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Recommended Citation

Agrawal, P., Kurcon, T., Pilobello, K. T., Rakus, J. F., Koppolu, S., Liu, Z., ... & Mahal, L. K. (2014). Mapping posttranscriptional regulation of the human glycome uncovers microRNA defining the glycocode. *Proceedings of the National Academy of Sciences*, 111(11), 4338-4343.

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Mapping posttranscriptional regulation of the human glycome uncovers microRNA defining the glycode

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Edited* by Carolyn R. Bertozzi, University of California, Berkeley, CA, and approved February 4, 2014 (received for review November 18, 2013)

Cell surface glycans form a critical interface with the biological milieu, informing diverse processes from the inflammatory cascade to cellular migration. Assembly of discrete carbohydrate structures requires the coordinated activity of a repertoire of proteins, including glycosyltransferases and glycosidases. Little is known about the regulatory networks controlling this complex biosynthetic process. Recent work points to a role for microRNA (miRNA) in the regulation of specific glycan biosynthetic enzymes. Herein we take a unique systems-based approach to identify connections between miRNA and the glycome. By using our glycomic analysis platform, lectin microarrays, we identify glycosylation signatures in the NCI-60 cell panel that point to the glycome as a direct output of genomic information flow. Integrating our glycomic dataset with miRNA data, we map miRNA regulators onto genes in glycan biosynthetic pathways (glycogenes) that generate the observed glycan structures. We validate three of these predicted miRNA/glycogene regulatory networks: high mannose, fucose, and terminal β -GalNAc, identifying miRNA regulation that would not have been observed by traditional bioinformatic methods. Overall, our work reveals critical nodes in the global glycosylation network accessible to miRNA regulation, providing a bridge between miRNA-mediated control of cell phenotype and the glycome.

glycan regulation | carbohydrate biosynthesis | systems biology | epigenetics | NCI-60

Cell surface glycans form a critical interface with the biological milieu, informing diverse processes from cellular migration to pathogen–cell interactions. Assembly of discrete carbohydrate structures requires coordination of a complex repertoire of proteins, including glycosyltransferases, glycosidases, and sugar nucleotide transporters, acting in tandem (1, 2). Little is known about the regulation of the complex biosynthetic networks required for glycosylation. MicroRNAs (miRNAs) are small non-coding RNAs that can bind the 3′-UTR of mRNA and inhibit mRNA stability or translation (3). Recent work points to a role for miRNA in modulating the levels of glycan biosynthetic enzymes (glycogenes) (4–11) with profound biological consequences, including promotion of tumor metastasis (5) and regulation of neuronal migration (9). Enrichment of glycogene mRNA in miRNA/mRNA/RNA-induced silencing complex-complexes during *Caenorhabditis elegans* development hints that miRNA may be a major regulator of glycosylation (12). Studying the relationship between the glycome and miRNA is complicated by the low abundance of glycogene transcripts, resulting in inaccuracies in microarray-based expression analysis, the basis for most miRNA target prediction algorithms (13, 14). These algorithms predict hundreds to thousands of gene targets with a precision between ~30% and 50% (15, 16). Target prioritization is often based on mRNA conservation across species (15), which presents a second issue for glycogenes as glycosylation is a rapidly evolving system (17). We reasoned that a systems-based approach integrating glycosylation patterns, the functional outcome of mRNA regulation, with miRNA expression would allow us to map miRNA onto

glycan biosynthetic pathways. Harnessing the power of lectin microarrays, our glycomic platform, we demonstrate that miRNAs are critical modulators of the human glycome and identify miRNA regulation of glycogenes elusive to current prediction algorithms.

