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ORIGINAL ARTICLE

# Suppression of osteoclastogenesis through phosphorylation of eukaryotic translation initiation factor 2 alpha

**Kazunori Hamamura · Nancy Tanjung · Hiroki Yokota**

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**Abstract** In response to various stresses including viral infection, nutrient deprivation, and stress to the endoplasmic reticulum, eukaryotic translation initiation factor 2 alpha (eIF2α) is phosphorylated to cope with stress induced apoptosis. Although bone cells are sensitive to environmental stresses that alter the phosphorylation level of eIF2α, little is known about the role of eIF2α mediated signaling during the development of bone-resorbing osteoclasts. Using two chemical agents (salubrinal and guanabenz) that selectively inhibit de-phosphorylation of eIF2α, we evaluated the effects of phosphorylation of eIF2α on osteoclastogenesis of RAW264.7 pre-osteoclasts as well as development of MC3T3 E1 osteoblast-like cells. The result showed that salubrinal and guanabenz stimulated matrix deposition of osteoblasts through upregulation of activating transcription factor 4 (ATF4). The result also revealed that these agents reduced expression of the nuclear factor of activated T cells c1 (NFATc1) and inhibited differentiation of RAW264.7 cells to multi-nucleated osteoclasts. Partial silencing of eIF2α with RNA interference reduced suppression of salubrinal/guanabenz-driven downregulation of NFATc1. Collectively, we demonstrated that the elevated phosphorylation level of eIF2α not only stimulates osteoblastogenesis but also inhibit osteoclastogenesis through regulation of ATF4 and NFATc1. The results suggest that eIF2α-mediated signaling might provide a novel therapeutic target for preventing bone loss in osteoporosis.

**Keywords** Osteoclasts · Salubrinal · Guanabenz · eIF2α · NFATc1

## Introduction

Osteoblasts and osteoclasts are the two major types of bone cells in bone remodeling. Osteoblasts are bone-forming cells originated from mesenchymal stem cells, while osteoclasts are bone-resorbing cells derived from hematopoietic stem cells. These two types of cells orchestrate a complex remodeling process, in which mineralized bone matrix is degraded by osteoclasts and newly formed by osteoblasts [1, 2]. In order to maintain proper bone mass, exercise and calcium rich diets are recommended. However, a failure of the coordinated action such as in osteoporosis, which is a common form of bone loss prevailing among postmenopausal women, increases risk of bone fracture [3]. In order to develop therapeutic drugs for treatment of osteoporosis, an understanding of signaling pathways that govern osteoclastogenesis—development of pre-osteoclasts (monocyte/macrophage) to multi-nucleated osteoclasts—is required. In this paper, we examined a signaling pathway for osteoclastogenesis that is mediated by eukaryotic translation initiation factor 2 alpha (eIF2α).

A protein complex, eIF2, is a heterotrimer essential for protein synthesis, and eIF2α is one of its major components together with eIF2β and eIF2γ [4]. In response to various stresses such as oxidation, radiation, and stress to the

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K. Hamamura (✉) · N. Tanjung · H. Yokota
Department of Biomedical Engineering, Indiana University-Purdue University Indianapolis, SL155, 723 West Michigan Street, Indianapolis, IN 46202, USA
e-mail: hamamurk@iupui.edu

H. Yokota
Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, IN 46202, USA

endoplasmic reticulum that potentially lead to cellular apoptosis, a serine residue of eIF2α is phosphorylated. This action would initiate a pro-survival program by lowering general translation efficiency except for a group of genes that includes activating transcription factor 4 (ATF4) [5]. The ATF4 is a transcription factor critical for osteoblastogenesis and bone formation [6]. In osteoblasts elevation of phosphorylated eIF2α (p-eIF2α) is reported to stimulate the expression of ATF4 [7, 8]. Little is known, however, about potential effects of p-eIF2α on development of osteoclasts.

Herein we addressed a question: Does elevation of p-eIF2α alter cellular fates of pre-osteoclasts? Osteoblasts and osteoclasts extensively interact through molecular pathways including RANK (receptor activator of nuclear factor kappa-B)/RANKL (RANK ligand)/OPG (osteoprotegerin) signaling [9, 10] and Wnt signaling [11]. Therefore, osteoclastogenesis is potentially regulated by signaling molecules that also affect osteoblastogenesis. Furthermore, osteoclastogenesis is influenced by various stresses such as estrogen deficiency and disuse or unloading [12]. Since elevation of p-eIF2α can provide stress-relieving effects on osteoblasts, we hypothesized that elevation of p-eIF2α suppresses differentiation of pre-osteoclasts to multi-nucleated osteoclasts.

In this study, we employed two chemical agents (salubrinal and guanabenz) and examined the effects of elevated p-eIF2α on osteoclastogenesis. These two agents selectively inhibit de-phosphorylation of p-eIF2α by interacting with protein phosphatase 1, PP1 [13, 14]. The signaling pathway, mediated by eIF2α, is not directly linked to known agents for osteoclastogenesis such as calcium binding agents and RANKL. Currently, the most common medications, prescribed for preventing bone loss in patients with osteoporosis, are bisphosphonates. Bisphosphonates preferentially bind to calcium in bone and induce apoptosis of osteoclasts [15]. Other medications using neutralizing antibodies targeted to RANKL would block osteoclastogenesis by mimicking OPG's binding to RANKL [16]. The RANKL is a cytokine belonging to the tumor necrosis factor family, and is involved in T cell-dependent immune responses as well as differentiation and activation of osteoclasts [9, 10]. To our knowledge, no therapeutic agents for osteoporosis have been targeted to eIF2α-mediated signaling.

We employed MC3T3 E1 osteoblast-like cells [17] and RAW264.7 cells [18] to evaluate osteoblastogenesis and osteoclastogenesis, respectively. In the presence and absence of salubrinal and guanabenz, MC3T3 E1 cells were cultured in an osteogenic medium for evaluation of matrix deposition, while RAW264.7 cells were cultured in an osteoclast differentiation medium for evaluation of multi-nucleation. Alizarin Red S staining was performed to evaluate osteoblast mineralization for MC3T3 E1 cells, and TRAP staining was conducted to determine multi-nucleated osteoclasts proliferation for RAW264.7 cells. To analyze molecular signaling pathways, quantitative real-time PCR and Western blot analysis were conducted. The mRNA levels of ATF4, osteocalcin, c-Fos [19], tartrate-resistant acid phosphatase (TRAP) [20], and osteoclast-associated receptor (OSCAR) [21] were determined. The protein expression levels of eIF2α, ATF4, and nuclear factor of activated T cells c1 (NFATc1) [22] were also determined. The NFATc1 is a transcription factor, which is critically important for development and activation of osteoclasts in response to RANKL. The RNA interference using siRNA specific to ATF4 and eIF2α was conducted to evaluate the role of ATF4 in osteoblastogenesis and eIF2α in osteoclastogenesis.

