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Essential role of RSK2 in c-Fos–dependent osteosarcoma development

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Introduction

Bone integrity depends on the proper balance between the activity of the bone-forming cells, osteoblasts, and the bone-resorbing cells, osteoclasts (1). c-Fos, a member of the heterodimeric transcription factor complex AP-1, has been shown to play key roles in modulating both osteoclast differentiation and osteoblast transformation (2, 3). Mice lacking c-Fos are osteopetrotic because of a complete block of osteoclast differentiation (4, 5). In contrast, mice overexpressing c-Fos develop osteosarcomas, a tumor affecting the osteoblastic lineage (6). Although not essential for osteoblast differentiation, c-Fos is expressed in osteoblasts, and osteogenic factors such as parathyroid hormone (PTH) or parathyroid hormone–related protein (PTHrP) transcriptionally induce c-fos mRNA (7, 8).

In addition to the transcriptional control, c-Fos is subject to extensive posttranslational modifications. Phosphorylation of c-Fos on threonine 325 by a not fully characterized kinase called Fos-regulating kinase (FRK) can stimulate its transcriptional activity (9). The role for ERK activation in the posttranscriptional regulation of c-Fos is better characterized. In vitro, both ERK and the ERK/P38–dependent kinase RSK2 can phosphorylate the C-terminal domain of c-Fos on serines 374 and 362, respectively. These 2 phosphorylation events stabilize the protein, allowing subsequent phosphorylation by ERK on threonine 325 and threonine 331, thereby modulating c-Fos–induced cell transformation (10–12). Recently, IFN-β has been shown to inhibit osteoclast differentiation by posttranscriptionally repressing c-Fos expression in osteoclast progenitors (13). Thus, c-Fos activity can be both transcriptionally and posttranscriptionally regulated in osteoblasts and in osteoclasts.

The putative c-Fos kinase RSK2 belongs to the group of broadly expressed protein kinases called ribosomal S6 kinases. RSKs are composed of 2 functional kinase domains connected by a linker, which are sequentially activated by ERK and by the PI3K–dependent kinase PDK1 (14). In addition to directly phosphorylating c-Fos, RSK2-mediated CREB phosphorylation has been shown to be required for c-fos transcriptional regulation in response to growth factor stimulation (15, 16). Furthermore, RSK2 appears to be involved in chromatin remodeling associated with the activation of the c-fos promoter by EGF (17). Importantly, Coffin-Lowry syndrome (CLS), a human X-linked mental retardation disorder associated with progressive skeletal abnormalities, is due to inactivating mutations of RSK2 (18). These observations prompted us to study the function of RSK2 in bone development and to investigate the functional relevance of the posttranscriptional modifications of c-Fos by RSK2 to osteoclast differentiation and to osteosarcoma formation.

Results

c-Fos phosphorylation by RSK2 appears to be dispensable in osteoclasts. Since serine 362 in the C-terminal domain of c-Fos has been proposed to be an RSK2 phosphorylation site essential for c-Fos transactivation activity, we first generated an antibody directed against c-Fos phosphorylated on serine 362 (pho–serine 362). This antibody recognized a band corresponding to the molecular weight of c-Fos in 3T3 fibroblasts. The reactive band was also detected when the adjacent serine 374 was mutated to alanine, but it was absent when serine 362 was mutated to alanine (Figure 1A). This antibody recognized a band corresponding to the molecular weight of c-Fos in 3T3 fibroblasts. The reactive band was also detected when the adjacent serine 374 was mutated to alanine, but it was absent when serine 362 was mutated to alanine (Figure 1A). Reblotting with a specific anti–c-Fos antibody confirmed that the protein recognized by the anti–phospho–serine 362 was indeed c-Fos (Figure 1A). Immunostaining of M-CSF–dependent bone marrow monocytes (M-BMMs) further showed that c-Fos was phosphorylated on serine 362 in response to M-CSF (Figure 1B). Moreover, using immunoprecipitation
RSK2, residual c-Fos phosphorylation on serine 362 was detected (Figure 1D). RNase protection assays demonstrated that c-fos transcriptional regulation by M-CSF was not altered in M-BMMs lacking RSK2 (Figure 1E). These data indicate that RSK2 contributes to but is not essential for the transcriptional and posttranscriptional regulation of c-Fos expression in osteoclast progenitors. As a consequence, Rsk2 deficiency had no effect on osteoclast differentiation or on the resorbing activity, when osteoclast differentiation was induced by M-CSF and RANKL in vitro (Figure 1F and data not shown).

