Role of GD2 and its biosynthetic enzyme GD3 synthase in prostate cancer tumorigenesis

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Running Title: GD2 in prostate cancer tumorigenesis

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Conflict of interest disclosure statement: Dr. H. Band and Dr. V. Band received funding from Nimbus Therapeutics for an unrelated project.

Word count: Abstract -213, Manuscript – Main text-5867, Methods- 1771
Figures: 06; **Supplementary Figures**: 05 and 1 table

ABSTRACT

While better management of loco-regional prostate cancer (PC) has greatly improved survival, advanced PC remains a major cause of cancer deaths. Identification of novel, targetable, pathways that contribute to tumor progression of PC could open new therapeutic options. The di-ganglioside GD2 is a target of FDA-approved antibody therapies in neuroblastoma, but the role of GD2 in PC has been only little explored. Here, we show that GD2 is expressed on a small subpopulation of PC cells in a subset of patients, especially in metastatic PC. Variable levels of cell surface GD2 expression are seen in most PC cell lines, and the expression is highly upregulated by experimental induction of lineage progression or enzalutamide resistance in CRPC cell models. $GD2^{high}$ cell fraction is enriched upon growth of PC cells as tumorspheres and $GD2^{high}$ fraction is enriched in tumorsphere growth. CRISPR-Cas9 knockout (KO) of the rate-limiting GD2 biosynthetic enzyme GD3 Synthase (GD3S) in GD2-high CRPC cell models led to marked impairment of their *in vitro* oncogenic traits, reduced cancer stem cell (CSC) and epithelial-mesenchymal transition (EMT) marker expression and growth as bone-implanted xenograft tumors. Our results support the potential role of GD3S and its product GD2 in promoting PC tumorigenesis by maintaining cancer stem cells and suggest the potential for GD2 targeting in advanced PC.

INTRODUCTION:

Despite gains from early diagnoses, the successful use of androgen derivation therapy (ADT) and the availability of androgen receptor (AR) signaling inhibitors (ARSIs) as targeted agents, prostate cancer (PC) remains a major killer of men and the low survival of patients with distant metastases remains a major clinical challenge. A key transition towards less controllable and incurable metastatic disease is the emergence of castration-resistant PC (CRPC). This has prompted search for targetable pathways that are key to CRPC tumorigenesis. Pathways that can

be targeted using existing agents with acceptable safety profiles in clinic are particularly attractive as their transition along the translational-clinical continuum can be expected to proceed more quickly to positively impact the course of advanced PC.

The cell-surface di-ganglioside GD2 is expressed on developing neuroectodermal cells and, at lower levels, in certain adult tissues like neurons and basal cells of the skin (1). Biochemically, GD2 is a b-series ganglioside generated from lactosyl-ceramide, which is serially converted to GM3, GD3 and GD2 by the actions of enzymes ST3GAL3 (GM3 synthase; GM3S), ST8SIA1 (GD3 synthase; GD3S) and B4GALNT1 (GM2/GD2 synthase; GD2S), respectively, with GD3S considered rate-limiting (2) (**Fig. 1A**). GD2 is highly expressed in nearly all neuroblastomas, most melanomas and retinoblastomas, and many Ewing sarcomas. It is also overexpressed on some cases of small cell lung cancer, gliomas, osteosarcomas, and soft tissue sarcomas (3).

Functionally, GD2 has been implicated in oncogenic signaling leading to increased cell proliferation, migration, and invasion, and designated by the NCI as the top 12th among 75 tumor antigens (4). Extensive studies have led to the development of GD2-targeted antibodies for maintenance therapy of neuroblastoma patients (5,6), with clinical studies demonstrating the safety and efficacy of this approach (7). A humanized anti-GD2 monoclonal antibody with a higher affinity for GD2 was recently approved for children and adults with relapsed or refractory high-risk neuroblastoma (8).

Validation of GD2 targeting as a therapeutic approach in neuroblastoma has prompted studies of other cancers (3). Significant insights have come from studies of breast cancer. Unlike neuroblastoma and other tumors characterized previously, where a majority of tumor cells express GD2, human breast cancer cell lines and patient samples harbor a small GD2^{high} fraction which

selectively formed tumorspheres *in vitro* and tumors when implanted *in vivo* and was enriched in triple-negative breast cancer (TNBC), a subtype known to be more aggressive and metastasis-prone. The $GD2^+$ breast cancer cells were $CD44^{hi}/CD24^{lo}$, indicating that GD2 is expressed in cancer stem cell (CSC) fraction, and GD3S knockdown reduced the CSC population and CSC-associated properties and abrogated tumor formation *in vivo* (9,10). Further, GD3S knockdown or inhibition of its expression by triptolide inhibited the EMT and migratory/invasive behavior *in vitro* and abrogated metastasis *in vivo* in claudin-low (mesenchymal) breast cancer cell lines (11). Finally, Dinutuximab inhibited experimental triple-negative breast tumor growth by targeting CSCs (12). Recently, O-acetyl-GD2 was also found to mark breast cancer CSCs (13). GD2 expression on breast cancer CSCs also allowed synergistic targeting by human γδ and $CD8^+$ T cells together with humanized anti-GD2 antibody hu14.18K322A (14). Further, GD2-CAR expressing T cells inhibited the orthotopic breast cancer xenograft growth and completely prevented lung metastasis by eliminating CSCs (15). In addition, GD2 expression correlated with CSC and EMT marker expression in bladder cancer cells (16). GD3S and GD3 (17) as well as O-acetyl-GD2 (17) were found to drive glioblastoma CSCs and tumorigenicity, with sensitization of tumor cells to temozolomide *in vitro* and *in vivo* by anti-O-acetyl-GD2 antibody. Thus, GD2 and its rate-limiting biosynthetic enzyme GD3S have emerged as key factors in promoting CSC behavior and as targets of CSC-directed therapeutic efforts (18,19). As CSCs are key to initiation and maintenance of tumors, and responsible for therapeutic resistance and metastasis (20), targeting of GD2 in this tumor cell subpopulation represents a significant shift in thinking about the role of this ganglioside and the enzymatic machinery responsible for its expression in non-neuroblastoma tumors.

Progression to CRPC is associated with increased CSC features and EMT (21). However, few, mostly older, studies have explored the status of GD2 in PC. An early study of a small number of prostate hyperplasia, prostate cancer, and normal prostate tissue samples by thin layer chromatography found that GD2 levels were reduced during tumorigenesis (22). Another study detected more GD2 expression by chromatography in AR-negative PC cell lines (HH870, DU145 and PC-3) than in AR^+ cell lines (LNCaP clones FGC and FGC-10), and immunofluorescence analyses showed the expression of cell surface GD2 on PC3 cells but not a normal prostate epithelial cells (23,24). A recent study found higher expression of a long non-coding RNA (lncRNA) MIR4435-2HG in PC cell lines (VCAP, LNCaP, DU145 and PC-3) compared to a normal prostate epithelial cell line and its knockdown reduced while ectopic overexpression increased the PC3 cell proliferation, migration and invasion *in vitro* and tumorigenesis *in vivo*. MIR4435-2HG was found to bind to GD3S mRNA and promote its expression, and GD3S knockdown (which by itself had no impact) was found to reduce the pro-tumorigenic effects of ectopic MIR4435-2HG overexpression (25). This study did not examine the ganglioside products of GD3S, and reported high GD3S expression in all 4 PC cell lines examined; the latter is not confirmed by our analysis of the mRNA expression of the same cell lines reported in a comprehensive epigenetics/transcriptomics study (26) and our analyses demonstrate little or no GD2 expression on some of these PC cell lines (See Results). Any functional role(s) of GD2 in PC remain unknown and its targeting in PC has never been explored despite considerable progress on the role of GD2 in other cancers and its successful targeting in neuroblastoma.

Here, we carry out analyses of GD2 expression in prostate cancer patient tissues and their metastases as well as in cell line models of CRPC progression and use CRISPR-KO of GD3S in a human and murine CRPC cell line models to establish that GD2 expression is a feature of a small

fraction of tumor cells enriched in tumorsphere growth *in vitro*, and that GD3S is required for tumorigenesis in CRPC.

MATERIALS AND METHODS:

Prostate cancer (PC) patient tissue microarrays and immunohistochemical analysis: Tissue microarrays (TMAs) composed of 320 cases of paired normal prostate and prostate cancer Gleason grades (3, 4 and 5) and or of 45 cases of bone and visceral metastatic samples with triplicate cores from Rapid Autopsy TMAs were obtained without any identifying patient information from the Prostate Cancer Biorepository Network (PCBN) repository under a materials transfer agreement. TMAs were stained for GD2 by immunohistochemistry (IHC) as described previously (27). Briefly, TMAs were deparaffinized in xylene, rehydrated in descending alcohols and heated in the antigen retrieval solution (vector, cat#H-3300) in a microwave to unmask the antigens. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 20 min. The sections were rinsed in phosphate buffered saline (PBS) and blocked in Protein-Block buffer (DakoCytomation #0909) for 15 min. The sections were then stained with anti-GD2 antibody (2 μg/ml) overnight at 4ºC. After rinsing in TBST, the sections were incubated for 15 min with an anti-mouse secondary antibody conjugated to a dextran-labeled polymer and HRP (DakoCytomation K4007), followed by incubation in DAB solution (DakoCytomation DAB # K4007, Solution 3a, b) for 5 min. The sections were counter-stained with hematoxylin and mounted under cover glasses using a xylene-based mounting medium. GD2 scoring was evaluated by a certified pathologist (SL) and staining scores were assigned as: 0 (negative), 1 (low positive), 2 (moderate positive) and 3 (strong positive). Four cores per case was analyzed for primary tumors. Histoscore was calculated by multiplying the staining intensity by % cells staining.

Histoscore distributions were assigned as follows: 0-5, negative; 5-25, low; 25-50, moderate; and >50, strong positive. Histoscore 5 was used as a cut off.

Analysis of *ST8SIA1* mRNA expression in transcriptomic data of CRPC patient-derived xenograft (PDX) tumors and organoid models. *ST8SIA1* mRNA expression was analyzed using publicly-available transcriptomic data on CRPC PDX and organoid models published by Tang et al., (26) (GEO accession GSE199190). As per the author classification, the CRPCs were grouped into 4 groups: Group 1, Androgen Receptor dependent (CRPC-AR); Group 2, Wnt dependent (CRPC-WNT); Group 3, neuroendocrine (CRPC-NE); and Group 4, stem cell like (CRPC-SCL) were analyzed. Gene expression is presented as normalized transcripts per million (TPM).

Cell lines and medium: 22Rv1 and RM-1 cell lines were cultured in RPMI-1640 (Hyclone; #SH30027.02) or DMEM (Gibco; #11965-092), respectively, with 10% fetal bovine serum (Gibco; #10437-028), 10 mM HEPES (Hyclone; #SH30237.01), 1 mM each of sodium pyruvate (Corning; #25-000-CI), nonessential amino acids (Hyclone; #SH30238.01), and L-glutamine (Gibco; #25030-081), 50 μM 2-ME (Gibco; #21985-023), and 1% penicillin/ streptomycin (#15140-122; Gibco). HEK-293T cells (ATCC CRL-3216) were cultured in complete DMEM medium. C4-2 parental, TP53 stable KD, RB1 KD cell lines, as well as the parental C4-2B and its enzalutamide resistant derivative line (C4-2BER) were grown as described previously (28). Cell lines were maintained for less than 30 days in continuous culture and were regularly tested for mycoplasma.

Reagents and Antibodies: Primary antibodies used for immunoblotting were as follows: anti-ST8SIA1/GD3S from Novus Biologicals (#NBP1-80750) or ProteinTech (#24918-1-AP); anti-SOX2 from ProteinTech (#11064-1-AP); anti-SOX9 from Abcam (#ab185966); anti-Snail from CST (#3879); anti-HSC70 (#sc-7298); anti-Notch1 (#sc-376403) from Santa Cruz Biotechnology;

anti-RB1 (BD Bioscience, #554136); anti-β-actin (#A5441) from Sigma; Anti-Stat3 (#9139S) from CST; anti-Lin28b (#ab191881 from Abcam); anti-Zeb1 (#3396S) from CST; anti-Slug (#ab27568) from Abcam and anti-N-cadherin (#13116S) from CST. The horseradish peroxidase (HRP)-conjugated Protein A (#101023) and HRP-conjugated rabbit anti-mouse secondary antibody (#31430) and HRP conjugated Goat anti-mouse IgM (#31440) for immunoblotting were from Thermo Fisher. APC conjugated anti-human ganglioside GD2 antibody (#357306; Biolegend) or APC-Mouse-IgG2a,κ isotype control (#400220; Biolegend) was used for IHC.

Generation of CRISPR/cas9 knockout and luciferase reporter cell lines: The following constructs were used to generate GD3S KO lines. For mouse RM-1, mouse *St8sia1* sgRNA CRISPR/Cas9 All-in-One Lentivector (pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro; #456551140595, Applied Biological Materials) with three target sequences Target 1-17: GGGCCCTACATACGTCCAGA (sgRNA1), Target 2-56: CTCGGAAATTCCCGCGTACC (sgRNA2), Target 3-141: CTACCGGCTGCCCAACGAGA (sgRNA3). For human 22Rv1 cells, GSGH1195-Edit-R Human All-in-one Lentiviral sgRNA (#GSGH1195-247582548 and #GSGH1195-247725854 designated as sgRNA2 and sgRNA3 respectively) from Horizon Discovery. Lentiviral supernatants generated in Lenti-x HEK-293T cells (TAKARA, #632180) using the 2nd generation packaging system (plasmids psPAX2, Addgene #12260 and pMD2.G, Addgene #12259) were applied to cells for 72h with polybrene (10 µg/ml, Sigma #H9268) and stable lines selected in 2 µg/ml puromycin. Clones obtained by limiting dilution were analyzed by western blotting for GD3S and FACS for GD2. 3 clones for each sgRNA, maintained separately, were pooled for experiments. The tdTomato-luciferase expressing RM-1 cells were generated using the pMuLE lentiviral system kit (Addgen#1000000060), as described (29). The mCherry-luciferase expressing 22Rv1 derivatives were generated using a lentiviral plasmid (pCDH-EF-

eFFly-T2A-mCherry; Addgene #104833). Cell lines with high reporter expression were obtained by FACS sorting.

