THE ROLE OF β-1,4-GLUCURONYLTRANSFERASE 1 IN α-DYSTROGLYCAN FUNCTION

by

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(Under the Direction of Lance Wells)

ABSTRACT

Alpha-dystroglycan (α-DG) is a necessary cell-surface receptor in diverse metazoan species and is particularly important in mammalian neural development and muscular structure. However, the complex cell biology, regulation and the non-template driven processing of this key peripheral membrane receptor has resulted in serious challenges in functional characterization. The role of α-DG in human disease including the congenital muscular dystrophies and arenavirus infections have spurred study even in the face of these challenges. We have undertaken studies aimed at further elucidating the requirements for the function of α-DG as well as at applying knowledge in the field toward developing treatments for arenavirus infection in particular. In the course of this work, we have principally characterized a key enzyme that post-translationally processes α-DG and is necessary for the various functions of α-DG. We further aim to apply this knowledge in the development of therapeutics for treating arenavirus infections.

INDEX WORDS: Congenital muscular dystrophy, O-mannosylation, alpha-dystroglycan, poly-N-acetyllactosamine, glucuronyltransferase, N-

acetylglucosaminyltransferase, B3GNT1, B4GAT1, LARGE, secondary dystroglycanopathies, Lassa fever virus, Arenavirus

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DEDICATION

Dedicated to a determination to change.

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By way of conclusion, the wisdom of Brooks and Wilder embodies a theme woven throughout the process of research and has often helped to stiffen my resolve. That, if science teaches us anything, it is to, with quiet dignity and with grace, accept our failures as well as our successes.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

This chapter outlines the structure of this dissertation and provides relevant background information. Brief sections on cell surface function including the role carbohydrates play and the function of the carbohydrate-modified protein α-dystroglycan (α-DG) at the cell surface in mammalian tissue are included in the present chapter. The following chapters consist of two previously published journal articles as well as a chapter detailing current progress toward the preparation of another research article. Each of these subsequent chapters expands on aspects of the biochemistry of α-DG, an indispensable cell-extracellular matrix adhesion glycoprotein in mammals with mechanical, developmental and cellular signaling roles.

Chapter 2 consists of a review article detailing O-mannosylation in mammals. O-mannosylation is required for the cell surface receptor function of α-DG. *In vivo*, extracellular binding partners of α-DG bind to an α-DG-specific repeating disaccharide polymer ligand synthesized as an extension of a phosphorylated O-mannose trisaccharide structure (termed functional glycosylation). Chapter 3 consists of a research article detailing our determination and characterization of the activity of an enzyme necessary for the functional glycosylation of α-DG. This enzyme, which we and a co-publishing lab have named β-1,4-glucuronyltransferase 1 (B4GAT1), transfers a glucuronic acid (GlcA) to putative nascent O-mannose functional glycans thus generating the substrate for synthesis of the repeating disaccharide ligand. Finally, chapter 4 reports on our progress toward preparation of a research article aimed at applying this discovery

for developing therapies directed against highly virulent arenaviruses that bind to functionally glycosylated α-DG in order to gain entry to mammalian (and in particular human) cells.

Cell surface, Carbohydrates

The surfaces of cells are fundamental interfaces in biology forming a key part of the boundary between the internal cellular compartment and the external environment. The centrality of this interface is clear given that all interactions between cells and their environment, including anchoring and motility, uptake and removal of metabolites and ions, and communication and signaling are mediated through this interface [1]. Many of these functions are provided by proteins and protein complexes called cell surface receptors. In mammalian biology most cell surface receptors are modified by covalently attached carbohydrates, termed glycans, that are often either directly or indirectly necessary for their function [2]. There are two predominant classes of glycans synthesized in mammals as categorized by linkage to the underlying protein. O-glycans that are linked, by displacement of the hydroxyl hydrogen, to a hydroxyl oxygen of a serine (Ser) or threonine (Thr) residue and N-glycans that are linked through the nitrogen of an asparagine residue [3]. The class of O-glycans can be further divided based on the identity of the initiating monosaccharide [3]. O-linked mannose (O-mannose) initiated glycans are central to the remainder of this dissertation however O-linked N-acetylgalactosamine (O-GalNAc) initiated glycans are also involved (possibly peripherally) in the biochemistry discussed herein (found on α-DG as well as reported on certain enzymes necessary for producing fully functional α-DG including POMGNT2 [4, 5] , see Chapter 2).

The ubiquity and importance of glycosylation of mammalian cell surface proteins is apparent upon analysis of curated entries in various high quality protein and post-translational modification databases compiled from the extant corpus of scientific literature [5-10] in

combination with data from recent large-scale glycoproteomics studies. These studies which integrate various analytical enrichment strategies with advanced shotgun glycoproteomics methods [9-12], most powerfully in addition to protein modification substitution (e.g. ^{18}O labeling) or genome editing-based reduction in glycan heterogeneity and size [4, 13, 14], have greatly expanded the proportion of the proteome with direct experimental evidence of glycosylation. While a single up-to-date reference consolidating this wealth of data is difficult to find, the process of generating custom views directed at specific questions has been continually improved by the efforts of database maintainers and authors publishing complete data sets as supplements. For example, a UniprotKB 2015_04 [6] search for mouse proteins with the manual annotations of "Plasmalemma [SL-0039]" and "transmem" (region) compared against the same search with an additional requirement for experimental evidence for glycosylation in combination with O-GalNAc site data from PhosphoSitePlus [5] and data from a recent large-scale mouse N-glycoproteomics study [10] produces lists identifying 257 of the 866 membrane proteins (29.7%) as being experimentally characterized as glycosylated. This is certainly an underestimate of true prevalence but is compelling. Furthermore, the functional importance of glycans is clear given the severity of phenotypes caused by any significant disruption of glycosylation machinery [15, 16], although the existence of degrees of redundancy in the various glycosylation pathways (e.g. polypeptide O-GalNAc transferases [17, 18], sialyltransferases [19, 20]) could be interpreted as evolutionarily highlighting or, at the level of individuals, partially genetically masking these effects. Categorized by type of glycosylation, defects in N-glycosylation (typically classified as congenital disorders of glycosylation or CDGs) result in pathological outcomes commonly including liver dysfunction, growth retardation, psychomotor delay and mental retardation [16]. Greater disruption of N-glycosylation is embryonic lethal. Defects in O-mannose glycan synthesis

can result in conditions traditionally referred to as dystroglycanopathic congenital muscular dystrophies (secondary dystroglycanopathies, CMDs) that also range in outcome from abnormal degeneration of muscle tissue that becomes pronounced in adulthood to causative of death in infancy or during embryogenesis [16]. These conditions vary with the level of function of the protein α-DG, dependent upon degree of synthesis of specific essential O-mannose glycan modifications.

O-Mannosylation

O-Mannose is added in an "α" stereochemical linkage to serines and threonines of substrate proteins by the activity of the protein-O-mannosyltransferase (POMT) enzyme complex consisting of the subunits POMT1 and POMT2 (see Chapter 2 or [3]). After addition, structural elaboration is carried out through the biosynthetic activities of an additional 15 currently known proteins (Tables 2.1 and 2.2, Figures 2.5 and 2.6). Many of these proteins and the underlying genes have been discovered through investigation of the glycosylation of α-DG particularly in relation to human disease. For many years the structure Siaα2–3Galβ1–4GlcNAcβ1–2Manα–Ser/Thr (often referred to as the classical tetrasaccharide, see Figure 2.2 structure 4) received the most attention in the literature. This structure is the most abundant O-mannose structure on α-DG and was hypothesized to be part of the epitope required for α-DG to bind to extracellular matrix (ECM) proteins since genetic ablation of POMGNT1 which adds β1,2-linked N-acetylglucosamine (GlcNAc) to O-mannose results in CMD phenotypes and a loss of ECM protein binding. It has now been shown that functional glycosylation of α-DG (as defined by binding activity toward ECM laminin globular-domain [LG] containing proteins) depends directly on synthesis of a repeating disaccharide consisting of glucuronic acid and xylose (GlcA-β3-Xyl-α3-) polymeric units by either of the dual-activity enzymes LARGE or LARGE2 [21]. This repeating disaccharide

has only been shown to be synthesized *in vivo* distal to the phosphate of a distinctive phospho-O-mannose trisaccharide (Figure 2.4, structure 21) covalently attached to α-DG. A preponderance of evidence suggests that it is this polymer chain alone ("LARGE-dependent glycan") that is essential for function and that α-DG primarily serves as the only natural *in vivo* substrate for synthesis in this regard. For example, the authors of the study that initially determined the activity of LARGE and the identity of its repeating disaccharide product noted that α-DG binds to LG4 and LG5 of laminin alpha chains and that one of the basic patches found in these domains is known to bind to chemically similar heparan sulfate-type acidic sugar chains [22]. Furthermore, the degree of LG domain binding correlates directly with degree of polymerization [21], and, reports in the literature (sometimes considered controversial) have provided evidence that overexpression of LARGE can result in "functionalization" of N-glycans and other non-native (as substrates) structures [23]. These results suggest that there is a lack of specificity for underlying polypeptide features in these interactions. Chapter 2 provides a broader survey of O-mannosylation including further details of synthesis of the LARGE-dependent glycan. Since the publication of this review, we, concomitantly with another lab, have added to this body of knowledge by determining the correct activity of B3GNT1 (B4GAT1) required for functional glycosylation of α-DG as reported in Chapter 3.

α -Dystroglycan

In mammals, alpha-dystroglycan (α-DG) is an extracellular peripheral membrane glycoprotein that forms the final link between the cellular dystrophin-associated protein complex (DAPC) and extracellular matrix (ECM) proteins. The DAPC together with the integrins comprise the two primary cell-ECM adhesion systems and exhibit a complementarity and interplay that, in concert with ECM components, provides a foundation for the architecture and function of mammalian tissues. It should be stressed that current literature highlights substantial flexibility

and modularity of the DAPC and a corresponding wide diversity of roles ranging from central mechanical functions in striated muscle tissue to key functions in development and patterning in the central and peripheral nervous systems. The physiological roles of α-DG are of great interest and accumulating evidence is suggestive of models in which the most severe pathologies depend on disruption of the basement membrane organizing functionality of α-DG and its key glycan modifications [24] as well as disruption in its role (through additional signaling effectors) in organizing, recruiting and modulating the cellular cytoskeleton in addition to other primary adhesion molecules such as the integrins [25, 26]. However, in this introduction, I will focus on the molecular properties of α-DG relevant to the remaining chapters and will only include brief coverage of other elements of the DAPC.

Human α-DG is encoded by the gene DAG1 (dystrophin associated glycoprotein-1), the translation product of which contains a diverse and rich array of sequence features including four identified polypeptide cleavage sites (including the signal peptide cleavage site), more than 34 mapped sites of glycosylation and an extensive set of protein-protein interaction regions. This set of features accounts for the incredible variability and diversity in function of the complex formed by α-dystroglycan (residues 30-653) and β-dystroglycan (β-DG, residues 654-895), the two principal protein products of DAG1 that result from serine endopeptidase activity encoded in the sequence of DAG1. β-DG is an approximately 43 kilodalton (kDa) single-pass type I transmembrane protein that is notable as a nexus of protein-protein interactions with wide variation across tissues and through development. For example, in striated muscle tissue, interactions with dystrophin and the sarcoglycan complex are essential for mechanical muscle cell membrane stability in this high stress environment. In the context of signaling, muscle tissue β-DG passes signals (indirectly) to neuronal nitric oxide synthase (nNOS) resulting in local dilation of blood

vessels that increases blood flow during muscular exertion. These functions are substantially dependent on forces and signals transduced by the extracellular binding partner of β-DG, α-DG.

Alpha-dystroglycan is frequently described, based on electron microscopy studies [27, 28] as an approximately 7.5 nm x 3 nm dumbbell shaped protein consisting of two globular domains (amino acid residues 1-315 and 486-653) connected by a central rod-shaped extensively glycosylated mucin-like domain (MLD, amino acids 316-485). *In vivo*, α-DG may exist as a mixture of this form and a likely more abundant form in which the globular N-terminal domain has been removed by a Furin-like convertase [29-32]. The role of each of domain in the ultimate function of α-DG can be partly dissected based on various studies. The C-terminal domain is necessary for α-DG binding to β-DG and consequent retention at the cell surface and mechanotransduction capability. Substantial evidence of additional functions does not appear in the literature and the C-terminal domain appears to otherwise be irrelevant in the synthesis of functional glycans on α-DG as well as in its resulting basement membrane organization and compaction functionality [30, 33]. This functionality is endowed by distinctive O-mannose glycans that are only synthesized at specific sites contained in the mucin-like domain (confined to the region 316 to 405) [30]. In particular, functional modification is localized primarily at Thr-317 and Thr-319 but also to lesser degrees at Thr-328 and Thr-329 [31] and almost certainly Thr-379 [34, 35]. Although the remainder of the MLD is substantially further O-glycosylated, the role of these glycans in the function of α-DG is less well-defined although certain classes, in particular β-1,2-linked O-mannose glycans (Figure 2.2) may help to facilitate synthesis of functional glycans *in vivo* (possibly through conformational effects or molecular interactions). A direct role for these glycans and N-glycans on α-DG in binding to ECM components has been ruled out [36, 37]. Finally, the N-terminal domain [38] is essential for synthesis of functional glycan structures

through necessary protein-protein interaction with (recruitment of) the enzyme LARGE that is responsible for synthesizing the key functional glycan epitope [30, 39]. However, the N-terminal domain is subsequently irrelevant for cell-surface binding of ECM ligands to α-DG crucial for its signaling, basement membrane organization and other functional roles [31]. Besides these features, α-DG exhibits extreme heterogeneity and a broad range of molecular weights due to extensive glycosylation [30, 40].

References

1. Lodish HF. Biomembranes: Structural Organization and Basic Functions. In: Molecular Cell Biology [Internet]. New York: W.H. Freeman; 2000. 4th ed. Section 5.3. Available from: http://www.ncbi.nlm.nih.gov/books/NBK21583/.

2. Varki A, Lowe JB. Biological Roles of Glycans. In: Essentials of Glycobiology [Internet]. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press; 2009. 2nd ed. Chapter 6. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1897/.

3. Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW, Etzler ME, editors. Essentials of Glycobiology. 2nd ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2009.

4. Steentoft C, Vakhrushev SY, Joshi HJ, Kong Y, Vester-Christensen MB, Schjoldager KT, Lavrsen K, Dabelsteen S, Pedersen NB, Marcos-Silva L, Gupta R, Bennett EP, Mandel U, Brunak S, Wandall HH, Levery SB, Clausen H. Precision mapping of the human O-GalNAc glycoproteome through SimpleCell technology. EMBO J. 2013;32(10):1478-88. doi: 10.1038/emboj.2013.79. PubMed PMID: 23584533; PMCID: 3655468.

5. Hornbeck PV, Zhang B, Murray B, Kornhauser JM, Latham V, Skrzypek E. PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. Nucleic acids research. 2015;43(Database issue):D512-20. doi: 10.1093/nar/gku1267. PubMed PMID: 25514926.

6. UniProt C. UniProt: a hub for protein information. Nucleic acids research. 2015;43(Database issue):D204-12. doi: 10.1093/nar/gku989. PubMed PMID: 25348405.

7. Baycin Hizal D, Wolozny D, Colao J, Jacobson E, Tian Y, Krag SS, Betenbaugh MJ, Zhang H. Glycoproteomic and glycomic databases. Clin Proteomics. 2014;11(1):15. doi: 10.1186/1559-0275-11-15. PubMed PMID: 24725457; PMCID: 3996109.

8. Dimmer EC, Huntley RP, Alam-Faruque Y, Sawford T, O'Donovan C, Martin MJ, Bely B, Browne P, Mun Chan W, Eberhardt R, Gardner M, Laiho K, Legge D, Magrane M, Pichler K, Poggioli D, Sehra H, Auchincloss A, Axelsen K, Blatter MC, Boutet E, Braconi-Quintaje S, Breuza L, Bridge A, Coudert E, Estreicher A, Famiglietti L, Ferro-Rojas S, Feuermann M, Gos A, Gruaz-Gumowski N, Hinz U, Hulo C, James J, Jimenez S, Jungo F, Keller G, Lemercier P, Lieberherr D, Masson P, Moinat M, Pedruzzi I, Poux S, Rivoire C, Roechert B, Schneider M, Stutz A, Sundaram S, Tognolli M, Bougueleret L, Argoud-Puy G, Cusin I, Duek-Roggli P, Xenarios I, Apweiler R. The UniProt-GO Annotation database in 2011. Nucleic acids research. 2012;40(Database issue):D565-70. doi: 10.1093/nar/gkr1048. PubMed PMID: 22123736; PMCID: 3245010.

9. Han D, Moon S, Kim Y, Min H, Kim Y. Characterization of the membrane proteome and N-glycoproteome in BV-2 mouse microglia by liquid chromatography-tandem mass spectrometry. BMC Genomics. 2014;15:95. doi: 10.1186/1471-2164-15-95. PubMed PMID: 24495382; PMCID: 3938046.

10. Zielinska DF, Gnad F, Wisniewski JR, Mann M. Precision mapping of an in vivo N-glycoproteome reveals rigid topological and sequence constraints. Cell. 2010;141(5):897-907. doi: 10.1016/j.cell.2010.04.012. PubMed PMID: 20510933.

11. Thaysen-Andersen M, Packer NH. Advances in LC-MS/MS-based glycoproteomics: getting closer to system-wide site-specific mapping of the N- and O-glycoproteome. Biochimica et biophysica acta. 2014;1844(9):1437-52. doi: 10.1016/j.bbapap.2014.05.002. PubMed PMID: 24830338.

12. Zielinska DF, Gnad F, Schropp K, Wisniewski JR, Mann M. Mapping N-glycosylation sites across seven evolutionarily distant species reveals a divergent substrate proteome despite a common core machinery. Mol Cell. 2012;46(4):542-8. doi: 10.1016/j.molcel.2012.04.031. PubMed PMID: 22633491.

13. Vester-Christensen MB, Halim A, Joshi HJ, Steentoft C, Bennett EP, Levery SB, Vakhrushev SY, Clausen H. Mining the O-mannose glycoproteome reveals cadherins as major O-mannosylated glycoproteins. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(52):21018-23. doi: 10.1073/pnas.1313446110. PubMed PMID: 24101494; PMCID: 3876253.

14. Steentoft C, Vakhrushev SY, Vester-Christensen MB, Schjoldager KT, Kong Y, Bennett EP, Mandel U, Wandall H, Levery SB, Clausen H. Mining the O-glycoproteome using zinc-finger nuclease-glycoengineered SimpleCell lines. Nat Methods. 2011;8(11):977-82. doi: 10.1038/nmeth.1731. PubMed PMID: 21983924.

15. Cummings RD, Pierce JM. The challenge and promise of glycomics. Chem Biol. 2014;21(1):1-15. doi: 10.1016/j.chembiol.2013.12.010. PubMed PMID: 24439204; PMCID: 3955176.

16. Freeze HH, Esko JD, Parodi AJ, Etzler ME, Varki A, Vacquier VD, Nizet V, Cummings RD, Turco S, Schachter H, Kannagi R, Toole BP. Glycans in Physiology and Disease. In: Essentials of Glycobiology [Internet]. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press; 2009. 2nd ed. Section V. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1949/.

17. Ten Hagen KG, Fritz TA, Tabak LA. All in the family: the UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferases. Glycobiology. 2003;13(1):1R-16R. doi: 10.1093/glycob/cwg007. PubMed PMID: 12634319.

18. Pratt MR, Hang HC, Ten Hagen KG, Rarick J, Gerken TA, Tabak LA, Bertozzi CR. Deconvoluting the functions of polypeptide N-alpha-acetylgalactosaminyltransferase family members by glycopeptide substrate profiling. Chem Biol. 2004;11(7):1009-16. doi: 10.1016/j.chembiol.2004.05.009. PubMed PMID: 15271359.

19. Dehnert KW, Baskin JM, Laughlin ST, Beahm BJ, Naidu NN, Amacher SL, Bertozzi CR. Imaging the sialome during zebrafish development with copper-free click chemistry. Chembiochem : a European journal of chemical biology. 2012;13(3):353-7. doi: 10.1002/cbic.201100649. PubMed PMID: 22262667; PMCID: 3385855.

20. Takashima S. Characterization of mouse sialyltransferase genes: their evolution and diversity. Biosci Biotechnol Biochem. 2008;72(5):1155-67. Epub 2008/05/08. doi: 10.1271/bbb.80025. PubMed PMID: 18460788.

21. Goddeeris MM, Wu B, Venzke D, Yoshida-Moriguchi T, Saito F, Matsumura K, Moore SA, Campbell KP. LARGE glycans on dystroglycan function as a tunable matrix scaffold to prevent dystrophy. Nature. 2013;503(7474):136-40. Epub 2013/10/18. doi: 10.1038/nature12605. PubMed PMID: 24132234; PMCID: 3891507.

22. Inamori K, Yoshida-Moriguchi T, Hara Y, Anderson ME, Yu L, Campbell KP. Dystroglycan function requires xylosyl- and glucuronyltransferase activities of LARGE. Science. 2012;335(6064):93-6. doi: 10.1126/science.1214115. PubMed PMID: 22223806; PMCID: 3702376.

23. Patnaik SK, Stanley P. Mouse large can modify complex N- and mucin O-glycans on alpha-dystroglycan to induce laminin binding. The Journal of biological chemistry. 2005;280(21):20851-9. doi: 10.1074/jbc.M500069200. PubMed PMID: 15788414.

24. Wright KM, Lyon KA, Leung H, Leahy DJ, Ma L, Ginty DD. Dystroglycan organizes axon guidance cue localization and axonal pathfinding. Neuron. 2012;76(5):931-44. doi: 10.1016/j.neuron.2012.10.009. PubMed PMID: 23217742; PMCID: 3526105.

25. Bello V, Moreau N, Sirour C, Hidalgo M, Buisson N, Darribere T. The dystroglycan: Nestled in an adhesome during embryonic development. Dev Biol. 2014. doi: 10.1016/j.ydbio.2014.07.006. PubMed PMID: 25050932.

26. Moore CJ, Winder SJ. Dystroglycan versatility in cell adhesion: a tale of multiple motifs. Cell Commun Signal. 2010;8:3. doi: 10.1186/1478-811X-8-3. PubMed PMID: 20163697; PMCID: 2834674.

27. Brancaccio A, Schulthess T, Gesemann M, Engel J. Electron microscopic evidence for a mucin-like region in chick muscle alpha-dystroglycan. FEBS letters. 1995;368(1):139-42. PubMed PMID: 7615068.

28. Kunz S, Calder L, Oldstone MB. Electron microscopy of an alpha-dystroglycan fragment containing receptor sites for lymphocytic choriomeningitis virus and laminin, and use of the receptoid body as a reagent to neutralize virus. Virology. 2004;325(2):207-15. doi: 10.1016/j.virol.2004.04.044. PubMed PMID: 15246261.

29. Singh J, Itahana Y, Knight-Krajewski S, Kanagawa M, Campbell KP, Bissell MJ, Muschler J. Proteolytic enzymes and altered glycosylation modulate dystroglycan function in carcinoma cells. Cancer Res. 2004;64(17):6152-9. doi: 10.1158/0008-5472.CAN-04-1638. PubMed PMID: 15342399.

30. Kanagawa M, Saito F, Kunz S, Yoshida-Moriguchi T, Barresi R, Kobayashi YM, Muschler J, Dumanski JP, Michele DE, Oldstone MB, Campbell KP. Molecular recognition by LARGE is essential for expression of functional dystroglycan. Cell. 2004;117(7):953-64. doi: 10.1016/j.cell.2004.06.003. PubMed PMID: 15210115.

31. Hara Y, Kanagawa M, Kunz S, Yoshida-Moriguchi T, Satz JS, Kobayashi YM, Zhu Z, Burden SJ, Oldstone MB, Campbell KP. Like-acetylglucosaminyltransferase (LARGE)-dependent modification of dystroglycan at Thr-317/319 is required for laminin binding and arenavirus infection. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(42):17426-31. doi: 10.1073/pnas.1114836108. PubMed PMID: 21987822; PMCID: 3198351.

32. Saito F, Saito-Arai Y, Nakamura A, Shimizu T, Matsumura K. Processing and secretion of the N-terminal domain of alpha-dystroglycan in cell culture media. FEBS letters. 2008;582(3):439-44. doi: 10.1016/j.febslet.2008.01.006. PubMed PMID: 18201566.

33. Akhavan A, Crivelli SN, Singh M, Lingappa VR, Muschler JL. SEA domain proteolysis determines the functional composition of dystroglycan. FASEB J. 2008;22(2):612-21. doi: 10.1096/fj.07-8354com. PubMed PMID: 17905726.

34. Yoshida-Moriguchi T, Yu L, Stalnaker SH, Davis S, Kunz S, Madson M, Oldstone MB, Schachter H, Wells L, Campbell KP. O-mannosyl phosphorylation of alpha-dystroglycan is required for laminin binding. Science. 2010;327(5961):88-92. doi: 10.1126/science.1180512. PubMed PMID: 20044576; PMCID: 2978000.

35. Campbell KP, Barresi R, inventors; University of Iowa Research Foundation, assignee. Increasing functional glycosylation of alpha-dystroglycan in the treatment of muscle degeneration. USA patent 8119766. 2012 2/21/2012.

36. Combs AC, Ervasti JM. Enhanced laminin binding by alpha-dystroglycan after enzymatic deglycosylation. The Biochemical journal. 2005;390(Pt 1):303-9. doi: 10.1042/BJ20050375. PubMed PMID: 15865602; PMCID: 1184583.

37. Kuga A, Kanagawa M, Sudo A, Chan YM, Tajiri M, Manya H, Kikkawa Y, Nomizu M, Kobayashi K, Endo T, Lu QL, Wada Y, Toda T. Absence of post-phosphoryl modification in dystroglycanopathy mouse models and wild-type tissues expressing non-laminin binding form of alpha-dystroglycan. The Journal of biological chemistry. 2012;287(12):9560-7. doi: 10.1074/jbc.M111.271767. PubMed PMID: 22270369; PMCID: 3308745.

38. Bozic D, Sciandra F, Lamba D, Brancaccio A. The structure of the N-terminal region of murine skeletal muscle alpha-dystroglycan discloses a modular architecture. The Journal of biological chemistry. 2004;279(43):44812-6. doi: 10.1074/jbc.C400353200. PubMed PMID: 15326183.

39. Hara Y, Balci-Hayta B, Yoshida-Moriguchi T, Kanagawa M, Beltran-Valero de Bernabe D, Gundesli H, Willer T, Satz JS, Crawford RW, Burden SJ, Kunz S, Oldstone MB, Accardi A, Talim B, Muntoni F, Topaloglu H, Dincer P, Campbell KP. A dystroglycan mutation associated with limb-girdle muscular dystrophy. The New England journal of medicine. 2011;364(10):939-46. doi: 10.1056/NEJMoa1006939. PubMed PMID: 21388311; PMCID: 3071687.

40. Durbeej M, Henry MD, Campbell KP. Dystroglycan in development and disease. Curr Opin Cell Biol. 1998;10(5):594-601. PubMed PMID: 9818169.

CHAPTER 2

THE MAMMALIAN O-MANNOSYLATION PATHWAY: GLYCAN STRUCTURES, ENZYMES, AND PROTEIN SUBSTRATES[1]

[1] Praissman JL, Wells L. Biochemistry. 2014;53(19):3066-78. doi: 10.1021/bi500153y. PubMed PMID: 24786756; PMCID: 4033628.

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Abstract

The mammalian O-mannosylation pathway for protein post-translational modification is intricately involved in modulating cell−matrix interactions in the musculature and nervous system. Defects in enzymes of this biosynthetic pathway are causative for multiple forms of congenital muscular dystophy. The application of advanced genetic and biochemical technologies has resulted in remarkable progress in this field over the past few years, culminating with the publication of three landmark papers in 2013 alone. In this review, we will highlight recent progress focusing on the dramatic expansion of the set of genes known to be involved in O-mannosylation and disease processes, the concurrent acceleration of the rate of O-mannosylation pathway protein functional assignments, the tremendous increase in the number of proteins now known to be modified by O-mannosylation, and the recent progress in protein O-mannose glycan quantification and site assignment. Also, we attempt to highlight key outstanding questions raised by this abundance of new information.

Introduction

Throughout biology, the addition of carbohydrates, or glycans, to extracellular and membrane proteins is an important post-translational modification involved in protein stability, quality control, cell-surface retention and ligand interactions [1]. Glycans modulate the biophysical properties of proteins and lipids and play particularly prominent roles in cellular interactions as the primary constituents of the often nanometers thick glycocalyces coating all mammalian cells. O-linked mannose (O-mannose) glycans are initiated by covalent linkage of mannose to the hydroxyl oxygen of a serine or threonine amino acid residue. O-mannose may then be extended by the addition of other monosaccharides and functional groups to form a variety of glycan structures.

In recent years, O-mannose glycans have been demonstrated to play critical roles in cellular interaction-based pathologies, including congenital muscular dystrophies (CMDs) [2-5] and cancers [6-9]. In particular, defects in the biosynthesis of O-mannose glycans often result in the hypoglycosylation of α-dystroglycan (α-DG), the most well characterized O-mannosylated mammalian protein. α-DG is a key part of the dystrophin-glycoprotein complex that links the extracellular matrix to the intracellular cytoskeleton. This linkage depends on the "functional glycosylation" of α-DG and its subsequent ability to bind to extracellular matrix proteins containing laminin globular (LG) domains [10, 11]. Hypoglycosylation of α-DG thus results in compromised tissue structure and robustness, causing CMDs termed secondary dystroglycanopathies [12].

In the past few years, glycomic advances have enabled increasingly rapid characterization of O-mannose glycans including further elucidation of the laminin-binding glycan structure [13, 14]. These results in conjunction with results from other studies have brought the complement of

observed O-mannose glycan structures to at least 23, some yet to be completely defined (Figures 2.2, 2.3 & 2.4). Glycoproteomic advances have dramatically increased our knowledge of proteins modified by O-mannosylation with a 2013 publication from the Clausen laboratory expanding the number of known O-mannosylated proteins from approximately 10 to more than 50 O-mannosylated glycoproteins, modified at a minimum of 235 sites, including most prominently the cadherins and plexins further cementing the role of O-mannosylation in cellular adhesion and interaction [15]. During the period from 2010 to mid-2013, the first papers mapping specific O-mannose glycans to distinct peptides and providing the first views of the actual sets of glycans co-synthesized *in vivo* were published [13, 16-18]. Additionally, a complementary series of quantitative glycomic and glycoproteomic studies of O-mannosylation in mammalian systems and pathologies were also published [16, 19, 20]. In parallel, advances in gene-based technologies in the past three years have allowed increasingly rapid characterization of the biosynthetic pathways involved. This has led to an expansion in the number of genes encoding proteins known to be directly involved in O-mannose structure synthesis from roughly 9 to 17 (Tables 2.1 & 2.2). In particular, gene trap insertion in a haploid mammalian cell line coupled with flow cytometry allowed the Brummelkamp laboratory to locate nearly all genes known to play a role in mammalian pathologies related to O-mannosylation (the work of roughly 15 years of biochemistry) as well as to identify previously undiscovered genes in a single publication [21]. Information about the "α-DG glycosylome" published in this paper integrated with biochemical information from the Campbell laboratory mapping a significant portion of key outstanding O-mannosylation pathway enzymatic activities [22] will play a prominent role in this review as we highlight the most recent results and synthesize information from across the field.

However, our understanding of mammalian O-mannosylation is far from complete. Glycan structures remain to be completely elucidated, details of the α-DG functional glycan epitope recognized by LG domains and the antibodies IIH6 and VIA4-1 [23, 24] remain to be conclusively resolved, and significant questions about the regulation and specificities of key enzyme activities and their functions across tissues and during development. Consequently, we will also attempt to highlight the most important outstanding questions in each section.

Nomenclature

In order to organize and conveniently discuss O-mannose glycans and glycosylation, first let us briefly consider nomenclature. In a recent paper from the Campbell laboratory elucidating biosynthesis of the "core" glycan structure that is ultimately extended so as to serve as the only known normal physiological acceptor of α-DG functional glycosylation, a set of core O-mannose structures were proposed (Figure 2.1) [22]. We will adopt this nomenclature when discussing the order-dependent biosynthesis of structures that represent base extensions beyond the N-acetylglucosamine (GlcNAc) residues directly attached to O-mannose. However, we also propose a corresponding set of named structures denoting O-mannose extended only by GlcNAc residues and will distinguish these smaller (sub)structures by the use of a lowercase m (Figure 2.1). This corresponding proposal more closely resembles the core structure naming convention adopted for O-GalNAc glycans [1]. Analogously, each core m structure is the most basic common core shared by a class of typically further extended O-mannose glycans. Consequently, this naming scheme allows for categorization of all of the known O-mannose structures observed to date (Figures 2.2, 2.3 & 2.4) and highlights possible biosynthetic fates dependent only on monosaccharides attached directly to O-mannose. We will use the core m nomenclature when discussing categories of glycans sharing a given core O-mannose structure.

Structures

O-mannose glycans account for up to 30% of all glycans O-linked to proteins in mammalian brain tissue [19, 25]. It is therefore understandable that O-mannose glycans have been shown to be essential for normal nervous system development that is dependent on neuron migration [3, 26, 27] and axon pathfinding [28] and to play a role in remyelination following myelin sheath damage [29]. Consequently, O-mannose glycans are tied to disorders causing cobblestone lissencephaly and mental retardation [30] as well as disorders such as multiple sclerosis in which myelin sheath destruction is a dominant factor [29]. O-mannose glycans also play a critical role in muscle structure and function, and defects in O-mannosylation can cause congenital muscular dystrophies (CMDs) that may or may not exhibit neurological involvement [30].

Protein-linked O-mannose glycans are initiated by the covalent attachment of mannose to serines and threonines via an α-linkage. Extended O-mannose glycans currently divide naturally into three categories based on known GlcNAc residue extensions of the initiating mannose (Figure 2.1, cores m1-m3). This set of core structures also captures the division in biological and biochemical roles ascribed to O-mannose structures (Figures 2.2, 2.3 & 2.4). O-mannose core structures may be extended in the Golgi by the addition of galactose (Gal) residues, N-acetylgalactosamine (GalNAc) residues, sialic acid (SA) terminals, sulfated glucuronic acid terminals forming human natural killer-1 epitopes, α1,3-linked fucose residues forming Lewis x structures, and the rather elusive core m3-specific post-phosphoryl laminin globular (LG) domain-binding moiety containing xylose (Xyl) and glucuronic acid (GlcA; Figure 2.4). To date, at least 23 distinct structures have been observed and characterized to varying degrees. Evidence indicates that O-mannose glycans show wide variation across tissue and cell types likely reflecting important

differences in function [5, 12, 16, 18, 19]. For years, α-DG was among the few protein substrates known until more recent studies suggested and confirmed a wide distribution of O-mannosylation across proteins [15, 19, 31, 32].

Core m1

The class of core m1 glycans consists of all O-mannose glycans in which O-mannose is extended with β1,2-linked GlcNAc but not with β1,6-linked GlcNAc (Figures 2.1, 2.2, 2.5). Core m1 glycans accounted for the largest fraction of O-mannose glycans released from mouse brain proteins, detected and quantified in a recent study, and constituted at least 15% of total brain protein O-glycans [19]. At least six different structures have been observed including notably the "classical tetrasaccharide" originally thought to be directly involved in laminin binding (structure 4, Figure 2.2) and a human natural killer-1 (HNK-1) epitope extended structure (structure 6, Figure 2.2). The current literature suggests that core m1 glycans do not directly bind key extracellular factors [19, 33] and that they play a less direct role at the cell surface in mammalian pathologies such as CMDs. Nevertheless, core m1 glycans are biologically essential becausesince core m1 itself is the precursor for core m2 and core m1 structures appear to be highly important for the maturation of core m3 glycans on α-DG (functional glycosylation). Core m1 glycans are abundant on the mucin domain of α-DG [16, 18] and contribute to the extended conformation adopted by this domain [34], a potential factor influencing core m3 glycan maturation. Loss of core m1 glycans correlates with a spectrum of CMDs [3, 35-37] perhaps because of the resulting disruption of the maturation of core m3 glycans on α-DG shown to underlie the molecular mechanism of these pathologies [13, 22]. Core m1 glycans are typically sialylated contributing to the charge state of the mucin domain of α-DG [18, 19].

