Determining the underlying mechanisms of macrophage colony-stimulating factor (M-CSF)-mediated osteoclast survival may be important in identifying novel approaches for treating excessive bone loss. This study investigates M-CSF-mediated MEK/ERK activation and identifies a downstream effector of this pathway. M-CSF activates MEK/ERK and induces MEK-dependent expression of the immediate early gene Egr2. Inhibition of either MEK1/2 or inhibition of Egr2 increases osteoclast apoptosis. In contrast, wild-type Egr2 or an Egr2 point mutant unable to bind the endogenous repressors Nab1/2 (caEgr2) suppresses basal osteoclast apoptosis and rescues osteoclasts from apoptosis induced by MEK1/2 or Egr2 inhibition. Mechanistically, Egr2 induces pro-survival Blc2 family member Mcl1 while stimulating proteasome-mediated degradation of pro-apoptotic Bim. In addition, Egr2 increased the expression of c-Cbl, the E3 ubiquitin ligase that catalyzes Bim ubiquitination. M-CSF, therefore, promotes osteoclast survival through MEK/ERK-dependent induction of Egr2 to control the Mcl1/Bim ratio, documenting a novel function of Egr2 in promoting survival.

Although bone in young adults is continually resorbed and rebuilt in a balanced manner, unbalanced bone loss results from increased bone resorption without concomitant replacement with an equal amount of new bone. Osteoclastic bone resorption is governed primarily by the numbers of osteoclasts present at the site of bone remodeling and the activity of those osteoclasts (1). Therefore, factors affecting osteoclastogenesis and osteoclast survival are key to regulating the amount of bone resorbed. Macrophage colony-stimulating factor (M-CSF) and receptor activator for nuclear factor-κB ligand (RANKL) are two cytokines both necessary and sufficient to moderate osteoclast differentiation from hematopoietic cells within the monocyte/macrophage lineage (2, 3). M-CSF binds to the receptor tyrosine kinase member c-fms, which then activates intracellular signaling through an autophosphorylation event (4). Although M-CSF is known to promote osteoclast survival, the mechanism by which M-CSF mediates survival is unknown. Mitogen-activated protein (MAP) kinases are specific protein kinases influencing cell proliferation, differentiation, and survival. All three MAP kinase pathways, namely the MEK/ERK, p38 MAPK, and c-Jun NH2-terminal kinase pathways, play roles in osteoclasts either in differentiation and/or in mediating osteoclast function and survival (5–9). Thus, activation of these pathways is crucial in modulating bone resorption rates. Chemical inhibition of MEK1/2, which inhibits the phosphorylation of the MAP kinases ERK1/2, increases osteoclast apoptosis and leads to a loss of cell polarity (5, 7). Once activated by MEK, ERK modulates cell cycle regulation and post-mitotic functions (10). In osteoclasts, ERK activation has been demonstrated to influence survival (5, 7, 9). Although the MEK/ERK pathway influences osteoclast survival, the mechanism of activation and downstream effectors of this pathway remain unresolved.

M-CSF promotes expression of the early gene response (Egr) family of transcription factors during macrophage differentiation (11). This immediate early gene family is part of the Kruppel-like zinc finger transcription factor family and is comprised of Egr1, Egr2, Egr3, Egr4, and the Wilms’ Tumor transcription factor. Egr1, Egr2, and Egr3 contain a repressor domain involved in binding of two corepressors, Nab1 and Nab2 (12, 13). Although the zinc finger binding domains of the Egr family members are virtually identical, the remaining domains differ significantly, implying unique functions for each of these transcription factors (14). Egr1 up-regulation in prostate cancer cells is thought to promote cell growth and transformation through increased expression of cyclin D1 (15, 16). However, Egr1 expression is either down-regulated or promotes apoptosis in other cancer cell lines (17–19), indicating cell-type specific responses. Egr2 expression has been primarily reported to promote apoptosis through transactivation of p53, Fasl, Bak, and BNIP3 or suppress proliferation through PTEN expression (20–22). To date, a pro-survival role for Egr2 has not been reported in any cell type.

Promotion of osteoclast apoptosis is triggered by an internal mechanism regulated by the release of cytochrome c from the
mitochondria and subsequent caspase activation (23). Cyto-
chrome c release is controlled by the influence Bcl2 family pro-
teins have on the stability of the mitochondrial membrane. The
two main classes of Bcl2 family proteins are divided by their
ability to either promote or inhibit apoptotic responses and
their structural similarity. Family members with the most
homology to Bcl2 antagonize apoptosis. These pro-survival
Bcl2 members include Bcl2, BclxL, Mcl1, and Bclw. In contrast
to Bcl2, there are also family members that promote apoptosis.
The pro-apoptotic members include Bax, Bak, Bim, Bad, Bik,
Blk, Hrk, Bid, Bok/Mtd, and Bcl-x<sub>-</sub>, a splice variant of BclxL.
Members of the Bcl2 family participate in both hetero-
and homodimerization. Although heterodimerization is not a
requirement for the function for the pro-survival members, it
is essential for the function of some members that promote apo-
ptosis (34). These members are localized within the cytosol and
must form a dimer with another member located on a mem-
brane to exert their action. Expression of Bcl2 promotes sur-
vival of osteoclasts, rescues cells from cycloheximide-induced
apoptosis, and reduces caspase 9 activation (23). In addition,
Bim deficiency leads to increased bone density and increased
osteoclast survival (35). However, endogenous responses medi-
ated by activation of the MEK/ERK pathway leading to altered
expression of pro-survival and/or pro-apoptotic Bcl2 family
members has yet to be identified.