Results

Glycomic Analysis of the NCI-60 Reveals Tissue Type-Specific Glycan Signatures. Lectin microarrays, in which carbohydrate-binding proteins are probes for glycan structure, provide a systems-level view of the glycome (Fig. 1A) (18–20). These microarrays give specific information on the repertoire of glycans present, e.g., high mannose epitopes, branching patterns, and terminal α -2,3- or α -2,6-sialic acids, in a high-throughput format. Unlike other glycomic methods, both the *N*- and *O*-linked glycome are observed (19), and the data are in the same format as mRNA and miRNA datasets. We analyzed the NCI-60, a cancer cell line panel containing 59 cell lines from nine tissue types, using our lectin microarray technology. This cell panel is a model for integrated analysis across multiple data types because of the availability of chemosensitivity profiles for these cells (21–23). The miRNA and mRNA profiles of the NCI-60 show the most coherent signatures for four tissue types: colon, leukemia, melanoma, and renal (22, 23). We first profiled the glycome of cells from these four tissue types for integration with miRNA datasets and observed clear segregation by tissue of origin (*SI Appendix, Fig. S1*). An expanded analysis of the cell panel displayed similar results and is shown in Fig. 1. Glycosylation patterns were confirmed by fluorescence microscopy (*SI Appendix, Fig. S2*). For both datasets, normalized data were subjected to hierarchical clustering by using the Pearson correlation coefficient (PearCC)

Significance

Carbohydrates (glycans) are complex cell surface molecules that control multiple aspects of cell biology, including cell–cell communication, cancer metastasis, and inflammation. Glycan biosynthesis requires the coordination of many enzymes, but how this is regulated is not well understood. Herein we show that microRNA (miRNA), small noncoding RNA, are a major regulator of cell surface glycosylation. We map miRNA expression onto carbohydrate signatures obtained by using lectin microarrays, a glycan analysis method. We identify and validate several miRNA–glycan networks, including a major decision point in *N*-linked glycan biosynthesis. Overall, our work provides insights into the “black box” of carbohydrate control mechanisms.

Author contributions: L.K.M. designed research; P.A., T.K., K.T.P., J.F.R., Z.L., B.S.B., W.S.E., K.-L.H., and Y.L. performed research; P.A., T.K., K.T.P., J.F.R., S.K., and L.K.M. analyzed data; and P.A., T.K., S.K., and L.K.M. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1321524111/-DCSupplemental.