Core m2

The class of core m2 glycans is initiated by β1,6-linked GlcNAc extension of core m1 (Figures 2.1 & 2.5). These structures are primarily found in brain and prostate tissue [6] and accounted for no more than 5% of brain protein-linked O-glycans quantified in a recent study [19]. Evidence of at least 13 different core m2-based structures exists (Figure 2.3) including HNK-1 epitope-containing structures that are linked to neural cell adhesion and migration [38]. Early studies demonstrated that an increased level of core m2 glycan synthesis in neuroblastoma cells leads to an increased level of integrin-dependent cell migration on laminin-coated plates [39, 40]. It was subsequently demonstrated that this effect depends on an increased level of tyrosine phosphorylation of β-catenin caused by core m2 glycan-based inhibition of receptor tyrosine phosphatase β (RPTPβ) activity. Further, the decrease in RPTPβ activity appears to depend on the increased level of HNK-1 epitope presentation caused by increased core m2 glycan levels [41]. Interestingly, however, a recent study of a mouse model lacking core m2 glycan synthesis revealed no obvious developmental nervous system defects [42] despite changes in integrin-dependent cell adhesion and migration noted in earlier *in vitro* studies [41]. In this study, it was also shown that the lack of core m2 glycans does not alter α-DG functional glycosylation. Core m2 glycans may however play a role in demyelination pathologies such as MS as demonstrated in a recent study showing inhibition of axon remyelination by core m2 glycans in model systems [29].29 An increased level of core m2 glycosylation has also recently been correlated with increased prostate cancer tumor growth and metastasis [6].

Core m3

The class of core m3 glycans is initiated by β1,4-linked GlcNAc extension of O-mannose (Figures 2.1 & 2.6). Apparently constituting a small and highly heterogeneous portion of the O-glycome, these structures have outsized biological effects as cell surface determinants of the

binding of α-DG to its ECM partners [19, 43]. In particular, defective core m3 glycosylation appears to be the common factor in secondary dystroglycanopathies [5, 12, 13, 44], in increased metastasis of carcinomas, including prostate and breast cancers [7-9, 45], and in various forms of aberrant neuronal migration [3, 27] and axon guidance [28] in mammals. On the other hand, properly extended core m3 glycans may contribute to more aggressive forms of melanomas [46] and can promote the entry of certain arenaviruses into cells [47]. The mechanisms involved are mediated through synthesis of a post-phosphoryl LG domain-binding extension apparently unique to core m3 glycans *in vivo* [13] containing -α3-GlcA-β3-Xyl- repeats [44] that has been shown to be a "tunable scaffold" regulating the avidity of cell surface receptors for ECM proteins [48]. In muscular dystrophies, shortening of this scaffold has deleterious effects on basement membrane compactness and structure and on neuromuscular junction formation [48]. A definitive structure of the post-phosphoryl LG domain-binding epitope remains an important question, although the -α3-GlcA-β3-Xyl- repeating structure is similar to known acidic sugar-containing LG domain-binding epitopes observed on glycosaminoglycans (GAGs) such as heparin [44]. These acidic GAG epitopes bind to basic residues in LG domains via electrostatic interactions [44]. Because core m3 glycans without LG domain-binding extensions are detected in other tissues, it is hypothesized that core m3 glycans lacking these extensions may play other roles [5].

Pathway – Genes and Enzymes

O-mannose glycan synthesis begins in the endoplasmic reticulum (ER) with addition of mannose to serine and threonine residues by the protein complex consisting of protein O-mannosyltransferase 1 (POMT1) and protein O-mannosyltransferase 2 (POMT2). Synthesis may then continue, producing various core structures and their elaborations (Figure 2.1, 2.5 & 2.6). Current data suggest that addition of 6-phosphate to O-mannose in the ER endoplasmic reticulum

(completing core M3) precludes the addition of β1,2-linked GlcNAc [49] in the cis-Golgi (Figure 2.1). As a consequence, β1,6-linked GlcNAc addition would also be prevented. However, the existence of other as yet undetected O-mannose glycan core structures cannot be ruled out. In this section, we focus on the synthesis and elaboration of core M3 glycans because of an abundance of recent discoveries, the remaining biochemical mysteries regarding their synthesis, and their centrality in human disease. We will also briefly discuss the synthesis of the other core structures and finally summarize information from the current literature pertaining to other genes that are involved in O-mannose glycan synthesis.

Protein O-Mannosylation (initiation)

Protein O-Mannosyltransferase 1 and 2 (POMT1 and POMT2, respectively)

POMT1 and POMT2 encode multipass membrane proteins that catalyze the transfer of mannose from dolichol-phosphate mannose (DPM) to serines and threonines in an O-linkage in the ER [50]. Mutations in these genes cause a spectrum of CMD phenotypes [35, 51-53]. Recent studies have provided evidence that supports the hypothesis [54] that phenotype severity correlates with the predicted degree of gene disruption [36] and inversely correlates with measurable enzymatic activity [55]. Knockout of either gene is embryonic lethal [56, 57] while a significant loss of function results in the most severe CMD phenotype, Walker-Warburg syndrome. POMT1 and -2 are located early in the secretory pathway anterior to most other glycosyltransferases and have been shown to significantly influence patterns of O-GalNAcylation in *in vitro* studies [58], increasing the level of interest in its specificity. This is due to the fact that O-GalNAcylation may greatly impact the biophysical properties of the α-DG mucin domain, particularly its conformational properties [58, 59], and thus processing of α-DG by other enzymes during secretory pathway traversal. A recent study showed that a 40-amino acid peptide region upstream

of α-DG O-mannose sites is involved in controlling specificity in EBNA-293 (human kidney) cells [60], however, another study found that specificity may be controlled by different currently unknown elements in lectican O-mannosylation in the brain [32]. Additional recent results of interest include the demonstration that N-glycosylation of POMT1 and -2 is necessary for activity [61] as well as the observation that POMT1 and -2 activity can be significantly reduced by defects in another gene, ISPD, encoding a putative nucleotidyltransferase [62].

Core M3 Synthesis

Genes encoding proteins involved in core M3 synthesis (Figure 2.6) have been identified over the past few years primarily on the basis of genetic studies conducted on patients presenting with congenital muscular dystrophy (CMD) phenotypes. These genes also appear prominently in the recently published α-DG glycosylome [21] and comprise three of the eight genes shown to be involved in the mammalian O-mannosylation pathway in the past three years (Table 2.1). The three genes involved had been annotated as glycosyltransferase-like domain-containing protein 2 (GTDC2, now POMGNT2), UDP-GalNAc: β-1,3-N-acetylgalactosaminyltransferase 2 (B3GALNT2) and probable inactive protein kinase-like protein SgK196 (SGK196, now POMK). POMGNT2 was identified in a paper employing exome sequencing of Walker-Warburg syndrome (WWS) patients and validated in zebrafish models [63]. B3GALNT2 was identified and validated similarly [64] with the addition of direct demonstration of α-DG hypoglycosylation accompanying B3GALNT2 deficiency. The *in vitro* activity of B3GALNT2 had been demonstrated previously, in 2004, but the *in vivo* function was not determined at that time [65]. Finally, POMK was identified as a cause of hydrocephalus and abnormal neuronal migration (WWS hallmarks) in genetically engineered mouse models in 2012 [66] but does not appear to have been directly implicated in muscular dystrophy biology and O-mannosylation until the publication of the

Brummelkamp laboratory α-DG glycosylome in 2013 [21]. The biochemistry of these genes was elucidated in a 2013 paper from the Campbell laboratory [22]. As discussed in the structures section, core M3 glycans are the only proven *in vivo* acceptors of the biologically critical LG domain-binding moiety of α-DG central to a number of disease processes including CMDs. This draws attention to the most important open question regarding core M3 glycan biosynthesis, the manner in which protein and site specificity is determined *in vivo*, which will also be discussed in this section.

Protein O-Linked Mannose N-Acetylglucosaminyltransferase 2 (POMGNT2 or GTDC2)

Experiments conducted in the Campbell laboratory demonstrated that POMGNT2 was ER-localized and that it transfers GlcNAc from UDP-GlcNAc to a synthetic version of an α-DG O-mannose peptide in a β1,4-linkage *in vitro* [22] (Figure 2.6). Ogawa and colleagues also demonstrated ER localization and found that CTD110.6, an antibody raised against O-GlcNAc, can cross react with β1,4-GlcNAc-extended O-mannose [67]. Because POMGNT2 is localized earlier in the secretory pathway than other O-mannose extending enzymes, it is noteworthy that prior studies of laminin binding reactivity, phosphorylation status [5, 12] and O-glycan sites of mammalian glycoproteins [13, 16-18, 68] suggest that core M3 structures are unique to a handful of sites of α-DG *in vivo*. In light of this, it is interesting that Yoshida-Moriguchi and colleagues demonstrated transfer of β1,4-linked GlcNAc to 4-methylumbelliferyl–α-D-mannoside [22], at least *in vitro*, and that Ogawa and colleagues demonstrated the CTD110.6 reactivity of a number of α-DG deletion and substitution mutants co-transfected with GTDC2 in HEK293T cells [67]. Consequently, it is currently unclear what structural or other biochemical determinants result in β1,4-GlcNAc modification of such a limited set of sites *in vivo* at this key biosynthetic point in the pathway.

*UDP-GalNAc: β-1,3-N-*Acetylgalactosaminyltransferase *2 (B3GALNT2)*

In 2013, B3GALNT2 was shown to be defective in some cases of congenital muscular dystrophy, to localize to the ER and was hypothesized to transfer GalNAc in a β1,3-linkage to GlcNAc-β1,4-Man-α on α-DG [64]. Significantly, B3GALNT2 is the first GalNAc-transferase that has been shown to localize primarily to the ER as opposed to relocating to the ER as a regulatory mechanism [69]. Yoshida-Moriguchi and colleagues demonstrated the apparent *in vivo* acceptor of B3GALNT2 activity for the first time in a paper published shortly thereafter [22]. B3GALNT2 transfers GalNAc in a β1,3-linkage to GlcNAc-β1,4-Man-α-R where R may be a peptide from α-DG or 4-methylumbelliferyl. Given the early position of B3GALNT2 activity in ER and Golgi trafficking, O-mannose glycans may provide the only acceptors for this enzyme *in vivo*.

Protein O-Mannose Kinase (POMK or SGK196)

In 2013, Yoshida-Moriguchi and colleagues established, through *in vitro* enzyme assays and high-performance liquid chromatography separation of fluorescently labeled substrates and products, that POMK is the kinase responsible for phosphorylating O-mannose at the 6-position completing the core M3 structure [22] (Figure 2.6). In contrast to the seeming lack of specificity *in vitro* of the previously discussed core M3 synthesizing enzymes, POMK could be shown to phosphorylate α-linked mannose only after the addition of GalNAc-β1,3-GlcNAcβ1,4 [22]. The discovery that POMK is a kinase is significant for two reasons. First, POMK lacks key catalytic residues found in other kinases characterized to date [22] and may therefore be the first discovered member of a new class of kinases. Second, extension of phosphate groups attached to sugars is an unusual, more difficult, chemistry for various reasons [70 p. 28-33] (see 10.4, DNA ligase). Therefore, the enzymatic activity stably extending the 6-position phosphate is of clear interest.

Core M3 Elaboration and LG Domain-Binding Related Genes

Core M3 glycans have been observed only on α-DG and were shown in 2010 to be the only glycans extended with the LG domain-binding moiety (functional glycan) of α-DG *in vivo* [13]. Because the Brummelkamp laboratory gene disruption screening methodology is based on the functional glycan status of α-DG, their screen should detect positive regulators of functional glycosylation [21]. Negative regulators are unlikely to be detected by this screen; however, one was discovered by another group recently using different methods [46]. The genes from these studies encoding proteins in the secretory pathway are likely to play direct roles in core M3 elaboration. The genes in Tables 2.1, 2.2 and 2.3 meeting these criteria are like-glycosyltransferase (LARGE), glycosyltransferase-like 1B (GYLTL1B or LARGE2), UDP-GlcNAc:βGalβ-1,3-N-acetylglucosaminyltransferase (B3GNT1), human natural killer-1 sulfotransferase (HNK-1ST), solute carrier family 35 (CMP-sialic acid transporter), member A1 (SLC35A1), fukutin (FKTN), fukutin-related protein (FKRP), transmembrane protein 5 (TMEM5), and base core M3 synthesis genes and POMGNT1 discussed in other sections. LARGE, FKTN, and FKRP have been extensively studied since the late 1990s, while B3GNT1, HNK-1ST, SLC35A1, and TMEM5 have only recently been implicated in the modification of α-DG. Recent major results include the elucidation of the enzymatic reactions carried out by LARGE and LARGE2 [44, 71, 72], the determination that B3GNT1 forms complexes with LARGE and LARGE2 critical to their activity [7], and the discovery that HNK-1ST negatively regulates LG domain-binding glycan synthesis [43, 46]. Further, all of these results have been tied either directly [43] or indirectly [13, 71-73] to the post-phosphoryl moiety of core M3 glycans . The roles of FKTN, FKRP, and TMEM5 remain less clear.

Like-glycosyltransferase (LARGE), Glycosyltransferase-like 1B (LARGE2), and N-acetyllactosamide β-1,3-N-Acetylglucosaminyltransferase (B3GNT1)

LARGE and LARGE2 encode proteins containing both a GT8 glycosyltransferase domain and a GT49 glycosyltransferase domain [74] and have been extensively studied using overexpression [2, 7, 43, 73, 75, 76] and glycosylation deficient cell lines [77-79]. Native LARGE modification of α-DG is regulated directly and/or indirectly by the activities of a number of other enzymes, including HNK-1ST, B3GNT1, and possibly SLC35A1 and POMGNT1 (see below). Overexpression of LARGE has been shown to lead to LARGE modification of non-native acceptors, including N-glycans and O-GalNAc glycans [73, 77] and, potentially as a consequence, to partially rescue functional glycosylation in cells derived from patients deficient in other O-mannosylation pathway activities [2, 75]. This has led to the suggestion that LARGE may be a particularly good target for gene therapy treatment strategies given that it can compensate for a variety of deficiencies [75].

In 2012, Inamori and colleagues determined by compositional sugar analysis that recombinant α-DG co-expressed with LARGE in HEK293 cells is modified by substantial quantities of xylose and glucuronic acid [44]. Competition assays and experiments conducted in UDP-Xyl synthesis deficient cell lines demonstrated that functional glycosylation of α-DG depended on xylose. Subsequent *in vitro* enzyme assays using tagged xylose and glucuronic acid acceptors established the reactions catalyzed by LARGE. Specifically, it was shown that the GT8 domain catalyzes the transfer of xylose (Xyl) in an α1,3-linkage to β1,3-linked glucuronic acid (GlcA) from UDP-Xyl and that the GT49 domain catalyzes the transfer of GlcA in a β1,3-linkage to α1,3-linked Xyl from UDP-GlcA [44]. Furthermore, Inamori and colleagues demonstrated that LARGE can build polymers consisting of –α3-GlcA-β3-Xyl- repeats without the presence or

action of other proteins. LARGE2 was subsequently shown to catalyze the same reaction [71, 72] although none of the currently published studies have established the initial acceptor for these activities *in vivo*. For example, Yoshida-Moriguchi and colleagues found that LARGE indeed appears to be unable to transfer directly to the 6-position phosphate on the core M3 structure [22]. The role of B3GNT1 is currently unknown, but it is interesting to note that it is the only B3GNT to cluster into CAZy family GT49.

Human Natural Killer-1 Sulfotransferase (HNK-1ST)

HNK-1ST encodes a sulfotransferase responsible for the sulfation of GlcA residues at position 3. In 2012, Nakagawa and colleagues observed an upregulation of HNK-1ST in S91 melanoma cells treated with the antitumor agent retinoic acid (RA) and demonstrated that the suppression of melanoma cell migration by RA depended on a reduction in the level of functional glycosylation of α-DG [46]. Furthermore, they demonstrated that the interaction between LARGE and α-DG was not disrupted by HNK-1ST and that the sulfo-transfer activity of HNK-1ST was the mechanism by which this enzyme modulates functional glycosylation of α-DG. In 2013, Nakagawa and colleagues demonstrated that HNK-1ST transfers sulfates to core M3 glycans in the post-phosphoryl moiety and that it is the activity of HNK-1ST in this post-phosphoryl moiety that is responsible for abolishing the functional glycosylation of α-DG [43]. Experiments utilizing the LARGE *in vitro* assay system established by Inamori and colleagues [44] coupled with HNK-1ST-based "pretransfer" of sulfates to α-DG suggest that the primary effect of sulfate transfer is the disruption of the ability of LARGE to build the repeating disaccharide [43]. These results taken together suggest a mechanism in which the relative activities between LARGE and HNK-1ST in a given cell compete to mediate the length of the disaccharide repeat and thus its affinity for ECM ligands. These results also strengthen the glycosaminoglycan analogy because HNK-1ST has also

been shown to negatively regulate GAG chain length by sulfate transfer [80]. An important distinction is the lack of HNK-1 reactivity of the resulting glycan on α-DG indicating that GlcA is not β1,3-linked to Gal.

Solute Carrier Family 35 (CMP-sialic acid transporter), Member A1 (SLC35A1)

SLC35A1 encodes the well-characterized Golgi CMP-sialic acid transporter in mammals [81, 82]. Mucin domains are heavily sialylated with significant consequences for protein conformation during and after trafficking [34]. Synthesis of LG domain-binding glycans on the mucin domain of α-DG clearly depends on structural features [83, 84] Furthermore, because sialidase treatment after synthesis of the functional glycans on α-DG results in increased laminin and IIH6 binding activity [33], a lack of concurrent sialylation of O-mannose and O-GalNAc glycans during DAG1 traversal of the compartments of the Golgi that contain LARGE may result in an unfavorable protein state for effective LARGE modification. Regardless of the exact mechanism, the lack of appearance of specific sialyltransferases (SiaTs) in the published α-DG glycosylome [21] suggests that redundancy exists and that multiple SiaTs are involved. As with many of the defects found in α-DG processing, LARGE overexpression has been shown to mitigate sialic acid deficiency-based aberrant glycosylation [77].

Fukutin (FKTN), Fukutin-Related Protein (FKRP), and Transmembrane Protein 5 (TMEM5)

The genes FKTN, FKRP, and TMEM5 encode the remaining positive regulators of α-DG functional glycosylation. These proteins localize to the secretory pathway and are potentially directly involved in α-DG functional glycan synthesis (Table 2.2). While it is likely that at least one of these genes encodes an activity synthesizing a key part of the linker between core M3 itself and the LARGE modification, and there has been extensive speculation with regard to FKTN and FKRP activities in particular [85-88], experiments to date have not revealed nucleotide-sugar

transfer activities [89] or other activities [90]. Recent studies have shown that at least a fraction of α-DG from FKTN and FKRP deficient models is partially extended on core M3 glycans post-phosphate [5, 12] and that mutation of the DXD motif of FKRP does not necessarily result in loss of functional glycosylation of α-DG [91]. Furthermore, functional glycosylation of α-DG has been observed in cell lines in which FKRP transcripts were not detected [7]. It has also been shown that some mutants of FKTN fail to fold properly and are retained in the ER, causing POMGNT1 to be retained as well [92] and that there may be direct interaction between FKTN and α-DG [90]. Thus, the role of FKTN and FKRP remains unclear, and they may not be directly involved in enzymatic synthesis. Recently, a study conducted in zebrafish has indicated that FKTN and FKRP may be required for appropriate folding and secretion of a set of proteins in a potentially non-glycosylation-dependent manner (viz. laminin-1) [93]. This model may explain the hypoglycosylation of α-DG in FKTN and FKRP deficient patients given the importance of the α-DG and LARGE binding interaction that depends on protein conformation [83]. This model is also consistent with the lower degree of correlation between α-DG hypoglycosylation and CMD phenotype severity in such patients [94] For example, studies including observations concerning a role for laminin-1 in muscular dystrophies have since been published [95]. Further evaluation of this model is needed. New animal models [12, 75, 96, 97] and biochemical tools [98] developed in the past few years should help researchers to further resolve the roles of FKTN and FKRP, which generally occur at low levels, can be difficult to detect, and may be differentially required among cell types and during development.

TMEM5 has only recently been identified as a cause of dystroglycanopathies, and no biochemical characterization appears in the literature at present. It has been noted that TMEM5

defects can cause cobblestone lissencephaly A, a severe phenotype associated most closely with POMT1 defects [99], placing TMEM5 centrally in α-DG functional glycan synthesis *in vivo*.

Core M1 and Core M2 Synthesis

Core M1 and core M2 glycans and their elaborated structures account for 20-30% of the O-glycans detected and quantified in various studies [19, 25]. Core M1 glycan synthesis is initiated by POMGNT1, which has long been implicated in CMDs (see the structures section). Core M2 glycan synthesis is initiated by GNT-VB (GNT-IX) and has been demonstrated to play a role in remyelination and potentially multiple sclerosis (see the structures section).

Protein O-Linked Mannose N-Acetylglucosaminyltransferase 1 (POMGNT1)

POMGNT1 encodes the enzyme that extends O-mannose with β1,2-linked GlcNAc in the cis-Golgi, an initial step in the synthesis of core M1-based and core M2-based structures (Figures 2.2 & 2.3). Although these structures are not directly involved in LG domain binding reactivity, POMGNT1 loss of function causes a loss of functional glycosylation of α-DG, a severe form of muscular dystrophy termed Muscle Eye Brain disease and a >10 kDa reduction in the molecular mass of α-DG in mouse skeletal muscle tissue [5]. This reduction is larger than that caused by deficiencies in other functional glycosylation genes and has potentially substantial structural consequences that may affect the activities of other enzymes implicated in CMDs. More directly, Nilsson et al. found strict core M1 glycan modification of the glycopeptide consisting of mucin domain residues 361−373 in human α-DG [18], a peptide that is directly N-terminal to the peptide on which the core M3 phospho-O-mannose-trisaccharide was mapped by Yoshida-Moriguchi and colleagues [13]. The only detected heterogeneity was in sialylation, and Harrison et al. detected core M3 (minus phosphate) on the corresponding mouse α-DG site followed by a core M1 classical tetrasaccharide attached to the two C-terminal threonine residues[17]. These regions contain

sequences that are significantly similar to the sequences of a region of α-DG most directly demonstrated to be highly important for laminin binding consisting of the first 24 residues of the mucin domain [68]. Specifically, paired threonine residues separated by a proline appear to be critical for LARGE-dependent functional glycosylation. Because enzymatic removal of core M1 glycans on α-DG from normal rabbit skeletal muscle causes an increase in laminin binding reactivity [33], one reasonable hypothesis is that specific sites of core M1 glycan modification could be a factor in the ability of LARGE to build the scaffold of the functional glycan structure of α-DG core M3 glycans. It has also been shown that CMD severity is inversely correlated with POMGNT1 activity [100]. Finally, one recent study found a correlation between POMGNT1 protein levels and glioma tissue grade [101] but further studies are needed to refine this finding and possible mechanisms.

α-1,6-Mannosylglycoprotein 6-β-N-Acetylglucosaminyltransferase B (GNT-VB or *GNT-IX)*

GNT-VB (GNT-IX) encodes the enzyme that extends O-mannose with β1,6-linked GlcNAc in the cis-Golgi that is dependent on prior β1,2-GlcNAc extension by POMGNT1. This activity is blocked when the β2 branch is further extended [102]. GNT-VB is active primarily in brain and prostate tissue [6] has been shown to negatively affect axon remyelination after neurotoxicant-induced myelin damage [29] and to promote prostate cancer metastasis [6]. These findings establish GNT-VB as a possible therapeutic target.

Additional Genes in O-Mannosylation

Isoprenoid Synthase Domain-Containing Protein (ISPD)

ISPD encodes a protein with an isoprenoid synthase domain most similar to 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase, an enzyme active in the non-mevalonate isoprenoid synthesis pathway found in bacteria that appears to be absent in mammals. The role of this gene

in dystroglycanopathies was discovered in 2012 through genetic screening and complementation analyses [62, 85, 99]. Defects in ISPD appear to be a common cause of dystroglycanopathies accounting for 10% of dystroglycanopathies in studies to date [99, 103] and 30% of the cases of Walker-Warburg syndrome examined in one study [62]. Willer and colleagues subsequently found that microsomal fractions from ISPD deficient Walker-Warburg syndrome patient fibroblasts showed significant reductions in O-mannose transfer to proteins [62]. This is an intriguing result considering that ISPD does not appear to contain a signal sequence and may have a cytosolic or possibly nuclear localization raising questions as to how ISPD might affect the activity of the protein O-mannosyltransferase complex in a microsomal fraction supplied with exogenous DPM. Although ISPD appears to associate most closely with more severe forms of CMD in studies to date, at least one study identified patients with mutations in ISPD and milder limb-girdle muscular dystrophy phenotypes [104].

α-(1,3)-Fucosyltransferase 9 (FUT9)

FUT9 encodes an α-1,3-fucosyltransferase that catalyzes synthesis of Lewis x glycans (Le^x). O-Mannose-initiated Le^x structures accounted for roughly 10% of the O-glycans released from mouse brain tissue and quantified in recent glycomic studies [19]. Studies have demonstrated that of the two fucosyltransferases capable of synthesizing Le^x glycans, FUT9 is substantially more active in such synthesis and is expressed in mouse brain at levels far higher than those of FUT4 [105]. Furthermore, $FUT9^{-/-}$ mice show a complete absence of detectable Le^x epitopes in brain tissue [106]. FUT9 is likely to encode the primary fucosyltransferase responsible for fucosylation of O-mannose glycans. Addition of fucose to GlcNAc has been shown in other glycan classes to block GlcA transfer and consequently HNK-1 epitope synthesis [107]. Given the prevalence of

HNK-1 epitopes in brain tissue and their previously noted functions, the interplay between fucosylation and glucuronylation may have a regulatory role.

Galactosylgalactosylxylosylprotein 3-β-Glucuronosyltransferase 1 (B3GAT1)

B3GAT1 encodes one of two primary HNK-1 epitope-synthesizing β1,3-glucuronyltransferases in mammals (GlcAT-P). In a recent paper, Morise et al. demonstrated that phosphacan is the major protein carrying the HNK-1 epitope in the developing mouse brain, that monoclonal antibody 6B4 specifically recognizes HNK-1-modified phosphacan, and that 6B4 reactivity is virtually completely abolished in B3GAT1 knockout mice [108]. Additionally, experiments involving co-transfection of phosphacan and GlcAT-P into COS-1 cells provided further evidence that GlcAT-P is required for HNK-1 epitope synthesis on O-linked glycan structures primarily attached to phosphacan [108].

Protein Substrates (O-Mannosylated Proteins)

O-Mannose modification was first detected in mammalian tissue in 1979 [109] and was subsequently shown to occur prominently on the protein α-DG from nervous [110, 111] and skeletal muscle tissues [112]. Several additional proteins were identified in the 2000s including RPTPβ [41], cd24 from mouse brain [113] and a human IgG2 light chain expressed in CHO cells [114], primarily in the context of biochemical studies directed at characterizing specific proteins. Efforts directed specifically at finding O-mannosylated proteins have been undertaken more recently, beginning with a study based on a bioinformatic search for a previously identified cis-peptide determinant of O-mannosylation on α-DG that resulted in the demonstration that neurofascin 186 is O-mannosylated [115]. Further studies utilizing large-scale enrichment and fractionation-based strategies resulted in the identification of the four lecticans (aggrecan, brevican, neurocan and versican) as an important class of O-mannosylated proteins [32]. And,

most recently, thirty-seven cadherins and six plexins were shown to be O-mannosylated using a "SimpleCell" system and Concanavalin A chromatography [15]. One of the most consistent themes observed in O-mannosylation is the association of O-mannosylation with proteins involved in cell-cell and cell-matrix adhesion. For example, O-mannosylation of RPTPβ, which is not directly involved in cell-cell or cell-matrix interactions, has been shown to modulate cell-cell interactions and to result in increased cell migration through an intracellular signaling mechanism [41]. The protein cd24 is likewise involved in cell adhesion in cancer biology, in immune system function, and in nervous system biology (potentially mediated in part through the presentation of the HNK-1 epitope on O-mannose glycans) [113]. In cancer biology, increased levels of cd24 correlate with more aggressive metastatic carcinomas [113]. Finally, the newly demonstrated importance of O-mannosylation in cadherin-mediated cell-cell adhesion and its crucial role in development [31] as well as the discovery of the prevalence of O-mannose glycans linked to cadherins [15] further illustrate this theme and open many avenues of additional study.

Conclusions

Progress in the field of O-mannosylation within the past few years has been substantial, culminating in the publication of three high-impact papers in 2013, the Brummelkamp laboratory α-DG glycosylome, the Clausen laboratory O-mannose glycoproteome, and the Campbell laboratory core M3 enzymes. Publication of the Brummelkamp laboratory α-DG glycosylome suggests that we may finally be able to define the borders of genetic causes of secondary dystroglycanopathies and may be close to determining genetic etiologies for most dystroglycanopathy patients without previously understood genetic defects. It will also allow for biochemical characterization of the proteins encoded by these genes ultimately allowing further development of treatments for dystroglycanopathies and other diseases caused by defects in O-

mannosylation. The Clausen laboratory O-mannose glycoproteome provides a significant addition to the set of known O-mannosylated proteins, helping to explain how O-mannose glycans can account for approximately 30% of O-glycans released and quantified from brain proteins even in mouse models lacking α-DG. Finally, the Campbell laboratory assigned three enzyme activities critical to functional glycosylation of α-DG that are dependent on O-mannose glycans. The field of mammalian O-mannosylation is at an exciting juncture with completion of such a solid framework upon which accelerated progress in attaining a deeper understanding, particularly clinically, may rest.

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References

1. Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW, Etzler ME, editors. Essentials of Glycobiology. 2nd ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2009.

2. Barresi R, Michele DE, Kanagawa M, Harper HA, Dovico SA, Satz JS, Moore SA, Zhang W, Schachter H, Dumanski JP, Cohn RD, Nishino I, Campbell KP. LARGE can functionally bypass alpha-dystroglycan glycosylation defects in distinct congenital muscular dystrophies. Nature medicine. 2004;10(7):696-703. doi: 10.1038/nm1059. PubMed PMID: 15184894.

3. Yoshida A, Kobayashi K, Manya H, Taniguchi K, Kano H, Mizuno M, Inazu T, Mitsuhashi H, Takahashi S, Takeuchi M, Herrmann R, Straub V, Talim B, Voit T, Topaloglu H, Toda T, Endo T. Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. Developmental cell. 2001;1(5):717-24. PubMed PMID: 11709191.

4. Brockington M, Blake DJ, Prandini P, Brown SC, Torelli S, Benson MA, Ponting CP, Estournet B, Romero NB, Mercuri E, Voit T, Sewry CA, Guicheney P, Muntoni F. Mutations in the fukutin-related protein gene (FKRP) cause a form of congenital muscular dystrophy with secondary laminin alpha2 deficiency and abnormal glycosylation of alpha-dystroglycan. American journal of human genetics. 2001;69(6):1198-209. Epub 2001/10/10. doi: 10.1086/324412. PubMed PMID: 11592034; PMCID: 1235559.

5. Kuga A, Kanagawa M, Sudo A, Chan YM, Tajiri M, Manya H, Kikkawa Y, Nomizu M, Kobayashi K, Endo T, Lu QL, Wada Y, Toda T. Absence of post-phosphoryl modification in dystroglycanopathy mouse models and wild-type tissues expressing non-laminin binding form of alpha-dystroglycan. The Journal of biological chemistry. 2012;287(12):9560-7. doi: 10.1074/jbc.M111.271767. PubMed PMID: 22270369; PMCID: 3308745.

6. Lange T, Ullrich S, Muller I, Nentwich MF, Stubke K, Feldhaus S, Knies C, Hellwinkel OJ, Vessella RL, Abramjuk C, Anders M, Schroder-Schwarz J, Schlomm T, Huland H, Sauter G, Schumacher U. Human prostate cancer in a clinically relevant xenograft mouse model: identification of beta(1,6)-branched oligosaccharides as a marker of tumor progression. Clinical cancer research : an official journal of the American Association for Cancer Research. 2012;18(5):1364-73. doi: 10.1158/1078-0432.CCR-11-2900. PubMed PMID: 22261809.

7. Bao X, Kobayashi M, Hatakeyama S, Angata K, Gullberg D, Nakayama J, Fukuda MN, Fukuda M. Tumor suppressor function of laminin-binding alpha-dystroglycan requires a distinct beta3-N-acetylglucosaminyltransferase. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(29):12109-14. PubMed PMID: 19587235.

8. de Bernabe DB, Inamori K, Yoshida-Moriguchi T, Weydert CJ, Harper HA, Willer T, Henry MD, Campbell KP. Loss of alpha-dystroglycan laminin binding in epithelium-derived cancers is caused by silencing of LARGE. The Journal of biological chemistry. 2009;284(17):11279-84. doi: 10.1074/jbc.C900007200. PubMed PMID: 19244252; PMCID: 2670132.

9. Esser AK, Miller MR, Huang Q, Meier MM, Beltran-Valero de Bernabe D, Stipp CS, Campbell KP, Lynch CF, Smith BJ, Cohen MB, Henry MD. Loss of LARGE2 disrupts functional glycosylation of alpha-dystroglycan in prostate cancer. The Journal of biological chemistry. 2013;288(4):2132-42. doi: 10.1074/jbc.M112.432807. PubMed PMID: 23223448; PMCID: 3554886.

10. Barresi R, Campbell KP. Dystroglycan: from biosynthesis to pathogenesis of human disease. Journal of cell science. 2006;119(Pt 2):199-207. doi: 10.1242/jcs.02814. PubMed PMID: 16410545.

11. Sato S, Omori Y, Katoh K, Kondo M, Kanagawa M, Miyata K, Funabiki K, Koyasu T, Kajimura N, Miyoshi T, Sawai H, Kobayashi K, Tani A, Toda T, Usukura J, Tano Y, Fujikado T, Furukawa T. Pikachurin, a dystroglycan ligand, is essential for photoreceptor ribbon synapse formation. Nature neuroscience. 2008;11(8):923-31. doi: 10.1038/nn.2160. PubMed PMID: 18641643.

12. Beedle AM, Turner AJ, Saito Y, Lueck JD, Foltz SJ, Fortunato MJ, Nienaber PM, Campbell KP. Mouse fukutin deletion impairs dystroglycan processing and recapitulates muscular dystrophy. The Journal of clinical investigation. 2012;122(9):3330-42. doi: 10.1172/JCI63004. PubMed PMID: 22922256; PMCID: 3428090.

13. Yoshida-Moriguchi T, Yu L, Stalnaker SH, Davis S, Kunz S, Madson M, Oldstone MB, Schachter H, Wells L, Campbell KP. O-mannosyl phosphorylation of alpha-dystroglycan is required for laminin binding. Science. 2010;327(5961):88-92. doi: 10.1126/science.1180512. PubMed PMID: 20044576; PMCID: 2978000.

14. Inamori K, Yoshida-Moriguchi T, Hara Y, Anderson ME, Yu L, Campbell KP. Dystroglycan function requires xylosyl- and glucuronyltransferase activities of LARGE. Science.335(6064):93-6. PubMed PMID: 22223806.

