Targeting Super-Enhancer associated oncogenes in esophageal squamous cell carcinoma

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SUPPLEMENTARY MATERIALS AND METHODS

Construction and Infection of CDK7 shRNA-Expressing Lentivirus

The pLKO.1-CDK7-shRNA was generated by inserting double-stranded oligonucleotides into pLKO.1-puro lentiviral vector, and was confirmed by DNA sequencing. Recombinant lentiviral vectors and packaging vectors (pCMV-dR8.91 and pMD2.G-VSVG) were co-transfected into 293T cells using Lipofectamine 2000 according to the manufacturer's instruction. Supernatants containing lentivirus expressing shRNA were harvested 48h after transfection, TE7 and KYSE510 cells were infected with the lentiviruses and supplemented with 8 mg/ml Polybrene (Sigma-Aldrich).

Cell Proliferation Assay

To measure proliferation, cells were seeded onto 96 well plates (2,000-5,000 cells per well), and exposed to either vehicle or different concentrations of inhibitors at indicated time points. Cell viability was assessed using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) staining method.

Cell-Cycle Analysis

Cells were harvested after 24 and 48 hr treatment with either vehicle or the inhibitor, and fixed (75% ethanol) overnight at - 20℃. Cell pellets were obtained by spinning at 15,000 RPM for 5 min, washed with cold PBS, and finally resuspended in PBS, with 0.1% triton, RNAse A and propidium iodide (PI). After 30 min of incubation, samples were analyzed by LSR II Flow Cytometer System (BD Biosciences, San Jose, CA).

Cell Apoptosis Analysis

Cells were harvested after exposure to either vehicle or inhibitor, washed twice with PBS, double-labeled with Annexin V-fluorescein isothiocyanate (FITC) and Propidium iodide (PI) using the Rh Annexin V/FITC kit (Bender Medsystem, San Bruno, CA) and measured by

LSR II Flow Cytometer System.

Colony Formation Assay

Colony formation assay was performed by plating cells in 6-well plates. After 2 weeks, cells were fixed with methanol and stained with crystal violet. The number of colonies was counted by ImageJ software. Data were presented as mean ± SD from 3 independent experiments in triplicate wells.

Antibodies and Chemicals

Antibodies: CDK7 (Cell Signaling Technology, 2916), CDK2 (Cell Signaling Technology, 2546), CDK4 (Cell Signaling Technology, 12790), CDK6 (Cell Signaling Technology, 13331), CDK9 (Cell Signaling Technology, 2316), RNAPII CTD S2 (Bethyl, A300-654A), RNAPII CTD S5 (Bethyl, A300-655A); RNAPII CTD S7 (Cell Signaling Technology, 13780); RNAPII (Santa Cruz, sc-899); DNAJB1 (Cell Signaling Technology, 4871S); YAP1 (Novus Biologicals, NB110-58358); SREBP2 (Abcam, ab30682); RUNX1 (Abcam, ab35962); Phospho-RUNX1 (Cell Signaling Technology, 4327); PAK4 (Cell Signaling Technology, 3242); Phospho-PAK4 (Santa Cruz, sc-135774); GAPDH (Abcam, ab46540); Anti-rabbit IgG (Cell Signaling Technology, 7074); Anti-mouse IgG (Cell Signaling Technology, 7076); H3K27ac (Abcam, ab4729); Rabbit anti-IgG (Abcam, ab46540).

Chemicals: THZ1 (ApexBio, A8882); AT7519 (Selleck Chem, S1524); SNS-032 (Selleck Chem, S1145); LEE011 (Selleck Chem, S7440); Flavopiridol (Selleck Chem, S1230); Roscovitine (Selleck Chem, S1153); JNJ-7706621 (Selleck Chem, S1249). KPT9274 (Karyopharm Therapeutics)

