C/EBPα transcription factor is regulated by the RANK cytoplasmic ^{535}IVVY^{538} motif and stimulates osteoclastogenesis more strongly than c-Fos

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Binding of receptor activator of NF-κB ligand (RANKL) to its receptor RANK on osteoclast (OC) precursors up-regulates c-Fos and CCAAT/enhancer–binding protein-α (C/EBPα), two critical OC transcription factors. However, the effects of c-Fos and C/EBPα on osteoclastogenesis have not been compared. Herein, we demonstrate that overexpression of c-Fos or C/EBPα in OC precursors up-regulates OC genes and initiates osteoclastogenesis independently of RANKL. However, although C/EBPα up-regulated c-Fos, c-Fos failed to up-regulate C/EBPα in OC precursors. Consistently, C/EBPα overexpression more strongly promoted OC differentiation than did c-Fos overexpression. RANK has a cytoplasmic ^{535}IVVY^{538} (IVVY) motif that is essential for osteoclastogenesis, and we found that mutation of the IVVY motif blocked OC differentiation by partly inhibiting expression of C/EBPα but not expression of c-Fos. We therefore hypothesized that C/EBPα overexpression might rescue osteoclastogenesis in cells expressing the mutated IVVY motif. However, overexpression of C/EBPα or c-Fos failed to stimulate osteoclastogenesis in the mutant cells. Notably, the IVVY motif mutation abrogated OC gene expression compared with a vector control, suggesting that the IVVY motif might counteract OC inhibitors during osteoclastogenesis. Consistently, the IVVY motif mutant triggered up-regulation of recombinant recognition sequence–binding protein at the Jκ site (RBP-J) protein, a potent OC inhibitor. Mechanistically, C/EBPα or c-Fos overexpression in the mutant cells failed to control the up-regulated RBP-J expression, leading to suppression of OC genes. Accordingly, RBP-J silencing in the mutant cells rescued osteoclastogenesis with C/EBPα or c-Fos overexpression with C/EBPα exhibiting a stronger osteoclastogenic effect. Collectively, our findings indicate that C/EBPα is a stronger inducer of OC differentiation than c-Fos, partly via C/EBPα regulation by the RANK ^{535}IVVY^{538} motif.

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This article contains Figs. S1–S4.

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Osteoclasts (OCs)[3] are multinucleated giant cells that are responsible for bone resorption (1–3). Through their bone-resorbing functions, these polykaryons carry essential roles in skeletal development and bone homeostasis. As such, deregulated OC differentiation has been regarded among the main causes of many osteolytic bone disorders (4, 5). OCs originate from the macrophage lineage upon stimulation by the macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB (RANK) ligand (RANKL) (5, 6). M-CSF acts through its receptor, colony-stimulating factor 1 receptor, to promote the proliferation of OC precursors, and RANKL activates its receptor RANK on OC precursors to mediate OC lineage commitment and differentiation. Importantly, RANK contains a cytoplasmic ^{535}IVVY^{538} (IVVY) motif that is critical for OC differentiation both *in vivo* and *in vitro* (7–10). Targeting of the RANK IVVY motif has been shown to prevent bone destruction in mouse models of inflammatory and ovariectomy-induced bone loss (10). These findings are underscored by a genetic study reporting that a truncating mutation causing the loss of a RANK region containing the IVVY motif results in osteopetrosis in humans (11).

RANKL mediates osteoclastogenesis by inducing the expressions of key transcription factors, including FBJ osteosarcoma oncogene (c-Fos) and nuclear factor of activated T cells, C1 (NFATc1) (12–15). NFATc1 is regarded as a master regulator of OC differentiation that plays essential roles in inducing the expressions of many OC genes, including cathepsin K (*Ctsk*) and tartrate-resistant acid phosphatase (*TRAP*) (16–18). Mice deficient in the *NFATc1* gene (*NFATc1*$^{-/-}$ mice) exhibit a severe osteopetrotic phenotype due to defective OC development (14). Likewise, mice deficient in the c-*Fos* gene

[3] The abbreviations used are: OC, osteoclast; ACTD, actinomycin D; C/EBP, CCAAT/enhancer–binding protein; CHX, cycloheximide; c-Fos, FBJ osteosarcoma oncogene; Ctsk, cathepsin K; Fas-RANK, a chimeric receptor system with the human Fas extracellular domain linked to the normal mouse RANK transmembrane and intracellular domains; Fas-mIVVY, a chimeric receptor system with the human Fas extracellular domain linked to the mouse RANK transmembrane and intracellular domains bearing an inactivating mutation in the cytoplasmic ^{535}IVVY^{538} motif; Hprt, hypoxanthine-guanine phosphoribosyltransferase; IRF-8, interferon-regulatory factor 8; ITAM, immunoreceptor tyrosine-based activation motif; MBM, mouse bone marrow; M-CSF, macrophage colony-stimulating factor; NFATc1, nuclear factor of activated T cells, C1; RANK, receptor activator of NF-κB; RANKL, receptor activator of NF-κB ligand; RBP-J, recombinant recognition sequence–binding protein at the Jκ site; TRAP, tartrate-resistant acid phosphatase; qPCR, quantitative real-time PCR; TSS, transcriptional start site; hFas, human Fas.

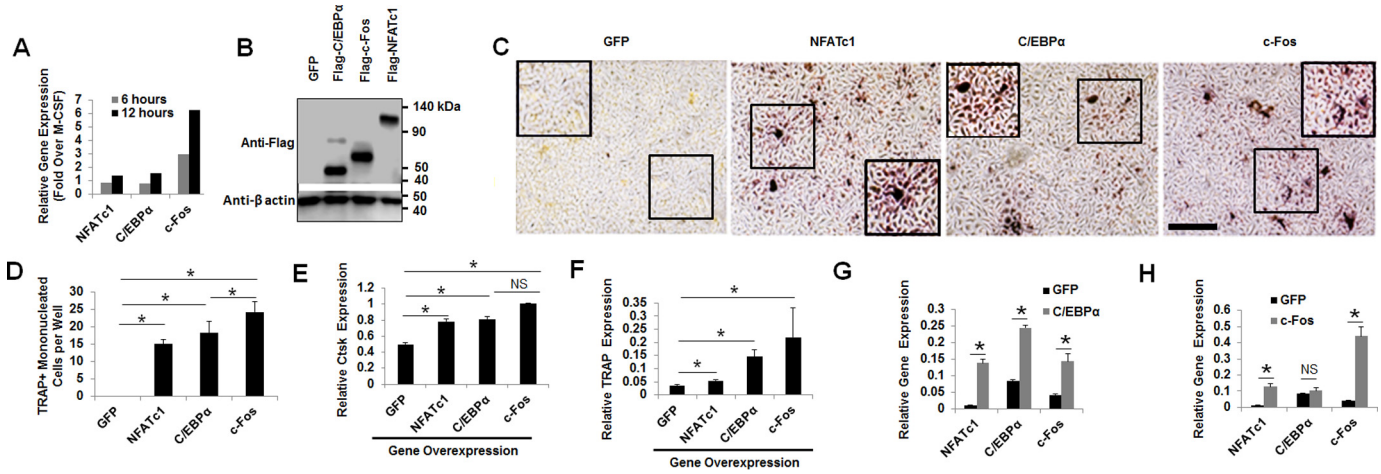

Figure 1. C/EBPα or c-Fos can initiate osteoclastogenesis independently of RANKL. *A*, MBM cells were transiently stimulated with M-CSF (10 ng/ml) or M-CSF (10 ng/ml) and RANKL (10 ng/ml) for 6 or 12 h. Gene expression was examined by qPCR. *B–D*, MBM cells expressing a GFP control, FLAG-NFATc1, FLAG-C/EBPα, or FLAG-c-Fos were treated with M-CSF (10 ng/ml) for 4 days for Western blot analysis using β-actin as a loading control from at least three independent experiments (*B*) or TRAP staining for osteoclastogenesis (*C*). *Scale bar*, 250 μm. Quantification for *C* is shown as mean number of mononucleated TRAP-positive cells per well from at least three independent experiments (*D*). *E* and *F*, MBM cells expressing GFP, NFATc1, C/EBPα, or c-Fos were cultured with M-CSF (10 ng/ml) for 4 days for analysis of the expressions of Ctsk (*E*) and TRAP (*F*) by qPCR. *G*, MBM cells expressing GFP or C/EBPα were treated with M-CSF (10 ng/ml) for 4 days. *H*, MBM cells expressing GFP or c-Fos were treated with M-CSF (10 ng/ml) for 4 days. Gene expressions in *A*, *D*, *E*, *F*, *G*, and *H* were assessed by qPCR using Hprt as a loading control in three independent experiments. *Error bars* show averages ± S.D. *, $p < 0.05$; *NS*, not significant.

(c-*Fos*$^{-/-}$ mice) also exhibit osteopetrosis from impaired OC development (13). However, unlike the *NFATc1*$^{-/-}$ mice, most c-*Fos*$^{-/-}$ mice show almost a normal lifespan despite other issues associated with delayed or absent gametogenesis, lymphocythemia, and altered behavior, indicating that c-Fos may not be required for the growth of most cell types during development. With regard to the molecular basis of its role during osteoclastogenesis, c-Fos can induce the expression of *NFATc1*, establishing *NFATc1* as a target gene of c-*Fos* in OC (19).

CCAAT/enhancer–binding protein-α (C/EBPα) is a transcription factor of the C/EBP family of transcription factors that is crucial for hematopoiesis through its ability to bind on gene promoters to regulate gene expression in a lineage-specific manner (20–22). Global deletion of the *C/EBPα* gene in mice (*C/EBPα*$^{-/-}$ mice) results in early death from defective granulocyte development and impaired homeostasis (23, 24). Recently, our group has reported that C/EBPα is strongly up-regulated by RANKL during osteoclastogenesis and carries a critical function in osteoclastogenesis by appointing OC precursor cells to the OC lineage (25). We showed that C/EBPα overexpression in OC precursors can induce the expressions of various OC markers, including NFATc1, independently of RANKL and thereby promotes lineage commitment. Moreover, we have recently reported that C/EBPα is also important for OC differentiation and function (26). Consistent with this posture, we revealed that newborn *C/EBPα*$^{-/-}$ mice are osteopetrotic due to impaired OC development (25).

Although the transcription factors C/EBPα and c-Fos are both critical for osteoclastogenesis *in vitro* and *in vivo* and are essential for the expressions of OC markers, including NFATc1, Ctsk, and TRAP, during OC differentiation (12, 13, 25, 27), the effects of their roles in osteoclastogenesis have not yet been compared. Moreover, although C/EBPα is critical for OC lineage commitment, a process that was shown to be regulated by the RANK IVVY motif (8), it remains unknown whether the RANK IVVY motif can also regulate *C/EBPα* expression during osteoclastogenesis. In the current study, we compared the roles of c-Fos and C/EBPα in OC differentiation by using a gain-of-function strategy in a RANK IVVY motif–dependent manner. This study not only enhances our understanding of the roles of c-Fos and C/EBPα in OC differentiation but also provides important insight into the role of RANK signaling and transcription factors in OC differentiation.