Western Blotting: Western blotting of whole cell extracts prepared in Triton-X-100 lysis buffer and quantified using the BCA assay kit (#23225; Thermo Fisher Scientific) was performed as described previously (29). 50 µg protein aliquots were run on SDS-7.5% polyacrylamide gels, transferred to polyvinylidene fluoride (PVDF) membrane, and immunoblotted with the indicated antibodies.

Flow cytometry: Cells seeded in regular medium for 48 h were released with trypsin-EDTA (#15400054; LifeTech (ThermoFisher)) and trypsinization stopped with soybean trypsin inhibitor (#17075029; LifeTech (ThermoFisher). Cells were washed thrice in ice-cold FACS buffer (2% FBS, 25mM HEPES in PBS), and live cells stained with APC anti-human GD2 (#357306; Biolegend) (isotype control, APC Mouse IgG2a, κ; #400220, Biolegend) or Anti-human/mouse CD49f antibody (#313620, Biolegend) (isotype control, Pacific Blue Rat IgG2a, κ; #400527, Biolegend). For GD3 FACS analysis, anti-GD3 (purified anti-GD3 (MEL-1) antibody, #917701 from Biolegend) with isotype control (purified mouse IgG3, κ; #401302) and secondary protein G, Alexa Fluor 488 (#P11065) was used. FACS analyses were performed on a BD LSRFortessa X50, BD LSRII or BD LSRII Green instruments and data analyzed using the FlowJo software.

Trans-well migration and invasion assay: Cells grown in 0.5% FBS-containing starvation medium for 24 hours were trypsinized and seeded at 2 x 10^4 on top chambers of 24-well plate trans-wells (Corning, cat# 353097 for migration; or Biocoat Matrigel invasion chambers #354480) in 400 µl of growth factor deprived medium. After 3 hours, medium containing 10% FBS was added to lower chambers and incubated for 16 hours. The non-migrated cells on the upper surface of membranes were removed with cotton swabs, migrated cells on the lower surface were

methanol-fixed and stained in 0.5% crystal violet in methanol. Six random 10x fields per insert were photographed, and cells counted using the ImageJ software. Each experiment was run in triplicates and repeated three times.

Cell proliferation assay: Cell proliferation was assayed as described (29), with 250 cells plated per well in 96-well flat-bottom plates in 100 µl medium and an equal volume of the CellTiter-Glo Luminescent Assay Reagent (#G7571; Promega) added at the indicated time-points. Luminescence was recorded using a GloMax® luminometer (Promega).

Tumorsphere assay: Tumorsphere cultures were done as described (30) with minor modifications. the tumorsphere medium contained DMEM/F12,Thermo Fisher; #1133032) supplemented with 1% penicillin/streptomycin, 4 µg/ml heparin (Stem cell technologies; #07980), 20 ng/ml Animal-Free Recombinant Human EGF (Peprotech; #AF-100-15), 10 ng/ml Recombinant Human FGF-basic (Peprotech; #100-18B), 1X N-2 supplement (Gibco; #17502-048), 1X B27 supplement (Gibco; #17504-044) and 4% Matrigel (BD Biosciences; #356234]. $2x10^4$ cells were seeded per well in 800 µl volume in ultra-low attachment 24-well plates. Tumorspheres were imaged under a phase contrast microscope after 1 week and quantified using the Image J software. All experiments were done in triplicates and repeated 3 times.

Intra-tibial tumorigenic growth: All animal experiments were performed with the approval of the UNMC Institutional Animal Care and Use Committee (IACUC Protocol# 21-004-06 FC). $4x10^4$ (in 20 µl cold PBS) parental (control) or GD3S-KO (sgRNA1 and sgRNA2) RM-1 cells engineered with lentiviral tdTomato-luciferase or $1x10^5$ (in 40 µl cold PBS) parental (control) or GDS3-KO (sgRNA1) 22Rv1 cells engineered with lentiviral mCherry-enhanced luciferase were injected in tibias of 8-weeks old castrated male C57BL/6 (for RM-1 model) or athymic nude (for 22Rv1 model) mice, respectively, and tumor growth monitored by bioluminescence imaging .

Mice were imaged weekly and followed until the control group reached pre-determined humane end points requiring anesthesia. At necropsy, hind limbs, lungs, spleen, and livers were harvested. For bioluminescence imaging, mice received 200 µl D-luciferin (15 mg/ml; Millipore Sigma #L9504) intraperitoneally 15 min before isoflurane anesthesia and were placed dorso-ventrally in the IVIS™ Imaging System (IVIS 2000). Images were acquired using the IVIS Spectrum CT and analyzed using the Living Image 4.4 software (PerkinElmer). The fold change in total Photon flux (p/s) over time was used to compare tumor growth between the groups.

Bone morphometry analysis: The harvested tibias were fixed in fresh 4% formalin for a day, transferred to 70% ethanol and scanned using a micro-CT scanner (Skyscan 1172, Bruker) (micro-CT scanning core facility, UNMC). The parameters were 55 kV, 593 181 µA, 0.5 mm aluminum filter, 9 µm resolution, 4 frames averaging, 0.4 rotation step, 180° scanning. The images were reconstructed using the NRecon software (version 1.7.4.6, Bruker microCT), registered and realigned using the DataViewer software (version 1.5.6.2, Bruker microCT) and analyzed using the CTAn software (version 1.18.8.0, Bruker microCT) for the percentage of bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp).

RNA isolation and Real Time qPCR analysis. Total RNA extracted using the TRIzol® Reagent (Ambion, Life Technology) was converted into cDNA with the Invitrogen SuperScript III system (18080-051) and subjected to real-time qPCR using the SYBR Green labeling method (Applied Biosystems; 4309155) on an Applied Bioscience QuantStudio thermocycler. The primer sequences used were: *ST8SIA1:* (forward) 5′-TCCCAGCATAATTCGGCAA-3′ reverse, 5′-ATCTGACAGTGTATAATAAACCCTC-3′); *B4GALNT1* (forward, 5′-TTCACTATCCGCATAAGACAC-3′; reverse, 5′-GTAACCGTTGGGTAGAAGC-3′);

ST3GAL3 (forward, 5′-ACATTGCTTGTGTTTGGAG-3′; reverse, 5′-GGACGACATTCCTTCTGC-3′); *TP53* (Forward, 5'-CCTCACCATCATCACACTGG-3'; reverse, 5'-CACAAACACGCACCTCAAAG-3'); RB1 (Forward, 5'-CTCTCACCTCCCATGTTGCT-3'; reverse, 5'-GGTGTTCGAGGTGAACCATT-3'); *GAPDH (*forward, 5'-CAGAACATCATCCCTGCCTCT GAPDH; reverse, 5'-GCTTGACAAAGTGGTCGTTGAG GAPDH). The fold change in expression was calculated relative to control (parental) cells using the ΔΔCt method and normalized to GAPDH reference gene expression.

RT^2 Profiler PCR Arrays: mRNA was isolated from RM-1 and its GD3S KO variant cells using QuantiTech Reverse Transcription Kit (#205310). The expression of specific genes was quantitated by qPCR using RT^2 Profiler PCR Array (PAMM-176ZA-6 RT^2 Profiler™ PCR Array (#330231) for mouse cancer stem cells with QuantiTech SYBR Green PCR Kit (#204141) on an Applied Bioscience QuantStudio thermocycler. The analysis was performed using the GeneGlobe online application (geneglobe.qiagen.com) and the fold regulation of the genes was represented against the control in the heatmap and volcano plot.

Statistical analysis: GraphPad Prism software (version 9) was used to perform the statistical analyses. The *in vitro* experimental data analysis used a two-tailed student's t test. A two-way ANOVA (mixed model) test was used to analyze the fold change in photon flux in xenograft studies. P values equal to or <0.05 were considered significant.

RESULTS:

Increased GD2 expression upon neuroendocrine differentiation of a CRPC cell line model.

To assess if GD2 overexpression is a feature of PC, we validated a commercially available anti-GD2 antibody (clone 14.G2; Biolegend) for immunostaining of known $GD2^+$ and $GD2^-$ cell lines. FACS as well as immunohistochemistry (IHC) analysis demonstrated that 14.G2 stained the known $GD2^+$ neuroblastoma cell line SK-N-BE-2 (31) but not the $GD2^-$ rhabdomyosarcoma cell line A204 (32), while the isotype control antibody showed similar background staining in both cell lines (**Supplementary Fig. S1**).

We surmised that GD2 overexpression might be a feature of CRPC with neuroendocrine differentiation (CRPC-NE), given the overexpression of GD2 in neuroectodermal lineage tumors (3), and the known acquisition of CSC features upon CRPC-NE differentiation (33). Depletion of RB1 or TP53 in androgen receptor-driven CRPC (CRPC-AR) cell lines (34) and in mouse models when combined with PTEN loss (35), is known to promote the NE trans-differentiation. Therefore, we compared the CRPC-AR cell line LNCaP C4-2 (referred to as C4-2), which has a mutational inactivation of PTEN (36), with its derivatives with stable knockdown (KD) of TP53 or RB1 (28) that have been shown to undergo NE differentiation (34, 34). We verified the expected TP53 or RB1 KD using q-PCR (**Fig. 1B**). FACS staining revealed low cell surface GD2 expression on parental C4-2 cells, but the size of this subpopulation and the levels of GD2 were markedly increased in RB1- or TP53-KD derivatives (**Fig. 1C**). IHC analyses confirmed these findings (**Fig. 1D**). Increased expression of GD2 upon NE-differentiation of C4-2 cells was associated with increases in the expression levels of the key GD2 biosynthesis enzymes GM3S, GD3S and GD2 synthase (GD2S) (**Fig. 1 E**).

Increased GD2 expression across PC cell lines, including CRPC cell lines driven by diverse mechanisms:

To assess if GD2 was more broadly expressed in PC cell models, we carried out FACS analysis of the cell surface expression of GD2 on a panel of established human and murine PC cell lines, using the C4-2 and its RB1 or TP53 KD derivatives characterized above as reference. Among the 18 cell lines analyzed, which included variants of some of the commonly used PC cell lines and two immortal prostate epithelial cell lines (PHPV18 and PSV40), 6 displayed little or no anti-GD2 staining above that with the control antibody (**Table 1, Supplementary Fig. S2A-C**). In contrast, low but clearly detectable levels of GD2 expression on a small subpopulation of cells was observed on 3 PC cell lines (ND1, C4-2 and C4-2B) and two immortal prostate epithelial cell lines (PHPV18 and PSV40) (**Table 1, Supplementary Fig. S2A-C**). Importantly, a high proportion of $GD2^+$ cells with high mean fluorescence intensities (MFI's) was observed in the human CRPC cell lines 22Rv1, which overexpresses wildtype AR and ARV7 splice variant (37), and C4-2B cells rendered enzalutamide-resistant (28), and on the murine CRPC cell line RM-1 (driven by Ras and Myc) (38) and transgenic adenocarcinoma mouse prostate (TRAMP)-derived TRAMP-C1 cell line (39) (**Table 1, Supplementary Fig. S2A-C**).

The GD3S enzyme (encoded by the ST8SIA1 gene) is rate-limiting for GD2 synthesis (2). Consistent with our staining data, the CCLE-DepMap mRNA expression data available for ten PC cell lines showed the highest value for the GD2-high 22Rv1 cell line, and the second lowest value for the $GD2^-$ PC3 cell line (**Table 1, Supplementary Fig. S2A-C)**. Further analysis of transcriptomics data of organoids and PDX tumors recently used to classify CRPC into 4 subtypes: CRPC-AR (AR-driven), CRPC-NE (neuroendocrine), CRPC-WNT (WNT pathway-driven) and CRPC-SCL (stem cell like) (26) demonstrated high *ST8SIA1* mRNA expression in the $GD2^{high}$ 22Rv1 cell line and nearly undetectable levels in the $GD2^-$ LNCaP cell line (**Supplementary Fig. S3, upper panel**). Using the levels in these cell lines as reference, >20% of CRPC organoids and

PDX tumors showed increased ST8SIA1 expression, and this was seen across the CRPC subtypes (**Supplementary Fig. S3**). Thus, our results suggested that a majority of established PC cell lines, including examples of CRPC, express low to high GD2 levels on a variable fraction of cells, reminiscent of studies with breast cancer cell models (9,10), and the ST8S1A1 expression results on organoid and PDX models supported the potential of GD2 expression in patient-derived PCs.

GD2 expression is seen on a small subset of tumor cells in primary PC patient tissues and a higher proportion of metastatic lesions.

To assess if GD2 overexpression on a subpopulation of cells in PC cell lines could be extended to human PC we used tissue microarrays (TMAs) from two PC patient cohorts (provided by the Prostate Cancer Biorepository Network; PCBN). IHC staining of a 320-sample TMA of paired normal prostate tissues and PC samples (Gleason scores of 3-5) demonstrated a statistically-significant overexpression of GD2 on primary prostate cancers compared to paired normal prostate tissue (**Fig. 2A-C**); unlike neuroblastoma, with nearly uniform $GD2^+$ tumor cells, but similar to triple-negative breast cancers, only a small subset of tumor cells was $GD2^+$. Overall, ~20% of primary PCs scored $GD2^+$ based on a cutoff of histoscores of less than 5 (**Fig. 2D**). Notably, the IHC analysis of a metastatic PC TMA (the 45-Case Bone and Visceral Metastasis from Rapid Autopsy TMA from PCBN) showed $GD2^+$ staining in about twice the percentage of metastatic lesions compared to primary tumors (**Fig. 2D**). The histoscore distribution analysis showed that metastatic tumors exhibited higher GD2 expression (**Fig. 2E-F**). These results indicate that GD2-expression is a feature of a subpopulation of tumor cells in PC, especially in PC metastasis.