RNA-Seq Analysis

Total RNA from each sample was extracted according to the manufacturer's instruction of RNeasy Mini kit (QIAGEN). RNA library was prepared using TruSeq Library Prep Kit (Illumina) according to the manufacturer's instructions and subjected to massive parallel

sequencing using Hiseq (Illumina) at Beijing Genomics Institute. To analyze the RNA-Seq results, we first aligned 100 bp paired-end sequencing reads to human reference genome (build GRCh37/hg19) using STAR aligner with ensemble gtf (v75) provided as junctions file[1]. Cufflinks were used to measure the expression in terms of FPKM (Fragments Per Kilobase of transcript per Million mapped reads) against Ensemble transcripts (v75)[2]. Those transcripts with mean expression of FPKM >1 were considered as actively transcribed and were used for subsequent analysis. Heatmaps were drawn with R software using log2 fold change values. Wiggle tracks for RNA-seq data were generated using rseqc (bam2wig.py command) and normalized for rpm[3].

Chromatin Immunoprecipitation

Cells were first crosslinked with 1% formaldehyde solution and incubated for 10 min at room temperature. Cells were neutralized by 1 mL 1.25 M glycine for 5 min, washed three times in ice-cold PBS, centrifuged at 2,000 rpm for 5 min to remove PBS and resuspended in nuclear extraction buffer A (10mM HEPES, 10mM KCL, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 0.5 Mm PMSF) by gentle pipetting or vortex, on ice for 15 min. 25ul of ice cold 10% NP-40 was added per 400 ul of Buffer A and vortexed for 10 sec, kept on ice for 1min and vortex again for 10 sec, centrifuged for 1-5 min at 16,000 ×g. 200 ul SDS lysis buffer was added to the pellet (0.5% SDS, 50 mM Tris, pH 8, 10 mM EDTA, 1 complete protease inhibitor (Roche). Chromatin was sheared in a Bioruptor Sonicator (Diagenode) with 26 cycle, 30 sec. ON, 30 sec. OFF, on high mode at 4℃. Sonication resulted in most small fragments being 200-250 bp in length. The sonicated lysates were collected and centrifuged for 10 min at 4 °C. Supernatants were collected and four parts of dilution buffer (1.25% Triton X-100, 12.5 mM Tris, pH 8, 187.5 mM NaCl, 1 complete protease inhibitor) were added. Sonicated lysates were cleared and incubated overnight at 4℃ with magnetic beads bound with antibody to enrich for DNA fragments bound by the indicated factor. Precipitated

immunocomplexes were washed, for 5 min each, as follows: once with low-salt buffer (0.1% SDS, 1% Triton X-100, 20 mM Tris, pH 8, 2 mM EDTA, 150 mM NaCl and 1 Complete protease inhibitor), once with high-salt buffer (0.1% SDS, 1% Triton X-100, 20 mM Tris, pH 8, 2mM EDTA, 500 mM NaCl and 1 Complete protease inhibitor), twice with LiCl buffer (0.7% sodium deoxycholate, 1% NP-40, 20 mM Tris, pH 8, 1 mM EDTA, 500 mM LiCl and 1 complete protease inhibitor) and once with Tris-EDTA buffer (with protease inhibitor). DNA was eluted with elution buffer (50 mM TrisHCl pH 8.0,10 mM EDTA, 1% SDS). Cross-links were reversed overnight. RNA and protein were digested using RNase A and Proteinase K, respectively and DNA was purified with QIAquick PCR spin kit (QIAGEN).

Gene Ontology Analysis

Gene Ontology analysis was performed using Goseq Bioconductor package[4]. All actively transcribed genes were considered as assayed genes (background) and genes with log2 fold change less than one (50nM THZ1 treatment at 6 hours) were considered as “THZ1-sensitive transcripts”. Similar analysis was performed for SE-associated genes.

