Adrenergic mediated increases in *INHBA* drive CAF phenotype and collagens

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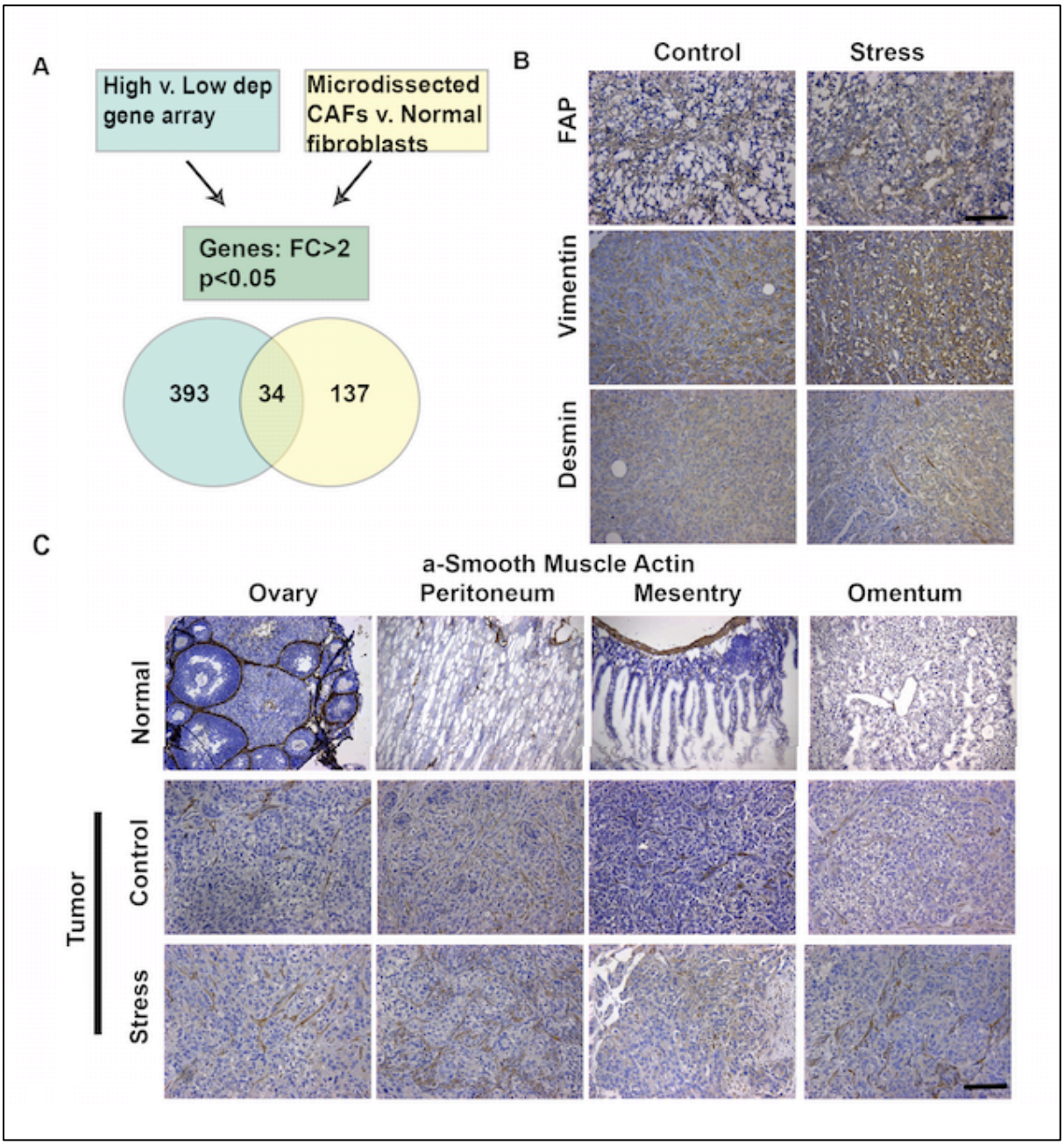
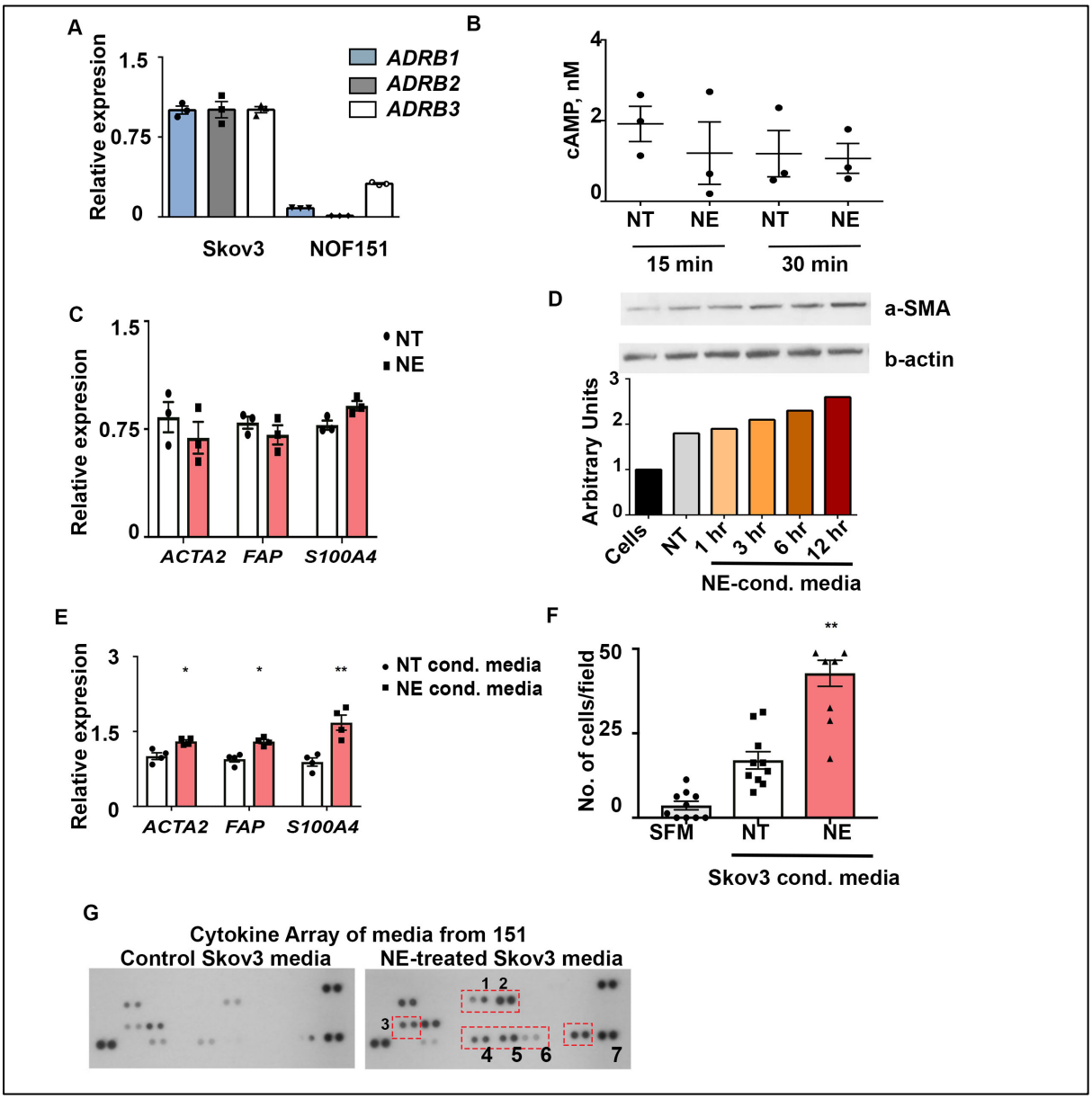
Supplementary Figures and Tables

Supplementary Figures



Supplementary Figure 1: Restraint stress increases CAF content in primary as well as metastatic sites.