Bim levels are regulated through degradation by the 26 S
proteasome (36), and events catalyzed by polyubiquitination
are mediated by the E3 ligase c-Cbl in osteoclasts (35). The
Cbl proteins, c-Cbl, Cbl-b, and Cbl-c, are RING-type E3
ubiquitin ligases (37, 38). c-Cbl regulates protein-tyrosine
kinase activity through receptor down-regulation, functions
as an adaptor protein, and regulates signal transduction
events involved in phosphatidylinositol 3-kinase and MAP
kinase signaling (39). In addition to controlling levels of Bim,
c-Cbl modulates osteoclast resorption, motility, and podo-
some formation through c-Src interactions and cytoskeletal
regulation (40–42).

Given the role M-CSF plays in supporting osteoclast survival,
the molecular signals through which M-CSF promotes survival
were investigated. The work reported here shows that M-CSF
transiently activates the MEK/ERK pathway to promote oste-
oclast survival. Activation of this pathway by M-CSF leads
to expression of Egr1 and Egr2. The specific roles Egr1 and Egr2
play in regulation of osteoclast survival were examined. Egr1
did not function to promote osteoclast survival, whereas the
findings reported here demonstrate a novel pro-survival func-
tion for Egr2 downstream of M-CSF-mediated MEK/ERK activ-
ation. In this report we also identify the mechanism of Egr2-
promoted osteoclast survival. Egr2 function is required for
expression of pro-survival Bcl2 family member Mcl1, whereas
Egr2 also promotes proteasome-mediated degradation of pro-
apoptotic Bim by regulating expression of the E3 ubiquitin
ligase c-Cbl. Thus, Egr2 functions through a novel pro-survival
mechanism in osteoclasts by increasing Mcl1 expression and
increasing targeted degradation of Bim through up-regulation
of c-Cbl.

**Egr2 Promotes Osteoclast Survival**

**EXPERIMENTAL PROCEDURES**

Unless otherwise noted, all chemicals were purchased form
Sigma-Aldrich.

**Osteoclast Differentiation**—Mouse marrow osteoclast pre-
cursors were obtained from female Balb/c mice (The Jackson
Laboratory, Bar Harbor, ME) as previously described (23). Long
bones of the hind limbs of 4–6-week-old mice were removed
after sacrifice, and the marrow was flushed out with sterile
phosphate-buffered saline. Marrow aspirates were plated at a
density of 2.9 × 10<sup>5</sup> in 100-mm dishes in αMEM supplemented
with 10% (v/v) fetal bovine serum (HyClone, Logan, UT) and 25
ng/ml M-CSF (R&D Systems, Minneapolis, MN) for 24 h. Non-
adherent cells were collected and plated in αMEM, 10% (v/v)
fetal bovine serum at a density of 2 × 10<sup>5</sup> cells/cm<sup>2</sup> in 24 well-
plates and supplemented with 25 ng/ml M-CSF and 100 ng/ml
recombinant RANKL. Cells were fed after 3 days in culture with
αMEM containing 10% (v/v) fetal bovine serum, 25 ng/ml
M-CSF, and 100 ng/ml recombinant RANKL. Once mature,
osteoclasts were treated as per experimental design.

**Real-time RT-PCR**—Osteoclasts were differentiated as above
and serum-starved for 1 h in αMEM. After serum starvation,
osteoclasts were harvested immediately for total RNA or cul-
tured with 25 ng/ml M-CSF for the indicated period, rinsed
with phosphate-buffered saline, and harvested for total RNA.
Total RNA was harvested in TRIzol reagent (Invitrogen), and
phenol/chloroform was extracted from which 1 µg of RNA was
reverse-transcribed using oligo(dT) primers. The resulting
cDNA samples were used in real-time RT-PCR analysis of Egr1
(gacgagtatccaggctacaagag, gcgcagaggaagagcagtg, atctcacggtgtcctggttc),
Egr2 (gaag-
gagaacgaagatcaatctctctgctgtgtc), Egr3 (agacgtggaggcc-
catctatc, gggaaaagattgctgtccaa), Mcl1 (gcagagcctgttgtgtg, agtggagaagagagagga),
Bim (ccctcctaggacctcccag, gcgcagaggaagagcagtg, atctcacggtgtcctggttc),
c-Cbl (ttttgccgatgtgaaatcaa, ccatggagaatggagag-
gtgaagagcacagggagga), and tubulin (gcgcagaggaagagcagtg, atctcacggtgtcctggttc),
Bim (ccctcctaggacctcccag, gcgcagaggaagagcagtg, atctcacggtgtcctggttc),
c-Cbl (ttttgccgatgtgaaatcaa, ccatggagaatggagag-
gtgaagagcacagggagga), and tubulin (tcgctcatcagagatcagag, gctataggttcctg-
cacag) expression as outlined in Karst et al. (43). Expression
of Egr1, Egr2, and Egr3 transcripts was normalized to tubulin
transcript levels for each sample. -Fold changes for each sample
as compared with time 0 were then calculated. Data are the
result of three replicate biological samples. Each experiment
was performed in triplicate.

**Western Blotting**—Osteoclasts were differentiated as above
and serum-starved in αMEM for 1 h. The MEK1/2 inhibitor
UO126 was used at a concentration of 10 µM where indicated
during serum starvation and subsequent treatments. The 26 S
proteasome inhibitor MG-132 was used at a concentration of
42 µM as indicated during serum starvation and culture. After
serum starvation, osteoclasts were either harvested immedi-
ae for Western blotting or cultured with 25 ng/ml M-CSF for
the indicated time period, rinsed with phosphate-buffered
saline, and harvested for Western blotting. Harvesting was
accomplished by scraping into SDS sample buffer lacking
β-mercaptoethanol and bromphenol blue. To ensure that equal
cell protein was analyzed, total protein was quantified using the
Bio-Rad Dc assay as per the manufacturer’s instructions, and
equal protein (60 µg) was loaded (Bio-Rad). Before loading, 1 µl of
β-mercaptoethanol and bromphenol blue were added to
each sample. Western blotting was carried out using antibodies

8056 JOURNAL OF BIOLOGICAL CHEMISTRY
VOLUME 283 • NUMBER 12 • MARCH 21, 2008
directed against total and phospho-MEK1/2 (Ser-217/221), total and phospho-ERK1/2 (Thr-202/Tyr-204), Egr1, Bim, ubiquitin (Cell Signaling, Beverly, MA), Egr2 (Santa Cruz Biotechnology, Santa Cruz, CA), Nab2 (Active Motif, Carlsbad, CA), Mcl1 (Abcam, Cambridge, MA), c-Cbl (BD Transduction Laboratories), and tubulin (E7, Hybridoma Bank, Iowa State, IA) at a 1:1000 dilution and the corresponding secondary antibodies (Cell Signaling) at a 1:10,000 dilution with chemiluminescent detection using the Pierce femto reagent. Blots were stripped and reprobed for total protein expression using a Western blot Recycling Kit (Alpha Diagnostics, San Antonio, TX). Each experiment was carried out a minimum of three times.

