Supplemental Information

A tightly controlled Src-YAP signaling axis determines therapeutic response to dasatinib in renal cell carcinoma

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Supplementary Methods

Cell viability assay

Cells were seeded in 96-well plates at 3000-5000 cells/well in triplicate. After 24 h, the cells were treated with different concentrations of the indicated kinase inhibitors and incubated at 37 °C. Then, the cells were cultured for 72 h, and the number of viable cells was measured by SRB assay. IC_{50} values were calculated based on a dose response curve.

Immunoblotting

Protein extracts were prepared by washing cells twice in cold PBS followed by lysis with SDS-lysis buffer (50 mM Tris-HCl, pH 7.4, 2% SDS). Cell lysates were boiled for 10 min and cleared by centrifugation at 14,000 × g for 5 min at 4 °C. The supernatant was collected and subsequently resolved by SDS-PAGE, transferred to nitrocellulose membranes, probed with the appropriate primary antibodies and then incubated with horseradish peroxidase-conjugated secondary antibodies. The immunoreactive proteins were detected using an ECL plus detection reagent (Pierce, Rockford, IL) and imaged by autoradiography. Antibodies used in immunoblotting were as follows: Phospho-Src Family Tyr416 (#2101, CST); Src (#2108, CST); Phospho-YAP Ser127 (#4911, CST); YAP (#14074, CST); TAZ (#4883, CST); Phospho-LATS1 Thr1079 (#8654, CST); LATS1 (#3477, CST); Phospho-SAPK/JNK Thr183/Tyr185 (#9255, CST); SAPK/JNK (#9252, CST); and LIMD1 (A303-182A-T, Bethyl).

Clonogenic assay

Cells were seeded in 6-well plates at 500 cells/well. After 24 h, cells were treated with the indicated drugs and allowed to grow for 10 days. Then, the cells were fixed with 1 mL of fixative solution (10% methanol, 10% acetic acid and 80% ddH_2O) and stained with 0.5% crystal violet solution.

EdU incorporation assay

Caki-1 or 769-P cells were seeded in 96-well plates at 3000 cells/well and treated with dasatinib for 24 h. Then, the cells were exposed to 50 μM of EdU solution (RiboBio, Guangzhou, China) for 2 h at 37 °C. Thereafter, cells were fixed in 4% PFA and permeabilized with 0.5% Triton-X 100. Then, the cells were treated with 1× Apollo reaction cocktail (RiboBio) for 30 min. Subsequently, the DNA contents of the cells were stained with Hoechst 33342 for 30 min and visualized under a laser scanning confocal microscopy.

Cell cycle analysis

Cells were treated with DMSO or dasatinib for 24 h. Both adherent and floating cells were harvested and fixed in cold 70% ethanol. Prior to FACS analysis, cells were washed with PBS and re-suspended in PBS containing 200 μg/mL RNase A (Beyotime, Shanghai, China) and 50 μg/mL PI (Beyotime, Shanghai, China) and incubated for 15 min at 37 °C in the dark. Quantification of the cell cycle distribution was evaluated using a Becton-Dickinson FACS Calibur flow cytometer (BD, San Jose, CA). The data were analyzed using ModFit LT (BD, San Jose, CA).

Quantitative real-time PCR

Quantitative real-time PCR was performed using ABI Prism VIIA7 Real-Time PCR System (Life Technologies, Carlsbad, CA). For the detection of gene expression, total RNA was extracted with TRIzol reagent and subjected to reverse transcription with PrimeScript® RT Reagent Kit (Takara, Mountain View, CA). PCR reactions were performed with SYBR® Premix Ex Taq™ Kit (Takara). Primers used were as follows: Cyr61 Forward, 5'-GCAGTTGGAAAAGGCAGCTC-3' and Reverse, 5'-GAAACGCTGCTTCATTGGCA-3'; YAP Forward, 5'-TAGCCCTGCGTAGCCAGTTA-3' and Reverse, 5'-TCATGCTTAGTCCACTGTCTGT-3'; and TAZ Forward,

5’-CACCGTGTCCAATCACCAGTC-3’ and Reverse, 5’-TCCAACGCATCAACTTCAGGT-3’.

