Osteoblastic molecular scaffold Gab1 is required for maintaining bone homeostasis

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Summary

The Grb2-associated binder 1 (Gab1), which serves as a scaffolding adaptor protein, plays a crucial role in transmitting key signals that control cell growth, differentiation and function from multiple receptors. However, its biological role in osteoblast activity and postnatal bone metabolism remains unclear. To elucidate the in vivo function of Gab1 in postnatal bone remodeling, we generated osteoblast-specific *Gab1* knockout mice. Disruption of Gab1 expression in osteoblasts led to decreased trabecular bone mass with a reduced bone formation rate and a decreased bone resorption. Bones from *Gab1* mutants also exhibited inferior mechanical properties. Moreover, primary osteoblasts from *Gab1* mutant mice demonstrated markedly suppressed osteoblast mineralization, increased susceptibility to apoptosis and decreased expression of receptor activator of NF-κB ligand (RANKL). Activation of serine-threonine Akt kinase and extracellular signal-regulated kinase in response to insulin and insulin-like growth factor 1 was attenuated in *Gab1* mutant osteoblasts. Our results show that Gab1-mediated signals in osteoblasts are crucial for normal postnatal bone homeostasis.

Key words: Gab1, Osteoblast, Bone mass, AKT, ERK, Apoptosis

Introduction

Dysregulation of osteoblast and osteoclast activities and consequent loss of bone homeostasis are associated with a range of pathological diseases including osteoporosis (Karsenty and Wagner, 2002). Previous studies have shown that insulin and insulin-like growth factor 1 (IGF-1) have anabolic effects on osteoblasts through serine-threonine Akt kinase (AKT) activation to maintain bone mass and turnover (Fujita et al., 2004; Fulzele et al., 2007; Scheid and Woodgett, 2001; Thomas et al., 1996). Human beings and animals with diabetes frequently exhibit impaired bone mineralization and defective bone strength (Thrailkill et al., 2005). However, the molecular mechanisms underlying the osteoblastic responses to IGF-1 and insulin during osteoblast development and function have not been fully investigated.

The growth factor receptor-bound protein 2 (Grb-2)-associated binder (Gab) proteins are scaffolding adapter molecules, consisting of Gab1, Gab2 and Gab3, which are engaged in signal relay from cytokine and growth factor receptors (Liu and Rohrschneider, 2002). On stimulation, Gab proteins undergo rapid tyrosine phosphorylation, creating multiple docking sites to recruit and activate Src homology-2 (SH2) domain-containing proteins such as Shp2 (Src homology 2 containing tyrosine phosphatase) and phosphoinositide 3-kinase (PI3K) (Holgado-Madruga et al., 1996; Takahashi-Tezuka et al., 1998). For Gab1 and Gab2, the Gab-SHP2 interaction is considered to be an essential component for extracellular signal-regulated kinase (ERK) activation, whereas the association between Gab and the p85 subunit of PI3K is important for mediating the PI3K-AKT signaling pathway (Gu and Neel, 2003). Reports on Gabs knockout mice have clearly demonstrated the crucial roles of the Gab proteins during development and in maintenance of tissue homeostasis (Bard-Chapeau et al., 2005; Bentires-Alj et al., 2006; Cai et al., 2002; Gu et al., 2001; Itoh et al., 2000; Nakaoka et al., 2007; Nishida et al., 2002; Sachs et al., 2000; Wada et al., 2005). Gab1 is ubiquitously expressed within various tissue cell types, and plays a crucial role in transmitting key signals that control a diverse set of biological responses (Bard-Chapeau et al., 2005; Cai et al., 2002; Holgado-Madruga et al., 1997; Ingham et al., 2001; Itoh et al., 2000; Sachs et al., 2000; Yamasaki et al., 2003). Disruption of the *Gab1* gene in mice results in embryonic lethality caused by impaired development of the heart, placenta and skin (Itoh et al., 2000). *Gab2* knockout mice exhibit impaired allergic responses and osteopetrosis caused by defective osteoclastogenesis (Gu et al., 2001; Wada et al., 2005). Although crucial roles of Gab1 involved in the signal transduction of many growth factors have been proposed, the function of Gab1 in postnatal bone metabolism remains poorly understood.

In this report, we show that Gab1 is required for osteoblast activity and bone homeostasis. Osteoblast-specific disruption of *Gab1* led to low trabecular bone mass with decreased bone formation and impaired bone mechanical properties. Furthermore, *Gab1* deficiency is associated with declined mineralization and increased susceptibility to apoptosis in osteoblasts. We also show that activation of AKT and ERK in response to IGF-1 and insulin is attenuated in *Gab1*-deficient osteoblasts.

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Results

Targeted ablation of *Gab1* in osteoblasts causes decreased bone mass

To elucidate the physiological role of Gab1 in osteoblast function and bone homeostasis, we generated an osteoblast-specific *Gab1* knockout mouse line by breeding the *Gab1*$^{flox/flox}$ mouse (Wada et al., 2005) with the *Osteocalcin-Cre* transgenic mouse (*OC-Cre*) (Tan et al., 2007). The *Gab1*$^{flox/flox}$*;OC-Cre* mice (*Gab1* cKO) were born normally and were fertile. Cre-mediated deletion of exon 3 of *Gab1* in different tissues isolated from a *Gab1*$^{flox/flox}$*;OC-Cre* mouse revealed that an efficient excision occurred exclusively in calvaria, femurs and spine, all of which were bone tissues containing osteoblasts (supplementary material Fig. S1A,B).

