Supplementary Information

GFRA1 Promotes Cisplatin-induced Chemoresistance in Osteosarcoma by Inducing Autophagy

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Figure S1

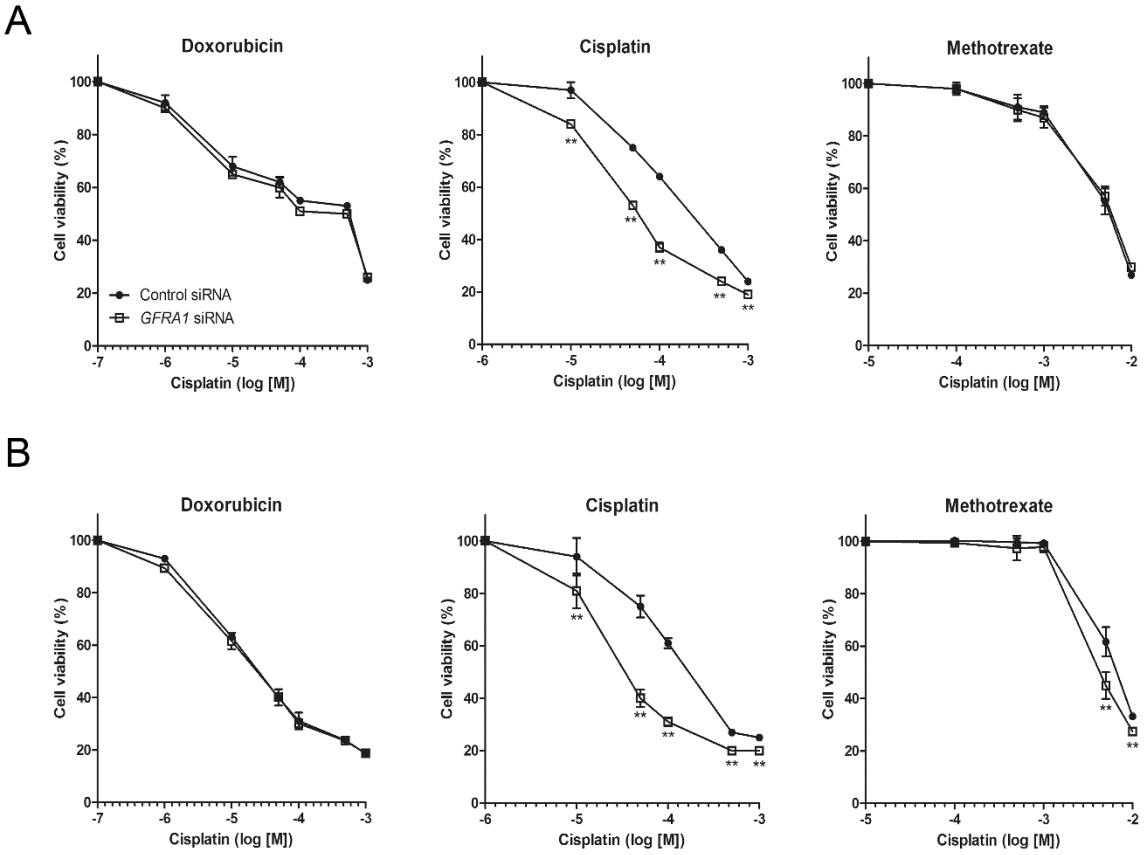

Figure S1. Cell viability of GFRA1-deficient osteosarcoma cells after treatment of chemotherapeutic agents. MG-63 and U-2 OS cells were transfected with either control siRNA or *GFRA1* siRNA for 48 h and then treated with different concentrations of doxorubicin, cisplatin, or methotrexate for 24 h. Cell viability was measured using the WST-1 assay. The values are presented as a mean ± s.d.m. (n=3). ** denotes $P < 0.05$. (**A**) MG-63 cells. (**B**) U-2 OS cells.

