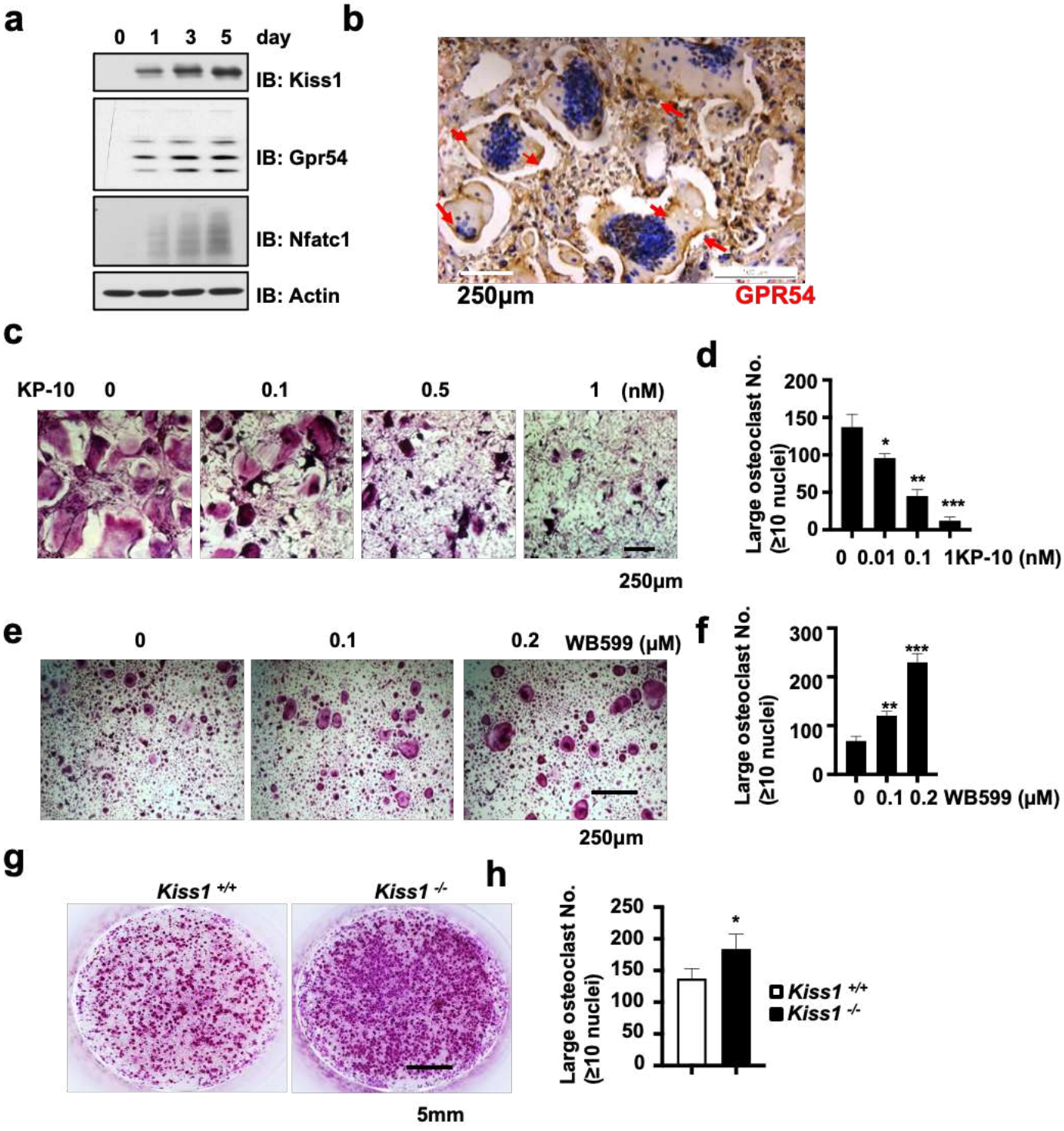
Supplementary Information

Kiss1/Gpr54 Protects Bone Mass through Src Dephosphorylation by Dusp18 in osteoclasts

Li et al.



Supplementary Figure 1. (Related to Figure 1).

a IB analysis of WCL derived from osteoclast in the process of differentiation.

b IHC staining in GCTB samples using the antibody of GPR54. The red arrow indicated the expression of GPR54 on the membrane of osteoclast-like multinucleated giant cells. Scale bar, 250 μm.

c Representative TRAP staining images (three biological replicates) show Kp-10 suppressed osteoclast formation in GCTB. Scale bar, 250 μm.

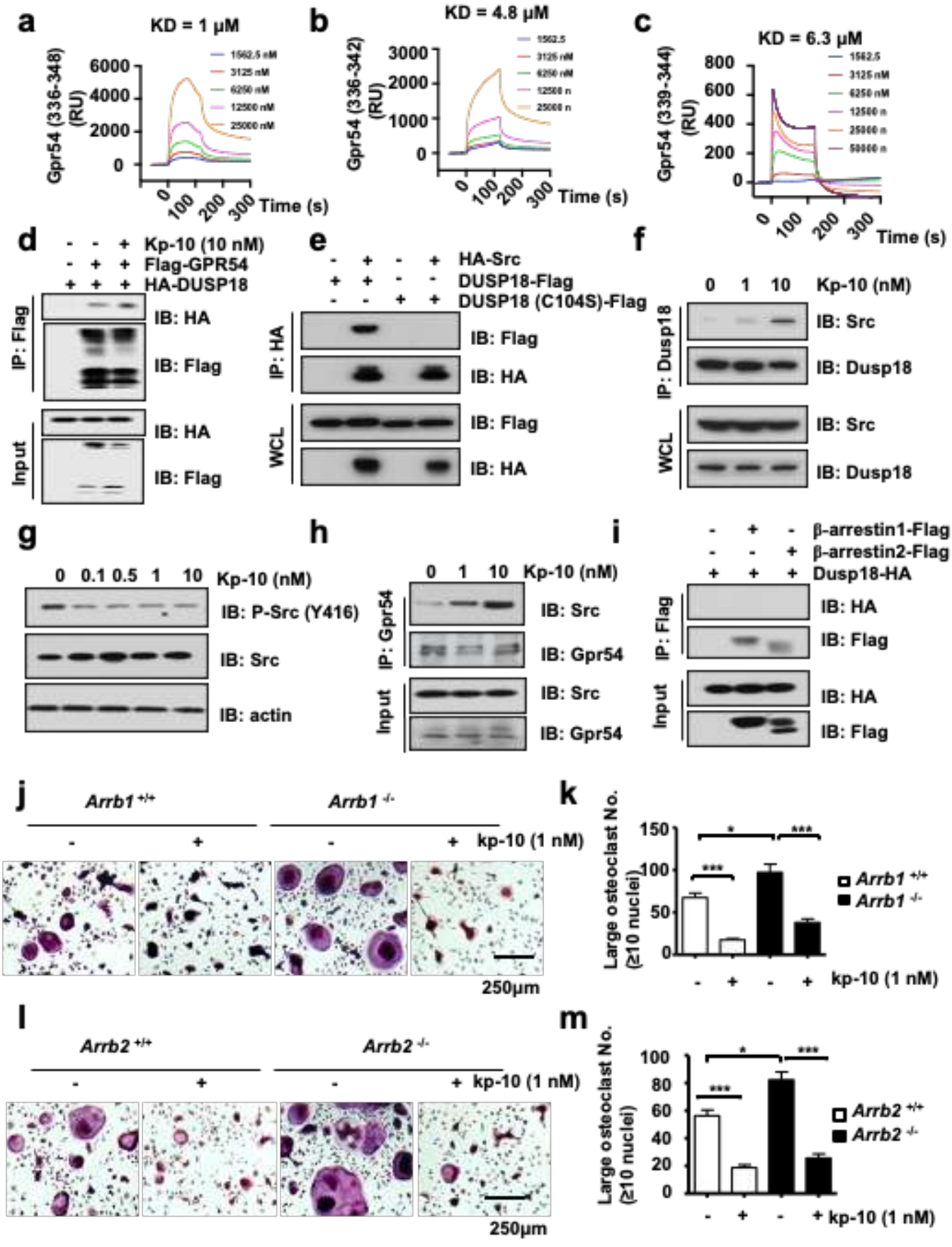
d The osteoclasts from Figure S1 c were counted. Error bars are standard errors of the mean (SEM). mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001. n = 3 per group.

