The immunomodulatory adapter proteins DAP12 and Fc receptor γ-chain (FcRγ) regulate development of functional osteoclasts through the Syk tyrosine kinase

Attila Mócsai*†‡, Mary Beth Humphrey‡§, Jessica A. G. Van Ziffle*, Yongmei Hu*, Andrew Burghardt¶, Steven C. Spusta§, Sharmila Majumdar¶, Lewis L. Lanier‖, Clifford A. Lowell*, and Mary C. Nakamura§**

*Department of Laboratory Medicine, §Department of Medicine and Department of Veterans Affairs Medical Center, ¶Magnetic Resonance Science Center, Department of Radiology, and ‖Department of Microbiology and Immunology and Cancer Research Institute, University of California, San Francisco, CA 94143; and †Department of Physiology, Semmelweis University School of Medicine, Budapest, Hungary

Communicated by Arthur Weiss, University of California, San Francisco, CA, March 7, 2004 (received for review January 20, 2004)

Osteoclasts, the only bone-resorbing cells, are central to the pathogenesis of osteoporosis, yet their development and regulation are incompletely understood. Multiple receptors of the immune system use a common signaling paradigm whereby phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs) within receptor-associated adapter proteins recruit the Syk tyrosine kinase. Here we demonstrate that a similar mechanism is required for development of functional osteoclasts. Mice lacking two ITAM-bearing adapters, DAP12 and the Fc receptor γ-chain (FcRγ), are severely osteopetrotic. *DAP12*$^{-/-}$*FcRγ*$^{-/-}$ bone marrow cells fail to differentiate into multinucleated osteoclasts or resorb bone *in vitro* and show impaired phosphorylation of the Syk tyrosine kinase. *syk*$^{-/-}$ progenitors are similarly defective in osteoclast development and bone resorption. Intact SH2-domains of Syk, introduced by retroviral transduction, are required for functional reconstitution of *syk*$^{-/-}$ osteoclasts, whereas intact ITAM-domains on DAP12 are required for reconstitution of *DAP12*$^{-/-}$ *FcRγ*$^{-/-}$ cells. These data indicate that recruitment of Syk to phosphorylated ITAMs is critical for osteoclastogenesis. Although DAP12 appears to be primarily responsible for osteoclast differentiation in cultures directly stimulated with macrophage-colony stimulating factor and receptor activator of NF-κB ligand cytokines, DAP12 and FcRγ have overlapping roles in supporting osteoclast development in osteoblast–osteoclast cocultures, which mirrors their overlapping functions *in vivo*. These results provide new insight into the biology of osteoclasts and suggest novel therapeutic targets in diseases of bony remodeling.

Osteoclasts are derived from hematopoietic precursor cells of the myeloid lineage. Although signals through the receptor activator of NF-κB (RANK)/RANKL (RANK ligand) and colony-stimulating factor 1 receptor/macrophage-colony-stimulating factor (M-CSF) receptor/ligand pairs are clearly required for osteoclastogenesis, regulation by other receptor-mediated signals is less well defined (1). Immunoreceptor tyrosine-based activation motif (ITAM)-mediated signaling is critical for receptors of the adaptive immune system (B cell receptors, T cell receptors, and Fc receptors), and innate immune receptors that couple to the ITAM-adapter proteins DAP12 and Fc receptor γ-chain (FcRγ) also regulate cellular differentiation and function in myeloid cells (2–4). The association of DAP12 deficiency with a human disease involving bony abnormalities (Nasu–Hakola disease or polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy) suggests that these receptors may play important roles in osteoclasts as well (5). In both humans and mice, loss of the DAP12 ITAM signaling adapter results in a significant defect in differentiation of osteoclast-like cells (OCLs) in cell culture (6–10) but does not completely block osteoclastogenesis *in vivo*. This observation suggests that other ITAM signaling adapter proteins, such as FcRγ, may also be involved in osteoclastogenesis. DAP12 and FcRγ are both transmembrane adapter proteins with ITAM domains that couple to downstream pathways through the Syk tyrosine kinase (4, 11). Thus, we studied mice doubly deficient in DAP12 and FcRγ and examined the functional role of Syk in osteoclasts.

Materials and Methods

Mice. We used offspring of *DAP12*$^{+/-}$*FcRγ*$^{-/-}$ × *DAP12*$^{-/-}$ *FcRγ*$^{+/-}$ matings (B6/129 mixed background) derived from intercrossing *DAP12*$^{-/-}$ (12) and *FcRγ*$^{-/-}$ (Taconic Farms) mice. Heterozygous animals were considered wild type given no suggestion of gene dosage effects for *DAP12* or *FcRγ* in prior analyses. *syk*$^{-/-}$ fetal liver from progeny of *syk*$^{+/-}$ C57BL/6 parents (13) was used for bone marrow transplantation as described (14).

Micro-Computed Tomography (CT) Analysis. Proximal tibias were scanned by high resolution micro-CT (μCT-20, Scanco Medical, Bassersdorf, Switzerland) with a cubic voxel size of 9 μm and with 3D structural parameters calculated (15, 16) (see *Supporting Methods*, which is published as supporting information on the PNAS web site). Groups were compared by a nonparametric Kruskal–Wallis with Dunn's post hoc test (INSTAT, GraphPad, San Diego).

Histologic Analysis and Immunofluorescence Microscopy. Proximal tibias were fixed in PBS plus 4% paraformaldehyde, decalcified in 0.5 M EDTA (pH 7.4), paraffin-embedded, sectioned at 6 μm, and stained by using standard techniques. Immunostaining was performed by using anti-Syk antibody (N-19, Santa Cruz Biotechnology), followed by Alexa Fluor-488-secondary antibody (Molecular Probes) and counterstaining with rhodamine-phalloidin.

***In Vitro* Osteoclast Cultures and Resorption Assays.** These assays were performed as described (10). Briefly, nonadherent bone marrow cells after 48 h in complete α-MEM (Invitrogen) with 10 ng/ml murine M-CSF (osteoclast precursors) were plated at 0.5 million per cm^2 and cultured 4–7 d in 70 ng/ml RANKL and 10 or 100 ng/ml M-CSF (R & D Systems). Tartrate resistant acid phosphatase (TRAP) staining was performed with a commercial kit (catalog no. 387-A, Sigma). For resorption assays, osteoclast precursors were plated on BioCoat Osteologic slides (BD Biosciences) or dentine discs (Immunodiagnostic Systems, Tyne and Wear, England), and cultured with RANKL/M-CSF for 10 d as described (10). Groups were compared by one-way ANOVA analysis with

Abbreviations: ITAM, immunoreceptor tyrosine-based activation motif; FcRγ, Fc receptor γ-chain; M-CSF, macrophage-colony-stimulating factor; RANK, receptor activator of NF-κB; OCL, osteoclast-like cell; CT, computed tomography; TRAP, tartrate resistant acid phosphatase; OB, osteoblast; MNC, multinucleated cells; SMI, structure model index; OSCAR, osteoclast-associated receptor.

