Negative Feedback Inhibition of NFATc1 by DYRK1A Regulates Bone Homeostasis**

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DYRK1A (dual specificity tyrosine-regulated kinase 1A) is a member of genetically conserved protein kinases that phosphorylate serine/threonine residues on substrates and autophosphorylate on tyrosine residues (1, 2). Most evidence has so far indicated that DYRK1A is mainly linked to brain development (3) and mental retardation associated with Down syndrome (4–6). However, the broad tissue distribution of DYRK1A (7) suggests a possibility of hitherto unknown function in addition to its role in neurodevelopment.

In the presence of macrophage colony-stimulating factor (M-CSF)3 and receptor activator of nuclear factor κB ligand (RANKL), macrophage/menocyte lineage of hematopoietic precursor cells differentiate into osteoclasts, cells specialized for bone resorption (8–10). M-CSF supports survival and proliferation of osteoclast precursors as well as expression of RANKL receptor (RANK). RANKL induces signaling cascades, including the recruitment of TNF receptor-associated factors, activation of mitogen-activated kinases, stimulation of NFκB and c-Fos transcription factors, and phospholipase C-γ-dependent calcium signaling, all of which culminate in the induction and activation of NFATc1, a “master” transcription factor for osteoclastogenesis (11–14).

Through a proteomic approach, we discovered that the expression of DYRK1 family protein was significantly up-regulated during osteoclastogenesis. Recent studies of Drosophila RNA interference screening and examination of genes in the Down syndrome critical region reported that DYRK1A facilitates nuclear export of NFAT (15, 16). Here we show that DYRK1A functions as a negative feedback regulator of NFATc1 in the modulation of bone homeostasis in vivo. Our results indicate a novel function of DYRK1A for bone cell regulation that might be applied to the treatment of bone-destructive diseases.

MATERIALS AND METHODS

Animals—Five-week-old ICR mice were obtained from Charles River Laboratories (Wilmington, MA). DYRK1A TG mice expressing human DYRK1A and WT littermates of C57BL/6 background were described previously (6). For the LPS-induced bone loss model, male WT or DYRK1A TG mice (6 weeks old) were injected intraperitoneally with PBS or LPS (Sigma; O111:B4; 5 mg/kg) dissolved in PBS of 200-μl volume at 7 and 3 days before sacrifice. For the ovarioectomy-induced bone loss model, female WT and DYRK1A TG mice (14 weeks old) were either ovarioctomized or sham-operated at 4 weeks before sacrifice. Animal experimental protocols were approved by the committees on the care and use of animals in research at Seoul National University and Inje University.

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3 The abbreviations used are: M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of nuclear factor κB ligand; BMM, bone marrow macrophage; TRAP, tartrate-resistant acid phosphatase; TG, transgenic; WT, wild type; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; Ab, antibody; CA, constitutively active; microCT, microcomputed tomography; BV, bone volume; TV, tissue volume.
Reagents—Recombinant human soluble RANKL, human M-CSF, and human BMP-2 were purchased from PeproTech (Rocky Hill, NJ). The leukocyte acid phosphatase assay kit was from Sigma. Lipofectamine 2000\textsuperscript{TM} was from Invitrogen. Antibodies (Abs) against DYRK1A (M01) and DYRK1B were from Abnova (Taipei, Taiwan) and Abgent (San Diego, CA), respectively. Abs against NFATc1 (7A6), Lamin B (M-20), and c-Fos (H-125) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The 7A6 anti-NFATc1 Ab was raised against a fragment of human NFATc1 that is conserved in all six known mouse isoforms. Anti-β-actin (AC-74) was from Sigma. Phosphospecific Abs for ERK (Thr\textsuperscript{202}/Tyr\textsuperscript{204}), JNK (Thr\textsuperscript{182}/Tyr\textsuperscript{185}), and p38 (Thr\textsuperscript{180}/Tyr\textsuperscript{182}) were from Cell Signaling Technology (Beverly, MA). Anti-phosphoserine Ab (3C171) was from Abcam (Cambridge, UK). Ionomycin and cyclosporin A were obtained from Calbiochem. All other chemicals were purchased from Sigma.

