Vitamin K-dependent carboxylation in osteoblasts regulates bone resorption through GAS6

Running title: Vitamin K-dependent carboxylation and bone resorption

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Contributions

M.F. and M.P. assembled the figures. M.F. wrote the manuscript with suggestions from the other authors. M.P., M.F., D.N.T.P., J.L., B.A.R. and Y.W.K., collected and analyzed data. M.F. conceived and designed the project and provided supervision to M.P., D.N.T.P., J.L., B.A.R. and Y.W.K. A.G.A.A. and M.M. collected and analyzed the µCT data. M.F. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of data and the accuracy of data analysis.

Abstract

Studies in humans suggest that vitamin K is involved in the regulation of bone remodeling, but the precise mechanism at play remains unknown. In cells, vitamin K functions as a co-factor for the γ-glutamyl carboxylase (GGCX), an enzyme responsible for the conversion of glutamic acid residues (Glu) into γ-carboxyglutamic acid (Gla) residues in secreted proteins. We aim here at determining the role of γ-carboxylation in bone remodeling and at identifying the Gla protein(s) involved. We show that mice lacking γ-carboxylation specifically in osteoblasts (*Ggcx*$^{flox/flox}$;*OCN-Cre*) have increased bone mass at 6 months of age due to a reduced number of multinucleated bone resorbing osteoclasts. In co-culture experiments, *Ggcx*-deficient osteoblasts were less effective than control osteoblasts at supporting the generation of osteoclasts. Among all known Gla proteins, we identify GAS6 as an osteoblast-secreted γ-carboxylated factor which signals to differentiating osteoclasts. The GAS6 receptors MerTK and AXL are expressed in pre-osteoclasts and pharmacological inhibitors of AXL and MerTK block osteoclast generation in co-culture. Conversely, recombinant γ-carboxylated GAS6 dose-dependently increases the size of osteoclasts and the number of nuclei per osteoclast in culture. GAS6 marginally affected the induction of osteoclast-specific genes during osteoclast differentiation but significantly increased pre-osteoclast fusion. Finally, increasing bone marrow GAS6 level in transgenic mice was sufficient to increase the number and size of osteoclasts and to decrease bone mass. This work identifies GAS6 as a novel osteoblast derived vitamin K-dependent protein regulating osteoclast maturation.

Keywords: Osteoclast; osteoblast; vitamin K; gamma-carboxylation; GAS6; GGCX

Introduction

The acquisition and maintenance of an adequate skeleton depend on the process of bone turnover, which requires the activity of two cell types: the osteoclast, responsible for the resorption of the mineralized bone extracellular matrix (ECM) and the osteoblast, responsible for the synthesis, secretion and mineralization of the bone ECM. An imbalance between the activities of these two cell types can result in severe bone diseases such as osteoporosis. Like many chronic aging disorders, osteoporosis appears to be a multifactorial disease. Hence, although several genetic variants may contribute to the physiopathology of osteoporosis [1,2], epigenetic, environmental and behavioral factors have also been implicated [3,4]. For instance, several micronutrients including vitamins are known to play a role in the acquisition and the maintenance of a healthy skeleton and could therefore influence the etiology of osteoporosis [4,5]. This is evidenced by the profound impact that vitamins C, D or B12 deficiency has on bone density or quality in humans [6-9]. Cross-sectional and prospective studies suggest that vitamin K (VK) might be another micronutrient playing a key role in the regulation of bone mass.

Longitudinal prospective studies have reported that lower VK intake or serum levels were associated with decreased bone mineral density (BMD) [10,11] or increased fracture risk in postmenopausal women [12-14]. There are two naturally occurring forms of VK: VK_1 or phylloquinone, synthesized in plants, and VK_2 or menaquinones (e.g., MK-4 and MK-7), present in animals and fermented foods [15]. Bone was shown to contain both VK_1 and MK-4 [16,17]. A number of clinical trials have tested the effects of VK_1 or VK_2 supplementation on BMD or fracture risk, but have led to conflicting results [15]. While some studies did find that MK-4, MK-7 or VK_1 increased femoral and/or vertebral BMD and/or reduced fracture risk, others found no

significant impact [18-22]. A few meta-analyses of interventional studies suggest that VK supplementation had a significant and positive effect on BMD at lumbar spine and forearm [18,19,23], while another suggested that VK prevents fractures without impacting BMD [24]. Therefore, whether VK is directly implicated in the regulation of bone mass or quality remains controversial. Moreover, the molecular and cellular mechanisms through which VK influences bone density and/or quality remain uncharacterized.

In vertebrates, the only well described cellular function of VK is to serve as a co-factor during the γ-carboxylation reaction that converts glutamic acid (Glu) residues to γ-carboxyglutamic acid (Gla) residues in specific proteins [25]. Hence, γ-carboxylated proteins (also called "Gla" proteins) are either secreted or transmembrane polypeptides with the potential of endocrine, paracrine or autocrine functions. Two enzymes are involved in γ-carboxylation: γ-glutamyl carboxylase (γ-carboxylase or GGCX) and vitamin K oxidoreductase (VKORC1) [26]. γ-carboxylase requires reduced VK (VKH_2) as an essential cofactor, which upon carboxylation is oxidized to VK epoxide (VKO). VKO is next reconverted to VKH_2 by VKORC1.

Osteocalcin (Ocn) is an osteoblast-derived Gla protein presents at high concentration in the bone ECM. Because γ-carboxylated Ocn has great affinity for hydroxyapatite, the mineral component of bone ECM, it was originally believed to play a role in bone mineralization [27]. The analysis of *Ocn*-deficient mice or rats (*Ocn*$^{-/-}$) suggested that the absence of osteocalcin may slightly increase bone formation and bone density [28,29]. However, two other studies involving different strains of *Ocn*$^{-/-}$ mice did not detected any impact on bone homeostasis [30,31]. Altogether, these genetic data do not support a major role for Ocn in bone mass accrual or turnover, suggesting that the

mechanism and the Gla protein(s) through which VK may influence bone health remain to be characterized.

We aim here at addressing specifically the role of γ-carboxylation in bone remodeling in vivo. We show that an osteoblast-specific inactivation of *Ggcx* in mice is associated with an increased bone density resulting from a reduced number of osteoclasts without significant impact on bone formation. We establish that *Ggcx*-deficient osteoblasts are less effective at supporting osteoclast formation ex vivo and identify GAS6 as an osteoblast-secreted γ-carboxylated factor which signals to osteoclast precursors. Finally, using cell-based assays and a gain-of-function model in mice, we shown that γ-carboxylated GAS6 promote pre-osteoclasts fusion. These data reveal an unexpected role for γ-carboxylation in coupling bone resorption to bone formation and provides new insights on the function of VK in bone homeostasis.

Results

The enzymes of the VK cycle are predominantly expressed in osteoblasts

To identify the bone cell type(s) mediating the potential impact of VK on bone, we examined the expression of GGCX and VKORC1 in osteoblasts and osteoclasts at the mRNA and protein levels. Quantitative PCR (qPCR) revealed that both *Ggcx* and *Vkorc1* expression levels are about 10-fold higher in mouse proliferating non-mineralized osteoblasts (pre-OB) compared to bone marrow derived monocytes (BMMC) or fully differentiated osteoclast (OCL) (**Fig.1A**). While *Vkorc1* mRNA level remains elevated in mineralized osteoblast culture (OB), the expression of *Ggcx* is reduced in the same cells compared to pre-OB cultures (**Fig.1A**). Western blotting confirmed that GGCX and VKORC1 are more abundant in primary osteoblast cultures compared to osteoclasts. Interestingly, the expression level of GGCX and VKORC1 in osteoblasts was comparable to the one observed in liver where these enzymes are known to play a critical role in the γ-carboxylation and activation of several coagulation factors (**Fig. 1B**).

Inactivation of GGCX in osteoblasts results in increased bone density in mice

Given the predominant expression of the VK-cycle enzymes in osteoblasts, we inactivated *Ggcx* specifically in this cell type and investigate the impact on bone remodeling. For this purpose, *Ggcx*$^{flox/flox}$ mice were bred with *Osteocalcin-Cre* (OCN-Cre) mice expressing the Cre recombinase in mature osteoblasts only [32]. We previously reported that *Ggcx* was specifically and efficiently inactivated in osteoblasts in *Ggcx*$^{fl/fl}$;*OCN-Cre* mice [33]. The percentage of circulating carboxylated osteocalcin, an osteoblast-specific VK-dependent protein, was reduced by 80% to 90% between 2 and 6 months of age in these mice, confirming efficient inactivation of VK-

dependent carboxylation in osteoblasts in vivo (Supplementary Figure 1). In a previous study, we reported that 3-month-old *Ggcx*$^{fl/fl}$;*OCN-Cre* male mice had normal trabecular bone density [33]. Since bone mass decreases with aging, we characterized the bone parameters of the *Ggcx*$^{fl/fl}$;*OCN-Cre* at 6 months of age when bone mass has already started to decline in mice. Histological analysis of lumbar vertebrae indicated that trabecular bone density was significantly increased by about 30% in 6-month-old *Ggcx*$^{fl/fl}$;*OCN-Cre* mice (**Fig. 1C-D**). Trabecular bone density was also increased by about 30% in the femur of *Ggcx*$^{fl/fl}$;*OCN-Cre* mice analyzed by microcomputed tomography (µCT), although this parameter did not reach statistical significance due to higher variation (**Fig. 1E-F**). However, trabecular bone surface density (BS/TV), was significantly increased in the same animals (**Fig. 1F**). In addition, trabecular spacing (Tb.S) was significantly reduced, while trabecular number (Tb.N) and connectivity density (Conn.D), a computational measure of the inter-connectivity among trabeculae, were both significantly increased (**Fig. 1G**). Trabecular thickness was unaffected in the *Ggcx*$^{fl/fl}$;*OCN-Cre* femur (**Fig. 1G**). Altogether, these data indicate that the absence of VK-dependent carboxylation in osteoblasts is associated with an increased bone mass at 6 months of age.

