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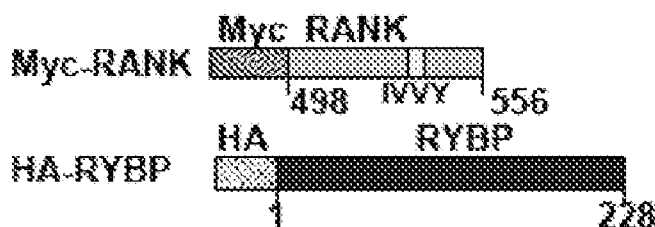


FIGURE 1C

(57) Abstract: The present invention provides a therapeutic composition, and method of use thereof, for improving bone mass, rigidity, or strength, or preventing and treating bone loss via modulation of the RANK signaling pathway. The therapeutic composition of the present invention comprises a RYBP peptide, or fragments thereof, that specifically interact with a motif of RANK to regulate osteoclastogenesis. The present invention further provides a composition, and method of use thereof, comprising a modulator that is capable of modulating the RYBP-RANK interaction, or modulating an effector in the RANK, signaling pathway through the RYBP-RANK interaction.



## COMPOSITIONS FOR IMPROVING BONE MASS

### CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims priority to U.S. Provisional Patent Application Serial No. 61/302,979 filed February 10, 2010, the entire contents of which  
5 are hereby incorporated by reference.

### GOVERNMENT SUPPORT STATEMENT

[0001] This invention was made with government support under Grant No. AR47830 awarded by National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS). The government has certain rights in the invention.

### 10 FIELD OF THE INVENTION

[0002] The present invention relates to the compositions and methods for improving bone mass, rigidity, or strength, or preventing and treating bone loss via RANK signaling pathway.

### BACKGROUND OF THE INVENTION

15 [0003] Osteoclasts, the sole bone-resorbing cell, not only play a pivotal role in skeletal development and maintenance but are also implicated in the pathogenesis of various bone disorders including postmenopausal osteoporosis and tumor bone metastasis (Teitelbaum, 2000; Raisz, 2005; Mundy, 2002). Osteoclasts are multinucleated giant cells that differentiate from mononuclear cells of the monocyte/macrophage lineage  
20 (Teitelbaum, 2000), thus involving both dramatic phenotypic changes and reprogramming of gene expression. Osteoclastogenesis requires two essential factors: the monocyte/macrophage-colony stimulating factor (M-CSF) and the receptor activator of NF- $\kappa$ B ligand (RANKL) (Suda et al., 1999; Teitelbaum, 2000; Boyle et al., 2003).

[0004] RANKL (also known as OPGL, ODF and TRANCE), a member of the  
25 tumor necrosis factor (TNF) family, was discovered independently by several groups (Anderson et al., 1997; Wong et al., 1997b; Lacey et al., 1998; Yasuda et al., 1998) in the late 1990s and thus far has been shown to regulate diverse physiological processes such as bone remodeling (Lacey et al., 1998; Yasuda et al., 1998), dendritic cell (DC) survival and

activation (Wong et al., 1997a; Josien et al., 1999; Josien et al., 2000), T-cell activation (Kong et al., 1999; Bachmann et al., 1999), lymph node organogenesis (Kong et al., 1999; Dougall et al., 1999; Kim et al., 2000a), B-cell differentiation (Kong et al., 1999; Dougall et al., 1999), mammary gland development (Fata et al., 2000), and thermoregulation in females or fever response inflammation (Hanada et al., 2009). RANKL regulates various biological functions by binding to and activating its receptor RANK (Hsu et al., 1999), which belongs to the TNF receptor (TNFR) family (Anderson et al., 1997). RANKL also has a decoy receptor, osteoprotegerin (OPG) (Simonet et al., 1997; Tsuda et al., 1997), which inhibits RANKL function by competing with RANK for binding RANKL (Yasuda et al., 1998; Lacey et al., 1998).

[0005] In bone, RANKL and RANK play important roles in osteoclastogenesis: Mice lacking either protein develop osteopetrosis due to failure to form osteoclasts (Kong et al., 1999; Dougall et al., 1999; Li et al., 2000; Kim et al., 2000b). Consistently, mice deficient for OPG develop early onset of osteoporosis due to elevated osteoclastogenesis (Bucay et al., 1998; Mizuno et al., 1998) whereas transgenic mice over-expressing OPG exhibit osteopetrosis, resulting from a decrease in late stages of osteoclastogenesis (Simonet et al., 1997).

[0006] The discovery of the RANKL/RANK/OPG axis was soon followed by an intensive investigation of RANK-activated intracellular signaling pathways involved in the regulation of the diverse functions. The initial efforts primarily focused on TNF receptor associated factor (TRAF)-dependent pathways since RANK was identified as a member of the TNF receptor (TNFR) family (Anderson et al., 1997) and members of the TNFR family, which lack intrinsic enzymatic activity, transduce intracellular signals by recruiting various TRAFs via specific motifs in the cytoplasmic domain (Locksley et al., 2001; Chung et al., 2002). Numerous biochemical and functional studies have established that RANK contains three functional TRAF-binding sites (PFQEP369-373, PVQEET559-564 and PVQEQG604-609) that redundantly play a role in osteoclast formation and function (Liu et al., 2004) (Liu et al., 2005; Hsu et al., 1999; Darnay et al., 1998; Wong et al., 1998; Kim et al., 1999; Darnay et al., 1999; Galibert et al., 1998). Collectively, through these functional TRAF-binding motifs, RANK activates six major signaling pathways NF- $\kappa$ B, JNK, ERK, p38, NFATc1 and Akt, which play important roles in

osteoclast formation, function and/or survival (Boyle et al., 2003; Liu et al., 2004; Feng, 2005).

[0007] On the other hand, several lines of evidence support that RANK may also activate a TRAF-independent signaling pathway(s) essential for osteoclastogenesis. It has been shown that TRAF6 acts as a key downstream signaling molecule for both RANK and IL-1R (Wu and Arron, 2003) and a single TRAF6-binding motif is able to promote osteoclastogenesis (Ye et al., 2002; Liu et al., 2004). However, administration of IL-1 to RANK-/- mice failed to induce any osteoclastogenesis *in vivo* (Li et al., 2000), indicating that an unidentified TRAF6-independent signaling pathway(s) is also required for osteoclastogenesis. Moreover, consistent with this *in vivo* finding, *in vitro* studies also demonstrated that IL-1 failed to stimulate osteoclastogenesis (Azuma et al., 2000; Kobayashi et al., 2000). Given that the TRAF independent signaling pathway(s) is most likely initiated by one or more motifs in the RANK cytoplasmic domain, a systematic structure/function study of the RANK cytoplasmic domain was carried out using a chimeric receptor approach (Xu et al., 2006). This study has led to an identification of a specific 4-a.a. RANK motif (IVVY535-538), which shares no homology with any of the known TRAF-binding sites but plays a crucial role in osteoclastogenesis by committing bone marrow macrophages (BMMs) to the osteoclast lineage (Xu et al., 2006). However, the precise molecular mechanism by which this RANK motif mediates the lineage commitment remains elusive.

[0008] RYBP (Ring1A and YY1 binding protein, also known as DEDAF and YEAF1, Genbank Accession No. BC080287) was initially identified as a protein interacting with the Polycomb group (PcG) proteins, Ring1A and M33, and the transcriptional factor YY1 in a two-hybrid screen and shown to mediate transcriptional repression in reporter assays (Garcia et al., 1999). It was later shown that RYBP also interacts with several members of the E2F family of transcription factors (Trimarchi et al., 2001; Schlisio et al., 2002), the transcriptional factor E4TF1/hGABP (Sawa et al., 2002) and ubiquitinated H2A (Arrigoni et al., 2006), a Ring1A/Ring1B-dependent chromatin mark associated with transcriptional repression (Li et al., 2007). RYBP knockout mice exhibited embryonic lethality, revealing its essential role in development (Pirity et al., 2005). In addition to embryonic lethality, either loss- or gain-of-function experiments revealed other developmental alterations including defects in neural tube closure and

formation of anterior eye structures (Pirity et al., 2005; Gonzalez et al., 2008). Intriguingly, RYBP was also identified in an independent two-hybrid screen as a protein interacting with death effector domain (DED)-containing proteins such as FADD, procaspase 8, and procaspase 10 and thus named differently as the death effector domain-associated factor (DEDAF) (Zheng et al., 2001). Moreover, RYBP has other interacting partners with roles in apoptosis, the viral apoptin protein and Hippi (Danen-van Oorschot et al., 2004; Stanton et al., 2007). Consistent with a proapoptotic function for RYBP, over-expression of RYBP in cell lines promote apoptosis (Zheng et al., 2001; Danen-van Oorschot et al., 2004). A recent study has revealed that RYBP interacts with MDM2 to alter the MDM2-p53 interaction, resulting in stabilization of p53, and thus may act as a tumor suppressor (Chen et al., 2009).

[0009] PcG proteins were originally identified in *Drosophila* as repressors of Hox genes, a family of transcription factors that control the anteroposterior segmentation of the fruitfly body (Schuettengruber et al., 2007). Homologues of *Drosophila* PcG proteins have subsequently been identified in vertebrates and plants and shown to be implicated in cell differentiation, stem cell identity, tumorigenesis and genomic imprinting (Schwartz and Pirrotta, 2008; Schwartz and Pirrotta, 2007; Kohler and Villar, 2008). PcG proteins form three major PcG complexes termed Polycomb repressive complexes (PRC) 1, PRC2 and PhoRC. The core components of the PRC1 complex include mammalian homologues of *Drosophila* Polycomb (PC), Posterior Sex Combs (PSC), Polyhomeotic (PH), and dRING. Specifically, RYBP interacts with Ring1A and Ring1B, two mammalian homologues of dRING, and M33 (also known as CBX2), a mammalian homologue of PC (Garcia et al., 1999; Gecz et al., 1995). The PRC2 complex primarily includes mammalian homologues of the E(Z) H3K27 methyltransferase, SU(Z)12, and Extra sex combs (ESC). PhoRC contains the mammalian transcription factor YY1, homologous to *Drosophila* Pleiohomeotic (PHO). The PRC2 complex is responsible for catalyzing the tri-methylation of lysine 27 on histone 3 (histone H3K27me3) in the PcG target genes, which is recognized by the PRC1 complex through the mammalian homologues of *Drosophila* PC (Cao and Zhang, 2004). Despite the ability of RYBP to associate with PcG proteins, the functional significance of the interaction in the regulation of PcG target genes is still unknown.

## SUMMARY OF THE INVENTION

[0010] The present invention provides a therapeutic composition for improving bone mass, rigidity, or strength, or preventing or treating bone loss caused by bone diseases, such as osteoporosis, bone erosion in rheumatoid arthritis, periodontal bone loss, and tumor (e.g., breast, prostate and multiple myeloma)-induced osteolysis (bone destruction) and bone metastasis, or other pathological conditions involving elevated osteoclast formation/activity, such as Paget disease and drug-induced bone loss. Such therapeutic composition comprises a therapeutically acceptable excipient and a peptide, or a fragment thereof, that specifically binds to a RANK fragment containing an IVVY (SEQ ID NO:4) motif so as to regulate osteoclastogenesis. In certain embodiments, the peptide derived from a portion or all of RYBP protein a protein interacting with components of the polycomb group (PcG) complexes (and also referred to by its accession number BC080287 in Genbank) is a RING1A and YY1 binding protein, encoded by a nucleic acid sequence as set forth in SEQ ID NO:1, and having at least a portion of the deduced amino acid sequence as set forth in SEQ ID NO:2 (Figure 8), or an analog thereof.

[0011] In certain embodiments, the fragment of the peptide comprises an amino acid sequence SRPRLKNVDRSTAQQLAVTVGNVTVIITDFKEKTRSSSTS (SEQ ID NO:3), or an analog or further fragment thereof, that specifically interacts with the IVVY (SEQ ID NO:4) motif of RANK. In certain embodiments, the fragment of the peptide comprises at least the four amino acid sequence VIIT (SEQ ID NO:5) or AVTV (SEQ ID NO:6), or an analog thereof, that specifically interacts with the IVVY (SEQ ID NO:4) motif of RANK.

[0012] The present invention further provides a method of improving bone mass, rigidity, or strength, or preventing or treating bone loss caused by bone diseases, such as osteoporosis, bone erosion in rheumatoid arthritis, periodontal bone loss, and tumor (e.g., breast, prostate and multiple myeloma)-induced osteolysis (bone destruction) and bone metastasis, or other pathological conditions involving elevated osteoclast formation/activity, such as Paget disease and drug-induced bone loss, comprising administering to a subject in need an effective amount of a therapeutic composition comprising a therapeutically acceptable excipient and RYBP, or a fragment thereof, that specifically interacts with the IVVY (SEQ ID NO:4) motif of RANK. In certain embodiments, the RYBP protein comprises the amino acid sequence as set forth in SEQ

ID NO:2 (Figure 8), or an analog thereof. In certain embodiments, the fragment of RYBP comprises an amino acid sequence as set forth in SEQ ID NO:3, or an analog or functional fragment thereof, that specifically interacts with the IVVY (SEQ ID NO:4) motif of RANK. In certain embodiments, the fragment of RYBP comprises at least the four amino acid sequence VIIT (SEQ ID NO:5) or AVTV (SEQ ID NO:6), or a natural or synthetic analog thereof, that specifically interacts with the IVVY (SEQ ID NO:4) motif of RANK.

[0013] The present invention also provides a therapeutic composition, or a method of regulating osteoclastogenesis thereof, comprising a small molecule, peptide, protein, drug, naturally-occurring, or chemically synthesized, known or later discovered, that is capable of modulating an interaction of RYBP, or a fragment thereof, with the IVVY (SEQ ID NO:4) motif of RANK, or modulating any downstream effectors in the RANK signaling pathway through the interaction of RYBP, or a fragment thereof, with the IVVY (SEQ ID NO:4) motif of RANK. The present invention provides that the IVVY (SEQ ID NO:4) motif of RANK engages RYBP, or a fragment thereof, to regulate osteoclastogenesis. The present invention provides that RYBP, or a fragment thereof, is a high affinity IVVY (SEQ ID NO:4) motif-binding protein functionally involved in osteoclastogenesis. RYBP is predominantly present in nuclei of osteoclast precursors. RANKL induces nucleocytoplasmic shuttling of RYBP in an IVVY (SEQ ID NO:4) motif dependent manner, leading to the activation of osteoclast genes. Therefore, RYBP, or a fragment thereof, is an important protein engaged in transmitting the IVVY (SEQ ID NO:4)-activated membrane-proximal signals to a mammalian cell nucleus to promote osteoclastogenesis via control of the expression of about 200 genes, including, but not limited to, osteoclast-related genes such as matrix metalloproteinase 9 (Mmp9), cathepsin K (Ctsk) and tartrate resistant acid phosphatase 5 (TRAP). Any drug, modulator, protein, peptide, small molecule, nucleic acid, siRNA, or oligonucleotide that is capable of modulating these gene or protein expressions via RYBP-RANK signalling is also within the scope of the present invention.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Figure 1 illustrates that RYBP interacts with the RANK IVVY (SEQ ID NO:4) Motif. (A) Schematic location of the RANK region used as bait in the two-hybrid screen. The mouse RANK is 625-a.a. long. The bait corresponds to a 59-a.a. RANK

cytoplasmic region (a.a. 498-556) which is highly conserved in a.a. sequence between the mouse and human and contains the IVVY (SEQ ID NO:4) (535-538) motif (Bejarano et al., 2005, Fig. 1 on page 1120 showing there are only two differences at residues 86 and 96 in the 226 a.a. RYBP protein and the two differences are located outside of the interacting region). TM: transmembrane domain; Ext: extracellular domain; Intr: intracellular domain. (B) Expression of RYBP during osteoclast differentiation. Primary BMMs were treated with MCSF (44ng/ml) and RANKL (100ng/ml) while macrophage-like RAW264.7 cells were stimulated RANKL (100ng/ml) alone to promote osteoclast formation. Cells were lysed at various time points for assessment of RYBP expression by Western blots with anti-RYBP antibody ( $\alpha$ -RYBP). Loading control was performed with anti- $\beta$ -actin antibody ( $\beta$ -actin). (C) Schematic structure of Myc-RANK and HA-RYBP. (D) RANK fragment 498-556 interacts with RYBP in mammalian cells. Myc-RANK, HA-RYBP and the two corresponding empty vectors (Myc-Vector and HA-Vector) were transiently transfected in different combinations into 293T cells. Cells were then lysed for co-immunoprecipitation assays (Co-IP) in which immunoprecipitation (IP) was performed with anti-HA antibody ( $\alpha$ -HA), followed by Western blots (WB) with anti-Myc antibody ( $\alpha$ -Myc) to assess the interaction between the RANK region and RYBP. The expression levels of the tagged proteins in transfected cells were determined by input WB with  $\alpha$ -HA,  $\alpha$ -RYBP and  $\alpha$ -Myc. (E) The interaction between RANK fragment 498-556 and RYBP was further demonstrated by performing reverse Co-IP assays, in which IP was performed with  $\alpha$ -HA, followed by WB with  $\alpha$ -Myc. (F) Myc-mRANK differs from Myc-RANK in that it contains inactivating mutations in the IVVY (SEQ ID NO:4) motif (IVVY (SEQ ID NO:4)  $\rightarrow$  IVAF (SEQ ID NO:7)). (G) The IVVY (SEQ ID NO:4) motif is specifically responsible for the interaction between RANK fragment 498-556 and RYBP. HA-RYBP was co-transfected with Myc-RANK or Myc-mRANK into 293T cells. IP was performed with  $\alpha$ -Myc, followed by WB with  $\alpha$ -HA. Input WB assays were performed as in (D).

[0015] Figure 2 provides identification of specific RYBP domains interacting with the RANK IVVY (SEQ ID NO:4) motif. (A) Schematic diagram of 11 deletion mutants (D1-D11) of HA-RYBP (WT). In each mutant construct, a region of around 20-aa was deleted. (B) Myc-RANK was co-transfected with HA-tagged WT or one mutant RYBP construct (HACo) as shown in left panel into 293T cells. IP was performed with  $\alpha$ -Myc, followed by WB with  $\alpha$ -HA. Input WB assays were performed as in Figure 1D. (C) Top

illustration depicts schematic location of the 40-a.a. RYBP region (deleted in D8 and D9) which was shown to be required for interacting with the RANK IVVY (SEQ ID NO:4) motif in (A). Bottom panel shows the sequence of the 40-a.a. region and schematic structure of 10 deletion mutants (S1-S10), each with 4 amino acid residues within the 40-a.a. region deleted. (D) Co-IP assays in (A) were repeated with S1-S10. (E) Location and sequence of two RYBP domains mediating the interaction with the RANK IVVY (SEQ ID NO:4) motif. Ext: extracellular domain; Intr: intracellular domain.

