Gα13 negatively controls osteoclastogenesis through inhibition of the Akt-GSK3β-NFATc1 signalling pathway

Mengrui Wu1, Wei Chen1, Yun Lu1, Guochun Zhu1, Liang Hao1 & Yi-Ping Li1

Many positive signalling pathways of osteoclastogenesis have been characterized, but negative signalling pathways are less well studied. Here we show by microarray and RNAi that guanine nucleotide-binding protein subunit α13 (Gα13) is a negative regulator of osteoclastogenesis. Osteoclast-lineage-specific Gna13 conditional knockout mice have a severe osteoporosis phenotype. Gna13-deficiency triggers a drastic increase in both osteoclast number and activity (hyper-activation), mechanistically through decreased RhoA activity and enhanced Akt/GSK3β/NFATc1 signalling. Consistently, Akt inhibition or RhoA activation rescues hyper-activation of Gna13-deficient osteoclasts, and RhoA inhibition mimics the osteoclast hyperactivation resulting from Gna13-deficiency. Notably, Gα13 gain-of-function inhibits Akt activation and osteoclastogenesis, and protects mice from pathological bone loss in disease models. Collectively, we reveal that Gα13 is a master endogenous negative switch for osteoclastogenesis through regulation of the RhoA/Akt/GSK3β/NFATc1 signalling pathway, and that manipulating Gα13 activity might be a therapeutic strategy for bone diseases.
Osteoclasts are the principal, if not exclusive, bone-resorbing cells. A balance between bone formation by osteoblasts and bone resorption by osteoclasts is critical to maintain normal bone density and mineral homeostasis. Osteoclastogenesis is initiated by signals transmitted by the receptor activator of nuclear factor-κB (RANK) ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). RANKL and M-CSF, binding to their receptors, RANK and cFms, respectively, on the surface of osteoclast precursors, activates many key transcription factors including nuclear factor of activated T-cells, c1 (NFATc1) and CCAAT-enhancer-binding protein alpha (C/EBPα). Our previous work on cathepsin K, Atp6i (refs 7,8), C/EBPα and RGS10 (ref. 10) has contributed to the discovery of positive regulatory machinery controlling osteoclast differentiation and function. Notably, excessive osteoclast activity is responsible for various bone diseases including osteoporosis, rheumatoid arthritis and Paget’s disease. Thus, we anticipate that hyperactivation of osteoclasts should be antagonized by intrinsic negative regulators, which have received limited attention, to maintain normal bone homeostasis. A decrease in the expression of negative osteoclast regulators should be antagonized by intrinsic negative regulators, to maintain normal bone homeostasis. A decrease in the expression of negative osteoclast regulators might trigger the bone loss associated with many pathological bone disorders.

Guanine nucleotide-binding protein subunit α13 (Gna13; encoded by Gna13) belongs to the G12 subfamily of the G protein superfamily. Gna13 regulates cell cytoskeleton organization by regulating the RhoGEF-RhoGTPase signalling pathway. Here we use a genome-wide screening strategy and characterize Gna13 as an intrinsic negative regulator of osteoclast formation and activity. Absence of Gna13 favours osteoclast formation and enlarges osteoclast size, leading to an osteopenia phenotype in mice. On the other hand, constitutively active Gna13 blocks the formation of multinucleated osteoclasts and their bone resorptive activity. We reveal that Akt-mediated signalling, regulated by RhoA, is critical for the function of Gna13 in osteoclasts.

Results

Gna13 silencing promotes osteoclastogenesis. Given the limited attention attributed to the investigation of negative regulators of osteoclastogenesis, we performed a genome-wide screening strategy to identify factors that negatively regulate osteoclast formation (Fig. 1). Towards this end, we compared gene expression in human blood monocyte-derived osteoclasts with that in their precursors, and found that Gna13 was not only drastically induced by RANKL, but also its expression pattern was comparable to various osteoclast genes (Fig. 1a), and much higher than the genes encoding several other G proteins (Fig. 1b). Moreover, gene expression analysis confirmed that Gna13 was highly expressed in osteoclasts and osteoclast-like cells derived from MCOP-5, an osteoclast precursor cell line that was generated in our lab, as compared with several tissues, such as heart, kidney, lung and intestine (Fig. 1c). Furthermore, using murine bone marrow monocytes (BMMs), which are widely used as primary osteoclast precursors, we demonstrated that whereas Gna13 expression was only mildly induced by M-CSF (~2-fold at both mRNA and protein level), its expression was strongly induced by combined stimulation with M-CSF and RANKL (~6-fold at mRNA level and 8-fold at protein level) (Fig. 1d,e). These results indicated that Gna13 might have important roles in osteoclasts. Hence, we then silenced Gna13 expression by lentiviral-mediated expression of short hairpin RNA against Gna13 in BMMs. The knockdown efficiency (~90%) was confirmed by immunoblotting as compared with control cells (Fig. 1f). Interestingly, our data showed that Gna13 silencing strongly increased osteoclast formation and also drastically enhanced osteoclast size (Fig. 1g,h). These results suggested that Gna13 might be a negative regulator of bone resorption.

Depletion of Gna13 causes osteopenia in mice. To further investigate the role of Gna13 in osteoclast formation and activity, we generated knock-out mice through specific deletion of Gna13 in the osteoclast lineage (Fig. 2). Mice bearing loxP sites encompassing the Gna13 exon2 (Gna13f/f mice) were crossed with those expressing Cre recombinase driven by the lysozyme M promoter (LysM-Cre) mice or the Cathepsin K promoter (Ctsk-Cre mice). The offspring were intercrossed to get Gna13f/f LysM-Cre mice or Gna13f/f Ctsk-Cre mice and wild-type (WT) mice. Mouse genotypes were confirmed by PCR (Supplementary Fig. 1a–c). The deletion of Gna13 in Gna13f/f LysM-Cre BMMS and Gna13f/f Ctsk-Cre pre-osteoclasts was confirmed by quantitative PCR and immunoblotting (Fig. 2a,b). While LysM-Cre deletes Gna13 in the osteoclast precursors (Fig. 2a,b), Ctsk-Cre works at a late stage of osteoclast differentiation (Supplementary Fig. 1d).

