Identification and characterization of a novel exo-glycosidase activity of NGLY1 on ENGase-digested N-GlcNAc proteins *in vitro*

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Abstract

N-Glycanase 1 (NGLY1) is known for its activity to completely remove conjugated N-glycans from glycoproteins. Early studies illustrated that NGLY1 defects were associated with a rare disease named NGLY1-related congenital disorder of glycosylation (NGLY1-CDDG). Despite extensive research has been conducted over the past decade on the biological impact of NGLY1's endo-glycosidase activity in cells and on disease, whether NGLY1 also exhibits an exo-glycosidase activity remains open. In this study, an exo-glycosidase activity of NGLY1 on the N-GlcNAc proteins was firstly reported and characterized *in vitro*. To distinguish for the conventional endo-glycosidase activity and the newly reported NGLY1's exo-glycosidase activity, the active sites of exo-NGLY1 were predicted and compared with those of endo-NGLY1. In addition, the correlation between NGLY1's exo-glycosidase activity *in vitro* and NGLY1-CDDG's clinical characterization was investigated and discussed.

Key words

N-Glycanase 1 (NGLY1), Exo-glycosidase activity, ENGase, N-GlcNAc proteins, Congenital disorders of glycosylation (CDG)

Introduction

N-Glycanase 1 (NGLY1) is an enzyme responsible for cleaving oligosaccharide moieties

from misfolded glycoproteins to enable their proper degradation(1). It could hydrolyze the amide bond between the proximal N-acetylglucosamine (GlcNAc) residue and the asparagine (Asn) side chain, thereby removing the N-glycans from the glycosylated proteins in the cytoplasm and releasing the complete oligosaccharide chain, converting the asparagine (Asn) on the peptide chain into aspartic acid (Asp)(1-3). In 1993, it was discovered that NGLY1 participated in the degradation of misfolded glycoproteins during the process of glycoprotein synthesis through the endoplasmic reticulum associated degradation (ERAD) pathway, thereby maintaining normal cell functions(4). In 2014, it was reported that NGLY1 deficiency indicated a congenital disorder of glycosylation (CDG), with the main clinical manifestations including developmental delay, intellectual disability, absence of tears and less sweating, abnormal liver function, and motor dysfunction(5).

Early studies suggested that NGLY1 was essential for ERAD, and the compromised *Ngly1* activity for ERAD could be partially complemented by *Engase* mediated deglycosylation of misfolded glycoproteins. In 2017, it was reported that diploid NGLY1 knockout was embryonic lethal in mice and this phenotype could be rescued by *Engase* knockout(6). To explain the phenotype, it was proposed that elevated ENGase activity may cause excess formation of N-GlcNAc proteins in cell, and the situation was bypassed in *Ngly1* and *Engase* double knockout(7). Although studies have shown that NGLY1 deficiency leads to N-GlcNAc proteins aggregation, it is still unclear which enzyme have the exo-glycosidase function to remove N-GlcNAc proteins.

This article investigated the new exo-glycosidase activity functions of NGLY1. It was discovered that NGLY1 had a new function of removing N-GlcNAc from glycosylated proteins by virtual molecular docking and lectin blot methods, as well as the sites that affected this new function. Additionally, we studied the influence of NGLY1's exo-glycosidase activity in NGLY1-CDDG.

Results

NGLY1 new function——exo-glycosidase activity

In this study, a prokaryotic expression system for NGLY1 was successfully constructed, enabling the purification of the NGLY1 protein from *E.coli* BL21 (DE3). Enzymatic activity assays demonstrated that NGLY1 exhibits endo-glycosidase activity, as evidenced by its action on the high-mannose type standard glycoprotein substrate RNase B which was a conventional substrate of NGLY1 (Fig. 1A). As controls, other N-glycosidases were employed, including the prokaryotic-expressed PNGase F and PNGase F-II from

Elizabethkingia meningoseptica discovered in 2015 by our group(8), both of which also had enzymatic activity toward RNase B (Fig. 1A).

Using Endo F1 (endo-beta-acetyl-glucosaminidase F1) derived from *Elizabethkingia meningoseptica* to cleave RNase B, the enzymatic products were N-GlcNAc proteins. This enzymatic products were used as the substrate for NGLY1. The results showed that NGLY1 could hydrolyze a single N-GlcNAc on protein. At the same time, PNGase F and PNGase F-II also had this new function (Fig. 1B).

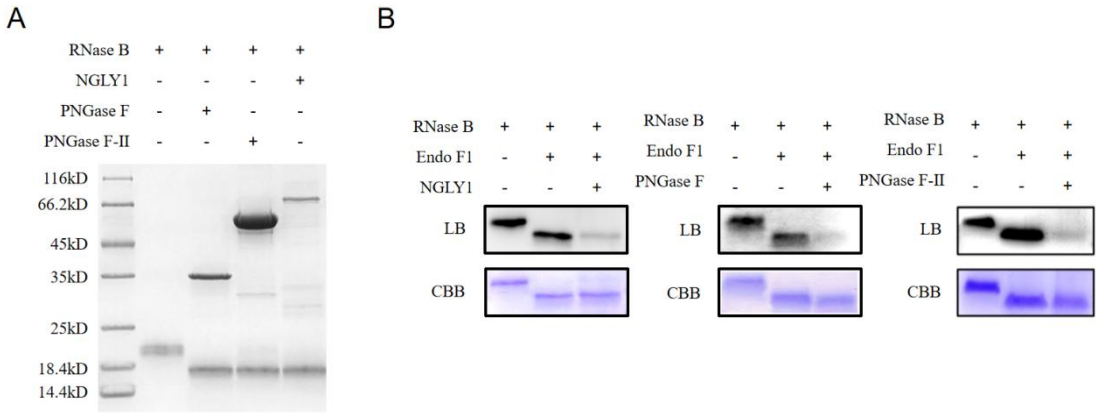


Figure1. N-glycosidase's endo-glycosidase activity and exo-glycosidase activity. A. N-glycosidase's endo-glycosidase activity. The first lane showed the band of RNase B alone; the second lane showed the bands of RNase B substrate treated with PNGase F, PNGase F-II and NGLY1 respectively. The downward shift of the RNase B band indicated that all three enzymes have deglycosylation functions. B. N-glycosidase's exo-glycosidase activity. The first lane showed the band of RNase B alone; the second lane showed the bands of RNase B substrate treated with Endo F1; the third lane showed the new substrate (enzymatic products of Endo F1 cleaving RNase B) treated with NGLY1, PNGase F and PNGase F-II respectively. CBB: Coomassie Brilliant Blue; LB: Lectin Blot. The darker the color of the band in the lectin blot, the higher the content of GlcNAc.

Prediction of the active sites of NGLY1

The three-dimensional structure of NGLY1 was predicted using AlphaFold2 (Fig. 2A), which contained three domains: PUB domain, PNG Core domain, and PAW domain. PNG Core domain was the main catalytic domain. The structures of molecular ligands were designed, and the molecular docking results with NGLY1 were visualized by PyMOL(Fig. 2B and 2C). It was found that the binding between the ligand and the protein mainly occurred in the middle region between PAW and PNG Core (the red box in Fig. 2A).

