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Emerging Branches of the N-End Rule Pathways are Revealing the Sequence Complexities of N-Termini Dependent Protein Degradation.

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Draft

Abstract

The N-end rule links the identity of the N-terminal amino acid of a protein to its *in vivo* half life as some N-terminal residues confer metabolic instability to a protein via their recognition by the cellular machinery that targets them for degradation. Since its discovery, the N-end rule has generally been defined as set of rules of whether an N-terminal residue is stabilizing or not. However, recent studies are revealing that the N-terminal code of amino acids conferring protein instability is more complex than previously appreciated as recent investigations are revealing that the identity of adjoining downstream residues can also influence the metabolic stability of N-end rule substrate. This is exemplified by the recent discovery of a new branch of N-end rule pathways that target proteins bearing N-terminal proline. In addition, recent investigations are demonstrating that the molecular machinery in N-termini dependent protein degradation may also target proteins for lysosomal degradation, in addition to proteasome dependent degradation. Herein, we describe some of the recent advances in N-end rule pathways discuss some of the implications regarding the emerging additional sequence requirements.

Keywords: N-end rule, protein degradation, arginylation, UBR domain, N-termini

Protein degradation is a major regulatory process in the maintenance of cellular proteostasis. The selective degradation of intracellular proteins controls diverse cellular and biochemical processes in all kingdoms of life. Not only is targeted protein degradation involved in controlling the levels of regulatory proteins but also in eliminating misfolded and any otherwise abnormal proteins. In mammalian cells, the intracellular selective protein degradation is mainly carried out by the Ubiquitin-Proteasome System (UPS) in addition to autophagy, and lysosomal proteolysis (Varshavsky 2011).

The N-end rule pathway

The first selective degradation signal discovered for proteins was the presence of destabilizing N-terminal amino acids on proteins (Bachmair et al. 1986; Bachmair and Varshavsky 1989; Gonda et al. 1989). This N-termini dependent protein degradation has been termed the N-end rule pathway, and encompasses the set of different N-terminal destabilizing amino acid residues that dictates the *in vivo* half-life of a given protein. The history and key milestones of our understanding of the N-end rule have been recently summarized (Varshavsky 2017) and investigations over the past years have revealed that the N-end rule pathway degrades a number of proteins that are central to a wide spectrum of cellular and biological processes in eukaryotes such as genome stability and repair (Hwang et al. 2009; Piatkov et al. 2012b; Rao et al. 2001), apoptosis (Ditzel et al. 2003; Eldeeb and Fahlman 2014; Piatkov et al. 2012a; Xu et al. 2012), autophagy (Cha-Molstad et al. 2017; Jiang et al. 2016; Yamano and Youle 2013), neurodegeneration (Brower et al. 2013), development (Weaver et al. 2017), and nitric oxide sensing of G-protein regulators (Hu et al. 2005; Lee et al. 2012).

While the biological roles of the N-end rule pathways have been mostly implicated because of the identification and characterization of particular protein substrates, the overall function of the

N-end rule in these processes is likely very complex as a result of the large number of various proteins that can be targeted by the pathway. For example, many pro-apoptotic protein fragments generated by proteolytic cleavage have been demonstrated to be targeted for degradation via the N-end rule pathway (Ditzel et al. 2003; Eldeeb and Fahlman 2016a; Eldeeb and Fahlman 2016b; Piatkov et al. 2012a; Piatkov et al. 2014; Xu et al. 2012). In addition to this, there are examples demonstrating that the inhibition of the N-end rule or knockouts of components of the N-end rule pathway sensitize cells to cell stress and cell death (Agarwalla and Banerjee 2016; Deka et al. 2016; Piatkov et al. 2012a). With these examples, it appears that the N-end rule plays an active role in inhibiting cell death, but additional reports have also demonstrated that the N-end rule also degrades anti-apoptotic protein fragments (Eldeeb and Fahlman 2014) and components of the N-end rule pathway promote cell death and cell cycle arrest (Kumar et al. 2016). These examples demonstrate that the biological function of the N-end rule pathway may not be simply defined as pro- or anti- apoptotic but appears to participate in the crucial balance of both pro- and anti- apoptotic proteins. This complexity in the role of the N-end rule pathway is exemplified by the Johanson-Blizzard syndrome, a genetic disease resulting from inactivating mutations to one of the redundant ubiquitin E3 ligases involved in the N-end rule pathway (Hwang et al. 2011; Zenker et al. 2005). The complexities of the Johanson-Blizzard syndrome being a multisystem congenital disorder effecting many tissues in the body when only a single redundant component of the N-end rule is mutated suggests key roles for this pathway in diverse tissues in the body.

