

Critical Review

RANKing Intracellular Signaling in Osteoclasts

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Summary

RANKL plays a pivotal role in the differentiation, function and survival of osteoclasts, the principal bone-resorbing cells. RANKL exerts the effects by binding RANK, the receptor activator of NF- κ B, in osteoclasts and its precursors. Upon binding RANKL, RANK activates six major signaling pathways: NFATc1, NF- κ B, Akt/PKB, JNK, ERK and p38, which play distinct roles in osteoclast differentiation, function and survival. Recent studies have not only provided more insights into RANK signaling but have also revealed that several factors, including INF- γ , IFN- β , and ITAM-activated costimulatory signals, regulate osteoclastogenesis via direct crosstalk with RANK signaling. It was recently shown that RANK contains three functional motifs capable of mediating osteoclastogenesis. Moreover, although both IFN- γ and IFN- β inhibit osteoclastogenesis, they exert the inhibitory effects by distinct

mechanisms. Whereas IFN- γ has been shown to block osteoclastogenesis by promoting degradation of TRAF6, IFN- β inhibits osteoclastogenesis by down-regulating c-fos expression. In contrast, the ITAM-activated costimulatory signals positively regulate osteoclastogenesis by mediating the activation of NFATc1 through two ITAM-harboring adaptors: FcR γ and DAP12. This review is focused on discussing the current understanding of RANK signaling and signaling crosstalk between RANK and the various factors in osteoclasts.

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INTRODUCTION

Osteoclasts, the body's principal bone-resorbing cells, not only play a critical role in skeletal development and maintenance but are also implicated in the pathogenesis of various bone diseases including postmenopausal osteoporosis (1). Osteoclasts are multinucleated giant cells of hematopoietic origin and osteoclast differentiation (osteoclastogenesis) requires two critical factors macrophage/monocyte-colony forming factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL) (2). RANKL, also known as OPGL, ODF and TRANCE, was identified as a member of the tumor necrosis factor (TNF) family in the late 1990s (3, 4, 5). To date, RANKL has been shown to play pivotal roles in regulating various biological processes such as bone homeostasis (1, 5), immune function (6, 7) and mammary gland development (8).

In bone, osteoblasts and bone marrow stromal cells serve as primary sources of RANKL as well as M-CSF (1). M-CSF and RANKL bind to their respective receptor c-fms and RANK (receptor activator of nuclear factor kappa B) expressed on osteoclast precursors to stimulate osteoclast formation. In mature osteoclasts, RANKL mediates osteoclast activation and survival (4). In addition, osteoblasts/stromal cells also produce a factor called osteoprotegerin (OPG), which is a decoy receptor for RANKL. OPG inhibits

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Abbreviations: DAP12, DNAX-activating protein 12; DD, death domain; ERK, extracellular signal-regulated kinase; FADD, Fas associated death domain; FcR γ , Fc receptor common γ subunit; IKK, Inhibitor of kappa B kinase; IFN, interferon; IRF-9, interferon regulatory factor 9; ITAM, immunoreceptor tyrosine-based activation motif; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; M-CSF, monocyte/macrophage-colony stimulating factor; NF- κ B, nuclear factor kappa B; NFAT, nuclear factor of activated T-cell; NIK, NF- κ B-inducing kinase; ODF, osteoclast differentiation factor; OPG, osteoprotegerin; OPGL, osteoprotegerin ligand; OSCAR, osteoclast-associated receptor; PI3-kinase, phosphoinositide-3OH kinase; PIP3, phosphatidylinositol-(3,4,5)-phosphate; PH, pleckstrin homology; PIR-A, paired immunoglobulin-like receptor A; PKB, protein kinase B; PLC γ , phospholipase C γ ; RANK, receptor activator of NF- κ B; RANKL, RANK ligand; SH2, Src homology 2; SIRP- β 1, signal-regulatory protein β 1; Stat, signal transducer and activator of transcription; TAK1, TGF- β -activated kinase 1; TNF, tumor necrosis factor; TNFR, TNF receptor; TRAF, TNFR associated factor; TRADD, TNFR associated death domain; TRANCE, TNF-related activation-induced cytokine; TREM, receptor expressed by myeloid cells.

