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A D-Amino Acid at the N-Terminus of a Protein Abrogates Its Degradation by the N-End Rule Pathway

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S *Supporting Information*

ABSTRACT: Eukaryotes have evolved the ubiquitin (Ub)/ proteasome system to degrade polypeptides. The Ub/ proteasome system is one way that cells regulate cytosolic protein and amino acids levels through the recognition and ubiquitination of a protein's N-terminus via E1, E2, and E3 enzymes. The process by which the N-terminus stimulates intracellular protein degradation is referred to as the N-end rule. Characterization of the N-end rule has been limited to only the natural L-amino acids. Using a cytosolic delivery platform derived from anthrax lethal toxin, we probed the stability of mixed chirality proteins, containing one D-amino acid on the N-terminus of otherwise all L-proteins. In all cases, we observed that one N-terminal D-amino acid stabilized the cargo protein to proteasomal degradation with respect to the N-end rule. We found that since the mixed chirality proteins were not polyubiquitinated, they evaded N-end-mediated proteasomal degradation. Evidently, a subtle change on the N-terminus of a natural protein can enhance its intracellular lifetime.
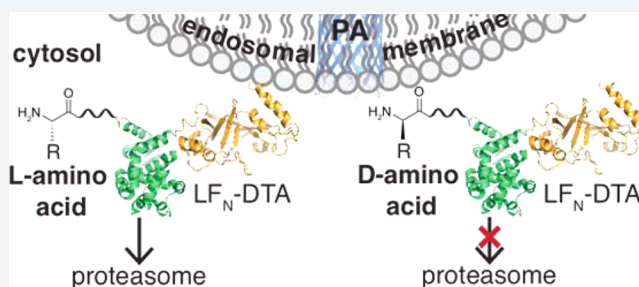
INTRODUCTION

The chirality of biomolecules in nature is critical for substrate recognition, protein binding, and product formation. While E3 ubiquitin (Ub) ligases of the Ub/proteasome system have promiscuous substrate binding sites, the chirality of protein substrates has never been investigated.[1−3] Since the homeostasis of a cell's protein and amino acid concentrations is regulated by the proteasome, perturbation of the proteasome's activity through substrate modification can affect the intracellular equilibrium. Here, we investigated the effect of one mirror image D-amino acid on the N-terminus of otherwise all L-proteins on proteasomal degradation after delivery of the mixed chirality proteins into the cytosol of cells.

Varshavsky and co-workers have characterized the N-end rule as it relates to the intracellular stability of proteins.[4−6] According to the N-end rule, the identity of the N-terminal amino acid mediates the selective degradation of specific proteins through the Ub/proteasome system. The N-terminal degradation signals are termed N-degrons, which can range from stabilizing to destabilizing residues. Key destabilizing residues include type 1 (R, K, and H) and type 2 (L, F, Y, W, and I) residues, while D, E, N, Q, and C can be destabilizing after modifications such as acetylation or arginylation. N-degrons are recognized by N-recognins, or E3 Ub ligases, which interact with E2 Ub conjugating enzymes to polyubiquitinate proteins for proteasomal degradation.[6] To date, the N-end rule has been defined only for L-amino acids.[7−10] In a recent study by Sriram et al. to identify inhibitors of the N-end rule, the authors demonstrated that two mixed chirality dipeptides

containing an N-terminal DArg did not affect protein stability to the same extent as dipeptides containing an N-terminal LArg.[3] While this study suggested that the N-end rule is stereospecific, it does not provide direct evidence that these findings will hold for an intact protein. The main challenge with probing the stability of proteins containing non-natural functionalities at the N-terminus is the delivery of such proteins into the cytosol. To overcome this challenge, we utilized a platform derived from nature that enables the delivery of different types of proteins into the cytosol of cells.

Anthrax lethal toxin from *Bacillus anthracis* utilizes the protective antigen (PA) pore to deliver lethal factor (LF) into the cytosol.[11] Protein translocation by anthrax lethal toxin has been extensively characterized. In short, to obtain entry into the cell, PA binds to an anthrax receptor on the cell surface and forms the PA prepore.[12−17] LF binds to the PA prepore, then the entire complex is endocytosed, and endosomal acidification triggers a conformational rearrangement of the PA prepore to form a pore.[18,19] LF translocates through the pore via a charge state dependent Brownian ratchet into the cytosol.[13,20] The N-terminal domain of LF (LF$_N$) is sufficient to bind to the PA prepore, but does not cause any intracellular toxicity.[21] The PA/LF$_N$ delivery system has been engineered to deliver various peptide, protein, and small molecule cargoes into the cell cytosol. Previous work has shown that numerous peptides and

proteins, including those composed of D-amino acids, can be efficiently delivered through the PA pore.[22−25]

The majority of eukaryotic proteins from ribosomal translation are composed of L-amino acids and achiral glycine. In order to study the intracellular stability of proteins containing D-amino acids, we used sortase A (SrtA) from *Staphylococcus aureus* or native chemical ligation (NCL) to ligate one D-amino acid onto the N-terminus of L-proteins that can then be delivered in a PA dependent manner.[26] Furthermore, we incorporated a cleavable linker that releases the cargo protein from LF_N after translocation into the cytosol further allowing us to characterize N-terminal D-amino acids on proteins other than LF_N, including A-chain of diphtheria toxin (DTA)[27,28] and a designed ankyrin repeats protein (DARPin).[29,30] We opted to use a hindered disulfide cleavable linker that was small in structure such that it could translocate through the PA pore efficiently and be readily cleaved in the reducing environment of the cytosol.[31]