As outlined in **Figure 1**, in eukaryotes there are a series of primary N-terminal destabilizing residues that include the type I positively charged Arg, Lys and His amino acids in addition to the type II large hydrophobic, Leu, Phe, Tyr, Trp, and Ile amino acids. Proteins with these two

types of N-terminal destabilizing residues are recognized by different protein domains on a series of E3 ubiquitin ligases in eukaryotes that include Ubr1 in *S. cerevisiae* (Bartel et al. 1990), Ubr11 in *S. pombe* (Fujiwara et al. 2013) and Ubr1, Ubr2, Ubr4 and Ubr5 in mammals (Tasaki et al. 2005). In addition to these primary destabilizing residues, N-terminal Asn and Gln serve as tertiary destabilizing N-terminal residues through their enzymatic deamidation via N-terminal amidohydrolases into the secondary destabilizing N-terminal residues Asp and Glu, respectively (**Figure 1B**) (Baker and Varshavsky 1995; Wang et al. 2009). The secondary destabilizing residues, Asp and Glu, are recognized by Ate1 which catalyzes the transfer of Arg (a primary N-terminal destabilizing residue) from Arg-tRNA_{Arg} to the N-termini of the protein (Balzi et al. 1990). For additional discussions regarding the molecular components of the N-end rule pathway, one is referred to two extensive reviews (Sriram et al. 2011; Tasaki et al. 2012).

Similar, yet distinct versions of N-end rule pathways are present in diverse organisms that have been investigated, including the bacterium *E. coli* (Tobias et al. 1991), yeast (Bachmair et al. 1986), plants (Potuschak et al. 1998) and mammals (Gonda et al. 1989).

Diversity of N-end rule pathways.

Although the general hierarchic structure and organization of the N-end rule is evolutionarily conserved from eubacteria to mammals, the actual specific enzymatic reactions and chemical modifications that mediate the recognition and subsequent targeting of N-terminal destabilizing residues are diverse. In higher eukaryotes an oxidized N-terminal cysteine is also a destabilizing residue as it is recognized by Ate1 for N-terminal arginylation (Kwon et al. 2002). In mammals, the generation of nitric oxide (NO) can lead to N-terminal cysteine oxidation and subsequent protein degradation (Hu et al. 2005). Conversely, in plants N-terminal cysteine oxidation is enzymatically catalyzed in the presence of molecular oxygen (White et al. 2017). In depth

discussions regarding the roles of N-end rule protein degradation in plants can be found in a number of recent reviews (Dissmeyer et al. 2017; Gibbs et al. 2016; van Dongen and Licausi 2015). As yeast lacks NO-synthases and N-terminal cysteine oxidases, N-terminal cysteines are stabilizing residues in these organisms.

Beyond eukaryotes, the N-end rule pathways are also diverse. For instance, in *E. coli* which is devoid of the ubiquitin-proteasome system, the N-end rule still exhibits both primary and secondary N-terminal destabilizing residues, but basic N-terminal Lys and Arg residues are secondary destabilizing residues (Tobias et al. 1991). In *E. coli*, proteins with basic N-termini are recognized by L/F-transferase, which catalyzes the transfer of Leu from an aminoacyl-tRNA to the N-termini of the protein, which is then targeted for degradation by the Clp protease system (Tobias et al. 1991). This biochemically determined N-terminal specificity by L/F-transferase may be oversimplified as the first *in vivo* substrate identified for the enzyme revealed that the protein was modified on an N-terminal methionine (Ninnis et al. 2009). Detailed discussions of the bacterial N-end rule are presented in a couple of reviews (Dougan et al. 2010; Fung and Fahlman 2015).

New N-end rule pathways.

Over the past several years there have been emerging examples of additional selectivity to N-termini dependent protein degradation. These include the emergence of N-terminal acetylation as a new branch of N-termini dependent degradation, the newly discovered proline N-termini

dependent degradation and a recent report that has brought N-terminal serine back into focus for N-termini dependent protein degradation.

