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Supplemental Information

FOXD3 Regulates VISTA Expression in Melanoma

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Supplementary Information

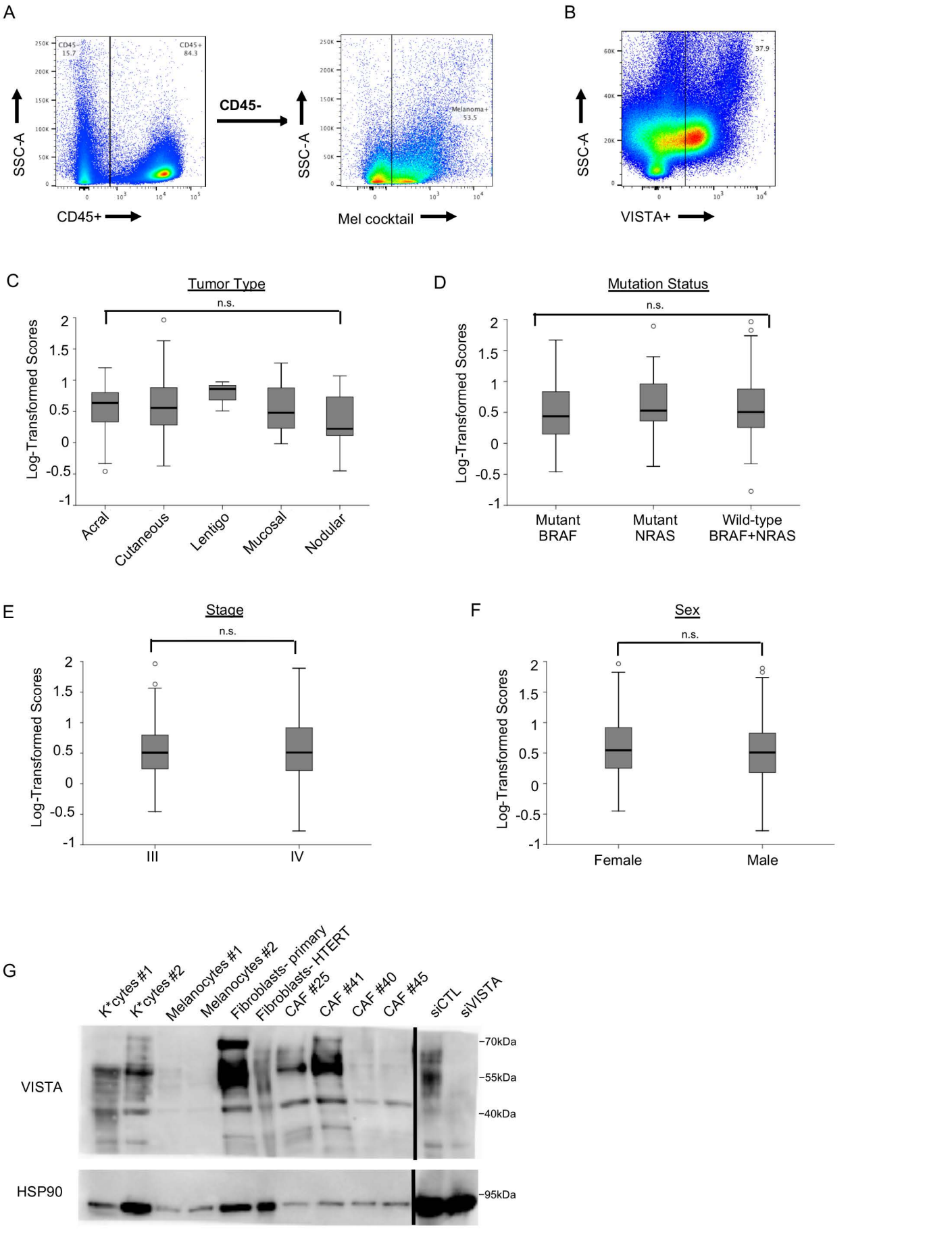
Supplementary Figures

Supplementary Figure 1, Related to Figures 1 and 2: VISTA is expressed in patient samples

A) Related to Figures 1 and 2, shown is the gating strategy. Cells were gated for live cells and CD45 positivity was used to select for immune cells. To select for melanoma cells, the CD45 negative population was additionally gated for positive staining of a melanoma antibody cocktail against GP100, Melan-A, and S100. B) VISTA gating was determined by comparing fluorescence levels to negative controls and by assessing the separation of positive and negative populations across the whole sample and within individual cell subsets. Once a gate was determined, it was applied to all cell subsets within the same sample. Shown is a representative gate of all Live Cells analyzed for VISTA staining. C-F) A tissue microarray of melanoma patient samples was stained for VISTA by immunohistochemistry. The number of VISTA-positive tumor cells was determined for each sample by a pathologist and the number of large, round nuclei was determined by Visiopharm. Each score value was divided by the number of nuclei in that sample, and log-transformed (-log10(score)). Shown are box plots average scores for all expressing (non-zero) patient samples comparing across C) Tumor Type D) Mutation Status E) Stage and F) Sex. Scores from patients with multiple samples were averaged. Patients with no listed mutation status were omitted from graph D. The dark line in each box plot represents the median of the scores for that group, and the circles represent outliers. Significance was tested using a non-parametric Mann-Whitney U Test. All graphs were created using IBM® SPSS software (v25). G) Cell lysates were generated from primary cultures of keratinocytes, melanocytes, and fibroblasts, and from immortalized fibroblasts (HTERT). Primary cultures of cancer-associated fibroblasts (CAFs) from different patients (each patient

indicated by a different #) were characterized by expression or upregulation of α-SMA, PDGFRα or FAP and lack of SOX10 or BRAF V600E and cell lysates were generated. VISTA expression was probed by Western blot. Lysates from siRNA knockdown of VISTA or a non-targeting control in 1205Lu cells were used as controls for each blot.

