Elucidating Human Milk Oligosaccharide biosynthetic genes through network-based multi-omics integration

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ABSTRACT

Human Milk Oligosaccharides (HMOs) are abundant carbohydrates fundamental to infant health and development. Although these oligosaccharides were discovered more than half a century ago, their biosynthesis in the mammary gland remains largely uncharacterized. Here, we used a systems biology framework that integrated glycan and RNA expression data to construct an HMO biosynthetic network and predict glycosyltransferases involved. To accomplish this, we constructed models describing the most likely pathways for the synthesis of the oligosaccharides accounting for >95% of the HMO content in human milk. Through our models, we propose candidate genes for elongation, branching, fucosylation, and sialylation of HMOs. We further explored selected enzyme activities through kinetic assay and their co-regulation through transcription factor analysis. These results provide the molecular basis of HMO biosynthesis necessary to guide progress in HMO research and application with the ultimate goal of understanding and improving infant health and development.

SIGNIFICANCE STATEMENT

With the HMO biosynthesis network resolved, we can begin to connect genotypes with milk types and thereby connect clinical infant, child and even adult outcomes to specific HMOs and HMO modifications. Knowledge of these pathways can simplify the work of synthetic reproduction of these HMOs providing a roadmap for improving infant, child, and overall human health with the specific application of a newly limitless source of nutraceuticals for infants and people of all ages.

1 INTRODUCTION

Human milk is the “gold standard” of nutrition during early life ¹⁻³. Beyond lactose, lipids, and proteins, human milk contains 11-17% (dry weight) oligosaccharides (Human Milk Oligosaccharides, HMOs)^{4,5}. HMOs are milk bioactives known to improve infant immediate and long-term health and development^{2,6}. HMOs are metabolic substrates for specific beneficial bacteria (e.g., *Lactobacillus* spp. and *Bifidobacter* spp.), and shape the infant’s gut microbiome ^{2,7}. HMOs also impact the infant’s immune system, protect the infant from intestinal and immunological disorders (e.g., necrotizing enterocolitis, HIV, etc.), and may aid in proper brain development and cognition ^{2,6,8,9}. In addition, recent discoveries show that some HMOs can be beneficial to humans of all ages, e.g. the HMO 2’-fucosyllactose (2’FL) protecting against alcohol-induced liver disease¹⁰.

The biological functions of HMOs are determined by their structures ⁶. HMOs are unconjugated glycans consisting of 3–20 total monosaccharides drawn from 3-5 unique monosaccharides: galactose (Gal, A), glucose (Glc, G), N-acetylglucosamine (GlcNAc, GN), fucose (Fuc, F) and the sialic acid N-acetyl-neuraminic acid (NeuAc, NN) (**Figure 1A**). All HMOs extend from a common lactose (Gal β 1-4Glc) core. The core lactose can be extended at the nonreducing end, with a β -1,3-GlcNAc to form a trisaccharide. That intermediate trisaccharide is quickly extended on its non-reducing terminus with a β -1,3-linked galactose to form a type-I tetrasaccharide (LNT) or a β -1,4-linked galactose to form a type-II tetrasaccharide (LNnT). Additional branching of the trisaccharide or tetrasaccharide typically occurs at the lactose core by addition of a β -1,6-linked GlcNAc to the Gal residue. Lactose or the elongated oligosaccharides can be further fucosylated in an α -1,2-linkage to the terminal Gal residue, or α 1,3/4-fucosylated on internal Glc or GlcNAc residues, and α -2,3-sialylated on the terminal Gal residue or α -2,6-sialylated on external Gal or internal GlcNAc residues^{6,8}(**Figure 1B**).

Figure 1 - HMO blueprint and synthesis (A) HMOs are built from a combination of the five monosaccharides D-glucose (Glc, blue circle), D-galactose (Gal, yellow circle), N-acetyl-glucosamine (GlcNAc, blue square), L-fucose (Fuc, red triangle), and sialic acid (N-acetyl-neuraminic acid (NeuAc), purple diamond). Lactose (Gal- β -1,4-Glc) forms the reducing end and can be elongated with several Lacto-N-biose or N-acetylglucosamine repeat units (Gal- β -1,3/4-GlcNAc). Lactose or the polylactosamine backbone can be fucosylated with α -1,2-, α -1,3-, or α -1,4- linkages or sialylated in α -2,3- or α -2,6- linkages². **(B)** Small HMOs can be fucosylated to make 2'FL while larger HMOs can be synthesized by the extension of the core lactose with N-acetylglucosamine (type-I) or lacto-N-biose (type-II) and subsequent decoration of the extended core with sialic acid to make more complex HMOs, such as DSLNT. **(C)** Three HMOs in this study: DSLNT, isomer 1 of DFLNT, isomer 6 of FDSLNT; isomer structures represent predictions from this study (see Methods, **Figure S 12**). Each monosaccharide-linking glycosidic bond is labeled (L1, L2,...L10) according to the linkage reactions listed in **Table 1**.

Despite decades of study, many details of HMO biosynthesis remain unclear. While the many possible monosaccharide addition events above are known, the order of the biosynthetic steps and many of the enzymes involved are not known (**Table 1**). For example, the lactose core is extended by alternating actions of β -1,3-N-acetylglucosaminyltransferases (b3GnT) and β -1,4-galactosaminyltransferases (b4GalT) while β -galactoside sialyltransferases (SGaT) and α -1,2-fucosyltransferases (including the FUT2 'secretor' locus) are responsible for some sialylation and fucosylation of a terminal galactose, respectively¹¹. However, each enzymatic activity in HMO extension and branching can potentially be catalyzed by multiple isozymes in the respective gene family. Direct evidence of the specific isozymes performing each reaction *in vivo* is extremely limited.

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88 **Table 1 - Glycosylation reactions examined.** We studied here several candidate glycosyltransferases
89 expressed in our samples to identify candidates for 10 elementary reactions (see Methods, **Table S 1**).
90 Acceptor, product and constraint are represented in LiCoRR¹²: monosaccharides include Gal (A), Fuc (F),
91 Glc (G), GlcNAc (GN), Neu5Ac (NN). Additionally, “)” and “(” indicate initiation and termination of a
92 branch respectively, “[X/Y]” indicates either monosaccharide, and “~” indicates a negation. An asterisk
93 “*” indicates an imperfect match between the EC number and reaction. Background colors correspond to
94 the monosaccharide added: GlcNAc (blue), Fuc (red), Neu5Ac (purple), and Gal (yellow).

Linkage	Reaction	EC Identifier	Acceptor {Constraint}	Product	Candidates
L1:b3GnT	b-1,3 N-acetylglucosamine	2.4.1.149	(A	(GNb3A	B3GNT2-6,8-9
L2:a2FucT	a-1,2 fucosyltransferase	(2.4.1.69,344)	(A	(Fa2A	FUT1-2
L3:a3FucT	a-1,3 fucosyltransferase	(2.4.1.152)	G/GN {~Ab3GN}	Fa3G/GN	FUT3-7,9-11
L4:ST3GalT	(b-Gal) a-2,3 sialyltransferase	(2.4.99.4)	(A	(NNA3A	ST3GAL1-6
L5:ST6GalT	(b-Gal) a-2,6 sialyltransferase	2.4.99.1	(A	(NNA6A	ST6GAL1-2
L6:b3GalT	b-1,3 galactotransferase	2.4.1.86	(GN	(Ab3GN	B3GALT1-2,4-5
L7:b4GalT	b-1,4 galactotransferase	2.4.1.90	(GN	(Ab4GN	B4GALT1-6
L8:b6GnT	b-1,6 N-acetylglucosamine	(2.4.1.150)	GNb3Ab4G	GNb3(GNb6)Ab4G	GCNT1-4,7
L9:a4FucT	a-1,4 fucosyltransferase	2.4.1.65	Ab3GNb3A {~GNb4Ab3GNb3A}	Ab3(Fa4)GNb3A	FUT3,5
L10:ST6GnT	(b-1,3-GlcNAc) a-2,6 sialyltransferase	(2.4.99.3,7)	Ab3GNb3A	Ab3(NNA6)GNb3A	ST6GALNAC1-6

