Statins inhibit onco-dimerization of the 4Ig isoform of B7-H3

Margie N. Sutton[1], Sarah E. Glazer[1], Ajlan Al Zaki[1], Arianna Napoli[1], Ping Yang[1], Priya Bhosale[2], Jinsong Liu[3], Seth T. Gammon[1], David Piwnica-Worms[1]*

[1]Department of Cancer Systems Imaging, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, USA

[2]Department of Abdominal Imaging, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, USA

[3]Department of Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, USA

Email: dpiwnica-worms@mdanderson.org

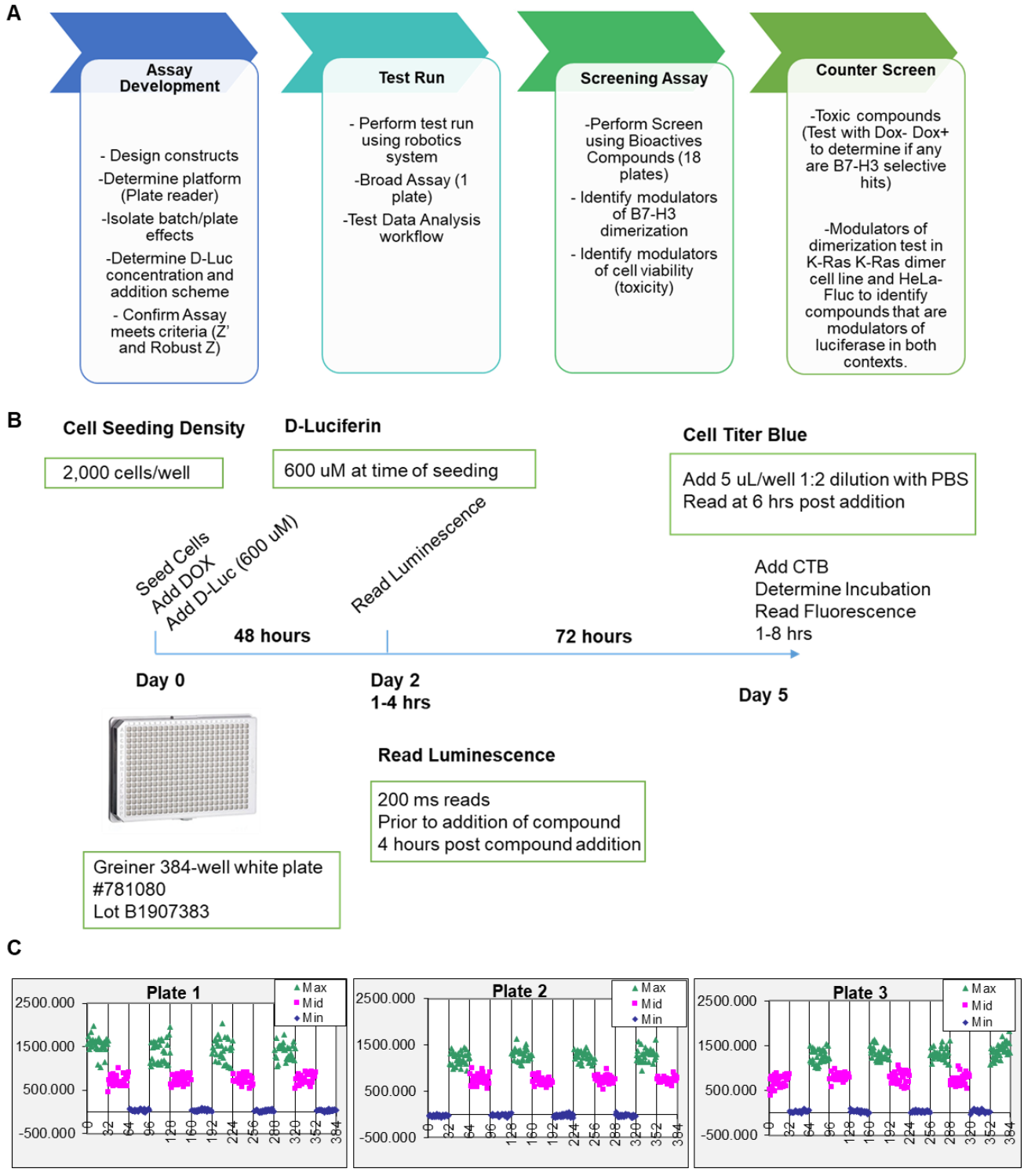
Author Contributions: M.N.S. S.T.G. and D.P.W. designed the study. M.N.S., S.E.G., A.A, A.N. and P.Y. carried out experiments. D.P.W. provided resources and reagents. J.L. provided TMA and pathology consult, P.B. provided clinical consult, M.N.S., S.T.G., S.E.G., A.A., and D.P.W. analyzed data, and M.N.S. and D.P.W. wrote the manuscript. All authors edited and approved the manuscript.

Competing Interest Statement: The authors declare no competing interests. The University of Texas MD Anderson Cancer Center has filed a patent application on an anti-B7-H3 antibody used in this report (S.T.G., and D.P.W., inventors). The technology is licensed in part to Radiopharm Ventures, LLC. The remaining authors have no conflicts of interest.

Keywords: B7-H3, CD276, protein dimerization, immune checkpoint, split-luciferase complementation, statins, immune modulation, gynecological cancers

*Corresponding author:
David Piwnica-Worms, M.D., Ph.D.
1881 East Road, Unit 1907
Houston, TX 77054
Email address:
dpiwnica-worms@mdanderson.org