RANKL function by competing with RANK for binding to RANKL (1). Intriguingly, the RANKL/RANK system has also been implicated in regulating bone formation in vivo in a mouse model (9).

Both RANKL and RANK are essential for the osteoclastogenic process since mice lacking the gene for either protein developed osteopetrosis due to failure to form osteoclasts (10, 11). Consistently, knockout mice deficient for OPG developed early onset of osteoporosis due to elevated osteoclastogenesis (12) whereas transgenic mice over-expressing OPG exhibited osteopetrosis, resulting from a decrease in late stages of osteoclast differentiation (13). Taken together, these data indicate that the RANKL/RANK system plays an essential role in skeletal development and bone remodeling. On the other hand, the RANKL/RANK system is also implicated in the pathogenesis of various bone diseases such as postmenopausal osteoporosis and bone loss in inflammatory conditions. Recently, it was shown that estrogen deficiency leads to the elevated expression of RANKL on both osteoblasts and lymphocytes and the increased RANKL expression plays a pathological role in the development of postmenopausal osteoporosis (14). Moreover, RANKL expressed on activated T-cells is also implicated in inducing bone loss and joint destruction in rheumatoid arthritis (15).

Since the discovery of the RANKL/RANK system in the late 1990s, intensive studies have been focused on elucidating RANK-initiated signaling involved in osteoclast differentiation, function and survival. Consequently, it has become clear that RANK activates six key signaling pathways in osteoclasts: nuclear factor of activated T-cell (NFAT) c1, nuclear factor kappa B (NF- κ B), Akt/protein kinase B (PKB), Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 (2). However, the precise signaling mechanisms underlying the activation of the various signaling pathways have not been fully elucidated. Recent studies have provided more insights into the molecular mechanism underlying the activation of these pathways. Especially, the new findings have revealed a higher degree of complexity of RANK signaling in osteoclasts than previously appreciated. Moreover, emerging evidence also indicates that interferon (IFN) γ , IFN- β and immunoreceptor tyrosine-based activation motif (ITAM) mediated signals regulate osteoclastogenesis by cross-talking with one or more RANK signaling pathways. In this review, I will provide a concise and updated overview of RANK signaling in osteoclasts. Then, I will discuss how INF- γ , IFN- β and ITAM-mediated signals regulate osteoclastogenesis by cross-talking with RANK signaling.

RANK SIGNALING IN OSTEOCLASTS

RANK was identified as a member of the tumor necrosis factor receptor (TNFR) family (6). Members of the TNFR family lack intrinsic enzymatic activity in their intracellular

domains (16). As a result, they transduce signals by recruiting adapter proteins, primarily death domain (DD)-containing proteins and members of the TNFR associated factor (TRAF) family. The DD-containing proteins include Fas associated death domain (FADD) and TNFR associated death domain (TRADD). These proteins link the DD-containing receptors to downstream proteases of the caspase family necessary for activation of apoptosis (16). The TRAF family contains six members (TRAFs 1, 2, 3, 4, 5 and 6), each containing a ring and zinc finger motif in their N-terminal and C-terminal domains that mediate self association and protein interaction (16). The TRAFs link either the DD-containing receptors (via other adapter proteins) or the receptor lacking a DD to activation of various signaling pathways such as NF- κ B, JNK, ERK and p38 (16).

