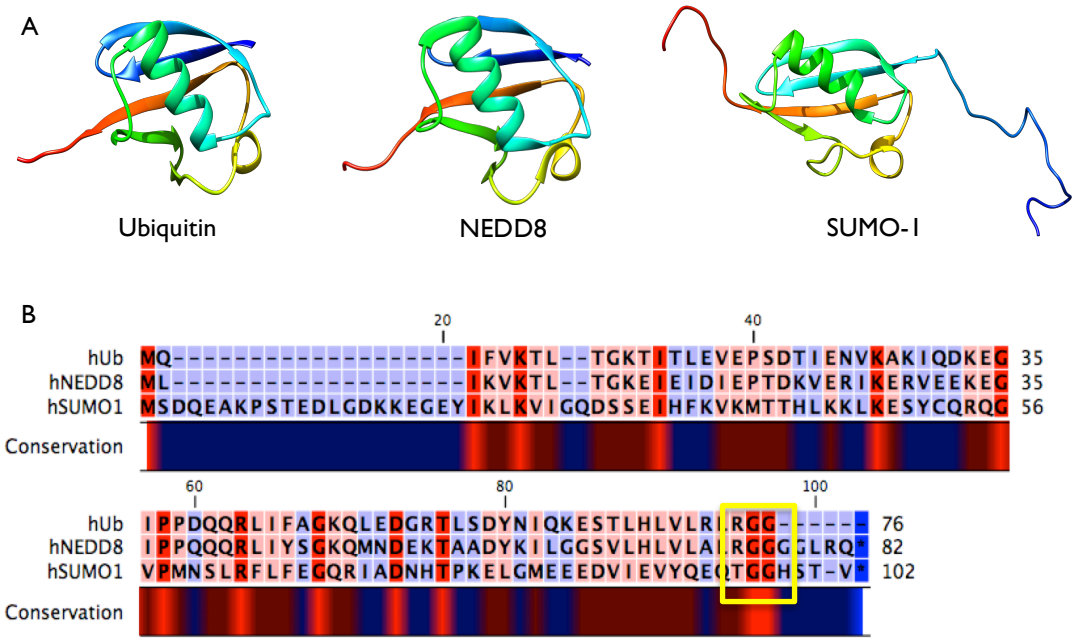


Figure 3. Ubiquitin and Ubiquitin like modifiers. (A) 3-D structures of Ubiquitin (1UBQ)[6] , NEDD8 (2KO3)[7] and SUMO1(1A5R)[8], showcasing Ubiquitin homology of a globular domain comprising a four-stranded mixed β-sheet and an α-helix (B) Protein sequence similarity between Ubiquitin, NEDD8 and SUMO1. Conserved residues are highlighted in red. Conserved di-glycine motif is indicated in yellow box.

1.3 NEDD8 and NEDD8 Cascade

In 1992, Kumar et all identified using subtractive cloning screening between a cDNA library derived from mouse neural precursor cells and mouse adult mRNA identified ten Neural precursor cell-Expressed Developmentally Down regulated

(NEDD) genes[12,13], one of these genes encoded protein (NEDD8)[4,5,14]. NEDD8 is a ~9KDa polypeptide (81 amino acid), which is 60% identical to ubiquitin sequence and 80% homologous to ubiquitin (Figure.3). From the tissue specific expression studies, NEDD8 message is highly enriched in the heart and skeletal muscle but much lower in all other tissues. NEDD8 message was seen highest in the day 11-mouse embryo and was observed to decrease on day 15 and 17 embryos[14]. Immunocytochemical analysis showed that NEDD8 expression was highly enriched in the nucleus and was much weaker in cytosol. In comparison, Ubiquitin expression was detectable equally in nucleus and cytosol[14].

Analogous to Ubiquitination, the NEDDylation pathway consists of E1, E2, E3 and deneddylating enzymes (Figure.4). The process is dynamic and reversible in nature[15-17]. The linkage between the NEDD8 and substrates is an isopeptide bond between the C-terminal carboxyl group of NEDD8 and the ε-amino group of lysine residue in substrate (similar as in ubiquitinylation). The pathway includes enzymes reactions that attach the NEDD8 to a specific substrate and then other peptidases cleave of the NEDD8 from the substrate. In case of NEDD8, NEDD8 is translated from mRNA as a large precursor protein. Pre-NEDD8 is recognized and processed by peptidases called Sentrin-specific proteases (SENPs) to remove its C-terminal extension, exposing a glycine-glycine motif in the mature NEDD8 to make it available for conjugation with the lysine side chains in the target proteins. NEDD8 pathway begins with a NEDD8-activation enzyme (called E1), which catalyzes an ATP-dependent activation of the NEDD8. NEDD8 E1 is a heterodimer: its subunits APPBP1 and Uba3. NEDD8 activation

by E1 can be divided into three steps, (i) the C-terminal carboxyl group of NEDD8 attacks the ATP forming a NEDD8-adenylate intermediate and releasing pyrophosphate, (ii) thiol group of the active site cysteine in the E1 attacks the NEDD8-adenylate intermediate releasing AMP and forming a high energy thioester intermediate and (iii) activated NEDD8 is transferred to cysteine of conjugating enzyme E2 (Ubc12). The transfer of activated NEDD8 from E1 to E2 result in the NEDD8-E2 intermediate, which can serve as donor in the final reaction of the NEDDylation of the target. The third step of the pathway includes the NEDD8 ligases (E3), which do not form covalent intermediates with SUMO; they bring E2-NEDD8 intermediate and substrate together. Different forms of protein-protein interactions take place inside the cell, non-covalent interactions, and covalent-bond formation, leading to different reactions and outcomes.

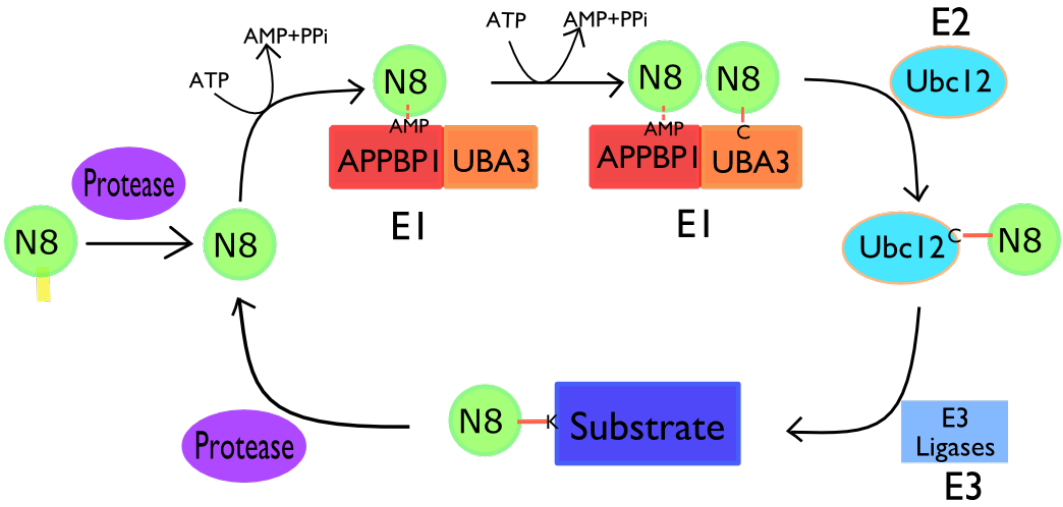


Figure 4. Schematic of NEDD8 enzymatic conjugation cascade. The pre-NEDD8's C-terminal tail is processed by action of peptidase, to produce mature NEDD8. The mature NEDD8 is then activated by heterodimer E1ligase, APPBP1-UBA3, by first adenylation of the C-terminal glycine followed by thioester linkage formation between NEDD8's glycine and UBA3's catalytic cysteine. The activated NEDD8 in then transferred to Ubc12,E2, in transthiolation reaction. With the help of E3 ligases, such as Rbx1, NEDD8 is conjugated to its substrate's lysine residues through an iso-peptide linkage.

1.4 NEDD8 targets and Biological Roles of NEDD8

An intact NEDD8 pathway is required for viability in *S. pombe, D. melanogaster*, *C.elegans* and mice[18]. A general elevation in the level of NEDD8 conjugation has been observed in oral squamous cell carcinoma cell lines where NEDD8 pathway inhibition decreased cell proliferation[19]. The components of Cullin-RING ligases (CRLs) are overexpressed, amplified or mutated in several human cancers, and many CRLs regulate the activity of numerous players in tumorgenesis[20](Figure.5). NEDD8 is expressed in proliferating cells and down regulated during cellular differentiation[21].

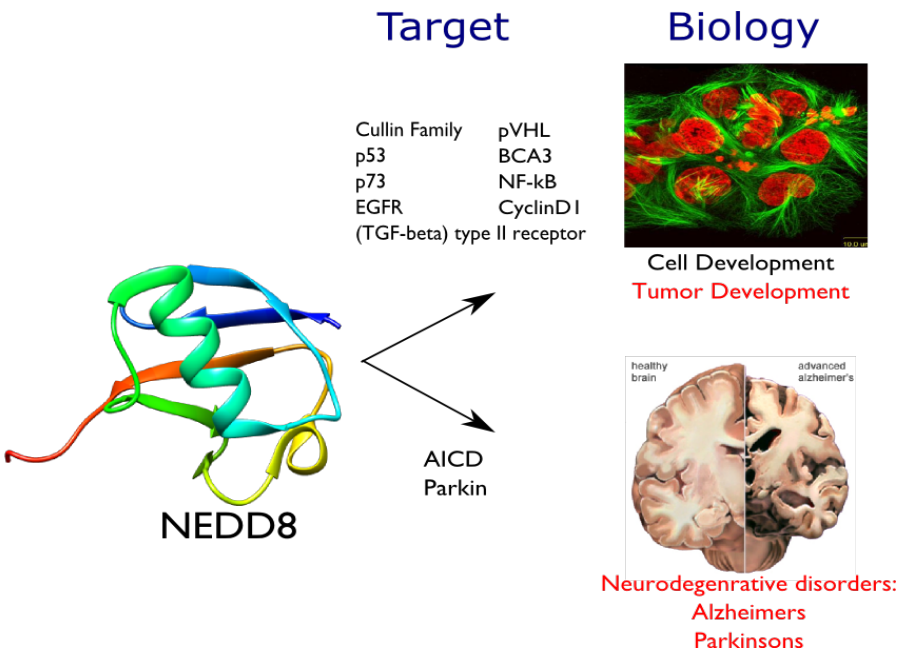


Figure 5. NEDD8 enzymatic cascade and diseases including cancer, neurodegenerative disorders associated with misregulation of NEDD8 cascade[2,4].

The first identified targets of NEDD8 were cdc53 in *S. cerevisiae*[15,22] and cul4a in human cells[23]. Both are members of the Cullin family of proteins—which has between three and six members in all eukaryotes from yeast to humans—suggesting that

Neddylation is an important mechanism for regulating Cullin function[5]. Indeed, all yeast and mammalian cullins—*S. cerevisiae*: cdc53, cul3 and rtt101; *S. pombe*: pcu1, pcu3 and pcu4; and mammalian: cul1, cul2, cul3, cul4a, cul4B and cul5—are neddylated on a conserved lysine in their c-terminal domain in vivo[17]. Cullins function as scaffolds for the assembly of multi-subunit ubiquitin E3s. They interact tightly with a ring-domain protein— Rbx1 or Rbx2 (for SCF) to enable the recruitment of ubiquitin E2s. In case of Cullin-1 (Cul1), NEDD8 conjugation to Lysine 720 residue in Cul1 by Rbx1 prevents association of Cul1 with the inhibitory protein CAND1 (Cullin-associated and Neddylation dissociated-1), thus maintain SCF activity for ubiquitin mediated proteolysis[5,10,18,24]. Several newly discovered NEDD8 substrates are established tumor suppressors or oncoproteins, including Cullin (1,2,3,4a-b, 5,7), VHL, p53, BCA3, p73, EGFR, PARC and MDM2[4,5,17,25] (Figure.5). MDM2 is E3 that mediates the conjugation of NEDD8 to p53, resulting in inhibition of transcriptional activity. Similar effects were observed for NEDD8 E3 ligase, FBOX11[5,10].

The most well-studies target of NEDD8, Cullin family, plays many critical roles in cellular processes, cell cycle, signal transduction, transcription, protein quality control, DNA replication, viral modulation, development, circadian clock[26]. Cullin Ring Ligases (CRLs), through controlling ubiquitination of various targets, controls the fate of many proteins, including p27 and CyclinE (cell cycle), Myc and pIkBa (transcription), Cdt-1 and E2F (DNA replication), b-catenin and AXR2/3 (signal transduction) , CD4(viral modulation)[26]. Cullins function as scaffolds for the assembly of multi-subunit ubiquitin E3s. It has been elucidated the mechanistic importance of Cullin NEDDylation to

enhance CRL ubiquitination[27]. One important aspect is that NEDDylation of the Cullin NEDDylation has been shown to result in conformational rearrangements in the CRL complex structure[28-31]. These structural rearrangements are important for CRL activity, providing proper geometric alignment of enzymatic component of CRL[30]. The presence of NEDD8 is also known to eliminate the CAND1 (CRL inhibitor) binding site from CRL[30].

1.5 Therapeutic Rational of potential of NEDD8 pathway inhibition

In recent study of NEDD8 pathway inhibition, it was estimated that CRs are responsible for ubiquitinating ~20% of all intracellular proteins that are marked for proteosomal degradation[32]. Furthermore a number of these proteins have been identified as having involvement with cancer[27]. A general elevation of NEDD8 conjugation has been observed in several carcinoma cells lines, where NEDD8 cascade inhibition decreased cell proliferation. The components of CRLS are overexpressed, amplified or mutated in several human cancers; also CRLS regulate the activity of many proteins, which play critical role in tumorgenesis[4,25,33]. Also, in humans, accumulating evidence shows that NEDD8 is overexpressed in neurodegenerative disorders[34,35].

For example, deregulation of p27 expression occurs in various cancer (prostate cancer, lung cancer, colorectal carcinoma) and levels of p27 are regulated by CRL1-Skp2. Also Skp2 overexpression is observed in various cancers. Also Cullin4a and 4b have shown ubiquitination of p27 independent of SCF-Skp2 in tumors. Another example is constitutive signaling of NF-kB in cancer cells. This activity is because of up regulation

of IKK activity, which makes the NF-kB inhibitor IkBa, a substrate of CRL mediated degradation[27,32]. Therefore, inhibition of NEDD8 cascade, which directly regulated CRL activity, will be able to aid in down regulating tumor cell[33].

Thus, through modulating CRLs, neddylation regulates many biological processes, including cell cycle progression, signal transduction and tumorigenesis. As from studies it is know that NEDD8 is overexpressed and CRLs are abnormally activated in many human cancers, targeting protein neddylation in general and cullin neddylation in particular appears to be an attractive anti-cancer approach. MLN4924, a small molecule inhibitor of NAE, was discovered[32] that inactivates CRLs[36] and causes accumulation of CRL substrates to suppress tumor cell growth both in vitro and in vivo[33]. Promising preclinical results advanced MLN4924 to several clinical trials for anticancer therapy. In preclinical settings, MLN4924 effectively suppresses tumor cell growth by inducing apoptosis, senescence, and autophagy, and causes sensitization to chemo-radiation therapies in a cellular context dependent manner. Signal molecules that determine the cell fate upon MLN4924 treatment, however, remain elusive. However, cancer cells develop MLN4924 resistance by selecting target mutations[2]. Therefore, we aim to develop a FRET based HTS platform for drug screening for NEDD8 cascade, to identify potential candidates for NEDD8 cascade inhibitors.

1.6 Förster Resonance Energy Transfer (FRET)

Cell is a very dynamic entity; molecular activities are ongoing in different subcellular domains or compartments of a cell. Different cells in the same tissue can

behave/exist differently. Understanding these subcellular molecular dynamics in quantitative manner is of extreme importance and in need for the field of systems biology. The identification and characterization of protein interactions is a key topic in current life science research; a huge variety of methodologies have been established in recent years to expedite research in this area.

In our studies, we are interested in deciphering various types of interactions and reaction intermediates to elucidate the Ubiquitin and Ubiquitin like modifiers enzymatic cascade's mechanisms. In the field of biotechnology, methodologies employed to study biochemical and cellular pathways are classified into three major catergories: Biochemical methods, genetics methods and biophysical methods. Under biochemical methods come the gold standards, Radiolabeling, Co-immunoprecipitation (Co-Ip), mass spectrometry, under genetic methods come, Yeast two-hybrid, mammalian two-hybrid, phage display, under biophysical methods come, Isothermal titration Calorimetry, Surface Plasmon Resonance, Förster Resonance Energy Transfer, BRET. Great technologies such as Mass spectrometry, Magnetic Resonance Imaging, are for monitoring ions, metabolites, however these methodologies have limited temporal and spatial resolution and are unable to provide local concentrations, intermediate formation, protein-protein interactions and flux rates. Classical biochemical methods, such as western blot and radiolabeling, do provide some basic information about the enzymatic reaction, but mainly gives information about the end point of a specific reaction and fails to elucidate the kinetics of a protein cascade[37,38]. Fluorescent ligands and proteins have revolutionized the field of cell biology imaging and are widely used for

addressing cell biological questions at the systems biology level. Fluorescent tags and proteins have been used in various biosensors for determining the signaling intermediates and metabolites in real time with subcellular localization[39]. Fluorescent tags and proteins have been used in various biosensors for determining the signaling intermediates and metabolites in real time with subcellular localization[40]. Genetically encoded fluorophores offer tools, which can be applied to probe molecular behavior *in vitro* and *in vivo*. Furthermore, genetically fused fluorophores, generally do not affect the structure of the protein of interest, as compared to the chemical conjugation of the fluorescent tags. Fluorescent protein tags have been employed in various different types of biosensors, ranging from studying the protein-protein interaction, cellular signals and assay metabolite response *in vitro* and *in vivo*[37-42].

Electronic excitation energy can be efficiently transferred between a fluorescent energy donor and a suitable energy accepter over distances as large as 70 Å. In 1948, Förster proposed a theory of this dipole-dipole energy transfer process which postulated that the rate of transfer depends on the transfer process which postulated that the rate of transfer depends on the inverse sixth power of the distance between the donor and acceptor[43]. This predicted distance dependence was verified by fluorescence studies of donor-acceptor pairs by know distance in well-defined model systems[44].

Energy transfer as defined by Vladimirov and Konev[45], namely "the radiation less transmission of an energy quantum from its site of absorption to the site of its utilization in the molecule, or system of molecules, the distances involved being considerably

greater that interatomic, without conversion to thermal energy of vibration, and without the donor and acceptor, between which the energy is transferred, coming in to kinetic collision". Fluorescence resonance energy transfer (FRET) is an electrodynamic phenomenon than can be explained using classical physics. FRET is a non-radiative process (Figure.6) whereby an excited state donor D (usually a fluorophore) transfers energy to a proximal ground state acceptor A through long-range dipole–dipole interactions[43,46]. FRET usually occurs over distances comparable to the dimensions of most biological macromolecules, that is, about 10 to 100Å. When the donor (D) and acceptor (A) are close to each other (2 to 10nm) with favorable orientations, the excitation of the donor can elicit an energy transfer to induce emission from the acceptor, results in quenching of donor and excitation of acceptor.

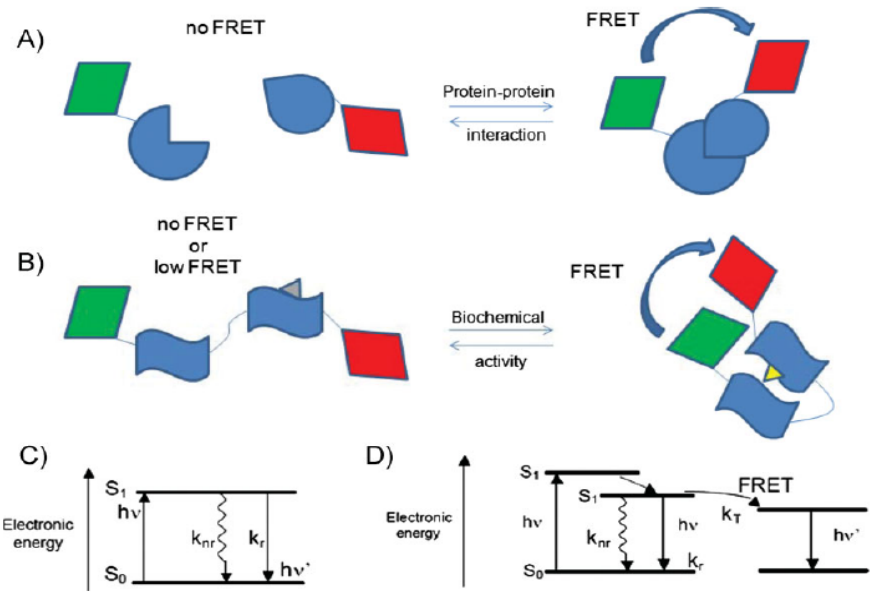


Figure 6. Basic principles of FRET. (A) The cartoon describes two proteins diffusing independently, one tagged with a green Fluorescent Protein (donor) and the other tagged with a red Fluorescent Protein (acceptor). When interaction occurs the distance and orientation of the tagged proteins allow FRET to occur. (B) A protein with two subunits is tagged with a green FP (donor) and a red FP (acceptor). When a conformational change occurs both donor and acceptor are close enough for FRET to happen. (C) Modified Perrin-Jablonski diagrams showing photonic processes: after initial excitation (~$10^{15 s-1}$), fluorescence occurs as a result of a photon being emitted during the decay of an excited electron from S1 to the ground level (S0) with a particular radiative kinetic constant (k_r ~$10^{9\ s-1}$). Note that other non-radiative phenomena for relaxation are also possible (k_{nr} ~ $10^{9\ s-1}$). (D) The process of FRET is described in this diagram; the process of energy transfer is indicated with a horizontal arrow (kT). In this case there is competition between three path-ways of relaxation: energy transfer (kT ~$10^{9\ s-1}$), fluorescence (k_r ~$10^{9\ s-1}$) and non- radiative phenomena (k_{nr} ~$10^{9\ s-1}$)[1,5].

Efficient energy transfer requires that the energy donor and acceptor be in resonance, which means that the fluorescence emission spectrum of the donor must overlap the absorption spectrum of the acceptor (Figure.7). The acceptor must absorb energy at the emission wavelength(s) of the donor, but does not necessarily have to remit the energy fluorescently itself (i.e. dark quenching). Energy transfer occurs without the appearance of a photon and is the result of long-range dipole-dipole

interactions between the donor and acceptor. The rate of energy transfer is highly dependent on many factors, such as the extent of spectral overlap, the relative orientation of the transition dipoles, and, most importantly, the distance between the donor and acceptor molecules[43,45,46].

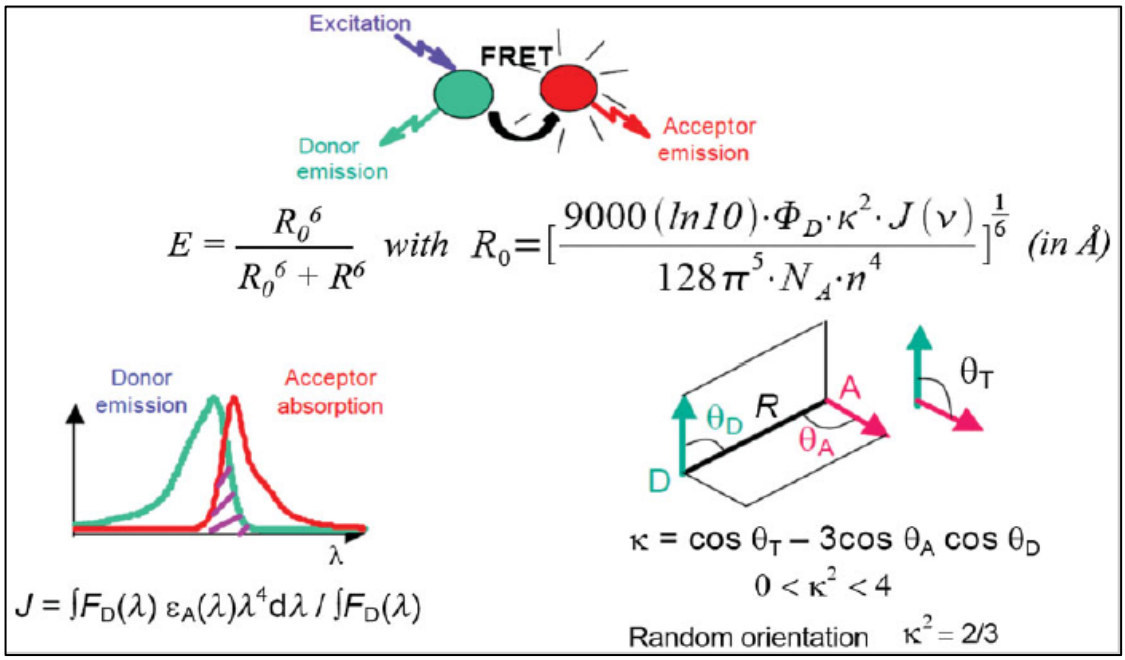


Figure.7 Basic Principles of FRET[1,2].

FRET efficiency can be calculated by using[47],

$E_{FRET} = 1/[1 + (r/R_o)^6]$ (Equation 1)

Förster Rate equation,

$k = 1/\tau_d * (R_o/r)^6$ (Equation 2)

$R_o^6 = 8.785 * 10^{-5} * (\kappa^2\phi_D J/ n^4)$ (Equation 3)

$J = \int F_d(\lambda)\,\varepsilon_A(\lambda)\lambda^4\, d\lambda$ (Equation 4)

Where τ_d is the donor lifetime in the absence of acceptor; **r** is the donor acceptor distance; R_o is the Förster or critical transfer distance at which the energy transfer rate is equal to the decay rate or the distance at which the transfer efficiency is 50%; κ^2 is the orientation factor between donor and acceptor; ϕ_D is the quantum yield of the donor in the absence of the acceptor; **n** is the index of refraction (generally taken to be 1.4); **J** is the overlap integral between donor and acceptor; F_d is the peak-normalized fluorescence spectrum of donor; ε_A is the molar absorption coefficient of the acceptor .

Structural and dynamic information of macromolecular assemblies is clearly important for understanding their functions. Dynamic interactions between proteins are thought to have key role in regulating most cellular signal transduction pathways. To study these interactions we need a platform, which elucidate interactions between these proteins. Resonance energy transfer as measured by steady state and time resolved fluorescence can provide important information n this area and therefore is an important phenomenon used by analytical biochemist and bioengineers[47]. FRET can overcome limitations which are posed by other techniques, like biochemical methods cannot detect weak or transient interactions, immunofluorescence microscopy cannot determine interaction at the protein scale (few nm as compare to several hundred nm), electron microscopy lacks the ability of precise labeling, western blot or radiolabel assays can not measure protein kinetics in real time, FRET is well suited to study of protein interactions in real time, which occur on a similar spatial scale 2-10 nm. FRET provides information about distances on the order of 10 to 100 A° and the efficiency of energy transfer is highly dependent (sixth-power) on the distance between donor and

acceptor and is thus suitable for investigating spatial relationships of interest in biochemistry. FRET-based techniques is convenient for studies of biological macromolecules, and has been extensively used in biological research including studying protein conformational change, identification of protein interactions, real-time monitoring of intracellular signaling activities, and high-throughput screening of bioactive molecules.

Ubiquitin and ubiquitin like protein family are responsible for various post-translational modifications (PTM), resulting in regulating cell cycle progression and development. Ubiquitin and UBLs undergo a dynamic enzymatic cascade in order to get conjugated to its target protein. Ubls shuffle through activation enzyme, conjugation enzyme and finally with aid of ligase enzyme is covalently linked to the substrate[3,9,17,48-50]. Different forms of interactions occur in cell, such as non-covalent interactions (in which two or more proteins come together form a complex) and covalent bond formations (such as phosphorylation of proteins, thioester linkages, isopeptide bonds). Understanding and differentiating between these different interactions are important, as it elucidate the mechanism of enzymatic/signaling cascade. Biosensors to understand and detect these different interactions are key for the field of systems biology and pharmokinetics. Ubiquitin and Ubiquitin like proteins undergo a dynamic multi-enzymatic cascade, involving different type of interactions (non-covalent and covalent). Ubiquitin and Ubiquitin related proteins perform post-translational modifications on various targets, which are critical for various physiological processes, such as transcription,

protein-DNA binding, protein-protein interactions, DNA repair, transcription regulation and genome organization[3,51-53].

