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Stk11 (Lkb1) deletion in the osteoblast lineage leads to high bone turnover, increased trabecular bone density and cortical porosity

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Abstract

The mTOR pathway couples energy homeostasis to growth, division and survival of the cell. Stk11/Lkb1 is a critical serine-threonine protein kinase in the inhibition of mTOR pathway action. In the mammalian skeleton, Stk11 regulates the transition between immature and hypertrophic chondrocytes. Here, we have focused on the action of Stk11 in the osteoblast lineage through osteoblast specific-removal of Stk11 activity. In the mouse model system, specification and primary organization of the neonatal boney skeleton is independent of Stk11. However, histological, molecular and micro-CT analysis revealed a marked perturbation of normal bone development evident in the immediate post-natal period. Cortical bone was unusually porous displaying a high rate of turnover with new trabeculae forming in the endosteal space. Trabecular bone also showed enhanced turnover and marked increase in the density of trabeculae and number of osteoclasts. Though mutants showed an expansion of bone volume and trabecular number their bone matrix comprised large amounts of osteoid and irregularly deposited woven bone highlighted by diffuse fluorochrome labeling. Additionally, we observed an increase in fibroblast-like cells associated with trabecular bone in Stk11 mutants. Stk11 down-regulates mTORC1 activity through control of upstream modulators of the AMP kinase family: an increase in the levels of the phosphorylated ribosomal protein S6, a target of mTORC1-mediated kinase activity, on osteoblast

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removal of *Stk11* suggests deregulated mTORC1 activity contributes to the osteoblast phenotype. These data demonstrate *Stk11* activity within osteoblasts is critical for the development of normally structured bone regulating directly the number and coordinated actions of osteoblasts, and indirectly osteoclast number.

Keywords

Serine/threonine kinase 11(*Stk11/Lkb1*); osteoblast differentiation; histomorphometry; peritrabecular marrow fibrosis; woven bone; mTOR signaling

Introduction

Endochondral ossification underlies axial and appendicular bone growth. In this process chondrocytes prefigure the shape of the skeletal element, which is mineralized and remodeled by osteoblasts [1] and [2]. Given the interplay between osteoblasts and chondrocytes, there is a tight coordination of their developmental programs. In the long bones, *Sox9* is a key determinant of the initial stages of skeletal development and a critical regulator of the chondrocyte [3], [4] and [5]. Down-regulation of *Sox9* allows bipotential, mesenchymal progenitors to differentiate into osteoblasts under the influence of Indian hedgehog (*Ihh*) secreted by adjacent pre-hypertrophic chondrocytes. *Ihh* triggers a *Runx2*-driven pathway of osteoblast development in which canonical Wnt-signal-mediated activation of *Osterix (Ox)* is a critical step in progression of osteoblast progenitors to maturing osteoblasts [6], [7], [8], [9], [10] and [11].

Stk11 (also known as *Lkb1*) is a multi-functional serine/threonine kinase that regulates cell cycle progression, cellular energy homeostasis and cell polarity through activation of AMP kinases [12] and [13]. *Stk11* is a known tumor suppressor: germ-line inactivating mutations result in Peutz-Jeghers syndrome (PJS), characterized by benign polyps in the gastrointestinal tract and an elevated increase risk for a variety of epithelial cancers [14] and [15]. *Stk11* is also essential for embryonic development. Mouse mutants lacking *Stk11* die at mid-gestation with profound vascular and neural tube deficiencies [16]. Conditional ablation of *Stk11* in specific cell populations has identified tissue specific actions for *Stk11* in a number of tissues [17], including the developing skeleton. In the skeleton, chondrocyte specific removal of *Stk11* highlights a critical role for *Stk11* in suppressing mTORC1 signaling in the transition of chondrocytes to post-mitotic, hypertrophic fates, and in so doing, suppresses cartilage tumor formation [18].

Here, we have extended our exploration of *Stk11* action in mammalian skeletal development to the osteoblast. Osteoblast specific removal of *Stk11* highlights a critical role for *Stk11* in regulating bone turnover and both the organization and number of cortical and trabecular osteoblast and osteoclast populations, and the microstructure and quality of the osteoblast-generated bone matrix.

