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# Kiss1/Gpr54 Prevents Bone Loss through Src Dephosphorylation by Dusp18 in Osteoclasts

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## Article

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# Kiss1/Gpr54 Prevents Bone Loss through Src Dephosphorylation

# by Dusp18 in Osteoclasts

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#### 1 SUMMARY

2 Osteoclasts are over-activated as we age, which results in bone loss. Src-deficient mice lead to 3 only one phenotype -severe osteopetrosis due to functional defect in osteoclasts, indicating that Src function is essential in osteoclasts. G-protein-coupled receptors (GPCR) have been targets for ~35% of 4 5 approved drugs. However, how Src kinase activity is negatively regulated by GPCRs remains largely elusive. Herein we report that Src is dephosphorylated at Tyr 416 by Dusp18 upon GPR54 activation by 6 7 its natural ligand Kp-10. Mechanically, both active Src and the Dusp18 phosphatase are recruited by 8 GPR54 through the proline/arginine-rich motif (PR motif) in the C terminus, which is dependent on the 9 Gaq signal pathway. As such, Kiss1, Gpr54, Dusp18 knockout mice all exhibit osteoclast 10 hyperactivation and bone loss. Accordingly, Kp-10 abrogated bone loss by suppressing osteoclasts 11 activity in vivo. Therefore, Kiss1/Gpr54 is a promising therapeutic strategy governing bone resorption 12 through Src dephosphorylation by Dusp18.

13

#### 14 INTRODUCTION

15 As we age, bone metabolism and homeostasis shift to favor over-activated osteoclasts, which leads to bone loss, a hallmark of human diseases such as osteoporosis <sup>1,2</sup>. Osteoclasts, which have the 16 17 only capacity to resorb bone, are formed from bone marrow monocytes induced by macrophage colony-18 stimulating factor (M-CSF) and the receptor activator of nuclear factor-kB ligand (RANKL) <sup>3,4</sup>. 19 Mechanically, both M-CSF and RANKL promote actin remodeling and bone resorption of osteoclasts, 20 which mostly depends upon induction of Src kinase activation (phosphorylation at Y416)<sup>5,6</sup>. Src consists 21 of 4 functional regions: Src homology 4 domain (SH4), SH3, SH2, SH1 (catalytic domain) and is activated through auto-phosphorylation at tyrosine 416 upon SH3 ligand binding 7-9. Two main SH3 22 23 domain-binding motifs, proline /arginine-rich motif (PR motif), have been identified: R/KxxPxxP (class I) and PxxPxR/K (class II) (where K is lysine and x is any amino acid) <sup>10-13</sup>. Even though Src is normally 24

present in a broad variety of cell types, genetic knockout of the *Src* gene in mice leads to only one major phenotype - severe osteopetrosis mainly due to impaired osteoclast function. This *in vivo* phenotype supports the notion that the Src kinase plays an essential biological function in osteoclasts 5,14-17. Therefore, inhibition of Src kinase activity has been considered as a useful therapeutic strategy for osteoclast overactivation-mediated bone loss <sup>18-22</sup>.

30 G protein-coupled receptors (GPCRs) are the most important drug targets. It is estimated that ~35 % of marketed drugs act directly on GPCRs  $^{23,24}$ . Src was activated by GPCRs through different 31 ways including direct binding with Src through SH3 binding motif in the intracellular domain <sup>25-28</sup>, 32 indirect phosphorylating Src at Y416 by Gas/i <sup>29</sup> or recruitment of Src via arrestins <sup>30</sup>. However, how 33 34 Src kinase activity is negatively regulated by GPCRs remains largely elusive. GPR54, also named as 35 KiSS1R (KiSS1 receptor), is a member of the GPCR superfamily <sup>31</sup>. Its natural ligands are Kisspeptins including Kp-54, -14, -13, -10 encoded by KiSS1 gene <sup>32</sup>. Biologically, KiSS1/GPR54 signaling in 36 hypothalamic neurons is the gatekeeper of puberty that controls hormones release via the hypothalamic-37 38 pituitary-gonadal axis <sup>33,34</sup>. GPR54 activation by Kisspeptins binding triggered signaling cascade 39 including primary  $G\alpha q/11$ -PLC $\beta$  (phospholipase C  $\beta$ )-PKC (protein kinase C)/Ca<sup>2+</sup> signal pathways and 40 subsequent signaling that is dependent on  $\beta$ -arrestin-1 and  $\beta$ -arrestin-2<sup>35</sup>.

41 There was a PR motif in the C terminus of human GPR54 (GPR54 CT) <sup>36</sup>. Comparing GPR54 42 with its homologous sequences from other species, we identified that this PR motif emerged and was conserved in terrestrial vertebrates. Furthermore, GPR54 recruited active Src and the phosphatase 43 44 Dusp18 through this PR motif upon activation by the ligand Kp-10, which is dependent on Gaq signaling. Finally, Src (Y416) was dephosphorylated by Dusp18 upon Gpr54 activation by Kp-10. Therefore, 45 46 osteoclasts' activity was negatively regulated by Kiss1/Gpr54 at least partially through the dephosphorylation of Src (Y416) by Dusp18. In vivo, both whole-body (Kiss1 -/-, Gpr54 -/-, Dusp18 -/-) 47 and osteoclast conditional knockout (Kiss1 cKO, Gpr54 cKO) mice exhibit bone loss and osteoclast 48

49 hyperactivation. Using a delivery system targeting bone surfaces consisting of six repetitive sequences 50 of aspartate, serine <sup>37</sup>, we developed the bone-targeting Kp-10 ((DSS)\*6-Kp-10) and found that 51 (DSS)\*6-Kp-10 protected bone loss in ovariectomy (OVX)-induced osteoporosis model through 52 blocking osteoclast activity. These results above suggest that Kiss1/Gpr54 plays a bone protection role 53 as a negative osteoclast modulator during bone metabolism and may be a potent therapeutic target for 54 the treatment of osteoclast-associated bone diseases.

55

#### 56 **RESULTS**

# 57 Kiss1/Gpr54 governs osteoclast formation and bone resorption mainly through Src 58 dephosphorylation.

59 Both Kiss1 and Gpr54 were highly expressed during osteoclasts formation by Western blotting 60 (Figure S1 a) and by immunohistochemistry (IHC) using GPR54 antibody in giant cell tumor of bone 61 (GCTB) samples characterized histologically by the presence of mononuclear osteoclast precursors and 62 osteoclast-like giant cells (Figure S1 b). Phosphorylation of Src (Y416) with RANKL treatment 63 dramatically increased when Gpr54 has deleted in bone marrow-derived macrophages (BMMs) 64 compared with MAPKs and NF-kB signal pathways that are the two important signaling branches to 65 control osteoclast differentiation (Figure 1a). Consistently, treatment of Kp-10 effectively reduced the phosphorylation of Src (Y416) in Gpr54-WT osteoclasts, but not in Gpr54 -/- osteoclasts (Figure 1b). 66 67 Furthermore, Src phosphorylation induced both by RANKL and M-CSF decreased when Gpr54 was activated by Kp-10 in WT osteoclasts, but not in *Gpr54<sup>-/-</sup>* osteoclasts (Figure 1c). Taken together, These 68 69 results suggested that Kiss1/Gpr54 likely played a role in the negative regulation of osteoclasts mainly 70 through Src dephosphorylation.

Compared with Gpr54 WT osteoclasts, Gpr54 -/- enhanced osteoclasts formation by TRAP 71 72 staining (Figure 1d) and the statistic of large osteoclasts ( $\geq 10$  nuclei) and small osteoclasts ( $\leq 5$  nuclei) 73 (Figure 1e), actin ring formation by phalloidin staining (Figure 1f) and the statistics of nuclei numbers 74 and area of actin ring (Figure 1g). In addition, osteoclast-mediated bone resorption was also promoted 75 when Gpr54 was deleted by pits assay (Figure 1h) and by statistics of pit depth (Figure 1i). On the 76 contrary, treatment of Kp-10 suppressed osteoclast formation (Figure 1d, 1e) and bone resorption (Figure 1h, 1i) in Gpr54-WT osteoclasts, but not in Gpr54 -/- osteoclasts. Remarkably, Gpr54 activation by Kp-77 78 10 suppressed osteoclast formation in primary cultured human giant cell tumor of bone cells (Figure S1 79 c) and the statistics of osteoclasts (Figure S1 d). Consistently, inhibition of Gpr54 by the antagonist of GPR54, WB599 (2-acylamino-4,6-diphenylpyridines) <sup>38</sup> enhanced osteoclast formation by TRAP staining (Figure S1 e) and the statistic of osteoclasts (Figure S1 f). In keeping with these results, we found that *Kiss1* loss also enhanced osteoclast formation by TRAP staining (Figure S1 g) and the statistic osteoclasts (Figure S1 h). These results indicated that Kiss1/Gpr54 suppressed actin organization in osteoclast, subsequently leading to impaired osteoclast formation, and bone resorption