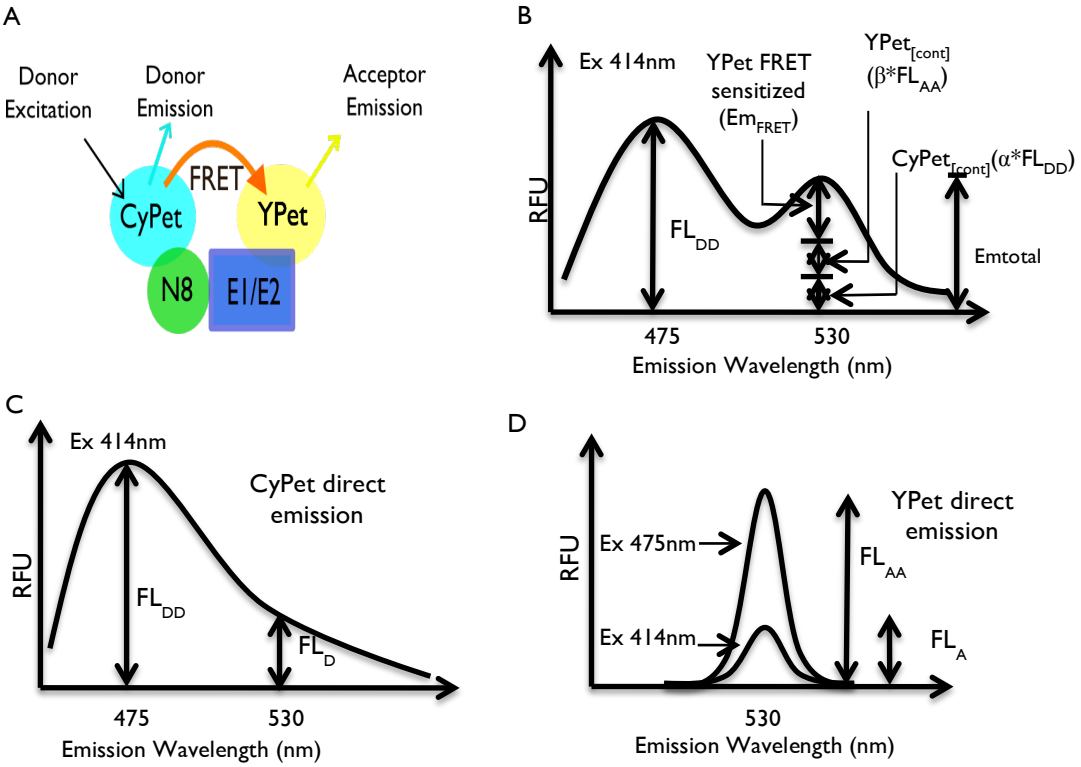
Researchers, in past, have used traditional biochemistry methodologies, such as western blot, radiolabeling or immuno-pull down assay, for determining various protein-protein interactions in Ubiquitin and Ubl proteins enzymatic cascade[16,54-56] and to understand the dynamics enzymatic cascade and they were successful in giving the basic information regarding the end point results of a reaction or to identify enzymes/protein targets in Ubiquitin, SUMO or NEDD8 cascades[54,57-62]. Researchers have used crystal structures to elucidate the biochemical and structural basis of Ub and Ubl proteins with their respective E1 and E2 enzyme[54,63-65]. FRET has been used as spectroscopic research tool in the field of biology, biotechnology and biophysics. A full detail understanding of specific cascade requires robust assay that can easily and quantitatively gauge the kinetics and dynamics of the various interactions undergoing in a reaction. Fluorescence has exceptional sensitivity for detecting low concentration of particles over broad spatial and temporal dimensions. FRET is applied as tool to determine molecular distances or to demonstrate the presence or absence of molecular complex[39-42].

There are several distinctive aspects regarding resonance energy transfer studies, a) Measurement of energy transfer is based on the fluorescence detection, thus ensuring high sensitivity, b) Structural information can be obtained at low resolution, regardless of the complexity or heterogeneity of the system, c) since time scale of resonance energy transfer is on the order of nanoseconds, many processes that time-averaged can be resolved[43,44,47,66].

There are a couple of research groups, who have developed FRET based assays for Ubiquitin and Ubiquitin like modifiers proteins[29,67-73]. Such as Boisclair *et.al* group which developed a FRET based inhibitor screening assay for Ubiquitin transfer from its E2 enzyme to HECT proteins[72], but their assays only reports the transfer of Ubiquitin from its E2 to E3 proteins. Also they use indirect labeling of the fluorophores for the FRET assay, which might lead to large molecular complex formation, resulting in incompetent FRET, because of the large distance between donor and acceptor fluorophores. Another group developed a colorimetric spectrophotometric assay for conjugation of Ubiquitin proteins[71], in which they quantify Ubiquitin conjugation by monitoring pyrophosphate released in the first enzymatic step of Ubiquitin transfer, which is the ATP dependent activation of Ubiquitin by its E1. This method is rapid and radiolabeling free, but this method is an indirect method to understand the Ubiquitination cascade and also it fails to monitor the dynamics of the Ubiquitin activation and conjugation steps in real time manner. Another group established a FRET based system for understanding the effect of NEDD8 conjugation on Cullin Ring ligases for Ubiquitination of target proteins[29]. They employ FRET based method and western blot methods to demonstrate the NEDD8 conjugation on Cullin Ring Ligases results in improved ubiquitination of target protein, by proposing that NEDD8 induced a structural change in Cullin Ring Ligase.

1.7 Quantitative FRET signal analysis by cross-wavelength emission and excitation method

In order to evaluate FRET signal quantitatively, our group recently developed a new spectrum analysis method to determine FRET signal based on cross-wavelength analysis of donor and acceptor[74]. In our mathematical model, the fluorescence signal at 530 nm emission (Em_{total}), when excited at 414 nm, includes three components: donor's direct emission ($CyPet_{[cont]}$), acceptor's direct emission ($YPet_{[cont]}$), and FRET sensitized YPet emission (Em_{FRET})(Figure.8A). The donor's (CyPet) direct emission at 530 nm is proportional to their emission at 475 nm, when excited at 414 nm, Fl_{DD}, by a constant ratio α (Figure.8B). The acceptor's (YPet) direct emission at 530 nm when excited at 414nm is proportional to its emission at 530 nm (Figure.8C), when excited at 475 nm, Fl_{AA}, by a constant ratio β (Figure.8D). The α and β, correlation coefficient values are 0.33 and 0.026 for our CyPet and YPet fusion proteins, respectively. Thus, we can determine the FRET-sensitized signal (Em_{FRET}) by subtracting $CyPet_{[cont]}$ and $YPet_{[cont]}$ from the Em_{total}. As Em_{FRET} is the FRET-sensitized YPet emission at 530 nm, Em_{FRET} can be used to quantify the FRET signal. When we mix the proteins involved in the NEDDylation process and initiate the NEDDylation pathway, the formation of protein complexes will result in an increase of Em_{FRET}. This absolute Em_{FRET} signal is dependent on the molecular events of protein-protein interactions.



E

$$\alpha = FL_{DA}/FL_{DD} \quad \beta = FL_{AD}/FL_{AA}$$

$$Em_{FRET} = (Em_{total})-(\alpha * FL_{DD})-(\beta * FL_{AA})$$

Figure 8. Design of FRET assay and FRET spectrum analysis. (A) CyPet-tagged SUMO1 interaction YPet-tagged SUMO ligases and substrate. (B) Dissection of emission spectra from a mixture of CyPet tagged and YPet tagged protein mixture. Fluorescent emission at acceptor, YPet, wavelength (530 nm; FL_{DA}) can be divided into three sections: FRET emission from YPet, direct emission of donor, CyPet, and direct emission of acceptor, YPet. (C) Donor emission at 475 nm (FL_{DD}) and 530 nm (FL_{DA}) when excited at 414 nm. (D) Acceptor emissions at 530 nm when excited at 414 nm (FL_{AD}) or 475 nm (FL_{AA}) E) Definition of Em_{FRET}.

In past, by employing our FRET based protein assay, we were able to investigate and understand the intermediates dynamics in SUMOylation cascade[75] and Ubiquitination cascade[76] in detail in real time. We can clearly differentiate between covalent conjugation and noncovalent complex formation between various proteins of Ubiquitin, SUMO and NEDD8[75-77]. We were able to shed light upon the SUMOylation cascade dynamics in terms of conformation changes and interactive partner switches that occur during the adenylation and thioester bond formation. We are the first group, which was able to study the Ubl-ligase thioester intermediate and dynamics transfer in real time. The quantitative estimation of different affinities for the transient complex in the conjugation cascade is hard to determine using approaches like western blot or radiolabeling, as the thioester bonds are high-energy bonds thus chemically unstable and have short half-life. Previously researchers have employed X-ray crystals approach for multi protein complex using non-hydrolysable adenylates mimics but these studies are incapable of revealing intermediates and real time dynamics of multi-enzyme cascade. We were able to extend our FRET based technology to Ubiquitin[76] and NEDD8[77] to understand if all the Ubiquitin and Ubiquitin like proteins have similar or different enzymatic reaction dynamics and affinities. More importantly, our studies demonstrate that our highly sensitive and quantitative FRET assay is a powerful tool for revealing specific transient molecular interactions and reaction intermediates involving cascades of multiple enzymatic reactions. My work pertains to develop FRET based protein assay to elucidate the NEDD8 enzymatic cascade and develop HTS assay for screening small chemicals as inhibitors for NEED8 cascade.

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Chapter 2: Development of linker strategy for sensitive trans-FRET assay to determine activation intermediate of NEDD8 conjugation cascade

2.1 Introduction

Ubiquitin and ubiquitin-like (Ubl) modifiers, such as NEDD8 (Neural precursor cell-Expressed Developmentally Down regulated gene 8), are conjugated to various target proteins through a conserved enzymatic pathway with their distinct specific enzymes[1,2]. Targeting these proteins with different Ubls affects the protein fate, such as cellular localizations, degradation, stabilization, conformation changes, changes in enzymatic activity or protein-protein interactions[3-7]. It is important to understand how different Ubls conjugate to the targets shuffling through their respective conserved enzymatic pathway.

10 NEDD genes were initially discovered through subtractive cloning screening between cDNA libraries derived from mouse neural precursor cells and mouse adult mRNA by Kumar group[8]. One of these genes encoded protein, NEDD8, which is 60% identical and 80% homologous to ubiquitin. The NEDD8 conjugation cascade involves E1, E2, E3 and the deneddylating enzymes (as mentioned in chapter1). Like ubiquitin, NEDD8 is translated as a precursor and processed by specific proteases at the conserved C-terminal Gly-76 to expose a conserved di-glycine motif. After the maturation step, the exposed C-terminal is adenylated by the E1 (NEDD8-activating enzyme (NAE)), a heterodimer of APPBP1 and UBA3, in an ATP-dependent reaction and transferred to catalytic cysteine of UBA3 via thioester linkage. Subsequently,

activated NEDD8 is transferred to the catalytic cysteine of NEDD8-conjugating enzyme (E2), Ubc12, forming thioester linkage. With the aid of E3 ligase, NEDD8 is transferred from E2 enzyme and conjugated to a conserved lysine residue of the target protein via an isopeptide linkage. NEDDylation is dynamic and reversible process in cells. Specific proteases, such as COP9 and NEDP1, have been shown to deconjugate NEDD8 from substrates[4].

NEDDylation plays important roles in several cellular processes, cell-cycle progression and regulation, the immune system through its critical role in mediating the ubiquitination by modulating Cullin-ring ligases of various targets. Deregulation of NEDD8-regulated CRLs' target is often associated with cancer. These targets include the cell-cycle regulators (Cyclin E, p27, c-Myc), transcription factors (NRF2, HIF-1α, c-Jun,), inhibitors of transcription (pIκBα), and regulation of DNA replication (Cdt-1)[3,9,10]. To elucidate molecular mechanisms underlining Ubl conjugation processes, FRET technology is very suitable for such complex events.

Genetic engineering has become an indispensable component for generating recombinant fusion proteins By genetically combining two or more genes together, the resulting fusion protein has improved bio-functionality and is multi-functional, due to the combination of individual component moieties. Fusion proteins have wide applications in biological research such as protein purification, imaging, protein assays and also biopharmaceuticals. For example, fusion proteins have been used to develop protease assays, using His-tag to improve the protein purification and many protein drugs are fused to Fc domains of antibodies or to carrier proteins. Fusion protein

engineering is also used for drug targeting and drug delivery. With the rapid advancement in the field of biotechnology, fusion protein technology will have increasing importance in generating novel proteins with improved functioning and performance for different applications[11].

The synthesis of a bioactive and functional recombinant fusion protein is difficult and requires both protein domains to be functional after fusion. For example, fusing two protein domains (at the genetic level) might result in improper folding of the protein or loss of domain function because of non-specific interaction between the domains or low protein production. In order to solve this problem, linker engineering (i.e., small peptide insertion) is employed to separate the domains. Researchers have applied linker-engineering technology, derived from naturally occurring multi-domain functional proteins, which are joined by linkers, for various applications to synthesize a functional protein system. These linker peptides serve to connect the protein moieties and also provide many other functions such as maintaining cooperative inter-domain interactions or preserving biological activity[11].

FRET (Förster Resonance Energy Transfer) is a phenomena in which energy is nonradiatively transferred upon electron excitation energy between two fluorophores, donor and acceptor, and have overlapped emission spectrum of donor and excitation spectrum of acceptor and are within a distance of 1-10nm. Because FRET can identify molecular interactive events *in vitro* and *in vivo*, FRET-based techniques have been extensively used in biological research in various settings[12-15]. The quantitative relationship between FRET efficiency and distance of the two fluorophores established

the use of FRET as a spectroscopic ruler to determine bio-molecular distance[16-19]. Additionally, the FRET signal is also correlated to the amount of donor-acceptor complexes and this quantitative relationship of FRET signal can be used to determine molecular interaction events quantitatively, such as protein interaction dissociation constant, K_d. Previous quantitative FRET analysis for the protein interaction affinity K_d in cells depended on determining FRET efficiency by a "three-cube" FRET analysis method[20-24]. This method has several assumptions and variations. No study directly links K_d determinations *in vitro* and *in vivo*. Our recent discovery of the highly sensitive engineered FRET pair, CyPet and YPet, led to novel FRET-based methodologies to determine the K_d and the protease kinetics parameter, K_{cat}/K_M, SUMO-enzyme thioester intermediates, and high-throughput screening (HTS) assays in SUMOylation *in vitro*[25-31].

In an effort to expand our technology to other Ubl pathways, we first adapted our method of FRET assays to reveal molecular events of NEDDylation. However, the fluorescent fusion protein, CyPet-NEDD8 generated according to our previous strategy of direct fusion of two proteins, had very low fluorescent signal and could not generate obvious FRET signals in our NEDDylation studies. We then attempted to develop protein-engineering method, linker strategy, to improve the fluorescent signal of fusion protein. Peptide linkers, which occur in native proteins or are engineered, function to fuse two or more protein functional domains to generate multi-functional proteins for various applications, such as new therapeutics, protein purification, *in vitro* or *in vivo* imaging, drug delivery, and bioassays[32-36]. Previous studies suggested that

mainly flexibility and hydrophilicity of the linker are important not to disturb the functions of the protein domains[37]. To enhance conformation flexibility, solubility resistance to proteolysis, and lessons learned from natural linkers, simple linkers, such as polyglycine, glycine-rich, and proline-rich peptides with loop and coil-coil structures, are most often used in fusion proteins[36,38-40]. However, Maeda *et al.*, 1997[41], observed that simply linking of the two moieties by a flexible linker did not retain the binding activity of the protein. Moreover, in other applications, such as quantitative FRET assays, we need to consider the length and the flexibility of the linker as, flexibility and distance are not preferred because the FRET efficiency is distance-sensitive and subsequent FRET signal is significantly affected by the distance of two fluorophores[17]. Previously peptide linkers have been employed to optimize the distance of linkers using fluorescent measurements[37]. Although early effort of peptide linker with alpha helix structure to optimize FRET signal of two fluorescent proteins in cis-configuration was developed, no strategy to improve FRET signal in trans-configuration has been reported so far[42]. In addition, the detection of unstable high-energy thioester intermediate of NEDD8-Ubc12 has not been reported before. In this chapter, we report a new strategy of engineering CyPet-tagged NEDD8 with linkers to improve the fluorescence signal of fusion protein and thus further improve the FRET signal that can distinguish non-covalent and covalent conjugation of NEDD8 to its E2 ligase, Ubc12, for the first time.

2.2 Results

Structural analysis of Nedd8, SUMO1 and YPet proteins. Ubiquitin and Ubl proteins, such as NEED8 and SUMO, have similar 3-D structures. However, their primary sequences are unique, thus providing specificity in both conjugation cascades and biological activities. In our initial effort to develop FRET-based assays to analyze NEDDylation cascade, a direct fusion of NEDD8 and CyPet led to a very low fluorescent protein when compared with our previous studies with CyPet-SUMO1 fusion protein[25,30,43]. Another obstacle was that the NEDD8 protein is naturally insoluble when expressed in the *E. coli* expression system[9]. To purify NEDD8, researchers have used methods based on denaturation conditions that involve urea or guanidium. Initially we developed a method to purify CyPet-tagged NEDD8 by using a NEDD8 resuspension buffer (see materials and method) that increased the solubility of NEDD8 (Figure.9B) and resulted in an easy purification without denaturation.

The fluorescence of CyPet-NEDD8 was low (Figure.10A) and resultantly we were unable to clearly detect and differentiate the NEDD8 to Ubc12 conjugation signal (+ATP) with respect to non-covalent interaction complex formation (-ATP) (Figure.10B and C). We speculate that the intermolecular interaction of the large basic face of NEDD8 with the acidic face of CyPet, resulting in attractions between them. Changes in conformations and/or misfolding of CyPet may have resulted in low fluorescence.

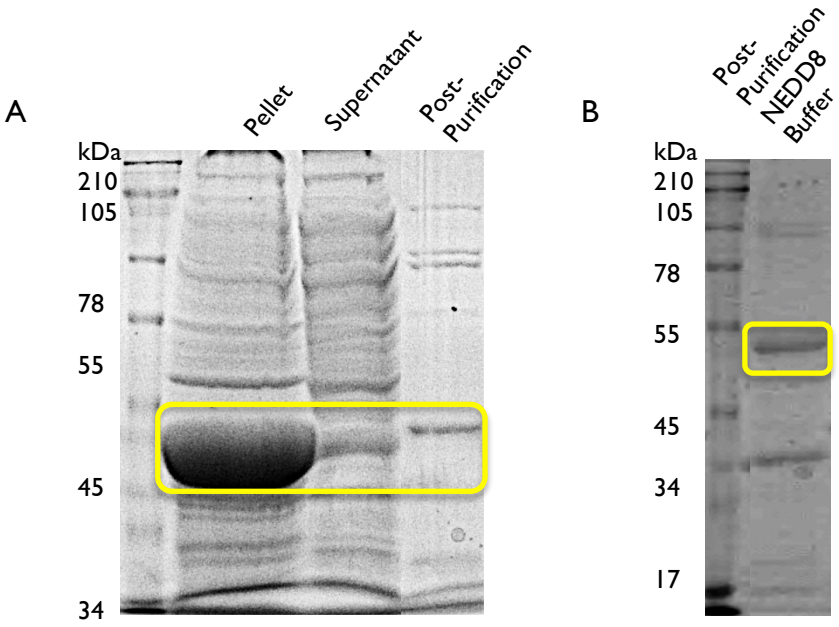


Figure 9. NEDD8 naturally insoluble when expressed in *E.coli I* expression system. A) Commassie stain showing major portion of CyPetNEDD8 expressed is part of the insoluble pellet as compared to supernatant or post-purification. B) Commassie staining of CyPetNEDD8 purified using NEDD8 buffer(50 mM Tris HCl, pH 8.0, 1 mM EDTA 20% glucose, 1% Triton).

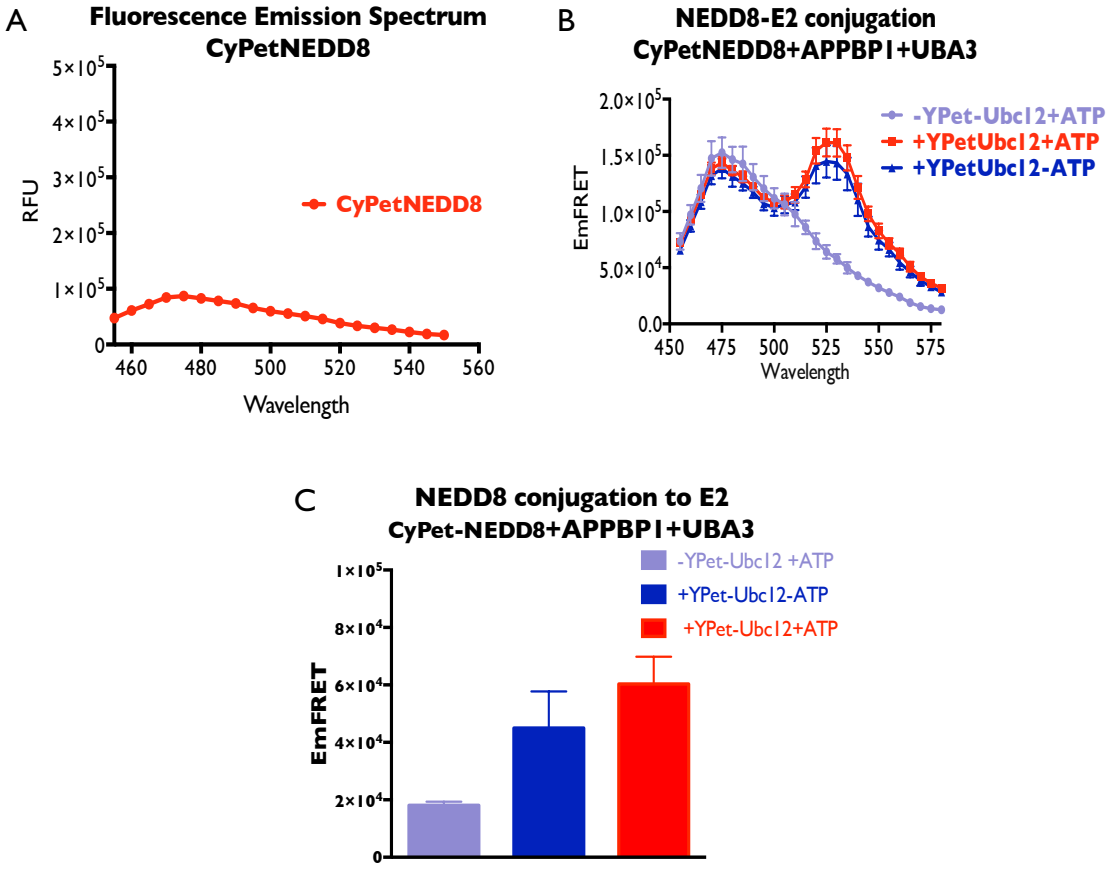


Figure 10. Fluorescence analysis of CyPetNEDD8. A) Fluorescence emission spectrum of CyPetNEDD8, when excited at 414nm. B) FRET emission spectrum of NEDD8 conjugation to Ubc12 in presence of ATP. C) Absolute FRET signal, Em_{FRET}, of NEDD8 conjugation to Ubc12.

We therefore analyzed the structures of NEDD8, SUMO1 and CyPet. Structures of CyPet, SUMO and NEDD8 are available in the PDB database, 3GEX, 1A5R and 2KO3 respectively. Molecular graphic images, for the electrostatic surfaces, were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081). We generated the generic surface of CyPet, SUMO and NEDD8 in Chimera, and with the coulombic surface coloring tool, we generated the coulombic surface for individual proteins (Figure.11).

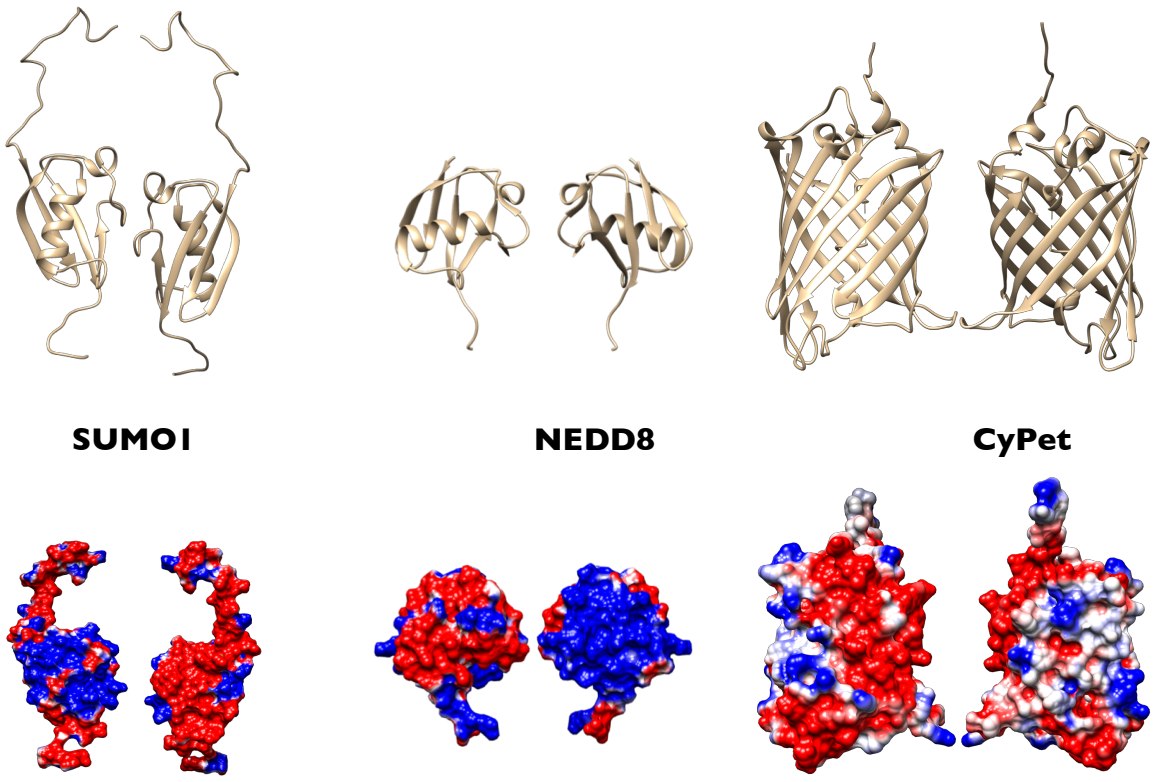


Figure 11. Structural analysis of Nedd8 and SUMO1 and CyPet structure. 3-D structures of SUMO1, NEDD8 and CyPet. Surface electrostatic potential of SUMO, NEDD8 and CyPet. Two views are shown for SUMO, NEDD8 and CyPet. The electrostatic potential is mapped on to corresponding molecular surfaces and is colored in the range -4kT/e (red) to +4kT/e (blue).

The coulombic surfaces of the NEDD8 and SUMO1 3-D structures show that the overall physiochemical properties of NEDD8 and SUMO1 are quite different. Their surfaces have different arrangements of acidic and basic faces. The beta strands of SUMO1 and NEDD8 are acidic and basic, respectively, and the alpha helical parts are basic and acidic, respectively (Figure.11). We speculated that the basic face of NEDD8 and acidic face of the CyPet have columbic clashes that might result in improper folding of directly linked fusion protein. On the other hand, SUMO1 has a long N-terminal tail, which separates the fused proteins. This separation allows for proper folding between the fused proteins so that the two fusion proteins can fold properly and therefore prevent any columbic clashes between the acidic face of SUMO1 and CyPet. Thus, based on the CyPet-SUMO1 structure, we hypothesized that, if we insert linkers of various lengths between the CyPet and NEDD8, it would prevent any columbic interactions between CyPet and NEDD8 and therefore lead to proper individually folded domains of fusion proteins.