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96 Here we leverage the heterogeneity in HMO composition and gene expression across human subjects
97 to refine our knowledge of the HMO biosynthetic network. Milk samples were collected from 11
98 lactating women across two independent cohorts between the 1st and 42nd day post-partum. (see
99 methods). Gene expression profiling of mammary epithelial cells was obtained from mRNA present
100 in the milk fat globule membrane interspace. Absolute and relative concentrations of the 16 most
101 abundant HMOs was measured. Starting from a scaffold of all possible reactions^{13–18}, we used
102 constraint-based modeling^{19,20} to reduce the network to a set of relevant reactions and most plausible

HMO structures when not known²¹ to form the basis for a mechanistic model^{13,14,22}. This resulted in a ranked ensemble of candidate biosynthetic pathway topologies. We then ranked 44 million candidate biosynthesis networks to identify the most likely network topologies and candidate enzymes for each reaction by integrating sample-matched transcriptomic and glycoproteomic data from the 11 subjects. For this we simulated all reaction fluxes and tested the consistency between changes in flux and gene expression to determine the most probable gene isoforms responsible for each linkage type. We followed with direct observations through fluorescence activity assays to confirm our predictions. Finally, we performed transcription factor analysis to delineate regulators of the system. The resulting knowledge of the biosynthetic network can guide efforts to unravel the genetic basis of variations in HMO composition across subjects, populations, and disorders using systems biology modeling techniques.

2 RESULTS

2.1 ABUNDANCES OF HMOs AND THEIR KNOWN ENZYMES DO NOT CORRELATE

While α -1,2-fucosylation of glycans in humans can be accomplished by both FUT1 and FUT2, only FUT2 is expressed in mammary gland epithelial cells (**Table S 1**). FUT2, the “secretor” gene, is essential to ABH antigens^{23–25} as well as HMO ^{2,26,27} expression. We confirmed that non-functional FUT2 in “non-secretor” subjects guarantees the near-absence of α -1,2-fucosylated HMOs like 2’FL and LNFP1 (Fig2C). But, examining only subjects with functional FUT2 (Secretors), we found FUT2 expression levels and the concentration (nmol/ml) of HMOs containing α -1,2-fucosylation do not correlate in sample-matched microarray and glycomic measurements by HPLC (**Figure 2**). Generalized Estimating Equations (GEE) showed no significant positive association (2’FL Wald p = 0.056; LNFP1 Wald p = 0.34). FUT1 could catalyze this reaction but its expression was not detected in these samples. We hypothesized that to successfully connect gene expression to HMO synthesis, one must account for all biosynthetic steps and not solely rely on direct correlations.

Figure 2 - FUT2 expression should increase 2'FL and LNFPI which require the enzyme but there is no significant positive association. Direct comparison of FUT2 gene expression and concentrations (nmol/mL) of α -1,2-fucose containing HMOs, 2'FL (A) and LNFPI (B), in sample-matched microarray and HPLC reveal no significant association in secretor women from cohort 1 sampled between day 1 and 42 post-partum. Trendlines and points are colored by subject. Linear trends were used to illustrate the intuition of the GEE approach used to estimate these associations across subjects. Non-secretor mothers were excluded due to non-functional FUT2. (C) A heatmap of all HMO concentrations across cohort 1 and cohort 2 (top-bar black and grey respectively). Known HMO structures are shown to the left of each row while uncharacterized structures are indicated with a black box. For proposed isomers of uncharacterized structures, see Figure S 12.

Figure 3 - Overview of Computational Methods for Model Assembly (A-F) and Assessment (G-I). (A) To build the candidate models of HMO biosynthesis, reaction rules were defined to specify all possible monosaccharide additions. (B) The Complete Network includes all oligosaccharides and reactions resulting from the iterative addition of monosaccharides to a root lactose. (C) Using Flux Variability Analysis, the Complete Network was trimmed, removing reactions that cannot reach experimentally-measured HMOs, to produce a (D) Reduced Network **Figure S 9**; red triangles are observed HMOs blue lines are “sink reactions” joining alternative isomers (**Figure S 12**). (E) From the Reduced Network, Mixed Integer Linear Programming (MILP) was used to extract Candidate Models, each representing a subnetwork capable of uniquely synthesizing the observed oligosaccharide profile using a minimal number of reactions; black clines are reactions retained in a candidate model. (F) Flux Balance Analysis was used to estimate flux through each reaction necessary to simulate the measured oligosaccharide concentrations. (G) Model scores were computed as the average maximum correlation between linkage-specific candidate genes and normalized flux through that linkage (**Figure S 11, S1.1.4**). (H) Model scores were parameterized on cohort 1 (left) and cohort 2 (right) data (see Methods). High-performing models, 95th percentile of scores, are highlighted in red. (I) Of the >40 million models considered (blue), 2.66 and 2.32 million models were high-performing when parameterized on data from cohort 1 or cohort 2, respectively. Nearly 250,000 models consistently explained the relationship between predicted flux and expression data from both cohort 1 and cohort 2. These commonly selected models were analyzed for common structural features.

2.2 HIGH-PERFORMING CANDIDATE BIOSYNTHETIC MODELS ARE SUPPORTED BY GENE EXPRESSION AND PREDICTED MODEL FLUX ACROSS SUBJECTS

We built and examined models for HMO biosynthesis in human mammary gland epithelial cells. From the basic reaction set (**Figure 3A**), we generated the complete reaction network (**Figure 3B**) containing

all possible reactions and HMOs with up to nine monosaccharides. The Complete Network was trimmed to obtain a Reduced Network (**Figure 3D**; **Figure S 9**, **Table S 2**) by removing reactions unnecessary for producing the observed oligosaccharides. Candidate models (**Figure 3E**) were built, capable of uniquely recapitulating the glycoprofiling data from milk using two independent cohorts - cohort 1 with 8 samples from 6 mothers between 6 hours and 42 days postpartum^{28,29} and cohort 2 with 2 samples per mother on the 1st and second day after birth³⁰. Mixed integer linear programming was used to identify subnetworks with the minimal number of reactions from the Reduced Network. We identified 44,984,988 candidate models that can synthesize the measured oligosaccharides. Each candidate model contains 43-54 reactions (19.5-24.4% of the reactions in the Reduced Network (**Table S 3**)). These models covered all the feasible combinations of HMO synthesis by the 10 known glycosyltransferase families (**Figure 1D**) that could describe the synthesis of the HMOs in this study.

To identify the most likely biosynthetic pathways for HMOs, we computed a model score for each candidate model using the glycoprofiling and transcriptomic data from the two independent cohorts, after excluding low-expression gene candidates. Genes were excluded when expression was undetected in over 75% of microarray samples and the independent RNA-seq³¹ measured low expression relative to the GTEx³²: TPM<2 and 75th percentile Lemay < GTEx Median TPM. Specificity and expression filtration reduced the candidate genes from 54 to 24 (see supplemental results, **Table S 1**, **Figure S 7**); three linkages (L2, L5 and L9) were resolved by filtration alone indicating that FUT2, ST6GAL1 and FUT3 respectively perform these reactions.

