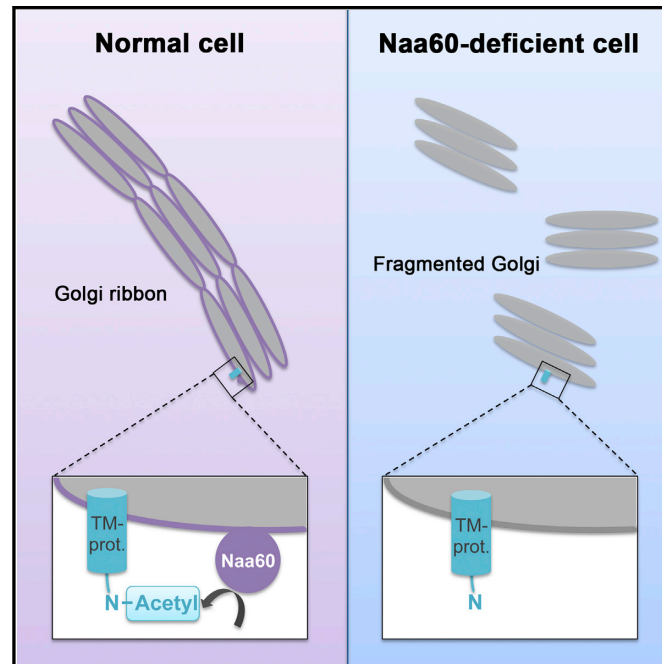


Cell Reports

An Organellar N α -Acetyltransferase, Naa60, Acetylates Cytosolic N Termini of Transmembrane Proteins and Maintains Golgi Integrity

Graphical Abstract



Authors

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In Brief

Aksnes et al. show that N-terminal acetylation, a common modification of soluble eukaryotic proteins, is also frequent among transmembrane proteins. They find Naa60 to be an organelle-associated N-terminal acetyltransferase, with cytosolic activity toward N termini of transmembrane proteins, likely involved in the maintenance of the Golgi's structural integrity.

Highlights

- Naa60 is an organelle N-terminal acetyltransferase, and it acts on the cytosolic face
- Most transmembrane proteins are Nt-acetylated, and Naa60 acts specifically on these
- Naa60 mainly localizes to the Golgi and is essential for Golgi ribbon structure
- PROMPT, a novel assay for membrane topology of proteins, is presented

An Organellar N α -Acetyltransferase, Naa60, Acetylates Cytosolic N Termini of Transmembrane Proteins and Maintains Golgi Integrity

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SUMMARY

N-terminal acetylation is a major and vital protein modification catalyzed by N-terminal acetyltransferases (NATs). NatF, or N α -acetyltransferase 60 (Naa60), was recently identified as a NAT in multicellular eukaryotes. Here, we find that Naa60 differs from all other known NATs by its Golgi localization. A new membrane topology assay named PROMPT and a selective membrane permeabilization assay established that Naa60 faces the cytosolic side of intracellular membranes. An Nt-acetylome analysis of *NAA60*-knockdown cells revealed that Naa60, as opposed to other NATs, specifically acetylates transmembrane proteins and has a preference for N termini facing the cytosol. Moreover, *NAA60* knockdown causes Golgi fragmentation, indicating an important role in the maintenance of the Golgi's structural integrity. This work identifies a NAT associated with membranous compartments and establishes N-terminal acetylation as a common modification among transmembrane proteins, a thus-far poorly characterized part of the N-terminal acetylome.

ACKNOWLEDGMENTS

This work was supported by grants from the Norwegian Cancer Society (to T.A.), The Bergen Research Foundation BFS (to T.A.), the Research Council of Norway (grants 197136 and 230865 to T.A.), and the Western Norway Regional Health Authority (to T.A.). P.V.D. acknowledges support from the Research Foundation - Flanders (FWO-Vlaanderen; project number G.0269.13N). Previous students in the Arnesen lab, Janniche Torsvik and Einar Birkeland, are thanked for earlier work on Naa60. Professors Rein Aasland and Anne-Marie Szilvay are acknowledged for the idea of specific protein cleavage as a tool for localization studies.

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EXPERIMENTAL PROCEDURES

HeLa, HeLaS3, A-431, and 293 cells were transfected with Eugene 6 (Roche/Promega), X-tremeGENE 9 (Roche), or Effectene (PROMPT validation experiments) according to the manufacturer's protocol. 0.25–0.5 μ g DNA was used per ml culture medium. Unless otherwise indicated, cells were used for downstream experiments 12–24 hr posttransfection. *NAA60* knockdown was performed by transfection using Dharmafect 1 (GE Dharmacon) or HiPerFect (QIAGEN) according to the respective manufacturer's protocols, with the addition of 5 nM pan-caspase inhibitor Z-VAD-FMK (renewed every 24 hr by replacing half of the culture medium). Cells were harvested or fixed approximately 72 or 96 hr post-siRNA transfection. Further cell culture details and material specifications on siRNA and shRNA are provided in the [Supplemental Experimental Procedures](#).

For preparation of crude A-431 cell fractions, cells were harvested and dissolved in homogenization buffer (0.25 M sucrose, 1 mM EDTA, 20 mM HEPES-NaOH [pH 7.4]), after which nuclei were removed by sedimentation at $3,000 \times g$ for 5 min and organelles and cytosol were separated by centrifugation at $17,000 \times g$ for 20 min. SILAC-labeled fractionated A-431 cells were used for N-terminal COFRADIC analysis, as previously described ([Staes et al., 2011](#); [Van Damme et al., 2009](#)) and detailed in the [Supplemental Experimental Procedures](#).

Standard procedures were followed for immunocytochemistry, and the buffers, antibodies, and incubation times are given in detail in the [Supplemental Experimental Procedures](#). Fluorescent images in [Figures 3B, 6A, and 6D](#) were obtained on a Leica TCS SP2 AOBS. Fluorescent images in [Figures 6A and 6D](#) were obtained on z stacks of 0.2- to 0.3- μ m-thick optical sections spanning the entire depth of Golgi complexes and are shown as maximum-intensity projections, and insets are presented as projections of two or three successive sections. All other fluorescent images were obtained on a Leica DMI6000 B wide field microscope. Further instrument details can be found in the [Supplemental Experimental Procedures](#). The acquired images were processed using the Photoshop CS5 image software (Adobe Systems).

Detailed information on additional procedures, including bioinformatics tools used and construction of plasmids, are available in the [Supplemental Experimental Procedures](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.01.053>.

AUTHOR CONTRIBUTIONS

H.A. wrote the paper and conceived and performed the majority of the bioinformatics, cloning, cellular, and microscopy work with the support of M.G.

and N.G.; P.V.D. conceived and performed COFRADIC analyses and protocol optimization, data interpretation, and bioinformatics analyses with contributions from K.G.; M.G. performed the carbonate wash assay and several knockdowns and validations; K.K.S. prepared cell fractions for COFRADIC and performed initial co-localization experiments with the help of C.H.; M.M. and T.V.K. acquired data on Golgi fragmentation upon *NAA60* knockdown; S.I.S. performed in vitro acetylation assays; M.N. developed the PROMPT assay with the contributions of M.Z, S.L., and C.F.; K.H. performed *NAA60* knockdown and antibody tests; and T.A. conceived, initiated, and supervised the project and wrote the paper together with H.A.

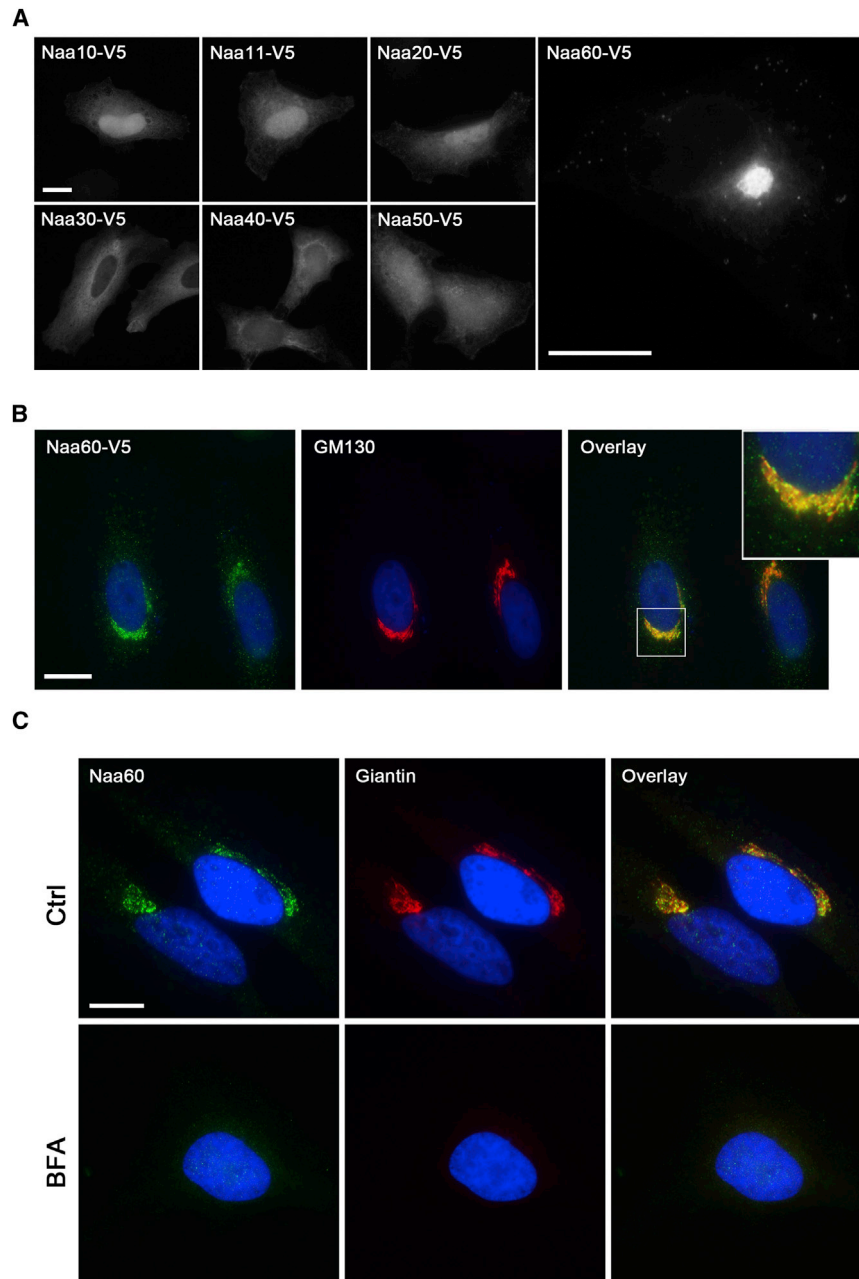


Figure 1. Naa60 Associates with Organelles as Opposed to the Other Known Human N α -Acetyltransferases and Co-distributes with Markers of the Golgi Apparatus

(A) Subcellular localization of all known human Naas. HeLa cells expressing C-terminally V5-tagged Naa10, Naa11, Naa20, Naa30, Naa40, Naa50, and Naa60 were subjected to immunocytochemistry. Only Naa60 showed localization to intracellular membranous compartments other than the nucleus.

(B) Naa60-V5 expressing HeLa cells were co-immunostained with anti-V5 and anti-GM130. Low-Naa60-V5-expressing cells are shown.

(C) HeLa cells were treated with brefeldin A (BFA) and co-immunostained with anti-Naa60₃₋₇₇ and antibodies toward the *cis/medial*-Golgi marker Giantin. As for Giantin, the perinuclear Golgi signal of Naa60 was lost after BFA treatment.

Scale bars, 10 μ m. See also Figures S1 and S3A.

