Supplementary Information

The interplay of cancer-associated fibroblasts and apoptotic cancer cells suppresses lung cancer cell growth through WISP-1-integrin αvβ3-STAT1 signaling pathway

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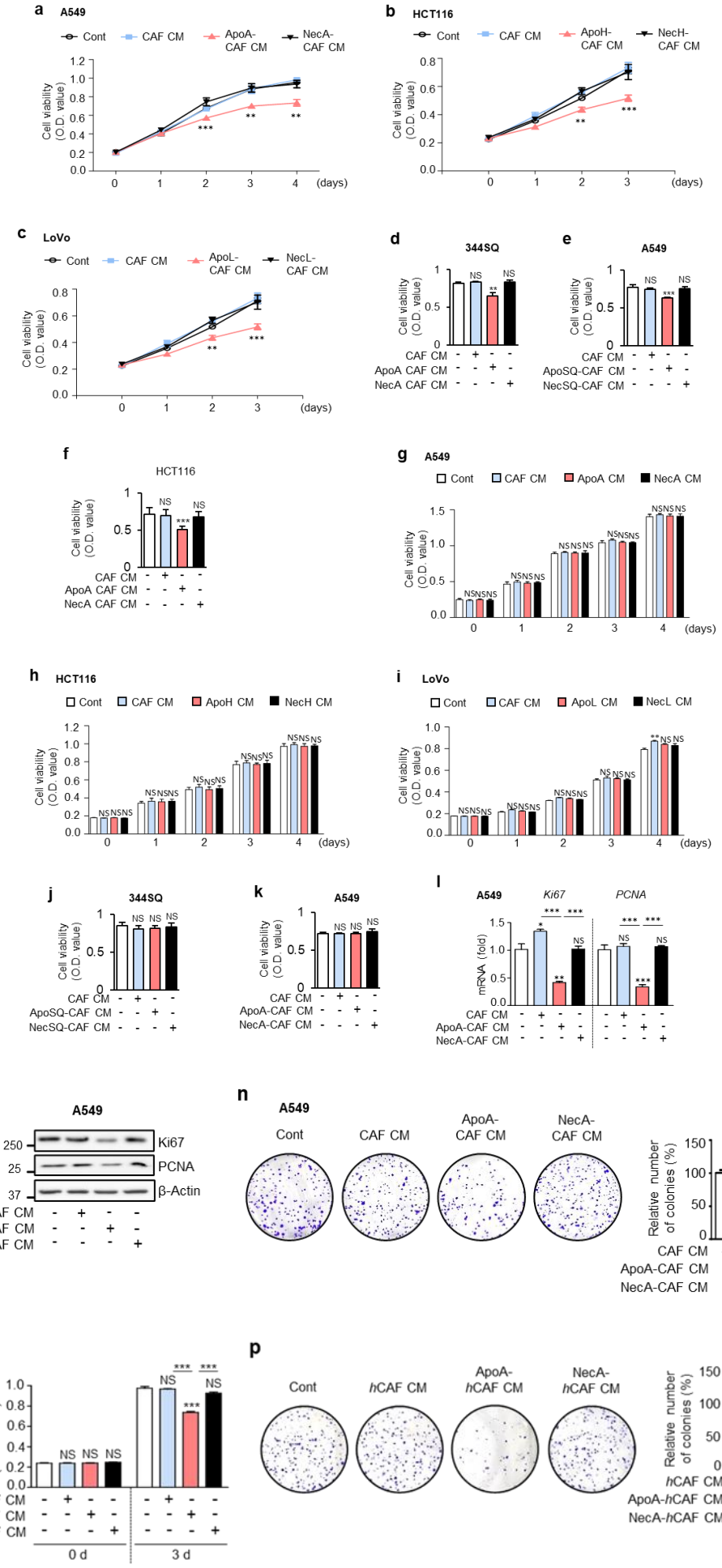
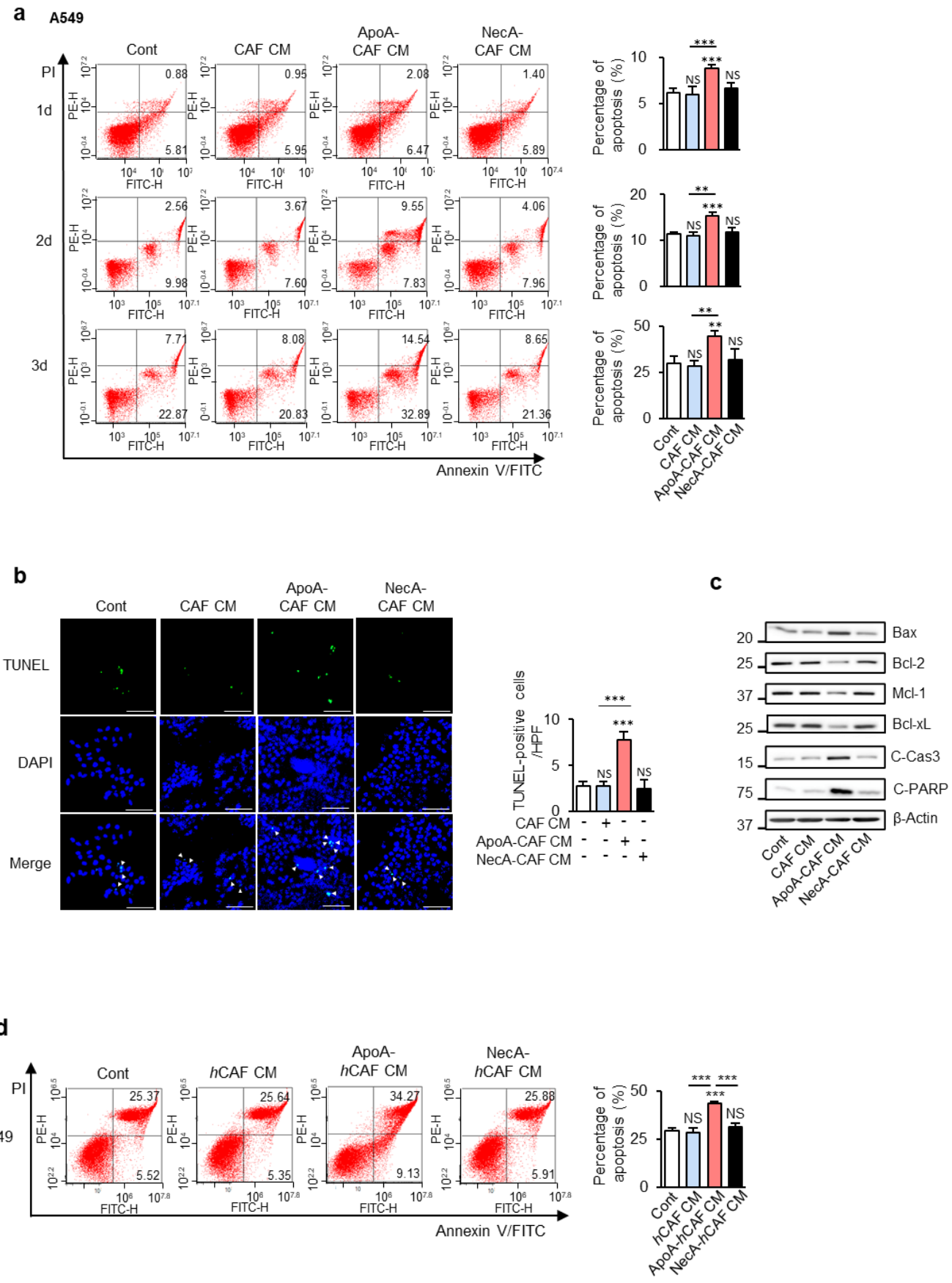
Supplementary Figure 1