[0016] Figure 3 illustrates functional involvement of RYBP in osteoclast differentiation. (A) Schematic illustration of pPower-siRNA retroviral vector. LTR: long terminal repeat; CMV: the cytomegalovirus promoter; mU6: the murine U6 promoter; PGK: the phosphoglycerate kinase promoter; Hygro: hygromycin. (B) BMMs were uninfected (UN) or infected with retrovirus expressing scramble control siRNA (Control: 3'-UCUAGGAGGAUCACUUCGA-5' (SEQ ID NO:8)) or siRNA (3'-UCUAGGAGGAUCACUUCGA-5' (SEQ ID NO:9)) targeting RYBP for 24 hrs. 2 days after infection, cells were lysed for Western blot analysis with anti-RYBP antibody. Loading control was performed with anti- $\beta$ -actin antibody ( $\beta$ -actin). (C) Uninfected BMMs (UN) and BMMs infected with retrovirus expressing scramble control siRNA (Control) or siRNA targeting RYBP (siRNA) described in (B) were treated with M-CSF (44ng/ml) and RANKL (100ng/ml) for 4 days. The culture was stained for TRAP. Top row is the image of the TRAP-stained culture plate. Bottom row shows the high power view of a representative area from each treatment of the TRAP-stained culture. (D) Strategy for knocking down the endogenous RYBP by siRNA without affecting the expression of exogenous HA-tagged WT RYBP (RYBP-HA) or mutant RYBP (dRYBP-HA). The selected siRNA targets the RYBP mRNA region which encodes DPPSEA (SEQ ID NO:10). The same region of RYBP-HA and dRYBP-HA constructs were altered so that they produce mRNA which are significantly different from siRNA but encode the same amino acid sequence. (E) BMMs were uninfected (Uninfected) or infected with retrovirus expressing siRNA (siRNA) targeting RYBP for 24 hrs. In addition, BMMs infected with retrovirus expressing siRNA were co-infected with virus expressing RYBP-HA (siRNA/RYPB-HA) or dRYBP-HA (siRNA/dRYBP-HA) for 24 hrs. 2 days after infection, cells were lysed for Western blot analysis with anti-RYBP antibody or anti-HA antibody. Loading control was performed with anti- $\beta$ -actin antibody ( $\beta$ -actin). (F)

Uninfected BMMs (UN) and BMMs infected with various viruses (siRNA, siRNA/RYPB-HA or siRNA/dRYBP-HA) as described in (E) were treated with M-CSF (44ng/ml) and RANKL (100ng/ml) for 4 days. The culture was stained for TRAP. Top row is the image of the TRAPstained culture plate. Bottom row shows the high power view of a representative area from each treatment of the TRAP-stained culture.

[0017] Figure 4 illustrates that the IVVY (SEQ ID NO:4) motif mediates the RANKL-induced intracellular translocation of RYBP during osteoclastogenesis. (A) RANKL stimulates intracellular translocation of RYBP during osteoclastogenesis. BMMs were treated with M-CSF (44ng/ml) alone (Control) or with M-CSF (44ng/ml) and RANKL (100ng/ml) (RANKL Treatment) for 1d, 2d, 3 day or 4 days. Cells were then fixed and stained with DAPI or immunostained with anti-RYBP antibody. (B) BMMs isolated from TNFR1-/-2-/- mice were infected with virus encoding a chimeric receptor consisting of the TNFR (SEQ ID NO:11) external domain linked to the transmembrane and intracellular domains of mouse wild-type RANK (WT) or virus encoding a chimeric receptor consisting of the TNFR (SEQ ID NO:11) external domain linked to the transmembrane and intracellular domains of mouse RANK containing inactivating mutation in the IVVY (SEQ ID NO:4) motif (Mu). Infected cells were treated with M-CSF (44ng/ml) and TNF (10ng/ml) for 1hrs, 2 hrs, 4hrs, 8hrs, 1d and 2d. Cells were then fixed and stained with DAPI staining or immunofluorescence staining with anti-RYBP antibody.

[0018] Figure 5 illustrates that the IVVY (SEQ ID NO:4) motif regulates gene expression in response to RANKL treatment. (A) Heat map of microarray assays. BMMs isolated from TNFR1-/-2-/- mice were infected with virus encoding a chimeric receptor consisting of the TNFR external domain linked to the transmembrane and intracellular domains of mouse wild-type RANK (WT) or virus encoding a chimeric receptor consisting of the TNFR external domain linked to the transmembrane and intracellular domains of mouse RANK containing inactivating mutation in the IVVY (SEQ ID NO:4) motif (Mu). Infected cells were treated with M-CSF (44ng/ml) and TNF (10ng/ml) for 1d. Total RNA was isolated from the treated cells for microarray analysis. (B) Statistical analysis of the data obtained from the microarray assays. (C) The confirmation of data on the up-regulation of the Mmp9, Car2, Ctsk and TRAP genes by the IVVY (SEQ ID NO:4) motif using semi-quantitative RT-PCR. TNFR1-/-2-/- BMMs expressing the wild-type

chimera (WT) and mutant chimera (Mu) were treated with M-CSF (44ng/ml) and TNF- $\alpha$  (10ng/ml) for 1d. Cells were lysed for total RNA isolation immediately after the 1d RANKL treatment or continued with M-CSF (44ng/ml) alone for 2 or 4 additional days before total RNA preparation for semi-quantitative RT-PCR assessment of the expression of the four genes. (D) BMMs were treated with M-CSF (44ng/ml) and RANKL (100ng/ml) for 1d. As in (C), cells were either lysed for total RNA isolation immediately after the 1d RANKL treatment or continued with M-CSF (44ng/ml) alone for 2 or 4 additional days before total RNA preparation for semiquantitative RT-PCR assessment of the expression of the four genes.

10 [0019] Figure 6 illustrates sequence and location of RANK cDNA region encoding the bait for two-hybrid screening. Human and mouse RANK cytoplasmic domains are shown and compared. The three boxed sequences are functional TRAF-binding sites. The IVVY (SEQ ID NO:4) motif that is essential for osteoclastogenesis is indicated by a black bar on top of the sequence. The 49-aa segment containing the IVVY (SEQ ID NO:4) motif, which is used as bait for two-hybrid screen, is shown in bold and underlined.  
15 hRANK: human RANK; mRANK: mouse RANK.

[0020] Figure 7 illustrates amino acid sequence and location of regions deleted in D1-D11. Both mouse RYBP amino acid sequence and corresponding coding sequence are shown. Numbers on left indicate the location of the amino acid residues. The deleted region in each mutant is specified d by vertical lines. The specific residues deleted in these mutants are: residues 4-23 in D1; residues 24-43 in D2; residues 44-63 in D3; residues 64-83 in D4; residues 84-103 in D5; residues 104-123 in D6; residues 124-143 in D7; residues 144-163 in D8; residues 164-183 in D9; residues 184-203 in D10; residues 204-228 in D11.

25 [0021] Figure 8 illustrates/underlined location and sequence of effective siRNA target in full length mouse RYBP cDNA, of SEQ ID NO:1 and deduced amino acid sequence of SEQ ID NO:2.

[0022] Figure 9 illustrates that the IVVY (SEQ ID NO:4) motif mediates the RANKL-induced intracellular translocation of RYBP during osteoclastogenesis during the first 24 hours. BMMs were treated with M-CSF (44ng/ml) alone (Control) or with M-CSF  
30

(44ng/ml) and RANKL (100ng/ml) (RANKL Treatment) for 1, 2, 4, 8, 16 or 24 hours. Cells were then fixed and stained with DAPI or immunostained with anti-RYBP antibody.

[0023] Figure 10 illustrates how a chimeric receptor can be used to determine whether IVVY mediates RYBP intracellular translocation in osteoclast precursors. (A) As a member of the TNFR family, RANK is activated by RANKL-induced trimerization. (B) As a surrogate, TNF $\alpha$  can induce the trimerization of the chimeric receptor to activate the RANK signaling required for osteoclast formation, function and/or survival. In addition, since TNF $\alpha$  can also regulate osteoclast formation and function, this approach requires the use of osteoclast precursors from TNFR1&R2 double knockout (TNFR1<sup>-/-</sup>R2<sup>-/-</sup>) mice to prevent potential signaling from TNFR. (C) Ch-WT is a chimeric receptor system consisting of the external domain of TNFR1 linked to the transmembrane and intracellular domains of wild-type RANK, while Ch-Mu is a mutant chimeric receptor system consisting of the external domain of TNFR1 linked to the transmembrane and intracellular domains of RANK containing inactivating mutations in the IVVY motif. Thus, this chimeric receptor approach can be used as a tool to determine whether IVVY mediates RYBP intracellular translocation in osteoclast precursors.

#### DETAILED DESCRIPTION OF THE INVENTION

[0024] The present invention provides a pharmaceutically administrable therapeutic composition, and method of use thereof, for promoting, or inhibiting, RANK signalling required for osteoclast formation, function and/or survival. The compositions and methods can be used for improving bone mass, rigidity, or strength, or preventing or treating bone loss caused by bone diseases, such as osteoporosis, bone erosion in rheumatoid arthritis, periodontal bone loss, and tumor (e.g., breast, prostate and multiple myeloma)-induced osteolysis (bone destruction) and bone metastasis, or other pathological conditions involving elevated osteoclast formation/activity, such as Paget disease and drug-induced bone loss. Such therapeutic composition comprises a therapeutically acceptable excipient and a peptide, or a fragment thereof, that specifically binds to a RANK fragment containing a IVVY (SEQ ID NO:4) motif so as to regulate osteoclastogenesis.

[0025] In certain embodiments, the peptide is RYBP protein having an amino acid sequence as set forth in SEQ ID NO:2, or fragments or analogs thereof. In certain

embodiments, the fragment of the peptide has, consists of, or comprises an amino acid sequence SRPRLKNVDRSTAQQLAVTVGNVTVIITDFKEKTRSSSTS (SEQ ID NO:3), or fragments or analogs thereof, that specifically interacts with the IVVY (SEQ ID NO:4) motif of RANK. In certain embodiments, the fragment of the peptide has, consists of, or  
5 comprises at least the four amino acid sequence VIIT (SEQ ID NO:5) or AVTV (SEQ ID NO:6), or homologs or analogs thereof, that specifically interacts with the IVVY (SEQ ID NO:4) motif of RANK.

[0026] The present invention also provides a therapeutic composition, or a method of regulating osteoclastogenesis thereof, comprising a small molecule, peptide, protein,  
10 drug, naturally-occurring, or chemically synthesized, known or later discovered, that is capable of modulating an interaction of RYBP, or a fragment thereof, with the IVVY (SEQ ID NO:4) motif of RANK, or modulating any downstream effectors in the RANK signaling pathway through the interaction of RYBP, or a fragment thereof, with the IVVY (SEQ ID NO:4) motif of RANK. The invention further provides a valuable research tool  
15 for screening and identifying additional molecules which modulate the interaction of RYBP, or a fragment thereof such as provided in SEQ ID NOS: 5 and 6, with the RANK motif IVVY (SEQ ID NO:4).

[0027] The present invention also provides isolated nucleotides, homologs and analogs that encode RYBP, or portions thereof, as set forth in SEQ ID NO:2, SEQ ID  
20 NO:3; SEQ ID NO:5, or SEQ ID NO:6; or hybridize at highly stringent conditions to the nucleotide sequence encoding the amino acid sequence as shown in SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6, or portions thereof. Moreover, the present invention provides nucleotides, homologs and analogs that comprise the nucleotide sequences encoding the amino acid sequence shown in SEQ ID NO:2, SEQ ID NO:3, SEQ  
25 ID NO:5, SEQ ID NO:6, portions, or complements thereof.

[0028] As used herein, the sequences of these peptides generally comprise about 3 to about 100 amino acids residues. As used herein, an "amino acid residue" refers to any naturally occurring amino acid, any amino acid derivative or any amino acid mimetic known in the art. Accordingly, the peptides encompass amino acid sequences comprising  
30 at least one of the 20 common amino acids found in naturally occurring proteins, or at least one modified or unusual amino acid known in the art or later derived.

[0029] The present invention also provides peptide analogs of RYBP, or a fragment thereof. As used herein, the term “analog” refers to two proteins or peptides that have the same or similar function, but that have evolved separately in unrelated organisms, such as murine and human analogs of the protein referred to herein as RYBP.

5 As used herein, the term “analog” further refers to a structural derivative of a parent compound that often differs from it by a single element. As used herein, the term “analog” also refers to any peptide modifications known to the art, including but are not limited to changing the side chain of one or more amino acids or replacing one or more amino acid with any non-amino acids.

10 [0030] Based on the amino acid sequences of the present invention, any peptides and their analogs comprising such sequences can be made by any techniques known to those of skill in the art, including but are not limited to the recombinant expression through standard molecular biological techniques, the conventional peptide/protein purification and isolation methods, and/or the synthetic chemical synthesis methods. The  
15 nucleotide and peptide sequences corresponding to various genes may be found at computerized databases known to those of ordinary skill in the art, for instance, the National Center for Biotechnology Information's Genbank and GenPept databases. Alternatively, various commercial preparations of proteins and peptides are known to those of skill in the art.

20 [0031] Because the length of the peptides of the present invention is relatively short, peptides and analogs comprising the amino acid sequences of these peptide inserts can be chemically synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. Short peptide sequences, usually from  
25 about 3 up to about 100 amino acids, can be readily synthesized by such methods. Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes a peptide and its analog of the present invention is inserted into an expression vector, transformed or transfected into an appropriate host cell, and cultivated under conditions suitable for expression.

30 [0032] Peptide mimetics may also be used for preparation of the peptides and their analogs of the present invention. Mimetics are peptide-containing molecules that mimic elements of protein secondary structure (Johnson et al., BIOTECHNOLOGY AND

PHARMACY, Pezzuto et al., Eds., Chapman and Hall, latest edition, New York). A peptide mimetic is expected to permit molecular interactions similar to the natural molecule, and may be used to engineer second generation molecules having many of the natural properties of the peptides, but with altered and even improved characteristics.

5 [0033] The present invention also provides chimeric or fusion peptides that comprise the amino acid sequences of RYBP, or a fragment thereof, of the present invention, as disclosed herein. As used herein, a "chimeric or fusion peptide" comprises the amino acid sequence corresponding to RYBP (SEQ ID NO:2), or a fragment, e.g., SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:6, or analogs thereof, operatively linked,  
10 preferably at the N- or C-terminus, to all or a portion of a second peptide or protein. As used herein, "the second peptide or protein" refer to a peptide or protein having an amino acid sequence which is not substantially identical to RYBP, a fragment, or analogs thereof, e.g., a peptide or protein that is different from SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6, or analogs thereof, and is derived from the same or a different  
15 organism. With respect to the fusion peptide, the term "operatively linked" is intended to indicate that the amino acid of RYBP, a fragment, or analogs thereof, and the second peptide or protein are fused to each other so that both sequences fulfill the proposed function attributed to the sequence used.

[0034] For example, fusions may employ leader sequences from other species to  
20 permit the recombinant expression of a protein in a heterologous host. Another useful fusion includes the addition of an immunologically active domain, such as an antibody epitope, to facilitate purification of the fusion protein. Inclusion of a cleavage site at or near the fusion junction will facilitate removal of the extraneous polypeptide after purification. Other useful fusions include linking of functional domains, such as active  
25 sites from enzymes, glycosylation domains, cellular targeting signals or transmembrane regions. In certain embodiments, the fusion proteins of the present invention comprise the peptide and/or analog comprising amino acid sequences of RYBP, or a fragment thereof, that is linked to another protein or peptide. Examples of proteins or peptides that may be incorporated into a fusion protein include cytostatic proteins, cytotoxic proteins, pro-  
30 apoptosis agents, anti-angiogenic agents, hormones, cytokines, growth factors, peptide drugs, antibodies, Fab fragments antibodies, antigens, receptor proteins, enzymes, lectins, MHC proteins, cell adhesion proteins and binding proteins. These examples are not meant

to be limiting and it is contemplated that within the scope of the present invention virtually any protein or peptide could be incorporated into a fusion protein comprising the peptides and analogs of the present invention.

[0035] Methods of generating fusion peptides/proteins are well known to those of skill in the art. Such peptides/proteins can be produced, for example, by chemical attachment using bifunctional cross-linking reagents, by de novo synthesis of the complete fusion peptide/protein, or by standard recombinant DNA techniques that involve attachment of a DNA sequence encoding the peptides of present invention, as disclosed herein, to a DNA sequence encoding the second peptide or protein, followed by expression of the intact fusion peptide/protein using. For example, DNA fragments coding for the peptide sequences of RYBP, fragments, or analogs thereof are ligated together in-frame in accordance with conventional techniques, for example by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers.

[0036] Alternatively, PCR amplification of gene fragments can be carried out using anchor primers that give rise to complementary overhangs between two consecutive gene fragments that can subsequently be annealed and re-amplified to generate a chimeric gene sequence (See, for example, Current Protocols in Molecular Biology, Eds. Ausubel et al., latest edition, John Wiley & Sons). Moreover, many expression vectors are commercially available that already encode a fusion moiety (e.g., a GST polypeptide). The nucleic acids encoding RYBP, fragments, or analogs thereof can be cloned into such an expression vector such that the fusion moiety is linked in-frame to these nucleic acids encoding peptides of RYBP, fragments, or analogs thereof.

[0037] In certain embodiments the peptides and analogs of the present invention may be isolated or purified. Protein purification techniques are well known in the art. These techniques involve, at one level, the homogenization and crude fractionation of the cells, tissue or organ to peptide and non-peptide fractions. The peptide/protein of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography,

gel exclusion chromatography, polyacrylamide gel electrophoresis, affinity chromatography, immunoaffinity chromatography and isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography (FPLC) or even HPLC.

- 5    **[0038]**       An isolated peptide is intended to refer to a peptide/protein that is purified to any degree relative to its naturally-occurring state. Therefore, an isolated or purified peptide refers to a peptide free from at least some of the environment in which it may naturally occur. Generally, "purified" will refer to a peptide composition that has been subjected to fractionation to remove various other components, and which composition
- 10   substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, or more of the peptides in the composition.
- 15   **[0039]**       Various methods for quantifying the degree of purification of the peptide are known in the art. These include, for example, determining the specific activity of an active fraction, or assessing the amount of peptides within a fraction by SDS/PAGE analysis. Various techniques suitable for use in peptide/protein purification are well known to those of skill in the art. These include, for example, precipitation with
- 20   ammonium sulphate, PEG, antibodies and the like, or by heat denaturation, followed by: centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of these and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain
- 25   steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

- [0040]**       There is no general requirement that the peptides, fragments, and their analogs always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification
- 30   may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or

in maintaining the activity of an expressed protein. The invention contemplates compositions comprising the peptides and a pharmaceutically acceptable carrier or excipients.

