N-Terminal Acetylation Inhibits Protein Targeting to the Endoplasmic Reticulum

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Abstract

Amino-terminal acetylation is probably the most common protein modification in eukaryotes with as many as 50%–80% of proteins reportedly altered in this way. Here we report a systematic analysis of the predicted N-terminal processing of cytosolic proteins versus those destined to be sorted to the secretory pathway. While cytosolic proteins were profoundly biased in favour of processing, we found an equal and opposite bias against such modification for secretory proteins. Mutations in secretory signal sequences that led to their acetylation resulted in mis-sorting to the cytosol in a manner that was dependent upon the N-terminal processing machinery. Hence N-terminal acetylation represents an early determining step in the cellular sorting of nascent polypeptides that appears to be conserved across a wide range of species.

Author Summary

The eukaryotic cell comprises several distinct compartments, called organelles, required to perform specific functions. The proteins in these compartments are almost always synthesised in the cytoplasm and so require complex sorting mechanisms to ensure their delivery to the appropriate organelle. Of course, not all proteins need to leave the cytoplasm since many remain there to perform cytoplasmic functions. It is well known that many proteins are modified by acetylation of their amino-terminus at a very early stage in their synthesis. We have discovered a profound difference between the likelihood of such a modification on cytoplasmic proteins and on those destined for one of the major organelles, the endoplasmic reticulum (ER): whereas cytoplasmic proteins are typically acetylated, those bound for the ER are largely unmodified. Moreover, when specific ER proteins were engineered to induce their acetylation we found that their targeting to the ER was inhibited. Our data suggest that N-terminal acetylation is a major determinant in protein sorting in eukaryotes.

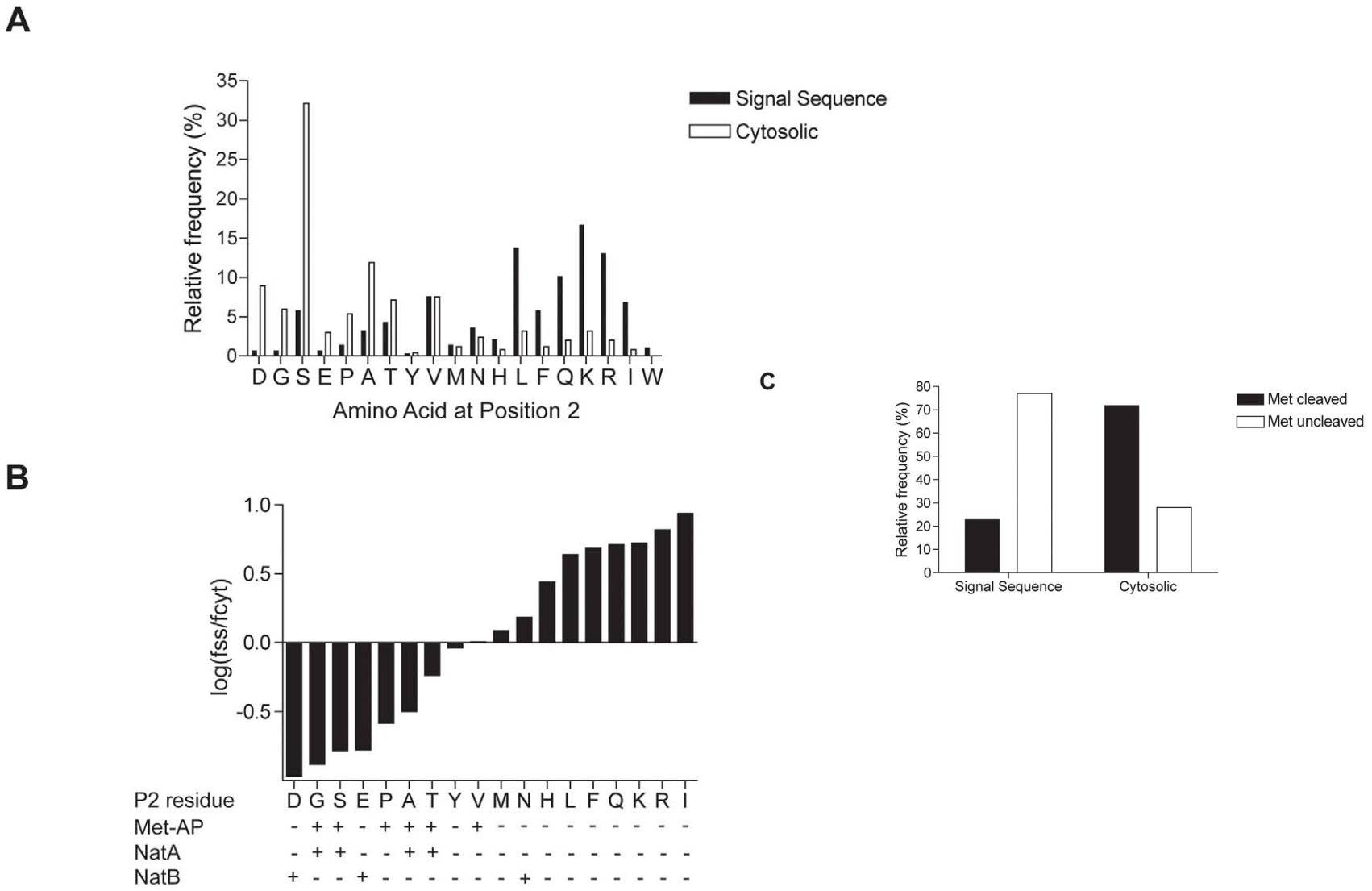

Figure 1. Amino acid frequency at P2 of signal sequences versus cytosolic proteins. (A) Relative frequency of amino acids at P2 of a filtered set of 277 signal sequence-containing proteins from *S. cerevisiae* was compared to a similar size group ($n = 252$) of randomly selected cytosolic proteins. Frequency distribution between the groups differed significantly ($p < 0.0001$, $\chi^2 = 207.3$ 18 *df*). (B) Ratio of relative frequency of P2 residues between signal sequence (f_{ss}) and cytosolic (f_{cyt}) proteins. Tryptophan was absent from the cytosolic group; therefore, no log(f_{ss}/f_{cyt}) value is plotted. P2 specificities of MetAP, NatA, and NatB are indicated. (C) Predicted methionine cleavage of signal sequence and cytosolic N-termini based on relative P2 frequency. For complete datasets, see Tables S1–S4.

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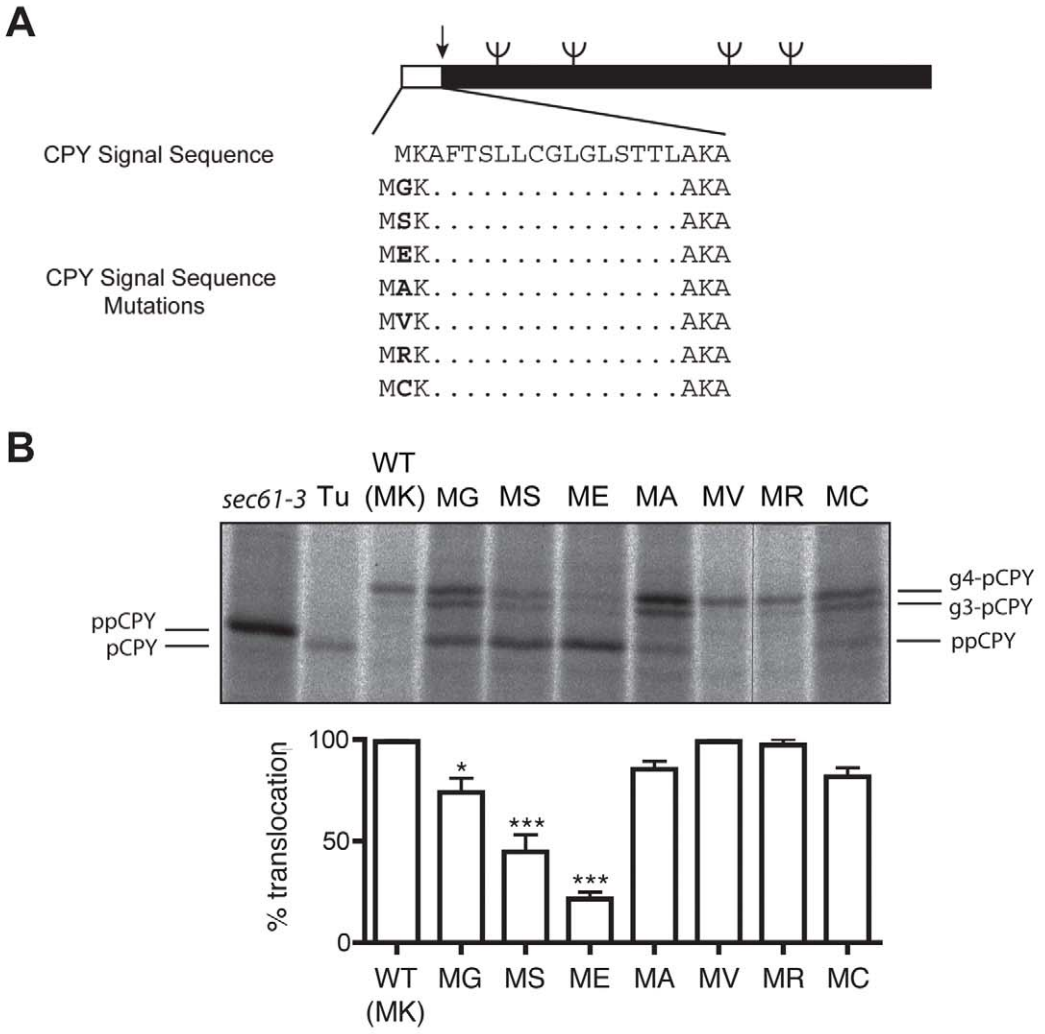

