Inactivating germ-line and somatic mutations in polypeptide *N*-acetylgalactosaminyltransferase 12 in human colon cancers

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Aberrant glycosylation is a pathological alteration that is widespread in colon cancer, and usually accompanies the onset and progression of the disease. To date, the molecular mechanisms underlying aberrant glycosylation remain largely unknown. In this study, we identify somatic and germ-line mutations in the gene encoding for polypeptide *N*-acetylgalactosaminyltransferase 12 (*GALNT12*) in individuals with colon cancer. Biochemical analyses demonstrate that each of the 8 *GALNT12* mutations identified inactivates the normal function of the GALNT enzyme in initiating mucin type O-linked protein glycosylation. Two of these inactivating *GALNT12* mutations were identified as acquired somatic mutations in a set of 30 microsatellite stable colon tumors. Relative to background gene mutation rates, finding these somatic *GALNT12* mutations was statistically significant at $P < 0.001$. Six additional inactivating *GALNT12* mutations were detected as germ-line changes carried by patients with colon cancer; however, no inactivating variants were detected among cancer-free controls ($P = 0.005$). Notably, in 3 of the 6 individuals harboring inactivating germ-line *GALNT12* mutations, both a colon cancer and a second independent epithelial cancer had developed. These findings suggest that genetic defects in the O-glycosylation pathway in part underlie aberrant glycosylation in colon cancers, and they contribute to the development of a subset of these malignancies.

cancer | *GALNT* | glycosylation

Protein glycosylation is a fundamental mechanism involved in multiple cellular processes. Aberrant glycosylation is a well-described hallmark of many human cancers, prominently including colon cancer, and has been shown to have multiple effects on cell growth, differentiation, transformation, adhesion, metastasis, and tumor immune surveillance (1). Although altered glycosylation in cancer has been attributed to changes in the levels of various glycosyltransferases (2), the underlying molecular basis of aberrant glycosylation in cancers remains largely unknown. The necessary first step in the pathway of mucin type O-linked glycosylation is the transfer of monosaccharide *N*-acetylgalactosamine (GalNAc) from UDP-GalNAc to the hydroxyl group of a serine or threonine residue (3). This reaction is catalyzed by a large family of polypeptide GalNAc-transferases (ppGalNac-Ts or GALNTs) that are normally located in the Golgi complex (3). In this study, we have examined the hypothesis that *GALNT* mutations contribute to human colon cancers, tumors in which aberrant glycosylation has prominently been described (1).

Results and Discussion

***GALNT12* Is a Mutational Target in Colon Cancers.** We initiated our investigation with analysis of *GALNT5*, because recently, our group reported finding 2 *GALNT5* somatic missense mutations in a study of primary breast cancers (4). Functional testing revealed that these 2 breast cancer derived mutations reduced *GALNT5* enzymatic activity to 0% and 45%, respectively (Fig. S1). However, sequencing of the coding exons of *GALNT5* in a cohort of 30 microsatellite stable (MSS) colon cancer cell lines did not detect any somatic *GALNT5* mutations in this disease.

We next examined *GALNT12*, another member of the *GALNT* gene superfamily, chosen because of its high level of expression in the normal colon (5). Sequencing of the *GALNT12* coding exons in a cohort of 30 MSS colon cancer cell lines revealed 2 somatic missense mutations, an E341D mutation in VACO-400 (V400), and a C479F mutation in V866 (Fig. 1*A*). Both of these mutations were confirmed as being present in the antecedent primary colon tumors from which these cell lines were established, and as being absent in normal colon tissues from these same patients (Fig. 1*A*). Based on the previously determined background rate of developing somatic nonsynonymous mutations in colon cancers (4), the detection of these 2 *GALNT12* missense mutations in a panel of 30 MSS colon cancer cell lines was statistically significant ($P < 0.001$). Also, these 2 somatic mutations respectively fell within the *GALNT12* catalytic and lectin binding domains (Table S1), whose proper folding is required for *GALNT* activity (6). Expression and biochemical assay of the corresponding *GALNT12* proteins revealed that both of these somatic mutations completely inactivated the *GALNT12* enzymatic activity (activity, <3% of WT) (Fig. 1*B*; Table S1). In both tumors with inactivating mutations of one *GALNT12* allele, the WT *GALNT12* allele was found to be retained and expressed. In total, of the 4 *GALNT* family somatic mutations identified in human breast and colon cancers, 3 were found to completely ablate *GALNT* enzymatic activity, whereas the fourth partially did so.