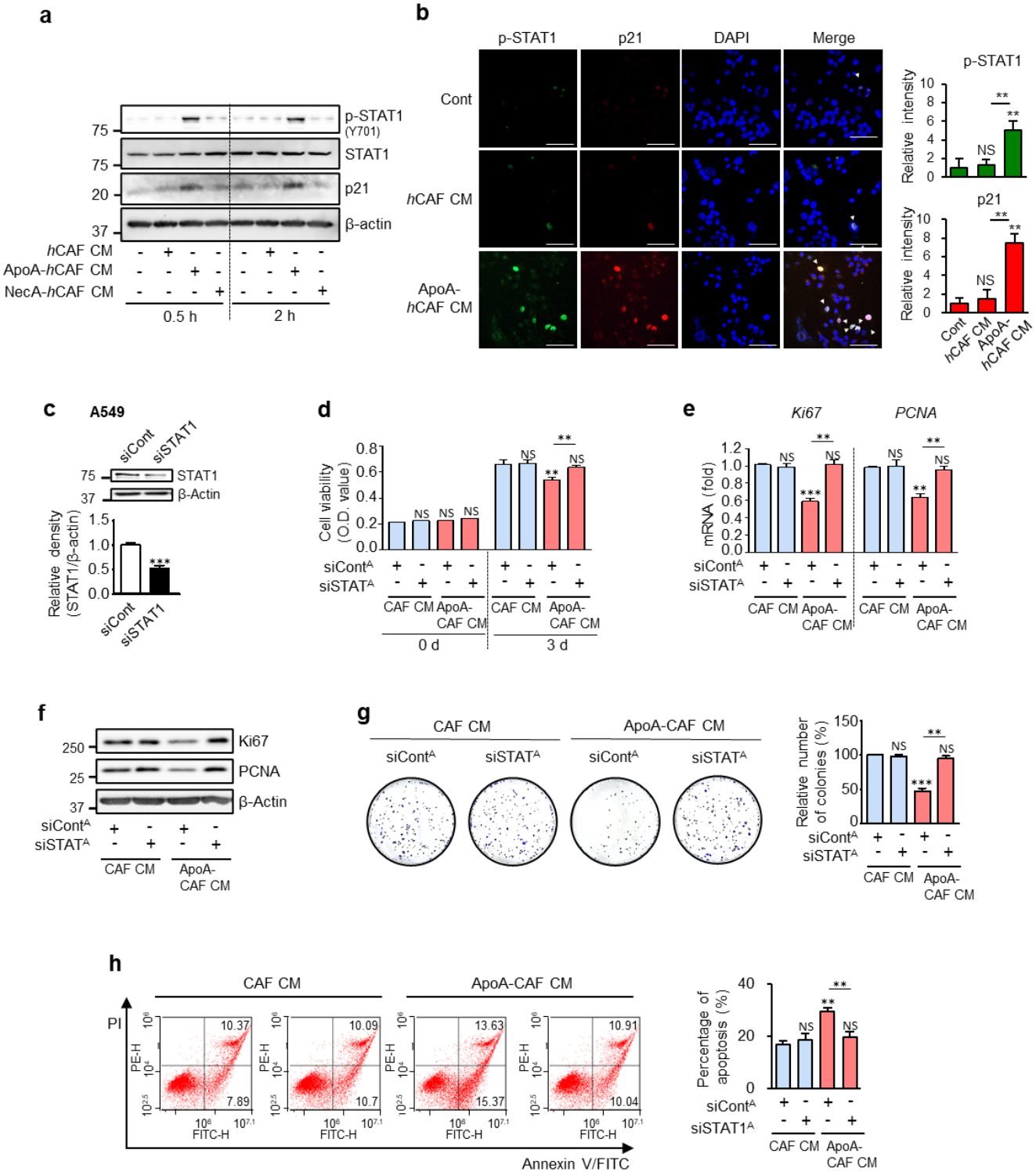
Supplementary Fig. S1. Conditioned medium from CAFs exposed to apoptotic cancer cells inhibits proliferation of cancer cells. (**a-k**) Cell viability assay of cancer cells, including A549 (**a, e**, **g**, **k**), HCT116 (**b**, **f**, **h**), LoVo (**c**, **i**), and 344SQ cells (**d**, **j**). (**a**-**c**) CAFs were exposed to apoptotic A549(ApoA), HCT116 (ApoH), and LoVo (ApoL) or necrotic cancer cells (NecA, NecH, and NecL) for 20 h. Conditioned medium from CAFs only (CAF CM), exposed to apoptotic cancer cells (ApoA-CAF CM, ApoH-CAF CM, and ApoL-CAF CM) or necrotic cancer cells (NecA-CAF CM, NecH-CAF CM, and NecL-CAF CM) was treated to corresponding cancer cells for the indicated days. (**d-f**) CAF CM, ApoA-CAF CM, or ApoSQ-CAF CM was treated to different types of cancer cells, including 344SQ cells (**d**), A549 cells (**e**), or HCT116 cells (**f**) for 3 days. (**g-i**) CAF CM or CM from apoptotic or necrotic cancer cells (ApoA CM, NecA CM, ApoH CM, NecH CM, ApoL CM, or NecL CM) was added to corresponding cancer cells for the indicated days. Apoptotic 344SQ (ApoSQ), necrotic 344SQ (NecSQ) (**j**), ApoA, or NecA (**k**) were seeded in the upper wells of a transwell system, while CAFs were seeded in the lower wells and cultured for 20 h. CAF CM, ApoSQ-CAF CM, NecSQ-CAF CM, ApoA-CAF CM, or NecA-CAF CM was added to corresponding cancer cells for 3 days. (**l**) qRT-PCR analysis of Ki67 and PCNA in A549 samples. (**m**) Immunoblot analysis of Ki67 and PCNA in A549 cell lysates. (**n**, **p**) *Left:* Representative images of colonies formed by A549 cells. *Right:* Quantitation of colony number formed by A549 cells. CAF CM, ApoA-CAF CM or NecA-CAF CM was treated to A549 cells for 3 (**l**, **m**) or 9 days (**n**). (**o**) Cell viability assay of A549 cells. CM from human CAFs only (*h*CAF CM), ApoA-*h*CAF CM, or NecA-*h*CAF CM was treated to A549 cells for 3 (**o**) or 9 days (**p**). NS, not significant; $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed Student's *t* test. Data are from one experiment representative of three independent experiments with similar results (**m**; **n** and **p** ***left***) or from three independent experiments (mean ± standard error; **a-l** and **o**; **n** and **p** ***right***).