GD2 overexpression defines a PC cell population with higher tumorigenic potential *in vitro.*

To assess the potential functional role(s) of GD2 overexpression in PC, we first compared the parental C4-2 cells ($GD2^{low}$) with their RB1- or TP53-KD lines that overexpress GD2 ($GD2^{high}$). Cell Titer-Glo assays showed that GD2-overexpressing, RB1- or TP53-KD derivatives exhibited higher and faster cell proliferation (**Fig. 3A, left panel**). Similarly, the $GD2^{high}$ enzalutamide resistant cell line, C4-2BER showed increased proliferation rate compared to $GD2^{low}$ control C4-2B cells (**Fig. 3A, right panel**). Transwell migration analyses indicated a higher migratory behavior of $GD2^{high}$ derivatives compared to $GD2^{low}$ C4-2 cells and C42-B parental cell lines, respectively (**Fig. 3B**). Importantly, the $GD2^{high}$ RB1- or TP53-KD derivatives exhibited a marked elevation of 3D tumor-sphere growth (**Fig. 3C, upper panel**), an *in vitro* correlate of CSC activity (40), and a similar elevation of tumor-sphere-forming ability was seen with $GD2^{high}$ C4-2BER compared to GD2-low C4-2B parental cells (**Fig. 3C, lower panel**).

Previous studies have shown that GD2-expressing cell fraction is enriched in tumorigenic behaviors (9). We therefore used FACS-sorting to isolate the $GD2^{high}$ and $GD2^{low}$ populations of 22Rv1 and RM-1 cell lines which spontaneously express high GD2 on a subpopulation of cells (**Table 1**). Consistent with previous studies demonstrating that GD2 expression is dynamic (41), the FACS-sorted populations drifted towards a mix of $GD2^{high}$ and $GD2^{low}$ populations upon continued 2D or 3D culture (**Fig. 3D**). Furthermore, FACS analysis revealed an enrichment for the $GD2^{high}$ fraction during tumorsphere growth vs 2-D culture with both $GD2^{high}$ and $GD2^{low}$ sorted fractions of 22Rv1, and RM-1 cell lines (**Fig. 3E**). A similar, time-dependent, increase in the $GD2^{high}$ population was observed upon tumorsphere vs. 2D growth of C4-2 ($GD2^{low}$) and its RB1 or TP53 KD lines ($GD2^{high}$) derivatives (**Supplementary Fig. S4**). Despite the GD2 expression being dynamic, the $GD2^{high}$ fraction of both 22Rv1 and RM-1 cell lines showed higher tumor-sphere-forming ability (**Fig. 3F**). Dual FACS staining of RM-1 and 22Rv1 cell lines grown as

tumorspheres for CD49f and GD2 demonstrated that a majority of GD2^{high} cells were within the CD49f^{high} fraction (**Fig. 3G**). These results suggested that GD2^{high} fraction defines a cell population with higher oncogenic potential and CSC-like behavior.

GD3 synthase, the rate-limiting enzyme in GD2 biosynthesis, is required for *in vitro* oncogenic attributes of GD2-high CRPC cell lines.

In view of our results above, we hypothesized that the GD2^{+} cell subpopulation is the major driver of the oncogenic attributes of PCs. To test this hypothesis, we targeted the rate-limiting GD2 biosynthesis enzyme GD3S (2) for CRISPR-Cas9 knockout (KO), using a single-vector Cas9/sgRNA expression system, in GD2-overexpressing mouse (RM-1) and human (22Rv1) CRPC cell lines and obtained clonal lines by serial limiting dilution cloning. Western blotting confirmed the loss of GD3S expression in GD3S sgRNA-targeted derivatives (**Fig. 4A, B**). These GD3S-KO clones indeed lacked the cell surface GD2 expression by FACS analysis (**Fig. 4C, D**). As GD3 is the immediate product of GD3S activity and a precursor of GD2, we also assessed the levels of GD3 in parental vs. GD3S-KO CRPC cell lines, using FACS. Relatively low to modest levels of GD3 were seen on parental cells and this expression was eliminated in KO cells (**Supplementary Fig. S5**).

In both the RM-1 and 22Rv1 cell line models, the GD3S-KO versions exhibited a marked decrease in cell proliferation, migration, and invasion (**Fig. 4 E-H**). GD3S-KO cell lines also exhibited marked impairment of their tumorsphere forming abilities compared to their parental non-targeted cells (**Fig. 5A**), supporting the importance of GD3S and its ganglioside products in promoting the CSC behavior of CRPCs. Further supporting this notion, FACS analysis of GD3S-KO grown cells showed a reduced proportion of CD49f^{hi} cells (**Fig. 5 B**), a marker associated with

PC CSCs (42). To confirm the linkage of GD3S and GD2 expression with CSC characteristics, we analyzed the mRNA expression levels of 84 mouse CSC signature genes using the RT^2 profiler quantitative PCR (qPCR) array in the control RM-1 vs its GD3S-KO derivative (**Fig. 5C, D).** This analysis revealed a significant downregulation of 17 genes including *Notch1, Stat3*, *Sox2, Pou5f1(Oct4), Mycn, Lin28b, Snai1, Zeb1, Epcam, Pecam1* and upregulation of 22 genes such as *CD24a*, *Mertk, Slug*, *Foxa2*, *CD44*, *Aldha1a*, *Klf4* and *Myc* in GD3S-KO cells (**Fig. 5D**). Western blotting analysis validated the qPCR-observed downregulation of Snail, ZEB1, Lin28b, Notch1, Stat3 (α, β), SOX2 and the observed upregulation of Slug in GD3S-KO RM-1 and 22Rv1 cells vs. their control cells (**Fig. 5E**). Additional Western blot analysis of CSC/EMT markers SOX9 and N-cadherin revealed their downregulation in GD3S-KO RM-1 and 22Rv1 cells vs. their control cells (**Fig. 5E).** Collectively, these result support the conclusion that the $GD2^{high}$ fraction in CRPC models defines a CSC-like population.

GD3S-KO impairs the tumorigenic ability of GD2-high CRPC cell lines.

To assess if the marked reduction in the pro-oncogenic traits upon GD3S-KO observed *in vitro* translates into impaired tumorigenesis *in vivo,* we used lentiviral infection to introduce a tdTomato-luciferase dual reporter into control and GD3S-KO RM-1 cell lines (the latter with two distinct sgRNAs). The tdTomato-high fractions were enriched by FACS-sorting and stable cell lines selected. We implanted the reporter-bearing control and GD3S-KO RM-1 cells in the tibias of castrated syngeneic C57BL/6 mice and monitored tumor growth (5 mice/group) over time by bioluminescence imaging. While control RM-1 cells generated robust tumors as seen by time-dependent increase in log10 photon flux in IVIS imaging, the GD3S-KO RM-1 cells produced significantly smaller tumors (**Fig. 6A, B**). Morphometric analysis of tibial bone by micro-CT

scanning showed reduced bone volume, trabecular number, thickness, and separation in mice implanted with control RM-1 cells, indicative of tumor-induced bone degradation, with a significant amelioration of these defects in tibias of mice implanted with GD3S-KO cells (**Fig. 6C-G**). To confirm these results, we also implanted the mCherry-luciferase expressing parental or GD3S-KO 22Rv1 cells intra-tibially in nude mice and analyzed these by IVIS imaging. Reduced tumorigenesis of GD3S-KO compared to parental tumor cells was confirmed in this model as well by IVIS imaging (**Fig. 6H, I**).

DISCUSSION:

Despite improvements in the diagnosis and management of early PC, progression to CRPC and metastatic disease represent lethal transitions responsible for high cancer burden from PC. Identifying targetable molecular pathways that either mark or contribute to these lethal states in PC can open new therapeutic avenues. Here, we use PC patient-derived tumor tissue analyses and as the PC cell line-based mechanistic approaches to demonstrate that the di-ganglioside GD2 marks a small but functionally-critical tumor cell subpopulation in PC with enrichment in more advanced disease states. Our studies using CRPC cell models establish that GD2 defines a subpopulation of tumor cells with cancer stem cell characteristics that contributes to oncogenic drive, thus raising the prospect of GD2 targeting in CRPC using clinically-approved therapeutic agents.

Our analyses of a large cohort of primary and metastatic PC TMAs demonstrated a small subpopulation of tumor cells that expresses GD2, with a significantly higher proportion of metastatic tumors harboring $GD2^+$ tumor cells (**Fig. 2D**). Notably, while a small percentage of $GD2^+$ cells were seen in normal prostate tissue, the proportion of such cells was significantly higher in tumors (**Fig. 2C**). In this regard, GD2 expression in PC is reminiscent of non-

neuroectodermal tumors like breast cancer where only a small proportion of tumor cells express GD2 (9), and the GD2 expression marks the rare stem cell population in normal tissues as well as in tumors (43). No prior studies have examined the GD2 expression at a histological level in PC tissues, but a limited number of prior studies, primarily using biochemical fractionation of glycolipids (22-24), are consistent with our analyses.

Our finding of GD2 expression in a subset of PCs was further substantiated by analyses of a panel of established PC cell lines, with a majority exhibiting GD2 expression on a small to substantial proportion of cells (**Table 1, Supplementary Fig. S2**). Notably, this pattern is similar to that seen in breast and other non-neuroectodermal tumor systems with GD2 expression on a subset of tumor cells (9). The $GD2^{high}$ and $GD2^{low}$ populations, however, appear to be in a dynamic equilibrium with each other, as seen by the re-emergence of mixed $GD2^{high}/GD2^{low}$ populations after the sorted pure $GD2^{high}$ and $GD2^{low}$ subsets were cultured *in vitro* (**Supplementary Fig. S4**), similar to that described in other tumor systems (41).

Notably, our analyses of in cell line models demonstrates that GD2 expression can be induced/elevated under scenarios of significance to the evolution of CRPC. First, GD2 could be dramatically induced by experimentally promoting the lineage plasticity of AR-driven C4-2 CRPC cell line by shRNA KD of TP53 or RB1, both leading to increased surface levels of GD2 and an increase in the proportion of $GD2^{+}$ cells (**Fig. 1C-D**), which was accompanied by a marked elevation in the expression of key GD2 biosynthetic enzymes GD3S and GD2S (**Fig. 1E**). A similar induction of GD2 expression was seen when GD2-low C4-2B CRPC cells were compared to their enzalutamide-resistant derivative C4-2BER (28) (**Table 1, Supplementary Fig. S2**). Aside from these experimental cell models, our analyses of the expression of the rate-limiting GD2 biosynthetic enzyme GD3S (*St8SIA1*) in publicly-available transcriptomic data of CRPC PDX and

tumor-derived organoid models showed that nearly a third of these, across the mechanistic subtypes (26), showed moderate to high GD3S expression (**Supplementary Fig. S3)**. These findings, together with our GD2 staining of primary and metastatic PC TMAs (**Fig. 2A, B, D)**, strongly support the likelihood that our findings are of direct relevance to human CRPC tumors.

The gain of GD2 expression upon genetic (RB1 or TP53 KD) or selection (enzalutamide resistance) events associated with key CRPC progression transitions (**Fig. 1C, D and Table-1**), and the expression of GD2 on a larger proportion of metastatic PC lesions (**Fig. 2D**), supported the likely biological significance of GD2 expression even when expressed on a subset of PC tumor cells. Our results strongly support this notion. Consistent with the $GD2^{+}$ cell population playing a key role in tumorigenic progression, the TP53- or RB1-KD induced lineage plasticity in C4-2 cells or selection of C4-2B cells for enzalutamide resistance markedly elevated their proliferation, migration and tumorsphere forming ability (**Fig. 3A-C**). Further supporting the role of $GD2^{+}$ cells in oncogenic drive, culturing RM-1, 22Rv1 (**Fig. 3D**) or C4-2BER (**Supplementary Fig. S4**) cell lines as tumorspheres led to marked enrichment for the $GD2^{high}$ population relative to cells grown in regular 2D conditions. Further, the sorted $GD2^{high}$ fraction of mouse RM-1 as well as of human 22Rv1 CRPC cell lines was significantly enriched in tumorsphere-forming ability (**Fig. 3D**). Directly supporting the role of GD2, CRISPR-Cas9 KO of GD3S in RM-1 and 22Rv1 cell lines, which eliminated their GD2 expression (**Fig. 4 A-D**), markedly impaired their proliferation, tumorsphere formation, and cell migration/invasion *in vitro* (**Fig. 4 E-H, Fig. 5A**). Notably, when examined as bone implants in syngeneic (RM-1) or nude mice (22Rv1), the GD3S-KO cell models were markedly impaired for tumorigenesis (**Fig. 6**).

That GD2 expression on only a subset of tumor cells was nonetheless critical for tumorigenesis is reminiscent of that seen in breast and other cancers where depletion of GD2 by

genetic or pharmacologic downregulation of GD3S expression (11,44) or by immunologic targeting the cell surface GD2 (12) led to impaired tumorigenesis. In these previous studies, GD2 expression has been shown to mark the CSC population, thus accounting for GD2's role in multiple tumorigenic traits. The strong co-staining of GD2 with high CD49f CSC marker (**Fig. 3G**), and the requirements of GD3S for tumorsphere growth (**Fig. 5A**) and for the enrichment of the CD49f^{hi} (CSC) population (**Fig. 5B**) are consistent with this idea. Further support for this idea is provided by our CSC gene signature qPCR analyses which demonstrated the downregulation of key CSC genes including *Sox2(45,46), Pou5f1(Oct4)(46), Mycn(47,48), Lin28b(49)*, Sox9(50), *Notch1(51)* and *Stat3(52)* in GD3S-KO CRPCs compared to their parental cells (**Fig. 5C-E**). The down regulation of the EMT related genes *Zeb1*, *Snail*, *N-cadherin* and *Epcam(53,54)* by GD3S-KO further supports the role of GD2 in promoting CSC characteristics and EMT. However, since our current assignment of a functional role to GD2 expression in PC cell models relied on the GD3S-KO, similar to the GD3S depletion approaches employed by others (11,44), it will be important to formally rule out a role of GD3, the other GD3S product and precursor to GD2 (2). Although the surface GD3 levels on the two cell models we targeted for GD3S-KO were relatively modest (**Supplementary Fig. S5**), further immunological targeting of GD2 and/or GD2S deletion should elucidate the functional role of GD2 vs. GD3 in PC tumorigenesis.

Collectively, our novel findings reveal that GD2 overexpression is a feature of a large subset of PCs, that GD2 may be further induced during transitions associated with PC progression, and that the GD2 biosynthetic machinery enzyme GD3S is essential for efficient tumorigenesis in CRPC models. Our findings raise the prospect of anti-GD2 targeting in advanced PC.

