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

Anna Shemorry,[1] Cheol-Sang Hwang,[2] and Alexander Varshavsky[1,*]
[1]Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA
[2]Department of Life Sciences, Phohang University of Science and Technology, Phohang, Gyeong-buk 790-784, South Korea
*Correspondence: avarsh@caltech.edu
http://dx.doi.org/10.1016/j.molcel.2013.03.018

ACKNOWLEDGMENTS

We thank R. Deshaies and K. Gould for gifts of plasmids. We are grateful to the present and former members of the Varshavsky laboratory for their assistance and advice. This work was supported by grants to C.-S.H. from the National Research Foundation of Korea (NRF-2011-0021975) and the Korea Healthcare Technology R&D Project (A111324), and to A.V. from the National Institutes of Health (DK039520, GM031530, and GM085371).

ALEXANDER VARSHAVSKY LAB ANNUAL REPORT 2012

CALIFORNIA INSTITUTE OF TECHNOLOGY

Smits Professor of Cell Biology:
Alexander Varshavsky

Professorial Awards and Honors:
2012 Otto Warburg Medal of the German Society for Biochemistry and Molecular Biology
2012 King Faisal International Prize
2012 BBVA Foundation Frontiers of Knowledge Award in Biomedicine

Research Assistant:
Elena Udartseva

Staff Scientists:
Christopher Brower, Konstantin Piatkov

Postdoctoral Scholars:
Yuan Liu, Jang-Hyun Oh

Graduate Students:
Anna Shemorry, Tri Vu, Brandon Wadas

Undergraduate:
Connor Rosen

Financial Support:
Howard and Gwen Laurie Smits Professorship in Cell Biology
March of Dimes Foundation
National Institutes of Health

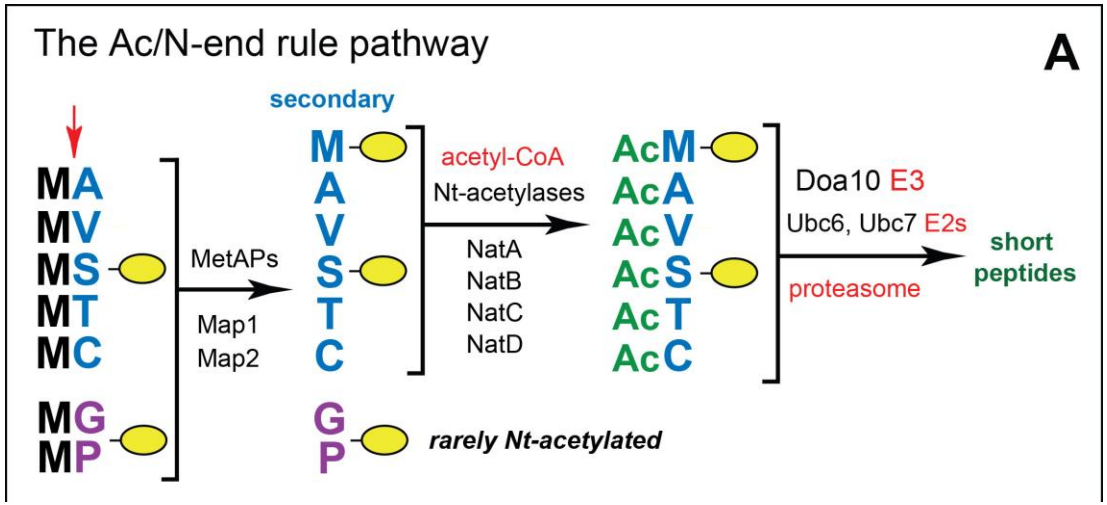
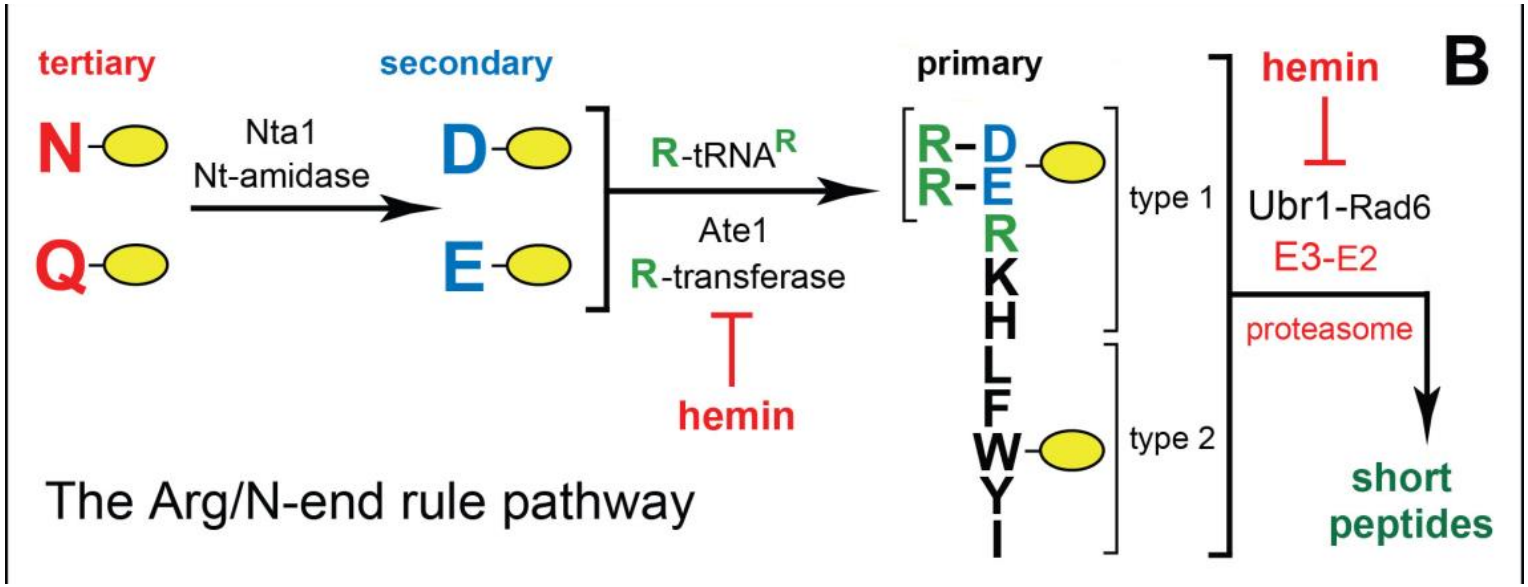


Figure S1. The Ac/N-End Rule Pathway, the Arg/N-End Rule Pathway, and the Steric Sequestration of Nα-Terminally Acetylated N-Termini of Cellular Proteins.
(A) The Ac/N-end rule pathway in the yeast Saccharomyces cerevisiae. N-terminal residues are indicated by single-letter abbreviations for amino acids. A yellow oval denotes the rest of a protein substrate. E3 ubiquitin (Ub) ligases of the N-end rule pathway are called N-recognins. The Doa10 E3 N-recognin physically binds to Nα-terminally acetylated (Nt-acetylated) polypeptides and contributes to the in vivo degradation of previously examined Nt-acetylated proteins. In the present study, we identified Not4 as a putative second N-recognin of the Ac/N-end rule pathway (Figure 2 and Discussion). Red arrow on the left indicates the removal of N-terminal Met by Met-aminopeptidases (MetAPs). N-terminal Met is retained if a residue at position 2 is nonpermissive (too large) for MetAPs. If the retained N-terminal Met or N-terminal Ala, Val, Ser, Thr, and Cys are followed by acetylation-permissive residues, the cited N-terminal residues are usually Nt-acetylated by the Nt-acetylases NatA-NatD, the bulk of which is associated with the ribosomes. Although second-position Gly or Pro can be made N-terminal by MetAPs and although the Doa10 E3

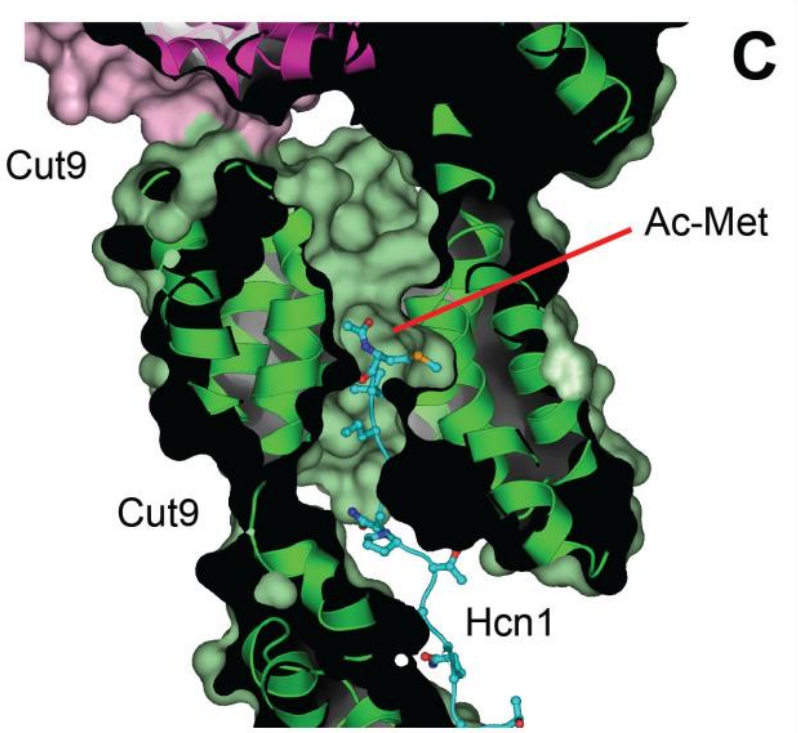
Figure S1. The Ac/N-End Rule Pathway, the Arg/N-End Rule Pathway, and the Steric Sequestration of Nα-Terminally Acetylated N-Termini of Cellular Proteins.
(A) The Ac/N-end rule pathway in the yeast Saccharomyces cerevisiae. N-terminal residues are indicated by single-letter abbreviations for amino acids. A yellow oval denotes the rest of a protein substrate. E3 ubiquitin (Ub) ligases of the N-end rule pathway are called N-recognins. The Doa10 E3 N-recognin physically binds to Nα-terminally acetylated (Nt-acetylated) polypeptides and contributes to the in vivo degradation of previously examined Nt-acetylated proteins. In the present study, we identified Not4 as a putative second N-recognin of the Ac/N-end rule pathway (Figure 2 and Discussion). Red arrow on the left indicates the removal of N-terminal Met by Met-aminopeptidases (MetAPs). N-terminal Met is retained if a residue at position 2 is nonpermissive (too large) for MetAPs. If the retained N-terminal Met or N-terminal Ala, Val, Ser, Thr, and Cys are followed by acetylation-permissive residues, the cited N-terminal residues are usually Nt-acetylated by the Nt-acetylases NatA-NatD, the bulk of which is associated with the ribosomes. Although second-position Gly or Pro can be made N-terminal by MetAPs and although the Doa10 E3 N-recognin can recognize Nt-acetylated Gly or Pro, few proteins with N-terminal Gly or Pro are Nt-acetylated. See Figure S2 for a summary of Nt-acetylation vis-à-vis specific N-terminal residues. The Nt-acetylation-mediated N-degrons are called Ac/N-degrons, to distinguish them from other N-degrons. The term “secondary” refers to the requirement for a modification (Nt-acetylation) of a destabilizing N-terminal residue before a protein can be recognized by a cognate N-recognin. Physiological functions of the Ac/N-end rule pathway are discussed in the main text.



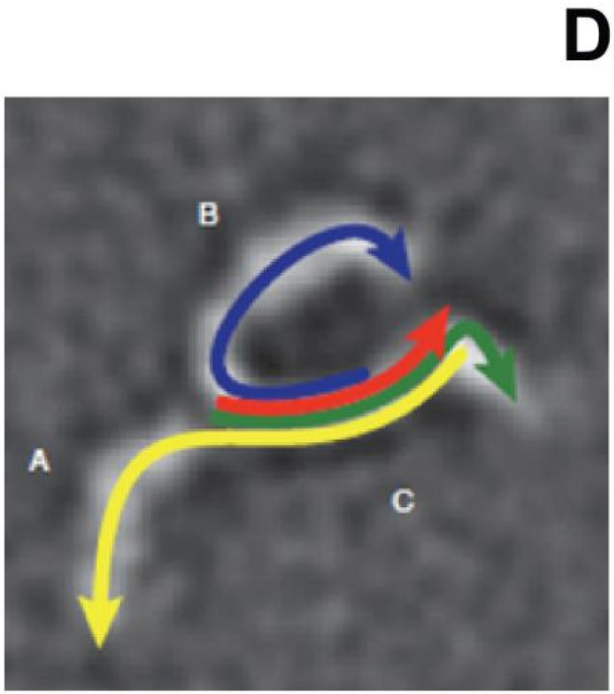
(B) The Arg/N-end rule pathway in S. cerevisiae (Varshavsky, 2011). The Ubr1/Rad6 E3-E2 N-recognin Ub ligase directly recognizes (binds to) the "primary" destabilizing N-terminal residues Arg, Lys, His, Leu, Phe, Tyr, Trp and Ile. In contrast, N-terminal Asn, Gln, Asp, and Glu (as well as Cys, under some metabolic conditions) are destabilizing owing to their preliminary enzymatic modifications. These include the Nt-deamidation of N-terminal Asn and Gln by the Nta1 Nt-amidase and the Nt-arginylation of N-terminal Asp and Glu by the Ate1 arginyltransferase (R-transferase), which can also Nt-arginylate oxidized Cys, either Cys-sulfinate or Cys-sulfonate. These derivatives of N-terminal Cys can form in cells that produce nitric oxide (NO) and may also form in S. cerevisiae. One aspect of the S. cerevisiae Arg/N-end rule pathway that is not illustrated in this diagram is a physical and functional interaction between the Ubr1 E3 of the Arg/N-end rule pathway and the Ufd4 E3 of the previously characterized Ub-fusion-degradation (UFD) pathway. Specifically, the targeting apparatus of the Arg/N-end rule pathway comprises a

(B) The Arg/N-end rule pathway in S. cerevisiae (Varshavsky, 2011). The Ubr1/Rad6 E3-E2 N-recognin Ub ligase directly recognizes (binds to) the "primary" destabilizing N-terminal residues Arg, Lys, His, Leu, Phe, Tyr, Trp and Ile. In contrast, N-terminal Asn, Gln, Asp, and Glu (as well as Cys, under some metabolic conditions) are destabilizing owing to their preliminary enzymatic modifications. These include the Nt-deamidation of N-terminal Asn and Gln by the Nta1 Nt-amidase and the Nt-arginylation of N-terminal Asp and Glu by the Ate1 arginyltransferase (R-transferase), which can also Nt-arginylate oxidized Cys, either Cys-sulfinate or Cys-sulfonate. These derivatives of N-terminal Cys can form in cells that produce nitric oxide (NO) and may also form in S. cerevisiae. One aspect of the S. cerevisiae Arg/N-end rule pathway that is not illustrated in this diagram is a physical and functional interaction between the Ubr1 E3 of the Arg/N-end rule pathway and the Ufd4 E3 of the previously characterized Ub-fusion-degradation (UFD) pathway. Specifically, the targeting apparatus of the Arg/N-end rule pathway comprises a physical complex of the RING-type E3 Ubr1 N-recognin and the HECT-type E3 Ufd4, together with their cognate E2 enzymes Rad6 and Ubc4 (or Ubc5), respectively. In addition to its two distinct binding sites that recognize type 1 (basic) and type 2 (bulky hydrophobic) destabilizing N-terminal residues, the S. cerevisiae Ubr1 N-recognin also contains (similarly to its counterparts in multicellular eukaryotes) at least one more binding site, which recognizes substrates that are targeted through their internal (non-N-terminal) degradation signals. One example of such a substrate is the Cup9 transcriptional repressor. Polyubiquitylated N-end rule substrates are processively destroyed to short peptides by the 26S proteasome. Hemin (Fe3+-heme) binds to R-transferase and inhibits its Nt-arginylation activity. Hemin also binds to Ubr1 and alters its functional properties, in ways that remain to be understood.