Decreased bone resorption in *Ggcx*$^{fl/fl}$;*OCN-Cre* mice

To get insight into the cellular mechanism behind the increased bone mass phenotype of the *Ggcx*$^{fl/fl}$;*OCN-Cre* mice, static and dynamic bone histomorphometry analyses were performed at 6 months of age. Mineral apposition rate (MAR), measured using double calcein labeling, was slightly but significantly reduced in absence of GGCX in osteoblasts (**Fig.2A-B**). However, bone formation rate (BFR/BS), osteoblast number and osteoblast surface were not significantly affected (**Fig.2C-E**). In contrast, both osteoclast number and surface were significantly decreased in *Ggcx*$^{fl/fl}$;*OCN-Cre* mice (**Fig. 2F-H**). Decrease circulating carboxy-terminal collagen crosslinks

(CTX) independently confirmed reduced osteoclast activity in the same animals (**Fig.2I**). Overall, these analyses suggest that the increased bone mass observed in the absence of γ-carboxylation in osteoblasts is mainly driven by a reduction in osteoclastic bone resorption.

Decreased osteoclastogenesis in absence of *Ggcx* in osteoblasts

Osteoclast differentiation is dependent on factors, such as RANKL and M-CSF, produced by cells of the osteoblast lineage including pre-osteoblast and osteocytes. Because of the reduction in osteoclasts number in the *Ggcx*$^{fl/fl}$;*OCN-Cre* mice, we hypothesized that *Ggcx*-deficient osteoblasts may be less efficient at supporting osteoclast differentiation or maturation. This was tested using co-cultures of wildtype bone marrow cells with either osteoblasts lacking GGCX (i.e., *Ggcx*$^{fl/fl}$ osteoblasts transduced with a CRE expressing adenovirus) or control osteoblasts (i.e., *Ggcx*$^{fl/fl}$ osteoblasts transduced with a GFP expressing adenovirus). We previously reported very efficient inactivation of GGCX in osteoblasts at the mRNA and protein levels using this approach [33] (see also **Fig. 1B**). The coculture was performed in the presence of 1,25 vitamin D_3 (VitD3) and prostaglandin E_2 (PGE2), which promote the expression of pro-osteoclastogenesis factors by osteoblasts. These experiments indicated that the number of osteoclasts was significantly reduced by approximately 3-fold in the co-cultures involving GGCX-deficient osteoblasts (**Fig.3A-B**). This result suggests that γ-carboxylation in osteoblasts may regulate the production or the activity of a pro-osteoclastogenic factor. We next tested if the absence of osteocalcin (OCN), a γ-carboxylated protein specifically secreted by osteoblasts and osteocytes, impacted osteoclasts formation in the same co-culture assay and we found that the absence of Ocn in osteoblast did not affect the number of osteoclasts obtained from WT bone marrow cells (**Fig. 3C-D**).

Cells from the osteoblast lineage produce factors that promote (i.e., RANKL and M-CSF) or inhibit (i.e., osteoprotegerin; OPG) osteoclasts generation. We assessed the expression of these factors in control and GGCX-deficient osteoblasts to determine if γ-carboxylation regulates the pro-osteoclastogenic potential of osteoblasts. qPCR revealed that as expected, VitD3 and PGE2, robustly increased the expression of *Rankl* and suppressed *Opg* expression (**Fig. 3E**). Consequently, the *Rankl/Opg* ratio, which is positively associated with osteoclast formation, was increased by more than 10-fold with VitD3 and PGE2. Importantly, the absence of *Ggcx* in osteoblasts had no significant impact on *Rankl, Opg* and the *Rankl/Opg* ratio in the pro-osteoclastogenic condition (**Fig. 3E**). The expression of *Csf1*, the gene encoding for M-CSF, was also unaffected in the absence of GGCX in osteoblasts (**Fig. 3E**). These data suggest that γ-carboxylation positively regulates osteoclast formation independently of osteocalcin and of the expression of known pro-osteoclastogenic factors.

The γ-carboxylated protein GAS6 is expressed by osteoblasts and activates its receptors on pre-osteoclasts

Based on the results obtained in vivo and in co-culture assays, we postulated the existence of a γ-carboxylated protein, different from osteocalcin, produced by osteoblasts and promoting osteoclast formation. Expression of all the known γ-carboxylated proteins encoding genes was measured by qPCR in primary mouse osteoblasts. As expected, we detected expression of the γ-carboxylated ECM proteins osteocalcin (*Ocn*), matrix Gla protein (*Mgp*) and periostin (*Postn*), previously shown to be produced by the osteoblastic lineage (**Fig.4A**) [34-36]. Previous studies have established that inactivation of *Mgp* or *Postn* in mice had no impact on osteoclast number and

surface [37,38], and our own data show that osteocalcin is no required for osteoclastogenesis in coculture assays (**Fig.3A-D**). We therefore excluded periostin, osteocalcin and MGP, as potential mediators of the positive effect of γ-carboxylation on osteoclasts formation. Of note, the γ-carboxylated coagulation factors and the Proline Rich and Gla domain 1 to 4 proteins (*Prrg1-4*) were undetectable in osteoblasts. However, the gene encoding Growth Arrest Specific 6 (*Gas6*) was expressed at a level comparable or higher to *Mgp*, *Ocn* and *Postn* (**Fig. 4A**).

GAS6 is a secreted γ-carboxylated protein that functions as a ligand for the TAM family of tyrosine kinase receptors that includes AXL, Tyro3 and MerTK. Given that GAS6 is a signaling molecule and that TAM receptors were previously shown to be present on myeloid cells in other tissues [39], we decided to assess the presence of functional GAS6 receptors on osteoclasts. The expression of TAM receptors was evaluated at different stages of bone marrow-derived monocytes (BMMC) differentiation into osteoclasts. Both *MerTK* and *Axl* were detected in primary BMMC (d0) and in differentiating osteoclasts (d2, d4 and d6), while *Tyro3* expression was below the detection limit in most samples (**Fig.4B**).

We previously established that purified recombinant GAS6 (recGAS6) produced in HEK293 cells in the presence of VK is fully γ-carboxylated and able to activate TAM receptors on muscle cells (Supplemental Fig. 2) [40]. We thus tested if this ligand could also elicit TAM receptors activation in primary pre-osteoclasts. For this purpose, BMMC were serum starved for 3 hours, stimulated for various times with recGAS6 (200ng/mL) and the phosphorylation of AXL and MerTK or their downstream target AKT [41] assessed by Western blotting. Following recGAS6 stimulation, we could detect an increase in the phosphorylation of MerTK (Y753) and AXL

(Y702) on a tyrosine residue previously shown to be a dominant autophosphorylation site in the tyrosine kinase domain of TAM receptors that leads to the activation of their kinase activity [42]. In the condition of this assay, recGAS6 also robustly induces the phosphorylation of AKT on serine 473, reflecting the activation of the phosphatidylinositol 3-kinase (PI3K) pathway. Together, these results support the existence of functional GAS6-TAM signaling coupling osteoblasts to osteoclasts.

GAS6 signaling promotes osteoclast formation ex vivo

We then tested if GAS6-TAM signaling influences osteoclast formation using two approaches. In a first set of experiments, we assessed osteoclastogenesis in co-cultures of WT bone marrow cells and WT osteoblasts in the presence of increasing concentrations of two pharmacological TAM inhibitors, LDC1267 and R428 (bemcentinib) [43,44]. As shown in Figure 5A, LDC1267, a pan-TAM inhibitor, dose dependently inhibits the formation of TRAP-positive multinucleated osteoclasts in these co-cultures, with an IC50 of ~750nM. R428, which is >50-100-fold more selective for AXL than MerTK and Tyro3, also inhibits osteoclast formation with a lower IC50 of ~250nM (**Fig.5B**). These data suggest that TAM receptor signaling promotes osteoclast formation in co-culture assay.

In a second approach, we tested if exogenous recGAS6 (25-200 ng/mL) impacts osteoclast formation in primary BMMC cultures in the presence of RANKL (20ng/mL) and M-CSF (10ng/mL). In these experiments, recGAS6 significantly and dose-dependently increases osteoclasts formation (**Fig.5C-E**). Quantification of the cultures following TRAP staining indicated that recGAS6 increased both the surface covered by TRAP+ osteoclasts (**Fig.5D**) and the number of multinucleated osteoclasts (**Fig. 5E)**, as early as 4 days of culture when small

multinucleated TRAP-positive osteoclasts begin to appear. In addition, the number of individual nuclei per osteoclast was significantly increased in presence of recGAS6 (**Fig.5F**), indicating the formation of larger osteoclasts. These results identify GAS6-TAM signaling as a modulator of osteoclastogenesis ex vivo.

GAS6 increases the fusion of pre-osteoclasts in culture

Osteoclast formation involves first, the transcriptional activation of a differentiation program in mononucleated pre-osteoclasts and second, the fusion of these pre-osteoclasts to form mature multinucleated osteoclasts. We sought to determine if GAS6 signaling increases osteoclast formation by promoting differentiation of BMMC into osteoclasts. The expression of genes encoding several markers of osteoclast differentiation was measured by qPCR at 2, 4 and 6 days of differentiation in the absence or presence of intermediate doses of recGAS6 (i.e., 50 and 100 ng/mL). The presence of recGAS6 in the media did not significantly impact the expression of *Acp5* (TRAP) and *Clcn7* which gradually increase between 2 and 6 days of differentiation as expected (**Fig.6A-B**). The highest dose of GAS6 (100ng/mL) induces a small and significant increase in the expression of *Ctsk*, the gene encoding cathepsin K, at day 4 and 6 of differentiation, and of *Dcstamp* at day 6 of differentiation (**Fig.6C-D**). Overall, this marginal impact of GAS6 on the expression of the osteoclast differentiation program cannot explain its strong positive effect on the generation of multinucleated osteoclasts in the same culture conditions. Indeed, 50 and 100 ng/mL of GAS6 increases by >50% the number of osteoclasts and the area of TRAP+ osteoclast (**Fig.5C-E**).