e BMMs isolated from eight-week-old WT mice were stimulated with M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 5-7 days with the indicated dose of the GPR54 antagonist, 2-acylamino-4,6-diphenylpyridines (WB599). Representative TRAP staining images are shown. Scale bar, 250 μm.

f The large osteoclasts (≥10 nuclei) from Figure S1 e were counted. Error bars are standard errors of the mean (SEM). mean ± s.d. **P < 0.01, ***P < 0.001. n = 3 per group.

g BMM cells isolated from eight-week-old *Kiss1* $^{-/-}$ mice and WT littermate controls were stimulated with M-CSF (10 ng/ml) and RANKL (50 ng/ml) for the indicated time. Representative TRAP staining images were shown. Scale bars, 5mm.

h The large osteoclasts (≥10 nuclei) from Figure S1 g were counted. Error bars are standard errors of the mean (SEM). mean ± s.d., *P < 0.05. n = 3 per group.


a
KD = 1 μM
Gpr54 (336-348) (RU)
Time (s)
b
KD = 4.8 μM
Gpr54 (336-342) (RU)
Time (s)
c
KD = 6.3 μM
Gpr54 (339-344) (RU)
Time (s)
d
Kp-10 (10 nM)
Flag-GPR54
HA-DUSP18
IP: Flag
IB: HA
IB: Flag
Input
IB: HA
IB: Flag
e
HA-Src
DUSP18-Flag
DUSP18 (C104S)-Flag
IP: HA
IB: Flag
IB: HA
WCL
IB: Flag
IB: HA
f
Kp-10 (nM)
IP: Dusp18
IB: Src
IB: Dusp18
WCL
IB: Src
IB: Dusp18
g
Kp-10 (nM)
IB: P-Src (Y416)
IB: Src
IB: actin
h
Kp-10 (nM)
IP: Gpr54
IB: Src
IB: Gpr54
Input
IB: Src
IB: Gpr54
i
β-arrestin1-Flag
β-arrestin2-Flag
Dusp18-HA
IP: Flag
IB: HA
IB: Flag
Input
IB: HA
IB: Flag
j
Arrb1 +/+
Arrb1 -/-
kp-10 (1 nM)
250μm
k
Large osteoclast No. (≥10 nuclei)
Arrb1 +/+
Arrb1 -/-
kp-10 (1 nM)
l
Arrb2 +/+
Arrb2 -/-
kp-10 (1 nM)
250μm
m
Large osteoclast No. (≥10 nuclei)
Arrb2 +/+
Arrb2 -/-
kp-10 (1 nM)

Supplementary Figure 2 (Related to Figure 3,4,5,6).

a SPR binding analysis of SH2-SH3 domain of Src and mouse Gpr54 (^{336}RVCPCCRQRQRRP^{348}) peptide with the binding affinity at 1 μM.

b SPR binding analysis of SH2-SH3 domain of Src and mouse Gpr54 (^{336}RVCPCCR^{342}) peptide. The binding affinity was measured at 4.8 μM.

c SPR binding analysis of SH2-SH3 domain of Src and mouse Gpr54 (^{339}PCCRQR^{344}) peptide. The binding affinity was measured at 6.3 μM.

d IB analysis of WCL and anti-Flag IP derived from 293T cells transfected with GPR54-Flag and DUSP18-HA and then treated with Kp-10 for 20 minutes.

e IB analysis of WCL and anti-Flag IP derived from 293T cells transfected with HA-Src and DUSP18-Flag or DUSP18(C104S)-Flag and then treated with Kp-10 for 20 minutes.

f IB analysis of WCL and antiAnti-Gpr54 IP were performed on WCL derived from RAW264.7 cells treated with or without Kp-10 stimulation for 20 minutes.

g IB analysis of WCL derived from MEFs isolated from *Arrb1* and *Arrb2* double knockout mice (Arrb*1/2* $^{-/-}$). MEFs were starved in serum-free α-MEM and treated with the indicated dose of Kp-10 for 20 minutes. IB analysis was carried out with antibodies as indicated.

h IB analysis of WCL and antiAnti-Gpr54 IP were performed on WCL derived from RAW264.7 cells treated with or without Kp-10 stimulation for 20 minutes.

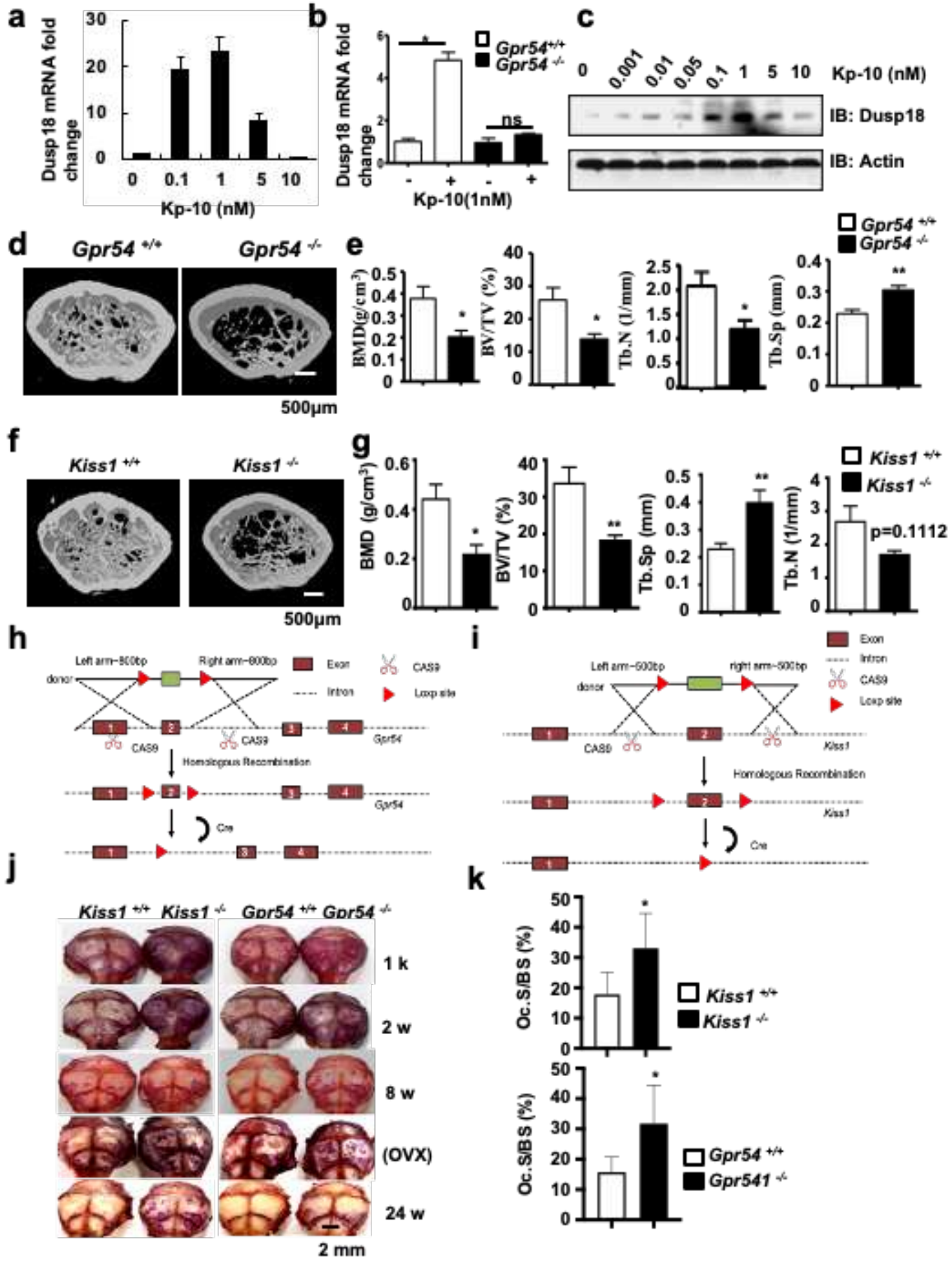
i IB analysis of WCL and anti-Flag IP derived from 293T cells transfected with HA-DUSP18-HA and either Flag- β-arrestin-1 or Flag-β-arrestin-2 constructs.