Regulated degradation of specific proteins by the Arg/N-end rule pathway mediates the



(C) Steric shielding of the Nt-acetylated N-terminal residue of a subunit in a protein complex. Shown here is a part of the crystal structure, by the Barford laboratory, of a complex between the Hcn1 and Cut9 subunits of the Schizosaccharomyces pombe APC/C Ub ligase (Zhang et al., 2010b). In this structure, the (indicated) Nt-acetylated N-terminal Met residue of Hcn1 is enclosed within a deep cleft formed by the Cut9 subunit, in the heterotetramer of Hcn1 and Cut9. The N-terminal region of Hcn1 is shown in cyan as a stick model, and Cut9 is depicted as a cut-out surface representation, to show the chamber's interior (Zhang et al., 2010b).



(D) Model of interactions, based on single-particle electron microscopy by the Hughson laboratory, among the subunits Cog1-Cog4 that form a specific subcomplex of the S. cerevisiae COG complex (Lees et al., 2010). The head of an arrow and its blunt end indicate the C-terminus and the N-terminus of a protein, respectively. The green, red, yellow, and blue arrows denote Cog1, Cog2, Cog3 and Cog4, respectively (Lees et al., 2010). Figure S1 refers to Figures 1, 3 and 5.

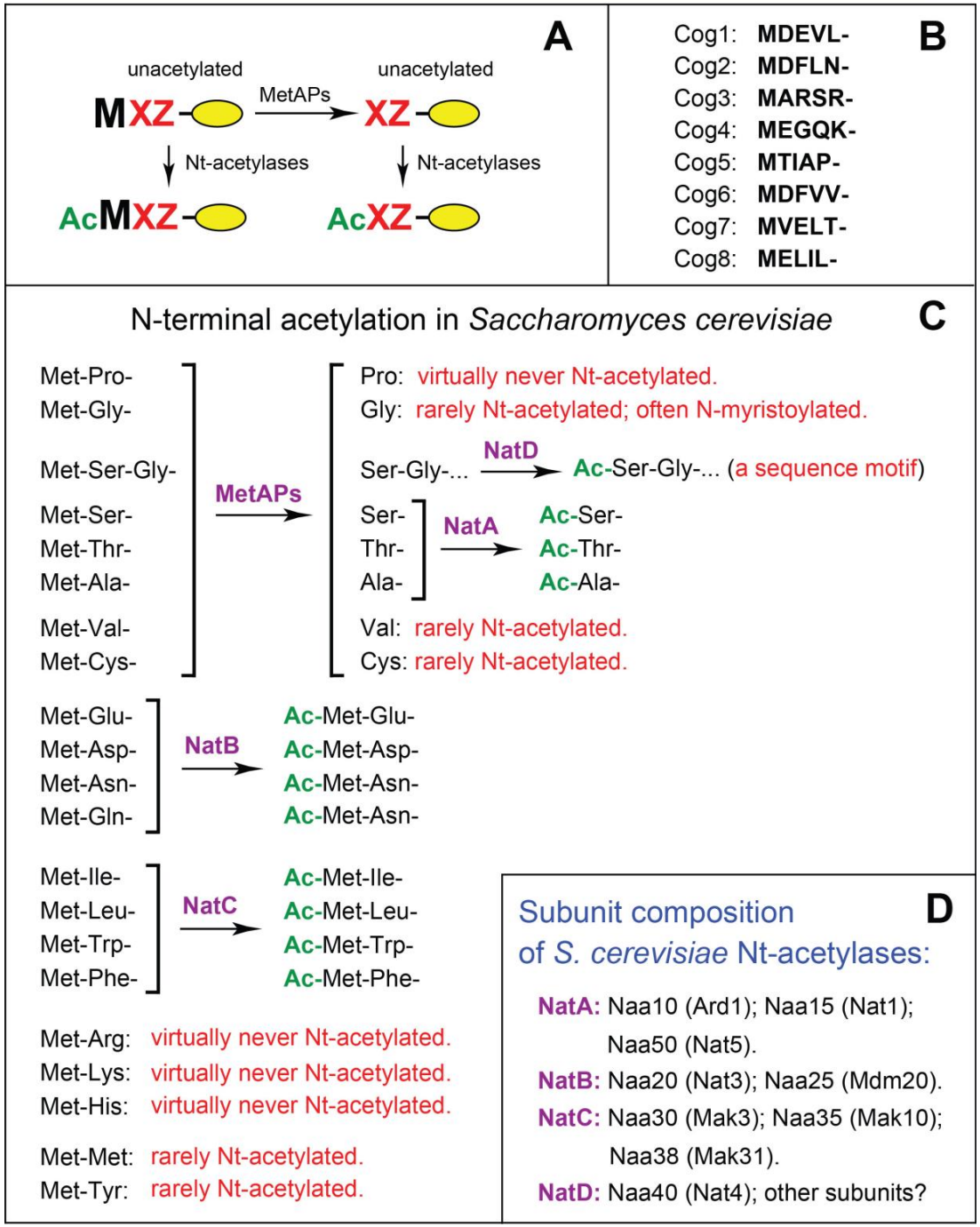


Fig. S2, Shemorry et al.

Figure S2. N-terminal Processing of Nascent Proteins, the N-termini of COG Subunits, and the Nα-Terminal Acetylation in S. cerevisiae.

(A) N-terminal processing of nascent cellular proteins by Nα-terminal acetylases (Nt-acetylases) and Met-aminopeptidases (MetAPs). "Ac" denotes the Nα-terminal acetyl moiety. M, Met. X and Z, single-letter abbreviations for any amino acid residue. Yellow ovals denote the rest of a protein.

(B) The first 5 encoded N-terminal residues of the Cog1-Cog8 subunits of the Conserved Oligomeric Golgi (COG) complex in S. cerevisiae (Miller and Ungar, 2012; Sztul and Lupashin, 2009). The Nt-acetylation status of the seven COG subunits (Cog2-Cog8) other than MD-Cog1wt remains to be determined. Save for Cog3, all of these subunits are candidates for Nt-acetylation in wild-type (wt) cells.

(C) Substrate specificities and subunit compositions of S. cerevisiae Nt-acetylases. This compilation is derived from data in the literature ((Arnesen et al., 2009; Helbig et al., 2010; Polevoda and Sherman, 2003; Starheim et al., 2012; Van Damme et al., 2012) and references therein). The present paper uses the revised nomenclature for specific subunits of Nt-acetylases (Polevoda et al., 2009) and cites the older names of these subunits in parentheses. Figure S2 refers to Figures 1 and 6.

Table S1: *S. cerevisiae* Strains Used in This Study

Strain	Relevant Genotype	Source
BY4742	*MATα his3-1 leu2-0 lys2-0 ura3-0 can1-100,*	Open Biosystems
BY10976	*ard1Δ::KanMX6 in* BY4742	Open Biosystems
BY15470	*mak3Δ::KanMX6 in* BY4742	Open Biosystems
BY15546	*nat3Δ::KanMX6 in* BY4742	Open Biosystems
BY17299	*doa10Δ:KanMX6 in* BY4742	Open Biosystems
BY4741	*MATa his3-1 leu2-0 met15-0 ura3-0*	Open Biosystems
BY4425	*rad6Δ:: KanMX4* in BY4741	Open Biosystems
BY14589	*cog1Δ::KanMX4* in BY4742	Open Biosystems
ASY101	COG1-3HA::HIS3MX6 in BY4742	This Study
ASY102	COG1-3HA::HIS3MX6 in BY17299	This Study
ASY103	COG1-3HA::HIS3MX6 in BY15546	This Study
ASY105	COG1-13MYC::HIS3MX6 in BY4742	This Study
YW05	*ubc1Δ:: HIS3* JD52	T. Sommers lab collection
BY4454	*mms2Δ::KanMX6* in BY4741	Open Biosystems
BY3994	*ubc5Δ::KanMX6* in BY4741	Open Biosystems
BY3219	*ubc4Δ::KanMX6* in BY4741	Open Biosystems
BBY67.3	*ubc6Δ::HIS3* in JD52	Varshavsky lab collection
BY597	*ubc7Δ::KanMX6* in BY4741	Open Biosystems
BY6577	*ubc8Δ:: KanMX6* in BY4741	Open Biosystems
BY4763	*ubc10Δ:: KanMX6* in BY4741	Open Biosystems
BY1636	*ubc11Δ:: KanMX6* in BY4741	Open Biosystems
BY5214	*ubc12Δ:: KanMX6* in BY4741	Open Biosystems
BY4027	*ubc13Δ:: KanMX6* in BY4741	Open Biosystems
AS104	*ubc6Δ::HIS3, ubc7Δ::KanMX6* in JD52	This Study
BY10568	*erg6Δ::KanMX6* in BY4742	Open Biosystems
RJD3268	*MATa, uba1::KANMX [pRS313 - UBA1-HIS], can1-100, leu2-3, -112, his3-11, -15, trp1-1, ura3-1, ade2-1*	(Ghaboosi and Deshaies, 2007)
RJD3269	*MATa, uba1*Δ*::KanMX* [*pRS313-uba1-204-HIS*], *can1-100, leu2-3, -112, his3-11, -15, trp1-1, ura3-1, ade2-1*	(Ghaboosi and Deshaies, 2007)
JD52	*MATa trp1- 63 ura3-52 his3- 200 leu2-3112. lys2-801*	(Hwang et al., 2010b)
CHY49	*pdr5Δ::KanMX6 in JD52*	(Dohmen et al., 1995)
CHY345	*ubr1Δ::LEU2* in BY4742	This Study
CHY346	*ubr1Δ::LEU2 doa10Δ::KANMX6* in BY4742	This Study
AS106	*not4Δ::HIS3MX6* in BY4742	This Study
AS107	*not4Δ::HIS3MX6 doa10Δ::KANMX6* in BY4742	This Study
AS108	*not4Δ::HIS3MX6 ubr1Δ::LEU2* in BY4742	This Study

Table S2: Plasmids Used in This Study

Plasmid	Description	Source
p316Cup1	pRS316 with P_{CUP1} promoter	This study
p313Cup1	pRS313 with P_{CUP1} promoter	This study
pAS101	Cog1-3flag in p316CUP1	This study
pAS117	MPCog1-3flag in p316Cup1	This study
pAS102	Cog2-3flag in p316CUP1	This study
pAS103	Cog1-3flag in p313CUP1	This study
pAS104	Cog1-3HA in p316CUP1	This study
pAS105	Cog1-HA in p316CUP1	This study
pAS106	Cog3-HA in p316CUP1	This study
pAS107	Cog4-HA in p316CUP1	This study
pAS108	Cog5-HA in p316CUP1	This study
pAS109	Cog6-HA in p316CUP1	This study
pAS110	Cog8-HA in p316CUP1	This study
pAS111	Cog1-3flag in p425GAL1	This study
p413MET25	pRS413 with P_{MET25} promoter	(Mumberg et al., 1994)
pAS112	Hcn1-3flag in p423CUP1	This study
pAS113	Hcn1-3flag in p413MET25	This study
p425Gal1	pRS425 with P_{GAL1} promoter	(Mumberg et al., 1994)
pAS114	Cut9-3flag in p425GAL1	This study
pAS115	Cog2-3flag, Cog3-HA in p423GAL1,10	This study
pAS116	Cog4-HA in p425GAL1,10	This study
pAS118	Cog1-3flag in YEPlac181 with pAdh1	This study
YEPlac181	2μ LEU2 plasmid	Varshavsky lab collection