X-ray and three-dimensional microcomputed tomography (micro-CT) scanning analyses of vertebrae and femora revealed that male *Gab1* mutant mice exhibited lower trabecular bone mass than their wild-type littermates at 6.5 months of age (Fig. 1A-C). Cortical bone thickness, the most important parameter for biomechanical strength, was reduced from 268.7±54.9 μm in controls to 199.9±32.5 μm in *Gab1* mutant mice at 6.5 months of age (Fig. 1D). Bone mineral density (BMD) of femurs from male mutant mice was about 20% lower than that of controls at 2 months and 6.5 months of age (Fig. 1E). Consistently, BMD of female mutant femurs exhibited a significant decrease as compared with sex-matched controls at 2 months and 15 months (Fig. 1F). Micro-CT analysis in trabeculae from female femurs also showed decreased bone mass at 2 months of age (supplementary material Fig. S2A-H). Detailed histological analysis of tibias revealed decreased bone mass in mutants at the age of 2 months (Fig. 1G). We also generated another osteoblast-specific *Gab1* knockout mouse by breeding the *Gab1*$^{flox/flox}$ mouse with the *Collagen1a1-Cre* transgenic mouse (*Col1a1-Cre*) (Zha et al., 2008). The *Gab1*$^{flox/flox}$*;Col1a1-Cre* mice exhibited similar phenotypes as *Gab1*$^{flox/flox}$*;OC-Cre* mice (supplementary material Fig. S3A-F). The results showed that targeted disruption of *Gab1* in osteoblasts resulted in osteopenia in mutant mice.

Gab1 deficiency leads to decreased bone formation

To determine the cellular basis underlying the bone mass loss in *Gab1* mutants, we performed bone histomorphometric analysis of the proximal tibias of male mice at 2 months of age. von Kossa staining showed a significantly decreased bone volume in the mutant mice (Fig. 2A-D). The ratio of trabecular bone volume to tissue volume (BV/TV) in tibias of *Gab1*-deficient mice was 33% lower than that of controls (Fig. 2G). The decreased bone volumes were correlated with decreased trabecular number (TbN) and increased trabecular separation (Tb.Sp) in mutants compared with controls (Fig. 2H,I). In addition, trabecular thickness was decreased in mutant mice (Fig. 2J). Number of osteoblasts per tissue area (N.Ob/T.Ar) and number of osteoblasts per bone perimeter (N.Ob/B.Pm) were significantly decreased in *Gab1* mutants (Fig. 2K,L). Double calcein labeling revealed a dramatically declined bone formation rate (BFR) in 2-month-old mutant mice as compared with controls (Fig. 2E,F). The ratio of bone formation rate to bone surface (BFR/BS) and the mineral appositional rate (MAR) were also markedly reduced in mutants (Fig. 2M,N). These results suggested that loss of osteoblastic *Gab1* resulted in impaired osteoblast function.

Loss of *Gab1* in osteoblasts causes reduced osteoclast activity

Decreased osteoblast activity might result in the reduction of osteoclast function. As expected, we found that *Gab1*-deficient mice had less numbers of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts on tibia sections at 2 months of age (Fig. 3A-D). Bone histomorphometric analyses revealed that all bone resorption parameters, the percentage of bone surface covered by mature osteoclasts (Oc.S/BS), the number of mature osteoclasts in tissue area (N.Oc/T.Ar) and the number of mature osteoclasts (N.Oc/B.Pm) were significantly decreased in *Gab1* mutant mice (Fig. 3E-G). Real-time PCR analysis showed that expression of genes encoding TRAP, cathepsin K (CathK) and matrix metalloproteinase 9 (MMP-9), lysosomal enzymes essential for osteoclastic bone resorption, were markedly reduced in femoral bones of *Gab1* mutant mice (Fig. 3H-J). Co-culture of calvarial osteoblasts and a common osteoclast progenitor population derived from the wild-type spleen showed that TRAP-positive cells were significantly reduced, when osteoblasts were derived from *Gab1* mutant mice as compared with those from controls (Fig. 3K,L).

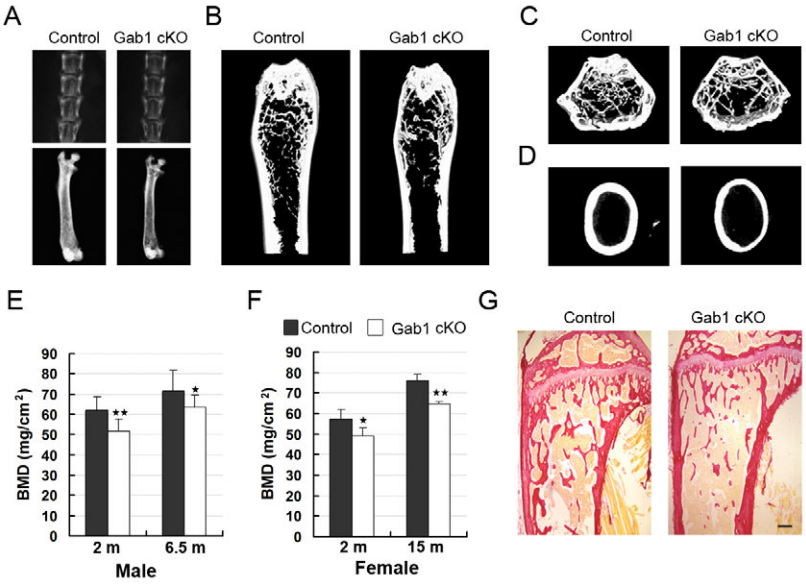

Fig. 1. Disruption of *Gab1* in osteoblasts leads to osteopenia. (**A**) Radiographic images of control (*Gab1*$^{flox/flox}$) and *Gab1* cKO (*Gab1*$^{flox/flox}$*;OC-Cre*) spine (upper) and tibia (lower) from 6.5-month-old mice. (**B**,**C**) 3D microCT images of femurs. (**D**) Cortical micro-CT in the midshaft on femur from control and *Gab1* cKO mice. (**E**) Femur BMD was remarkably decreased in both 2-month-old and 6.5-month-old male *Gab1* mutant mice. (**F**) Femur BMD was decreased in both 2-month-old and 15-month-old female *Gab1* mutant mice. In E and F, **P*<0.05, ***P*<0.01. All values are mean ± s.d. from five control (black bars) or *Gab1* mutant mice (white bars). (**G**) Histological analysis of tibias bone in *Gab1* mutant and control mice. Scale bar: 300 μm.