j BMM cells isolated from eight-week-old *Arrb1* knockout mice and WT littermate controls were stimulated with M-CSF (10 ng/ml) and RANKL (50 ng/ml) for the indicated time. Representative TRAP staining images (three biological replicates) are shown. Scale bar, 250 μm.

k The large osteoclasts (⩾10 nuclei) from Figure S2 j were counted. mean ± s.d. *P < 0.05, ***P < 0.001. n = 3 per group.

l BMM cells isolated from eight-week-old *Arrb2* $^{-/-}$ knockout mice WT littermate controls were stimulated with M-CSF (10 ng/ml) and RANKL (50 ng/ml) for the indicated time. Representative TRAP staining images (three biological replicates) are shown. Scale bar, 250 μm.

m The large osteoclasts (⩾10 nuclei) from Figure S2 l were counted. mean ± s.d. *P < 0.05, ***P < 0.001. n = 3 per group.


a
Dusp18 mRNA fold change
Kp-10 (nM)
b
Dusp18 mRNA fold change
Gpr54+/+
Gpr54 -/-
ns
Kp-10(1nM)
c
Kp-10 (nM)
IB: Dusp18
IB: Actin
d
Gpr54 +/+
Gpr54 -/-
500μm
e
BMD(g/cm3)
BV/TV (%)
Tb.N (1/mm)
Tb.Sp (mm)
f
Kiss1 +/+
Kiss1 -/-
500μm
g
BMD (g/cm3)
BV/TV (%)
Tb.Sp (mm)
Tb.N (1/mm)
p=0.1112
h
Left arm~800bp
Right arm~800bp
donor
Exon
Intron
CAS9
Loxp site
Gpr54
Homologous Recombination
Cre
i
Left arm~500bp
right arm~500bp
donor
Kiss1
Homologous Recombination
Cre
j
Kiss1 +/+
Kiss1 -/-
Gpr54 +/+
Gpr54 -/-
1 k
2 w
8 w
(OVX)
24 w
2 mm
k
Oc.S/BS (%)
Kiss1 +/+
Kiss1 -/-
Oc.S/BS (%)
Gpr54 +/+
Gpr541 -/-

Supplementary Figure 3. (Related to Figure 5, 6).

a RAW264.7 cells were treated with the indicated dose of Kp-10 for 30 minutes. Total RNAs were extracted for quantitative real-time RT-PCR analyses for relative RNA levels of Dusp18.

b BMM cells isolated from eight-week-old *Gpr54* $^{-/-}$ mice and WT littermate controls were stimulated with 1 nM Kp-10 for 30 minutes. Total RNAs were extracted for quantitative real-time RT-PCR analyses for relative RNA levels of Dusp18.

c IB analysis of WCL derived from RAW264.7 cells treated with the indicated dose of Kp-10 stimulation for 1 hour.

d Representative micro-CT images of 8-week-old WT and *Gpr54* $^{-/-}$ mice (n = 6 per group. Scale bar, 500 μm.

e Bone mass parameter analysis of 8-week-old WT and *Gpr54* $^{-/-}$ mice. BMD, bone mineral density; BV/TV, bone volume as a fraction of total bone volume; Tb. Th, trabecular thickness; Tb. N, trabecular number; Tb.Sp, trabecular separation. mean ± s.d. $*P < 0.05$, $**P < 0.01$, unpaired two-tailed Student's t-test. n = 6 per group.

f Representative micro-CT images of 8-week-old WT and *Kiss1*$^{-/-}$ mice (n=6). Scale bar, 500 μm.

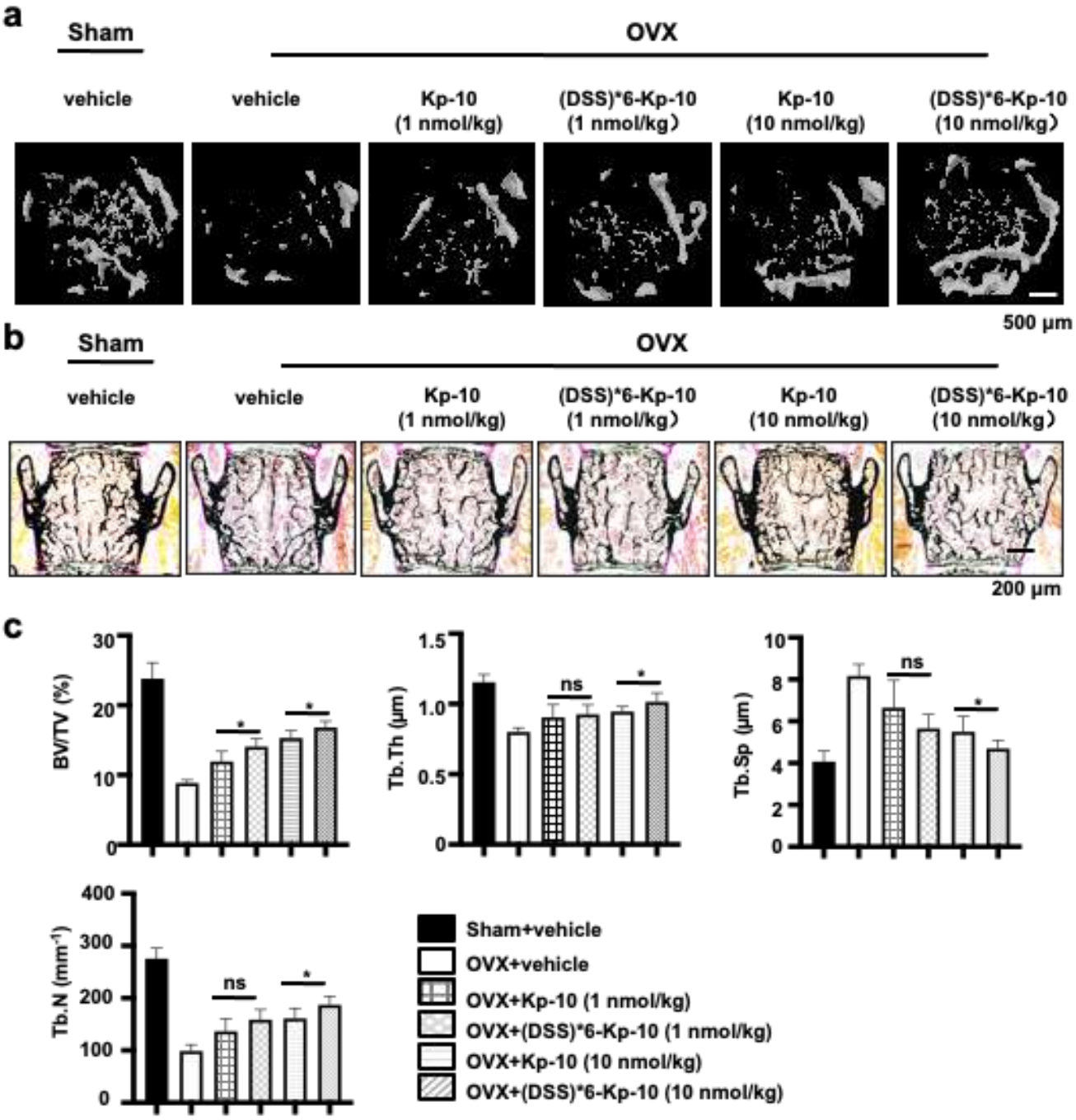
g Bone mass parameter analysis of WT and *Kiss1*$^{-/-}$ mice. BMD, bone mineral density; BV/TV, bone volume as a fraction of total bone volume; Tb. Th, trabecular thickness; Tb. N, trabecular number; Tb.Sp, trabecular separation. mean ± s.d. $*P < 0.05$, $**P < 0.01$, unpaired two-tailed Student's t-test. n = 6 per group.