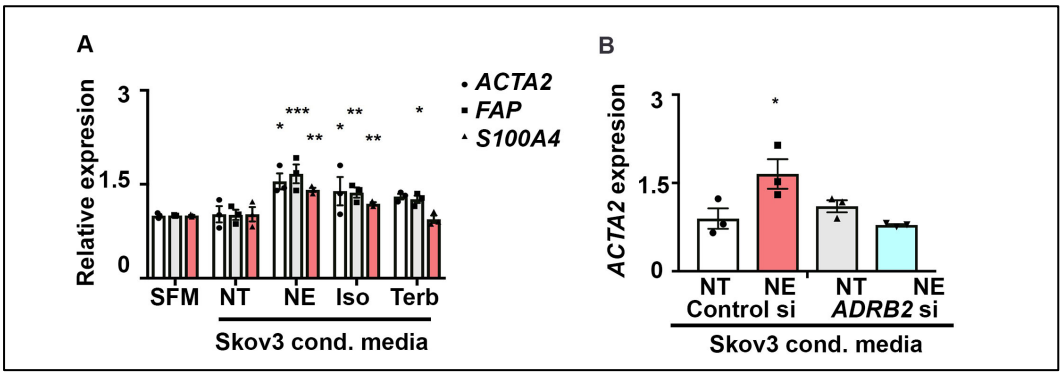
(a) Venn diagram showing comparison of genes that are upregulated 2-fold in tumor samples from ovarian cancer patients with a high depression (dep) score compared to those with a low depression score and in microdissected cancer-associated fibroblasts (CAFs) compared to normal fibroblasts in primary ovarian cancer. FC, fold-change. (b) Expression of CAF marker fibroblast activated protein (FAP), desmin and vimentin in micrographs of representative tumors from control and restraint-stressed mice in the adrenergic-receptor positive Skov3-ip1 model. (c) Expression of CAF marker alpha–smooth muscle actin (α-SMA) in micrographs of representative normal tissues and matched metastatic tumors from the Skov3-ip1 mouse model. Scale bars, 100μM, n=5/group for FAP, Desmin, Vimentin data; n=3/group for metastatic data.



Supplementary Figure 2: Conditioned media from NE-treated cancer cells accelerate transformation of normal ovarian fibroblasts

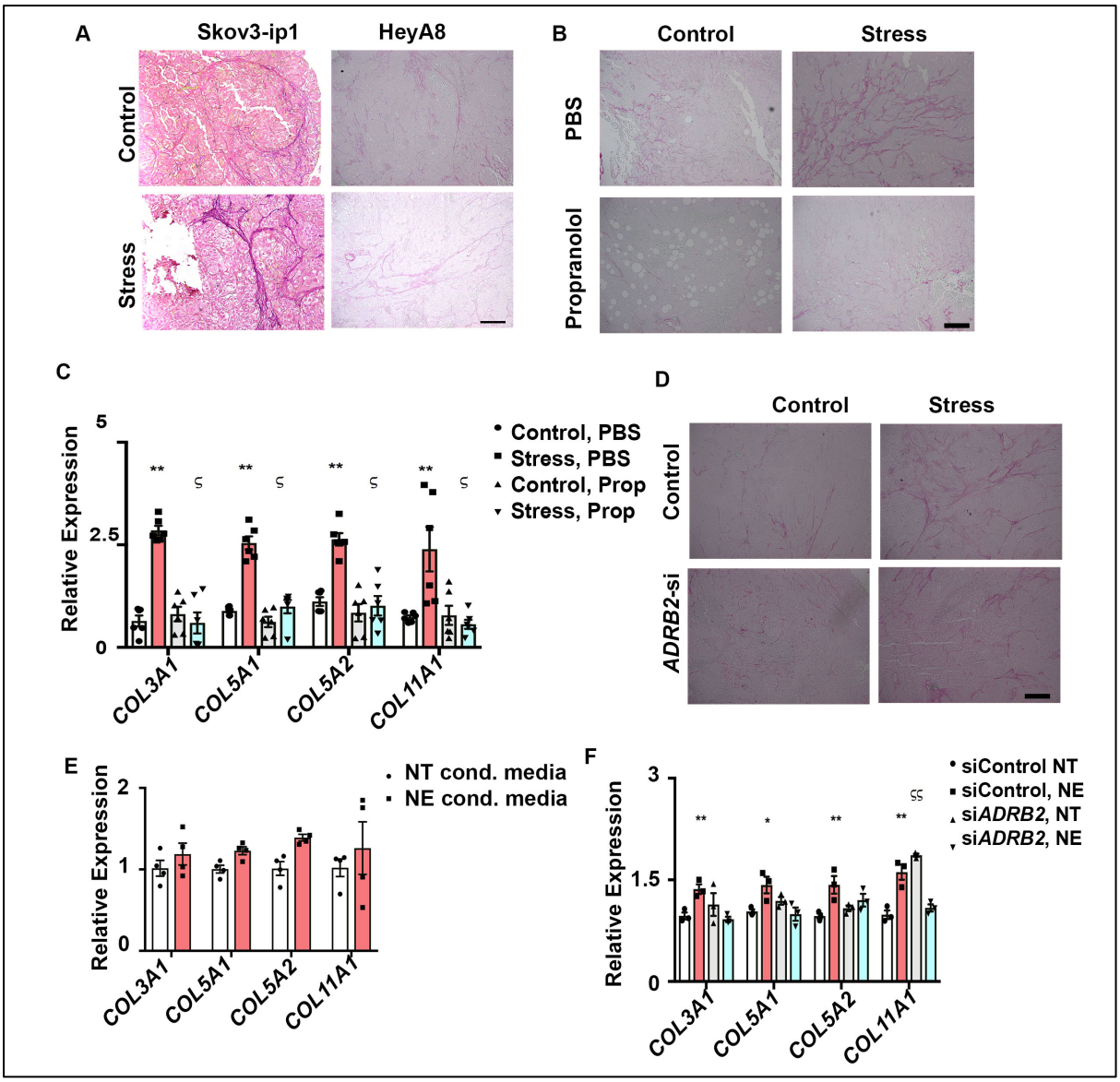
(a) Expression of beta-adrenergic receptors (ADRB) in NOF151 normal fibroblasts relative to ADRB-positive Skov3-ip1 cells. (b) ELISA for cAMP in NOF151 treated with norepinephrine (NE) for 15 or 30 min or not treated (NT). (c) Expression of CAF markers *ACTA2, S100A4,* and *FAP* in NOF151 cells after treatment with NE (NT: non-treated controls). (d) Expression of CAF markers *ACTA2, S100A4,* and *FAP* in NOF151 cells after exposure to medium conditioned by Skov3-ip1 cells treated with NE or control (NT). (e) Expression of CAF marker alpha–smooth muscle actin (α-SMA) in NOF151 cells after exposure to medium conditioned by NE-treated Skov3-ip1 cells. (f) *In vitro* migratory potential of NOF151 cells toward serum-free medium (SFM), medium conditioned by untreated Skov3-ip1 cells, or medium conditioned by NE-treated SKov3-ip1 cells through 0.1% gelatin over 6 h was assessed by fixing and counting the number of cells migrated per high-power field (10 fields/group). (g) Immunoblot multiplex assay for pro-inflammatory cytokines in NOF151 cells exposed to medium conditioned by NE-treated or NT Skov3-ip1 cells. (1: GCSF, 2:GM-CSF, 3:IL1a, 4: CCL2, 5: MIF, 6: CCL3, 7: PAI1). Data are presented as mean±s.e.m. of n=3 for