Since recGAS6 increases the number of nuclei per OCL (**Fig.5F**), we next tested if GAS6 promotes pre-osteoclast fusion. For this purpose, we established a pre-osteoclast fusion assay in

which an equal number of *Ctsk*$^{Cre/+}$ and *Rosa26*$^{CAG\text{-}lox\text{-}stop\text{-}lox\text{-}tdTomato}$ BMMC were induced to differentiate into osteoclasts. In this experimental setting, the fluorescent protein tdTomato (Tom) is only expressed in cells resulting from the fusion of at least one *Ctsk*$^{Cre/+}$ and one *Rosa26*$^{CAG\text{-}lox\text{-}stop\text{-}lox\text{-}tdTomato}$ pre-osteoclasts (**Fig.6E**). We then monitored using time-lapse microscopy the appearance of fluorescent Tom+ positive cells in the presence or absence of recGAS6. As shown in Figure 6F, Tom+ cells begin to appear in osteoclast culture at day 4 and their number increases considerably at day 5 and 6, validating our approach. Interestingly, the number of Tom+ cells was significantly higher in the presence of recGAS6 in the culture media even with the lowest tested dose of 50 ng/mL, suggesting that GAS6 signaling promotes osteoclast fusion.

GAS6 promotes osteoclast formation and bone resorption in vivo

In addition to GAS6, protein S (Pros1) was shown to activate TAM receptors [45]. Our data indicate that osteoblasts also express Pros1 (**Fig. 4A**) and that both AXL and MerTK are present in pre-osteoclasts (**Fig. 4B**). This redundancy in the TAM signaling system prevented us from testing its importance in osteoclast formation through a genetic loss-of-function model. Instead, a gain-of-function approach was employed to determine the impact of raising GAS6 on osteoclastic bone resorption in vivo. We took advantage of a previously described transgenic mouse line expressing GAS6 under the human *ApoE* promoter [40]. In these *ApoE-Gas6*^{Tg} mice, GAS6 is produced by hepatocytes and therefore fully carboxylated and secreted in the circulation, leading to a 4-fold increase in active GAS6 in the serum (**Fig.6A**). Since the skeleton, and particularly the bone marrow cavity is highly vascularized, we reasoned that this rise in circulating GAS6 will translate into an increased concentration of this protein in the bone marrow space, in which osteoclast differentiation takes place. Accordingly, the concentration of GAS6 in the bone

marrow space was increased by 2-fold in *ApoE-Gas6*^{Tg} mice (**Fig.7B**). We next investigated bone density and remodeling in this GAS6 gain-of-function mouse model.

Histology and µCT revealed a reduced trabecular bone volume (BV/TV) in vertebrae and femur of 6-month-old *ApoE-Gas6*^{Tg} male mice compared to WT littermates (**Fig.7C-F**). The µCT analyses showed that trabecular bone surface density (BS/TV), was also significantly reduced in the same animals (**Fig. 7G**). Trabecular spacing (Tb.S) was significantly increased and trabecular number (Tb.N) and connectivity density (Conn.D) significantly decreased, while trabecular thickness was unaffected in *ApoE-Gas6*^{Tg} mice (**Fig. 7H**). Static and dynamic bone histomorphometry indicated that *ApoE-Gas6*^{Tg} mice were characterized by a significant increase in both the number and surface of osteoclasts in the trabecular bone (**Fig. 7I-J**). Importantly, bone formation parameters were unaffected in the same animals (**Supplementary Figure 3**). Altogether, these data indicate that increased concentration of GAS6 in bone is sufficient to stimulate the formation of osteoclast in vivo, resulting in reduced bone density.

Discussion

Whether VK supplementation has any beneficial impact on skeletal health remains unclear since interventional studies in humans have led to conflicting results [22]. Our data suggest that VK-dependent carboxylation in osteoblasts is not required for normal bone formation and mineralization by this cell type. Instead, we uncover that γ-carboxylation, through GAS6, impacts the capacity of osteoblasts to regulate osteoclasts formation. Taken at face value, these findings suggest that VK should negatively regulate bone mass, potentially explaining the limited impact of VK supplementation on bone density in humans. However, osteoclastic bone resorption also plays a critical role in bone turnover and repair of microdamage to the bone structure [46]. Consequently, the production of active γ-carboxylated GAS6 by osteoblasts may be important for the fine tuning of osteoclast activity during bone repair processes, thereby promoting the maintenance of a healthy skeleton and preventing fractures.

Vitamin K-dependent carboxylation in osteoblast may influence the activity of several Gla proteins, including osteocalcin and MGP. The conclusion that γ-carboxylation in osteoblasts impact bone resorption through GAS6 is supported by several lines of evidence. First, the bone phenotype of the *ApoE-Gas6*^{Tg} mice, a GAS6 gain-of-function model, is the mirror image of the one observed in *Ggcx*$^{fl/fl}$;*OCN-Cre* mice. Second, in line with previous reports, we show that GAS6 is strongly expressed in osteoblasts [47,48]. Third, recombinant carboxylated GAS6 promotes the formation of larger osteoclasts in culture, while pharmacological inhibition of its receptors reduces osteoclastogenesis. Fourth, osteocalcin-deficient osteoblasts fully support osteoclast formation, while *Mgp*-deficient mice have normal bone resorption [38].

In line with our findings, a recent study reported that whole body inactivation of MerTK in mice results in higher bone density with concomitant reduction in bone resorption [49]. TAM receptors signaling may also play a role in osteoblasts function since it was reported that MerTK inactivation specifically in osteoblasts using the *Col1a1-Cre* driver caused increased bone formation and bone density, without affecting bone resorption [48]. Importantly, *Ggcx*$^{fl/fl}$;*OCN-Cre* mice did not show this increase in bone formation, suggesting that osteoblasts-derived carboxylated GAS6 does not suppress osteoblast activity through MerTK. Both protein S and GAS6 can activate MerTK, while AXL is activated exclusively by GAS6 [45]. According to our own data and previous reports [48,50], osteoblasts express protein S. We also found that pre-osteoclast and differentiated osteoclasts express comparable level of AXL and MerTK. Due to this redundancy in ligands and receptors, assessing the role of the Pros1/GAS6/TAM axis in bone resorption using loss-of-function mouse models will likely require the generation of cell-specific double knockout mice.

In our primary culture assays, low to intermediate doses of GAS6 (i.e., 25 to 100 ng/mL) significantly increased osteoclasts number and surface at 5 and 6 days of culture. The same concentration also increased the average number of nuclei per osteoclast. However, dose of 50 to 100 ng/mL had limited impact on the expression of the osteoclast differentiation program. Together with the results of our fusion assay, these data suggest that GAS6 does not regulate osteoclast differentiation but rather promotes the fusion of pre-osteoclasts.

To optimally activate TAM receptors, GAS6 requires its Gla domain, even though this domain is not required for binding to the ectodomain of TAM [45]. In the context of its myeloid function, GAS6 Gla domain binds to phosphatidylserines (PtdSer) exposed at the surface of a phagocytic

target (e.g., an apoptotic cell), and although the precise mechanism is not fully understood, this PtdSer binding induces a conformational change in GAS6 that results in the activation of the TAM receptor on the phagocytic cells [51]. Interestingly, recent studies have established that PtdSer are exposed on the outer leaflet of the plasma membrane during pre-osteoclast differentiation and that this exposure mediates the fusion of osteoclast precursors [52-54]. Therefore, GAS6 may be contributing to the fusion process by binding PtdSer at the surface of one pre-osteoclast while activating TAM receptors at the surface of another pre-osteoclast. Future studies will focus on understanding how the signal downstream of TAM in pre-osteoclast contributes to the fusion.

In conclusion, this work uncovers a new vitamin K-dependent carboxylation pathway in osteoblasts involved in the regulation of osteoclast formation.

Materials and Methods

Animals

All the mouse strains used in this study were backcrossed or generated on a pure C57BL6/J genetic background. The generation of *Ggcx*$^{fl/fl}$;*OCN-Cre* mice was previously described [33]. Briefly, *Ggcx*$^{flox/flox}$ mice were bred with *Osteocalcin-Cre* (OCN-Cre) mice, which express the Cre recombinase in mature osteoblasts only [32]. We previously established that *Ggcx* was specifically and efficiently inactivated in osteoblasts in *Ggcx*$^{fl/fl}$;*OCN-Cre* mice [33]. The generation and characterization of *ApoE-Gas6*^{Tg} mice expressing mouse *Gas6* under the control of the liver specific human APOE promoter and hepatic enhancers was also previously reported [40]. The *tdTomato*$^{flox/flox}$ strain expressing the tdTomato reporter gene from the *Rosa26* locus was obtained from the Jackson Laboratory (*B6.Cg-Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze/J}$; stock 007914). The *Ctsk-Cre* (*Ctsk*$^{tm1(cre)Ska}$) strain was previously described [55]. Osteocalcin-deficient mice (*Ocn-/-*) in which both osteocalcin genes (*Bglap1/Ocn1* and *Bglap2/Ocn2*) are inactivated were previously reported [29]. C57BL6/J newborn or adult mice (Jackson Laboratory; strain 000664) were used as a source of primary wildtype (WT) osteoblasts and bone marrow cells. All strains were maintained in an IRCM specific pathogen-free animal facility under 12-hour dark/12-hour light cycles. Mice were fed ad libitum a normal chow diet (Teklad global 18% protein rodent diet; 2918; Envigo). Male mice were used in all experiments. All animal use complied with the guideline of the Canadian Committee for Animal Protection (CCAP) and was approved by IRCM institutional animal care committee.

Biochemical measurements

Serum carboxylated (Gla) and total osteocalcin were assessed using previously described ELISA assays [56]. Serum C-terminal telopeptide of collagen type I (CTX) was measured using a

commercially available assay (RatLaps; IDS, AC-06F1). Serum GAS6 was quantified using a mouse GAS6 ELISA (DuoSet ELISA, R&D Systems, DY986) as we previously described [40]. To measure GAS6 in bone marrow, the bone marrow of one femur per mouse was flushed using 1mL of PBS1X and the concentration of GAS6 quantified using the same ELISA assay. To estimate the concentration of GAS6 in the BM, the amount of GAS6 per femur (i.e, GAS6 concentration × 1mL) was divided by 20µL, the estimated volume for a femur BM cavity of a 6-month-old male mouse based on previous µCT data.