## Materials and methods

### Cell culture

The MC3T3 E1 mouse osteoblast-like cells (clone 14—MC3T3 E1-14; and no clonal cells in supplementary figures), and RAW264.7 mouse pre-osteoclast (monocyte/macrophage) cells were cultured in αMEM containing 10 % fetal bovine serum and antibiotics (50 U/ml penicillin, and 50 μg/ml streptomycin; Life Technologies, Grand Island, NY, USA). Cells were maintained at 37 °C and 5 % $CO_2$ in a humidified incubator. Cell mortality and live cell numbers were determined 24 h after the treatment with 20 ng/ml RANKL (PeproTech, Rocky Hills, NC, USA) in response to 0.1–20 μM salubrinal or 1–20 μM guanabenz acetate (Tocris Bioscience, Ellisville, MO, USA). Cells were stained with trypan blue and the numbers of live and dead cells were counted using a hemacytometer.

### Quantitative real-time PCR

Total RNA was extracted using an RNeasy Plus mini kit (Qiagen, Germantown, MD, USA). Reverse transcription was conducted with high capacity cDNA reverse transcription kits (Applied Biosystems, Carlsbad, CA, USA), and quantitative real-time PCR was performed using ABI 7500 with Power SYBR green PCR master mix kits (Applied Biosystems). We evaluated mRNA levels of ATF4, Osteocalcin (OCN), NFATc1, c-Fos, tartrate-resistant acid phosphatase (TRAP), and osteoclast-associated receptor (OSCAR) with the PCR primers listed in Table 1. The GAPDH was used for internal control. The relative mRNA abundance for the selected genes with respect to the level of GAPDH mRNA was expressed as a

**Table 1** Real-time PCR primers used in this study

| Target | Forward primer | Backward primer |
|---|---|---|
| ATF4 | 5′-TGGCGAGTGTAAGGAGCTAGAAA-3′ | 5′-TCTTCCCCCTTGCCTTACG-3′ |
| OCN | 5′-CCGGGAGCAGTGTGAGCTTA-3′ | 5′-AGGCGGTCTTCAAGCCATACT-3′ |
| NFATc1 | 5′-GGTGCTGTCTGGCCATAACT-3′ | 5′-GCGGAAAGGTGGTATCTCAA-3′ |
| c-Fos | 5′-AGGCCCAGTGGCTCAGAGA-3′ | 5′-CCAGTCTGCTGCATAGAAGGAA-3′ |
| TRAP | 5′-TCCTGGCTCAAAAAGCAGTT-3′ | 5′-ACATAGCCCACACCGTTCTC-3′ |
| OSCAR | 5′-ACACACACACCTGGCACCTA-3′ | 5′-GAGACCATCAAAGGCAGAGC-3′ |
| GAPDH | 5′-TGCACCACCAACTGCTTAG-3′ | 5′-GGATGCAGGGATGATGTTC-3′ |

ratio of $S_{\text{treated}}/S_{\text{control}}$, where $S_{\text{treated}}$ is the mRNA level for the cells treated with chemical agents, and $S_{\text{control}}$ is the mRNA level for control cells [23].

### Western immunoblotting

Cells were lysed in a radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and phosphatase inhibitors (Calbiochem, Billerica, MA, USA). Isolated proteins were fractionated using 10–15 % SDS gels and electro-transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). The membrane was incubated for 1 h with primary antibodies followed by 45 min incubation with goat anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase (Cell Signaling, Danvers, MA, USA). We used antibodies against ATF4, NFATc1 (Santa Cruz), p-eIF2α (Thermo Scientific, Waltham, MA, USA), eIF2α, caspase 3, cleaved caspase 3, p38 and p-p38 mitogen activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and p-ERK, nuclear factor kappa B (NFκB) p65 and p-NFκB p65 (Cell Signaling), and β-actin (Sigma). Protein levels were assayed using a SuperSignal west femto maximum sensitivity substrate (Thermo Scientific), and signal intensities were quantified with a luminescent image analyzer (LAS-3000, Fuji Film, Tokyo, Japan).

### Knockdown of ATF4 and eIF2α by siRNA

Cells were treated with siRNA specific to ATF4 and eIF2α (Life Technologies). Selected target sequences for knockdown of ATF4 and eIF2α were: ATF4, 5′-GCU GCU UAC AUU ACU CUA A-3′; and eIF2α, 5′-CGG UCA AAA UUC GAG CAG A-3′. As a nonspecific control, a negative siRNA (Silencer Select #1, Life Technologies) was used. Cells were transiently transfected with siRNA for ATF4, eIF2α or control in Opti-MEM I medium with Lipofectamine RNAiMAX (Life Technologies). Six hours later, the medium was replaced by regular culture medium. The efficiency of silencing was assessed with immunoblotting or quantitative PCR 48 h after transfection.

### Mineralization assay

Mineralization of extracellular matrix was assayed by Alizarin Red S staining. The MC3T3-E1 cells were plated in 6-well plates. When cells were confluent, 50 μg/ml of ascorbic acid (Wako Chemicals, Richmond, VA, USA) and 5 mM β-glycerophosphate (Sigma) were added. The medium was changed every other day, and staining was conducted after 3 weeks. Cells were washed with PBS twice and fixed with 60 % isopropanol for 1 min at room temperature, followed by rehydration with distilled water for 3 min at room temperature. They were stained with 1 % Alizarin red S (Sigma) for 3 min and washed with distilled water.

### Osteoclastogenesis in vitro and TRAP (Tartrate-resistant acid phosphatase) staining

The RAW264.7 cells were plated at a density of $5 \times 10^3/\text{cm}^2$ into a 12-well or a 60 mm dish, and cultured with 20 ng/ml RANKL in the presence and absence of salubrinal or guanabenz. The culture medium was replaced every 2 days. After 5 days of culture, the cells were stained for TRAP staining using an acid phosphatase leukocyte kit (Sigma). The number of TRAP-positive cells containing three or more nuclei was determined.

### Statistical analysis

Three or four-independent experiments were conducted and data were expressed as mean ± SD. For comparison among multiple samples, ANOVA followed by post hoc tests was conducted. Statistical significance was evaluated at $p < 0.05$. The single and double asterisks and daggers indicate $p < 0.05$ and $p < 0.01$. To determine intensities in immunoblotting and areas of Alizarin red S staining, images were scanned with Adobe Photoshop CS2 (Adobe Systems, San Jose, CA, USA) and quantified using Image J.

