Supplemental information:

Antibodies and constructs used in this study: Antibodies were obtained from the following companies: Cell signaling (Danvers, MA): cleaved caspase-9 (Asp330, human specific), cleaved PARP (Asp214, human specific), cleaved caspase-8, Bid (human specific), Bcl-xL, phospho-Bad (Ser136), PPIA4 (i.e., Cyclophilin A), phospho-Akt Ser473, Akt1 (C73H10). Santa Cruz Biotechnology Inc. (Santa Cruz, CA): phospho-AR (4H24 Ser 213/210), actin (C-11) HRP-linked, Fas (DX2), Fas-L (C-178), DR4 (B-N28), DR5 (DJR2-4). Epitomics: caspase-3 (active), Akt1(c-terminal), phospho-Akt1(pS473), IkappaB-α. Millipore (Billercia, MA): phospho-IkBα(ser32/ser36), Bad (N-term), p85 PI3-kinase (both mouse and rabbit antibodies). Sigma Aldrich (St. Luis, MO): FADD. Novocastra Leica Microsystems (Bannockburn, IL): RHAMM (CD168). Thermo Fischer Scientific (Fremont, CA): androgen receptor Ab-1 (clone AR441). BD Biosciences Pharmingen (San Diego, CA): Bcl-2 monoclonal antibody, Bax. Abcam (Cambridge, MA: DR5 (clone 45B872.1; ab22044). pNF-κB-luc (p-NF-κB-Luc cis reporter plasmid) was purchased from Stratagene, La Jolla, CA. Myr-HA-Akt1 plasmid (myr-Akt; Addgene, Inc, Cambridge, MA). ON-TARGETplus RHAMM siRNA and CD44 siRNA, and Dharmafect 2 transfection reagent were obtained from Thermo Fischer Scientific –Dharmacon RNAi Technologies (Fremont, CA). Lipofectamine 2000 was obtained from Invitrogen, Carlsbad, CA. VEGF ELISA kit was obtained from Research Diagnostics Inc., R&D Systems.

Reagents and kits: HA12K: Lifecore Biomedical Chaska, MN; HA8K: Genzyme Corp., Cambridge, MA; IETD-CHO: EMD Biosciences USA; LY29400: Cayman Chemical Company, Michigan; Cell Death ELISA Plus kit: Roche Diagnostics; Pleasanton, CA; VEGF ELISA kit: Research Diagnostics Inc., Minneapolis, MN R&D Systems; Myr-HA-Akt1 plasmid: Addgene Inc, Cambridge; pNF-κB-luc: Stratagene LA Jolla, CA; iQ SYBR Green Supermix: BioRad, Hercules, CA;

Detailed description of motility and invasion assays: Matrigel™ invasion assay was carried out as described previously (17-19) except that sHA was added in both chambers of the Transwell but not in Matrigel™. The cells in the top chamber were resuspended in RPMI 1640 + ITS (insulin, transferrin and selenium, Sigma Aldrich, St Luis, MO) medium and the bottom chamber contained growth medium as the chemoattractant. For motility assay, 8-μm pore Transwell with similar experimental set up was used and the migration of the cells was assayed after 18 h incubation. In both assays, HA12K (50 μg/ml) was added to the top and the bottom chambers of some wells. The cells in the top and bottom chambers were determined using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), assay (17-19); the cells adhered to the lower surface of the filter, were included in the bottom chamber fraction. To neutralize the effect of sHA on cell growth, percent invasion or motility were calculated as (O.D. bottom chamber ÷ O.D. (top + bottom chambers)) x100.

