Supporting Information

Guha et al. 10.1073/pnas.1202653110

SI Materials and Methods

Cell Culture. Androgen-independent prostate cancer (PCa) cell line PC3 (ATCC) and PC3 cells expressing a luciferase reporter (PC3-Luc) (kind gift from Patrick J. Casey, Duke University, Durham, NC) were cultured in a mixture of DMEM (Sigma) and F-12 (Sigma) (1:1) supplemented with 10% (vol/vol) FBS (Quality Biologicals), 100 units/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate (Sigma). Human umbilical vein endothelial cells (HUVECs) (Lonza) were maintained in EGM2 basal medium supplemented with Bullet kit (Lonza). The human T-cell line MOLT-4 (ATCC), Jurkat (gift of Agnes Azimzadeh, University of Maryland, Baltimore), and B16 melanoma-specific T-cell transgenic D90.1 $CD8^+$ T cells (pmel T cells) were grown in RPMI medium 1640 containing 2 mM glutamine, penicillin-streptomycin, and 10% FBS. All cells were cultured in the presence of 5% CO_2 at 37°C.

Generation of gal3$^{-/-}$ PC3-Luc Cells. Confluent PC3-Luc cells were treated with hexadimethrine bromide (7 mg/mL) followed by the addition of 50 mL of galectin-3 MISSION shRNA Lentiviral Transduction Particles (SHCLNV-NM_002306; Sigma) or Mission shRNA Control Transduction Particles (SHC002V; Sigma). On the next day, cells were exchanged with fresh medium, and a day later, puromycin selection at a final concentration of 0.75 μg/mL was initiated to generate stable gal3 knockout cell line. After 10 d of puromycin selection, gal3 knockout (KO) and negative control KO cells were generated and subjected to Western blot to investigate gal3 expression.

Purification of Thomsen-Friedenreich disaccharide-Containing Compounds from Pacific Cod. Thomsen-Friedenreich disaccharide (TFD)-containing compounds were purified either from extraction of Pacific cod purchased from local store or from cod-derived antifreeze glycoproteins purchased from A/F Protein. Extraction of cod (900 g) was performed in 1 L of Tris-buffered saline (TBS; 50 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ at pH 8.0) containing 100 μM PMSF, and after centrifuging at 8,000 × *g* for 30 min at 4 °C, the clear supernatant was passed through a column of peanut agglutinin (PNA)-agarose (Sigma) (that was preequilibrated with the TBS). The column was washed with TBS until no protein was detected in the washing (monitored at 214 nm), and the bound protein was eluted with 100 mM lactose in the TBS. The lactose eluate was dialyzed with water and further fractionated by fast performance liquid chromatography (Amersham) on a size exclusion column Superdex 75. Inhibitory activity of each peak was investigated on galectin-3 binding to asialofetuin on a 96-well plate (see later). The purified TFD-containing glycoprotein corresponding to ~100 kDa (designated as TFD_{100}) was used in angiogenesis, tumor–endothelial cell interaction, and apoptosis experiments.

Preliminary investigation on the nature of the carbohydrates and their activities to inhibit gal3 binding was performed after size fractionation of crude antifreeze glycoproteins (purchased from A/F Protein) by using Centricon membrane devices of various molecular mass cutoff such as 50 kDa, 30 kDa, 10 kDa, and 3 kDa (2 mL of capacity) (Millipore). Briefly, 50 mg of crude glycoproteins in 2 mL of water was taken in a Centricon device of 50-kDa cutoff and centrifuged at 5,000 × *g* to ~100 μL (~1 h) at 4 °C by using J20 rotor (Beckman centrifuge). The retentate on the centricon membrane was diluted to 2 mL with water and recentrifuged. This process of dilution and centrifugation was continued two more of times. At the end of this process, the retentate (designated as fraction 1) was collected and saved for further characterization. The filtrate was processed for further fractionation on 30-kDa molecular mass cutoff centricon device and so on as described above. Thus, the partial purification resulted five fractions (Fr) as follows: Fr1 (>50 kDa); Fr2 (<50 kDa, but >30 kDa); Fr3 (<30 kDa, but >10 kDa); Fr4 (<10 kDa, but >3 kDa); and Fr5 (<3 kDa). Each fraction was freeze dried and stored at −20 °C until further characterization. The Fr1 was further purified on a PNA-agarose column (Sigma) as described above.