Figure 2. Removal of the N-terminal methionine inhibits ER translocation of CPY. (A) Schematic of wild-type CPY and P2 mutants. Signal peptide sequence, position of N-glycosylation (ψ), and signal peptidase cleavage (↓) sites are indicated. (B) Yeast cells (*Δpep4,Δprc1*) expressing either wild-type or mutant CPY were pulse-labelled with [^{35}S]methionine/cysteine, then CPY immunoprecipitated, and analysed by SDS-PAGE and phosphorimaging. Positions of glycosylated CPY (g4-pCPY and g3-pCPY) are indicated as are the untranslocated ppCPY and signal-sequence cleaved, non-glycosylated CPY (pCPY) observed in *sec61-3* cells and in tunicamycin-treated wild-type cells (Tu), respectively. Translocation efficiency was determined by quantification of ppCPY and g3- and g4-pCPY from three independent experiments. Error bars represent standard error of the mean. Asterisks represent $p<0.05$ (*) and $p<0.001$ (***) according to the one-way analysis of variance with Tukey's multiple comparison test. (C) CPY translocation was analysed as in (B), in a wild-type (*Δpep4,Δprc1*) and isogenic *Δmap1* strain in the presence and absence of the Map2 inhibitor fumagillin (for quantification, see Figure S1).

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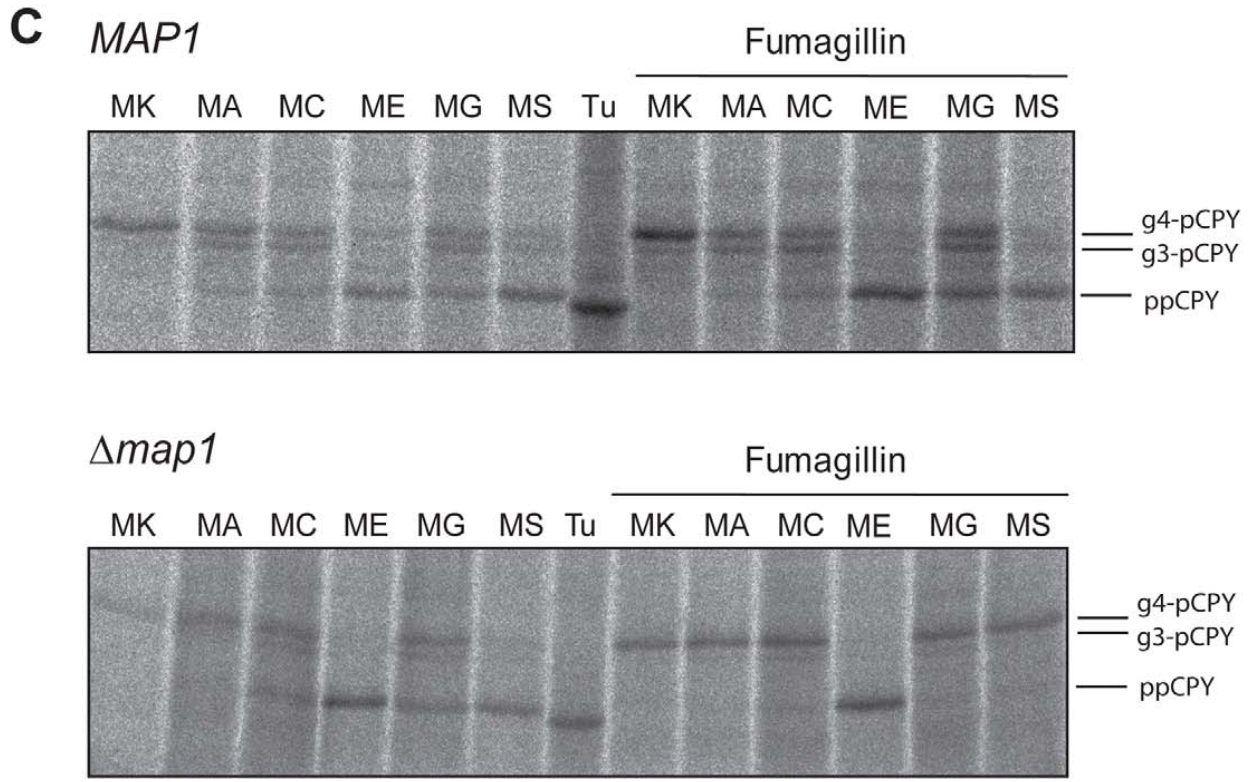

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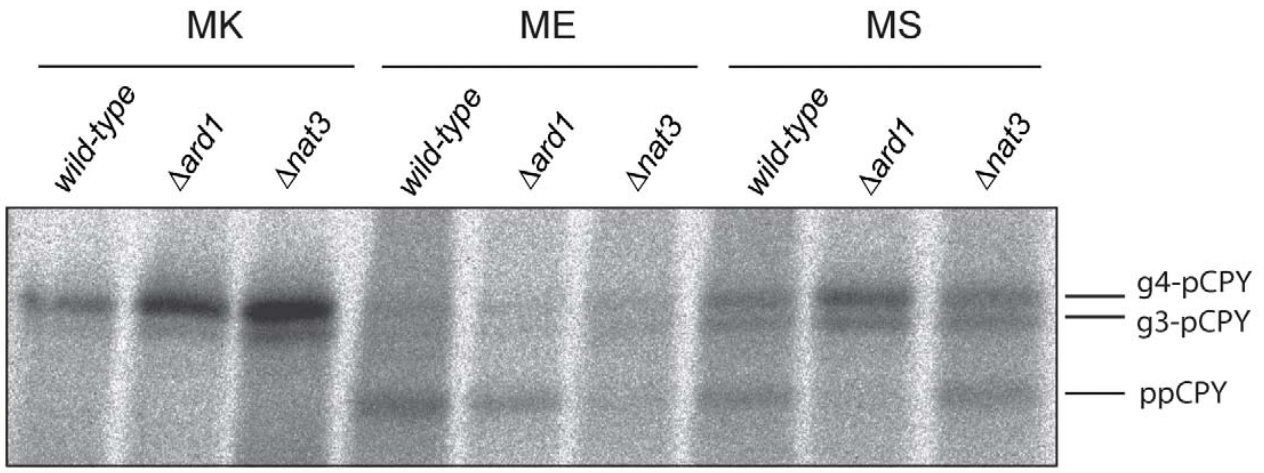

Figure 3. N-terminal acetylation blocks protein translocation. Translocation of wild-type, MS, and ME mutants of CPY was examined z(as in Figure 2B) in wild-type and *Δard1* and *Δnat3* strains, which lack NatA and NatB activity, respectively. Data are representative of three independent experiments.