Results

C/EBPα overexpression shows a stronger osteoclastogenic effect than c-Fos overexpression

c-Fos is a critical transcription factor for osteoclastogenesis (12, 13). Likewise, C/EBPα is also an essential transcription factor for osteoclastogenesis (25, 26). In comparing the roles of C/EBPα and c-Fos in osteoclastogenesis, we first compared their requirements for OC lineage commitment by using their target gene, *NFATc1*, as a positive control (Fig. 1). Transient stimulation of mouse bone marrow (MBM) cells, widely used as primary OC precursors, with M-CSF and RANKL was shown to up-regulate NFATc1, c-Fos, and C/EBPα during osteoclastogenesis (14–16, 25). We recapitulated this finding and confirmed that stimulation of MBM cells by M-CSF and RANKL for 12 h could up-regulate the expressions of *NFATc1*, *C/EBPα*, and c-*Fos* as compared with M-CSF alone (Fig. 1*A*), supporting their involvement in early stages of osteoclastogenesis. In line with this notion, *NFATc1* (14), c-*Fos* (12), and *C/EBPα* (25) were each shown to be able to appoint OC precursors to the OC lineage. Hence, we examined the effect of overexpressing C/EBPα or c-Fos in inducing OC lineage priming as compared with NFATc1 overexpression (Fig. 1, *B–D*). In overexpressing the genes in OC precursor cells, we used the 293GPG retroviral system, which has been extensively used to study various genes in OC biology through gene overexpression by using the pMX retroviral plasmid (28, 29). Using a pMX-puro-GFP control

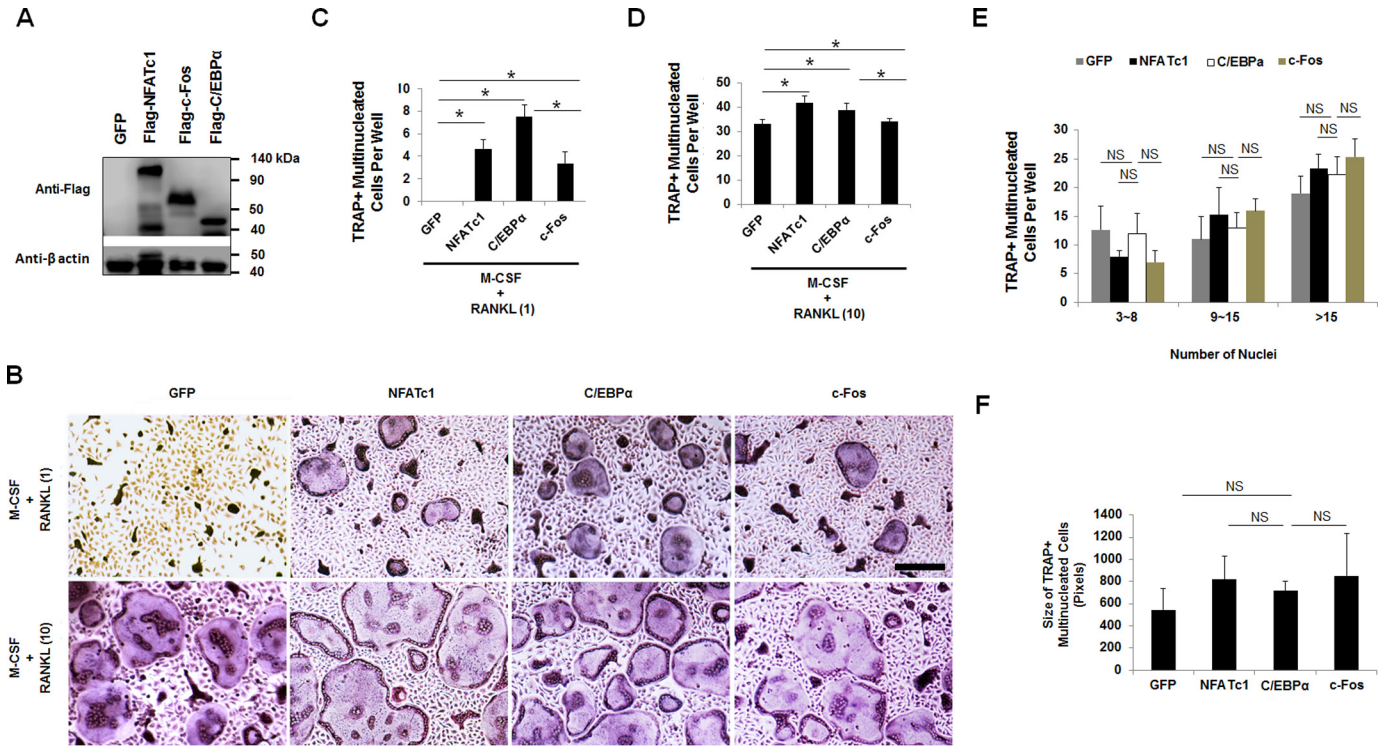

Figure 2. C/EBPα is a stronger inducer of OC differentiation than c-Fos. *A*, MBM cells expressing a GFP control, FLAG-NFATc1, FLAG-C/EBPα, or FLAG-c-Fos were cultured with M-CSF (10 ng/ml) for 4 days for Western blot analysis using β-actin as a loading control from at least three independent assays. *B*, MBM cells expressing GFP, FLAG-NFATc1, FLAG-C/EBPα, or FLAG-c-Fos were cultured with M-CSF (10 ng/ml) and RANKL (1 ng/ml) or M-CSF (10 ng/ml) and RANKL (10 ng/ml) for 4 days and then stained for TRAP activity. *Scale bar*, 250 μm. *C* and *D*, quantifications for *B* are shown for M-CSF and RANKL (1 ng/ml) (*C*) and M-CSF and RANKL (10 ng/ml) (*D*) as mean number of multinucleated TRAP-positive cells per well from at least three independent assays. *E* and *F*, quantifications of OC size for M-CSF and RANKL (10 ng/ml) shown in *B* via the number of nuclei (*E*) or area (*F*) of multinucleated TRAP-positive cells. The *numbers in parentheses* show concentrations in ng/ml. *Error bars* show averages ± S.D. *, $p < 0.05$; *NS*, not significant.

vector, we generated a retrovirus encoding the GFP cDNA to infect MBM cells. We showed that GFP was highly expressed on days 2, 3, and 4 postinfection (Fig. S1), confirming that this retroviral system can sustain high gene expression for our standard osteoclastogenesis assays (6, 25, 26). We showed that overexpression of C/EBPα, c-Fos, or NFATc1 in MBM cells, as confirmed by Western blot analysis (Fig. 1*B*), could initiate osteoclastogenesis by generating TRAP-positive mononucleated cells without RANKL stimulation as compared with a GFP control (Fig. 1, *C* and *D*). This finding recapitulates the previous reports that C/EBPα (25), c-Fos (12), and NFATc1 (14) can all induce lineage priming. In addressing the molecular basis of this finding, we showed that overexpression of C/EBPα, c-Fos, or NFATc1 could significantly up-regulate the OC genes encoding Ctsk (Fig. 1*E*) and TRAP (Fig. 1*F*) as compared with a GFP control in the absence of RANKL (27). These results confirmed the previous reports that C/EBPα and c-Fos could induce the expressions of OC marker genes and thereby promote OC lineage commitment.

Next, in comparing the abilities of C/EBPα and c-Fos to mediate OC lineage commitment, we examined their abilities to up-regulate each other independently of RANKL. Whereas *C/EBPα* overexpression could induce the expressions of c-*Fos* and its target gene *NFATc1* in MBM cells without RANKL stimulation (Fig. 1*G*), c-*Fos* overexpression could only induce the expression of its target gene *NFATc1*, but not that of *C/EBPα*, in the absence of RANKL (Fig. 1*H*). This finding indicated that c-*Fos* is a target gene of *C/EBPα* during osteoclastogenesis. As a control, NFATc1 overexpression in MBM cells was unable to up-regulate C/EBPα and c-Fos in the absence of RANKL (data not shown), confirming that NFATc1 is a target of C/EBPα and c-Fos during OC differentiation and was thus unable to up-regulate these genes.

Subsequently, we compared the roles of C/EBPα and c-Fos in OC differentiation by using a gain-of-function strategy (Fig. 2). Treatment of MBM cells with permissive levels (one-tenth of the amount required for normal osteoclastogenesis) of RANKL was shown to mediate OC lineage commitment but was insufficient to promote OC differentiation (30, 31). We utilized this approach to examine the roles of C/EBPα and c-Fos overexpression in OC differentiation as compared with NFATc1 overexpression. Our standard osteoclastogenesis assay requires the treatment of MBM cells with M-CSF and 10 ng/ml RANKL for 4 days (25, 26). Therefore, we overexpressed C/EBPα or c-Fos in the presence of M-CSF and RANKL (1 ng/ml) to promote OC differentiation with RANKL-evoked lineage priming. The data showed that overexpression of C/EBPα in MBM cells, as confirmed by Western blotting (Fig. 2*A*), could promote OC differentiation with permissive RANKL dosages as compared with a GFP control (Fig. 2*B*), confirming our recent report that C/EBPα is not only involved in OC lineage priming but can also promote OC differentiation (26). Comparatively, we noted that although overexpression of C/EBPα, c-Fos, or NFATc1 in MBM cells could each promote OC differentiation with 1 ng/ml RANKL (Fig. 2*B*), the C/EBPα overexpressers generated significantly more OCs than c-Fos overexpression or the GFP control