Following low-expression filtering, we compared flux-expression correlation. Leveraging sample-matched transcriptomics and glycomics datasets, we computed model scores indicating the capacity of each candidate gene to support corresponding reaction flux. The model score was computed by first identifying for each reaction, the candidate gene that shows the best Spearman correlation between gene expression and normalized flux; flux was normalized as a fraction of the input flux to limit the influence of upstream reactions (**Figure S 11**, S1.1.4). The highest gene-linkage scores, for each reaction, for each model were averaged to obtain a model score (**Figure 3G**, see Methods). The model scores indicate consistency between gene expression and model-predicted flux. The high-performing models ($z(\text{model score}) > 1.646$) were selected for further examination (**Figure 3H**, see Methods). Though quantile-quantile plots indicated the model score distributions were pseudo-gaussian, variation in skew resulted in slightly different numbers of high-performing models for the two different subject cohorts. Specifically, we found 2,658,052 high-performing models from cohort 1 and 2,322,262 high-performing models using cohort 2 (**Figure 3I**, **Table S 4**). We found 241,589 high-performing models common to cohort 1 and cohort 2. The model scores of commonly high-performing models are significantly correlated (Spearman $R_s = 0.2$, $p < 2.2e-16$) and a hypergeometric enrichment of cohort 1 and cohort 2 selected models shows the overlap is significant relative to the background of 44 million models ($p < 2.2e-16$). We analyzed these 241,589 commonly high-performing models and determined which candidate genes were common in high-performing models.

To determine the most important reactions (**Figure 4**) in the reduced network, we asked which reactions were most significantly and frequently represented among the top 241,589 high-performing models. We then filtered to retain only the top 5% of most important paths from lactose

to each observed HMO (see Methods). The most important reactions form the Summary Network (Figure 4). Here, HMO biosynthesis naturally segregates into type-I backbone structures, with β -1,3-galactose addition to the GlcNAc-extended core lactose, and type-II structures, with β -1,4-galactose addition to the GlcNAc-extended core lactose. As expected, LNFPI, LNFPII, LSTb and DSLNT segregate to the type-I pathway while LNFPIII and LSTc are found in the type-II pathway (see Methods for HMO definitions). The Summary Network suggests resolutions to large structurally ambiguous HMOs (FLNH5, DFLNT2, DFLNH7, and DSLNH2) by highlighting their popularity in high-performing models. The Summary Network also shows three reactions of high comparable strength projecting from GlcNAc- β 1,3-lactose to LNT, LNnT and a bi-GlcNAc-ylated lactose (HMO8, Figure 4, Table S 2) suggesting LNT may be bypassed through an early β -1,3-GlcNAc branching event; a previously postulated alternative path³³. We checked for consistency with previous work³⁴ and found that (1) the single fucose on the reducing-end Glc residue is always α -1,3 linked, (2) for monofucosylated structures, the non-reducing terminal β -1,3-galactose is α -1,2-fucosylated, (3) all galactose on the β -1,6-GlcNAc is always β -1,4 linked while all galactose on the β -1,3-GlcNAc are either β -1,3/4 linked. With the exception FDSLNH1, (4) no fucose is found at the reducing end of a branch and (5) all α -1,2-fucose appear on a β -1,3-galactose and not β -1,4-galactose in monofucosylated structures with more than four monosaccharides; suggesting that FDSLNH1 is an unlikely isomer. The summary network also suggests that most HMOs have type-I LacNAc backbones. To address the potential over-representation of type-I HMOs in our models, we examined the distribution of type-I and type-II in tetra- and pentasaccharides with known structures. Across samples, the median abundance of type-II HMOs, LNnT, LNFPIII and LSTc were 3.33%, 0.041%, and 2.68% of total nmol/mL while type-I HMOs of the same size, LNT, LNFPI, LNFPII, and LSTc, was 15.3%, 9.39%, 7.45% and 0.45% respectively. This confirms the greater abundance of type-I HMOs compared with the type-II structures in the glycomic profiles (Figure 2C). This Summary Network thus provides orientation in this underspecified space.

Figure 4 Summary Network of the most important reactions in the reduced network. Observed, intermediate and candidate HMOs most important to commonly high-performing networks were selected from the Reduced Network (Figure 3D; Supplemental Methods S1.1.2). (A) Several ambiguous isomers (Figure S 12) were preferred (Figure S 1) in the commonly high-performing models. (B) A summary network was constructed from reaction importance; an aggregation of the proportion of high-performing models that include a reaction, and the enrichment of a reaction in the high-performing model set (see Methods). Line weight indicates the relative importance of each reaction. Line color corresponds to the monosaccharide added at each step and line type corresponds to the linkage type. The Summary Network naturally segregates into type-I and type-II backbone structures. For measured HMO definitions (e.g. FDSLNT and DSLNT) see Methods, for intermediate HMO definitions (e.g. 8, 10, or 25) see Table S 2, for uncertain structures (e.g. DFLNT7, FLNH5) see Figure S 12.

Figure 5 – Gene expression correlation with model flux predicts enzymes involved in HMO biosynthesis. (A) To determine the gene expression that best explains flux through each reaction in each glycomics-transcriptomics matched sample, we examined the proportion of high-performing models where each gene was most flux-correlated (PROP, **Figure S 4**), we also examined the gene-linkage score (GLS) and Model Score Contribution (MSC). For this visual, each measure was max-min normalized between 0 and 1. Genes were selected based on high performance on all three measures across cohorts (line type). (B) We summarize the three performance scores from panel A across cohorts into a single support score (see Methods). Briefly, “Support” is p-value for the sum of PROP, GLS and MSC z-scores (relative to a permuted background), Fisher-pooled across cohorts then False Discovery Rate (FDR) corrected across genes (see Methods). Unmeasured genes appear below the plot in the Not Determined (N.D.) box. Genes selected by default (purple, “*”) as the only measured gene candidate (**Table 1**)

2.3 GLYCOSYLTRANSFERASES ARE RESOLVED BY RANKING REACTION CONSISTENCY ACROSS SEVERAL METRICS

We further analyzed the high-performing models to identify the glycosyltransferases responsible for each step in HMO biosynthesis (**Table 1**). As previously described, not all members of a gene family were examined in this analysis. Some genes were excluded due to their well characterized irrelevance (e.g. FUT8) and others, like FUT1, were excluded due to low expression in lactating breast epithelium (see **Table S 1**, methods and supplemental results for the detailed inclusion criteria). To determine the genes preferred for each reaction, we used three metrics to quantify the association between candidate gene expression and predicted flux. These were (1) *proportion* (*PROP* - the relative proportion of models best explained by a candidate gene, **Figure S 4**), (2) *gene linkage score* (*GLS* - the average Spearman correlation between gene expression and flux), and (3) *model score contribution* (*MSC* - an estimate of the gene-influence indicated by the Pearson correlation between model score and gene linkage score) (**Figure 5A**, **Figure S 5**). For each candidate gene, we generated a reaction support score (**Figure 5B**, see Methods); the pooled significance of the maxima of PROP, GLS and MSC across both cohorts.

Three reactions, L2 (FUT2), L5 (ST3GAL1) and L9 (FUT3), were matched to genes by default as they were the only gene candidates remaining following gene expression filtering (**Table S 1**, Supplementary Results). At least one gene showed significant support ($q < 0.1$) for each remaining reaction. GCNT3 shows highly significant support ($q < 0.001$) and nearly 100% of models selected this isoform over GCNT2C or GCNT1 (**Figure S 4**). B4GALT4 is the most significantly supporting gene for the L7: b4GalT reaction (**Figure 5B**). In both cohort 1 and 2, B4GALT4 outperforms all other isoforms in all three metrics. B4GALT4 expression best explains flux in 62% and 80% (PROP) of high-performing models using cohort 1 and 2 data respectively (**Figure S 4**). B4GALT4 also has the highest MSC and GLS ($z > 5.6$) of any isoforms. Interestingly, while B4GALT1 is highly expressed and fundamental to lactose synthesis in the presence of α -lactalbumin and lactation in general^{35,36}, it showed negligible support for the L7 reaction (**Figure 5B**). Considering the reaction support score, all linkages show at least one gene for each reaction that significantly explains behavior across cohorts (**Figure 5B**).

