Consequences of the Endogenous *N*-Glycosylation of Human Ribonuclease 1

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NNN (*P. pastoris*): ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCC
NNQ (*P. pastoris*): ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCC
NQN (*P. pastoris*): ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCC
QNN (*P. pastoris*): ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCC
NQQ (*P. pastoris*): ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCC
QNQ (*P. pastoris*): ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCC
QQN (*P. pastoris*): ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCC
QQQ (*P. pastoris*): ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCC
NNN (*E. coli*):
QQQ (*E. coli*):

NNN (*P. pastoris*): GCATTAGCT**AAG**GAGAGTAGAGCTAAAAAGTTCCAACGTCAACATATG
NNQ (*P. pastoris*): GCATTAGCT**AAG**GAGAGTAGAGCTAAAAAGTTCCAACGTCAACATATG
NQN (*P. pastoris*): GCATTAGCT**AAG**GAGAGTAGAGCTAAAAAGTTCCAACGTCAACATATG
QNN (*P. pastoris*): GCATTAGCT**AAG**GAGAGTAGAGCTAAAAAGTTCCAACGTCAACATATG
NQQ (*P. pastoris*): GCATTAGCT**AAG**GAGAGTAGAGCTAAAAAGTTCCAACGTCAACATATG
QNQ (*P. pastoris*): GCATTAGCT**AAG**GAGAGTAGAGCTAAAAAGTTCCAACGTCAACATATG
QQN (*P. pastoris*): GCATTAGCT**AAG**GAGAGTAGAGCTAAAAAGTTCCAACGTCAACATATG
QQQ (*P. pastoris*): GCATTAGCT**AAG**GAGAGTAGAGCTAAAAAGTTCCAACGTCAACATATG
NNN (*E. coli*): **ATG**AAAGAATCTCGTGCTAAAAAATTCCAGCGTCAGCATATG
QQQ (*E. coli*): **ATG**AAAGAATCTCGTGCTAAAAAATTCCAGCGTCAGCATATG

NNN (*P. pastoris*): GATTCCGACTCCTCTCCATCTTCATCTTCCACATATTGTAATCAAATG
NNQ (*P. pastoris*): GATTCCGACTCCTCTCCATCTTCATCTTCCACATATTGTAATCAAATG
NQN (*P. pastoris*): GATTCCGACTCCTCTCCATCTTCATCTTCCACATATTGTAATCAAATG
QNN (*P. pastoris*): GATTCCGACTCCTCTCCATCTTCATCTTCCACATATTGTAATCAAATG
NQQ (*P. pastoris*): GATTCCGACTCCTCTCCATCTTCATCTTCCACATATTGTAATCAAATG
QNQ (*P. pastoris*): GATTCCGACTCCTCTCCATCTTCATCTTCCACATATTGTAATCAAATG
QQN (*P. pastoris*): GATTCCGACTCCTCTCCATCTTCATCTTCCACATATTGTAATCAAATG
QQQ (*P. pastoris*): GATTCCGACTCCTCTCCATCTTCATCTTCCACATATTGTAATCAAATG
NNN (*E. coli*): GACTCTGACTCTTCTCCGTCTTCTTCTTCTACTTACTGCAACCAGATG
QQQ (*E. coli*): GACTCTGACTCTTCTCCGTCTTCTTCTTCTACTTACTGCAACCAGATG

Aglycosylated Gln codons in red

NNN (*P. pastoris*): ATGAGAAGGAGAAATATGACCCAAGGTCGTTGTAAACCTGTTAATACG
NNQ (*P. pastoris*): ATGAGAAGGAGAAATATGACCCAAGGTCGTTGTAAACCTGTTAATACG
NQN (*P. pastoris*): ATGAGAAGGAGAAATATGACCCAAGGTCGTTGTAAACCTGTTAATACG
QNN (*P. pastoris*): ATGAGAAGGAGA**cag**ATGACCCAAGGTCGTTGTAAACCTGTTAATACG
NQQ (*P. pastoris*): ATGAGAAGGAGAAATATGACCCAAGGTCGTTGTAAACCTGTTAATACG
QNQ (*P. pastoris*): ATGAGAAGGAGA**cag**ATGACCCAAGGTCGTTGTAAACCTGTTAATACG
QQN (*P. pastoris*): ATGAGAAGGAGA**cag**ATGACCCAAGGTCGTTGTAAACCTGTTAATACG
QQQ (*P. pastoris*): ATGAGAAGGAGA**cag**ATGACCCAAGGTCGTTGTAAACCTGTTAATACG
NNN (*E. coli*): ATGCGTCGTCGTAACATGACTCAGGGTCGTTGCAAACCGGTTAACACT
QQQ (*E. coli*): ATGCGTCGTCGT**caa**ATGACTCAGGGTCGTTGCAAACCGGTTAACACT