■ RESULTS

Sortase A Attaches One D-Amino Acid onto the N-Terminus of LF_N-DTA. The X-LF_N-DTA_{mut} constructs were produced through enzyme-mediated ligation of XALPSTGG onto the N-terminus of the LF_N-DTA_{mut}. The N-terminal amino acid (X) represents a natural L-amino acid (^{L}X) or its mirror image D-amino acid (^{D}X), while the remaining residues were L-stereochemistry (Figure 1a). Each XALPSTGG peptide
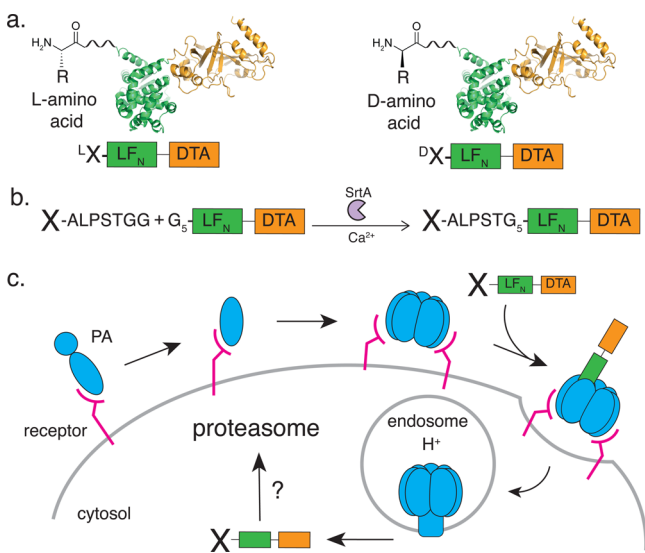
Figure 1. Intracellular stability was monitored for X-LF_N-DTA constructs delivered through protective antigen pore. (a) ^{L}X- (left) and ^{D}X- (right) amino acids ligated to the N-terminus of LF_N-DTA (LF_N, green, pdb is 1J7N; DTA, orange, pdb is 1DTP). (b) XALPSTGG peptides, where X represents either an L- or D-amino acid, are ligated onto G_5-LF_N-DTA using sortase A (SrtA) to form X-LF_N-DTA constructs. (c) Translocation of X-LF_N-DTA constructs is achieved using protective antigen (PA) of anthrax toxin.

was ligated to G_5-LF_N-DTA_{mut} using *Staphylococcus aureus* sortase A to yield XALPSTG$_5$-LF_N-DTA_{mut} constructs (X-LF_N-DTA_{mut}; Figure 1b). A one-pot ligation scheme was used for each reaction.[22,32]

One N-Terminal D-Amino Acid Stabilizes LF_N-DTA to Proteasomal Degradation. We used the A chain of diphtheria toxin (DTA) as a first measure of proteasomal

degradation inside cells with less protein synthesis inhibition, inferring that the cargo was degraded more rapidly. Similar assays have been used to understand how the N-end rule affects toxin stability in the cytosol of cells.[7] For our experiments, we used a mutant form of DTA (E148S; DTA_{mut}) that is 300-fold less active than wild-type DTA,[33] allowing us to detect differences in cytosolic lifetime of each X-LF_N-DTA_{mut} over a wider dynamic range. Since wild-type DTA can neutralize its substrate in minutes, DTA_{mut} enabled analysis of various substrates after a 6-h time period. DTA inhibits protein synthesis by ADP ribosylating elongation factor-2.[27,28] To corroborate our observations with DTA as the read-out, we used an orthogonal assay based on Western blot analysis of the cytosolic fraction (Figure 1c).[34]

For the protein synthesis inhibition assay, Chinese hamster ovary (CHO-K1) cells were treated with 10-fold serial dilutions of each construct for 6 h to allow for sufficient buildup of the translocated material in the cytosol and to observe DTA_{mut} activity. After translocation, the cells were washed and treated with ^{3}H-Leu in leucine-free medium for 1 h to detect DTA_{mut} activity. The fraction of protein synthesis with respect to DTA_{mut} was measured with a scintillation counter (Figure S1 and Table S1). According to Figure 2a, regardless of chirality, stabilizing amino acids such as A and V on the N-terminus of LF_N-DTA_{mut} had similar EC_{50} values as the positive control (LF_N-DTA_{mut}), which contains ^{L}A at the N-terminus. Furthermore, destabilizing residues like ^{L}W on the N-terminus of LF_N-DTA_{mut} had significantly higher EC_{50} values than the control (i.e., less DTA activity) while the ^{D}W-LF_N-DTA_{mut} construct displayed activity comparable to the control, which suggests that D-amino acids act as stabilizing residues.

To further confirm that our observations were a result of proteasomal degradation, we used Western blot analysis. The X-LF_N-DTA_{mut} constructs were delivered into CHO-K1 cells, lysed using digitonin lysis buffer, and analyzed by Western blot. Digitonin is a nonionic detergent used to permeabilize the plasma membrane, while the membrane-bound organelles remain intact. CHO-K1 cells were treated with X-LF_N-DTA_{mut} constructs in the presence of PA for 6 h and were lysed using a digitonin lysis buffer and then analyzed by Western blot. The Western blot was immunostained with an LF antibody for stability analysis, and then stained for the cytosolic proteins, Erk1/2, and the early endosomal protein, Rab5. A low level of Rab5 and a high level of Erk1/2 demonstrate efficient cytosolic extraction. Based on the findings in Figure 2b, the Western blot results corroborated the protein synthesis inhibition data and showed significant differences in cytosolic protein levels, where D-amino acids proved to be stabilizing regardless of the side chain identity. These data support the N-end rule for N-terminal L-amino acids, while all N-terminal D-amino acids stabilized X-LF_N-DTA_{mut} to degradation.