ACKNOWLEDGEMENTS

We thank Dr. Ian Frew for Multiple Lentiviral Expression System Kit and Dr. Irmela Jeremias for the pCDH-EF1a-eFFLy-mCherry (mCherry-luciferase reporter) plasmid, obtained through Addgene. This research was funded by: Pilot grants from the Fred & Pamela Buffett Cancer Center (HB & VB); Department of Defense grants W81XWH-17-1-0616 and W81XWH-20-1-0058 to HB and W81XWH-20-1-0546 to VB; the NIH grants R21CA241055 and R03CA253193 to VB; the NIH Pathway to Independence Award R00 GM1287671 and the NIH MIRA award R35 GM147467 (to M.J.R.); the Raphael Bonita Memorial Fund; and support to UNMC core facilities from the NCI Cancer Center Support Grant (P30CA036727) awarded to Fred & Pamela Buffett Cancer Center and from the Nebraska Research Initiative. AMB, SC and IM received the University of Nebraska Medical Center Graduate Student Fellowships. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH, Department of Defense, or other funding agencies.

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Figure Legends:

Fig. 1: **Induction of high GD2 expression in of GD2 in CRPC cell line models with neuroendocrine (NE) differentiation. (A)** Schematic of key GD2 biosynthetic steps displaying the enzymes (*ST3GAL3, ST8SIA1* and *B4GALNT1*) and intermediate products (GM3 and GD3) [Adapted from (55)]. C4-2 cells with stable knockdown (KD) of TP53 or RB1, previously shown to undergo NE differentiation, were compared with parental cells. **(B)** qPCR analysis for *TP53* and *RB1* mRNA expression shows TP53 KD or RB1 KD cells relative to C4-2 cells. Data represent mean ± SEM with two-tailed unpaired *t* test. n = 3; ***, $P < 0.001$. (C) Cell surface GD2 expression analyzed by live cell surface staining with anti-GD2 vs isotype control antibodies followed by flow cytometry. X-axis, fluorescence intensity; Y-axis, Events normalized to mode. FACS plots display % cells staining positive and mean fluorescence intensity (MFI) of staining with anti-GD2 (blue) and isotype control (red). (D) Immunohistochemistry (IHC) staining confirmation of increased GD2 expression on TP53 KD or RB1 KD C4-2 cells relative to parental cells. **(E)** qPCR analysis shows increased expression of *ST3GAL3, ST8SIA1* and *B4GALNT1 mRNAs* in TP53 KD *and* RB1 KD cells compared to parental C4-2 cell line. Data are mean ± SEM with two-tailed unpaired *t* test. n = 3; *, $P < 0.05$.

Fig. 2: GD2 expression on a subset of tumor cells in primary prostate cancer tissue with higher expression in metastatic lesions. Tissue microarrays (TMAs) composed of paired normal prostate and prostate cancer Gleason grades (3, 4 and 5) (n = 320) or 45 cases of bone and visceral metastatic samples (both obtained from the Prostate Cancer Biorepository Network) were analyzed by IHC staining for GD2. Histoscores were calculated by multiplying the staining intensity (0, negative; 1, low; 2, moderate positive; 3, strong positive) by the % tumor cells staining positive.

(**A, B**) Representative examples of negative, low, moderate, and high GD2 staining in **(A)** primary and **(B)** bone metastatic tumor samples. (**C)** Scattered plot shows histoscore distribution of primary tumors and normal prostate. GD2 expression is significantly higher in primary tumors than normal prostate. Data represents 34 cases of GD2 expression in tumors vs 24 in normal tissue, considering histoscore >25 as positive. Mann-Whitney unpaired t-test used as statistical analysis; *, $P < 0.05$. **(D)** Pie charts displaying the % of cases that were $GD2^+$ or $GD2^-$ in primary and metastatic tumors samples. Histoscores of 0-5 were considered negative while scores higher than 5 were considered positive. (**E, F**) Frequency distribution plots histoscores plotted against **(F)** percentage of patients and **(F)** number of patients. The indicated histoscore grouping are: 0-5 (negative); 5-25 (low), 25-50 (moderate); and >50 (strong).

Fig. 3: GD2 overexpression promotes the *in vitro* oncogenic traits: (**A**) Analysis of cell proliferation. The parental C4-2 and its TP53 KD or RB1 KD cells and C4-2B and its enzalutamide resistant variant C4-2BER were plated in 96-well plates and cell proliferation was assessed on the indicated days (Day 1 to Day 5) using the CellTiter-Glo luminescent cell viability assay. The Y-axis represents the fold increase in relative luminescence units (RLU) relative to Day 1. Data are mean ± SEM with analysis by multiple unpaired *t* test. n = 3 with six replicates each; **, $P < 0.01$; ***, $P < 0.001$. (**B**) Analysis of cell migration. 20,000 C4-2 parental, TP53 KD or RB1 KD and C4-2B and C4-2BER cells plated in low serum medium in top chambers of trans-well chambers were allowed to migrate for 16 hours towards serum-containing medium in the bottom chambers. Left, representative images. Right, quantification. Data represents mean ± SEM with unpaired t test. n = 3 with three replicates each; ***, $P < 0.001$. (**C**) Analysis of tumorsphere forming ability. The indicated cell lines were plated in 24-well ultra-low attachment plates in 4% Matrigel and

images were obtained on Day 7. Left, representative images; scale bar, 1000 μm. Right, quantified tumorsphere numbers per 4X microscopic field. Data represents mean ± SEM with unpaired t test. n = 3 with three replicates each and 6 images per well; ***, $P < 0.001$. (**D**) $GD2^{high}$ and $GD2^{low}$ population were FACS sorted in RM-1 and 22Rv1 cells and grown as 2D and tumorspheres. After Day 5, the cells were reanalyzed for GD2 fractions in both $GD2^{high}$ and $GD2^{low}$ post sorted cells. In both tumorspheres and 2D culture the $GD2^{low}$ cells re-expressed GD2. (**E**) Enrichment of $GD2^{high}$ cells in tumorspheres of CRPC cell lines. The indicated cell lines were cultured on plastic in regular two-dimensional (2D) cultures or were seeded in tumorsphere cultures. Cells harvested at the indicated times were analyzed for cell surface GD2 expression (vs. isotype control) at the indicated times. % $GD2^{+}$ cells and MFI are indicated. Left, representative FACS images; Right, quantitation of data. Mean ± SEM with two-way ANOVA test, **, $P < 0.01$; ***, $P < 0.001$. (**F**) $GD2^{high}$ fraction of CRPC cell lines is enriched for tumorsphere-forming ability. Constitutively GD2-expressing RM-1 and 22Rv1 CRPC cell lines were FACS sorted for $GD2^{high}$ and $GD2^{low}$ populations, grown on 2D overnight and plated at 20,000 cells per well of 24-well ultra-low attachment plates in tumorsphere media containing 4% Matrigel. Tumorspheres were imaged on Day 5. Left, representative images; scale bar, 2000 μm. Right, Quantification of tumorspheres (>250 μm and <250 μm) per 2X magnification field. Data represents mean ± SEM with unpaired t test. n = 3 with three replicates each and 6 images per well; ns, not significant; **, $P < 0.01$; ***, $P < 0.001$. (**G**) FACS analysis of tumorsphere-grown RM-1 and 22Rv1 cell lines after anti-GD2 and anti-CD49f double-staining shows a majority of $GD2^{+}$ cells to be $CD49f^{hi}$. % GD2 and/or CD49f +/- populations are shown in the respective quadrants.

Fig. 4: *GD3S* knockout in CRPC cell lines impairs the in vitro tumorigenic and pro-metastatic traits. (**A-D**) Generation of GD3S knockout of RM-1 and 22Rv1 CRPC cell lines. Cells were transduced with lentiviral All-in-One CRISPR/Cas9 constructs and stable clones analyzed by Western blotting with anti-GD3S antibody; Hsc70, loading control. Known $GD2^+$ (9464D) and $GD2^-$ (975A2) mouse neuroblastoma cell lines were used as controls. (**C-D**) Complete loss of cell surface GD2 expression in GD3S-KO RM-1 and 22Rv1 clones. The parental cell lines and their GD3S-KO clones were live-cell stained with anti-GD2 (blue) or isotype control (red) antibody followed by FACS analysis. FACS plots indicate mean fluorescence intensity (MFI) on X-axis vs. Events Normalized to Mode on Y-axis. % positive cells and MFIs are indicated in FACS plots. (**E-F**) Reduced CRPC cell proliferation upon GD3S-KO. Three clones of parental and GD3S-KO cell lines maintained separately were mixed plated at 1,000 cells per well in 96-well plates and live cells quantified at the indicated times using the CellTiter-Glo cell viability assay. The Y-axis represents the fold increase in relative luminescence units (RLU) relative to Day 1. Data are mean ± SEM with analysis by multiple unpaired t test. n = 3 with six replicates each; *$p<0.05$, **$p<0.01$. (**G-H**) Reduced CRPC cell migration (**G**) and invasion (**H**) upon GD3S-KO. 20,000 parental or GD3S-KO RM-1 or 22Rv1 cells were plated in low serum medium in top chambers of trans-well chambers without (migration) or with (invasion) Matrigel coating and allowed to migrate or invade for 16 hours towards serum-containing medium in the bottom chambers. Left, representative images. Right, quantification. Data represents mean ± SEM with unpaired *t* test. n = 3 with three replicates each and 6 images per well; ; ***$p<0.001$. (**I-J**) Reduced CRPC tumorsphere forming ability upon GD3S-KO. The indicated cell lines were plated in 24-well ultra-low attachment plates in 4% Matrigel and images were obtained on Day 7. Left, representative images; scale bar, 1000 μm. Right, quantified tumorsphere numbers per 4X

microscopic field. Data represents mean ± SEM with unpaired *t* test. n = 3 with three replicates each and 6 images per well; , unpaired *t-test* ; ***p<0.001.

Fig. 5: Impairment of cancer stem cell (CSC)-associated traits in CRPC cell lines upon GD3S-KO. (**A**) Reduced CRPC tumorsphere forming ability upon GD3S-KO. The indicated cell lines were plated in 24-well ultra-low attachment plates in 4% Matrigel and images were obtained on Day 7. Left, representative images; scale bar, 1000 µm. Right, quantified tumorsphere numbers per 4X microscopic field. Data represents mean ± SEM with unpaired t test. n = 3 with three replicates each and 6 images per well; unpaired *t-test* ; ***p<0.001. **(B)** Reduced expression of CSCs markers in GD3S-KO CRPC cell lines. The indicated cell lines were subjected to live cell staining followed by Left, representative FACS plots display CD49F staining (MFI's) on X-axis and forward scatter on Y-axis. Right, quantitation of % of CD49f-high population. Data represents mean ± SEM, unpaired *t test*; n=3; *p<0.05, **p<0.01. (**C**) Volcano plot of the mouse cancer stem cell RT^2 profiler quantitative PCR array analysis of RM-1 controls vs. GD3S-KO cells. X-axis, log fold change; Y-axis, -log p-value. Green dots represent the downregulated genes and red dots display the upregulated one with a log1.5-fold change cut-off. (**D**) The heatmap shows the significantly upregulated (red) and downregulated (green) genes in RM-1 GD3S KO cells compared to control cells.; n=3 independent experiments, p<0.05 is deemed significant. (**E**) Reduced expression of cancer stem cells and epithelial-mesenchymal transition (EMT) markers in GD3S-KO CRPC cell lines. Lysates of the parental vs. GD3S-KO RM-1 or 22Rv1 cell lines were subjected to western blotting with antibodies against the indicated proteins, with Hsc70 or β-actin used as loading controls. Densitometric quantification is shown on top of each blot.

Fig. 6: Impairment of in vivo bone-implanted CRPC tumorigenesis by GD3S KO. (**A**) Parental (Control) RM-1 cells or their edited versions carrying sgRNA1 or sgRNA2 (GD3S-KO) were engineered with tdTomato-luciferase and 4 x 10^4 cells injected in tibias of castrated male C57/BL6 mice (8/group) and primary tumor growth was monitored by bioluminescence imaging at the indicated time points in mice (5/group) with detectable bioluminescent signals on day 8. (**B**) Bioluminescence signals of RM-1 tumors over time are shown as log fold-change in photon flux over the time. Data are mean ± SEM, n=5; Two-way ANOVA (mixed model); *p<0.05. (**C**) Micro-CT scanned images of tibias isolated from mice implanted with the indicated RM-1 cell lines. µCT analyses of the trabecular bone was used to calculate the: (**D**) bone volume fraction (BV/TV%); (**E**) trabecular thickness (µm); (**F**) trabecular number (per µm); and (**G**) trabecular separation (µm). Data represent mean ± SEM, with unpaired t-test used for statistical analysis; *p<0.05 and ** p<0.01. (**H**) Impairment of tumorigenesis of 22Rv1 CRPC cells by GD3S-KO. 1 x 10^5 control or GD3S KO (sgRNA1) 22Rv1 cells engineered with mCherry-luciferase were injected in tibias of castrated male athymic nude mice (6/group) and tumor growth was monitored by bioluminescence imaging in mice (5/group) with detectable bioluminescent signals on day. (**I**) Bioluminescence signals of 22Rv1 xenografts over time are shown as log fold-change in photon flux over time. Data are mean ± SEM, n=5, with analysis by Two-way ANOVA (mixed model); **p<0.01.

Supplementary Figure Legends:

Fig. S1. Validation of anti-GD2 antibody. (A) FACS analysis of $GD2^-$ A-204 Rhabdomyosarcoma and $GD2^+$ SK-N-BE(2) human neuroblastoma cell lines stained with anti-GD2 vs. isotype control. (B) Immunohistochemistry (IHC) staining of A-204 and SK-N-BE(2) cell lines with anti-GD2 or isotype control. Scale bar, 50 µm. Insets show a higher magnification

of regions within each image. GD2 antibody selectively stains the SK-NB-2 but not the A-204 cell line.

Fig. S2: FACS plots of cell surface GD2 expression on human and mouse prostate cancer (PC) cell lines. The indicated human and mouse prostate cancer cell lines were live cell stained using anti-GD2 vs. isotype control and analyzed by FACS. GD2, blue; isotype control, red. Percentage % population and MFI are indicated in FACS plots. (**A**) Cell lines with essentially undetectable GD2 (all human). (**B**) $GD2^{low}$ PC cell lines (all human). (**C**) $GD2^{high}$ human (22Rv1 and C4-2BER) and mouse PC cell lines (RM-1 and Tramp-C1) are shown.