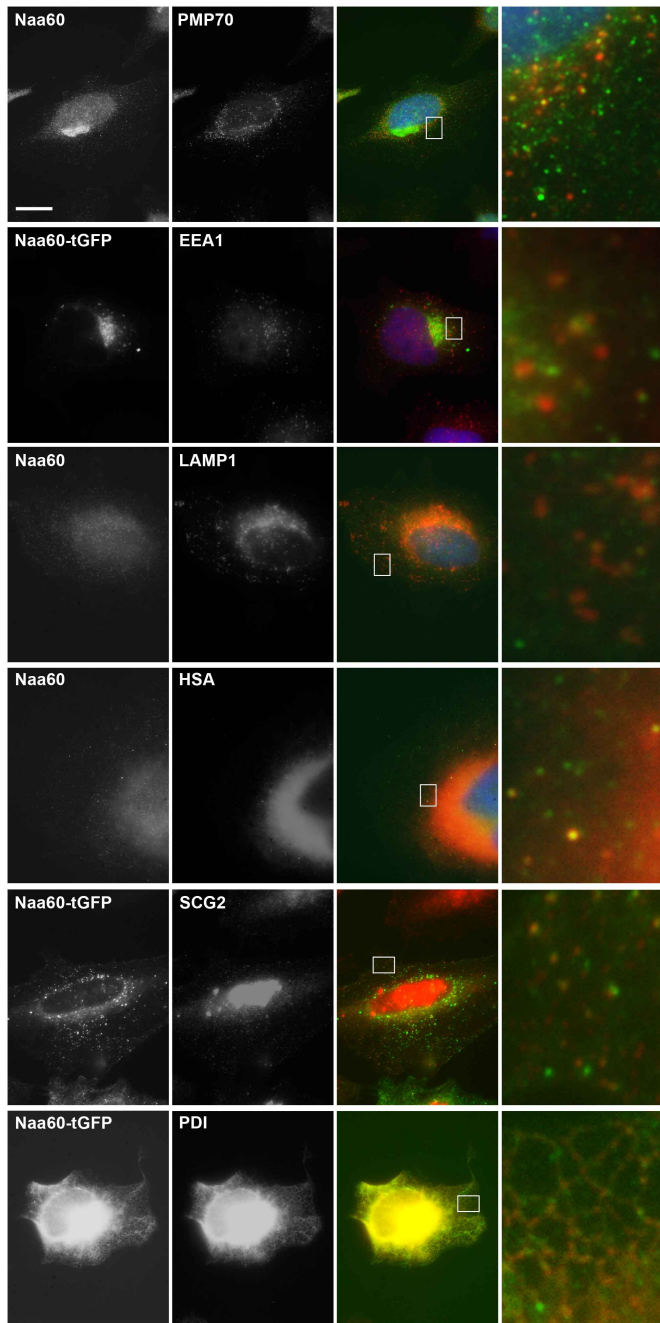


Figure S1. Additional co-localization studies related to Figure 1.

Figure S1. Additional co-localization studies related to Figure 1. Weak to absent co-localizations were observed for tagged and endogenous Naa60 with various markers for the secretory and endocytic pathway. The first column shows either endogenous or overexpressed tagged Naa60 as indicated; the second column shows the subcellular markers; and the third column shows an overlay and the rightmost column a zoomed-in area marked in the preceding. Note that there is a difference the localization pattern of endogenous Naa60 (Naa60), which was more confined to the Golgi, and overexpressed tagged Naa60 (Naa60-tGFP), which was more widely distributed to other intracellular organelles. The following organelle markers were used: PMP70: peroxisomes; EEA1: early endosomes; LAMP1: lysosomes; HSA (human serum albumin): secretory vesicles; SCG2 (secretogranin II): secretory vesicles; PDI: ER. Scale bar: 10 μ m.

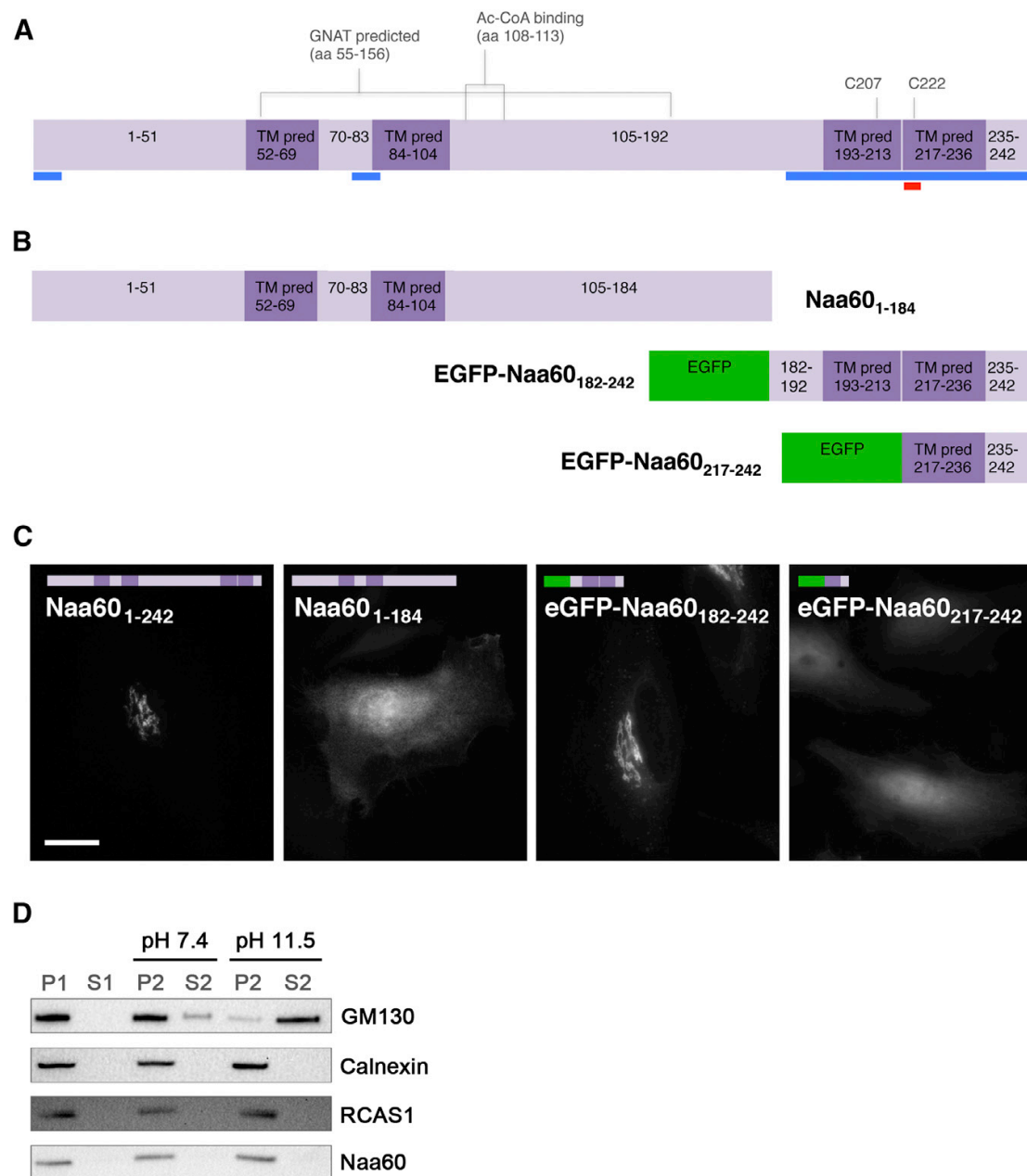


Figure 2. The Naa60 C Terminus Is Required and Sufficient for Membrane Localization and Integration

(A) The Naa60 protein sequence with predicted motifs and domains indicated. Dark purple, transmembrane domains (predicted with low probability); blue underlined, regions unique for Naa60 among the catalytic Naas (aa 1–13, 78–87, and 180–242); red underlined, putative endo/lysosomal targeting signal, QAHSLL (aa 216–221); C207 and C222 were predicted as putative S-palmitoylation sites by CCS-Palm 3.0. The primary sequence constituting the GNAT-domain together with the core of the Ac-CoA-binding motif Q/RxxGxG/A is indicated above.

(B) Naa60 constructs used for localization studies. Color-coding as in (A).

(C) HeLa cells were transfected with the indicated constructs and fixed. The membrane localization observed for the full-length Naa60 (Naa60_{1–242}) was lost by deleting the last 58 amino acids (Naa60_{1–184}). eGFP-Naa60_{182–242} showed a localization pattern resembling that of full-length C-terminally tagged Naa60 whereas a shorter construct, eGFP-Naa60_{217–242} localized to the cytosol and nucleus. Naa60_{1–242} and Naa60_{1–184} were expressed from plasmid without tag and detected with anti Naa60_{3–77}. Scale bar, 10 μ m. See also Figure S2.

(D) HeLa cells expressing untagged Naa60 were subjected to subcellular fractionation yielding an organellar pellet (P1), which was dissolved in either sucrose buffer (pH 7.4) or sodium carbonate buffer (pH 11.5) prior to a second centrifugation yielding two sets of P2s and S2s. Naa60 shared its western blot profile with the non-extractable integral membrane proteins Calnexin and RCAS1, as opposed to the peripheral membrane protein GM130, which was extractable by the alkaline buffer.

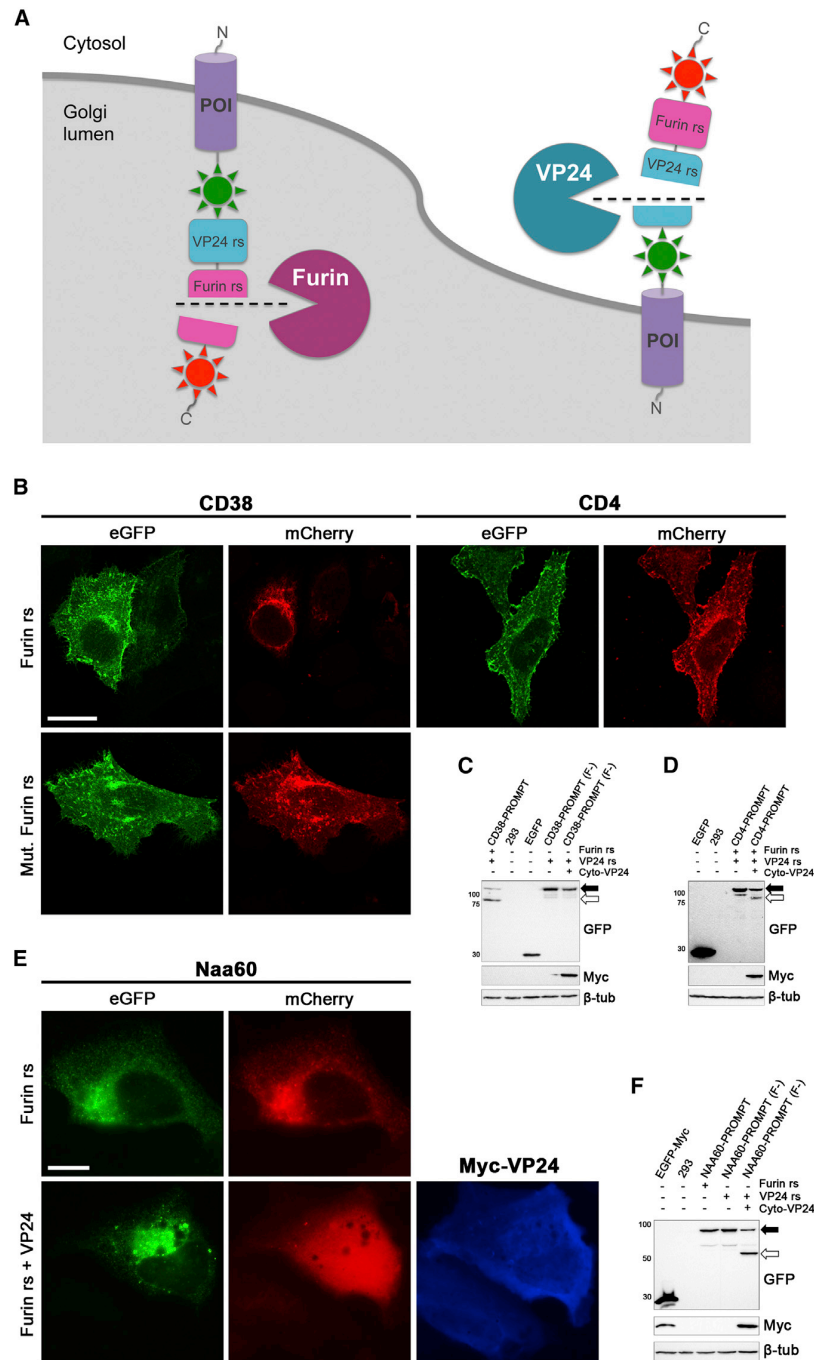


Figure 3. PROtease Assay for Membrane Protein Topology, or PROMPT

(A) Principle and constructs of PROMPT. The protein of interest (POI) is expressed with a C-terminal double-fluorescent reporter tag composed of eGFP (green) and mCherry (red). PROMPT determines the membrane topology of the POI by organelle-specific proteolytic cleavage of the red fluorescent portion from the PROMPT construct. The endogenous Golgi-localized protease, Furin, will find its recognition site (rs) when the POI C terminus faces the Golgi (left), whereas the ectopic-expressed VP24 will cleave PROMPT constructs when the C terminus of the POI is cytosolic (right). Controls include experiments performed in the absence of VP24 or with a POI construct harboring a mutated Furin recognition site.

(B–D) Validation of PROMPT assay. HeLa S3 (B) or 293 cells (C and D) expressing the indicated constructs were fixed and subjected to fluorescence microscopy (B) or harvested for western blotting (C and D). Furin-mediated cleavage occurred on the CD38-construct only in the presence of an intact Furin recognition site (B and C). The CD4 construct was cleaved not by Furin but by the co-expressed cytosolic VP24 (B and D).