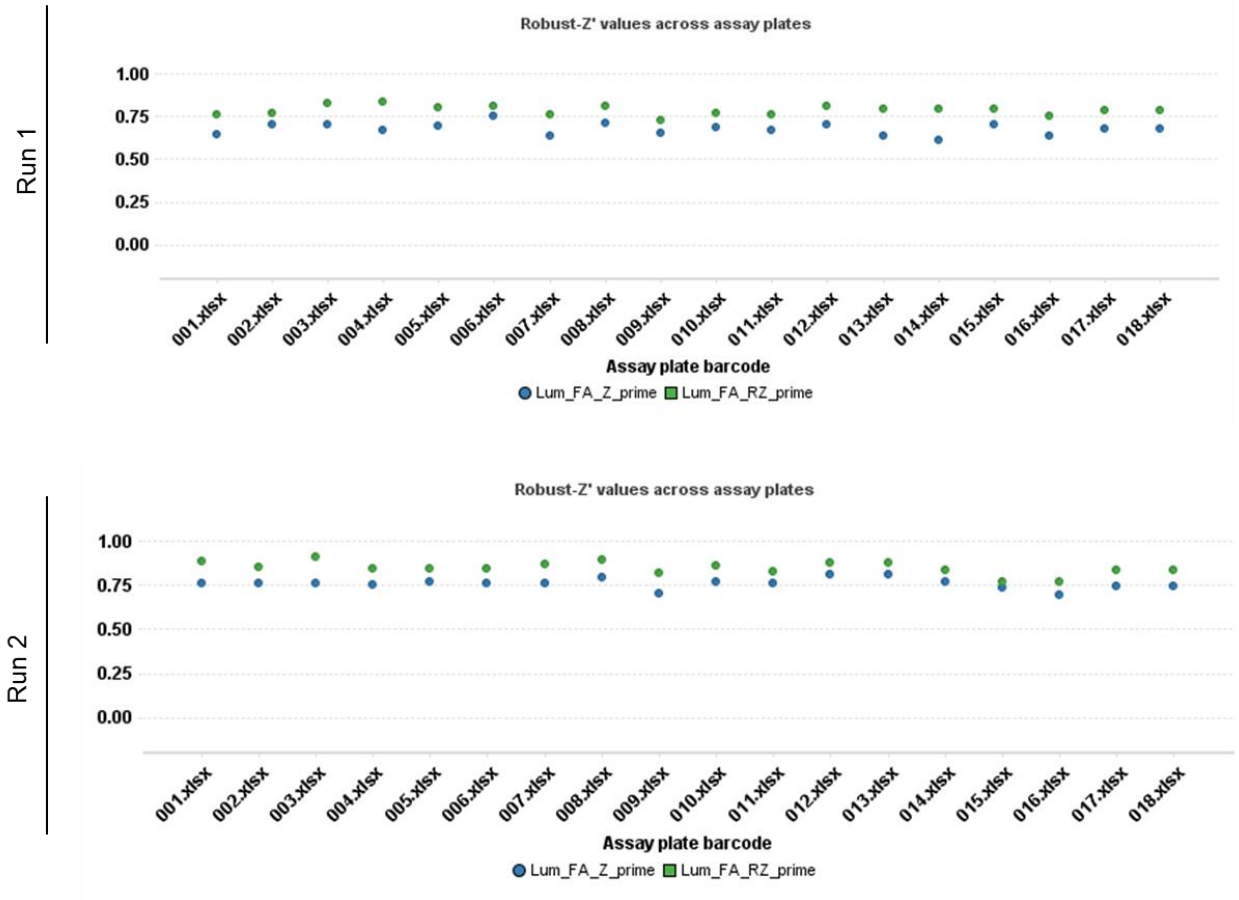
Supplemental Figure 1



Supplemental Figure 1. High throughput screening conditions. A. Schematic of the screening process is outlined taking into account the criteria being tested at each step to

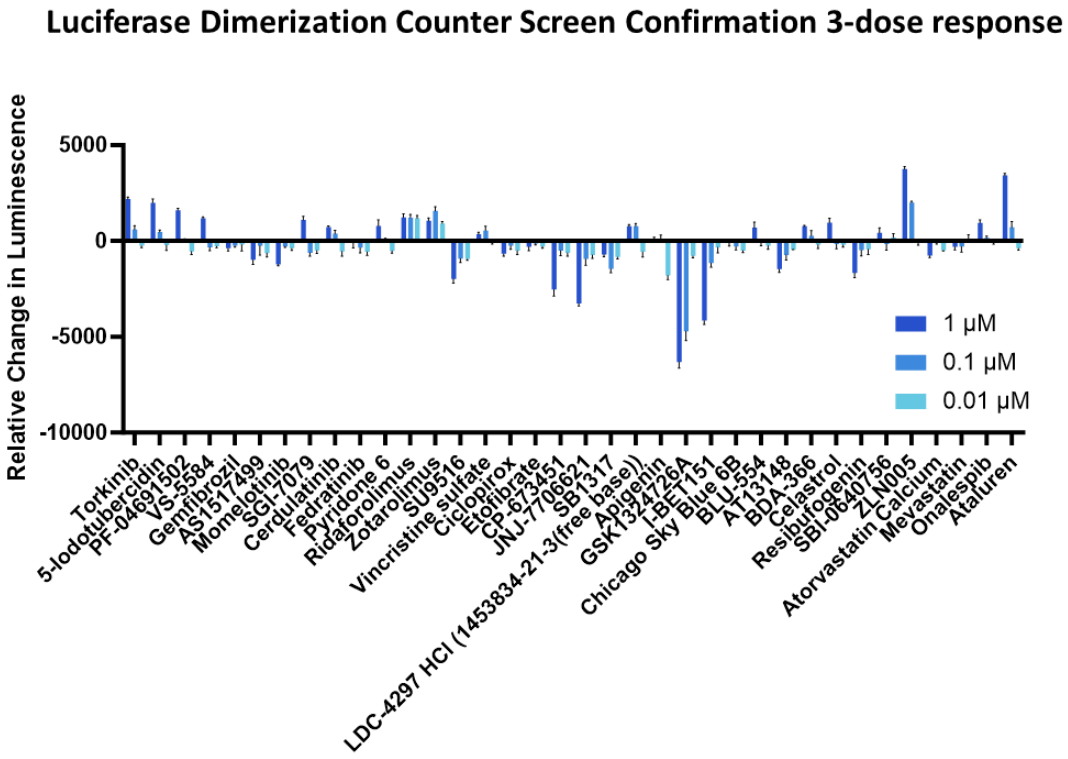
develop the HTS. **B.** Assay specific conditions and methods are detailed for each plate. **C.** Luminescence readouts for interleaved-signal format plates using a min, mid and max signal concentration of doxycycline to induce 4Ig-B7-H3 expression and dimerization as observed by split-luciferase complementation.

Supplemental Figure 2



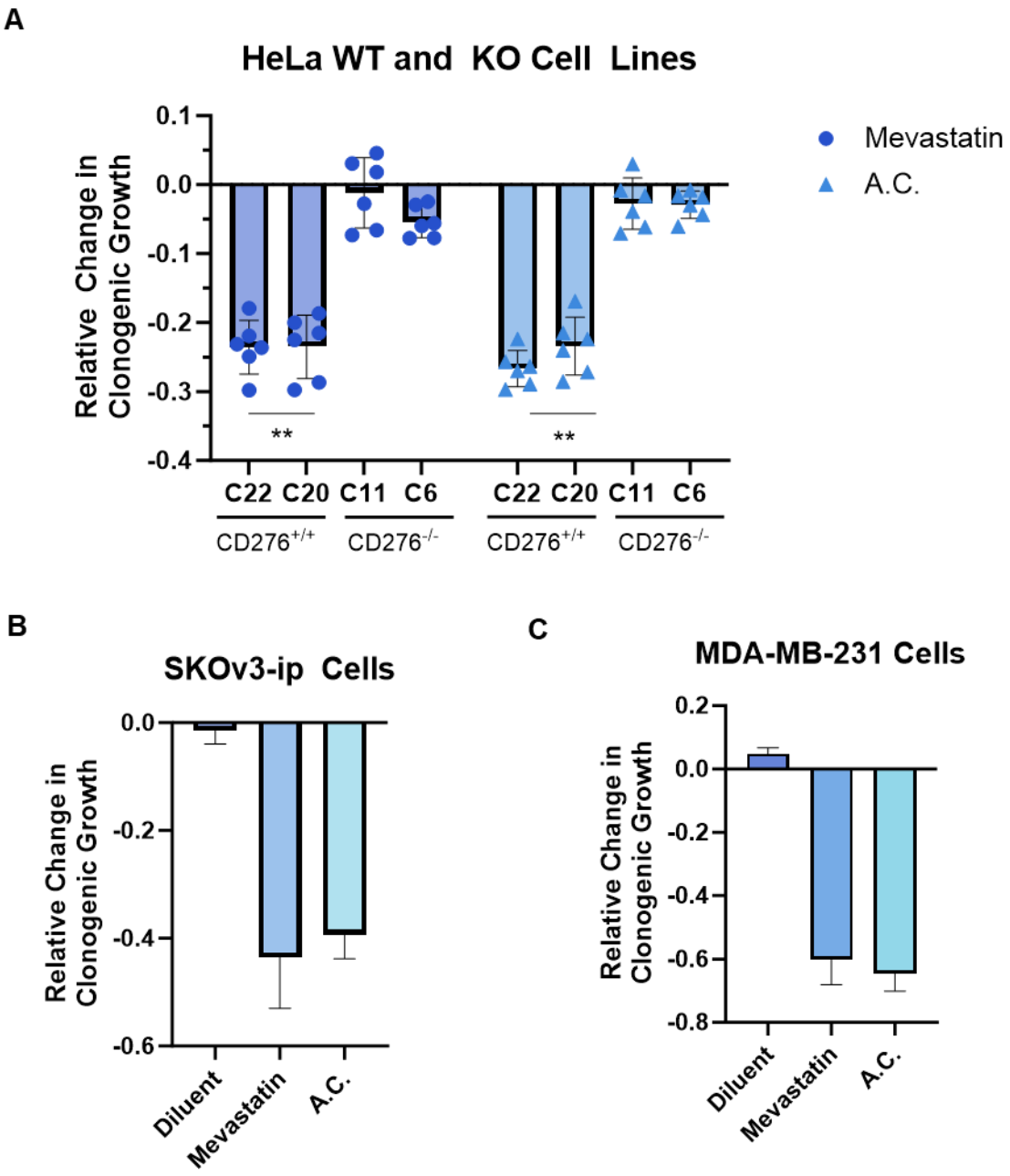
Supplemental Figure 2. The high-throughput screen was robust across all plates and biological replicates. Z' and Robust Z' values for each plate were calculated and plotted across biological replicates (Run 1 and Run 2) of the HTS.