As a control, CHO-K1 cells were treated with select conjugates (^{L}V-, ^{D}V-, ^{L}A-, ^{D}A-, ^{L}W, and ^{D}W-LF_N-DTA_{mut}) in the presence of lactacystin, a proteasome inhibitor. The samples treated with lactacystin all showed strong anti-LF bands, indicating that the proteasome played a key role in degrading the ^{L}X-LF_N-DTA_{mut} constructs but had no observable effect on the ^{D}X-LF_N-DTA_{mut} constructs. Furthermore, these data indicated that each construct translocated efficiently into the cells, regardless of the N-terminal amino acid.

To verify the mechanism of translocation and endosome escape, we incubated the X-LF_N-DTA_{mut} constructs with a mutant PA (PA[F427H]),[35] a vacuolar H^+-ATPase inhibitor
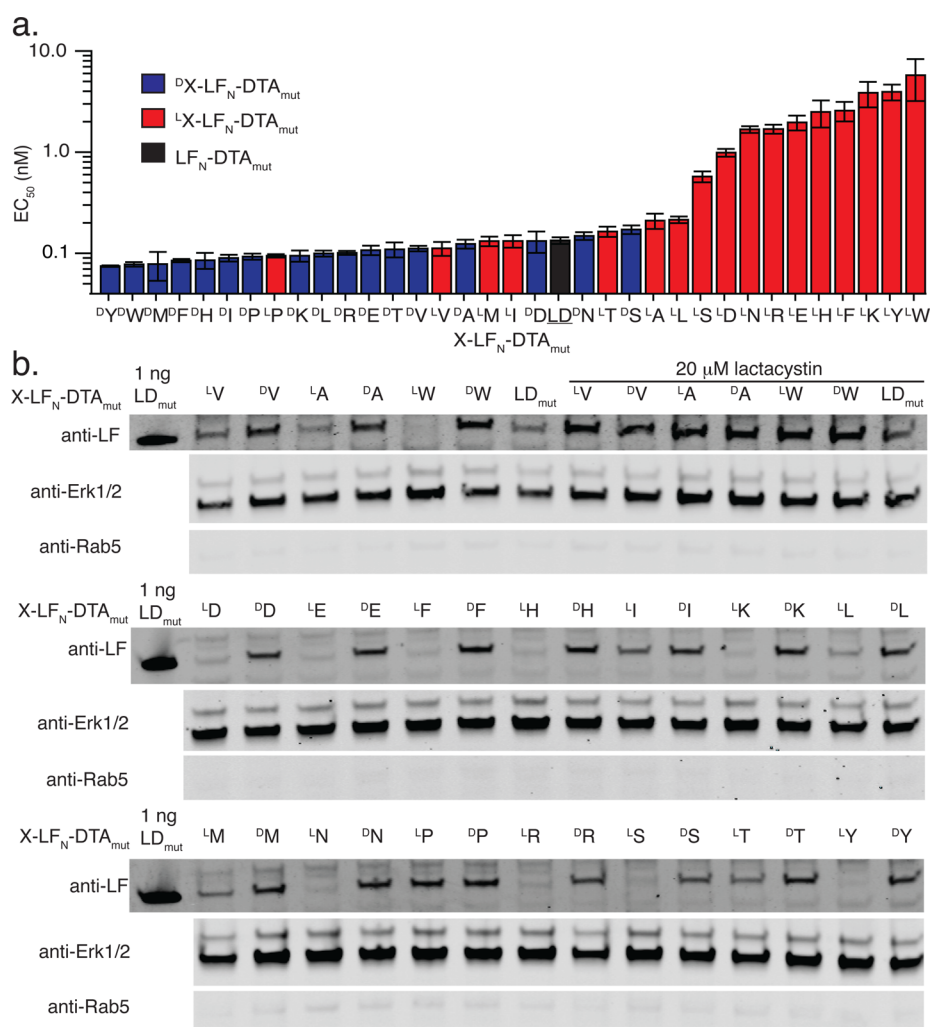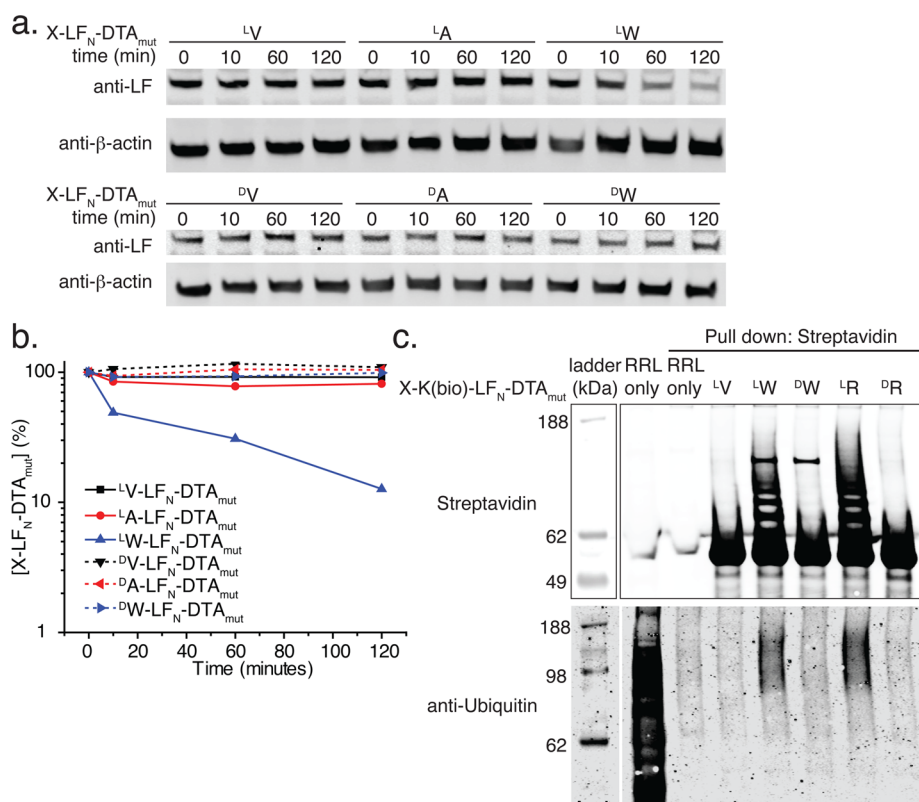
Figure 2. One N-terminal D-amino acid on LF$_N$-DTA enhances protein stability. (a) Translocation X-LF$_N$-DTA constructs was analyzed by protein synthesis inhibition assay in CHO-K1 cells after 6 h ($n = 3$). EC$_{50}$ values from the protein synthesis inhibition assay were graphed for all ^{L}X-LF$_N$-DTA or ^{D}X-LF$_N$-DTA constructs. EC$_{50}$ values (and error bars) were determined using a Boltzmann distribution fit. (b) ^{L}V-, ^{D}V-, ^{L}A-, ^{D}A-, ^{L}W-, and ^{D}W-LF$_N$-DTA$_{mut}$ were translocated into CHO-K1 cells in the presence of 20 nM PA for 6 h, then extracted using digitonin lysis buffer, and analyzed by Western blot. As a proteasomal inhibitor, 20 μM lactacystin was used. Translocation of all ^{L}X-LF$_N$-DTA or ^{D}X-LF$_N$-DTA constructs was analyzed by Western blot.