Bone histology and histomorphometry

Mice were injected intraperitoneally twice at a 3-day interval with 0.2 mL of a solution of calcein (0.25% in a solution of 0.15M NaCl and 2% $NaHCO_3$). Following euthanasia, the skeletal tissue was fixed in 4% formaldehyde in PBS 1X and processed for histomorphometry and micro-CT. For non-decalcified histology, the vertebrae were embedded in methyl methacrylate resin (MMA) as previously described [57] and sectioned (5 µm and 7µm thickness). Following deplastification, the sections were stained with von Kossa and van Gieson (7µm), with Toluidine blue (5 µm) or TRAP (5 µm). Bone histomorphometric analyses were completed using the OsteoMeasure Analysis System (OsteoMetrics) connected to a light and fluorescent microscope (DM4000B LED; Leica) with a 40× objective (HCX PL FLUOTAR 40×; NA = 0.75).

Micro-CT

Ex vivo micro-computed tomography (µCT) scans were performed on harvested distal femurs using the Skyscan 1272 system (Bruker, Kontich, Belgium) at 62 kV, 156 µA, and 6 µm isotropic resolution with a 0.5 mm aluminum filter. Images were reconstructed with NRecon (beam

hardening correction: 50%; ring artifact reduction: 7) and analyzed using CTAn software (Bruker, Skyscan). The trabecular bone region of interest (ROI) was defined 0.75 mm proximal to the growth plate and extended 1 mm proximally. Morphometric parameters were calculated following published guidelines for rodent bone analysis using μCT [58].

Production and Purification of Recombinant GAS6

Recombinant γ-carboxylated mouse GAS6 was produced as previously described [40]. Briefly, HEK293 Flp-In T-REx cells (Thermo Fisher Scientific) stably transfected with a tetracycline-inducible plasmid encoding mouse GAS6 with a C-terminal 6xHis tag were maintained in DMEM (Wisent) supplemented with 10% fetal bovine serum (FBS; Sigma) and 200 μg/mL hygromycin for selection. Cells were expanded and the expression of GAS6 induced with 1 μg/mL tetracycline in presence of 22 μM vitamin K_1 (Millipore Sigma, V3501) in serum-free DMEM for 36 hours. The culture supernatant was collected, centrifuged, and filtered (0.22 μm) to remove cells and debris. Recombinant GAS6 was purified by nickel affinity chromatography using a HisTrap HP column (Cytiva) connected to an ÄKTAprime FPLC system (GE Healthcare). Elution was performed using an imidazole-containing buffer, and the purified protein was dialyzed against PBS. Protein purity and concentration were assessed by SDS-PAGE followed by Coomassie staining (see **Supplemental Figure 2**).

Osteoblast cultures

Mouse calvaria pre-osteoblasts were isolated from 3-day-old pups and cultured as previously described [33]. Briefly, dissected calvarias were digested at 37°C twice for 10 minutes and twice for 30 min with a solution of collagenase type 2 (0.1 mg/mL; Worthington Biochemical Corporation,

LS004176) containing 0.25% trypsin. Only the last two digestions were kept and cultured in alpha modified minimum essential medium (α-MEM, Sigma, M0644) supplemented with 10% fetal bovine serum (FBS; Sigma) and 1% penicillin-streptomycin (Wisent) to obtain proliferating pre-osteoblasts (pre-OB). Differentiation into mineralizing osteoblasts (OB) was induced by culturing these cells in the same media supplemented with 5mM β-glycerophosphate and 100 μg/mL L-ascorbic acid for 21 days. Media was changed every 2 days. To generate control and *Ggcx*$^{-/-}$ pre-osteoblasts ex vivo, *Ggcx*$^{flox/flox}$ cells were transduced with either GFP- or Cre-expressing adenovirus (University of Iowa) at a MOI of 200 [33].

Bone marrow monocytes and osteoclasts cultures

Primary bone marrow cells were isolated from the femurs and tibias of 6- to 10-week-old C57BL6/J mice. Bones were cleaned of soft tissue and flushed with α-MEM supplemented with 10% FBS and 1% penicillin-streptomycin using a 25G needle. Bone marrow-derived monocytes (BMMC) were obtained by culturing the marrow cell suspension in non-adherent 10 cm dishes in M-CSF–rich medium (20% L929 cell-conditioned medium; ATCC) for 5 days. Non-adherent cells were removed by vigorous washing with PBS, and adherent cells were harvested by incubation with 0.02% EDTA in PBS.

For osteoclast-like cell (OCL) differentiation, BMMCs were seeded at 2×10^4 cells per well in 24-well plates and cultured in medium supplemented with 20 ng/mL RANKL and 10 ng/mL M-CSF, with or without recombinant γ-carboxylated GAS6 at various concentrations (25-200 ng/mL). Multinucleated mature osteoclasts were visualized by TRAP staining [59]. In selected experiments, nuclei were stained with DAPI, and both brightfield and fluorescence images were

acquired (DM4000B LED; Leica) with a 5× objective (HCX PL FLUOTAR 5×; AP = 0.15). The number of nuclei per osteoclast was quantified using ImageJ. In co-culture assays, 6×10^6 bone marrow cells were added to confluent osteoblasts cultures in 12 well plates in αMEM supplemented with 10% FBS, prostaglandin E_2 (PGE_2; 10^{-6} M) and 1,25 vitamin D_3 ($VitD_3$; 10^{-8} M). In some experiments, TAM receptors inhibitors R428 or LDC1267 (Selleckchem, S2841 and S7638) were added to the cultures at the indicated concentrations. After 8 days of coculture, the cells were fixed and TRAP staining performed. Osteoclasts culture pictures were acquired using a SteREO Discovery.V12 microscope (Zeiss) using Zen software. Osteoclasts number and surface area were quantified from TRAP-stained cell cultures using ImageJ software.

Fusion assay

An equal number of *tdTomato*$^{flox/flox}$ and *Ctsk-Cre* BMMCs (1×10^4 cells each per well) were seeded in 24-well plates and cultured in medium supplemented with 20 ng/mL RANKL and 10 ng/mL M-CSF, with or without recombinant γ-carboxylated GAS6 at various concentrations. Plates were imaged using the Incucyte Live-Cell Analysis System (Sartorius). Fluorescence (tdTomato) and phase contrast images were acquired every 24 h starting from day 3 of culture using a 10× objective (16 fields per well). Images were analyzed using ImageJ and each tdTomato-positive (red) cell with 2 or more nuclei was counted as a fusion event.

Gene expression

Total RNA was extracted from cells or tissues as previously [60]. mRNA was reverse transcribed using M-MLV as previously reported [61]. Relative gene expression was measured by quantitative PCR using PowerUp SYBR Green Master Mix (A25741; Applied Biosystems) with gene-

specific primers (Supplemental Table 1) and ViiA7 Real-Time PCR System (Applied Biosystems).

Western blot

Proteins from cells or tissues were extracted with a lysis buffer containing 20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM EDTA (pH 8.0), 1mM EGTA, 2.5mM NaPyrophosphate, 1mM β-glycerophosphate, 10mM NaF, 1% Triton, 1 mM vanadate, 1mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors (Roche Diagnostics, 4693132001). Proteins were detected by Western blot with the indicated primary antibodies overnight at 4°C and quantified by densitometry analyses using Image Lab software (Bio-Rad Laboratories). The antibodies used are listed in Supplemental Table 2.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism version 10.4.0. Results are given as mean ± SEM. Unpaired two-tailed Student's *t* test was used to compare two groups. For experiments involving multiple groups, one- or two-way ANOVA, followed by Bonferroni's multiple comparisons test was used. In all figures, $*$ $P < 0.05$; $**$ $P < 0.01$; $***$ $P < 0.001$. All experiments were repeated at least 3 times or performed on at least 3 independent animals.

Data and Resource Availability

The data sets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request. The *ApoE-Gas6*^{Tg} transgenic and the *Ggcx*$^{flox/flox}$ mouse lines, the recombinant GAS6 expressing cell line, the phosphorylated AXL (Y702)

antibody and the mouse VKORC1 antibody used during the current study are available from the corresponding author upon reasonable request.

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Conflict of interests

No potential conflicts of interest relevant to this article were reported.

Contributions

M.F. and M.P. assembled the figures. M.F. wrote the manuscript with suggestions from the other authors. M.P., M.F., D.N.T.P., J.L., B.A.R. and Y.W.K., collected and analyzed data. M.F. conceived and designed the project and provided supervision to M.P., D.N.T.P., J.L., B.A.R. and Y.W.K. A.G.A.A. and M.M. collected and analyzed the μCT data. M.F. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of data and the accuracy of data analysis.

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FIGURES LEGENDS

Figure 1. Increased bone mass in mice lacking γ-carboxylation in osteoblasts. (A) Gene expression analysis by qPCR of *Ggcx* and *Vkorc1* in bone marrow derived monocytes (BMMC), osteoclasts (OCL), proliferating pre-osteoblasts (pre-OB) and mineralized osteoblast (OB) cultures (n=2-3). **(B)** Protein expression in liver (Liv) and bone cells by Western blot. GGCX was analyzed on a 7.5% SDS Tris Glycine gel using 20µg of extracts, while VKORC1 was resolved on a 10% SDS Tris Tricine gel using 10µg of extracts. **(C-I)** Six-month-old *Ggcx*^{ff} and *Ggcx*^{ff}*;OCN-Cre* male mice were analyzed (n=7-10). **(C)** Representative pictures of sections from lumbar vertebrae stained with von Kossa and van Gieson. **(D)** Quantification of trabecular bone volume over tissue volume (BV/TV) from the L4 and L5 lumbar vertebrae sections. **(E)** Representative µCT images of the distal femur trabecular bones. **(F)** Quantification of trabecular bone volume (BV/TV) and trabecular bone surface density (BS/TV) from the µCT data. **(G)** Trabecular bone µCT derived data. Tb.Sp., Tb.N., and Tb.Th., trabecular spacing, number, and thickness respectively; Conn.Dn., connectivity density. Unpaired, 2-tailed Student's t test was used in (D), (F), and (G). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, ns : non-significant.