Supplemental Figure 3



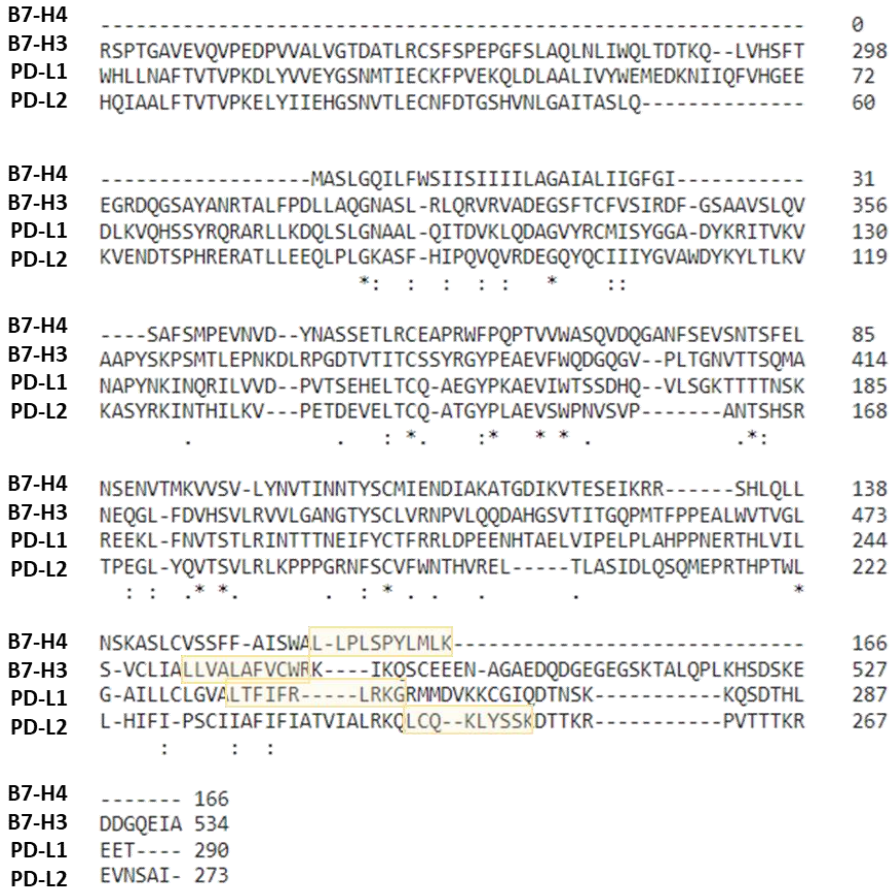
Supplemental Figure 3. 4Ig-B7-H3 dimerization counter screen was performed using a 3-dose titration curve for all "active compounds". U2OS-4Ig-B7-H3 cells were treated with active compounds for 4 hrs at 0.1 -1.0 µM concentrations. Relative change in luminescence is plotted where bars represent the mean ± SD. The experiment was performed in technical triplicate.

Supplemental Figure 4



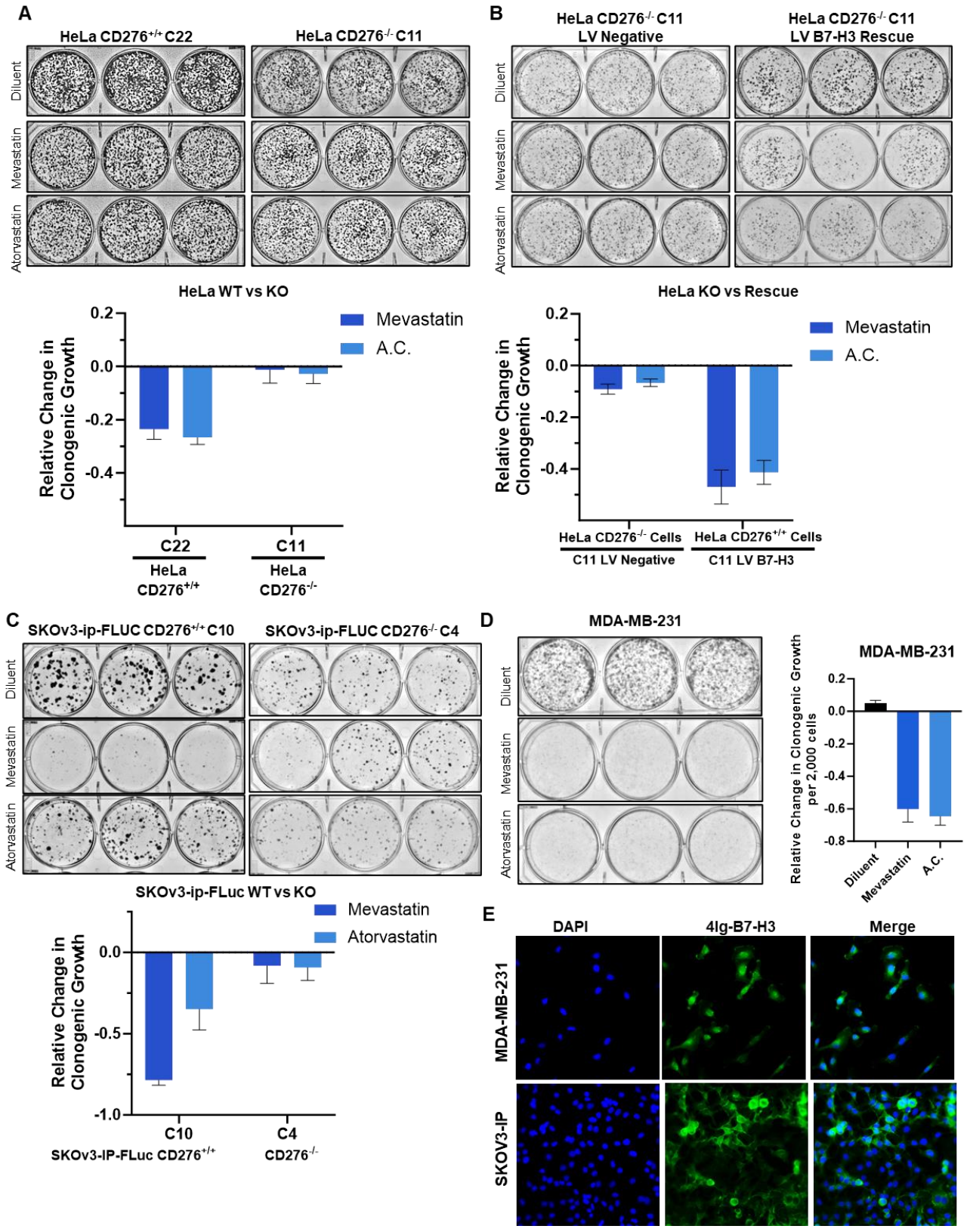
Supplemental Figure 4. Statins inhibitors reduce clonogenic growth in multiple 4Ig-B7-H3 positive cancer cell lines. A. 2,000 HeLa WT or KO cells were seeded and without statin treatment (1µM treatment with mevastatin, atorvastatin calcium) on day 1 and day 4 and clonogenic growth was measured after 10 days. Colonies were fixed and stained with Coomassie blue on day 10. Clonogenic growth was quantified using ImageJ and represented in the bar graph (mean ± SD). The experiment was performed in technical triplicate with three biological replicates, ***p<0.001. **B-C.** Clonogenic growth was also measured for SKOv3-ip (5,000 cells/well seeded) and MDA-MB-231 (2,000 cells/well).