(E and F) Determination of Naa60's C-terminal topology by the PROMPT assay. NAA60 was subcloned into the PROMPT constructs shown in (A) and expressed in 293 cells, which were fixed and subjected to fluorescence microscopy (E) or western blotting (F). The Naa60 construct was subject to cleavage not by Furin but by the co-expressed cytosolic VP24.

Scale bars, 20 μ m (B) and 10 μ m (E). rs, recognition site; Mut., mutated; -PROMPT, POI-eGFP-[VP24 rs]-[Furin rs]-mCherry; -PROMPT (F-), POI-eGFP-[VP24 rs]-[Mut. Furin rs]-mCherry; filled arrow, intact fusion protein; framed arrow, cleaved fusion protein.

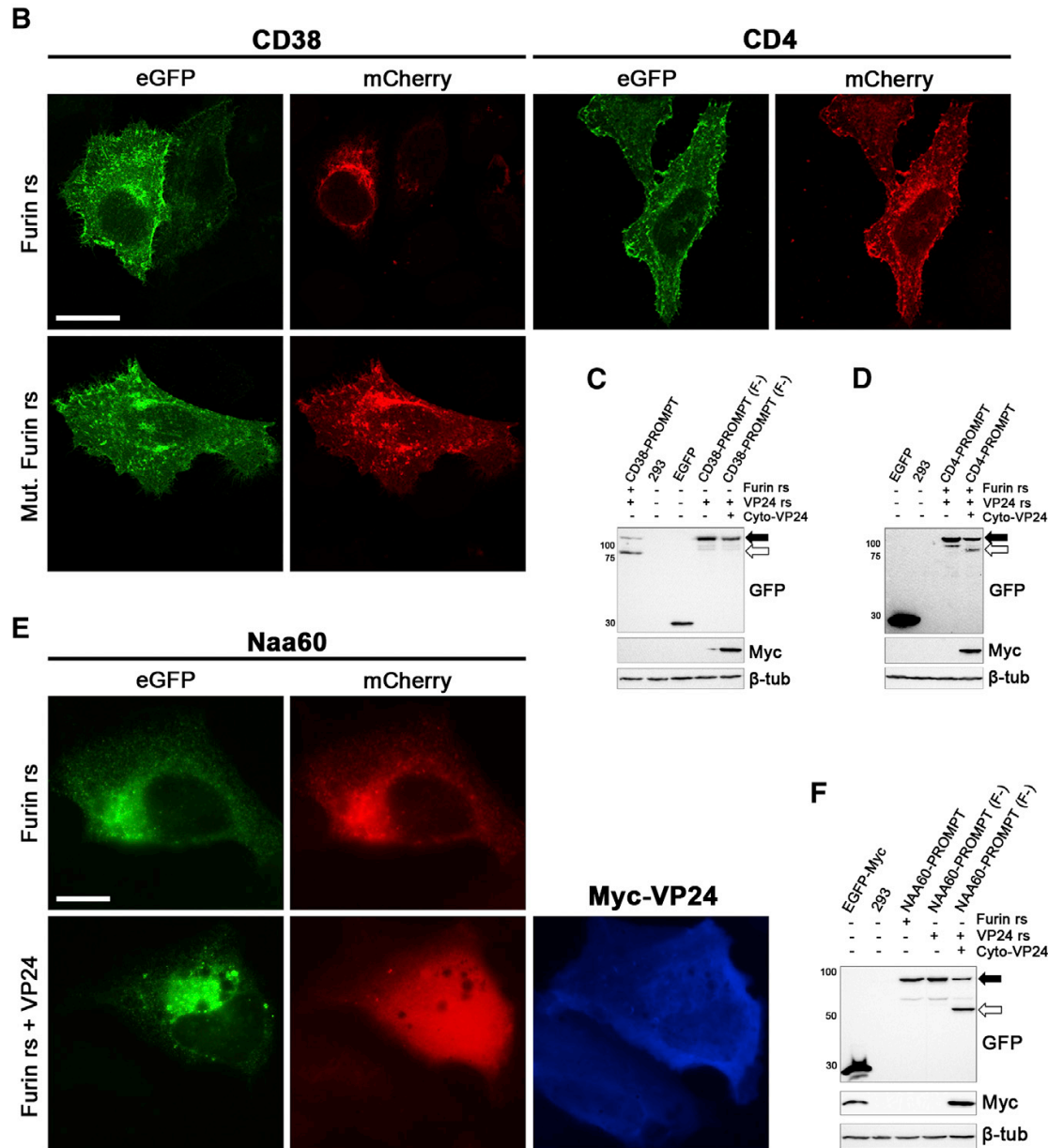


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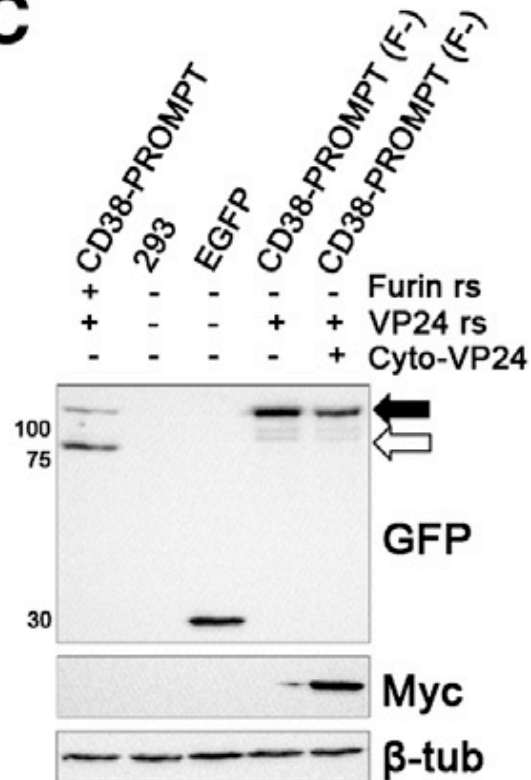
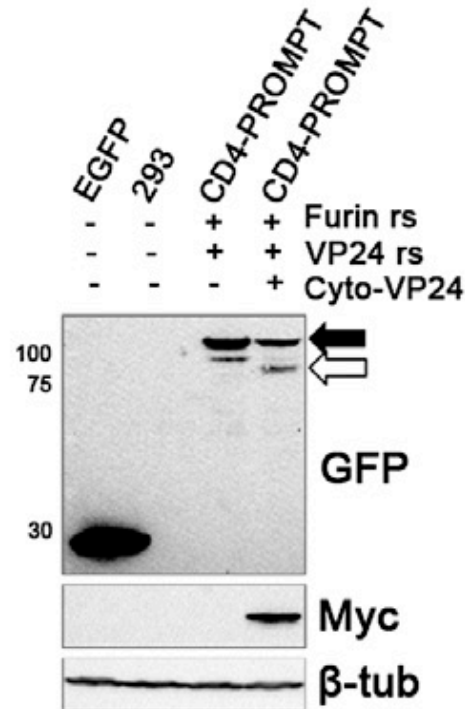
C**D**

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Scale bars, 20 μm (B) and 10 μm (E). rs, recognition site; Mut., mutated; -PROMPT, POI-eGFP-[VP24 rs]-[Furin rs]-mCherry; -PROMPT (F-), POI-eGFP-[VP24 rs]-[Mut. Furin rs]-mCherry; filled arrow, intact fusion protein; framed arrow, cleaved fusion protein.

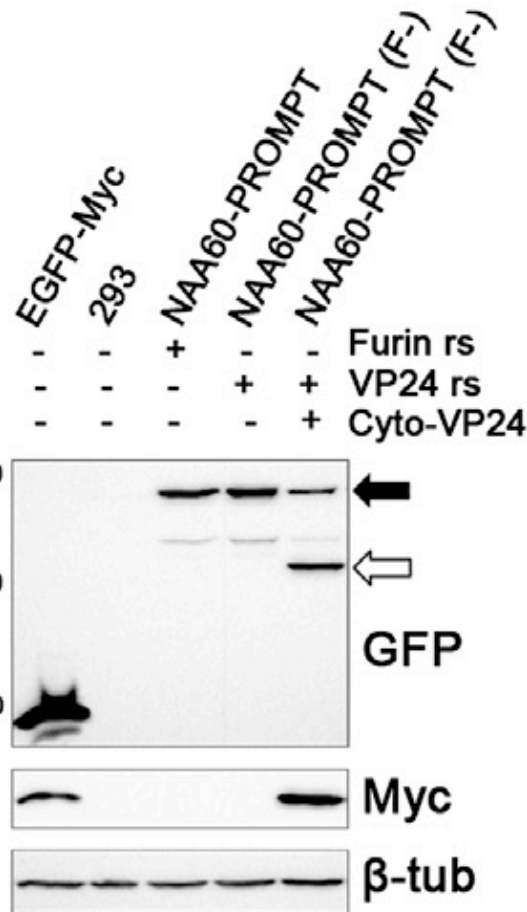


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(E and F) Determination of Naa60's C-terminal topology by the PROMPT assay. *NAA60* was subcloned into the PROMPT constructs shown in (A) and expressed in 293 cells, which were fixed and subjected to fluorescence microscopy (E) or western blotting (F). The Naa60 construct was subject to cleavage not by Furin but by the co-expressed cytosolic VP24.

Scale bars, 20 μm (B) and 10 μm (E). rs, recognition site; Mut., mutated; -PROMPT, POI-eGFP-[VP24 rs]-[Furin rs]-mCherry; -PROMPT (F-), POI-eGFP-[VP24 rs]-[Mut. Furin rs]-mCherry; filled arrow, intact fusion protein; framed arrow, cleaved fusion protein.

Figure S2. Minor or no impact on Naa60 membrane localization for the additional regions shown in Figure 2A. HeLa cells expressing the indicated constructs.

(A) N-terminal truncation (Naa60₁₄₋₂₄₂-tGFP) and N-terminal tagging (eGFP-Naa60) interfered somewhat with the localization pattern of Naa60. Note also that, expressing N-terminally tagged Naa60 resulted in increased appearance of enlarged misshaped vacuoles.

(B) Deletion of aa 78-87 putative loop (Naa60₇₈₋₈₇Δ-tGFP) did not perturb Naa60 membrane localization. Mutation (Naa60_{L220/221A}-tGFP) or deletion (Naa60₂₁₆₋₂₂₁Δ-tGFP) of the QAHSLL putative endo/lysosomal targeting signal did not have any detectable effect on Naa60 membrane localization.

(C) Naa60 Cys-Ser mutations did not disrupt Naa60 membrane localization. Several variants of single and multiple mutations were performed. The most extensive are shown.

(D) 2-bromo palmitate (2-BP)-treatment did not detach Naa60 from organelles but somewhat affected Naa60 subcellular distribution, as vesicles appeared typically larger as well as less abundant in the cell periphery. Scale bar: 10 μm.

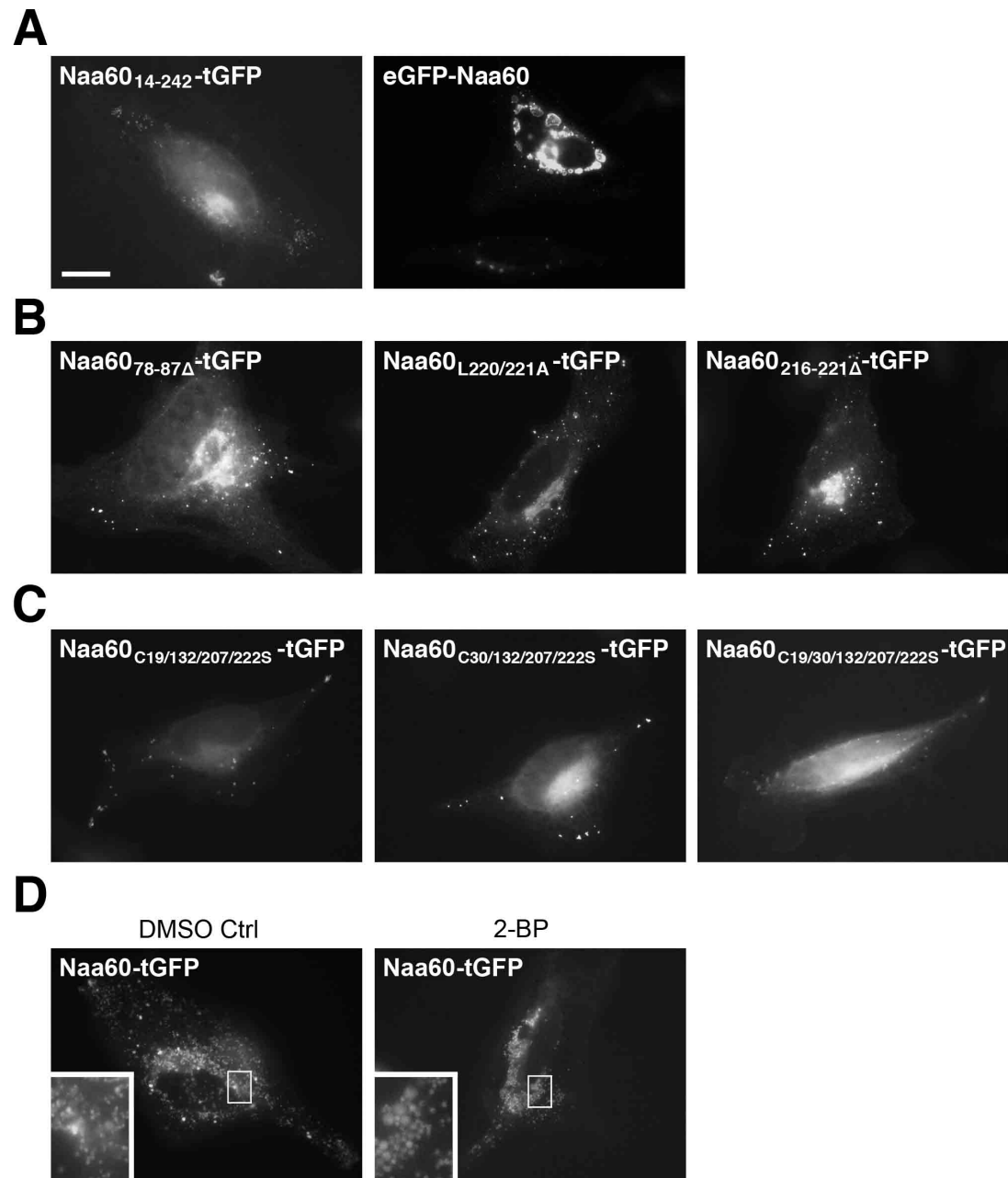


Figure S2. Minor or no impact on Naa60 membrane localization for the additional regions shown in Figure 2A.