Figure 2. Reduced osteoclasts number and surface in *Ggcx*^{ff}*;OCN-Cre* mice. (A-H) Bone histomorphometry analysis of lumbar vertebrae in six-month-old *Ggcx*^{ff} and *Ggcx*^{ff}*;OCN-Cre* male mice (n=6-10). **(A)** Representative pictures of calcein double labeling and toluidine blue staining. **(B)** Mineral apposition rate (MAR). **(C)** Bone formation rate over bone surface (BFR/BS). **(D)** Number of osteoblasts per bone perimeter (N.Ob/B.Pm). **(E)** Osteoblasts surface over bone surface (Ob.S/BS). **(F)** Representative pictures of TRAP staining. **(G)** Number of osteoclasts per bone perimeter (N.Oc/BPm). **(H)** Osteoclast surface over bone surface (Oc.S/BS). **(I)** Fasting serum CTx levels (n=12-17). Results represent the mean ± SEM. Unpaired, 2-tailed Student's t test was used in (B-E) and (G-I). **$p < 0.01$, *$p < 0.05$, ns : non-significant.

Figure 3. *Ggcx* inactivation impairs the ability of osteoblasts to support osteoclastogenesis ex vivo. (A) Representative TRAP staining of osteoblasts (OB) and bone marrow cells (BM) co-cultures at day 8 in the presence of prostaglandin E_2 (PGE_2; 10^{-6} M) and 1,25 vitamin D_3 ($VitD_3$; 10^{-8} M). *Ggcx*^{ff} osteoblasts were transduced with either Ad-GFP (control) or Ad-Cre (knockout) before the addition of the WT bone marrow cells. **(B)** Quantification of the number of TRAP+

osteoclasts per well (Nb of OCL/well) (n=3). **(C)** Representative TRAP staining of osteoblasts and bone marrow cells co-cultures at day 8. Control (*Ocn+/+*) or osteocalcin-deficient (*Ocn-/-*) osteoblasts were cultured with WT bone marrow cells. **(D)** Quantification of the number of TRAP+ osteoclasts per well (Nb of OCL/well) (n=3). **(E)** Gene expression analysis by qPCR in *Ggcx*^{ff} + Ad-GFP and *Ggcx*^{ff} + Ad-Cre osteoblasts cultured in presence (+) or absence (-) of PGE_2 and $VitD_3$. Results represent the mean ± SEM. Unpaired, 2-tailed Student's t test was used in (B) and (D). Two-way ANOVA with Bonferroni's post tests was used in (E). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, ns : non-significant.

Figure 4. The γ-carboxylated protein GAS6 is expressed by osteoblasts and activates its receptors on pre-osteoclasts. (A) Expression analysis by qPCR of genes encoding the known γ-carboxylated proteins. *Gas6* is highlighted in red. **(B)** Gene expression analysis by qPCR of the TAM receptors *Axl*, *Mertk* and *Tyro3* in bone marrow derived monocytes (d0: day 0) and in differentiating osteoclasts cultures in the presence of RANKL and M-CSF (d2-d6: day 2 to 6). **(C)** Western blot analysis of the phosphorylation (P) of AKT (S473) and TAM (Y702 in AXL and Y753 in MerTK) in bone marrow derived monocytes (BMMC) serum starved for 3h and treated with GAS6 (200ng/mL) for the indicated times. Total AKT, MerTK and AXL were used as loading controls. **(D)** Quantification of P-AKT (S473), P-MerTK (Y753) and P-AXL (Y702) normalized to the amount of total protein (n=2-3). Results represent the mean ± SEM.

Figure 5. Carboxylated GAS6 signaling promotes osteoclasts formation in culture. (A-B) Representative TRAP staining (left) and quantification of the TRAP+ osteoclast area (right) in WT osteoblasts (OB) and bone marrow cells (BM) co-cultures at day 8 in the presence of PGE_2 and $VitD_3$, with or without the TAM inhibitors LDC1267 (A) or R428 (B), at the indicated concentrations. **(C-F)** Bone marrow derived monocytes (BMMC) were cultured in the presence of RANKL (20 ng/mL) and M-CSF (10 ng/mL) with or without recombinant carboxylated GAS6 at the indicated concentrations for up to 6 days. **(C)** Representative TRAP staining at day 5 and 6 of differentiation. **(D)** Quantification of the TRAP+ osteoclast area at day 4, 5 and 6 of differentiation. **(E)** Quantification of the number of TRAP+ multinucleated osteoclasts at day 4, 5 and 6 of differentiation. **(F)** Quantification of the number of nuclei per osteoclast at day 5 of

differentiation. Results represent the mean ± SEM. One-way ANOVA with Bonferroni's post tests was used in (A-B) and (D-F). ***p < 0.001, **p < 0.01, *p < 0.05, ns : non-significant.

Figure 6. Carboxylated GAS6 impact on osteoclasts differentiation and fusion. (A-D) Gene expression analysis by qPCR of osteoclasts differentiation markers *Acp5* (TRAP), *Clcn7*, *Ctsk* and *Dcstamp*. Bone marrow derived monocytes (BMMC) were cultured in the presence of RANKL (20 ng/mL) and M-CSF (10 ng/mL) with or without recombinant carboxylated GAS6 at the indicated concentrations for 2, 4 and 6 days (n=4 per condition). **(E)** Schematic representation of the assay used to assess the impact of GAS6 on pre-osteoclasts fusion in culture using a conditionally activated tdTomato (Tom) reporter. **(F)** Representative pictures of live osteoclasts cultures at the indicated time and concentration of recombinant GAS6. The stars indicate the presence of fusion events (Tom+ cells) in the GAS6 conditions at Day 4. **(G)** Quantification of the number of fusion events per 10 mm^2 at the indicated time of osteoclasts differentiation (n=16 fields per condition). Results represent the mean ± SEM. One-way ANOVA with Bonferroni's post tests was used in (A-D) and (G). ***p < 0.001, **p < 0.01.

Figure 7. GAS6 promotes osteoclasts formation and bone resorption in vivo. (A-J) Six-month-old *WT* (non-transgenic littermates) and *ApoE-Gas6*^{Tg} male mice were analyzed. **(A-B)** GAS6 concentration in the serum (A) and bone marrow cavity (B) (n=4). **(C)** Representative pictures of sections from lumbar vertebrae stained with von Kossa and van Gieson. **(D)** Quantification of trabecular bone volume over tissue volume (BV/TV) from the L4 and L5 lumbar vertebrae sections (n=15-17). **(E-H)** µCT analysis of the distal femur trabecular bone (n=12). **(E)** Representative µCT images. **(F)** Quantification of trabecular bone volume (BV/TV). **(G)** Quantification of trabecular bone surface density (BS/TV). **(H)** Trabecular bone µCT derived data. Tb.Sp., Tb.N., and Tb.Th., trabecular spacing, number, and thickness, respectively; Conn.Dn., connectivity density. **(I-J)** Bone histomorphometry analysis of lumbar vertebrae in six-month-old *WT* and *ApoE-Gas6*^{Tg} male (n=19-20). **(I)** Representative pictures of TRAP staining. **(J)** Number of osteoclasts per bone perimeter (N.Oc/BPm) and osteoclasts surface over bone surface (Oc.S/BS). Results represent the mean ± SEM. Unpaired, 2-tailed Student's t test was used in (A,B, D, F, G and J). ***p < 0.001, **p < 0.01, *p < 0.05, ns : non-significant.

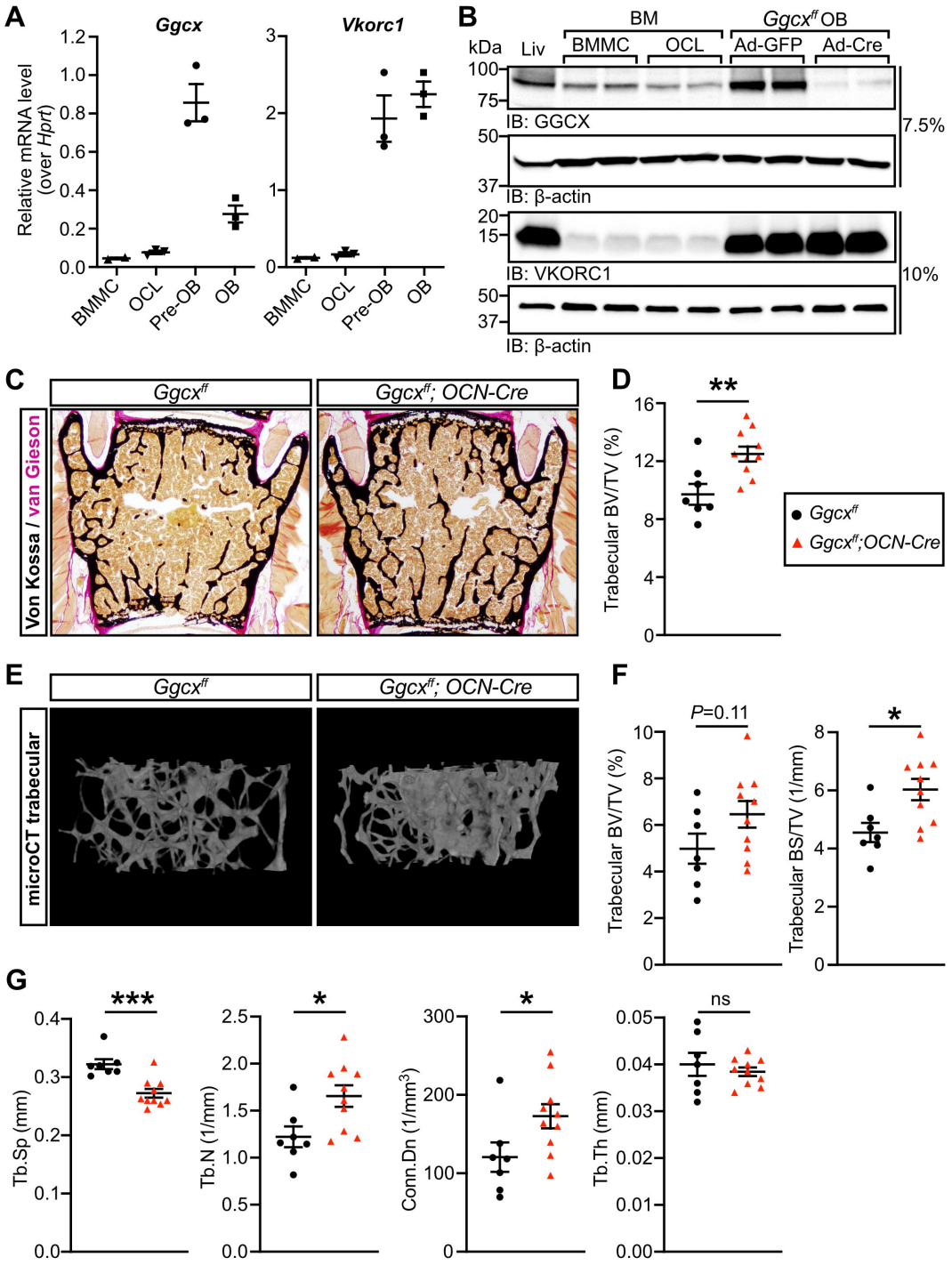
A
Ggcx
Vkorc1
Relative mRNA level (over Hprt)
BMMC
OCL
Pre-OB
OB
B
kDa
Liv
BM
BMMC
OCL
Ggcx^ff OB
Ad-GFP
Ad-Cre
IB: GGCX
IB: β-actin
IB: VKORC1
IB: β-actin
7.5%
10%
C
Ggcx^ff
Ggcx^ff; OCN-Cre
Von Kossa / van Gieson
D
Trabecular BV/TV (%)
Ggcx^ff
Ggcx^ff;OCN-Cre
E
Ggcx^ff
Ggcx^ff; OCN-Cre
microCT trabecular
F
P=0.11
Trabecular BV/TV (%)
Trabecular BS/TV (1/mm)
G
Tb.Sp (mm)
Tb.N (1/mm)
Conn.Dn (1/mm3)
Tb.Th (mm)
ns