Supplemental Figure 5



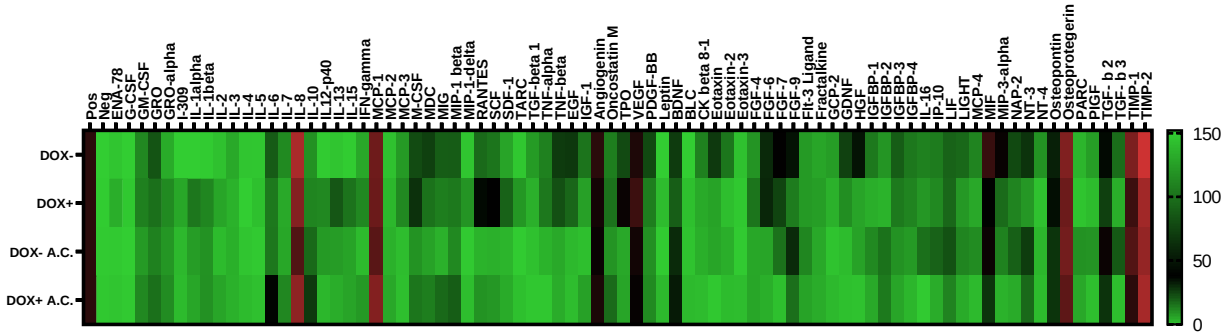
Supplemental Figure 5. CRAC/CARC domains are present in the transmembrane region of many B7-family members. Sequence alignment of B7-H4, B7-H3, PD-L1 and PD-L2 highlight the presence of predicted CRAC/CARC domains within all four B7-family member proteins. The stars, dots and colons below the alignment indicate degree of conservation. "*" indicate columns of identical residues, colons and dots indicate columns where there is some conservation of the biochemical character of the side chains.

Supplemental Figure 6



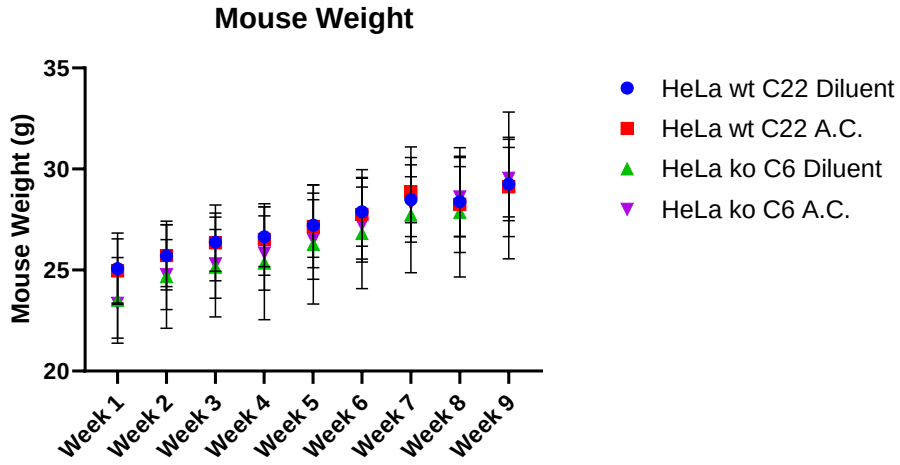
Supplemental Figure 6. Statins inhibit clonogenic growth in a 4Ig-B7-H3 dependent fashion. A-B. 2,000 HeLa wildtype or *CD276* knockout cells, or rescued cells **(B)** were seeded and allowed to grow for 10 days, changing the media every 72 hours. Cells were subjected to 1µM treatment with mevastatin or atorvastatin calcium on day 1 and day 4. Plates were stained with Coomassie blue dye on day 10 and representative images of the plates are shown. **C.** 5,000 SKOv3-ip wildtype or *CD276* knockout cells were seeded and allowed to grow for 10 days, changing the media every 72 hours. Cells were subjected to 1µM treatment with mevastatin or atorvastatin calcium on day 1 and day 4. Plates were stained with Coomassie blue dye on day 10 and representative images of the plates are shown. **A-C.** Clonogenic growth was quantified using ImageJ and represented in the bar graph (mean ± SD). The experiment was performed in technical triplicate with three biological replicates, ***p<0.001, significance was reached when comparing each individual treatment between the B7-H3 expressing group and the non-expressing group (KO). **D.** 2,000 MDA-MB-231 WT cells were seeded and allowed to grow for 10 days, changing the media every 72 hours. Cells were subjected to 1µM treatment with mevastatin or atorvastatin calcium on day 1 and day 4. Plates were stained with Coomassie blue dye on day 10 and representative images of the plates are shown. Clonogenic growth was quantified using ImageJ and represented in the bar graph (mean ± SD). The experiment was performed in technical triplicate with three biological replicates, ***p<0.001. **E.** Endogenous expression of B7-H3 as determined by immunofluorescence staining using SKOv3-ip and MDA-MB-231 cancer cells.

