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Control of Protein Quality and Stoichiometries by N-Terminal Acetylation and the N-End Rule Pathway

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SUMMARY

N^{α}-terminal acetylation of cellular proteins was recently discovered to create specific degradation signals, termed Ac/N-degrons and targeted by the Ac/N-end rule pathway. We show that Hcn1, a subunit of the APC/C ubiquitin ligase, contains an Ac/N-degron that is repressed by Cut9, another APC/C subunit and the ligand of Hcn1. Cog1, a subunit of the Golgi-associated COG complex, is also shown to contain an Ac/N-degron. Cog2 and Cog3, direct ligands of Cog1, can repress this degron. The subunit decoy technique was used to show that the long-lived endogenous Cog1 is destabilized and destroyed via its activated (unshielded) Ac/N-degron if the total level of Cog1 increased in a cell. Hcn1 and Cog1 are the first examples of protein regulation through the physiologically relevant transitions that shield and unshield natural Ac/N-degrons. This mechanistically straightforward circuit can employ the demonstrated conditionality of Ac/N-degrons to regulate subunit stoichiometries and other aspects of protein quality control.

Keywords

proteolysis; N-degron; ubiquitin; Not4; Cog1; Hcn1

INTRODUCTION

Approximately 90% of human proteins are N^{α}-terminally acetylated (Nt-acetylated) (Arnesen et al., 2009; Polevoda and Sherman, 2003; Van Damme et al., 2012). Nt-acetylation is apparently irreversible, in contrast to acetylation-deacetylation of internal lysines in cellular proteins (Starheim et al., 2012). Nt-acetylation can occur posttranslationally but the bulk of it is cotranslational, being mediated by ribosome-associated Nt-acetylases (Gautschi et al., 2003; Park and Szostak, 1992).

We discovered that one biological function of Nt-acetylation is the creation of specific degradation signals (degrons) in cellular proteins (Hwang et al., 2010b). Nt-acetylated proteins are targeted by a specific branch of the N-end rule pathway. This pathway

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SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Figures S1-S5, and Tables S1 and S2.

recognizes proteins containing N-terminal degradation signals called N-degrons, polyubiquitylates these proteins and thereby causes their degradation by the proteasome (Figure S1A, B). The main determinant of an N-degron is a destabilizing N-terminal residue of a protein. Recognition components of the N-end rule pathway are called N-recognins. In eukaryotes, N-recognins are E3 ubiquitin (Ub) ligases that can target N-degrons. Regulated destruction of specific proteins by the N-end rule pathway mediates a strikingly broad range of biological functions, cited in the legend to Figure S1 (Dougan et al., 2011; Mogk et al., 2007; Tasaki et al., 2012; Varshavsky, 2008, 2011).

In eukaryotes, the N-end rule pathway comprises two branches, the Arg/N-end rule pathway and the Ac/N-end rule pathway (Figure S1A, B). The Arg/N-end rule pathway targets specific unacetylated N-terminal residues. N-terminal Arg, Lys, His, Leu, Phe, Tyr, Trp, and Ile are directly recognized by E3 N-recognins. In addition, N-terminal Asn, Gln, Asp, and Glu (as well as Cys, under some metabolic conditions) are destabilizing owing to their preliminary enzymatic modifications, which include Nt-deamidation and Nt-arginylation (Figure S1B) (Hwang et al., 2010a; Piatkov et al., 2012; Varshavsky, 2011).

In contrast to the Arg/N-end rule pathway, the Ac/N-end rule pathway targets proteins through their Nt-acetylated residues, largely Nt-acetylated Met, Ala, Val, Ser, Thr, or Cys. These degradation signals are called Ac/N-degrons, to distinguish them from other N-degrons. In the first study of these degradation signals, 7 out of 9 natural Nt-acetylated proteins in the yeast *Saccharomyces cerevisiae* (MATα2, His3, Tbf1, Slk1, Aro8, Hsp104, and Ymr090w) were shown to contain Ac/N-degrons (Hwang et al., 2010b). (Because the assays employed were definitive only in positive cases, the other 2 proteins may also contain Ac/N-degrons.) In addition, Cog1 and Hcn1, the proteins of this study, have also been found to contain Ac/N-degrons, as described below. Given the quasi-random choices of the 11 natural Nt-acetylated proteins examined so far, it is likely that Ac/N-degrons are present in many (possibly most) Nt-acetylated proteins. We suggested that the biological roles of Ac/N-degrons encompass protein quality control, including the regulation of input stoichiometries of subunits in oligomeric proteins (Hwang et al., 2010b; Varshavsky, 2011). In the latter process, a free subunit of a protein complex is short-lived until the subunit becomes a part of the cognate complex.

Consider an oligomeric protein in which a subunit's Nt-acetylated residue is sterically shielded. The initial destruction, through an Ac/N-degron, of some of this subunit's newly formed molecules would be halted by formation of the oligomer and the resulting sequestration of the Ac/N-degron. If this shielding occurs through intramolecular folding of a subunit or through homo-oligomeric interactions, such an Ac/N-degron would become repressed by default, i.e., regardless of the level of subunit's expression, either during translation of the subunit's polypeptide chain or soon thereafter. In contrast, if the shielding involves hetero-oligomeric interactions, the control of the Ac/N-degron would require a sufficiently low level of subunit expression. Above that level, some molecules of this subunit would remain short-lived, owing to titration of protein ligands that normally sequester the subunit's Ac/N-degron.

In this mechanism, the regulation of input stoichiometries of subunits in protein complexes involves the degradation of uncomplexed (e.g., overproduced) subunits through their Ac/N-degrons. As mentioned above, this regulation would be relevant largely to subunits in which Ac/N-degrons can be sequestered through hetero-oligomeric interactions, because the shielding of a protein's Ac/N-degron through homo-oligomeric interactions would occur even if this protein is overproduced. The conditionality of Ac/N-degrons may also mediate, through the same degron-shielding mechanism, the selective destruction of misfolded proteins, those among them that cannot sequester their Ac/N-degrons. Because most Ac/N-

degrons are formed cotranslationally, the degradation of a protein by the Ac/N-end rule pathway would occur, in part, concurrently with the synthesis of a nascent polypeptide or shortly after its completion, for example during the cotranslational assembly of protein complexes, or in the context of a polypeptide's interaction with chaperones (Brandman et al., 2012; Duncan and Mata, 2011; Halbach et al., 2009; Hartl et al., 2011).

The same logic applies to any conditional degradation signal. Indeed, specific internal degrons participate in proteolysis-mediated homeostasis, including the destruction of abnormal proteins (Finley et al., 2012; Fredrickson and Gardner, 2012; Guerriero and Brodsky, 2012; Ravid and Hochstrasser, 2008; Varshavsky, 2011, 2012; Wolf and Stolz, 2012). Note, however, that compared to other degradation signals, Ac/N-degrons are the earliest degrons to form in nascent polypeptide chains. Ac/N-degrons are also likely to be unusual in their high prevalence.

To explore Ac/N-degrons and the Ac/N-end rule pathway, we focused on Cog1 and Hcn1. *S. cerevisiae* Cog1 is a 48 kDa subunit of the Conserved Oligomeric Golgi (COG) complex, which comprises the subunits Cog1-Cog8, resides in the cytosol, associates with membranes, and participates in Golgi transport (Miller and Ungar, 2012; Sztul and Lupashin, 2009). Cog1 is a bridging subunit between the lobes of the COG complex (Fotso et al., 2005; Lees et al., 2010). Hcn1 (Cdc26) of the yeast *Schizosaccharomyces pombe* is a 9 kDa subunit of the Anaphase-Promoting Complex/Cyclosome (APC/C) Ub ligase, which mediates the degradation of mitotic regulators and other proteins (Barford, 2011; Hershko, 2010; Pines, 2011).

We show here that both Cog1 and Hcn1 contain Ac/N-degrons. In addition, we identified Not4 (Mot2) as an E3 Ub ligase that targets Cog1. We also show, using the subunit decoy technique, that the Ac/N-degrons of Cog1 and Hcn1 are regulated through interactions between these proteins and their protein ligands. These results constitute the first evidence for the physiologically relevant conditionality of Ac/N-degrons and for the steric sequestration basis of this conditionality. Hcn1 and Cog1 are the first examples of protein regulation through transitions that shield and unshield natural Ac/N-degrons.

