N-terminal methionine excision of proteins creates tertiary destabilizing N-degrons of the Arg/N-end rule pathway

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All organisms begin protein synthesis with methionine (Met). The resulting initiator Met of nascent proteins is irreversibly processed by Met aminopeptidases (MetAPs). N-terminal (Nt) Met excision (NME) is an evolutionarily conserved and essential process operating on up to two-thirds of proteins. However, the universal function of NME remains largely unknown. MetAPs have a well-known processing preference for Nt-Met with Ala, Ser, Gly, Thr, Cys, Pro, or Val at position 2, but using CHX-chase assays to assess protein degradation in yeast cells, as well as protein-binding and RT-qPCR assays, we demonstrate here that NME also occurs on nascent proteins bearing Met–Asn or Met–Gln at their N termini. We found that the NME at these termini exposes the tertiary destabilizing Nt residues (Asn or Gln) of the Arg/N-end rule pathway, which degrades proteins according to the composition of their Nt residues. We also identified a yeast DNA repair protein, MQ-Rad16, bearing a Met–Gln N terminus, as well as a human tropomyosin–receptor kinase–fused gene (TFG) protein, MN-TFG, bearing a Met–Asn N terminus as physiological, MetAP-processed Arg/N-end rule substrates. Furthermore, we show that the loss of the components of the Arg/N-end rule pathway substantially suppresses the growth defects of nua20Δ yeast cells lacking the catalytic subunit of NatB Nt acetylase at 37 °C. Collectively, the results of our study reveal that NME is a key upstream step for the creation of the Arg/N-end rule substrates bearing tertiary destabilizing residues in vivo.

Virtually all polypeptides emerging from ribosomes begin with methionine (Met), as this is the residue dictated by the translation initiation codons. The initiator N-terminal (Nt)-Met of nascent polypeptides is co-translationally and irreversibly excised by ribosome-bound Met-aminopeptidases (MetAPs) if it includes a penultimate residue with a small and uncharged side chain (Ala, Gly, Ser, Cys, Thr, Pro, or Val) (1). Evolutionarily conserved Nt-Met (Nt-M) excision (NME) is applied to more than 50% of all nascent proteins, thus profoundly contributing to Nt-proteome diversity, enzyme activity, cell growth and viability, free Met or GSH homeostasis, etc. Nonetheless, the universal role of this massive NME process remains largely elusive (1–3).

In ~60% of nascent yeast proteins and 80% of nascent human proteins, the retained Nt-M (Nt-M) and neo-Nt residues after NME very frequently undergo N-terminal acetylation (Nt-acetylation) (4). The Nt-M acetylation state of nascent proteins can be a primary decision-making step for the retention or removal of Nt-M, as it dynamically competes with NME on ribosomes by blocking MetAPs via steric hindrance (1, 4, 5). Moreover, numerous lines of evidence have uncovered that Nt-acetylation substantially contributes to protein stability, activity, folding, localization, and interaction, thus affecting cell proliferation, apoptosis, development, etc. (4, 6). Furthermore, Nt-acetylation elicits specific protein degradation signals (degrons), which are recognized directly by an Nt-acetylation–targeting proteolytic system, termed the Ac/N-end rule pathway (Fig. S1B) (6–8).

The common characteristic of the N-end rule pathways relies on the direct detection of protein Nt-degrons (N-degrons) by specific recognition components (N-recognins) for proteolysis (9). A critical and indispensable determinant of N-degrons is destabilizing residues, which are yielded either through their steric unmasking (via defects in the protein’s folding, interaction, or localization, etc.) or subsequent Nt modifications (via acetylation, formylation, deamidation, oxidation, arginylation, endoproteolytic cleavage, etc.) (6, 9). In eukaryotes, specific ubiquitin (Ub) ligases or the autophagy adaptor SQSTM1 (p62) is a dedicated N-recognin of the N-end rule pathways and directly recognizes protein N-degrons, thereby mediating their degradation by 26S proteasomes or lysosomes (9, 10). The previously established N-end rule pathways in Saccharomyces cerevisiae are the Arg/N-end rule, Ac/N-end rule, Pro/N-end rule, and fMet/N-end rule pathways (see below) (Fig. S1, A–D) (6, 9, 11, 12).

The canonical N-end rule pathway is the Arg/N-end rule pathway, in which Nt-unacetylated destabilizing residues are endoplasmic reticulum; ERAD, ER-associated protein degradation; Y2H, yeast two-hybrid.
targeted through a highly organized hierarchy (9). In *S. cerevisiae*, the primary destabilizing residues of the Arg/N-end rule pathway are type-1 basic (Arg, Lys, and His), type-2 bulky hydrophobic (Leu, Trp, Phe, Ile, and Tyr), or Met-Φ (a hydrophobic amino acid) Nt-residues (Fig. S1A and C) (9, 13). The tertiary destabilizing residues Nt-Asn (Nt-N) and Nt-Gln (Nt-Q) are converted to the secondary destabilizing residues Asp and Glu, respectively, by Nta1 amidase (Fig. S1A). The resulting Nt-Asp (Nt-D) and Nt-Glu (Nt-E) are argylated by Ate1 arginyltransferase. The sole N-recognin Ubr1 E3 Ub ligase (in concert with Ufd4 E3 Ub ligase) directly detects Nt-unacyetylated type-1 basic and type-2 bulky hydrophobic residues for Ub-dependent and proteasome-mediated destruction in *S. cerevisiae* (9, 14). The Arg/N-end rule pathway performs a broad range of functions in protein quality control, small peptide sensing, chromosome segregation, DNA repair, stress responses, etc. (9, 15–21).