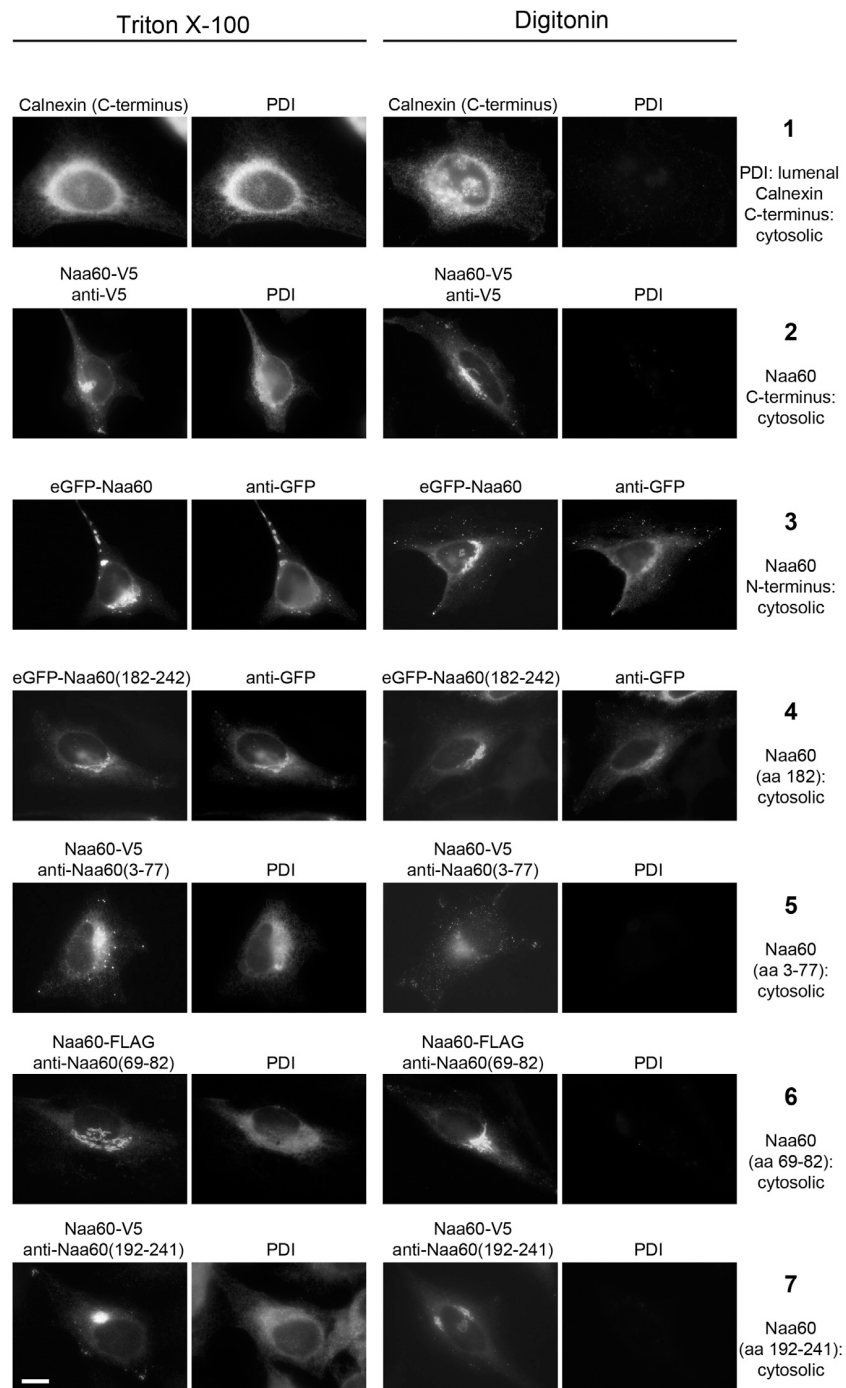


Figure 4. Naa60 Faces the Cytosol

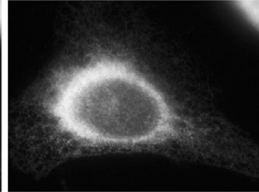
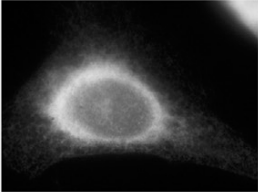
HeLa cells were subjected to selective membrane permeabilization immunofluorescence. All probed regions of Naa60 were mapped to the cytosolic side: panel 2, C-terminally tagged Naa60-V5 detected with anti-V5; panel 3, N-terminally tagged eGFP-Naa60 detected with anti-GFP; panel 4, Naa60 amino acid 182 detected with antibody toward N-terminal tag on truncated NAA60 (eGFP Naa60₁₈₂₋₂₄₂); panel 5, Naa60 aa 3–77 detected with antibody toward Naa60 peptide sequence 3–77; panel 6, Naa60 aa 69–82 detected with antibody toward Naa60 peptide sequence 69–82; and panel 7, Naa60 aa 192–241 detected with antibody toward Naa60 peptide sequence 192–241. Luminal PDI and cytosolic Calnexin controls (panel 1) were performed in all experiments. In addition, the PDI control was applied in all cells where this combination of antibodies and/or fluorophores was compatible. Scale bar, 10 μ m.

Triton X-100

Digitonin

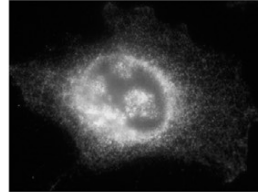
Calnexin (C-terminus)

PDI



Calnexin (C-terminus)

PDI

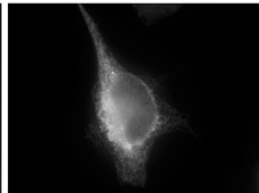
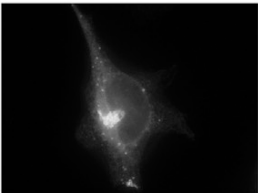


1

PDI: luminal
Calnexin
C-terminus:
cytosolic

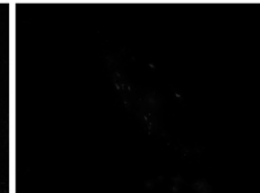
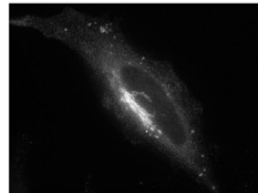
Naa60-V5
anti-V5

PDI



Naa60-V5
anti-V5

PDI

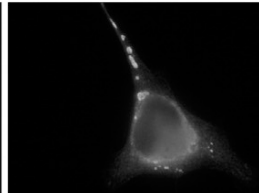
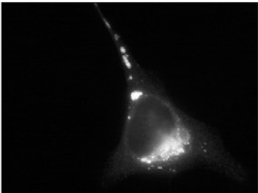


2

Naa60
C-terminus:
cytosolic

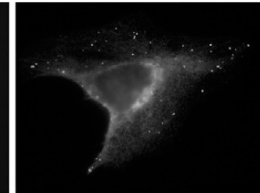
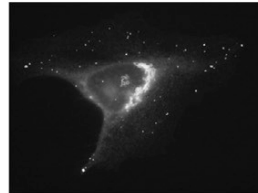
eGFP-Naa60

anti-GFP



eGFP-Naa60

anti-GFP

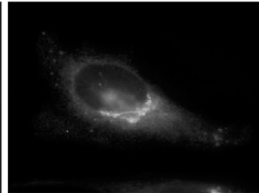
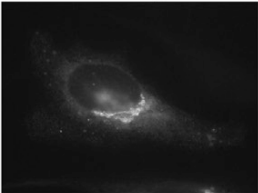


3

Naa60
N-terminus:
cytosolic

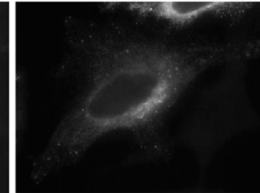
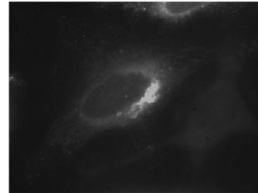
eGFP-Naa60(182-242)

anti-GFP



eGFP-Naa60(182-242)

anti-GFP



4

Naa60
(aa 182):
cytosolic

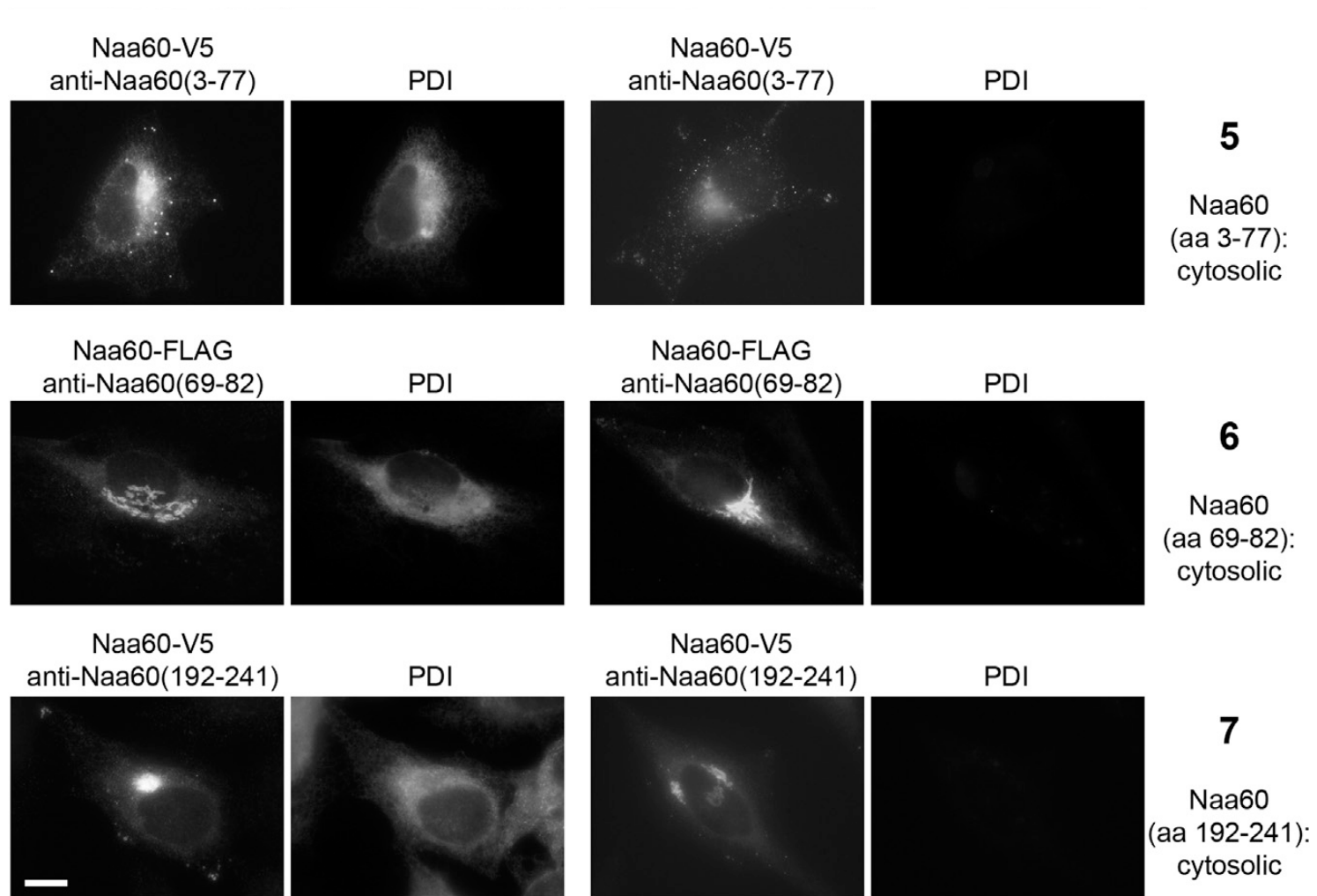


Table 1. Naa60 Substrates Identified in the COFRADIC Analysis of Fractionated siCTRL and siNAA60 A-431 Cells