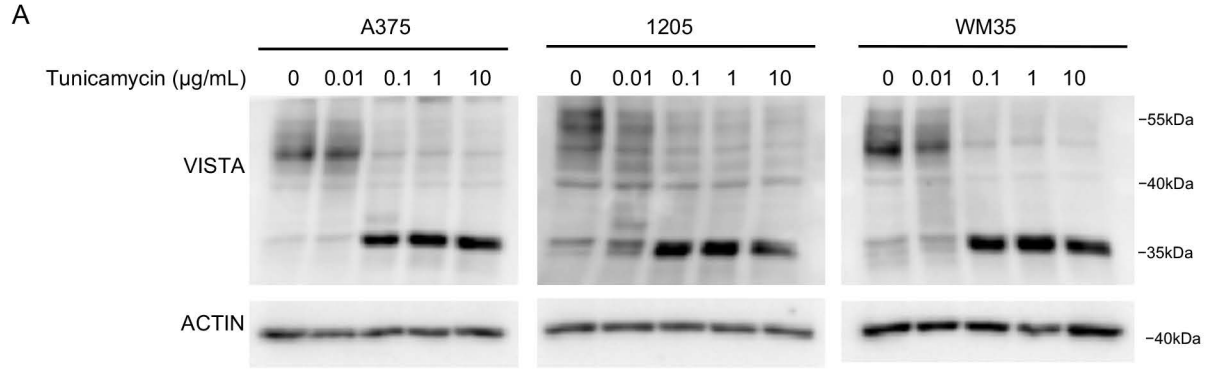
Supplementary Figure 1, Related to Figures 1 and 2

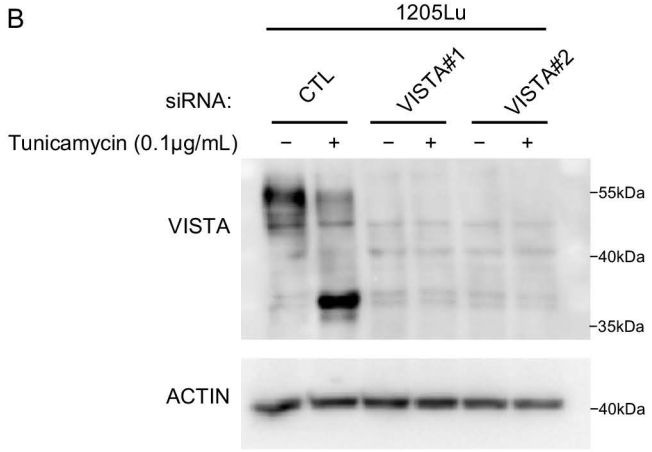


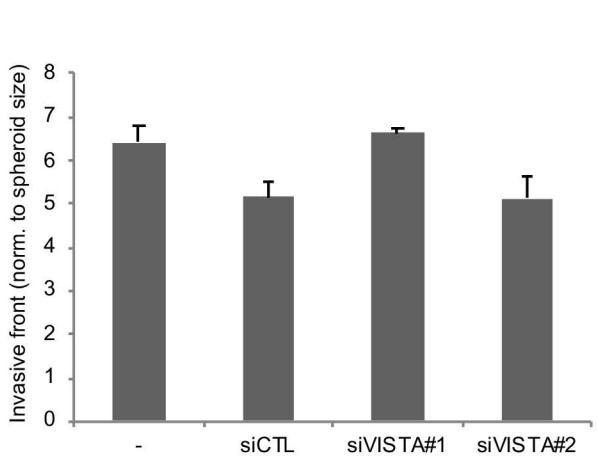
Supplementary Figure 2, Related to Figures 2 and 3: VISTA is N-linked glycosylated in melanoma cell lines, and has minor effects on wound closure and growth

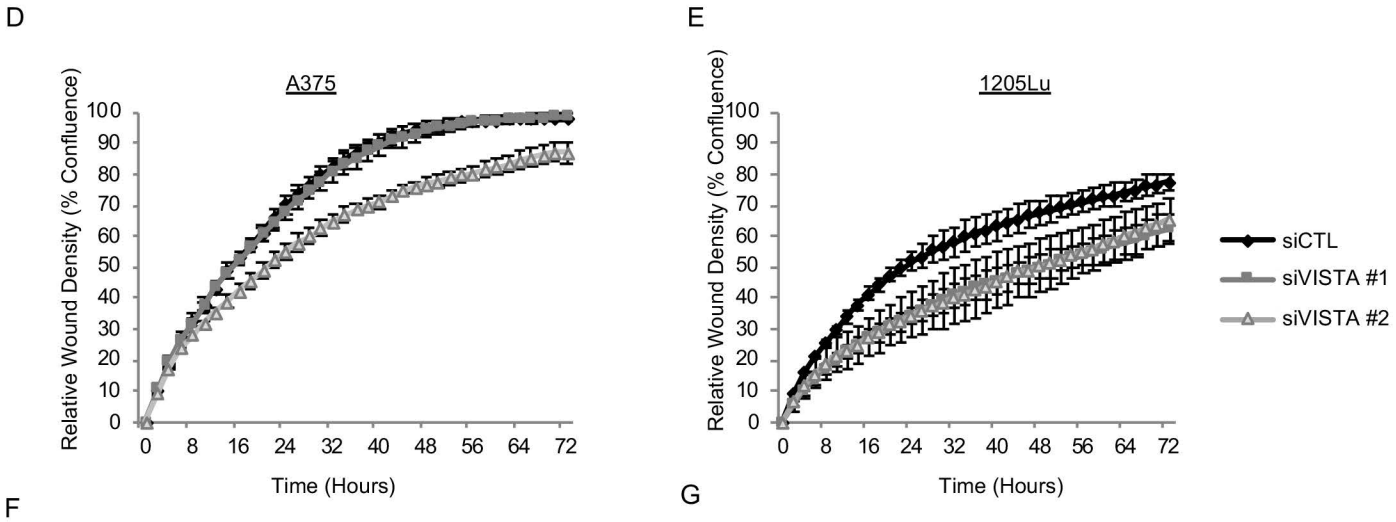
A) Cells were treated with varying concentrations of the N-linked glycosylation inhibitor tunicamycin for 24 hours. Cell lysates were analyzed by western blot. B) Cells were transfected with two different siRNAs targeting VISTA or a non-targeting control for 72 hours. Cells were treated with or without 0.1µg/mL tunicamycin for an additional 24 hours, then lysates were probed by western blot. C) siRNA transfection was utilized to knockdown VISTA. 24 hours post transfection, cells were plated onto agarose to form spheroids. Cells were allowed to form spheroids for 4 days, then placed into 3D collagen matrix and incubated for an additional 2 days. Cells were incubated with calcein to mark live cells, and images were taken. The area of the invasive front was quantified using ImageJ and normalized to the area of the spheroid. D-E) siRNA transfection was utilized to knockdown VISTA. 72 hours post transfection, cells were plated onto a 96-well plate. The Incucyte® WoundMaker™ was utilized to create a uniform scratch across all wells. Cells were imaged by the Essen Incucyte® Zoom Live cell imager and relative wound density was quantified as percentage confluence within the original scratch. F-G) siRNA transfection was utilized to knockdown VISTA. 72 hours post transfection, cells were re-plated and imaged by the Essen Incucyte® Zoom Live cell imager and cell growth was quantified as percent plate confluence.