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#### GPR54 activation by the ligand Kp-10 recruits the Src kinase through the PR motif in GPR54 CT 86 87 By comparing multiple homologs of GPR54, we found that the SH3 binding motif was ubiquitous 88 in the C-terminus of GPR54 in terrestrial vertebrates (Figure 2a). Human GPR54 contains the most 89 redundant SH3-binding sequence including two class I RxxPxxP motifs (336RVCPCAP342 and <sup>350</sup>RPGPSDP<sup>356</sup>) and two class II PxxPxR motifs (<sup>339</sup>PCAPRR<sup>344</sup> and <sup>345</sup>PRRPRR<sup>350</sup>). Furthermore, these 90 91 key proline and arginine residues are conserved in the first half of the peptide indicating that the first 92 half of the PR motif may play an even important function, for example, in their recruiting or binding of 93 SH3 domain-containing proteins. SH3 domains are found in proteins of signaling pathways regulating the cytoskeleton, the Ras protein, the Src kinase and many others <sup>39</sup>. Using proteins microarray assay for 94 95 the screening of SH3 domain interactions, we found that the GPR54 CT interacted with Src most 96 obviously in this experimental setting (Figure 2b). Of note, by surface plasmon resonance (SPR) analysis, 97 we confirmed that GPR54 CT dose-dependently bound with Src, and the binding affinity (kinetic 98 analysis) was calculated to be approximately 2.6 nM (Figure 2c). Then, we co-crystallized the human GPR54 (<sup>336</sup>RVCPCAPRRPRRPRRPGPSDP<sup>356</sup>) peptide with the Src SH3 domain. According to the 99 electric densities, we can model the first half of the peptide (<sup>333</sup>AFRRVSPSAPRR<sup>344</sup>) whereas densities 100 101 for residues 345 to 356 are missing, indicating the latter residues do not bind to the SH3 domain. The GPR54 peptide adopts a class I RxxPxxP motif (<sup>336</sup>RVCPCAP<sup>342</sup>) and parts of class II PxxPxR motifs 102 (<sup>339</sup>PCAPRR<sup>344</sup>) with the key residues R336, P339 and P342 each forms a hydrogen bond with SH3 103

104 domain residues Y90, Y136 and W118, respectively (Figure 2d), The structure characterization of 105 involvement of the N-terminal half of the PR motif is consistent with the above analysis that the N-106 terminal region is conserved among species. Consistently, SPR assay showed that Src bound with the 107 peptide encompassing the PR-motif of human GPR54 (<sup>336</sup>RVCPCAPRRPRRPGPSDP<sup>356</sup>) with 108 binding affinities of 0.7 µM (Figure 2e), 8.3 µM (the left-hand half of PR motif peptide <sup>333</sup>AFRRVCPCAPRR<sup>344</sup>, Figure 2f), but weakly interacted with the right-hand half of PR motif peptide 109 110 ( $^{342}$ PRRPRRPRRPGPSDP $^{356}$ ) with the binding affinity of 498  $\mu$ M (data not show). Furthermore, the 111 C-terminus of mouse Gpr54 (<sup>336</sup>RVCPCCRQRQRRP<sup>348</sup>) contains two putative SH3 binding motifs including RxxPxxR (<sup>336</sup>RVCPCCR<sup>342</sup>) and PxxRxR (<sup>339</sup>PCCRQR<sup>344</sup>) in the PR motif of mouse Gpr54 112 (<sup>336</sup>RVCPCCRQRQRRP<sup>348</sup>) (Figure 2a). By SPR assay, peptides encompassing the PR motif of mouse 113 Gpr54 interacted with Src with the binding affinity of 1 µM (Figure S2 a), 4.8 µM (<sup>336</sup>RVCPCCR<sup>342</sup>, 114 Figure S2 b), 6.3 µM (<sup>339</sup>PCCROR<sup>344</sup>, Figure S2 c). By pull-down assay, the R336A&P339A mutant of 115 116 human GPR54 CT, which damaged the key proline and arginine in the RxxPxxR motif (<sup>336</sup>RVCPCCR<sup>342</sup>) and PxxRxR motif (<sup>339</sup>PCCRQR<sup>344</sup>), dramatically dampened the interaction of Src with Gpr54 CT. 117 118 Furthermore, the  $\triangle 339-344$  (deletion sign:  $\triangle$ ) mutant of GPR54 CT, which destroyed the putative SH3 119 binding motifs, completely abolished the binding of Src with GPR54 CT (Figure 2g).

Consistently, the binding of Src with GPR54 was enhanced by Kp-10 treatment in 293T cells transfected with HA-Src and GPR54-FLag (Figure 2h). Notably, the interaction of endogenous Gpr54 with Src was enhanced after treatment of Kp-10 in pre-osteoclasts (Figure 2i). In addition, immunofluorescence (IF) staining showed that under ectopic expression conditions, Src co-localized on the membrane with GPR54 especially after Kp-10 stimulation in 293T cells (Figure 2j). These findings coherently showed that GPR54 bound with Src through PR motif, suggesting that GPR54 may be an upstream modulator of Src kinase in the osteoclast setting.

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#### 128 The DUSP18 phosphatase was recruited by GPR54 upon activation by the ligand Kp-10 through

#### 129 the PR motif in GPR54 CT

130 GPCRs cannot dephosphorylate kinases directly by themselves. Therefore, we hypothesized that 131 some phosphatases may participate in Src dephosphorylation mediated by Kiss1/Gpr54. In support of 132 this notion, we examined the total phosphatase activity co-immunoprecipitated with the Gpr54 antibody 133 and observed a significant increase after Kp-10 stimulation (Figure 3a). Mass spectrometry analysis of 134 proteins co-immunoprecipitated with GPR54 antibody identified Dusp18 as a protein that strongly 135 interacted with Gpr54 upon activation by Kp-10 (Figure 3b). Furthermore, Co-IP analysis also validated 136 that Dusp18 was recruited by Gpr54 in RAW264.7 cells (Figure 3c). IF staining showed that Dusp18 137 colocalized with Gpr54 in the plasma membrane especially after treatment of Kp-10 in RAW264.7 cells 138 (Figure 3d). Consistently, the binding of Dusp18 with GPR54 was enhanced after Kp-10 stimulation in 139 293T cells transfected with Dusp18-HA and GPR54-Flag (Figure S2 d).

140 By SPR analysis, we observed that the human GPR54 CT dose-dependently bound with Dusp18 141 with the binding affinity of 40 nM (Figure 3e). Furthermore, the peptide of the RxxPxxR motif (<sup>336</sup>RVCPCCR<sup>342</sup>) in mouse Gpr54 CT bound with Dusp18 with the binding affinity of 1.8 µM (Figure 142 3f), 9.2 µM (PxxRxR motif (<sup>339</sup>PCCRQR<sup>344</sup>) peptide (Figure 3g). Furthermore, when we introduced 143 point mutations R336A&P339A, which damaged the RxxPxxP motif (<sup>336</sup>RVSPSAP<sup>342</sup>) and the PxxPxR 144 motif (<sup>339</sup>PSAPRR<sup>344</sup>), obviously dampened the interaction of Dusp18 with human GPR54 CT, and 145 146  $\triangle$ 339-344 mutant of human GPR54 CT, which destroyed the two SH3 binding motifs in the left hand, abolished the binding of DUSP18 with human GPR54 CT (Figure 3h). Therefore, GPR54 interacted with 147 148 Dusp18 also through the left-hand side of the PR motif in GPR54 CT. Of note, by competitive pull-down 149 assay, we found that DUSP18 competed with Src for binding to the C terminus of human GPR54 (Figure 4i). Hence, these results coherently supported that the C-terminus of GPR54 recruited both Dusp18 and 150 151 Src through PR motif and suggested that Dusp18 may play a key role in the process of Src

152 dephosphorylation mediated by Kiss1/Gpr54.

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#### 154 Src was dephosphorylated by DUSP18 when GPR54 was activated by its ligand Kp-10 155 By SPR analysis, we found that DUSP18 interacted with Src with a binding affinity of 5.9 nM 156 (Figure 4a). Interestingly, in contrast to WT-DUSP18, a mutation in the catalytic center of DUSP18 157 (C104S) eliminated the interaction with Src by Co-IP (Figure 4b, and S2 e). Bacterially expressed, 158 purified WT-DUSP18 potently reduced the phosphorylation of Src (Y416) purified from Sf9 insect cells 159 in vitro, but the enzymatically dead DUSP18 (C104S) mutant had no activity (Figure 4c). IF staining 160 showed that Dusp18 colocalized with Gpr54 in the plasma membrane especially after treatment of Kp-161 10 in RAW264.7 cells (Figure 4d). Notably, the formation of the heterotrimer including DUSP18, Src, and GPR54, was enhanced after Kp-162 163 10 stimulation in 293T cells transfected with HA-Src, GPR54-Myc, and DUSP18-Flag, but not when the 164 catalytic center of DUSP18 was dead (Figure 4e). Consistently, the interaction of Dusp18 with Src was enhanced after Kp-10 stimulation in pre-osteoclasts using Co-IP analysis (Figure 4f, and S2 f). In 165 166 keeping with it, the phosphorylation of Src (Y416) was enhanced when *Dusp18* was deleted in RAW264.7 cells (Figure 4g). Furthermore, Kp-10 blocked Src phosphorylation in WT pre-osteoclasts 167 168 but not in *Dusp18* loss pre-osteoclasts (Figure 4h). Therefore, bone resorption was suppressed in *Dusp18* WT osteoclasts but not in Dusp18 -/- osteoclasts by pit formation assay (Figure 4i) and quantitation of 169 170 the depth of pit (Figure 4i). These findings supported the notion that Dusp18 is a phosphatase of Src in 171 this experimental setting, and Src was dephosphorylated through Dusp18 when Gpr54 was activated by 172 Kp-10. Therefore, Kiss1/Gpr54 signaling suppresses osteoclast formation and bone resorption partially 173 through Src dephosphorylation by its upstream phosphatase Dusp18.