***GALNT12* Mutations in the Germ Lines of Colon Cancer Cases.** We next examined whether germ-line *GALNT12* mutations might also contribute to colon cancer development in the population. The complete coding sequence of *GALNT12* was determined in DNA

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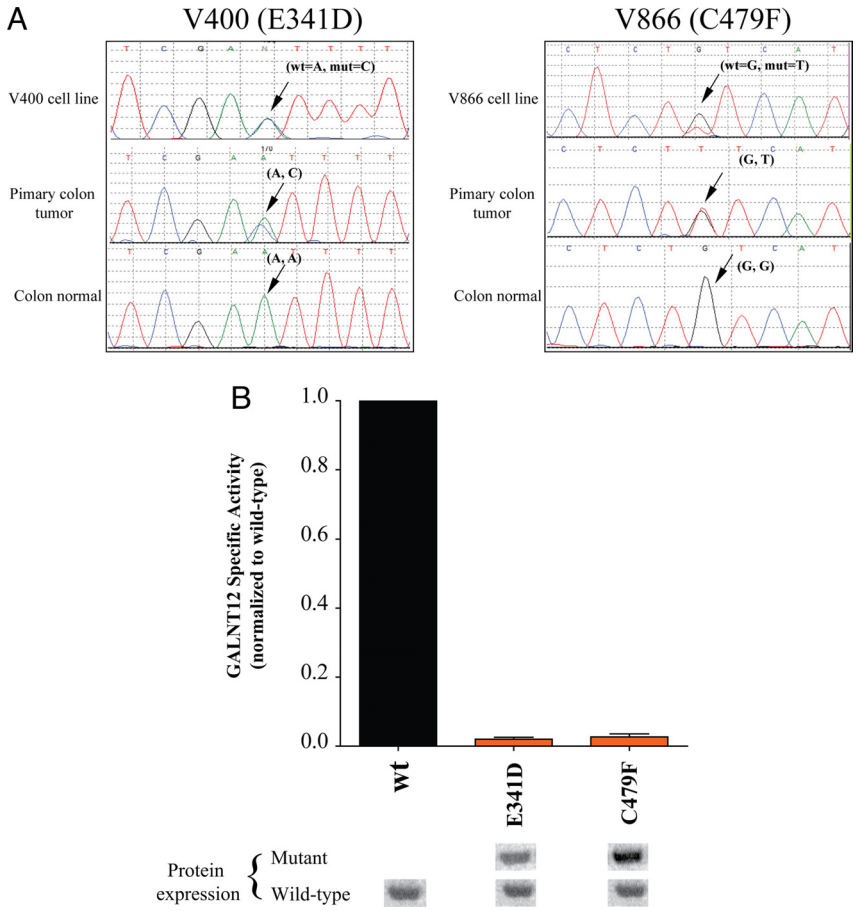

Fig. 1. Somatic mutations inactivating *GALNT12* in V400 and V866 colon cancers. (*A*) DNA sequencing chromatograms depict *GALNT12* missense mutations detected in the V400 and V866 colon cancer cell lines and their matched primary colon tumors, but not in matched normal colon tissue from these same individuals. Arrows indicate positions of mutations, with ''mut'' designating mutant alleles. The mutations shown were confirmed by sequencing individual cloned *GALNT12* PCR products amplified from the V400 and V866 primary colon cancers. (*B*) Quantitation of enzymatic activity of mutant *GALNT12* proteins. (*Lower*) Expression in transfection assays of epitope-tagged WT and mutant *GALNT12* proteins as determined by Western blot analysis. (*Upper*) Matching quantitation of the specific enzyme activities normalized to the activity of the WT protein. Each bar represents the average activity values from 3 independent biological replicates (see *Materials and Methods*). The error bars represent the SEM. Note the loss of functional activity of the 2 somatic *GALNT12* mutants (<3%) compared with the WT protein.

samples from normal cells of 272 colon cancer patients as compared with 192 control individuals that were all older than 70 years of age, and without any history of having had cancer. We identified 7 *GALNT12* sequence variants that were present exclusively among the cancer patients (each detected in an individual patient), and that were not detected among any controls (Table 1; Table S1). First, a *GALNT12* sequence variant that changed the initiating methionine codon (M1I, ATG>ATA) was noted present in a single cancer patient who had been affected by a colon cancer as well as a breast cancer (Table 1; Table S1). As was expected from the nature of this mutation, multiple attempts to express this variant through transfection of an expression vector were unsuccessful; i.e., the next in-frame methionine (codon 65) could not substitute as an efficient start codon (Fig. 2). Next, a nonsense mutation (Y395X) was