Nt Sequence	Start	Nt-Ac siCTRL (%)	Nt-Ac siNAA60 (%)	Δ Nt-Ac (%)	Protein	Full Protein Name	Annotated TM	(Predicted) TMD	N-Term Topology	Subcellular Localizations
MAPKG	1	87	16	71	TRAP- ^a	translocon-associated protein subunit gamma	✓	4	in	ER-M
MKQYQ	34	79	25	54	TM222	transmembrane protein 222	✓	3	in	PM
MGPGP ^b	1	71	20	51	MFSD12 ^a	major facilitator superfamily domain- containing protein 12	✓	9/9+(1)	out/in	ER-M
MVSMS	1	85	42	43	LAPTM4A ^a	lysosomal-associated transmembrane protein 4A	✓	4	in	ES
MVISE	1	76	38	38	UfSP2 ^a	Ufm1-specific protease 2		1	in	Cyto, ER
MQGKK	1	66	29	37	OATP3A ^a	solute carrier organic anion transporter family member 3A1	✓	12/12+(1)/ 12+(1)/12+(2)	in	PM
MAPKG	1	94	60	34	TM208 ^a	transmembrane protein 208	✓	2	in	ER-M
MLAAK ^b	26	70	39	31	LAT1	large neutral amino acids transporter small subunit 1	✓	12/12+(1)	in	C; APM
MLPST	1	100	72	28	UNC50	protein unc-50 homolog	✓	5	in	NIM; GA-M
MAPDP	1	95	69	26	FADS1	Fatty acid desaturase 1	✓	3	in	ER-M
MKAVQ	148	88	64	24	LRC59	leucine-rich repeat-containing protein 59	✓	1	out	MI M; ER-M
MVGFG	1	90	68	22	CASC4	protein CASC4	✓	1	in	M
AAGGS ^c	2	80	62	18	GSCR2	glioma tumor suppressor candidate region gene 2 protein		no	—	NU; NO
MIEES	1	79	62	18	DTN-B	dystrobrevin beta		(1)	out	C
AANYS	2	75	58	17	TMM43	transmembrane protein 43	✓	3/3+(1)	in	ER; NIM
MGELP	1	65	49	17	SFXN3	sideroflexin-3	✓	4/4+(1)	out/in	Mito-M
MTQGK	1	96	82	14	HIAT1	hippocampus abundant transcript 1 protein	✓	11	in	M
MYAPG	1	97	85	12	TM127	transmembrane protein 127	✓	4	in	PM; C
MMGLG	1	95	83	11	GOLM1	Golgi membrane protein 1	✓	1	in	GA; cis-GA-M
AATAL ^c	2	61	50	11	PTPM1	phosphatidylglycerophosphatase and protein-tyrosine phosphatase 1		no	—	MIM
MVSKA	1	14	3	11	UXS1	UDP-glucuronic acid decarboxylase 1	✓	1	in	GA-M
AAVAA	2	33	22	10	HACD2	very-long-chain (3R)-3-hydroxyacyl-[acyl-carrier protein] dehydratase 2	✓	9/4+(1)	in/out	ER-M
MAPMG	1	8	0	8	IMP3	inositol monophosphatase 3	✓	1/1+(1)/1+(2)	in	M

See also [Table S1](#) for a complete list of all N termini identified. Abbreviations: Δ Nt-Ac, difference in degree of Nt-acetylation between siCTRL and siNAA60 samples; M, membrane; ES, endo-membrane system; PM, plasma membrane; C, cytoplasm/cytosol; APM, apical membrane; NIM, nucleus inner membrane; GA, Golgi apparatus; MI, microsome; NU, nucleus; NO, nucleolus; Mito, mitochondrion; MIM, mitochondrion inner membrane.

^aSubstrates used in follow-up studies ([Figure S3E](#)).

^bN termini whose mass spectrometry spectra are shown in [Figure S3C](#).

^cPossibly indirectly affected by NAA60 knockdown.

Figure S3. COFRADIC-related validations, representative MS spectra, *in vitro* peptide acetylation, and substrate localization analysis, supporting Table 1.

(A) Validation of the subcellular fractionation procedure to generate organelle- and cytosol-enriched proteome fractions. A-431 cells were subjected to crude subcellular fractionation by means of differential centrifugation. Endogenous Naa60 co-segregated with the organelle marker LAMP1 (Pellet (3×)), whereas Naa10 and Naa30 were present in the cytosolic fraction (Super (1×)). 3x refers to 3 equivalents of lysate input to improve immunodetection.

(B) Validation of samples prepared for COFRADIC. A-431 cells grown in $^{13}\text{C}_6$ $^{15}\text{N}_4$ L-arginine-containing medium were treated with non-targeting control siRNA (siCTRL, -) and cells grown in $^{12}\text{C}_6$ L-arginine-containing medium were treated with siNAA60 (+). After 72 hours cells were subjected to crude subcellular fractionation as in A and subjected to COFRADIC followed by LC-MS/MS analysis. Sample aliquots were analysed by Western blot.

(C) Representative MS spectra of two database annotated and initiator methionine starting N-termini identified as Naa60 substrates by N-terminal COFRADIC. *Upper panel*: MS spectrum of the MFSD12 (CS028) N-terminus (M₁GPGP-) showing a ΔNt-Ac of 51% between the siCTRL (71%) and siNAA60 (20%) setups. *Lower panel*: MS spectrum of the LAT1 N-terminus (M₁LAAG-) showing a ΔNt-Ac of 31% between the siCTRL (70%) and siNAA60 (39%) setups.

(D) NAT activity of recombinant Naa60 towards synthetic oligopeptides. Purified recombinant His-SUMO-Naa60 was mixed with 500 μM Acetyl CoA and 500 μM of the indicated oligopeptides in reaction buffer and incubated at 37°C. Product formation was measured indirectly by adding DTNB and measuring the absorbance at 412 nm. The NAT presumed to be responsible for the different N-terminal peptide sequences *in vivo* is indicated below.

* indicate an N-terminus previously determined as a Naa60 substrate ([Van Damme et al., 2011](#)) and # indicate N-termini identified as putative Naa60 substrates in the present study (Table 1), where MVSM and MKQY are verified as substrates while AANY is not. (E) Six of the NatF substrates identified by means of N-terminal COFRADIC (Table 1) were tested for potential Nt-acetylation dependent effects on subcellular localization. The criteria for selecting these candidates were: *i*) at least 30% absolute shift in the degree of Nt-acetylation between the two setups analyzed (8 proteins); *ii*) an N-terminal sequence matching the defined substrate specificity of Naa60/NatF (8 proteins); *iii*) a natural N-terminus (starting with a database-annotated iMet) (6

were treated with siNAA60 (+). After 72 hours cells were subjected to crude subcellular fractionation as in A and subjected to COFRADIC followed by LC-MS/MS analysis. Sample aliquots were analysed by Western blot.

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(D) NAT activity of recombinant Naa60 towards synthetic oligopeptides. Purified recombinant His-SUMO-Naa60 was mixed with 500 μ M Acetyl CoA and 500 μ M of the indicated oligopeptides in reaction buffer and incubated at 37°C. Product formation was measured indirectly by adding DTNB and measuring the absorbance at 412 nm. The NAT presumed to be responsible for the different N-terminal peptide sequences *in vivo* is indicated below. * indicate an N-terminus previously determined as a Naa60 substrate ([Van Damme et al., 2011](#)) and # indicate N-termini identified as putative Naa60 substrates in the present study (Table 1), where MVSM and MKQY are verified as substrates while AANY is not. (E) Six of the NatF substrates identified by means of N-terminal COFRADIC (Table 1) were tested for potential Nt-acetylation dependent effects on subcellular localization. The criteria for selecting these candidates were: *i*) at least 30% absolute shift in the degree of Nt-acetylation between the two setups analyzed (8 proteins); *ii*) an N-terminal sequence matching the defined substrate specificity of Naa60/NatF (8 proteins); *iii*) a natural N-terminus (starting with a database-annotated iMet) (6 proteins); *iv*) established or predicted as transmembrane (6 proteins); and *v*) an N-terminus with a predicted N-in topology (6 proteins). This left TRAP γ (an ER-protein that constitutes part of the TRAP-complex ([Hartmann et al., 1993](#))), MFSD12 (a less well studied protein whose related proteins localize to the ER ([Angers et al., 2008](#))), LAPTM4A (localizes to endosomes and lysosomes ([Hogue et al., 2002](#))), Ufsp2 (not experimentally proven as transmembrane, but may localize to the ER ([Ha et al., 2011](#))), OATP3A1 (localizes to the plasma membrane ([Kindla et al., 2011](#))), and TM208 (localizes to the ER ([Zhao et al., 2013](#))). A-431 cells were transfected with WT or proline mutant versions (indicated in red) of C-terminally tGFP-tagged substrates as indicated. Scale bar: 10 μ m.

