



The N-End Rule Pathway: Its Physiological Importance and Role in Disease Pathology

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Accepted on: 22-09-2013; Finalized on: 31-10-2013.

ABSTRACT

The N-end rule pathway is an ubiquitin dependent proteolytic system. It targets proteins for degradation by recognizing their N-terminal amino acid residues (N-degrons). N-degrons, recognized by N-recognins, undergo ubiquitination and 26S-mediated selective proteolysis. This pathway controls in-vivo half-life of many proteins regulating many crucial physiological functions such as, import of peptides, regulation of cell cycle, viral and bacterial infections, cardiovascular development, apoptosis, etc. Over a decade and more N-end rule physiological substrates are deciphered to be characteristically involved in various disease pathologies, which necessitates development of drug-like molecules counteracting these pathologic extremisms. This review attempts to showcase N-end rule's physiological importance in the light of understanding certain diseases, and exemplifies potential, N-end rule "druggable" targets.

Keywords: Proteolysis, N-end rule, N-degron, N-recognin, Inhibitor.

INTRODUCTION

The N-end rule proteasomal pathway determines the metabolic stability and fate of cellular proteins and hence, their in-vivo half-lives. The target protein is identified and degraded by 26S proteasome in ubiquitin dependent manner. The target protein, which is recognized and degraded by the identity of its destabilizing N-terminal amino acid residue, is called N-degron. Hence, the proteasomal degradation pathway is called N-end rule pathway. Degradable N-degrons need their physical binding with a class of proteins, called N-recognins.^{1, 2} N-recognins belongs to E3 ubiquitin ligase proteins family. Similar type of N-end rule degradation pathway is present in all examined organisms, including mammals to bacteria and fungi but in distinct fashion.³⁻⁵

The cellular protein degradation pathway involves ubiquitin/proteasome system. Ubiquitination and successive degradation of target proteins involve enzymatic reactions controlled by three types of enzymes: Ubiquitin (Ub)-activating enzyme (E1), Ubiquitin-carrier enzyme (E2) and Ubiquitin-ligating enzyme (E3). Two discrete and successive steps are involved in degradation of target proteins by the ubiquitin mediated proteolysis pathway: (a) covalent attachment of multiple Ub molecules to the target protein, called ubiquitination and (b) degradation of Ub-tagged proteins by 26S proteasome complex. In ubiquitination step, at first, E1 binds to Ub in an ATP-dependent manner. E1 form a high energy thioester bond between its own internal cystein residue (active site of E1) and the C-terminal Glycine (G76) of ubiquitin protein.^{6, 7} E1 then transfer the activated Ub moiety to E2 enzyme, where another thioester bond forms between the cystein residue at the active site of E2 and the C-terminal Glycine (G76) of Ub. On the other hand, target protein is recognized by the binding sites of E3 enzyme. Now, E2-Ub

complex gets associated with E3-target protein moiety to ubiquitinate target protein. Ub makes an 'isopeptide bond' with an internal lysine residue of target protein.⁸ Once, mono-ubiquitination occurs, poly-ubiquitination happens successively in which C-terminal amino acid residue of each Ub makes an iso-peptide bond with ϵ -amino group internal lysine residue (most commonly K48) of previously attached Ub. Poly-ubiquitination is necessary for a protein to create a proteasomal degradation signal. So, poly-ubiquitinated target protein is rapidly recognized and degraded by 26S proteasome.⁹ However, a de-ubiquitinating (Dub) enzyme is also involved in this proteasomal pathway to maintain the balance of ubiquitination of proteins and their subsequent degradation. Dub enzymes are also responsible for recycling of Ub by converting their polymeric form to monomers and depositing them into the pool of free Ub within the cell.¹⁰ [Figure 1].

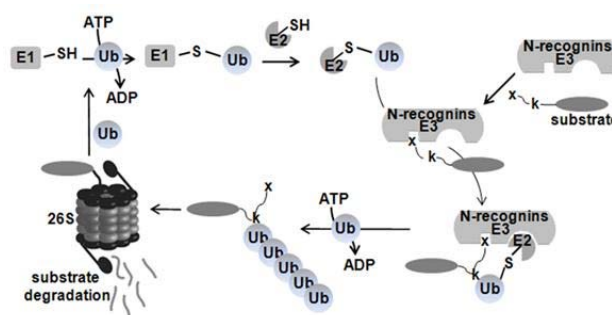


Figure 1: Schematic diagram of N-end rule ubiquitin proteasome system.

