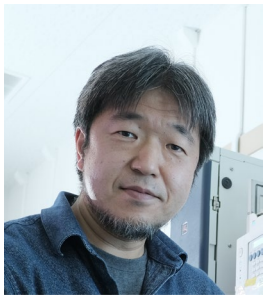
Glycometabolic Biochemistry Laboratory（2021）

Chief Scientist: Tadashi Suzuki (D.Sci.)



(0) Research field

CPR Subcommittee**:** Biology

Keywords: glycoproteins, asparagine-linked glycans, metabolism, peptide:*N*-glycanase, Ngly1

(1) Long-term goal of laboratory and research background

Peptide:*N*-glycanase (PNGase) releases asparagine-linked (*N*-linked) glycans from glycoproteins/glycopeptides. The cytoplasmic PNGases (NGLY1/Ngly1 in human/mouse or rat), ubiquitously found throughout eukaryotes, are now widely recognized as a component implicated in the ERAD (ER-associated degradation) process, which constitute one of the quality control machineries for newly synthesized misfolded glycoproteins exported out of the ER lumen. While the biosynthetic pathway for *N*-glycans has been clarified in detail, the catabolic pathway for the "free" *N*-glycans, released by the cytoplasmic PNGase or other activities, remains largely unknown. Although this "non-lysosomal" metabolic path for *N*-glycan may represent one of the very basic biological phenomena in eukaryotes, there are still many more enzymes/transporters that remains to be identified. We are currently trying to identify other players involved in this process, and also taking a number of approaches to analyze the physiological importance of this non-lysosomal metabolic pathway.

(1) Current research activities (FY2021) and plan (until Mar. 2025)

N-glycanase 1 (NGLY1) deficiency, an autosomal recessive disorder caused by mutations in the *NGLY1* gene, is characterized by developmental delay, hypolacrima or alacrima, seizure, intellectual disability, movement disorders and other neurological phenotypes. In a collaboration with Takeda Pharmaceutical Co. (T-CiRA program), we successfully generated the systemic *Ngly1*-KO rats, which recapitulated disease symptoms of NGLY1 deficiency, such as developmental delay, movement disorder, somatosensory impairment and scoliosis. This year, we went on to develop viable *Ngly1*-KO mice, using two inbred strains, i.e. C57BL/6 (B6) mice and JF1 mice. While *Ngly1*-KO mice in each strain were found to be embryonic lethal, hydrid F1 *Ngly1*-KO mice (JF1/B6F1 mice) escaped embryonic lethality, and showed developmental delay and motor dysfunction similar to human patients. Accumulation of ubiquitinated proteins were evidenced in specific tissues in central nervous system. JF1/B6F1 *Ngly1*-KO mice also showed increased levels of plasma/urinary Asn-GlcNAc, a potential biomarker for NGLY1 deficiency, indicating that Asn-GlcNAc can be widely used as a marker for NGLY1 deficiency subjects/animals. B6/JF1F1 *Ngly1*-KO mice should serve as yet another valuable tool for preclinical studies for NGLY1 deficiency as an isogenic *Ngly1*-KO model animal [1].

We also utilized *Ngly1*-KO rat and single intracerebroventricular injection of AAV9 expressing human NGLY1 cDNA was applied to the *Ngly1*-KO rats during the weaning period. As a result, expression of human NGLY1 protein was evident in tissues of central nervous system. Surprisingly, this treatment drastically improved motor phenotypes of *Ngly1*-KO rats, as judged by the rota-rod test and gait analysis. These results clearly indicate that at least some of phenotypes found in *Ngly1*-KO rats are reversible, further supporting the idea that our *Ngly1*-KO rat is useful for evaluating various therapeutic options in pre-clinical studies. Our study also demonstrates that central nervous system, including brain, can be the primary therapeutic target organs for NGLY1 deficiency [2].