all experimental groups. Statistical significance was obtained using the 1-way ANOVA: *p < 0.05, **p < 0.01.



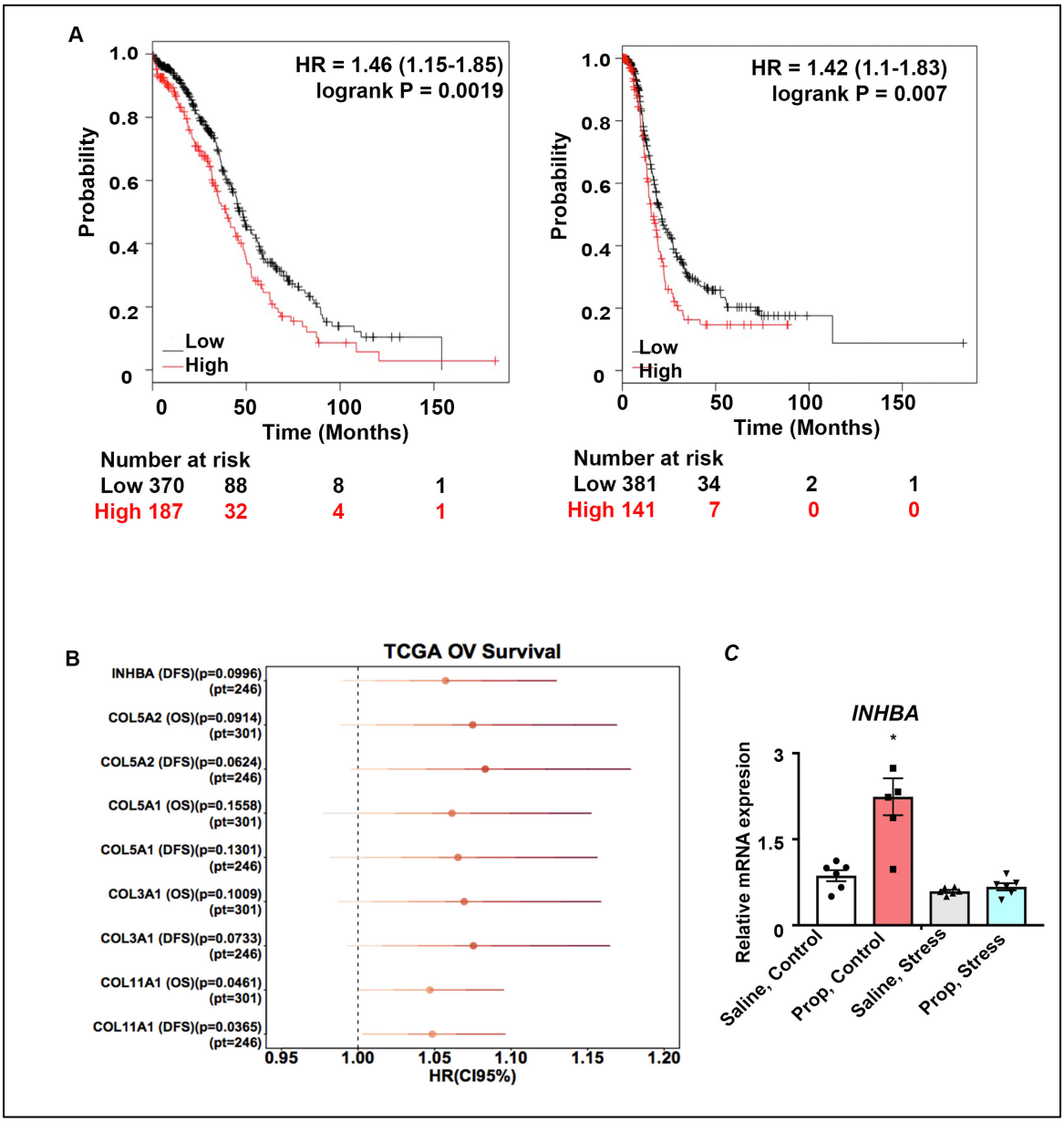
Supplementary Figure 3: Restraint stress increases αSMA in an ADRB2-dependent manner

(a) Expression of CAF markers *ACTA2, S100A4,* and *FAP* in NOF151 cells exposed to serum-free medium (SFM) or to medium conditioned by Skov3-ip1 cells that were untreated (NT) or treated with norepinephrine (NE), nonspecific beta-agonist isoproterenol (Iso), or ADRB2-specific agonist terbutaline (Terb). (b) ACTA2 expression in NOF151 treated with ADRB2 or control siRNA and conditioned with NT or NE-treated Skov3-ip1 cells. Data are presented as mean±s.e.m. of n=3 for all experimental groups. Statistical significance was obtained using the 1-way ANOVA: *p < 0.05, **p < 0.01, ***p<0.001.