Two-month-old WT and Gna13f/f LysM-Cre or Gna13f/f Ctsk-Cre mice have no obvious gross morphological changes. As assessed by X-ray analysis, skeletal mass was decreased in distal femurs of both male and female Gna13f/f LysM-Cre mice as compared with those of WT littermates (Fig. 2c). Quantitative microtomography (μ-CT) analysis showed that Gna13f/f LysM-Cre mice exhibited ~30% reduction in bone volume/tissue volume (BV/TV) and trabecular number (Tb.N) as well as ~40% increase in trabecular space (Tb.Sp), ~50% decreased in bone mineral density (BMD) as compared with WT littermates (Fig. 2d,e). Tartrate-resistant acid phosphatase (TRAP) activity was markedly increased in the primary spongiosea of 2-month old Gna13f/f LysM-Cre mouse femur as compared with that of WT littermates (Fig. 2f–h). Histomorphometric parameters of distal femurs showed that, in the trabecular bone area, Gna13f/f LysM-Cre mice had ~3-fold increased in osteoclast numbers per bone surface (N.Oc/BS) as compared with WT littermates (Fig. 2g). Interestingly, osteoclast number was even more drastically increased in the cortical bone area in the mutant mice (Fig. 2f,g). Similarly, X-ray and μ-CT analyses showed that skeletal mass was decreased in distal femurs of 2-month-old Gna13f/f Ctsk-Cre mice (Fig. 2i), Supplementary Fig. 2c). TRAP staining showed ~6-fold increase in osteoclast numbers in femoral sections of Gna13f/f Ctsk-Cre mice as compared with that of WT controls (Fig. 2k, Supplementary Fig. 1d–f). Histomorphometry based on Trichrome staining showed that trabecular bone number was reduced without much change of osteoblast number and osteoblast surface per bone surface in the Gna13f/f Ctsk-Cre mice, confirming that the reduced bone density was not caused by decreased bone formation (Supplementary Fig. 2a,b). In fact, as measured by calcine labelling (Supplementary Fig. 2d), mineral apposition rate was increased by about 35% in Gna13f/f Ctsk-Cre mice. Consistently, serum alkaline phosphatase (ALP) was also increased by about 35% in Gna13f/f Ctsk-Cre mice (Supplementary Fig. 2e). The accelerated bone formation and remodelling might be caused by robust osteoclast formation. These results show that osteoclast-lineage-specific Gna13 deletion promotes osteoclastic bone resorption leading to lower bone density in vivo.

Gna13-depletion hyper-activates osteoclast. Given our data that Gna13 deficiency in vivo led to an increase in osteoclast development (Fig. 2), we examined the effects of using lower doses of RANKL in osteoclastogenesis under Gna13 deficiency. Unlike WT BMMs, Gna13-deficient cells formed osteoclasts when attended by permeisive RANKL dose (one tenth of the optimum 10 ng ml−1 RANKL) (Fig. 3a) and formed 25% more
osteoclast nuclei number per mm². Results in Gna13-infected BMMs (Mock) and BMMs infected with lentivirus expressing scrambled short hairpin RNA (shRNA) (sh-src) or shRNA targeting Gna13 (sh-Gna13) under the treatment of M-CSF or M-CSF and RANKL, and its quantification in lower panel; N ≥ 4. (e) Western blot to analyse time-course expression of Gna13 in BMMs under the treatment of M-CSF or M-CSF and RANKL, and its quantification in lower panel; N ≥ 4. (f) Western blot to analyse Gna13 expression in non-infected BMMs (mock) and BMMs infected with lentivirus expressing scrambled short hairpin RNA (shRNA) (sh-src) or shRNA targeting Gna13 (sh-Gna13). (g) TRAP staining to detect osteoclast formation of mock, sh-src and sh-Gna13 BMMs. (h) Quantification of cell number (OC/N/well and nuclei.N/mm²) from human PBC to OC. **P ≤ 0.01; ***P ≤ 0.001 (Student’s t-test). Scale bars, 200 μm.

**Figure 1 | Gna13 which is induced by RANKL and M-CSF inhibits osteoclastogenesis.** (a,b) Microarray profile of gene expression during osteoclast differentiation. OC, osteoclasts; PBM, peripheral blood monocytes. (c) Semi-quantitative reverse transcription–PCR to detect Gna13 mRNA level in murine tissues and cells. (d) Quantitative reverse transcription–PCR to analyse time-course expression of Gna13, normalized to Hprt1, in murine bone marrow monocytes (BMMs) under the treatment of M-CSF; or M-CSF and RANKL; N ≥ 4. (e) Western blot to analyse time-course expression of Gna13 in BMMs under the treatment of M-CSF or M-CSF and RANKL, and its quantification in lower panel; N ≥ 4. (f) Western blot to analyse Gna13 expression in non-infected BMMs (mock) and BMMs infected with lentivirus expressing scrambled short hairpin RNA (shRNA) (sh-src) or shRNA targeting Gna13 (sh-Gna13). (g) TRAP staining to detect osteoclast formation of mock, sh-src and sh-Gna13 BMMs. (h) Quantification of cell number (OC/N/well and nuclei.N/mm²) from human PBC to OC. **P ≤ 0.01; ***P ≤ 0.001 (Student’s t-test). Scale bars, 200 μm.

osteoclasts at 10 ng ml⁻¹ RANKL (Fig. 3a,b). Further, BMMs deficient in Gna13 generated osteoclast earlier (∼3 day 3) than WT BMMs (∼day 5) (Supplementary Fig. 3a,b). Gna13 deficiency not only increased the sensitivity of BMMs to RANKL, but also increased the size of osteoclasts (Fig. 3a,b). More osteoclasts with 3–8 nuclei or >13 nuclei were observed in the Gna13-deficient group (increased by 50% and 100%, respectively) (Fig. 3b). Consistently, gene expression analysis revealed that the expression of the osteoclast genes encoding NFATc1, Ctsk, Acp5, Atp6v0d2 and dc-stamp was higher in Gna13⁻/⁻ LysM-Cre, but not in WT, BMMs cultured with M-CSF and RANKL for 3 days (Fig. 3c). Importantly, among the aforementioned genes, dc-stamp and Atp6v0d2 are known to have roles in osteoclast fusion15,16, indicating that Gna13 negatively regulates osteoclast formation by also promoting the fusion of mononucleated osteoclasts into multinucleated osteoclasts.

To further analyse the role of Gna13 in osteoclastic bone resorption, we performed in vitro bone resorption assays and analysed bone resorption pits by wheat germ agglutinin (WGA) staining and scanning electron microscope (Fig. 3d). Data showed that the total resorption area by Gna13⁻/⁻ LysM-Cre osteoclasts was significantly higher than that of WT osteoclasts (Fig. 3e). Collagen I C-terminal telopeptide (CTX-I), released during bone resorption17, were drastically increased in Gna13⁻/⁻ LysM-Cre cell culture medium (Fig. 3f). In addition, WGA-fluorescein isothiocyanate (FITC) staining showed that pits resorbed by Gna13⁻/⁻ LysM-Cre osteoclasts are also larger (∼4-fold) and deeper (∼3-fold) than those by WT cells (Fig. 3g,h, Supplementary Fig. 4). Collectively, the results showed that Gna13 negatively regulates osteoclastic bone resorption.

Next, we co-stained the osteoclasts on bone slides with Rhodamine-conjugated-Phalloidin (red), to analyse F-actin ring formation, a critical structure of mature osteoclasts18, anti-Ctsk antibody (green) and Hoechst (blue) (Fig. 3i,j). Data showed that Gna13-deficient osteoclasts exhibited 1.5-fold increased F-actin ring formation (Fig. 3i), and the average size of F-actin rings was also about threefold larger in these cells than control cells (Fig. 3j,k).

Furthermore, immunostaining showed a more polarized location of Ctsk in Gna13⁻/⁻ LysM-Cre cells (Fig. 3i), indicating a change in Ctsk transportation and secretion. We thus analysed intracellular and medium Ctsk by western blot (Fig. 3m,n). Data showed a slight increase (∼1.5-fold) in intracellular Ctsk level in Gna13⁻/⁻ LysM-Cre osteoclasts (Fig. 3m). However, secreted Ctsk concentration in the culture medium by Gna13⁻/⁻ LysM-Cre osteoclasts was drastically elevated (∼4-fold) (Fig. 3n). These results demonstrate that Gna13-deficient osteoclasts had enlarged actin ring with increased Ctsk secretion, both of which would contribute to enhanced bone resorption besides enhanced osteoclast formation.