Figure S2

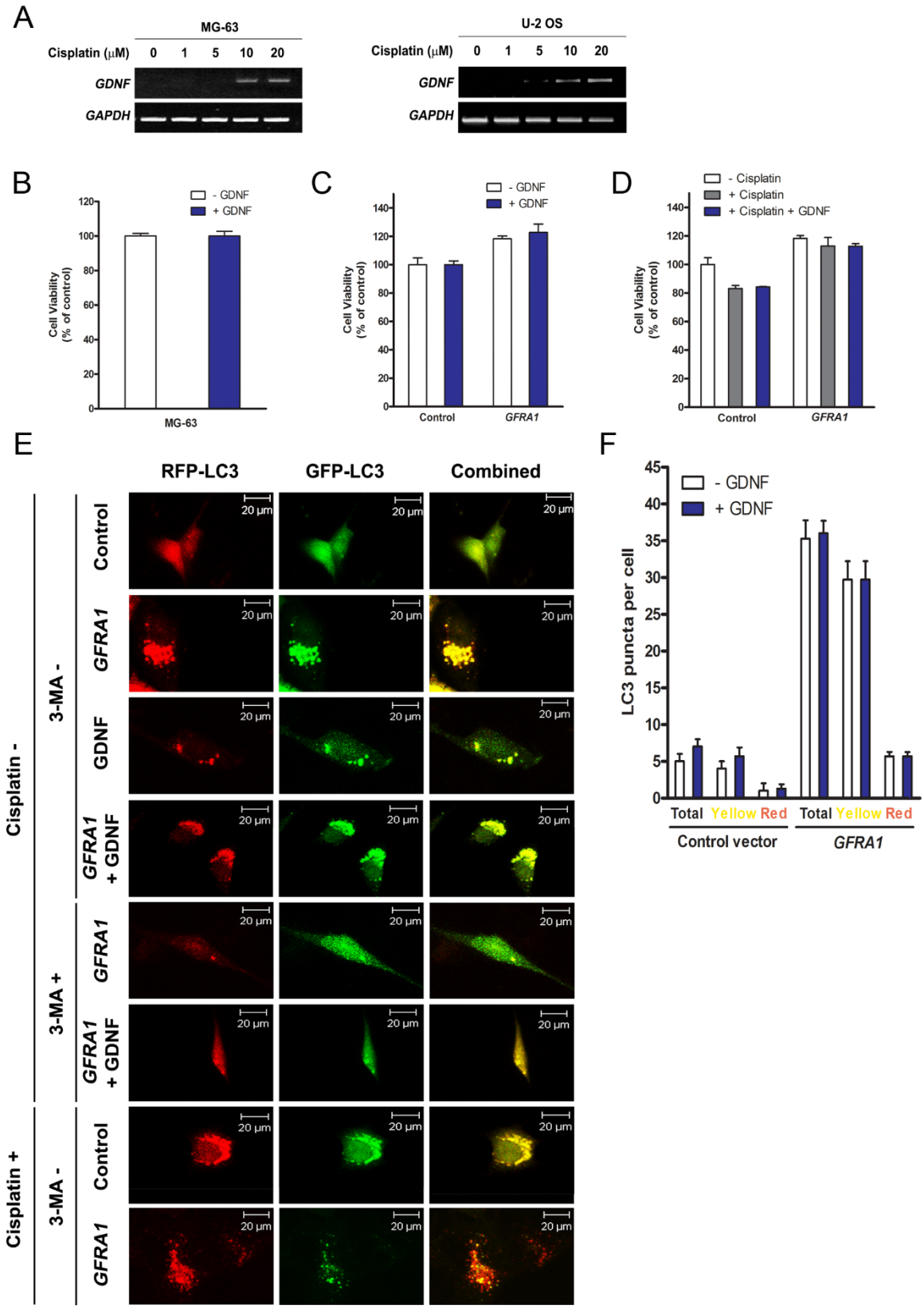
A
MG-63
Cisplatin (μM) 0 1 5 10 20
GDNF
GAPDH
U-2 OS
Cisplatin (μM) 0 1 5 10 20
GDNF
GAPDH
B
- GDNF
+ GDNF
Cell Viability (% of control)
MG-63
C
- GDNF
+ GDNF
Cell Viability (% of control)
Control
GFRA1
D
- Cisplatin
+ Cisplatin
+ Cisplatin + GDNF
Cell Viability (% of control)
Control
GFRA1
E
RFP-LC3
GFP-LC3
Combined
Cisplatin -
3-MA -
Control
GFRA1
GDNF
GFRA1 + GDNF
3-MA +
GFRA1
GFRA1 + GDNF
Cisplatin +
3-MA -
Control
GFRA1
20 μm
F
- GDNF
+ GDNF
LC3 puncta per cell
Total Yellow Red Total Yellow Red
Control vector
GFRA1

Figure S2. GFRA1-mediated chemoresistance of osteosarcoma cells is independent of GDNF. (**A**) Quantitative real-time PCR of *GDNF* mRNA expression after cisplatin treatment. MG-63 and U-2 OS cells were treated with different concentrations of cisplatin for 24 h. (**B to D**) Cell viability was measured using the WST-1 assay. The values are represented as a mean ± s.d.m. (n=3). (**B**) MG-63 cells were treated with PBS or GDNF (50 ng/ml) for 24 h. (**C**) Control and GFRA1-overexpressing cells were cultured and treated with PBS or GDNF for 24 h. (**D**) Control and GFRA1-overexpressing cells were treated and cultured with PBS only, cisplatin, or cisplatin + GDNF (50 ng/ml) for 24 h, respectively. (**E**) Representative images of mRFP-LC3 and GFP-LC3 puncta. Scale bar: 20 μm. Control and GFRA1-overexpressing MG-63 cells were transiently transfected with a mRFP-GFP tandem fluorescent-tagged LC3 plasmid (m*RFP-GFP-LC3*) and then treated with PBS (Cisplatin -) or cisplatin (20 μM; Cisplatin +) for 24 h. Control and GFRA1-overexpressing MG-63 cells were also transiently transfected with a mRFP-GFP tandem fluorescent-tagged LC3 plasmid (m*RFP-GFP-LC3*) and then treated with PBS or GDNF for 24 h in the absence (3-MA -) or presence (3-MA +) of 3-MA. Scale bar: 20 μm. (**F**) Quantitative analysis of the number of yellow puncta and the number of mRFP-LC3 puncta in the combined images of Control and GFRA1-overexpressing MG-63 cells treated with GDNF (50 ng/ml). The values are presented as a mean ± s.d.m. (n=3).