Materials and Methods

Animal breeding and animal procedures

As an initial step to generate an *Stk11* conditional (c) knockout in osteoblasts, *Osx-GFP::Cre* mice were mated with *Stk11^{c/c}* mice to obtain *Osx-GFP::Cre;Lkb1^{c/+}* driver males. Intercrossing these with a homozygous *Stk11^{c/c}* strain enabled the collection of experimental (*Osx-GFP::Cre; Lkb1^{c/c}*) and control genotypes as documented in the text. All experiments and procedures were performed with the approval of the Animal and Care and Use committees at Harvard University and the University of Southern California. Table 1 provides information on the experimental cohorts.

Skeletal staining, histology, and immunostaining

Skeletons were stained with alizarin red and alcian blue as described previously to visualize non-mineralized cartilage, and mineralized cartilage and bone matrices, respectively [19]. Hematoxylin and eosin staining, alizarin red staining and TRAP staining were performed according to standard protocols. Immunostaining was performed according to standard protocols: primary antibodies employed in the study are listed in Table 2. A Zenon kit (Zenon® Alexa Fluor® 647 Rabbit IgG Labeling Kit, Life Technologies) was used to distinguish two primary antibodies raised in the same species. For fluorescent visualization, secondary antibodies were conjugated with a variety of Alexa fluors (Life Technologies). For immunohistochemistry, antigen retrieval was performed with boiling citrate buffer. HRP-conjugated secondary antibodies, ABC kit and DAB substrate (Vector labs) were used for visualization.

Histomorphometry

Twenty-eight days old mice were subcutaneously injected with 40 mg/kg demeclocycline and 20 mg/kg calcein, 1 and 3 days before necropsy, respectively. Tibiae were dehydrated in acetone, infiltrated, and embedded without demineralization in methyl methacrylate. Histomorphometric analysis of longitudinal 5 μ m unstained and toluidine blue stained sections was carried out with the OsteoMeasure system (OsteoMetric) measuring across the width of the secondary spongiosa, 400 μ m below the growth plate. All parameters were expressed according to standardized nomenclature [20]. Osteoblast number per tissue area (N.Ob/T.Ar./mm²), osteoclast number per tissue area (N.Oc/T.Ar./mm²), and fibroblast surface per bone surface (Fb.S/BS, %) were quantified. Bone volume per tissue volume (BV/TV, %), trabecular thickness (Tb.Th, μ m), trabecular separation (Tb.Sp, μ m), and trabecular number (Tb.N./mm) were measured.

Micro-computed tomographic (micro-CT) imaging was performed on the distal metaphysis and mid-diaphysis of the femur of mice from each group using a high-resolution desktop imaging system (μ CT40, Scanco Medical AG, Bruttisellen, Switzerland). The methods used were in accordance with guidelines for the use of μ CT in rodents [21]. Transverse slices were acquired with 12 μ m³ isotropic voxel size, 70 kVp peak x-ray tube potential, 200 ms integration time, and were subjected to Gaussian filtration. The length of the region of interest (ROI) for each group was adjusted based on the average femur length for each genotype. Trabecular bone microarchitecture was evaluated in the distal metaphysis in a

