

# Suppression of Osteoclastogenesis via Upregulation of Zfyve21 and Ddit4 by Salubrinal and Guanabenz

Kazunori Hamamura<sup>1,\*</sup>, Nancy Tanjung<sup>2</sup>, Andy Chen<sup>3</sup>, Hiroki Yokota<sup>2</sup>, and Akifumi Togari<sup>1</sup>

<sup>1</sup>Department of Pharmacology, School of Dentistry, Aichi-Gakuin University, Nagoya, Japan

愛知学院大学歯学部薬理学講座

<sup>2</sup>Department of Biomedical Engineering, Indiana University Purdue University Indianapolis,  
Indianapolis, IN 46202 USA

<sup>3</sup>Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN 47907 USA

Running Title: Inhibition of Osteoclastogenesis by Salubrinal and Guanabenz

\*Corresponding Author

Kazunori Hamamura, PhD/DDS ([hamak@dpc.agu.ac.jp](mailto:hamak@dpc.agu.ac.jp))

Department of Pharmacology

School of Dentistry

Aichi-Gakuin University

1-100 Kusumoto-Cho, Chikusa-Ku

Nagoya 464-8650, Japan

Phone: +81-52-757-6743

Fax: +81-52-752-5988

## 対訳

Salubrinal と guanabenz は、eukaryotic translation initiation factor 2 alpha (eIF2 $\alpha$ )の脱リン酸化阻害剤として知られていて、それらは、破骨細胞分化のマスター分子である nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1)を低下させ、破骨細胞分化を抑制する。NFATc1 の抑制メカニズムはあまり解明されていない。そこで、ゲノムワイドのマイクロアレー解析を用いて、私たちは、特に salubrinal および guanabenz が作用する破骨細胞分化に関与する分子レギュレーターを研究した。私たちは、2つの遺伝子群を同定した。1つは、receptor activator of nuclear factor kappa-B ligand (RANKL)によって亢進され、salubrinal および guanabenz によって抑制される遺伝子群。もう1つは、RANKL によって抑制され、salubrinal および guanabenz によって亢進される遺伝子群。マイクロアレーおよび qPCR の結果により、zinc finger protein, FYVE domain containing 21 (Zfyve21) と DNA-damage-inducible transcript 4 (Ddit4)が、RANKL によって抑制され、salubrinal および guanabenz によって亢進されることが明らかになった。また、Zfyve21 あるいは Ddit4 の部分的なサイレンシングが salubrinal および guanabenz による NFATc1 の抑制を軽減させた。総括すると、この研究により Zfyve21 および Ddit4 が破骨細胞分化の阻害分子であることが明らかになった。このことより、私たちは、それらが骨粗鬆症などの骨疾患から生じる骨量低下を抑制する新規標的分子になりうることを期待する。

Key words : 破骨細胞、Zfyve21、Ddit4、salubrinal、guanabenz

## **Abstract**

Salubrinal and guanabenz are two known inhibitors of de-phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2 $\alpha$ ), and they suppress osteoclastogenesis through downregulating nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), a master molecule of osteoclastogenesis. The mechanism of NFATc1 suppression is not well understood. Using genome-wide microarray analysis, we investigated molecular regulators of osteoclastogenesis, in particular, in response to salubrinal and guanabenz. We identified two sets of genes: a set of genes that were upregulated by receptor activator of nuclear factor kappa-B ligand (RANKL) and downregulated by salubrinal and guanabenz; and the other set of genes that were downregulated by RANKL and upregulated by salubrinal and guanabenz. The microarray and qPCR result revealed that a zinc finger protein, FYVE domain containing 21 (Zfyve21), as well as DNA-damage-inducible transcript 4 (Ddit4), were suppressed by RANKL and upregulated by salubrinal and guanabenz. A partial silencing of Zfyve21 or Ddit4 attenuated salubrinal- and guanabenz-driven suppression of NFATc1. Collectively, this study demonstrates that Zfyve21 and Ddit4 are two inhibitors of osteoclastogenesis. We expect that they may potentially serve as novel targets for preventing bone loss from skeletal diseases such as osteoporosis.

Key words : osteoclasts, Zfyve21, Ddit4, salubrinal, guanabenz

## Introduction

Stress to the endoplasmic reticulum (ER) is induced by a multitude of causes, including UV, viral infection, and protein misfolding<sup>1, 2)</sup>. The ER stress is known to generate an integrated stress response (ISR), in which the general rate of protein synthesis is reduced by elevating the phosphorylation level of eukaryotic translation initiation factor 2 alpha (eIF2 $\alpha$ )<sup>3)</sup>. Severe ER stress leads to cellular apoptosis, and the progression of various diseases are reported to be linked to the ER stress and regulation of eIF2 $\alpha$  phosphorylation<sup>4- 6)</sup>. Salubrinal and guanabenz are small synthetic agents, which bind to a protein phosphatase 1 (PP1) complex and inhibit dephosphorylation of eIF2 $\alpha$ <sup>7, 8)</sup>. It has been shown in pre-clinical studies that their administration improves varying symptoms of diseases such as neuronal disorders, inflammatory diseases, and cancer, primarily via alleviating detrimental effects caused by the ER stress<sup>9-14)</sup>.

