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## A Liver-Bone Endocrine Relay by IGFBP1 Promotes Osteoclastogenesis and Mediates FGF21-Induced Bone Resorption

Xunde Wang<sup>1,\*</sup>, Wei Wei<sup>1,\*</sup>, Jing Y. Krzeszinski<sup>1</sup>, Yubao Wang<sup>1</sup>, and Yihong Wan<sup>1</sup>

<sup>1</sup>Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

### SUMMARY

Fibroblast growth factor 21 (FGF21) promotes insulin sensitivity but causes bone loss. It elevates bone resorption by an undefined non-osteoclast-autonomous mechanism. We have detected a pro-osteoclastogenic activity in the hepatic secretome that is increased by FGF21, and largely attributed to insulin-like growth factor binding protein 1 (IGFBP1). *Ex vivo* osteoclast differentiation and *in vivo* bone resorption are both enhanced by recombinant IGFBP1 but suppressed by an IGFBP1-blocking antibody. Anti-IGFBP1 treatment attenuates ovariectomy-induced osteoporosis, and abolishes FGF21-induced bone loss while maintaining its insulin-sensitizing metabolic benefit. Mechanistically, IGFBP1 functions via its RGD domain to bind to its receptor integrin  $\beta 1$  on osteoclast precursors, thereby potentiating RANKL-stimulated Erk-phosphorylation and NFATc1 activation. Consequently, osteoclastic integrin  $\beta 1$  deletion confers resistance to the resorption-enhancing effects of both IGFBP1 and FGF21. Therefore, the hepatokine IGFBP1 is a critical liver-bone hormonal relay that promotes osteoclastogenesis and bone resorption, as well as an essential mediator of FGF21-induced bone loss.

### Graphical Abstract

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Correspondence and requests for materials should be addressed to Y.Wan (yihong.wan@utsouthwestern.edu).

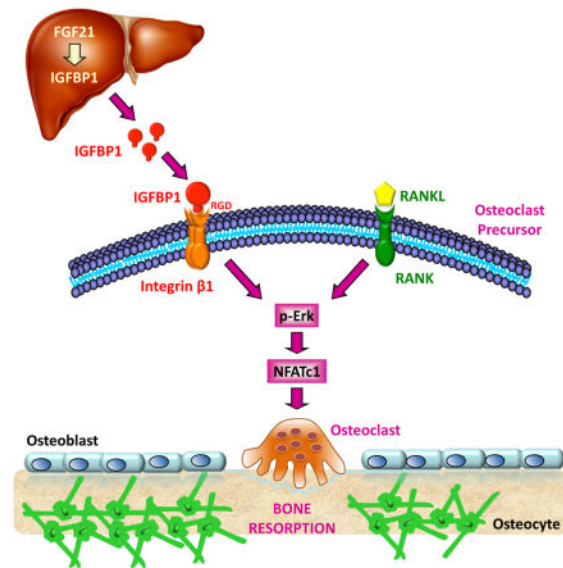
\*These authors contributed equally to this work

#### AUTHOR CONTRIBUTIONS

X.W., W.W. and Y.Wan conceived the project and designed the experiments. X.W. and W.W. conducted most of the experiments and data analyses. J.Y.K. assisted with ovariectomy. Y.Wang assisted with co-immunoprecipitation. Y.Wan wrote the manuscript.

The authors declare that they have no financial conflict of interest.

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## INTRODUCTION

Osteoclasts, the professional bone resorbing cells, are essential for bone turnover and skeletal regeneration (Novack and Teitelbaum, 2008). However, excessive osteoclast activity can lead to diseases such as osteoporosis, arthritis and cancer bone metastasis (Novack and Teitelbaum, 2008). Osteoclastogenesis is the differentiation of osteoclasts from hematopoietic progenitors in response to receptor activator of nuclear factor kappa-B ligand (RANKL), which can be regulated by endocrine hormones and metabolic signals. It can also be stimulated by pharmacological agents such as rosiglitazone, a widely used drug for diabetes (Wan et al., 2007). New knowledge of how osteoclastogenesis and bone resorption are regulated will provide key insights into disease pathology as well as better treatment.

FGF21 is a powerful regulator of glucose and lipid metabolism, thus a potential new drug for obesity and diabetes that is currently in clinical trials (Canto and Auwerx, 2012; Potthoff et al., 2012). We have recently identified FGF21 as a physiologically and pharmacologically significant negative regulator of bone mass (Wei et al., 2012), suggesting that skeletal fragility may be an undesirable consequence of chronic FGF21 administration. Thus, the identification of the cellular and molecular mechanisms for how FGF21 controls bone homeostasis will both enhance our fundamental understanding of skeletal physiology and illuminate potential strategies to separate its metabolic benefits from its detrimental bone loss side effects.

FGF21 induces bone loss by simultaneously decreasing bone formation and increasing bone resorption (Wei et al., 2012). However, the mechanism for how FGF21 enhances bone resorption was unclear. Our previous findings show that FGF21 does not directly regulate osteoclast differentiation from hematopoietic progenitors (Wei et al., 2012), indicating that FGF21 acts on other tissues and cell types to indirectly promote osteoclastogenesis and bone resorption. Here we have identified IGFBP1 as an endocrine hormone from the liver that

directly promotes RANKL-mediated osteoclastogenesis via its receptor integrin  $\beta 1$ , as well as an essential mediator of FGF21-induced bone resorption and bone loss.

## RESULTS

### IGFBP1 is an FGF21-Induced Pro-Osteoclastogenic Hepatokine

Because FGF21 is highly expressed in the liver, we hypothesize that it may induce the secretion of endocrine factor(s) from the liver that can directly enhance osteoclastogenesis. To test this hypothesis, we collected liver-cell-derived conditioned medium (LCM) from WT or FGF21-Tg mice and determined their effects on RANKL-mediated and rosiglitazone-stimulated osteoclast differentiation from WT bone marrow cells. Compared with mock treatment, osteoclast differentiation was significantly augmented by LCM from WT mice and further enhanced by LCM from FGF21-Tg mice, quantified by the expression of osteoclast markers such as TRAP (tartrate-resistant acid phosphatase) (Figure 1A). These results indicate that WT liver secretes pro-osteoclastogenic factor(s) in response to physiological levels of FGF21, which is enhanced by pharmacological FGF21 over-expression.

To identify this pro-osteoclastogenic hepatokine, we searched for liver-specific secreted factors that are up-regulated by FGF21. Because IGFBP1 is an FGF21-inducible liver-specific factor (Inagaki et al., 2008); and osteoclast differentiation can be enhanced by the predominantly osteoblast-residing IGFBP2 (DeMambro et al., 2012), we postulate that IGFBP1 may be the pro-osteoclastogenic hepatokine in the LCM that is responsible for FGF21-induced bone resorption. We found that IGFBP1 mRNA was high and FGF21-inducible in the liver but absent in the bone (Figure 1B). Liver IGFBP1 mRNA was lower in FGF21-KO mice (Figure S1A), suggesting that FGF21 induction of IGFBP1 is physiologically relevant. IGFBP1 mRNA was the highest in the liver compared to other tissues by >100 fold (Figure 1C), supporting its role as a hepatokine. Moreover, IGFBP1 protein levels in both liver and serum were increased in FGF21-Tg mice compared to WT controls (Figure 1D). These results show that FGF21 induces IGFBP1 expression and secretion from the liver.

To investigate whether IGFBP1 is required for the pro-osteoclastogenic activity in WT LCM, we tested whether the activity can be eliminated by an IGFBP1-blocking antibody. Compared to an IgG control, anti-IGFBP1 abolished the osteoclast-enhancing effects of WT LCM (Figure 1E). Western blot confirmed that anti-IGFBP1 did not cross-react with IGFBP2 (Figure S1B). These results indicate that IGFBP1 is the major pro-osteoclastogenic factor in the liver secretome.

To determine if IGFBP1 is sufficient to stimulate RANKL-mediated osteoclastogenesis in the absence of other factors in the liver secretome, we next treated WT osteoclast differentiation cultures with recombinant IGFBP1. When bone marrow osteoclast differentiation was induced with 100ng/ml RANKL, IGFBP1 significantly stimulated osteoclast differentiation at 2ng/ml, and the effects were enhanced in a dose-dependent manner, shown by the increased TRAP expression (Figure 1F), number and size of mature osteoclasts, as well as resorptive activity (Figure 1G). A titration of anti-IGFBP1 antibody

showed that the pro-osteoclastogenic effects by 2ng/ml IGFBP1 could be completely blocked by 4ng/ml of anti-IGFBP1 (Figure S1C). Moreover, RANKL-induced osteoclast differentiation from the RAW264.7 mouse macrophage cell line was also enhanced by both mouse and human IGFBP1 (Figure 1H).