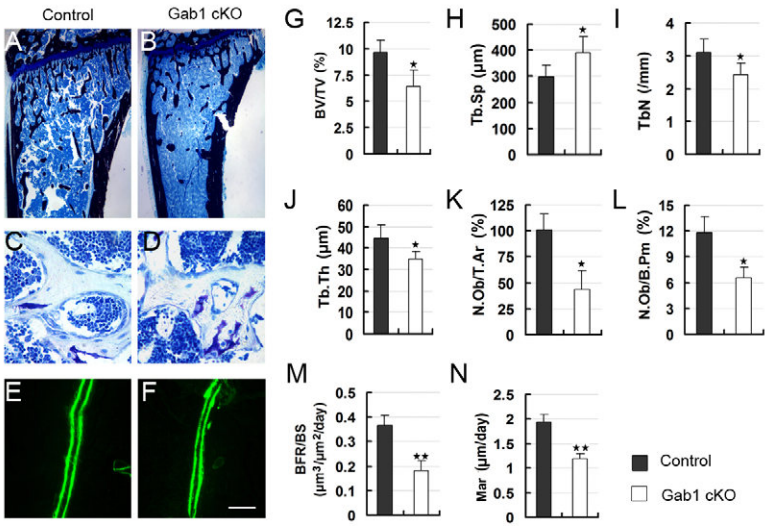

Fig. 2. Decreased bone formation in *Gab1* mutant mice. (**A**,**B**) von Kossa staining of the proximal tibia sections of *Gab1* cKO (B) and control male mice (A). (**C**,**D**) Toluidine blue staining showed a decrease in the number of *Gab1* cKO osteoblasts. (**E**,**F**) Representative calcein-labeled sections of tibia from control (E) and *Gab1* cKO male mice (F). (**G-N**) Quantitative histomorphometric measurements were performed on the spongiosa at the proximal tibias of 2-month-old male mice. (G) BV/TV, bone volume/tissue volume; (H) Tb.Sp, trabecular separation; (I) TbN, trabecular number; (J) Tb.Th, trabecular thickness; (K) N.Ob/T.Ar, osteoblast number/tissue area; (L) N.Ob/B.Pm, osteoblast number/bone perimeter; (M) BFR/BS, bone formation rate/bone surface; (N) MAR, mineral apposition rate. All values are mean ± s.d. from five control (black bars) or *Gab1* cKO mice (white bars). **P*<0.05, ***P*<0.01. Scale bars: 600 μm (A,B); 50 μm (C-D); 100 μm (E-F).

Furthermore, expression of the receptor activator of NF-κB ligand (RANKL) was markedly downregulated in *Gab1* mutant osteoblasts, whereas the expression of osteoprotegerin (OPG) was not changed (Fig. 3M,N). Quantitative analysis of mouse serum RANKL concentrations also showed a reduction in the *Gab1* mutant mice as compared with controls (Fig. 3O). These results indicated that the osteoclastogenic activity of the *Gab1* mutant osteoblasts was significantly decreased.

Loss of *Gab1* in osteoblasts results in inferior biomechanical properties

The mechanical properties of bone are determined by the amount and structure of bone. To investigate the role of Gab1 in the macromechanical properties of bone, we performed a three-point bending test whereby right femoral mid-diaphyses were deflected to failure by a loaded bar from above (Fig. 4A). Femurs from *Gab1*-deficienct mice exhibited altered mechanical properties, indicated

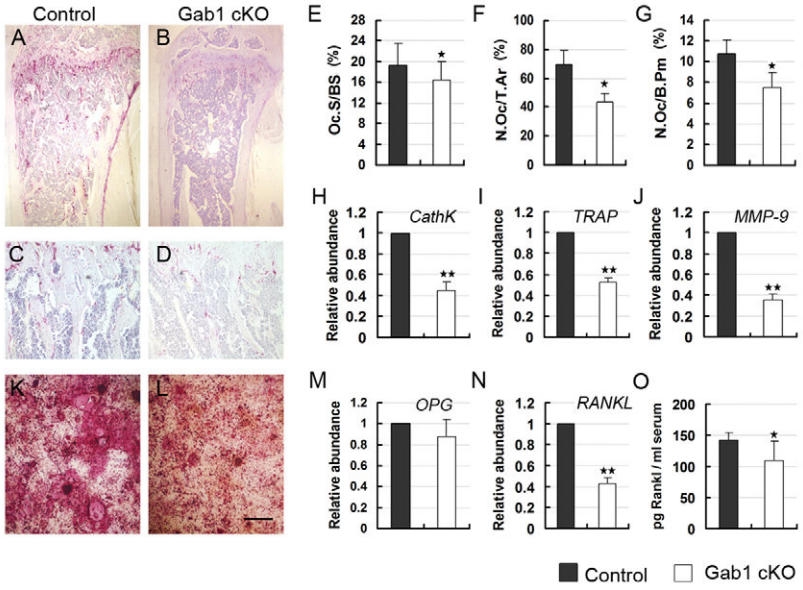

Fig. 3. Deletion of *Gab1* in osteoblasts results in decreased bone resorption. (**A-D**) Representative images of TRAP-stained proximal tibias of 2-month-old control (A,C) and *Gab1* cKO male mice (B,D). C and D show higher magnification. (**E-G**) Bone histomorphometry shows that *Gab1* mutant mice have lower ratios of osteoclast surface to bone surface (Oc.S/BS, E), number of mature osteoclasts in tissue area (N.Oc/T.Ar, F) and number of mature osteoclasts in bone perimeter (N.Oc/B.Pm, G) on the spongiosa at the proximal tibias. (**H-J**) Real-time PCR analyses of genes encoding CathK (H), TRAP (I) and MMP-9 (J) in control and *Gab1* mutant femoral bone mRNA of 2-month-old mice. (**K**,**L**) TRAP staining was performed following co-culture of osteoclast progenitor cells derived from the wild-type spleen with control (K) or *Gab1* mutant primary calvarial osteoblasts (L). (**M**,**N**) Gene expression of *OPG* (M) and *RANKL* (N) was examined by real-time PCR using the RNA isolated from the calvarial osteoblast cultures at day 7. (**O**) Detection of RANKL in the serum by ELISA revealed a reduction of RANKL in the mutant mice. All values in E-J are mean ± s.d. from five control (black bars) or *Gab1* cKO mice (white bars). Values in M and N from control (black bars) or *Gab1* mutant primary calvarial osteoblasts (white bars) are at least repeated three times. Values in O are from six wild-type or *Gab1* cKO mice. **P*<0.05, ***P*<0.01. Scale bars: 600 μm (A,B); 150 μm (C,D); 100 μm (K,L).