Apart from other ramifications of these advances, they also clarify, in hindsight, the cause of the long-held assumption in the field (before the 2010 discovery of Ac/N-degrons) that Nt-acetylation protects intracellular proteins from degradation. Specifically, many previously studied Nt-acetylated proteins appeared to be long-lived, a correct in part but fundamentally incomplete conclusion. This is shown here through a deeper understanding of the conditionally long-lived endogenous Cog1, its reversible interactions with other COG subunits, and the ensuing shielding and unshielding of its Ac/N-degron. The resulting view is two-fold. First, an Ac/N-degron in a protein precludes the targeting of this protein by proteolytic systems (e.g., the Arg/N-end rule pathway) that require the unmodified N-terminus. Second, a molecule of an Ac/N-degron-containing protein that becomes long-lived enters this state once this molecule ceases (through steric shielding) to be targeted by the Ac/N-end rule pathway. Whether this hiatus from being vulnerable to degradation is long-lasting or transient (and recurring) is determined by the in vivo dynamics of the complex that sequesters the protein's Ac/N-degron.

RESULTS

The Ac/N-Degron of Cog1, a Subunit of the COG Complex

S. cerevisiae Cog1 is Nt-acetylated (Arnesen et al., 2009; Van Damme et al., 2012). The encoded Met-Asp (MD) N-terminal sequence of wild-type (wt) Cog1, termed MD-Cog1^{wt}, implied that its (retained) N-terminal Met is Nt-acetylated by the NatB Nt-acetylase (Figure

S2B–D). MD-Cog1^{wt} was expressed in yeast using low copy plasmids, the P_{CUP1} promoter, and C-terminal epitope tagging.

Cycloheximide (CHX)-chases showed that MD-Cog1^{wt} was short-lived in wt cells ($t_{1/2} \ll 1$ hr) (Figure 1A). Our previous work identified the Doa10 E3 Ub ligase as an N-recognin of the Ac/N-end rule pathway (Hwang et al., 2010b). However, MD-Cog1^{wt} remained short-lived in *doa10Δ* cells (Figures 1A, S3A and S5D, E), prompting the search (described later in the paper) for another N-recognin. In agreement with the presence of an Ac/N-degron in MD-Cog1^{wt}, it was stabilized, including its increased levels at time zero (the beginning of the chase) in *naa20Δ* (*nat3Δ*) cells, which lacked the catalytic subunit of the cognate NatB Nt-acetylase (Figures S2D and S3A, C, G). In contrast, the instability of MD-Cog1^{wt} did not require non-cognate Nt-acetylases (Figures S2C, D and S3C).

In agreement with CHX-chases (which monitor both “young” and “old” protein molecules), the newly formed MD-Cog1^{wt}, analyzed by ^{35}S-pulse-chases, was also short-lived in wt and *doa10Δ* cells ($t_{1/2} \approx 10$ min) but was nearly completely stabilized in *naa20Δ* cells lacking the NatB Nt-acetylase (Figures 1B,C, D and S2D).

We also constructed the MK-Cog1^{D2K} and P-Cog1 mutants. In MK-Cog1^{D2K}, wt Asp2 (Figure S2B) was replaced by Lys. In P-Cog1, Pro was inserted before Asp2. The N-terminal sequences of mutant proteins were Met-Lys (MK) and Pro-Asp (PD), respectively, the latter after the cotranslational removal of N-terminal Met. Either N-terminal Pro followed by any residue or N-terminal Met followed by a basic residue are not Nt-acetylated in *S. cerevisiae* (Figure S2C) (Arnesen et al., 2009; Goetze et al., 2009). In agreement with the presence of an Ac/N-degron in MD-Cog1^{wt}, the non-Nt-acetylatable MK-Cog1^{D2K} and P-Cog1^{D2P} were long-lived in CHX-chases, in addition to their increased levels at the beginning of the chase (Figure 1A and S3B). P-Cog1^{D2P} was also stabilized in ^{35}S-pulse-chases (Figure 1B, C, D).

The degradation of MD-Cog1^{wt} was mediated largely by the proteasome, as shown using either *pdr5Δ S. cerevisiae*, which allowed for the intracellular retention of MG132, a proteasome inhibitor (Figure S3D, E), or through the use of wt cells made permeable to MG132 by low levels of SDS (Figure S3G). MD-Cog1^{wt} was also examined using the conditional *uba1-204* allele of the Uba1 Ub-activating (E1) enzyme (Ghaboosi and Deshaies, 2007). MD-Cog1^{wt} was short-lived in wt and *uba1-204* cells at 30°C and remained short-lived in wt cells at the nonpermissive temperature of 37°C (Figure S3H). In contrast, MD-Cog1^{wt} was stabilized at 37°C in *uba1-204* cells, including its increased levels at time zero (Figure S3H). Thus, the degradation of MD-Cog1^{wt} requires the Ub-activating enzyme.

Antibody Specific for Nt-Acetylated Cog1

The Nt-acetylated N-terminal peptide of MD-Cog1^{wt}, Ac-MDEVLPLFRDS, was used to produce a rabbit antibody, termed anti-Cog1^{AcNt}, that recognized the Nt-acetylated peptide but not its unacetylated counterpart (Figure S4A–C). This antibody detected Nt-acetylated MD-Cog1^{wt} in extracts from wt cells either at the beginning of the chase (Figure 1E) or under steady-state conditions (Figure S4B). In contrast, anti-Cog1^{AcNt} detected, at most, trace amounts of MD-Cog1^{wt} in extracts from *naa20Δ* (*nat3Δ*) cells lacking the cognate NatB Nt-acetylase (Figure 1E), thereby providing independent (immunological) confirmation of the mass spectrometric evidence (Arnesen et al., 2009; Van Damme et al., 2012) for Nt-acetylation of MD-Cog1^{wt} in wt cells. The same immunoblot re-probed with anti-Flag antibody revealed comparable levels of MD-Cog1^{wt} (C-terminally tagged with 3 flag epitopes) in both wt and *naa20Δ* cells at the beginning of the chase, thereby confirming the specificity of anti-Cog1^{AcNt} (Figure 1E, F). In agreement with previously described

chase assays (Figures 1A–D and S3A–C), MD-Cog1^{wt} was short-lived in wt cells but was stabilized in *naa20Δ* cells (Figure 1E–H), whereas P-Cog1 was nearly stable under all conditions (Figure 1E, F, H).

Together, CHX-chases, ^{35}S-pulse-chases, and anti-Cog1^{AcNt} results with wt versus mutant *S. cerevisiae* and with mutants of MD-Cog1^{wt} (Figures 1, S3 and S4) identified this subunit of the COG complex as a short-lived substrate of the Ac/N-end rule pathway.

Cog1 Interactions with Other COG Subunits and Membranes

Cog1 contributes to interactions of the COG complex with the Golgi membrane (Sztul and Lupashin, 2009). In a test that did not distinguish between Golgi and other membranes, cell extracts were fractionated into soluble ("cytosol") and insoluble ("membrane") parts. The absence of Nt-acetylation of MD-Cog1^{wt} in *naa20Δ* cells did not affect its partitioning between membrane and soluble fractions (Figure S4D–E).

Schulman and colleagues showed that the Nt-acetyl moiety can significantly contribute to the affinity between an Nt-acetylated protein and its protein ligand (Monda et al., 2012; Scott et al., 2011). We found that both Cog3$_{ha}$ and Cog4$_{ha}$ could be coimmunoprecipitated (co-IPed) with MD-Cog1^{wt} from both wt and *naa20Δ* extracts (Figure S4G, H). Thus, Nt-acetylation of MD-Cog1^{wt} is not strictly required for its direct or indirect interactions with Cog3 and Cog4, i.e., a possibly lower affinity of unacetylated MD-Cog1^{wt} (in *naa20Δ* cells) for its protein ligands was still sufficient for co-IP. We also found that the apparent M_r of the 93-kDa Cog3 was increased by ~8 kDa either in *naa20Δ* cells or in the absence of overexpressed MD-Cog1^{wt}. Upshifted Cog3 could be co-IPed with MD-Cog1^{wt} (Figure S4G). One interpretation of these results is that Cog3 is monoubiquitylated under the above conditions.