‡A.M. and M.B.H. contributed equally to this work.

**To whom correspondence should be addressed at: Immunology/Arthritis Section, Department of Veterans Affairs Medical Center, University of California, 111R, 4150 Clement Street, San Francisco, CA 94121. E-mail: marynak@itsa.ucsf.edu.

www.pnas.org/cgi/doi/10.1073/pnas.0401602101

Bonferroni's correction for multiple comparisons with INSTAT software.

Osteoblasts (OB) isolated by described methods (17) were used for coculture experiments. Briefly, OB were allowed to migrate out of collagenase II (Sigma) and trypsin/versene-treated femur and calvaria fragments harvested from adult wild-type mice over 10 d in OB media [complete α-MEM with 50 μg/ml L-ascorbic acid (Fisher)]. Confluent cultures were subcultured by plating 8,000 OB per 96-well plate. On day 2, 5×10^4 osteoclast precursors were seeded onto the OB monolayer, and the cocultures were incubated for 7 d, with OB media changed every 3 d.

Detection of Osteoclast Specific Gene Transcripts. Total RNA was obtained, reverse transcribed, and amplified by using murine primers for GAPDH, calcitonin receptor, cathepsin K, integrin β_3, osteoclast-associated receptor (OSCAR), and RANK, as described (10).

Western Blot Analysis. OCLs cultured 5 d in 70 ng/ml RANKL and 10 ng/ml M-CSF or macrophages cultured 5 d in 10 ng/ml M-CSF were lysed in RIPA buffer followed by precipitation (14) with anti-Syk (N-19), anti-FcRγ (Upstate Biotechnology, Lake Placid, NY) antibodies, an anti-DAP12 antiserum (generous gift of T. Takai, Tohoku University, Sendai, Japan), or a GST fusion protein of the tandem SH2-domains of murine Syk (from A. DeFranco, University of California, San Francisco). Blots of whole-cell lysates (20 μg per sample) or precipitates were probed with anti-Syk, anti-DAP12, anti-FcRγ, anti-phosphotyrosine (4G10), anti-CD11b (M-19), anti-actin (C-2) antibodies (Santa Cruz Biotechnology) or anti-phospho-Syk (Y519/520; Cell Signaling Technology) and horseradish peroxidase-conjugated secondary reagents (Amersham Pharmacia).

Retroviral Reconstitution. Retroviruses generated by using pMIG-W vector alone (from Y. Rafaeli, University of California, San Francisco) or pMIG-W encoding murine Syk or a Syk SH2 mutant (R194A) were used to transduce *syk*$^{-/-}$ osteoclast precursors. Retrovirus generated by using pMX-pie vector or PMX-pie encoding FLAG-tagged DAP12 or mutated DAP12 at Y65F and/or Y76F was used to infect *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ osteoclast precursors as described (10). Cells were then cultured with RANKL/M-CSF as above. See *Supporting Methods* for further details.

Results

***DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ Mice Have Severe Osteopetrosis.** *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ mice develop normally but are smaller than their wild-type littermates, with a rounded face and thickened, shortened femurs (data not shown), characteristic of osteopetrosis. Notably, *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ mice develop teeth, distinguishing their phenotype from Src- or RANKL-deficient animals (18, 19). We confirmed the osteopetrotic phenotype by micro-CT analysis of the proximal tibia (Fig. 1*A*). *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ mice had a relative bone volume of 88 ± 3% ($n = 4$), whereas wild-type mice averaged 15 ± 2% ($n = 3$) ($P < 0.001$). *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ tibias showed markedly increased trabecular number, trabecular thickness, and decreased trabecular separation compared with wild type. The marked negative value of the structure model index (SMI) in the *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ mice (SMI = −17.6 ± 4.1) indicates an overwhelmingly concave structure, solid with tube-like channels of marrow space, rather than the rod-like trabecular structure in wild-type animals (SMI = 1.7 ± 0.1). Parameters from bones of *FcR*$\gamma^{-/-}$ animals ($n = 3$) did not differ from wild type, whereas *DAP12*$^{-/-}$ mice demonstrated marginally increased bone mass ($n = 2$), as described (8, 10). Histological examination of *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ bones showed large areas of unresorbed bone with cartilagenous streaks and small bone marrow cavities in comparison with those from wild-type, *DAP12*$^{-/-}$, or *FcR*$\gamma^{-/-}$ animals (Fig. 1*B*).