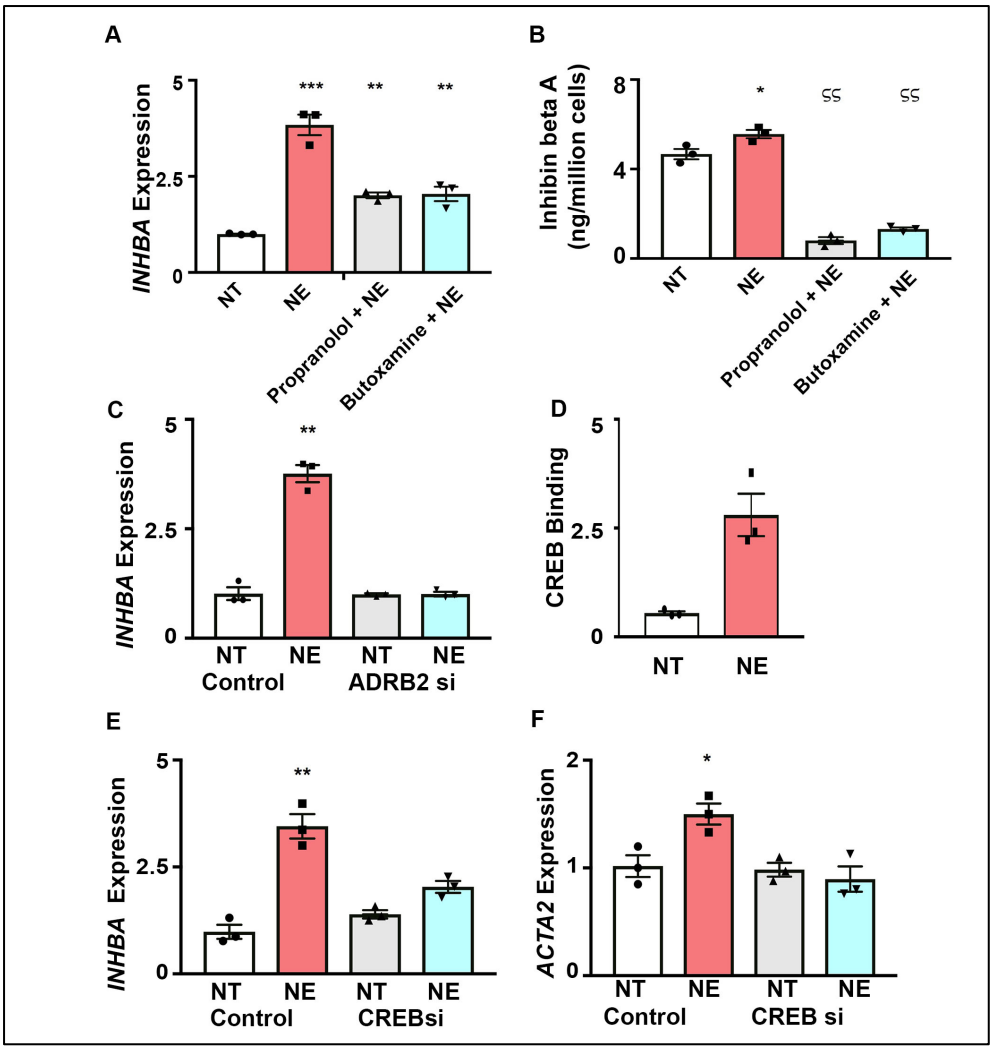
Supplementary Figure 4: Restraint stress increases collagen levels in tumors in an ADRB2 dependent manner

(a) Expression of collagen detected by Sirius staining in micrographs of representative Skov3-ip1 and HeyA8 tumors from control and restraint-stressed mice. (b) Expression of collagen detected by Sirius staining on micrographs of representative HeyA8 tumors from control and stressed mice treated with nonspecific beta-blocker propranolol or PBS. (c) Expression of collagen genes in Skov3-ip1 tumors from control and stressed mice treated with nonspecific beta-blocker propranolol (Prop) or Saline. (d) Expression of collagen detected by Sirius staining in micrographs of representative HeyA8 tumors from control and stressed mice treated with control or ADRB2 siRNA. (e) Expression of collagen genes in NOF151 cells exposed to medium conditioned by untreated (NT) or norepinephrine (NE)-treated Skov3-ip1 cells. (f) Expression of collagen genes in NOF151 cells exposed to medium conditioned by untreated (NT) or norepinephrine (NE) and ADRB2 silenced Skov3 cells. Scale bars, 100μM. Data are presented as mean±s.e.m. of n=3 for all experimental groups. Statistical significance was obtained using the 1-way ANOVA: *p < 0.05, **p < 0.01 compared to controls, $^{\varsigma}$p<0.05 compared to stress or NE-treated cells.



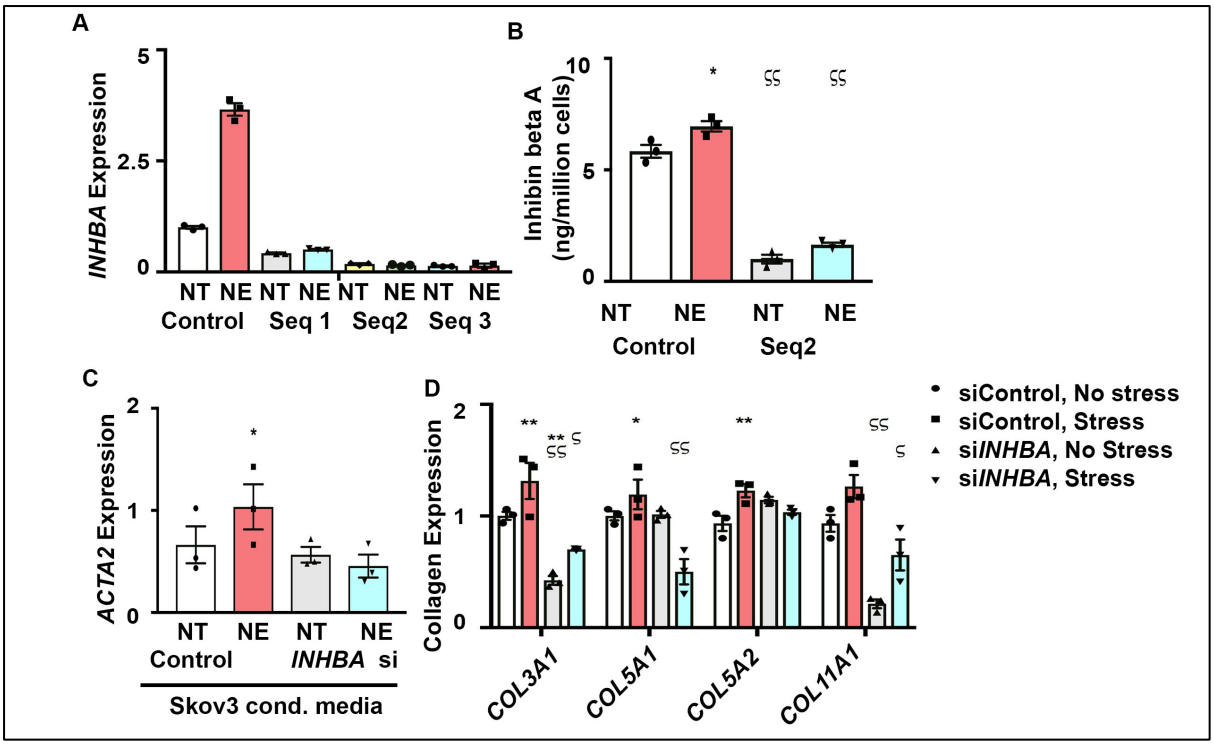
Supplementary Figure 5: INHBA in ovarian cancer is associated with worse PFS and OS.

(a) Kaplan-Meier plots for overall survival (left) and progression-free survival (right) in patients with ovarian cancer, based on expression level of *INHBA*. Data were extracted from The Cancer Genome Atlas database. (b) General plots showing the hazard ratio, confidence interval and p-value for ovarian cancer using TCGA data. (c) Expression of *INHBA* in HeyA8 tumors from control and restraint-stressed mice treated with nonspecific beta-blocker propranolol (Prop) or PBS. Data are presented as mean±s.e.m. of n=5 for all experimental groups. *p < 0.05, compared to controls.



