# JNK1 modulates osteoclastogenesis through both c-Jun phosphorylation-dependent and -independent mechanisms

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## Summary

**Phosphorylation of the N-terminal domain of Jun by the Jun kinases (JNKs) modulates the transcriptional activity of AP-1, a dimeric transcription factor typically composed of c-Jun and c-Fos, the latter being essential for osteoclast differentiation. Using mice lacking JNK1 or JNK2, we demonstrate that JNK1, but not JNK2, is specifically activated by the osteoclast-differentiating factor RANKL. Activation of JNK1, but not JNK2, is required for efficient osteoclastogenesis from bone marrow monocytes (BMMs). JNK1 protects BMMs from RANKL-induced apoptosis during differentiation. In addition, BMMs from mice carrying a mutant of c-Jun phosphorylation sites (JunAA/JunAA), as well as cells lacking either c-Jun or JunD, which is another JNK substrate, revealed that c-Jun phosphorylation and c-Jun itself, but not JunD, are essential for efficient osteoclastogenesis. Moreover, JNK1-dependent c-Jun phosphorylation in response to RANKL is not involved in the anti-apoptotic function of JNK1. Thus, these data provide genetic evidence that JNK1 activation modulates osteoclastogenesis through both c-Jun-phosphorylation-dependent and -independent mechanisms.**

Key words: Jun-N-terminal kinase, c-Jun, Osteoclasts, RANKL, Apoptosis

## Introduction

Proliferation, differentiation and cell death signals converge on the mitogen-activated protein kinases (MAP kinases), which act as sensors of the environment. The MAP kinases include the extracellular regulated kinases (ERKs), the p38 stress kinases and the c-Jun N-terminal kinases (JNKs) (Chang and Karin, 2001; Davis, 2000). The type of activating stimuli, the combinatorial use of the various MAP kinases and their activating upstream kinases associated with specific scaffolding proteins determine the specificity of the substrates phosphorylated (Davis, 2000). ERKs are mainly activated by mitogenic stimuli, whereas p38 and JNKs, which belong to the stress-activated protein kinases (SAPKs), are activated in response to inflammatory cytokines, ultraviolet irradiation, heat shock or osmotic shock (Chang and Karin, 2001). The function of each subgroup of MAP kinases has been extensively studied in vitro, but the results were difficult to interpret owing to the complexity of the system (Davis, 2000). Recently, MAP kinase function was analyzed in cells isolated from knockout mice (Davis, 2000).

The complexity of the signaling network is well illustrated by the JNKs, which are composed of at least 10 different isoforms encoded by three different genes: *Jnk1*, *Jnk2* and *Jnk3*. Mice with these three genes individually inactivated are viable and morphologically normal, indicating that individual JNKs are dispensable for normal development. By contrast, the combined deletion of *Jnk1* and *Jnk2* leads to embryonic lethality owing to the failure of neural tube closure (Davis, 2000). Both *Jnk1*- and *Jnk2*-deficient mice are immunodeficient owing to a defect in T cell function (Constant et al., 2000; Dong et al., 1998; Sabapathy et al., 1999a; Sabapathy et al., 2001; Yang et al., 1998).

Some of the best characterized substrates of the JNKs are the components of AP-1, a dimeric transcription factor formed by the association of Fos proteins (c-Fos, FosB, Fra-1 and Fra-2) or ATF proteins (ATF-2, ATF-a) with Jun proteins (c-Jun, JunB and JunD). Phosphorylation of c-Jun on serines 63 and 73 by JNKs increases AP-1 transcriptional activity (Chang and Karin, 2001; Davis, 2000). Mice harbouring mutation of the phosphorylation sites of c-Jun phenocopy the *Jnk3*-deficient mice, suggesting that c-Jun phosphorylation by JNK3 mediates stress-induced neuronal apoptosis (Behrens et al., 1999; Yang et al., 1997). c-Jun was also found to be the target of JNK1- and JNK2-mediated apoptosis in immature thymocytes, whereas NF-ATs were proposed as substrates of JNK1 and JNK2 function in mature T cells (Behrens et al., 2001; Dong et al., 1998; Sabapathy et al., 2001). These data suggest that the phosphorylation of a given substrate by JNK is tissue specific and is dependent on the stage of differentiation of the cell.

The differentiation of osteoclasts, the bone resorbing cells derived from the bone marrow is controlled by two cytokines: macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) (Karsenty and Wagner,

2002). RANKL acts by binding to its receptor RANK, which belongs to the superfamily of TNF receptors. In vitro, RANKL can be replaced by TNF-α to induce osteoclast differentiation. RANKL and TNF-α are potent activators of the JNK pathways (Suda et al., 1999). Multiple reports have associated RANKL-mediated osteoclast differentiation with JNK-induced c-Jun phosphorylation, and a correlation has been established between a decrease in osteoclast differentiation and a decrease in RANKL-induced JNK activation (Shevde et al., 2000; Srivastava et al., 1999; Takayanagi et al., 2000). Moreover, osteoclast differentiation is blocked in mice lacking c-Fos (Jochum et al., 2001). The study of c-Jun and JunB function in osteoclasts was compromised by the early embryonic lethality in mice lacking *c-Jun* or *JunB* (Jochum et al., 2001). The early lethality of c-*jun* deletion can be overcome by the use of mice harboring a conditional allele of c-*jun* flanked by *loxP* sites, allowing tissue-specific deletion (Behrens et al., 2002). Mice lacking *JunD*, the main Jun component found in osteoclasts (David et al., 2001), are viable and do not display any obvious bone phenotype (Thepot et al., 2000).

To address the role of Jun phosphorylation by the JNKs in osteoclasts, we analyzed the differentiation potential of hematopoietic cells isolated from mice lacking JNK1 (*Jnk1*$^{-/-}$), JNK2 (*Jnk2*$^{-/-}$) or mice expressing a mutated form of c-Jun (*JunAA/JunAA*) that cannot be phosphorylated by the JNKs. We found that osteoclastogenesis of cells derived from *JunAA/JunAA* and *Jnk1*$^{-/-}$ but not *Jnk2*$^{-/-}$ mice is impaired. A similar defect was also found in cells lacking c-*Jun* but not in cells lacking *JunD*. Only JNK1, but not JNK2, is activated by RANKL in M-CSF-dependent osteoclast progenitors. We also found that, in osteoclast progenitors, JNK1 plays a protective role that is independent of c-Jun phosphorylation against RANKL-induced apoptosis. Our data demonstrate that JNK1-mediated apoptosis and c-Jun phosphorylation regulate osteoclastogenesis.

## Materials and Methods

### Mice

The mice used in this study have been previously described (Behrens et al., 1999; Behrens et al., 2002; Sabapathy et al., 1999a; Sabapathy et al., 2001; Thepot et al., 2000).

### Cell culture

Co-cultures were performed in 24-well dishes by plating primary osteoblasts ($10^5$ per well) isolated from wild-type newborn mice with spleen or bone marrow cells ($10^6$ per well) in α-MEM containing 10% FCS, $10^{-8}$ M 1α,25 $(OH_2)D_3$-dihydroxyvitamin $D_3$ and $10^{-7}$ M dexamethasone (Calbiochem). Osteoclast differentiation was evaluated by TRAP staining using the Leukocyte Acid Phosphatase kit (Sigma). The resorption assay was performed on bovine cortical bone slices; resorption pits were stained with toluidine blue and quantified using NIH image software. For RANKL or TNF-α-induced osteoclast differentiation, bone marrow cells were plated overnight and non-adherent cells were recovered and plated in 24-well dishes ($5\times10^5$ cells per well) in the presence of M-CSF (20 ng/ml) (R&D) and RANKL (50 ng/ml) (Insight Biotechnology) or TNF-α (50 ng/ml) (R&D).