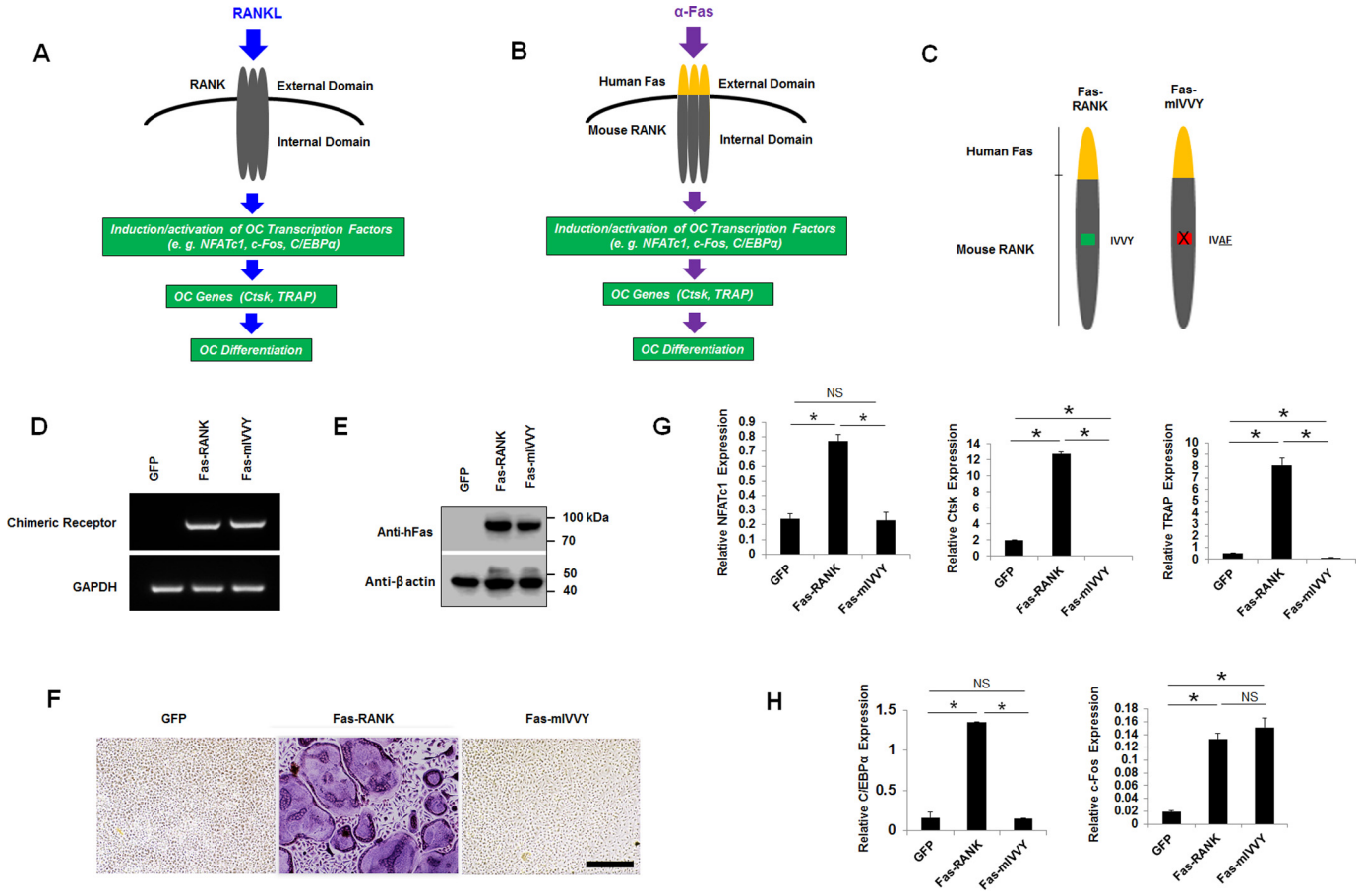

Figure 3. Mutational inactivation of the RANK cytoplasmic IVVY motif blocks osteoclastogenesis and inhibits C/EBPα, but not c-Fos, expression. *A*, a schematic of RANK activation by RANKL to induce OC differentiation. *B*, a schematic of the chimeric receptor system, which can be specifically activated by α-Fas to stimulate OC differentiation. *C*, schematics of Fas-RANK and Fas-mIVVY. *D* and *E*, MBM cells expressing a GFP control, Fas-RANK, or Fas-mIVVY were treated with M-CSF (10 ng/ml) for 4 days for gene expression analysis by RT-PCR (*D*) and Western blotting (*E*) using GAPDH and β-actin as loading controls, respectively. *F*, MBM cells expressing GFP, Fas-RANK, or Fas-mIVVY were treated with M-CSF (10 ng/ml) and α-Fas (100 ng/ml) for 4 days. Cultures from at least three independent assays were then stained for TRAP activity. *Scale bar*, 250 μm. *G* and *H*, MBM cells expressing GFP, Fas-RANK, or Fas-mIVVY were treated with M-CSF (10 ng/ml) and α-Fas (100 ng/ml) for 2 days before being submitted to qPCR analysis using Hprt as a loading control in three independent repeats. *Error bars* show averages ± S.D. *, $p < 0.05$; *NS*, not significant.

(Fig. 2*C*). Consistently, the C/EBPα overexpressers also formed significantly more OCs with stimulation by 10 ng/ml RANKL than the cells overexpressing c-Fos or expressing GFP control (Fig. 2, *B* and *D*). These results indicated that C/EBPα exhibited a stronger role than c-Fos in mediating OC differentiation from the RANKL-precommitted cells. However, we found that C/EBPα overexpression did not influence OC size as compared with the GFP control as well as c-Fos or NFATc1 overexpression (Fig. 2, *E* and *F*).

Mutational inactivation of the RANK IVVY motif blocks OC differentiation and inhibits C/EBPα, but not c-Fos, expression

Activation of the RANK IVVY motif, through RANKL-induced RANK activation, induces the expressions of Ctsk and TRAP during OC differentiation (8–10). In further comparing the roles of C/EBPα and c-Fos in OC differentiation, we investigated whether the RANK IVVY motif could regulate their expressions during OC differentiation (Fig. 3). RANK and Fas are members of the tumor necrosis factor receptor superfamily, which are activated by ligand-induced receptor trimerization (32). Upon binding to RANK, RANKL triggers RANK trimerization, leading to signal transduction for induction of OC transcription factors and genes to promote OC differentiation (Fig. 3*A*) (7, 32). Xu *et al.* (33) developed a chimeric receptor system that consists of the human Fas extracellular domain linked to the transmembrane and cytoplasmic domains of mouse RANK to study RANK signaling during osteoclastogenesis. The chimeric receptor system can be exclusively activated by a human Fas-activating antibody (α-Fas) in a similar fashion as RANKL to induce OC transcription factors and genes for OC differentiation (Fig. 3*B*). In this study, we utilized two chimeras (Fas-RANK and Fas-mIVVY) that were previously generated and validated by Xu *et al.* (8). Fas-RANK is constructed with the human Fas extracellular domain linked to the transmembrane and intracellular domains of normal mouse RANK, and Fas-mIVVY has the human Fas extracellular domain linked to the transmembrane and intracellular domains of mouse RANK containing an inactivating mutation in the IVVY motif (Fig. 3*C*). Following retrovirally induced gene expression, the chimeras were shown to be highly expressed on the cell surface of OC precursors and respond successfully to stimulation by α-Fas and M-CSF to promote OC differentiation in a similar fashion as the RANKL/RANK system (8, 33–37). MBM cells expressing a GFP control, Fas-RANK, or Fas-mIVVY, as confirmed by

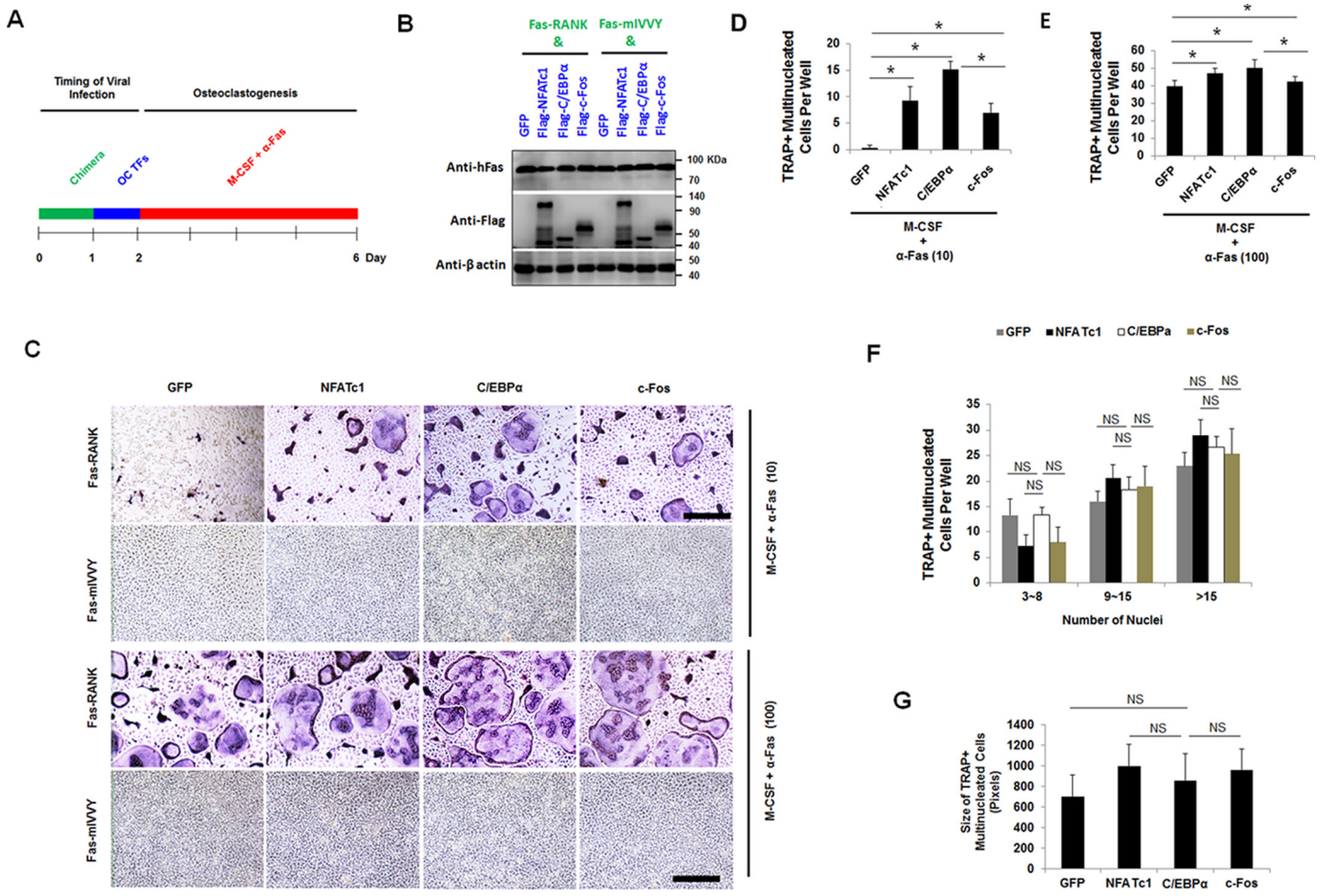

Figure 4. C/EBPα or c-Fos fails to mediate osteoclastogenesis with mutational inactivation of the RANK cytoplasmic IVVY motif. *A*, experimental strategy. *B*, MBM cells doubly expressing Fas-RANK or Fas-mIVVY and a GFP control, FLAG-NFATc1, FLAG-C/EBPα, or FLAG-c-Fos were cultured with M-CSF (10 ng/ml) for 4 days for Western blotting using β-actin as a loading control. *C*, MBM cells were cultured as in *B* but treated with M-CSF (10 ng/ml) and α-Fas (10 ng/ml) or M-CSF (10 ng/ml) and α-Fas (100 ng/ml) for 4 days to stimulate OC differentiation. The cultures were then stained for TRAP activity. *Scale bars*, 250 μm. *D* and *E*, quantifications for *C* are shown for M-CSF and α-Fas (10 ng/ml) (*D*) or M-CSF and α-Fas (100 ng/ml) (*E*) as mean number of multinucleated TRAP-positive cells per well from at least three independent assays. *F* and *G*, quantification of OC size for M-CSF and α-Fas (100 ng/ml) for the Fas-RANK expressers shown in *C* via the number of nuclei (*F*) or area (*G*) of multinucleated TRAP-positive cells per well. The *numbers in parentheses* show concentrations in ng/ml. *Error bars* show averages ± S.D. *, $p < 0.05$; *NS*, not significant. *TFs*, transcription factors.

reverse transcription (RT)-PCR (Fig. 3*D*) and Western blotting (Fig. 3*E*), were stimulated with M-CSF and α-Fas to promote OC differentiation (Fig. 3*F*). We recapitulated the findings of the previous studies and showed that only the cells expressing Fas-RANK, but not the Fas-mIVVY expressers, could generate OCs with stimulation by M-CSF and α-Fas (Fig. 3*F*) (8–10). Furthermore, we used the macrophage RAW264.7 cell line and confirmed that RAW264.7 cells expressing Fas-RANK, but not the Fas-mIVVY expressers, could mediate osteoclastogenesis with α-Fas stimulation (Fig. S2). At the molecular level, our quantitative real-time PCR (qPCR) analysis confirmed that mutational inactivation of the RANK IVVY motif significantly abrogated the expressions *NFATc1*, *Ctsk*, and *TRAP* during OC differentiation (34–36) (Fig. 3*G*). Importantly, we also found that inactivation of the RANK IVVY motif significantly attenuated the expression of *C/EBPα*, but exhibited no overt effect on c-*Fos* expression, during OC differentiation (Fig. 3*H*). These results indicated that the RANK IVVY motif promoted OC differentiation in part by regulating *C/EBPα* expression but was dispensable to c-*Fos* expression.