We performed TUNEL staining on day-6 osteoclasts (Supplementary Fig. 3c,e) and cytokine/serum-starved day-5
Figure 2 | Osteoclast-specific Gna13-CKO mice displayed osteoporosis. (a) Quantitative reverse transcription–PCR confirmed deletion of Gna13 mRNA in day-1 Gna13\(^{fl/fl}\)LysM-Cre (\(f/f;\)LysM-Cre) and day-4 Gna13\(^{fl/fl}\)Ctsk-Cre (\(f/f;\)Ctsk-Cre) pre-osteoclasts; \(N=6\). (b) Western blot confirmed deletion of G\(\alpha\)13 protein in Gna13\(^{fl/fl}\)LysM-Cre BMMs and day-5 Gna13\(^{fl/fl}\)Ctsk-Cre pre-osteoclasts. Quantification is co-presented in the right panels; \(N=6\). (c) Radiographs of two-month-old wild type (WT) and Gna13\(^{fl/fl}\)LysM-Cre male and female mice femurs. (d) \(\mu\)-CT analysis of two-month-old WT and Gna13\(^{fl/fl}\)LysM-Cre female mice femurs. (e) Quantification from (d); \(N=6\). BMD, bone mineral density; BV, bone volume; Tb.Th, trabecular bone thickness; Tb.N, trabecular bone number; Tb.Sp, trabecular bone space; TV, tissue volume. (f) TRAP staining of 2-month-old WT and Gna13\(^{fl/fl}\)LysM-Cre female mice femurs. (g) High magnification images of (f). (h) Quantification of N.Oc/BS (osteoclast number / bone surface) in (f); \(N=6\). (i) Radiographs of two-month-old WT and Gna13\(^{fl/fl}\)Ctsk-Cre female mice femurs. (j) \(\mu\)-CT analysis of two-month-old WT and Gna13\(^{fl/fl}\)Ctsk-Cre female mice femurs. (k) TRAP staining of 2-month-old WT and Gna13\(^{fl/fl}\)Ctsk-Cre female mice femurs. WT, wild-type. Results were expressed as mean ± s.d.; *\(P\leq0.05\); **\(P\leq0.01\); ***\(P\leq0.001\); ns, not significant (Student’s t-test). Scale bars in (d,f,j,k) 200 \(\mu\)m; Scale bar in (g) 20 \(\mu\)m.
Figure 3 | Loss of Gna13 promotes osteoclast formation and function. (a) TRAP staining to detect osteoclast formation from WT and Gna13f/f/LysM-Cre (ff/LysM-Cre) BMMs treated by M-CSF and different doses of RANKL for 5 days. (b) Quantification of cell number and relative cell size in a. RANKL = 10 ng ml\(^{-1}\) group; N = 6. TRAP* MNC, TRAP positive multinucleated cell. (c) Quantitative reverse transcription-PCR analysis of act6v0d2, dc-stamp, atp6i, nfatc1, ctsk and trap expression in WT and Gna13f/f/LysM-Cre osteoclasts; N = 6. (e) WGA (wheat germ agglutinin) staining and SEM (scanning electron microscope) analysis of bone slides to detect bone resorption pits. (f) Quantification of bone resorption area in e; N = 6. (g) Confocal microscopy of FITC-WGA stained bone resorption pits. (h) Quantification of bone resorption pit depth and relative pit size in g. Culture medium CTX-I (type I collagen carboxy-terminal peptide) concentration measured by ELISA. (i) Fluorescence microscopy of F-actin ring stain in mature osteoclasts. (j) Confocal microscopy of F-actin ring (red)/Ctsk (green)/nuclei (blue) staining in mature osteoclasts. (k) Quantification of F-actin ring number in i; N = 6. (l) Quantification of F-actin ring volume in j; N = 30. (m) Western blot to detect intracellular Ctsk protein level, and its quantification (lower panel); N = 3. (n) Western blot to detect Ctsk concentration in the culture medium, and its quantification (right panel); N = 3. Results are expressed as mean ± s.d.; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001 (Student’s t-test or ANOVA analysis). Scale bars in a, d, 200 μm; Scale bar in g, 5 μm.
osteoclasts (Supplementary Fig. 3d,f), and similar apoptosis nuclei ratio are observed in the WT and mutant cells. Similar survival rate was observed in the WT and Gna13\(^+/\)LysM-Cre cells when the osteoclasts were extensively cultured for 7 days (Supplementary Fig. 3h,i). Data indicated that Gna13-deficiency might not influence osteoclast apoptosis. Further, acridine Orange staining showed that acidification is similar between WT and Gna13\(^+/\)LysM-Cre osteoclasts (Supplementary Fig. 3g).

**Gz13-RhoA brakes Akt-GSK3\(\beta\)-NFATc1 signalling** RANKL and M-CSF orchestrate multiple signalling pathways to promote osteoclast formation\(^1\)–\(^3\). Expression of RANKL receptor (RANK) and M-CSF receptor (cFms) were similar in WT and Gna13-deficient cells (Supplementary Fig. 3j,k). To analyse signal transduction in response to RANKL or M-CSF stimulation (including Akt, p38, JNK and Erk activation), BMMs were starved for 5 h and then stimulated with RANKL or M-CSF for 5–60 min (Fig. 4a,b). Akt phosphorylation was enhanced in Gna13\(^+/\)LysM-Cre cells by either RANKL or M-CSF as compared with WT BMMs (Fig. 4a,b). Nonetheless, activation of the p38, JNK and Erk signalling pathways were similar between WT and Gna13\(^+/\)LysM-Cre cells (Supplementary Fig. 5).

Given the drastic increase in Akt activation, we then applied the Akt inhibitor MK2206 2HCl to attenuate Akt activity, which inhibited osteoclastogenesis in a dose-dependent manner (Fig. 4c–e). Notably, Gna13-deficient cells were more resistant to the Akt inhibitory effect on osteoclastogenesis. In the presence of 0.1–0.2\(\mu\)M MK2206 2HCl (Fig. 4d,e), Gna13-deficient cells formed osteoclasts at normal level comparatively to WT cells. The results indicate that Gna13 deficiency promotes osteoclastogenesis by increasing Akt activity.

Studies have demonstrated that Gz13 activates RhoA through RhoGEF (p115RhoGEF, LARG)\(^11\),\(^12\), and RhoA can inhibit Akt activity\(^19\). Consistently, RhoA activity was dramatically downregulated in Gna13-deficient cells (Fig. 4f). In all, 250 ng ml\(^{-1}\) Rho activator II corrected the hyper-activation of Gna13-deficient osteoclasts (Fig. 4g–i). In addition, Rho activator II could attenuate Akt activation in osteoclasts (Fig. 4m). On the other hand, in the presence of 6–12 ng ml\(^{-1}\) Rho inhibitor, cell-permeable C3 toxin, osteoclast differentiation was enhanced (Fig. 4j–l) and Akt activation was also increased (Fig. 4n). Thus, Gz13 regulated Akt activity through RhoA so as to promote osteoclastogenesis.