Detailed description of PI3-kinase immunoprecipitation and PI3-kinase activity assay: LNAI and DU145 cells were treated with 5 μg/ml and 30 μg/ml sHA, respectively. Following incubation, $2x10^6$ cells in control and sHA treated samples were solubilized in RIPA buffer (50 mM Tris.HCl, pH7.4, 137 mM NaCl, 0.06% bovine serum albumin and 1% NP40, 0.1 mM sodium orthovanadate, 1 mM $MgCl_2$, 1 mM $CaCl_2$). The cell lysates were clarified by centrifugation at 14,000 rpm for 10 minutes and then sequentially incubated with a rabbit anti-p85 PI3 kinase antibody (5-μl/ml; Millipore Corporation; Catalog # 06-195) at 4^0 C for 16 -18 hours and 60 μl of Protein A –agarose (Sigma Chemical Co). As control, the cell lysates were immunoprecipitated using rabbit IgG. The immunoprecipitates were sequentially washed in three times each in RIPA buffer, 50 mM Tris.HCl pH7.4, 150 mM NaCl, 5 mM LiCl, 0.1 mM orthovanadate. The beads were washed once with PI3 kinase reaction buffer (Echelon

Biosciences). PI3-kinase activity assay was performed as per the manufacturer's instructions (PI3-kinase pico assay) in duplicate. PI3-kinase activity was expressed as: pmole PI(3,4,5)P3/10^6 cells. Alternatively, the immunoprecipitates were washed once in 50 mM Tris.HCl, pH 7.4 buffer instead of the kinase assay buffer and incubated at room temperature in 1 X SDS sample buffer for 30 minutes with frequent gentle shaking to release the immunoprecipitated proteins. The beads were removed by centrifugation and the immunoprecipitates were subjected to immunoblotting using a mouse anti-p85 PI3-kinase (Millipore Corp), anti-CD44 or anti-RHAMM antibody under reduced conditions.

Detailed description of transient transfection assays with myr-Akt plasmid and CD44 and RHAMM siRNAs: LNAI cells (50,000 – 65,000 cells/12-well plates) were transiently transfected with myr-HA-Akt plasmid (Addgene, Inc., Cambridge, MA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Twenty-four hour following transfection, cells were trypsinized, counted and then replated for cell proliferation, apoptosis and immunoblot and gene expression analyses, in the presence of sHA (0-20 μg/ml). For NFκB-luciferase and PSA-luciferase reporter assays, 24 hour following transfection with myr-Akt or vector, the cells were transfected with pNF-κB-luc, PSAe1p/Luc (28) or pGL4.74[*hRluc*/TK] plasmids. Following 24 hour incubation, the cells were trypsinized, and replated in the presence of sHA. The firefly luciferase and Renilla luciferase activities were assayed 24 hours following sHA treatment using Dual-Glo® Luciferase Assay system (Promega Corp., Madison, SI). For siRNA transfections, DU145 and LNAI (30,000 – 50,000) cells were transfected with On-Targetplus CD44 and/or RHAMM siRNAs or control Non-targeting siRNA using Dharmafect 2 transfection reagent (Thermo Fischer-Dharmacon RNAi Technologies, Fremont, CA). Twenty-four hour following transfection, the cells were trypsinized, and replated for proliferation, apoptosis, and protein and gene expression analysis, as described in the manuscript.

MVD and TUNEL assay: Staining of microvessels was performed as described previously (17-19). Microvessel density (MVD) was determined by counting microvessels in 10 high power fields and expressed as mean ± S.D. using a Nikon H550L microscope with a video screen camera (17-19). Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed to determine the extent of apoptosis in tumor tissues, using the *In situ* Cell Death detection kit (Roche Diagnostics; Indianapolis, IN). The apoptosis index was calculated by counting the number of TUNEL positive cells in three high power fields for each specimen in the vehicle and in the sHA-treated groups and expressed as mean ± S.D.

Supplement Table 1: Primers for Q-PCR: Sequences for the forward and reverse complementary PCR primers used in the Q-PCR analyses of the specific genes are indicated along with the GenBank accession number for the gene. CD44s forward primer is within exon 5 and the CD44s reverse primer is a junction primer between exon 5 and 15, since in CD44-standard form a part of exon 5 is spliced to a part of exon 15. Both CD44v primers are in exon 14 (or variant exon 10).

