Supplementary Figures

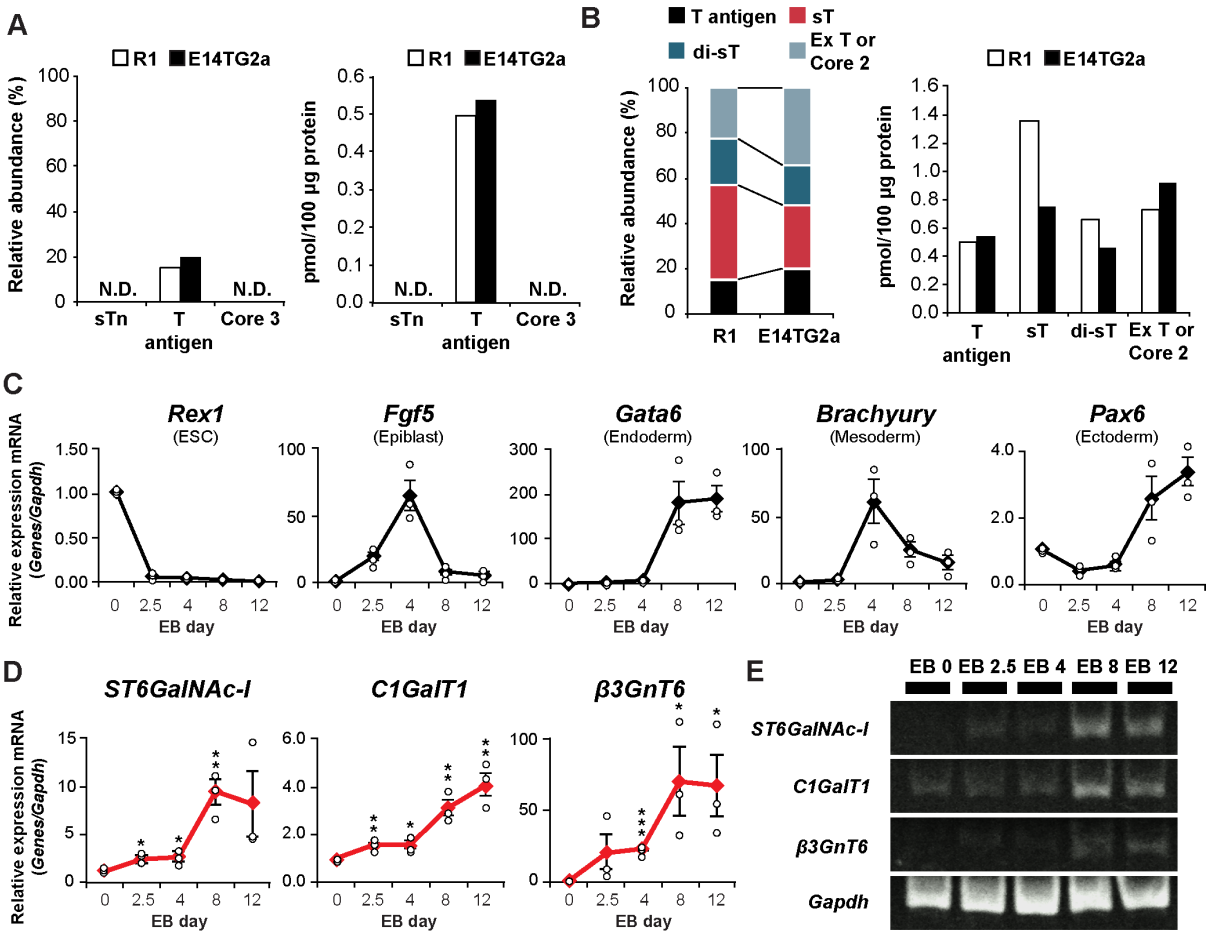


Figure S1. *C1GalT1* is the most highly expressed in ESCs and during ESCs early differentiation.

(A): Relative (left panel) and absolute amount (right panel) of sTn antigen (sTn), T antigen, and Core 3 structure (Core 3) by mass spectrometry in R1 (white box) and E14TG2a cell lines (black box). **(B):** Relative (left panel) and absolute amount (right panel) of *O*-glycan structures detected by mass spectrometry in R1 and E14TG2a cell lines. T antigen (black box), and C1GalT1-mediated elongation pathway modifications; sT antigen (sT, red box), disialyl T antigen (di-sT, dark blue box), and extended T antigen or Core 2 structure (Ex T or Core 2, light blue box). The data were obtained from a single technical and biological replicate. N.D. not detected. **(C):** Real-time PCR analysis of *Rex1, Fgf5, Gata6, Brachyury,* and *Pax6* in embryoid bodies (EB) at day 0 (ESC), 2.5, 4, 8, and 12 normalized against *Gapdh*. **(D):** Real-time PCR analysis of *ST6GalNAc-I, C1GalT1,* and *β3GnT6* in EB at day 0 (ESC),

2.5, 4, 8, and 12 normalized against *Gapdh*. **(E):** *ST6GalNAc-I, C1GalT1, β3GnT6,* and *Gapdh* mRNA quantification by PCR. mRNA was extracted from the EB at day 0 (ESC), 2.5, 4, 8, and 12 and amplified by PCR using 25 cycles to avoid saturation, and separated on polyacrylamide gel. The values are shown as means ± s.e.m. from three independent experiments**.** Significant values are indicated as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