Cog1 Is Targeted by the Not4 (Mot2) E3 Ubiquitin Ligase

The Doa10 E3 N-recognin physically binds to Nt-acetylated polypeptides and contributes to the in vivo degradation of previously examined Nt-acetylated proteins (Hwang et al., 2010b). However, Doa10 was not required for the degradation of MD-Cog1^{wt} (Figures 1A, B, S1A, S3A and S5D). Consistently, MD-Cog1^{wt} was not stabilized in *ubc6Δ ubc7Δ* cells lacking two cognate E2s of the Doa10 E3 (Figure S5F, G). Null mutants in 9 other E2s also did not stabilize MD-Cog1^{wt} (Figure S5A–C). In addition, MD-Cog1^{wt} remained short-lived in *ubr1Δ* and *san1Δ* cells, which lacked, respectively, the E3 N-recognin of the Arg/N-end rule pathway (Figure S1B) and a nuclear E3 that targets misfolded proteins (Fredrickson and Gardner, 2012) (Figure S5D, E). Given these negative results, we examined the stability of MD-Cog1^{wt} in a *not4Δ* mutant, because the Not4 (Mot2) E3 Ub ligase interacts, in particular, with a ribosome-bound chaperone called the Nascent Polypeptide-Associated Complex (NAC) (Collart et al., 2013).

Remarkably, MD-Cog1^{wt} was strongly stabilized in *not4Δ* cells, including its increased levels at the beginning of the chase (Figure 2A, B). In addition, the observed stabilization of MD-Cog1^{wt} in the absence of Not4 was not significantly affected in *not4Δ doa10Δ* and *not4Δ ubr1Δ* double mutants that also lacked either Doa10, an N-recognin of the Ac/N-end rule pathway, or Ubr1, the N-recognin of the Arg/N-end rule pathway (Figures 6A, B and S1A, B). The same results (dependence of the MD-Cog1^{wt} degradation on the presence of Not4) were obtained in CHX-chase assays in which MD-Cog1^{wt} was detected by immunoblotting with anti-Cog1^{AcNt} antibody, which selectively recognized Nt-acetylated MD-Cog1^{wt} (Figure 2C). The discovery that Not4 is the putative second N-recognin of the Ac/N-end rule pathway opens up a separate direction of studies (see Discussion).

Short-Lived Cog1 Is Stabilized by Coexpression of Cog2-Cog4

Both the conjectured conditionality of Ac/N-degrons (Hwang et al., 2010b) and electron microscopy of the yeast Cog1-Cog4 subcomplex (Figure S1D) (Lees et al., 2010) suggested that the Ac/N-degron of MD-Cog1^{wt} may be at least partially sequestered in the subcomplex. Cog2 and Cog3 are direct binding ligands of MD-Cog1^{wt} (Sztul and Lupashin, 2009). We asked whether the short-lived, moderately overexpressed (from the P_{CUP1} promoter on a low copy plasmid) MD−Cog1$^{wt}_{3f}$ could be stabilized by expressing, from the galactose-inducible $P_{GAL1\text{-}10}$ promoter on a high copy plasmid, the COG subunits Cog2, Cog3 and Cog4. Indeed, the short-lived MD−Cog1$^{wt}_{3f}$ was strongly stabilized by coexpression of either Cog2 and Cog3 or of Cog2, Cog3 and Cog4 (Figure 3C–F). We conclude that the Ac/N-degron of MD-Cog1^{wt} is conditional in a physiologically relevant manner (see Discussion).

Endogenous Cog1 Is Long-Lived If No Other Cog1 Is Coexpressed In a Cell

To address the degradation of endogenous Cog1, we replaced, through in vivo recombination, the chromosomal *COG1* with an otherwise identical DNA segment that expressed the C-terminally triply ha-tagged MD-Cog1^{wt} (MD−Cog1$^{wt}_{3ha}$) from the endogenous P_{COG1} promoter. Remarkably, in cells expressing solely the endogenous MD−Cog1$^{wt}_{3ha}$, it was much more stable than the triply flag-tagge MD−Cog1$^{wt}_{3f}$ overexpressed from the P_{CUP1} promoter. Specifically, less than 10% of the endogenous MD−Cog1$^{wt}_{3ha}$ was degraded during 2 hrs of CHX-chase, in contrast to degradation of the bulk of overexpressed MD−Cog1$^{wt}_{3f}$ (Figure 3A vs., for example, Figure 1A, F). In addition, the metabolic stability of endogenous MD−Cog1$^{wt}_{3ha}$ remained unaltered in *naa20Δ* cells lacking the cognate NatB Nt-acetylase, in contrast to a strong stabilization of the (short-lived) overexpressed MD−Cog1$^{wt}_{3f}$ in the absence of NatB (Figure 3A, B vs. Figures 1C, S3A, and S3C). The reason for this striking difference in stability between the endogenous Cog1 and overexpressed Cog1 was identified through the subunit decoy technique, as described below.

Subunit Decoy Technique Reveals the Cause of Stability of Endogenous Cog1

The results described so far suggested that the stability of endogenous MD-Cog1^{wt} (Figure 3A) stems from sequestration of its Ac/N-degron by other COG subunits during the assembly of the degradation-resistant COG complex. We addressed this possibility by an approach termed the subunit decoy technique. First, a C-terminally epitope-tagged protein of interest is expressed at its endogenous level from its native chromosomal location and transcriptional promoter. Second, the same protein but C-terminally tagged with a different epitope is also expressed in these cells, as described in Figure 4A.

Cells expressing wt levels of MD−Cog1$^{wt}_{13myc}$ (C-terminally tagged with 13 myc epitopes) from the chromosomal *COG1* locus were transformed either with a vector alone or with a plasmid expressing the otherwise identical MD−Cog1$^{wt}_{3f}$ (C-terminally tagged with 3 flag epitopes) from the constitutive P_{ADH1} promoter (Figure 4B). Remarkably, whereas the endogenous MD−Cog1$^{wt}_{13myc}$ was stable in the absence of ectopically expressed MD−Cog1$^{wt}_{3f}$, the same endogenous MD−Cog1$^{wt}_{13myc}$ became a short-lived protein in the presence of the (also short-lived) MD−Cog1$^{wt}_{3f}$ (Figures 3A, B and 4B, C). In another design of the subunit decoy experiment, cells expressing wt levels of MD−Cog1$^{wt}_{3ha}$ (C-terminally tagged with 3 ha epitopes) from the chromosomal *COG1* locus were transformed with a plasmid expressing the otherwise identical MD−Cog1$^{wt}_{3f}$ from the galactose-inducible P_{GAL1} promoter. In

glucose medium, where MD−Cog1$^{wt}_{3f}$ was not expressed, the endogenous MD−Cog1$^{wt}_{3ha}$ was long-lived ($t_{1/2} \gg 1$ hr), as evidenced by its stability in a CHX-chase and the absence of a significant change in *naa20Δ* cells lacking the cognate NatB Nt-acetylase (Figure 3B). In striking contrast, the same endogenous MD−Cog1$^{wt}_{3ha}$ became short-lived ($t_{1/2} \ll 30$ min) in the presence of galactose, which induced MD−Cog1$^{wt}_{3f}$ (Figure 4D–F). As described in Discussion, these technically robust results are likely to be of general significance (i.e., far beyond the subject of Cog1), because the conditional sequestration model of Ac/N-degrons (see Introduction) predicts the observed destabilization of endogenous MD-Cog1^{wt} in the presence of additional Cog1 molecules in a cell.

The Ac/N-Degron of Hcn1, a Subunit of the APC/C Ubiquitin Ligase

In the crystal structure, by Barford and colleagues, of the complex between the APC/C subunits Hcn1 and Cut9 of *S. pombe* (Zhang et al., 2010), the Nt-acetylated Met residue of Hcn1 was found to be enclosed within a chamber of the folded Cut9 subunit (Figure S1C). Zhang et al. (2010) suggested that this configuration might be the first structurally explicit example of the sequestration of Ac/N-degrons proposed by Hwang et al. (2010b).

We addressed this possibility as follows. The encoded Met-Leu (ML) N-terminal sequence of the 9-kDa wt Hcn1, termed ML-Hcn1^{wt}, implied that its (retained) N-terminal Met was Nt-acetylated by the NatC Nt-acetylase (Figure S2C). ML-Hcn1^{wt} was C-terminally tagged with 3 flag epitopes and expressed in *S. cerevisiae* from the P_{CUP1} promoter. As determined using both CHX-chases and ^{35}S-pulse-chases, ML-Hcn1^{wt} was indeed short-lived in wt cells, with $t_{1/2} < 40$ min in the CHX-chase (Figure 5A) and $t_{1/2}$ of ~10 min in the ^{35}S-pulse-chase (Figure 5B, C). In contrast, ML-Hcn1^{wt} was virtually completely stabilized in *naa30Δ* (*mak3Δ*) cells lacking the catalytic subunit of the cognate NatC Nt-acetylase (Figure 5A–C). We conclude that the degradation of ML-Hcn1^{wt} is mediated by its Ac/N-degron.