5 [0041] In certain embodiments, the RYBP peptide, fragments, and analogs thereof, of the present invention may be attached to imaging agents including but are not limited to fluorescent, and/or radioisotopes including but are not limited to <sup>125</sup>I, for imaging, diagnosis and/or therapeutic purposes. Many appropriate imaging agents and radioisotopes are known in the art, as are methods for their attachment to the peptides.

10 [0042] The present invention also provides isolated nucleic acids/nucleotides, homologs and analogs that comprise the nucleotide sequences encoding the RYBP amino acid sequence, fragments, or analogs thereof. As used herein, the "nucleic acids/nucleotides" may be derived from genomic DNA, complementary DNA (cDNA) or synthetic DNA. The term "nucleic acid/nucleotide" also refer to RNA or DNA that is linear or branched, single or double stranded, chemically modified, or a RNA/DNA hybrid  
15 thereof. It is contemplated that a nucleic acid within the scope of the present invention may comprise 10-150 or more nucleotide residues in length. Where incorporation into an expression vector is desired, the nucleic acid may also comprise a natural intron or an intron derived from another gene. Less common bases, such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine, and others can also be used.

20 [0043] An "isolated" nucleic acid molecule is one that is substantially separated from other nucleic acid molecules which are present in the natural source of the nucleic acid (i.e., sequences encoding other polypeptides). Preferably, an "isolated" nucleic acid is free of some of the sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in its naturally occurring replicon. For  
25 example, a cloned nucleic acid is considered isolated. A nucleic acid is also considered isolated if it has been altered by human intervention, or placed in a locus or location that is not its natural site, or if it is introduced into a cell by agroinfection. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be free from some of the other cellular material with which it is naturally associated, or culture medium when  
30 produced by recombinant techniques, or chemical precursors or other chemicals when chemically synthesized.

[0044] As used herein, "homologs" are defined herein as two nucleic acids or peptides that have similar, or substantially identical, nucleic acids or amino acid sequences, respectively. The term "homolog" further encompasses nucleic acid molecules that differ from one of the nucleotide sequences due to degeneracy of the genetic code and thus encodes the same amino acid sequences. In one of the preferred embodiments, homologs include allelic variants, orthologs, paralogs, agonists, and antagonists of nucleic acids encoding RYBP (SEQ ID NO:2), fragments, e.g., SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:6, or analogs thereof, as defined hereafter.

[0045] As stated above, the present invention includes RYBP (SEQ ID NO:2), or fragments (e.g., SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:6), and which are intended to include functional equivalents such as homologs and analogs thereof. To determine the percent sequence identity of two amino acid sequences (e.g., one of the sequences of SEQ ID NO: 2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6, and a mutant form thereof), the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of one polypeptide for optimal alignment with the other polypeptide or nucleic acid). The amino acid residues at corresponding amino acid positions are then compared. When a position in one sequence is occupied by the same amino acid residue as the corresponding position in the other sequence, then the molecules are identical at that position. The same type of comparison can be made between two nucleic acid sequences.

[0046] The percent sequence identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., percent sequence identity numbers of identical positions/total numbers of positions x100). Preferably, the isolated amino acid homologs included in the present invention are at least about 50-60%, preferably at least about 60-70%, and more preferably at least about 70-75%, 75-80%, 80-85%, 85-90%, or 90-95%, and most preferably at least about 96%, 97%, 98%, 99%, or more identical to an entire amino acid sequence of RYBP (SEQ ID NO:2) or fragments (e.g., SEQ ID NOs:3, 5, or 6). In certain embodiments, the amino acid homologs have sequence identity over at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, or more contiguous amino acid residues of the sequence disclosed herein including SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:6.

[0047] In other embodiments, an isolated nucleic acid homolog of the invention comprises a nucleotide sequence which is at least about 40-60%, preferably at least about

60-70%, more preferably at least about 70-75%, 75-80%, 80-85%, 85-90%, or 90-95%, and even more preferably at least about 95%, 96%, 97%, 98%, 99%, or more identical to a nucleotide sequence encoding amino acid sequences disclosed herein including SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:6.

- 5 [0048] The determination of the percent sequence identity between two nucleic acid or peptide sequences is well known in the art. For instance, the Vector NTI 6.0 (PC) software package (InforMax, 7600 Wisconsin Ave., Bethesda, Md. 20814) to determine the percent sequence identity between two nucleic acid or peptide sequences can be used. In this method, a gap opening penalty of 15 and a gap extension penalty of 6.66 are used  
10 for determining the percent identity of two nucleic acids. A gap opening penalty of 10 and a gap extension penalty of 0.1 are used for determining the percent identity of two polypeptides. All other parameters are set at the default settings. For purposes of a multiple alignment (Clustal W algorithm), the gap opening penalty is 10, and the gap extension penalty is 0.05 with blosum62 matrix. It is to be understood that for the purposes  
15 of determining sequence identity when comparing a DNA sequence to an RNA sequence, a thymidine nucleotide is equivalent to a uracil nucleotide.

- [0049] In another aspect, the present invention provides an isolated nucleic acid comprising a nucleotide sequence that hybridizes to the nucleotides encoding the amino acid sequence of RYBP (SEQ ID NO:2), fragments (e.g., SEQ ID NOs:3, 5, or 6),  
20 homologs, or analogs thereof, respectively under stringent conditions. As used herein with regard to hybridization for DNA to a DNA blot, the term "stringent conditions" refers to hybridization overnight at 60°C in 10xDenhart's solution, 6xSSC, 0.5% SDS, and 100µg/ml denatured salmon sperm DNA. Blots are washed sequentially at 62°C for 30 minutes each time in 3xSSC/0.1% SDS, followed by 1xSSC/0.1% SDS, and finally  
25 0.1xSSC/0.1% SDS. As also used herein, in certain embodiments, the phrase "stringent conditions" refers to hybridization in a 6xSSC solution at 65°C. In another embodiment, "highly stringent conditions" refers to hybridization overnight at 65°C in 10x Denhart's solution, 6xSSC, 0.5% SDS and 100µg/ml denatured salmon sperm DNA. Blots are washed sequentially at 65°C for 30 minutes each time in 3xSSC/0.1% SDS, followed by  
30 1xSSC/0.1% SDS, and finally 0.1xSSC/0.1% SDS. Methods for nucleic acid hybridizations are described in Meinkoth and Wahl, Anal. Biochem. 138:267-284; Current Protocols in Molecular Biology, Chapter 2, Ausubel et al., eds., latest edition, Greene

Publishing and Wiley-Interscience, New York; and Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology: Hybridization with Nucleic Acid Probes, Part 1, Chapter 2, latest edition, Elsevier, New York.

[0050] Using the above-described methods, and others known to those of skill in the art, one of ordinary skill in the art can isolate homologs of RYBP peptide, fragments thereof, comprising amino acid sequence shown in SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:6. One subset of these homologs are allelic variants. As used herein, the term “allelic variant” refers to a nucleotide sequence containing polymorphisms that lead to changes in the amino acid sequences of RYBP (SEQ ID NO:2) or fragments (e.g., SEQ ID NOs:3, 5, or 6) thereof without altering the functional activities. Such allelic variations can typically result in 1-5% variance in nucleic acids encoding RYBP, or fragments thereof.

[0051] In addition, the skilled artisan will further appreciate that changes can be introduced by mutation into a nucleotide sequence that encodes the amino acid sequence of RYBP (SEQ ID NO:2), fragments (e.g., SEQ ID NOs: 3, 5, or 6), or analogs thereof. For example, nucleotide substitutions leading to amino acid substitutions at “non-essential” amino acid residues can be made in a sequence encoding the amino acid sequence of RYBP (SEQ ID NO:2), fragments (e.g., SEQ ID NOs: 3, 5, or 6), or analogs thereof. A “non-essential” amino acid residue is a residue that can be altered without altering the activity of said peptide, whereas an “essential” amino acid residue is required for desired activity of such peptide.

[0052] Accordingly, another aspect of the invention pertains to nucleic acid molecules encoding RYBP peptide, fragments or analogs thereof, that contain changes in amino acid residues that are not essential for the peptide activity. In one embodiment, the isolated nucleic acid molecule comprises a nucleotide sequence encoding peptide, wherein the peptide comprises an amino acid sequence at least about 50%, 60-70%, 70-75%, 75-80%, 80-85%, 85-90%, 90-95%, or 95-100% identical to an amino acid sequence, such as the sequence of RYBP (SEQ ID NO:2) or fragments (e.g., SEQ ID NOs: 3, 5, or 6) thereof.

[0053] An isolated nucleic acid molecule encoding a peptide having sequence identity with an amino acid sequence of RYBP (SEQ ID NO:2) or fragments (e.g., SEQ

ID NOs: 3, 5, or 6) thereof can be created by introducing one or more nucleotide substitutions, additions, or deletions into a nucleotide encoding such peptide sequences, respectively, such that one or more amino acid substitutions, additions, or deletions are introduced into the encoded peptide and/or the side chain of the amino acids constituting the encoded peptides. Mutations can be introduced into the nucleic acid sequence encoding RYBP (SEQ ID NO:2) or fragments (e.g., SEQ ID NOs: 3, 5, or 6) thereof by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain.

[0054] Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine), and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in RYBP peptide or fragments thereof is preferably replaced with another amino acid residue from the same side chain family. Alternatively, in another embodiment, mutations can be introduced randomly along all or part of the RYBP peptide sequence or fragments thereof, such as by saturation mutagenesis, and the resultant mutants can be screened for RANK protein interactions. Following mutagenesis of the nucleic acid sequence encoding the RYBP peptide sequence or fragments thereof, the encoded peptide can be expressed recombinantly and the RANK binding activity of the peptide can be determined.

[0055] The nucleotides of the present invention may be produced by any means, including genomic preparations, cDNA preparations, in vitro synthesis, RT-PCR, and in vitro or in vivo transcription. It is contemplated that the peptides, their variations and mutations, or fusion peptides/proteins may be encoded by any nucleic acid sequence that encodes the appropriate amino acid sequence. The design and production of nucleic acids encoding a desired amino acid sequence is well known to those of skill in the art based on standardized codons. In preferred embodiments, the codons selected for encoding each

amino acid may be modified to optimize expression of the nucleic acid in the host cell of interest. Codon preferences for various species of host cell are well known in the art.

[0056] The present invention further provides a therapeutic composition, or a method of regulating osteoclastogenesis thereof, comprising a pharmaceutically or therapeutically acceptable excipients, and a modulator, small molecule, peptide, protein, drug, known or later discovered, that is capable of modulating an interaction of RYBP, or a fragment thereof, with the IVVY (SEQ ID NO:4) motif of RANK, or modulating any downstream effectors in the RANK signaling pathway through the interaction of RYBP, or a fragment thereof, with the IVVY (SEQ ID NO:4) motif of RANK. As used herein, the term "modulator" refers to any naturally-occurred or chemically synthesized, known or later discovered, compounds, small molecules, peptides, proteins, enzymes, and drugs.

[0057] In certain embodiments, the pharmaceutically or therapeutically acceptable excipients, carriers or vehicles in the composition of the present invention comprise any acceptable materials, and/or any one or more additives known in the art. Furthermore, the composition of the present invention can also be made in any suitable solutions and/or formulations for oral, parenteral, transdermal or transmucosal administrations.

[0058] The present invention may be understood more readily by reference to the following detailed description of the preferred embodiments of the invention and the Examples included herein. However, before the present peptides, compounds, compositions, and methods are disclosed and described, it is to be understood that this invention is not limited to specific nucleic acids, specific peptides or proteins, specific cell types, specific host cells, specific conditions, or specific methods, etc., as such may, of course, vary, and the numerous modifications and variations therein will be apparent to those skilled in the art. It is also to be understood that the terminology used herein is for the purpose of describing specific embodiments only and is not intended to be limiting. It is also to be understood that as used in the specification and in the claims, "a" or "an" can mean one or more, depending upon the context in which it is used. Thus, for example, reference to "a cell" can mean that at least one cell can be utilized.

[0059] Throughout this application, various publications are referenced. The disclosures of all of these publications and those references cited within those publications

in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

[0060] It should also be understood that the foregoing relates to preferred embodiments of the present invention and that numerous changes may be made therein without departing from the scope of the invention. The invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof, which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

#### EXAMPLES

##### *Cell lines, Mice and Preparation of Primary BMMs*

[0061] 293T and RAW264.7 cells were obtained from The American Type Culture Collection (ATCC). 293GPG and Plat-E retroviral packaging cells were kindly provided by Dr Daniel Ory (Washington University) and Dr Toshio Kitamura (University of Tokyo), respectively. C57BL/6 and TNFR1-/-R2-/- double KO mice were purchased from Harlan Industries (Indianapolis, IN) and The Jackson Laboratory (Bar Harbor, ME), respectively. Animals were bred and maintained in accordance with the regulations of the UAB institutional animal care and use committee. BMMs were isolated from long bones of 4-8 week old C57BL/6 or TNFR1-/-R2-/- mice as previously described (Feng et al., 2001).

##### *Construction of an Osteoclast Precursor cDNA Library*

[0062] An osteoclast precursor cDNA library was constructed using CloneMiner™ cDNA Library Construction System from Invitrogen (Carlsbad, CA). Briefly, mRNA was isolated from mouse primary BMMs and used as template for cDNA synthesis. Synthesized cDNA was size fractionated by column chromatography and the desired size of cDNA (>500bp) was pooled and cloned into pDONOR™222 vector with BP Clonase™ to make Osteoclast Precursor cDNA Entry Library. pGBKT7 AD Vector of MATCHMAKER Two-Hybrid System 3 (Invitrogen) was converted to a Gateway® destination vector using Gateway® Vector Conversion System (Invitrogen). The whole set

of cDNA clones was then transferred from the Osteoclast Precursor cDNA Entry Library into the modified pGBKT7 AD Vector to prepare an Osteoclast Precursor Two-Hybrid cDNA Library.

*Two-Hybrid Screen*

- 5 [0063] A 49-aa RANK segment (residues 498-546) containing the IVVY motif (residues 535-538) was used as bait in the two-hybrid screening. A mouse cDNA region encoding this 49-aa RANK fragment was cloned in frame into pGBKT7 DNA-BD Vector of the MATCHMAKER Two-Hybrid System 3 (Invitrogen). The resulting plasmid was named as pGBKT7 DNA-BD-RANK. 8ml of competent yeast cells (strain AH109),  
10 freshly prepared according to the protocol in the instruction manual, was combined with a freshly prepared DNA mixture containing 500µg of pGBKT7 DNA-BD-RANK, 250µg of the Osteoclast Precursor Two-Hybrid cDNA Library and 20mg of herring testes carrier DNA. The yeast-DNA complex was thoroughly mixed by vortexing and subsequent transformation steps were performed following the instructions in the manual. AH109  
15 transformants were selected under medium stringency condition by plating the transformation mixtures on the medium-stringency medium (SD/-His/-Leu/-Trp medium) in 150-mm plates. The plates were incubated upside down at 30°C until blue colonies appeared and/or grown big enough for further analysis. DNA was isolated from blue yeast colonies for transformation into E. Coli. DNA was then isolated from a large number of  
20 bacterial colonies derived from each blue yeast colony and sequenced to reveal the identity of the gene(s) encoding the potential RANK interacting partners.

*Construction of Deletion Mutants and Site-directed Mutagenesis*

- [0064] Construction of internal deletion and site-directed mutagenesis were performed using the QuickChange™ Site-directed Mutagenesis Kit (Stratagene). The  
25 deleted regions and point mutations were confirmed by sequencing and other regions in the cDNA were sequenced to confirm that no mutations were introduced by PCR amplification during the experimental manipulation.

*Co-Immunoprecipitation Assays*

- [0065] 293T cells were cultured in DMEM containing 10% heat-inactivated fetal bovine serum (HI-FBS) and penicillin/streptomycin (culturing medium). Cells grown near  
30 confluence were lifted and replated at density of  $5 \times 10^6$  cells/dish in 100-mm tissue

culture dishes the day before transfection. Total 8µg plasmid was transfected into 293T cells using Lipofectamine<sup>TM</sup> in combination of Plus<sup>®</sup> Reagent (Invitrogen). Briefly, 8µg plasmid was mixed with 20µl Plus<sup>®</sup> Reagent before addition of 350µl DMEM. The mixture was incubated at RT for 15min and then added with 380µl freshly prepared

5 Lipofectamine<sup>TM</sup> Reagent (Lipofectamine<sup>TM</sup> Reagent + 350µl DMEM). The complete transfection solution was further incubated at RT for 15min before being added to one dish of 293T cells which was washed with DMEM twice immediately before the addition of the solution. The cells were incubated at 37°C for 3hrs before addition of 6.5ml culturing medium and the incubation continued overnight. Next day, the medium containing the

10 DNA/transfection reagents was replaced with fresh culturing medium. The transfected cells were cultured for one more day before lysis with IP Lysis Buffer (0.5M EDTA, 1M Tris-HCl pH8.0, 5M NaCl, 10% NP-40 and 10% Glycerol) for Co-IP assays. 30µg protein from each lysate was mixed and then incubated with 3µg anti-HA antibody or anti-Myc antibody from Clontech Laboratories, Inc (Mountain View, CA) at 4°C on a rotating apparatus overnight. Next day, 50µl of Protein-G beads, which was freshly washed with IP

15 Buffer and then resuspended with IP Buffer (1:1 ratio), was added to the mixture and the incubation continued at 4°C on a rotating apparatus for 2 hrs. Then, the beads were spun down and washed with IP buffer 5 times. Every time, the beads resuspended in IP buffer were incubated at 4°C on a rotating apparatus for 15 min before being spun down again.

20 After the final washing, supernatant was removed and the beads were resuspended with 35µl of SDS Sample Buffer from Cell Signaling Technology (Danvers, MA). The samples were boiled for 5 min for Western blot analysis as described below.

#### *Western Blot Analysis*

[0066] Western blot analysis was performed as previously described (Xu et al., 2006; Liu et al., 2004). RAW264.7 cells cultured in DMEM containing 10% HI-FBS and penicillin/streptomycin were treated with RANKL (100ng/ml) for various times before being lysed for Western blot analysis with anti-RYBP antibody from Abcam Inc (Cambridge, MA). Primary BMMs were normally cultured in  $\alpha$ -MEM containing 10% HI-FBS and penicillin/streptomycin in the presence of M-CSF (44ng/ml). Uninfected BMMs

25 or BMMs infected with retrovirus encoding siRNA and/or tagged-RYBP were cultured in the presence of M-CSF (44ng/ml) with or without RANKL (100ng/ml) for various times and then lysed for Western blot analysis. For Western blot analysis of IP complex, all 35µl

30

of the eluted proteins from each IP assay was loaded for Western blot analysis with anti-HA antibody (Clontech Laboratories, Inc), anti-Myc antibody (Clontech Laboratories, Inc), anti-RYBP (Abcam Inc) antibody and anti- $\beta$ -actin antibody (Santa Cruz Biotechnology).