Table 1. Colon cancer associated mutations inactivating *GALNT12*

Sample	Mutation	Type	Ethnicity	Sex	Cancer phenotype
V866	C479F	Somatic	Caucasian	Male	Proximal (caecum) colon cancer
V400	E341D	Somatic	Caucasian	Male	Proximal colon cancer
931-22	M1I (start codon) ATG>ATA	Germ line	African American	Female	(*i*) Proximal (transverse) colon carcinoma (*ii*) Ductal carcinoma in situ right breast
2105	T491M	Germ line	Caucasian	Male	(*i*) Proximal (caecum) colon cancer (*ii*) Colon cancer at the splenic flexure
X452	R297W	Germ line	African American	Female	(*i*) Proximal (caecum) colon cancer (*ii*) Invasive carcinoma right breast (*iii*) Intraductal carcinoma left breast
Hx018	Y395X	Germ line	Caucasian	Male	Cancer in the rectum
Hx196	R373H	Germ line	Caucasian	Female	Cancer in the sigmoid colon
Hx208	R382H	Germ line	Caucasian	Female	Proximal (caecum) colon cancer

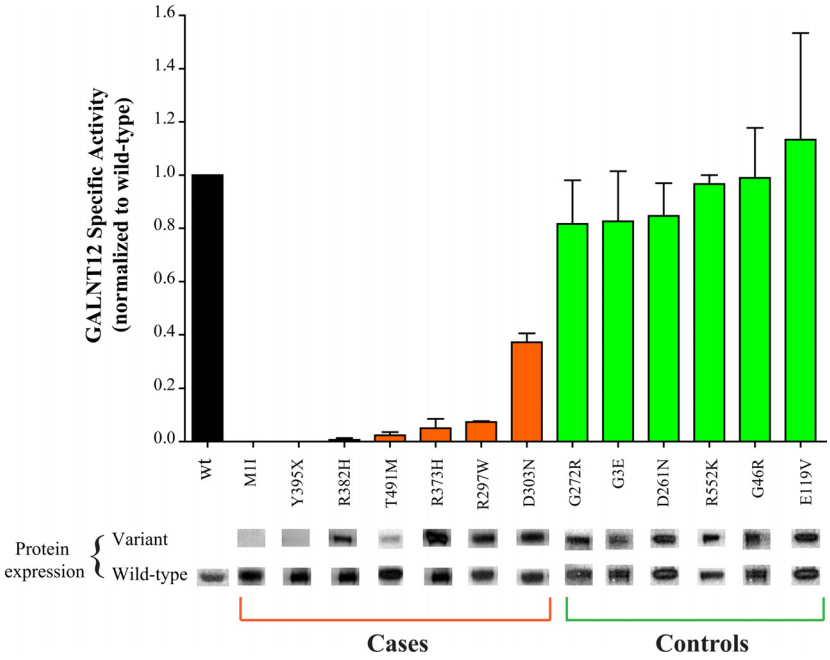

Fig. 2. Functional characterization of germ-line *GALNT12* variants. Shown are the *GALNT12* enzyme activities of variants identified exclusively in the germ lines of individuals with colon cancer (Cases), versus that of variants found among controls (Controls) (Table S1). (*Lower*) Expression in transfection assays of epitope-tagged *GALNT12* proteins. In each experiment, the variant and WT *GALNT12* proteins were assayed in parallel transfections. (*Upper*) Matching quantitation of the specific enzyme activities of *GALNT12* variants normalized to the activity of the WT protein. Noted for each variant is the amino acid alteration. Note the marked loss of functional activity of the cancer specific variants compared with variants carried by controls ($P = 0.001$). The error bars represent the SEM. The nucleotide change for each variant and the protein domain location of these variants are listed in Table S1.

