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Supplemental Information

Targeting Human Cancer by a Glycosaminoglycan Binding Malaria Protein

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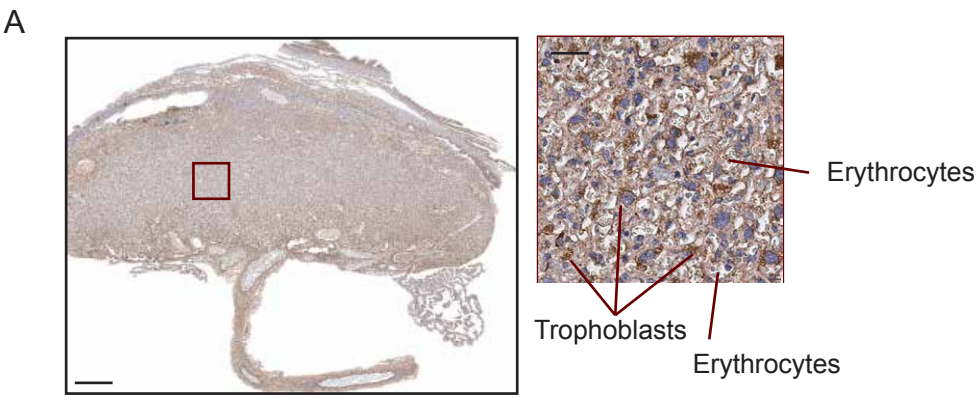
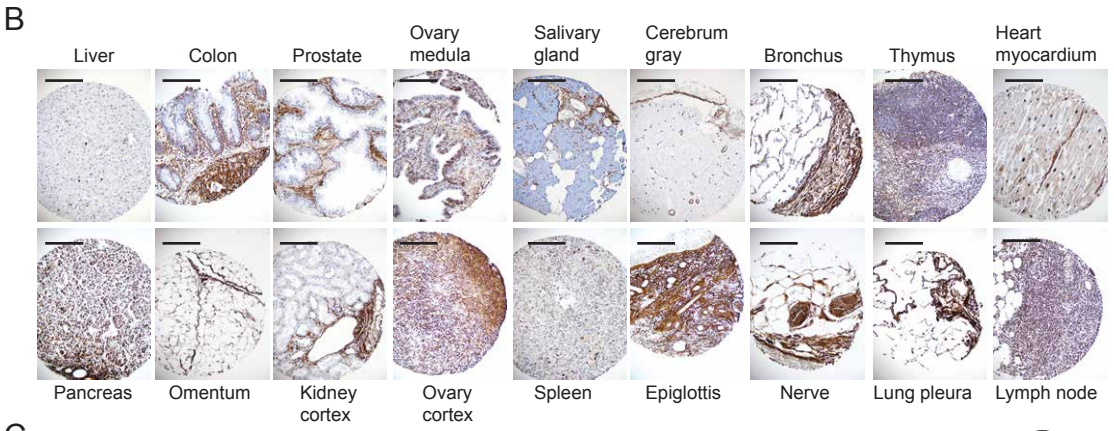
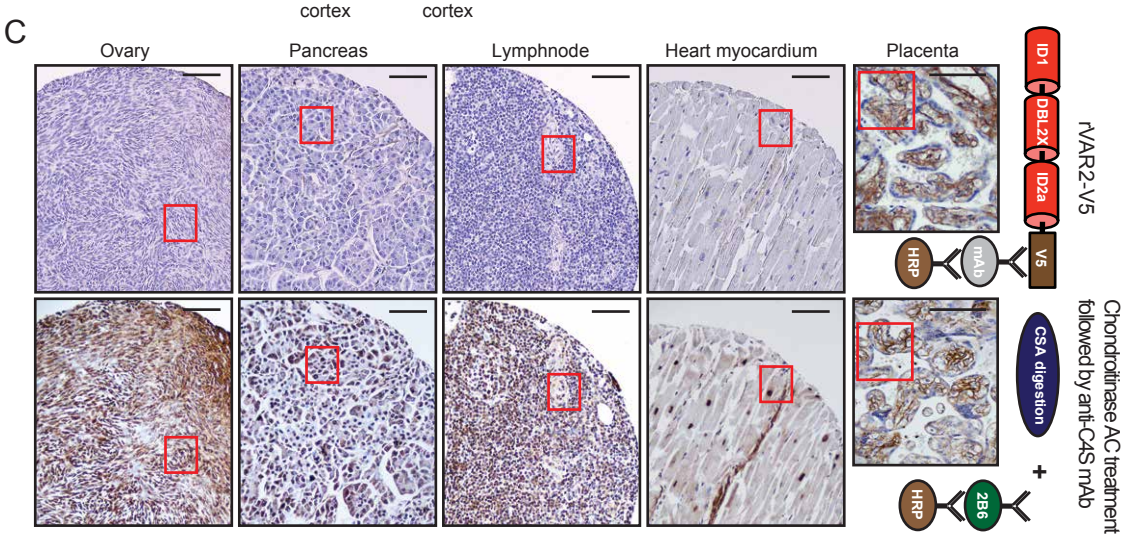
SUPPLEMENTAL DATA







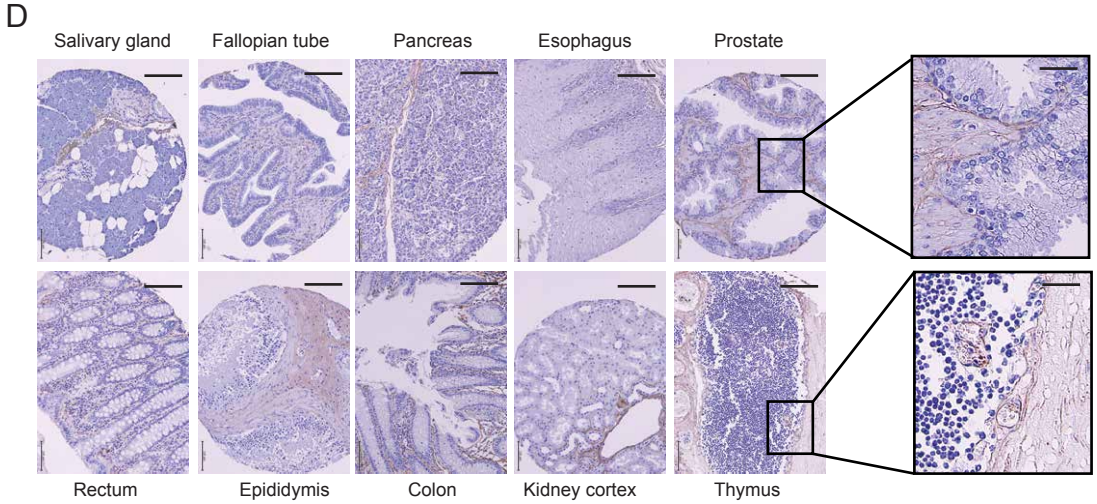


Figure S1, related to Figure 1

(**A**) 4x (left - scale bar 1 mm) and 40x (right - scale bar 150 µm) magnified images of murine placenta incubated with 500 picomolar rVAR2 and 1:700 anti-V5-HRP. Red box represents magnifications of the indicated area.
(**B**) Representative 10x (scale bar 300 µm) magnified images of indicated normal tissue cores incubated with 1 U/ml Chondroitinase AC followed by 1:20 dilution of anti-C4S (2B6) antibody.
(**C**) Representative images of indicated normal tissue specimens stained with 500 picomolar recombinant VAR2CSA and 1:700 anti-V5-HRP (upper panel) or as describe in **C** (lower panel) (Scale bar 200 µm). Red boxes represents areas presented in **Figure 1E**.
(**D**) Representative 10x (Scale bar 250 µm) magnified images of indicated normal tissue specimens incubated with 500 picomolar rVAR2 and 1:700 anti-V5-HRP. Black boxes represent magnifications of the indicated areas (Scale bar 100 µm).