#### 5 *In vitro* Osteoclastogenesis Assays

[0067] Primary BMMs were cultured in 24-well tissue culture plates (5×10<sup>4</sup> cells/well) with  $\alpha$ -MEM containing 10% heat-inactivated FBS in the presence of 0.02 volume of culture supernatant of M-CSF-producing cells (final M-CSF concentration 44 ng/ml) (Takeshita et al., 2000) and 100ng of GST-RANKL (Lam et al., 2000). Osteoclasts  
10 began to form on day 3 and cultures were stained for TRAP activity on day 5 using a commercial kit (Sigma, 387-A).

#### *Retroviral Infection of primary BMMs*

[0068] 293GPG cells were cultured in DMEM with 10% HI-FBS supplemented with tetracycline, puromycin, G418 and penicillin/streptomycin as previously described  
15 {2222}. Plat-E cells were cultured in DMEM with 10% HI-FBS supplemented with puromycin, blasticidin and penicillin/streptomycin as previously described {Morita, 2000 3155 /id}. The retroviral vectors were transiently transfected into 293GPG cells or Plat-E cells using Lipofectamine Plus Reagent (Invitrogen). Virus supernatant was collected at 48, 72 and 96 h after transfection. Cells were then infected with virus for 24 hours in the  
20 presence of M-CSF (220ng/ml) and 8  $\mu$ g/ml polybrene. Cells were further cultured in the presence of M-CSF (220ng/ml) and 2  $\mu$ g/ml puromycin for selection and expansion of transduced cells. Selected cells were subsequently used for various studies.

#### *Immunofluorescence Assays*

[0069] 1×10<sup>5</sup> BMMs were plated on polylysine coated coverslips in 6-well plates  
25 and the cultures were treated with M-CSF (44 ng/ml) alone or M-CSF (44 ng/ml) plus RANKL (100 ng/ml) for various times. TNFR1-/-R2-/- BMMs infected with virus encoding chimeric receptors were treated with MCSF (44 ng/ml) alone or M-CSF (44 ng/ml) plus TNF- $\alpha$  (10 ng/ml) for various times. Cells were fixed with 3% formaldehyde solution in PBS for 45 min at RT, and treated with 0.5% Triton X-100 in PBS for 2 min,  
30 and blocked with 1% BSA for 30 min at RT. Cells were then incubated with a Rabbit anti-RYBP polyclonal antibody from Abcam Inc (Cat# ab5976, 1:250 dilution in Blocking

Buffer) for 1 hrs at RT, washed with PBS for 5 min (3 times) and blocked again with 1% BSA for 30 min at RT. Cells were further incubated with the Goat polyclonal to Rabbit IgG-H&L (FITC) second antibody from Abcam Inc (Cat# ab6717, 1:500 dilution in Blocking Buffer). Coverslips were washed with PBS for 5 min (3 times) and mounted  
5 with VECTASHIELD Mounting Medium with DAPI with DAPI (Cat# H-1200) from Vector Laboratories (Burlingame, CA). Coverslips were then subjected to confocal imaging using Leica DMIRBE Inverted UV SP1 Confocal Microscope System with Leica Confocal Software at UAB Imaging facility. Recombinant mouse TNF $\alpha$  (410-TRNC-050) was from R&D Systems (Minneapolis, MN).

#### 10 *Microarray Analysis*

[0070] BMMs expressing Ch-WT or Ch-IVVY were plated in six 60-mm tissue culture dishes and treated with M-CSF (44ng/ml) and TNF $\alpha$  (10ng/ml) for 24 hours. Total RNA was isolated from the six dishes and pooled. The RNA sample preparation was repeated independently two more times. Three sets of total RNA samples prepared from  
15 three independent assays were subject to microarray analysis using Mouse Genome 430 2.0 Array at the UAB Microarray Shared Facility. The raw data sets were obtained through GeneChip Operating Software (GCOS, Affymetrix, CA, USA) and loaded into the ArrayAssist and GeneSpring WG software (Agilent Technologies, CA, USA) for background subtraction and normalization by GC-robust multichip analysis (RMA) (Wu et  
20 al., 2004). The p-values were obtained by an unpaired t-test assuming unequal variance. Various gene lists with different fold changes or functions criteria were further filtered by minimum intensity and by presence/absence (P/A) calls. The resulting lists were used to generate heatmap and clusters through hierarchical clustering methods.

#### *Semi-quantitative RT-PCR*

25 [0071] Primary BMMs were treated with M-CSF (44ng/ml) and RANKL (100ng/ml) for 24 hours. Cells were either lysed for total RNA isolation immediately after the 24-hrs treatment or were further cultured with lysed M-CSF (44ng/ml) for 2 or 4 more days before lysis. Primary BMMs expressing Ch-WT or Ch-Mu were treated with M-CSF (44ng/ml) and TNF (10ng/ml) for 24 hours. Similarly, cells were either lysed for total  
30 RNA isolation immediately after the 24-hrs treatment or were further cultured with lysed M-CSF (44ng/ml) for 2 or 4 more days before lysis. Reverse transcription was performed

with reverse transcriptase from Invitrogen using 1µg total RNA. 1/30 of the reverse transcription reaction mixture was used to perform PCR to assess the levels of mRNA for Mmp9, Ctsk, TRAP and Car2 genes.

*Identification and Characterization of RYBP as a Protein Interacting with the RANK IVVY Motif*

[0072] The RANK IVVY (SEQ ID NO:4) motif plays a pivotal role in mediating the osteoclast lineage commitment by activating an essential downstream signaling pathway(s) through recruiting a signaling molecule (Xu et al., 2006). To identify the signaling protein, the two-hybrid screen was performed. In order to ensure success in obtaining the novel protein, it is critical to use an osteoclast precursor cDNA library for two-hybrid screening. Given that no such library was commercially available, an osteoclast precursor cDNA library was constructed using CloneMiner™ cDNA Library Construction System from Invitrogen (Carlsbad, CA). A mouse cDNA region encoding a 49-aa RANK fragment (residues 498-546), which contains the RANK motif (IVVY, SEQ ID NO:4, 535-538), was cloned into pGBKT7 DNA-BD Vector of MATCHMAKER Two-Hybrid System 3 as bait for two-hybrid screening (Figure 1A) (Figure 6). Three rounds of screening (a total of  $3.6 \times 10^6$  independent clones) of the osteoclast precursor cDNA library yielded 14 positive clones encoding proteins capable of interacting with the RANK motif in yeast. Additional assays involving transformation of the bait construct and each of the isolated clones back into yeast revealed that 3 out of the 14 clones were false positives. The remaining 11 clones were also sequenced, revealing that several clones encode the same proteins (Table 1).

Table 1. Prioritization of Candidate Proteins Encoded by Clones from Two-Hybrid Screening

| Clone Name         | Clones became blue on the same day colony formation |      |      |      |      |      |                                      |      | Clones became blue one day after colony formation |                                          |                                      |
|--------------------|-----------------------------------------------------|------|------|------|------|------|--------------------------------------|------|---------------------------------------------------|------------------------------------------|--------------------------------------|
|                    | S1-1                                                | S1-2 | S1-3 | S2-1 | S3-1 | S3-2 | S1-4                                 | S3-3 | S1-5                                              | S1-6                                     | S3-4                                 |
| Candidate Proteins | GB: BC080287<br>Ring1 and YY1 bind Protein (RYBP)   |      |      |      |      |      | GB: BC050102<br>ATP-binding Cassette |      | GB: AC120837<br>A novel gene in Chromosome 11     | GB: BC006220<br>E2F Transcription Factor | GB: BC006722<br>Heat Shock Protein 8 |
| Prioritized Order  | #1                                                  |      |      |      |      |      | #2                                   |      | #3                                                | #4                                       | #5                                   |

[0073] Moreover, some clones became blue on the same day when they formed on the plates while others became blue one day after colony formation. This indicates that proteins encoded by these clones exhibit different affinities for the bait (the novel RANK motif) since the time a clone takes to become blue should be proportional to its affinity for the bait. Based on the affinity of these proteins for the bait and the number of clones encoding them, these proteins were prioritized in numerical order with #1 as the protein which is most likely to be the functional one involved in the RANK motif-mediated signaling (Table 1).

[0074] RYBP was assigned #1 since it has the highest affinity for the bait and was represented in 6 independently isolated clones (Table 1). As such, the potential of RYBP as a signaling molecule interacting with the IVVY (SEQ ID NO:4) motif was examined to regulate osteoclastogenesis. Western blots showed that RYBP is highly expressed in osteoclast precursors and during osteoclast differentiation (Figure 1B). It was then determined whether the interaction between the RANK fragment and RYBP seen in yeast also occurs in mammalian cells. The cDNA region encoding the bait was cloned into pCMV-Myc vector (BD Biosciences Clontech) to generate Myc-RANK (Figure 1C). The full-length RYBP cDNA was subcloned into pCMV-HA (BD Biosciences Clontech) vector to prepare HA-RYBP (Figure 1C). Myc-RANK, HA-RYBP and two empty control vectors (HA-Vector and Myc-Vector) were cotransfected into 293 cells in different combinations (Figure 1D). Transfected cells were lysed for coimmunoprecipitation with anti-HA antibody. The precipitates were then subject to Western analysis with anti-Myc antibody. While the assays cotransfected with HA-Vector plus Myc-Vector, HA-Vector

plus Myc-RANK, or HA-RYBP plus Myc-Vector failed to coimmunoprecipitate HA-RYBP (lanes 1, 2 and 3 in Figure 1D), the assay with both HA-RYBP and Myc-RANK gave rise to a positive band for HA-RYBP (lane 4 in Figure 1D), indicating that RANK interacts with RYBP in mammalian cells. To further verify the interaction between  
5 RANK and RYBP, the same cell lysates were coimmunoprecipitated with anti-Myc antibody and then the precipitates were analyzed by Western analysis with anti-HA antibody (Figure 1E). In this assay, a band was also recognized by anti-HA antibody in the precipitate from cells cotransfected with HA-RYBP and Myc-RANK (lane 4 in Figure 1E), but not in those from the control cells (lanes 1, 2 and 3 in Figure 1E), providing  
10 additional evidence that RANK interacts with RYBP.

[0075] Next, it was determined whether the IVVY (SEQ ID NO:4) motif in the RANK region is specifically responsible for interaction with RYBP. Myc-mRANK was constructed in which the same RANK cDNA region bearing the inactivating mutation in the novel motif (IVAF, SEQ ID NO:7, L535-538) was subcloned into pCMV-Myc vector  
15 (Figure 1F). HA-RYBP was cotransfected with either Myc-RANK or Myc-mRANK into 293 cells. Cell lysates were coimmunoprecipitated with anti-Myc antibody and then the precipitates were analyzed by Western analysis with anti-HA antibody. While cotransfection of HA-RYBP with Myc-RANK yielded a positive band (lane 1, Figure 1G), the assay with HA-RYBP and Myc-mRANK failed to do so (lane 2, Figure 1G), indicating  
20 that the interaction between RANK region and RYBP is specifically mediated by the IVVY motif.

*Identification of Specific RYBP Domains Responsible for Interaction with the RANK IVVY (SEQ ID NO:4) Motif*

[0076] To identify specific RYBP domains mediating the interaction with the  
25 RANK IVVY (SEQ ID NO:4) motif, 11 deletion mutants of RYBP (D1-D11) were generated and around 20-aa region was deleted in each mutant (Figure 2A)(Figure S2). Deletion of a small region was intended to minimize the effect of the deletion on the structural integrity of RYBP since the 3-dimensional structure may be important for the interaction. In addition, a systematic generation of the 11 deletion mutants would enable  
30 us to identify the region mediating the interaction in an unbiased fashion. These deletion mutants were subcloned into pCMV-HA vector. These constructs were cotransfected with Myc-RANK into 293T cells to perform coimmunoprecipitation assays as described above

in Figure 1. The data show that D8 and D9 were unable to interact with RANK (lanes 9 and 10, Figure 2B), indicating that the segments deleted in these two constructs are important for the interaction.

[0077] The sequence of the 40-aa region deleted in D8 and D9 is shown in Figure 2C. To further locate the specific domain(s) responsible for the interaction, 10 more deletion mutants were prepared in each of which a 4-aa segment is deleted (S1-S10) (Figure 2C). Coimmunoprecipitation assays were performed with the small deletion mutants and Myc-RANK. The assays involving immunoprecipitation with anti-Myc antibody followed by Western analysis with anti-HA antibody demonstrated that while S5 and S7 exhibited reduced capacity to interact with RANK, the amino acid residues deleted in S7 are more critical than those deleted in S5 in mediating the interaction with RANK (Figure 2D). The location and sequence of the two functional domains are depicted in Figure 2E.

*RYBP Is Functionally Involved in RANKL-induced Osteoclastogenesis*

[0078] To functionally determine RYBP is involved in the IVVY (SEQ ID NO:4) motif-mediated osteoclastogenesis, RYBP expression in primary BMMs was knocked down using the RNAi technology. While primary BMMs are extremely difficult to transfect, they can be efficiently infected by retrovirus. Several retrovirus-based expression systems including the  $\Delta$ U3nlsLacZ-293GPG system were used to express exogenous genes in primary BMMs (Ory et al., 1996; Feng et al., 2001; Xu et al., 2006). To achieve a high level expression of siRNA to efficiently down-regulate RYBP expression, a retroviral siRNA expression vector named pPower-siRNA based on  $\Delta$ U3nlsLacZ (Ory et al., 1996)(Figure 3A) was developed.  $\Delta$ U3nlsLacZ contains a strong CMV promoter in place of U3 of 5' LTR to attain higher levels of transcripts than most natural 5' LTR when transfected into 293 cells-based packaging cells (Ory et al., 1996). More than 20 sequences in the gene encoding RYBP were selected for potential siRNA target sequences by the siRNA target finder provided by Ambio, Inc (Austin, TX) and subcloned them into pPower-siRNA between Xho and Bam HI. Retrovirus encoding these siRNAs were prepared by transfecting these as described in (Ory et al., 1996; Feng et al., 2001) and used to infect primary BMMs. The infected cells were lysed for Western analysis to examine the downregulation of RYBP expression. Several functional siRNA

sequences were obtained and the one with the highest efficiency in suppression of RYBP gene expression was chosen for the subsequent assays (Figure 3A) (Figure 8).

[0079] A scrambled siRNA sequence was designed to use as control (Figure 3A). BMMs were uninfected or infected with retrovirus expressing scrambled RNA or RYBP siRNA. An aliquot of infected cells were lysed for assessment of levels of RYBP expression, whereas the remaining cells were plated and treated with M-CSF and RANKL to stimulate osteoclastogenesis. As shown in Figures 3B and 3C, the selected siRNA significantly suppressed RYBP expression in osteoclast precursors and the siRNA-mediated downregulation of RYBP expression inhibited osteoclastogenesis. In contrast, neither reduction of RYBP expression nor failure in osteoclastogenesis was seen in uninfected cells or those infected with retrovirus encoding the scrambled control sequence. These data indicate that RYBP is functionally involved in the RANK motif-mediated osteoclastogenesis.

[0080] To further investigate the functional role of RYBP in osteoclastogenesis, rescue experiments were carried out, which aimed to address two important issues: 1) off-target effect of siRNA and 2) the functional significance of the RYBP domain shown to mediate its interaction with the RANK IVVY (SEQ ID NO:4) domain. This rescue study requires an effective strategy to reduce the endogenous RYBP in BMMs and simultaneously express an exogenous WT or mutant RYBP. RNAi is a highly sequence-specific process. It has been established that a single-nucleotide mismatch can significantly render a siRNA inactive in mammalian cells (Hall, 2004; Amarzguioui et al., 2003; Pusch et al., 2003). Thus, this can be achieved by introducing multiple mutations within the targeting sequence in the constructs encoding exogenous WT or mutant RYBP and these mutations are selected so that the resulting sequence encode the same amino acids. Based on these principles, we prepared two constructs: RYBP-HA and dRYBP-HA (Figure 3D). RYBP-HA encodes WT RYBP linked containing a HA tag at its C-terminus whereas dRYBP-HA expresses a HA-tagged mutant RYBP in which the domain (VIIT168-171) responsible for interacting with RANK was deleted (Figure 3D). Moreover, in both constructs, the coding region targeted by the siRNA was considerably mutated (7 out of 19 nt) in a manner that the introduced mutation do not alter the amino acid sequence (Figure 3D).

[0081] These constructs were cloned into retroviral expression vectors for expression in BMMs. As shown in Figure 1E, while retrovirally expressed RYBP siRNA dramatically knocked down endogenous RYBP expression, addition of the retrovirus encoding HA-tagged WT RYBP or mutant RYBP efficiently increased RYBP levels. The re-expressed RYBP are HA-tagged exogenous RYBP since HA antibody recognizes the protein (Figure 1E). Importantly, the re-expression of WT RYBP-HA effectively rescued osteoclastogenesis while the dRYBP-HA exhibited impaired capacity to restore osteoclastogenesis (Figure 1F), further demonstrating that RYBP plays a functional role in the RANK-mediated osteoclastogenesis. Specifically, these data indicate that the failed osteoclastogenesis from BMMs retrovirally transduced with RYBP siRNA directly result from the reduced levels of RYBP, not due to the off-target effects. Secondly, the data demonstrated that the RYBP domain identified by co-IP experiments in Figure 2 is functionally involved in the RANK-mediated osteoclastogenesis.

15 *IVVY (SEQ ID NO:4) Motif Regulates RYBP Intracellular Translocation during RANKL-mediated Osteoclastogenesis*

[0082] Having established that RYBP not only interacts with the IVVY (SEQ ID NO:4) motif but is also functionally involved in osteoclastogenesis, the molecular mechanism by which RYBP modulates RANKL-mediated osteoclastogenesis were not determined. RYBP has been shown to be a nuclear protein and regulate gene expression by acting as a protein interacting with members of the PcG and various transcription factors (Garcia et al., 1999; Trimarchi et al., 2001; Schlisio et al., 2002; Sawa et al., 2002). However, given that the current work has demonstrated that RYBP is capable of interacting with RANK, which is a well-documented transmembrane protein, suggesting that RYBP may also be present in the cytoplasm and acts as a signaling molecule to transmit the membrane-proximal signal to regulate nuclear events. Immunofluorescence staining assays were performed to examine the subcellular location of RYBP during the 4-day in vitro osteoclastogenesis.