Supplementary Figure 2



Supplementary Fig. S2. Conditioned medium from CAFs exposed to apoptotic A549 cells enhances apoptosis of A549 cells. (**a**, **d**) *Left:* Flow cytometry analysis after Annexin V−FICT/PI dual staining was employed to evaluate the cell apoptosis of A549 cells. *Right*: Apoptotic cells were quantified as the sum of the percentages of early and late stages of apoptosis. (**b**) Representative images of apoptosis in A549 cells by TUNEL assay (original magnification: x200). Nuclei were observed by DAPI staining. Scale bar = 100 μm. *Below:* Quantitation of the number of TUNEL−positive cells (number/HPF) in the different groups. (**c**) Immunoblot analysis of Bax, Bcl-2, Mcl-1, Bcl-xL, cleaved caspase-3, Cleaved PARP, and β-actin in A549 lysates. (**a-c**) A549 cells were treated with CAF CM, ApoA-CAF CM, or NecA-CAF CM for 1-3 days for measuring apoptosis by flow cytometry, 2 days for TUNEL assay, and 3 days for immunoblot assay. (**d**) A549 cells were treated with *h*CAF CM, ApoA-*h*CAF CM, or NecA-*h*CAF CM for 3 days. NS, not significant; $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed Student's *t* test. Data are from one experiment representative of three independent experiments with similar results (**a**, **b**, and **d** ***left***; **c**) or from three independent experiments (mean ± standard error; **a**, **b**, and **d** ***right***).

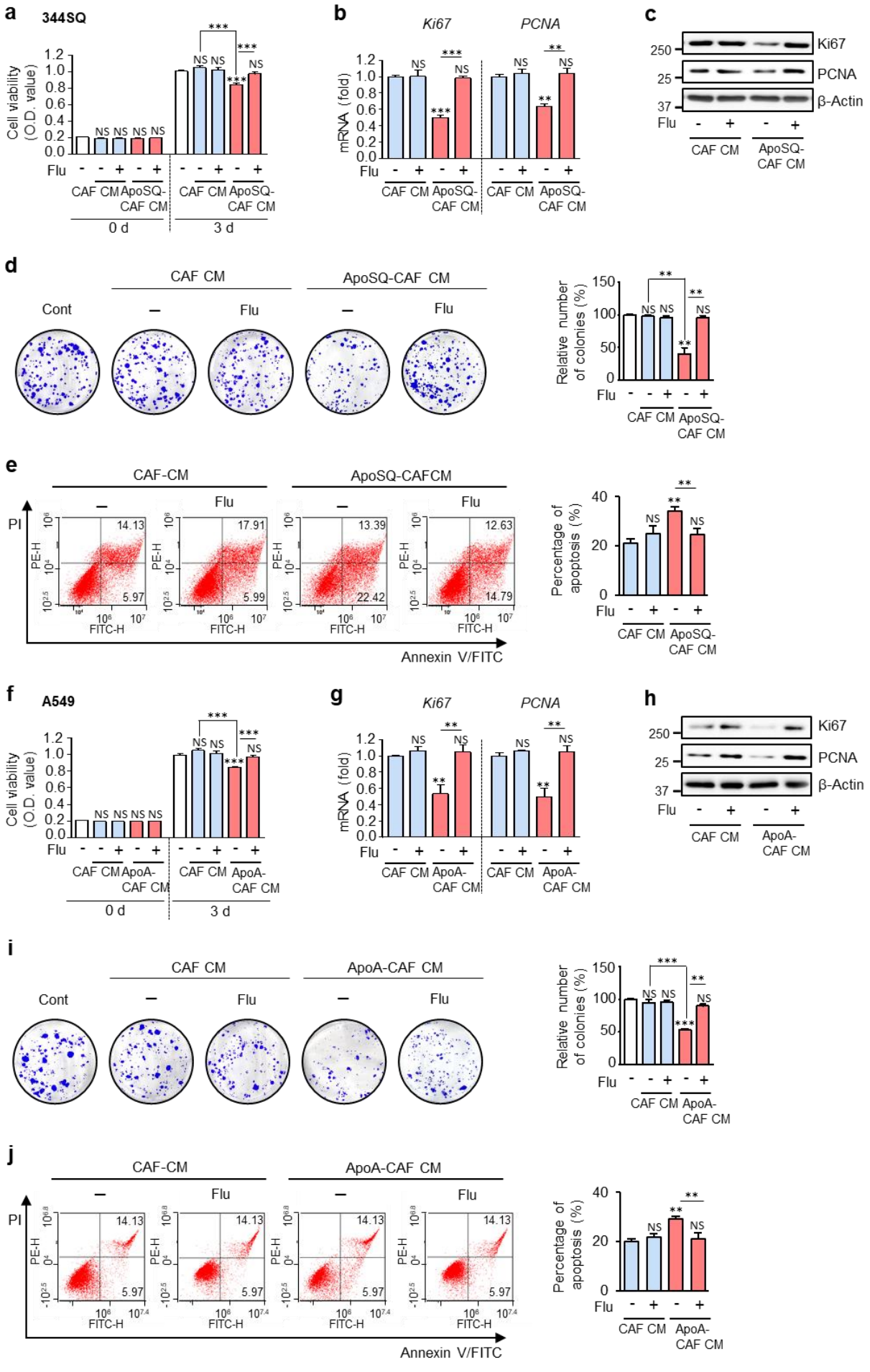
Supplementary Figure 3



Supplementary Fig. S3. STAT1 signaling is required for the antiproliferative and pro-apoptotic effects in A549 dells. (**a**) Immunoblot analysis of the indicated proteins in A549 cells treated with *h*CAF CM, ApoA-*h*CAF CM, or NecA-*h*CAF CM for 0.5 and 2 h. (**b**) Immunofluorescence staining for phosphorylated STAT1 and p21 (*Left*) and quantification (*right*) in A549 cells for 2 h after treatment with *h*CAF CM or ApoA-*h*CAF CM. The imaging medium was