Overexpression of C/EBPα or c-Fos in cells expressing mutated RANK IVVY motif fails to mediate OC differentiation

Given that inactivation of the RANK IVVY motif blocked OC differentiation and inhibited *NFATc1* and *C/EBPα*, but not c-*Fos*, expression (Fig. 3), we reasoned that overexpression of C/EBPα or NFATc1 might rescue OC differentiation in MBM cells expressing the mutated RANK IVVY motif. In addressing this issue, MBM cells doubly expressing Fas-RANK or Fas-mIVVY and a GFP control, NFATc1, C/EBPα, or c-Fos (Fig. 4*A*), as confirmed by Western blotting (Fig. 4*B*), were stimulated with permissive (10 ng/ml) or optimum (100 ng/ml) levels of α-Fas in the presence of M-CSF for 4 days as validated in previous studies to promote OC differentiation (34–36) (Fig. 4*C*). As expected, MBM cells doubly expressing Fas-RANK and C/EBPα, c-Fos, or NFATc1 generated numerous OCs with either 10 (Fig. 4, *C* and *D*) or 100 ng/ml (Fig. 4, *C* and *E*) α-Fas as compared with the GFP control. Notably, overexpression of C/EBPα in the Fas-RANK expressers generated significantly more OCs than the c-Fos and Fas-RANK double expressers (Fig. 4, *D* and *E*), further confirming that C/EBPα exhibited a

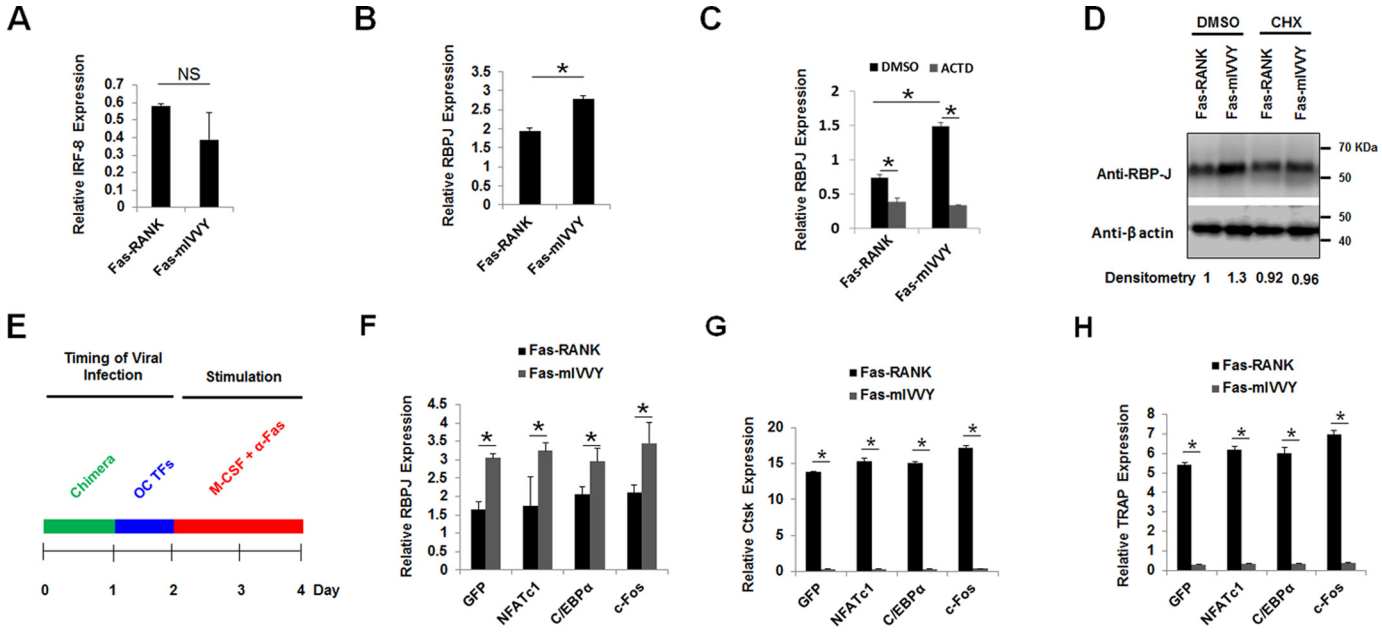

Figure 5. Mutational inactivation of the RANK cytoplasmic IVVY motif represses OC genes but up-regulates RBP-J. *A* and *B*, MBM cells expressing a GFP control, Fas-RANK, or Fas-mIVVY were treated with M-CSF (10 ng/ml) and α-Fas (100 ng/ml) for 2 days. The expressions of *IRF-8* (*A*) and *RBP-J* (*B*) were examined by qPCR. *C*, MBM cells expressing Fas-RANK or Fas-mIVVY were cultured with M-CSF (10 ng/ml) for 3 days before being treated with ACTD (1 μg/ml) for 12 h while undergoing stimulation by M-CSF (10 ng/ml) and α-Fas (100 ng/ml). The expression of *RBP-J* was then examined by qPCR. *D*, MBM cells expressing Fas-RANK or Fas-mIVVY were cultured with M-CSF (10 ng/ml) for 3 days before being treated with CHX (5 μg/ml) for 12 h while undergoing stimulation by M-CSF (10 ng/ml) and α-Fas (100 ng/ml). The expression of RBP-J was then examined by Western blotting using β-actin as a loading control in three independent experiments. *E*, experimental strategy for *F–H*. *F–H*, MBM cells doubly expressing Fas-RANK or Fas-mIVVY and GFP, FLAG-NFATc1, FLAG-C/EBPα, or FLAG-c-Fos were treated with M-CSF (10 ng/ml) and α-Fas (100 ng/ml) as indicated in *E*. The expressions of *RBP-J* (*F*), *Ctsk* (*G*), and *TRAP* (*H*) were examined by qPCR. The qPCR analyses in *A*, *B*, *C*, *F*, *G*, and *H* were performed using Hprt as a loading control in three independent experiments. *Error bars* show averages ± S.D. *, $p < 0.05$; *NS*, not significant. *TFs*, transcription factors.

stronger osteoclastogenic effect than c-Fos. However, we found that MBM cells doubly expressing Fas-mIVVY and C/EBPα, c-Fos, or NFATc1 failed to generate OCs with 10 (Fig. 4, *C* and *D*) or even 100 ng/ml (Fig. 4, *C* and *E*) α-Fas as compared with cells expressing the chimeric receptor with normal RANK, indicating that gene overexpression could not rescue osteoclastogenesis from inactivation of the RANK IVVY motif. Moreover, we found that, similarly to the RANKL-induced osteoclastogenesis assays (Fig. 2, *E* and *F*), *C/EBPα* or c-*Fos* overexpression showed no overt effect on OC size from the α-Fas-mediated osteoclastogenesis assays (Fig. 4, *F* and *G*). In confirming this finding, we demonstrated that RAW264.7 cells doubly expressing Fas-RANK and *C/EBPα*, c-*Fos*, or *NFATc1* could promote OC differentiation with both permissive (10 ng/ml) and optimum (100 ng/ml) α-Fas stimulation as compared with the GFP control (Fig. S3, A–E). Nevertheless, forced expression of *C/EBPα*, c-*Fos*, or *NFATc1* in RAW264.7 cells expressing the mutated RANK IVVY motif failed to promote OC differentiation (Fig. S3, A–E). Moreover, we demonstrated that C/EBPα overexpression showed no overt effect on OC size as compared with cells overexpressing c-Fos or expressing the GFP control (Fig. S3F).

Mutational inactivation of RANK IVVY motif up-regulates recombinant recognition sequence–binding protein at the Jκ site (RBP-J)

Our data thus far demonstrated that although the RANK IVVY motif could regulate the expressions of *C/EBPα* and *NFATc1*, but not that of c-*Fos*, during OC differentiation (Fig. 3) overexpression of C/EBPα, NFATc1, or c-Fos in cells expressing the mutated RANK IVVY motif could not mediate OC differentiation (Fig. 4). We were surprised that although overexpression of C/EBPα or c-Fos in MBM cells could initiate osteoclastogenesis independently of RANKL (Fig. 1) and promote OC differentiation (Fig. 2) overexpression of C/EBPα or c-Fos in cells expressing the mutated RANK IVVY motif was unable to promote osteoclastogenesis. Notably, a closer look at the role of the RANK IVVY motif in regulating the expressions of Ctsk and TRAP during osteoclastogenesis revealed that the inactivation of the RANK IVVY motif led to a significantly lower *Ctsk* or *TRAP* expression as compared with the GFP control (Fig. 3*G*). The result suggested that the RANK IVVY motif, besides positively regulating the expressions of OC genes, might also counteract the expressions of OC inhibitors during OC differentiation. This is consistent with the notion that deregulated expressions of potent negative regulators of osteoclastogenesis in the context of inactivation of the RANK IVVY motif may negatively affect the abilities of NFATc1, C/EBPα, or c-Fos to promote OC differentiation (38). Among the known factors that can negatively regulate OC differentiation, RBP-J (39–41) and interferon-regulatory factor 8 (IRF-8) (42, 43) have been regarded as the leading factors. Hence, we investigated the ability of the RANK IVVY motif to regulate the expressions of *RBP-J* and *IRF-8* during OC differentiation. The data showed that mutational inactivation of the RANK IVVY motif displayed no significant effect on *IRF-8* expression (Fig. 5*A*) but triggered a significant increase in *RBP-J* expression (Fig. 5*B*),

indicating that the RANK IVVY motif could negatively regulate *RBP-J* expression during OC differentiation. For additional insight into the role of the RANK IVVY motif in regulating *RBP-J* expression, we used the RNA synthesis inhibitor actinomycin D (ACTD) and the protein synthesis inhibitor cycloheximide (CHX) to examine the effects of IVVY inactivation on *RBP-J* expression at the transcriptional and translation levels. As expected, treatment of MBM cells expressing Fas-mIVVY with DMSO, used as a control, triggered a significant increase in *RBP-J* mRNA levels as compared with the Fas-RANK expressers upon stimulation by M-CSF and α-Fas (Fig. 5*C*). However, we noted that the Fas-RANK and Fas-mIVVY expressers showed comparable *RBP-J* mRNA levels upon treatment with ACTD to inhibit RNA synthesis, indicating that the RANK IVVY motif induced *RBP-J* expression at the transcriptional level (Fig. 5*C*). Furthermore, treatment of the Fas-mIVVY expressers with DMSO triggered a 30% increase in RBP-J protein levels as compared with the Fas-RANK expressers upon stimulation by M-CSF and α-Fas (Fig. 5*D*). However, treatment of Fas-mIVVY expressers with CHX to inhibit protein synthesis showed similar RBP-J protein levels as the Fas-RANK expressers upon activation by M-CSF and α-Fas. These results indicated that mutational inactivation of the RANK IVVY motif triggered a drastic increase in *RBP-J* mRNA levels, which translated into increased protein levels.

RBP-J was shown to suppress OC differentiation by inhibiting the immunoreceptor tyrosine-based activation motif (ITAM)–associated receptor costimulatory signaling, which is required for osteoclastogenesis (39–41, 44). Accordingly, we found that overexpression of *C/EBPα*, c-*Fos*, or *NFATc1* failed to control the up-regulated *RBP-J* expression from the inactivation of the RANK IVVY motif as compared with cells expressing the normal RANK cytoplasmic domain (Fig. 5, *E* and *F*). Additionally, consistent with the inability of gene overexpression to rescue OC differentiation in cells expressing the mutated RANK IVVY motif (Fig. 4), overexpression of *C/EBPα*, c-*Fos*, or *NFATc1* in MBM cells expressing the mutated RANK IVVY motif failed to induce the expressions of Ctsk (Fig. 5, *E* and *G*) and TRAP (Fig. 5, *E* and *H*), mimicking the reported role of RBP-J in inhibiting gene expression during OC differentiation (39). Collectively, these results indicated that the inability of *NFATc1*, *C/EBPα*, or c-*Fos* to mediate OC differentiation in the context of inactivation of the RANK IVVY motif stemmed in part from transcriptionally up-regulated *RBP-J* expression, which negatively affected the expressions of OC marker genes.