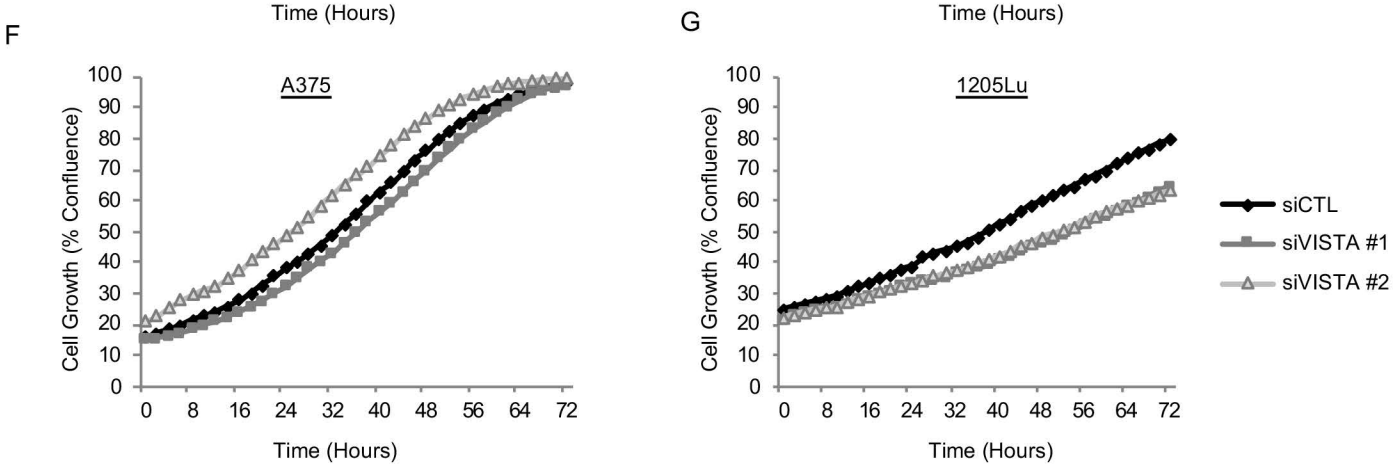
Supplementary Figure 2, Related to Figures 2 and 3







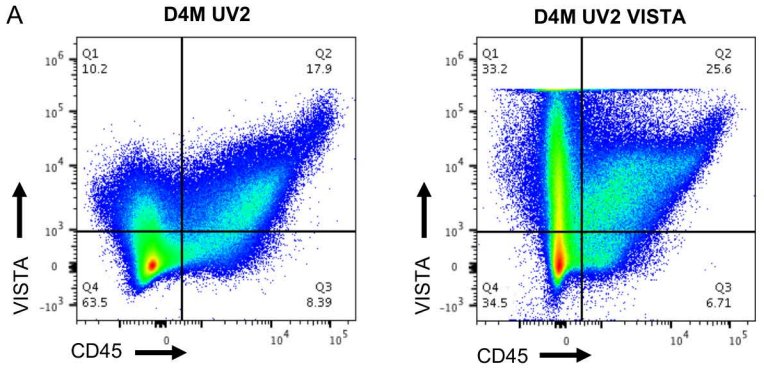




Supplementary Figure 3, Related to Figure 3: Tumor-specific expression of VISTA does not affect long-term survival of mice but alters tumor growth at early time points

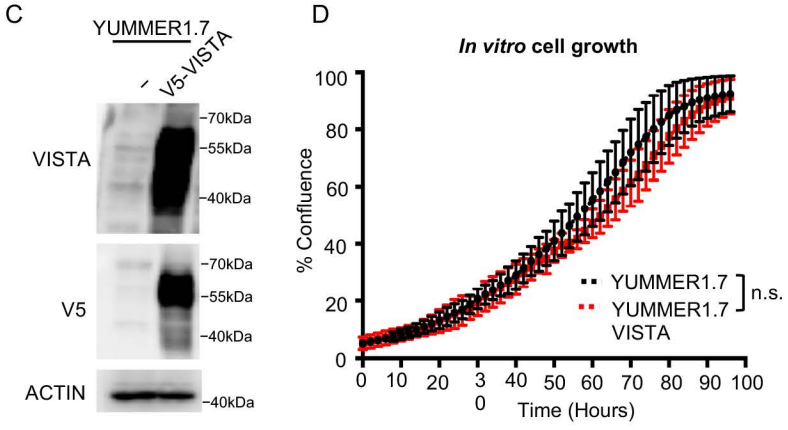
A) Related to Figure 3; *in vivo*, CD45- cells express VISTA in D4M UV2 tumors. Both expression level and percentage of CD45- cells that stain positive for VISTA increase in tumors from VISTA-expressing D4M UV2 cells. B) Related to Figures 3D and E, shown is the tumor incidence ratio, recorded as the number of tumors recorded as >50mm^3 on each measurement day out of the total number of animals injected. C) YUMMER1.7 cells were engineered to express VISTA, and expression was verified by western blot. D) *In vitro* cell growth of YUMMER1.7 cells expressing VISTA was evaluated using the Incucyte® Live Cell Imager. No significant difference in cell growth was found. Data are representative of three independent experiments. E) Related to Figure 3F, shown is the tumor incidence ratio, recorded as the number of tumors >50mm^3 on each measurement day out of the total number of animals injected. F) The poorly immunogenic cell lines, D4M and YUMM1.7, were engineered to express VISTA. G) D4M cells (3X10^5) were injected and tumor growth was tracked every 2-3 days. Tumors were considered fully formed when they exceeded 50mm^3. No significant difference was observed in time to tumor formation. Data were collected from a total of 7 mice per group, combined from two independent experiments. H) YUMM1.7 cells (3X10^5) were injected as in (G). No significant difference was observed in time to tumor formation. Data were collected from a total of 6 mice per group. I) Tumors from Figure 3D-E were allowed to grow until they exceeded 750mm^3. Only early time points (days 2, 5, and 7) showed significant differences in growth (n=6 mice per group). *p<0.05, **p<0.01, ***p<0.001. J) Related to I, shown is a Kaplan Meier Survival curve.