region that began 240 μm (20 slices) above the peak of the distal growth plate and extended proximally 1.8 mm (150 slices) for the *Stk11^{c/c}* and *Osx-GFP::Cre; Stk11^{c/+}* genotypes and 1.2 mm (100 slices) for the *Osx-GFP::Cre; Stk11^{c/c}* genotype. Cortical bone was evaluated in the mid-diaphysis in a region that started 55% of the bone length below the femoral head and extended 600 μm (50 slices) distally for the *Stk11^{c/c}* and *Osx-GFP::Cre; Stk11^{c/+}* genotypes and 420 μm (35 slices) for the *Osx-GFP::Cre; Stk11^{c/c}* genotype. Thresholds of 187 and 527 mg HA/cm^3 were used for the evaluations of trabecular and cortical bone, respectively. For the trabecular bone region, an adaptive-iterative thresholding (AIT) algorithm was used to determine the average threshold for the control group [22] and [23], and the same threshold was applied to the mutants. For the cortical region, a threshold was selected that gave a reproducible segmentation at the midshaft; the same threshold was applied to both the control and mutant mice. Cancellous bone outcomes included trabecular bone volume fraction (BV/TV, %), trabecular thickness (Tb.Th, μm), trabecular number (Tb.N, mm^{-1}), trabecular separation (Tb.Sp, μm), connectivity density (Conn.D, mm^{-3}), and structural model index (SMI). Cortical bone outcomes included cortical tissue mineral density (Ct.TMD, mg HA/mm^3), cortical thickness (Ct.Th, μm), bone area (Ct.Ar, mm^2), total area (Tt.Ar, mm^2), medullary area (Ma.Ar, mm^2), cortical bone area fraction (Ct.Ar/Tt.Ar, mm^2), cortical porosity (Ct.Po, %), polar moment of inertia (J, mm^4), and the maximum and minimum moments of inertia (I_{max} and I_{min} , mm^4). Since several specimens contained large amounts of trabecular bone in the medullary region at the femoral mid-diaphysis, cortical bone analysis involved two steps: (1): evaluation of an ROI containing both cortical and trabecular bone (to identify the Tt.Ar), and (2): evaluation of an ROI containing just cortical bone (to obtain Ct.Th, Ct.Po, Ct.TMD, and biomechanical parameters). This two-step process was applied to all specimens.

Quantitative real-time RT-PCR

Calvaria from P25 mice were dissected and subjected to serial enzymatic digestion at 37°C with Liberase TM Research grade (Roche). The digestion duration for fraction 1 was 10min and was discarded. Fraction 2 and 3 was 45min each, and were collected for RNA isolation with RNeasy micro kit (Qiagen). cDNA was prepared with qScript cDNA super mix (Quanta), and analyzed with SYBR Premix Ex Taq II (Clontech). Relative expression for each gene was calculated by the $\Delta\Delta\text{Ct}$ method with Gapdh as the normalization gene. Primer sequences were: Rankl-F: TTGCACACCTCACCATCAAT; Rankl-R: TCCGTTGCTTAACGTCATGT; Opg-F: ACCTCACCACAGAGCAGCTT; Opg-R: GCTCGATTTGCACGTCTTTC; Gapdh-F: AGGTCGGTGTGAACGGATTG; Gapdh-R: TGTAGACCATGTAGTTGAGGTCA.

Statistical analyses

All statistical analyses used GraphPad Prism 6 (GraphPad Software); samples were considered statistically significant when the p-value was ≤ 0.05 . Data obtained from histomorphometric analysis are presented as mean $\pm$ standard error of mean (s.e.m.) and micro-CT analysis are presented as mean $\pm$ standard deviation (s.d.). Each set of data was analyzed by one-way analysis of variance (ANOVA) followed by Fisher's Protected Least Significant Difference (PLSD) test. All other statistical analysis employed the Student's t-test; here data are presented as mean $\pm$ s.d..

Results

Conditional removal of *Stk11* in osteoblast precursors

Stk11 is ubiquitously expressed in all mammalian tissues, and removal of *Stk11* results in embryonic lethality [16]. To investigate the potential role of *Stk11* during bone development and homeostasis, we used a previously described conditional allele of *Stk11* (*Stk11^c*), and osteoblast precursor specific expression of Cre recombinase directed by an *Osx*-GFP::Cre transgene to specifically remove *Stk11* activity from osteoblast precursors in *Osx*-GFP::Cre; *Stk11^{c/c}* individuals [11] and [24]. Hereafter, we refer to this genetic combination as “*Stk11* mutants. Control mice with one active *Stk11* allele (*Osx*-GFP::Cre; *Stk11^{c/+}*) were phenotypically similar to wild-type littermates.