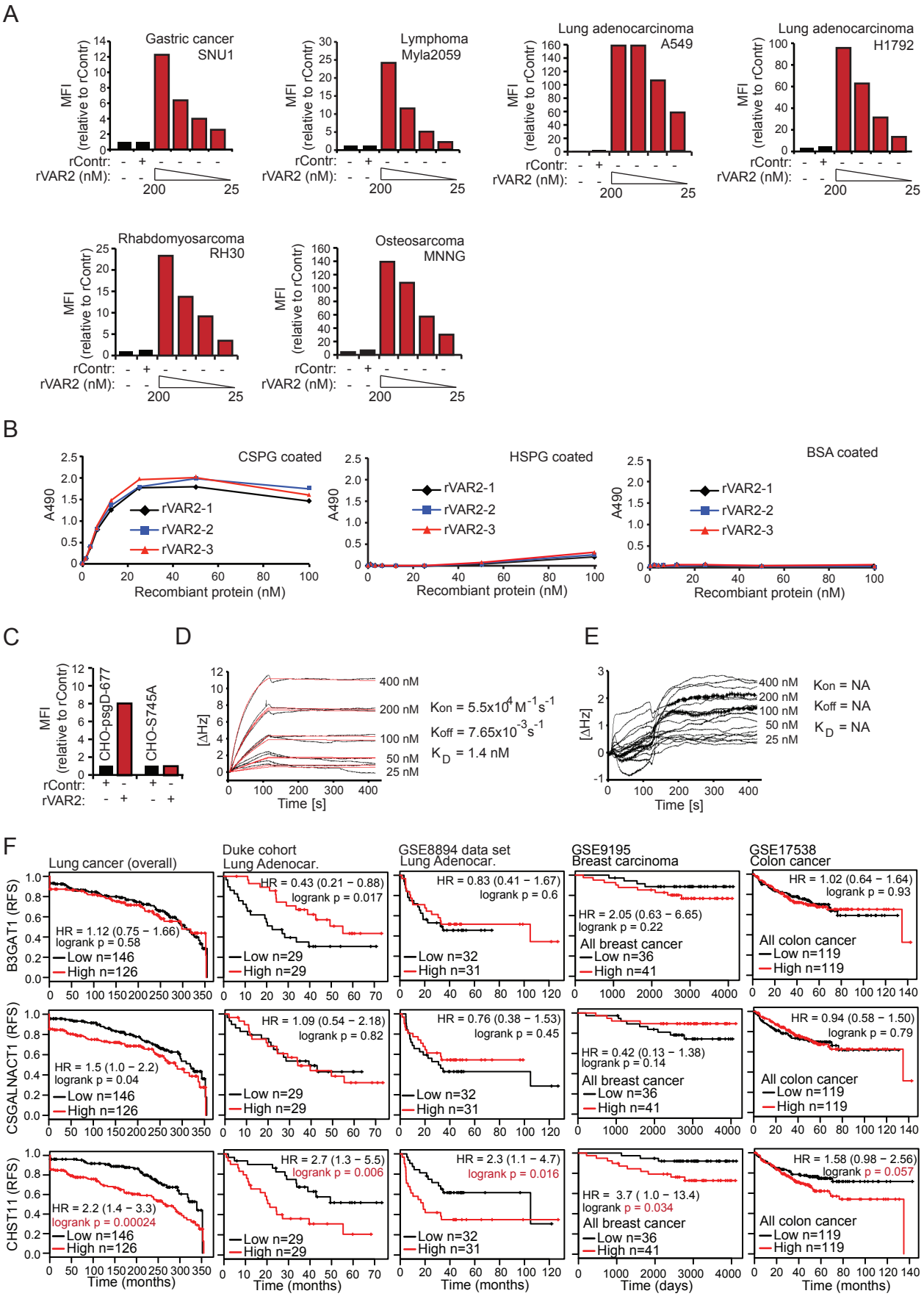

A
Gastric cancer
SNU1
Lymphoma
Myla2059
Lung adenocarcinoma
A549
Lung adenocarcinoma
H1792
Rhabdomyosarcoma
RH30
Osteosarcoma
MNNG
MFI (relative to rContr)
rContr:
rVAR2 (nM):
200
25
B
CSPG coated
HSPG coated
BSA coated
A490
rVAR2-1
rVAR2-2
rVAR2-3
Recombiant protein (nM)
C
CHO-psgD-677
CHO-S745A
rVAR2:
D
[ΔHz]
400 nM
200 nM
100 nM
50 nM
25 nM
Kon = 5.5x10^4 M^-1s^-1
Koff = 7.65x10^-3s^-1
K_D = 1.4 nM
Time [s]
E
Kon = NA
Koff = NA
K_D = NA
F
Lung cancer (overall)
Duke cohort
Lung Adenocar.
GSE8894 data set
Lung Adenocar.
GSE9195
Breast carcinoma
GSE17538
Colon cancer
B3GAT1 (RFS)
CSGALNACT1 (RFS)
CHST11 (RFS)
HR = 1.12 (0.75 − 1.66)
logrank p = 0.58
Low n=146
High n=126
HR = 0.43 (0.21 − 0.88)
logrank p = 0.017
Low n=29
High n=29
HR = 0.83 (0.41 − 1.67)
logrank p = 0.6
Low n=32
High n=31
HR = 2.05 (0.63 − 6.65)
logrank p = 0.22
All breast cancer
Low n=36
High n=41
HR = 1.02 (0.64 − 1.64)
logrank p = 0.93
All colon cancer
Low n=119
High n=119
HR = 1.5 (1.0 − 2.2)
logrank p = 0.04
HR = 1.09 (0.54 − 2.18)
logrank p = 0.82
HR = 0.76 (0.38 − 1.53)
logrank p = 0.45
HR = 0.42 (0.13 − 1.38)
logrank p = 0.14
HR = 0.94 (0.58 − 1.50)
logrank p = 0.79
HR = 2.2 (1.4 − 3.3)
logrank p = 0.00024
HR = 2.7 (1.3 − 5.5)
logrank p = 0.006
HR = 2.3 (1.1 − 4.7)
logrank p = 0.016
HR = 3.7 (1.0 − 13.4)
logrank p = 0.034
HR = 1.58 (0.98 − 2.56)
logrank p = 0.057
Time (months)
Time (days)

