Osteoclasts are specialized macrophages that resorb bone. Mice lacking the AP-1 component c-Fos are osteopetrotic because of a lack of osteoclast differentiation and show an increased number of macrophages. The nature of the critical function of c-Fos in osteoclast differentiation is not known. Microarray analysis revealed that Nfatc1, another key regulator of osteoclastogenesis, was down-regulated in Fos−/− osteoclast precursors. Chromatin immunoprecipitation assay showed that c-Fos bound to the Nfatc1 and Acp5 promoters in osteoclasts. In vitro promoter analyses identified nuclear factor of activated T-cells (NFAT)/AP-1 sites in the osteoclast-specific Acp5 and CalcR promoters. Moreover, in Fos−/− precursors gene transfer of an active form of NFAT restored transcription of osteoclast-specific genes in the presence of receptor activator of the NF-κB ligand (RANKL), rescuing bone resorption. In the absence of RANKL, however, Fos−/− precursors were insensitive to NFAT-induced osteoclastogenesis unlike wild-type precursors. These data indicate that lack of Nfatc1 expression is the cause of the differentiation block in Fos−/− osteoclast precursors and that transcriptional induction of Nfatc1 is a major function of c-Fos in osteoclast differentiation.

AP-1 refers to a family of dimeric transcription factors composed of Fos (c-Fos, Fra1, Fra2, and FosB) and Jun proteins (1, 2). Transcription factors such as nuclear factor-κB (NF-κB) and AP-1, both critically involved in osteoclast differentiation, are activated in the presence of macrophage/colony-stimulating factor (M-CSF, also known as CSF-1) and receptor activator of NF-κB ligand (RANKL) (3, 4). These cytokines induce signals via multiple pathways including mitogen-activated protein kinases, phosphatidylinositol 3-kinase and calcium (3, 5, 6). The essential role for c-Fos during osteoclast differentiation (7–9) is partially explained by the observations that the expression of all Fos family proteins is down-regulated in Fos−/− precursors and that other Fos proteins such as Fra1 can rescue the differentiation of these precursors (10, 11). Therefore, a role of c-Fos appears to enhance production of Fos proteins during osteoclastogenesis. c-Fos also transcriptionally induces β-interferon, which then negatively regulates osteoclastogenesis by down-regulating Fos at the protein level (12). Beyond the Fos family, however, c-Fos target genes that rescue osteoclastogenesis in Fos−/− precursors are not known.

Nfatc1 is a member of the NFAT family of activated T-cells (NFATc1) family of transcription factors (NFATc1, NFATc2, NFATc3, and NFATc4, as accepted by HUGO and the Genome Data Base, corresponding to NFAT2, NFAT1, NFAT4, and NFAT3, respectively) (13–15). It has been shown to be up-regulated following RANKL treatment and is important for osteoclast differentiation (5, 16, 17). In this study, we explore the cause of the differentiation block in Fos−/− precursors by analyzing transcriptional target genes of c-Fos, especially Nfatc1, during osteoclast differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture—ST2-T cells were established by infecting the mouse stromal line ST2 with the retroviral vector expressing RANKL (18). For co-culture, bone marrow cells or splenocytes were seeded at 6 × 10^4 cells/cm² with 6 × 10^5/cm² ST2-T cells and cultured in the presence of 10−8 M 1,25-dihydroxyvitamin D3 and 10−7 M dexamethasone. For osteoclast-free culture, non-adherent hematopoietic precursor cells were cultured in the presence of 10 ng/ml recombinant human M-CSF (Genzyme) and 10–30 ng/ml recombinant mouse RANKL (R&D Systems). RAW264.7 cells were obtained from ATCC (TIB-71). Transient transfection was performed using LipofectAMINE (Invitrogen). Microarray—Oligonucleotide microarrays (GeneChip Murine Genome U74Av2, Affymetrix) were used to monitor the relative abundance of transcripts. Gene Expression Omnibus accession numbers: Fos+/− splenocytes (GSM10341), Fos−/− splenocytes (GSM10342), Fos+/− bone marrow (GSM10343), Fos−/− splenocytes expressing ΔNFAT (GSM10344), Fos−/− splenocytes expressing green fluorescent protein (GFP) (GSM10345).