Gene	Accession #	Forward Primer	Reverse Primer
CD44s	NM_001001391.1	5'CTGTACACCCCATCCCA GAC3'	5'TGTGTCTTGGTCTCTGGTAGC3'
CD44v	NM_001001390.1	5'CAGGTGGAAGAAGAGACCC AA3'	5'GCTGAGGTCACTGGGATGAA3'
PPIA4	BC137058.1	5'ATGGTCAACCCCACCGTGT3'	5'TCTGCTGTCTTTGGGACCTT GTC3'
RHAMM	NM_012484.1	5'CAGCTGGAAGATGAAGAAG GA3'	5'GCATGTAGTTGTAGCTGAAAAGG 3'
VEGF-A	GQ129408.1	5'GCACCCATGGCAGAAGG3'	5'CTCGATTGGATGGCAGTAGCT3'
p53	NM_000546	5' TCAACAAGATGTTTTGCC AACTG 3'	5' ATGTGCTGTGACTGCTTGTAG ATG 3'
Cytokeratin 5	NM_000424.3	5' ATGGCCACTTACCGCA AGCTGCTGGAGGG 3'	5' AAACACTGCTTGTGACAACA GAG 3'
Cytokeratin 8	NM_002273.3	5' CCGTGGTTGTGAAGAAG ATC 3'	5' TGTTCACTTGGGCAGGAC 3'
Cytokeratin 18	NM_000224.2	5'GAGTATGAGGCCCTGCTGAA CATCA3'	5'GCGGGTGGTGGTCTTTTGGAT3'
Androgen Receptor	NM_000044.2	5' TCAACTCCAGGATGCTCT AC3'	5' ACATCCGGGACTTGTGCATG3'

Supplement Table 2: Characterization of prostate cancer cell lines. Cell line characterization: LNCaP and its derivatives (C42B, LNAI) are AR, PSA, p53, and cytokeratin 8, and 18 positive and cytokeratin 5 and CD44 negative (40,45). LAPC-4 cells are AR, PSA, cytokeratin 5, 8 and 18 positive and CD44 negative (45). DU145 cells are AR, PSA, p53 negative and cytokeratin 5, 8, and 18 and CD44 positive. PC-3 cells are AR, PSA negative and cytokeratin 5, 8, and 18 and CD44 positive. PC3-ML is a metastatic PC-3 cell line. Total RNA isolated from prostate cancer cells was subjected to Q-PCR for phenotypic characterization. The transcript levels were normalized to PPIA4. The values shown are normalized mRNA levels; Mean ± S.D.

Transcript	LNCaP	LNAI	C4-2B	LAPC-4	DU145	PC3-ML
Cytokeratin 5	0.018	0.014	0.031	2.33	1.19	0.26
Cytokeratin 8	33.22	20.81	30.7	114.47	57.24	374.51
Cytokeratin 18	86.76	27.84	94.93	5.56	81.79	597.94
Androgen receptor	35.85	3.68	25	3.85	0.002	0.002
P53	7.54	7.20	24.06	65.75	6.79	3.22
CD44s	0.017	0.047	0.26	0.01	26.61	365.53
CD44v	0.008	0.03	0.006	0.07	1.18	7.38

Supplement Table 3: Serum from vehicle and sHA treated animals was sent for analysis to the University of Miami's Pathology Research Resource facility. The data shown are mean ± SD (n = 5 for each group).