Ctsk is a direct target of repression by RBP-J

We next examined whether the inhibition of OC genes, such as *Ctsk*, was due to a direct transcriptional repression by RBP-J (Fig. 6). Using an online transcription factor–binding site predictive tool (alggen.lsi.upc.es),[4] we identified three binding sites for RBP-J on the 4-kb promoter region upstream of the murine *Ctsk* gene (Fig. 6*A*). Two of these binding regions were approximated 2628 (Site I) and 1841bp (Site 2) upstream of the Ctsk transcriptional start site (TSS) (−1841 to +1 bp relative to TSS and −2628 to +1 bp relative to TSS, respectively) (Fig. 6*A*). The third binding region was ~587 bp (Site 3) upstream of the Ctsk TSS (−587 to +1 bp relative to TSS) (Fig. 6*A*). Consistently, chromatin immunoprecipitation (ChIP) sequencing analysis demonstrated that Site 1 is a major binding site for RBP-J on the Ctsk promoter, whereas Site 2 and Site 3 were minor binding sites (Fig. 6*B*). Next, we performed luciferase assays using a reporter carrying different fragments of the Ctsk promoter (Fig. 6, *C* and *D*). RAW cells expressing RBP-J, as confirmed by Western blotting (Fig. 6*D*), were cotransfected with different Ctsk promoter fragments carrying the luciferase gene (Fig. 6*C*). The data showed that coexpression of two Ctsk promoter regions (−2445 to +1 bp relative to TSS and −2232 to +1 bp relative to TSS) containing Site 1 and Site 2 resulted in a drastic increase in luciferase activity as compared with three other regions (−1825 to +1 bp relative to TSS, −1630 to +1 bp relative to TSS, and −717 to +1 bp relative to TSS) containing only Site 1 (Fig. 6*C*). As controls, transfection of RAW cells with a pGL3-Control vector (positive control) showed a drastic increase in luciferase activity, whereas transfection of RAW cells with pGL3-Basic vector (negative control) failed to induce luciferase activity (Fig. 6*C*). Collectively, the data indicated that RBP-J could directly bind on Ctsk promoter to repress its expression in OC precursors.

RBP-J silencing rescues osteoclastogenesis in cells expressing mutated RANK IVVY motif

Next, we investigated the effect of silencing the *RBP-J* gene on rescuing osteoclastogenesis in the Fas-mIVVY expressers. We were able to silence the *RBP-J* gene using an shRNA construct that was purchased from Sigma-Aldrich as assessed by Western blot analysis (Fig. 7*A*). We found that, consistent with the reported role of RBP-J in suppressing osteoclastogenesis (39, 41), RBP-J silencing in MBM cells drastically enhanced RANKL-induced OC differentiation (Fig. 7, *B* and *C*). Accordingly, *RBP-J* silencing in MBM cells doubly expressing Fas-RANK and *C/EBPα*, c-*Fos*, or *NFATc1* (Fig. 7, *D* and *E*) formed more OCs than *RBPJ*-depleted cells expressing the GFP control and Fas-RANK as well as MBM cells triply expressing the scrambled shRNA control, Fas-RANK, and *C/EBPα*, c-*Fos*, or *NFATc1* (Fig. 7*F*, *top two rows*). Interestingly, *RBP-J* silencing in MBM cells doubly expressing Fas-mIVVY and *C/EBPα*, c-*Fos*, or *NFATc1* could partially rescue osteoclastogenesis as compared with the scrambled shRNA control doubly expressing Fas-mIVVY and *C/EBPα*, c-*Fos*, or *NFATc1* (Fig. 7*F*, *last two rows*). Consistently, the C/EBPα overexpressers doubly expressing the *RBP-J* shRNA construct and Fas-RANK or Fas-mIVVY generated significantly more OCs than the c-Fos overexpressers doubly expressing the *RBP-J* shRNA construct and Fas-RANK or Fas-mIVVY (Fig. 7, *G* and *H*), further confirming the stronger osteoclastogenic effect of C/EBPα than of c-Fos. Notably, *RBP-J* silencing in cells doubly expressing Fas-mIVVY and the GFP control could also generate a few TRAP-positive multinucleated cells as compared with cells triply expressing the Fas-mIVVY, the GFP control, and the scrambled shRNA control (Fig. 7, *F* and *H*), indicating that *RBP-J* silencing could initiate osteoclastogenesis in the cells expressing the mutated RANK IVVY motif. We further confirmed this finding by

[4] Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party-hosted site.

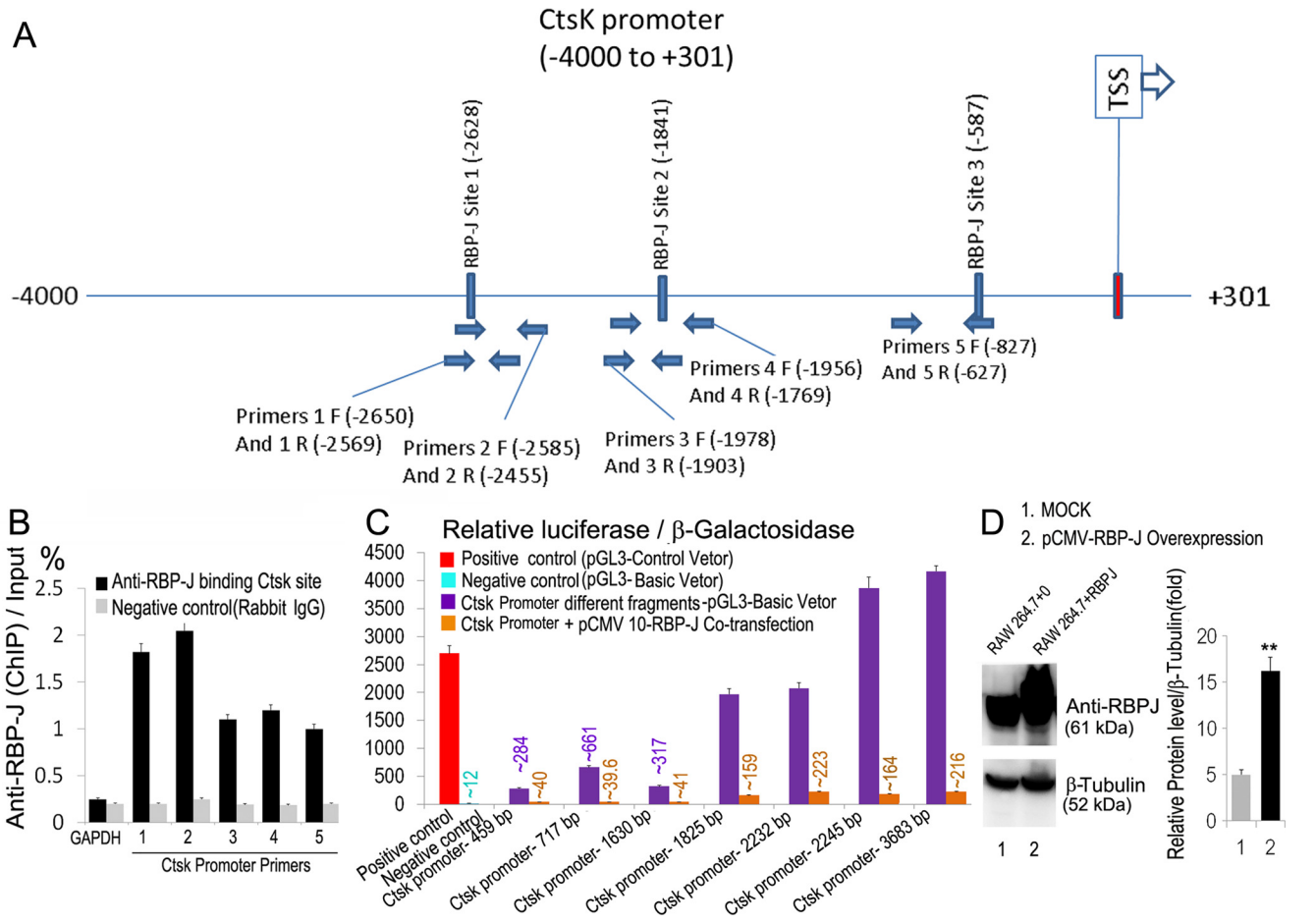

Figure 6. Ctsk is a direct target of repression by RBP-J. *A*, scheme of a 4-kb region of the murine Ctsk promoter showing the conserved RBP-J– binding sites and the location of the primers used for ChIP. *B*, ChIP qPCR assay using 293T cells, anti-RBP-J antibody, rabbit IgG (negative control), and primers as indicated in *A*. The data are shown as percent input from three independent experiments. *C*, luciferase activity from the Ctsk promoter with the indicated deletions in the presence or absence of RBP-J protein in RAW cells is shown from three independent repeats. RAW cells expressing pGL3-Control vector or pGL3-Basic vector served as positive and negative controls, respectively. *D*, Western blot analysis of RAW cells expressing pCMV-RBP-J or empty vector (*MOCK*) with anti-RBP-J antibody using anti-tubulin as a loading control. *Error bars* show averages ± S.D. **, $p < 0.01$.

silencing the *RBP-J* gene in the RAW cells (Fig. S4). First, we showed that *RBP-J* depletion in RAW cells led to significantly more OCs than the scrambled shRNA control upon RANKL stimulation (Fig. S4, A–D). Moreover, *RBP-J* silencing in RAW cells doubly expressing Fas-RANK and *C/EBPα*, c-*Fos*, or *NFATc1* formed more OCs than *RBPJ*-depleted cells expressing the GFP control and Fas-RANK as well as RAW cells triply expressing the scrambled shRNA control, Fas-RANK, and *C/EBPα*, c-*Fos*, or *NFATc1* (Fig. S4, E–G). *RBP-J* silencing in the RAW cells doubly expressing Fas-mIVVY and *C/EBPα*, c-*Fos*, or *NFATc1* could also partially rescue OC differentiation (Fig. S4, E and F). Moreover, the *C/EBPα* overexpressers doubly expressing the *RBP-J* shRNA construct and Fas-RANK or Fas-mIVVY generated significantly more OCs than the c-Fos overexpressers doubly expressing the *RBP-J* shRNA construct and Fas-RANK or Fas-mIVVY (Fig. S4, G and H). Consistently, *RBP-J* silencing in RAW cells doubly expressing Fas-mIVVY and the GFP control could also generate a few TRAP-positive multinucleated cells as compared with cells triply expressing Fas-mIVVY, the GFP control, and the scrambled shRNA control (Fig. S4, F and H). Taken together, the results indicated that *RBP-J* silencing could initiate osteoclastogenesis in cells expressing the mutated RANK IVVY motif, and overexpression of *C/EBPα*, c-*Fos*, or *NFATc1* in the mutant cells could further enhance OC differentiation.

Discussion

In this study, we compared the roles of C/EBPα and c-Fos in OC differentiation by using a gain-of-function strategy.