Ac-N-End rule: An emerging major branch in N-termini dependent protein degradation is dependent on N-terminal acetylation, and has been referred to as the Ac-N-end rule pathway (Hwang et al. 2010). This selective targeted degradation has been demonstrated to be via E3 ubiquitin ligases that include Doa10 in yeast (Kim et al. 2014) and Teb4 in mammals (Park et al. 2015). As it has been estimated that ~60% of the yeast proteome and ~90% of the mammalian proteomes are N-terminally acetylated (Arnesen et al. 2009), this pathway is predicted to have a widespread impact on cellular proteostasis. Emerging lines of evidence are revealing that the selective degradation of proteins by the Ac-N-end rule may be more complex than initially appreciated. A recent proteomic analysis on NatB, an N-terminal acetylase, has reported that the global protein abundance of NatB substrates is not significantly altered in NatB mutants (Gao et al. 2016). This apparent dichotomy may be rationalized in several ways, which include sufficient residual acetylation in the mutant, additional unforeseen sequence specificity of the Ac-N-end rule pathway or that many of the N-termini are inaccessible for the Ac-N-end rule pathway as this pathway has been reported to degrade protein subunits that are in excess of their target complex (Shemorry et al. 2013). In support of a model where many of these acetylated N-termini may not be directly accessible for the Ac-N-end rule pathway is the recent report that demonstrates this pathway is involved in the degradation of several HSP90 client proteins (Oh et al. 2017). While much is left to be explored regarding this interplay between N-terminal acetylation and protein stability, some of its implications and details have been discussed in detail (Eldeeb and Fahlman 2016a; Lee et al. 2016).

The Pro/N-End rule pathway:

Recently another branch of N-termini dependent protein degradation has been reported, the N-terminal Proline-degron, that targets the gluconeogenic enzymes fructose-1, 6-bisphosphatase (Fbp1), isocitrate lyase (Icl1), and malate dehydrogenase for N-termini dependent degradation (Chen et al. 2017). In this report, Varshavsky and colleagues demonstrated that Gid4 subunit of the GID E3 ubiquitin ligase is the major recognin responsible for targeting substrates with an N-terminal proline. This report builds upon previous investigations on protein degradation of these enzymes during the switch from gluconeogenesis to glycolysis which had identified the dependence of protein degradation on the N-terminal proline (Hammerle et al. 1998) and suggested this was dependent on Gid1 (Menssen et al. 2012; Santt et al. 2008). While these key elements were previously identified, the identification and determination of the essential role of Gid4 in substrate recognition was not reported. This is in light of previous work where what appeared to be an N-terminal proline targeting a protein for degradation was in fact modulating phosphorylation dependent degradation (Sheng et al. 2002). The recent work by Chen et. al. (Chen et al. 2017) now delineates the specificity of Gid4 as a Pro/N-recognin and elucidated the molecular elements that bring about the binding of Gid4 to its target substrates of Fbp1 and other gluconeogenic enzymes.

Pro/N-end rule degradation may be specific for a defined subset of proteins with proline N-termini as additional sequences are required for recognition. The extent of tolerance in interaction between Gid4 and its target substrates was elegantly addressed by series of mutants in the first 6 amino acids of Fbp1 coupled to yeast two-hybrid assay (Chen et al. 2017). It was demonstrated that variations in these amino acids leads to either permissive (allowed), suboptimal or restrictive (disallowed) binding. This and other related results prompted the authors to suggest that the deep span and flexibility of binding groove of Gid4 towards its

substrates is reminiscent of the binding groove of antigen-presenting MHC proteins. Aspects of the sequence specificity and significance of the Pro/N-end rule has been discussed previously (Dougan 2017), but additional insights will require the characterization of the diversity of physiological substrates of this pathway to determine whether it is highly distinct for regulating the identified glucogenic enzymes or targets a more global set of proteins for degradation.