Fig. S3: High *ST8SIA1* (GD3S) mRNA expression in a substantial proportion of CRPC patient-derived xenograft (PDX) tumors and organoid models. Publicly available transcriptomic data published by Tang et al.(26) (GEO accession GSE199190) on CRPC PDX and organoid models were queried for *ST8SIA1* mRNA expression. The CRPC groups are as per Tang et al: Group 1, Androgen Receptor dependent (CRPC-AR, peach); Group 2, Wnt dependent (CRPC-WNT, green); Group 3, neuroendocrine (CRPC-NE, blue); and Group 4, stem cell like (CRPC-SCL, purple). Gene expression is shown as normalized transcripts per million (TPM). The horizontal red line in left panel demarcates the negative/positive cutoff based on $GD2^{-}$ LNCaP cells.

Fig. S4: Enrichment of $GD2^{high}$ cells in tumorspheres of CRPC cell lines:

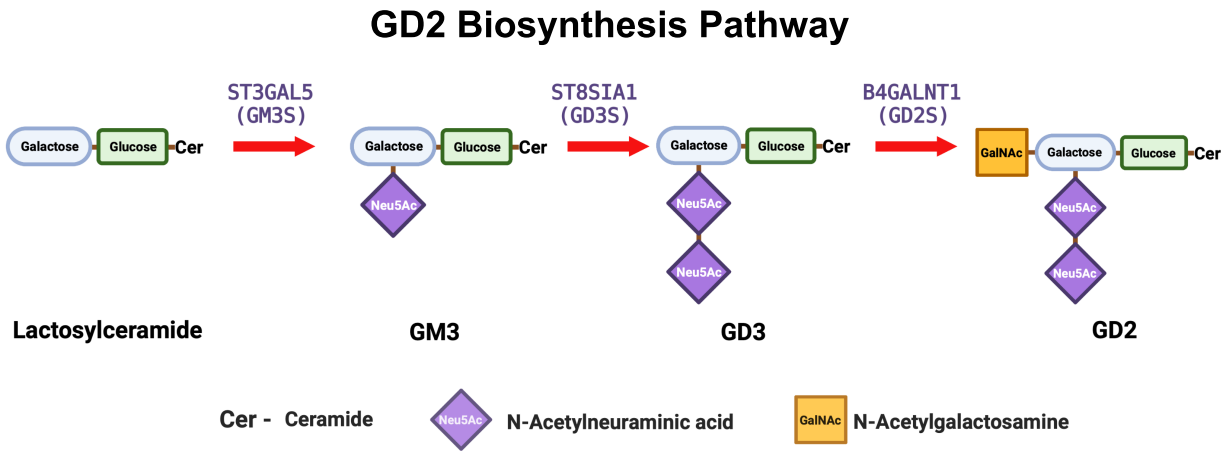
Enrichment of $GD2^{high}$ cells in tumorspheres of CRPC cell lines. The C4-2 parental and RB1 KD cells were cultured in regular two-dimensional (2D) cultures or were seeded in tumorsphere

cultures. Cells harvested at the indicated times were analyzed for cell surface GD2 expression (vs. isotype control) at the indicated times. % $GD2^+$ cells and MFI are indicated. Left, representative FACS images; Right, quantitation of data. Mean ± SEM with two-way ANOVA test, **, $P < 0.01$; ***, $P < 0.001$.

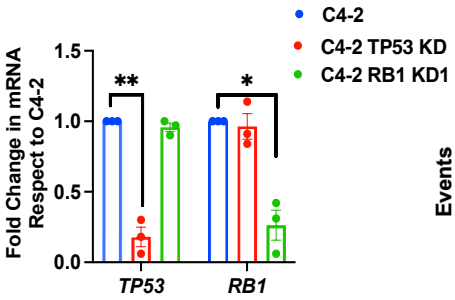
Fig. S5: GD3 expression in GD3S knockout RM-1 cells. Live cell FACS analysis of murine RM-1 and its GD3S KO variants (sgRNA1, sgRNA2 & sgRNA3) and human 22Rv1 and its GD3S KO variants (sgRNA2 & sgRNA3) show decreased expression of GD3 synthesis in the GD3S KO variants and is displayed as over lay FACS plots. The percentage of GD3 and MFI are indicated in FACS plots. The names of the cell lines and antibodies are mentioned on the right side of the FACS plots.

Fig. 1

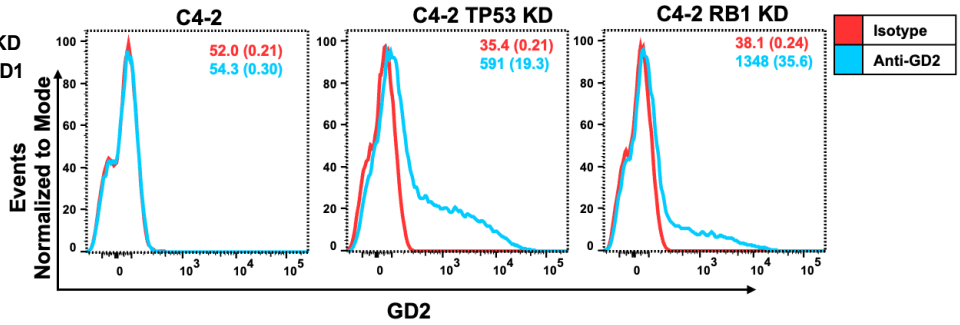
A


GD2 Biosynthesis Pathway
ST3GAL5 (GM3S)
ST8SIA1 (GD3S)
B4GALNT1 (GD2S)
Galactose
Glucose
Cer
Neu5Ac
GalNAc
Lactosylceramide
GM3
GD3
GD2
Cer - Ceramide
N-Acetylneuraminic acid
N-Acetylgalactosamine

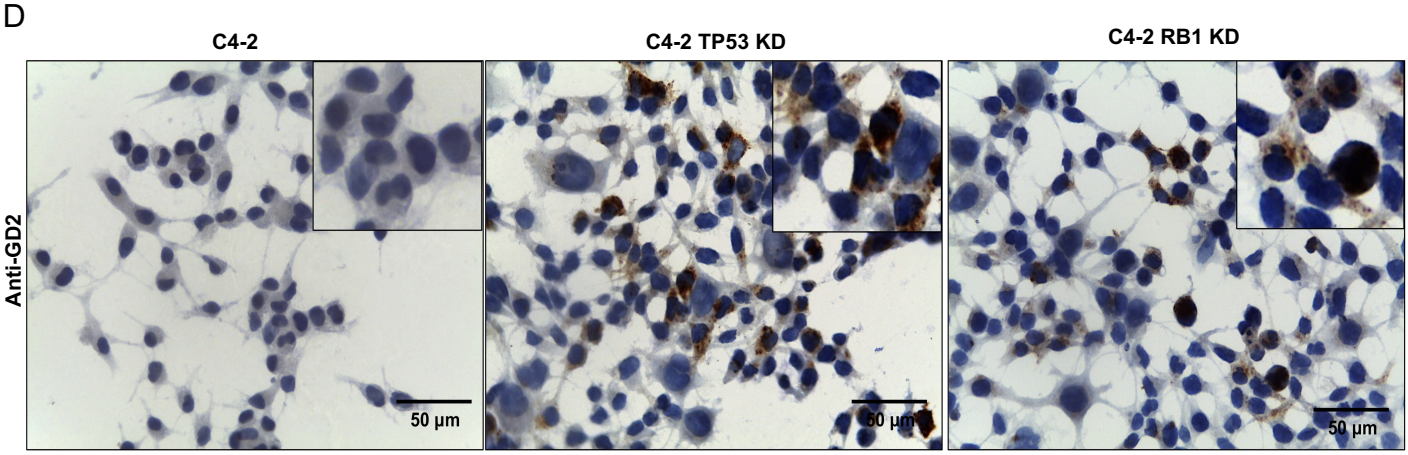
B


C4-2
C4-2 TP53 KD
C4-2 RB1 KD1
Fold Change in mRNA Respect to C4-2
1.5
1.0
0.5
0.0
**
*
TP53
RB1

C


C4-2
C4-2 TP53 KD
C4-2 RB1 KD
52.0 (0.21)
54.3 (0.30)
35.4 (0.21)
591 (19.3)
38.1 (0.24)
1348 (35.6)
Isotype
Anti-GD2
Events Normalized to Mode
GD2

D


C4-2
C4-2 TP53 KD
C4-2 RB1 KD
Anti-GD2
50 µm
50 µm
50 µm

E

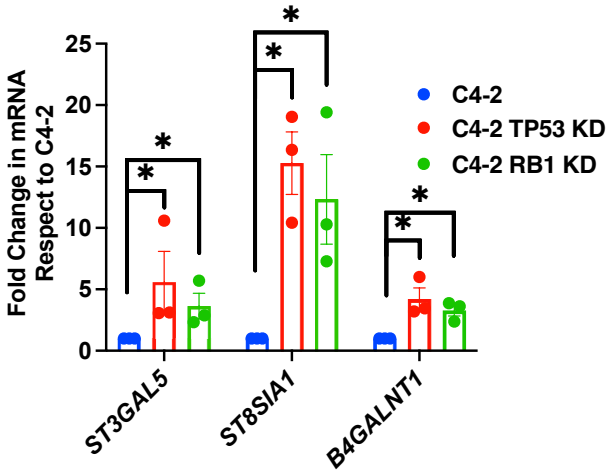

C4-2
C4-2 TP53 KD
C4-2 RB1 KD
Fold Change in mRNA Respect to C4-2
25
20
15
10
5
0
*
ST3GAL5
ST8SIA1
B4GALNT1

Fig. 2

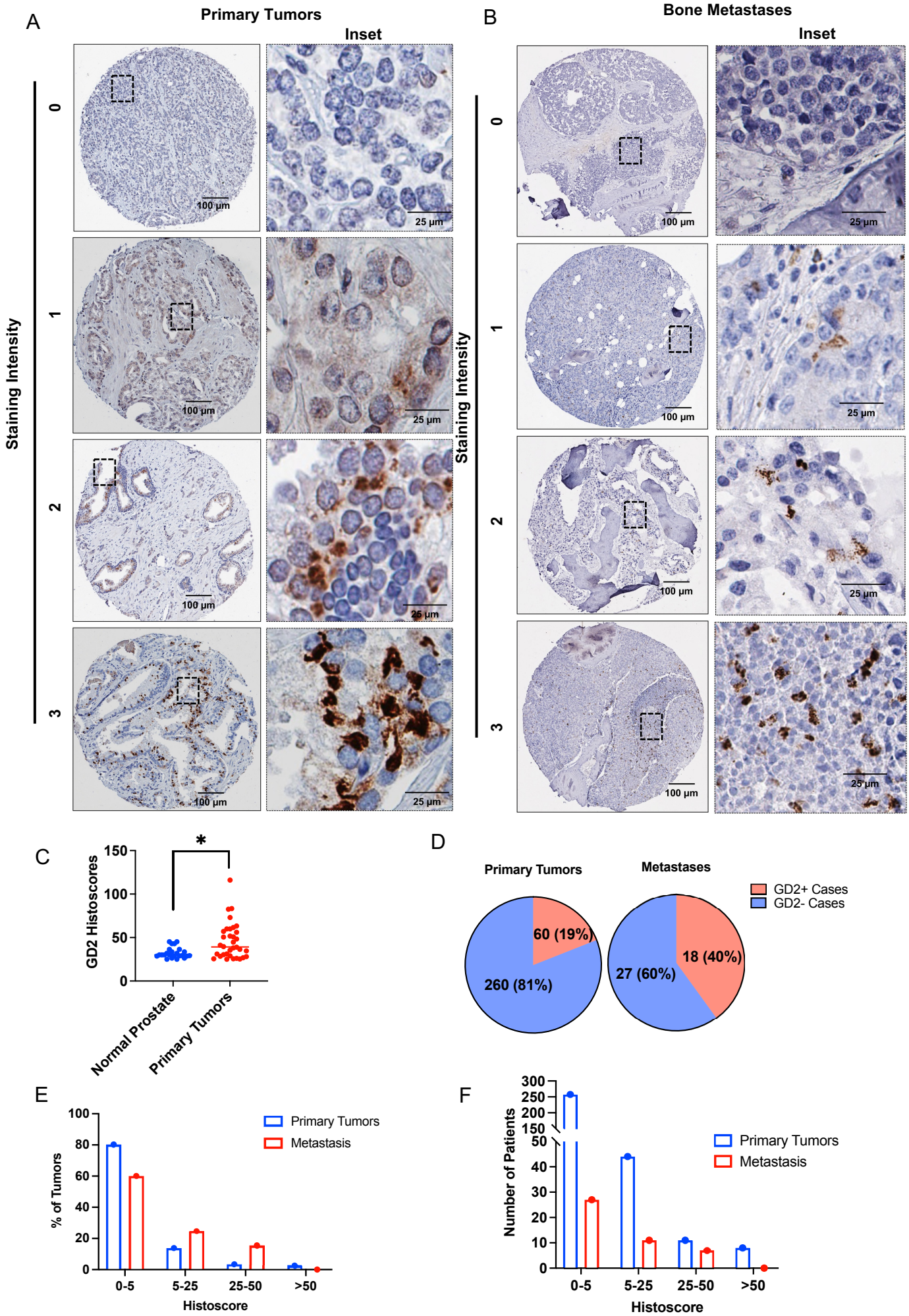

A
Primary Tumors
Inset
Staining Intensity
0
1
2
3
100 µm
25 µm
B
Bone Metastases
Inset
Staining Intensity
0
1
2
3
100 µm
25 µm
C
GD2 Histoscores
150
100
50
0
*
Normal Prostate
Primary Tumors
D
Primary Tumors
Metastases
GD2+ Cases
GD2- Cases
60 (19%)
260 (81%)
18 (40%)
27 (60%)
E
% of Tumors
100
80
60
40
20
0
Primary Tumors
Metastasis
0-5
5-25
25-50
>50
Histoscore
F
Number of Patients
300
250
200
150
50
40
30
20
10
0
Primary Tumors
Metastasis
0-5
5-25
25-50
>50
Histoscore