h Targeting strategy of *Gpr54.* Genomic structure of the WT mouse *Gpr54* gene. Exon 2 was flanked by Loxp sequences. The modified *Gpr54* locus after homologous recombination and the deleted *Gpr54* gene after Cre-mediated excision of exon 2 is shown.

i Targeting strategy of *Kiss1.* Genomic structure of the WT mouse *Kiss1* gene. Exon 2 was flanked by Loxp sequences. The modified *Kiss1* locus after homologous recombination and the deleted *Kiss1* gene after Cre-mediated excision of Exon 2 is shown.

j Representative TRAP staining images of the whole calvaria from, *Kiss1*$^{-/-}$, *Gpr54*$^{-/-}$ mice compared with indicated wild-type mice. Scale bar, 2 mm. OVX indicated that 1-week-old indicated mice (n=3 per group) were ovariectomized and then 8-week-old OVX mice have conducted TRAP staining.

k Parameter analysis of the whole calvaria from Figure S2 g, mean ± s.d. **P* < 0.05, unpaired two-tailed Student's t-test. n = 6 per group.



Supplementary Figure 4. (Related to Figure 7).

a OVX mice model (n= 9 per group). Representative micro-computed tomography (micro-CT) images of indicated group (n=3 per group). Scale bars, 500 μm.

b Representative histomorphometry image of L3 lumbar of indicated group (n=6 per group). Scale bars, 200 μm.

c Parameter analysis of trabecular bone of L3 lumbar (n=6 per group). BV/TV, bone volume as a fraction of total bone volume; Tb.Th, trabecular thickness; Tb. N, trabecular number; Tb.Sp, trabecular separation. mean ± s.d. *$P < 0.05$, unpaired two-tailed Student's t-test. n = 6 per group.