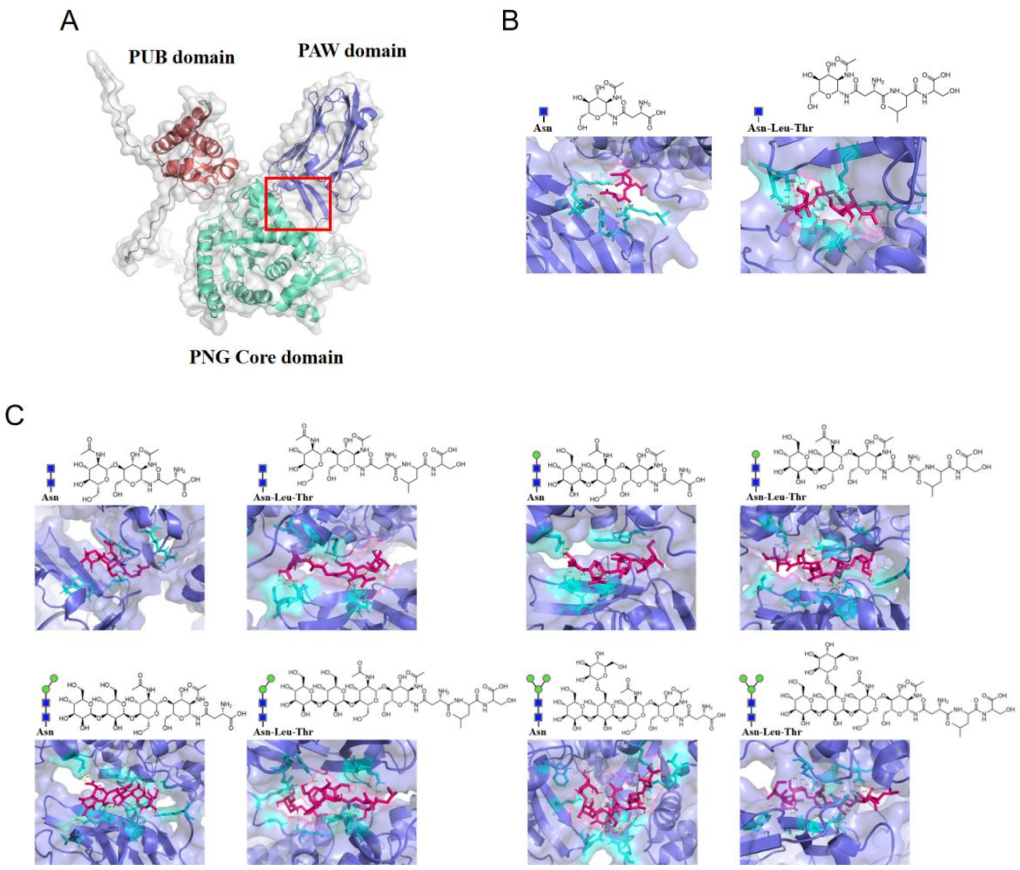


Figure2. The molecular docking of ligands and NGLY1. A. The 3D structure of NGLY1. NGLY1 contained three domains: PUB domain, PNG Core domain, and PAW domain. The red box was the docking pocket. B. The ligand structure and molecular docking picture to predict the active sites that affected exo-glycosidase activity. Each group of pictures included a ligand schematic diagram, the molecular structure of the ligand, and a molecular docking diagram. In the molecular docking diagram, the bright pink area represented the structure of the ligand. C. The ligand structure and molecular docking picture to predict the active sites that affected endo-glycosidase activity. Blue square: N-acetylglucosamines (GlcNAc); Green circle: Mannose.

The 2D docking profiles and associated non-covalent interactions were visualized by Discovery Studio, and the amino acid sites of molecular docking were obtained (Table 1). Venn diagram-based analysis was conducted on the intersection of the docking sites of the two ligands (Fig. 3). It was found that although no overlapping binding sites were observed between Asn-GlcNAc and Thr-Leu-Asn-GlcNAc in the molecular docking results, their respective binding sites were present in the cross-combination of other ligands (the sites in blue box). Following Venn diagram-based analysis, these amino acid residues were candidate sites: LYS-238, TRP-244, TRP-369, ARG-458, TRP-517, GLU-518, ARG-529, and THR-533. These sites were selected for single-amino-acid site-directed mutagenesis studies.

Table1. Sites of various ligand binding to NGLY1.

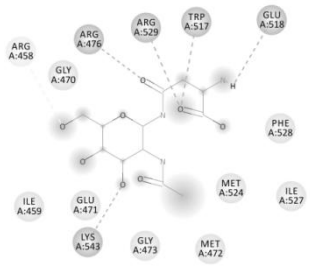
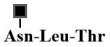
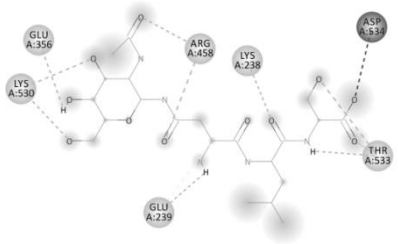
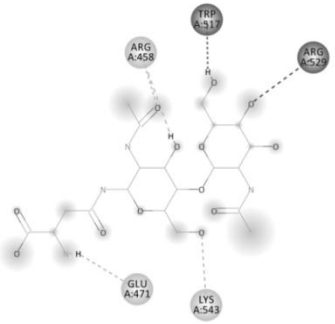
Ligand diagram	2D docking diagram	Non-covalent interaction	Docking scores	Docking amino acid sites
Asn		5 H–bond	-5.6	ARG-476、TRP-517、GLU-518、ARG-529、LYS-543
 Asn-Leu-Thr		9 H–bond	-7.3	LYS-238、GLU-239、GLU-356、ARG-458、LYS-530、THR-533、ASP-534
Asn		4 H–bond	-6.9	ARG-458、GLU-471、TRP-517、ARG-529、LYS-543

Table1. (continued)

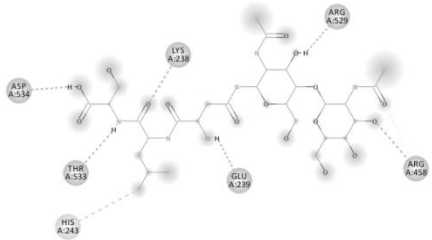
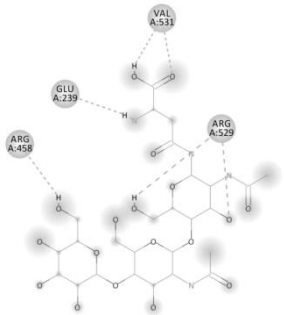
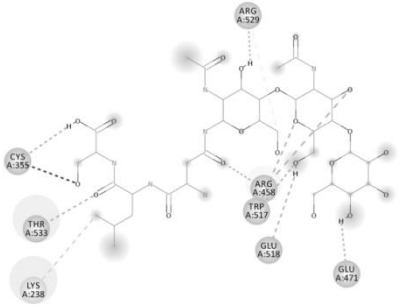
Ligand diagram	2D docking diagram	Non-covalent interaction	Docking scores	Docking amino acid sites
Asn-Leu-Thr		7 H–bond 1 Pi-Alkyl	-7.1	LYS-238、GLU-239、HIS-243、ARG-458、ARG-529、THR-533、ASP-534
Asn		6 H–bond	-7.1	GLU-239、ARG-458、ARG-529、VAL-531
Asn-Leu-Thr		8 H–bond 1 C-H Bond 1 Alkyl	-7.9	LYS-238、CYS-355、ARG-458、GLU-471、TRP-517、GLU-518、ARG-529、THR-533

Table1. (continued)