The Ac/N-end rule pathway destroys Nt-acetylated proteins via direct contact of their Nt-acetyl tag with the endoplasmic reticulum (ER)/nuclear transmembrane Doa10 (TEB4, also known as MARCH6, in mammals) or the cytosolic and nuclear Ndt4 E3 Ub ligases (Fig. S1B) (8, 22, 23). The Ac/N-end rule pathway has been implicated in the regulation of protein subunit stoichiometry, G-protein signaling–mediated blood pressure, circadian rhythms, plant stress responses, lipid droplet maintenance, etc. (13, 22–26).

Another branch of the N-end rule pathways is the Pro/N-end rule pathway, which directly targets Nt-Pro (Nt-P) or Pro at position 2 of a given protein, along with the adjacent sequences. The Pro/N-end rule pathway exploits Gid4 as a Pro/N-recognin, thereby degrading several gluconeogenic enzymes in *S. cerevisiae* (Fig. S1D) (11). The molecular structure and substrate specificity of human GID4 as the Pro/N-recognin have been recently determined (27).

The fMet/N-end rule pathway targets proteins carrying N-terminal methionine (fMet) for degradation. We have recently found that fMet can be used to initiate protein synthesis in the cytosol of *S. cerevisiae* (12). The resulting fMet can also act as fMet/N-degron, in agreement with our previous prediction (8) and actual demonstration of its existence in bacteria (28). Note, the eukaryotic fMet/N-end rule pathway recruits the Psh1 E3 Ub ligase as an fMet/N-recognin, thereby degrading cytosolic fMet-containing proteins via the Ub/proteasome system (Fig. S1E) (12).

Given their strong hydrolytic preference for Nt-Met-Gly (MG), Met-Ala (MA), Met-Ser (MS), Met-Cys (MC), Met-Thr (MT), Met-Val (MV), and Met-Pro (MP), MetAPs are generally postulated not to excise unmodified Nt-M bearing the tertiary-destabilizing residue Asn or Gln at position 2 (1). MetAP-generated neo-Nt residues are therefore widely assumed to be stabilizing in the context of the Arg/N-end rule pathway, with the exception of the Nt-C residue that becomes destabilizing through oxidation under certain circumstances (29–32). Interestingly, it has also been suggested that the prevention of NME may increase the protein life span because some plastid-encoded proteins become shorter-lived under conditions of reduced deformylation (33). On the contrary, this study reveals that NME substantially and discernibly occurs on proteins with tertiary-destabilizing residues, thus causing their degradation via the Arg/N-end rule pathway in vivo.

**Results**

**Ubr1 mediates degradation of Nt-unacytlated MN-α2 fusions**

The previously dissected reporter MN-α2^{3–67}−ε^K−ha-Ura3 (denoted as MN-α2-ε^K−ha-Ura3) (8), generated by co-translational deubiquitylation of UbR^{48}, MN-α2-ε^K−ha-Ura3, contained Ub with a Lys-48 to Arg mutation (UbR^{48}), Nt-MN, α2 (N-terminal 3–67 residues of a transcription repressor Mata2), a 44-residue ε^K fragment (stemming from *Escherichia coli* LacI protein), the ha (YPYDVPDYA) tag, and the Ura3 (orotidine 5′-phosphate decarboxylase) reporter (Fig. 1A). The MN-α2-ε^K−ha-Ura3 is Nt-acetylated in *vivo* by the NatB Nt-acetylase complex, which consists of a catalytic subunit Naa20 as well as an ancillary subunit Naa25, and is subsequently targeted for degradation by Doa10, an Ac/N-recognin of the Ac/N-end rule pathway (Fig. 1A) (8). Thus, *ura3Δ* (uracil auxotrophic) *S. cerevisiae* cells that expressed MN-α2-ε^K−ha-Ura3 from the *CUP1* promoter on a low-copy-number plasmid with a TRP1 auxotrophic marker grew very poorly on the synthetic complete SC-Trp-Ura (both tryptophan and uracil-lacking) medium because of the rapid degradation of the MN-α2-ε^K−ha-Ura3 reporter (Fig. 1B). In agreement with our previous finding that Doa10 mediates degradation of MN-α2-ε^K−ha-Ura3 by targeting its Nt-acetyl group (8), the loss of Doa10 notably rescued the growth of *ura3Δ* *S. cerevisiae* cells with MN-α2-ε^K−ha-Ura3 on the same selective medium (Fig. 1B). Unexpectedly, the growth defect of the *ura3Δ* yeast cells expressing MN-α2-ε^K−ha-Ura3 was also markedly abolished by the absence of Ubr1, an N-recognin of the *S. cerevisiae* Arg/N-end rule pathway (Fig. 1B). Moreover, CHX-chase assays of protein degradation showed that MN-α2-ε^K−ha-Ura3 was greatly stabilized in both *doa10Δ* cells (half-life (t_{1/2}) ≈74 min) and *ubr1Δ* cells (t_{1/2} ≈81 min) compared with WT cells (t_{1/2} ≈28 min) (Fig. 1, C and D).

The observed Ubr1- or Doa10 (via Nt-acetylation)-mediated degradation of MN-α2-ε^K−ha-Ura3 was also retained in the otherwise identical reporter MN-α2^{3–67}−GST (denoted as MN-α2-GST), containing GSH-$S$-transferase (GST) instead of the ε^K−ha-Ura3 C-terminal moiety (Fig. 2A).