## Results

### Enhanced mineralization of MC3T3 E1-14 cells by salubrinal

Prior to examining the effects of salubrinal on osteoclastogenesis, we tested its effects on the development of osteoblasts focusing on cell viability, phosphorylation of eIF2α (p-eIF2α), expression of ATF4 and osteocalcin, and matrix mineralization. Administration of 5–20 μM salubrinal to MC3T3 E1-14 cells did not increase cell mortality or inhibit cell proliferation (Fig. 1a). Unlike application of 10 nM thapsigargin, which is a stress inducer to the endoplasmic reticulum that elevates p-eIF2α, incubation with 10 μM salubrinal for 24 h did not elevate the expression level of cleaved caspase 3 (Fig. 1b). After 3-week incubation in an osteogenic medium, Alizarin red S staining area showed that salubrinal enhanced mineralization of MC3T3 E1-14 cells in a dose dependent manner (Fig. 1c). The enhanced mineralization was also observed in non-clonal MC3T3 E1 cells (Supplementary Fig. S1).

### ATF4-mediated elevation of osteocalcin mRNA in MC3T3 E1-14 cells

Salubrinal is an inhibitor of de-phosphorylation of eIF2α. Administration of 5 μM salubrinal to MC3T3 E1-14 cells elevated phosphorylation of eIF2α, followed by an increase in ATF4 expression (Fig. 2a). Furthermore, the level of osteocalcin mRNA was increased 3.3 ± 0.5 fold (24 h) and 3.3 ± 0.3 fold (32 h) (Fig. 2b). When expression of ATF4 was significantly reduced by RNA interference (Fig. 2c, d), however, salubrinal-driven elevation of the osteocalcin mRNA level was suppressed (Fig. 2e). Non-clonal MC3T3 E1 cells also presented elevation of p-eIF2α and ATF4, together with an increase in the mRNA levels of ATF4 and osteocalcin (Supplementary Fig. S2). In addition, administration of guanabenz to MC3T3 E1-14 elevated the mRNA level of osteocalcin in a dose dependent manner, consistent with an increase in p-eIF2α and ATF4 (Supplementary Fig. S3).

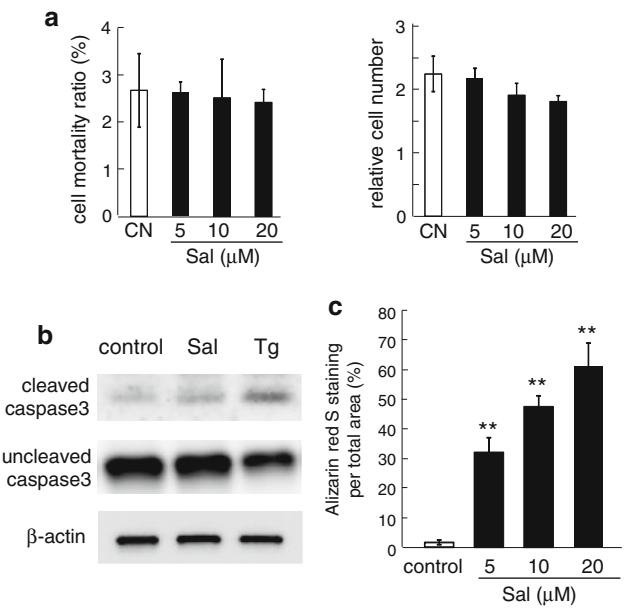


**Fig. 1** Osteogenic effects of salubrinal on MC3T3 E1 (clone 14) osteoblast cells. *CN* control, *Sal* salubrinal, and *Tg* thapsigargin. The *double asterisk* indicates $p < 0.01$ in comparison to CN. **a** Cell mortality ratio and relative cell numbers. **b** No activation of cleaved caspase 3 by salubrinal. **c** Alizarin red S staining area in response to 5, 10, and 20 μM salubrinal

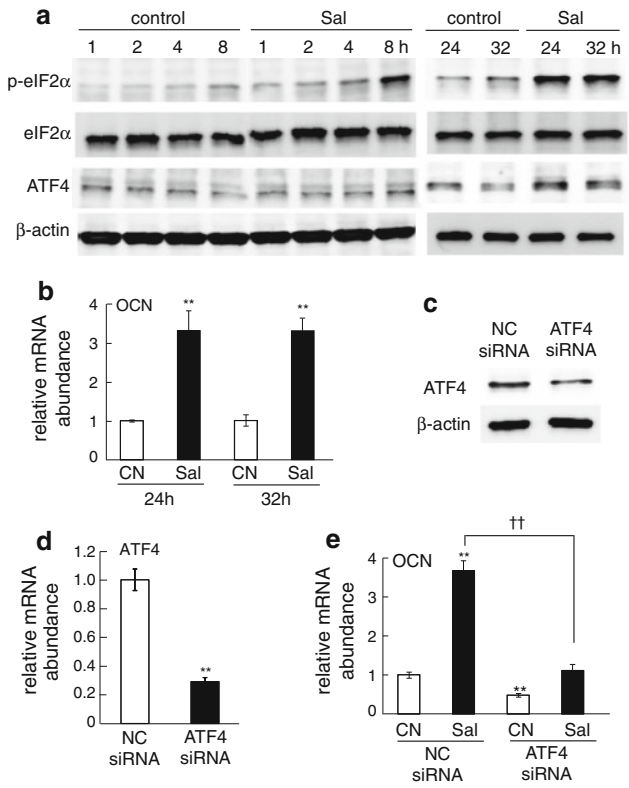


**Fig. 2** Upregulation of p-eIF2α, ATF4 and osteocalcin by salubrinal in MC3T3 E1 (clone 14) osteoblast cells in response to 5 μM salubrinal. *CN* control, *Sal* salubrinal, and *NC* non-specific control siRNA. The *double asterisk* indicates $p < 0.01$ in comparison to CN or NC. The *double dagger* indicates with $p < 0.01$ in comparison to the salubrinal-treated NC siRNA cells. **a** Western blot analysis of p-eIF2α and ATF4. **b** Salubrinal driven elevation of osteocalcin mRNA level. **c** ATF4 level after transfecting siRNA specific to ATF4. **d** Relative mRNA levels of ATF4 in response to RNA interference with ATF4 siRNA and non-specific control (NC) siRNA. **e** Relative mRNA levels of osteocalcin (OCN). The *asterisk* is for the comparison to the control with NC siRNA, and the *dagger* is the comparison between the samples transfected with ATF4 siRNA

### Inhibition of osteoclastogenesis of RAW264.7 cells by salubrinal

The primary aim of this study is to evaluate the effects of salubrinal on osteoclastogenesis. In response to 0.1–20 μM salubrinal for 24 h, we examined cell mortality and live cell numbers of RAW264.7 pre-osteoclasts. Cell mortality ratio did not present statistically significant differences in the presence and absence of RANKL (Fig. 3a). The number of live cells was increased by ~50 % by incubation with RANKL, and administration of 10–20 μM salubrinal reduced the numbers approximately by 10 % (Fig. 3b). Consistent with the stimulatory role of RANKL, the number of TRAP-positive multi-nucleated cells was substantially increased by the addition of RANKL. However, administration of 0.5–20 μM salubrinal reduced the number of TRAP-positive cells in a dose dependent manner (Fig. 3c, d).