**Bim Ubiquitination Assay**—Osteoclasts were differentiated as above, serum-starved for 1 h, and either harvested in radioimmune precipitation assay buffer (Santa Cruz Biotechnology) containing sodium orthovanadate, phenethylsulfonyl fluoride, and a protease inhibitor mixture or incubated with 25 ng/ml M-CSF as indicated. After M-CSF treatment, osteoclasts were harvested in radioimmune precipitation assay buffer as above. A 500-µg sample of the resulting cell lysates was incubated with total Bim antibody for 15 min at 4 °C, after which a 60-µl aliquot of 50% protein A-agarose slurry was added and incubated with the samples overnight at 4 °C. A 60-µg sample of total cell lysate was saved for total tubulin levels as assayed by Western blotting. After overnight incubation, beads were pelleted and washed 2 times with each of 10 mM Tris, pH 8.0, 150 mm NaCl, 0.5% Nonidet P-40, 0.05% SDS; 10 mM Tris pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 0.05% SDS, 0.5% dodecylmaltoside, and 10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% SDS. SDS sample buffer containing bromphenol blue and β-mercaptoethanol was then added to the beads. Samples were boiled and pelleted. The resulting supernatant was used in a Western blot for ubiquitin and stripped and reprobed for total Bim.

**Adenovirus Infections**—Adenoviral constructs for Nab2, wild type, and caEgr1 were a kind gift from Dr. M. Ehrengruber (65). The adenoviral construct for dominant negative MEK1 was received from L. F. Parada. Wild-type Egr2 virus was purchased from Vector Biolabs, Philadelphia, PA. An expression construct for caEgr2 containing an I268N transversion, a gift from Dr. J. Milbrandt, was used in a custom construction of the corresponding adenovirus (Vector Biolabs). Viruses were expanded and titered according to standard procedures as needed. Mature osteoclasts were infected with each indicated virus at a multiplicity of infection of 100 for 18 h before the experimental procedures.

**Statistical Analysis**—Data obtained are the mean ± S.D. and are representative of three replicate experiments. The effect of treatment was compared with control values using Student’s t test to assess significant differences using Microsoft Excel Apple software.

**RESULTS**

**M-CSF Transiently Activates the MEK/ERK Pathway to Promote Osteoclast Survival**—Mature osteoclasts were serum-starved and treated with 25 ng/ml M-CSF for 0–30 min (Fig. 1A). A rapid increase in the phosphorylation of both MEK1/2 and ERK1/2 was induced by M-CSF. To determine whether
Egr2 Promotes Osteoclast Survival

blocking MEK could block M-CSF-mediated activation of this pathway, MEK activity was blocked through chemical inhibition of MEK1/2 by UO126 (Fig. 1B). M-CSF stimulated activation of ERK after 5 min, and chemical inhibition of MEK1/2 blocked ERK1/2 activation induced by M-CSF administration (Fig. 1B). Because these data confirmed M-CSF-mediated MEK/ERK activation, the influences of M-CSF-mediated activation of the MEK/ERK pathway on osteoclast survival were examined. To examine the role of MEK in M-CSF-mediated osteoclast survival, osteoclasts were treated with M-CSF in the presence of the MEK1/2 inhibitor, vehicle control, or no treatment. As documented previously, examination of nuclear condensation clearly delineated apoptotic osteoclasts (Fig. 1C) (5).

Using this basis, the percentage of apoptotic osteoclasts associated with each treatment were determined. M-CSF treatment sustained osteoclast survival under serum-free conditions as previously described (Fig. 1D) (44). Blocking MEK1/2 activity through UO126 treatment abolished the pro-survival effects of M-CSF and increased osteoclast apoptosis (Fig. 1D). Thus, M-CSF-promoted osteoclast survival can be blocked through chemical inhibition of MEK1/2.

The Immediate Early Genes Egr1 and Egr2 Are Induced by M-CSF-mediated Activation of the MEK/ERK Pathway—Because activation of the MEK/ERK pathway in response to M-CSF is crucial in supporting osteoclast survival, potential downstream targets of this pathway were next examined. The Egr (early gene response) family genes Egr1, Egr2, and Egr3 are induced by M-CSF during macrophage differentiation (11). For this reason, Egr1, Egr2, and Egr3 were identified as candidate downstream effectors of the MEK/ERK pathway potentially involved in osteoclast survival. To explore this possibility, expression of the Egr transcription factors in response to M-CSF treatment of mature osteoclasts was evaluated. Mature osteoclasts were serum-starved and treated with M-CSF as indicated in Fig. 2. After M-CSF treatment, total RNA was harvested and analyzed by real-time RT-PCR for Egr1, Egr2, and Egr3 transcripts. As indicated in Fig. 2A, M-CSF treatment led to a robust and transient increase in Egr1 and Egr2 transcripts. A change in Egr3 transcript levels was not observed with M-CSF addition (data not shown). Because transcript levels of Egr1 and Egr2 were up-regulated by M-CSF, MEK-dependent protein expression of Egr1 and Egr2 downstream of M-CSF was tested. The addition of M-CSF after serum starvation increased expression of both Egr1 and Egr2 (Fig. 2B). This response was blocked through chemical inhibition of MEK1/2 (Fig. 2C). These data demonstrate that M-CSF treatment of mature osteoclasts leads to MEK-dependent expression of two Kruppel-like transcription factors, Egr1 and Egr2.