Improving the fluorescent signals of fusion proteins by incorporating linkers in fusion proteins of CyPet-Linker-NEDD8.

To improve the folding and fluorescence of fusion proteins, we inserted linkers of different lengths and amino acids sequence between the CyPet and NEDD8 proteins to increase the distance between CyPet and NEDD8 (Table 2). We designed linker based on previous research from different groups or based on amino acid structural propensity. We designed Linker1 and 4 based on the amino acids' natural structural

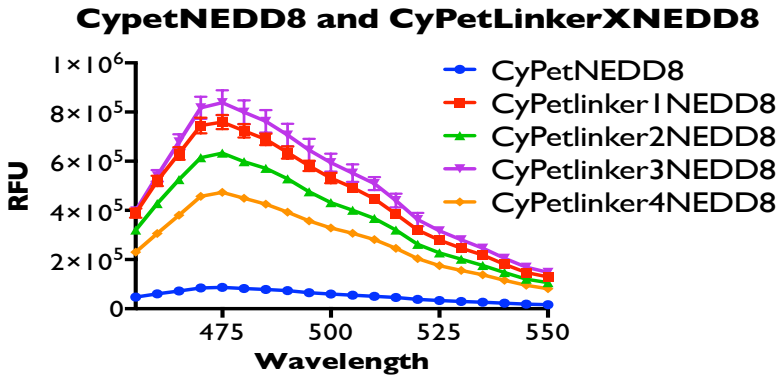
propensities. Linker2 peptide sequence was adapted from the pool of linkers that were either flexible or a helix to separate two fluorescent proteins in cis-configuration for a FRET assay[42]. Linker3 sequence design was directly taken from the Ohashi research group[44], who have developed several linkers to study protein structures with FRET technology.

Table 2. Characteristics of various linkers. Over all structures were predicted from PEP-FOLD and length of linkers were calculate using Chimera computational tools.

Name	Sequence	No. of aa	Length of linker	Overall structure
Linker1	AAAAAA	6	6 Å	Random coil
Linker2	LAEAAAKEAA	10	14 Å	Alpha helix
Linker3	TSGSPGLQEFGT	12	29 Å	Random coil and alpha helix
Linker4	LAAALAAA	8	11 Å	Alpha helix

After genetically engineering CyPet-NEDD8 fusion protein with linkers, we observed that CyPet-Linker3-NEDD8 had the maximum fluorescence emission, followed by CyPet-Linker1-NEDD8, CyPet-Linker2-NEDD8, CyPet-Linker4-NEDD8 and CyPet-NEDD8 (decreasing order of fluorescence emission at 475 nm) (Figure.12 A and B). The higher emission of CyPet at 475 should mainly reflect longer spatial distance between CyPet and NEDD8 and better protein folding, thus reducing the mis-folding because of coulombic interactions.

A



B

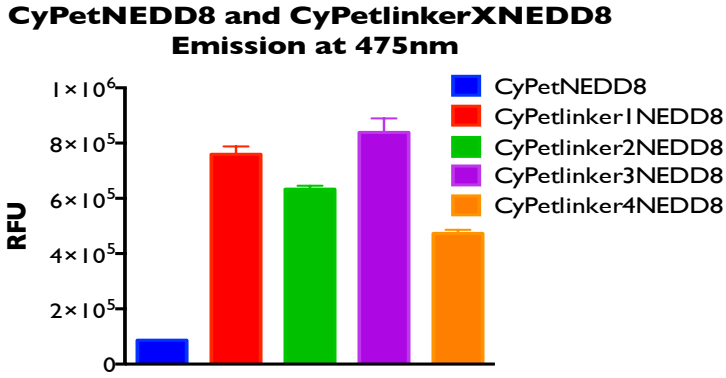


Figure 12. CyPet-NEDD8 and CyPet-LinkerX-NEDD8 fluorescence analysis. A. Emission spectrum of CyPet-NEDD8 and CyPet-LinkerX-NEDD8 when excited at 414em. B. Emission max of CyPet-NEDD8 and CyPet-LinkerX-NEDD8 when excited at 414 nm and Em(max) at 475nm.

We further investigated the structures of the linkers with PEP-FOLD to generate secondary structures (Figure.13), to determine their effect on fluorescence improvement. Specifically, *de novo* approach was used to predict 3D peptide structure from sequence information[45]. PEP-FOLD predicted, based on *de novo* approach, Linker1 to be random coil. This makes the linker very flexible, thus decreasing the

distance between CyPet and NEDD8 and explaining the lower fluorescence. PEP-FOLD predicted, based on *de novo* approach, Linker2 to be mostly helical. This makes the linker less flexible and decreased the length of the Linker2 (Table.2), thus decreasing the distance between CyPet and NEDD8 and explaining the lower fluorescence. For Linker3, the predicted structure was partially helical and random coil, making the linker more flexible, and along with larger distance between two domains, further reducing the steric hindrance between CyPet and NEDD8 and providing the greatest fluorescence emission. The structure of linker4 predicted by PEP-FOLD was mainly random coil and partially helical, and so it was flexible but also the second shortest linker. These observations explain its low emission among the CyPetNEDD8 proteins with engineered linkers.

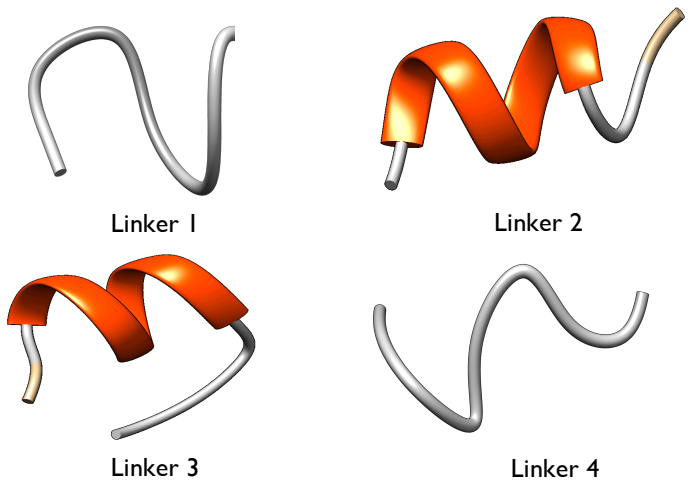


Figure 13. Computationally predicted linker structure using PEP-FOLD.

Quantitative FRET signal analysis by cross-wavelength emission and excitation method. In order to evaluate FRET signal quantitatively, our group recently developed a new spectrum analysis method to determine FRET signal based on cross-wavelength analysis of donor and acceptor[25]. In our mathematical model, the fluorescence signal at 530 nm emission (Em_{total}), when excited at 414 nm, includes three components: donor's direct emission ($CyPet_{[cont]}$), acceptor's direct emission ($YPet_{[cont]}$), and FRET sensitized YPet emission (Em_{FRET}) (Figure.8 from chapter 1). We measure and calculate FRET signal as mentioned in Chapter 1. When the proteins involved in the NEDDylation process are mixed and the conjugation cascade is initiated, the formation of protein complexes will lead to an increase of the Em_{FRET} whereas disruption of protein complexes will result in a decrease of the Em_{FRET}.

Determine non-covalent interaction and covalent conjugation of Nedd8 peptide to its E2 conjugating enzyme, Ubc12, by high fluorescent CyPet-Linkers-Nedd8. Our main aim was to develop sensitive FRET assays to study various protein interactions in NEDDylation pathway for mechanistic studies, including non-covalent and covalent intermediates. To do this, the donor fluorescent protein and acceptor fluorescent protein must have high fluorescence efficiency. In earlier studies with CyPet-NEDD8 for the E2 conjugation assay, the FRET signals were very difficult to detect due to low fluorescence emission of CyPet (Figure.10). We successfully improved the fluorescence emission by engineering our CyPet-NEDD8 with linker.

To test our linker-based protein engineering strategy for CyPet-NEDD8 fusion protein, we used the NEDD8-E2 (Ubc12) conjugation as our first model system. We mixed the CyPet-NEDD8 or CyPet-LinkerX-NEDD8 with YPetUbc12 in the presence of E1 heterodimer, APPBP1 and Uba3, in the buffer with or without ATP. After a 10-min incubation at 37°C, the solution was excited at 414 nm, and fluorescence emission spectrum at 455 nm–580 nm was scanned. CyPet-NEDD8 or CyPet-LinkerX-NEDD8 showed a very weak interaction with YPetUbc12 in absence of ATP. However, CyPet-NEDD8 did not show much FRET signal in the presence of ATP. In contrast, ~3-fold stronger FRET signals were observed from CyPET-LinkerX-NEDD8 with ATP because of the formation of a thioester linkage between NEDD8 and Ubc12 (Figure.14). After quantifying sensitized FRET signals by our methodology, the FRET signals (Em_{FRET}) were determined (Figure.15). CyPet-Linker3-NEDD8 showed the highest signal for covalent conjugation to Ubc12 (Figure.15). This correlates with its highest fluorescent signal by itself before conjugation (Figure.12).

These results suggest that improved fluorescent signal of fusion protein itself can lead to improved FRET signal in biological assays. This is also the first time we can differentiate non-covalent interaction and covalent conjugation of NEDD8 to its E2 ligase. Thus we have successfully developed a high sensitive FRET based protein assay for studying the mechanistic mechanisms of the NEDDylation pathway in real time manner.

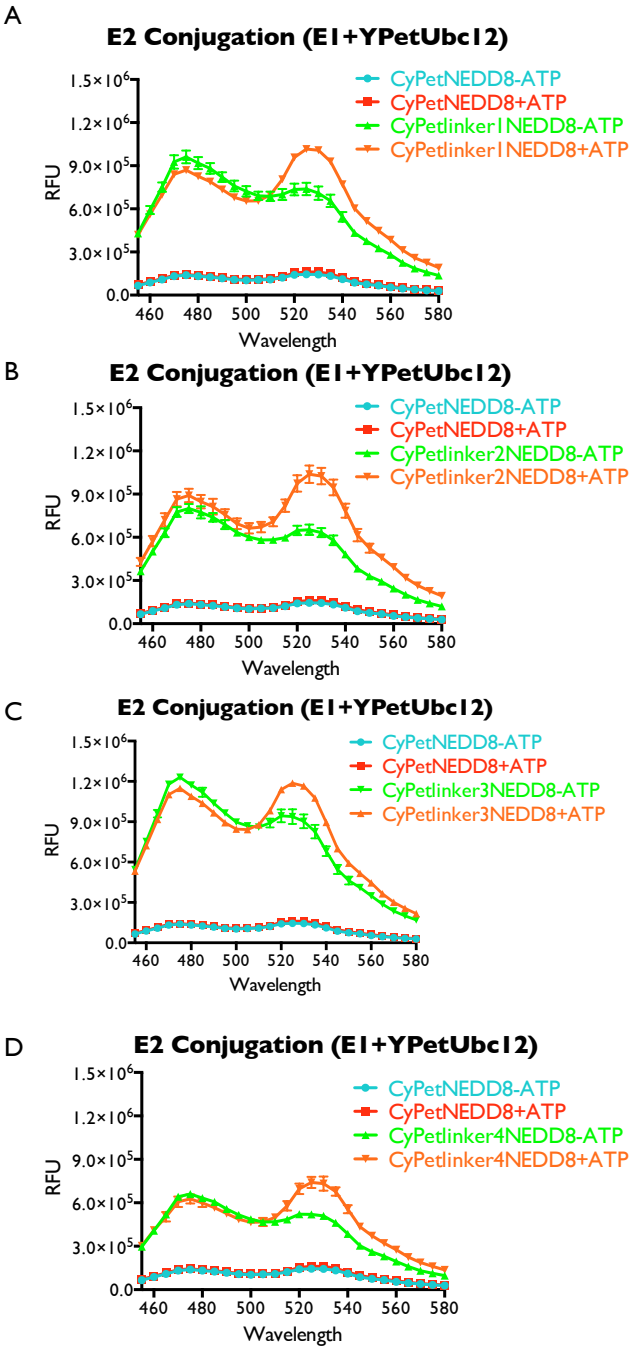


Figure 14. FRET emission spectrum comparison of NEDD8 conjugation to Ubc12. A) CyPetNEDD8 and CyPetLinker1NEDD8. B) CyPetNEDD8 and CyPetLinker2NEDD8. C) CyPetNEDD8 and CyPetLinker3NEDD8. D) CyPetNEDD8 and CyPetLinker4NEDD8.

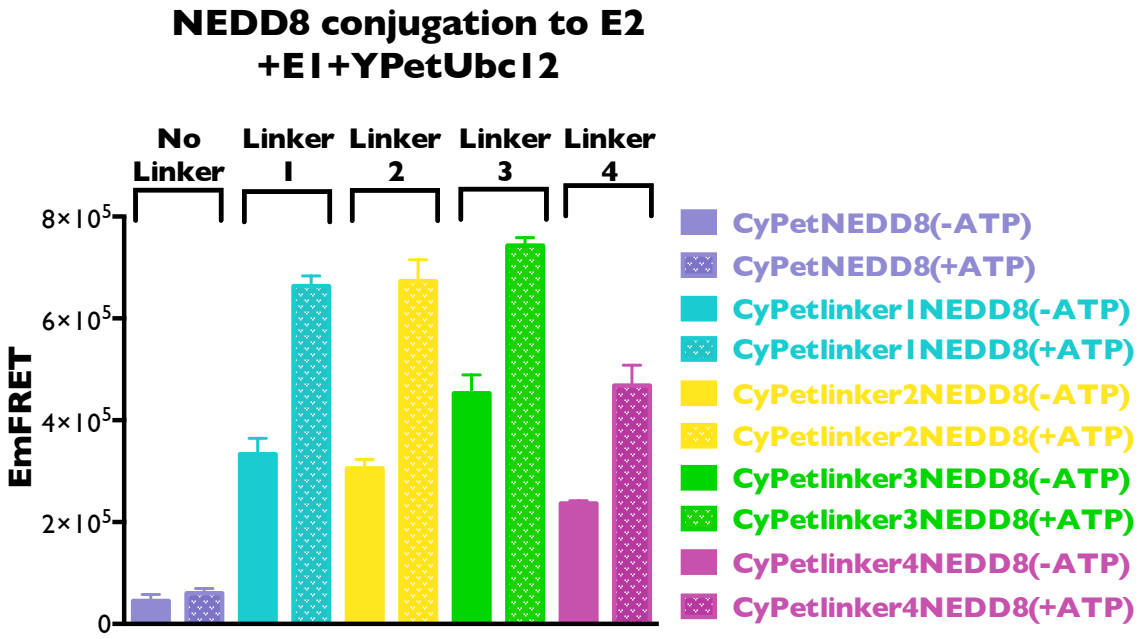


Figure.15 Absolute FRET signal, Em_{FRET}, of engineered NEDD8 conjugation to Ubc12.

2.3 Discussion

Herein, we showed that our strategy to add a linker between fluorescent proteins and proteins of interest is an effective approach to improve fluorescent and FRET signals of fusion proteins. This improvement allowed us to follow the covalent conjugation of NEDD8 to its E2 ligase in the presence of E1 and ATP. This general methodology can improve trans-FRET signals to effectively determine biochemical reaction intermediates.

There are at least two possibilities that the linkers produce significant higher fluorescent signals. First, NEDD8 is more positively charged than other Ubl proteins, such as SUMO (Figure.11). We speculate that the basic side of NEDD8 might interact with the acidic side of CyPet (Figure.11), causing columbic interactions between CyPet

and NEDD8 and further resulting in misfolding of the proteins. Therefore, it decreases the fluorescence of CyPet and further results in low FRET signal. The linkers can eliminate the columbic clash of two fusion proteins. Second, the linkers provide more space to allow two proteins of CyPet and NEDD8 to fold independently no matter the charge differences of CyPet and NEDD8. This hypothesis can be partially verified in CyPet and SUMO1 model in the future.

Data from Linker 3 partially support the second hypothesis. The fusion protein with Linker 3 enhanced the fluorescence emission (at 475 nm) and FRET signal for NEDD8's conjugation to its E2, Ubc12. There is a correlation between fluorescent signal and the length of linkers (Table 2 and Figure.12). This hypothesis can be verified by generating equal length of alpha helix peptide and random coil in the future. In addition, it is also possible that the overall random coil structure (as predicted from PEP-FOLD)(Figure.13) provide not only more distance but also flexibility between CyPet and NEDD8.

Although there is no comprehensive study for linkers, a variety of fusion proteins encompassing functions derived from different domains have been developed with linkers. For some fusion proteins, protein domain fusion without any linker may result in a non-functional protein; instead, the insertion of a linker may rescue the activity[11,46-49]. For example, a fusion protein of streptococcal protein G with *Vargula* luciferase for fluorescent immune assay (FIA) recovered the antibody binding activity when a linker was inserted between these two proteins[50]. For most of fusion proteins, linkers would improve activities of fusion proteins. To achieve the structural flexibility

of fusion proteins, most current linkers have loop structures[38]. A few cases, peptides with alpha helix structure were used successfully for fusion proteins. In the case of streptococcal protein G with *Vargula* luciferase fusion protein, a linker containing three alpha helices from protein A was used to rescue the fusion protein activity[50]. Several alpha helix peptides were used to separate two fluorescent protein domains, such as EGFP and EBFP, when fusion together as cis-FRET sensor[39,42,51,52].

Our linker engineering strategy with relatively rigid linker structure for the fusion protein, CyPet-linker-NEDD8 resulted in a significant improved fluorescence and subsequently FRET signals for studying NEDD8-E2 thioester intermediate. These results will help us to dissect different reaction steps of NEDDylation process and reaction intermediate and develop FRET-based high-throughput screening assays for drug discovery for this pathway. The rigid structure of linkers in our studies can provide additional critical benefits for future quantitative FRET analysis because it can restrict the conformational changes within fusion proteins resulting in stable FRET signal. The results clearly suggested the ability of the linkers to control the distance and reduce the interference between the CyPet and NEDD8. To our knowledge, this is the first time that linker strategy is used for improving trans-FRET assay. This study also suggests a great potential of linker engineering strategy for improving fluorescent fusion protein signals, in addition to engineering fluorescent protein itself, in various biological and biomedical research and applications.

2.4 Materials and Methods

DNA constructs

The open reading frames of CyPet and YPet were amplified using primers containing NheI and SalI sites, and the open reading frame of NEDD8 was augmented by PCR with primers containing SalI and NotI sites. The DNA fragments encoding the designed linkers were prepared using PCR and the appropriate oligonucoletide primers and NEDD8 as a template with the cleavage site Sal1 and Bamh1. All PCR products were cloned into the pCRII-TOPO vector (Invitrogen). After their sequences were verified, the genes encoding NEDD8 and linker-NEDD8 were ligated into linearized pCRII-CyPet. After the sequences were confirmed, the cDNAs encoding fusion proteins were digested with NheI/NotI and cloned into the NheI/NotI sites of the pET28 (b) vector (Novagen). The open reading frames of APPBP1, Uba3, and Ubc12 were augmented by PCR with primers containing SalI and NotI sites. All PCR products were cloned into the pCRII-TOPO vector (Invitrogen). After their sequences were verified, the genes encoding ligases were ligated into linearized pCRII-YPet plasmids. After those sequences were confirmed, the cDNAs encoding fusion proteins were digested with NheI/NotI and cloned into the NheI/NotI sites of the pET28 (b) vector (Novagen). To obtain non-fluorescent tagged proteins, the open reading frames were instead digested by SalI/NotI and cloned into those corresponding sites on the pET28 (b) vector. The amino acid sequences of the designed linkers were: Linker1, AAAAAA, Linker2, LAEAAAKEAA (10aa), Linker3, TSGSPGLQEFGT (12aa), Linker4, LAAALAAA (8aa).

Protein expression and purification

BL21 *Escherichia coli* cells were transformed with pET28 vectors encoding CyPet-NEDD8, CyPet-LinkerX-NEDD8, APPBP1, Uba3 and YPet-Ubc12. The transformed bacterial cells were plated onto LB agar plates containing 50mg/mL kanamycin, and a single colony for each clone was picked up for starter culture and inoculated in several flasks of 2xYT medium and grown at 37°C for 3 hours. Expression of polyhistidine-tagged recombinant proteins was induced with 0.1 mM isopropyl b-D-1-thiogalactopyranoside at 25°C overnight. All Bacterial cells were collected by centrifugation at 8,000 rpm for 5 min, resuspended, other than CyPet-NEDD8 cell cultures, in a buffer of 20 mM Tris-HCl, pH 7.5, 500 mM NaCl and 5 mM imidazole, and sonicated with an ultrasonic liquid processor (Misonix, Farmingdale, NY). CyPet-NEDD8 cell cultures were resuspended in NEDD8 buffer (50 mM Tris HCl, pH 8.0, 1 mM EDTA 20% glucose, 1% Triton). Cell lysates containing recombinant proteins were cleared by centrifugation at 25,000g for 30 min. The recombinant proteins were then bound to Ni^{2+}-NTA agarose beads (QIAGEN, Valencia, CA) and eluted with a buffer of 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 150 mM imidazole. Afterwards, the proteins were dialyzed into a buffer of 20 mM Tris-HCl pH 7.5, 50 mM NaCl, and 1 mM DTT. Protein purity was confirmed by SDS-PAGE and Coomassie blue staining, and concentrations were determined by the Coomassie Plus Protein Assay (Pierce). CyPet- and YPet-tagged protein concentrations were determined by their fluorescence.

FRET assay

CyPetNEDD8 or CyPet-LinkerX-NEDD8 was mixed with YPet-Ubc12 in the presence or absence of ATP and NEDDylation buffer (50mM Tris-HCl, 50mM NaCl, 10mM $MgCl_2$ 1mM DTT) and incubated for 10minutes at 37°C. Fluorescence signals were determined by the fluorescent plate reader, Flexstation II384 (Molecular Devices). Emission intensities were collected at three wavelengths: 475 nm and 530 nm at an excitation wavelength of 414 nm, and 530 nm at an excitation wavelength of 475 nm.

FRET spectrum analysis

After the emission intensities were corrected by subtraction of background fluorescence from the plate, the EmFRET for the reactions were calculated and compared. To use FRET to monitor the change of protein-protein interaction status, our lab defined the EmFRET ($Em_{FRET} = Em_{total} - Cypet(cont) - Ypet(cont)$) (Figure 3). When we mixed the proteins involved in the NEDDylation pathway and initiated the conjugation cascade by adding ATP, the formation of protein complexes led to an increase of the FRET signal. Disrupting the protein complexes resulted in a decrease of the FRET signal[25,26].

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Chapter 3: Development of FRET-based assay for reaction dynamics, interaction switches and specificities for NEDDylation cascade

3.1 Introduction

Post-translational modification by NEDD8 is an important mechanism that regulates protein activities and stability in eukaryotes. The conjugation of NEDD8 to its target substrates (described in chapter 1 and 2) employs a series of sequential enzymatic steps, which involve: activation enzyme E1, conjugation enzyme E2 and ligation enzyme E3. E1 (APPBP1-UBA3) activates NEDD8 in two steps: i) Mg^{2+}-dependent hydrolysis of ATP between the α and β phosphoryl groups driving the formation of tightly bound NEDD8 adenylated at Gly-76, ii) followed by NEDD8 forming a thioester linkage with catalytic cysteine's sulfhydryl group of E1. Followed by NEDD8 C-terminus activation, a transthiolation reaction then takes place between APPBP1-UBA3 and Ubc12, thus resulting in the formation of thioester bond between E2 catalytic cysteine and NEDD8. In combination with a variety of E3s, which are generally considered to determine substrate specificity in vivoNEDD8 are finally conjugated to lysine residues of their substrates[1-4].

In any cellular mechanism or cascade, such as non-covalent interactions (in which two or more proteins come together in order to form a complex) and covalent bond formations (such as phosphorylation of proteins, thioester linkages, isopeptide bonds). Understanding and differentiating between these different interactions are important, as it elucidates the mechanism of enzymatic/signaling cascade. Biosensors to understand

and detect these different interactions are key for the field of systems biology and pharmokinetics. Ubiquitin (Ub) and Ubiquitin-like (Ubl) proteins take part in a dynamic multi-enzymatic cascade, involving different type of interactions (non-covalent and covalent). Ubiquitin and Ubiquitin-related proteins perform post-translational modifications on various target which are critical for various physiological processes, such as transcription, protein-DNA binding, protein-protein interactions, DNA repair, transcription regulation and genome organization[1,2,4-7]. Researchers, in past, have used traditional biochemistry methodologies, such as western blot, radiolabeling or immuno-pull down assay, for determining various protein-protein interactions in Ub and Ubl proteins enzymatic cascade and to understand the dynamics enzymatic cascade and they were successful in giving the basic information regarding the end point results of a reaction or to identify enzymes/protein targets in Ubiquitin, SUMO or NEDD8 cascades[3,8-17]. Researchers have used crystal structures to elucidate the biochemical and structural basis of Ub and Ubl proteins with their respective E1 and E2 enzyme[8,9,12,14,17,18].

In this chapter, I will describe the development of a Forster Resonance Energy Transfer (FRET)-based method to detect the conjugation of NEDD8 to its E1 and E2 enzymes in a real-time manner. In this method, NEDD8 and a complement protein, APPBP1, UBA3 or Ubc12, are genetically attached to CyPet and YPet, respectively. Upon forming a complex consisting of either NEDD8-E1 or NEDD8-E2 causes an increase in the FRET signal between CyPet and YPet. Using our FRET-based protein assay, we determined the intermediates and reaction dynamics of NEDD8 with its E1s and E2 ligases and revealed the mechanistic mechanisms of NEDD8 activation and

intermediate switches among ligases. Herein, I report the detailed investigation of NEDDylation dynamics and interaction switches and specificities of NEDD8 among its ligases and other Ubl ligases using novel quantitative *in vitro* FRET-based protein assay.

3.2 Results

Design of FRET based protein assay and Fluorescence Spectrum Analysis of FRET Signal. There are different types of interactions between macromolecules in cell, and understanding these interactions gives a better insight about that particular signaling cascade system. To identify these different interactions in NEDD8 conjugation cascade, we employ our recently developed quantitative and high sensitive FRET assay (Figure.8)[19,20]. In our FRET based model, FRET signal is proportional to the amount of bound FRET pair; therefore our method can detect and differentiate covalent conjugation from non-covalent interactions, based on how signal strength and stability. There are two types of interactions between NEDD8 and E1 and NEDD8 and E2, non-covalent interactions and covalent conjugation (thioester linkage). In the NEDDylation cascade, the non-covalent interactions bring proteins together to form pre-complex and thioester linkages are important for the activation and transfer of NEDD8 from E1 to E2 and finally form isopeptide linkage between NEDD8 and target substrate, in the NEDDylation pathway[11].

To observe non-covalent or covalent conjugation of NEDD8 with E1 or E2, we used a Forster Resonance Energy Transfer (FRET) technique, which involves the high efficient FRET pair CyPet-NEDD8 and YPet-labeled, APPBP1 (E1 subunit), Uba3 (E1

subunit), Ubc12 (E2 subunit). We measured and calculated FRET signal as described in Chapter 1 (Figure.8). When we mix the proteins involved in ubiquitination or SUMOylation and initiate the conjugation cascade, the formation of protein complexes will lead to an increase of the FRET signal, whereas disruption of protein complexes will result in a decrease of the FRET signal.

Detection of NEDD8-E1 protein complex by FRET. In Ubl enzymatic cascade, such as NEDD8 pathway, the first step of NEDD8 activation is very critical, as the E1 enzyme is responsible for identifying and priming the correct Ubl. The co-crystal of APPBP1-UBA3-NEDD8-ATP[11] showcases NEDD8 interacting with both APPBP1 and UBA3. E1 activates NEDD8 C-terminal glycine in two steps: first activates NEDD8's glycine by forming an adenylated with help of ATP and then secondly form a thioester bond formation between catalytic cysteine of UBA3 and glycine of NEDD8 (Figure.4, Chapter 1). Therefore we speculated some intrinsic affinity of NEDD8 towards its E1 subunits. We first employed our FRET assay to determine the complex formation between NEDD8 and its E1 heterodimer (APPBP1 and UBA3).