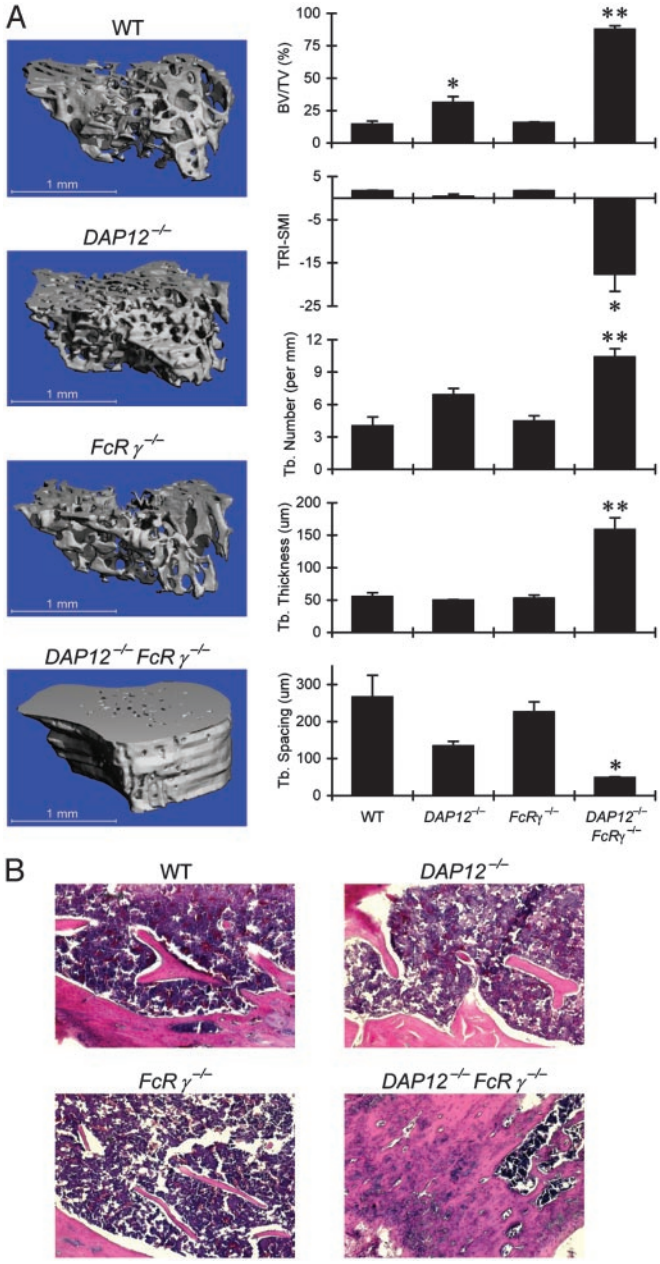

Fig. 1. Osteopetrosis in mice lacking DAP12 and FcRγ. (*A*) Three-dimensional reconstitution of micro-CT scans of proximal tibia and 3D trabecular (Tb.) quantitative parameters (mean ± SEM) of bone structure. Significant differences from wild-type are shown (*, $P < 0.05$; **, $P < 0.001$). (*B*) Hematoxylin-eosin staining of decalcified sections of the primary spongiosa of proximal tibias. BV/TV, relative bone volume. TRI-SMI, 3D reconstruction image SMI.

Syk Colocalizes with Actin in Osteoclasts and Fails to Be Phosphorylated in *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ Cells. In other cells, phosphorylation of ITAM tyrosines recruits and activates Syk through binding to its SH2 domains (2, 3, 11). We found that Syk is expressed in wild-type OCLs generated by 70 ng/ml RANKL and 10 ng/ml M-CSF *in vitro*, and it colocalizes with actin at the cell periphery (Fig. 2*A*). OCLs express a significantly higher amount of Syk than macrophages (Fig. 2*B*). By using a GST fusion protein containing the tandem SH2 domains of Syk [GST-Syk(SH2)$_2$], we show that Syk

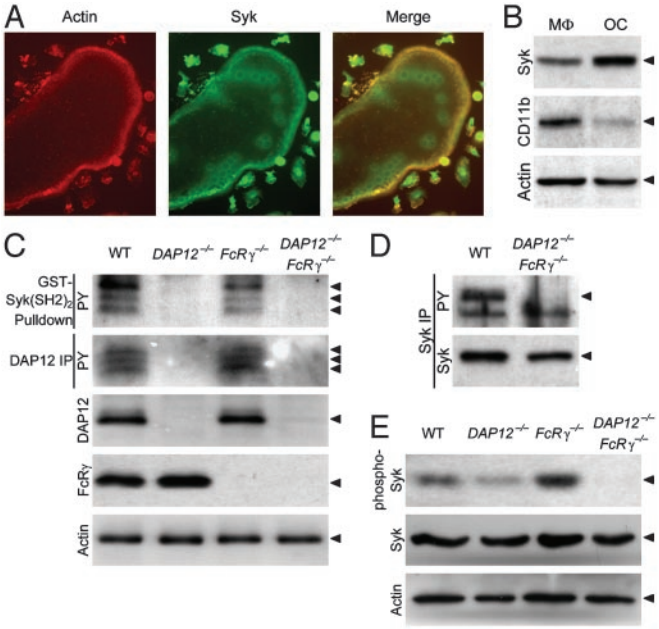

Fig. 2. Lack of Syk phosphorylation in *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ osteoclast-like cells. (*A*) OCLs stained with anti-Syk and phalloidin. (*B*) Expression of Syk in *in vitro* OCLs (OC) and macrophages (MΦ) compared with the macrophage marker CD11b and actin by immunoblotting. (*C*) Precipitation of whole-cell lysates with GST–Syk fusion protein containing the SH2 domains of Syk or DAP12 antiserum probed with anti-phosphotyrosine antibody. Whole-cell lysates show expression of DAP12, FcRγ, and actin. (*D*) Immunoprecipitation (IP) with anti-Syk followed by phosphotyrosine (PY) immunoblot. (*E*) Immunoblot of whole-cell lysate for Y519/520 phosphorylated Syk, total Syk, or actin.

can associate with tyrosine-phosphorylated proteins in osteoclast lysates consistent in size and phosphorylation pattern with DAP12 (Fig. 2*C*). No phosphorylated proteins are seen associated with GST-Syk(SH2)$_2$ in cells from *DAP12*$^{-/-}$ *or DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ mice. Immunoprecipitation of Syk from OCL lysates demonstrates that Syk is tyrosine-phosphorylated (Fig. 2*D*), and this phosphorylation is notably absent in OCLs from *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ animals. Whole-cell lysates of wild-type OCLs show the phosphorylation of Syk at activation loop tyrosine residues (Y519/520), which is partially decreased in *DAP12*$^{-/-}$ (but not *FcR*$\gamma^{-/-}$) OCLs and nearly completely absent in *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ OCLs (Fig. 2*E*), suggesting that FcRγ can partially compensate for the lack of DAP12 in maintaining Syk kinase activity in OCLs.

DAP12, FcRγ, and Syk Are Required for *in Vitro* Generation of Osteoclasts. In concert with the severe osteopetrosis in *DAP12*$^{-/-}$ *FcR*$\gamma^{-/-}$ mice, RANKL/M-CSF-treated *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ osteoclast precursors showed defective *in vitro* osteoclast differentiation (Fig. 3*A*). *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ OCLs were mononuclear, although clearly positive for the osteoclast marker TRAP. Single mutant *DAP12*$^{-/-}$ OCLs showed a similar phenotype, as previously described (6–10), whereas *FcR*$\gamma^{-/-}$ OCLs were indistinguishable from wild type. Similar to *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ cells, OCLs from *syk*$^{-/-}$ precursors [obtained from bone marrow chimeras generated by using *syk*$^{-/-}$ fetal liver cells (14)] also failed to differentiate normally *in vitro*. Interestingly, mononuclear TRAP$^+$ OCLs from *DAP12*$^{-/-}$, *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$, or *syk*$^{-/-}$ cells all expressed markers generally associated with mature osteoclasts, including cathepsin K, β_3 integrin, calcitonin receptor, OSCAR, and RANK (Fig. 3*B*), suggesting that the block in differentiation *in vitro* is at an intermediate to late stage.