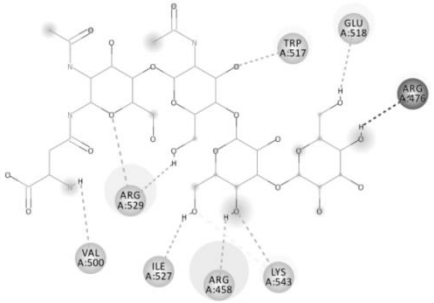
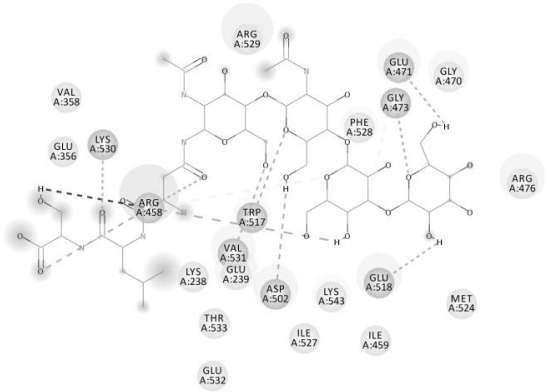
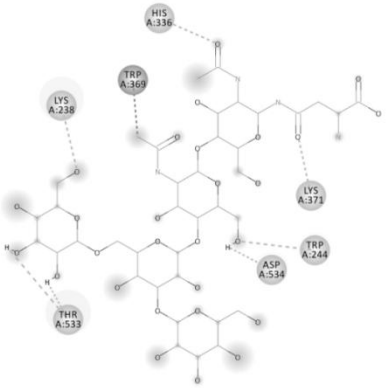
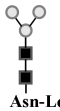
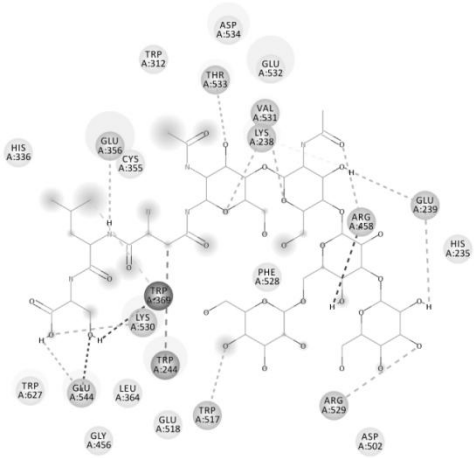
Ligand diagram	2D docking diagram	Non-covalent interaction	Docking scores	Docking amino acid sites
Asn		8 H–bond	-7.2	ARG-476 、 VAL-500 、 TRP-517 、 GLU-518 、 ILE-527 、 ARG-529、LYS-543
Asn-Leu-Thr		10 H–bond 2 C-H Bond	-7.6	ARG-458 、 GLU-471 、 GLY-473 、 TRP-517 、 ASP-502 、 GLU-518、LYS-530、VAL-531

Table1. (continued)

Ligand diagram	2D docking diagram	Non-covalent interaction	Docking scores	Docking amino acid sites
Asn		5 H–bond 1 Pi-Sigma	-7.1	LYS-238、TRP-244、HIS-336、TRP-369、LYS-371、THR-533、ASP-534
 Asn-Leu-Thr		11 H–bond 1 C-H Bond 1 Pi-Sigma 1 Pi-Alkyl	-8.0	LYS-238 、 TRP-244 、 GLU-239 、 GLU-356 、 TRP-369 、 ARG-458 、 TRP-517 、 ARG-529 、 LYS-530 、 THR-533 、 GLU-544

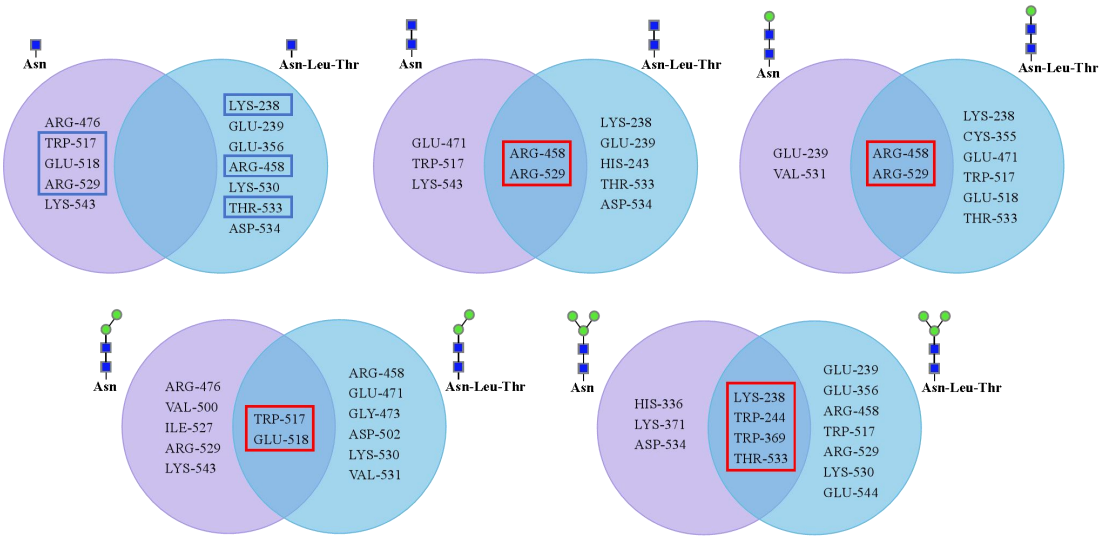


Figure3. Venn diagram-based intersection screening of the docking sites. Asn and Asn-Leu-Thr were crossed by connecting two ligands of the same glycan chains respectively. The intersection sites were marked by red boxes, and the blue box showed the existence of the sites in the red boxes in the first Venn diagram.

The active sites associated with exo-glycosidase and endo-glycosidase activity of NGLY1

Based on the 8 predicted active sites obtained through screening, the amino acids were specifically mutated to the amino acids with opposite charges. The successful construction of mutants was determined by sequencing of the recombinant plasmid genes (Fig. S1). The mutants of NGLY1 (K238D, W244R, W369E, R458E, W517R, E518K, R529E and T533A) were purified by Ni-NTA affinity chromatography.

The exo-glycosidase activity of K238D and E518K were affected. Compared with the NGLY1 WT group, exo-glycosidase activity of K238D was completely affected (Fig. 4A) and E518K exhibited lower exo-glycosidase activity (Fig. 4B). Regarding endo-glycosidase activity, K238D lacked endo-glycosidase activity while E518K retained it.

The exo-glycosidase activity of W244R, W517R and W369E were not affected but their endo-glycosidase activity were influenced (Fig. 4C1 and 3C2 and 3C3). The enzymatic activities of W244R and W517R towards RNase B were lost, whereas W369E had a lower enzymatic activity against RNase B.

In addition, it was found that the remaining screening intersection sites R458E, R529E and T533A had no effect on endo-glycosidase activity nor exo-glycosidase activity (Fig. 4D1, 4D2 and 4D3).