When NEDD8 and APPBP1 were tagged with CyPet and YPet respectively, no significant FRET was observed between CyPet-NEDD8 and YPet-APPBP1 without ATP and UBA3 (Figure.16A, purple circles). However, when both UBA3 and ATP were added to the protein mixture, a large increase of FRET signal was observed, however the signal started to decrease after about 10minutes (Figure.16A, red circles). This suggests that interaction between NEDD8 and APPBP1 requires the presence of both

UBA3 and ATP. The dependence of FRET increase between CyPet-NEDD8 and YPet-APPBP1 on ATP indicates that the FRET signal we observed is due to the formation of complex between NEDD8 and E1 upon NEDD8 activation (ATP dependence), but whether it is via non-covalent interaction with E1 subunits or covalent conjugation to Uba3 still remains unclear. To elucidate this interaction, we generated a mutant of UBA3 with its catalytic cysteine mutated to alanine (C237A), which lacks the ability to form a thioester bond with NEDD8. We observed from our FRET results, CyPet-NEDD8 and YPet-APPBP1 were not able to form a stable complex intermediate, as NEDD8 is unable to form thioester linkage with UBA3, (Figure.16A, green circles). Also when UBA3 (C237A) is present, the FRET signal is slightly higher with respect to the FRET signal of NEDD8 and APPBP1 by itself, suggesting that NEDD8 and APPBP1 non-covalent interaction requires the presence of UBA3 (Figure.16A, green circles with respect to purple circles). We further tested if presence of Ubc12 stabilizes the NEDD8-E1 complex. Wild-type activation complexes containing an APPBP1-UBA3, NEDD8 and Ubc12 are not stable, because thioester-bonding NEDD8 is readily passed from the E1's catalytic cysteine to the catalytic cysteine of E2, and the intermediate of NEDD8 and E2 is not stable either, because it is readily passed to substrates. Therefore, an activation complex trapping strategy was used to determine if Ubc12 aided in stability of NEDD8-E1 complex, by employing UBA3 (C237A). The NEDD8-APPBP1-UBA3 active complex reached a peak concentration in the presence of ATP then decayed to the background fluorescence of two separated proteins or non-covalent complex over 10 minutes (Figure.16B, red circles). Interestingly, in the presence of Ubc12 and ATP,

the FRET signal of NEDD8-E1 active complex (with UBA3 (C237A)), started even higher, than the absence of Ubc12 and gradually decreased over 15-20 minutes, much slower, as compared to NEDD8-E1 activation in absence of Ubc12 (Figure.16B, green triangles). Furthermore, in presence of Ubc12 and absence of ATP, the FRET signal of NEDD8-E1-E2 non-covalent complex was slightly higher that FRET signal of NEDD8-E1 non-covalent complex. These results suggest that, Ubc12 stabilizes the NEDD8-E1 activation complex.

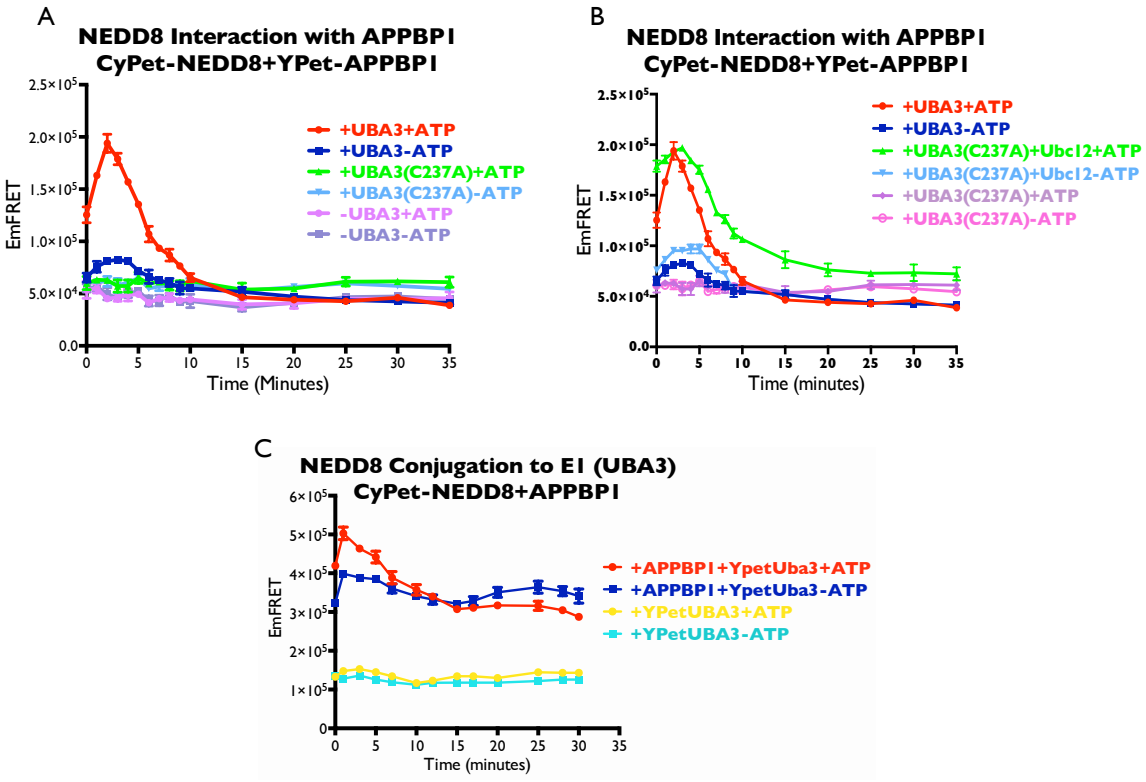

Figure 16. NEDD8-E1 intermediate formation. A and C) NEDD8 activation requires APPBP1, UBA3 and ATP. B) Ubc12 stabilizes NEDD8 and E1 complex.

We further tested NEDD8's E1 thioester, by probing CyPet-NEDD8 and YPet-UBA3 (with APPBP1) conjugation in presence or absence of ATP. In this case, we were not able to clearly differentiate between covalent conjugation and non-covalent complex formation (Figure.16C). However it was indicative of strong interaction between CyPet-NEDD8 and YPet-UBA3, which were observed only after APPBP1 and ATP were added. But the reason for low FRET signal could be ascribed to the distance between CyPet and YPet – that is, the distance increases when YPet is tagged with UBA3. As in the previous CyPet-NEDD8 and YPet-APPBP1 conjugation cascade assay, the difference between NEDD8 and APPBP1 conjugation was clearly distinguishable (Figure.16A).

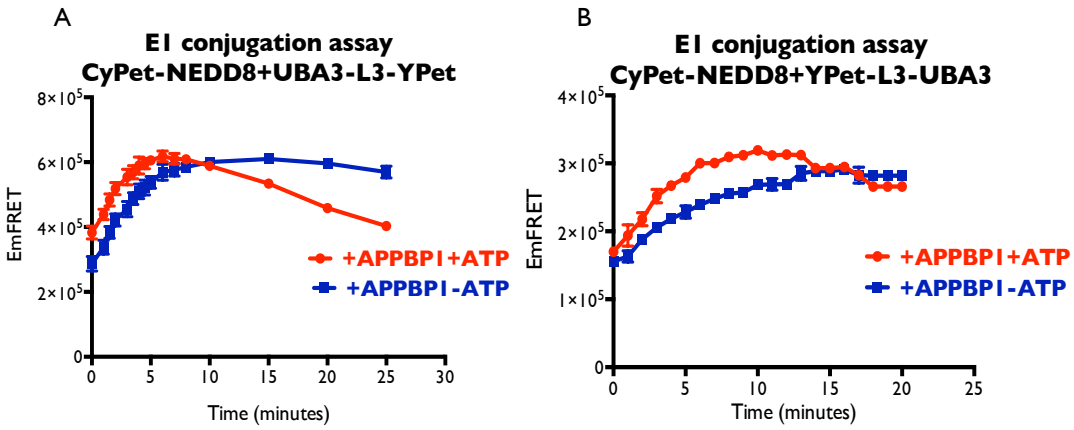

Figure.17 Linker Engineering Strategy to improve FRET signal for NEDD8-UBA3 conjugation. A) CyPetNEDD8 and UBA3-Linker3-YPet. B) CyPetNEDD8 and YPet-Linker3-UBA3. (Linker3=TSGSPGLQEFGT)

To improve the FRET signal of NEDD8 and UBA3 conjugation, we engineered YPet-UBA3 with linker3 (Linker3=TSGSPGLQEFGT, refer to Chapter2), generating two different variant of engineered YPet-tagged UBA3, YPet-linker3-UBA3 and UBA3-linker3-YPet. Unfortunately, linker engineering did not solve the problem of low FRET

signal (Figure.17 A and B). We further tested, the interaction of NEDD8 and UBA3 in absence of APPBP1, we observed that, in absence of APPBP1, NEDD8 and UBA3 have low FRET signal suggesting that, APPBP1 and ATP are required for formation of strong E1 covalent and non-covalent complex (Figure.16C). However, later we observed that APPBP1 is not required for NEDD8 activation and Ubc12 conjugation (Chapter 4).

Detection of NEDD8-E2 protein complex by FRET. In NEDD8 conjugation cascade, E1 mediated NEDD8 is followed by the transfer of NEDD8 to its conjugation enzyme, E2, Ubc12, in a transthiolation reaction, forming a thioester linkage between catalytic cysteine of Ubc12 and glycine of NEDD8. The E2 conjugating enzyme for ubiquitin and other Ubls, NEDD8 and SUMO1 has critical roles. It receives activated ubiquitin or Ubls, from the activation enzyme E1, as a new thioester intermediate and, with the help of E3 igase in vivo, transfers the ubiquitin or Ubls to its specific substrates[8]. We expanded our FRET based assay to examine this second step of NEDD8 conjugation cascade involving NEDD8, E1 and E2.

In the NEDD8, E1 and E2 complex, there are two types of interaction, covalent bond formation (thioester linkage) and non-covalent interactions (complex formation). To determine and differentiate between these interactions, we analyzed the interaction between NEDD8 and Ubc12 by employing our FRET based protein assay. From the FRET assay we could clearly see the difference in the FRET sensitized (EmFRET) of YPet emission at 530 of CyPet-NEDD8 and YPet-Ubc12 covalent conjugation (+ATP) and that of CyPet-NEDD8 and YPet-Ubc12 non-covalent interaction (-ATP), with its E1

complex (Figure.18A, red circles and blue squares, respectively). NEDD8 and Ubc12 have very low affinity for each other in absence of E1 heterodimer (APPBP1 and UBA3) (Figure.18A, green triangles as compared to red circles). In order to confirm that NEDD8 and Ubc12 form a stable complex with E1 in presence of ATP is due to thioester linkage between NEDD8's glycine and Ubc12's catalytic cysteine, we further explored the pair, and found that YPet-Ubc12 (C111A) was unable to form a stable complex due to the inability to form the thioester linkage because of mutated Cysteine (Figure.18B, green triangles). This also showcases that NEDD8 and Ubc12 intrinsic affinity is not required for NEDD8 to Ubc12 conjugation, but presence of E1 is required for NEDD8 and Ubc12 to interact and conjugate. The NEDD8-Ubc12 thioester bond is more stable then NEDD8-UBA3 thioester bond (Figure16C with respect to Figure18B).

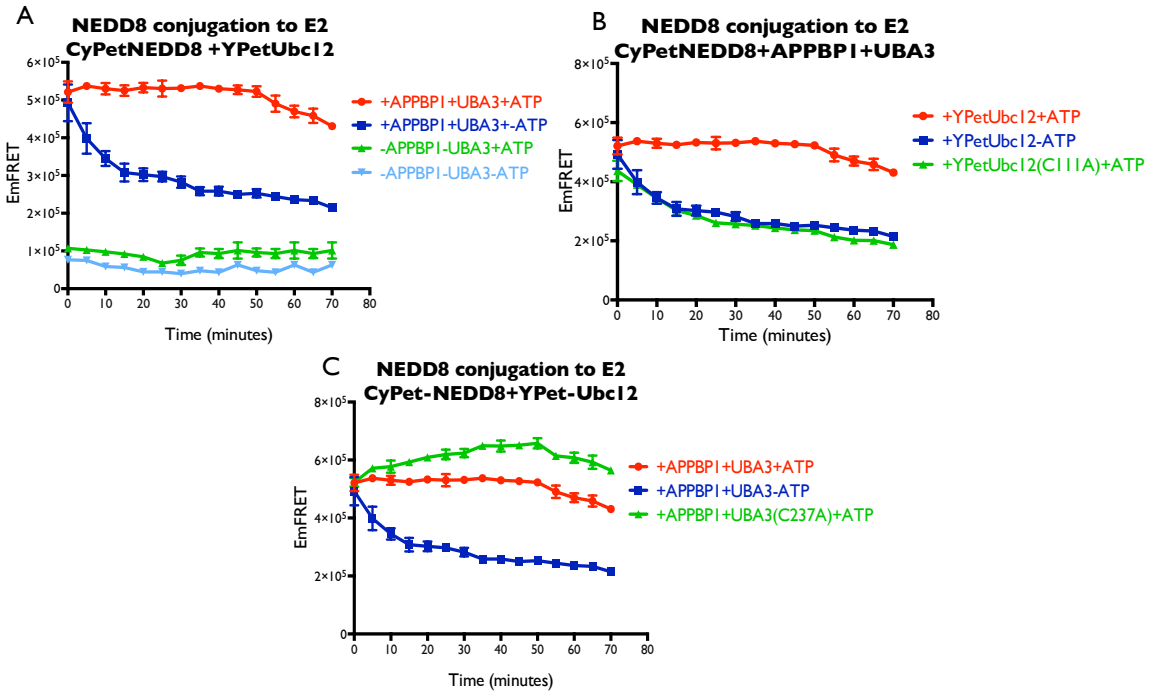

Figure.18 NEDD8 E2 intermediate determination. A) Thioester bond-mediated interaction of NEDD8 and Ubc12 requires E1, ATP. B) FRET reveals non-covalent and covalent inter-actions between NEDD8 and Ubc12 in the presence of E1 and ATP. C) Ubc12 stabilizes the E1 and NEDD8 intermediate complex.

From the biochemistry-based studies, by employing western blot and radiolabeling, researchers have been able to discover NEDD8 conjugation cascade is more linear cascade than dynamic in nature. However, cell is a very dynamic entity and all the enzymatic and signaling cascades are dynamic at multi-levels. Keeping that in mind, we wanted to test if Ubc12 aided in the stability of NEDD8-E1 complex by forming of pre-complex. Wild-type activation complexes containing an APPBP1-UBA3, NEDD8 and Ubc12 are not stable, because thioester-bonding NEDD8 is readily passed from the E1's catalytic cysteine to the catalytic cysteine of E2, and the intermediate of NEDD8 and E2 is not stable either, because it is readily passed to substrates. Therefore,

an activation complex trapping strategy was used to determine if Ubc12 aided in stability of NEDD8-E1 complex, by employing UBA3 (C237A). We observed that, E2, Ubc12, aids in the stability of the NEDD8 and E1 complex, (Figure. 18C). UBA3 (C237A) is unable to form thioester intermediate with NEDD8, but presence of Ubc12, stabilizes the complex of NEDD8, E1 and E2, and gives a higher FRET signal with respect to when wild type UBA3 is present. In previous studies, based on X-ray structures they speculate the E2 binding domain in E1 is hidden, and UBA3~NEDD8 thioester intermediate is required for E1-E2 binding. But from our real time FRET data, suggests that Ubc12 can bind to E1-NEDD8 complex, even in the absence of UBA3~NEDD8 thioester bond formation (Figure.18C) and further stabilized the NEDD8-E1-E2 complex.

E1 and E2 specificity in Ubl cascades. Ubiquitin and Ubiquitin-like proteins have sequence and structural similarity. Furthermore they share similar activation and conjugation mechanism, with their respective E1s and E2s sharing structural and functional similarity[3,4,8]. We wanted to investigate any cross reactivity between E1s and E2s of Ubiquitin and SUMO1 with NEDD8. We observed that E1s are specific to their own Ubls (Figure.19A), which is consistent with the fact that E1 identify their specific Ubl for the downstream activation[11]; therefore NEDD8 was able to form thioester intermediate with only UBA3, and not UBA1 (Ubiquitin's E1) or Uba2 (SUMO1's E1) (Figure.19A). Also NEDD8 had higher non-covalent affinity for UBA3 as compared to UBA1 or Uba2 (orange, with respect to purple and blue, Figure. 19A). Similarly, from our FRET data NEDD8 was able to form thioester bond intermediate with only Ubc12,

and not with Ubc9 or UbcH5a (SUMO1's or Ubiquitin's E2 respectively) (Figure.19B). Therefore suggesting the specificity of NEDD8 to its own E2 with no cross reactivity with SUMO's or Ubiquitin's E2.

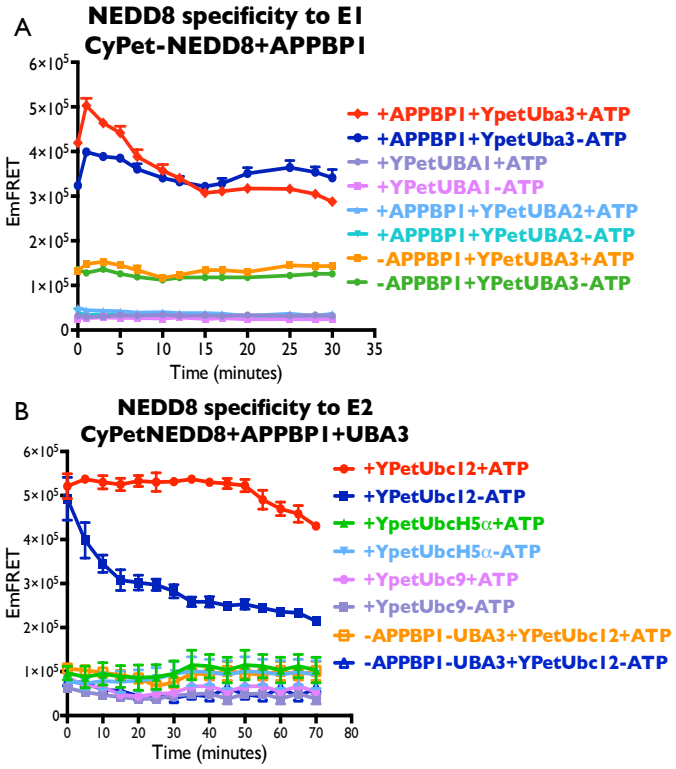

Figure.19 NEDD8's E1 and E2 specificity. FRET revealsNEDD8s' specificity towards its own E1 (A) and E2 (B).

3.3 Discussion

Our results, for the first time have revealed the reaction intermediates and affinity switches of NEDD8 with its E1 and E2 in the NEDD8 enzymatic conjugation cascade. In NEDD8 conjugation cascade, just like any Ubl conjugation cascade, NEDD8 shuffles through different binding partners (E1, E2 or E3) and interacts with these enzymes through both covalent and non-covalent interactions. Our FRET based real

time studies have showcased various interaction switches between NEDD8 and its conjugation cascade enzymes. The FRET signal between NEDD8 and APPBP1 or UBA3 is observed only in the presence of ATP and the other counter E1 subunit, UBA3 or APPBP1, respectively, for adenylation and thioester intermediate formation, indicating that heterodimer formation is important for NEDD8 activation (Figure. 15). Also showing that the presence of NEDD8, results in conformational change in E1 heterodimer complex, which aids in the formation of the NEDD8-E1 thioester intermediate, which otherwise is not possible if one of the E1 subunits are missing (Figure. 15). The thioester linkage between NEDD8 and UBA3 is not stable over time, thioester linkage being high energy bond, can be seen by the gradual decrease of FRET signal between CyPetNEDD8 and YPetAPPBP1 or YPetUBA3, in presence of E1 and ATP (Figure.15). Interestingly, a less-stable complex between NEDD8 and E1 (APPBP1-UBA3 (C237A)) is formed (Figure.15A), in which UBA3 (C237A) is incapable of forming a thioester intermediate with NEDD8's C-terminal glycine, showcasing that NEDD8~UBA3 thioester intermediate in key for a stable NEDD8-E1 complex formation. However, interestingly, a more-stable complex between NEDD8 and E1 (APPBP1- UBA3 (C237A)) is formed in presence of Ubc12 (Figure.15B and 18C), in which UBA3 (C237A) is incapable of forming a thioester intermediate with NEDD8's C-terminal glycine, but still is able to form a stronger complex. This also indicates the fact that thioester bond formation between NEDD8 and UBA3 is required for NEDD8 to be transferred to Ubc12. Furthermore, NEDD8 and Ubc12 thioester intermediate is much more stable over time, as compared to the NEDD8-E1 thioester intermediate

(Figure.16 and 18A). Moreover, NEDD8 has low intrinsic affinity for the Ubc12 in the absence of E1 heterodimer (Figure.18A), supported by our Kd data for NEDD8 and Ubc12 pair (Kd=20μM, data not shown), where as NEDD8 has more intrinsic affinity towards UBA3 (Kd=0.7μM, data not shown), also indicating the fact that NEDD8 should have affinity for E1, for its correct priming by its E1. Another aspect need be considered in our i*n vitro* FRET-based protein assay is that inside the cell volume, protein concentration and environments are different from *in vitro* system. The catalytic reaction systems are diffusion limited in side the cellular environment and concentration of proteins are considerably low with respect to protein concentration used in an *in vitro* system.

Our studies also demonstrate an interesting example that the tracking of FRET signals can reveal molecular interactions and dynamics of the reaction cascade involving multiple proteins. We here for the first time have been able to dissect these different interactions in NEDD8 conjugation cascade in real time. In past, researchers have used traditional biochemist methodologies, which have provided with the basic overview of Ubiquitin and Ubiquitin like modifications. The quantitative estimation of different affinities for the transient complexes in the Ubiquitin and Ubl conjugation cascade is hard to determine in real time using other approaches because the thioester intermediates are chemically not stable.

FRET has been used as spectroscopic research tool in the field of biology, biotechnology and biophysics. A full detail understanding of specific cascade requires robust assay that can easily and quantitatively gauge the kinetics and dynamics of the

various interactions undergoing in a reaction. Fluorescence has exceptional sensitivity for detecting low concentration of particles over broad spatial and temporal dimensions. FRET is applied as tool to determine molecular distances or to demonstrate the presence or absence of molecular complex[21-24]. There are a couple of research groups who have developed FRET based assays for Ubiquitin and Ubiquitin like modifiers proteins[25-29]. Boisclair *et.al* group developed a FRET based inhibitor screening assay for Ubiquitin transfer from its E2 enzyme to HECT proteins, but their assays only reports the transfer of Ubiquitin from its E2 to E3 proteins[30]. Furthermore they use indirect labeling of the fluorophores for the FRET assay, which might lead to large molecular complex formation, resulting in incompetent FRET, because of the large distance between donor and acceptor fluorophores. Another group developed a colorimetric spectrophotometric assay for conjugation of Ubiquitin proteins, in which they quantify Ubiquitin conjugation by monitoring pyrophosphate released in the first enzymatic step of Ubiquitin transfer, which is the ATP dependent activation of Ubiquitin by its E1[15]. This method is rapid and radiolabeling free, but this method is an indirect method to understand the Ubiquitination cascade and also it fails to monitor the dynamics of the Ubiquitin activation and conjugation steps in real time manner. Another group established a FRET based system for understanding the effect of NEDD8 conjugation on Cullin Ring ligases for Ubiquitination of target proteins[31]. They employ FRET based method and western blot methods to demonstrate the NEDD8 conjugation on Cullin Ring Ligases results in improved ubiquitination of target protein, by proposing that NEDD8 induced a structural change in Cullin Ring Ligase.

Deciphering what kind of interactions occurs in NEDDylation can give us detailed understanding about the specificity and, in future, aid in drug discovery. To study these interactions, the methods available, such as western blot, radiolabelling are very time consuming and labor intensive and expensive. Although much knowledge about the Ub/Ubl handoff among ligases has been gained from these studies, the reaction intermediates and dynamics in the sequential formation of transient complexes along the E1–E2–E3 conjugation cascade have not been observed in a real dynamic mode. In addition, mechanisms of NEDD8 activation and switches between E1 and E2 ligases have not been completely determined. Therefore our study provides great insights about the NEDD8 conjugation cascade in real time manner.

3.4 Materials and Methods

Molecular cloning of DNA constructs

The open reading frames of CyPet and YPet were amplified by using primers containing NheI and SalI restriction sites, respectively. The open reading frames of NEDD8, APPBP1, Uba3 and Ubc12 were amplified by PCR using primers containing SalI and NotI restriction sites, respectively. All PCR products were cloned into the pCRII-TOPO vector (Invitrogen). After their sequences were confirmed from DNA seqeuncing, the genes encoding NEDD8 and NEDD8 ligases were ligated into linearized pCRII-CyPet or pCRII-YPet plasmids. After the sequences were confirmed, the cDNAs encoding fusion proteins were digested with NheI/NotI and cloned into the NheI/NotI sites of the pET28(b) vector (Novagen). To get non-fluorescent tagged proteins, the

open reading frames of NEDD8 and NEDD8 enzymes were extracted by SalI/NotI digestion and cloned into the SalI/NotI sites of the pET28(b) vector. The mutant forms of Uba3, and Ubc12 were created by PCR site-directed mutagenesis and cloned into the pET28(b) vector, following the protocol described above. For YPet-linker3-UBA3 and UBA3-linker3-YPet constructs, the DNA fragment encoding the designed linker3 was prepared by PCR with the appropriate oligonucoletide primers and UBA3 or YPet as a template with the cleavage site Sal1 and Bamh1 and cloned as described above.

Protein expression and purification

BL21(DE3) *Escherichia coli* cells were transformed with pET28 vectors encoding NEDD8,APPBP1, Uba3, Ubc12, their mutants, and fluorescent protein-labeled versions.. The transformed bacteria cells were plated on LB agar plates containing 50mg/mL kanamycin, and a single colony was picked up and inoculated in 2xYT medium. The expression of polyhistidine-tagged recombinant proteins, other than CyPetNEDD8, was induced with 0.2 mM IPTG at 25°C overnight. Bacterial cells were collected by centrifugation at 8,000rpm 5min, resuspended in buffer containing 20mM Tris–HCl, pH 7.5, 500 mM NaCl and 5 mM imidazole, and sonicated with an ultrasonic liquid processor (Misonix, Farmingdale, NY). Cell lysate containing recombinant proteins was cleared by centrifugation at 35,000g for 30min. The recombinant proteins were then bound to Ni^{2+}-NTA agarose beads (QIAGEN, Valencia, CA) and eluted with buffer containing 20mM Tris–HCl, pH 7.5, 200mM NaCl, and 150mM imidazole. After the proteins were dialyzed into buffer containing 20mM Tris–HCl pH 7.5, 50mM NaCl, and

1mM DTT. SDS confirmed the purity of the proteins–PAGE and Coomassie blue staining, and concentrations were determined by Coomassie Plus Protein Assay (Thermo, Barrington, IL).

FRET measurements

After the proteins labeled by CyPet or YPet are mixed in the presence or absence of other protein members or cofactors, their fluorescence was determined by the fluorescent plate reader Flexstation II^{384} (Molecular Devices) in a 384-well plate. The emission intensities at three wavelengths were collected: 475 nm and 530 nm at an excitation wavelength of 414 nm, and 530 nm at an excitation wavelength of 475 nm.

Protein interaction and NEDDylation assay

All reagents were purchased from Research Products International Corp unless otherwise specified. For the interaction of NEDD8 with E1, 1 μM CyPet-NEDD8 and 1 μM of YPet-APPBP1 or YPet-UBA3 were mixed in the presence or absence of 1 μM of UBA3 or APPBP1, respectively (buffered solution containing 50 mM Tris-HCl (pH=7.4), 1 mM DTT and 4mM MgCl2). For E1 specificity assay, 1 μM YPet-UBA1 or YPet-Uba2 was added. For mutant UBA3 assay, 1 μM wild type or mutant UBA3 was added. Samples were incubated at 37°C and fluorescence emission was monitored after ATP (2mM final concentration) or H2O was added at time zero. For NEDD8 and UBA3 (linker engineered) protein assay, 1 μM CyPet-NEDD8 and 1 μM of YPet-linker3-UBA3 or UBA3-linker3-YPet were mixed in the presence of 1 μM of APPBP1, (buffered

solution containing 50 mM Tris-HCl (pH=7.4), 1 mM DTT and 4mM MgCl2). Samples were incubated at 37°C and fluorescence emission was monitored after ATP (2mM final concentration) or H2O was added at time zero.