To determine whether IGFBP1 also regulates osteoclast differentiation in human cells, we treated human peripheral blood mononuclear (PBMN) precursors with human RANKL, in the presence of human IGFBP1 or vehicle control. Osteoclast differentiation from human PBMN was also augmented by hIGFBP1, shown by the higher TRAP expression (Figure 1I), as well as the increased number, size and activity of mature osteoclasts (Figure 1J).

In contrast, IGFBP1 affected neither bone marrow osteoblast differentiation (Figure 1K) nor RANKL/OPG expression in osteoblasts (Figure 1L). When osteoblast-derived conditioned medium was applied to osteoclast cultures, osteoclastogenesis was enhanced only when IGFBP1 was added to osteoclasts but not osteoblasts, further indicating that IGFBP1 acts directly on osteoclasts rather than indirectly through osteoblasts (Figure 1M). Collectively, these findings indicate that IGFBP1 is a pro-osteoclastogenic hepatokine that is induced by FGF21 and functions as an endocrine hormone upon the skeleton.

### **Bone Resorption is Enhanced by rIGFBP1 but Blocked by Anti-IGFBP1 Antibody**

We next investigated whether IGFBP1 gain- or loss-of-function regulates bone resorption *in vivo*. For gain-of-function, we administered recombinant IGFBP1 into WT mice with a daily IP injection at 0.015mg/kg/day for 14 days. For loss-of-function, we administered an IGFBP1 blocking antibody into WT mice with a daily IP injection at 0.03mg/kg/day for 14 days. ELISA analysis of a bone resorption marker C-terminal telopeptide fragments of the type I collagen (CTX-1) revealed that bone resorption was increased by 110% by rIGFBP1 and decreased by 42% by anti-IGFBP1 compared with control (Figure 2A). Consistent with our *ex vivo* observation that IGFBP1 does not affect osteoblast differentiation (Figure 1L), the bone formation marker N-terminal propeptide of type I procollagen (P1NP) was unaltered by either rIGFBP1 or anti-IGFBP1 treatment (Figure 2B). Histomorphometry showed that osteoclast numbers and surface, as well as eroded surface, were increased by rIGFBP1 and decreased by anti-IGFBP1 (Figure S2A), whereas osteoblast numbers and surface, as well as bone formation rate and mineral apposition rate, were unaltered (Figure S2B–C). Consequently, microcomputed tomography ( $\mu$ CT) analysis of the proximal tibiae revealed that IGFBP1 treatment caused a significant bone loss; in contrast, anti-IGFBP1 treatment protected bone leading to a significantly higher bone mass (Figure 2C–D, S2D).

Two weeks of rIGFBP1 treatment elevated serum IGFBP1 by 47% (Figure 2E), which was similar to the IGFBP1 induction by 2 weeks of FGF21 treatment (59%) (Figure 2F). Interestingly, we found that ovariectomy (OVX) - a widely used model for postmenopausal osteoporosis - also elevated serum IGFBP1 levels in female mice to a similar degree by 41% compared with sham controls (Figure 2G). This stemmed from a 49% increase in hepatic IGFBP1 expression (Figure 2G, *top*) as IGFBP1 expression in other tissues were >100-fold lower and unaffected by OVX. This is consistent with human studies showing elevated plasma IGFBP1 levels in osteoporotic patients (Jehle et al., 2003; Salminen et al., 2008).

Conversely, two weeks of anti-IGFBP1 treatment reduced serum IGFBP1 by 71% (Figure 2H).

Importantly, serum levels of free and total IGF1 were unaltered by rIGFBP1 or anti-IGFBP1 treatment at the dose applied (Figure 2I), consistent with the result that bone formation was unchanged. This observation is in line with the fact that IGFBP3 is the most abundant circulating IGFBP that accounts for 80–90% of all IGF binding (Endogenous et al., 2010; Stolzenberg-Solomon et al., 2004), and IGFBP1 only plays a minor role in IGF1 sequestration. Moreover, the anti-IGFBP1 antibody exhibits <1% cross-reactivity with other IGFBPs such as IGFBP-2, IGFBP-3, IGFBP-5, IGFBP-6 and IGFBP-7 (R&D Systems and Figure S1B). Therefore, our pharmacological approaches to acutely increase or decrease IGFBP1 levels during adulthood at a physiologically relevant dose presents a unique window of opportunity to examine the intrinsic function of IGFBP1 *in vivo* that is independent of IGF1, revealing it as a potent resorption-enhancing liver hormone.

### IGFBP1 Blockade Abolishes OVX-Induced Bone Resorption

To further explore the therapeutic potential of IGFBP1 blockade, we examined whether anti-IGFBP1 treatment can attenuate the elevated bone resorption during postmenopausal osteoporosis in a mouse disease model. To simulate the estrogen loss in postmenopausal women, we performed OVX in female WT mice. Three days after OVX or sham surgery, we injected anti-IGFBP1 or an IgG negative control at 0.03mg/kg and three times per week for 5 weeks. Uterine weight was reduced by ~80% in all OVX mice compared to sham controls, indicating effective estrogen depletion (Figure 3A). Serum CTX-1 was increased by 55% in OVX mice treated with IgG control compared to sham control mice treated with IgG (Figure 3B). In contrast, the OVX-induced bone resorption was abolished by anti-IGFBP1 treatment, which instead led to a 28% reduction in CTX-1 compared to sham controls treated with IgG (Figure 3B). Although bone formation was unaffected by anti-IGFBP1 treatment (Figure 3C), OVX-induced bone loss was significantly rescued (Figure 3D). Body weight in OVX mice was unaffected by anti-IGFBP1 treatment (not shown). These results reveal a previously unrecognized additional mechanism for OVX-induced bone resorption: estrogen loss exerts not only a direct effect of enhancing osteoclast survival (Krum, 2011; Nakamura et al., 2007), but also an indirect effect of increasing osteoclast differentiation via elevating circulating IGFBP1 levels, suggesting that IGFBP1 blockade may be a potential treatment for osteoporosis.

### IGFBP1 Blockade Abolishes FGF21-Induced Bone Resorption

We also tested if anti-IGFBP1 could ameliorate the bone resorption induced by FGF21. Under chow-diet feeding, anti-IGFBP1 (0.03mg/kg/day for 14 days) effectively reduced serum CTX-1 in FGF21-Tg mice to a similar level as in WT controls (Figure 3E), without affecting serum P1NP (Figure 3F), leading to a significant rescue of the FGF21-induced bone loss (Figure 3G). Histomorphometry showed that this anti-IGFBP1 treatment abolished the increased osteoclast surface and number in FGF21-Tg mice (Figure S3A), without significantly affecting osteoblast surface/number, bone formation rate or mineral apposition rate (Figure S3B–C). Similarly, under high-fat-diet (HFD) feeding, anti-IGFBP1 also prevented FGF21 induction of CTX-1 (Figure 3H), without affecting FGF21 reduction of

P1NP (Figure 3I), leading to the attenuation of FGF21-induced bone loss (Figure 3J). Importantly, insulin tolerance test (ITT) showed that the improved insulin sensitivity in FGF21-Tg mice was intact following anti-IGFBP1 treatment (Figure 3K). This indicates that IGFBP1 blockade may represent an exciting strategy to prevent the bone loss side effects while retaining the metabolic benefits of FGF21.

### **IGFBP1 Potentiates RANKL-Stimulated Erk Phosphorylation and NFATc1 Activation**

We next investigated the molecular mechanisms for how IGFBP1 enhances osteoclastogenesis. Our bone marrow osteoclast differentiation scheme allowed us to specifically dissect the effects of IGFBP1 on precursor proliferation during the first 3 days of MCSF treatment (d1-3), and osteoclast differentiation during the latter 3 days of RANKL + MCSF treatment (d4-6) (Figure 4A). When IGFBP1 treatment was limited to d4-6 only, osteoclast differentiation was enhanced to a similar extent as when IGFBP1 treatment was throughout the entire 6 days (d1-6) (Figure 4B, S1D). In contrast, osteoclast differentiation was unaffected when IGFBP1 treatment was limited to d1-3 (Figure 4B, S1D). Consistent with these observations, MTT assay showed that IGFBP1 did not alter cell proliferation on either d3 or d6 (Figure 4C). These results indicate that IGFBP1 promotes osteoclastogenesis by enhancing RANKL-mediated differentiation without affecting MCSF-mediated precursor proliferation.