doi:10.1371/journal.pbio.1001073.g003

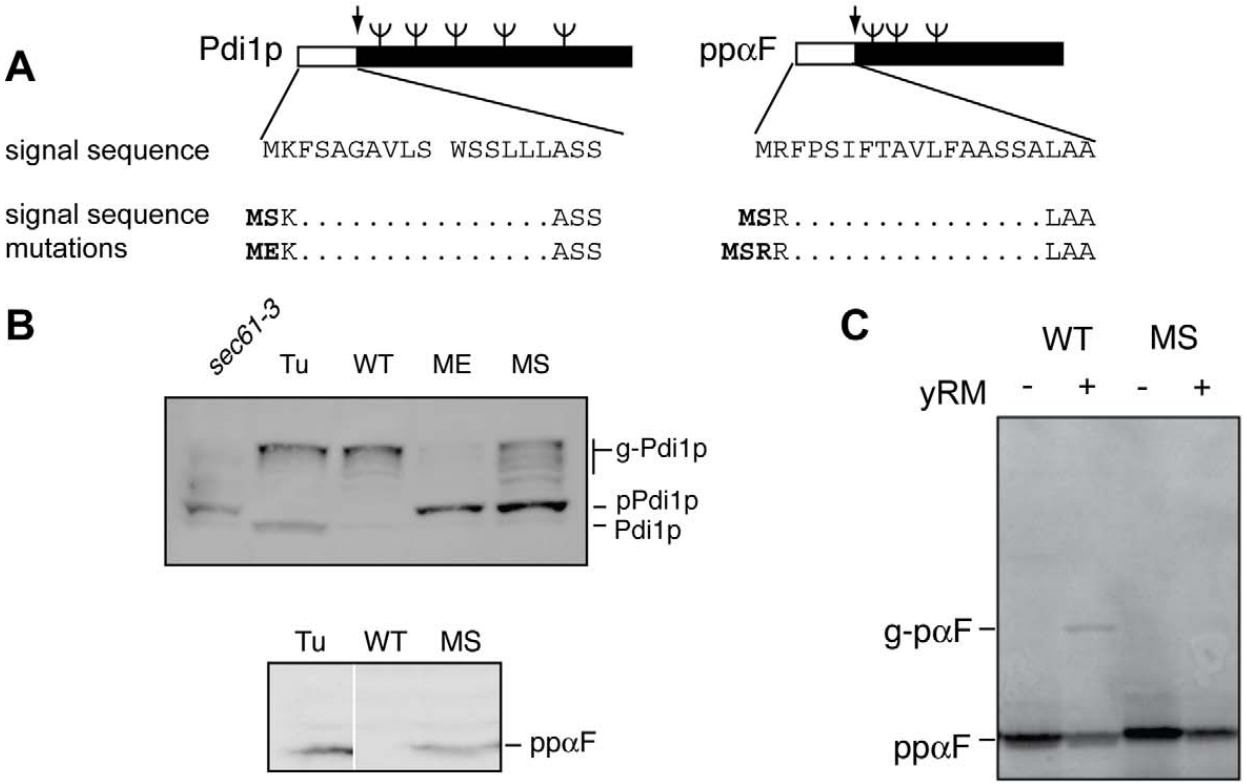

Figure 4. Protein N-acetylation inhibits ER translocation both in vivo and in vitro. (A) Schematic of wild-type and P2 signal sequence mutants of Pdi1p and preproα-factor. Position of N-glycosylation (ψ) and signal peptidase cleavage ($\downarrow$) sites are indicated. (B) Wild-type and indicated mutants of myc-tagged Pdi1p and ppαF were expressed in wild-type (*Δpep4*) or *sec61-3* strains, and treated, where indicated, with Tunicamycin (Tu). Steady-state levels of protein were determined by preparation of cell extracts from these strains and analysis by Western blot with anti-myc antibodies. (C) Wild-type (MR) and MS forms of lysine-less ppαF (where all lysines had been mutated to arginine) were translated in vitro, then incubated with yeast microsomes (yRM). Position of non-translocated (ppαF) and signal-sequence cleaved, glycosylated (g-pαF) are indicated. (D) Lysine-less forms of both wild-type (MR) and MS ppαF were translated in vitro in the presence of either [^{35}S] methionine or [^{14}C] acetyl-CoA and immuno-precipitated with anti-ppαF antibodies before analysis by either scintillation counting or SDS-PAGE. Error bars represent standard deviation; three asterisks indicate $p<0.001$ according to the two-tailed student's *t* test. (E) Wild-type (MR) and MS ppαF with lysine residues at positions 5 and 12 were translated in vitro in the presence of [^{35}S] methionine and TDBA-lysyl-tRNA. Targeting to microsomes was performed in the absence of ATP and then cross-linking induced by uv-irradiation. Where indicated, samples were denatured and immuno-precipitated with Sec61 antisera.
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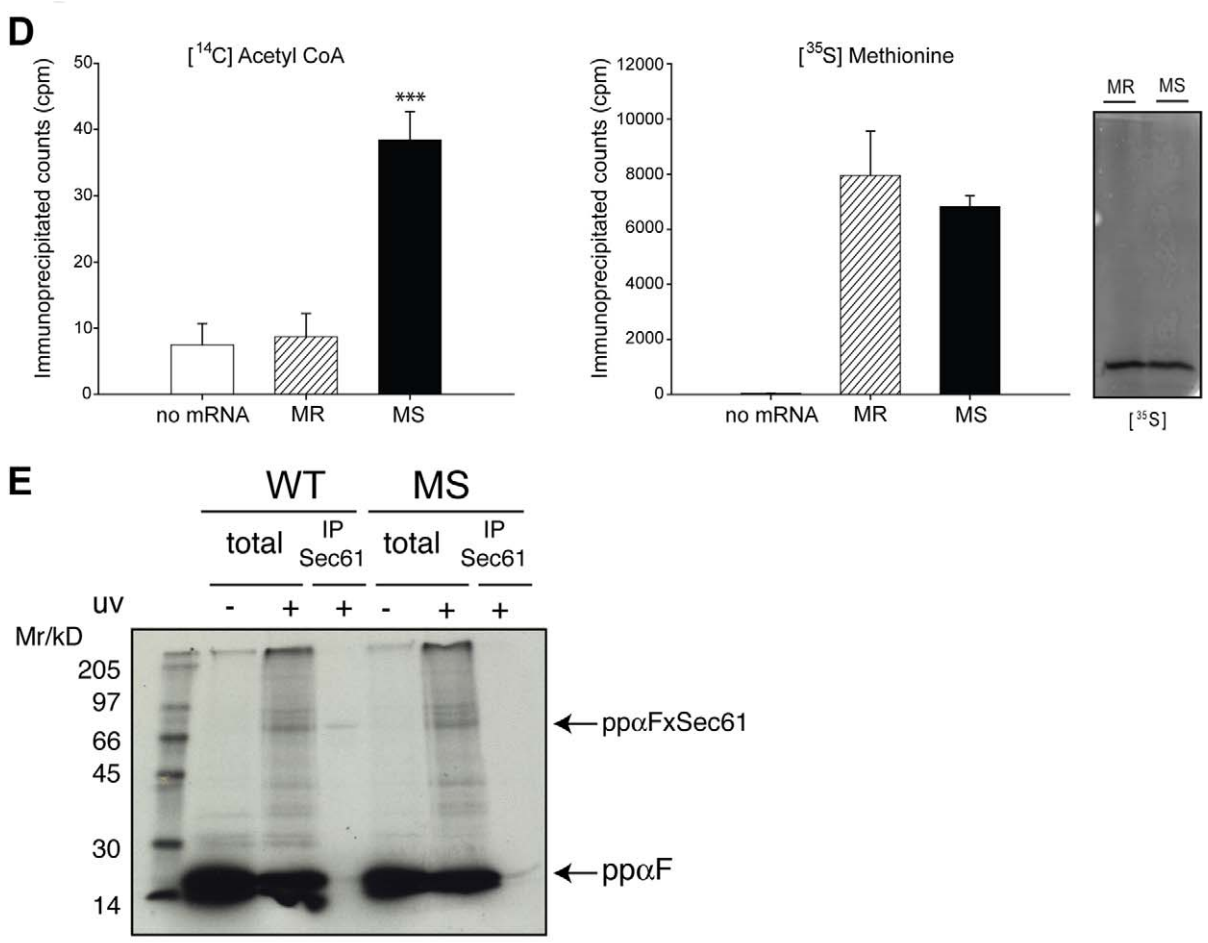