NNN (*P. pastoris*): TTTGTGCACGAACCATTGGTGGACGTACAGAACGTTTGCTTCCAAGAG
NNQ (*P. pastoris*): TTTGTGCACGAACCATTGGTGGACGTACAGAACGTTTGCTTCCAAGAG
NQN (*P. pastoris*): TTTGTGCACGAACCATTGGTGGACGTACAGAACGTTTGCTTCCAAGAG
QNN (*P. pastoris*): TTTGTGCACGAACCATTGGTGGACGTACAGAACGTTTGCTTCCAAGAG
NQQ (*P. pastoris*): TTTGTGCACGAACCATTGGTGGACGTACAGAACGTTTGCTTCCAAGAG
QNQ (*P. pastoris*): TTTGTGCACGAACCATTGGTGGACGTACAGAACGTTTGCTTCCAAGAG
QQN (*P. pastoris*): TTTGTGCACGAACCATTGGTGGACGTACAGAACGTTTGCTTCCAAGAG
QQQ (*P. pastoris*): TTTGTGCACGAACCATTGGTGGACGTACAGAACGTTTGCTTCCAAGAG
NNN (*E. coli*): TTCGTTCATGAACCGCTGGTTGACGTTCAGAACGTTTGCTTCCAGGAA
QQQ (*E. coli*): TTCGTTCATGAACCGCTGGTTGACGTTCAGAACGTTTGCTTCCAGGAA

NNN (*P. pastoris*): AAAGTCACATGTAAAAATGGACAAGGTAATTGCTACAAGAGTAATTCC
NNQ (*P. pastoris*): AAAGTCACATGTAAAAATGGACAAGGTAATTGCTACAAGAGTAATTCC
NQN (*P. pastoris*): AAAGTCACATGTAAAAATGGACAAGGTAATTGCTACAAGAGTcaaTCC
QNN (*P. pastoris*): AAAGTCACATGTAAAAATGGACAAGGTAATTGCTACAAGAGTAATTCC
NQQ (*P. pastoris*): AAAGTCACATGTAAAAATGGACAAGGTAATTGCTACAAGAGTcaaTCC
QNQ (*P. pastoris*): AAAGTCACATGTAAAAATGGACAAGGTAATTGCTACAAGAGTAATTCC
QQN (*P. pastoris*): AAAGTCACATGTAAAAATGGACAAGGTAATTGCTACAAGAGTcaaTCC
QQQ (*P. pastoris*): AAAGTCACATGTAAAAATGGACAAGGTAATTGCTACAAGAGTcaaTCC
NNN (*E. coli*): AAAGTTACTTGCAAAAACGGTCAGGGTAACTGCTACAAATCTAACTCT
QQQ (*E. coli*): AAAGTTACTTGCAAAAACGGTCAGGGTAACTGCTACAAATCTcagTCT