Parameter	Vehicle (Mean ± SD)	sHA (50 mg/kg) (Mean ± SD)	t-test	Normal Range
Glucose	129 ± 33	135.4 ± 28.6	P > 0.05	90 - 193
Blood urea nitrogen	20 ± 6.9	24.4 ± 4.3	P > 0.05	18 - 29
Creatinine	0.24 ± 0.05	0.32 ± 0.1	P > 0.05	0.1 – 0.4
Calcium	8.6 ± 2.8	9.6 ± 0.46	P > 0.05	8.6 – 10.1
Protein	4.5 ± 1.5	4.8 ± 0.42	P > 0.05	4.6 – 6.9
Alkaline aminotransferase	45.4 ± 9.7	60.2 ± 19	P > 0.05	29 - 191

Supplement Figure Legends:

Supplement Figure 1: A: Effect of dihydrotestosterone on the growth of LNAI cells. LNAI cells (20,000 cells/well) were exposed to various concentrations of dihydrotestosterone in RPMI 1640 medium containing 10% charcoal stripped fetal bovine serum. Following 48 hour incubation, the cells were counted. As a control, the cells were cultured in complete growth medium and cell counts at 48 hours in this medium were 4.98 ± 0.35. **B: HAase ELISA like assay:** Twenty four hour cultures of LNAI and DU145 cells were incubated in RPMI1640 + ITS medium. Forty eight hours later the conditioned medium was collected and the cells were counted. The conditioned medium was subjected to HAase ELISA-like assay in the presence of various concentrations of sHA as described previously (23). The HAase activity was expressed as unitx10^{-4}/10^{6} cells (Data: Mean ±SD). **C. Time course of sHA effect on cell proliferation.** LNAI cells were treated with sHA (5-μg/ml) and counted every 24 hour. **D. Cell cycle analysis:** LNAI cells were treated with 5 μg/ml sHA for 24 hours. Following incubation the untreated and treated cells were solubilized in a hypotonic PI-dye solution and then analyzed in an EPICS XL flow cytometer, equipped with a long pass red filter, FL3 (630 nm). The FL3 histograms were analyzed for estimating cell cycle phase distribution by Modfit Easy (Lite) program (Veritas Software, ME) (16). The results are an average of duplicate measurements. Control each case indicates samples not treated with sHA. **E: Effect of a cell permeable caspase-8 inhibitor on sHA induced growth inhibition.** LNAI cells (25,000 cells/well) cultured in the growth medium, were incubated with IETD-CHO (caspase-8 inhibitor) for six hours before the addition of sHA. Following 48 hour incubation, cells were counted (and subjected to apoptosis assay using the Cell Death ELISA kit in triplicate. In the right panel % No treatment means apoptosis in no IETD-CHO and no sHA sample was considered as 100% and the results were expressed as % No treatment (Data: Mean ±SD).

Supplement Figure 2: Semi-quantitative PCR analysis of CD44v9 variant in prostate cancer cells. A: Total RNA isolated from prostate cancer cells was reverse transcribed and subjected to PCR using CD44 Exon13 (v9) forward primer: CAGAGCTTCTCTACATCACA and CD44 Exon17 reverse primer: ATGCAAACTGCAAGAATC under following conditions: 95^0C 2 min (1 cycle); 30 cycles of 95^0 C, 1 min, 55^0 C, 1 min, 72 C, 1 min; 1 cycle of 72^0 C 5 min. The PCR product(s) were analyzed by 1.2% agarose gel electrophoresis and eithidium bromide staining. **B: Co-immunoprecipitation and immunoblot analysis of PI3-kinase and associated HA receptors.** LNAI and DU145 cells were treated with sHA (5 μg/ml : LNAI; 30 μg/ml: DU145) for 48 hours. p85 subunit of PI3-kinase was immunoprecipitated from control and sHA treated cells as described in Materials and Methods (Supplemental information). Immunoprecipitates were subjected to immunoblot analysis using mouse anti-p85 PI3-kinase, anti-CD44 or anti-RHAMM antibodies, along with the total cell lysate (shown in the figure is DU145 cell lysate: positive control) under reduced conditions. **C: PI3-kinase activity assay:** The PI3-kinase activity assay was performed using the PI3-kinase pico assay kit as described in Materials and Methods (Supplement information). The pmoles of PIP3 are normalized to immunoprecipitated generated by immunoprecipitating 10^6 cells in each sample. **D: Effect of LY29400 and sHA on cell growth.** LNAI cells (25,000 cells/well) cultured in growth medium were exposed to LY29400 (10 μM) and sHA (0-5 μg/ml). Following incubation the cells were counted (Data: Mean ±sd).