Figure 6 - Results of the CMP-Glo™ Glycosyltransferase Assay to test GT candidates on relevant HMO acceptors. Average luminescence below 10,000 is considered weak activity, and activity above 200,000 is considered very high activity. Reported luminescence values were background corrected and 95% confidence intervals are shown. For complete details see **Table S 6**. Shapes correspond to ST3GALT isoforms

2.4 KINETIC ASSAYS CONFIRM SELECTED GENES AND EXPAND OUR SCOPE

Towards validating and expanding our gene-reaction predictions, glycosyltransferase enzyme activity assays were performed using the NTP-Glo™ Glycosyltransferase assay format from Promega. We used linkage L1:b3GnT and L10:ST6GnT to validate our selections and examined every plausible isoform of the ST3GAL for its ability to perform the linkage L4:ST3GalT reaction. Five acceptors were used: (1) lactose to examine activity on the initial HMO acceptor, (2) LNT and (3) LNnT to establish which enzymes would act on larger type-I and type-II tetrasaccharides, (4) Gal β 1,3-GalNAc to determine specificity for non-HMO O-type glycans, and (5) a GlcNAc- β 1,3-Gal- β 1,4-GlcNAc- β 1,3-Gal- β 1,4-Glc pentasaccharide structure to test the formation of a non-reducing terminal type-I (Gal-b1,3-

) cap on a longer acceptor. We explored the activities of various gene products to perform specific glycosyltransferase reactions crucial to HMO biosynthesis (**Figure 6, Table S 5**).

In the cross-cohort aggregate analysis (**Figure 5B**), B3GNT2 is selected as a reasonable candidate to catalyze flux through the L1:b3GnT reaction. The B3GNT2 support score is nearly 100 times more significant than B3GNT8, the next most associated gene. Consistent with the predictions that b3GnT should convert lactose into the precursor to LNT and LNnT, the UDP-Glo™ assay showed B3GNT2 had high activity toward lactose as an acceptor. We further found that B3GNT2 could add a β 1,3-GlcNAc to LNnT as is necessary for poly-lacNAc HMOs. The cross-cohort aggregate analysis (**Figure 5B**) selected ST6GALNAC2 to perform L10, the α 2,6 addition of sialic acid to the internal β 1,3-GlcNAc; necessary for the biosynthesis of LSTb from LNT and possibly DSLNT from LSTa. However, the CMP-GLO™ assay highlighted a negligible activity of ST6GALNAC2 toward LNT even at very high enzyme input indicating that this enzyme does not convert LNT to LSTb. We did not test if it can convert LSTa to DSLNT. In contrast, ST6GALNAC5 was effectively able to use LNT as an acceptor, although we did not confirm the formation of the LSTb structure. ST6GALNAC5 could not be considered in the support score calculation because it was only measured in cohort 2; expression was greater than zero in 1 of 12 samples.

Finally, we tested the affinities of plausible ST3GAL isoforms to sialylate LNT, LNnT or β 1,3-GlcNAc (**Table S 5**). The multi-cohort analysis (**Figure 5B**) implicates ST3GAL1 as the best candidate for this reaction. The CMP-Glo™ assay indicated that ST3GAL1 has limited activity toward LNT but high activity toward Gal β 1,3-GlcNAc suggesting ST3GAL1, *in vitro*, is more involved in non-HMO O-type glycan biosynthesis. ST3GAL2 showed a similar but less substantial pattern. ST3GAL3 showed the strongest activity for sialylation both LNT and LNnT suggesting it could synthesize LSTa from LNT. ST3GAL6 shares a similar but lesser activity for LNT and LNnT.

We analyzed the original expression profiles to determine which genes were sufficiently expressed to actuate this activity. STGAL1, 3 and 5 were strongly expressed in nearly 100% of samples across both cohorts; ST3GAL2 and 4 show zero expression in 75% of samples in at least one cohort (**Figure S 6**). ST3GAL3 was highly expressed and effective at catalyzing the L4 reaction for LNT and LNnT while ST3GAL1 was highly expressed and weakly catalyzed sialylation of LNT making ST3GAL3 the most likely candidate for L4 reaction on LNT and LNnT.

Figure 7 - de novo promoter-enriched TF motifs and IPA predicted TFs using differential expression analyses with respect to 16 HMOs. (A) MEME identified TF motifs and 5 known TFs (ETV4, ETS1, EGR1, SP1, and ERG) associated with them (see **Table S 6**). MEME-discovered TFs were cross-referenced with known TF binding sites using TOMTOM. Logos for the matched known and discovered motifs are shown in the top and bottom of each subpanel; the p-value is a logo matching significance calculated by TOMTOM. (B) Subset of a biclustering of activation z-score computed by IPA indicating the likelihood that a TF activates ($z > 0$) or inhibits ($z < 0$) an HMO concentration signature (gene expression associated with changes in HMO concentration). The full biclustering can be found in the supplement (**Figure S 16**)

Table 2 - TF motif (MEME) and IPA upstream regulator integrated results

LINKAGE	REACTION SUPPORT SCORE SELECTED CANDIDATE	MEME TF MOTIF (P-VALUE)*1	JASPAR TF (P-VALUE)*2	IPA PREDICTED TF*3
L1:B3GNT	B3GNT2	TF Motif-II (1.39e-12)	SP1 (4.96e-05) EGR1 (2.17e-05)	Y
L2:A2FUCT	FUT2	TF Motif-III (3.63e-16)	IKZF1 (7.62e-04)	Y
L3:A3FUCT	FUT11	TF Motif-II (1.00e-7)	SP1 (4.96e-05) EGR1 (2.17e-05)	Y
L4:ST3GAL T	ST3GAL1	TF Motif-I (2.76e-11)	ETV4 (1.24e-03) ETS1 (3.01e-04) ERG (3.50e-04)	Y Y Y
L7:B4GALT	B4GALT4	TF Motif-II (7.67e-11)	SP1 (4.96e-05) EGR1 (2.17e-05)	Y Y
L10: ST6GNT	ST6GALNAC2	TF Motif-II (1.08e-7)	SP1 (4.96e-05) EGR1 (2.17e-05)	Y Y

*1. The P-value (see **Figure S 15**) is the significance of the selected GT to the MEME identified TF motif.

*2. The P-value (see **Table S 7**) is the significance of known TF associated with the MEME identified TF motif.

*3. The IPA upstream regulator analyses were conducted on the three different sets of DEGs: 16 HMOs, 19 glycan motifs, and 4 differential motifs (see Methods for details). Based on the Z-score predicted by IPA using the gene expression data, we selected the significant TFs with IPA predicted activation score $|Z \text{ value}| \geq 3$ in this study. Note, 'Y' denotes the known TF is presented in the indicated dataset (HMO (**Figure 7**, **Figure S 16**), Motif (**Figure S 17**), or differential motif (**Figure S 18**)) of the IPA predicted TF and 'N' means the TF doesn't present in the dataset of IPA predicted TF.