Supplementary Figure 3, Related to Figure 3



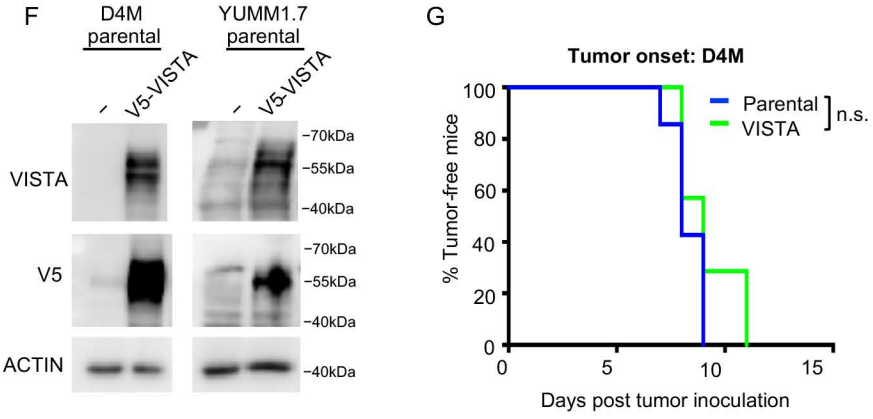
B

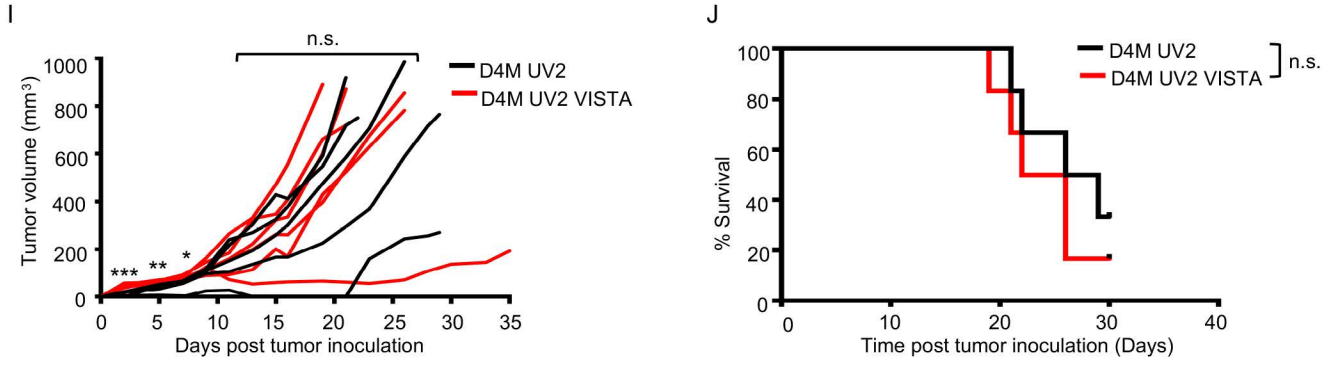
Tumor incidence ratio (mice with tumors/total injected mice)		
Days	D4M UV2	D4M UV2 VISTA
2	0 / 18	2 / 18
5	1 / 18	11 / 18
7	10 / 18	15 / 18
9	14 / 18	17 / 18
11	14 / 18	17 / 18
13	15 / 18	17 / 18
23	17 / 18	17 / 18



E

Tumor incidence ratio (mice with tumors/total injected mice)		
Days	YUMMER1.7	YUMMER1.7 VISTA
5	0 / 6	0 / 6
7	0 / 6	1 / 6
8	1 / 6	3 / 6
9	2 / 6	5 / 6
10	3 / 6	6 / 6
21	4 / 6	6 / 6
39	4 / 6	6 / 6



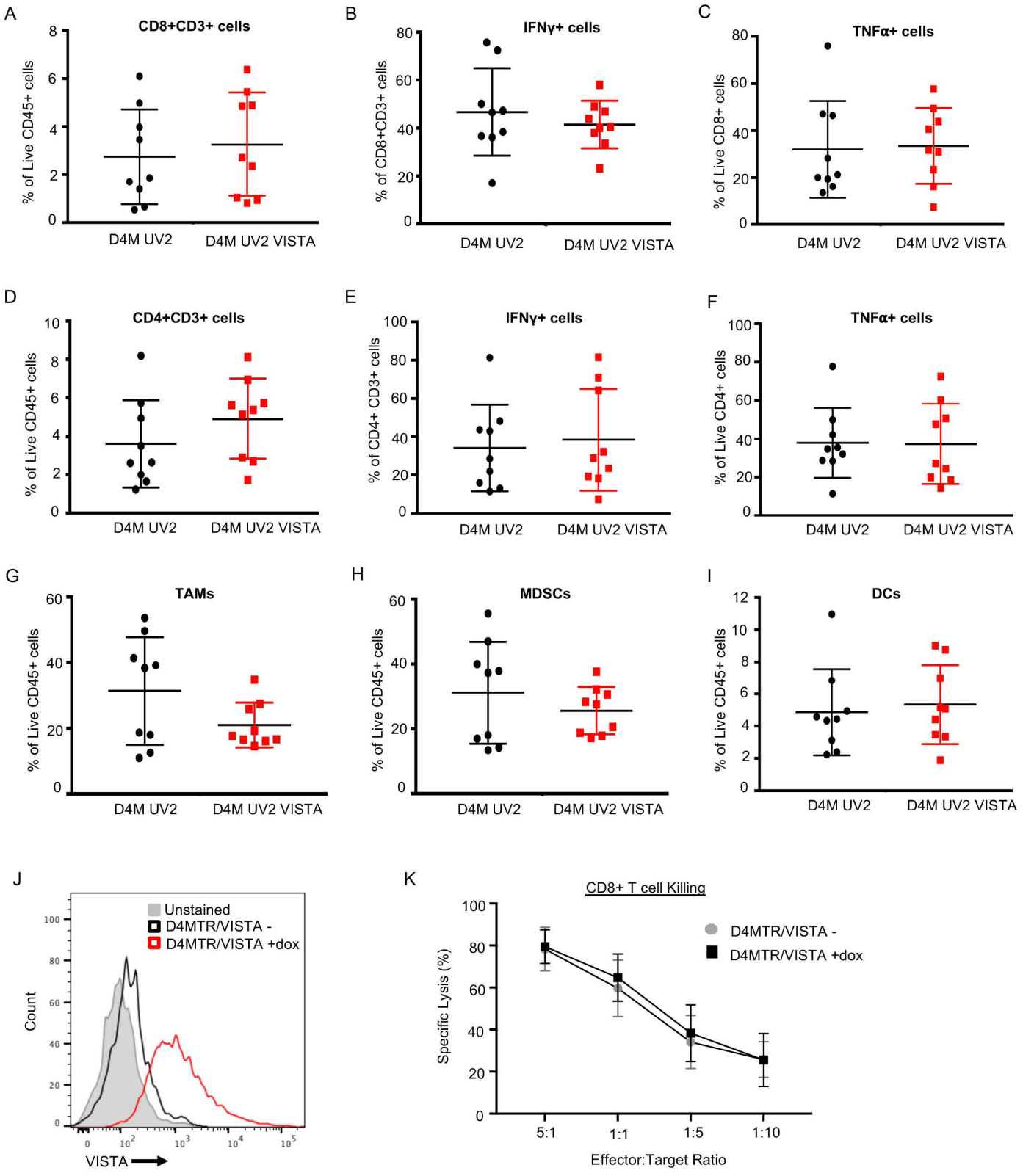


Supplementary Figure 4, Related to Figure 4: Tumor-specific expression of VISTA does not significantly alter T cell activation potential or infiltration of T cells and innate immune cells