Regarding skeletal diseases, salubrinal and guanabenz are reported to present multiple beneficial effects<sup>11, 15-20)</sup>. First, they stimulate differentiation of bone-forming osteoblasts, through the upregulation of activating transcription factor 4 (ATF4) and osteocalcin<sup>16)</sup>. Second, they attenuate differentiation of bone-resorbing osteoclasts, through downregulating nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1)<sup>17, 18)</sup>. Third, salubrinal presents chondroprotective effects by suppressing inflammatory cytokines and matrix metalloproteinase 13 (MMP13)<sup>19, 20)</sup>. Lastly, salubrinal improves inflammatory symptoms of anti-collagen antibody-induced arthritis via suppression of dual-specificity phosphatase 2 (Dusp2)<sup>11)</sup>.

In this study, we conducted genome-wide expression analysis using RAW264.7 pre-osteoclast cells and investigated a regulatory mechanism that is involved in salubrinal- and guanabenz-

driven suppression of NFATc1. This microarray analysis led us to focus on two potential regulators: a zinc finger protein, FYVE domain containing 21 (Zfyve21), and DNA-damage-inducible transcript 4 (Ddit4). Both were suppressed by receptor activator of nuclear factor kappa-B ligand (RANKL), while upregulated by salubrinal and guanabenz. To evaluate the involvement of Zfyve21 and Ddit4 in expression of NFATc1, we employed RNA interference with specific siRNAs. Herein, we demonstrate the novel role of Zfyve21 and Ddit4 in RANKL-induced regulation of NFATc1.

## **Materials and Methods**

### **Cell Culture**

RAW264.7 mouse pre-osteoclast cells were cultured in  $\alpha$ MEM containing 10% fetal bovine serum and antibiotics (50 units/ml penicillin and 50  $\mu$ g/ml streptomycin; Life Technologies, Grand Island, NY, USA). Cells were maintained at 37°C and 5% CO<sub>2</sub> in a humidified incubator.

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

RAW264.7 cells were plated at  $2.0 \times 10^4$  and  $1.0 \times 10^5$  cells into a 12-well and 60 mm dish, respectively, and cultured with 20 or 50 ng/ml RANKL (PeproTech, Rocky Hills, NC, USA) in the presence and absence of 10 or 20  $\mu$ M salubrinal or guanabenz (R&D Systems, Minneapolis, MN, USA)<sup>16</sup>. After 5-day incubation with RANKL, cells were treated for TRAP staining using an acid phosphatase leukocyte kit (Sigma, St. Louise, MO, USA). The number of TRAP-positive cells containing three or more nuclei was determined.

### **Quantitative real-time PCR**

Total RNA was extracted using an RNeasy Plus mini kit (Qiagen, Germantown, MD, USA). Reverse transcription was conducted with a high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA), and quantitative real-time PCR (qPCR) was performed using ABI 7500 with Power SYBR green PCR master mix kits (Applied Biosystems). We evaluated mRNA levels of TRAP, cathepsin K, Dscr1, Dusp2, Hipk2, Ptpn22, Tnip1, Agap1, Ddit4, Pla2g15, Ttf2, and Zfyve21 with the PCR primers listed in Table 1. GAPDH was used for an internal control.

### **Western blot analysis**

Cells were lysed in a radioimmuno-precipitation 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% SDS gels and electro-transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). The membrane was incubated with primary antibodies followed by goat anti-mouse IgG conjugated with horseradish peroxidase (Cell Signaling, Danvers, MA, USA). We used antibodies against NFATc1 (Santa Cruz) and  $\beta$ -actin (Sigma). The level of proteins was detected using a SuperSignal west femto maximum sensitivity substrate (Thermo Scientific, Waltham, MA, USA).

### **Microarray analysis**

A microarray experiment was conducted using RAW264.7 cells. We employed 4 groups (3 samples per group; Illumina MouseWG-6 v2.0): CN (control), RANKL (20 ng/ml RANKL), Sal (20 ng/ml RANKL with 20  $\mu$ M salubrinal), and Gu (20 ng/ml RANKL with 20  $\mu$ M guanabenz). Cells were harvested 4 h after treatment with the above agents. Genes that were differentially expressed after addition of salubrinal and guanabenz were identified by calculating fold change. Note that all of the chosen genes had raw signal values greater than 100. Of particular interest were the genes that were upregulated by addition of RANKL and downregulated by addition of salubrinal and guanabenz, and the genes that were downregulated by addition of RANKL and upregulated by addition of salubrinal and guanabenz. Statistical significance of the regulation was assessed by Student's *t*-test.

**Knockdown of NFATc1, Dscr1, Ptpn22, Zfyve21, and Ddit4 by siRNA**

RAW264.7 cells were treated with siRNA specific to NFATc1 (5'-CGG UUA CUU GGA GAA UGA A- 3'; Life Technologies), Dscr1 (5'-CGU CAU AAA UUA CGA UCU U-3'; Life Technologies), Ptpn22 (5'-GUU UGA AGA UUU UUC GAA A-3'; Life Technologies), Zfyve21 (5'-AGG CGG AGU UUU AUG ACA A-3'; Life Technologies), Ddit4 (5'-GCU AAG UAC CGG CUU CAG A- 3'; Life Technologies), or a nonspecific control (NC) (5'-UGU ACU GCU UAC GAU UCG G-3', Life Technologies). Cells were transiently transfected with siRNA in Opti-MEM I medium with Lipofectamine RNAiMAX (Life Technologies). The efficiency of RNA silencing was assessed with immunoblotting or qPCR 48 h after transfection.