Previously, we identified ENGase, another deglycosylating enzyme acting on *N*-glycans, as a factor to escape the embryonic lethality of B6 *Ngly1*-KO mice, and it has been shown that *Ngly1 Engase* double KO mice partially escaped the embryonic lethality, and most of them could manage to survive for more than 1 year. As they age, however, they develop several detrimental phenotypes such as tremor, bent spine, that mimic disease symptoms of NGLY1 deficiency, strongly indicating that suppression effect of *Engase*-deletion is only partial. On the other hand, our collaboration with Dr. Yukiko Yoshida (Tokyo Metropolitan Institute of Medical Science) showed that deletion of *Fbs2*, a gene encoding a sugar-recognizing subunit for ubiquitin ligase, resulted in almost complete escape of embryonic lethality for *Ngly1*-KO mice. Moreover, the survived *Fbs2 Ngly1* double KO mice showed normal motor functions, and exhibited, despite the defective *Ngly1* gene, no disease-related phenotypes. Our cell-based analysis also showed that, when FBS2 was overexpressed in human-derived cultured cells, cells could not form colonies. Further detailed analyses indicated that, upon overexpression of FBS2 in *NGLY1*-KO cells, abnormally-ubiquitinated glycoproteins, which were supposed to be recognized and degraded by the proteasome under normal conditions, were accumulated, which somehow caused proteasome dysfunctions, leading to cell death (Figure). This study not only clarifies the disease mechanism for NGLY1 deficiency, but also raises the possibility that functional inhibitor for FBS2 could serve as a promising drug for NGLY1 deficiency, especially because that *Fbs2*-KO mice did not exhibit any detrimental phenotype [3].