Fig. 3

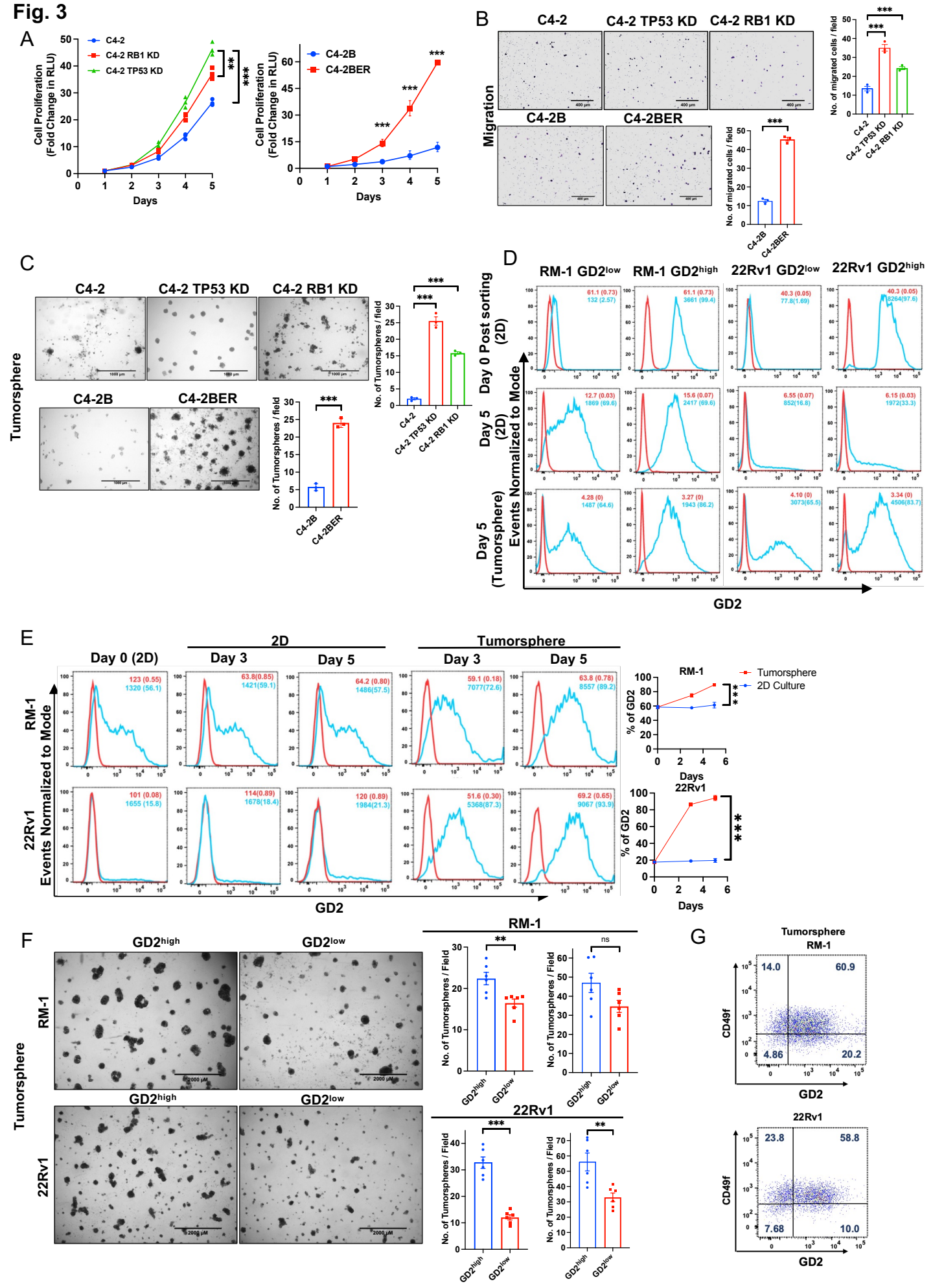

A
C4-2
C4-2 RB1 KD
C4-2 TP53 KD
Cell Proliferation (Fold Change in RLU)
Days
C4-2B
C4-2BER
B
Migration
C4-2
C4-2 TP53 KD
C4-2 RB1 KD
C4-2B
C4-2BER
No. of migrated cells / field
C
Tumorsphere
No. of Tumorspheres / field
D
RM-1 GD2low
RM-1 GD2high
22Rv1 GD2low
22Rv1 GD2high
Day 0 Post sorting (2D)
Day 5 (2D)
Day 5 (Tumorsphere)
Events Normalized to Mode
GD2
E
Day 0 (2D)
2D
Tumorsphere
Day 3
Day 5
RM-1
22Rv1
% of GD2
Days
Tumorsphere
2D Culture
F
GD2high
GD2low
No. of Tumorspheres / Field
ns
G
Tumorsphere
CD49f
GD2

Fig. 4

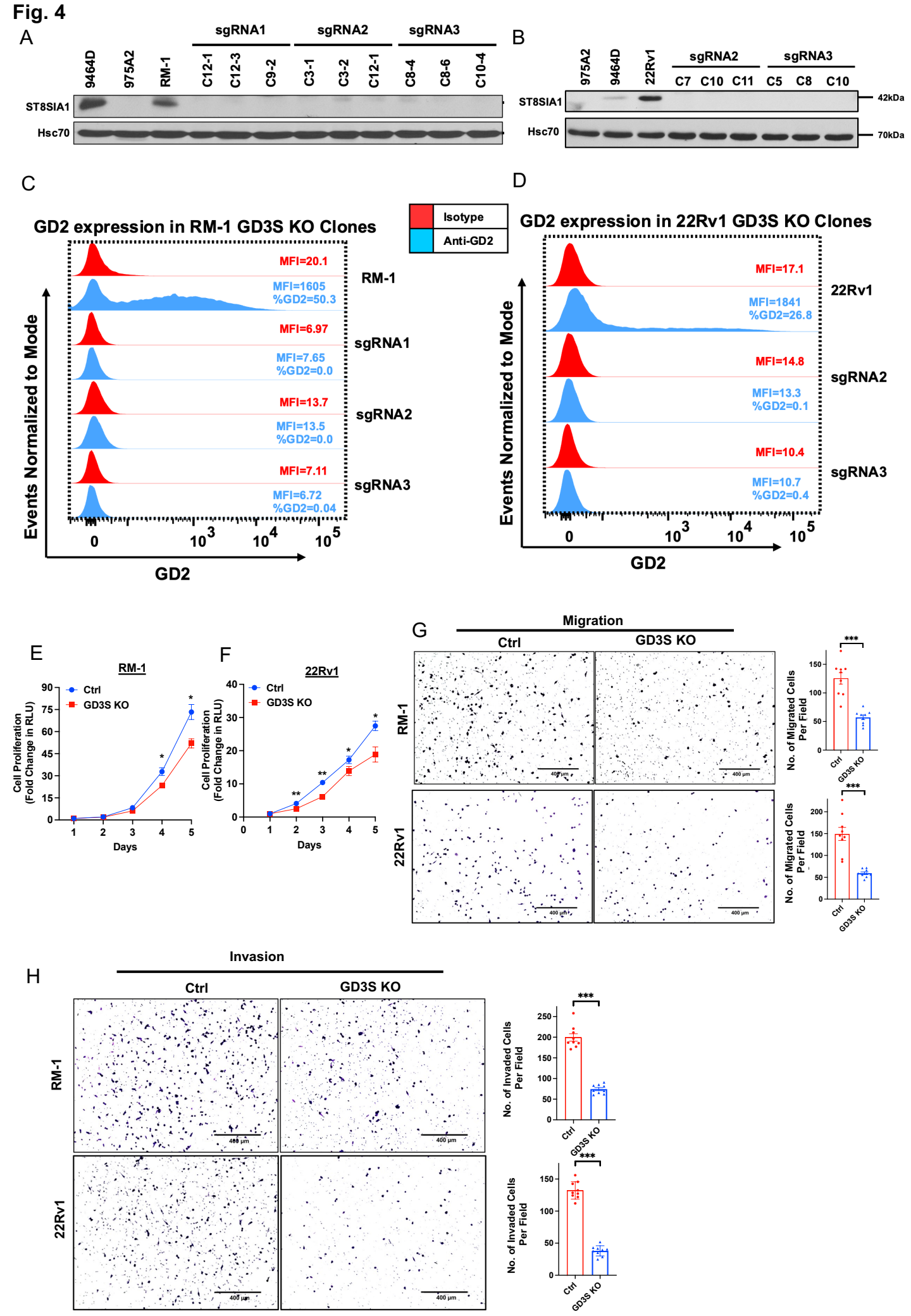

A
sgRNA1
sgRNA2
sgRNA3
9464D
975A2
RM-1
C12-1
C12-3
C9-2
C3-1
C3-2
C12-1
C8-4
C8-6
C10-4
ST8SIA1
Hsc70
B
975A2
9464D
22Rv1
sgRNA2
sgRNA3
C7
C10
C11
C5
C8
C10
ST8SIA1
Hsc70
42kDa
70kDa
C
GD2 expression in RM-1 GD3S KO Clones
Isotype
Anti-GD2
MFI=20.1
MFI=1605
%GD2=50.3
RM-1
MFI=6.97
MFI=7.65
%GD2=0.0
sgRNA1
MFI=13.7
MFI=13.5
%GD2=0.0
sgRNA2
MFI=7.11
MFI=6.72
%GD2=0.04
sgRNA3
Events Normalized to Mode
GD2
D
GD2 expression in 22Rv1 GD3S KO Clones
MFI=17.1
MFI=1841
%GD2=26.8
22Rv1
MFI=14.8
MFI=13.3
%GD2=0.1
sgRNA2
MFI=10.4
MFI=10.7
%GD2=0.4
sgRNA3
Events Normalized to Mode
GD2
E
RM-1
Ctrl
GD3S KO
Cell Proliferation (Fold Change in RLU)
Days
F
22Rv1
Ctrl
GD3S KO
Cell Proliferation (Fold Change in RLU)
Days
G
Migration
Ctrl
GD3S KO
RM-1
22Rv1
No. of Migrated Cells Per Field
Ctrl
GD3S KO
H
Invasion
Ctrl
GD3S KO
RM-1
22Rv1
No. of Invaded Cells Per Field
Ctrl
GD3S KO

Fig. 5

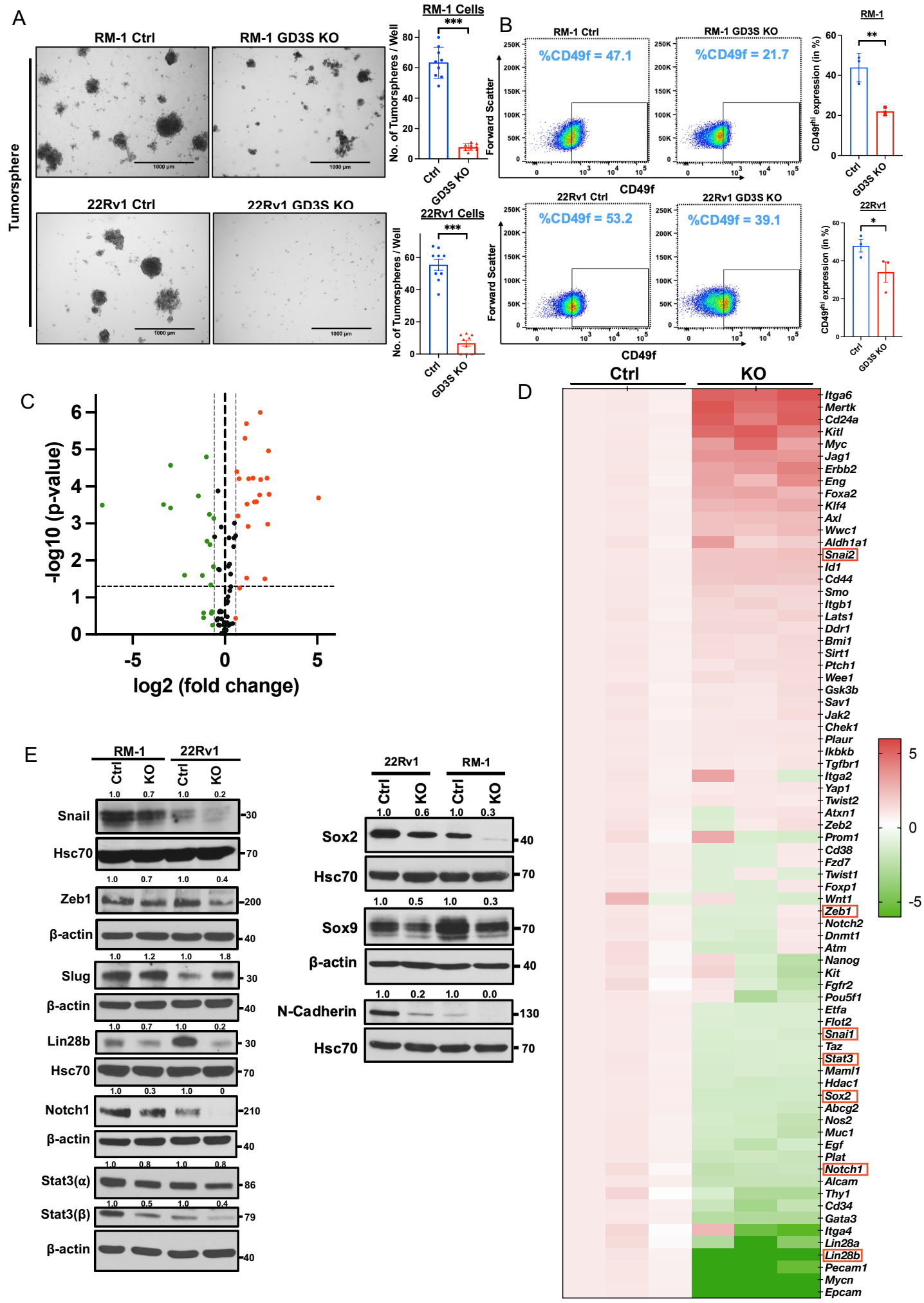

A
RM-1 Ctrl
RM-1 GD3S KO
Tumorsphere
22Rv1 Ctrl
22Rv1 GD3S KO
1000 µm
RM-1 Cells
22Rv1 Cells
No. of Tumorspheres / Well
***
Ctrl
GD3S KO
B
%CD49f = 47.1
%CD49f = 21.7
%CD49f = 53.2
%CD49f = 39.1
Forward Scatter
CD49f
CD49f^hi expression (in %)
**
*
C
-log10 (p-value)
log2 (fold change)
D
Ctrl
KO
Itga6
Mertk
Cd24a
Kitl
Myc
Jag1
Erbb2
Eng
Foxa2
Klf4
Axl
Wwc1
Aldh1a1
Snai2
Id1
Cd44
Smo
Itgb1
Lats1
Ddr1
Bmi1
Sirt1
Ptch1
Wee1
Gsk3b
Sav1
Jak2
Chek1
Plaur
Ikbkb
Tgfbr1
Itga2
Yap1
Twist2
Atxn1
Zeb2
Prom1
Cd38
Fzd7
Twist1
Foxp1
Wnt1
Zeb1
Notch2
Dnmt1
Atm
Nanog
Kit
Fgfr2
Pou5f1
Etfa
Flot2
Snai1
Taz
Stat3
Maml1
Hdac1
Sox2
Abcg2
Nos2
Muc1
Egf
Plat
Notch1
Alcam
Thy1
Cd34
Gata3
Itga4
Lin28a
Lin28b
Pecam1
Mycn
Epcam
E
RM-1
22Rv1
Ctrl
KO
Snail
Hsc70
Zeb1
β-actin
Slug
Lin28b
Notch1
Stat3(α)
Stat3(β)
Sox2
Sox9
N-Cadherin