Conditional removal of *Stk11* in osteoblast precursors results in increased osteoblast number and activity

Stk11 mutants were born at the expected Mendelian ratio, and were indistinguishable from littermates at birth. Whole mount skeletal staining with alcian blue and alizarin red at embryonic day (E) 16.5 and postnatal day (P) 0.5 revealed no obvious difference between control and mutant (Fig. 1A–D). However, histological analysis of skeletal sections from mutant neonates revealed an increase in alizarin red stained mineralized matrix (Fig. 1E–F) at P 0.5. Immunostaining to identify Runx2+ and Osx+ osteoblast precursors and osteoblasts demonstrated a marked increase in cells of the osteoblast lineage in both the bone collar (Fig. 1G–H), and emerging trabeculae (Fig. 1I–L). Examination of collagen (I) deposition high-lighted a disorganized bone matrix surrounding this population (Fig. 1M–N). The marked increase in postnatal osteoblasts led to a parallel decrease in the bone-free zone of the diaphyseal marrow space within the shaft of the long bones (Fig. 1E–N).

Stk11 mutants show increased turnover, woven bone formation and porous cortical bone

By P15, *Stk11* mutants exhibited pronounced growth retardation; compulsory euthanasia was required around P30. As at earlier stages, in P25 mice the gross features of skeletal organization were normal except that limbs were shorter and thicker than those of controls, and the distal femurs of the *Stk11* mutant stained more darkly in whole mount preparations (Fig. 2). We also noticed the mutant calvaria had a rough surface compared to a smooth surface of the control calvaria (Fig. 2B). More detailed analysis showed that the numbers of Osx+ and Runx2+ cells were dramatically elevated at this stage, and consequently, the presumptive marrow space was almost completely filled by a collagen (I)-containing bone matrix (Fig. 3, 4A–B, 6A–B). Histological sections showed a striking increase in trabeculae both in the trabecular bone area and along the cortex in the midshaft (Fig 4A and 5), with an overall disruption of the distinction between cancellous and cortical bone.

To examine osteoblast activity, we performed dynamic histomorphometry with sequential labeling of newly synthesized bone through incorporation of intraperitoneal injected calcein (P25) and demeclocycline (P27). When long bones were analyzed at P28 (Fig. 4C), incorporation of the two fluorochromes was evident as distinct lines in the bones of control littermates. In contrast, mutants displayed irregular and diffuse fluorochrome labeling, a characteristic feature of immature woven bone.

Stk11 mutants showed a 7-fold increase in trabecular bone volume (BV/TV) compared to controls (Fig. 4D). Trabeculae were 1.5-fold thicker and 4-fold more numerous (Tb. N and Tb. Th); consequently, trabecular separation (Tb. Sep.) in mutants was only 15% of that of controls. TRAP staining of osteoclast revealed a 2-fold increase in osteoclast number (corrected for total area, N.Oc/T.Ar) in mutants, as best illustrated after TRAP staining (Fig. 4B, 5A–B, Table 3). Consistently, the Rankl/Opg ratio was significantly increased in mutant osteoblasts (Fig. 5C–D). Similarly, N.Ob/TAr tended to increase together with bone density but at this time point it was only moderately increased. The trabecular bone surface, which is normally lined with cuboidal osteoblasts, showed an unexpected increase in elongated fibroblast-like cells displaying low levels of Osx activity. This population lined 70% of the bone surface, clustering in the mutant bone shaft (Fig. 4B, 6C–D, Table 1). Cells within these clusters were Ki67+ indicating high rates of proliferative activity (Fig. 6E–F). In addition to an expansion of osteoblast-like cells with abnormal Osx levels, adipocytes, which were rarely observed in the marrow shaft of control littermates, were prominent in mutants at P30 (Fig. 4A').

