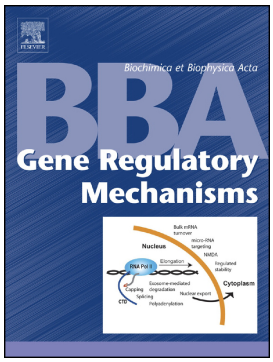

Epigenetic silencing of the synthesis of immunosuppressive Siglec ligand glycans by NF-κB/EZH2/YY1 axis in early-stage colon cancers

Hsiang-Chi Huang, Chia-Chun Chao, Po-Han Wu, Hsiao-Yu Chung, Hsueh-Yi Lee, Ching-Shu Suen, Ming-Jing Hwang, Bi-He Cai, Reiji Kannagi

PII: S1874-9399(18)30341-9
DOI: https://doi.org/10.1016/j.bbagrm.2019.01.002
Reference: BBAGRM 94353

To appear in: *BBA - Gene Regulatory Mechanisms*

Received date: 20 August 2018
Revised date: 7 December 2018
Accepted date: 7 January 2019

Please cite this article as: H.-C. Huang, C.-C. Chao, P.-H. Wu, et al., Epigenetic silencing of the synthesis of immunosuppressive Siglec ligand glycans by NF-κB/EZH2/YY1 axis in early-stage colon cancers, BBA - Gene Regulatory Mechanisms, https://doi.org/10.1016/j.bbagrm.2019.01.002

Epigenetic silencing of the synthesis of immunosuppressive Siglec ligand glycans by NF-κB/EZH2/YY1 axis in early-stage colon cancers

Hsiang-Chi Huang [1,2], Chia-Chun Chao [1], Po-Han Wu [1], Hsiao-Yu Chung [1], Hsueh-Yi Lee [1], Ching-Shu Suen [1], Ming-Jing Hwang [1], Bi-He Cai [1,3] and Reiji Kannagi [1,3]

[1] *Institute of Biomedical Sciences, Academia Sinica, Taipei 115, Taiwan.*
[2] *Taiwan International Graduate Program in Molecular Medicine, National Yang-Ming University and Academia Sinica, Taipei 115, Taiwan.*

[3] Corresponding authors at: Institute of Biomedical Sciences, Academia Sinica, 128 Sec. 2, Academia Rd. Nankang, Taipei 115, Taiwan. Tel.: +81-886-2-2652-3976.
E-mail addresses: bigbiha@ibms.sinica.edu.tw (Bi-He Cai), and rkannagi@ibms.sinica.edu.tw (Reiji Kannagi).

Email addresses for all authors:
Hsiang-Chi Huang hsiangchi2009@gmail.com
Chia-Chun Chao smoldwolf@gmail.com
Po-Han Wu hank726jr@gmail.com
Hsiao-Yu Chung shirleychung1987@gmail.com
Hsueh-Yi Lee sakon@ibms.sinica.edu.tw
Ching-Shu Suen csuen@gate.sinica.edu.tw
Ming-Jing Hwang mjhwang@ibms.sinica.edu.tw
Bi-He Cai bigbiha@ibms.sinica.edu.tw
Reiji Kannagi rkannagi@ibms.sinica.edu.tw

Running title:
NF-κB and epigenetic silencing of Siglec ligands

List of abbreviations;
ChIP, chromatin immunoprecipitation; DAPA, DNA affinity precipitation assay; PRC2, polycomb repressive complex 2; shRNA, short hairpin RNA; Siglec, sialic acid–binding immunoglobulin-like lectin; TCGA, The Cancer Genome Atlas; DTDST, diastrophic dysplasia sulfate transporter gene. ST6GalNAc6, ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 6; EZH2, Enhancer of zeste homolog 2; SUZ12, Suppressor of Zeste 12; YY1, Yin-Yang 1.

Abstract

Normal colonic epithelial cells express sialyl 6-sulfo Lewis^{x} and disialyl Lewis^{a} on their cell surface, which are ligands for the immunosuppressive molecule Siglec-7. Expression of these normal glycans is frequently lost upon malignant transformation by silencing *DTDST* and *ST6GalNAc6* at the early stage of colorectal carcinogenesis, and leads to production of inflammatory mediators that facilitate carcinogenesis. Indeed, by querying The Cancer Genome Atlas datasets, we confirmed that the level of *DTDST* or *ST6GalNAc6* mRNA is substantially decreased at the early stage of colorectal carcinogenesis. Cultured colon cancer cell lines were used in this study including DLD-1, HT-29, LS174T and SW620. Their promoter regions were strongly marked by repressive mark H3K27me3, catalyzed by EZH2 that was markedly upregulated in early stage of colorectal carcinogenesis. Suppression of EZH2 substantially downregulated H3K27me3 mark and upregulated *DTDST* and *ST6GalNAc6* as well as expression of normal glycans and Siglec-binding activities. Transcription factor YY1 was vital for the recruitment of PRC2-containing EZH2 to both promoters. Inhibition of NF-κB substantially reduced *EZH2* transcription and restored their mRNAs as well as the production of normal Siglec ligand glycans in the results obtained from in vitro studies on cultured colon cancer cell lines.

These findings provide a putative mechanism for promotion of carcinogenesis by loss of immunosuppressive molecules by epigenetic silencing through NF-κB-mediated EZH2/YY1 axis.

(216 words)

Key Words:

Disialyl Lewis^{a}, *DTDST,* PRC2 (polycomb repressive complex 2), Sialyl 6-sulfo Lewis^{x}, *ST6GalNAc6*.

1. Introduction

Cancer is the leading cause of death globally and was responsible for 8.8 million deaths in 2015 [1]. Aberrant glycosylation occurs in all types of human cancers, and many glycan epitopes serve as tumor-associated antigens [2,3]. Sialyl Lewis^x and sialyl Lewis^a are typical cancer-associated glycans. Sialyl Lewis^a frequently appears in the cancers of the pancreas and digestive organs, and sialyl Lewis^x frequently appears in breast and lung cancers and cancers of the digestive organs. Sialyl Lewis^a and sialyl Lewis^x in serum samples are often utilized for clinical diagnosis of these cancers.

We have previously investigated the mechanisms involved in the induction of sialyl Lewis^x and sialyl Lewis^a in human cancers. Normal colonic epithelial cells express a normal glycan called disialyl Lewis^a, which has a more complex structure than sialyl Lewis^a, and we demonstrated that the silencing of *ST6GalNAc6*, which is involved in its synthesis, upon malignant transformation leads to the appearance of sialyl Lewis^a [4]. Similarly, normal colonic epithelial cells also express a normal glycan, sialyl 6-sulfo Lewis^x, which has a more complex structure than sialyl Lewis^x [5], and the silencing of *DTDST*, which is involved in its synthesis, upon malignant transformation leads to the appearance of sialyl Lewis^x [6]. Roles of these genes in biosynthesis of disialyl Lewis^a and sialyl 6-sulfo Lewis^x are shown in Supplementary Figure S1. Sialyl Lewis^a and sialyl Lewis^x are ligands for endothelial selectins that are present on endothelial cells of the vascular bed and mediate hematogenous metastasis of cancers [7-11]. In contrast, we found that disialyl Lewis^a and sialyl 6-sulfo Lewis^x are specific ligands for Siglec-7 and/or Siglec-9, composing a family of immunosuppressive molecules present in colonic mucosal macrophages/dendritic cells, and proposed that they prevent excess activation of mucosal immune cells and maintain immune homeostasis of the colonic mucosal membranes by suppressing production of pro-cancer inflammatory molecules such as COX2 [4,12] [1] . Both *ST6GalNAc6* and *DTDST* play pivotal roles, not only in affecting the production of cell surface glycans but also in altering their biological functions to switch on selectin-mediated cell adhesion and switch off immunosuppressive glycan expression on the surface of epithelial cells.

Histone modifications such as H3K9me3, H3K27me3 and H4K20me3 serve as epigenetic markers for gene silencing [13-15]. H3K9 methyltransferase G9a and H3K27 methyltransferase EZH2 (Enhancer of zeste) are elevated in colorectal cancers [16,17]. However, the mechanisms of epigenetic silencing of *DTDST* and *ST6GalNAc6* remain to be fully explored. As our preliminary results indicated that H3K27me3 is the most prominent histone modification in *DTDST* and *ST6GalNAc6* in cultured colon cancer cells, we explored the mechanisms involved in epigenetic silencing of these two genes, focusing on the H3K27 methyltransferase EZH2.

2. Materials and Methods

2.1. Cultured normal intestinal epithelial cells, **human colon cancer cells and treatments with small-molecule inhibitors** Normal intestinal epithelial cells HIEC-6 (ATCC) were maintained in OptiMEM 1 (Gibco Catalog No. 31985), supplemented with 20 mM HEPES, 10 mM GlutaMAX (Gibco Catalog No. 35050), 10 ng/mL Epidermal Growth Factor and 4% FBS (Invitrogen). Human cultured colon cancer cell lines DLD-1, HT-29, LS174T and SW620 (ATCC, Manassas, VA, USA) were maintained in DMEM or RPMI1640 medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% FBS (Invitrogen). Small-molecule inhibitors BIX01294, UNC0631, DZNeP (3-deazaneplanocin A), EPZ-6438 (Tazemetostat), UNC0379 and Bay11-7085 were obtained from Selleckchem (Houston, TX, USA). Chromatin immunoprecipitation (ChIP) assays and quantitative RT-PCR were used for evaluation of *DTDST* and *ST6GalNAc6* mRNA induction by small-molecule inhibitors and/or shRNAs. Primers used for PCR are listed in Supplementary Table S1.

2.2 RNA extraction, reverse transcription (RT)-PCR, and Real-Time Quantitative PCR (RT-qPCR)

Total RNA was extracted by Trizol reagent (Invitrogen). Total RNA was reverse transcribed into cDNA using ToolsQuant II Fast RT Kit (TOOLS, Taiwan). Real-time quantitative PCR (RT-qPCR) was performed in Bio-Rad CFX Connect system using Evagreen master mix (Bio-Rad, Hercules, CA, USA) with cDNA serving as template. The primers used are listed in Supplementary Table S1. GAPDH is used as the internal control.

2.3. The Cancer Genome Atlas (TCGA) data analysis

The RNA expression (mRNA-Seq) data for normal and cancer tissues as well as the expression data for seven transcript isoforms of *ST6GALNAc6* were downloaded from Broad Institute TCGA GDAC Firehose (https://gdac.broadinstitute.org/) [18]. The TCGA datasets collected in this study were from Illumina HiSeq level_3 (v2) platforms, and the values had been normalized using RNA-Seq by Expectation-Maximization (RSEM). The cancer stages of colon (COAD) and rectal adenocarcinoma (READ) patients were extracted from TCGA clinical data. PERL scripts were used to parse all files and extract the values.