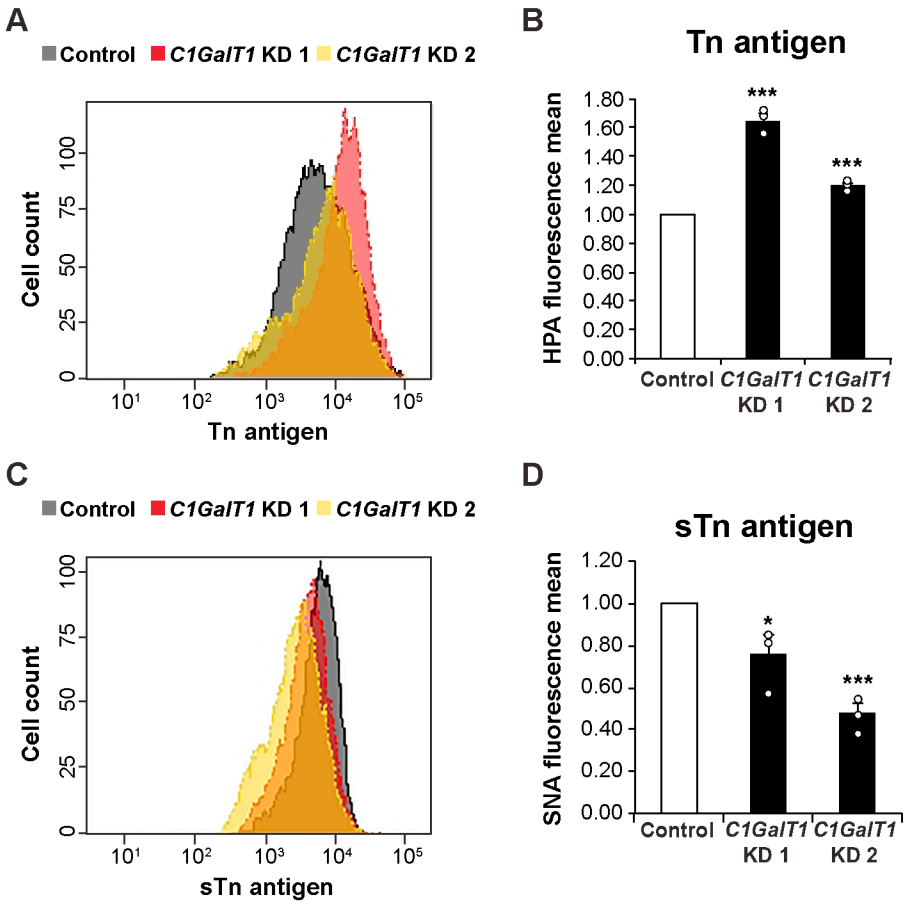


Figure S2. *C1GalT1* KD ESCs have higher levels of Tn antigen.

(A): FACS analysis in *C1GalT1* KD cells after HPA-FITC staining. **(B):** Histogram representing fluorescence mean intensity of image (**A**). **(C):** FACS analysis in *C1GalT1* KD cells after SNA-FITC staining. **(D):** Histogram representing fluorescence mean intensity of image (**C**). The fold change is relative to that of control cells. The values are shown as means ± s.e.m. of three independent experiments. Significant values are indicated as * $P < 0.05$, and *** $P < 0.001$.