15. Vester-Christensen MB, Halim A, Joshi HJ, Steentoft C, Bennett EP, Levery SB, Vakhrushev SY, Clausen H. Mining the O-mannose glycoproteome reveals cadherins as major O-mannosylated glycoproteins. Proceedings of the National Academy of Sciences of the United States of America. 2013. doi: 10.1073/pnas.1313446110. PubMed PMID: 24101494.

16. Stalnaker SH, Hashmi S, Lim JM, Aoki K, Porterfield M, Gutierrez-Sanchez G, Wheeler J, Ervasti JM, Bergmann C, Tiemeyer M, Wells L. Site mapping and characterization of O-glycan structures on alpha-dystroglycan isolated from rabbit skeletal muscle. The Journal of biological chemistry. 2010;285(32):24882-91. doi: 10.1074/jbc.M110.126474. PubMed PMID: 20507986; PMCID: 2915724.

17. Harrison R, Hitchen PG, Panico M, Morris HR, Mekhaiel D, Pleass RJ, Dell A, Hewitt JE, Haslam SM. Glycoproteomic characterization of recombinant mouse alpha-dystroglycan. Glycobiology. 2012;22(5):662-75. doi: 10.1093/glycob/cws002. PubMed PMID: 22241827; PMCID: 3311285.

18. Nilsson J, Nilsson J, Larson G, Grahn A. Characterization of site-specific O-glycan structures within the mucin-like domain of alpha-dystroglycan from human skeletal muscle. Glycobiology. 2010;20(9):1160-9. doi: 10.1093/glycob/cwq082. PubMed PMID: 20507882.

19. Stalnaker SH, Aoki K, Lim JM, Porterfield M, Liu M, Satz JS, Buskirk S, Xiong Y, Zhang P, Campbell KP, Hu H, Live D, Tiemeyer M, Wells L. Glycomic analyses of mouse models of congenital muscular dystrophy. The Journal of biological chemistry. 2011;286(24):21180-90. doi: 10.1074/jbc.M110.203281. PubMed PMID: 21460210; PMCID: 3122180.

20. Gomez Toledo A, Raducu M, Cruces J, Nilsson J, Halim A, Larson G, Ruetschi U, Grahn A. O-Mannose and O-N-acetyl galactosamine glycosylation of mammalian alpha-dystroglycan is conserved in a region-specific manner. Glycobiology. 2012;22(11):1413-23. doi: 10.1093/glycob/cws109. PubMed PMID: 22781125.

21. Jae LT, Raaben M, Riemersma M, van Beusekom E, Blomen VA, Velds A, Kerkhoven RM, Carette JE, Topaloglu H, Meinecke P, Wessels MW, Lefeber DJ, Whelan SP, van Bokhoven H, Brummelkamp TR. Deciphering the glycosylome of dystroglycanopathies using haploid screens for lassa virus entry. Science. 2013;340(6131):479-83. doi: 10.1126/science.1233675. PubMed PMID: 23519211.

22. Yoshida-Moriguchi T, Willer T, Anderson ME, Venzke D, Whyte T, Muntoni F, Lee H, Nelson SF, Yu L, Campbell KP. SGK196 is a glycosylation-specific O-mannose kinase required for dystroglycan function. Science. 2013;341(6148):896-9. doi: 10.1126/science.1239951. PubMed PMID: 23929950.

23. Ervasti JM, Campbell KP. Membrane organization of the dystrophin-glycoprotein complex. Cell. 1991;66(6):1121-31. Epub 1991/09/20. PubMed PMID: 1913804.

24. Ervasti JM, Campbell KP. A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin. The Journal of cell biology. 1993;122(4):809-23. Epub 1993/08/01. PubMed PMID: 8349731; PMCID: 2119587.

25. Chai W, Yuen CT, Kogelberg H, Carruthers RA, Margolis RU, Feizi T, Lawson AM. High prevalence of 2-mono- and 2,6-di-substituted manol-terminating sequences among O-glycans released from brain glycopeptides by reductive alkaline hydrolysis. European journal of biochemistry / FEBS. 1999;263(3):879-88. PubMed PMID: 10469154.

26. Beltran-Valero de Bernabe D, Currier S, Steinbrecher A, Celli J, van Beusekom E, van der Zwaag B, Kayserili H, Merlini L, Chitayat D, Dobyns WB, Cormand B, Lehesjoki AE, Cruces J, Voit T, Walsh CA, van Bokhoven H, Brunner HG. Mutations in the O-mannosyltransferase gene POMT1 give rise to the severe neuronal migration disorder Walker-Warburg syndrome. American journal of human genetics. 2002;71(5):1033-43. doi: 10.1086/342975. PubMed PMID: 12369018; PMCID: 419999.

27. Satz JS, Ostendorf AP, Hou S, Turner A, Kusano H, Lee JC, Turk R, Nguyen H, Ross-Barta SE, Westra S, Hoshi T, Moore SA, Campbell KP. Distinct functions of glial and neuronal dystroglycan in the developing and adult mouse brain. The Journal of neuroscience : the official

journal of the Society for Neuroscience. 2010;30(43):14560-72. doi: 10.1523/JNEUROSCI.3247-10.2010. PubMed PMID: 20980614; PMCID: 2979314.

28. Wright KM, Lyon KA, Leung H, Leahy DJ, Ma L, Ginty DD. Dystroglycan organizes axon guidance cue localization and axonal pathfinding. Neuron. 2012;76(5):931-44. doi: 10.1016/j.neuron.2012.10.009. PubMed PMID: 23217742; PMCID: 3526105.

29. Kanekiyo K, Inamori K, Kitazume S, Sato K, Maeda J, Higuchi M, Kizuka Y, Korekane H, Matsuo I, Honke K, Taniguchi N. Loss of branched O-mannosyl glycans in astrocytes accelerates remyelination. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2013;33(24):10037-47. doi: 10.1523/JNEUROSCI.3137-12.2013. PubMed PMID: 23761899.

30. Martin PT. Congenital muscular dystrophies involving the O-mannose pathway. Current molecular medicine. 2007;7(4):417-25. PubMed PMID: 17584082; PMCID: 2855644.

31. Lommel M, Winterhalter PR, Willer T, Dahlhoff M, Schneider MR, Bartels MF, Renner-Muller I, Ruppert T, Wolf E, Strahl S. Protein O-mannosylation is crucial for E-cadherin-mediated cell adhesion. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(52):21024-9. Epub 2013/12/04. doi: 10.1073/pnas.1316753110. PubMed PMID: 24297939; PMCID: 3876218.

32. Pacharra S, Hanisch FG, Muhlenhoff M, Faissner A, Rauch U, Breloy I. The lecticans of Mammalian brain perineural net are o-mannosylated. Journal of proteome research. 2013;12(4):1764-71. Epub 2013/02/23. doi: 10.1021/pr3011028. PubMed PMID: 23428289.

33. Combs AC, Ervasti JM. Enhanced laminin binding by alpha-dystroglycan after enzymatic deglycosylation. The Biochemical journal. 2005;390(Pt 1):303-9. doi: 10.1042/BJ20050375. PubMed PMID: 15865602; PMCID: 1184583.

34. Thornton DJ, Rousseau K, McGuckin MA. Structure and function of the polymeric mucins in airways mucus. Annual review of physiology. 2008;70:459-86. doi: 10.1146/annurev.physiol.70.113006.100702. PubMed PMID: 17850213.

35. Godfrey C, Clement E, Mein R, Brockington M, Smith J, Talim B, Straub V, Robb S, Quinlivan R, Feng L, Jimenez-Mallebrera C, Mercuri E, Manzur AY, Kinali M, Torelli S, Brown SC, Sewry CA, Bushby K, Topaloglu H, North K, Abbs S, Muntoni F. Refining genotype phenotype correlations in muscular dystrophies with defective glycosylation of dystroglycan. Brain : a journal of neurology. 2007;130(Pt 10):2725-35. doi: 10.1093/brain/awm212. PubMed PMID: 17878207.

36. Mercuri E, Messina S, Bruno C, Mora M, Pegoraro E, Comi GP, D'Amico A, Aiello C, Biancheri R, Berardinelli A, Boffi P, Cassandrini D, Laverda A, Moggio M, Morandi L, Moroni I, Pane M, Pezzani R, Pichiecchio A, Pini A, Minetti C, Mongini T, Mottarelli E, Ricci E, Ruggieri A, Saredi S, Scuderi C, Tessa A, Toscano A, Tortorella G, Trevisan CP, Uggetti C, Vasco G, Santorelli FM, Bertini E. Congenital muscular dystrophies with defective glycosylation of dystroglycan: a population study. Neurology. 2009;72(21):1802-9. doi: 10.1212/01.wnl.0000346518.68110.60. PubMed PMID: 19299310.

37. Raducu M, Baets J, Fano O, Van Coster R, Cruces J. Promoter alteration causes transcriptional repression of the POMGNT1 gene in limb-girdle muscular dystrophy type 2O. European journal of human genetics : EJHG. 2012;20(9):945-52. doi: 10.1038/ejhg.2012.40. PubMed PMID: 22419172; PMCID: 3421125.

38. Kizuka Y, Oka S. Regulated expression and neural functions of human natural killer-1 (HNK-1) carbohydrate. Cellular and molecular life sciences : CMLS. 2012;69(24):4135-47. doi: 10.1007/s00018-012-1036-z. PubMed PMID: 22669261.

39. Lee I, Guo HB, Kamar M, Abbott K, Troupe K, Lee JK, Alvarez-Manilla G, Pierce M. N-acetylglucosaminyltranferase VB expression enhances beta1 integrin- dependent PC12 neurite outgrowth on laminin and collagen. Journal of neurochemistry. 2006;97(4):947-56. doi: 10.1111/j.1471-4159.2006.03785.x. PubMed PMID: 16606368.

40. Abbott KL, Troupe K, Lee I, Pierce M. Integrin-dependent neuroblastoma cell adhesion and migration on laminin is regulated by expression levels of two enzymes in the O-mannosyl-linked glycosylation pathway, PomGnT1 and GnT-Vb. Experimental cell research. 2006;312(15):2837-50. doi: 10.1016/j.yexcr.2006.05.022. PubMed PMID: 16857188.

41. Abbott KL, Matthews RT, Pierce M. Receptor tyrosine phosphatase beta (RPTPbeta) activity and signaling are attenuated by glycosylation and subsequent cell surface galectin-1 binding. The Journal of biological chemistry. 2008;283(48):33026-35. doi: 10.1074/jbc.M803646200. PubMed PMID: 18838383; PMCID: 2586282.

42. Lee JK, Matthews RT, Lim JM, Swanier K, Wells L, Pierce JM. Developmental expression of the neuron-specific N-acetylglucosaminyltransferase Vb (GnT-Vb/IX) and identification of its in vivo glycan products in comparison with those of its paralog, GnT-V. The Journal of biological chemistry. 2012;287(34):28526-36. doi: 10.1074/jbc.M112.367565. PubMed PMID: 22715095; PMCID: 3436567.

43. Nakagawa N, Takematsu H, Oka S. HNK-1 sulfotransferase-dependent sulfation regulating laminin-binding glycans occurs in the post-phosphoryl moiety on alpha-dystroglycan. Glycobiology. 2013;23(9):1066-74. doi: 10.1093/glycob/cwt043. PubMed PMID: 23723439.

44. Inamori K, Yoshida-Moriguchi T, Hara Y, Anderson ME, Yu L, Campbell KP. Dystroglycan function requires xylosyl- and glucuronyltransferase activities of LARGE. Science. 2012;335(6064):93-6. doi: 10.1126/science.1214115. PubMed PMID: 22223806; PMCID: 3702376.

45. Tsuboi S, Hatakeyama S, Ohyama C, Fukuda M. Two opposing roles of O-glycans in tumor metastasis. Trends in molecular medicine. 2012;18(4):224-32. doi: 10.1016/j.molmed.2012.02.001. PubMed PMID: 22425488; PMCID: 3356160.

46. Nakagawa N, Manya H, Toda T, Endo T, Oka S. Human natural killer-1 sulfotransferase (HNK-1ST)-induced sulfate transfer regulates laminin-binding glycans on alpha-dystroglycan. The Journal of biological chemistry. 2012;287(36):30823-32. doi: 10.1074/jbc.M112.363036. PubMed PMID: 22801424; PMCID: 3436325.

47. Kunz S, Rojek JM, Kanagawa M, Spiropoulou CF, Barresi R, Campbell KP, Oldstone MB. Posttranslational modification of alpha-dystroglycan, the cellular receptor for arenaviruses, by the glycosyltransferase LARGE is critical for virus binding. Journal of virology. 2005;79(22):14282-96. doi: 10.1128/JVI.79.22.14282-14296.2005. PubMed PMID: 16254363; PMCID: 1280193.

48. Goddeeris MM, Wu B, Venzke D, Yoshida-Moriguchi T, Saito F, Matsumura K, Moore SA, Campbell KP. LARGE glycans on dystroglycan function as a tunable matrix scaffold to prevent dystrophy. Nature. 2013;503(7474):136-40. Epub 2013/10/18. doi: 10.1038/nature12605. PubMed PMID: 24132234; PMCID: 3891507.

49. Mo KF, Fang T, Stalnaker SH, Kirby PS, Liu M, Wells L, Pierce M, Live DH, Boons GJ. Synthetic, structural, and biosynthetic studies of an unusual phospho-glycopeptide derived from alpha-dystroglycan. Journal of the American Chemical Society. 2011;133(36):14418-30. doi: 10.1021/ja205473q. PubMed PMID: 21812486; PMCID: 3176502.

50. Manya H, Chiba A, Yoshida A, Wang X, Chiba Y, Jigami Y, Margolis RU, Endo T. Demonstration of mammalian protein O-mannosyltransferase activity: coexpression of POMT1 and POMT2 required for enzymatic activity. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(2):500-5. PubMed PMID: 14699049.

51. Akasaka-Manya K, Manya H, Endo T. Mutations of the POMT1 gene found in patients with Walker-Warburg syndrome lead to a defect of protein O-mannosylation. Biochemical and biophysical research communications. 2004;325(1):75-9. doi: 10.1016/j.bbrc.2004.10.001. PubMed PMID: 15522202.

52. van Reeuwijk J, Janssen M, van den Elzen C, Beltran-Valero de Bernabe D, Sabatelli P, Merlini L, Boon M, Scheffer H, Brockington M, Muntoni F, Huynen MA, Verrips A, Walsh CA, Barth PG, Brunner HG, van Bokhoven H. POMT2 mutations cause alpha-dystroglycan hypoglycosylation and Walker-Warburg syndrome. Journal of medical genetics. 2005;42(12):907-12. doi: 10.1136/jmg.2005.031963. PubMed PMID: 15894594; PMCID: 1735967.

53. Mercuri E, D'Amico A, Tessa A, Berardinelli A, Pane M, Messina S, van Reeuwijk J, Bertini E, Muntoni F, Santorelli FM. POMT2 mutation in a patient with 'MEB-like' phenotype. Neuromuscular disorders : NMD. 2006;16(7):446-8. doi: 10.1016/j.nmd.2006.03.016. PubMed PMID: 16701995.

54. van Reeuwijk J, Maugenre S, van den Elzen C, Verrips A, Bertini E, Muntoni F, Merlini L, Scheffer H, Brunner HG, Guicheney P, van Bokhoven H. The expanding phenotype of POMT1 mutations: from Walker-Warburg syndrome to congenital muscular dystrophy, microcephaly, and mental retardation. Human mutation. 2006;27(5):453-9. doi: 10.1002/humu.20313. PubMed PMID: 16575835.

55. Lommel M, Cirak S, Willer T, Hermann R, Uyanik G, van Bokhoven H, Korner C, Voit T, Baric I, Hehr U, Strahl S. Correlation of enzyme activity and clinical phenotype in POMT1-associated dystroglycanopathies. Neurology. 2010;74(2):157-64. doi: 10.1212/WNL.0b013e3181c919d6. PubMed PMID: 20065251.

56. Willer T, Prados B, Falcon-Perez JM, Renner-Muller I, Przemeck GK, Lommel M, Coloma A, Valero MC, de Angelis MH, Tanner W, Wolf E, Strahl S, Cruces J. Targeted disruption of the Walker-Warburg syndrome gene Pomt1 in mouse results in embryonic lethality. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(39):14126-31. doi: 10.1073/pnas.0405899101. PubMed PMID: 15383666; PMCID: 521095.

57. Hu H, Li J, Gagen CS, Gray NW, Zhang Z, Qi Y, Zhang P. Conditional knockout of protein O-mannosyltransferase 2 reveals tissue-specific roles of O-mannosyl glycosylation in brain development. The Journal of comparative neurology. 2011;519(7):1320-37. doi: 10.1002/cne.22572. PubMed PMID: 21452199; PMCID: 3634366.

58. Tran DT, Lim JM, Liu M, Stalnaker SH, Wells L, Ten Hagen KG, Live D. Glycosylation of alpha-dystroglycan: O-mannosylation influences the subsequent addition of GalNAc by UDP-GalNAc polypeptide N-acetylgalactosaminyltransferases. The Journal of biological chemistry. 2012;287(25):20967-74. doi: 10.1074/jbc.M112.370387. PubMed PMID: 22549772; PMCID: 3375520.

59. Liu M, Borgert A, Barany G, Live D. Conformational consequences of protein glycosylation: preparation of O-mannosyl serine and threonine building blocks, and their incorporation into glycopeptide sequences derived from alpha-dystroglycan. Biopolymers. 2008;90(3):358-68. doi: 10.1002/bip.20847. PubMed PMID: 17868094.

60. Breloy I, Schwientek T, Gries B, Razawi H, Macht M, Albers C, Hanisch FG. Initiation of mammalian O-mannosylation in vivo is independent of a consensus sequence and controlled by peptide regions within and upstream of the alpha-dystroglycan mucin domain. The Journal of biological chemistry. 2008;283(27):18832-40. doi: 10.1074/jbc.M802834200. PubMed PMID: 18456664.

61. Manya H, Akasaka-Manya K, Nakajima A, Kawakita M, Endo T. Role of N-glycans in maintaining the activity of protein O-mannosyltransferases POMT1 and POMT2. Journal of biochemistry. 2010;147(3):337-44. doi: 10.1093/jb/mvp170. PubMed PMID: 19880378.

62. Willer T, Lee H, Lommel M, Yoshida-Moriguchi T, de Bernabe DB, Venzke D, Cirak S, Schachter H, Vajsar J, Voit T, Muntoni F, Loder AS, Dobyns WB, Winder TL, Strahl S, Mathews KD, Nelson SF, Moore SA, Campbell KP. ISPD loss-of-function mutations disrupt dystroglycan O-mannosylation and cause Walker-Warburg syndrome. Nature genetics. 2012;44(5):575-80. doi: 10.1038/ng.2252. PubMed PMID: 22522420; PMCID: 3371168.

63. Manzini MC, Tambunan DE, Hill RS, Yu TW, Maynard TM, Heinzen EL, Shianna KV, Stevens CR, Partlow JN, Barry BJ, Rodriguez J, Gupta VA, Al-Qudah AK, Eyaid WM, Friedman JM, Salih MA, Clark R, Moroni I, Mora M, Beggs AH, Gabriel SB, Walsh CA. Exome sequencing and functional validation in zebrafish identify GTDC2 mutations as a cause of Walker-Warburg syndrome. American journal of human genetics. 2012;91(3):541-7. doi: 10.1016/j.ajhg.2012.07.009. PubMed PMID: 22958903; PMCID: 3512000.

64. Stevens E, Carss KJ, Cirak S, Foley AR, Torelli S, Willer T, Tambunan DE, Yau S, Brodd L, Sewry CA, Feng L, Haliloglu G, Orhan D, Dobyns WB, Enns GM, Manning M, Krause A, Salih MA, Walsh CA, Hurles M, Campbell KP, Manzini MC, Consortium UK, Stemple D, Lin

YY, Muntoni F. Mutations in B3GALNT2 cause congenital muscular dystrophy and hypoglycosylation of alpha-dystroglycan. American journal of human genetics. 2013;92(3):354-65. doi: 10.1016/j.ajhg.2013.01.016. PubMed PMID: 23453667; PMCID: 3591840.

65. Hiruma T, Togayachi A, Okamura K, Sato T, Kikuchi N, Kwon YD, Nakamura A, Fujimura K, Gotoh M, Tachibana K, Ishizuka Y, Noce T, Nakanishi H, Narimatsu H. A novel human beta1,3-N-acetylgalactosaminyltransferase that synthesizes a unique carbohydrate structure, GalNAcbeta1-3GlcNAc. The Journal of biological chemistry. 2004;279(14):14087-95. doi: 10.1074/jbc.M310614200. PubMed PMID: 14724282.

66. Vogel P, Read RW, Hansen GM, Payne BJ, Small D, Sands AT, Zambrowicz BP. Congenital hydrocephalus in genetically engineered mice. Veterinary pathology. 2012;49(1):166-81. doi: 10.1177/0300985811415708. PubMed PMID: 21746835.

67. Ogawa M, Nakamura N, Nakayama Y, Kurosaka A, Manya H, Kanagawa M, Endo T, Furukawa K, Okajima T. GTDC2 modifies O-mannosylated alpha-dystroglycan in the endoplasmic reticulum to generate N-acetyl glucosamine epitopes reactive with CTD110.6 antibody. Biochemical and biophysical research communications. 2013;440(1):88-93. doi: 10.1016/j.bbrc.2013.09.022. PubMed PMID: 24041696.

68. Hara Y, Kanagawa M, Kunz S, Yoshida-Moriguchi T, Satz JS, Kobayashi YM, Zhu Z, Burden SJ, Oldstone MB, Campbell KP. Like-acetylglucosaminyltransferase (LARGE)-dependent modification of dystroglycan at Thr-317/319 is required for laminin binding and arenavirus infection. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(42):17426-31. doi: 10.1073/pnas.1114836108. PubMed PMID: 21987822; PMCID: 3198351.

69. Gill DJ, Chia J, Senewiratne J, Bard F. Regulation of O-glycosylation through Golgi-to-ER relocation of initiation enzymes. The Journal of cell biology. 2010;189(5):843-58. doi: 10.1083/jcb.201003055. PubMed PMID: 20498016; PMCID: 2878949.

70. Soderberg T. Organic Chemistry with a Biological Emphasis2010.

71. Ashikov A, Buettner FF, Tiemann B, Gerardy-Schahn R, Bakker H. LARGE2 generates the same xylose- and glucuronic acid-containing glycan structures as LARGE. Glycobiology. 2013;23(3):303-9. doi: 10.1093/glycob/cws153. PubMed PMID: 23135544.

72. Inamori K, Hara Y, Willer T, Anderson ME, Zhu Z, Yoshida-Moriguchi T, Campbell KP. Xylosyl- and glucuronyltransferase functions of LARGE in alpha-dystroglycan modification are conserved in LARGE2. Glycobiology. 2013;23(3):295-302. doi: 10.1093/glycob/cws152. PubMed PMID: 23125099; PMCID: 3555503.

73. Zhang P, Hu H. Differential glycosylation of alpha-dystroglycan and proteins other than alpha-dystroglycan by like-glycosyltransferase. Glycobiology. 2012;22(2):235-47. doi: 10.1093/glycob/cwr131. PubMed PMID: 21930648; PMCID: 3255506.

74. Grewal PK, McLaughlan JM, Moore CJ, Browning CA, Hewitt JE. Characterization of the LARGE family of putative glycosyltransferases associated with dystroglycanopathies. Glycobiology. 2005;15(10):912-23. doi: 10.1093/glycob/cwi094. PubMed PMID: 15958417.

75. Kanagawa M, Nishimoto A, Chiyonobu T, Takeda S, Miyagoe-Suzuki Y, Wang F, Fujikake N, Taniguchi M, Lu Z, Tachikawa M, Nagai Y, Tashiro F, Miyazaki J, Tajima Y, Takeda S, Endo T, Kobayashi K, Campbell KP, Toda T. Residual laminin-binding activity and enhanced dystroglycan glycosylation by LARGE in novel model mice to dystroglycanopathy. Human molecular genetics. 2009;18(4):621-31. doi: 10.1093/hmg/ddn387. PubMed PMID: 19017726; PMCID: 2638827.

76. Rojek JM, Campbell KP, Oldstone MB, Kunz S. Old World arenavirus infection interferes with the expression of functional alpha-dystroglycan in the host cell. Molecular biology of the cell. 2007;18(11):4493-507. doi: 10.1091/mbc.E07-04-0374. PubMed PMID: 17761532; PMCID: 2043543.

77. Patnaik SK, Stanley P. Mouse large can modify complex N- and mucin O-glycans on alpha-dystroglycan to induce laminin binding. The Journal of biological chemistry. 2005;280(21):20851-9. doi: 10.1074/jbc.M500069200. PubMed PMID: 15788414.

78. Hu Y, Li ZF, Wu X, Lu Q. Large induces functional glycans in an O-mannosylation dependent manner and targets GlcNAc terminals on alpha-dystroglycan. PloS one. 2011;6(2):e16866. doi: 10.1371/journal.pone.0016866. PubMed PMID: 21347376; PMCID: 3036717.

79. Aguilan JT, Sundaram S, Nieves E, Stanley P. Mutational and functional analysis of Large in a novel CHO glycosylation mutant. Glycobiology. 2009;19(9):971-86. doi: 10.1093/glycob/cwp074. PubMed PMID: 19470663; PMCID: 2720279.

80. Nakagawa N, Izumikawa T, Kitagawa H, Oka S. Sulfation of glucuronic acid in the linkage tetrasaccharide by HNK-1 sulfotransferase is an inhibitory signal for the expression of a chondroitin sulfate chain on thrombomodulin. Biochemical and biophysical research communications. 2011;415(1):109-13. doi: 10.1016/j.bbrc.2011.10.023. PubMed PMID: 22020094.

81. Gerardy-Schahn R, Oelmann S, Bakker H. Nucleotide sugar transporters: biological and functional aspects. Biochimie. 2001;83(8):775-82. PubMed PMID: 11530210.

82. Zhao W, Chen TL, Vertel BM, Colley KJ. The CMP-sialic acid transporter is localized in the medial-trans Golgi and possesses two specific endoplasmic reticulum export motifs in its carboxyl-terminal cytoplasmic tail. The Journal of biological chemistry. 2006;281(41):31106-18. doi: 10.1074/jbc.M605564200. PubMed PMID: 16923816.

83. Kanagawa M, Saito F, Kunz S, Yoshida-Moriguchi T, Barresi R, Kobayashi YM, Muschler J, Dumanski JP, Michele DE, Oldstone MB, Campbell KP. Molecular recognition by LARGE is essential for expression of functional dystroglycan. Cell. 2004;117(7):953-64. doi: 10.1016/j.cell.2004.06.003. PubMed PMID: 15210115.

84. Hara Y, Balci-Hayta B, Yoshida-Moriguchi T, Kanagawa M, Beltran-Valero de Bernabe D, Gundesli H, Willer T, Satz JS, Crawford RW, Burden SJ, Kunz S, Oldstone MB, Accardi A, Talim B, Muntoni F, Topaloglu H, Dincer P, Campbell KP. A dystroglycan mutation associated with limb-girdle muscular dystrophy. The New England journal of medicine. 2011;364(10):939-46. doi: 10.1056/NEJMoa1006939. PubMed PMID: 21388311; PMCID: 3071687.

85. Roscioli T, Kamsteeg EJ, Buysse K, Maystadt I, van Reeuwijk J, van den Elzen C, van Beusekom E, Riemersma M, Pfundt R, Vissers LE, Schraders M, Altunoglu U, Buckley MF, Brunner HG, Grisart B, Zhou H, Veltman JA, Gilissen C, Mancini GM, Delree P, Willemsen MA, Ramadza DP, Chitayat D, Bennett C, Sheridan E, Peeters EA, Tan-Sindhunata GM, de Die-Smulders CE, Devriendt K, Kayserili H, El-Hashash OA, Stemple DL, Lefeber DJ, Lin YY, van Bokhoven H. Mutations in ISPD cause Walker-Warburg syndrome and defective glycosylation of alpha-dystroglycan. Nature genetics. 2012;44(5):581-5. doi: 10.1038/ng.2253. PubMed PMID: 22522421; PMCID: 3378661.

86. Kuchta K, Knizewski L, Wyrwicz LS, Rychlewski L, Ginalski K. Comprehensive classification of nucleotidyltransferase fold proteins: identification of novel families and their representatives in human. Nucleic acids research. 2009;37(22):7701-14. doi: 10.1093/nar/gkp854. PubMed PMID: 19833706; PMCID: 2794190.

87. Harnett W, Rzepecka J, Houston KM. How do nematodes transfer phosphorylcholine to carbohydrates? Trends in parasitology. 2010;26(3):114-8. doi: 10.1016/j.pt.2009.12.003. PubMed PMID: 20083435.

88. Van Reeuwijk J, Olderode-Berends MJ, Van den Elzen C, Brouwer OF, Roscioli T, Van Pampus MG, Scheffer H, Brunner HG, Van Bokhoven H, Hol FA. A homozygous FKRP start codon mutation is associated with Walker-Warburg syndrome, the severe end of the clinical spectrum. Clinical genetics. 2010;78(3):275-81. doi: 10.1111/j.1399-0004.2010.01384.x. PubMed PMID: 20236121.

89. Xiong H, Kobayashi K, Tachikawa M, Manya H, Takeda S, Chiyonobu T, Fujikake N, Wang F, Nishimoto A, Morris GE, Nagai Y, Kanagawa M, Endo T, Toda T. Molecular interaction between fukutin and POMGnT1 in the glycosylation pathway of alpha-dystroglycan. Biochemical and biophysical research communications. 2006;350(4):935-41. PubMed PMID: 17034757.

90. Yamamoto T, Kawaguchi M, Sakayori N, Muramatsu F, Morikawa S, Kato Y, Shibata N, Kobayashi M. Intracellular binding of fukutin and alpha-dystroglycan: relation to glycosylation of alpha-dystroglycan. Neuroscience research. 2006;56(4):391-9. doi: 10.1016/j.neures.2006.08.009. PubMed PMID: 17005282.

91. Esapa CT, Benson MA, Schroder JE, Martin-Rendon E, Brockington M, Brown SC, Muntoni F, Kroger S, Blake DJ. Functional requirements for fukutin-related protein in the Golgi apparatus. Human molecular genetics. 2002;11(26):3319-31. PubMed PMID: 12471058.

92. Tachikawa M, Kanagawa M, Yu CC, Kobayashi K, Toda T. Mislocalization of fukutin protein by disease-causing missense mutations can be rescued with treatments directed at folding amelioration. The Journal of biological chemistry. 2012;287(11):8398-406. doi: 10.1074/jbc.M111.300905. PubMed PMID: 22275357; PMCID: 3318729.

93. Lin YY, White RJ, Torelli S, Cirak S, Muntoni F, Stemple DL. Zebrafish Fukutin family proteins link the unfolded protein response with dystroglycanopathies. Human molecular genetics. 2011;20(9):1763-75. doi: 10.1093/hmg/ddr059. PubMed PMID: 21317159; PMCID: 3071672.

94. Jimenez-Mallebrera C, Torelli S, Feng L, Kim J, Godfrey C, Clement E, Mein R, Abbs S, Brown SC, Campbell KP, Kroger S, Talim B, Topaloglu H, Quinlivan R, Roper H, Childs AM, Kinali M, Sewry CA, Muntoni F. A comparative study of alpha-dystroglycan glycosylation in dystroglycanopathies suggests that the hypoglycosylation of alpha-dystroglycan does not consistently correlate with clinical severity. Brain pathology (Zurich, Switzerland). 2009;19(4):596-611. PubMed PMID: 18691338.

95. Sztal TE, Sonntag C, Hall TE, Currie PD. Epistatic dissection of laminin-receptor interactions in dystrophic zebrafish muscle. Human molecular genetics. 2012;21(21):4718-31. doi: 10.1093/hmg/dds312. PubMed PMID: 22859503.

96. Blaeser A, Keramaris E, Chan YM, Sparks S, Cowley D, Xiao X, Lu QL. Mouse models of fukutin-related protein mutations show a wide range of disease phenotypes. Human genetics. 2013;132(8):923-34. doi: 10.1007/s00439-013-1302-7. PubMed PMID: 23591631.

97. Kawahara G, Guyon JR, Nakamura Y, Kunkel LM. Zebrafish models for human FKRP muscular dystrophies. Human molecular genetics. 2010;19(4):623-33. doi: 10.1093/hmg/ddp528. PubMed PMID: 19955119; PMCID: 2807370.

98. Lynch TA, Lam le T, Man N, Kobayashi K, Toda T, Morris GE. Detection of the dystroglycanopathy protein, fukutin, using a new panel of site-specific monoclonal antibodies. Biochemical and biophysical research communications. 2012;424(2):354-7. doi: 10.1016/j.bbrc.2012.06.147. PubMed PMID: 22771323.

99. Vuillaumier-Barrot S, Bouchet-Seraphin C, Chelbi M, Devisme L, Quentin S, Gazal S, Laquerriere A, Fallet-Bianco C, Loget P, Odent S, Carles D, Bazin A, Aziza J, Clemenson A, Guimiot F, Bonniere M, Monnot S, Bole-Feysot C, Bernard JP, Loeuillet L, Gonzales M, Socha K, Grandchamp B, Attie-Bitach T, Encha-Razavi F, Seta N. Identification of mutations in TMEM5 and ISPD as a cause of severe cobblestone lissencephaly. American journal of human genetics. 2012;91(6):1135-43. doi: 10.1016/j.ajhg.2012.10.009. PubMed PMID: 23217329; PMCID: 3516603.

100. Clement EM, Godfrey C, Tan J, Brockington M, Torelli S, Feng L, Brown SC, Jimenez-Mallebrera C, Sewry CA, Longman C, Mein R, Abbs S, Vajsar J, Schachter H, Muntoni F. Mild POMGnT1 mutations underlie a novel limb-girdle muscular dystrophy variant. Archives of neurology. 2008;65(1):137-41. doi: 10.1001/archneurol.2007.2. PubMed PMID: 18195152.

101. Lan J, Guo P, Chen M, Wu B, Mao Q, Qiu Y. O-linked mannose beta-1,2-N-acetylglucosaminyltransferase 1 correlated with the malignancy in glioma. The Journal of craniofacial surgery. 2013;24(4):1441-6. doi: 10.1097/SCS.0b013e318295378b. PubMed PMID: 23851827.

102. Inamori K, Endo T, Gu J, Matsuo I, Ito Y, Fujii S, Iwasaki H, Narimatsu H, Miyoshi E, Honke K, Taniguchi N. N-Acetylglucosaminyltransferase IX acts on the GlcNAc beta 1,2-Man

alpha 1-Ser/Thr moiety, forming a 2,6-branched structure in brain O-mannosyl glycan. The Journal of biological chemistry. 2004;279(4):2337-40. PubMed PMID: 14617637.

103. Cirak S, Foley AR, Herrmann R, Willer T, Yau S, Stevens E, Torelli S, Brodd L, Kamynina A, Vondracek P, Roper H, Longman C, Korinthenberg R, Marrosu G, Nurnberg P, Consortium UK, Michele DE, Plagnol V, Hurles M, Moore SA, Sewry CA, Campbell KP, Voit T, Muntoni F. ISPD gene mutations are a common cause of congenital and limb-girdle muscular dystrophies. Brain : a journal of neurology. 2013;136(Pt 1):269-81. doi: 10.1093/brain/aws312. PubMed PMID: 23288328; PMCID: 3562076.

104. Tasca G, Moro F, Aiello C, Cassandrini D, Fiorillo C, Bertini E, Bruno C, Santorelli FM, Ricci E. Limb-girdle muscular dystrophy with alpha-dystroglycan deficiency and mutations in the ISPD gene. Neurology. 2013;80(10):963-5. doi: 10.1212/WNL.0b013e3182840cbc. PubMed PMID: 23390185.