2.4. Chromatin immunoprecipitation (ChIP) assays

The ChIP assays were performed as previously described [6]. The chromatin samples were prepared from cultured human colon cancer cells with a ChIP-IT Express kit (Active Motif, Carlsbad, CA). The chromatin samples (100 µl) were incubated with

antibodies (5 μg) for 16 h at 4°C. Primers used to detect the *DTDST* and *ST6GalNAc6* promoter regions are shown in Supplementary Table S1, using Phusion Flash PCR master mix with 35 cycles of amplification. Antibodies specific for ChIP assays included H4K20m3 (ab9053), G9a (ab40542), YY1 (ab38422), polymerase II (39233), H3K9m3 (39765), H3K27me3 (39155), SUZ12 (39357) and EZH2 (39901; all from Active Motif).

2.5. Use of shRNA for DTDST, ST6GalNAc6, G9a, EZH2, SUZ12, SUV420H1, SUV420H2 and p65

The pLKO_TRC005 -based lentiviral shRNA vectors for DTDST (TRCN0000273646) and the pLKO.1-based lentiviral shRNA vectors for ST6GalNAc6 (TRCN0000035324), EHMT2 (G9a) (TRCN0000115667, TRCN0000115669), EZH2 (TRCN0000010475, TRCN0000040076), SUZ12 (TRCN0000038727, TRCN0000038728), SUV420H1(TRCN0000130388), SUV420H2(TRCN0000141258) and p65 (RELA) (TRCN0000014684) were purchased from the National RNAi Core Facility Platform at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica, and embryonic kidney cells-293T cells were obtained from Invitrogen, Carlsbad, CA, USA. The pLKO.1-puro plasmid, psPAX2 packaging plasmid and envelope plasmid pMD2.G were simultaneously transfected into 293T cells to produce virus using X-tremeGENE HP DNA Transfection Reagent (Roche, Mannheim, Germany). The medium was replaced with fresh medium 16 h after transfection, and the viral supernatants were collected in two aliquots after 24 h and 48 h. The cells (1.0×10^6 cells/60-mm culture dish) were transduced with virus plus 8 μg/ml polybrene. The medium was changed 24 h after infection, and the cells were cultured for an additional 24 h. Cells expressing shRNA were selected by culturing with 5 μg/ml puromycin for 1 week.

2.6. Flow cytometric analysis and binding assays for recombinant Siglecs

Cell surface expression of glycans was analyzed by flow cytometric analysis using our panel of glycan-specific monoclonal antibodies. The antibody G72 (murine IgM) was prepared as described [19] and was used for evaluation of sialyl 6-sulfo Lewis^x expression. The antibody FH7 (murine IgG3) [20] was a gift from Dr. Sen-itiroh Hakomori, Pacific Northwest Research Institute, Seattle, WA and was used for evaluation of disialyl Lewis^a expression.

Binding of recombinant Siglecs to HT-29, SW620, LS174T and/or EPZ-6438-treated cells was ascertained by flow cytometric analyses as described [21]. Recombinant Siglec-7 was preincubated with biotinylated rabbit anti–human IgG at 4°C for 1 h, followed by incubation with phycoerythrin-labeled streptavidin at room temperature for 15 min before being incubated with cells. For flow cytometry, we

used class-matched Ig for negative control, and LS12 cells [22] for positive control for sialyl 6-sulfo Lewis x, and SW1083 for positive control for disialyl Lewis a [12]. For siglec-7 binding assays we used ECV304 cells for negative control, and LS12 cells [22] for positive control.

2.7. Plasmids, promoter constructs and luciferase assays

The complete coding sequences of human TFAP2C, CDX2 and PKNOX2 were each constructed into the pcDNA3 expression vector. pBabe-Puro-IKBalpha-mut (super repressor), a gift from William Hahn (Addgene plasmid #15291) [23], is a dominant-negative mutant NF-κB inhibitor (IkBm) and inhibits NF-κB activity; IkBm was also cloned into pcDNA3. The human *DTDST* and *ST6GalNAc6* promoters were amplified by PCR. Genomic DNA was prepared from the human colon cancer cell line HT-29 by using the Molecular Research Center Genomic DNA purification kit and used as a template. The full-length reporter gene constructs for *DTDST* (−835/−1) and *ST6GalNAc6* (−1304/−1) and all their deletion constructs were generated by PCR amplification. The mutated YY1 construct (YY1m) had a mutation at bases −426 to −419 (from TGATGGGG to TGATAAAA) in the *DTDST* promoter (p(-569): −569/−1) and a mutation at bases −136 to −129 (from TGATGGGA to TGATTTGA) in the *ST6GalNAc6* promoter (p(-581): −581/−1). The PCR products were purified with a high PCR product purification kit (GeneJET Plasmid Miniprep kit, Thermo Fisher Scientific, Vilnius, Lithuania EU) and cloned into the pGL3-basic firefly luciferase vector (Promega, Madison, WI, USA). The sequence of each cloned promoter region was confirmed by sequencing (Applied Biosystems 3730XL system). For reporter assays, cells were cotransfected with the firefly luciferase construct pRL-SV40 Renilla vector (Promega) and the *DTDST* or *ST6GalNAc6* promoter construct vector. For the reporter assay, an expression vector (pcDNA3) encoding TFAP2C, CDX2 or PKNOX2 and a vector with the *DTDST* or *ST6GalNAc6* promoter construct were co-transfected into colon cancer cells using X-tremeGENE HP DNA Transfection Reagent (Roche). After 24 h, luciferase activity was measured by using the Dual-Luciferase Reporter Assay System kit (Promega).

2.8. Transfection with YY1 siRNA

The colon cancer cell lines were cultured in RPMI 1640 or DMEM medium containing 10% FBS. The YY1 siRNA sense sequence was 5’-AGCUUUUGUUGAGAGUUCATT-3’ and antisense sequence was 5’-UGAACUCUCAACAAAAGCUTT-3’. A non-silencing siRNA was used as a negative control. Cultured cells (8×10^6) were added to 800 μl opti-MEM medium and 2 μL (5 nmol) YY1 siRNA or non-silencing siRNA. The resulting mixture was transferred into a

4-mm BTX Gap Cuvette and electroporated (250 V, 13 ms, two pulses, 1-s interval) using a BTX Gemini X2 Electroporation system (BTX, Holliston, MA). The cells were harvested 24 h after siRNA transfection to evaluate YY1 protein and 48 h after siRNA transfection to evaluate *DTDST* and *ST6GalNAc6* mRNA.

2.9. DNA affinity precipitation assay (DAPA)

Biotinylated DNA probes were used to interact with nuclear proteins from colon cancer cell line HT-29, and the resulting DNA-protein complexes were precipitated with streptavidin-coated magnetic beads (Invitrogen). The collected beads were washed, and proteins were eluted with SDS-PAGE sample buffer. The binding proteins were separated by SDS-PAGE and analyzed by western blotting. Antibodies specific for western blot and/or DAPA assays included EZH2 (21800-1-AP; Proteintech), SUZ12 (MA5-11188; Thermo Fisher Scientific) and YY1 (ab12132; Abcam).

2.10. Statistical analysis

Statistical differences between two groups were assessed with a t-test (two-tailed) by Prism 5 software (GraphPad, La Jolla, CA, USA). $p < 0.05$ was considered statistically significant (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

3. Results

3.1. Identification of repressive histone markers that occupy promoter regions of *DTDST* and *ST6GalNAc6* and roles of methyltransferases

We previously showed that *DTDST* and *ST6GalNAc6* mRNA is reduced in colon cancer tissues as compared with non-malignant epithelial cells from the same patients; this significant decrease is evident at relatively early stages of cancer, although the number of examined patients was small [4,6]. Here we confirmed these results with large-scale RNA-Seq transcriptome analyses of cancer and normal tissues from TCGA datasets. There was a sharp and prominent decrease in mRNA levels of both *ST6GalNAc6* and *DTDST* in cancers, and this was highly statistically significant at $p < 0.001$ (Fig. 1A). The decrease was present in stage I samples, but there were no significant differences among stage I–IV tissues, suggesting that the decrease specifically occurred during the early stage of colon carcinogenesis.

When we used ChIP analyses to screen cultured human colon cancer cells for repressive histone markers in the *DTDST* and *ST6GalNAc6* promoter regions, we detected several repressive histone markers, including H3K9me3, H3K27me3 and H4K20me3, and H3K27me3 was most frequently observed (Fig. S2).

To identify which histone methyltransferase is responsible for silencing of *DTDST* and *ST6GalNAc6*, we analyzed changes in their mRNAs by treating the colon cancer

cells with a specific inhibitor of the H3K9 methyltransferase G9a (BIX-01294), H3K27 methyltransferase EZH2 (DZNeP and EPZ-6438) and the H4K20 methyltransferase SETD8 (UNC0379). Those histone methyltransferase inhibitors impact on depleting their respective histone markers (Fig. S3). DZNeP is not strictly specific to EZH2. It is an inhibitor for S-adenosylmethionine-dependent methyltransferases. The EZH2 inhibitors DZNeP and EPZ-6438 significantly induced mRNAs for both genes (Fig. 1B). Effects of these EZH2 inhibitors were dose dependent (Fig. S4). In contrast, the G9a inhibitor (BIX-01294) moderately increased only *ST6GalNAc6* mRNA but had no effect on *DTDST* (Fig. 1B), and the SETD8 inhibitor (UNC0379) failed to show any inducing effects (Fig. 1B). These results strongly suggested that EZH2 is the major histone methyltransferase that suppresses transcription of *DTDST* and *ST6GalNAc6*.