We next examined how IGFBP1 potentiates RANKL signaling. We found that IGFBP1 enhanced both basal and RANKL-induced Erk phosphorylation in osteoclast differentiation cultures (Figure 4D–E). In contrast, IGFBP1 did not affect basal or RANKL-stimulated c-Jun phosphorylation, Akt phosphorylation or I $\kappa$ B $\alpha$  degradation (Figure 4F). To identify which RANKL downstream transcription factor(s) is regulated by IGFBP1 signaling, we transfected RAW267.4 cells with a luciferase reporter driven by the response elements for NFATc1, AP-1 or NF $\kappa$ B. As expected, RANKL treatment activated these endogenous transcription factors, leading to the induction of their corresponding luciferase reporter (Figure 4G). IGFBP1 co-treatment selectively potentiated the RANKL-induction of NFATc1 reporter, but not AP-1 or NF $\kappa$ B reporters (Figure 4G). In line with the published reports that Erk activation promotes NFATc1 signaling and osteoclast differentiation (Choi et al., 2014; Kim et al., 2007), our results showed that IGFBP1 enhancement of NFATc1 and osteoclastogenesis was abolished by an Erk inhibitor (Figure 4H). Consistently, IGFBP1 also increased NFATc1 expression in osteoclast differentiation cultures (Figure 4I). These results indicate that IGFBP1 promotes RANKL-mediated osteoclastogenesis by enhancing Erk phosphorylation and NFATc1 activation.

### **IGFBP1 Functions through the Integrin $\beta$ 1 Receptor in the Osteoclast Lineage**

We next set out to identify the IGFBP1 receptor on osteoclast precursors. An RGD (Arginine-Glycine-Aspartic acid) integrin recognition motif in the C-terminus of IGFBP1 is highly conserved among mammals including human, pig, mouse and rat (Figure 5A), indicating that IGFBP1 may function as a ligand for integrin receptors. A previous study using Chinese hamster ovary (CHO) cells reports that integrin  $\alpha$ 5 $\beta$ 1 is the only cell surface receptor that can bind to IGFBP1 in an RGD-dependent but IGF1-independent fashion (Jones et al., 1993). We found that both IGFBP1 stimulation of osteoclastogenesis (Figure



5B) and IGFBP1 induction of Erk phosphorylation (Figure 5C) were abolished by the addition of a synthetic RGD-containing competing peptide to WT osteoclast differentiation culture. This indicates that the RGD binding motif in IGFBP1 is functionally required; suggesting that integrin  $\alpha 5 \beta 1$  may be the IGFBP1 receptor for its pro-osteoclastogenic activity. Integrin  $\alpha 5$  can partner with several integrin  $\beta$  subunits including integrin  $\beta 3$  that can promote osteoclast cytoskeleton remodeling (Novack and Teitelbaum, 2008). Thus, the specificity of IGFBP1 binding likely resides in integrin  $\beta 1$  (Itgb1).

To determine the *in vivo* requirement of Itgb1 for IGFBP1 stimulation of bone resorption, we generated osteoclast-specific Itgb1 knockout mice (Oc-Itgb1-KO) by breeding Itgb1 flox mice (Raghavan et al., 2000) with lysozyme-cre transgenic mice (Clausen et al., 1999). *Ex vivo* bone marrow osteoclast differentiation assay showed that Itgb1 expression was reduced by 93% in the Oc-Itgb1-KO cultures, indicating efficient Itgb1 deletion (Figure 5D). Compared to control cultures, Oc-Itgb1-KO cultures were completely resistant to IGFBP1 potentiation of osteoclast differentiation, but still sensitive to RANKL-mediated and rosiglitazone-stimulated osteoclast differentiation (Figure 5E–F). Furthermore, IGFBP1 induction of Erk-phosphorylation was completely abolished in Oc-Itgb1-KO osteoclast precursors, whereas RANKL stimulation of Erk-phosphorylation remained intact (Figure 5G). Co-immunoprecipitation (Co-IP) assay showed that IGFBP1 physically interacted with Itgb1 in osteoclasts, which can be effectively blocked by anti-IGFBP1 or RGD peptide (Figure 5H). In contrast, knockdown of Itgb3 or Itgb5 did not affect the ability of IGFBP1 to potentiate osteoclast differentiation (Figure 5I). These findings reveal a previously unrecognized functional diversity for how the  $\beta$  integrin family regulates osteoclastogenesis: Itgb1 promotes the early stage of osteoclast differentiation in response to IGFBP1, whereas other  $\beta$  integrins such as Itgb3 mainly promote the late stage of cytoskeleton remodeling (Novack and Teitelbaum, 2008).

### Osteoclastic Itgb1 Deletion Abolishes IGFBP1-induced Resorption and Bone Loss

We then analyzed the *in vivo* consequences by comparing Oc-Itgb1-KO mice with littermate controls, treated with IGFBP1 or vehicle control (0.015mg/kg/day for 14 days). Consistent with the *ex vivo* observations, IGFBP1-induced bone resorption (Figure 6A), as well as osteoclast numbers and surface (Figure S4A), were completely abolished in Oc-Itgb1-KO mice, indicating that Itgb1 is required for the pharmacological effects of IGFBP1. Moreover, basal bone resorption (Figure 6A), as well as osteoclast numbers and surface (Figure S4A), were also significantly lower in Oc-Itgb1-KO mice compared to control mice, indicating that Itgb1 is also required for the physiological regulation by IGFBP1. In contrast, the bone formation marker P1NP (Figure 6B), osteoblast numbers/surface, bone formation rate and mineral apposition rate (Figure S4B–C), were unaffected in either IGFBP1-treated mice or Oc-Itgb1-KO mice. Consequently, Oc-Itgb1-KO mice had higher basal bone mass and were refractory to IGFBP1-induced bone loss (Figure 6C–D, S4D). These results indicate that Itgb1 is the major and essential  $\beta$  integrin receptor for IGFBP1 that accounts for its pro-osteoclastogenic function.

## Deletion of the IGFBP1-Itgb1 Axis Abolishes FGF21-induced Resorption and Bone Loss

To further investigate whether the IGFBP1-Itgb1 signaling pathway is required for FGF21-induced bone resorption and bone loss, we next examined the skeletal effects of FGF21 in Oc-Itgb1-KO mice. Oc-Itgb1-KO or control mice were fed with HFD for 5 weeks, and treated with FGF21 (1mg/kg/day) or vehicle control during the last 14 days. In control mice, FGF21 elevated bone resorption (Figure 6E) and osteoclast number/surface (Figure S5A), leading to a lower bone mass (Figure 6F–G). In contrast, these effects were abolished in Oc-Itgb1-KO mice (Figure 6E–G, S5). ITT assay revealed that FGF21-mediated insulin sensitization was intact in Oc-Itgb1-KO mice (Figure 6H). These data indicate that Itgb1 deletion in the osteoclast lineage specifically abolishes FGF21-induced bone loss detrimental effects while retaining FGF21-mediated metabolic benefits. These findings further support that Itgb1 functions as a key IGFBP1 receptor in the osteoclast lineage to mediate the resorption-enhancing effects of the FGF21-IGFBP1 axis. In accordance with our pharmacological findings, genetic rescue experiments using IGFBP1-KO/FGF21-Tg compound mutants showed that IGFBP1 deletion conferred a lower basal bone resorption and a higher basal bone mass, as well as a complete resistance to FGF21-induced bone resorption and bone loss (Figure 6I–K, S6). These results further strengthen the conclusion that IGFBP1 is a physiologically significant regulator of bone resorption, and the key Itgb1 ligand that mediates FGF21-induced bone resorption.

## DISCUSSION

This study uncovers a previously unrecognized liver-bone endocrine relay, as well as a key mechanism for FGF21-induced bone resorption. Physiological or pharmacological elevation of FGF21 induces IGFBP1 expression in the liver and its secretion into circulation. In turn, IGFBP1 acts directly on the osteoclast precursors by binding to integrin  $\beta$ 1 receptor via its RGD motif. Consequently, IGFBP1 stimulates osteoclast differentiation and bone resorption by potentiating RANKL-induced Erk phosphorylation and NFATc1 activation, leading to lower bone mass (Figure 7). Thus, we have identified IGFBP1 as an endocrine hormone that is secreted from the liver in response to metabolic cues to regulate bone resorption and skeletal homeostasis.

Our findings reveal that an anti-IGFBP1 antibody can suppress bone resorption and increase bone mass, highlighting pharmacological IGFBP1 blockade as a potential strategy for the treatment of osteoporosis. Moreover, we have also identified the IGFBP1-Itgb1 pathway as an essential and specific mediator of FGF21-induced bone resorption; either an IGFBP1 blocking antibody or osteoclastic Itgb1 deletion confers resistance to FGF21-induced bone loss without compromising its insulin-sensitizing benefits. These findings suggest that pharmacological IGFBP1 blockade may also represent a potential avenue to ameliorate the detrimental skeletal effects of FGF21 while preserving its beneficial metabolic actions. Our previous study shows that FGF21 inhibits osteoblast differentiation via a direct effect on the mesenchymal lineage by favoring marrow adipogenesis (Wan, 2013; Wei et al., 2012). Here we show that FGF21 stimulates osteoclast differentiation via an indirect liver-bone hormonal relay by IGFBP1. Therefore, two distinct mechanisms mediate the two different aspects of FGF21 regulation of bone remodeling.