105. Nishihara S, Iwasaki H, Nakajima K, Togayachi A, Ikehara Y, Kudo T, Kushi Y, Furuya A, Shitara K, Narimatsu H. Alpha1,3-fucosyltransferase IX (Fut9) determines Lewis X expression in brain. Glycobiology. 2003;13(6):445-55. doi: 10.1093/glycob/cwg048. PubMed PMID: 12626397.

106. Kudo T, Fujii T, Ikegami S, Inokuchi K, Takayama Y, Ikehara Y, Nishihara S, Togayachi A, Takahashi S, Tachibana K, Yuasa S, Narimatsu H. Mice lacking alpha1,3-fucosyltransferase IX demonstrate disappearance of Lewis x structure in brain and increased anxiety-like behaviors. Glycobiology. 2007;17(1):1-9. doi: 10.1093/glycob/cwl047. PubMed PMID: 16973732.

107. Kakuda S, Sato Y, Tonoyama Y, Oka S, Kawasaki T. Different acceptor specificities of two glucuronyltransferases involved in the biosynthesis of HNK-1 carbohydrate. Glycobiology. 2005;15(2):203-10. doi: 10.1093/glycob/cwi001. PubMed PMID: 15470230.

108. Morise J, Kizuka Y, Yabuno K, Tonoyama Y, Hashii N, Kawasaki N, Manya H, Miyagoe-Suzuki Y, Takeda S, Endo T, Maeda N, Takematsu H, Oka S. Structural and biochemical characterization of O-mannose-linked human natural killer-1 glycan expressed on phosphacan in developing mouse brains. Glycobiology. 2014;24(3):314-24. Epub 2013/12/20. doi: 10.1093/glycob/cwt116. PubMed PMID: 24352591.

109. Finne J, Krusius T, Margolis RK, Margolis RU. Novel mannitol-containing oligosaccharides obtained by mild alkaline borohydride treatment of a chondroitin sulfate proteoglycan from brain. The Journal of biological chemistry. 1979;254(20):10295-300. PubMed PMID: 39937.

110. Chiba A, Matsumura K, Yamada H, Inazu T, Shimizu T, Kusunoki S, Kanazawa I, Kobata A, Endo T. Structures of sialylated O-linked oligosaccharides of bovine peripheral nerve alpha-dystroglycan. The role of a novel O-mannosyl-type oligosaccharide in the binding of alpha-dystroglycan with laminin. The Journal of biological chemistry. 1997;272(4):2156-62. PubMed PMID: 8999917.

111. Smalheiser NR, Haslam SM, Sutton-Smith M, Morris HR, Dell A. Structural analysis of sequences O-linked to mannose reveals a novel Lewis X structure in cranin (dystroglycan) purified

from sheep brain. The Journal of biological chemistry. 1998;273(37):23698-703. PubMed PMID: 9726975.

112. Sasaki T, Yamada H, Matsumura K, Shimizu T, Kobata A, Endo T. Detection of O-mannosyl glycans in rabbit skeletal muscle alpha-dystroglycan. Biochimica et biophysica acta. 1998;1425(3):599-606. PubMed PMID: 9838223.

113. Bleckmann C, Geyer H, Lieberoth A, Splittstoesser F, Liu Y, Feizi T, Schachner M, Kleene R, Reinhold V, Geyer R. O-glycosylation pattern of CD24 from mouse brain. Biological chemistry. 2009;390(7):627-45. doi: 10.1515/BC.2009.044. PubMed PMID: 19284289.

114. Martinez T, Pace D, Brady L, Gerhart M, Balland A. Characterization of a novel modification on IgG2 light chain. Evidence for the presence of O-linked mannosylation. Journal of chromatography A. 2007;1156(1-2):183-7. doi: 10.1016/j.chroma.2007.04.050. PubMed PMID: 17493628.

115. Pacharra S, Hanisch FG, Breloy I. Neurofascin 186 is O-mannosylated within and outside of the mucin domain. Journal of proteome research. 2012;11(8):3955-64. doi: 10.1021/pr200996y. PubMed PMID: 22746206.

116. Yuen CT, Chai W, Loveless RW, Lawson AM, Margolis RU, Feizi T. Brain contains HNK-1 immunoreactive O-glycans of the sulfoglucuronyl lactosamine series that terminate in 2-linked or 2,6-linked hexose (mannose). The Journal of biological chemistry. 1997;272(14):8924-31. Epub 1997/04/04. PubMed PMID: 9083013.

Table 2.1. Genes Encoding Established O-Mannose Glycan Synthesis Activities

Gene(s)	**Protein Function**	**Core Glycans**
POMT1 and POMT2	Mannose transfer from DPM to Ser/Thr	M1, M2, M3
POMGNT1	β1,2-GlcNAc transfer to O-mannose	M1, M2
POMGNT2 (GTDC2)	β1,4-GlcNAc transfer to O-mannose	M3
GNT-VB (GNT-IX)	β1,6-GlcNAc transfer to O-mannose	M2
B3GALNT2	β1,3-GalNAc transfer to GlcNAc-β1,4-Man-α	M3
POMK (SGK196)	6-phosphorylation of O-mannose	M3
LARGE	Xyl-GlcA repeat polymerization	M3
LARGE2	Xyl-GlcA repeat polymerization	M3
HNK-1ST	GlcA sulfation, including core M3 glycan Xyl-GlcA polymer sulfation	M1, M2, M3
FUT9	α1,3-Fucose transfer	M1, M2
B3GAT1	β1,3-Glucuronyl transfer	M1, M2

Table 2.2. Genes Encoding Hypothesized O-Mannose Glycan Synthesis Activities

Gene(s)	**Protein Function**	**Core Glycans**
ISPD	Role in O-mannose transfer activity	M1, M2, M3
FKTN	Protein Maturation?	M3
FKRP	Protein Maturation?	M3
B3GNT1	β1,3-GlcNAc transfer?	M3
TMEM5	Unknown	M3

Table 2.3. Genes Encoding Proteins Involved in Precursor Supply

Gene(s)	**Protein Function**	**Core Glycans**
MPDU1	Mannose supply	M1, M2, M3
PMM2	Mannose supply	M1, M2, M3
GPMBB	Synthesis of GDP-Man	M1, M2, M3
DPM1, DPM2 and DPM3	GDP-Man to DPM transfer	M1, M2, M3
UGDH	UDP-Glc to UDP-GlcA conversion	M1, M2, M3
UXS1	UDP-GlcA to UDP-Xyl conversion	M3
SLC35A1	Golgi CMP-sialic acid antiporter	M1, M2, M3

Table 2.4. Genes Encoding Proteins Involved in ER and Golgi Trafficking

Gene(s)	**Protein Function**	**Core Glycans**
COG4	Vesicle trafficking	?
COG5	Vesicle trafficking	?
COG7	Vesicle trafficking	?
COG8	Vesicle trafficking	?
PTAR1	Protein trafficking	?

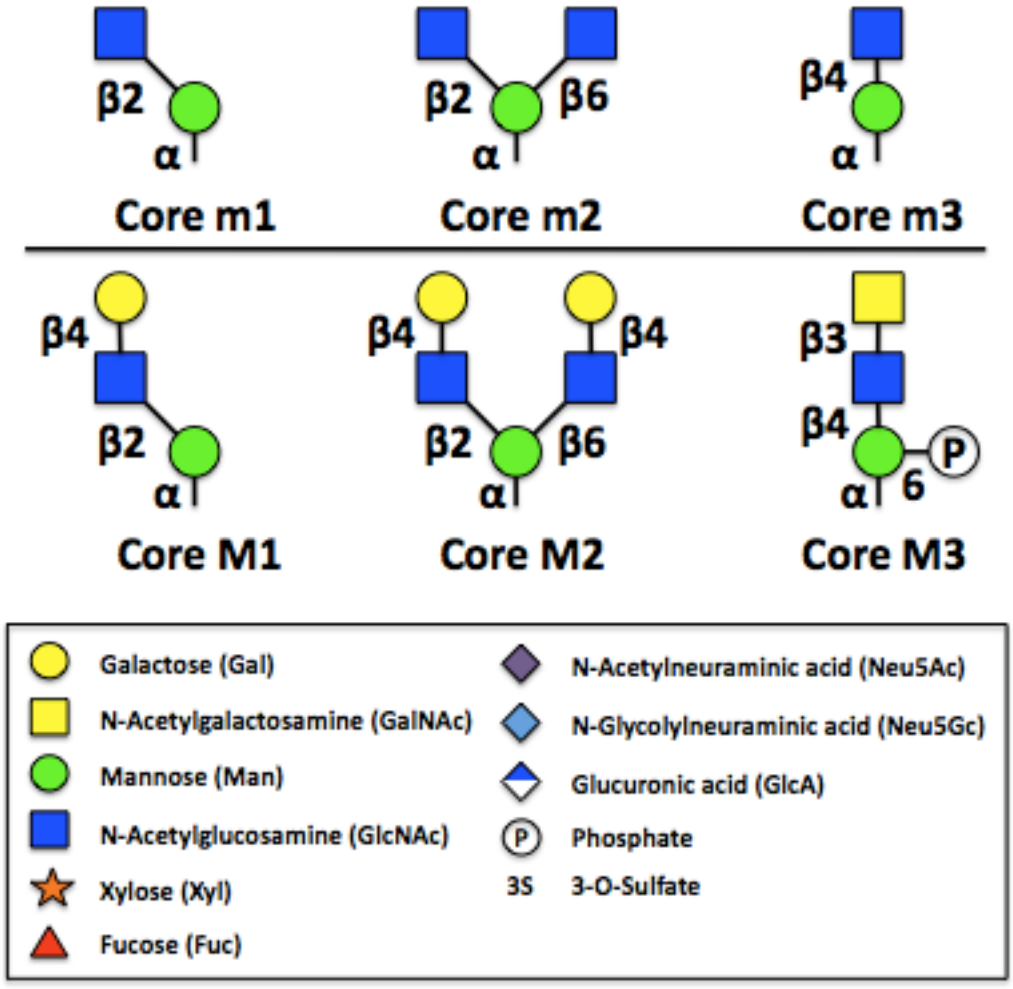
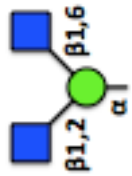
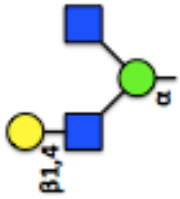
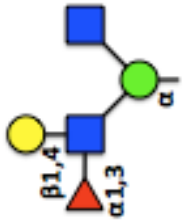
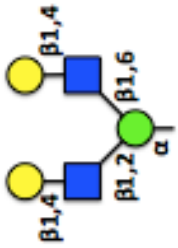
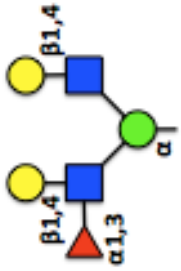
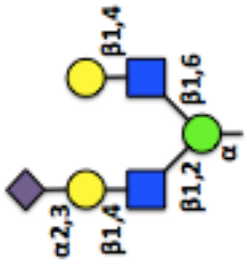


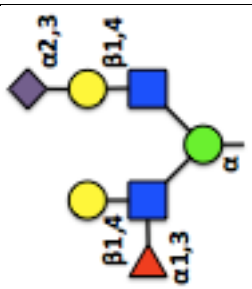
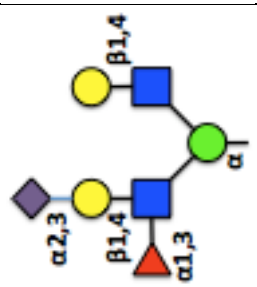
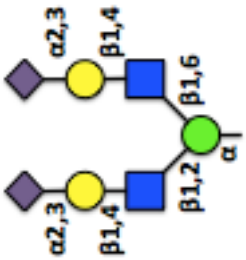
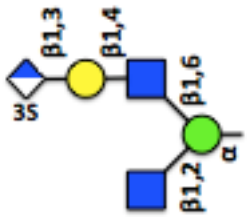
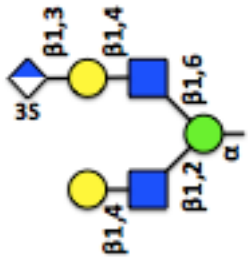
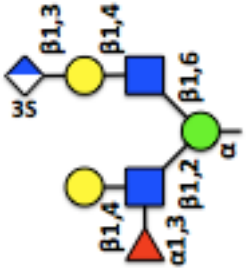
Figure 2.1. Core structures of O-mannose glycans. The naming scheme used in the bottom row was proposed in [22] while the top row contains our proposed naming scheme for substructures. Essentials of Glycobiology symbolic representations of monosaccharides and other molecules are described in the box [1].

#	Structure	References
1		[16, 19, 25, 113]
2		[16, 19, 25, 111, 113]
3		[19, 25, 111, 113]
4		[16, 19, 25, 111, 113]
5		[16, 19, 110]
6		[116]

Figure 2.2. Core m1 Glycans Found in Mammals.

See Figure 2.1 for symbol legend. Core m1 glycans account for more than 15% of protein-linked O-glycans in mouse brain [19]. Core m1 glycans are necessary for α-DG functional glycosylation and modulate O-GalNAcylation. Note that O-mannose without extension has also been observed [19].

#	Structure	References
7		[42]
8		[42]
9		[42, 113]
10		[42, 113]
11		[42, 113]
12		[42, 113]
13		[19, 25, 42]

14		[19]
15		[113]
16		[16, 19, 25, 42]
17		[116]
18		[116]
19		[116]

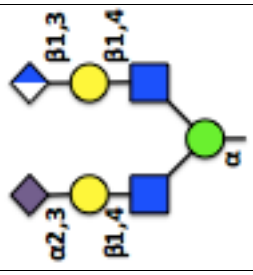
20		[113]

Figure 2.3. Core m2 Glycans Found in Mammals.

See Figure 2.1 for symbol legend. Core m2 glycans account for ~5% of protein linked O-glycans in mouse brain [19]. Core m2 glycans are involved in the inhibition of RPTPβ activity, which causes an increased level of integrin-dependent cell migration.

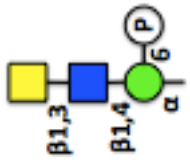
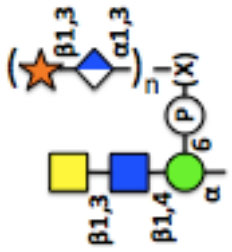
#	Structure	References
21		[13]
22		[44]

Figure 2.4. Core m3 Glycans Found in Mammals.

See Figure 2.1 for symbol legend. Core m3 glycans account for an unknown but probably small fraction of protein-linked O-glycans in mouse brain. Core m3 glycans include the α-DG functional glycan (structure 22), are involved in LG domain-binding and in the entry of viruses into cells. (X) depicts unknown elements, and a subscript n indicates polymer repeats.

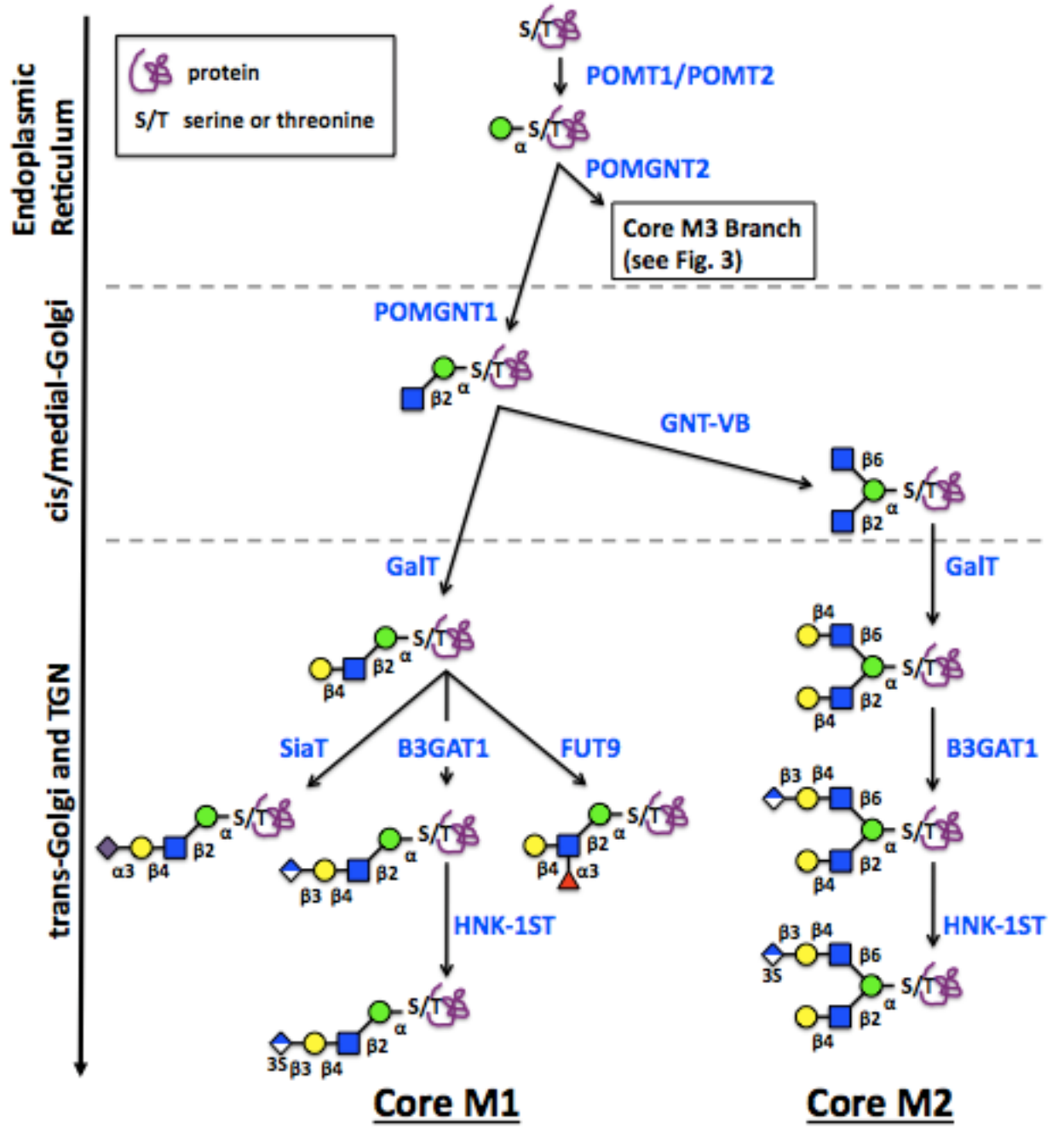


Figure 2.5. Synthesis of representative structures along the Golgi-centric core M1 and M2 glycan synthesis pathways. FUT9 and B3GAT1 (GlcAT-P) have been demonstrated in certain tissues and models to be the primary enzymes responsible for the steps indicated, although other fucosyl- and glucuronyltransferases may be present. Abbreviations: GalT, β1,4-galactosyltransferase; SiaT, α2,3-sialyltransferase. See Figure 2.1 for the monosaccharide code legend.

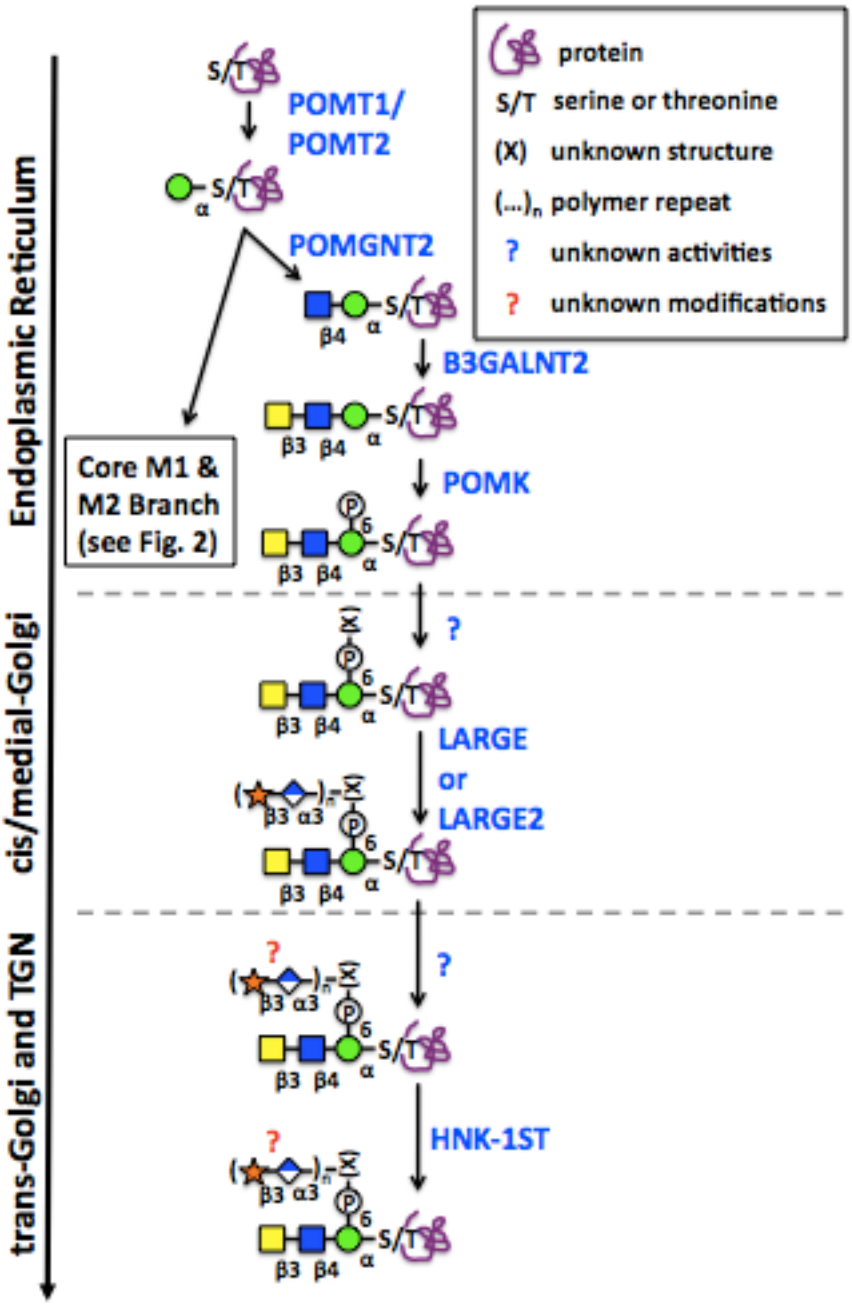


Figure 2.6. Synthesis of representative core M3 structures that are dependent on multiple initial activities based in the ER. Evidence suggests that 6-position phosphorylation of O-mannose that is dependent on β1,4-GlcNAc extension precludes β1,2-GlcNAc addition in the Golgi [49]. Core M3 is the only core known to be modified with the α-DG functional glycan structure. Various steps

as well as the structures ultimately built have not been fully elucidated. See Figure 2.1 for the monosaccharide code legend.

CHAPTER 3

B4GAT1 IS THE PRIMING ENZYME FOR THE LARGE-DEPENDENT FUNCTIONAL GLYCOSYLATION OF α-DYSTROGLYCAN[1]

[1] Praissman JL, Live DH, Wang S, Ramiah A, Chinoy ZS, Boons GJ, Moremen KW, Wells L. Elife. 2014;3. doi: 10.7554/eLife.03943. PubMed PMID: 25279697; PMCID: 4227051.

Abstract

Recent studies demonstrated that mutations in B3GNT1, an enzyme proposed to be involved in poly-N-acetyllactosamine synthesis, were causal for congenital muscular dystrophy with hypoglycosylation of α-dystroglycan (secondary dystroglycanopathies). Since defects in the O-mannosylation protein glycosylation pathway are primarily responsible for dystroglycanopathies and with no established O-mannose initiated structures containing a β3 linked GlcNAc known, we biochemically interrogated this human enzyme. Here we report this enzyme is not a β-1,3-N-acetylglucosaminyltransferase with catalytic activity towards β-galactose but rather a β-1,4-glucuronyltransferase, designated B4GAT1, towards both α- and β-anomers of xylose. The dual-activity LARGE enzyme is capable of extending products of B4GAT1 and we provide experimental evidence that B4GAT1 is the priming enzyme for LARGE. Our results further define the functional O-mannosylated glycan structure and indicate that B4GAT1 is involved in the initiation of the LARGE-dependent repeating disaccharide that is necessary for extracellular matrix protein binding to O-mannosylated α-dystroglycan that is lacking in secondary dystroglycanopathies.

Introduction

Glycosylation is the most abundant and diverse post-translational modification of proteins [1]. The synthesis of complex glycans is catalyzed by the action of over 200 individual glycosyltransferases in humans [2]. For most of the enzymes studied to date, there is exceptional selectivity for the donor sugar nucleotide and the underlying acceptor glycan as well as stereo- and linkage-specificity for the catalyzed additions [2]. Mutations in the genes encoding many of these glycosyltransferases have been established as causal for a variety of human diseases including congenital muscular dystrophy, specifically the secondary dystroglycanopathies [3-6].

At least a dozen enzymes/proteins are involved in the synthesis of O-mannose-initiated glycans that when defective lead to various forms of congenital muscular dystrophy, termed secondary dystroglycanopathies, that range from the phenotypically mild Limb-Girdle to the severe Walker-Warburg muscular dystrophies [5, 7-9]. Until recently [10-12], α-dystroglycan, a central component of the dystrophin-glycoprotein complex that serves to connect the cytoskeleton inside the cell with the extracellular matrix outside the cell, was the only well-established O-mannoslyated mammalian protein [3, 13]. The proper O-mannosylation of α-dystroglycan is essential for its ability to bind to components of the extracellular matrix including laminin [3, 14]. In recent years, significant progress has been made in defining the multitude of O-mannose structures produced in multicellular animals including partial elucidation of the LARGE-dependent functional glycan structure [15-24]. Recently, reports in the literature have connected mutations in B3GNT1 with congenital muscular dystrophies [25-27] and specifically to the defective glycosylation of α-dystroglycan [28] even though O-mannose glycan structures containing a β3-GlcNAc have yet to be elucidated [5].

In 1997, B3GNT1 (also known as iGnT) was reported to be the first successfully cloned β-1,3-N-acetylglucosaminyltransferase involved in the synthesis of poly-N-acetyllactosamine [29]. Poly-N-acetyllactosamine chains consist of the repeating disaccharide -β3-GlcNAc-β4-Gal-, a structure termed the i-antigen when unbranched [30, 31]. Poly-N-acetyllactosamine is a prevalent glycan substructure found in N-glycans and O-glycans on proteins whose abnormal levels have been associated with human diseases including cancer [30-33]. The Fukuda group used an expression cloning strategy enriching for plasmids containing cDNA inserts that substantially increased poly-N-acetyllactosamine, as judged by antibody binding, on the surfaces of Namalwa KJM-1 cells [29]. Shortly thereafter, work carried out by Hennet's and Sasaki's groups led to the cloning of three other β-1,3-N-acetylglucosaminyltransferases, B3GNT2, 3, and 4 [34, 35]. However, papers as recently as 2008 continued to use conflicting nomenclature for B3GNT2 by calling it B3GNT1 causing some confusion within the field [36]. Importantly, B3GNT2, B3GNT3 and B3GNT4 were found to share motifs with β3-galactosyl-transferases as well as β3-GalNAc-transferases [34, 35, 37]. These three enzymes are in the same Carbohydrate-Active Enzymes database (CAZy) family and group together based on primary sequence similarity and motifs hypothesized to play a role in forming the β3 linkage [34, 35, 37]. B3GNT1 lacks these motifs and is in a different CAZy family, interestingly one shared with LARGE and LARGE2 that contain both xylosyl- and glucoronsyl-transferase activity and which functionally modify O-mannosylated glycans [21, 22, 38]. Furthermore, it has been reported that the B3GNT1 enzyme is a binding partner for LARGE in mammalian cells [39].

The combination of data suggesting substantial divergence of B3GNT1 from other B3GNTs and the similarity in primary sequence and disease association with LARGE led us to reexamine the enzymatic activity of B3GNT1 to resolve the apparent inconsistencies and establish

its potential enzymatic role in O-mannosylation and secondary dystroglycanopathies. Here we report that B3GNT1 is in fact B4GAT1 (β-1,4-glucuronyltransferase 1) that generates the substrate for the LARGE-dependent repeating disaccharide that is required for interaction of O-mannosylated α-dystroglycan with extracellular matrix proteins.

Results

B3GNT1 (B4GAT1), unlike B3GNT2, is not a N-acetylglucosaminyltransferase

A secreted (lacking the trans-membrane domain) epitope-tagged form of human B3GNT1 was recombinantly expressed in HEK293F cells, purified, and the epitope tag removed before enzymatic characterization. Purity and enzyme identity was assessed by Coomassie G-250 staining of SDS-PAGE separated protein as well as by shotgun proteomics using reverse-phase liquid chromatography-nanospray tandem mass spectrometry (LC-MS/MS) following tryptic digestion (Figure 3.2, Table 3.1). Incubation of the purified protein with UDP-[^{3}H]GlcNAc and an enzymatically synthesized and purified substrate, Gal-β4-GlcNAc-β-pNP (Figure 3.3), under previously reported buffering conditions for B3GNTs [34, 35] failed to result in a detectable product as measured by incorporation of radioactivity into substrate (Figure 3.1). In order to validate our acceptor sugar and buffering conditions, we expressed, purified, and characterized a secreted epitope-tagged form of human B3GNT2 (Figure 3.2, Table 3.1). Side-by-side extended (overnight) incubations of B3GNT1 and the B3GNT2 enzyme preparations with acceptor and sugar nucleotide showed clear N-acetylglucosaminyltransferase activity toward the acceptor only with B3GNT2, as assayed by radioactive incorporation of GlcNAc into the N-acetyllactosamine acceptor (Figure 3.1**,** glycans displayed in symbolic representations [40]).

B3GNT1, but not B3GNT2, is a glucuronyltransferase that uses xylose as an acceptor

Given that B3GNT1 clustered into CAZy family GT49, the family containing the glucuronyltransferase domain of LARGE, we performed a multiple sequence alignment (Figure 3.4A). B3GNT1 aligned strongly with LARGE and LARGE2 in the glucuronyltransferase domain DXD-motif region typically involved in metal ion dependent sugar nucleotide binding. This DXD-motif was shown to be necessary for the glucuronyltransferase activity of LARGE and LARGE2 consistent with probable sugar nucleotide binding in this region [22]. B3GNT2 did not align well with B3GNT1 or the LARGE proteins (Figure 3.4A). We hypothesized that B3GNT1 might have glucuronyltransferase activity contrary to its proposed enzymatic function and thus carried out a screen using various tagged monosaccharide acceptors. Incubation of B3GNT1 and UDP-GlcA with multiple glycan acceptors resulted in significant transfer only to α- and β-xylopyranosides, as measured by radioactive transfer from UDP-[^{14}C]GlcA (Figure 3.4B). B3GNT2 did not transfer GlcA to either anomeric form of xylopyranoside tested (data not shown).

B3GNT1, unlike LARGE, is not selective for the stereochemistry of the acceptor xylose

We next expressed, purified and characterized a secreted form of human LARGE (Figure 3.2, Table 3.1) whose activity we investigated. Our results confirm previous reports that LARGE transfers GlcA to α-xylopyranosides but not β-xylopyranosides (Figure 3.4C**,** [21, 22]). In contrast, B3GNT1 does not appear to be hampered by the stereochemistry of the acceptor sugar (Figure 3.4B, C). We also confirmed the disaccharide products by LC-MS/MS (Figure 3.4D-F).

Soluble forms of B3GNT1 and LARGE do not form a complex *in vitro*

In order to investigate whether a complex formed between our soluble enzymes, we incubated the purified forms of the two enzymes together with only the LARGE enzyme being epitope tagged. Following re-purification of LARGE based on the epitope tag, we were unable to

detect the presence of B3GNT1 suggesting that the soluble forms of the proteins do not interact *in vitro* (Figure 3.5).

B3GNT1 is more catalytically efficient than LARGE with monosaccharide acceptors

We performed kinetic analysis of B3GNT1 and LARGE using the α-xylopyranoside as an acceptor and determined only minor differences (3.0 and 6.0 mM, respectively) in Km values for the acceptor (Table 3.2). However, at 2 mM UDP-GlcA, B3GNT1 has a specific activity that is 48 times higher than LARGE for α-xylopyranoside as the acceptor (Table 3.2). For B3GNT1, we were also able to use β-xylopyranoside as an acceptor and determined that the enzyme was more than twice as efficient with this acceptor as opposed to its anomer, α-xylopyranoside, though the Km value for the acceptor (4.0 mM) was only slightly altered (Table 3.2). Thus, B3GNT1 possesses a significantly higher specific activity, turnover rate and catalytic efficiency than LARGE toward the monosaccharide α-xylopyranoside acceptor and is even more efficient with the β-anomer that LARGE is not able to utilize (Table 3.2).

B3GNT1 is a B4GAT unlike LARGE that harbors B3GAT activity

To further characterize the product of B3GNT1 compared to LARGE, we carried out large-scale transfer reactions with the proven substrates of each enzyme. Disaccharide products were purified using reverse phase C18 HPLC and analyzed by multiple NMR-based experiments (Figure 3.6, 3.7 and Table 3.3). Glycosidic linkages and anomeric configurations were determined by NMR [41-43] that clearly demonstrates that the B3GNT1 and LARGE products share the same stereochemistry but different linkages of the terminal glucuronic acid to the underlying xylose (Figure 3.6, 3.7 and Table 3.3). From this analysis, we determined that B3GNT1 is a xylopyranoside β-1,4-glucuronyltransferase that we designate, using standard convention,

B4GAT1 and, as previously described, we confirmed that LARGE contains xylopyranoside β-1,3-glucuronyltransferase activity [21].

The B4GAT1 disaccharide products are substrates for the xylosytransferase activity of LARGE

Since B4GAT1 transfers GlcA to both anomers of xylopyranoside in contrast to LARGE and produces a β1,4-linkage as opposed to a β1,3-linkage, we sought to determine if the xylosyltransferase domain of LARGE would transfer to products of B4GAT1. We tested GlcA-β4-Xyl-α-pNP and GlcA-β4-Xyl-β-MU (products of B4GAT1) as well as GlcA-β3-Xyl-α-pNP (the product of LARGE as a positive control), each of which we produced and then purified by reverse phase C18 HPLC before use. Reactions were carried out overnight at 37^{o}C with UDP-Xyl as the donor. Substrates and products were desalted by reverse phase C18 spin columns and then subjected to C18 reverse-phase HPLC. Distinct substrate and product peaks were detected for each of the three potential acceptors tested and the identity of each peak was confirmed by LC-MS/MS (Figure 3.8). Hence, LARGE can extend both disaccharide products of B4GAT1 and its own disaccharide product with α3-linked xylose.

Trisaccharides are not efficient substrates for B4GAT1, unlike the glucuronyltransferase activity of LARGE

Having produced three different trisaccharides with non-reducing end terminal xylose, Xyl-α3-GlcA-β4-Xyl-α-pNP, Xyl-α3-GlcA-β4-Xyl-β-MU (products sequentially of B4GAT1 and xylosyltransferase activity of LARGE), and Xyl-α3-GlcA-β3-Xyl-α-pNP (the product of the bifunctional glucuronyltransferase and xylosyltransferase activities of LARGE), we tested each for elongation by B4GAT1 and LARGE. Incubation of the trisaccharides with UDP-GlcA and LARGE produced tetrasaccharide products in all cases, which were observed by HPLC and

confirmed by LC-MS/MS (Figure 3.9). In sharp contrast, we were unable to produce any observable tetrasaccharide with B4GAT1 using any of the three trisaccharides as acceptors (Figure 3.9), though the enzyme was active for transfer to monosaccharide xylopyranosides (Figure 3.4).