Figure S2, related to Figure 2

(**A**) Relative mean fluorescence intensity (MFI) of the denoted cell lines incubated with recombinant control protein (rContr) or VAR2CSA (rVAR2) as indicated and detected by flow cytometry using anti-V5-FITC.
(B) Enzyme-linked immunosorbent assay (ELISA) showing concentration dependent rVAR2 binding of three independently synthesized protein batches (rVAR2-1,2,3) to immobilized CSPG (left), HSPG (middle), and BSA (right). (**C**) Relative mean fluorescence intensity (MFI) of Immortalized CHO-psgD-677 and CHO-S745A cells incubated with recombinant control protein (rContr) or VAR2CSA (rVAR2) as indicated and detected by flow cytometry using anti-V5-FITC.
(**D-E**) Sensorgram showing binding between recombinant VAR2CSA and immobilized CHO-psgD-677 (D) and CHO-S745A (E) cells measured in delta Hertz [ΔHz] as a function of time (in seconds) using the indicated concentrations of recombinant protein. Black lines represent data, and red lines represent fitted curves attained by a 1:1 binding model. Black box summarizes K_{on}, K_{off}, and the calculated K_D value.
(F) Kaplan-Meier plots of the expression of key CS enzymes CHST11, B3GAT1 and CSGALNACT1 linked to overall survival or recurrence free survival (RFS) in indicated datasets of overall lung cancer, the Duke and GSE8894 cohort of lung adenocarcinoma, the GSE9195 breast cancer cohort and the GSE17538 colon cancer cohort. High and low expression was defined as using the median value of the selected gene as separation point. Hazard ratio (HR) was calculated using Cox proportional hazards regression, and p value was calculated using the logrank test.

Table S1, Related to Figure 2 [1]

Cancer of epithelial lineage		
A549	Lung adenocarcinoma	++
H1792	Lung adenocarcinoma	+++
BeWo	Placenta choriocarcinoma	++
C32	Melanoma	++
LCC6	Melanoma	++
Colo 205	Colorectal adenocarcinoma	+++
ES-2	Ovarian clear cell carcinoma	++
LNCap	Prostate adenocarcinoma	++
PC-3	Prostate adenocarcinoma	+++
MDA-MB-231	Breast adenocarcinoma	++
T47D	Breast ductal carcinoma	+
SNU-1	Gastric carcinoma	++
T24	Bladder transitional cell carcinoma	++
UM-UC-6	Bladder transitional cell carcinoma	+++
Cancer of mesenchymal lineage		
CW9019	Rhabdomyosarcoma	+
RH30	Rhabdomyosarcoma	++
MG63	Osteosarcoma	++
MNNG	Osteosarcoma	+++

U2os	Osteosarcoma	++
TC32	Ewing sarcoma	+
TC71	Ewing sarcoma	++
Cancer of hematopoietic lineage		
FARAGE	Diffuse Large B-cell lymphoma	0
SU-DHL-8	Diffuse Large B-cell lymphoma	+
KG-1	Acute Myelogenous Leukemia	+++
MOLP-2	Multiple Myeloma	++
MyLa 2059	Cutaneous T cell lymphoma	++
NALM-6	B cell precursor acute lymphoblastic leukemia	++
NU-DHL-1	B cell lymhphoma	++

[1] Patient-derived cancer cell lines bound by rVAR2 sorted according to cancer type. 0: no binding; +: low binding; ++: medium binding; +++: high binding. In total, 111 cancer cell lines were tested in flow cytometry of which 106 (95%) cells were positive for VAR2 binding.