Long ago, the N-end rule proteasomal degradation pathway was discovered as an ubiquitin mediated pathway but its physiological importance is lately realized. In the last decade, many components of the mammalian N-end rule pathway have been identified, including a substrate recognition component; E3-

ubiquitin ligase protein family (N-recognins) and their substrates. These discoveries have provided new insights into the components, functions and mechanism of this unique proteasomal degradation system. The genetic dissection in mammals revealed N-end rule's importance in various vital physiological processes. Through the regulation of many cellular proteins N-end rule regulates various aspects of eukaryotic cellular functions or biological processes, including cell cycle progression, oncogenesis, import of peptides, chromosomal instability, cardiovascular development, and apoptosis.^{11, 12} E3-ubiquitin ligases are key components in N-end rule pathway as they recognize target proteins and their in-vivo half-lives. Therefore, N-recognins have become attractive and potentially "druggable" molecular targets for treatment of various diseases including cardiovascular disease and cancer.

COMPONENTS OF N-END RULE PATHWAY

N-end rule E3s (N-recognins) recognize destabilizing N-terminal residue of target protein and participate in the formation of a protein-linked poly-ubiquitination. N-recognins E3 family proteins contain a 70-residue zinc-finger domain, called UBR box.^{13, 14} The sets of UBR proteins vary depend on organism. Mammalian genome encodes at least seven UBR box proteins, termed UBR1-UBR7, whereas yeast *S. Cerevisiae* encodes only two UBR1 and UBR2. Heterogeneous UBR proteins are different in size and sequence but except UBR4, they contain a unique substrate-recognition subunit of E3 complex. UBR1 (E3 α), UBR2 and UBR3 contain RING (Really Interesting New Gene) domain; UBR5 contains HECT (Homologous to the E6-AP Carboxyl Terminal) domain; UBR6 & UBR7 contain F-box and PHD domain respectively.^{14, 15} In mammals, only four N-recognins (UBR1, UBR2, UBR4, & UBR5) have been characterized and shown to bind to destabilizing N-terminal residues.¹⁴⁻¹⁶ UBR3, UBR6 & UBR7 are not characterized as N-recognins yet.

In plants, like *Arabidopsis*, at least two types of N-degrons are present depending on N-recognins. Proteolysis6 (ptr6), which is a UBR-box containing E3 ligase, recognizes and binds with only basic N-terminal amino acid residues. Ptr1 recognizes aromatic N-terminal amino acid residues and it doesn't contain UBR-box E3 ligase.^{17, 18}

As mentioned earlier, the N-end rule is a relation between the metabolic stability, hence the fate of a protein, to the identity of its N-terminal residue. The target proteins bearing either basic N-terminal residues or bulky hydrophobic N-terminal residues generate degradation signals to be recognized by N-recognins. These target proteins with degradation signals are called N-degron. All the characterized N-recognins have at least two binding sites for recognizing basic N-terminal and bulky hydrophobic destabilizing amino acid residues.^{1, 2} N-recognins recognize not only destabilizing N-terminal amino acid residues of target proteins (N-degrons) but

also internal destabilizing amino acid residues, called I-degron inserted in the substrate protein's frame.¹⁹

There are three main sub-types of N-degrons depending on their destabilizing N-terminal amino acid residues: (a) Primary N-degrons (N-d^p): These residues are recognized by N-recognins without any post-translational modification and their physical association depends on the recognition sites of N-recognins. In eukaryotes, type 1 binding site binds a set of positively charged N-terminal residues Arg, Lys or His (N-d^{p1}); type 2 binding site binds a set of bulky hydrophobic N-terminal residues Phe, Leu, Ile, Tyr, Trp (N-d^{p2}). In *E. coli*, only type 2 residues are obtained. Herein N-d^p residues are Phe, Leu, Trp, and Tyr. In mammals, Ala, Ser and Thr N-terminal residues which were previously classified as type 3 N-d^p¹¹, now have been characterized as stabilizing residues.^{14, 15} (b) Secondary N-degrons (N-d^s): These residues need post-translational modification to be recognized. In mammals, Asp, Glu, and Cys are this type of residues whereas in *S. cerevisiae* Asp & Glu are N-d^s. They are converted to N-d^p by an enzyme Arg-tRNA-protein transferase (R-transferase encoded by *ATE1* gene). In case of *E. coli* Arg and Lys are N-d^s. These N-d^s residues need Leu/Phe-tRNA-protein transferase (L/F-transferase) to be converted into its N-d^p residues; (c) Tertiary N-degrons (N-d^t): These residues also need modification to be converted into N-d^p. N-terminal Asn, Gln and Cys are denoted as N-d^t. N-terminal amidohydrolase converts them into N-d^s followed by the addition of Arg to form N-d^p to be recognized by N-recognins. Cys is first oxidized by O₂ and NO and is converted into N-d^p by the conjugation of Arg by the action of *ATE1*.²³ [Figure 2]

In eukaryotes, synthesized long-lived proteins contain methionine (Met) as a stabilizing residue at their N-terminal. In order to make those proteins short-lived, post-translational modification is necessary. Stabilizing residue Met is cleaved off by the action of MetAPs (Methionine aminopeptidase) to expose second residue. MetAPs cannot generate primary or secondary destabilizing residues in both eukaryotes and prokaryotes. In mammals, MetAPs generate either tertiary destabilizing residue Cys or stabilizing residues like Val, Gly, Ser, Pro, Thr by the removal of N-terminal Met [Figure 3].^{12, 20} For example, MetAPs chop out Met from N-terminal of RGS proteins to expose Cys as tertiary destabilizing residue.³²