Western Blotting—Total cell extracts were prepared in a standard SDS lysis buffer. Nuclear extracts were prepared as described (19).
Immunofluorescence—Mature osteoclasts were prepared from femurs of 3-day-old wild-type mice on day 6 by culturing with a scalpel into medium. After 1 h incubation, cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 and 1% bovine serum albumin in phosphate-buffered saline. Primary antibodies used were anti-NFATc1 (7A6) or anti-NFATc2 (G1-D10).

Plasmids—Mouse Acp5-luciferase reporter plasmid was constructed in pGL3 vector (Promega) by transferring the promoter region (−1453 down to the end of intron 1) from pKB5 (a gift from D. Roodman), and the NFAT site mutation was introduced by using the QuikChange kit (Stratagene). ANFAT was constructed by assembling PCR-amplified fragments encoding amino acids 1–239 of enhanced GFP (Clontech) followed by Ser-Arg (an XbaI site) and amino acids 317–902 of human NFATc1 (20). The 2.5-kb ANFAT fragment was cloned into both the cytomegalovirus-driven expression vector pRK5 and the retroviral vector pMX (21). pBJ5-human NFATc1 expression plasmid (pSH102) was cytomegalovirus-driven expression vector pRK5 and the retroviral vector pMX (21). pBJ5-human NFATc1 expression plasmid (pSH102) was a gift from G. R. Crabtree. The tor pMX (21). pBJ5-human NFATc1 expression plasmid (pSH102) was cytomegalovirus-driven expression vector pRK5 and the retroviral vector pMX (21). pBJ5-human NFATc1 expression plasmid (pSH102) was a gift from G. R. Crabtree. The tor pMX (21). pBJ5-human NFATc1 expression plasmid (pSH102) was cytomegalovirus-driven expression vector pRK5 and the retroviral vector pMX (21). pBJ5-human NFATc1 expression plasmid (pSH102) was a gift from G. R. Crabtree. The tor pMX (21). pBJ5-human NFATc1 expression plasmid (pSH102) was cytomegalovirus-driven expression vector pRK5 and the retroviral vector pMX (21). pBJ5-human NFATc1 expression plasmid (pSH102) was a gift from G. R. Crabtree.

RESULTS

NFATc1 Is Down-regulated in Fos−/− Osteoclast Precursors—We set out to identify genome-wide novel c-Fos target genes in the osteoclast lineage. Three osteoclastogenic cultures derived from wild-type and Fos−/− spleoncytes and wild-type bone marrow were prepared and co-cultured with stromal ST2-T cells. By day 6, the wild-type cultures produced abundant multinucleated osteoclasts, which were tartrate-resistant acid phosphatase (TRAP)-positive, whereas no such cells were generated in the Fos−/− culture (data not shown). The co-cultured cells were harvested in toto, and gene expression was analyzed by microarrays. The genes in which expression was detectable in wild-type cultures but absent or very low in the Fos−/− culture are summarized in Table I. The numbers for wild-type spleoncytes (Sp+/+) and Fos−/− spleoncytes (Sp−/−) and wild-type bone marrow cells (Bm+/+), GeneChips scores indicating RNA levels. In the Fos−/− culture, the expression of Nfatc1 was undetectable, and the expression of known osteoclast marker genes was reduced. These include Acp5 (encoding TRAP), Ctsk (cathepsin K), Car2 (carbonic anhydrase 2), Mmp9 (matrix metalloproteinase 9), and Calcr (calcinon receptor). However, Marco, a macrophage receptor, was not reduced in the Fos−/− culture (Table I).