In vivo osteoclastogenesis is known to be supported by cells of the osteoblastic lineage that synthesize osteoclastogenic molecules such as RANKL. We therefore analyzed the osteoclastogenic properties of Rsk2-deficient osteoblasts. No change in osteoclast differentiation could be observed when wild-type bone marrow cells were cocultured with primary osteoblasts isolated from the calvaria of newborn Rsk2−/− mice compared with osteoblasts from wild-type littersmates (data not shown). Thus, RSK2 is essential neither for osteoclast differentiation nor for the osteoclastogenic function of osteoblasts in vitro.

RSK2 regulates bone mass. Since humans carrying inactivating mutations of RSK2 suffer from multiple bone defects, we studied the role of RSK2 in bone development in vivo by performing histomorphometric analyses of male and female Rsk2-deficient mice. While no change in bone mass was detectable in the absence of Rsk2 in 2- and 4-week-old males, we observed a significant decrease in bone mass in older mice (Figure 2A). A similar phenotype was also present in females, where the bone defect was first observed at 4 weeks of age (data not shown). No differences in the number of osteoclasts were detected, and osteoclast differentiation was also unaffected, as shown by similar numbers of osteoblasts and osteocytes found in 7-month-old Rsk2−/− wild-type littermates (Figure 2B). The low-bone-mass phenotype was of functional importance, since a significant decrease in the diameter of the long bones and a decrease in biomechanical competence of Rsk2-deficient bones were measured (Figure 2C). Dynamic histomorphometry demonstrated a substantial decrease in bone-formation rate in these mice (Figure 2D), whereas no defect in resorption by osteoclasts was detected by measurement of the amount of deoxypyridinoline in the urine (data not shown). Finally, no change in the width and in the organization of the growth plate was observed in Rsk2-deficient mice, which indicates that the bone defect is not secondary to a developmental defect affecting chondrocyte differentiation (Figure 2E). These data suggest that Rsk2-deficient mice are osteopenic because of a functional defect of osteoblasts.

We next analyzed the expression of markers of osteoblast differentiation in adult mice by in situ hybridization. The expression of
type I collagen, osteocalcin, and osteopontin was not affected by the absence of RSK2 in the growth plate, either in the trabecular bone of the primary spongiosa or in the cortex (Figure 3A and data not shown). In contrast, the expression of Phex, an endopeptidase involved in the regulation of mineralization (21, 22), was undetectable in the cortex of Rsk2-deficient mice (Figure 3A). To determine whether decreased Phex expression could in part explain the bone defect observed in the absence of RSK2, we compared the mineralization in the cortex of Rsk2−/− mice with that in the cortex of Hyp−/− mice carrying an inactivating mutation of Phex (23). A significant proportion of bone matrix remained unmineralized in the cortex but not in the trabecular bone of Rsk2-deficient mice (Figure 3B). A similar but more severe mineralization defect, which was also more pronounced in the cortex than in the trabecular bone, was observed in Hyp−/− mice (Figure 3B and data not shown). However, no significant changes in phosphate contents were detected in the serum and urine of Rsk2-deficient mice (data not shown), which indicates that the mineralization defect is not secondary to a defect in renal phosphate reabsorption but is likely due to decreased Phex expression in cortical bone.