2.5 SELECTED GLYCOSYLTRANSFERASES SHARE TRANSCRIPTIONAL REGULATORS ACROSS INDEPENDENT PREDICTIONS

To explore the transcriptional regulation during lactation, we used two orthogonal approaches for transcription factor (TF) discovery. We used Ingenuity Pathway Analysis (IPA) to predict upstream regulatory factors based on differential expression associated with each HMO. IPA analyzed all genes differentially expressed with HMO abundance, not only HMO glycogenes; these differential expression patterns formed HMO specific gene expression signatures. Additionally, we used MEME for *de novo* motif discovery in the promoter regions of HMO glycogenes and TOMTOM to map those discovered motifs to known TFs. We validated these predictions by examining transcriptional regulators selected by both MEME and IPA (**Figure S 13**, see Methods).

IPA discovered 57 TFs significantly ($|z| \geq 3$; $p < 0.001$) associated with the 16 HMO-specific gene expression signatures. We performed differential expression on HMO substructure abundance and substructure abundance ratios¹⁷; IPA found 66 and 49 TFs significantly ($|z| \geq 3$; $p < 0.001$) associated with HMO substructure and substructure ratio specific gene expression signatures. Using MEME, we

identified three putative TF regulatory sites (TF motifs I, II and III) for 6 selected glycosyltransferases responsible for the HMO biosynthesis (**Table 2** and **Figure S 15**). TOMTOM calculated that these putative binding sites were significantly associated with six known TFs (IKZF1, SP1, EGR1, ETS1, ETV4 and ERG) that were also predicted by IPA as regulators of gene signatures associated with HMO concentration (**Figure 7**, **Figure S 16**) or HMO glycan substructures abundance (**Figure S 17**). SP1, EGR1, ETS1, ETV4 and ERG are all predicted to positively influence expression associated with the biosynthetically related HMOs: 3'SL, 3FL, LSTb and DSLNT; 3'SL and 3FL share a common substrate (lactose) while LSTb is a likely precursor to DSLNT. The motif-level analysis showed opposing regulation between IKZF1: upregulating gene expression signatures associated with the 3'SL and LSTb substructure abundance¹⁷ (X34 and X62 respectively, see **Figure S 19**) and downregulating gene expression associated with GlcNAC-lactose, LNT and LNFPI substructure abundance (X18, X40 and X65 respectively, see **Figure S 19**), while EGR1, ERG and ETS1 have the opposite predicted impact (**Figure S 17**). The motif-level predictions are consistent with the HMO-level predictions of upregulation on 3'SL and LSTb while adding an additional point of contrast. While EGR1, ERG and ETS1 are predicted to increase production of sialylated HMOs, they may have the opposite impact on LNFPI. Thus, we detect signatures of multiple transcription factors that could coordinate the regulation of the genes we identified to contribute to HMO biosynthesis (see supplemental discussion).

3 DISCUSSION

By integrating sample-matched quantitative oligosaccharide measurements and gene expression data using computational models of HMO biosynthesis, we resolved genes responsible for 10 elementary reactions in human mammary gland epithelial cells. The modeling-based strategy was essential since simple correlations failed to capture the simplest HMO-gene associations, given the complex interactions of glycosyltransferases in the HMO biosynthetic pathway. Because the pathway characterization is still incomplete, we built >44 million candidate models that uniquely recapitulate glycoprofiling data in two independent cohorts. Candidate model flux, i.e. activity of each reaction, was predicted for each model and compared to sample-matched gene expression data. We used the consistency between gene expression and predicted flux across cohorts in high-performing models to select genes for each fundamental reaction. Analysis of these models suggested glycosyltransferase genes, thus providing a clearer picture of the enzymes and regulators of HMO biosynthesis in mammary epithelial cells. The clarification of the pathways and enzymes involved in HMO biosynthesis will be an invaluable resource to help (1) discover the maternal genetic basis of health-impacting^{1,2,5,6,37-46} HMO composition heterogeneity^{7,26,47,48} and (2) drive chemoenzymatic synthesis⁴⁹⁻⁵³ and metabolic engineering for manufacturing HMOs for food ingredients, supplements and potential therapeutics⁵⁴⁻⁵⁹ (see supplemental discussion).

Of the three fucosylation reactions, two were determined using expression data alone while the third required additional insight from the flux-expression comparison or, support score. Consistent with studies in blood²³⁻²⁵ and milk^{26,47,60} types, we selected FUT2 as the gene supporting the α 1,2-fucosylation (L2:a2FucT) linkage reaction. FUT1 was ruled out due to non-expression (**Table S 1**,

supplemental results). In the second fucosylation reaction, FUT3, FUT4 and FUT11 all show significant support for α 1,3-fucosylation (L3:a3FucT) linkage formation. FUT11 is more commonly considered an N-glycan-specific transferase⁶¹ and therefore a less likely candidate. Both FUT3 and FUT4 prefer to fucosylate the inner GlcNAc of a type-I polylactosamine⁶². FUT3 prefers neutral type-I polylactosamine while FUT4 also fucosylates the sialylated form^{63,64}; the charge preferences are inverted for type-II polylactosamine acceptors⁶⁵. Prudden et. al.⁵² used FUT9 to perform this reaction, consistent with its ability to transfer α 1,3 fucose to the distal GlcNAc of a neutral polylactosamine⁶¹⁻⁶³. The four HMO structures with α 1,3-Fucose in the Summary Network (**Figure 4**) include 3FL (neutral inner fucosylation), LNFPIII (neutral distal fucosylation), DFLNT2 (neutral inner fucosylation), and FDSLNH2 (sialylated and neutral distal fucosylation). FUT9 showed negligible expression in RNA-Seq (3rd Quartile TPM=0.37, **Table S 1**), yet it is highly expressed (TPM>10) brain and stomach³². Therefore, it is likely that the distal fucosylation is conducted by another enzyme *in vivo* while the inner fucosylation is likely performed by either FUT3 or FUT4. FUT3 was also chosen for the α 1,4-fucosylatoin (L9:a4FucT) by default due to the non-expression of FUT5, confirmed by RNA-Seq (**Table S 1**, supplemental results). FUT3 adds an α 1,4-fucose to the GlcNAc of a neutral type-I chain to form the Lewis-A or Lewis-B group and adds an α 1,3-fucose to the GlcNAc of a type-II chain^{63,64}. Usage of FUT3 would provide a parsimonious explanation for the fucosylation of both type-I and type-II HMOs like LNFPII (Fuc- α 1,4-LNT (type-I)) and LNFPIII (Fuc- α 1,3-LNnT (type-II)).