To confirm the differential expression at the protein level, Western blot analysis was performed using total protein extracts prepared on day 6 from the osteoclastogenic co-cultures. Consistent with the RNA data, Nfatc1 was not detectable in the Fos−/− culture (Fig. 1A). The size variation of NFATc1 in the wild-type bone marrow culture may be caused by either degradation products of different isoforms of Nfatc1 in mature osteoclasts freshly isolated from the femurs of wild-type mice. Immunofluorescence microscopy showed nuclear staining of NFATc1 but not Nfatc2 in mature multinucleated osteoclasts generated in vitro (Fig. 1B). Western blot analysis of nuclear extracts from the macrophage-osteoclast precursor RAW264.7 cells demonstrated that nuclear NFATc1 was detectable only after RANKL stimulation (Fig. 1C). These data suggest that RANKL stimulates Nfatc1 synthesis via c-Fos. c-Fos binds to the Nfatc1 Promoter—Putative c-Fos binding sites have been mapped in the promoter region of Nfatc1 (Fig. 2A) and (28). To examine whether the Nfatc1 promoter could be directly regulated by c-Fos in osteoclast precursors, we performed a chromatin immunoprecipitation assay using primary wild-type bone marrow cells and Fos−/− spleoncytes treated with RANKL. The Nfatc1-P1 promoter fragment containing the distal block of homology between human and mouse sequences (27) was specifically precipitated with an anti-c-Fos antibody in samples prepared from wild-type cells treated with RANKL (Fig. 2B). The proximal block of homology could not be analyzed because of difficulty in PCR amplification. We also tested whether the Acp5 promoter (3A) was precipitated by anti-c-Fos antibody (2B). In wild-type cells, c-Fos is present on the Acp5 promoter in the absence of RANKL, and c-Fos occupancy of the Acp5 promoter increases after RANKL treatment.

| Accession No. | Gene        | Sp+/+ | Sp−/− | Bm+/+ | Nfatc1/GFP |
|--------------|-------------|-------|-------|-------|------------|
| M99054       | Acp5        | 3836  | 462   | 2580  | 4.0        |
| AJ006033     | Ctsk        | 2962  |       | 649   | 2495       |
| M25944       | Car2        | 971   |       | 14    | 719        |
| A0F8743      | Nfatc1      | 752   | A     | 325   | 3.5        |
| U65353       | Sema4d      | 707   | A     | 309   | 2.0        |
| AV92870      | Mmp9        | 620   | 80    | 451   | NC         |
| AA656014     | Tam7sf      | 593   | 30    | 295   | NC         |
| AW125713     | Unknown      | 544   | 60    | 331   | NC         |
| U87814       | Pstph1      | 528   | A     | 207   | NC         |
| AV251613     | Unknown      | 357   | A     | 120   | 6.5        |
| U18542       | Calcr       | 337   | A     | 385   | 12.2       |
| X53929       | Den         | 320   | A     | 321   | NC         |
| L22545       | Col11a1     | 254   | A     | 188   | 7.4        |
| AFO29215     | Antigen-2   | 214   | A     | 111   | 2.7        |
| AFO42487     | Kcnn4       | 208   | A     | 117   | NC         |
| U18424       | Marco       | 108   | 761   | 223   | NC         |