NNN (*P. pastoris*): TCAATGCATATCACAGACTGTCGTCTGACTAATGGAAGCAGATACCCC
NNQ (*P. pastoris*): TCAATGCATATCACAGACTGTCGTCTGACTcagGGAAGCAGATACCCC
NQN (*P. pastoris*): TCAATGCATATCACAGACTGTCGTCTGACTAATGGAAGCAGATACCCC
QNN (*P. pastoris*): TCAATGCATATCACAGACTGTCGTCTGACTAATGGAAGCAGATACCCC
NQQ (*P. pastoris*): TCAATGCATATCACAGACTGTCGTCTGACTcagGGAAGCAGATACCCC
QNQ (*P. pastoris*): TCAATGCATATCACAGACTGTCGTCTGACTcagGGAAGCAGATACCCC
QQN (*P. pastoris*): TCAATGCATATCACAGACTGTCGTCTGACTAATGGAAGCAGATACCCC
QQQ (*P. pastoris*): TCAATGCATATCACAGACTGTCGTCTGACTcagGGAAGCAGATACCCC
NNN (*E. coli*): TCTATGCATATCACTGACTGCCGTCTGACTAACGGTTCTCGTTACCCG
QQQ (*E. coli*): TCTATGCATATCACTGACTGCCGTCTGACTcaaGGTTCTCGTTACCCG

NNN (*P. pastoris*): AACTGTGCATACAGAACTTCTCCAAAGGAAAGGCACATTATCGTTGCT
NNQ (*P. pastoris*): AACTGTGCATACAGAACTTCTCCAAAGGAAAGGCACATTATCGTTGCT
NQN (*P. pastoris*): AACTGTGCATACAGAACTTCTCCAAAGGAAAGGCACATTATCGTTGCT
QNN (*P. pastoris*): AACTGTGCATACAGAACTTCTCCAAAGGAAAGGCACATTATCGTTGCT
NQQ (*P. pastoris*): AACTGTGCATACAGAACTTCTCCAAAGGAAAGGCACATTATCGTTGCT
QNQ (*P. pastoris*): AACTGTGCATACAGAACTTCTCCAAAGGAAAGGCACATTATCGTTGCT
QQN (*P. pastoris*): AACTGTGCATACAGAACTTCTCCAAAGGAAAGGCACATTATCGTTGCT
QQQ (*P. pastoris*): AACTGTGCATACAGAACTTCTCCAAAGGAAAGGCACATTATCGTTGCT
NNN (*E. coli*): AACTGCGCTTACCGTACTTCTCCGAAAGAACGTCATATCATCGTTGCT
QQQ (*E. coli*): AACTGCGCTTACCGTACTTCTCCGAAAGAACGTCATATCATCGTTGCT

NNN (*P. pastoris*): TGTGAGGGTTCACCTTATGTCCCTGTTCATTTTGATGCCTCGGTGGAA
NNQ (*P. pastoris*): TGTGAGGGTTCACCTTATGTCCCTGTTCATTTTGATGCCTCGGTGGAA
NQN (*P. pastoris*): TGTGAGGGTTCACCTTATGTCCCTGTTCATTTTGATGCCTCGGTGGAA
QNN (*P. pastoris*): TGTGAGGGTTCACCTTATGTCCCTGTTCATTTTGATGCCTCGGTGGAA
NQQ (*P. pastoris*): TGTGAGGGTTCACCTTATGTCCCTGTTCATTTTGATGCCTCGGTGGAA
QNQ (*P. pastoris*): TGTGAGGGTTCACCTTATGTCCCTGTTCATTTTGATGCCTCGGTGGAA
QQN (*P. pastoris*): TGTGAGGGTTCACCTTATGTCCCTGTTCATTTTGATGCCTCGGTGGAA
QQQ (*P. pastoris*): TGTGAGGGTTCACCTTATGTCCCTGTTCATTTTGATGCCTCGGTGGAA
NNN (*E. coli*): TGCGAAGGTTCTCCGTACGTTCCGGTTCATTTCGACGCTTCTGTTGAA
QQQ (*E. coli*): TGCGAAGGTTCTCCGTACGTTCCGGTTCATTTCGACGCTTCTGTTGAA

NNN (*P. pastoris*): GATAGCACT
NNQ (*P. pastoris*): GATAGCACT
NQN (*P. pastoris*): GATAGCACT
QNN (*P. pastoris*): GATAGCACT
NQQ (*P. pastoris*): GATAGCACT
QNQ (*P. pastoris*): GATAGCACT
QQN (*P. pastoris*): GATAGCACT
QQQ (*P. pastoris*): GATAGCACT
NNN (*E. coli*): GACTCTACT
QQQ (*E. coli*): GACTCTACT

Figure S1. DNA sequences that encode the proteins used in this work. DNA that encodes a leader sequence is in a gray box. The codon for the initial amino acid of each mature protein is in bold typeface.

