Liver kinase b1 (Lkb1) protein kinase activity regulates cell growth and cell polarity. Here, we show Lkb1 is essential for maintaining a balance between mitotic and postmitotic cell fates in development of the mammalian skeleton. In this process, Lkb1 activity controls the progression of mitotic chondrocytes to a mature, postmitotic hypertrophic fate. Loss of this Lkb1-dependent switch leads to a dramatic expansion of immature chondrocytes and formation of enchondroma-like tumors. Pathway analysis points to a mammalian target of rapamycin complex 1-dependent mechanism that can be partially suppressed by rapamycin treatment. These findings highlight a critical requirement for integration of mammalian target of rapamycin activity into developmental decision-making during mammalian skeletogenesis.

Growth of the endochondral skeleton is dependent on a cartilaginous growth plate. In the growth plate, mitotic chondrocytes transition to a postmitotic, terminal hypertrophic chondrocyte fate (Fig. S1A). Reciprocal signaling between prehypertrophic chondrocyte-derived Indian hedgehog (Ihh) and epiphyseal secreted parathyroid hormone-related peptide (Pthrp; also known as Pthlh) controls the spatial positioning of the hypertrophic transition and the normal growth properties of the skeleton (1–3). The present study demonstrates an unexpected role for liver kinase b1 (Lkb1; also known as Stk11) in growth plate regulation.

Lkb1 is a multifunctional serine/threonine kinase inhibitor of mTOR signaling whose activity regulates cell cycle progression, cellular energy homeostasis, and cell polarity (4, 5). Mouse embryos lacking Lkb1 die at midgestation with vascular and neural tube defects (6), and germ-line inactivating mutations of Lkb1 in the human population underlie Peutz–Jeghers syndrome, characterized by development of benign polyps in the gastrointestinal tract, and an increased risk of various types of epithelial cancers (7, 8). Conditional ablation of Lkb1 in pancreatic, vascular, neural and cardiac tissue links Lkb1 to tissue-specific actions in a variety of organ systems (9). Here, we provide evidence that Lkb1 regulation of mammalian target of rapamycin complex 1 (mTORC1) action is a critical step in the transition of mitotic chondrocytes to postmitotic hypertrophic fates suppressing cartilaginous tumor-like growths in the postnatal mammalian skeleton.

Results

Removal of Lkb1 in Chondrocytes Results in Expansion of Columnar Mitotic Chondrocytes, Delayed Hypertrophic Development, and Formation of Enchondroma-Like Tumors. We established a potential link between Lkb1 activity and mammalian skeletogenesis unexpectedly through conditional removal of Lkb1 activity in a large region of the caudal mouse embryo. Given the pleiotropic activity of the original Cre-driver line, we intercrossed mice carrying a Cre-dependent conditional Lkb1 allele (Lkb1fl/fl) (10) with a Col2a1-Cre transgenic strain (11); here, skeletal Cre-activity is initiated in immature, mitotic, and early postmitotic chondrocytes (Fig. S1B). Through these crosses, mice were generated that lacked Lkb1 activity specifically within chondrocytes of the endochondral skeleton (Col2a1-Cre;Lkb1fl/fl; hereafter referred to as Lkb1 mutants). In contrast to littermates that retained an active Lkb1 allele (Col2a1-Cre;Lkb1+/+; hereafter referred to as control littermates), Lkb1 mutants displayed a prominent postnatal phenotype.