**Statistical analysis**

Three or four independent *in vitro* experiments were conducted, and data were expressed as mean  $\pm$  S.D. For comparison among multiple samples, statistical significance was evaluated at  $p < 0.05$ . The single and double asterisks indicate  $p < 0.05$  and  $p < 0.01$ , respectively.



## **Results**

### **Suppression of osteoclastogenesis by salubrinal and guanabenz**

The number of TRAP-positive multinucleated osteoclasts was significantly reduced by 10 or 20  $\mu$ M salubrinal and guanabenz in RAW264.7 cells (Fig. 1A & 1B). The level of RANKL-driven NFATc1 protein was also significantly attenuated by salubrinal and guanabenz (Fig. 2A & 2B). A partial silencing of NFATc1 by its siRNA suppressed the mRNA levels of TRAP and cathepsin K compared to those treated with non-specific control (NC) siRNA (Fig. 2C & 2D). The reduction ratio of the mRNA levels of TRAP and cathepsin K was higher than that of NFATc1 mRNA. This difference might be caused by time dependent peak/bottom expression levels among NFATc1, TRAP, and cathepsin K.

### **Microarray data analysis in response to salubrinal/guanabenz**

We identified two sets of genes (the first set of genes that were upregulated by RANKL but downregulated by salubrinal and guanabenz; and the second set of genes that were downregulated by RANKL but upregulated by salubrinal and guanabenz). Five genes were chosen for each set (Table 2 & 3). The first set of genes such as Dscr1, Dusp2, Hipk2, Ptpn22, and Tnip1 were upregulated by RANKL, while the second set of genes such as Agap1, Ddit4, Pla2g15, Ttf2, and Zfyve21 were downregulated by RANKL. In qPCR, four genes (Dscr1, Ptpn22, Ddit4, and Zfyve21) were confirmed to follow the same trend to the microarray result (Fig. 3A & 3B). Hereafter, we focused on the role of Dscr1, ptpn22, Zfyve21, and Ddit4.

### **Effects of Dscr1, Ptpn22, Zfyve21 and Ddit4 on NFATc1**

In order to examine the role of Dscr1, Ptpn22, Zfyve21 and Ddit4 in regulation of RANKL-driven NFATc1, we employed RNA interference using siRNA specific for Dscr1, Ptpn22, Zfyve21 or Ddit4 (Fig. 4). RAW264.7 cells, transfected with NC siRNA, showed a reduction of NFATc1 in response to salubrinal and guanabenz. A partial silencing of Dscr1 and Ptpn22 did not affect expression of RANKL-induced NFATc1 (Fig. 4B & 4D). However, a partial silencing of Zfyve21 and Ddit4 attenuated salubrinal/guanabenz-driven suppression of NFATc1 (Fig. 4F & 4H).

## Discussion

We present in this study that salubrinal and guanabenz, which inhibit de-phosphorylation of eIF2 $\alpha$  and attenuate the ER stress, suppress osteoclastogenesis via downregulating NFATc1. The genome-wide mRNA profiling using RAW264.7 cells reveals that Zfyve21 and Ddit4 are downregulated by RANKL and this downregulation is suppressed by salubrinal/guanabenz. The expression patterns of Zfyve21 and Ddit4 in response to RANKL and salubrinal/guanabenz were verified by qPCR, and the involvement of Zfyve21 and Ddit4 in expression of NFATc1 was validated using RNA interference.

Microarray analysis followed by qPCR verification indicates that four genes are responsive to salubrinal and guanabenz and potentially novel regulators of osteoclastogenesis. RANKL elevates the mRNA levels of Dscr1 and Ptpn22 and their elevation is suppressed by salubrinal and guanabenz, while the mRNA levels of Zfyve21 and Ddit4 are downregulated by RANKL and its downregulation is upregulated by salubrinal and guanabenz. A partial silencing of Dscr1 and Ptpn22 revealed that they do not significantly alter expression of NFATc1. Therefore, in this study, we focused on the role of Zfyve21 and Ddit4 as potential inhibitors of osteoclastogenesis.

Zfyve21 is a member of a protein family that consists of a phosphatidylinositol 3-phosphate-binding FYVE domain<sup>21</sup>). This gene is reported to be involved in cell migration and cancer metastasis via disassembling focal adhesions<sup>21, 22</sup>). It is possible that Zfyve21 is inhibitory in a process of multi-nucleation of osteoclasts, but further analysis is necessary to understand its role in osteoclastogenesis. Ddit4 is a transcriptional target of p63 and p53, as well as a hypoxia-inducible factor 1-responsive gene<sup>23, 24</sup>). It is reported that transcription of Ddit4 is stimulated by

ATF4 that is upregulated by the elevated phosphorylation of eIF2 $\alpha$ <sup>25</sup>). Ddit4 regulates mTORC1 signaling, and its expression level is upregulated during pathological conditions such as hypoxia, cancer cachexia, and diabetes<sup>26</sup>). Since salubrinal and guanabenz elevate the phosphorylation level of eIF2 $\alpha$ , our result with Ddit4 is consistent with the role of eIF2 $\alpha$  in osteoclast development.