Supplemental Figure 7



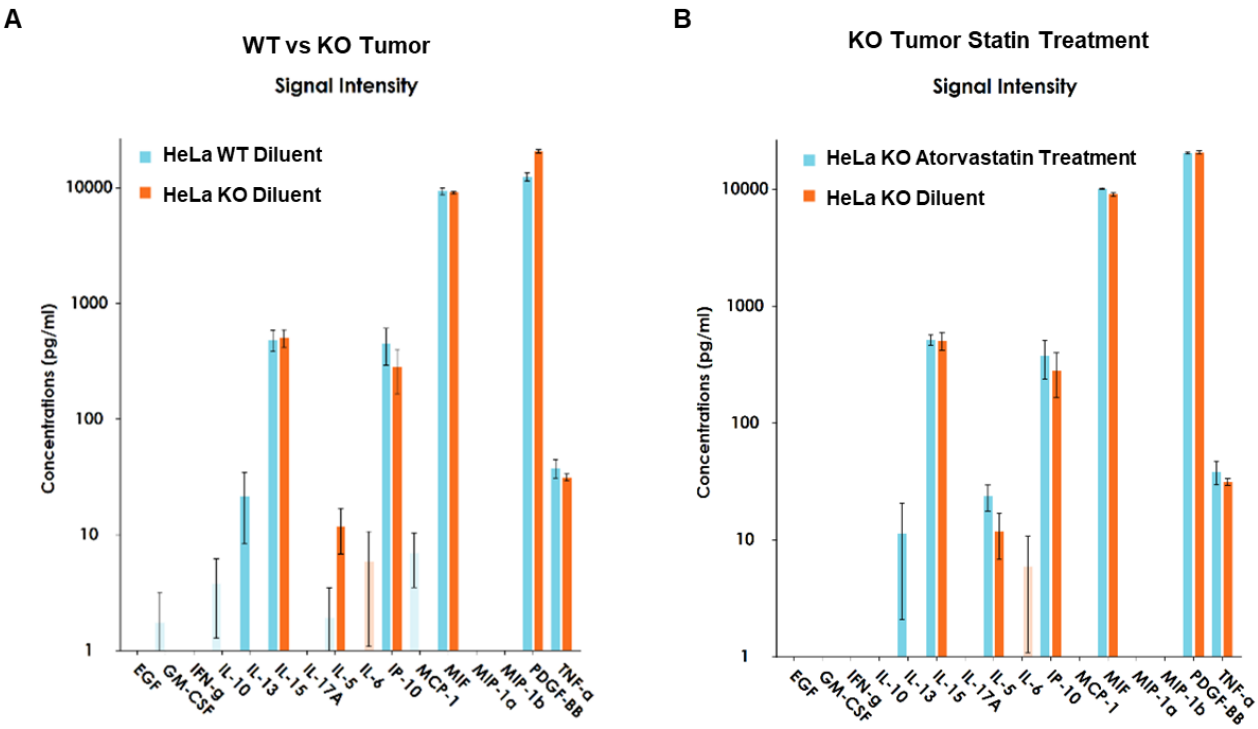
Supplemental Figure 7. Statin treatment alters cytokine release, some of which occur in a 4Ig-B7-H3-dependent fashion. Heatmap cytokine protein levels are plotted for media collected from 1.0 x 10^5 U2OS-4Ig-B7-H3 cells seeded and treated with or without Doxycycline for 48 hours followed by treatment with diluent or atorvastatin (1 μM) for 24 hours. Relative cytokine levels were plotted for each condition; DOX-, DOX+, DOX- Atorvastatin treatment, DOX+ Atorvastatin treatment.

Supplemental Figure 8



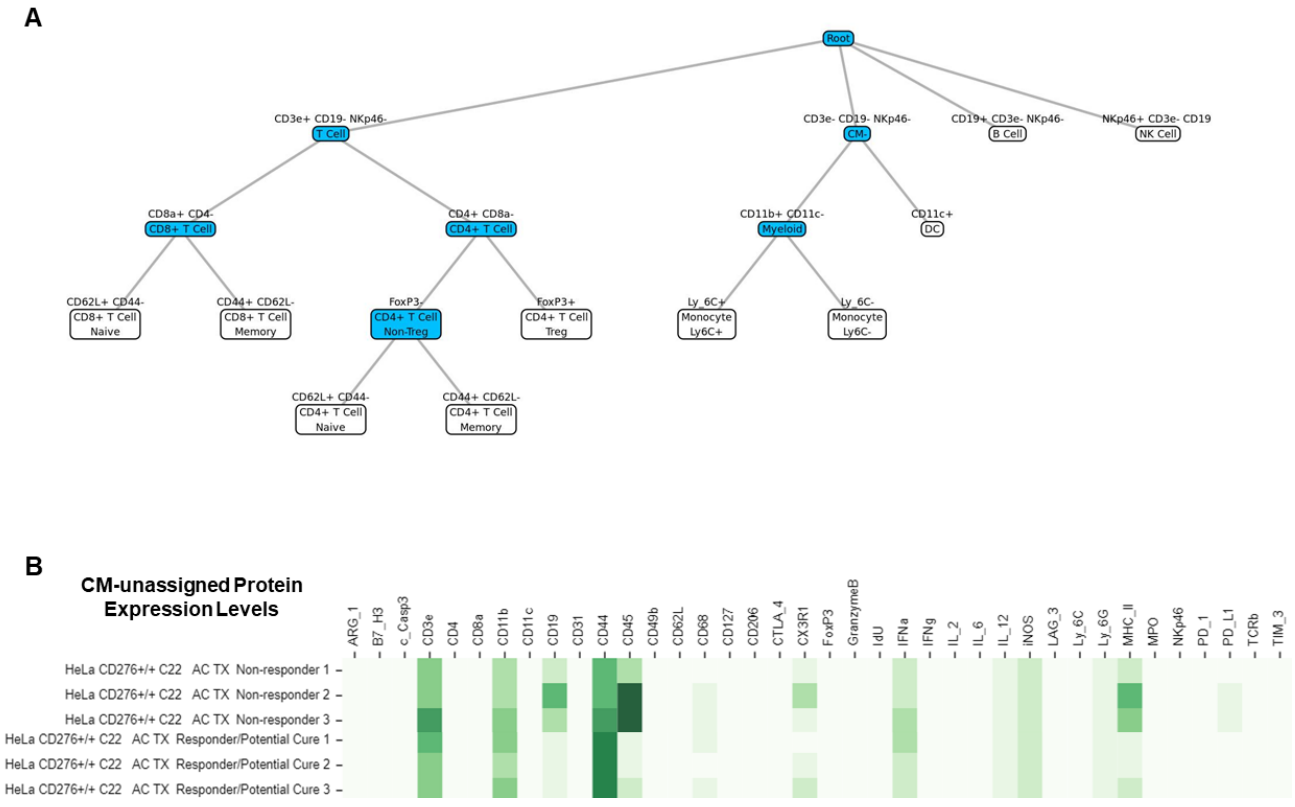
Supplemental Figure 8. Treatment of HeLa WT or HeLa KO xenografts with statins or diluent did not significantly alter mouse weight over the course of the study. Mouse weight for each mouse was measured weekly, mean ± SD, for each treatment group is presented.