Lkb1 mutants were born at the expected Mendelian ratio, and appeared superficially normal at birth. However, marked growth retardation was evident by weaning, and, as a result of this growth deficiency, and a lethargic phenotype, mutants were euthanized by postnatal day (P) 40 to satisfy institutional guidelines on humane animal care. Histological analysis of long bones after weaning (at P30) revealed a profound disorganization of the Lkb1 mutant skeleton (Fig. 1 and Fig. S2A). Alcian blue staining of long bones from normal individuals highlights nonhypertrophic chondrocytes within the cartilaginous growth plate localized close to the epiphysis (Fig. S2A). Chondrocytes organize into stratified tiers of mitotic Col2a1+/Sox9+ proliferative chondrocytes in the growth plate before transitioning to Col2a1−/Sox9−/Runx2+ postcolumnar, postmitotic, hypertrophic chondrocytes that undergo cell death and replacement by bone-forming osteoblasts (Fig. L4 and Fig. S2B). Hypertrophic chondrocytes and osteoblasts of the outer cortical and inner trabecular region of the main shaft of the long bones in the Lkb1 mutants showed a dramatic expansion of immature chondrocytes and formation of enchondroma-like tumors. Pathway analysis points to a mammalian target of rapamycin complex 1-dependent mechanism that can attenuate the energy-sensing mammalian target of rapamycin complex 1 pathway critical for switching chondrocyte states. A failure of mTORC1 suppression in Lkb1 mutants leads to a dramatic disruption of the skeletal growth plate and the formation of cartilage tumors comprising undifferentiated chondrocytes that display differential sensitivity to two key cartilage growth regulators, Indian hedgehog and Igf. The study highlights the interconnection between energy sensing pathways, normal growth control, and tumorigenesis in the skeletal program.

Significance

The transition from a mitotic to a postmitotic, hypertrophic chondrocyte is a key regulatory event in the growing vertebrate skeleton. By using genetic approaches, cell culture, and cell transplantation models, we provide compelling evidence that attenuating the energy-sensing mammalian target of rapamycin complex 1 (mTORC1) pathway is critical for switching chondrocyte states. A failure of mTORC1 suppression in Lkb1 mutants leads to a dramatic disruption of the skeletal growth plate and the formation of cartilage tumors comprising undifferentiated chondrocytes that display differential sensitivity to two key cartilage growth regulators, Indian hedgehog and Igf. The study highlights the interconnection between energy sensing pathways, normal growth control, and tumorigenesis in the skeletal program.
bone are mineralized and highlighted by Alizarin red (Fig. S2A). In contrast to control littermates, Lkb1 mutants displayed prominent Alcian blue staining within normally bone-restricted regions of the enchondroma-like skeleton (Fig. S2A). Detailed histological analyses of the Lkb1 mutant from P10 to P30 revealed a mass of proliferating immature Col2a1+/Sox9+ chondrocytes deep within the shaft of the long bone (Fig. 1B). At P10, the growth plate was markedly disorganized: ectopic hypertrophic chondrocytes were observed at the core of the growth plate and next to the groove of Ranvier (Fig. S3). By P20, proliferating chondrocytes formed columns perpendicular to the normal longitudinal axis of growth. Tumor-like cell nodules were also found close to the primary bone marrow (Fig. S3). Analysis at P30, showed that these are largely made up of Sox9+/Osterix+ chondrocytes that displayed low levels of collagen (X) indicative of immature chondrocytes (Fig. 1B and Fig. S3).

To investigate the genesis of this phenotype, we focused on the period preceding the overt change in body size in Lkb1 mutants: P3 and earlier (Fig. 1 and Fig. S2D). At P3, analysis of Alcian blue and Alizarin red staining revealed that skeletal growth was similar between Lkb1 mutants and control littermates, but the axial (vertebrae) and appendicular (long bone) skeleton was markedly deficient in mineralized matrix (Fig. 1 F-K). In line with expectations from the genetic model, the osteoblast program was not primarily affected (Alizarin red and von Kossa stains; Fig. S4A). In contrast, femur and vertebral sections (Fig. 1 M, N, P, Q, S, and T) revealed a dramatic expansion of the growth plate region in Lkb1 mutants reflected by an extended domain of Alcian blue-stained immature cartilage (Fig. S4A).