It has been reported that salubrinal and guanabenz induce cell apoptosis in cancer cells<sup>13, 14</sup>). For instance, apoptosis is induced in breast cancer cells by salubrinal or guanabenz at a dose above 20  $\mu$ M<sup>13</sup>). Salubrinal and guanabenz at a dose below 20  $\mu$ M, however, are not linked to cell mortality in RAW264.7 cells<sup>16</sup>). Therefore, it is unlikely that Zfyve21 and Ddit4 are directly involved in cell mortality.

Both salubrinal and guanabenz are known to reduce the ER stress and inhibit de-phosphorylation of eIF2 $\alpha$ <sup>7, 8</sup>). However, there are differences in their functions. In pre-clinical studies, salubrinal and not guanabenz reduces MMP13 activity via reducing the phosphorylation level of NF $\kappa$ B, and attenuates the progression of osteoarthritis<sup>19, 20</sup>). On the other hand, guanabenz and not salubrinal is known as an agonist of  $\alpha$ 2 adrenergic receptor and is used for treatment of hypertension<sup>27</sup>). The common feature of salubrinal and guanabenz is their interactions with PP1 subunits, while the different feature is their unique binding partners in PP1 complex<sup>7, 8</sup>).

In this study, we specifically analyzed the involvement of Zfyve21 and Ddit4 in regulation of NFATc1 from a list of ten genes in Table 2 & 3. Further analysis is recommended to evaluate

Zfyve21's and Ddit4's actions, using primary macrophages and animal models. In summary, Zfyve21 and Ddit4 are identified as two novel regulators that inhibit expression of NFATc1 and suppress osteoclastogenesis. We expect that this study contributes to our basic understanding of osteoclastogenesis and development of a new therapeutic target for bone diseases such as osteoporosis.

### **Acknowledgements**

The authors appreciate M. Hamamura for technical assistance. All authors state that they have no conflicts of interest. This study was supported by grant DOD W81XWH-11-1-0716.

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### Figure Legends

Figure 1. Inhibitory effects of salubrinal and guanabenz on development of osteoclasts in the presence of 20 ng/ml RANKL. Sal = salubrinal, and Gu = guanabenz. (A&B) Suppression of TRAP-positive multinucleated osteoclasts by 10 or 20  $\mu$ M salubrinal and guanabenz in RAW264.7 cells, respectively.

Figure 2. Suppression of RANKL-driven osteoclast-linked genes in response to salubrinal/guanabenz in RAW264.7 cells. Sal = 20  $\mu$ M salubrinal, and Gu = 20  $\mu$ M guanabenz. (A) Salubrinal induced inhibition of NFATc1 on day 2 in RAW264.7 cells. (B) Guanabenz induced inhibition of NFATc1 on day 2 in RAW264.7 cells. (C) Partial knockdown of NFATc1 in the presence and absence of RANKL in RAW264.7 cells. (D) Levels of TRAP and cathepsin

K mRNAs in response to non-specific control (NC) siRNA and NFATc1 siRNA. The double asterisks indicate  $p < 0.01$ . Note that 20 and 50 ng/ml RANKL was used in (A&B) and (C&D), respectively.

Figure 3. Prediction of molecular regulators in osteoclastogenesis. (A) Potential activators of NFATc1, suppressible by 20  $\mu$ M salubrinal/guanabenz. (B) Potential inhibitors of NFATc1, inducible by 20  $\mu$ M salubrinal/guanabenz. Note that 20 ng/ml RANKL was used.

Figure 4. Salubrinal- and guanabenz-driven upregulation in Zfyve21 and Ddit4. (A&B) Undetectable effect of Dscr1 siRNA on NFATc1. (C&D) Undetectable effect of Ptpn22 siRNA on NFATc1. (E) Zfyve21 mRNA level after transfecting siRNA specific to Zfyve21. (F) NFATc1 protein level in the presence and absence of 20  $\mu$ M salubrinal/guanabenz in response to Zfyve21 siRNA treatment. (G) Ddit4 mRNA level after transfecting siRNA specific to Ddit4. (H) NFATc1 protein level in the presence and absence of 20  $\mu$ M salubrinal/guanabenz in response to Ddit4 siRNA treatment. The double asterisks indicate  $p < 0.01$ . Note that 20 ng/ml RANKL was used in (B, D, F, and H).

Figure 5. Proposed mechanism of salubrinal and guanabenz' action on osteoclastogenesis.

**Table**

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

target	forward primer	backward primer
TRAP	5'-TCCTGGCTCAAAAAGCAGTT-3'	5'-ACATAGCCCACACCGTTCTC-3'
cathepsin K	5'-CAGCTTCCCCAAGATGTGAT-3'	5'-AGCACCAACGAGAGGAGAAA-3'
Dscr1	5'-CCCGACAAACAGTTCCTCAT-3'	5'-CACACTGGGAGTGGTGTCTG-3'
Dusp2	5'-TGGAATCTTGCCCTACCTG-3'	5'-CTCCTGGAACCAGGCACTTA-3'
Hipk2	5'-CTTCCAGCACAAGAACCACA-3'	5'-ACCTTCACTCGGTACGGTTG-3'
Ptpn22	5'-ATAGCAACCCACACGACTCC-3'	5'-TGCTCCAAATGTTACCACCA-3'
Tnip1	5'-AAGAGGAGGAGAAGGCCAAG-3'	5'-CCTTGTAGGCATGGTGAGGT-3'
Agap1	5'-CGAAGATGCTTTCGTGAACA-3'	5'-CCCTTCATCCCTAATCAGCA-3'
Ddit4	5'-CTCTGGGATCGTTTCTCGTC-3'	5'-GACACCCCATCCAGGTATGA-3'
Pla2g15	5'-TGCTTGTATGGCACTGGTGT-3'	5'-TCCAGAAGCACACGTTTCAG-3'
Ttf2	5'-GCCCAAACTGAGAAAGCTG-3'	5'-ACCAAAGCTGGGTTTCCTT-3'
Zfyve21	5'-GGGGACAGTCATCGTAAAAT-3'	5'-AGCTTGGTAGCCTTGTGCAT-3'
GAPDH	5'-TGCACCACCAACTGCTTAG-3'	5'-GGATGCAGGGATGATGTTTC-3'