Figure 1

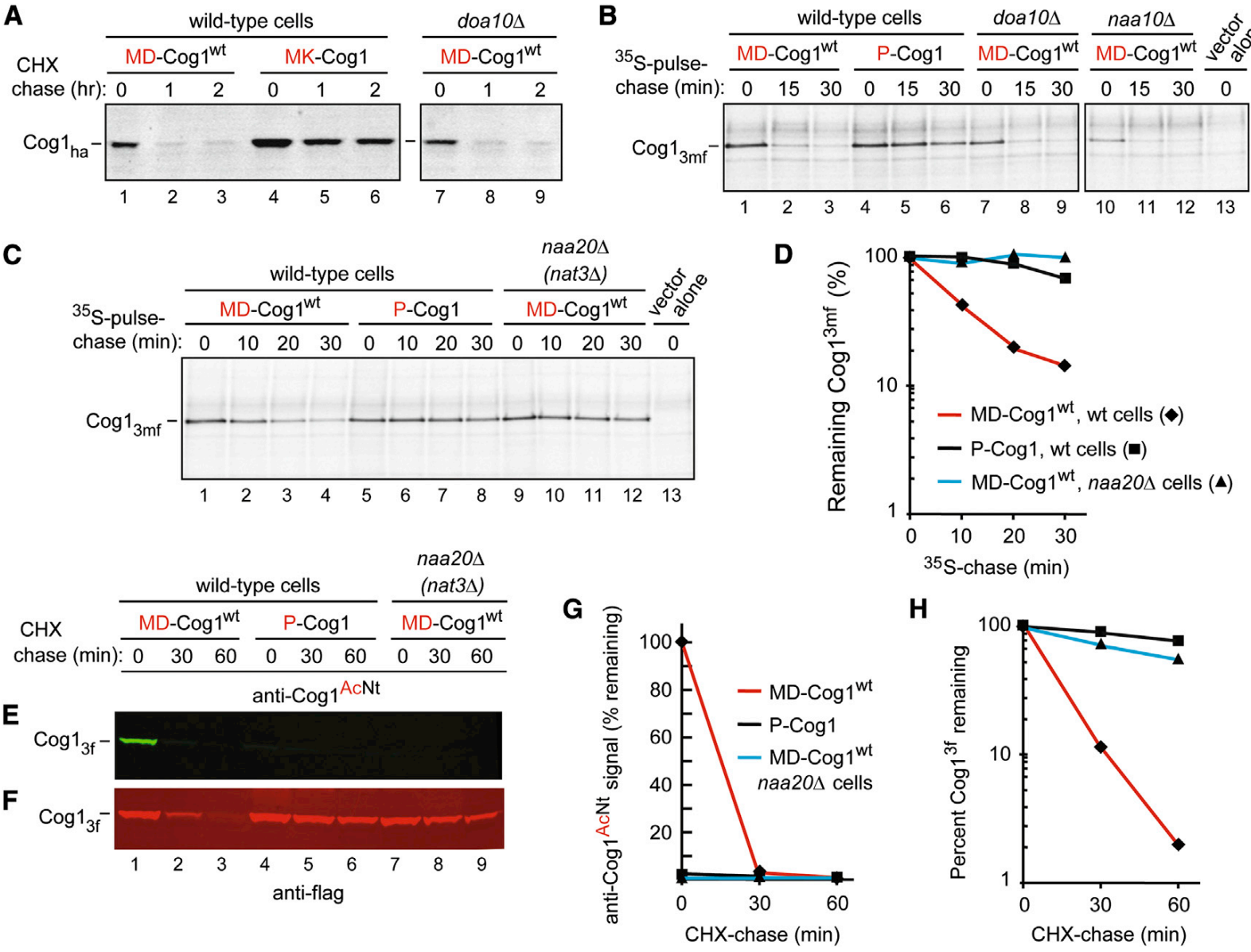

A
wild-type cells
doa10Δ
CHX
MD-Cog1wt
MK-Cog1
MD-Cog1wt
chase (hr): 0 1 2 0 1 2 0 1 2
Cog1ha
1 2 3 4 5 6 7 8 9
B
wild-type cells
doa10Δ
naa10Δ
35S-pulse-
MD-Cog1wt
P-Cog1
MD-Cog1wt
MD-Cog1wt
vector alone
chase (min): 0 15 30 0 15 30 0 15 30 0 15 30 0
Cog13mf
1 2 3 4 5 6 7 8 9 10 11 12 13
C
wild-type cells
naa20Δ (nat3Δ)
35S-pulse-
MD-Cog1wt
P-Cog1
MD-Cog1wt
vector alone
chase (min): 0 10 20 30 0 10 20 30 0 10 20 30 0
Cog13mf
1 2 3 4 5 6 7 8 9 10 11 12 13
D
Remaining Cog13mf (%)
100
10
1
MD-Cog1wt, wt cells (◆)
P-Cog1, wt cells (■)
MD-Cog1wt, naa20Δ cells (▲)
0 10 20 30
35S-chase (min)
wild-type cells
naa20Δ (nat3Δ)
CHX
MD-Cog1wt
P-Cog1
MD-Cog1wt
chase (min): 0 30 60 0 30 60 0 30 60
anti-Cog1AcNt
E
Cog13f
F
Cog13f
1 2 3 4 5 6 7 8 9
anti-flag
G
anti-Cog1AcNt signal (% remaining)
100
80
60
40
20
MD-Cog1wt
P-Cog1
MD-Cog1wt
naa20Δ cells
0 30 60
CHX-chase (min)
H
Percent Cog13f remaining
100
10
1
0 30 60
CHX-chase (min)

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 noncognate (for MD-Cog1^{wt}) NatA Nt-acetylase (Figure S2). Cog1 proteins were C-terminally tagged with three 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 Figures S4A–S4C) was used for immunoblotting in CHX-chase assays with MD-Cog1^{wt} and P-Cog1 (C-terminally tagged with three 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 semilog plot of the Flag-specific Cog1 immunoblotting patterns in (F). Same designations as in (G). See also Figure S1, Figure S2, Figure S5, Table S1, and Table S2.

Figure 2

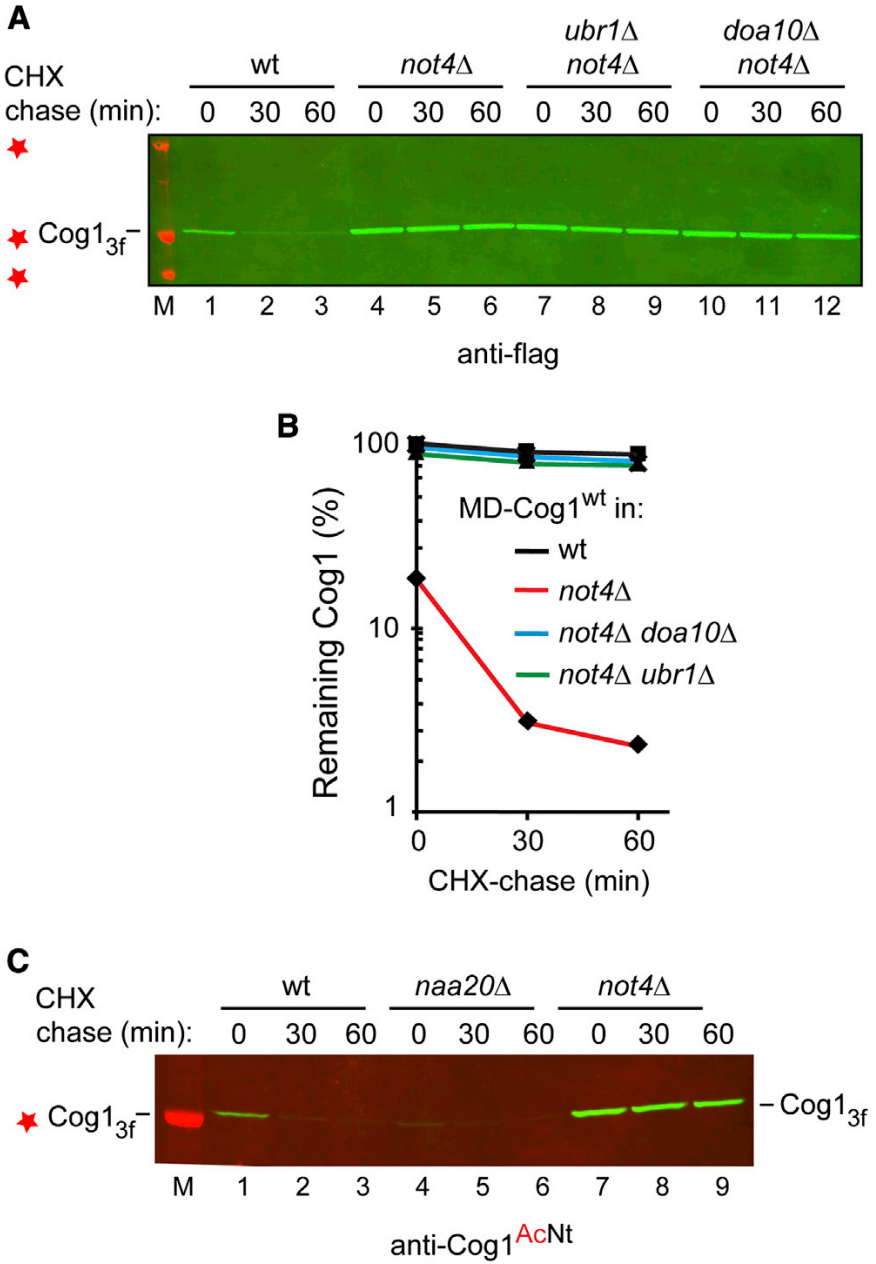

A
CHX
chase (min):
wt
not4Δ
ubr1Δ not4Δ
doa10Δ not4Δ
0 30 60 0 30 60 0 30 60 0 30 60
Cog1 3f
M 1 2 3 4 5 6 7 8 9 10 11 12
anti-flag
B
Remaining Cog1 (%)
100
10
1
MD-Cog1 wt in:
wt
not4Δ
not4Δ doa10Δ
not4Δ ubr1Δ
0 30 60
CHX-chase (min)
C
CHX
chase (min):
wt
naa20Δ
not4Δ
0 30 60 0 30 60 0 30 60
Cog1 3f
Cog1 3f
M 1 2 3 4 5 6 7 8 9
anti-Cog1 AcNt

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

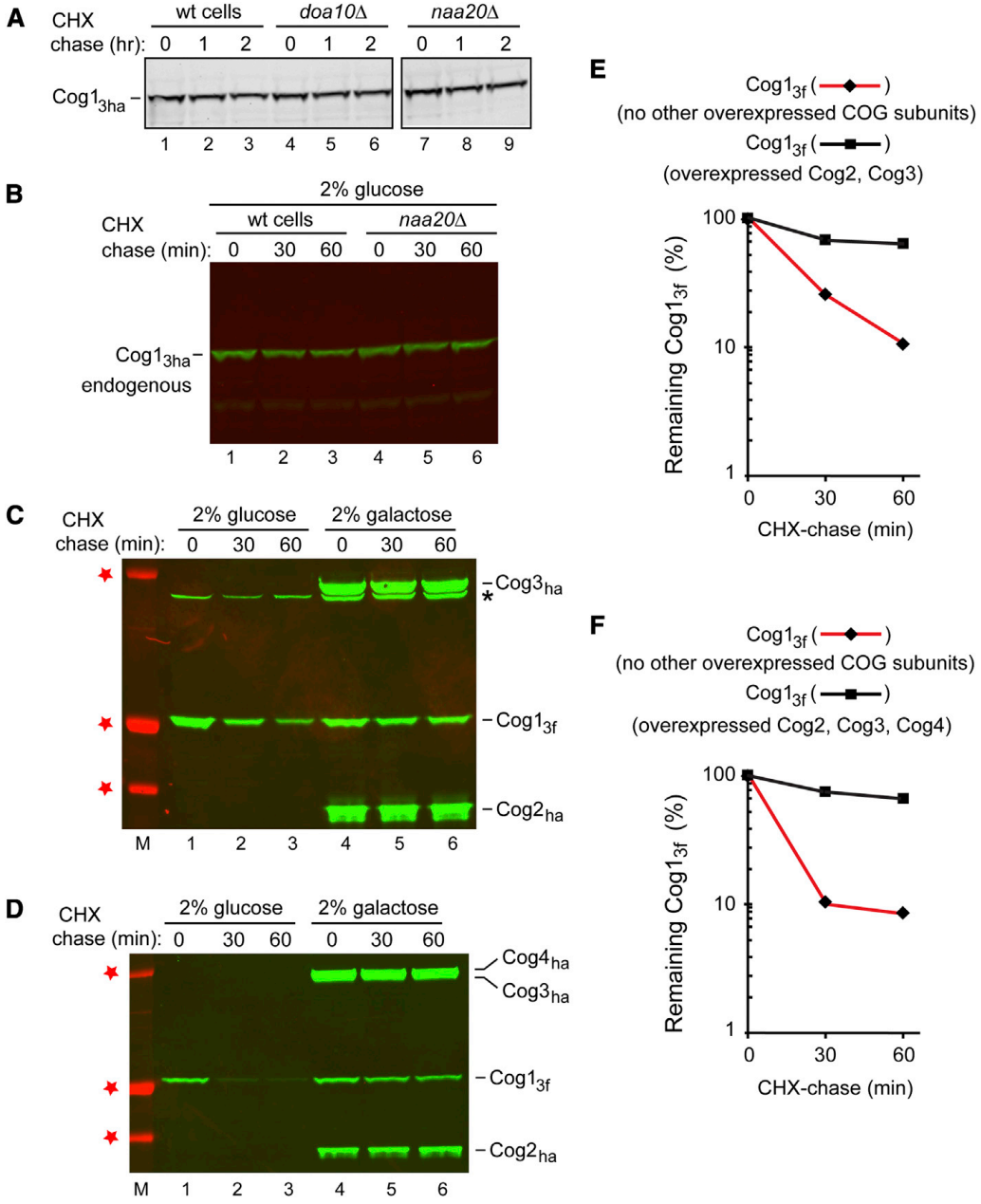

A
CHX
chase (hr): 0 1 2 0 1 2 0 1 2
wt cells
doa10Δ
naa20Δ
Cog1 3ha
1 2 3 4 5 6 7 8 9
B
2% glucose
CHX
chase (min): 0 30 60 0 30 60
wt cells
naa20Δ
Cog1 3ha
endogenous
1 2 3 4 5 6
C
CHX
chase (min): 0 30 60 0 30 60
2% glucose
2% galactose
Cog3 ha
*
Cog1 3f
Cog2 ha
M 1 2 3 4 5 6
D
CHX
chase (min): 0 30 60 0 30 60
2% glucose
2% galactose
Cog4 ha
Cog3 ha
Cog1 3f
Cog2 ha
M 1 2 3 4 5 6
E
Cog1 3f (◆—)
(no other overexpressed COG subunits)
Cog1 3f (■—)
(overexpressed Cog2, Cog3)
Remaining Cog1 3f (%)
100
10
1
0 30 60
CHX-chase (min)
F
Cog1 3f (◆—)
(no other overexpressed COG subunits)
Cog1 3f (■—)
(overexpressed Cog2, Cog3, Cog4)
Remaining Cog1 3f (%)
100
10
1
0 30 60
CHX-chase (min)

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 three 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 three 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.