To analyze the cellular mechanisms underlying the osteoblast defect in Rsk2-deficient mice, primary osteoblasts were isolated from wild-type and Rsk2-deficient littersmates. Treatment of primary osteoblasts with known bone anabolic molecules such as IGF-1, PTH 1–34, and with BMP2 resulted in a rapid activation of RSK2 and ERK (Figure 3C and data not shown). No difference in cell doublings and Ki67 staining was observed (Figure 3, D and E), and TUNEL staining revealed no changes in apoptosis between wild-type and mutant osteoblasts (Figure 3E). However, in the absence of RSK2, alkaline phosphatase activity was significantly affected, and a drastic reduction in the number of mineralized bone nodules as measured by alizarin red staining was observed (Figure 3F). Moreover, the induction of mineralization by osteoblasts stimulated by IGF-1 was impaired in the absence of RSK2 (Figure 3G). These data indicate that the osteopenia in Rsk2-deficient mice is likely due to a cell-autonomous functional defect of osteoblasts, rather than an effect on osteoblast proliferation or survival.

RSK2 is essential for development of c-fos-induced osteosarcomas. The osteoblastic phenotype observed in Rsk2-deficient mice and the fact that RSK2 can phosphorylate c-Fos on serine 362 suggested that c-Fos phosphorylation by RSK2 could be involved in the development of c-Fos–induced osteosarcomas. Therefore, we generated c-fos transgenic mice lacking RSK2 (Figure 4A). As previously described, H2-c-fosLTR transgenic mice develop osteosarcomas in all bones of the body (6). Interestingly, osteosarcomas were initially also detected in all bones of H2-c-fosLTR/Rsk2−/− mice (Figure 4, B and C). Whereas osteosarcomas increased in size with age in H2-c-fosLTR mice, the tumor area did not significantly increase in mice lacking RSK2 (Figure 4B). Microcomputed tomography and quantitative analyses confirmed that, while the tumor incidence was similar in H2-c-fosLTR and H2-c-fosLTR/Rsk2−/− mice, the tumor burden was drastically reduced in the absence of RSK2 (Figure 4C). Moreover, von Kossa staining revealed that the mineralization inside and outside the tumors was severely impaired, confirming the function of RSK2 in the regulation of bone mineralization (Figure 4D). These data indicated that, while overexpression of c-Fos in osteoblasts does not overcome the RSK2-dependent bone mineralization defect, RSK2 activity is essential for c-Fos-
mediated tumor development. It is worth noting that, despite unchanged expression of RANKL and OPG, the small tumors lacking RSK2 had decreased numbers of osteoclasts (Supporting Figure 1; supplemental material available online with this article; doi:10.1172/JCI200522877DS1), whereas increased osteoclast numbers were proposed to be involved in the development of c-Fos–induced osteosarcomas (6, 24). This suggests that active bone resorption is necessary for the progression of osteosarcomas.

It has recently been shown that long-term treatment with PTH might lead to the development of osteosarcomas in rats (25). Therefore, we measured the level of circulating PTH in H2-c-fos LTR, Rsk2-deficient, and wild-type mice. We did not detect any difference in PTH levels in the blood of c-fos transgenic mice or Rsk2-deficient mice compared with wild-type mice (data not shown). Thus, the development and the progression of osteosarcomas in c-fos transgenic mice are not due to increased circulating PTH levels.