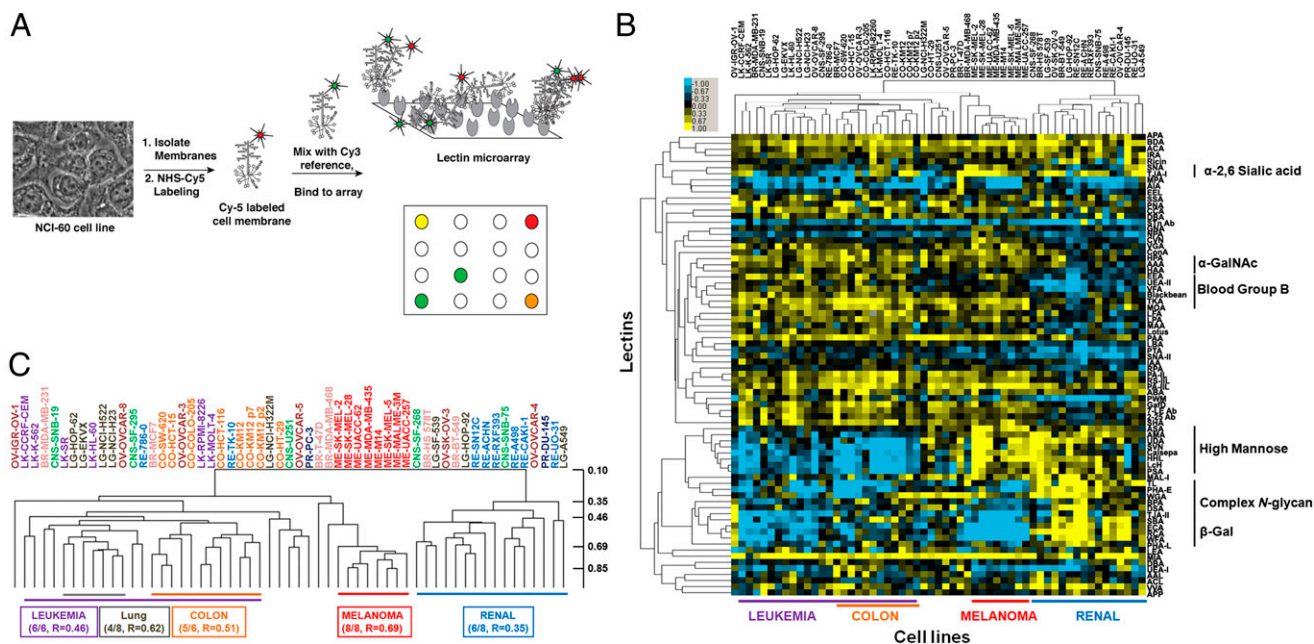


Fig. 1. Ratiometric comparison of NCI-60 cell lines. (A) Experimental scheme. Equal amounts of Cy5-sample (S) and Cy3-reference (R) were analyzed on the lectin microarray. (B) Median-normalized \log_2 ratios (S/R) for 55 cell lines of the NCI-60 were hierarchically clustered by using centered PearCC as the distance metric and average linkage analysis ($n = 76$ lectins). Heat map is shown. Yellow, $\log_2(S/R) > \log_2(S_{\text{median}}/R_{\text{median}})$; blue, $\log_2(S_{\text{median}}/R_{\text{median}}) > \log_2(S/R)$. (C) Dendrogram from B. As expected, biological replicates for KM12 clustered tightly. PearCC scale is shown at right.

as the distance metric and average linkage analysis. Melanoma and renal cell lines showed enrichment in high mannose [ASA, AMA, UDA, scytovirin (SVN), Calsepa, HHL; see *SI Appendix*,

Tables S1 and S2 for full lectin names] but were distinguished by differing levels of complex multiantennary N-glycans (PHA-L, PHA-E; renal, high; melanoma, low). In contrast, leukemia and