Egr2 Function Suppresses Osteoclast Apoptosis—Given the rapid, transient MEK-dependent expression of these immediate early genes upon M-CSF treatment, potential roles for this gene family in suppression of osteoclast apoptosis were explored. To inhibit the function of all Egr family members, the Egr family corepressor Nab2, which binds to a specific repressor domain shared by Egr1, Egr2, and Egr3, was employed (12, 13). We also determined if additive effects caused by MEK inhibition and Nab2 expression were evident, as this would imply separate mechanisms. An increase in osteoclast apoptosis with Nab2 expression as compared with vector infection was observed (Fig. 3A), supporting a role for the Egr transcription factors in promotion of osteoclast survival. Although both Nab2 and MEK inhibition increased osteoclast apoptosis, a further increase in osteoclast apoptosis with Nab2 expression in combination with UO126 treatment was not evident (Fig. 3A).

In addition, numbers of total osteoclasts were also determined (Fig. 3B). A difference in overall total numbers of osteoclasts was not observed. A trend toward decreased overall cell numbers in osteoclasts treated with UO126 in combination with Nab2 infection as compared with either treatment alone was observed (Fig. 3B). These data suggest a role for Egr1 and/or Egr2 in M-CSF-suppressed osteoclast apoptosis and demonstrate the necessity of Egr family members in the promotion of MEK-dependent osteoclast survival.

Because Nab2 expression increased osteoclast apoptosis and both Egr1 and Egr2 were expressed in response to M-CSF in mature osteoclasts, Egr1 and Egr2 were evaluated as potential regulators of osteoclast apoptosis. Wild-type forms of both Egr1 and Egr2 as well as two point mutants (caEgr1 and caEgr2) were used to test this possibility. This point mutation occurs within the repressor domain of each transcription factor and abolishes the interaction between Egr1/2 and Nab2/1/2. Osteoclast survival, osteoclasts were treated with M-CSF as indicated in the presence of the MEK1/2 inhibitor UO126 or vehicle control. Western blotting for Egr1, Egr2, and tubulin was performed. C, Egr1 and Egr2 protein expression induced by M-CSF is dependent on MEK/ERK activation; osteoclasts were serum-starved and treated with M-CSF as indicated in the presence of the MEK1/2 inhibitor UO126 or vehicle control. Western blotting for Egr1, Egr2, and tubulin was then performed from the resulting cell lysate. DMSO, Me₂SO.

FIGURE 2. M-CSF induces MEK-dependant expression of two immediate early genes, Egr1 and Egr2, in mature day 4 osteoclasts. A, M-CSF induces Egr1 and Egr2 transcript production. Osteoclasts were treated with M-CSF and assayed for Egr1 and Egr2 transcript production using real-time PCR. Values are representative of three replicate samples. *, p < 0.05. B, Egr1 and Egr2 protein expression is increased by M-CSF. Osteoclasts were serum-starved for 2 h and treated with M-CSF as indicated. Western blotting for Egr1, Egr2, and tubulin was performed. C, Egr1 and Egr2 protein expression induced by M-CSF is dependent on MEK/ERK activation; osteoclasts were serum-starved and treated with M-CSF as indicated in the presence of the MEK1/2 inhibitor UO126 or vehicle control. Western blotting for Egr1, Egr2, and tubulin was then performed from the resulting cell lysate. DMSO, Me₂SO.
Egr2 were infected with each respective adenovirus and treated with M-CSF after serum starvation. Expression of either wild type or caEgr1 did not significantly influence osteoclast apoptosis compared with vector infection alone (Fig. 3C). In contrast, expression of either the wild-type or caEgr2 repressed basal osteoclast apoptosis when compared with vector infection alone (Fig. 3C). Measures for total osteoclasts obtained with each treatment were also evaluated (Fig. 3D). An overall change in total osteoclast numbers with each treatment was not observed, indicating that expression of either Egr1 or Egr2 virus alone had no adverse effects overall. Because numbers of total osteoclasts were unchanged, apoptotic tartrate-resistant acid phosphatase-positive mononuclear cells were also assessed to determine the effects of Egr1 and Egr2 on pre-osteoclasts. Although the basal rate of mononuclear cell apoptosis was minimal, no significant difference from vector control infection was observed with either Egr1 or Egr2 expression (data not shown). The ability of either wild-type Egr2 or caEgr2 to abolish Nab2-promoted osteoclast apoptosis was next evaluated. Expression of caEgr2 suppressed the increase in osteoclast apoptosis observed with Nab2 expression when both viruses were used in combination (Fig. 3E). The total number of osteoclasts with expression of caEgr2 when used alone or in combination with Nab2 expression did not change (Fig. 3F).

Erg2 Promotes Osteoclast Survival through Increased Mcl1 Expression and Decreasing Bim Levels—Because expression of caEgr2 suppressed osteoclast apoptosis, we sought to determine whether Egr2 expression could alleviate the effects of chemical inhibition of MEK1/2 by UO126. Not only did expression of wild type and caEgr2 decrease osteoclast apoptosis as compared with vector infection, both the wild-type and point mutant forms of Egr2 were able to repress the effects of UO126 and return levels of osteoclast apoptosis to basal rates (Fig. 4, A and B). Numbers of total osteoclasts were also assessed. An overall decrease in cell numbers was observed in osteoclasts treated with UO126 in combination with caEgr2 infection as compared with vehicle-treated vector-infected cells but not when compared with vector-infected cells treated with UO126 (Fig. 4B).