To elucidate the involvement of Ubr1 in the degradation of the reporter protein in more detail, we performed CHX-chase assays of MN-α2-GST in WT, *ubr1Δ*, *doa10Δ*, *ubr1Δ* *doa10Δ*, *naa20Δ*, and *naa20Δ* *ubr1Δ* cells via immunoblotting using anti-GST antibodies (which detect all species of MN-α2-GST variants). Indeed, analogous to our previous findings (8) and the above results with MN-α2-ε^K−ha-Ura3 (Fig. 1), the MN-α2-GST was rapidly degraded in WT cells (t_{1/2} ≈10 min), but partially stabilized in *ubr1Δ* (t_{1/2} ≈30 min), *doa10Δ* (t_{1/2} ≈21 min), or *naa20Δ* cells (t_{1/2} ≈22 min) (Fig. 2, B–F). Strikingly, the short-lived MN-α2-GST was almost completely stabilized in either double-mutant *ubr1Δ* *doa10Δ* cells or *naa20Δ* *ubr1Δ* cells, demonstrating that the Arg/N-end rule and Ac/CN-end rule pathways co-mediated the degradation of MN-α2-GST redundantly (Fig. 2, B–F).

To understand the impact of Nt-acetylation on the Ubr1-mediated degradation of MN-α2-GST more clearly, N-termi-
Figure 1. Ubr1 mediates degradation of MN-α2-e-K-ha-Ura3. A, scheme of the degradation of MN-α2-e-K-ha-Ura3 by the Ac/N-end rule pathway. Co-translational deubiquitylation of Ub-36-MN-α2-e-K-ha-Ura3 yields MN-α2-e-K-ha-Ura3 in vivo. Degradation of the resulting MN-α2-e-K-ha-Ura3 involves NatB Nt-acetylase and Doa10 E3 Ub ligase. B, growth assays of WT, doa10Δ, or ubr1Δ cells expressing MN-α2-e-K-ha-Ura3. The indicated cells were cultured to A600 ~1, serially diluted 5-fold, and then spotted on uracil-containing (SC–Trp) or uracil-lacking (SC–Trp–Ura) plates. The plates were incubated at 30 °C for 3 days. C, CHX chases of MN-α2-e-K-ha-Ura3 in WT, doa10Δ, or ubr1Δ S. cerevisiae for 0, 30, and 60 min. Cell extracts were separated by SDS/10% Tris-glycine PAGE, followed by immunoblotting with anti-ha and anti-tubulin antibodies. D, quantitation of data in C. Data show mean ± S.D. of three independent experiments.

N-terminal Met excision for the Arg/N-end rule pathway

followed by immunoblotting with anti-ha and anti-tubulin antibodies. MIN- inactive fUb1C1220S (Fig. 2) acted with fUb1 and more strongly interacted with catalytically inactive fUb1C1220S, but not in those of Nt-acetylated variants, but not in those of Nt-acetylated fUb1C1220S (Fig. 2). Indeed, CHX-chase assays showed that the induction of 4UBr1 in the galactose-containing culture led to a significant decrease in the levels of total MN-α2-GST derivatives (Nt-acetylated, Nt-unacylated, and other modified glutamate) variants, but not in those of Nt-acetylated AcMN-α2-GST that was detected via immunoblotting with anti-α2AcM antibodies (Fig. 2F) (8). Additionally, Nt-unacylated MN-α2-GST (which, in fact, bore Nt-unacylated or potentially processed MN-α2-GST derivatives. See below for details.) in nnaa20Δ ubr1Δ doa10Δ cells (lacking NatB Nt-acetylase) became markedly destabilized by the expression of 4UBr1, but not by that of the catalytically inactive mutant 4UBr1C1220S (Fig. 2G). Furthermore, co-immunoprecipitation assays demonstrated that MN-α2-GST marginally, but detectably, interacted with 4UBr1 and more strongly interacted with catalytically inactive 4UBr1C1220S (Fig. 2H). In actuality, the physical binding of 4UBr1C1220S to MN-α2-GST was more profound in nnaa20Δ ubr1Δ doa10Δ cells (bearing Nt-acylated MN-α2-GST because of their lack of NatB Nt-acetylase) than ubr1Δ doa10Δ cells (bearing mostly Nt-acylated MN-α2-GST but little, if any, Nt-unacylated MN-α2-GST) (Fig. 2H). These results indicate that Ubr1 mediates degradation of Nt-acylated MN-α2-GST, whereas Doa10 targets Nt-acylated AcMN-α2-GST (Fig. 2J) in agreement with our previous identification of Doa10 as an Ac/N-recognition (8).

Ubr1-dependent degradation of MN-α2-GST or MQ-α2-GST requires Ate1 and Nta1

Nt-sequence analysis using Edman degradation revealed that MN-α2-GST purified from nnaa20Δ ubr1Δ doa10Δ cells contained mixtures of ~64% Arg–Asp–Lys–Ile–Pro (RDKIP) and ~36% DNA-encoded Met–Asn–Lys–Ile–Pro (MNKIP) (Fig. 3A). Thus, we presumed that the detected RD-α2-GST in nnaa20Δ ubr1Δ doa10Δ cells arose from NME, followed by Nt-deamidation and subsequent Nt-arginylation in compliance with the previously well-established hierarchy of the Arg/N-end rule pathway (Fig. S1A). Indeed, CHX-chases showed that Nt-unacylated MN-α2-GST in nnaa20Δ cells was virtually completely stabilized by the absence of either Ate1 or Nta1 (Fig. 3, D–G).