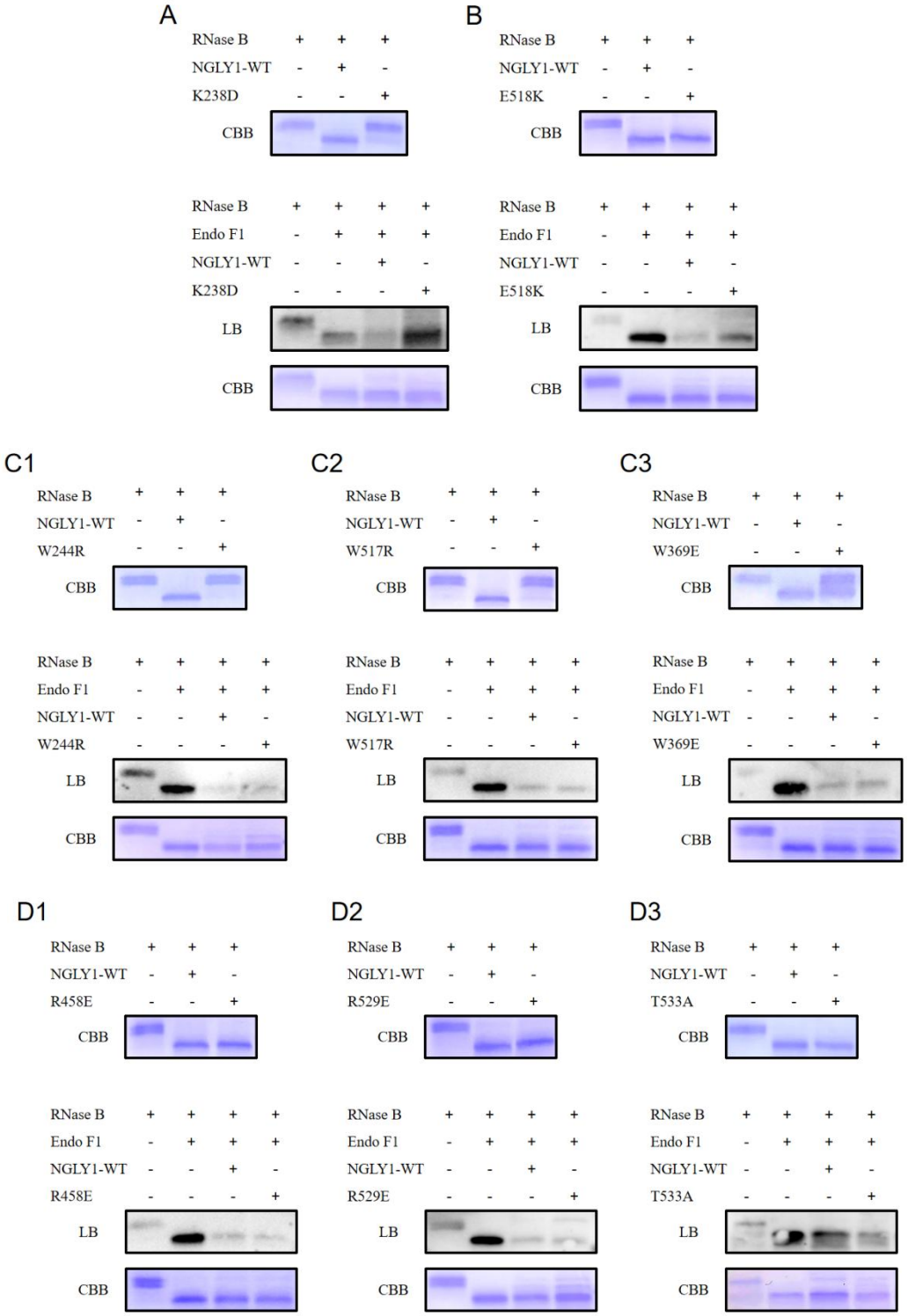


Figure4. SDS-PAGE and lectin blot results of NGLY1 mutations' endo-glycosidase activity and exo-glycosidase activity. A. K238D retains some endo-glycosidase activity and has no exo-glycosidase activity. B. E518K has endo-glycosidase activity and retains some exo-glycosidase activity. C1. W244R has no endo-glycosidase activity and retains exo-glycosidase activity. C2. W517R has no endo-glycosidase activity and retains exo-glycosidase activity. C3. W369E retains some endo-glycosidase activity and has exo-glycosidase activity. D1. R458E has endo-glycosidase activity and exo-glycosidase activity. D2. R529E has endo-glycosidase activity and exo-glycosidase activity.

D3. T533A has endo-glycosidase activity and exo-glycosidase activity.

The correlation between NGLY1's exo-glycosidase activity *in vitro* and NGLY1-CDDG's clinical characterization

In this study, the effects of several patient mutants on the activities of NGLY1 endo-glycosidase and exo-glycosidase activity were investigated. The R328G mutant was completely inactive towards RNase B and the single GlcNAc, which meant R328G had no endo-glycosidase activity nor exo-glycosidase activity(Fig. 5A). The Y342C and E356G mutants had partial defects in the enzymatic activity of RNase B, and compared with the NGLY1 WT group, their exo-glycosidase activity also had defects (Fig. 5B and 5D). The C355R mutant caused the loss of enzymatic activity of RNase B, while maintaining enzymatic activity towards a single GlcNAc (Fig. 5C).

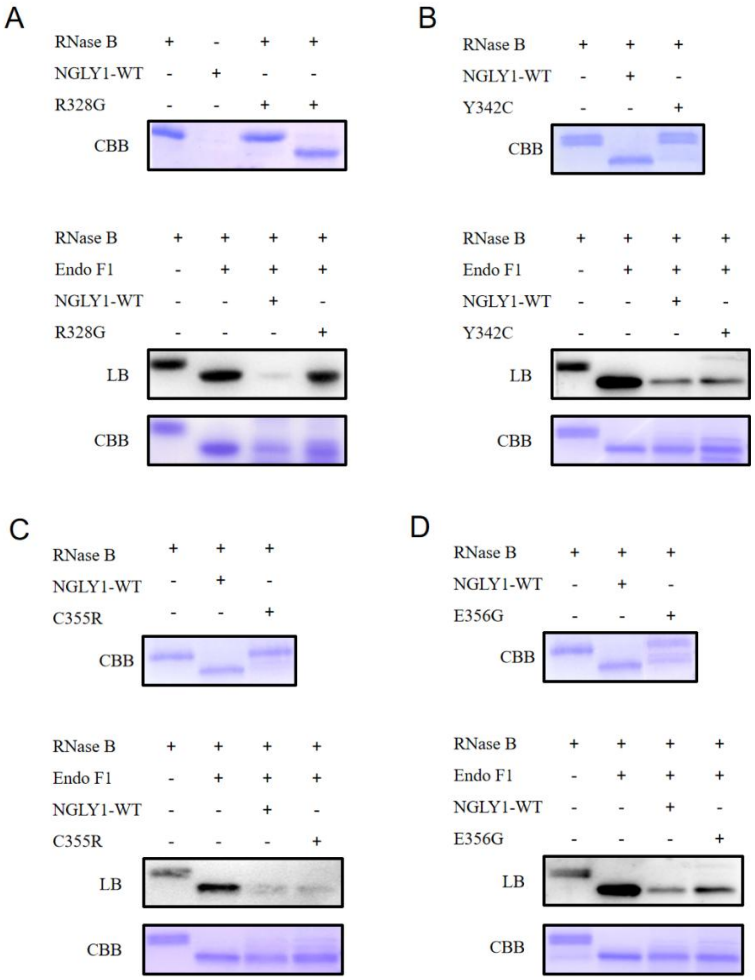


Figure5. The endo-glycosidase and exo-glycosidase activity of the reported NGLY1 patients' mutations. A. R328G has no endo-glycosidase activity nor exo-glycosidase activity. B. Y342C remains some endo-glycosidase activity and has no exo-glycosidase activity. C. E356G remains some endo-glycosidase activity and has no exo-glycosidase activity. D. C355R have no endo-glycosidase activity and have exo-glycosidase activity.