Supplementary Figure 5. Uncropped Original Scans

Figure 1g

Figure 2b

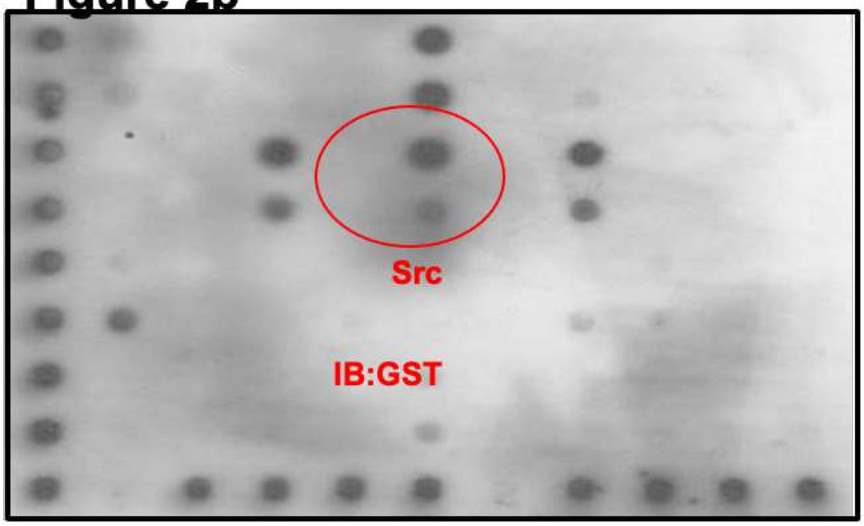


Figure 1h

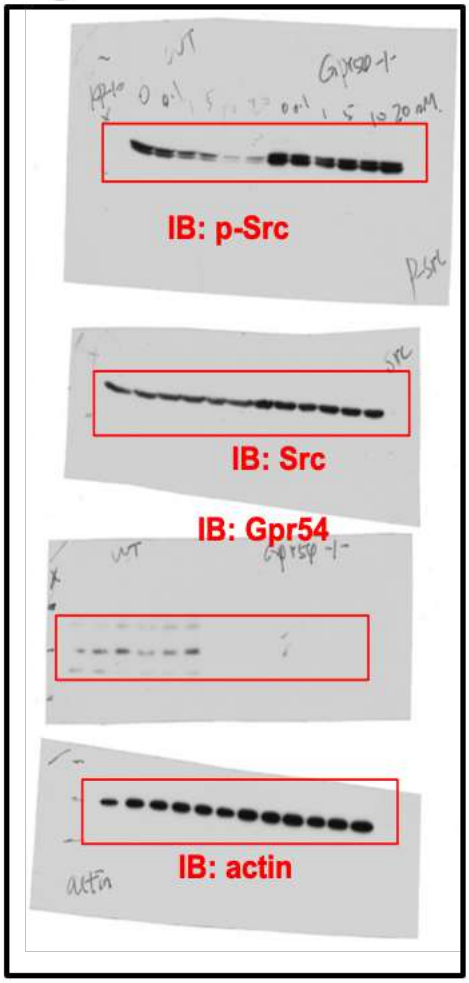


Figure 1i

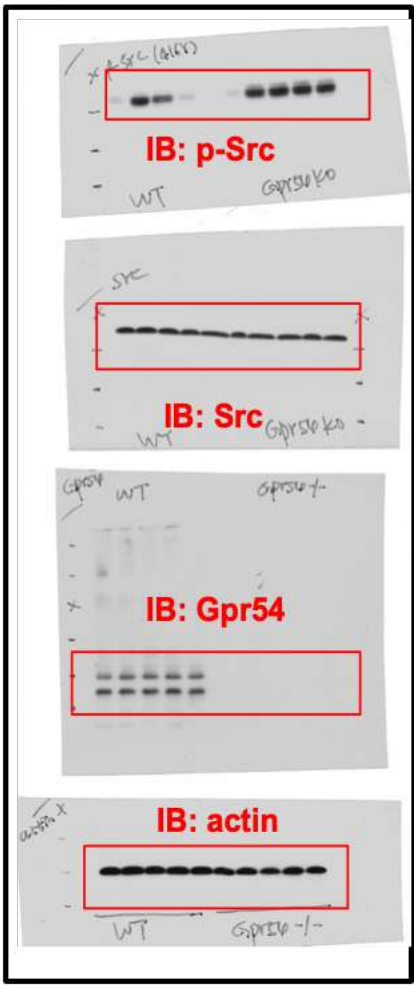


Figure 3g

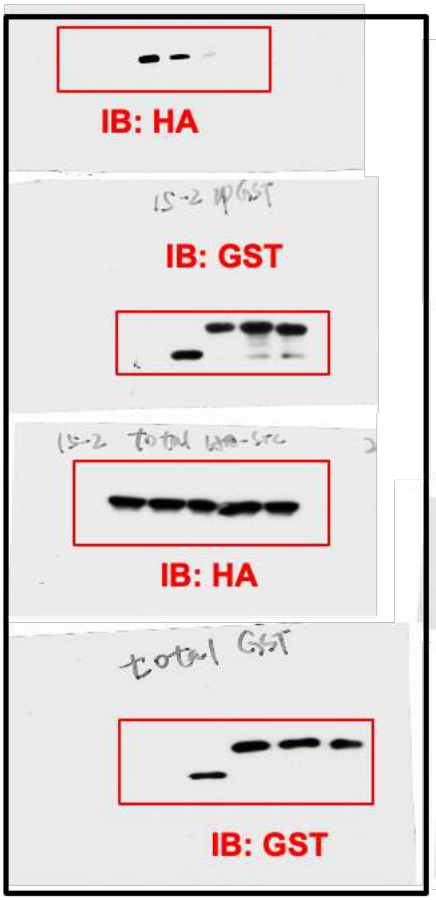


Figure 3h

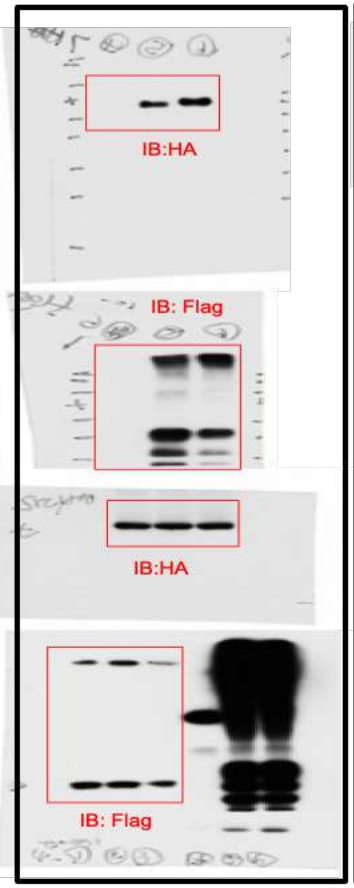


Figure 3i

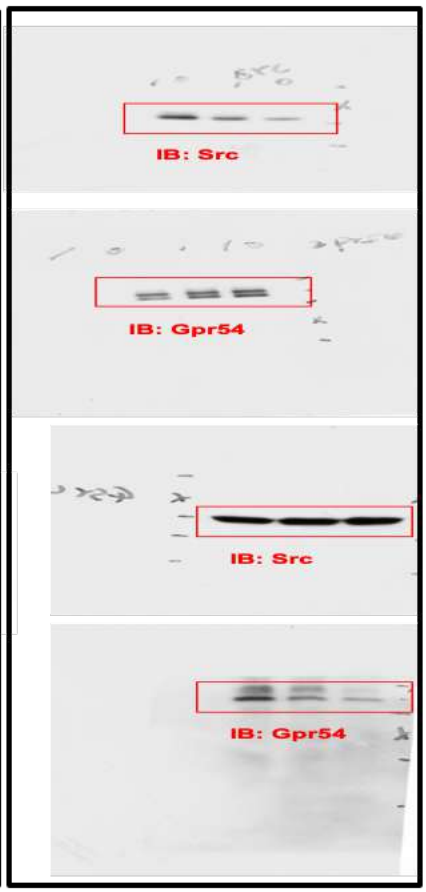


Figure 4c

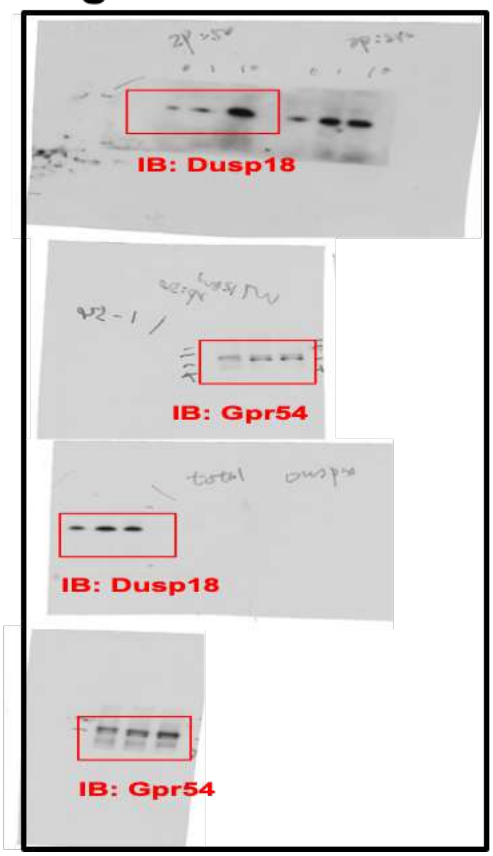


Figure 4h

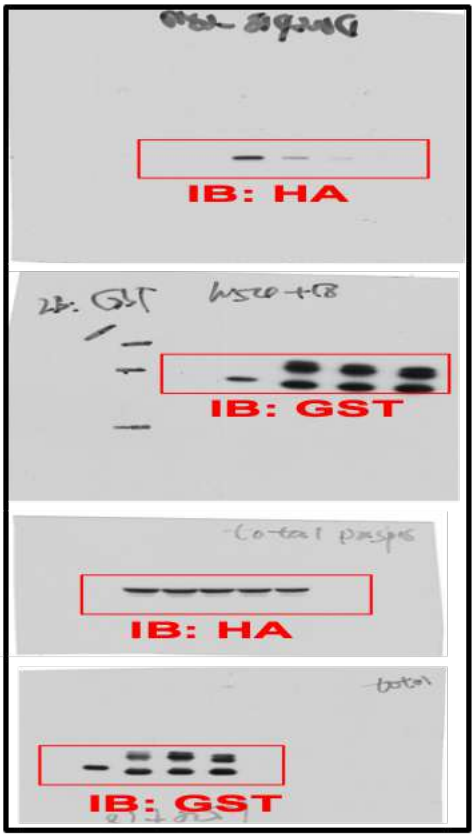


Figure 4i

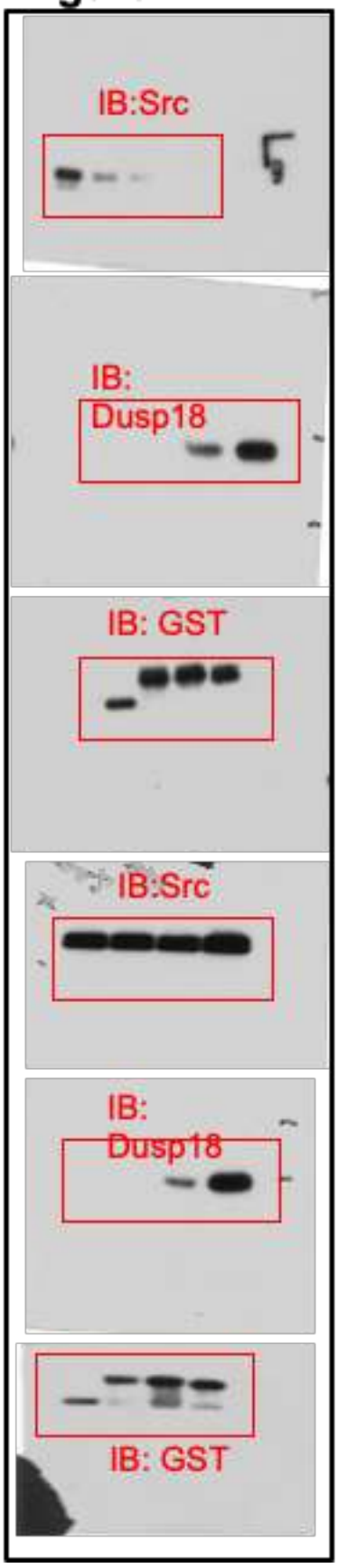


Figure 5b

Figure 5c

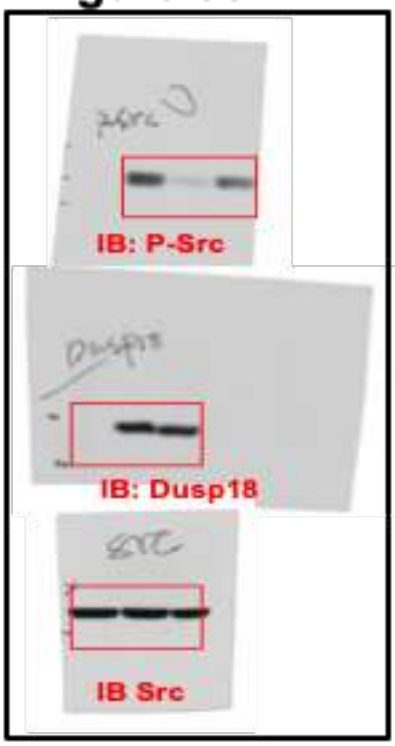


Figure 5f

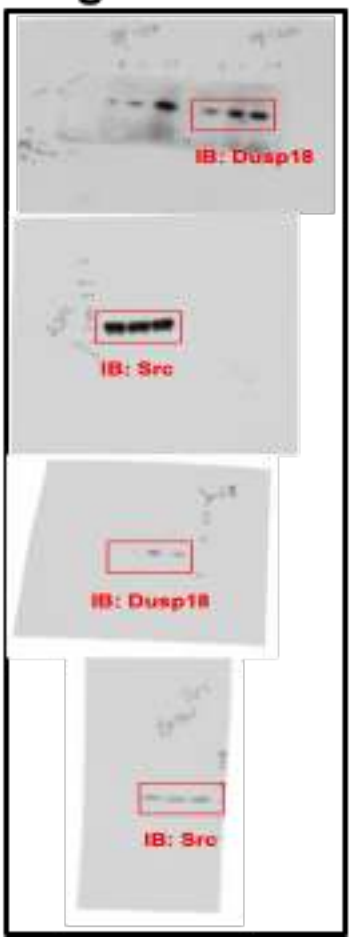


Figure 5g

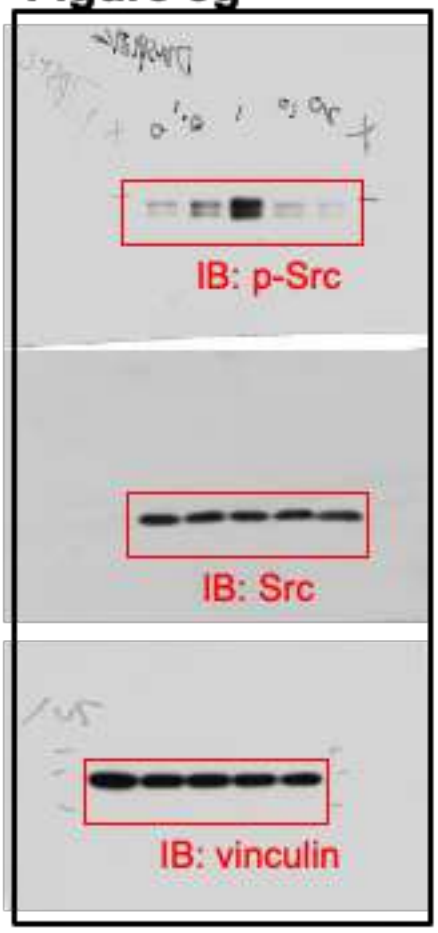


Figure 5e

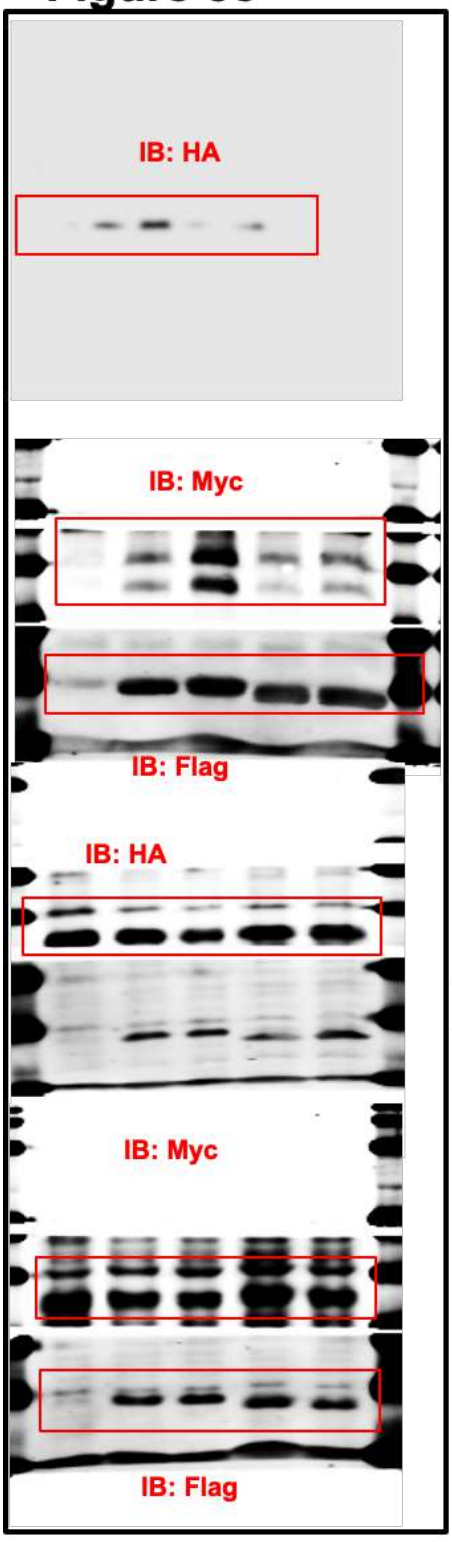


Figure 5h

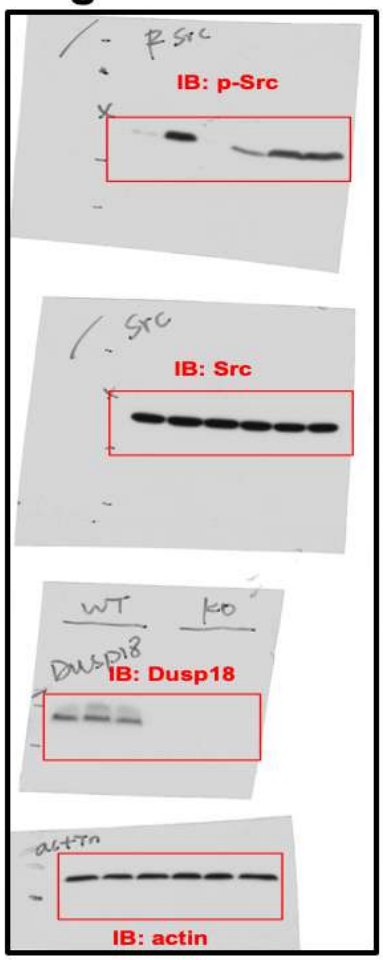


Figure 6b

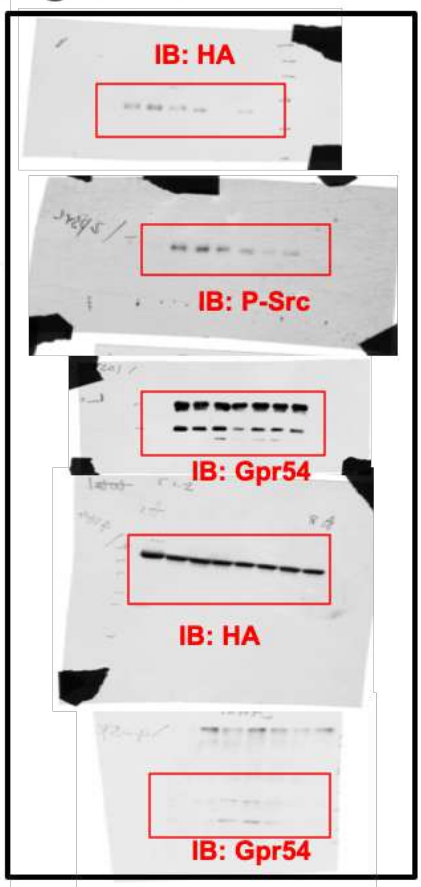