TABLE I Identification of Fos target genes by transcriptional profiling ΔNfatc1/GFP—fold increase between ΔNfat- and GFP-expressing Fos−/− spleoncytes in osteoclastogenic cultures. A, absent; NC, no change.
Fig. 1. Expression of NFATc1. A, NFATc1 is undetectable by Western blotting in Fos−/− osteoclastogenic culture. Splenocytes (Sp) and bone marrow cells (Bm) were co-cultured with ST2-T cells (st) for 6 days under osteoclastogenic conditions. Protein extracts in toto were analyzed using anti-NFATc1 monoclonal antibody (7A6). +/+ and −/− indicate wild-type and Fos−/− cultures, respectively. r, positive control. Ramos cell extract. B, nuclear localization of NFATc1 in freshly isolated mature multinucleated osteoclasts. Immunofluorescence microscopy was performed with the anti-NFATc1 and anti-NFATc2 (G1-D10) antibodies. TRAP, TRAP activity stain; DAPI, 4′,6-diamidino-2-phenylindole (a nuclear stain). Note that the NFATc2-positive cell is TRAP-negative. C, Western blot analysis of nuclear extracts prepared from RAW264.7 cells cultured in the absence (−) or presence (+) of RANKL for 4 days using the anti-NFATc1 antibody.

These results suggest that c-Fos binds to the NFATc1 and Acp5 promoters during osteoclastogenesis.

Acp5 and Calcr Promoters Contain Functional NFAT/AP-1 Sites—It is known that AP-1 composed of Fos/Jun dimers and NFAT transcription factors can cooperatively bind to promoter regions of various genes including the IL-2 gene (29, 30). To study the molecular mechanisms by which NFAT is involved in the regulation of osteoclast-specific gene expression we examined promoter sequences of Acp5 and Calcr, two potential c-Fos target genes (Table I). First we searched for such composite binding sites for NFAT and AP-1 (NFAT/AP-1 sites) in mouse, human, and pig Acp5 promoter sequences. Two short stretches were highly conserved around the multiple transcription start sites, which we termed Acp5-160 and Acp5-120, respectively (Acp5 elements located at −160 and −120 relative to the 3′ end of exon 1). Sequences of the conserved Acp5-120 were found to be similar to the prototypical 15-bp NFAT/AP-1 site in the IL-2 promoter (15) (Fig. 3A). Acp5-160 contains the binding site for the microphthalmia transcription factor (31). From EMSA results, the binding activity at Acp5-120 was indistinguishable from that observed with the IL-2 site (Fig. 3B). Binding of c-Fos to the Acp5 promoter in osteoclasts was demonstrated by chromatin immunoprecipitation assay (Fig. 2B). Then we mutated the putative NFAT site in Acp5-120 from GGAAAAT to GGC-CGC in Acp5-luciferase reporter plasmids. Both human and mouse wild-type Acp5-luciferase constructs were most efficiently activated with NFATc4 compared with NFATc1, NFATc2, and NFATc3 in transient transfection assays (data not shown). Thus we constructed a constitutively active nuclear form of NFATc1, ΔNFAT (20), fused to GFP. When the wild-type and mutant reporter plasmids were co-transfected with a ΔNFAT expression plasmid into RAW264.7 cells, ΔNFAT activated the wild-type Acp5 promoter more efficiently than the mutant promoter (Fig. 3C). Furthermore, the Acp5 promoter activity was enhanced by a co-transfected c-Fos expression vector and suppressed by small interfering RNA vectors for c-Fos in transient transfection assays (Fig. 3D). These data suggest that Acp5-120 is a functional NFAT/AP-1 binding site in the Acp5 promoter.

Next we searched for NFAT/AP-1 sites in the mouse osteoclast-specific Calcr-P3 promoter (22) and found eight putative NFAT/AP-1 sites in the −797 Calcr-P3 promoter (Fig. 4A). Co-transfection of both the Calcr-P3-luciferase reporter plasmids and the ΔNFAT-expression vector into RAW264.7 cells resulted in a 30-fold increase in promoter activity above constitutive levels. Sequential 5′ deletion of the Calcr-P3 promoter demonstrated that the −178 construct containing the putative NFAT/AP-1 sites 1–4 was sufficient for full activity. Site-specific mutagenesis of each NFAT site from GGAAAAN to GGC-CGC revealed that site 2 at −93 was critical and that sites 1, 3, and 4 appear to cooperate with site 2 (Fig. 4A). In EMSA, site 2 was bound by NFATc1 using either RANKL-stimulated RAW264.7 nuclear extracts or in vitro translation products (Fig. 4B). Next we transfected RAW264.7 cells with Calcr-P3-luciferase reporters and treated them with the calcium ionophore A23187. We observed that the Calcr promoter activities were enhanced only when site 2 was present presumably...
through activation of endogenous NFAT (Fig. 4C). Furthermore, the stimulatory effect of A23187 was blunted by pretreatment of the cells with cyclosporin A (Fig. 4D). These data suggest that site 2 is the critical NFAT site in the Acp5 promoter.