It has been established that the PI3K/Akt-GSK3\(\beta\)-NFATc1 signalling cascade is critical for osteoclast differentiation\(^2\)–\(^12\). Specifically, Akt can inhibit GSK3\(\beta\), which can promote NFATc1 translocation from the nucleus into the cytoplasm and thereby abrogate osteoclast differentiation. Thus, we characterized GSK3\(\beta\) phosphorylation level and NFATc1 nuclei translocation in WT and Gna13\(^+/\)LysM-Cre cells. We found that GSK3\(\beta\) phosphorylation was hyper-activated in Gna13\(^+/\)LysM-Cre monocyte/macrophage in response to RANKL and M-CSF (Fig. 4o). NFATc1 expression increased during osteoclastogenesis in WT and Gna13\(^+/\)LysM-Cre cells, and more quickly in Gna13\(^+/\)LysM-Cre cells (Fig. 4p). Consistently, while similar amounts of NFATc1 were observed in the cytoplasm, much more NFATc1 was observed in the Gna13\(^+/\)LysM-Cre nucleus than WT (Fig. 4q). The results demonstrated that Gz13 mediates Akt-GSK3\(\beta\)-NFATc1 signalling to promote osteoclast gene expression.

**Gz13CA inhibits osteoclastogenesis.** To confirm the inhibitory effect of Gz13 on osteoclastogenesis, we overexpressed a constitutively active form of Gz13 (Gz13CA) in BMMs and RAW264.7 cells (Fig. 5). Moreover, we overexpressed Gz13CA in WT and Gna13\(^+/\)LysM-Cre pre-osteoclasts using lentivirus\(^22\). A successful infection (>90% infection) was assessed by green fluorescent protein expression in control groups (Supplementary Fig. 6a,c), and Gz13CA overexpression was confirmed by western blot (Supplementary Fig. 6b,d,e). Importantly, Gz13CA overexpression drastically blocked osteoclast fusion, inhibited bone resorption and impaired F-actin ring formation in WT and Gna13\(^+/\)fLysM-Cre cells (Fig. 5a–f). Additionally, the secretion of Ctsk was decreased after Gz13CA overexpression in WT and Gna13-deficient osteoclasts (Fig. 5g,h). Ctsk and NFATc1 protein levels were also decreased after Gz13CA overexpression (Fig. 5i,j). Furthermore, Gz13CA overexpression attenuated Akt activation in Gna13\(^+/\)LysM-Cre cells, but did not affect the activation of p38 and ERK (Fig. 5k,l; Supplementary Fig. 7a). Gz13CA overexpression in WT BMMS also drastically inhibited osteoclastogenesis (Fig. 5m left panels; 5n). Interestingly, overexpression of a constitutively active form of Akt (AktCA) could partially rescue the inhibitory effects of Gz13CA (Fig. 5m right panels; 5n). These results demonstrate that Gz13CA inhibited osteoclastogenesis by inhibiting Akt activation in BMMs.

Furthermore, we infected RAW264.7 cells with the same retrovirus we used in BMMs in Fig. 5m,o. Similarly, Gz13CA overexpression inhibited osteoclast-like cell formation (Fig. 5o left panels; 5p), which could be rescued by AktCA overexpression (Fig. 5o right panels; 5p). Akt activation was greatly decreased in RAW264.7 cells overexpressing Gz13CA (Fig. 5q,r), while the activation of p38 and ERK remains unchanged (Supplementary Fig. 7b). The overexpression of Gz13CA and AktCA was confirmed by western blot (Fig. 5s,t). The results indicated that Gz13CA also inhibited osteoclast-like cells formation from the RAW264.7 cells by inhibiting Akt phosphorylation.

**Gz13CA protects mice from pathological bone loss.** To investigate Gz13 clinical implication potential, we overexpressed Gz13CA locally in mice using adeno-associated virus (AAV) gene expression to investigate its effects in protecting against bone degradation. AAV vectors have advantages compared with many other viral and non-viral based gene delivery platforms in both function and safety for correcting genetic-based diseases\(^23\).

To test the use of AAV-Gz13CA, we utilized three disease models of bone loss: the human tumour-necrosis factor \(\alpha\) (hTNF\(\alpha\))-transgene expressing mouse model of autoimmune arthritis\(^24\) (named TNF\(\alpha\)-RA) (Fig. 6), ovariectomized (OVX) animal model of osteoporosis, (Fig. 7a–d) and calvaria-adjacent lipopolysaccharide (LPS)-injection mouse model of osteolysis (Fig. 7e,f)\(^25\). Successful infection of AAV in vivo was monitored by yellow fluorescent protein (YFP) expression (Supplementary Figs 8 and 9a). Successful overexpression of G13CA in vivo was confirmed by immunofluorescence staining (Supplementary Fig. 9b).

TNF\(\alpha\)-RA mice were accompanied by excessive osteoclast formation and bone destruction (Fig. 6). Local administration of AAV-Gz13CA to ankles greatly reduced bone loss, as assessed by X-ray in the TNF\(\alpha\)-RA mice as compared with those injected with AAV-YFP (Fig. 6c,d). Not surprisingly, osteoclast number was dramatically decreased in TNF\(\alpha\)-RA mice with local injection of AAV-Gz13CA to ankles (Fig. 6g,h). Unexpectedly, local administration of AAV-Gz13CA also relieved ankle swelling (Fig. 6a,b), cartilage destruction (Fig. 6e lower panels, Fig. 6f) and inflammation cell infiltration (Fig. 6e upper and middle panels, Fig. 6f) in TNF\(\alpha\)-RA mice. Hence, reduced bone loss and decreased osteoclast number might be a combined effect of Gz13CA and inhibited inflammation.
Figure 4 | Knockout of Gna13 enhances Akt-GSK3β-Nfatc1 signalling through RhoA. (a) Western blot analysis to detect Akt phosphorylation induced by RANKL in WT and Gna13f/fLysM-Cre (f/fLysM-Cre) monocytes/macrophages. N = 3. (b) Western blot analysis to detect Akt phosphorylation induced by M-CSF in WT and Gna13f/fLysM-Cre monocytes/macrophages. Lower panel in a-b, Quantification of phospho-Akt level versus total Akt level; N = 3. (c) TRAP staining to detect osteoclastogenesis of WT and Gna13-deficient osteoclasts treated by MK2206 2HCl at different doses during differentiation. (d) Quantification of TRAP+ MNC number per well in c; N = 6. (e) Quantification of TRAP+ nuclei number per mm² in j; N = 6. (f) RhoA activity in WT and Gna13f/fLysM-Cre pre-osteoclasts upon RANKL and M-CSF stimulation, and its quantification; N = 3. (g) TRAP staining of WT and Gna13f/fLysM-Cre osteoclasts treated by Rho activator II at different doses during differentiation. (h) Quantification of TRAP+ MNC number per well in g; N = 6. (i) Quantification of TRAP+ nuclei number per mm² in n; N = 6. (j) TRAP staining of WT and Gna13f/fLysM-Cre osteoclasts treated by different doses of RhoA inhibitor (C3 toxin) (0, 6, 12 ng ml⁻¹) during differentiation. (k) Quantification of TRAP+ MNC number per well in j; N = 6. (l) Quantification of TRAP+ nuclei number per mm² in j; N = 6. (m) Akt phosphorylation in pre-osteoclasts treated different doses of Rho activator II. (n) Akt phosphorylation in pre-osteoclasts treated different doses of C3 toxin (0, 6, 12 ng ml⁻¹). Lower panel in m-n, Quantification of phospho-Akt level versus total Akt level; N = 3. (o) GSK3β phosphorylation induced by RANKL and M-CSF. Lower panel, Quantification of phospho-GSK3β level versus total GSK3β level; N = 3. (p) Western blot analysis of Nfatc1 expression in WT and Gna13f/fLysM-Cre osteoclast precursors. (q) Nfatc1, GAPDH (cytoplasm loading control) and p84 (nuclei loading control) in Nuclei and cytoplasm lysates of WT and Gna13f/fLysM-Cre pre-osteoclasts. Right panels in p-q, Quantification of Nfatc1 level versus loading control; N = 3. Results were expressed as mean ± s.d.; *P≤0.05; **P≤0.01; ***P≤0.001 (Student’s t-test or ANOVA analysis). Scale bars, 200 μm.