Neither B4GAT1 nor LARGE appear to possess branching activity

The inability of B4GAT1 to modify trisaccharides ending with xylose does not rule out the possibility that B4GAT1 may possess a branching activity. We tested whether B4GAT1 or LARGE were capable of transferring glucuronic acid to any of the tagged purified disaccharides GlcA-β4-Xyl-α-pNP, GlcA-β4-Xyl-β-pNP or GlcA-β3-Xyl- α-pNP. We did not detect any transfer of GlcA in these *in vitro* assays (Figure 3.10). Thus, none of the GlcA-capped products of the enzymes appear to be substrates for the other enzyme.

Formation of disaccharide polymer lags in the absence of B4GAT1

To further characterize the interplay between LARGE and B4GAT1, we examined production of polymer by LARGE both with and without B4GAT1. Incubation of Xyl-α-pNP or Xyl-β-pNP with either B4GAT1 alone, LARGE alone, or B4GAT1 and LARGE with both sugar nucleotide donors resulted in substantially different polymer production time courses as assayed by radioactive transfer (Figure 3.11). Time points were taken at 1, 2, 14 and 36 hours. B4GAT1 rapidly completes transfer to substrate. ~1200 DPM represents complete transfer to the 10 nmol of substrate present. LARGE is unable to transfer to Xyl-β-pNP (Figure 3.11B) and shows a significant lag in initial transfer to Xyl-α-pNP before the formation of polymer. In contrast, B4GAT1 and LARGE together demonstrate robust initial transfer activity and formation of polymer using either substrate. Analysis of the synthesized polymer via mass spectrometry shows extensive polymerization is achievable following incubation with both enzymes (Figure 3.12). These data provide further evidence that LARGE possesses poor initiating activity but works efficiently in polymer formation once B4GAT1 is added for initial transfer of GlcA.

Discussion

O-mannosylation of α-dystroglycan is required for its proper function and when disrupted is a significant cause of a subset of congenital muscular dystrophies referred to as secondary dystroglycanopathies [3-9] A phosphorylated O-mannose trisaccharide (core M3, [5]) attached to α-dystroglycan and its extension by LARGE after poorly defined intermediate biosynthetic steps (Figure 3.13) has been shown to be directly involved in the required ECM ligand binding activity [16, 21, 44]. Here, we have established that B4GAT1 is a β-1,4-glucuronyltransferase, not a B3GNT (Figure 3.1), with activity toward monomeric α- and β- xylopyranosides (Figure 3.4, 3.6**)**. We have gone on to show that LARGE can extend the resulting products of B4GAT1 (Figure 3.8). Prior reports indicated that B4GAT1 (B3GNT1) activity is necessary for expression of LARGE-dependent functional glycosylation of α-dystroglycan and that B4GAT1 (B3GNT1) and LARGE form a complex [25-27, 39]. We were unable to confirm this latter point using our soluble forms of the enzymes (Figure 3.5). Since we have shown that the specific activity and catalytic efficiency of B4GAT1 towards the tested monosaccharide xylosides is greater by more than an order of magnitude compared to LARGE catalysis (Table 3.2), this suggests that B4GAT1 is an initiating enzyme for LARGE-dependent glycan synthesis. This conclusion is further strengthened by data showing a significant lag in the initial rate for synthesis of or a complete failure to produce polymer in the absence of B4GAT1 (Figure 3.11). It also appears that B4GAT1 serves exclusively as a priming enzyme since we could not detect B4GAT1 modification of LARGE extended trisaccharides (Figure 3.9). We also demonstrate that neither LARGE nor B4GAT1 is capable of adding GlcA to a disaccharide that has been capped with GlcA suggesting that branching is not occurring (Figure 3.9, Figure 3.10). This leads to the conclusion that there is only one type of repeating disaccharide (Xyl-α3-GlcA-β3) attached to α-dystroglycan that is synthesized by LARGE. This data also serves to highlight the differences in structural isomers by showing both

anomeric configuration (α and β, Figure 3.4C and 3.11) as well as linkage position (-3 and -4, Figure 3.9) of isomeric substrates influences their subsequent ability to be elongated by enzymes (LARGE and B4GAT1). In sum, our data provide a basis for further attempts to fully elucidate the functional LARGE-dependent O-mannose-initiated structure(s) and suggests that at least one of the remaining incompletely characterized genes implicated in functional glycosylation of α-dystroglycan (FKTN, FKRP, ISPD, and TMEM5, [6, 28]) likely encodes a xylosyltransferase to provide a substrate for B4GAT1. Given that B4GAT1 is essential for proper glycosylation of α-dystroglycan [25-27, 39] and that LARGE is incapable of transferring GlcA to a β-xylopyranoside, (Figure 3.4**,** [21, 22]) or generating a polymer from pNP-β-Xyl without B4GAT1 present (Figure 3.11), our findings strongly suggest that the underlying xylose is in a β-linkage on α-dystroglycan. This work also clarifies why deficiencies in this enzyme would be associated with loss of functional glycosylation and laminin binding of α-dystroglycan and be causal for congenital muscular dystrophy [25-27, 39]. Further, our results likely clarify how loss of expression of B4GAT1 (B3GNT1), similar to loss of expression of LARGE [45], can lead to loss of laminin-binding α-dystroglycan and promote metastasis in certain cancers [39]. Finally, potential complex interactions between poly-N-acetyllactosamine, as often measured by the i-antigen antibody, and the LARGE-dependent repeating disaccharide, often measured on α-dystroglycan by the IIH6 antibody, need to be reexamined in the context of the newly defined enzymatic activity of B4GAT1 (B3GNT1) [25-27, 30, 39, 46-48]. For example, B4GAT1 has previously been observed to be in complex with B4GALT1, an enzyme required for poly-N-acetyllactosamine synthesis [46]. This finding suggested that interaction between these two enzymes, which were thought to generate the repeating disaccharide, potentially influenced poly-N-acetyllactosamine synthesis. In lieu of our findings, this extrapolated conclusion from the interaction data is no longer valid. Instead, the

relationship between poly-N-acetyllactosamine synthesis and the functional O-mannose glycan pathway needs to be evaluated, especially given that both have been implicated in cancer metastasis [25-27, 30, 39, 46-48].

We propose renaming B3GNT1 according to its defined activity as B4GAT1 (Figure 3.1, Figure 3.4, Figure 3.6). This designation is following in the tradition of the three other known non-proteoglycan glycoprotein glucuronyltransferases that all add GlcA in a β3-linkage (B3GAT1-3, [49]). Interestingly, the only established glycoprotein glycoslytransferases capable of transferring GlcA in a β4-linkage are the dual-activity exostoses enzymes involved in heparan sulfate (HS) proteoglycan polymerization that build the repeating disaccharide (-GlcNAc-α4-GlcA-β4-) backbone of the glycosaminoglycan [50]. Similarities exist between HS (and other proteoglycan) biosynthesis pathways and the LARGE-dependent functional O-mannose glycan assembly pathway built on α-dystroglycan (Figure 3.13). Included in these similarities are that they both contain non-reducing end repeating disaccharides (GlcA and GlcNAc for HS and GlcA and Xyl for the functional O-Man glycan structure), they both contain an acidic glycan (GlcA), there is a copolymerase that has dual-enzymatic activity (exostoses enzymes for HS and LARGE for the functional O-Man glycan structure), both require a specific underlying core structure, and each contains specific priming glycosyltransferases (EXTLs for HS [51, 52], B4GAT1 for the functional O-Man glycan structure) that adds one of the sugars found in the repeat to the underlying core structure to initiate elongation. Here we have established that B4GAT1, previously referred to as B3GNT1, is a xylopyranoside β1,4-glucuronyltransferase that appears to be the priming enzyme for the LARGE copolymerase for building the functional O-Man structure on α-dystroglycan that when defective causes CMD.

Materials and Methods

Reagents

Tagged monosaccharide glycosides were purchased from Sigma-Aldrich (St. Louis, MO) at ≥97% purity as were UDP-GlcA trisodium salt, UDP-GlcNAc disodium salt and UDP-Gal disodium salt. HPLC solvents were also purchased from Sigma-Aldrich (Chromasolv grade). UDP-Xyl was purchased from CarboSource Services (Athens, GA). Mini-protean TGX PAGE gels were purchased from Bio-Rad. UDP-[^{3}H]GlcNAc was purchased from American Radiolabeled Chemicals (St. Louis, MO) and UDP-[^{14}C]GlcA was from PerkinElmer (Waltham, MA).

Enzyme Production

The catalytic domains of human B3GNT1 (amino acid residues 54-415, UniProt O43505), B3GNT2 (amino acid residues 35-397, UniProt Q9NY97), and LARGE (amino acid residues 91-756, UniProt O95461) were expressed as soluble, secreted fusion proteins by transient transfection of HEK293 suspension cultures [53]. The coding regions were amplified from Mammalian Gene Collection Gerhard, 2004 #4 clones using primers that appended a tobacco etch virus (TEV) protease cleavage site [54] to the NH_2-terminal end of the coding region and attL1 and attL2 Gateway® adaptor sites to the 5' and 3' terminal ends of the amplimer products. The amplimers were recombined via BP clonase reaction into the pDONR221 vector and the DNA sequences were confirmed. The pDONR221 clones were then recombined via LR clonase reaction into a custom Gateway adapted version of the pGEn2 mammalian expression vector [53, 55] to assemble a recombinant coding region comprised of a 25 amino acid NH_2-terminal signal sequence from the *T. cruzi* lysosomal α-mannosidase [56] followed by an 8xHis tag, 17 amino acid AviTag [57], "superfolder" GFP [58], the nine amino acid sequence encoded by attB1 recombination site,

followed by the TEV protease cleavage site and the respective glycosyltransferase catalytic domain coding region.

Suspension culture HEK293f cells (Life Technologies, Grand Island, NY) were transfected as previously described [53] and the culture supernatant was subjected to Ni-NTA superflow chromatography (Qiagen, Valencia, CA). Enzyme preparations eluted with 300 mM imidazole were concentrated to ~1 mg/ml using an ultrafiltration pressure cell membrane (Millipore, Billerica, MA) with a 10 kDa molecular weight cutoff.

Enzymatic Reactions

All reactions for figures 3.4 through 3.10 were performed in 0.1 M MES pH 6.5, 10 mM MnCl2, 5 mM $MgCl_2$. Reactions summarized in Figure 3.1 were performed with omission of $MgCl_2$, conditions that more closely match those in the original literature [29, 35]. Non-radioactive nucleotide sugar donors were included at 2 mM for analytical procedures excluding the polymer production assays in which both UDP-GlcA and UDP-Xyl were included at 10 mM. Nucleotide sugar donors were included at up to 8 mM for preparative scale production of material for NMR or purification for further reactions. Radioactive nucleotide sugar donors were included at approximately 40,000 DPM per sample. Substrate concentrations for analytical procedures were kept constant at 2 mM except in the kinetics assays. All incubations were carried out at 37ºC. Incubation times for kinetics were set at 2 hr for B4GAT1 and 16 hr for LARGE based on time course curves used to ensure adequate transfer while maintaining the initial rate condition required. All other analytical reactions were performed for 16–18 hr while preparative reactions involving LARGE were carried out for upwards of 24 hr with occasional addition of enzyme due to the low specific activity of LARGE with respect to certain substrates.

Enzyme Assays and Product Purification

Enzymatic reactions were stopped by boiling for 5 min, acidified to 0.1% TFA, and glycoside acceptors were separated from sugar nucleotide using reverse-phase C18 spin (The Nest Group, Inc., Southborough, MA) columns. Transfer was determined by scintillation counting or HPLC with LC-MS/MS verification of species. For scintillation counting, a PerkinElmer (Waltham, MA) Tri-Carb 2910TR liquid scintillation counter was used with ScintSafe Plus 50% scintillation cocktail under standard settings for the isotope in question. HPLC was carried out on an Agilent (Santa Clara, CA) 1100 LC system equipped with variable wavelength absorbance detector set for monitoring pNP and MU derivatives (310 nm). Quantification was by peak area. Buffer A was 50 mM ammonium formate pH 4.3 and buffer B was 20% buffer A in 80% acetonitrile for all assays discussed. Separations were carried out by isocratic elution using a Grace Vydac 218TP C18 column (5 μm particle size, 2.1 mm × 150 mm). All disaccharide products were separated using 14%B. Trisaccharide and tetrasaccharide separations were noted to be most strongly influenced by the identity of the underlying labeled disaccharide. Hence, all longer chain products and substrates extending GlcA-β4-Xyl-α-pNP were separated at 8%B, all products and substrates extending GlcA-β4-Xyl-β-MU were separated using 10%B, and all products and substrates extending GlcA-β3-Xyl-α-pNP were separated at 5%B. LC-MS and LC-MS/MS of reaction products was performed in positive mode on either a Thermo Fisher (Waltham, MA) Orbitrap XL or a Thermo Fisher Orbitrap Fusion utilizing short linear gradients from 0.1% formic acid in water to 0.1% formic acid in 80% acetonitrile. Full MS was acquired in the Orbitrap for accurate mass determination while glycoside MS/MS fragmentation spectra were obtained in the linear ion trap and assigned manually.

Shotgun Proteomics and Protein SDS-PAGE

Shotgun proteomics was performed on tryptic digests of purified enzyme samples according to our standard protocol on a Thermo Fisher Orbitrap XL [59]. Data was searched in Proteome Discoverer 1.4 using Sequest HT with the percolator node set at a 1% peptide false-discovery rate and a recent human reference proteome from Uniprot to which common contaminant protein sequences had been added. SDS-PAGE was also carried out according to standard protocols using the Bio-Rad (Hercules, CA) Precision Plus Protein Kaleidoscope molecular weight ladder. Gels were Coomassie Brilliant Blue G-250 stained and visualized.

***In Vitro* Complex Formation Assay**

Uncleaved LARGE and TEV protease cleaved B4GAT1 were combined at equimolar concentrations in 20 mM HEPES pH 7 with 150 mM NaCl and 10 mM imidazole. Final protein concentration was 0.5 mg/ml. After incubation at 4°C for 15 min, the mixture was applied to Novagen Ni-NTA resin, washed three times with 20 mM imidazole in PBS pH 7 and eluted with 250 mM imidazole in PBS pH 7. The input and collected fractions were analyzed by SDS-PAGE and silver staining [59].

Enzyme Kinetics

Time course experiments were carried out to ensure that the initial rate condition required for Michaelis-Menten kinetic analysis was met. A 2 hr incubation at 37°C was found to be acceptable for B4GAT1 whereas at 16 hr incubation period was judged suitable for LARGE. We compared the activity of B4GAT1 to that of LARGE while varying monosaccharide acceptor concentration. Concentrations ranging from 200 nM to 2 mM were tested to generate typical Michaelis-Menten curves for each enzyme against the substrates Xyl-α-pNP and Xyl-β-pNP. UDP-GlcA was supplied at 2 mM in all reactions. Reactions were terminated by boiling and processed as described above with quantification by HPLC. A Lineweaver-Burk plot was

generated in Microsoft Excel and kinetic parameters determined from it. We were unable to detect the presence of product for LARGE at 2 mM donor and acceptor after 16 hr with Xyl-β-pNP.

NMR

Samples of the products from action of LARGE and B4GAT1 with UDP-GlcA on Xyl-α-pNP were purified as described above, and, for each, ~1 mg was dissolved in 0.5 ml D_2O. NMR spectra of each of these samples were obtained on a VNMRS 600 MHz spectrometer with a triple resonance HCN cold probe. 2-Dimensional ^{1}H double quantum COSY, and TOCSY, and ^{1}H-^{13}C HSQC and HMBC spectra [42] were recorded using standard pulse sequences in the Agilent VnmrJ 4.3 software and processed in that software. The ^{1}H and ^{13}C peak assignments were made based on analysis of these data using the single and multiple bond connectivities these experiments reveal. The glycosidic linkages were established based on ^{1}H,^{13}C HMBC correlations in both directions across the glycosidic linkages (Figure 3.7). Anomeric stereochemistry was determined from the 1-bond C-H coupling of the anomeric sites where a value of less than 170 Hz indicates a β-linkage and one of more than 170 Hz indicates an α-linkage [41]. These were further confirmed from the $^{3}J_{H1,H2}$ couplings of the respective sugar residues. ^{1}H chemical shifts were calibrated relative to the HDO signal at 4.77 ppm at 25°C and the ^{13}C shifts were then determined by the indirect referencing approach from the proton reference frequency [43].

LARGE synthesized polymer Analysis by Mass Spectrometry

Following a protocol developed to significantly enhance spectra acquired for highly acidic carbohydrates [60], Nafion® 117 solution was applied to a Bruker MSP 96 ground steel target and allowed to dry. Samples of polymerization reaction mixtures were mixed 1:1 with 2,5-DHB resuspended in 50% acetonitrile at 20 mg/ml and spotted on the dried Nafion® 117 membrane.

Matrix-assisted laser desorption ionization with time-of-flight detection mass spectrometry spectra were acquired using a Bruker Microflex.

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References

1. Varki A. Evolutionary forces shaping the Golgi glycosylation machinery: why cell surface glycans are universal to living cells. Cold Spring Harbor perspectives in biology. 2011;3(6). Epub 2011/04/29. doi: 10.1101/cshperspect.a005462. PubMed PMID: 21525513; PMCID: 3098673.

2. Moremen KW, Tiemeyer M, Nairn AV. Vertebrate protein glycosylation: diversity, synthesis and function. Nature reviews Molecular cell biology. 2012;13(7):448-62. Epub 2012/06/23. doi: 10.1038/nrm3383. PubMed PMID: 22722607; PMCID: 3934011.

3. Barresi R, Campbell KP. Dystroglycan: from biosynthesis to pathogenesis of human disease. Journal of cell science. 2006;119(Pt 2):199-207. doi: 10.1242/jcs.02814. PubMed PMID: 16410545.

4. Freeze HH. Congenital Disorders of Glycosylation: CDG-I, CDG-II, and beyond. Current molecular medicine. 2007;7(4):389-96. Epub 2007/06/23. PubMed PMID: 17584079.

5. Praissman JL, Wells L. Mammalian O-mannosylation pathway: glycan structures, enzymes, and protein substrates. Biochemistry. 2014;53(19):3066-78. doi: 10.1021/bi500153y. PubMed PMID: 24786756; PMCID: 4033628.

6. Wells L. The o-mannosylation pathway: glycosyltransferases and proteins implicated in congenital muscular dystrophy. The Journal of biological chemistry. 2013;288(10):6930-5. Epub 2013/01/19. doi: 10.1074/jbc.R112.438978. PubMed PMID: 23329833; PMCID: 3591603.

7. Muntoni F, Torelli S, Wells DJ, Brown SC. Muscular dystrophies due to glycosylation defects: diagnosis and therapeutic strategies. Current opinion in neurology. 2011;24(5):437-42. Epub 2011/08/10. doi: 10.1097/WCO.0b013e32834a95e3. PubMed PMID: 21825985.

8. Mercuri E, Muntoni F. The ever-expanding spectrum of congenital muscular dystrophies. Annals of neurology. 2012;72(1):9-17. Epub 2012/07/26. doi: 10.1002/ana.23548. PubMed PMID: 22829265.

9. Dobson CM, Hempel SJ, Stalnaker SH, Stuart R, Wells L. O-Mannosylation and human disease. Cellular and molecular life sciences : CMLS. 2013;70(16):2849-57. Epub 2012/11/02. doi: 10.1007/s00018-012-1193-0. PubMed PMID: 23115008; PMCID: 3984002.

10. Vester-Christensen MB, Halim A, Joshi HJ, Steentoft C, Bennett EP, Levery SB, Vakhrushev SY, Clausen H. Mining the O-mannose glycoproteome reveals cadherins as major O-mannosylated glycoproteins. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(52):21018-23. Epub 2013/10/09. doi: 10.1073/pnas.1313446110. PubMed PMID: 24101494; PMCID: 3876253.

11. Lommel M, Winterhalter PR, Willer T, Dahlhoff M, Schneider MR, Bartels MF, Renner-Muller I, Ruppert T, Wolf E, Strahl S. Protein O-mannosylation is crucial for E-cadherin-mediated cell adhesion. Proceedings of the National Academy of Sciences of the United States of America.

2013;110(52):21024-9. Epub 2013/12/04. doi: 10.1073/pnas.1316753110. PubMed PMID: 24297939; PMCID: 3876218.

12. Winterhalter PR, Lommel M, Ruppert T, Strahl S. O-glycosylation of the non-canonical T-cadherin from rabbit skeletal muscle by single mannose residues. FEBS letters. 2013;587(22):3715-21. Epub 2013/10/12. doi: 10.1016/j.febslet.2013.09.041. PubMed PMID: 24113656.

13. Endo T, Manya H. O-mannosylation in mammalian cells. Methods Mol Biol. 2006;347:43-56. Epub 2006/10/31. doi: 10.1385/1-59745-167-3:43. PubMed PMID: 17072003.

14. Ervasti JM, Campbell KP. A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin. The Journal of cell biology. 1993;122(4):809-23. Epub 1993/08/01. PubMed PMID: 8349731; PMCID: 2119587.

15. Stalnaker SH, Hashmi S, Lim JM, Aoki K, Porterfield M, Gutierrez-Sanchez G, Wheeler J, Ervasti JM, Bergmann C, Tiemeyer M, Wells L. Site mapping and characterization of O-glycan structures on alpha-dystroglycan isolated from rabbit skeletal muscle. The Journal of biological chemistry. 2010;285(32):24882-91. doi: 10.1074/jbc.M110.126474. PubMed PMID: 20507986; PMCID: 2915724.

16. Yoshida-Moriguchi T, Yu L, Stalnaker SH, Davis S, Kunz S, Madson M, Oldstone MB, Schachter H, Wells L, Campbell KP. O-mannosyl phosphorylation of alpha-dystroglycan is required for laminin binding. Science. 2010;327(5961):88-92. doi: 10.1126/science.1180512. PubMed PMID: 20044576; PMCID: 2978000.

17. Hara Y, Kanagawa M, Kunz S, Yoshida-Moriguchi T, Satz JS, Kobayashi YM, Zhu Z, Burden SJ, Oldstone MB, Campbell KP. Like-acetylglucosaminyltransferase (LARGE)-dependent modification of dystroglycan at Thr-317/319 is required for laminin binding and arenavirus infection. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(42):17426-31. doi: 10.1073/pnas.1114836108. PubMed PMID: 21987822; PMCID: 3198351.

18. Stalnaker SH, Aoki K, Lim JM, Porterfield M, Liu M, Satz JS, Buskirk S, Xiong Y, Zhang P, Campbell KP, Hu H, Live D, Tiemeyer M, Wells L. Glycomic analyses of mouse models of congenital muscular dystrophy. The Journal of biological chemistry. 2011;286(24):21180-90. doi: 10.1074/jbc.M110.203281. PubMed PMID: 21460210; PMCID: 3122180.

19. Stalnaker SH, Stuart R, Wells L. Mammalian O-mannosylation: unsolved questions of structure/function. Current opinion in structural biology. 2011;21(5):603-9. Epub 2011/09/29. doi: 10.1016/j.sbi.2011.09.001. PubMed PMID: 21945038; PMCID: 3356693.

20. Harrison R, Hitchen PG, Panico M, Morris HR, Mekhaiel D, Pleass RJ, Dell A, Hewitt JE, Haslam SM. Glycoproteomic characterization of recombinant mouse alpha-dystroglycan. Glycobiology. 2012;22(5):662-75. doi: 10.1093/glycob/cws002. PubMed PMID: 22241827; PMCID: 3311285.

21. Inamori K, Yoshida-Moriguchi T, Hara Y, Anderson ME, Yu L, Campbell KP. Dystroglycan function requires xylosyl- and glucuronyltransferase activities of LARGE. Science. 2012;335(6064):93-6. doi: 10.1126/science.1214115. PubMed PMID: 22223806; PMCID: 3702376.

22. Inamori K, Hara Y, Willer T, Anderson ME, Zhu Z, Yoshida-Moriguchi T, Campbell KP. Xylosyl- and glucuronyltransferase functions of LARGE in alpha-dystroglycan modification are conserved in LARGE2. Glycobiology. 2013;23(3):295-302. doi: 10.1093/glycob/cws152. PubMed PMID: 23125099; PMCID: 3555503.

23. Live D, Wells L, Boons GJ. Dissecting the molecular basis of the role of the O-mannosylation pathway in disease: alpha-dystroglycan and forms of muscular dystrophy. Chembiochem : a European journal of chemical biology. 2013;14(18):2392-402. Epub 2013/12/10. doi: 10.1002/cbic.201300417. PubMed PMID: 24318691; PMCID: 3938021.

24. Panin VM, Wells L. Protein O-mannosylation in metazoan organisms. Current protocols in protein science / editorial board, John E Coligan [et al]. 2014;75:Unit 12 Epub 2014/02/11. doi: 10.1002/0471140864.ps1212s75. PubMed PMID: 24510673; PMCID: 3984005.

25. Buysse K, Riemersma M, Powell G, van Reeuwijk J, Chitayat D, Roscioli T, Kamsteeg EJ, van den Elzen C, van Beusekom E, Blaser S, Babul-Hirji R, Halliday W, Wright GJ, Stemple DL, Lin YY, Lefeber DJ, van Bokhoven H. Missense mutations in beta-1,3-N-acetylglucosaminyltransferase 1 (B3GNT1) cause Walker-Warburg syndrome. Human molecular genetics. 2013;22(9):1746-54. Epub 2013/01/30. doi: 10.1093/hmg/ddt021. PubMed PMID: 23359570; PMCID: 3613162.

26. Shaheen R, Faqeih E, Ansari S, Alkuraya FS. A truncating mutation in B3GNT1 causes severe Walker-Warburg syndrome. Neurogenetics. 2013;14(3-4):243-5. Epub 2013/07/24. doi: 10.1007/s10048-013-0367-8. PubMed PMID: 23877401.

27. Czeschik JC, Hehr U, Hartmann B, Ludecke HJ, Rosenbaum T, Schweiger B, Wieczorek D. 160 kb deletion in ISPD unmasking a recessive mutation in a patient with Walker-Warburg syndrome. European journal of medical genetics. 2013;56(12):689-94. Epub 2013/10/15. doi: 10.1016/j.ejmg.2013.09.014. PubMed PMID: 24120487.

28. Jae LT, Raaben M, Riemersma M, van Beusekom E, Blomen VA, Velds A, Kerkhoven RM, Carette JE, Topaloglu H, Meinecke P, Wessels MW, Lefeber DJ, Whelan SP, van Bokhoven H, Brummelkamp TR. Deciphering the glycosylome of dystroglycanopathies using haploid screens for lassa virus entry. Science. 2013;340(6131):479-83. doi: 10.1126/science.1233675. PubMed PMID: 23519211.

29. Sasaki K, Kurata-Miura K, Ujita M, Angata K, Nakagawa S, Sekine S, Nishi T, Fukuda M. Expression cloning of cDNA encoding a human beta-1,3-N-acetylglucosaminyltransferase that is essential for poly-N-acetyllactosamine synthesis. Proceedings of the National Academy of Sciences of the United States of America. 1997;94(26):14294-9. Epub 1998/02/07. PubMed PMID: 9405606; PMCID: 24948.

30. Ujita M, Misra AK, McAuliffe J, Hindsgaul O, Fukuda M. Poly-N-acetyllactosamine extension in N-glycans and core 2- and core 4-branched O-glycans is differentially controlled by i-extension enzyme and different members of the beta 1,4-galactosyltransferase gene family. The Journal of biological chemistry. 2000;275(21):15868-75. Epub 2000/04/05. doi: 10.1074/jbc.M001034200. PubMed PMID: 10747980.

31. Zhou D. Why are glycoproteins modified by poly-N-acetyllactosamine glyco-conjugates? Current protein & peptide science. 2003;4(1):1-9. Epub 2003/02/07. PubMed PMID: 12570780.

32. Ho WL, Che MI, Chou CH, Chang HH, Jeng YM, Hsu WM, Lin KH, Huang MC. B3GNT3 expression suppresses cell migration and invasion and predicts favorable outcomes in neuroblastoma. Cancer science. 2013;104(12):1600-8. Epub 2013/10/15. doi: 10.1111/cas.12294. PubMed PMID: 24118321.

33. Lu CH, Wu WY, Lai YJ, Yang CM, Yu LC. Suppression of B3GNT7 gene expression in colon adenocarcinoma and its potential effect in the metastasis of colon cancer cells. Glycobiology. 2014;24(4):359-67. Epub 2014/01/15. doi: 10.1093/glycob/cwu002. PubMed PMID: 24418929.

34. Shiraishi N, Natsume A, Togayachi A, Endo T, Akashima T, Yamada Y, Imai N, Nakagawa S, Koizumi S, Sekine S, Narimatsu H, Sasaki K. Identification and characterization of three novel beta 1,3-N-acetylglucosaminyltransferases structurally related to the beta 1,3-galactosyltransferase family. The Journal of biological chemistry. 2001;276(5):3498-507. Epub 2000/10/24. doi: 10.1074/jbc.M004800200. PubMed PMID: 11042166.

35. Zhou D, Dinter A, Gutierrez Gallego R, Kamerling JP, Vliegenthart JF, Berger EG, Hennet T. A beta-1,3-N-acetylglucosaminyltransferase with poly-N-acetyllactosamine synthase activity is structurally related to beta-1,3-galactosyltransferases. Proceedings of the National Academy of Sciences of the United States of America. 1999;96(2):406-11. Epub 1999/01/20. PubMed PMID: 9892646; PMCID: 15149.

36. Biellmann F, Henion TR, Burki K, Hennet T. Impaired sexual behavior in male mice deficient for the beta1-3 N-acetylglucosaminyltransferase-I gene. Molecular reproduction and development. 2008;75(5):699-706. Epub 2007/11/17. doi: 10.1002/mrd.20828. PubMed PMID: 18008318.

37. Togayachi A, Sato T, Narimatsu H. Comprehensive enzymatic characterization of glycosyltransferases with a beta3GT or beta4GT motif. Methods in enzymology. 2006;416:91-102. Epub 2006/11/23. doi: 10.1016/S0076-6879(06)16006-1. PubMed PMID: 17113861.

38. Goddeeris MM, Wu B, Venzke D, Yoshida-Moriguchi T, Saito F, Matsumura K, Moore SA, Campbell KP. LARGE glycans on dystroglycan function as a tunable matrix scaffold to prevent dystrophy. Nature. 2013;503(7474):136-40. Epub 2013/10/18. doi: 10.1038/nature12605. PubMed PMID: 24132234; PMCID: 3891507.

39. Bao X, Kobayashi M, Hatakeyama S, Angata K, Gullberg D, Nakayama J, Fukuda MN, Fukuda M. Tumor suppressor function of laminin-binding alpha-dystroglycan requires a distinct beta3-N-acetylglucosaminyltransferase. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(29):12109-14. PubMed PMID: 19587235.

40. Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW, Etzler ME. Essentials of Glycobiology. 2nd Edition ed. New York: Cold Spring Harbor Laboratory Press; 2009 2009.

41. Bock K, Pedersen C. Study of C-13H Coupling-Constants in Hexopyranoses. J Chem Soc-Perkin Transactions 2. 1974;3:293-9.

42. Van de Ven FJM. Multidimensional NMR in Liquids. New York: Wiley-VCH; 1995 1995.

43. Wishart DS, Bigam CG, Yao J, Abildgaard F, Dyson HJ, Oldfield E, Markley JL, Sykes BD. 1H, 13C and 15N chemical shift referencing in biomolecular NMR. Journal of biomolecular NMR. 1995;6(2):135-40. Epub 1995/09/01. PubMed PMID: 8589602.

44. Yoshida-Moriguchi T, Willer T, Anderson ME, Venzke D, Whyte T, Muntoni F, Lee H, Nelson SF, Yu L, Campbell KP. SGK196 is a glycosylation-specific O-mannose kinase required for dystroglycan function. Science. 2013;341(6148):896-9. doi: 10.1126/science.1239951. PubMed PMID: 23929950.

45. de Bernabe DB, Inamori K, Yoshida-Moriguchi T, Weydert CJ, Harper HA, Willer T, Henry MD, Campbell KP. Loss of alpha-dystroglycan laminin binding in epithelium-derived cancers is caused by silencing of LARGE. The Journal of biological chemistry. 2009;284(17):11279-84. doi: 10.1074/jbc.C900007200. PubMed PMID: 19244252; PMCID: 2670132.

46. Lee PL, Kohler JJ, Pfeffer SR. Association of beta-1,3-N-acetylglucosaminyltransferase 1 and beta-1,4-galactosyltransferase 1, trans-Golgi enzymes involved in coupled poly-N-acetyllactosamine synthesis. Glycobiology. 2009;19(6):655-64. Epub 2009/03/06. doi: 10.1093/glycob/cwp035. PubMed PMID: 19261593; PMCID: 2682609.

47. Wright KM, Lyon KA, Leung H, Leahy DJ, Ma L, Ginty DD. Dystroglycan organizes axon guidance cue localization and axonal pathfinding. Neuron. 2012;76(5):931-44. doi: 10.1016/j.neuron.2012.10.009. PubMed PMID: 23217742; PMCID: 3526105.

48. Yoneyama T, Angata K, Bao X, Courtneidge S, Chanda SK, Fukuda M. Fer kinase regulates cell migration through alpha-dystroglycan glycosylation. Molecular biology of the cell. 2012;23(5):771-80. Epub 2012/01/13. doi: 10.1091/mbc.E11-06-0517. PubMed PMID: 22238358; PMCID: 3290637.

49. Morita I, Kizuka Y, Kakuda S, Oka S. Expression and function of the HNK-1 carbohydrate. Journal of biochemistry. 2008;143(6):719-24. Epub 2007/11/21. doi: 10.1093/jb/mvm221. PubMed PMID: 18024472.

50. Zak BM, Crawford BE, Esko JD. Hereditary multiple exostoses and heparan sulfate polymerization. Biochimica et biophysica acta. 2002;1573(3):346-55. Epub 2002/11/06. PubMed PMID: 12417417.

51. Kim BT, Kitagawa H, Tamura J, Saito T, Kusche-Gullberg M, Lindahl U, Sugahara K. Human tumor suppressor EXT gene family members EXTL1 and EXTL3 encode alpha 1,4- N-

acetylglucosaminyltransferases that likely are involved in heparan sulfate/ heparin biosynthesis. Proceedings of the National Academy of Sciences of the United States of America. 2001;98(13):7176-81. Epub 2001/06/08. doi: 10.1073/pnas.131188498. PubMed PMID: 11390981; PMCID: 34642.

52. Kitagawa H, Shimakawa H, Sugahara K. The tumor suppressor EXT-like gene EXTL2 encodes an alpha1, 4-N-acetylhexosaminyltransferase that transfers N-acetylgalactosamine and N-acetylglucosamine to the common glycosaminoglycan-protein linkage region. The key enzyme for the chain initiation of heparan sulfate. The Journal of biological chemistry. 1999;274(20):13933-7. Epub 1999/05/13. PubMed PMID: 10318803.

53. Meng L, Forouhar F, Thieker D, Gao Z, Ramiah A, Moniz H, Xiang Y, Seetharaman J, Milaninia S, Su M, Bridger R, Veillon L, Azadi P, Kornhaber G, Wells L, Montelione GT, Woods RJ, Tong L, Moremen KW. Enzymatic basis for N-glycan sialylation: structure of rat alpha2,6-sialyltransferase (ST6GAL1) reveals conserved and unique features for glycan sialylation. The Journal of biological chemistry. 2013;288(48):34680-98. Epub 2013/10/25. doi: 10.1074/jbc.M113.519041. PubMed PMID: 24155237; PMCID: 3843080.

54. Phan J, Zdanov A, Evdokimov AG, Tropea JE, Peters HK, 3rd, Kapust RB, Li M, Wlodawer A, Waugh DS. Structural basis for the substrate specificity of tobacco etch virus protease. The Journal of biological chemistry. 2002;277(52):50564-72. Epub 2002/10/16. doi: 10.1074/jbc.M207224200. PubMed PMID: 12377789.