Figure S3

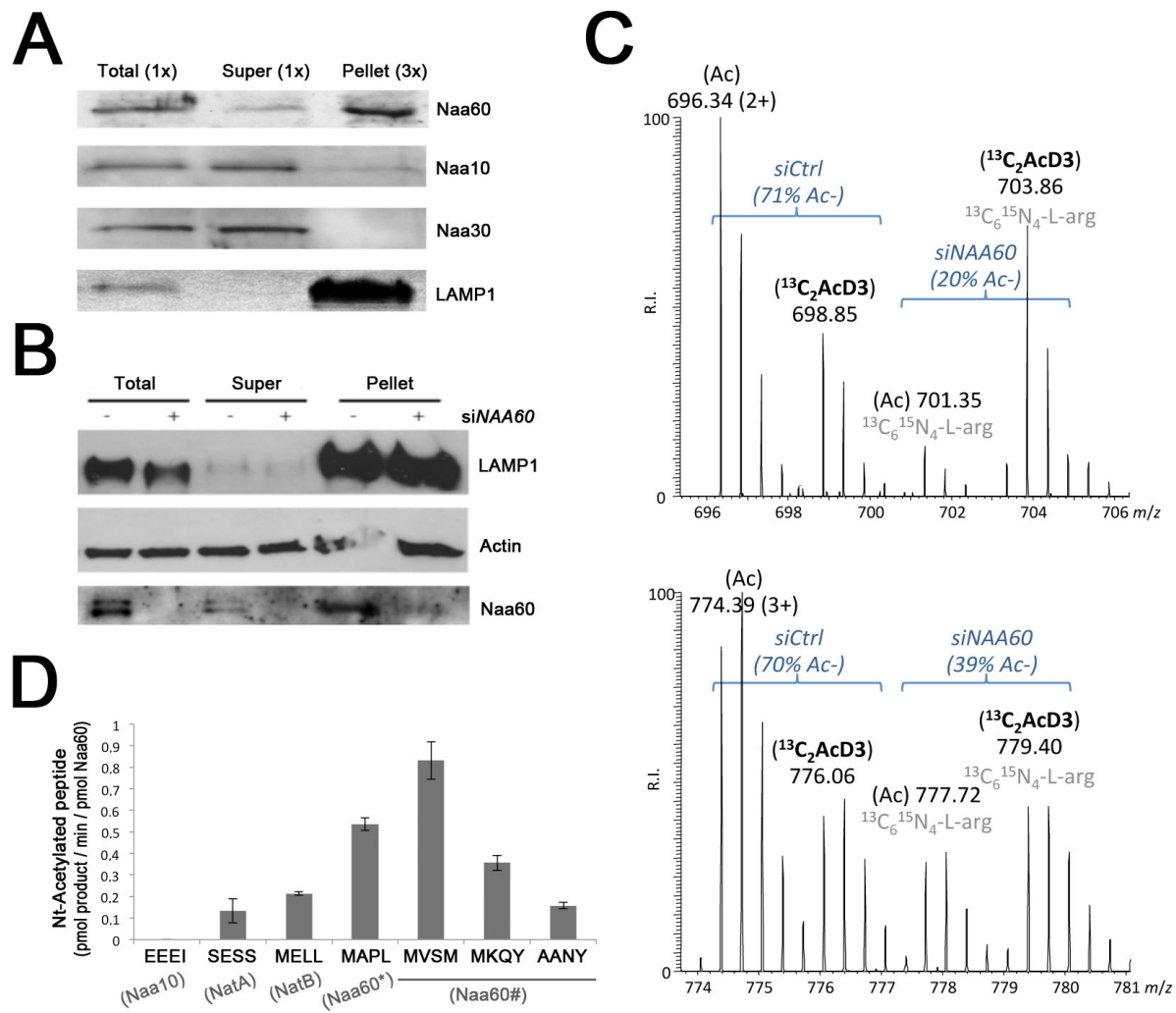


Figure S3

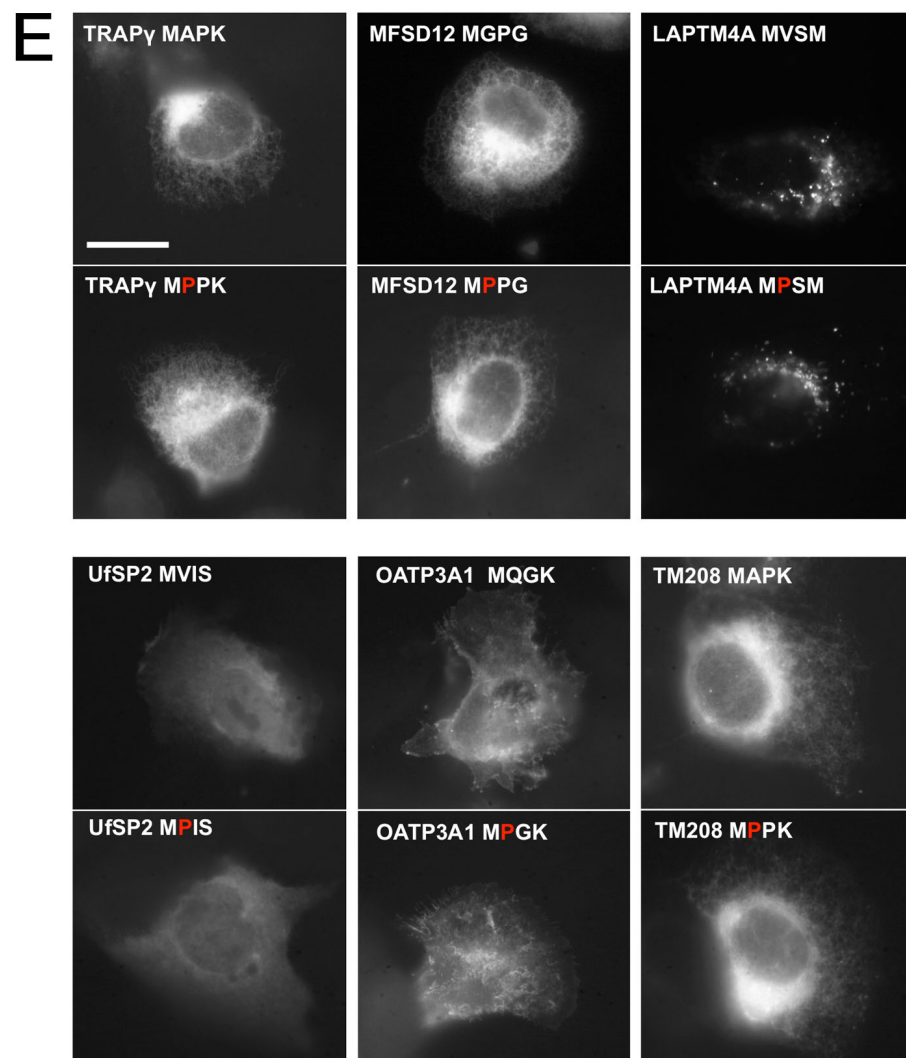
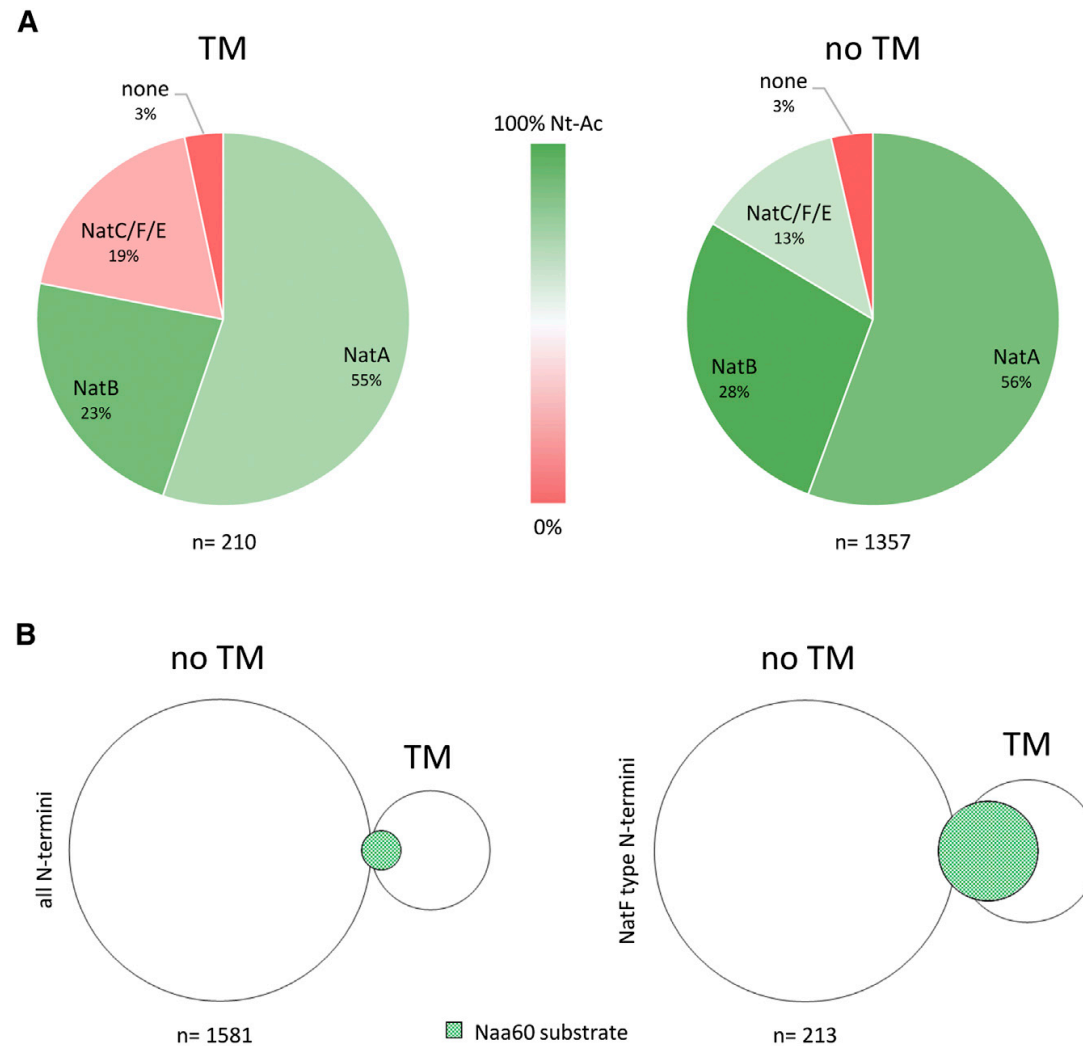


Figure S3. COFRADIC-related validations, representative MS spectra, *in vitro* peptide acetylation, and substrate localization analysis, supporting Table 1.



C

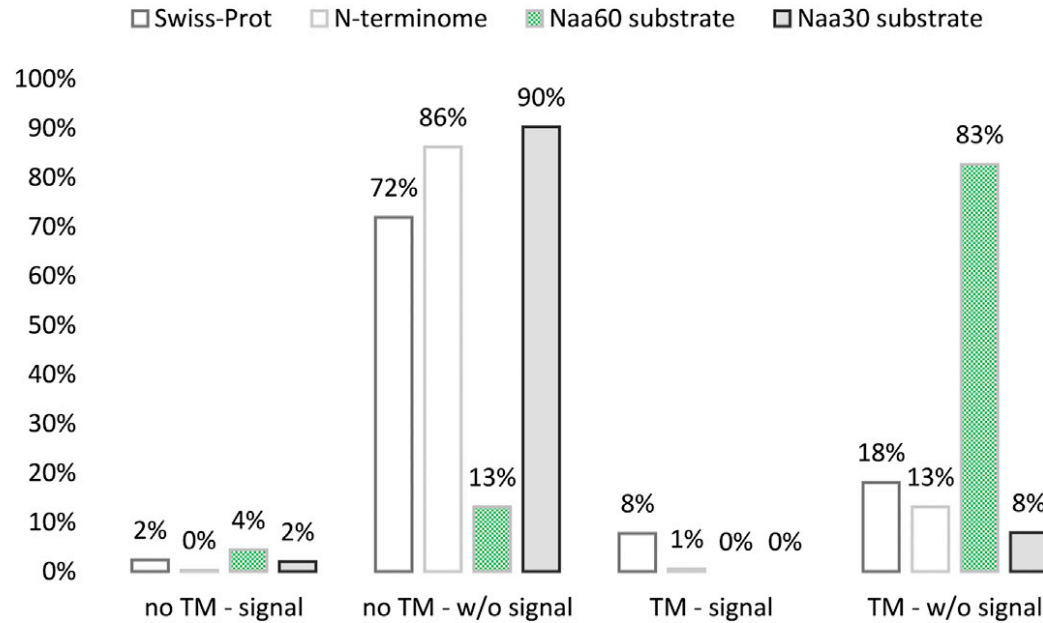


Figure 5. The Naa60/NatF Substrate Category Is Enriched for Transmembrane Proteins

(A) All unique N termini identified in the siCTRL setup, for which the degree of Nt-acetylation could be determined, were grouped into transmembrane (TM) or non-transmembrane protein N-termini (no TM) and categorized according to their NAT specificity profile. The “none” category consists of (M)P- starting N termini that are typically refractory to Nt-acetylation. The color code and intensity reflects the average percentage of Nt acetylation as determined in the siCTRL setup; green and red coloring indicates a degree of Nt acetylation above and below 50%, respectively.

(B) Venn diagrams of all N termini (left) and all Naa60/NatF type N termini (right) for which the Nt-acetylation status could be determined in both the siCTRL and siNAA60 setups, grouped into transmembrane and non-transmembrane proteins.

(C) Bar chart plotting the occurrence of N termini (i.e.: all Swiss-Prot N termini, all N termini identified in this study, Naa60 substrate N termini identified in this study, and Naa30 substrate N termini; T.V.K., P.V.D., K.K.S., K.G., T.A., V. Jonckheere, L.M. Myklebust, G. Bjørkøy, and J.E. Varhaug, unpublished data) for non-transmembrane and signal-containing proteins, non-transmembrane proteins without signal sequence, transmembrane and signal-containing proteins, and transmembrane proteins without signal sequence.