Although overexpression of C/EBPα or c-Fos can initiate osteoclastogenesis independently of RANKL, C/EBPα overexpression shows a stronger osteoclastogenic effect than c-Fos

C/EBPα and c-Fos are induced early during osteoclastogenesis, suggesting their involvement in OC lineage priming. Accordingly, we showed that overexpression of C/EBPα or c-Fos in MBM cells could up-regulate OC genes and thereby initiate osteoclastogenesis independently of RANKL. This finding is in agreement with other studies showing that C/EBPα or NFATc1 overexpression can initiate osteoclastogenesis independently of RANKL (14, 25). However, to our knowledge, this is the first report on the role of c-Fos overexpression in initiating osteoclastogenesis independently of RANKL. In

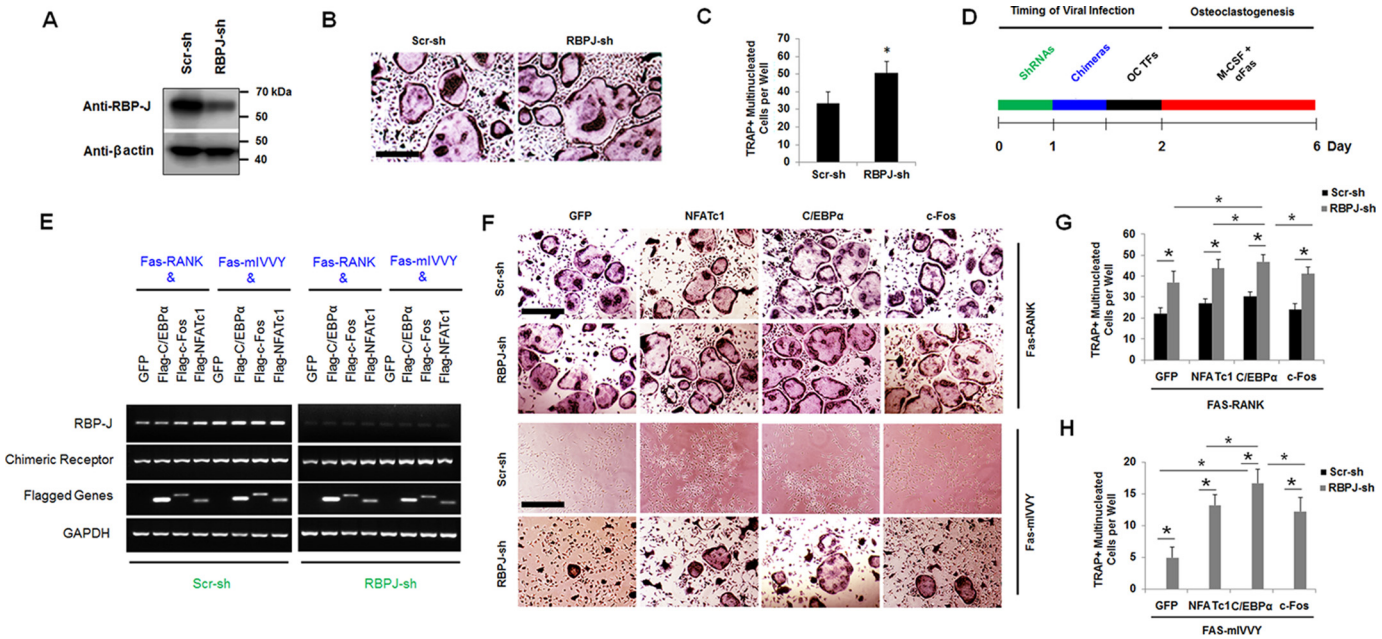

Figure 7. RBP-J silencing rescues osteoclastogenesis from the mutational inactivation of the RANK cytoplasmic IVVY motif. *A*, MBM cells expressing an *RBP-J* shRNA construct (*RBPJ-sh*) or a scrambled shRNA control (*Scr-sh*) were cultured with M-CSF (10 ng/ml) for 4 days. Gene silencing was examined by Western blotting using β-actin as a loading control in three independent experiments. *B*, MBM cells expressing *RBP-J* shRNA construct or scrambled shRNA control were treated with M-CSF and RANKL (10 ng/ml) for 4 days to stimulate OC differentiation. *Scale bar*, 250 μm. *C*, quantification for *B* is shown as mean number of multinucleated TRAP-positive cells per well in three independent assays. *D*, experimental strategy for *E–H*. *E*, MBM cells triply expressing an shRNA construct (*RBP-J* shRNA or scrambled shRNA control), a chimera (Fas-RANK or Fas-mIVVY), and a GFP control, FLAG-NFATc1, FLAG-C/EBPα, or FLAG-c-Fos were treated with M-CSF for 4 days for gene expression analysis by RT-PCR using GAPDH as a loading control. *F*, the triple expressers as in *E* were stimulated with M-CSF (10 ng/ml) and α-Fas (100 ng/ml) to promote osteoclastogenesis. The cultures were then stained by TRAP. *Scale bars*, 250 μm. *G* and *H*, quantifications for *F* are shown for the Fas-RANK (*G*) and Fas-mIVVY (*H*) expressers as mean number of multinucleated TRAP-positive cells per well from three independent assays, respectively. *Error bars* show averages ± S.D. *, $p < 0.05$; *NS*, not significant. *TFs*, transcription factors.

the absence of RANKL, we found that although *C/EBPα* could up-regulate c-*Fos*, c-*Fos* failed to up-regulate *C/EBPα* in OC precursors. This finding confirms our previous report that c-*Fos* is a target gene of *C/EBPα* during osteoclastogenesis (25), which is further supported by the fact that C/EBPα overexpression displays a stronger osteoclastogenic effect than does c-*Fos* overexpression. We believe that the ability of *C/EBPα* to up-regulate c-*Fos* may in part account for its stronger effect in mediating OC differentiation than c-*Fos*.

RANK IVVY motif mediates osteoclastogenesis in part by regulating C/EBPα, but not c-Fos, expression

We confirmed that the RANK IVVY motif is essential for osteoclastogenesis through regulation of OC genes, including *NFATc1*, *Ctsk*, and *TRAP* (9, 10, 34, 35), during OC differentiation. We demonstrated that inactivation of the RANK cytoplasmic IVVY motif inhibited the expressions of the aforementioned OC genes and thereby blocked OC differentiation. Importantly, we revealed that inactivation of the RANK IVVY motif repressed C/EBPα but was dispensable for c-Fos expression during OC differentiation, suggesting that the stronger ability of C/EBPα than of c-Fos to promote OC differentiation may be mediated through regulation by the RANK IVVY motif. The fact that *NFATc1*, a target gene of *C/EBPα* and c-*Fos*, is also repressed with inactivation of the RANK IVVY motif despite normal c-Fos expression indicates that C/EBPα may be a major factor responsible for NFATc1 up-regulation during OC differentiation. It will be important to further investigate this issue in future studies. The RANK IVVY motif was initially identified due to its essential role in OC lineage commitment (8) and was later shown to promote TNF-α– and IL-1–mediated OC differentiation (34, 35). The fact that c-Fos can promote OC lineage priming although it is not regulated by the RANK IVVY motif suggests that c-Fos overexpression may be unable to fully activate the osteoclastogenic machinery during the early stages of osteoclastogenesis, which is critical for cell differentiation. Nevertheless, it is also possible that the RANK IVVY motif by itself may be insufficient to up-regulate C/EBPα during osteoclastogenesis because it was reported that the IVVY motif functions by cooperating with two other RANK cytoplasmic motifs (^{559}PVQEET^{564} and ^{604}PVQEQG^{609}) to induce OC genes during OC differentiation (36). We therefore anticipate that the IVVY motif may function with other RANK motifs to up-regulate C/EBPα during OC differentiation.

Overexpression of NFATc1, C/EBPα, or c-Fos cannot mediate osteoclastogenesis in cells bearing inactivated RANK IVVY motif

Given the role of the RANK IVVY motif in inducing the expressions of *NFATc1* and *C/EBPα* during OC differentiation, we reasoned that overexpression of C/EBPα or NFATc1, unlike c-Fos, might rescue OC differentiation in cells expressing the mutated RANK IVVY motif. However, we later found that gene overexpression failed to induce OC differentiation in the mutant cells despite the ability of *C/EBPα*, c-*Fos*, or *NFATc1* to initiate osteoclastogenesis independently of RANKL and promote OC differentiation. Notably, we confirmed this finding in both primary cells and the RAW264.7 cell line. Moreover, in

deciphering the molecular mechanism for this finding, we showed that *C/EBPα*, *NFATc1*, or c-*Fos* overexpression was unable to induce OC genes with inactivation of the RANK IVVY motif. This finding prompted the assumption that the RANK IVVY motif might also down-regulate OC inhibitors, which might counteract the effect of NFATc1, c-Fos, or C/EBPα in promoting OC differentiation with inactivation of the RANK IVVY motif (38, 44). This is consistent with the idea that inactivation of the RANK IVVY motif might trigger dysregulation of potent OC inhibitors (*e.g.* RBP-J and IRF-8) in inhibiting OC differentiation. We revealed that *RBP-J*, but not *IRF-8*, was significantly up-regulated in cells expressing the mutated RANK IVVY motif, and overexpression of *NFATc1*, *C/EBPα*, or c-*Fos* was unable to control the up-regulated *RBP-J* expression in the mutant cells, which prevented C/EBPα, c-Fos, and NFATc1 from inducing OC genes. Our finding is in agreement with other studies showing that RBP-J can directly repress gene expression in various cell types. Boggs *et al.* (45) showed that RBP-J binds to the p53 promoter to repress p53 gene expression in fibroblasts. Moreover, RBP-J binds to Sox9, a critical transcription factor for chondrogenesis, to repress Sox9 expression and thereby inhibits chondrocyte differentiation (46). Furthermore, Rozenberg *et al.* (47) demonstrated that methylation-dependent binding of RBP-J to a GC repressor element negatively regulates smooth muscle myosin heavy chain promoter activity and inhibits smooth muscle cell marker genes. Consistent with these studies, we showed that the mechanism by which RBP-J inhibits OC differentiation involves its direct binding on an OC gene promoter to repress gene expression during osteoclastogenesis. Remarkably, whereas NFATc1 can bind on Ctsk promoter to induce its expression (48), NFATc1, C/EBPα, or c-Fos overexpression was unable to induce Ctsk expression with RBP-J up-regulation from inactivation of the RANK IVVY motif. This suggests that binding of RBP-J on Ctsk promoter may prevent the osteoclastic transcription factors from accessing the promoter to promote gene expression. Future study is warranted to further address the mechanistic basis of the inhibitory effect of RBP-J on OC genes.

Notably, we demonstrated that inactivation of the RANK IVVY motif led to increased *RBP-J* mRNA levels, which translated into increased protein levels. Although this finding indicates that the RANK IVVY motif regulates *RBP-J* at mainly a transcriptional level, it does not exclude the possibility that the RANK IVVY motif may also regulate RBP-J by other means, including protein stability. Hence, the mechanism by which the RANK IVVY motif regulates RBP-J remains incomplete. With regard to the transcriptional regulation, we anticipate that the RANK IVVY motif may activate some unknown factors that can bind on the RBP-J promoter to down-regulate its expression and that inactivation of this motif may release this inhibitory brake on *RBP-J* expression. Importantly, RBP-J can suppress ITAM-mediated costimulatory signaling and limits the cross-talk between ITAM and RANK signaling, which ultimately inhibit OC differentiation through inhibition of the Ca^{2+}/calmodulin signaling pathway (39). Consistently, we showed that *RBP-J* silencing could partially rescue osteoclastogenesis in both primary cells and RAW6264.7 cells doubly expressing the mutated RANK IVVY motif and C/EBPα or c-Fos with C/EBPα exhibiting a stronger osteoclastogenic effect.