We next examined, in nnaa20Δ cells, the degradation of MZ-α2-GSTs that were otherwise identical but had different residues at position 2 (Z = Asn, Gln, Asp, Glu, Lys). Among these, MN-α2-GST (t1/2 ~23 min) and MQ-α2-GST (t1/2 ~20 min) were markedly short-lived in contrast to the relatively long-lived MD-α2-GST (t1/2 ~35 min), ME-α2-GST (t1/2 ~50 min), or MK-α2-GST (t1/2 ~53 min) (Fig. 3, B and C). Moreover, the short-lived MQ-α2-GST of nnaa20Δ cells was also greatly stabilized by the absence of either Nta1 or Ate1 (Fig. 3, F and G), as was MN-α2-GST (Fig. 3, D and E), indicating that the MQ-starting reporters also necessitate Nt-deamidation and subsequent Nt-arginylation prior to their Ubr1-mediated degradation.
MetAPs cleave the Nt-M of MN-α2-GST and MQ-α2-GST for degradation

*S. cerevisiae* contains two MetAPs (Map1 and Map2), which redundantly cut off the Nt-M of nascent proteins, but only if the residue at position 2 is no larger than Val (34). Nonetheless, given the evident involvement of Ubr1, Ate1, and Nta1 in the degradation of MN-α2-GST or MQ-α2-GST (Figs. 2 and 3), we presumed that MetAPs might process the Nt-M of the reporters before they are targeted by the Arg/N-end rule pathway (Fig. 4A). To test this possibility, we expressed MN-α2-GST or MQ-α2-GST from the P_{CUP1} promoter on a low-copy-number CEN plasmid in *map1Δ* naa20Δ cells in either the presence or absence of the specific Map2 inhibitor, fumagillin. Tellingly, short-lived MN-α2-GST and MQ-α2-GST were strongly stabilized in *map1Δ naa20Δ* cells by fumagillin-mediated Map2 inactivation (Fig. 4, B and C). Altogether, we conclude that MetAPs can noticeably cleave the Nt-M of proteins, even with tertiary destabilizing Asn or Gln present at position 2, thus triggering their degradation by the Arg/N-end rule pathway in vivo (Fig. 4A).

Arg/N-end rule pathway mediates degradation of human MN-TFG

The observation of the Arg/N-end rule-dependent degradation of MN-α2-GST and MQ-α2-GST (Fig. 4) post-NME prompted us to search for native MQ/MN-starting proteins that are targeted for degradation by the Arg/N-end rule pathway. Surprisingly (and unexpectedly), our independent yeast two-hybrid (Y2H) screen using a C-terminally truncated human UBR11–1031 fragment with the previously defined substrate-binding sites (35) repeatedly isolated a human tropomyosin–receptorkinase–fused gene (TFG) as its binding partner (Fig. 5, A and C). Interestingly, the screened TFG-harboring prey vectors retained a stop codon just upstream of TFG ORF (ORF) following the GAD-coding sequence (Fig. 5A); GAD encodes the Gal4 transcription–activation domain, and native
TFG ORF comprises an MN N terminus and an intrinsic transcription-activation domain (36). Hence, we postulated that the resulting MN-TFG would activate the expression of the Y2H reporters through a direct interaction with the Gal4-binding domain–UBR11–1031 fusion, even in situations lacking N-terminally positioned GAD (Fig. 5, A and C). To verify this possibility, we further mapped the TFG-binding site of UBR1 by repeating the Y2H assays using the truncated UBR1 derivatives as baits and found that TFG bound to the UBR box (type 1 site)–containing UBR11–1031, UBR11–632, UBR11–191, UBR193–221, or UBR193–191, but not to the UBR box lacking UBR193–157 and UBR1167–1031 (Fig. 5, B and C). The identical MN-starting sequences of MN-TFG and MN-H9251 suggested that MN-TFG would be subject to NME, followed by Nt-deamidation and subsequent Nt-arginylation before its targeting by UBR1. Indeed, loss of either Nta1 or Ate1 almost completely abrogated the binding of UBR1 to MN-TFG in Y2H assays (Fig. 5 D).

Given these results, we sought to examine further whether the Arg/N-end rule pathway mediated the degradation of MN-TFG in mammalian cells by expressing the C-terminal triply FLAG-tagged human MN-TFG (MN-TFGf3) in Ate1/H11001/WT mouse fibroblast (MEF) cells and Ate1/H11002/KO MEF cells. Upon CHX chases, MN-TFGf3 became short-lived in WT Ate1/H11001/WT MEF cells, but greatly stabilized in Ate1/H11002/KO MEF cells (Fig. 5, E and F), despite almost no significant changes in the levels of TFGf3 mRNA between Ate1/H11002/KO MEF cells and Ate1/H11002/KO MEF cells (Fig. 5G). Therefore, the observed strong augmentation of the MN-TFGf3 level in Ate1/H11002/KO MEF cells suggested the Arg/N-end rule pathway.
MEF cells most likely arise from alterations in the rate of its initial proteolytic decay (especially in accordance with many previous supporting studies) for the substantial co-translational degradation of nascent proteins (37–39). Of note, the degradation of MN-TFG also requires the 26S proteasome because the steady-state level of MN-TFG is greatly up-regulated by the presence of the proteasome inhibitor MG-132 (Fig. 5H). Overall, these results suggest that MN-TFG would be degraded by the Arg/N-end rule pathway involving consecutive reactions of NME, Nt-deamidation, as well as subsequent Nt-arginylation in mammalian cells.

**Figure 5. Human MN-TFG is degraded by the Arg/N-end rule pathway.** A, schematic representation of human UBR11–1031 in Y2H bait vector and human TFG in Y2H prey vectors to be screened. B, UBR box, the N-domain and the RING domain of human UBR1. Fragments of UBR1 used to map its TFG-binding region are depicted below the diagram. C, interaction of TFG with UBR1 fragments upon Y2H assays. D, in vivo detection of UBR11–1031–TFG interactions in ATE1 NT4, ate1Δ, and nta1Δ S. cerevisiae using Y2H assay. S. cerevisiae cells co-expressing bait and prey plasmids were serially diluted 5-fold and spotted on SC (−Leu/Trp) or SC (−Leu/Trp/His/Ade) plates (see “Experimental procedures” for details). E, CHX chases of C-terminal triply FLAG-tagged human TFG in ATE1 NTA1, nta1Δ KO MEF cells for 0, 4, 8, and 12 h. F, graph represents quantitation of data in E with mean ± S.D. of three independent experiments. G, relative levels of human TFG mRNAs in WT and Ate1Δ/Δ KO MEF cells using RT-qPCR. The data presented are mean ± S.D. in triplicate for each sample. H, steady-state levels of TFG in HeLa cells with or without the proteasome inhibitor MG132.