### Conditional deletion of *c-jun*

Mice harboring an allele of c-*jun* flanked by *loxP* sites along with the inducible Mx-Cre transgene were used to delete c-*jun* in the monocytic compartment (Behrens et al., 2002). 11-week-old mice were injected twice at seven day intervals with 5 μg of pIpC per gram of body weight. Osteoclast differentiation was performed two weeks after the second injection. The efficiency of the deletion was controlled by PCR using DNA isolated from the cultured cells.

### TUNEL assay

To determine the apoptotic index, bone marrow monocytes (BMMs) isolated from mice of each genotype were plated for two days in the presence of M-CSF (20 ng/ml) or M-CSF and RANKL (50 ng/ml). The TUNEL assay was performed 12 hours after restimulation of the cells by the cytokines. The total number of cells was determined by DAPI staining. The apoptotic index was calculated as the ratio of TUNEL-positive cells to DAPI-positive cells.

### Western blot

Western blot analysis was performed using 50 μg of whole cell extract from primary M-CSF-dependent bone marrow monocytes. The samples were separated on SDS-PAGE, transferred to nitrocellulose and probed with anti-JNK (666.8) (BD Pharmingen), anti-phospho-S63c-Jun (a gift from M. Yaniv), anti-TRAF2, anti-TRAF6 (Santa Cruz Biotechnology) or anti-phospho p38 and phospho ERK (Cell Signalling) antibodies.

### JNK kinase assay

Immunprecipitation kinase assays were performed using 50 μg of whole cell extract from primary M-CSF-dependent monocytes and GST-c-Jun (1-79) as a substrate. JNK1 and JNK2 were immunoprecipitated overnight with polyclonal antibodies directed against JNK1 and JNK2 (Santa Cruz Biotechnology). A specific antibody directed against JNK1 (333.8) (BD Pharmingen) was used for JNK1 immunodepletion. Kinase assays were performed as previously described (Sabapathy et al., 1999a).

### Northern blot

10 μg of total RNA isolated from M-CSF-dependent bone marrow monocytes using Trizol reagent (Life Technologies) was transferred to Porablot Membrane (Gene X press) and hybridized with the full-length mouse TRAF2 cDNA.

### Electrophoretic mobility shift assay (EMSA)

10 μg of nuclear extract prepared from M-CSF-dependent bone marrow monocytes were used for the binding reaction on the NF-κB-binding site (5′-TCGAGGGCTGGGGTTCCCCATCTC-3′).

### Statistical analysis

An unpaired two-tailed assuming unequal variances student's T test was used to determine the statistical significance of the data (***$P$<0.0001, **$P$<0.001, *$P$<0.005; ns, non-significant). For each figure, the error bars indicate the standard deviation.

## Results

### Impaired osteoclastogenesis of *Jnk1*$^{-/-}$ but not of *Jnk2*$^{-/-}$ hematopoietic precursors

To determine whether JNK1 and JNK2 regulate osteoclast differentiation and activity, bone marrow or spleen cells were isolated from *Jnk1*$^{-/-}$, *Jnk2*$^{-/-}$ and wild-type litter mates and co-cultured with wild-type osteoblasts from newborn mice.

The lack of JNK1 resulted in a 50% reduction in osteoclast differentiation (Fig. 1A,C), and a similar reduction was observed on the surface of resorbed bone (Fig. 1E), indicating that JNK1 modulates osteoclastogenesis. By contrast, the lack of *Jnk2* did not affect osteoclast differentiation nor the ability of the cells to resorb bone, indicating that JNK2 is not involved in osteoclastogenesis (Fig. 1B,D,F).

## JNK1 is specifically activated by RANKL in osteoclast progenitors

To analyze the mechanisms responsible for the specificity of JNK1 function in osteoclasts, we examined whether the addition of RANKL alters the expression of JNK1 and JNK2 in M-CSF-dependent bone marrow monocytes (BMMs). Two major forms of JNK migrating at 46 and 55 kDa were expressed in osteoclast progenitors, and their levels of expression were not significantly regulated by RANKL (Fig. 2A). We used JNK1- and JNK2-deficient cells to identify which isoforms of JNK are expressed in BMMs. The 55 kDa isoform was identified as JNK2 and the 46 kDa as JNK1 (Fig. 2A). We therefore analyzed whether the functional difference between JNK1 and JNK2 could be explained by differential activation of their kinase activities by RANKL. BMMs were isolated from *Jnk1*$^{-/-}$, *Jnk2*$^{-/-}$ and wild-type littermates, and the kinetics of JNK activation by RANKL were examined by immunoprecipitation kinase assay. Treatment of wild-type cells by RANKL resulted in a time-dependent increase in JNK activity, peaking around 15 minutes after RANKL addition (Fig. 2B,C). Both the basal and induced JNK activities were completely abolished in JNK1-deficient cells, whereas deletion of JNK2 did not affect the response (Fig. 2B,C). Furthermore, JNK activity was not recovered in JNK1-immunodepleted wild-type extracts (Fig. 2D). To determine whether the JNK2 isozyme expressed in osteoclast progenitors is functional, wild-type and *Jnk1*$^{-/-}$ BMMs were exposed to UV, a known activator of both JNK1 and JNK2. UV exposure induced JNK activity in *Jnk1*$^{-/-}$ cells, indicating that osteoclast progenitors expressed a functional JNK2 (Fig. 2E). These data demonstrate that JNK1 is specifically activated by the osteoclastogenic factor RANKL and that JNK2 is unable to compensate for the lack of JNK1.

## Impaired TRAF2 upregulation in *Jnk1*$^{-/-}$ BMMs

JNK activation by the TNF-α family of cytokines is dependent on the recruitment of downstream adaptors belonging to the TNF-receptor-associated factor (TRAF) family (Inoue et al., 2000). Among them, TRAF2 was shown to be necessary for JNK activation (Lee et al., 1997; Yeh et al., 1997; Zhang et al., 2001), whereas TRAF6 was shown to be necessary for osteoclast differentiation (Inoue et al., 2000). We hypothesized that the lack of a compensatory effect of JNK2 in the absence of JNK1 could be due to a defect in TRAF-mediated signaling. Thus, we analyzed whether the