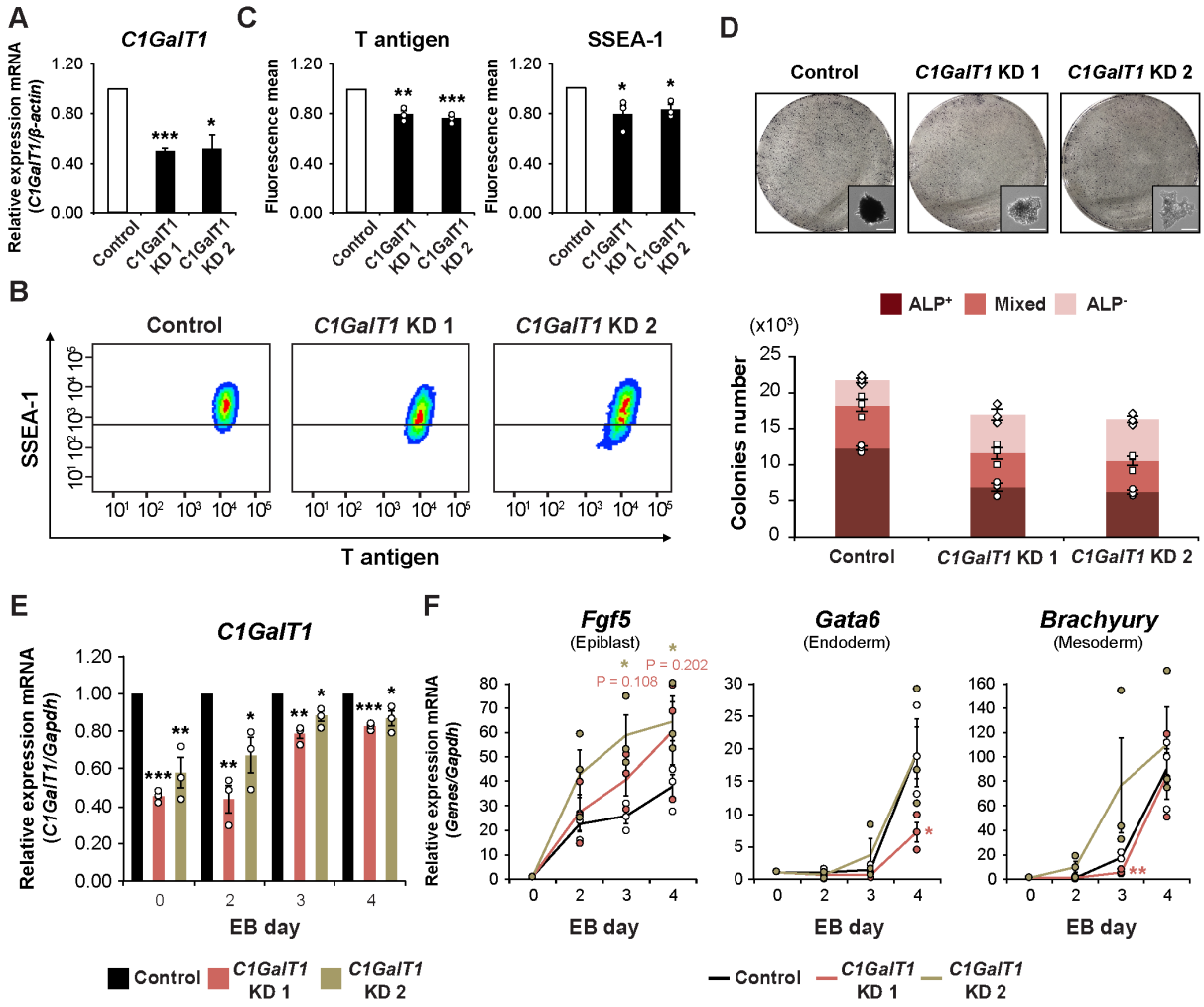


Figure S3. *C1GalT1* KD results in enhanced differentiation potential.

(A): Real-time PCR analysis of stable *C1GalT1* KD cells. The amount of *C1GalT1* was normalized against that of *β-actin*. **(B):** Density plot by FACS of stable *C1GalT1* KD cells stained with anti-SSEA-1-PE Ab and PNA-FITC. The dark line separates the SSEA-1^{+} population (upper side) and the SSEA-1^{-} population (lower side). **(C):** Histogram representing PNA-FITC and SSEA-1-PE fluorescence mean intensity of image (**B**). The fold change is relative to that of control cells. **(D):** Clonogenicity assay of stable *C1GalT1* KD cells. Scale bar, 25 µm. **(E):** Real-time PCR analysis of *C1GalT1* in embryoid bodies (EB) from transient *C1GalT1* KD ESCs plated 2 days post transfection and collected at day 0 (ESC), 2, 3, and 4 normalized against *Gapdh*. **(F):** Real-time PCR analysis of *Fgf5, Gata6,* and *Brachyury* in embryoid bodies (EB) from transient *C1GalT1* KD ESCs plated 2 days post transfection and collected at day 0 (ESC), 2, 3, and 4 normalized against *Gapdh*. The values are shown as means ± s.e.m. of three independent experiments. Significant values are indicated as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

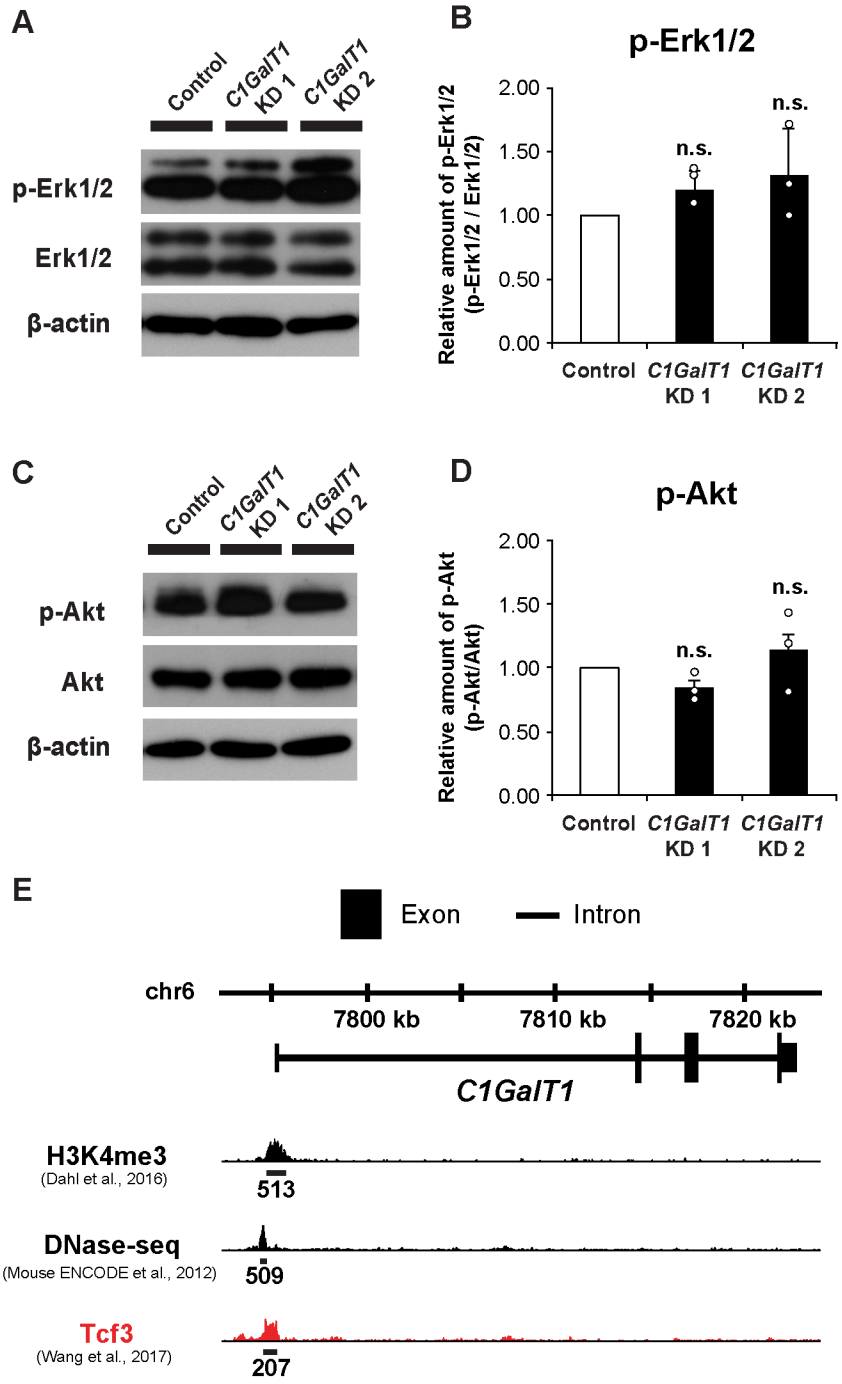


Figure S4. Knockdown of *C1GalT1* does not affect Fgf and Akt signaling. **(A):** Representative image of western blot analysis using antibodies against p-Erk1/2 and Erk1/2 in *C1GalT1* KD cells. **(B):** p-Erk1/2 western blot band intensities normalized against Erk1/2 and shown as fold change relative to control cells. **(C):** Representative image of western blot analysis using antibodies against p-Akt and Akt in *C1GalT1* KD cells. **(D):** p-Akt western blot band intensities normalized against Akt and shown as fold change relative to control cells. The values are shown as