Reference

1. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013;**29**(1):15-21
2. Trapnell C, Roberts A, Goff L, et al. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nature protocols 2012;**7**(3):562-78
3. Wang L, Wang S, Li W. RSeQC: quality control of RNA-seq experiments. Bioinformatics 2012;**28**(16):2184-5
4. Young MD, Wakefield MJ, Smyth GK, Oshlack A. Gene ontology analysis for RNA-seq: accounting for selection bias. Genome biology 2010;**11**(2):R14

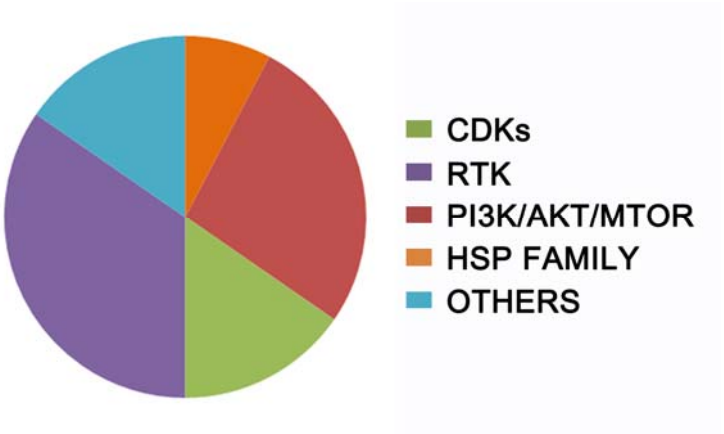
Supplementary Table 1. IC50 (nM) values of 104 inhibitors in ESCC cells

RANKING	INHIBITOR	TARGET	TE7	TE5	KYSE30	KYSE180
1	Elesclomol	HSP70	8	10	9	7
2	Flavopiridol	CDKS	7	6	399	1001
3	Velcade	Proteasome	7	7	196	693
4	BEZ235	PI3K/mTOR	99	33	7	514
5	YM-155	Survivin	45	547	8	42
6	Crenolanib	PDGFR	7	8	4787	9334
7	Dasatinib	Src/ Abl/kit	310	326	800	1000
8	MK-2206	AKT	9	8	10000	10000
9	Ponatinib	Abl, PDGFRα, VEGFR2, FGFR1 and Src	24	115	153	129
10	SNS-032	CDKS	1623	1623	310	408
11	Sunitinib	VEGFR/PDGFR	520	918	1000	1000
12	Vismodegib	Hedgehog	7	6	10000	10000
13	BI-2536	PLK1	1821	1906	693	3163
14	17-AAG	HSP90	1515	5496	2090	1446
15	A-674563	AKT	3632	3891	709	1073
16	AT7519	CDKS	2400	3021	183	538
17	Foretinib	HGFR/VEGFR	3389	8914	1550	1906
18	H-89	PKA	693	205	10000	10000
19	MGCD-265	VEGFR	340	662	10000	10000
20	Midostaurin	PKCα/VEGFR	18	92	5013	3312
21	PD173955	Bcr-Abl / Src	1863	5496	1231	2952
22	PI-103	PI3K	3891	7944	408	1260
23	Roscovitine	CDKS	1821	1414	10000	10000
24	ABT-737	Bcl family	10000	10000	10000	10000
25	Afatinib	EGFR/HER2	3803	6027	2240	2693
26	Alisertib	Aurora A	10000	10000	4075	10000
27	Arsenic Trioxide	N/A	10000	10000	10000	10000
28	Axitinib	VEGFR, c-kit, PDGFR	10000	10000	10000	10000
29	Azacytidine	DNA methyltransferase	10000	10000	10000	10000
30	AZD1480	JAK1/2	10000	10000	10000	10000
31	Barasertib	Aurora B	10000	10000	10000	10000
32	BMS-345541	IKK	10000	10000	7245	6762
33	Bosutinib	Src/Abl	3716	6762	4787	3632
34	Canertinib	pan-ErbB	8914	5624	10000	2819
35	Carbozantinib	c-MET and VEGFR2	10000	10000	10000	10000