Table2. The activity of NGLY1 mutants and NGLY1-CDDG patients' clinical charaterization.

	Mutation	R328G(9)	Y342C(9)	C355R(10)	E356G(11)
Glycosidase activity	Endo-glycosidase/Exo-gl-ycosidase	-/-	-/±	-/+	±/±
Clinical Characteriz-ation	Global developmental delay	Yes	Yes	Yes	Yes
	Neuromotor developmental delay/abnormalities	Yes	Yes	Yes	Yes
	Hyperkinetc movement abnormalities	Yes	Yes	Yes	Yes
	Liver dysfunction	Yes	Yes	Yes	No
	Alacrima/ hypohidrosis	Yes	Yes	Yes	No
	Microcephaly	Yes	Yes	/	No
	Ocular dysfunction	Yes	No	/	Yes
	Ptosis	Yes	No	/	/
	Small hands/feet	Yes	No	/	Yes
	Anhydrosis	Yes	No	/	/
	Vomiting	Yes	No	/	/
	Peripheral neuropathy	Yes	/	/	Yes
	Seizures	No	Yes	Yes	Yes
	Diminished reflexes	Yes	No	/	/
	Abnormal brain imaging	Yes	No	/	Yes

+: normal enzymatic activity; -: no enzymatic activity; ±: enzyme activity partially defects; /: not available or not applicable.

Furthermore, the clinical symptoms of these NGLY1-CDDG patients and the endo-glycosidase and exo-glycosidase activity of their mutants were summarized (Table2.). The exo-glycosidase and endo-glycosidase activities of R328G were completely absent. Correspondingly, the symptoms of patients with the R328G mutation appeared to be more severe, with additional symptoms such as abnormal brain imaging, diminished reflexes, anhidrosis, vomiting, ocular dysfunction, ptosis and small hands/feet compared to Y342C patients. This might be because, the NGLY1 in Y342C mutation patients, although mutated, still retained some exo-glycosidase activity, which could remove N-GlcNAc proteins in body to a certain extent, thereby exerting a detoxification effect. The exo-glycosidase activity of C355R was completely retained, and the symptoms of patients with the C355R mutation were

slightly milder. For instance, symptoms such as microcephaly, ocular dysfunction, abnormal brain imaging, and anhidrosis have not been reported in C355R patients. E356G not only retained partial exo-glycosidase activity but also partial endo-glycosidase activity. Its symptoms were milder than those of other patients, with no liver dysfunction, alacrima/hypohidrosis, or microcephaly. It was speculated that the dual glycosidase activity of NGLY1 might be functioning simultaneously in the patients' body.

Discussion

Previously, it has been proposed that NGLY1 is a key component of the ERAD process for newly synthesized proteins. This process was designated as ER-assisted digestion of improperly folded glycoproteins(4). By predicting protein interactions through the STRING website (https://cn.string-db.org/), a connection between NGLY1 and ENGase was found (Fig. S3). *Ngly1* knockout studies conducted in mice demonstrated that the embryonic lethal phenotype of *Ngly1* could be rescued by a second knockout of *Engase*(6). As both ENGase and NGLY1 work on the same or similar substrates, the down-regulation of NGLY1 may stimulate an up-regulation of ENGase and generate more ENGase products (N-GlcNAc proteins), releasing the ERAD stress caused by NGLY1 deficiency. The accumulation of N-GlcNAc proteins could result in the aggregation and elimination of functional proteins within the cell. Based on early studies and our own results generated from *C. elegans*, we hypothesized that ENGase products may represent an index of stress, including the ones from environmental and genetics(12). Therefore, we suggested that there may be an exo-glycosidase activity that could relieve the protein stress resulting from increased ENGase activity.

An *in vitro* assay was conducted for this activity by using the ENGase products as substrates. To our surprise, an exo-glycosidase activity of NGLY1 was detected. In addition, the similar activity was also detected in PNGase F and PNGase F-II from *E. meningoseptica.* We also illustrated that NGLY1, PNGase F and PNGase F-II had no enzymatic cleavage effect on single amino acid linked with GlcNAc (Asn-GlcNAc) (Fig. S2), suggesting that peptide N-glycosidase exerted exo-glycosidase activity by recognizing N-GlcNAc on proteins only.

In this study, the discovery of NGLY1's exo-glycosidase activity has created a new double-hit mechanism for NGLY1-CDDG. *In vitro* results generated from NGLY1-CDDG associated mutations were consistent with the dual-function model. The defects in endo/exo -glycosidase of NGLY1 were detected in most of the mutations, indicating a correlation between NGLY1's exo-glycosidase activity and clinical symptoms of NGLY1-CDDG.

In a previous study, the active sites of NGLY1 were predicted, and the mutations on these sites were listed as high risk mutants associated with rare disease NGLY1-CDDG(13). In this study, additional high risk sites were added based on the results generated from assays for exo-glycosidase of NGLY1. The addition may provide a new reference value for the diagnosis and treatment of NGLY1-CDDG. Firstly, the results generated from the dual enzyme activities assay established in this study could be used for subtyping of NGLY1-CDDG. Secondly, although our data demonstrated that NGLY1-CDDG were associated with defects of exo-glycosidae activity of NGlY1, the results did not exclude the possibility that other enzymes may also involved in the detoxification process for N-GlcNAc proteins. The possibilities are under the investigation. Finally, the predicted active site mutations without reported NGLY1-CDDG may open a discussion on other symptoms or diseases associated with this gene.

Experimental procedures

Purification of NGLY1

The NGLY1 protein inducible expression and purification system has been established and the methods used in this study were based on the previously published articles of our research group(13,14).

Detection on endo-glycosidase activity of NGLY1

The enzyme activity assay of NGLY1 has been established in advance(13). Mix the NGLY1 (2 μg) with the 100°C denatured substrate RNase B (New England Biolabs, Ipswich, Massachusetts, USA) (2 μg), and add PBS to make the total reaction system (20 μL). Incubate at 37°C for 12-16 hours, then separate by 15% SDS-PAGE and conduct Coomassie blue staining. The molecular weight of RNase B is approximately 17 kDa. After NGLY1-mediated de-sulfation, the molecular weight of RNase B drops to approximately 13.7 kDa. When the activity of NGLY1 is inhibited, the molecular weight of RNase B remains at approximately 17 kDa.

Detection on exo-glycosidase activity of NGLY1

The product of RNase B cleaved by Endo F1 was used as the substrate for verifying the NGLY1's exo-glycosidase activity. After RNase B (2 μg) was subjected to a 100°C denaturation treatment for 10 minutes, Endo F1 (2 μg) was added for enzymatic digestion for 12-16 hours at 37°C. After 10 minutes of denaturation at 100°C, it was cooled on ice. Equal

amounts of enzyme (4 μg) were added, and enzymatic digestion is carried out for 12-16 hours. Then, loading buffer was added for denaturation treatment.