NFAT Rescues Osteoclast Differentiation in Fos\(^{−/−}\) Precursors—To test whether NFAT activity could rescue osteoclastogenesis in the absence of c-Fos, we introduced GFP or the GFP fusion \(\Delta\)NFAT into Fos\(^{−/−}\) spleenocytes by retroviral gene transfer. Infected cells were co-cultured with the ST2-T cells under osteoclastogenic conditions. At day 6, mRNA was harvested and microarray analysis was performed to compare gene expression between GFP and \(\Delta\)NFAT virus-infected Fos\(^{−/−}\) cells. Strikingly, expression of \(\Delta\)NFAT activated about two-thirds of the genes that failed to be induced in Fos\(^{−/−}\) cells including Acp5, Calcr, Ctsk, and endogenous Nfatc1 (Table I, \(\Delta\)NFAT/GFP). This indicated that the differentiation block was to a large extent overcome by \(\Delta\)NFAT in the absence of c-Fos when RANKL from ST2-T cells was present. Indeed, whereas abundant GFP-positive cells were observed by day 6 with both viruses, TRAP-positive cells were generated only with \(\Delta\)NFAT virus (Fig. 5A). Next we tested whether the \(\Delta\)NFAT-expressing Fos\(^{−/−}\) osteoclasts could resorb bone. Although resorption pits were not visible on bone slices in cultures of GFP virus-infected Fos\(^{−/−}\) splenocytes, co-cultures containing \(\Delta\)NFAT virus-infected Fos\(^{−/−}\) cells generated multiple resorption pits (bone surface resorbed, 2.3 ± 0.5%) (Fig. 5B). Next we compared the rescue efficiency in two types of osteoclastogenic cultures, co-culture using ST2-T cells and osteoblast-free cultures using only soluble M-CSF and RANKL. In co-cultures, the rescue with \(\Delta\)NFAT was comparable with that with c-Fos as judged by
Calcr expression, TRAP-positive cell numbers, and resorption (Fig. 5C, st). In contrast, in the absence of stromal cells the rescue with ΔNFAT was lower than that of c-Fos based on all three parameters (Fig. 5C, M+R). Apart from the differences in efficiency in both cultures, ΔNFAT substituted at least in part for the osteoclastogenic function of c-Fos to the extent that Fos−/− splenocytes formed bone resorption pits in the presence of RANKL. In addition, gene transfer of the human full-length NFATc1 also rescued Fos−/− osteoclastogenesis in vitro (data not shown). These data collectively indicate that the lack of NFATc1 is a major reason for the differentiation block in Fos−/− osteoclast precursors.

**NFAT Rescue of Fos−/− Precursors Is RANKL-dependent—** To examine the role of RANKL in NFAT-induced osteoclast formation, we introduced the GFP fusion ΔNFAT into RAW264.7 cells by transient transfection. This resulted in the induction of the endogenous Acp5 and Calcr genes as early as 1 day after transfection even in the absence of RANKL (Fig. 6A). This is consistent with the reported osteoclastogenic activity of NFATc1 in the absence of RANKL (5). Next, we tested whether ΔNFAT could rescue Fos−/− precursors in the absence of RANKL. ΔNFAT produced bone-resorbing TRAP-positive cells from wild-type precursors (Bm+/+, Sp+/+) in the absence or presence of RANKL (Fig. 6B). However, Fos−/− precursors (Sp−/−) hardly produced any TRAP-positive cells upon introduction of ΔNFAT in the absence of RANKL (Fig. 6B), and no bone resorption pits were observed (data not shown). These data suggest that the rescue of Fos−/− cells with NFAT activity requires receptor activator of NF-κB (RANK) signaling.

