OMTN, Volume *10*

Supplemental Information

MicroRNA-300 Regulates the Ubiquitination of PTEN through the CRL4B^{DCAF13} E3 Ligase in Osteosarcoma Cells

Zhi Chen, Wei Zhang, Kaibiao Jiang, Bin Chen, Kun Wang, Lifeng Lao, Canglong Hou, Fei Wang, Caiguo Zhang, and Hongxing Shen

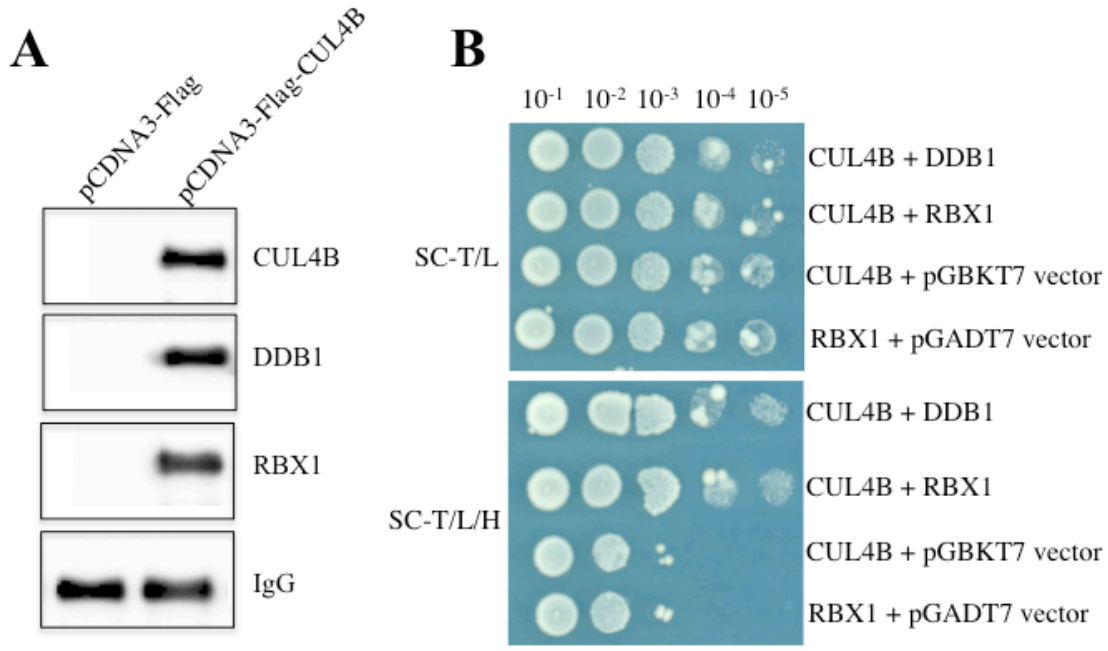

Fig. S1 CUL4B formed a complex with DDB1 and RBX1 *in vivo* and *in vitro*.

(A) CUL4B formed a complex with DDB1 and RBX1 *in vivo*. The *pCDNA3-Flag-CUL4B* vector was transfected into U2OS cells; then, Flag-tagged CUL4B was *i*mmunoprecipitated. The protein levels of CUL4B, DDB1, and RBX1 were determined by western blot analysis. The *pCDNA3-Flag* empty vector was used as a negative control, and IgG was used as a loading control. **(B)** CUL4B formed a complex with DDB1 and RBX1 in yeast cells. The *pGADT7-CUL4B* plasmid was co-transformed with *pGBKT7-DDB1* or *pGBKT7-RBX1* into AH109 cells. Cell growth was determined in media without Trp and Leu (SC-T/L) (top panel) or without Trp, Leu and His (SC-H/T/L) (bottom panel). Columns in each panel represent the serial decimal dilutions.