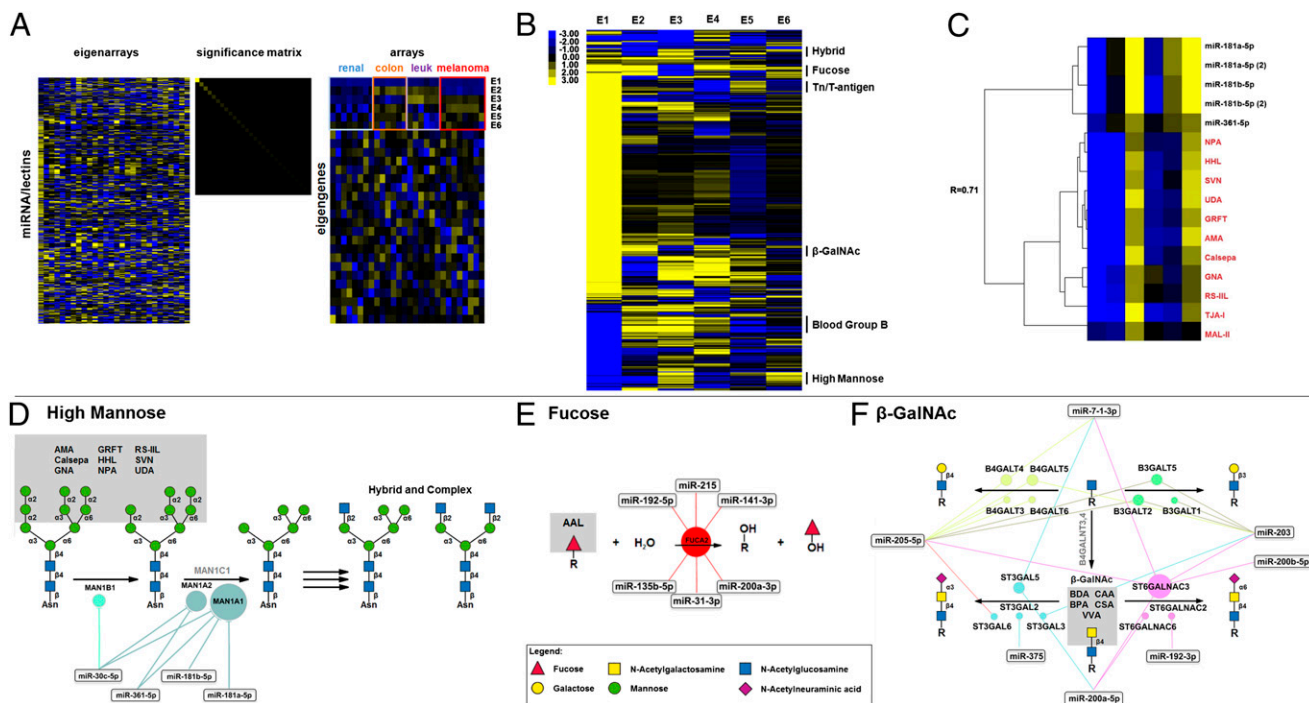


Fig. 2. Mapping of miRNA/glycosylation networks. (A) SVD of lectin and miRNA data for renal, colon, leukemia (leuk), and melanoma cell lines. The first six eigenvalues (E1–E6) are indicated. (B) Hierarchical clustering of the projection correlation values for E1–E6 using uncentered PearCC as the distance metric and average linkage analysis. Select clusters are annotated by glycan specificity. (C) A detailed representation of the high mannose cluster ($R = 0.71$; one-tailed $P = 0.06$; $n = 6$). Lectins are shown in red. (D–F) Networks derived from lectin/miRNA clusters: (D) high mannose network, (E) fucose network, (F) β -GalNAc network. Gray boxes indicate epitopes recognized by lectins. Bubbles indicate predicted miRNA targets, with bubble size reflecting number of miRNA targeting gene. Lines connect miRNA targets; genes in silver are genes in the pathway that are not targeted.