Egr2 Is a Downstream Effector of the MEK/ERK Pathway Controlling Osteoclast Apoptosis—Because Egr2 suppressed osteoclast apoptosis, the role for Egr2 as a downstream effector of the MEK/ERK pathway was examined. To investigate this possibility, a bypass experiment was performed to determine whether Egr2 expression could alleviate the effects of chemical inhibition of MEK1/2 by UO126. Not only did expression of wild type and caEgr2 decrease osteoclast apoptosis as compared with vector infection, both the wild-type and point mutant forms of Egr2 were able to repress the effects of UO126 and return levels of osteoclast apoptosis to basal rates (Fig. 4, A and B). Numbers of total osteoclasts were also assessed. An overall decrease in cell numbers was observed in osteoclasts treated with UO126 in combination with caEgr2 infection as compared with vehicle-treated vector-infected cells but not when compared with vector-infected cells treated with UO126 (Fig. 4B).
mine the mechanism of Egr2-promoted osteoclast survival. To determine whether Egr2 affected osteoclast apoptosis through altered expression of Bcl2 family members, modulation of transcript levels for this family of genes by Egr2 inhibition was first assayed. Osteoclasts were infected with the Nab2 adenovirus or vector control. Real-time RT-PCR was then performed to determine how Egr2 inhibition altered gene expression of Bcl2 family members. An increase in Mcl1 transcript expression was observed after M-CSF treatment and was unaffected by expression of caEgr2 (Fig. 5D). Moreover, influences of Egr2 inhibition on Mcl1 and Bim protein levels were evaluated. After infection with Nab2 or vector control, osteoclasts were serum-starved and treated with M-CSF as indicated. Nab2 expression decreased levels of Mcl1 while increasing Bim levels in the presence of M-CSF (Fig. 5E). The impact of caEgr2 expression on Mcl1 and Bim protein levels was evaluated next. Expression of caEgr2 increased Mcl1 expression after M-CSF addition (Fig. 5F). In contrast to Bim transcript levels, caEgr2 expression decreased Bim protein (Fig. 5F).

Egr2 Promotes Ubiquitin-dependent Degradation of Bim by Regulating Levels of the E3 Ligase c-Cbl—Because caEgr2 expression decreased Bim levels in the absence of effects on Bim transcript levels, we next determined if Egr2 functioned to promote Bim protein turnover. Osteoclasts were serum-starved and treated with M-CSF in the presence of the 26 S proteasome inhibitor MG-132. Because Bim is the target of ubiquitin-dependent degradation and inhibition of the proteasome increased levels of Bim, we determined if expression of caEgr2 promoted Bim ubiquitination. Osteoclasts were infected with the caEgr2 adenovirus or vector control, serum-starved, and treated with M-CSF for the indicated times. Ubiquitinated forms of Bim were, therefore, examined by immunoprecipitation with antibodies directed toward total Bim followed by Western blotting with antibodies directed toward ubiquitin. Consistent with increased Bim turnover promoted by caEgr2 expression, caEgr2 expression increased Bim polyubiquitination compared with vector infection after M-CSF treatment (Fig. 6B). There are three different Bim isoforms, BimEL (23
kDa), BimL (15 kDa), and BimS (12 kDa). The ubiquitinated forms with a molecular mass of 19, 22, and 30 kDa would be the monoubiquitinated forms of BimS, BimL, and BimEL, respectively. Expression of caEgr2 increased the monoubiquitinated 12 kDa BimS and 15 kDa BimL as well (Fig. 6B).

Because Egr2 promoted Bim ubiquitination and degradation, the ability of Egr2 to modulate expression of the ubiquitin E3 ligase responsible for Bim ubiquitination, c-Cbl, was determined. The impact of caEgr2 expression on c-Cbl expression was examined. Osteoclasts were infected with either the caEgr2 adenovirus or vector control. Expression of c-Cbl was assayed through real-time RT-PCR. Expression of caEgr2 increased transcript levels of c-Cbl (Fig. 6C). Moreover, expression of caEgr2 increased expression of c-Cbl protein (Fig. 6D). These data indicate a role for Egr2 in promotion of M-CSF-suppressed osteoclast apoptosis through increased expression of pro-survival Bcl2 family member McI1 and the E3 ligase c-Cbl, thereby increasing the ratio of McI1/Bim levels.

**DISCUSSION**

M-CSF promotes the survival of cells within the monocyte/macrophage cell lineage, including bone-resorbing osteoclasts. Thus, understanding the mechanisms of M-CSF-induced osteoclast survival may contribute toward the treatment of conditions resulting in increased bone loss such as osteoporosis and tumor-induced bone loss (osteolysis). This study examined downstream effectors of M-CSF signaling in prevention of osteoclast apoptosis. M-CSF treatment transiently activated MEK/ERK. Transient activation of the MEK/ERK pathway has been implicated as a survival response in contrast to the sustained activation observed during differentiation (45, 46). M-CSF treatment also led to MEK-dependent induction of two Kruppel-like transcription factors, Egr1 and Egr2. Because M-CSF induced MEK-dependent expression of Egr1 and Egr2, the roles of these transcription factors in M-CSF-promoted osteoclast survival were examined.

Because inhibition of these transcription factors through expression of Nab2 increased osteoclast apoptosis, roles for Egr1 and Egr2 as effectors of M-CSF-mediated osteoclast survival were further explored. Egr1 did not repress osteoclast apoptosis, whereas expression of either wild-type or an Egr2 point mutant lacking interaction with endogenous repressors Nab1/2 (caEgr2) decreased osteoclast apoptosis. Because numbers of total osteoclasts and late pre-osteoclasts were unaffected by expression of either Egr1 or Egr2, these data suggest that Egr1 and Egr2 are not involved in early stage osteoclast differentiation. In addition, Egr2 did promote osteoclast precursor survival, supporting a selective effect of Egr2 on fully differentiated osteoclasts. Although expression of Nab2 promoted osteoclast apoptosis, this effect was abolished through the expression of caEgr2.
Egr2 Promotes Osteoclast Survival

These data demonstrate the ability of Egr2 to rescue osteoclasts from Nab2-induced apoptosis. Expression of caEgr2 and wild-type Egr2 also overcame osteoclast apoptosis promoted by inhibition of MEK1/2. Taken together, these data indicate a function for Egr2 as part of a transcriptional complex mediating the anti-apoptotic effects downstream of M-CSF-induced MEK/ERK activation.