Supplement Figure 3: Measurement of the transcript levels of HA receptors in siRNA transfectants. DU145 and LNAI cells transfected with control (ctr) CD44 and/or RHAMM siRNAs were treated with sHA for 48 hours. Following incubation, the total RNA isolated from the transfectants was subjected to Q-PCR for measuring HA receptor transcripts. The transcript

levels were normalized to PPIA4 transcript levels. DU145 cells: **A:** RHAMM; **B:** CD44. C: LNAI cells, RHAMM levels.

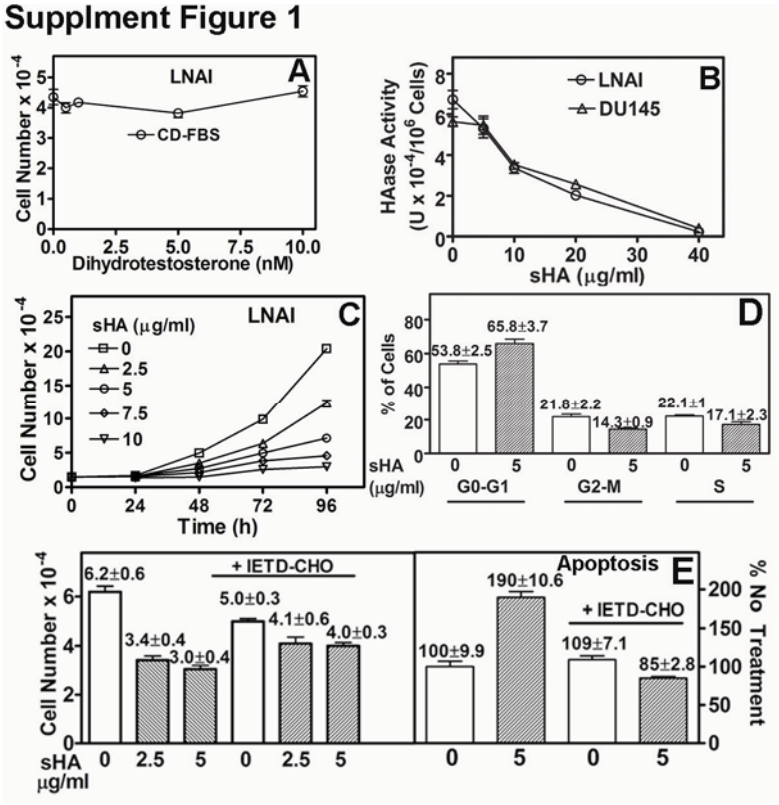
Supplement Figure 4: Effect of CD44, RHAMM and CD44+RHAMM overexpression on sHA induced growth inhibition. cDNA of CD44s isoform was PCR amplified and cloned into pCDNA3.1-v5-His vector. RHAMM cDNA in the same expression vector was kindly provided by Dr. Eva Turley. The cells were transiently transfected with RHAMM, CD44 or CD44+RHAMM expression vectors or the vector using Lipofectamine 2000, as per the manufacturer's protocol. For the CD44+RHAMM co-transfection, half the amount of DNA for each construct was used when compared to the amount used for single transfection. Twenty four hours following transfection, the cells were exposed to sHA and 48 hours later the cells were counted (Data: Mean ±sd). **A and B:** Q-PCR analysis showing RHAMM (A) and CD44 (B) mRNA levels in various transient transfectants. **C:** Cell counts of transfectants exposed to different concentrations of sHA.