As a TNFR family member lacking DD(6), RANK presumably transduces intracellular signals by utilizing TRAF proteins. Numerous early in vitro binding studies showed that RANK directly interacts with different TRAF proteins (TRAFs 1, 2, 3, 5 and 6) (17–22), suggesting that RANK may contain multiple TRAF-binding motifs that regulate osteoclast differentiation, function and/or survival. Consistently, recent functional studies indicated that RANK indeed contains multiple domains that are able to mediate osteoclast formation and function (23, 24). More specifically, three RANK cytoplasmic motifs, PFQEP^{369–373} (Motif 1), PVQEET^{559–564} (Motif 2) and PVQEQG^{604–609} (Motif 3), are capable of independently mediating osteoclast formation and function (Fig. 1). Notably, Motif 2 and Motif 3 are more potent than Motif 1 in promoting osteoclast formation and function (24).

Motif 1 activates NF- κ B and three mitogen-activated protein kinase (MAPK) pathways (JNK, ERK and p38) in response to RANKL stimulation (24). Motif 1 binds TRAF6

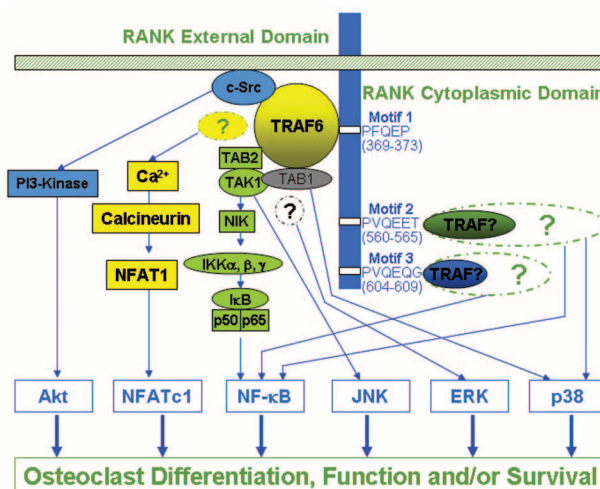


Figure 1. RANK signaling in osteoclast differentiation, function and survival.

(25). Thus, activation of these pathways by Motif 1 involves the formation of a protein complex containing TRAF6, TGF- β -activated kinase 1 (TAK1) and an adaptor protein TAB2 (Fig. 1). TAB2 facilitates the formation of the complex by linking TAK1 to TRAF6 while the RANKL-induced formation of the complex containing TRAF6, TAK1 and TAB2 activates TAK1 (26), which will then phosphorylate NF- κ B-inducing kinase (NIK) to activate the I κ B kinase (IKK) $\alpha\beta\gamma$ complex (27), leading to the activation of the NF- κ B pathway (Fig. 1). In addition, activated TAK1 also activates the JNK pathway (28). Activation of p38 is mediated by TAB1 which has been shown to be able to bind and recruit p38 to the TRAF6-TAK1 complex (29). How Motif 1 activates the ERK pathway remains unclear but it is likely that TRAF6 is also involved in the process (Fig. 1).

RANKL activates the Akt/PKB pathway through TRAF6 (30). This is achieved by RANKL-induced formation of a signaling complex containing both c-Src and TRAF6 at Motif 1 (Fig. 1). TRAF6 enhances the kinase activity of c-Src in the signaling complex to activate phosphoinositide-3-OH kinase (PI3-Kinase) (30). Activated PI3-kinase stimulates the formation of phosphatidylinositol-(3,4,5)-phosphate (PIP3) at the plasma membrane, where Akt/PKB is recruited via its pleckstrin homology (PH) domain and then activated (30) (Fig. 1). Moreover, TRAF6 is also involved in the activation of transcription factor, NFATc1, which plays an important role in osteoclastogenesis (31). RANKL-induced recruitment of TRAF6 mobilizes intracellular calcium, which results in the activation of calcineurin (31). Activated calcineurin in turn dephosphorylates and activates NFAT1, resulting in its translocation into nuclei to form a ternary complex with c-Fos and c-Jun at the promoter for NFATc1 gene to stimulate the expression of NFATc1 (32) (Fig.1). Although the role of TRAF6 in NFATc1 activation is well established, how TRAF6 mobilizes intracellular calcium remains unknown (Fig. 1). In addition, Motif 1/TRAF6-mediated signal only partially activates NFATc1 since the activation of NFATc1 is not completely impaired in osteoclast precursors lacking TRAF6 (31). As discussed below, full activation of NFATc1 also requires signaling crosstalk between RANK signaling and ITAM-mediated signals.