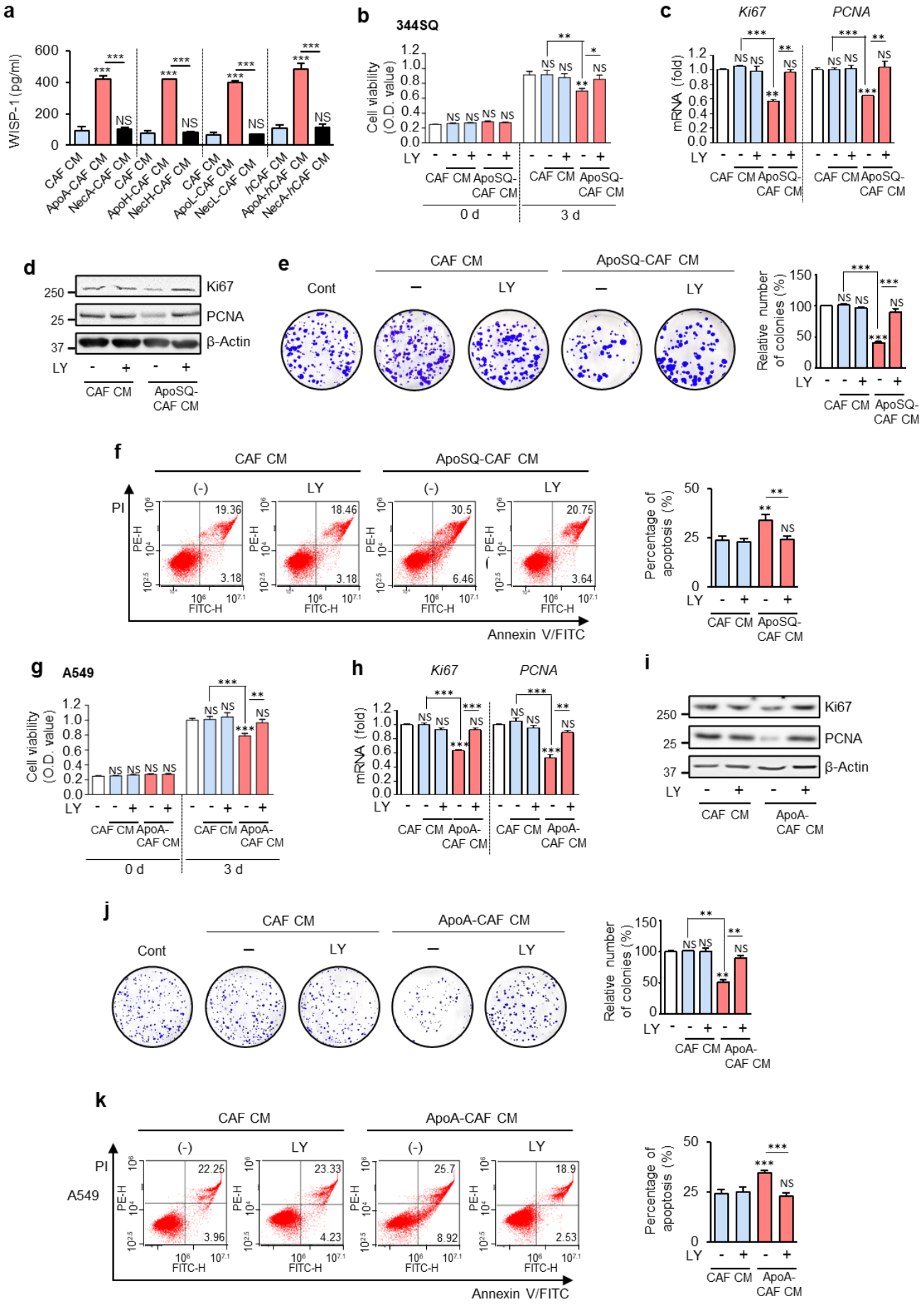
VECTASHIELD fluorescence mounting medium containing DAPI. Original magnification: ×200. Scale bars = 20 μm. (**c**) Immunoblot analysis of STAT1 in A549 cells transfected with control or STAT1 siRNA (*upper*). Densitometric analysis of the relative STAT1 abundance (*lower*). (**d**) Cell viability assay of A549 cells. (**e**) qRT-PCR analysis of Ki67 and PCNA in A549 samples. (**f**) Immunoblot analysis of Ki67 and PCNA in A549 cell lysates. (**g**) *Left:* Representative images of colonies formed by A549 cells. *Right:* Quantitation of colony number formed by A549 cells. (**h**) *Left:* Flow cytometry analysis after Annexin V−FICT/PI dual staining was employed to evaluate the cell apoptosis of A549 cells. *Right*: Apoptotic cells were quantified as the sum of the percentages of early and late stages of apoptosis. (**d**-**h**) A549 cells were transfected with control or STAT1 siRNA before treatment with CM. CAF CM, or ApoA-CAF CM was treated to A549 cells for 3 (**d-f**, **h**) or 9 days (**g**). NS, not significant; $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed Student's *t* test. Data are from one experiment representative of three independent experiments with similar results (**a**, **f**; **b**, **g,** and **h** ***left***; **c** ***upper***) or from three independent experiments (mean ± standard error: **b**, **g**, and **h** ***right***, **c** ***lower***; **d**, **e**).

Supplementary Figure 4



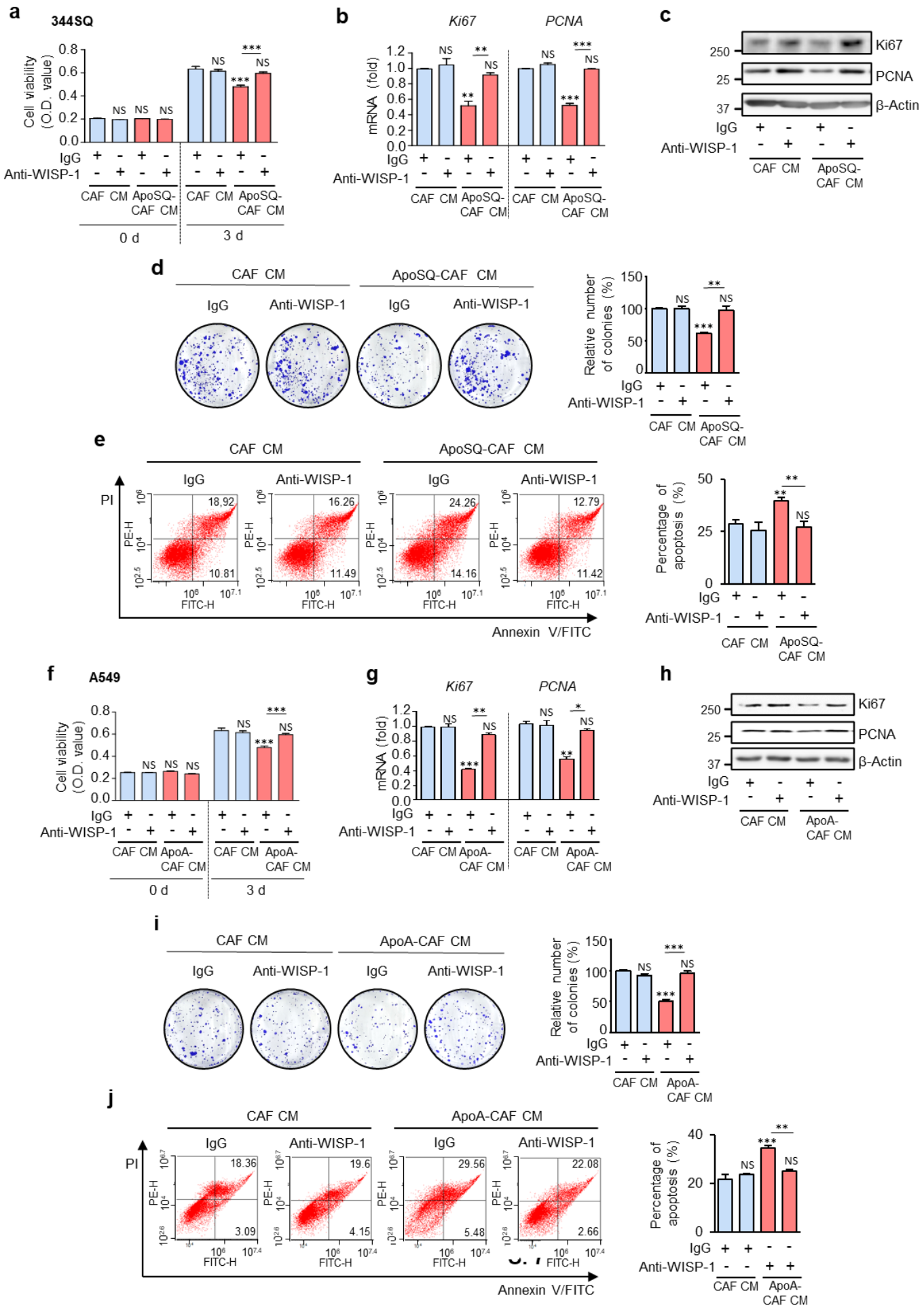
Supplementary Fig. S4. Pharmacological inhibition of STAT1 activation reverses the antiproliferative and pro-apoptotic effects. Cell viability assay of 344SQ (**a**) and A549 cells (**f**). qRT-PCR analysis of Ki67 and PCNA in 344SQ (**b**) and A549 samples (**g**). Immunoblot analysis of Ki67 and PCNA in 344SQ (**c**) and A549 cell lysates (**h**). *Left:* Representative images of colonies formed by 344SQ (**d**) and A549 cells (**i**). *Right:* Quantitation of colony number formed by 344SQ and A549 cells. *Left:* Flow cytometry analysis after Annexin V−FICT/PI dual staining was employed to evaluate the cell apoptosis of 344SQ (**e**) and A549 cells (**j**). *Right*: Apoptotic cells were quantified as the sum of the percentages of early and late stages of apoptosis. (**a-j**) 344SQ and A549 cells were pretreated with the STAT1 inhibitor fludarabine (0.5 μM) before treatment with CM. CAF CM, ApoSQ-CAF CM or ApoA-CAF CM was treated to corresponding cancer cells for 3 (**a-c**, **e**, **f-h**, **j**) or 8 days (**d**, **i**). NS, not significant; $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed Student's *t* test. Data are from one experiment representative of three independent experiments with similar results (**c**, **h**; **d**, **e, i**, and **j** ***left***) or from three independent experiments (mean ± standard error: **a**, **b**, **f**, **g**; **d**, **e**, **i**, and **j** ***right***).