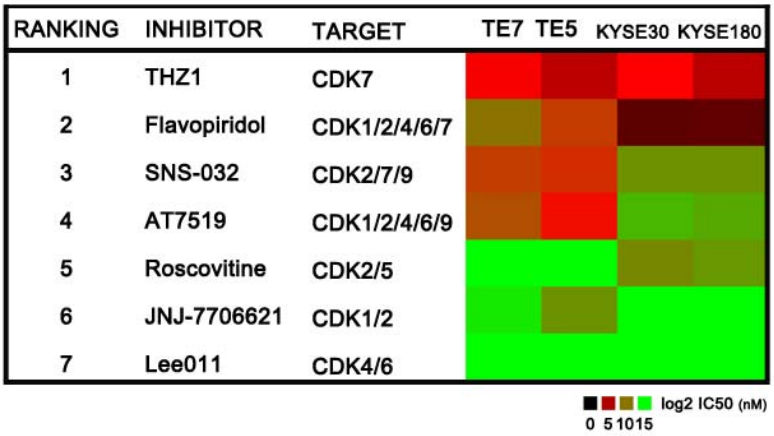
36	Cediranib	VEGFR/ c-kit	10000	8914	4572	8512
37	CHIR-99021	GSK-3α/β	3632	6027	10000	10000
38	CI-1040	MEK	10000	10000	10000	10000
39	CPI-267203	BET domain	10000	10000	10000	10000
40	Crizotinib	c-Met/ALK	3632	6027	7944	10000
41	CYT387 (momelotinib)	JAK1/2 inhibitor	6608	10000	8129	10000
42	DBZ	Notch γ-secretase	10000	10000	10000	10000
43	Doramapimod	p38 MAPK	10000	10000	10000	10000
44	Dovitinib	FGFR	4075	4468	7763	10000
45	Erlotinib	EGFR	9334	10000	10000	9121
46	GDC-0879	B-Raf	10000	10000	10000	10000
47	GDC-0941	PI3K	4075	4468	3632	2952
48	Gefitinib	EGFR	10000	10000	10000	10000
49	GS-1101	PI3K delta	5249	9551	10000	10000
50	GSK-1838705A	IGF-IR	10000	10000	2952	4787
51	GSK-1904529A	IGF-IR	2189	3163	10000	10000
52	GSK690693	Akt	10000	10000	7763	10000
53	GW-2580	CSF1R/Fms	10000	10000	10000	10000
54	Ibrutinib	BTK	10000	10000	10000	2513
55	Imatinib	v-Abl/PDGFR	10000	10000	10000	10000
56	JAK Inhibitor I	JAK	5496	9551	10000	10000
57	JNJ-28312141	CSF-1R	10000	10000	10000	10000
58	JNJ-38877605	c-Met	10000	10000	10000	10000
59	JNJ-7706621	CDK1/2	10000	10000	1661	6919
60	KI20227	c-fms	10000	10000	10000	10000
61	KU-55933	ATM	10000	10000	10000	10000
62	KW-2449	FLT3/ABL/ABL-T315I	10000	10000	9334	10000
63	Lapatinib	EGFR/HER2	10000	9121	10000	10000
64	Linifanib	VEGFR/PDGFR	10000	10000	10000	7944
65	LY-333531	PKC	10000	10000	10000	10000
66	Masitinib	Kit/PDGFRα/β	10000	10000	10000	10000
67	MLN120B	IκB Kinase β	5000	5000	5000	5000
68	MLN8054	Aurora A	10000	10000	10000	10000
69	Motesanib	VEGFR	10000	10000	10000	10000
70	Neratinib	HER2 and EGFR	3891	4787	5013	2400
71	NF-kB Activation Inhibitor	NF-kB	576	10000	10000	10000
72	Nilotinib	Bcr-Abl	6027	7	10000	10000
73	NVP-ADW742	IGF-1R	10000	10000	2952	10000
74	NVP-TAE684	ALK	8512	7414	1951	10000
75	Pazopanib	VEGFR/PDGFR, FGFR, c-Kit and c-Fms	10000	10000	10000	10000