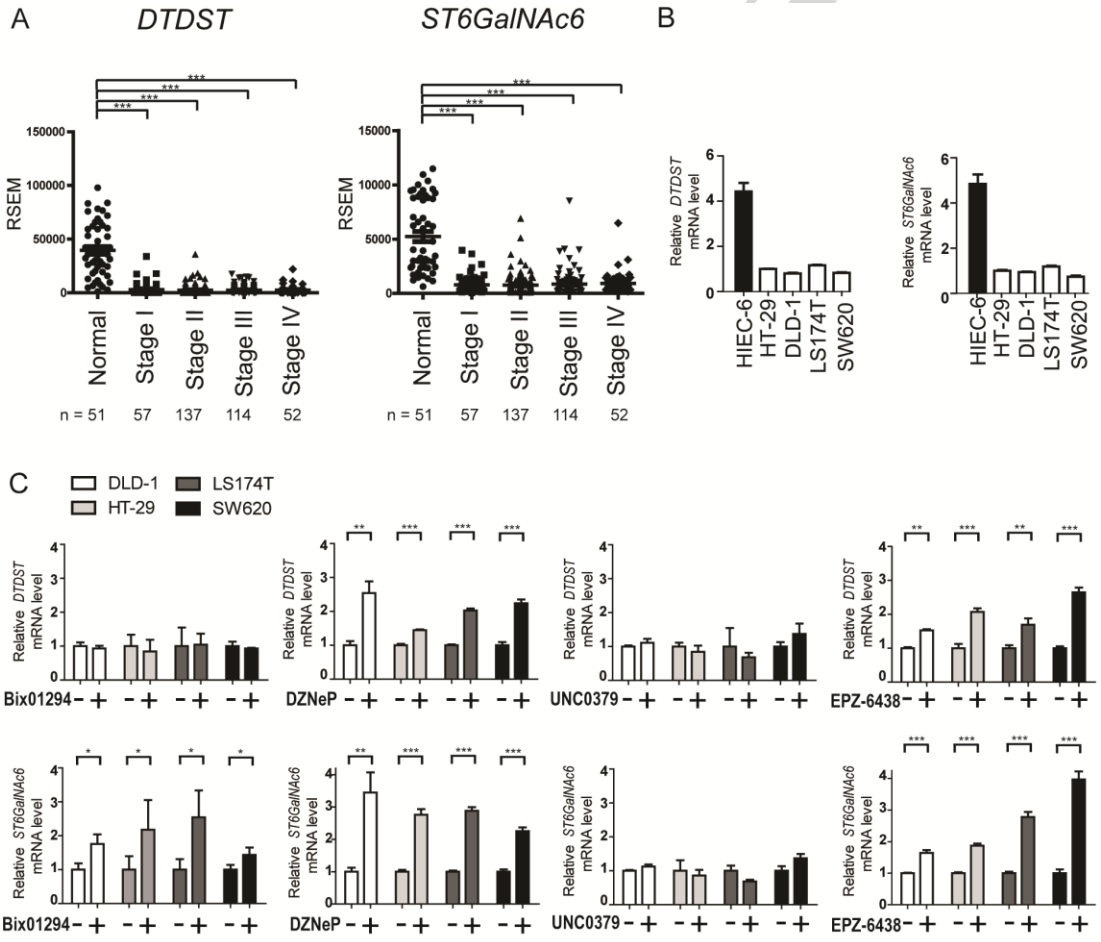

Figure 1. Decreased *DTDST* and *ST6GalNAc6* mRNA in colorectal cancer patients and the roles of repressive histone methyltransferase inhibitors in cultured colon cancer cells. (A) Large-scale RNA-Seq transcriptome analysis of *DTDST* and *ST6GalNAc6* in normal colorectal tissues and in tissues from four stages of colorectal cancer from the TCGA database. N, number of patients; ***, $p < 0.001$. (B) Relative expression of *DTDST* and *ST6GalNAc6* mRNA between normal intestinal epithelial cells (HIEC-6) and colon cancer cells. *DTDST* and *ST6GalNAc6* mRNAs were analyzed

by RT-qPCR and are shown as relative mRNA levels with respect to expression in HT-29 cell. (C) Induction of *DTDST* (upper panels) and *ST6GalNAc6* (lower panels) mRNA in cultured colon cancer cells (DLD-1, HT-29, LS174T and SW620 cells) after treatment with histone methyltransferase inhibitors. Cells were treated with 1 μg/ml BIX-01294 (G9a inhibitor), 2.5 μg/ml DZNeP (EZH2 inhibitor), 5 μg/ml UNC0379 (SETD8 inhibitor) or 5 μg/ml EPZ-6438 (EZH2 inhibitor) for 72 h. *DTDST* and *ST6GalNAc6* mRNAs were analyzed by RT-qPCR and are shown as relative mRNA levels with respect to expression in untreated cells. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ ($n = 3$ with mean ± SD shown).

3.2. Identification of PRC2 containing EZH2 as a major histone repressive complex in silencing *DTDST* and *ST6GalNAc6*

EZH2 is active as a methyltransferase only in the form of the PRC2 complex [24]. We thus determined whether inhibition of PRC2 activity is capable of restoring *DTDST* and *ST6GalNAc6* mRNA with a knockdown approach. We targeted SUZ12 because it is essential for PRC2 activity and is highly expressed in colon cancers [25,26]. Introduction of shRNAs for SUZ12 dramatically decreased the target protein levels as did shRNA for EZH2 (Fig. 2A) and significantly increased *DTDST* and *ST6GalNAc6* mRNA levels (Fig. 2B).

Consistently, introduction of shEZH2 or shSUZ12 significantly decreased H3K27me3 levels in the promoter regions of both *DTDST* and *ST6GalNAc6* based on ChIP analysis (Fig. 2C). DZNeP and EPZ-6438 showed similar effects in decreasing H3K27me3 levels (Fig. 2D). ChIP-Seq data from the DataBase of Transcriptional Start Sites (DBTSS) [27] also supported enrichment of H3K27me3 in the promoter regions of *ST6GalNAc6* and *DTDST* in colon cancer cells (Fig. S5). Thus H3K27me3 modifications in the *DTDST* and *ST6GalNAc6* regulatory regions were produced by EZH2 as a component of PRC2.

A large-scale RNA-Seq transcriptome analysis from TCGA datasets showed that EZH2 mRNA is elevated in colorectal cancers as compared with its expression in normal tissue (Fig. 2E). The mRNA increase was observed between normal colon and stage I samples, with no differences in expression among stages I–IV, suggesting that this increase occurred during the early stage of carcinogenesis.

This increase in EZH2 mRNA showed a close chronological correspondence with the sharp decrease in *ST6GalNAc6* and *DTDST* mRNAs between normal colon and stage I samples. The inverse correlation was significant for both genes: $r = –3976$ and $p < 0.001$ for EZH2 and *ST6GalNAc6* mRNAs and $r = –3987$ and $p < 0.001$ for EZH2 and *DTDST* mRNAs (Fig. S6A). These results suggest that a quantitative increase in EZH2 during the early stage of cancer is at least one of the major mechanisms for silencing

ST6GalNAc6 and *DTDST* expression. RNA-Seq transcriptome analysis from TCGA datasets indicated that mRNA for SUZ12, another PRC2 component [25,26], was also significantly increased in colorectal cancers, similar to the expression changes seen for EZH2 (Fig. 2E). mRNAs for the H3K27 demethylases KDM6A and KDM6B decreased to 76% and 82%, respectively, in stage I colon cancer tissues relative to their expression in normal colon tissue samples (Fig. 2F). It is not clear if this moderate decrease has a biological significance, but it may contribute to the enhanced tri-methylation of H3K27 in cancers.

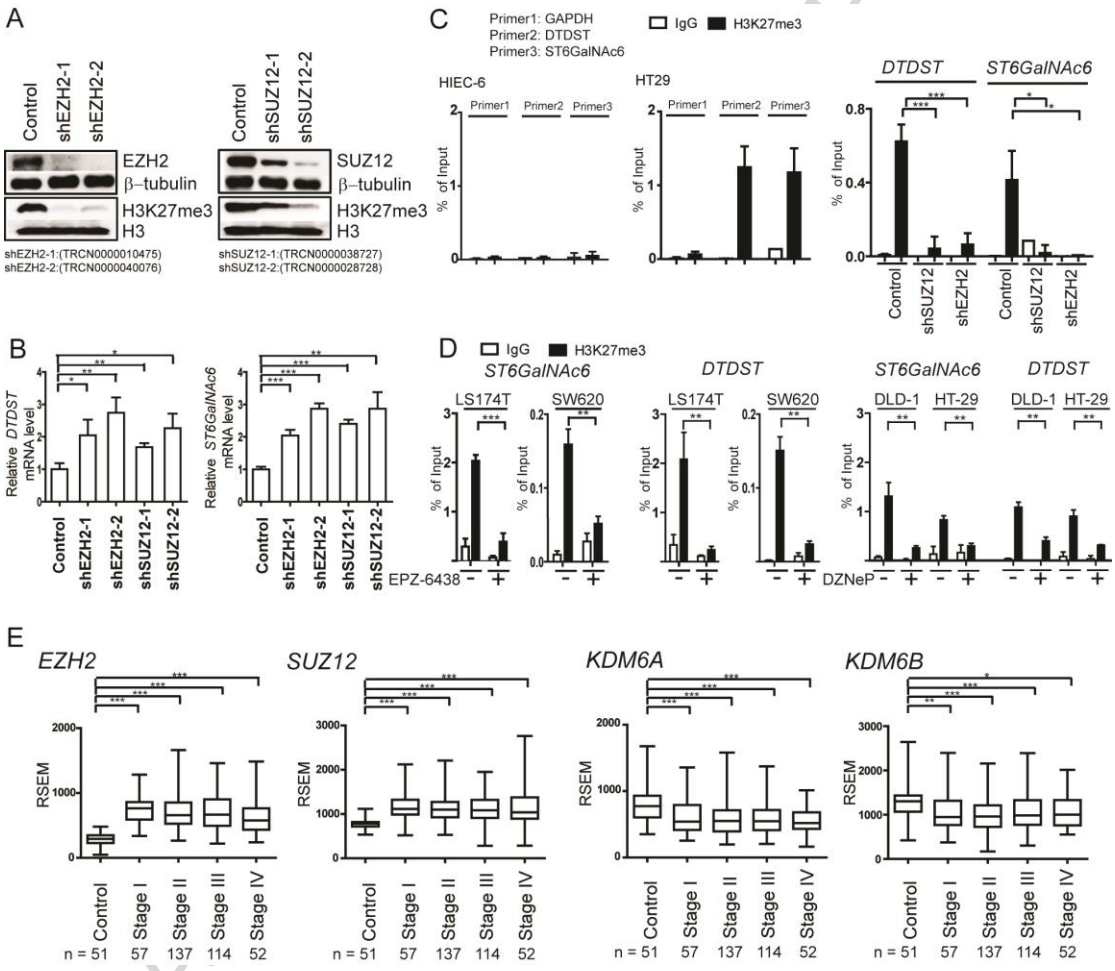

Figure 2. EZH2 in PRC2 is a major histone methyltransferase for the silencing of *DTDST* and *ST6GalNAc6*. (A) Western blot analysis of SUZ12, EZH2 and H3K27me3 in HT-29 cells after introduction of shRNAs for EZH2 or SUZ12. The cells transfected with pLKO scrambled shRNA (pLAS.Void) were used as control. (B) Induction of *DTDST* and *ST6GalNAc6* mRNA in HT-29 cells after introduction of shRNAs for EZH2 or SUZ12. (C) Left two panels show ChIP assays of H3K27me3 levels in the promoter regions of *GAPDH* (off target region), *DTDST* and *ST6GalNAc6* in HIEC-6 and HT-29 cells. Right panel, ChIP assays of H3K27me3 levels in the promoter regions of *DTDST* and

ST6GalNAc6 in HT-29 cells receiving pLKO scrambled shRNA (pLAS.Void) or shRNAs for SUZ12 or EZH2. (D) ChIP assays of H3K27me3 levels in *DTDST* and *ST6GalNAc6* promoter regions in DLD-1, HT-29, LS174T and SW620 cells. Cells were treated with 2.5 μg/ml DZNeP or 5 μg/ml EPZ-6438 for 96 h. H3K27me3 levels were analyzed by RT-qPCR and are shown as a percentage relative to the pre-immunoprecipitation input. (E) Large-scale RNA-Seq transcriptome analysis of EZH2, SUZ12, KDM6A and KDM6B in normal colorectal samples and four stages of colorectal cancer from the TCGA database. In panels B, C and D, ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ ($n = 3$ with mean ± SD shown).