Figure S3

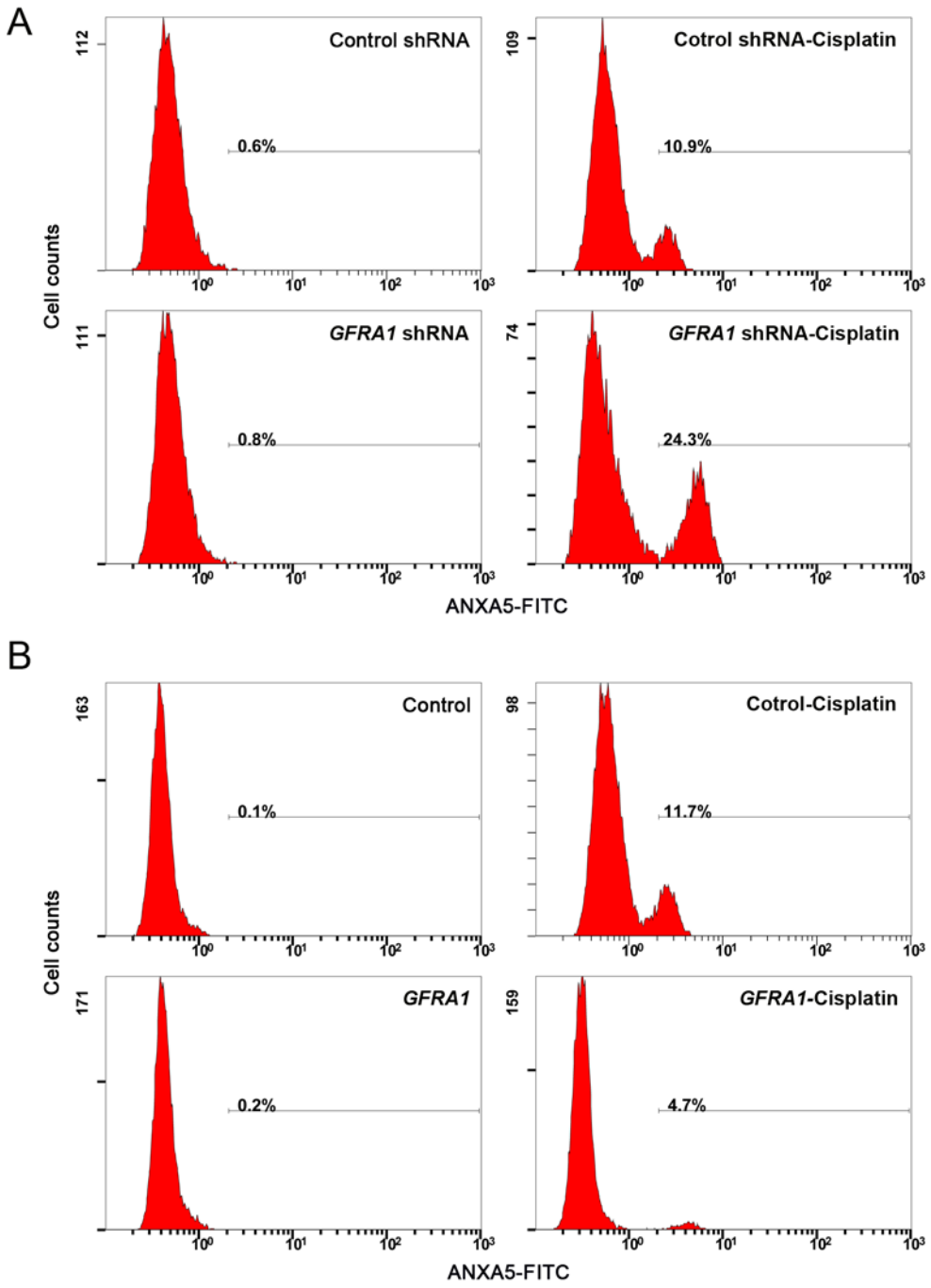

Figure S3. Effect of GFRA1 on cisplatin-induced apoptosis in MG-63 cells. (**A**) Apoptotic cells were counted in control or GFRA1-deficient MG-63 cells by flow cytometry 24 h after cisplatin treatment. (**B**) Under the same conditions, apoptotic cells were counted in control or GFRA1-overexpressing MG-63 cells.