OVX-induced bone loss and osteoclast number increased due to estrogen depletion (Fig. 7a–d, sham + PBS group compared with OVX + YFP group). OVX mice were subjected to calvaria-adjacent subcutaneous injection of AAV-YFP or AAV-Gz13CA. As assessed by X-ray and u-CT, application of AAV-Gz13CA reduced OVX-induced bone loss (Fig. 7a,b). As assessed by TRAP staining of whole calvarial and calvarial sections, AAV-Gz13CA dramatically reduced OVX-induced osteoclast number increase (Fig. 7c,d) as compared with YFP group.

In the LPS-injection model, both WT and Gna13f/fLysM-Cre mice were subjected to calvaria-adjacent subcutaneous injection of LPS or PBS control (Fig. 7e,f). Compared with local injection of PBS, LPS induced a dramatic increase in osteoclast number and bone resorption (Fig. 7e,f). The absence of Gz13 further promoted the induction of osteoclastogenesis and bone loss in both PBS and LPS treated mice (Fig. 7e,f). Local administration of AAV-Gz13CA to calvaria had a marked therapeutic effect on osteoclast formation and bone destruction by LPS, compared with local injection of AAV-YFP control (Fig. 7e,f). These results confirm that it is possible to overexpress Gz13CA in vivo to target pathologic bone loss.

Full images of western blots are presented in Supplementary Fig. 10.

Discussion
We proposed a model that Gz13-RhoA antagonizes osteoclast formation and activity by attenuating the Akt-GSK3β-Nfatc1 pathway and suppressing RhoA activity in pre-osteoclasts. Further studies are needed to address the underlying mechanisms and identify potential therapeutic targets for the treatment of osteoporosis.
Figure 5 | Gna13 gain-of-function inhibits osteoclast formation and function in vitro. (a) TRAP staining to detect osteoclastogenesis of WT and Gna13f/fLysM-Cre (f/fLysM-Cre) cells transfected with lentivirus overexpressing green fluorescent protein (GFP) and Gna13CA. (b) Quantification of TRAP+ MNC number per mm² in a; N = 6. (c) WGA staining to detect bone resorption of WT and Gna13f/fLysM-Cre cells overexpressing GFP and Gna13CA. (d) Quantification of bone resorption area per total area in c; N = 6. (e) Rhodamine-conjugated-Phalloidin staining to detect F-actin ring formation of WT and Gna13f/fLysM-Cre cells transfected with lentivirus overexpressing GFP and Gna13CA. (f) Quantification of F-actin ring number per view in e; N = 6. (g,l.k) Western blot to detect medium Ctsk (g), osteoclast-specific gene expression (i) Akt phosphorylation induced by RANKL and M-CSF (k) in WT and Gna13f/fLysM-Cre osteoclasts (overexpressing GFP or Gna13CA). (h,j) Quantification of protein level in g-l; N = 3. (m,o) TRAP staining to detect osteoclastogenesis of BMMs (m) and RAW264.7 cells (o), which were transfected with control retrovirus and retrovirus overexpressing 3xFLAG-Gna13CA, AktCA or 3xFLAG-Gna13CA + AktCA. (n,p) Quantification of TRAP+ MNC per well in n.m and in p.o; N = 6. (q,r) Western blot analysis of RANKL induced Akt phosphorylation in RAW264.7 cells transfected with control retrovirus and retrovirus overexpressing Gna13CA, and its quantification; N = 3. (s,t) Western blot confirmation of AktCA and Gna13CA overexpression in RAW264.7 cells, and its quantification; N = 3. EV, empty vesicle control. Results were expressed as mean ± s.d.; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001 (Student’s t-test or ANOVA analysis). Scale bars, 200 µm.
signalling axis (Fig. 8). Briefly, as a downstream of RANK and c-Fms, Akt phosphorylates and inactivates GSK3β, which has a role in NFATc1 nuclei exportation, so as to promote osteoclast differentiation; Meanwhile, Gz13 activates RhoA which in turn inhibits Akt phosphorylation and activity, so as to antagonize the over-activation of osteoclast (Fig. 8). The critical role of PI3K-Akt activity in osteoclast differentiation and activation is well-documented\(^{20,21,26–29}\). Although several endogenous factors that oppose osteoclast differentiation were described, most are downregulated under osteoclastogenic condition. In contrast, Gz13 is induced by the combined stimulation of RANKL and M-CSF, and in turn inhibit osteoclast differentiation so as to avoid over-activated osteoclast and excessive bone loss. Hence, our data reveal that Gz13 is an intrinsic control mechanism during osteoclastogenesis. In addition, we comprehensively study and report a signalling cascade (Gz13-RhoA-Akt-GSK3β-NFATc1) that negatively regulates osteoclastogenesis and osteoclast function. Most importantly, our results from both loss-of-function and gain-of-function strategies proved that Gz13 is a key negative regulator and main switch in osteoclastogenesis.

It was reported that RhoA activity is essential for podosome formation and osteoclastic bone resorption\(^{30}\). On the other hand, it was also reported that increased RhoA activity destabilizes the sealing zone in osteoclasts, and inhibition of RhoA stabilizes the sealing zone correlating with acetylation and stabilization of the microtubule network in these cells\(^{31–34}\). Our study showed that inhibition of RhoA activity through Gz13 knockout, RhoA inhibitor and RNA interference favours osteoclastogenesis, while RhoA activator inhibits osteoclastogenesis. Hence, these studies suggest that RhoA is the downstream effector of Gz13 to negatively regulate osteoclastogenesis. Our finding is underscored by genetic evidences that have suggested an important role for the RhoGEF-RhoGTPase (for example, Arhgef3-RhoA) pathway in osteoporosis\(^{35–38}\). Genome-wide linkage studies have identified chromosome region 3p14-p21, in which RHOA gene was located, as a quantitative trait locus for BMD\(^{35,36}\). One RHOA single-nucleotide polymorphism (SNP) rs17595772, and one ARHGEF3 SNP were reported to be significantly associated with decreased BMD in postmenopausal women\(^{37,38}\). However, the mechanisms underlying the correlation between RhoGEF-RhoGTPase pathway and BMD remain to be explored.