A) Related to Figure 4, mice were sacrificed 7 days after tumor injection. Immune infiltrates were analyzed by flow cytometry. Live CD45+ cells were analyzed for the presence of CD8+CD3+ T cells and quantified as a percentage of total Live CD45+ cells. B) Cells were stimulated with PMA + ionomycin + golgi plug for 5 hours and then analyzed by flow cytometry staining. Live CD8+CD3+ cells were analyzed for IFNγ positivity and quantified as a percentage of CD8+ T cells. C) As in B, Live CD8+CD3+ cells were analyzed for TNFα positivity and quantified as a percentage of CD8+ T cells. D) As in A, Live CD45+ cells were analyzed for the presence of CD4+CD3+ T cells. E) As in B, Live CD4+CD3+ cells were analyzed for IFNγ positivity and quantified as a percentage of CD4+ T cells. F) As in C, Live CD4+CD3+ cells were analyzed for TNFα positivity and quantified as a percentage of CD4+ T cells. G) As in A, cells were gated as Live CD11b+F4/80+CD3-CD45+for Tumor Associated Macrophages (TAMs) and quantified as a percentage of total Live CD45+ cells. H) As in A, cells were gated as Live CD11b+GR-1+CD3-CD45+for Myeloid-Derived Suppressor Cells (MDSCs) and quantified as a percentage of total Live CD45+ cells. I) As in A, cells were gated as Live F480-CD11c+MHCII^{hi}CD3-CD45+ for dendritic cells (DCs) and quantified as a percentage of total Live CD45+ cells. No statistical significance ($p<0.05$) was observed in A-I. J) D4MTR/VISTA cells were treated with or without doxycycline (100ng/mL) for 48 hours to induce expression of VISTA. Levels of VISTA were analyzed by flow cytometry at the conclusion of the T cell cytotoxicity assay. K) CD8+ T cells were expanded from OT-1 transgenic mouse spleens (specific for OVA peptide) and activated *in vitro* with OVA peptide (257-264) and IL-2. D4MTR/VISTA cells were treated with or without doxycycline for 48 hours prior to the assay to induce VISTA expression. Cancer cells were pulsed with or without OVA peptide (257-264), and labeled with high (15μM) or low (5μM) concentrations of carboxyfluorescein succinimidyl ester

(CSFE) to distinguish pulsed versus non-pulsed cells. Pulsed and non-pulsed cancer cells were plated at a 1:1 ratio, and incubated with T cells at varying ratios for 24 hours. The ratio of $CSFE_{hi}$ to $CSFE_{low}$ cells was analyzed by flow cytometry, and specific lysis (% Specific Lysis = [1-(Non-transferred control ratio/Experimental ratio)] x 100) was calculated at each effector:target ratio tested.

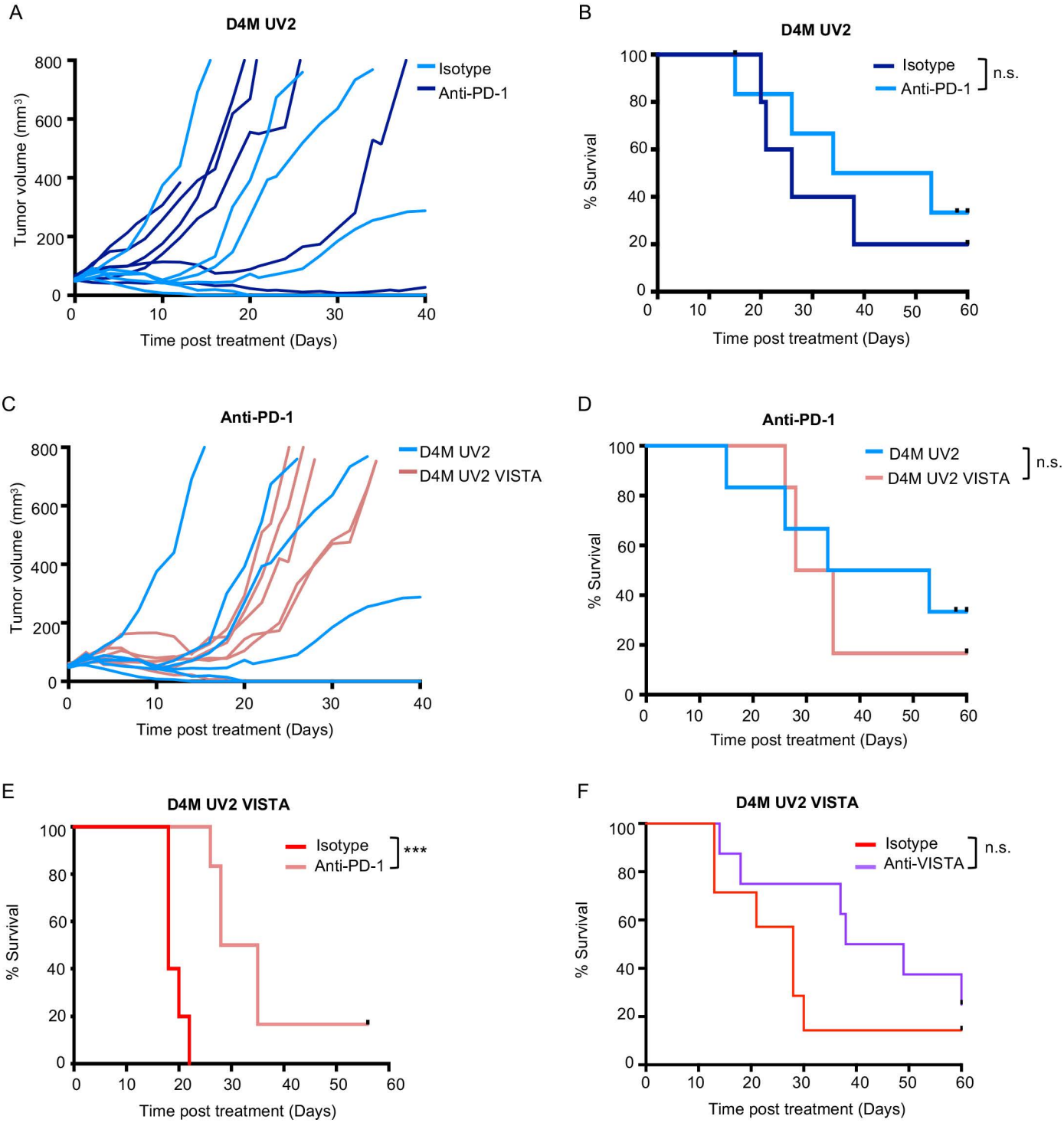
Supplementary Figure 4, Related to Figure 4



Supplementary Figure 5, Related to Figure 4: Tumor-specific expression of VISTA does not significantly alter response to PD-1

A-B) Related to Figure 4G, shown is the growth of each tumor and survival of anti-PD-1 treated mice bearing D4M UV2 parental tumors. The difference in survival was not statistically significant. C-D) Survival and tumor growth differences between anti-PD-1-treated D4M UV2 and D4M UV2 VISTA. Differences in survival were not statistically significant. E) Related to Figure 4G, shown is the survival of anti-PD-1 treated mice bearing D4M UV2 VISTA tumors; ***$p<0.001$. F) Related to Figure 4F, shown is the survival of anti-VISTA treated mice bearing D4M UV2 VISTA tumors. The difference in survival was not statistically significant ($p=0.196$).