The role of RANK IVVY motif in osteoclastogenesis remains complex

Whereas the RANK IVVY motif can up-regulate NFATc1 and C/EBPα, but not c-Fos, and down-regulate RBP-J during OC differentiation, overexpression of *NFATc1*, *C/EBPα*, or c-*Fos* can only partially rescue OC differentiation in *RBPJ*-depleted cells expressing the mutated RANK IVVY motif. These results indicated that the main mechanism(s) by which the RANK IVVY motif mediates osteoclastogenesis remains incomplete. Furthermore, despite its role in OC formation, the RANK IVVY motif is dispensable for the activation of the RANKL-induced signaling pathways (ERK, p38, JNK, and NF-κB) (8, 9). Our results indicate that the RANK IVVY motif can mediate OC differentiation by orchestrating a cascade of critical functions, which include induction of transcription factors, up-regulation of OC genes, and down-regulation of OC suppressors. Further studies on the role of the RANK IVVY motif in osteoclastogenesis should generate important insights into this issue. A key long-standing question is to unambiguously identify the protein(s) that is recruited by the RANK IVVY motif to mediate osteoclastogenesis. We anticipate that the RANK IVVY motif may recruit different factors at different stages of osteoclastogenesis and thereby exhibits distinct roles in different stages of osteoclastogenesis.

In conclusion, we demonstrated that C/EBPα exhibits a stronger osteoclastogenic effect than c-Fos in part through regulation by the RANK cytoplasmic IVVY motif. Moreover, the IVVY motif promotes osteoclastogenesis by positively regulating OC markers and negatively regulating *RBP-J*. It is unknown whether the stronger effect of C/EBPα than of c-Fos in mediating OC differentiation *in vitro* also applies *in vivo*. It will be important to validate this finding *in vivo* through comparative analyses of bone phenotypes in *C/EBPα*$^{-/-}$ mice and c-*Fos*$^{-/-}$ mice in conjunction with gene rescuing strategies. Nevertheless, our study provides important comparative insight into the roles of transcription factors and RANK signaling in OC differentiation.

Experimental procedures

Chemical and biological reagents

All chemicals were obtained from Sigma-Aldrich. Anti-FLAG antibody (catalogue number F1804–1 mg) was purchased from Sigma-Aldrich. Recombinant mouse RANKL (catalogue number 462-TEC) and M-CSF (catalogue number 416-ML) were purchased from R&D Systems. Anti-β-actin (catalogue number SC-81178) antibody was from Santa Cruz Biotechnology, and anti-human Fas-activating antibody (α-Fas; catalogue number 05–201) was purchased from Millipore. ACTD and CHX were purchased from Sigma-Aldrich.

Construct generation and retroviral transduction

We previously constructed the pMX-puro-3xFLAG vector by cloning a synthesized 3xFLAG oligonucleotide into the pMX-puro vector (26, 28). The pMX-puro-3xFLAG-C/EBPα

(FLAG-C/EBPα), pMX-puro-3xFLAG-c-Fos (FLAG-c-Fos), and pMX-puro-3xFLAG-NFATc1 (FLAG-NFATc1) constructs were prepared by first amplifying the mouse C/EBPα, c-Fos, and NFATc1 cDNAs from the pSport6-C/EBPα (Addgene), pSport6-c-Fos (Addgene), and pSport6-NFATc1 (Addgene) vectors, respectively. The amplified cDNAs were then subcloned in-frame with the 3xFLAG sequence into the pMx-puro-3xFLAG vector. The constructs were confirmed by sequencing. The pMX-puro-GFP (GFP), pMX-puro-hFas-RANK (Fas-RANK), and pMX-puro-hFas-mIVVY (Fas-mIVVY) were generated in previous studies (8, 33). For retrovirus generation, the 293GPG retroviral packaging cell line was cultured in DMEM with 10% heat-inactivated FBS, G418, tetracycline, penicillin/streptomycin, and puromycin (29) before transient transfection with pMX retroviral constructs using the calcium phosphate precipitation method. The retroviral supernatant was harvested at 48, 72, and 96 h post-transfection and then utilized to infect cells for osteoclastogenesis assays (35).

Lentiviral transduction

RBP-J shRNA or scrambled shRNA lentiviral construct (Sigma-Aldrich) and packaging constructs were co-transfected into HEK-293T cells using the calcium phosphate precipitation method (49). The lentiviral supernatant was collected at 60 h post-transfection and then utilized to infect cells for osteoclastogenesis.

In vitro osteoclastogenesis assays from MBM cells

MBM cells were isolated from long bones of 4–6-week-old C57BL/6 mice and cultured as described previously (50–52). Briefly, MBM cells (5×10^4 cells/well) were cultured in 24-well culture dishes in α-minimal essential medium with 10% heat-inactivated FBS and M-CSF (20 ng/ml) for 24 h. Some cells were directly differentiated into OCs as indicated in individual experiments. Other cells were infected with a retrovirus/lentivirus in the presence of M-CSF (10 ng/ml) and Polybrene (8 μg/ml) for 24 h before being cultured as indicated in related experiments for OC formation. The cultures were stained for TRAP activity using a leukocyte acid phosphatase kit (catalogue number 387-A, Sigma) according to the manufacturer's instructions at the end of the assays to examine OC differentiation. The osteoclastogenesis assays were quantified by counting and/or assessing the size of multinucleated TRAP-positive cells (more than three nuclei) in a representative area under a microscope. The experiments involving mice were approved by the University of Alabama at Birmingham institutional animal care and use committee.

In vitro osteoclastogenesis assays from RAW264.7 cells

The macrophage RAW264.7 cell line (1.5×10^4cells/well) was cultured in 24-well culture dishes in DMEM with 10% heat-inactivated FBS for 24 h before infection with a retrovirus/lentivirus in the presence of Polybrene (8 μg/ml) for 24 h followed by osteoclastogenesis assays as indicated in individual experiments. At the end of the assays, the cultures were stained for TRAP activity as indicated above. The assays were quantified by counting and/or assessing the size of multinucleated TRAP-positive cells (more than three nuclei) in a representative area under a microscope.

Western blot analysis

Western blot analysis was carried out as described previously (53). Briefly, cells were cultured as indicated in individual experiments before protein collection for gel electrophoresis. The membranes were washed, and enhanced chemiluminescence detection was carried using Luminata Forte HRP substrate from Millipore. The membranes were visualized using a C-DiGit® blot scanner and Image Studio software from LI-COR Biosciences.

qPCR analysis

qPCR analysis was performed as described previously (54). Cells were cultured as indicated in individual experiments, and total RNA was collected using TRIzol reagent (Life Technologies). 1 μg of total RNA was transcribed into cDNA using the ProtoScript® first strand cDNA synthesis kit (New England Biolabs) according to the manufacturer's instructions. qPCRs were carried using Fast SYBR® Green Master Mix reagent (Life Technologies) using hypoxanthine-guanine phosphoribosyltransferase (Hprt) as an endogenous control for normalization. PCR conditions and primer sequences are available upon request.

RT-PCR analysis

Cells were cultured as indicated in individual experiments, and total RNA was collected for cDNA synthesis as indicated above. Gene amplification analysis was carried using *Taq* DNA polymerase (catalogue number E001, Novo Protein) as indicated previously (34). RT-PCR primers used to detect the chimeric receptors (Fas-RANK and Fas-mIVVY) were 5′-ATGCTGGGCATCTGGACCCTCCTA-3′ for the human Fas extracellular domain (forward) and 5′-GAAGTCACAGCCCTCAGAATC-3′ for the mouse RANK intracellular domain (reverse). RT-PCR primers used to examine the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as an endogenous control, were 5′-TCATTGAGAGCAATGCCAGC-3′ (forward) and 5′-ACATCATCCCTGCATCCACTG-3′ (reverse). 20 μl of the PCR was loaded on a 2% agarose gel for electrophoretic analysis.

ChIP

ChIP was performed as described in a previous study (55). After immunoprecipitation using anti-RBP-J antibody (catalogue number 5313, Cell Signaling Technology) and DNA extraction, quantitative PCR was performed using the primers in the promoter region of the murine *Ctsk* gene. PCR conditions and primer sequences are available upon request.

Promoter-luciferase assay

The luciferase assay was carried as described in a previous study (55). Briefly, different fragments of the promoter region of the murine *Ctsk* gene were amplified by PCR using a Ctsk bacterial artificial chromosome clone. The promoter regions were then inserted into the pGL3-Basic vector to construct the pGL3-Ctsk promoter regions. Subsequently, RAW cells were

transiently transfected with a DNA mixture containing the pGL3-Ctsk construct (0.3 μg) and β-gal-expressing plasmids (0.03 μg) with or without RBP-J– expressing vector (pCMV-RBP-J; 0.3 μg) using FuGENE 6. Luciferase activity was examined using the Dual-Glo luciferase assay system (Promega) 48 h post-transfection. The β-gal activity of the cell lysates was analyzed using the β-galactosidase Enzyme Assay System (Promega). The level of luciferase activity was normalized to the level of β-gal activity.

Statistical analysis

Data are reported as averages ± S.D. Statistical significance was assessed using Student's *t* test. *p* values less than 0.05 were considered significant.

Author contributions—Y.-P. L. designed the study. J. J. and W. C. carried out experiments. J. J., W. C., X. F., and Y.-P. L. analyzed data and prepared the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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C/EBPα transcription factor is regulated by the RANK cytoplasmic ^{535}IVVY^{538} motif and stimulates osteoclastogenesis more strongly than c-Fos

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Supplemental Information

C/EBPα is Regulated by the RANK Cytoplasmic IVVY535-538 Motif and Stimulates Osteoclastogenesis more Strongly than c-Fos