identified in a different cancer patient (Table 1). Multiple attempts to express this variant through transfection of an expression vector were also unsuccessful; i.e., the truncated protein encoded by this mutation was unstable (Fig. 2). Five *GALNT12* missense variants were additionally identified, all detected exclusively among the cancer cases (each detected in an individual patient), and all not detected among any of the controls (Table 1; Table S1). These 5 additional variants resembled the somatic *GALNT12* mutations in all falling within either the catalytic or the lectin binding domain (Fig. 3; Table S1). Functional testing of proteins expressed from these missense variants showed that 4 encoded virtually inactive enzymes (R382H, 1% activity; T491M, 2% activity; R373H, 5% activity; and R279W, 7% activity) (Fig. 2; Table S1). The remaining variant allele (D303N) encoded an enzyme with a reduced activity (37%) (Fig. 2; Table S1). Thus, 6 of 7 total germ-line variants identified in cancer patients encoded essentially inactive *GALNT12* enzymes. A corresponding examination of the complete *GALNT12* coding sequence among all control individuals identified 6 additional *GALNT12* sequence variants, all carried either exclusively among the control cohort or carried jointly by both patients and controls (Fig. 2; Table S1). Functional testing of proteins expressed from the 6 variants present in the control group showed that they encoded enzymes with activities ranging from 81% to 113% of WT (Fig. 2; Table S1).

These findings may be compared in several ways. Although inactivating *GALNT12* mutations are infrequent in the germ-line, they are found significantly more often among colon cancer patients (6 of 272 cases) than among cancer free controls (none of 192 controls) ($P = 0.044$). Alternatively viewed, of the 7 germ-line *GALNT12* variant sequences that were detected only in cancer patients, 6 encoded inactive enzymes; whereas in contrast, none of the 6 *GALNT12* sequence variants identified among control individuals inactivated this enzyme ($P = 0.005$). Last, the median activity of the 7 *GALNT12* variants identified in the cancer patients was 2.3%, a level that was significantly lower than the 91% median activity of the 6 *GALNT12* variants found among the control cohort ($P = 0.001$).

Biological Associations of *GALNT12* Mutations. Individuals with inactivating germ-line mutations in *GALNT12* developed colon cancers later in life (median age 71.5, range 56–93 years of age). This observation is consistent with the hypothesis that multiple rare germ-line gene variants account for many of the cases of colon

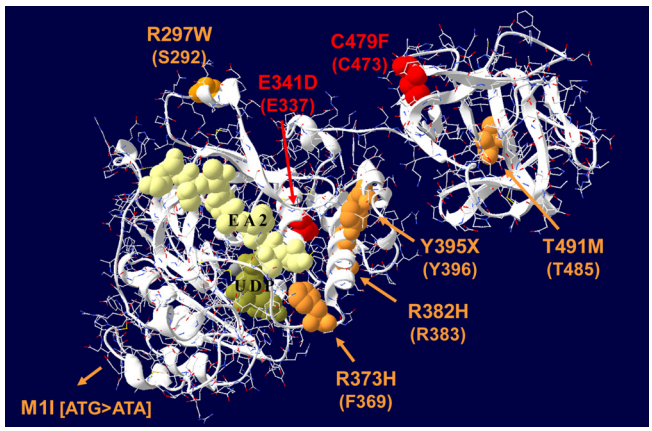

Fig. 3. Structural mapping of inactivating *GALNT12* mutations. Colon cancer associated mutations inactivating *GALNT12* are depicted by homology mapping to the X-ray crystal structure of *GALNT2* bound to an EA2 substrate peptide (EA2) and to UDP (15). The upper right domain is the lectin domain, whereas the lower left domain is the catalytic domain. Inactivating germ-line mutations are in orange, and somatic mutations in red. Residues in parenthesis designate the position of the homologous *GALNT2* residues. The *GALNT2* X-ray crystal structure does not include the first 75 residues of the N-terminal transmembrane and stem domains; thus, the label for the *GALNT12* start codon mutation (M1I, ATG>ATA) is placed to the left of the crystal structure.

neoplasia that arise in the population (7). Complete medical records were available for review from 3 of the patients in whom we detected inactive germ-line *GALNT12* variants (activity, ≤7%). Consistent with *GALNT12* inactivation imparting susceptibility to cancer, each of these 3 individuals had notably been affected by 2 independent epithelial cancers (Table 1). The patient carrying the M1I mutation (ATG>ATA), had developed both a colon cancer and a breast cancer; the patient carrying the T491M mutation (2% activity) had developed 2 separate colorectal cancers; and the patient carrying the R297W mutation (7% activity) had developed bilateral breast cancers in addition to a primary colorectal cancer (Table 1).

