Supporting Information for

Original article

The FAPα-activated prodrug Z-GP-DAVLBH inhibits the growth and pulmonary metastasis of osteosarcoma cells by suppressing the AXL pathway

Geni Ye[a,b,†], Maohua Huang[a,b,c,†], Yong Li[a,b], Jie Ouyang[a,b], Minfeng Chen[a,b], Qing Wen[a,b], Xiaobo Li[a,b], Huhu Zeng[a,b], Pei Long[a,b], Zepei Fan[d], Junqiang Yin[d,*], Wencai Ye[a,b,*], Dongmei Zhang[a,b,*]

[a]*College of Pharmacy, Jinan University, Guangzhou 510632, China*

[b]*Guangdong Province Key Laboratory of Pharmacodynamic Constituents of Traditional Chinese Medicine and New Drugs Research, Jinan University, Guangzhou 510632, China*

[c]*Integrated Chinese and Western Medicine Postdoctoral Research Station, Jinan University, Guangzhou 510632, China*

[d]*Department of Musculoskeletal Oncology, the First Affiliated Hospital of Sun Yat-sen University, Guangzhou 510080, China*

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[*]Corresponding authors. Tel.: +86 20 85222653, +86 20 85220004, +86 20 87755766.

E-mail addresses: dmzhang701@jnu.edu.cn (Dongmei Zhang), chywc@aliyun.com (Wencai Ye), yinjunq@mail.sysu.edu.cn (Junqiang Yin).

[†]These authors made equal contributions to this work.

Supporting figures

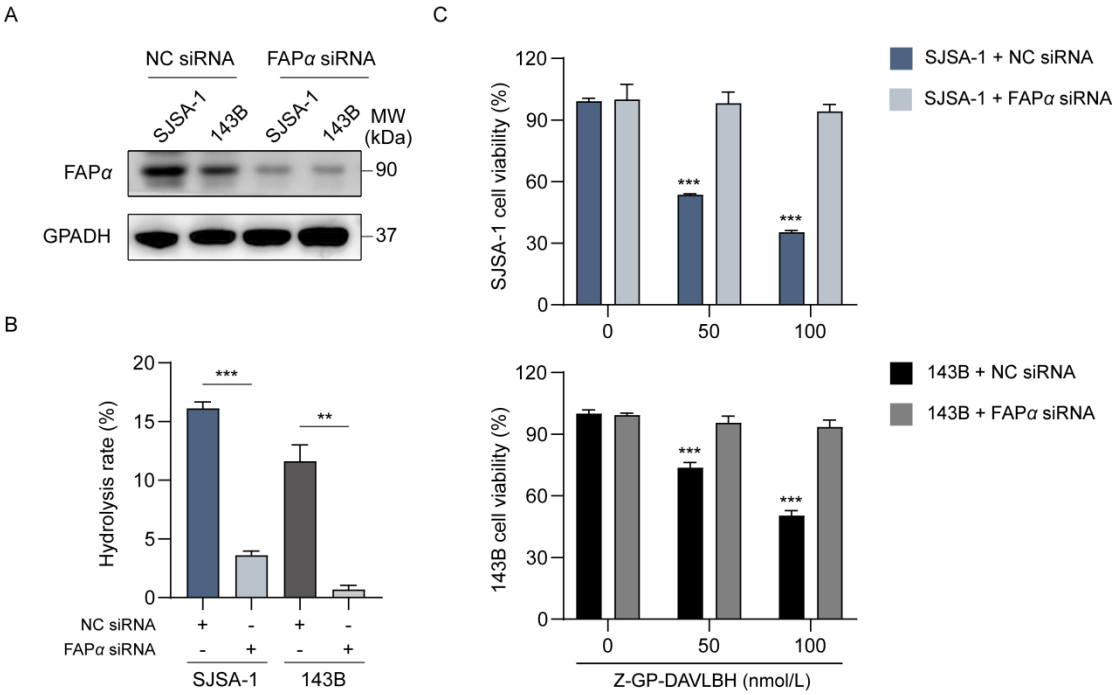

Figure S1 Z-GP-DAVLBH shows an attenuated ability to inhibit the viability of osteosarcoma cells with low expression of FAPα. Osteosarcoma cells were transfected with FAPα siRNA to mimic the cells with low expression of FAPα. (A) Western blotting analysis was conducted to determine the expression of FAPα in osteosarcoma cells (SJSA-1 and 143B cells) after transfection with FAPα siRNA. (B) The hydrolysis efficiency of Z-GP-DAVLBH of osteosarcoma cells was analyzed by LC–MS. Data are presented as mean ± SEM, $n = 3$; $^{**}P < 0.01$ and $^{***}P < 0.001$. (C) The effect of Z-GP-DAVLBH on osteosarcoma cell viability was evaluated by MTT assay. Data are presented as mean ± SEM, $n = 3$; $^{***}P < 0.001$ *vs.* the untreated (0 nmol/L, 0.1% DMSO) group.