Immunoprecipitation and Immunoblotting—Immunoprecipitates, whole cell lysates, or nuclear extracts from the same number of cells were subjected to 10% SDS-PAGE followed by immunodetection using chemiluminescence (17).

Quantitative Real-time PCR Analysis—The mRNA expression of genes was quantified by the methods described previously (17). Primer sequences for real-time PCR analyses were listed in supplemental Table S1.

Osteoclast Differentiation—BMMs (2 × 10\textsuperscript{4} cells/well in 48-well plastic plates) were incubated with 30 ng/ml M-CSF and 100 ng/ml RANKL for 3–4 days. At the end of incubation, osteoclast differentiation was determined by staining for TRAP activity using a leukocyte acid phosphatase assay kit (Sigma), and cells were photographed as previously described (17). In some experiments, BMMs were cocultured with osteoblasts (10\textsuperscript{4}/well) in the presence of 10 nm 1,25-dihydroxyvitamin D\textsubscript{3} and 1 μM prostaglandin E\textsubscript{2}.

Osteoblast Differentiation—Calvarial cells were prepared from 1-day-old mice (18). Osteoblast differentiation was induced by treating cells (10\textsuperscript{4}/well in 48-well plates) with either 10 μM β-glycerophosphate plus 100 μM ascorbic acid or 300 μg/ml BMP-2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. After 6 days, cells were stained for alkaline phosphatase activity. The intensity of alkaline phosphatase staining was quantified by scanning of cell culture plates (PowerLook 1100 scanner) followed by measuring the integrated density using the ImageJ program (version 1.40; available on the National Institutes of Health web site). Alternatively, the mineralization was visualized by alizarin red staining after 2–3 weeks of culture.

Plasmids—pcDNA3.1-DYRK1A was prepared from mouse brain cDNA (19). pMX-NFATc1 WT and pMSCV-NFATc1 constitutive active (CA) mutant were also used. For DYRK knockdown experiments, oligonucleotides for small interfering RNA were generated by targeting a 21-base sequence of mouse Dyrk1a and Dyrk1b. The resulting oligonucleotide sequences (supplemental Table S2) were annealed and ligated into pSuper-retro vector (Oligoengine, Seattle, WA) using BamHI and HindIII sites.

Retroviral Gene Transfer—Retroviruses were packaged by transfecting plasmids into Plat-E cells. Retroviral infection of cells was performed as described previously (17).

Luciferase Assay—RAW264.7 cells were transfected with pcDNA3.1 vector or pcDNA3.1-DYRK1A and incubated with 100 ng/ml RANKL for 2 days. The cells were transfected with the reporter plasmid containing the 0.8-kb proximal region of NFATc1 promoter (20). At 24 h after reporter transfection, cells were lysed in Glo\textsuperscript{TM} lysis buffer. Luciferase activity was measured using Bright-Glo\textsuperscript{TM} luciferase assay system (Promega, Madison, WI) in a FLUOStar Optima luminometer (BMG Labtech GmbH, Offenburg, Germany).

Calcein-Xylenol Orange Double Labeling—WT or DYRK1A TG mice (8 weeks old) were injected intraperitoneally with 25 mg/kg calcein (Sigma; dissolved in 200 μl of 2% NaHCO\textsubscript{3}, 150 mM NaCl) followed by 90 mg/kg xylenol orange (Sigma) at day 1 and day 7, respectively. Mice were sacrificed at day 10, and femurs were fixed and embedded in resin (BMP Lab, Seoul, Korea). Sections were observed under a Zeiss LSM 5 PASCAL laser-scanning microscope (Carl Zeiss Microimaging GmbH, Goettingen, Germany) with a ×40 objective (C-Apochromat/1.2 W Corr). The distance between calcein and xylenol orange deposition was calculated from five different regions of the same image in 4 slides/group using the LSM 5 image browser (version 3.1).