Table 2. Genes upregulated by RANKL and downregulated by salubrinal/guanabenz.

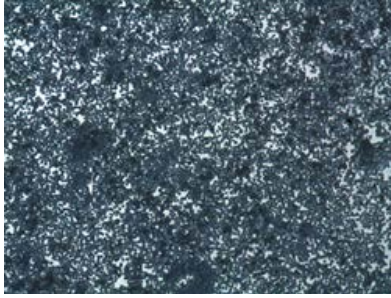
Gene symbol	Fold Change [G/R]	P value [G/R]	Fold Change [S/R]	P value [S/R]	Fold Change [R/C]	P value [R/C]
Dscr1	-1.53	8.11E-06	-1.24	1.18E-03	1.91	2.30E-07
Dusp2	-1.62	6.87E-04	-1.33	3.73E-02	1.82	6.84E-04
Hipk2	-1.33	2.33E-02	-1.29	2.46E-02	1.44	5.61E-03
Ptpn22	-1.87	1.12E-04	-1.48	1.69E-02	2.11	8.07E-04
Tnip1	-1.43	5.63E-03	-1.34	3.78E-02	2.66	9.41E-04

Table 3. Genes downregulated by RANKL and upregulated by salubrinal/guanabenz.

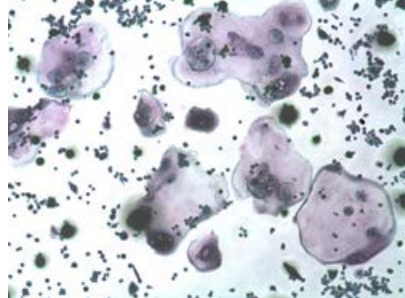
Gene symbol	Fold Change [G/R]	P value [G/R]	Fold Change [S/R]	P value [S/R]	Fold Change [R/C]	P value [R/C]
Agap1	1.57	3.01E-04	1.19	1.62E-02	-1.72	1.96E-02
Ddit4	1.10	1.91E-02	1.22	1.70E-03	-1.39	2.10E-03
Pla2g15	1.64	1.33E-03	1.17	4.00E-02	-1.80	1.45E-04
Ttf2	1.42	1.15E-02	1.20	3.42E-02	-1.59	1.44E-03
Zfyve21	1.36	2.38E-03	1.25	6.38E-03	-1.52	2.66E-03

A

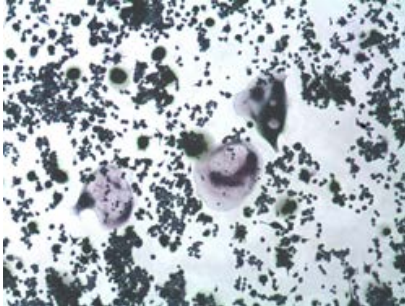
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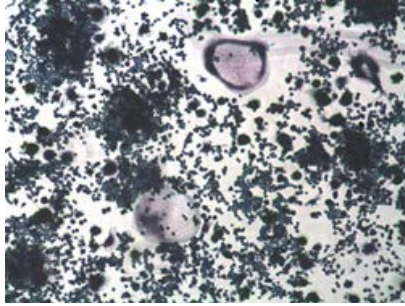
RANKL