To complement the histomorphometric analysis and further examine the bone microstructure, we performed micro-computed tomographic (micro-CT) imaging on femur samples at P28, focusing analysis on the distal metaphysis and mid-diaphysis regions (Fig. 7, Table 4). Examining trabecular bone properties in the distal metaphysis, we observed a significant increase in bone volume (4-fold), trabecular number (2-fold), and trabecular thickness (2-fold) (BV/TV, Tb.N, Tb.Th), and a concomitant decrease in trabecular separation (Tb.Sp). Structure model index (SMI), a measurement related to the architecture of the bone trabeculae, typically ranges from 0 to 3 (0 indicating a plate structure and 3 a cylindrical rod structure). The SMI value for control samples was 2.0; in mutants this was notably a negative value indicating a bone structure with many closed cavities, and dense, plate-like bone. To assess the cortical bone properties, we focused on the mid-diaphysis region. Surprisingly, the apparent cortical thickness was dramatically reduced in the mutant. It was only 30% of that of the control, and was probably due to the markedly increased cortical porosity compared to control littermates and the presence of woven trabeculae along the endosteal surface of the cortex. When we considered the bone volume and bone mineral density of the whole cross section of the midshaft, BV/TV was significantly increased with a small increase in BMD in the mutant compared to the control (Table 2). The polar moment of inertia (pMOD), an indicator of torsional rigidity in the mutant was less than 50% of that of the control. Taken together, data from both the histomorphometric and micro-CT analyses clearly show that Stk11 is essential for postnatal bone homeostasis.

Mechanistic analysis of the Stk11 mutant

To investigate the mechanism underlying the *Stk11* action in osteoblasts, we focused on the mTORC1 pathway, a critical target downstream of Stk11-dependent AMP kinases. Examination of mTORC1 catalytic substrates: eukaryotic translation initiation factor 4E-binding protein 1 (4e-bp1) and ribosomal protein S6 (rpS6), with antibodies specific to phospho-forms of each protein detected phosphorylated rpS6, but not phosphorylated 4e-bp1, in Osx+ osteoblasts lining the bone surface of control femurs at P15 (Fig. 8). These cells were dramatically increased in the mutants whereas phosphorylation of 4e-bp1 was not

altered. These data suggest that *Stk11* suppression of mTORC1/rpS6 signaling accounts for a component of *Stk11* activity in normal bone formation.

Discussion

We have demonstrated that *Stk11* is required for normal development and homeostasis of mammalian bone. Removal of *Stk11* from *Osx*⁺ osteoblast precursors had an anabolic effect on the bone program during embryonic development, increasing the number of osteoblasts. In postnatal mice, loss of *Stk11* resulted in deregulated bone formation and increased bone turnover. Trabecular bone volume was significantly increased, and ectopic mineralized trabeculae penetrated deep into normal bone-free marrow space of the midshaft. Thus, although the cortex appeared to be thinner by microCT, the overall thickness of the cortex was increased, with the trabecular features of the endosteal extension giving a very porous aspect to the cortex, and contributing to the observed decreased strength of the long bones.

Trabeculae were surrounded by a markedly increased number of *Osx*⁺ cells though many of these cells displayed a fibroblastic appearance and showed reduced levels of *Osx*. The total number of osteoclasts was also increased and likely the expansion of their numbers contributed to the observed increased in bone turnover. Overall, the bone of *Stk11* mutants shares features with bone of patients with hyperparathyroidism and Jansen's metaphyseal chondrodysplasia, a rare genetic disorder caused by mutations resulting in constitutively-active parathyroid hormone (PTH) receptor [25] and [26].

Molecular and structural analysis of bone deposition indicates that *Stk11* deficient osteoblasts synthesize a disorganized woven bone matrix, the sign of a very high rate of bone formation, and a possible reflection of an immature state of osteoblast development. Woven bone is observed during the embryonic stage and in pathological conditions in adult stages such as patients with Paget's disease and during fracture repair. Persistent woven bone deposition is consistent with the model that *Stk11* mutant osteoblasts fail to transition to a fully mature state.

Peritrabecular marrow fibrosis is another hallmark observation of hyperparathyroidism. Previous studies have demonstrated that PTH is a potent stimulator of fibroblast proliferation [27]. It is speculated that osteoblasts respond to PTH to release paracrine factors such as Igfs and Pdgfs that modulate adjacent fibroblastic cell types [28] considered to be preosteoblastic cells based on their location and expression of osteoblast marker proteins [29] and [30]. In our study, some fibroblast-like cells express low level of *Osx* and could, therefore, represent an immature, preosteoblastic cell type.