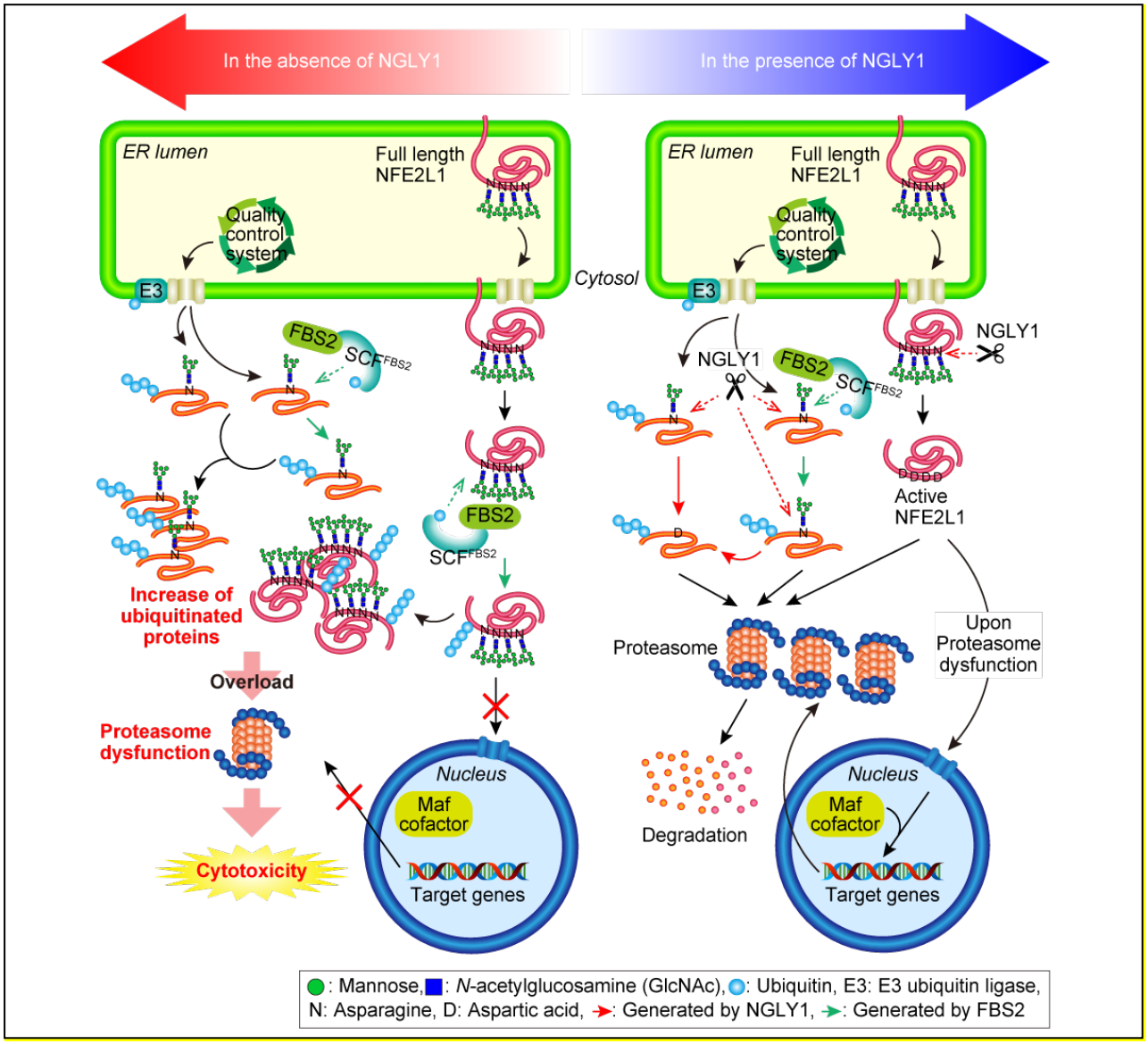


Figure Proposed mechanism for toxic effect of FBS2 in NGLY1-defective cells [3].
(right) Under normal NGLY1 functions, glycoprotein substrates for ERAD, including a transcription factor NFE2L1, undergo ubiquitination, followed by proteasomal degradation. On the other hand, (left) glycoprotein substrates for ERAD are abnormally ubiquitinated by FBS2-containing ubiquitin ligase (SCF^{FBS2}), and their accumulation somehow led to proteasomal dysfunction. Cells then try to activate NFE2L1 to induce expression of genes encoding proteasome subunits, but NFE2L1 itself is a glycoprotein and therefore ubiquitinated by SCF^{FBS2} and accumulated. As a result, proteasomal dysfunction persists, eventually leading to cell death. Figure is from Fujihira, et al. (2022) **J. Biochem.** 171, 161-167).

Since the discovery of NGLY1 deficiency in 2012, more than 100 cases have been identified worldwide, and numerous disease alleles have been reported. Correlation between the severity of patients' symptoms and the extent of the reduction in NGLY1 activity in these patients, however, remains unknown, due to the lack of a facile, quantitative assay system for NGLY1, especially when crude extract was used as an enzyme source. We expressed 27 disease-associated mutants and their activities were tested using fluorescence-labeled glycopeptides, and found that three mutants retained 30-70% of the activity of wild-type NGLY1. We also developed a method for measuring endogenous NGLY1 activity in crude extracts using a glycosylated cyclopeptide that exhibited resistance to the endogenous proteases in the extract. This new assay method using GCP could be applicable for a facile, early diagnosis of NGLY1 deficiency [4].

Free *N*-glycans (FNGs), formed by NGLY1 or other activities, are normally accumulated intracellularly (intracellular FNGs). On the other hand, we recently identified extracellular sialyl FNGs in various animal sera. While most of intracellular FNGs have a single GlcNAc at their reducing termini (Gn1-type), extracellular FNGs have an *N,N'*-diacetylchitobiose at their reducing termini (Gn2-type). We reported on an improved

method for isolating free glycans from animal se from animal sera, and unexpectedly found that, not only sialyl FNGs, but there are other types of free glycans such as Gn1-type FNGs, neutral FNGs, or small sialyl glycans such as sialyl lactose/LacNAc, structures often found in milk oligosaccharides. Our results indicate that there are varieties of free oligosaccharides in animal sera, and the mechanism responsible for their formation is much more complicated than currently envisaged [5].

In the future, we will continue to aim at clarifying the molecular mechanism for the catabolism of *N*-glycans and their precursors (dolichol-linked oligosaccharides). We will also aim at unveiling the species-specific glycan biosynthetic and degradation pathway, to provide insight into the functional importance of glycans from the standpoint of "comparative glycobiology". In addition, we will clarify the pathophysiology of *Ngly1*-KO mice and also contribute to develop the therapeutic means for NGLY1 deficiency through T-CiRA program.

(3) Members

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(Technical Staff 1)
Reiko Fujinawa
Junichi Seino
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(Assistant)
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(Research Part Time Worker II)
Ritsuko Oka
Tsugiyo Matsuda