Gene expression microarray analysis

Caki-1 and 769-P cells were treated with DMSO or 1 μM dasatinib for 6, 12 and 24 h. Total RNA was extracted with TRIzol reagent (Invitrogen, Grand Island, NY), then purified, amplified and labeled to obtain biotin-labeled cRNA. Affymetrix PrimeView GeneChip hybridization was performed according to the manufacturer’s protocol (Affymetrix, CA, USA). Differentially expressed genes (exhibiting at least two fold changes in expression) were screened via moderated-t test ($P < 0.001$). GSEA was performed against MSigDB v5.1 with 10^5 permutations on gene sets. The original and normalized microarray data of this study can be accessed at GSE81235 in NCBI GEO Datasets.

Co-immunoprecipitation

Caki-1 cells were seeded into 10 cm dishes at 80% confluence. Then, the cells were treated with DMSO or dasatinib for 3 h and lysed with NP-40 lysis buffer (Beyotime, Shanghai, China) supplemented with protease and phosphatase inhibitors (Merck, Darmstadt, Germany). Equal amounts of proteins in lysis buffer were incubated with the indicated antibody or control IgG separately overnight at 4 °C, following by immunoprecipitation with protein A/G-conjugated agarose beads (Santa Cruz, CA, USA) at 4 °C for 3 h. Samples were centrifuged at 3000 rpm for 3 min at 4 °C and washed with NP-40 lysis buffer 6 times and once with cold PBS. The precipitates were boiled in SDS-lysis buffer for 10 min, and the supernatants were collected and subjected to immunoblotting analysis. The input samples represented ~ 1% of protein amounts used for co-IP. The antibodies for used immunoprecipitation were as follows: YAP1 Mouse mAb (#12395, CST) and LATS1 Rabbit mAb (#3477,

CST).

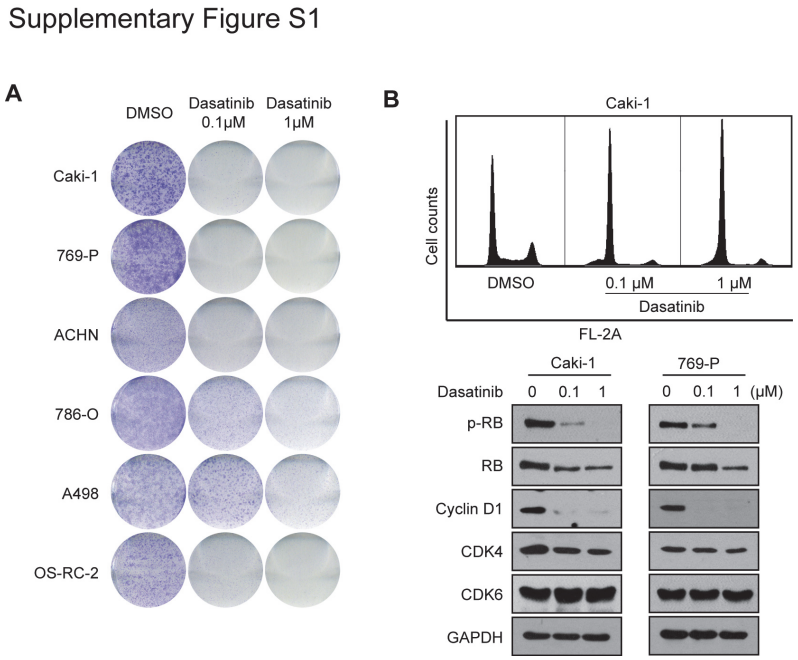
Supplementary Table

Supplementary Table 1: KinaseProfiler^{TM} screening analysis of the human kinases that inhibited by Dasatinib at molecular level.