Supplement Figure 5: Effect of sHA treatment on animal weight. A: Tumor bearing animals (Figure 6) were weighed weekly during the course of the experiment. Each point represents average weight of animals in each group at each time point. **B:** H&E staining of formalin fixed liver, kidney and lung from vehicle and sHA-treated animals.

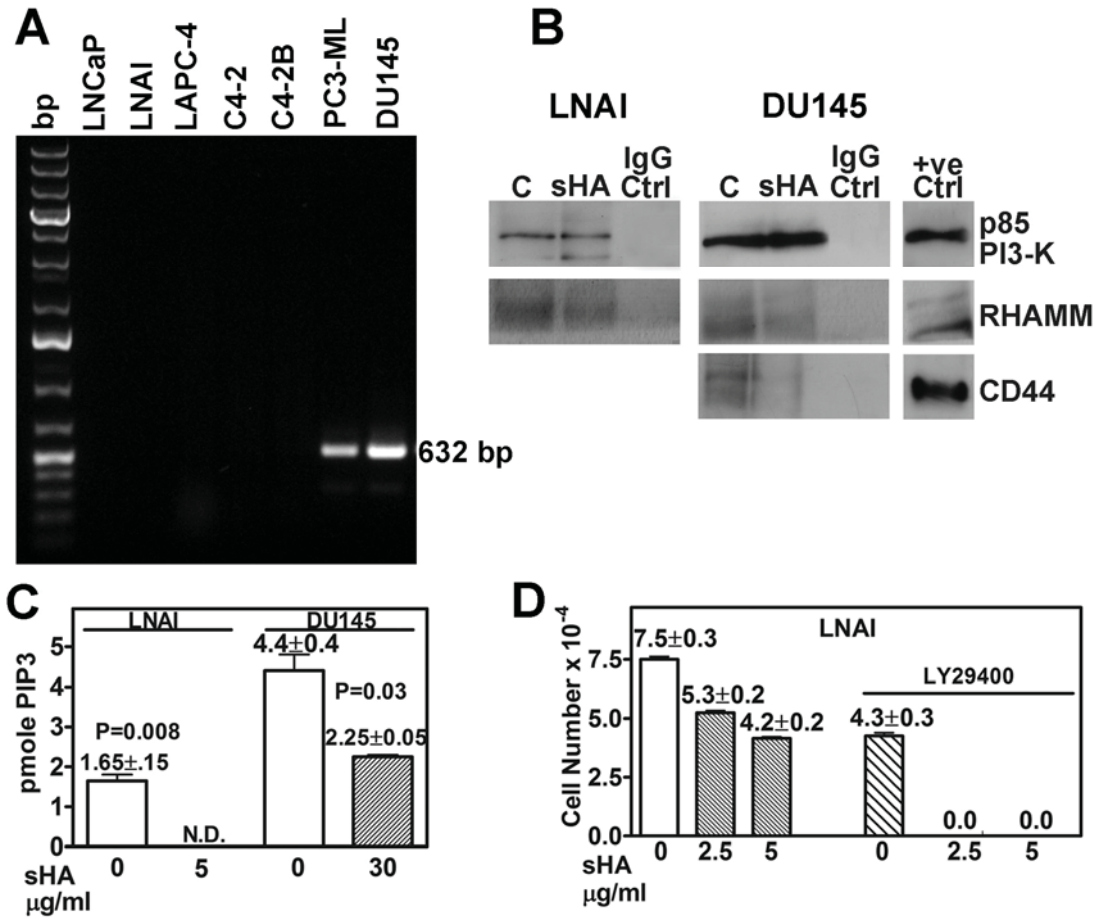
Supplement Figure 6: A: Working model of the tumor-associated HA-HYAL-1 system. HYAL-1 expressed by tumor cells degrades extracellular matrix-associated HA that is synthesized by tumor stroma and tumor cells. Degradation of HA generates angiogenic fragments which bind to HA receptors RHAMM and/or CD44. Please note that RHAMM is not a transmembrane protein and it is exported outside of the cell by unconventional transport mechanism (50). RHAMM associates with CD44 or other proteins on the cell surface (50).