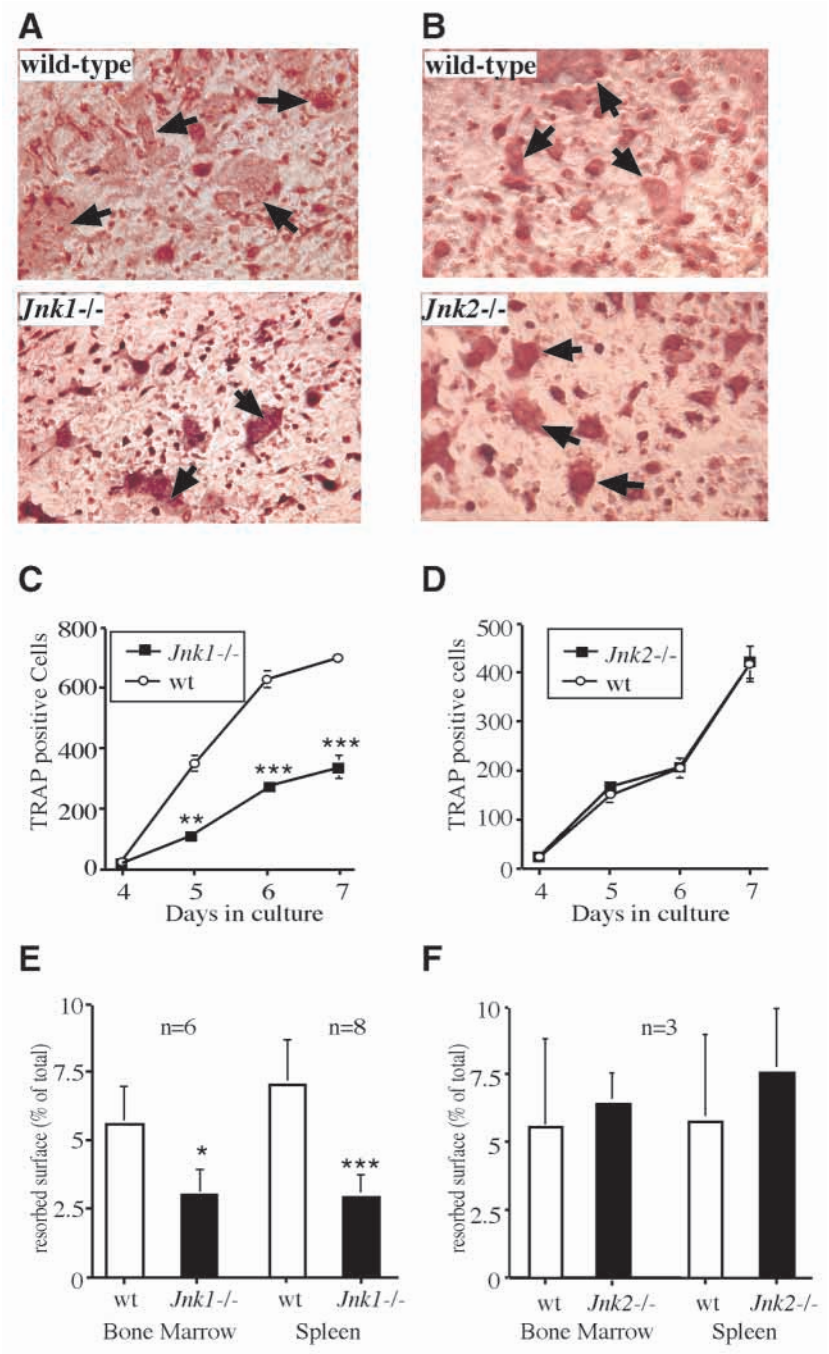


**Fig. 1.** Osteoclastogenesis is impaired in *Jnk1*$^{-/-}$ but not *Jnk2*$^{-/-}$ mice. Bone marrow hematopoietic precursors were isolated from *Jnk1*$^{-/-}$ (A,C,E), *Jnk2*$^{-/-}$ (B,D,F) and wild-type littermates and co-cultured with wild-type primary osteoblasts in osteoclastogenic conditions. Osteoclastogenesis was monitored by TRAP staining (red color). (A,B) TRAP staining after 6 days of culture; the arrows indicate multinucleated TRAP-positive cells. (C,D) Time course of osteoclast differentiation. (E,F) Osteoclast activity measured by pit assay on bovine cortical bone slices. The method for statistical analysis is described in the materials and methods, the stars indicate the statistical significance: (***) $P<0.0001$, (**) $P<0.001$, (*) $P<0.005$, ($n$) number of slices analyzed.

lack of JNK1 affects the expression of TRAF2 and TRAF6. At the protein level, we found that RANKL did not alter TRAF2 expression within the first 6 hours of stimulation (data not shown). However, we found that RANKL-mediated TRAF2 upregulation is abolished in BMMs from *Jnk1*$^{-/-}$ but not from *Jnk2*$^{-/-}$ mice that were cultured for 3 days (Fig. 3A). By contrast, *Jnk1* deletion did not affect TRAF6 expression (Fig. 3B). Moreover, a rapid upregulation of TRAF2 was found in *Jnk1* $^{-/-}$ BMMs 1 hour after UV irradiation, which activates JNK2 (Fig. 3A). Thus, the lack of a compensatory effect of JNK2 in the absence of JNK1 may be due to the impaired regulation of TRAF2. To determine if the regulation of TRAF2 in osteoclast progenitors occurred at the transcriptional level, TRAF2 mRNA expression was analyzed in wild-type and *Jnk1*$^{-/-}$ osteoclast precursors. Upregulation of TRAF2 mRNA by RANKL was not observed in wild-type or *Jnk1*$^{-/-}$ osteoclast precursors (Fig. 3C), suggesting that the JNK1-dependent TRAF2 upregulation is post-transcriptional.

### Impaired c-Jun phosphorylation in *Jnk1*$^{-/-}$ BMMs

We next investigated whether c-Jun phosphorylation is JNK1 dependent in osteoclast progenitors exposed to osteoclastogenic signals. *Jnk1*$^{-/-}$ or wild-type BMMs were stimulated by different combinations of M-CSF, RANKL and TNF-α for 1 hour or exposed to UV. c-Jun phosphorylation was induced in RANKL or/and TNF-α-treated wild-type cells (Fig. 4A) but was totally abolished in *Jnk1*$^{-/-}$ cells, although it was still observed following UV exposure (Fig. 4A). c-Jun was expressed in both wild-type and *Jnk1*$^{-/-}$ cells, excluding the hypothesis that the lack of phosphorylation in response to osteoclastogenic signals was due to a lack of c-Jun expression (Fig. 4B). Thus, c-Jun phosphorylation in response to osteoclastogenic stimulation is strictly dependent on JNK1 expression and activation. Finally, we analyzed whether the lack of JNK1 affects the activation of the other signalling cascades activated by M-CSF and RANKL in osteoclast progenitors. Both the activation of ERK and p38 (Fig. 4C) and the activation of NF-κB were found to be unaffected in *Jnk1*$^{-/-}$ BMMs (Fig. 4D).

### Impaired osteoclastogenesis in *JunAA/JunAA* mice and in mice lacking c-Jun but not in mice lacking JunD

If phosphorylation of c-Jun by JNK1 modulates osteoclastogenesis, then progenitor cells isolated from *JunAA/JunAA* mice should present a similar