174

175 The formation of the complex (active Src, Dusp18, and GPR54) induced by Kp-10 was dependent

#### 176 **on Gαq signaling**

Next, we intend to explore whether Src dephosphorylation mediated by GPR54 was dependent on 177 178  $\beta$ -arrestin-1/2 or not. Our results showed that the phosphorylation of Src can still be suppressed after Kp-179 10 stimulation in Arrb1 and Arrb2 double-knockout MEFs (Figure S2 g), and the interaction of Gpr54 180 with Src was also enhanced in Arrb1 and Arrb2 double-knockout MEFs (Figure S2 h). In addition, 181 Dusp18 did not bind with β-arrestin-1 or β-arrestin-2 (Figure S2 i). Moreover, Kp-10 still blocked 182 osteoclasts formation by TRAP staining ether in Arrb1 -/- BMMs (Figure S2 j) or in Arrb2 -/- BMMs (Figure S2 l), and by the statistics of osteoclasts in Arrb1 -/- BMMs (Figure S2 k) or in Arrb2 -/- BMMs 183 184 (Figure S2 m). Therefore, the results above suggested that Src dephosphorylation upon GPR54 activation 185 by Kp-10 was not dependent on  $\beta$ -arrestin-1 and  $\beta$ -arrestin-2. 186 Interestingly, Gpr54 activation by Kp-10 upregulated the mRNA expression of Dusp18 in a short 187 time as revealed by real-time RT-PCR experiments (Figure S3 a, Figure S3 b, Figure 5 a), but not in Gpr54 loss BMMs (Figure S3 b). The protein level of Dusp18 was induced by Kp-10 as revealed by 188

189 western blot experiments in Raw264.7 (Figure S3 c). We screened  $G\alpha q/11$  downstream signals using 190 inhibitors including PLC inhibitor (U73122), PKC inhibitor (Staurosporine), ERK inhibitor 191 (LY3214996), and P38 inhibitor (SB203580) that may take part in the regulation of Dusp18 expression. 192 Notably, PKC or ERK inhibitor decreased the expression of Dusp18 by real-time RT-PCR experiments 193 (Figure 5a). These results supported the notion that Kiss1/Gpr54 upregulates Dusp18 expression at least 194 partially through the Gaq-PKC-ERK-dependent signal pathway, but the underlying molecular 195 mechanism requires additional in-depth studies in the future. Using different inhibitors of  $G\alpha q/11$ dependent pathway of GPR54 including Ca<sup>2+</sup>/CaMKII inhibitor (KN-93) and Src family inhibitor 196 197 (Saracatinib), we tested the interaction of GPR54 with Src in 293T cells transfected with HA-Src and 198 GPR54-Flag (Figure 5b), the interaction of GPR54 with DUSP18 in 293T cells transfected with 199 DUSP18-HA and GPR54-Flag (Figure 5c) and the interaction of Src with DUSP18 in 293T cells 200 transfected with HA-Src, DUSP18-Flag, and GPR54-myc (Figure 5d). Our results showed that all the 201 above interactions were abolished by Src family kinase inhibitor (Saracatinib). Therefore, only 202 phosphorylated Src is involved in the complex formation induced by Kp-10. Furthermore, in Gaq loss 203 RAW264.7 cells, the interaction of Dusp18 with Src was dramatically decreased (Figure 5e). Consistently, Kp-10 inhibited the phosphorylation of Src in WT RAW264.7 cells, but not in *Gag* loss 204 205 RAW264.7 cells (Figure 5f). Taken together, our finding suggested that Dusp18 was upregulated in a 206 short time by Kiss1/Gpr54, and both active Src and the Dusp18 phosphatase are recruited to GPR54 207 through PR motif in its C terminus upon activation by Kp-10, which are dependent on Gaq signaling 208 (Figure 5g).

209

#### 210 Mice deficient in *Kiss1*, *Gpr54*, or *Dusp18* exhibited bone loss and osteoclasts hyperactivation

211 To test our hypothesis that under physiological conditions, the Kiss1/Gpr54 signaling axis 212 protects bones through Dusp18 by suppressing osteoclast activity, we examined bone mass and 213 osteoclast activity in osteoclast conditional knockout mice of Kiss1 (Kiss1 cKO), and Gpr54 (Gpr54 cKO). as well as whole-body knockout mice of Kiss1 (Kiss1-/-), Gpr54 (Gpr54-/-), and Dusp18 (Dusp18 214 -/-). The loss of bone mass happened in *Gpr54* cKO mice (Figure 6a, 6b) and *Gpr54* -/- mice (Figure 3S 215 d, 3S e), Kiss1 cKO mice (Figure 6c, 6d), and Kiss1 -/- mice (Figure 3S f, 3S g), Dusp18 -/- mice (Figure 216 217 6e, 6f) by micro-computed tomography (micro-CT) analysis and the statistical analysis of parameters of 218 trabecular bone separately. The targeting strategy of Gpr54 cKO (Figure 3S h) and Kiss1 cKO (Figure 219 3S i) was shown. Furthermore, osteoclasts were hyperactivated in femurs of Gpr54 cKO mice (Figure 6g, 6h), femurs of Kiss1 cKO mice (Figure 6i, 6j), calvaria of Kiss1 -/- mice, and Gpr54 -/- mice (Figure 220 S3 j, S3 k), femurs of *Dusp18<sup>-/-</sup> mice* (Figure 6k, 6l) by TRAP staining and the statistical analysis of 221 222 parameters of osteoclast. Taken together, our data provide strong evidence to support a bone-protective 223 role for Kiss1/Gpr54 signaling in vivo.

224

#### 225 Kp-10 ameliorated OVX-induced bone loss

226 Our data above showed that Kp-10/Gpr54 negatively regulated osteoclast activity through Src 227 dephosphorylation by Dusp18. Therefore, we intended to examine whether Kp-10 could ameliorate bone 228 loss in vivo. In ovariectomized mice, after intravenous injection of Kp-10 (1, 10, 50 nmol/kg) and bone 229 targeting Kp-10 ((DSS)\*6-Kp-10) (1, 10, 50 nmol/kg) twice a week for two months, we observed that 230 mice with 1 or 10 nmol/kg (DSS)\*6-Kp-10 showed better bone protection effect than 1 or 10 nmol/kg 231 Kp-10 correspondingly by micro-CT analysis (Figure S4 a), Von Kossa staining (Figure S4 b) and 232 quantitative statistical analysis of parameters of trabecular bone (Figure S4 c). Furthermore, 50 nmol/kg 233 (DSS)\*6-Kp-10 blocked OVX-induced bone loss by micro-CT analysis (Figure 7a) and statistical 234 analysis of parameters of trabecular bone (Figure 7b). Consistent with these results, 50 nmol/kg 235 (DSS)\*6-Kp-10 treatment suppressed OVX-induced osteoclast activation by TRAP staining (Figure 7c) 236 and the measurement of osteoclast parameters (Figure 7d). Thus, these data coherently suggested that 237 the Kiss1/Gpr54 signaling axis played a bone protective role both in vitro and in vivo through blocking 238 osteoclastic bone resorption.

239

#### 240 **DISCUSSION**

241 Src was activated by GPCRs through different ways including direct interaction via SH3 binding motif in the intracellular domain <sup>25-28</sup>, or indirect activation of Src via and arrestins <sup>30</sup> or Gas/i <sup>29</sup>. Gas 242 243 and Gai did stimulate autophosphorylation of Src (Tyr416) directly, but Gaq, Ga12, and GBy can not. 244 Our results revealed a Gog dependent mechanism of Src inhibition by GPCR. Both active Src and 245 Dusp18 phosphatase were recruited by the PR motif in Gpr54 CT after treatment with Kp-10, which is 246 dependent on Gaq signaling. However, whether and how Gaq regulates the complex formation (active 247 Src, Dusp18, Gpr54) is still unclear. The particularity mechanism of GPR54 for its inhibitory function 248 remains an open question and warrants future in-depth investigation.

249 Kiss1/Gpr54 signaling in hypothalamus neurons is the switch of puberty through the 250 hypothalamic-pituitary-gonadal axis. Gonadotropin-releasing hormone (GnRH) was stimulated by 251 Kiss1/Gpr54 and then act on the anterior pituitary triggering the release of luteinizing hormone (LH), 252 and follicle-stimulating hormone (FSH). In addition, FSH and LH prompt the ovaries to begin producing 253 the hormone estrogen and work together to get the testes to begin producing testosterone <sup>31,32</sup>. Both estrogen and androgen have been shown to play a role in bone protection <sup>45-47</sup>. Our results showed that 254 255 Kiss1/Gpr54 negatively regulated osteoclast formation and bone resorption directly. However, whether 256 Kiss1/Gpr54 showed bone protection through modulating osteoblast needs to be explored in the future. Osteoclast-specific knockout mice (Kiss1 cKO, Gpr54 cKO) exhibit osteoclast hyperactivation and bone 257 258 loss, which indicated that osteoclastic Kiss1 and Gpr54 directly play an important role in bone protection. 259 Despite that, the role of Kp-10 from the hypothalamus in bone metabolism is likely to be important and 260 should be addressed in the future. In conclusion, Kiss1/Gpr54 prevented bone loss through Src 261 dephosphorylation by Dusp18 in osteoclasts. Our findings provide an insight into a new drug target of 262 osteoporosis and may lead to the development of a novel therapeutic strategy for osteoclast-related 263 disease

#### **FIGURE LEGENDS**

# Figure 1. Kiss1/Gpr54 governs osteoclasts formation and bone resorption mainly through Src dephosphorylation.

- a IB analysis of WCL derived from BMMs isolated from eight-week-old wild-type (WT) and *Gpr54* -/- mice. BMMs were starved in serum-free α-MEM for 4 hours and treated with 100 ng/mL RANKL for another 30 minutes. Blots were probed with antibodies as indicated.
- b IB analysis of WCL derived from BMMs isolated from eight-week-old WT and *Gpr54* -/- mice. BMMs were starved in serum-free α-MEM and the presence of indicated dose of Kp-10 for 20 minutes. IB analysis was carried out with antibodies as indicated.
- c IB analysis of WCL derived from BMMs isolated from eight-week-old WT and *Gpr54* -/- mice. BMMs were starved in serum-free α-MEM and treated with indicated doses of Kp-10 for 20 minutes and followed by the addition of 50 ng/mL M-CSF, 100 ng/mL RANKL for another 30 minutes. IB analysis was carried out with antibodies as indicated.
- d BMMs isolated from eight-week-old WT and *Gpr54<sup>-/-</sup>* mice were stimulated with M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 5-7 days. Representative TRAP staining images (with three biological replicates) are shown. Scale bar, 250 μm.
- e The number of osteoclasts from Figure 2D was counted. Mean ± SEM; \*\*\*\* P<0.0001; \*\* 0.05<P<</li>
  0.01; ns, not significant,.
- f BMMs isolated from eight-week-old WT and *Gpr54<sup>-/-</sup>* mice were stimulated with M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 5-7 days. Phalloidin staining of F-actin ring structures in WT and *Gpr54*<sup>-/-</sup> osteoclasts is shown. Scale bar, 250 μm.
- **g** The number of nuclei per actin ring and the area per actin ring from Figure 2F were counted. Mean  $\pm$  SEM; \*\*\* P< 0.001.
- **h** BMMs isolated from eight-week-old WT and *Gpr54* <sup>-/-</sup> mice were seeded on bone slices and stimulated with M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 5-7 days. Pits were scanned by

confocal microscopy (x, y, and z section). Scale bar, 125  $\mu$ m.