3.3. Recovery of expression of normal glycans and restoration of Siglec ligand activity by inhibition of EZH2 and PRC2

Next, we examined whether inhibition of EZH2 can rescue the expression of the normal glycans disialyl Lewis^a and sialyl 6-sulfo Lewis^x on the cell surface. Introduction of shEZH2 and/or shSUZ12 (Fig. 3A) or treatments with an EZH2 inhibitor EPZ-6438 (Fig. 3B) led to expression of normal glycans on colon cancer cells. When we analyzed expression of normal glycans after treatments with inhibitors for H3K9 and H4K20 methyltransferases in cultured colon cancer cells, G9a inhibitor restored disialyl Lewis^a expression only moderately but did not restore sialyl 6-sulfo Lewis^x expression (Fig. 3C). The H4K20 methyltransferase inhibitor had no effect on either glycan (Fig. 3C). The shRNAs for these methyltransferase genes also showed similar results (Fig. 3D). These results confirmed that inhibition of only EZH2 substantially recovered expression of normal glycans. Treatment with the EZH2 inhibitor EPZ-6438 induced significant binding activity to Siglec-7 of colon cancer cells (Fig. 3E). Moreover, we examined siglec-7 binding activity by introduction of shRNAs for *ST6GalNAc6* or *DTDST* and then treated with EPZ-6438 for 12 days. Introduction of shRNAs for *ST6GalNAc6* or *DTDST* decreased the target mRNA levels (Fig. S7). The results of siglec-7 binding study indicated that recovery siglec-7 binding activity was significantly suppressed compared to controls (Fig. 3F). Thus inhibition of EZH2 restored expression of normal glycans, which is in line with the induction of mRNAs encoded by *DTDST* and *ST6GalNAc6*.

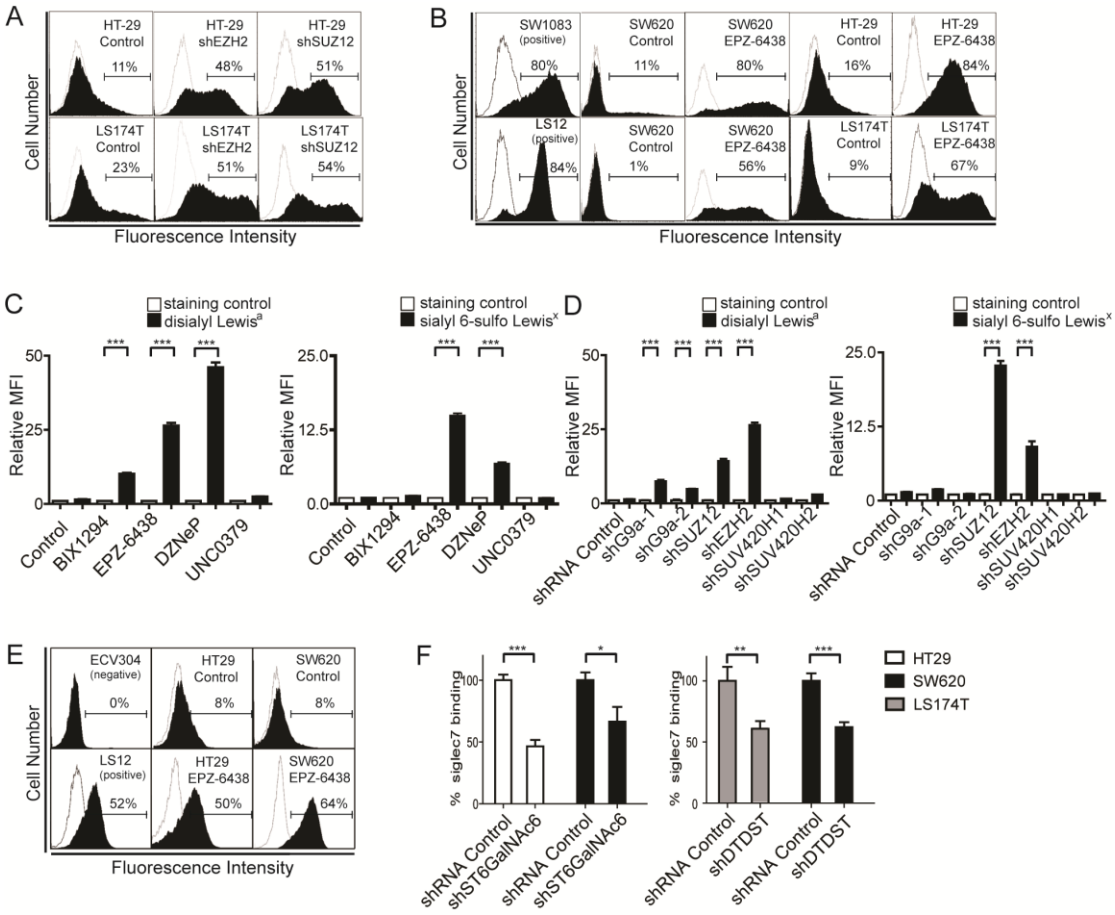

Figure 3. Recovery of normal glycans disialyl Lewis[a] and sialyl 6-sulfo Lewis[x] by inhibition of EZH2. (A) Flow cytometric analysis of levels of disialyl Lewis[a] (upper panels) and sialyl 6-sulfo Lewis[x] (lower panels) on HT-29 and LS174T cells, respectively, after introduction of shRNAs for EZH2 or SUZ12. The cells transfected with pLKO scrambled shRNA (pLAS.Void) were used as control. (B) Flow cytometric analysis of expression levels of disialyl Lewis[a] (upper panels) and sialyl 6-sulfo Lewis[x] (lower panels) on colon cancer cells after treatment with 5 μg/ml EPZ-6438 for 14 d. Thin lines in (A) and (B) indicate staining control. SW1083 transfected with ST6GalNAc6 served for positive control for disialyl Lewis[a], and LS12 cells served for positive control for sialyl 6-sulfo Lewis[x]. (C) Flow cytometric analysis of levels of disialyl Lewis[a] and sialyl 6-sulfo Lewis[x] on SW620 cells after treatment with histone methyltransferase inhibitors. Cells were treated with 1 μg/ml BIX-01294, 2.5 μg/ml DZNeP, 5 μg/ml EPZ-6438 or 5 μg/ml UNC0379 for 8 d. (D) Flow cytometric analysis of levels of disialyl Lewis[a] and sialyl 6-sulfo Lewis[x] on SW620 cells after introduction of shRNAs for G9a, EZH2, SUZ12 and SUV420H1/H2. The relative levels of disialyl Lewis[a] and sialyl 6-sulfo Lewis[x] are shown as the relative mean fluorescence intensity (MFI) compared with the control group IgG or IgM. ***, $p < 0.001$. (E) Flow cytometric analyses of binding of recombinant Siglec-7 to colon cancer cells treated with an EZH2 inhibitor. HT-29 or SW620 cells were treated with 5 μg/ml EPZ-6438 for

12 d. Control shows binding of recombinant Siglec-7 to non-treated colon cancer cells. Thin lines are staining control without recombinant Siglec-7. For siglec-7 binding assays we used ECV304 cells for negative control, and LS12 cells for positive control. (F) Inhibition of siglec-7 binding by induction of shST6GalNAc6 or shDTDST in colon cancer cells, which were treated with 5 μg/ml EPZ-6438 for 12 d.

3.4. Identification of *ST6GalNAc6* variant 3 as a major variant silenced by EZH2 in colon cancers

ST6GalNAc6 transcription results in seven variants—variant 1 (V1)–V6, which are processed into mRNA, whereas V7 is a non-coding RNA (Fig. S8A). Large-scale RNA-Seq transcriptome analysis from TCGA indicated that *ST6GalNAc6* V3 is present in normal colon tissue (Fig. S8B). To assess whether *ST6GalNAc6* V3 is highly repressed by PRC2, we looked for this specific variant after treatment with shEZH2 or EZH2 inhibitors. Inhibition of EZH2 selectively restored *ST6GalNAc6* V3 mRNA (Fig. S8C and D).

Expressed sequence tag (EST) data from TCGA (Fig. S9) and transcription start site information from the DBTSS (Fig. S10) also supported *ST6GalNAc6* V3 as the dominant form in normal colon and rectum [27,28]. Taken together, these findings demonstrate that *ST6GalNAc6* V3 is the major variant expressed in normal colon and is absent as a result of EZH2 activity in colon cancer cells.

3.5. Identification of YY1 as a crucial transcription factor that recruits EZH2-containing PRC2 complexes to *DTDST* and *ST6GalNAc6* V3 promoter regions

We next looked for transcription factors that modulate *DTDST* and *ST6GalNAc6* V3 activity. In the *DTDST* promoter region, binding sites for two potential activators, TFAP2C and CDX2 from the JASPAR database (http://jaspar.binf.ku.dk/), and one binding site for a repressor, YY1, which is based on the CCAT core type (Fig. S11) [29]. Reporter assays using sequentially deleted reporter constructs supported these activities (Fig. 4A). Similarly, potential TFAP2C-, PKNOX2-, and CDX2- based on the JASPAR database, and a YY1-binding site [29] were identified in the *ST6GalNAc6* promoter, and their activities were also supported by reporter constructs (Fig. 4A). Overexpression of the two (for DTDST) or three (for *ST6GalNAc6* V3) activators induced significant promoter activities in reporter assays (Fig. 4B). Mutations in the putative YY1-binding sites in the p(–569) construct for *DTDST* and in the p(–581) construct for *ST6GalNAc6* V3 significantly induced reporter activity (Fig. 4C), strongly supporting its repressive role in the transcriptional regulation of both genes.