Stk11 is a critical regulator of the mTOR pathway acting through direct control of AMP kinase activity. Activation of the mTOR pathway is associated with increased cell proliferation and cell growth that is largely mediated by two downstream substrates: 4e-bp1 and rpS6. A recent study has demonstrated the critical role of *Stk11*/mTORC1 pathway in controlling chondrocyte differentiation. The work here establishes a broader role for *Stk11* in the mammalian skeleton. Elevated phosphor-rpS6 levels in *Stk11* deficient osteoblasts points to ectopic activation of the mTORC1 pathway upon removal of *Stk11*. Interestingly,

phosphor-rpS6+ cells are not confined to the *Osx*+ population suggesting that there may be non-cell autonomous consequences from *Stk11* removal in *Osx*+ osteoblasts; Igf signaling is known to activate mTORC1/rpS6 and could be one mechanism for future exploration [18].

Recent findings have demonstrated that mTORC1 signaling mediates the anabolic effects of Wnt7b and parathyroid hormone on bone. The Wnt7b-dependent increase of bone matrix formation is abolished on removal of *Raptor* (a key component of the mTORC1 complex) in osteoblasts [31]. In addition, the anabolic effect of parathyroid hormone is significantly suppressed by rapamycin, a selective mTORC1 inhibitor [32]. Given the highly similar bone phenotype of the *Stk11* mutant and that of hyperparathyroidism, it is likely that mTOR signaling mediates at least some of the effects of PTH in osteoblasts. A more detailed investigation of the roles of mTOR signaling in the regulation of osteoblast programs is warranted given the significance of altered bone morphology to the functional properties of bone observed in our study of *Stk11*.

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Highlights

- Osteoblast specific removal of *Stk11* results in a marked perturbation of post-natal bone development.
- Specification and primary organization of the neonatal skeleton is independent of the *Stk11* activity.
- *Stk11* mutants have a significant increase in the number of osteoblasts and osteoclasts, and a marked increase in bone deposition.
- Enhanced bone turnover in *Stk11* mutants was associated with woven bone formation and increased porosity of cortical bone.
- Elevated levels of phosphorylated ribosomal protein S6 implicate mTORC1 misregulation as a component of the *Stk11* mutant phenotype.

Figure 1. Characterization of the embryonic skeletal phenotype of *Stk11* controls and mutants Whole skeletons were isolated on E16.5 (A, B) and P0.5 (C, D), and stained with Alcian blue and alizarin red. Histological analysis of sections through P0.5 tibiae stained with alizarin red (E, F). P0.5 femur sections were immunostained with antibodies specific to Osterix (G–J), Runx2 (K, L), and collagen (I) (M, N). Nuclei were visualized with DAPI. Yellow lines in G and H indicate the thickness of the periosteum.

Figure 2. Whole mount skeleton staining of postnatal *Stk11* controls and mutants
 Whole skeletons were isolated on P25, and stained with Alcian blue and alizarin red (A). Higher magnification of the skull (B), rib cage (C), and hindlimb (D) are shown. Green arrows in B2 indicate the rough surface of the mutant calvaria, and the blue arrow in D2 points to the darker staining of the mutant metaphysis.

Figure 3. Molecular characterization of the postnatal *Stk11* controls and mutants

Immunofluorescence was performed on P25 femur sections with antibodies specific to Osterix (A, B), Runx2 (C, D), and collagen (I) (E, F). The distal femur (yellow box) and midshaft region (green box) were shown in a higher magnification. Nuclei were visualized with DAPI.

Figure 4. Histomorphometric analysis of postnatal Stk11 controls and mutants

Undecalcified tibia sections were stained with von Kossa (A, with higher magnification in A'), Toluidine blue (B) and calcein/demeclocycline (C). (D) Bar graphs showing selected data obtained from the histomorphometric analysis with error bars indicating the standard errors (* p 0.05). Blue dot-lined area in A' shows a cluster of adipocytes. Green arrowheads and red arrowheads in B indicate elongated fibroblast-like cells and osteoclasts, respectively.