Pit depth from Figure 2H was quantified by confocal microscopy. Error bars are standard errors of the mean (SEM). Mean ± SEM; \*\*\*\* P< 0.0001; ns, not significant.</li>

#### Figure 2. GPR54 recruited Src through the PR motif in GPR54 CT

- **a** Sequence analysis of the proline and arginine-rich motif in multiple species of GPR54 CT.
- b SH3 domain protein-binding array using the TranSignal<sup>™</sup> SH3 Domain Array kit. GST-GPR54 CT was used as the probe (GST protein as the control). Spots with stronger intensities indicate higher binding affinity of GST-GPR54 CT with various SH3 Domains. The principal Src binding region is circled in red evident (1 Src; 2 the dilution of Src in half).
- c SPR binding-affinity measurement of Src and GPR54 CT. Src and GPR54 CT binding affinity is 2.6 nM (kinetic analysis).
- d Crystallization structure analysis of the SH2-SH3 domain of Src and the human GPR54 PR motif (<sup>336</sup>RVCPCAPRRPRRPRPGPSDP<sup>356</sup>). Src (G85-V247)-GPR54 (333A-357A with C338S and C340S mutation) recombinant fusion protein was purified from *E. coli*. Crystals were generated from purified recombinant proteins, subjected to X-ray crystallography, and the resulting model is shown. GPR54 CT bound with the SH3 domain of Src mainly through the motif <sup>333</sup>AFRRVCPCAPRR<sup>344</sup>.
- e SPR binding-affinity measurement of Src and peptide of human GPR54 PR motif
  (<sup>336</sup>RVCPCAPRRPRRPRRPGPSDP<sup>356</sup>) with a KD value (0.7 μM).
- f SPR binding-affinity measurement of Src and peptide of human GPR54 (333AFRRVCPCAPRR344)with a KD value (8.3 μM).
- g Immunoblots (IB) of total samples and GST pull-downs using GST proteins purified from *E. coli;* GST proteins were incubated separately with the whole-cell lysis (WCL) of 293T cells transfected with HA-Src.
- h IB analysis of WCL and anti-GPR54 immunoprecipitants (IP) assays derived from 293T cells

transfected with GPR54-flag and HA-Src after Kp-10 induced for 20 minutes.

- IB analysis of WCL and anti-GPR54 IP assays derived from RAW264.7 cells induced by Kp-10 for 20 minutes.
- j 293T cells transfected with GPR54-flag and HA-Src for 12 hours, then treated with 10 nM Kp-10 for 20 minutes. IF staining was carried out using indicated antibodies. Representative images are shown. Scale bar, 10 μm.

# Figure 3. DUSP18 phosphatase was recruited by GPR54 after Kp-10 stimulation through the PR motif

- a Anti-Gpr54 IP was performed on WCL derived from RAW264.7 cells treated with or indicated dose of Kp-10 for 20 minutes. Beads were analyzed by phosphatase activity assay. Mean ± SEM; \* P< 0.05; \*\* P< 0.01.</li>
- Anti-Gpr54 IP was performed on WCL derived from RAW264.7 cells treated with or without Kp-10 for 20 minutes and then analyzed by mass spectrometry assay.
- c IB analysis of WCL and anti-GPR54 IP of cell lysates from RAW264.7 cells treated with Kp-10 for 20 minutes.
- d IF staining was carried out using indicated antibodies on RAW264.7 cells treated with or without Kp 10 for 20 minutes. Scale bar, 10 μm.
- e SPR binding analysis of DUSP18 and GPR54 CT. DUSP18 interacted with human GPR54 CT with the binding affinity measured at 40 nM.
- f SPR binding analysis of DUSP18 and mouse Gpr54 ( $^{336}$ RVCPCCR $^{342}$ ) peptide. The binding affinity was measured at 1.8  $\mu$ M.
- g SPR binding analysis of DUSP18 and mouse Gpr54 ( $^{339}PCCRQR^{344}$ ) peptide. The binding affinity was measured at 9.2  $\mu$ M.
- **h** IB of total samples and GST pull-downs using GST proteins purified from *E. coli;* GST proteins were

incubated separately with the WCL of 293T cells transfected with DUSP18-HA.

IB analysis of total samples and GST pull-downs using His-SRC proteins purified from Sf9 cells,
 His-DUSP18, and GST proteins purified from *E. coli*.

#### Figure 4. Src was dephosphorylated by DUSP18 when GPR54 was activated by Kp-10

- a SPR binding analysis of DUSP18 and Src. The binding affinity of DUSP18 and Src was measured at 5.9 nM.
- B analysis of WCL and anti-Flag IP derived from 293T cells transfected with HA-Src and either
  Dusp18-Flag or Dusp18 (C104S)-Flag constructs.
- **c** Dusp18 dephosphorylated Src at Y416 *in vitro*. His-Dusp18 and His-Dusp18 (C103S) proteins purified from *E. coli* and SRC proteins purified from Sf9 insect cells were incubated in the phosphatase buffer at 30°C for 30 minutes. IB analysis of the protein phosphatase reaction products with antibodies as indicated.
- **d** IF staining of RAW264.7 cells treated with 10 nM Kp-10 for 20 minutes was carried out using the indicated antibodies.
- e IB analysis of WCL and anti-Flag IP derived from 293T cells transfected with HA-Src, GPR54-myc, and either Dusp18-Flag or Dusp18 (C104S)-Flag constructs.
- f IB analysis of WCL and anti-Src IP derived from RAW264.7 cells treated with or without Kp-10 for 20 minutes.
- g IB analysis of WCL a derived from *Dusp18<sup>-/-</sup>* RAW264.7 cells treated with or without Kp-10 for 20 minutes.
- h BMMs isolated from eight-week-old WT and Dusp18<sup>-/-</sup> mice were cultured in the absence of serum and with indicated doses of Kp-10 for 20 minutes, and then treated with 100 ng/ml RANKL for 30 minutes. Cells were lysed and blots probed with indicated antibodies.
- i BMMs isolated from eight-week-old WT and Dusp18<sup>-/-</sup> mice were seeded on bone slices and

stimulated with M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 5-7 days. Pits were scanned by confocal microscopy (XY and z section). Scale bars,  $125 \mu m$ .

**j** Pit depths were measured by confocal microscopy. Mean  $\pm$  SEM; \*\* P< 0.01; \*\*\* P< 0.01; ns, not significant.

# Figure 5. The formation of the complex (active Src, Dusp18, and GPR54) induced by Kp-10 was partially dependent on Gaq signaling

- RAW264.7 cells were treated with indicated inhibitors including PLC inhibitor (U73122, 10 μM),
  PKC inhibitor (Staurosporine, 0.25 μM), ERK inhibitor (LY3214996, 10 μM), P38 inhibitor (SB203580, 10 μM) for 1 hours and then added with 10 nM Kp-10 for 30 minutes. Total RNAs were extracted for quantitative real-time RT-PCR analyses for relative RNA levels of Dusp18.
- b IB analysis of WCL and anti-Flag IP derived from 293T cells transfected with GPR54-flag and Src-HA constructs for 24 hours. Cells were pre-treated with indicated inhibitors including PLC inhibitor (U73122, 10 μM), PKC inhibitor (Staurosporine, 0.5 μM), ERK inhibitor (LY3214996, 10 μM), Ca<sup>2+</sup>/CaMKII inhibitor (KN-93, 10 μM), Src family kinase (Saracatinib, 10 μM) for 1 hour and then added with 10 nM Kp-10 for 20 minutes.
- c IB analysis of WCL and anti-Flag IP derived from 293T cells transfected with GPR54-flag and Dusp18-HA constructs for 24 hours. Cells were pre-treated with indicated inhibitors including PLC inhibitor (U73122, 10 μM), PKC inhibitor (Staurosporine, 0.5 μM), ERK inhibitor (LY3214996, 10 μM), Ca<sup>2+</sup>/CaMKII inhibitor (KN-93, 10 μM), Src family kinase inhibitor (Saracatinib, 10 μM) for 1 hour and then added with 10 nM Kp-10 for 20 minutes.
- d IB analysis of WCL and anti-Flag IP derived from 293T cells transfected with GPR54-myc, Src-HA, and Dusp18-flag constructs for 24 hours. Cells were pre-treated with indicated inhibitors including PLC inhibitor (U73122, 10  $\mu$ M), PKC inhibitor (Staurosporine, 0.5  $\mu$ M), ERK inhibitor (LY3214996, 10  $\mu$ M), Ca<sup>2+</sup>/CaMKII inhibitor (KN-93, 10  $\mu$ M), Src family kinase (Saracatinib, 10

 $\mu$ M) for 1 hour and then added with 10 nM Kp-10 for 20 minutes.

- e IB analysis of WCL and anti-Flag Src from  $G\alpha q$  knockout RAW264.7 cells treated with or without Kp-10 for 20 minutes.
- **f** IB analysis of WCL derived from WT RAW264.7 cells and *Gαq* knockout RAW264.7 cells treated with or without Kp-10 for 20 minutes.
- **g** The working model of Kiss1/Gpr54 mediated Src dephosphorylation.