Supplementary Figure 5



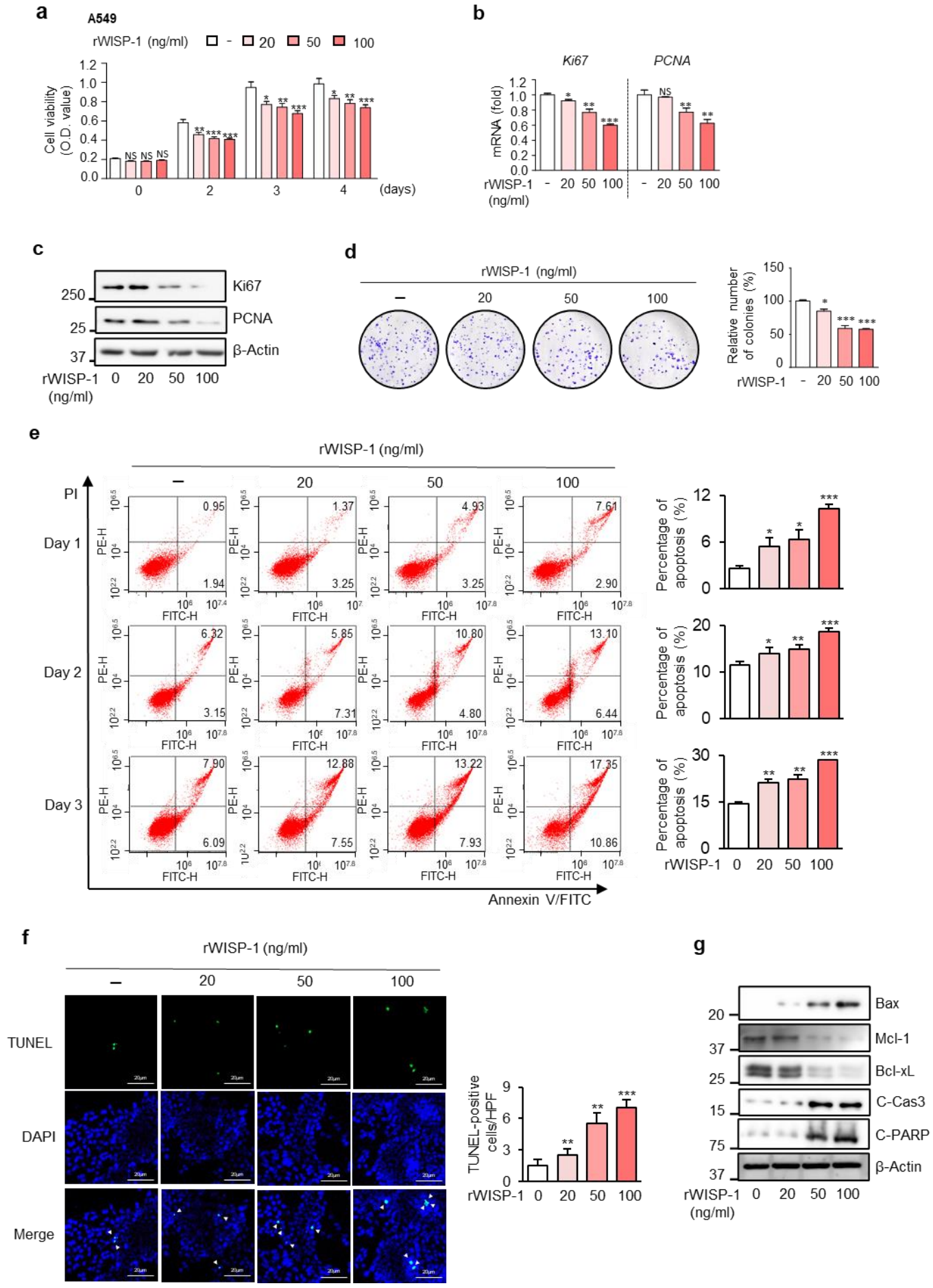
Supplementary Fig. S5. Inhibition of Notch1 signaling pathway in CAFs reverses the antiproliferative and pro-apoptotic effects in lung cancer cells. (**a**) ELISA of WISP-1 in CM from CAFs and *h*CAFs exposed to apoptotic A549 (ApoA), necrotic A549 (NecA), apoptotic HCT116 (ApoH), necrotic HCT116 (NecH), apoptotic LoVo (ApoL), or necrotic Lovo (NecL) for 20 h. Cell viability assay of 344SQ (**b**) and A549 cells (**g**). qRT-PCR analysis of Ki67 and PCNA in 344SQ (**c**) and A549 samples (**h**). Immunoblot analysis of Ki67 and PCNA in 344SQ (**d**) and A549 cell lysates (**i**). *Left:* Representative images of colonies formed by 344SQ (**e**) and A549 cells (**j**). *Right:* Quantitation of colony number formed by these cancer cells. Flow cytometry analysis after Annexin V−FICT/PI dual staining was employed to evaluate apoptosis of 344SQ (**f**) and A549 cells (**k**). *Right*: Apoptotic cells were quantified as the sum of the percentages of early and late stages of apoptosis. (**b**-**k**) CAFs were treated with the Notch inhibitor LY3039478 (10 μM) for 20 h, before exposure to ApoSQ (**b**-**f**) or ApoA (**g**-**k**). CAF CM, ApoSQ-CAF CM, or ApoA-CAF CM was added to corresponding lung cancer cells for 3 (**b**-**d**, **f, g**-**i, k**) or 8 days (**e**, **j**). NS, not significant; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, two-tailed Student's *t* test. Data are from one experiment representative of three independent experiments with similar results (**d**, **i**; **e**, **f**, **j**, and **k** ***left***) or from three independent experiments (mean ± standard error; **a**-**c**, **g**, **h**; **e**, **f**, **j**, and **k** ***right***).