Figure 6d

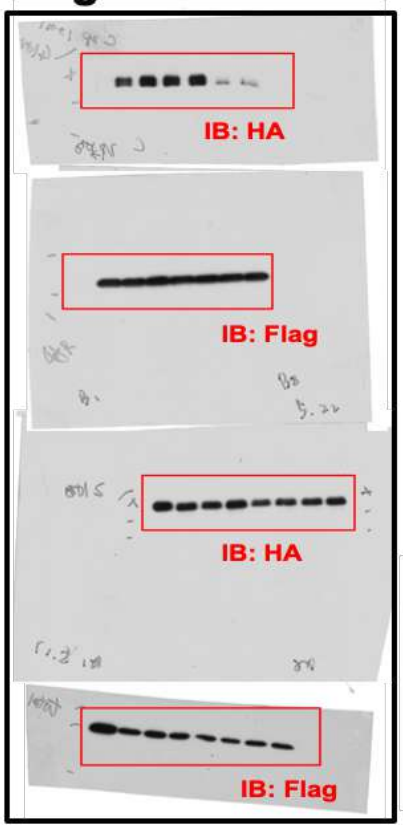


Figure 6c

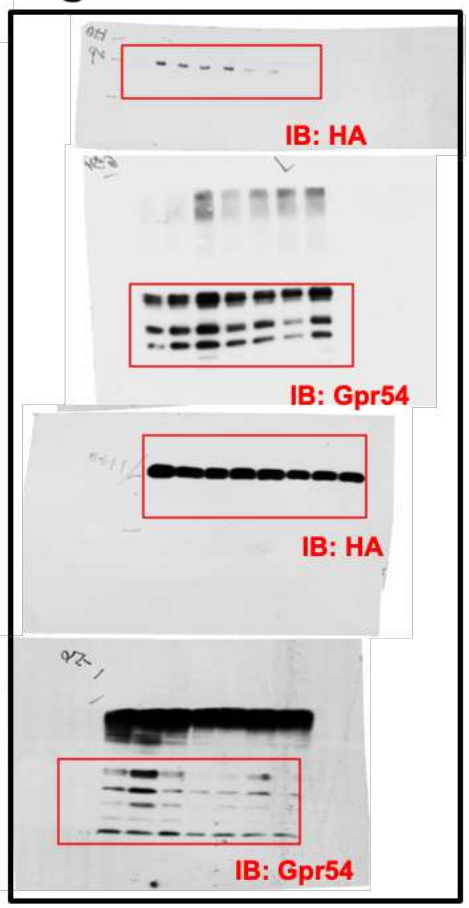


Figure 6e

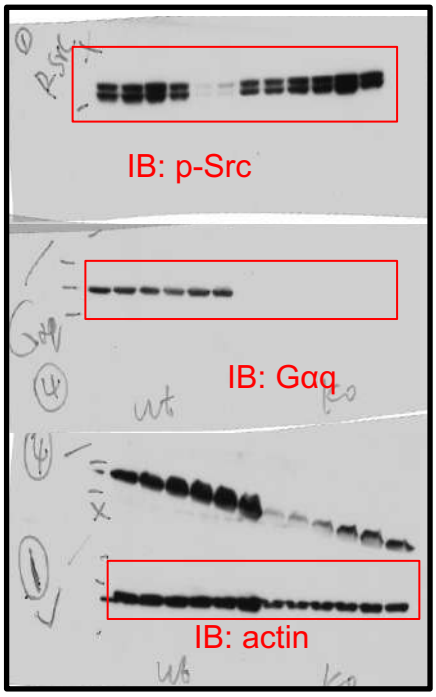


Figure 6f

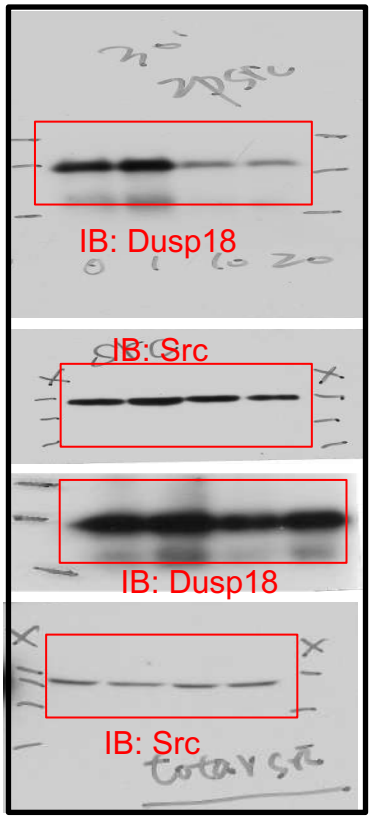


Figure S1 a

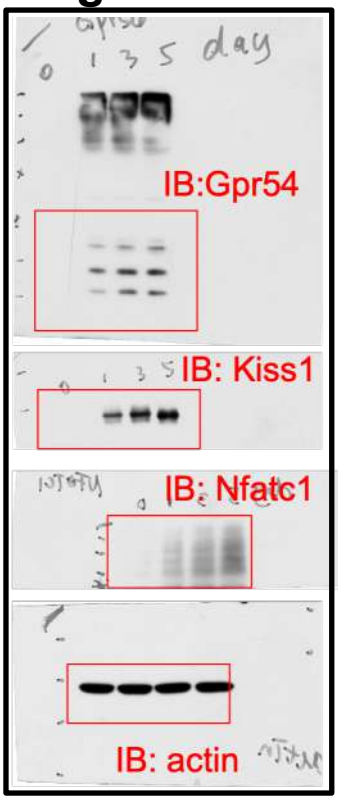


Figure S3 d

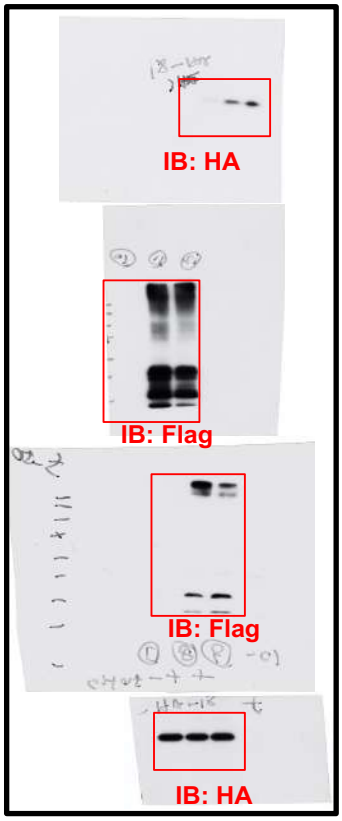


Figure S3 e

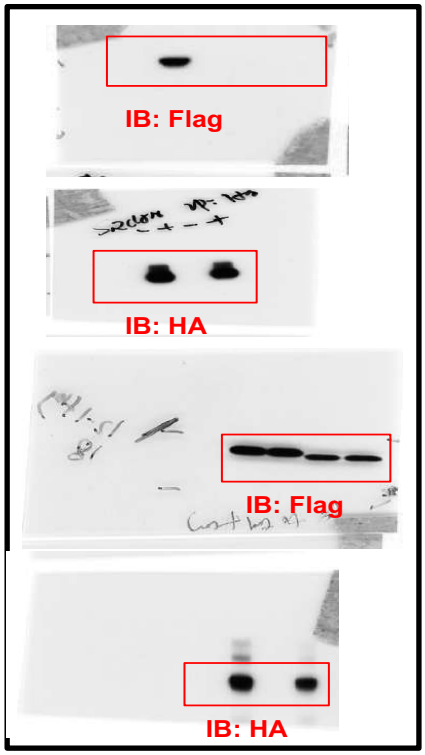


Figure S3 f

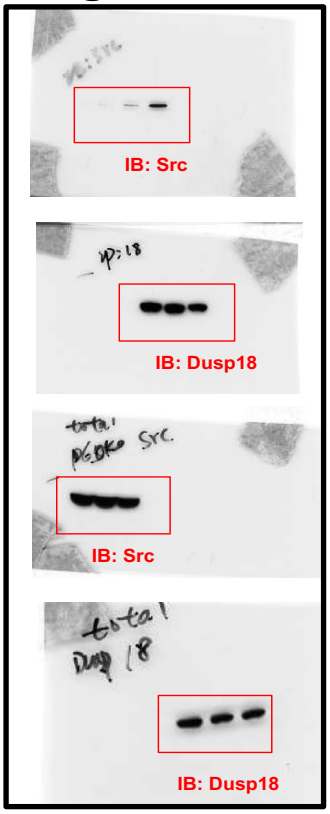


Figure S3g

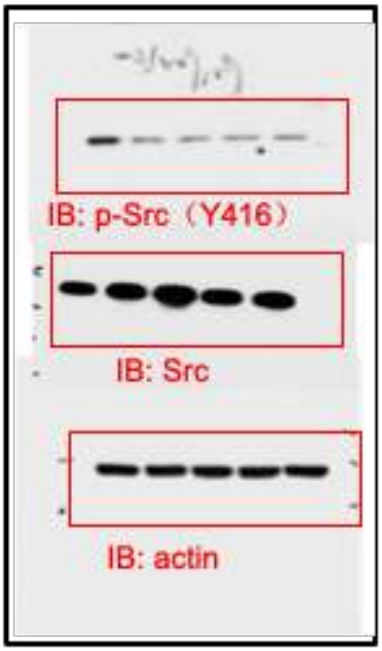


Figure S3h

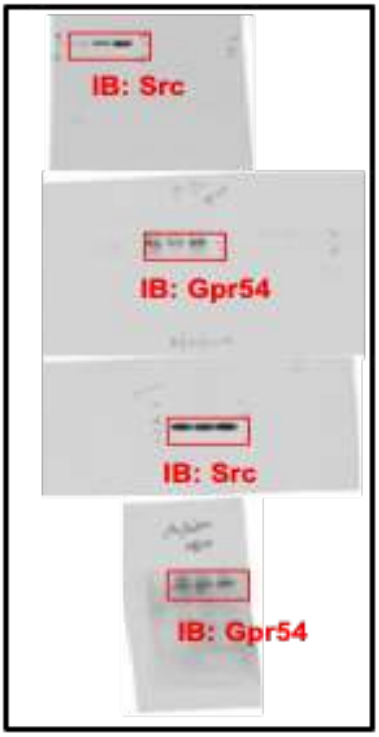


Figure S3i

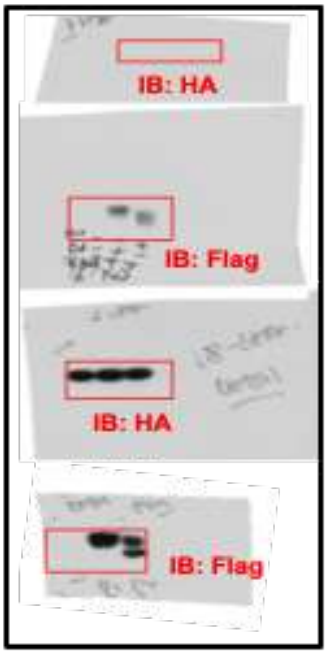


Figure S4c

Table 1. TranSignal™ SH3 Domain Array kit information.

CRK	Itk	SJHUA	Lyn1	Yes1	MLPK3	Cortactin	SPCN	CCB4	Amphiphysin
CRK	Itk	SJHUA	Lyn1	Yes1	MLPK3	Cortactin	SPCN	CCB4	Amphiphysin
NOF	VAV2	Hck	FYB	Src	Nebulin	SLK	FGR	EMP55	Dlg2