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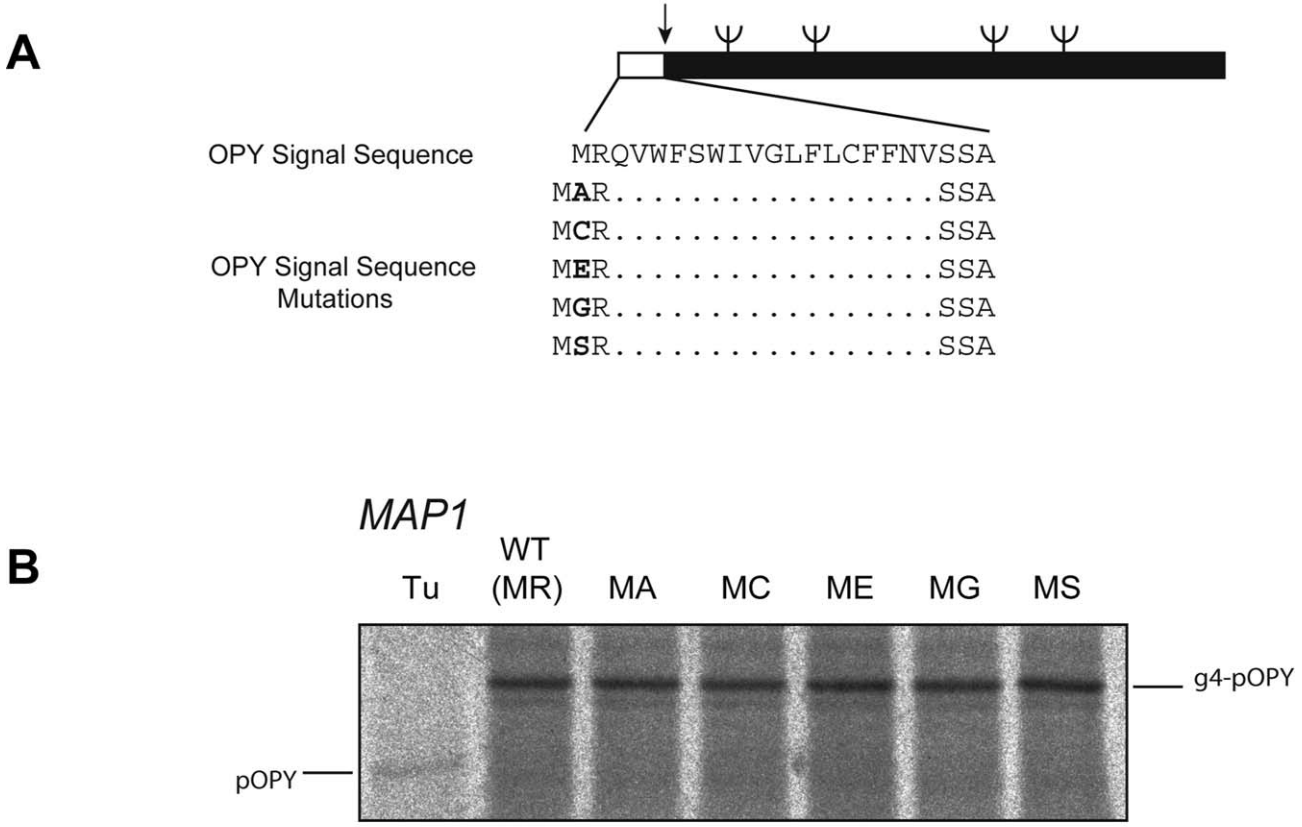

Figure 5. An SRP-dependent precursor is refractory to N-acetylation. (A) Schematic of wild-type OPY (CPY with the endogenous signal sequence replaced by that of Ost1) and corresponding P2 signal sequence mutants. (B) Wild-type and mutant OPY translocation in vivo was monitored by pulse-labelling and immunoprecipitation as in Figure 2B. (C) Lysine-less wild-type (MR) and MS opαF (ppαF with the signal sequence replaced with that of Ost1p and all lysines mutated to arginine) were translated in vitro in the presence of [^{35}S] methionine, denatured, and modified with amine-reactive sulfo-NHS-SS-biotin. Biotinylated proteins were re-isolated on immobilized-streptavidin and analysed by SDS-PAGE and phosphorimaging.

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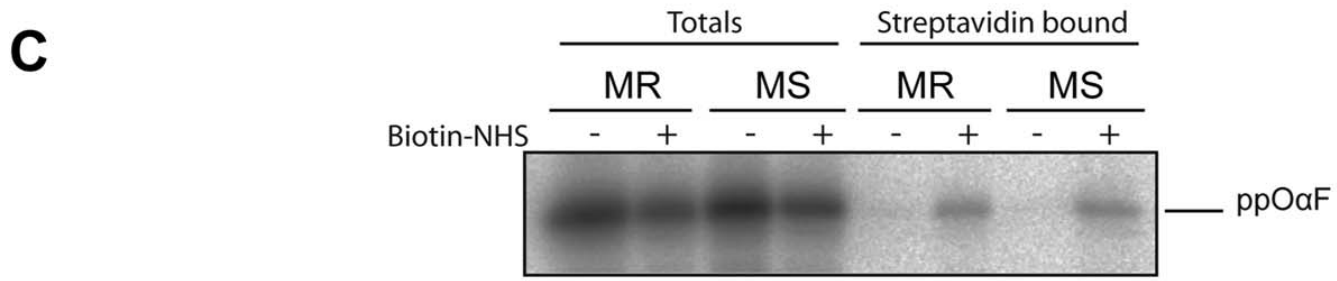

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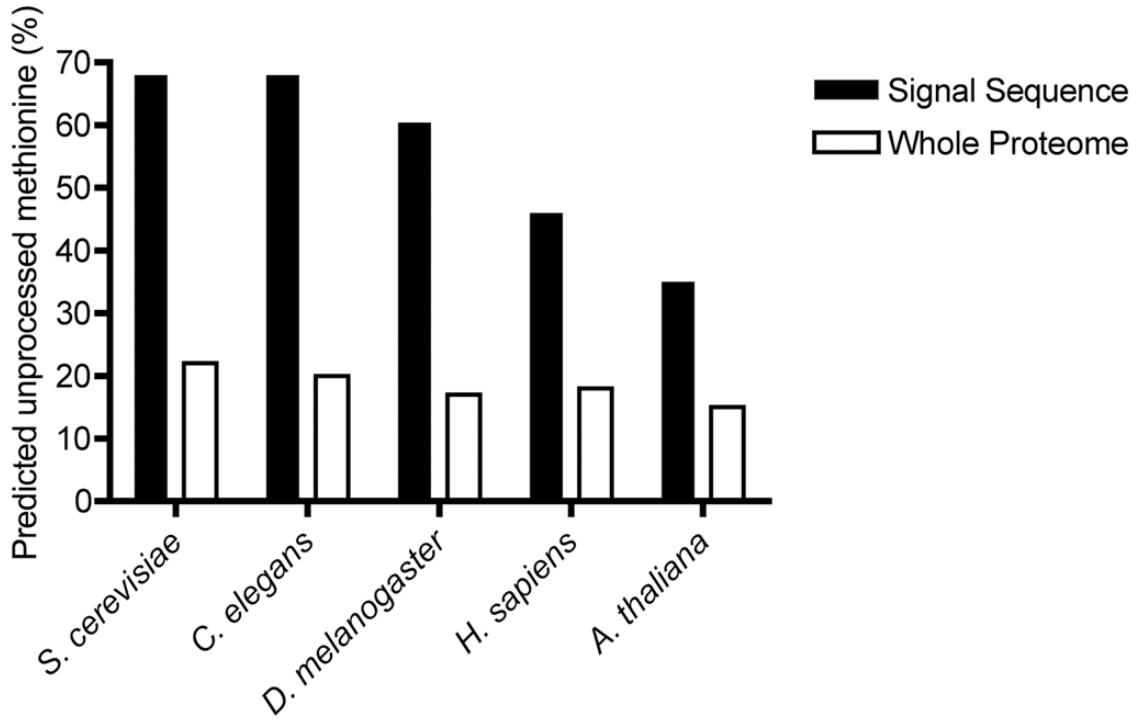