Another intriguing aspect of the Pro/N-end rule pathway is the dichotomous targeting of the substrates via GID-proteasome degradation and the alternative autophagy-related targeted degradation pathway via vacuole import and degradation (VID). It remains to be understood why eukaryotic cells have evolved two unique mechanisms (proteasome-related and autophagy-related) to conditionally degrade specific proteins that have a Pro/N-degron. What are the specific molecular inputs that trigger the transition between proteasomal targeting and VID-based degradation? It has yet to be determined how the Gid4 recognition of Pro/N-degron could be exploited by VID-based degradation. In line with this alternative path to degradation, it is noteworthy to mention that the canonical Arg-N-rule pathway has been shown to target proteins for degradation via the canonical ubiquitin–proteasome dependent pathway (Piatkov et al. 2012a) and through autophagy (macro-autophagy)-related pathway (Jiang et al. 2016).

A return of serine to the forefront of N-termini dependent protein degradation.

Recent work by Justa-Schuch *et al.* unveiled a crucial role of N-terminal serine in regulating metabolic stability of the Syk tyrosine kinase in B-cells (Justa-Schuch et al. 2016). Notably, Syk is activated upon B-cell receptor activation, and its activity can be counteracted via the activity of phosphatases including PTPRO and Shp-1 (Chen et al. 2006), and through proteasomal degradation mediated by the E3 ubiquitin ligase Cbl (Paolini et al. 2001). Justa-Schuch et al. (2016) demonstrate evidence for a role of Dpp9, a serine aminopeptidase, as a crucial negative

regulator of Syk tyrosine kinase that impacts Syk metabolic stability via N-termini dependent degradation (Justa-Schuch et al. 2016). The inhibition of DPP9 led to an increased half-life of Syk and higher levels of actively phosphorylated Syk pY352, indicating that DPP9 may trigger the targeting of the active form of Syk for proteasomal degradation. Crucially, the authors revealed that processing of Syk by Dpp9 exposes a Ser neo N-terminal residue. Mitigation of Dpp9 activity or mutation of the Dpp9 cleavage site in Syk (SykD2A) augmented the stability of Syk. The key role of the newly exposed Ser for Syk stability was demonstrated by mutagenesis of this residue to the otherwise stabilizing Gly or Val residues, where the mutants exhibited significantly longer half-lives. Based on this, the authors conclude that Syk is a novel substrate for N-termini dependent degradation and Dpp9 may act as a crucial peptidase that has the potential to modulate the stability of many proteins in a similar fashion.

While it was determined that the N-terminal serine mediated degradation of Syk is dependent on the E3 ubiquitin ligase c-Cbl (Justa-Schuch et al. 2016), it remains unclear whether this is an independent branch of N-termini dependent degradation or whether there are other factors involved that direct degradation by either Ac-N-end rule degradation or the canonical N-end rule machinery.

This report on Syk is not the first example of serine being proposed to be a destabilizing N-terminus. Initial work on the N-end rule revealed that while a reporter protein with a serine N-termini was stable in yeast it was degraded in rabbit reticulocyte extract (Gonda et al. 1989). From these initial investigations Ser, Thr and Ala have been grouped as Type III destabilizing N-termini (Varshavsky 2003) (**Figure 1**), for which much is still unknown. It has been demonstrated that the degradation of proteins with these N-termini are not dependent on the UBR domain containing proteins as has been demonstrated for both type I and type II

destabilizing N-termini (Tasaki et al. 2005; Tasaki et al. 2009). It is possible that the degradation of protein with type III N-termini maybe in fact explained by the Ac-N-end rule pathway but this has yet to be fully explored and is potentially complicated by the potential for an N-terminal Ser being accessible to post-translational modifications, such as phosphorylation.

Higher levels of sequence specificity in N-terminal dependent protein degradation.

A key finding of Pro/N-end rule was the requirement for additional sequence elements in the N-terminal peptide sequence (Chen et al. 2017). This additional sequence dependence on N-terminal dependent degradation is not unique to this pathway as reviewing the literature on the canonical Arg-N-end rule pathway also reveals a variety of additional sequence dependencies. In the case of cysteine oxidation by NO in mammals, there is a requirement for the penultimate amino acid to be a basic residue to enable the chemical S-nitrosylation of the N-terminal Cys by NO (Hu et al. 2005).