Endopeptidases, like caspases, calpains, separases, cleave long-lived proteins internally and generate C-terminal fragments. The resulting C-terminal fragments contain stabilizing residues or primary, secondary, tertiary destabilizing residues in mammals. [Figure 3]

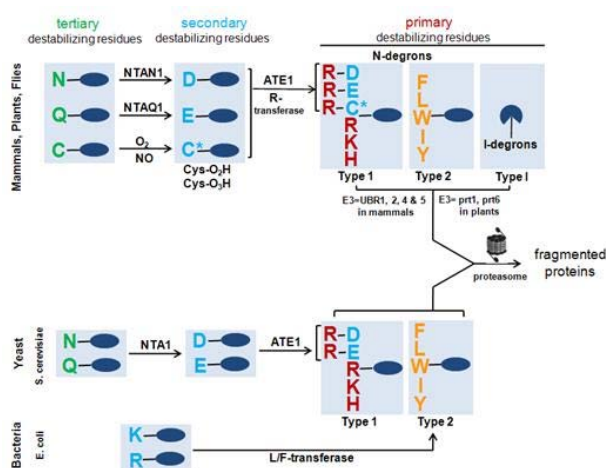


Figure 2: The hierarchy of N-end rule pathway in mammals, plants, flies, yeast and bacteria. Single letters are abbreviation of N-terminal amino acid of proteins and blue ovals denote the rest of proteins.

Stabilizing residues

The N-terminal amino acid residues of target proteins which are not identified and efficiently bound by N-recognins, called stabilizing residues. They do not get modified also. Gly, Val, Met, Pro, Ser, ala and Thr are this type of residues. N-terminal stabilizing residues bearing proteins are not substrates of N-end rule pathways. [Figure 3]

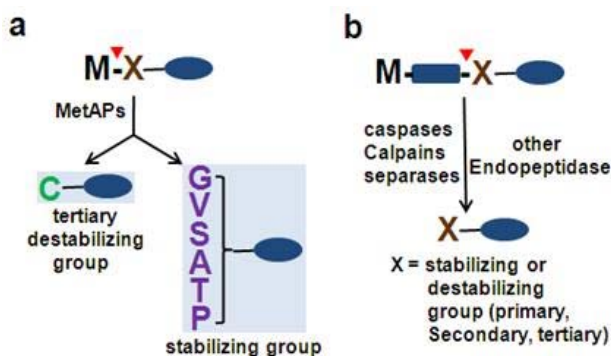


Figure 3: Formation of N-degrons. Two types of mechanism involved. a) MetAPs remove N-terminal Met to expose stabilizing or destabilizing residues. b) Endoproteolytic cleavage of proteins yield C-terminal fragments bearing stabilizing or destabilizing residues.

PHYSIOLOGICAL ROLES OF N-END RULE PATHWAY INVOLVEMENT OF N-DEGRONS

Muscle wasting during diseases

Skeletal muscle size and function depend on the balance of the rate of synthesis and degradation of muscle proteins. Many pathological states like cancer, sepsis, diabetes, metabolic acidosis, fasting, denervation, and hyperthyroidism accompany loss of muscles resulting in muscle atrophy due to increased rate of muscle's protein degradation via ubiquitin proteasomal pathway.^{21, 22} It was found that the rate of ubiquitin conjugation with substrate proteins is high in tumor-bearing or sepsis rats. Ub-conjugation and hence protein breakdown was shown

high in insulin deficient acute diabetic rats or in fasting condition leading to muscle wasting. Induced insulin deficient rats showed up to 50% enhanced rates of Ub-conjugation with endogenous muscle proteins. Lecker et al showed that a specific substrate β -lactalbumin, bearing N-terminal Lys residue was ubiquitinated faster in diabetic rats. These enhanced ubiquitination happen through N-end rule pathway involving Ub-ligase E3.²¹ Competitive di-peptide (Lys-Ala) inhibitors of E3 Ub-ligases decreased the rates of Ub-conjugation in muscle protein extracts from sepsis or tumor-bearing rats.²²