Microcomputed Tomography (microCT)—Femurs from mice were subjected to microCT analyses using a SkyScan 1172 scanner (SkyScan, Aartselaar, Belgium; 40 kV, 250 μA, 7.9 μm pixel size). Images obtained from a 1-mm-thick distal femoral area, starting from 1 mm below the growth plate, were analyzed by the CT-analyzer program (version 1.7; SkyScan) to calculate bone volume and bone parameters. A three-dimensional reconstitution of images was performed by CT-volume software (version 1.11; SkyScan).

Histology and Histomorphometry—Femurs were fixed, decalcified for 1 month in 10% EDTA, and embedded in paraffin. Sections of 5 μm thickness were prepared using Leica microtome RM2145 (Leica Microsystems, Bannockbrun, IL) and were subjected to TRAP and hematoxylin staining. Histomorphometric analysis was performed as described (21) using the Bioquant OsteoII program (Bioquant Image Analysis Corp., Nashville, TN).

Statistics—Student’s t test was used to determine the significance of difference between two groups. Comparison of results composed of more than two groups was done by one-way analysis of variance followed by Student-Newman-Keuls post hoc tests. Differences with p < 0.05 were regarded as significant. A detailed description of methods is provided in the supplemental material.

RESULTS

DYRK1A Negatively Regulates Osteoclastogenesis—DYRK1A expression significantly increased upon stimulation of mouse bone marrow macrophages (BMMs) with RANKL (Fig. 1A). A real-time PCR analysis revealed that RANKL significantly up-regulated Dyrk1a mRNA expression (Fig. 1B). To gain insights into the role of DYRK1A during RANKL-induced osteoclastogenesis, we down-regulated DYRK1A protein expression by
introducing small interfering RNA into BMMs via retroviral infection (supplemental Fig. S1A). Osteoclast differentiation was dramatically enhanced in DYRK1A down-regulated BMMs, suggesting a negative regulatory role of DYRK1A during osteoclastogenesis (Fig. 1C). Furthermore, when DYRK1A was overexpressed in RAW264.7 macrophages (supplemental Fig. S1B), a significant decrease in osteoclastogenesis was observed (Fig. 1D). On the other hand, DYRK1B did not dramatically affect osteoclastogenesis, although its expression was significantly increased by RANKL (supplemental Fig. S2).

**DYRK1A Is a Negative Feedback Inhibitor of NFATc1 Activity—** To uncover the RANKL-dependent transcription factor(s) that might induce DYRK1A expression in osteoclasts, BMMs were infected with retroviruses to overexpress transcription factors known to be activated downstream of RANKL. Although c-Fos only slightly stimulated DYRK1A expression, both WT and constitutive active NFATc1 dramatically increased DYRK1A in the absence of RANKL (Fig. 2A), suggesting that NFATc1 might be a regulator of DYRK1A expression. Although the RANKL-dependent DYRK1A expression was suppressed by NFκB inhibitors (supplemental Fig. S3A), it was most likely due to the inhibition of NFATc1 expression because NFκB inhibitors did not reduce DYRK1A expression in the presence of constitutive active NFATc1 (supplemental Fig. S3B). It was suggested that DYRK1A might phosphorylate and thereby exclude NFAT from the nucleus (15, 16). Indeed, there was a significant increase in the phosphorylation of NFATc1 in DYRK1A-overexpressing 293FT cells (Fig. 2B). Similar enhanced phosphorylation of NFATc1 was observed in RAW264.7 cells overexpressing DYRK1A (supplemental Fig. S3C). The accumulation of nuclear NFATc1 in response to RANKL treatment was prominently suppressed in DYRK1A-overexpressing cells (Fig. 2C). Similarly, NFATc1 in the nuclear fraction from DYRK1A-overexpressing cells did not increase upon stimulation with ionomycin (Fig. 2D). In addition, it was evident that there was little activation of NFATc1 promoter activity by RANKL in DYRK1A-overexpressing RAW264.7 cells, compared with that in control cells (Fig. 2E). DYRK1A also suppressed NFATc1-dependent IL-4 promoter activity (supplemental Fig. S3D). In accordance with the reduced NFATc1 promoter activation, RANKL-induced increase in total NFATc1 protein was significantly impaired in DYRK1A-overexpressing cells (Fig. 2F). Taken together, we suggest that DYRK1A inhibits its NFATc1 activity and expression via a negative feedback regulation.