Current anti-resorptive agents (for example, bisphosphonates and denosumab) are effective but far from ideal. The major problem of bisphosphonates is their permanent deleterious affect on normal bone remodelling. Agents preventing bone resorption by inhibiting late differentiation of osteoclasts without affecting normal bone remodelling are more desired, which are unlikely to interfere with the coupling of osteoblastic bone formation to osteoclastic bone resorption, critical for maintaining normal bone homeostasis\(^{39}\). Here we showed that overexpression of Gz13CNA markedly inhibits osteoclast formation and bone resorption both in vitro and in vivo. Importantly, although multi-nuclear cell formation was blocked, Gz13CNA over-expressing monocytes were TRAP\(^+\) stained (Fig. 5a), indicating that Gz13CNA regulated osteoclast differentiation at a late stage and could be a good target for bone loss without affecting normal bone remodelling. Interestingly, Gz13 might have dual functions in inflammatory disease models (TNF-α–RA and LPS-injection models), by concurrently inhibiting osteoclast differentiation and inflammation so as to achieve the ultimate outcome of bone

**Figure 6 | Gz13 protects Rheumatoid arthritis mice from inflammatory bone loss. (a–h)** Histology analysis of 18-week-old male WT and hTNFtg Rheumatoid arthritis mouse ankles. hTNFtg mouse ankles were injected with AAV-Gz13CA or AAV-YFP. (a) Photographic images before and after AAV treatment. The red arrows showed paw swelling is relieved after AAV-Gz13CA treatment. (b) Quantification of hind paw volume in a; N = 8. (c,d) Radiographic images; White arrows mark bone destruction. (d) Quantification of bone destruction (Larsen grade) in a; N = 8. (e) H&E staining (black arrows mark monocyte infiltration) and Safranin O (SO) staining (black arrows mark articular cartilage damage). (f) Quantification of cartilage damage (OARSI grade) and inflammation (infiltration score) in e; N = 8. (g) TRAP staining; black arrows mark TRAP positive cells. (h) Quantification of osteoclast number (Oc.N) in g; N = 8. Results were expressed as mean ± s.d.; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001 (Student’s t-test). scale bars in e,g 200 μm.
Figure 7 | Gα13 protects mice from OVX- and LPS-induced bone loss. (a–d) Histology analysis of calvarial bones from 13-week-old female sham-operated mice (control), and ovariectomized (OVX) mice injected with AAV-YFP or AAV-Gα13CA. (a) Radiographic (higher panels) and μ-CT analysis (lower panels). High-magnification images are co-presented in the right corners. (b) Quantification of bone volume per tissue volume ($BV/TV$); $N=3$. (c) Whole calvaria TRAP staining; Quantification of relative TRAP positive area is co-presented on the right panel; $N=4$. (d) TRAP staining of calvaria frozen sections. Quantification of osteoclast and bone resorption metrics are presented in lower panel; OC.N/B.Pm, osteoclast number per bone perimeter; OC.N/BS, osteoclast number per bone surface; ES/BS, eroded surface per bone surface; $N=4$. (e) TRAP staining using frozen sections of calvarial bones from 8-week-old male WT and Gna13f/fLysM-Cre (f/f;LysM-Cre) mice treated with PBS, LPS, LPS + AAV-YFP or LPS + AAV-Gα13CA. (f) Quantification of TRAP-positive cells per bone surface and marrow cavity area in e; $N=6$. Results were expressed as mean ± s.d.; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$ (Student’s t-test or ANOVA analysis). Scale bars in a,c 200 μm; scale bars in d,e, 1 mm.
In vitro osteoclast differentiation assay. Mature osteoclasts were generated as described\(^2\). Briefly, isolated BMMs from C57BL/6 mice were cultured in MEM (pH 6.9) containing 10% FBS, 10 ng ml\(^{-1}\) recombinant RANKL and 10 ng ml\(^{-1}\) recombinant M-CSF for 5 days. Recombinant murine M-CSF and RANKL were obtained from R&D Systems, Inc. Cell culture medium was obtained from Gibco, Life Technologies Corporation. Mature osteoclasts were characterized by staining for TRAP activity using a commercial kit (Sigma-Aldrich, 387A-1KT) and TRAP\(^{+}\) MNCs were enumerated per well in a 24-well plate.

In vitro bone resorption assay. For bone resorption assay, osteoclasts were seeded on bovine bone slides. Concentration of bovine cross-linked C-telopeptide of type I collagen (CTX-I) in the medium was measured using CrossLaps for Culture ELISA (CTX-I) kit (Immunodiagnostics Systems Limited) following the manufacturer’s instructions. Bone resorption pits were sonicated in PBS, stained with 2 μg ml\(^{-1}\) WGA-lectin (Sigma-Aldrich, L-4895) and then incubated with mouse anti-mouse Ctsk primary antibody (1:100, Santa Cruz sc-48353, London) for two hours and then with FITC goat anti-mouse IgG (H+L) secondary antibody (Immunodiagnostic Systems Limited). Nuclei were visualized by 1 μg ml\(^{-1}\) DAPI (4’,6-diamidino-2-phenylindole; Sigma-Aldrich, D9542). Three-dimensional images were taken by confocal microscopy and constructed by Imaris software\(^4\).

Cellular immunofluorescent staining. For F-actin ring staining, osteoclasts on bovine bone slides were incubated with 2 μl ml\(^{-1}\) Texas Red-X Phalloidin (Life Technologies, T7471). For anti-TRAP immunofluorescent staining, osteoclasts on bovine bone slides were fixed by 4% formaldehyde, permeabilized with 0.2% Triton X-100, blocked with 5% goat serum and 100 μl mg\(^{-1}\) un-conjugated AfinnPure Fab Fragment Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Labs), incubated with mouse anti-mouse Ctsk primary antibody (1:100, Santa Cruz sc-48353, London) and then with FITC goat anti-mouse IgG (H+L) secondary antibody (Immunodiagnostic Systems Limited). Nuclei were visualized using 1 μg ml\(^{-1}\) DAPI (4’,6-diamidino-2-phenylindole; Sigma-Aldrich, D9542). Three-dimensional images were taken by confocal microscopy and constructed by Imaris software\(^4\).

Quantitative reverse transcription-polymerase chain reaction (qPCR) assay. Total RNA was isolated from cultured cells with TRizol reagent (Life Technologies, 15596018). Mouse complementary DNA (cDNA) was reverse-transcribed from 0.5 μg total RNA with SuperScript VILO Master Mix (Life Technologies, 11755050). Quantitative reverse transcription–PCR performed on StepOne Real-Time PCR System (Life Technologies, Applied Biosystems) using the TaqMan Gene Expression assays (Life Technologies, Applied Biosystems) or SYBR Green reagents (Fisher Scientific Inc) was used. Western blot was performed using following antibodies: anti-p84 mouse IgG (GeneTex GTX70220, 1:1,000), anti-IκBα rabbit IgG (Cell Signaling Technology 2831S, 1:1,000) for the phosphorylation assay, BMMs and osteoclasts were starved for 5 h and stimulated with RANKL or M-CSF for different times. Protein expression was observed by autoradiography. The data are represented as mean ± standard deviation.