In the colon cancer arising in the individual with the germ-line R382H inactivating mutation (Table 1), the WT *GALNT12* allele was lost. However, the WT *GALNT12* allele was found as retained in tumor DNA available from 4 of the remaining individuals bearing germ-line *GALNT12* mutations, and expression of both a WT and a mutant *GALNT12* transcript was noted in RNA extracted from frozen tumor tissue available from 2 of these patients. To test the possibility that missense *GALNT12* mutations might impart dominant negative activity, we cotransfected expression vectors that encoded the C479F *GALNT12* mutation and WT *GALNT12*, respectively. The GALNT12 protein pool recovered from these cells showed a specific enzymatic activity of 56% of WT, consistent with that half of the GALNT12 protein pool encoded by the C479F mutation being inactive, but not with this mutant protein having dominant negative activity. Similarly, the C479F *GALNT12* mutation tested as negative for conferring any transforming activity, as assayed by expression of this mutation in NIH 3T3 cells (Table S2). These findings, along with the inability to stably express either the M1I or the Y395X mutant proteins, suggest that colon cancer associated *GALNT12* mutations are most likely simple null alleles. Although the in vivo protein targets that are glycosylated by *GALNT12* are unknown, the expectation would be that reduction of *GALNT* activity would increase the amounts of improperly glycosylated or unglycosylated proteins produced in colon cancers. Consistent with this expectation, immunohistochemistry with an antibody reactive only with the unglycosylated core MUC1 peptide revealed a marked increase in levels of unglycosylated MUC1 protein detected in 3 of 3 colon cancers that bear *GALNT12* mutations, and for which paraffin-embedded tissue sections were available to us (Fig. S2). Although these colon cancers showed a marked increase in the levels of unglycosylated MUC1 when compared with matched normal colon mucosa, their expression of total MUC1 protein remained similar to normal levels (Fig. S2), which is consistent with the increase in unglycosylated MUC1 reflecting a defect in mucin type O-linked glycosylation in these cancers.

The 8 *GALNT12* mutations listed in Table 1 are evidence that this gene may have a role in colorectal cancers. These data also provides evidence that mutations of *GALNT12* may be detected in the normal tissues of individuals who develop cancer. Although these germ-line variants are uncommon, their association with development of colon cancer is statistically significant. Also, the dramatic effects of these mutations on the functional activity of the encoded proteins, coupled with the finding of inactivating somatic *GALNT12* mutations in colon cancers, provide further support for the conclusion that germ-line *GALNT12* mutations likely contribute to cancer predisposition. Also, supporting this interpretation is that 3 of the 6 individuals carrying inactivating *GALNT12* mutations demonstrated unusual cancer phenotypes, all developing 2 independent epithelial cancers. These findings additionally suggest that the aberrant glycosylation commonly seen in colon and in other cancers may in some instances represent a primary abnormality resulting from mutations of glycosyl-transferase genes. Although the physiologic in vivo substrates of *GALNT12* are not known, this gene is highly expressed in the colon, in which synthesis of O-glycosylated mucins necessarily requires the biochemical activity of GALNT enzymes (5, 8). Also, genetic knockout of murine *Muc2* mucin alleles induces spontaneous development of colonic tumors, with the tumor susceptibility imparted by this defective mucin suggested to be mediated by induction of increased proliferation and occult inflammation in the colonic mucosa (9, 10). Therefore, our findings support the idea that defects in mucin-type glycosylation can contribute to tumorigenesis. *GALNT12* belongs to a large family of multiple genes encoding enzymes that participate in synthesis of O-linked glycoproteins, and it will be of interest to investigate the role of these other enzymes in tumors of the colon and other organs.

Materials and Methods

Colon Cancer Cell Lines. The VACO series of colon cancer cell lines and xenografts were propagated as described (11). DNA from 30 of these colon cancer cell lines was examined for alterations in *GALNT5* and *GALNT12*. DNA from the corresponding colon cancer tumors and from corresponding normal colon tissues was further examined to determine the origins of the *GALNT* sequence alterations detected in the VACO cell lines. The SW480 cell line was obtained from American Type Culture Collection and grown in MEM (Gibco) with additives as described (11).