76	Pelitinib	EGFR	2189	3389	4170	2043
77	PHA-665752	Met	3632	2513	9334	3549
78	PHT-427	Akt/PDPK1	10000	10000	10000	10000
79	PP242	mTOR	4366	3312	852	3891
80	PRT062607	SYK	10000	7587	9551	10000
81	Quizartinib	FLT3	10000	10000	4075	8711
82	RAF265	B-Raf /VEGFR2	10000	8711	10000	2631
83	Rapamycin	mTOR	10000	10000	36	10000
84	Regorafenib	VEGFR/PDGFRβ/Kit/ RET/ Raf-1	10000	10000	10000	10000
85	Ruxolitinib	JAK1/2	10000	10000	10000	10000
86	S31-201	Stat3	10000	10000	10000	10000
87	Saracatinib	c-Src/Abl	6027	6167	3163	9334
88	SB-431542	ALK	10000	5249	10000	10000
89	Selumetinib	MEK1	10000	10000	10000	10000
90	SGX-523	Met	10000	10000	8914	10000
91	Sorafenib	Raf/VEGFR/PDGFR	4787	6762	10000	10000
92	STO609	CaM-KK	10000	10000	10000	10000
93	SU11274	c-Met	10000	10000	10000	10000
94	TG100-115	PI3K	10000	10000	10000	10000
95	Tivozanib	VEGFR	10000	10000	4572	10000
96	Tofacitinib	JAK1/2	10000	9121	10000	10000
97	Tozasertib	Aurora/FLT3/ Bcr-Abl	10000	10000	10000	10000
98	Trametinib	MEK1/2	3312	10000	760	10000
99	Vandetanib	VEGFR2	5496	5249	10000	2400
100	Vargatef	VEGFR, FGFR, PDGFR	10000	10000	5013	9334
101	Vatalanib	VEGFR2/KDR	10000	10000	10000	10000
102	Vemurafenib	BRAF	10000	10000	10000	10000
103	VX-745	p38α/β	10000	10000	10000	10000
104	XAV-939	TNKS1/2	10000	10000	10000	10000

Supplementary Figure 1. Pie chart of top 23 ranked inhibitors classified based on their molecular targets.

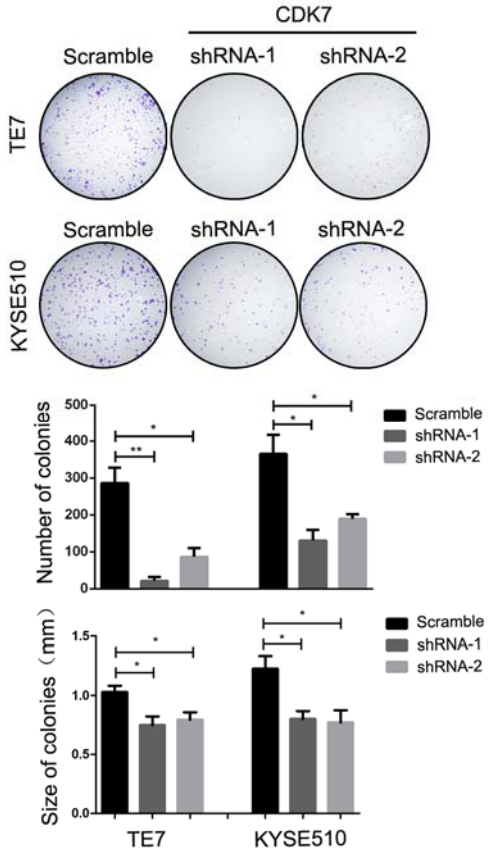


Supplementary Figure 2. Identification of CDK7 as a druggable kinase in ESCC cells