Biacore Kinetics and Affinity Analysis. The binding kinetics and affinity of a few carbohydrates for gal3 were measured in surface plasmon resonance-based binding assays by using a Biacore T200 instrument (GE Healthcare). Biotinylated gal3 was attached to Biacore Sensor Chip SA (GE Healthcare) according to the instructions. For this purpose, biotinlyated gal3 was diluted into HBS-EP+ (GE Heathcare) to a concentration of 1 μg/mL and captured onto the streptavidin surface to a final level of 1750 resonance units (RU). A reference surface was prepared with biotin captured onto the streptavidin. Binding kinetics and affinity of carbohydrate ligands (such as lactose, *N*-acetyllactosamine, TFD, and TFD_{100}) were measured by using a titration of concentrations in twofold dilutions that spanned the dissociation constant (K_D). The binding response of small carbohydrate ligands were measured in resonance units in real time at 10 Hz. These carbohydrates were injected for 30 s at 50 μL/min, and 30 s of dissociation data were collected. For TFD_{100} binding assay, lower density surface of 550 RU of biotinlyated gal3 was prepared. The binding of TFD_{100} exhibited significantly slower kinetics and required injections for 120 s, and collection of 600 s of dissociation data. The slow dissociation of TFD_{100} prompted regeneration of the gal3 surface by using a 60-s injection of 100 mM lactose. Raw sensorgram data were reference subtracted and blank subtracted before kinetic and affinity analysis. Biacore T200 Evaluation software was used for steady-state affinity analysis and kinetic analysis. The association and dissociation rates for lactose binding approached the resolution limit of the instrument, therefore a plot of response at equilibrium versus concentration was used for steady-state analysis of the K_D. The association and dissociation rates, as well as the K_D for TFD_{100} binding, were determined by using a simple 1:1 binding model.

Analytical Procedures. Crude, membrane-fractionated, and purified TFD containing glycoproteins were investigated for the presence of TFD by using a glycan differentiation kit (Roche) following the manufacturer's instructions. The carbohydrate content (neutral hexose) was measured by phenol-sulfuric acid assay (1). Analytical PAGE in the presence of SDS (2%) was carried out on a NuPAGE Novex 4–12% Bis-Tris gels (Invitrogen) under reducing or nonreducing conditions as reported (2). The protein content was measured at 214 nm by using BSA as a standard. In some cases, protein concentration was measured with Coomassie blue dye as described (3).

Solid Phase Binding-Inhibition Assay. The inhibitory activity of TFD_{100}, other TFD-containing fractions, and alkaline treated TFD_{100} on gal3 binding was determined by using a solid phase assay as described (3). Briefly, asialofetuin (0.5 μg/100 μL per well) in 0.1 M Na_2CO_3/0.02% NaN_3 (pH 9.6) was adsorbed onto the wells of microtiter plates (Immulon; Dynatech Laboratories) at 37 °C for 3 h, and the bound glycoprotein was fixed with 2% formaldehyde in PBS (10 mM phosphate and 140 mM NaCl at pH 7.5) at 37 °C for 30 min. The plates were washed three times with

PBS (azide-free)/0.05% Tween 20 and incubated with the gal3-biotin conjugate (10 ng/100 μL per well for binding assays) or with preincubated mixture of equal volume of conjugate and varying concentrations of test ligands (for binding-inhibition assays). After incubation for 1 h at 4 °C, the plates were washed with ice-cold azide-free PBS-Tween 20, and the bound conjugate was allowed to interact with peroxidase-labeled streptavidin (0.05 μg/100 μL per well) in azide-free PBS-Tween for 1 h at 4 °C. The plate was washed with ice-cold azide free PBS-Tween, and the bound peroxidase activity was assayed with peroxidase substrate diammonium 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (KPL) (3). To prepare gal3-biotin conjugate, 0.5 mg of the purified recombinant gal3 as described (4) in 0.5 mL of azide-free PBS/0.1 M lactose was mixed with 0.5 mg of EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) in 50 μL of PBS. After incubation for 2 h on ice, the mix was dialyzed with PBS and purified by affinity chromatography on lactosyl-sepharose (3). The purified gal3-biotin conjugate was dialyzed with PBS and stored in 1% BSA-50% glycerol at −20 °C until further use. For removal of *O*-glycan from TFD_{100}, purified TFD_{100} was subjected to standard β-elimination reaction. Briefly, 100 μL of the TFD_{100} (15 μg/mL) was incubated with 2 M sodium borohydride (100 μL in 0.1 M sodium hydroxide) at 37 °C for 20 h. The reaction mixture was neutralized with 100 μL of 0.3 M acetic acid, and the cleaved sugars were membrane separated by using Microcon 10.

Binding of TFD_{100} with Other Galectins. The inhibitory activity of TFD_{100} on binding of other galectins, such as gal4, and N-terminal of gal9 was determined on solid phase assay as described above. Briefly, galectin (for binding) or mixture of fixed amount of galectin and varying amount of TFD_{100} (for binding inhibition) was added to asialofetuin-adsorbed wells, and after washing, the bound galectin was mixed with the antigalectin antibody followed by the addition of secondary antibody-conjugated HRP and development with ABTS substrate as above.

Immunoassay to Quantitate gal3 in Normal and PCa Patient Sera (gal3-Containing and gal3-Depleted). For immunoassay, purified gal3 was coated on a 96-well plate (0.1 μg per well/100 μL of coating buffer as described above), and the fixed amount of purified anti-gal3 antibody premixed with varying amount of purified gal3 or unknown sample (normal or patient serum as well as gal3-depleted sera) was then added as described (5). After washing the well, the bound antibody was detected with a secondary antibody conjugated with horseradish peroxidase followed by addition of ABTS substrate (3). The amount of gal3 in the sample was measured from a standard curve. For preparation of gal3-depleted serum, each serum was passed through a column of anti-gal3 antibody-sepharose and flow through was collected.