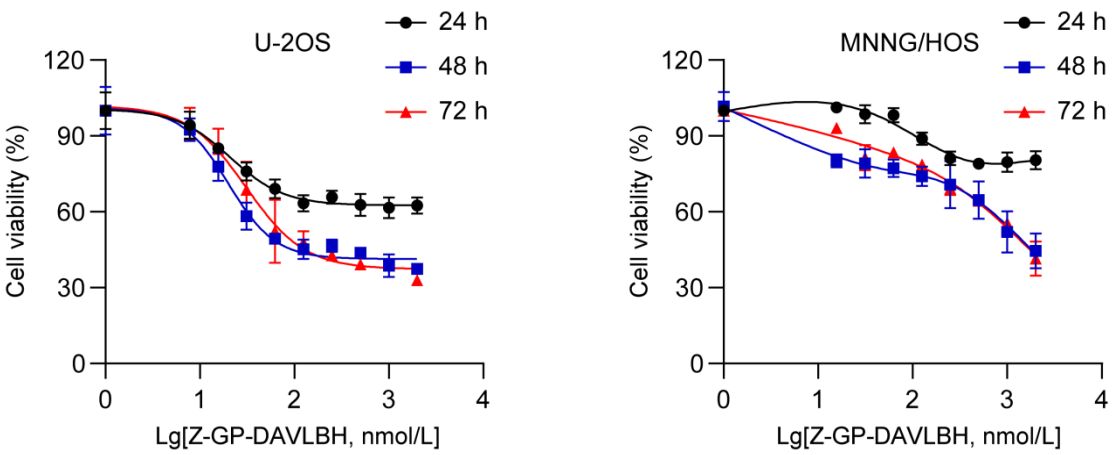

Figure S2 Z-GP-DAVLBH decreases the cell viability of U-2OS and MNNG/HOS cells. U-2OS and MNNG/HOS cells were treated with various concentrations of Z-GP-DAVLBH for 24, 48, and 72 h. Cell viability was detected by an MTT assay. Data are presented as mean ± SEM, $n = 3$.

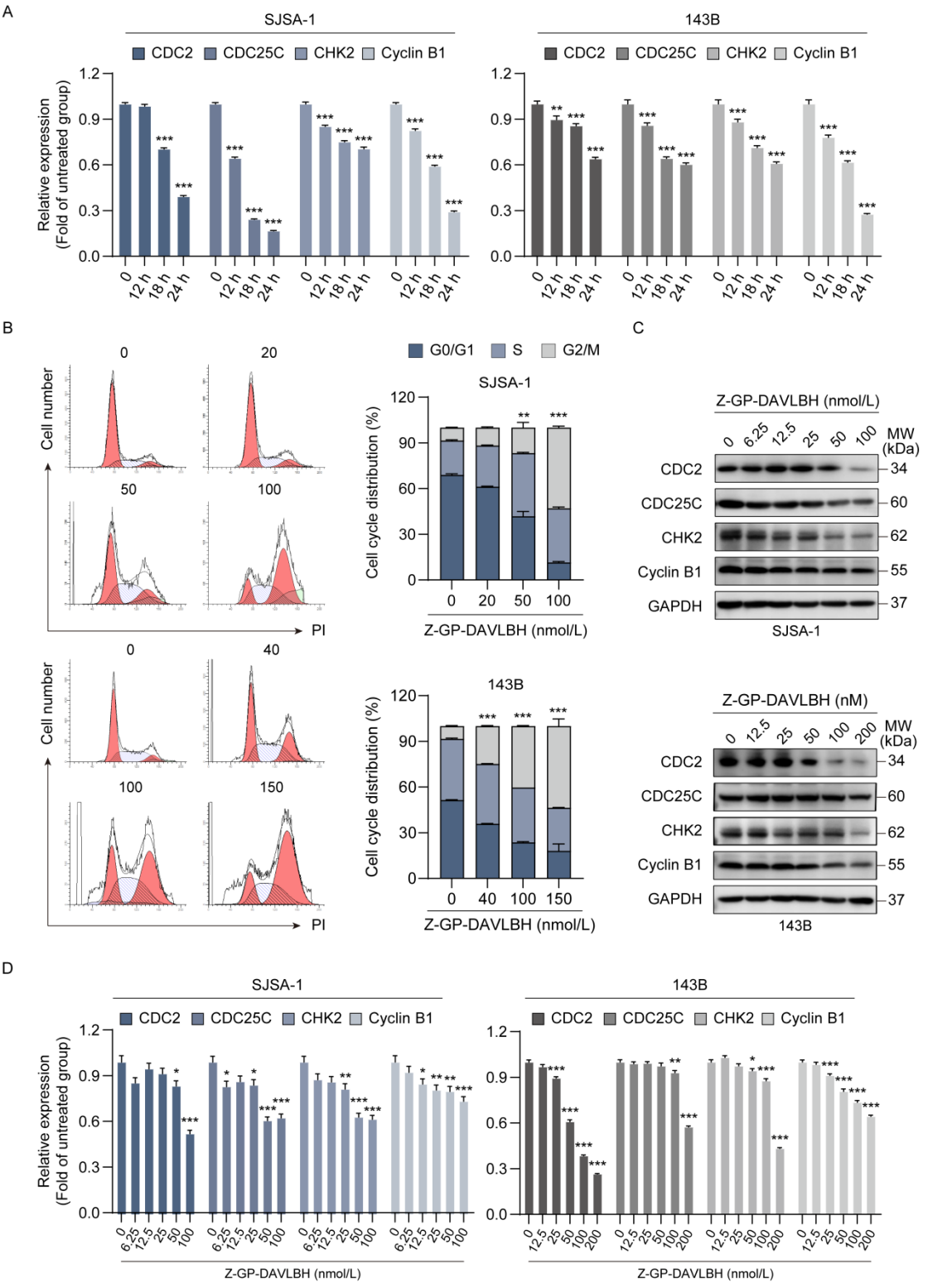

Figure 3 Z-GP-DAVLBH induces cell cycle arrest in the G2/M phase in osteosarcoma cells. (A) SJSA-1 (50 nmol/L) and 143B (100 nmol/L) cells were treated with Z-GP-DAVLBH for the indicated times. Quantification of the expression of CDC2, CDC25C,

CHK2, and Cyclin B1 in osteosarcoma cells is shown. (B)–(D) Osteosarcoma cells (SJSA-1 and 143B) were exposed to various concentrations of Z-GP-DAVLBH for 24 h. (B) The cell cycle distribution was evaluated by flow cytometric analysis. (C) Cell cycle-associated proteins were determined by Western blotting analysis. Representative blots are shown. (D) Quantification of the blots in (C) is shown. Data are presented as mean ± SEM, $n = 3$; $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* the untreated (0 nmol/L, 0.1% DMSO) group.