Expression of caEgr2 decreased overall cell numbers when used in combination with the MEK1/2 inhibitor UO126. The decrease in overall cell numbers seen with combination treatment may be reflective of an incomplete rescue of MEK1/2 inhibition. Data presented here demonstrated Egr2 expression downstream of MEK/ERK activation mediated by M-CSF and decreased osteoclast apoptosis; however, expression of Egr2 alone may not be sufficient to fully restore all MEK/ERK targets promoting survival. This result is not surprising given several published observations. ERK activation has been previously implicated in the control of osteoclast survival mediated by pro-survival cytokines such as M-CSF. In accord with these findings, we found that Egr2 alone does not promote osteoclast survival. The Egr2 knock-out mouse, in contrast to the Egr1 mouse knock-out, has a striking phenotype. These mice exhibit perinatal lethality and show a deficiency in hindbrain and bone development. Thus, because these mice exhibit a broad bone phenotype, one critical function of Egr2 may be as a primary target for the control of osteoclast survival mediated by pro-survival cytokines such as M-CSF. In accord with the phenotype of the Egr2 knock-out, inhibition of this family of transcription factors through Nab2 expression leads to decreased osteoclast survival.

The data presented here demonstrate a function for Egr2 as a mediator of osteoclast survival. These findings correspond well given the phenotype of each respective knock-out animal model. The Egr1 knock-out mouse has an overt phenotype, including no deficiency in macrophage numbers, despite the proposed role for Egr1 in differentiation. This discrepancy may be due to redundancy between Egr1, Egr2, and Egr3. However, the possibility of redundancy between Egr1 and Egr2 as well as Egr1 and Egr3 in macrophage differentiation was refuted by studies of Carter and Tourtellotte (11). These findings correspond well given the phenotype of each respective Egr family member.
stream of pathway activation (23, 47). Here we have shown that inhibition of the Kruppel-like transcription factor Egr2 increases osteoclast apoptosis and expression of a c-EBPα. We, therefore, investigated the mechanism for this novel function of Egr2 in promotion of osteoclast survival. Expression of the pro-survival Bcl2 family member Mcl1 at both transcript and protein levels was also inhibited by Nab2-mediated repression of Egr2. Mcl1 has been previously identified as a crucial regulator of cells within the hematopoietic system. Although Mcl1 deficiency leads to early embryonic lethality, conditional knock-out of Mcl1 within the immune system causes decreased numbers of hematopoietic stem cells, lymphocytes as well as mature B and T cells (51, 52). Because Mcl1 plays a central role in the development and maintenance of the immune system, Mcl1 was a candidate as a downstream effector of Egr2-promoted osteoclast survival. Although basal turnover of pro-survival family members such as Bcl2 is slow, Mcl1 levels are dynamic and inducible (53–59). Given the rapid turnover, blocking protein translation decreases overall levels of Mcl1 protein (60). Inhibition of protein translation leads to rapid and substantial osteoclast apoptosis, which is blocked by Bcl2 overexpression (23, 47). We did not find significant changes in other pro-survival Bcl2 family members such as Bcl2 and Bcl-x expression associated with either Egr2 inhibition or caEgr2 expression (data not shown). In contrast, the observed increase in Mcl1 expression promoted by caEgr2 supports a pro-survival response, most likely dependent on new protein synthesis. Thus, expression of Mcl1 downstream of Egr2 is a critical regulator of osteoclast survival.

In previous studies Akiyama et al. (61) identified a role for the pro-apoptotic Bcl2 family member Bim in inhibition of osteoclast survival (35), and expression of constitutively active MEK decreased Bim expression. Bim is a BH3-only member of the Bcl2 family, and therefore, its apoptosis-inducing function is blocked through binding to pro-survival members such as Mcl1 (30, 62). Because the MEK/ERK pathway has been demonstrated to decrease levels of Bim and Mcl1 has been shown to be a critical regulator of Bim function, we also examined Egr2-mediated regulation of Bim. We observed a decrease in Bim protein modulated by Egr2 function with no concomitant effect on Bim expression (63, 64). We did not observe changes in expression of pro-apoptotic Bcl2 family members Bax and Bak. In addition, we show novel regulation of the Bim E3 ubiquitin ligase c-Cbl by Egr2. These data provide a mechanistic insight as to the pro-survival functions of Egr2 downstream of MEK/ERK activation and also show a relationship between Egr2 and c-Cbl in osteoclast function. Egr2 regulates Bim by influencing levels of the Bim ubiquitin E3 ligase c-Cbl, thus promoting ubiquitin-dependent turnover of Bim. These results link decreased expression of Bim seen with caMEK expression observed by Akiyama et al. (61) to expression of Egr2 and its regulation of c-Cbl. Because c-Cbl has been previously identified as a critical regulator of bone development, osteoclast migration, resorption, and survival (40–42), the identification of Egr2 as a novel c-Cbl regulator provides an additional mechanistic insight into the function of Egr2 in promotion of cell survival.

We have identified a novel function of Egr2 in promotion of cell survival. Herein we also examined the mechanism for Egr2-promoted osteoclast survival and identified two important functions for Egr2. First, we have demonstrated novel regulation of Mcl1 expression and effect correlated with increased cell survival. Second, we have shown Egr2 to also function in regulation of c-Cbl, an important modulator of osteoclast function and bone development. This regulation of c-Cbl by Egr2 reduces Bim protein levels by increased ubiquitin-dependent protein turnover. Thus, activation of the MEK/ERK pathway not only decreases levels of pro-apoptotic Bim as previously reported but also increases expression of the Bim-antagonist Mcl1 downstream of Egr2 expression. In summary, because previous work defining the role of Egr2 in cell survival has delineated only a pro-apoptotic role for Egr2, this study defines a novel role for Egr2 in control of MEK-mediated survival through regulation of mitochondrial-dependent apoptosis by increasing the ratio of Mcl1 expression to Bim expression.