means ± s.e.m. of three independent experiments; n.s. not significant. **(E):** ChIP-seq datasets were browsed by using ChIP-Atlas (https://chip-atlas.org). The datasets were obtained from wild-type/untreated ESCs precipitated using an anti-H3K4me3 Ab (SRX1204276) and DNase-seq (SRX191012), to identify the active promoter region and open chromatin, respectively, and by an anti-Tcf3 Ab (SRX1080398). The threshold for statistical significance was calculated by peak-caller MACS2 set as 50 ($q < 1E-05$).

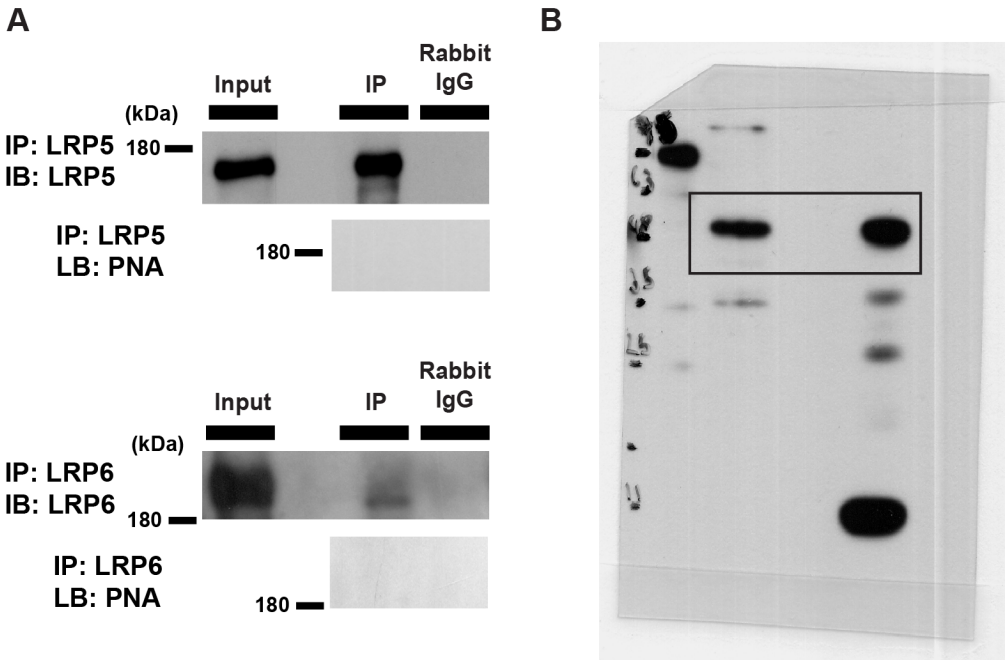


Figure S5. Frizzled co-receptors LRP5/6 do not carry T antigen.

(A): Representative images of western blot (IB) using an anti-LRP5 Ab (upper panel), or an anti-LRP6 Ab (lower panel), and lectin blot (LB) using PNA-HRP, on the immunoprecipitated fraction (IP) precipitated with an antibody against LRP5 (upper panel), or LRP6 (lower panel). The input represents the total ESC lysate. Similar results were obtained from three independent experiments. **(B):** Uncropped gel/blot relative to Fig. 5A.