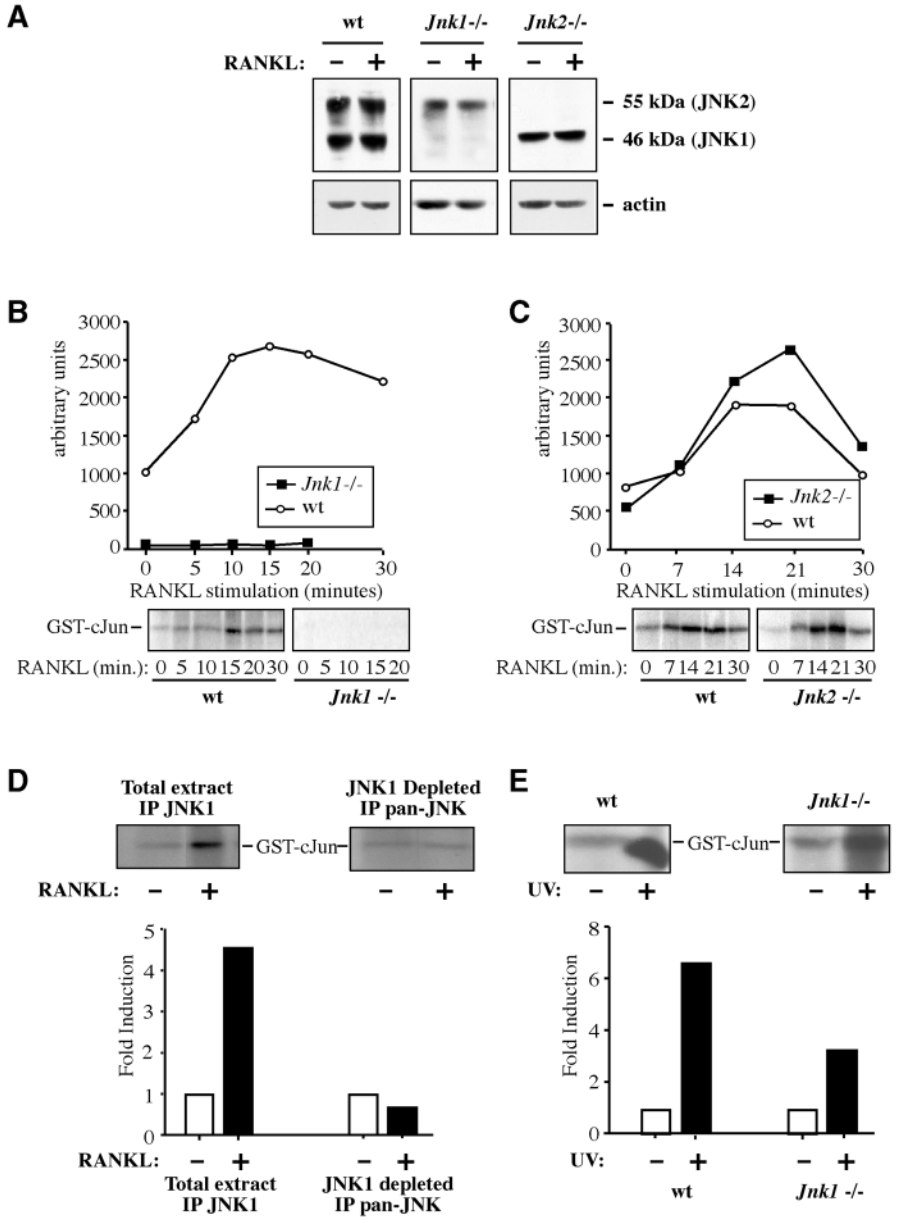


**Fig. 2.** JNK1 but not JNK2 is activated by RANKL in osteoclast progenitors. (A) The expression of JNK isoforms was analyzed in bone marrow monocytes (BMMs) isolated from wild-type, *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ mice and cultured for 3 days in the presence of M-CSF alone (20 ng/ml) (–) or M-CSF and RANKL (50 ng/ml) (+). The expression of JNK isoforms was determined by western blotting using an antibody that recognizes both JNK1 and JNK2. (B,C) Time-dependent activation of JNK by RANKL. M-CSF-dependent monocytes were isolated from wild-type, *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ mice and treated with RANKL (50 ng/ml) for the indicated times. The kinase activity was assayed following JNK immunoprecipitation using GST-N-terminal-c-Jun fusion protein as a substrate. The phosphorylation of GST-c-Jun was quantified after SDS PAGE separation and phosphoimager analysis. (D) JNK1 immunodepletion. Wild-type M-CSF-dependent monocytes were treated with RANKL (50 ng/ml) for 15 minutes. JNK1 and JNK2 were sequentially immunoprecipitated, and JNK activity was assayed in both fractions. (E) Activation of JNK2 in *Jnk1*$^{-/-}$ BMMs. Wild-type and *Jnk1*$^{-/-}$ M-CSF-dependent monocytes were exposed to UV irradiation (40 J/m$^2$). Kinase activity was measured following JNK immunoprecipitation.

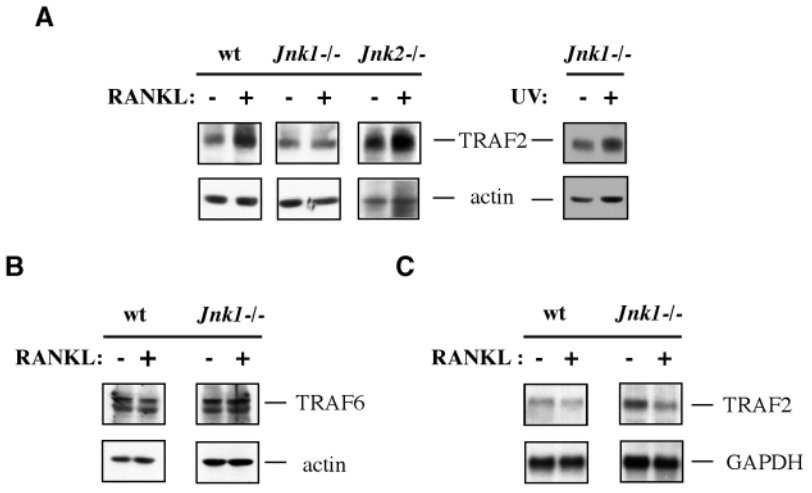


**Fig. 3.** TRAF2 upregulation by RANKL is abolished in *Jnk1*$^{-/-}$ osteoclast progenitors. Purified BMMs were isolated and treated for 3 days in the absence (–) or presence (+) of RANKL (50 ng/ml). The expression of TRAF2 (A) or TRAF6 (B) protein was analyzed by western blotting. TRAF2 expression was also analyzed 1 hour following UV irradiation of JNK1-deficient BMMs (UV +). Loading was controlled by reblotting with an anti-α actin antibody. (C) The expression of TRAF2 mRNA was analyzed by northern blotting. RNA loading was controlled by probing with GAPDH.

osteoclast differentiation defect to *Jnk1*$^{-/-}$ cells. Indeed, when co-cultured with wild-type osteoblasts, the efficiency of osteoclast differentiation of *JunAA/JunAA* cells was also reduced compared with wild-type cells (Fig. 5A). To exclude the possibility that the impaired differentiation of *JunAA/JunAA* cells is limited to the co-culture conditions, we replaced the supportive osteoblasts by soluble M-CSF and RANKL. As in co-culture experiments, the efficiency of differentiation of *JunAA/JunAA* cells was impaired compared with cells isolated from heterozygote littermates (Fig. 5B). The efficiency of differentiation was also reduced when RANKL was replaced by TNF-α (Fig. 5C). Thus, c-Jun N-terminal phosphorylation is necessary for efficient osteoclastogenesis induced by RANKL or TNF-α.

We next analyzed whether JunD, which is expressed in BMMs (data not shown), could also mediate JNK1 function in osteoclast differentiation. The differentiation of osteoclast progenitors isolated from JunD-deficient mice was examined. No significant effects on RANKL-induced osteoclast differentiation could be detected in the absence of JunD (Fig. 5D). Furthermore, the lack of JunD did not significantly affect the osteoclastogenesis defect observed with *JunAA/JunAA* mice, as determined with cells isolated from double mutant *JunD*$^{-/-}$ *JunAA/JunAA* mice (Fig. 5E). Thus, c-Jun appears to be the main mediator of JNK1 function during osteoclast differentiation.

To confirm the importance of c-Jun in osteoclastogenesis, c-*Jun* was inactivated in the hematopoietic compartment using mice carrying a floxed c-*jun* allele and expressing the Cre recombinase under the inducible Mx promoter (Behrens et al., 2002). The efficiency of deletion was analyzed by PCR of DNA isolated from M-CSF-dependent BMMs (data not show). BMMs isolated following the induction of c-*Jun* deletion in vivo showed an impaired efficiency of differentiation when compared with wild-type or heterozygote littermates (Fig. 5F), indicating that c-Jun affects osteoclastogenesis.