Binding of angiogenic HA fragments to HA receptors induces a complex formation between PI3 kinase and HA receptors, and activates PI3-kinase. PI3-kinase activation induces activation of Akt and downstream signaling. Activation of Akt up-regulates HA receptor expression, which allows continued signaling through angiogenic HA fragment and HA receptor interaction, through a feedback loop. Continuous HA-receptor and PI3-kinase/Akt signaling promotes cell growth, cell survival, NFkB-activation, VEGF expression and cell motility and invasion. **B:** The presence of sHA inhibits angiogenic HA fragment formation, which results in the inhibition of Akt signaling. This in turn down regulates HA receptor expression. Down regulation of the HA-receptor PI3-kinase/Akt feedback loop causes induction of apoptosis, cell cycle arrest, inhibition of cell motility and invasion and downregulation of VEGF expression.

Supplment Figure 1



Supplemental Figure 2



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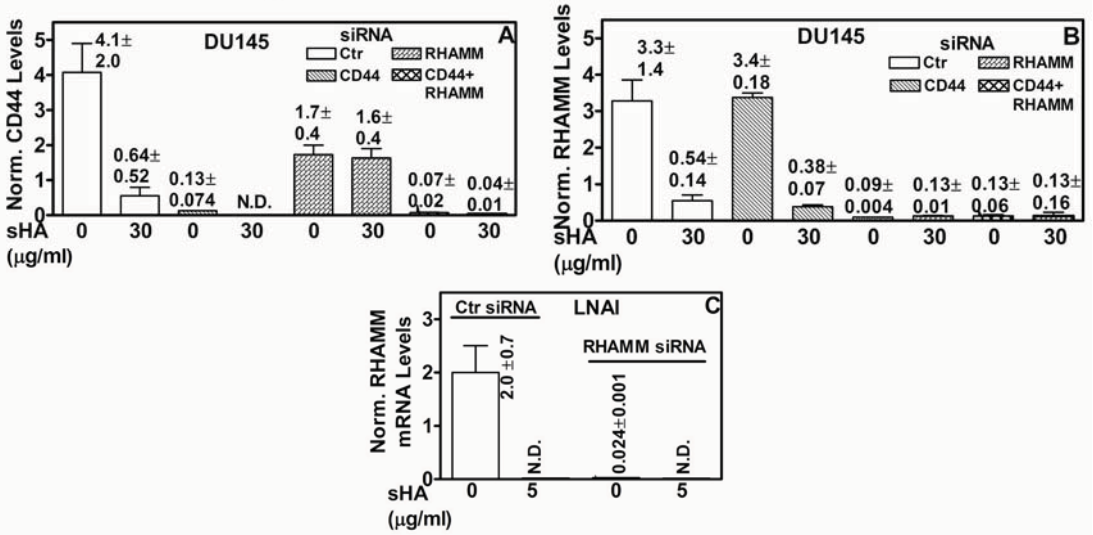