Kinase	Activity% (Dasatinib, 0.1 μM)	Kinase	Activity% (Dasatinib, 1 μM)
Abl(h)	-5	Abl(h)	-4
EphA2(h)	-3	EphA2(h)	-3
Fgr(h)	-3	EphA4(h)	-2
EphA4(h)	-2	CSK(h)	-2
EphA5(h)	-1	Yes(h)	-2
EphB2(h)	-1	EphB3(h)	-2
Abl (Q252H) (h)	0	EphA5(h)	-1
ACK1(h)	0	EphB2(h)	-1
EphA3(h)	0	Abl (Q252H) (h)	-1
Lyn(h)	0	cKit(h)	-1
Blk(h)	0	ACK1(h)	0
cSRC(h)	0	EphA3(h)	0
EphB1(h)	0	Lyn(h)	0
PTK5(h)	0	Bmx(h)	0
CSK(h)	1	EphB4(h)	0
Bmx(h)	1	Src(1-530)(h)	0
EphB4(h)	1	PDGFRα(V561D)(h)	0
Arg(h)	1	Fgr(h)	1
BRK(h)	1	Blk(h)	1
Tec(h) activated	1	cSRC(h)	1
Txk(h)	1	EphB1(h)	1
Hck(h)	1	Arg(h)	1
Src(1-530)(h)	2	BRK(h)	1
BTK(h)	2	Tec(h) activated	1
EphA8(h)	2	Txk(h)	1
Lck(h) activated	2	BTK(h)	1
cKit(V560G)(h)	2	EphA8(h)	1
Fms(h)	2	Lck(h) activated	1
Lck(h)	2	Fyn(h)	1
Abl(Y253F)(h)	2	PTK5(h)	2
Hck(h) activated	2	cKit(V560G)(h)	2
EphA1(h)	2	Fms(h)	2
Yes(h)	3	Lck(h)	2
PDGFRα(V561D)(h)	3	DDR1(h)	2
Fyn(h)	3	EGFR(L861Q)(h)	2
Fms(Y969C)(h)	3	ErbB4(h)	2
SIK(h)	3	Abl(Y253F)(h)	3
DDR1(h)	4	Hck(h) activated	3
BTK(R28H)(h)	4	Fms(Y969C)(h)	3
Abl (H396P) (h)	6	Abl (H396P) (h)	3