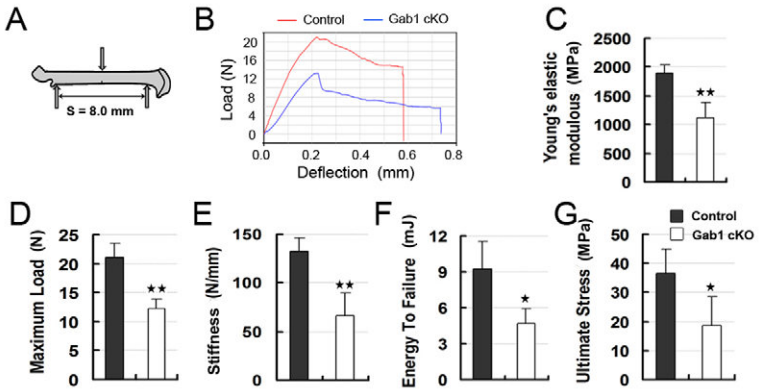

Fig. 4. Mechanical testing of femur shaft shows decreased bone strength in *Gab1* cKO mice. (**A**) Diagram of three-point bending test of femurs. (**B**) Representative image of load-deflection diagram demonstrating the differences in the mechanical properties of bone from control and *Gab1* cKO mice. (**C-G**) Impairment of biochemical properties in *Gab1* cKO mice is indicated by indices of bone strength (Young's elastic modulus, maximum load, stiffness, energy-to-failure, ultimate stress). All values in C-G are mean ± s.d. from six control (black bars) or *Gab1* cKO male mice (white bars). **P*<0.05, ***P*<0.01.

by the load-deflection diagram (Fig. 4B). Young's elastic modulus in mutant mice exhibited a 41% decrease relative to that of wild-type mice (Fig. 4C). Femurs from 2-month-old *Gab1* mutant mice showed a 42% decrease in bone strength (maximum load) as compared with wild-type controls (Fig. 4D), consistent with the decreased cortical bone area. In addition, stiffness and energy-to-failure were reduced significantly (by 49% and 50%, respectively) relative to wild-type controls (Fig. 4E,F). Ultimate stress (the stress causing bone fracture) of *Gab1*-deficient bone decreased by nearly 50% compared with controls (Fig. 4G). These results demonstrated that loss of osteoblastic *Gab1* eventually affected the intrinsic bone mechanical properties.

Gab1 deficiency suppresses osteoblast proliferation and mineralization

To explore the cellular mechanism underlying the osteopenia phenotype of *Gab1* mutant mice, primary osteoblast proliferation and function were measured in in vitro culture systems. The *Gab1* mutant calvarial osteoblasts isolated from *Gab1*$^{flox/flox}$;*Col1a1-Cre* mice showed a decreased percentage of Ki-67-positive osteoblasts as compared with that of controls (35.6±7.87% in wild type versus 21.1±1.8% in mutants) (Fig. 5A,B). Early osteoblast differentiation was evaluated by alkaline phosphotase (ALP) staining. In order to avoid the effect of reduced proliferation, we analyzed osteoblast differentiation at high density in cultures. ALP staining and relative ALP activity in cell extracts were unchanged in mutants compared with controls at all stages examined (Fig. 5C,D). Furthermore, the amount of ALP in serum revealed no obvious difference between *Gab1* mutant mice and control mice (Fig. 5E). These data suggested that *Gab1* was not necessary for early osteoblast differentiation. However, when late differentiation of osteoblast was assayed by Alizarin Red staining and von Kossa staining, the deposition of mineralized extracellular matrix (bone nodules) was reduced in *Gab1* mutants at day 18 in culture (Fig. 5F). Next, in order to identify candidate genes that might be responsible for the impaired osteoblast maturation, the expression of osteoblast marker genes was analyzed by real-time PCR in cultures at day 7. Expression levels of transcripts for collagen type 1a (*Col1a*), osteocalcin (*OC*) and bone sialoprotein (*Bsp*) were significantly reduced, whereas mRNA expression of *ALP* was not changed (Fig. 5G). In 2-month-old total bone extracts, the reduction of *Col1a*, *OC* and *Bsp* were also confirmed by real-time PCR (data not shown), which was in agreement with reduced bone mineralization in vivo. We also performed in situ hybridization on bone sections from 2-month-old mice and immunofluorescence staining on primary osteoblasts to confirm that the expression of *Col1a* was indeed decreased in *Gab1* mutants (supplementary material Fig. S4A-D). In addition, we studied whether silencing of *Gab1* in a preosteoblastic cell line could mimic the defective mineralization phenotype of osteoblasts derived from *Gab1* mutant mice. Western blot suggested that *Gab1* was knocked down efficiently in MC3T3-E1 cells (Fig. 5H). We found that interference of *Gab1* reduced cell proliferation (data not shown), decreased bone nodule formation but did not influence early differentiation (Fig. 5I). These results imply that Gab1 is essential for late differentiation and mineralization of osteoblasts.

Loss of *Gab1* in osteoblasts increases susceptibility to apoptosis

To investigate the role of *Gab1* in mediating survival signals to osteoblasts, osteoblastic apoptosis was detected using the TUNEL (terminal dUTP nick-end labeling) assay and Annexin-V flow cytometry analysis. The rate of apoptotic cells was significantly increased in cultured primary *Gab1* mutant osteoblasts relative to control cells, as revealed by the TUNEL assay (Fig. 6A,B). In addition, the percentage of Annexin-V-positive osteoblasts was significantly increased under serum-free induction (Fig. 6C,D). To confirm the anti-apoptotic role of Gab1, we performed the TUNEL assay on *Gab1*-silenced MC3T3E1-S14 preosteoblastic cells. Consistently, *Gab1*-silenced preosteoblasts also showed enhanced susceptibility to serum-deprivation-induced cell apoptosis (Fig. 6E,F). These results suggested that *Gab1* deficiency in osteoblasts enhanced their susceptibility to apoptosis.