High-Dose M-CSF Partially Restores the Developmental Defect in *DAP12*$^{-/-}$, *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$, and *syk*$^{-/-}$ Osteoclasts. Supraphysiologic stimulation of myeloid precursors with M-CSF has been shown to partially rescue osteoclastogenesis in mice lacking β_3 integrins (20, 21). We examined osteoclast differentiation from *DAP12*$^{-/-}$, *FcR*$\gamma^{-/-}$, *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$, and *syk*$^{-/-}$ precursors *in vitro* with 10-fold excess (100 ng/ml) of M-CSF. In high-concentration M-CSF, wild-type and *FcR*$\gamma^{-/-}$ precursors formed extremely large, highly multinucleated OCLs, and formation of TRAP$^+$ multinucleated OCLs from *DAP12*$^{-/-}$, *DAP12*$^{-/-}$ *FcR*$\gamma^{-/-}$, and *syk*$^{-/-}$ cells was partially restored (Fig. 3*A*).

***DAP12*$^{-/-}$, *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$, and *syk*$^{-/-}$ Osteoclasts Fail to Resorb Mineralized Matrix.** Next we examined functional resorption by the mutant OCLs *in vitro*. In contrast to wild-type or *FcR*$\gamma^{-/-}$ OCLs, *DAP12*$^{-/-}$, *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$, or *syk*$^{-/-}$ OCLs failed to digest an artificial calcium phosphate substrate and formed barely detectable pits on dentin (Fig. 4). In cultures with high-concentration M-CSF with partially restored TRAP$^+$ multinucleated cell formation, resorption on calcium phosphate substrate by *DAP12*$^{-/-}$, *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$, and *syk*$^{-/-}$ OCLs was still minimal (Fig. 4*A*), suggesting that the ITAM signaling pathway may play a role in functional

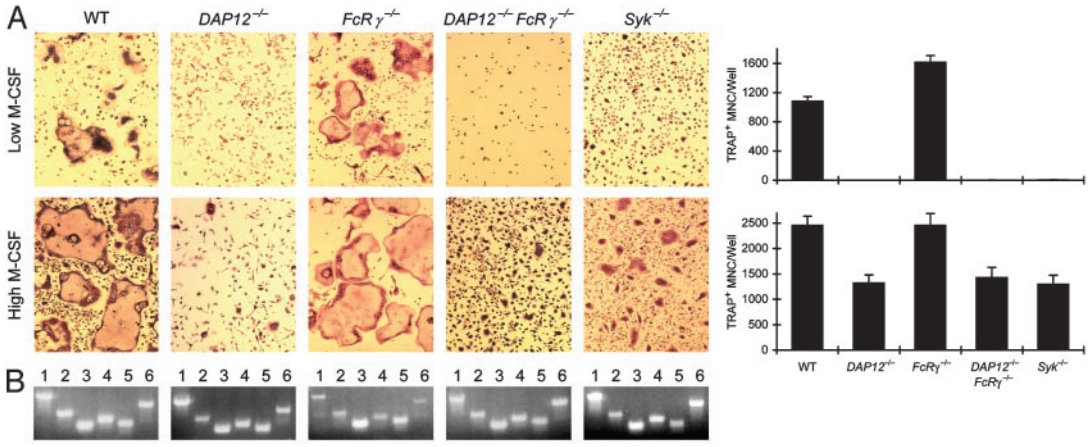

Fig. 3. DAP12/FcRγ and Syk are required for *in vitro* generation of osteoclast-like cells. (*A*) TRAP-stained OCLs generated in RANKL and 10 (''low'') or 100 ng/ml (''high'') M-CSF. TRAP$^+$ multinucleated cells (MNC = 3 or more nuclei per cell) enumerated as mean ± SEM of 3 wells (2 cm^2 per well). (*B*) RT-PCR analysis of OCLs cultured in RANKL and 10 ng/ml M-CSF. 1, GAPDH; 2, calcitonin receptor; 3, cathepsin K; 4, integrin β_3; 5, OSCAR; 6, RANK. *DAP12*$^{-/-}$, *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$, and *syk*$^{-/-}$ groups were statistically different ($P < 0.001$) from wild type in both conditions.