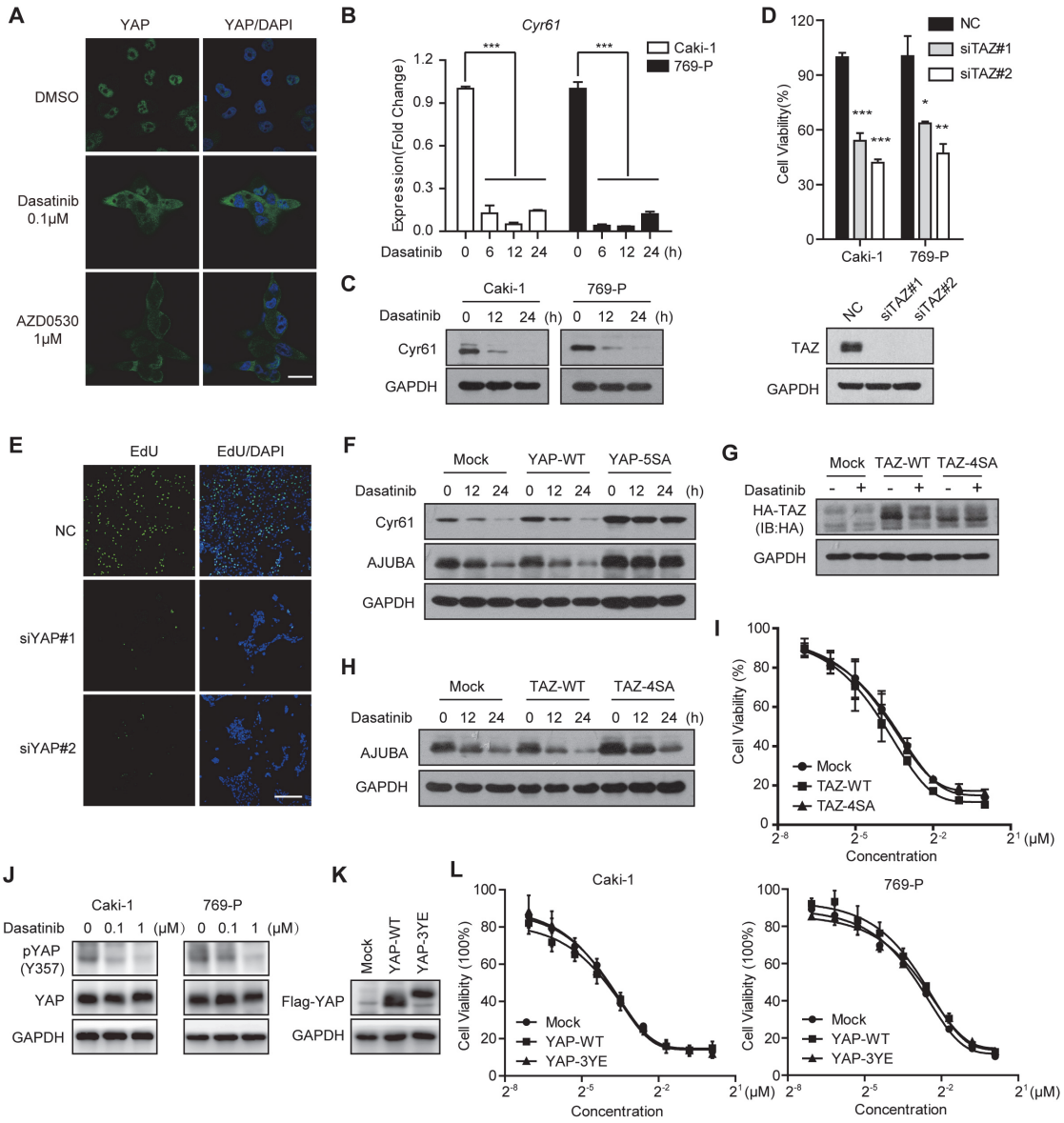
Abl (M351T)(h)	6	DDR2(h)	3
cKit(D816H)(h)	7	PDGFRα(D842V)(h)	3
cKit(h)	9	PDGFRα(h)	3
DDR2(h)	9	SIK(h)	4
PDGFRα(D842V)(h)	9	cKit(D816H)(h)	4
cKit(V654A)(h)	12	cKit(V654A)(h)	4
EGFR(L861Q)(h)	21	Hck(h)	5
ErbB4(h)	28	BTK(R28H)(h)	5
EGFR(L858R)(h)	29	Abl (M351T)(h)	6
cKit(D816V)(h)	38	EGFR(L858R)(h)	7
FGFR2(N549H)(h)	40	PDGFRβ(h)	7
RIPK2(h)	42	EphA1(h)	8
PDGFRβ(h)	43	FGFR2(N549H)(h)	8
ALK4(h)	44	cKit(D816V)(h)	11
EphB3(h)	50	ALK4(h)	12
PDGFRα(h)	50	EGFR(h)	16
		LIMK1(h)	19
		SAPK2a(h)	20
		ALK1(h)	23
		RIPK2(h)	26
		B-Raf(V599E)(h)	32
		TGFBR1(h)	34
		NLK(h)	39
		MLK1(h)	41
		Src(T341M)(h)	41
		FGFR2(h)	44
		Wee1(h)	46
		Abl(T315I)(h)	48
		MST3(h)	48
		B-Raf(h)	49