Western blotting and phosphorylation assay. Cell lysates preparation and SDS-polyacrylamide gel electrophoresis (western blotting analysis) were carried out according to a standard protocol\(^2\). For the phosphorylation assay, BMMs and primary osteoclasts derived from BMMs with 2-day treatment of RANKL and M-CSF were starved for 5 h and stimulated with RANKL or M-CSF for different times. Proteins were harvested in cell lysis buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich Co. LLC, 1:100) and phosphatase inhibitor cocktail 2 (P-5726, Sigma-Aldrich Co. LLC, 1:100). To extract nuclear and cytoplasmic proteins, Chromatin Immunoprecipitation Reagent Kit (Thermo Fisher Scientific Inc) was used. Western blot was performed using following antibodies: anti-p84 mouse IgG (GeneTex GTX70220, 1:1,000), anti-IκBα rabbit IgG (Cell Signaling Technology 4812S, 1:1,000), anti-phospho-IκBα (Ser32) rabbit IgG (Cell Signaling Technology 2839S, 1:1,000), anti-p38 rabbit IgG (Cell Signaling Technology 9212S, 1:1,000), anti-phospho-p38 (Thr180/Tyr182) rabbit IgG (Cell Signaling Technology 4631S, 1:1,000), anti-Erk rabbit IgG (Cell Signaling Technology 4370S, 1:1,000), anti-phospho-Erk (Thr202/Tyr204) rabbit IgG (Cell Signaling Technology 4693S, 1:1,000), anti-Akt rabbit IgG (Cell Signaling Technology 4685S, 1:1,000), anti-phospho-Akt (Thr308) rabbit IgG (Cell Signaling Technology 2665S, 1:1,000), anti-phospho-JNK rabbit IgG (Cell Signaling Technology 9251S, 1:1,000), anti-phospho-JNK (Thr183/Tyr185) rabbit IgG (Cell Signaling Technology 9258S, 1:1,000), anti-c-Jun rabbit IgG (Cell Signaling Technology 9165S).
GSK3

IRES-Bla (referred to as pHR in the following text) was a gift from Dr Ling Tian in (pCMV-VSV-G and pCMV-Dr8.92) were purchased from Addgene. pHR-EF-anti-GAPDH mouse IgG (Santa Cruz sc-166574, 1:500), anti- G12

ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms13700

TRAP staining and immunofluorescent staining.

For lentivirus production, lentivirus vectors (with 10% pLB vector or 10% pHR vector) and packaging plasmids were co-transfected into HEK-293T cells using a retrovirus vector (titer 109-10 ml-1) expressing YFP or G13CA. Mice were harvested 5 weeks after injection of LPS (sigma) at 25 mg kg-1 body weight or PBS and analysed after 35 days after the first injection. Samples were harvested 35 days after the first injection, and Safranin O (SO) stain, H&E stain analysis were performed before and 35 days after the first injection. Samples were harvested 35 days after the first injection, and Safranin O (SO) stain, H&E stain and TRAP staining were performed. Hind paw volume was quantified by water displacement method55. Bone destruction in X-ray was quantified by Larsen method in RA lesion area56. Cartilage destruction was assessed using safranin O staining and was measured as OARSI grade in ankle joint area55.

Statistics. Data represent mean ± s.d. Analysis was performed using GraphPad Prism, version 5. Statistical significance was assessed using an two-tailed Student’s t-test or ANOVA analysis, as indicated in the figure legend, considering a P value ≤0.05 as significant.

Data availability. The authors declare that the data supporting the findings of this study are available within the article and its Supplementary Information files.

References

1. Takayanagi, H. New immune connections in osteoclast formation. Nat. Rev. Immunol. 11, 1197–1202 (2011).

2. Boyle, W. J., Simonet, W. S. & Lacey, D. L. Osteoclast differentiation and activation. Nature 423, 375–378 (2003).

3. Teitelbaum, S. L. & Ross, F. P. Genetic regulation of osteoclast development and function. Nat. Rev. 4, 638–649 (2003).

4. Chen, W. et al. C/EBPα regulates osteoclast lineage commitment and function. Nat. Cell Biol. 11, 7294–7299 (2013).

5. Chen, W. et al. Novel pycnodysostosis mouse model uncovers cathepsin K function as a potential regulator of osteoclast apoptosis and senescence. Hum. Mol. Genet. 16, 410–423 (2007).

6. Li, Y. P. et al. Cloning and complete coding sequence of a novel human cathepsin K homolog expressed in giant cells of osteoclastomas. J. Bone Miner. Res. 10, 1197–1202 (1995).

7. Li, Y. P., Chen, W. & Stashenko, P. Molecular cloning and characterization of a novel human cathepsin K. J. Biol. Chem. 268, 20275–20280 (1993).

8. Zhuo, Y., Gauthier, J. Y., Black, W. C., Percival, M. D. & Duong, L. T. Inhibition of bone resorption by the cathepsin K inhibitor odanacatib is fully reversible. Biochem. Biophys. Res. Commun. 281, 723–728 (2001).

9. Chen, W. et al. C/EBPs regulate osteoclast lineage commitment. Proc. Natl Acad. Sci. U.S.A. 110, 7294–7299 (2013).

10. Yang, S. L. & Li, Y. P. RGS10-null mutation impairs osteoclast differentiation resulting from the loss of [Ca2+]i oscillation regulation. Genes Dev. 21, 1803–1816 (2007).

11. Sielher, S. Regulation of RhoGEF proteins by G12/13-coupled receptors. Br. J. Pharmacol. 158, 41–49 (2010).

12. Wozefeld, T., Wettstuchrek, N. & Offermanns, S. G12(G13)-mediated signalling in mammalian physiology and disease. Trends Pharmacol. Sci. 29, 582–589 (2008).

13. Chen, W. & Li, Y. P. Generation of mouse osteoclastogenic cell lines immortalized with SV40 large T antigen. J. Bone Miner. Res. 13, 1112–1123 (1998).

14. Moers, A. et al. G13 is an essential mediator of platelet activation in hemostasis and thrombosis. Nat. Med. 9, 1418–1422 (2003).

15. Yagi, M. et al. DC-STEMP is essential for cell-cell fusion in osteoclasts and foreign body giant cells. J. Exp. Med. 202, 345–355 (2005).

16. Lee, S. H. et al. v-ATPase V0 subunit d-2-deficient mice exhibit impaired osteoclast fusion and increased bone formation. Nat. Med. 12, 1403–1409 (2006).

17. Zhou, Y., Gauthier, J. Y., Black, W. C., Percival, M. D. & Duong, L. T. Inhibition of bone resorption by the cathepsin K inhibitor odanacatib is fully reversible. Bone 67, 269–280 (2014).

18. Kanemitsu, J. et al. A beta subunit of F-actin containing podosomes is involved in bone resorption. Bone 117, 287–299 (2015).

19. Muppidi, J. R. et al. Loss of signalling via Gα13 in germline C-cell-derived lymphoma. Nature 516, 254–258 (2014).

20. Moon, J. B. et al. Akt induces osteoclast differentiation through regulating the GSK3β/β-NFATc1 signaling cascade. J. Immunol. 188, 163–169 (2012).