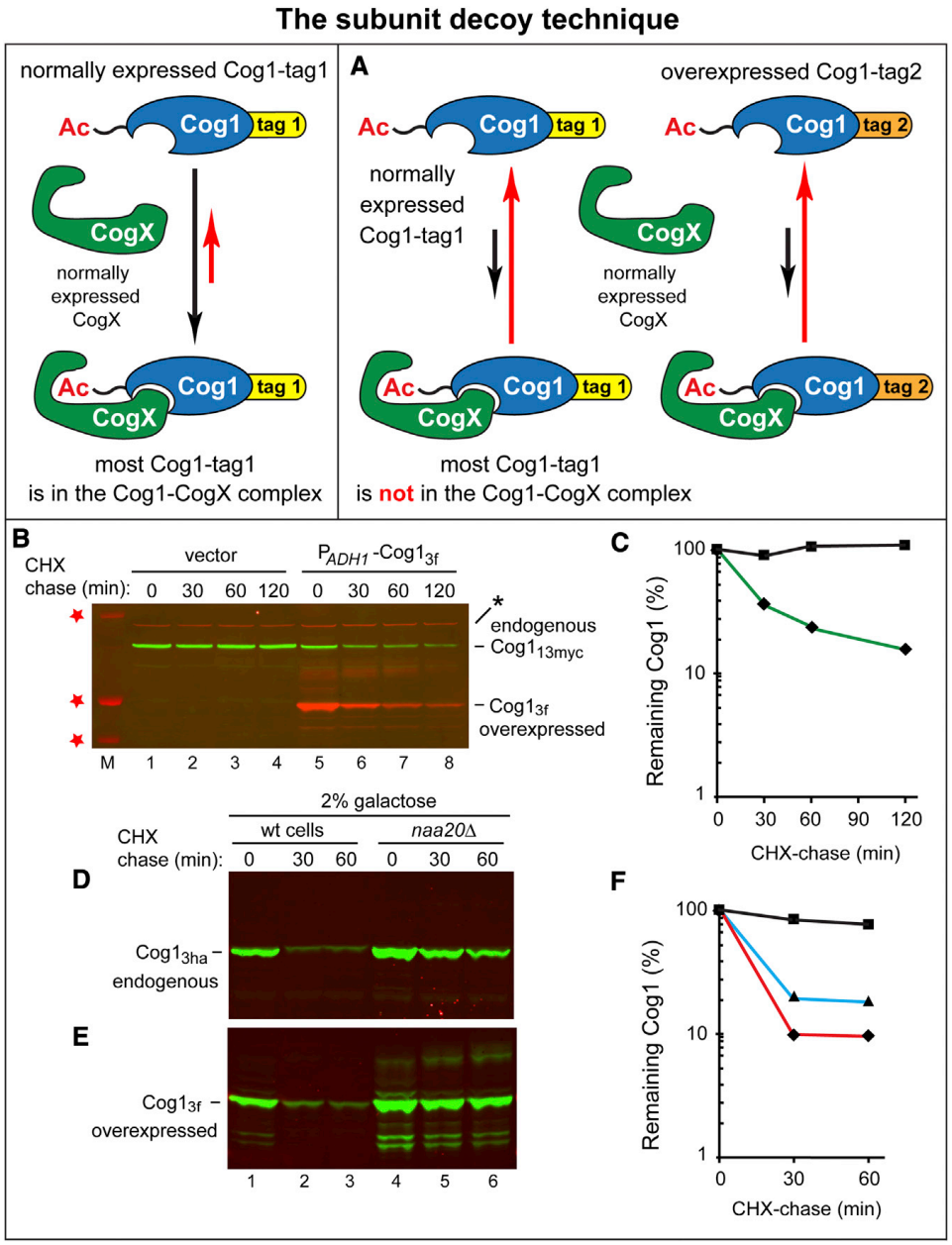


Figure 4. Subunit Decoy Technique and the Cause of Stability of Endogenous Cog1

(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 three 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 cross-reacting 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) and (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 Figure S4 and Figure S5.

Figure 4

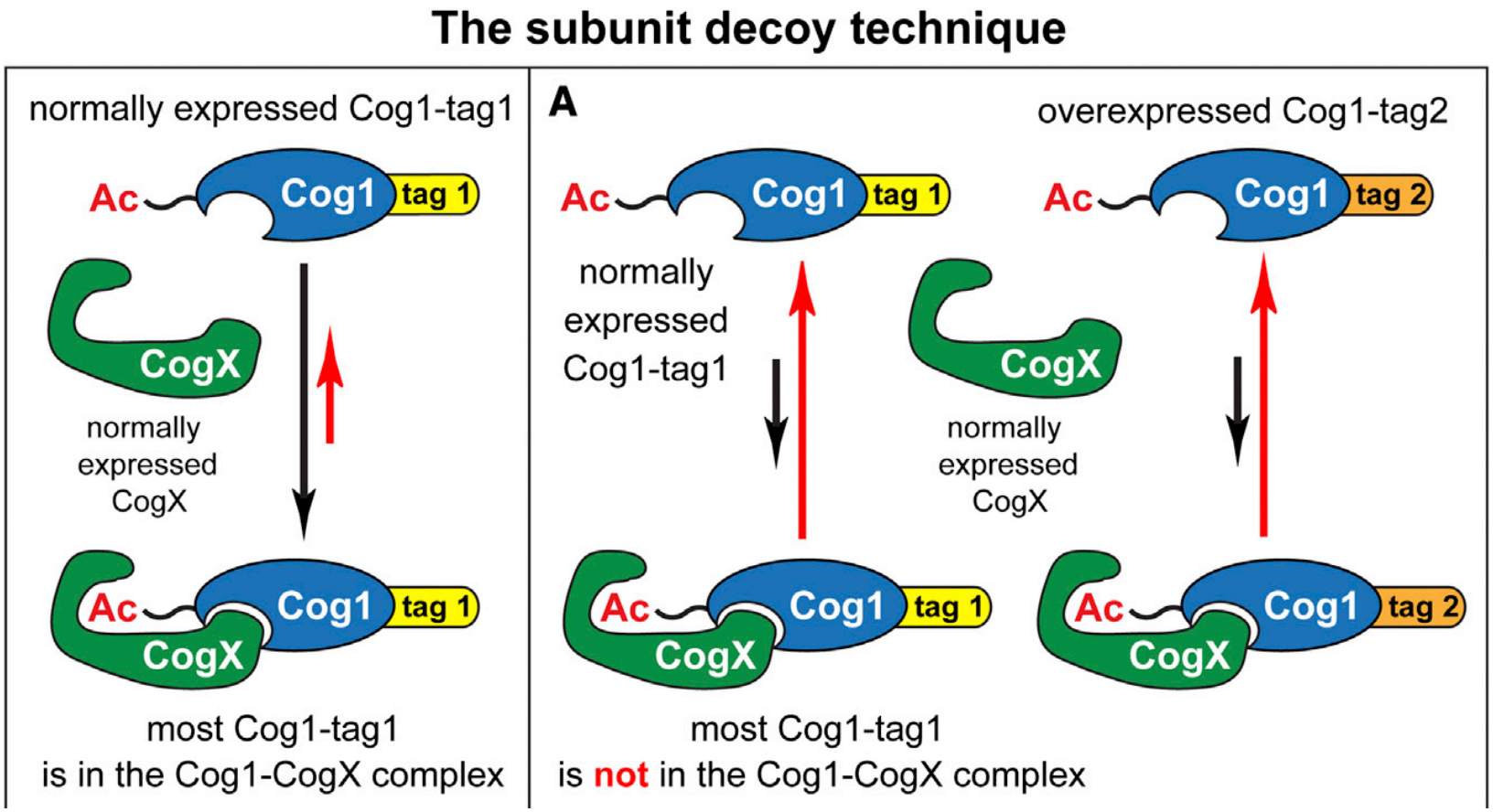

The subunit decoy technique
normally expressed Cog1-tag1
A
overexpressed Cog1-tag2
Ac
Cog1
tag 1
tag 2
CogX
normally expressed CogX
most Cog1-tag1 is in the Cog1-CogX complex
most Cog1-tag1 is not in the Cog1-CogX complex

Figure 4

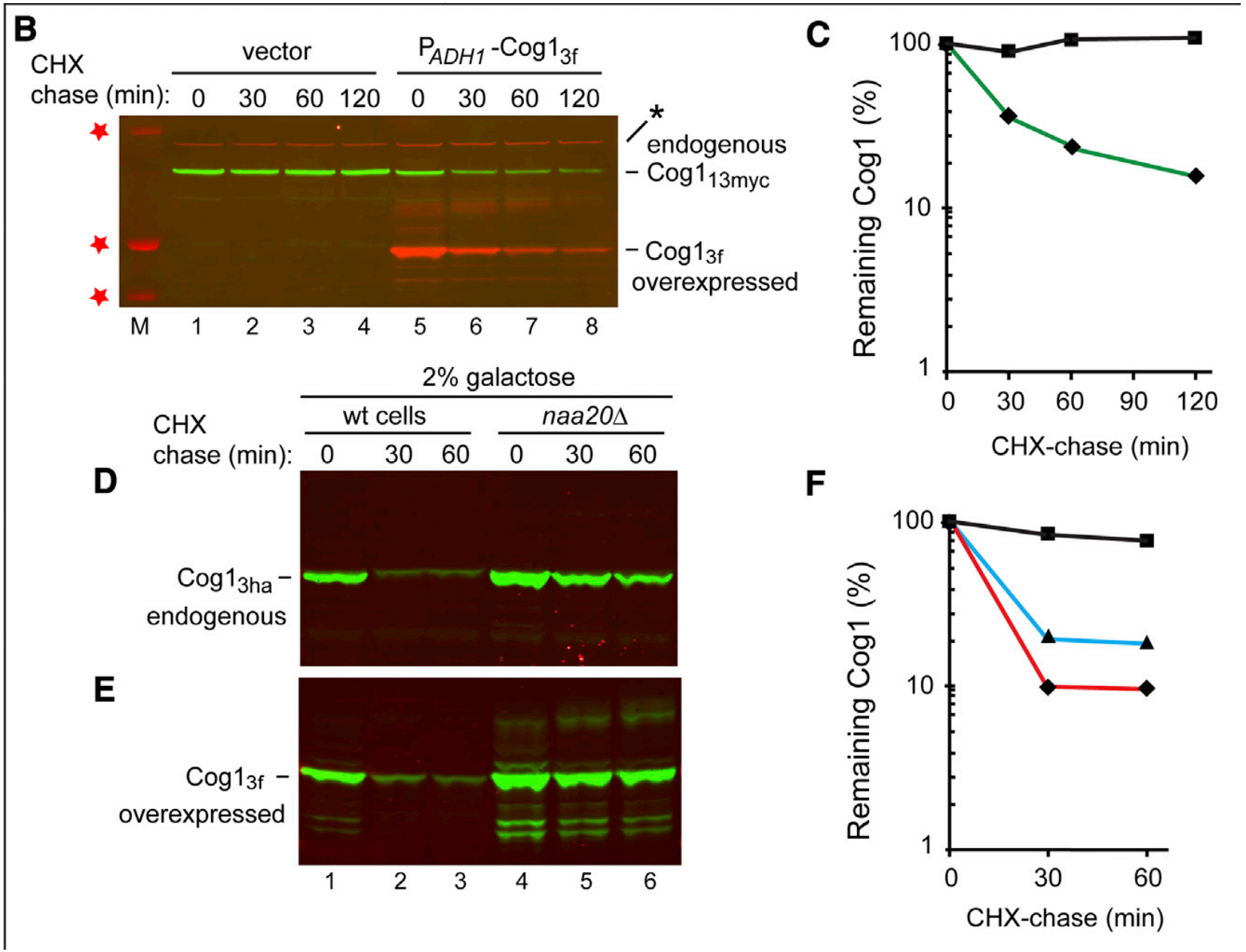

B
CHX
chase (min):
vector
P_{ADH1}-$Cog1_{3f}$
0 30 60 120 0 30 60 120
*
endogenous
– $Cog1_{13myc}$
– $Cog1_{3f}$
overexpressed
M 1 2 3 4 5 6 7 8
C
Remaining Cog1 (%)
100
10
1
0 30 60 90 120
CHX-chase (min)
2% galactose
CHX
chase (min):
wt cells
naa20Δ
0 30 60 0 30 60
D
$Cog1_{3ha}$ –
endogenous
E
$Cog1_{3f}$ –
overexpressed
1 2 3 4 5 6
F
Remaining Cog1 (%)
100
10
1
0 30 60
CHX-chase (min)