Cardiovascular development

N-end rule pathway plays an important role in cardiovascular system. G-proteins are the regulator of cardiac growth and angiogenesis. G-protein-coupled receptors (GPCRs) present on the plasma membrane of organ cells and interact with heterotrimeric (G α , G β , and G λ) G proteins to regulate intra-cellular signaling pathways. G α subunit becomes activated by the ligand stimulation of GPCRs to exchange GDP with GTP and upon GTP binding, the inactivated heterotrimeric G-proteins form two activated subunits, G β and G λ . These two activated subunit keep the signal on until the hydrolysis of GTP from G α .²³ The G α -subunit is divided into four families: Gs, Gi, Gq and G12. G α q and G α i pathways play critical role in proliferation and differentiation of myocardial cells. The activators of Gq- or Gi-coupled receptors stimulate cardiomyocytes hypertrophy and hence increase the size of cardiomyocytes as well as heart weight.^{24, 25} The regulators of G protein signaling (RGS) proteins are connected as negative controllers of cardiovascular Gi and Gq signaling pathways.²⁶ RGS, as GTPase-activating proteins increase the rate of hydrolysis of GTP from Gi and Gq.²⁷ These lead to deactivation of various down-stream effectors like PKC, Ras and MAPK those are responsible for myocardial cell growth.²⁸ Among RGS protein family, RGS4, RGS5 and RGS16, which belong to the R4 subfamily, have been shown as negative regulators of Gi and Gq-mediated cardiovascular signaling pathways.²⁹

Lee et al showed that RGS4 and RGS5 are in-vivo substrates of N-end rule proteasomal pathway whereas RGS16 was shown as in-vitro substrate.²³ The degradation of these proteins needs Arg-transferase enzymes. ATE1 gene encodes a family of Arg-tRNA-protein transferases (R-transferases) that mediate conjugation of Arg to N-terminal Asp, Glu, and Cys of proteins in eukaryotes. This yield N-terminal Arg that acts as a degradation signal for Ub-dependent N-end rule pathway.^{30, 31} RGS4, RGS5, and RGS16 require sequential modification to be recognized as N-end rule substrate by UBR1 and UBR2 as they bear Cys-2 residue at their N-terminals after dissociation of Met from N-terminal by the enzyme MetAP. Cys-2 requires oxidation prior to arginylation by ATE1. Cys-2 is oxidized into CysO₃H through a transiently oxidized form of CysO₂H in the presence of O₂ and NO. The oxidized form of Cys is then recognized by ATE1 Arg-transferase, as

CysO₂H has structural similarity with Asp,^{23, 32} yielding N-terminal degradation signal (N-degrons). The mouse embryos lacking *ATE1* gene (*ATE1*^{-/-}) die due to defects in heart development and late impaired angiogenesis,³¹ indicating that *ATE1*-dependent proteolysis has a critical role in the regulatory mechanism of myocardial cell growth and formation of new blood vessels. In mouse embryonic fibroblast cells lacking *UBR1*^{-/-} or *UBR2*^{-/-} the degradation of RGS4 and RGS5 was inhibited and the strong stabilization of these two substrates happened in *UBR1*^{-/-} /*UBR2*^{-/-} cells. This indicates that in mammals *UBR1* and *UBR2* act as functionally overlapping E3 ligase for RGS4, RGS5 and possibly for RGS16. Altogether it indicates that for the degradation of RGS4, RGS5 and RGS16 *ATE1-UBR1/UBR2*-mediated N-end rule degradation pathway is involved in mammals and NO plays an important role in this process.^{23, 31, 33}

Development of hetero-bivalent inhibitors against N-end rule E3 ligase

Dipeptides are used widely as N-end rule pathway inhibitors as a useful tools to study this pathway. As N-recognins have at least two types of well characterized binding sites, it had been shown that dipeptides (e.g. Arg-Ala, Phe-Ala) bearing either type 1 or 2 N-terminal residues inhibit degradation of N-end rule substrates.³⁴⁻³⁶ However, mono-valent dipeptides have very low affinity toward type 1 & 2 binding sites of N-recognins and they are unstable due to the action of endo-peptidase in cells.³⁷ But the extent of inhibition increased by co-administration of type 1 & 2 N-terminal amino acid residue bearing dipeptides¹⁹ and significantly increased by enzyme (β-galactosidase)-based macromolecular bivalent inhibitors containing both type 1 and type 2 N-terminal residues.³⁸ The enzyme based inhibition study revealed the spatial proximity of two binding sites of N-recognins.³⁸ This hypothesized the necessity of conjugation of multiple low-affinity ligands (like dipeptides) into a high-affinity multivalent molecule providing high selectivity and affinity in a ligand-protein interaction.

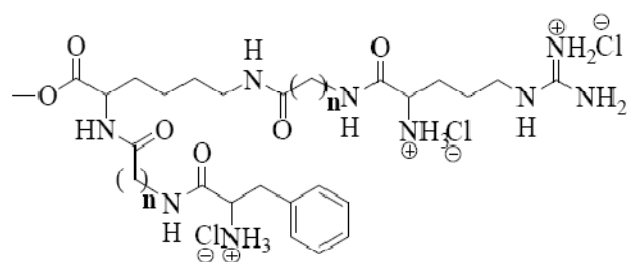
Based on the enzyme-based hetero-divalent inhibitor one lipid based small molecule, RF-C11 was designed in our lab and characterized.¹⁶ As a lipid-based molecule RF-C11 is resistant to proteolytic degradation by cellular endo-peptidase. Hence, this acts as stable inhibitor unlike dipeptide inhibitors.