Colon Cancer and Control Patient Samples. DNA was examined from an additional 242 individuals with colon cancer. This sample set included DNA from 160 Caucasian and 41 African Americans that was purified from either blood samples ($n = 20$) or from normal colon tissue samples ($n = 181$) that were collected under an Institutional Review Board (IRB) approved protocol at the Case Medical Center, plus DNA from 41 individuals, including 30 Caucasians and 8 African Americans, which were collected under an IRB approved protocol at the Johns Hopkins Medical Center. DNA from these patients' tumors was tested for microsatellite instability by comparison of microsatellite alleles in tumor and matched normal colon DNA at microsatellite markers: BAT26, BAT40, D2S123, D5S346, and D17S250 (12). DNA from 192 control individuals, including 125 Caucasians and 67 African Americans, was purified from blood samples that also were collected under an IRB approved protocol at the Case Medical Center. All controls were age 70 or above, and all were free of any history of colon cancer, colon adenomas, or any noncolonic cancer. Patients' medical histories were confirmed by review of medical records. DNA samples were analyzed by PCR amplification and DNA sequencing (Table S3).

Immunoshistochemical Detection of Unglycosylated MUC1 in Colon Cancers. See *SI Methods*.

Vector Constructs. ***Generation of secreted* pPROTA-*and* pIHV-GALNT12 *constructs.*** Using PCR, we generated a cDNA fragment corresponding to amino acids 38–581 of human *GALNT12* (NM_024642), which begins with the first codon immediately after the N-terminal transmembrane domain, and ends with the termination codon (Fig. S3). The forward and reverse PCR primers (Table S3) were engineered to contain EcoRI restriction enzyme sites, facilitating cloning into the *pPROTA* vector that includes an in-frame rat MMP3 signal peptide that directs secretion of the recombinant protein into the tissue culture medium, and that includes the N-terminal IgG binding domain of *Staphylococcus aureus* protein A that enables protein detection and purification (13), or into a modified SV40 promoter-driven pZeoSV2 vector (*pIHV*) (Invitrogen) that was engineered to contain an insulin secretion signal to direct the secretion of the recombinant protein into the cell culture medium (14), and an N-terminal His_6 and V5 epitope tags to facilitate purification and detection of the recombinant protein (Fig. S3). The *GALNT12* cDNA fragments encoding mutant alleles were generated either by direct PCR amplification of cDNA from samples with *GALNT12* mutations, or by incorporating specific mutations in primers followed by PCR amplification using WT *GALNT12* cDNA as the template. ***Generation of secreted* pIHV-GALNT5 *constructs.*** A cDNA fragment corresponding to amino acids 41–940 of human *GALNT5* (NM_0145680) was generated by PCR. The forward and reverse PCR primers were engineered to contain EcoRI restriction enzyme sites, and the PCR fragment was cloned into a modified SV40 promoter-driven pZeoSV2 vector (*pIHV*) (Invitrogen) that was engineered to contain an insulin secretion signal to direct the secretion of the recombinant protein into the cell culture medium (14), and an N-terminal His_6 and V5 epitope tags to facilitate purification and detection of the recombinant protein (Fig. S3). The *GALNT5* cDNA fragments encoding mutant alleles were generated either by direct PCR amplification of cDNA from samples with *GALNT5* mutations, or by incorporating specific

mutations in primers followed by PCR amplification using WT *GALNT5* cDNA as the template.

Generation of the C-terminally tagged* GALNT12 *mutant constructs. To generate the expression vectors for the N-terminally located *GALNT12* mutations, a full-length *GALNT12* ORF without the C-terminal stop codon was amplified by RT-PCR from RNA from a normal control. The G3E and G46R mutants were generated by incorporating the respective base pair change into the forward PCR primer, and the start codon ATG>ATA variant was amplified from the 931–22 individual with the mutation. The resulting PCR products were cloned by using the TA-cloning method into the *pcDNA3.1/V5-His TOPO* cloning vector (Invitrogen), in frame with C-terminal V5 and His_6 epitope tags (Fig. S3). The constructs were verified by sequencing.

DNA Transfection. Transfection was performed by using Fugene transfection reagent (Roche) according to standard protocol. Briefly, 10^6 Cos7 cells (American Type Culture Collection), grown in DMEM (HyClone), were plated per 100-mm dish ≈24 h before transfection, and incubated in 5% CO_2 at 37 °C overnight; 4 μg of plasmid DNA was used per 100-mm dish, with DNA:Fugene ratio of 1:3. Effectene transfection reagent (Qiagen) was used for SW480 transfections; 10^6 cells were plated per 100-mm dish ≈24 h before transfection, and incubated in 5% CO_2 at 37 °C overnight; 2 μg of plasmid DNA was used per 100-mm dish.