## Increased RANKL-induced apoptosis in JNK1-deficient BMMs

We next analyzed the cellular mechanism that could explain the decreased osteoclastogenesis observed with JNK1 deficient

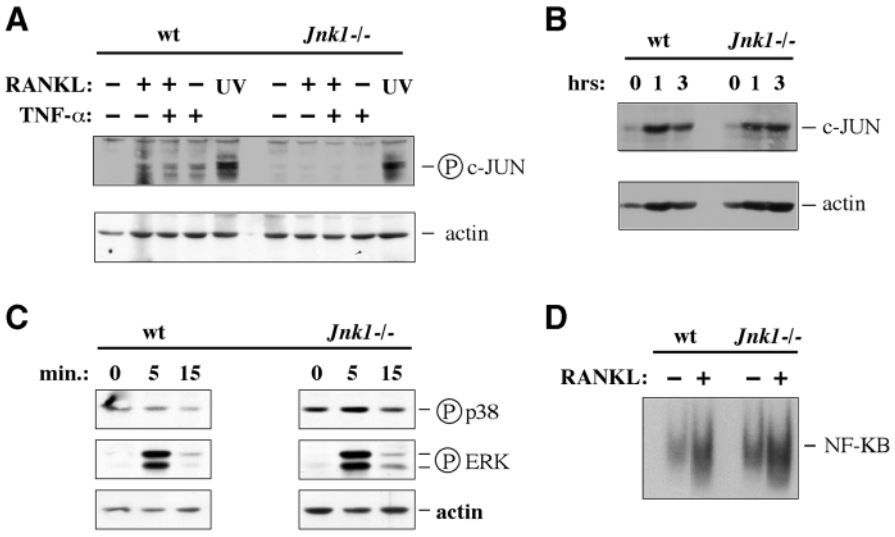


**Fig. 4.** c-Jun phosphorylation by osteoclastogenic signals is JNK1 dependent. (A) The phosphorylation of c-Jun in BMMs was monitored by western blotting using a specific antibody directed against the phosphorylated form of c-Jun (phospho-Ser 63), 1 hour after the addition of M-CSF (20 ng/ml), RANKL (50 ng/ml), TNF-α (50ng/ml) or UV irradiation (40 J/m$^2$). (B) The expression of c-Jun was analyzed in M-CSF- (20ng/ml) and RANKL- (50 ng/ml) stimulated BMMs isolated from wild-type (wt) or *Jnk1*$^{-/-}$ mice. (C) The activation of p38 and ERK in response to M-CSF and RANKL was analyzed by western blotting using specific anti-phosphoantibodies. (D) The activation of NF-κB was analyzed by EMSA using nuclear extracts isolated from BMMs treated for 30 minutes with RANKL (+) compared with untreated cells (–).

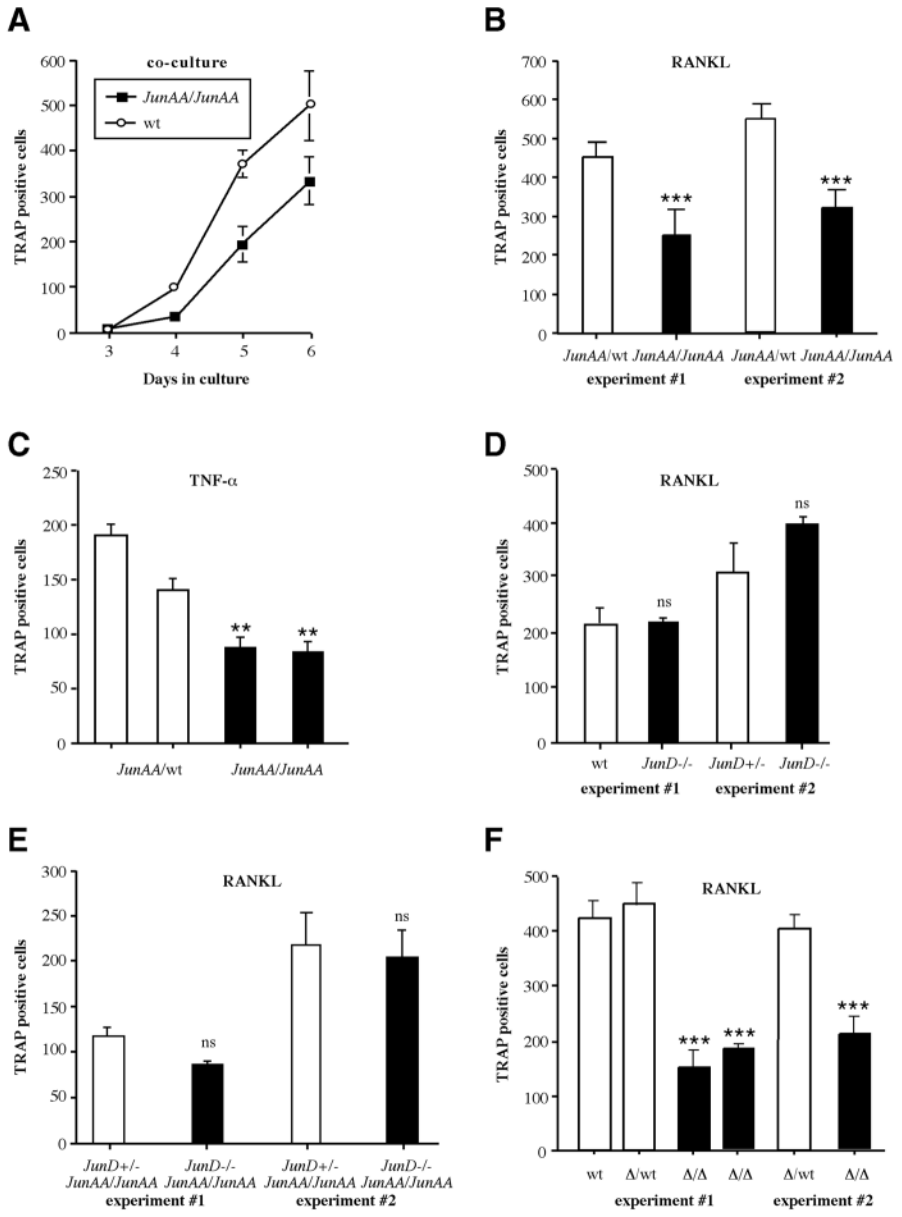


**Fig. 5.** Osteoclastogenesis is affected in cells from *JunAA/JunAA* mice and in cells lacking c-Jun but not lacking JunD. (A) Co-culture of *JunAA/JunAA* or wild-type hematopoietic precursors with wild-type osteoblasts. (B) Osteoclast differentiation of bone marrow precursors treated with M-CSF (20 ng/ml) and RANKL (50 ng/ml), or M-CSF (20 ng/ml) and TNF-α (50 ng/ml) (C). The data represent results obtained from two experiments (B) or with pairs of littermate mice (C). (D) The function of JunD in osteoclastogenesis was addressed by direct stimulation of bone-marrow precursors isolated from wild-type, $JunD^{+/-}$ or $JunD^{-/-}$ mice by M-CSF and RANKL. (E) RANKL-mediated differentiation of double mutated cells $JunD^{-/-}$; *JunAA*/*JunAA* compared with $JunD^{+/-}$; *JunAA*/*JunAA* cells. (F) RANKL-mediated differentiation of BMMs lacking c-*Jun* (Δ/Δ) compared with wild-type (wt) or heterozygous controls (Δ/wt). Osteoclast differentiation was monitored by TRAP staining from day 3 to 6 (A) or at day 4 (C,D,E,F) of culture. The deletion of c-Jun was measured by PCR analysis of DNA isolated from the cultured cells (data not shown). The stars indicate the statistical significance: (***) $P<0.0001$, (**) $P<0.001$; (ns) non-significant.

cells. As JNK activity has been involved in the control of cell survival in numerous cellular models, the apoptotic index was determined in BMMs isolated from JNK1-deficient, JNK2-deficient or wild-type mice. A three-fold increase in apoptosis was found in JNK1-deficient cells treated with RANKL and isolated from male or female mice (Fig. 6A-C). *Jnk2* deletion did not affect the survival of the cells (Fig. 6B). To determine whether c-Jun phosphorylation mediates the anti-apoptotic effect of JNK1 in BMMs, we determined the apoptotic index of BMMs isolated from *JunAA*/*JunAA* mice compared with *JunAA*/wt. No significant increase in apoptosis was found in *JunAA*/*JunAA* BMMs treated with RANKL (Fig. 6D). Thus, the protection of osteoclast progenitors from RANKL-induced apoptosis is independent of c-Jun phosphorylation.