NOF	VAV2	Hck	FYB	Src	Nebulin	SLK	FGR	EMP55	Dlg2
Abl(2)	HS1	Tim	PS095	RasGAP	BTK	PEXD	Y124	NCK (3)	VAV
Abl(2)	HS1	Tim	PS095	RasGAP	BTK	PEXD	Y124	NCK (3)	VAV
		GST	TXK	ITSN (1)	ITSN (2)	Riz	PLCγ	Abl(1)	BLK
		GST	TXK	ITSN (1)	ITSN (2)	Riz	PLCγ	Abl(1)	BLK

Table 2. Data collection and refinement statistics

	Src(SH3+SH2)-54CT(PR motif)
Data collection	
Space group	$P3_1\,21$
Cell dimensions	
a, *b*, *c* (Å)	95.80, 95.80, 123.38
Resolution (Å)	82.99 - 3.54 (3.66- 3.54) *
R_{merge} (%)	2.7(35.3)
CC (1/2) (%)	99.9(76.2)
$I / \sigma I$	13.3(2.3)
Completeness (%)	97.4(99.9)
Redundancy	2.0(2.0)
Refinement	
Resolution (Å)	82.48 - 3.50
No. reflections	8115
R_{work} / R_{free}	0.268 / 0.304
Ramachandran	
Favored (%)	95.34
Allowed (%)	4.37
Outlier (%)	0.29
R.m.s. deviations	
Bond lengths (Å)	0.002
Bond angles (°)	0.459

*Values in parentheses are for the highest-resolution shell.