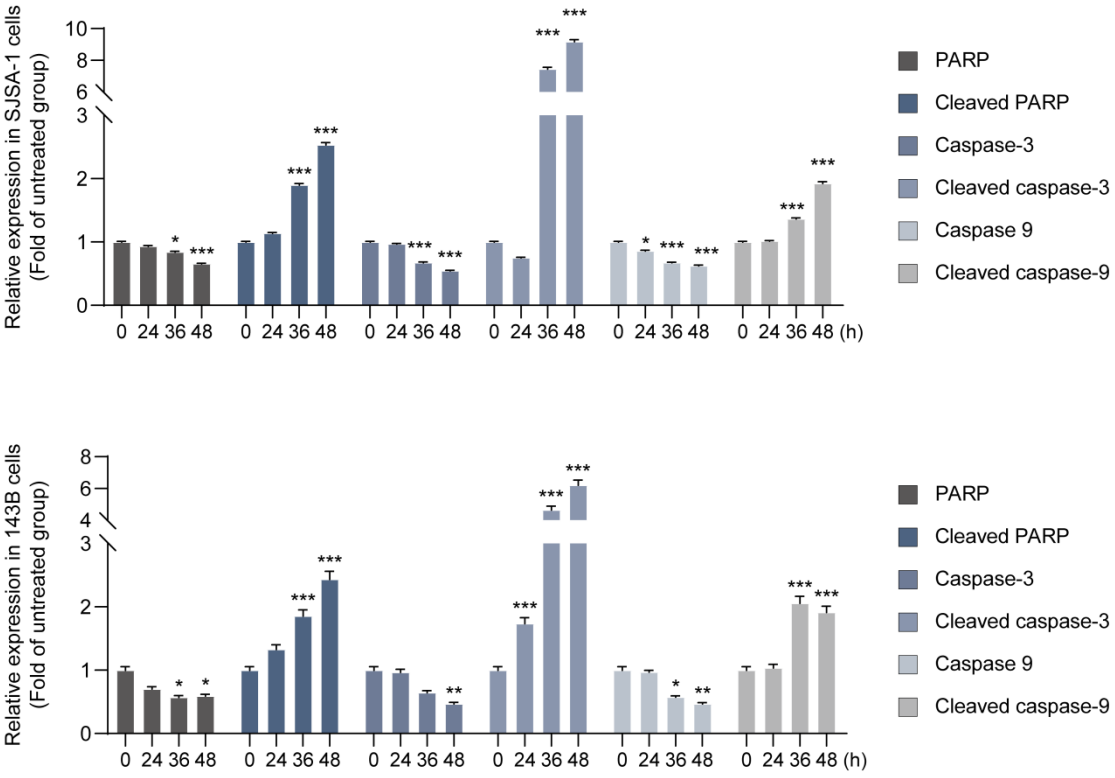

Figure 4 Z-GP-DAVLBH upregulates the expression of apoptosis-related proteins in osteosarcoma cells. SJSA-1 (50 nmol/L) and 143B (100 nmol/L) cells were treated with Z-GP-DAVLBH for the indicated times. Quantification of the expression of PARP, cleaved PARP, caspase-3, cleaved caspase-3, caspase 9, and cleaved caspase-9 in osteosarcoma cells is shown. Data are presented as mean ± SEM, $n = 3$;$^{**}P < 0.01$ and

$^{***}P < 0.001$ *vs.* the untreated (0 nmol/L, 0.1% DMSO) group.

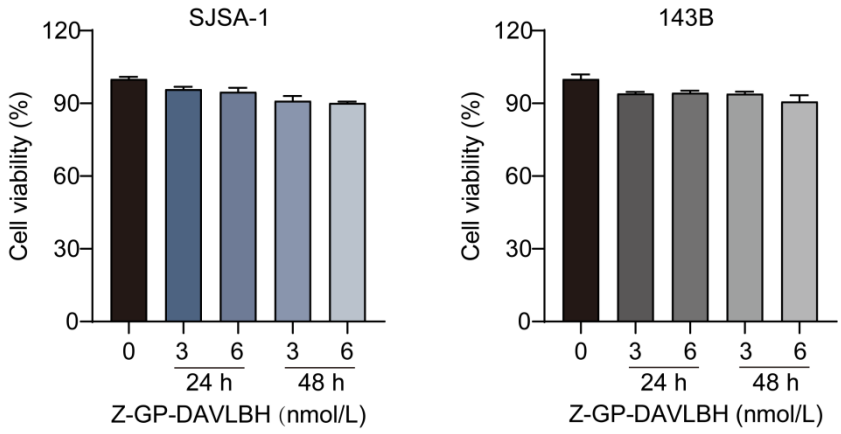

Figure 5 Effect of Z-GP-DAVLBH on cell viability. Following treatment for 24 and 48 h, MTT assay was used to detect the effects of Z-GP-DAVLBH (3 and 6 nmol/L) on the viability in SJSA-1 and 143B cells.