Supplementary Figure 6



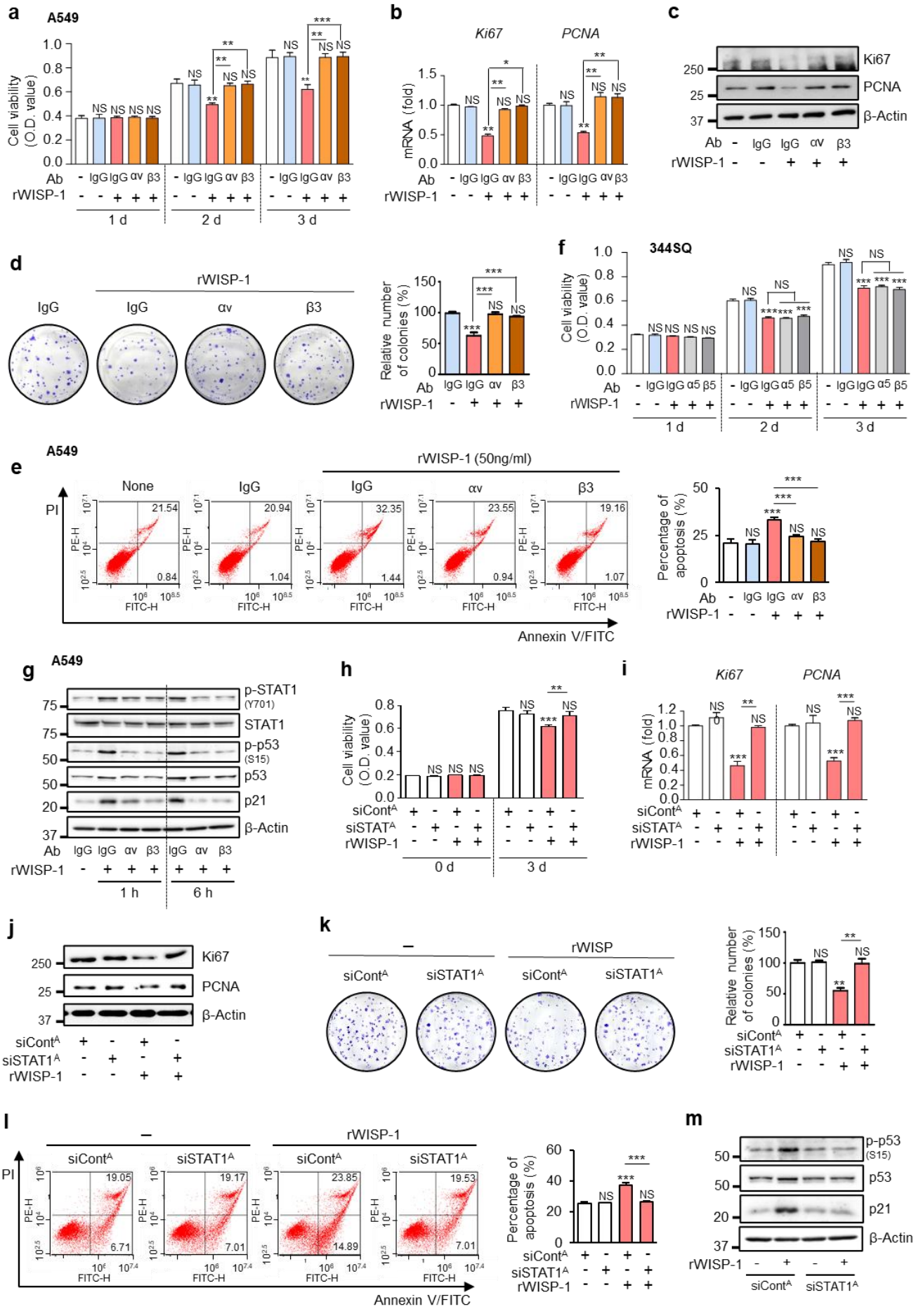
Supplementary Fig. S6. WISP-1 mediates the antiproliferative and pro-apoptotic effects in lung cancer cells. Cell viability assay of 344SQ (**a**) and A549 (**f**) cells. qRT-PCR analysis of Ki67 and PCNA in 344SQ (**b**) and A549 (**g**) samples. Immunoblot analysis of Ki67 and PCNA in 344SQ (**c**) and A549 (**h**) cell lysates. *Left:* Representative images of colonies formed by 344SQ (**d**) A549 (**i**) cells. *Right:* Quantitation of colony number formed by these cancer cells. *Left:* Flow cytometry analysis after Annexin V−FICT/PI dual staining was employed to evaluate the apoptosis of 344SQ (**e**) and A549 (**j**) cells. *Right*: Apoptotic cells were quantified as the sum of the percentages of early and late stages of apoptosis. CAF CM, ApoSQ-CAF CM, or ApoA-CAF CM was pretreated with neutralizing anti-WIPS-1 Ab (10 μg/ml) or isotype IgG before addition to 344SQ (**a**-**e**) and A549 (**f**-**j**) cells. CM was added to lung cancer cells for 3 (**a**-**c**, **e**, **f-h**, **j**) or 8 days (**d**, **i**). NS, not significant; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, two-tailed Student's *t* test. Data are from one experiment representative of three independent experiments with similar results (**c**, **h**; **d**, **e**, **i**, and **j** ***left***) or from three independent experiments (mean ± standard error: **a**, **b, f, g**; **d**, **e**, **i**, and **j** ***right***).