Figure S4

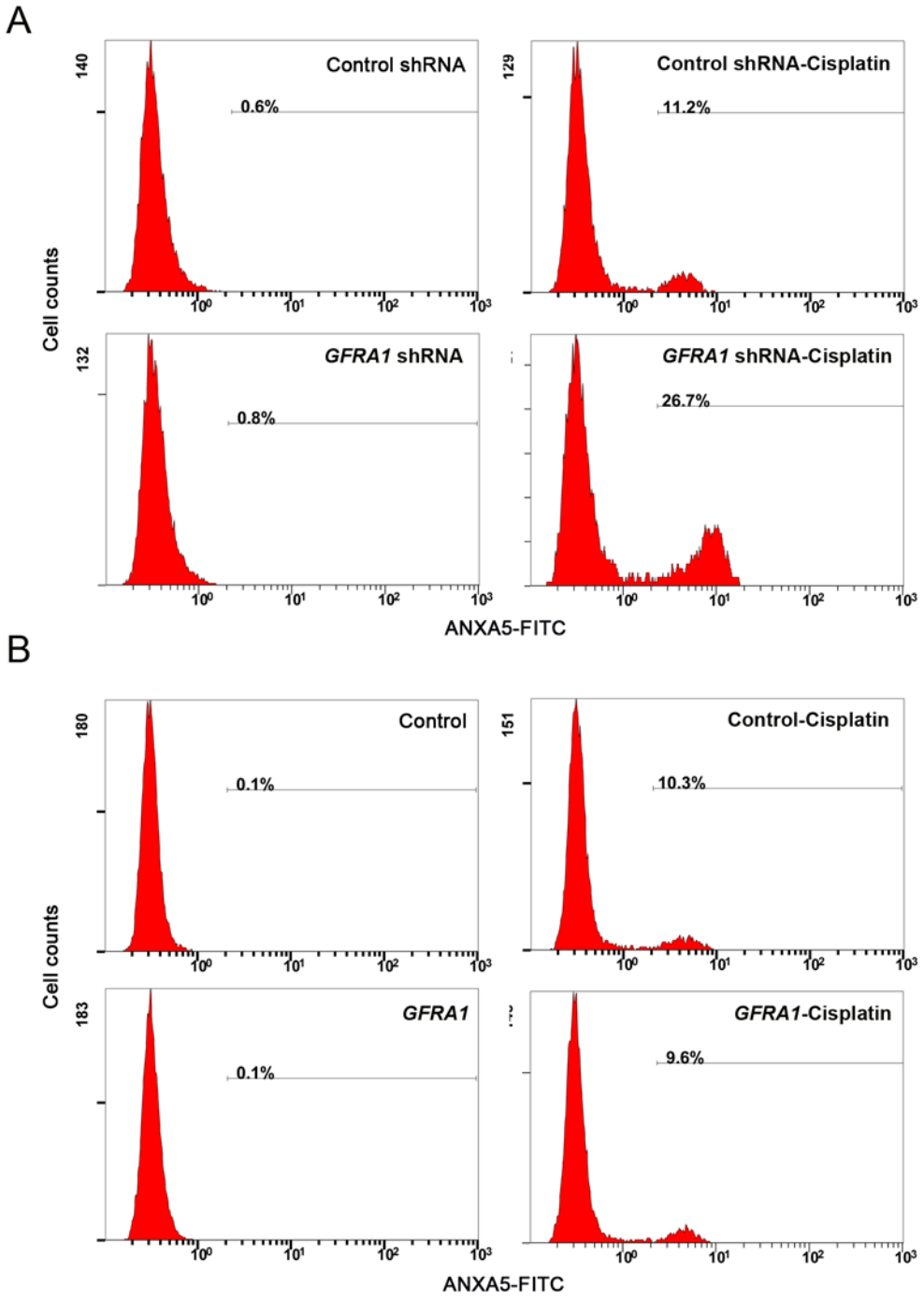

Figure S4. Effect of GFRA1 on cisplatin-induced apoptosis in U-2 OS cells. (**A**) Apoptotic cells were counted in control and GFRA1-deficeint U-2 OS cells by flow cytometry 24 h after cisplatin treatment. (**B**) Under the same conditions, apoptotic cells were counted in control or GFRA1-overexpressing U-2 OS cells.