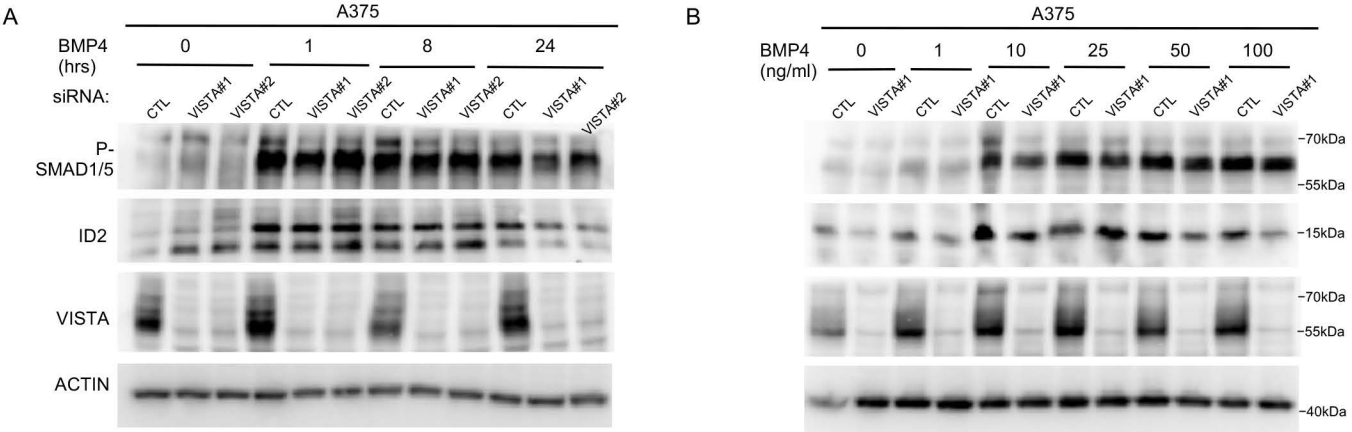
Supplementary Figure 5, Related to Figure 4

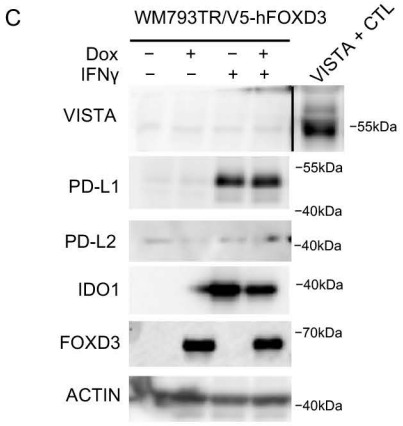


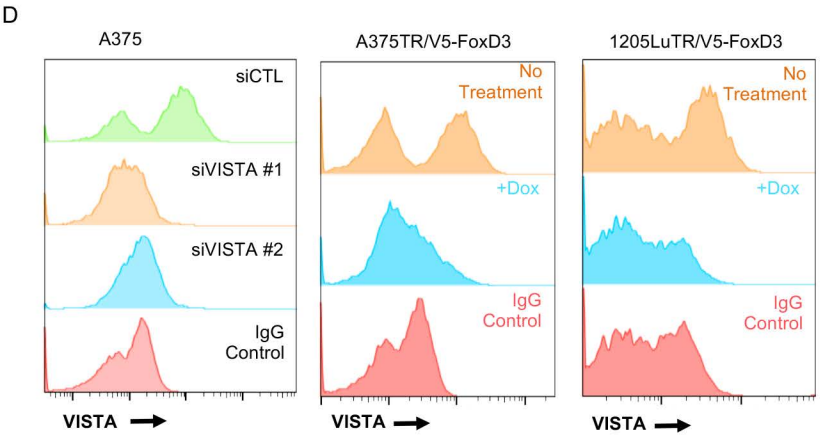
Supplementary Figure 6, Related to Figure 5: VISTA knockdown does not influence BMP4 signaling in melanoma cells, and is co-expressed but differentially regulated from PD-L1

A) siRNA transfection was utilized to knockdown VISTA. 72 hours post transfection, cells were treated with 25ng/mL BMP4 for 0-24 hours, and subsequently lysed. Activation of SMAD1/5 by phosphorylation and upregulation of ID2 induced by BMP4 signaling were probed by western blot. B) siRNA transfection was utilized to knockdown VISTA. 72 hours post transfection, cells were treated with varying concentrations of BMP4 for 8 hours, and subsequently lysed. Activation of SMAD1/5 by phosphorylation and upregulation of ID2 induced by BMP4 signaling were probed by western blot. C) Related to Figure 5, WM793 cells do not express VISTA. WM793 cells also do not exhibit FOXD3-dependent regulation of PD-L1 or IDO1. D) Related to Figure 5, reagents used to detect VISTA expression by flow cytometry were validated by VISTA knockdown. Cells were transfected with non-targeting control or two independent siRNAs targeting VISTA transcripts for 72 hours, and VISTA expression was analyzed by flow cytometry. Shown are representative histograms of VISTA expression analyzed by flow cytometry and quantified in Figure 5. E) TCGA RNA-seq data were evaluated for correlation between levels of VISTA and levels of several common immune checkpoint proteins. F) Patient tumor samples were stained with fluorescent antibodies for flow cytometry analysis. Cells were gated for CD45 positivity to select for immune cells, and VISTA and PD-1 expression levels were determined. Cells were gated for CD45 negativity, then gated for S100/MELANA/GP100 positivity to select for melanoma cells, and VISTA and PD-L1 expression levels were determined. G) Cells were treated with 100ng/mL IFNγ for 48 hours, and lysates were probed by western blot. H) Cells were treated for 48 hours with 5ng/mL TGFβ1, 20ng/mL TNFα, 100ng/mL IL-6, 10ng/mL IL-1α, or 100ng/mL IFNγ, and lysates were probed by western blot. siRNA knockdown controls were transfected with VISTA targeting siRNA #1 or non-targeting control for 72 hours before lysis.