Despite the fact that Motif 2 and Motif 3 are more potent than Motif 1 in promoting osteoclast formation, the signaling pathways activated by these two motifs still remain obscure. Previous binding studies suggested that Motif 2 and Motif 3 may bind TRAF proteins other than TRAF6 (22). In line with this finding, Galibert et al. showed that Motif 2 interacts with TRAF3 and Motif 3 is capable of binding TRAF1, TRAF2 and TRAF5 (22). But, another *in vitro* study demonstrated that neither TRAF1 nor TRAF3 interacts with RANK (17). Given the uncertainty, additional studies are needed to functionally identify TRAF proteins that specifically bind to these RANK motifs. Moreover, it is likely that the initiation of downstream signaling from these two motifs may require a

specific TRAF protein as well as additional signaling molecules, which together form a signaling complex similar to that utilized by Motif 1 (Fig. 1). Nevertheless, it has been shown that Motif 2 activates NF- κ B and p38 pathways in osteoclast precursors whereas Motif 3 activates only the NF- κ B pathway (24) (Fig. 1).

In summary, the functional identification of three distinct RANK motifs that are capable of mediating osteoclast formation and function has further advanced our understanding of RANK signaling in osteoclasts. Notably, the previous prevailing view was that RANK-initiated signaling is primarily mediated through TRAF6 (2). The functional involvement of three different RANK motifs in osteoclast formation and function points to an additional complexity of RANK signaling that was not previously appreciated (23, 24). Future studies will need to be especially focused on delineating the signaling pathways initiated by Motif 2 and Motif 3 since the signaling mechanisms employed by these two motifs largely remain unknown (Fig. 1).

SIGNALING CROSSTALK BETWEEN RANK AND OTHER FACTORS IN OSTEOCLASTS

Like most membrane-bound receptors, RANK does not act alone in transmitting intracellular signals. Since the unraveling of the RANKL/RANK system, it has become clear that many factors, which were previously known to be able to regulate osteoclast differentiation, function and survival, actually exert their effects on osteoclasts/osteoclast precursors by either synergizing with or cross-talking with RANK signaling pathways. Most notably, it has been recently shown that several factors critically involved in regulating immune function such as IFN- γ , IFN- β and ITAM-mediated signals play important roles in modulating osteoclastogenesis by cross-talking with RANK signaling.

IFNs

IFNs are a family of cytokines that play important roles in the immune response and are classified into two major types, primarily based on receptor specificity and sequence homology (33). The type I IFNs consist of four subtypes: IFN- α , IFN- β , IFN- ω and IFN- τ . The type I IFNs are structurally related and share a common heterodimeric receptor comprising IFNAR1 and IFNAR2 chains. Both IFNAR1 and IFNAR2 chains are able to transduce signals and mediate the biological effects of type I IFNs (33) (Fig. 2). In contrast, IFN- γ is the only Type II IFN identified so far. IFN- γ is structurally unrelated to type I IFNs and it exerts its biological functions by binding to a tetrameric receptor consisting of two ligand-binding IFNGR1 chains and two signal-transducing IFNGR2 chains (33) (Fig. 2). Among these distinct members of the IFN family, only two IFNs, IFN- γ and IFN- β , have been shown to be able to modulate osteoclastogenesis.