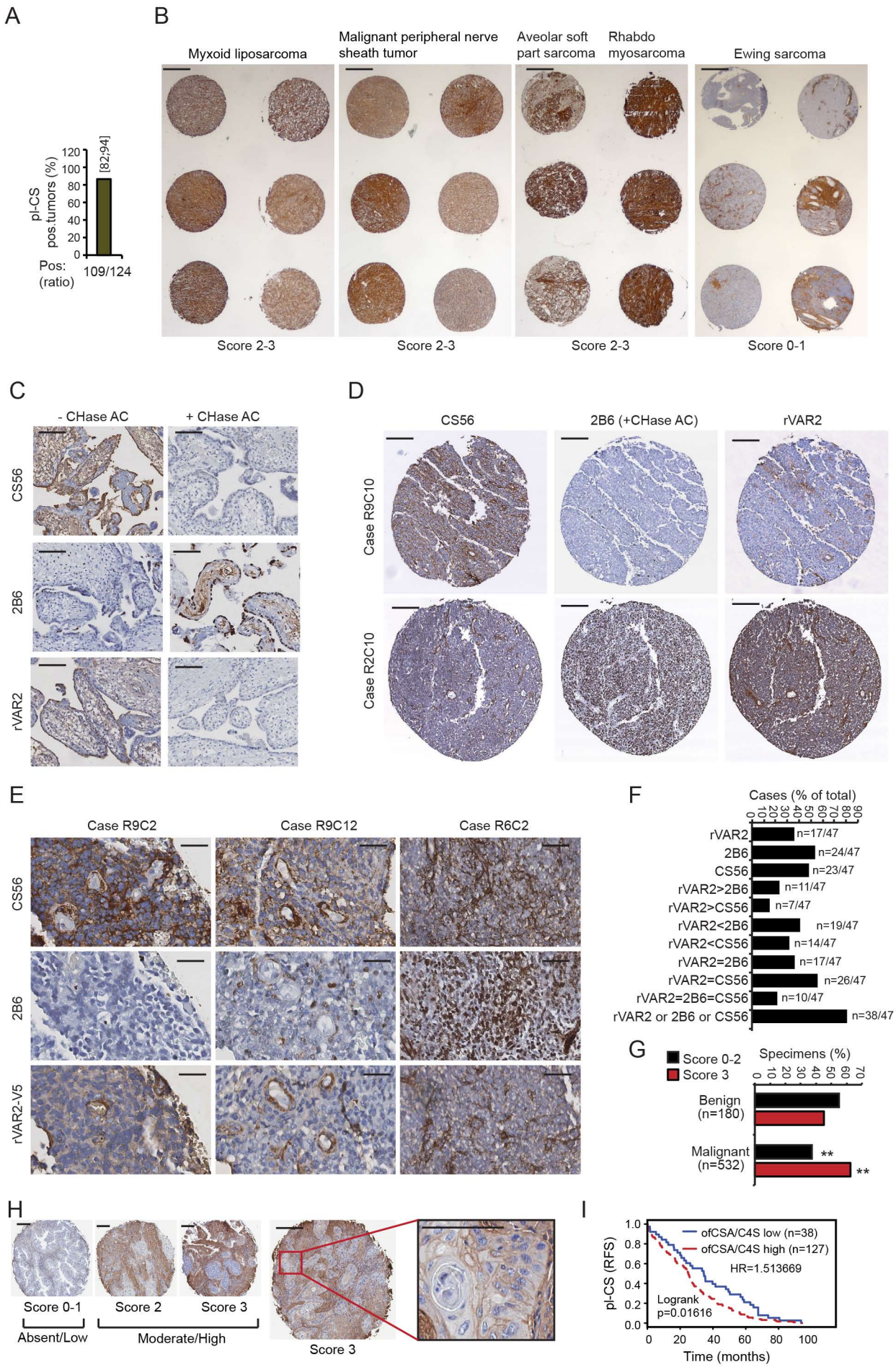

A
pl-CS pos.tumors (%)
120
100
80
60
40
20
0
[82;94]
Pos: (ratio)
109/124
B
Myxoid liposarcoma
Malignant peripheral nerve sheath tumor
Aveolar soft part sarcoma
Rhabdo myosarcoma
Ewing sarcoma
Score 2-3
Score 2-3
Score 2-3
Score 0-1
C
- CHase AC
+ CHase AC
CS56
2B6
rVAR2
D
CS56
2B6 (+CHase AC)
rVAR2
Case R9C10
Case R2C10
E
Case R9C2
Case R9C12
Case R6C2
CS56
2B6
rVAR2-V5
F
Cases (% of total)
0 10 20 30 40 50 60 70 80 90
rVAR2 n=17/47
2B6 n=24/47
CS56 n=23/47
rVAR2>2B6 n=11/47
rVAR2>CS56 n=7/47
rVAR2<2B6 n=19/47
rVAR2<CS56 n=14/47
rVAR2=2B6 n=17/47
rVAR2=CS56 n=26/47
rVAR2=2B6=CS56 n=10/47
rVAR2 or 2B6 or CS56 n=38/47
G
Score 0-2
Score 3
Specimens (%)
0 10 20 30 40 50 60 70
Benign (n=180)
Malignant (n=532)
**
**
H
Score 0-1
Score 2
Score 3
Absent/Low
Moderate/High
Score 3
I
pl-CS (RFS)
1.0
0.8
0.6
0.4
0.2
0.0
ofCSA/C4S low (n=38)
ofCSA/C4S high (n=127)
HR=1.513669
Logrank p=0.01616
0 20 40 60 80 100
Time (months)

Figure S3, related to Figure 3

(**A**) Column graph representation of placental-like CS (pl-CS) staining intensity of mixed breast cancer subtypes (n=124) scored (0-3) for binding to recombinant VAR2CSA. Column shows percentage and exact binomial 95% confidence interval of placental-like CS positive (score 2-3) tumors.
(**B**) Representative 2x (Scale bar 500 μm) magnified overview images of the soft tissue mesenchymal tumor tissue microarray (Fig. 3G) incubated with recombinant VAR2CSA (rVAR2-V5) and anti-V5-HRP and scored on a 0-3 scale for plasma membrane staining intensity where score 2 equals that of placenta.
(**C**) Representative images of human placenta specimens stained for CS expression with CS56 antibody, 2B6 antibody, and rVAR2 protein (Scale bar 100 μm). When indicated, specimens were pre-treated with chondroitinase AC to depolymerize the C4S and C6S chains down to 1 single CS unit (stub), which if C4S creates an epitope for the 2B6 antibody while removing binding of the CS56 and rVAR2 (control for binding specificity to CS).
(**D**) Representative images (4x magnification - scale bar 125 μm) of 2 (out of 47) tissue micro arrayed cases of mixed adulthood and pediatric Ewing's sarcoma treated and stained as in **C**.
(**E**) Three cases of Ewing sarcoma tissue specimens stained with CS56, 2B6 or rVAR2 as in **C** (Scale bar 150 μm).
(**F**) Ewing's sarcoma TMA (n=47) stained with the 3 reagents (CS56, 2B6, and rVAR2) where analyzed for relative staining intensity as follows **i**) rVAR2 (score 2-3) vs. 2B6 (score 0-1) (rVAR2>2B6); **ii**) rVAR2 (score 2-3) vs. CS56 (score 0-1) (rVAR2>CS56); **iii**) rVAR2 (score 0-1) vs. 2B6 (score 2-3) (rVAR2<2B6); **iv**) rVAR2 (score 0-1) vs. CS56 (score 2-3) (rVAR2<CS56); **v**) rVAR2 and 2B6 same score (rVAR2=2B6); **vi**) rVAR2 and CS56 same score (rVAR2=CS56); rVAR2 and 2B6 and CS56 same score (rVAR2=2B6=CS56); **vii**) at least one of the reagents with a score 2-3 (rVAR2 or 2B6 or CS56).
(**G**) Column graph representation of the distribution in staining intensity between benign vs. malignant soft-tissue lesions. **:p<0.0001.
(**H**) Representative images of a (n=165) non-small cell lung cancer TMA stained with recombinant VAR2CSA (rVAR2). Red box marks the amplified area of a score 3 squamous cell carcinoma. (Scale bar 250 μm).
(**I**) Kaplan-Meier plot of non-small cell lung tumor TMA stained for placental-like CSA showing recurrence free survival (RFS) of patients with low (score 0-1, n=38) or high (score 2-3, n=127) expressing tumors. Hazard ratio (HR) was calculated using Cox proportional hazards regression, and p value was calculated using the logrank test.

Table S2, related to Figure 3

Melanoma	Score 0	Score 1	Score 2	Score 3
Benign	14/49 (28.6%)	17/49 (34.7%)	17/49 (34.7%)	1/49 (2.0%)
Clark 1	0/7 (0.0%)	1/7 (14.3%)	2/7 (28.6%)	4/7 (57.1%)
Clark 2	5/32 (15.7%)	10/32 (31.3%)	8/32 (25.0%)	9/32 (28.1%)
Clark 3	2/24 (8.3%)	6/24 (25.0%)	12/24 (50.0%)	4/24 (16.7%)
Clark 4	3/23 (13.0%)	6/23 (26.1%)	8/23 (34.8%)	6/23 (26.1%)
Clark 5	0/3 (0.0%)	1/3 (33.3%)	0/3 (0.0%)	2/3 (66.7%)
Recurrent/ Metastatic	3/21 (14.3%)	1/21 (4.8%)	8/21 (38.1%)	9/21 (42.9%)