Figure 5. TRAP staining and Rankl/Opg expression of postnatal Stk11 control and mutant femur sections

Femur sections isolated from P25 controls and mutants were stained with TRAP staining (A). Bar graph showing the number of pixels within the yellow-box that are TRAP+ (B). Rankl and Opg expression in P25 calvarial osteoblasts was determined with quantitative real-time RT-PCR (C, D). Error bars indicate standard deviations. (* p < 0.05).

Figure 6. Immunohistochemical analysis of postnatal *Stk11* controls and mutants
Immunohistochemistry was performed on adjacent femur sections isolated from P30 controls and mutants with specific antibodies against osterix (A–D) and Ki67 (E–F). Green arrowheads in D1 indicate peritrabecular fibroblast-like cells. The red dot-lined and blue dot-lined area in D and F indicate the bone marrow and cell cluster of elongated fibroblast-like cells, respectively.

Figure 7. Micro-CT analysis of postnatal Stk11 controls and mutants
Micro-CT analysis was performed on femurs isolated from P28 controls and mutants. Selected data of the cortical bone properties were plotted in bar graphs (C) Error bars indicate standard deviations (* $p < 0.05$).

Figure 8. mTORC1 signaling analysis of postnatal *Stk11* controls and mutants

Co-immunostaining was performed on femur sections from P15 *Stk11* controls and mutants with specific antibodies against osterix and phosphor rpS6 (p235/p236) (A), phosphor rpS6 (p240/p244) (B), or phosphor 4e-bp1 (p37/p46) (C). Nuclei were visualized with DAPI.

Table 1

Experiment design

Age	Experiments/analyses performed	Samples (Sample size)	Gender
E16.5	Whole skeleton staining with Alcian blue and alizarin red	control (3)/mutant (3)	ND
P0.5	Whole skeleton staining with Alcian blue and alizarin red Histology Immunofluorescence	control (3)/mutant (3)	ND
P15	Immunofluorescence	control (3)/mutant (3)	males and females
P25	Whole skeleton staining with Alcian blue and alizarin red Immunofluorescence TRAP staining Rankl and Opg expression	control (3)/mutant (3)	males and females
P28	Histomorphometry	Stk11 ^{ec} (4)/Ossx-GFP::Cre;Stk11 ^{ec/+} (4)/Ossx-GFP::Cre;Stk11 ^{ec} (4)	all females
	Micro-CT analysis	Stk11 ^{ec} (3)/Ossx-GFP::Cre;Stk11 ^{ec/+} (3)/Ossx-GFP::Cre;Stk11 ^{ec} (3)	all females
P30	Immunohistochemistry	control (3)/mutant (3)	males and females

ND: not determined
control: Ossx-GFP::Cre;Stk11^{ec/+}
mutant: Ossx-GFP::Cre;Stk11^{ec/c}

Table 2

List of Antibodies used

Antibody	Vendor	Catalogue N.	Dilution
Collagen (I)	Rockland	600-401-103	1/200
Ki67	Vector labs	VP-RM04	1/200
Osterix	Abcam	ab22552	1/5000
phosphor 4e-bp1 (Thr37/46)	Cell Signaling Technology	2855	1/100
phosphor rpS6 (Ser235/236)	Cell Signaling Technology	2211	1/100
phosphor rpS6 (Ser240/244)	Cell Signaling Technology	5364	1/100
Runx2	Santa Cruz	sc10758	1/100

Histomorphometric analysis showed increased osteoblast number, osteoclast number and bone deposition in Stk11 mutants

Table 3

	Histomorphometric analysis (mean±s.e.m.)				p-value	
	Stk11 (c/c)	Oss-Cre;Stk11(c/+)	Oss-Cre;Stk11(c/c)	ANOVA	Fisher's PLSD	
BV/TV (%)	4.52±0.97	5.2±1.75	31.69±4.85 ^a	0.0002	0.0002	
Tb.Th (µm)	24.79±3.02	22.63±2.65	38.44±2.71 ^a	0.0063	0.0031	
Tb.N (/mm)	1.83±0.38	2.11±0.57	8.1±0.83 ^a	<0.0001	<0.0001	
Tb. Sep (µm)	588±111	640±250	90±18	0.0688	ND	
N.Ob./T.Ar (/mm ²)	82.2±17.41	67.63±19.93	109±36	0.542	ND	
N.Oc./T.Ar (/mm ²)	7.11±0.42	10.42±3.65	22.9±7.83	0.115	ND	
Fb.S/BS (%)	0±0	0±0	70.68±9.08 ^a	<0.0001	<0.0001	