Supplemental Figure 9



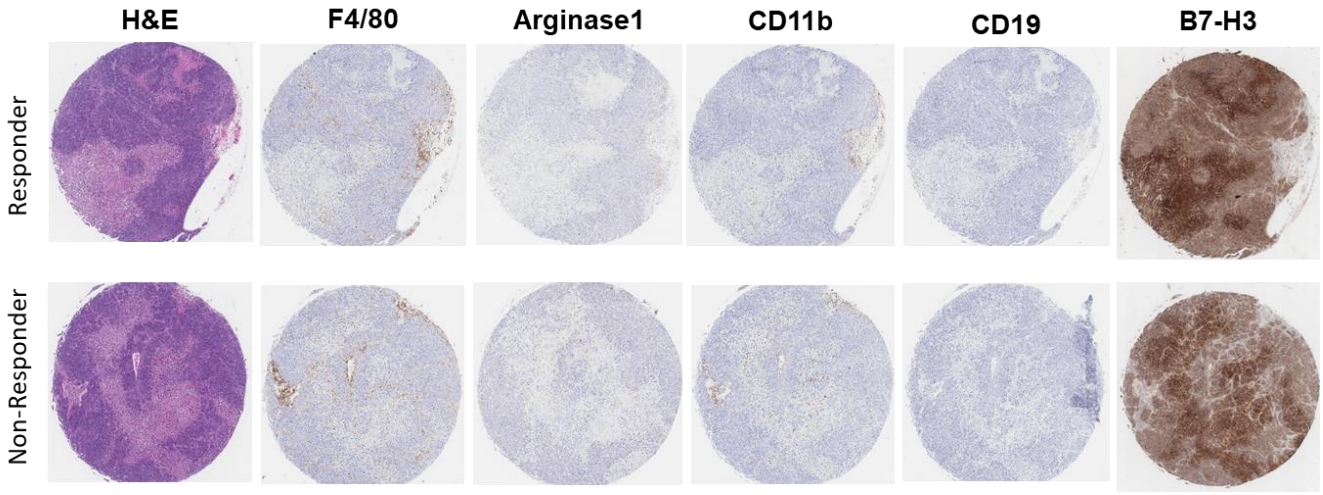
Supplemental Figure 9. Serum cytokine levels for *in vivo* tumor types (WT and KO) and response to statin treatment. Circulating cytokines were measured from plasma collected at experimental pre-endpoint, where mice bearing WT or KO HeLa xenografts were treated with diluent or atorvastatin (as described in Figure 6). Cytokine concentrations (pg/mL) are represented as mean ± SD for 3 individual mice/group.

Supplemental Figure 10



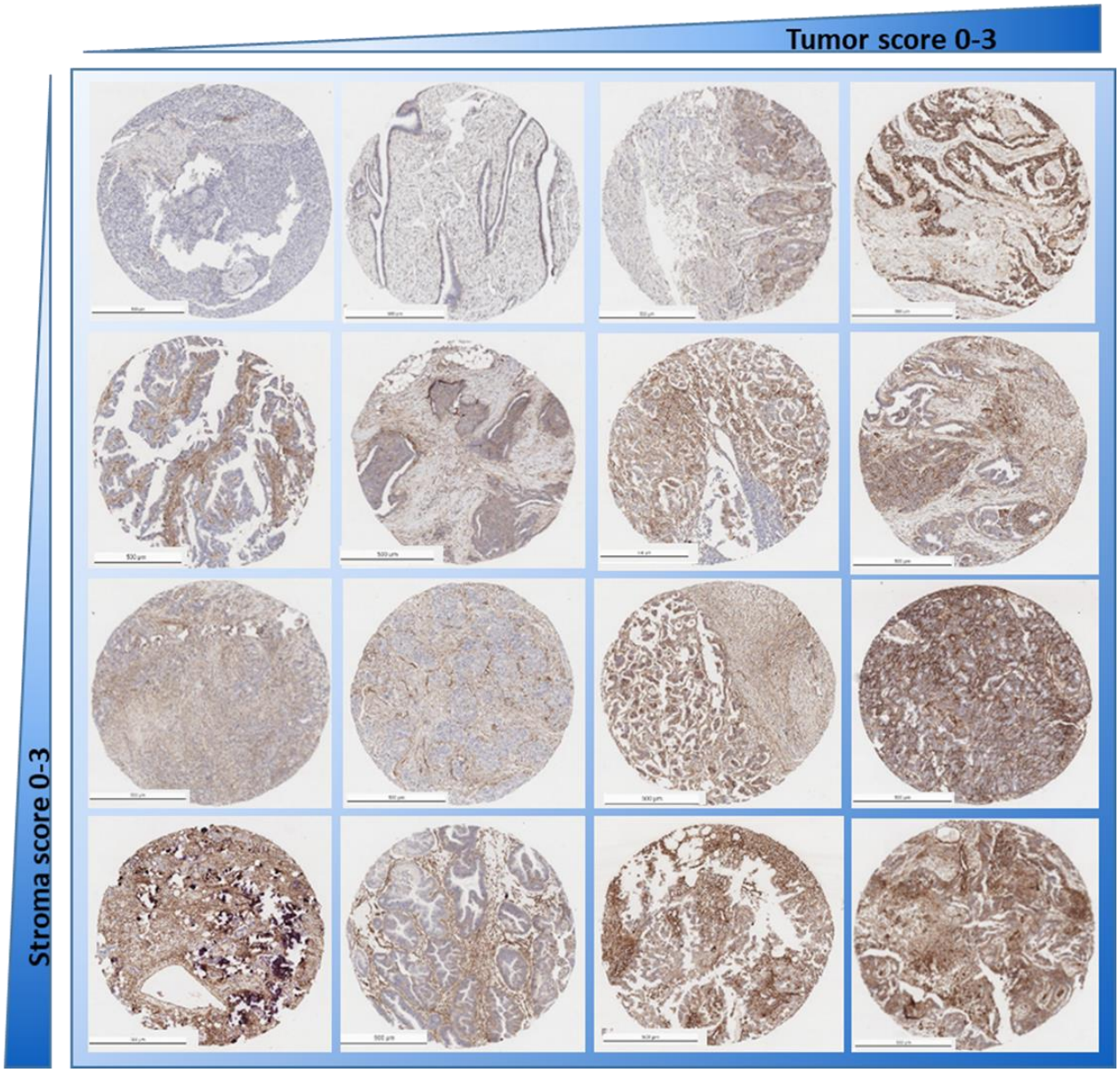
Supplemental Figure 10. Astrolabe hierarchy of cell identification and protein content within the CM-unassigned cell class. A. Schematic documenting Astrolabe hierarchy of cell identification markers. **B.** Heatmap containing protein content levels for markers within the CM-Unassigned cell populations contained within our panel. Each mouse is represented per row; likely non-responders are grouped on top with potential responders grouped below.

Supplemental Figure 11.



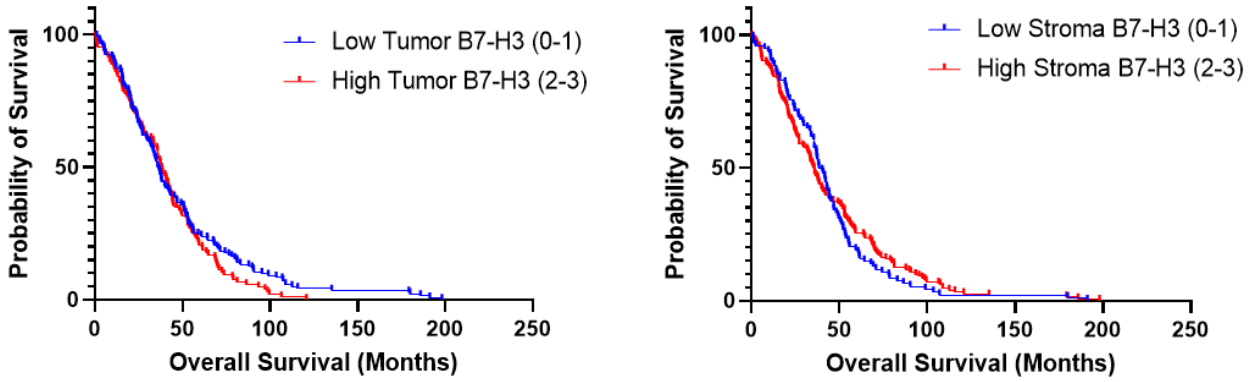
Supplemental Figure 11. Immunohistochemical staining of tumor immune infiltrates in the tumor of responders vs. non-responders to atorvastatin treatment.