NIH 3T3 Transformation Assay. The assay is described in *SI Methods*.

GALNT Recombinant Protein Purification. The vectors described above were used to transfect either Cos7 or SW480 colon cancer cell lines. For *pPROTA-GALNT12* constructs, medium was collected for 48–72 h, and recombinant protein was immunoprecipitated from 8 mL of medium by using goat anti-rabbit agarose beads (Sigma). Agarose beads with bound recombinant protein were washed twice with T12 wash buffer: 50 mM Tris, pH 7.5/150 mM NaCl/1 mM $CaCl_2$/1 mM $MnCl_2$/EDTA-free protease inhibitor pellets (Roche). For *pIHV-GALNT5* and *pIHV-GALNT12* constructs, conditioned medium was collected 48–72 h, and recombinant protein was immunoprecipitated from 8 mL of medium by using anti-V5 agarose beads (Sigma). The *GALNT12* beads were processed with T12 wash buffer as described above. For *GALNT5* immunoprecipitates, agarose beads with bound recombinant protein were washed twice with T5 wash buffer: 50% glycerol/50 mM NaCl/50 mM sodium cacodylate, pH 6.5/EDTA-free protease inhibitor pellets (Roche).

For *pcDNA3.1* constructs, the cell lysates were made in T12 wash buffer supplemented with 0.3% CHAPS. The cell monolayers were washed twice with ice-cold PBS and incubated with lysis buffer for 15 min on ice. After scraping, the lysates were clarified by centrifugation 15 min at maximal speed. The recombinant protein was immunoprecipitated from the lysates by using anti-V5 agarose beads (Sigma) and washed with T12 wash buffer as described above.

Western Blot Analysis. After immunoprecipitations, a 1/10 fraction of the recombinant protein was mixed with equal volume of Laemmli sample buffer (Bio-Rad) at 95 °C for 5 min, and loaded onto a Bis-Tris SDS/4–12% polyacrylamide gel (Invitrogen). After SDS/PAGE, proteins were transferred onto Immobilon-P PVDF membranes (Millipore). Membranes were blocked for 1 h with 5% nonfat milk, and incubated with appropriate dilution of mouse anti-V5 antibody conjugated to horseradish peroxidase (Invitrogen) to detect the V5-tagged proteins (*pIHV* and *pcDNA3.1* constructs), or with normal mouse IgGs followed by incubation with donkey anti-mouse horseradish peroxidase (*pPROTA* constructs) (Jackson ImmunoResearch). Enhanced Chemiluminescence Plus (Amersham Biosciences) and a STORM 840 phosphoimager were used to detect and quantitate the protein bands.

GALNT5 and GALNT12 Enzyme Activity Assays. Transferase bound beads (50–100 μL settled volume) were added to transferase-specific reaction mixtures (250 μL) in 600-μL Eppendorf microcentrifuge tubes. Transferase-specific reaction mixtures were as follows: ppGalNAc T5, 40 mM sodium cacodylate, pH 6.5, 4 mM 2-mercaptoethanol, 0.1% Triton X-100, 10 mM $MnCl_2$, 1 mM UDP-GalNAc (containing 0.1 μCi of [^{3}H]UDP-GalNAc (American Radiolabeled Chemicals), and 0.5–2 mM EA2 peptide substrate (PTTDSTTPAPTTK) (12); ppGalNAc T12, 25 mM Tris buffer, pH 7.4, 0.2% Triton X-100, 5 mM $MnCl_2$, 1 mM UDP-GalNAc, and 0.05 mM Muc5Ac peptide (GTTPSPVPTTSTTSA) (5). Reagent reaction mixtures were combined with the transferase bound beads and shaken at 37 °C in a thermostated microplate shaker (Taitec Microincubator M-36) to maintain the beads in suspension. Aliquots of suspended beads (70–100 μL) were removed at 1, 2, and 3 h for T5, and 3, 7, and overnight for T12. Each time point was quenched with an equal volume of 250 mM EDTA and frozen on dry ice for later processing. After dilution by water (20-fold), the samples were resuspended, and an aliquot removed for counting on a Beckman LS5801 scintillation counter. The remainder of the sample was passed through a fresh 2-mL Dowex 1 × 8 column (HCl− form, 100–200 mesh), and an aliquot was removed for post-Dowex counting. Relative transferase activity at each time point was obtained from the post-Dowex counts after subtracting counts obtained from an empty vector control incubation. Under these conditions, the reported activity represents the combined transfer to peptide activity and UDP-GalNAc hydrolysis activity of the expressed transferase. Activities were scaled to protein content obtained by affinity tag Western blot analysis as described above. Mutant transferase-specific activities were normalized to the WT transferase to yield an approximate relative specific activity of each mutant. The chosen time points for both GALNT12 and GALNT5 were typically within the linear range of enzymatic activity versus time. Time point values that deviated from linearity were not used. Individual incubations were repeated 3 or more times for each recombinant GALNT preparation. Also, the specific activity of each mutant/variant and WT GALNT was assayed in 3 independent preparations, with the final enzyme activity values expressed as the average of these 3 biological replicates.