Through the SDS-PAGE electrophoresis method, the proteins in the sample were separated according to their molecular weights. The separated proteins were transferred onto a PVDF membrane. The transfer conditions were 220V for 100 minutes. The membrane was treated with 5% bovine serum albumin (BSA, Beyotime, Shanghai, China) blocking solution at room temperature on a shaker for 2 hours to block non-specific binding sites. The membrane was incubated with 1:200 diluted Wheat Germ Agglutinin (WGA) (Vector Laboratories, San Francisco Bay Area, USA) at 4℃ on a shaker overnight (12-16 hours) to allow the lectin to bind to the target carbohydrate chains on the membrane proteins. After the lectin fully binds, the membrane was washed 1x TBST for 10 minutes, repeated three times, and the free lectin is removed. Then, 1:2000 diluted horseradish peroxidase-labeled Streptavidin (Beyotime, Shanghai, China) was added at room temperature on a shaker for 2 hours. 1× TBST is washed for 10 minutes, repeated three times, and the ECL chemiluminescent substrate reagent (biosharp, Hefei, Anhui, China) was added to the membrane. The fluorescence signal was detected by luminescence imaging to determine the presence and relative content of the target carbohydrate chain.

Computer virtual molecular docking of NGLY1 and ligands

The three-dimensional structure of NGLY1 was previously predicted using the amino acid sequence of NGLY1 through the online tool AlphaFold2 (website: https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb?pli=1#scrollTo=kOblAo-xetgx, accessed on January 10, 2022).

The three-dimensional structures of 10 ligands were drawn, generated the 3D structures in OpenBabel, and removed water molecules and hydrogen atoms. Using the "AutoDock vina" function in AutoDock software, we performed molecular docking of these 10 ligands with NGLY1 respectively. NGLY1 consists of three domains: the PUB domain, the PNG core domain, and the PAW domain. Among them, the PNG core domain plays the main catalytic role. Center the receptor pocket around the PNG core domain of the NGLY1 protein (coordinates x = -4.054, y = -4.87, z = 2.613), and set this receptor pocket as a cubic grid with side lengths (113×70×112A). AutoDock Vina uses BFGS local optimization and genetic algorithms to generate 9 conformations. The lowest energy conformation were selected and their interaction with NGLY1 were analyzed using the PyMOL (version 3.1.3) .

Construction of NGLY1 amino acid mutation and enzymatic activity analysis

The pET28a-NGLY1 (wild type) plasmid is kept in our laboratory. All NGLY1 mutants were constructed using the pET28a-NGLY1 (wild type) plasmid as the template and reverse complementary primers through PCR (Takara, Beijing, China). The constructed pET28a-NGLY1 (wild type) plasmid and pET28a-NGLY1 (mutant) plasmids were respectively transformed into Escherichia coli BL21(DE3) (Tiangen, Beijing, China) for protein expression. The primer sequences are shown in Table 3. The purification of NGLY1 mutants and the detection methods of endo-glycosidase and exo-glycosidase activities were the same as those for NGLY1.

Table3. PCR primer sequences for constructing NGLY1 mutants.

Mutant			Primer 5′-3'
K238D	AAG→GAC	Forward	CACCCAGTGAAAAAATTCTTCGTCAAACCAGTGCAAAAGCTCCAG
		Reverse	CTGGAGCTTTTGCACTGGTTTGACGAAGAATTTTTTCACTGGGTG
W244R	TGG→CGG	Forward	CTGCACAAAACGTTATTCACCCGGTGAAAAAATTCTTCCTTAAAC
		Reverse	GTTTAAGGAAGAATTTTTTCACCGGGTGAATAACGTTTTGTGCAG
W369E	TGG→GAG	Forward	AAAGCTTCTTGCCCTCTCCTATTTCATAAAGGAGTGGCTTGTC
		Reverse	GACAAGCCACTCCTTTATGAAATAGGAGAGGGCAAGAAGCTTT
R458E	AGA→GAA	Forward	GCCACTGACCCAGATATTTCTCCCCCAAGTTCTCCAGG
		Reverse	CCTGGAGAACTTGGGGGAGAAATATCTGGGTCAGTGGC
W517R	TGG→CGG	Forward	CACGCCATTCTCCCGTCCAGAAATGGTTTGATTGTT
		Reverse	AACAATCAAACCATTTCTGGACGGGAGAATGGCGTG
E518K	GAG→AAG	Forward	TTTTCCACACGCCATTCTTCCATCCAGAAATGGTTTG
		Reverse	CAAACCATTTCTGGATGGAAGAATGGCGTGTGGAAAA
R529E	AGA→GAA	Forward	CATGTGCCAGTCTGTTTCAACTTTTTCGAATATAGATTCCATTTTCCACACG
		Reverse	CGTGTGGAAAATGGAATCTATATTCGAAAAAGTTGAAACAGACTGGCACATG
T533A	ACA→GCA	Forward	CCATGTGCCAGTCTGCTTCAACTTTTCTGAATATAGATTCCATT
		Reverse	AATGGAATCTATATTCAGAAAAGTTGAAGCAGACTGGCACATGG
R328G	CGC→GGC	Forward	AATCCCAAACATAGCCAGCTTCAAACCCTACAGCT
		Reverse	AGCTGTAGGGTTTGAAGCTGGCTATGTTTGGGATT

Table3. (continued)

Mutant			Primer 5′-3'
Y342C	TAT→	Forward	GCTGCTGAGAAGGAGAACAGACTTCTGTCCAGACA
	TGT	Reverse	TGTCTGGACAGAAGTCTGTTCTCCTTCTCAGCAGC
C355R	TGT→	Forward	GTCACAGACATCTTCACGTGCATCACAGTGCAGCC
	CGT	Reverse	GGCTGCACTGTGATGCACGTGAAGATGTCTGTGAC
E356G	GAA→	Forward	GCTTGTCACAGACATCTCCACATGCATCACAGTGC
	GGA	Reverse	GCACTGTGATGCATGTGGAGATGTCTGTGACAAGC

Data availability

All data are included in the manuscript and supporting information.

Supporting Information

This article contains supporting information.

Funding and additional information

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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A

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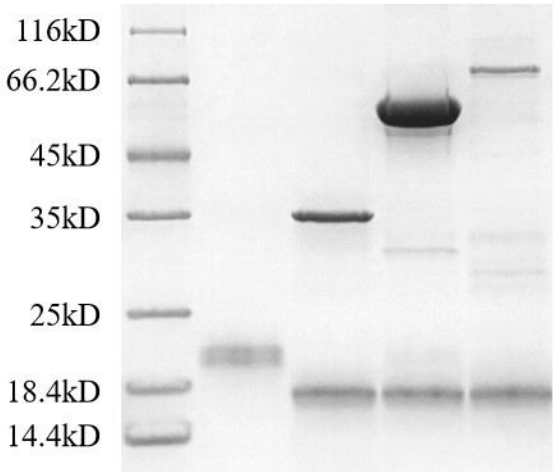
NGLY1	-	-	-	+
PNGase F	-	+	-	-
PNGase F-II	-	-	+	-



B

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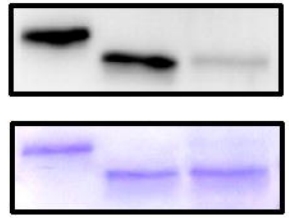
Endo F1	-	+	+
NGLY1	-	-	+



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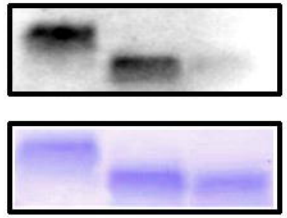
PNGase F	-	-	+