## Discussion

Here we demonstrate that the activation of JNK1 is necessary for efficient osteoclastogenesis but not for the bone resorbing activity of these cells. The lack of compensation by JNK2 in the absence of JNK1 during osteoclast differentiation cannot be explained by a lack of expression of JNK2, since both JNK1 and JNK2 are expressed in osteoclast progenitors. These data indicate that JNK1, but not JNK2, is specifically activated by the osteoclastogenic cytokine RANKL. JNK1 appears to play two different functions during osteoclast differentiation. The first one, protection against RANKL-induced apoptosis, is independent of c-Jun phosphorylation. The second is dependent on c-Jun phosphorylation, as demonstrated by the decreased osteoclastogenesis observed in *JunAA*/*JunAA* mice and as confirmed by the deletion of c-*Jun*. Thus, JNK1 appears to regulate osteoclast differentiation at different levels.

The pro- or the anti-apoptotic functions of JNK1 and JNK2 depend on the stage of development (Kuan et al., 1999; Sabapathy et al., 1999b), on the stage of cell differentiation (Sabapathy et al., 2001) and on the type of apoptotic stress

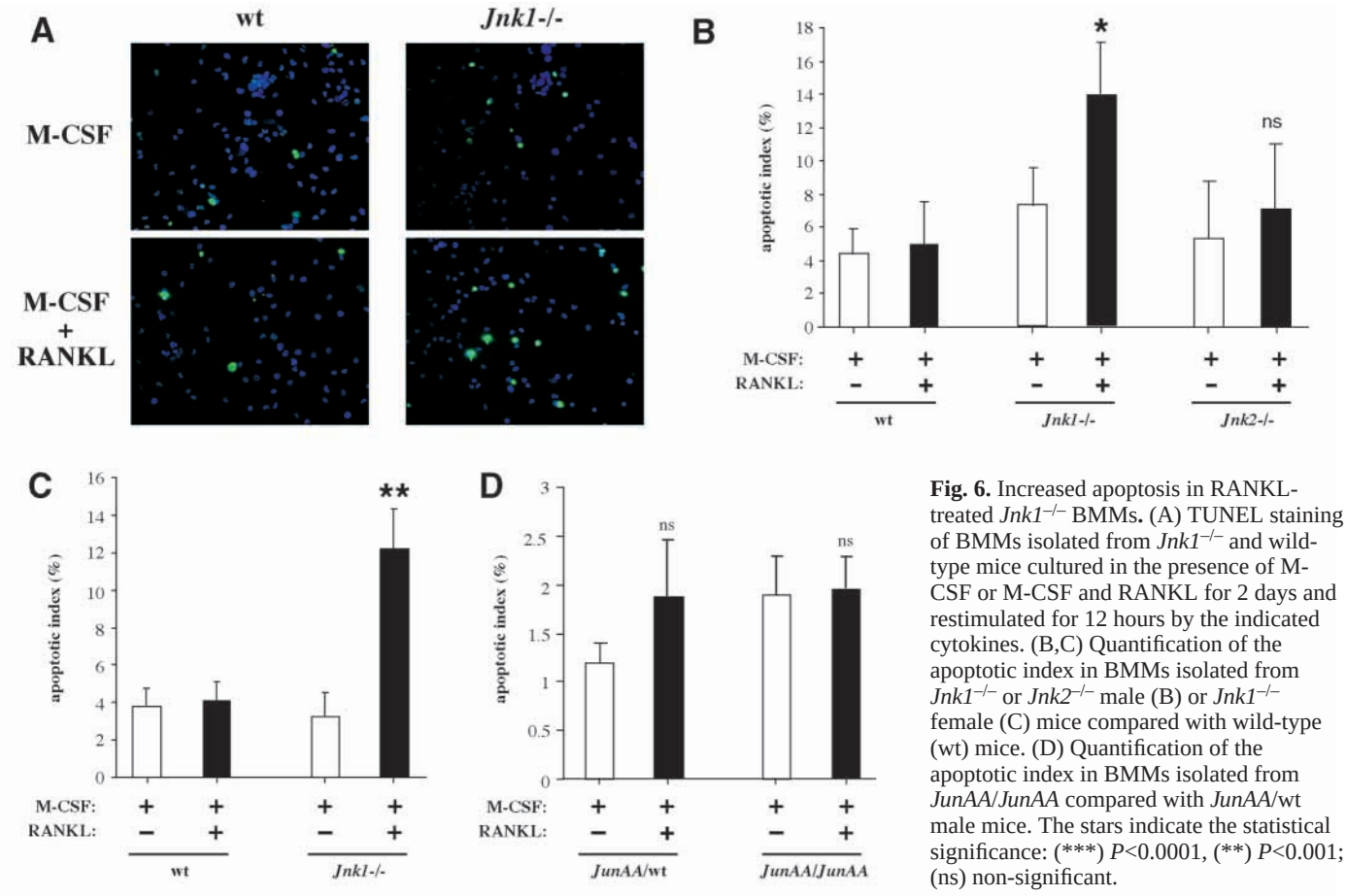


**Fig. 6.** Increased apoptosis in RANKL-treated *Jnk1*$^{-/-}$ BMMs**.** (A) TUNEL staining of BMMs isolated from *Jnk1*$^{-/-}$ and wild-type mice cultured in the presence of M-CSF or M-CSF and RANKL for 2 days and restimulated for 12 hours by the indicated cytokines. (B,C) Quantification of the apoptotic index in BMMs isolated from *Jnk1*$^{-/-}$ or *Jnk2*$^{-/-}$ male (B) or *Jnk1*$^{-/-}$ female (C) mice compared with wild-type (wt) mice. (D) Quantification of the apoptotic index in BMMs isolated from *JunAA*/*JunAA* compared with *JunAA*/wt male mice. The stars indicate the statistical significance: (***) $P<0.0001$, (**) $P<0.001$; (ns) non-significant.

applied to the cells (Hochedlinger et al., 2002; Tournier et al., 2000). Our data demonstrate that specific activation of JNK1, but not JNK2, by RANKL protects monocytes from RANKL-induced apoptosis during osteoclast differentiation. This is in agreement with the observation that JNK1, but not JNK2, is involved in the survival of activated T cells (Arbour et al., 2002). Similarly, both *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ fibroblasts were found to be more sensitive to TNF-α- and sorbitol-induced cell death independently of c-Jun phosphorylation (Hochedlinger et al., 2002). We observed that TRAF2 upregulation in response to RANKL is impaired in JNK1-deficient BMMs. The same observation was made in TNF-α receptor-1-deficient BMMs in which osteoclastogenesis in response to TNF-α is also impaired (Zhang et al., 2001). Although TRAF2-deficient mice are severely growth retarded, no bone phenotype has been reported and its function in osteoclasts is not known (Yeh et al., 1997). However, TRAF2-deficient cells show increased sensitivity to TNF-α-induced cell death (Lee et al., 1997; Yeh et al., 1997). Therefore, the decreased expression of TRAF2 in *Jnk1*$^{-/-}$ osteoclast progenitors might explain their increased sensitivity to RANKL-induced apoptosis. How can JNK activation regulate TRAF2 expression? JNK activation has been shown to affect the stability of target proteins (Chang and Karin, 2001; Davis, 2000). It was recently shown that the ubiquitin-mediated degradation of TRAF6 is correlated with decreased JNK activation and is responsible for the suppression of RANKL-mediated osteoclast differentiation by interferon γ (Takayanagi et al., 2000). The induction of TRAF2 degradation by non-death-domain TNF-receptor superfamily members has been reported in the case of TNF-RII, CD30 and CD40 (Brown et al., 2001; Chan and Lenardo, 2000; Duckett and Thompson, 1997; Li et al., 2002). Thus, JNK1 might be indirectly involved in the mechanisms regulating TRAF2 stability.