Although less marked, this phenotype was evident before birth, at embryonic day (E) 18.5 (Fig. 1 L, O, and R and Fig. S2B). Measurement of specific cartilage domains showed similar proportions of round resting zone and postcolumnar chondrocytes between control littermates and Lkb1 mutants (Fig. 1U), but a grossly extended domain of immature columnar chondrocyte in mutants (Fig. 1U). At E16.5, no clear phenotype was evident in the femur, although a delay mineralization was evident in posterior vertebrae (Fig. S2B and C). In summary, in the absence of Lkb1, mutant chondrocytes retained an immature identity whereby normal chondrocytes transition to a terminal hypertrophic fate. An expanded growth rate is likely the underlying event in the establishment of tumor masses in the later skeleton.

**Lkb1 Is Essential for Switching Between Chondrocyte States.** To investigate these regulatory events further, we examined key markers of chondrocyte identity. Col2a1 [collagen (II)]-producing non-hypertrophic chondrocytes were expanded in the E18.5 Lkb1 mutant femur (Fig. 2A and D), whereas the number of Col10a1 [collagen (X)]-expressing hypertrophic chondrocytes was markedly reduced, and Col10a1 protein was not detected (Fig. 2B, E, I, and L). In addition, late-stage, Mmp13+ hypertrophic chondrocytes were entirely absent from long bones of mutants at E18.5 (Fig. 2C and F; note Mmp13+ osteoblasts were not affected by Lkb1 removal). Production of transcriptional regulators linked to chondrocyte developmental programs displayed a similar temporal and spatial displacement. Mef2c and Runx2, key determinants of hypertrophic differentiation, are activated together with Osterix and Ihh in prehypertrophic chondrocytes. In Lkb1 mutants, expression of all of these genes was first observed within chondrocytes at an extended position relative to the periacicular surface indicative of a marked delay in chondrocyte differentiation (Fig. 2 G, H, I, and K and Fig. S4B).

To examine cell proliferation, we visualized cyclin D1, a key regulator of the G1-to-S phase transition, and the incorporation of exogenously supplied 5-ethyl-2′-deoxyuridine (EdU) or BrdU, to identify chondrocytes undergoing DNA replication. Both approaches highlight an expanded domain of proliferating undifferentiated chondrocytes (Fig. 2 M–R). However, the fraction of cells undergoing DNA replication within this domain was not altered, suggesting that the excessive number of flattened chondrocytes likely reflects delayed hypertrophic differentiation rather than an increased rate of division (Fig. S4D). Collagen (X) protein was detected by P3; consequently, Lkb1 is not essential for the hypertrophic transition, but rather Lkb1 activity controls the normal developmental timing of this key cellular transition within the growth plate (Fig. S4C).

The mTOR Pathway Mediates the Effects of Lkb1 in Chondrocytes. The mTOR pathway balances cell growth and proliferation with the energy level of the cell (12), and is negatively regulated when conditions are unfavorable (13, 14). To address mTOR signaling in chondrocytes, and to distinguish between mTOR action within mTORC1 and mTORC2 complexes, we examined phosphorylation of two key mTORC1 substrates, ribosomal protein S6 (rpS6) and eukaryotic initiation factor 4e-binding protein

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**Fig. 1.** Expansion of columnar mitotic chondrocytes results in formation of enchondroma-like structure. (A and B) P30 femur sections stained with H&E. Immunohistochemistry was performed on adjacent sections with specific antibodies recognizing collagen (II) (A2 and B2) and Sox9 (A2′ and B2′). Areas boxed in red in A and B are magnified in A1, A2, B1, and B2, respectively. Areas boxed in green in A2 and B2 are magnified in A2′ and B2′, respectively. (C–K) P3 skeletal preparations stained with Alcian blue and Alizarin red, with higher-magnification views of the femur (F–I) and vertebra (I–K). Colored bars indicate the length of mineralized regions within the skeletal element. (L–T) Histological sections through the femur and lumbar vertebrae at E18.5 and P3, stained with H&E. (U) Bar graphs displaying the length of the zones of round, columnar, and postcolumnar chondrocytes. Error bars indicate the SD of the means (n > 3; *P < 0.01 between columnar regions of Lkb1fi/+ and Col2a1-Cre;Lkb1fi/+ on E18.5. **P < 0.001 between columnar regions of Lkb1fi/+ and Col2a1-Cre;Lkb1fi/+ at P3). (Scale bars: A and B, 1 mm; C–E, 0.5 cm; I–K, 1 mm; L–U, 200 μm.)
(4e-bp1) and phosphorylation of serine 473 of Akt, a hallmark of mTORC2 complex activity.