**DISCUSSION**

It has been established that the lack of c-Fos expression results in a differentiation block in the osteoclast lineage (7–9). However, whether this is a cumulative effect of numerous deregulated c-Fos target genes or an effect of one critical c-Fos target gene is unclear. Our results show that the absence of Nfatc1 expression in Fos−/− precursors is the major cause of the differentiation block because an active form of NFAT alone rescued osteoclast-specific gene expression and bone resorptive function.

We have identified NFAT/AP-1 sites in the Acp5 and Calcr promoters. EMSA showed that NFAT and AP-1 cooperatively bind to the Acp5 NFAT/AP-1 site, and the chromatin immunoprecipitation assay indicated that c-Fos binds to the Acp5 promoter in osteoclasts. These observations are consistent with the idea that NFATc1 and c-Fos synergize to activate the Acp5 promoter (5). On the other hand, the rescue of osteoclastogenesis by ΔNFAT alone in the absence of c-Fos clearly demonstrates that c-Fos is not essential for activation of the Acp5 promoter. To activate these promoters in Fos−/− precursors, ΔNFAT may interact with Jun-Jun homodimers (32) or may act alone in the absence of cooperative partners (33). EMSA using Fos−/− cell extract in combination with in vitro translated NFATc1 will help to address this issue. Although binding of

**FIG. 5. Rescue of osteoclast formation with ΔNFAT.** A, TRAP staining of osteoclastogenic co-cultures derived from wild-type (+/+) splenocytes and GFP and ΔNFAT virus-infected Fos−/− splenocytes (−/−). Below, GFP fluorescence and TRAP stain are shown in higher magnification. The bars represent 100 μm. B, bone resorption assay. Infected cells were co-cultured with ST2-T cells on bovine bone slices for 10 days, and pit formation was examined in a double-blind study with backscattered electron imaging. The bars represent 0.75 mm. Arrows indicate bone resorption pits. C, rescue activity of ΔNFAT virus relative to that of c-Fos virus. st, osteoclastogenic co-cultures using ST2-T cells. M+R, osteoblast-free cultures using soluble M-CSF and RANKL. Fos−/− splenocytes were infected with GFP, c-Fos, or ΔNFAT viruses. Calcr transcripts were quantitated by real-time PCR; TRAP-positive multinucleated cells (MNC) were counted, and the resorbed area (%) was measured on bone surfaces.

**FIG. 6. Rescue of osteoclast formation with ΔNFAT in the absence of RANKL.** A, transient transfection of ΔNFAT-expression vector induces endogenous Acp5 and Calcr expression in RAW264.7 cells untreated or treated with 30 ng/ml RANKL as indicated and harvested at the days depicted. Osteoclasts generated in co-culture served as positive control (lane 8) and no RNA as negative control (lane 1). Transcripts were analyzed by reverse transcripase-PCR with primers for Acp5, Calcr (all isoforms), and glyceraldehyde-3-phosphate dehydrogenase (Gapdh). The reverse transcripts-PCR products for Calcr were also detected by hybridization with an internal probe (hyb). B, rescue of TRAP-positive Fos−/− cells by ΔNFAT requires RANKL. M-CSF-dependent macrophages derived from wild-type and Fos−/− splenocytes (Sp+/+ and Sp−/−) and wild-type bone marrow cells (Bm+/+) were infected with the GFP ΔNFAT virus and then were cultured for 3 more days in the presence or absence of RANKL. The bottom row shows expression of GFP ΔNFAT fusion protein.
NFAT to the Calcr promoter was unambiguously demonstrated by EMSA, binding of AP-1 to the Calcr promoter needs to be rigorously tested in the future.