### Downregulation of NFATc1 in RAW264.7 cells by salubrinal

The NFATc1 is a transcription factor critical for activating osteoclastogenesis. Addition of RANKL to the culture medium significantly induced NFATc1 expression at day 2 and maintained its elevated level on day 4 (Fig. 4). The RANKL-induced expression of NFATc1 was reduced by administration of 5–20 μM salubrinal on both days, and the effect of salubrinal was dose dependent (Fig. 4).

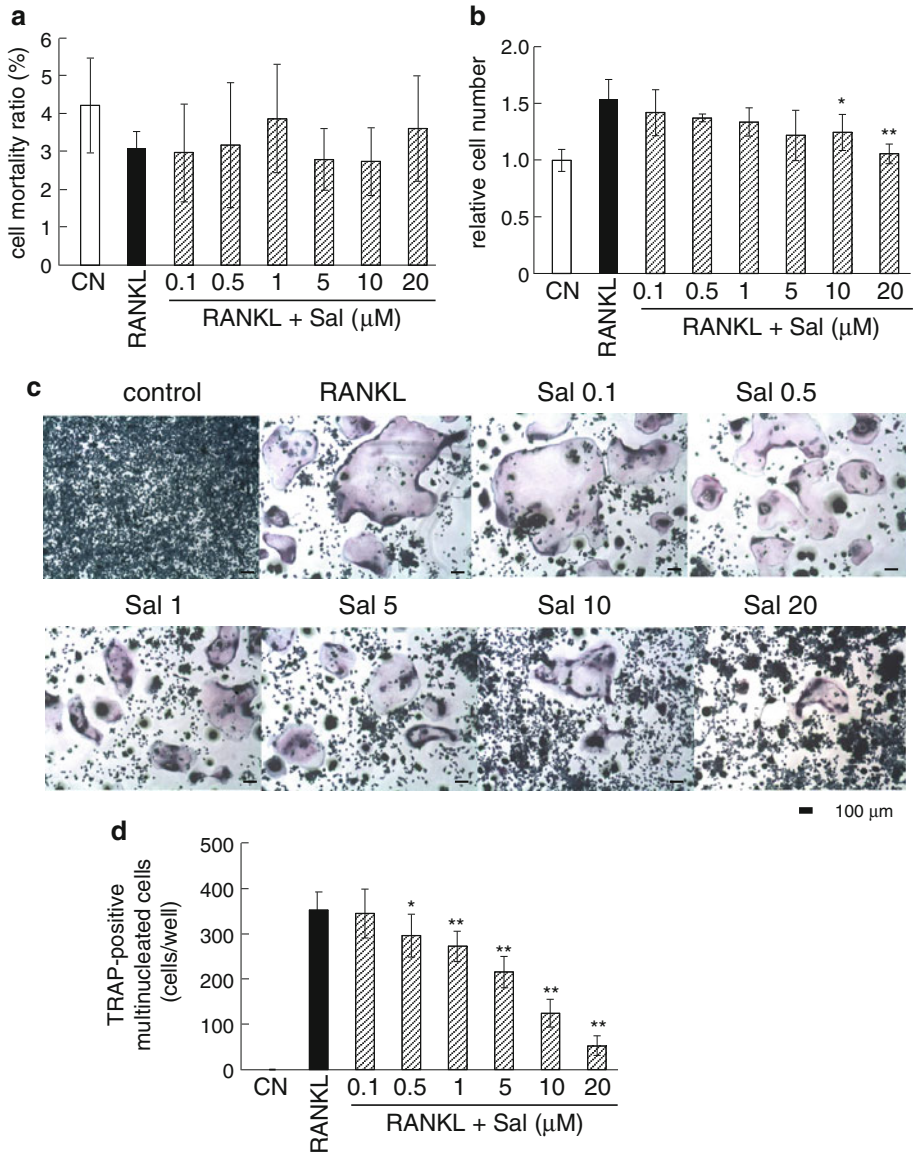


**Fig. 3** Inhibitory effects of salubrinal on RAW264.7 pre-osteoclasts. *CN* control, and *Sal* salubrinal. The *single and double asterisks* indicate $p < 0.05$ and $p < 0.01$ in comparison to the RANKL-treated cells, respectively. **a** Cell mortality ratio. **b** Relative cell numbers. **c** Dose-dependent suppression of RANKL driven activation of osteoclasts by salubrinal. **d** Dose-dependent suppression of TRAP-positive multi-nucleated cells by salubrinal

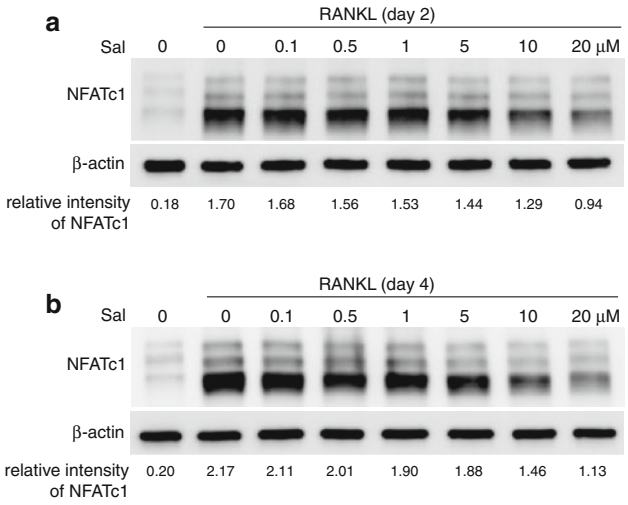


**Fig. 4** Reduction of RANKL-induced NFATc1 expression by salubrinal. *Sal* salubrinal. The relative intensity of NFATc1 to β-actin is shown. **a** Expression of NFATc1 (2 days after RANKL administration). **b** Expression of NFATc1 (4 days after RANKL administration)