Supplementary Figure 6, Related to Figure 5

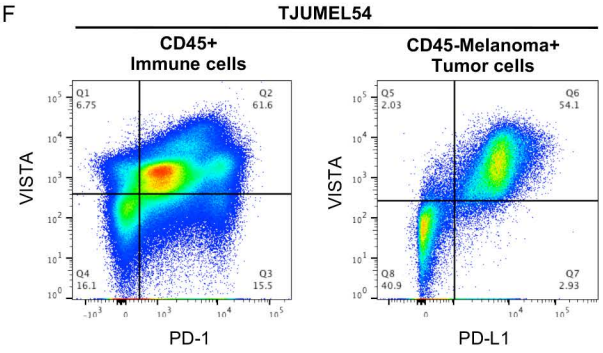


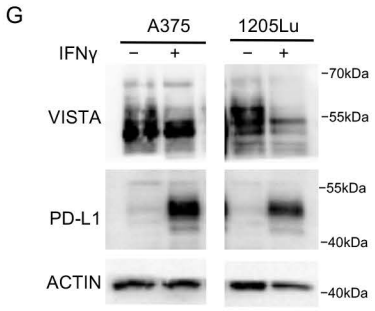


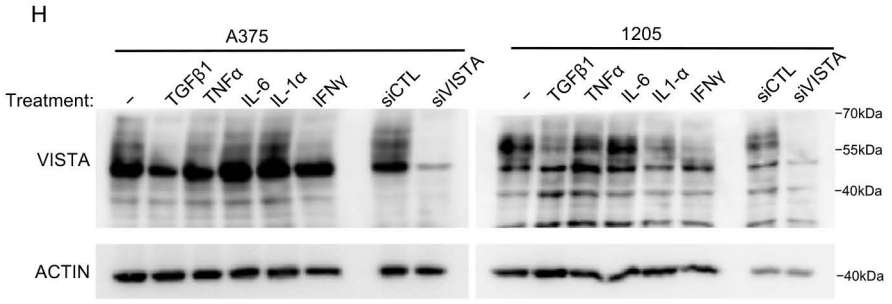


E

Immune Regulatory Protein	Correlation with VISTA	P value
PD-L1	0.477	4.00E-28
PD-L2	0.665	1.82E-61
PD-1	0.693	1.15E-68
CTLA-4	0.503	1.25E-31
TIM-3	0.743	6.99E-84
TIGIT	0.703	1.67E-71
LAG-3	0.653	1.71E-58
OX40L	0.318	1.52E-12
OX40	0.641	6.15E-56



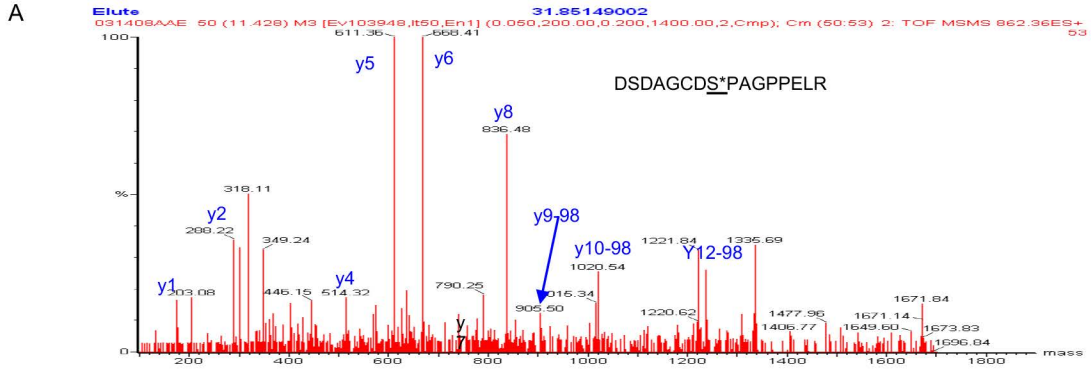


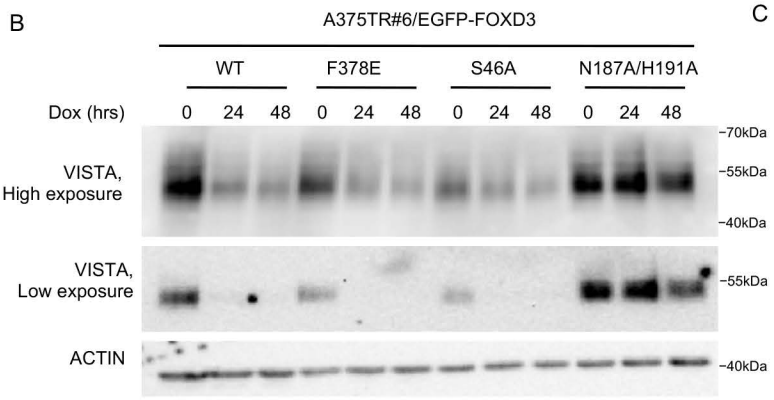


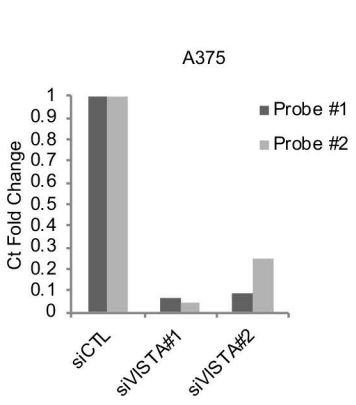
Supplementary Figure 7, Related to Figure 5: FOXD3 directly binds DNA to suppress VISTA transcription