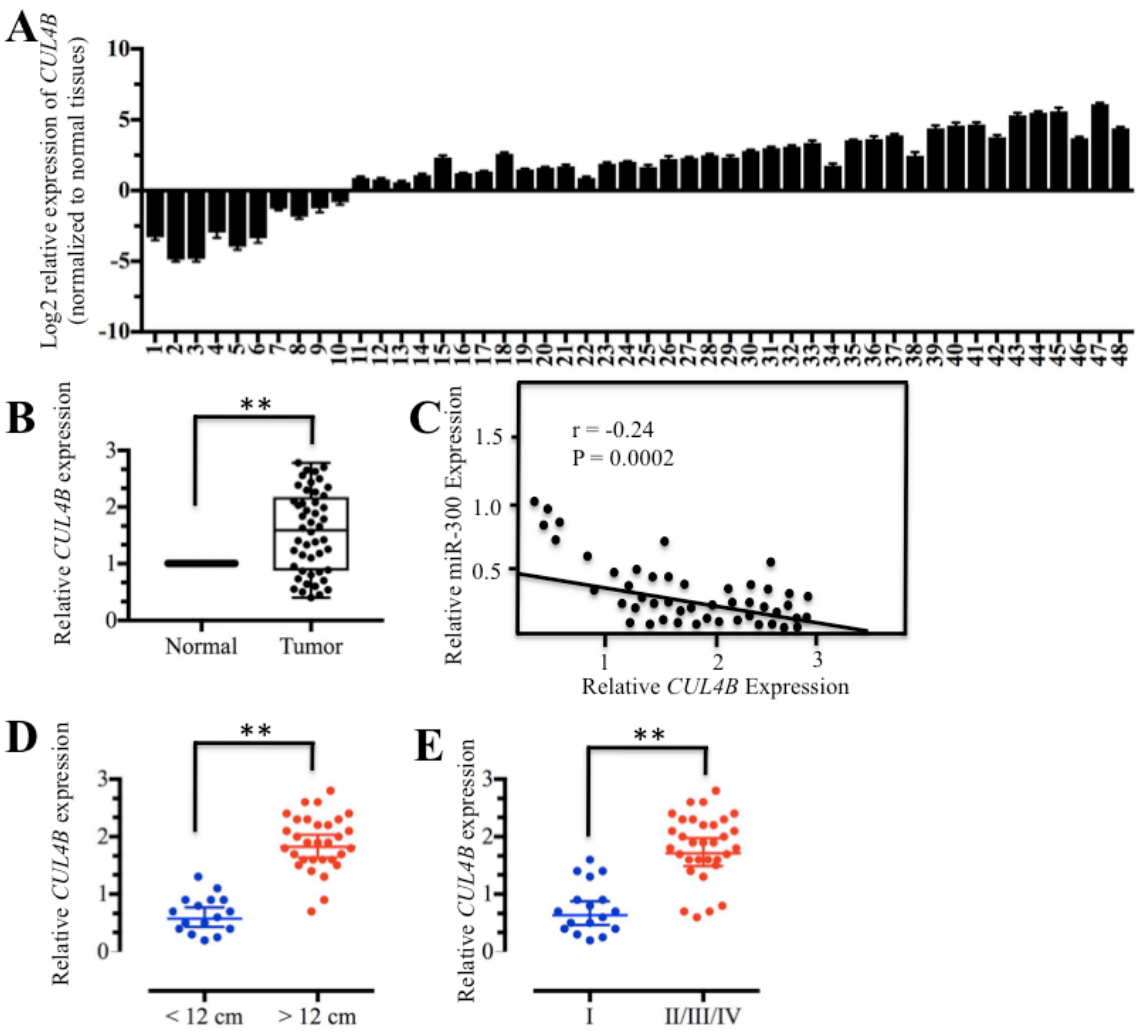

Fig. S2 CUL4B expression was negatively associated with miR-300 level.

(A-B) Expression of *CUL4B* in osteosarcoma cancerous tissues is shown. Relative expression of *CUL4B* in osteosarcoma tumors (n = 48) was normalized to corresponding adjacent normal tissues (n = 48). **(C)** The expression of *CUL4B* was negatively correlated with miR-300 level. **(D-E)** The expression of *CUL4B* was positively correlated with osteosarcoma tumor size and MSTS stage. The expression of *CUL4B* was significantly higher in larger tumors (tumor maximal diameter ≥ 12 cm) **(D)** and was significantly higher in osteosarcoma patients with advanced MSTS stages (II/III/IV) than in those with an early MSTS stage (I) **(E)**. ** $P < 0.001$.

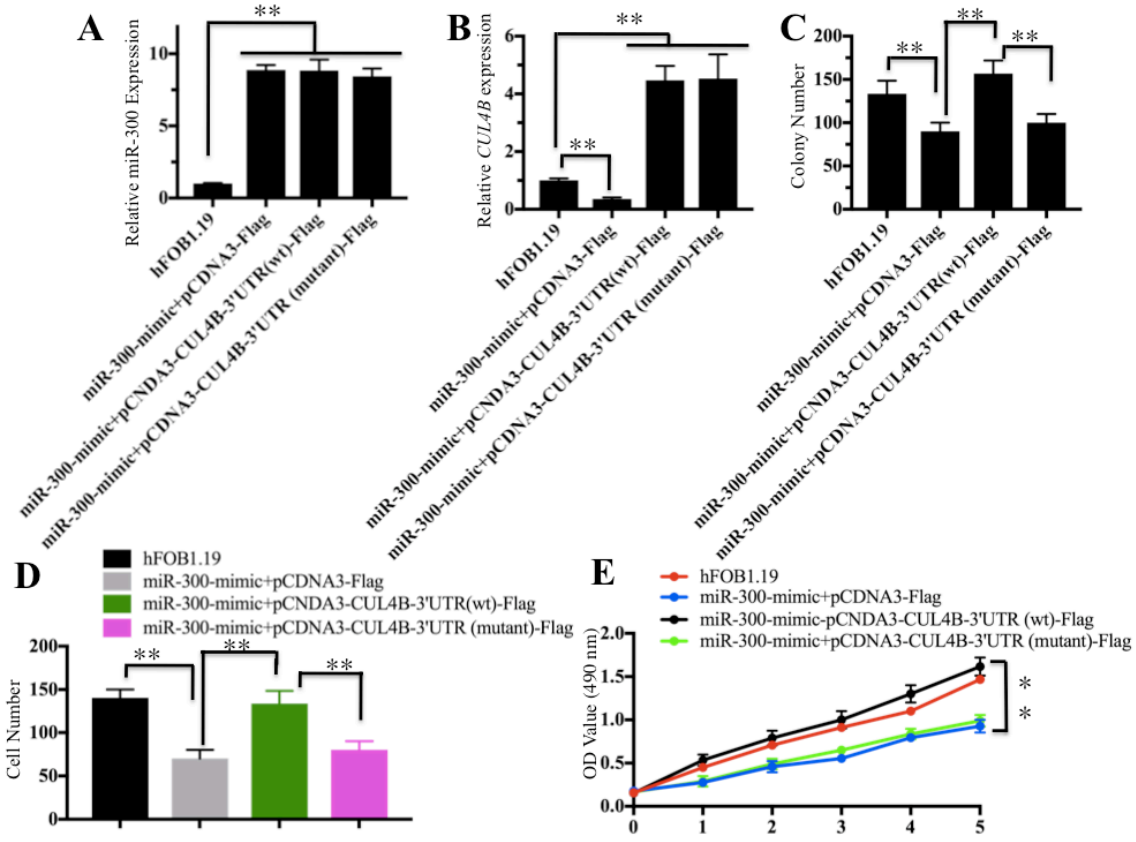

Fig. S3 miR-300 specifically bond to the 3'UTR of CUL4B.