[0083] As shown in Figure 4A, while RYBP is present both in the cytoplasm and in the nucleus of BMMs treated with M-CSF only, the nucleus has significantly more RYBP than the cytoplasm (left panel). Moreover, the spindle-like shape of the cells and the pattern of RYBP subcellular location remain unchanged during the 4-day M-CSF treatment. In contrast, one-day RANKL treatment induced the translocation of RYBP from

the nucleus to the cytoplasm (right panel, Figure 4A). In particular, a significant amount of RYBP moved to the plasma membrane, presumably resulting from the interaction with RANK. Interestingly, on day 2, a portion of RYBP moved back to the nucleus and they appear to be located primarily at the nuclear periphery (right panel, Figure 4A). On day 3, cells had begun to fuse to form multinucleated osteoclasts in which RYBP was seen at the plasma membrane. In addition, nuclei also appeared to move to plasma membrane. On day 4, most nuclei had lined up together with the plasma membrane. On the last two days, RYBP was exclusively located at the plasma membrane and the nuclear membrane. It is also noted that RYBP was localized speckled nuclear bodies in cells treated with M-CSF for one day but RYBP was then distributed throughout the Nucleoplasm with longer M-CSF treatment (left panel, Figure 4A).

[0084] To further investigate RYBP intracellular translocation during the first 24 hours of the RANKL treatment, immunofluorescence staining assays were repeated with M-CSF (Control) or M-CSF plus RANKL for 1, 2, 4, 8, 16 and 24 hrs (Figure 9). The assays indicated that a notable amount of RYBP proteins had moved out of the nucleus 8 hrs after RANKL treatment and a significant portion of RYBP proteins were located in the cytoplasm at the 16-hr RANKL treatment point. Similar to the previous assays in Figure 4A, some of RYBP proteins had been localized at the plasma membrane 24 hrs after RANKL treatment (right panel, Figure 9). In MCSF-treated control cells, RYBP was present in both the cytoplasm and the nucleus and there was no significant intracellular translocation of RYBP. However, cells were largely spherical at 1-hr M-CSF treatment but cells gradually become spindle-shaped with longer M-CSF treatment (Left panel, Figure 9). In contrast, while cells treated with both M-CSF and RANKL during the first 16 hrs had a similar shape as those treated with M-CSF only, some of them become spherical again 24 hrs after RANKL treatment.

[0085] Next, to investigate whether the IVVY (SEQ ID NO:4) motif specifically mediates the RYBP translocation was investigated, a chimeric receptor system consisting of the external domain of TNFR1 linked to the transmembrane and intracellular domains of RANK was used (Figure S5) (Liu et al., 2004; Xu et al., 2006). Briefly, as a member of the TNFR family, RANK is activated by RANKL-induced trimerization (Figure 10A). As a surrogate, TNF can induce the trimerization of the chimeric receptor to activate the RANK signaling required for osteoclast formation, function and/or survival (Figure 10B)

(Liu et al., 2004; Xu et al., 2006). Since TNF can also regulate osteoclast formation and function, osteoclast precursors BMMs from TNFR1&R2 double knockout (TNFR1<sup>-/-</sup>R2<sup>-/-</sup>) mice were used to prevent potential signaling from TNFR.

[0086] BMMs expressing Ch-WT or Ch-Mu were prepared using the retroviral approach as described previously (Liu et al., 2004; Xu et al., 2006) (Figure 10B). The surface expression of the chimeric receptors was assessed by flow cytometric analysis (data not shown). Infected cells with similar levels of the chimeric receptor surface expression were treated with M-CSF (44ng/ml) and TNF (10ng/ml) for 1hr, 2 hrs, 4 hrs, 8 hrs, 1 day or 2 days. The data demonstrated that Ch-WT gave rise to a similar pattern of RYBP intracellular translocation in response to TNF treatment (right panel, Figure 4B) as endogenous RANK did in response to RANKL treatment (right panel, Figure 4A and right panel, Figure 4B). However, Ch-Mu, in which IVVY (SEQ ID NO:4) motif was inactivated, failed to induce RYBP intracellular translocation (left panel, Figure 4B). These data indicate that the IVVY (SEQ ID NO:4) motif plays a critical role in mediating RYBP intracellular translocation, establishing the functional significance of the interaction between RYBP and the RANK IVVY motif.

*Identification of IVVY (SEQ ID NO:4)-regulated Genes by Microarray analysis*

[0087] As a protein interacting with members of the PcG family, RYBP has been shown to mediate transcriptional repression in reporter assays (Garcia et al., 1999). Moreover, RYBP also interacts with various transcription factors including YY1 (Garcia et al., 1999), members of the E2F family (Trimarchi et al., 2001; Schlisio et al., 2002), E4TF1/hGABP (Sawa et al., 2002) and ubiquitinated H2A (Arrigoni et al., 2006), which is associated with transcriptional repression (Li et al., 2007). These observations support that RYBP functions as a regulator of gene expression. Given that the data have established that the IVVY (SEQ ID NO:4) motif plays a critical role in osteoclastogenesis by interacting with RYBP, it is likely that RYBP may transmit the IVVY (SEQ ID NO:4) motif initiated signal to regulate gene expression required for osteoclastogenesis.

[0088] To determine whether the IVVY (SEQ ID NO:4) motif regulates gene expression in BMMs. BMMs expressing the chimeric receptor containing wild type RANK intracellular domain (Ch-WT) or mutant RANK bearing inactivating mutations in the IVVY (SEQ ID NO:4) motif (Ch-Mu) (Figure 10B) were treated with M-CSF

(44ng/ml) and TNF (10ng/ml) for 24 hrs. Total RNA was isolated for microarray analysis at the Microarray Shared Facility at the University of Alabama at Birmingham (UAB). The 3 chips in wild type groups and 3 in mutant group were clustered based on their expressions (Figure 5A). The number of genes activated based different p values and fold changes were also listed (Figure 5B). The microarray assays indicate that 203 genes that are significantly regulated by the IVVY (SEQ ID NO:4) motif (i.e.  $\geq 1.1$  fold change with p value  $< 0.05$ ) (Figure 5B). The detailed information on these genes is provided in Table 2.

Table 2: List of genes regulated by the IVVY motif

| Gene Title                                                                | Gene Symbol                 | Chromosome Number | Fold Change | Up or Down |
|---------------------------------------------------------------------------|-----------------------------|-------------------|-------------|------------|
| matrix metalloproteinase 9                                                | Mmp9                        | chr2              | 52.957066   | up         |
| matrix metalloproteinase 9                                                | Mmp9                        | chr2              | 35.26287    | up         |
| carbonic anhydrase 2                                                      | Car2                        | chr3              | 15.665942   | up         |
| cathepsin K                                                               | Ctsk                        | chr3              | 15.22314    | up         |
| chemokine (C-X-C motif) ligand 3                                          | Cxcl3                       | chr5              | 13.967028   | up         |
| acid phosphatase 5, tartrate resistant                                    | Acp5/TRAP                   | chr9              | 12.049807   | up         |
| Cd200 antigen                                                             | Cd200                       | chr16             | 9.085004    | up         |
| G protein-coupled receptor 68                                             | Gpr68                       | chr12             | 9.067647    | up         |
| serine incorporator 2 /// hypothetical protein LOC100044221               | LOC100044221<br>/// Serinc2 | chr4              | 8.342579    | up         |
| Tnf receptor-associated factor 1                                          | Traf1                       | chr2              | 8.335335    | up         |
| transmembrane 7 superfamily member 4                                      | Tm7sf4                      | chr15             | 7.1823263   | up         |
| vascular endothelial growth factor C                                      | Vegfc                       | chr8              | 7.1291      | up         |
| vascular endothelial growth factor C                                      | Vegfc                       | chr8              | 6.6310782   | up         |
| vascular endothelial growth factor C                                      | Vegfc                       | chr8              | 5.5311403   | up         |
| versican                                                                  | Vcan                        | chr13             | 5.449619    | up         |
| glypican 1                                                                | Gpc1                        | chr1              | 5.397397    | up         |
| versican                                                                  | Vcan                        | chr13             | 5.2082253   | up         |
| myosin ID                                                                 | Myo1d                       | chr11             | 5.0793095   | up         |
| adrenergic receptor kinase, beta 2                                        | Adrbk2                      | chr5              | 4.936684    | up         |
| RIKEN cDNA 4833422F24 gene                                                | 4833422F24Rik               | chr2              | 4.8180194   | up         |
| Rab38, member of RAS oncogene family                                      | Rab38                       | chr7              | 4.580771    | up         |
| RIKEN cDNA 2610528A11 gene                                                | 2610528A11Rik               | chr14             | 4.5504866   | up         |
| cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)                 | Cdkn2b                      | chr4              | 4.53978     | up         |
| Jun dimerization protein 2                                                | Jundm2                      | chr12             | 4.353514    | up         |
| Na <sup>+</sup> /H <sup>+</sup> exchanger domain containing 2             | Nhedc2                      | chr3              | 4.1892962   | up         |
| myosin IB                                                                 | Myo1b                       | chr1              | 4.1877007   | up         |
| chaperone, ABC1 activity of bcl complex like (S. pombe)                   | Cabc1                       | chr1              | 4.010523    | up         |
| met proto-oncogene                                                        | Met                         | chr6              | 3.9681833   | up         |
| Rous sarcoma oncogene                                                     | Src                         | chr2              | 3.7441297   | up         |
| adrenergic receptor kinase, beta 2                                        | Adrbk2                      | chr5              | 3.7051468   | up         |
| A kinase (PRKA) anchor protein 6                                          | Akap6                       | chr12             | 3.6483796   | up         |
| macrophage stimulating 1 receptor (c-met-related tyrosine kinase)         | Mst1r                       | chr9              | 3.4987073   | up         |
| purinergic receptor P2X, ligand-gated ion channel, 5                      | P2rx5                       | chr11             | 3.34429     | up         |
| acid phosphatase, prostate                                                | Acpp                        | chr9              | 3.1458142   | up         |
| lymphocyte antigen 6 complex, locus 1                                     | Ly6i                        | chr15             | 3.1208453   | up         |
| nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 | Nfatc1                      | chr18             | 3.054388    | up         |
| G protein-coupled receptor 132                                            | Gpr132                      | chr12             | 2.9648147   | up         |
| metallothionein 2                                                         | Mt2                         | chr8              | 2.5575764   | up         |
| orosomucoid 1                                                             | Orm1                        | chr4              | 2.438179    | up         |
| SMAD specific E3 ubiquitin protein ligase 1                               | Smurf1                      | chr5              | 2.4253285   | up         |
| N-myc downstream regulated gene 4                                         | Ndr4                        | chr8              | 2.3484612   | up         |
| SLIT-ROBO Rho GTPase activating protein 3                                 | Srgap3                      | chr6              | 2.2787986   | up         |
| RIKEN cDNA 9030425E11 gene                                                | 9030425E11Rik               | chr9              | 2.2157958   | up         |
| Gardner-Rasheed feline sarcoma viral (Fgr) oncogene homolog               | Fgr                         | chr4              | 2.1882703   | up         |
| calcitonin receptor                                                       | Calcr                       | chr6              | 2.1615038   | up         |

| Gene Title                                                                                                                       | Gene Symbol   | Chromosome Number | Fold Change | Up or Down |
|----------------------------------------------------------------------------------------------------------------------------------|---------------|-------------------|-------------|------------|
| nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1                                                        | Nfatc1        | chr18             | 2.1178071   | up         |
| expressed sequence AW049765                                                                                                      | AW049765      | chr17             | 2.1107264   | up         |
| metallothionein 3                                                                                                                | Mt3           | chr8              | 2.0902364   | up         |
| Nur77 downstream gene 2                                                                                                          | Ndg2          | chr10             | 2.0589802   | up         |
| gasdermin domain containing 1                                                                                                    | Gsdmcl        | chr15             | 1.9001833   | up         |
| peroxisomal biogenesis factor 11a                                                                                                | Pex11a        | chr7              | 1.8860891   | down       |
| glutathione synthetase                                                                                                           | Gss           | chr2              | 1.8565459   | up         |
| RIKEN cDNA 1810029B16 gene                                                                                                       | 1810029B16Rik | chr8              | 1.8382206   | up         |
| arginine vasopressin-induced 1                                                                                                   | Avpi1         | chr19             | 1.7936711   | up         |
| Nur77 downstream gene 2                                                                                                          | Ndg2          | chr10             | 1.7673057   | up         |
| interferon gamma inducible protein 30                                                                                            | Ifi30         | chr8              | 1.7602197   | up         |
| 0 day neonate skin cDNA, RIKEN full-length enriched library, clone:4632424N07 product:unclassifiable, full insert sequence       |               | chr13             | 1.7456527   | up         |
| 7 days embryo whole body cDNA, RIKEN full-length enriched library, clone:C430014D18 product:unclassifiable, full insert sequence |               | chr1              | 1.6371719   | up         |
| Rho family GTPase 1                                                                                                              | Rnd1          | chr15             | 1.6316801   | up         |
| proviral integration site 3                                                                                                      | Pim3          | chr15             | 1.5919101   | up         |
| transcription factor EC                                                                                                          | Tefec         | chr6              | 1.5889215   | up         |
| adenylate kinase 2 /// similar to adenylate kinase 2                                                                             | Ak2 ///       | chr4              | 1.5666348   | up         |
| calreticulin 3                                                                                                                   | Calr3         | chr8              | 1.5528721   | up         |
| forkhead box P4                                                                                                                  | Foxp4         | chr17             | 1.5294316   | up         |
| sulfiredoxin 1 homolog (S. cerevisiae)                                                                                           | Srxn1         | chr2              | 1.5259216   | up         |
| lipoprotein lipase                                                                                                               | Lpl           | chr8              | 1.518442    | down       |
| acyl-CoA synthetase long-chain family member 1                                                                                   | Acs11         | chr8              | 1.5109522   | up         |
| peroxisome biogenesis factor 16                                                                                                  | Pex16         | chr2              | 1.5087851   | up         |
| proviral integration site 3                                                                                                      | Pim3          | chr15             | 1.5076823   | up         |
| Hypothetical protein LOC100042016                                                                                                | LOC100042016  | chr11             | 1.5062494   | down       |
| RIKEN cDNA 2810046L04 gene                                                                                                       | 2810046L04Rik | chr3              | 1.502087    | down       |
| chr11                                                                                                                            | 1.5018126     |                   | down        |            |
| RIKEN cDNA 2810025M15 gene                                                                                                       | 2810025M15Rik | chr1              | 1.4973557   | up         |
| eukaryotic translation initiation factor 5A2                                                                                     | Eif5a2        | chr3              | 1.496838    | down       |
| HEAT repeat containing 1                                                                                                         | Heatr1        | chr13             | 1.496193    | down       |
| EH-domain containing 1                                                                                                           | Ehd1          | chr19             | 1.4889908   | up         |
| mitochondrial ribosomal protein L45                                                                                              | Mrpl45        | chr11             | 1.4728131   | up         |
| ubiquitin associated domain containing 2                                                                                         | Ubac2         | chr14             | 1.4714627   | up         |
| CDK2-associated protein 2                                                                                                        | Cdk2ap2       | chr19             | 1.4631894   | up         |
| exosome component 1                                                                                                              | Exosc1        | chr19             | 1.459716    | down       |
| RAB20, member RAS oncogene family                                                                                                | Rab20         | chr8              | 1.456158    | up         |
| annexin A4                                                                                                                       | Anxa4         | chr6              | 1.4501132   | up         |
| EH-domain containing 1                                                                                                           | Ehd1          | chr19             | 1.4483279   | up         |
| CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase like 2                                         | Ctdspl2       | chr2              | 1.4445461   | down       |
| RIKEN cDNA 2400009B08 gene                                                                                                       | 2400009B08Rik | chr8              | 1.4394982   | up         |
| RIKEN cDNA 2810002O09 gene                                                                                                       | 2810002O09Rik | chrX              | 1.4358203   | up         |
| 0 day neonate cerebellum cDNA, RIKEN full-length enriched library, clone:C230031C13 product:unclassifiable, full insert sequence |               | chr13             | 1.4317822   | down       |
| chromodomain protein, Y chromosome-like 2                                                                                        | Cdy12         | chr8              | 1.429501    | up         |
| Tnf receptor-associated factor 2                                                                                                 | Tnf12         | chr2              | 1.4162527   | up         |
| 12 days embryo male wolffian duct includes surrounding region                                                                    |               | chr14             | 1.4105098   | down       |

| Gene Title                                                                                  | Gene Symbol                                        | Chromosome Number | Fold Change | Up or Down |
|---------------------------------------------------------------------------------------------|----------------------------------------------------|-------------------|-------------|------------|
| cDNA, RIKEN full-length enriched library, clone:6720430M22                                  |                                                    |                   |             |            |
| product:unclassifiable, full insert sequence                                                |                                                    |                   |             |            |
| DENN/MADD domain containing 2D                                                              | Dennd2d                                            | chr3              | 1.404753    | down       |
| zinc finger protein 28                                                                      | Zfp28                                              | chr7              | 1.4026449   | down       |
| death inducer-obliterator 1                                                                 | Dido1                                              | chr2              | 1.3924606   | up         |
| golgi associated, gamma adaptin ear containing, ARF binding protein 2                       | Gga2                                               | chr7              | 1.3818957   | up         |
| Transcribed locus                                                                           |                                                    | chr4              | 1.3627918   | up         |
| Cd99 antigen-like 2                                                                         | Cd99l2                                             | chrX              | 1.3593745   | up         |
| acyl-Coenzyme A dehydrogenase family, member 11                                             | Acad11                                             | chr9              | 1.3416152   | up         |
| transmembrane protein 30A                                                                   | Tmem30a                                            | chr9              | 1.3382797   | down       |
| paired-Ig-like receptor A1 /// paired-Ig-like receptor A11 /// paired-                      | Lilrb3 ///<br>LOC100038908<br>///                  | chr7              | 1.3368992   | up         |
| zinc finger CCCH type containing 12C                                                        | Zc3h12c                                            | chr9              | 1.2695061   | up         |
| nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha               | Nfkb1a                                             | chr12             | 1.2683418   | up         |
| PR domain containing 2, with ZNF domain                                                     | Prdm2                                              | chr4              | 1.2645257   | up         |
| eukaryotic translation initiation factor 4E binding protein 1                               | Eif4ebp1                                           | chr8              | 1.2629627   | up         |
| RIKEN cDNA 1110003F05 gene                                                                  | 1110003F05Rik                                      | chr17             | 1.2587925   | down       |
| RIKEN cDNA D230040J21 gene                                                                  | D230040J21Rik                                      | chr5              | 1.2540071   | down       |
| kelch-like 7 (Drosophila)                                                                   | Klhl7                                              | chr5              | 1.2528863   | down       |
| solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 4 | Slc25a4                                            | chr8              | 1.2489876   | up         |
| RIKEN cDNA B230337E12 gene                                                                  | B230337E12Rik                                      | chr1              | 1.2462453   | down       |
| LEM domain containing 3 /// similar to LEM domain containing 3                              | Lemd3 ///<br>LOC100044466                          | chr10             | 1.2456872   | down       |
| steroid 5 alpha-reductase 2-like /// hypothetical protein LOC100044230                      | LOC100044230<br>/// Srd5a21                        | chr5              | 1.2416695   | up         |
| RIKEN cDNA 6330416G13 gene                                                                  | 6330416G13Rik                                      | chr4              | 1.2402455   | up         |
| GLE1 RNA export mediator-like (yeast)                                                       | Gle1l                                              | chr2              | 1.2331597   | down       |
| solute carrier family 25, member 39                                                         | Slc25a39                                           | chr11             | 1.2330384   | up         |
| ATPase, H+ transporting, lysosomal V0 subunit C /// similar to vacuolar H(+)-ATPase         | Atp6v0c ///<br>LOC100039636<br>///<br>LOC100046757 | chr6              | 1.2311169   | up         |
| ATPase, H+ transporting, lysosomal V0 subunit B                                             | Atp6v0b                                            | chr4              | 1.2288831   | up         |
| sorcin                                                                                      | Sri                                                | chr5              | 1.2287564   | up         |
| cyclin H                                                                                    | Ccnh                                               | chr13             | 1.2249198   | down       |
| ubiquitin-conjugating enzyme E2D 2                                                          | Ube2d2                                             | chr18             | 1.2231128   | down       |
| splicing factor, arginine/serine-rich 6                                                     | Sfrs6                                              | chr2              | 1.2213966   | down       |
| mitofusin 1                                                                                 | Mfn1                                               | chr3              | 1.2205157   | up         |
| 3-oxoacyl-ACP synthase, mitochondrial                                                       | Oxsm                                               | chr14             | 1.2186738   | up         |
| negative regulator of ubiquitin-like proteins 1                                             | Nub1                                               | chr5              | 1.2164594   | up         |
| Vcell division cycle 73, Paf1/RNA polymerase II complex component, homolog (S. cerevisiae)  | Cdc73                                              | chr1              | 1.215644    | down       |
| peptidyl-prolyl isomerase G (cyclophilin G)                                                 | Ppig                                               | chr2              | 1.2147061   | down       |
| SUMO1 activating enzyme subunit 2                                                           | Sae2                                               | chr7              | 1.2131295   | down       |
| eukaryotic translation initiation factor 4E binding protein 1                               | Eif4ebp1                                           | chr8              | 1.2103645   | up         |
| intraflagellar transport 20 homolog                                                         | Ift20                                              | chr11             | 1.2086008   | down       |