Coexpression of Cut9 with Hcn1 Represses the Ac/N-degron of Hcn1

Given the sequestration of the Nt-acetylated Met of ML-Hcn1^{wt} in the cleft of Cut9 (Figure S1C) and the demonstrated degradation of ML-Hcn1^{wt} (in the absence of Cut9) by the Ac/N-end rule pathway (Figure 5A–C), we asked whether coexpression of Cut9 would inhibit the degradation of ML-Hcn1^{wt}. Several independent CHX-chase assays have shown that the short-lived ML-Hcn1^{wt} was virtually completely stabilized by Cut9 upon its overexpression from the galactose-inducible P_{GAL1} promoter (Figure 5D, E). These results were analogous to the metabolic stabilization of the short-lived MD-Cog1^{wt} by its coexpressed ligands Cog2-Cog4 (Figure 3C–F), with the added advantage of knowing that the observed inhibition of the degradation of ML-Hcn1^{wt} by Cut9 (Figure 5D, E) can be visualized and understood at atomic resolution, owing to the crystal structure of the Hcn1-Cut9 complex (Figure S1C) (Zhang et al., 2010).

DISCUSSION

This study extended key ramifications of the discovery of Ac/N-degrons and the Ac/N-end rule pathway (Figure S1A) (Hwang et al., 2010b). We proposed that the functions of Ac/N-degrons are based on their conditionality and encompass the control of protein quality, including the regulation of input stoichiometries of subunits in oligomeric protein complexes (Hwang et al., 2010b; Varshavsky, 2011). Until now, these were merely suggestions about possible steric sequestration and biological functions of Ac/N-degrons.

To address these functions, we chose two unrelated natural Nt-acetylated proteins, Cog1 (MD-Cog1^{wt}) and Hcn1 (ML-Hcn1^{wt}), described in Introduction. CHX-chase and pulse-chase experiments with MD-Cog1^{wt} and its mutants expressed in wt or mutant *S. cerevisiae*,

as well as the development of an antibody, anti-Cog1^{AcNt}, that selectively recognized Nt-acetylated MD-Cog1^{wt}, identified this subunit of the COG complex as a short-lived substrate of the Ac/N-end rule pathway (Figures 1 and S3–S5). Experiments with ML-Hcn1^{wt} also showed it to be an unstable protein targeted through its Ac/N-degron (Figure 5).

Through a different line of inquiry in this study, we discovered that at least Cog1 and also, presumably, other proteins containing Ac/N-degrons are targeted for degradation by the Not4 (Mot2) E3 Ub ligase (Figure 2). The Not4 E3 is a part of the large (>1 MDa) Ccr4-Not complex. It is present in the nucleus and cytosol, and interacts, in particular, with a ribosome-bound NAC chaperone (Collart et al., 2013). The apparent preference of distinct E3 N-recognins of the Ac/N-end rule pathway such as Doa10 and Not4 for specific (possibly overlapping) classes of substrates containing Ac/N-degrons remains to be understood. One possibility is that different N-recognins may differ in their requirements for determinants of Ac/N-degrons that are additional to the Nt-acetyl group of a substrate protein. Such determinants may include, for example, the location and/or flexibility of a substrate's internal Lys residue(s) that has to be polyubiquitylated by an E3-associated E2 enzyme (Varshavsky, 2011). Despite the clarity of molecular genetic evidence for the involvement of Not4 in the degradation of Cog1 (Figure 2), a comprehensive identification of Not4 as an N-recognin of the Ac/N-end rule pathway would still require, among other things, a demonstration that Not4 specifically binds to Nt-acetylated polypeptides. Being outside the main focus of the present work (see Introduction), this exciting advance is being developed further in a separate study.

ML-Hcn1^{wt} is an *S. pombe* protein and was, therefore, not expected to have ligands in *S. cerevisiae* that might shield its Nt-acetylated Met and thereby repress its Ac/N-degron. The disposition was different with *S. cerevisiae* MD-Cog1^{wt}, in that some newly formed molecules of overexpressed MD-Cog1^{wt} were expected to associate with Cog1-binding subunits of the COG complex and thereby repress, through steric shielding, the Ac/N-degron of MD-Cog1^{wt}. However, a majority of newly formed molecules of overexpressed MD-Cog1^{wt} would be unable to experience such a fate, as they would "run out" of cognate COG protein ligands to bind to. If so, overexpressed MD-Cog1^{wt} and ML-Hcn1^{wt} would be expected to be stabilized by coexpression of their cognate protein ligands.

This prediction was shown to be correct. A short-lived, overexpressed MD-Cog1^{wt} became a long-lived protein when Cog2 and Cog3 (direct ligands of Cog1) or Cog2, Cog3 and Cog4 were coexpressed with MD-Cog1^{wt} (Figure 3C–F). Analogously, the short-lived ML-Hcn1^{wt} was stabilized upon coexpression of its cognate ligand Cut9 (Figure 5D, E). Thus, the Ac/N-degrons of MD-Cog1^{wt} and ML-Hcn1^{wt} are conditional in a physiologically relevant manner, as they can be repressed through the previously demonstrated interactions between these proteins and their ligands.

Further analyses utilized an approach we termed the subunit decoy technique. In this method, a C-terminally epitope-tagged protein is expressed at its endogenous (wt) level in the absence or presence of the same protein that is alternatively tagged and overexpressed (Figure 4A). Several studies over the last two decades reported that an overexpressed protein could diminish the expression of its endogenous counterpart, and that this repression could be posttranscriptional, i.e., it involved changes in either translation or protein degradation, through degrons that remained unknown (Chen and Archer, 2005).

When the C-terminally ha-tagged MD−Cog1$^{wt}_{3ha}$ was expressed from its chromosomal locus at wt levels and in the absence of other versions of Cog1 in the cell, it was found to be long-lived, in striking contrast to overexpressed MD-Cog1^{wt}, the bulk of which was degraded through the Ac/N-degron (Figures 1A and 3A). The subunit decoy technique revealed the

mechanism of this phenomenon in the context of Ac/N-degrons. Specifically, when the long-lived endogenous MD−Cog1$^{wt}_{3ha}$ was expressed in the presence of the otherwise identical but flag-tagged and overexpressed MD−Cog1$^{wt}_{3f}$ decoy, the previously stable MD−Cog1$^{wt}_{3ha}$ became short-lived (Figure 4). The conditional sequestration model of Ac/N-degrons (see Introduction) predicts this outcome. Specifically, endogenous (i.e., sufficiently low) expression levels of MD−Cog1$^{wt}_{3ha}$ would be comparable to endogenous levels of the protective subunits of the COG complex such as Cog2 and Cog3 (Figure 3). As a result, most (though not necessarily all) molecules of newly formed MD−Cog1$^{wt}_{3ha}$ escape the degradation by the Ac/N-end rule pathway through the formation of complexes with COG subunits that shield the Ac/N-degron of MD−Cog1$^{wt}_{3ha}$. In contrast, the "additional" expression of the flag-tagged MD−Cog1$^{wt}_{3f}$ decoy results in total levels of MD-Cog1^{wt} that outstrip the supply of endogenous Cog1-binding subunits of the COG complex. Consequently, most newly formed molecules of ha-tagged MD−Cog1$^{wt}_{3ha}$ are no longer forming degradation-resistant COG-based complexes and therefore remain short-lived, alongside the also short-lived molecules of the flag-tagged MD−Cog1$^{wt}_{3f}$ decoy (Figure 4).

The subunit decoy experiments produced yet another insight. It was the bulk (not just a small subset) of the endogenous MD−Cog1$^{wt}_{3ha}$ that was destabilized by the coexpression of the MD−Cog1$^{wt}_{3f}$ decoy (Figure 4). In other words, this destabilization of endogenous MD−Cog1$^{wt}_{3ha}$ included its older molecules that were the long-lived components of the COG complex, before the expression of the MD−Cog1$^{wt}_{3f}$ decoy. These findings strongly suggested that the COG complex is dynamic in vivo, with dissociations-reassociations of COG subunits that repeatedly unshield (activate) and later sequester the Ac/N-degron of MD−Cog1$^{wt}_{3ha}$. The fact that endogenous MD−Cog1$^{wt}_{3ha}$ is relatively long-lived in the absence of the MD−Cog1$^{wt}_{3f}$ decoy (Figure 3A, B) implies that the probability of destruction of MD−Cog1$^{wt}_{3ha}$ upon a single cycle of its transient (and rapidly reversed) dissociation from the COG complex is low. However, when the COG complex dissociates in the presence of coexpressed MD−Cog1$^{wt}_{3f}$ decoy, the latter, being in excess, would prevent, through direct competition, an efficacious re-incorporation of MD−Cog1$^{wt}_{3ha}$ into the reassembled COG complex, thereby greatly increasing the probability of destruction of the "orphaned" (competed out) MD−Cog1$^{wt}_{3ha}$ by the Ac/N-end rule pathway, as was observed in these experiments (Figure 4).