The hFOB1.19 cells were transfected with the following combinations of plasmids: miR-300-mimic + pCDNA3-Flag; miR-300-mimic + pCDNA3-CUL4B-3'UTR (wt)-Flag, or miR-300-mimic + pCDNA3-CUL4B-3'UTR (mutant)-Flag, respectively. After 48 h, multiple studies were performed: miR-300 expression **(A)** and *CUL4B* mRNA **(B)**, colony formation ability **(C),** cell invasion ability **(D)** and cell proliferation **(E).** hFOB1.19 cell line was used as a control. ** $P < 0.001$.

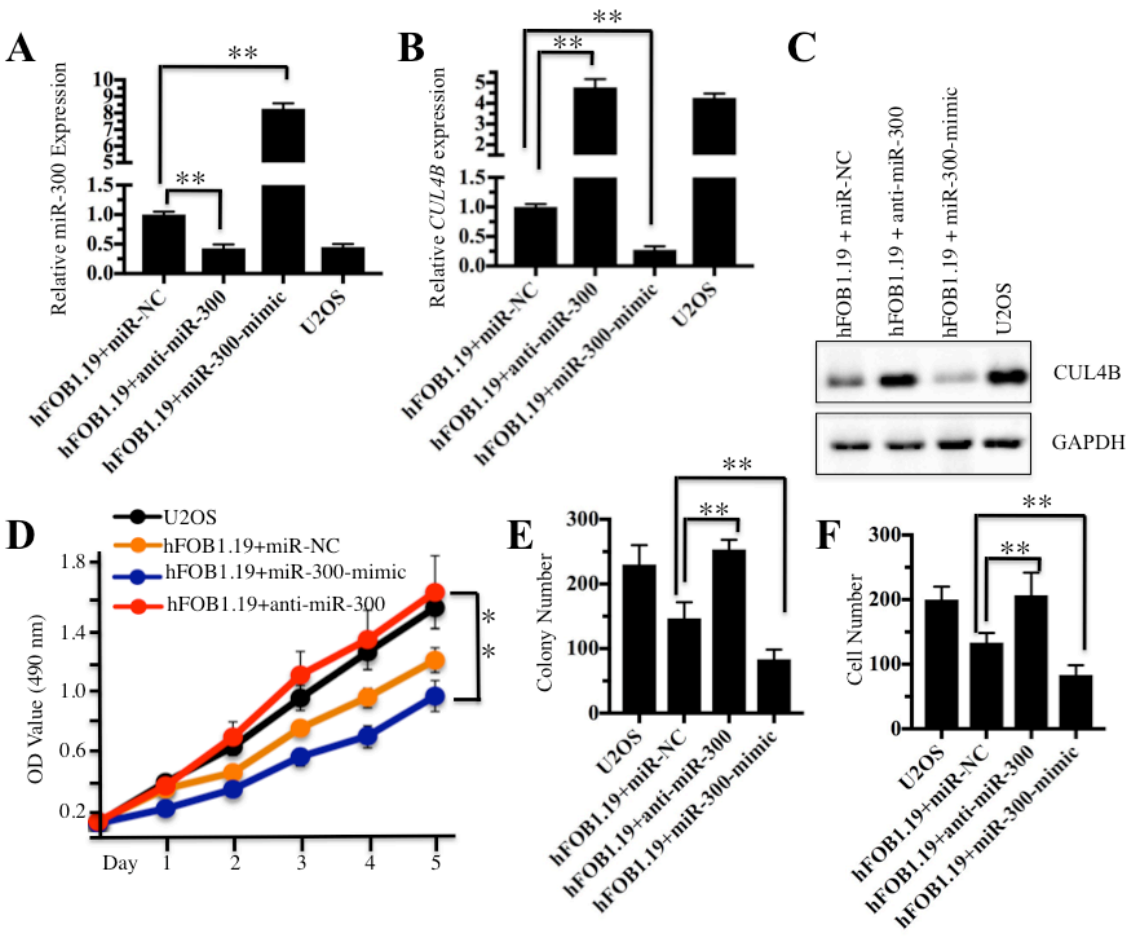

Fig. S4 Downregulation of miR-300 in hFOB1.19 cells caused similar phenotypes to U2OS cells.

The hFOB1.19 cells were transfected with miR-NC, anti-miR-300, or miR-300-mimic, respectively. After 48 h, multiple studies were performed: miR-300 expression **(A)** and mRNA **(B)** and protein levels **(C)** of CUL4B, cell proliferation **(D)**, colony formation ability **(E),** as well as cell invasion ability **(F).** U2OS cells were used as control in these studies. ** $P < 0.001$.