In the interaction assay of NEDD8 and Ubc12, 1 μM CyPet-NEDD8, 1 μM wild type or mutant YPet-Ubc12 were mixed in presence of 1 μM APPBP1 and 1 μM UBA3 (buffered solution containing 50 mM Tris-HCl (pH=7.4), 1 mM DTT and 4 mM MgCl2). For mutant UBA3 assay, 1 μM wild type or mutant UBA3 was added. Samples were incubated at 37°C and fluorescence emission was monitored after ATP (1mM final concentration) or H2O was added at time zero. For E2 specificity assay, 1 μM YPet-Ubc9 or YPet-UbcH5α was added, in presence of E1. For mutant UBA3 assay, 1 μM wild type or mutant UBA3 was added. Samples were incubated at 37°C and fluorescence emission was monitored after ATP (2mM final concentration) or H2O was added at time zero.

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Chapter 4: Deciphering the distinct roles of E1 heterodimer subunits, APPBP1 and UBA3, in NEDD8 conjugation cascade

4.1 Introduction

Post-translational modification by ubiquitin and ubiquitin-like protein family has surfaced as an important cellular mechanism by which to regulate various cellular processes, such as cell division, signal transduction, embryonic development, immune response, DNA transcription and replication, endocytic trafficking, biosynthetic pathways, cancer and many other biological processes[1-5]. Family of ubiquitin-like modifiers (UBLs) consist of 17 proteins, NEDD8, SUMO, ISG15, FUB1, FAT10, Atg8, Atg12, Urm1, and UFM1 that have been identified to conjugate to its target proteins in a similar fashion to ubiquitin[1-6]. In 1992, Kumar et all identified using subtractive cloning screening between a cDNA library derived from mouse neural precursor cells and mouse adult mRNA identified ten Neural precursor cell-Expressed Developmentally Down regulated (NEDD) genes, one of these genes encoded protein (NEDD8) which was 60% identical to ubiquitin's sequence and 80% homologous to ubiquitin's structure[7].

Ubiquitin and Ubiquitin-like modifiers conjugate to various target proteins through a conserved but distinct enzymatic pathway with their own specific enzymes. Generally speaking, Ubls have one or two E1s, one or multiple E2s and E3s[1,8-10]. Targeting of these various proteins by different Ubls can lead to different protein fate, cellular localization, conformation change, change in enzymatic activity, protein's half-life or protein-protein interactions[4,5]. As there are different consequences of target proteins after their Ubl conjugation, thus Ubl conjugation to their specific targets through the

coordinating enzymatic cascade is very important. It is critical to understand how different Ubls, shuffle through their respective conserved enzymatic pathway, conjugate to the targets. Different Ubls are conjugated to their respective targets by a conserved mechanism but distinct cascades of modular enzymes, which sequentially involve, activating enzyme: E1, conjugation enzyme: E2 and ligases: E3. Ubl conjugation cascades are dynamic and reversible (Scheme.1). These enzymes (E1, E2 and E3) are modular in nature, with conserved domains carrying out specific related functions in different pathways, but have shown to have specificity to their own Ubl[10]. Briefly, similar to Ubiquitin, NEDD8 and SUMO is translated from mRNA as a large precursor protein. Ubl is recognized and processed by peptidases called Sentrin-specific proteases (SENPs) to remove its C-terminal extension, exposing a glycine-glycine motif in the mature Ubl to make it available for conjugation with the lysine side chains in the target proteins. Ubl pathway begins with an activation enzyme (called E1), which catalyzes an ATP-dependent activation of the Ubl. In case of Ubiquitin, E1 is single polypeptide, Uba1, and in case of NEDD8 and SUMO1, E1 is a heterodimer: its subunits APPBP1 and UBA3 and AOS1 and Uba2, respectively. Ubl activation by E1 can be divided into three steps, (i) the C-terminal carboxyl group of Ubl attacks the ATP forming a Ubl-adenylate intermediate and releasing pyrophosphate, (ii) thiol group of the active site cysteine in the E1 attacks the Ubl-adenylate intermediate releasing AMP and forming a high energy thioester intermediate and (iii) activated Ubl is transferred to cysteine of conjugating enzyme E2 (many in case of ubiquitin; Ubc12 and Ube2F in case of NEDD8; Ubc9 in case of SUMO) forming thioester linkage[9,11]. The transfer of activated Ubl from E1 to E2

result in the Ubl-E2 intermediate, which can serve as donor in the final reaction of the NEDDylation of the target. The third step of the pathway includes the Ubl ligases (E3), which may or may not form covalent intermediates with Ubl; they bring E2-Ubl intermediate and substrate together[12](Figure.4). Different forms of protein-protein interactions take place inside the cell, non-covalent interactions, and covalent-bond formation, leading to different reactions and outcomes. The linkage between the Ubl and substrates is an isopeptide bond between the C-terminal carboxyl group of Ubl and the ε-amino group of lysine residue in substrate (similar as in ubiquitination). The linkage between Ubl's glycine and E1's catalytic cysteine is a high-energy thioester linkage. Ubl interacts with its E1, E2 and E3 non-covalently thorough conserved residues maintaining specificity[2,8,13-15]. Thus the pathway includes enzymatic reactions that attach the specific Ubl to a specific substrate and then other peptidases cleave of the Ubl from the substrate.

Ubiquitin and Ubls and their activating enzymes appear to have evolved from primitive biosynthetic pathway, MoaD-MoeB (molybdopterin biosynthesis) and ThiS-Thif (thiamin biosynthesis) pathway[10,16,17]. MoaD and ThiS are structurally homologous to ubiquitin. Like Ubiquitin, MoaD and ThiS are adenylated at their C-termini by MoeB and ThiF enzymes respectively, but the only difference is that MoaD and ThiS do not get conjugated to a target protein at the end of the pathway, but instead are employed for carrying atoms at their C-termini for their respective biosynthetic pathways[16-19]. Also, MoeB and ThiF, the activation enzymes for MoaD and ThiS, respectively, have sequence and structural homology to E1s of Ubiquitin and Ubls[16,20-22]. In MoeB and ThiF, the two

structural homology regions are separated out by short sequences[8,10,20,22], but in case of UBA1, APPBP1 and UBA3 and AOS1 and Uba2, there are insertions between these two structural regions. And it can be understood that these evolutionary insertions are for the evolved E1- specific functions, such as binding to their cognate E2 and transfer of Ubl to the catalytic cysteine of respective E2[9-11]. Sequence homology among E1s and E2s of Ubiquitin, NEDD8, SUMO and ISG15 results in similar mechanism for their activation and conjugation step. However, E1s for Atg8p/Atg12p and Urm1p have different sequences from Ubiquitin's E1, thus imply having a divergent mechanisms[8-10].

Ubiquitin and Ubls have their own set of E1, E2s and E3s for conjugating to their respective target proteins in cell. A lot of research has been done elucidating the biochemical mechanisms in the Ubl conjugation enzymatic cascade using conventional biochemistry tools such as western blots and Xray crystallography[8-11,14,15,23-25]. But much more needs to be understood and discovered about these Ubl and their respective conjugation cascades, such as new targets, new candidates for E1s, E2s and E3s, protein affinities between these enzymes in multi-enzymatic cascade and intermediates dynamics of these enzymatic steps, which in future can aid in the understanding the links between various signaling cascades and drug discovery. With that in mind, our lab is motivated to develop new technologies, which aid in investigating different signaling cascades reaction intermediate dynamics and affinities in a real time, sensitive, fast and high throughput manner, in order to understand these complex enzymatic cascades. In past, we have developed novel high sensitive FRET based quantitative tool to understand the reaction dynamics of Ubiquitination[26] and SUMOylation[27] cascade. Our FRET based tool cannot

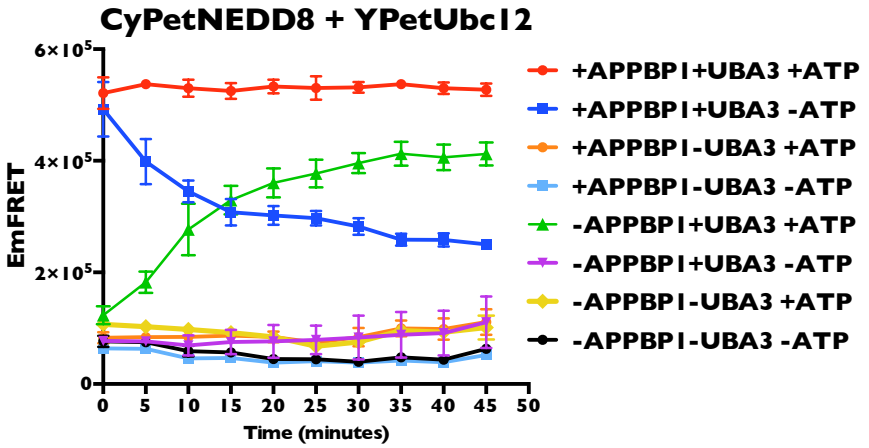
only dissect FRET signal, but also measure the disassociation constant (K_d) for interaction of SUMO and Ubc9[28] (E2) and k_{cat}/K_m of different SENPs (proteases) and SUMO paralogs[29]. In addition, our FRET based method can also quantify and dissect various intermediate formations in the Ubiquitin[26], SUMO[27] and NEDD8[30] enzymatic cascade. We here for the first report an interesting finding and study, of APPBP1 acting more as scaffold protein than have enzymatic properties.

4.2 Results

APPBP1 accelerates, but is not absolutely required for Nedd8 activation. Interestingly, as every year passes, researchers around the world decipher new findings about cell biology and its complex networks. New discoveries lead to a deep and broad understanding about different signaling pathways, cellular processes and diseases. Similarly over the years it has been discovered that NEDD8 conjugates to various important targets such as CRL and p53 depends upon the chronological action of its activation enzyme (E1), conjugation enzyme (E2) and ligases (E3). As discussed above, E1 (APPBP1 and UBA3 heterodimer) primes NEDD8's C-terminal glycine and prepares NEDD8 for E2 (Ubc12 conjugation)(Figure. 4). To understand NEDD8 and its conjugation cascade in detail, we employed FRET based protein assay (Figure.8), which involves CyPet and YPet fluorescent proteins. While investigating the intermediate formation and NEDD8 conjugation cascade dynamics, we first investigated the activation step of NEDD8 using CyPet-NEDD8/YPet-Ubc12 thioester intermediate formation as a signal of final product in presence or absence of E1 heterodimer and ATP. The

formation of CyPet-NEDD8/YPet-Ubc12 thioester intermediate leads to a high Em_{FRET} signal as described above. In the presence of APPBP1, UBA3 and ATP, the conjugation of CyPet-NEDD8 to YPet-Ubc12 led to a high FRET signal in a very rapid reaction (red circles, Figure.20A). To our surprise, in the absence of APPBP1, CyPet-NEDD8 could also be conjugated to YPet-Ubc12 over a long time period of more than 45 minutes of our experiment (green triangles, Figure.20A). This indicates that the covalent thioester intermediate of CyPet-NEDD8/YPet-Ubc12 complex could be formed without APPBP1, which may play an accelerating role in the reaction. Interestingly, in the absence of ATP, CyPet-NEDD8 and YPet-Ubc12 could form a unstable complex in the presence of APPBP1 and UBA3 (Blue square in Figure.20A). This may indicate that transient non-covalent complex may be formed, but dissociated quickly. In the absence of UBA3 or ATP, either the covalent and non-covalent intermediates could not be formed. The covalent thioester intermediate of CyPet-NEDD8/YPet-Ubc12 was also confirmed by biochemical assay. The covalent thioester intermediate of CyPet-NEDD8/YPet-Ubc12 was observed in the western-blot, but could be dissociated by DTT (lane 1 and 2 in Figure.20B). In the absence of APPBP1, the covalent thioester intermediate of CyPet-NEDD8/YPet-Ubc12 could also be formed (lane 5-10 in Figure.20B). These interesting discoveries motivated us to investigate the roles of APPBP1 in NEDD8 activation.

A.



B.

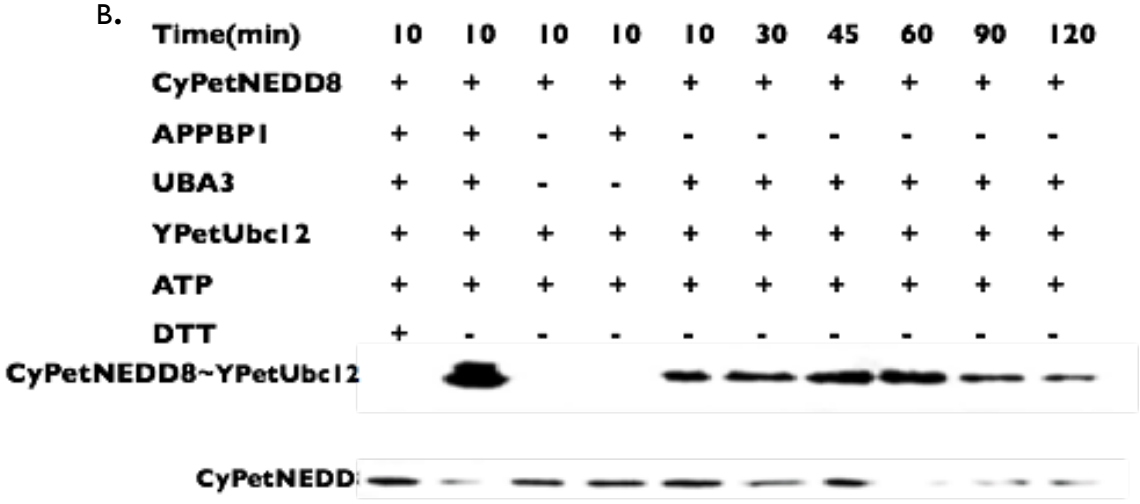


Figure.20 E1 requirement for NEDD8 conjugation to E2. (A) FRET based protein assay for determining E1 requirement of APPBP1 and UBA3 for NEDD8 activation and conjugation to Ubc12(data are represented as mean +/- SD). (B) Western blotting confirms the ability of UBA3 by itself to activate NEDD8 and conjugate NEDD8 to Ubc12

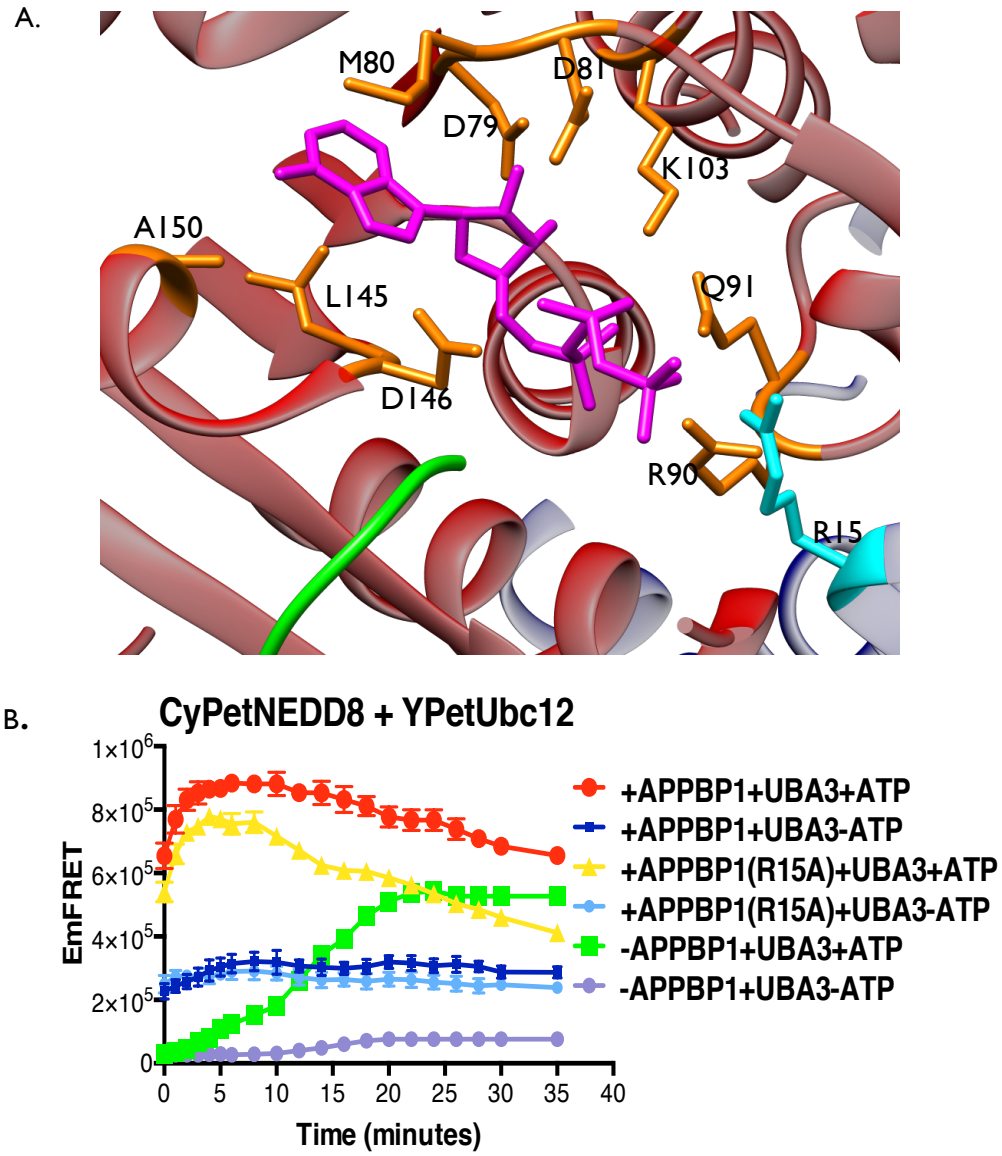


Figure.21 Importance of APPBP1-ATP binding. (A) Close-up view of ATP binding pocket in E1 heterodimer of NEDD8 (PDB ID: 1R4N), UBA3 shown in red, APPBP1 in blue,NEDD8 in green, ATP in magenta, residue from UBA3 participating in ATP binding pocket in orange and from APPBP1 in cyan (parts of APPBP1 and UBA3 are hidden for clarity). (B) APPBP1's ATP binding residue (Arg15) is not critical for NEDD8 conjugation to Ubc12 (data are represented as mean +/- SD).

ATP binding activity of APPBP1 accelerates, but not required, for rapid NEDD8 activation. The co-crystal structure of APPBP1 with UBA3, NEDD8 and ATP shows that APPBP1 interacts with all three other components of E1 NEDD8 complex, UBA3, NEDD8 and ATP[31]. We decided to investigate how each interaction contributes to the NEDD8 activation. We first examine the ATP binding pocket of NEED8 E1 activating enzyme in the APPBP1-UBA3-NEDD8 complex (Figure.21A). In the ATP binding pocket, ATP has close proximity to NEDD8's C-terminus[31]. There are 15 residues that interact with ATP, out of which one residue, Arg15, is from APPBP1 and the rest residues are from UBA3 (Figure.21A).). We therefore decided to mutate the Arg15 of APPBP1to Ala. The mutant, APPBP1 (R15A), did not completely affect the NEDD8 activation and resulting conjugation to Ubc12, but with slower activation kinetics and activity (comparing the red and yellow curves in Figure.21B). The mutant APPBP1 (R15A) did promote a low affinity complex of NEDD8 with Ubc12, which was not promoted by UBA3 itself (comparing purple and light blue curves in Figure.21B). These discoveries suggest that Arg15 in APPBP1 contributes partially to the ATP binding, but not essential for catalytic activity of ATP hydrolysis. This may be explained by the fact that only one residue from APPBP1 participates in interacting with ATP, where as 14 residues of UBA3 interacts with ATP.

The binding of APPBP1 with Nedd8 is not required for its full activation, but contributes to its rapid activation. In the E1-NEDD8-ATP complex, both APPBP1-UBA3 interacts with NEDD8. We then investigate the requirement of the interaction between APPBP1 with NEDD8. By analyzing the interacting interface of APPBP1 with NEDD8, three mutant sets of APPBP1 with increasing mutations, N273A, T236A.K240A, T236A.K240A.N273A, were generated by PCR-mediated site-directed mutagenesis. Not too surprisingly, all three mutants, APPBP1 (N273A), APPBP1 (T236A.K240A), and APPBP1 (T236A.K240A.N273A), did not affect the activation of NEDD8 (Fig. 22A-C), however reaching the same plateau as wild type APPBP1. Within three mutant sets, APPBP1 (T236A.K240A.N273A) mutant resulted in the most delay in NEDD8 activation (Figure. 22C). Mutations in APPBP1 interface interacting with NEDD8 did not drastically affect NEDD8 and APPBP1 non-covalent interactions (Figure. 22D). However, these results do portray that APPBP1 and NEDD8 interaction may be important for the initial NEDD8-E1 complex formation but are not important for NEDD8s activation, as upon mutating the APPBP1-NEDD8 binding interface, the activation is delayed but not inhibited or lowered.

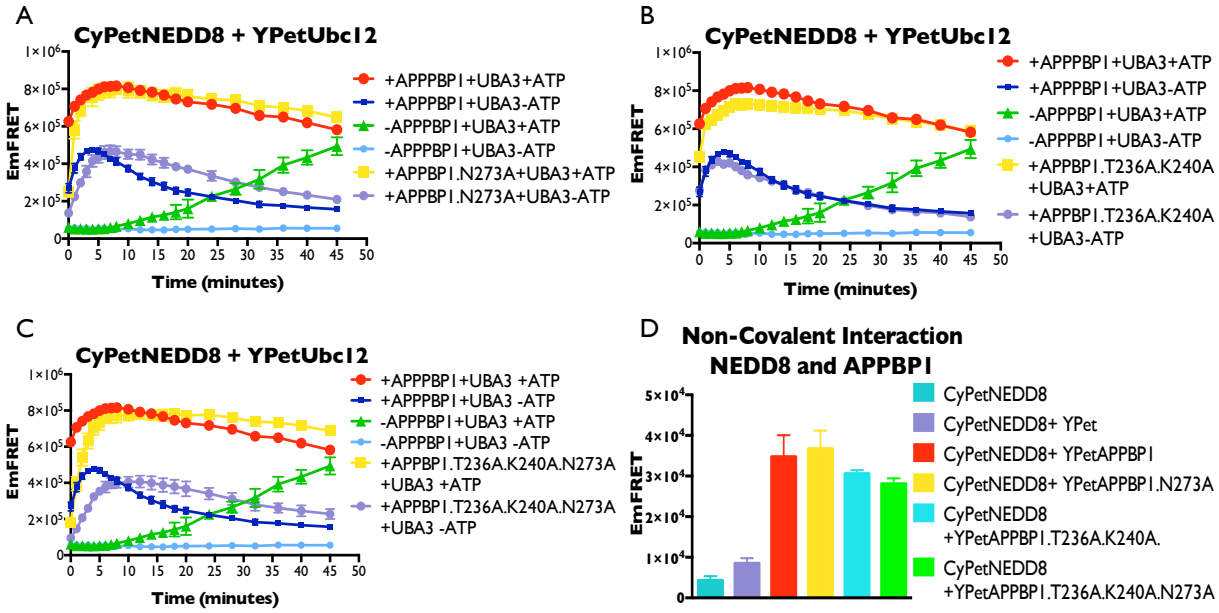


Figure 22. Noncovalent interactions between APPBP1 and NEDD8 are not critical for NEDD8 activation and conjugation. (A-C) FRET based protein assay for determining importance of APPBP1 and NEDD8 non-covalent interface for NEDD8 activation and conjugation to Ubc12. (D) Effects of mutations in APPBP1 on APPBP1-NEDD8 non-covalent interactions (data are represented as mean +/- SD).

The binding of UBA3 to Nedd8 is required for its activation. From the crystal structure of NEDD8 and E1 complex, we can clearly see, how beautifully NEDD8 C-terminal tail embarks into the UBA3 grove, close to the nucleotide-binding pocket[31,32]. We wanted to investigate if the NEDD8 interactions with UBA3 are important for NEDD8 activation. Three mutant sets of UBA3, V344A, V344A.Y352A, V344A.Y352A.E266A, were generated. UBA3 (V344A.Y352A.E266A) mutant completely prevented NEDD8 activation, even in the presence of APPBP1 and ATP (Fig.23C). Where as UBA3 (V344A.Y352A) mutant partially repressed the NEDD8 activation, as the FRET signal was even lower with respect to FRET signal of NEDD8 activation when

APPBP1 was not present (Fig.23B). UBA3 (V344A) mutant was partially able to slower the NEDD8 activation (Fig.23A), but V344A mutation in combination with E266A and Y352A mutations, does prevent NEDD8 activation completely (Fig.23C). Mutations in UBA3 interface interacting with NEDD8 did affect NEDD8 and UBA3 non-covalent interactions (Fig. 23D). These results showcase that UBA3 and NEDD8 non-covalent interactions are critical for NEDD8 activation, which is the most important and first step in the NEDD8 conjugation cascade.

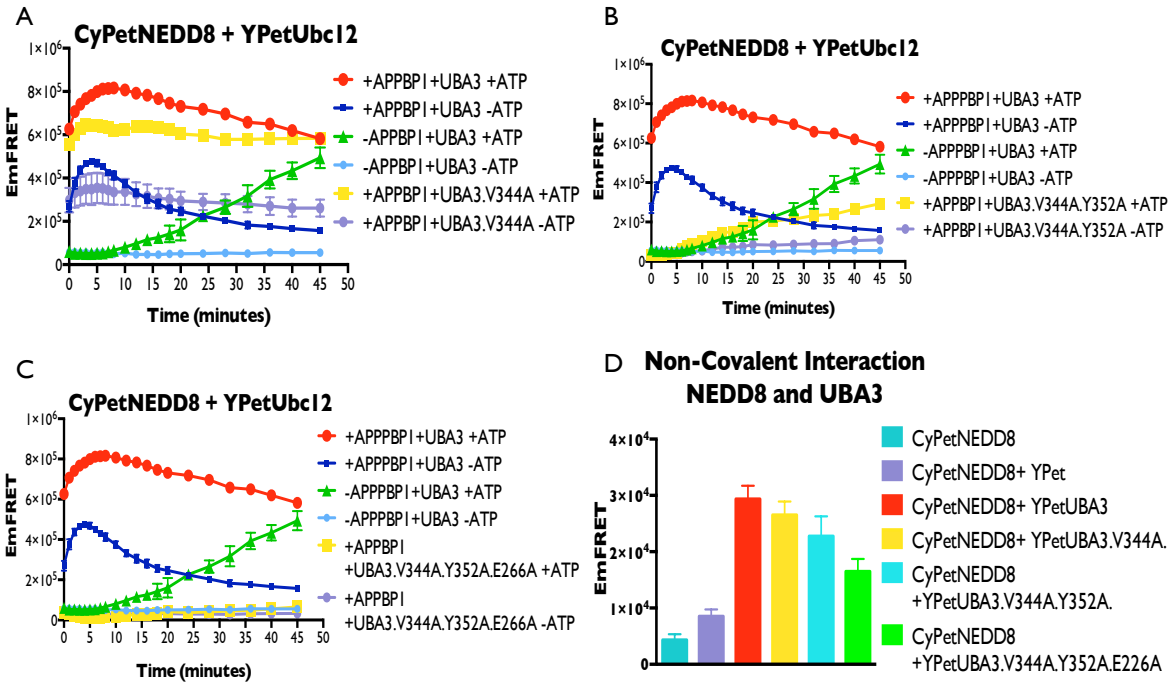


Figure 23. Non-covalent interactions between UBA3 and NEDD8 are critical for NEDD8 activation and conjugation. (A-C) FRET based protein assay for determining importance of UBA3 and NEDD8 non-covalent interface for NEDD8 activation and conjugation to Ubc12. (D) Effects of mutations in UBA3 on UBA3-NEDD8 non-covalent interactions (data are represented as mean +/- SD).