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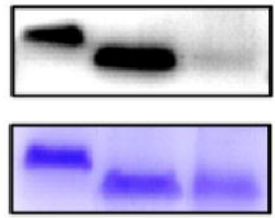
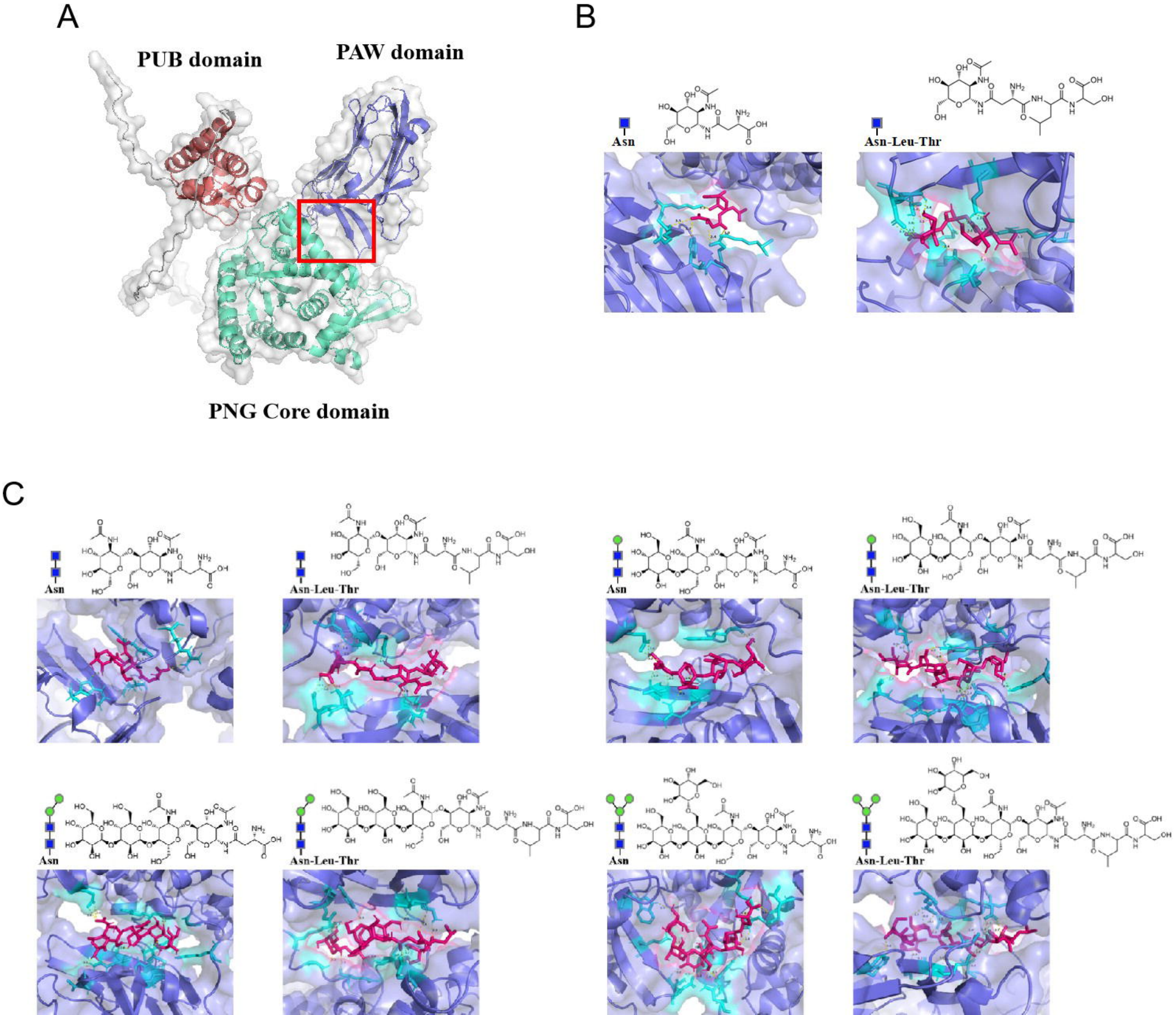
Endo F1	-	+	+
PNGase F-II	-	-	+