Impairment of AKT and ERK activation in *Gab1*-deficient osteoblasts

It has been shown that Gab1 serves as a scaffolding protein downstream of several receptor protein-tyrosine kinases, including the insulin and epidermal growth factor (EGF) receptors. On stimulation of receptors, Gab proteins recruit the p85 subunit of PI3K and Shp2, resulting in activation of both AKT and ERK, respectively (Cunnick et al., 2000; Ingham et al., 2001; Koyama et al., 2008). So, we next examined whether AKT and ERK signal transduction cascades were affected by *Gab1* deficiency. We found that phosphorylation of AKT and ERK significantly decreased in primary mutant osteoblasts isolated from calvaria (Fig. 7A). Phosphorylation of AKT and ERK stimulated by IGF-1 and insulin in *Gab1*-deficient cells was in all cases lower than that of controls (Fig. 7B,C). By contrast, the levels of activation of AKT and ERK induced by basic fibroblast growth factor (bFGF) and EGF were not significantly different between *Gab1*-deficient and control

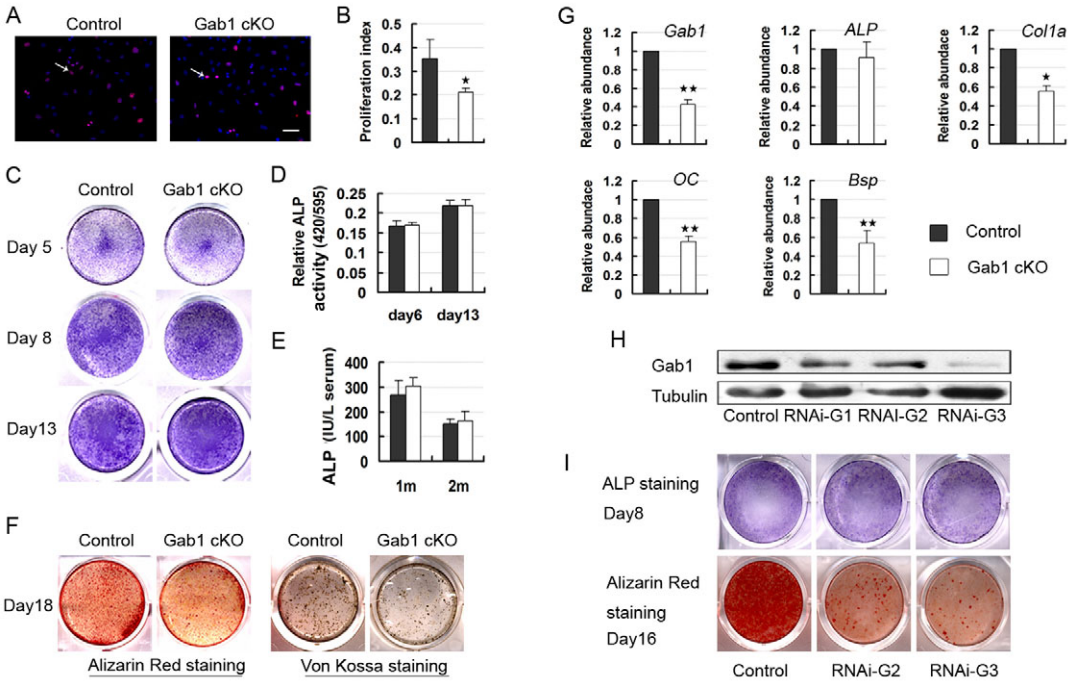

Fig. 5. Impaired osteoblast proliferation and maturation, but not early differentiation, in *Gab1* mutants. (**A**) Fluorescent immunohistochemistry of Ki-67 (red, arrows) in the primary calvarial osteoblasts. Scale bar: 100 μm (A). (**B**) The percentages of Ki-67-positive cells in *Gab1* mutant and control primary calvarial osteoblasts; n=6, *P<0.05. (**C**) Normal early osteoblast differentiation of the primary calvarial osteoblasts was shown by ALP staining at indicated stages. (**D**) Relative ALP activity was quantified between control and *Gab1* mutant osteoblasts after 6 (n=4, P=0.851) and 13 (n=6, P=0.984) days of culture. (**E**) Serum ALP levels (IU/l) in 1-month-old and 2-month-old control and $Gab1^{flox/flox}$;*OC-Cre* mice; n=5, P>0.05. (**F**) Alizarin Red (left) and von Kossa (right) staining of the calvarial osteoblast cultures demonstrates the impaired maturation of osteoblasts (mineralization) in *Gab1*-deficient mice. (**G**) Real-time PCR analysis of *Gab1*, *ALP*, *OC*, *Col1a2*, and *Bsp* mRNA expression in control and *Gab1* mutant calvarial osteoblasts after 7 days differentiation. Expression levels were normalized to *GAPDH* expression and are presented as relative expression; n=3, *P<0.05, **P<0.01. (**H**) Expression of Gab1 in MC3T3-E1 preosteoblastic cells treated with scrambled control siRNA and *Gab1* siRNAs (RNAi-G1-3). Tubulin is shown as a loading control. (**I**) ALP (upper, performed at day 8) and Alizarin Red (lower, performed at day 16) staining show that downregulation of *Gab1* by siRNA did not affect differentiation but decreased mineralization in MC3T3-E1 preosteoblastic cells. In B, D, E and G, control (black bars), *Gab1* mutant mice (white bars).

osteoblasts during the time course of the experiment (Fig. 7D,E). These data indicate that *Gab1* is involved in mediating IGF-1- and insulin-stimulated activation of AKT and ERK in osteoblasts.