YY1 is reportedly up-regulated in colorectal cancers and cell lines [30], and YY1

binding to DNA recruits EZH2 [31,32]. Accordingly, we validated YY1 binding motifs in both repression regions by DNA affinity precipitation assay (DAPA), and the results showed that YY1 was physically associated with PRC2 elements including EZH2 and SUZ12 (Fig. 4D). Binding of YY1 and PRC2 elements was dramatically decreased when a mutation was introduced into the YY1 binding motifs. Our ChIP results also supported that YY1 is enriched in *DTDST* and *ST6GalNAc6* V3 promoter regions (Fig. 4E).

Introduction of a YY1-specific siRNA (siYY1) into colon cancer cells successfully decreased YY1 mRNA and its protein levels (Fig. 5A and B) and significantly increased the *DTDST* and *ST6GalNAc6* mRNA levels (Fig. 5C). The results of ChIP assays further indicated that depletion of YY1 decreased not only H3K27me3 levels but also EZH2 and SUZ12 recruitment to the promoter regions of both genes (Fig. 5D). These results strongly suggested that the transcription factor YY1 serves as a crucial factor for recruiting PRC2 containing EZH2 to the YY1-binding sites in the regulatory regions of both *DTDST* and *ST6GalNAc6* V3.

Unexpectedly, YY1 mRNA levels in colorectal cancers showed no significant difference relative to normal tissue in the TCGA datasets at any stage of cancer (Fig. 5E), which is not consistent with a previous report [30]. This was in clear contrast to the sharp increase in EZH2 mRNA shown in Fig. 2E. Accordingly, there was no significant correlation between YY1 mRNA levels and *DTDST* or *ST6GalNAc6* mRNA levels (Fig. S6B).

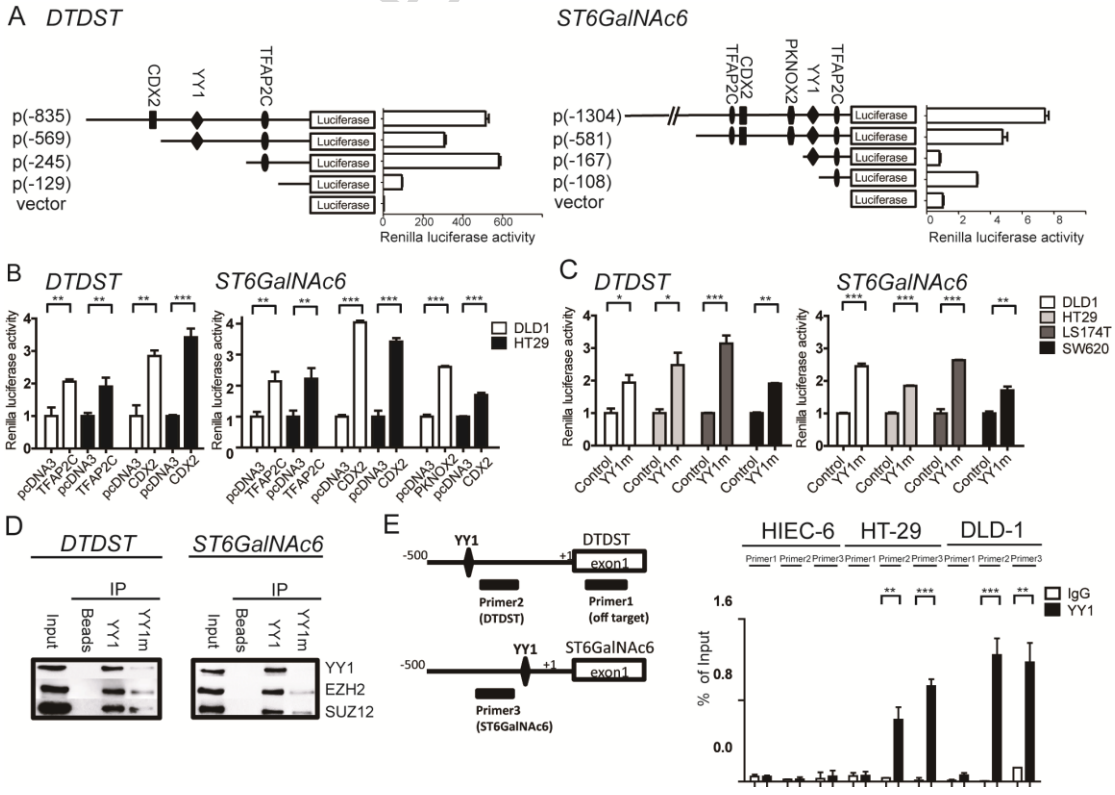

Figure 4. Recruitment of PRC2 containing EZH2 to the *DTDST* and *ST6GalNAc6* promoters by YY1. (A) Deletion analysis assays are shown for the human *DTDST* (YY1-, TFAP2C- and CDX2-binding sites) and *ST6GalNAc6* (YY1-, TFAP2C-, PKNOX2- and CDX2-binding sites) promoters. Renilla luciferase activity is shown as fold increases relative to the pGL3 basic promoter construct (vector). LS174T cells were used for *DTDST* assays, and HT-29 cells were used for *ST6GalNAc6* assays. (B) Promoter analysis of overexpression of TFAP2C or CDX2 using the *DTDST* promoter p(−569) (left panel) and PKNOX2, TFAP2C or CDX2 using the *ST6GalNAc6* promoter p(−581) (right panel). (C) Mutational analysis of the YY1-binding site in the *DTDST* p(−569) and *ST6GalNAc6* p(−581) promoter. The mutated YY1 construct (YY1m) had a mutation of YY1 binding site at bases −426 to −419 (from TGATGGGG to TGATAAAA) in *DTDST* and at bases −136 to −129 (from TGATGGGA to TGATTTGA) in *ST6GalNAc6*. The control and mutated YY1 constructs were transiently expressed in colon cancer cells for luciferase assays. Firefly luciferase activity was normalized to Renilla luciferase activity in(B) and (C). Data are presented as the relative fold increase compared with pcDNA3. In panels A, B, and C, ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ ($n = 3$ with mean ± SD shown). (D) DAPAs of the YY1-binding sites in the *DTDST* (−437 to −407, CTGGAATCTTTGATGGGGAATGAGTTATAG) and *ST6GalNAc6* (−118 to −147, TGGAGTTCAAATCCCATCAGAACCAGTGGA) promoters. YY1 binding to biotinylated oligonucleotides containing either wild-type (YY1) or a mutated YY1-binding sequence (YY1m) was assayed by DNA affinity precipitation and analyzed by western blotting using nuclear extracts of HT-29 cells. The mutated YY1 sequence had a mutation at bases −426 to −419 (from TGATGGGG to TGATAAAA) in *DTDST* and at bases −136 to −129 (from TGATGGGA to TGATTTGA) in *ST6GalNAc6*. Input, 10% of nuclear proteins; beads, the assay contained beads and nuclear protein but no probe. (E) ChIP analysis of YY1 in the promoter regions of *DTDST* and *ST6GalNAc6* in normal intestinal epithelial cells and colon cancer cells. Left panel shows illustration of location of analyzed ChIP regions in DTDST exon1 (off target region), DTDST and ST6GalNAc6.

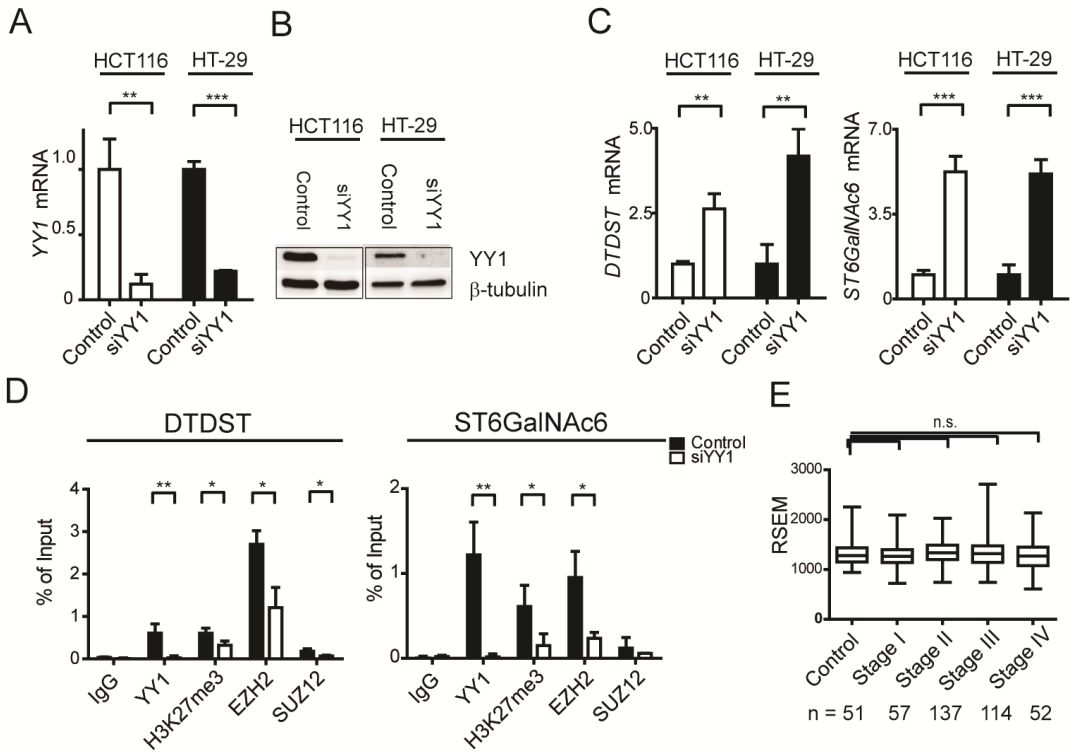

Figure 5. Effects of YY1-specific siRNA on expression of *DTDST* and *ST6GalNAc6*. (A) YY1 mRNA in colon cancer cells after introduction of an siRNA targeting YY1 (siYY1) by electroporation for 1 d, as ascertained by RT-qPCR (n = 3 with mean ± SD shown). (B) Western blot analysis of YY1 in colon cancer cells after introduction of siRNA for YY1 (siYY1). (C) Analysis of *DTDST* and *ST6GalNAc6* mRNAs in cultured colon cancer cells after introduction of siYY1 by electroporation and incubation for 2 d. Results of RT-qPCR analysis are shown as relative mRNA levels compared with the expression of untreated cells (n = 3 with mean ± SD shown). (D) ChIP analysis of enrichment of YY1, H3K27me3, EZH2 and SUZ12 in *DTDST* and *ST6GalNAc6* promoters after incorporation of siYY1 by electroporation and incubation for 3 d. The enrichment levels were analyzed by RT-qPCR and are shown as the percent of input relative to the input before immunoprecipitation (n = 3 with mean ± SD shown). (E) Large-scale RNA-Seq transcriptome analysis of YY1 across four stages of colorectal cancer based on data from the TCGA database.