Figure 5

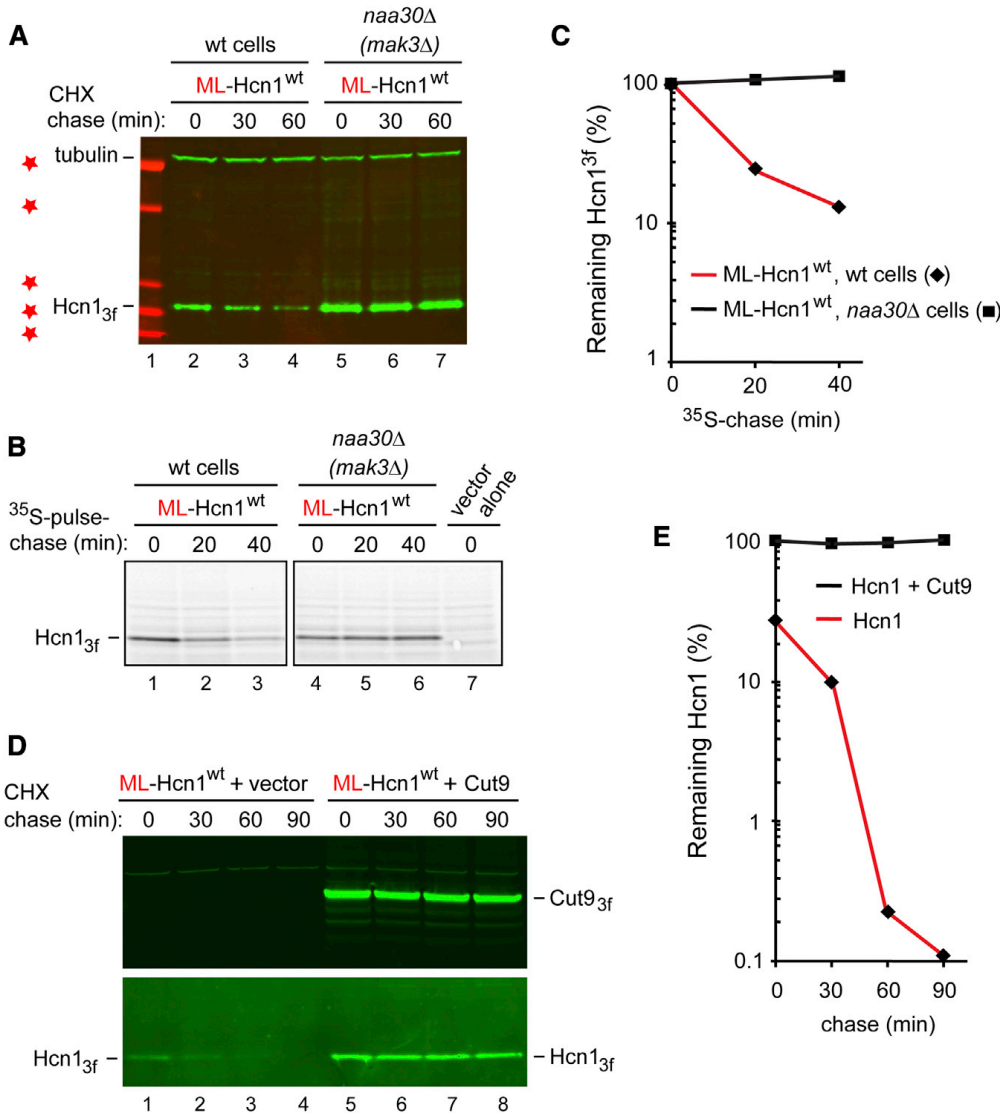


Figure 5. Hcn1 and Repression of Its Ac/N-Degron by Cut9

(A) CHX-chases in WT or *naa30Δ* (*mak3Δ*) *S. cerevisiae* expressing the WT *S. pombe* Hcn1, termed ML-Hcn1^{wt}, C-terminally tagged with three 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 three 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 coexpressed Cut9. ■, ML-Hcn1^{wt} in the presence of coexpressed Cut9.

See also Figure S1.

Figure 5

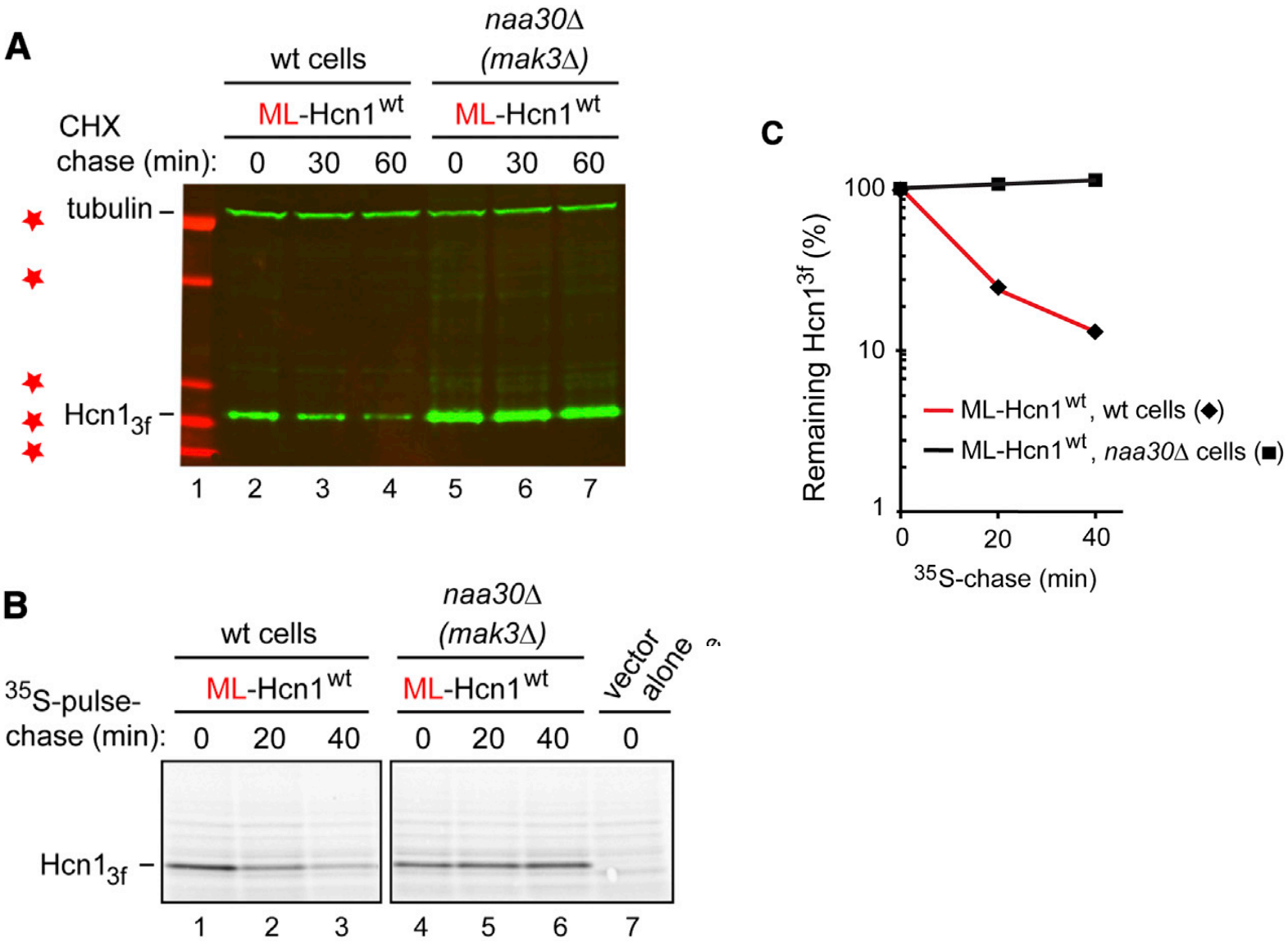

A
wt cells
naa30Δ (mak3Δ)
ML-Hcn1wt
ML-Hcn1wt
CHX chase (min):
0 30 60 0 30 60
tubulin –
Hcn1 3f –
1 2 3 4 5 6 7
B
wt cells
naa30Δ (mak3Δ)
ML-Hcn1wt
ML-Hcn1wt
vector alone
35S-pulse-chase (min):
0 20 40 0 20 40 0
Hcn1 3f –
1 2 3 4 5 6 7
C
Remaining Hcn1 3f (%)
100
10
1
0 20 40
35S-chase (min)
ML-Hcn1wt, wt cells (◆)
ML-Hcn1wt, naa30Δ cells (■)

Figure 5

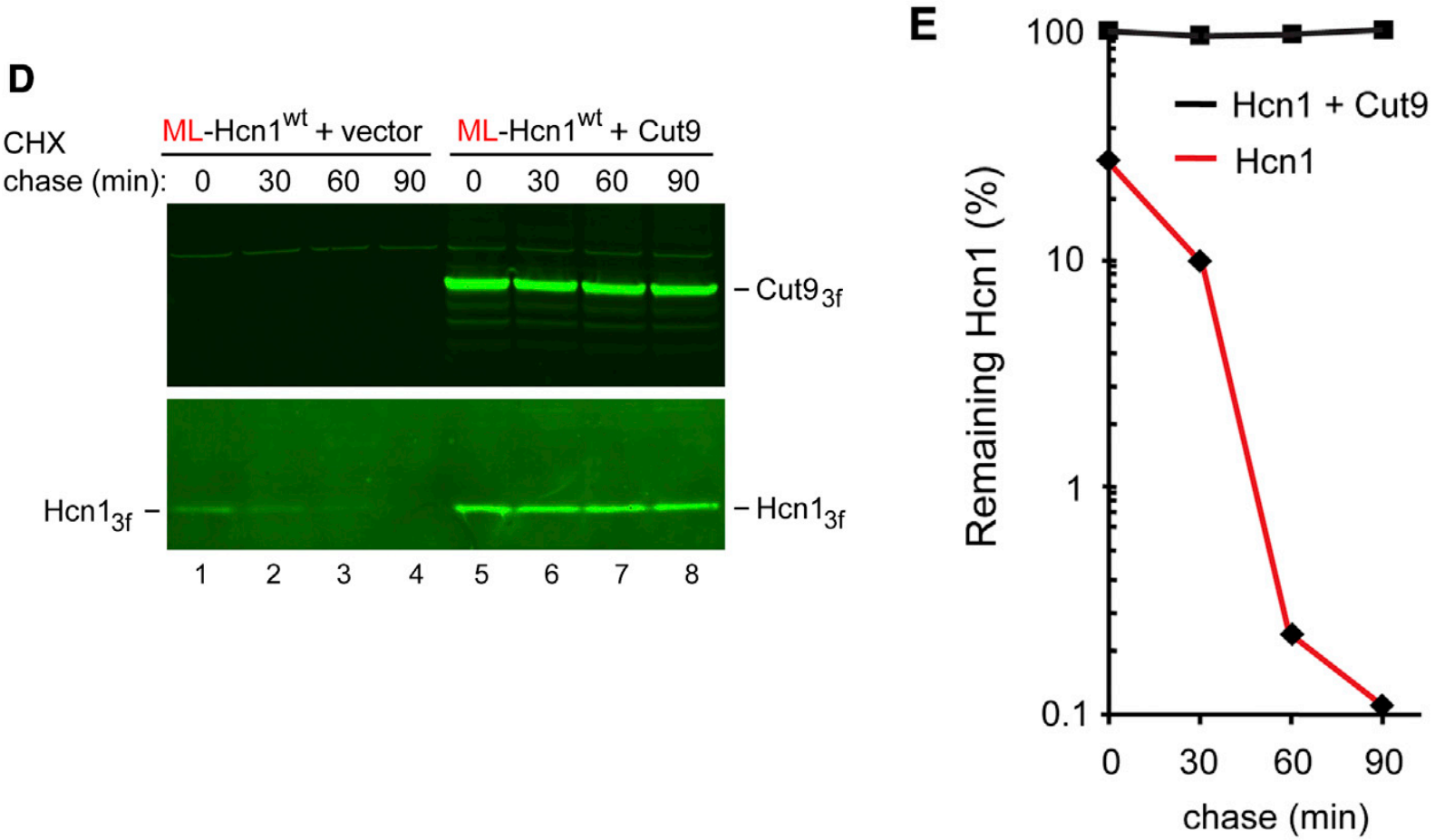

D
CHX
chase (min): 0 30 60 90 0 30 60 90
ML-Hcn1wt + vector
ML-Hcn1wt + Cut9
– Cut93f
Hcn13f –
– Hcn13f
1 2 3 4 5 6 7 8
E
100
10
1
0.1
Remaining Hcn1 (%)
Hcn1 + Cut9
Hcn1
0 30 60 90
chase (min)

Figure 6

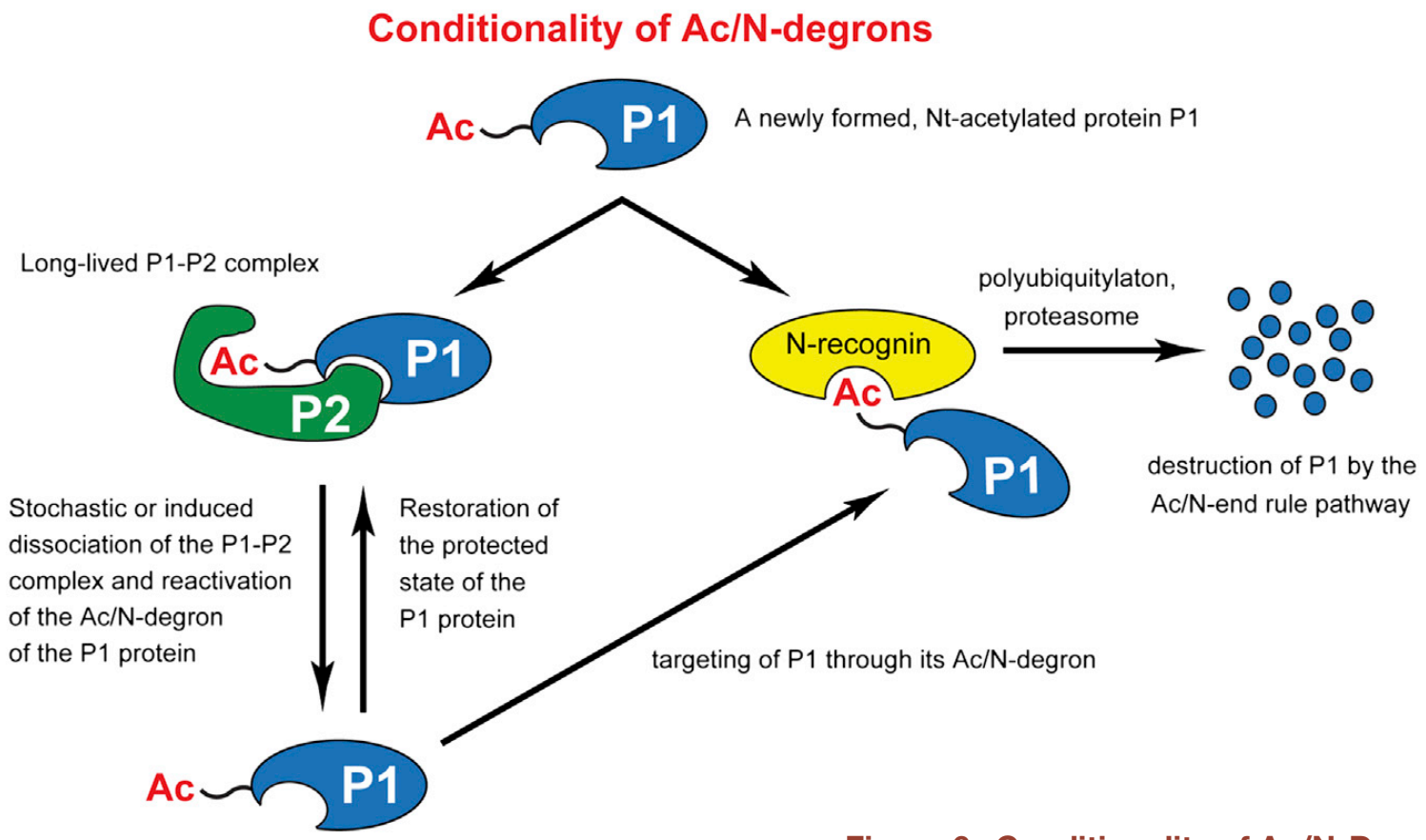


Figure 6. Conditionality of Ac/N-Degrons

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.