One of two sialyltransferases was clearly resolved with expression data alone, the other required additional examination. ST6GAL1 was chosen by default to support the α 2,6-sialylation (L5:ST6GalT) reaction due to the non-expression of ST6GAL2 (**Table S 1**). ST6GAL1 sialylates galactose in HMOs⁵². For the second sialylation reaction, our flux-expression comparison selected ST6GALNAC2 and ST6GALNAC6 as the significant supporters of α 2,6 sialylation (L10:ST6GnT). Through a kinetic assay, we confirmed that ST6GALNAC2 (previously shown to accept core-1 O-glycans^{66,67}) fails to sialylate LNT. Though our kinetic assay shows that ST6GALNAC5 (known to sialylate GM1b⁶⁸) can sialylate LNT, it was not expressed in this context (**Table S 1**, supplemental results). ST6GALNAC3 expression was not observed in microarrays but could not be ruled out due to RNA-Seq expression (**Table S 1**, supplemental results); it sialylates the GalNAc of NeuAc- α 2,3-Gal- β 1,3-GalNAc- α 1-O-Ser/Thr and NeuAc- α 2,3-Gal- β 1,3-GalNAc- β 1,4-Gal- β 1,4-Glc- β 1-Cer when the inner galactose is not sialylated (e.g. GD1a or GT1b)⁶⁹⁻⁷² but has not been shown to transfer to a GlcNAc. The last ganglioside-accepting family gene, ST6GALNAC6, has broader activity accepting several gangliosides (GM1b, GD1a, and GT1b)⁶⁹ and sialylating the GlcNAc of LNT-ceramide⁷³. Considering the broader activity, clear expression and computational selection, ST6GALNAC6 is the most likely candidate, though ST6GALNAC3 should not be ruled out. In the third reaction, ST3GAL1 shows significant support for α 2,3-sialylation (L4:ST3GalT) reactions while ST3GAL3 shows negligible consistency in the flux-expression comparison. Yet, *in vitro*, ST3GAL3 was most effective at sialylating both LNT and LNnT in kinetic assays while ST3GAL1 weakly sialylated LNT. ST3GAL4, which prefers type-II acceptors⁷⁴⁻⁷⁶, was used previously to perform this reaction *in vitro*⁵², but it was not expressed on the microarrays nor RNA-Seq. ST3GAL3 can accept type-I, type-II and type-III acceptors including LNT and prefer type-I acceptors^{74,75,77} while ST3GAL1 accepts type-I, type-III and core-1 acceptors but not type-II^{74,75,78}. The kinetic assays and previous literature show ST3GAL3 is more capable than ST3GAL1 at catalyzing this reaction, while ST3GAL1 expression was found to be the only plausible candidate

based on estimated flux through this reaction. If ST3GAL1 were responsible for this reaction, its inability to sialylate type-II HMO could partially explain the lack of sialylation and larger structures in the type-II HMO branch. Both ST3GAL1 and ST3GAL3 remain plausible candidate genes, and further *in vivo* studies are needed. Both galactosylation reactions required further examination of flux-expression relationships. We found B3GALT4 to significantly support the type-I β -1,3-galactose addition (L6:b3GalT). B3GALT4 can transfer a galactose to GalNAc in the synthesis of GM1 from GM2⁷⁹. Unlike B3GALT5, there is no evidence that B3GALT4 can transfer galactose to a GlcNAc⁸⁰. B3GALT5, has been shown to transfer a β -1,3-galactose to GlcNAc to form LNT *in vitro*⁸¹. B3GALT5 expression measured for cohort 1 microarray was much lower than expression in cohort 2 and the independent RNA-Seq³¹ suggesting that the probes in the first microarray may have failed (Table S 1, supplemental results). While both B3GALT4 and B3GALT5 seem plausible, given the historical failures of B3GALT4 to perform this reaction and our likely failure to measure and evaluate B3GALT5, B3GALT5 may be the stronger candidate for this reaction. In the second galactosylation reaction, the flux-expression comparison found B4GAL4 and B3GALT3 most significantly supports the type-II definitive β -1,4-galactose addition (L7:b4GalT). These gene-products can synthesize LNnT-ceramide⁸². Additionally, in the presence of α -lactalbumin (highly expressed during lactation), B4GALT4 shows an increased affinity for GlcNAc acceptors suggesting during lactation it is more likely to perform the L7 reaction^{82,83}. B4GALT1 and B4GALT2 synthesize lactose in the presence of α -lactalbumin during lactation^{35,36}, but B4GALT1 expression was not correlated with L7 flux and B4GALT2 was not expressed (Table S 1). We note that associations between B4GALT1 expression L7 flux may be masked due to its consistent high. Therefore, flux-expression correlation should not be used to exclude B4GALT1 as a candidate for the L7 reaction. Doing so, B4GALT4, B4GALT3 and possibly B3GALT1 remain the most plausible candidates.

Finally, both GlcNAc additions required flux-expression examinations. B3GNT2 showed significant support in the flux-expression comparison. In our kinetic assays, B3GNT2 demonstrated high activity towards lactose as an acceptor. Previously, B3GNT2 has performed the β -1,3-GlcNAc addition (L1:b3GnT) on multiple glycan types including several HMOs: lactose, LNnT, polylactosamine-LNnT⁸⁴. The agreement of literature, kinetic assays and flux-expression analysis indicate B3GNT2 is an appropriate choice for this reaction. In the second GlcNAc reaction, GCNT3 and GCNT1 most significantly support the branching β -1,6-GlcNAc addition (L8:b6GnT). While GCNT2B can effectively transfer the branching GlcNAc to the inner galactose of LNnT^{52,85}, it was not expressed in the cohort microarrays or independent RNA-Seq. GCNT1 transfers a branching GlcNAc to the GalNAc of a core-1 O-glycan^{86,87} while GCNT3 acts on core-1 and the galactose of the LNT-like core-3 structure^{88,89}. GCNT3 is also specifically expressed in mucus-producing tissues^{88,89} like lactating mammary gland epithelium. Interestingly, GCNT3 acts on galactose of the GlcNAc- β 1,3-Gal- β 1,4-Glc trisaccharide (predistally) while GCNT2 acts on the central galactose of the LNnT or LNT tetrasaccharide (centrally)⁸⁵. Therefore, reliance on GCNT3 for the branching reaction would explain the noncanonical branched tetrasaccharide (HMO8, Figure 4) suggesting a third major branch from GlcNAc- β 1,6-lactose, distinct from LNT and LNnT. Predistal addition of the branched GlcNAc may also explain the lack of branched type-II structures since B4GALT4 cannot act on branched core-4 structures⁹⁰. HMO biosynthesis with GCNT3 and B4GALT4 could explain the type-I bias seen in the Summary Network (Figure 4).

Our results show consistency with experimental validation here and the published literature. Further direct empirical studies will be invaluable to confirm each gene-reaction association and the complete biosynthesis network. Such studies would include further clinical cohort studies and the development of mammary organoid models capable of producing HMOs. Such experimental systems can clarify the impact of mammary-tissue specific genes, cofactors, and HMO chaperones like α -lactalbumin^{82,83} on glycosyltransferase activity. Therefore, further development of authentic *in vitro* cell and organoid models will be invaluable to finalizing our model of HMO biosynthesis.

4 CONCLUSION

By using systems biology approaches, different omics data can be integrated, as shown here to predict gene-reaction relations even in highly uncertain and underdetermined networks. Of the ten fundamental reactions we aimed to resolve and reduce (**Table 1**), we succeeded in narrowing the candidate substantially for each one. The newly reduced space of HMO biosynthetic pathways and knowledge of the enzymes and their regulation will enable mechanistic insights into the relationship of maternal genotype and infant development. Finally, once essential HMOs are identified, the knowledge presented here on the HMO biosynthetic network can provide insights for large-scale synthesis of HMOs as ingredients, supplements, or potential therapeutics to further help improve the health of infants, mothers, and people of all ages.

5 AUTHOR CONTRIBUTION

BK, AR, LB and NEL designed and performed the study and wrote the manuscript. ABB performed preliminary analysis. AR performed modeling analyses. BK analyzed and interpreted the models analyses. MAM and MWH provided samples. DC, JYY, JN, KM and LB performed expression, purification of glycosyltransferases and kinetic assays. AR, BK, and NK performed literature surveys to determine appropriate candidate genes for each reaction. BK and BB performed motif-level analysis. BK and AWTC performed transcription factor analysis.