Acknowledgments—We thank Dr. Beth Lee for the gift of the glutathione S-transferase RANKL expression construct. We also thank Drs. Patricia Collin-Osdoby and Philip Osdoby for advice on the glutathione S-transferase RANKL purification.

REFERENCES

1. Rodan, G. A., and Martin, T. J. (2000) Science 289, 1508–1514
2. Takahashi, N., Yamana, H., Yoshi, S., Roodman, G. D., Mundy, G. R., Jones, S. J., Boyle, A., and Suda, T. (1988) Endocrinology 122, 1373–1382
3. Udagawa, N., Takahashi, N., Akatsu, T., Sasaki, T., Yamaguchi, A., Kodama, H., Martin, T. J., and Suda, T. (1989) Endocrinology 125, 1805–1813
4. Jaworski, A., Christy, E., Yusoff, P., Byrne, R., and Hamilton, J. A. (1996) Biochem. J. 320, 1011–1016
5. Giorgis, A., Bradley, E., Shaw, A., and Oursler, M. J. (2003) J. Cell. Biol. 89, 165–179
6. Li, X., Udagawa, N., Itoh, K., Suda, K., Murase, Y., Nishihara, T., Suda, T., and Takahashi, N. (2002) Endocrinology 143, 3105–3113
7. Nakamura, H., Hirata, A., Tsuji, T., and Yamamoto, T. (2003) J. Bone Miner. Res., 18, 1198–1205
8. Miyazaki, T., Katagiri, H., Kanegae, Y., Takayanagi, H., Sawada, Y., Yamamoto, A., Pando, M. P., Asano, T., Verma, I. M., Oda, H., Nakamura, K., and Tanaka, S. (2000) J. Cell Biol. 148, 333–342
9. Satoh, S., Fujita, N., and Tsuruo, T. (2004) J. Biol. Chem. 279, 33759–33767
10. Johnson, G. L., and Lapadat, R. (2002) Science 298, 1911–1912
11. Carter, J. H., and Tourtellotte, W. G. (2004) J. Immunol. 178, 3038–3047
12. Russo, M. W., Sevetson, B. R., and Milbrandt, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6873–6877
13. Svan, J., Sevetson, B. R., Apel, E. D., Zimonjic, D. B., Popescu, N. C., and Milbrandt, J. (1996) Mol. Cell. Biol. 16, 3545–3553
14. Donovan, K. J., Tourtellotte, W. G., Milbrandt, J., and Baraban, J. M. (1999) Trends Neurosci. 22, 167–173
15. Xiao, D., Chinnappan, D., Pestell, R., Albanese, C., and Weber, H. C. (2005) Cancer Res. 65, 9934–9942
16. Baron, V., De Gregorio, G., Krones-Herzig, A., Virolle, T., Calogero, A., Urcis, R., and Mercolla, D. (2003) Oncogene 22, 4194–4204
17. Ke, J., Gururajala, M., Kumar, A., Simmons, A., Turcios, L., Chelvarajan, R. L., Cohen, D. M., Wiest, D. L., Monroe, J. G., and Bondada, S. (2006) J. Biol. Chem. 281, 39806–39818
18. Lim, J. H., Park, J. W., Min, D. S., Chang, J. S., Lee, Y. H., Park, Y. B., Choi, K. S., and Kwon, T. K. (2007) Apoptosis 12, 411–421
19. Lucerna, M., Pymje, J., Mechtcheriakova, D., Kalld, A., Gruber, F., Bilban, M., Sobanov, Y., Schabbauer, G., Breuss, J., Wagner, O., Bischoff, M., Clauss, M., Binder, B. R., and Hofer, E. (2006) Cancer Res. 66, 6708–6713
20. Droin, N. M., Pinkoski, M. J., Dejardin, E., and Green, D. R. (2003) Mol.}

MARCH 21, 2008•VOLUME 283•NUMBER 12
JOURNAL OF BIOLOGICAL CHEMISTRY 8063
Egr2 Promotes Osteoclast Survival