Figure S3

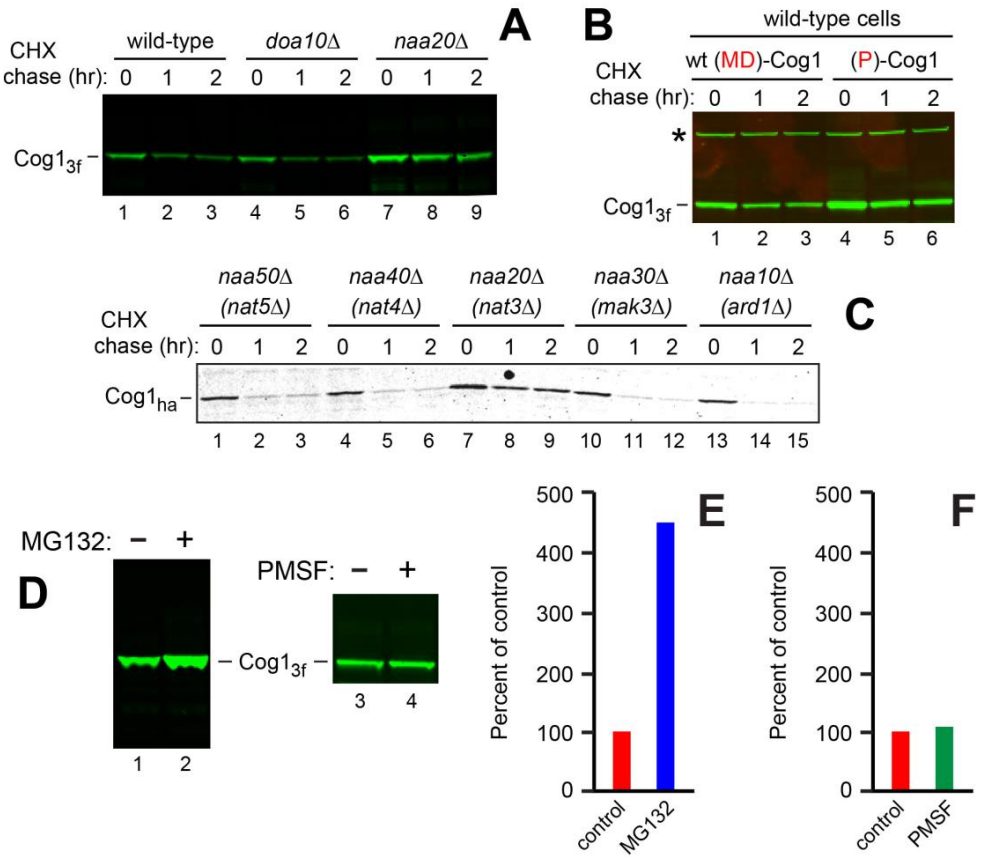


Figure S3, Shemorry et al.

Figure S3

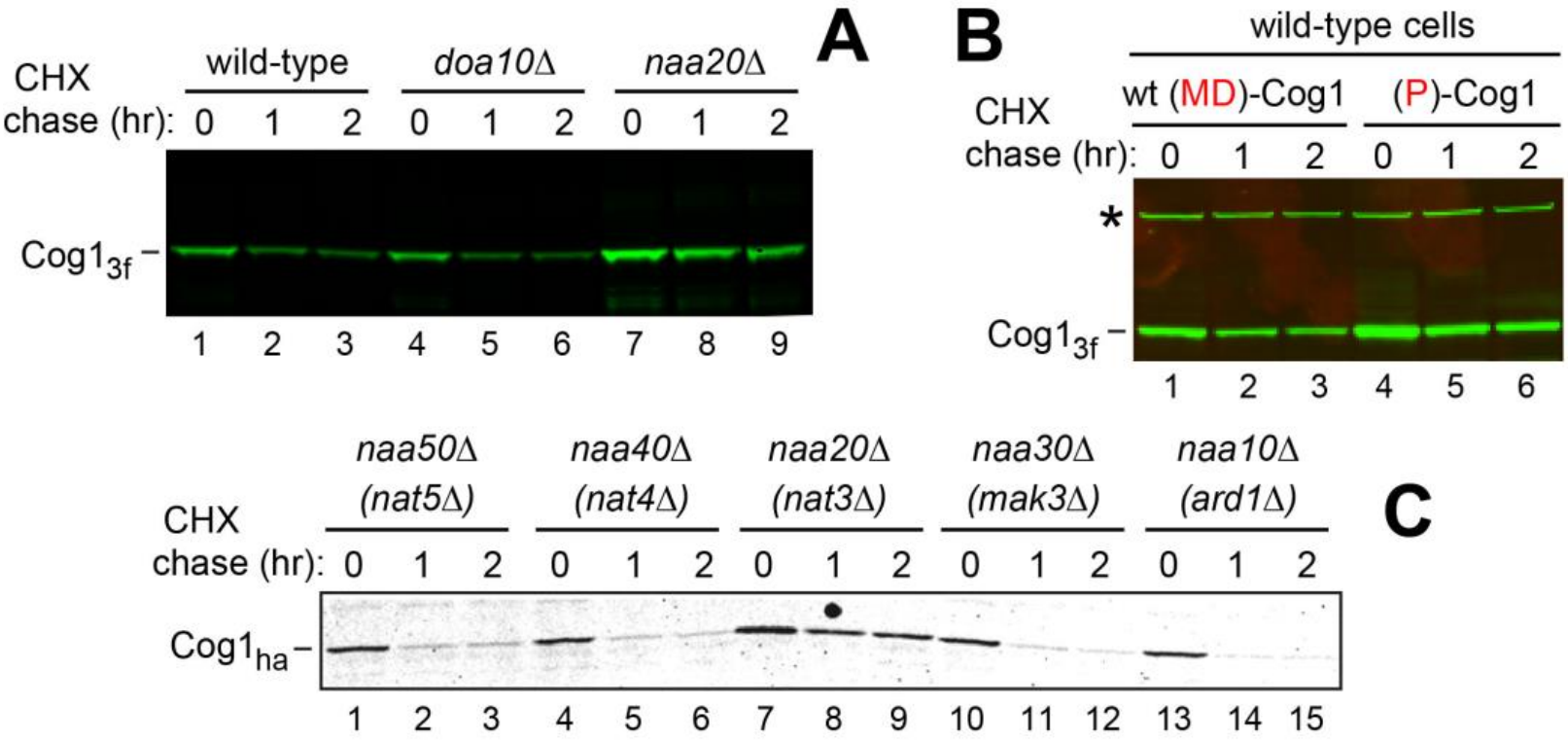

A
CHX
chase (hr): 0 1 2 0 1 2 0 1 2
wild-type
doa10Δ
naa20Δ
Cog1 3f
1 2 3 4 5 6 7 8 9
B
wild-type cells
wt (MD)-Cog1
(P)-Cog1
CHX
chase (hr): 0 1 2 0 1 2
*
Cog1 3f
1 2 3 4 5 6
C
naa50Δ (nat5Δ)
naa40Δ (nat4Δ)
naa20Δ (nat3Δ)
naa30Δ (mak3Δ)
naa10Δ (ard1Δ)
CHX
chase (hr): 0 1 2 0 1 2 0 1 2 0 1 2 0 1 2
Cog1 ha
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure S3

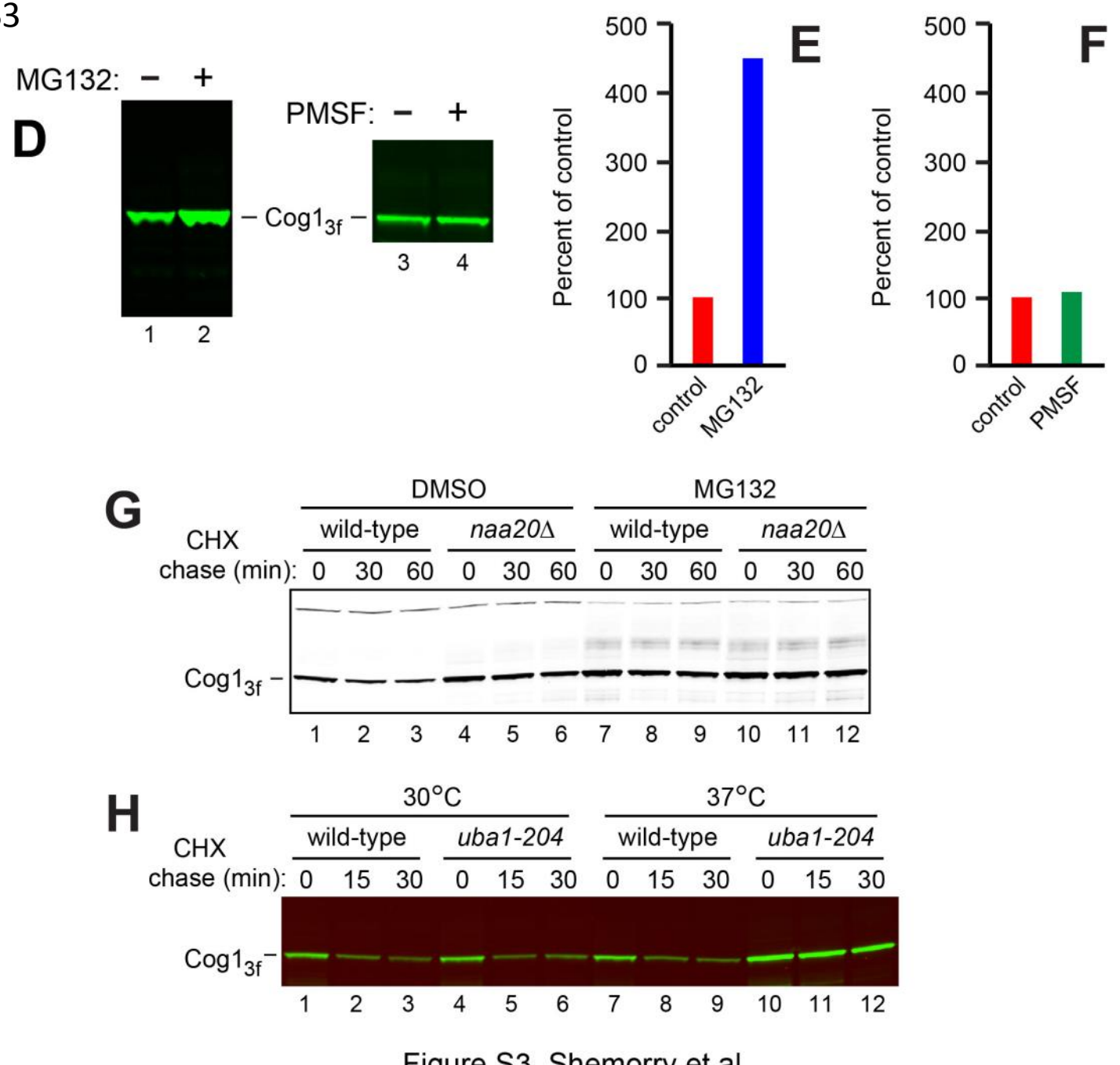


Figure S3, Shemorry et al.

Figure S3:

Figure S3. Degradation of Cog1 by the Ac/N-End Rule Pathway.

(A) Cycloheximide (CHX)-chases (related to the ones described in Figure 1) were carried out at 30°C with wt (lanes 1-3), doa10Δ (lanes 4-6) and naa20Δ (nat3Δ) (lanes 7-9) S. cerevisiae strains that expressed wt Cog1, termed MD-Cog1wt, which was C-terminally tagged with three flag epitopes. At the indicated times of chase, proteins in cell extracts were fractionated by SDS-PAGE and assayed by immunoblotting with anti-Flag antibody.

(B) CHX-chases as in A, with wt cells that expressed MD-Cog1wt (lanes 1-3) or its non-Nt-acetylatable P-Cog1 mutant (lane 4-6), both of which were C-terminally tagged with three flag epitopes. See the main text for descriptions of P-Cog1. Asterisk on the left denotes a crossreacting protein.

(C) CHX-chases as in A, with MD-Cog1wt C-terminally ha-tagged and examined in naa50Δ (nat5Δ) (lanes 1-3), naa40Δ (nat4Δ) (lanes 4-6), naa20Δ (nat3Δ) (lanes 7-9), naa30Δ (mak3Δ) (lanes 10-12), and naa10Δ (ard1Δ) (lanes 13-15) S. cerevisiae strains. Each of these strains lacked the activity of a specific Nt-acetylase (see Figure S2C), including the cognate (for MD-Cog1wt) NatB Nt-acetylase (lanes 7-9).