IFN- γ and Osteoclastogenesis: while IFN- γ is best known as an important cytokine involved in immune function, it was shown almost 20 years ago that IFN- γ also exerts an inhibitory effect on osteoclastogenesis (34). However, the molecular mechanism by which IFN- γ blocks osteoclastogenesis remained obscure until recently. In 2000, Takayanagi and co-workers demonstrated that IFN- γ suppresses osteoclastogenesis by inducing rapid degradation of TRAF6 (35). As shown in Fig. 2, binding of IFN- γ to IFNGR1 (ligand binding chain of the IFN- γ receptor) leads to the recruitment of the signal transducer and activator of transcription (Stat) 1 to IFNGR2 (signaling chain of the IFN- γ receptor), leading to activation of Stat1. Activated Stat1 will translocate into the nucleus to stimulate the expression of the proteasome activators PA28 α and PA28 β to activate proteasome (35) (Fig. 2). In addition, activated Stat1 also activates the expression of an unknown gene(s) whose product(s) is/are required to mediate the poly-ubiquitination of TRAF6 (Fig. 2). Together, these two biological events trigger rapid degradation of TRAF6, leading to blockage of osteoclastogenesis (Fig. 2).

The work of Takayanagi and co-workers provides an interesting explanation of how IFN- γ inhibits osteoclastogenesis. This explanation is primarily based on the premise that TRAF6 is essential for osteoclastogenesis. However, it has been controversial whether TRAF6 is essential for osteoclastogenesis. Two TRAF6 knockout mice were generated by different laboratories and the two groups reached opposite conclusions regarding the role of TRAF6 in osteoclastogenesis (36, 37). Both groups showed that their TRAF6^{-/-} mice developed osteopetrosis due to impaired bone resorption, supporting an important role for TRAF6 in bone remodeling. But, one group showed that deletion of TRAF6 completely blocked osteoclastogenesis (37) while the other group demonstrated that the absence of TRAF6 impaired only osteoclast

function without affecting osteoclastogenesis (36). The recent identification of three RANK motifs capable of independently mediating osteoclastogenesis supports that TRAF6 is involved in but not essential for osteoclastogenesis, because only Motif 1 has been shown to utilize TRAF6 (Fig. 1). A functional assay showed that mutation of the TRAF6-binding RANK motif (Motif 1) did not affect osteoclast formation *in vitro* (24). Given the uncertainty, additional studies, better done by other independent groups, are needed to further clarify the role of TRAF6 in osteoclastogenesis. More studies may also be necessary to further investigate the molecular mechanism by which IFN- γ blocks osteoclastogenesis.

IFN- β and Osteoclastogenesis: while it has been known almost for 20 years that IFN- γ plays an inhibitory role in osteoclastogenesis, the involvement of IFN- β in osteoclastogenesis was only recently elucidated. The investigation of the functional role of IFN- β in osteoclast biology was prompted primarily by an *in vitro* finding that the IFN- β gene is one of the RANKL-inducible genes in osteoclast precursors (38). Subsequent *in vivo* studies with knockout mice deficient for IFNAR1 or IFN- β demonstrated a reduction in bone mass due to an elevated number of osteoclasts in these mice, revealing an inhibitory role of IFN- β in osteoclastogenesis (38).

Consistent with the *in vivo* observations, IFN- β exhibited a strong inhibitory effect on *in vitro* osteoclastogenesis from primary osteoclast precursors treated with RANKL and M-CSF (38). A series of *in vitro* and *in vivo* experiments further revealed that IFN- β negatively regulates osteoclastogenesis by a negative feedback mechanism involving c-fos (38), a critical factor involved in osteoclastogenesis (39). Specifically, IFN- β inhibits osteoclastogenesis by down-regulating c-fos expression (38). The IFN- β -mediated suppression of c-fos results from inhibition of c-fos synthesis rather than from transcriptional down-regulation. Figure 2 shows the detailed pathway leading to blockage of c-fos expression. RANKL activates the expression of c-fos by an unknown mechanism. c-fos then transcriptionally stimulates the expression of IFN- β in osteoclast precursors. As a result, IFN- β is then produced and secreted by osteoclast precursors and secreted IFN- β in turn binds to and activates IFN- β receptor in osteoclast precursors. Activated IFN- β receptor then recruits and activates the transcription factor ISGF3 (a heterotrimeric complex comprising Stat1, Stat2 and interferon regulatory factor (IRF)-9). Activated ISGF3 will induce the expression of PKR (double-stranded-RNA-activated protein kinase), an inhibitor of protein synthesis. PKR then inhibits translation of c-fos from its mRNA (38), ultimately leading to inhibition of osteoclastogenesis (Fig. 2).