Substitution	Forward (5′→3′)	Reverse (3′→5′)
Q34N	GAGAAATATGACCCAAGGTCGTTGTAAA	ACATTAGTTTACTACTCTTCCTCT**TTA**TACT
Q76N	GAGTAATTCCTCAATGCATATCACAGACTG	CCTGTTCCATTAACGATGTTCTCA**TTA**AGG
Q88N	TCGTCTGACTAATGGAAGCAGATACCCCAA CTGTGCATAC	GAGTTACGTATAGTGTCTGACAGCAGACTG A**TTA**CCTTCGTC

Figure S2. Oligonucleotides used for site-directed mutagenesis. To effect the Q34N substitution, the CAG codon CAG codon of Gln34 was replaced with an AAT codon (underlined) of asparagine (reverse complement shown in bold). To effect the Q76N substitution, the CAA codon of Gln76 was replaced with an AAT codon (underlined) of asparagine (reverse complement shown in bold). To effect the Q88N substitution, the CAG codon of Gln88 was replaced with an AAT codon (underlined) of asparagine (reverse complement shown in bold).

NNN (*P. pastoris*): MRFPSIFTAVLFAASSALAKESRAKKFQRQHMDSDSSPSSSSTYCNQ
NNQ (*P. pastoris*): MRFPSIFTAVLFAASSALAKESRAKKFQRQHMDSDSSPSSSSTYCNQ
NQN (*P. pastoris*): MRFPSIFTAVLFAASSALAKESRAKKFQRQHMDSDSSPSSSSTYCNQ
QNN (*P. pastoris*): MRFPSIFTAVLFAASSALAKESRAKKFQRQHMDSDSSPSSSSTYCNQ
NQQ (*P. pastoris*): MRFPSIFTAVLFAASSALAKESRAKKFQRQHMDSDSSPSSSSTYCNQ
QNQ (*P. pastoris*): MRFPSIFTAVLFAASSALAKESRAKKFQRQHMDSDSSPSSSSTYCNQ
QQN (*P. pastoris*): MRFPSIFTAVLFAASSALAKESRAKKFQRQHMDSDSSPSSSSTYCNQ
QQQ (*P. pastoris*): MRFPSIFTAVLFAASSALAKESRAKKFQRQHMDSDSSPSSSSTYCNQ
NNN (*E. coli*): MKESRAKKFQRQHMDSDSSPSSSSTYCNQ
QQQ (*E. coli*): MKESRAKKFQRQHMDSDSSPSSSSTYCNQ

NNN (*P. pastoris*): MMRRRNMTQGRCKPVNTFVHEPLVDVQNVCFQEKVTCKNGQGNCYKS
NNQ (*P. pastoris*): MMRRRNMTQGRCKPVNTFVHEPLVDVQNVCFQEKVTCKNGQGNCYKS
NQN (*P. pastoris*): MMRRRNMTQGRCKPVNTFVHEPLVDVQNVCFQEKVTCKNGQGNCYKS
QNN (*P. pastoris*): MMRRRQMTQGRCKPVNTFVHEPLVDVQNVCFQEKVTCKNGQGNCYKS
NQQ (*P. pastoris*): MMRRRNMTQGRCKPVNTFVHEPLVDVQNVCFQEKVTCKNGQGNCYKS
QNQ (*P. pastoris*): MMRRRQMTQGRCKPVNTFVHEPLVDVQNVCFQEKVTCKNGQGNCYKS
QQN (*P. pastoris*): MMRRRQMTQGRCKPVNTFVHEPLVDVQNVCFQEKVTCKNGQGNCYKS
QQQ (*P. pastoris*): MMRRRQMTQGRCKPVNTFVHEPLVDVQNVCFQEKVTCKNGQGNCYKS
NNN (*E. coli*): MMRRRNMTQGRCKPVNTFVHEPLVDVQNVCFQEKVTCKNGQGNCYKS
QQQ (*E. coli*): MMRRRQMTQGRCKPVNTFVHEPLVDVQNVCFQEKVTCKNGQGNCYKS