Figure 6. Osteoclast conditional knockout mice of *Gpr54* mice (Gpr54 cKO) and *Kiss1* mice (*Kiss1* cKO), whole-body knockout mice of Dusp18 (*Dusp18* -<sup>/-</sup>) exhibited bone loss and osteoclasts hyperactivation.

- (a, c, e) Representative micro-CT images of (a) 12-week-old WT and *Gpr54* cKO mice (n = 6 per group),
  - (b) 12-week-old WT and *Kiss1* cKO mice (n=6 per group) and (c) 8-week-old WT and Dusp18
    -/- mice (n=6 per group). Scal bar, 500 μm.
- (b, d, f) Bone parameters of WT and *Gpr54* cKO mice (b, n=6 per group), WT and *Kiss1* cKO mice (d, n=6 per group), WT and Dusp18 <sup>-/-</sup> mice (f, n=6 per group). 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  $\pm$  s.d.; \*\*\* *P* < 0.000; \*\*0.01< *P* < 0.001; \*0.05< *P* < 0.01; ns, not significant. unpaired two-tailed Student's t test.
- (g, i, k) Representative TRAP staining images of 12-week-old WT and *Gpr54* cKO mice (g, Scale bar, 20 μm), 12-week-old WT and *Kiss1* cKO mice (i, Scale bar, 500 μm) and 8-week-old WT and Dusp18 <sup>-/-</sup> mice (k, Scale bar, 50 μm).
- (h, j, l) Osteoclast parameters from 12-week-old WT and *Gpr54* cKO mice (h, n=6 per group), 12-week-old WT and *Kiss1* cKO mice (j, n=6 per group), 8-week-old WT and Dusp18<sup>-/-</sup> mice (l, n=6 per group). mean  $\pm$  s.d. ; \*\*\* *P* < 0.000; \*\*0.01< *P* < 0.001; \*0.05< *P* < 0.01; ns, not significant. unpaired two-tailed Student's t test.

#### Figure 7. Kp-10 ameliorated OVX-induced bone loss.

- a Representative micro-CT images of control mice treated with vehicle (Sham, n=6), ovariectomized mice (OVX, n=6), and OVX mice treated with (DSS)\*6-Kp-10 (OVX+(DSS)\*6-Kp-10, n=8). Scale bar, 500 μm.
- **b** Parameter analysis of trabecular bone from Figure 7a. BMD, bone mineral density; BV/TV, bone volume as a fraction of total bone volume. mean  $\pm$  s.d. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, unpaired two-tailed Student's T test. (n=6 in Sham or OVX group, n=8 in OVX+(DSS)\*6-Kp-10 group).
- **c** Representative TRAP staining images of femurs from Figure 7a.
- d Histomorphometry analysis of the femurs from Figure 7a. Oc.S/BS, osteoclast surface per bone surface; N.Oc/B.Pm, osteoclast bone surface density; ES/BS, eroded surface per bone surface. mean  $\pm$  s.d. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, unpaired two-tailed Student's t test.

#### **METHODS**

**Cell Culture, transfection.** 293T and RAW264.7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 mg/ml streptomycin. Sf9 cells were grown in GIBCO® insect culture media supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 mg/ml streptomycin. Arrb1 and Arrb2 double knockout MEFs were a gift from Dr. Robert J. Lefkowitz (Duke University). All cell lines were tested to be negative for mycoplasma contamination.

**Kp-10 modification.** Modified Kp-10 (D-Tyr–Asn-(D-Trp)-Asn-Ser-Phe-(azaGly)-Leu-Arg (Me)-Phe-NH2) was designed as previously described <sup>40</sup>, which showed not only high metabolic stability but also excellent GPR54 agonistic activity. Bone targeting Kp-10 (Asp-Ser-Ser-Asp-Ser-Ser-Asp-Ser-Ser-Asp-Ser-Ser-Asp-Ser-Ser-Asp-Ser-Ser-D-Tyr–Asn-(D-Trp)-Asn-Ser-Phe-(azaGly)-Leu-Arg (Me)-Phe-NH2) was designed using six repetitive sequences of aspartate, serine, and serine, which is a bone surfacetargeting delivery system as previously described <sup>37</sup>

**Site-directed Mutagenesis.** Site-directed mutagenesis to generate various Dusp18 and C-terminus of GPR54 mutants was performed using the QuikChange XL Site-Directed Mutagenesis Kit (Agilent) according to the manufacturer's instructions.

SH3 domain protein-binding array. SH3 domain protein-binding array using TranSignal<sup>™</sup> SH3 Domain Array kit (Cat. #MA3010). The kit was purchased from Panomics, probed using GST-GPR54 CT. Purified different SH3 domain-containing proteins including the Src kinase family were spotted in duplicate on membrane filter and tested for their binding to GST-GPR54 CT. Briefly, membrane filters were incubated with 30 mg/ml GST-GPR54 CT protein overnight at 4 °C. After washing the membrane, the GST antibody was incubated overnight at 4 °C. Protein binding was visualized by incubation with an HRP-conjugated antibody. Spots with stronger intensities indicated a higher binding affinity of GST-GPR54 CT with the SH3 domain-containing protein (s).

Surface plasmon resonance (SPR). SPR was determined using a Biacore T200 instrument (GE). SRC or

DUSP18 protein was immobilized on the sensor chip (CM5) using the amine-coupling method according to standard protocols. SRC protein or Dusp18 was diluted in 10 mM acetate buffer, pH 5.5. Immobilization was performed according to the manufacturer's recommendations. The kinetics and affinity assay were examined at 25 °C at a flow rate of 30 µl/minute using PBS buffer. Diluted DUSP18, Src, and GPR54 CT protein were kept at 25 °C and placed into the rack tray before injection. The KD values were calculated with the kinetics and affinity analysis option of Biacore T200 evaluation software. The interaction of GPR54 CT, peptides of PR motifs with Src or DUSP18, and the interaction of DUSP18 and Src were analyzed respectively by regeneration with pH 2.0 Gly-HCl buffer.

Protein expression and purification. The C terminus of human GPR54 (329H-398L) and mouse Gpr54 (331R-396L) cDNAs were subcloned into a pGEX-4T2 vector with an N-GST tag and the related mutants were performed using the Ouick-change XL Site-Directed Mutagenesis Kit (Agilent). The protein was expressed in E. coli C41(Rosetta). Cells were harvested after being induced by 1 mM isopropyl β-Dthiogalactopyranoside (IPTG) for 4 h at 37 °C. The cell pellets were lysed in lysis buffer (20 mM Tris 8.0, 500 mM NaCl, 1% Triton X-100, 1 mM DTT, protease inhibitors) on ice. The supernatant after centrifugation at 14000 rpm for 30 min was filtered and applied to the GST Sep glutathione agarose column. The beads were washed with 10 column volumes of wash buffer (20 mM Tris 8.0, 500 mM NaCl. 1 mM DTT, protease inhibitors) and then eluted with 5 column volumes of elution buffer (20 mM Tris 8.0, 500 mM NaCl, 20 mM GSH, 1 mM DTT, protease inhibitors). The proteins were then purified by size exclusion using a Superdex 200 Increase 10/300 GL column (GE Healthcare) with SEC buffer (1xPBS, pH 7.4, 3mM DTT). The purified proteins were then concentrated to ~5 mg/ml using a 30 kDa molecular mass cut-off centrifuge concentrator. For purification of full-length WT human DUSP18 and DUSP18 (C104S) proteins, DUSP18 was cloned into pMCSG7 vector with an N-terminal 6xHis tag, and DUSP18 (C104S) mutant was made using the Ouick-change XL Site-Directed Mutagenesis Kit (Agilent). The proteins were expressed in BL21 (DE3) with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 16 h at 16 °C. The collected cells were disrupted by sonication in lysis buffer (20 mM Tris 8.0, 500 mM NaCl, 1% Triton X-100, 1 mM DTT, protease inhibitors) on ice. The lysate was then centrifuged and subjected to nickelaffinity chromatography. The beads were washed with 10 column volumes of wash buffer (20 mM Tris 8.0, 500 mM NaCl, 20 mM imidazole, protease inhibitors) and eluted with 5 column volumes of elution buffer (20 mM Tris 8.0, 500 mM NaCl, 300 mM imidazole, protease inhibitors). The proteins were then purified by size-exclusion chromatography using a Superdex 200 Increase 10/300 GL column (GE Healthcare) with SEC buffer (1xPBS, pH 7.4, 3 mM DTT). The purified proteins were then concentrated to ~13 mg/ml using a 10 kDa molecular mass cut-off centrifuge concentrator. For purification of Src (G85-L536) protein, cDNAs of human Src were subcloned into pFastBac HT B vector with an N-terminal 6xHis tag. The protein was expressed in Sf9 cells (Expression Systems) using the Bac-to-Bac Baculovirus Expression System (Invitrogen) for 48 h. His-tagged Src was purified from Sf9 cell lysis with the Ni–NTA system as above. The purified proteins were then concentrated to  $\sim 5 \text{ mg}/\text{ml}$  using a 30 kDa molecular mass cut-off centrifuge concentrator. For purification of the Src (G85-V247)-GPR54 (333A-357A with C338S, C340S mutation) recombinant fusion protein, the C-terminus of GPR54 (333A-357A with C338S and C340S mutation) was linked to the C terminus of Src SH3-SH2 domain (G85-V247), which was subcloned into pMCSG7 vector with a 6xHis tag and a TEV protease recognition site at the N terminus before the receptor sequence. The proteins were expressed and purified with the Ni–NTA system as above. A PD MiniTrap G-25 column was used to remove imidazole. The protein was then treated overnight with His-tagged TEV protease to remove the N-terminal His tag. His-tagged TEV protease cleaved His-tag and uncleaved protein was removed from the sample by passing the sample over an equilibrated nickel-affinity column chromatography. The receptor was then concentrated to 8-13mg/ml with a 30 kDa molecular mass cut-off centrifuge concentrator (Sartorius Stedim).