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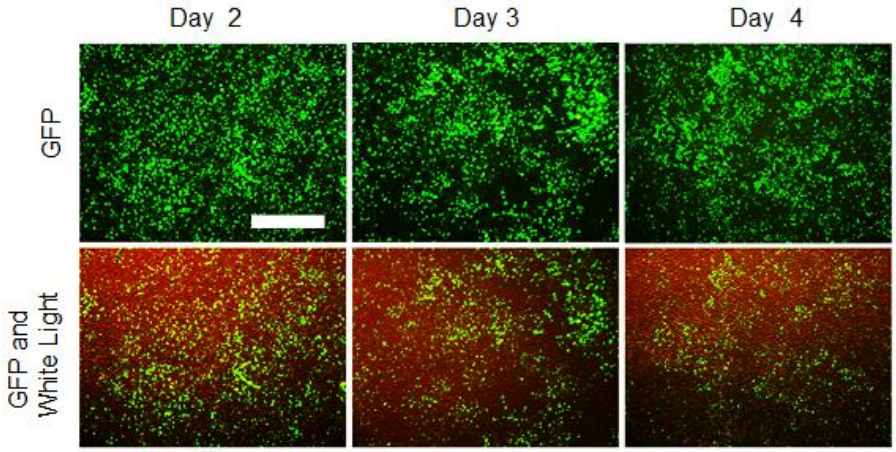
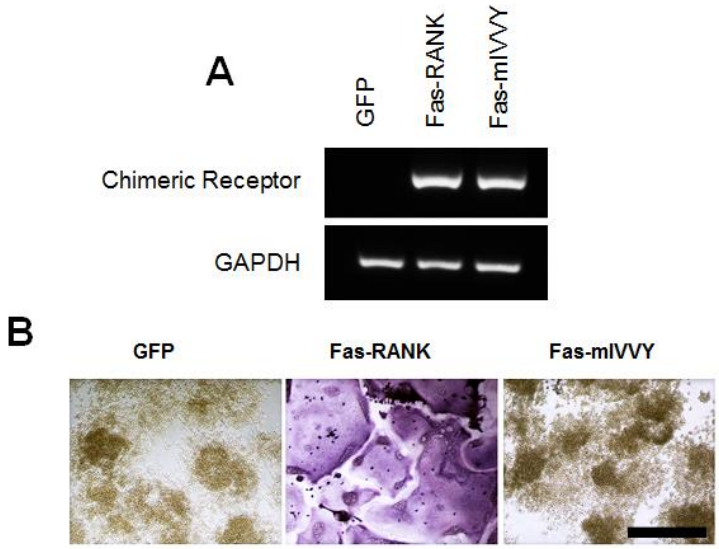
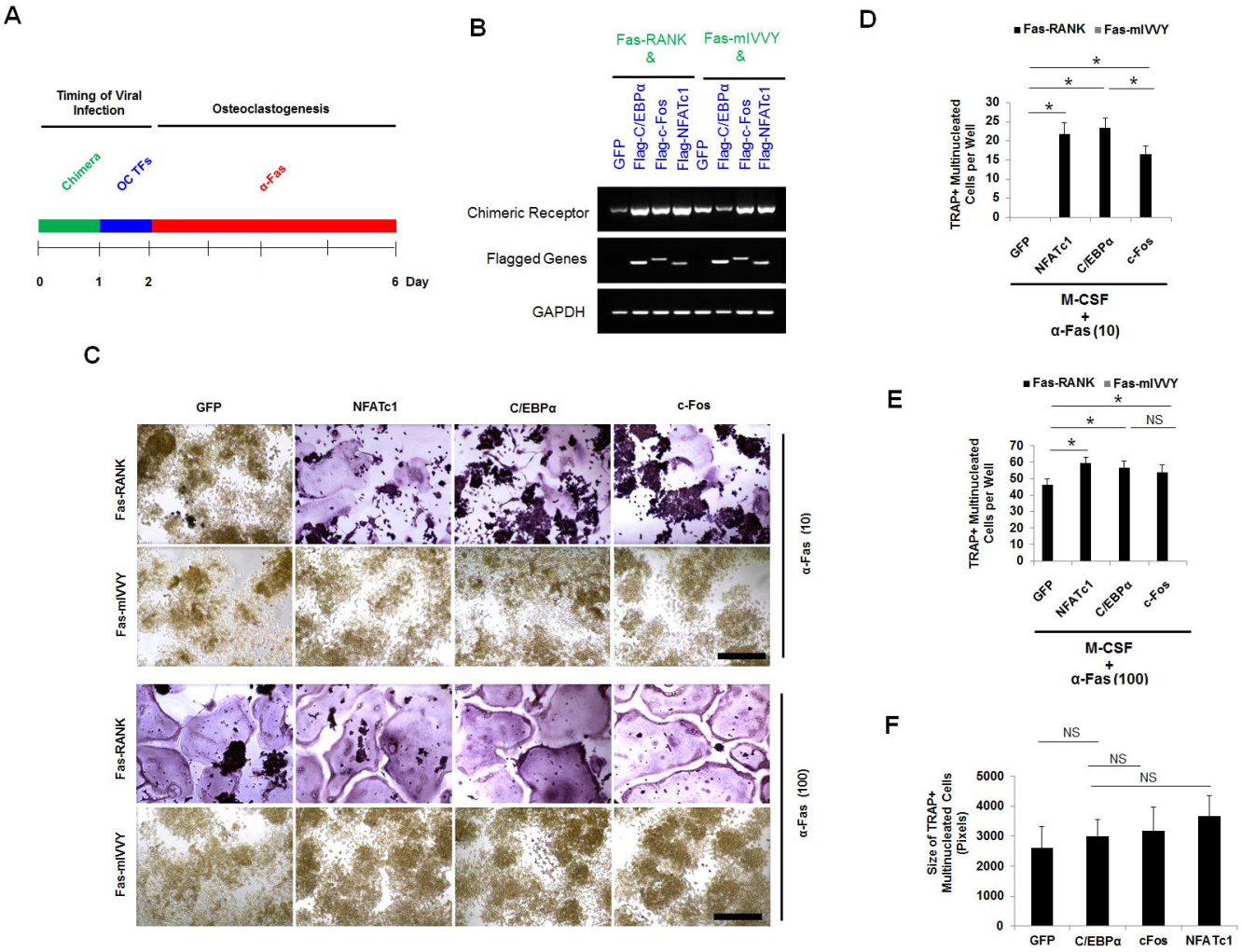


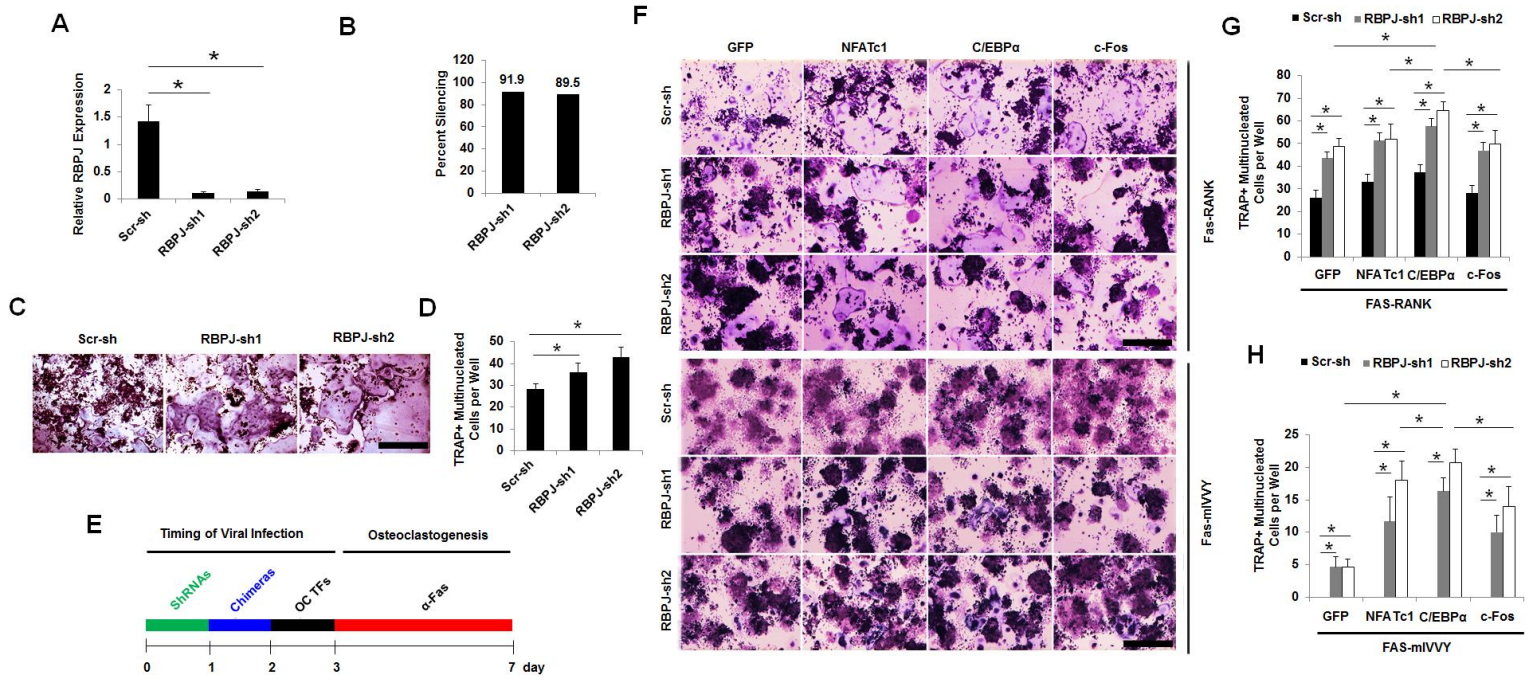
SUPPLEMENTAL FIGURE 1**. Analysis of infected MBM cells by GFP expression.** MBM cells were infected with a retrovirus encoding the GFP cDNA, and GFP expression was examined on days 2, 3, and 4 post infection. Scale bar = 250 μm. GFP was highly expressed by the infected MBM cells.



SUPPLEMENTAL FIGURE 2. **Mutational inactivation of the RANK cytoplasmic IVVY motif blocks osteoclastogenesis in the RAW264.7 cell line**. *A*, RAW cells expressing a GFP control (GFP), Fas-RANK, or Fas-mIVVY were cultured for 4 days without RANKL for gene expression analysis by RT-PCR using GAPDH as a loading control. *B*, RAW cells expressing GFP, Fas-RANK, or Fas-mIVVY were cultured with α-Fas (100 ng/ml) for 4 days to stimulate OC differentiation. TRAP staining was carried for analysis of OC differentiation from at least three independent experiments. Scale bar = 250 μm.



SUPPLEMENTAL FIGURE 3. **C/EBPα or c-Fos fails to mediate osteoclastogenesis with mutational inactivation of the RANK cytoplasmic IVVY motif in RAW264.7 cells.** A, experimental strategy. *B,* RAW cells doubly expressing Fas-RANK or Fas-mIVVY plus a GFP control (GFP), FLAG-NFATc1 (NFATc1), FLAG-C/EBPα (C/EBPα), or FLAG-c-Fos (c-Fos) were cultured for 4 days without RANKL for to gene expression analysis by RT-PCR using GAPDH as a loading control. *C,* RAW cells doubly expressing Fas-RANK or Fas-mIVVY plus GFP, NFATc1, C/EBPα, or c-Fos were cultured with α-Fas (10 ng/ml or 100 ng/ml) for 4 days to stimulate OC differentiation. TRAP staining was carried at the end of the assay for analysis of OC differentiation. Scale bar = 250 μm. *D and E,* quantifications for C are shown for α-Fas (10 ng/ml) (D) or α-Fas (100 ng/ml) (E) as mean number of multinucleated TRAP-positive cells per well from at least three independent assays. *F,* quantification of OC size for the Fas-RANK expressers shown in C. The numbers in parentheses show concentrations in nanograms per milliliter. Error bars show averages ± S.D. *, $p < 0.05$. NS, not significant.



SUPPLEMENTAL FIGURE 4. **RBP-J silencing rescues osteoclastogenesis in RAW cells from the mutational inactivation of the RANK cytoplasmic IVVY motif.** *A*, RAW264.7 cells expressing two different *RBP-J* shRNA constructs (*RBP-J-sh1* and *RBP-J-sh2*) or a Scramble shRNA control (Scr-sh) was cultured for 4 days without RANKL. Gene silencing was examined by qPCR using Hprt as a loading control in three independent experiments. *B*, quantification for A is shown as percent *RBP-J* silencing. *C*, RAW264.7 expressing RBPJ-sh or Scr-sh were treated with RANKL (10 ng/ml) for 4 days to stimulate OC differentiation. TRAP staining was carried at the end of the assay. Scale bar = 250 μm. *D*, quantification for C is shown as mean number of multinucleated TRAP-positive cells per well from three independent assays. *E*, experimental strategy for F-H. *F*, RAW cells triply expressing the shRNA constructs (*RBPJ-sh* or Scr-sh) and chimeras (Fas-RANK or Fas-mIVVY) plus a GFP control (GFP), FLAG-NFATc1 (NFATc1), FLAG-C/EBPα (C/EBPα), or FLAG-c-Fos (c-Fos) were treated with α-Fas (100 ng/ml) for 4 days. TRAP staining was carried at the end of the assay to examine OC differentiation. Scale bars = 250 μm *G and H*, quantifications for F are shown for the Fas-RANK (G) and Fas-mIVVY (H) expressers as mean number of multinucleated TRAP-positive cells per well from three independent assays. Error bars show averages ± S.D. *, $p < 0.05$.