Progression of osteosarcomas requires RSK2-dependent c-Fos posttranscriptional regulation. We next assessed whether decreased tumor formation in the absence of Rsk2 was due to a decrease in c-Fos expression in vivo. RSK2 was found to be expressed in H2-c-fosLTR transgenic tumors but was absent in tumors from H2-c-fosLTR/Rsk2–/y mice (Figure 5A). Importantly, c-Fos was detected in tumors from H2-c-fosLTR transgenic mice and was found phosphorylated on serine 362 (Figure 5A). In contrast, c-Fos expression was drastically decreased and no phosphorylation on serine 362 was detected in tumors isolated from H2-c-fosLTR/Rsk2–/y mice (Figure 5A). To better characterize the cellular mechanisms that could explain the reduction of c-Fos–induced osteosarcomas in the absence of Rsk2, we quantified proliferation and apoptosis within the tumors. Ki67 staining revealed that cell proliferation was decreased in tumors from H2-c-fosLTR/Rsk2–/y mice compared with control littermates (Figure 5B). Furthermore, TUNEL staining demonstrated increased numbers of apoptotic cells in Rsk2-deficient osteosarcomas (Figure 5B). We next analyzed c-Fos phosphorylation on serine 362 in c-Fos–dependent osteosarcomas. Western blot analysis indicated that c-Fos was expressed and phosphorylated on serine 362 in exponentially growing wild-type primary osteoblasts even upon IGF-1 or PTH treatment.
littermates (Figure 5C). Northern blot analyses demonstrated that dent osteosarcomas in the absence of RSK2 (Figure 5B). Thus, impaired development of c-Fos–dependent osteosarcomas of c-Fos–transformed osteoblasts (Figure 5F), which is similar to the in vivo situation. Impaired development of c-Fos–dependent osteosarcomas in the absence of RSK2 likely results from the combined effect of decreased proliferation and increased apoptosis of c-Fos–transformed osteoblasts. RSK2 is essential for c-Fos–induced tumor progression. (A) X-ray analysis of 7-month-old H2-c-fosLTR and H2-c-fosLTR/Rsk2−/− littermates. Histological analyses of bone sections from vertebral bodies and tibiae. White arrowheads indicate the osteosarcomas. Magnification, ×25. (B) Kinetic analysis of the development of osteosarcomas in H2-c-fosLTR/Rsk2−/− and H2-c-fosLTR/Rsk2−/− littermates; n indicates the number of tumors measured in tibiae at the indicated age. (C) Top: Microcomputed tomography analysis of vertebral bodies of 7-month-old H2-c-fosLTR/Rsk2−/− and H2-c-fosLTR/Rsk2−/− littermates. Magnification, ×5. Bottom: Quantification of tumor incidence (percentage of affected vertebrae) and tumor burden (tumor volume relative to tissue volume) in vertebral bodies; n indicates the number of tumor-affected vertebrae measured for tumor burden. (D) Top: Analysis of osteoid volume in tumors of wild-type and Rsk2−/− littermates. Magnification, ×200. Bottom: Quantification of the osteoid volume in the tumors and in the cortex. *P < 0.05.