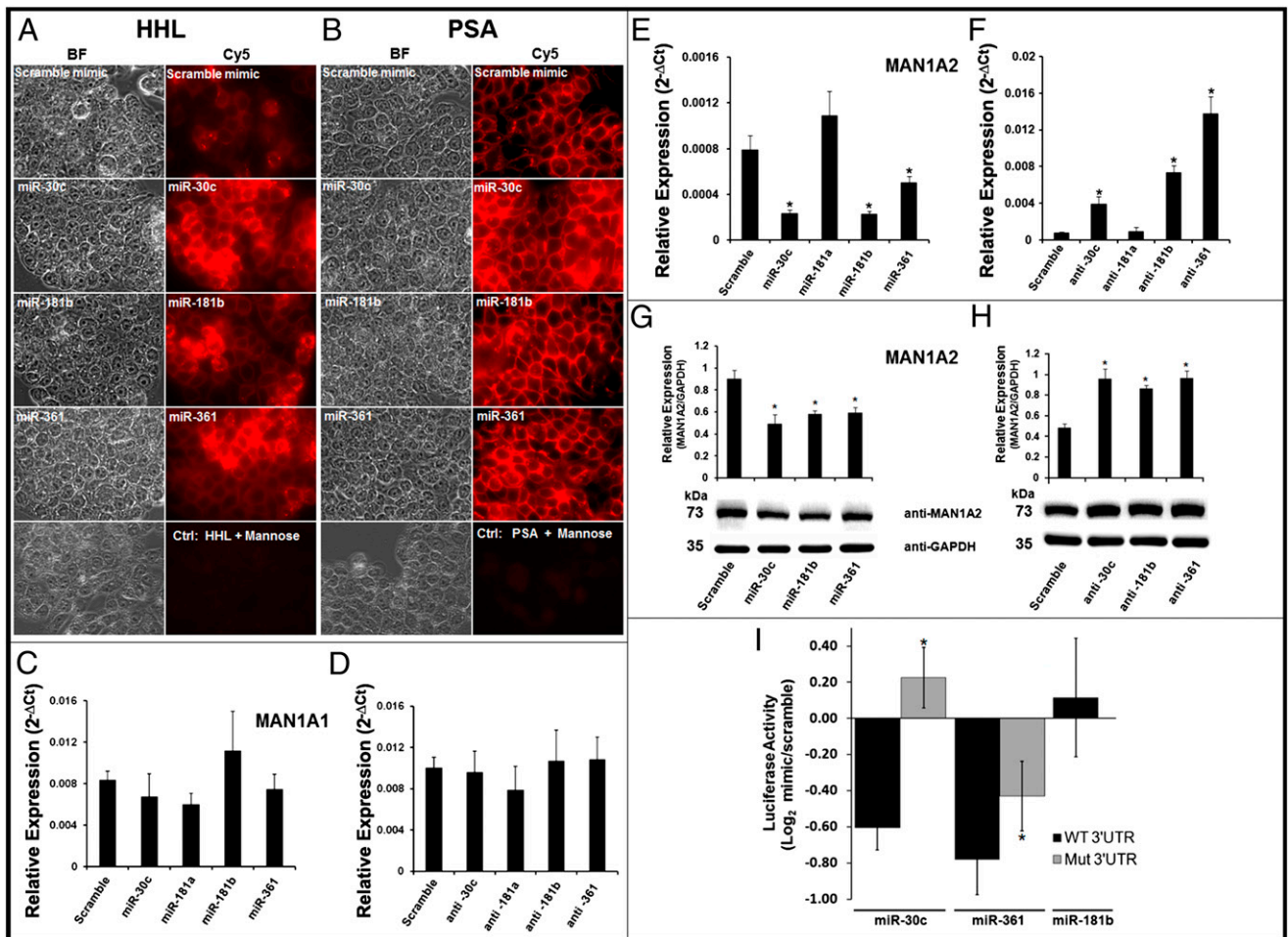


Fig. 3. Validation of the high mannose network. (A and B) Representative fluorescence images of HT-29 cells stained with high mannose lectins (A) HHL or (B) PSA following 96 h treatment with miR-30c, -181b-5p, -361-5p, or scramble mimics. Monosaccharide control for staining is shown. Data are representative of three biological replicates. Statistical analysis of the staining and additional lectin data for GNA, NPA, and the LcH control is shown in *SI Appendix, Fig. S5*. (C–F) Real-time qPCR analysis of indicated glyco genes in HT-29 cells treated with indicated miRNA mimics (50 nM; C and E) or corresponding inhibitors (anti-, 25 nM; D and F) for 72 h. Scrambled sequences are used as a control (scramble). Graphs show average relative expression normalized to *GAPDH* of three biological replicates. (C and D) *MAN1A1*. (E and F) *MAN1A2*. (G and H) Western blot analysis of *MAN1A2* samples treated as described with (G) miRNA mimics or (H) inhibitors. Graphs are of average signal normalized to *GAPDH* for three biological replicates. Representative images corresponding to the graphs are shown. (I) Graphical representation of luciferase activity from *MAN1A2* constructs cotransfected with miR-30c, -181b-5p, -361-5p, or scramble mimics (60 nM) in HEK-293T/17 cells. Mut, miR-30 mutant or miR-361-5p mutant construct as indicated (*SI Appendix, Fig. S8A and Table S3*). Luciferase data were normalized to scramble control. Error bars denote SD (* $P < 0.05$, Student *t* test).

colon had lower levels of high mannose and divergent levels of complex multiantennary glycans (colon, high; leukemia, low) and β -GalNAc epitopes (BPA, SBA; colon, high; leukemia, low). In the larger NCI-60 dataset, no discrete glycan signatures were observed for the remaining cell types. Our data are consistent with previous mRNA- and miRNA-based profiles (22, 24). Overall, our data suggest that the glycome of a cell is a direct representation of the complex flow of genetic information that encodes cell type.

Multidimensional Singular Value Decomposition Analysis Identifies Regulatory miRNA/Glycosylation Networks. To examine the relationship between miRNAs and the observed glycan signatures, we integrated data from our original glycomic analysis (*SI Appendix, Fig. S1*) with the miRNA expression data from Liu et al. (22) by using singular value decomposition (SVD), an unsupervised matrix method (25). SVD decomposes datasets into unique “eigenarrays” and “eigengenes,” related by a significance matrix, which can map onto biological phenotype. We observed five eigengenes (E2–E6; Fig. 2A), reflecting cell type-dependent patterns, which, together with the first eigengene (E1), account for 94% of the variation in the data. Projection of the lectins and

miRNA onto individual eigengenes resulted in sets of lectins with mixed specificities, confounding our mapping of miRNA/glycan associations. We reasoned that miRNA that tightly associate with a particular glycan structure via regulation of underlying glyco genes should covary with lectin binders in their contributions to the eigengenes. Therefore, we performed a multidimensional analysis of miRNA and lectin contributions to the eigengenes by hierarchically clustering projection scores for the first six eigengenes by using PearCC as the distance metric and average linkage analysis (Fig. 2B). This improved segregation of lectins and identified multiple miRNA/lectin clusters that map onto biosynthetic pathways. We identified regulatory networks for high mannose (Fig. 2C and D and *SI Appendix, Fig. S3A*), fucose (Fig. 2E and *SI Appendix, Fig. S3B*), terminal β -GalNAc (Fig. 2F and *SI Appendix, Fig. S3C*), Tn and T-antigens (*SI Appendix, Figs. S3D and S4A*), hybrid-N-glycans (*SI Appendix, Figs. S3E and S4B*), and blood group B (*SI Appendix, Figs. S3F and S4C*), suggesting widespread control of glycan biosynthetic pathways by miRNA. We validated three of these networks—high mannose, fucose, and terminal β -GalNAc—as subsequently discussed in detail, identifying seven previously unknown miRNA/glycogene interactions and providing strong evidence that