This deeper understanding (Figures 1, 2–4, and 6) led to a clarifying reinterpretation of statements, in a number of studies over the last two decades, that most Nt-acetylated proteins were long-lived in vivo. Experimental support of such statements stemmed primarily from assays that would not be expected to detect a transient degradation of a protein of interest. Given the logic of the deeper understanding produced with Cog1 in the present work, we conclude that perception, in earlier studies, about the in vivo stability of Nt-acetylated proteins was correct in part but fundamentally incomplete at least for some and possibly for many of these proteins. Specifically, the targeting, by the Ac/N-end rule pathway, of an Ac/N-degron-containing protein such as Cog1 is (reversibly) prevented once Cog1 becomes a part of the COG complex. Although this complex is stable enough for in vitro studies (Sztul and Lupashin, 2009), it is clearly dynamic in vivo (Figure 4 and discussion above), similarly to many other protein complexes (Barford, 2011; Hartl et al., 2011). Thus, an Ac/N-degron of an "intermittently sheltered" protein molecule can become available for proteolytic targeting repeatedly during the lifetime of this molecule in a cell.

Because the bulk of Nt-acetylation is cotranslational, i.e., it involves nascent proteins, it is possible (we suggest it is likely) that every nascent and/or newly formed protein that acquires an Ac/N-degron is targeted for destruction by the Ac/N-end rule pathway through this degron, with a varying (from protein to protein) but nearly always non-zero probability of degradation. This initial vulnerability results in a fraction of each protein's molecules being destroyed cotranslationally or nearly so. Previous work introduced the in vivo Ub sandwich technique and employed it to demonstrate the existence and significant extent of the cotranslational degradation of nascent proteins that contained N-degrons of the Arg/N-end rule pathway (Turner and Varshavsky, 2000). It should now be possible to measure, using analogous methods, the extent of initial (including cotranslational) degradation of specific Nt-acetylated proteins by the Ac/N-end rule pathway.

The degradation of a nascent or newly formed protein that contains an Ac/N-degron is a part of quality control in that the degradation ceases if and when this degron is shielded from the Ac/N-end rule pathway. For example, during the assembly of the COG complex the initially active Ac/N-degron of Cog1 would be (reversibly) sequestered through its interactions with specific COG subunits such as Cog2 and Cog3 (Figure 3), thereby yielding a degradation-resistant intermediate that coalesces with other COG subunits to yield the COG complex. Because some protein complexes assemble largely cotranslationally and some largely posttranslationally, and because many proteins undergo chaperone-assisted folding before they can productively encounter their cognate protein ligands (Hartl et al., 2011), specific routes through which a nascent protein bearing an Ac/N-degron would sequester it from the Ac/N-end rule pathway are expected to vary from one protein to another. The speed of sequestration is expected to vary as well, even for normal proteins in unstressed cells. Consequently, the extent of the initial (and usually transient) destruction of a protein that accompanies the cotranslational formation of its Ac/N-degron is expected to vary significantly among different proteins.

There are, fundamentally, three routes to the steric shielding of an Ac/N-degron: through intramolecular folding of a polypeptide containing this degron; through homo-oligomeric interactions among identical protein subunits; and through hetero-oligomeric interactions in which an Ac/N-degron of one subunit of a complex is shielded by another protein that interacts with this subunit (Figures 6 and S1C). The sequestration of an Ac/N-degron that occurs through intramolecular folding or homo-oligomeric interactions is not "saturable", i.e., Ac/N-degrons of this class would become extinguished regardless of whether a protein containing such a degron is expressed normally or overexpressed. In contrast, if the shielding involves hetero-oligomeric interactions, the self-regulation of Ac/N-degron would require a sufficiently low level of subunit expression. Above that level, some subunit molecules would remain short-lived, owing to titration of protein ligands that can sequester the subunit's Ac/N-degron. Such is the case with both Cog1 and Hcn1 of the present study, as their Ac/N-degrons are repressed through hetero-oligomeric interactions (Figures 3–6 and S1C, D).

The mechanics of Ac/N-degrons can underlie both the input stoichiometries of specific subunits in protein complexes and the selective destruction of misfolded proteins, those among them that cannot sequester their Ac/N-degrons rapidly enough. In the present study, Cog1 vis-à-vis the COG complex and Hcn1 vis-à-vis the APC/C cyclosome (Figures 6 and S1C, D) are likely examples of the physiological control of input stoichiometries through the Ac/N-end rule pathway. It is unknown, so far, whether Ac/N-degrons play important roles in the selective elimination of misfolded or otherwise abnormal proteins. It should be also noted that the function of the Nt-acetyl group as a key determinant of Ac/N-degrons is compatible with other known aspects of Nt-acetylation, including its contributions to protein-membrane interactions, to physical affinities between specific proteins, and to the

negative regulation of protein translocation into the endoplasmic reticulum (ER) (Starheim et al., 2012).

The apparent irreversibility of Nt-acetylation (see Introduction) is curious because it is likely that evolution could have readily produced an Nt-deacetylase, given the existence of efficacious enzymes that deacetylate acetylated internal lysines in many proteins. The absence of Nt-deacetylases suggests an adaptive value of retaining an Ac/N-degron in a protein molecule that acquired it, most likely because this degron can become active (unshielded) repeatedly during the molecule's sojourn in a cell, as discussed above in the context of Cog1 and the COG complex.

One setting of likely physiological relevance vis-à-vis Ac/N-degrons is aneuploidy, in which the chromosome number in a cell is not an exact multiple of the haploid number. Physiological defects in aneuploid cells are caused, at least in part, by maladaptive molar ratios of newly formed proteins in such cells, given their deviations from normal gene dosages. This may account for the hypersensitivity of aneuploid cells to proteasome inhibitors and for other manifestations of proteotoxic stress in such cells (Oromendia et al., 2012). Now that the conditionality of Ac/N-degrons is no longer a conjecture (Figures 4–6), the relevance of the Ac/N-end rule pathway to protection from stress caused by aneuploidy can be explored through approaches described in the present work.

The ER-embedded transmembrane Doa10 E3 Ub ligase is a previously identified N-recognin of the Ac/N-end rule pathway (Figure S1A) (Hwang et al., 2010b). Either "soluble" or transmembrane proteins that enter the ER can be retrotranslocated to the cytosol and destroyed if these proteins are recognized by the quality control system termed ER-Associated Degradation (ERAD) (Guerriero and Brodsky, 2012; Wolf and Stolz, 2012). The Doa10 E3 contributes to the targeting of proteins through ERAD, in addition to being an N-recognin of the Ac/N-end rule pathway. Consequently, Ac/N-degrons and the Ac/N-end rule pathway may also contribute to ERAD, a verifiable proposition.

We conclude by noting that the physiologically relevant conditionality of Ac/N-degrons that has been demonstrated and analyzed in the present study is fundamentally analogous to conditional properties of other degradation signals, including internal degrons. These aspects of internal degrons, while clearly present and probably critical to their physiology (Ravid and Hochstrasser, 2008), are largely unexplored.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, and Genetic Techniques

Tables S1 and S2 describe *S. cerevisiae* strains and plasmids. Standard techniques were employed for strain and plasmid construction.

Cycloheximide-Chase and Pulse-Chase Assays

They were carried out largely as described (Hwang et al., 2010b). The Odyssey near-infrared scanner (Li-Cor) was used for immunoblotting analyses and quantification.

Antibody Specific for Nt-Acetylated Cog1

The Nt-acetylated peptide AcMDEVLPLFRDSC and its unacetylated counterpart MDEVLPLFRDSC were synthesized by Abgent (San Diego, CA). Production of the anti-Cog1^{AcNt} antibody is described in Supplemental Experimental Procedures.

Coimmunoprecipitation of Cog1 with Cog2-Cog4

S. cerevisiae coexpressing flag-tagged MD-Cog1^{wt} and either Cog3 or Cog4 (ha-tagged) were lysed, and clarified extracts were subjected to co-IP using anti-flag magnetic beads.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- N-terminal acetylation of cellular proteins can create specific Ac/N-degrons.
- Hcn1 and Cog1 are shown to contain specific Ac/N-degrons.
- Ac/N-degrons of Cog1 and Hcn1 are repressed by their ligands Cog2/3 and Cut9.
- Conditionality of Ac/N-degrons can mediate the control of protein stoichiometries.