**Arg/N-end rule pathway mediates degradation of S. cerevisiae MQ-Rad16**

The positive Y2H interaction of the human UBR box with Nt-arginylated TFG bearing an intrinsic transcription activation domain (without GAD) (Fig. 5, B and C) prompted us to search for Nt-arginylated proteins in S. cerevisiae, in which ~9% of DNA-encoded proteins contain an MQ/MN-starting N terminus according to the Saccharomyces Genome Database (https://www.yeastgenome.org/). To this end, we repeated the Y2H screen using a human UBR11–1031 containing only a UBR box (type-1 binding) site as a bait and a yeast genomic DNA library (as preys) in the ubr1Δ/Δ S. cerevisiae cells co-expressing bait and prey plasmids for 0, 4, 8, and 12 h. F, graph represents quantitation of data in E with mean ± S.D. of three independent experiments. G, relative levels of human TFG mRNAs in WT and Ate1Δ/Δ KO MEF cells using RT-qPCR. The data presented are mean ± S.D. in triplicate for each sample. H, steady-state levels of TFG in HeLa cells with or without the proteasome inhibitor MG132.
MQ-Rad16 was abolished by the loss of either Ate1 or Nta1 upon Y2H assays (Fig. 6A).

Consistent with the results observed for MN-α2-GST and MQ-α2-GST (see Fig. 4 for details), fumagillin-mediated inactivation of Map2 also caused a discernible stabilization of C-terminally ha-tagged MQ-Rad16 (MQ-Rad16<sub>ha</sub>) in map1Δ naa20Δ cells (Fig. 6B), indicating the involvement of NME in the degradation of MQ-Rad16<sub>ha</sub> in <i>S. cerevisiae</i>. Moreover, CHX chases showed that MQ-Rad16<sub>ha</sub> was short-lived in WT cells, but partially stabilized in both single-mutant naa20Δ cells or <i>nta1Δ</i> cells (Fig. 6C). MQ-Rad16<sub>ha</sub> was more stabilized in double-mutant <i>naa20Δ ubr1Δ</i> cells (Fig. 6C). Furthermore, MQ-Rad16<sub>ha</sub> was also long-lived in <i>ate1Δ nta20Δ</i> cells or <i>nta1Δ nta20Δ</i> cells (Fig. 6, D and E), indicating that MQ-Rad16<sub>ha</sub> was preliminarily processed via NME, Nt-deamidation, and subsequent Nt-arginylation before the targeting of the protein by Ubr1 (Fig. 6).

**Discussion**

Because of the strong processing activity of MetAPs toward Nt-M bearing small residues at position 2, NME is assumed to yield only stabilizing Nt-residues (Nt-G, -A, -S, -C, -T, -V, or -P) of the Arg/N-end rule pathway in <i>S. cerevisiae</i> (1, 9). As opposed to the typical substrate specificities of MetAPs, this study shows that MetAPs can produce the tertiary destabilizing MN/MQ-starting proteins because of their vulnerable degradation at higher temperatures via the Arg/N-end rule pathway (Fig. 7B).

In line with our present findings, global proteomic analyses bear out that the Asn or Gln at the 2nd position in some native proteins is exposed to the N terminus without the retention of the initiator Met (42). Additionally, <i>S. cerevisiae</i> Map1 processes, to an inefficient but significant extent, the Nt-M of a synthetic peptide with a MN N terminus in vivo (43, 44). Knop and co-workers (45) also reported, using multiplexed protein stability profiling for the quantitative and systematic mapping of degrons in the yeast Nt-proteome, that 10 MN-starting reporters and an Nt-truncated isoform of the mannosyltransferase MN-Ktr2 (with Met–Asn N terminus) may involve Map1-mediated NME for the Arg/N-end rule pathway.

Strikingly, Varshavsky and co-workers (46) first identified the engineered reporter Met–Gln–Leu–Ser–Ile–Ile–Asp–
Pro–Asp–Gly–Thr (MQLSIIIDPDGT)-eK-ha-Ura3 (23Q-Ura3) with Gln at position 2, whose degradation requires Nta1, Ate1, and Ubr1. However, the absence of either Map1 or Map2 does not significantly affect the degradation of 23Q-Ura3, suggesting the overlapping action of MetAPs or unknown dedicated aminopeptidases (46). Furthermore, Finley and co-workers (47) isolated an MH-starting PB12 reporter that undergoes NME, thereby exposing the penultimate His (a primary destabilizing residue of the Arg/N-end rule pathway) at its N terminus. Of note, the Ubr1-mediated degradation of the PB12 reporter is also unaffected in *map1*/*H9004* cells. Therefore, the possibility cannot be excluded that not only MetAPs but also as yet unknown aminopeptidases can participate in the NME of MN/MQ-starting proteins according to their Nt-sequence context or structural conformation.