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7 MATERIALS AND METHODS

7.1 MILK SAMPLE COLLECTION

Samples were collected following Institutional Review Board approval (Baylor College of Medicine, Houston, TX). Lactating women 18-35 years of age with uncomplicated singleton pregnancy, vaginal delivery at term (>37 weeks), Body Mass Index <26 kg/m² without diabetes, impaired glucose tolerance, anemia, or renal or hepatic dysfunction were given informed consent before sample collection. Description of the protocols used to collect milk samples and the diversity of subjects present in both datasets. Cohort 1 consists of 8 samples for each of the 6 subjects (48 samples total) including milk from 4 secretor mothers and 2 non-secretor mothers spanning from 6 hrs to 42 days postpartum. Sample collection was previously described^{28,29}. Cohort 2 consists of 2 samples over each of the 5 (10 samples total) including samples from 4 secretor mothers and 1 non-secretor mother spanning 1 to 2 days postpartum. Sample collection was previously described³⁰.

7.2 ILLUMINA MRNA MICROARRAYS & GLYCOPROFILING

All expression and glycoprofiling measurements were sample-matched. Therefore, comparisons across data-types occurred within each individual sample described in the previous section. Not all samples in these studies have both microarray and glycoprofile measurements, only the samples described in the previous section have matched glycomics and transcriptomics data.

mRNA was isolated from TRIzol-treated milk fat in each sample. Expression in cohort 1 was measured using HumanHT-12 v4 Expression Beadchip microarrays (Illumina, Inc.) with ~44k probes. Extraction of mRNA and measurement of expression in milk samples was performed as previously described^{28,29}. Gene expression data for cohort 1 were retrieved from the Gene Expression Omnibus at accession: GSE36936. Cohort 2 gene expression data were measured using a Human Ref-8 BeadChip array (Illumina, Inc) with ~22k probes. Extraction of mRNA and related methods were previously described³⁰. Expression data for cohort 1 can be accessed at accession: GSE12669. Both microarrays were background corrected. The cohort 1 microarray was normalized using cubic spline normalization and the cohort 2 microarray was normalized using the robust spline normalization.

As previously described^{41,91}, HMO composition and abundance data were collected using high-performance liquid chromatography (HPLC) with 2-aminobenzamide (CID: 6942) derivatization and a raffinose (CID:439242) standard. 16 HMOs were measured using retention time and commercial standards including 2-fucosyllactose (2'FL), 3-fucosyllactose (3FL), 3-sialyllactose (3'SL), lacto-N-tetraose (LNT), lacto-N-neotetraose (LNnT), lacto-N-fucopentaose (LNFP1, LNFP2 and LNFP3), sialyl-LNT (LSTb and LSTc), difucosyl-LNT (DFLNT), disialyllacto-N-tetraose (DSLNT), fucosyl-lacto-N-hexaose (FLNH), difucosyl-lacto-N-hexaose (DFLNH), fucosyl-disialyl-lacto-N-hexaose (FDSLNH) and disialyl-lacto-N-hexaose (DSLNH). Technicians were blinded to sample metadata. HMO composition and abundance measurement for cohort 1 were fully described in¹⁷. Measurements for cohort 2 are previously unpublished and used the same methodology.

7.3 SOFTWARE

Modeling of HMO biosynthesis was performed in Matlab 2016b using the CobraToolbox⁹². All analysis of biosynthetic models, interpretation and statistics were performed in R v3.5 and v3.6. In R, we used *bigmemory*, *bigalgebra* and *biganalytics* to handle the millions of models and associated statistics⁹³. We used *metap* for pooling p-values⁹⁴.

7.4 GENERATION AND SCORING OF GLYCOSYLATION NETWORK MODELS

Here we attempt to determine the genes responsible for making HMOs through the construction and interrogation of models of their biosynthesis. Similar to the other biosynthetically constrained glycomic models like the milk metaglycome²¹, Cartoonist⁹⁵ and several N-glycome simulations^{13,96–98}, we began with a set of elementary reactions. Enumerating all feasible permutations of the elementary reaction (**Figure 3A**; S1.1.1), we delineated every possible reaction series from lactose to each of the 16 most abundant HMOs. Of the measured HMOs, 11 have fully determined molecular structures, while the remaining five have multiple candidate structures (**Figure 1C**, **Figure S 12**)^{6,8,34,99–101}. The set of all possible reactions leading to characterized and ambiguous structures formed the Complete Network (**Figure 3B**; Supplemental Methods S1.1.1). Though non-lysosomal glycosidase^{102–104} reactions are not explicitly specified, they are implicitly encoded in the flux. To reduce the Complete Network to a more manageable size, we identified and removed all reactions that do not lead to observed oligosaccharides using Flux Variability Analysis (FVA; Supplemental Methods S1.2.4;^{105–107}). This trimming (**Figure 3C**; Supplemental Methods S1.1.2) defines the Reduced Network (**Figure 3D**; Supplemental Methods S1.1.2). The Reduced Network describes many candidate models that can uniquely simulate the HMO abundance collected through High-Performance Liquid Chromatography (HPLC). A mixed integer linear programming (Supplemental Methods S1.2.5;^{108,109}) approach was employed to extract candidate models from the Reduced Network capable of uniquely recapitulating the HPLC data with minimal reactions (**Figure 3E**; Supplemental Methods S1.1.3). The reactions of each candidate model were parameterized to determine the necessary flow of material (flux) through each reaction to reproduce the measured oligosaccharide profiles (**Figure 3F**; Supplemental Methods S1.1.3; S). The models were ranked by the consistency between the predicted flux and the expression of genes believed to be associated with each reaction (**Figure 3G**; Supplemental Methods S1.1.4). This consistency is evaluated by the Spearman correlation of changes in flux and gene expression across subjects (**Figure 3H**; Supplemental Methods S1.1.4.1).

7.5 CANDIDATE MODEL RANKING, MODEL SELECTION AND SELECTION VALIDATION

Model scores, indicating the consistency between flux and gene expression (S1.1.4.1), were used to rank candidate models (S1.1.4.2). The distribution of model scores computed from each dataset were approximately normal, as evidenced by their linear Q-Q plots. This permitted the construction of a background normal distribution of model scores (**Figure S 20**). We then selected high-performing models, those with z-score normalized model scores greater than 1.646 (i.e., greater than the top 5% of scores from a normal distribution) for further study. Model selection was performed on scores computed independently for cohort 1 and cohort 2. Commonly high-performing models were those that perform well in both cohort 1 and cohort 2. Hypergeometric enrichment was used to confirm

that the top cohort 1 and cohort 2 models significantly overlapped. (see Supplemental Methods S1.1.4.2)

7.6 SUMMARY NETWORK EXTRACTION FROM THE REDUCED NETWORK

The Summary Network relates a heuristic selection of the most important reactions in the HMO biosynthesis network as measured by proportion of inclusion in the commonly high-performing models and enrichment in the commonly high-performing models relative to the background. Paths drawn from observed HMOs to the root lactose were scored for their aggregate importance. The top 5% of paths leading to each observed HMO were retained to form the Summary Network (Supplemental Methods see S1.1.4.3).

7.7 AMBIGUOUS GENE SELECTION

We aimed to match 10 elementary glycosyltransferase reactions to the supporting genes (**Table 1**). Candidate genes were filtered from the relevant gene families to exclude gene products well known to perform unrelated reactions (**Table 1**). Candidate genes were first evaluated for expression in breast epithelium samples including microarrays in this study, independent RNA-Seq (GSE45669)³¹ and comparison to global expression distributions in GTEx³²; genes unmeasured by microarray in at least 75% of microarray samples (3rd Quartile, Q3) within each cohort were excluded unless they were non-negligibly expressed in the independent RNA-Seq ($\text{TPM}_{\text{Lemay}} > 2$ or $\text{TPM}_{\text{Lemay}} > \text{Median}(\text{TPM}_{\text{GTEx}})$) (see supplemental results, **Table S 1**, **Figure S 7**).