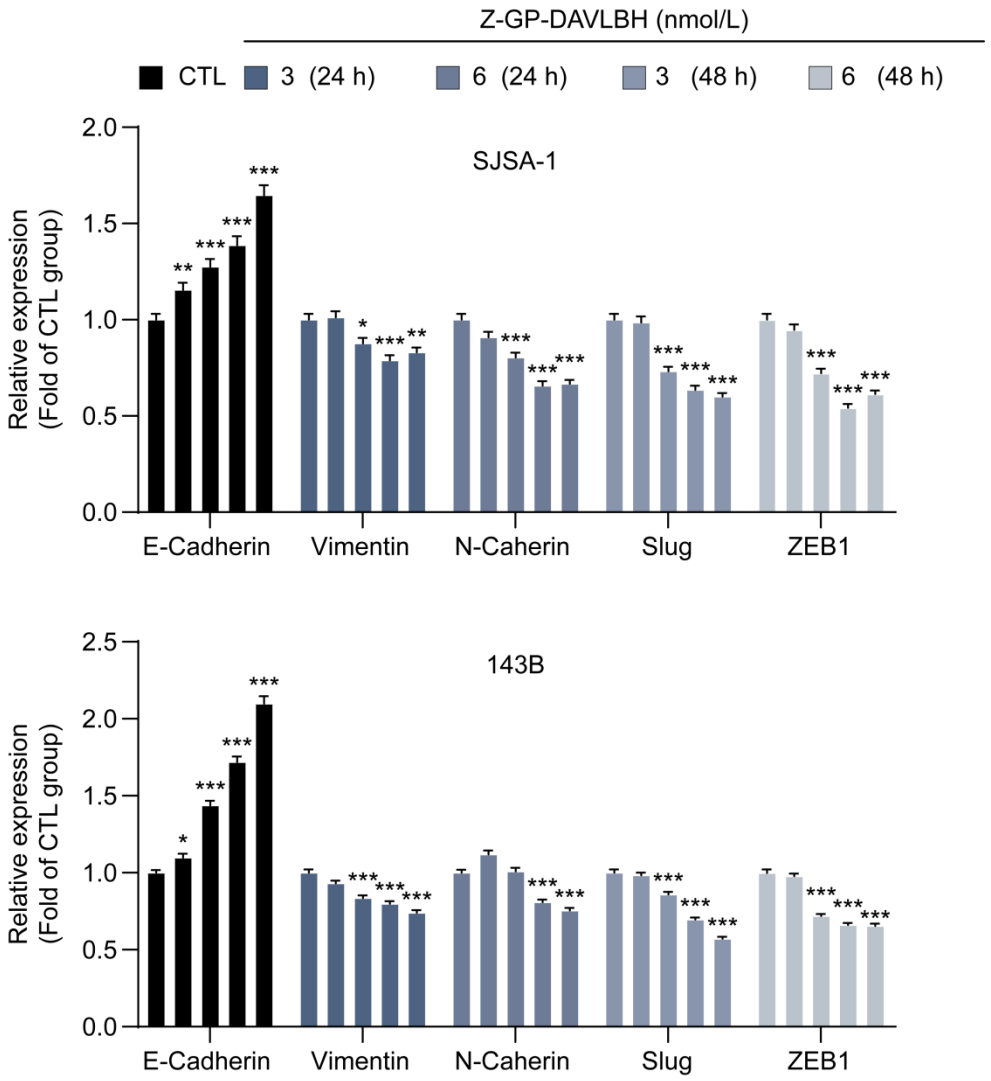

Figure 6 Z-GP-DAVLBH inhibits EMT in osteosarcoma cells. SJSA-1 and 143B cells treated with Z-GP-DAVLBH (3 or 6 nmol/L) for 24 or 48 h and the expression of E-cadherin, Vimentin, N-cadherin, Slug, and ZEB1 in the treated cells was determined by Western blotting analysis. Quantification of blots is shown. Data are presented as mean ± SEM, $n = 3$; $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* the CTL (0 nmol/L, 0.1% DMSO) group.