Figure 6. A bias against N-terminal processing of signal sequences is conserved across eukaroytes. Predicted frequency of an unprocessed initiating methionine in signal sequences from *S. cerevisaie* ($n = 277$), *C. elegans* ($n = 378$), *Drosophila* ($n = 448$), human ($n = 595$), and *Arabidopsis* ($n = 500$) compared to the respective proteomes as a whole [2]. For complete datasets, see Tables S5 and S6.
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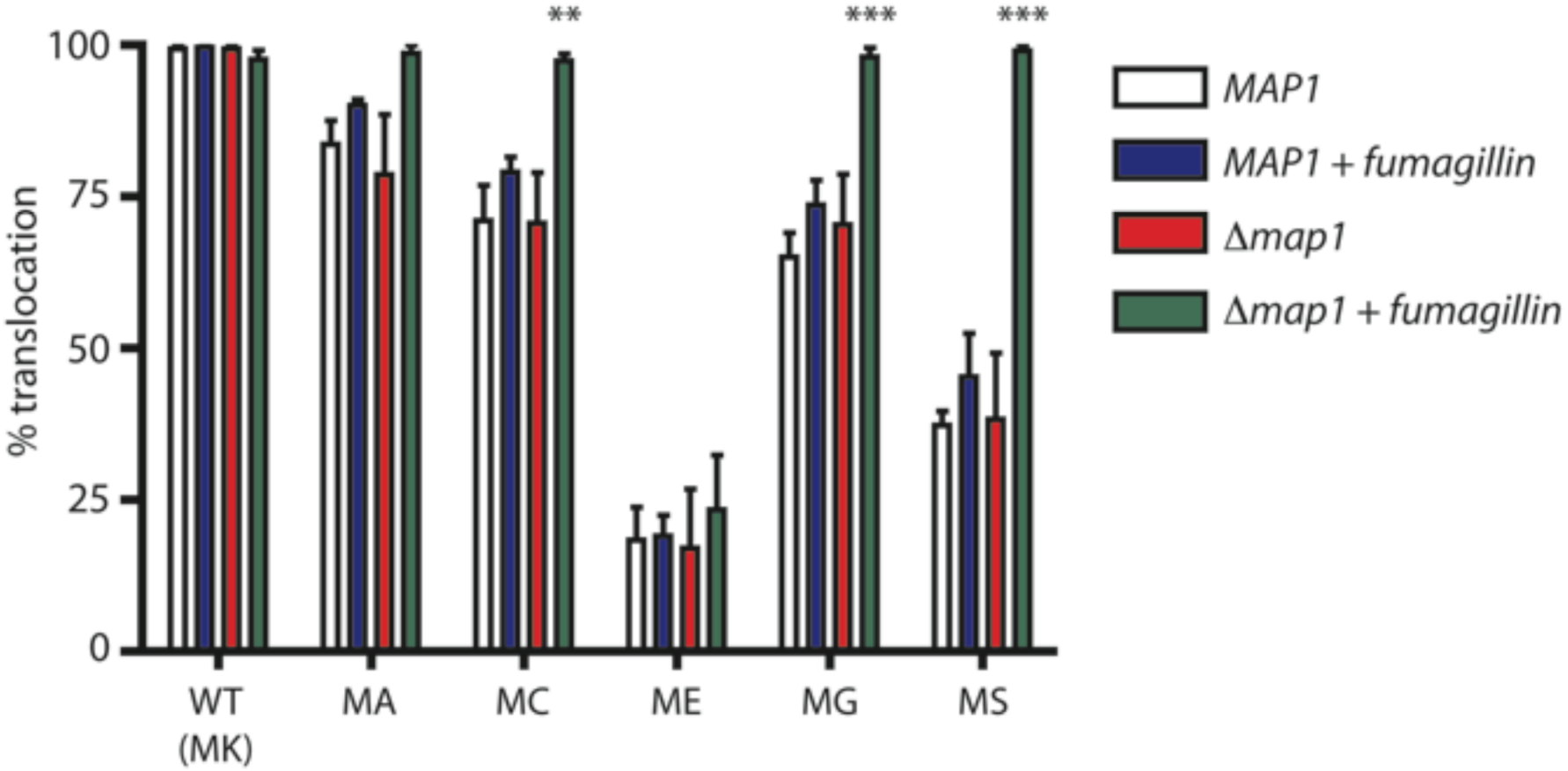
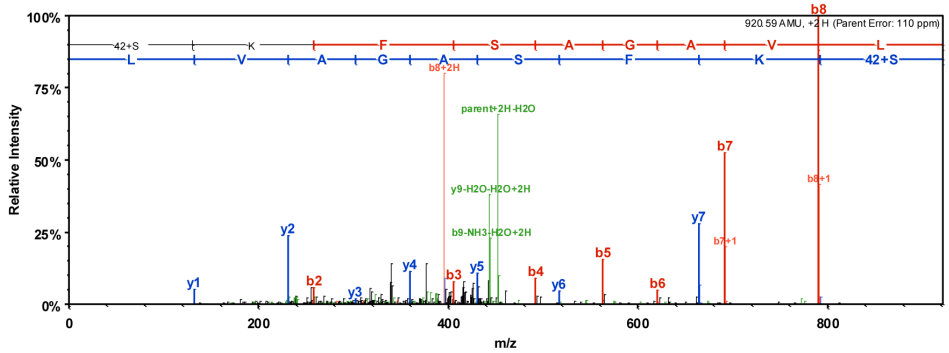


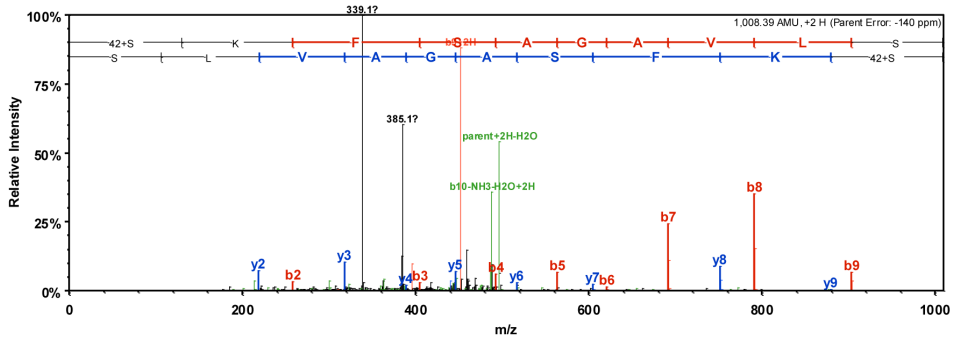
Figure S1 Quantification of CPY translocation in the presence and absence of MetAP activity. Pulse-labelling of WT (MK) CPY and mutants with A, C, E, G, and S inserted at P2 was performed in wild-type (*MAP1 Δprc1 Δpep4*) and *Δmap1* (*Δprc1 Δpep4*) yeast cells in the presence and absence of the Map2 inhibitor fumagillin. CPY was immunoprecipitated and analysed by SDS-PAGE and phosphorimaging (see Figure 2). Translocation efficiency was determined from quantification of the relative amounts of glycosylated-CPY and non-translocated pCPY. The data are displayed graphically and represent the means of three independent experiments. Error bars represent the standard error of the mean. Asterisks represent statistically significant differences to the untreated wild-type (*MAP1*) strain with $p<0.01$ (**) and $p<0.001$ (***) according to the two-way analysis of variance.
(TIF)

A Ac-SKFSAGAVL



B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	130.0			112.0	S+42	921.5	461.3	904.5	903.5	9
2	258.1	129.6	241.1	240.1	K	792.5	396.7	775.4	774.5	8
3	405.2	203.1	388.2	387.2	F	664.4			646.4	7
4	492.2	246.6	475.2	474.2	S	517.3			499.3	6
5	563.3	282.1	546.3	545.3	A	430.3				5
6	620.3	310.7	603.3	602.3	G	359.2				4
7	691.3	346.2	674.3	673.3	A	302.2				3
8	790.4	395.7	773.4	772.4	V	231.2				2
9	921.5	461.3	904.5	903.5	L	132.1				1

B Ac-SKFSAGAVLS



B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	130.0			112.0	S+42	1,008.5	504.8	991.5	990.5	10
2	258.1	129.6	241.1	240.1	K	879.5	440.3	862.5	861.5	9
3	405.2	203.1	388.2	387.2	F	751.4			733.4	8
4	492.2	246.6	475.2	474.2	S	604.3			586.3	7
5	563.3	282.1	546.3	545.3	A	517.3			499.3	6
6	620.3	310.7	603.3	602.3	G	446.3			428.3	5
7	691.3	346.2	674.3	673.3	A	389.2			371.2	4
8	790.4	395.7	773.4	772.4	V	318.2			300.2	3
9	903.5	452.3	886.5	885.5	L	219.1			201.1	2
10	1,008.5	504.8	991.5	990.5	S	106.0			88.0	1

Figure S2 MS-pPdi1p is Methionine-cleaved and N-acetylated in vivo. MS-pPdi1p-myc was affinity purified from yeast cells with anti-myc antiserum and analysed by SDS-PAGE and staining with Coomassie brilliant blue (Text S1). The MS-pPdi1p-myc precursor band was excised, digested with elastase, and analysed by LC-MS/MS (Text S1). Product ion spectra and associated fragmentation tables, which list all the fragment ions observed (highlighted), are shown for two N-terminal peptides. No peptides corresponding to an unmodified N-terminus were detected in the analysis.
(TIF)