55. Barb AW, Meng L, Gao Z, Johnson RW, Moremen KW, Prestegard JH. NMR characterization of immunoglobulin G Fc glycan motion on enzymatic sialylation. Biochemistry. 2012;51(22):4618-26. Epub 2012/05/12. doi: 10.1021/bi300319q. PubMed PMID: 22574931; PMCID: 3447994.

56. Vandersall-Nairn AS, Merkle RK, O'Brien K, Oeltmann TN, Moremen KW. Cloning, expression, purification, and characterization of the acid alpha-mannosidase from Trypanosoma cruzi. Glycobiology. 1998;8(12):1183-94. Epub 1998/12/22. PubMed PMID: 9858640.

57. Beckett D, Kovaleva E, Schatz PJ. A minimal peptide substrate in biotin holoenzyme synthetase-catalyzed biotinylation. Protein science : a publication of the Protein Society. 1999;8(4):921-9. Epub 1999/04/22. doi: 10.1110/ps.8.4.921. PubMed PMID: 10211839; PMCID: 2144313.

58. Pedelacq JD, Cabantous S, Tran T, Terwilliger TC, Waldo GS. Engineering and characterization of a superfolder green fluorescent protein. Nature biotechnology. 2006;24(1):79-88. Epub 2005/12/22. doi: 10.1038/nbt1172. PubMed PMID: 16369541.

59. Porterfield M, Zhao P, Han H, Cunningham J, Aoki K, Von Hoff DD, Demeure MJ, Pierce JM, Tiemeyer M, Wells L. Discrimination between Adenocarcinoma and normal pancreatic ductal fluid by proteomic and glycomic analysis. Journal of proteome research. 2014;13(2):395-407. Epub 2013/12/18. doi: 10.1021/pr400422g. PubMed PMID: 24328148; PMCID: 3946306.

60. Jacobs A, Dahlman O. Enhancement of the quality of MALDI mass spectra of highly acidic oligosaccharides by using a nafion-coated probe. Anal Chem. 2001;73(3):405-10. PubMed PMID: 11217739.

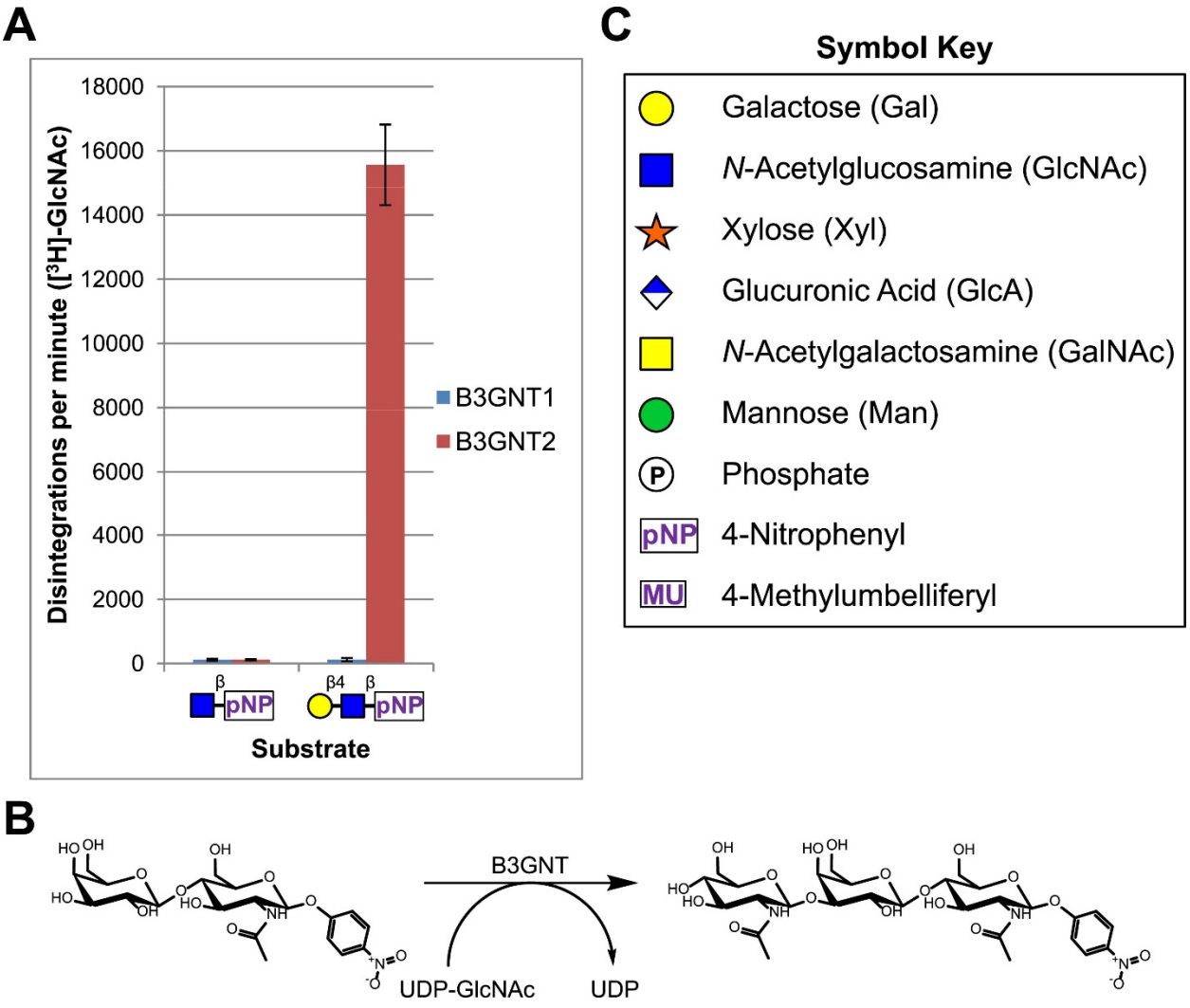

Figure 3.1. B3GNT1 does not possess β-1,3-N-acetylglucosaminyltransferase activity.

(A) The β-1,3-N-acetylglucosaminyltransferase activity of B3GNT1 towards pNP tagged N-acetyllactosamine was compared to that of B3GNT2 (reaction scheme presented in (B)). Incubations were carried out overnight at 37°C in 0.1 M MES pH 6.5 containing 10 mM $MnCl_2$, 40,000 DPM UDP-[^{3}H]GlcNAc and 2 mM non-radioactive UDP-GlcNAc. pNP-sugars were isolated from sugar nucleotide by reverse-phase C18 spin columns. UDP-[^{3}H]GlcNAc transfer to Gal-β4-GlcNAc-β-pNP was measured by liquid scintillation counting. Averaged results from three independent experiments with error bars indicating standard deviation are shown. GlcNAc-β-pNP

was used as a negative control acceptor. (C) Relevant sugar code symbols from ‘Essentials of Glycobiology’ as well as other symbols used throughout the paper are shown.

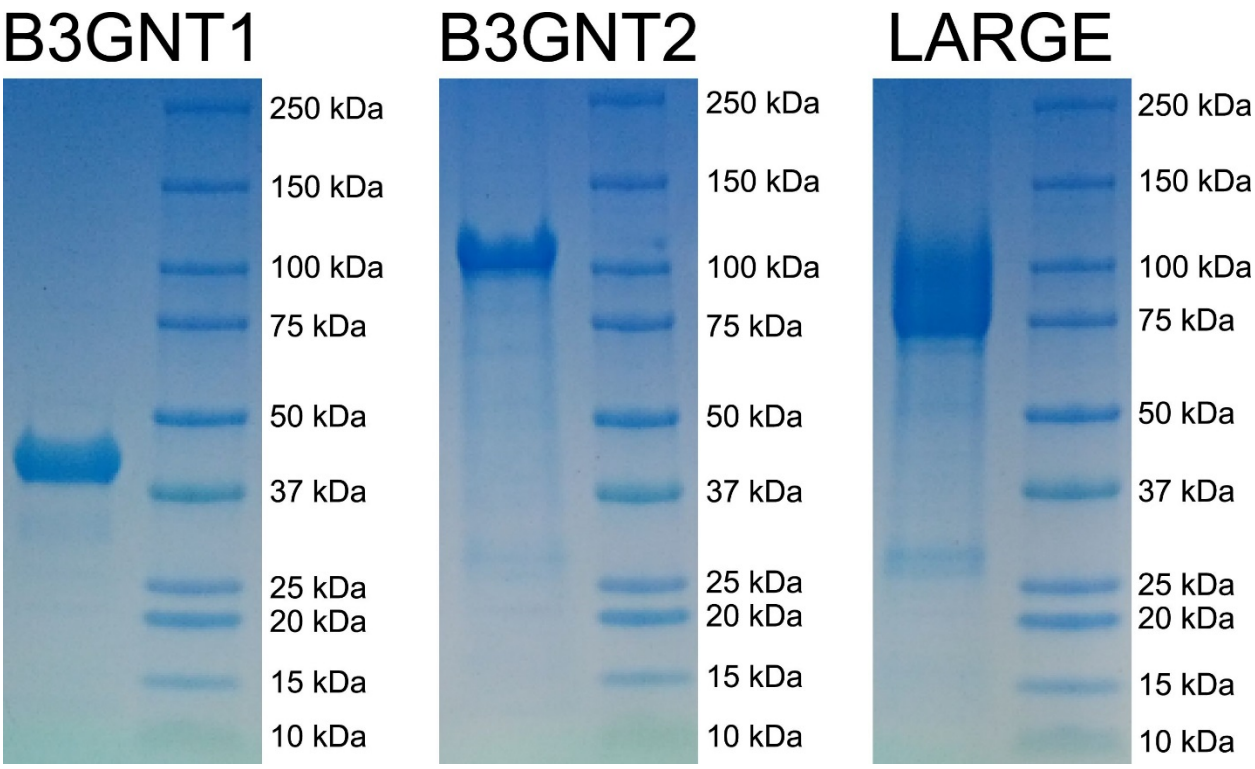

Figure 3.2. Coomassie brilliant blue G-250 stained SDS-PAGE gels of purified enzyme samples. The prominent bands appear at the appropriate molecular weights for our constructs. B3GNT1 was cleaved from tag leaving only the catalytic domain while the other enzymes were not cleaved from expression construct elements (affinity tag and GFP).

Table 3.1. Top 5 protein search results in each purified enzyme sample

LC-MS/MS was carried out on tryptic digests of purified enzyme samples. Results filtered to peptide 1% false-discovery rate are displayed from a Proteome Discoverer 1.4 Sequest HT search against the Uniprot human database along with total score and number of peptide spectral matches for each protein assignment (# PSMs).

Sample	Accession	Description	Score	# PSMs
B3GNT1	O43505	N-acetyllactosaminide beta-1,3-N-acetylglucosaminyltransferase [B3GN1_HUMAN]	17768.32	5015
	136429	TRYPSIN PRECURSOR.	187.25	60
	P04264	Keratin, type II cytoskeletal 1 [K2C1_HUMAN]	153.93	45
	Trypa5	Promega Trypsin Artifact 5	162.69	34
	547748	KERATIN, TYPE I CYTOSKELETAL 9 [gi|545257]	102.89	34
B3GNT2	Q9NY97-2	Isoform 2 of UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 2 [B3GN2_HUMAN]	6829.93	2025
	P08107	Heat shock 70 kDa protein 1A/1B [HSP71_HUMAN]	1016.32	270
	P08238	Heat shock protein HSP 90-beta [HS90B_HUMAN]	380.11	121
	P60709	Actin, cytoplasmic 1 [ACTB_HUMAN]	394.4	113
	P24821	Tenascin [TENA_HUMAN]	322.19	111
LARGE	O95461	Glycosyltransferase-like protein LARGE1 [LARGE_HUMAN]	14187.98	4658
	E9PE77	Uncharacterized protein [E9PE77_HUMAN]	888.62	236
	P08107	Heat shock 70 kDa protein 1A/1B [HSP71_HUMAN]	667.31	180
	E9PC84	Uncharacterized protein [E9PC84_HUMAN]	277.79	86
	P60709	Actin, cytoplasmic 1 [ACTB_HUMAN]	266.18	78

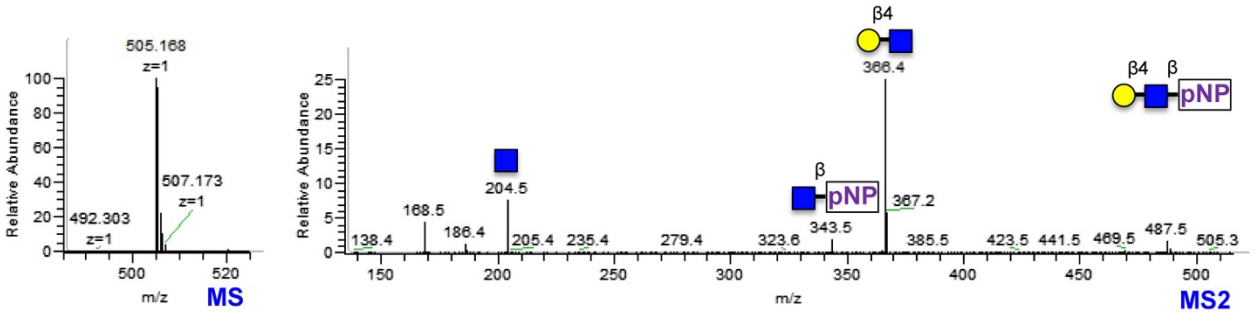

Figure 3.3: Gal-β4-GlcNAc-β-pNP synthesized using bovine B4GALT1 LC-MS and LC-MS/MS spectra of Gal-β4-GlcNAc-β-pNP produced using B4GALT1.

Low m/z fragments include diagnostic GlcNAc oxonium ions at 186 and 168.

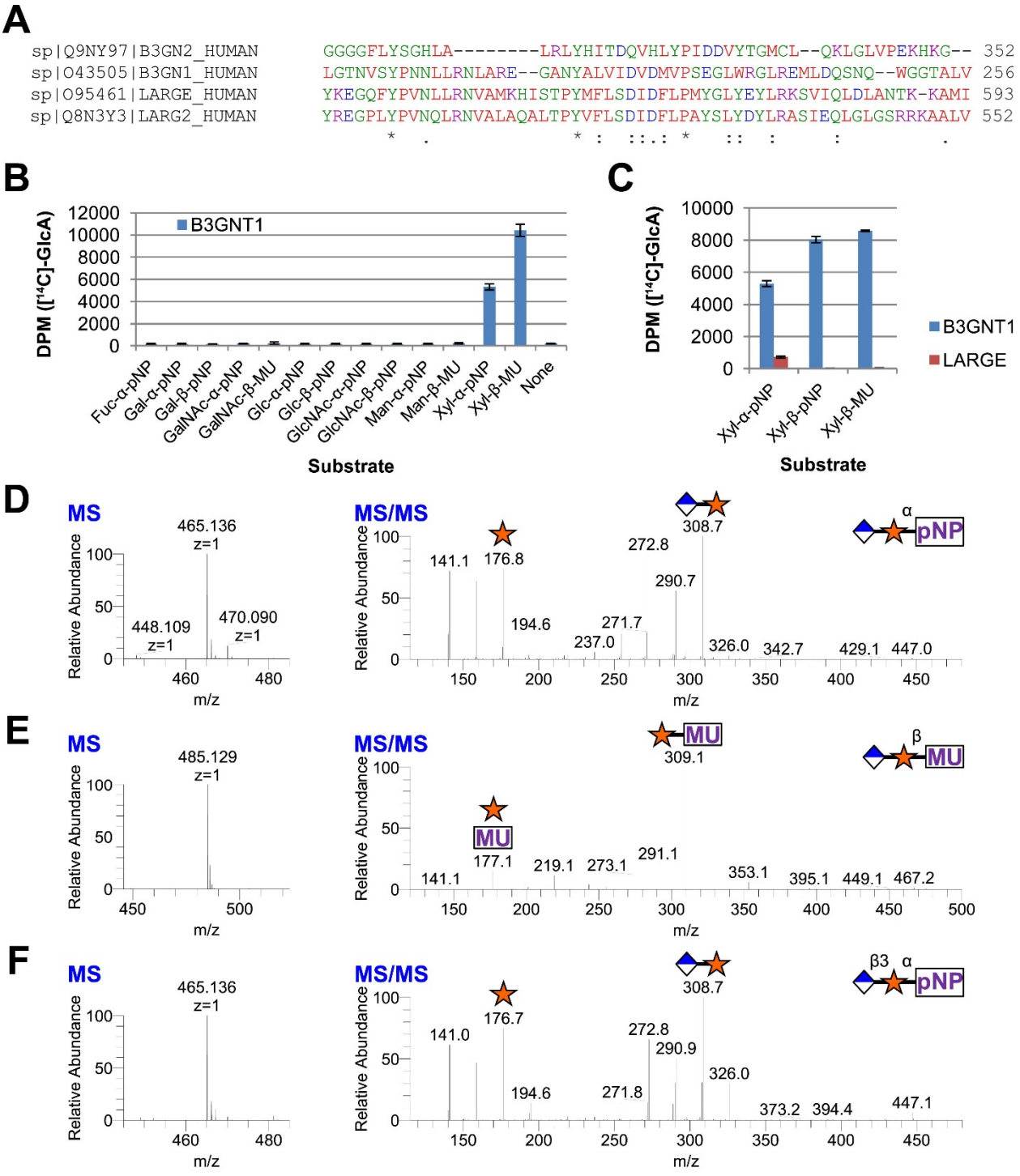

Figure 3.4. B3GNT1 is a glucuronyltransferase that uses xylose as an acceptor.

B3GNT1 is in CAZy family GT49 along with LARGE and LARGE2. (A) Clustal Omega multiple sequence alignment excerpt showing the strong alignment of B3GNT1 with the

glucuronyltransferase domain of LARGE and LARGE2, including the DXD motif shown to be important for activity. B3GNT2 does not align well with the other inputs. (B) UDP-[^{14}C]GlcA transfer screen assayed by liquid scintillation counting (disintegrations per minute–DPM), averaged results of three independent experiments with error bars indicating standard deviation. B3GNT1 transfers to xylose in both anomeric configurations. (C) LARGE transfers only to α-linked xylose as previously reported. (D) LC-MS and LC-MS/MS data showing B3GNT1 transfer to Xyl-α-pNP. The ammonium adduct is the dominant species (465.136) however the protonated species is observable at 448.109. The majority of unlabeled fragment peaks represent losses of water from labeled peaks. (E) LC-MS and LC-MS/MS data showing B3GNT1 transfer of UDP-GlcA to Xyl-β-MU. (F) LC-MS and LC-MS/MS data showing LARGE transfer to Xyl-α-pNP. Reactions were carried out overnight in 0.1 M MES pH 6.5 containing 10 mM $MnCl_2$, 5 mM $MgCl_2$, 2 mM substrate, 40,000 DPM UDP-[^{14}C]GlcA and 2 mM non-radioactive donor.

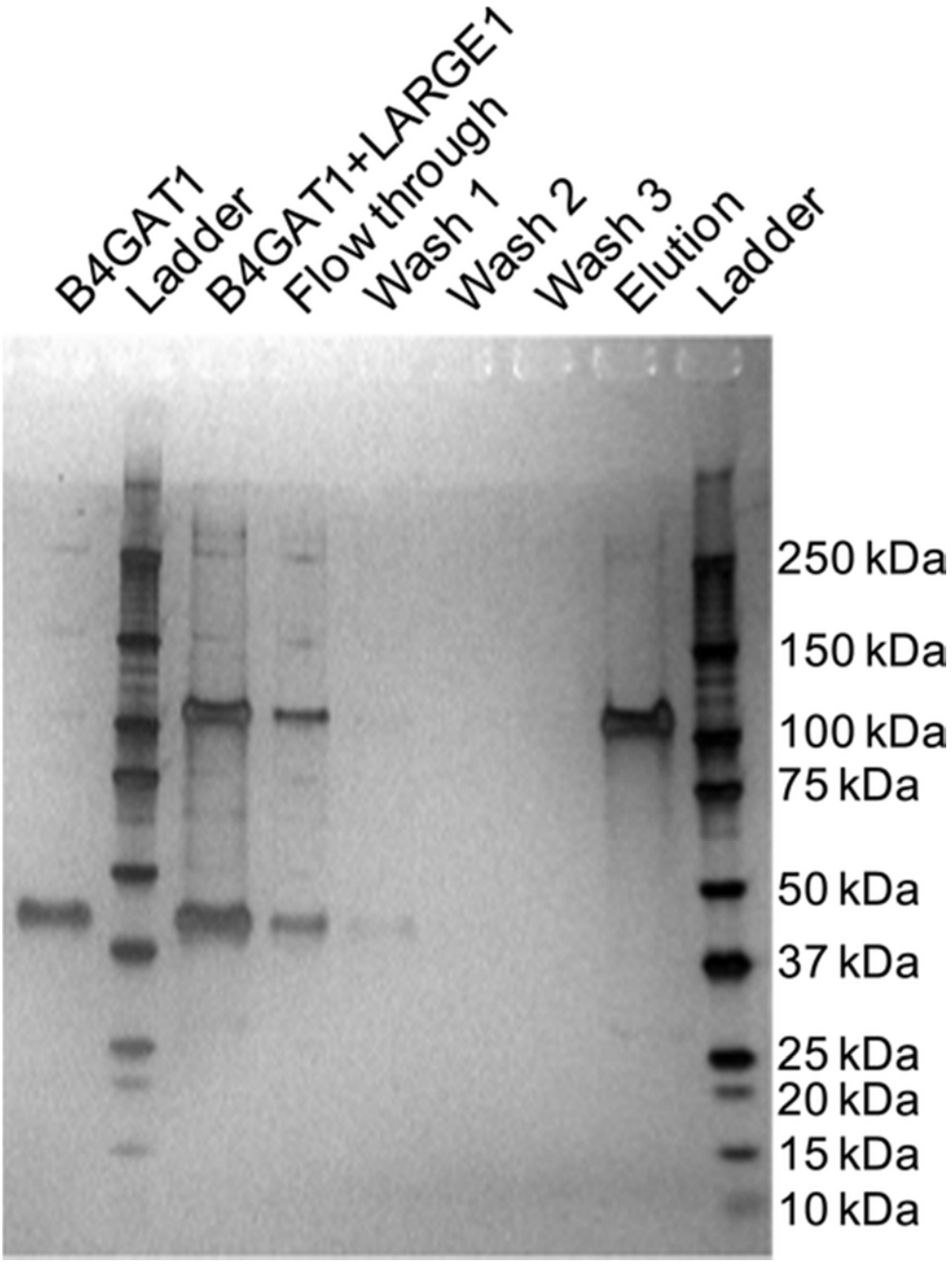

Figure 3.5. Silver stained SDS-PAGE gel of complex formation assay samples.

B4GAT1 (lacking an 8xHis tag) and LARGE1 (fused to an 8xHis tag) were mixed together and incubated for 15 min at 4°C. After incubation, the mixture was bound to Ni-NTA resin, washed three times with 20 mM imidazole containing PBS pH 7 and eluted in 250 mM imidazole in PBS

pH 7. Aliquots were run by SDS-PAGE and silver stained. Evidence for *in vitro* complex formation was not observed as is seen by the lack of a B4GAT1 band in the elution fraction.

Table 3.2: Kinetics at 2mM UDP-GlcA

NM = not measurable (below limit of detection)

Enzyme	Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	Specific Activity (pmol/min/µg)
B3GNT1					
	Xyl-α-pNP	3.0	0.087	29	130
	Xyl-β-pNP	4.0	0.25	63	380
LARGE					
	Xyl-α-pNP	6.0	0.005	0.84	2.7
	Xyl-β-pNP	NM	NM	NM	NM

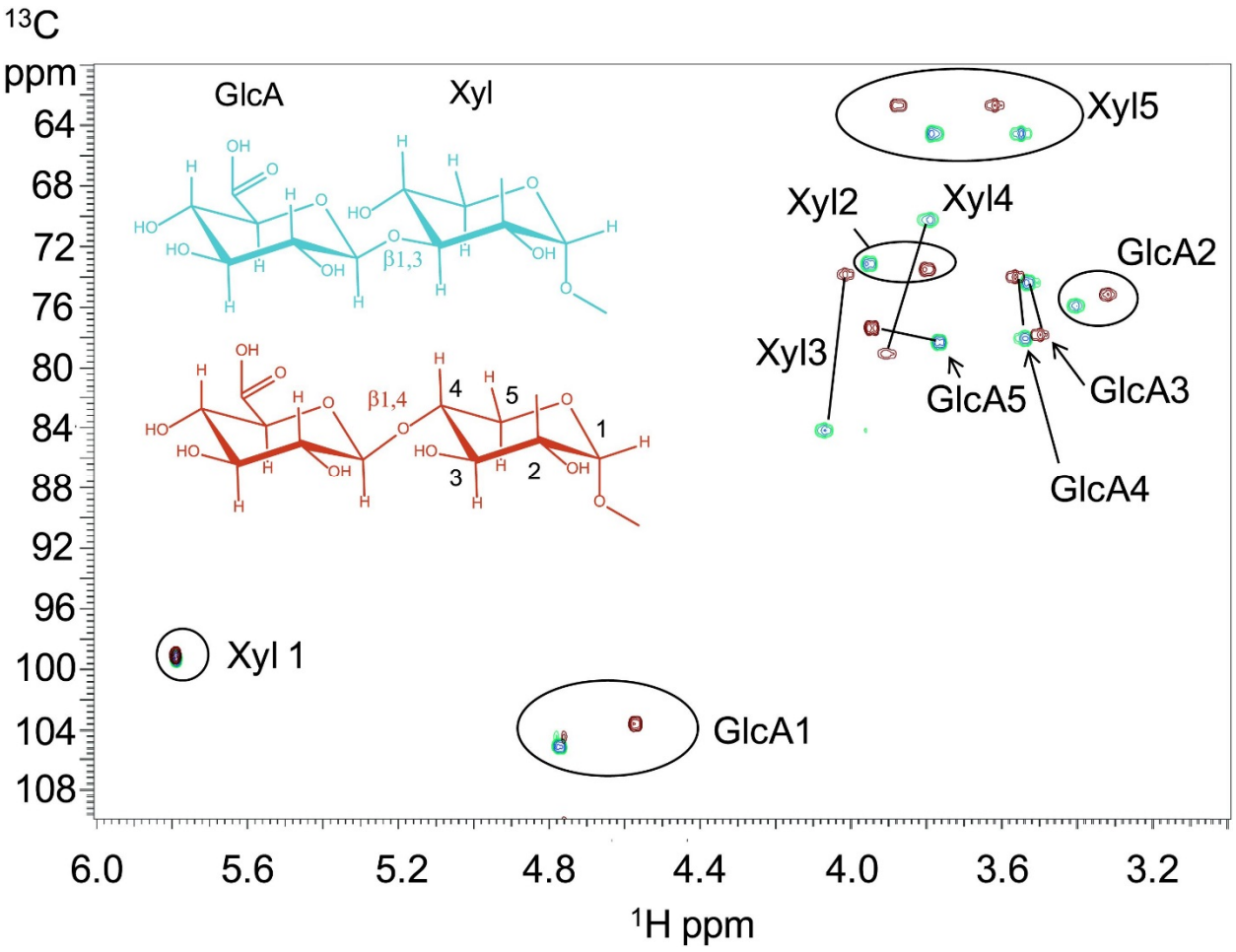

Figure 3.6. B3GNT1 is a β1,4-glucuronyltransferase in contrast to LARGE which is a β1,3-glucuronyltransferase.

^{1}H–^{13}C HSQC spectrum with peak assignments and structures of the products resulting from the action of B4GAT1 and UDP-GlcA on Xyl-α-pNP (red) and LARGE and UDP-GlcA on the same substrate (blue). Cross peaks are at the intersection of the ^{13}C and ^{1}H shifts of the partners in each C-H pair. The changes in the spectrum between the two compounds reflect the differences in the site of the glycosidic linkage formed, and particularly the major differences in the carbon shifts for the Xyl 3 and Xyl 4 sites between the two disaccharides is diagnostic of the change in their participation in the two different respective glycosidic linkages. The β configuration of the GlcA

1 site is confirmed by the 1-bond $^{1}H–^{13}C$ couplings of 166 Hz for these sites, as well as couplings between the H1 and H2 protons, 7.9–8.0 Hz. (data not shown).

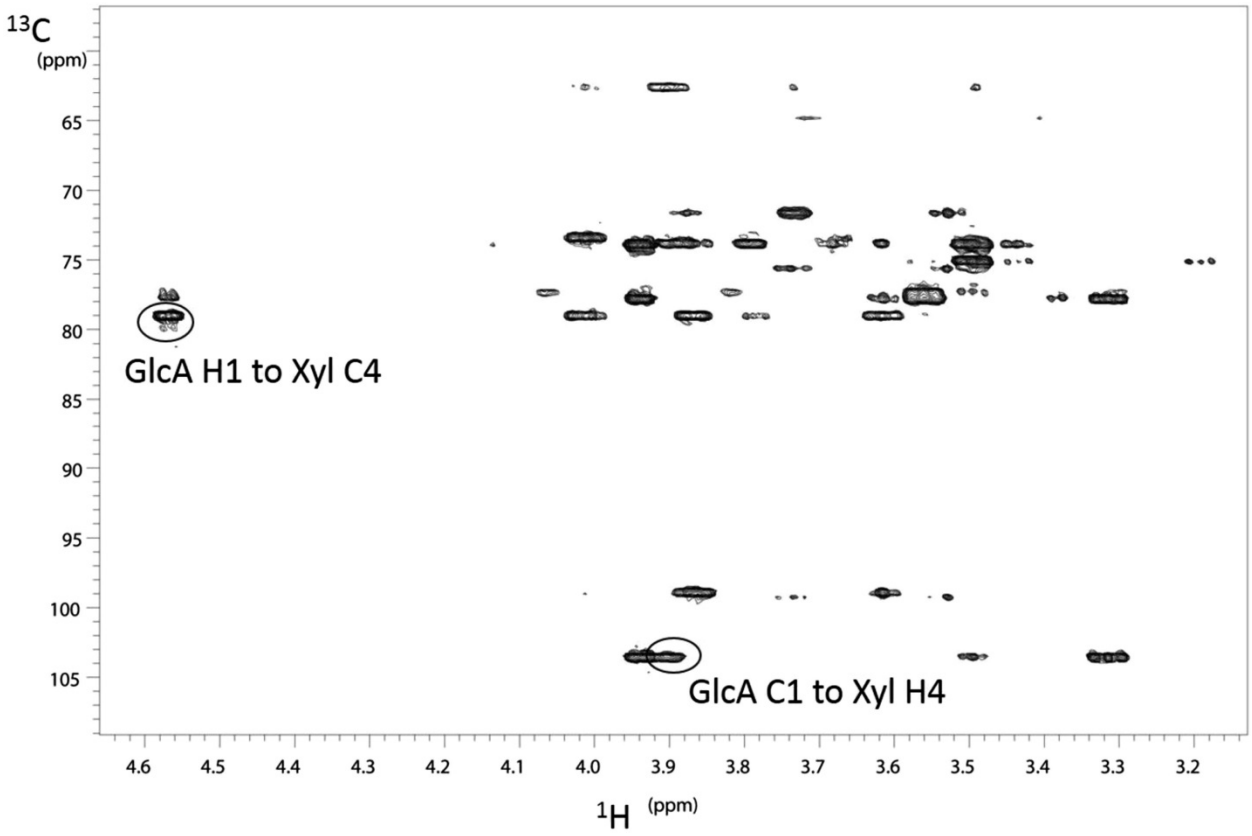

Figure 3.7. NMR determination of the 1,4 glycosidic linkage.

Section of the $^{1}H–^{13}C$ HMBC spectrum of the B4GAT1 product of GlcA addition to Xyl-α-pNP with both of the key through bond $^{1}H–^{13}C$ connections across the glycosidic linkage highlighted, unequivocally identifying the 1,4 glycosidic linkage.

Table 3.3. Chemical shifts of disaccharides. Chemical shifts of the disaccharide portion of the products of LARGE and B4GAT1 addition of GlcA to Xyl-α-pNP. Proton shifts are referenced to the HDO signal, 4.77 ppm at 25°C relative to DSS, and ^{13}C shifts relative to DSS at 0 ppm were then determined using indirect referencing to the proton standard.

	Xyl1	**Xyl2**	**Xyl3**	**Xyl4**	**Xyl5**	**GlcA1**	**GlcA2**	**GlcA3**	**GlcA4**	**GlcA5**
LARGE	**product**									
^{1}H	5.789	3.952	4.072	3.790	3.549,3.780	4.771	3.404	3.532	3.538	3.766
^{13}C	99.22	73.26	84.26	70.27	64.59	105.01	75.91	74.41	78.05	78.33
$^{1}J_{CH}$	176					163				
B4GAT1	**product**									
^{1}H	5.790	3.795	4.015	3.904	3.617,3.873	4.568	3.316	3.496	3.563	3.943
^{13}C	99.09	73.51	73.84	79.12	62.65	103.61	75.19	77.79	74.03	77.37
$^{1}J_{CH}$	176					162				

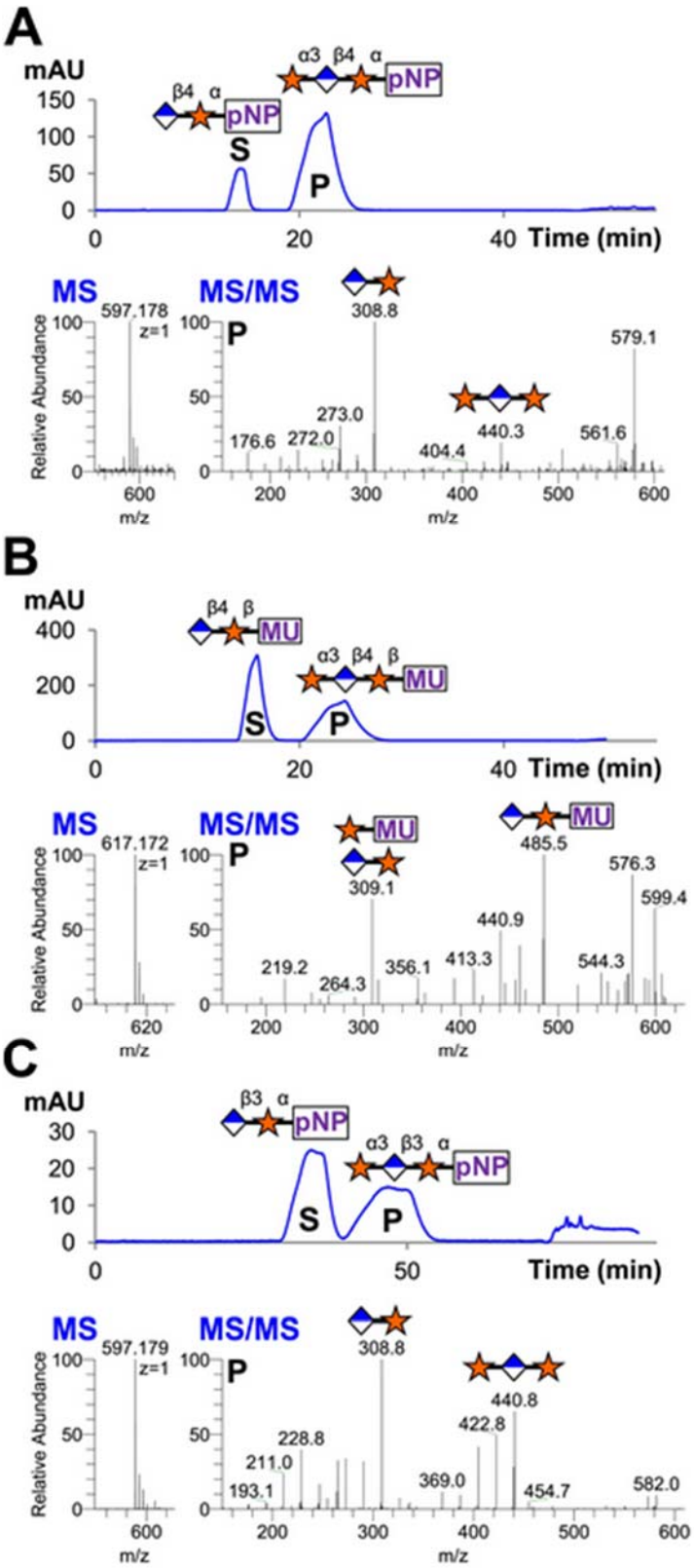
A
mAU
150
100
50
0
β4 α
pNP
α3 β4 α
pNP
S
P
0
20
40 Time (min)
MS
MS/MS
597.178
z=1
308.8
P
579.1
273.0
440.3
561.6
176.6
272.0
404.4
Relative Abundance
600
m/z
200
300
400
500
600
m/z
B
mAU
400
200
0
β4 β
MU
α3 β4 β
MU
S
P
0
20
40 Time (min)
MS
MS/MS
617.172
z=1
MU
309.1
MU
485.5
576.3
599.4
440.9
219.2
264.3
356.1
413.3
544.3
Relative Abundance
620
m/z
200
300
400
500
600
m/z
C
mAU
30
20
10
0
β3 α
pNP
α3 β3 α
pNP
S
P
0
50
Time (min)
MS
MS/MS
597.179
z=1
308.8
P
440.8
422.8
228.8
211.0
193.1
369.0
454.7
582.0
Relative Abundance
600
m/z
200
300
400
500
600
m/z

Figure 3.8. B4GAT1 disaccharide products are substrates for the xylosyltransferase activity of LARGE.