**Protein crystallization and structure determination.** For crystallization, the C-terminus of GPR54 (333-356 with mutations C338S and C340S) was inserted into the C-terminus of Src SH3-SH2 domains (G85-V247), this fragment (Src<sup>G85-V247</sup>–GPR54<sup>333-356</sup>) was then subcloned into pMCSG7 vector with a 6xHis tag and a TEV protease recognition site at the N terminus before the chimera sequence. Two cysteines

(C338&C340) in the peptide are mutated to serine to avoid potential oxidation during protein purification. The mutated GPR54 residues, S338 and S340, both do not involve any side-chain interaction with the SH3 domain, except the main chain interaction between 340 and SH3 residue N135. The proteins were expressed and purified with the Ni-NTA system as above. A PD MiniTrap G-25 column was used to remove imidazole. The protein was then treated overnight with His-tagged TEV protease to remove the Nterminal His tag. Uncleaved protein and TEV were removed from the sample by passing the sample over equilibrated nickel-affinity chromatography. The fusion protein was then applied to size exclusion chromatography (Superdex 75) and the homogeneous sample was concentrated to 8-13 mg/ml with a 30 kDa molecular mass cut-off centrifuge concentrator (Sartorius Stedim). The concentrated fusion protein was set up for crystallization using a hanging drop with NT8 (Formulatrix). Diffraction-quality crystals were produced at 18 °C in 0.1 M MES and PEG 20000. Diffraction data for the chimera protein (Src<sup>G85-</sup> <sup>V247</sup>–GPR54<sup>333-356</sup>) were collected at a wavelength of 0.979 Å at beamline BL17U1 at SSRF, and indexed, integrated, and scaled using the automatic XIA2 software package <sup>41</sup>. The structure was solved by the molecular replacement method using SH3 and SH2 domains of Src as a searching model. Refinement was carried out using Phenix <sup>42</sup> and with manual adjustments with Coot <sup>43</sup>. Refinement parameters were summarized in Table 2.

**Immunoblots (IB).** For Gpr54 detection, The collected cells were disrupted by sonication in 1x RIPA buffer (25mM Tris, pH7.5, 150mM NaCl, 0.5% Sodium deoxycholate, 1% Triton-X-100) supplemented with protease inhibitors (Complete Mini, Roche) and phosphatase inhibitors (phosphatase inhibitor cocktail set I and II, Calbiochem). Cell lysates were put on the shaker at 4 °C for 1 hour, then centrifuged at 12000 rpm for 30 min, The supernatants were diluted with 3x loading buffer (6.7% SDS, 33% Glycerol, 300 mM DTT) and incubated at room temperature for 30 minutes without boiling in 100°C before being resolved by SDS-PAGE and immunoblotted with an antibody for Gpr54 (Cell Signaling Technologies, 13776, 1:1,000). For detection of other proteins, cells were lysates in 1x RIPA buffer as above. Cell lysis was put on the shaker at 4 °C for 1 hour, then centrifuged at 12000 rpm for 30 min. The supernatants were diluted at 12000 rpm for 30 min. The supernatants were diluted with an antibody for Gpr54 (Cell Signaling Technologies, 13776, 1:1,000). For detection of other proteins, cells were lysates in 1x RIPA buffer as above. Cell lysis was put on the shaker at 4 °C for 1 hour, then centrifuged at 12000 rpm for 30 min. The supernatants were diluted

with 3x loading buffer, boiled at 100°C for 10 minutes before being resolved by SDS-PAGE, and immunoblotted with indicated antibodies.

**Immunoprecipitation.** Cells were lysed in 1x RIPA buffer supplemented with protease inhibitors and phosphatase inhibitors. Cell lysates were put on the shaker at 4 °C for 1 hour. The protein concentrations of whole-cell lysates were measured using a Beckman Coulter DU-800 spectrophotometer and Bio-Rad protein assay reagent. Equal amounts of whole-cell lysates were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotted with indicated antibodies. For Co-IP analysis, 1,000  $\mu$ g lysates were incubated with the indicated antibody (1-2  $\mu$ g) for 3-4 hours at 4 °C followed by 3-4 hours incubation with Protein A/G sepharose beads (GE Healthcare). The recovered immunocomplexes were washed five times with NETN buffer (20 mM Tris, pH 8.0), 150 mM NaCl, 1 mM EDTA and 0.5% NP-40) before being resolved by SDS-PAGE and immunoblotted with indicated antibodies. For Gpr54 Co-IP analysis, the recovered immunocomplexes were washed five times with NETN buffer and incubated with 1x loading buffer for 30 minutes without boiling at 100 °C.

**Osteoclast differentiation.** For osteoclast differentiation analyses *in vitro*, we isolated bone marrow monocytes (BMMs) from femurs and tibias of 8-week-old WT and *Kiss1*<sup>-/-</sup>, *Gpr54*<sup>-/-</sup> and *Dusp18*<sup>-/-</sup> mice using a 1ml syringe with a 26 G needle. The differentiation experiments were conducted in triplicate. BMMs were seeded into 48-well plates at a concentration of  $1 \times 10^4$  cells per well. Cells were incubated with 50 ng/ml RANKL (R&D, 462-TEC) and 10 ng/ml M-CSF (R&D, 416-ML), and the differentiation medium was changed every other day for 5-7 days. Osteoclasts were fixed and stained using the TRAP staining kit (Sigma-Aldrich, 387A-1KT). For Actin-ring formation assay and pits assay, BMMs ( $5 \times 10^3$  cells/well) were seeded into 96-well plates with a bone slice and incubated with M-CSF (10 ng/ml) and RANKL (50 ng/ml) with or without Kp-10, and the culture medium was changed every other day for 5-7 days. Osteoclasts were fixed with 4% Glutaraldehyde and stained with 1% toluidine blue, and pit depth was examined by laser-scanning confocal microscopy. For primary cultures of giant-cell tumor of bone (GCTB) cells, the use of all patient-derived tumor

specimens was approved by the Institutional Review Board and the research ethics committee of Shanghai Changzheng Hospital, which appeared in the proceedings of the meeting of the Ethics Committee on 18 November 2014. Informed consent was obtained from all tissue donors. The GCTB cells were isolated from tumor samples derived from tumor resections in Shanghai Changzheng Hospital,  $1 \times 10^6$  GCTB cells were seeded in a 24-well plate. Cells were stimulated with indicated doses of Kp-10 for 5–7 days. The medium was changed every 2 days. Osteoclasts were fixed and stained using the TRAP-staining kit (Sigma-Aldrich, 387A-1KT).

*In vitro* phosphatase activity assay. For the total phosphatase activity assay, the Co-IP experiment was carried out using the Gpr54 antibody after Kp-10 treatment of RAW264.7 cells for 20 minutes. Beads were washed with phosphatase assay buffer of the PP2A phosphatase assay kit (Sigma-Aldrich, 17-313) and incubated with p-Nitrophenyl Phosphate (PNPP) (NEB, P0757) for 30 minutes at 37 °C. The assay was performed according to the phosphatase assay kit (Sigma-Aldrich,17-313). For the DUSP18 phosphatase activity assay, His-DUSP18 and His-DUSP18 (C103S) were purified from *E Coli*, and His-Src was purified from Sf9 insect cells. His-DUSP18 and His-DUSP18 (C103S) were incubated with His-Src in 1x phosphatase buffer (20 mM HEPES, 20 mM MgCl2, 0.03% β-mercaptoethanol) <sup>44</sup> for 30 minutes at 30 °C with gentle shaking. The reactions were stopped by the addition of 3× SDS sample buffer followed by boiling for 10 min and subjected to Western blotting using specific antibodies.

**Mass spectrometry analyses.** For mass spectrometry (MS) analysis, anti-Gpr54 IP was performed with the whole-cell lysates derived from two 10-cm dishes of RAW264.7 cells with or without Kp-10 treatment for 20 minutes. The protein complexes after Co-IP were extensively washed with PBS, followed by onbead digestion. The beads were suspended in 8M urea containing 10 mM dithiothreitol and incubated at 37 °C for 30 minutes. The solution was cooled to room temperature and iodoacetamide was added to a final concentration of 15 mM. Beads were incubated at room temperature for 20 minutes in dark. The solution was then diluted to a final urea concentration of 2 M with 100 mM Tris-HCl, followed by in-solution trypsin digestion at 37 °C overnight. The reaction was terminated by adding formic acid (FA) to 5% and centrifuged. Half of the peptide supernatant was used for liquid chromatography coupled with mass spectrometry analysis to identify proteins. Peptides were then resuspended in 2% acetonitrile with 1% FA and loaded onto a micro-capillary column packed in-house with 15 cm of reversed-phase MagicC18 material (5 µm, 200 Å, Michrom Bioresources, Inc., Auburn, CA) using a built-in autosampler in a Proxeon NanoEasy LC 1000 HPLC system (Thermo Scientific, Bremen, Germany). The HPLC separation was performed with a 10–35% acetonitrile (1% FA) gradient over 80 minutes followed by a 35-98% acetonitrile (1% FA) gradient over 20 minutes, then a 25 min isocratic loading at 2% acetonitrile and 1% FA. Mass spectra were acquired on a Q-Exactive mass spectrometer (Thermo Scientific, Bremen, Germany), with each duty cycle containing an MS scan at 70,000 resolution (at 200 m/z) followed by 10 MS/MS scans at 15,000 resolution (at 200 m/z).

The raw mass spectrometry data were searched against the mouse IPI databases (version 3.86, released on June 28, 2012) using the Proteome Discoverer software suite (Thermo Scientific, San Jose, USA) utilizing a label-free quantification feature. A static modification of 57.021 Da on-cysteine residues and differential oxidation (+15.995 Da) on methionine residues was permitted. The protein level false discovery rate was set to be less than 1%. At least one peptide was required for the quantification.