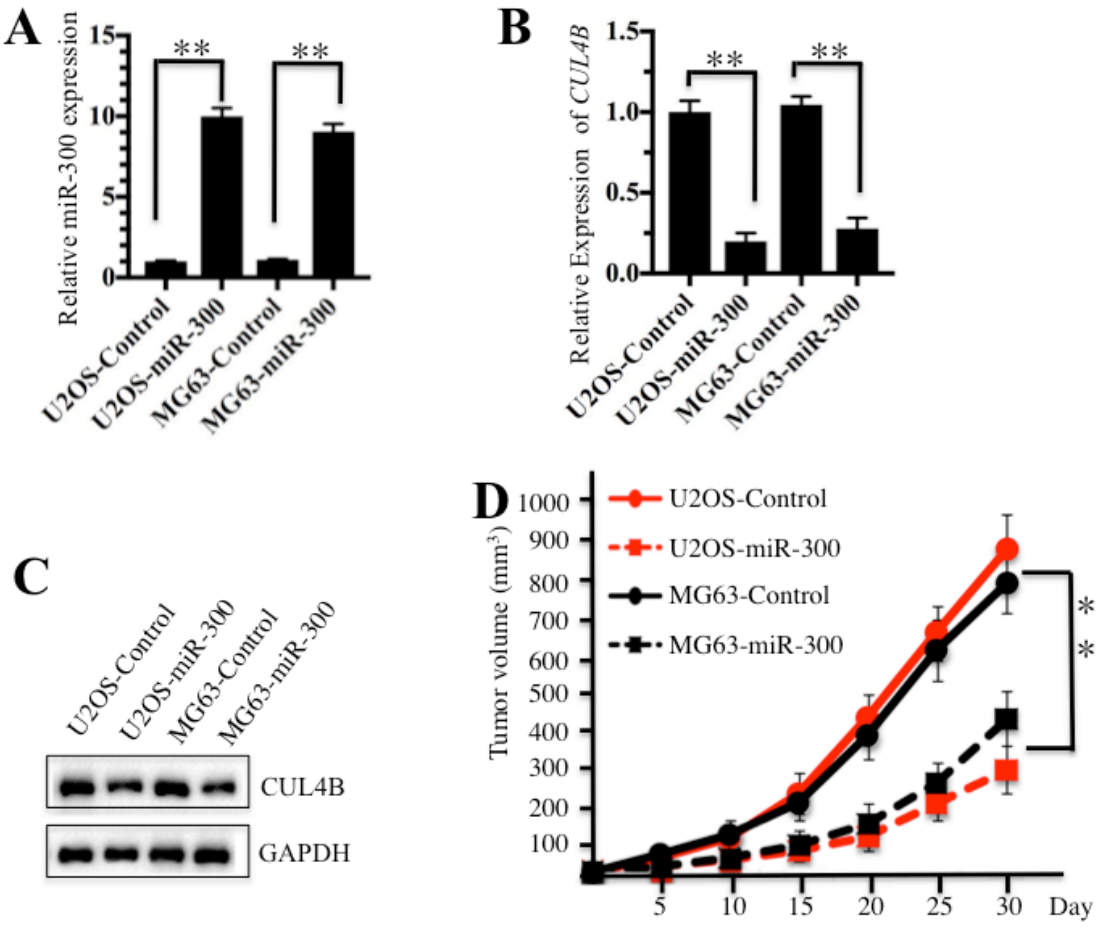

Fig. S5 Overexpression of miR-300 in osteosarcoma cells caused significant growth inhibition.

The U2OS and MG63 cells overexpressing pmR-ZsGreen1-miR-300 plasmid (U2OS-miR-300 and MG63-miR-300), and their relative controls transfected with pmR-ZsGreen1 empty vector (U2OS-Control and MG63-Control), were subjected to multiple studies including miR-300 expression **(A)** and mRNA **(B)** and protein levels **(C)** of CUL4B, as well as in vivo tumor formation assay **(D)**. ** $P < 0.001$.

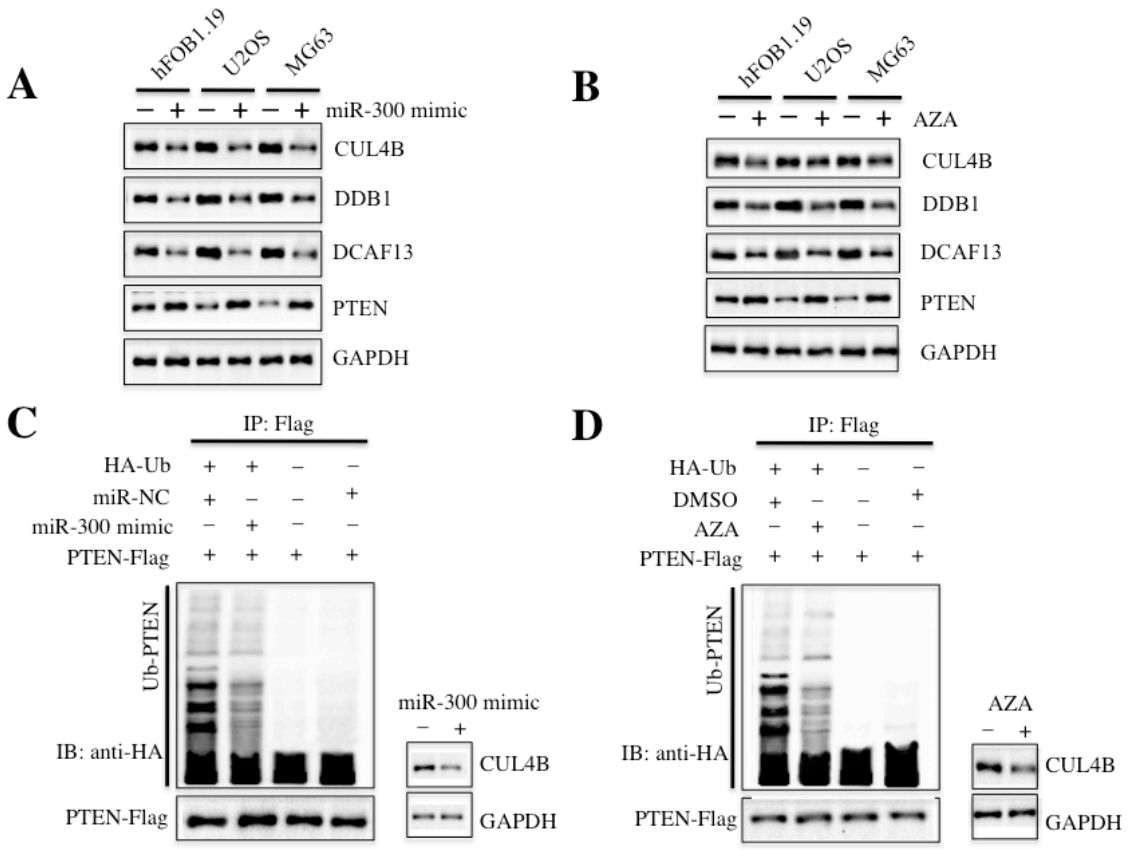

Fig. S6 Overexpression of miR-300 or AZA treatment decreased the ubiquitination level of PTEN.