S indicates substrate peaks, P indicates product peaks (xylose added). Separation was carried out by isocratic C18 reverse-phase HPLC with absorbance monitoring at 310 nm. Products were confirmed by accurate mass and MS/MS fragmentation shown below with assignment of key characteristic peaks. (A) P = Xyl-α3-GlcA-β4-Xyl-α-pNP. (B) P = Xyl-α3-GlcA-β4-Xyl-β-MU. (C) The product of the sequential dual enzymatic activity of LARGE as a positive control; P = Xyl-α3-GlcA-β3-Xyl-α-pNP. Reactions were carried out overnight in 0.1 M MES pH 6.5 containing 10 mM $MnCl_2$, 5 mM $MgCl_2$, 2 mM substrate and 2 mM UDP-Xyl.

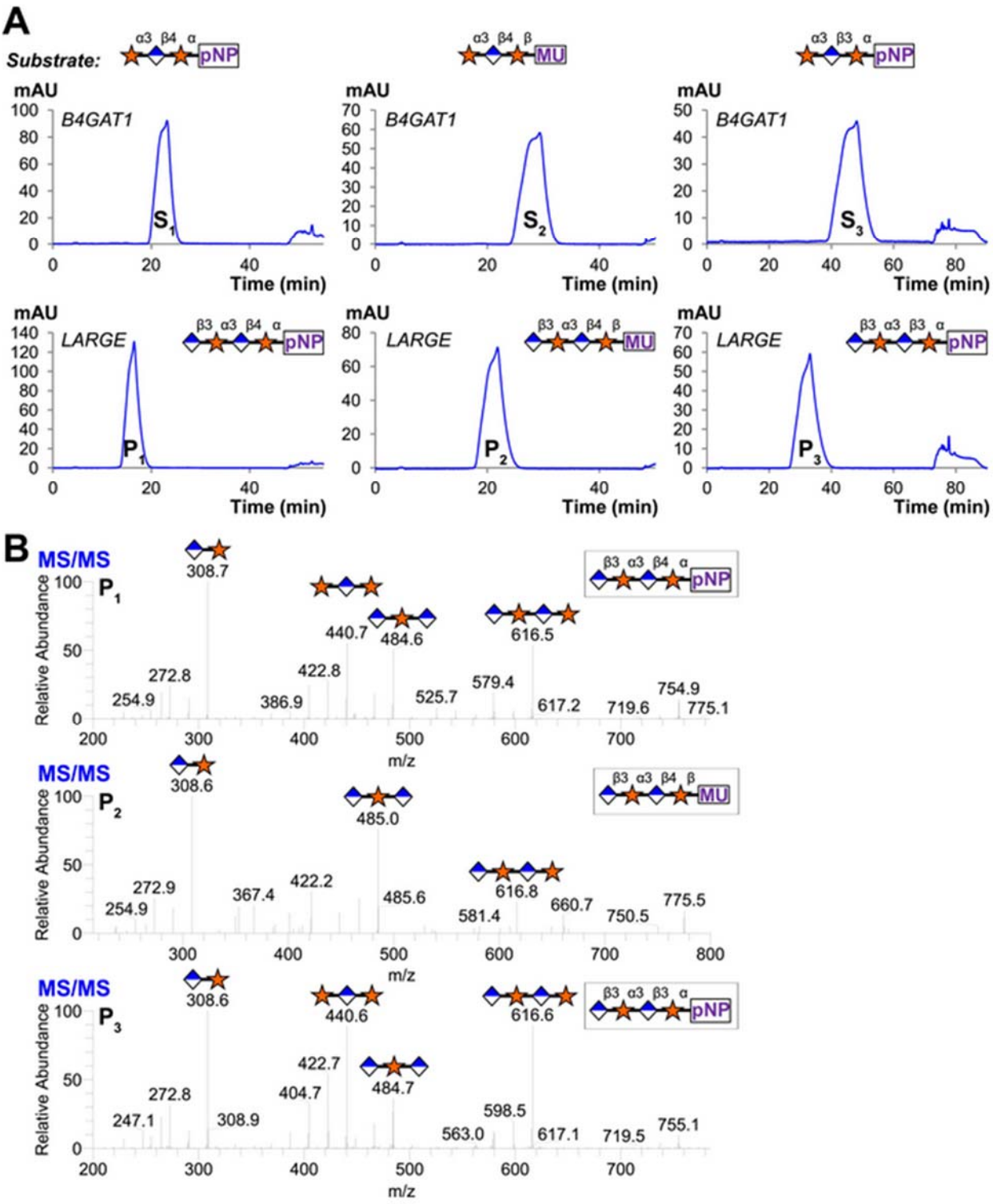

Figure 3.9. Trisaccharides terminating in an a3-xyloside are inefficient substrates for B4GAT1 in contrast to LARGE.

Purified trisaccharides were incubated with LARGE or B4GAT1 plus UDP-GlcA overnight and then separated by isocratic reverse-phase C18 HPLC. (A) P with a subscript indicates product (addition of GlcA) whereas S with a subscript indicates an unmodified trisaccharide substrate. The top chromatogram in each pair shows the result of incubation with B4GAT1, the bottom shows the result of incubation with LARGE. (B) Results were confirmed by MS/MS fragmentation spectra, only product spectra are shown. Reactions were carried out overnight in 0.1 M MES pH 6.5 containing 10 mM $MnCl_2$, 5 mM $MgCl_2$, 2 mM substrate and 2 mM UDP-GlcA.

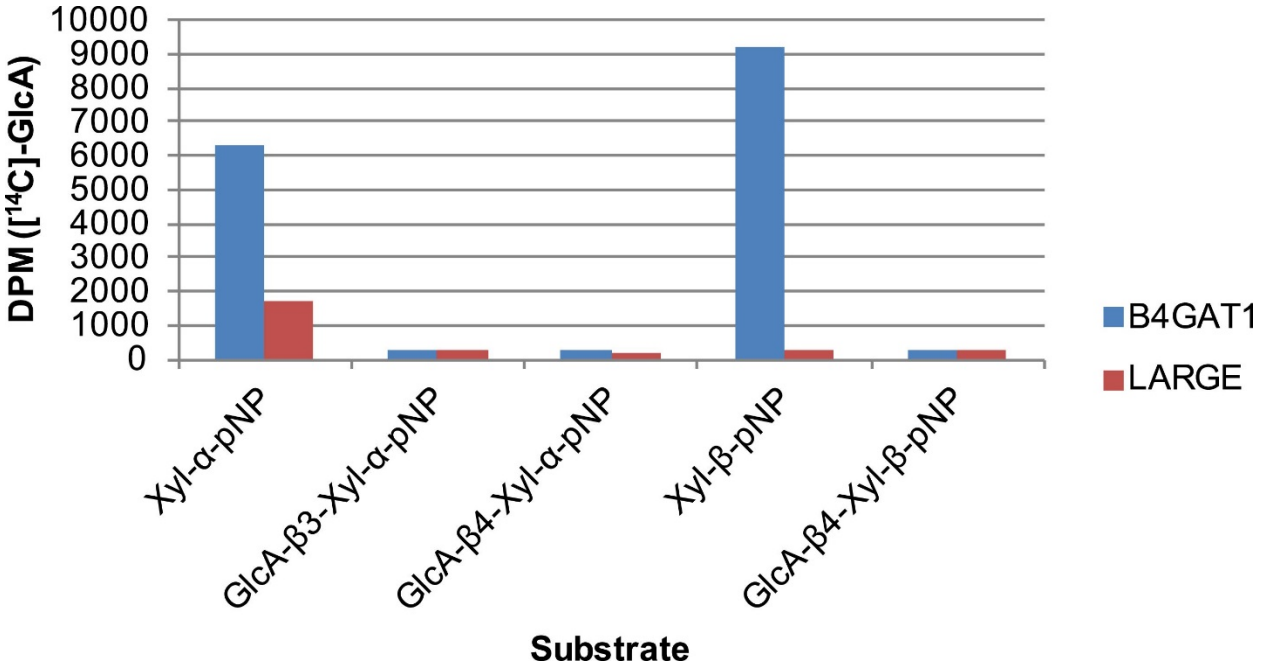

Figure 3.10. B4GAT1 and LARGE do not appear to possess branching activity.

Using purified disaccharide products of B4GAT1 or LARGE, we investigated B4GAT1 and LARGE for potential branching activity. Xyl-α-pNP and Xyl-β-pNP were included as controls. Transfer of GlcA by B4GAT1 or LARGE to disaccharides containing a terminal GlcA residue was not detected by radioactive transfer assay. Reactions were carried out overnight in 0.1 M MES pH 6.5 containing 10 mM $MnCl_2$, 5 mM $MgCl_2$, 2 mM substrate, 40,000 DPM UDP-[^{14}C]GlcA and 2 mM non-radioactive donor.

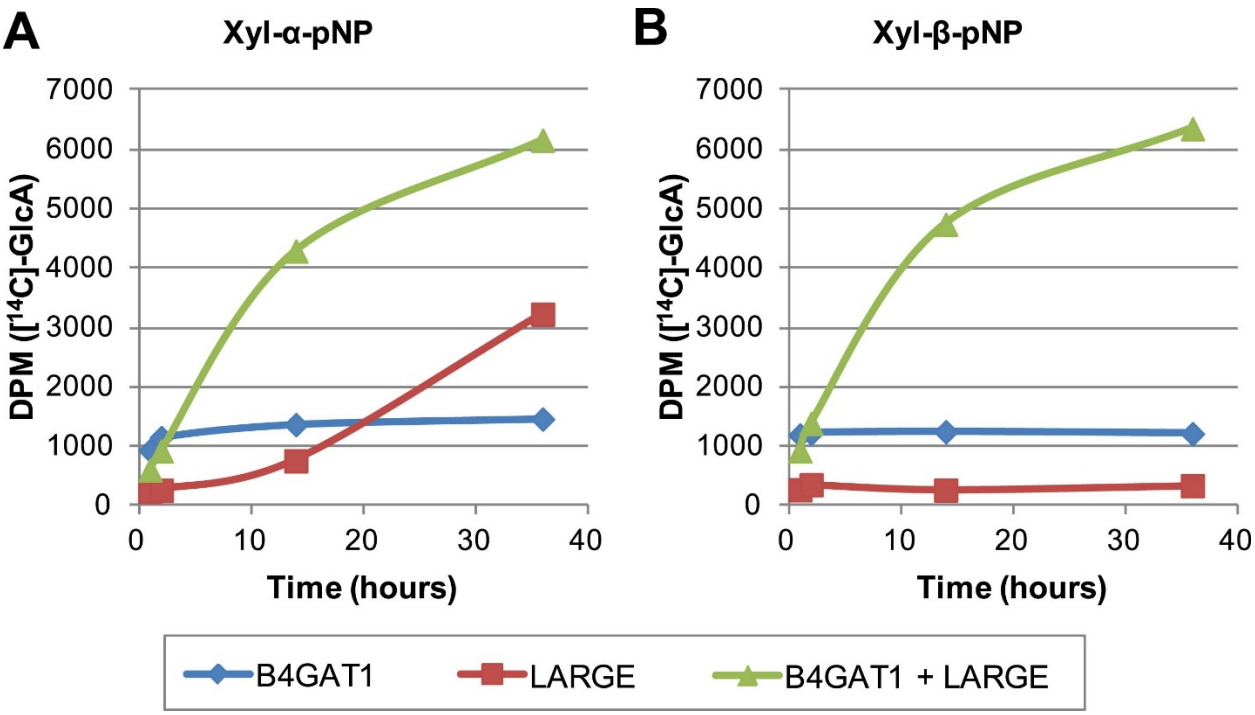

Figure 3.11. The slow reaction velocity of LARGE is rescued by addition of B4GAT1. Reactions were carried out in 0.1 M MES pH 6.5 with 10 mM $MnCl_2$, 5 mM $MgCl_2$, 40,000 DPM UDP-[^{14}C]GlcA and 10 mM non-radioactive UDP-GlcA and UDP-Xyl each. Aliquots were removed at the displayed time points, boiled, and processed using RP C18 spin columns to separate untransferred donor from substrate. (A) The initial transfer of GlcA by LARGE to Xyl-α-pNP is slow in the absence of B4GAT1 but after transfer of the first GlcA polymerization rates increase to mirror those of LARGE in the presence of B4GAT1. (B) With Xyl-β-pNP as substrate, B4GAT1, which only possesses glucuronyltransferase activity, transfers a single GlcA per molecule of substrate. LARGE is unable to transfer GlcA to Xyl-β-pNP. Polymerization is only observed with the addition of both enzymes to the reaction mixture.

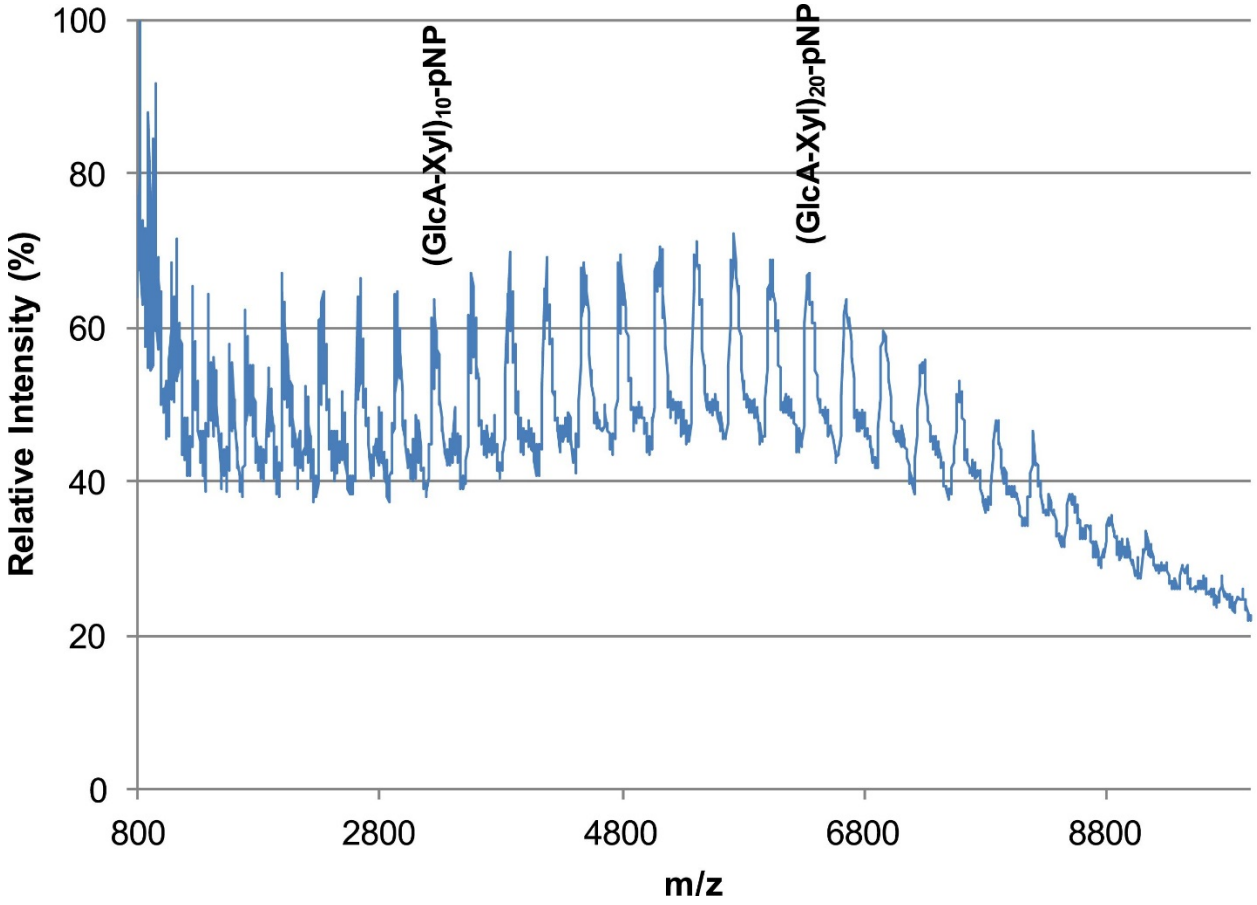

Figure 3.12. B4GAT1 and LARGE combined produce extended GlcA-Xyl polymer chains. B4GAT1 and LARGE were incubated together for 36 hr in 0.1 M MES pH 6.5 containing 10 mM $MnCl_2$, 5 mM $MgCl_2$, 10 mM UDP-GlcA, 10 mM UDP-Xyl and 1 mM Xyl-α-pNP. Mass spectra were acquired on a Bruker microflex and depict polymer formation. Note, due to likely decreased ionization efficiency with increasing polymerization, the sizes of observed peaks are unlikely to represent true abundances of each polymer species in the mixture.

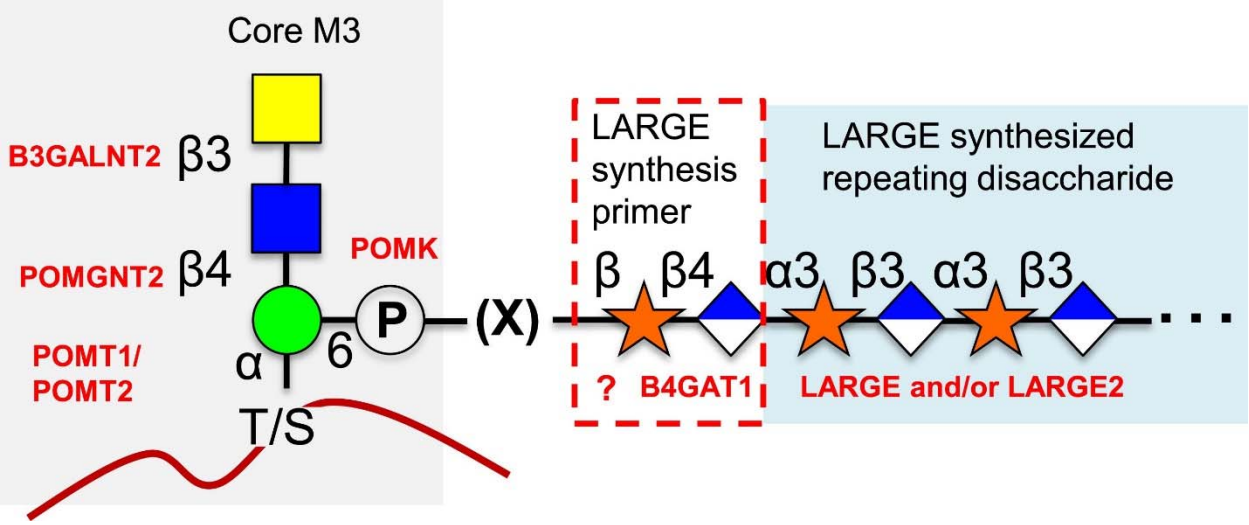

Figure 3.13. Proposed role of B4GAT1 in the O-mannosylation pathway.

The molecular link between the phospho-O-mannose trisaccharide synthesized on α-dystroglycan and the LARGE synthesized repeating disaccharide crucial for laminin binding reactivity is still not fully characterized (represented as [X]). B4GAT1 appears to possess a priming activity for LARGE and likely adds to an underlying β-xylose that is added by an as yet undefined glycosyltransferase (represented with a question mark).

CHAPTER 4

TREATMENT OF ARENAVIRAL INFECTION USING ALPHA-DYSTROGLYCAN FUNCTIONAL GLYCAN MIMICS

Introduction

The arenavirus family is one of four virus families containing members known to cause viral hemorrhagic fevers (VHF) [1]. Hemorrhagic fever virus infections, including those caused by Ebola virus (EBOV) that have resulted in recent outbreaks extensively covered in the media [2-9], are characterized by fever development, hemorrhage (bleeding) in a significant proportion of patients, and multiple organ system involvement [1, 10]. Although the various Ebolavirus species have received the most attention in the popular press [11], the arenavirus VHF agent Lassa virus (LASV) has been far more destructive year-to-year. LASV infects upwards of 300,000 people per year, results in 5-10,000 deaths and produces significant sequelae among survivors including incidence of hearing loss at near 20% [10, 12, 13]. Some researchers provide evidence that these commonly cited statistics in fact underrepresent the true extent of morbidity and mortality. However, LASV is considered to be one of the most neglected tropical diseases with only one approved drug treatment (ribavirin) and no currently approved vaccines [1, 14]. At the same time, LASV is not simply an issue for developing countries. LASV is endemic in rodent populations that easily move (and become established) across national and continental boundaries, may be unintentionally carried by asymptomatic travelers leading to the potential for rapid dispersal via air travel and represents a possible serious threat as a bioterrorism agent due to its effective transmission in aerosol form (LASV is classified as a Category A threat by the CDC) [15].

Arenaviruses, including LASV and lymphocytic choriomeningitis virus (LCMV), are host cell membrane enveloped negative-strand ambisense RNA viruses encoded by deceptively simple genomes typically consisting of only four genes [10]. Arenavirus virion particles possess a structure shared by other such enveloped viruses. Specifically, the genome is packaged by nucleocapsid proteins that also interact with a "matrix" protein (Z) involved in anchoring host cell derived membrane to the particle [16, 17]. Viral glycoprotein complex (GP or GP-C) trimer "spikes" protrude through this shroud and are the ligands for virus adsorption to host cells necessary for cell binding and uptake. Although arenaviruses are structurally relatively simple, the multifunctional nature and flexibility of their component proteins as well as details of their infection cycle can produce a high level of pathogenicity and have often hampered efforts to develop effective treatments. Of specific note, LASV is extremely well adapted to avoiding innate cell immunity mechanisms and is highly resistant to adaptive immune responses as well. LASV is highly resistant to interferon based antiviral defenses which are key in early acute phase response to infectious agents. LASV also possesses protein/epitope flexibility that has markedly increased the difficulty of producing effective vaccines or utilizing neutralizing antibodies [18]. Furthermore, one of the most potent effectors of LASVs immunosuppression is its use of functionally glycosylated α-dystroglycan (α-DG) as its primary cell surface receptor which confers the advantage of extremely effective cooption of key dendritic immune cells (antigen-presenting cells) [19] on which more than 99% of the total α-dystroglycan present in the immune system is found [10]. The use of this receptor is also associated with disruption of cell signaling through displacement of α-dystroglycans extracellular matrix ligands among the more virulent strains. Although strength of affinity for α-dystroglycan among Old World and New World Clade C arenaviruses contributes substantially to their effectiveness in immune system suppression and

ultimately to their pathogenicity, as with similar adaptations observed in other agents of disease including lymphoma cells, this type of increased short-term fitness represents something of a double-edged sword. Fitness achieved in this manner favors increased host death (in opposition to potential establishment of persistent infections) and also necessarily creates better targets for drug-based strategies and for potential pathogen scrubbing via dialysis.

Although the factors just discussed suggest that disruption of initial virus-cell interaction is a particularly attractive target for drug development, the majority of specific molecular details of the epitopes required for LASV GP binding and the details of their biosynthesis have only been substantially revealed in the past few years. Hence, previous studies aimed at developing viral entry inhibitors have largely relied on very broad small molecule screens. Between 2012 and 2014 studies revealed that a unique repeating disaccharide consisting of xylose and glucuronic acid is synthesized by the enzyme LARGE (functional glycosylation) and is required by α-DG ligands including arenaviruses, that the glycan in the absence of protein is sufficient for ligand binding [20], and that this glycan structure can be efficiently synthesized by the concerted action of soluble secreted forms of the enzymes LARGE and B4GAT1 [21]. Based on this work, we set out to test whether enzymatically synthesized forms of this functional glycan epitope utilized by a-DG dependent arenaviruses for cell entry might serve as the basis for a new set of viral entry inhibitor drugs and related therapies for treatment of arenavirus infection. Expected advantages of such drugs include limited toxicity and allergenicity compared to compounds like ribavirin due to the fact that the molecular species is already synthesized and is present endogenously (possibly dependent on half-life and dose requirements) [22-24], based on expected potent inhibition (low IC_{50}) and high specificity, and due to the chemical stability and expected cost and infrastructure

requirements of such therapeutics versus interventions based on immune plasma or protein biologicals (e.g. antibodies).

While we expect our proposed therapeutics to possess certain improved characteristics in comparison to those characterized in prior studies, it is important to note that previous studies have already demonstrated the efficacy of viral entry inhibition as a general strategy for treatment of arenavirus infection. For example, small molecule screens that identified compounds that block pH-dependent membrane fusion after endocytosis were followed by studies that demonstrated significant reduction in mortality in a guinea pig model of LASV infection upon administration of a selected compound from that screen. Of particular interest are studies based on the use of amphipathic DNA polymers with similar properties to our proposed drug that disrupt cell entry of the related and mechanistically highly similar LCMV viruses, although likely in a non-competitive manner [25]. A significant body of work on arenavirus infections has shown that simply slowing the course of viral progression even after the appearance of symptoms through the use of viral entry inhibitors can reduce mortality [26, 27] and that viral entry inhibitor drugs are likely even more valuable in a combinatorial approach to treatment [25].

The present work centers on synthesis and analytical characterization of potential inhibitors as well as human cell culture characterization of synthesized potential inhibitors through the use of pseudotyped viruses that are well-recognized platforms for the study of viral entry.

Materials and Methods

Reagents, Enzymes, Polymer Synthesis

Reagents, enzyme production and enzymatic reactions remain as described in [21]. At present, (GlcA-β3 Xyl-α3-)$_n$-GlcA-β4-Xyl-β-pNP was produced utilizing B4GAT1 and LARGE

and UDP-GlcA and UDP-Xyl included at 10mM each and assessed by MALDI-TOF as previously described [21]. This characterized heterogeneous polymer mixture was tested in inhibition assays. aDGd340, a fusion construct encoding amino acids 1-340 of human α-dystroglycan (Uniprot accession number: Q14118) and additional elements including superfolder GFP as introduced by gateway cloning into the pGEc2-DEST vector was produced and purified via the same system used for enzyme production and previously described [21, 28]. aDGd340 was confirmed to be functionally glycosylated via IIH6C4 (Millipore Corporation, Billerica, MA) western blot.

Cell Lines

The near-haploid HAP1 cell line (Horizon Genomics GmbH, Vienna, Austria), a derivative of the chronic myelogenous leukemia KBM-7 cell line, and HAP1-based knockout lines including HAP1-DAG1-KO, HAP1-LARGE-KO, and HAP1-B3GNT1-KO (i.e. HAP1-B4GAT1-KO) cells were cultured in IMDM supplemented with L-glutamine, 10% FBS, penicillin-streptomycin and sodium pyruvate (HAP1 culture medium) at 37°C with 10% CO2.

Pseudotyped Virus Production

Media samples of pseudotyped recombinant vesicular stomatitis viruses rVSV-G, rVSV-GP-EBOV, rVSV-GP-LASV and rVSV-GP-LCMV were kindly provided by Dr. Sean Whelan and have been previously described [29, 30]. Briefly, each virus strain is recombinantly engineered to express eGFP. In addition, the virus strains rVSV-GP-EBOV, rVSV-GP-LASV and rVSV-GP-LCMV are engineered such that the native VSV glycoprotein (GP) is replaced by the EBOV, LASV or LCMV glycoproteins respectively. Virus expansion was carried out in Vero-MARU cells grown in MEM with 5% FBS. Media was harvested at ~16 hours post-infection and frozen at -80°C. End-point dilution assays using Vero-MARU cells were used to establish the virus titer ($TCID_{50}$ measure) in harvested media.

Shotgun Proteomics

Shotgun proteomics was performed on tryptic digests of viral-production media according to a standard protocol. LC-MS/MS with CID fragmentation was carried out using a Thermo Fisher Orbitrap XL. Data was searched using a Proteome Discoverer 1.4 workflow specifying a Sequest HT search of essentially all spectra funneled into the percolator node set at a 1% peptide false-discovery rate. The database searched consisted of UniprotKB 2015_01 reviewed primate entries, Chlorocebus entries, in-silico translations of mRNAs encoded in the plasmids used to generate the rVSV viruses and common contaminants.

Viral Infection Assays

Approximately 18-24h prior to each infection experiment, HAP1 cells were plated in 96-well plates in fresh HAP1 culture medium. Plating density was determined such that the cells would be between 55 and 70% confluent at the time of viral challenge. Serial dilutions of virus in HAP1 medium were prepared from thawed virus-production media aliquots. Dilutions were either pre-mixed with potential inhibitors and incubated for 10m at ambient temperature or incubated for 10m without addition of inhibitor. Media was removed from each well and replaced by either fresh HAP1 culture media, media containing virus or media containing virus and a potential inhibitor. After 1h, media was again replaced with fresh HAP1 culture media without virus or inhibitors and cells were incubated for 4-5h at 37°C before assessment for eGFP expression. Infection was quantified as percentage of eGFP positive cells using either a Nexcelom cellometer or via manual inspection using a fluorescence microscope.

Results and Discussion

The near-haploid human derived cell line HAP1 as well as knockout HAP1 cell lines for various genes integral to the production of functionally glycosylated α-DG were selected for use

in studying viral entry inhibition. HAP1 cells produce functionally glycosylated α-DG, are particularly genetically tractable with respect to creation of complete gene knockouts due to their near-haploid status and were previously used to carry out screens that produced a genome-wide map of genes critical for the functional glycosylation of α-DG [30]. For studying viral entry, we adopted the recombinant vesicular stomatitis virus (rVSV) model that is well established as a BSL-2 platform for studying viral entry through substitution of the glycoprotein (pseudotyping) responsible for initial viral adsorption to the host cell surface. The rVSVs used in the present study were engineered to encode eGFP allowing for simple determination of cell infection via fluorescence signal detection. Prior to carrying out infectivity assays, we confirmed the identity of the pseudotyped viruses via shotgun proteomics (Table 4.1).

To examine the role of functionally glycosylated α-DG in the uptake of specific pseudotyped rVSVs and to validate our system with respect to prior literature we performed a viral uptake assay of serial dilutions of the rVSVs in each of the HAP1 cell lines of interest. Figure 4.1-4.4 shows the results of these assays as a function of the $TCID_{50}$ reported for the viral stocks in Vero-MARU cells. The HAP1 cell lines are named so as to indicate the gene knocked out in that line (e.g. HAP1-LARGE-KO cells are LARGE1 knockouts). The rVSV pseudotyped viruses are named so as to indicate the identity of the viral glycoprotein that mediates initial cell surface adsorption (e.g. rVSV-GP-LASV encodes a Lassa virus glycoprotein in place of the vesicular stomatitis virus glycoprotein). The data obtained for rVSV-G, rVSV-GP-EBOV and rVSV-GP-LASV largely exhibited the expected pattern. Infection by rVSV-G and rVSV-GP-EBOV was unaffected by disruption of the functional glycosylation of α-DG in accordance with the previously established lack of use of a-DG as a (primary) cell-surface receptor by the corresponding virus glycoproteins (Figure 4.2 and 4.3). rVSV-GP-LASV which uses functionally glycosylated α-DG

as its primary cell-surface receptor showed markedly reduced viral uptake in the DAG1 and LARGE knockout lines as expected (Figure 4.4). Somewhat surprisingly, HAP1-B4GAT1-KO showed a less pronounced reduction in uptake. Based on the recent literature [21, 31], it is unclear why the clone of HAP1-B4GAT1-KO we assayed does not result in similar disruption of viral uptake. Future testing of cell lysates for B4GAT activity (i.e. transfer of glucuronic acid to beta-linked xylose) might provide an answer or a starting point for further experiments. Assays of rVSV-GP-LCMV also produced initially surprising results given that many strains of LCMV require functionally glycosylated α-DG for efficient uptake (Figure 4.5). On further inspection (sequence alignments) it appears that the glycoprotein present in the viral stocks provided by our collaborators may be from the "Armstrong" strain of LCMV, a strain with low affinity binding to functionally glycosylated α-DG [32]. This strain may not use α-DG as its primary cell surface receptor. The results of these assays provide the basic information for the inhibitor studies we are now carrying out.

Preliminary inhibitor assays have been performed, however, the results with enzymatically synthesized compounds have been negative in the low- to mid-nM range tested (data not shown). However, these inhibition assays as originally attempted were not performed with pre-incubation of virus with inhibitor nor was the media removed after an initial period of (ideally) "synchronous" infection. These factors are now better appreciated and will be incorporated into the design of future experiments. TEV-cleaved functionally glycosylated aDGd340 (aDGd340cTEV) produced in HEK293F cells was tested given that previous studies have indicated that LASV GP mediated uptake by cells could be inhibited by incubation of virus with soluble functionally glycosylated forms of α-DG prior to viral challenge of cells [32]. We have reproduced that result using aDGd340cTEV (Figure 4.6 and 4.7). The degree of functional glycosylation of aDGd340 has not

yet been quantified although the molar protein concentration required for viral uptake inhibition in the data reported in Figure 4.7 suggest a low level functional glycosylation. Solid-phase binding assays of various biotinylated functional glycan repeating disaccharide polymers and of aDGd340 will be used to establish quantitative comparisons [33].

Conclusions

The present study is part of an effort to build on our paper (chapter 3) in which we determined the enzymatic activity of B4GAT1. In these studies, we are focusing on the role of B4GAT1 and associated enzymes in the function of α-DG as a cell surface receptor. We hope to gain further insight into functional aspects of α-DG in human cells and to apply this information toward the creation of potential therapeutics for certain arenavirus infections.

References

1. Paessler S, Walker DH. Pathogenesis of the viral hemorrhagic fevers. Annu Rev Pathol. 2013;8:411-40. doi: 10.1146/annurev-pathol-020712-164041. PubMed PMID: 23121052.

2. Merino JG. Response to Ebola in the US: misinformation, fear, and new opportunities. BMJ. 2014;349:g6712. doi: 10.1136/bmj.g6712. PubMed PMID: 25380659.

3. Chan M. Ebola virus disease in West Africa--no early end to the outbreak. The New England journal of medicine. 2014;371(13):1183-5. doi: 10.1056/NEJMp1409859. PubMed PMID: 25140856.

4. Boehlert E. The Media's Ebola Coverage: The More You Watch, The Less You Know? Blog - Media Matters for America. 2014.

5. The medium and the message of Ebola. Lancet. 2014;384(9955):1641. doi: 10.1016/S0140-6736(14)62016-X. PubMed PMID: 25441179.