**Osteoporotic Bone Phenotype of DYRK1A Transgenic Mice Despite Impaired Osteoclastogenesis—** The in vitro inhibition of osteoclastogenesis by DYRK1A led us to analyze the bone phenotype of DYRK1A BAC transgenic (DYRK1A TG) mice that overexpress human DYRK1A by 1.5-fold under the control of endogenous promoter to mimic the increased gene dosage of Down syndrome (6). Although we detected no emphatic skele-

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**FIGURE 1. Negative regulation of osteoclast differentiation by DYRK1A.** A, BMMs from ICR mice were treated with 100 ng/ml RANKL for 2 days in the presence of 30 ng/ml M-CSF. Whole cell lysates were subjected to Western blotting. B, real-time PCR analysis to assess mRNA expression of Dyrk1a relative to that of Hprt1 (hypoxanthine guanine phosphoribosyltransferase 1). C, control or DYRK1A down-regulated BMMs were stimulated with 100 ng/ml RANKL for 4 days in the presence of 30 ng/ml M-CSF and stained for TRAP activity. D, vector- or DYRK1A-transfected RAW264.7 cells were stimulated with 200 ng/ml RANKL for 4 days and stained for TRAP activity. All data are representative of at least three independent experiments. Data in B–D are mean ± S.E. of triplicate assays. **, p < 0.01 versus control.
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FIGURE 2. Reduced activity and expression of NFATc1 by DYRK1A via a negative feedback mechanism. A, BMMs were infected with retroviruses containing c-Fos, WT NFATc1, or CA mutant of NFATc1 and further incubated for 48 h in the absence or presence of 100 ng/ml RANKL. Cell lysates were subjected to Western blotting. B, 293FT cells were co-transfected with pMX-NFATc1 and pcDNA vector or pcDNA-DYRK1A constructs. At 24 h after transfection, the NFATc1 immunoprecipitates were subjected to Western blotting using anti-phosphoserine Ab. C, control or DYRK1A-transfected RAW264.7 cells were stimulated with 200 ng/ml RANKL for 48 h. The nuclear fraction was examined for NFATc1 by Western blotting. D, control or DYRK1A-transfected RAW264.7 cells were cultured with 200 ng/ml RANKL for 48 h before stimulation with 10 μM ionomycin for 30 min. Nuclear NFATc1 was examined. E, NFATc1 promoter activity was measured in RAW264.7 cells in the presence or absence of excess DYRK1A. F, control or DYRK1A-transfected RAW264.7 cells were stimulated with 200 ng/ml RANKL for 2 days. Whole cell lysates were analyzed for NFATc1 expression. Data are representative of at least three independent experiments. Data in E are mean ± S.E. of triplicate assays. **, p < 0.01 versus control.