Discussion

Our results firmly establish that Gab1 has a function in bone metabolism that is distinct from that of Gab2, although there is a high degree of homology and structural similarity between Gab1 and Gab2 (Nishida and Hirano, 2003). Gab2 has been shown to couple RANK to downstream signaling required for osteoclastogenesis, and has a negative regulatory role for osteoblast differentiation (Itoh et al., 2007; Wada et al., 2005). We have shown for the first time that disruption of *Gab1* in mature osteoblasts resulted in a low-bone-turnover osteopenia phenotype at 2 months of age, demonstrating an essential function of *Gab1* in osteoblasts. Altered skeleton caused by *Gab1* deletion leads to poor mechanical properties. It is conceivable that the osteopenic phenotype of osteoblast-specific *Gab1* mutant mice is largely due to the impaired activation of AKT and ERK after *Gab1* ablation. Supportively, $Akt1^{-/-}$ and $Akt2^{-/-}$ mice exhibit skeletal abnormalities with decreased bone mass (Kawamura et al., 2007), whereas activation of AKT by *Pten* deletion in osteoblasts leads to the osteopetrosis phenotype (Liu et al., 2007). In addition, ERK-MAPK activation has been demonstrated to stimulate osteoblast differentiation and skeletal development through a pathway involving RUNX2 (Ge et al., 2007).

Gab1 appears to positively regulate osteoblast maturation. Osteoblastic differentiation undergoes several stages, including proliferation, extracellular matrix deposition, matrix maturation, and mineralization (Stein and Lian, 1993). Surprisingly, Gab1 has little effect on early osteoblast differentiation, as suggested by comparable early marker ALP-positive osteoblasts in both control and *Gab1* mutant mice. *Gab1* mutants suffered from a decreased bone formation rate and a defect in late differentiation and bone mineralization. These results were confirmed by a decreased number of mineralized nodules in cultured *Gab1* mutant calvarial osteoblasts and osteoblastic cell line MC3T3-E1. Consistently, expression of *OC* and *Bsp*, late markers of osteoblast differentiation, were significantly reduced in both *Gab1* mutant bones and primary osteoblasts. In addition, synthesis of extracellular matrix *Col1a* was markedly decreased. Impaired late differentiation of osteoblast and insufficient expression of the bone matrix components might account for the low-bone-mass phenotype and the impaired mechanical properties of bone from *Gab1* mutant mice.

Gab1 is essential for regulating osteoblast survival. Gab1 has been shown to play an important positive role in cell survival. Mutant Gab1 lacking PI3K-binding sites induces apoptosis and overexpression of Gab1 in PC12 cells alone is sufficient to protect

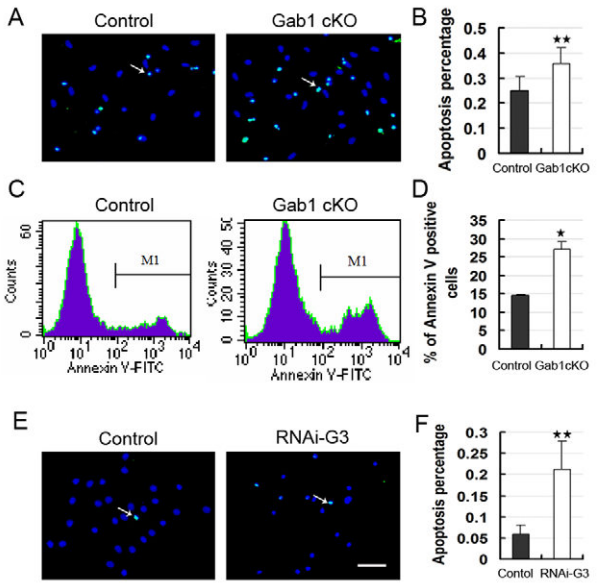

Fig. 6. Disruption of *Gab1* is associated with increased apoptosis. (**A**) TUNEL assay of primary calvarial osteoblasts isolated from control and *Gab1*-deficient mice. The primary osteoblasts were cultured in a serum-free medium for 24 hours before analysis. (**B**) The percentages of TUNEL-positive cells in control and mutant primary calvarial osteoblasts is shown; n=3, $^{**}P$<0.01. (**C**) Flow cytometric analyses of Annexin-V expression in primary osteoblasts isolated from control and *Gab1* mutant mice. A representative experiment is shown. (**D**) Quantification shows that the percentage of Annexin-V-positive cells is increased in *Gab1* mutant osteoblasts. $^{*}P$<0.05. (**E**) TUNEL assay shows that downregulation of Gab1 by siRNA increased apoptosis in MC3T3-E1 preosteoblastic cells. (**F**) The percentages of TUNEL-positive cells in *Gab1* knockdown and control MC3T3-E1 preosteoblastic cells is shown; n=5, $^{**}P$<0.01. Scale bar: 100 μm (A,E).

cells from apoptosis (Holgado-Madruga et al., 1997; Korhonen et al., 1999). The Shp2 interaction with Gab1 has been well studied, and deletion of *Shp2* in trophoblast stem cells leads to increased apoptosis (Yang et al., 2006). Inhibition of PI3K and AKT and of MAPK and ERK activities strongly increased osteoblast apoptosis under serum-free conditions (Liang et al., 2008). In this study, we showed that both primary *Gab1*-deficient osteoblasts and *Gab1* knockdown pre-osteoblastic cells exhibited increased apoptosis, providing evidence to demonstrate that Gab1 played a protective role in osteoblast apoptosis.

More importantly, disruption of adaptor Gab1 in osteoblasts resulted in decreased expression of RANKL, which in turn affected osteoclast differentiation. The OPG-RANKL system has long been known as a key regulator that couples the function of osteoblasts and osteoclasts (Kearns et al., 2008). Recently, it has been shown that the Wnt signaling pathway in osteoblasts determines osteoclastgenesis through regulating expression of OPG (Boyce et al., 2005; Glass et al., 2005; Holmen et al., 2005), whereas Hedgehog signaling in osteoblasts promotes osteoclast formation by upregulating parathyroid hormone-related protein (PTHrP) and RANKL expression (Mak et al., 2008). We, and others, have also revealed that TGF-β signaling plays a crucial role in the regulation of the RANKL-OPG axis (Hofbauer et al., 1998; Murakami et al., 1998; Okamoto et al., 2006; Tan et al., 2007). Here, we have demonstrated that, in mature osteoblasts, signals mediated by Gab1 are required for osteoclastgenesis through positive regulation of RANKL expression.