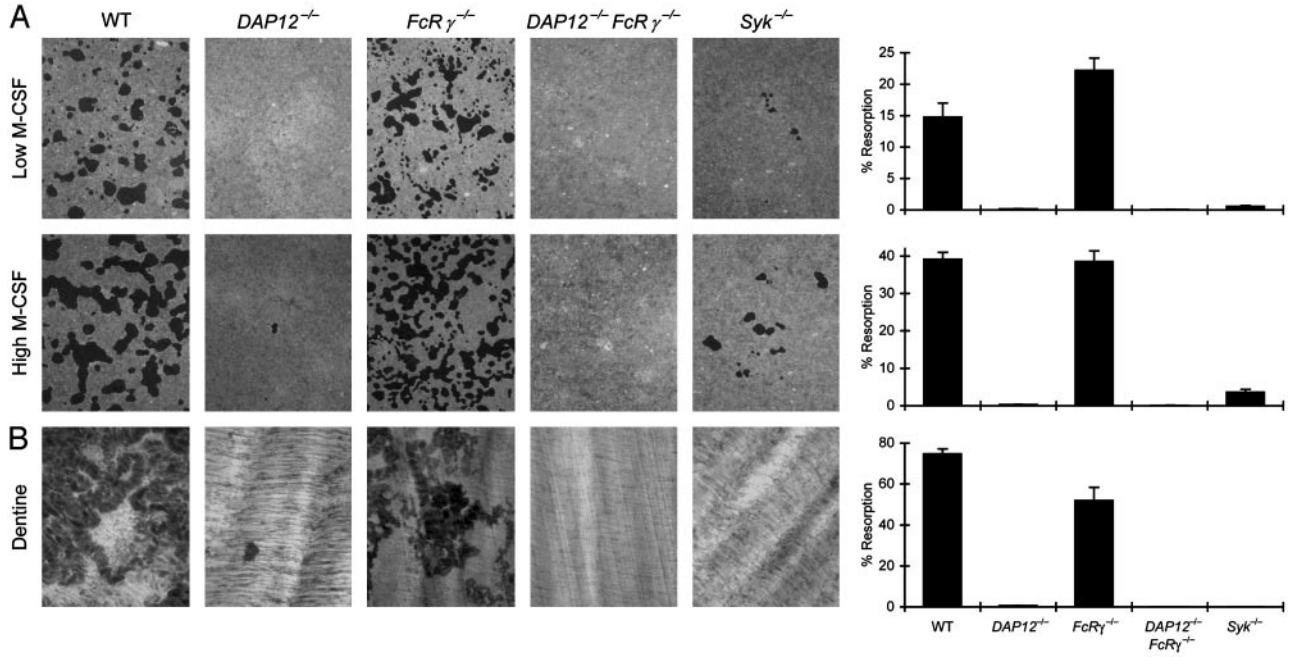

Fig. 4. DAP12/FcRγ and Syk are required for functional resorption of mineralized matrix. (*A*) OCLs generated in RANKL and M-CSF (10 or 100 ng/ml) on an artificial calcium phosphate substrate. The percentage of the resorption of substrate (dark areas) was quantified by dark-field microscopy and expressed as the mean ± SEM of three samples. (*B*) Bone resorption by OCLs on dentine slices with RANKL and 10 ng/ml M-CSF, visualized by toluidine blue staining and light microscopy. Resorption in the $DAP12^{-/-}$, $DAP12^{-/-}FcR\gamma^{-/-}$, and $syk^{-/-}$ groups was statistically different ($P < 0.001$) from wild type in all conditions.

resorption by osteoclasts in addition to their role in differentiation to $TRAP^+$ multinucleated OCLs.

SH2 Domains of Syk are Required for Osteoclast Development and Mineralized Matrix Resorption. Recruitment of Syk to ITAM domains depends on the binding of its SH2 domains to phosphorylated tyrosines within the ITAM. In other cell types, the R194 residue in the distal SH2 domain of Syk is critical for ITAM-mediated signaling (22). We examined the effect of this mutation in osteoclastogenesis by using retroviral expression of wild-type and R194A Syk in $syk^{-/-}$ precursor cells. Reconstitution of Syk expression in $syk^{-/-}$ cells partially restored the *in vitro* formation and function of $syk^{-/-}$ OCLs (Fig. 5 *A* and *B*). Although numbers of OCLs formed from *syk*-transduced $syk^{-/-}$ cells remained lower than those in wild-type cell cultures, the difference correlated with lower expression levels of Syk in the retrovirally reconstituted samples (Fig. 5*C*) and the efficiency of retroviral transduction (30–40% as assessed by GFP expression; data not shown). Importantly, an SH2 domain mutant (R194A) of Syk that fails to bind to phosphorylated ITAM-containing chains did not reconstitute either phenotype or resorbing function of $syk^{-/-}$ OCLs when expressed at levels equivalent to the retrovirally reconstituted wild-type Syk. These data indicate that Syk function in osteoclast formation requires SH2-phosphotyrosine binding.

Reintroduction of Intact DAP12 ITAM Is Required for Development and Function of $DAP12^{-/-}FcR\gamma^{-/-}$ Osteoclasts. We similarly examined the requirement for phosphorylated tyrosines within the DAP12 ITAM for *in vitro* osteoclastogenesis. Reconstitution of wild-type mouse DAP12 but not single tyrosine (Y65 or Y76) or double tyrosine (Y65/Y76) ITAM mutants can partially restore the formation (Fig. 5*D*) and resorptive function (Fig. 5*E*) of $DAP12^{-/-}FcR\gamma^{-/-}$ OCLs. Full restoration is likely not achieved because of a 25–30% transduction of $DAP12^{-/-}FcR\gamma^{-/-}$ precursors. Equivalent expression of mouse DAP12 and the DAP12 ITAM mutants is demonstrated by cell surface expression of the FLAG epitope on FLAG-tagged DAP12 and the FLAG-tagged DAP12 ITAM mutants (Fig. 5*F*). These results indicate that DAP12 is critical for osteoclastogenesis *in vitro* through phospho-ITAM-mediated recruitment of SH2 domain-containing proteins.

Coculture with OB Partially Restores *in Vitro* Osteoclast Formation in $DAP12^{-/-}$ Cells. To further examine the role of the adapter proteins in osteoclastogenesis, we examined $DAP12^{-/-}$, $FcR\gamma^{-/-}$, $DAP12^{-/-}FcR\gamma^{-/-}$, or $syk^{-/-}$ precursors under alternate conditions for differentiation by using coculture of osteoclast precursors with OB. Coculture of $DAP12^{-/-}$ osteoclast precursors with wild-type murine OB resulted in partial normalization of OCL formation (Fig. 6*A*), and these OCLs resorbed an artificial bone matrix, although less than did wild-type OCLs (Fig. 6*B*). In contrast, *in vitro* OCL development or function of $DAP12^{-/-}FcR\gamma^{-/-}$ or $syk^{-/-}$ precursors remained severely defective under coculture conditions, indicating a requirement for ITAM adapters and Syk. These results suggest that FcRγ can partially compensate for the lack of DAP12 under osteoclast–OB coculture conditions. This finding may contribute to the lack of *in vivo* osteopetrosis seen in the $DAP12^{-/-}$ single mutants compared with the $DAP12^{-/-}FcR\gamma^{-/-}$ mice.