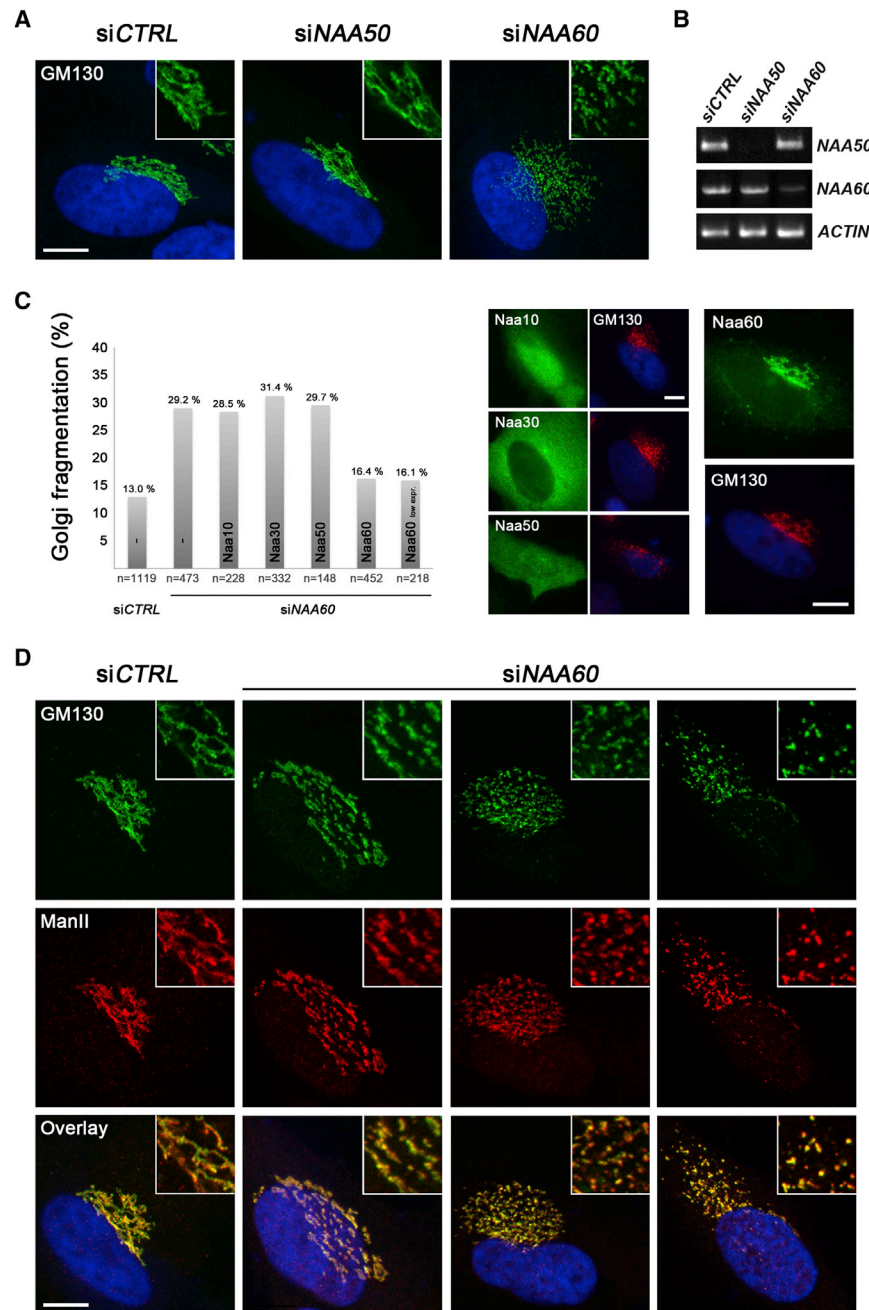


Figure 6. NAA60 Knockdown Affects Golgi Morphology

(A) Abnormal Golgi morphology was observed in siNAA60-treated, but not siNAA50-treated, HeLa cells immunostained for the *cis*-Golgi marker GM130 and compared to siCTRL. Images are based on cell counts showing fragmented Golgi in 14% of siCTRL ($n = 338$), 14% of siNAA50 ($n = 263$), and 38% of siNAA60 ($n = 231$). See also Figure S4A for additional statistics.

(B) Reduction in mRNA level after treatment of HeLa cells with smart pool siRNA used in (A). NAA50, NAA60, and ACTIN PCRs were performed on cDNA synthesized from RNA isolates from cells treated with siCTRL, siNAA50 and siNAA60 cells as in (A). This confirmation was performed in all siRNA experiments.

(C) Left: the siNAA60-induced Golgi phenotype could be rescued by Naa60-V5 expression, but not by expressing any of the cytosolic NATs (Naa10-, Naa30-, or Naa50-V5). Cells were treated with siNAA60-4 and, 3–4 hr later, transfected with V5 plasmids encoding the indicated proteins. Naa60-V5 contained silent mutations enabling expression in siNAA60-4-treated cells. The % of cells displaying a fragmented Golgi was counted among V5-positive cells only, except in the siCTRL and the siNAA60-4 samples (bars 1 and 2). Additionally, to exclude plasmid expression efficacies as a confounding variable, an expression control (Naa60-low expr.) was performed where the “siNAA60-4 + Naa60-V5” condition was recounted, only considering the weakest half of the V5-positive cell population. Right: immunofluorescence images from the same experiment as to the left.

(D) siCTRL- and siNAA60-treated cells co-immunostained for GM130 and Mannosidase II (ManII) revealed that these *cis*- and *medial*-Golgi markers cohered equally well in control and Golgi phenotypic cells. Three differing degrees of Golgi structural effects observed in the siNAA60 cells are shown.

All scale bars represent 10 μ m. See also validating data in Figure S4 and additional data in Figure S5.

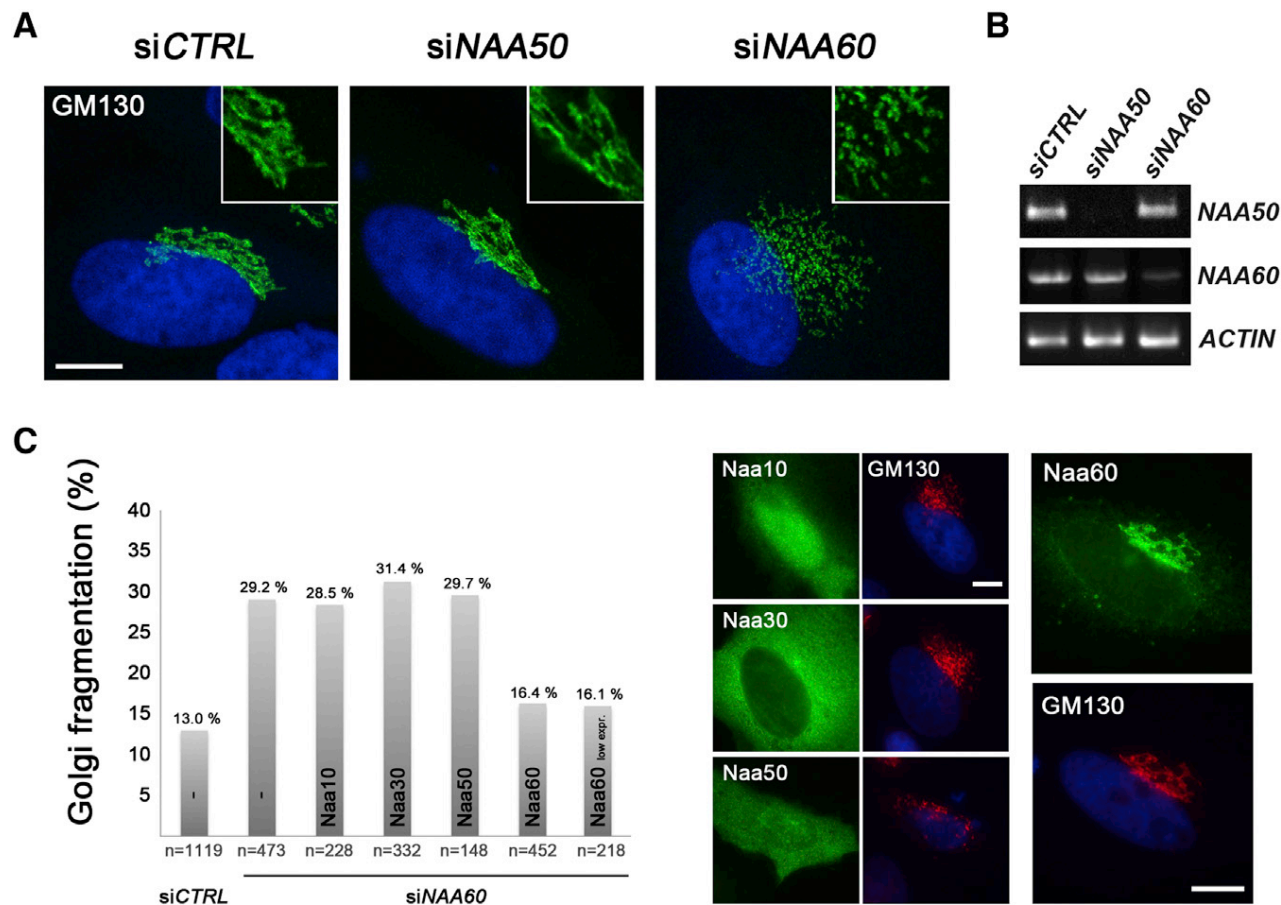


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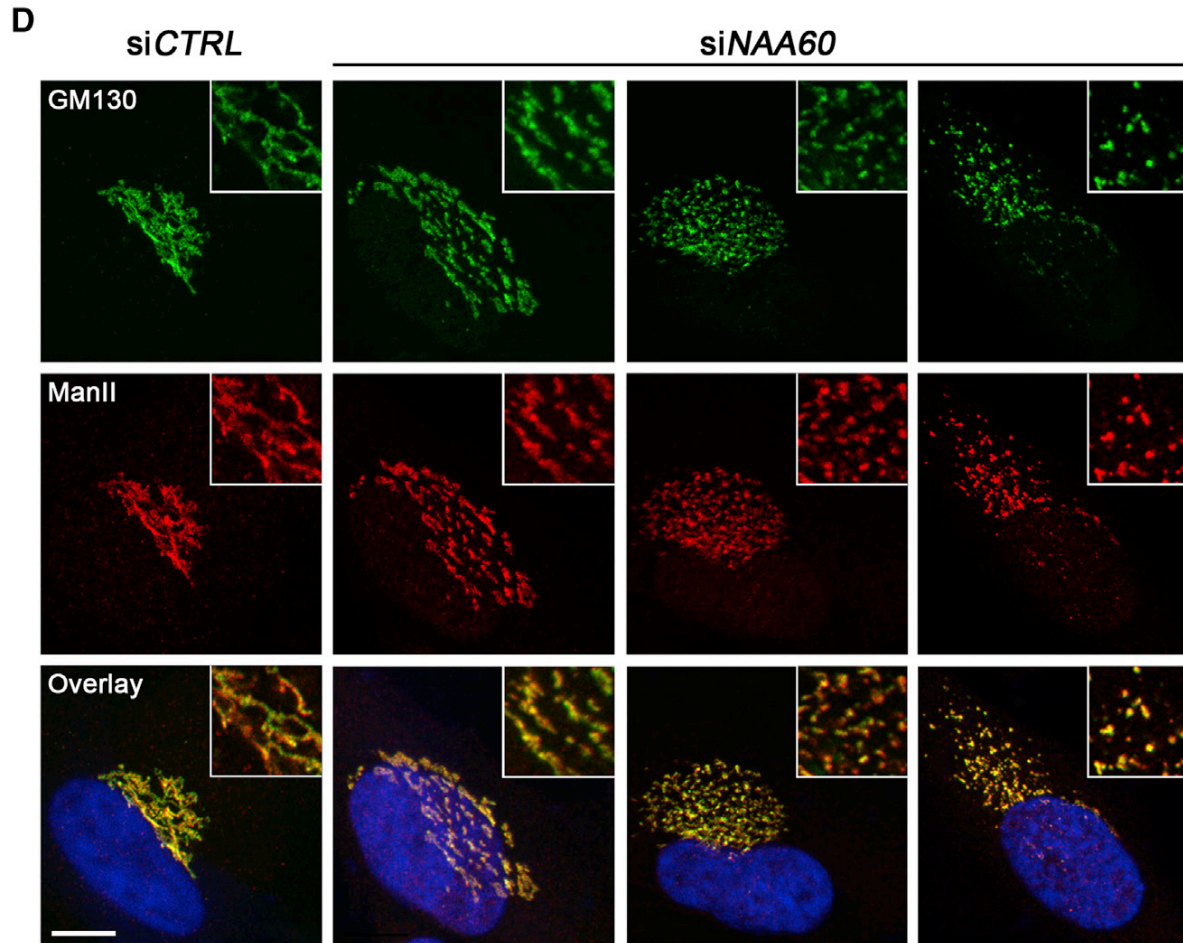


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All scale bars represent 10 μm . See also validating data in Figure S4 and additional data in Figure S5.

Figure S4. Experiments supporting the specificity of Golgi phenotype in *NAA60* knockdown cells presented in Figure 6.

(A) *Left panel:* CAL-62 cells immunostained for GM130 after si*NAA60* treatment (smart pool). *Right panel:* quantifications based on cell counting from samples shown in the left panel. Data are from three individual experiments (n=100-150 cells per sample per experiment) for both cell lines. Error bars denote the standard deviations of three independent experiments. si*NAA60* significantly differs from siCTRL in both cell lines with ($p < 0.05$, ANOVA, Post Hoc).

(B-C) The *NAA60* knockdown Golgi phenotype was verified by two alternative independent siRNAs, si*NAA60*-3 (30%, n=292) and si*NAA60*-4 (31%, n=231) (B), as well as an additional knockdown approach where *NAA60* shRNA is expressed from an RFP-vector, sh*NAA60* (sh*NAA60*-89) vs. shCTRL (non-targeting control shRNA-15)

(C). In shRNA-positive cells, the fragmented Golgi phenotype was observed in 16% of shCTRL-15 (n=161) and 52% of sh*NAA60*-89 (n=185) amongst the RFP-positive cells. Scale bars: 10 μm (A, C); 5 μm (B).