The interaction of APPBP1 with UBA3 is not required for its activation, but contributes to its rapid activation. In Ubl conjugation cascade, in case of bacteria or eukaryotes, we have seen that nature has chosen either one or two modular enzymes, which are required for the Ubl activation in the enzymatic cascade. As seen, in case of MoaD-MoeB pathway, two MoeB come together to form a homodimer, and act as the activation enzyme for MoaD. In case of Ubiquitin and ISG15, one single polypeptide, which mimics modularity of the MoeB homodimer structure and in case of SUMO and NEDD8, two enzymes come together to form a heterodimer, in order to mimic the modularity of the E1 enzyme. This indicates that, these two N-terminal domain and C-terminal domain of the E1 family are some how important and required for their activity. So we wanted to examine if the E1 heterodimer (APPBP1 and UBA3) interaction important for the NEDD8 activation. To assess, whether this interaction was important, we mutated the UBA3 interaction interface in APPBP1. Four mutant sets of APPBP1, D331A, E43A.D331A, D331A.K506A, E43A.D331A.K506A, were generated. APPBP1 (D331A) mutant slowed the initial NEDD8 activation, but it was not able to completely abolish NEDD8 activation and conjugation (Figure.24B). Where as APPBP1 (D331A.K506A) and APPBP1 (E43A.D331A), resulted in partial repression of NEDD8 activation (Figure.24C-D). However, interestingly APPBP1 (D331A.E43A.K506A) mutant was able to constrain NEDD8 activation to great extent as compared to the NEDD8 activation in absence of APPBP1 but presence of UBA3 (Figure.24E). This is an interesting finding as well, as in absence of APPBP1, UBA3 by itself is still able to activate NEDD8 and conjugate to its

E2, Ubc12. However, in presence of APPBP1 (D331A.E43A.K506A) mutant, UBA3 is partially able to activate NEDD8. Mutations in APPBP1 interface interacting with UBA3 did affect APPBP1 and UBA3 non-covalent interactions as well (Fig. 24F), with APPBP1 (D331A.E43 A.K506A) mutant reducing the affinity for UBA3 the most.

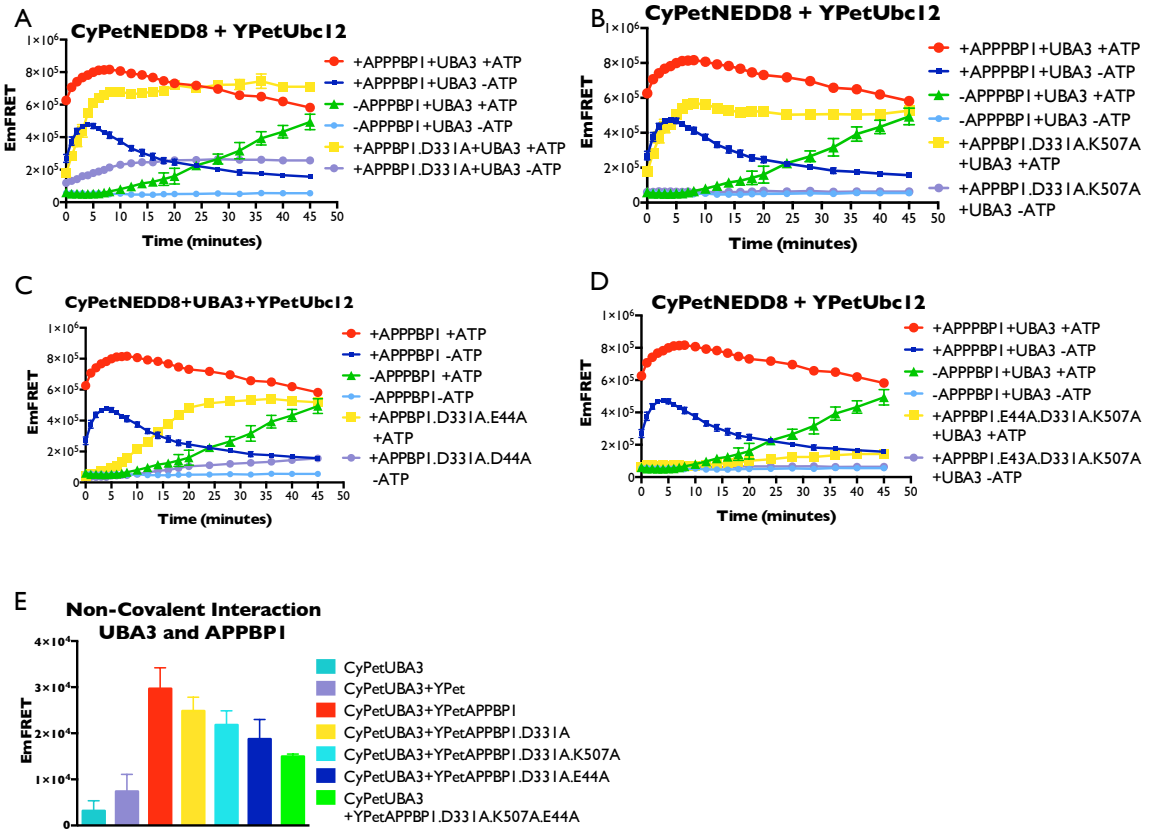


Figure 24. Non-covalent interactions between APPBP1 and UBA3 are partially critical for NEDD8 activation and conjugation. (A-D) FRET based protein assay for determining importance of APPBP1 and UBA3 non-covalent interface for NEDD8 activation and conjugation to Ubc12 F) Effects of mutations in APPBP1 on APPBP1-UBA3 non-covalent interactions (data are represented as mean +/- SD).

4.3 Discussion

Our results, for the first time, provide a systematic and detail investigations about the requirements of each subunit of NEDD8's E1 heterodimer in NEDD8 activation. Here we report, APPBP1, a subunit of E1 heterodimer, is not absolutely critical for NEDD8 activation, but rather plays a scaffold role that helps the kinetics of activation. As from the structural homology studies, it has been shown that APPBP1 is homologous to the N-terminal domains of Uba1 and AOS1[9,10]. Uba1 is single polypeptide as Ubiquitin E1 activating enzyme, where as heterodimers of AOS1 and Uba2, and APPBP1 and UBA3, for SUMO and NEDD8 activations, respectively, together form the counterparts of Uba1. We have previously reported, that AOS1 and Uba2 both are required for the SUMO activation and its transfer to Ubc9 (SUMO E2)[27]. Thus, the capability of UBA3 being able to activate and conjugate NEDD8 to its E2 conjugating enzyme in the absence of APPBP1, although with slower kinetics, is unique to NEDD8 conjugation cascade (Figure.20). This discovery is consistent to previous report that in plant system, ECR1 (UBA3 counterpart in plants) is able to generate the NEDD8-adenylate intermediate in the absence of AXR1 (APPBP1 counterpart in plants)(del Pozo and Estelle, 1999). But in these studies, the roles of ECR1 and AXP1 and molecular contributions to NEDD8 activation were not dissected in detail. Thus, even all the E1 being structurally and functionally homologous, UBA3 being able to activate and conjugate NEDD8 to its E2 in the absence of APPBP1, although with slower kinetics, is unique to NEDD8 conjugation cascade.

Our results indicate that, APPBP1 is more of scaffold protein, where as UBA3 has all the critical catalytic activity. As in case of the E1's ATP binding pocket, only one residue (Arginine 15) from APPBP1 and rest 14 residues from UBA3 contribute to ATP binding pocket (Figure.21). Also APPBP1 and NEDD8's non-covalent interactions are not that important for the NEDD8 activation, as seen from the NEDD8 activation and conjugation FRET based assay and NEDD8-APPBP1 non-covalent FRET assay (Figure.22). By mutating the APPBP1 interface (interacting with NEDD8), does not drastically effect on NEDD8 activation and subsequent conjugation to its E2, Ubc12, it only results in some initial delay in the complex formation, but reaches the same FRET signal plateau as compared to when both APPBP1 (wt) and UBA3 (wt) are present. Where as UBA3 and NEDD8 non-covalent interactions play a critical role for NEDD8 activation (Figure.23). In both cases of NEDD8 conjugation assay and non-covalent interaction assay, when UBA3 interface (interacting with NEDD8) was mutated, it resulted in complete inhibition of NEDD8 activation and conjugation to its E2 and also resulted in decrease in affinity between NEDD8 and UBA3. UBA3 plays important role of initial binding with NEDD8 (Ubl identification), adenylation and priming NEDD8 for E2 conjugation and binding to E2, even in the absence of APPBP1. As APPBP1 by itself cannot activate NEDD8 and cannot result in E2 conjugation, but the presence of APPBP1 accelerates the activation reaction, indicating that APPBP1 has scaffold properties than enzymatic function.

However, the interaction between APPBP1 and UBA3 is important for stabilization of the complex. In the NEDD8-E2 conjugation assay, when amino acids on APPBP1 interface interacting with UBA3 were mutated (APPBP1 D331A, E43A, K506A), the NEDD8 activation and conjugation to its E2 was almost completely abolished (Figure.24D). This was an interesting finding, as when APPBP1 is not present UBA3 can activate NEDD8 and aid in NEDD8-E2 conjugation (Figure.20). This phenomenon can be partially explained by the structural analysis of the NEDD8-UBA3-APPBP1 complex. The mutations in APPBP1 result in some unfavorable local microenvironment for residues in UBA3. The E44 of APPBP1 interacts with the K56 of UBA3 through a salt bridge (distance is ~3.5Å), but when E44 is mutated to Alanine, it may result in another unfavorable interaction between UBA3's K56 and APPBP1's H491, leading to conformational changes of Uba3 and its activities (Figure.25A). Similarly, in case of the other two mutations K507A and D331A in APPBP1, they may cause a similar perturbation in the microenvironment of UBA3's D326 and R223, respectively (Figure.25 B, C). Also interestingly, we further noticed that UBA3's K56 is close to ATP binding pocket, R223 is close to catalytic Cysteine and D326 is close to NEDD8's c-terminal tail binding pocket. We therefore reasoned that, combination of these three residues of APPBP1 (D331, K507, E44) are important for stabilizing APPBP1 and UBA3 complex and UBA3 activity, as when the three charged residues in APPBP1 were mutated to a neutral amino acid, alanine, it may results in conformational changes for UBA3 and thus further effects its enzymatic activity.

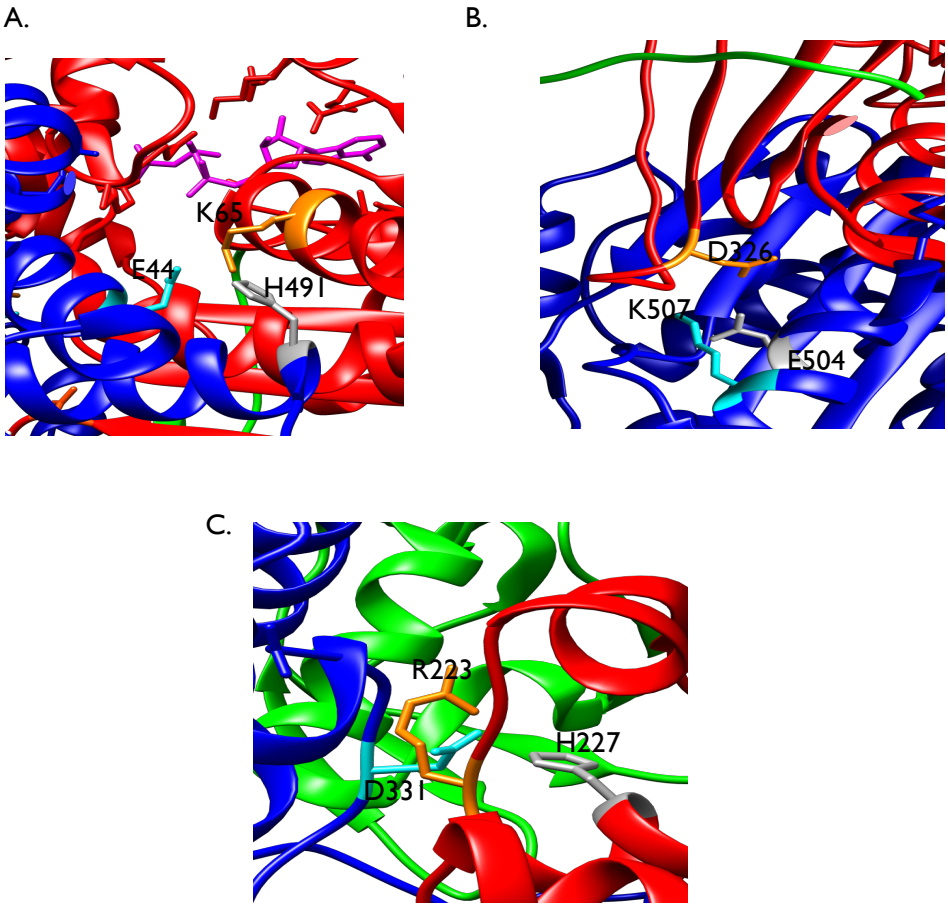


Figure.25 Close up view of APPBP1 and UBA3 interface, (PDB ID: 1R4N), UBA3 shown in red, APPBP1 in blue, NEDD8 in green, ATP in magenta, APPBP1 residue in cyan, UBA3 residue in orange, possible unfavorable interaction residue in grey. (A) Possible interactions interface between APPBP1's E44 and UBA3's K65. In case of E44A mutation, UBA3's K56 and APPBP1's H491 have unfavorable interactions. (B) Possible interactions interface between APPBP1's K507 and UBA3's D326. In case of K507A mutation, UBA3's D326 and APPBP1's E504 have unfavorable interactions. (C) Possible interactions interface between APPBP1's D331 and UBA3's R223. In case of D331A mutation, UBA3's R223 and UBA3's H227 have unfavorable interactions.

Our discoveries also provide valuable clues for potential evolution pathway of Ubl conjugating cascades, such as Ubiquitin, SUMO, NEDD8 and ISG15. Ubiquitin and Ubls. Their activating enzymes appear to have evolved from primitive biosynthetic pathway, MoaD-MoeB (molybdopterin biosynthesis) and ThiS-Thif (thiamin biosynthesis) pathway[10,16,17]. MoaD and ThiS are structurally homologous to ubiquitin. Like Ubiquitin, MoaD and ThiS are adenylated at their C-termini by MoeB and ThiF, respectively, instead of being conjugated to substrates, they are employed for carrying atoms at their C-termini for their respective biosynthetic pathways[16-19]. MoeB and ThiF, the activation enzymes for MoaD and ThiS, respectively, have sequence and structural homology to E1s of Ubiquitin and Ubls[16,20-22]. The sequences and structures of MoeB and Ubl-activating enzymes suggest that they are modular enzymes with a common domain involved in Ubl adenylation and specific domains to carry out other particular functions in case of Ubl E1s. The common feature between MoeB and Ubl E1s is C-terminal adenylation of an Ubl. The interesting point to be noted is that MoeB and ThiF, form a functional homodimer for their enzymatic activities, where as Ubiquitin's E1 and ISG15's E1 evolved as single polypeptide and SUMO's and NEDD8's E1 evolved as heterodimer of two polypeptide, all mimicking the structural modules of MoeB and ThiF (Figure.26). Also in MoeB and ThiF, the two structural homology domains are separated out by short sequence of eight residue[8,10,20,22], but in case of UBA1, APPBP1 and UBA3, AOS1 and Uba2, there are large insertions between these two structural regions, which are homologous to MoeB. And it can be understood that these evolutionary insertions correspond to new domains, which are for the evolved E1- specific functions, such as

forming a thioester bond with activated Ubl, binding to their cognate E2 and transfer of Ubl to the catalytic cysteine of respective E2 [9-11].

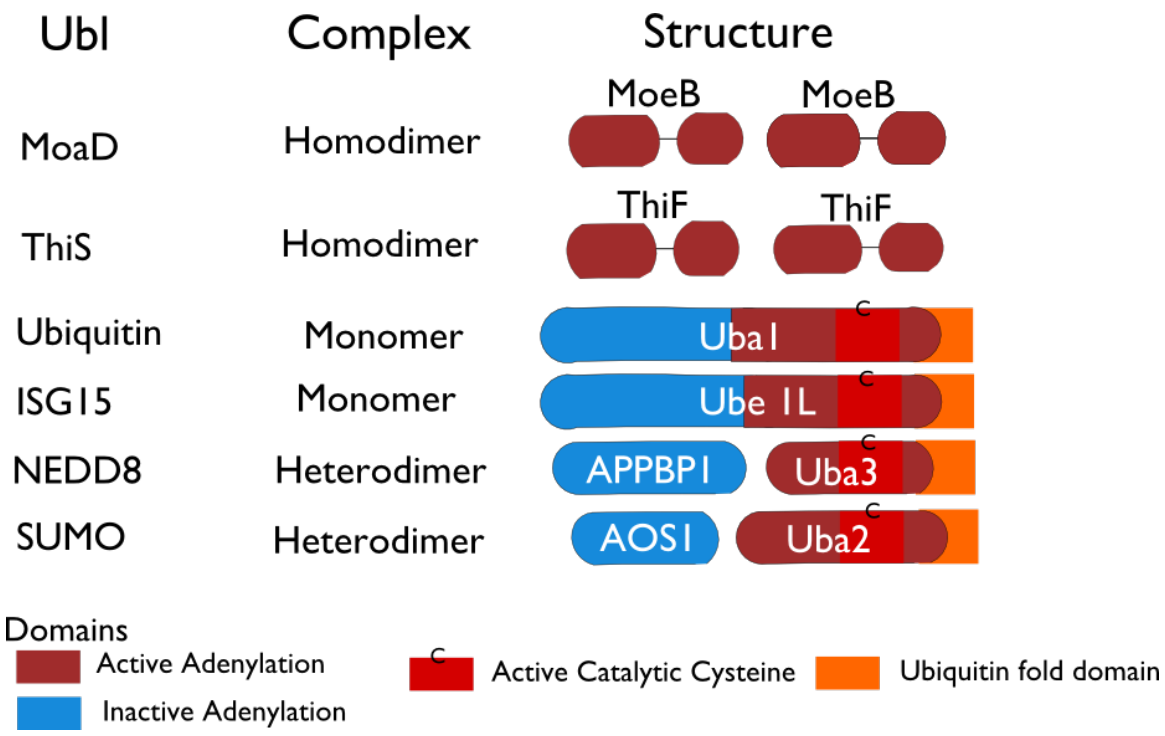


Figure.26 Comparison of activating enzyme for Ubiquitin and Ubiquitin like proteins with MoaD and ThiF activating enzyme. The four ubls whose E1s are most closely related are ubiquitin, SUMO, NEDD8 and ISG15 and their family members. The Ubl E1s are 110-120kDa and are either single polypeptide or heterodimer of two polypeptides, corresponding to the sequence and structure of MoeB. The E1 of Ubiquitin and ISG15 are single polypeptides. In case of heterodimeric E1s (SUMO and NEDD8 cascade), one subunit corresponds to N terminal half of the single-chain E1s and other subunit of the heterodimer corresponds to C-terminal of half. The N and C-terminal halves of the E1s for ubiquitin, SUMO, NEDD8 and ISG15 are partially homologous to each other, and the region of sequence homology between the two halves is also the region of sequence homology to MoeB and ThiF.

The crystal structures of E1 of NEDD8 and MoeB indicate that Ubl E1s have three domains: an evolutionary conserved domain resembling the MoeB (for identifying Ubl and Ubl c-terminal activation) and two evolved eukaryotic E1-specific domains (for thioester linkage formation with its catalytic cysteine and binding to its E2 and transfer

of Ubl to E2's catalytic cysteine)[8-10,15,22]. Sequence homology among E1s and E2s of Ubiquitin, NEDD8, SUMO and ISG15 suggests potential similar mechanism for their activation and conjugation step. However, E1s for Atg8p/Atg12p and Urm1p have different sequences from Ubiquitin's E1, thus implying potential divergent mechanisms[8-10]. The different extensions and insertions in the MoeB/ThiF-like sequences of Ubl-activating enzymes reflect upon the addition of different functionalities required for the different Ubl-conjugation cascades.

It is beautiful how nature evolves different modules for specific functions as required by the environment, as in case of bacterial, MoeB and ThiF function in basic biosynthetic pathways, where as in case of eukaryotes, Ubl's activation enzyme play very critical role of activating "right" Ubl for downstream conjugation, which further have a essential roles in different cell cycle regulation and development. This study leads to an interesting evolutionary link between the evolutions of E1 activation enzymes of Ubiquitin and Ubiquitin like proteins. Ubiquitin and Ubiquitin like proteins have a conserved mechanism of activation and conjugation to their respective targets. Ubiquitin and ISG15 have a single polypeptide E1 activation enzyme (Uba1 and Ube1L respectively) and in case of NEDD8 (APPBP1 and UBA3) and SUMO1 (AOS1 and Uba2), two polypeptides form a heterodimer mimicking N-terminal and C-terminal domain of single polypeptide. Here we showcase that APPBP1 is more of scaffold protein, but in case of SUMO both AOS1 and Uba2 are required for SUMO activation and in case of Ubiquitin, Uba1 is a single polypeptide. This leads to an open-end question about evolution link in the E1 enzymes, leading two scenarios of gene-split: Uba1 gene

split to two functional genes leading to NEDD8's E1 and SUMO1's E1 or gene fusion: NEDD8s E1 (APPBP1 and UBA3) fuse to lead to UBA1 or further evolved to lead to SUMO1's E1 (AOS1 and Uba2).

Our FRET based technology is able to decipher the mechanism of Ubiquitin and Ubiquitin like proteins (SUMO and NEDD8) in detail, differentiating covalent conjugation from non-covalent interactions and understanding the effects of mutation on protein-protein interactions in the enzymatic cascade in real time. In past, we have deciphered Ubiquitination and SUMOylation cascade intermediates and dynamics in real time[26,27]. To reveal the intermediate transient complex in the conjugation cascade is hard to determine using traditional approaches like western blot or radiolabeling, as the thioester bonds are high-energy bonds thus chemically unstable and have short half-life. We have successfully employed our quantitative FRET-based technology to determine enzymatic reaction dynamics, reaction intermediates, substrate/enzyme specificity, transient molecular interactions, and conformational changes in Ubiquitin, SUMO and NEDD8 conjugation cascades.

4.4 Material and Methods

DNA constructs

The open reading frames of CyPet and YPet were amplified by using forward and reverse primers containing NheI and SalI sites, respectively, and the open reading frames of NEDD8, APPBP1, UBA3 and Ubc12 were amplified by PCR using forward and reverse primers containing SalI and NotI sites, respectively. All PCR products were

cloned into the pCRII-TOPO vector (Invitrogen). After their sequences were confirmed, the genes encoding NEDD8 and NEDD8 ligases were ligated into linearized pCRII-CyPet or pCRII-YPet plasmids. After the sequences were confirmed, the cDNAs encoding fusion proteins were digested with NheI/NotI and cloned into the NheI/NotI sites of the pET28(b) vector (Novagen). To get non-fluorescent tagged proteins, the open reading frames of NEDD8 and NEDD8 enzymes were extracted by SalI/NotI digestion and cloned into the SalI/NotI sites of the pET28(b) vector. CyPet-linker3-NEDD8 was cloned as described earlier[30]. In our study, we used full-length cDNA for all NEDD8, APPBP1, UBA3 and Ubc12. The mutant forms of APPBP1and UBA3 were created by PCR site-directed mutagenesis and cloned into the pET28(b) vector, following the protocol described above. The mutations in APPBP1 for NEDD8 interface and in UBA3 for NEDD8 interface were selected by closely investigating the X-ray crystal structure (1R4N)[31] and mutations in APPBP1 for UBA3 interface and one mutation in UBA3 and NEDD8 were selected based on AESOP analysis, described below.

We selected mutations in the protein interfaces based on electrostatic calculations. Calculations were performed using integrated Analysis of Electrostatic Similarities of Proteins (AESOP) framework to delineate the role of charged amino acids in binding[33-44]. Such an approach is essential for elucidating protein-protein interactions by performing computational mutagenesis and alanine scans as a means of perturbation to quantitatively assess the impact of each mutation to the stability of protein complexes. The coordinates of the complex were obtained from the Protein Data Bank

(PDB code: 1R4N). The program PDB2PQR was used to add atomic radii and partial charges to the atomic coordinate file using the PARSE force field[36,44]. The radii and charges were used to calculate the electrostatic potential and free energies by numerically solving the linearized Poisson-Boltzmann equation with the program Adaptive Poisson-Boltzmann Solver (APBS)[35].

The molecular (dielectric boundary) and ion accessibility surfaces were determined using spherical probes and radii set to 1.4 and 2.0 Å respectively. The APBS calculations were performed on a grid containing 225 x 225 x 225 grid points with grid dimensions of 204 Å x 202 Å x 154 Å. The dielectric coefficient for the solvent and protein interior for each complex were set to 78.54 and 20, respectively[40]. Two electrostatic potential calculations were performed with different counter ion concentrations of 0 mM and 150 mM.

We then use the electrostatic calculation to calculate the pairwise similarities following electrostatic similarity distance (ESD) equation[33,38],

$$ESD = \frac{1}{N}\sum_{i,j,k}\frac{|\varphi_B(i,j,k)-\varphi_A(i,j,k)|}{\max(|\varphi_B(i,j,k),\varphi_A(i,j,k)|)} \qquad \text{(Equation 1)}$$

In Eq. (1), φ_A and φ_B refer to electrostatic potential at grid point (i, j, k) in proteins A and B, respectively, and N represents the total number of grid points at which electrostatic potential has been calculated. A pairwise comparison of the distance matrix was generated. An ESD value of 0 denotes identical electrostatic potentials, and as the ESD value increases, the dissimilarity in electrostatic potential increases.

The resulting complexes were hierarchically clustered in a dendrogram depending on their ESD value. Free energies of association were also calculated according to the

thermodynamic cycle illustrated in (Figure.27). To incorporate the effect of association and solvation, $\Delta G^{solution}$ is calculated according to the following equations[38,40,39].

$$\Delta G^{solu} - \Delta G^{ref} = \Delta G_{AB}^{solvation} - \Delta G_{A}^{solvation} - \Delta G_{B}^{solvation} = \Delta\Delta G^{solvation}$$

(Equation 2)

$$\Delta G^{solu} = \Delta\Delta G^{solvation} + \Delta G^{coulomb}$$ (Equation 3)

In these equations, AB refers to the protein complex APPBP1 and the heterodimer NEDD8-UBA3. Similarly mutation E266A was selected in UBA3 (for NEDD8 interface). Mutations in APPBP1 for UBA3 interface and one mutation in UBA3 and NEDD8 were selected based on AESOP analysis (Figure.28 A and B respectively)

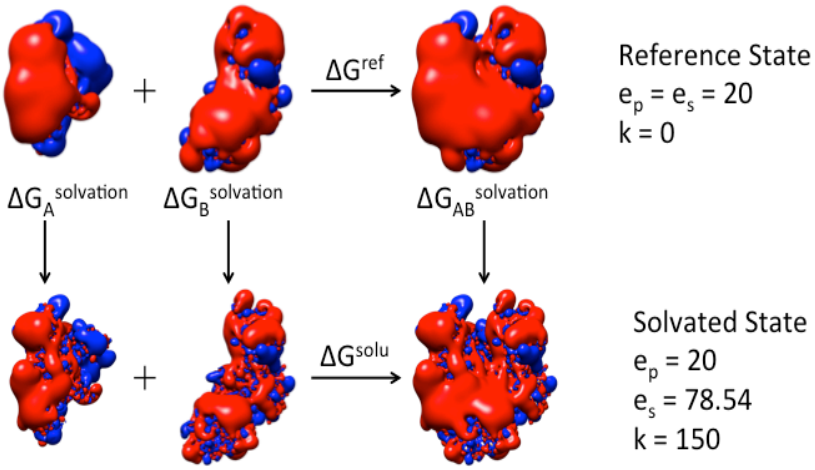


Figure.27 Thermodynamic cycle for calculation of electrostatic free energy of association and solvation. The iso-potential contours illustrated are the spatial distribution of electrostatic potential. Blue and red surfaces represent iso-values of ±1*k**B**T*/e, respectively, where *k**B*** is the Boltzmann constant, T is temperature, and e is the unit charge of an electron. The proteins A, B, and AB in the figure are APPBP1, UBA3, and APPBP1-UBA3 complex respectively. The top process models the reference state (dielectric coefficient of 20 for both proteins in interior and exterior and no counter ions) and the bottom process models the association step in a solvated state with dielectric coefficient of 20 for protein interior, 78.54 for protein exterior, and 150mM concentration of counter ions. The three vertical processes show solvation of each free protein and complex.