Discussion

These studies suggest a critical role for ITAM signals through the Syk tyrosine kinase during osteoclastogenesis and further illustrate the importance of this signaling pathway in the differentiation of hematopoietic cells toward highly specialized functions. ITAM-mediated signals dependent on Syk kinase or the related kinase ZAP-70 are known to play essential roles in the development and function of the adaptive immune system, particularly in T cells and B cells (2). The importance of ITAM-dependent receptors is also recognized in innate immune cells, including macrophages, neutrophils, dendritic cells, natural killer cells, and mast cells (2, 3, 14, 23). Syk may play a broader role in cellular regulation in that it can be directly activated through ligation of surface integrins and has been shown to be critical for specific integrin-mediated functions in macrophages, neutrophils, and platelets (14, 24, 25). Recent find-

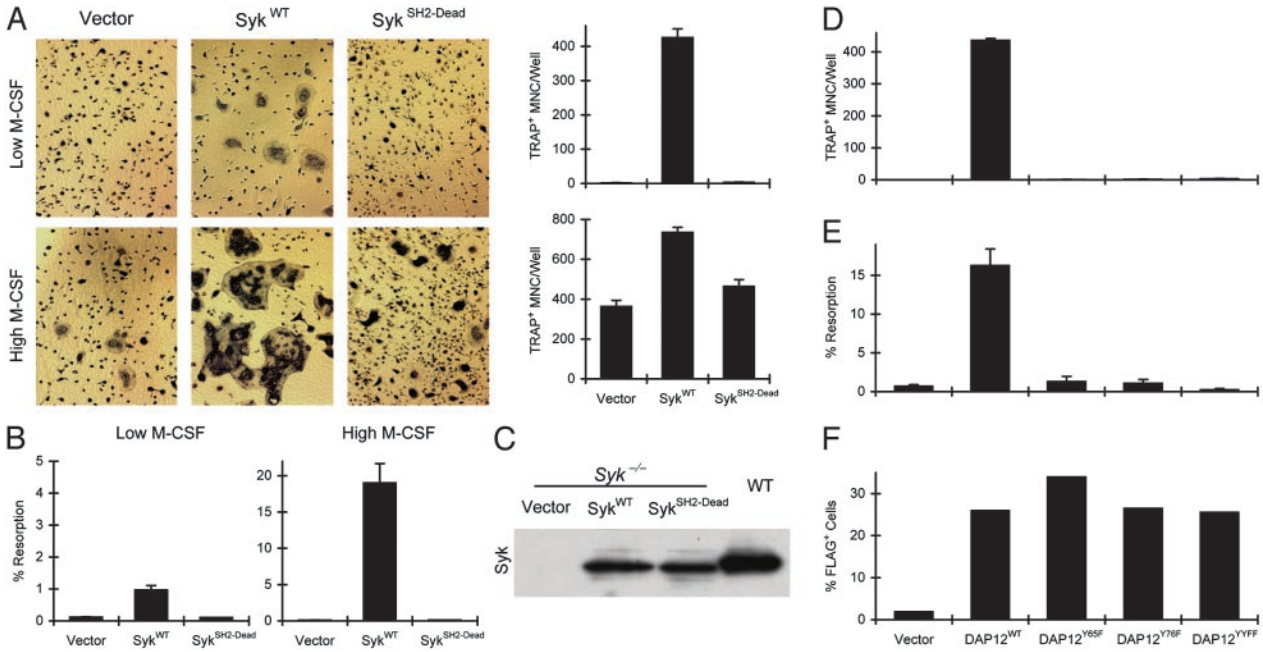

Fig. 5. SH2 domains of Syk and an intact DAP12 ITAM are required for *in vitro* osteoclast differentiation and function. TRAP$^+$ MNC (*A*) and the percent resorption (*B*) of calcium phosphate substrate by *syk*$^{-/-}$ precursors infected with retrovirus encoding vector alone, wild-type *syk*, or a SH2-dead mutant (R194A) of *syk* at indicated M-CSF concentrations. In both conditions, TRAP$^+$ MNC number and percent resorption in the Syk^{WT} groups was statistically different ($P < 0.01$) from vector or Syk$^{SH2\text{-}Dead}$, with no difference between vector and Syk$^{SH2\text{-}Dead}$ groups ($P > 0.05$). (*C*) Anti-Syk blot of whole-cell ligands from retrovirally transduced or wild-type OCLs. TRAP$^+$ MNC (*D*) and percent resorption (*E*) from *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ precursors infected with virus-encoding vector alone, wild-type DAP12, or ITAM tyrosine mutants (Y65, Y76, or both) of DAP12 cultured in 10 ng/ml M-CSF. (*F*) Expression of FLAG epitope on cells retrovirally transduced with FLAG-tagged DAP12 or FLAG-tagged DAP12 mutants.

ings that Syk is associated with a modified ITAM in ERM (ezrin, radixin, and moesin) proteins has also suggested its role in the cytoskeletal changes mediated by these proteins (26).

In the developing osteoclast, Syk may contribute to several of these pathways, given the importance of integrins and cytoskeletal rearrangements that take place during osteoclastogenesis (1, 27). The finding that the *in vitro* developmental defect but not the bone-resorbing capacity of OCLs from *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ or *syk*$^{-/-}$ precursors can be partially restored by treatment with high-dose M-CSF is highly reminiscent of the recent studies on β_3 integrin-deficient cells (20, 21) and a recent report on *DAP12*$^{-/-}$ cells (9). Similar to the report on *DAP12*$^{-/-}$cells (9), we found that *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ and *syk*$^{-/-}$ preosteoclasts phosphorylated extracellular response kinase normally in response to M-CSF (data not shown), demonstrating that other signaling pathways are intact. The degree to which the deficiency of DAP12, FcRγ, or Syk directly affects $\alpha_V\beta_3$ integrin function in osteoclasts has not been fully explored. Syk-deficient neutrophils, macrophages, and platelets have been reported to show impaired signaling through integrins (14, 24, 25, 28), and Syk can associate with the cytoplasmic domain of integrin β-chains (28). Supporting our hypothesis that ITAM signaling is linked to integrins, Faccio *et al.* (9) recently reported that *DAP12*$^{-/-}$ OCLs fail to migrate to osteopontin, an $\alpha_V\beta_3$ integrin ligand, whereas *syk*$^{-/-}$ OCLs fail to phosphorylate Pyk2 or Src on adherence to osteopontin. Thus, it is possible that ITAM adapters may couple to cell surface integrins to provide osteoclast differentiation signaling through Src-family and Syk kinases. Such signaling likely cooperates with RANK and M-CSF receptor to provide optimal differentiation responses. Signals downstream of Syk (Cbl and Pyk2) (14) have been identified in osteoclast formation and function (21, 29–31). ITAM signaling in other cells also stimulates phospholipase Cγ and Ca^{2+}-flux (2, 11), leading to activation of nuclear factor of activated T cells transcription factors (NFAT). Signaling through intracellular pathways involving NFATc1 are also required during osteoclast differentiation (32). Interestingly, despite the clear defect in differentiation in development and function observed in *DAP12*$^{-/-}$, *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$, and *syk*$^{-/-}$ OCLs, we found that they express markers traditionally associated with late-stage differentiated osteoclasts, including calcitonin receptor, integrin β3, and OSCAR. Faccio *et al.* reported that *DAP12*$^{-/-}$ OCLs had reduced expression of osteoclast markers at low concentrations of M-CSF at days 2 and 4 of culture (9). Our examination of *DAP12*$^{-/-}$ OCL at day 7 of culture did not show