tal anomaly in gross examination by soft x-ray (supplemental Fig. S4A), microCT analysis of femurs revealed that trabecular bone volume was markedly decreased in TG mice compared with WT (supplemental Fig. S4B). Analyses of bone parameters from microCT data further confirmed an osteoporotic bone phenotype of both male and female DYRK1A TG mice (Fig. 3A). The bone volume/tissue volume (BV/TV) in male mice was 12.7 ± 0.6% in WT and 10.1 ± 0.9% in TG femurs. Similarly, BV/TV in female mice was reduced from 12.6 ± 0.5% in WT to 10.9 ± 0.3% in TG mice. The analyses of trabecular thickness (supplemental Fig. S4C), trabecular number (supplemental Fig. S4D), and trabecular separation (supplemental Fig. S4E) indicated reduced bone mass in TG mice. However, it seemed that the extra DYRK1A did not affect cortical bone thickness of femurs (supplemental Fig. S4, F and G). The RANKL-stimulated expression of NFATc1 was significantly reduced in DYRK1A TG BMMs (Fig. 3B). We observed no significant defect in RANKL-dependent mitogen-activated kinase activation (supplemental Fig. S5A), indicating that the signaling pathways known to regulate RANKL-dependent NFATc1 expression in BMMs were not affected. Both proliferation (supplemental Fig. S5B) and RANKL-induced osteoclastogenesis (Fig. 3C and supplemental Fig. S5C) were significantly impaired in DYRK1A TG BMMs. A real-time PCR analysis revealed significant reduction of osteoclast differentiation markers Acp5, Calcr, and, to a lesser extent, Ctsk in TG BMMs following RANKL stimulation (Fig. 3D). In agreement with the reduced osteoclast differentiation, the resorption activity of TG osteoclasts was significantly lower than that of WT osteoclasts (data not shown). However, when DYRK1A TG BMMs were forced to express WT or constitutive active NFATc1, defective osteoclastogenesis was restored (Fig. 3E).

Defective Osteoblastogenesis in DYRK1A TG Mice—Since the impaired osteoclastogenesis contradicted the reduced bone mass in DYRK1A TG mice, we next investigated the function of osteoblasts in TG mice. Stimulation of osteoblast differentiation in mouse calvarial cells with β-glycerophosphate and ascorbic acid significantly induced DYRK1A expression (Fig. 4A). Interestingly, the induction of DYRK1A paralleled NFATc1 expression. Overexpression of NFATc1 in calvarial cells induced DYRK1A expression (Fig. 4B), suggesting that DYRK1A might be under the regulation of negative feedback loop as in osteoclasts. A markedly reduced NFATc1 induction by BMP-2 was observed in TG osteoblasts (Fig. 4C), whereas extra DYRK1A did not affect BMP-2-dependent Smad phosphorylation (supplemental Fig. S6A). Treatment of osteoblasts with BMP-2 increased nuclear NFATc1 in WT cells but not in DYRK1A TG cells (Fig. 4D). In addition, proliferation (supplemental Fig. S6B) and differentiation (Fig. 4E) of calvarial osteoblasts were significantly impaired in DYRK1A TG mice. The inhibition of NFATc1 by a calcineurin inhibitor cyclosporine A resulted in a significant attenuation of osteoblast differentiation, suggesting a role for NFATc1 in osteoblasts (supplemental Fig. S6C). A real-time PCR analysis revealed that the
mRNA expression of osteoblast differentiation markers, including Bglap1, Alpl, and Col1a1, was significantly reduced in DYRK1A TG osteoblasts (Fig. 4F). Interestingly, however, the expression of Tnfsf11 was consistently higher in TG than in WT osteoblasts. DYRK1A TG osteoblasts formed less mineralized nodules (Fig. 4G). In vivo bone formation, measured by the distance between the two fluorescence labels after sequential labeling of femurs with calcein and xylene orange, was visibly impaired in TG mice (Fig. 4H). Accordingly, mineral apposition rate was significantly lower in TG mice (supplemental Fig. S6D). However, presumably due to the compensatory effect of increased RANKL (encoded by Tnfsf11) expression in TG osteoblasts, impaired TG osteoblast function by extra DYRK1A did not affect osteoclastogenesis in an osteoblast-BMM coculture system (Fig. 4I and supplemental Fig. S6E). Taken together, DYRK1A plays a negative regulatory role during normal osteoblast differentiation. In DYRK1A TG mice, impaired osteoblast function seemingly overrode impeded osteoclast function, resulting in the reduced bone mass.