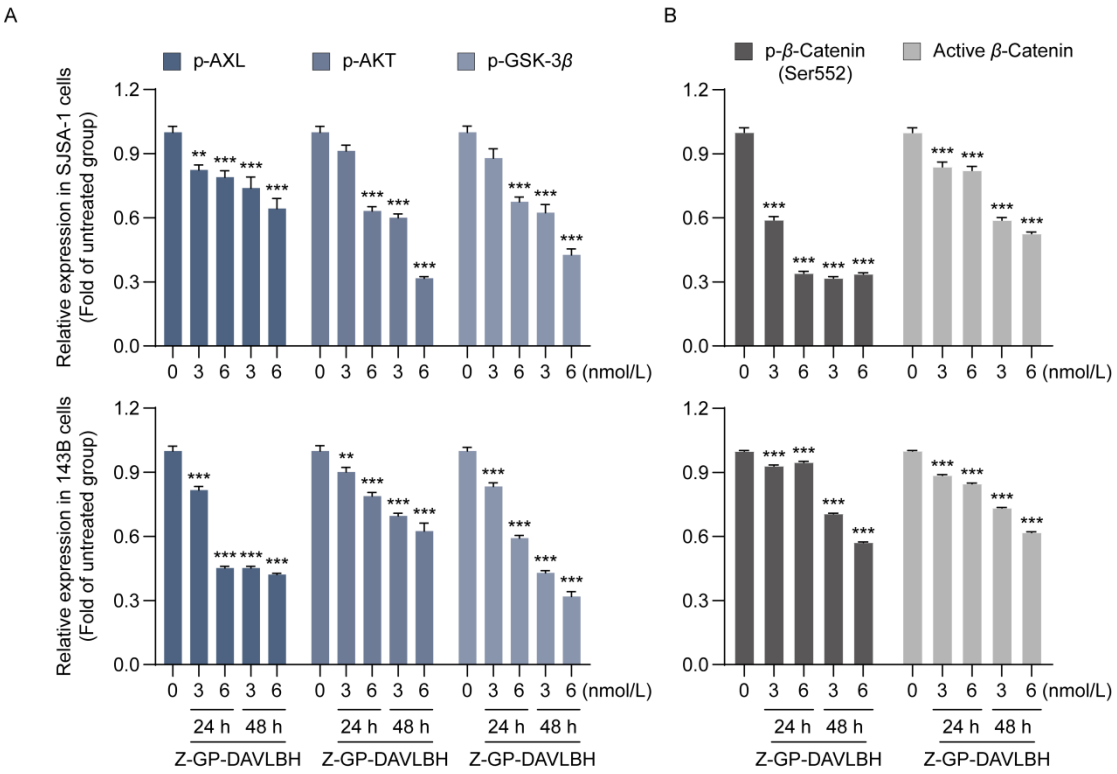

Figure 7 Z-GP-DAVLBH inhibits the AXL/AKT/GSK-3*β*/*β*-catenin pathway in osteosarcoma cells. SJSA-1 and 143B cells treated with Z-GP-DAVLBH (3 or 6 nmol/L) for 24 or 48 h, and then the expression of p-AXL (Tyr779), AXL, p-AKT (Ser473), AKT, p-GSK-3*β* (Ser9), and GSK-3*β* in the treated cells were determined by Western blotting analysis. (A) Quantification of the blots in Fig. 5A. (B) Quantification of the blots in Fig. 5B. Data are presented as mean ± SEM, $n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* the untreated (0 nmol/L, 0.1% DMSO) group.

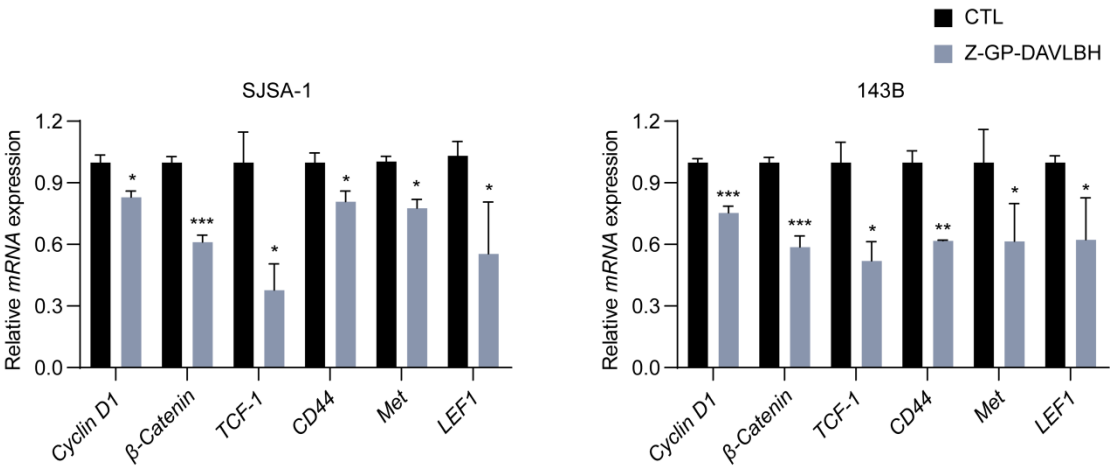

Figure 8 Z-GP-DAVLBH downregulates *β*-catenin target genes in osteosarcoma cells. SJSA-1 and 143B cells were treated with Z-GP-DAVLBH for 48 h and total RNAs was harvested and analyzed by RT-PCR assay. Quantification of the mRNA levels of Cyclin D1, *β*-catenin, *TCF-1, CD44, MET*, and *LEF1* in osteosarcoma cells. Data are presented as mean ± SEM, $n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* the CTL (0.1% DMSO) group.