(bafilomycin A1), or at 4 °C with CHO-K1 for 6 h. In all cases, no material was found to translocate into the cytosol by Western blot (Figure S2). These controls indicated that delivery of the X-LF$_N$-DTA$_{mut}$ constructs into the cytosol is dependent on functional endocytic machinery and PA. Moreover, we found that our observations were not cell-specific. After translocation, we observed protein stabilization in human embryonic kidney cells (HEK-293T) and human cervical cancer cells (HeLa), similar to the stabilization observed in CHO-K1 cells (Figure S3).

Proteasomal Stabilization Is Not an Artifact of the Sortag. SrtA ligation adds a short linkage (i.e., LPSTG$_5$) between the N-terminal amino acid (X) and the start of the protein. We used native chemical ligation (NCL)[36] to prepare constructs with native N-terminal sequences for comparison with sortagged proteins. For our analysis, LF$_N$-DTA$_{mut}$ was synthesized containing L-alanine at the N-terminus (wild-type) and compared to NCL synthesized constructs containing ^{D}A, ^{L}W, or ^{D}W.[37] Each NCL construct was translocated in CHO-K1 cells, and their protein stability was compared to that of the sortagged conjugates using Western blot. For the NCL reaction

we installed a Cys residue at position 17 that was later alkylated with bromoacetamide, which does not affect translocation. Based on the Western blot of the translocated material in Figure S4, both native and sortagged constructs containing ^{L}A, ^{D}A, and ^{D}W had similar protein stability, while ^{L}W in both cases was degraded. These observations indicated that stabilization of LF$_N$-DTA$_{mut}$ through the incorporation of one N-terminal D-amino acid is not an artifact of the sortag.

LF$_N$-DTA with One N-terminal D-Amino Acid Is Stable In Vitro. To support our cytosolic studies, we analyzed the in vitro rates of degradation of the X-LF$_N$-DTA$_{mut}$ constructs in rabbit reticulocyte lysate (RRL). Pure X-LF$_N$-DTA$_{mut}$ proteins were incubated in the presence of 70% RRL at 37 °C. Samples at various time points were pulled and analyzed by Western blot using LF and β-actin antibodies (Figure 3a). As indicated in Figure 3b, only ^{L}W-LF$_N$-DTA$_{mut}$ experienced significant protein degradation after 120 min. These data further support the in vivo protein synthesis inhibition and Western blot analyses, which collectively suggest that N-terminal D-amino acids stabilize LF$_N$-DTA$_{mut}$ to protein degradation.

Figure 3. One N-terminal D-amino acid prevents ubiquitination of LF_N-DTA. (a) The stability of ^LV-, ^DV-, ^LA-, ^DA-, ^LW-, and ^DW-LF_N-DTA_{mut} (4 ng) was monitored in 70% RRL over time at 37 °C and then analyzed by Western blot. (b) The concentration of X-LF_N-DTA_{mut} (%) was plotted against time, based on the Western blot in panel a. (c) X-K(bio)-LF_N-DTA_{mut} constructs (1 μM; X represents ^LV, ^LW, ^DW, ^LR, and ^DR) were incubated in 70% RRL for 10 min at 37 °C and then pulled down using streptavidin beads for 1 h. Elution samples were analyzed by Western blot (streptavidin and anti-ubiquitin staining).