RF-C11 was synthesized as a model compound, L₁L₂-C11, which is composed of three replaceable components: ligand (L₁L₂), linker (Cn), and core (lysine) [Figure 4]. In RF-C11, two C10 hydrocarbon chains were conjugated to two ligands; type 1 (Arg) and type 2 (Phe) amino acid residues, while hydrocarbon chains are attached to core lysine. RF-C11 is the first synthetic inhibitor which showed the scope to regulate, to be precise, inhibit N-recognins E3 ligase of N-end rule pathway. Lee & Pal et al. comprehensively demonstrated that a hetero-bivalent inhibitor (RF-C11) of E3 component inhibits the rate of degradation of N-end rule physiological substrate RGS4 in

mammalian cells. RF-C11 containing both type1 (Arg) and type 2 (Phe) ligands binds more specifically and with higher affinity with the type 1 & 2 substrate binding sites of E3 ubiquitin ligase simultaneously and hence delays the degradation rate of RGS4.¹⁶ Proliferation and hypertrophy of cardiomyocytes, isolated from mouse embryonic heart cardiac are reduced significantly by RF-C11.¹⁶

However, this study did not give a complete picture by SAR (structure activity relationship) study towards understanding the effect of chain length of this lipid-based inhibitor on E3 ligase affinity. If the non-specific interaction of ligands (Arg/Phe) is ignored then the binding ability of model molecule RF-Cn (where 'n' is no. of carbons) to UBR box protein's binding sites will greatly depend on linker length in the inhibitor molecule. A shorter linker is thermodynamically favorable as it has lower conformational entropy on binding and higher value of effective concentration (*C_{eff}*). *C_{eff}* is enhanced by the local concentration of ligands near the binding sites. During protein-ligand interaction one bound ligand (e.g. Phe) facilitates the binding of other ligand (e.g. Arg) in the same molecule by increasing its local concentration within a hemisphere of radius roughly equivalent to the molecule's linker length and vice-versa. So, the efficient and strong binding efficacy of ligands of hetero-bivalent inhibitor varies with the linker chain length, until it matches the distance between two binding pockets.³⁹

A series of molecules (RF-Cn; RF-C2 to RF-C16) have been developed with varying chain length (n = 1 to 15) [Figure 4] and their N-recognin binding efficacies have been examined (40). RF-C5, a structural analogue of RF-C11 with varying chain length, has been shown to inhibit N-end rule substrate (RGS4) degradation more efficiently *in vitro* mammalian cells. RF-C5 and RF-C11 inhibit N-end rule pathway directly in mammalian cells where RF-C5 has better efficacy to block the degradation of RGS4 than RF-C11.⁴⁰



RF-Cn (where n = 1, 2, 4, 7, 8, 10 & 14)

Figure 4: Chemical structure of hetero-bivalent inhibitors

However, RF-C5 with shorter chain length could not self-aggregate whereas RF-C11 self-aggregated readily.⁴⁰ This implied that RF-C11 with self-aggregation property may be used as a physiological amphipathic drug-type molecule as emulsion which can take care of its own *in vivo* delivery.

Role in apoptosis

Apoptosis, a process of programmed cell death, turns out to eradicate damaged, abnormal or unusually proliferative cells from multicellular organism. Dysregulation of cellular process occur in many diseases like autoimmune and immunodeficiency diseases, neurodegenerative disorders, and cancer as well. Thus, proteins involved in apoptosis regulation are of intense biological interest and many serve as attractive therapeutic targets. The inhibitors of apoptosis (**IAPs**) are a family of protein, first discovered in baculoviruses. They are shown to be involved in suppressing the host cell death response to viral infection.⁴¹ IAP family proteins are characterized by a conserved domain baculoviral IAP repeat (BIR) consists of ~70 amino acids. Within the known IAP proteins family of viruses and animal species up to three tandem copy of BIR can occur. Structural-functional studies of IAP family proteins showed that at least one BIR domain is required for the suppression of apoptosis, although other domains may also be needed under certain conditions in some species.⁴² IAPs have been shown to bind directly to specific caspases, and hence suppress the caspase activity and apoptotic cell death. In *Drosophila*, Reaper, Grim and Hid act as activators of apoptosis whereas in mammals this role is played by Smac/DIABLO. Activators or effectors of apoptosis neutralize the activity of IAPs by binding into the same pockets in IAPs which are meant to bind caspases in a competitive manner and thus increase the level of caspases.¹¹ IAPs, in addition to their caspase binding ability, act as Ub ligases bearing a RING domain. They mediate either self-ubiquitination or ubiquitination of other proteins bound to them.⁴³