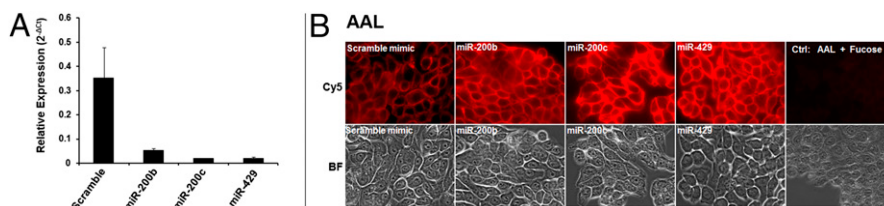


Fig. 5. MiR-200f members down-regulate *FUCA2* expression and increase fucosylation in HT-29. (A) Real-time qPCR analysis of *FUCA2* mRNA expression in cells treated with miR-200b-3p (200b), -200c-3p (200c), -429, or scramble mimic. Data were generated as in Fig. 3 C and E. (B) Representative fluorescence images of cells treated as in Fig. 3 A and B with indicated miR-200f mimics or scramble and stained with AAL. Monosaccharide inhibition control is shown (Ctrl). Data are representative of three biological replicates; *SI Appendix, Fig. S9B* provides statistical quantification of staining.

catalyzes addition of sialic acid to β -GalNAc, is a predicted target of five of the seven miRNAs in this complex network. By using an *ST6GALNAC3*-3'-UTR luciferase reporter assay, we examined the ability of three miRNAs from the cluster [miR-200a-5p (200a*), 200b-5p (200b*), and miR-205-5p] to regulate enzyme expression (Fig. 6 and *SI Appendix, Fig. S8C*). Of the three mimics tested, only miR-200b* inhibited luciferase expression. No effect was observed for miR-200a* or miR-205-5p. miR-200a* and -200b* have identical seed regions but differ in their flanking bases, arguing that these are important in determining target specificity. Mutation of the miR-200b* binding region abrogated the effect of mimic on luciferase expression, validating the predicted binding site (Fig. 6). The miR-200b* binding site in *ST6GALNAC3* is not conserved across species and would not be prioritized by prediction algorithms. By integrating miRNA and glycomic data, our analysis prompted us to validate this glycoenzyme as a target of miR-200b*.

Conclusions

The sheer complexity of the glycome, with its multiple potentially redundant enzymes, low glycoenzyme expression levels, rapid evolution, and dendritic structures, makes identification of critical control mechanisms difficult. Herein we harness the power of our lectin microarray platform to demonstrate that the glycome communicates the genomic information flow governing tissue type and cell state at the surface of the cell. miRNAs are master regulators of this information flow and control the glycan biosynthetic network through direct interaction with glycoenzyme transcripts, as we show in this work. miRNAs are known to target multiple genes concurrently, opening up the possibility that the glycome may be modulated along several pathways (e.g., N-linked, O-linked, glycolipid) simultaneously by the same miRNA. Although cancer cells were used in this work, miRNAs regulate normal biological processes ranging from T-cell development to embryogenesis, opening a window into glycoenzyme regulation and involvement in these systems. Overall, our work begins to map glycans onto critical regulatory networks controlling cell phenotype, providing a means to deconstruct the glycode.

Materials and Methods

Cell Lines. The NCI-60 cell set was from the Division of Cancer Treatment and Diagnosis Tumor Repository (National Cancer Institute, Frederick, MD). Cell lines were grown in RPMI-1640 (Lonza) supplemented with 10% (vol/vol) FBS (Atlanta Biologicals) and 2 mM L-glutamine (Lonza) at 37 °C in 5% CO₂.