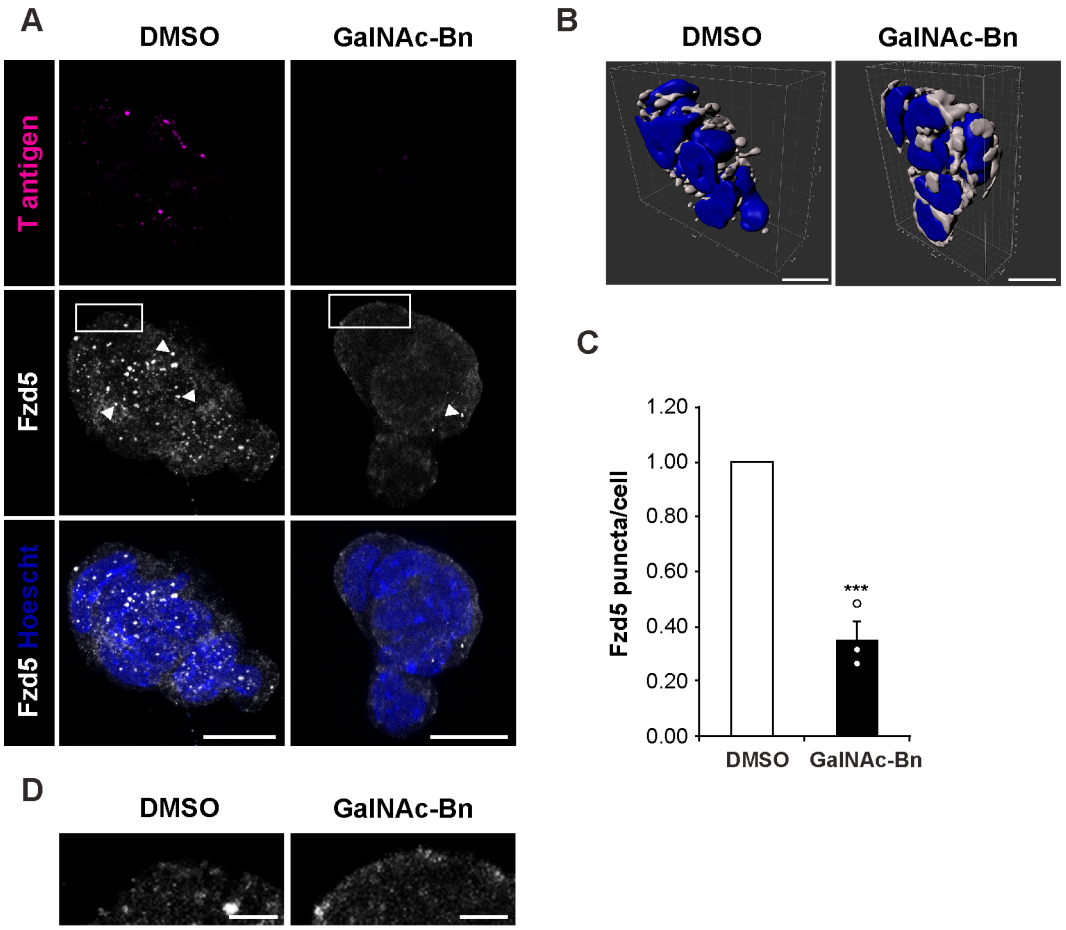


Figure S6. Mucin-type *O*-glycosylation inhibitor GalNAc-Bn reduces Frizzled-5 internalization.

(A): Representative image of a maximum intensity projection of intracellular molecules using PNA-biotin and an anti-Fzd5 Ab in the presence or absence of 2 mM GalNAc-Bn for 48 hr. Nuclei were stained with Hoechst. Arrowheads indicate Fzd5 puncta staining. Scale bar, 10 µm. **(B):** 3D reconstruction of images in **(A)** using Imaris version 9.3.1. Scale bar, 10 µm. **(C):** Quantification of Fzd5 puncta staining normalized against the number of nuclei and shown as a fold change relative to control. **(D):** Magnification of highlighted areas in image **(A)**. Scale bar, 2.5 µm. The values are shown as means ± s.e.m. of three independent experiments. Significant values are indicated as *** $P < 0.001$.

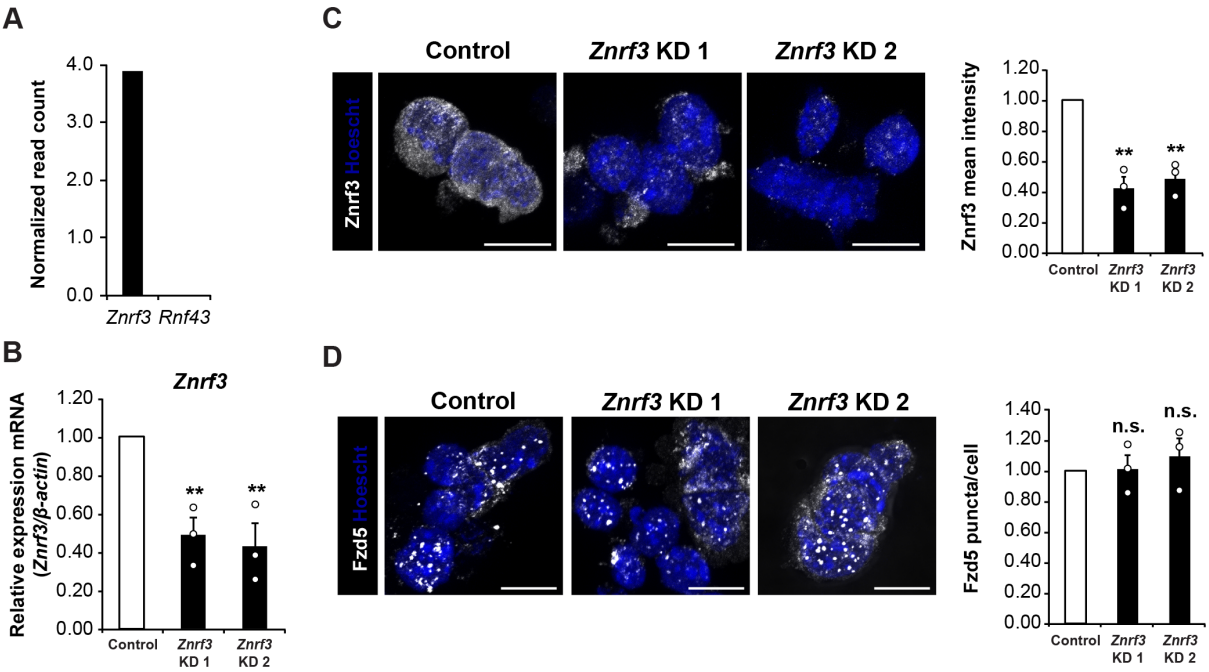
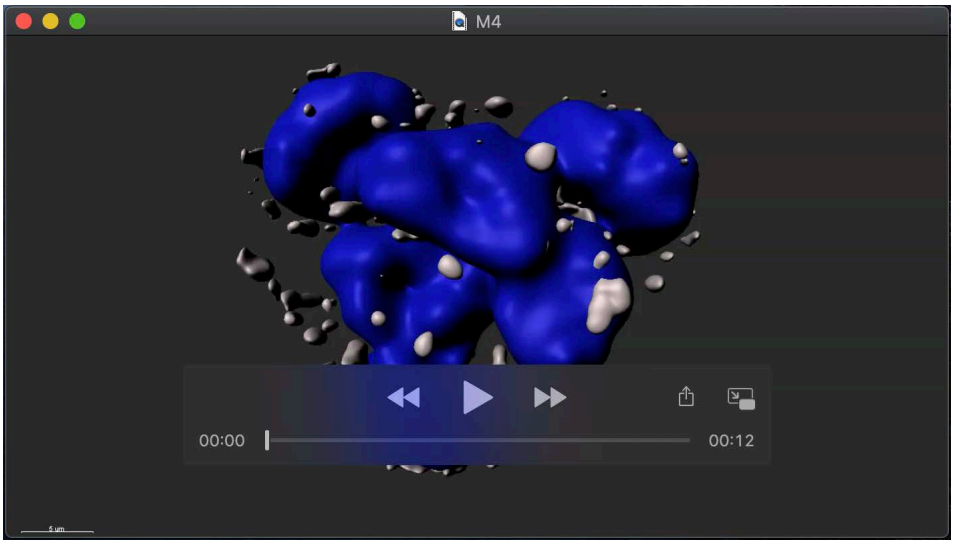