Cell. Biol. 23, 7638–7647
21. Unoki, M., and Nakamura, Y. (2001) Oncogene 20, 4457–4465
22. Unoki, M., and Nakamura, Y. (2003) Oncogene 22, 2172–2185
23. Oursler, M. J., Bradley, E. W., Ellering, T., and Giulivi, C. (2005) Am. J. Physiol. Cell Physiol. 288, 156–168
24. Boyd, J. M., Gallo, G. J., Ebb, R. G., Subramanian, T., Chittenden, T., Lutz, R. J., et al. (1995) Oncogene 11, 1921–1928
25. Hsu, S. Y., Kaipia, A., McGee, E., Lobelle, M., and Hsueh, A. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12401–12406
26. Hegde, R., Srivinasula, S. M., Ahmad, M., Fernandes-Alnemri, T., and Alnemri, E. S. (1998) J. Biol. Chem. 273, 7783–7786
27. Inohara, N., Ding, L., Chen, S., and Nunez, G. (1997) EMBO J. 16, 1686–1694
28. Inohara, N., Ekhterae, D., Garcia, I., Carrio, R., Merino, J., Levy, J., Gailit, J., Bowtell, D., and Baron, R. (2001) J. Cell. Biol. 152, 1815–1822
29. Inohara, N., Ekhterae, D., Garcia, I., Carrio, R., Merino, J., Levy, J., Gailit, J., Bowtell, D., and Baron, R. (2001) J. Cell. Biol. 152, 1815–1822
30. O’Connor, L., Strasser, A., O’Reilly, L. A., Haussmann, G., Adams, J. M., Cory, S., and Huang, D. C. (1998) EMBO J. 17, 384–395
31. Oltvai, Z. N., Milliman, C. L., and Korsmeyer, S. J. (1993) Cell 74, 609–619
32. Wang, K., Yin, X. M., Chao, D. T., Milliman, C. L., and Korsmeyer, S. J. (1996) Genes Dev. 10, 2859–2869
33. Yang, E., Zhou, Y., Jockel, J., Boise, L. H., and Thompson, C. B., and Korsmeyer, S. J. (1995) Cell 80, 285–291
34. Yin, X. M., Oltvai, Z. N., and Korsmeyer, S. J. (1994) Nature 369, 321–323
35. Akiyama, T., Bouillet, P., Miyazaki, T., Kadono, Y., Chikuda, H., Chung, U. I., Fukuda, A., Hikita, A., Seto, H., Okada, T., Inaba, T., Sanjay, A., Baron, R., Kawaguchi, H., Oda, H., Nakamura, K., Strasser, A., and Tanaka, S. (2003) EMBO J. 22, 6653–6664
36. Ley, R., Balmain, K., Hadfield, K., Weston, C., and Cook, S. J. (2003) J. Biol. Chem. 278, 18811–18816
37. Levkovitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tsygankov, A. Y., Alroy, I., Lavi, I., Iwai, K., Reiss, Y., Ciechanover, A., Lipkowitz, S., and Yarden, Y. (1999) Mol. Cell 4, 1029–1040
38. Joazeiro, C. A., Wing, S. S., Huang, H., Leverage, J. D., Hunter, T., and Liu, Y. C. (1999) Science 286, 309–312
39. Swaminathan, G., and Tsygankov, A. Y. (2006) J. Cell. Physiol. 209, 21–43
40. Tanaka, S., Amling, M., Neff, L., Peayman, A., Uhlmann, E., Levy, J. B., and Baron, R. (1996) Nature 383, 528–531
41. Sanjay, A., Houghton, A., Neff, L., D’Iorio, D., Bardelay, C., Antoine, E., Levy, J., Gaillot, J., Bowtell, D., Horne, W. C., and Baron, R. (2001) J. Cell Biol. 152, 181–195
42. Faccio, R., Novack, D. V., Zallone, A., Ross, F. P., and Teitelbaum, S. L. (2003) J. Cell Biol. 162, 499–509
43. Karst, M., Gornay, G., Galvin, R. J., and Oursler, M. J. (2004) J. Cell. Physiol. 200, 99–106
44. Fuller, K., Owens, J. M., Jagger, C. I., Wilson, A., Moss, R., and Chambers, T. J. (1993) J. Exp. Med. 178, 1733–1744
45. Hase, I., Plotkin, L. I., Han, L., Jilka, R. L., Kostenui, S., Bellido, T., and Manolagas, S. (2005) J. Biol. Chem. 280, 4632–4638
46. Carter, J. H., LeFever, J. M., Wiest, D. L., and Tourtellotte, W. G. (2007) J. Immunol. 178, 6796–6805
47. Glantschnig, H., Fisher, J. E., Wesołowski, G., Rodan, G. A., and Reszka, A. A. (2003) Cell Death Differ. 10, 1165–1177
48. Bouyer, P., Sakai, H., Ikotawa, T., Kawano, T., Fulton, C. M., Born, W. F., and Insogna, K. L. (2007) Endocrinology 148, 831–840
49. Laslo, P., Spooner, C. J., Warmflash, A., Lancki, D. W., Lee, H. J., Sciammas, R., Gantner, B. N., Diner, A. R., and Singh, H. (2006) Cell 126, 755–766
50. Levi, G., Topilko, P., Schneider-Maunoury, S., Lasagna, M., Mantero, S., Cancedda, R., and Charnay, P. (1996) Development 122, 113–120
51. Opferman, J. T., Iwasaki, H., Ong, C. C., Suh, H., Mizuno, S., Akashi, K., and Korsmeyer, S. J. (2005) Science 307, 1101–1104
52. Opferman, J. T., Letai, A., Beard, C., Sorcinelli, M. D., Ong, C. C., and Korsmeyer, S. J. (2003) Nature 426, 671–676
53. Opferman, J. T. (2006) Cell Death Differ. 13, 1260–1262
54. Weng, C., Li, Y., Xu, D., Shi, Y., and Tang, H. (2005) J. Biol. Chem. 280, 10491–10500
55. Dimmel, S., Breitschopf, K., Haendeler, J., and Zeiher, A. M. (1999) J. Exp. Med. 189, 1815–1822
56. Kozopas, K. M., Yang, T., Buchan, H. L., Zhou, P., and Craig, R. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3516–3520
57. Nijhawan, D., Fang, M., Traer, E., Zhong, Q., Gao, W., Du, F., and Wang, X. (2003) Genes Dev. 17, 1475–1486
58. Zhan, Q., Bieszczad, C. K., Bae, I., Forsan, A. J., and Craig, R. W. (1997) Oncogene 14, 1031–1039
59. Yang, T., Buchan, H. L., Townsend, K. J., and Craig, R. W. (1996) J. Cell. Physiol. 166, 523–536
60. Adams, K. W., and Cooper, G. M. (2007) J. Biol. Chem. 282, 6192–6200
61. Akiyama, T., Miyazaki, T., Bouillet, P., Nakamura, K., Strasser, A., and Tanaka, S. (2005) Biochem. Biophys. Res. Commun. 341, 48–59
62. Kelekar, A., and Thompson, C. B. (1998) Trends Cell Biol. 8, 324–330
63. Zhuang, S., and Insogna, K. L. (2007) Cell Death Differ. 13, 1263–1267
64. Clohessy, J. G., Zhuang, J., de Boer, J., Gil-Gomez, G., and Brady, H. J. (2006) J. Biol. Chem. 281, 5750–5759
65. Ehrenreiter, M. U., Muhlebach, S. G., Sohrman, S., Leutenegger, C. M., Lester, H. A., and Davidson, N. (2000) Gene 258, 63–69