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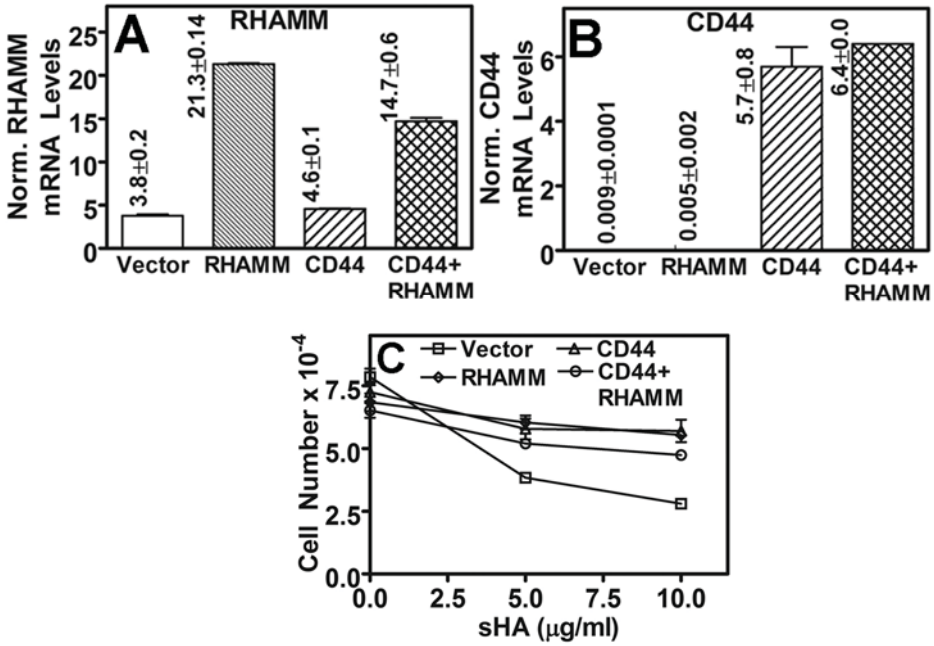

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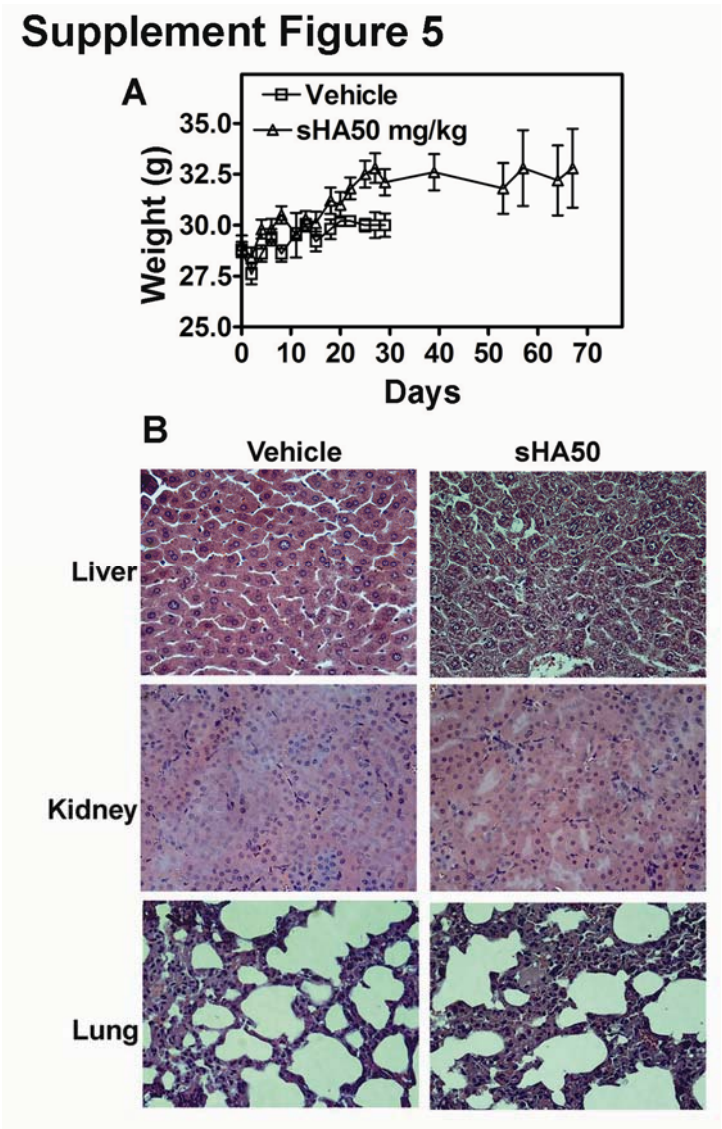

Supplement Figure 6