^a p 0.05 comparing OssGFP::Cre; Stk11^{c/+} and OssGFP::Cre; Stk11^{c/c} with Fisher's PLSD
ND: Fisher's PLSD not determined when p-value of ANOVA >0.05

Micro-CT analysis showed the increased trabecular bone volume and porous cortical bone in Stk11 mutants

Table 4

Distal Femur (Trabecular Bone Architecture) (mean±s.d.)				p-value	
	Stk11 (c/c)	Osx-Cre;Stk11(c/+)	Osx-Cre;Stk11(c/c)	ANOVA	Fisher's PLSD
BV/TV (%)	15.18 ± 3.71	16.02 ± 4.92	71.46 ± 3.15^a	<0.0001	<0.0001
Conn-Dens. (1/mm ³)	147.71 ± 44.23	146.62 ± 49.84	248.97 ± 91.53	0.196	ND
SMI	2.19 ± 0.16	1.98 ± 0.19	-6.98 ± 1.77^a	<0.0001	<0.0001
Tb.N (1/mm)	3.96 ± 0.91	3.97 ± 1.02	9.50 ± 0.40^a	0.0002	0.0002
Tb.Th (mm)	0.053 ± 0.00	0.055 ± 0.00	0.105 ± 0.02^a	0.0008	0.0006
Tb.Sp (mm)	0.269 ± 0.07	0.272 ± 0.08	0.090 ± 0.00^a	0.018	0.0116
Femoral Midshaft (Cortical Bone Properties) (mean±s.d.)					
	Stk11 (c/c)	Osx-Cre;Stk11(c/+)	Osx-Cre;Stk11(c/c)		
Cortical Thickness (mm)	114.00 ± 4.24	97.00 ± 8.54	35.00 ± 2.65^a	<0.0001	<0.0001
Cortical Density 2 (mgHA/ccm)	987.51 ± 10.24	954.39 ± 6.42	834.40 ± 11.58^a	<0.0001	<0.0001
BArea[mm ²]	0.51 ± 0.00	0.45 ± 0.06	0.38 ± 0.21	0.607	ND
TArea[mm ²]	1.65 ± 0.07	1.67 ± 0.20	1.73 ± 0.48	0.965	ND
MArea[mm ²]	1.14 ± 0.07	1.22 ± 0.14	1.35 ± 0.28	0.557	ND
BA/TA (%)	30.97 ± 1.42	26.86 ± 0.94	21.06 ± 6.04	0.090	ND
pMOI[mm ⁴]	0.223 ± 0.01	0.203 ± 0.05	0.08 ± 0.05^a	0.037	0.0278
Imax[mm ⁴]	0.14 ± 0.01	0.12 ± 0.03	0.05 ± 0.03^a	0.026	0.019
Imin[mm ⁴]	0.09 ± 0.00	0.08 ± 0.02	0.03 ± 0.02^a	0.045	0.031
Porosity (%)	1.73 ± 2.4	2.73 ± 0.79	47.32 ± 7.21^a	0.0001	<0.0001
Femoral Midshaft (Whole bone section) (mean±s.d.)					
	Stk11 (c/c)	Osx-Cre;Stk11(c/+)	Osx-Cre;Stk11(c/c)		
BV/TV (%)	35.91±1.56	31.99±0.82	71.46±16.61^a	0.0044	0.0024
BMD (mgHA/ccm)	296.10±4.44	263.11±6.42	335.63±68.89	0.165	ND

^a p 0.05 comparing OsxGFP::Cre; Stk11(c/+) and OsxGFP::Cre; Stk11 (c/c) with Fisher's PLSD
ND: Fisher's PLSD not determined when p-value of ANOVA >0.05