| Gene Title                                                                                                                     | Gene Symbol              | Chromosome Number | Fold Change | Up or Down |
|--------------------------------------------------------------------------------------------------------------------------------|--------------------------|-------------------|-------------|------------|
| (Chlamydomonas)                                                                                                                |                          |                   |             |            |
| ATPase, H <sup>+</sup> transporting, lysosomal V0 subunit B                                                                    | Atp6v0b                  | chr4              | 1.2080344   | up         |
| transmembrane BAX inhibitor motif containing 4                                                                                 | Tmbim4                   | chr10             | 1.2065625   | up         |
| translocase of outer mitochondrial membrane 70 homolog A (yeast)                                                               | Tomm70a                  | chr16             | 1.2040935   | up         |
| coiled-coil domain containing 93                                                                                               | Ccdc93                   | chr1              | 1.2035478   | up         |
| RIKEN cDNA 2610301B20 gene                                                                                                     | 2610301B20Rik            | chr4              | 1.1994959   | down       |
| isocitrate dehydrogenase 3 (NAD <sup>+</sup> ) beta                                                                            | Idh3b                    | chr2              | 1.1974369   | up         |
| ATP synthase, H <sup>+</sup> transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 3                          | Atp5g3                   | chr2              | 1.1937019   | up         |
| RIKEN cDNA 1810013D10 gene                                                                                                     | 1810013D10Rik            | chr5              | 1.1921476   | up         |
| translocase of outer mitochondrial membrane 20 homolog (yeast)                                                                 | Tomm20                   | chr2              | 1.190947    | up         |
| zinc finger and BTB domain containing 48                                                                                       | Zbtb48                   | chr4              | 1.19065     | down       |
| Ewing's tumor-associated antigen 1                                                                                             | Etaa1                    | chr11             | 1.1876866   | down       |
| dyncin light chain LC8-type 2                                                                                                  | Dynl12                   | chr11             | 1.185562    | down       |
| tripartite motif protein 23                                                                                                    | Trim23                   | chr13             | 1.1853576   | down       |
| splicing factor, arginine/serine-rich 2, interacting protein                                                                   | Sfrs2ip                  | chr15             | 1.1847556   | down       |
| solute carrier family 25 (mitochondrial carnitine/acylcarnitine translocase), member 20                                        | Slc25a20                 | chr9              | 1.18337     | up         |
| ankyrin repeat and zinc finger domain containing 1                                                                             | Ankzf1                   | chr1              | 1.1801457   | down       |
| RIKEN cDNA C330006K01 gene                                                                                                     | C330006K01Rik            | chr5              | 1.1782492   | up         |
| RIKEN cDNA 2310035K24 gene                                                                                                     | 2310035K24Rik            | chr2              | 1.1772768   | up         |
| mitochondrial ribosomal protein L9                                                                                             | Mrpl9                    | chr3              | 1.1764377   | up         |
| RIKEN cDNA 1600012H06 gene                                                                                                     | 1600012H06Rik            | chr17             | 1.1758627   | down       |
| BCL2-antagonist/killer 1                                                                                                       | Bak1                     | chr17             | 1.1748867   | up         |
| RIKEN cDNA 1200011O22 gene                                                                                                     | 1200011O22Rik            | chr19             | 1.1721143   | up         |
| squamous cell carcinoma antigen recognized by T-cells 1                                                                        | Sart1                    | chr19             | 1.172027    | down       |
| leucyl-tRNA synthetase                                                                                                         | Lars                     | chr18             | 1.171722    | down       |
| eukaryotic translation initiation factor 4, gamma 2                                                                            | Eif4g2                   | chr7              | 1.1678882   | down       |
| S-phase kinase-associated protein 1A                                                                                           | Skp1a                    | chr11             | 1.1656432   | up         |
| glycerol phosphate dehydrogenase 2, mitochondrial                                                                              | Gpd2                     | chr2              | 1.1632929   | down       |
| vav 1 oncogene                                                                                                                 | Vav1                     | chr17             | 1.1601086   | down       |
| APAF1 interacting protein /// similar to MMRP19                                                                                | Apip ///<br>LOC100044135 | chr2              | 1.1600349   | up         |
| splicing factor, arginine/serine-rich 6                                                                                        | Sfrs6                    | chr2              | 1.158874    | down       |
| 10 days neonate cortex cDNA, RIKEN full-length enriched library, clone:A830030M19 product:unclassifiable, full insert sequence |                          | chr3              | 1.1568067   | down       |
| RAB22A, member RAS oncogene family                                                                                             | Rab22a                   | chr2              | 1.1567866   | up         |
| mitochondrial ribosomal protein S14                                                                                            | Mrps14                   | chr1              | 1.1528465   | down       |
| synaptophysin-like protein                                                                                                     | Sypl                     | chr12             | 1.1516387   | down       |
| AT hook containing transcription factor 1                                                                                      | Ahctf1                   | chr1              | 1.1466129   | up         |
| suppressor of hairy wing homolog 3 (Drosophila)                                                                                | Suhw3                    | chrX              | 1.1445446   | down       |
| ring finger protein (C3H2C3 type) 6                                                                                            | Rnf6                     | chr5              | 1.1389387   | up         |
| heterogeneous nuclear ribonucleoprotein K                                                                                      | Hnrpk                    | chr13             | 1.1362003   | down       |
| DnaJ (Hsp40) homolog, subfamily C, member                                                                                      | Dnajc14                  | chr10             | 1.1335106   | up         |

| Gene Title                                                                                                     | Gene Symbol               | Chromosome Number | Fold Change | Up or Down |
|----------------------------------------------------------------------------------------------------------------|---------------------------|-------------------|-------------|------------|
| 14                                                                                                             |                           |                   |             |            |
| Transcribed locus                                                                                              |                           | chr19             | 1.1277171   | down       |
| ERO1-like beta ( <i>S. cerevisiae</i> )                                                                        | Ero1lb                    | chr13             | 1.1264199   | down       |
| transmembrane protein 18                                                                                       | Tmem18                    | chr12             | 1.125084    | down       |
| dynein cytoplasmic 2 light intermediate chain 1 /// similar to Dynein cytoplasmic 2 light intermediate chain 1 | Dync2li1 /// LOC100048514 | chr17             | 1.1209211   | up         |
| G1 to S phase transition 1                                                                                     | Gspt1                     | chr16             | 1.1209048   | down       |
| actin related protein 2/3 complex, subunit 1A                                                                  | Arpc1a                    | chr5              | 1.120718    | up         |
| Rab9 effector protein with kelch motifs                                                                        | Rabepk                    | chr2              | 1.1180472   | up         |
| ubiquitin-conjugating enzyme E2 variant 1                                                                      | Ube2v1                    | chr3              | 1.1133846   | up         |
| DnaJ (Hsp40) homolog, subfamily B, member 4                                                                    | Dnajb4                    | chr3              | 1.11245     | down       |
| mitochondrial ribosomal protein L50                                                                            | Mrpl50                    | chr4              | 1.1107732   | down       |
| myoneurin                                                                                                      | Mynn                      | chr3              | 1.1068301   | down       |
| mitochondrial ribosomal protein L48                                                                            | Mrpl48                    | chr4              | 1.1060202   | up         |

[0089] Notably, a number of known osteoclast-related genes such as matrix metalloproteinase 9 (Mmp9), carbonic anhydrase 2 (Car2), cathepsin K (Ctsk) and tartrate resistant acid phosphatase 5 (Acp5/TRAP) were up-regulated more than 10 fold by the

5 IVVY (SEQ ID NO:4) motif. RT-PCR analysis confirmed that the IVVY (SEQ ID NO:4) motif is involved in the transcriptional activation of Mmp9, Car2, Ctsk and TRAP genes (Figure 5C). It was shown that the IVVY (SEQ ID NO:4) motif plays a crucial role in committing BMMs into the osteoclast lineage and it takes only 24 hours for the IVVY (SEQ ID NO:4) motif to fully commit BMMs into the osteoclast lineage (Xu et al., 2006).

10 Moreover, once cells are fully committed by the IVVY (SEQ ID NO:4) motif, the TRAF-dependent signaling pathways are sufficient to mediate the remaining osteoclastogenic process.

[0090] These data suggest that the IVVY (SEQ ID NO:4) motif can permanently activate some of its target genes. To investigate this possibility, BMMs were treated for 24

15 hours and the transcriptional activation of the Mmp9, Car2, Ctsk and TRAP genes was assessed by RT-PCR immediately, 2 days or 4 days after the 24-hour RANKL treatment (Figure 5D). The data indicate that while the levels of the Mmp9, Car2 and TRAP mRNA decline 2 days after the 24-hour RANKL treatment, the RANKL-induced increase in the Ctsk mRNA levels persist 4 days after the RANKL treatment, suggesting that the

20 RANKL-mediated activation of the Ctsk gene is permanent.

- [0091] Shortly after the discovery of the RANKL/RANK system in the late 1990s, it was reported that RANK, upon binding by RANKL, activates a unique pathway(s) that distinguish itself from other members of the TNFR to regulate osteoclastogenesis (Yeh et al., 1999). This report was re-enforced by the recent identification of a novel RANK cytoplasmic motif which plays a crucial role in osteoclast lineage commitment (Xu et al., 2006). In the current studies, the long-sought RANK signaling pathway in osteoclastogenesis were elucidated to deal with two important issues: a) whether the IVVY (SEQ ID NO:4) motif mediates the osteoclast lineage commitment by recruiting an intracellular protein to activate a novel signaling pathway and b) whether the IVVY (SEQ ID NO:4) motif induces cell differentiation by regulating gene expression. These studies have revealed that the IVVY (SEQ ID NO:4) motif plays a critical role in osteoclastogenesis by interacting with RYBP, a protein interacting with members of the PcG family and various transcription factors including YY1 (Garcia et al., 1999), members of the E2F family (Trimarchi et al., 2001; Schlisio et al., 2002), E4TF1/hGABP (Sawa et al., 2002) and ubiquitinated H2A (Arrigoni et al., 2006), which is associated with transcriptional repression (Li et al., 2007). Moreover, specific RYBP domains involved in interacting with the RANK IVVY (SEQ ID NO:4) motif were identified. Furthermore, the data have also demonstrated that the IVVY (SEQ ID NO:4) motif/RYBP, or fragments thereof, regulates osteoclastogenesis via the activation of a large number of genes.
- [0092] Osteoclasts play a pivotal role in both skeletal development and adult skeletal maintenance. During bone development, osteoclasts are required to degrade the cartilage matrix for vascular invasion in endochondral ossification (Lee and Einhorn, 2001). Furthermore, osteoclasts are also involved in bone growth and shape modification in endochondral ossification (bone modeling) (Baron, 2003). After skeletal maturation, osteoclasts continue to play a critical role in bone maintenance by participating in bone remodeling. Bone remodeling is a lifelong process of bone renewal in which old bone is resorbed by osteoclasts and then replaced by new bone formed by osteoblasts (Martin and Rodan, 2001). The constant remodeling of bone is required not only for maintaining healthy skeleton but also for regulating calcium homeostasis (Martin and Rodan, 2001).
- [0093] Nonetheless, aberration in osteoclast formation and activity resulting in elevated bone resorption is implicated in the pathogenesis of postmenopausal osteoporosis (Teitelbaum, 2000), causes bone erosion in various inflammatory conditions including RA

(Goldring, 2003), and plays a role in breast tumor bone metastasis (Mundy, 2002). Consequently, effective inhibition of osteoclast formation and activity has long been regarded as an attractive strategy for preventing and treating these bone diseases. However, currently available antiresorptive therapeutic agents such as estrogen, selective  
5 estrogen receptor modulators (SERMs), bisphosphonates and calcitonin either lack satisfactory efficacy or have potential to cause serious side effects in clinical management of postmenopausal osteoporosis and bone loss associated with other pathological conditions (Stepan et al., 2003; Lufkin et al., 2004; Marcus et al., 2002). Thus, development of new antiresorptive therapeutic drugs with higher potency and specificity is  
10 imperative.

[0094] The discovery of RANKL and its two receptors RANK and osteoprotegerin (OPG) in the late 1990's has created huge enthusiasm for developing new antiresorptive agents targeting the RANKL/RANK/OPG regulatory axis. So far, efforts have been undertaken in developing OPG, soluble RANK-Fc, and anti-RANKL antibodies as new  
15 antiresorptive therapeutics to treat bone diseases (Doggrell, 2003; Zhang et al., 2003; Bekker et al., 2004). However, all of these agents have an inherited drawback as therapeutic drugs, primarily due to the fact that their action lacks specificity. The RANKL/RANK system is not only involved in osteoclast formation and function (Hsu et al., 1999), but is also a critical mediator of other biological processes such as dendritic cell  
20 (DC) survival and activation (Wong et al., 1997a; Josien et al., 1999; Josien et al., 2000), Tcell activation (Kong et al., 1999; Bachmann et al., 1999), lymph node organogenesis (Kong et al., 1999; Dougall et al., 1999; Kim et al., 2000a), B-cell differentiation (Kong et al., 1999; Dougall et al., 1999), mammary gland development (Fata et al., 2000), and thermoregulation in females or fever response inflammation (Hanada et al., 2009).

25 [0095] The RANK IVVY (SEQ ID NO:4) motif/RYBP, or fragments thereof, pathway represents a new antiresorptive drug target for the following three reasons. First, the IVVY (SEQ ID NO:4) motif/RYBP pathway plays an essential role in osteoclastogenesis (Xu et al., 2006) (Figure 3). As a result, therapeutic blockage of this new signaling pathway in osteoclast precursors would result in a dramatic reduction in  
30 osteoclastogenesis. Secondly, the IVVY (SEQ ID NO:4) motif/RYBP pathway also represents a specific antiresorptive target for treating bone diseases involving osteoclasts. It has been well established that TNF family members play crucial roles in various

immune functions primarily by engaging TRAF proteins (Arron et al., 2002; Xie et al., 2008) (Kobayashi et al., 2003). However, the IVVY (SEQ ID NO:4) motif recruits RYBP to activate a TRAF-independent pathway. As such, therapeutic targeting of the IVVY (SEQ ID NO:4)/RYBP pathway should have minimal effect on immune response and associated side effects. Moreover, the specific RYBP domains identified which mediate interaction with the RANK IVVY (SEQ ID NO:4) motif provide potential therapeutic candidates and targets and/or facilitate development of either biochemical or cell-based assays for identifying small molecules targeting the interaction between the IVVY (SEQ ID NO:4) motif and RYBP protein.

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## CLAIMS

What is claimed is:

1. A pharmaceutically administerable therapeutic composition for improving bone mass, rigidity, or strength, or preventing or treating bone loss comprising a pharmaceutically acceptable excipient and a RYBP peptide having an amino acid sequence as set forth in SEQ ID NO:3 or a sequence at least 90% identical to SEQ ID NO:3, or a fragment thereof, that specifically interacts with a RANK peptide comprising an amino acid sequence of IVVY (SEQ ID NO:4) to regulate osteoclastogenesis.  
5
- 10 2. The therapeutic composition of Claim 1, wherein said RYBP fragment has an amino acid sequence as set forth in SEQ ID NO:2, or a sequence at least 90% identical to SEQ ID NO:2.
3. The therapeutic composition of Claim 1, wherein said RYBP fragment comprises an amino acid sequence as set forth in SEQ ID NO:5, or a sequence at least 75% identical to SEQ ID NO:5.  
15
4. The therapeutic composition of Claim 1, wherein said RYBP fragment comprises an amino acid sequence as set forth in SEQ ID NO:6, or a sequence at least 75% identical to SEQ ID NO:6.
5. The therapeutic composition of Claim 1, wherein said RYBP fragment comprises an amino acid sequence of SEQ ID NO:3.  
20
6. The therapeutic composition of Claim 1, wherein said RYBP peptide comprises an amino acid sequence of SEQ ID NO:5 or SEQ ID NO:6.
7. A method for improving bone mass, rigidity, or strength, or preventing or treating bone loss comprising administering to a subject in need a therapeutically effective amount of the therapeutic composition of Claim 1.  
25
8. The method of Claim 7, wherein the RYBP fragment has an amino acid sequence as set forth in SEQ ID NO:2 or a sequence at least 90% identical to SEQ ID NO:2.
9. The method of Claim 7, wherein the RYBP fragment comprises an amino acid sequence as set forth in SEQ ID NO:5, or a sequence at least 75% identical to SEQ ID NO:5.  
30

10. The method of Claim 7, wherein the RYBP fragment comprises an amino acid sequence as set forth in SEQ ID NO:6, or a sequence at least 75% identical to SEQ ID NO:6.
- 5 11. The method of Claim 7, wherein the RYBP fragment comprises an amino acid sequence of SEQ ID NO:3, SEQ ID NO:5 or SEQ ID NO:6.
12. The method of Claim 7, wherein said bone loss is caused by abnormally elevated osteoclast formation or activity.
13. The method of Claim 7, wherein said bone loss is caused by osteoporosis.
14. The method of Claim 7, wherein said bone loss is caused by bone erosion in  
10 rheumatoid arthritis.
15. The method of Claim 7, wherein said bone loss is caused by periodontal bone loss.
16. The method of Claim 7, wherein said bone loss is caused by tumor-induced osteolysis, bone destruction or bone metastasis.
17. The method of Claim 7, wherein said bone loss is caused by Paget disease.
- 15 18. The method of Claim 7, wherein said bone loss is a drug-induced bone loss.
19. A method for regulating osteoclastogenesis comprising modulating interaction of RYBP, or a fragment thereof, with an amino acid sequence of IVVY (SEQ ID NO:4) of a RANK peptide.
- 20 20. A therapeutic composition for improving bone mass, rigidity, or strength, or preventing or treating bone loss comprising a pharmaceutically acceptable excipient and a modulator that is capable of increasing an interaction of RYBP, or a fragment thereof, with the amino acid sequence of IVVY (SEQ ID NO:4) of a RANK peptide, or increasing an effector in the RANK signaling pathway through the RYBP-RANK interaction.