Table 3. Information of genotyping PCR primers sequence.

Gene	Primer	Sequence
Kiss1	sense	GATTCCGTTGCCGACCGTAT
	antisense	GGGACATGGCATGGTACTTCA
Gpr54	WT	GCCTAAGTTTCTCTGGTGGAGGATG
	both	CGCGTACCTGCTGGATGTAGTTGAC
	mutant	GTGGGATTAGATAAATGCCTGCTCT
Kiss1 ^{floxed}	Left sense	TCTGGCAAGCACTTGAAAG
	Right antisense	ATACCGCGATTCCTTTTCC
Gpr54^{floxed}	Left sense	CCAAGGCAGGGAGCTTCTA
	Right antisense	CCTCAGTGGCCGAGTTTCT
	Left sense	TGTCTTATGGACGTGATAGCC
	Right antisense	GCGGATGCTGGAAGATGG
Dusp18	Left sense	CCTTCACCGCACTCCTCTA
	Right antisense	CACAGACCCTGACCACAACA
	sense	GTCCCTTCCATTGTTCACGG
	antisense	TCAAACGGGTCTCCTTCTCG
Arrb1	sense	CCTAGTGCTGGGATTACAAG
	antisense	CATAGCCTGAAGAACGAGAT
Arrb2	Left WT	ACAGGGTCCACTTTGTCCA
	mutant	GGGGGTGGGGAGGGGTGTTAG
	Right WT	GCTAAAGCGCATGCTCCAGA
LysM-Cre	WT	TTACAGTCGGCCAGGCTGAC
	common	CTTGGGCTGCCAGAATTTCTC
	mutant	CCCAGAAATGCCAGATTACG