Figure S5

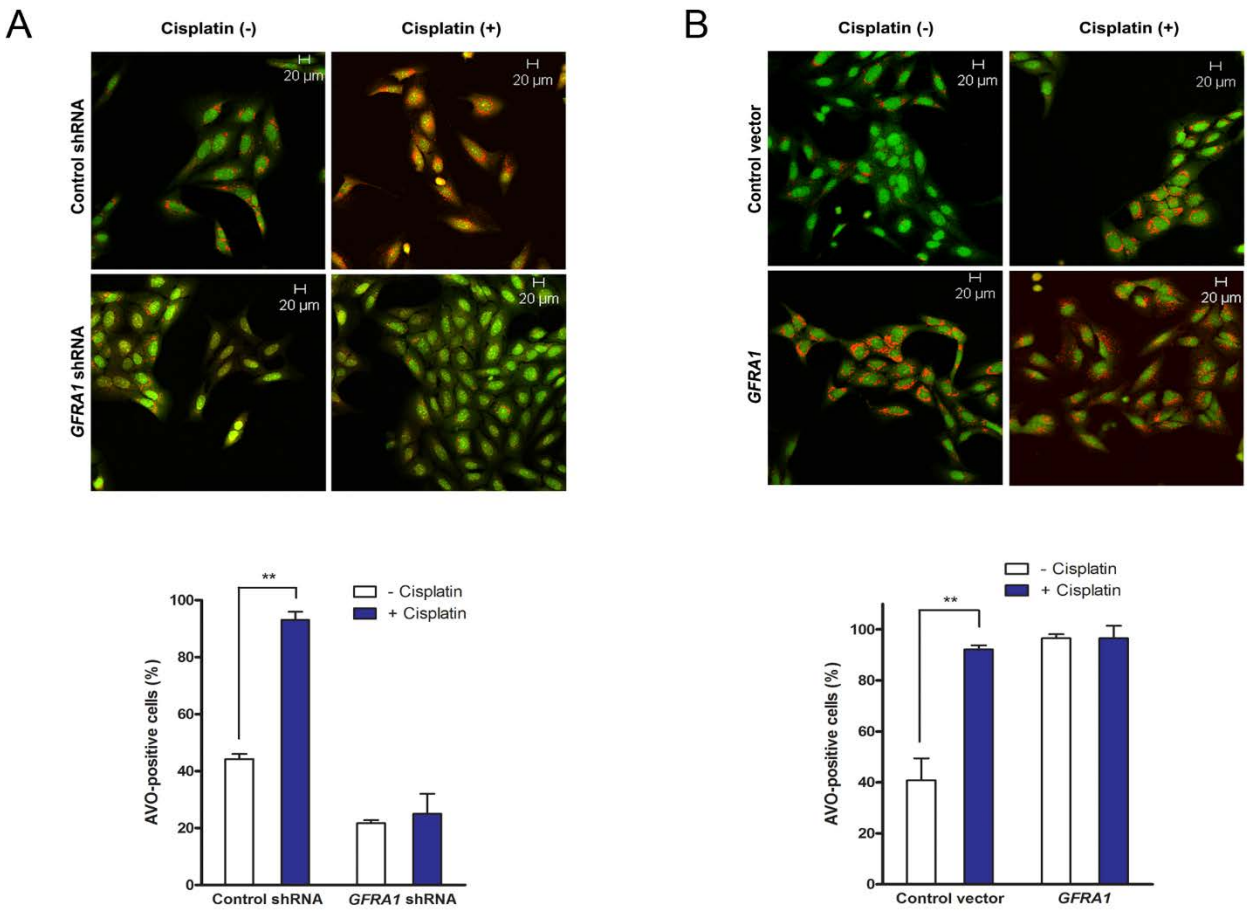

Figure S5. Acridine orange staining of GFRA1-deficient and GFRA1-overexpressing U-2 OS cells after cisplatin treatment. (**A**) Control and GFRA1-deficient U-2 OS cells were treated with cisplatin (20 μM) for 24 h and then stained with acridine orange (0.5 μg/ml) for 15 min. Top, representative images of cells stained with acridine orange. Scale bar: 20 μm. Bottom, quantitative analysis of the number of AVOs. The values are represented as a mean ± s.d.m. (n=3). ** denotes $P < 0.05$. (**B**) Control and GFRA1-overexpressing U-2 OS cells were treated with cisplatin (20 μM) for 24 h and then stained with acridine orange. Top, representative images of cells stained with acridine orange. Scale bar: 20 μm. Bottom, quantitative analysis of the number of AVOs. The values are represented as a mean ± s.d.m. (n=3). ** denotes $P < 0.05$.