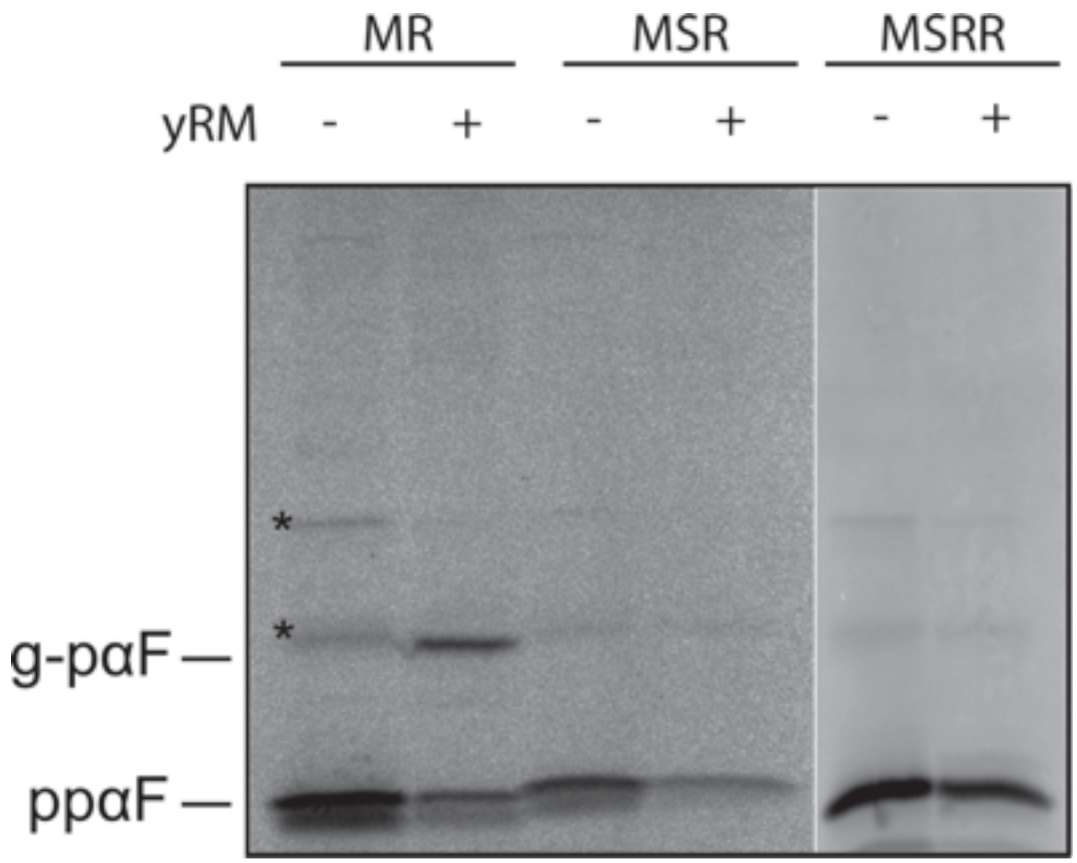


Figure S3 N-acetylation of ppαF blocks translocation in vitro. Wild-type (MR), MSR, and MSRR ppαF were translated in vitro in rabbit reticulocyte lysate and then incubated with yeast microsomes (yRM). Position of non-translocated (ppαF) and signal-sequence cleaved, glycosylated (g-pαF) are indicated. (*) Ubiquitinylated ppαF generated in the absence of microsomes.
(TIF)

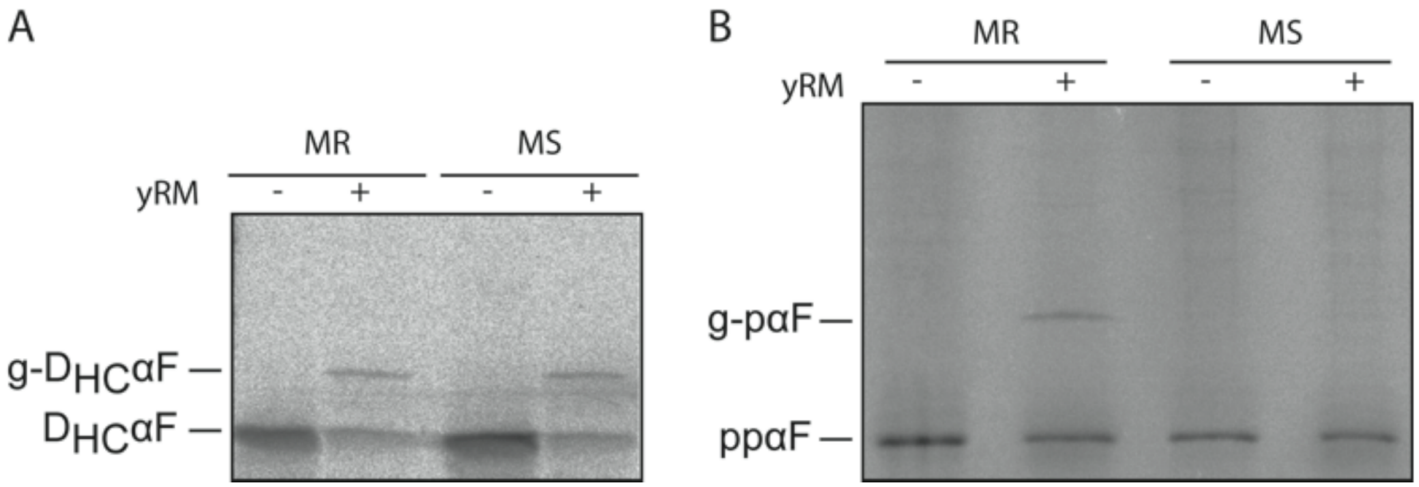


Figure S4 D_{HC}-αF translocation is insensitive to a P2 residue that can promote N-acetylation. (A) D_{HC}-αF comprises ppαF with the hydrophobic core of the signal sequence replaced with that of DPAP B, creating an SRP-dependent substrate. D_{HC}-αF with the endogenous P2 residue (MR) or with a serine inserted at position 2 (MS) were translated in vitro in a yeast extract supplemented with [^{35}S] methionine in the presence or absence of yeast microsomes (yRM). Translated proteins were immunoprecipitated with anti-αF antibodies prior to analysis by SDS-PAGE and phosphorimaging. Positions of the unprocessed (D_{HC}αF) and glycosylated (g-D_{HC}αF) forms of the protein are indicated. (B) WT and MS ppαF were translated in yeast extract in the presence of [^{35}S] methionine and incubated with or without yeast microsomes.
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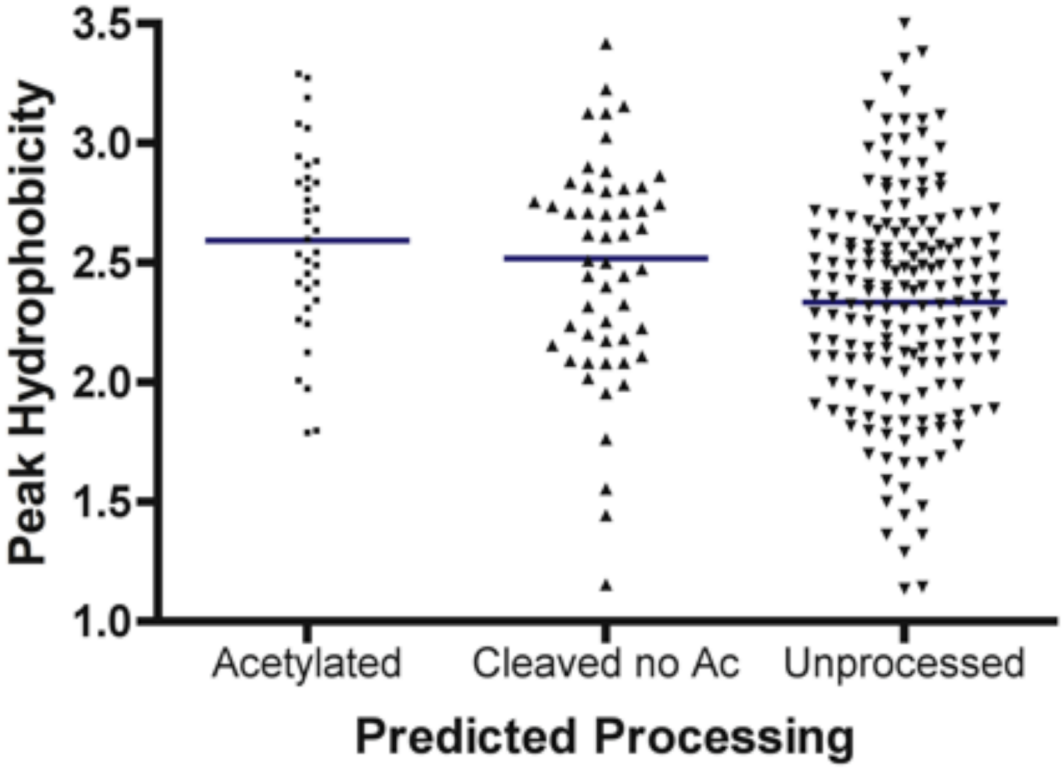