Supplemental Figure 12



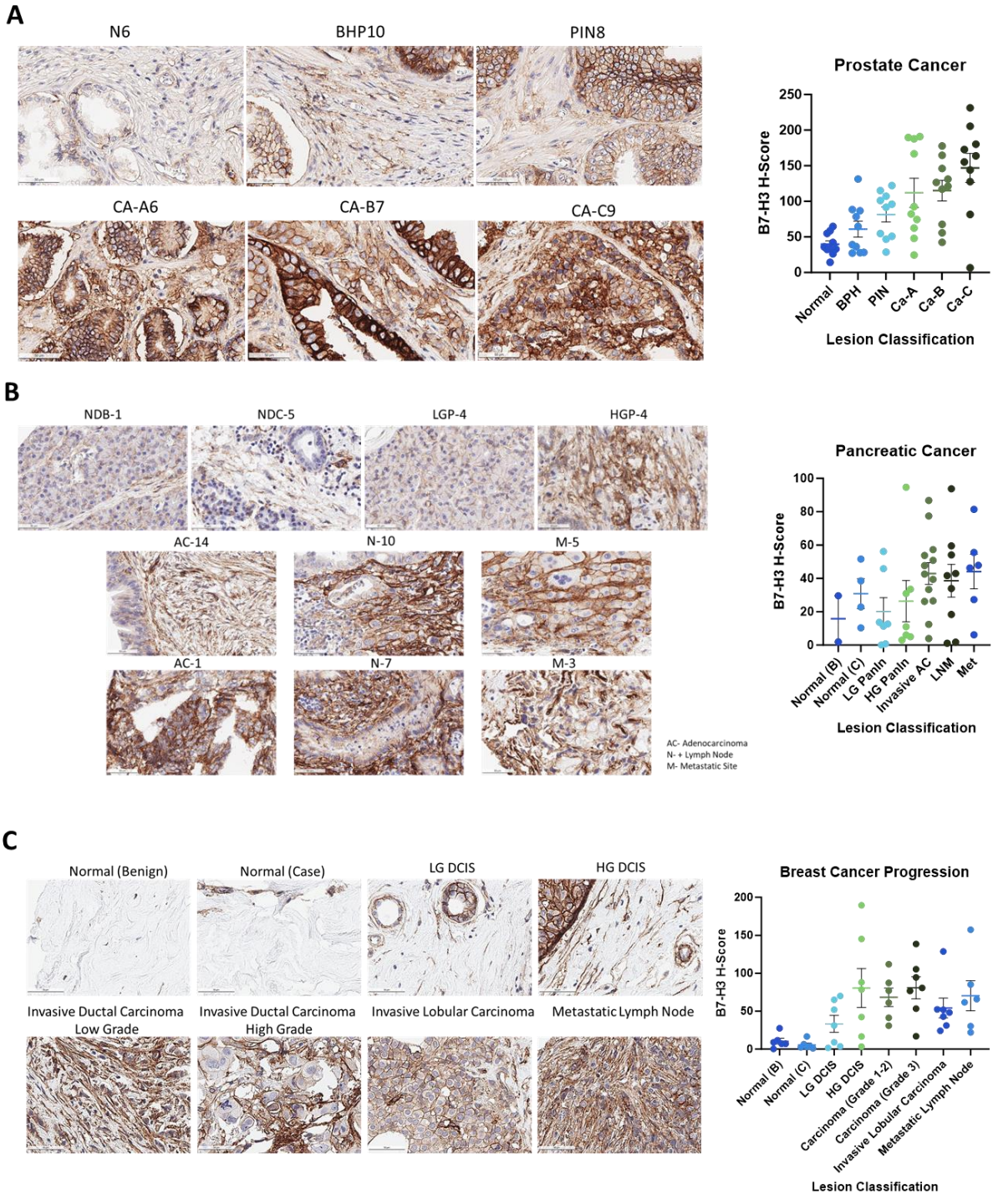
Supplemental Figure 12. Immunohistochemical staining for B7-H3 of patient tissue microarrays, representative images for scored tumor and stroma from 0-3 are depicted. Scale bar represents 250 µm.

Supplemental Figure 13



Supplemental Figure 13. Kaplan-Meier curves for patient survival based on tumor and stromal staining scores of B7-H3 protein.

Supplemental Figure 14



Supplemental Figure 14. Immunohistochemical staining of B7-H3 of patient tissue microarrays for tumor progression across multiple disease sites. Representative images of B7-H3 staining across progression of disease and corresponding H-Score per arrayed tissue spot as determined by automated quantification of the staining for prostate cancer (A), pancreatic cancer (B), and breast cancer (C). Scale bar represents 50 μm.

Supplemental Table 1. Wiki 2019 Pathway Analysis list of p-Values for by term for pathway enrichment.

Term	P-value
miRNA targets in ECM and membrane receptors WP2911	0.000239501
Alzheimer's disease WP2059	0.003394807
Regulation of Wnt/B-catenin Signaling by Small Molecule Compounds WP3664	0.017707727
Hypothesized Pathways in Pathogenesis of Cardiovascular Disease WP3668	0.025937094
Statin inhibition of cholesterol production WP430	0.030027116
Matrix Metalloproteinases WP129	0.031047063
Photodynamic therapy-induced HIF-1 survival signaling WP3614	0.038158147
Metabolic reprogramming in colon cancer WP4290	0.043207035
Glycolysis and Gluconeogenesis WP534	0.046224242
PI3K-Akt signaling pathway WP4172	0.048926293

Supplemental Table 2. Clinicopathological Variables for Ovarian Cancer TMA

Clincopathological variable		n
Histologic types		
Low-grade serous carcinoma		22
High-grade serous carcinoma		250
Other/Unknown		12
Age at diagnosis (years)		(STD)
Mean	60.45	12.3
Median	61.2	
Range	21.7-92.4	
FIGO Stage		
Stage I		18
Stage II		11
Stage III		182
Stage IV		62
Unknown		11
Diagnosis		
Clear Cell		8
Endocervical		2
Endometrial Papillary Serous Carcinoma		1
Endometrioid Adenocarcinoma		15
Malignant Mixed Mullerian Tumor (MMMT)		6
Mixed Type Carcinoma		38
Mucinous		12
Serous Carcinoma		189
Transitional Cell Carcinoma		5
Undifferentiated Carcinoma		8

Supplemental Table 3. CyTOF panel antibodies.