Our data show that IGFBP1 alone can stimulate Erk phosphorylation and further enhance RANKL-induced Erk phosphorylation (Figure 4D–E, 5G), without affecting c-Jun phosphorylation, Akt phosphorylation or  $\text{I}\kappa\text{B}\alpha$  degradation (Figure 4F). The reason that IGFBP1 only stimulates osteoclastogenesis in the presence of RANKL is that although pErk can promote osteoclast differentiation, it is not sufficient to trigger the entire program of differentiation, which also requires the other RANKL downstream signaling pathways. Thus, in the absence of RANKL, IGFBP1 can stimulate pErk but not osteoclast differentiation, but in the presence of RANKL, IGFBP1-potentiated pErk can enhance RANKL-mediated osteoclast differentiation.

Our findings reveal that IGFBP1 functions as a pro-osteoclastogenic liver hormone via the *Itgb1* receptor in osteoclasts but independent of IGF1, as well as a key mediator of FGF21-induced bone resorption and bone loss. A previous study shows that IGFBP2 can also enhance osteoclast differentiation (DeMambro et al., 2012). Although IGFBP2 is highly expressed in osteoblasts, it is also expressed in liver, suggesting that its resorption-enhancing activity may partially stem from the liver, which has never been previously recognized and still awaits future investigation. Moreover, IGFBP2 also contains a similar RGD region, suggesting that its pro-osteoclastogenic function may be also mediated by integrin receptors. The robust induction of liver IGFBP1 expression by FGF21 has been well described (Inagaki et al., 2008), although FGF21 induction of IGFBP2 has also been reported (Emanuelli et al., 2014). We found that liver IGFBP2 mRNA were not significantly altered by 3 days of FGF21 treatment vs. vehicle control (1.2 fold,  $p=0.25$ ), neither was serum IGFBP2 levels (1.06 fold,  $p=0.49$ ). Importantly, the complete abolishment of FGF21-induced bone resorption both in IGFBP1-KO mice and anti-IGFBP1 treated mice demonstrate that IGFBP1 is the major mediator of FGF21 enhancement of osteoclastogenesis, whereas IGFBP2 plays only a minor role, if any. Although basal bone formation is unaffected by IGFBP1 blockade or osteoclastic *Itgb1* deletion, FGF21 inhibition of bone formation is dampened (Figure S3B–C, S5B–C, S6B–C), which in combination with the impairment of FGF21 augmentation of bone resorption alleviates the uncoupling effects and FGF21-induced bone loss.

The increase in circulating IGFBP1 levels upon OVX and the abolishment of OVX-induced bone resorption by IGFBP1 blockade suggest that estrogen may protect bone by suppressing IGFBP1-mediated pathological bone resorption. Although the major physiological effect of estrogen is to inhibit bone resorption, bone formation also changes following estrogen deficiency. In our experiments in mice, we observed a reduction in bone formation 5 weeks after OVX compared with sham controls (Figure 3C). A previous study in rat reported that bone formation was decreased during the first week post-OVX and then increased during third and fourth weeks post-OVX (Tanizawa et al., 2000). We do not know why this discrepancy occurs.

An early study failed to detect any osteoclast differentiation defects in *Itgb1*-deficient cells (Schmidt et al., 2011). Potential explanations for this discrepancy are: 1) The *Itgb1*-deficient mice in this early study were generated with MX1-cre after a single dose of pI/pC injection, and it was unclear how efficient *Itgb1* was deleted. 2) The heat-inactivated FCS they used in the differentiation cultures might have much less active IGFBP1 than the FBS we used. 3)

Their study was entirely *in vitro* without any *in vivo* examination of bone resorption, osteoclast number or bone mass. A recent study showed that deletion of DAP12 had no effect on bone mass relative to WT nor did additional deletion of *Itgb1* in osteoclast lineage cells on a DAP12<sup>-/-</sup> background (Zou and Teitelbaum, 2015). The source of this discrepancy is unclear.

Integrin receptors are important effectors of not only cell adhesion and migration but also cell differentiation. Particularly, integrin  $\beta 1$  has been reported to regulate neuronal and astrocytic differentiation (Pan et al., 2014; Tate et al., 2004); keratinocyte differentiation (Hotchin et al., 1995; Levy et al., 2000), type II lung epithelial cell differentiation (Sanchez-Esteban et al., 2006), chondrogenesis (Singh and Schwarzbauer, 2012), and mammary epithelium differentiation (Naylor et al., 2005). Here we uncover IGFBP1 as a functional circulating ligand for integrin  $\beta 1$ , in addition to the previously reported extracellular matrix ligands such as fibronectin. Importantly, our current genetic and biochemical studies have established integrin  $\beta 1$  as a critical IGFBP1 receptor that is essential for its pro-osteoclastogenic function.

Several human diseases including osteoporosis and diabetes are associated with higher serum IGFBP1 levels and at the same time bone loss (Jehle et al., 2003; Miao et al., 2005; Moyer-Mileur et al., 2008; Rosen, 2008; Ruan and Lai, 2010; Salminen et al., 2008; Schwartz et al., 2001), although a lack of correlation has also been reported (Pye et al., 2011). Nonetheless, the functional significance and molecular mechanisms underlying IGFBP1 actions in these clinical observations have been long elusive. This study challenges the dogma that IGFBP1 regulates physiology solely as an IGF1 binder by revealing its function as an endocrine hormone. A quarter of century after its cloning, IGFBP1 is now recognized as a metabolic signal sent from the liver to the bone to directly stimulate osteoclastogenesis and bone resorption via the receptor integrin  $\beta 1$ . This discovery paves the road for future investigations of how IGFBP1 is regulated under various physiological and pathological conditions. In addition to FGF21, IGFBP1 levels may be also modulated by other upstream signals - the observation that ovariectomy induces IGFBP1 indicates that estrogen may directly or indirectly suppress liver IGFBP1 expression. Moreover, this discovery provides key insights for how skeletal homeostasis may be modulated via changes in IGFBP1. For example, IGFBP1 levels is suppressed by insulin and increased in type 1 diabetes (Lee et al., 1993); it is also stimulated by glucocorticoids (Goswami et al., 1994). Intriguingly, our findings indicate that IGFBP1 blockade may specifically prevent the excessive bone resorption without completely eliminating RANKL-mediated physiological basal bone resorption, which is essential for bone regeneration and fracture repair. Hence, the discovery of IGFBP1 as an FGF21-induced pro-osteoclastogenic liver hormone opens an exciting new path to the fundamental understanding of the physiological and pathological connection between energy metabolism and skeletal homeostasis, as well as the development of new pharmacological treatments of bone and metabolic diseases.

## EXPERIMENTAL PROCEDURES

### Mice

FGF21-Tg mice (Ding et al., 2012; Inagaki et al., 2007) and IGFBP1-KO mice (Leu et al., 2003) on a C57BL6 background have been described. Osteoclastic integrin  $\beta$ 1 KO mice were generated by crossing Integrin  $\beta$ 1 flox mice (Raghavan et al., 2000) with lysozyme-cre mice (Clausen et al., 1999) (Jackson laboratory). Ovariectomy or sham operation was performed as described (Wei et al., 2011). Insulin tolerance test was performed as described (Ding et al., 2012). Mice were fed standard chow containing 4% fat ad libitum unless stated otherwise. For diet-induced-obesity, mice were fed a high fat diet containing 60% kcal from fat (Research Diets Inc. #D12492). All experiments were conducted using littermates. Sample size estimate was based on power analyses performed using SAS 9.3 TS X64\_7PRO platform at the UTSW Biostatistics Core. With the observed group differences and the relatively small variation of the *in vivo* measurements, a sample size of four per group (n=4) and three per group (n=3) will provide >90% and >80% power at type I error rate of 0.05 (two-sided test), respectively. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center.

### Reagent

Recombinant mouse IGFBP1 (1588-B1-025), human IGFBP1 (871-B1-025), mouse RANKL (462-TR), human RANKL (390-TN-010), mouse MCSF (416-ML), as well as anti-IGFBP1 antibody (AF1240) were from R&D Systems. RGD-containing peptide (Gly-Arg-Gly-Asp-Thr-Pro) was from Sigma. Antibodies for p-ERK (#9101), total ERK (#4695), p-Akt (#4058), total-Akt (#2920), p-c-Jun (#9164) and total c-Jun (#9165) were from Cell Signaling; antibodies for I $\kappa$ B $\alpha$  (sc-371) and Itgb1 (sc-8978) were from Santa Cruz Biotechnology. RAW264.7 mouse macrophage cell line was from ATCC (TIB-71). Serum and cellular IGFBP1 was quantified by ELISA (Abnova, KA3054). Total and free serum IGF1 was quantified by ELISA (ALPCO, 22-IG1MS-E01).