## Partial suppression of mRNA levels of NFATc1, c-Fos, TRAP, and OSCAR by salubrinal

Addition of RANKL increased the mRNA levels of NFATc1, c-Fos, TRAP, and OSCAR, and administration of 20 μM salubrinal significantly reduced their mRNA levels. On day 2, for instance, the RANKL-driven increase was 9.4 ± 0.5 fold (NFATc1), 1.9 ± 0.1 fold (c-fos), 165 ± 4.2 fold (TRAP), and 467 ± 22 fold (OSCAR). The reduction by 20 μM salubrinal was 46 % (NFATc1), 32 % (c-fos), 35 % (TRAP), and 21 % (OSCAR) (Fig. 5a). Consistent with the observed dose response, administration of salubrinal at 0.1–1 μM did not contribute to significant reduction in these mRNA levels except for NFATc1 and c-fos on day 4 (Fig. 5b).

## Temporal profile of p-eIF2α and NFATc1

The temporal expression profile revealed that addition of RANKL transiently reduced the phosphorylation level of eIF2α (2–8 h) and elevated NFATc1 by 13.4 ± 3.2 fold (24 h) (Fig. 6). This induction of NFATc1 was partially suppressed by salubrinal with an increase in the level of p-eIF2α. In the early period (2–4 h), administration of 20 μM salubrinal increased the level of p-eIF2α but did not alter the level of NFATc1. In the later period (8–24 h), however, the level of NFATc1 was significantly reduced by 48 % (8 h) and 44 % (24 h). Administration of 20 μM salubrinal did not significantly alter the phosphorylation level of ERK, p38 MAPK, and NFκB (Fig. 6). Note that the normalized level of "1" in Fig. 6c was defined as the level for the cells that were not treated with RANKL without administration of guanabenz.

## Inhibitory effects of guanabenz on osteoclastogenesis of RAW264.7 cells

To further examine a potential involvement of p-eIF2α in regulation of osteoclastogenesis, we employed guanabenz that also acts as an inhibitor of de-phosphorylation of eIF2α. Administration of 1 and 5 μM guanabenz did not alter cell mortality and the number of live cells, although its administration at 10 and 20 μM reduced the number of live cells in 24 h (Fig. 7a, b). Consistent with salubrinal's inhibitory action, guanabenz also attenuated osteoclastogenesis of RAW264.7 cells in a dose dependent manner (Fig. 7c, d). Compared to the number of TRAP-positive multi-nucleated cells of 377 ± 39 (RANKL only), guanabenz reduced the number of differentiated osteoclasts to 364 ± 38 (1 μM), 288 ± 51 (5 μM), 189 ± 25 (10 μM), and 73 ± 16 (20 μM).

## Reduction of RANKL-induced NFATc1, c-Fos, TRAP, and OSCAR by guanabenz

The induction of NFATc1 by RANKL was suppressed by guanabenz in a dose dependent manner (Fig. 8a). The mRNA levels of NFATc1, c-Fos, TRAP, and OSCAR were also reduced by administration of 20 μM guanabenz. Lower concentrations of guanabenz, 5 and 10 μM, were effective in reducing the levels of TRAP and OSCAR mRNA (Fig. 8b). The temporal expression profile of p-eIF2α and NFATc1 in response to 20 μM guanabenz revealed that p-eIF2α was upregulated in 2 h and NFATc1 was partially suppressed in 8 h (Fig. 9). The normalized level of "1" was defined as the level for the cells that were not treated with RANKL without administration of guanabenz. In the absence of RANKL administration, however, either salubrinal or guanabenz did not significantly alter cell mortality and expression of NFATc1 and TRAP (Supplementary Fig. S4).

## Reduction in salubrinal/guanabenz-driven suppression of NFATc1 expression by RNA interference for eIF2α

To evaluate the effects of eIF2α on the expression level of NFATc1, we employed RNA interference specific for eIF2α together with a non-specific control (NC) (Fig. 10). In response to 20 μM salubrinal, RAW264.7 cells transfected with the control siRNA demonstrated a reduction of NFATc1 by 56 %. However, the expression of NFATc1 was reduced only by 20 % in the cells transfected with eIF2α siRNA. Furthermore, 20 μM guanabenz decreased the level of NFATc1 by 43 % in the cells transfected with