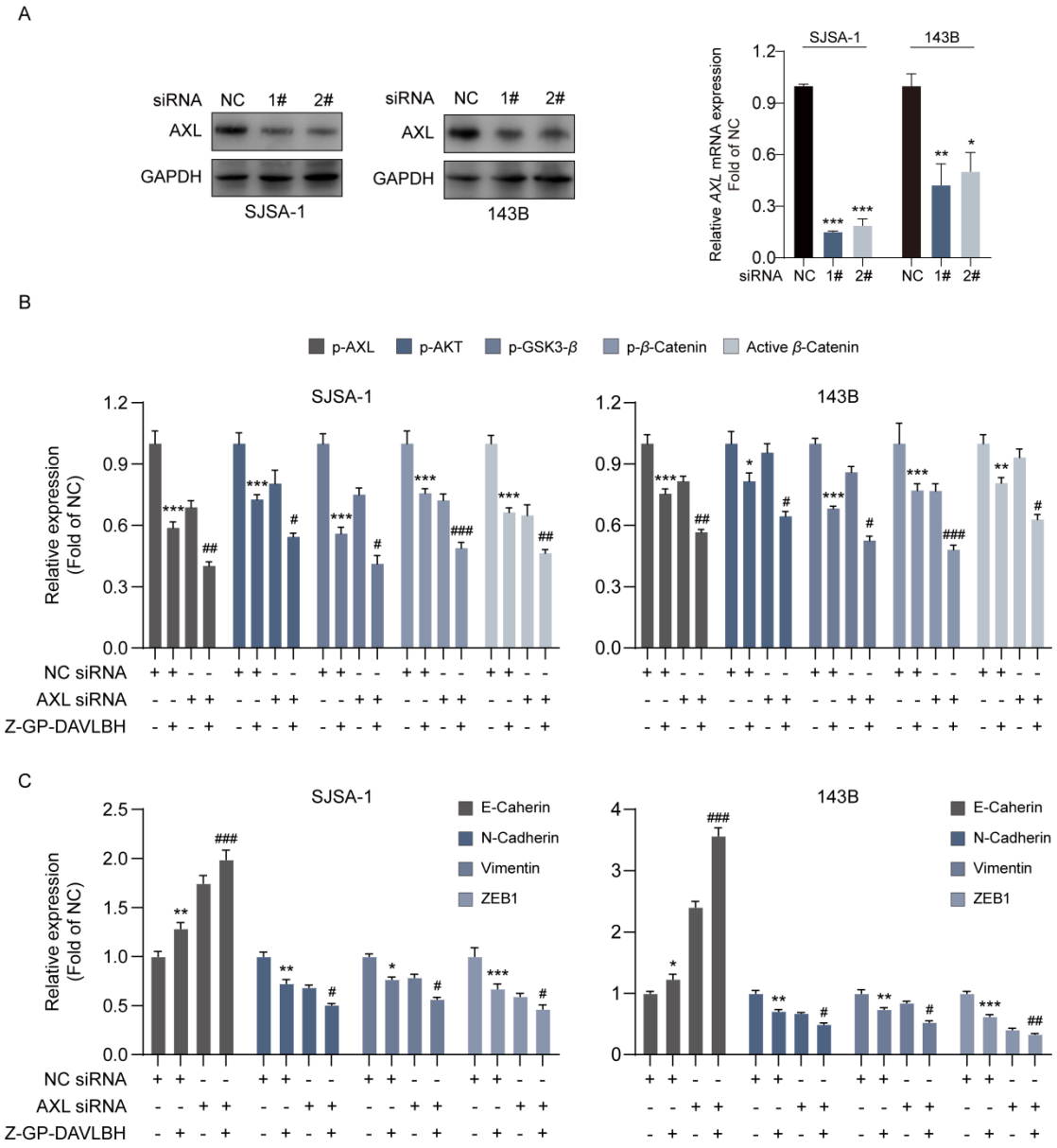

Figure 9 Silencing AXL increases the ability of Z-GP-DAVLBH to inhibit the AXL/AKT/GSK-3*β*/*β*-catenin pathway and EMT in osteosarcoma cells. Osteosarcoma cells (SJSA-1 and 143B) were transfected with NC or AXL siRNA. (A) AXL mRNA and protein levels in osteosarcoma cells were determined by RT-PCR assay and Western blotting analysis. (B) Quantification of the blots in Fig. 5C. (C) Quantification of the blots in Fig. 5D. Data are presented as mean ± SEM, $n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* the vehicle (0.1% DMSO) group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* the

Z-GP-DAVLBH-treated group.

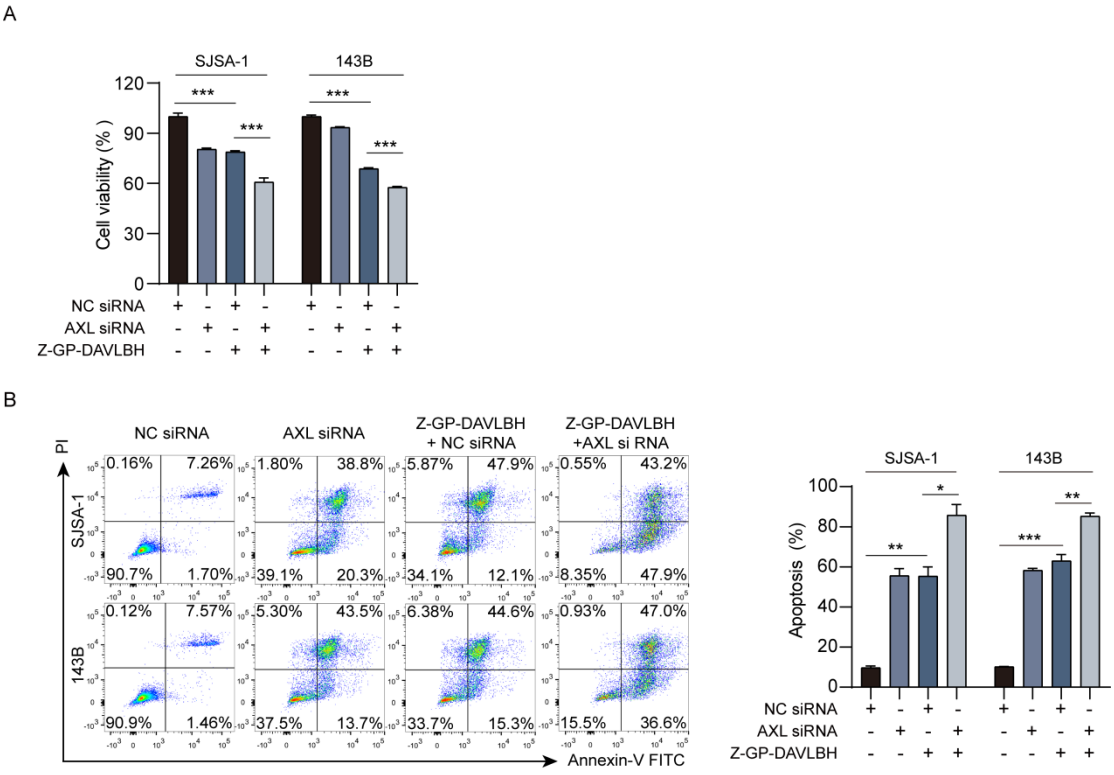

Figure 10 Silencing AXL increases the ability of Z-GP-DAVLBH to decrease the viability and induces apoptosis in osteosarcoma cells. SJSA-1 (50 nmol/L) and 143B (100 nmol/L) were treated with Z-GP-DAVLBH for 48 h. (A) An MTT assay was conducted to evaluate the effect of Z-GP-DAVLBH on osteosarcoma cell viability. (B) Representative images of apoptotic cell populations and quantification of apoptotic cells. Data are presented as mean ± SEM, $n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* the indicated group.