TaggedAbs.description	Target	Label	IntracellularStaining	Clone	Specificities	Source	Catalog
Ly-6G 141Pr	Ly-6G	141Pr	FALSE	1A8	Ms	DVS-Fluidigm	3141008B
Caspase 3(Cleaved) 142Nd	Caspase 3, cleaved	142Nd	TRUE	D3E9	Hu, Rt, Ms, crossed	DVS-Fluidigm	3142004A
TCRb(Ms) 143Nd (MDA)	TCRbeta	143Nd	FALSE	H57-597	Ms	Tonbo	70-5961-U100
IL-2(Ms) 144Nd	IL-2	144Nd	TRUE	JES6-5H4	Ms	DVS-Fluidigm	3144002B
CD274(Ms) 145Nd	CD274, PD-L1, B7-H1	145Nd	FALSE	10F.9G2	Ms	Tonbo	70-1243-U100
CD8a(Ms) 146Nd (MDA)	CD8a	146Nd	FALSE	53-6.7	Ms	BioLegend	100702
CD223(Ms) 147Sm	CD223, LAG-3	147Sm	FALSE	C9B7W	Ms	BioLegend	125202
CD11b 148Nd	CD11b	148Nd	FALSE	M1/70	Ms, Hu	DVS-Fluidigm	3148003B
CD19(Ms) 149Sm (MDA)	CD19	149Sm	FALSE	4D5	Ms	BioLegend	115502
Ly-6C 150Nd	Ly-6C	150Nd	FALSE	HK1.4	Ms	DVS-Fluidigm	3150010B
CD335(Ms) 151Eu	CD335, NKp46	151Eu	FALSE	29A1.4	Ms	BioLegend	137625
CD3e 153Eu	CD3e	153Eu	FALSE	D4V8L	Ms	CST	99940BF
IFNa(Ms) 154Sm	IFNa	154Sm	TRUE	RMMA-1	Ms	PBL	22100-1
MPO 155Gd	MPO	155Gd	TRUE	EPR20257	Hu, Ms, Rt	Abcam	ab221847
CD4(Ms) 156Gd	CD4	156Gd	FALSE	GK1.5	Ms	Tonbo	70-0041-U100
Foxp3(Ms) 158Gd	Foxp3	158Gd	TRUE	FJK-16s	Ms, Rt, Bv, Cn, Po, Fe	DVS-Fluidigm	3158003A
IL-12(Ms) 159Tb	IL-12	159Tb	TRUE	C15.6	Ms	BD	554477
CD44 160Gd	CD44	160Gd	FALSE	IM7	Hu, Ms, Ch, Rh	BioLegend	103002
iNOS(Ms) 161Dy	iNOS	161Dy	TRUE	CXNFT	Ms	DVS-Fluidigm	3161011B
TIM-3(Ms) 162Dy	TIM3, CD366	162Dy	FALSE	RMT3-23	Ms	DVS-Fluidigm	3162029B
CD152(Ms) 163Dy	CD152, CTLA-4	163Dy	FALSE	9H10	Ms	BioLegend	106202
CD62L(Ms) 164Dy	CD62L	164Dy	FALSE	MEL-14	Ms	DVS-Fluidigm	3164003B
IFNg(Ms) 165Ho	IFNg	165Ho	TRUE	XMG1.2	Ms	DVS-Fluidigm	3165003B
Arginase-1 166Er	Arginase-1	166Er	TRUE	Polyclonal	Hu, Ms	DVS-Fluidigm	3166023B
IL-6(Ms) 167Er	IL-6	167Er	TRUE	MP5-20F3	Ms	DVS-Fluidigm	3167003B
CD206(Ms) 169Tm	CD206, MMR	169Tm	TRUE	C068C2	Ms	DVS-Fluidigm	3169021B
CD49b(Ms) 170Er	CD49b, Integrin Î±2	170Er	FALSE	HMÎ±2	Ms	DVS-Fluidigm	3170008B
CD279(Ms) 171Yb (29F.1A12)	CD279, PD-1	171Yb	FALSE	29F.1A12	Ms	BioLegend	135202
Granzyme B 173Yb	Granzyme B	173Yb	TRUE	GB11	Hu, Ms	DVS-Fluidigm	3173006B
I-A/I-E(Ms) 174Yb	I-A/I-E, MHC-II	174Yb	FALSE	M5/114.15.2	Ms	DVS-Fluidigm	3209006B
CD127(Ms) 175Lu	CD127, IL-7Ra (Ms)	175Lu	FALSE	A7R34	Ms	DVS-Fluidigm	3175006B
CX3CR1(Ms) 176Yb	CX3CR1	176Yb	FALSE	SA011F11 SA011F11 SA011F11	Ms	BioLegend	149002
CD11c(Ms) 209Bi	CD11c	209Bi	FALSE	N418	Ms	DVS-Fluidigm	3209005B
CD45(Ms) 89Y	CD45(Ms)	89Y	FALSE	30-F11	Ms	DVS-Fluidigm	3089005B
B7-H3	CD276 (B7-H3)		FALSE	AF1397	Hu, Ms	R&D Systems	

Supplemental Table 4. HTS Screening Parameters

Category	Parameter	Description
Assay	Type of assay	Split Luciferase Complementation, Cell Viability
	Target	4Ig-B7-H3 homodimerization
	Primary measurement	Change in luminescence
	Key reagents	D-Luc, Doxycycline,
	Assay protocol	30,000 cells were pretreated with doxycycline (500 µg/mL) and D-luciferin (600µM) for 48 hours. Luminescence was read using a Tecan Infinite M1000, prior to the addition of compounds in nanoliter volumes using the LabCyte Echo 550 acoustic transfer platform. Following 4 hr incubation at 37°C, luminescence was re-read. Final cell viability was measured 72 hours post compound addition using CellTiter-Blue (Promega) fluorescence assay.
	Additional comments	Final DMSO concentrations were maintained at 0.3% for all wells excluding those containing cells with medium alone
Library	Library size	5362 compounds
	Library composition	Small molecules with
	Source	Texas A&M IBT High Throughput Screening Core
	Additional comments	
Screen	Format	384-well plate
	Concentration(s) tested	1.0 µM
	Plate controls	DMSO, DOX-, DOX+
	Reagent/ compound dispensing system	LabCyte Echo 550
	Detection instrument and software	Tecan Infinite M1000, Magellan software
	Assay validation/QC	Assay QC was performed using interleaved-signal format to assess inter- and intra-assay variation over multiple days. Coefficients of variation were calculated for each signal on each plate and all found to be less than 20% Signal windows and Z' factors for each plate were ≥ 2 or ≥ 0.4, respectively.
	Correction factors	Screen was performed in biological duplicate, a counter screen was performed to eliminate inhibitors of split luciferase complementation. Validation was performed in an orthogonal luminescence readout, and using time- and dose-dependent Removal of compounds where the variation in pre-read between duplicate assays was ≥ 10%
	Normalization	Robust Z-scores were used to normalize the change in luminescence across plates for untreated, and DMSO control wells.
	Additional comments	Active compounds were further refined to "On-target" compounds following the counter screen using K-Ras split-luciferase ReBil 2.0 assay.
Post-HTS analysis	Hit criteria	Change in Robust Z-score
	Hit rate	0.65%
	Additional assay(s)	CellTiter-Blue for cell viability and general toxicity
	Confirmation of hit purity and structure	Replicate assays were confirmed using commercially purchased compounds, validated through NRM
	Additional comments	Secondary assays (Homo- and HeteroFRET-FLIM) were performed to confirm the inhibition of 4Ig-B7-H3 dimerization with statins.