The key caspases must be activated to a significant threshold level to induce a cell into apoptotic death. *Drosophila* DIAP1 is cleaved by activated caspases at position 20, producing an almost full fragment of DIAP1, bearing Asparagine (Asn) at N-terminal. This fragment is an N-end rule substrate and degraded by *Arg-ATE1* N-end rule pathway after the conjugation of Arg by *ATE1*. To suppress the pro-apoptotic activity of Reaper the fragment of DIAP1 plays an essential role and thus reduces the apoptotic threshold of cells.⁴⁴ On the other hand, anti-apoptotic activity of DIAP1 is maintained by its association and co-degradation with pro-apoptotic signals like Reaper, caspase. The self-ubiquitination activity of DIAP1 or N-end rule pathway may be involved here for this co-degradation of DIAP1 and pro-apoptotic factors.¹¹ XIAP (X-linked IAP), a mammalian counterpart of DIAP1, is known to be cleaved by activated caspases. Among caspases, activated caspase-3 & 7 were more efficient to cleave XIAP at position 242 bearing aspartic acid. The resulting C-terminal fragment of XIAP bears N-terminal Ala at position 243.⁴² Ala, was previously described as type 3 N-end rule substrate, which is degraded via an uncharacterized Ub ligase,¹¹ now characterized as stabilizing residue.

In mammalian cells, full length XIAP degradation is also observed via N-end rule pathway. Du et al. showed that UBR1 bears a third substrate binding site which remains blocked by its C-terminal residues. This binding site recognizes degrons internally ('i' degron) instead of recognizing their N-terminal residue. The third binding site (type i) becomes open upon inhibition of type 1 & 2 binding sites of UBR1 by dipeptides like Arg-Ala for type 1 and Leu-Ala for type 2.¹⁹ In a recent study, we hypothesized that if we trigger simultaneous inhibition of both type 1&2 binding site, we may accomplish as efficient down-regulation of type i substrate. Towards this, we chose to use hetero-bivalent inhibitor RF-C11 against cancer cells. Upon RF-C11 treatment we find very efficient down-regulation of XIAP, thereby increasing apoptotic threshold of cancer cells to externally co-treated drugs. This leads to enhance sensitization of cancer cells. Inhibition of type 1 & 2 binding sites of UBR1 by a synthesized hetero-bivalent inhibitor RF-C11¹⁶ helps the degradation of full length of XIAP possibly via type i binding site (S.K.P. & R.B. manuscript under revision).

Active caspases cleave numerous numbers of pro-apoptotic proteins in the process of regulation of apoptosis. Fragmented pro-apoptotic proteins like BRCA1 (N-Asp), RIPK1 (N-Cys), TRAF1 (N-Cys), BCL_{xL} (N-Asp), BID (N-Arg) which are conserved in evolution, contain destabilizing N-terminal residues. These fragments have been shown to be degraded via N-end rule pathway. One such fragment of RIPK1, bearing Cys at its N-terminal, has been shown as N-end rule substrate and it degrades via *Arg-ATE1* N-end rule pathway. The metabolic stabilization of this fragment by mutation at N-terminal using a stabilizing residue Val or partial deletion of *Arg-ATE1* pathway make cells sensitize to apoptosis.⁴⁵ BRCA1, breast cancer susceptibility type 1, is cleaved by active caspase-3 and C-terminal fragment, bearing Asp, is degraded by N-end rule pathway in cells. The metabolic stabilization of BRCA1 happens in cells lacking *ATE1* gene or if N-terminal Asp is replaced by any stabilizing residue. This N-end rule mediated degradation of BRCA1 is also independent of cell type. Experiment with different human cell lines like HEK 293T, MDA-MB-231, and MDA-MB-468 showed similar results in the C-terminal fragment degradation via *Arg-ATE1*-N-end rule pathway.⁴⁶

Role in cell cycle and genomic stability

Mitosis and meiosis are two processes involved in cell division of somatic and germ cells respectively. Eukaryotic cells pass their genetic information by passing genomes from one cell generation to the next. Cell replicates their entire nuclear DNA during S phase of cell cycle and then segregate the resulting sister chromatids from each other later during mitosis or meiosis phase. During segregation the spindle tools hold the replicated chromosomes in a bipolar fashion and move the sister chromatids into opposite directions so that two genetically identical daughter cells can be formed. Physical connection, cohesion, between sister chromatid is essential for chromosome segregation. A chromosome-associated



multi-subunit protein complex, **cohesin**, keeps the sister chromatids connected to each other during metaphase. Cohesin is highly conserved in eukaryotes and has close homologs in bacteria.⁴⁷ Cohesion core complex consists of four subunits Scc1, Scc3, Smc1 and Smc3. RAD21 is mammalian counterpart of Scc1.