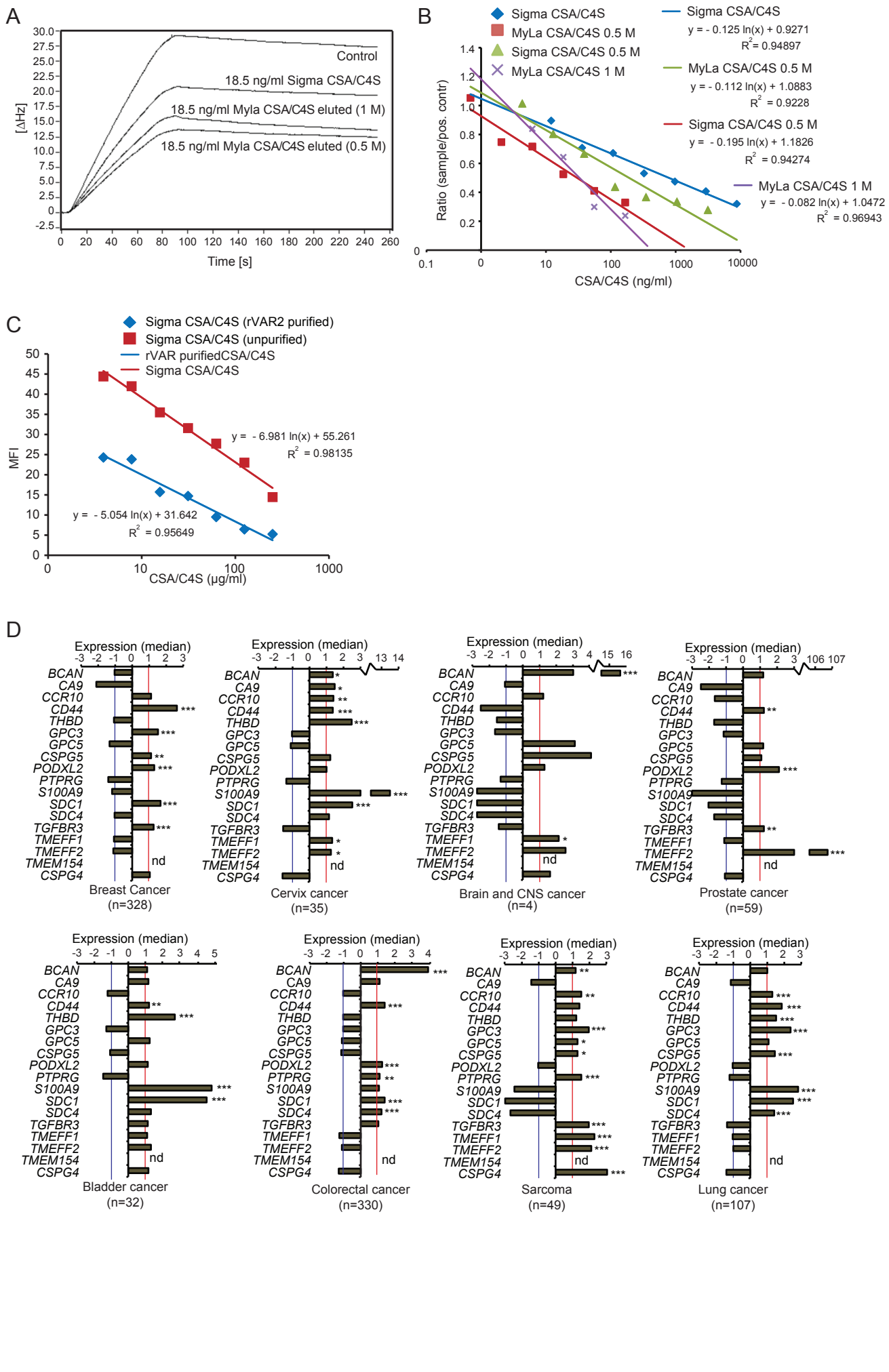

A
Control
18.5 ng/ml Sigma CSA/C4S
18.5 ng/ml Myla CSA/C4S eluted (1 M)
18.5 ng/ml Myla CSA/C4S eluted (0.5 M)
[ΔHz]
Time [s]
B
Sigma CSA/C4S
MyLa CSA/C4S 0.5 M
Sigma CSA/C4S 0.5 M
MyLa CSA/C4S 1 M
Sigma CSA/C4S
y = - 0.125 ln(x) + 0.9271
R2 = 0.94897
MyLa CSA/C4S 0.5 M
y = - 0.112 ln(x) + 1.0883
R2 = 0.9228
Sigma CSA/C4S 0.5 M
y = - 0.195 ln(x) + 1.1826
R2 = 0.94274
MyLa CSA/C4S 1 M
y = - 0.082 ln(x) + 1.0472
R2 = 0.96943
Ratio (sample/pos. contr)
CSA/C4S (ng/ml)
C
Sigma CSA/C4S (rVAR2 purified)
Sigma CSA/C4S (unpurified)
rVAR purifiedCSA/C4S
Sigma CSA/C4S
y = - 6.981 ln(x) + 55.261
R2 = 0.98135
y = - 5.054 ln(x) + 31.642
R2 = 0.95649
MFI
CSA/C4S (µg/ml)
D
Expression (median)
BCAN
CA9
CCR10
CD44
THBD
GPC3
GPC5
CSPG5
PODXL2
PTPRG
S100A9
SDC1
SDC4
TGFBR3
TMEFF1
TMEFF2
TMEM154
CSPG4
nd
Breast Cancer
(n=328)
Cervix cancer
(n=35)
Brain and CNS cancer
(n=4)
Prostate cancer
(n=59)
Bladder cancer
(n=32)
Colorectal cancer
(n=330)
Sarcoma
(n=49)
Lung cancer
(n=107)

Figure S4, related to Figure 4

(**A**) Representative sensorgram from the binding analysis (IC_{50} values) of high- and low affinity rVAR2-bound CS purified from crude bulk trachea CSA (Sigma) and from MyLa 2059 T-cell Lymphoma cells. Binding of 30 nM rVAR2 pre-incubated with the indicated CS concentrations is measured as delta Hertz [ΔHz] as a function of time (seconds). Inhibitory capacity was assessed as a decrease in peak response levels compared to the positive control.

(**B-C**) IC_{50} values were calculated from the biosensor (**B**) and FACS (**C**) analysis using linear regression. Concentration of CS is plotted against rVAR2 binding given as a ratio to the nearest positive control (B) or MFI (C). Linear regression was performed in Excel. The equations and corresponding R2 values are given in the plots, while the resulting IC_{50} values are shown in table S4.

(D) Expression of the indicated genes encoding VAR2CSA plasma membrane binding placental-like CS-modified proteoglycans Figure 4H in the indicated patient specimens extracted from the Oncomine Bitner array and divided into cancer groups. Blue and red lines represent median cut-off for average expression across the entire dataset. *p<0.05, **p<0.01, ***p<0.001. nd: not determined (missing probe).

Table S3, related to Figure 4 [1]

	Myla2059		KG-1	
Composition	Input	rVAR2 purified	Input	rVAR2 purified
Non-sulfated	1.9±1.4%	0%	2.1±1.5%	0%
Mono-sulfated	98.1±1.4%	100%	98.1±1.4%	100%
C4S	93.9%	95.8%	95.1%	95.5%
C6S	6.1%	4.2%	4.9%	4.5%
Di-sulfated	0%	0%	0%	0%

[1] Compositional analysis of extracted CS species before and after rVAR2 affinity purification. Table shows degree of sulfation as determined by LC-MS and ratio of C4S versus C6S as determined by tandem MS. Selected data is summarized graphically in Fig 5B-E.