Discussion

The growth factor–regulated ribosomal S6 kinase RSK2 appears to be essential for osteoblast function, since mice lacking RSK2 are osteopenic because of a cell-autonomous defect in osteoblast activity. In contrast, osteoclast differentiation was unaffected in vivo and in vitro in the absence of RSK2. However, the development of c-Fos–induced osteosarcomas was found to be dependent on RSK2-mediated c-Fos posttranscriptional regulation. Thus, important functions of c-Fos are determined by RSK2-mediated posttranscriptional mechanisms, which may also be required in human osteosarcomas and in CLS. Humans carrying mutations of RSK2 suffer from CLS, an X-linked mental retardation condition associated with progressive skeletal deformities and osteopenia, delayed bone age, and delayed fontanelle closure (18). It has been shown that mice lacking RSK2 also have impaired learning and cognitive functions (19). Interestingly, mice lacking c-fos in the CNS have similar learning and behavioral deficits (27). Moreover, Rsk2-deficient mice are osteopenic, and it has been shown that some of the skeletal phenotypes observed in CLS are caused by the lack of phosphorylation of AT4 by RSK2 (20). We now demonstrate that the osteopenia in Rsk2-deficient mice is due to a postdevelopmental defect in osteoblast activity. In adult Rsk2-deficient mice, the expression of osteoblast markers of differentiation was not affected, whereas the expression of the mineralization marker Phex was abolished in cortical bone. The Phex mutation in humans is responsible for the development of X-linked hypophosphatemic rickets and of adult-onset vitamin D–resistant hypophosphatemic osteomalacia (21, 22). These diseases
Bone mineralization of Rsk2-deficient osteoblasts was affected in vivo and in vitro. Phex is mainly expressed in osteocytes and in selected osteoblasts (29), and its expression in bone is known to decrease with age, reaching a very low level in the adult (30, 31). It is conceivable that decreased Phex expression might be responsible for decreased mineralization observed in the cortical bones of Rsk2-deficient mice. Whether RSK2-dependent Phex expression is also involved in the reduced bone mass and reduced bone formation in the trabecular bone remains to be established. It has recently been suggested that the reduced bone mass in Rsk2-deficient trabecular bone is caused by decreased type I collagen synthesis (20). These data were supported by in vitro studies showing a posttranscriptional decrease in type I collagen synthesis, when Rsk2- or ATF4-deficient osteoblasts were cultured in media lacking nonessential amino acids (20). While a possible role for altered Phex expression in CLS patients or impaired RSK2 activation in the development of X-linked hypophosphatemia should be explored in future experiments, these data provide substantial evidence that the progressive bone defect associated with CLS is most likely due to a cell-autonomous defect in the function and not in the differentiation of osteoblasts. The development of osteosarcomas in c-fos transgenic mice and the expression of c-Fos in human osteosarcomas (32, 33) suggest that c-Fos could be the substrate...
mediating RSK2 function in osteoblasts. However, c-Fos is likely not the main substrate mediating RSK2 function in osteoblasts, since c-fos–deficient osteoblasts appear to be functional (34). Moreover, c-Fos protein was barely detectable in exponentially growing and in IGF-1– or PTH-stimulated primary osteoblasts, while osteogenic molecules can induce c-fos transcription in osteoblasts (7, 8). Importantly, proliferation and cell death observed in c-Fos–induced osteosarcomas were dependent on RSK2, although the absence of RSK2 did not affect these parameters in nontransformed osteoblasts. In addition, overexpression of c-Fos did not rescue the RSK2-dependent osteopenia outside the tumors. Whereas c-Fos phosphorylation by RSK2 may not be important for normal osteoblast function, RSK2 is essential for osteosarcoma development to maintain high levels of c-Fos protein. The c-Fos protein is known to be unstable and can be rapidly degraded by a proteasome-dependent mechanism (35), although it can be stabilized by posttranscriptional modification. In particular, the C-terminal domain of c-Fos has been shown to be important for protein stability (36). Replacement of serine 362 and serine 374 in the c-Fos C-terminal domain with the phospho-mimetic amino acid aspartic acid protected c-Fos from being degraded, while mutation of these serines to the nonphosphorylatable amino acid alanine decreased c-Fos stability (11, 12, 36). Importantly, c-Fos protein, which was nearly undetectable in vivo in Rsk2-deficient osteosarcomas as well as in cells isolated from bone tumors of c-fos transgenic mice lacking RSK2, was posttranscriptionally regulated by proteasome-mediated degradation. These data suggest that in transformed osteoblasts c-Fos stabilization can be achieved via an RSK2-dependent mechanism. It remains to be determined whether both phosphorylation sites are necessary for c-Fos stabilization as described for fibroblasts (10, 11).