NNN (*P. pastoris*): NSSMHITDCRLTNGSRYPNCAYRTSPKERHIIVACEGSPYVPVHFDA
NNQ (*P. pastoris*): NSSMHITDCRLTQGSRYPNCAYRTSPKERHIIVACEGSPYVPVHFDA
NQN (*P. pastoris*): QSSMHITDCRLTNGSRYPNCAYRTSPKERHIIVACEGSPYVPVHFDA
QNN (*P. pastoris*): NSSMHITDCRLTNGSRYPNCAYRTSPKERHIIVACEGSPYVPVHFDA
NQQ (*P. pastoris*): QSSMHITDCRLTQGSRYPNCAYRTSPKERHIIVACEGSPYVPVHFDA
QNQ (*P. pastoris*): NSSMHITDCRLTQGSRYPNCAYRTSPKERHIIVACEGSPYVPVHFDA
QQN (*P. pastoris*): QSSMHITDCRLTNGSRYPNCAYRTSPKERHIIVACEGSPYVPVHFDA
QQQ (*P. pastoris*): QSSMHITDCRLTQGSRYPNCAYRTSPKERHIIVACEGSPYVPVHFDA
NNN (*E. coli*): NSSMHITDCRLTNGSRYPNCAYRTSPKERHIIVACEGSPYVPVHFDA
QQQ (*E. coli*): QSSMHITDCRLTQGSRYPNCAYRTSPKERHIIVACEGSPYVPVHFDA

NNN (*P. pastoris*): SVEDST
NNQ (*P. pastoris*): SVEDST
NQN (*P. pastoris*): SVEDST
QNN (*P. pastoris*): SVEDST
NQQ (*P. pastoris*): SVEDST
QNQ (*P. pastoris*): SVEDST
QQN (*P. pastoris*): SVEDST
QQQ (*P. pastoris*): SVEDST
NNN (*E. coli*): SVEDST
QQQ (*E. coli*): SVEDST

Figure S3. Amino acid sequences of the proteins used in this work. Leader sequences are in a gray box. Asparagine-to-glutamine substitutions are indicated in red typeface.

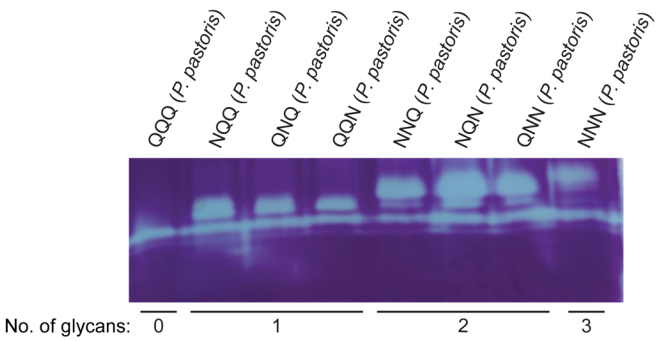

Figure S4. Zymogram of human RNase 1 glycoforms. Conditioned medium from *P. pastoris* cultures were evaluated for ribonucleolytic activity. Macroheterogeneity was observable in *N*-glycosylated samples as additional bands.