Table S4, related to Figure 4 [1]

	Sigma CSA/C4S	Sigma CSA/C4S	MyLa2059 CSA/C4S	Placenta CSA/C4S
Affinity	Input	rVAR2 purified	rVAR2 purified	rVAR2 purified
IC_{50} µg/ml (Biosensor)	0.79	0.19	0.033	0.063
IC_{50} µg/ml (Cancer cell flow cytometry)	99.6	14.7	n.d.	n.d.
Composition				
Non-sulfated	10.5±0.5%	1.4±1.1%	0%	
Mono-sulfated	89.5±0.5%	98.6±1.1%	100%	
C4S	79.6%	90.3%	95.8%	
C6S	20.4%	9.7%	4.2%	
Di-sulfated	0%	0%	0%	

[1] Summary of the analysis of the capacity of BT-CSA, before and after rVAR2 affinity purification, and rVAR2 affinity purified Myla2059 CS and placental CS, to inhibit rVAR2 binding to immobilized CSPG in the Attana biosensor and binding to cancer cells in FACS. The composition of the different sources of CS (as in table S2) is listed below. N.d. means that experiment could not be performed due to insufficient amounts of material. Binding inhibition is shown as the concentration needed to block 50% of the binding (IC_{50} values) between rVAR2 and the cells measured by biosensor. Data is summarized in Fig 5F-G.

Table S5, related to Figure 4[1].

Gene symbol	Protein	Presence of CSA chain(s)	Known function in Cancer
BCAN	Brevican	Yes	Cell adhesion and motility. Isoforms highly expressed on cell surface in glioma (Theocharis et al., 2010)
CA9	Carbonic anhydrase IX	Proteoglycan but not previously shown to be CSA conjugated.	Involved in cell proliferation and transformation, and solid tumor acidification. Expressed in all clear-cell renal cell carcinoma, but is not detected in normal kidney (Takacova et al., 2013). Also expressed in various other tumours including breast cancers.
CCR10	Chemokine receptor 10	Not known	Chemokine receptor. Overexpressed in lymphoma, cutaneous squamous cell carcinoma (Kai et al., 2011) and melanoma. Suggested to be involved in metastasis (Murakami et al., 2004).
CD44	CD44 molecule	Yes on splicevariants	Cell adhesion and migration. Associated with a wide range of cancers and cancer stem cells (Naor et al., 1997)
THBD	Thrombomodulin	Yes	Binds thrombin. Differently expressed in a wide range of cancers including carcinoma, adenocarcinoma and glioma. Loss of TM expression correlates with certain cancer progressions. Progression of cancer correlates with serum levels of TM and high expression promotes angiogenesis and

			metastasis (Hanly et al., 2005).
GPC3	Glypican 3	HS proteoglycan but not previously shown to present CSA	Binds ECM proteins and growth factors. Has been described as an oncofetal antigen in hepatocellular carcinoma (Iozzo and Sanderson, 2011).
GPC5	Glypican 5	Yes	Binds ECM proteins and growth factors. Overexpressed in several tumors. Has been demonstrated to increase tumour proliferation in rhabdomyosarcomas by potentiating the action of FGF-2, HGF and Wnt1A (Williamson et al., 2007).
CSPG5	Neuroglycan	Yes	ND
PODXL2	Podocalyxin-like 2	Yes	Cell adhesion. Overexpressed in several tumors including breast and prostate cancer, malignant brain tumours, testicular, hepatocellular and renal cell carcinoma Overexpression has been shown to be an independent predictor of prognosis in breast, renal and colorectal cancers (Larsson et al., 2011).
PTPRG	Protein tyrosin phosphatase, receptor type, G	Proteoglycan with CS acceptor site	Enzyme involved in cell growth, differentiation, mitotic cycle, and transformation by oncogenes. Gene methylation and protein activity is associated with tumor suppression (Della Peruta et al., 2010).

S100A9	S100 calcium binding protein A9	No	A calcium- and zinc-binding protein important in the regulation of inflammatory processes and immune response. Upregulated in various cancers including hepatocellular carcinoma (HCC) (Wu et al., 2013).
SDC1 **&** ***SDC4***	Syndecan 1 & Syndecan 4	Yes	Regulates cell proliferation, differentiation, adhesion and migration. In particular Syndecan-4 is a focal adhesion component in a range of cell types and mediates breast cancer cell adhesion and spreading. Overexpressed in many cancers including prostate and breast and is key to spread of cancer. Expression correlations with tumor recurrence and poor prognosis (Theocharis et al., 2010).
TGFBR3	Transforming growth factor, beta receptor III. Also called Betaglycan	Yes (Part time)	Binds TGF. Plays a role in multiple cancers. Has been shown to decrease cancer cell motility (Mythreye and Blobe, 2009).
TMEFF1/TENB1	Transmembrane protein with EGF-like and two follistin-like domain 1 also called Tomoregulin-1	Proteoglycan with unknown modification but has several putative CS acceptor sites	Associated with prostate cancer as well as gastric cancer (Uchida et al., 1999).
TMEFF2/TENB2	Transmembrane protein with EGF-like and two follistin-like domain 1 Tomoregulin-2	Yes	Diagnostic marker for prostate cancer (Zhao et al., 2008).
TMEM154	Transmembrane protein 154	Not known but has	ND

		putative CS acceptor site	

[1]VAR2SCA plasma membrane binding-proteins identified by a functional gain-of-binding Retrogenix
screen. ND: No data available.

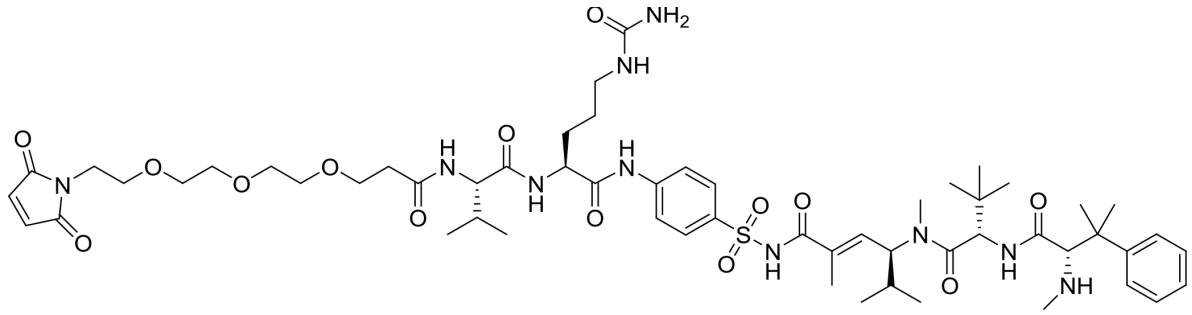


Figure S5, related to Figure 7
Structure of the protease cleavable linker-molecule MTvc886 used to conjugate KT886 to rVAR2.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell lines