3.6. Roles of NF-κB in epigenetic silencing of *DTDST* and *ST6GalNAc6* v3

Our findings suggest that recruitment of EZH2 to the regulatory region of *DTDST* and *ST6GALNAc6* by YY1 is the major mechanism for epigenetic silencing of these genes during early stages of colonic carcinogenesis, but it is still not clear what would be the trigger mechanisms for the EZH2/YY1 axis. Increased EZH2 mRNA levels offer one possibility. As NF-κB induces EZH2 transcription [33,34], we examined whether silencing of *DTDST* and *ST6GalNAc6* activities is triggered through NF-κB–mediated

EZH2 transcription in colon cancer cells. First, we examined EZH2 mRNA levels after administration of NF-κB inhibitors Bay11-7085 and TPCA-1 in colon cancer cells. Both NF-κB inhibitors significantly decreased EZH2 mRNA levels (Fig. 6A) and notably increased *DTDST* and *ST6GalNAc6* mRNA levels (Fig. 6B). These NF-κB inhibitors also dramatically recovered expression of normal glycans in colon cancer cells (Fig. 6C) and markedly enhanced the Siglec-7 binding to the cells (Fig. 6D). However, the situation may be more complicated, because we found that the proteins in PRC2 other than EZH2, which play essential roles in supporting the methyltransferase activity of EZH2, also have NF-κB binding sites in the regulatory region of the genes (data not shown).

Furthermore, knockdown of NF-κB p65 (Fig. 6E) significantly decreased EZH2 mRNA (Fig. 6F) and significantly increased *DTDST* and *ST6GalNAc6* mRNA (Fig. 6G). Transfection of a dominant-negative mutant NF-κB inhibitor (IkBm) confirmed that NF-κB has a role in silencing *DTDST* and *ST6GalNAc6* activities (Fig. 6H-J).

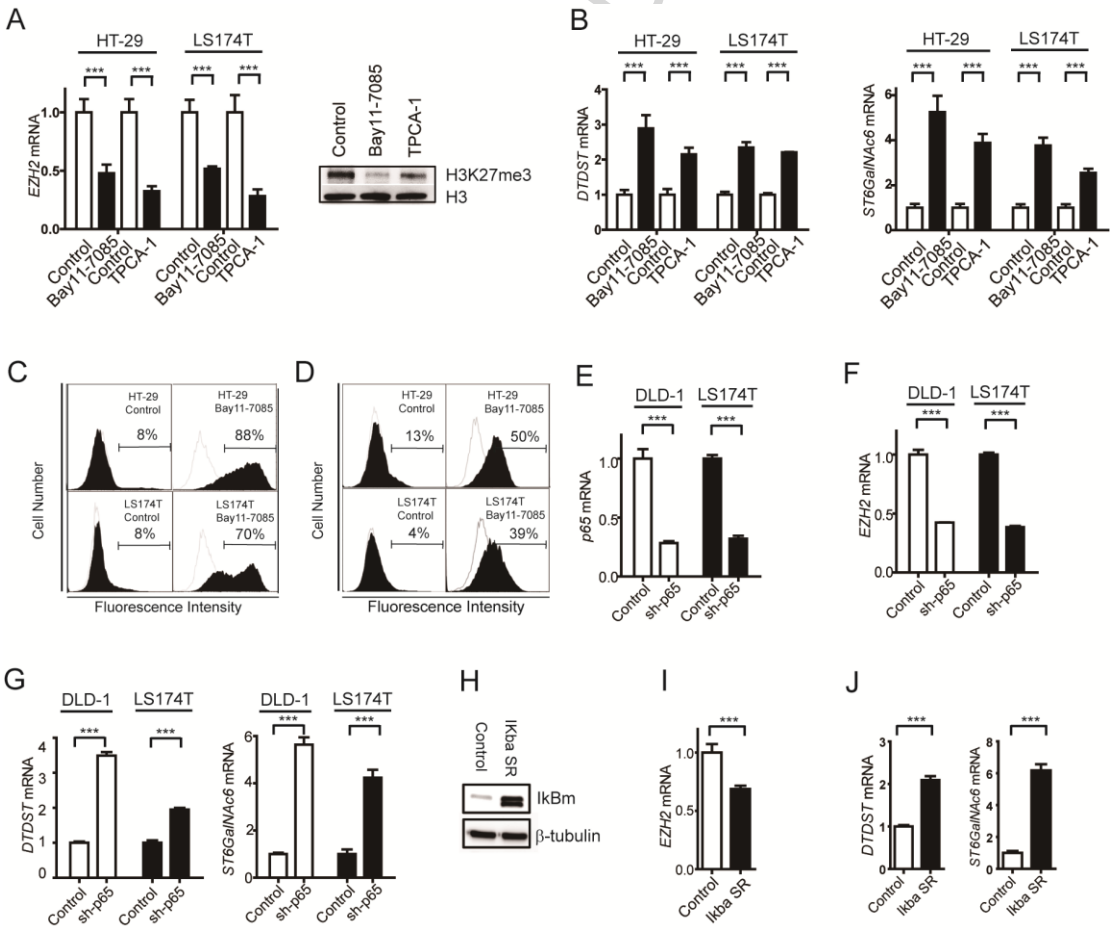

Figure 6. NF-κB modulates both *DTDST* and *ST6GalNAc6* activity in colon cancer cells. (A) EZH2 mRNA in colon cancer cells after treatment with NF-κB inhibitors Bay11-7085 (10 μg/ml) and TPCA-1 (5 μg/ml) for 6 h as ascertained by RT-qPCR. (left

panel) and Western blot analysis of H3K27me3 (right panel) in HT29 cell after treatment with NF-κB inhibitors Bay11-7085 (10 μg/ml) or TPCA-1 (5 μg/ml) for 3 d. (B) *DTDST* and *ST6GalNAc6* mRNA in colon cancer cells after treatment with NF-κB inhibitors Bay11-7085 (10 μg/ml) and TPCA-1 (5 μg/ml) for 1 d as ascertained by RT-qPCR. (C) Flow cytometric analysis of expression of disialyl Lewis^a (upper panel) and sialyl 6-sulfo Lewis^x (lower panel) on colon cancer cell lines after administration of 10 μg/ml Bay11-7085 for 12 d. (D) Flow cytometric analysis of binding of recombinant Siglec-7 to colon cancer cells treated with Bay11-7085 as in (C). Thin lines in (C) and (D) indicate staining control. (E) Gene expression analysis of p65 mRNA in colon cancer cells after introduction of shRNAs for p65. (F, G) EZH2 mRNA (F) and *DTDST* and *ST6GalNAc6* mRNA (G) in colon cancer cells after introduction of shRNAs specific for p65. (H) Western blot analysis after transfection of dominant-negative mutant NF-κB inhibitor (IkBm) in DLD-1 cells for 12 h. (I) EZH2 mRNA after transfection of IkBm in DLD-1 cells for 12 h. (J) Gene expression analysis of *DTDST* and *ST6GalNAc6* mRNA by transfection of IkBm in DLD-1 cells. In panels A, B, E, F, G, I and J, ***, $p < 0.001$ ($n = 3$ with mean ± SD shown).

4. Discussion

We previously showed that disialyl Lewis^a and sialyl 6-sulfo Lewis^x glycans are normal glycans that are preferentially expressed in non-malignant colonic epithelial cells and tend to disappear in colon cancer cells [4,5]. We proposed that this is due to silencing of *ST6GalNAc6* and *DTDST* by showing a significant decrease in their mRNA levels in colon cancers as compared with non-malignant colonic epithelial cells and that this leads to the appearance of the well-known cancer-associated glycans sialyl Lewis^a and sialyl Lewis^x in cancer cells [4,6]. Silencing of *ST6GalNAc6* and *DTDST* was also confirmed in this study by the bioinformatics analysis of the TCGA datasets, which showed a prominent decrease in *ST6GalNAc6* and *DTDST* transcription that was evident during the very early stages of colon cancer.

We also showed that the normal glycans disialyl Lewis^a and sialyl 6-sulfo Lewis^x serve as ligands for immunosuppressive molecules, namely Siglecs [4,12], whereas the cancer-associated glycans sialyl Lewis^a and sialyl Lewis^x serve as ligands for endothelial selectins [7-9]. Siglecs have dual roles in cancer progression, some of which are pro-oncogenic and others of which are anti-oncogenic. Loss of Siglecs can enhance cancer-promoting inflammation within the tumor microenvironment by expanding the number of M2 macrophages, which facilitate the growth of tumors, whereas at other times loss of Siglecs leads to killing of tumor cells in vivo and enhanced immunosurveillance of autologous tumors [35]. MUC1 molecules with aberrant Siglec ligand glycans repolarize resident macrophages into tumor-associated macrophages and promote carcinogenesis [36]. The dual roles of Siglecs may reflect

the dual function of macrophages in cancer progression. Our previous findings confirm that the interaction of Siglecs with their glycan ligands suppresses production of pro-oncogenic inflammation mediators such as COX2 by macrophages [12], and their loss will promote colon carcinogenesis. It is notable that normal epithelial cells express substantial amounts of Siglec ligands, which are lost during the course of carcinogenesis because of silencing of specific genes such as *ST6GalNAc6* and *DTDST*.

Suppression of *ST6GalNAc6* and *DTDST* transcription is suggested to occur during relatively early stages of colon carcinogenesis, because the decrease in their mRNAs was already statistically significant in stage I samples from the TCGA database and because the appearance of sialyl Lewis^a occurs during the early phase of the adenoma-carcinoma sequence. This implies that the loss of Siglec ligand activity occurs during a relatively early stage of colon carcinogenesis. APC mutation contributes to the formation of benign colonic adenoma, but production of additional pro-oncogenic inflammatory mediators such as COX2 is required for malignant transformation of adenoma cells, and thus COX2 inhibitors may prevent colon cancers [37-39]. We previously showed that Siglec polymerization suppresses COX2 production induced by inflammatory stimuli and proposed that loss of Siglec ligand glycans would contribute to promoting colon carcinogenesis [12]. Epigenetic silencing of *ST6GalNAc6* and *DTDST* induced by EZH2 may facilitate malignant transformation of colonic epithelial cells, and suppression of this epigenetic silencing could be beneficial for chemoprevention of colon cancers, providing a better therapeutic strategy to alleviate the problem of COX2 inhibitor–associated side effects.