RANKL + 10  $\mu$ M Sal

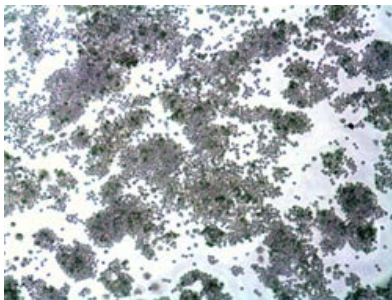


RANKL + 20  $\mu$ M Sal

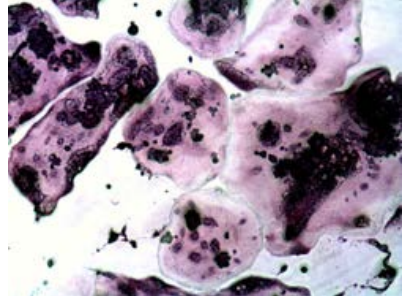


B

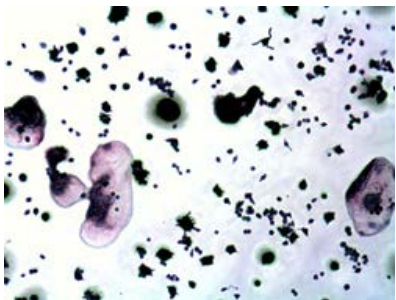
control



RANKL



RANKL + 10  $\mu$ M Gu



RANKL + 20  $\mu$ M Gu

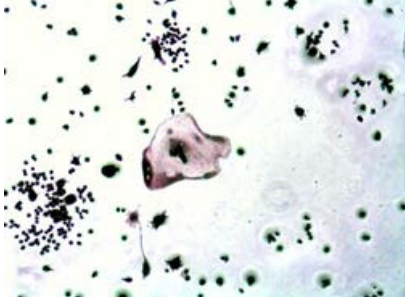


Figure 1

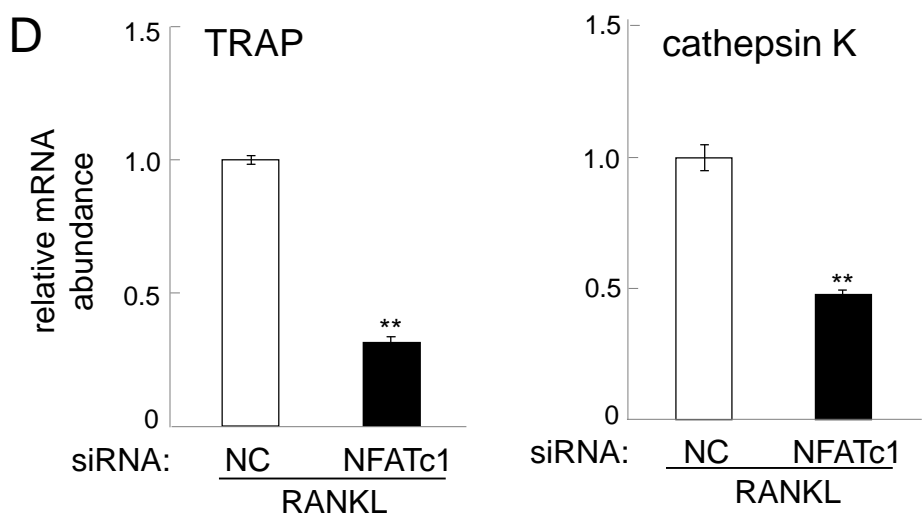
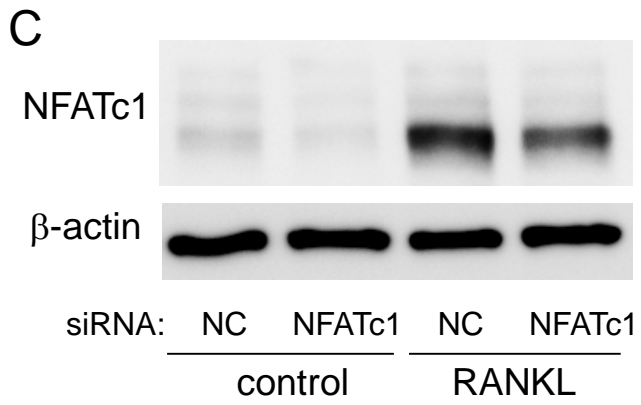
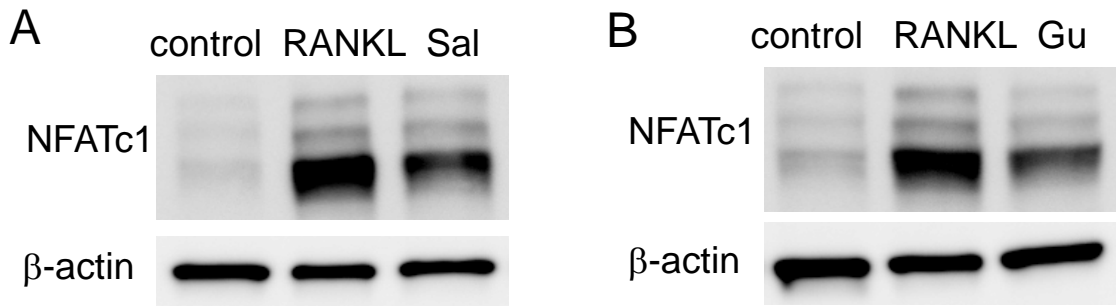