Figure S6

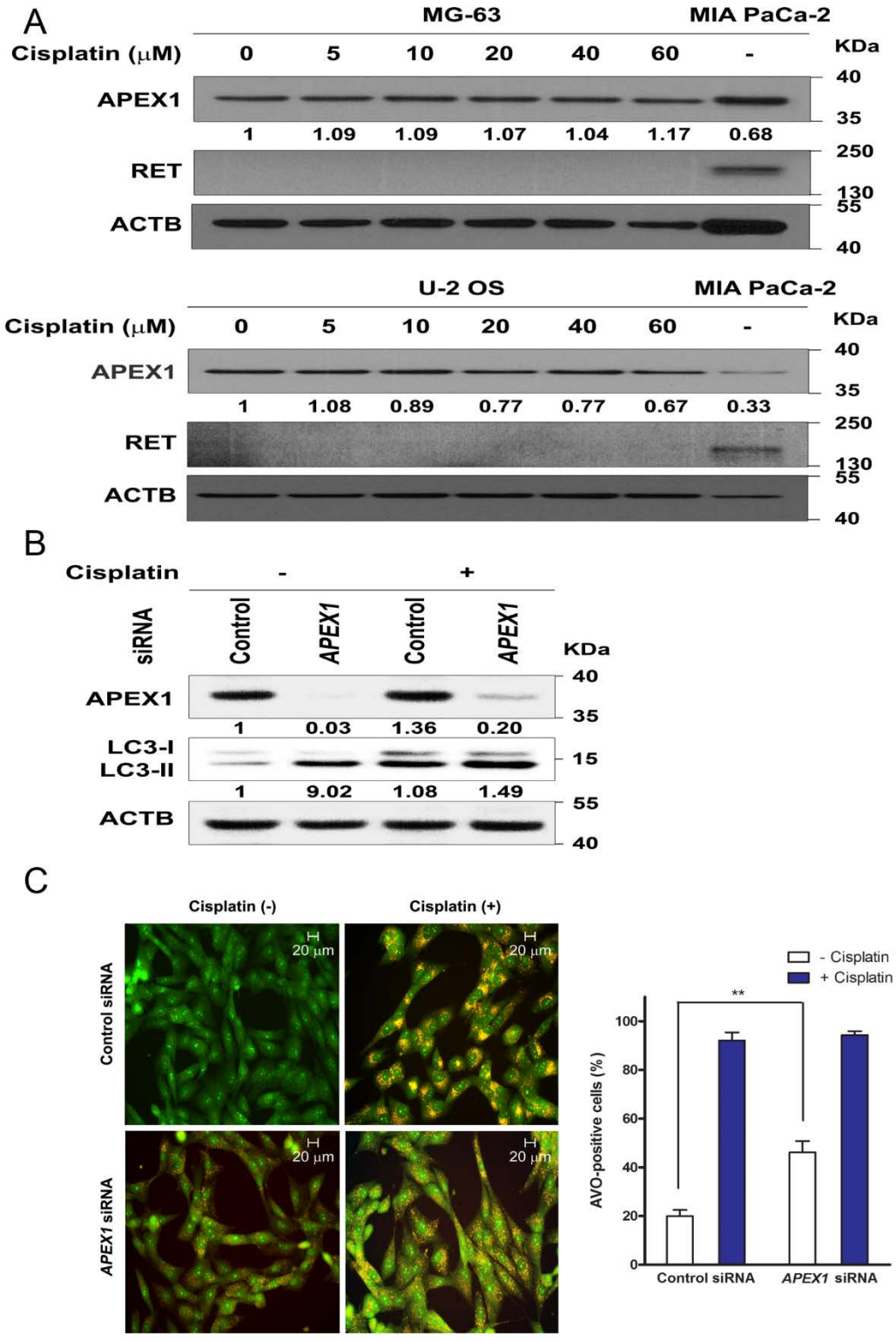
A
MG-63
MIA PaCa-2
Cisplatin (μM)
0
5
10
20
40
60
-
KDa
APEX1
40
35
1
1.09
1.09
1.07
1.04
1.17
0.68
RET
250
130
ACTB
55
40
U-2 OS
MIA PaCa-2
Cisplatin (μM)
0
5
10
20
40
60
-
KDa
APEX1
40
35
1
1.08
0.89
0.77
0.77
0.67
0.33
RET
250
130
ACTB
55
40
B
Cisplatin
-
+
siRNA
Control
APEX1
Control
APEX1
KDa
APEX1
40
35
1
0.03
1.36
0.20
LC3-I
LC3-II
15
1
9.02
1.08
1.49
ACTB
55
40
C
Cisplatin (-)
Cisplatin (+)
Control siRNA
APEX1 siRNA
H
20 μm
- Cisplatin
+ Cisplatin
**
AVO-positive cells (%)
100
80
60
40
20
0
Control siRNA
APEX1 siRNA

Figure S6. GFRA1-mediated chemoresistance of osteosarcoma cells is independent of APEX1 and RET signaling. (**A**) Immunoblot analysis of MG-63 or U-2 OS cell lysates with antibodies specific for APEX1, RET and ACTB. Cells were treated with the indicated concentrations of cisplatin for 24 h. The cell lysates of GDNF-treated MIA PaCa-2 were used as positive control for APEX1 and RET expression. The numbers below the lanes indicate densitometric quantification of APEX1 relative to ACTB control. (**B**) Immunoblot analysis of control and MG-63 cell lysates with antibodies specific for APEX1, LC3B and ACTB. MG-63 cells were cultured with control or *APEX1* siRNA for 48 h and then treated with cisplatin (20 μM) for 24 h. (**C**) Control and APEX1-deficient MG-63 cells were treated with cisplatin (20 μM) for 24 h and then stained with acridine orange (0.5 μg/ml) for 15 min. Top, representative images of cells stained with acridine orange. Scale bar: 20 μm. Bottom, quantitative analysis of the number of AVOs. The values are presented as a mean ± s.d.m. (n=3). ** denotes $P < 0.05$.