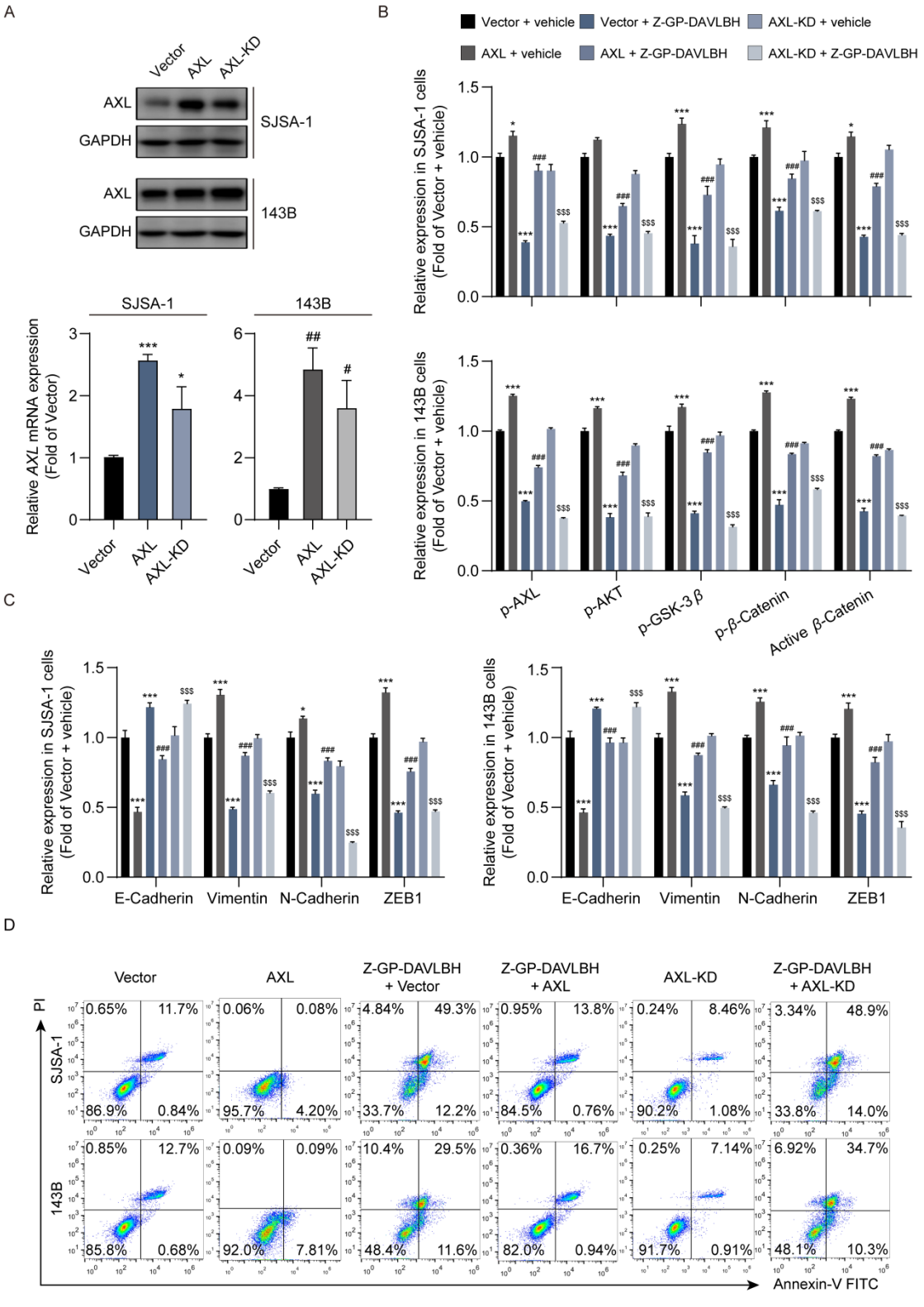

Figure 11 AXL overexpression attenuates the ability of Z-GP-DAVLBH against the AXL/AKT/GSK-3*β*/*β*-catenin pathway and EMT in osteosarcoma cells. Following transfection with AXL, AXL-KD, and their corresponding vector, osteosarcoma cells

were treated with Z-GP-DAVLBH. (A) RT-PCR and Western blotting analyses were conducted to measure the mRNA and protein levels of AXL in osteosarcoma cells. Data are presented as mean ± SEM, n = 3; $^{*}P < 0.05$ and $^{***}P < 0.001$ *vs.* SJSA-1 cells transfected with vector; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *vs.* 143B cells transfected with vector. (B) Quantification of the blots in Fig. 6A. (C) Quantification of the blots in Fig. 6B. (D) Representative images of apoptotic cell populations. Data are presented as mean ± SEM, n = 3; $^{*}P < 0.05$ and $^{***}P < 0.001$ *vs.* the cells transfected with vector; $^{\#\#\#}P < 0.001$ *vs.* the cells transfected with AXL overexpression plasmid; $^{\$\$\$}P<0.001$ *vs.* the cells transfected with AXL-KD overexpression plasmid.