(D) Left panel: Expression of MD-Cog1wt C-terminally tagged with three flag epitopes in pdr5Δ S. cerevisiae. Cells were incubated for 1 hr in SD medium containing either 0.5% dimethylsulfoxide (DMSO) (the solvent for a stock solution of the proteasome inhibitor MG132) (lane 1), or both 50 μM MG132 and 0.5% DMSO (lane 2). The incubation was followed by preparation of extracts, SDS-PAGE and immunoblotting with anti-Flag antibody. Right panel: same procedures as in experiments of the left panel but with erg6Δ S. cerevisiae incubated in SD containing either 1% isopropanol (the solvent for a stock solution of phenylmethylsulfonyl fluoride (PMSF), an inhibitor of serine proteases) (lane 1), or both 1 mM PMSF and 1% isopropanol
(lane 2).
(E) Quantification of data in D, left panel.
(F) Quantification of data in D, right panel.
(G) CHX-chases with wt (lanes 1-3, 7-9) or naa20Δ (nat3Δ) (lanes 4-6, 10-12) S. cerevisiae strains expressing MD-Cog1wt C-terminally tagged with three flag epitopes. Cells were grown for 3 hrs in SD medium containing 0.003% SDS (to allow for the entry of MG132) and either 0.5% DMSO (control, lanes 1-6) or both 50 μM MG132 and 0.5% DMSO. Note the metabolic stabilization of MD-Cog1wt in wild-type cells by MG132 (lanes 1-3 vs. lanes 7-9) and the metabolic stabilization of MD-Cog1wt in naa20Δ cells (lacking the NatB Nt-acetylase) irrespective of the absence or presence of MG132 (lanes 4-6 vs. lanes 10-12).
(H) CHX-chases with either wt or uba1-204 S. cerevisiae (the latter containing a temperature-sensitive mutant of the Ub-activating (E1) enzyme (Ghaboosi and Deshaies, 2007)) expressing MD-Cog1wt C-terminally tagged with three flag epitopes. Lanes 1-3, wt cells at 30°C. Lanes 4-5, uba1-204 cells at 30°C. Lanes 7-8, same as in lanes 1-3 but at 37°C (nonpermissive temperature for uba1-204 cells). Lanes 10-12, same as lanes 4-6 but at 37°C. Note the metabolic stabilization of MD-Cog1wt in uba1-204 cells at 37°C (lanes 10-12). Figure S3 refers to Figure 1.

Figure S4

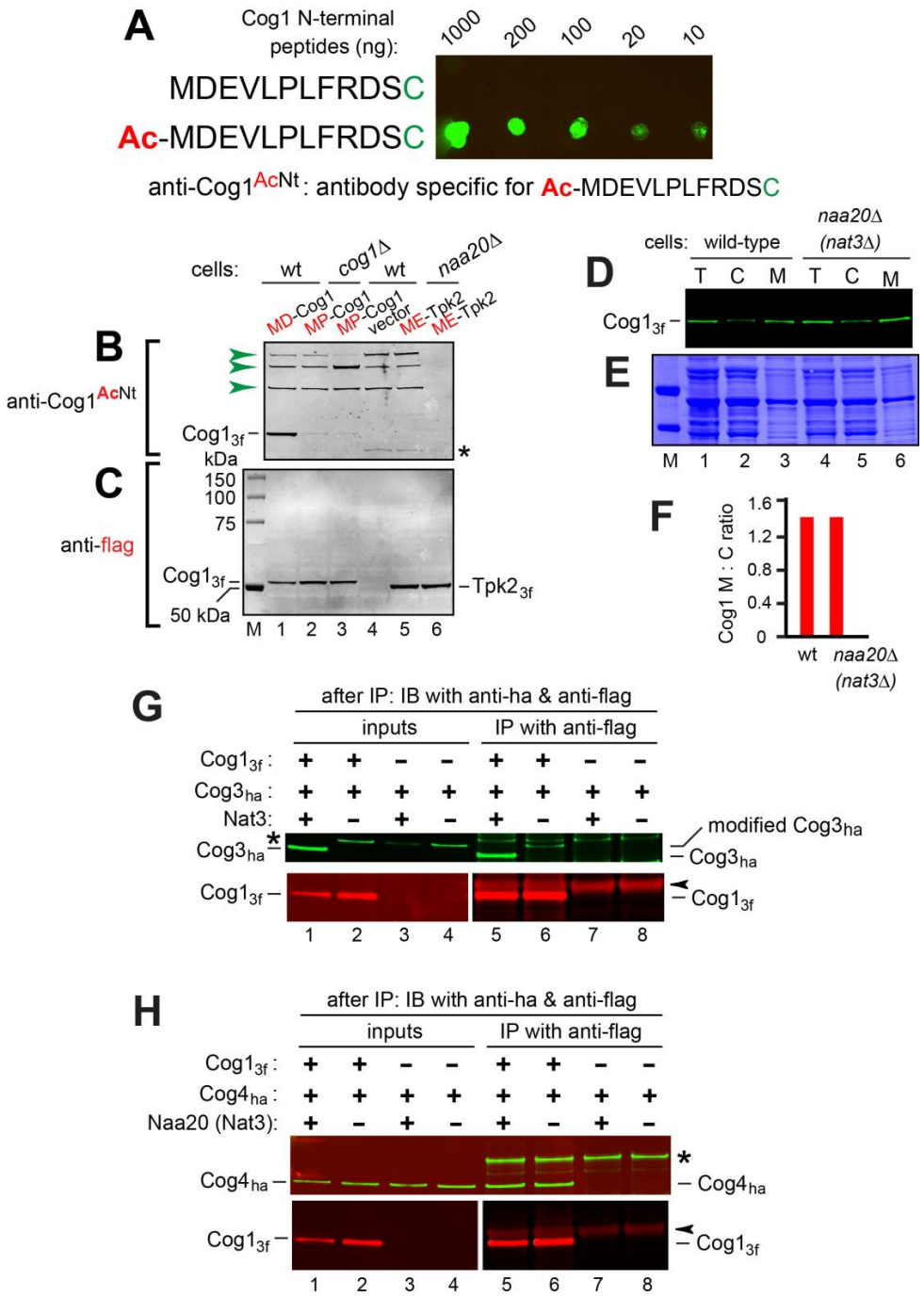


Figure S4, Shemorry et al.

Figure S4

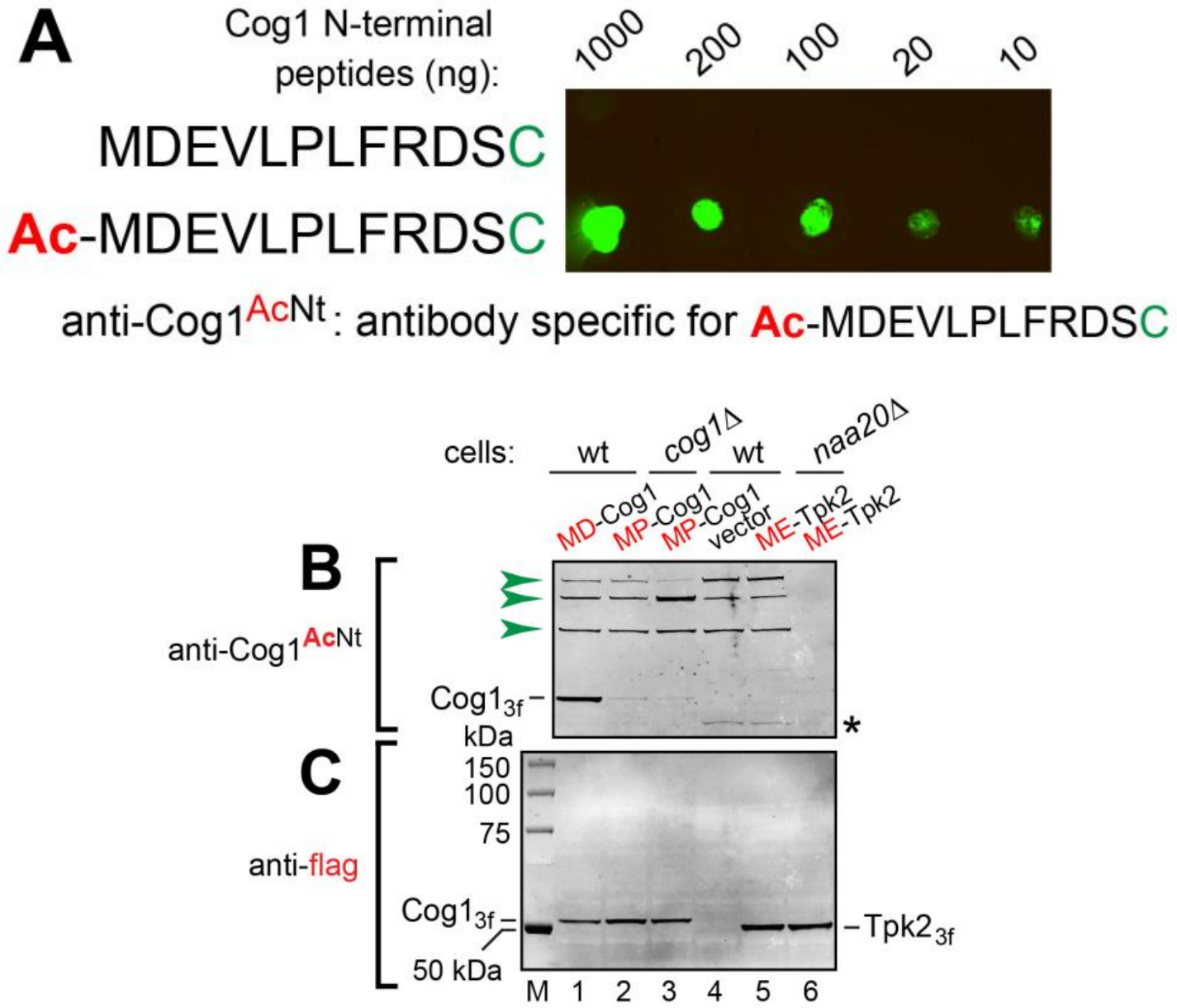

A
Cog1 N-terminal peptides (ng):
1000
200
100
20
10
MDEVLPLFRDSC
Ac-MDEVLPLFRDSC
anti-Cog1AcNt: antibody specific for Ac-MDEVLPLFRDSC
cells:
wt
cog1Δ
wt
naa20Δ
MD-Cog1
MP-Cog1
MP-Cog1
vector
ME-Tpk2
ME-Tpk2
B
anti-Cog1AcNt
Cog13f
kDa
*
C
150
100
75
anti-flag
Cog13f
50 kDa
Tpk23f
M 1 2 3 4 5 6

Figure S4

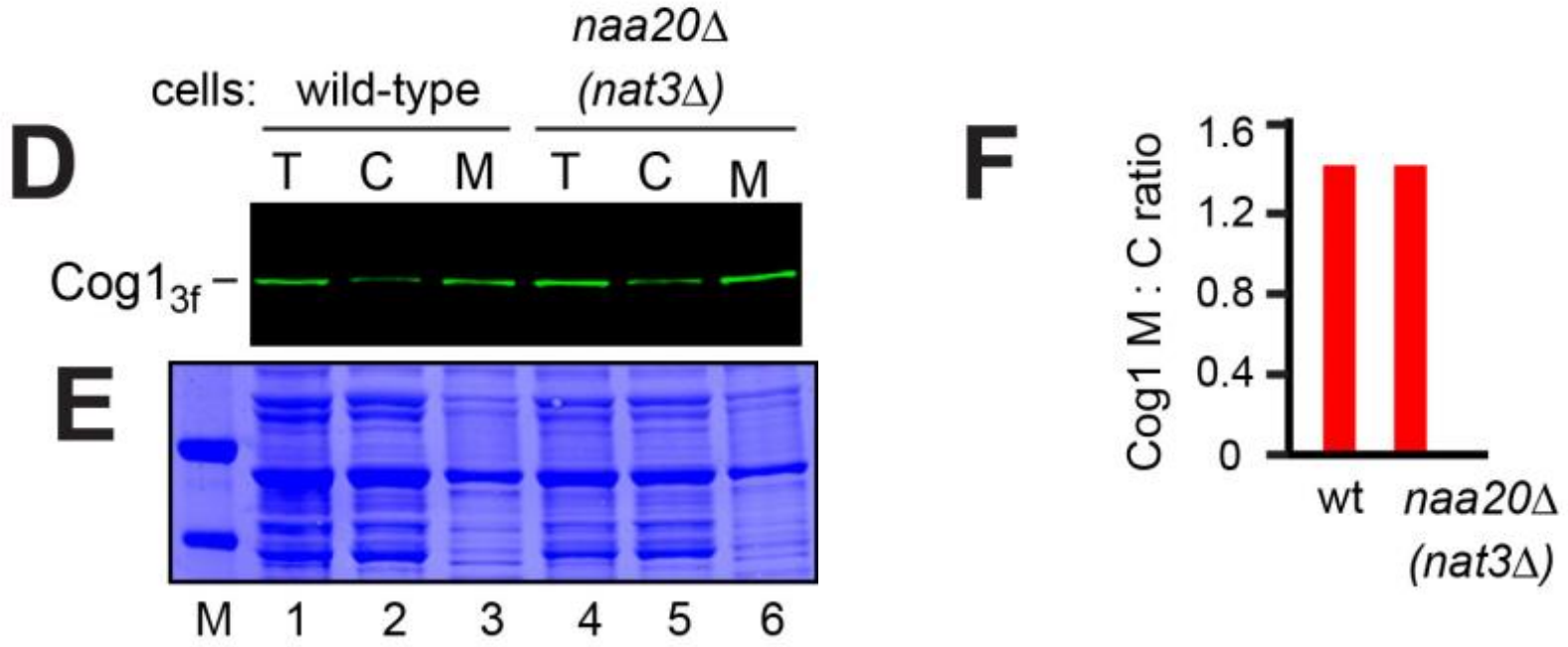

D
cells: wild-type
naa20Δ (nat3Δ)
T C M T C M
Cog1 3f
E
M 1 2 3 4 5 6
F
Cog1 M : C ratio
1.6
1.2
0.8
0.4
0
wt
naa20Δ (nat3Δ)