We used the model score definition, which quantifies how well the genes explain a model, i.e., if the expression of the genes are best correlated to the normalized flux of the reaction (**Figure S 11**, S1.1.4) they are proposed to support. We examined each gene contribution to the overall model score in three ways to determine a consensus support score for each gene-reaction association (see S1.1.5.2).

The first metric we examined was the proportion (PROP) of commonly high-performing models best explained by an isoform relative to the proportion of background models that select that same isoform. The second metric was the average gene-linkage score (GLS) in high-performing models, i.e., the Spearman correlation between the normalized flux (**Figure S 11**, S1.1.4) and gene expression of corresponding candidate genes. The gene-linkage score is a continuous measure of the consistency between each gene with the flux it was proposed to support. Because it considers every gene, not just the most flux-consistent gene, it is helpful for judging performance when the most flux-consistent gene is more ambiguous. The third metric was the model-score contribution (MSC). MSC quantifies the Pearson correlation between the gene-linkage score, the gene expression consistency with the normalized flux, and the overall model score (i.e., the average correlation of all most-flux-consistent genes). The model score indicates the frequency with which a gene is the most flux-consistent gene normalized by its contribution relative to the other most flux-consistent genes in that model.

An aggregate reaction support score was constructed to describe performance within each individual score (PROP, GLS, and MSC) and consistency across cohorts. To measure significance, the gene-linkage score matrix (i.e., Spearman correlation between each candidate gene and the corresponding normalized flux for each model) was shuffled ($n=27$) and all analyses rerun on each shuffle to generate a permuted background distribution for PROP, GLS and MSC; shuffling of the GLS matrix was done using a perfect minimal hash to remap all entries back to the GLS matrix in a random

order¹¹⁰. Performance within each independent cohort was described as the sum of z-scores for each of three measures; z-score was calculated relative to the mean and standard deviations of these scores in the permutation results. Consistency across cohorts was determined by pooling p-values using the Fisher's log-sum method^{94,111}. The score presented in **Figure 5B** is the $-\log_{10}(\text{FDR}(\text{cohort-pooled-p}))$.

7.8 *IN VITRO* GLYCOSYLTRANSFERASE ACTIVITY ASSAYS

Recombinant forms of the respective glycosyltransferases were expressed and purified as previously described¹¹². Enzyme activity was determined using the UDP-GloTM or UMP/CMP-GloTM Glycosyltransferase Assay (Promega) that determined UDP/CMP concentration formed as a by-product of the glycosyltransferase reaction. Assays were performed according to the manufacturer's instructions using reactions (10 μL) that consisted of a universal buffer containing 100 mM each of MES, MOPS, and TRIS, pH 7.0, donor (1 mM UDP-GlcNAc (Promega) for B3GNT2; 1 mM UDP-Gal (Promega) for B3GALT2; 0.2 mM CMP-SA (Nacalai USA Inc.) for ST3GAL1-6, ST6GALNAC2, and ST6GALNAC5), 1 mM acceptor (lactose (Sigma) and lacto-N-neotetraose (LNnT) (Carbosynth) for B3GNT2; lacto-N-tetraose (LNT, Bode lab) and pentasaccharide (GlcNAc-b1,3-Gal-b1,4-GlcNAc-b1,3-Gal-b1,4-Glc, Boons lab, University of Georgia) for B3GALT2; LNnT, LNT, and Gal- β 1,3-GalNAc (Carbosynth) for ST3GAL1-6; LNT for ST6GALNAC2 and ST6GALNAC5. The B3GNT2 and B3GALT2 assays also contained 1 mg/ml BSA and 5 mM MnCl_2 . Assays were carried out for 1 h (B3GNT2, B3GALT2, ST6GALNAC2, and ST6GALNAC5) or 30 min (ST3GAL1-6) at 37 °C. Reactions (5 μL) were stopped by mixing with an equal volume of Detection Reagent (5 μL) in white polystyrene, low-volume, 384-well assay plates (Corning) and incubated for 60 min at room temperature. After incubation, luminescence measurements were performed using a GloMax Multi Detection System plate reader (Promega). The average luminescence was subtracted from the average luminescence of respective blank to correct for background. Background and reaction measurements were performed in triplicate.

7.9 DIFFERENTIAL EXPRESSION (DE) ANALYSIS

The differential expression analysis was conducted on three different datasets: 1) 16 different HMOs (2'FL, 3'SL, 3FL, FLNH, LNT, LNnT, LSTb, LNFP-III, LNFP-II, LNFP-I, DFLNT, LSTc, DSLNT, FDSLNH, DSLNH, DFLNH), 2) 19 glycan motifs (X18, X32, X34, X35, X37, X40, X62, X63, X64, X65, X66, X94, X106, X113, X120, X127, X141, X142, X143, see **Figure S 19**), and 3) 4 differential motifs for the difference ("conversion rate") between related motifs (X65-X40, X106-X62, X63-X37, X62-X40, see **Figure S 19**). Substructure abundance for glycan motifs and conversion ratios were computed using Glycompare v1¹⁷. The gene expression data were downloaded from the Gene Expression Omnibus¹¹³ (GSE36936). Specifically, for each HMO, motif or differential motif, we used concentration (e.g., HMO-3FL) as the predictor for gene expression in the differential expression analysis (e.g., "gene expression ~ [3FL]"). The differential expression analysis was performed by fitting linear models using empirical Bayes method as implemented in the *limma* v3.40.6 in R v3.6.1 package¹¹⁴ and p-values were adjusted for multiple testing using Benjamini-Hochberg (BH) method¹¹⁵. In this way, we determined gene-expression signatures indicative of each HMO and motif abundance.

7.10 INGENUITY PATHWAYS ANALYSIS (IPA) UPSTREAM REGULATOR

Differential expression signatures indicative of differential abundance in 16 HMOs, 19 motifs and 4 differential motifs were analyzed to predict upstream regulators using Ingenuity Pathway Analysis (IPA, QIAGEN Inc.). Gene expression signatures indicative of HMO and motif abundance were defined as genes differentially expressed with abundance in the previous *limma* analysis (FDR $q < 0.05$ and $|\text{Fold Change}| > 1.5$).

7.11 DE NOVO TF BINDING SITE MOTIFS DISCOVERY AND KNOWN TF BINDING SITE IDENTIFICATION

We downloaded promoter sequences (file: “*upstream1000.fa.gz*”; version: GRCH38) from UCSC Genome Browser public database (<https://genome.ucsc.edu/>) for the O-glycosyltransferase genes used in this study (Table S 1). These promoter sequences included 1,000 bases upstream of annotated transcription starts of RefSeq genes with annotated 5' UTRs. To conduct *de novo* TF binding site motifs discovery, we first applied the motif discovery program MEME¹¹⁶ to identify candidate TF binding site motifs on the downloaded promoter sequences with default parameters. The 10 TF binding site motifs found by MEME were analyzed further for matches to known TF binding sites for mammalian transcription factors in the motif databases, JASPAR Vertebrates¹¹⁷, via motif comparison tool, TOMTOM¹¹⁸. The resulting discovered TF binding site motifs and their significantly associated known TF binding sites (Table S 6, Table S 7) for mammalian transcription factors were used further to compare with the IPA predicted upstream regulators.

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A **B** **C**

A. Reaction Rules

Substrates

Products

B. Complete Network

C. Trimmed Complete Network (FVA)

D. Reduced Network

E. Candidate Models (MILP)

F. Model Flux (FBA)

G. Model Score

H. Model Score Distributions

I. Commonly Selected Models

A

Examined DFLNT Isomers

Galactose (Gal)

Glucose (Glc)

N-Acetylglucosamine (GlcNAc)

Fucose (Fuc)

Sialic Acid (Neu5Ac)

B

A.

ETV4
ETS1
ERG
EGR1
SP1

B.