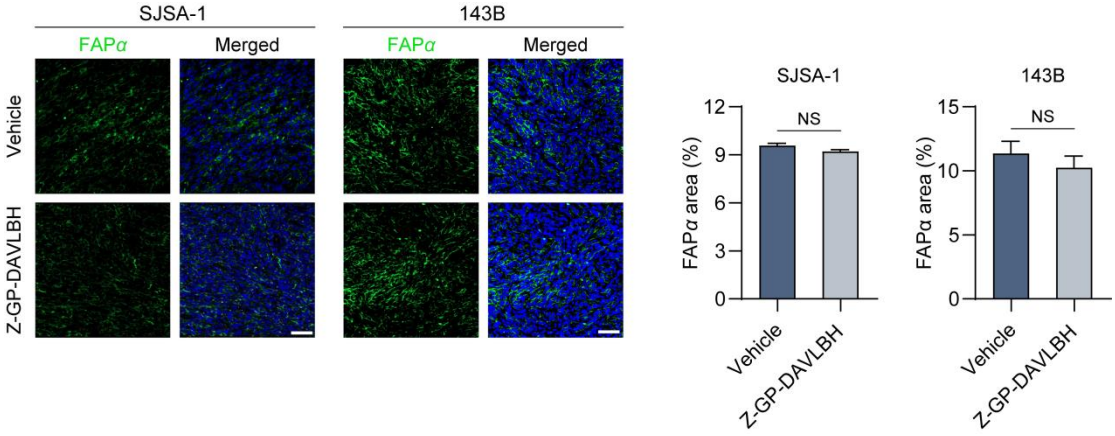

Figure 12 Z-GP-DAVLBH shows a negligible effect on FAPα expression in osteosarcoma xenograft tumors. SJAJ-1 or 143B tumor-bearing mice were treated with either vehicle (0.9% NaCl solution containing 1% DMSO) or Z-GP-DAVLBH (2 mg/kg, i.v.) every other day. Immunofluorescence staining of human FAPα in SJSA-1 and 143B xenograft tumors. Scale bar: 50 μm. Quantification of FAPα expression in tumor tissues is shown. Data are presented as mean ± SEM, n = 6; NS = no significance.

Supporting Tables

Table S1 Primer sequences used in RT-PCR assay.

Gene name	Forward/Reverse	Sequence 5′ to 3′
E-Cadherin	Forward	TCTCTCACGCTGTGTCATCC
	Reverse	CACTGGATTTGTGGTGACGA
Vimentin	Forward	GTCCGTGTCCTCGTCCTCCTAC
	Reverse	AGTTGGCGAAGCGGTCATTCAG
N-Cadherin	Forward	AGGCGTCTGTAGAGGCTTCTGG
	Reverse	TCTGCTGACTCCTTCACTGACTCC
Slug	Forward	CCTGGTCAAGAAGCATTTCAACGC
	Reverse	GGAGGAGGTGTCAGATGGAGGAG
TWIST	Forward	GCCGGAGACCTAGATGTCATT
	Reverse	CCCACGCCCTGTTTCTTTGA
MMP9	Forward	AATCTCACCGACAGGCAGCT
	Reverse	CCAAACTGGATGACGATGTC
ZEB1	Forward	CCCATTACAGGCAACCAGTTCTCC
	Reverse	GAAGTTGGCTAGGCTGCTCAAGAC
ZO-1	Forward	GGCGGATGGTGCTACAAGTGATG
	Reverse	AGGCTCAGAGGACCGTGTAATGG
Cyclin D1	Forward	AAAGAATTTGCACCCCGCTG
	Reverse	GACAGACAAAGCGTCCCTCA
β-Catenin	Forward	GAAACGGCTTTCAGTTGAGC
	Reverse	CTGGCCATATCCACCAGAGT
TCF1	Forward	GCTCATCACCGACACCACCAAC
	Reverse	AGGCTGCTGGAGGACACTGTG
CD44	Forward	AGTCACAGACCTGCCCAATG
	Reverse	AACCTCCTGAAGTGCTGCTC
MET	Forward	TGGGCACCGAAAGATAAACCT
	Reverse	TCGGACTTTGCTAGTGCCTC
LEF1	Forward	TGCATCAGGTACAGGTCCAA
	Reverse	TGTTCCTTTGGGGTCGACTG
AXL	Forward	GAACCTTCAACTCCTGCCTTCTCG
	Reverse	TTCATCGTCTTCACAGCCACCTTG
ACTB	Forward	TCTTCCAGCCTTCCTTCCTG
	Reverse	CCTGCTTGCTGATCCACATC

Table S2 The IC_{50} values of Z-GP-DAVLBH, doxorubicin, and cisplatin for SJSA-1 and 143B cells.

Cell line	Time (h)	IC_{50} (nmol/L)[a]		
		Z-GP-DAVLBH	Doxorubicin	Cisplatin
SJSA-1	24	346.1 ± 155.7	2535.0 ± 87.0	5064.0 ± 159.0
	48	121.6 ± 37.0	724.5 ± 81.6	3665.5 ± 168.6
	72	55.3 ± 8.2	322.0 ± 7.4	2410.0 ± 89.8
143B	24	2549.3 ± 663.0	13352.0 ± 163.0	6147.5 ± 330.5
	48	122.0 ± 13.4	3943.5 ± 458.5	3984.3 ± 171.0
	72	112.0 ± 8.1	2650.0 ± 3.3	3020.5 ± 72.3

[a]SJSA-1 and 143B cells were treated with Z-GP-DAVLBH, doxorubicin, and cisplatin for the indicated times, and cell viability was determined by MTT assay. Doxorubicin and platinum were used as positive controls. The data are presented as mean ± SEM.