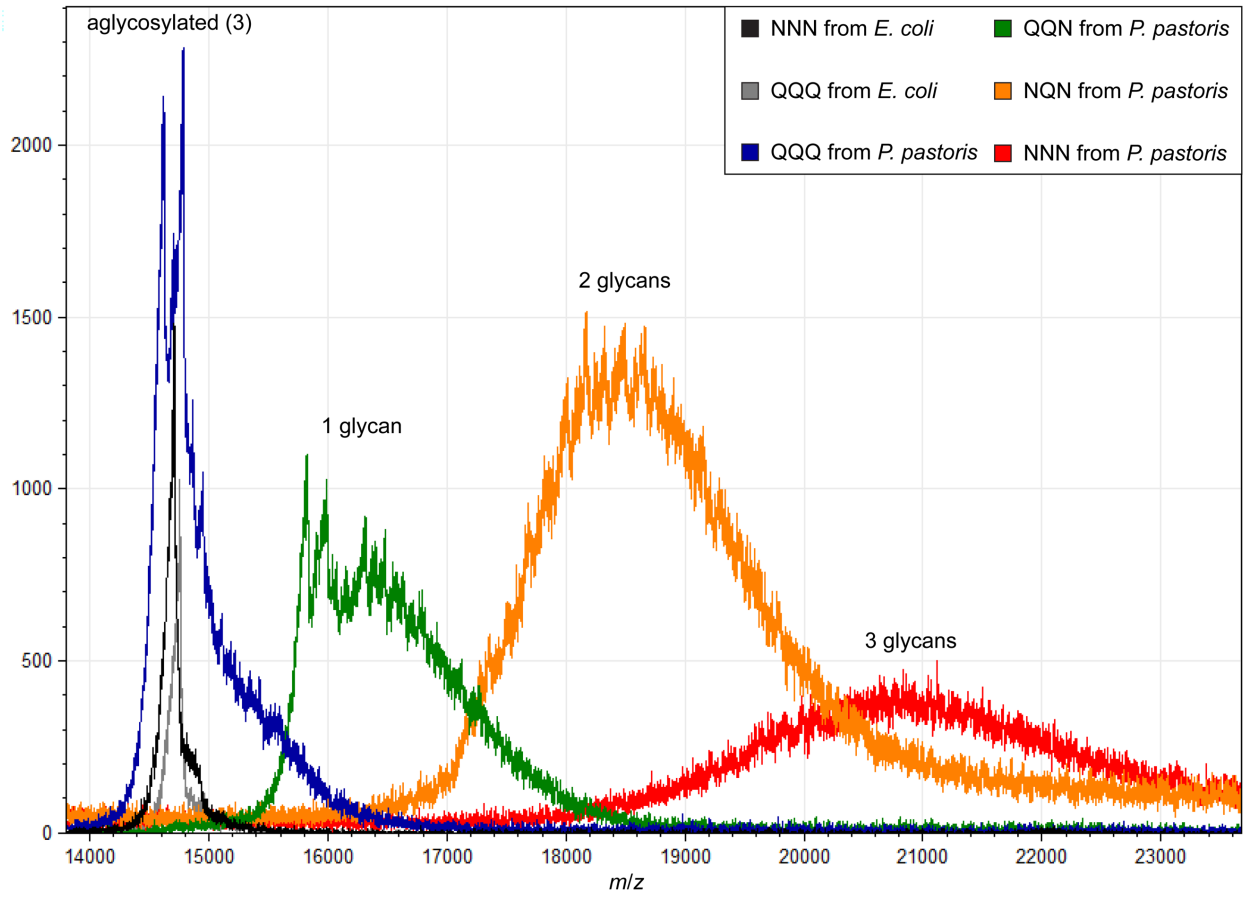

Figure S5. Representative MALDI–TOF mass spectra of human RNase 1 glycoforms.