Figure S7

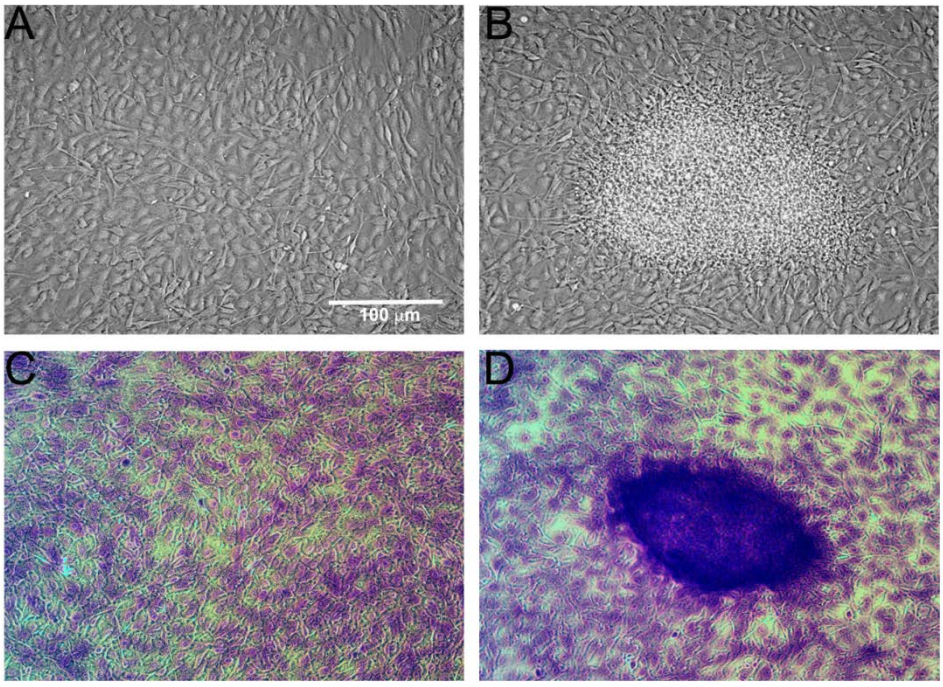

Figure S7. Cellular transformation of NIH3T3 cells by GFRA1. NIH3T3 cells ($1x10^6$) were cocultured with NIH3T3 cells ($1x10^3$) expressing empty vector or *GFRA1*. (**A and B**) Representative images of phase-contrast microscopy (**C and D**) Representative images of crystal violet staining. Scale bar: 100 µm.