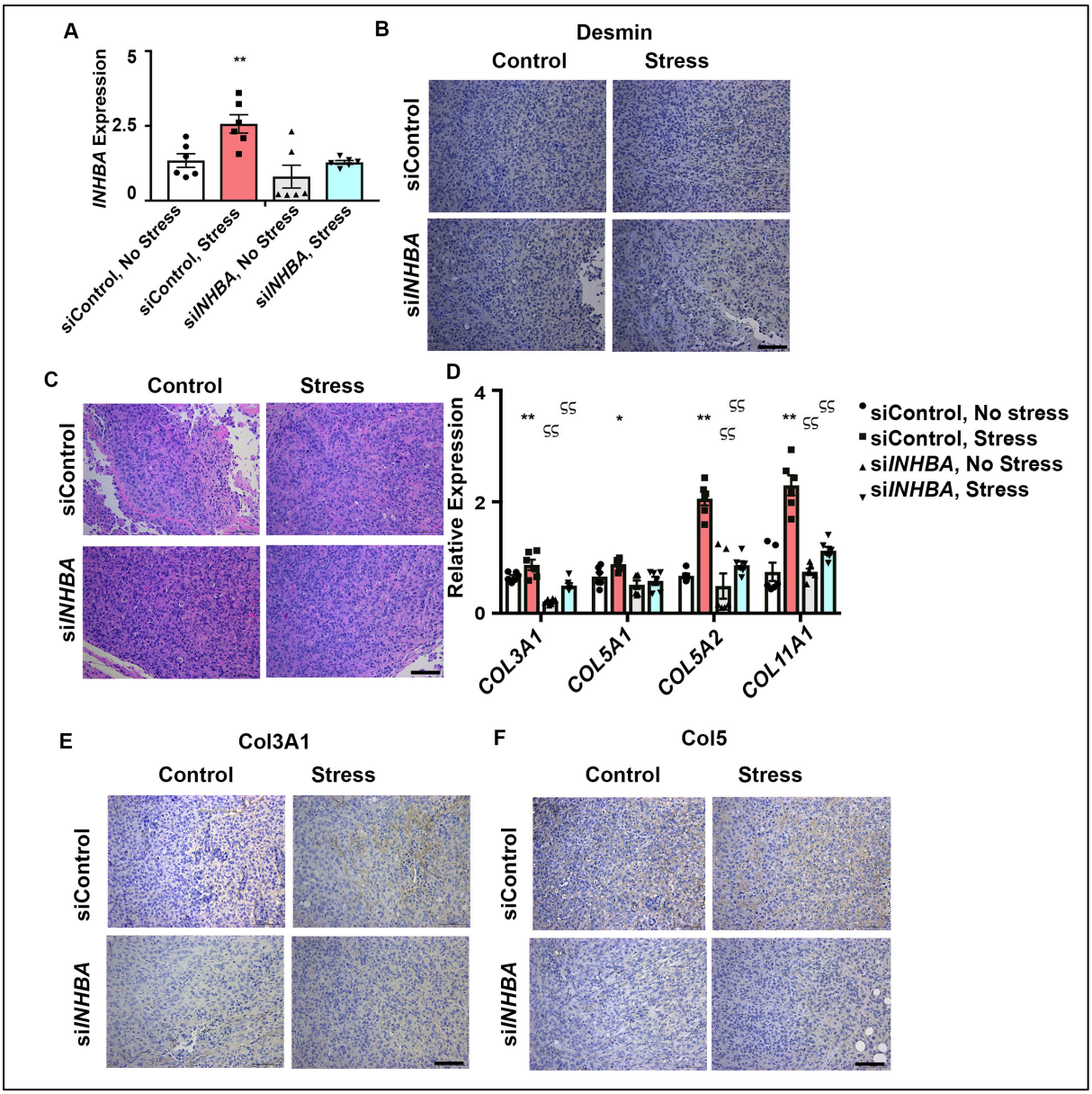
Supplemental Figure 6: INHBA expression in tumor cells is mediated by ADRB2 and CREB

(a) Expression of *INHBA* in Skov3-ip1 cells after no treatment (NT) or treatment with norepinephrine (NE) with or without propranolol or ADRB2-specific blocker butoxamine. (b) Concentration of Inhibin Beta A in HeyA8 cells after no treatment or treatment with NE with or without propranolol, or butoxamine. (c) Effect of silencing ADRB2 on *INHBA* expression in Skov3 cells not treated or treated with NE. (d) Chromatin immunoprecipitation analysis for CREB binding to the *INHBA* promoter in Skov3-ip1 cells not treated or treated with NE. (e) Effect of silencing CREB on *INHBA* expression in Skov3 cells not treated or treated with NE.(f) Effect of medium conditioned by CREB1 siRNA– or control siRNA–treated cells not treated or treated with NE on CAF marker ACTA2 expression in NOF151 cells Data are presented as mean±s.e.m. of n=3 for all experimental groups. Statistical significance was obtained using the 1-way ANOVA: *p < 0.05, **p < 0.01, ***p<0.001. ^{ss}p<0.01 compared to stress or NE-treated cells.



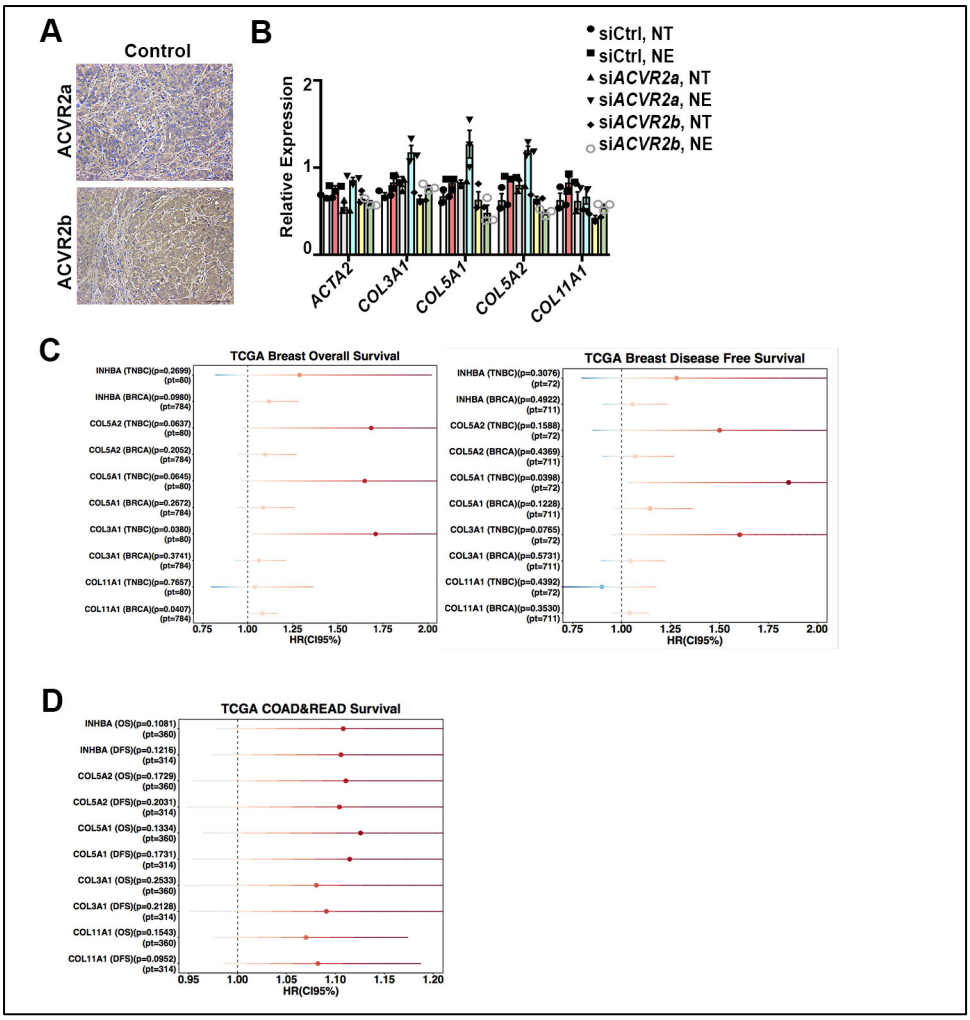
Supplementary Figure 7: Conditioned media from INHBA-silenced tumor cells decrease CAF-phenotype and collagens in NOF151