(A) Effect of overexpression of miR-300 on the protein levels of CUL4B, DDB1, DCAF13 and PTEN. The hFOB1.19, U2OS, and MG63 cell lines were transfected with miR-300-mimic or its negative control miR-NC for 48 h, followed by detection of CUL4B, DDB1, DCAF13 and PTEN levels by western blotting. **(B)** Effect of AZA treatment on the protein levels of CUL4B, DDB1, DCAF13 and PTEN. The hFOB1.19, U2OS, and MG63 cell lines were primarily treated with DMSO or AZA (1 μM), followed by detection of CUL4B, DDB1, DCAF13 and PTEN levels by western blotting. **(C** and **D)** Overexpression of miR-300 or AZA treatment decreased the ubiquitination level of PTEN *in vivo*. U2OS cells were first co-transfected with PTEN-Flag and HA-Ubiquitin, followed by miR-300 overexpression **(C)** or AZA treatment. After 48 h of incubation, cells were lysed, immunoprecipitated with anti-Flag antibody, and then probed with anti-HA antibody to detect ubiquitinated PTEN. The protein level of CUL4B

after these treatments is also indicated in the right panels of each figure.

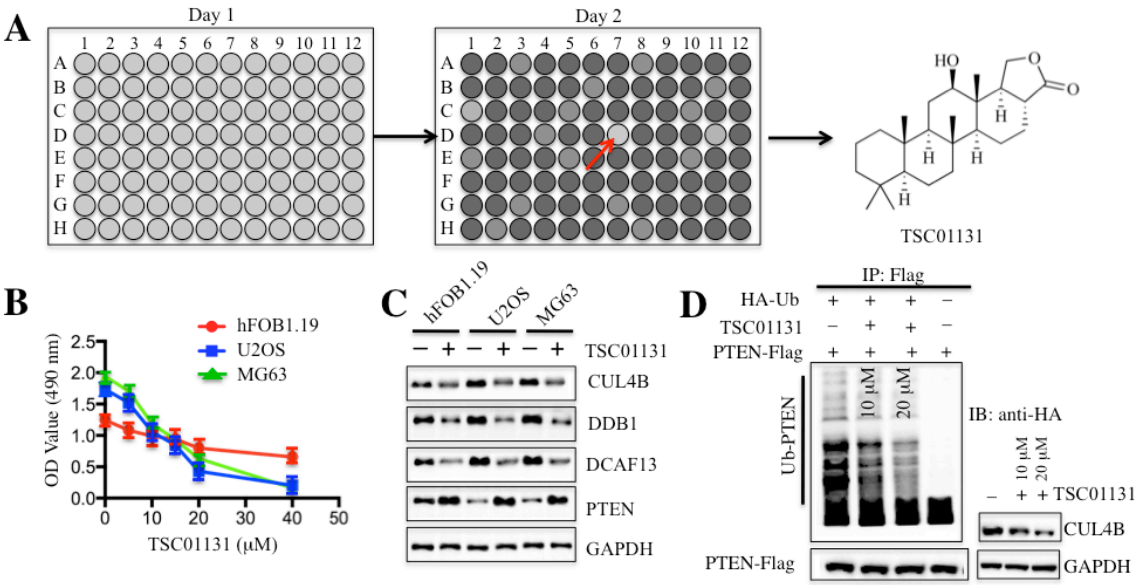

Fig. S7 TSC01131 could specifically inhibit the CUL4B-DDB1 interaction.

(A) Schematic representation of the screen for small molecules that disrupt the CUL4B-DDB1 interaction. The AH109 yeast cells expressing *pGADT7-CUL4B* and *pGBKT7-DDB1* were grown in 96-well plates at a density of OD600=0.1, and the SC-H/T/L medium was supplemented with the individual compound in each well. After 18 h of incubation at 30°C, cell density was measured at 600 nm with the Synergy HTX Multi-Mode Reader. Compounds that significantly inhibited cell growth (OD600<0.2) were selected. The chemical structure of TSC01131 is indicated. **(B)** Effect of TSC01131 on cell proliferation. Different concentrations of TSC01131 (0, 5, 10, 15, 20 and 40 μM) were added into DMEM medium to evaluate the degree of cell proliferation, and cell viability was determined at 490 nm. **(C)** Effect of TSC01131 treatment on the protein levels of CUL4B, DDB1, DCAF13 and PTEN. The hFOB1.19, U2OS, and MG63 cell lines were primarily treated with DMSO or TSC01131 (10 μM), followed by the detection of CUL4B, DDB1, DCAF13 and PTEN levels by western blotting. **(D)** Treatment with TSC01131 decreased the ubiquitination level of PTEN *in vivo*. U2OS cells were first co-transfected with PTEN-Flag and HA-Ubiquitin for 48 h, followed by

treatment with TSC01131 for 6 h. Then, the cells were lysed, immunoprecipitated with anti-Flag antibody, and probed with anti-HA antibody to detect ubiquitinated PTEN.