Importantly, osteoclast-specific gene expression is not entirely rescued with ΔNFAT. Those genes for which expression is not rescued, for example Mmp9, may be more strictly dependent on c-Fos or additional c-Fos-dependent transcription factors. Curiously, the rescue activity of ΔNFAT was similar to that of c-Fos when Fos<sup>−/−</sup> precursors were co-cultured with ST-2 but was lower than that of c-Fos when soluble M-CSF and RANKL were used. Therefore, c-Fos dependence appears to ST-2 but was lower than that of c-Fos when soluble M-CSF and RANKL (5), NFATc1 induce osteoclast differentiation even in the absence of RANKL (5), ΔNFAT expression in Fos<sup>−/−</sup> precursors failed to rescue osteoclast differentiation in the absence of RANKL. This suggests that in the absence of RANKL, NFAT requires c-Fos, presumably as a binding partner or possibly indirectly to exert its osteoclastogenic function. It also suggests that RANKL may induce an alternative partner for NFAT that can substitute for c-Fos in function in Fos<sup>−/−</sup> cells.

Taken together, these results demonstrate that a major function of c-Fos during osteoclast formation is to trigger a transcriptional regulatory cascade by producing and cooperating with NFATc1, thereby activating a number of target genes involved in osteoclast differentiation and function. These yet to be identified novel target genes together with NFATc1 may provide additional drug targets for bone diseases including osteoporosis and rheumatoid arthritis.