Figure S5 Peak hydrophobicity analysis of Yeast Signal Sequences. Mean peak hydrophobicity of yeast signal sequences group according to their predicted N-terminal processing. Peak hydrophobicity determined based on Kyte-Doolittle [57] with a window size of 11. The "acetylated," "methionine cleaved not acetylated," and "non-processed" groups had mean peak hydrophobicities of 2.593±0.0657 (SEM), 2.518±0.0673, and 2.333±0.0352, respectively. The "acetylated" and "cleaved not acetylated" groups differed significantly from the "unprocessed" group ($p<0.01$ and $p<0.05$, respectively, one-way ANOVA with Tukey's multiple comparison test). The acetylated and cleaved group were not significantly different. Note that only two signal sequences of the acetylated group (<6%) had a peak hydrophobicity of less than 2, the threshold for interaction with SRP [30]. (TIF)

Table S3: Relative Amino Acid Frequency at Position 2 by Compartment in Yeast

P2 Residue	Signal Sequence	Cytosol	f(ss)/f(cyt)
A	3.25	10.32	0.317
C	0.00	0.00	∞
D	0.72	6.75	0.108
E	0.72	4.37	0.167
F	6.50	1.19	4.887
G	0.72	5.56	0.131
H	2.17	0.79	2.749
I	6.86	0.79	8.705
K	16.61	3.17	5.269
L	13.72	3.17	4.353
M	1.44	1.19	1.222
N	3.61	2.38	1.527
P	1.44	5.56	0.262
Q	10.11	1.98	5.132
R	13.00	1.98	6.598
S	5.78	35.32	0.165
T	4.33	7.54	0.579
V	7.58	7.54	1.013
W	1.08	0.00	∞
Y	0.36	0.40	0.916
Total	100.00	100.00	
n	277	251	

Table S5 Relative P2 Frequency of Signal Sequences from different Organisms

P2 residue	Human	C. elegans	S. cerevisiae	D.melanogaster	A.thaliana
A	18.99	1.59	2.94	7.81	25.00
C	0.34	1.85	0.00	0.89	0.40
D	1.85	1.06	1.10	1.34	3.57
E	5.04	1.32	1.10	2.23	7.74
F	2.02	4.23	6.62	6.47	0.79
G	9.41	2.12	0.74	4.02	8.33
H	1.01	2.65	2.21	2.01	0.40
I	3.03	7.41	6.62	2.46	1.39
K	9.92	12.96	16.18	18.08	18.45
L	8.07	7.94	11.40	8.48	3.17
M	1.51	2.38	1.84	3.57	2.78
N	1.68	8.47	4.78	6.70	2.98
P	2.86	2.65	1.10	3.57	0.79
Q	3.70	4.23	11.76	4.91	0.99
R	13.28	23.54	13.24	10.04	6.15
S	5.21	5.82	5.51	7.14	8.53
T	2.69	5.56	4.04	3.57	4.37
V	6.05	1.85	7.72	2.46	3.57
W	2.18	1.32	0.74	2.01	0.20
Y	1.18	1.06	0.37	2.23	0.40
n	595	378	277	448	500

Table S6 Predicted Frequency of N-terminal processing of Signal Sequences from different organisms

	Predicted N-terminal Processing				
Organism	Unprocessed	Met-cleaved	Uncleaved & acetylated	Met-cleaved & acetylated	n
S. ceverevisiae	66	16	11	7	277
C. elegans	68	15	11	6	378
D. melanogaster	60	17	10	13	448
Human	46	23	23	8	595
A. thaliana	34	24	14	28	500

Table S7 ***Yeast strains used in this study***

Name	*Genotype*	**Reference**
MWY63	*Mat***a** *prc1::Kan*MX *leu2 his3 ura3 ade2 pep4-3 sec61-3*	[1]
Δprc1	*Matα prc1::KanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	[2]
Δpep4	*Mat***a** *pep4::KanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	[2]
GFY3	*Mat***a** *prc1::KanMX4 pep4:: KanMX4 his3Δ1 leu2Δ0 ura3Δ0*	This study
GFY7	*Mat***a** *prc1::KanMX4 pep4:: KanMX4 map1::HIS3MX6 his3Δ1 leu2Δ0 ura3Δ0*	This study
GFY11	*Matα prc1::KanMX4 ard1::hph his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	This study
GFY12	*Matα prc1::KanMX4 nat3:: hph his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	This study

References

[1] Willer M, Forte GM, Stirling CJ (2008) Sec61p is required for ERAD-L: genetic dissection of the translocation and ERAD-L functions of Sec61p using novel derivatives of CPY. J Biol Chem. 283: 33883-33888.

[2] Winzeler EA, Shoemaker DD, Astromoff A, Liang H, Anderson K, et al. (1999) Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science 285: 901-906.

Table S8 ***Oligonucleotides used in this study***

Name	Sequence	Description
Map1 KO F	TCCTAGCAAGAAAAAAATAAGCAAAAAAATTGTATAATCGGATCCCCGGGTTAATTAA	*HIS3MX6* disruption *MAP1*
Map1 KO R	GTACAAGTTCAAGTTTTTTATTGGTTTCTATATGTATAAGATAAGAATTCGAGCTCGTTTAAAC	*HIS3MX6* disrupt *MAP1*
Ard1/A KO F2	AAATACATACGATCAAGCTCCAAAATAAACTTCGTCAACCCGGATCCCCGGGTTAATTAA	Hph disruption *ARD1*
Ard1/A KO R	CCTGGATGAAAATATACTACGTTTATATAGGTTGATTTAAAATACGACTCACTATAGGGAG	Hph disruption *ARD1*
Nat3/B KO F2	ATTGAGAATATTCAAGGAAAGAGACAGGAGGATTCGAGAACGGATCCCCGGGTTAATTAA	Hph disruption *NAT3*
Nat3/B KO R	ATTATTATGTTCTGAGTATGAGGACGAGGTAATACATACCAATACGACTCACTATAGGGAG	Hph disruption *NAT3*
CPY-A-F	CAACTTAAAGTATACATACGCTATGGCTAAAGCATTCACCAGTTTACTATG	SDM of pMW346
CPY-A-R	CATAGTAAACTGGTGAATGCTTTAGCCATAGCGTATGTATACTTTAAGTTG	"
CPY-C-F	CAACTTAAAGTATACATACGCTATGTGTAAAGCATTCACCAGTTTACTATG	"
CPY-C-R	CATAGTAAACTGGTGAATGCTTTACACATAGCGTATGTATACTTTAAGTTG	"
CPY-E-F	CAACTTAAAGTATACATACGCTATGGAAAAAGCATTCACCAGTTTACTATG	"
CPY-E-R	CATAGTAAACTGGTGAATGCTTTTTCCATAGCGTATGTATACTTTAAGTTG	"
CPY-G-F	CAACTTAAAGTATACATACGCTATGGGTAAAGCATTCACCAGTTTACTATG	"
CPY-G-R	CATAGTAAACTGGTGAATGCTTTACCCATAGCGTATGTATACTTTAAGTTG	"
CPY-R-F	CAACTTAAAGTATACATACGCTATGAGAAAAGCATTCACCAGTTTACTATG	"
CPY-R-R	CATAGTAAACTGGTGAATGCTTTTCTCATAGCGTATGTATACTTTAAGTTG	"
CPY-S-F	CAACTTAAAGTATACATACGCTATGTCCAAAGCATTCACCAGTTTACTATG	"
CPY-S-R	CATAGTAAACTGGTGAATGCTTTGGACATAGCGTATGTATACTTTAAGTTG	"
CPY-V-F	CAACTTAAAGTATACATACGCTATGGTCAAAGCATTCACCAGTTTACTATG	"
CPY-V-R	CATAGTAAACTGGTGAATGCTTTGACCATAGCGTATGTATACTTTAAGTTG	"
OPY-A-F	GGTGCTGAAAAAATGGCTAGGCAGGTTTGGTTC	SDM of pOPY
OPY-A-R	GAACCAAACCTGCCTAGCCATTTTTTCAGCACC	"
OPY-C-F	GGTGCTGAAAAAATGTGTAGGCAGGTTTGGTTC	"
OPY-C-R	GAACCAAACCTGCCTACACATTTTTTCAGCACC	"
OPY-E-F	GGTGCTGAAAAAATGGAAAGGCAGGTTTGGTTC	"
OPY-E-R	GAACCAAACCTGCCTTTCCATTTTTTCAGCACC	"
OPY-G-F	GGTGCTGAAAAAATGGGTAGGCAGGTTTGGTTC	"
OPY-G-R	GAACCAAACCTGCCTACCCATTTTTTCAGCACC	"
OPY-S-F	TTGGTGCTGAAAAAATGTCTAGGCAGGTTTGGTTCTCTTGG	"
OPY-S-R	CCAAGAGAACCAAACCTGCCTAGACATTTTTTCAGCACCAA	"
PDI-F	ACTTAAAGTTATAACATACGCTATGAAGTTTTCTGCTGGTGCCGTC	Myc-tagging PDI1
PDI-S-F	ACTTAAAGTTATAACATACGCTATGTCTAAGTTTTCTGCTGGTGCCGTC	SDM of pPDI1-myc
PDI-E-F	ACTTAAAGTTATAACATACGCTATGGAGAAGTTTTCTGCTGGTGCCGTC	"
PDI-myc-R	ACCACCGTGGATCCATTCCTACAAATCTTCTTCAGAAATCAATTTTTGTTCCAATTCATCGTGAA TGGCATC	Myc-tagging PDI1
PPαF-F	CAACTTAAAGTATACATACGCTATGAGATTTCCTTCAAT	Myc-tagging ppαF
PPαF-S-F	CAACTTAAAGTATACATACGCTATGTCTAGATTTCCTTCAAT	SDM of ppAF-2myc
PPαF-2myc-R	ACCACCGTGGATCCATTCTTACAAATCTTCTTCAGAAATCAATTTTTGTTCCAGGTCCTCCTCG CTGATCAGCTTCTGCTCGTACATTGGTTGGCCGGG	Myc-tagging ppαF
ppαF wt sp6 F	ATTTAGGTGACACTATAGACGATTAAAAGAATGAGATTTC	MR mRNA template
ppαF S sp6 F	ATTTAGGTGACACTATAGACGATTAAAAGAATGTCCAGATTTCCTTCAAT	MSR mRNA template
ppαF msrr F	ATTTAGGTGACACTATAGACGATTAAAAGAATGTCCAGAAGATTTCCTTC	MSRR mRNA template
pEH3 R	GCCTGCAGGTCGACTCTAGAGTCG	pEH3 mRNA template
ppalpha k5 R	GCATGCCTGCAGGTCGACTTTGTTAC	pGF22 mRNA template
Ost1 ss EcoRI-f	GCAAGCTGAATTCCTTCTTTGACAAGTACCCGATTGC	PCR OST1 signal sequence