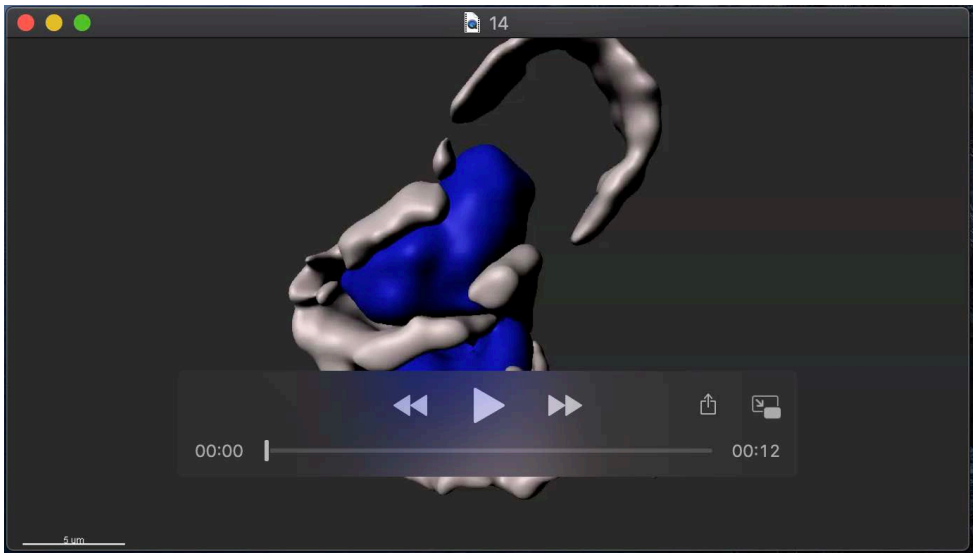
Figure S7. Znrf3 is not involved in Frizzled-5 endocytosis in ESCs.

(A): *Znrf3* and *Rnf43* expression in ESCs analyzed by RNA-seq shown as normalized read count. The data were obtained from a single technical and biological replicate. **(B):** Real-time PCR analysis of *Znrf3* KD cells. The amount of *Znrf3* was normalized against that of *β-actin*. **(C):** Representative image of a maximum intensity projection of internal molecules in *Znrf3* KD cells after immunostaining using anti-Znrf3 Ab. Nuclei were stained with Hoechst. Scale bar, 10 µm. Znrf3 mean intensity is shown as fold change relative to that of the control. (right histogram). **(D):** Representative image of a maximum intensity projection of internal molecules in *Znrf3* KD cells after immunostaining using anti-Fzd5 Ab. Nuclei were stained with Hoechst. Scale bar, 10 µm. Quantification of Fzd5 puncta staining normalized against the number of nuclei and shown as a fold change relative to control (right histogram). The values are shown as means ± s.e.m. of three independent experiments. Significant values are indicated as ** $P < 0.01$; n.s. not significant.



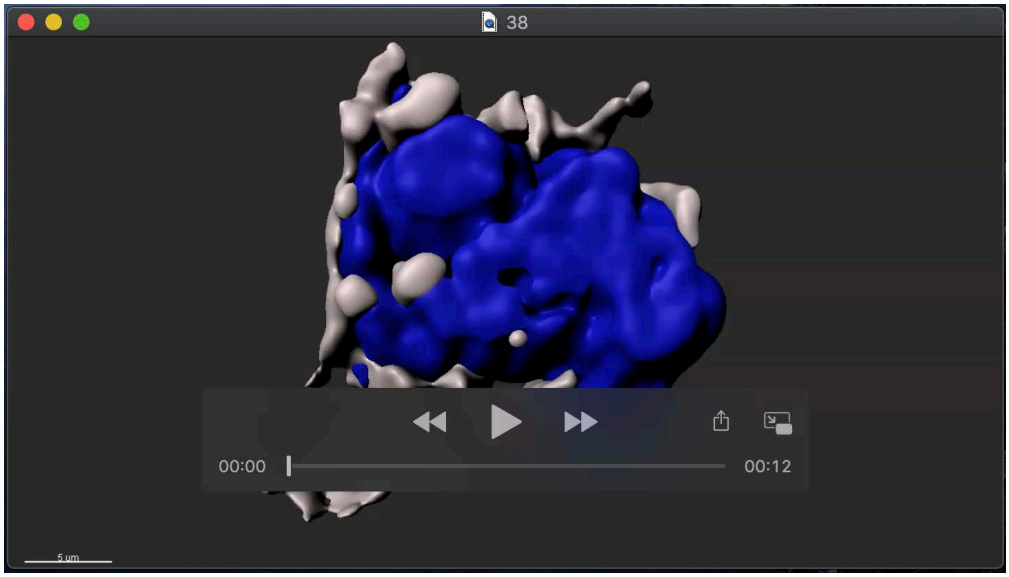
Movie 1. T antigen on Frizzled-5 regulates its endocytosis

Fzd5 puncta staining observed in control cells by 3D reconstruction. Fzd5 staining and nuclei are shown in white and blue, respectively. Scale bar 5 µm.