The efficiency of c-Fos–dependent osteosarcoma formation was earlier reported to be determined by the levels of c-Fos (24). Moreover, c-Fos has been demonstrated to be necessary for the malignant progression but not for the initiation of skin tumors induced by v-Ha-ras, a strong activator of the ERK pathway (37). We now show that reduced levels of c-Fos affect not the initiation but the progression of osteosarcomas. Increased cell proliferation and reduced cell death were found to be dependent on RSK2 and correlated with c-Fos phosphorylation on serine 362 in osteosarcomas, thereby accounting for the oncogenic activity of c-Fos. The absence of an apparent osteoclast defect in Rsk2-deficient mice and the residual phosphorylation on serine 362 in Rsk2-deficient osteosarcomas suggest that the decreased osteoclast numbers in c-Fos–induced osteosarcomas are not mediated via c-Fos phosphorylation by RSK2. Moreover, Rsk2 deficiency did not significantly modify the expression of RANKL and its decoy receptor OPG, both in vivo and in vitro cultures of primary osteoblasts. Therefore, decreased c-Fos levels and the reduced bone formation in the tumor may affect the expression of factors within the tumor, which could account for the observed decrease in osteoclast numbers.

These data demonstrate important roles for RSK2 in normal bone development as well as in c-Fos–dependent osteosarcoma formation. Further analyses of osteoblasts isolated from Rsk2-deficient mice and CLS patients will be required to identify additional RSK2 targets other than ATF4 and c-Fos that are responsible for the observed bone defects. The design of small molecules positively regulating RSK2 activity might allow the generation of therapeutic tools for the treatment of diseases affecting bone remodeling due to decreased osteoblast activity, whereas molecules inhibiting RSK2 activity could be used to treat human osteosarcomas.

**Methods**

Mice. All animal studies were approved by the Magistratsabteilung 58, Vienna, Austria. Rsk2 knockout mice (20) were maintained on a C57BL/6J background, and littermates were used for analyses. Homozygous H2-c-fos-LTR transgenic mice (32) were crossed with the Rsk2 knockout mice, and most analyses were done on males heterozygous for the fos transgene in the presence or absence of Rsk2.

Cell culture. Primary osteoblasts and osteoclasts were cultured as previously described (38, 39). Bone nodule mineralization was analyzed at day 21 of the osteoblast culture by alizarin red staining (Sigma-Aldrich) or staining for alkaline phosphatase activity (ALP kit; Sigma-Aldrich). M-BMMs were generated as previously described (39) in the presence of M-CSF (20 ng/ml) (R&D Systems Inc.). To induce osteoclast differentiation in coculture, total bone marrow cells (10⁶ cells per well) were plated with primary osteoblasts (10⁵ cells per well) in 24-well plates in α-modified MEM. The differentiation was induced by addition to the media of dexamethasone (10⁻⁷ M) and d-ilhydroxyvitamin D₃ (1,25(OH)₂D₃) (10⁻⁸ M). Immortalized osteosarcoma-derived cell lines and subclones were described elsewhere (6). Primary cells were isolated according to the same procedure from long bone tumors of 10-week-old H2-c-fosLTR/ Rsk2⁻/⁻ and H2-c-fosLTR/Rsk2⁺/⁺ littermates.

Western blot and immunoprecipitation. Western blot analyses were performed as previously described (39), and the blots were probed with anti–phospho-RSK2, anti–c-Fos (EMD Biosciences Inc. or Santa Cruz Biotechnology Inc.), or anti-β-actin (Sigma-Aldrich). The polyclonal anti–phospho–serine 362 c-Fos antibodies were generated against phosphorylated c-Fos C-terminal peptides. For immunoprecipitation, total cell extracts were incubated overnight with a mix of an anti-RSK2 antibody (Santa Cruz Biotechnology Inc.) and corresponding agarose-conjugated secondary antibody beads (Sigma-Aldrich).

Immunostaining and immunohistology. For immunostaining, M-BMMs were grown on coverslips, fixed, and stained as previously described (40). Immunoreactivity was revealed using Alexa Fluor–coupled secondary antibodies (Invitrogen Corp.), and DNA was counterstained with DAPI. For immunohistology, 5-μm-thick sections of paraffin-embedded, decalcified bones were incubated overnight at 60°C, deparaffinized, and boiled twice in antigen-retrieval buffer (DakoCytomation) in a microwave oven (500 watts for 5 minutes). Nonspecific staining was blocked using PBS-containing 2% BSA and 10% normal goat serum.