Figure 1

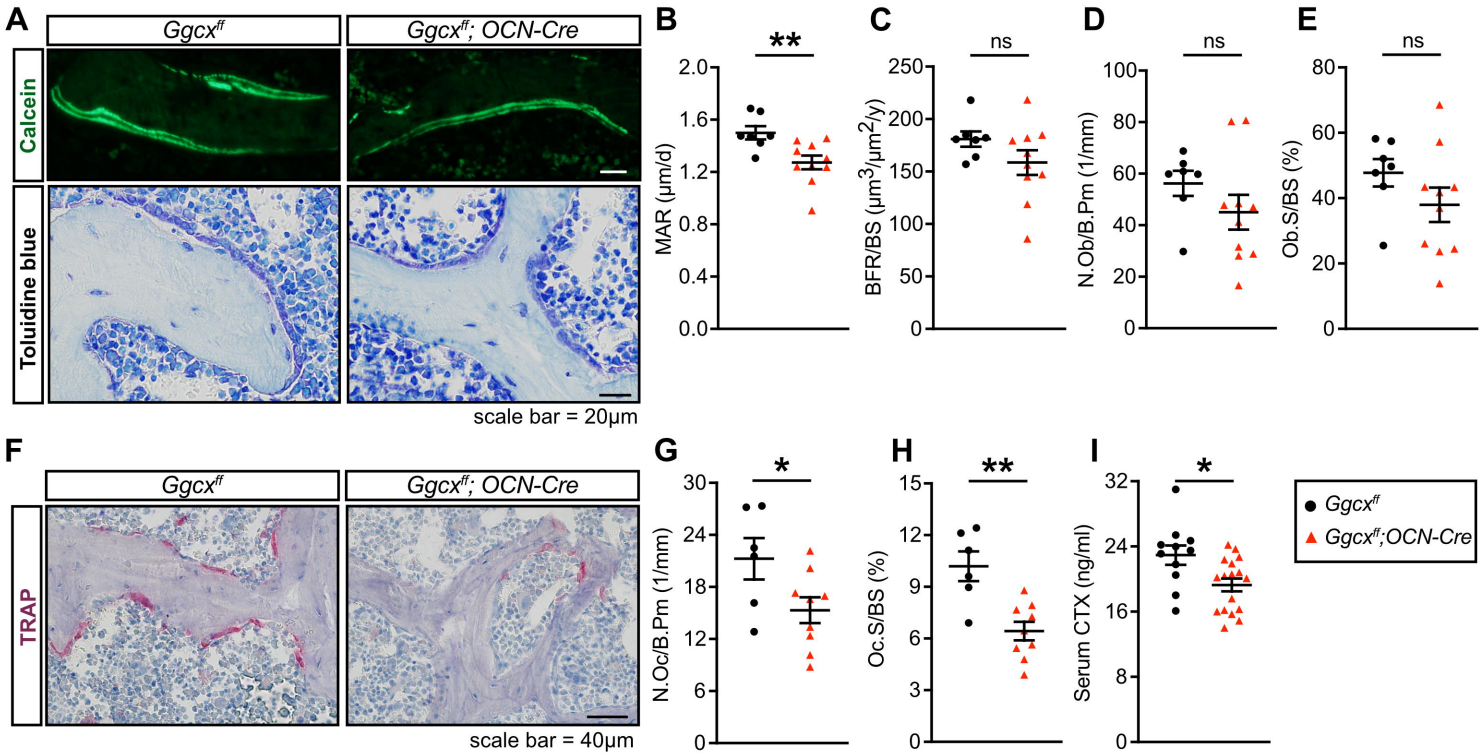
A
Ggcx^ff
Ggcx^ff; OCN-Cre
Calcein
Toluidine blue
scale bar = 20μm
B
**
MAR (μm/d)
C
ns
BFR/BS (μm³/μm²/y)
D
ns
N.Ob/B.Pm (1/mm)
E
ns
Ob.S/BS (%)
F
Ggcx^ff
Ggcx^ff; OCN-Cre
TRAP
scale bar = 40μm
G
*
N.Oc/B.Pm (1/mm)
H
**
Oc.S/BS (%)
I
*
Serum CTX (ng/ml)
Ggcx^ff
Ggcx^ff;OCN-Cre

Figure 2

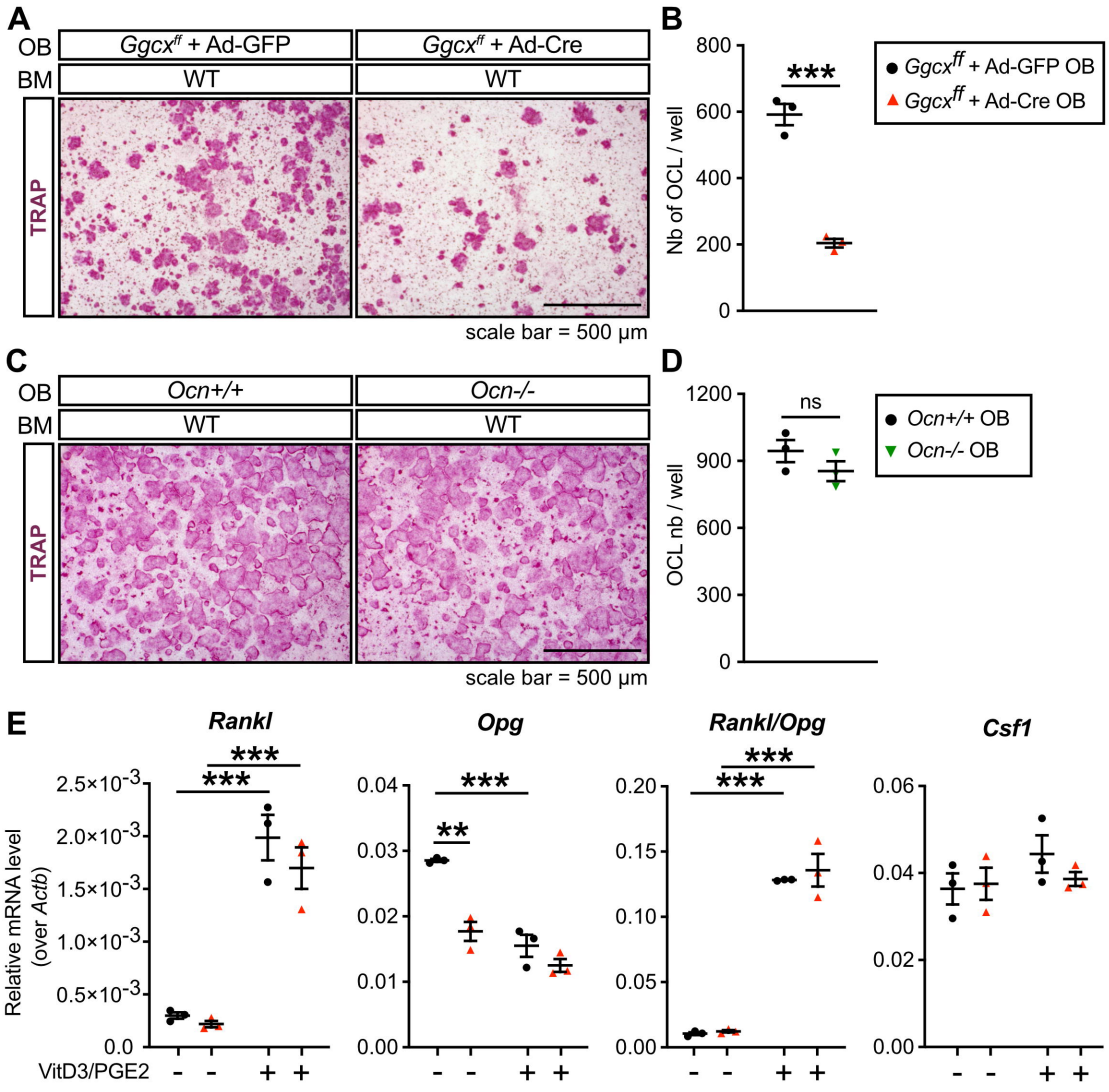
A
OB
BM
Ggcx^ff + Ad-GFP
Ggcx^ff + Ad-Cre
WT
WT
TRAP
scale bar = 500 µm
B
Nb of OCL / well
800
600
400
200
0
***
Ggcx^ff + Ad-GFP OB
Ggcx^ff + Ad-Cre OB
C
OB
BM
Ocn+/+
Ocn-/-
WT
WT
TRAP
scale bar = 500 µm
D
OCL nb / well
1200
900
600
300
0
ns
Ocn+/+ OB
Ocn-/- OB
E
Rankl
Opg
Rankl/Opg
Csf1
Relative mRNA level (over Actb)
2.5×10-3
2.0×10-3
1.5×10-3
1.0×10-3
0.5×10-3
0.0
0.04
0.03
0.02
0.01
0.00
0.20
0.15
0.10
0.05
0.00
0.06
0.04
0.02
0.00
***
**
VitD3/PGE2
- - + +