Expression of EZH2 was significantly increased in stage I colon cancer samples in the TCGA database. There was a sharp increase between normal colon samples and stage I cancers, whereas the levels did not show appreciable changes from stage I to stage IV. The increase in EZH2 expression during the early stage of cancer is impressive and is consistent with the timing of epigenetic silencing of *ST6GalNAc6* and *DTDST*, which showed a sharp decrease between normal colon samples and stage I cancers. This underscores the role of EZH2 during early stages of colon carcinogenesis.

EZH2 is involved in various aspects of cancer biology [40], including cancer cell proliferation, histologic grade, invasion, metastasis, cancer stem cells, epithelial-mesenchymal transition, chemoresistance and patient prognosis, but the role of EZH2 during early stages of cancer has not, thus far, attracted enough attention from researchers. EZH2 expression is increased in many cancers, and various factors modulate its expression, including pRB-E2F [41], MEK-ERK-ELK1 [42], KRAS mutations [43] and hypoxia-inducible factor HIF-1α [44], but the recently described role of NF-κB [33,34] appears to be most pertinent in inducing EZH2 in the context of facilitating malignant transformation by pro-inflammatory factors during

early stages of carcinogenesis. NF-κB can be activated by bacterial stimuli in the colon [45,46], and this may induce EZH2, which suppresses expression of Siglec ligand glycans. Some colonic microbiota, such as *Bacteroides fragilis* and *Fusobacterium nucleatum*, are especially potent for stimulating NF-κB activity [45,46]. Our findings suggest that NF-κB, as well as EZH2, is a candidate target for chemoprevention of colon carcinogenesis.

NF-κB is involved in many crucial physiological pathways. Our findings suggest NF-κB trigger EZH2 to suppress *ST6GalNAc6* and *DTDST* activities. However, the situation may be more complicated, because we found SUZ12, which plays essential roles in supporting the methyltransferase activity of EZH2 in PRC2, also have NF-κB binding sites in the regulatory region of the genes (data not shown). In colon cancer tissues, the mRNA levels of H3K27 demethylases KDM6A and KDM6B are moderately decreased compare to their expression in normal colon tissue samples. KDM6A, but not KDM6B, expression was also moderately increased by NF-κB inhibitor treatment (data not shown), but right now we do not know its biological significance.

We also demonstrated that YY1 is a critical transcription factor for recruiting PRC2 to the promoter regions of *ST6GalNAc6* and *DTDST* and in silencing their activities. The C terminus of YY1 directly binds EED in PRC2 [47], and the WD-repeat domain of EED binds to the N-terminal α-helix of EZH2 [48]. ChIP assays indicated that YY1-binding sites are present in the promoter regions of both *ST6GalNAc6* and *DTDST* and are associated with PRC2 and EZH2. Knock-down of YY1 using siRNA depleted PRC2 and EZH2 from the YY1-binding sites and successfully recovered mRNA levels of *ST6GalNAc6* and *DTDST*. We suggest that YY1 is a vital transcription factor to silence *ST6GalNAc6* and *DTDST* by recruiting PRC2 containing EZH2. Although there was no significant increase in YY1 in colon cancer samples in the TCGA datasets, our findings suggest that YY1 is another target for chemoprevention of colon carcinogenesis. These findings provide a putative mechanism for promotion of carcinogenesis by loss of immunosuppressive molecules by epigenetic silencing, and suggest that targeting NF-κB mediated EZH2/YY1 axis holds promise for prevention of the onset of colorectal cancers.

Acknowledgements

We thank Dr. Sen-itiroh Hakomori, Pacific Northwest Research Institute, Seattle, WA, for the gift of antibody FH7. This work was supported by research grants from Academia Sinica and the Ministry of Science and Technology of Taiwan (MOST 107-0210-01-19-01, MOST 106-0210-01-15-02, and MOST 106-2320-B-001-015).

Conflict of interest

The authors declare they have no conflicts of interest.

References

[1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2017, CA Cancer J. Clin., 67 (2017) 7-30. https://doi.org/10.3322/caac.21387

[2] S.I. Hakomori, Glycosynaptic microdomains controlling tumor cell phenotype through alteration of cell growth, adhesion, and motility, FEBS Lett., 584 (2010) 1901-1906. https://doi.org/10.1016/j.febslet.2009.10.065. Epub 2009 Oct 27.

[3] S.S. Pinho, C.A. Reis, Glycosylation in cancer: mechanisms and clinical implications, Nat. Rev. Cancer., 15 (2015) 540-555. https://doi.org/10.1038/nrc3982

[4]K. Miyazaki, K. Ohmori, M. Izawa, T. Koike, K. Kumamoto, K. Furukawa, T. Ando, M. Kiso, T. Yamaji, Y. Hashimoto, A. Suzuki, A. Yoshida, M. Takeuchi, R. Kannagi, Loss of disialyl Lewis^{a}, the ligand for lymphocyte inhibitory receptor Siglec-7, associated with increased sialyl Lewis^{a} expression on human colon cancers, Cancer Res., 64 (2004) 4498-4505. https://doi.org/10.1158/0008-5472.CAN-03-3614

[5] M. Izawa, K. Kumamoto, C. Mitsuoka, A. Kanamori, K. Ohmori, H. Ishida, S. Nakamura, K. Kurata-Miura, K. Sasaki, T. Nishi, R. Kannagi, Expression of sialyl 6-sulfo Lewis x is inversely correlated with conventional sialyl Lewis x expression in human colorectal cancer, Cancer Res., 60 (2000) 1410-1416.

[6] A. Yusa, K. Miyazaki, N. Kimura, M. Izawa, R. Kannagi, Epigenetic silencing of the sulfate transporter gene *DTDST* induces sialyl Lewis^{x} expression and accelerates proliferation of colon cancer cells, Cancer Res., 70 (2010) 4064-4073. https://doi.org/10.1158/0008-5472.CAN-09-2383

[7] J.B. Lowe, L.M. Stoolman, R.P. Nair, R.D. Larsen, T.L. Berhend, R.M. Marks, ELAM-1-dependent cell adhesion to vascular endothelium determined by a transfected human fucosyltransferase cDNA, Cell, 63 (1990) 475-484.

[8] M.L. Phillips, E. Nudelman, F.C.A. Gaeta, M. Perez, A.K. Singhal, S. Hakomori, J.C. Paulson, ELAM-1 mediates cell adhesion by recognition of a carbohydrate ligand, sialyl-Le^{x}, Science, 250 (1990) 1130-1132.

[9] A. Takada, K. Ohmori, T. Yoneda, K. Tsuyuoka, A. Hasegawa, M. Kiso, R. Kannagi, Contribution of carbohydrate antigens sialyl Lewis A and sialyl Lewis X to adhesion of human cancer cells to vascular endothelium, Cancer Res., 53 (1993) 354-361.

[10]R. Kannagi, M. Izawa, T. Koike, K. Miyazaki, N. Kimura, Carbohydrate-mediated cell adhesion in cancer metastasis and angiogenesis, Cancer Sci., 95 (2004) 377-384.

[11]R. Kannagi, K. Sakuma, K. Miyazaki, K-T. Lim, A. Yusa, J. Yin, M. Izawa, Altered expression of glycan genes in cancers induced by epigenetic silencing and tumor hypoxia: Clues in the ongoing search for new tumor markers, Cancer Sci., 101 (2010) 586-593. https://doi.org/10.1111/j.1349-7006.2009.01455.x

[12]K. Miyazaki, K. Sakuma, Y.I. Kawamura, M. Izawa, K. Ohmori, M. Mitsuki, T. Yamaji, Y. Hashimoto, A. Suzuki, Y. Saito, T. Dohi, R. Kannagi, Colonic epithelial cells express specific ligands for mucosal macrophage immunosuppressive receptors, siglec-7 and -9, J. Immunol., 188 (2012) 4690-4700. https://doi.org/10.4049/jimmunol.1100605

[13]R. Cao, L. Wang, H. Wang, L. Xia, H. Erdjument-Bromage, P. Tempst, R.S. Jones, Y. Zhang, Role of histone H3 lysine 27 methylation in Polycomb-group silencing, Science, 298 (2002) 1039-1043. https://doi.org/10.1126/science.1076997

[14]G. Schotta, M. Lachner, K. Sarma, A. Ebert, R. Sengupta, G. Reuter, D. Reinberg, T. Jenuwein, A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin, Genes Dev., 18 (2004) 1251-1262. https://doi.org/10.1101/gad.300704

[15]Y. Kondo, Targeting histone methyltransferase EZH2 as cancer treatment, J. Biochem., 156 (2014) 249-257. https://doi.org/10.1093/jb/mvu054

[16]J. Zhang, P. He, Y. Xi, M. Geng, Y. Chen, J. Ding, Down-regulation of G9a triggers DNA damage response and inhibits colorectal cancer cells proliferation, Oncotarget, 6 (2015) 2917-2927. https://doi.org/10.18632/oncotarget.2784

[17]A. Benard, I.J. Goossens-Beumer, A.Q. van Hoesel, H. Horati, H. Putter, E.C. Zeestraten, C.J. van de Velde, P.J. Kuppen, Prognostic value of polycomb proteins EZH2, BMI1 and SUZ12 and histone modification H3K27me3 in colorectal cancer, PLoS. One, 9 (2014) e108265. https://doi.org/10.1371/journal.pone.0108265

[18]Broad Institute TCGA Genome Data Analysis Center. Analysis-ready standardized TCGA data from Broad GDAC Firehose 2016_01_28 run. Broad Institute of MIT and Harvard. Dataset. https://doi.org/10.7908/C11G0KM9 (17 February 2016).

[19]C. Mitsuoka, M. Sawada-Kasugai, K. Ando-Furui, M. Izawa, H. Nakanishi, S. Nakamura, H. Ishida, M. Kiso, R. Kannagi, Identification of a major carbohydrate capping group of the L-selectin ligand on high endothelial venules in human lymph nodes as 6-sulfo sialyl Lewis x, J. Biol. Chem., 273 (1998) 11225-11233.

[20]R. Kannagi, A. Kitahara, S. Itai, K. Zenita, K. Shigeta, T. Tachikawa, A. Noda, H. Hirano, M. Abe, S. Shin, Y. Fukushi, S. Hakomori, H. Imura, Quantitative and qualitative characterization of human cancer-associated serum glycoprotein antigens expressing epitopes consisting of sialyl or sialyl-fucosyl type 1 chain, Cancer Res., 48 (1988) 3856-3863.