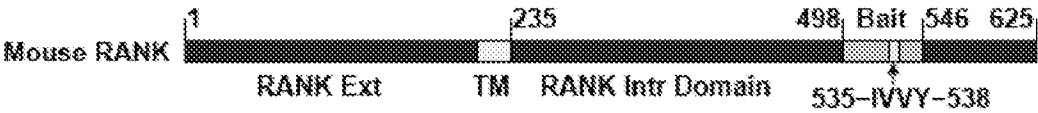


FIGURE 1A

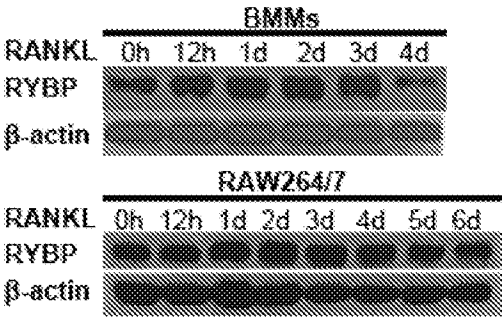


FIGURE 1B

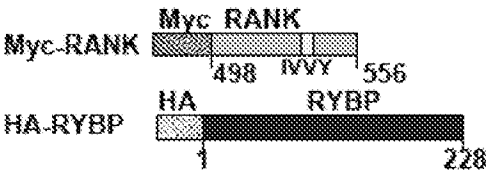


FIGURE 1C

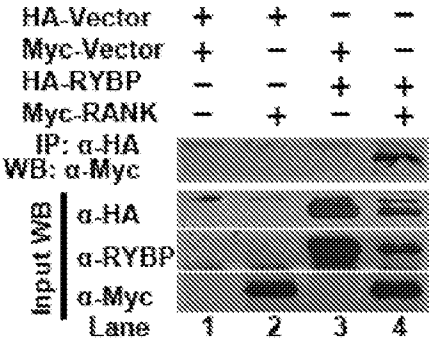


FIGURE 1D

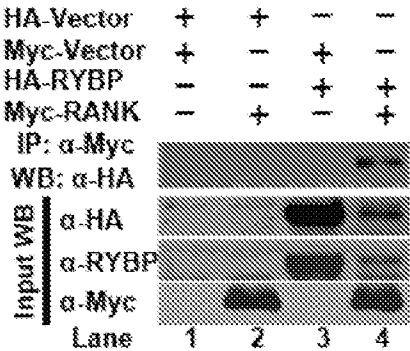


FIGURE 1E

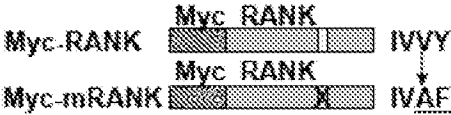


FIGURE 1F

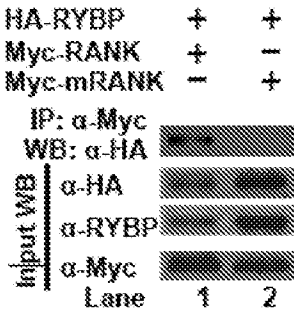


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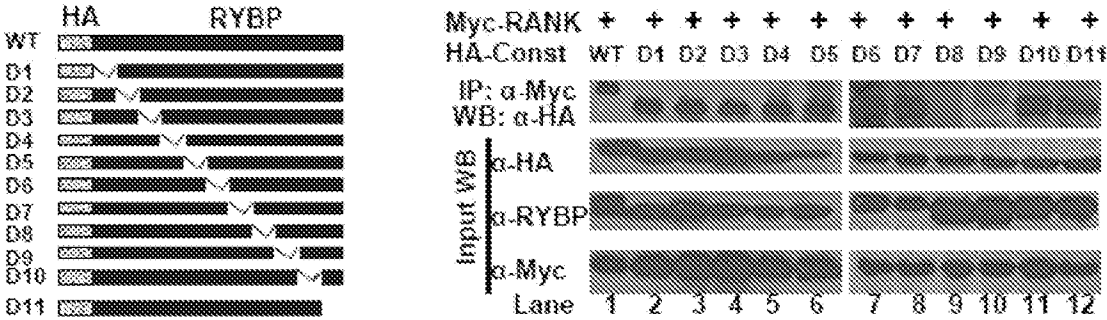


FIGURE 2A

FIGURE 2B

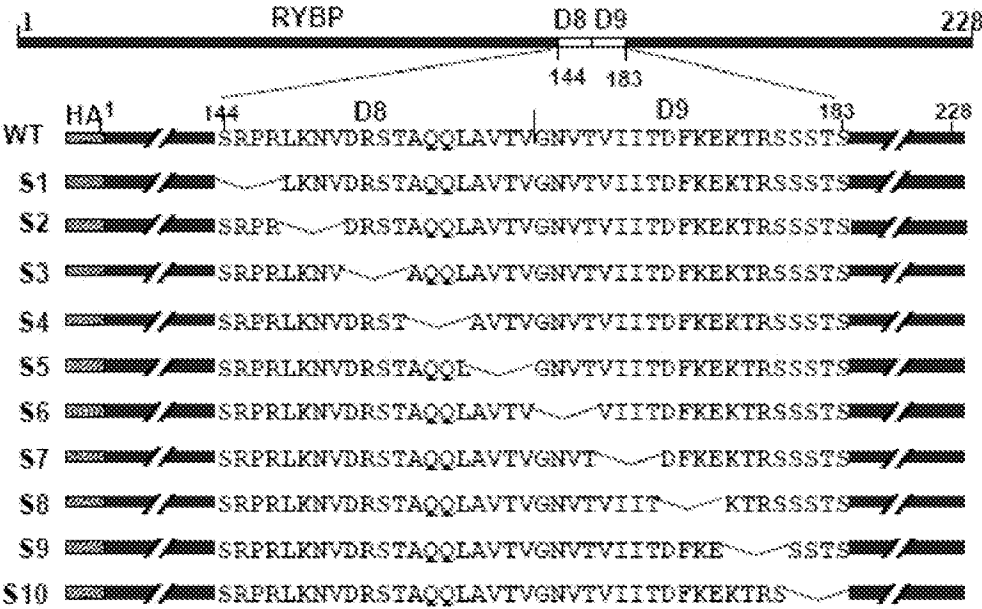


FIGURE 2C

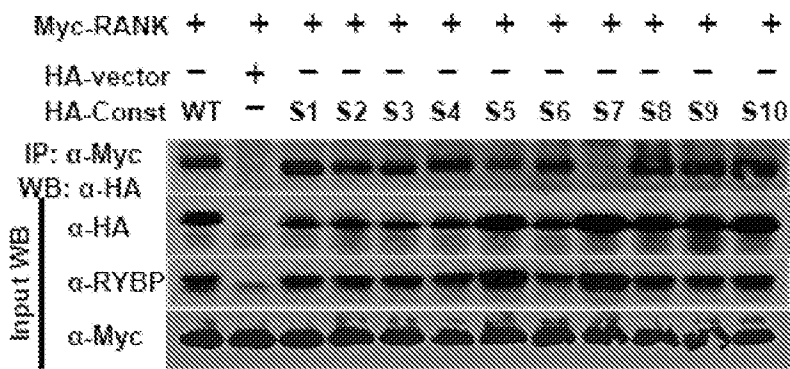


FIGURE 2D

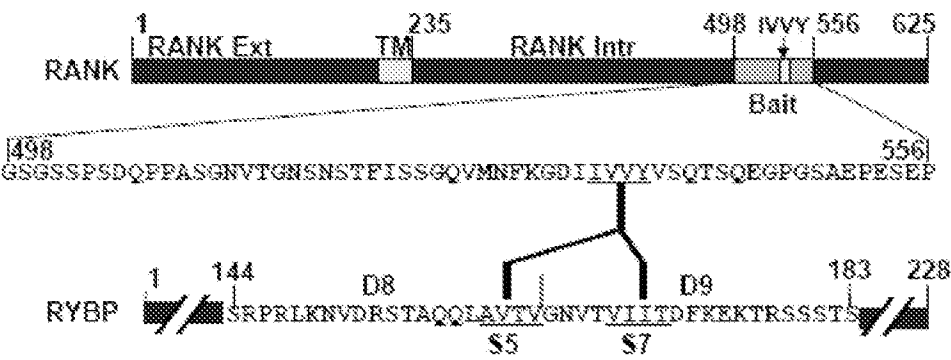


FIGURE 2E

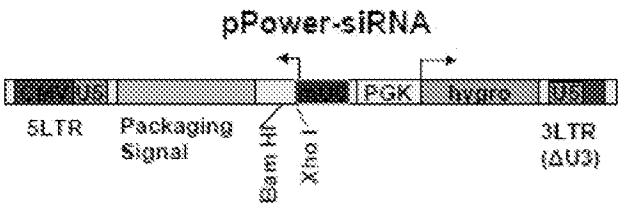


**FIGURE 3A**

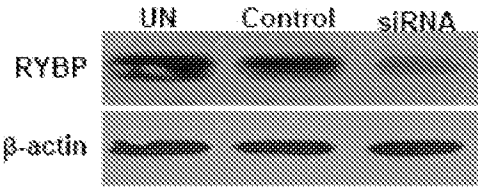


**FIGURE 3B**

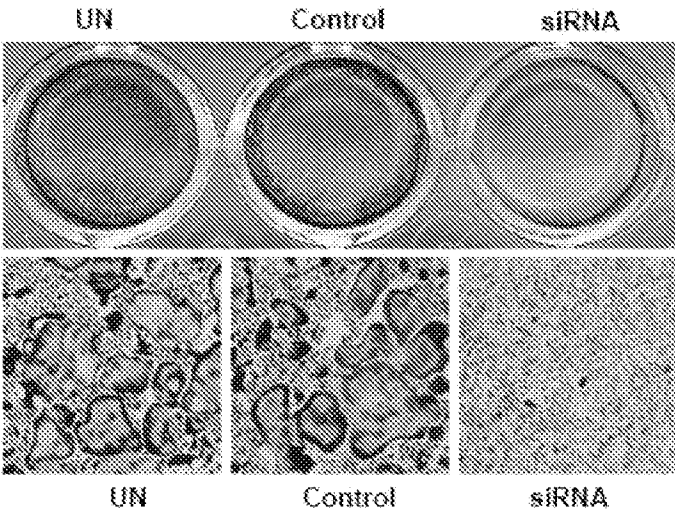


**FIGURE 3C**

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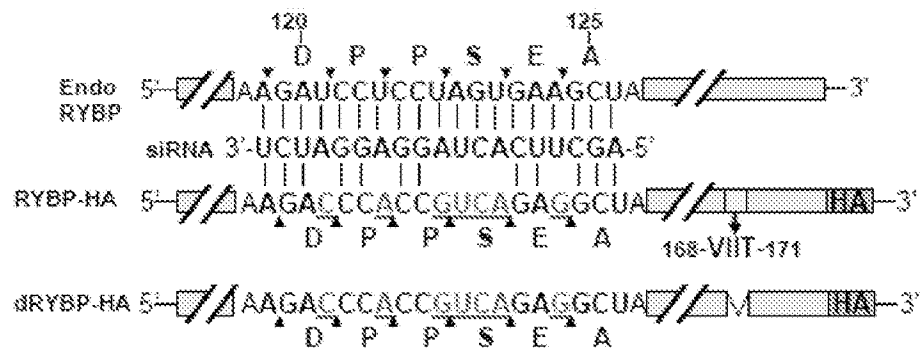