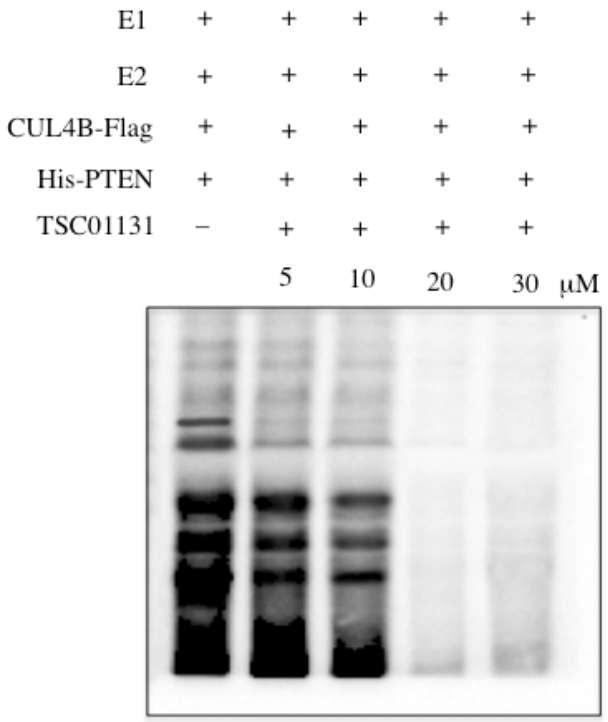

Fig. S8 TSC01131 specifically inhibited the *in vitro* ubiquitination of PTEN.

The purified His-PTEN protein was incubated with E1, E2, and CUL4B-Flag in ubiquitination reaction buffer, followed by treating with different concentrations of TSC01131 (5, 10, 20 or 30 μM). The uniquitination of PTEN was determined by immunoblotting with anti-PTEN.

Supplementary Table-1. The candidate proteins interacting with DCAF13 in human osteosarcoma cells

Accession Number	Protein ID	Symbol	Molecular Weight	MASCOT Score
P60484	Phosphatase and tensin homolog deleted on chromosome 10	PTEN	47 kDa	912
Q13620	Cullin 4B	CUL4B	104 kDa	835
Q16531	Damage Specific DNA Binding Protein 1	DDB1	127 kDa	719
Q13601	Small Subunit Processome Component Homolog	KRR1	44 kDa	578
Q15269	Periodic tryptophan protein 2 homolog	PWP2	102 kDa	404
O43818	Ribosomal RNA Processing 9	RRP9	52 kDa	315
P62877	RING-Box 1	RBX1	12 kDa	224
Q9BVI4	Nucleolar Complex-Associated Protein 4-Like Protein	NOC4L	58 kDa	154
Q92466	Damage Specific DNA Binding Protein 2	DDB2	48 kDa	89
Q15843	Neural Precursor Cell Expressed, Developmentally Downregulated 8	NEDD8	9 kDa	87
Q9Y2X3	Nucleolar Protein 58	NOP58	60 kDa	86
P62081	Ribosomal Protein S7	RPS7	22 kDa	85
Q96RS0	Trimethylguanosine Synthase	TGS1	97 kDa	85
Q9UG63	ATP Binding Cassette Subfamily F Member 2	ABCF2	71 kDa	83
Q9ULW3	Activator Of Basal Transcription 1	ABT1	31 kDa	82

P56377	Adaptor Related Protein Complex 1 Sigma 2 Subunit	AP1S2	19 kDa	80
P35226	B Lymphoma Mo-MLV Insertion Region 1	BMI1	37 kDa	78
Q8IY81	FtsJ Homolog 3	FTSJ3	97 kDa	78
P22087	Fibrillarin	FBL	34 kDa	77
Q9NVP1	DEAD-Box Helicase 18	DDX18	75 kDa	77
P62273	Ribosomal Protein S29	RPS29	7 kDa	75
Q9BRS2	RIO Kinase 1	RIOK1	66 kDa	74
Q9ULX3	NIN1/PSMD8 Binding Protein 1	NOB1	47 kDa	73
Q8TED0	U3 small nucleolar RNA-associated Protein 15	UTP15	58 kDa	73
Q9BVJ6	U3 Small Nucleolar RNA-Associated Protein 14 Homolog A	UTP14A	88 kDa	71
Q12788	Transducin Beta Like 3	TBL3	89 kDa	70
Q96B26	Exosome Component 8	EXOSC8	30 kDa	70
Q9H4L4	SUMO1/Sentrin/SMT3 Specific Peptidase 3	SENP3	65 kDa	68
Q13868	Exosome Component 2	EXOSC2	33 kDa	67
Q9NQT5	Exosome Component 3	EXOSC3	30 kDa	67
Q9NPD3	Exosome Component 4	EXOSC4	26 kDa	65
P62249	Ribosomal Protein S16	RPS16	16 kDa	65
P62280	Ribosomal Protein S11	RPS11	18 kDa	64
Q9UET6	FtsJ RNA Methyltransferase Homolog 1	FTSJ1	36 kDa	63
P55769	Non-Histone Chromosome Protein 2-Like 1	NHP2L1	14 kDa	63
P62241	Ribosomal Protein S8	RPS8	24 kDa	60
P23396	Ribosomal Protein S3	RPS3	27 kDa	60