The segregation of sister chromatids occurs through the cleavage of Scc1/RAD21 subunit by 'separase' at metaphase-anaphase transition to open the ring structure of cohesin complex. The degradation of this cohesin subunit occurs via N-end rule pathway and hence it is essential to maintain chromosomal stability. This is an example of subunit-specific proteolytic degradation of N-end rule pathway, where a subunit is degraded without affecting the bigger oligomeric complex. In *S. cerevisiae*, it was found that at metaphase-anaphase transition separase cleaves the Scc1 of molecular mass 63kD. The resulting 33kD C-terminal fragment of Scc1 is an N-end rule substrate bearing an N-terminal destabilizing group Arg. Though the N-terminal residues of Scc1/RAD21 C-terminal fragment vary among species (Glu in mammals, Cys in drosophila), they are degraded via *Arg-ATE1* N-end rule pathway.⁴⁸ In *UBR1*^{-/-} *S. cerevisiae* cells frequent chromosome loss is observed due to failure of Scc1 degradation and genomic instability increases in *ATE1*^{-/-} mammalian cells.⁴⁹

The genomic stability is maintained by N-end rule pathway as it involves in DNA repair mechanism. DNA polymerase- β resynthesizes excised and damaged DNA strand during DNA repairing. In eukaryotes, PCNA (Proliferating Cell Nuclear Antigen) acts as processivity factor for DNA polymerase- α .^{50, 51} Mono-ubiquitinated PCNA up-regulates the activity of DNA polymerase- β and hence, maintains the DNA repair mechanism. A deubiquitin enzyme Usp1, ubiquitin specific peptidase 1, regulates genomic stability by de-ubiquitinating PCNA. Auto-cleaved Usp1 generates C-terminal fragment bearing N-terminal Glutamine, which is degraded by N-end rule pathway. The enhancer of Usp1 (Uaf1) binds to C-terminal fragment of Usp1 and delays the degradation of this fragment. By doing this Uaf1 keeps de-ubiquitinated activity of Usp1 up. The metabolic stability of Gln-bearing C-terminal fragment of Usp1 increases the de-ubiquitinating activity of Usp1 and hence negatively regulates genomic stability.⁵²

Regulation of peptide transport

One important function of N-end rule pathway in *S. cerevisiae* is the control of peptide import through the regulation of CUP9, a transcriptional repressor of the di- and tripeptide transporter PTR2.¹⁹ This is another example of subunit specific proteolytic degradation via N-end rule. CUP9 is a short-lived component of transcriptional repressor complex along with other long-lived component like Ssn6-Tup1.^{53, 54} Peptides are main source of amino acids and nitrogen for cellular requirement in all organisms. Cellular growth and development are largely dependent on the import of

peptides. CUP9, a 35kDa homeodomain protein, contains non-N-terminus internal destabilizing residue which is identified by a third 'type i' binding site of UBR1. This type i binding site which remains covered by C-terminus residues of UBR1, becomes uncovered by the binding of di/tri-peptides bearing N-terminal destabilizing residues with type 1 & 2 sites of UBR1. So, blocking of type 1/2 binding sites of UBR1 allosterically activates the action of type I binding site and hence, activates the degradation of CUP9. The expression of peptide transporter PTR2 increases due to CUP9 degradation. This allows *S. cerevisiae* to sense extracellular peptides and to increase peptide uptake.^{19, 48, 53} CUP9 also transcriptionally regulate the activity of OPT2 gene that encodes oligopeptides' importer.⁵⁵

Regulation of viral and bacterial infection

During infection in host cells retroviruses replicate themselves via reverse-transcription. Viral genome i.e. RNA is reverse-transcribed into cDNA, which is incorporated into host cell genome by an enzyme called integrase. HIV-1 (Human Immunodeficiency Virus-1) integrase also inserts its genome into the genome of host mammalian cells using this mechanism. During infection, HIV-1 integrase which is derived from a viral polyprotein by protease 'in virion' is released into cytoplasm of host cells. This cleaved integrase bears a primary type 2 destabilizing residue Phe and is degraded via N-end rule pathway.^{56, 57} Metabolic stabilization of HIV-1 integrase causes impaired replication of virus followed by ineffective early phase infection where reverse-transcription and integration of viral cDNA into host cell's genome is involved. This could be due to the inhibition property of HIV-1 integrase against reverse-transcriptase enzyme.⁵⁷

Like HIV-1 integrase, polyproteins in other viruses are also cleaved by viral proteases and give rise to protein fragments which are N-end rule substrates. Sindbid virus, responsible for sindbid fever in humans, produces four non-structural proteins named nsP1-4 from polyprotein upon infection. Among them nsP4 acts as viral RNA polymerase⁵⁸ and is short-lived in infected host cells. Tight regulation of nsP4 is important for the cell cycle of virus in host cells. Among three different stages of regulation of nsP4, N-end rule pathway is one regulation mechanism where nsP4 is identified and degraded by its N-terminal type 2 destabilizing residue Tyr. This substrate (sindbid virus RNA polymerase) was one of the earliest identified natural substrate of N-end rule pathway.⁵⁹ Replacement of Tyr by a stabilizing group like Ala causes the partial metabolic stabilization of nsP4⁵⁹ and it may be exploited to deregulate the cell cycle of sindbid viruses.