Proliferation and apoptosis assays. For proliferation curves, triplicate cultures of calvarial osteoblasts were seeded in medium containing 10% FCS, and cells were counted at the indicated time points. Proliferation indices were determined in vivo or in vitro by immunofluorescence staining using antibodies directed against Ki67 (Novocastra Laboratories Ltd.). Apoptotic indices were determined by TUNEL staining using the In Situ Cell Death Detection Kit (Roche Diagnostics GmbH).

RNase protection assay, Northern blotting, and RT-PCR. Total RNA was isolated using TRizol reagent (Invitrogen Corp.). c-Fos expression was analyzed using the RNase protection kit mFos/Jun protooncogene template set according to the manufacturer’s instructions (BD Biosciences — Pharmingen). For Northern blotting, mRNA was purified using the Oligotex Kit (QIAGEN GmbH), and Northern blotting was performed as already described (6). RT-PCR was performed using the following primers: for RANKL, ACCAGCATCAAATCTCCCAAG and GGAAGGGAACAGTAGC; for OPG, GGAACCCGAGGAGGACACAGT and CTTCTCCAGCCAGGCTTCCCAT; for β-tubulin, CAACGT-CAAGACGCGGTGTTG and GACAGAGGCAAACGTGACCC.

In situ hybridization. In situ hybridization was performed on sections of decalcified long bones of 2-month-old mice according to standard protocols. Digoxigenin-labeled riboprobes of type I collagen, osteocalcin, osteopontin, and Phex were hybridized overnight at 65°C. Alkaline phosphatase–conjugated anti-Digoxigenin (Roche Diagnostics GmbH) incubation was performed overnight at 4°C in a humidified chamber. The signal was visualized using BM Purple AP STRAP (Roche Diagnostics GmbH).

Histology and histomorphometry. After whole-animal contact radiation (Faxitron Cabinet X-Ray System; Faxitron X-Ray Corp.), bones were dissected out and fixed in 3.7% formaldehyde. After dehydration, undecalcified spines and tibiae were embedded in methyl methacrylate, and 5-μm sections were cut in sagittal plane on a rotation microtome. Histomorphometry was performed as previously described (41). For comparative histomorphometry, samples from Rsk2−/− males, Rsk2−/− females, and sex-matched wild-type mice of the indicated ages were used. Analysis of bone volume (percentage), osteoclast number per bone perimeter (per millimeter), osteocyte number per bone matrix area (per square millimeter), osteoclast number per bone perimeter (per millimeter), cortical thickness (microns), and bone formation rate (cubic micrometers per square micrometer per year) was carried out according to standardized protocols (42) using the OsteoMeasure histomorphometry system (OsteoMetrics Inc.). For the determination of tumor burden, sections of 6 vertebrae of the H2-c-fosLTR/Rsk2−/− group and 8 vertebrae of the H2-c-fosLTR/Rsk2−/− group were evaluated for tumor area per tissue area (ImageTool; The University of Texas Health Science Center at San Antonio). Statistical analysis was performed using unpaired, 2-sided t test; P < 0.05 was accepted as significant. Error bars represent the SD. Microcomputed tomography (μCT) analysis was used for 3D histomorphometry and visualization of the vertebral bone structure. Lumbar vertebra L6 of 7-month-old mice was scanned in a μCT 40 (SCANCO Medical AG) at a resolution of 6 μm. Raw data were manually segmented, analyzed with μCT Evaluation Program version 4.4A, and displayed in μCT Ray version 3.0 (SCANCO Medical AG). To assess the femoral thickness, 15 planes per femur were scanned at the midshaft. Longitudinal femoral thickness was measured with the Distance3D tool of μCT Evaluation Program version 4.4A. Tumor incidence was evaluated by scanning of 20 tail vertebrae per group. For biomechanical testing, femurs were dissected and a 3-point bending was performed as previously described (41).

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