Histochemical Analyses of Normal and Tumor Prostate Tissues. Immunohistochemical detection of gal3 using specific anti-gal3 antibody was performed on 5-μm-thick paraffin-embedded sections containing the most representative tumor areas. In brief, sections were deparaffinized in xylene and hydrated through graded concentrations of ethanol and then with distilled water. Samples were heated in a microwave oven in 1× Target Retrieval solution and then washed with PBS for 5 min. All sections were incubated in 3% hydrogen peroxide to inhibit endogenous peroxidase. Protein A-sepharose purified anti-gal3 antibody (10 μg/mL) (4) was applied to the slides and incubated for 30 min at room temperature in a humidified chamber. Protein A-sepharose purified preimmune rabbit serum was used as negative control. Sections were incubated with postprimary block for 15 min and polymer for 15 min (NovoLink Polymer kit; Novocastra, Vision BioSystems). Staining was visualized with the diaminobenzidine chromogen and counterstained with Mayer's hematoxylin.

To investigate TFD expression, prostate tissue (normal and tumor) array (US Biomax) was subjected to mouse anti-TFD antibody (Abcam; Ag A78G/A7) followed by anti-mouse IgG-FITC conjugate as described above. Expression of TFD was also investigated by binding with peanut lectin-FITC (EY Laboratories) and, after washing, the binding was visualized under fluorescence microscope.

Angiogenesis. In vitro induction of angiogenesis in the presence of gal3 and the inhibition of angiogenesis in the presence of TFD_{100} or lactose were performed by using Chemicon's In Vitro Angiogenesis Kit (Millipore) following the manufacturer's instructions. Briefly, 5×10^4 HUVECs were seeded in matrigel-coated 96 wells in the presence or absence of gal3, TFD_{100}, or lactose alone or in combination with gal3 + TFD_{100} or gal3 + lactose. After 5 h, the microvessel formation was analyzed under phase contrast microscope at the 10× magnification. For quantitation of tube formation, the number of branching was counted in six areas (each 25 nm^2) of each well and an average value was taken.

For in vivo induction of blood vessels, black mice (strain C57/BL6) were administered with 0.5 mL of matrigels in the presence or absence of gal3 and TFD100 under skin at the abdomen in the following four groups (5 mice per group): Group 1, Matrigel+VEGF (20 ng); Group 2, Matrigel+VEGF (20 ng) + gal3 (500 ng); Group 3: Matrigel+VEGF (20 ng) + TFD_{100} (100 ng); and Group 4, Matrigel+VEGF (20 ng) + gal3 (500 ng) + TFD_{100} (100 ng). After a week, the matrigel plugs were removed and sectioned (5 μm) for immunostaining with anti-CD31 antibody (BD Biosciences) as described (6).

Extracellular Localization of gal3 and TFD in PC3 Cells and HUVECs. The extracellular localization of gal3 in the PC3 cells was investigated with anti-gal3 antibody on a flow cytometer (Becton Dickinson FACSCanto II). Briefly, confluent cells were washed with PBS-5 mM EDTA, separated from the plate, and incubated with 10 μg/mL protein A-sepharose purified polyclonal rabbit anti-gal3 antibody (4) for 30 min at 4 °C. After washing with PBS for three times by using centrifugation (200 × *g*) for 5 min each, the cells were incubated with 0.8 μg/mL DyLight 649 labeled goat anti-rabbit IgG (KPL). The washed cells were then subjected to the flow cytometry and acquired at FL4 channel. The extracellular localization of TFD in the PC3 cells and HUVECs was investigated with PNA-FITC (EY Laboratories) and subjected to the flow cytometry. The cells were analyzed through FL1 channel. The presence of TFD-containing glycoprotein in PC3 cells and HUVECs was also investigated by using glycan differentiation kit (Roche) as described above.

Tumor–Endothelial Cell Interactions. For adhesion to endothelial cells, HUVECs was grown to confluence and single-cell suspension of calcein labeled (eBioscience) PC3 cells were allowed to bind to HUVECs in the presence of TFD_{100}, siRNAs, lactose, and various antibodies as described (7). Inhibition of tumor-endothelial cells was performed in three different ways. (*i*) HUVECs were treated with various reagents, and the labeled PC3 cells were allowed to bind to the washed HUVECs. (*ii*) The calcein-labeled PC3 cells were treated with various reagents and, after washing, the cells were allowed to bind to HUVECs. (*iii*) The interaction of labeled PC3 cells to HUVECs was performed in the presence of TFD_{100} and gal3 siRNA. The binding or binding-inhibition in each well was examined by phase contrast microscopy at 10× magnification and quantitated on a spectrofluorimeter (SpectraMax M5 Multimode Microplate Reader from Molecular Devices) by using calcein AM Ex/Em -495/515.

Apoptosis of T Cells (MOLT-4, Jurkat, and $CD8^+$ Cells). Gal3-mediated apoptosis of MOLT-4 cells in the presence or absence of TFD_{100} was assessed by measuring apoptotic cells by annexin V binding (Oncogene) on a flow cytometer. Jurkat cells were subjected to TUNEL assay.