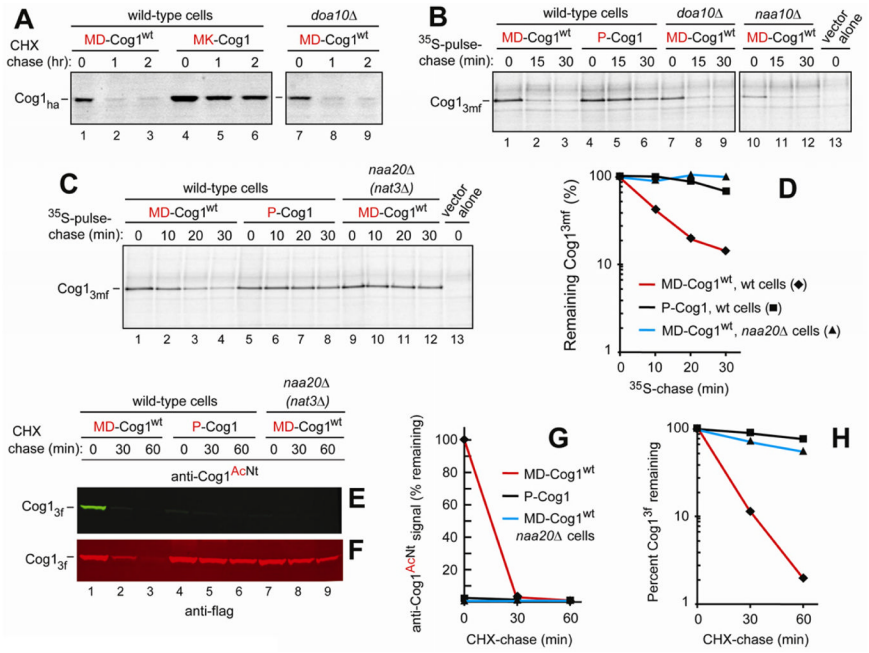

Figure 1. The Ac/N-degron of Cog1

(A) Cycloheximide (CHX)-chases were performed at 30°C with wt or *doa10Δ S. cerevisiae* expressing either wt Cog1, termed MD-Cog1^{wt}, or its MK-Cog1 derivative in which Asp2 was replaced with Lys2. Both proteins were C-terminally ha-tagged. At the indicated times of chase, proteins in cell extracts were fractionated by SDS-PAGE and assayed by immunoblotting with anti-ha antibody.

(B) ^{35}S-pulse-chase with MD-Cog1^{wt} or P-Cog1 in wt, *doa10Δ*, or *naa10Δ* (*ard1Δ*) *S. cerevisiae*, the latter strain lacking the catalytic subunit of the non-cognate (for MD-Cog1^{wt}) NatA Nt-acetylase (Figure S2). Cog1 proteins were C-terminally tagged with 3 flag epitopes modified to contain a Met residue in each epitope, to increase ^{35}S-Met in Cog1.

(C) Same as in B but another ^{35}S-pulse-chase. It included *naa20Δ* (*nat3Δ*) *S. cerevisiae* lacking the catalytic subunit of the cognate (for MD-Cog1^{wt}) NatB Nt-acetylase (Figure S2).

(D) Quantification of data in C.◆, MD-Cog1^{wt} in wt cells. ▲, MD-Cog1^{wt} in *naa20Δ* cells. ■, P-Cog1 in wt cells.

(E) Anti-Cog1^{AcNt} antibody specific for Nt-acetylated MD-Cog1^{wt} (see Figure S4A–C) was used for immunoblotting in CHX-chase assays with MD-Cog1^{wt} and P-Cog1 (C-terminally tagged with 3 flag epitopes) in either wt or *naa20Δ* (*nat3Δ*) *S. cerevisiae*.

(F) Same as in E, except that membrane was reprobed with anti-flag antibody.

(G) Quantification of anti-Cog1^{AcNt}-specific immunoblotting patterns in E using a linear scale, with the level of MD-Cog1^{wt} at time zero in wt cells taken as 100%. ◆, MD-Cog1^{wt} in wt cells. ▲, MD-Cog1^{wt} in *naa20Δ* cells. ■, P-Cog1 in wt cells.

(H) Same as in G but a semi-log plot of the flag-specific Cog1 immunoblotting patterns in F. Same designations as in G. See also Figures S1, S2, S5, and Tables S1 and S2.

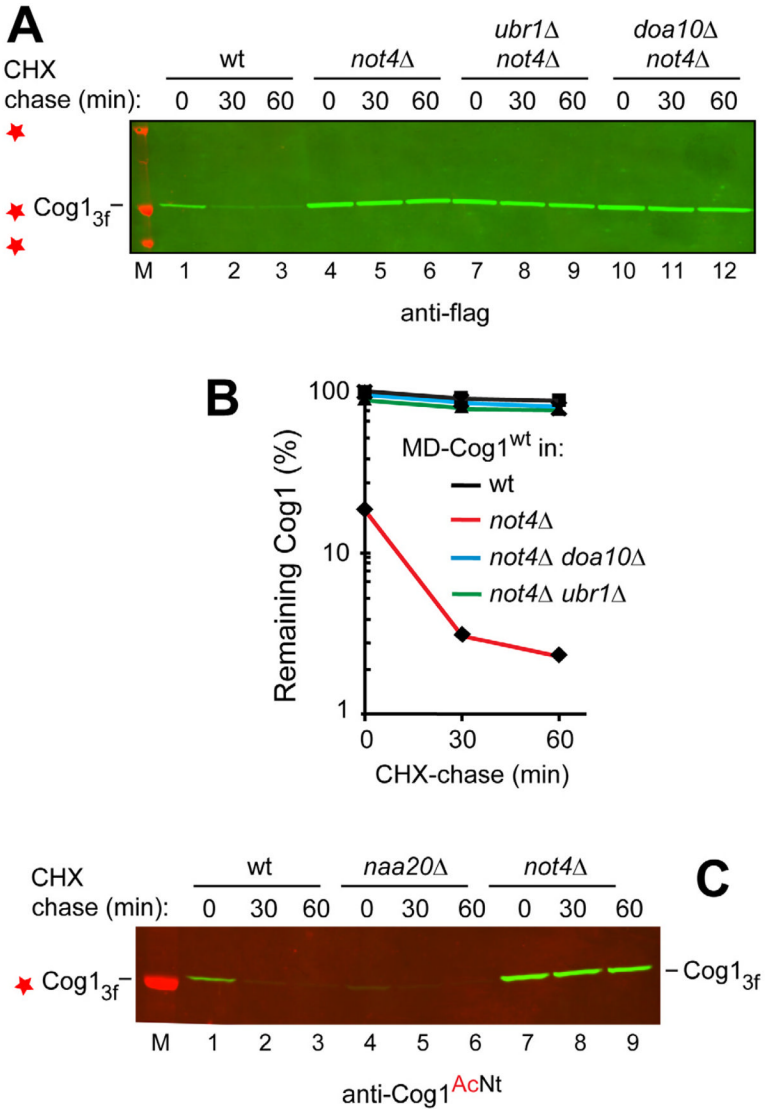

Figure 2. Stabilization of Cog1 in *S. cerevisiae* Lacking the Not4 E3 Ubiquitin Ligase

(A) CHX-chases with yeast expressing MD-Cog1^{wt} C-terminally tagged with three flag epitopes. Lane M and red stars, M_r markers of 37, 50, and 100 kDa, respectively. MD-Cog1^{wt} in wt yeast (lanes 1–3), and in *not4Δ* (lanes 4–6), *not4Δ ubr1Δ* (lanes 7–9), and *not4Δ doa10Δ* mutants (lanes 10–12).

(B) Quantification of immunoblots in H, with the level of MD-Cog1^{wt} in *not4Δ* cells at the beginning of chase taken as 100%. ■, MD-Cog1^{wt} C in *not4Δ* cells (black curve). ◆, MD-Cog1^{wt} in wt cells (red curve).▲, MD-Cog1^{wt} in *not4Δ ubr1Δ* cells (green curve). **X**,MD-Cog1^{wt} in *not4Δ doa10Δ* cells (blue curve).

(C) Same as in A but an independent CHX-chase, and immunoblotting with anti-Cog1^{AcNt} antibody specific for Nt-acetylated MD-Cog1^{wt}. Lane M and red star, an M_r marker of 50 kDa. MD-Cog1^{wt} in wt yeast (lanes 1–3), and in *naat20Δ* (*nat3Δ*) (lanes 4–6), and *not4Δ* mutants lanes 7–9). See also Figure S5.