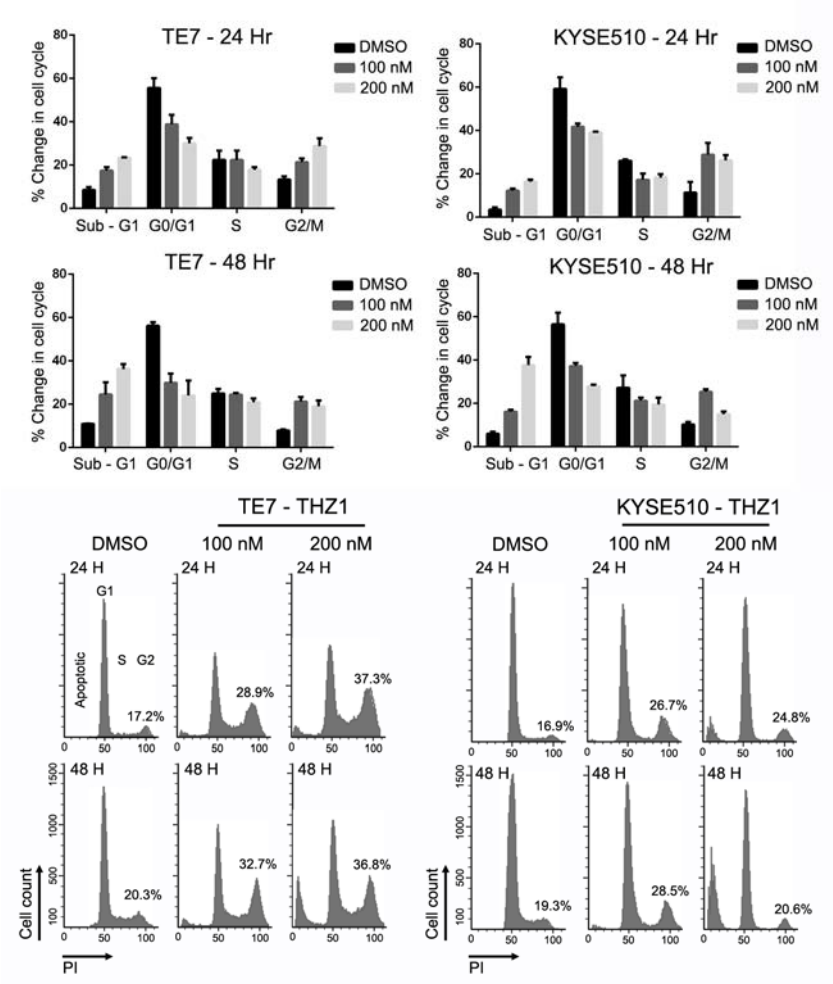
Heatmap showing the sensitivity of TE7, TE5, KYSE30 and KYSE180 cells to a panel of 7 different CDK inhibitors.

Supplementary Figure 3. CDK7 silencing decreased cell proliferation of ESCC cells.



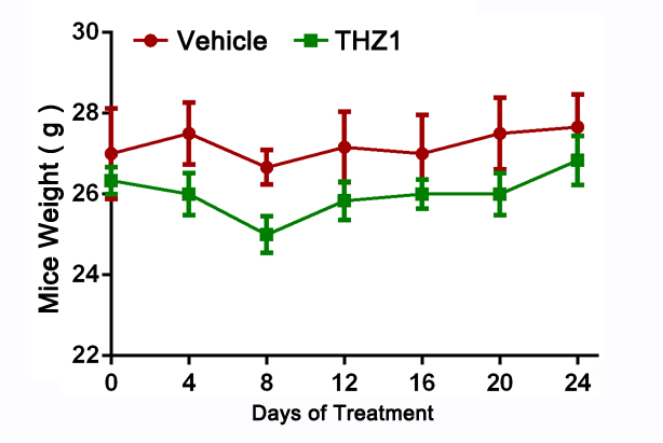
Colony formation assay showed significant decrease in both the colony numbers and size upon CDK7 knockdown compared with Scramble group (* P < .05, ** P < .01).