LF_N-DTA with One N-Terminal D-Amino Acid Is Not Ubiquitinated. Polyubiquitination of proteins by the E1, E2, and E3 enzymes is a critical step before proteasomal degradation.[38] In order to identify the mode in which proteins with N-terminal D-amino acids are stabilized, we used a pull-down assay. Protein constructs containing biotin (X-K(bio)-LF_N-DTA_{mut}, where K(bio) represents biotinylated lysine and X represents ^LV, ^LW, ^DW, ^LR, or ^DR) were synthesized (Figure S5 and Figure S6). Each construct was incubated with 70% RRL for 10 min to allow for polyubiquitination followed by pull-down using streptavidin beads and Western blot analysis (Figure 3c). Streptavidin and anti-ubiquitin staining indicated that only the constructs containing N-terminal ^LW and ^LR were ubiquitinated, while the negative control ^LV- as well as both ^DW- and ^DR-K(bio)-LF_N-DTA_{mut} constructs contained no detectable ubiquitination. These results indicate that destabilizing amino acids like ^LW and ^LR are recognized by the Ub/proteasome system and are readily degraded, while ^DW and ^DR are not ubiquitinated (Figure 3d).

N-Terminal Stabilization Is Not Protein-Specific. In order to study the stabilization of proteins other than LF_N, we incorporated a cleavable linker to separate the attached cargo from LF_N once the entire construct translocated into the cytosol. The cleavable linker allowed for the intracellular stabilization of different types of cargo to be explored using the PA/LF_N delivery platform. We used a hindered disulfide cleavable linker to increase its stability toward reduction outside of the cell.[31] While hindered disulfide bonds have a wide range of reduction rates, the penicillamine−cysteine bond was chosen

since it is more stable than unhindered disulfide bonds, but can be readily reduced in the cytosol over the time scale of our experiments (Figure S7).

Enabled by the hindered disulfide cleavable linker, we analyzed the stability of X-DTA_{mut} and X-DARPin protein cargo after translocation into the cell cytosol. For our analyses, **1-X** (Figure 4a) and **2-X** (Figure 4b) conjugates were synthesized containing X-DTA_{mut} and X-DARPin linked to LF_N through a hindered disulfide, respectively, where X represents ^LV, ^DV, ^LA, ^DA, ^LW, and ^DW. The protein stability of X-DTA_{mut} was analyzed using the protein synthesis inhibition assay after translocation in CHO-K1 cells for 6 h. According to the results in Figure S8, the protein synthesis inhibition of X-DTA_{mut} was stabilized through the addition of one N-terminal D-amino acid. These data were confirmed through the use of Western blot analysis. CHO-K1 cells were treated and lysed using the same conditions previously described. According to the anti-LF immunostaining in Figure 4c, there was no detectable full-length material (**1-X**) after the 6 h incubation. These results indicated sufficient cleavage of the hindered disulfide material in the reducing environment of the cytosol after translocation. The bands corresponding to cleaved LF_N further demonstrated the reduction. The presence of bands corresponding to DTA in Figure 4c corroborated the protein synthesis inhibition assay data; the cleaved X-DTA_{mut} proteins in which X represents a D-amino acid were stabilized to degradation upon cleavage from LF_N. A similar analysis was made for the X-DARPin protein cargo (**2-X**), in which we demonstrated the stabilization of biotinylated DARPin using
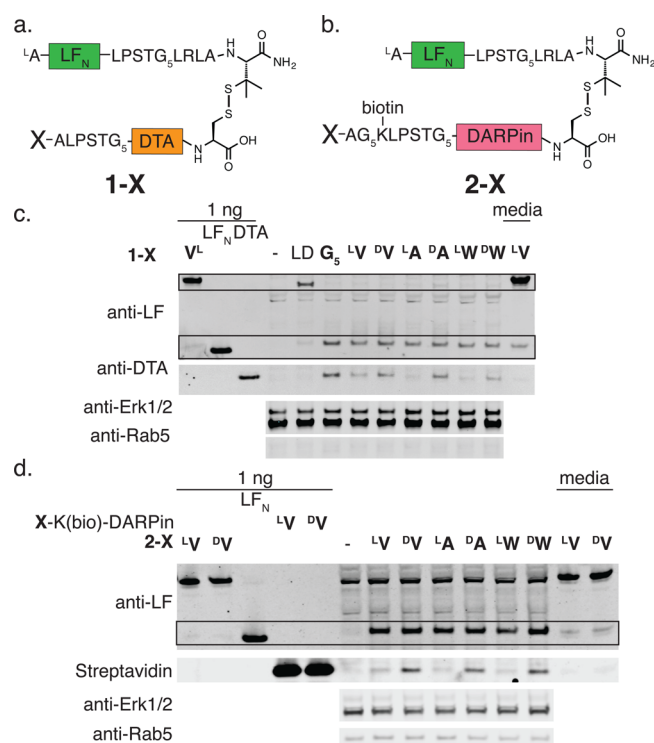
Figure 4. N-terminal D-amino acid stabilization is not limited to LF$_N$. (a) Molecular composition of X-DTA$_{mut}$ conjugated to LF$_N$ through a hindered disulfide (**1-X**), where X represents G$_5$, ^{L}V, ^{D}V, ^{L}A, ^{D}A, ^{L}W, or ^{D}W. (b) Molecular composition of X-DARPin conjugated to LF$_N$ through a hindered disulfide (**2-X**), where X represents ^{L}V, ^{D}V, ^{L}A, ^{D}A, ^{L}W, or ^{D}W. (c) CHO-K1 cells were treated with 100 nM **1-X** conjugates in the presence of 20 nM PA for 6 h, then extracted using digitonin lysis buffer, and analyzed by Western blot. The absence of full-length material suggests that each construct was appropriately reduced in the cytosol. Furthermore, LF$_N$ (^{L}A as the native N-terminus) and X-DTA$_{mut}$ bands indicated cleavage and stabilization of the X-DTA$_{mut}$ cargo with one N-terminal D-amino acid. The postincubation medium was analyzed by Western blot to indicate the stability of the hindered disulfide over the time of the experiment. (d) CHO-K1 cells were treated with 100 nM **2-X** conjugates in the presence of 20 nM PA for 6 h, then extracted using digitonin lysis buffer, and analyzed by Western blot using anti-LF and streptavidin staining. LF$_N$ and X-DARPin bands indicated cleavage and stabilization of the X-DARPin cargo with one N-terminal D-amino acid.