We, as well as others, have already identified c-Jun as a mediator of JNK function in thymocytes (Behrens et al., 2001; Dong et al., 1998; Sabapathy et al., 2001). c-Jun was also shown to mediate JNK3-dependent stress-induced neuronal apoptosis (Behrens et al., 1999). We now demonstrate that hematopoietic cells lacking c-*jun* as well as hematopoietic cells isolated from *JunAA/JunAA* mice partially phenocopy the osteoclastogenesis defect observed in *Jnk1*$^{-/-}$ cells. The other JNK substrate belonging to the Jun family, JunD, is also expressed in osteoclast progenitors, but the osteoclastogenic defect is not observed in cells isolated from *JunD*$^{-/-}$ mice. This provides genetic evidence that c-Jun is the main AP-1 target mediating JNK1 function in osteoclasts and suggests that c-Jun could be one partner of c-Fos during osteoclast differentiation. Preliminary data from mice lacking the third Jun member JunB in the osteoclast lineage indicate that osteoclastogenesis of bone marrow monocytes lacking JunB is affected (J.-P.D. and E.F.W., unpublished), suggesting that both c-Jun and JunB are involved in efficient osteoclast differentiation. However, no obvious phenotype resembling the c-*fos* deletion was observed in the individual Jun knockout mice. As all three forms of Jun are expressed in osteoclast progenitors, it is most likely that all

three Jun members can substitute for each other during osteoclast differentiation.

How can one explain the specificity of activation of JNK1? The use of specific scaffolding proteins that complex different components of the signaling cascade, leading to a specific functional module, could be a one possibility. Such a model has been demonstrated in the case of MP1 (MEK partner 1), which specifically connects ERK1, but not ERK2, to MEK1 (Schaeffer et al., 1998). Similarly, several scaffolding proteins such as the JNK-interacting proteins (JIPs) linking components of the JNK pathway have been identified (Davis, 2000). Our data suggest the presence of a specific osteoclastogenic JNK1 functional module.

Although *Jnk1*$^{-/-}$ and *JunAA/JunAA* mice display a reduced body size (Behrens et al., 1999) (K.S. and E.F.W., unpublished), an apparent bone phenotype is not detectable. The integrity of bone is dependent on the proper maintenance of the balance between bone formation and bone resorption, which is controlled by multiple local and systemic factors (Manolagas, 2000). Since AP-1 activity can regulate both osteoblast and osteoclast function (Jochum et al., 2001), one possibility would be that the lack of c-Jun phosphorylation affects both bone formation and bone resorption. In agreement with this hypothesis, the development of c-Fos-dependent osteosarcoma is impaired in *JunAA/JunAA* mice (Behrens et al., 2000). Another possibility would be that factors that inhibit osteoclast differentiation are downregulated in *Jnk1*$^{-/-}$ mice. Indeed, T cells lacking JNK1 produced less interferon γ, a strong inhibitor of osteoclastogenesis (Dong et al., 1998; Takayanagi et al., 2000). The JNK pathway is also activated by cytokines, such as TNF-α and IL-1, which mediate the increase in osteoclast differentiation observed in rheumatoid arthritis, fracture healing, osteolysis, tumor invasion of bone and post-menopausal osteoporosis (Suda et al., 1999). In agreement with our data, Abu-Amer et al. showed that mice lacking TNF-α type 1 receptors do not have any obvious bone phenotype in vivo, but the differentiation of osteoclasts in response to TNF-α is impaired in vitro (Abu-Amer et al., 2000). These mice are protected against osteolysis and fail to respond to LPS-induced osteoclastogenesis in vivo (Abu-Amer et al., 1997). Furthermore, it was recently reported that the decrease in osteoclast differentiation following estradiol treatment resulted from a block in RANKL-induced JNK activation (Shevde et al., 2000; Srivastava et al., 1999). Moreover, high levels of JNK1 expression were found in synovial tissues from patients suffering from rheumatoid arthritis. Interestingly, using an adjuvant-induced arthritis model, the administration of a specific JNK inhibitor protected rats from bone destruction (Han et al., 2001). However, *Jnk2*$^{-/-}$ mice were only partially protected against bone destruction, suggesting that JNK1 is also involved in arthritis-induced bone destruction (Han et al., 2002). These observations strongly suggest that c-Jun phosphorylation by JNK1 is a key event regulating osteoclastogenesis in pathological conditions. Thus, specific inhibition of JNK1 could be useful in clinical treatment of bone disorders associated with increased osteoclast differentiation.

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## References

**Abu-Amer, Y., Ross, F. P., Edwards, J. and Teitelbaum, S. L.** (1997). Lipopolysaccharide-stimulated osteoclastogenesis is mediated by tumor necrosis factor via its P55 receptor. *J. Clin. Invest.* **100**, 1557-1565.

**Abu-Amer, Y., Erdmann, J., Alexopoulou, L., Kollias, G., Ross, F. P. and Teitelbaum, S. L.** (2000). Tumor necrosis factor receptors types 1 and 2 differentially regulate osteoclastogenesis. *J. Biol. Chem.* **275**, 27307-27310.

**Arbour, N., Naniche, D., Homann, D., Davis, R. J., Flavell, R. A. and Oldstone, M. B.** (2002). c-Jun NH(2)-terminal kinase (JNK)1 and JNK2 signaling pathways have divergent roles in CD8(+) T cell-mediated antiviral immunity. *J. Exp. Med.* **195**, 801-810.

**Behrens, A., Sibilia, M. and Wagner, E. F.** (1999). Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. *Nat. Genet.* **21**, 326-329.

**Behrens, A., Jochum, W., Sibilia, M. and Wagner, E. F.** (2000). Oncogenic transformation by ras and fos is mediated by c-Jun N-terminal phosphorylation. *Oncogene* **19**, 2657-2663.

**Behrens, A., Sabapathy, K., Graef, I., Cleary, M., Crabtree, G. R. and Wagner, E. F.** (2001). Jun N-terminal kinase 2 modulates thymocyte apoptosis and T cell activation through c-Jun and nuclear factor of activated T cell (NF-AT). *Proc. Natl. Acad. Sci. USA* **98**, 1769-1774.

**Behrens, A., Sibilia, M., David, J. P., Mohle-Steinlein, U., Tronche, F., Schutz, G. and Wagner, E. F.** (2002). Impaired postnatal hepatocyte proliferation and liver regeneration in mice lacking c-jun in the liver. *EMBO J.* **21**, 1782-1790.

**Brown, K. D., Hostager, B. S. and Bishop, G. A.** (2001). Differential signaling and tumor necrosis factor receptor-associated factor (TRAF) degradation mediated by CD40 and the Epstein-Barr virus oncoprotein latent membrane protein 1 (LMP1). *J. Exp. Med.* **193**, 943-954.