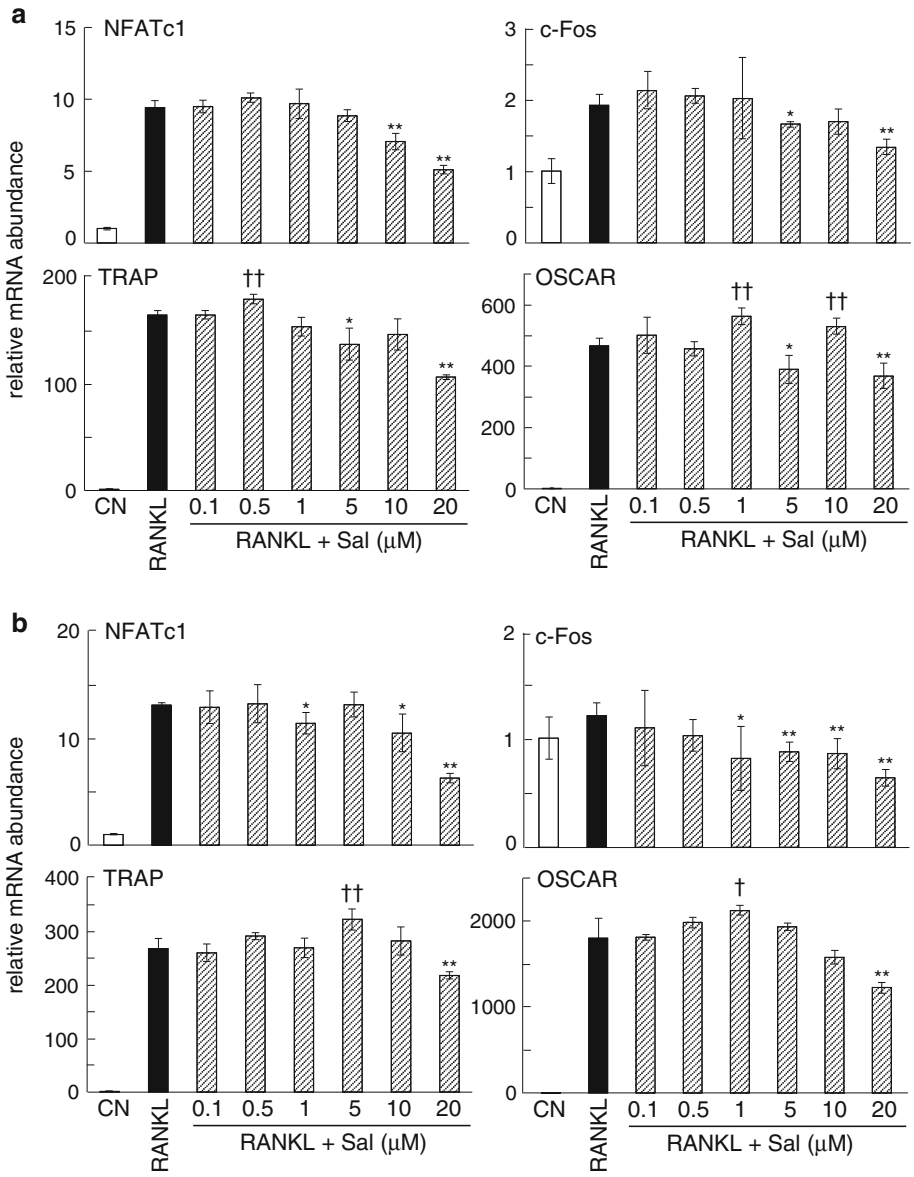


**Fig. 5** Effects of salubrinal on mRNA expression levels of NFATc1, c-Fos, TRAP, and OSCAR. *CN* control. The *single and double asterisks* indicate significant decreases with $p < 0.05$ and $p < 0.01$ in comparison to the RANKL-treated cells, respectively. The *single and double daggers* indicate significant increases with $p < 0.05$ and $p < 0.01$ in comparison to the RANKL-treated cells, respectively. **a** Messenger RNA levels (2 days after RANKL administration). **b** Messenger RNA levels (4 days after RANKL administration)

the control siRNA but the transfection of eIF2α siRNA abolished the suppressive effect of guanabenz. The phosphorylation level of NFκB was not significantly altered by transfection with eIF2α siRNA.

## Discussion

In this study we demonstrate that differentiation of RAW264.7 pre-osteoclasts to multi-nucleated osteoclasts is inhibited by administration of salubrinal and guanabenz, which block de-phosphorylation of eIF2α and elevate the level of p-eIF2α. The growth area covered by multi-nucleated cells is significantly reduced by salubrinal and guanabenz in a dose dependent manner. Partially silencing eIF2α using RNA interference significantly suppressed salubrinal/guanabenz-driven reduction of NFATc1 expression. Together with the stimulated development of MC3T3 E1 osteoblasts by an increase in ATF4 expression, the results herein suggest that eIF2α mediated signaling may play a physiological role in osteoclastogenesis and osteoblastogenesis.

Both salubrinal and guanabenz interact with PP1 and inhibit its activity of de-phosphorylating p-eIF2α. Guanabenz is reported to bind to PP1R15A subunit [14], while the exact binding site of salubrinal is not known. Guanabenz is also known as an $\alpha_2$-adrenergic receptor agonist and used to treat hypertension [24]. The observed

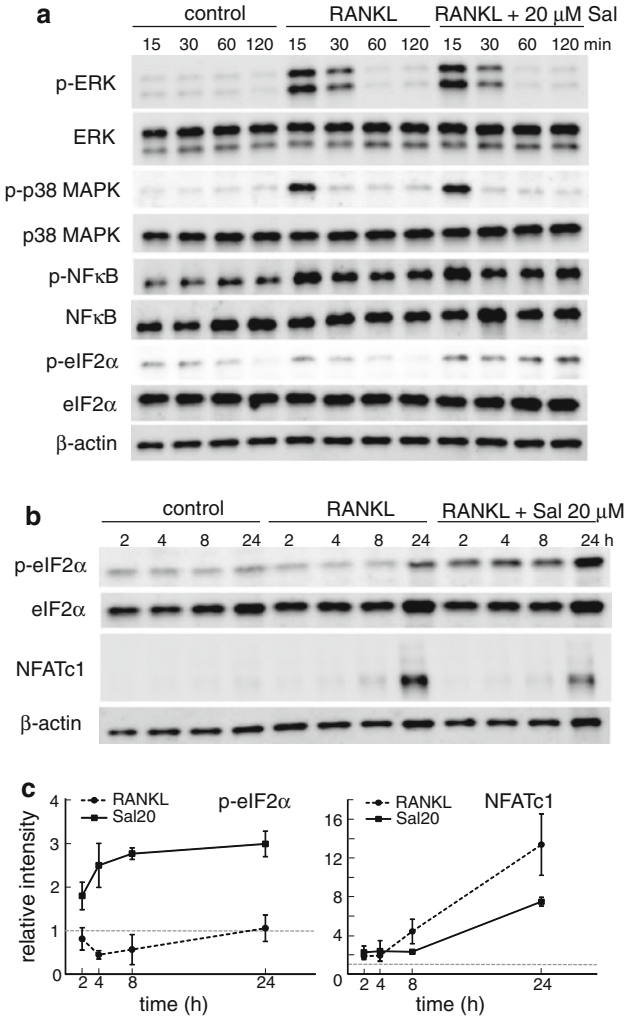


**Fig. 6** Temporal expression profile of p-ERK, p-p38 MAPK, p-NFκB, p-eIF2α and NFATc1 in the presence and absence of 20 μM salubrinal. **a** Western blot analysis of p-ERK, p-p38 MAPK, p-NFκB, and p-eIF2α at 15, 30, 60 and 120 min. **b** Western blot analysis of eIF2α-p and NFATc1. **c** Comparison of the expression level of eIF2α-p and NFATc1 with and without 20 μM salubrinal. The normalized level of "1" was defined as the level for the cells that were not treated with RANKL without administration of salubrinal