one N-terminal D-amino acid (Figure 4d). Both DTA$_{mut}$ and DARPin proteins were stabilized using one N-terminal D-amino acid suggesting that this phenomenon is not protein-specific.

■ DISCUSSION

We have demonstrated that one D-amino acid at the N-terminus of a protein abrogates its proteasomal degradation by the N-end rule pathway. This phenomenon was evident for LF$_N$-DTA$_{mut}$, DTA$_{mut}$, and DARPin proteins delivered into the cytosol using the PA/LF$_N$ delivery system. Our findings suggest that stabilization using D-amino acids at the N-terminus of proteins that follow the N-end rule is not protein-specific. Thus, we update the N-end rule to include D-amino acids as stabilizing residues. Since proteins with an N-terminal D-amino acid were not polyubiquitinated, our observations hold for proteins that follow the N-end rule and rely on ubiquitination for degradation. Further investigation of the promiscuity of E3 Ub ligases using noncanonical L-amino acids such as

selenocysteine or hydroxyproline would provide insight into E3 substrate specificity. We believe that the inclusion of N-terminal D-amino acids can be expanded to stabilize biologics prone to degradation via the N-end rule as well as to extend the intracellular half-lives of therapeutic proteins.

In addition to D-amino acids, we observed that ^{L}Pro effectively abrogated the degradation of LF$_N$-DTA$_{mut}$. The original N-end rule studies were performed in vitro and relied on protein fusions such as beta-galactosidase to the C-terminus of ubiquitin.[39] After translation, deubiquitinating enzymes (DUBs) cleaved the protein fusions, allowing each derivative to be analyzed. Although proline is a naturally occurring amino acid, the ubiquitin−proline bond is inefficiently cleaved by naturally occurring DUBs.[40] Using the PA/LF$_N$ delivery platform, we delivered both ^{L}P-LF$_N$-DTA and ^{D}P-LF$_N$-DTA. Our results demonstrated that both L- and D-stereoisomers had an equivalent stabilizing effect. As a result, proteins can be stabilized to the same extent as those containing N-terminal D-amino acids through the natural ^{L}Pro residue.

In the Ub/proteasome system, an E3 Ub ligase forms a complex with an E2 Ub conjugating enzyme, in order to conjugate ubiquitin onto the fated protein.[41,42] Specifically, the UBR box domain within E3 ubiquitin ligases recognizes protein substrates containing type 1 destabilizing residues, while the N-domain recognizes protein substrates containing type 2 destabilizing residues. The K_d for the interaction between an E3 Ub ligase from *Saccharomyces cerevisiae* (Ubr1) and peptide substrates containing type 1 N-terminal destabilizing residues has been determined to be ~1 μM.[2] This low affinity makes binding experiments challenging and our attempts to investigate binding unsuccessful. Nevertheless, analysis of crystal structures of the UBR box domains from the Ubr1 and Ubr2 E3 ubiquitin ligases revealed critical hydrogen bonds between the first two residues of the substrates.[43,44] We hypothesized that inverted stereochemistry at the α carbon of the N-terminal residue would interrupt the hydrogen bonding network, prevent substrate binding in the UBR box, and inhibit ubiquitination. Consistent with this hypothesis, through a pull-down assay, we demonstrated that LF$_N$-DTA$_{mut}$ constructs containing one N-terminal D-amino acid were not ubiquitinated, while the constructs containing an N-terminal L-amino acid were polyubiquitinated. These experiments provide direct evidence for the N-end rule in nature. In this study, each construct contained the same residue in the second position (i.e., ^{L}Ala). Based on analyses of the Ubr crystal structures, it is clear that the second amino acid plays a supportive role in substrate recognition. Exploration into the effect of the second position amino acid's identity (e.g., stereochemistry) is underway.

The biological properties of mixed chirality proteins have previously been unexplored due to the plasma membrane, which acts as a barrier between the extracellular and intracellular environments. The development of the PA/LF$_N$ delivery system has provided access to a new chemical space within the cytosol of cells. The PA/LF$_N$ delivery system has been used to deliver a variety of cargoes into the cytosol. Many examples of delivery incorporate the protein, peptide, or small molecule cargo on the C-terminus of LF$_N$, leaving cargo attachment to the N-terminus of LF$_N$ relatively unexplored.[22,23,25,45] Through these studies, we have found that the PA pore can tolerate short peptide modifications at the N-terminus of LF$_N$. Furthermore, we analyzed the intracellular stability of two different proteins, DTA$_{mut}$ and DARPin, by

installing a hindered disulfide cleavable linker between LF_N and the protein cargo. The hindered disulfide was shown to be readily reduced in the cell cytosol, freeing the cargo for interactions with intracellular substrates. This type of cleavable linker permits any cargo to be tethered onto LF_N for delivery followed by traceless release inside the cell. Previous explorations of translocation have involved the delivery of proteins from N- to C-termini. For the first time, by use of a cleavable linker, we demonstrated that the PA pore is capable of translocating protein cargo from C- to N-termini, providing further support to PA's payload promiscuity. Utilizing the PA/LF_N delivery system, studies are ongoing to explore the effect of mirror image amino acids on ubiquitin-independent protein degradation including unstructured or destabilizing regions of proteins.[23]