The present study overtly reveals NME as another critical process of the Arg/N-end rule pathway in the creation of tertiary destabilizing residues by demonstrating that Ubr1-mediated recognition of MN/MQ-starting proteins necessitates consecutive reactions of NME, Nt-deamidation, and subsequent Nt-arginylation (Figs. 2–6 and 7B). Accordingly, the resulting NME enormously increases the number of Arg/N-end rule substrates, considering that MN/MQ-starting proteins encompass about 9% of all nuclear DNA-encoded proteins. Nonetheless, it should be noted that the previously overlooked or underestimated requirement of NME in the degradation of MN/MQ-starting substrates by the Arg/N-end rule pathway most likely stems from two redundant (overlapping) MetAPs (Map1 and Map2) and the alternative proteolytic route by the Ac/N-end rule pathway, because the ablation effects of either single MetAP or the Arg/N-end rule pathway can be efficiently suppressed by the other MetAP or the Ac/N-end rule pathway, respectively.

Notably, Nt-acetylation confers the opposite impacts on protein stability, particularly because it not only creates Ac/N-degrons of the Ac/N-end rule pathway, but also concomitantly precludes the destruction of proteins by the Arg/N-end rule pathway (6, 7, 9). Hence, many MN/MQ-starting proteins are susceptible to the NME-mediated Arg/N-end rule pathway, and also, alternatively, to the Ac/N-end rule pathway through their Nt-acetylated Met, as demonstrated in the degradation patterns of MN-α2-eK-ha-Ura3, MN-α2-GST, MQ-α2-GST, or MQ-Rad16ha (Figs. 1–3 and 6).

In addition and analogous to our previous finding that Nt-acetylation transforms an MΦ (a hydrophobic residue)/N-degron into an AcMΦ/N-degron, thus switching the targeting route of an MΦ-starting protein to the Ac/N-end rule pathway (Fig. S1C) (6, 13, 48), the NatB-mediated Nt-acetylation of MN/MQ-starting proteins would not only preclude their NME-mediated degradation by the Arg/N-end rule pathway, but also provoke their alternative proteolytic route via the Ac/N-end rule pathway. Consequently, dual or alternative targeting of MN/MQ-starting proteins by both the Arg/N-end rule pathway and the NatB-mediated Ac/N-end rule pathway would degrade these proteins cooperatively, irrespective of their Nt-acetylation states (Fig. 7B). Indeed and in a similar outcome to that of MΦ/N-degron–containing proteins (13), MN/MQ-starting proteins, such as MN-α2-GST, MQ-α2-GST, and MQ-Rad16na, were more strongly stabilized in the double mutant *ubr1Δ nqa20Δ* cells than in the single mutant *ubr1Δ* or *nqa20Δ* cells (Figs. 2, 3, and 6), indicating the co-targeting of MN/MQ-starting substrates by the Arg/N-end rule...
and the NatB-mediated Ac/N-end rule pathways. However, the actual targeting mechanism of MN/MQ-starting proteins by Ubr1 is substantially different from that of MΦ-starting proteins. In particular, Ubr1 recognizes MN/MQ-starting proteins after their preliminary modifications (consisting of NME, N-terminal deamidation, and subsequent Nt-arginylation) in contrast to its direct binding to the nonacetylated Nt-M of MΦ-starting proteins (Fig. 7B) (6, 13, 48).

It is also noteworthy that Nt-acetylation also modulates the activities of E3 Ub ligases, 26S proteasomes, and molecular chaperones (6). Furthermore, the antagonistic (triggering and restraining) impacts of Nt-acetylation on the Arg/N-end rule and the Ac/N-end rule pathways, respectively, increase the complexities of intracellular protein degradation more profoundly than those previously assumed, thereby making it particularly difficult to identify or predict the specific proteolytic pathway of a given protein using specific genetic studies harnessing a single gene deletion or knockdown, etc. (6, 7, 48).

Deg1 represents the first 67 residues of Mato2 (49). In this study, we also demonstrated here that MN-α2-GST (Deg1-GST) was significantly and substantially stabilized in nna20Δ cells that lacked a catalytic subunit of NatB Nt-acetylase (Fig. 2B) in agreement with previous observations of MN-α2-eK-ha-Ura3 (Deg1-eK-ha-Ura3) and MN-α2-Leu2M1Δ (another Deg1 fusion protein) (8, 50). Conversely, it is also reported that the loss of Naa20 very weakly affects the degradation of endogenous Mato2, as well as other Deg1 fusions such as MN-α2-FLAG-Ura3 (Deg1-FLAG-Ura3) (49). The cited study interpreted these results as an indication that Nt-acetylation has little effect on the recognition of Doa10 for its substrates (49), in contrast to our previous finding that Doa10 works as an Ac/N-recognin (8). Although these discrepancies in the Nt-acetylation–dependent degradation of Deg1 fusions remain to be further examined, we infer that the shifting rate or extent of the NatB-mediated Ac/N-end rule pathway to the NME-mediated Arg/N-end rule pathway may cause distinct proteolytic outcomes, particularly according to the sequence context of Deg1 substrates or nna20Δ strains that are used in our studies and those of others (8, 49, 50).

Strikingly, and with conceptual similarity to the results with nna20Δ cells, dfm1Δ cells (lacking the rhomboid derlin Dfm1) are reported to have very dissimilar proteolytic patterns (51). For instance, some studies do not detect any defects in the degradation of ER-associated protein degradation (ERAD) substrates in dfm1Δ cells (52, 53), whereas others reveal the significant stabilization of a subset of ERAD substrates in the absence of Dfm1 (54, 55). More recently, Hampton and co-workers (51) resolved the controversial role of Dfm1 in ERAD by demonstrating that dfm1Δ cells rapidly suppress and thereby compromise ERAD by up-regulating the alternative proteolytic pathway.