**Immunofluorescence (IF) staining.** For external IF staining, 293T cells were seeded on glass coverslips in a 24-well plate and transfected with Flag-GPR54 and HA-Src for 12 hours. Cells were subsequently incubated with or without Kp-10 for 20 min and fixed in 10% Trichloroacetic acid and incubated with anti-Flag (mouse1:200), anti-HA(rabbit 1:200) at 4 °C overnight. Confocal images were acquired using a Zeiss LSM 880 Upright Laser Scanning Confocal Microscope. For endogenous IF staining, RAW264.7 cells were seeded on glass coverslips in a 24-well plate overnight. Cells were subsequently incubated with or without Kp-10 for 20 minutes. Cells were fixed in 10% TCA and incubated with anti-Gpr54 (Cell Signaling Technologies, 13776, 1:100), anti-Dusp18 (Santa Cruz., sc-376923, 1:50), or anti-Src (Cell Signaling Technologies, 2108, 1:100) overnight. IF staining of Dusp18 was detected by a streptavidin-biotin-fluorochrome (sABC) signal amplification system as described previously <sup>45</sup>. The images were obtained

by laser-scanning confocal inverted microscope (Fluoview FV10i, Olympus).

**Micro-CT analyses.** 3D micro-CT analyses were performed as previously described <sup>46</sup>. We scanned the femur using *in vitro* X-ray microtomography (Skyscan 1272, Bruker micro CT) at a pixel size of 9 µm, and analyzed the results according to the manufacturer's instructions. Region-of-interest (ROI) was defined from 10 to 110 image slices, where the growth plate slice was defined as 0 mm. The contrast was defined from 68–255; 3D analysis, BMD, and 3D models were analyzed using CTAn software (Bruker micro CT). 3D models were adjusted in CT Vox software (Bruker micro CT).

Mice. Generation of Gpr54<sup>-/-</sup> mice (strain C57/BL/6) was previously described <sup>47</sup>. Generation of Gpr54 floxed mice (strain C57/BL/6) and Kiss1 floxed mice (strain C57/BL/6) were performed using the CRISPR/Cas9 system in the C57BL/6J mouse strain from the Animal Center of East China Normal University (ECNU), and described in Figure S6 A (Gpr54 floxed mice) and Figure S6 B (Kiss1 floxed mice). Generation of Kiss1<sup>-/-</sup> mice was performed using the CRISPR/Cas9 system in the C57BL/6J mouse strain from the Animal Center of East China Normal University (ECNU). A sgRNA targeting CCTGGATCCACAGGTACGCAC of the Kiss1 gene was designed and 13 bp (CCTGGATCCACAG) of the *Kiss1* gene was deleted. Genotyping was performed by PCR as described in Table S4. Generation of Dusp18<sup>-/-</sup> mice was performed using the CRISPR/Cas9 system in the C57BL/6J mouse strain from the Animal Center of East China Normal University (ECNU). Two 20-bp sgRNAs targeting TGCGAGAGGCCTCTGATCGAAGG and GCGACGGGCGCATCGACCACAGG were designed and 164 bp between 406 bp and 569 bp of the Dusp18 gene was deleted. Genotyping was performed by PCR as described in Table S4. Generation of Arrb1<sup>-/-</sup> and Arrb2<sup>-/-</sup> mice (strain C57/BL/6) were described previously <sup>48,49</sup>. LvsM-Cre mice (strain C57BL/6) were described in reference <sup>50</sup>. Both male and female mice were used in all experiments, except for the OVX model that only female mice were used. All of the mice were randomly assigned to groups. Maintenance, use, and treatment of all animals were under the accepted standards of the Ethics Committee at ECNU.

Immunohistochemistry (IHC) assay. Femurs were fixed with 4% formalin, decalcified with 0.5 M EDTA

for 1–2 weeks, embedded with paraffin, sectioned, and stained using the TRAP staining kit (Sigma-Aldrich, 387A-1KT). Osteoclast numbers, osteoclast surface area, and eroded surface area were assessed by the OsteoMeasure Analysis System (Osteometrics, Atlanta, GA, USA) according to standard criteria. The third lumbar spine vertebrae (L3) were fixed with 4% formalin, sectioned, and subjected to Von Kossa staining as described previously <sup>51,52</sup>. Histomorphometric measurements were made on sections of lumbar vertebrae (L3) using the OsteoMeasure Analysis System (Osteometrics, Atlanta, GA, USA) according to standard criteria.

**Treatment with Kp-10 and bone targeting Kp-10 ((DSS)\*6-Kp-10) in vivo.** For animal studies in vivo, mice were randomized for weight. For the OVX-induced bone loss model, we sham-operated or ovariectomized 3-month-old C57BL/6 mice to induce osteoporosis for 1 month. Mice were randomly divided into six groups including Sham+vehicle (n=6), OVX+vehicle (n=6), OVX+ Kp-10 (1 nmol/kg, n=6), OVX+Kp-10 (1 nmol/kg, n=6), OVX+(DSS)\*6-Kp-10 (1 nmol/kg, n=6), OVX+Kp-10 (1 nmol/kg, n=6). To analyze the therapeutic effect of 50 nmol/kg (DSS)\*6-Kp-10, ovariectomized mice were randomly divided into three groups including Sham+vehicle (n=6), OVX +vehicle (n=6), OVX+(DSS)\*6-Kp-10 (50 nmol/kg, n=8). Vehicle indicated dose of Kp-10 or (DSS)\*6-Kp-10 were injected into the tail vein twice one week. After two months of treatment, the femurs and the L3 lumbrae were isolated for micro-CT or histomorphometric analysis. 3D micro-CT analyses were performed according to a standard protocol. BMD and bone volume were analyzed by CT-analysis software (CTAn, Bruker micro CT, Kontich, Belgium) and images were reconstituted by CT-volume software (CTvol, skyscan, CTAn, Bruker micro CT, Kontich, Belgium).

**Statistical analyses.** Data are represented as mean  $\pm$  s.d. The statistical significance of differential values between experiments and controls was calculated by GraphPad Prism V7 using the homoscedastic two-tailed student's T-test. Significance was considered to be *P* < 0.05. Results are representative examples of more than two independent experiments. Investigators were not blinded during animal experiments.

#### Data availability

Full scans of the gels and blots are available in Supplementary Fig. 8. All relevant data are available from the corresponding author.

#### AUTHOR CONTRIBUTIONS

Z.L. performed most of the experiments with assistance from X.Y., J.J., Z.W., Z.W., S.X., B.R., W.L., G.M., S.H., P.W., M.Q., H.W., J.Z., Y.C., H.Z., Y.C., and Z.L., J.X. designed the experiments, supervised the study and wrote the manuscript with help from S.S., G.L., H.I., W.W., G.S., J.L., M.L.. All authors commented on the manuscript.

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# Figures



# Figure 1

Kiss1/Gpr54 governs osteoclasts formation and bone resorption mainly through Src dephosphorylation. a IB analysis of WCL derived from BMMs isolated from eight-week-old wild-type (WT) and Gpr54 -/- mice. BMMs were starved in serum-free  $\alpha$ -MEM for 4 hours and treated with 100 ng/mL RANKL for another 30 minutes. Blots were probed with antibodies as indicated. b IB analysis of WCL derived from BMMs isolated from eight-week-old WT and Gpr54 -/- mice. BMMs were starved in serum-free a-MEM and the presence of indicated dose of Kp-10 for 20 minutes. IB analysis was carried out with antibodies as indicated. c IB analysis of WCL derived from BMMs isolated from eight-week-old WT and Gpr54 -/- mice. BMMs were starved in serum-free a-MEM and treated with indicated doses of Kp-10 for 20 minutes and followed by the addition of 50 ng/mL M-CSF, 100 ng/mL RANKL for another 30 minutes. IB analysis was carried out with antibodies as indicated. d BMMs isolated from eight-week-old WT and Gpr54 -/- mice were stimulated with M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 5-7 days. Representative TRAP staining images (with three biological replicates) are shown. Scale bar, 250 µm. e The number of osteoclasts from Figure 2D was counted. Mean ± SEM; \*\*\*\* P< 0.0001; \*\* 0.05<P< 0.01; ns, not significant, f BMMs isolated from eight-week-old WT and Gpr54 -/- mice were stimulated with M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 5-7 days. Phalloidin staining of F-actin ring structures in WT and Gpr54 -/- osteoclasts is shown. Scale bar, 250 µm. g The number of nuclei per actin ring and the area per actin ring from Figure 2F were counted. Mean ± SEM; \*\*\* P< 0.001. h BMMs isolated from eight-week-old WT and Gpr54 -/- mice were seeded on bone slices and stimulated with M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 5-7 days. Pits were scanned by confocal microscopy (x, y, and z section). Scale bar, 125 µm. i Pit depth from Figure 2H was quantified by confocal microscopy. Error bars are standard errors of the mean (SEM). Mean ± SEM; \*\*\*\* P< 0.0001; ns, not significant.





Probe: GPR54 CT



#### Figure 2

GPR54 recruited Src through the PR motif in GPR54 CT a Sequence analysis of the proline and argininerich motif in multiple species of GPR54 CT. b SH3 domain protein-binding array using the TranSignal<sup>™</sup> SH3 Domain Array kit. GST-GPR54 CT was used as the probe (GST protein as the control). Spots with stronger intensities indicate higher binding affinity of GST-GPR54 CT with various SH3 Domains. The principal Src binding region is circled in red evident (1 Src; 2 the dilution of Src in half). c SPR bindingaffinity measurement of Src and GPR54 CT. Src and GPR54 CT binding affinity is 2.6 nM (kinetic analysis). d Crystallization structure analysis of the SH2-SH3 domain of Src and the human GPR54 PR motif (336RVCPCAPRRPRRPRPGPSDP356). Src (G85-V247)-GPR54 (333A-357A with C338S and C340S mutation) recombinant fusion protein was purified from E. coli. Crystals were generated from purified recombinant proteins, subjected to X-ray crystallography, and the resulting model is shown. GPR54 CT bound with the SH3 domain of Src mainly through the motif 333AFRRVCPCAPRR344. e SPR binding-affinity measurement of Src and peptide of human GPR54 PR motif

(336RVCPCAPRRPRRPRRPGPSDP356) with a KD value (0.7  $\mu$ M). f SPR binding-affinity measurement of Src and peptide of human GPR54 (333AFRRVCPCAPRR344) with a KD value (8.3  $\mu$ M). g Immunoblots (IB) of total samples and GST pull-downs using GST proteins purified from E. coli; GST proteins were incubated separately with the whole-cell lysis (WCL) of 293T cells transfected with HA-Src. h IB analysis of WCL and anti-GPR54 immunoprecipitants (IP) assays derived from 293T cells transfected with GPR54-flag and HA-Src after Kp-10 induced for 20 minutes. i IB analysis of WCL and anti-GPR54 IP assays derived from RAW264.7 cells induced by Kp-10 for 20 minutes. j 293T cells transfected with GPR54-flag and HA-Src for 12 hours, then treated with 10 nM Kp-10 for 20 minutes. IF staining was carried out using indicated antibodies. Representative images are shown. Scale bar, 10  $\mu$ m.