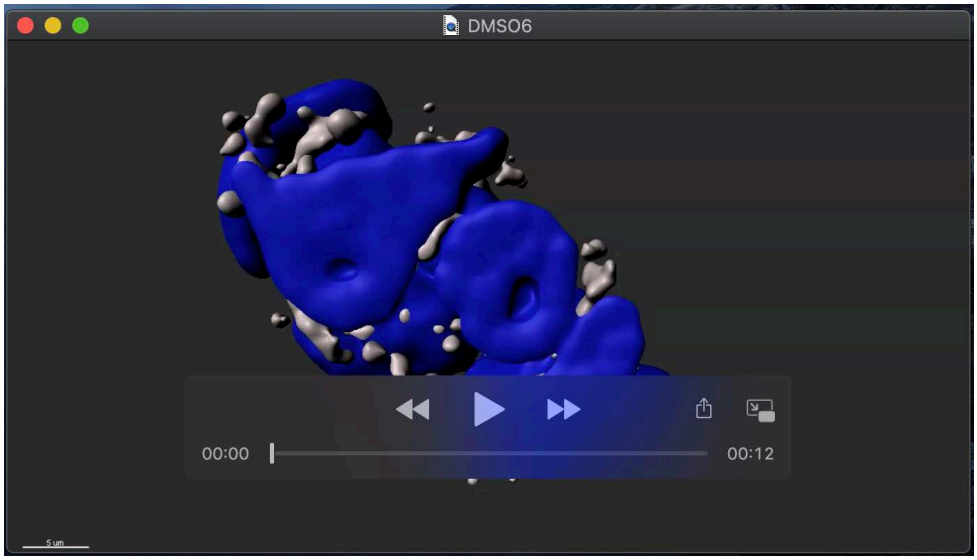
Movie 2. T antigen on Frizzled-5 regulates its endocytosis

Fzd5 membrane localization observed in *C1GalT1* KD 1 cells by 3D reconstruction. Fzd5 staining and nuclei are shown in white and blue, respectively. Scale bar 5 µm.



Movie 3. T antigen on Frizzled-5 regulates its endocytosis

Fzd5 membrane localization observed in *C1GalT1* KD 2 cells by 3D reconstruction. Fzd5 staining and nuclei are shown in white and blue, respectively. Scale bar 5 µm.

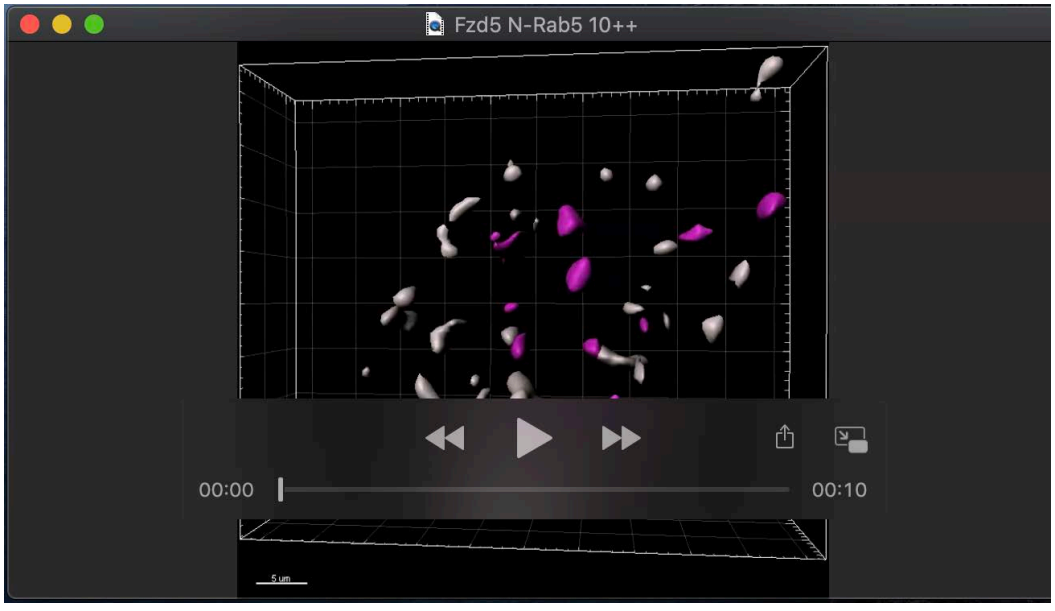


Movie 4. Mucin-type *O*-glycosylation inhibitor GalNAc-Bn reduces Frizzled-5 internalization.

Fzd5 puncta staining observed in cells treated with DMSO by 3D reconstruction. Fzd5 staining and nuclei are shown in white and blue, respectively. Scale bar 5 µm.