6. Fung IC, Tse ZT, Cheung CN, Miu AS, Fu KW. Ebola and the social media. Lancet. 2014;384(9961):2207. doi: 10.1016/S0140-6736(14)62418-1. PubMed PMID: 25625391.

7. Drazen JM, Kanapathipillai R, Campion EW, Rubin EJ, Hammer SM, Morrissey S, Baden LR. Ebola and quarantine. The New England journal of medicine. 2014;371(21):2029-30. doi: 10.1056/NEJMe1413139. PubMed PMID: 25347231.

8. Ebola opportunity. Nature. 2014;515(7528):465-6. doi: 10.1038/515465b. PubMed PMID: 25428460.

9. Sack K, Fink S, Belluck P, Nossiter A. How Ebola Roared Back. NYTimescom Feed. 2014 30 December;Sect. Health.

10. Oldstone MB, Campbell KP. Decoding arenavirus pathogenesis: essential roles for alpha-dystroglycan-virus interactions and the immune response. Virology. 2011;411(2):170-9. doi: 10.1016/j.virol.2010.11.023. PubMed PMID: 21185048; PMCID: 3071849.

11. Parsi K. Dr. Fauci Or: How I Learned To Stop Worrying And Hate The Media's Coverage Of Ebola [Internet]. 2014 [cited 2015 4/9/2015]. Available from: http://www.bioethics.net/2014/10/dr-fauci-or-how-i-learned-to-stop-worrying-and-hate-the-medias-coverage-of-ebola/.

12. Nunberg JH, York J. The curious case of arenavirus entry, and its inhibition. Viruses. 2012;4(1):83-101. doi: 10.3390/v4010083. PubMed PMID: 22355453; PMCID: 3280523.

13. National Center for Emerging and Zoonotic Infectious Diseases. (2014). *Lassa Fever*. Retrieved from http://www.cdc.gov/vhf/lassa/resources/Lassa-FactSheet.pdf.

14. Ruz C. Ebola intensifies the struggle to cope with Lassa fever. BBC. 2014.

15. Centers for Disease Control and Prevention. Bioterrorism Agents/Diseases [updated 2/6/2015]. Available from: http://emergency.cdc.gov/agent/agentlist-category.asp.

16. Loureiro ME, D'Antuono A, Levingston Macleod JM, Lopez N. Uncovering viral protein-protein interactions and their role in arenavirus life cycle. Viruses. 2012;4(9):1651-67. doi: 10.3390/v4091651. PubMed PMID: 23170177; PMCID: 3499824.

17. Eichler R, Strecker T, Kolesnikova L, ter Meulen J, Weissenhorn W, Becker S, Klenk HD, Garten W, Lenz O. Characterization of the Lassa virus matrix protein Z: electron microscopic study of virus-like particles and interaction with the nucleoprotein (NP). Virus Res. 2004;100(2):249-55. doi: 10.1016/j.virusres.2003.11.017. PubMed PMID: 15019244.

18. Olschlager S, Flatz L. Vaccination strategies against highly pathogenic arenaviruses: the next steps toward clinical trials. PLoS Pathog. 2013;9(4):e1003212. doi: 10.1371/journal.ppat.1003212. PubMed PMID: 23592977; PMCID: 3623805.

19. Pannetier D, Faure C, Georges-Courbot MC, Deubel V, Baize S. Human macrophages, but not dendritic cells, are activated and produce alpha/beta interferons in response to Mopeia virus infection. Journal of virology. 2004;78(19):10516-24. doi: 10.1128/JVI.78.19.10516-10524.2004. PubMed PMID: 15367618; PMCID: 516411.

20. Goddeeris MM, Wu B, Venzke D, Yoshida-Moriguchi T, Saito F, Matsumura K, Moore SA, Campbell KP. LARGE glycans on dystroglycan function as a tunable matrix scaffold to prevent dystrophy. Nature. 2013;503(7474):136-40. Epub 2013/10/18. doi: 10.1038/nature12605. PubMed PMID: 24132234; PMCID: 3891507.

21. Praissman JL, Live DH, Wang S, Ramiah A, Chinoy ZS, Boons GJ, Moremen KW, Wells L. B4GAT1 is the priming enzyme for the LARGE-dependent functional glycosylation of alpha-dystroglycan. Elife. 2014;3. doi: 10.7554/eLife.03943. PubMed PMID: 25279697; PMCID: 4227051.

22. Nahmias AJ, Kibrick S. Inhibitory effect of heparin on herpes simplex virus. J Bacteriol. 1964;87(5):1060-6. PubMed PMID: 4289440; PMCID: 277146.

23. Yang HS, La WG, Bhang SH, Jeon JY, Lee JH, Kim BS. Heparin-conjugated fibrin as an injectable system for sustained delivery of bone morphogenetic protein-2. Tissue Eng Part A. 2010;16(4):1225-33. doi: 10.1089/ten.TEA.2009.0390. PubMed PMID: 19886733.

24. Lee Y, Nam JH, Shin HC, Byun Y. Conjugation of low-molecular-weight heparin and deoxycholic acid for the development of a new oral anticoagulant agent. Circulation. 2001;104(25):3116-20. PubMed PMID: 11748110.

25. Lee AM, Rojek JM, Gundersen A, Stroher U, Juteau JM, Vaillant A, Kunz S. Inhibition of cellular entry of lymphocytic choriomeningitis virus by amphipathic DNA polymers. Virology. 2008;372(1):107-17. doi: 10.1016/j.virol.2007.10.016. PubMed PMID: 18022208; PMCID: 2821746.

26. Vela E. Animal models, prophylaxis, and therapeutics for arenavirus infections. Viruses. 2012;4(9):1802-29. doi: 10.3390/v4091802. PubMed PMID: 23170184; PMCID: 3499831.

27. Cashman KA, Smith MA, Twenhafel NA, Larson RA, Jones KF, Allen RD, 3rd, Dai D, Chinsangaram J, Bolken TC, Hruby DE, Amberg SM, Hensley LE, Guttieri MC. Evaluation of Lassa antiviral compound ST-193 in a guinea pig model. Antiviral Res. 2011;90(1):70-9. doi: 10.1016/j.antiviral.2011.02.012. PubMed PMID: 21371508; PMCID: 3319460.

28. Meng L, Forouhar F, Thieker D, Gao Z, Ramiah A, Moniz H, Xiang Y, Seetharaman J, Milaninia S, Su M, Bridger R, Veillon L, Azadi P, Kornhaber G, Wells L, Montelione GT, Woods RJ, Tong L, Moremen KW. Enzymatic basis for N-glycan sialylation: structure of rat alpha2,6-sialyltransferase (ST6GAL1) reveals conserved and unique features for glycan sialylation. The Journal of biological chemistry. 2013;288(48):34680-98. Epub 2013/10/25. doi: 10.1074/jbc.M113.519041. PubMed PMID: 24155237; PMCID: 3843080.

29. Wong AC, Sandesara RG, Mulherkar N, Whelan SP, Chandran K. A forward genetic strategy reveals destabilizing mutations in the Ebolavirus glycoprotein that alter its protease dependence during cell entry. Journal of virology. 2010;84(1):163-75. doi: 10.1128/JVI.01832-09. PubMed PMID: 19846533; PMCID: 2798398.

30. Jae LT, Raaben M, Riemersma M, van Beusekom E, Blomen VA, Velds A, Kerkhoven RM, Carette JE, Topaloglu H, Meinecke P, Wessels MW, Lefeber DJ, Whelan SP, van Bokhoven H, Brummelkamp TR. Deciphering the glycosylome of dystroglycanopathies using haploid screens for lassa virus entry. Science. 2013;340(6131):479-83. doi: 10.1126/science.1233675. PubMed PMID: 23519211.

31. Willer T, Inamori K, Venzke D, Harvey C, Morgensen G, Hara Y, Beltran Valero de Bernabe D, Yu L, Wright KM, Campbell KP. The glucuronyltransferase B4GAT1 is required for initiation of LARGE-mediated alpha-dystroglycan functional glycosylation. Elife. 2014;3. doi: 10.7554/eLife.03941. PubMed PMID: 25279699; PMCID: 4227050.

32. Spiropoulou CF, Kunz S, Rollin PE, Campbell KP, Oldstone MB. New World arenavirus clade C, but not clade A and B viruses, utilizes alpha-dystroglycan as its major receptor. Journal of virology. 2002;76(10):5140-6. PubMed PMID: 11967329; PMCID: 136162.

33. Michele DE, Barresi R, Kanagawa M, Saito F, Cohn RD, Satz JS, Dollar J, Nishino I, Kelley RI, Somer H, Straub V, Mathews KD, Moore SA, Campbell KP. Post-translational disruption of dystroglycan-ligand interactions in congenital muscular dystrophies. Nature. 2002;418(6896):417-22. doi: 10.1038/nature00837. PubMed PMID: 12140558.

Table 4.1. rVSV glycoprotein hits in viral-production media samples

Shotgun proteomics was carried out on tryptic digests of media samples and, for each sample, the identity of all viral glycoproteins (GPs) detected was extracted into this table – each sample contained only the expected viral glycoprotein. Additional proteins detected in each sample included the remaining viral proteins (nucleoprotein, matrix protein, etc.) as well as FBS and Vero-MARU derived proteins.

Sample	Description	Coverage	# Peptides	# PSMs	# AAs	MW [kDa]
rVSV-G	VSV GP	43.25%	17	60	511	57.4
rVSV-GP-EBOV	EBOV GP	36.09%	18	53	507	56.8
rVSV-GP-LASV	LASV GP	8.96%	5	8	491	55.8
rVSV-GP-LCMV	LCMV GP	24.50%	12	29	498	56.2

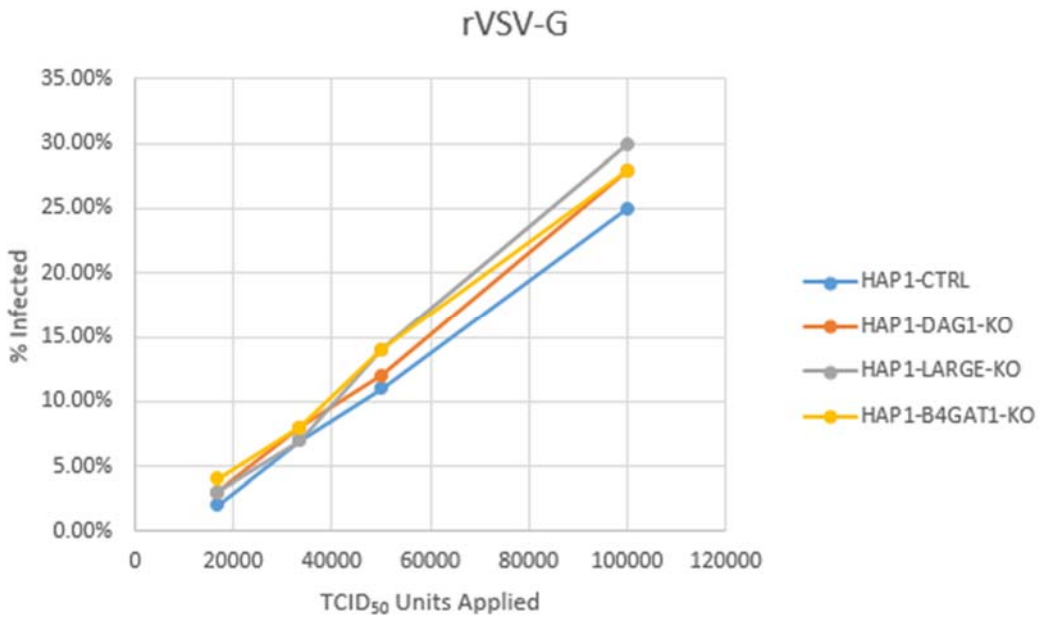

Figure 4.1. rVSV-G infection of HAP1 cell lines

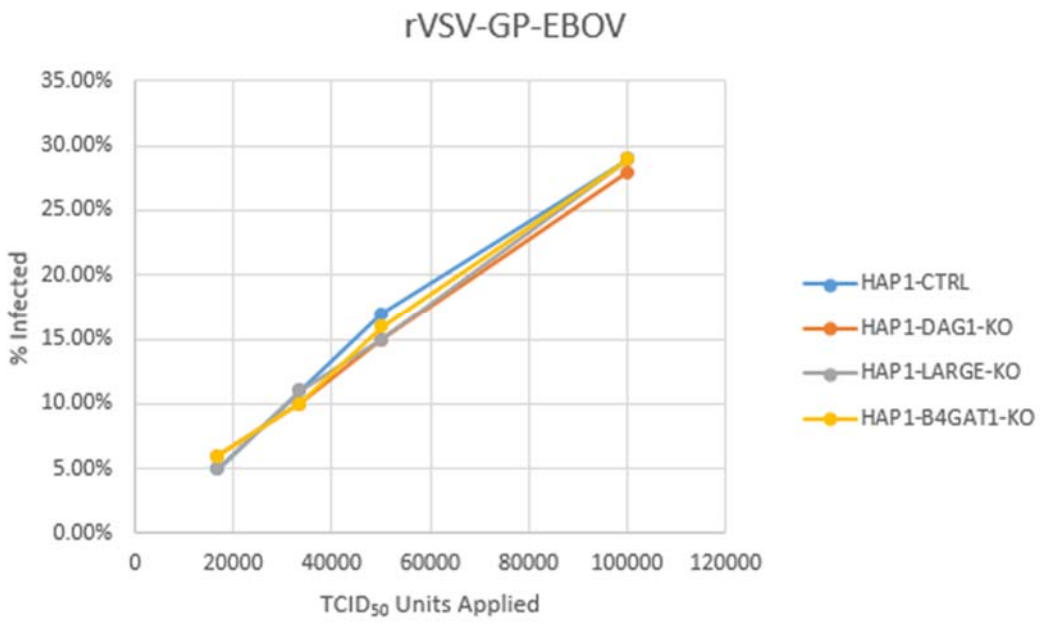

Figure 4.2. rVSV-GP-EBOV infection of HAP1 cell lines

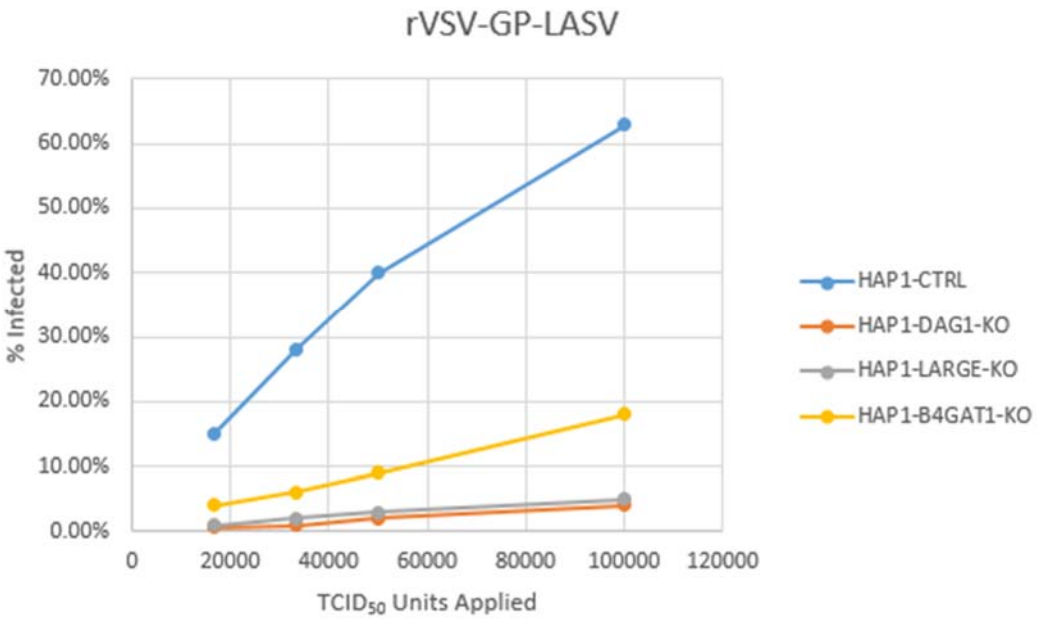

Figure 4.3. rVSV-GP-LASV infection of HAP1 cell lines

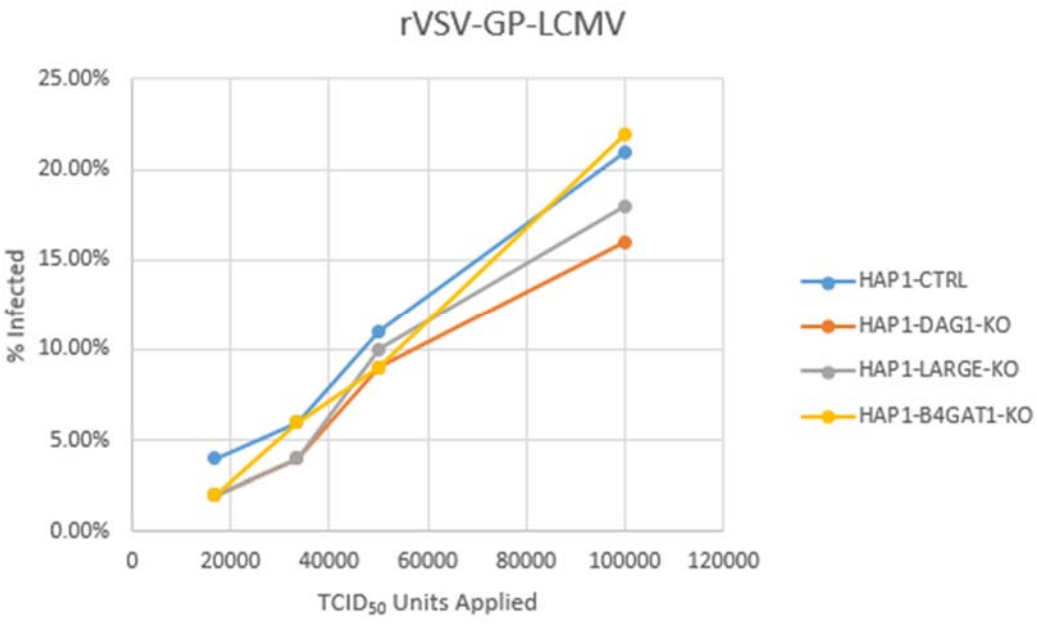

Figure 4.4 rVSV-GP-LCMV infection of HAP1 cell lines

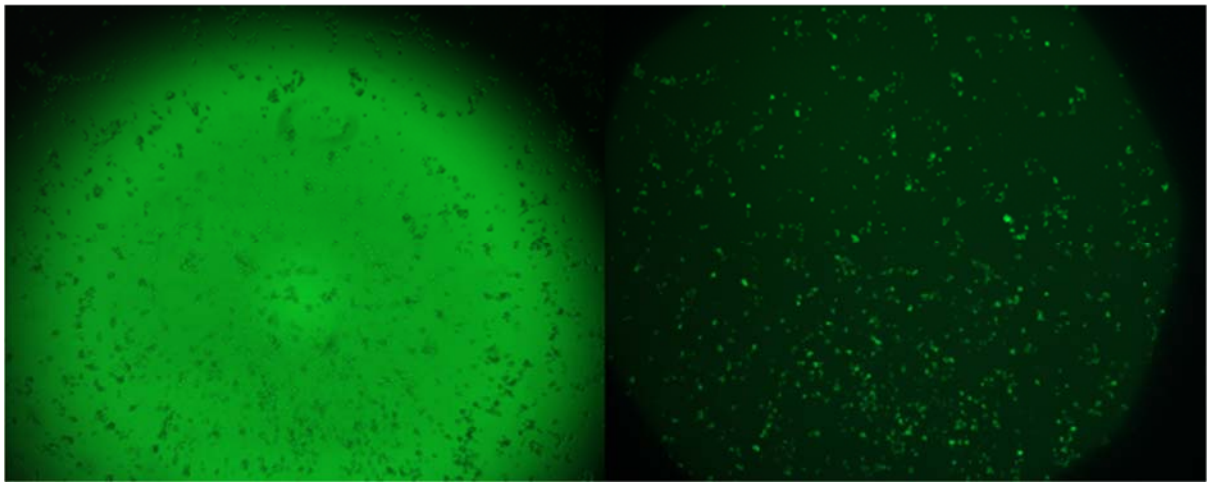

Figure 4.5. rVSV-GP-LASV infection without aDGd340 treatment (bright-field and green-fluorescence images).

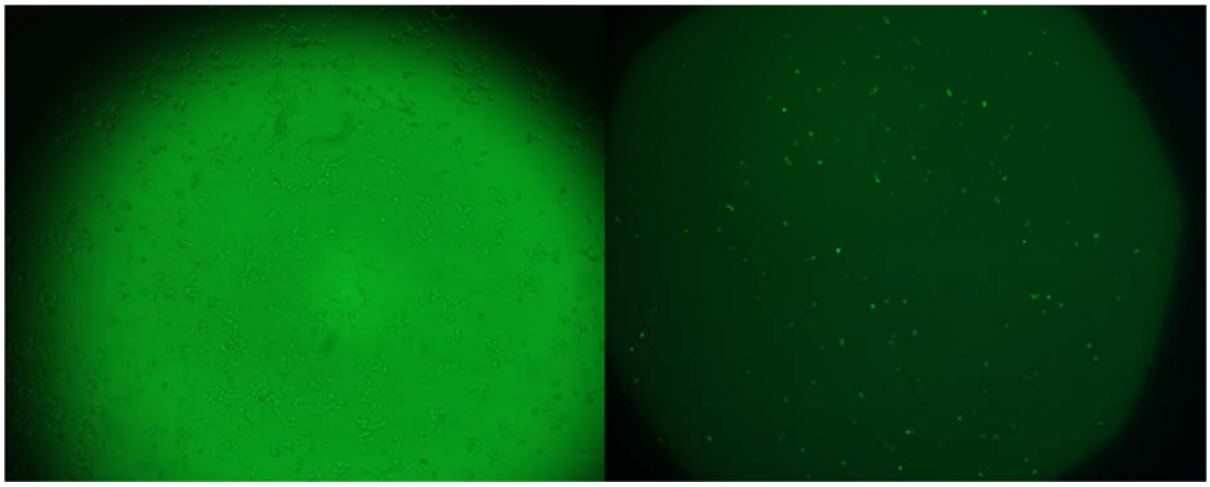

Figure 4.6. rVSV-GP-LASV infection with aDGd340cTEV treatment (bright-field and green-fluorescence images).

CHAPTER 5

CONCLUSIONS

Alpha-dystroglycan has proven to be of great functional significance, both developmentally and in pathogenic processes, while also possessing regulatory and molecular properties that have greatly increased the challenge of its characterization. In this work, my collaborators and I have worked to further characterize α-DG and to apply knowledge gained in its characterization toward human health. Specifically, we have added to knowledge of the enzymes and structure required for the function of α-DG and are currently turning our attention toward application of this knowledge to the development of viral therapeutics. However, α-DG presents numerous possibilities for further characterization which I will now discuss further.

During the past five years, our understanding of the molecular basis for the cell-surface receptor functionality of α-DG has dramatically expanded. The acidic carbohydrate polymer ligand (matriglycan) of ECM and other α-DG binding partners has been determined [1] and the enzymatic activities of six of the twelve genes implicated in direct building block transfer to nascent core M3 functional glycan chains have been determined (see Table 5.1, Figure 2.1, Figure 2.6 and Figure 3.13). Furthermore, powerful genetic screen technology has solidified the boundaries of genetic determinants of functional glycosylation suggesting that there are likely only two or three uncharacterized activities remaining until we have completed the core M3 functional glycan synthesis pathway [2, 3]. However, these remaining activities, characterization of additional regulatory factors at the molecular level and the application of this knowledge in treating diseases constitute continuing challenges. In this final chapter, I will focus on these challenges and present

a current perspective and thoughts toward addressing them. I will begin with the challenge of completing the basic characterization of the pathway and conclude with a brief section discussing more speculative possibilities for application in treating arenaviral-based diseases.

Completing the Pathway, Determination of "X"

Completing the pathway for core M3 functional glycan synthesis *in vivo* is of great interest. I am focusing on leveraging improvements in databases (e.g. domain and metabolite databases), genetic and cell culture technologies, and analytical techniques toward this goal. Each of these areas has experienced remarkable developments in cost-effectiveness and availability within just the past five years. The overall strategy we intend to employ is based on initial analytical determination of the remaining structural components of core M3 functional glycans followed by experiments aimed at connecting each molecular component to the protein or proteins required to catalyze its synthesis. The first section discusses the analytical determination of the putative unknown molecular components of core M3 functional glycans that connect the phosphorylated O-mannosyl trisaccharide to the β-linked xylose we discovered to be required for initiation of "matriglycan" [4] (LARGE, -α3-GlcA- β3-Xyl-) chain synthesis. The second section proposes several experimental approaches that may be employed to complete basic characterization of the protein factors in the pathway based on successful completion of analytical characterization.

Analytical Structure Determination

I will discuss my priorities and proposed experiments proceeding from approaches employed in cell culture and during protein production through to analytical and data processing techniques. During the cell culture, protein production and initial processing stage, the focus is on reducing molecular heterogeneity and obtaining adequate quantities of relevant molecular species for analytical characterization. To this end, the primary strategy already in place is the engineering

and production of a truncated and tagged fusion protein containing human α-DG residues 1 to 340 (dubbed aDGd340) in a human-derived cell suspension culture system (FreeStyle HEK293F) that greatly facilitates high-yield (milligram) and high-purity protein production (see Chapter 3). One pitfall to overexpression under control of a strong constitutive promoter is possible capacity limits of the secretory pathway for post-translational modification. However, we have already confirmed that a portion of the material thus produced is functionally glycosylated (by IIH6 western blot) and expect that a reasonable proportion of the remaining material is extended post-core M3 phosphate to lesser degrees. This might be confirmed by various techniques including anion-exchange chromatographic separation of cold aqueous HF released phospho-O-mannosyl glycans. Ideally, we would prefer to isolate a fraction consisting of all post-phosphoryl extended aDGd340 and glycosidase treat to remove -α3-GlcA-β3-Xyl- chain heterogeneity, however, a suitable endoglycosidase or α3-linked exo-xylosidase has not been found. Secondary strategies at this stage of production include the use of knockout cell lines (specifically HAP1-B4GAT1-KO and HAP1-LARGE-KO, see Chapter 4) for protein production or the combined transfection of a suitable HNK-1ST construct and production in the suspension culture system. Either approach should greatly decrease heterogeneity and would allow for potential radiolabeling (e.g. by B4GAT1) coupled with chromatography to purify and further simplify samples for further analytical characterization.

At the analytical level, the primary current approach centers on application of a panel of LC-MS/MS-based glycoproteomic techniques to the 28 residue O-mannosylated peptide region of human α-DG that may be separately recovered and concentrated after aDGd340 protein production. O-GalNAc glycans are being removed using exo-glycosidases to reduce some of the heterogeneity and the resulting material is being analyzed by HCD, ETD and pseudo-neutral loss

approaches to identify glycopeptides that may be post-phosphoryl O-mannose trisaccharide extended. Through the use of accurate mass and fragmentation spectra we expect to be able to determine the chemical nature of post-phosphoryl structures. In parallel, I am carrying out database searches that utilize the Wildcard search™ feature implemented in Byonic [5] and also examining peptide sequence tags [6] manually to identify peptides of interest by fragmentation that may be used to locate precursors with unknown modifications and thus unanticipated mass shifts. I also plan to use cold aqueous hydrofluoric acid to release phospho-diester linked glycans [7] which may then be characterizable through glycomic techniques compatible with acidic, phosphorylated and possibly sulfated carbohydrates (certain permethylation protocols result in the loss of these glycans). Complementary cleanup and separation steps may include the use of IMAC for phosphate-species purification and anion-exchange chromatography for more refined separation which is applicable to acidic carbohydrates under relatively low pH (4.0) conditions [8]. Finally, secondary techniques may include MALDI-TOF (see Chapter 4), FTIR and other spectrometric and spectroscopic techniques that might reveal any unique characteristics of the final uncharacterized moiety.

Gene Function Determination

Assuming success in analytical characterization, protein activity determination will be significantly simplified. Techniques used to establish the post-phosphoryl structure linking the core M3 O-mannose trisaccharide to the matriglycan chain will provide an assay that might be combined with knockout cell line aDGd340 production to try to determine function. Depending on the stability of the chemistry employed and also possible protein-protein interaction requirements (for example POMGNT1 and FKTN [9]), *in vitro* assays may provide simpler functional characterization. There are a limited number of metabolites present in the Golgi

apparatus as can be conveniently viewed using a database such as the Human Metabolome Database [10] and it may be hypothesized, based on the homology of FKTN and FKRP as well as on the organic chemistry of phosphates and requirements for a xylosyltransferase, that the relevant metabolites will not be overly exotic. An *in vitro* assay using an appropriate synthesized O-mannosylated peptide (enzymatically treated with POMGNT2, B3GALNT2 and likely POMK) and appropriate combinations of engineered versions of FKTN, FKRP, TMEM5 and POMGNT1 may demonstrate product formation from which individual roles might be teased out in additional experiments.

Whether our group succeeds in this work or it is first reported by another group, I expect the basic pathway to be fully elucidated within a year.

Application Possibilities for Arenaviral Infection Prevention and Treatment

In Chapter 4, I presented the basis for a paper aimed at the use of enzymatically synthesized matriglycan for arenaviral infection treatment. The genesis of this idea comes from a strategy already extensively used by the human body to prevent infection, namely the production of mucins ("decoys" [11]). To conclude, I will offer a table (Table 5.2) containing proposed specific implementations for viral capture or interference using synthetic matriglycan. Proposals are grouped into categories (on columns) of "prevention" and "remediation" and sub-grouped based on valence of presentation (on rows) which may significantly impact effectiveness through the binding enhancement that multivalence can provide [11]. The range of proposals covers many different scenarios, for example, inexpensive face masks might be useful to at-risk populations including agricultural workers and might provide a level of protection against accidental exposure to infected rodent biological material aerosols. The proposals might be combined with additional effectors such as nanotechnology-based antiseptic surfaces [12].We are focusing on proving the

basic matriglycan strategy in cell culture and then rapidly moving into mouse models in collaboration with Dr. Daniel Mead (UGA) once we have promising therapy candidates. We will also explore implementations as proposed in the table in collaboration with Dr. Jason Locklin (UGA).

References

1. Inamori K, Yoshida-Moriguchi T, Hara Y, Anderson ME, Yu L, Campbell KP. Dystroglycan function requires xylosyl- and glucuronyltransferase activities of LARGE. Science. 2012;335(6064):93-6. doi: 10.1126/science.1214115. PubMed PMID: 22223806; PMCID: 3702376.

2. Jae LT, Raaben M, Riemersma M, van Beusekom E, Blomen VA, Velds A, Kerkhoven RM, Carette JE, Topaloglu H, Meinecke P, Wessels MW, Lefeber DJ, Whelan SP, van Bokhoven H, Brummelkamp TR. Deciphering the glycosylome of dystroglycanopathies using haploid screens for lassa virus entry. Science. 2013;340(6131):479-83. doi: 10.1126/science.1233675. PubMed PMID: 23519211.

3. Jae LT, Raaben M, Herbert AS, Kuehne AI, Wirchnianski AS, Soh TK, Stubbs SH, Janssen H, Damme M, Saftig P, Whelan SP, Dye JM, Brummelkamp TR. Virus entry. Lassa virus entry requires a trigger-induced receptor switch. Science. 2014;344(6191):1506-10. doi: 10.1126/science.1252480. PubMed PMID: 24970085; PMCID: 4239993.

4. Yoshida-Moriguchi T, Campbell KP. Matriglycan: a novel polysaccharide that links dystroglycan to the basement membrane. Glycobiology. 2015. doi: 10.1093/glycob/cwv021. PubMed PMID: 25882296.

5. Bern M, Kil YJ, Becker C. Byonic: advanced peptide and protein identification software. Curr Protoc Bioinformatics. 2012;Chapter 13:Unit13 20. doi: 10.1002/0471250953.bi1320s40. PubMed PMID: 23255153; PMCID: 3545648.

6. Mortz E, O'Connor PB, Roepstorff P, Kelleher NL, Wood TD, McLafferty FW, Mann M. Sequence tag identification of intact proteins by matching tanden mass spectral data against sequence data bases. Proceedings of the National Academy of Sciences of the United States of America. 1996;93(16):8264-7. PubMed PMID: 8710858; PMCID: 38658.

7. Yoshida-Moriguchi T, Yu L, Stalnaker SH, Davis S, Kunz S, Madson M, Oldstone MB, Schachter H, Wells L, Campbell KP. O-mannosyl phosphorylation of alpha-dystroglycan is required for laminin binding. Science. 2010;327(5961):88-92. doi: 10.1126/science.1180512. PubMed PMID: 20044576; PMCID: 2978000.

8. Cook BJ, Clay RP, Bergmann CW, Albersheim P, Darvill AG. Fungal polygalacturonases exhibit different substrate degradation patterns and differ in their susceptibilities to polygalacturonase-inhibiting proteins. Mol Plant Microbe Interact. 1999;12(8):703-11. doi: 10.1094/MPMI.1999.12.8.703. PubMed PMID: 10432636.

9. Xiong H, Kobayashi K, Tachikawa M, Manya H, Takeda S, Chiyonobu T, Fujikake N, Wang F, Nishimoto A, Morris GE, Nagai Y, Kanagawa M, Endo T, Toda T. Molecular interaction between fukutin and POMGnT1 in the glycosylation pathway of alpha-dystroglycan. Biochemical and biophysical research communications. 2006;350(4):935-41. PubMed PMID: 17034757.

10. Wishart DS, Jewison T, Guo AC, Wilson M, Knox C, Liu Y, Djoumbou Y, Mandal R, Aziat F, Dong E, Bouatra S, Sinelnikov I, Arndt D, Xia J, Liu P, Yallou F, Bjorndahl T, Perez-

Pineiro R, Eisner R, Allen F, Neveu V, Greiner R, Scalbert A. HMDB 3.0--The Human Metabolome Database in 2013. Nucleic acids research. 2013;41(Database issue):D801-7. doi: 10.1093/nar/gks1065. PubMed PMID: 23161693; PMCID: 3531200.

11. Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW, Etzler ME, editors. Essentials of Glycobiology. 2nd ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2009.

12. Haldar J, Chen J, Tumpey TM, Gubareva LV, Klibanov AM. Hydrophobic polycationic coatings inactivate wild-type and zanamivir- and/or oseltamivir-resistant human and avian influenza viruses. Biotechnol Lett. 2008;30(3):475-9. doi: 10.1007/s10529-007-9565-5. PubMed PMID: 17972018.

Table 5.1. Genes Encoding Proteins with Known or Hypothesized (Direct) Core M3 Glycan Synthesis Activities

Gene(s)	**Protein Function**
POMT1 and POMT2	Mannose transfer from DPM to Ser/Thr
POMGNT2 (GTDC2)	β1,4-GlcNAc transfer to O-mannose
B3GALNT2	β1,3-GalNAc transfer to GlcNAc-β1,4-Man-α
POMK (SGK196)	6-phosphorylation of O-mannose
FKTN	Unknown
FKRP	Unknown
TMEM5	Unknown
B4GAT1	β1,4-GlcA transfer to β-Xyl
LARGE	Xyl-GlcA repeat polymerization
LARGE2	Xyl-GlcA repeat polymerization
POMGNT1	β1,2-GlcNAc transfer to O-mannose, interaction with FKTN

Table 5.2. Proposed Implementations for Arenaviral Capture or Treatment Using Synthetic Matriglycan

Presentation Valence	**Prevention**	**Treatment**
Monovalent	Inhaler	Intravenous drug administration
Multivalent	Coated face mask	Dialysis membranes
	Coated screen	Liposome-based approaches
	Inhaler	Intravenous drug administration