Supplementary Figure 4. Cell-cycle analysis of TE7 and KYSE510 cells treated with THZ1



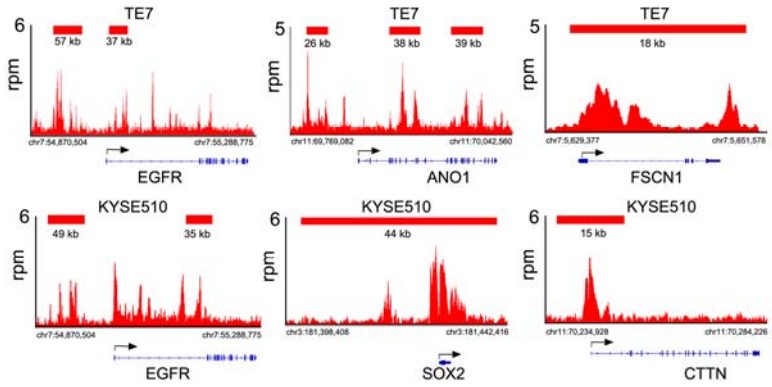
TE7 and KYSE510 cells were treated with indicated concentrations of THZ1 for either 24 or 48 hr and analyzed by flow cytometry by staining with propidium iodide (PI) .

Supplementary Figure 5. THZ1 treatment did not cause loss of weight of NSG mice

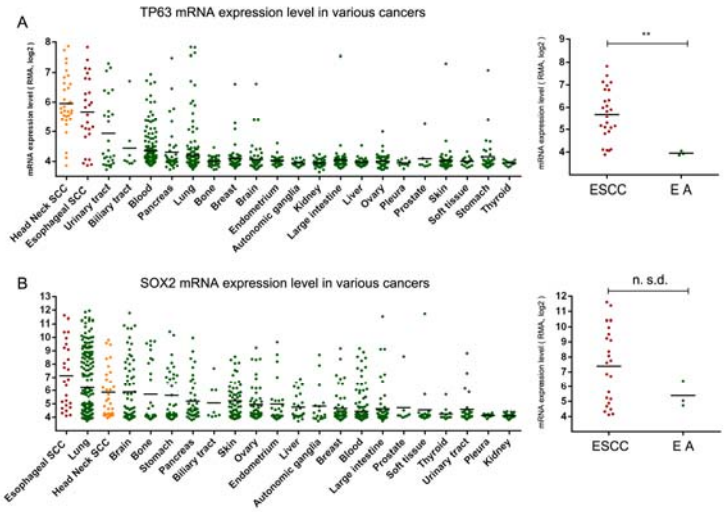


During 24 days of THZ1 treatment (twice daily, 10 mg/kg), no significant loss of weight was observed in the mice (Mean ± SD of 12 mice).

Supplementary Figure 6. H3K27ac ChIP-seq profiles of representative SE-associated genes in TE7 and KYSE510 cells

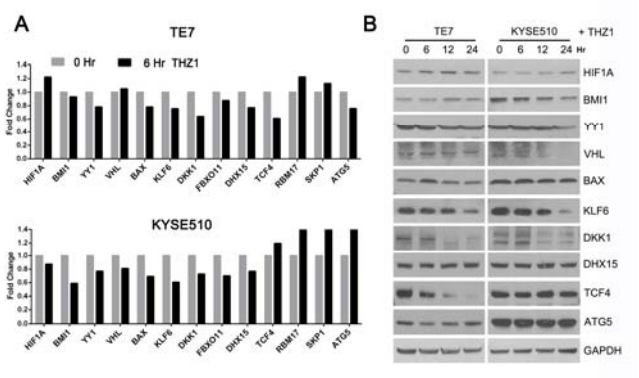


Supplementary Figure 7. mRNA expression levels of TP63 and SOX2 in various types of cancer cells



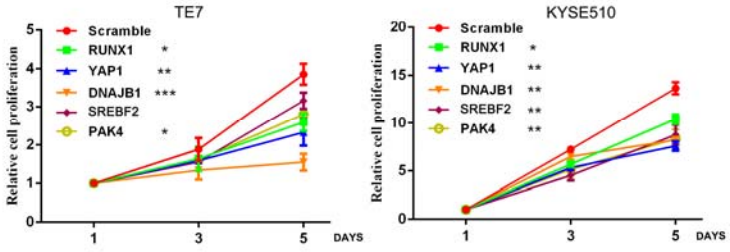
(A) Left, mRNA expression of TP63 was the highest in ESCC and head and neck squamous cancers among various types of cancer cell lines. Right, TP63 expression was higher in ESCC than EA cell lines (** $P < .01$). (B) SOX2 expression was the highest in ESCC and ranked 3rd in head and neck squamous cancers. SOX2 expression was higher in ESCC than EA, albeit it did not achieve statistical significance (n.s.d). Data were retrieved from CCLE project.