The unraveling of the signaling crosstalk between the RANKL/RANK system and IFN- β represents an important step forward in the understanding of the molecular mechanism underlying osteoclast differentiation. More importantly, the new finding also supports that IFN- β may be used as a new therapeutic agent for treating bone loss associated with

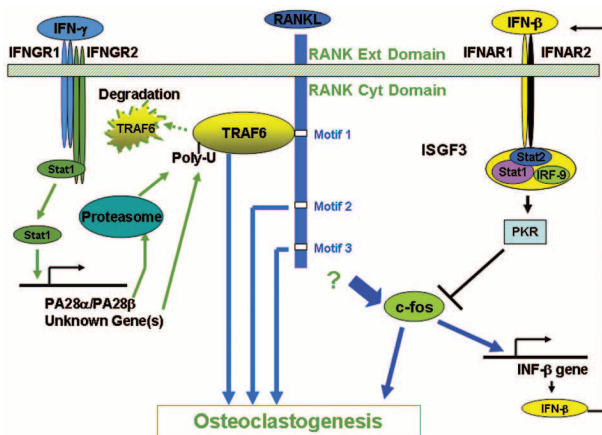


Figure 2. Signaling crosstalk between RANK and IFN- γ /IFN- β . Poly-U: poly-ubiquitination; Ext: external; Cyt: cytoplasmic.

various diseases. Notably, although the expression $\text{INF-}\alpha$ is not regulated by RANKL, $\text{INF-}\alpha$ may also serve as an additional therapeutic agent for treating bone diseases since $\text{INF-}\beta$ and $\text{INF-}\alpha$ share the same signaling receptor. Furthermore, given that ISGF3 is critically involved in $\text{INF-}\gamma$ -mediated inhibition in osteoclastogenesis, future investigation of skeletal abnormality in mice lacking Stat1 and IRF-1 will provide additional evidence supporting the functional role of $\text{INF-}\beta$ in osteoclastogenesis.

ITAM-Activated Costimulatory Signals

ITAM is a unique and semi-conserved protein module existing in a large number of immunoreceptors and signaling adaptors (40). The module consists of two repeats of the conserved sequence Tyr-X-X-Leu/Ile spaced by six-to-eight residues and it functions as ‘on and off’ switch that transmitting intracellular signaling. The engagement of ITAM-containing receptors/adaptors results in a rapid and transient phosphorylation of the ITAM’s tyrosine residues that function as temporal scaffolds for Src homology 2 (SH2) domains of downstream signaling proteins. Recruitment and binding of these signaling proteins to phospho-ITAMs initiate a cascade of signaling events that lead to the modulation of immune cells. ITAM-mediated costimulatory signals play important roles in immune functions and one of the major pathways activated by ITAM-mediated costimulatory signals is the transcription factor NFAT (41).

The investigation of the potential role of ITAM-mediated costimulatory signals in osteoclastogenesis was triggered by an earlier study showing that NFATc1 is an essential factor involved in osteoclastogenesis. In addition, RANKL can only lead to a partial activation of NFATc1 in osteoclast precursors (31), suggesting that other unidentified signaling receptors/adaptors may work in concert with RANKL to induce full activation of NFATc1. Based on a recent study by Koga and coworkers (42), ITAM in two adaptor molecules, Fc receptor