Figure S4

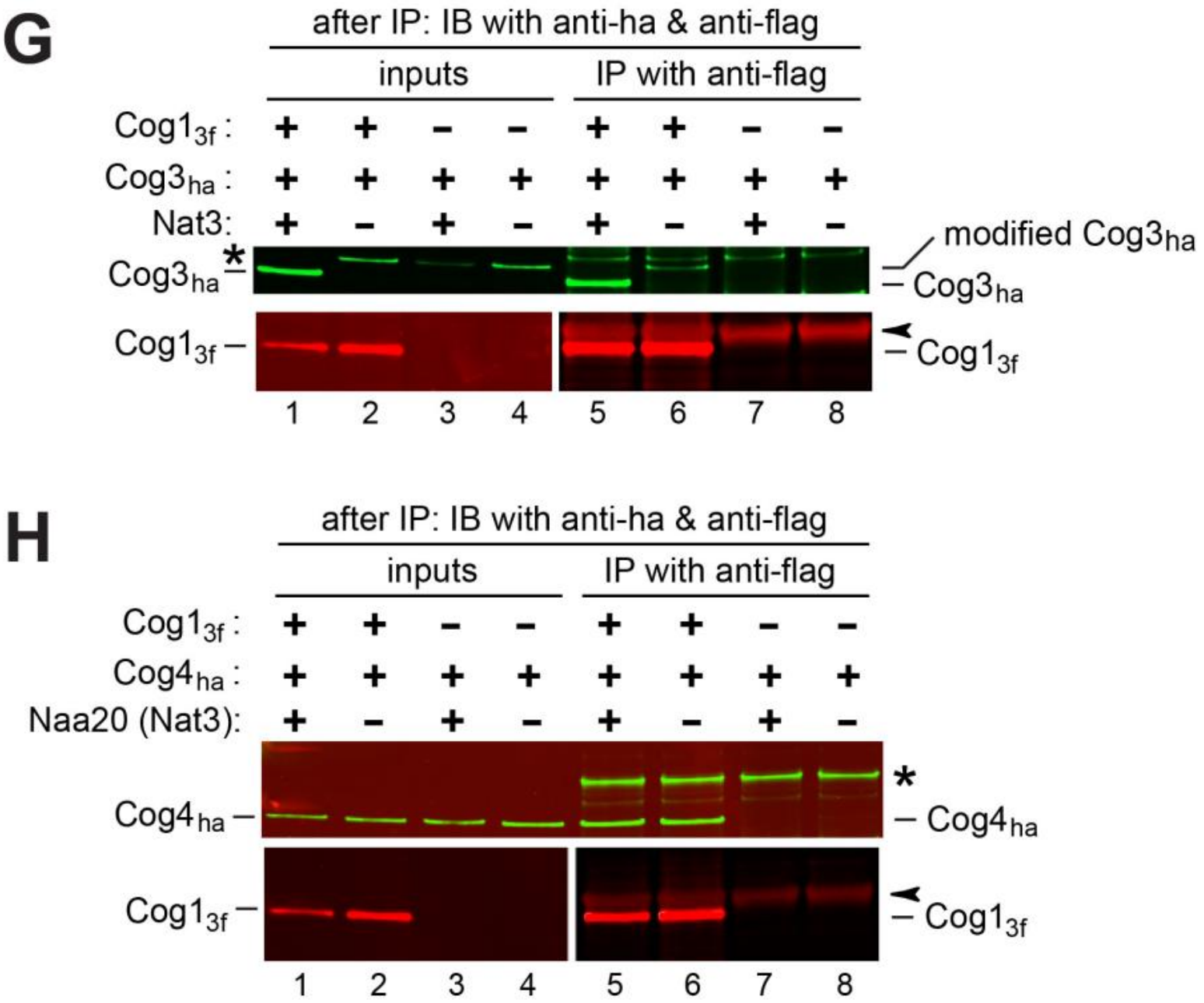

G
after IP: IB with anti-ha & anti-flag
inputs
IP with anti-flag
Cog1 3f :
Cog3 ha :
Nat3:
Cog3 ha *
Cog1 3f
modified Cog3 ha
Cog3 ha
Cog1 3f
1 2 3 4 5 6 7 8
H
after IP: IB with anti-ha & anti-flag
inputs
IP with anti-flag
Cog1 3f :
Cog4 ha :
Naa20 (Nat3):
Cog4 ha
Cog1 3f
*
Cog4 ha
Cog1 3f
1 2 3 4 5 6 7 8

Figure S4. Antibody Specific for Nt-Acetylated Cog1, and Interactions of Nt-Acetylated and Unacetylated Cog1 with Subunits of the COG Complex or with Membranes.

(A) Characterization of the anti-AcNtCog1 antibody using a dot immunoassay. Increasing amounts of the Nt-acetylated Ac-MDEVLPLFRDSC peptide and its non-acetylated counterpart MDEVLPLFRDSC were spotted onto a nitrocellulose membrane, followed by immunoblotting with the rabbit anti-AcNtCog1 antibody that was raised against the Ac-MDEVLPLFRDSC peptide and was then affinity-purified both "positively" (against Ac-MDEVLPLFRDSC) and "negatively" (against MDEVLPLFRDSC) (see Extended Experimental Procedures).

(B) Wt, cog1Δ, and naa20Δ (nat3Δ) S. cerevisiae strains overexpressed either MD-Cog1wt, P-Cog1, or ME-Tpk2 (an Nt-acetylated protein whose N-terminal sequence is different from that of MD-Cog1wt; a negative control) from the PCUP1 promoter on low copy plasmids. Equal amounts of total protein in the extracts were fractionated by SDS-PAGE and immunoblotted with the anti- AcNtCog1 antibody. Lane 1, MD-Cog1wt (C-terminally tagged with three flag epitopes) was expressed in wt cells. Lane 2, same as in lane 1 but the identically tagged P-Cog1 (MP-Cog1). Lane 3, same as in lane 2 but in cog1Δ cells. Lane 4, same as in lane 1 but vector alone (no exogenously expressed MD-Cog1wt). Lane 5, ME-Tpk2 (C-terminally tagged with three flag epitopes) was expressed in wt cells. Lane 6, same as in lane 5 but ME-Tpk2 was expressed in naa20Δ (nat3Δ) cells lacking the cognate NatB Nt-acetylase.

Anti-AcNtCog1 detected the band of Nt-acetylated MD-Cog1wt in lane 1. Consistently, there was virtually no signal in other lanes, except for the barely detectable band in lanes 4 and 5 (marked by asterisk on the right) that is likely to be the endogenous Nt-acetylated MD-Cog1wt (endogenous MD-Cog1wt was expressed at levels significantly below those of exogenous MD-Cog1wt). Consistent with the absence of three flag epitopes in the endogenous MD-Cog1wt, its band migrated faster than the band of the exogenous (tagged) MD-Cog1wt (lane 1 vs. lanes 4 and 5). Note the absence of crossreaction of anti-AcNtCog1 with Nt-acetylated ME-Tpk2 in wt cells (lane 5). The anti-AcNtCog1 antibody also detected proteins larger than MD-Cog1wt; they are marked by green arrowheads on the left. These proteins were not derivatives of MD-Cog1wt, as they were present in cells not expressing MD-Cog1wt (lane 4). The three proteins were Nt-acetylated by NatB, as they were absent in naa20Δ (nat3Δ) cells (lane 6). A likely and parsimonious interpretation is that the anti-AcNtCog1 antibody detected three specific Nt-acetylated proteins whose cognate Nt-acetylase (NatB) is the same as the one that Nt-acetylates MD-Cog1wt and whose N-terminal sequences are sufficiently close to that of MD-Cog1wt to have resulted in a crossreaction.

(C) Same as in B, but the same membrane was re-probed with anti-flag antibody, to detect the bulk of triply flag-tagged MD-Cog1wt and ME-Tpk2.
(D) Equal amounts of total detergent-free cell extracts from wt or naa20Δ (nat3Δ) S. cerevisiae were fractionated to yield the cytosolic (C) and membrane (M) fractions, followed by SDS-PAGE and immunoblotting with anti-flag antibody to detect the triply flag-tagged MD-Cog1wt. Lanes 1-3, wt total (T) extract and its C and M fractions, respectively. Lanes 4-6, same as in lanes 1-3 but from naa20Δ (nat3Δ) cells.
(E) Coomassie Blue staining of membrane probed by anti-flag in D.
(F) Quantification of the ratio of MD-Cog1wt in the membrane versus cytosolic fractions in panel D, using Odyssey (Li-Cor) (see Extended Experimental Procedures).
(G) Coimmunoprecipitation of MD-Cog1wt and Cog3 in the presence and absence of Nt-acetylation. Wt and naa20Δ (nat3Δ) S. cerevisiae strains carried either a PCUP1 wt promoter-containing low copy plasmid, or the otherwise identical plasmid expressing MD-Cog1 (C-terminally tagged with three flag epitopes), or the plasmid expressing Cog3 (C-terminally ha-tagged), in the indicated combinations of test proteins and genetic backgrounds of strains in which they were expressed. Extracts from these strains were immunoprecipitated using anti-flag beads, followed by SDS-PAGE and immunoblotting with anti-ha (to detect Cog3; the upper panel, green color) or with anti-flag (to detect MD-Cog1wt; the lower panel, red color). As described in the main text, most Cog3 in naa20Δ (nat3Δ) cells was converted into a derivative of lower electrophoretic mobility, indicated on the right as “modified Cog3”. The asterisk on the left indicates a protein crossreacting with anti-ha antibody. The arrowhead on the right marks the position of the heavy IgG chain, above the band of immunoprecipitated MD-Cog1wt.
(H) Same as in G (including the same notations), but coimmunoprecipitation of MD-Cog1wt (C-terminally tagged with three flag epitopes) and Cog4 (C-terminally ha-tagged). The asterisk in the top panel on the right indicates a protein crossreacting with anti-ha. Figure S4 refers to Figures 1 and 4.

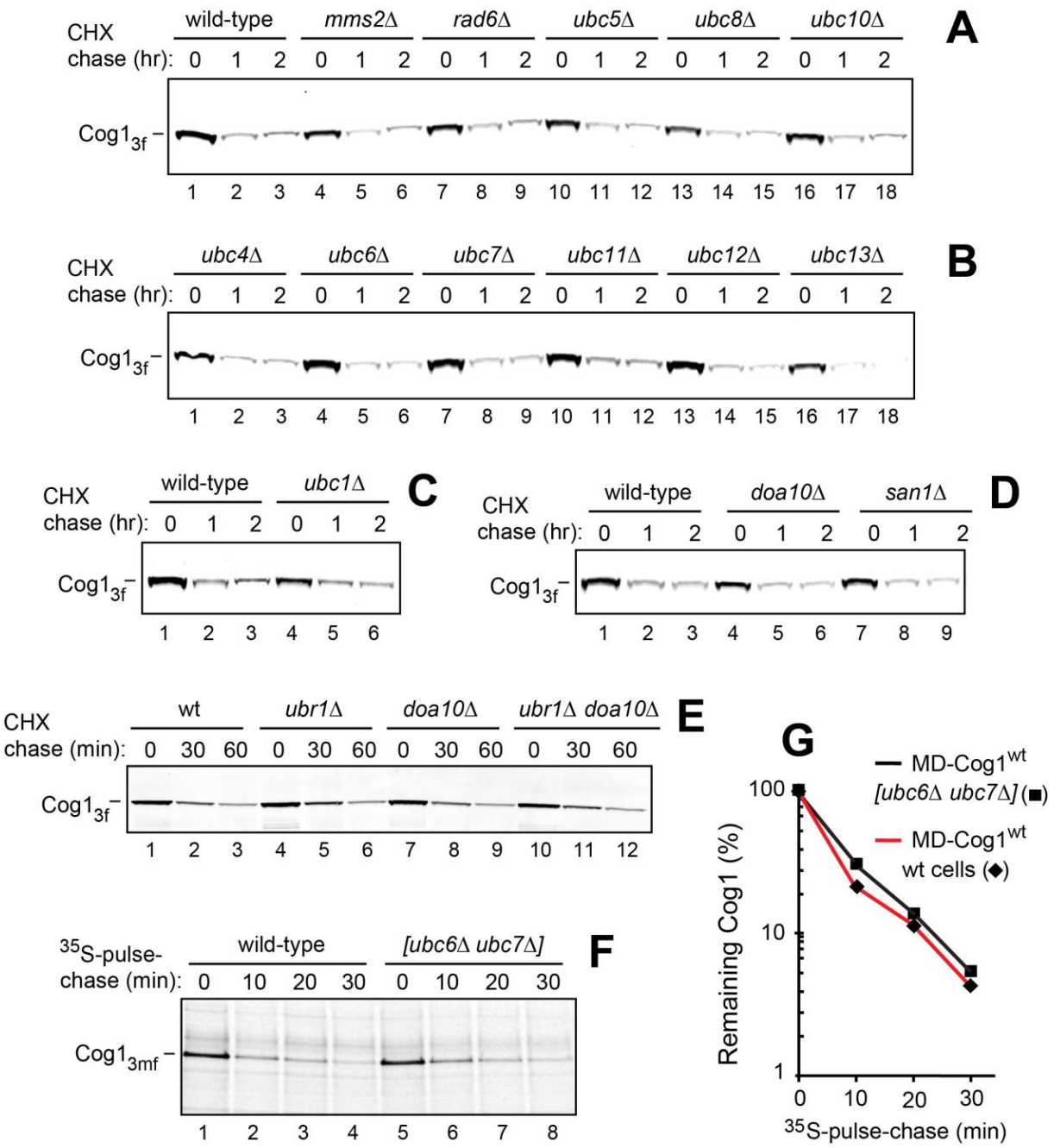


Figure S5, Shemorry et al.

Figure S5. Degradation of MD-Cog1wt by the Ac/N-End Rule Pathway in S. cerevisiae Mutants Lacking Specific E2 or E3 enzymes.

(A) CHX-chases with yeast mutants in specific Ub-conjugating (E2) enzymes that expressed MD-Cog1wt C-terminally tagged with three flag epitopes. Wt S. cerevisiae (lanes 1-3) and mms2Δ (lanes 4-6), rad6Δ (lanes 7-9), ubc5Δ (lanes 10-12), ubc8Δ (lanes 13-15), and ubc10Δ (lanes 16-18) mutants.

(B) Same as in A but with S. cerevisiae mutants ubc4Δ (lanes 1-3), ubc6Δ (lanes 4-6), ubc7Δ (lanes 7-9), ubc11Δ (lanes 10-12), ubc12Δ (lanes 13-15), and ubc13Δ (lanes 16-18).

(C) Same as in A but with wt (lanes 1-3) and ubc1Δ (lanes 4-6) S. cerevisiae.

(D) CHX-chases with mutants in two specific E3 enzymes. Same as in A but with wt (lanes 1-3), doa10Δ (lanes 4-6), and san1Δ (lanes 7-9) S. cerevisiae strains.

(E) Same as in D but the chases for 30 and 60 min with wt (lanes 1-3), ubr1Δ (lanes 4-6), doa10Δ (lanes 7-9), and double-mutant ubr1Δ doa10Δ (lanes 10-12) S. cerevisiae strains.

(F) Lanes 1-4, 35S-pulse chase with wt S. cerevisiae and MD-Cog1wt (C-terminally tagged with three flag epitopes modified to contain a Met residue in each epitope, to increase 35S in Cog1; see Extended Experimental Procedures). Lanes 5-8, same as lanes 1-4 but with a double mutant ubc6Δ ubc7Δ.

(G) Quantification of 35S-pulse-chase data in F.