21. Jaffe, L. M., Lee, L. Y., Smith, D. L., Wright, C. & Lee, S. Y. Phosphorylation of the Akt/GSK-3beta axis during RANKL signaling. Bone 55, 126–131 (2013).

22. Zhang, W. et al. TRIB3 mediates glucose-induced insulin resistance via a mechanism that requires the hexosamine biosynthetic pathway. Diabetes 62, 4192–4200 (2013).

23. Dayal, S. & Berns, K. J. Gene therapy using adeno-associated virus vectors. Clin. Microbiol. Rev. 21, 583–593 (2008).

24. Keffer, J. et al. Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. EMBO J. 10, 4025–4031 (1991).
25. Chiang, C. Y., Kyritsis, G., Graves, D. T. & Amar, S. Interleukin-1 and tumor necrosis factor activities partially account for calvarial bone resorption induced by lipopolysaccharide in rat osteoblasts in vitro. J. Bone Miner. Res. 13, 688–694 (1998).

26. Nakamura, I. et al. Phosphatidylinositol-3 kinase is involved in ruffled border formation in osteoclasts. J. Cell. Physiol. 172, 230–239 (1997).

27. Nakamura, I. et al. Phosphatidylinositol 3-kinase regulates osteoclastic bone resorption through protein kinase B-mediated vesicle transport. J. Bone Miner. Res. 27, 2464–2475 (2012).

28. Takeshita, S. et al. SHIP-deficient mice are severely osteoporotic due to increased numbers of hyper-resorptive osteoclasts. Nat. Med. 8, 943–949 (2002).

29. Chellaiah, M. et al. Rho-A is critical for osteoclast podosome organization, motility, and bone resorption. J. Biol. Chem. 275, 11993–12002 (2000).

30. Destaing, O. et al. Novel Rho-mDia2-HDAC6 pathway controls podosome patterning through microtubule acetylation in osteoclasts. J. Cell Sci. 118, 2901–2911 (2005).

31. Ory, S., Brazier, H., Pawlak, G. & Blangy, A. Rho GTPases in osteoclasts: orchestrators of podosome architecture. Eur. J. Cell Biol. 87, 469–477 (2008).

32. Nakamura, I. et al. Phosphatidylinositol-3-kinase regulates osteoclasts in vivo by local injection of lipopolysaccharide. J. Cell. Physiol. 230, 230–239 (2015).

33. Nakamura, I. et al. Phosphatidylinositol-3-kinase regulates osteoclasts in vivo by local injection of lipopolysaccharide. J. Cell. Physiol. 230, 230–239 (2015).

34. Granot-Attas, S., Luxenburg, C., Finkelshtein, E. & Elson, A. Protein tyrosine phosphatase epsilon regulates integrin-mediated podosome stability in osteoclasts by activating Src. Mol. Biol. Cell 20, 4324–4334 (2009).

35. Ioannidis, J. P. et al. Meta-analysis of genome-wide scans provides evidence for sex- and site-specific regulation of bone mass. J. Bone Miner. Res. 22, 173–183 (2007).

36. Wilson, S. G. et al. Comparison of genome screens for two independent cohorts provides replication of suggestive linkage of bone mineral density to 3p21 and 1p36. Am. J. Hum. Genet. 72, 144–155 (2003).

37. Mullin, B. H. et al. Further genetic evidence suggesting a role for the RhoGTPase-RhoGEF pathway in osteoporosis. Bone 45, 387–391 (2009).

38. Mullin, B. H. et al. Identification of a role for the ARHGEF3 gene in postmenopausal osteoporosis. Am. J. Hum. Genet. 82, 1262–1269 (2008).

39. Martin, T. J. & Sims, N. A. Osteoclast-derived activity in the coupling of bone formation to resorption. Trends Mol. Med. 11, 76–81 (2005).

40. Nakamura, T. et al. Estrogen prevents bone loss via estrogen receptor alpha and induction of Fas ligand in osteoclasts. Cell 130, 811–823 (2007).

41. Su, X. et al. The ADP receptor P2RY12 regulates osteoclast function and pathologic bone remodeling. J. Clin. Invest. 122, 3579–3592 (2012).

42. Zhao, H. et al. Synaptotagmin VII regulates bone remodeling by modulating osteoclast and osteoblast secretion. Dev. Cell 14, 914–925 (2008).

43. DeSelms, C. J. et al. Autophagy proteins regulate the secretory component of osteoclastic bone resorption. Dev. Cell 21, 966–974 (2011).

44. Yang, D. Q. et al. V-ATPase subunit ATP6API (Ac45) regulates osteoclast differentiation, extracellular acidification, lysosomal trafficking, and protease exocytosis in osteoclast-mediated bone resorption. J. Bone Miner. Res. 27, 1695–1707 (2012).

45. Feng, S. et al. Atp6v1c1 is an essential component of the osteoclast proton pump and F-actin ring formation in osteoclasts. Biochem. J. 417, 195–203 (2009).

46. Ory, D. S., Neugeboren, B. A. & Mulligan, R. C. A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. Proc. Natl Acad. Sci. USA 93, 11400–11406 (1996).

47. Asagiri, M. et al. Cathepsin K-dependent toll-like receptor 9 signaling revealed in experimental arthritis. Science 319, 624–627 (2008).

48. Boini, S. & Guillouin, F. Radiographic scoring methods as outcome measures in rheumatoid arthritis: properties and advantages. Ann. Rheum. Dis. 60, 817–827 (2001).

49. Glasson, S. S., Chambers, M. G., Van Den Berg, W. B. & Little, C. B. The OARSI histopathology initiative—recommendations for histological assessments of osteoarthritis in the mouse. Osteoarthr. Cartil. 18, S17–S23 (2010).

Acknowledgements

We would like to thank Dr S. Offermanns from Max-Planck-Institute for kindly giving us the Gna13floxed mice. We would like to thank Dr Garvey from UAB for kindly giving us the pHK-ER-FRER-BlA vector, and Dr Xu Feng from UAB for kindly giving us the pMXs-3xFLAG-ires-puro retroviral vector and 293GPG retroviral packaging cell line. We also would like to thank Dr Joel Jules, Matthew McConnell and Dr Jay M. McDonald for their extensive reading, discussion and excellent assistance with this manuscript. We appreciate the assistance of the Center for Metabolic Bone Disease at the University of Alabama at Birmingham (UAB) (P30 AR046031). We are also grateful for the assistance of the UAB Small Animal Phenotyping Core, the UAB Metabolism Core, the UAB Vector Production Facility and the UAB Neuroscience Molecular Detection Core Laboratory (P30 NS047466). This work was supported by NIH grants R01-AR-044741 (Y.-P.L.) and R01-DE-023813 (Y.-P.L.).

Author contributions

Y.-P.L., M.W. and W.C. designed the experiments. M.W., W.C., Y.L., G.Z. and L.H. conducted the studies. M.W., W.C., Y.L., G.Z., I.H. and Y.-P.L. collected the data and analysed the data. M.W., W.C. and Y.-P.L. drafted the manuscript. All authors approved the final version of the manuscript for submission.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Wu, M. et al. Gna13 negatively controls osteoclastogenesis through inhibition of the Akt-GSK3β-NFATc1 signalling pathway. Nat. Commun. 8, 13700 doi: 10.1038/ncomms13700 (2017).

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.