D-Amino acid incorporation into polypeptides occurs in nature, albeit infrequently when compared to L-amino acid incorporation. Select organisms including bacteria and some eukaryotes utilize racemases to convert L- to D-amino acids or nonribosomal protein synthetases to site specifically insert a D-amino acid within a growing peptide chain.[46,47] The precise roles of naturally occurring D-amino acid containing polypeptides remain an area of investigation; however, early studies have shown that polypeptides containing D-amino acids are often more active compared to their L-counterparts.[46] Furthermore, naturally occurring cyclic peptides which would also evade the N-end rule have been demonstrated to have enhanced stability in cells.[48] Perhaps nature has evolved unexpected ways to circumvent the N-end rule.

■ METHODS

Materials. Peptides were synthesized using Fmoc-protected L- and D-amino acids, N,N,N',N'-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), and 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]-pyridinium 3-oxid hexafluorophosphate (HATU) purchased from Creosalus and ChemImpex. Dimethylformamide, piperidine, diisopropylethylamine, trifluoroacetic acid, and triisopropylsilane were purchased from VWR or Sigma-Aldrich. All cloning was accomplished using the QuikChange Lightning kit (Agilent) or HiFi DNA Taq Polymerase (LifeTechnologies) and pET SUMO Champion kit (LifeTechnologies). All proteins were expressed in BL21(DE3) from LifeTechnologies. All medium for tissue culture was from LifeTechnologies, and fetal bovine serum was from Sigma-Aldrich. For Western blots, nitrocellulose membranes (GE), filters (BioRad), and PBS blocking buffer (LI-COR) were used. We used the following primary and secondary antibodies: LF (Santa Cruz), DTA (abcam), Erk1/2 (Cell Signaling), Rab5 (Cell Signaling), β-actin (Sigma-Aldrich), ubiquitin (Santa Cruz), donkey anti-goat IRdye800 (LI-COR), donkey anti-goat IRdye680 (LI-COR), goat anti-mouse IRdye800 (LI-COR), goat anti-mouse IRdye680 (LI-COR), goat anti-rabbit IRdye680, goat anti-rabbit IRdye800 (LI-COR), and streptavidin IRdye680 (LI-COR). Unless specified otherwise, all other reagents were purchased from VWR, Sigma-Aldrich, or LifeTechnologies.

Sortase A Mediated Ligation of X-LF_N-DTA_{mut} Constructs. The X-LF_N-DTA_{mut} constructs were synthesized using the one-pot SrtA-mediated ligation strategy with an evolved SrtA (SrtA*), as reported in Liao et al.[22,26] G_5-LF_N-DTA_{mut} was ligated onto each XALPSTGG peptide using the following conditions: 50 μM G_5-LF_N-DTA_{mut}, 1 mM XALPSTGG, and 5 μM SrtA* in SrtA buffer (50 mM Tris pH 7.5, 150 mM NaCl

and 10 mM $CaCl_2$). These conditions were optimized to maximize the amount of product formed with respect to G_5-LF_N-DTA_{mut}. The sortagging reactions were incubated at room temperature for 25 min, then NiNTA was added to each reaction mixture, and the mixtures were rotated for an additional 5 min to remove the SrtA* from the reaction mixtures. At the completion of the reaction, the samples were spin filtered at 4 °C and then buffer exchanged three times into 20 mM Tris pH 7.5 and 150 mM NaCl to remove the excess peptide. LC–MS was used to analyze the purity of each X-LF_N-DTA_{mut} construct.

Protein Synthesis Inhibition Assay with X-LF_N-DTA_{mut} Constructs. Chinese hamster ovary (CHO-K1) cells (ATCC) were grown in F-12K medium containing 10% (v/v) fetal bovine serum and 1× penicillin−streptomycin at 37 °C and 5% CO_2. For the protein synthesis inhibition assay, 20,000 CHO-K1 cells were plated per well in 96-well plates 16 h before the assay. Each X-LF_N-DTA_{mut} construct was diluted 10-fold and in triplicate, and then PA was added to each well for a final concentration of 20 nM. The plates were incubated for 6 h at 37 °C and 5% CO_2. After incubation, the medium was removed and the cells were washed three times with PBS. Leucine-free F-12K containing ^{3}H-leucine (1 μCi mL^{-1}, PerkinElmer) was added to each well and incubated for 1 h at 37 °C and 5% CO_2. The radioactivity was removed, and the cells were washed three times with PBS and suspended in scintillation fluid. Scintillation counting was used to measure the amount of ^{3}H-Leu present, which is indicative of DTA activity (i.e., fraction of protein synthesis). For each sample, the scintillation counts were normalized to a PA only control. The data were fitted with a sigmoidal Boltzmann fit using OriginLab software.