Figure 3

# Figure 3

DUSP18 phosphatase was recruited by GPR54 after Kp-10 stimulation through the PR motif a Anti-Gpr54 IP was performed on WCL derived from RAW264.7 cells treated with or indicated dose of Kp-10 for 20 minutes. Beads were analyzed by phosphatase activity assay. Mean ± SEM; \* P< 0.05; \*\* P< 0.01. b Anti-Gpr54 IP was performed on WCL derived from RAW264.7 cells treated with or without Kp-10 for 20 minutes and then analyzed by mass spectrometry assay. c IB analysis of WCL and anti-GPR54 IP of cell

lysates from RAW264.7 cells treated with Kp-10 for 20 minutes. d IF staining was carried out using indicated antibodies on RAW264.7 cells treated with or without Kp- 10 for 20 minutes. Scale bar, 10  $\mu$ m. e SPR binding analysis of DUSP18 and GPR54 CT. DUSP18 interacted with human GPR54 CT with the binding affinity measured at 40 nM. f SPR binding analysis of DUSP18 and mouse Gpr54 (336RVCPCCR342) peptide. The binding affinity was measured at 1.8  $\mu$ M. g SPR binding analysis of DUSP18 and mouse Gpr54 (339PCCRQR344) peptide. The binding affinity was measured at 9.2  $\mu$ M. h IB of total samples and GST pull-downs using GST proteins purified from E. coli; GST proteins were incubated separately with the WCL of 293T cells transfected with DUSP18-HA. i IB analysis of total samples and GST pull-downs using His-SRC proteins purified from Sf9 cells, His-DUSP18, and GST proteins purified from E. coli.



# Figure 4

Src was dephosphorylated by DUSP18 when GPR54 was activated by Kp-10 a SPR binding analysis of DUSP18 and Src. The binding affinity of DUSP18 and Src was measured at 5.9 nM. b IB analysis of WCL and anti-Flag IP derived from 293T cells transfected with HA-Src and either Dusp18-Flag or Dusp18 (C104S)-Flag constructs. c Dusp18 dephosphorylated Src at Y416 in vitro. His-Dusp18 and His-Dusp18 (C103S) proteins purified from E. coli and SRC proteins purified from Sf9 insect cells were incubated in

the phosphatase buffer at 30oC for 30 minutes. IB analysis of the protein phosphatase reaction products with antibodies as indicated. d IF staining of RAW264.7 cells treated with 10 nM Kp-10 for 20 minutes was carried out using the indicated antibodies. e IB analysis of WCL and anti-Flag IP derived from 293T cells transfected with HA-Src, GPR54-myc, and either Dusp18-Flag or Dusp18 (C104S)-Flag constructs. f IB analysis of WCL and anti-Src IP derived from RAW264.7 cells treated with or without Kp-10 for 20 minutes. g IB analysis of WCL a derived from Dusp18-/- RAW264.7 cells treated with or without Kp-10 for 20 minutes. h BMMs isolated from eight-week-old WT and Dusp18-/- mice were cultured in the absence of serum and with indicated doses of Kp-10 for 20 minutes, and then treated with 100 ng/ml RANKL for 30 minutes. Cells were lysed and blots probed with indicated antibodies. i BMMs isolated from eight-week-old WT and Dusp18 -/- mice were seeded on bone slices and stimulated with M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 5-7 days. Pits were scanned by confocal microscopy (XY and z section). Scale bars, 125 µm. j Pit depths were measured by confocal microscopy. Mean ± SEM; \*\* P< 0.01; \*\*\* P< 0.01; ns, not significant.



# Figure 5

The formation of the complex (active Src, Dusp18, and GPR54) induced by Kp-10 was partially dependent on Gaq signaling a RAW264.7 cells were treated with indicated inhibitors including PLC inhibitor (U73122, 10  $\mu$ M), PKC inhibitor (Staurosporine, 0.25  $\mu$ M), ERK inhibitor (LY3214996, 10  $\mu$ M), P38 inhibitor (SB203580, 10  $\mu$ M) for 1 hours and then added with 10 nM Kp-10 for 30 minutes. Total RNAs were extracted for quantitative real-time RT-PCR analyses for relative RNA levels of Dusp18. b IB analysis of

WCL and anti-Flag IP derived from 293T cells transfected with GPR54-flag and Src- HA constructs for 24 hours. Cells were pre-treated with indicated inhibitors including PLC inhibitor (U73122, 10 µM), PKC inhibitor (Staurosporine, 0.5 µM), ERK inhibitor (LY3214996, 10 µM), Ca2+/CaMKII inhibitor (KN-93, 10 µM), Src family kinase (Saracatinib, 10 µM) for 1 hour and then added with 10 nM Kp-10 for 20 minutes. c IB analysis of WCL and anti-Flag IP derived from 293T cells transfected with GPR54-flag and Dusp18-HA constructs for 24 hours. Cells were pre-treated with indicated inhibitors including PLC inhibitor (U73122, 10 µM), PKC inhibitor (Staurosporine, 0.5 µM), ERK inhibitor (LY3214996, 10 µM), Ca2+/CaMKII inhibitor (KN-93, 10 µM), Src family kinase inhibitor (Saracatinib, 10 µM) for 1 hour and then added with 10 nM Kp-10 for 20 minutes. d IB analysis of WCL and anti-Flag IP derived from 293T cells transfected with GPR54-myc, Src-HA, and Dusp18-flag constructs for 24 hours. Cells were pre-treated with indicated inhibitors including PLC inhibitor (U73122, 10 µM), PKC inhibitor (Staurosporine, 0.5 µM), ERK inhibitor (LY3214996, 10 µM), Ca2+/CaMKII inhibitor (KN-93, 10 µM), Src family kinase (Saracatinib, 10µM) for 1 hour and then added with 10 nM Kp-10 for 20 minutes. e IB analysis of WCL and anti-Flag Src from Gag knockout RAW264.7 cells treated with or without Kp-10 for 20 minutes. f IB analysis of WCL derived from WT RAW264.7 cells and Gag knockout RAW264.7 cells treated with or without Kp-10 for 20 minutes. g The working model of Kiss1/Gpr54 mediated Src dephosphorylation.



### Figure 6

conditional knockout mice of Gpr54 mice (Gpr54 cKO) and Kiss1 mice (Kiss1 cKO), whole-body knockout mice of Dusp18 (Dusp18 -/-) exhibited bone loss and osteoclasts hyperactivation. (a, c, e) Representative micro-CT images of (a) 12-week-old WT and Gpr54 cKO mice (n = 6 per group), (b) 12-week-old WT and Kiss1 cKO mice (n=6 per group) and (c) 8-week-old WT and Dusp18 -/- mice (n=6 per group). Scal bar, 500 µm. (b, d, f) Bone parameters of WT and Gpr54 cKO mice (b, n=6 per group), WT and Kiss1 cKO mice

(d, n=6 per group), WT and Dusp18 -/- mice (f, n=6 per group). 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  $\pm$  s.d.; \*\*\* P < 0.000; \*\*0.01< P < 0.001; \*0.05< P < 0.01; ns, not significant. unpaired two-tailed Student's t test. (g, i, k) Representative TRAP staining images of 12-week-old WT and Gpr54 cKO mice (g, Scale bar, 20 µm), 12-week-old WT and Kiss1 cKO mice (i, Scale bar, 500 µm) and 8-week-old WT and Dusp18 -/- mice (k, Scale bar, 50 µm). (h, j, l) Osteoclast parameters from 12-week-old WT and Gpr54 cKO mice (h, n=6 per group), 12-weekold WT and Kiss1 cKO mice (j, n=6 per group), 8-week-old WT and Dusp18-/- mice (l, n=6 per group). mean  $\pm$  s.d.; \*\*\* P < 0.000; \*\*0.01< P < 0.001; \*0.05< P < 0.01; ns, not significant. unpaired two-tailed Student's t test.



### Figure 7

Kp-10 ameliorated OVX-induced bone loss. a Representative micro-CT images of control mice treated with vehicle (Sham, n=6), ovariectomized mice (OVX, n=6), and OVX mice treated with (DSS)\*6-Kp-10 (OVX+ (DSS)\*6-Kp-10, n=8). Scale bar, 500  $\mu$ m. b Parameter analysis of trabecular bone from Figure 7a. BMD, bone mineral density; BV/TV, bone volume as a fraction of total bone volume. mean ± s.d. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, unpaired two-tailed Student's T test. (n=6 in Sham or OVX group, n=8 in OVX+(DSS)\*6-

Kp-10 group). c Representative TRAP staining images of femurs from Figure 7a. d Histomorphometry analysis of the femurs from Figure 7a. Oc.S/BS, osteoclast surface per bone surface; N.Oc/B.Pm, osteoclast bone surface density; ES/BS, eroded surface per bone surface. mean  $\pm$  s.d. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, unpaired two-tailed Student's t test.

# Supplementary Files

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