Likewise, in nna20Δ cells, the ablation of the NatB-mediated Ac/N-end rule pathway would rapidly trigger the other NME/NatB-mediated Arg/N-end rule pathway, thus exhibiting the observed comparable degradation of Deg1 fusions in both WT cells and nna20Δ cells (49), which remains to be tested. Furthermore, our most recent genetic and biochemical experiments reveal that TEB4 (a mammalian homolog of Doa10) (23) more preferentially binds to Nt-acetylatable native proteins, such as RGS2 and PLIN2, than to their nonacetylatable counterparts, which is in agreement with our previous identification of TEB4 (Doa10) as an Ac/N-recognin (26).

This study also demonstrates that human TFG is a substrate of the NME-mediated Arg/N-end rule pathway. Endogenous TFG is involved in the spatial coordination of the early secretory event from endoplasmic reticulum to Golgi by binding to the coat protein complex II (COPII) (56).

Remarkably, the TFG gene is frequently identified as a chromosomally translocated chimeric gene with many oncogenes, such as NTRK1 (a neurotrophic receptor tyrosine kinase) in thyroid papillary carcinomas, ALK (anaplastic lymphoma kinase), NORT (a nuclear orphan receptor), NEMO (NF-κB essential modulator), TANK (TRAF-associated NF-κB activator), and TEC (translocated in extraskeletal genes in some cancer cells), etc. (36, 57–59). Interestingly, the resulting TFG-fusion oncogenic proteins contain TFG primarily at their N-terminal region. Therefore, the present finding that the MetAPs-mediated Arg/N-end rule pathway degrades MN-TFG suggests that the N-terminal TFG of these oncogenic fusion proteins would act as a portable degron for the NME-mediated Arg/N-end rule pathway. As a result, the TFG-mediated dysregulation of oncogenic proteins most likely promotes tumorigenesis. Elucidating whether Nt-fused TFG can trigger the degradation of oncogenic fusion proteins via the MetAPs-mediated Arg/N-end rule pathway in malignant cancer cells is therefore of great interest.

**Experimental procedures**

**Miscellaneous reagents and antibodies**

Cycloheximide (C7698), yeast protease inhibitor mixture (P8215), phenylmethylsulfonyl fluoride (93482), and fumagillin (F6771) were purchased from Sigma. Chicken egg white lysozyme (LDB0308) was purchased from Bio Basic (Markham, Ontario, Canada), and MG-132 (474790) was from Calbiochem. Anti-FLAG M2 (F1804), anti-ha (H9658), and anti-tubulin (T5168) antibodies were purchased from Sigma. Anti-GST (A00865) antibodies were sourced from GenScript (Piscataway, NJ). Secondary antibodies for immunoblotting were horseradish peroxidase–conjugated goat anti-rabbit (170-6515, Bio-Rad) or anti-mouse (170-6516, Bio-Rad) antibodies, with detection using Clarity Western ECL substrate (170-5061, Bio-Rad), according to the manufacturer’s instructions. Dynabeads protein A and G (10001D and 10003D, ThermoFisher Scientific, Waltham, MA) and GSH-Sepharose 4B (17-0756-05, GE Healthcare) were used for co-immunoprecipitation and pulldown assays.

**Yeast strains, culture media, and genetic methods**

*S. cerevisiae* strains used in this study are described in Table 1. Strain construction and transformation were conducted using standard techniques (60). S. cerevisiae CHY367, CHY368, CHY860, CHY907, CHY908, CHY2009, CHY2014, CHY1015, CHY2016, CHY2017, CHY3129, CHY3186, CHY5052, CHY5091, CHY5092, CHY5093, CHY5094, and CHY5141 were constructed through the PCR-mediated gene disruption method using pFA6a-KanMX6, pFA6a-HphNT1, or pFA6a-NatNT2 modules. *S. cerevisiae* cells were cultured in YPD (1%
yeast extract, 2% peptone, and 2% glucose), SC (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose, and specific compounds to support the cell growth of specific auxotrophic strains), SRaf (SC with 2% raffinose instead of 2% glucose), or SGal (SC with 2% galactose instead of 2% glucose) media.

**Plasmid construction**

Tables 2 and 3 describe the plasmids and oligomers used in this study. To construct the pCH1525 vector that expressed Ub\(^{MN-\alpha2^{67-67}}\)-GST from the \(P_{CUP1}\) promoter on a low-copy-number pRS314 vector, the \(MN-\alpha2^{67-67}\) and GST genes were PCR-amplified from pCH535 and pEG-KG using primer pairs OCH5067/OCH992 and OCH613/OCH614. The resulting PCR products were digested with SacII/EcoRI and ligated into SacII/EcoRI-cut pCH535.

To construct pCH1591 that expressed human UBR1\(^{1-1031}\) from a Y2H expression bait vector, human UBR1\(^{1-1031}\) DNA was PCR-amplified from pCH432 carrying human UBR1 cDNA using the primers OCH5001/OCH5003, digested with SfiI/Sall, and then cloned into SfiI/Sall-cut pGBKTT7. To create pCH5003 that expressed TFG\(_{top}\), the human TFG gene was PCR-amplified from pCH5131, which was derived from the Y2H screen, using the primer pair OCH5017/OCH5018. The resulting PCR fragment was digested with BamHI/XbaI and inserted into BamHI/XbaI-cut pDNA3.1 (+). To construct pCH5054 that expressed MQ-Rad16\(_{ba}\) from \(P_{CUP1}\) promoter on a low-copy-number pRS316 vector, RAD16 was PCR-amplified from yeast genomic DNA using the primer pair OCH5067/OCH5069, digested with SpeI/SacI, and then ligated into SpeII/Sacl-cut pCH692.