Figure 3

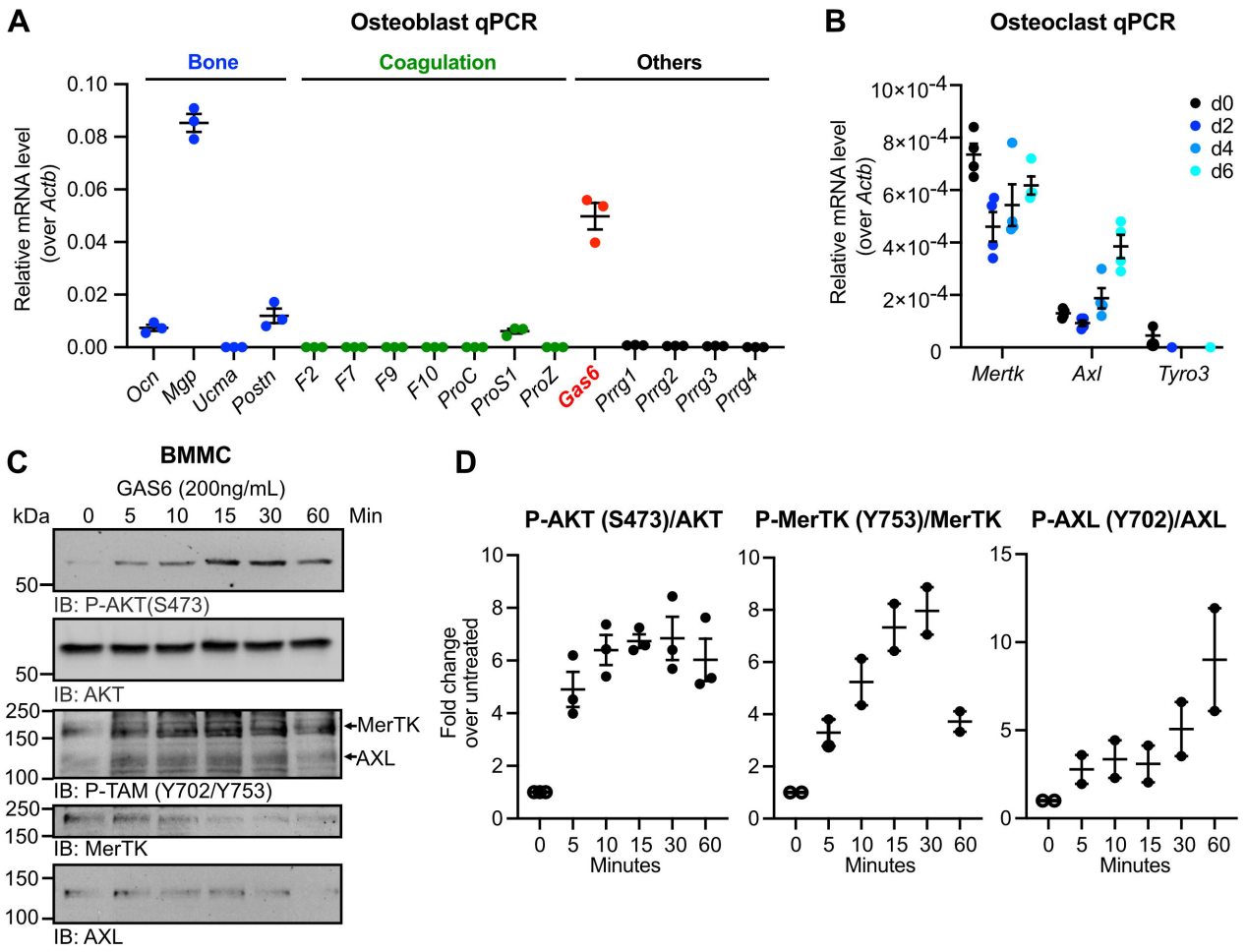
A
Osteoblast qPCR
Bone
Coagulation
Others
Relative mRNA level (over Actb)
0.10
0.08
0.06
0.04
0.02
0.00
Ocn
Mgp
Ucma
Postn
F2
F7
F9
F10
ProC
ProS1
ProZ
Gas6
Prrg1
Prrg2
Prrg3
Prrg4
B
Osteoclast qPCR
Relative mRNA level (over Actb)
10×10-4
8×10-4
6×10-4
4×10-4
2×10-4
0
d0
d2
d4
d6
Mertk
Axl
Tyro3
C
BMMC
GAS6 (200ng/mL)
kDa
0
5
10
15
30
60
Min
50
IB: P-AKT(S473)
50
IB: AKT
250
150
100
MerTK
AXL
IB: P-TAM (Y702/Y753)
250
150
IB: MerTK
150
100
IB: AXL
D
P-AKT (S473)/AKT
P-MerTK (Y753)/MerTK
P-AXL (Y702)/AXL
Fold change over untreated
Minutes

Figure 4

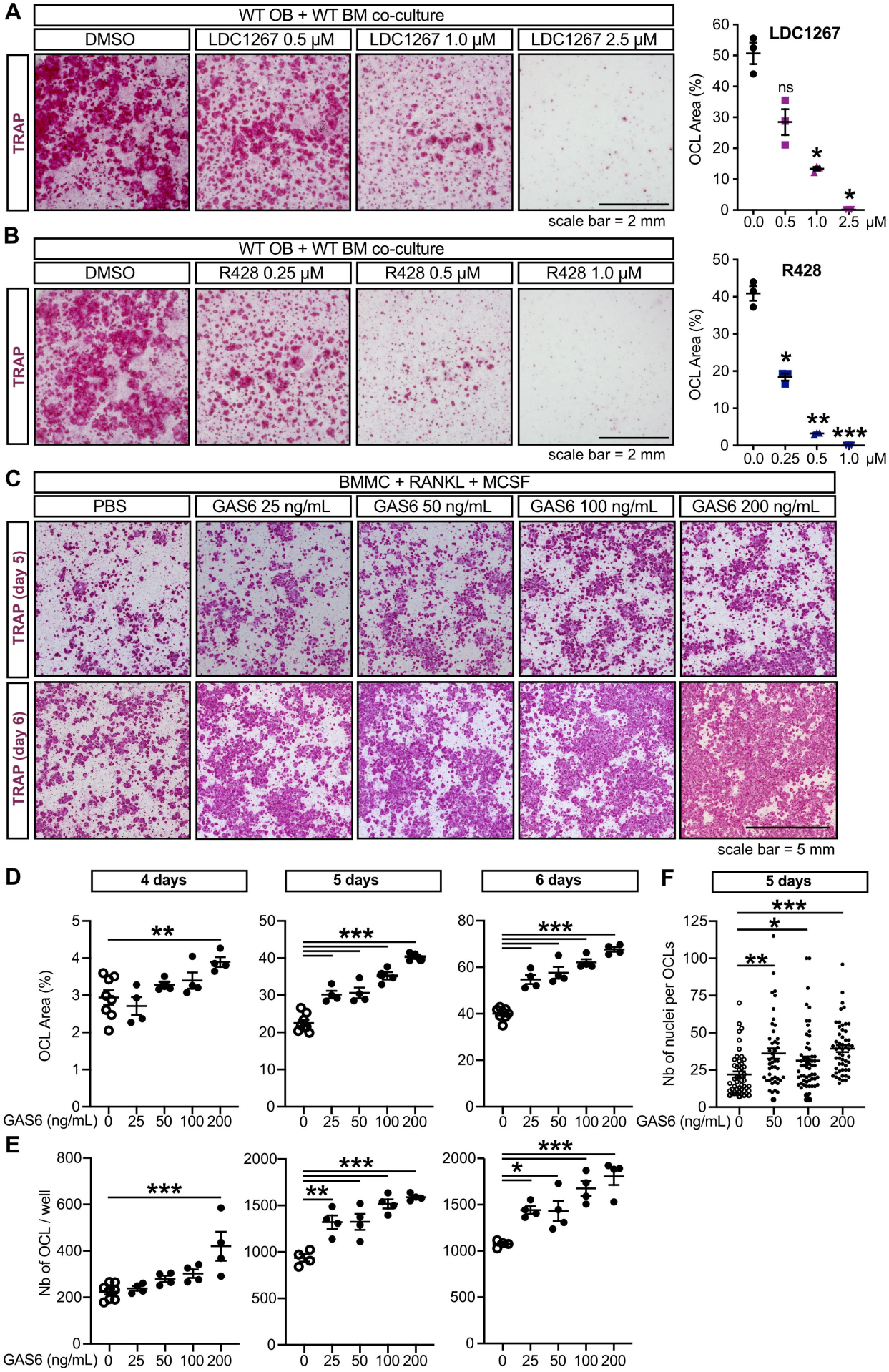
A
WT OB + WT BM co-culture
DMSO
LDC1267 0.5 µM
LDC1267 1.0 µM
LDC1267 2.5 µM
TRAP
scale bar = 2 mm
LDC1267
OCL Area (%)
ns
0.0 0.5 1.0 2.5 µM
B
WT OB + WT BM co-culture
DMSO
R428 0.25 µM
R428 0.5 µM
R428 1.0 µM
TRAP
scale bar = 2 mm
R428
OCL Area (%)
0.0 0.25 0.5 1.0 µM
C
BMMC + RANKL + MCSF
PBS
GAS6 25 ng/mL
GAS6 50 ng/mL
GAS6 100 ng/mL
GAS6 200 ng/mL
TRAP (day 5)
TRAP (day 6)
scale bar = 5 mm
D
4 days
5 days
6 days
OCL Area (%)
GAS6 (ng/mL) 0 25 50 100 200
F
5 days
Nb of nuclei per OCLs
GAS6 (ng/mL) 0 50 100 200
E
Nb of OCL / well
GAS6 (ng/mL) 0 25 50 100 200