Human Cancer cell lines BxPC-3‡, NCI-N87‡, HCC1954‡, HCT-15‡, Capan-2‡, AsPC-1‡, Jurkat‡, MiaPaCa-2‡, OVCAR-3‡, Karpas299†, H1975◦, NCI-H358‡, SK-BR-3‡, MCF-7‡, DLD-1‡, NCI-H1437◦, HPAF-II‡, Colo205◦, MG63◦, PC-3‡, T47D‡, MDA-MB-231‡, MDA-MB-468‡, A549‡, 253J B-V◦, UM-UC-3‡, K562*, Rh30◦, U2OS◦ ,U138MG‡, A172‡, MNNG/HOS◦, JIMT-1◦, OE19†, DU145‡, HepG2‡, SKOV-3‡, KG1a‡, H292‡, and HCC1806‡ were obtained from ATCC‡, Sigma-Aldrich†, the University of Copenhagen*, or the Vancouver Prostate Centre/British Columbia Cancer Agency◦ and were cultured as instructed by the supplier. The Myla2059 Lymphoma cell lines were graciously donated by Niels Ødum at the University of Copenhagen. FARAGE, SU-DHL-8, KG-1 and NALM-6 were a kind gift from Karen Dybkaer, Aalborg University Hospital, Denmark.

Enzyme-linked immunosorbent assay (ELISA)

Plastic wells were coated with either Chondroitin Sulfate Proteoglycan (CSPG), Heparan Sulfate Proteoglycan (HSPG), or Bovine Serum Albumine (BSA) as indicated and binding of rVAR2 were detected using anti-his HRP antibody.

Immunohistochemistry

Normal tissue and clinico-pathological diagnosed tumor specimens from human patients were obtained from Origen and from tissue microarrays containing a wide variety of mesenchymal tumors (Pacheco and Nielsen, 2012). Lung cancer and melanoma samples

were obtained from patients who underwent total tumor resection. Specimen blocks chosen for the TMA met the criteria of nonnecrotic, nonirradiated, or chemo-treated lung cancer tissue. The TMA contains 231 NSCLC specimens (127 adenocarcinoma and 104 squamous cell carcinoma) and 159 mixed staged melanoma specimens. Samples were double punched 0.6 um diameter using an indexed manual arrayer with an attached stereomicroscope under the direction of a certified pathologist, who also reviewed and verified the tumor content. Test for association of CSA (low or high) with RFS in the lung TMA was performed using a one-tail Mann-Whitney test, and CSA scores with melanoma progression was performed using Goodman-Kruskal-Gamma test. $p < 0.05$ was considered significant. Using the Ventana Discovery platform, sectioned paraffin-embedded tissue samples were stained with 500 picomolar V5-tagged rVAR2 without antigen retrieval followed by 1:700 monoclonal anti-V5 step, and a anti-mouse-HRP detection step. Mounted and stained specimens were subsequently scored for membranous staining intensity on a 0-3 scale. Score 0-1 represented absent/weak staining and score 2-3 positive staining, where score 2 reflects a staining intensity equal to that of placenta (included as a positive control in each staining run). Within each cancer group the percentage and exact binomial 95% confidence interval was calculated using STATA 12 software.

Retrogenix Technology

Screening for VAR2CSA binding proteins was performed using the Retrogenix Cell Microarray technology (www.retrogenix.com). Initially 3550 expression vectors, each encoding a unique human plasma membrane protein and the fluorescent ZsGreen1 protein, were pre-spotted on glass slides. Human HEK293 cells were grown over the arrays and

reverse-transfected, resulting in the over-expression of each membrane protein and ZsGreen1. Slides were then incubated with 5 µg/ml V5-tagged VAR2 DBL1-ID2a followed by an AlexaFluor647-labeled anti-V5 antibody (AbD Serotec). VAR2CSA-binding proteins were identified by fluorescence imaging (ZsGreen1 and AlexaFluor647) and ImageQuant software (GE). For validation and specificity testing, expression vectors encoding the VAR2CSA binding proteins were re-spotted on new slides, and HEK293 cells were reverse-transfected. Slides were then treated with V5-tagged rVAR2 with or without 400 µg/ml CSA or following a 30 min chondroitinase ABC (CHase; Sigma) pre-treatment, or treated with rContr or with no ligand. Interactions were analysed using the AlexaFluor647-labeled anti-V5 antibody and fluorescence imaging. After fluorescence imaging of the slides, spot and background intensities were quantified from at least 6 high power field images representing at least 2 independent experiments using ImageQuant software (GE).

Bioinformatics

The Bittner multicancer dataset is available at http://www.oncomine.org/main/index.jsp (Rhodes et al., 2004). The dataset was analyzed for median expression of identified genes encoding VAR2CSA plasma-membrane binding proteins relative to a calculated average median expression across the entire dataset. Survival and microarray analysis were performed using the R statistical environment. Affymetrix microarray datasets linked to outcome were normalized using the Robust Multichip Array (RMA) method from affy package (Gautier et al., 2004) and optimal probe sets were selected with the Jetset package (Li et al., 2011). For the Duke and the Bild lung cancer datasets, survival analysis was

performed with the overall survival time. For all the others datasets the outcome variable is the time of recurrence free survival. Survival curves were calculated using the Kaplan-Meier method. High and low expression patients were divided using the median value of the selected gene as separation point. Hazard ratio was calculated using Cox proportional hazards regression, and p value was calculated using the logrank test.

Immunoprecipitation

Membrane proteins were extracted by lysing C32 cells with EBC lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 2.5 mM MgCl2, 1 mM EDTA, 1% CHAPS and a protease inhibitor cocktail (Roche). The lysate was loaded onto a Hitrap NHS HP column (GE) containing immobilized rVAR2 or DBL4 control protein. The column was washed extensively in Lyses buffer as well as lysis buffer containing 250 mM NaCl. Bound protein was eluded with 0.5 M NaCl in lysis buffer and concentrated on a vivaspin column (MWCO 10.000 kDa). Protein samples and a high-molecular weight marker (LC5699, Life Technologies) were loaded onto a NuPAGE Tris-acetate gel (Life Technologies). Proteins were subsequently transferred to a nitrocellulose overnight at 4C at 75 mA. The membranes were stained with anti-CSPG4 antibody (LHM2, Abcam) or Anti-panCD44 (2C5, RnD systems).

Mass Spectrometry

Denaturing SEC-HRMS for intact mass analysis of recombinant VAR2 and VAR2 drug conjugates was performed on a Waters Acquity H Class UPLC with PDA detection at 280 nm utilizing an Acquity UPLC BEH 200 SEC column (1.7 uM, 4.6 mm x 150 cm).

High resolution mass spectrometry detection was achieved using a MicroMass Q-TOF Premier with a scan range from 250-4900 m/z. The analysis was performed using an isocratic elution at 0.25 ml/min over 11 min with 70/30 H20/ACN with 0.1% TFA and 0.1% FA. Data collection and analysis was done with MassLynx 4.1 with spectral deconvolution using MaxEnt1.