To investigate whether tumor-associated gal3 can induce tumor specific $CD8^+$ T cells, we used pmel T cells as a model system (8). After harvesting T cells from mouse spleen, cells were activated with mouse gp100(25-33) peptide (EGSRNQDWL; GenScript) (8), mixed with a monolayer of B16 melanoma cells, and apoptosis was measured by Annexin V binding after gating with anti-CD8 and anti-CD25 antibodies. To investigate whether patient serum-associated gal3 could induce apoptosis of activated $CD8^+$ cells, PCa patient serum (wild type or gal3-depleted) was mixed with activated pmel T cells and apoptosis was measured in the presence or absence of TFD_{100} (1 nM).

Experimental Metastasis Assay. Confluent culture of PC3-Luc or $gal3^{-/-}$ PC3-Luc cells was scraped and resuspended at 1.5×10^7 cells per mL in PBS. Cells (3×10^6 in 200 μL of PBS) were then injected i.v. into the tail vein of 20 nude mice (3–4 wk old, Strain NCRNU-F, Taconic), 10 mice per cell type, by using tail vein injection apparatus (Braintree Scientific). Mice of each cell type were separated into two groups. One group received TFD_{100} (50 μg/kg body weight) i.p. twice a week for a period of 5 wk, whereas the other group received only PBS. Before imaging, mice were injected i.p. with Luciferin (150 mg/kg; Xenogen) and returned to their cages for 5 min to allow for biodistribution. Mice were anesthetized with 2% isoflurane gas (integrated within the Xenogn IVIS-200 system) and imaged 5 min for a single-photon emission. Regions of interest were created and measured as area flux, defined by radiance (photons per second per square centimeter per steradian) according to the manufacturer's calibration (Xenogen). Total photon flux was calculated and corrected for tissue depth by spectral imaging by using Living Image 3.0 software (Xenogen). After 5 wk, mice were euthanized and India ink (15%) was injected into their lungs through trachea. The lungs were fixed in Fekete's solution (100 mL of 70% ethanol, 10 mL of formalin, and 5 mL of glacial acetic acid) and after destaining with Fekete's solution, lungs were photographed. For immunohistochemical analysis, unstained lungs were sectioned (5 μm) and stained with hematoxylin and eosin (H&E) and anti-human Ki67 antibody (BioLegend), and the sections were examined by light microscopy.

Statistical Analysis. The statistical analyses were performed by using one-way ANOVA followed by Turkey–Kramer multiple comparisons (GraphPad InStat, version 3). The differences were considered significant when $P < 0.05$.

1. Dubois M, Gilles K, Hamilton JK, Rebers PA, Smith F (1951) A colorimetric method for the determination of sugars. *Nature* 168(4265):167.
2. Laemmli UK, Favre M (1973) Maturation of the head of bacteriophage T4. I. DNA packaging events. *J Mol Biol* 80(4):575–599.
3. Ahmed H, Pohl J, Fink NE, Strobel F, Vasta GR (1996) The primary structure and carbohydrate specificity of a β-galactosyl-binding lectin from toad (*Bufo arenarum* Hensel) ovary reveal closer similarities to the mammalian galectin-1 than to the galectin from the clawed frog Xenopus laevis. *J Biol Chem* 271(51):33083–33094.
4. Ahmed H, Cappello F, Rodolico V, Vasta GR (2009) Evidence of heavy methylation in the galectin 3 promoter in early stages of prostate adenocarcinoma: Development and validation of a methylated marker for early diagnosis of prostate cancer. *Transl Oncol* 2(3):146–156.
5. Allen HJ, Sharma A, Ahmed H, Piver MS, Gamarra M (1993) Galaptin and galaptin-binding glycoconjugates in serum and effusions of carcinoma patients. *Tumour Biol* 14(6):360–368.
6. Bagley RG, et al. (2008) Human endothelial precursor cells express tumor endothelial marker 1/endosialin/CD248. *Mol Cancer Ther* 7(8):2536–2546.
7. Glinsky VV, et al. (2001) The role of Thomsen-Friedenreich antigen in adhesion of human breast and prostate cancer cells to the endothelium. *Cancer Res* 61(12):4851–4857.
8. Geng D, et al. (2010) Amplifying TLR-MyD88 signals within tumor-specific T cells enhances antitumor activity to suboptimal levels of weakly immunogenic tumor antigens. *Cancer Res* 70(19):7442–7454.

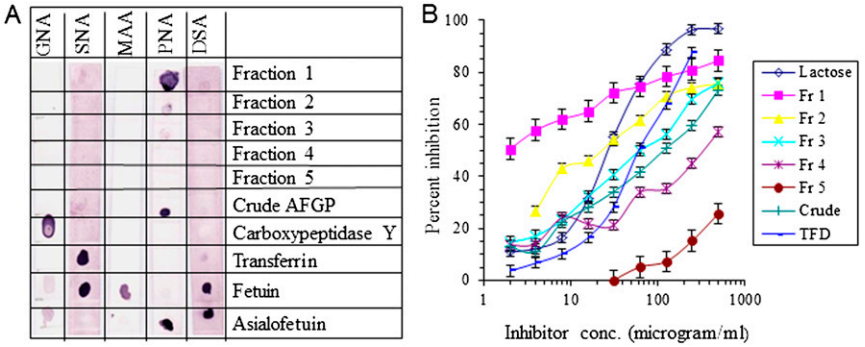

Fig. S1. Characterization of various TFD-containing compounds. (*A*) Glycan analysis. Positive reaction with GNA (*Galanthus nivalis* agglutinin) indicates mannose, terminally linked. Positive reaction with *Sambucus nigra* agglutinin (SNA) indicates sialic acid, terminally linked (2–6) to galactose (SAα2,6Gal). Positive reaction with *Maackia amurensis* agglutinin (MAA) indicates SAα2,3Gal. Positive reaction with PNA indicates Galβ1,3GalNAc. Positive reaction with *Datura strammonium* agglutinin (DSA) indicates Galβ1,4GlcNAc. (*B*) Inhibition of gal3 binding to asialofetuin with TFD-containing compounds. Each compound was tested in triplicate to inhibit gal3 binding to asialofetuin in 96-well plate as described in *Materials and Methods*.