(a) Validation of multiple sequences of *INHBA* siRNA in HeyA8 cells not treated (NT) or treated with norepinephrine (NE). (b) Concentration of Inhibin Beta A in HeyA8 cells after *INHBA* was silenced via siRNA sequence 2 and no treatment or treatment with NE. (c) Effect of conditioned medium from INHBA siRNA– or control siRNA–treated Skov3 cells on expression of CAF marker ACTA2 in untreated or NE-treated NOF151 cells. (d) Effect of conditioned medium from ADRB2 siRNA– or INHBA siRNA– NE-treated Skov3 cells on collagen expression in NOF151 cells. Data are presented as mean±s.e.m. of n=3 for all experimental groups. Statistical significance was obtained using the 1-way ANOVA: *p < 0.05, **p < 0.01 compared to control, $^{\varsigma}$p<0.05 , $^{\varsigma\varsigma}$p<0.01 compared to NE-treated cells.



Supplementary Figure 8: **Silencing INHBA in tumor cells during restraint stress decreases CAFs and collagen content**

Effects of silencing *INHBA in vivo* in orthotopic HeyA8 tumor-bearing mice subjected or not subjected (control) to daily restraint stress and treated twice per week with either control siRNA or *INHBA* siRNA. (a) Validation of knockdown of *INHBA* by qRT-PCR. (b) Expression of desmin in micrographs of representative tumors from control and restraint stressed mice treated with control or *INHBA* siRNA. (c) H&E micrographs of representative tumors from control and restraint stressed mice treated with control or *INHBA* siRNA. (d) Expression of ACTA2 and collagens after INHBA silencing. (e-f) Expression of specific collagens in micrographs of representative tumors from control and restraint stressed mice treated with control or *INHBA* siRNA. Scale bars, 100μM. Data are presented as mean±s.e.m. of n=3 for all experimental groups. Statistical significance was obtained using the 1-way ANOVA: *p < 0.05, **p < 0.01 compared to siControl-no stress, ^{ss}p<0.01 compared to siControl-Stress.



Supplementary Figure 9: **Role of ACVR receptor and Survival data for breast and colon cancers.**

(a) Expression of ACVR2a and ACVR2b in ovarian tumor stroma. (b) Effect of NE-conditioned media after silencing ACVR2a and ACVR2b in NOF151 cells on ACTA2 and collagen expression. (c-d) General plots showing the hazard ratio, confidence interval and p-value for breast and colon cancers using TCGA data. Data are presented as mean±s.e.m. of n=3 for all experimental groups. Scale bars, 100μM.

Supplementary Tables

Supplementary Table 1: Gene co-expression with *INHBA* in TCGA ovarian cancer samples (highlighted probes common with Table S1)

Gene Symbol	Reporter ID	Correlation
INHBA	210511_s_at	1
THBS2	203083_at	0.90183127
COL11A1	204320_at	0.8865286
COL11A1	37892_at	0.8865286
FAP	209955_s_at	0.8479447
CTSK	202450_s_at	0.8479447
VCAN	211571_s_at	0.8479447
SPARC	200665_s_at	0.8177246
SPARC	212667_at	0.8177246
AEBP1	201792_at	0.8177246
COL1A2	202403_s_at	0.8177246
COL1A2	202404_s_at	0.8177246
COL6A3	201438_at	0.8177246
-	211161_s_at	0.8177246
COL3A1	215076_s_at	0.8177246
COL3A1	201852_x_at	0.8177246
COL1A1	202310_s_at	0.8177246
COL1A1	202311_s_at	0.8177246
COL5A1	203325_s_at	0.8177246
COL5A1	212489_at	0.8177246
COL5A1	212488_at	0.8177246
-	221729_at	0.8177246
COL5A2	221730_at	0.8177246
MMP2	201069_at	0.81008625
SNAI2	213139_at	0.8011878
FBN1	202765_s_at	0.8011878
FBN1	202766_s_at	0.8011878
FN1	211719_x_at	0.7849791
FN1	210495_x_at	0.7849791
FN1	216442_x_at	0.7849791
FN1	212464_s_at	0.7849791
COL10A1	217428_s_at	0.7731993
-	205941_s_at	0.7731993
CDH11	207172_s_at	0.7619725
CDH11	207173_x_at	0.7619725
DCN	209335_at	0.7619725
DCN	201893_x_at	0.7619725
DCN	211813_x_at	0.7619725
DCN	211896_s_at	0.7619725

LUM	201744_s_at	0.7619725
SERPINF1	202283_at	0.731467
CRISPLD2	221541_at	0.72007775
ASPN	219087_at	0.72007775
POSTN	210809_s_at	0.72007775
TMEM158	213338_at	0.7155617
OLFML2B	213125_at	0.7155617
ADAM12	213790_at	0.7155617
ADAM12	202952_s_at	0.7155617
NTM	222020_s_at	0.7155617
ECM1	209365_s_at	0.7155617
LRRC15	213909_at	0.7155617
MMP11	203876_s_at	0.7155617
MMP11	203878_s_at	0.7155617
COPZ2	219561_at	0.71121013
PCOLCE	202465_at	0.6899231
THBS1	201108_s_at	0.68605226
THBS1	201109_s_at	0.68605226
THBS1	201110_s_at	0.68605226
GLT8D2	221447_s_at	0.68131775
-	221019_s_at	0.68131775
MMP19	204575_s_at	0.67816544
COL6A1	212091_s_at	0.6591419
COL6A1	213428_s_at	0.6591419
COL6A2	209156_s_at	0.6591419
ANGPTL2	213004_at	0.6519295
ANGPTL2	213001_at	0.6519295
ITGA5	201389_at	0.6519295
LOXL2	202998_s_at	0.6519295
RAB31	217762_s_at	0.6445346
RAB31	217764_s_at	0.6445346
RAB31	217763_s_at	0.6445346
PLAU	205479_s_at	0.6445346
PLAU	211668_s_at	0.6445346
VCAM1	203868_s_at	0.6445346
LPPR4	213496_at	0.6307415
ETV1	221911_at	0.6307415
COL16A1	204345_at	0.6217892
BGN	213905_x_at	0.6217892
BGN	201261_x_at	0.6217892
C1QTNF3	220988_s_at	0.6091054
TIMP3	201150_s_at	0.6091054
TIMP3	201148_s_at	0.6091054
TIMP3	201149_s_at	0.6091054
TIMP3	201147_s_at	0.6091054