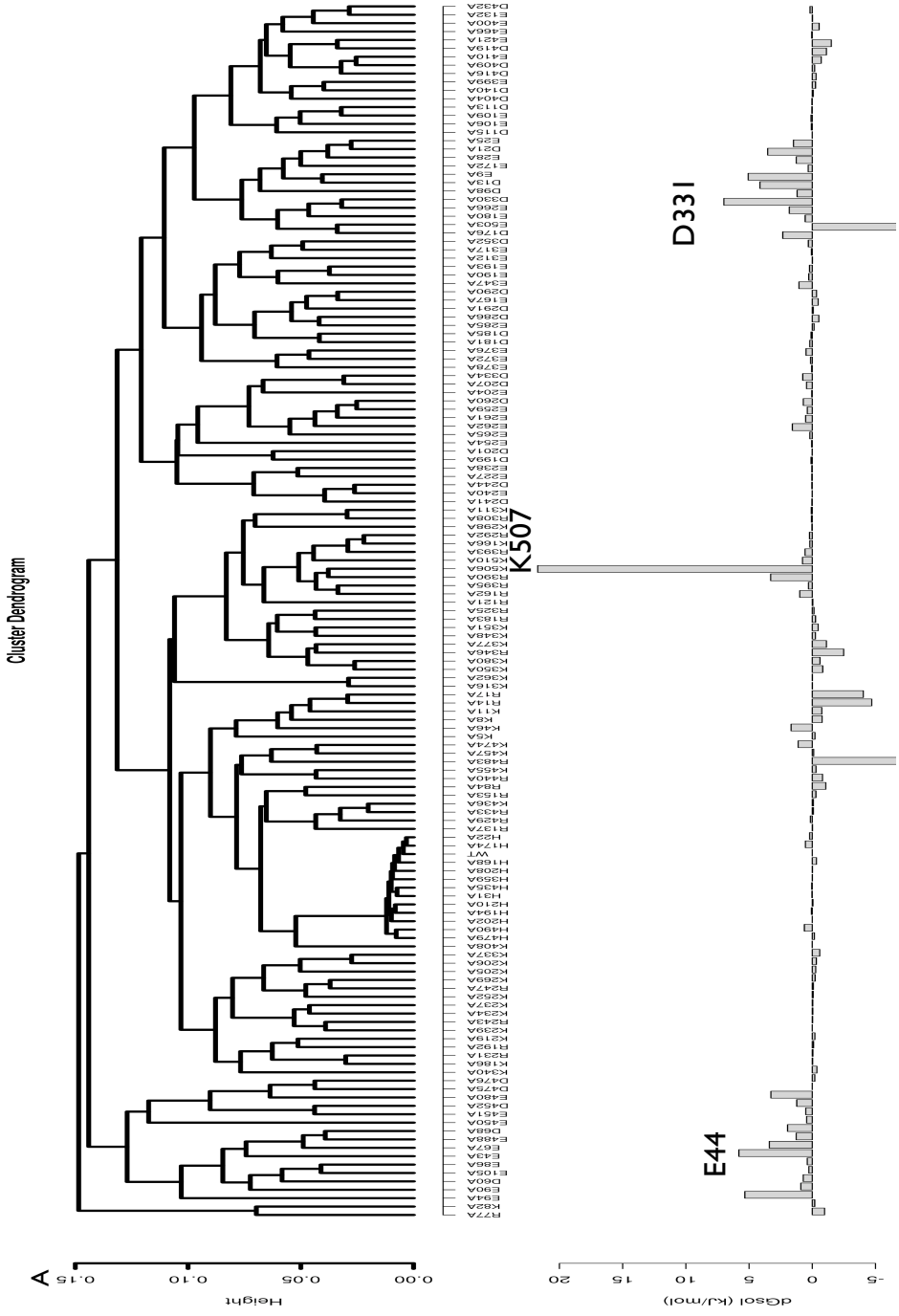


Figure.28 (A) Alanine scan electrostatic clustering and free energies of association of APPBP1 and UBA3. Clustering dendrogram of the alanine scan mutants of APPBP1 using the average weighted difference ESD. Free energy of mutants ordered according to average weighted difference clustering.

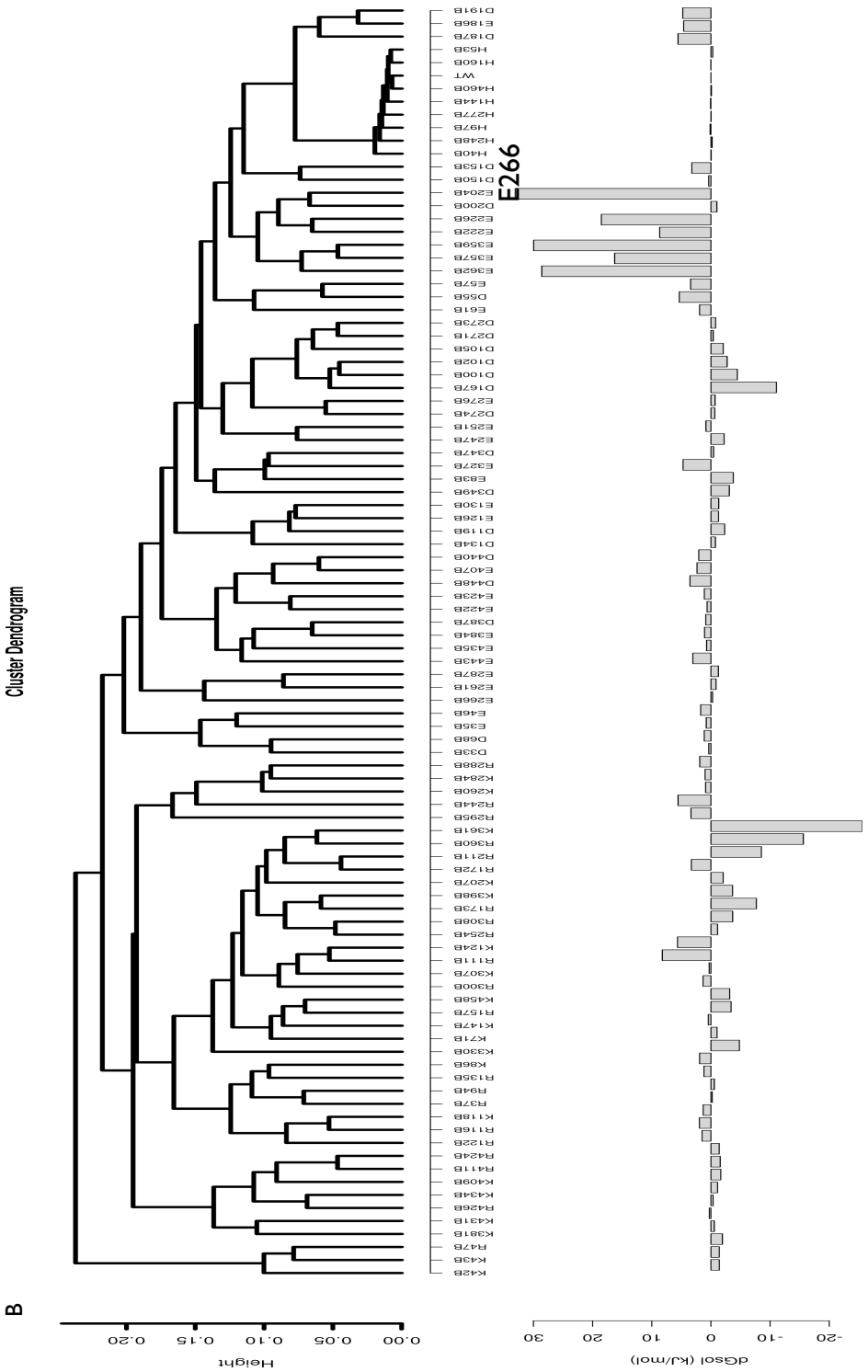


Figure.28 (B) Alanine scan electrostatic clustering and free energies of association of UBA3 and NEDD8. Clustering dendrogram of the alanine scan mutants of UBA3 using the average weighted difference ESD. Free energy of mutants ordered according to average weighted difference clustering.

Protein expression and purification

BL21(DE3) Escherichia coli cells were transformed with pET28 vectors encoding NEDD8, APPBP1, UBA3, Ubc12, their mutants, and fluorescent protein-labeled versions. The transformed bacteria cells were plated on LB agar plates containing 50μg/mL kanamycin, and a single colony cultures were inoculated in 2xYT medium. The expression of poly histidine-tagged recombinant proteins, was induced with 0.2 mM IPTG at 25°C overnight. Bacterial cells were collected by centrifugation at 8,000rpm 5min, resuspended in buffer containing 20mM Tris–HCl, pH 7.5, 500mM NaCl and 5mM imidazole, and sonicated with an ultrasonic liquid processor (Misonix, Farmingdale, NY). Cell lysate containing recombinant proteins was cleared by centrifugation at 35,000g for 30min. The recombinant proteins were then bound to Ni^{2+}-NTA agarose beads (QIAGEN, Valencia, CA) and eluted with buffer containing 20mM Tris–HCl, pH 7.5, 200mM NaCl, and 150mM imidazole. After the proteins were dialyzed into buffer containing 20mM Tris–HCl pH 7.5, 50mM NaCl, and 1mM DTT. SDS–PAGE and Coomassie blue staining confirmed the purity of the proteins. Protein concentrations were determined using Coomassie Plus Protein Assay (Pierce) and concentrations of fluorescent-tagged proteins were determined using fluorescent protein concentration standard curves.

FRET measurements

After the proteins labeled by CyPet or YPet were mixed in the presence or absence of other protein members or cofactors, their fluorescence was determined by

FRET measurements

After the proteins labeled by CyPet or YPet were mixed in the presence or absence of other protein members or cofactors, their fluorescence was determined by the fluorescent plate reader Flexstation II^{384} (Molecular Devices) in a 384-well plate. The emission intensities at three wavelengths were collected: 475 nm and 530 nm at an excitation wavelength of 414 nm, and 530 nm at an excitation wavelength of 475 nm. After the emission intensities were corrected by subtraction of background fluorescence from the plate, the EmFRET for the reactions were calculated and compared. To use FRET to monitor the change of protein-protein interaction status, we defined the EmFRET ($Em_{FRET} = Em_{total} - Cypet_{(cont)} - Ypet_{(cont)}$), (Figure.8, chapter.1)[27,28].

Protein interaction and in vitro NEDDylation assay

All reagents were purchased from Research Products International Corp unless otherwise specified. For all the respective FRET assay, 1μM CyPetNEDD8 was mixed with 1μM YPetUbc12, in presence or absence of APPBP1, APPBP1(mutants), UBA3 and UBA3(mutants)(1μM), in NEDDylation buffer (50mM Tris-HCl, 50mM NaCl, 10mM $MgCl_2$, 1mM DTT). Samples were incubated at 37°C and fluorescence emission was monitored right after buffer with or without ATP was added (2mM final concentration).

Western blot assay for NEDD8 conjugation to Ubc12

For the western-blot assay, 0.25μM CyPetNEDD8, 0.1μM APPBP1, 0.1μM UBA3 and 0.25μM YPetUbc12 were mixed in a buffered solution containing 50mM Tris–HCl

(pH 7.4), 50mM NaCl, 1mM DTT, 10mM MgCl2 and 1mM ATP with a total volume of 50μl. Samples were incubated at 37 °C for 10-120 minutes. The reactions were terminated by addition of non-reducing 4M urea/SDS-gel loading buffer. After they were separated by 10% SDS-PAGE and transferred to a NC membrane, the NEDD8 proteins were recognized by incubation with mouse anti-NEDD8 antibody (Santa Cruz Biotechnology) followed by HRP-labeled goat anti-mouse IgG antibody (Sigma Aldrich). The chemiluminescence was developed using the Supersignal® West Dura Extended Duration Substrate (Thermo Scientific) and two different exposure times, 60minutes for CyPetNEDD8~YPetUbc12 and 5 minutes for CyPetNEDD8, and captured by the Biospectrum® imaging system (UVP, LLC).

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Chapter 5: Engineering of Cullin1 towards FRET based high-throughput screening assay for NEDDylation cascade

5.1 Introduction

The cell is always in a dynamic state, having different signaling cascades (e.g., in response to various stimuli), and biosynthetic processes (e.g., DNA synthesis/ protein synthesis/ protein degradation), however the details underlying these mechanisms are still not completely understood. Proteosomal based degradation is one the complex signaling mechanisms. The Ubiquitin-proteasome system (UPS) is responsible for protein turnover and plays a critical role in maintaining cellular protein homeostasis[4,5]. Protein degradation plays an important role in maintaining specific protein levels and removing damaged/unwanted proteins[4]. Proteins that are degraded by the UPS, play a critical role in processes such as cell cycle regulation, cell growth and proliferation, intracellular signaling in response to external stimuli, membrane receptor regulation, and apoptosis. Therefore down-regulation of UPS signaling system can result in uncontrolled cell proliferation or up-regulation can increase certain pro-survival signaling and potentially result in the progression of cancer or disease[5,7].

Proteins targeted for UPS based degradation are usually marked by covalent attachment of a polyubiquitin chain. Ubiquitination of target substrate is achieved by systematic enzymatic cascade (involving E1, E2 and E3)[8-11], as discussed in Figure. 29. Ubiquitin has 2 E1s, several E2s and several 100 E3 ligases[12]. E3 ligases belong to 3 different classes based on the domain found in their core protein: i) HECT (homologous to E6-AP carboxyl terminus), ii) RING (Really Interesting New Gene) finger and iii) U-

box E3s. Within the RING finger E3s there are two subclasses, the simple RING finger E3s contain the E2 binding RING domain and substrate binding domain within the same polypeptide. The Cullin-RING ligases[2] (CRL) which are multicomponent complexes based upon a Cullin protein core tightly associated with the RING finger domain contain proteins and various adaptor and receptor proteins for substrate binding[8,11-15].

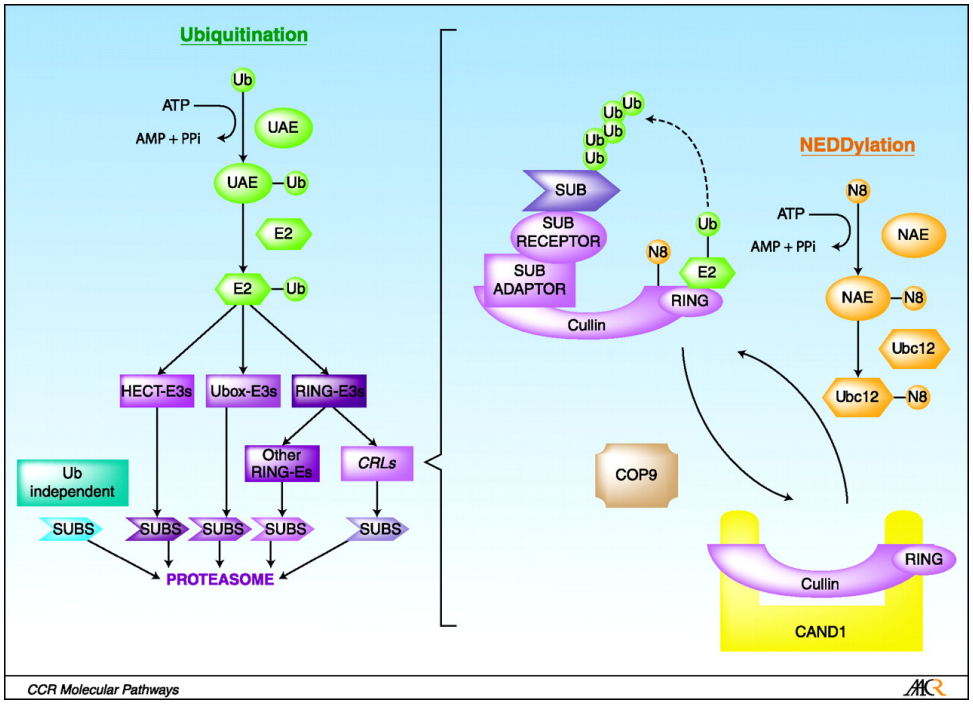


Figure 29. NEDD8 modification is required to activate cullin-RING ubiquitin ligases (CRLs). Protein degradation by the UPS is a highly regulated process. Proteasomes degrade proteins that are tagged with poly-ubiquitin chains. The formation of the poly-ubiquitin chain on a protein is catalyzed by a pyramidal cascade of enzymes; a E1-activating enzyme, E2 conjugation enzymes that in turn collaborate with specific E3 ligases to catalyze the formation of a poly-ubiquitin chain on a substrate protein recruited by that E3. E3 ligases can be subdivided into three broad subclasses; HECT, U-box, and RING-finger domain E3s with CRLs representing the largest subfamily of RING-finger E3s. A key feature of CRLs is that the cullin subunit must be modified on a conserved lysine by the ubiquitin-like protein NEDD8 to activate holoenzyme activity. NEDD8 activation and conjugation to cullin proteins is catalyzed via an enzymatic cascade that is homologous to ubiquitination. Removal of NEDD8 from Cullin is catalyzed by the COP9 signalosome. Deneddylation facilitates dissociation of CRL components. The cullin-RING core is sequestered in an inactive state by binding to CAND1 until it is recruited to form a new CRL[11].

CRLs were first identified in 1998[16,17] and CRLs regulate an array of eukaryotic cellular and organismal processes. CRLs are modular in nature and have an extended, rigid structure.; there are 8 different Cullins (Cul1, 2, 3, 4a, 4b, 5, 7 and 9(or PARC))[18], each has a curved yet rigid architecture and nucleates a multi-subunit ligase complex for ubiquitination and contains a conserved Cullin homology domain[2,13]. The adaptor protein binds to the N-terminal region of Cullin. Where as the RING finger domain protein (Rbx1/Rbx2) binds to the C-terminal of Cullin. The adaptor protein recruits substrate-binding receptor, where as the RING subunit recruits ubiquitin-conjugating enzyme (E2) to ubiquitinate target substrate[2,16,17,19,20].

It has been estimated that there are more that 300 CRLs combinations in human cells and they are responsible for designating 20% of cellular proteins for proteosomal degradation after Ubiquitin modification of the target substrate[21]. The 300 CRLs are organized through different combinations of Cullins, adaptor proteins, and substrate receptors (Figure.30)[2,17]. Adaptor proteins recruit substrate receptors and different Cullins bind to their own set of adaptor proteins, in turn each adaptor protein binds to various substrate receptor, which increase the number of substrate a single Cullin can affect.

The most well-studies target of NEDD8, Cullin family[22], plays many critical roles in cellular processes, cell cycle, signal transduction, transcription, protein quality control, DNA replication, viral modulation, development, circadian clock[3,17]. By controlling ubiquitination of various targets, Cullin Ring Ligases (CRLs) regulates the ubiquitination of various targets thereby controlling the fate of many proteins, including p27 and

CyclinE (cell cycle), Myc and pIkBa (transcription), Cdt-1 and E2F (DNA replication),b-catenin and AXR2/3 (signal transduction) , CD4(viral modulation)[14,17,23,24].

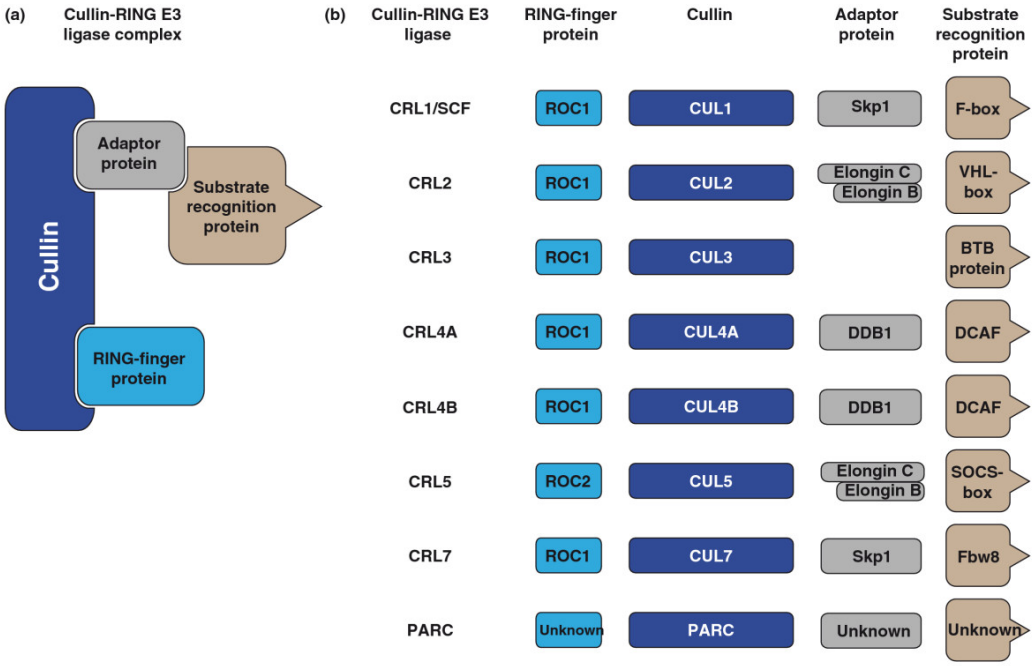


Figure 30. Modularity of cullin-RING E3 ligases. Cullin proteins are molecular scaffolds that assemble multi-subunit cullin-RING E3 ubiquitin ligase (CRL) complexes. The mammalian cullin protein family comprises eight members (CUL1 to CUL7 and PARC). In CRL, a cullin protein tethers both a substrate-recognition subunit, often through an adaptor protein, and the RING finger component. The cullin-organized CRL thus positions a substrate in close proximity to the RING-bound E2 ubiquitin-conjugating enzyme (not shown), which catalyzes the transfer of ubiquitin to the substrate. (a) General CRL composition. (b) Specific composition of the CRLs 1 to 7 and PARC. Abbreviations: BTB, Bric-a-brac, Tramtrack, Broad-complex domain; DCAF, DDB1-CUL4 associated factor; DDB1, DNA damage-binding protein 1; Fbw8, F-box and WD repeat domain containing protein 8; PARC, p53-associated parkin-like cytoplasmic protein; SOCS, Suppressors of cytokine signaling; Skp1, S-phase kinase-associated protein 1; VHL-Box, von Hippel-Lindau box[2,6].

The best-characterized substrate of NEDD8 cascade is the Cullin family of proteins[18,25]. All of the Cullins known so far are modified by the covalent attachment of NEDD8 to a conserved lysine residue in the Cullin-homology domain[26]. NEDDylation of

Cullin enhances the Cullin-Ring-Ligase activity[1,17,26,27]. Genetic studies also indicate that NEDDylation is important for he functions of Cul1, Cul2 and Cul3[17]. Each Cullin contain a key lysine residue at its C-terminus for targeted NEDDylation, which is required for CRL activity. Rbx and Dcn1 proteins are found to act as NEDD8 E3 ligases for Cullin[21].

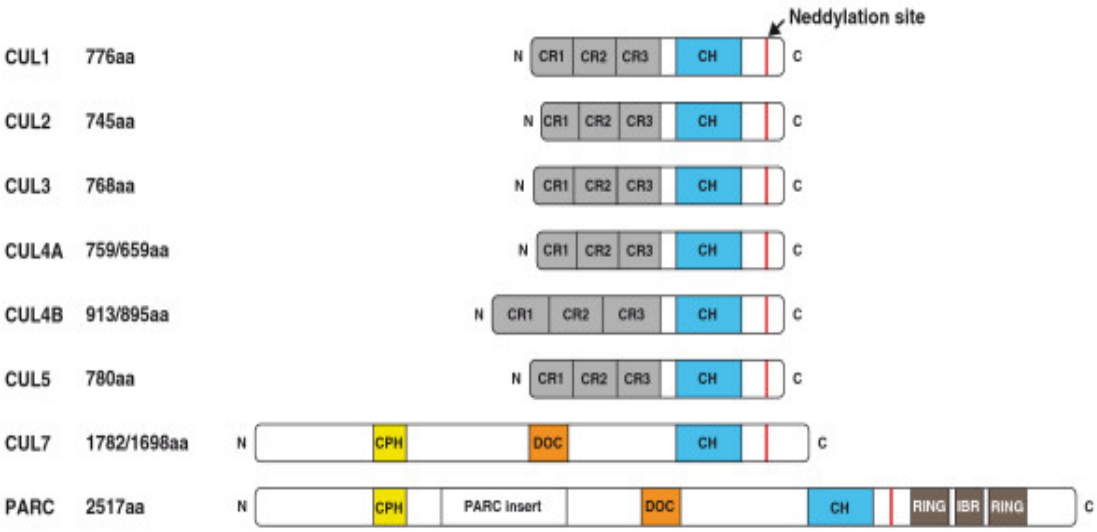


Figure 31. Cullin protein domain organization in humans. Cullin repeat 1 (CR1) anchors the cognate adaptor proteins, and the cullin homology domain (CH) at the carboxyl terminus is critical for binding of the RING-finger protein. The red line indicates the position of the NEDDylation site. Abbreviations: aa, amino acids; CH, cullin homology domain; CPH, conserved domain in CUL7, PARC and HERC2; CR, cullin repeat; DOC, a domain similar to the DOC1 domain of the anaphase-promoting complex/cyclosome but of unknown function; IBR, in between RING; RING, really interesting new gene[2,3].

Cullin NEDDylation is a process known to enhance the CRL activity, thus further resulting in ubiquitination of various substrates[1,26,27]. In the down-regulated mode, CAND1 are bound to non-NEDDylated Cullin, and block the assembly of substrate receptor adaptor module via its N-terminus. NEDDylation of Cullin at its C-terminal lysine residue, results in a conformational changes, which causes CAND1 to dissociate[19,20] (Figure.29). This dissociation a) increases and stabilizes ubiquitin-charged

E2 recruitment to CRLs[25], b) bridges a gap, approximately 50 Å, between the substrate docking site and the E2 active site[19,20] which greatly facilitates the initiation of ubiquitin transfer[25], and c) enhances the rate of ubiquitin chain elongation, suggesting that this conformation change also improves the activated E2 access to the end of the nascent polyubiquitin chain [25,27,28].

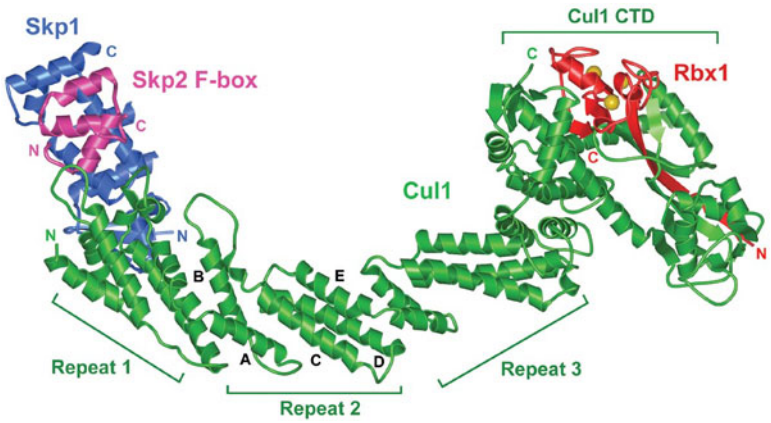


Figure 32. Structure of the Cul1–Rbx1–Skp1–F boxSkp2 SCF ubiquitin ligase complex[1].

As NEDDylation plays a critical role in many cellular processes, by modulating CRL functionality, and impairment of NEDDylation is a reason for different diseases[10,15,21,26,29], our goal is to develop FRET based high-throughput screening to identify bioactive chemical compounds, which can inhibit the activity of NEDD8 pathway. In order to develop a FRET based assay for NEDD8 conjugation to Cullin-1, our first aim was to develop a FRET based assay to detect covalent conjugation of NEDD8 to Cullin-1. Here we report engineering of Cullin1 for FRET based protein assay for HT-platform for drug screening for NEDDylation cascade.