**CHX-chase assays of protein degradation**

*S. cerevisiae* cells were cultured to a 600 nm absorbance value (\(A_{600}\)) of \(\approx 1.0\) in YPD or SC media at 30 °C and then treated with CHX (at a final concentration of 0.2 mg/ml). Cell samples (equivalent to 1 ml of cell suspension at an \(A_{600}\) of 1) were collected at the indicated times via centrifugation for 2 min at

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### Table 1

*S. cerevisiae* strains used in this study

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<th>Strains</th>
<th>Relevant genotypes</th>
<th>Sources</th>
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### Table 2

Plasmids used in this study

<table>
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<th>Plasmids</th>
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<td>2μ-based vector with a Leu marker</td>
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<td>pACT2</td>
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<td>Yeplac181</td>
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<td>pBAM</td>
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<tr>
<td>pCH5054</td>
<td>MQ-Rad16(_{ba}) in p314CUP1</td>
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11,200 × g and resuspended in 1 ml of 0.2 M NaOH for 20 min on ice, followed by centrifugation for 2 min at 11,200 × g. Pelleted cells were resuspended in 50 μl of HU buffer (8 M urea, 5% SDS, 1 mM EDTA, 0.1 M DTT, 0.005% bromophenol blue, 0.2 M Tris-HCl, pH 6.8) containing 1 × protein inhibitor mixture (Sigma) and incubated for 10 min at 70 °C. After centrifugation for 5 min at 11,200 × g, 10 μl of the resulting supernatants were separated via Tris-glycine SDS-PAGE, followed by immunoblotting with anti-GST (1:2,000), anti-ha (1:2,000), or anti-tubulin (1:4,000) antibodies.

For the CHX-chase assays of TFGβ3, Ate1−/− WT MEF cells and Ate1−/− KO MEF cells were maintained in DMEM supplemented with 10% FBS and 1 × streptomycin/penicillin in a 5% CO2 incubator at 37 °C. On day 1, 1 × 105 cells were seeded onto 12-well culture plates and then transfected with 0.5 μg of pCH5003 and 3 μl of polyethyleneimine (PEI) (1 mg/ml stock solution in water, pH 7.0). After transfecting for 48 h, cells were treated with CHX (the final amount, 100 μg/ml) before harvesting and lysing in RIPA buffer (89900, Thermofisher Scientific) at the indicated time points. Cell lysates were incubated on ice for 30 min and centrifuged for 15 min at 4 °C with 15,000 × g. Supernatants were collected, and the protein concentration was measured using the Bradford assay (500001, Bio-Rad), according to the manufacturer’s protocol. Five hundred ng of total RNAs were extracted using an RNeasy mini kit (74104, Qiagen, Germantown, MD), according to the manufacturer’s protocol. Five hundred ng of total RNAs were converted into cDNA using a TOPscript™ cDNA synthesis kit (Enzymics, EZ0055, South Korea) in 20-μl reactions. Ten ng of cDNA from each sample were used for quantitative real-time RT-qPCR using a model 492 cLC precinct protein micro-sequencer (Applied Biosystems, Gmbh) at the Protein Sequencing Laboratory (Seoul, South Korea).

**Table 3**

<table>
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<th>Examples of PCR oligomers used in this study</th>
<th>Name</th>
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<td>OCH1005</td>
<td>5′-ACATCTAGATCGATAACCAGGTCCAGGTTGGGT-3′</td>
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</table>

**N-terminal Met excision for the Arg/N-end rule pathway**

Approximately 5 μg of MN-a2-GST protein partially purified from nna20Δ ubr1Δ doa10Δ cells was separated on a Tris-SDS-10% polyacrylamide gel at 80 V for 90 min in SDS-PAGE running buffer. After electrophoresis, the gel was equilibrated in CAPS transfer buffer (10% methanol, 10 mM CAPS, pH 11) for 10 min before electroblotting onto a PVDF membrane (Immobilon P, Millipore, Billerica, MA). Electroblotting was performed at 80 mA overnight at 4 °C. The PVDF membrane was washed in distilled water for 10 min, stained with Coomassie Blue R-250 (0.1% R-250 in 50% methanol) for 10 min, and then destained twice for 15 min in a destaining buffer (50% methanol, 10% acetic acid). The relevant protein band of MN-α2-GST was cut and analyzed by Edman degradation

**Total RNA extraction and real-time RT-qPCR**

For extraction of total RNA, 1 × 105 Ate1−/− WT MEF cells or Ate1−/− KO MEF cells were seeded onto a 12-well plate in DMEM, 10% FBS plus streptomycin/penicillin on day 1, and then transfected with 0.5 μg of PCH5003 and 3 μl of PEI. After a 48-h incubation, total RNA was extracted using an RNaseasy mini kit (74104, Qiagen, Germantown, MD), according to the manufacturer’s protocol. Five hundred ng of total RNAs were converted into cDNA using a TOPscript™ cDNA synthesis kit (Enzymics, EZ0055, South Korea) in 20-μl reactions. Ten ng of cDNA from each sample were used for quantitative real-time RT-qPCR using a StepOnePlus Real-Time System (ThermoFisher Scientific), and Power SYBR Green PCR primer pairs OCH6874/OCH6875 for human TFG and OCH8104/OCH8105 for ACTB (encoding β-actin) were designed using NCBI Primer-BLAST. The RT-qPCR cycles were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and...
N-terminal Met excision for the Arg/N-end rule pathway

59°C for 1 min. To calculate relative TFG expression, the ∆∆CT method was used.

Statistical analysis
To calculate significant differences (p values), two-tailed paired Student’s t tests were used through Microsoft Excel 2016. A p value of <0.05 was considered statistically significant. All the values are presented as mean ± S.D.


Acknowledgments—We are grateful to Y.-T. Kwon (Seoul National University, South Korea) for providing Ate1 paper.

References

N-terminal Met excision for the Arg/N-end rule pathway