Our results suggest that adaptor Gab1 might participate in mediating IGF-1- and insulin-triggered AKT and ERK activation in osteoblasts. IGF-1 and insulin have been shown to play important roles in the anabolic regulation of bone metabolism through effects on collagen synthesis (Kream et al., 1985; Rosen and Luben, 1983; Thomas et al., 1996) and ALP production (Canalis, 1983). Mice with osteoblast-specific deletion of IGF-1 receptor exhibited osteopenia phenotype with normal size and weight (Zhang et al., 2002). Insulin receptor substrate-1 (*Irs1*)-deficient mice show low turnover osteopenia, with the impairment of osteoblast proliferation and differentiation, and support of osteoclastogenesis (Ogata et al., 2000). Our *Gab1* mutant phenotypes are similar to those of mice deficient in IGF-1 receptor and IRS-1, described above. Overexpression of Gab1 in *Irs1*-deficient fibroblasts can partially restore biological effects mediated by IGF-1 signaling (Winnay et al., 2000). In the current study, we found that the phosphorylation levels of AKT and ERK stimulated by IGF-1 or insulin, but not by bFGF or EGF, were significantly diminished in *Gab1*-deficient osteoblasts as compared with controls, suggesting that decreased bone mass in *Gab1* mutant mice is possibly due to the reduction in sustained activation of AKT and ERK of osteoblasts in response to IGF-1 and insulin.

In summary, we have provided first genetic evidence demonstrating that osteoblastic Gab1 is essential for maintaining normal postnatal bone homeostasis and bone mechanical property. We show that selective deletion of *Gab1* in osteoblasts results in decreased proliferation, defective mineralization and increased apoptosis. Moreover, differentiation of osteoclasts is also decreased

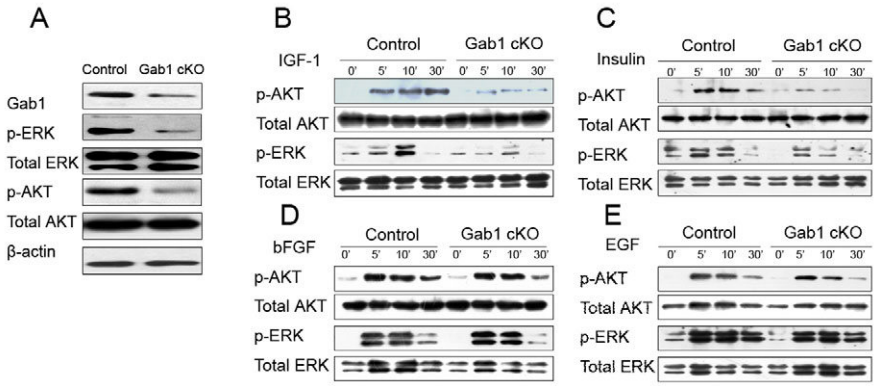

Fig. 7. Impairment of AKT and ERK activation in *Gab1*-deficient osteoblasts. (**A**) Expression of AKT and ERK phosphorylation was determined by western blotting. β-actin is shown as a loading control. (**B-E**) Reduced activation of AKT and ERK kinase is detected in *Gab1*-deficient osteoblastic cells in response to IGF-1 (B) and insulin (C), but not bFGF (D) and EGF (E). Wild-type and *Gab1*-deficient primary osteoblasts were serum-starved and then stimulated for 5, 10 and 30 minutes with 10 nM IGF-1, 100 nM insulin, 1nM bFGF or 100 ng/ml EGF.

due to impaired capacity of *Gab1* mutant osteoblasts in supporting osteoclastogenesis. Mechanistically, we show that the activation of AKT and ERK is significantly attenuated in *Gab1*-deficient osteoblasts in response to IGF-1 and insulin.

Materials and Methods

Generation of osteoblast-specific *Gab1* knockout mice

Mice lacking *Gab1* in ostoblasts were generated by breeding homozygous carrying the floxed *Gab1* alleles ($Gab1^{flox/flox}$) (Bard-Chapeau et al., 2005) with *OC-Cre* (Tan et al., 2007) or *Col1a1-Cre* (Zha et al., 2008) transgenic mice. $Gab1^{flox/flox}$*;OC-Cre* or $Gab1^{flox/flox}$*;Col1a1-Cre* mice were obtained by breeding the $Gab1^{flox/flox}$ mice with $Gab1^{flox/flox}$*;OC-Cre* or $Gab1^{flox/flox}$*;Col1a1-Cre* mice. The $Gab1^{flox/flox}$ littermates were used as controls. Animals were handled in accordance with institutional guidelines. Genotyping for *Gab1* locus and *Cre* transgene were detected by PCR analysis on genomic DNA extracted from mouse tails using primers described previously (Bard-Chapeau et al., 2005; Tan et al., 2007). Primers used to detect the specific deletion of exon 3 of *Gab1* in the bone by PCR were described previously (Bard-Chapeau et al., 2005).

Bone mineral density measurements and micro-CT

Radiographic analyses were carried out using a soft X-ray system (Contour Plus). BMD of the left femur was measured by dual energy X-ray absorptiometry with a Piximus Mouse Densitometer (GE Lunar Medical System). The femurs were scanned and reconstructed with 8 μm isotropic voxel size on a microcomputed tomography analysis (micro-CT) system (eXplore Locus SP, GE Medical Systems). The reconstructed 3D imagines of femurs were analyzed using Microviewer (GE Medical Systems). A fixed threshold (1600) was used to separate the bone and marrow phases. Specifically, trabecular bone architecture was analyzed at the distal femoral metaphysis, whereas cortical bone morphology was evaluated at the femoral midshaft.