Protection against LPS- and Ovariectomy-induced Bone Loss in DYRK1A TG Mice—To examine the effect of extra DYRK1A on bone resorption in TG mice, we adopted an LPS challenge and ovariectomy model that emulates inflammation- and hormone deprivation-induced bone loss. A three-dimensional visualization of distal femoral area showed massive loss of trabecular bone following LPS treatment in WT mice (Fig. 5A). On the other hand, no further significant loss of trabecular bone was observed in DYRK1A TG mice challenged with LPS. This was clearly demonstrated when bone volume was analyzed from microCT data (Fig. 5B). The BV/TV was 9.2 ± 0.2% for the PBS-treated group and 7.3 ± 0.2% for the LPS-treated group in WT mice. However, there was no statistical difference in the bone volume of femurs between PBS- and LPS-challenged TG mice (8.1 ± 0.2% for the PBS-treated group and 8.0 ± 0.2% for the LPS-treated group). Analyses of trabecular thickness and trabecular number also revealed that LPS did not induce trabecular bone loss in DYRK1A TG mice (supplemental Fig. S7, A and B). Consistent with the microCT results, histological analyses revealed significant loss of trabecular bone accompanied by increased TRAP activity in femoral sections from LPS-treated WT mice compared with PBS-treated groups (Fig. 5C). On the other hand, no such bone loss and increase of TRAP-positive osteoclasts were observed in DYRK1A TG mice upon LPS treatment. Assessment of the osteoclast and osteoblast numbers also indicated that DYRK1A TG mice were protected from LPS effects (Fig. 5D and supplemental Fig. S7C). A three-
dimensional reconstitution of microCT images (supplemental Fig. S8A) and an analysis of microCT data (Fig. 5E) from ovariectomized mice also revealed that ovariectomy reduced bone volume by more than 30% in WT mice. The BV/TV was 15.6 \pm 0.5% in sham-operated and 10.4 \pm 0.6% in ovariectomized WT mice. However, the femoral bone volume of \textit{DYRK1A} TG mice was not affected by ovariectomy (13.5 \pm 0.1% in sham-operated and 13.4 \pm 0.4% in ovariectomized mice) (Fig. 5E). Analyses of trabecular thickness (supplemental Fig. S8B) and trabecular number (supplemental Fig. S8C) also indicated that ovariectomy resulted in a significant trabecular bone loss only in WT mice. Histological analyses were consistent with the microCT results, showing no increase of TRAP-positive osteoclasts and bone loss in \textit{DYRK1A} TG mice following ovariectomy (supplemental Fig. S8D). The number of osteoblasts in sham-operated and ovariectomized TG mice was significantly smaller than that of corresponding WT mice (supplemental Fig. S8E). Although osteoclast number per bone perimeter dramatically increased following ovariectomy in WT mice, the increase was insignificant in TG mice (Fig. 5F). Taken together, these results suggest that \textit{DYRK1A} TG mice are apparently resistant to LPS- and ovariectomy-induced bone loss.