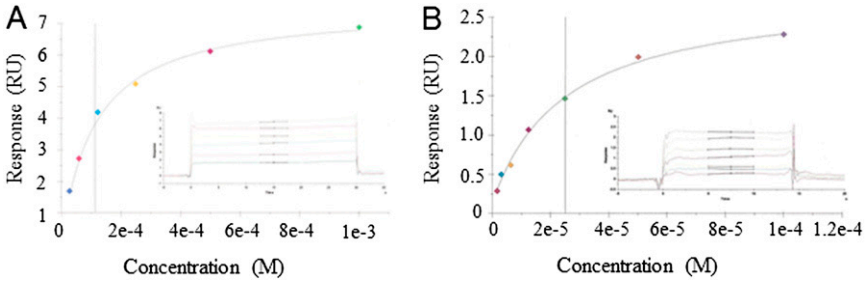

Fig. S2. Surface plasmon resonance assay on Biacore. Binding kinetics and affinity of lactose (*A*) and *N*-acetyllactosamine (*B*) for gal3 were measured by using a titration of concentrations in twofold dilutions that spanned the K_D. Lactose was measured from 31 μM to 1,000 μM, and the K_D was determined to be 1.1^{e-4} M or 110 μM. *N*-Acetyllactosamine was measured from 1.56 μM to 100 μM, and the K_D was determined to be 2.5^{e-5} M or 25 μM.

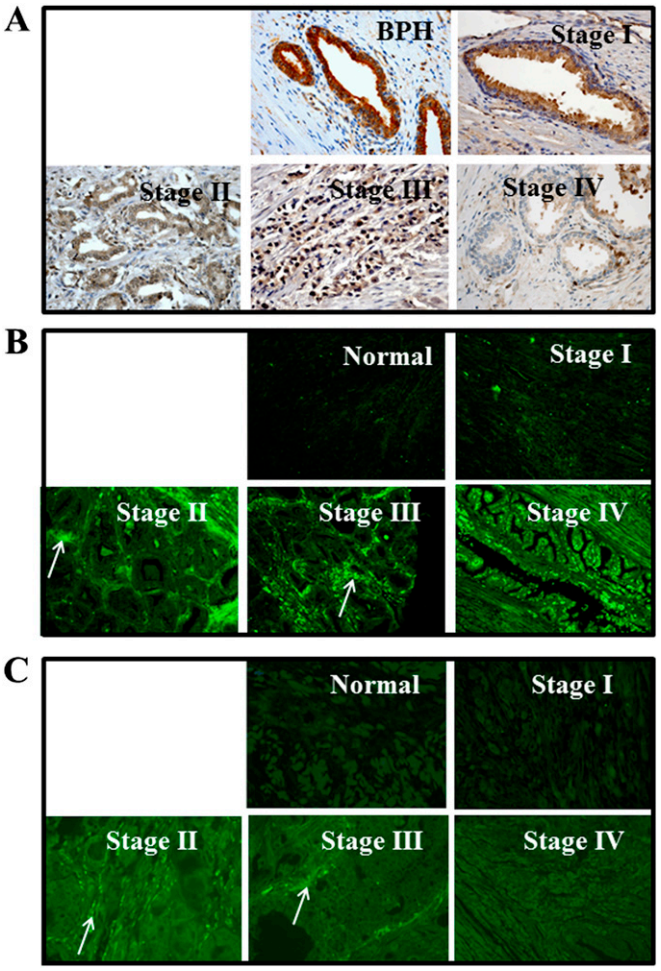

Fig. S3. Expression of gal3 and TFD in prostate tissues from at least four normal individuals and four of each stage of PCa patients. Shown are representative results. (*A*) Expression of gal3 as determined by immunostaining with affinity purified anti-gal3 antibodies. BPH, benign prostatic hyperplasia; stage I, T1N0Mx; stage II, T2N0Mx; stage III, T3N0Mx; and stage IV, T4N1Mx. (Magnification: 400×.) (*B* and *C*) Expression of TFD as determined by immunostaining with anti-TFD antibody (*B*) or binding with PNA-FITC (*C*). Stage I, T1N0Mx; stage II, T2N0Mx; stage III, T3N0Mx; and stage IV, T4N1Mx. (Magnification: 400×.)