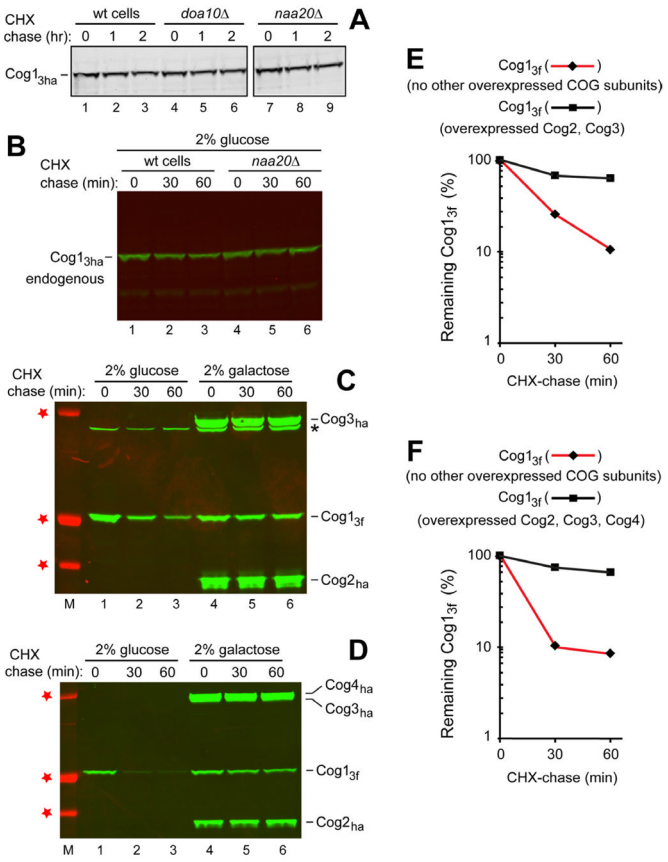

Figure 3. Stabilization of Overexpressed, Short-Lived Cog1 by Coexpressed Cog2-Cog4

(A) CHX-chases with endogenous MD–Cog1$^{wt}_{3ha}$ (C-terminally tagged with 3 ha epitopes) expressed from the chromosomal *COG1* locus and the native P$_{COG1}$ promoter in wt, *doa10Δ*, and *naa20Δ* (*nat3Δ*) cells.

(B) Same as in A but an independent CHX-chase. *S. cerevisiae* (in 2% glucose) expressing endogenous MD–Cog1$^{wt}_{3ha}$ and carrying a plasmid that could express the MD–Cog1$^{wt}_{3f}$ decoy but only in the presence of galactose. Lanes 4–6, same as lanes 1–3 but in *naa20Δ* cells.

(C) Stabilization of overexpressed MD-Cog1^{wt} (C-terminally tagged with 3 flag epitopes) by coexpressed Cog2 and Cog3. Lane M and red stars, M$_r$ markers of 37, 50, and 100 kDa, respectively. Lanes 1–3, wt *S. cerevisiae* in 2% glucose, expressing MD-Cog1^{wt} from the P$_{CUP1}$ promoter on a low copy plasmid and carrying a high copy plasmid that expressed, only in galactose, both Cog2 and Cog3 (C-terminally tagged with ha) from the bidirectional P$_{GAL1/10}$ promoter. Lanes 4–6, same as lanes 1–3 but with cells in 2% galactose. Asterisk on the right indicates a protein crossreacting with anti-ha.

(D) Same as in C but cells also carried a second high copy plasmid expressing Cog4 (C-terminally tagged with ha) from the P$_{GAL1/10}$ promoter.

(E) Quantification of data in C for MD-Cog1^{wt}. ◆, MD-Cog1^{wt} in cells that did not coexpress other COG subunits. ■, MD-Cog1^{wt} in cells that coexpressed (in galactose) Cog2 and Cog3.

(F) Quantification of data in D for MD-Cog1^{wt}. ◆, MD-Cog1^{wt} in cells that did not coexpress other COG subunits. ■, MD-Cog1^{wt} in cells that coexpressed (in galactose) Cog2-Cog4. See also Figure S1.

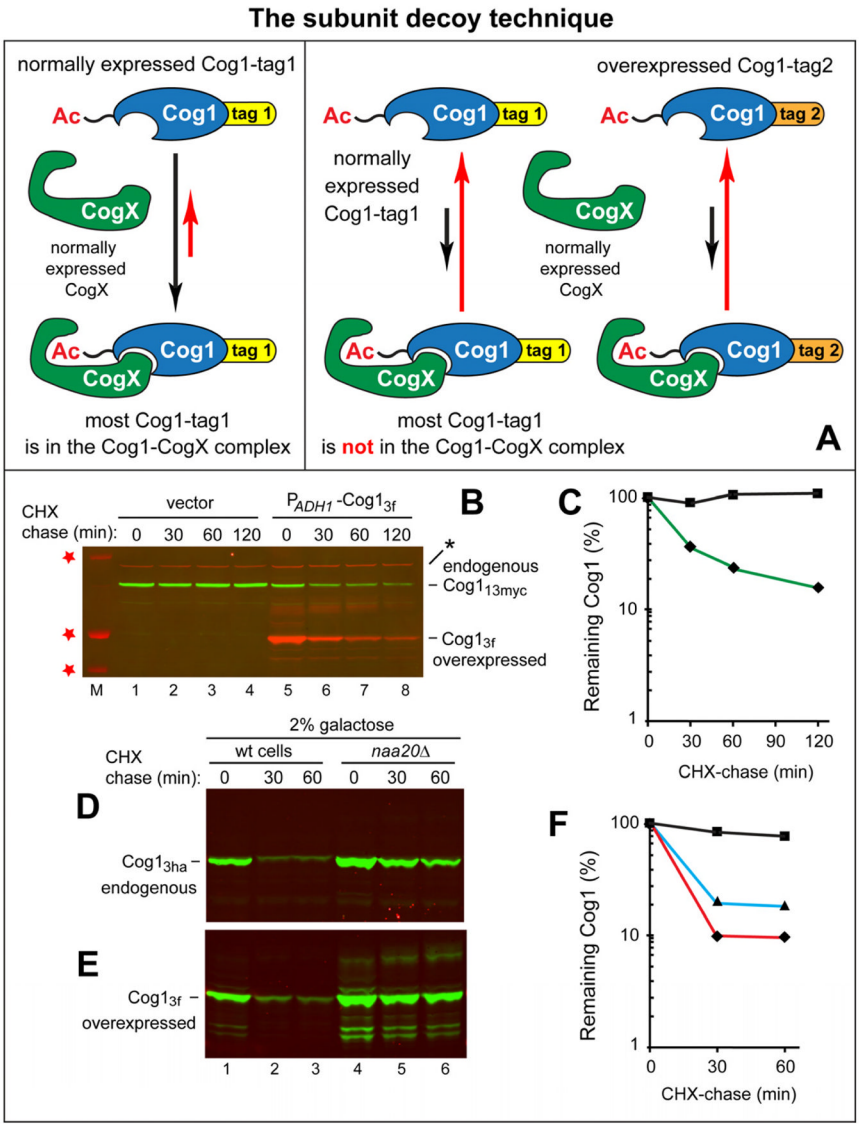

Figure 4. Subunit Decoy Technique and the Cause of Stability of Endogenous Cog1

(A) The subunit decoy technique. "CogX" denotes a Cog1-interacting COG subunit (Cog2 or Cog3) that can shield the Ac/N-degron of Cog1. "Normally expressed" refers to levels of expression from endogenous promoters and chromosomal loci. The normally expressed Cog1-tag1 bears a C-terminal tag denoted as "tag1", whereas the otherwise identical but overexpressed Cog1-tag2 decoy bears a different C-terminal tag ("tag2"). In the absence of decoy, the bulk of (normally expressed) Cog1-tag1 would occur as a CogX-Cog1-tag1 complex in which the Ac/N-degron of Cog1 is largely sequestered. By contrast, in the presence of overexpressed Cog1-tag2 decoy, the bulk of both Cog1-tag1 and Cog1-tag2 would not be in the complex with CogX (i.e., their Ac/N-degron would be active), given relatively low levels of a (normally expressed) CogX "shielding" protein.