common μ subunit ($\text{FcR}\gamma$) and DNAX-activating protein (DAP) 12, play an important role in the activation of NFATc1 in osteoclast precursors (42) (Fig. 3). To this end, $\text{FcR}\gamma$ may associate with OSCAR (osteoclast-associated receptor) and/or PIR-A (paired immunoglobulin-like receptor) while DAP12 may partner with TREM-2 (triggering receptor expressed by myeloid cells) and/or SIRP β 1 (signal-regulatory protein β 1) (42). Full activation (phosphorylation) of these ITAMs requires signals from both the immunoreceptors and RANK signaling. However, the specific tyrosine kinase(s) activated by RANKL to phosphorylate ITAM remain unknown (Fig. 3). Phosphorylated ITAMs serve as docking sites for SH2 containing signaling molecule Syk, which will be recruited to and then activated by the ITAM. Activated Syk will promote activation of the phospholipase $\text{C}\gamma$ ($\text{PLC}\gamma$)-calcium pathway, eventually leading to the activation of NFATc1 (Fig. 3).

The revelation of functional involvement of ITAM-mediated signals in osteoclastogenesis helps explain why three RANK motifs can independently mediate osteoclastogenesis. It has been established that NFATc1 is essential for osteoclastogenesis (31). However, among the three functional RANK motifs, only Motif 1 can interact with TRAF6, which is able to mediate a partial activation of NFATc1 (31) (Fig. 3). Motif 2 and Motif 3 must employ different mechanisms to activate NFATc1. It is reasonable to hypothesize that Motif 2 and Motif 3 recruit a TRAF protein (TRAF 1, 2, 3, or 5) to activate a common or different tyrosine kinase(s) that phosphorylates and activates ITAM in $\text{FcR}\gamma$ and DAP12. As such, each of the three functional RANK motifs can independently mediate a partial activation of NFATc1. However, full activation of NFATc1 requires signals from all these three motifs. This is consistent with the functional study demonstrating that each of the three RANK motifs is only able to mediate a partial osteoclastogenesis (24). Future studies aimed at testing this hypothesis and/or identifying the tyrosine kinase(s) will greatly advance our understanding of RANK signaling in osteoclast biology.

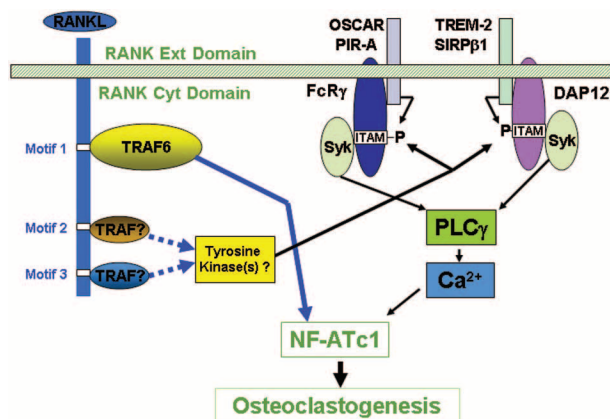


Figure 3. Signaling crosstalk between RANK and ITAM-harboring immunoreceptors.

CONCLUDING REMARKS

In the past several years, we have witnessed many great advances in our understanding of the molecular mechanism controlling osteoclast differentiation, function and survival. In particular, recent findings revealed additional complexity of RANK signaling in osteoclasts. Moreover, emerging evidence has also elucidated significant signaling crosstalk between RANK signaling and those activated by immunoreceptor/adaptors. Nevertheless, the signaling pathways activated by RANK and the various immunoreceptor/adaptors in osteoclasts and/or its precursors have not been fully elucidated and many critical questions remain to be answered. For instance, it has been established that the RANKL/RANK system is involved in activating c-fos expression in osteoclast precursors, but the precise mechanism underlying the RANK-mediated c-

fos expression has not been elucidated. Furthermore, how exactly RANK stimulates the phosphorylation of the tyrosine residues in ITAMs also remains unclear. Thus, future efforts should be focused on addressing these important questions. Notably, previous studies have also created a few considerable controversies regarding the signaling by RANK. Therefore, future studies may also need to be directed to resolve these controversies.

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