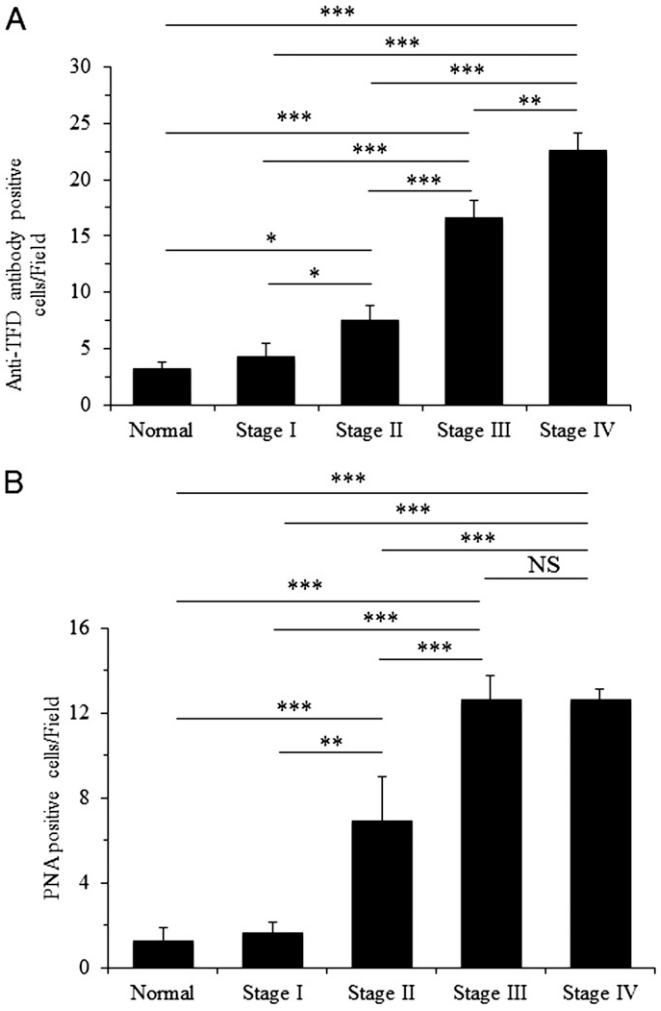

Fig. S4. Quantitation of TFD expression as determined by anti-TFD antibody staining (*A*) and PNA staining (*B*). The data are shown as the means ± SD from three determinations. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ANOVA. NS, not significant.

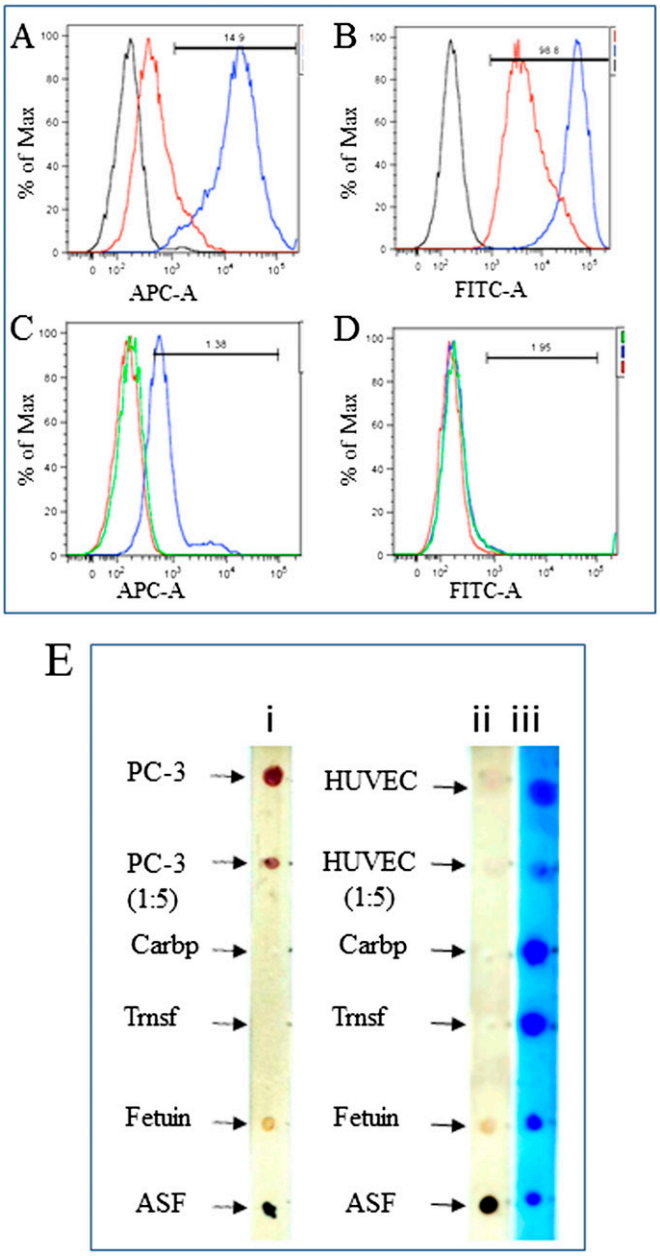

Fig. S5. Extracellular localization of gal3 and TFD in PC3 cells (*A* and *B*) and HUVECs (*C* and *D*). Expression of gal3 in PC3 cells (*A*) and HUVECs (*C*). (*A*) Black, unstained cells, mean fluorescence unit 205; blue, with gal3 Ab, mfu 25851; and red, with preimmune IgG, mfu 759. (*C*) Red, unstained cells, mfu 97; blue, with gal3 Ab, mfu 296; and green, cells stained with preimmune IgG, mfu 95. Expression of TFD in PC3 cells (*B*) and HUVECs (*D*). (*B*) Black, unstained cells, mfu 183; blue, with PNA-FITC, mfu 59320; and red, with PNA-FITC plus lactose, mfu 8386. (*D*) Red, unstained cells, mfu 97; blue, with PNA-FITC, mfu 95; and green, with PNA-FITC plus lactose, mfu 97. (*E*) Glycan differentiation analysis of PC3 (*i*) and HUVEC (*ii*) cell extract with PNA binding. (*E, iii*) Dot blot of HUVEC extract and standard proteins/glycoproteins stained with Coomassie showing protein/glycoprotein load.