Fig. 6

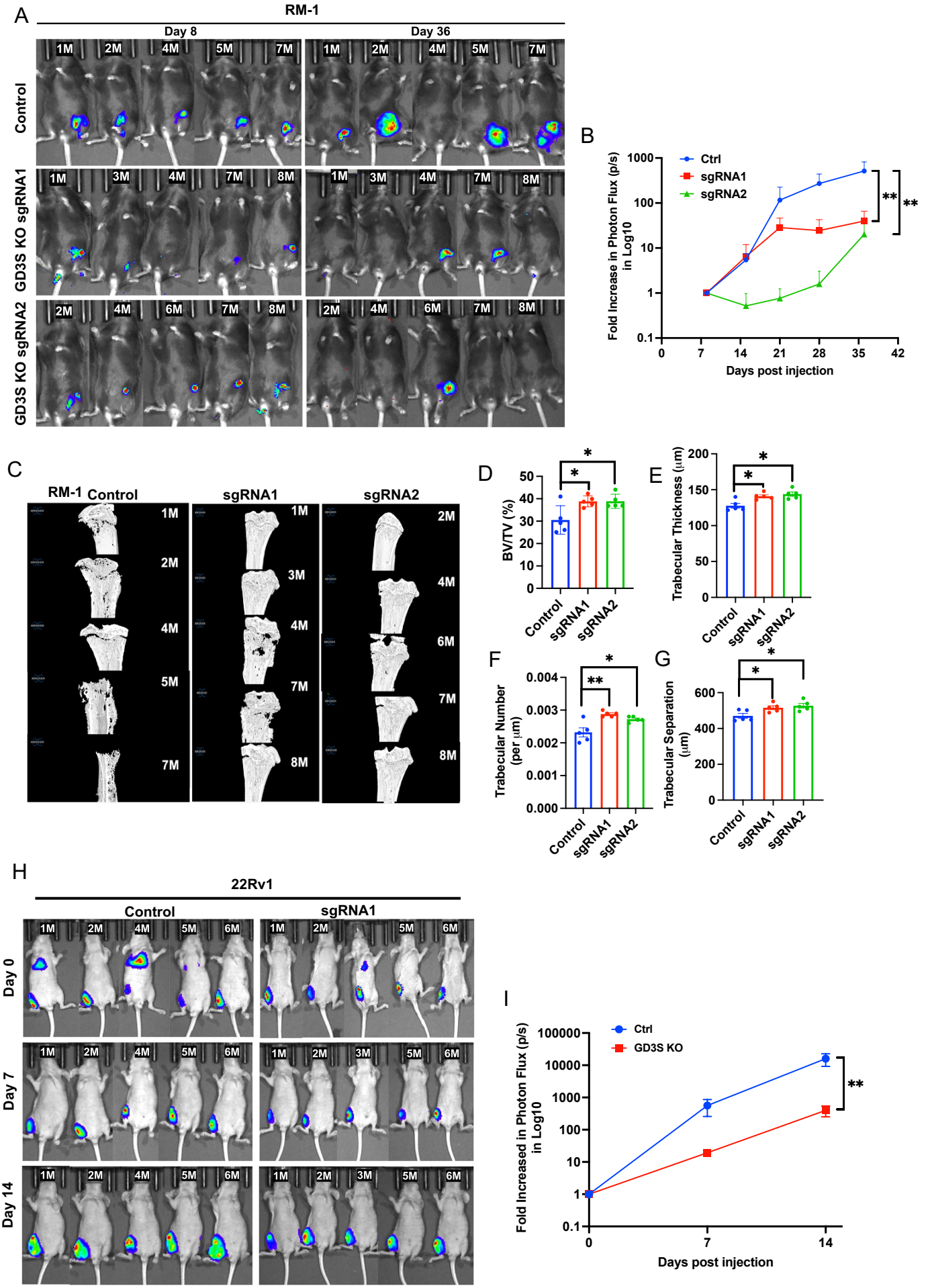

A
RM-1
Day 8
Day 36
Control
GD3S KO sgRNA1
GD3S KO sgRNA2
B
Ctrl
sgRNA1
sgRNA2
Fold Increase in Photon Flux (p/s) in Log10
Days post injection
C
RM-1 Control
sgRNA1
sgRNA2
D
BV/TV (%)
E
Trabecular Thickness (μm)
F
Trabecular Number (per μm)
G
Trabecular Separation (μm)
Control
sgRNA1
sgRNA2
H
22Rv1
Control
sgRNA1
Day 0
Day 7
Day 14
I
Ctrl
GD3S KO
Fold Increased in Photon Flux (p/s) in Log10
Days post injection

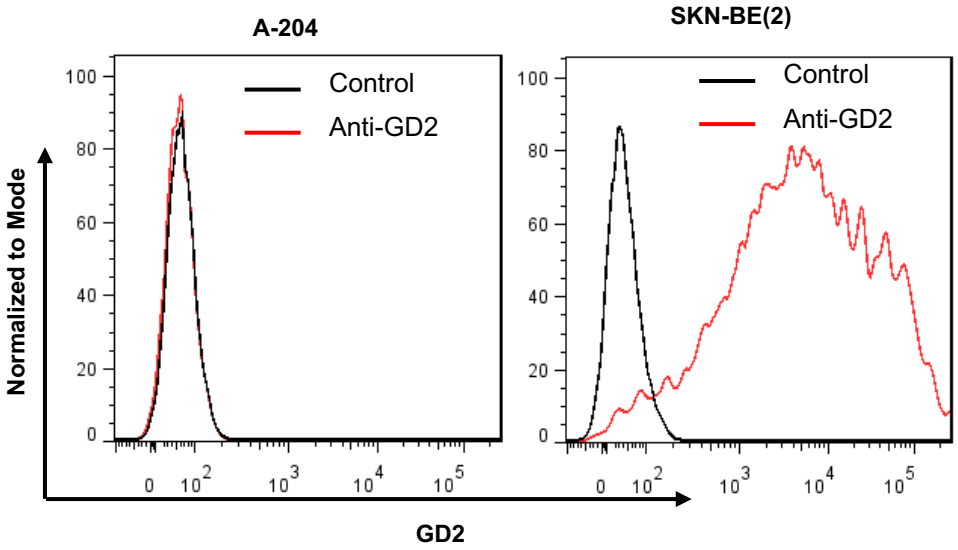
Table-1: Prostate cancer cell lines show % GD2 on live cell FACS analysis using anti-GD2 staining.

Cell Line Names	% GD2
PC3	0
PC3M	0
PC3M LN4	0
LNCaP (Low Passage)	0
MDA PCa2b (Low Passage)	0
NE1.3	0
DU145	0
ND1	~0 - 2
LNCaP C4-2	~0 - 2
LNCaP C4-2B	~0 - 2
PHPV18	~2 - 3
PSV40	~4 - 6
22Rv1	~15 - 30
LNCaP C4-2 RB1 KD	~10 - 20
LNCaP C4-2 p53 KD	~10 - 35
LNCaP C4-2BER (Enzalutamide Resistant)	~25 - 40
TRAMP-C1	~40 - 60
RM-1	~45 - 70

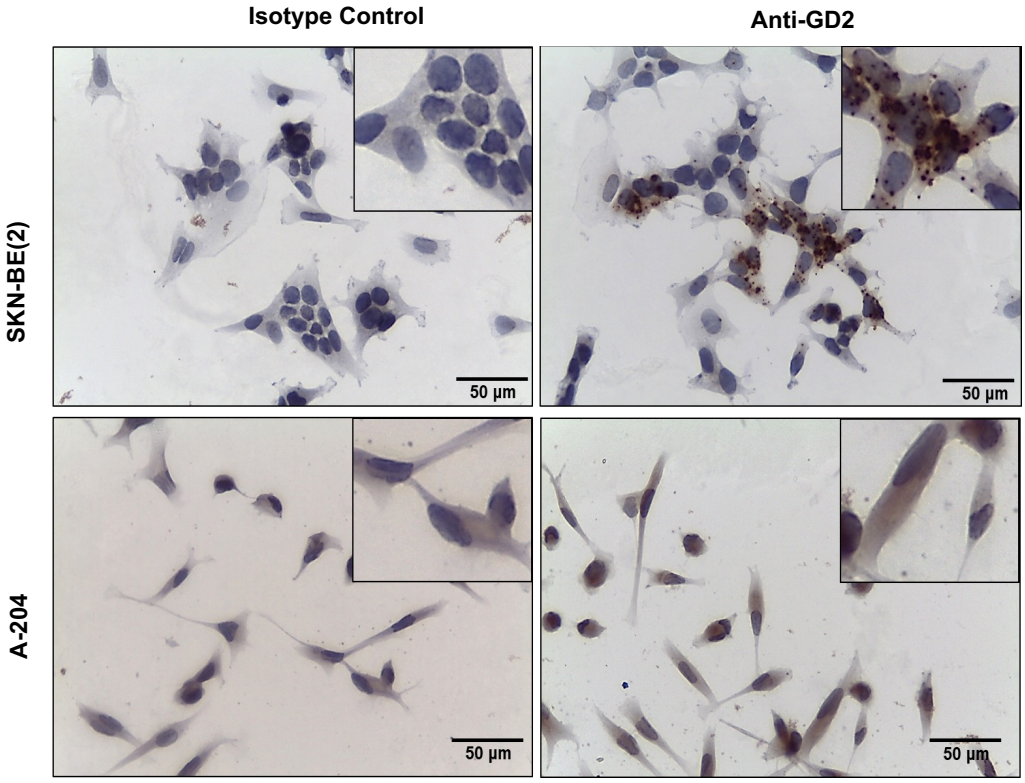
Supplementary Figures

Supplementary Fig. S1.

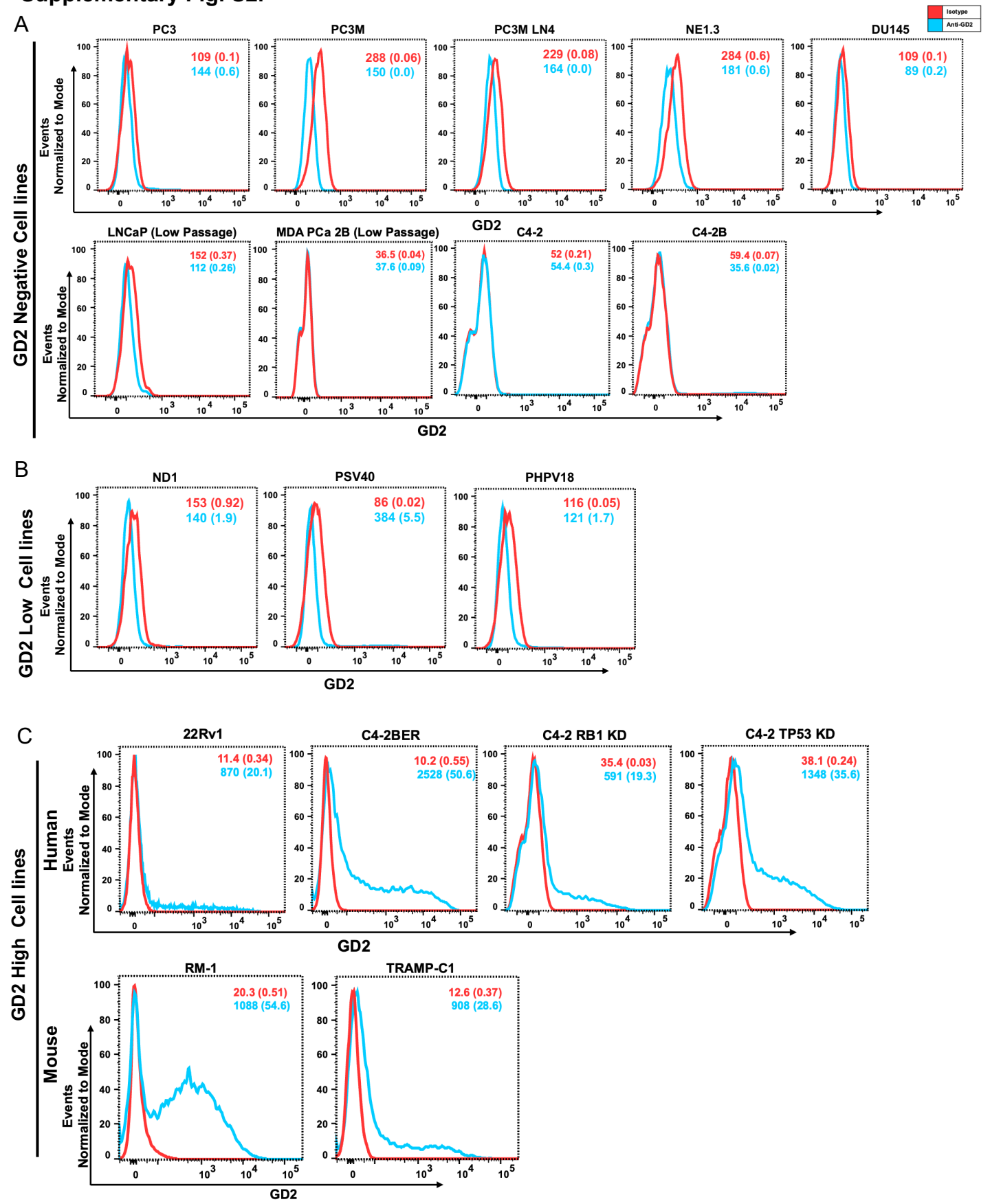
A.