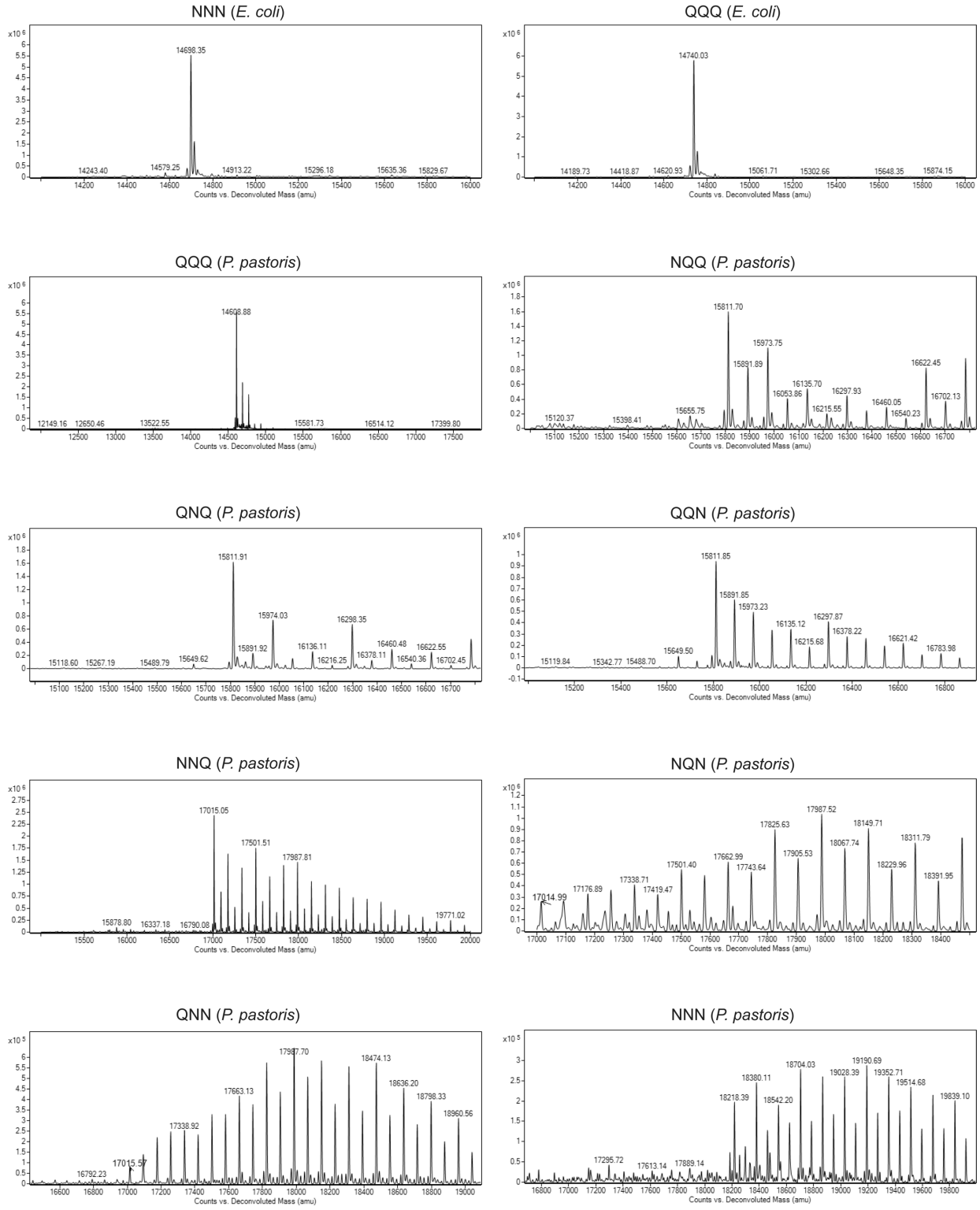

Figure S6. Deconvoluted ESI mass spectra of human RNase 1 glycoforms. Each D-mannose unit has a mass of 162.05 Da. The mass of the lightest glycoform is listed in Table 1.