**DISCUSSION**

In the present report, we investigated the role of \textit{DYRK1A} in bone homeostasis. Intriguingly, in an effort to delineate signaling pathways responsible for the induction of \textit{DYRK1A}, we discovered that NFATc1 stimulated the expression of \textit{DYRK1A} in both osteoclast precursors and osteoblasts. Since NFATc1 activity and expression were inhibited by \textit{DYRK1A}, it is likely that there is a negative feedback mechanism. In this hypothesis, increased NFATc1 upon pro-osteoclastogenic signals in osteo-
Osteoclast precursors induce DYRK1A expression, which in turn attenuates NFATc1 activity and expression, thereby limiting excessive osteoclastogenesis. DYRK1A inhibited NFATc1 nuclear localization and activity via phosphorylation. Importantly, DYRK1A also reduced NFATc1 promoter activity, suggesting a crucial role for DYRK1A in the regulation of NFATc1 autoamplification process (22). The schematic diagram in Fig. 6, A and B, depicted this DYRK1A-dependent negative feedback regulation of osteoclastogenesis. Similarly, bone formation-inducing signals, such as BMP-2, induce NFATc1 expression in osteoblasts. Enhanced DYRK1A by NFATc1 turns down NFATc1 signaling to prevent uncontrolled bone formation. Thus, the DYRK1A-NFATc1 negative feedback loop might serve as a mechanism to maintain bone homeostasis and to prevent pathologic bone destruction or bone formation.

The role of DYRK1A in Down syndrome has been of particular interest due to its location on human chromosome 21q22.2 region, denoted as the “Down syndrome critical region,” which is believed to contain crucial genes for Down syndrome pathogenesis (23). The BAC DYRK1A TG mice used in this study have benefits as a clinically relevant model because these mice carry only one copy of human DYRK1A under the control of the endogenous promoter region (see the supplemental material for detailed discussion). As a result, these mice exhibited ~1.5-fold overexpression of DYRK1A protein, providing an optimal model to study the role of DYRK1A in Down syndrome pathogenesis (6, 19, 24). Several groups have reported that the bone mineral density in Down syndrome patients is significantly lower than that in normal population. Importantly, the reduced bone density was observed in both males and females even in young age groups (25–29). This was in sharp contrast with the prevalence of osteoporosis in elderly women in normal population. However, the etiology of osteoporosis among Down syndrome patients has remained ambiguous and under debate until the present. The current explanations range from the existence of an additional gene(s) on extra chromosome 21 to malnutrition and sedentary lifestyle among Down syndrome patients. Here we showed for the first time that the 1.5-fold overdose of DYRK1A alone was sufficient to induce significant reduction in bone volume (21% in male mice and 13% in female mice) (Fig. 3A), reminiscent of the Down syndrome-associated osteoporosis. Inhibition of NFATc1 activity by additional DYRK1A resulted in a compromised osteoblast proliferation, differentiation, and mineralization, supporting a suggested

**FIGURE 5.** Protection against LPS- and ovariectomy-induced bone loss in DYRK1A TG mice. A, 6-week-old male mice were challenged with PBS or LPS to induce bone loss. Femurs were subjected to microCT analyses, and representative three-dimensional reconstruction images of femurs (1-mm thickness starting from 2 mm below the growth plate) are shown. B, BV/TV was analyzed from microCT images by the CT-analysis program. C, sections of decalcified femurs were stained for TRAP activity and hematoxylin. D, the number of osteoclasts was counted from images in C. E, sections of decalcified femurs were stained for TRAP activity and hematoxylin. F, the number of osteoclasts was counted from femoral sections after TRAP staining. Results in B, D, E, and F are mean ± S.E. of 3 mice/group. **, p < 0.01; *, p < 0.05 versus PBS-treated WT. ‡, p < 0.01 between values linked by lines. NS, not significant.
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role of NFATc1 in osteoblastogenesis (30, 31). At the same
time, the proliferation, differentiation, and function of osteo-
clasts were also diminished in Dyrk1a TG mice. From these
results, we suggest that extra DYRK1A in Dyrk1a TG mice
and Down syndrome patients sets a new equilibrium for
NFATc1 signaling in bone homeostasis, reducing bone turn-
over (Fig. 6C). In concert with this hypothesis, we observed
consistently lower numbers of osteoblasts and osteoclasts in
bones from Dyrk1a TG mice in accordance with the reported
"adynamic" bone phenotype in Down syndrome patients (32).
Notably, the Down syndrome critical region also contains
dscr1, which encodes a calcineurin inhibitor. As a result, the
phosphorylated state of NFATc1 might be further sustained by
inhibition of calcineurin (16), possibly magnifying the effect of
DYRK1A on NFATc1 activity and bone homeostasis in Down
syndrome.