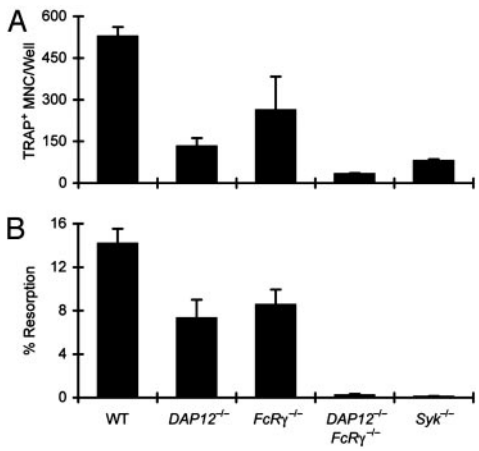

Fig. 6. Coculture partially restores *in vitro* osteoclast formation in *DAP12*$^{-/-}$ cells but not *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$ or *syk*$^{-/-}$ cells. OB from wild-type mice were used to stimulate osteoclast precursors from wild-type, *DAP12*$^{-/-}$, *FcR*$\gamma^{-/-}$, *DAP12*$^{-/-}$*FcR*$\gamma^{-/-}$, or *syk*$^{-/-}$ mice. The number of TRAP$^+$ MNC (*A*) and the percent resorption (*B*) on calcium phosphate substrate is shown.

distinct differences, although it remains possible that expression of these markers is delayed.

DAP12-deficient cells show a nearly complete defect of osteoclast development and *in vitro* bone resorption when OCLs are generated from precursors in the presence of M-CSF and RANKL. DAP12 is clearly phosphorylated in such wild-type osteoclast cultures, whereas we were not able to detect phosphorylation of FcRγ under identical conditions (not shown). Furthermore, phosphoproteins that associate with the tandem SH2-domains of Syk are present in wild-type and $FcR\gamma^{-/-}$ but not in $DAP12^{-/-}$ OCLs. These results indicate that DAP12, rather than FcRγ, is primarily responsible for supporting the development of osteoclasts under *in vitro* culture conditions where osteoclasts are present without OB.

An apparent paradox is raised by the observation that both $DAP12^{-/-}$ and $DAP12^{-/-}FcR\gamma^{-/-}$ OCLs show a severe defect *in vitro*, but only the $DAP12^{-/-}FcR\gamma^{-/-}$ mice manifest severe osteopetrosis *in vivo*. Comparison of the *in vitro* phenotypes of cytokine-treated osteoclast cultures with that of osteoclast–OB cocultures may provide a possible explanation for this difference. In osteoclast–OB cocultures, we observed development of multinucleated OCLs from $DAP12^{-/-}$ but not from $DAP12^{-/-}FcR\gamma^{-/-}$ precursors, suggesting that FcRγ is able to compensate for the lack of DAP12 in the presence of OB. A possible scenario could be that OB promote osteoclastogenesis by a mechanism requiring FcRγ in osteoclasts (through, for example, the recently described OSCAR receptor, which is expected to associate with FcRγ). Such compensation may occur *in vivo* and explain the nearly normal bone density and the presence of multinuclear osteoclasts in $DAP12^{-/-}$ mice *in vivo* (8, 10). Furthermore, although the *in vitro* morphology of $DAP12^{-/-}$ versus $DAP12^{-/-}FcR\gamma^{-/-}$ OCLs (in the absence of OB) was very similar, we consistently observed some *in vitro* bone resorption by $DAP12^{-/-}$ but not by $DAP12^{-/-}FcR\gamma^{-/-}$ cells, and the phosphorylation of Syk was also further decreased in $DAP12^{-/-}FcR\gamma^{-/-}$ compared with in $DAP12^{-/-}$ cells. Thus, FcRγ in $DAP12^{-/-}$ osteoclasts may allow a level of *in vivo* bone resorption sufficient for nearly normal bone density, even in the absence of additional signals from OB. An FcRγ-dependent signal may also originate from other (nonosteoblastic) components of the bony microenvironment (e.g., stromal cells and soft tissue matrix) that are not present *in vitro*. Additionally we have not ruled out that the lack of both DAP12 and FcRγ could lead to increased bone formation by OB, exacerbating the *in vivo* phenotype.

Although our study demonstrates the importance of the DAP12 and FcRγ adapter proteins in osteoclast development and function, the full spectrum of associated receptors and their ligands has not been completely defined. It is likely that, similar to other innate immune cells, osteoclasts express a number of different receptors and associated ITAM-containing signaling adapters, which provide a diverse means of regulating osteoclastogenesis in response to local changes and cellular interactions. Differences in receptor or adapter expression between mice and humans likely explain the different phenotypic consequences of DAP12 deficiency between species. The identification of the receptor/ligand interactions involved will be critical to identifying the role of these receptors and adapters in normal and pathological bony remodeling.

Osteoporosis has been linked to dysregulation of osteoclast function, placing this cell type in the center of pathogenesis of the disease. The signaling proteins and molecular interactions described here may provide novel therapeutic approaches for the pharmacological treatment of osteoporosis or other diseases involving bony remodeling. Small molecule inhibitors of Syk are already in development for use in treatment of allergic diseases. Our results may suggest their possible utility in bone diseases.

We thank V. Tybulewicz for $syk^{+/-}$ mice; Hong Yu, G. Cassafer, and E. Niemi for technical support; A. DeFranco and Y Rafaeli for DNA plasmids; and T. Takai for DAP12 antiserum. A.M. is a Bolyai Postdoctoral Fellow of the Hungarian Academy of Sciences, M.B.H. is an Abbott Scholar in Rheumatology Research, L.L.L. is an American Cancer Society Research Professor, C.A.L. is a Scholar of the Leukemia and Lymphoma Society, and M.C.N. is an American Cancer Research Scholar. This work was supported by the Department of Veterans Affairs, National Institutes of Health Grants DK58066 (to C.A.L.), CA89294 (to L.L.L.), and AG17762 (to S.M.), Medical Research Council of Hungary Grant 044/2002 (to A.M.), and the Rosalind Russell Center for Arthritis Research.