Systematic analysis of substrate selectivity of the aminoacyl-tRNA transferases, which catalyze the addition of destabilizing amino acids to the N-termini of secondary destabilizing N-termini (**Figure 2A**), have also revealed some selectivity for the second amino acid for both the bacterial (Kawaguchi et al. 2013) and eukaryotic (Wadas et al. 2016b) enzymes. With the eukaryotic aminoacyl-tRNA transferase, Ate1, investigations revealed a general low preference for proline and tryptophan at the penultimate position for the four-different alternative spliced isoform of Ate1 tested. In addition, there were differences between the isoforms, for example the Ate1_{1B7B} spliced isoform exhibits an additional low specificity for aspartate and glutamate penultimate amino acids (Wadas et al. 2016b) (**Figure 2B**).

Structural and biochemical investigations of the UBR domain from the E3 ligases which bind Type I destabilizing N-termini, have also revealed a role for the penultimate amino acid in the binding of peptides to this protein (Choi et al. 2010; Matta-Camacho et al. 2010; Munoz-Escobar et al. 2017). Intriguingly, while the Ubr proteins are highly conserved within eukaryotes there appears to be some divergence in the binding specificity as a result of the identity of the penultimate amino acid for the yeast (Choi et al. 2010) and human (Munoz-Escobar et al. 2017) UBR domains.

With the interconnection of Ate1 products being substrates for the Ubr proteins, a systematic comparison of the reported penultimate amino acid specificity is presented (**Figure 2B**). With Ate1 recognizing acidic N-terminal residues the products it generates have a acidic penultimate residue as dictated by its substrate specificity. When one then compares this to the reported specificity of the Ubr proteins, it appears that the yeast UBR1 protein exhibits a low specificity for acidic penultimate residues (Choi et al. 2010), such that Ate1 products in yeast are sub-optimal substrates for UBR1. Further investigations may reveal this apparent dichotomy may be to tune the half-life of protein substrates or may correlate with emerging alternative roles for protein arginylation (Cha-Molstad et al. 2015; Cornachione et al. 2014; Jiang et al. 2016; Saha et al. 2010; Zhang et al. 2015).

The implications of these additional sequence requirements may have evolved to regulate the specific half life of individual proteins with destabilizing N-termini. Furthermore, it shall provide the molecular basis for a potential cellular mechanism that can regulate the degradation of a protein via post translational modifications that may be analogously identified in the future as we have previously demonstrated for an internal phosphorylation site modulating the degradation of the BMX kinase by the N-end rule pathway (Eldeeb and Fahlman 2016b).

Conclusions

In summary, the discovery of new N-end rule pathways is revealing an increasing diversity of N-termini that can be recognized for targeted protein degradation. The biological implications of these protein degradations pathways are still to be fully realized by the identification and characterization of their substrate repertoires. The revelations of sequence specificity and context of the components of these pathways make the simple prediction of substrates insufficient, as the prediction of substrate stability in cells has demonstrated additional complexities(Wadas et al. 2016a). Additionally, the elucidation of sequence specificities of the components of these N-end rule pathways may eventual reveal potential mechanisms of regulation where reversible post translational modifications may modulate their recognition by the components of these pathways.

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Figure Legends

Figure 1. Destabilizing N-Terminal Amino Acids. A) A summary of the destabilizing amino acids in eukaryotes with the exclusion of the complexities of the Ac-N-end rule pathway (Park et al. 2015). Blue highlights the stable N-terminal residues. The different shades of orange highlight the distinct destabilizing residues recognized by the canonical Arg-N-end Rule pathway; the tertiary, secondary and Type I and Type II primary destabilizing residues. Gold highlights the recent identification of Pro as an N-terminal destabilizing residue and yellow emphasizes the poorly defined type III N-termini. B) Schematic of the hierarchical structure of the canonical mammalian N-End Rule pathway and the key steps leading to proteasomal dependent degradation.

Figure 2. Summary of penultimate amino acid effects. A) Schematics of biochemical investigations used for investigating the effects of penultimate residues (X) on arginylation by Ate1 or binding to UBR protein domains. B) Summary of the quantified data reported for penultimate amino acid (X) effects for the enzymatic activity of human Ate1 (Wadas et al. 2016b), the binding affinity for the UBR domain from human Ubr2 (Munoz-Escobar et al. 2017) or the UBR domain from yeast Ubr1 (Choi et al. 2010). The areas of the circles presented in the dot blot are proportional to the relative activity for Ate1 or binding for the UBR proteins.

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51x20mm (600 x 600 DPI)

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96x54mm (300 x 300 DPI)