Supplementary Figure 7



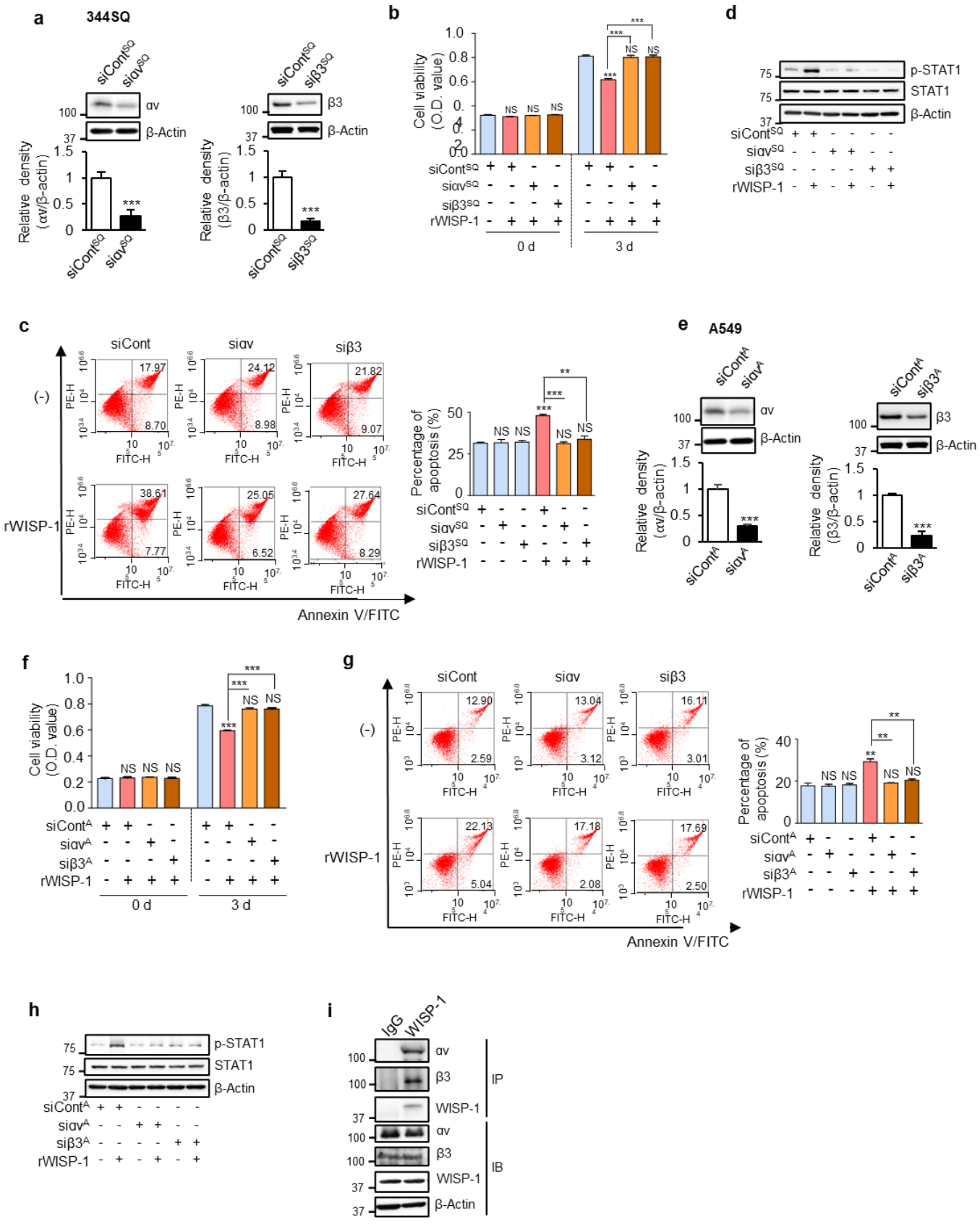
Supplementary Fig. S7. rWISP-1 suppresses proliferation and promotes apoptosis in A549 cells. (**a**) Cell viability assay of A549 cells. (**b**) qRT-PCR analysis of Ki67 and PCNA in A549 samples. (**c**) Immunoblot analysis of Ki67 and PCNA in A549 cell lysates. (**d**) *Left:* Representative images of colonies formed by A549 cells. *Right:* Quantitation of colony number formed by A549 cells. (**e**) *Left:* Flow cytometry analysis after Annexin V−FICT/PI dual staining was employed to evaluate the cell apoptosis of A549 cells. *Right*: Apoptotic cells were quantified as the sum of the percentages of early and late stages of apoptosis. (**f**) *Left:* Representative images of apoptosis in A549 cells by TUNEL assay (original magnification: x200). Nuclei were observed by DAPI staining. Scale bar = 20 μm. *Right:* Quantitation of the number of TUNEL−positive cells (number/HPF) in the different groups. (**g**) *Left*: Immunoblot analysis of Bax, Mcl-1, Bcl-xL, cleaved caspase-3, cleaved PARP, and β-actin in A549 lysates. A549 cells were treated with rWISP-1 (20, 50, and 100 ng/ml) for 2-4 (**a**), 3 (**b**, **c, g**), 8 (**d**), 1-3 (**e**), or 2 days (**f**). NS, not significant; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, two-tailed Student's *t* test. Data are from one experiment representative of three independent experiments with similar results (**c**, **g**; **d-f *left***) or from three independent experiments (mean ± standard error: **a**, **b; d-f *right***).

Supplementary Figure 8



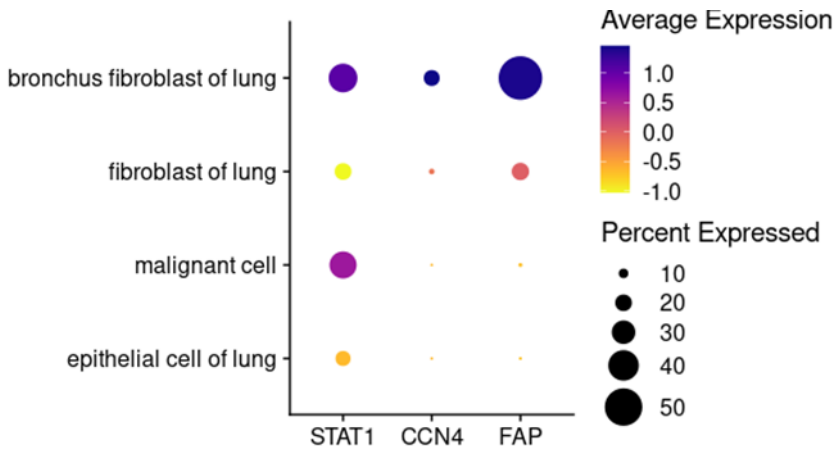
Supplementary Fig. S8. WISP-1 acts through integrin αvβ3/STAT1 signaling pathway to inhibit proliferation and promote apoptosis in lung cancer cells. Cell viability assay of A549 (**a**, **h**) and 344SQ cells (**f**). (**b**, **i**) qRT-PCR analysis of Ki67 and PCNA in A549 samples. (**c, g, j, m**) Immunoblot analysis of the indicated proteins in A549 lysates. (**d**, **k**) *Left:* Representative images of colonies formed by A549 cells. *Right:* Quantitation of colony number formed by A549 cells. (**e**, **l**) *Left:* Flow cytometry analysis after Annexin V−FICT/PI dual staining was employed to evaluate the apoptosis of A549 cells. *Right*: Apoptotic cells were quantified as the sum of the percentages of early and late stages of apoptosis. (**a-e**, **g**) A549 cells were pretreated with an anti-integrin blocking antibody (3 μg/ml; anti-integrin αv or β3) or corresponding IgG isotype control for 30 min before treatment with 50 ng/ml rWISP-1 for the indicated time (**a**, **g**), 3 (**b**, **c**, **e**), or 8 days (**d**). (**f)** 344SQ cells were pretreated with an anti-integrin blocking antibody (3 μg/ml; anti-integrin α5 or β5) or corresponding IgG isotype control for 30 min before treatment with 50 ng/ml rWISP-1 for the indicated day. (**h**-**m**) A549 cells were transfected with control or STAT1 siRNA before treatment with 50 ng/ml rWISP-1 for 1 h (**m**), 3 (**h**, **i**, **j**, **l**), or 8 days (**k**). NS: not significant; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, two-tailed Student's *t*-test. The data are from one experiment representative of three independent experiments with similar results (**c**, **g**, **j**, **m**; **d**, **e**, **k**, and **l** ***left***) or from three independent experiments (mean ± standard error in **a**, **b**, **f**, **h**, **i**; **d**, **e**, **k**, and **l** ***right***).

Supplementary Fig. S9.



Supplementary Fig. S9. WISP-1 signals through integrin αvβ3/STAT1 pathway to induce antiproliferative and pro-apoptotic effects. Immunoblot analysis of integrin αv or β3 in 344SQ (**a**) and A549 cells (**e**) transfected with control, integrin αv, or β3 siRNA (*upper*). Densitometric

analysis of the relative integrin αv or β3 abundance (*lower*). Cell viability assay of 344SQ (**b**) and A549 cells (**f**). *Left:* Flow cytometry analysis after Annexin V−FICT/PI dual staining was employed to evaluate the apoptosis of 344SQ (**c**) and A549 cells (**g**). *Right*: Apoptotic cells were quantified as the sum of the percentages of early and late stages of apoptosis. Immunoblot analysis of phosphorylated/total STAT1 and β-actin in 344SQ (**d**) and A549 lysates (**h**). 344SQ (**b-d)**, and A549 cells (**f-h**) were transfected with control, integrin αv or β3 siRNA 20 h before treatment with 50 ng/ml rWISP-1 for 1 h (**d**, **h**) or 3 days (**b**, **c**, **f**, **g**). (**i**) CoIP assays of protein interaction in A549 cells. Cell lysates were immunoprecipitated (IP) with anti-WISP-1 and then immunoblotted with anti-integrin αv and anti-integrin β3 antibodies. NS, not significant; $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed Student's *t* test. Data are from one experiment representative of three independent experiments with similar results (**a** and **e** ***upper***; **d**, **h**, **i**; **c** and **g** ***left***) or from three independent experiments (mean ± standard error: **a** and **e** ***below***; **b**, **f**; **c** and **g** ***right***).