Table S9 ***Plasmids used in this study***

Name	Description	Source
pMW346	ppCPY *LEU2* pRS315	[1]
pCPY-A	SDM of pMW346 Alanine inserted at position 2 of signal sequence	This study
pCPY-C	SDM of pMW346 Cysteine inserted at position 2 of signal sequence	This study
pCPY-E	SDM of pMW346 Glutamate inserted at position 2 of signal sequence	This study
pCPY-G	SDM of pMW346 Glycine inserted at position 2 of signal sequence	This study
pCPY-R	SDM of pMW346 Arginine inserted at position 2 of signal sequence	This study
pCPY-S	SDM of pMW346 Serine inserted at position 2 of signal sequence	This study
pCPY-V	SDM of pMW346 Valine inserted at position 2 of signal sequence	This study
pOPY	pMW346 modified so that Ost1 signal sequence replaced that of CPY	This study
pOPY-A	SDM of pOPY Alanine inserted at position 2 of signal sequence	This study
pOPY-C	SDM of pOPY Cysteine inserted at position 2 of signal sequence	This study
pOPY-E	SDM of pOPY Glutamate inserted at position 2 of signal sequence	This study
pOPY-G	SDM of pOPY Glycine inserted at position 2 of signal sequence	This study
pOPY-S	SDM of pOPY Serine inserted at position 2 of signal sequence	This study
pPDI-myc	pMW346 with CPY ORF replaced with C-terminal myc-tagged *PDI1*	This study
pPDI-myc-S	pMW346 with CPY ORF replaced with C-terminal myc-tagged *PDI1*-MS mutant	This study
pPDI-myc-E	pMW346 with CPY ORF replaced with C-terminal myc-tagged *PDI1*-ME mutant	This study
pPPαF-2myc	pMW346 with CPY ORF replaced with C-terminal 2xmyc-tagged ppαF	This study
pPPαF-2myc-S	pMW346 with CPY ORF replaced with C-terminal 2xmyc-tagged ppαF ME mutant	This study
pA11-K5K14	K5 ppαF in pAlter, all lysines codons in *wild-type* ppαF altered to arginine and lysines introduced at positions 5 and 14 of signal sequence	[2]
pEH3	pGEM3-ppαF	[3]
pGF22	pGEM3-ppαF with all lysines codons in *wild-type* ppαF altered to arginine	This study
pGF23	pGF22 cut SalI klenow filled and relegated to remove one HincII site	This study
pGF24	O-αFactor pGF22 signal sequence of ppαF replaced with that of OST1	This study
pGF25	As pGF24 except for insertion of serine at position 2 of OST1 signal sequence	This study
pGF28	As for pGF22 but with the signal sequence replaced by that of D_{HC} from pJD96	This study
pJD96	p-D_{hc}αF (codon-bias optimised)	[4]

References

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[2] Plath K, Mothes W, Wilkinson BM, Stirling CJ, Rapoport TA (1998) Signal sequence recognition in posttranslational protein transport across the yeast ER membrane. Cell 94: 795-807.

[3] Steel GJ, Brownsword J, Striling CJ (2002) Tail-Anchored Protein Insertion into Yeast ER requires Novel a Posttranslational Mechanism Which is Independent of the SEC Machinery. Biochemistry 41: 11914-11920.

[4] Mason N, Ciufo LF, Brown JD (2000) Elongation arrest is a physiologically important function of the signal recognition particle. EMBO J. 19: 4164-4174.

Supporting Methods

Sample preparation

Wild-type *(GFY3)* yeast harbouring plasmid pPdi1myc-S were grown at 30°C in 200 mL minimal media to an OD_{600} of ≈1.8. Cells were washed with dH_2O and then lysed with glass beads in 2 mL of ice cold lysis buffer (50 mM Hepes-KOH pH 7.6, 1 mM EDTA, 0.1 % [v/v] Triton X-100, 0.2 M KCl, 5 mM 2-mercaptoethanol, 10% [v/v] glycerol, 10 μg.mL^{-1} leupeptin, 10 μg.mL^{-1} pepstatin, 0.5 mM PMSF, 1X complete EDTA-free protease inhibitor pill [Roche]). Cellular debris and glass beads were removed by centrifugation (2min, 1200 *g,* 4°C) and then the extracts cleared by further centrifugation (20 min, 16000 *g,* 4°C). Extracts were then preadsorbed for 30 min with 150 μL insoluble Protein A (Sigma), and then rotated with 30 μg anti-myc antibodies (Clone 4A6, Millipore) prebound to 60 μL Protein G-sepharose (Zymed) for 3h in the cold. Beads were then washed twice with lysis buffer, twice with 20 mM Tris-HCl pH7.6, 500 mM NaCl, 0.4% (v/v) NP-40 and once with 10 mM Tris-HCl pH 7.6 prior to elution with SDS-PAGE sample buffer. Eluted material was then separated on a 7.5% Tris-glycine SDS-PAGE gel, stained with Instant Blue (Expedeon) and the MS-pPdi1p-myc precursor band excised. The sample was reduced, alkylated with iodoacetamide, and then digested overnight at 37°C with elastase.

Mass-spectrometry

Digested samples were analysed by LC-MS/MS using a NanoAcquity LC (Waters, Manchester, UK) coupled to a LTQ Velos (Thermo Fisher Scientific, Waltham, MA). Peptides were concentrated on a pre-column (20 mm x 180 μm i.d, Waters). The peptides were then separated using a gradient from 99% A (0.1% formic acid in water) and 1% B (0.1% formic acid in acetonitrile) to 30% B, in 40 min at 300 nL.min^{-1}, using a 75 mm x 250 μm i.d. 1.7 μM BEH C18, analytical column (Waters). Peptides were selected for fragmentation automatically by data-dependant analysis.

Data analysis

Peak lists were generated from the data using the MSN_Extract utility via Mascot Daemon software (Matrix Science, UK). The peak-list file was edited to include four spectra from a previous trypsin digestion and searched against a yeast-specific database with trypsin as the enzyme and fixed carbamidomethyl modification of cysteines using Mascot (v 2.2, Matrix Science, UK). The additional spectra allowed Mascot to identify PDI and the data was then resubmitted using the error-tolerant option required for matching elastase-generated data. Acquired data were further validated using Scaffold (v 3.0, Proteome Software, Portland, OR). Matches were considered significant if they had greater than 95.0% probability as specified by the Peptide Prophet algorithm[1]. Matches were further validated by manual inspection to compare between the highest match and the nearest alternative.

Reference

1. Keller A, Nesvizhskii AI, Kolker E, Aebersold R (2002) Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. Anal Chem 74: 5383-5392.