Supplementary Figure 8. TE-associated transcripts were not sensitive to THZ1 treatment.



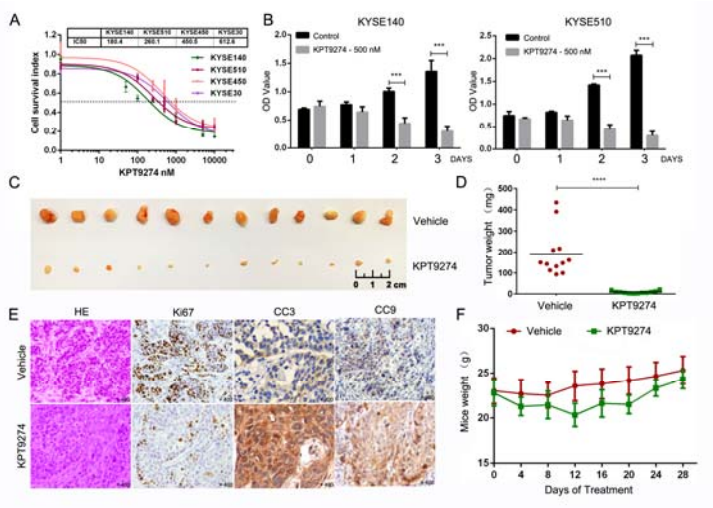
Thirteen TE-associated transcripts were randomly selected, and the alterations of their mRNA and protein levels upon THZ1 treatment were quantified by qRT-PCR (A) and immunoblotting assays (B)

Supplementary Figure 9. SE-associated candidate oncogenes were required for the proliferation in both TE7 and KYSE510 cells



Proliferation rate (MTT assay) of TE7 and KYSE510 cells was measured upon knockdown of the candidate genes by siRNAs (* $P < .05$, ** $P < .01$, *** $P < .001$, compared with Scramble group).

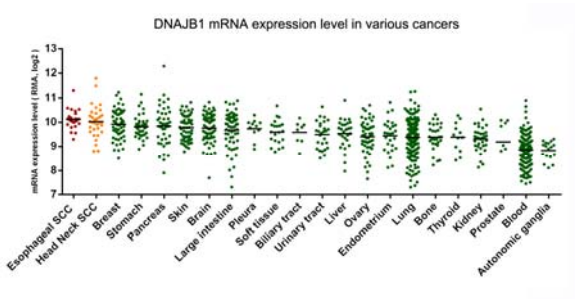
Supplementary Figure 10. Identification of PAK4 as a SE-associated drug target in ESCC



(A) Dose-response curves of ESCC cell lines (high PAK4 expression) to treatment with KPT9274 (PAK4 inhibitor). Data are represented as mean ± SD of three

replicates. (B) Proliferation assay (MTT) shows effects of KPT9274 treatment on ESCC cell lines at indicated time points. Bars represent mean ± SD (*** $P < .001$). (C) Photographs and (D) Tumor weights of KYSE510 xenografts from both vehicle and KPT9274 (150 mg/kg, twice daily, 5 days/week, orally) treatment groups at the endpoint of experiment (28 days) (**** $P < .0001$). (E) H&E and IHC staining of tumor tissue sections. Cell proliferation and apoptosis was examined by Ki67, CC3 and CC9 staining, respectively. Original magnification, X 400. (F) No significant difference in the weight of mice between vehicle and KPT9274 groups was observed during the 28 days of treatment.

Supplementary Figure 11. mRNA expression levels of DNAJB1 in various types of cancer cell lines



mRNA expression of DNAJB1 was the highest in ESCC and head/neck squamous cancers among various types of cancer cell lines. Data were retrieved from CCLE project.