Lectin Microarray. Sample preparation. Cell samples were prepared and Cy5-labeled as previously described (36). Reference was prepared by mixing equal amounts (by protein) of membrane samples from HS-578T, PC-3, OVCAR-3, OVCAR-4, LOX IMVI, and SNB-19 before labeling with Cy3.

Printing, hybridization, and analysis. Lectins were purchased from E. Y. Laboratories or Vector Laboratories with the following exceptions: recombinant cyanovirin, SVN and griffithsin were gifts from B. O'Keefe (Frederick National Laboratory for Cancer Research, Frederick, MD); recombinant Gaf-D, PA-IL, PA-IIL, and RS-IIL were made as previously described (37); TJA-I and TJA-II were from NorthStar Bioproducts. All antibodies were purchased from Abcam. *SI Appendix, Tables S1 and S2*, summarize the print lists and buffers. Printing, hybridization and data analysis were performed as previously described (20, 36). Our data were normally distributed as determined by the

Lilliefors test in MATLAB. Lectins were excluded from analysis if they did not meet our minimal threshold for activity (20).

SVD and Multidimensional Analysis. The normalized miRNA dataset from Liu et al. (22) was downloaded from CellMiner [RNA; Agilent Human microRNA (V2); <http://discover.nci.nih.gov/cellminer/>]. miRNA and lectin microarray data (*SI Appendix, Fig. S1*) were combined and ordered by tissue of origin for the four tissue types. SVD and the projection correlation using the dot product were performed by using the built-in "SingularValueDecomposition" function in Mathematica 8 on the collated dataset. For multidimensional analysis, projection correlation values for each lectin or miRNA vector were calculated by taking the dot product of the expression values with each of the first six eigengenes, which account for 94% of data variance. These correlation values were hierarchically clustered by using the PearCC (uncentered) as the distance metric and average linkage analysis in Cluster 3.0 and visualized in Java TreeView. Clusters were chosen for annotation if they met the following criteria: (i) >50% of lectins in the cluster had overlapping specificity toward a defined epitope, (ii) PearCC > 0.51 ($P \leq 0.15$, single-tailed t test), and (iii) miRNAs in the cluster were annotated in microRNA.org and mapped onto a clear biosynthetic pathway. The centered PearCC as the distance metric gave different results, generally segregating lectins from miRNA; however, the high mannose cluster was observed and included miR-30c, which we validated (*SI Appendix, Fig. S3A*).

Mapping miRNA Onto Glycosylation Pathways. Biosynthetic pathways for relevant glycan epitopes were mapped by using the Kyoto Encyclopedia of Genes and Genomes (38) and the work of Nairn et al. (2). The pathway data were then compared with the complete set of all predicted glycoenzyme targets from the downloaded microRNA.org database (14) for miRNA within the corresponding cluster. miRNA glycoenzyme targets were overlaid onto the appropriate pathways.

Transfection of miRNA Mimics and Inhibitors. Cells were seeded in 12-well or 35-mm glass-bottom dish (1×10^5 cells), cultured 24 h, and transfected with miRNA mimics (50 nM) or inhibitors (25 nM, miRIDIAN; Dharmacon; *SI Appendix, Table S3*) with Lipofectamine 2000 (Invitrogen). Samples were analyzed 72 h (real-time qPCR and Western) or 96 h (microscopy) posttransfection.

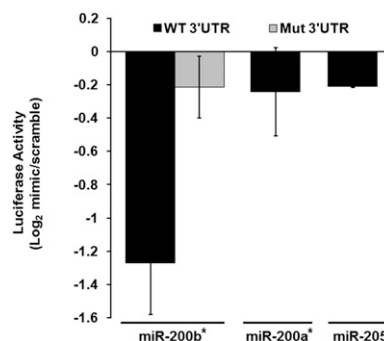


Fig. 6. Validation of β -GalNAc network. Graphical representation of luciferase activity from *ST6GALNAC3* constructs cotransfected with miR-200a-5p, -200b-5p, -205, or scramble mimics. Mut, *ST6GALNAC3* mutant (*SI Appendix, Table S3*). Data were generated as in Fig. 3I.