FIGURE 3D

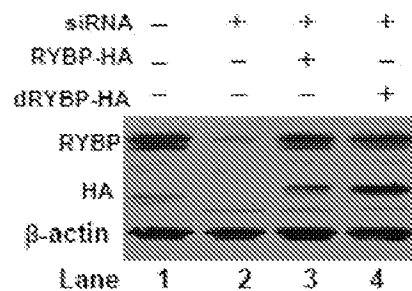


FIGURE 3E

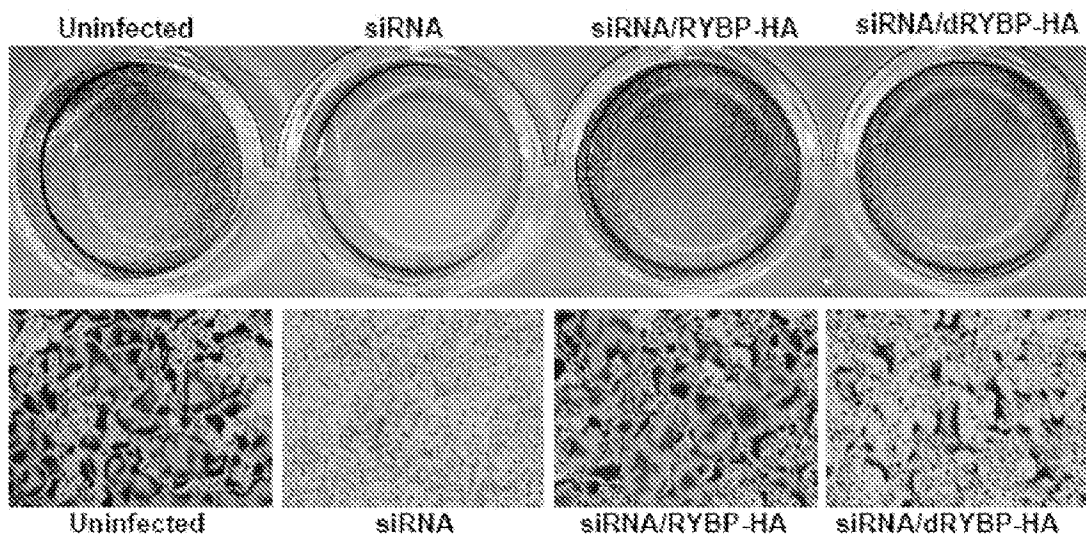


FIGURE 3F

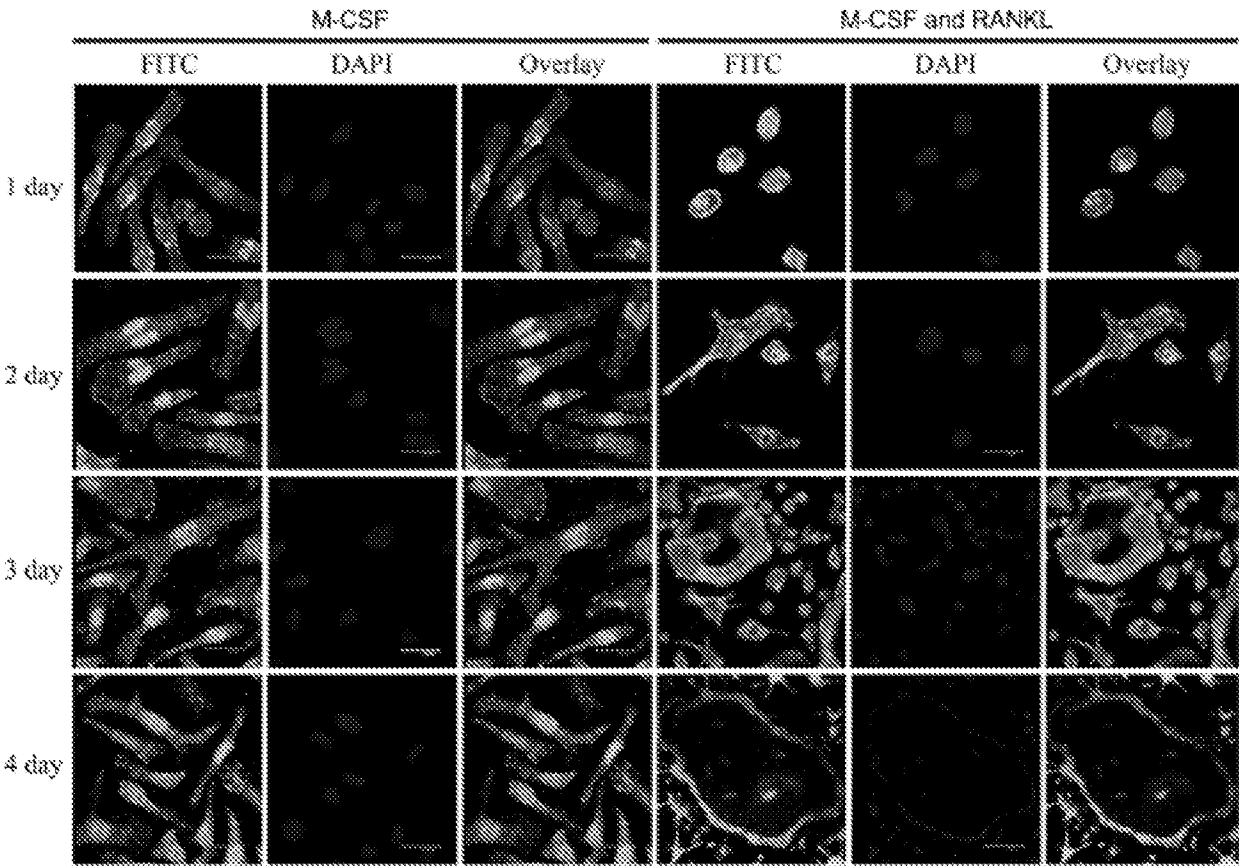


FIGURE 4A

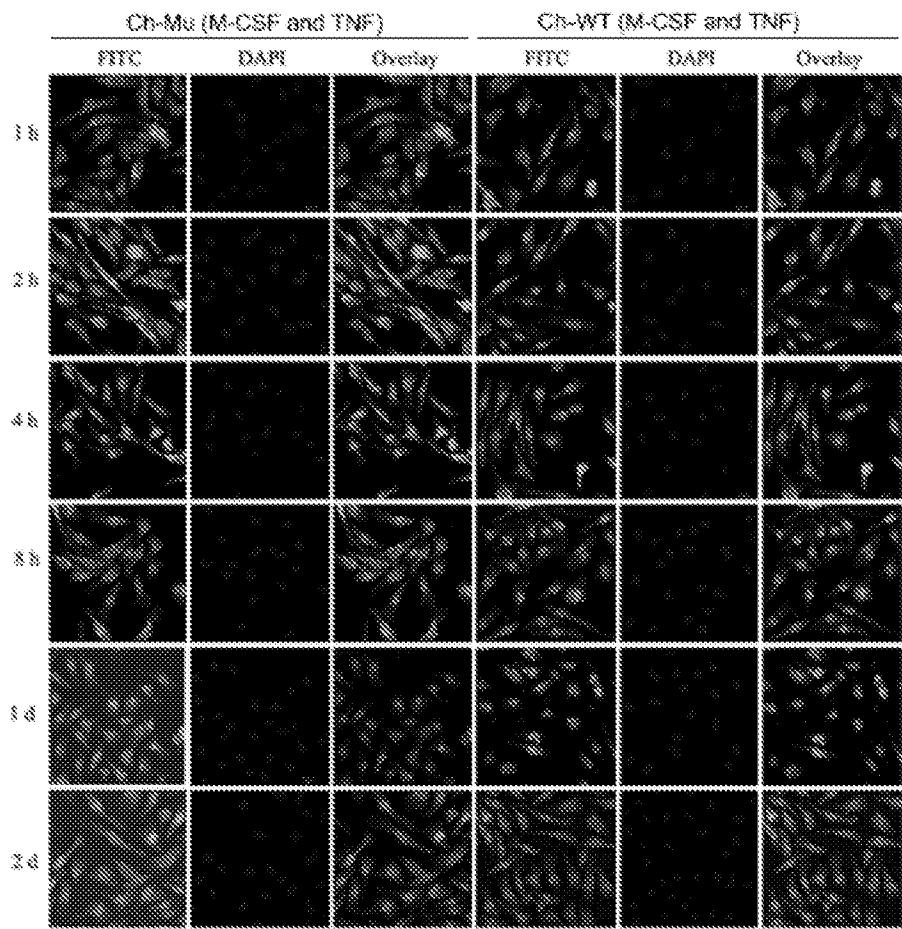


FIGURE 4B

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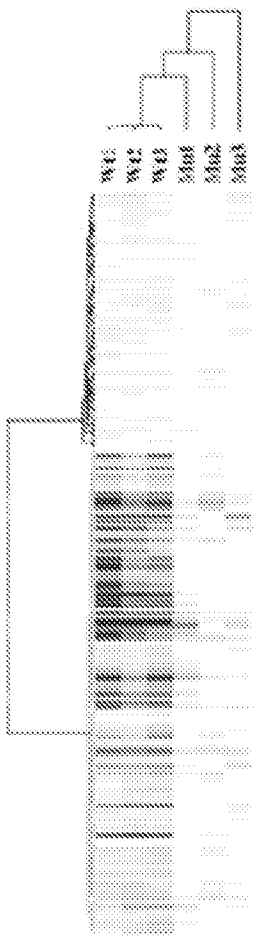


FIGURE 5A

|        | p<0.05 | p<0.02 | p<0.01 | p<0.005 |
|--------|--------|--------|--------|---------|
| FC>1.1 | 303    | 79     | 41     | 23      |
| FC>1.5 | 72     | 36     | 21     | 9       |
| FC>2.0 | 48     | 26     | 16     | 8       |
| FC>3.0 | 36     | 20     | 12     | 6       |

FIGURE 5B

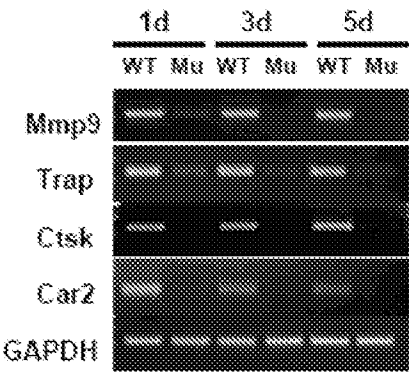


FIGURE 5C

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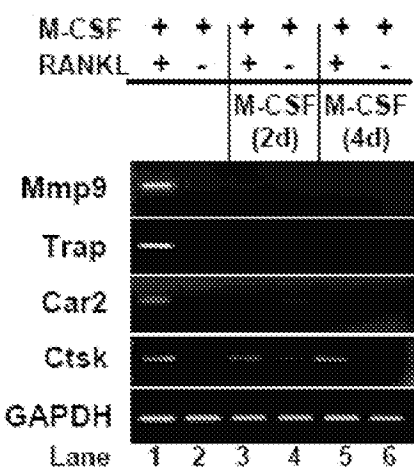


FIGURE 5D

```

234 CYRKEKFKALTANLQHWINEACGRHSLSGDKESSGDSVCVSTHTANTGQQGACGVLVLLTLEEK hRANK
    ||| ||||| ||| ||| ||||| ||| ||| ||| ||| |||
235 TYREGGKALTANLQWVNDACSSLSGDKESSGDRCAQGHSAISSQQIVCEGILVLTREEK mRANK

294 TPEEDNCTPLDGGVVDGGTGVGGCPYAQCEHARMLSLVSKTEIIEDETFQMPTEDEYDMORF hRANK
    ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
295 MVPE-----DGAGVCGPVCAAGCPHAEVDSERTFTLVSEVETQCDLERKIPTEDEYTDORF mRANK

354 SQETDQLFLITEPGSKSTHFFSERLEVGENDSLSQCFIGTQSTVGSRESNCCTEPLCETOW hRANK
    ||| ||| ||| ||| ||||| ||||| ||||| ||| ||| ||| |||
355 SQETGSELLLIQQGSKSTHFFQERLEVGENDSLSQCFITCTESTVDSIGCDFTPEPPTDS mRANK

414 TPMSSENYLQKEVDSGHCPHKAASPSINWADVCTGCRNPFGEEDCEPLVGSFKRGPLDQCA hRANK
    ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
415 MPVSPERNLTKETEGDSCLPWVY--SSNSTDGYTGSGNTPGEDHPEFPGSLKOGPLDQCA mRANK

474 YGMCLPSETEEAERTEARDQVEDCAGRLPESARACAGSCNSHCCQSPASGNVTGNSHSTF hRANK
    ||| ||| ||| ||| ||| ||| ||| ||||| ||||| ||||| |||||
488 YSMGFPSEAAAAMAEAGVRDQDRAE-----RCASGSCNSHSDQSPASGNVTGNSHSTF mRANK

534 ISSGQVHNFKGLIIVVYVSQTSQEGAAAA--AEEMERPVQHEETLAHRDSEAGNGERFPO hRANK
    ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
532 ISSGQVHNFKGLIIVVYVSQTSQEGPGSREDESEFVERPVQHEETLAHRDSEAGTAGERFPO mRANK

    Bait

591 PCGGPREG-----REPEKASRPVQEQGAKA 616 hRANK
    ||| ||| ||| ||||| |||
592 VCATGAGLOEQGAPPEKFDGTSRPVQEQGGAOTSLHTQGSGQCAE 625 mRANK

```

**FIGURE 6**

|     |             |            |             |             |             |             |     |
|-----|-------------|------------|-------------|-------------|-------------|-------------|-----|
|     | gggggggggc  | gagcagctgt | ggggcgccgg  | ggggcagccc  | gtccATGacc  | atggggcgaca | D1  |
|     |             |            |             |             | 1 M T       | M G D       |     |
| 6   | agaagagccc  | gaccaggcca | aaaagacaag  | cgaaacctgc  | cgcagacgaa  | ggtcttttggg | D2  |
|     | K K S P     | T R P      | K R Q       | A K P A     | A D E       | G F W       |     |
| 26  | attgtagcgt  | ctgcaccttt | aggaacagcg  | cgaagcctt   | taaattgcagc | atctgcgatg  | D3  |
|     | D C S V     | C T P      | R N S       | A E A F     | K C S       | I C D       |     |
| 46  | tgcggaaagg  | cacctccacc | aggaaaacctc | gcattcaattc | tcagctgggtg | gcacagcagg  | D4  |
|     | V R K G     | T S T      | R K P       | R I N S     | Q L V       | A Q Q       |     |
| 66  | tggcacagca  | gtacgccact | ccacctcccc  | ctaagaagga  | gaagaaggag  | aaggtcgaaa  | D5  |
|     | V A Q Q     | Y A T      | P P P       | P K K E     | K K E       | K V E       |     |
| 86  | agcctgacaa  | agaaaagcca | gagaaagaca  | aggacattag  | ccccagtgtc  | accaagaaaa  | D6  |
|     | K P D K     | E E P      | E E D       | K D I S     | P S V       | T K E       |     |
| 106 | acaccaacaa  | gaaaaacaaa | ccaaagtctg  | atattctgaa  | agatcctcct  | agtgaagcta  | D7  |
|     | N T N K     | K T K      | P K S       | D I L K     | D P P       | S E A       |     |
| 126 | acagcataca  | gtctgctaac | gtacaacaa   | agaccagcga  | aacaaaccac  | acctcaaggg  | D8  |
|     | N S I Q     | S A N      | A T T       | K T S E     | T N H       | T S R       |     |
| 146 | cccggctgaa  | gaatgtggac | aggagcaccg  | cacagcagtt  | ggcagtaact  | gtggggcaacg | D9  |
|     | P E L K     | N V D      | R S T       | A Q Q L     | A V T       | V G N       |     |
| 166 | tcacogtcac  | tatcacagac | tttaaggaaa  | agactcgtctc | ctcctccaca  | tcctcttcca  | D10 |
|     | V T V I     | I T D      | F K E       | K T R S     | S S T       | S S S       |     |
| 186 | cagtgaacctc | cagtgcaggg | tcagaacagc  | agaaccagag  | cagctcgggc  | tcagagagca  | D11 |
|     | T V T S     | S A G      | S E Q       | Q N Q S     | S S G       | S E S       |     |
| 206 | cagacaaagg  | ctcctcccg  | tcctccacgc  | caaagggcga  | catgtccagca | gtgaatgatg  |     |
|     | T D K G     | S S E      | S S T       | P K G D     | M S A       | V N D       |     |
| 226 | aatctttctG  | Agattgcaca | tggaattgtg  | aaactatgaa  | tcaggytatg  | agattcaaac  |     |
|     | E S P       |            |             |             |             |             |     |

**FIGURE 7**

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01 Rotabte View  
 121  
 132 K X B S T R Q M R G A N E A A D E C F W  
 242 H C B V C T P R N S A D A T K C S I C D  
 352 V R N G T S T R R F R I N S Q L V A Q Q  
 462 U R Q Q X A T O P R R R E R R E R V E  
 572 K R U R R R R R R R R R R R R R R R R R  
 682 Offensive SIGMA Target  
 792  
 802  
 912  
 1021  
 1142  
 1201  
 1262  
 1322  
 1382  
 1442  
 1502  
 1562  
 1622  
 1682  
 1742  
 1802  
 1862  
 1922

**FIGURE 8**

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1891 accaatgcagttgttagtntttttttttaaaacctactatggctcatctttattaaacagg  
 2041 ggttcgtcaacotcacatcagaaactgacacagagagatctctccagtcctctggaggttaagttct  
 2101 taagtctctctgtgtggtgaaagagatcgcaggggttgcatgggctgagcttgcctggggaagctcaca  
  
 2161 tctccaggagaggtgaaatgaaataggtaggtgtctctctgttgaactgaaactcttgaaagctctct  
 2221 catcttcacacacatctctaggggttagggagataaagaaagaaacactgataaataaggtcttatcatctc  
 2281 catcccaagttctcagtgaaactcctgctctctctctctctctctctctctctctctctctctctct  
 2341 cttagctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 2401 ctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 2461 ctggtggttaaaagaaacgggtctctctctctctctctctctctctctctctctctctctctctctct  
 2521 caagaaacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacac  
 2581 actaaacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacac  
 2641 ggtctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 2701 ggtggtgaaaggtctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 2761 agtgaacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacac  
 2821 acttttaaaacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacac  
 2881 aatattctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 2941 gagggtgttaaggaactctctctctctctctctctctctctctctctctctctctctctctctctctct  
 3001 caaatctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 3061 gaacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacac  
 3121 gctgggaacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacac  
 3181 ctggtgtctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 3241 ggaacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacac  
 3301 cagtcagatctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 3361 tgaataggtctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 3421 ctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 3481 ctacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacac  
 3541 agatttaaaacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacac  
 3601 ctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 3661 ctggtgaacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacac  
 3721 ctctggtctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 3781 ctgctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 3841 atttatctggaacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacac  
 3901 atggtctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 3961 gtagctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 4021 atttctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 4081 attctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 4141 aaggtctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 4201 gtaggtctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 4261 cacaggtctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 4321 aaaggtctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 4381 ctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctct  
 4441 aatcaaacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacac

FIGURE 8  
(Continued)

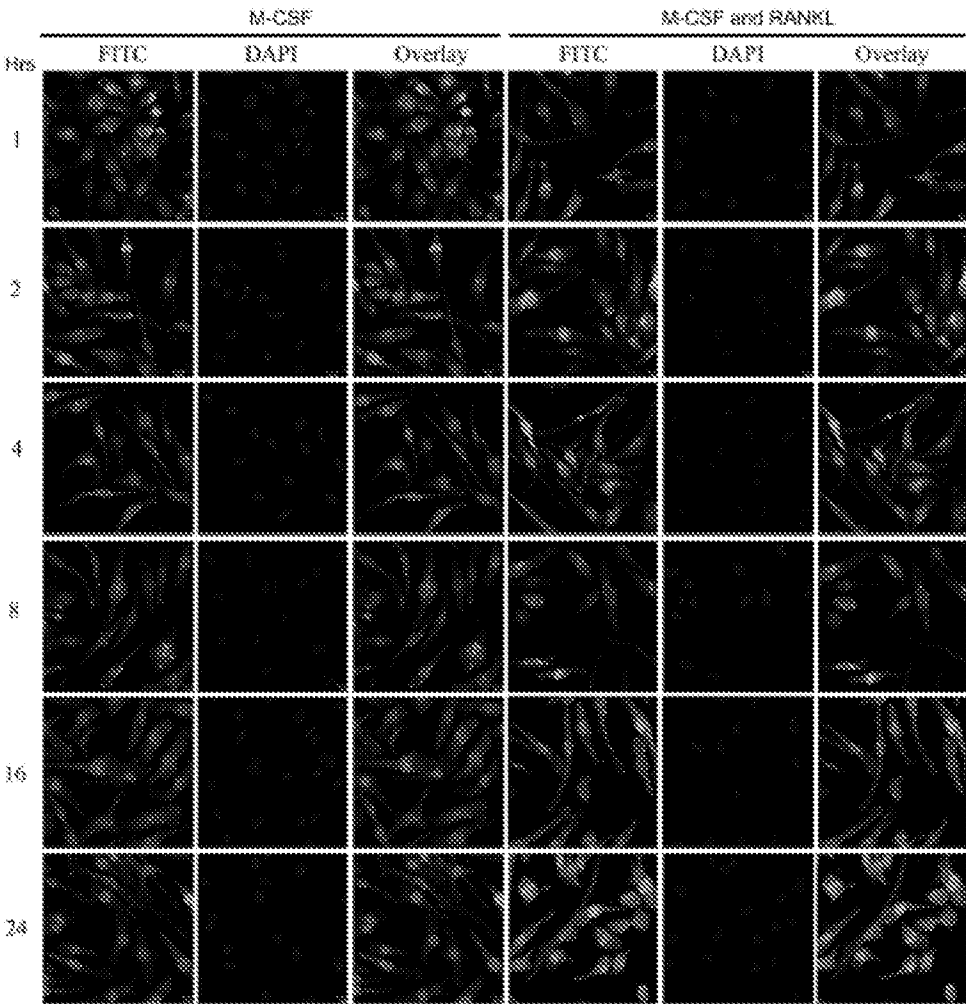


FIGURE 9

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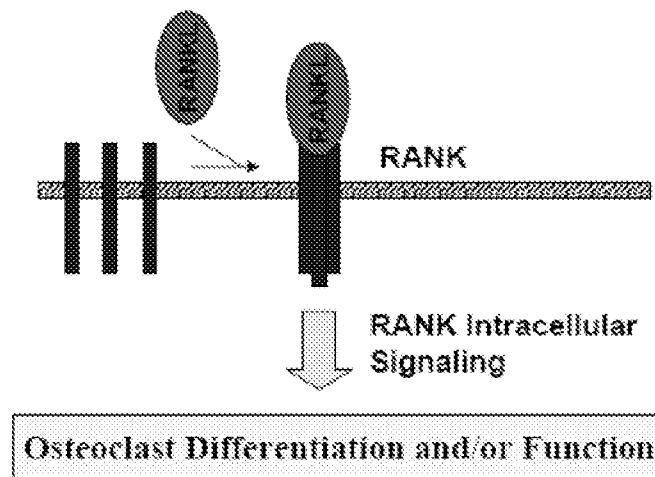


FIGURE 10A

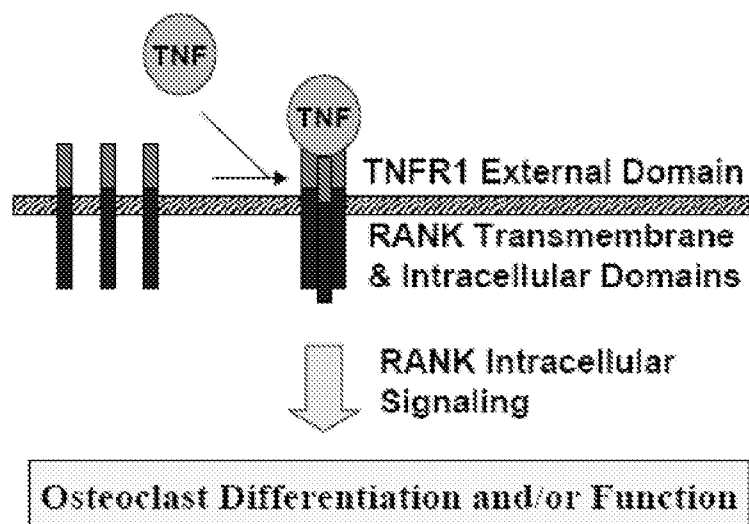


FIGURE 10B

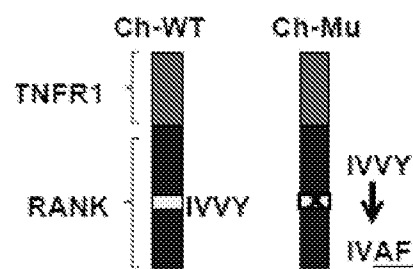


FIGURE 10C