1. Boyle, W. J., Simonet, W. S. & Lacey, D. L. (2003) *Nature* **423,** 337–342.
2. Turner, M., Schweighoffer, E., Colucci, F., Di Santo, J. P. & Tybulewicz, V. L. (2000) *Immunol. Today* **21,** 148–154.
3. Lanier, L. L. & Bakker, A. B. (2000) *Immunol. Today* **21,** 611–614.
4. Takai, T., Li, M., Sylvestre, D., Clynes, R. & Ravetch, J. V. (1994) *Cell* **76,** 519–529.
5. Paloneva, J., Kestila, M., Wu, J., Salminen, A., Bohling, T., Ruotsalainen, V., Hakola, P., Bakker, A. B., Phillips, J. H., Pekkarinen, P., *et al*. (2000) *Nat. Genet.* **25,** 357–361.
6. Cella, M., Buonsanti, C., Strader, C., Kondo, T., Salmaggi, A. & Colonna, M. (2003) *J. Exp. Med.* **198,** 645–651.
7. Paloneva, J., Mandelin, J., Kiialainen, A., Bohling, T., Prudlo, J., Hakola, P., Haltia, M., Konttinen, Y. T. & Peltonen, L. (2003) *J. Exp. Med.* **198,** 669–675.
8. Kaifu, T., Nakahara, J., Inui, M., Mishima, K., Momiyama, T., Kaji, M., Sugahara, A., Koito, H., Ujike-Asai, A., Nakamura, A., *et al*. (2003) *J. Clin. Invest.* **111,** 323–332.
9. Faccio, R., Zou, W., Colaianni, G., Teitelbaum, S. L. & Ross, F. P. (2003) *J. Cell Biochem.* **90,** 871–883.
10. Humphrey, M. B., Ogasawara, K., Yao, W., Spusta, S. C., Daws, M. R., Lane, N. E., Lanier, L. L. & Nakamura, M. C. (2004) *J. Bone Miner. Res.* **19,** 224–234.
11. McVicar, D. W., Taylor, L. S., Gosselin, P., Willette-Brown, J., Mikhael, A. I., Geahlen, R. L., Nakamura, M. C., Linnemeyer, P., Seaman, W. E., Anderson, S. K., *et al*. (1998) *J. Biol. Chem.* **273,** 32934–32942.
12. Bakker, A. B., Hoek, R. M., Cerwenka, A., Blom, B., Lucian, L., McNeil, T., Murray, R., Phillips, L. H., Sedgwick, J. D. & Lanier, L. L. (2000) *Immunity* **13,** 345–353.
13. Turner, M., Mee, P. J., Costello, P. S., Williams, O., Price, A. A., Duddy, L. P., Furlong, M. T., Geahlen, R. L. & Tybulewicz, V. L. (1995) *Nature* **378,** 298–302.
14. Mocsai, A., Zhou, M., Meng, F., Tybulewicz, V. L. & Lowell, C. A. (2002) *Immunity* **16,** 547–558.
15. Hildebrand, T. & Ruegsegger, P. (1997) *J. Microsc. (Oxford)* **185,** 67–75.
16. Hildebrand, T. & Ruegsegger, P. (1997) *Comput. Methods Biomech. Biomed. Eng.* **1,** 15–23.
17. Bakker, A. & Klein-Nulend, J. (2003) *Methods Mol. Med.* **80,** 19–28.
18. Soriano, P., Montgomery, C., Geske, R. & Bradley, A. (1991) *Cell* **64,** 693–702.
19. Kong, Y. Y., Yoshida, H., Sarosi, I., Tan, H. L., Timms, E., Capparelli, C., Morony, S., Oliveira-dos-Santos, A. J., Van, G., Itie, A., *et al*. (1999) *Nature* **397,** 315–323.
20. Faccio, R., Takeshita, S., Zallone, A., Ross, F. P. & Teitelbaum, S. L. (2003) *J. Clin. Invest.* **111,** 749–758.
21. Faccio, R., Novack, D. V., Zallone, A., Ross, F. P. & Teitelbaum, S. L. (2003) *J. Cell Biol.* **162,** 499–509.
22. Gao, J., Zoller, K. E., Ginsberg, M. H., Brugge, J. S. & Shattil, S. J. (1997) *EMBO J.* **16,** 6414–6425.
23. Bouchon, A., Hernandez-Munain, C., Cella, M. & Colonna, M. (2001) *J. Exp. Med.* **194,** 1111–1122.
24. Vines, C. M., Potter, J. W., Xu, Y., Geahlen, R. L., Costello, P. S., Tybulewicz, V. L., Lowell, C. A., Chang, P. W., Gresham, H. D. & Willman, C. L. (2001) *Immunity* **15,** 507–519.
25. Obergfell, A., Eto, K., Mocsai, A., Buensuceso, C., Moores, S. L., Brugge, J. S., Lowell, C. A. & Shattil, S. J. (2002) *J. Cell Biol.* **157,** 265–275.
26. Urzainqui, A., Serrador, J. M., Viedma, F., Yanez-Mo, M., Rodriguez, A., Corbi, A. L., Alonso-Lebrero, J. L., Luque, A., Deckert, M., Vazquez, J. & Sanchez-Madrid, F. (2002) *Immunity* **17,** 401–412.
27. Teitelbaum, S. L. (2000) *J. Bone Miner. Metab.* **18,** 344–349.
28. Woodside, D. G., Obergfell, A., Talapatra, A., Calderwood, D. A., Shattil, S. J. & Ginsberg, M. H. (2002) *J. Biol. Chem.* **277,** 39401–39408.
29. Sanjay, A., Houghton, A., Neff, L., DiDomenico, E., Bardelay, C., Antoine, E., Levy, J., Gailit, J., Bowtell, D., Horne, W. C. & Baron, R. (2001) *J. Cell Biol.* **152,** 181–195.
30. Tanaka, S., Amling, M., Neff, L., Peyman, A., Uhlmann, E., Levy, J. B. & Baron, R. (1996) *Nature* **383,** 528–531.
31. Lakkakorpi, P. T., Bett, A. J., Lipfert, L., Rodan, G. A. & Duong le, T. (2003) *J. Biol. Chem.* **278,** 11502–11512.
32. Takayanagi, H., Kim, S., Koga, T., Nishina, H., Isshiki, M., Yoshida, H., Saiura, A., Isobe, M., Yokochi, T., Inoue, J., *et al*. (2002) *Dev. Cell* **3,** 889–901.