(B) Lane M and red stars, M_r markers of 37, 50, and 100 kDa, respectively. Lanes 1–4, stability of endogenous $MD{-}Cog1^{wt}_{13myc}$ (C-terminally tagged with 13 myc epitopes) in the absence of the $MD{-}Cog1^{wt}_{3f}$ decoy (C-terminally tagged with 3 flag epitopes). CHX-chase with $MD{-}Cog1^{wt}_{13myc}$ expressed from the chromosomal *COG1* locus and the native P_{COG1}

promoter in wt cells in the presence of vector alone. Lanes 5–8, same as lanes 1–4 but cells carried a plasmid that expressed the MD−Cog1$^{wt}_{3f}$ decoy from the P$_{ADH1}$ promoter. An asterisk denotes a protein crossreacting with anti-flag.

(C) Quantification of data in B. ◆, endogenous MD−Cog1$^{wt}_{13myc}$ in wt cells that did not express the MD−Cog1$^{wt}_{3f}$ decoy. ■, endogenous MD−Cog1$^{wt}_{13myc}$ in wt cells that expressed MD−Cog1$^{wt}_{3f}$.

(D) Same as in C but with wt and *naa20Δ* cells expressing the endogenous MD−Cog1$^{wt}_{3ha}$ in 2% galactose, i.e., in the presence of the coexpressed MD−Cog1$^{wt}_{3f}$ decoy. Immunoblotting with anti-ha, specific for MD−Cog1$^{wt}_{3ha}$.

(E) Same as in D but also probed (in a parallel immunoblot) with anti-flag, specific for MD−Cog1$^{wt}_{3f}$.

(F) Quantification of data in D, E. ◆, endogenous MD−Cog1$^{wt}_{3ha}$ in wt cells grown in 2% glucose. ■, endogenous MD−Cog1$^{wt}_{3ha}$ in wt cells grown in 2% galactose, i.e., in the presence of the MD−Cog1$^{wt}_{3f}$ decoy. ▲, MD−Cog1$^{wt}_{3f}$ decoy. See also Figures S4 and S5.

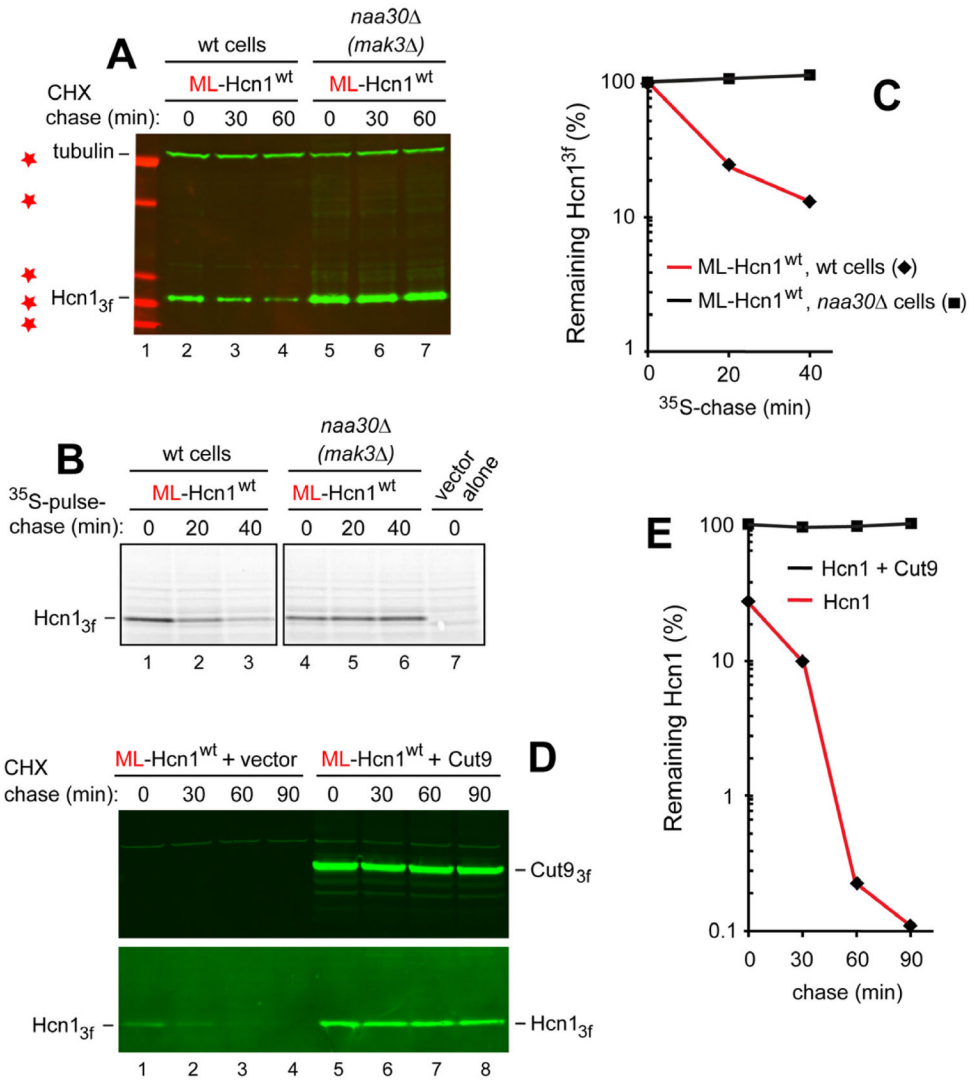

Figure 5. Hcn1 and Repression of Its Ac/N-degron by Cut9

(A) CHX-chases in wt or *naa30Δ* (*mak3Δ*) *S. cerevisiae* expressing the wt *S. pombe* Hcn1, termed ML-Hcn1^{wt}, C-terminally tagged with 3 flag epitopes. *naa30Δ* cells lacked the catalytic subunit of the cognate NatC Nt-acetylase (Figure S2). Lane 1 and red stars, M_r markers of 10, 15, 20, 37, and 50 kDa, respectively.

(B) ^{35}S-pulse-chase with ML-Hcn1^{wt} in wt and *naa30Δ* (*mak3Δ*) *S. cerevisiae*. Lane 7, vector alone.

(C) Quantification of data in B. ◆, ML-Hcn1^{wt} in wt cells. ■, ML-Hcn1^{wt} in *naa30Δ* cells.

(D) Lanes 1–4, CHX-chase with wt cells in 2% galactose (and without methionine) that expressed ML-Hcn1^{wt} from the methionine-repressible P_{MET25} promoter on a low copy plasmid and carried a vector alone (no Cut9 expression). Note the metabolic instability of ML-Hcn1^{wt} (lower panel). Lanes 5–8, same as lanes 1–4 but with a low copy plasmid (instead of control vector) expressing Cut9 from the galactose-inducible P_{GAL1} promoter, with both ML-Hcn1^{wt} and Cut9 C-terminally tagged with 3 flag epitopes. Note the metabolic stabilization of ML-Hcn1^{wt}(lower panel), including a strong increase of its level at the beginning of the chase.

(E) Quantification of data in D. ◆, ML-Hcn1^{wt} in the absence of co-expressed Cut9. ■, ML-Hcn1^{wt} in the presence of co-expressed Cut9.

See also Figure S1.

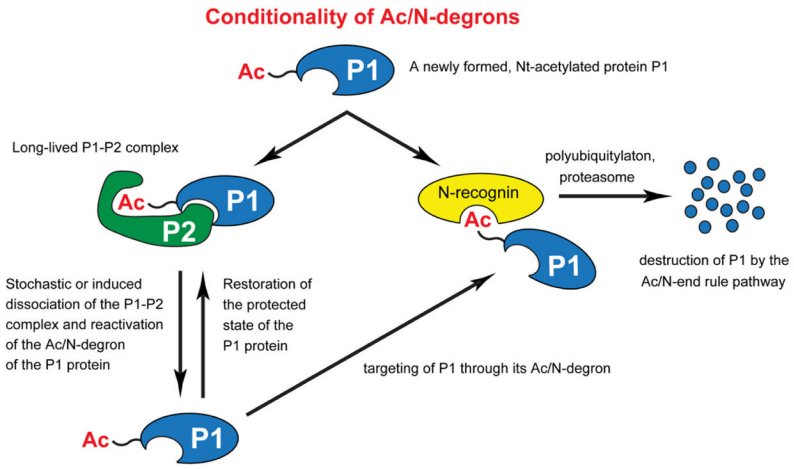

Figure 6. Conditionality of Ac/N-degrons

This diagram summarizes the functional understanding of the dynamics of Nt-acetylated proteins vis-à-vis the Ac/N-end rule pathway attained in the present study, in conjunction with results that initially revealed the Ac/N-end rule pathway (Hwang et al., 2010b). See also Figure S1.