Statistical Methods. The *P* value for observing 2 somatic *GALNT12* mutations in 30 colon cancer cell lines was calculated by evaluating the likelihood ratio statistics (LRT) for the null hypothesis that mutations occur at the passenger rates. The *P* value is the chance of observing an LRT as big as or bigger than the one seen empirically, and it is evaluated by simulations. Both the LRT and the simulation account for the gene size and composition (4). The *P* value for comparing the number of inactivating mutations in colon cancer cases versus controls was calculated by using Fisher's exact test. The *P* value for comparing the numbers of *GALNT12* variant alleles encoding either active or inactive enzymes in patients versus controls was calculated by using Fisher's exact test. The *P* value for comparing the median enzymatic activity of the variant alleles detected in cancer cases versus that of variant alleles detected in controls was calculated by using an exact Wilcoxon test.

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1. Brockhausen I (2006) Mucin-type O-glycans in human colon and breast cancer: Glycodynamics and functions. *EMBO Rep* 7:599–604.
2. Brockhausen I (1999) Pathways of O-glycan biosynthesis in cancer cells. *Biochim Biophys Acta* 1473:67–95.
3. Ten Hagen KG, Fritz TA, Tabak LA (2003) All in the family: The UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferases. *Glycobiology* 13:1R–16R.
4. Wood LD, et al. (2007) The genomic landscapes of human breast and colorectal cancers. *Science* 318:1108–1113.
5. Guo JM, et al. (2002) Molecular cloning and characterization of a novel member of the UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase family, pp-GalNAc-T12. *FEBS Lett* 524:211–218.
6. Tenno M, Saeki A, Kezdy FJ, Elhammer AP, Kurosaka A (2002) The lectin domain of UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase 1 is involved in O-glycosylation of a polypeptide with multiple acceptor sites. *J Biol Chem* 277:47088–47096.
7. Fearnhead NS, et al. (2004) Multiple rare variants in different genes account for multifactorial inherited susceptibility to colorectal adenomas. *Proc Natl Acad Sci USA* 101:15992–15997.
8. Ten Hagen KG, et al. (1998) Cloning and expression of a novel, tissue specifically expressed member of the UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase family. *J Biol Chem* 273:27749–27754.
9. Velcich A, et al. (2002) Colorectal cancer in mice genetically deficient in the mucin Muc2. *Science* 295:1726–1729.
10. Yang K, et al. (2008) Interaction of Muc2 and Apc on Wnt signaling and in intestinal tumorigenesis: Potential role of chronic inflammation. *Cancer Res* 68:7313–7322.
11. Markowitz S, et al. (1995) Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* 268:1336–1338.
12. Umar A, et al. (2004) Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. *J Natl Cancer Inst* 96:261–268.
13. Larsen RD, Ernst LK, Nair RP, Lowe JB (1990) Molecular cloning, sequence, and expression of a human GDP-L-fucose:β-D-galactoside 2-α-L-fucosyltransferase cDNA that can form the H blood group antigen. *Proc Natl Acad Sci USA* 87:6674–6678.
14. Hagen FK, et al. (1997) cDNA cloning and expression of a novel UDP-N-acetyl-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase. *J Biol Chem* 272:13843–13848.
15. Fritz TA, Raman J, Tabak LA (2006) Dynamic association between the catalytic and lectin domains of human UDP-GalNAc:polypeptide alpha-N-acetylgalactosaminyltransferase-2. *J Biol Chem* 281:8613–8619.