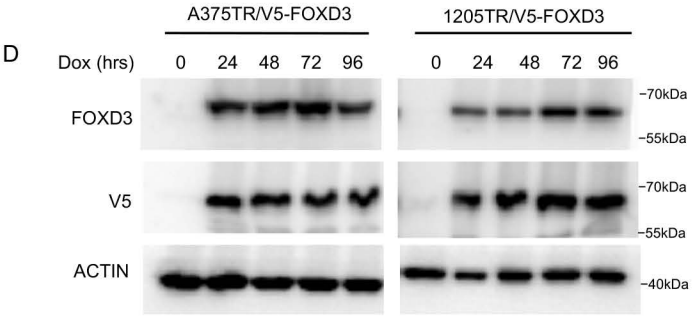
A) We purified expressed FOXD3 from melanoma cells for characterization of phosphorylation sites. We used TiO2-based immobilized metal ion affinity chromatography (IMAC) to enrich the signal of phosphopeptides followed by LC-MS/MS. This resulted in the identification of serine 46 as a major phosphorylation site on FOXD3. Shown is MS/MS spectrum obtained from the fragmentation of the precursor ion at m/z 862.36. The doubly charged precursor of m/z = 862.36 was identified as phosphorylated DSDAGCDSpPAGPPELR. The phosphorylation site is identified as serine-46 by a neutral loss of H_3PO_4 from y_9 fragment after β-elimination. y's or b's represent collision induced fragmentation from either the peptide C terminus or N terminus respectively. B) FOXD3 mutants were generated by site-directed mutagenesis of FOXD3 expression plasmid. Wild-type (WT) or mutant FOXD3 expression was induced by doxycycline treatment (100ng/mL), and lysates were probed for VISTA expression. C) Related to Figure 6, taqman probes used for detection of VISTA by qRT-PCR were validated by siRNA knockdown of VISTA. Cells were transfected with a non-targeting control, or 2 independent sequences targeting VISTA. After 72 hours, RNA was isolated and probed by qRT-PCR using two independent Taqman probes. D) Inducible expression of V5-FOXD3 was probed in a time course of doxycycline (10ng/mL) treatment, as related to Figure 6C. E) Cells were treated with 5mM of the autophagy inhibitor 3-Methyladenine (3MA) for varying lengths of time, and lysates were probed by western blot. F) A375TR and 1205TR were induced to express FOXD3 by doxycycline treatment for 48 hours. Cells were then treated with the proteasomal inhibitor MG132 (10μM) for varying lengths of time, and lysates were probed by western blot. P53 was included as a positive control that is negatively regulated by the proteasome. G) ChIP success was verified by testing samples evaluated in Figure 6E for a known FOXD3 target: ERBB3. Data are representative of three independent experiments.

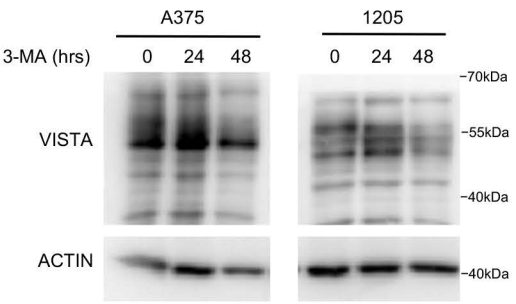
Supplementary Figure 7, Related to Figure 6

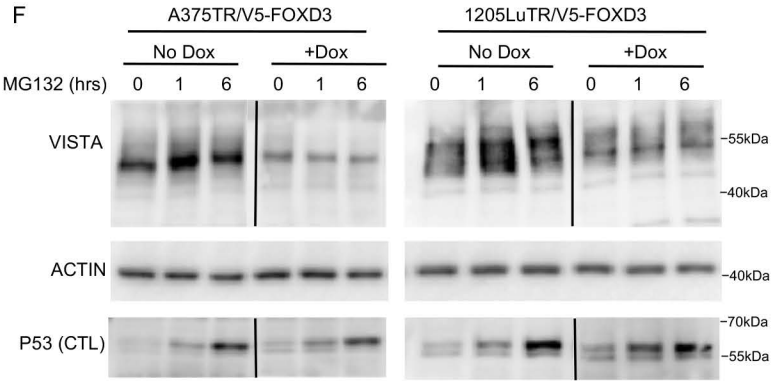


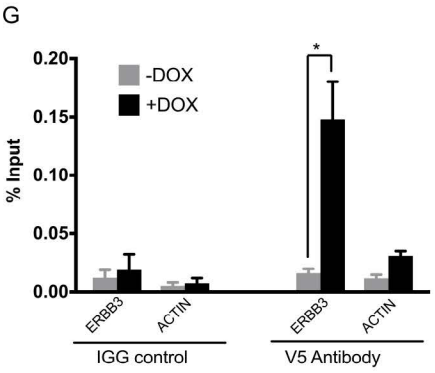












Supplementary Table 1: Patient sample information

Listed are the patient samples analyzed by western blot, IHC, and/or flow cytometry in Figures 1 and 2. Sanger sequencing was completed for the BRAF V600 and NRAS Q61 loci, and mutational status is listed for each sample. When available, tumor site information is also reported. Related to Figure 2, qualitative assessment of tumor-specific VISTA staining by IHC was observed by a pathologist and reported.

Supplementary Table 1, Related to Figures 1 and 2

Sample	Mutation Status	Sex	Tumor site	Tumor-specific VISTA staining
TJUMEL27A	BRAF V600E	Male	Bowel met	Moderate/Low
TJUMEL27B	BRAF V600E	Male	Bowel met	Negative
TJUMEL28	BRAF V600K	Male	Chest nodule punch biopsy	Moderate/Low
TJUMEL28R	BRAF V600K	Male	progression on chemotherapy	Moderate/Low
TJUMEL28RC	BRAF V600K	Male	progression on BRAFi after several treatments including anti-PD-1 and BRAFi/MEKi	Moderate/Low
TJUMEL29	WT/WT	Male	Cervical neck lymph node	Not determined
TJUMEL30	BRAF V600E	Male	Thigh punch biopsy	Not determined
TJUMEL31	BRAF V600E	Female	Thigh subcutaneous met	Moderate/Low
TJUMEL33	BRAF V600E	Female	Arm met	High
TJUMEL36	NRAS Q61R	Female	Superficial inguinal node	Not determined
TJUMEL42	BRAF V600E	Male	Intestinal met	High
TJUMEL43	BRAF V600K	Male	Abdominal met	Moderate/Low
TJUMEL44	NRAS Q61R	Female		Not determined
TJUMEL45	WT/WT	Female	Axiliary node met	Not determined
TJUMEL46	NRAS Q61R	Female	Back axillary met	Not determined
TJUMEL52	NRAS Q61R	Male	Cervical lymph node	Moderate/Low
TJUMEL53	NRAS Q61K	Female	Lymph node met	Moderate/Low
TJUMEL54	BRAF V600E	Female	Axiliary node met	High
TJUMEL55	NRAS Q61K	Male	Axiliary met	Not determined
TJUMEL56	WT/WT	Female	Auxiliary node met	High

Supplementary Table 2: ChIP primers

Primers were designed to amplify a 150bp-250bp region of DNA. A single peak was observed in the melt curve of each primer set, indicating proper detection of the desired amplicon.

Supplementary Table 2, Related to Figure 6

Target	Fwd Primer	Rev Primer
β- ACTIN	AGTGTGGTCCTGCGACTTCTAAG	CCTGGGCTTGAGAGGTAGAGTGT
VSIR Intron 1 Peak #1	CAGGCCAGCACTGCAGACAG	GGACCTTGGTAAATATTTGTGGAATG
VSIR Intron 1 Peak #2	ACTGGGGCTGCCAAGGCTAA	CGCTTCCTTCAATAGAGCCT
VSIR Intron 1 Peak #3	ACTCAAATAAAAGAATGAGTAGATTTAAAG	CACAAACACACACTTCCCCT
ERBB3	ATCCCACCCTCAGTAGACAC	CAACTTTGATTACCCTCCTC