Histological and histomorphometric analysis

Tissues were fixed in 10% formalin overnight, decalcified in 15% EDTA-PBS and embedded in paraffin. Sections 6 μm thick were cut and stained with hematoxylineosin and TRAP according to standard methods. For in vivo fluorescent labeling, 2-month-old animals were injected with calcein (20 mg/kg body weight) intraperitoneally at days 7 and 2 before tissue collection. After dehydration, the undecalcified tibiae were embedded in methylmethacrylate, and 5-μm sections were cut and stained with von Kossa stain or toluidine blue. Histomorphometric analyses were performed using OsteoMeasure (OsteoMetrics, Decatur, GA) bone analysis software. The regions of interest for trabecular bone data collection were measured in an area 1.5 mm in length, from 0.3 mm below the growth plate of the proximal tibias. All histomorphometric parameters are reported in accordance with standard criteria (Parfitt et al., 1987).

Femur biomechanical testing

To test for differences in bone failure properties between *Gab1* mutant and control mice, femur from 2-month-old mice were rehydrated at room temperature in phosphate buffered saline (PBS) and femoral biomechanical properties assessed by three-point bending. Strength tests were performed at the right femur midshaft with a displacement rate of 6 mm/minute (span length, 8 mm) using a mechanical testing machine (model 5865; Instron, Norwood, MA). Whole-bone mechanical properties, including maximum load, stiffness, energy-to-failure and Young's elastic modulus, were determined using load-deflection diagrams.

Primary cell culture

Primary osteoblasts were isolated from calvariae of 3-day-old neonatal $Gab1^{flox/flox}$*;Col1a1-Cre* mice by serial digestion using 0.1% collagenase I (Gibco) as previously described (Tan et al., 2007). Cells were grown in α-MEM containing 10% FBS until confluent. Cells were then re-plated for differentiation at 8×10^4 cells per well, or for proliferation at 2×10^4 cells per well in a 24-well plate. Osteoblasts were grown on the glasses in a 24-well plate and the Ki-67 (Abcam) assay used to detect proliferation. For differentiation, medium was supplemented with 50 mg/ml L-ascorbic acid and 10 mM β-glycerophosphate at day 2 of culture. Staining of cultures for ALP activity was conducted using Sigma Kit 86R. Bone nodules were identified morphologically by staining with Alizarin Red S (Sigma-Aldrich) and von Kossa stain. Osteoblast-osteoclast co-culture experiments were performed as described (Jochum et al., 2000). Briefly, calvarial osteoblasts (5×10^4 per 24-well culture dish) were co-cultured with nonadherent monocyte/macrophage progenitor cells derived from wild-type spleen (5×10^5 per 24-well culture dish) in medium supplemented with 10 nM 1,25-dihydroxy vitamin D3. TRAP staining was performed after 8 days of culture.

Cell death analyses

To determine the osteoblast apoptosis, primary osteoblasts and MC3T3-E1 cell line were grown on glasses in a 24-well plate and then the culture medium changed to a serum-free medium for 24 hours. Apoptotic osteoblasts were then further identified by an in situ cell-death detection kit (Promega) according to the manufacturer's instructions. FITC-labeled Annexin-V staining was used to confirm the presence of apoptosis. Osteoblasts were plated on 100-mm plates (1×10^6 per plate) and cultured for 1 day, and then cultured in serum-free medium for 24 hour before harvesting for Annexin-V–FITC staining. After staining with Annexin-V–FITC, osteoblasts were analyzed by FACS and 20,000 cells were counted.

RNA interference in MC3T3-E1

To inhibit the expression of *Gab1* in MC3T3-E1 pre-osteoblasts, three oligoribonucleotides specifically targeting mouse *Gab1* were synthesized by Invitrogen. The coding sequences were: RNAi-G1, 5′-CCTAACAGAACC-CTCTTTG-3′; RNAi-G2, 5′-ATGATGTATGACTGCCCAC-3′; and RNAi-G3, 5′-AGCCACATCCAACTCATGA-3′. The targeted oligoribonucleotides were introduced into pSUPERretro Vector, and retroviral vector pSUPERretro-Gab1 was transfected into PLAT-E cells with FuGene6 (Roche, Basel, Switzerland) to be packaged. The infection of MC3T3-E1 by retrovirus was described previously (Sun et al., 2008).

RNA isolation and real-time PCR

Total RNA was isolated from mouse bones and osteoblasts using the TRizol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (5 μg) was reverse transcribed to cDNA with the use of the first-strand cDNA synthesis kit (Invitrogen). Quantitative PCR was performed to measure the relative mRNA levels using the LightCycler system (Roche) with SYBR Green. The primers have been described previously (Tan et al., 2007).

Western blot analysis

Primary osteoblasts were plated on 60-mm plates (5×10^5 per plate) and cultured for 2 days. Culture medium was changed to α-MEM without serum for 6 hours and then stimulated with insulin (100 nM), IGF-1 (10 nM), EGF (100 ng/ml) or bFGF (1 nM). Total cell proteins were extracted by sonication in RIPA buffer containing protease inhibitors (Roche). After measurement of the protein content according to the BCA protein assay kit (Thermo Scientific, Rockford, IL), protein samples were separated on SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Primary antibodies used included: Gab1, phosphorylated ERK, phosphorylated AKT, total ERK, total AKT (Cell Signaling), β-actin (Sigma) and tubulin (Santa Cruz Biotechnology).

In situ hybridization and immunofluorescence

In situ hybridization was performed as previously described (Yang et al., 2008). For immunofluorescence, primary osteoblasts cultured for 7 days were fixed in 4% PFA for 15 minutes, washed in PBS containing 0.5% Triton X-100, blocked for 1 hour at room temperature using goat serum, and incubated with collegen antibody (1:500; Abcam) for 2 hours at room temperature. After washing, FITC-conjugated goat anti-rabbit antibody was used to detect the primary antibody. For nuclear staining, osteoblasts were treated with DAPI (Sigma).

ELISA assay

Sera were collected from 2-month-old female mice and RANKL levels determined using the Quantikine mouse RANKL ELISA kit (R&D Systems).

Statistical analysis

All results were expressed as mean ± standard deviation. All statistical analyses were performed using the SPSS software package. In the Student's *t*-test, $P<0.05$ was accepted as significant.

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