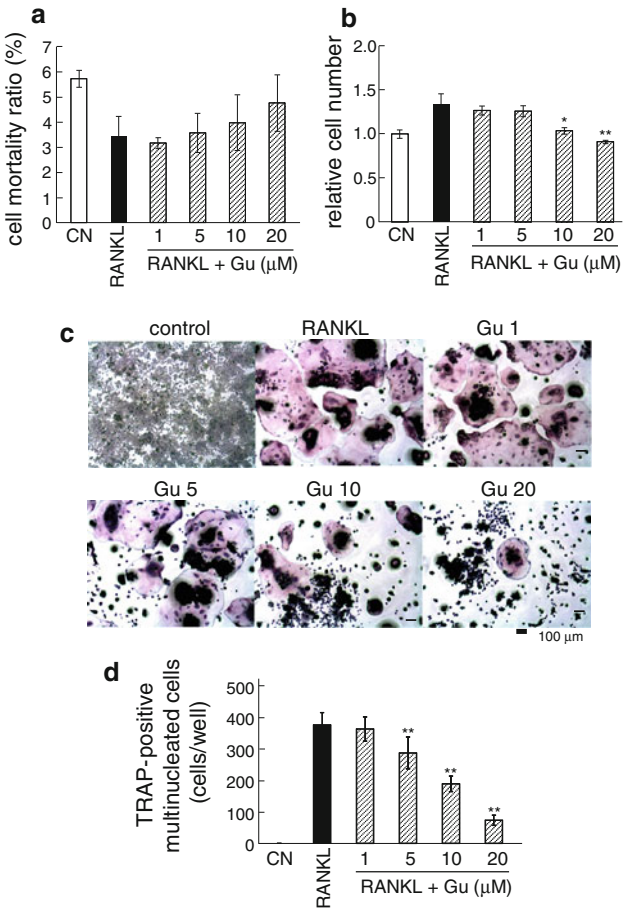


**Fig. 7** Inhibitory effects of guanabenz on development of RAW264.7 pre-osteoclasts. *CN* control, *Gu* guanabenz. The *single and double asterisks* indicate significant decreases with $p < 0.05$ and $p < 0.01$ in comparison to the RANKL-treated cells, respectively. **a** Cell mortality ratio. **b** Relative cell numbers. **c** Dose-dependent suppression of RANKL driven activation of osteoclasts by guanabenz. **d** Dose-dependent suppression of TRAP-positive multi-nucleated cells by guanabenz

stimulation of osteoblastogenesis as well as attenuation of osteoclastogenesis by both agents strongly indicates that eIF2α-mediated signaling is central to regulation of ATF4 and NFATc1. This result is also supported by the salubrinal-driven alterations in the mRNA levels of osteocalcin and TRAP, which are representative in development of osteoblasts and osteoclasts, respectively. Osteocalcin is synthesized solely by osteoblasts for matrix mineralization and calcium homeostasis [25], while TRAP is highly expressed in osteoclasts and its overexpression has been observed to cause bone loss in transgenic mice [26].

The elevation of p-eIF2α is reported to enhance the development of osteoblasts and mineralization of extracellular matrix. In response to various stresses such as oxidation, radiation, and stress to the endoplasmic reticulum, cells undergo either survival or an apoptotic pathway [27]. As part of a pro-survival program, the level of p-eIF2α is raised followed by diminished translational efficiency to all proteins except for a limited group including ATF4 [5]. Salubrinal's action mimics the induction of a pro-survival program without imposing damaging stresses, which result in the upregulation of ATF4 without inducing apoptosis. Since ATF4 is a transcription factor critical for osteoblastogenesis and bone formation, we examined the effects of the administration of

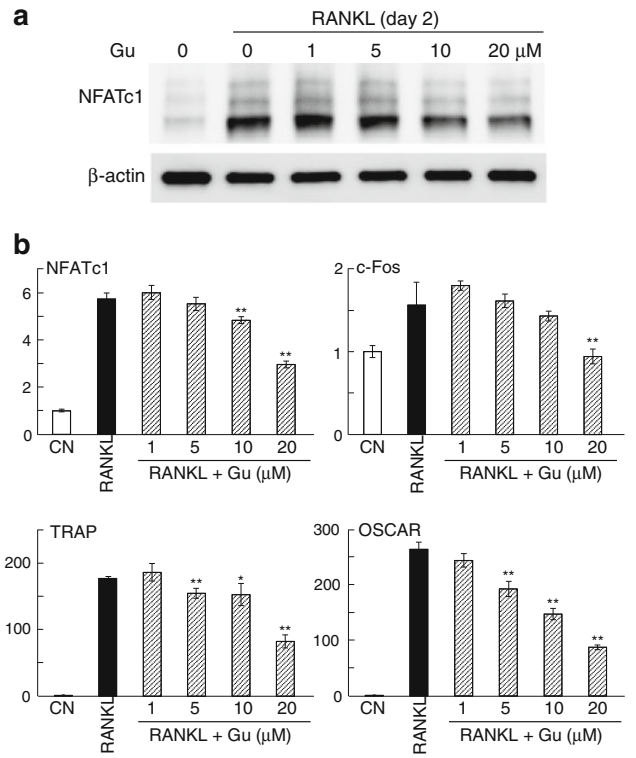


**Fig. 8** Reduction of RANKL-induced NFATc1 expression by guanabenz. *CN* control, *Gu* guanabenz. The *single and double asterisks* indicate significant decreases with $p < 0.05$ and $p < 0.01$ in comparison to the RANKL-treated cells, respectively. **a** Expression of NFATc1 (2 days after RANKL administration). **b** Messenger RNA levels of NFATc1, c-Fos, TRAP, and OSCAR (2 days after RANKL administration)

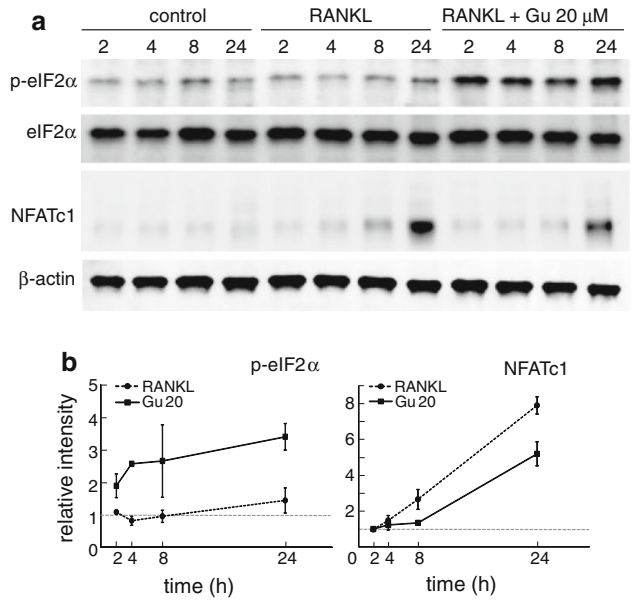


**Fig. 9** Temporal expression profile of p-eIF2α and NFATc1 in the presence and absence of 20 μM guanabenz. *Gu* guanabenz. **a** Western blot analysis of eIF2α-p and NFATc1. **b** Comparison of the expression level of eIF2α-p and NFATc1 with and without 20 μM guanabenz. The normalized level of "1" was defined as the level for the cells that were not treated with RANKL without administration of guanabenz

salubrinal and guanabenz on the mRNA level of osteocalcin as well as the mineralization of the extracellular matrix. Silencing ATF4 using RNA interference significantly suppressed salubrinal-driven upregulation of osteocalcin expression. Thus, the result here is consistent with the previously reported role of salubrinal that stimulates new bone formation in the healing of bone wound [8].