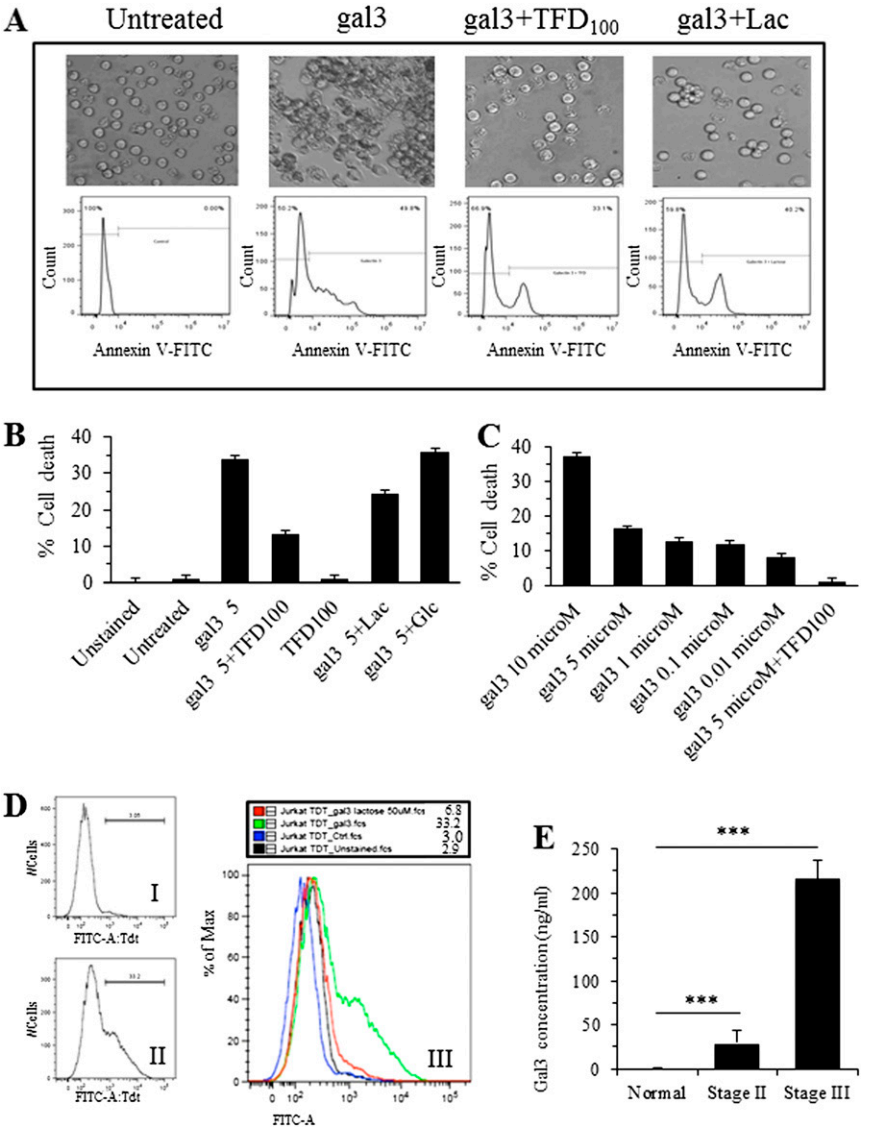

Fig. S6. (*A*) Apoptosis of MOLT-4 T cells in the presence of gal3 (5 μM), gal3 plus TFD_{100} (3.5 nM), and gal3 plus lactose (50 μM). (*B* and *C*) Apoptosis of Jurkat cells. (*B*) Gal3-mediated apoptosis of Jurkat cells and their inhibition with 3.5 nM TFD_{100}. (*C*) Gal3 dose-dependent apoptosis of Jurkat cells. (*D*) Gal3-mediated apoptosis of Jurkat cells as measured by TUNEL assay. (*DI*) Cytogram of untreated Jurkat cells. (*D, II*) Cytogram of Jurkat cells in the presence of gal3. (*D, III*) Overlay of cytograms showing TUNEL assays for Jurkat cells in the presence of gal3 or gal3 plus lactose. (*E*) Statistical analyses of gal3 concentrations in sera of PCa patients (stage II and III) compared with that from normal serum. ***$P < 0.001$ ANOVA.