Supplementary Figures



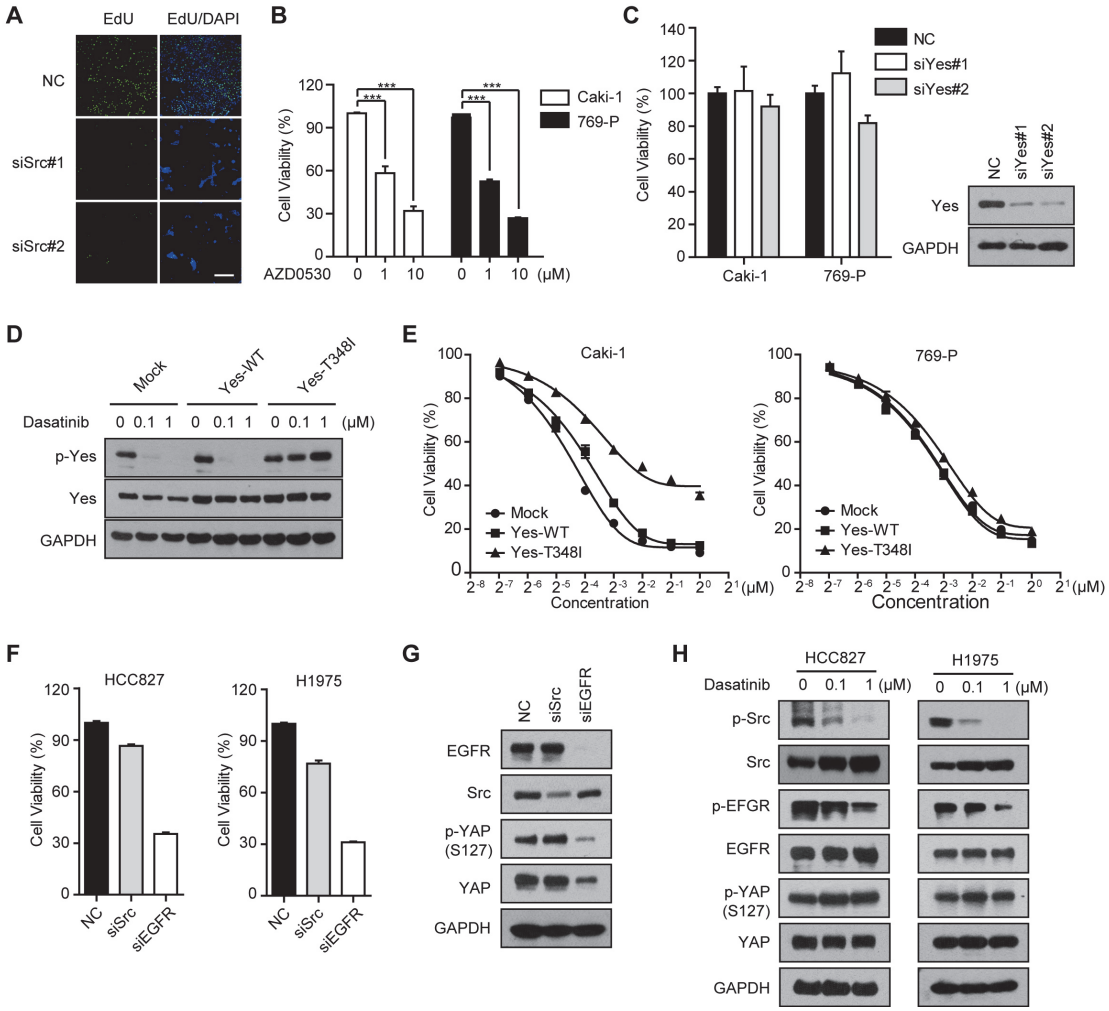
Supplementary Figure S1. Dasatinib notably suppresses the survival of RCC cells by inducing G1/S cell cycle arrest. (A) The growth inhibition effects of dasatinib (0.1 and 1 μM) were tested using the clonogenic assay. **(B)** Caki-1 cells were treated with DMSO or dasatinib at 0.1 and 1 μM for 24 h. Cell cycle distribution was analyzed by flow cytometry. Cell cycle regulators were analyzed by immunoblotting.

Supplementary Figure S2



Supplementary Figure S2. Dasatinib impairs YAP transcriptional activity in sensitive RCC cell lines. (A) Caki-1 cells were treated with 0.1 μM dasatinib or 1 μM AZD0530 for 3 h. The subcellular location of YAP was determined by immunofluorescence. Scale bar, 30 μm. **(B-C)** Caki-1 cells were treated with dasatinib at 1 μM for 6, 12 and 24 h. Cyr61 expression was measured by real-time PCR (B) and immunoblotting (C). Bars, mean ± SD. ***$P < 0.001$. **(D)** Caki-1 and 769-P cells were transfected with scramble or TAZ siRNAs for 72 h. Cell viability was measured using SRB assay. Bars, mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. **(E)** Caki-1 cells were treated with dasatinib for 24 h, and cell cycle progression was measured via