-	214927_at	0.6091054
ITGBL1	205422_s_at	0.6091054
EPYC	206439_at	0.6091054
PRRX1	205991_s_at	0.60363436
EDNRA	216235_s_at	0.60363436
EDNRA	204463_s_at	0.60363436
EDNRA	204464_s_at	0.60363436

Supplementary Table 2: Gene co-expression with *IL6* in TCGA ovarian cancer samples (highlighted genes common with Table S1)

Gene Symbol	Reporter ID	Correlation
IL6	205207_at	1
HBEGF	203821_at	0.533625
CH25H	206932_at	0.492976
THBD	203887_s_at	0.492976
NR4A3	209959_at	0.492976
GEM	204472_at	0.492976
RGS2	202388_at	0.492976
SLC2A14	216236_s_at	0.391042
SLC2A3	202497_x_at	0.391042
SGK1	201739_at	0.334213
PTGS2	204748_at	0.304811
NR4A2	204621_s_at	0.293695
DUSP2	204794_at	0.293695
BTG2	201236_s_at	0.293695
ZEB1	208078_s_at	0.293695
RHOB	212099_at	0.293695
JUN	201464_x_at	0.293695
IER2	202081_at	0.293695
JUNB	201473_at	0.293695
KLF6	208961_s_at	0.293695
GADD45B	207574_s_at	0.293695
PPP1R15A	37028_at	0.293695
EGR2	205249_at	0.293695
CYR61	201289_at	0.293695
CTGF	209101_at	0.293695
ATF3	202672_s_at	0.293695
DUSP1	201044_x_at	0.293695
EGR3	206115_at	0.293695
NR4A1	202340_x_at	0.293695
FOSB	202768_at	0.293695
ZFP36	201531_at	0.293695
EGR1	201694_s_at	0.293695
FOS	209189_at	0.293695
CEBPD	203973_s_at	0.293695
EDN1	218995_s_at	0.293695
C10orf10	209182_s_at	0.293695
GADD45A	203725_at	0.293695
KLF10	202393_s_at	0.293695
BHLHE40	201169_s_at	0.293695
PLK3	204958_at	0.293695

DUSP5	209457_at	0.293695
LIF	205266_at	0.293695
MAFF	205193_at	0.293695
IER3	201631_s_at	0.293695
C8orf4	218541_s_at	0.293695

Supplementary Table 3: Gene co-expression with *INHBA* in TCGA breast and colorectal cancer samples (highlighted genes common with Table S1)

	Breast Cancer	
INHBA	A_23_P122922	1
PPAPDC1A	A_24_P810284	0.847718
COL11A1	A_23_P11806	0.847718
KIF26B	A_23_P63541	0.804722
MMP11	A_23_P57417	0.79355
COL10A1	A_23_P214140	0.79355
GJB2	A_23_P407042	0.77325
FN1	A_24_P85539	0.756792
MMP13	A_23_P138931	0.713044
HSD17B6	A_23_P25030	0.704811
SPOCK1	A_24_P354689	0.675556
COL12A1	A_24_P291814	0.675556
CTHRC1	A_23_P111886	0.675556
COL3A1	A_24_P935491	0.675556
ASPN	NM_017680_2_2198	0.675556
AEBP1	A_23_P145918	0.675556
NOX4	A_23_P47147	0.675556
COL8A1	A_23_P69030	0.675556
COL1A2	A_24_P265274	0.675556
LOC651721	A_24_P282266	0.675556
WISP1	NKI_NM_003882	0.675556
LOC100128844	A_32_P141365	0.675556
P4HA3	A_23_P127956	0.675556
TMEM90B	NM_024893_1_1555	0.675556
LRRC15	A_24_P827032	0.675556
CDH11	A_23_P152305	0.675556
POSTN	A_24_P347411	0.675556
ADAM12	A_23_P202327	0.675556
COL6A3	NM_004369_1_9684	0.675556
VCAN	A_23_P144959	0.675556
COL1A1	A_23_P207521	0.675556
COL5A2	A_23_P10391	0.675556
COL5A1	A_23_P158590	0.675556
THBS2	A_23_P253652	0.675556
FAP	A_23_P56746	0.675556
TNFSF4	A_23_P126836	0.655745
LOC401097	A_23_P305243	0.655745
CILP2	A_23_P108238	0.642901
CORIN	A_23_P81131	0.634304
TLL2	A_23_P404778	0.610464
ERMN	A_23_P102017	0.609356
PLAU	A_23_P24103	0.609356

SULF1	A_23_P43165	0.609356
KIAA1199	A_23_P324754	0.609356
GREM1	A_23_P432945	0.571732
COMP	A_23_P90436	0.565048
PPEF1	A_23_P125505	0.543588
GRM8	A_32_P8221	0.543588
MATN3	NM_002381_2_2496	0.527919
C20orf103	A_23_P40294	0.527919
GRP	A_23_P101134	0.527919
ST6GAL2	A_32_P126157	0.527919
RGS4	A_23_P200737	0.510739
LOC285548	A_24_P892494	0.50405
NKX3-2	A_23_P386254	0.50405

Colorectal Cancer		
INHBA	A_23_P122922	1
COL11A1	NM_080629_1_6174	0.889562
ADAM12	NM_003474_2_4854	0.826843
NOX4	A_23_P47148	0.826843
CTHRC1	A_23_P111886	0.826843
FAP	A_23_P56746	0.826843
COL10A1	A_23_P214140	0.826843
COL5A2	A_32_P218731	0.811893
WISP1	NKI_NM_003882	0.809216
PDPN	A_23_P201322	0.809216
COL8A1	A_23_P69030	0.786018
ITGA11	A_23_P206022	0.786018
P4HA3	A_23_P127956	0.786018
SULF1	A_23_P43165	0.786018
THBS2	A_23_P253651	0.786018
PPAPDC1A	A_24_P810284	0.786018
COL1A1	A_23_P207521	0.786018
COL12A1	A_24_P291810	0.786018
COL6A3	NM_004369_1_9860	0.786018
COL1A2	A_23_P255244	0.786018
SPARC	A_23_P7642	0.786018
ZNF469	A_23_P335080	0.786018
CDH11	A_23_P152305	0.786018
KAL1	A_23_P429948	0.754175
LOC651721	A_24_P282266	0.744667
HS3ST3A1	A_23_P66523	0.72371
POSTN	A_23_P205111	0.72371
LOX	NM_002317_3_1867	0.72371
TWIST1	A_23_P71067	0.715534
ST6GALNAC5	A_23_P33093	0.698786
LUM	A_32_P28664	0.698786
TMEM90B	A_24_P190504	0.698786
FBN1	A_24_P152553	0.698786
ANTXR1	A_24_P131522	0.698786
NTM	A_23_P84060	0.698786
SPOCK1	A_23_P81598	0.698786
HTRA3	A_23_P395438	0.698786
TNFSF4	A_23_P126833	0.672474
MXRA5	A_24_P282355	0.662766
ADAMTS6	A_23_P213319	0.632247
KIF26B	A_23_P63541	0.632247
CNIH3	A_23_P384044	0.625946
SHISA2	A_32_P55236	0.625946