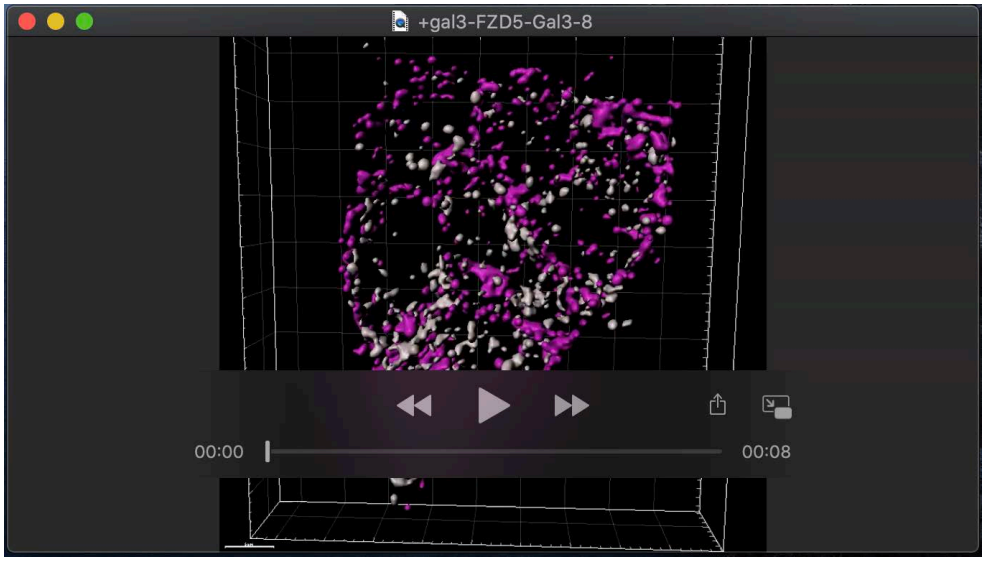
Movie 5. Mucin-type *O*-glycosylation inhibitor GalNAc-Bn reduces Frizzled-5 internalization.

Fzd5 membrane localization observed in cells treated with 2 mM GalNAc-Bn for 48h by 3D reconstruction. Fzd5 staining and nuclei are shown in white and blue, respectively. Scale bar 5 µm.



Movie 6. T antigen on Frizzled-5 regulates its endocytosis

Fzd5 and Rab5 colocalization observed in ESCs by 3D reconstruction. Fzd5 and Rab5 staining are shown in white and magenta, respectively. Scale bar at the bottom.



Movie 7. Frizzled-5 endocytosis is mediated by galectin-3

Fzd5 and Lgals3 colocalization observed in ESCs treated with 15 µg/mL Lgals3 for 30 minutes by 3D reconstruction. Fzd5 and Lgals3 staining are shown in white and magenta, respectively. Scale bar at the bottom.

Supplementary Table

Table S1 Primer sets

Gene	Forward primer	Reverse primer
C1GalT1	5'- GCAAGGCATTCAGATGACAA -3'	5'- ATGTTGGCTGGAATCTGCAT -3'
ST6GalNAc-I	5'- GAGAGGCAGTCCAAGGAGAGC -3'	5'- TGAGGATTCTCTGGTGCTGGC -3'
β3GnT6	5'- AGTCCCACGACACTGGCTTTC -3'	5'- CCTGCCTGTGTTCCTGGAGG -3'
Oct3/4	5'- CTCACCCTGGGCGTTCTCT -3'	5'- AGGCCTCGAAGCGACAGA -3'
Sox2	5'- ACCAGAAGAACAGCCCGGA -3'	5'- CCCGGGACCATACCATGA -3'
β-catenin	5'- GTTAAACTCCTGCACCCACCA -3'	5'- AAAGGGCAAGGTTTCGAATCA -3'
Axin2	5'- GGGAGCAGTTTTGTGGCAGCA -3'	5'- AGGGTCCTGGGTAAATGGGTGAG -3'
Fgf5	5'- GCAGCCCACGGGTCAA -3'	5'- CGGTTGCTCGGACTGCTT -3'
Otx2	5'- CATGATGTCTTATCTAAAGCAACCG -3'	5'- GTCGAGCTGTGCCCTAGTA-3'
Cdx2	5'- GAGCTGGCTGCCACACTTG -3'	5'- GCTTCTTCTTGATTTTCCTCTCCTT -3'
Gata3	5'- CCATTACCACCTATCCGCCC -3'	5'- TCGACTTACATCCGAACCCG -3'
Gata4	5'- TCCATGTCCCAGACATTCAGACT -3'	5'- AGCAGACAGCACTGGATGGAT -3'
Gata6	5'- CCCCTCATCAAGCCACAGAA -3'	5'- GTGACAGTTGGCACAGGACAGT -3'
Brachyury	5'- TGCTGCAGTCCCATGATAACTG -3'	5'- ATGACTCACAGGCAGCATGCT -3'
Mixl1	5'- GCACGTCGTTCAGCTCGGAGCAGC -3'	5'- AGTCATGCTGGGATCCGGAACGTGG -3'
FoxA2	5'- AGCCGTGAAGATGGAAGGG -3'	5'- CTCCGCGTAGTAGCTGCTCC -3'
Sox17	5'- GCACAACGCAGAGCTAAGCA -3'	5'- CTGCCAAGGTCAACGCCT -3'

Pax6	5'- AACCTGGCTAGCGAAAAGCA -3'	5'- CCCGTTCAACATCCTTAGTTTATCA-3'
Nestin	5'- TGCAGACACCTGGAAGAAGTTC -3'	5'- CCCAAGGAAATGCAGCTTCA -3'
Rex1	5'- GCTCCTGCACACAGAAGAAA -3'	5'- GTCTTAGCTGCTTCCTTCTTGA -3'
Znrf3	5'- CGGCGACTATACCACCCAC -3'	5'- GGGGTCCAATTCTGGCTGTT -3'
Gapdh (qPCR/T-vector)	5'- TGCACCACCAACTGCTTAGC -3'	5'- GGCATGGACTGTGGTCATGAG -3'
β-actin	5'- GCTCTGGCTCCTAGCACCAT -3'	5'- GCCACCGATCCACACAGAGT -3'
C1GalT1 (T-vector)	5'- CGAGAAGAGGCTGCCATTC -3'	5'- AGCATCCAGGACCCTCTAT -3'
ST6GalNAc-I (T-vector)	5'- AAGGCTGAGCCCCAAGTAC -3'	5'- GCAGTGAAGCCATAGAAGGA -3'
β3GnT6 (T-vector)	5'- AGCAGCCGCAGGTTCAAG -3'	5'- GTCGATGGGGAAGAGCGG -3'