Q969U6	F-Box And WD Repeat Domain Containing 5	FBXW5	64 kDa	60
P27694	Replication Protein A1	RPA1	68 kDa	57
Q9H0A0	N-Acetyltransferase 10	NAT10	116 kDa	56
P42224	Signal Transducer And Activator Of Transcription 1	STAT1	87 kDa	55
P15884	Transcription Factor 4	TCF4	71 kDa	55
Q9NR30	DExD-Box Helicase 21	DDX21	87 kDa	55
Q562E7	WD Repeat Domain 81	WDR81	211 kDa	53
Q9BVP2	G Protein Nucleolar 3	GNL3	62 kDa	52
O76021	Ribosomal L1 Domain Containing 1	RSL1D1	55 kDa	52
Q9BQ67	Glutamate Rich WD Repeat Containing 1	GRWD1	49 kDa	52
O43147	Small G Protein Signaling Modulator 2	SGSM2	113 kDa	52
Q9BZG8	Diphthamide Biosynthesis 1	DPH1	49 kDa	52
O00541	Pescadillo Ribosomal Biogenesis Factor 1	PES1	68 kDa	50
Q13823	G Protein Nucleolar 2	GNL2	84 kDa	50
Q53HL2	Cell Division Cycle Associated 8	CDCA8	31 kDa	50
Q14209	E2F Transcription Factor 2	E2F2	48 kDa	50
Q12830	Bromodomain PHD Finger Transcription Factor	BPTF	338 kDa	50
O15381	Nuclear VCP-Like	NVL	95 kDa	50
Q14872	Metal Regulatory Transcription Factor 1	MTF1	81 kDa	50

Supplementary Table-2. The predicted miRNAs that target the 3'-UTR of CUL4B in miRDB

Target Rank	Target Score	miRNA Name	miRNA Sequence
1	100	miR-300	CCUGAGAAAAGGGCCAA
2	99	miR-4531	AUGGAGAAGGCUUCUGA
3	99	miR-3977	GUGCUUCAUCGUAAUUAACCUUA
4	99	miR-6830-5p	CCAAGGAAGGAGGCUGGACAUC
5	97	miR-4659a-3p	UUUCUUCUUAGACAUGGCAACG
6	97	miR-545-5p	UCAGUAAAUGUUUAUUAGAUGA
7	97	miR-153-5p	UCAUUUUUGUGAUGUUGCAGCU
8	97	miR-4659b-3p	UUUCUUCUUAGACAUGGCAGCU
9	96	miR-561-3p	CAAAGUUUAAGAUCCUUGAAGU
10	96	miR-381-3p	AUACAAGGGCAAGCUCUCUGU
11	96	miR-300	UAUACAAGGGCAGACUCUCUCU
12	94	miR-4776-3p	CUUGCCAUCCUGGUCCACUGCAU
13	94	miR-6885-3p	CUUUGCUUCCUGCUCCCCUAG
14	94	miR-7159-5p	UUCAACAAGGGUGUAGGAUGG
15	94	miR-8060	CCAUGAAGCAGUGGGUAGGAGGAC
16	93	miR-3691-3p	ACCAAGUCUGCGUCAUCCUCUC
17	91	miR-3664-5p	AACUCUGUCUUCACUCAUGAGU
18	91	miR-4451	UGGUAGAGCUGAGGACA
19	90	miR-3671	AUCAAAUAAGGACUAGUCUGCA
20	90	miR-4482-3p	UUUCUAUUUCUCAGUGGGGCUC
21	88	miR-495-3p	AAACAAACAUGGUGCACUUCUU
22	88	miR-5688	UAACAAACACCUGUAAAACAGC
23	88	miR-4279	CUCUCCUCCCGGCUUC
24	87	miR-4668-5p	AGGGAAAAAAAAAAGGAUUUGUC
25	86	miR-181a-2-3p	ACCACUGACCGUUGACUGUACC

26	86	miR-5003-3p	UACUUUUCUAGGUUGUUGGGG
27	86	miR-4645-3p	AGACAGUAGUUCUUGCCUGGUU
28	85	let-7a-2-3p	CUGUACAGCCUCCUAGCUUUCC
29	85	let-7g-3p	CUGUACAGGCCACUGCCUUGC
30	85	miR-5584-5p	CAGGGAAAUGGGAAGAACUAGA
31	85	miR-4766-5p	UCUGAAAGAGCAGUUGGUGUU
32	85	miR-4753-3p	UUCUCUUUCUUUAGCCUUGUGU
33	84	miR-4496	GAGGAAACUGAAGCUGAGAGGG
34	83	miR-544a	AUUCUGCAUUUUUAGCAAGUUC
35	82	miR-194-5p	UGUAACAGCAACUCCAUGUGGA
36	82	miR-340-5p	UUAUAAAGCAAUGAGACUGAUU
37	82	miR-4261	AGGAAACAGGGACCCA
38	82	miR-6848-3p	GUGGUCUCUUGGCCCCCAG
39	82	miR-5582-3p	UAAAACUUUAAGUGUGCCUAGG
40	82	miR-421	AUCAACAGACAUUAAUUGGGCGC
41	82	miR-6843-3p	AUGGUCUCCUGUUCUCUGCAG
42	81	miR-3121-3p	UAAAUAGAGUAGGCAAAGGACA
43	80	miR-589-3p	UCAGAACAAAUGCCGGUUCCCAGA
44	80	miR-548x-3p	UAAAAACUGCAAUUACUUUC
45	80	miR-548aj-3p	UAAAAACUGCAAUUACUUUUA
46	80	miR-548am-3p	CAAAAACUGCAGUUACUUUUGU
47	80	miR-548j-3p	CAAAAACUGCAUUACUUUUGC
48	80	miR-548aq-3p	CAAAAACUGCAAUUACUUUUGC
49	80	miR-548ae-3p	CAAAAACUGCAAUUACUUUCA
50	80	miR-548ah-3p	AAAAACUGCAGUUACUUUUGC
51	79	miR-3617-3p	CAUCAGCACCCUAUGUCCUUUCU
52	79	miR-767-3p	UCUGCUCAUACCCCAUGGUUUCU
53	79	miR-4503	UUUAAGCAGGAAAUAGAAUUUA
54	79	miR-651-3p	AAAGGAAAGUGUAUCCUAAAAG
55	78	miR-3680-3p	UUUUGCAUGACCCUGGGAGUAGG