Historically, it was of great interest to search for a gene(s) on
human chromosome 21 that is responsible for specific Down
syndrome pathogenesis. In this context, the possible involve-
ment of Dyrk1a in Down syndrome skeletal anomalies in
addition to the osteoporotic bone phenotype might be worthy of pur-
suit. Notably, however, we could not observe significant gross malforma-
tions in the skeletal system, such as a craniofacial defect in Dyrk1a TG
mice, in contrast to Down syn-
drome patients and trisomy-16
mice (33, 34). Indeed, mice trisomic
only for the Down syndrome critical
region did not show a distinctive
facial phenotype (35), suggesting
that genes in this region may not be
involved. Alternatively, mice over-
expressing Ets2 that is located out-
side of the Down syndrome critical
region had defects in cranial and
cervical skeleton formation (36).
However, NFATc1, a substrate of
DYRK1A, has been linked to cranio-
facial anomalies in mice. Recent
reports observed craniofacial mal-
f ormations in Nfatc1−/− mice that
express the constitutive active form
of NFATc1 (31), viable Nfatc1−/−/−
mice (31), and Nfatc2−/−/−/− Nfatc4−/−
double knock-out mice (16), sug-
gest ing possible gene dosage-
dependent mechanisms for NFATc
proteins in the regulation of skeletal
development. Taken together,
although it is believed that the regu-
lation of NFATc1 by DYRK1A alone
is sufficient to control bone homeo-
stasis, skeletal development might
be governed by additional mecha-

Notwithstanding the deleterious effect on bone mass in
unchallenged Dyrk1a TG mice, extra DYRK1A was beneficial
in protecting these mice from experimental bone loss induced
by inflammation or hormone deprivation. As summarized in
Fig. 6C, bone destruction by LPS or ovariectomy was prevented
because the negative-regulation of NFATc1 reduced osteoclas-
togenesis in Dyrk1a TG mice. In efforts to develop a strategy
to prevent osteoporosis, NFATc1 has been suggested as a target
molecule due to its role in osteoclastogenesis (22, 37). We sug-
gest that targeting DYRK1A instead of directly modulating
NFATc1 might provide an efficient strategy for treating bone-
destructive diseases, because only a 50% increase in the
DYRK1A protein level was enough to completely prevent bone
loss in animal models.

To summarize, we have discovered a negative feedback
mechanism for Dyrk1a-NFATc1 regulation, which affects
bone homeostasis. Our results provide an insight into the
novel role for Dyrk1a in the regulation of bone mass in

FIGURE 6. Regulation of bone mass by Dyrk1a. A, during the early phase of osteoclastogenesis, binding of
RANKL to RANK on osteoclast precursors initiates signaling events, including the activation of calcineurin.
Dephosphorylated NFATc1 translocates to nucleus and stimulates the induction of osteoclastogenic genes,
including Nfatc1. NFATc1 also initiates Dyrk1a transcription. B, increase in the Dyrk1a protein during osteo-
clastogenesis results in the phosphorylation of NFATc1 and inhibition of nuclear translocation, thereby reduc-
ing the transcription of osteoclastogenic genes, including Nfatc1. Similar negative feedback regulation of
NFATc1 by Dyrk1a is also observed during BMP-2-dependent osteoblastogenesis. C, Dyrk1a TG mice show
osteoporotic phenotype due to the reduced bone turnover. However, these mice are protected from the
pathologic bone destruction by inflammation or hormone deprivation.
normal and pathologic conditions, including Down syndrome-associated osteoporosis, as well as prospects for therapeutic applications for bone-destructive diseases.

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