### Bone Analyses

$\mu$ CT was performed to evaluate bone volume and architecture using a Scanco  $\mu$ CT-35 instrument (SCANCO Medical) as described (Wei et al., 2012; Wei et al., 2010). Bone histomorphometry was performed as described (Wei et al., 2012). As a bone resorption marker, serum CTX-1 was measured with the RatLaps<sup>TM</sup> EIA kit (Immunodiagnostic Systems) (Wei et al., 2012). As a bone formation marker, serum PINP was measured with the Rat/Mouse PINP EIA kit (Immunodiagnostic Systems) (Wei et al., 2012).

### Bone Marrow Osteoclast Differentiation

Osteoclasts were differentiated from mouse bone marrow cells as described (Wan et al., 2007; Wei et al., 2010). Briefly, hematopoietic bone marrow cells were purified with 40 $\mu$ m cell strainer, and differentiated with 40ng/ml of mouse M-CSF (R&D Systems) in  $\alpha$ -MEM containing 10% FBS for 3 days, then with 40ng/ml of mouse MCSF and 100ng/ml of mouse RANKL (R&D Systems) for 3–9 days, with or without rosiglitazone (1 $\mu$ M). The FBS contained bovine IGFbps including IGFBP1, and IGFBP3-bound IGF1. Mature osteoclasts

were identified as multinucleated (>3 nuclei) TRAP<sup>+</sup> cells. Osteoclast differentiation was quantified by the RNA expression of osteoclast marker genes using RT-QPCR, normalized by the L19 ribosomal protein gene. For osteoclast resorptive function analyses, osteoclast differentiation was conducted in OsteoAssay bone plates (Lonza) (n=8 wells per condition), and osteoclast activity was quantified as calcium release from bone into culture medium using CalciFluo ELISA assay (Lonza); “-RANKL” and “+RANKL” served as negative and positive controls, respectively. Each experiment was repeated for three times and representative results are shown. Osteoblast differentiation was conducted as described (Wei et al., 2012). For all bone cell differentiation, similar results were observed using bone marrow cells from male or female mice, and representative results from male mice are shown. To determine the effects of liver-secreted factors on osteoclast differentiation, fresh liver was minced in culture medium ( $\alpha$ -MEM+10% FBS) and dispersed by gentle vortex for 5 min; cells were pelleted by centrifugation at 10K rpm for 10min; supernatant was collected as liver-cell-derived conditioned medium (LCM) and used to treat osteoclast differentiation culture. Human PBMN cells (ReachBio) were differentiated into osteoclasts in  $\alpha$ -MEM containing 10% FBS, 25ng/ml MCSF, 50ng/ml hRANKL, 1 $\mu$ M Dexamethasone and 1 $\mu$ M rosiglitazone for 14 days, in the presence of 5ng/ml human IGFBP1 or PBS control.

### Statistical Analyses

All statistical analyses were performed with Student's t-Test and presented as mean  $\pm$  standard deviation (SD) unless stated otherwise. For *in vivo* experiments with  $\geq 3$  groups, statistical analyses were performed with ANOVA followed by the post hoc Tukey pairwise comparisons. The p values were designated as: \*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.005; \*\*\*\*, p<0.001; n.s. non-significant (p>0.05).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

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### Highlights

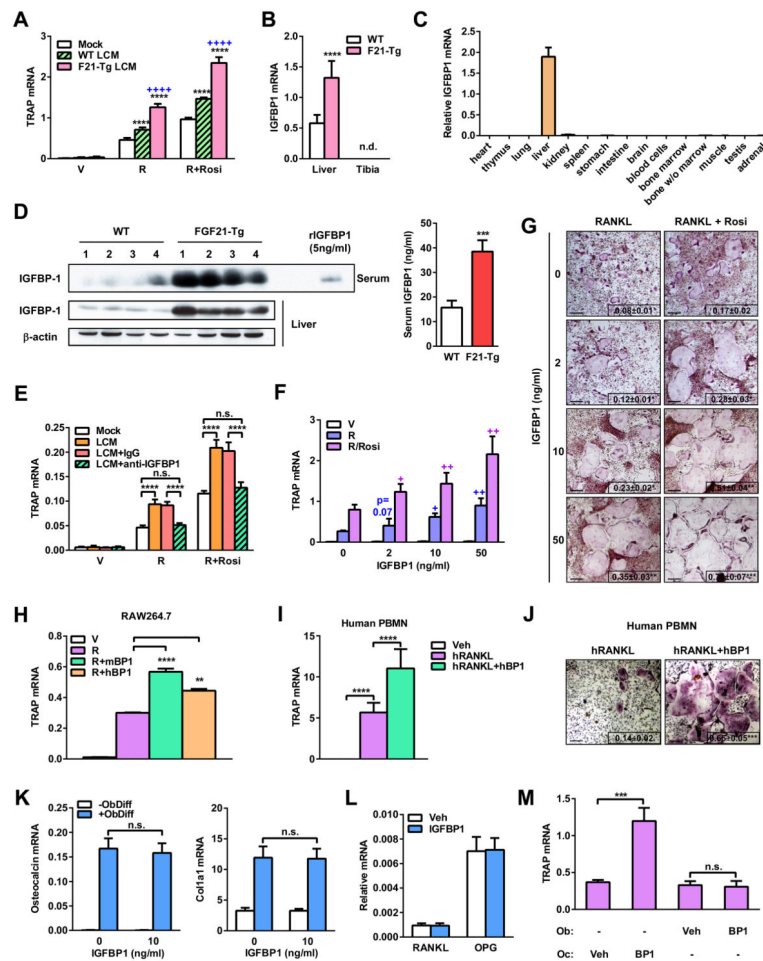
- IGFBP1 is an FGF21-induced pro-osteoclastogenic liver hormone
- IGFBP1 functions through Integrin  $\beta$ 1 receptor in the osteoclast lineage
- IGFBP1 potentiates RANKL signaling and NFATc1 activation
- IGFBP1 blockade abolishes FGF21-induced bone resorption but not insulin sensitization

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### Figure 1. IGFBP1 is an FGF21-Induced Pro-Osteoclastogenic Hepatokine

(A) Effects of liver-cell-derived conditioned media (LCM) from WT or FGF21-Tg mice (2-month-old, male,  $n=4$ ) on osteoclast differentiation from WT bone marrow cells, quantified by the mRNA of a representative osteoclast marker TRAP ( $n=4$ ); \* compares LCM treatment with mock controls; + compares LCM from FGF21-Tg mice with LCM from WT control mice. V, vehicle; R, RANKL; Rosi, rosiglitazone.

(B) IGFBP1 mRNA levels in the liver and tibia (bone + marrow) from WT and FGF21-Tg mice ( $n=3$ ); n.d., not detected.

(C) IGFBP1 mRNA levels in various tissues ( $n=3$ ).

(D) Left, western blot of IGFBP1 protein in the serum (top) and liver (bottom) of WT and FGF21-Tg mice (2-month-old, male,  $n=4$ ). Equal volume (20 $\mu$ l) of each sample and rIGFBP1 was loaded; the concentration of rIGFBP1 used (5ng/ml) is shown. Right, ELISA of serum IGFBP1 levels in WT and FGF21-Tg mice (2-month-old, male,  $n=6$ ).

(E) The pro-osteoclastogenic activity of WT LCM was abolished by an IGFBP1-blocking antibody (anti-IGFBP1, 100ng/ml) ( $n=3$ ). IgG served as a negative control.

(F–G) Recombinant mouse IGFBP1 enhanced the RANKL-mediated and rosiglitazone-stimulated osteoclast differentiation from WT bone marrow cells in a dose-dependent manner.

(F) Quantification of TRAP mRNA (n=3); + compares IGFBP1 treatment with no IGFBP1 controls.

(G) Representative images of TRAP-stained differentiation cultures showing that IGFBP1 increased the number and size of mature osteoclasts at day 4 after RANKL treatment. Mature osteoclasts were identified as multinucleated (>3 nuclei) TRAP<sup>+</sup> (purple) cells. Scale bar, 25µm. Inset shows the quantification of resorptive activity by calcium release from bone into medium (mM) (n=8); \* compares with no IGFBP1 control.

(H) Osteoclast differentiation from RAW264.7 mouse macrophage cell line was induced by RANKL and further enhanced by mouse and human IGFBP1, quantified by TRAP mRNA (n=3). R, RANKL; BP1, IGFBP1.