Figure S8

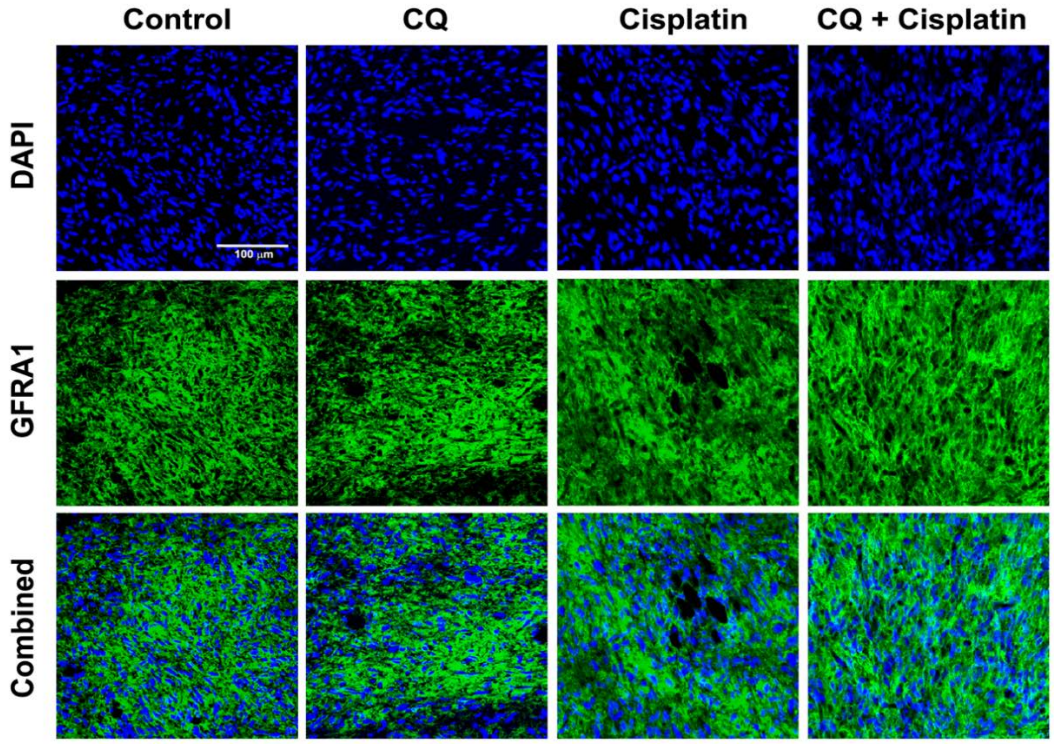

Figure S8. Representative images of immunofluorescence staining of GFRA1 in tumor sections generated from mice injected with MG-63 cells containing a GFRA1 expression vector and then treated with PBS, CQ, cisplatin, or cisplatin + CQ. Scale bar: 100 μm.

Table S1. Information of 27 osteosarcoma patients.

Case No.	Gender	Age	Diagnosis	Chemotherapy					
				Before			After		
				DAPI Staining	GFRA1	HMGB1	DAPI Staining	GFRA1	HMGB1
1	M	15	Osteosarcoma, chondroblastic type	+	-	-	+	-	-
2	M	15	Osteosarcoma, chondroblastic type	+	-	-	+	-	-
3	M	7	Parosteal osteosarcoma	+	-		+	-	-
4	M	7	Osteosarcoma, osteoblastic type	+	-	-	+	+	+
5	F	76	Osteosarcoma, osteoblastic type	+	-	-	+	-	-
6	M	20	Osteosarcoma, osteoblastic type	+	-	-	+	+	+
7	F	3	Osteosarcoma, osteoblastic type	+	-	-	+	+	+
8	F	17	Osteosarcoma, osteoblastic type	+	-	-	+	+	+
9	M	20	Osteosarcoma, osteoblastic type	+	-	-	+	-	-
10	M	16	Osteosarcoma, telangiectatic type	+	-	-	-	ND	ND
11	M	24	Parosteal osteosarcoma	+	-	-	-	ND	ND
12	F	8	Osteosarcoma, chondroblastic type	+	-	-	-	ND	ND
13	M	12	Osteosarcoma, chondroblastic type	+	-	-	-	ND	ND
14	F	10	Osteosarcoma, osteoblastic type	+	-	-	-	ND	ND
15	M	10	Osteosarcoma, osteoblastic type	+	-	-	-	ND	ND
16	F	15	Osteosarcoma, osteoblastic type	+	-	-	-	ND	ND
17	F	6	Osteosarcoma, osteoblastic type	+	-	-	-	ND	ND
18	M	32	Osteosarcoma, chondroblastic type	+	-	-	-	ND	ND
19	F	26	Osteosarcoma, osteoblastic type	+	-	-	-	ND	ND
20	M	13	Osteosarcoma, osteoblastic type	+	-	-	-	ND	ND
21	M	14	Osteosarcoma, osteoblastic type	+	-	-	-	ND	ND
22	F	10	Osteosarcoma, osteoblastic type	+	-	-	-	ND	ND
23	M	11	Osteosarcoma, osteoblastic type	+	-	-	-	ND	ND
24	F	17	Osteosarcoma, osteoblastic type	+	-	-	-	ND	ND
25	M	16	Osteosarcoma, osteoblastic type	+	-	-	-	ND	ND
26	M	14	Osteosarcoma, osteoblastic type	+	-	-	-	ND	ND
27	F	10	Osteosarcoma, osteoblastic type	+	-	-	-	ND	ND

Nine patients (cases No. 1 to 9) showed chemoresistance after treatment. Tissue samples from patients are collected and analyzed for GFRA1 and HMGB1 immunostaining before and after chemotherapy. ND, not determined. No DAPI-positive cells were observed from samples of 18 patients (cases No. 10 to 27) and the expression of GFRA1 and HMGB1 was not determined.

Table S2. Clinicopathological characteristics of 9 osteosarcoma patients that showed chemoresistance.

Parameter	n	GFRA1 Expression n (%)	HMGB1 Expression n (%)
Gender			
Female	3	2 (66.7)	2 (66.7)
Male	6	2 (33.3)	2 (33.3)
Age			
> 20 years old	1	0 (0)	0 (0)
≤ 20 years old	8	4 (50.0)	4 (50.0)
Tumor site			
Distal femur	6	3 (50)	3 (50)
Proximal femur	1	1 (100)	1 (100)
Others	2	0 (0)	0 (0)
Histological classification			
Osteoblastic	6	4 (66.7)	4 (66.7)
Chondroblastic	2	0 (0)	0 (0)
Others	1	0 (0)	0 (0)
Treatment period (weeks)			
< 4	2	0 (0)	0 (0)
4 – 15	7	4 (57.1)	4 (57.1)
Metastatic status			
Non-metastatic	5	0 (0)	0 (0)
Metastatic (lung)	4	4 (100)	4 (100)