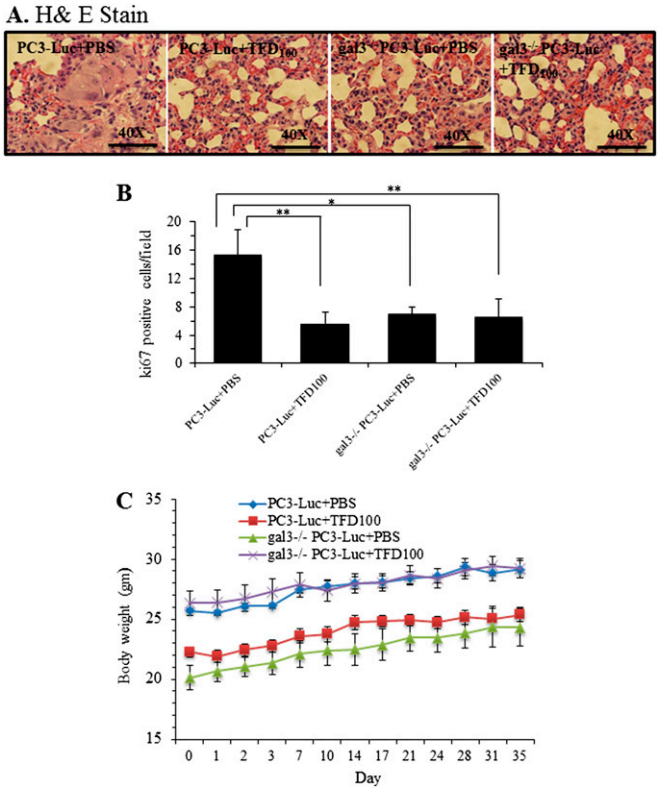

Fig. S7. (*A*) Representative diagrams showing H&E stain of lung sections from untreated or TFD_{100}-treated mice. (*B*) Bar diagram showing ki-67–positive cells in lungs of vehicle treated and TFD_{100}-treated mice. (*C*) Body weight of vehicle and TFD_{100}-treated mice. $*P < 0.05$; $**P < 0.01$; ANOVA.

Table S1. Characterization of various fractions from centricon centrifugation

Sample	Molecular size	% yield	% protein	% hexose (neutral)	Type of glycan	I_{50} value, μg/mL	RIA
Lactose	360 Da	NA	NA	NA	NA	27	1
TFD	383 Da	NA	NA	NA	NA	58	0.47
Crude extract	—	NA	1.1	46	Galβ1,3GalNAc-	108	0.25
Fraction 1	>50 kDa	3.3	2.6	23	Galβ1,3GalNAc-	2.5	10.5
Fraction 2	<50 kDa, >30 kDa	1.9	0.7	20	Galβ1,3GalNAc-	27	1
Fraction 3	<30 kDa, >10 kDa	18.4	1.0	39	Galβ1,3GalNAc-	82	0.33
Fraction 4	<10 kDa, >3 kDa	35.0	0.6	48	ND	300	0.09
Fraction 5	<3 kDa	41.3	0.5	9	ND	>500	<0.06

NA, not applicable; ND, no glycan detected with Glycan Differentiation Kit; % protein, by Coomassie staining (1); RIA, Relative inhibitory activity compared with lactose (taken as 1); % yield, by dry weight.

1. Ahmed H, Pohl J, Fink NE, Strobel F, Vasta GR (1996) The primary structure and carbohydrate specificity of a β-galactosyl-binding lectin from toad (*Bufo arenarum* Hensel) ovary reveal closer similarities to the mammalian galectin-1 than to the galectin from the clawed frog Xenopus laevis. *J Biol Chem* 271(51):33083–33094.

Table S2. Inhibition of galectin binding to asialofetuin by lactose and TFD_{100}

Galectin	Lac I_{50} value, μM	TFD_{100} I_{50} value, nM	RIA
gal3	100	0.25	400,000
gal4	100	1.2	83,333
gal9N	100	1.5	66,667

gal9N, N-terminal of gal9; RIA, relative inhibitory activity compared with lactose taken as 1.

Table S3. Summary of gal3 concentration in normal and PCa patients sera

Serum	Pathological stage	Gleason score	Conc of gal3, ng/mL	Conc of gal3 after gal3 depletion, ng/mL
Normal	Not applicable	—	1.6	0.01
P17	III, pT3bN0Mx	4 + 5 = 9	212	10
P19	III, pT3bN0Mx	3 + 5 = 8	237	0
P22	III, pT3aN0Mx	4 + 4 = 8	202	0
P27	III, pT3aN0Mx	4 + 5 = 9	18	0
P42	II, pT2cN0Mx	3 + 4 = 7	16	0
P50	II, pT2cN0Mx	4 + 5 = 9	36	0
P56	II, pT2cN0Mx	3 + 4 = 7	40	0

Table S4. Serum chemistry of the TFD_{100}-treated mice

Test	Result value	Normal range	Unit of measure
Albumin	3.03 ± 0.05	2.5–4.6	g/dL
Alanine aminotransferase	51.33 ± 2.51	35–222	U/L
Total bilirubin	0.1 ± 0.00	0.0–0.9	mg/dL
Calcium	10.83 ± 1.19	6.0–13.0	mg/dL
Total protein	4.76 ± 0.20	3.9–6.4	g/dL
Blood urea nitrogen	23.33 ± 5.50	9–33	mg/dL