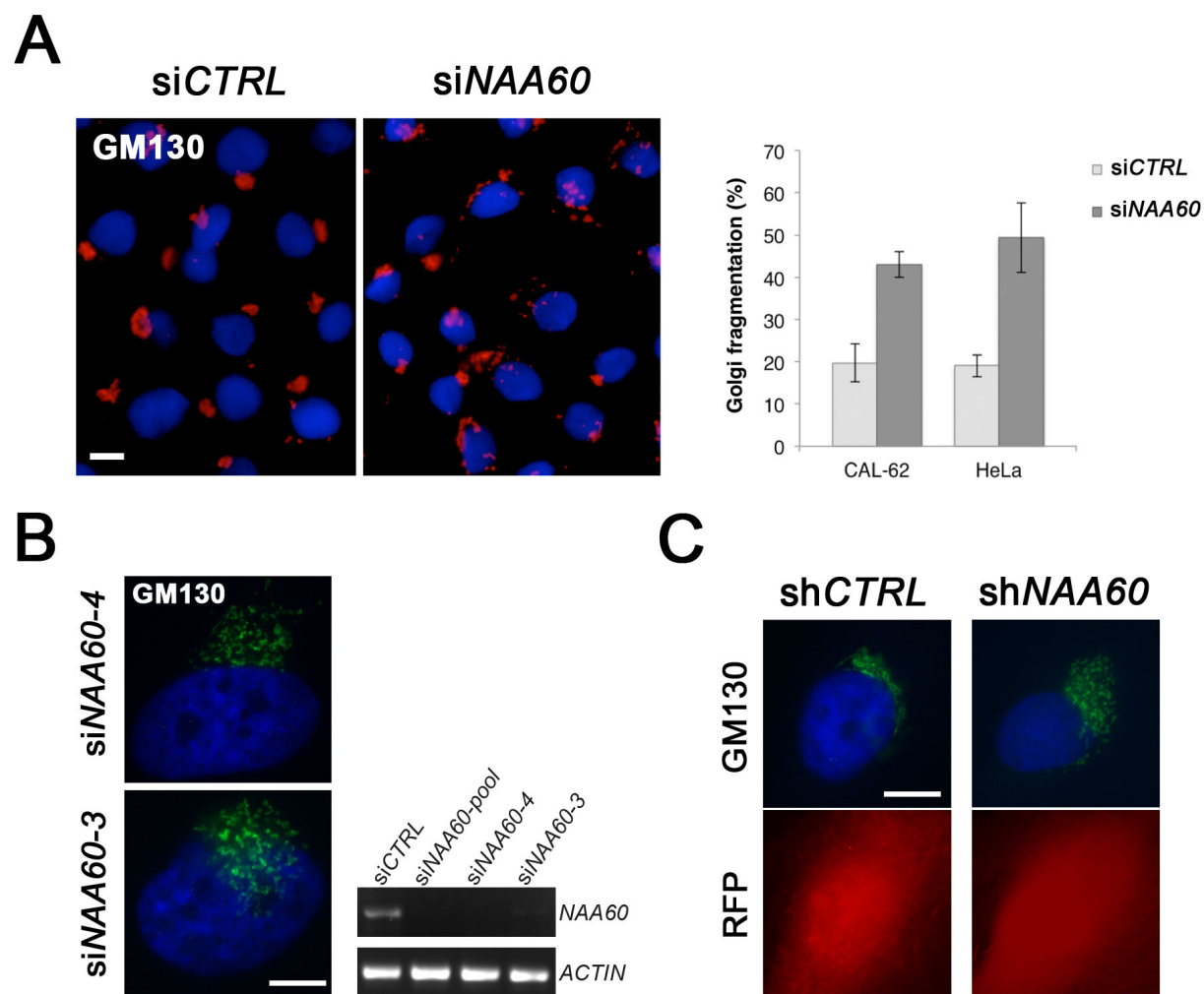


Figure S4. Experiments supporting the specificity of Golgi phenotype in *NAA60* knockdown cells presented in Figure 6.

Figure S5. *NAA60* knockdown did not cause cytoskeletal disassembly or structural perturbation of organelles other than the Golgi fragmentation shown in Figure 6.

(A) The Golgi-localized Giantin was shown to display the same phenotype as GM130, thus verifying Giantin as a marker for knockdown cells similarly as GM130.

(B) The si*NAA60*-induced structural effect was specific to the Golgi and no alterations were observed for other organelles or for the F-actin or microtubule cytoskeleton. PDI: ER-marker; COX IV: mitochondrial marker; PMP70: peroxisome marker; EEA1: endosomal marker; LAMP1: lysosomal marker; Rhodamine Phalloidin: actin cytoskeleton stain; and β -tubulin: microtubule cytoskeleton. Giantin was used as a Golgi marker when combined with rabbit antibody against EEA1. In all other panels mouse-derived antibodies were used and GM130 was used as Golgi marker. Scale bar: 10 μ m.

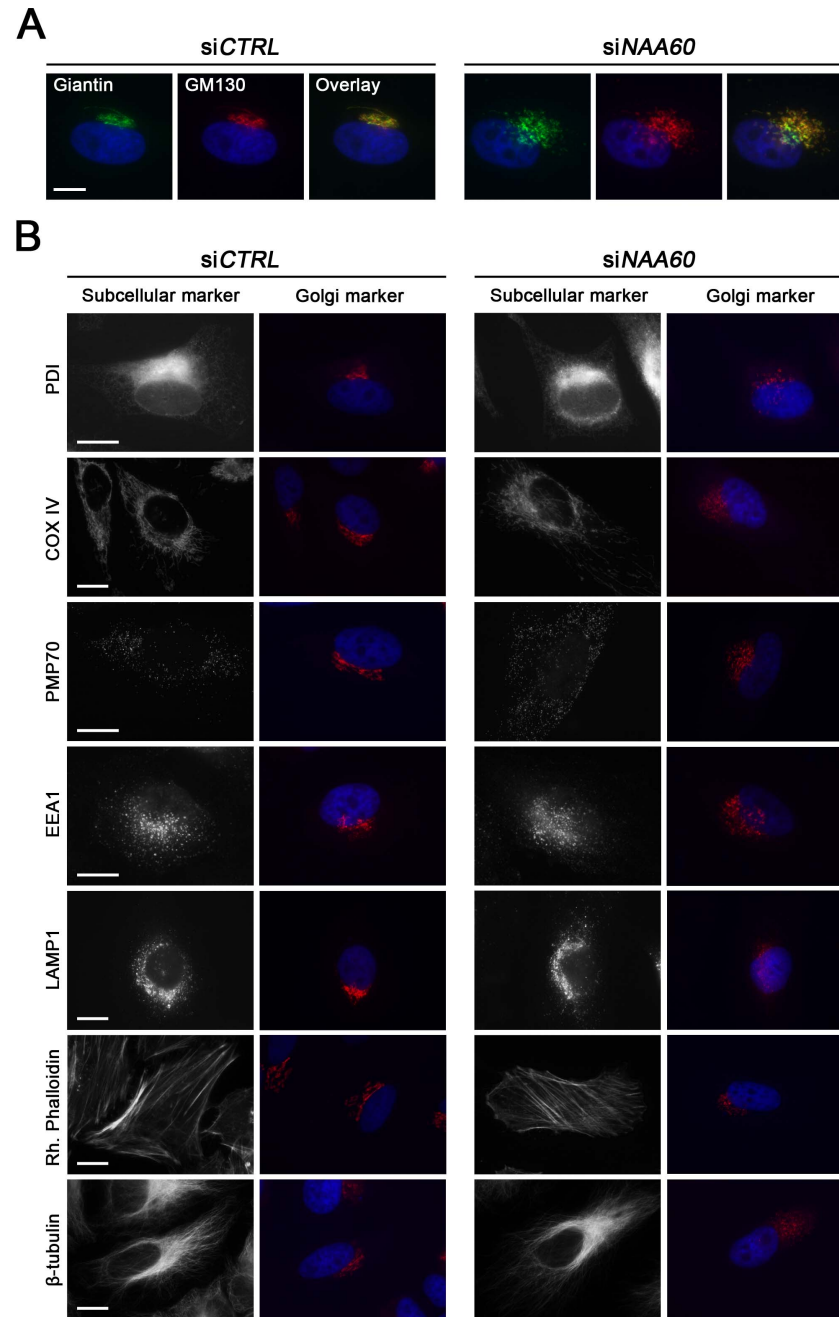


Figure S5. *NAA60* knockdown did not cause cytoskeletal disassembly or structural perturbation of organelles other than the Golgi fragmentation shown in Figure 6.

Table S1. List of 1,699 unique human N-terminal peptides identified, related to Table 1

List of 1,699 human N-termini identified in the cytoplasmic and organellar-enriched proteome fractions of *siCTRL* and *siNAA60*-treated human A-431 cells. Swiss-Prot accession, identified peptide sequence, start and end positions, and experiment number(s) in which the peptide was identified (1 and 2 indicate cytoplasmic and organelle-enriched fractions, respectively) are shown. NatF substrate, amino acid residue preceding the identified N-terminus (P1; if any), N-terminal modification status/states confirmed by tandem mass spectrometry (MS/MS) (per setup), degree (%) of Nt-acetylation (per setup/difference between setups/in total), number of identified spectra (per setup/in total), and global Nt-modification status in the *siCTRL* setup(s) (N-termini found to be less than 2% Nt-Ac and more than 98% Nt-Ac) were considered as 100% free and 100% Nt-Ac, respectively, when considering their global Nt-Ac status. N.D. (not determined) indicates N-termini where acetylation status could not be calculated. UniProt database primary accession number, protein description, gene symbols, protein length (aa), Gene Ontology terms, subcellular localizations, and, whenever a peptide matched to multiple members of a protein family (redundancy) isoforms are given for all uniquely identified yeast N-termini. Maximum Mascot ion score, delta threshold (= maximum score – minimum threshold), minimum threshold score, confidence level of identification, and C-terminal modification status/states confirmed by MS/MS (in total) are also indicated. N-termini are ranked alphabetically. (Table uploaded as a separate Excel file)

Table S2. Nt-acetylation status of transmembrane versus non-transmembrane protein N-termini, related to Table 1 and S1 and Figure 5.

Transmembrane						
NAT ^a	Start with Met	Amino 1	Nt-Ac			
			Free	Full	Partial	Total
NatA	0	A	9 %	70 %	21 %	91 %
		C	0 %	100 %	0 %	100 %
		G	80 %	0 %	20 %	20 %
		S	4 %	87 %	9 %	96 %
		T	60 %	20 %	20 %	40 %
		V	83 %	0 %	17 %	17 %
NatB	1	D	0 %	100 %	0 %	100 %
		E	0 %	86 %	14 %	100 %
		N	0 %	57 %	43 %	100 %
		Q	50 %	0 %	50 %	50 %
NatC	1	I	0 %	100 %	0 %	100 %
		L	20 %	40 %	40 %	80 %
Other	0	F	0 %	100 %	0 %	100 %
		N	0 %	100 %	0 %	100 %
		P	86 %	0 %	14 %	14 %
	1	A	14 %	14 %	71 %	86 %
		G	0 %	0 %	100 %	100 %
		K	57 %	0 %	43 %	43 %
		M	0 %	0 %	100 %	100 %
		T	33 %	33 %	33 %	67 %
		V	27 %	18 %	55 %	73 %
		Y	50 %	0 %	50 %	50 %
	Grand Total			18 %	58 %	24 %

Non-transmembrane						
NAT	Start with Met	Amino 1	Nt-Ac			
			Free	Full	Partial	Total
NatA	0	A	3 %	92 %	5 %	97 %
		C	50 %	50 %	0 %	50 %
		G	52 %	22 %	26 %	48 %
		S	1 %	99 %	1 %	99 %
		T	10 %	73 %	18 %	90 %
		V	79 %	3 %	17 %	21 %
NatB	1	D	0 %	99 %	1 %	100 %
		E	0 %	99 %	0 %	100 %
		N	0 %	96 %	4 %	100 %
		Q	0 %	80 %	20 %	100 %
NatC	1	F	29 %	57 %	14 %	71 %
		I	29 %	29 %	43 %	71 %
		L	7 %	62 %	31 %	93 %
Other	0	D	0 %	100 %	0 %	100 %
		E	0 %	100 %	0 %	100 %
		F	0 %	100 %	0 %	100 %
		I	100 %	0 %	0 %	0 %
		K	100 %	0 %	0 %	0 %
		N	0 %	100 %	0 %	100 %
		P	96 %	4 %	0 %	4 %
	1	A	0 %	100 %	0 %	100 %
		G	0 %	20 %	80 %	100 %
		H	0 %	100 %	0 %	100 %
		K	34 %	11 %	55 %	66 %
		M	0 %	80 %	20 %	100 %
		P	100 %	0 %	0 %	0 %
		S	17 %	33 %	50 %	83 %
		T	0 %	84 %	16 %	100 %
		V	15 %	35 %	50 %	85 %
Y	0 %	100 %	0 %	100 %		
Grand Total			10 %	82 %	8 %	90 %

^a N-termini are categorized according to their NAT substrate class. Bold numbers indicate categories with notable differences in Nt-acetylation levels between transmembrane and non-transmembrane proteins. (See also Table S1 for a complete list of all N-termini identified).