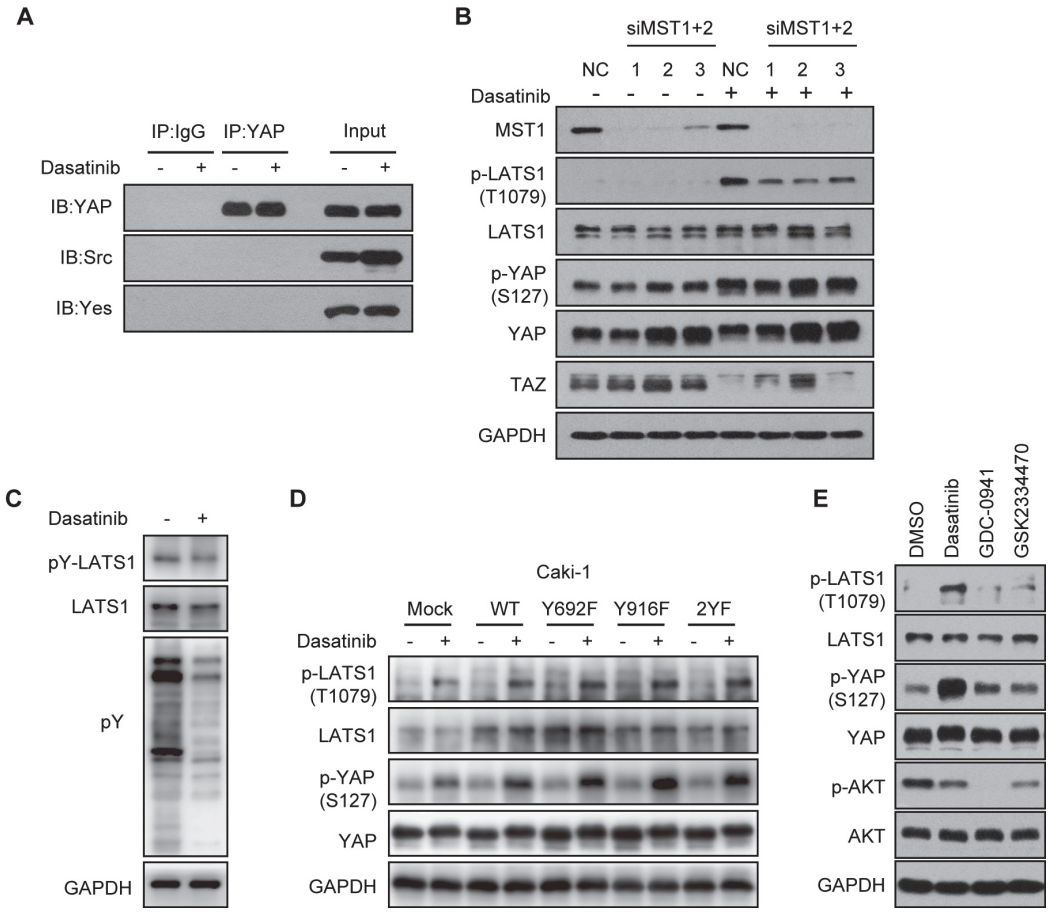
EdU incorporation assay. Scale bar, 300 μm. **(F)** Caki-1 cells transfected with YAP or YAP-5SA were treated with dasatinib, and YAP/TAZ target genes were assessed by immunoblotting. **(G-I)** Caki-1 cells were stably transfected with retrovirus expressing either empty vector, TAZ or TAZ-4SA. HA-TAZ expression was determined by immunoblotting (G). Cells were treated with dasatinib, and YAP/TAZ target genes were assessed by immunoblotting (H). Cell viability after dasatinib treatment for 72 h was assessed by SRB assay. Bars, mean ± SD (I). **(J)** Caki-1 and 769-P cells were treated with dasatinib for 3 h and phosphorylation of YAP Y357 was indicated with immunoblotting. **(K-L)** RCC cells were transfected with either empty vector, Flag-YAP-WT or Flag-YAP-3YE. The expression of Flag was determined by immunoblotting (K). Cell viability after dasatinib treatment for 72 h was assessed by SRB assay (L).