(I–J) Osteoclast differentiation from human peripheral blood mononuclear (PBMN) cells was induced by human RANKL and further enhanced by human IGFBP1.

(I) Quantification of TRAP mRNA (n=3).

(J) Representative images of TRAP-stained differentiation cultures. Scale bar, 25µm. Inset shows resorptive activity by calcium release from bone into medium (mM) (n=8); \* compares IGFBP1 with vehicle control.

(K–M) IGFBP1 does not affect osteoblast differentiation or RANKL/OPG expression.

(K–L) Bone marrow osteoblast differentiation cultures were treated with 0 or 10ng/ml of IGFBP1.

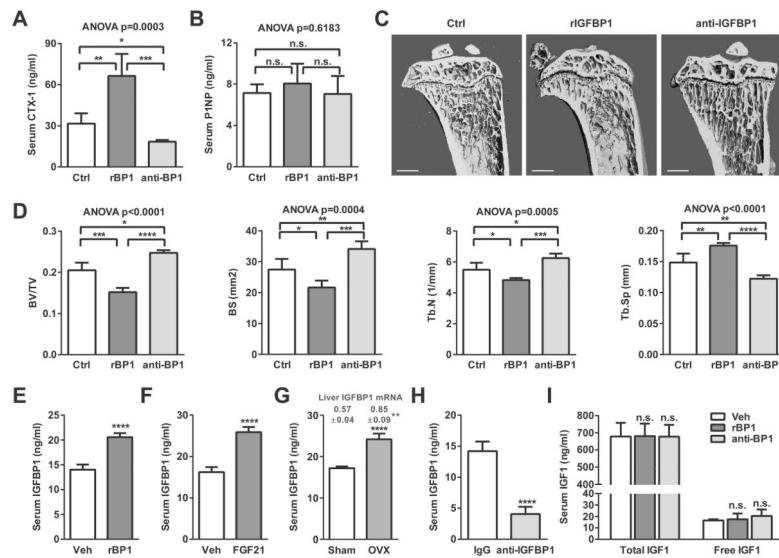
(K) Osteoblast differentiation was unaltered, quantified by the mRNA of osteoblast markers osteocalcin and Col1a1 (n=3). ObDiff, osteoblast differentiation cocktail.

(L) RANKL and OPG expression in osteoblasts were unaltered by IGFBP1 (n=3).

(M) Bone marrow osteoblast differentiation cultures were treated with 50ng/ml IGFBP1 or vehicle control (Veh) for 18 days, on the last day, the cells were cultured in fresh medium without IGFBP1 for 16 hrs and osteoblast conditioned medium was collected, and added to osteoclast differentiation cultures together with 50ng/ml IGFBP1 or Veh control, in the presence of 20ng/ml RANKL. Osteoclast differentiation was quantified as TRAP expression (n=3).

Statistical analyses were performed with Student's t-Test and are shown as mean ± SD. \*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.005; \*\*\*\*, p<0.001; n.s. non-significant (p>0.05).

See also Figure S1.



### Figure 2. IGFBP1 Enhances Bone Resorption *In Vivo*

(A–D) Effects of rIGFBP1 and anti-IGFBP1 treatment on bone. WT C57BL6 mice (10-week-old, male, n=6) were treated with IGFBP1 (0.015mg/kg/day), anti-IGFBP1 antibody (0.03mg/kg/day) or control by daily IP injection for 14 days. PBS and IgG controls showed similar results, and representative data for PBS control are shown.

(A) Serum CTX-1 bone resorption marker.

(B) Serum PINP bone formation marker.

(C) Representative  $\mu$ CT images of the entire proximal tibiae (scale bar, 1mm).

(D) Quantification of trabecular bone volume and architecture. BV/TV, bone volume/tissue volume ratio; BS, bone surface; Tb.N, trabecular number; Tb.Sp, trabecular separation.

(E–H) ELISA analyses of serum IGFBP1 levels.

(E) WT C57BL6 mice (10-week-old, male, n=6) were treated with PBS vehicle control (Veh) or IGFBP1 (0.015mg/kg/day) for 14 days.

(F) WT C57BL6 mice (3-month-old, male, n=6) were fed with HFD for 5 weeks and treated with vehicle or FGF21 (1mg/kg/day) for the last 14 days.

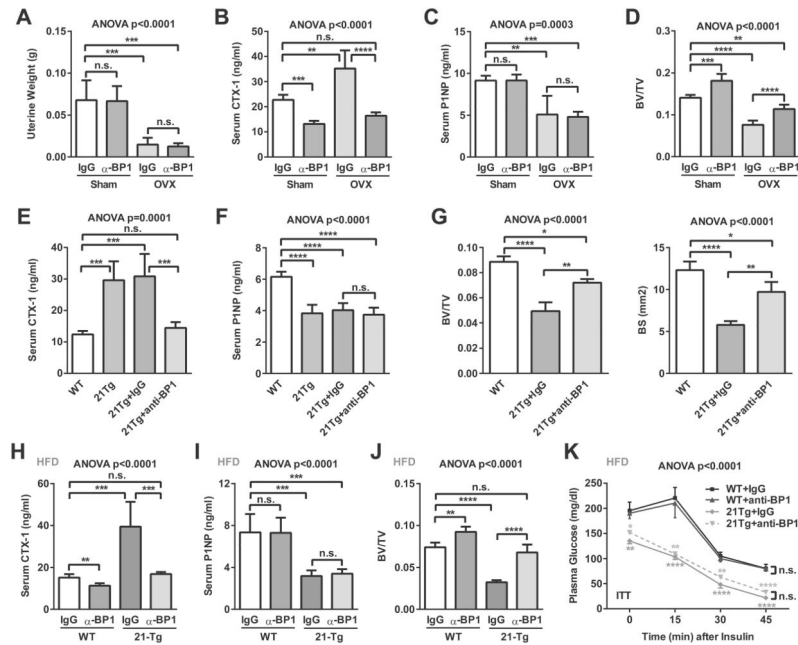
(G) WT C57BL6 mice (4-month-old, female, n=6) were ovariectomized (OVX) or sham-operated, and serum was collected 5 weeks later. Liver IGFBP1 mRNA level was also higher in OVX mice compared with sham controls (shown on top). IGFBP1 mRNA in other tissues such as kidney, heart, bone, bone marrow, spleen, thymus, lung, stomach, intestine, brain and muscle were below 0.005 and not significantly different between OVX and sham.

(H) Serum IGFBP1 was reduced by anti-IGFBP1 treatment. WT C57BL6 mice (10-week-old, male, n=6) were treated with IgG control or anti-IGFBP1 (0.03mg/kg/day) for 14 days. Antibody-bound IGFBP1 in the serum was removed by protein A/G plus agarose, and then unbound IGFBP1 was quantified.

(I) Serum levels of total IGF1 and free IGF1 were unaffected by rIGFBP1 or anti-IGFBP1 treatment, quantified by ELISA. WT C57BL6 mice (10-week-old, male, n=6) were treated with rIGFBP1 (0.015mg/kg/day), anti-IGFBP1 (0.03mg/kg/day) or vehicle control for 14 days.

Statistical analyses were performed with ANOVA followed by the post hoc Tukey pairwise comparisons (A–D) or Student's t-Test (E–I); error bars indicate SD. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.005$ ; \*\*\*\*,  $p < 0.001$ ; n.s. non-significant ( $p > 0.05$ ).  
See also Figure S2.





### Figure 3. IGFBP1 Blockade Abolishes OVX- and FGF21-Induced Bone Resorption

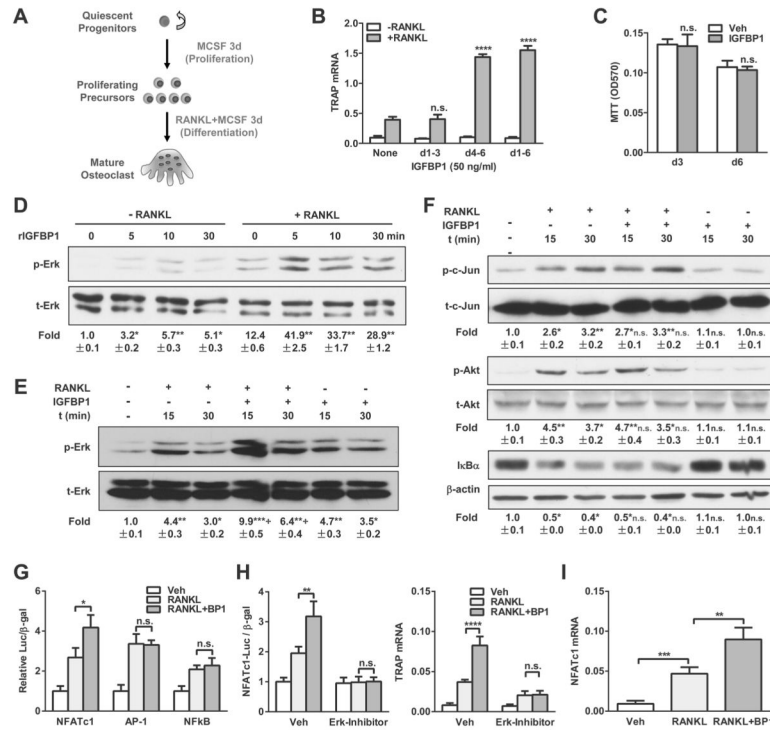
(A–D) Anti-IGFBP1 treatment attenuates ovariectomy (OVX)-induced bone resorption and bone loss. WT C57BL/6 mice (18-week-old, female, n=6) were ovariectomized or sham-operated. Three days after surgery, they were treated with anti-IGFBP1 (anti-BP1) or IgG control at 0.03mg/kg/day and 3 times per week for 5 weeks. (A) Uterine weight. (B) Serum CTX-1. (C) Serum P1NP. (D) BV/TV by  $\mu$ CT.