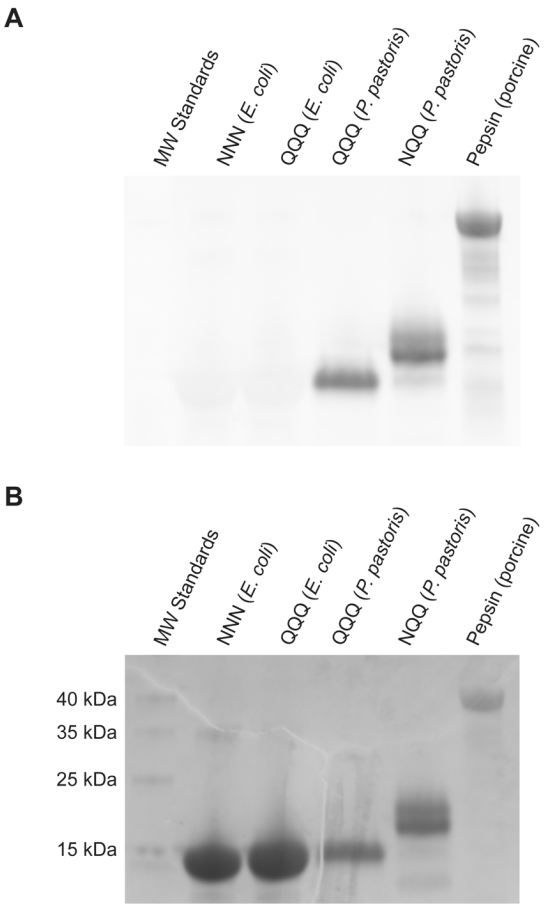

Figure S7. SDS–PAGE gels showing the phosphorylation of human RNase 1 by *P. pastoris*. Representative RNase 1 glycoforms as well as a phosphorylated pepsin standard were subjected to electrophoresis in a polyacrylamide gel (15% w/v) and visualized by staining for either phosphorylated proteins with Pro-Q™ Diamond phosphoprotein gel stain (A) or total protein with Coomassie blue (B).

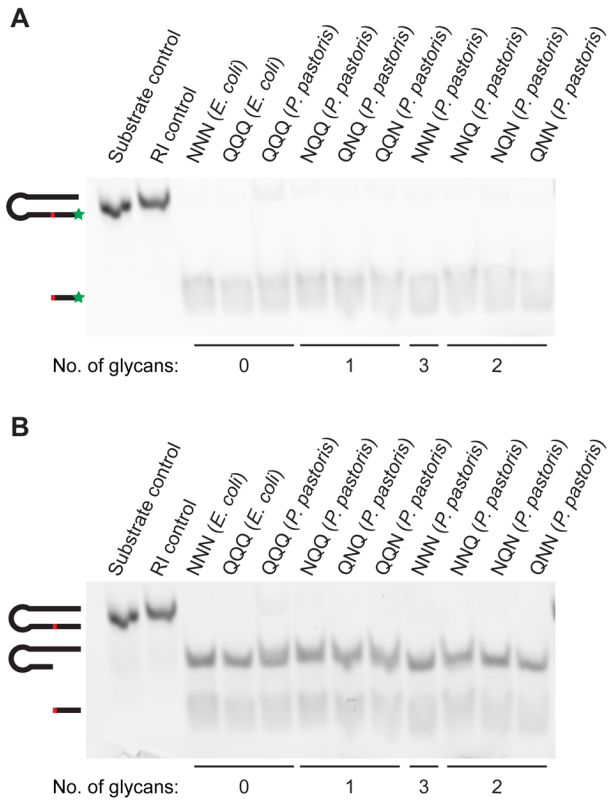

Figure S8. Native gel showing cleavage of a double-stranded RNA by RNase 1 glycoforms. The substrate is a DNA hairpin containing a single RNA residue (red) and a fluorophore (green) at its 5′ end. The gel was visualized for fluorescence (A) and for total nucleic acids with SYBR Gold (B).

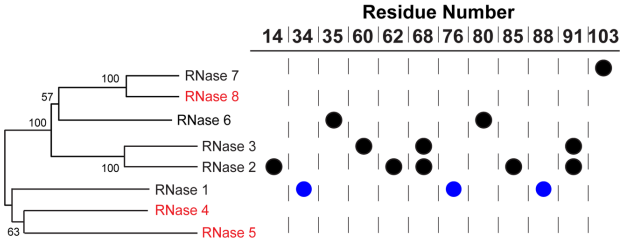

Figure S9. Putative *N*-glycosylation sites in human ptRNases 1–8. Circles indicate an asparagine residue within an *N*-glycosylation sequon. The neighbor-joining phylogenetic tree is adapted from ref. 1 and shows bootstrap values >50. The sequon positions are adapted from ref. 2 and are aligned with the sequence for human RNase 1.

REFERENCES

(1) Zhang, J., Dyer, K. D., and Rosenberg, H. F. (2002) RNase 8, a novel RNase A superfamily ribonuclease expressed uniquely in placenta, *Nucleic Acids Res. 30*, 1169–1175.

(2) Sorrentino, S. (2010) The eight human "canonical" ribonucleases: Molecular diversity, catalytic properties, and special biological actions of the enzyme proteins, *FEBS Lett. 584*, 2194–2200.