Fluorescence Microscopy. Fluorescence microscopy was used to confirm lectin microarray and miRNA data as it causes minimal perturbation to adherent cells. Cells were cultured in glass-bottom dishes, fixed in 4% (wt/vol) paraformaldehyde in HBSS (5 min, room temperature) and incubated with biotinylated lectins (10 μ g/mL in PBS solution, 45 min, 37 °C; Vector Laboratories), followed by streptavidin-Cy5 staining (1:100 vol/vol in PBS solution, 30 min, 37 °C; Invitrogen) in the dark. NCI-60 validation samples were stained with DAPI (600 nM) to test for permeabilization, which was not observed under these fixation conditions. miRNA mimic-treated samples were not stained with DAPI. Samples were imaged by fluorescence microscopy (60 \times PlanFluor objective, NA 0.3, Eclipse TE 2000-U; Nikon) and a minimum of 10 fluorescence (excitation/emission, 625–650 nm/670 nm) and bright-field (phase contrast) image pairs were obtained. All samples stained with the same lectin were imaged under identical conditions. Lectins were preincubated with monosaccharide inhibitors (30 min, 200 mM mannose for HHL, PSL, GNA, NPA, LcH; 200 mM fucose for AAL) as a staining control. Images were analyzed in MetaMorph (Molecular Devices). For statistical analysis, images were background-subtracted and six random areas per image were selected in bright field. Regions with aggregated lectin or internal staining (as observed by bright fluorescent aggregates and/or DAPI staining where applicable) were excluded from the statistical analysis and alternative regions selected. For NCI-60 validation studies, the average fluorescence of six areas was normalized to cell count (>29 cells per image, bright field) for four random images and averaged to generate graphs. For miRNA validation, data from 10 images per replicate (three biological replicates, 30 images total, 180 random areas) were averaged to generate graphs.

RNA Extraction and Real-Time qPCR. Total RNA was extracted from samples (miRNeasy Mini Kit; Qiagen), quantified by using a NanoDrop ND-1000 device, and reverse-transcribed (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems). Transcripts were quantified by real-time qPCR using Power SYBR Green PCR Master Mix (Applied Biosystems) in a LightCycler 480 (Roche).

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Primers were designed by using PrimerSelect (*SI Appendix, Table S3*). Cycle threshold values were normalized to the housekeeping gene *GAPDH*. The average for three biological replicates was plotted as relative transcript abundance.

Western Blotting. Cells were lysed in cold RIPA buffer supplemented with protease inhibitors. Equal amounts of protein were resolved by 10% SDS/PAGE, transferred onto nitrocellulose membranes, and blocked in block buffer [5% (wt/vol) BSA, PBST (PBS, pH 7.4, 0.05% Tween-20), 1 h, room temperature]. Antibodies were diluted in block buffer; primary antibodies were as follows: α -MAN1A2 (1:1,000; Novagen), α -MAN1A1 (1:1,000; Abcam), α -GAPDH (1:5,000; Abcam); secondary antibodies corresponding to the primary antibodies were as follows: α -mouse or α -rabbit-HRP (1:5,000; Bio-Rad). Blots were developed by using SuperSignal West Pico (Thermo Scientific).

Luciferase Reporter Assay. *MAN1A2*-3'UTR and *ST6GALNAC3*-3'UTR reporter plasmids were obtained from SwitchGear Genomics. *FUCA2*-3'UTR was cloned from HT-29 cDNA and inserted into pLightSwitch-MT vector (SwitchGear Genomics). Mutagenesis of seed regions was performed with Phusion Hot Start Flex (New England Biolabs) and 5'-phosphorylated primers (*SI Appendix, Table S3*, shows primers and constructs). Plasmids were purified using Endo-Free Plasmid Maxi Kit (Qiagen) for transfection. Each plasmid (250 ng DNA) was cotransfected with 60 nM miRNA or scramble mimic in HEK 293T/17 cells in a 96-well plate by using Lipofectamine 2000 (Life Technologies). After 24 h, luminescence was developed by using LightSwitch Assay Reagent (SwitchGear Genomics) and read on a SynergyHT microplate reader. All luciferase data were normalized to scramble control.

ACKNOWLEDGMENTS. We thank Drs. E. Hernando-Monge and J. Ribeiro for critical reading of the manuscript and Boval Biosolutions for lyophilized protease- and IgG-free bovine serum albumin (no. LY-0081). This work was supported by National Institutes of Health Grant 7 DP2 OD004711-02.

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