Supplementary Figure S3. Src kinase is the most dominant therapeutic target of dasatinib that regulates YAP activity in RCC cells. (A) Caki-1 cells were treated with scramble or Src siRNAs for 72 h, and cell cycle progression was measured by an EdU incorporation assay. Scale bar, 300 μm. **(B)** Caki-1 and 769-P cells were treated with AZD0530 (1 and 10 μM) for 72 h, and cell viability was measured by SRB assay. Bars, mean ± SD. ***$P < 0.001$. **(C)** Caki-1 cells and 769-P cells were transfected with scramble or Yes siRNAs for 72 h, and cell viability was measured by SRB assay. Bars, mean ± SD. **(D-E)** Caki-1 and 769-P cells were stably transfected with retrovirus expressing either empty vector, Yes-WT or Yes-T348I. Cells were treated

with dasatinib for 3 h, and Yes phosphorylation was measured by immunoblotting (D). Cell viability after dasatinib treatment for 72 h was assessed by SRB assay. Bars, mean ± SD (E). **(F-G)** HCC827 and H1975 cells were treated with scramble, Src siRNA or EGFR siRNA for 72 h. Cell viability was measured by SRB assay (F). p-YAP expression was detected using immunoblotting (G). **(H)** HCC827 and H1975 cells were treated with dasatinib for 3 h and then subjected to immunoblotting with the indicated antibodies.

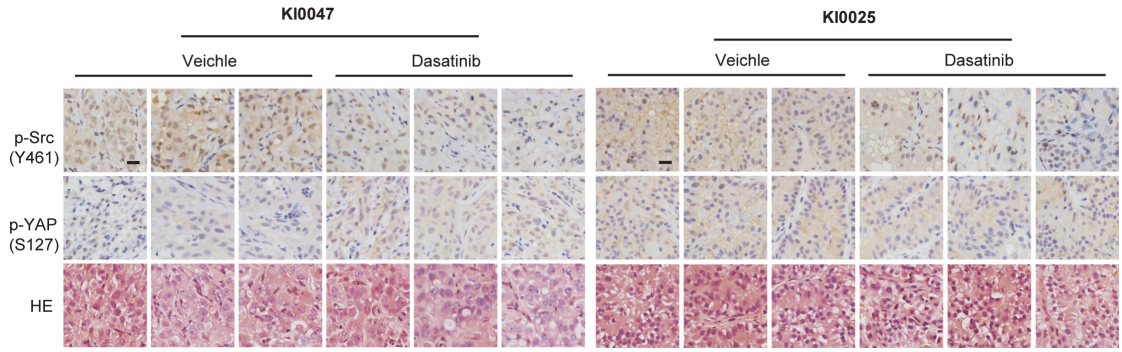
Supplementary Figure S4



Supplementary Figure S4. The JNK-LIMD1-LATS1 signaling cascade mediates the modulation of YAP following Src inhibition in RCC cells. (A) The interactions between YAP and Src, Yes were determined by co-immunoprecipitation. **(B)** 769-P cells were treated with scramble or MST1/2 siRNAs for 72 h, and the cells were

exposed to dasatinib at 1 μM for 1 h. The proteins lysates were subjected to immunoblotting analysis with the indicated antibodies. **(C)** Caki-1 cells were treated with dasatinib for 3 h and the tyrosine phosphorylation of LATS1 was detected by immunoprecipitated and subsequent immunoblotting. **(D)** Caki-1 cells were transfected with LATS1-WT, Y692F, Y916F and 2YF for 48 h and then treated with dasatinib for 3 h. Then cells were lysed for immunoblotting with indicted antibodies. **(E)** Caki-1 cells were treated with dasatinib, PI3K inhibitor (GDC-0941, 1 μM) or PDK1 inhibitor (GSK2334470, 1 μM) for 3 h followed by immunoblotting.

Supplementary Figure S5



Supplementary Figure S5. YAP alteration correlates with the sensitivity of RCC cells to dasatinib. Nude mice bearing patient-derived KI0047 or KI0025 xenografts were administered either vehicle control or dasatinib (50 mg/kg) once daily for 28 or 23 consecutive days (n = 6 for each group). The expression of p-Src and p-YAP in different tumor tissues was assessed by IHC.