56	77	miR-7154-5p	UUCAUGAACUGGGUCUAGCUUGG
57	75	miR-6776-3p	CAACCACCACUGUCUCUCCCCAG
58	75	miR-6768-5p	CACACAGGAAAAGCGGGGCCCUG
59	75	miR-548e-5p	CAAAAGCAAUCGCGGUUUUUGC
60	74	miR-4311	GAAAGAGAGCUGAGUGUG
61	74	miR-5190	CCAGUGACUGAGCUGGAGCCA
62	73	miR-584-3p	UCAGUUCCAGGCCAACCAGGCU
63	72	miR-664b-3p	UUCAUUUGCCUCCCAGCCUACA
64	72	miR-579-3p	UUCAUUUGGUAUAAACCGCGAUU
65	72	miR-4282	UAAAAUUUGCAUCCAGGA
66	71	miR-527	CUGCAAAGGGAAGCCCUUUC
67	71	miR-450b-5p	UUUUGCAAUAUGUUCCUGAAUA
68	71	miR-561-5p	AUCAAGGAUCUUAAACUUUGCC
69	71	miR-518a-5p	CUGCAAAGGGAAGCCCUUUC
70	70	miR-5002-5p	AAUUUGGUUUCUGAGGCACUUAGU
71	70	miR-34a-3p	CAAUCAGCAAGUAUACUGCCCU
72	69	miR-548c-3p	CAAAAAUCUCAAUUACUUUUGC
73	66	miR-5094	AAUCAGUGAAUGCCUUGAACCU
74	66	miR-581	UCUUGUGUUCUCUAGAUCAGU
75	66	miR-648	AAGUGUGCAGGGCACUGGU
76	65	miR-6874-3p	CAGUUCUGCUGUUCUGACUCUAG
77	65	miR-148b-5p	AAGUUCUGUUAUACACUCAGGC
78	64	miR-3646	AAAAUGAAAUGAGCCCAGCCCA
79	63	miR-3653-5p	CCUCCUGAUGAUUCUUCUUC
80	62	miR-3065-5p	UCAACAAAAUCACUGAUGCUGGA
81	61	miR-1273e	UUGCUUGAACCCAGGAAGUGGA
82	61	miR-567	AGUAUGUUCUUCCAGGACAGAAC
83	59	miR-6797-3p	UGCAUGACCCUUCCCUCCCCAC
84	59	miR-6076	AGCAUGACAGAGGAGAGGUGG
85	59	miR-876-3p	UGGUGGUUUACAAAGUAAUUCA

86	58	miR-2053	GUGUUAAUUAAACCUCUAUUUAC
87	58	miR-6762-3p	UGGCUGCUUCCCUUGGUCUCCAG
88	58	miR-4253	AGGGCAUGUCCAGGGGGU
89	58	miR-4302	CCAGUGUGGCUCAGCGAG
90	58	miR-6862-5p	CGGGCAUGCUGGGAGAGACUUU
91	57	miR-154-3p	AAUCAUACACGGUUGACCUAUU
92	57	miR-5011-5p	UAUAUAUACAGCCAUGCACUC
93	57	miR-487a-3p	AAUCAUACAGGGACAUCCAGUU
94	56	miR-647	GUGGCUGCACUCACUUCCUUC
95	56	miR-942-5p	UCUUCUCUGUUUUGGCCAUGUG
96	56	miR-134-3p	CCUGUGGGCCACCUAGUCACCAA
97	56	miR-7158-5p	GGCUCAAUCUCUGGUCCUGCAGCC
98	55	miR-6809-3p	CUUCUCUUCUCUCCUUCCCAG
99	55	miR-6826-3p	CUCCCCUCUCUUUCCUGUUCAG
100	54	miR-4483	GGGGUGGUCUGUUGUUG
101	54	miR-4680-5p	AGAACUCUUGCAGUCUUAGAUGU
102	54	miR-1293	UGGGUGGUCUGGAGAUUUGUGC
103	54	miR-6832-5p	AGUAGAGAGGAAAAGUUAGGGUC
104	52	miR-9-5p	UCUUUGGUUAUCUAGCUGUAUGA
105	52	miR-5197-3p	AAGAAGAGACUGAGUCAUCGAAU
106	52	miR-4764-5p	UGGAUGUGGAAGGAGUUAUCU

Supplementary Table-3. The clinicopathological futures of 48 osteosarcoma patients and miR-300 expression

Characteristics	Number of cases	Relative expression of miR-449c		*P*-value
		Low (<0)	High (≥0)	
Gender				
Male	24	18	6	0.7223
Female	24	20	4	
Age				
≥20	10	7	3	0.7154
<20	38	31	7	
Tumor size				
≥12 cm	13	13	0	0.034*
<12	35	25	10	
MSTS stages				
I	14	4	10	<0.0001**
II/III/IV	34	34	0	

*P < 0.05, **P < 0.001

Supplementary Table-4. Primers used for qRT-PCR

Gene	Forward	Reverse
β−Actin	5’-CACCAACTGGGACGACAT-3’	5’- ACAGCCTGGATAGCAACG-3’
CUL4B	5’-GGAGAACACTGCAGTCATTAG-3’	5’-GCAGCATCAATTTGATACTGTCTG-3’

Supplementary Table-5. Primers used for qMSP

Gene	Forward	Reverse
GAPDH	5’- CGCTTTCTTTCCTTTCGC-3’	5’- TGCCCATTCATTTCCTTCC-3’
Island	5’-TTTTTTTGTAATTTGTGAATATATAATTGT -3’	5’-AAACTAACCTAAAACCAAACTAACC-3’