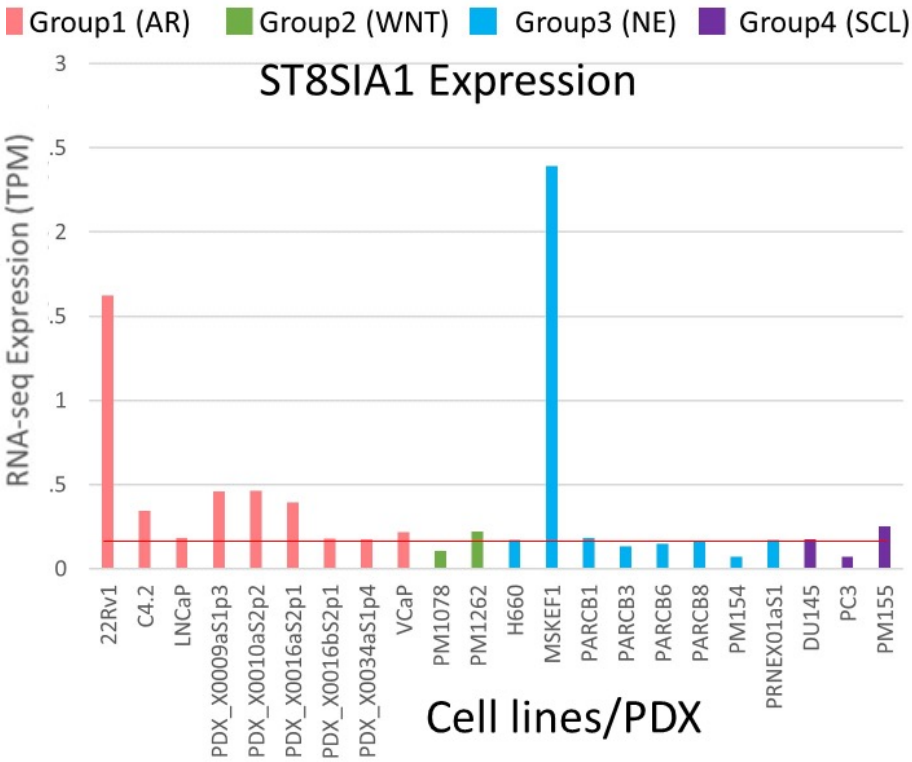
B.

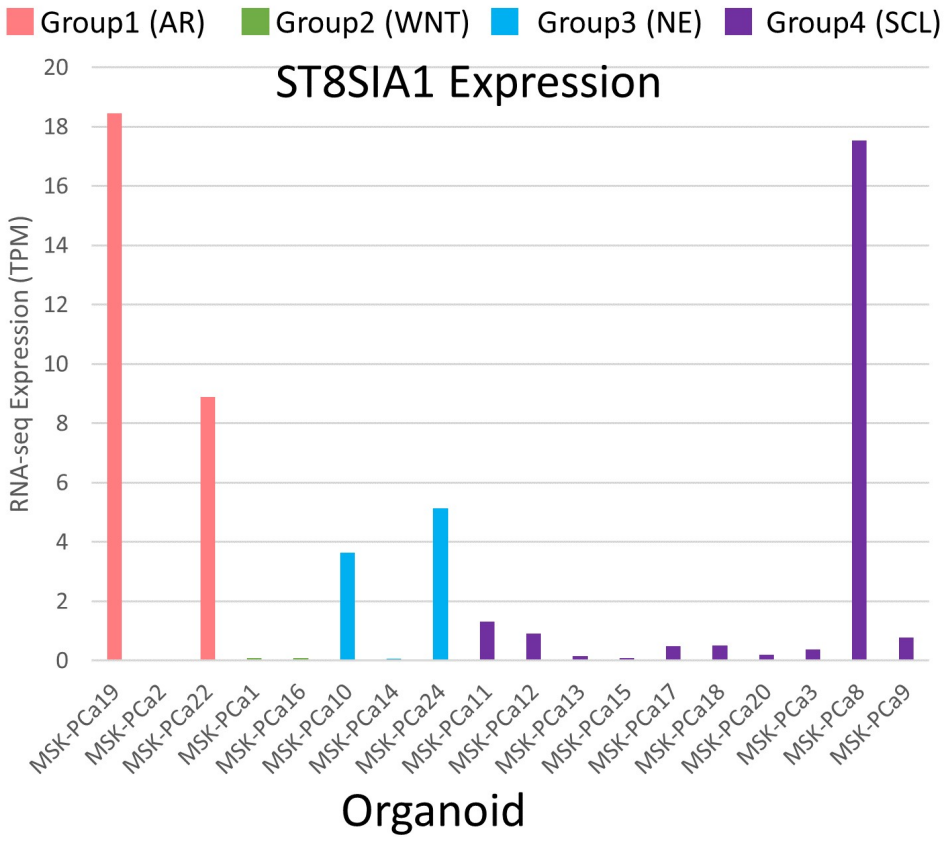


Supplementary Fig. S2.

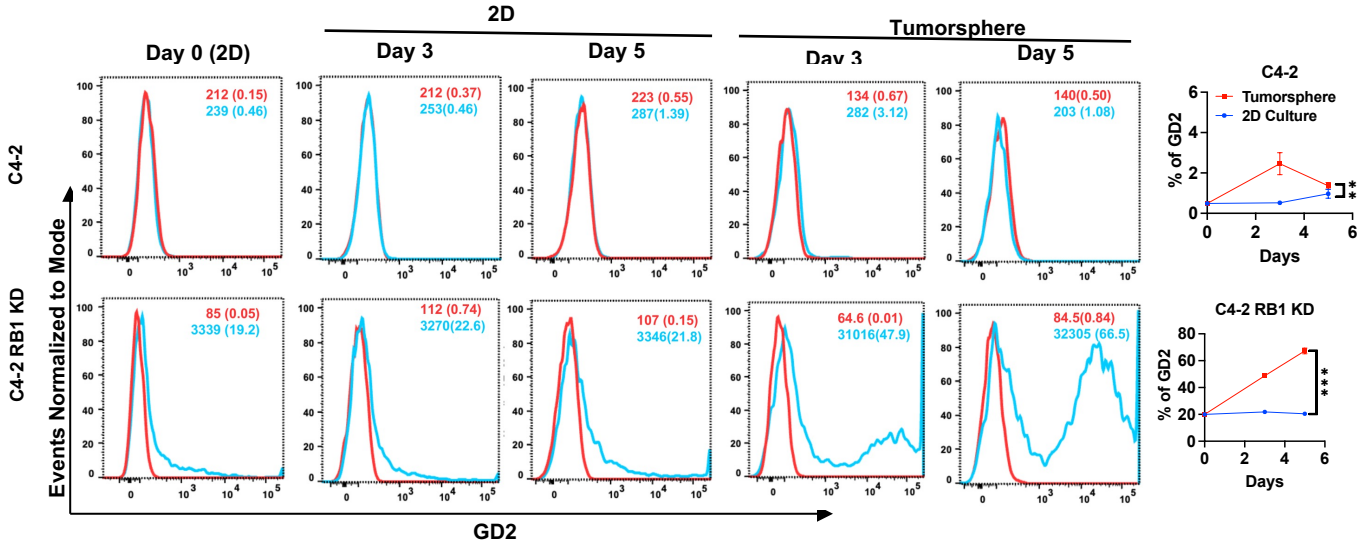
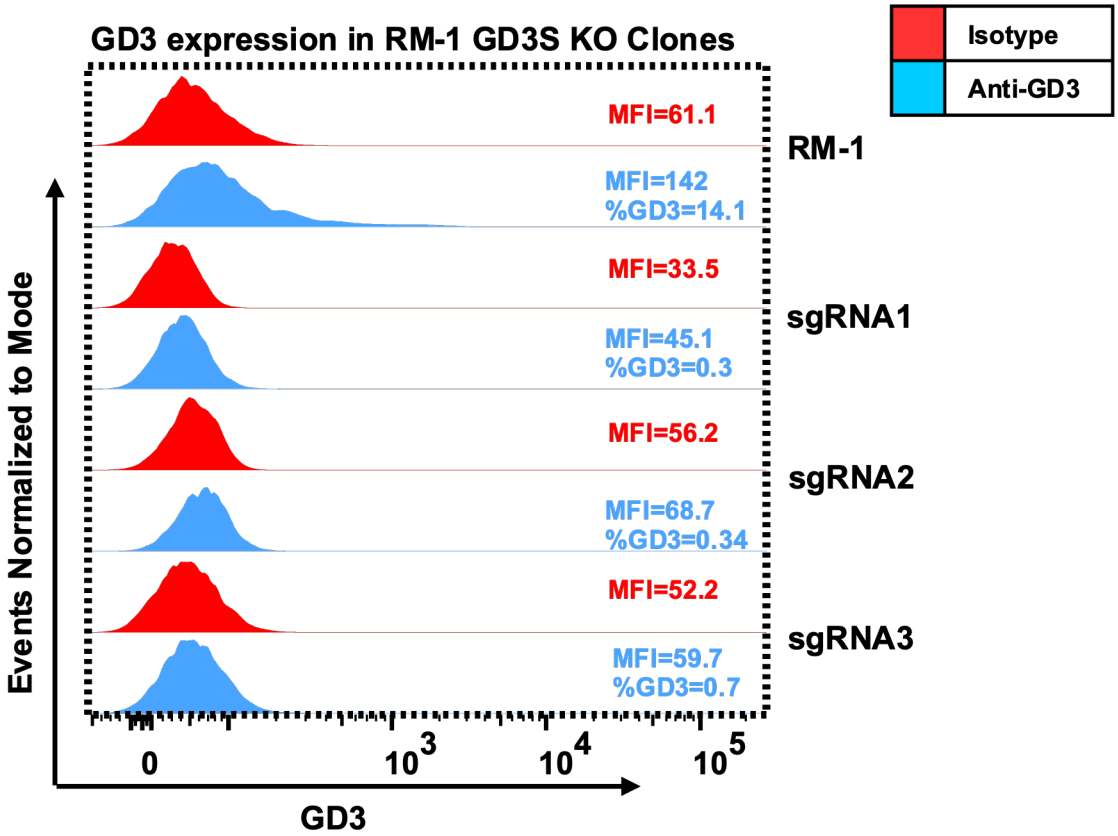
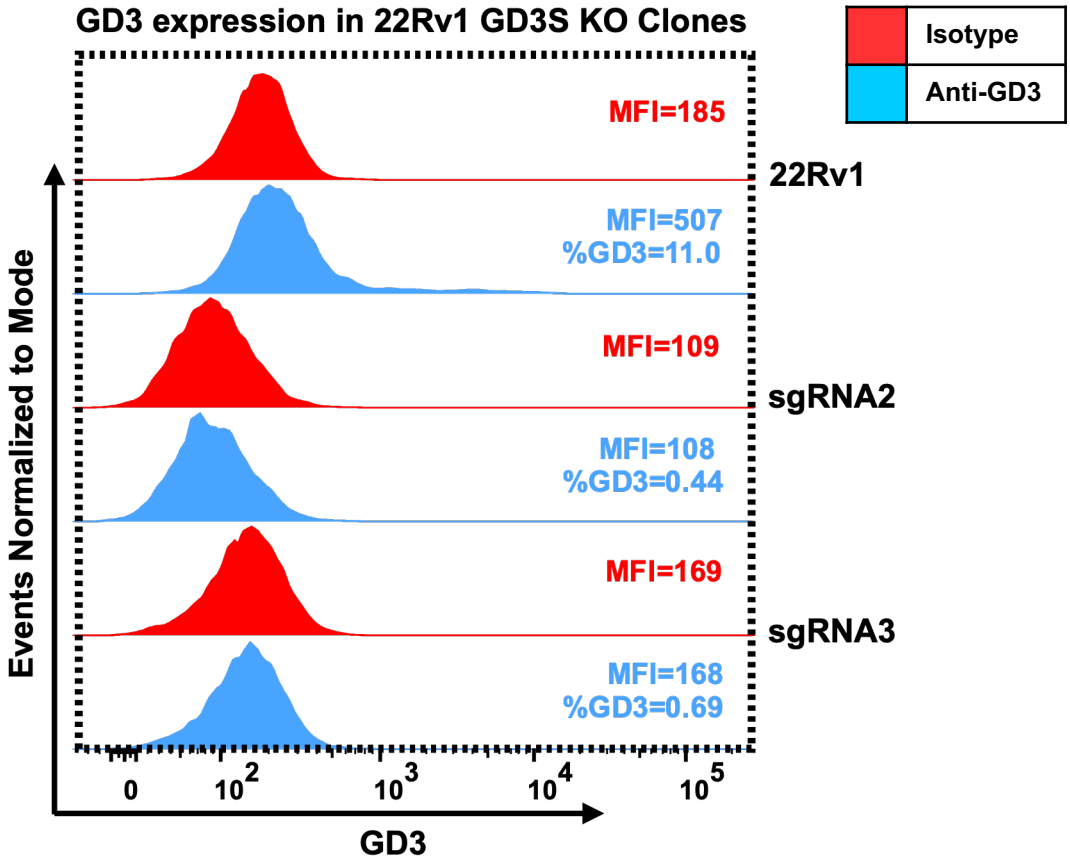


Supplementary Fig. S3.





Supplementary Fig. S4



Supplementary Fig. S5