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REFERENCES

1. Chinenov, Y., and Kerppola, T. K. (2001) Oncogene 20, 2438–2452
2. Shaulian, E., and Karin, M. (2002) Nat. Cell Biol. 4, E131–E136
3. Teitelbaum, S. L., and Ross, P. F. (2003) Nat. Rev. Genet. 4, 638–649
4. Karsenty, G., and Wagner, E. F. (2002) Dev. Cell 2, 389–406
5. Takayanagi, H., Kim, S., Koga, T., Nishina, H., Isshiki, M., Yoshida, H., Suzuki, A., Isohe, M., Yokochi, T., Inoue, J., Wagner, E. F., Mak, T. W., Kodama, T., and Taniguchi, T. (2002) Dev. Cell 3, 889–901
6. Komarova, S. V., Pilkington, M. P., Weidema, A. F., Dixon, S. J., and Sims, S. M. (2003) J. Biol. Chem. 278, 8286–8293
7. Wang, Z. Q., Ovtit, C., Grigoriadis, A. E., Mohle-Steinlein, U., Ruther, U., and Wagner, E. F. (1992) Nature 360, 741–744
8. Johnson, R. S., Spiegelman, B. M., and Papainannou, V. (1992) Cell 71, 577–586
9. Grigoriadis, A. E., Wang, Z. Q., Ceechini, M. G., Hofbatter, W., Felix, R., Fleisch, H. A., and Wagner, E. F. (1994) Science 266, 443–448
10. Matsuo, K., Owens, J. M., Tonko, M., Elliott, C., Chambers, T. J., and Wagner, E. F. (2000) Nat. Genet. 24, 184–187
11. Fleischmann, A., Hafezi, F., Elliott, C., Reme, C. E., Ruther, U., and Wagner, E. F. (2000) Genes Dev. 14, 2705–2710
12. Takayanagi, H., Kim, S., Matsuoka, S., Suzuki, H., Suzuki, T., Sato, K., Yokochi, T., Oda, H., Nakamura, K., Ida, N., Wagner, E. F., and Taniguchi, T. (2002) Nature 416, 744–749
13. Hoey, T., Son, Y. L., Williamson, K., and Xu, X. (1995) Immunity 2, 461–472
14. Crabtree, G. R., and Olsen, E. N. (2002) Cell 109, suppl. 867–879
15. Hogan, P. G., Chen, L., Nardone, J., and Rao, A. (2003) Genes Dev. 17, 2025–2032
16. Ishida, N., Hayashi, K., Hoshiba, M., Ogawa, T., Koga, S., Miyatake, Y., Kamagawa, M., Kimura, T., and Takeya, T. (2002) J. Biol. Chem. 277, 41147–41156
17. Hirotani, H., Tohyo, N. A., Woo, J.-T., Stern, P. H., and Citlone, N. A. (2004) J. Biol. Chem. 279, 13894–13992
18. Lean, J. M., Mates, R., Fox, S. W., Fuller, K., Gibson, F. M., Draycott, G., Wani, M. R., Bayley, K. E., Wong, B. R., Choi, Y., Wagner, E. F., and Chambers, T. J. (2000) Bone 27, 29–40
19. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) Nucleic Acids Res. 17, 6149
20. Molkentin, J. D., Lu, J. R., Antos, C. I., Markham, B., Richardson, J., Robbins, J., Grant, S. R., and Olson, E. N. (1996) Cell 83, 215–228
21. Oishi, M., Kinosita, S., Morikawa, Y., Shibuya, A., Phillips, J., Lanier, L. I., Gorman, D. M., Nolan, G. P., Miyajima, A., and Kitamura, T. (1996) Exp. Hematol. 24, 324–329
22. Anusaksathien, O., Laplace, C., Li, X., Ren, Y., Peng, L., Goldring, S. R., and Galson, D. L. (2001) J. Biol. Chem. 276, 22663–22674
23. Barton, G. M., and Medzhitov, R. (2002) Rev. Natl. Acad. Sci. U. S. A. 99, 14934–14945
24. Weinmann, A. S., and Farnham, P. J. (2002) Methods (Orlando) 26, 37–47
25. Lacey, D. L., Timme, E., Van, H. L., Kelley, M. J., Dunstan, C. R., Burgess, T., Elliott, R., Colombo, A., Elliott, G., Seuly, S., Hsu, H., Sullivan, J., Hardins, N., Dave, E., Capparelli, C., Eli, A., Qian, Y. X., Kaufman, S., Sarosi, I., Shalhoub, V., Senaldi, G., Gus, J., Delaney, J., and Boyle, W. J. (1998) Cell 93, 165–176
26. Bogman, M. A., Powell, D. R., Weiss, D. L., and Brown, M. A. (1999) J. Immunol. 162, 2820–2828
27. Chevrel, S., Jankevics, E., Tyrsin, D., Akinmihanov, A., Moroz, D., Jha, M. K., Schluze-Luehrmann, J., SANTNER-NANAN, B., FEOKTISTOVA, E., Konig, T., AVOTS, A., Schimpl, A., BERBERICH-SIEBELT, F., SCHRIMPL, A., and Solberg, E. (2002) Immunity 16, 881–895
28. Zhou, B., Cron, R. Q., Wu, B., Genin, A., Wang, Z., Liu, S., Robson, P., and Baldwin, H. S. (2002) J. Biol. Chem. 277, 10794–10711
29. Chen, L., Glover, J. N., Hogan, P. G., Rao, A., and Harrison, S. C. (1998) Nature 392, 42–48
30. Diebold, R. J., Rajaram, N., Leonard, D. A., and Kerppola, T. K. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7915–7920
31. Luchin, A., Suchting, S., Merson, T., Rosol, T. J., Hume, D. A., Cassady, A. I., and Ostrowski, M. C. (2001) J. Biol. Chem. 276, 36703–36710
32. Macian, F., Lopez-Rodriguez, C., and Raeo, A. (2001) Oncogene 20, 2476–2489
33. Stroud, J. C., and Chen, L. (2003) J. Mol. Biol. 334, 1009–1022
34. Kim, N., Takami, M., Rho, J., Josien, R., and Choi, Y. (2002) J. Exp. Med. 195, 281–289