Translocation and Western Blot Analysis with X-LF_N-DTA_{mut} Constructs. For Western blot analysis, 200,000 CHO-K1 cells were plated per well in 12-well plates 16 h prior to treatment. Cells were treated with 100 nM X-LF_N-DTA_{mut} construct in the presence of 20 nM PA in serum-containing F-12K for 6 h at 37 °C and 5% CO_2. In select experiments, lactacystin was used to inhibit the proteasome. For this treatment, cells were preincubated with 20 μM lactacystin for 1 h at 37 °C and 5% CO_2 and then subsequently treated with the X-LF_N-DTA_{mut} constructs in the presence of PA. After translocation, the medium was removed and 0.25% trypsin−EDTA was added to each well for 5 min at 37 °C and 5% CO_2 to remove any nonspecifically bound material from the cell surface as well as lift the cells from the plate. The cells were washed twice with PBS at 500g for 2 min at room temperature. In order to obtain the cytosolic fraction, cells were lysed according to the conditions previously reported. In brief, the cells were lysed using 50 μg mL^{-1} digitonin in buffer containing 75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose, and protease inhibitor cocktail (Roche) for 10 min on ice and then spun down at 4 °C for 10 min.

The extracted lysates were analyzed by Western blot. Nitrocellulose membrane and filters were soaked in buffer containing 48 mM Tris-HCl, 39 mM glycine, 0.0375% SDS (v/v), and 20% methanol (v/v). Proteins from the gel were transferred to the membrane at 17 V for 1 h using a TE 70 semi-dry transfer unit (GE Healthcare). After transfer, the membrane was blocked for 2 h at room temperature with blocking buffer (LI-COR) and then incubated with the appropriate primary antibody (LF, Erk1/2, or Rab5) in TBST (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20 (v/v)) overnight at 4 °C. The membranes were washed three

times with TBST, then stained with a secondary antibody, and imaged using an Odyssey infrared imaging system (LI-COR). The efficiency of lysis was analyzed by anti-Erk1/2 (cytosolic protein) and anti-Rab5 (early endosome) immunostaining.

In Vitro Stability of X-LF$_N$-DTA$_{mut}$ Constructs. The in vitro stability of X-LF$_N$-DTA$_{mut}$ constructs (where X represents ^{L}V, ^{D}V, ^{L}A, ^{D}A, ^{L}W, or ^{D}W) was analyzed in rabbit reticulocyte lysate (RRL). Each X-LF$_N$-DTA$_{mut}$ construct (4 ng) was incubated in a 70% RRL solution for up to 120 min. Time points were pulled at 0, 10, 60, and 120 min. At each time point, 2 μL of each sample was added to 20 μL of 1× loading dye and flash frozen. Time points were analyzed by Western blot, which was immunostained with LF and β-actin antibodies. The bands were quantified using LI-COR Image Studio software. The rate of degradation graph was plotted according to normalized values.

Streptavidin Pulldown of Ubiquitinated Constructs. In order to analyze the ubiquitination of the biotinylated constructs, 1 μM X-K(bio)-LF$_N$-DTA$_{mut}$ was incubated in 70% RRL (20 μL total volume) at 37 °C for 10 min. The samples were then incubated with 20 μL of Dynabeads MyOne Streptavidin C1 beads (LifeTechnologies; washed twice with 200 μL of 50 mM HEPES pH 7.1, 200 mM KCl, 10% glycerol, 0.02% NP-40) for 1 h at room temperature. After incubation, the beads were washed twice with the same HEPES buffer and then eluted in 20 μL of 2× loading dye for 10 min at 95 °C. Samples were analyzed by Western blot, which was stained with streptavidin and ubiquitin antibody.

Stabilization of X-DTA$_{mut}$ or X-DARPin after Translocation. The hindered disulfide conjugates (**1-X** and **2-X**) were synthesized through C-terminal penicillamine (C*) on LF$_N$ (LF$_N$-C*) and a C-terminal cysteine (C) on X-DTA$_{mut}$ or X-DARPin. A three-step ligation strategy was optimized for the synthesis of the hindered disulfide conjugates: 1, sortagging to form LF$_N$-C*; 2, sortagging to form X-DTA$_{mut}$-C-Ellman's X-DARPin-C-Ellman's; and 3, oxidation to form **1-X** or **2-X** conjugates, where X is any amino acid on the N-terminus of DTA$_{mut}$ or DARPin. Complete ligation details can be found in the Supporting Information.

As a first measure of X-DTA$_{mut}$'s protein stability, we used the protein synthesis inhibition assay with **1-X** conjugates. CHO-K1 cells were treated with 10-fold dilutions of **1-X** conjugates for 6 h in the presence of 20 nM PA for 6 h at 37 °C and 5% CO$_2$. After 6 h, the cells were washed three times with PBS and then treated with leucine-free F-12K medium containing ^{3}H-leucine (1 μCi mL^{-1}, PerkinElmer) for 1 h at 37 °C and 5% CO$_2$. The cells were washed three times and then resuspended in scintillation fluid, and ^{3}H radioactivity was counted. For each sample, the scintillation counts were normalized to a PA only control.

The protein stability of both X-DTA$_{mut}$ and X-DARPin was determined using Western blot analysis. CHO-K1 cells were treated with 100 nM **1-X** or **2-X** (where X represents ^{L}V, ^{D}V, ^{L}A, ^{D}A, ^{L}W, or ^{D}W) in the presence of 20 nM PA for 6 h at 37 °C and 5% CO$_2$. After 6 h, the medium was removed and 0.25% trypsin–EDTA was added to each well for 5 min at 37 °C and 5% CO$_2$. The cells were washed twice with PBS at 500g for 2 min at room temperature. The cytosolic fraction was extracted using the digitonin lysis conditions and analyzed by Western blot as previously described. The membrane was stained with LF, DTA or streptavidin, Erk1/2, and Rab5 antibodies and then stained with the appropriate secondary antibodies prior to imaging.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.5b00308.

Western blot analyses, rate of reduction of **1-X**, and LC–MS characterization (PDF)

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Notes
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