(4) Representative research achievements

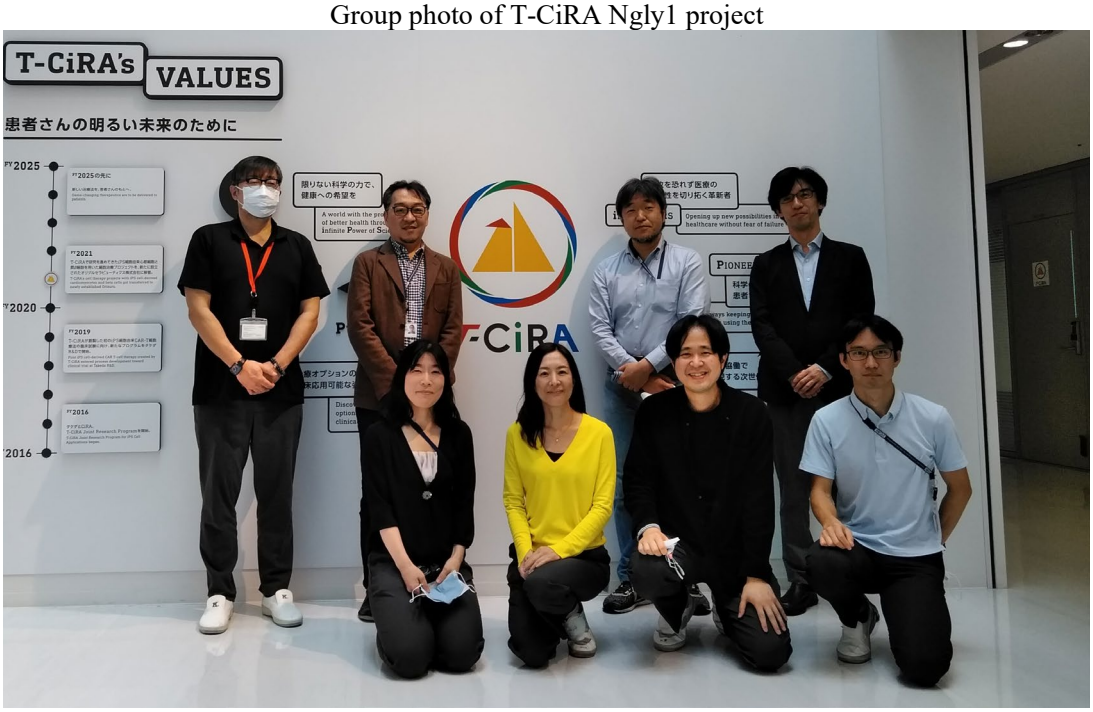
1. M. Asahina, R. Fujinawa, H. Fujihira, Y. Masahara-Negishi, T. Andou, R. Tozawa, and T. Suzuki*. (2021) JF1/B6F1 *Ngly1-/-* mouse as an isogenic animal model of NGLY1 deficiency. ***Proc. Japan Acad. Ser. B Phys. Biol. Sci.*** **97,** 89-102 (doi: 10.2183/pjab.97.005)
2. Y. Yoshida*, M. Asahina, A. Murakami, J. Kawasaki, M. Yoshida, R. Fujinawa, K. Iwai, R. Tozawa, N. Matsuda, K. Tanaka*, and T. Suzuki* (2021) Loss of peptide:*N*-glycanase causes proteasome dysfunction mediated by a sugar-recognizing ubiquitin ligase. ***Proc. Natl. Acad. Sci. USA*** **118,** e2102902118 (doi: 10.1073/pnas.2102902118)
3. M. Asahina, R. Fujinawa, H. Hirayama, R. Tozawa, Y. Kajii, and T. Suzuki* (2021) Reversibility of motor dysfunction in the rat model of NGLY1 deficiency. ***Mol. Brain*** **14,** 91 (doi: 10.1186/s13041-021-00806-6).
4. H. Hirayama, Y. Tachida, J. Seino, and T. Suzuki* (2022) A method for assaying peptide:*N*-glycanase/*N*-Glycanase 1 activities in crude extracts using an *N*-glycosylated cyclopeptide. ***Glycobiology*** **32,** 110-122 (doi: 10.1093/glycob/cwab115).
5. C. Huang, J. Seino, H. Fujihira, K. Sato, R. Fujinawa, Z. Sumer-Bayraktar, N. Ishii, I. Matsuo, S. Nakaya, and T. Suzuki* (2022) Occurrence of free *N*-glycans with a single GlcNAc at the reducing termini in animal sera. ***Glycobiology*** **32,** 314-332 (doi: 10.1093/glycob/cwab124)

*=corresponding author

Supplementary

Group photo of RIKEN Glycometabolic Biochemistry Laboratory

Group photo of T-CiRA Ngly1 project



Laboratory Homepage

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