A gram-positive bacterium *Listeria monocytogenes* infects and causes listeriosis in human and other animals also. Listeriosis is a food-borne life-threatening disease affecting central nervous system. During infection bacteria release pore-forming toxin Listeriolysin O (LLO) to enter into cytoplasm from phagosome of host cells.



The bacterial secretion i.e. LLO bear an N-terminal type 1 destabilizing residue Lys. LLO is a mammalian N-end rule substrate and the replacement of Lys by a stabilizing group Val increases its half-life. The metabolic stabilization of LLO decreases virulence of this bacterium.⁶⁰

INVOLVEMENT OF N-RECOGNINS IN PHYSIOLOGICAL CONDOTION

N-recognins, important components of N-end rule pathway, recognizes N-degrons. Recent studies revealed roles of N-recognins involved in various physiological processes in mammals. In mammal, seven UBR box proteins, namely from UBR1-UBR7 have been identified though only four of them are well characterized.

UBR2 maintain chromosomal stability by the ubiquitination of histone in spermatocytes as well as somatic cells.⁶¹ UBR2^{-/-} male mice are infertile due to the arrest of spermatocytes at meiotic stage. During meiosis, UBR2 protein accumulates at a specific region of chromosomes to maintain the level of ubiquitinated histone and to act as transcriptional silencer of many genes associated with X & Y chromosomes.⁶² UBR2 function to ubiquitinate H2A & H2B but not H3 or H4. In UBR2 deficient mice, double strand breaking (DSB) repair mechanism was impaired due to insufficient ubiquitination of histone at meiotic stage. In somatic cells also, UBR2 mediates chromatin associated ubiquitination upon DNA damage.⁶¹ UBR2 up-regulation occurring in tumor bearing mice leads to muscle proteolysis. Tumor cell-induced up-regulation of UBR2 was observed in C2C12 myotubes treated with medium from LLC (Lewis Lung Carcinoma) or colon adenocarcinoma cells. The up-regulation of UBR2 was inhibited upon treatment of a p38^{MAPK} inhibitor molecule. It has been shown that the inhibition of p38^{MAPK} isoform of MAPK is necessary and sufficient to block the up-regulation of UBR2. The activation of p38^{MAPK} helps to activate the promoter region of UBR2 gene leading to up-regulation of UBR2 protein responsible for tumor induced muscle proteolysis.⁶³ UBR1 and UBR2 regulate mammalian target of rapamycin (m-TOR) signaling pathway by binding amino acid leucine. m-TOR mediated phosphorylation of S6K1 and 4E-BP is enhanced by leucine and hence the biosynthesis of proteins. Over-expression of UBR1/2 decreases m-TOR dependent phosphorylation of S6K1, but UBR1/2 deficient amino acid starved human 293T cells shows the up-regulation of S6K1 phosphorylation. So, UBR1 and UBR2 play an important role in leucine-m-TOR signaling pathway and regulate protein synthesis.⁶⁴

Another N-recognin UBR4 has been shown to regulate autophagy. UBR4/p600 protein remains connected with cellular cargoes which are engulfed by autophagic vacuoles and degraded by lysosomal degradation system. The loss of UBR4 causes multiple dysregulation in autophagic pathway and increases autophagy marker protein light chain-3 (LC3).⁶⁵ UBR4 not only helps proteolysis of short-lived proteins by proteasome, but

also mediates lysosomal bulk degradation where it absorbs maternal proteins from yolk sack and converts them into amino acids. UBR1^{-/-} mice die because of impaired vascular development in yolk sack during embryogenesis.⁶⁶

UBR1 is involved in an autosomal recessive disorder, called Johanson-Blizzard syndrome that includes dysregulation of pancreas, malformation and mental retardation. It has been shown that UBR1^{-/-} mice possesses abnormal behavior with weight loss and they are susceptible to pancreatic injury.⁶⁷ UBR1-deficient mice show less spontaneous activity and take longer time in learning process, suggesting that UBR1 is also involved in learning, memory and possibly other cognitive responses.⁶⁸

CONCLUSION

N-end rule proteasomal degradation pathway is still an emerging field, though many of its components are well characterized. Various studies showed that this pathway has crucial physiological roles in different organisms and mammals, though many remain undiscovered. Some synthetic inhibitors have been developed against UBR1 E3 ligase. Those inhibitors potentially regulate the metabolic fate of some proteins involved in crucial physiological process. Clearly, with the above examples it is evident that a potential N-end rule inhibitor can find numerous roles in multiple pathological conditions. So, the exploitation of this pathway by inhibitors or by some other means would be of great interest to understand regulates many down-stream physiological pathways, in normal and pathophysiological state. Hence, we believe that N-end rule pathway regulating molecules/inhibitors will play a bigger role in developing novel therapeutics against multitude of challenging diseased conditions.

Acknowledgement: SKP acknowledges CSIR, Govt. of India for doctoral fellowship. RB acknowledges CSIR 'Young Scientist Award' grant & CSIR-IICT Institute grant OLP 0003 for research.

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Source of Support: Nil, Conflict of Interest: None.