Figure 2

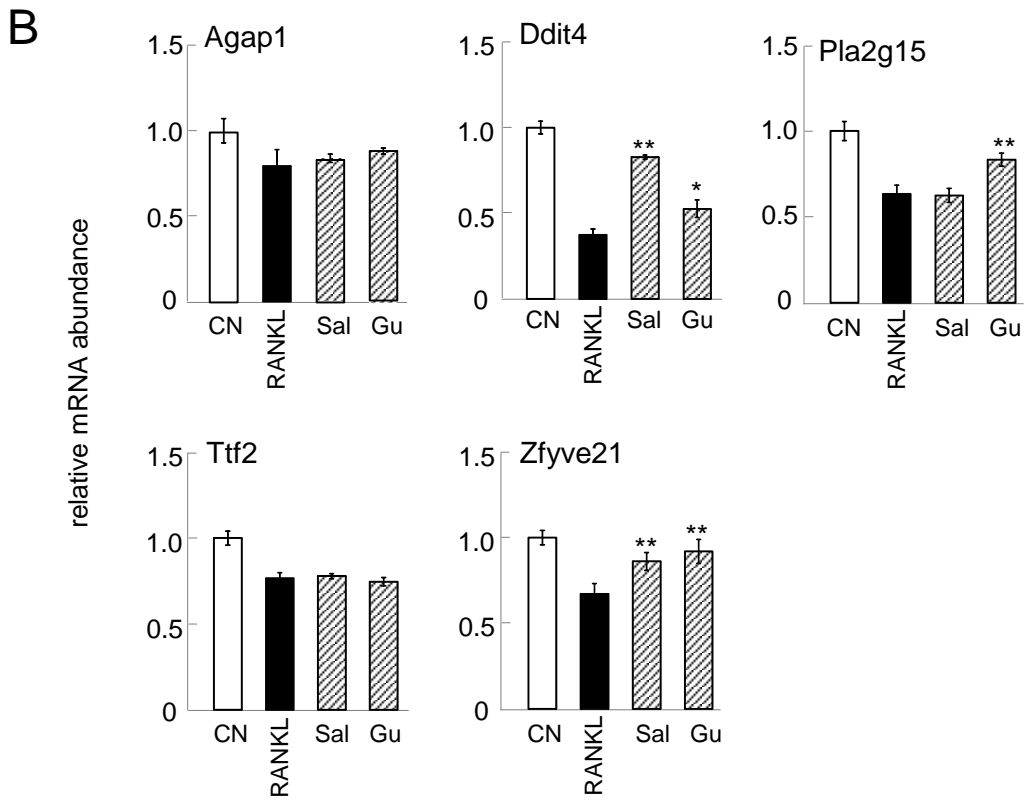
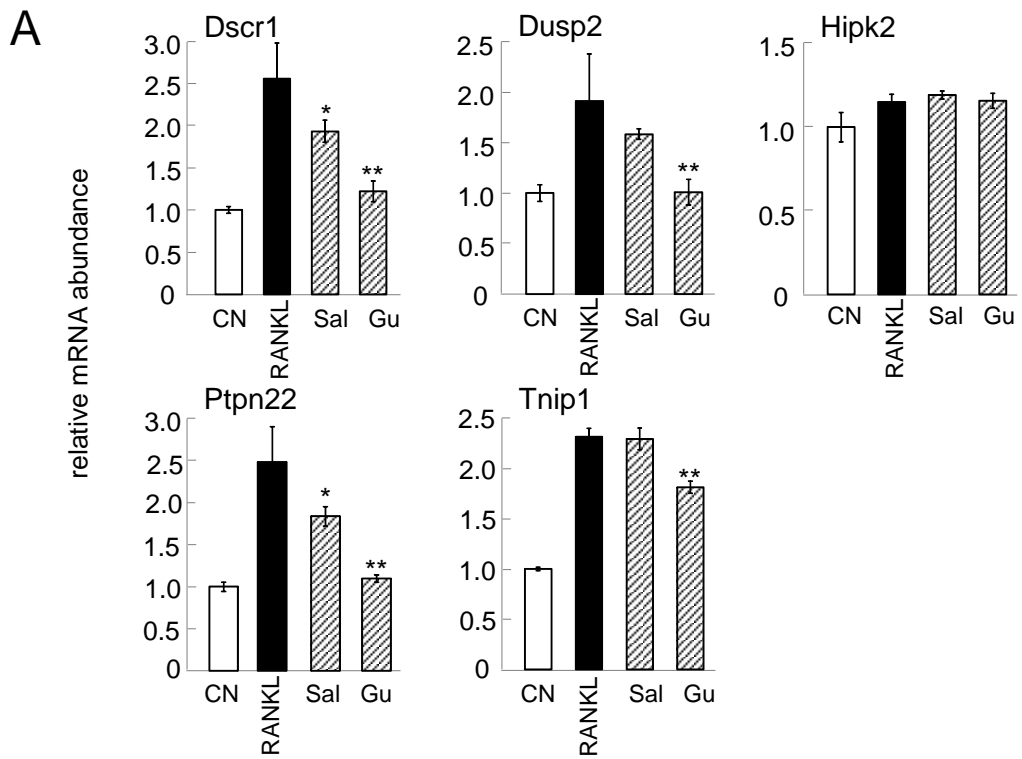