**Chan, F. K. and Lenardo, M. J.** (2000). A crucial role for p80 TNF-R2 in amplifying p60 TNF-R1 apoptosis signals in T lymphocytes. *Eur. J. Immunol.* **30**, 652-660.

**Chang, L. and Karin, M.** (2001). Mammalian MAP kinase signalling cascades. *Nature* **410**, 37-40.

**Constant, S. L., Dong, C., Yang, D. D., Wysk, M., Davis, R. J. and Flavell, R. A.** (2000). JNK1 is required for T cell-mediated immunity against Leishmania major infection. *J. Immunol.* **165**, 2671-2676.

**David, J. P., Rincon, M., Neff, L., Horne, W. C. and Baron, R.** (2001). Carbonic anhydrase II is an AP-1 target gene in osteoclasts. *J. Cell Physiol.* **188**, 89-97.

**Davis, R. J.** (2000). Signal transduction by the JNK group of MAP kinases. *Cell* **103**, 239-252.

**Dong, C., Yang, D. D., Wysk, M., Whitmarsh, A. J., Davis, R. J. and Flavell, R. A.** (1998). Defective T cell differentiation in the absence of Jnk1. *Science* **282**, 2092-2095.

**Duckett, C. S. and Thompson, C. B.** (1997). CD30-dependent degradation of TRAF2: implications for negative regulation of TRAF signaling and the control of cell survival. *Genes Dev.* **11**, 2810-2821.

**Han, Z., Boyle, D. L., Chang, L., Bennett, B., Karin, M., Yang, L., Manning, A. M. and Firestein, G. S.** (2001). c-Jun N-terminal kinase is required for metalloproteinase expression and joint destruction in inflammatory arthritis. *J. Clin. Invest.* **108**, 73-81.

**Han, Z., Chang, L., Yamanishi, Y., Karin, M. and Firestein, G. S.** (2002). Joint damage and inflammation in c-Jun N-terminal kinase 2 knockout mice with passive murine collagen-induced arthritis. *Arthritis Rheum.* **46**, 818-823.

**Hochedlinger, K., Wagner, E. F. and Sabapathy, K.** (2002). Differential effects of JNK1 and JNK2 on signal specific induction of apoptosis. *Oncogene* **21**, 2441-2445.

**Inoue, J., Ishida, T., Tsukamoto, N., Kobayashi, N., Naito, A., Azuma, S. and Yamamoto, T.** (2000). Tumor necrosis factor receptor-associated factor (TRAF) family: adapter proteins that mediate cytokine signaling. *Exp. Cell Res.* **254**, 14-24.

**Jochum, W., Passegue, E. and Wagner, E. F.** (2001). AP-1 in mouse development and tumorigenesis. *Oncogene* **20**, 2401-2412.

**Karsenty, G. and Wagner, E. F.** (2002). Reaching a genetic and molecular understanding of skeletal development. *Dev. Cell* **2**, 389-406.

**Kuan, C. Y., Yang, D. D., Samanta Roy, D. R., Davis, R. J., Rakic, P. and Flavell, R. A.** (1999). The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. *Neuron* **22**, 667-676.

**Lee, S. Y., Reichlin, A., Santana, A., Sokol, K. A., Nussenzweig, M. C. and Choi, Y.** (1997). TRAF2 is essential for JNK but not NF-kappaB activation and regulates lymphocyte proliferation and survival. *Immunity* **7**, 703-713.

**Li, X., Yang, Y. and Ashwell, J. D.** (2002). TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2. *Nature* **416**, 345-347.

**Manolagas, S. C.** (2000). Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *Endocr. Rev.* **21**, 115-137.

**Sabapathy, K., Hu, Y., Kallunki, T., Schreiber, M., David, J. P., Jochum, W., Wagner, E. F. and Karin, M.** (1999a). JNK2 is required for efficient T-cell activation and apoptosis but not for normal lymphocyte development. *Curr. Biol.* **9**, 116-125.

**Sabapathy, K., Jochum, W., Hochedlinger, K., Chang, L., Karin, M. and Wagner, E. F.** (1999b). Defective neural tube morphogenesis and altered apoptosis in the absence of both JNK1 and JNK2. *Mech. Dev.* **89**, 115-124.

**Sabapathy, K., Kallunki, T., David, J. P., Graef, I., Karin, M. and Wagner, E. F.** (2001). c-Jun NH2-terminal kinase (JNK)1 and JNK2 have similar and stage-dependent roles in regulating T cell apoptosis and proliferation. *J. Exp. Med.* **193**, 317-328.

**Schaeffer, H. J., Catling, A. D., Eblen, S. T., Collier, L. S., Krauss, A. and Weber, M. J.** (1998). MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. *Science* **281**, 1668-1671.

**Shevde, N. K., Bendixen, A. C., Dienger, K. M. and Pike, J. W.** (2000). Estrogens suppress RANK ligand-induced osteoclast differentiation via a stromal cell independent mechanism involving c-Jun repression. *Proc. Natl. Acad. Sci. USA* **97**, 7829-7834.

**Srivastava, S., Weitzmann, M. N., Cenci, S., Ross, F. P., Adler, S. and Pacifici, R.** (1999). Estrogen decreases TNF gene expression by blocking JNK activity and the resulting production of c-Jun and JunD. *J. Clin. Invest.* **104**, 503-513.

**Suda, T., Takahashi, N., Udagawa, N., Jimi, E., Gillespie, M. T. and Martin, T. J.** (1999). Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocr. Rev.* **20**, 345-357.

**Takayanagi, H., Ogasawara, K., Hida, S., Chiba, T., Murata, S., Sato, K., Takaoka, A., Yokochi, T., Oda, H., Tanaka, K. et al. (**2000). T-cell-mediated regulation of osteoclastogenesis by signalling cross- talk between RANKL and IFN-gamma. *Nature* **408**, 600-605.

**Thepot, D., Weitzman, J. B., Barra, J., Segretain, D., Stinnakre, M. G., Babinet, C. and Yaniv, M.** (2000). Targeted disruption of the murine junD gene results in multiple defects in male reproductive function. *Development* **127**, 143-153.

**Tournier, C., Hess, P., Yang, D. D., Xu, J., Turner, T. K., Nimnual, A., Bar-Sagi, D., Jones, S. N., Flavell, R. A. and Davis, R. J.** (2000). Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science* **288**, 870-874.

**Yang, D. D., Kuan, C. Y., Whitmarsh, A. J., Rincon, M., Zheng, T. S., Davis, R. J., Rakic, P. and Flavell, R. A.** (1997). Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. *Nature* **389**, 865-870.

**Yang, D. D., Conze, D., Whitmarsh, A. J., Barrett, T., Davis, R. J., Rincon, M. and Flavell, R. A.** (1998). Differentiation of CD4+ T cells to Th1 cells requires MAP kinase JNK2. *Immunity* **9**, 575-585.

**Yeh, W. C., Shahinian, A., Speiser, D., Kraunus, J., Billia, F., Wakeham, A., de la Pompa, J. L., Ferrick, D., Hum, B., Iscove, N. et al. (**1997). Early lethality, functional NF-kappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. *Immunity* **7**, 715-725.

**Zhang, Y. H., Heulsmann, A., Tondravi, M. M., Mukherjee, A. and Abu-Amer, Y.** (2001). Tumor necrosis factor-alpha (TNF) stimulates RANKL-induced osteoclastogenesis via coupling of TNF type 1 receptor and RANK signaling pathways. *J. Biol. Chem.* **276**, 563-568.