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PAW domain
PNG Core domain
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C
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Asn-Leu-Thr
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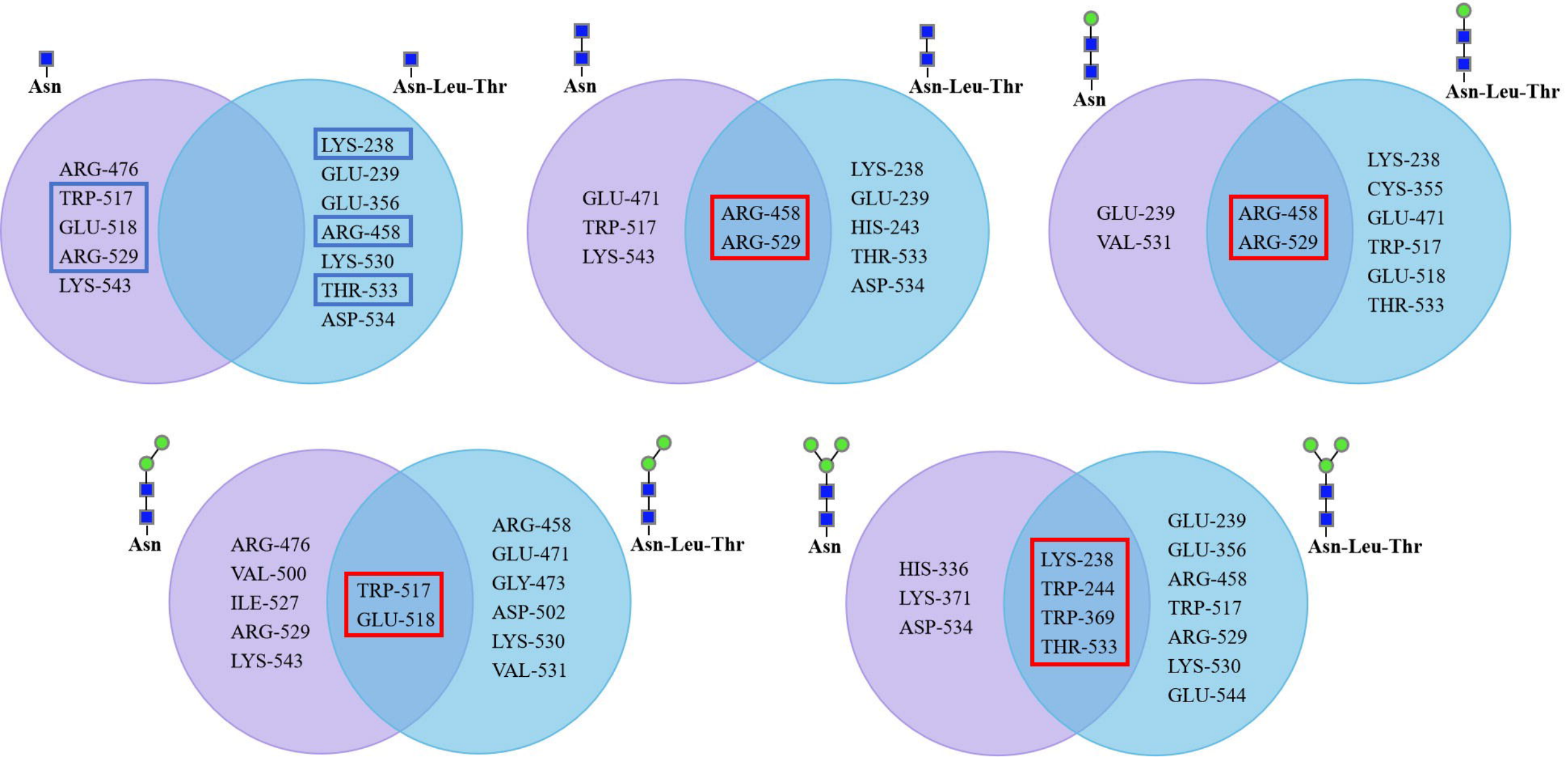
Asn-Leu-Thr
Asn
Asn-Leu-Thr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LYS-530
THR-533
ASP-534
Asn
Asn-Leu-Thr
GLU-471
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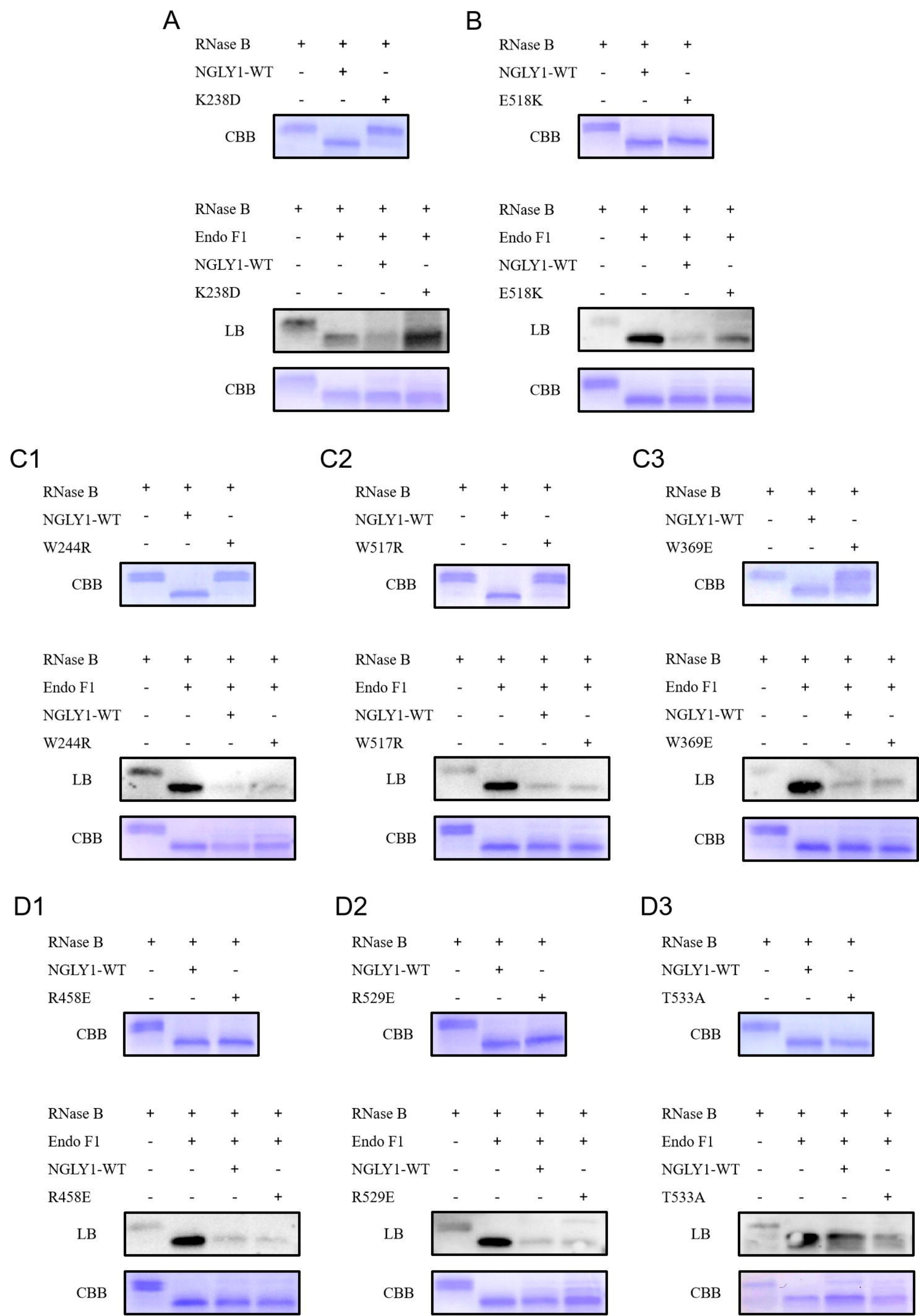
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K238D - - +
CBB
RNase B + + + +
Endo F1 - + + +
NGLY1-WT - - + -
K238D - - - +
LB
CBB
B
RNase B + + +
NGLY1-WT - + -
E518K - - +
CBB
RNase B + + + +
Endo F1 - + + +
NGLY1-WT - - + -
E518K - - - +
LB
CBB
C1
RNase B + + +
NGLY1-WT - + -
W244R - - +
CBB
RNase B + + + +
Endo F1 - + + +
NGLY1-WT - - + -
W244R - - - +
LB
CBB
C2
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NGLY1-WT - + -
W517R - - +
CBB
RNase B + + + +
Endo F1 - + + +
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W517R - - - +
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C3
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D1
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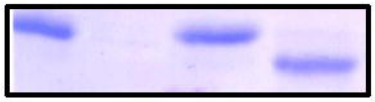
RNase B + + + +
Endo F1 - + + +
NGLY1-WT - - + -
T533A - - - +
LB
CBB

A

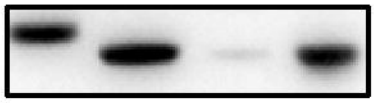
RNase B	+	-	+	+
NGLY1-WT	-	+	-	-
R328G	-	-	+	+

CBB



RNase B	+	+	+	+
Endo F1	-	+	+	+
NGLY1-WT	-	-	+	-
R328G	-	-	-	+

LB



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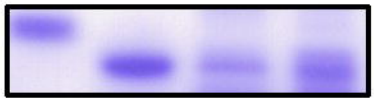


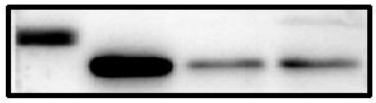
B

RNase B	+	+	+
NGLY1-WT	-	+	-
Y342C	-	-	+

CBB

RNase B	+	+	+	+
Endo F1	-	+	+	+
NGLY1-WT	-	-	+	-
Y342C	-	-	-	+

LB



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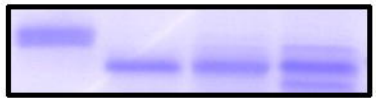


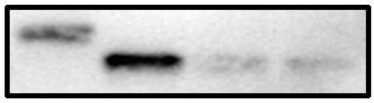
C

RNase B	+	+	+
NGLY1-WT	-	+	-
C355R	-	-	+

CBB

RNase B	+	+	+	+
Endo F1	-	+	+	+
NGLY1-WT	-	-	+	-
C355R	-	-	-	+

LB



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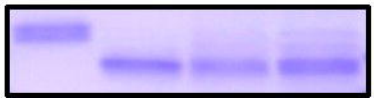


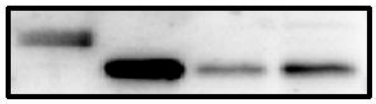
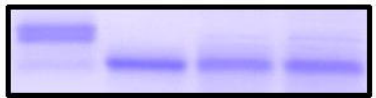
D

RNase B	+	+	+
NGLY1-WT	-	+	-
E356G	-	-	+

CBB

RNase B	+	+	+	+
Endo F1	-	+	+	+
NGLY1-WT	-	-	+	-
E356G	-	-	-	+

LB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