Figure 3

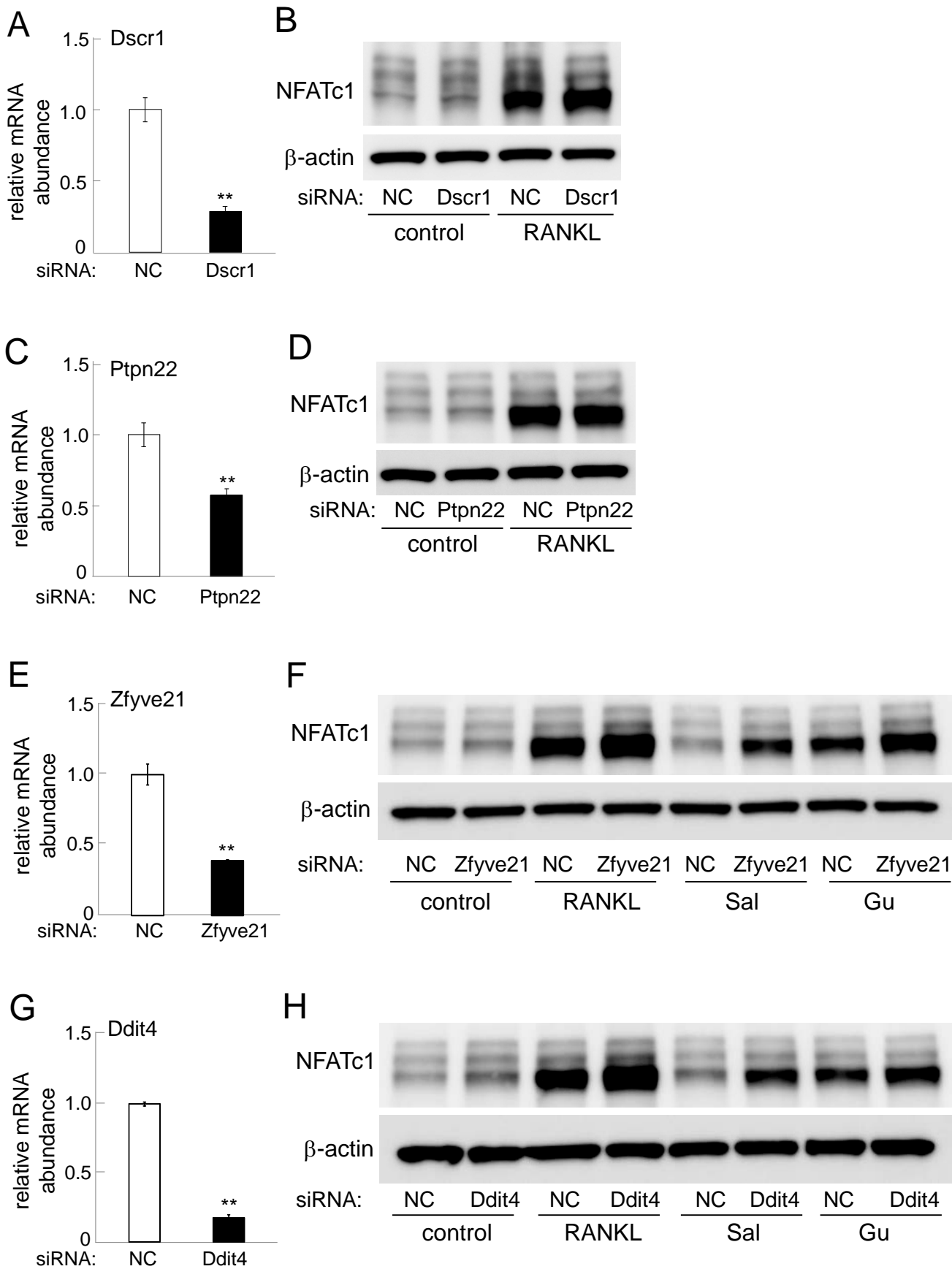


Figure 4



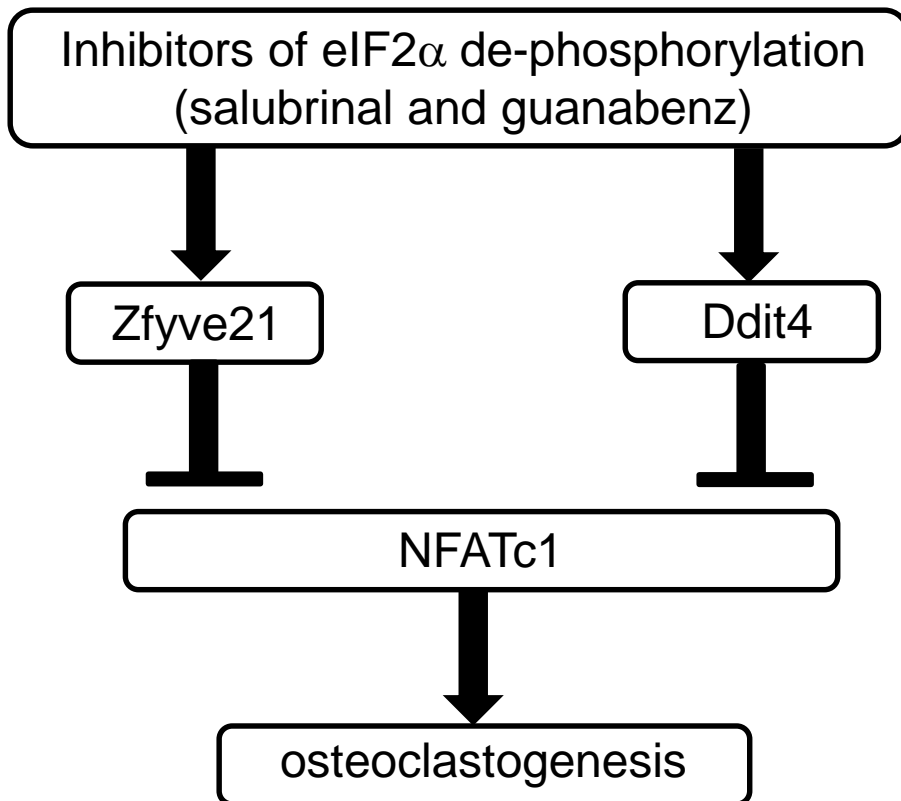


Figure 5