[21]K. Ohmori, F. Fukui, M. Kiso, T. Imai, O. Yoshie, H. Hasegawa, K. Matsushima, R. Kannagi, Identification of cutaneous lymphocyte-associated antigen as sialyl 6-sulfo Lewis x, a selectin ligand expressed on a subset of skin-homing helper memory T cells, Blood, 107 (2006) 3197-3204. https://doi.org/10.1182/blood-2005-05-2185

[22]N. Kimura, C. Mitsuoka, A. Kanamori, N. Hiraiwa, K. Uchimura, T. Muramatsu, T. Tamatani, G. S. Kansas, and R. Kannagi, Reconstitution of functional L-selectin ligands on a cultured human endothelial cell line by cotransfection of $\alpha 1\rightarrow 3$ fucosyltransferase VII and newly cloned GlcNAcβ:6-sulfotransferase cDNA, Proc. Natl. Acad. Sci. U. S. A., 96 (1999) 4530-4535. https://doi.org/10.1073/pnas.96.8.4530

[23]J.S. Boehm, J.J. Zhao, J. Yao, S.Y. Kim, R. Firestein, I.F. Dunn, S.K. Sjostrom, L.A. Garraway, S. Weremowicz, A.L. Richardson, H. Greulich, C.J. Stewart, L.A. Mulvey, R.R. Shen, L. Ambrogio, T. Hirozane-Kishikawa, D.E. Hill, M. Vidal, M. Meyerson, J.K. Grenier, G. Hinkle, D.E. Root, T.M. Roberts, E.S. Lander, K. Polyak, W.C. Hahn, Integrative genomic approaches identify IKBKE as a breast cancer oncogene, Cell, 129 (2007) 1065-1079. https://doi.org/10.1016/j.cell.2007.03.052

[24]L. Jiao, X. Liu, Structural basis of histone H3K27 trimethylation by an active polycomb repressive complex 2, Science, 350 (2015) aac4383. https://doi.org/10.1126/science.aac4383

[25]R. Cao, Y. Zhang, SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex, Mol. Cell, 15 (2004) 57-67. https://doi.org/10.1016/j.molcel.2004.06.020

[26]A. Kirmizis, S.M. Bartley, A. Kuzmichev, R. Margueron, D. Reinberg, R. Green, P.J. Farnham, Silencing of human polycomb target genes is associated with methylation of histone H3 Lys 27, Genes Dev., 18 (2004) 1592-1605. https://doi.org/10.1101/gad.1200204

[27]A. Suzuki, H. Wakaguri, R. Yamashita, S. Kawano, K. Tsuchihara, S. Sugano, Y. Suzuki, K. Nakai, DBTSS as an integrative platform for transcriptome, epigenome and genome sequence variation data, Nucleic Acids Res., 43 (2015) D87-D91. https://doi.org/10.1093/nar/gku1080

[28]R. Yamashita, S. Sugano, Y. Suzuki, K. Nakai, DBTSS: DataBase of transcriptional start sites progress report in 2012, Nucleic Acids Res., 40 (2012) D150-D154. https://doi.org/10.1093/nar/gkr1005

[29]S.R. Yant, W. Zhu, D. Millinoff, J.L. Slightom, M. Goodman, D.L. Gumucio, High affinity YY1 binding motifs: identification of two core types (ACAT and CCAT) and distribution of potential binding sites within the human β globin cluster, Nucleic Acids Res., 23 (1995) 4353-4362.

[30]N. Zhang, X. Li, C.W. Wu, Y. Dong, M. Cai, M.T. Mok, H. Wang, J. Chen, S.S. Ng, M. Chen, J.J. Sung, J. Yu, microRNA-7 is a novel inhibitor of YY1 contributing to colorectal tumorigenesis, Oncogene, 32 (2013) 5078-5088.

https://doi.org/10.1038/onc.2012.526
[31]A. Basu, F.H. Wilkinson, K. Colavita, C. Fennelly, M.L. Atchison, YY1 DNA binding and interaction with YAF2 is essential for Polycomb recruitment, Nucleic Acids Res., 42 (2014) 2208-2223. https://doi.org/10.1093/nar/gkt1187
[32] X. Pan, M. Papasani, Y. Hao, M. Calamito, F. Wei, W. J. Quinn, A. Basu, J. Wang, S. Hodawadekar, K. Zaprazna, H. Liu, Y. Shi, D. Allman, M. Cancro, M. L. Atchison, YY1 controls Igκ repertoire and B-cell development, and localizes with condensin on the Igκ locus, EMBO J., (2013) 32, 1168-1182 https://doi.org/10.1038/emboj.2013.66
[33]D. Fujikawa, S. Nakagawa, M. Hori, N. Kurokawa, A. Soejima, K. Nakano, T. Yamochi, M. Nakashima, S. Kobayashi, Y. Tanaka, M. Iwanaga, A. Utsunomiya, K. Uchimaru, M. Yamagishi, T. Watanabe, Polycomb-dependent epigenetic landscape in adult T-cell leukemia, Blood, 127 (2016) 1790-1802. https://doi.org/10.1182/blood-2015-08-662593
[34]H. Rajabi, M. Hiraki, A. Tagde, M. Alam, A. Bouillez, C.L. Christensen, M. Samur, K.K. Wong, D. Kufe, MUC1-C activates EZH2 expression and function in human cancer cells, Sci. Rep., 7 (2017) 7481. https://doi.org/10.1038/s41598-017-07850-0
[35]H. Laubli, O.M. Pearce, F. Schwarz, S.S. Siddiqui, L. Deng, M.A. Stanczak, A. Verhagen, P. Secrest, C. Lusk, A.G. Schwartz, N.M. Varki, J.D. Bui, A. Varki, Engagement of myelomonocytic Siglecs by tumor-associated ligands modulates the innate immune response to cancer, Proc. Natl. Acad. Sci. U. S. A., 111 (2014) 14211-14216. https://doi.org/10.1073/pnas.1409580111. Epub 2014 Sep 15.
[36]R. Beatson, V. Tajadura-Ortega, D. Achkova, G. Picco, T.D. Tsourouktsoglou, S. Klausing, M. Hillier, J. Maher, T. Noll, P.R. Crocker, J. Taylor-Papadimitriou, J.M. Burchell, The mucin MUC1 modulates the tumor immunological microenvironment through engagement of the lectin Siglec-9, Nat. Immunol., 17 (2016) 1273-1281. https://doi.org/10.1038/ni.3552
[37]M. Oshima, M.M. Taketo, COX selectivity and animal models for colon cancer, Curr. Pharm. Des., 8 (2002) 1021-1034.
[38]H. Oshima, M. Oshima, The inflammatory network in the gastrointestinal tumor microenvironment: lessons from mouse models, J. Gastroenterol., 47 (2012) 97-106. https://doi.org/10.1007/s00535-011-0523-6
[39]A.M. Fajardo, G.A. Piazza, Chemoprevention in gastrointestinal physiology and disease. Anti-inflammatory approaches for colorectal cancer chemoprevention, Am. J. Physiol Gastrointest. Liver Physiol., 309 (2015) G59-G70. https://doi.org/10.1152/ajpgi.00101.2014
[40]M. Yamagishi, K. Uchimaru, Targeting EZH2 in cancer therapy, Curr. Opin. Oncol., 29 (2017) 375-381. https://doi.org/10.1097/CCO.0000000000000390
[41]A.P. Bracken, D. Pasini, M. Capra, E. Prosperini, E. Colli, K. Helin, EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer, EMBO J., 22 (2003) 5323-5335. https://doi.org/10.1093/emboj/cdg542
[42]S. Fujii, K. Tokita, N. Wada, K. Ito, C. Yamauchi, Y. Ito, A. Ochiai, MEK-ERK pathway regulates EZH2 overexpression in association with aggressive breast cancer subtypes, Oncogene, 30 (2011) 4118-4128. https://doi.org/10.1038/onc.2011.118
[43]E. Riquelme, C. Behrens, H.Y. Lin, G. Simon, V. Papadimitrakopoulou, J. Izzo, C. Moran, N. Kalhor, J.J. Lee, J.D. Minna, I.I. Wistuba, Modulation of EZH2 Expression by MEK-ERK or PI3K-AKT signaling in lung cancer is dictated by different KRAS oncogene mutations, Cancer Res., 76 (2016) 675-685. https://doi.org/10.1158/0008-5472.CAN-15-1141
[44]S. Mahara, P.L. Lee, M. Feng, V. Tergaonkar, W.J. Chng, Q. Yu, HIFI-α activation underlies a functional switch in the paradoxical role of Ezh2/PRC2 in breast cancer, Proc. Natl. Acad. Sci. U. S. A., 113 (2016) E3735-E3744. https://doi.org/10.1073/pnas.1602079113
[45]Z. Wei, S. Cao, S. Liu, Z. Yao, T. Sun, Y. Li, J. Li, D. Zhang, Y. Zhou, Could gut microbiota serve as prognostic biomarker associated with colorectal cancer patients' survival? A pilot study on relevant mechanism, Oncotarget, 7 (2016)

46158-46172. https://doi.org/10.18632/oncotarget.10064

[46] A.D. Kostic, E. Chun, L. Robertson, J.N. Glickman, C.A. Gallini, M. Michaud, T.E. Clancy, D.C. Chung, P. Lochhead, G.L. Hold, E.M. El Omar, D. Brenner, C.S. Fuchs, M. Meyerson, W.S. Garrett, Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment, Cell Host Microbe., 14 (2013) 207-215. https://doi.org/10.1016/j.chom.2013.07.007

[47] D.P. Satijn, K.M. Hamer, J. den Blaauwen, A.P. Otte, The polycomb group protein EED interacts with YY1, and both proteins induce neural tissue in Xenopus embryos, Mol. Cell Biol., 21 (2001) 1360-1369. https://doi.org/10.1128/MCB.21.4.1360-1369.2001

[48] Z. Han, X. Xing, M. Hu, Y. Zhang, P. Liu, J. Chai, Structural basis of EZH2 recognition by EED, Structure, 15 (2007) 1306-1315. https://doi.org/10.1016/j.str.2007.08.007

Highlights

DTDST and *ST6GalNAc6* synthesize Siglec ligand glycans in normal colonic epithelium

Expression of the *DTDST* and *ST6GalNAc6* genes is suppressed at stage 1 colon carcinoma

This is due to epigenetic silencing through the H3K27me3 modification by EZH2 in PRC2

NF-kappaB and YY1 markedly contribute to epigenetic silencing of *DTDST* and *ST6GalNAc6*

Inhibition of EZH2 or NF-kappaB recovers normal glycans and Siglec binding activity