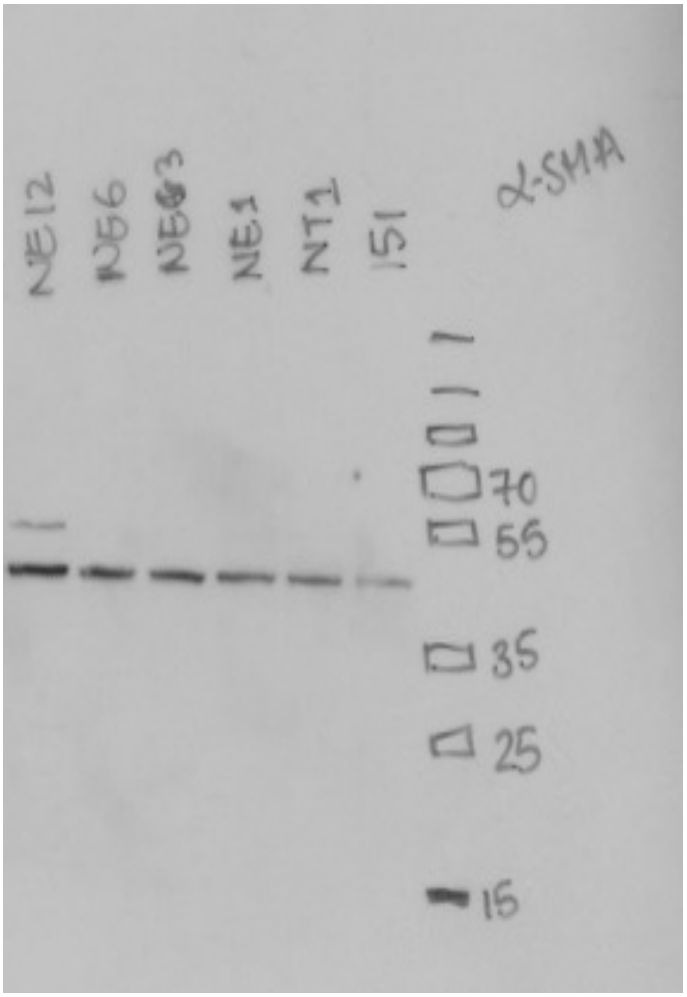
MMP11	A_23_P57417	0.625946
PPEF1	A_23_P125503	0.625946
MFAP2	A_23_P1027	0.625946
LRRC15	A_24_P827037	0.616798
HMCN1	A_23_P148991	0.616798
CRISPLD1	A_23_P59958	0.616798
COMP	A_23_P90430	0.616798
ITGBL1	A_23_P113777	0.616798
SFRP4	A_23_P215320	0.616798
KCNE4	A_23_P392574	0.604307
DIO2	A_23_P48736	0.581334
CLEC5A	A_23_P304356	0.550313
SLN	A_23_P150343	0.550313
C5orf46	A_23_P19176	0.550313
FGF1	A_23_P213330	0.550313
OLR1	A_24_P124624	0.550313
SPP1	A_23_P7311	0.550313
MMP13	A_23_P138931	0.54776
ALPK2	A_23_P15876	0.54776
PRRX1	A_23_P502731	0.54776
FN1	NM_002026_1_7942	0.541192
DKK2	A_23_P155847	0.52731
GRP	A_23_P101134	0.52731
EDNRA	A_24_P217572	0.521915

Supplementary Table 4: Primer Sequences

Human Sequences		
B-actin	AGCCTCGCCTTTGCCGA	CTGGTGCCTGGGGCG
ACTA2	CCAGAGCCATTGTCACACAC	CAGCCAAGCACTGTCAGG
INHBA	ATCTCGAAGTGCAGCGTCTT	GGAGGGCAGAAATGAATGAA
S100A4	TGTTGCTGTCCAAGTTGCTC	AACTAAAGGAGCTGCTGACCC
FAP	TCAGTGTGAGTGCTCTCATTGTAT	GCTGTGCTTGCCTTATTGGT
ADRB2	TCCACCTGGCTAAGGTTCTG	TGTCCTTCTACGTTCCCCTG
ACVR2a	GAAAGCCCAGTTGCTTAACG	GAAAGCCCAGTTGCTTAACG
ACVR2b	TGAGTACATGCTGCCCTTTG	TAATGGTGGGCCTCATCTTC
COL3A1	GATGGGGTCAAATGAAGGTG	GTGTGTTTCGTGCAACCATC
COL5A1	AGGATTTCCTGGACCAAAGG	TCTTGCCTTGGAAACCAGTC
COL5A2	CGGTGAAGAAGGCAAAAGAG	TTCTCCTTGAGCACCCTTTG
COL11A1	TCCTGGTGAAAAAGGACCAC	TTCTTTCCCAGGATGACCAG
CREB pos 1*	ACAACCCTTCACCGTTCTTG	ATAGGGGTAAAGCTGGTCAGG
CREB neg *	GAGATTCTAAAGACCTGGGAAGG	CGACCCCAACCAACTTACAC
Murine Sequences		
B-actin	GCTACAGCTTCACCACCACA	TCTCCAGGGAGGAAGAGGAT
ACTA2	GTTCAGTGGTGCCTCTGTCA	ACTGGGACGACATGGAAAAG
S100A4	TTTGTGGAAGGTGGACACAA	CAGCACTTCCTCTCTCTTGG
FAP	CTTTGTGTTTCCTTCAGGTTTG	CTTTGGAGTTACCACCCTGG
ACVR2a	GGCGACATTGTTTTGCTACC	AGCCAACAACCTTGCTTCAC
ACVR2b	CTTTAAGCCCTTGCCTTTCC	TCACAGCCACAAAGTCGTTC
COL3A1	AGGATCTGTCCTTTGCGATG	TCTCCAAATGGGATCTCTGG
COL5A1	TGAACAGATGAAGCGACCAC	TATTCGCCATCTGGGAAGTC
COL5A2	TCCTCAGGGAATTGATGGAG	GCCATCTGAGCTGAAAAAGG
COL11A1	TGGTCATCCTGGGAAAGAAG	ACCCTTTTCGCCTTTAGAGC

*ChIP primers

Uncut blot for Supplementary Figure 2d
aSMA expression in NOF151 after exposure to conditioned media.
Antibody: ab5694 (alpha smooth muscle actin, Abcam)
Observed band: 42kDa



Uncut blot for Supplementary Figure 2d
aSMA expression after exposure to conditioned media.
Antibody: A5316 (beta-actin, Sigma Aldrich)
Observed band: 42kDa