(E–G) Anti-IGFBP1 treatment attenuates FGF21-induced bone resorption and bone loss. (E–G) All mice were 7–8 month old females on chow diet (n=6). FGF21-Tg mice were treated with anti-IGFBP1 (anti-BP1) or IgG control at 0.03mg/kg/day daily for 14 days, and compared with WT mice or untreated FGF21-Tg mice. (E) Serum CTX-1. (F) Serum P1NP. (G) Trabecular BV/TV and BS in proximal tibiae by  $\mu$ CT.

(H–K) All mice were 9–10 month old females that were on high-fat-diet for 5 weeks (n=6). WT or FGF21-Tg mice were treated with anti-IGFBP1 or IgG control at 0.03mg/kg/day daily for the last 14 days. (H) Serum CTX-1. (I) Serum P1NP. (J) BV/TV. (K) Insulin tolerance test (ITT); \* compares with IgG-treated WT mice.

Statistical analyses were performed with ANOVA followed by the post hoc Tukey pairwise comparisons; error bars indicate SD. \*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.005; \*\*\*\*, p<0.001; n.s. non-significant (p>0.05).

See also Figure S3.



**Figure 4. IGFBP1 Potentiates RANKL Signaling by Enhancing Erk-phosphorylation and NFATc1 Activation**

(A) A diagram of the time course of the bone marrow osteoclast differentiation assay, d, day.

(B) IGFBP1 functions at the RANKL-induced differentiation stage. IGFBP1 was added to WT osteoclast differentiation cultures on day 1–3 only, day 4–6 only, or the entire day 1–6; differentiation was quantified by TRAP mRNA (n=3). \* and n.s. compare with “no IGFBP1 treatment”.

(C) IGFBP1 does not affect cell proliferation in the osteoclast differentiation culture by MTT assays (n=6).

(D–F) IGFBP1 induces ERK phosphorylation in synergy with RANKL.

(D) WT bone marrow cells were cultured with MCSF for 5 days, with or without RANKL during the last 2 days. The cells were treated with IGFBP1 (50ng/ml) for the indicated amount of time, the levels of p-ERK and total ERK (t-ERK) were analyzed by western blot. The p-ERK/t-ERK ratio was quantified as fold changes compared to lane 1 (n=3). \* compares with “0 min” time point in each group.

(E–F) WT bone marrow cells were cultured with MCSF for 3 days, and then treated with IGFBP1 (50ng/ml) and/or RANKL (100ng/ml) for the indicated amount of time. (E) The p-ERK/t-ERK ratio was quantified as fold changes compared to lane 1 (n=3). (F) IGFBP1 does not affect basal or RANKL-induced c-Jun phosphorylation (Ser73), Akt phosphorylation or IκBα degradation. The p-c-Jun/t-c-Jun ratio, p-Akt/t-Akt ratio, and IκBα/β-actin ratio were quantified as fold changes compared to lane 1 (n=3). \* compares with lane 1; + or n.s. compares with “no IGFBP1” under the same treatment condition.

(G) IGFBP1 specifically enhances RANKL-induced NFATc1 activation. RAW264.7 cells were transfected with NFATc1-Luc, AP1-Luc or NFκB-Luc reporter together with a CMV-

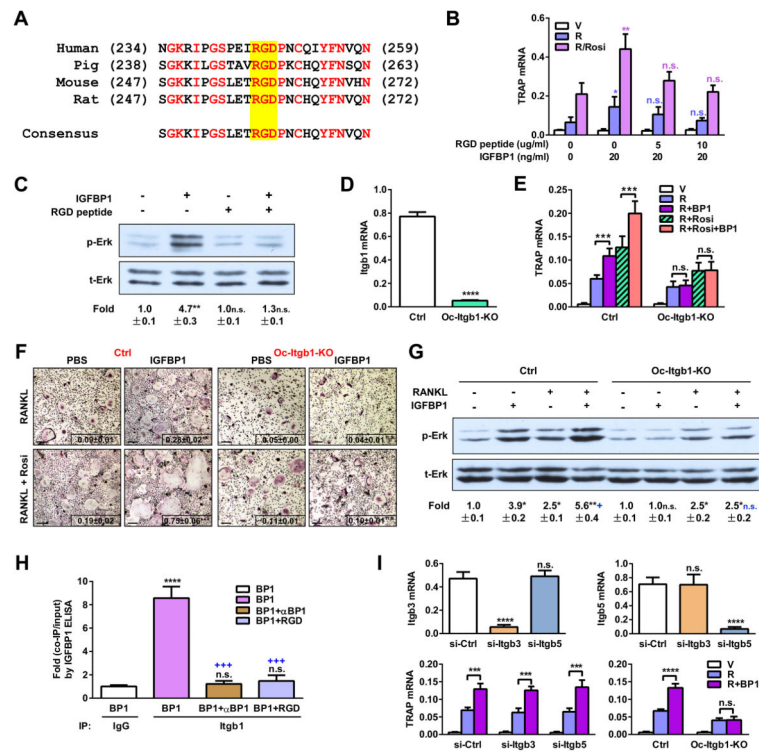
$\beta$ gal reporter as internal control for 24hr, and then treated with RANKL (100ng/ml) and/or IGFBP1 (50ng/ml) for 24hr before reporter assays (n=6).

(H) IGFBP1 enhancement of NFATc1-luc reporter (left) and bone marrow osteoclast differentiation (right) was abolished by Erk inhibitor U0126 (n=3, 10 $\mu$ M). Vehicle control (Veh) was PBS+DMSO.

(I) IGFBP1 increased NFATc1 expression in osteoclast differentiation cultures (n=3).

Statistical analyses were performed with Student's t-Test and are shown as mean  $\pm$  SD. \*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.005; \*\*\*\*, p<0.001; n.s. non-significant (p>0.05).

See also Figure S1.



### Figure 5. IGFBP1 Functions via Integrin $\beta$ 1 Receptor in Osteoclasts

(A) Amino acid sequence alignment of the C-termini of IGFBP1 from human, pig, mouse and rat. The conserved amino acids are highlighted in red, the RGD motifs are highlighted in yellow.

(B) An RGD-containing peptide dose-dependently abolished the pro-osteoclastogenic activity of IGFBP1, quantified by TRAP mRNA ( $n=3$ ). \* and n.s. compare with “no IGFBP1 treatment” (the group on the most left side) under the same RANKL (R) or Rosi treatment condition.

(C) IGFBP1-induced ERK phosphorylation was abolished by RGD-containing peptide. WT bone marrow cells were cultured with MCSF for 5 days, pretreated with RGD-containing peptide (20  $\mu$ g/ml) for 15 min, and then treated with IGFBP1 (50 ng/ml) for 5 min. The p-ERK/t-ERK ratio was quantified as fold changes compared to lane 1 ( $n=3$ ); \* compares with lane 1.

(D) Itgb1 expression was effectively diminished in osteoclast precursors from Oc-Itgb1-KO mice ( $n=3$ ).

(E–F) IGFBP1 enhancement of osteoclast differentiation was abolished in Oc-Itgb1-KO cultures.

(E) TRAP mRNA ( $n=3$ ). (F) Images of TRAP-stained differentiation cultures. Scale bar, 25  $\mu$ m. Inset shows resorptive activity by calcium release from bone into medium (mM) ( $n=8$ ); \* and n.s. compare IGFBP1 with PBS control in the same genotype and culture condition.