Figure 5

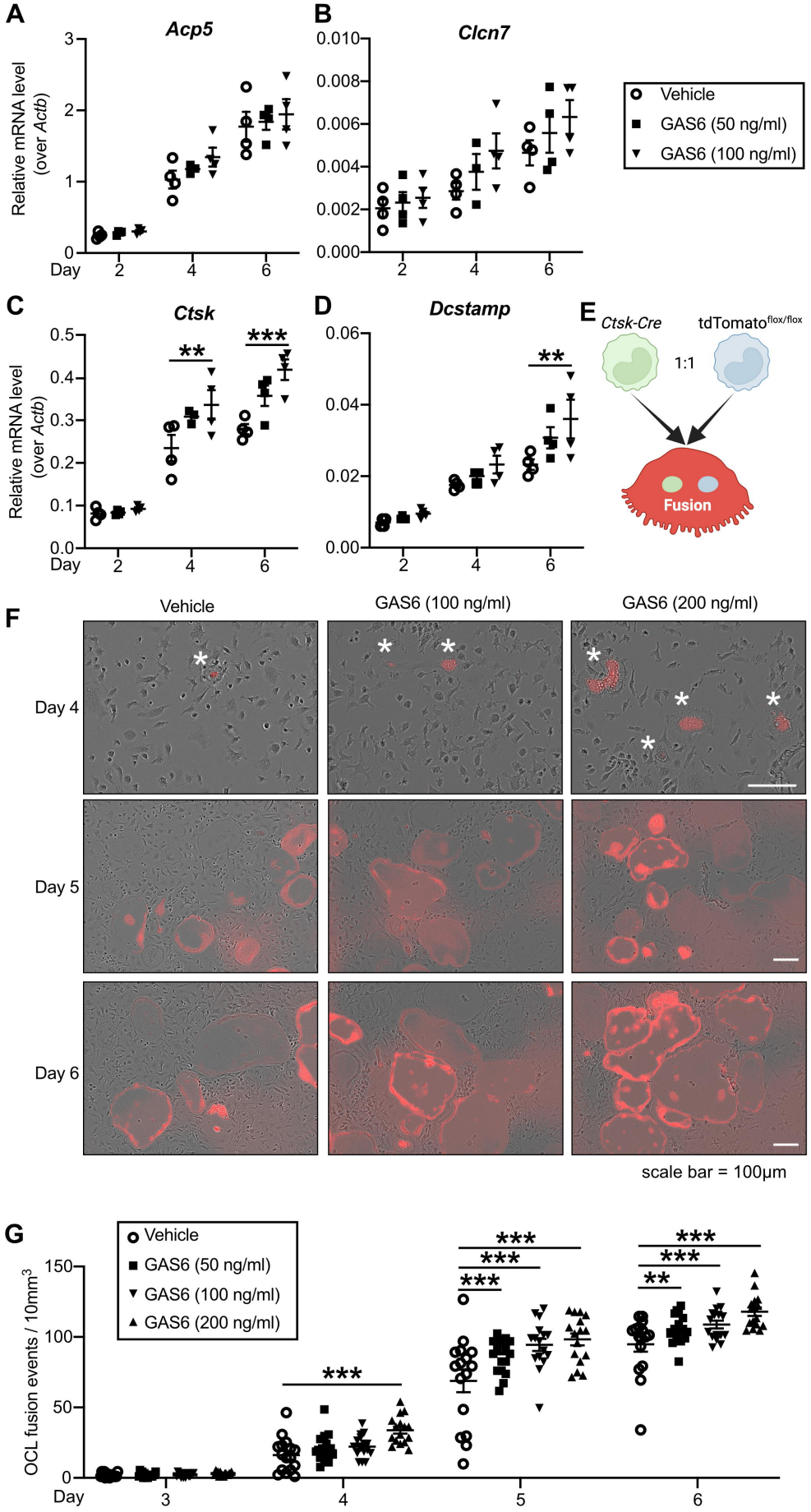
A
Acp5
Relative mRNA level (over Actb)
Day
B
Clcn7
Vehicle
GAS6 (50 ng/ml)
GAS6 (100 ng/ml)
C
Ctsk
D
Dcstamp
E
Ctsk-Cre
tdTomato flox/flox
1:1
Fusion
F
Vehicle
GAS6 (100 ng/ml)
GAS6 (200 ng/ml)
Day 4
Day 5
Day 6
scale bar = 100μm
G
GAS6 (200 ng/ml)
OCL fusion events / 10mm3

Figure 6

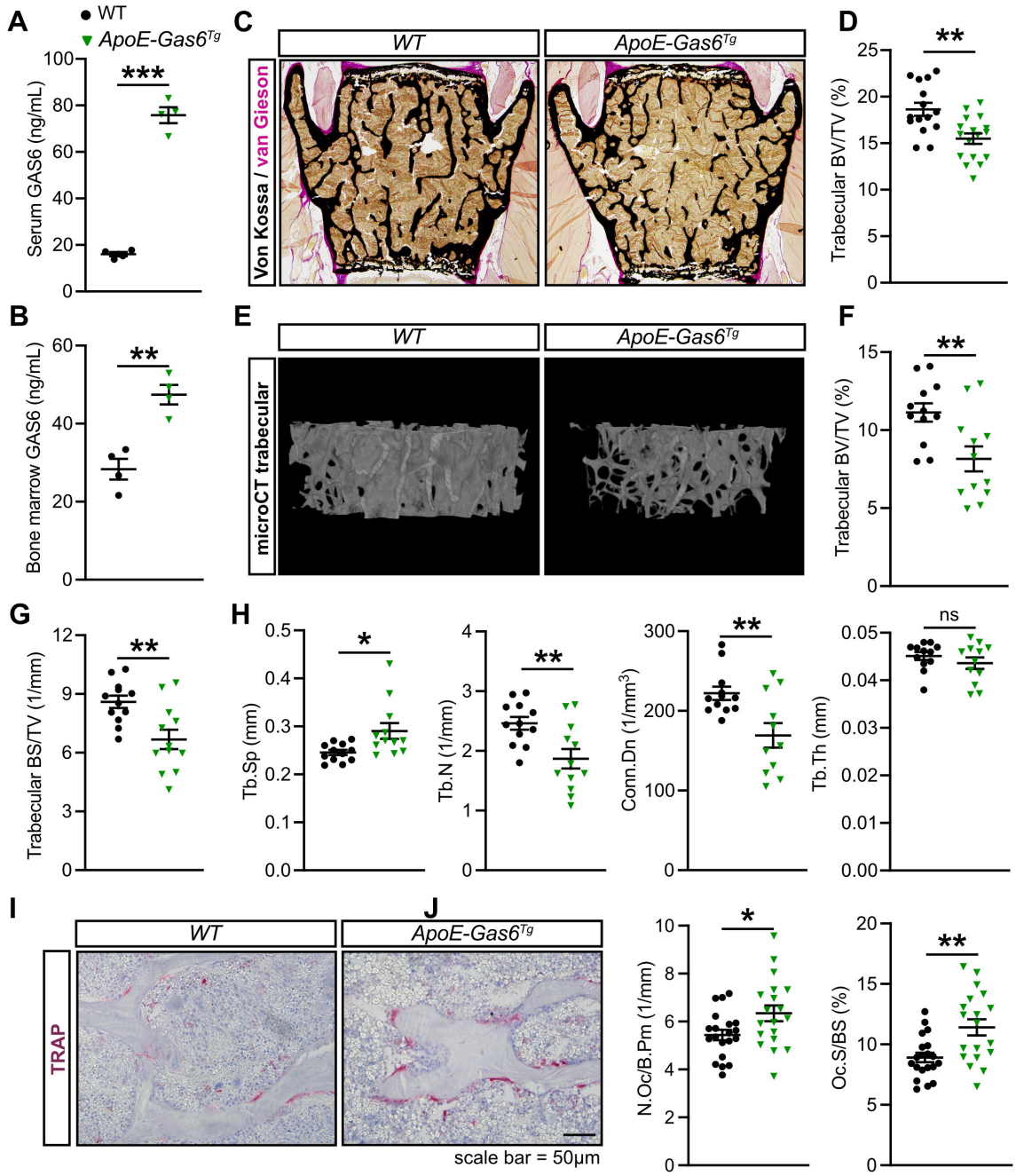
A
WT
ApoE-Gas6Tg
Serum GAS6 (ng/mL)
***
B
Bone marrow GAS6 (ng/mL)
**
C
WT
ApoE-Gas6Tg
Von Kossa / van Gieson
D
Trabecular BV/TV (%)
**
E
WT
ApoE-Gas6Tg
microCT trabecular
F
Trabecular BV/TV (%)
**
G
Trabecular BS/TV (1/mm)
**
H
Tb.Sp (mm)
*
Tb.N (1/mm)
**
Conn.Dn (1/mm3)
**
Tb.Th (mm)
ns
I
WT
ApoE-Gas6Tg
TRAP
scale bar = 50μm
J
N.Oc/B.Pm (1/mm)
*
Oc.S/BS (%)
**

Figure 7