Subcutaneous PC3 and B16 tumor model

PC-3M-Luc-C6 cells ($2x10^6$ cells) were injected subcutaneously into the right flank of $Foxn1^{nu}$ mice on day -12. The mice were divided into 3 groups with 10 mice in each, and were treated with intravenous injections of vehicle (saline), a control DT388 fusion protein (rContr-DT), or an rVAR2 DT388 fusion protein (rVAR2-) respectively, on day 0, 2 and 6 at 0.6 mg/Kg doses. Tumor growth was monitored using a caliper-measuring tool, and the 3 longest perpendicular axes in the x/y/z plane of each tumor were measured. Tumor volume was calculated according to the standard formula: volume = xy^2*0.5236 (Janik et al., 1975). Saline and rVAR2-DT treated mice were also injected with luciferine and scanned for tumor chemiluminescence using IVIS at day 3 and 13 after 1^{st} dose (saline or rVAR2-DT). Control mice were given PBS or 0.33 mg/Kg rVAR2-NIR before sacrifice, and tumors were extracted and scanned by IVIS.

Karpas299 Xenograft Study

The in vivo efficacy study was performed at the Experimental Therapeutics & Animal Resource Centre at the BC Cancer Research Centre. Female C.B-17/IcrHsd-Prkdcscid mice (Harlan Laboratories) were anesthetized using isoflurane and were then implanted subcutaneously in the back with $1x10^6$ Karpas299 human T cell lymphoma tumour cells per mouse. Tumours were established over a period of 19 days, and test animals were then grouped according to tumour volume such that each group (n=7) had an equal distribution of tumour volumes. Dimensions of established tumours were measured with calipers. Tumour volumes were calculated according to the equation (Length x $Width^2$ /2) with the length (millimeters) being the longer axis of the tumour. The mean tumour volume on treatment day was greater than 150 mm^3. Intravenous test article administration began on Day 21, and continued every two to three days (total of three injections) at the doses indicated in the figure legend. Animals were injected based on individual body weights using a 28 G needle. Dilation of the lateral tail vein was achieved by holding the animals under a heating lamp for 1-2 min. The animals were briefly restrained (approximately 1 min) and injection was delivered into the lateral tail vein. Animal health was assessed acutely using Post Injection Clinical Observation Record (PICOR) forms. Body weights and tumour volumes were measured every Monday, Wednesday, and Friday. Animals remained on study until their tumours reached 800 mm^3 in size or they otherwise required euthanasia due to achieving a humane endpoint.

4T1 syngeneic bone metastasis model

Five to 6 weeks old C57black/6 female mice were maintained under isoflurane anesthesia and (5×10^5) 4T1-luciferase cells suspended in 100 µl of PBS solution were injected into the left ventricle under ultrasound guidance using a 30 gauge needle. The location of the tip of the needle in the left ventricle was confirmed by pulsatile blood flow in the hub of the needle. Animals were monitored until 8 weeks after injection using IVIS imaging system. Metastasis sites were collected at day of sacrifice and fixed in formalin for pathology studies.

Preparation of Drug Conjugate

To a solution of DBL1-ID2a (25.7 mg; 225 nmol) in ice cold PBS, pH 7.4 (34 ml) was added MTvc-KT886 (180 µl of a 10 mM dmso stock solution). The protein solution was mixed gently and allowed to stand on ice for a period of 70 min prior to concentration to a final volume of 12 ml by passage over an Amicon Ultra Centrifugal Filter (3000 xg; ~25 mins; 4°C; 50 kDa MWCO; Millipore product UFC905096). The concentrated protein solution was next purified over Zeba Spin Desalting Columns (40 kDa MWCO; 10 ml size; Thermo Scientific product 87772) preconditioned with sterile PBS. Concentration of the recovered materials was estimated by BCA assay using DBL1-ID2a as a standard. Composition and purity of the VDC were assessed by SDS-PAGE and SEC-UPLC-Esi-MS.

In vitro Cytotoxicity Assay of VAR2-Drug Conjugates using human Cancer Cell lines

Cells were removed from their culture vessel using Gibco® Trypsin-EDTA (Invitrogen # 25300-054). Detached cells were diluted in respective growth medium (Invitrogen #: 10313-021, A10491-01, 16600-082, 12561-056, 35050-061, 11415-064) + 10% Fetal bovine serum (Corning #: 35-015-CV) to 25000 cells/mL such that 100 µl/well will dispense 2500 cells/well. Cells were seeded into black walled, flat bottomed 96-well plates (Costar # 3904). Adherent cell lines cells were incubated for one night at 37 ^{0}C in a 5% CO2 atmosphere (No CO2 for MDA-MB-231) to allow the cells to attach to the microtitre plate surface, while suspension cells were seeded immediately before use. Test compounds were diluted directly in the appropriate cell growth medium at five-times the desired final concentration. These compounds were then titrated 1:3, over eight steps. A control with no test compound present (growth medium alone) was included on each microtiter plate in sextuplicate. 25 µl/well of the prepared titrations was added in triplicate to each cell line assayed. The cells and titrations were incubated at 37 ^{0}C / 5% CO2 for five nights. After the incubation, cell viability was measured using CellTiter-Glo® (Promega #G7572) reagent by adding thirty µl of prepared CellTiter-Glo® to each assay well. The mixtures were incubated for at least twenty min in the dark prior to measuring emitted luminescence using a microplate luminometer (500 ms integration time). The collected relative luminescence units (RLU) were converted to % cytotoxicity using the RLU values measured from the growth medium alone control as follows: % Cytotoxicity = 1 - [Well RLU/average medium alone control RLU]. Data (% Cytotoxicity vs. Concentration of

ADC (log10 [nM]) were plotted and were analyzed by non-linear regression methods using GraphPad Prism software v. 5.02 to obtain EC_{50} estimates.

***In vitro* toxicity assay using DT-VAR2**

The cells were seeded and incubated 3-5 days with toxin coupled rVAR2 or control protein. 400 µg/ml CSA was used as a specificity control. Following incubation, cells were washed in PBS and stained with Methylene Blue in Methanol or WST1 (Roche). Color was dissolved in 0.2 M Sodium Citrate in 50% Ethanol and quantified by absorbance at 450 nm. IC_{50} values were calculated as concentration of toxin at 50% survival. For a description of the in vitro tox assay using VCD886 please see supplementary methods.

Tolerability Study

Female CD-1 mice (Harlan Laboratories) were injected with the test articles and doses indicated in the figure legend using the housing/restraint/injection methods indicated above. Monitoring for acute toxicity effects was facilitated using the Post Injection Clinical Observation Record (PICOR) to assess morbidity and help determine humane endpoints. A PICOR was only completed for an animal in the event that a moribund animal was observed. Mice were monitored and weighed 3 times weekly for 12 days. Dose escalation occurred following favourable assessment of acute tolerability.

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