A schematic diagram illustrating the proposed pathway of eIF2α-mediated signaling in osteoblastogenesis and osteoclastogenesis is presented (Fig. 11). Through inhibition of de-phosphorylation of eIF2α, salubrinal and guanabenz are capable of enhancing bone formation by activating ATF4, as well as reducing bone resorption by down-regulating NFATc1. Osteoclastogenesis is a complex developmental process, in which active interactions take place between osteoblasts and osteoclasts. In the RANK/RANKL/OPG signaling pathway, for instance, osteoblasts provide RANKL that stimulates osteoclastogenesis. Since binding of RANKL to RANK is known to activate MAPKs and NFκB [28, 29], we evaluated a potential role of ERK, p38, and NFκB in the eIF2α-mediated signaling. In response to administration of 20 μM salubrinal, we examined the levels of p-ERK, p-p38 MAPK, and p-NFκB together with p-eIF2α. However, no detectable changes in the levels of their phosphorylated form were observed. It is possible that salubrinal may activate transcription factors such as MafB (V-maf musculoaponeurotic fibrosarcoma oncogene homolog B), IRF8 (interferon regulatory factor 8), and Bcl6 (B cell lymphoma 6), which are known to be inhibitors of NFATc1 [30–32]. Alternatively, microRNA and epigenetic changes such as histone modification regulate expression of NFATc1 might be involved [33, 34]. For instance, H3K27 demethylase is reported to demethylate the site of H3K27me3 of NFATc1 and stimulate RANKL-induced osteoclastogenesis [34]. The results herein require further analysis on a regulatory mechanism that links elevation of p-eIF2α to the suppression of NFATc1.

A recent study independently reported that salubrinal alters the fate of osteoclasts and bone resorption through eIF2α-mediated translational regulation [35]. Herein, we further examined the regulatory mechanism using not only salubrinal but also guanabenz, which are the inhibitors of PP1. The results revealed that these agents can also regulate expression of NFATc1 at a transcriptional level. A separate in vivo study as well as in vitro studies using

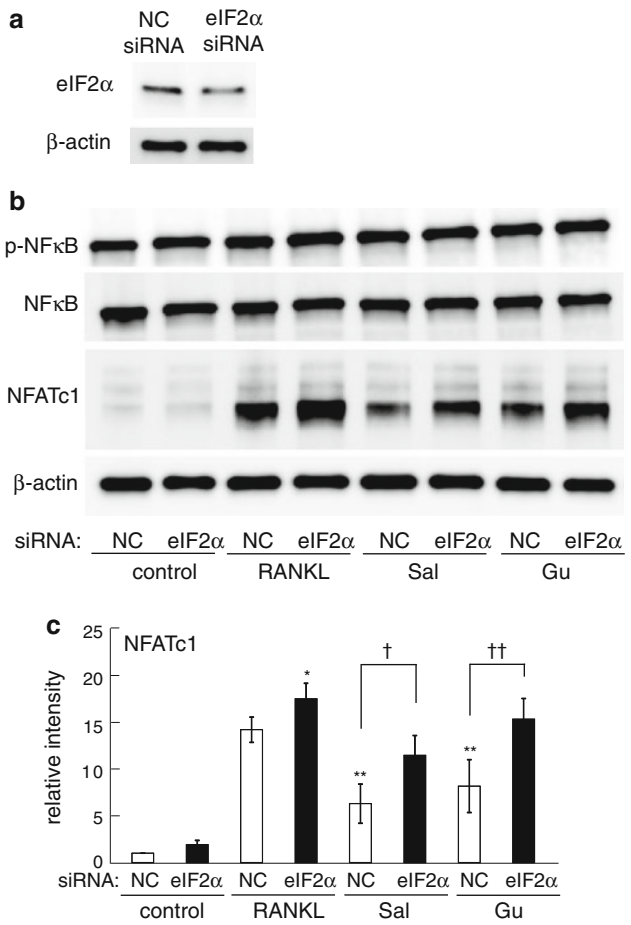


**Fig. 10** Reduction in salubrinal/guanabenz driven suppression of NFATc1 expression by RNA interference specific for eIF2α. *Sal* salubrinal, *Gu* guanabenz, and *NC* non-specific control siRNA. The *single and double asterisks* indicate significant changes to the RANKL-treated NC siRNA cells with $p < 0.05$ and $p < 0.01$, respectively. The *single and double daggers* indicate significant changes to the salubrinal or guanabenz-treated NC siRNA cells with $p < 0.05$ and $p < 0.01$, respectively. **a** eIF2α level after transfecting siRNA specific to eIF2α. **b** Western blot analysis of p-NFκB and NFATc1. **c** Comparison of the expression level of NFATc1 between control siRNA and eIF2α siRNA

primary bone marrow derived cells support salubrinal's efficacy on inhibition of bone resorption. In summary, we demonstrate that elevation of p-eIF2α stimulates osteocalcin expression through upregulation of ATF4 in osteoblasts and inhibits TRAP expression via downregulation of NFATc1 in pre-osteoclasts. Silencing eIF2α with RNA interference reduced suppression of salubrinal/guanabenz-driven downregulation of NFATc1. The results in this study support the possibility of regulating bone remodeling through eIF2α-mediated signaling for combatting bone loss in osteoporosis.

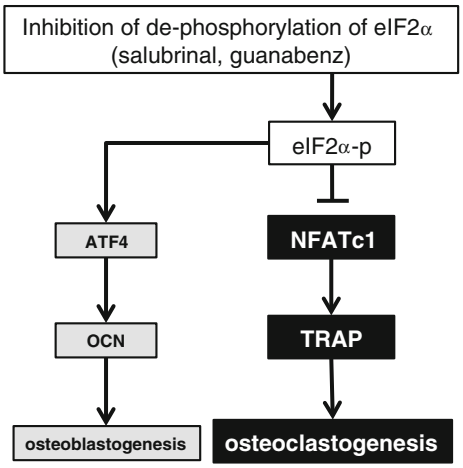


**Fig. 11** Proposed mechanism of eIF2α signaling on osteoclastogenesis through NFATc1

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**Conflict of interest** All authors state that they have no conflicts of interest.

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