5.2 Results

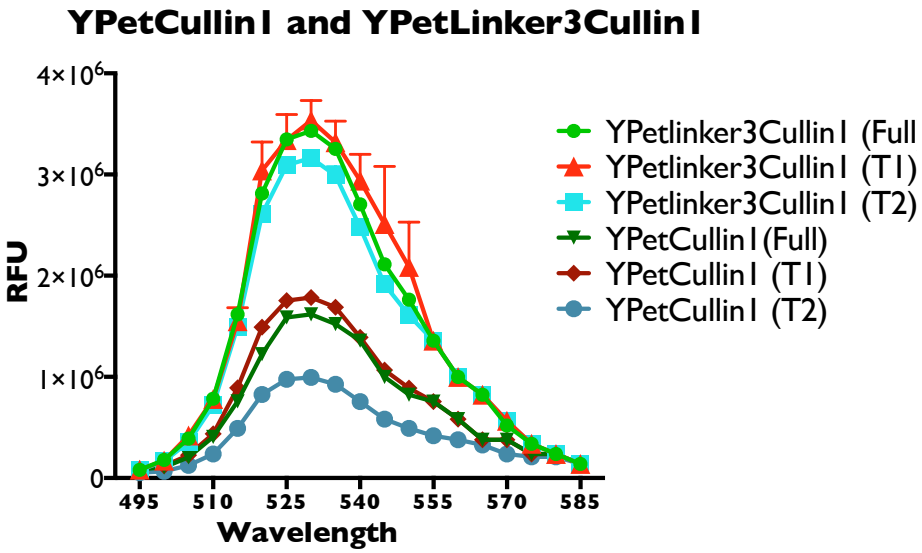
Structural analysis of Cullin1 and Engineering of Cullin1. In pursuit of developing a FRET based assay to detect NEDD8 conjugation to its substrate Cullin1, a direct fusion of Cullin1 to YPet led to low fluorescence (Figure.33), which was in big contrast to our previous attempt with SUMO target protein YPet-RanGap[30]. The low fluorescence of YPet-tagged to Cullin1 may be due to two reasons: i) large size of Cullin-1 protein, ii) improper folding of YPet-Cullin1 or both. Furthermore, using our FRET based protein assay we were not able to detect the NEDD8 conjugation to Cullin1 (in presence of E1, E2, E3 and ATP) (Figure.34). We speculated that the large size of Cullin1 and YPet conjugation at the N-terminal of Cullin1, might hinder the FRET signal because of the distance dependency. Therefore, to improve FRET signal we engineered Cullin1, by shortening the N-terminal region, to produce 324-776 (T1) and 534-776 (T2), polypeptides. We chose these truncation lengths to maintain proper function and structural properties of Cullin1 (Figure.31 and 32).

By following the first strategy of truncating Cullin1 to two shorter lengths, the fluorescence signal of YPet-Cullin1 (all length variants) had no drastic improvement, YPet-Cullin1(T1) being slightly better that YPet-Cullin1(Full)(Figure.33). Also the FRET signal of NEDD8 conjugation in presence of ATP and all the enzymatic machinery was not clearly distinguishable from the FRET signal in absence of ATP (Figure.34). Unfortunately truncating Cullin1 did not improve the FRET signal for NEDD8 conjugation to Cullin1.

Improving the fluorescent signals of fusion proteins by incorporating linkers in fusion proteins of CyPet-Linker3-Cullin1. We further investigated the Cullin1 structure and noticed that Cullin1 is U-shaped rigid protein. In chapter II we developed an improved FRET based assay to aid in understanding and studying different interactions in NEDD8 cascade, by engineering CyPet-NEDD8 with a peptide linker[31], which improved the fluorescence of CyPet-tagged NEDD8 and further aided in development of FRET based protein assay for NEDD8-E2 conjugation. Linker3 engineering of CyPet-NEDD8 resulted in highest fluorescent emission from all other linker engineering. Therefore we hypothesized that, the insertion of linker 3 (TSGSPGLQEFGT) may help in proper folding of YPet-Cullin1 fusion protein and resultantly increase the fluorescence of YPet-Cullin1 and further improve our FRET based assay for NEDD8 Cullin1 conjugation.

To improve the folding and fluorescence of fusion proteins, we inserted linker3 between YPet and Cullin1 protein (all three length variants, Full; T1;T2) to allow proper folding of YPet and Cullin1. After genetically engineering YPet-Cullin1 with linker3, we observed that YPet-linker3-Cullin1 (all length variants) had improved fluorescence emission as compared to YPet-Cullin1 (all length variants) (Figure.33 A and B). YPet-linker3-Cullin1 has the highest emission maximum at 530nm, when excited at 475nm.

A



B

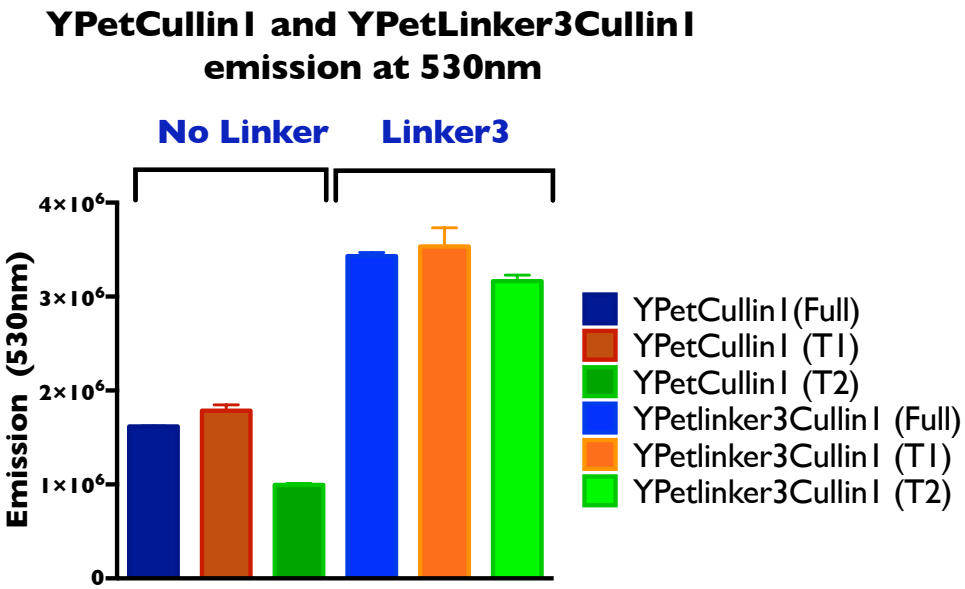


Figure 33. YPet-Cullin1 and YPet-linker3-Cullin1 fluorescence analysis. A. Emission spectrum of YPet-Cullin1 and YPet-linker3-Cullin1 when excited at 475em. B. Emission max of CyPet-NEDD8 and CyPet-LinkerX-NEDD8 when excited at 475 nm and Em(max) at 530nm.

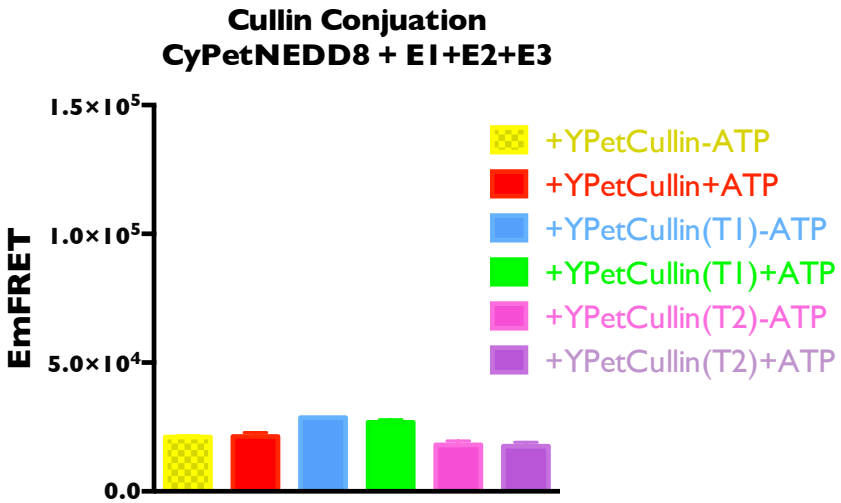


Figure 34. Absolute FRET signal, EmFRET, of covalent conjugation of NEDD8 to Cullin1 (all three length variants), in presence of E1, E2 and E3.

Determine covalent conjugation of NEDD8 peptide to its substrate Cullin1. Our main aim was to develop FRET based high throughput screening assay for NEDDylation cascade, with Cullin1 as final target for NEDD8. We employed our FRET based assay to detect the covalent conjugation of NEDD8 to Cullin1, in presence of E1, E2, E3 and ATP. The fluorescence signal of YPet-tagged Cullin was improved by engineering YPet-linker3-Cullin1; however, the detection of the NEDD8-Cullin1 conjugation using our FRET based assay was unsuccessful (Figure.35). The major reason for this problem is that RBX1, the E3 ligase, know to be important for the NEDD8 conjugation to Cullin1, is naturally insoluble in *E.coli* expression system, which resulted in the expression of inactive RBX1. The NEDD8 binding buffer (refer to chapter.2),

which had improved the solubility of CyPet-NEDD8 expressed in *E.coli*, was used to improve solubility of RBX1. However the purified RBX-1 was not active.

In the future, insect cell or mammalian cell expression systems for RBX-1 expression will be utilized to synthesize active RBX1. Resultantly, we will be able to develop our FRET based HTS-platform using our engineered YPet-linker3-Cullin1.

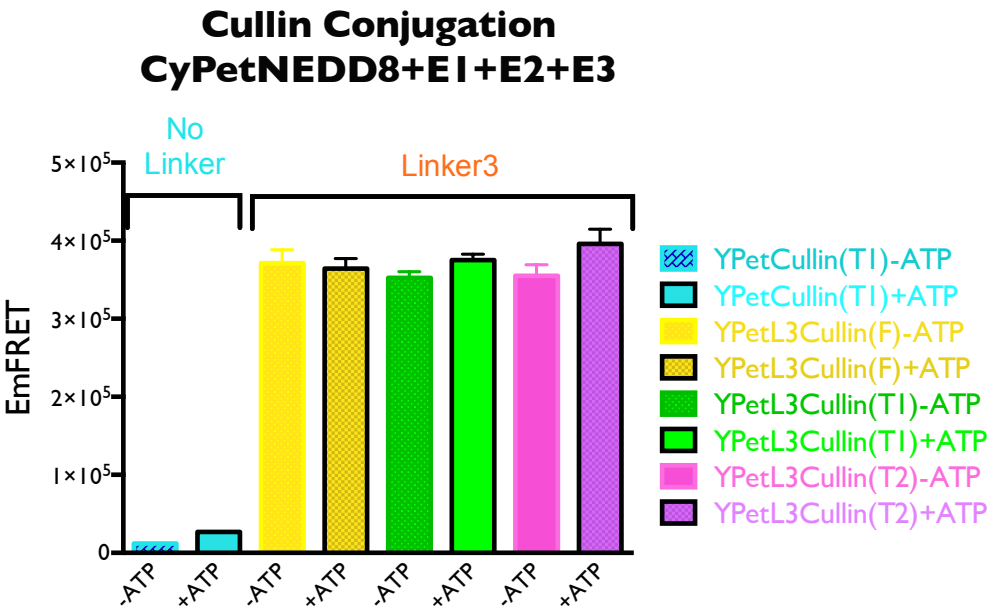


Figure 35. Absolute FRET signal, Em_{FRET}, of engineered Cullin1 conjugation to NEDD8. L3 is Linker3 polypeptide (TSGSPGLQEFGT).

5.3 Discussion

Here we showcase that linker engineering strategy of adding distance between two proteins aids in improved fluorescence of YPet fluorescent protein tagged to Cullin1. The reason behind the increase in fluorescence after linker engineering of YPet-Cullin1 may be because of proper folding of these individual polypeptides and resulting

in proper structure and functional properties, in our case increased fluorescence (Figure.33). Data from linker3 engineering of YPetCullin1 clearly showcases and support the hypothesis of proper folding and improvement of functional properties of individual polypeptide.

By genetically fusing two or more protein domains together, the fusion protein product may obtain many distinct functions derived from each of their component moieties. In past researchers have employed linkers to fuse different domains with respective properties and linkers have shown increasing importance in the construction of stable, bioactive fusion proteins. Empirical linkers designed by researchers are generally classified into 3 categories according to their structures: flexible linkers, rigid linkers, and in vivo cleavable linkers[32]. For some fusion protein domains fused together without any spacer between them might result in non-functional proteins, however addition of linker may help in rescue the protein function and activity[32-36]. For example, a fusion protein of streptococcal protein G with *Vargula* luciferase for fluorescent immune assay (FIA) recovered the antibody binding activity when a linker was inserted between these two proteins[37]. Many researchers have employed linkers as protease sensitive or ligand responsive reporters in fluorescence cis-FRET based assays[38,39]. Several alpha helix peptides were used to separate two fluorescent protein domains, such as EGFP and EBFP, when fusion together as cis-FRET sensor[39-42].

Our linker engineering strategy with relatively rigid linker structure for the fusion protein, CyPet-linker-NEDD8, resulted in a significant improved fluorescent and subsequently FRET signals for studying NEDD8-E2 thioester intermediate[31]. We

employed same linker3 for engineering YPetCullin1, because the rigid structure of linkers in our studies can provide additional critical benefits for future quantitative FRET analysis because it can restrict the conformational changes within fusion proteins resulting in stable FRET signal. The results clearly suggested the ability of the linkers to control the distance and reduce the interference between the YPet and Cullin1. This study also suggests a great potential of linker engineering strategy for improving fluorescent fusion protein signals, in addition to engineering fluorescent protein itself, in various biological and biomedical research and applications. This engineering strategy will aid us in development of FRET based protein assay for detection of NEDD8-Cullin1 conjugation.

5.4 Materials and Methods

DNA constructs

CyPet-linker3-NEDD8 was cloned as described in chapter II. The open-reading frames of YPet were amplified by PCR with primers containing NheI/BamHI sites or BamH1/NotI sites (Cullin1) or SalI/NotI sites (APPBP1, UBA3, Ubc12, Rbx1 and DCN1), and the PCR products were cloned into pCRII-TOPO vector (Invitrogen). The fragments encoding Cullin-1 were extracted by BamHI/NotI digestion and inserted into the BamHI/NotI sites of pCRII-YPet. For expression of recombinant proteins in bacteria, the cDNAs encoding CyPet-Linker3-NEDD8, APPBP1, UBA3, Ubc12, RBX-1, DCN-1 and YPetCullin-1 were cloned into the NheI/NotI sites or SalI/NotI of pET28(b) vector (Novagen). For generating two different lengths of Cullin-1 (324-776 and 534-776), PCR

with specifically designed primers were used to truncate full length Cullin-1 in two different sizes. For generating YPet-Linker3-Cullin1, same strategy was employed as for CyPet-Linker3-Cullin1 (Chapter II).

Protein Expression

BL21(DE3) *Escherichia coli* (*E. coli*) cells were transformed with pET28 vectors encoding CyPet-linker3-NEDD8, YPetCullin1 (All three types with and without Linker engineering), APPBP1, UBA3, Ubc12, Rbx1 and Dcn1. After the transformed bacteria were plated on LB agar plates containing 50 μg/mL kanamycin, single colony was picked and inoculated in 2xYT medium. The expression of polyhistidine-tagged recombinant proteins was induced with 0.1 mM IPTG at 25°C overnight. Bacterium cells were centrifuged at 6000 rpm 15 min, resuspended in buffer containing 20 mM Tris-HCl (pH=7.5), 0.5M NaCl and 5 mM imidazole, and sonicated with an ultrasonic liquid processor (Misonix). Cell lysate containing recombinant proteins was cleared by centrifugation at 25000g 30min. The recombinant proteins were then bound to Ni2+-NTA agarose beads (QIAGEN) and eluted in buffer containing 20 mM Tris-HCl (pH=7.5), 0.2 M NaCl and 150 mM imidazole. The proteins recombinant proteins were then bound to Ni2+-NTA agarose beads (QIAGEN) and eluted in buffer containing 20 mM Tris-HCl (pH=7.5), 0.2 M NaCl and 150 mM imidazole. The proteins were later dialyzed in buffer containing 20 mM Tris-HCl (pH=7.5), 50 mM NaCl and 1 mM DTT. The purity of the proteins was confirmed by SDS-PAGE followed by Coomassie blue staining, and their concentrations was determined by Coomassie Plus Protein Assay (Thermo) using known quantities of bovine serum albumin as standards.

FRET spectrum measurements

All reagents were purchased from Research Products International Corp unless otherwise specified. Fluorescence emissions of YPetCullin1 (all length variants of Cullins and with linker length variants) were determined by the fluorescent plate reader Flexstation II384 (Molecular Devices). The emission spectrum of YPet tagged proteins, when excited at 475nm from 490nm – 580 nm was measured. For the entire respective FRET assay, 1μM CyPetNEDD8 was mixed with 1μM YPetCullin1 (all variants), in presence or absence of APPBP1, UBA3, Ubc12, Rbx1 and Dcn1 in NEDDylation buffer (50mM Tris-HCl, 50mM NaCl, 10mM $MgCl_2$, 1mM DTT). Samples were incubated at 37°C and fluorescence emission was monitored right after adding buffer with or without ATP was added (2mM final concentration) for 60 minutes, every 15 minutes.

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Chapter 6: Conclusion and Future Perspectives

Research advancements using new technologies have improved our understanding of signaling mechanisms, biochemical pathways, and cell regulation mechanisms. The importance of understanding the signaling cascades aids in understanding causes of different diseases, prognosis of diseases and drug development of various diseases. With this aim in mind, we are moving towards the development of sensitive and high-throughput methods to understand signaling cascades related to Ubiquitin and Ubiquitin like modifiers. Ubiquitin and Ubiquitin like modifiers are know to regulate critical cell processes, such as DNA replication, transcription and repair, cell cycle progression, signal transduction, metabolism regulation, protein stability, fighting viral infections, etc. My Ph.D. dissertation pertains to systemic dissection and understanding of NEDD8 conjugation cascade by employing our quantitative FRET based technology. In my research, I have successfully engineered fluorescent-tagged NEDD8 with linker, for the first time, for development of a trans-FRET protein assay to determine activation intermediate of NEDDylation cascade. This linker engineering has aided us to detect and clearly differentiate different types of protein-protein interactions in NEDDylation cascade, which was not possible before.

Furthermore, for the first time, I have been successfully able to systematically understand the activation and conjugation reaction intermediates in NEDD8 conjugation cascade. Most importantly, during the study the of reaction intermediates in NEDD8

conjugation cascade, we came across the unique finding for the E1 heterodimer requirement in NEDD8 pathway. We came across a unique finding that, UBA3 by it self can result in the NEDD8 conjugation to Ubc12 in presence of ATP and that APPBP1 is not absolutely required for NEDD8 activation and E2 conjugation, but when present it accelerates the NEDD8 activation and E2 conjugation. Our results, for the first time, provide a systematic and detail investigations about the requirements of each subunit of NEDD8's E1 heterodimer in NEDD8 activation. Here we report, APPBP1, a subunit of E1 heterodimer, is not absolutely critical for NEDD8 activation, but rather plays a scaffold role that helps the kinetics of activation.

Additionally, we have been able to successfully engineer YPet-tagged Cullin with linker peptide 3, to improve the fluorescence of YPet-tagged Cullin. This will aid in the development of high-sensitive FRET based HT-screening assay platform for inhibitors of NEDD8 conjugation cascade. In the future, we will establish insect lines or mammalian cell expression system to express proteins insoluble in *E.coli* expression system, such as Rbx1 and PIAS1 (E3 ligases for NEDD8 and SUMO1), which will are required for the NEDD8 and SUMO1 conjugation cascade.

NEDD8 conjugation cascade, as discussed in this dissertation, plays a crucial role in regulating various cellular processes, such as transcription, signal transduction, cell cycle regulation, and is also know to be misregulated in various cancers and diseases. Therefore discovering new drugs to regulate NEDD8 conjugation cascade is important,

thus making the development of HT-screening assay for drug discovery critical. In future, this HTS-platform can also be employed for different targets proteins, other than Cullin family, such as p53, EGFR, MDM2, PARC, BCA3, and VHL. Furthermore, we can employ our FRET based platform for identifying potential E1 activation enzymes, E2 conjugation enzyme, E3 ligation enzyme and targets of Ubiquitin and Ubiquitin like modifier cascades.

Appendix H: Curriculum Vitae

Harbani Kaur Malik-Chaudhry, Ph.D.
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Employment

University of California, Riverside Riverside, CA (2008-2014)
Ph.D Scientist, Department of Bioengineering, Fall 2008-March 2014
Teaching Assistant, Fall 2009- Fall 2012

MedImmune Vaccines Inc. Mountain View, CA (Summer 2007)
Summer Intern, Vaccine Analytical Science Group

Dabur Research Foundation New Delhi, India (Summer 2006)
Summer Intern, Department of Vaccine development

Education

University of California, Riverside Riverside, CA (September 2008 – March 2014)
Ph.D. in Bioengineering

Jaypee Institute of Information Technology NOIDA, India (July 2004 - June 2008)
Bachelors Tech. Biotechnology

Research Experience

University of California, Riverside Riverside, CA (2010-2014)
Ph.D. Scientist

- **Development of FRET based HTS platform for NEDDylation cascade**
  Development of FRET- based high-throughput screening assay for discovering small molecule inhibitors of NEDD8 cascade. Involving molecular biology, protein engineering, fluorescent protein, FRET and biochemistry techniques.
- **FRET based protein assay to detect and differentiate different forms of protein interactions in NEDDylation pathway**
  Detailed investigation of NEDDylation dynamics and interaction switches and specificities of NEDD8 among its ligases (E1, E2 and E3) using FRET based protein assay. Involving molecular biology, protein engineering, fluorescent protein, FRET and biochemistry techniques.
- **Engineering High Efficient Fluorescent-Tagged NEDD8 for FRET based protein assay**
  Using linker engineering and recombinant DNA technology we engineered fluorescent-tagged NEDD8 to aid in developing protein-protein interaction FRET based assay. Involving molecular biology, protein engineering, fluorescent protein, FRET and biochemistry techniques.
- **Protein Engineering for secretory proteins in bacterial expression system**
  Engineered proteins with various secretion systems, such as TAT and SRP, to aid in protein expression and solubility of high molecular weight proteins and improve the downstream processing.

MedImmune Vaccines Inc, Mountain View, CA (Summer 2007)
Summer Intern

- **Develop antibody-based assay for characterization of Vero Host Cell Proteins**
  Prepared and quantified specific labeled antibodies for the development of antibody based protein assay for characterization of Vero-Host Cell proteins.

Jaypee Institute of Information Technology NOIDA, India (2007-2008)
Undergrad Researcher

- **Characterize Encephalitis viral protein interactions using yeast two-hybrid system**
  Cloning and expression of Encephalitis virus genes and further characterize protein-protein interaction using Yeast two-hybrid system.

Dabur Research Foundation New Delhi, India (Summer 2006)
Summer Intern

- **Antibody expression for vaccine development**
  Employing molecular biology and biochemistry tools for Antibodies Expression and characterization for vaccine development.

Teaching and Training Experience

University of California, Riverside Riverside, CA (2009-2013)
Teaching Assistant

- Intro: Cell and Molecular Biology (2 QTRS) (100 students, lecture: 4 hours/week)
- Biotechnology Laboratory (3 QTRS) (~90 students, laboratory: 10 hours/week)
- Bimolecular Engineering (1 QTR) (~40 students, lecture: 3 hours/week)
- Biotechnology and Molecular Bioengineering (2 QTRS) (~90 students, lecture: 3 hours/week)

Managed coordination of lesson plans in collaboration with professor.

University of California, Riverside Riverside, CA (2010-2014)
Lab Manager

- Trained and managed ~20 undergrad and ~10 graduate students over the course of 3 years
- Oversaw the development of research projects and managed teams of undergrad researchers in various projects
- Coordinate, planned, assigned, and directed work in the laboratory to assist faculty in teaching research programs
- Oversaw operational and safety aspects of the laboratory
- Managed all safety data

Relevant Skills and Qualifications

- Proficient with **molecular cloning** techniques such as **PCR, DNA ligations/digestions**, and to all that pertains
- Proficient with **gene expression** techniques within ***E.Coli* (bl21-DE3), Yeast and mammalian cell (HEK293)**
- Proficient in **protein purification and characterization methods** (Affinity Chromatography& Western Blots)
- Proficient with **VisionWorks®LS Analysis Software**
- Proficient with **High Performance Liquid Chromatography** (HPLC)
- Proficient with **Fluorescent based protein assays** and **High-throughput screening assays**
- Proficient with **Flex station II™** and **Softmax Pro Software** (High-throughput plate reader)
- Proficient with **Beckman Coulter Biomek® FX Laboratory Automation Workstation and Software**
- Proficient with **DNA sequencing technology** such as MiSeq and HiSeq-2000 and 2500
- Proficient with **Biacore™**(SPR Technology)
- Proficient with **Yeast-two hybrid system**
- Computer Software/OS: **COMSOL™, Matlab™, LabView™, Inkscape™**, and windows/Linux

Computer Experience

- **Image and Data Analysis**: ImageJ, Prism
- **Molecular Modeling:** SwissPDB, Chimera, MolMol, VMD, APBS
- **Microsoft Office products:** Word, Excel, PowerPoint, Publisher, Access, Outlook
- **Adobe products:** Photoshop, Illustrator, Acrobat, Flash
- **Web design:** Created and manage website

Peer Reviewed Publications

- "**Amyloid Histology Stain for Rapid Bacterial Endospore Imaging**"
  Journal of Clinical Microbiology 2011, 49(8), 2966-2975; Xia, B.; Upadhyayula, S.; Nunez, V.; Landsman, P.; Lam, S.; **Malik, H**.; Gupta, S; Sarshar, M.; Hu, J.; Anvari, B.; Jones, G.; Vullev, V. I.

- "**Target SUMOylation and other UBL pathways for drug discovery**"
  Current Topics in Biotechnology 2012, 7(21), 0972-8126X**; Malik-Chaudhry, H**.; Wiryawan, H.; Liao, J.

- "**A Linker Strategy for Trans-FRET Assay to Determine Activation Intermediate of NEDDylation Cascade**"
  Biotechnology & Bioengineering, 2014, doi: 10.1002/bit.25183; **Malik-Chaudhry, H.K.**; Saavedra, A.; Liao, J.

- "**Inspecting E1 requirement of NEDD8 activation using quantitative FRET based protein assay**", Molecular Cell, submitted, **Malik-Chaudhry, H**.; Saavedra, A.; Reyes, M.; Gaieb, Z., Morikis, D., Liao, J.

- "**Development of FRET based sensitive protein assay to detect and differentiate different forms of protein interactions in NEDDylation pathway**", Methods in Molecular Biology, *in progress*, **Malik-Chaudhry, H**.; Saavedra, A.; Reyes, M.S.; Kung, R.; Lee, F.; Liao, J.

- **"Quantitative and high-sensitive FRET sensor for bio-reaction intermediate and dynamics determination",** Sensors, *in progress*, **Malik-Chaudhry, H**., Song, Y., Shultz, J., Liao, J.

Conference Abstracts Accompanied by Oral and Poster Presentations

- UC Bioengineering Symposium 2010 Davis, CA
  "Development of Novel Microarray for the study of SUMO pathway" (Poster presentation)
- UC SPIE Symposium 2012 Riverside, CA
  "FRET based Quantitative Systems Biology Approach for NEDDylation Pathway Characterizations" (Poster Presentation)
- UC Bioengineering Symposium 2012 Berkeley, CA
  "Development of High-Sensitive FRET-based Assay to Study Protien-Protein Interactions in NEDD8 conjugation pathway" (Poster Presentation)
- UC Bioengineering Symposium 2013 San Diego, CA
  "FRET based Quantitative Systems Biology Approach for NEDDylation pathway characterizations" (Oral Presentation)
- UC Bioengineering Symposium 2013 San Diego, CA
  "Toward Completion of determining full SUMOylation Pathway Kinetics using Novel Quantitative FRET Technologies" (Poster Presentation)