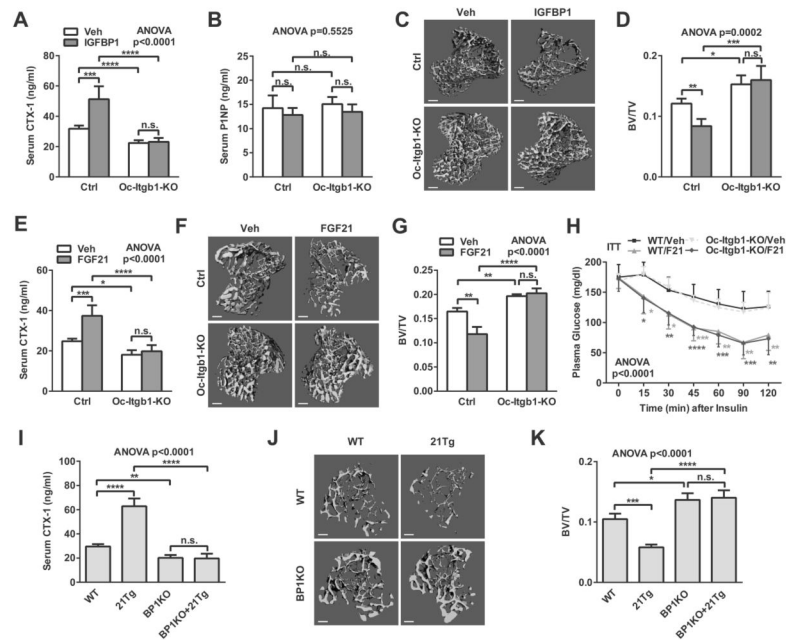
(G) IGFBP1 induction of Erk phosphorylation was abolished in Oc-Itgb1-KO cultures ( $n=3$ ). Bone marrow cells from Oc-Itgb1-KO mice or littermate controls were cultured with MCSF for 5 days, with or without RANKL during the last 2 days, and then stimulated with

IGFBP1 (50 ng/ml) or vehicle control for 5 min. The p-ERK/t-ERK ratio was quantified as fold changes compared to lane 1 in each group of the same genotype (n=3); black \* compares with lane 1 in each group; blue \* or n.s. compares with “no IGFBP1, RANKL only” in lane 3 in each group.

(H) Co-IP analysis of IGFBP1 binding to Itgb1 in osteoclasts, and its blockade by anti-IGFBP1 and RGD peptide. Co-IP was performed with anti-Itgb1 or IgG control; IGFBP1 was released from the complex and then quantified by ELISA (n=3). Black \* and n.s. compare with column 1; blue + compares with column 2.

(I) Knockdown of Itgb3 or Itgb5 does not affect IGFBP1 induction of osteoclast differentiation. Bone marrow osteoclast differentiation cultures were transfected with siRNA before RANKL induction, and expression of Itgb3 (top left), Itgb5 (top right) and TRAP differentiation marker (bottom) was quantified by RT-QPCR (n=6). TRAP mRNA in Oc-Itgb1-KO cells serves as a positive control.

Statistical analyses were performed with Student's t-Test and are shown as mean  $\pm$  SD. \*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.005; \*\*\*\*, p<0.001; n.s. non-significant (p>0.05).



**Figure 6. Osteoclastic Itgb1 Deletion Abolishes IGFBP1- and FGF21-induced Bone Resorption and Bone Loss**

(A–D) IGFBP1 induction of bone resorption and bone loss was abolished in Oc-Itgb1-KO mice. Oc-Itgb1-KO mice or littermate controls (2-month-old, male, n=6) were treated with IGFBP1 (0.015mg/kg/day) or PBS for 14 days. (A) Serum CTX-1. (B) Serum PINP. (C)  $\mu$ CT images of the trabecular bone of the tibial metaphysis. Scale bar, 10 $\mu$ m. (D) Trabecular BV/TV.

(E–H) Osteoclastic Itgb1 deletion abolishes FGF21-induced bone loss while retaining FGF21-induced insulin sensitization. Oc-Itgb1-KO mice or littermate controls (3-month-old, male, n=6) were fed with HFD for 5 weeks, and treated with FGF21 (1mg/kg/day) during the last 14 days.

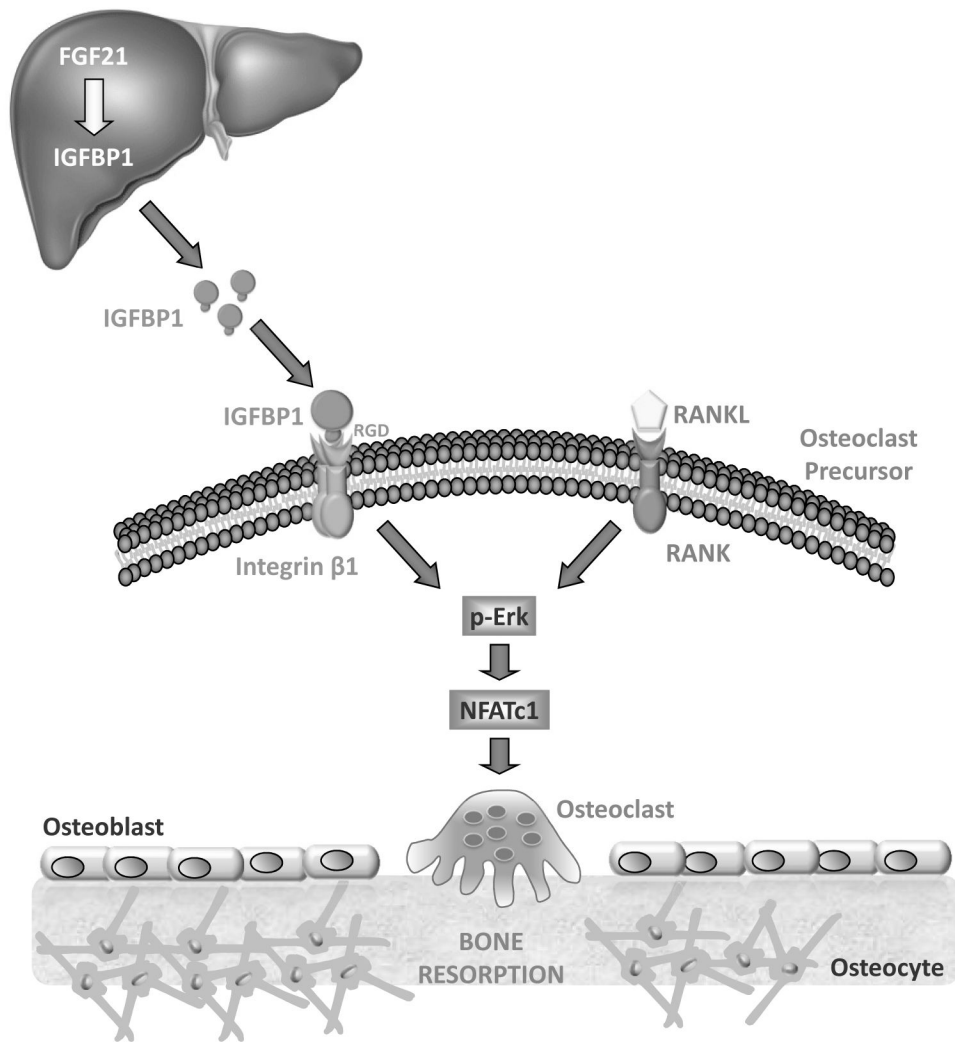
(E) Serum CTX-1. (F)  $\mu$ CT images of the trabecular bone of the tibial metaphysis. Scale bar, 10 $\mu$ m. (G) Trabecular BV/TV. (H) ITT assay. Mice were fasted for 4hr, and then received a single IP injection of FGF21 (1mg/kg) or vehicle control, co-injected with insulin (0.75U/kg).

(I–K) Comparison of WT, FGF21-Tg, IGFBP1-KO and IGFBP1-KO/FGF21-Tg mice (2-month-old, male, n=6). (I) Serum CTX-1. (J)  $\mu$ CT images of the trabecular bone of the tibial metaphysis. Scale bar, 10 $\mu$ m. (K) Trabecular BV/TV.

Statistical analyses were performed with Student's t-Test or ANOVA followed by the post hoc Tukey pairwise comparisons; error bars indicate SD. \*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.005; \*\*\*\*, p<0.001; n.s. non-significant (p>0.05).

See also Figure S4, S5 and S6.





**Figure 7. A simplified model for how the liver hormone IGFBP1 regulates osteoclast differentiation and mediates FGF21-induced bone resorption**

FGF21 induces liver expression and secretion of IGFBP1. IGFBP1 in turn functions as an endocrine hormone by binding to its receptor integrin  $\beta 1$  on the osteoclast precursors, leading to the potentiation of RANKL signaling and enhanced osteoclast differentiation. Consequently, a physiological or pharmacological elevation of FGF21 levels results in a higher circulating IGFBP1 level, leading to increased bone resorption and bone loss.