Supplementary Fig. S10. Expression levels of STAT1 and CCN4 in non-small cell lung cancer (NSCLC) single-cell datasets. Expression levels of STAT1, CCN4, and FAP in lung fibroblasts, epithelial cells, and malignant cells from NSCLC single-cell datasets.

Table S1. List of antibodies used for this study.

Antigen	Vendor	Cat.No	Source	Species cross-reactivity	Application	Dilution
Arg1	Cell signaling	93668	Rb	H, M, R	IB	1:1000
Bax	Cell signaling	2772	Rb	H, M, R	IB	1:1000
Bcl-xL	Cell signaling	2764	Rb	H, M, R	IB	1:1000
Bcl-2	Cell signaling	3498	Rb	H, M	IB	1:1000
C-Cas3	Cell Signaling	9661	Rb	H, M, R, Mk	IB	1:1000
					IHC	1:100
Integrin αv	Cell signaling	60869	Rb	H, M, R	IB	1:1000
	Invitrogen	14-0512-85	Rb	H, M	Neutralizing	5ug/ml
Integrin β3	Cell signaling	13166	Rb	H, M, R	IB	1:1000
	Invitrogen	MA1-35264	M	H	Neutralizing	5ug/ml
Ki67	abcam	ab16667	Rb	H, M	IB	1:1000
	Invitrogen	MA5-14520	Rb	H, M, R	IHC	1:200
Mcl-1	Cell signaling	5453	Rb	H, M	IB	1:1000
Notch1	abcam	ab52627	Rb	H, M	IB	1:1000
p-STAT-1	Cell signaling	7649	Rb	H, M, R	IB	1:1000
	Santa cruz	sc-7988	G	H, M	ICC	1:200
					IHC	1:200
p21	Santa cruz	sc-6246	M	H, M, R	IB	1:1000
					ICC	1:200
p53	Cell signaling	2524	M	H, M, R, Hm, Mk	IB	1:1000
p-p53	Cell signaling	9284	Rb	H, M, R, Mk	IB	1:1000
PCNA	abcam	ab29	M	H, M, R	IB	1:1000
STAT-1	Cell signaling	9172	Rb	H, M, R	IB	1:1000
WISP-1	Abcam	ab260036	Rb	M, R	IB	1:1000
	Santa cruz	sc-133126	M	H	IB	1:1000
					IP	5μg/ml
α-tubulin	AbClon	AbC-2001	M	H, M, R	IB	1:3000
β-actin	Santa cruz	sc-69879	M	Broad species	IB	1:1000
Goat IgG (Alexa 488)	Thermo fisher scientific	A11055	D	Not applicable	ICC	1:1000
IgG	R&D Systems	MAB005	R	H, M	Neutralizing	5ug/ml
IgG	Invitrogen	08-6599	M	H, M	Neutralizing	5ug/ml
IgG	Santa cruz	sc-2027	Rb	Broad species	Neutralizing	5ug/ml
Mouse IgG (Alexa 568)	Thermo fisher scientific	A11061	Rb	Not applicable	ICC	1:1000
Mouse IgG (HRP)	GeneTex	GTX213111	G	Not applicable	IB	1:5000
Mouse IgG(HRP)	GeneTex	GTX213111	G	Not applicable	IB	1:5000
Rabbit IgG (Alexa 488)	Thermo fisher scientific	A11008	G	Not applicable	ICC	1:1000
Rabbit IgG (HRP)	GeneTex	GTX213110	G	Not applicable	IB	1:5000
Rabbit IgG (HRP)	GeneTex	GTX213110	G	Not applicable	IB	1:5000

Abbreviation: H-Human, M-Mouse, R-Rat, Rb-Rabbit, G-Goat, B-Bovine, C- C. Elegans, Pm-Primate, RM-Rhesus Macaque, Mk-Monkey, D-Donkey, Hm-Hamster IB-Immunoblotting, IHC-Immunoprecipitation,IP-Immunoprecipitation, IF-Immunofluorescence, FACS-Fluorescence activated cell sorting

Table S2. Sequences of qRT-PCR primer.

Target gene	Forward (5'→ 3')	Reverse (5'→ 3')
Human *Ki67*	AAGCCCTCCAGCTCCTAGTC	GCAGGTTGCCACTCTTTCTC
Mouse *Ki67*	AATCCAACTCAAGTAAACGGGG	TTGGCTTGCTTCCATCCTCA
Human *Pcna*	CCTGCTGGGATATTAGCTCCA	CAGCGGTAGGTGTCGAAGC
Mouse *Pcna*	GGCGTGAACCTCACCAGTAT	TTCTCCTGGTTTGGTGCTTC
Hprt	CAGACTGAAGAGCTACTGTAATG	CCAGTGTCAATTATATCTTCAAC

Table S3. List of siRNA.

Target gene	Sense	Antisense
Human *Stat1*	5'-CUGGAUAUAUCAAGACUGA-3'	5'-UCAGUCUUGAUAUAUCCAG-3'
Mouse *Stat1*	5'-CACAGUUUUAUCCUGAG-3'	5'-UCUCAGGAUAAAACUGUG-3'
Mouse Notch1	5'-′CCCUUUGAGUCUUCAUACA-3'	5′-UGUAUGAAGACUCAAAGGG-3′
Mouse Wisp-1	5′-GGAAUCCUAACGAUAUCUU-3'	5′- AAGAUAUCGUUAGGAUUCC-3′
Human *integrin αv*	5′-CCGAAACAAUGAAGCCUUA-3'	5′-UAAGGCUUCAUUGUUUCGG-3'
Mouse *integrin αv*	5′-GCUUAAAGGCCAGAUGGCAAUU-3'	5′-UUGCCAUCUGCCUUUAAGCUU-3'
Human *integrin β3*	5′-UUACUGCCGUGACGAGAUU-3'	5′-AAUCUCGUCACGGCAGUAA-3'
Mouse *integrin β3*	5′-GAUGUAACCUGAAGGAGAA-3'	5′-UUCUCCUUCAGGUUACAUC-3'

Table S4. The number of extracted cells in a single-cell RNA sequencing dataset

	number of cells
bronchus fibroblast of lung	1,662
fibroblast of lung	4,050
epithelial cell of lung	16,175
malignant cell	20,746
Total	42,633

Table S5. Correlation of Stat1 (encoded by STAT1) and Wisp-1 (CCN4) in bulk lung cancer expression data

Parameter	**Pearson correlation coefficient (r)**	**r^2**	**P-value**	**Sample size (n)**
TCGA CCN4 and STAT1 (RNA-seq)	0.361	0.130	0	510
CPTAC CCN4 and STAT1 (RNA-seq)	0.282	0.080	0.00282	110
CPTAC CCN4 (RNA-seq) and STAT1 (protein abundance)	0.390	0.152	0.00002	110
CPTAC CCN4 (RNA-seq) and STAT1 S727 (phospo-protein abundance)	0.276	0.076	0.00347	110
CPTAC CCN4 (RNA-seq) and tumor purity	-0.520	0.271	6.73E-09	109
CPTAC CCN4 (RNA-seq) and stromal score	0.650	0.422	1.55E-14	110