In control littermates, phosphorylation of rpS6 and 4e-bp1 was evident in the proliferating columnar chondrocytes within the long bones, but their phosphorylation state was markedly reduced on commitment to the hypertrophic chondrocyte program (Fig. 3 A–C; note that rpS6 displays a later burst of phosphorylation within mature hypertrophic chondrocytes marked by an asterisk in Fig. 3C). In Lkb1 mutants, mTOR expression was not altered; however, mTORC1 activity, highlighted by rpS6 and 4e-bp1 phosphorylation, extended into regions where hypertrophic development should normally have initiated (Fig. 3 D–G). In contrast, phosphorylation of Akt (Ser473) was unaltered in Lkb1 mutants, lending support for an mTORC1-specific role in the skeletal phenotype (Fig. 3 D and H).

To explore mTORC1 action, the mTORC1 inhibitor rapamycin was introduced into dams harboring Lkb1 mutant embryos between 16.5 and 18.5 d of development. Interestingly, rapamycin treatment decreased phosphorylation of mTORC1 substrates (Fig. S5), normalized proliferation and differentiation of chondrocytes in Lkb1 mutants, and restored a cyclinD1−/Oxs−/Col10a1+ hypertrophic chondrocyte zone by E18.5 (Fig. 3 I–T). Collectively, these data indicate that an Lkb1-dependent attenuation of mTORC1 action is critical for the normal progression of chondrocytes to a terminal hypertrophic fate.

Loss of Lkb1 Results in Chondrocyte Apoptosis at the Core of the Growth Plate. By P3, the cell density at the core of the Lkb1 mutant growth plate was noticeably lower. To determine whether cells are dying in this region, we performed TUNEL assay and examined activation of caspase-3 to visualize apoptotic cells. TUNEL and cleaved caspase-3–positive cells localize within the core of the extended growth plate (Fig. S6). EF5 staining (a chemical indicator of hypoxia) and Vegfa expression (a hypoxia-induced target gene) indicate that the region surrounding the area of cell death was markedly hypoxic, suggesting that low
oxygen levels likely underlie the observed apoptosis (Fig. S6). TUNEL-positive cells were detected in a similar region of the Lkb1 mutant growth plate at P10, but, by P22, when the growth plate is highly disorganized, scattered apoptotic cells predominantly localized at the edge of the cartilaginous zone abutting bone-forming areas (Fig. S6).

**Loss of Lkb1 Results in Enchondroma-Like Tumors in the Postnatal Skeleton.** To investigate the tumorigenic properties of the enchondroma-like mass that forms postnatally in Lkb1 mutant long bones, we assayed chondrocyte growth in anchorage-independent conditions in vitro, and growth following transplantation into immune deficient [NOD scid gamma (NSG)] mice. Whereas control chondrocytes occasionally generated small colonies in nonadherent agar cultures, Lkb1 mutant chondrocytes consistently formed prominent colonies under identical conditions (Fig. 4 A–C). When Lkb1 mutant chondrocytes were transplanted to the flank of NSG mice, safranin O-stained cartilage matrix-secreting cells were recovered at the site of injection 3 mo later (six of eight experiments; Fig. 4 G, I, and M). In contrast, no cartilage nodules were observed in the only tissue recovered from one of four control chondrocyte transplants (Fig. 4 F, I, and L). Consistent with an mTORC1 action, rapamycin inhibited growth of Lkb1 mutant chondrocytes in nonadherent agar culture and following in vivo transplantation (Fig. 4 D, E, H, K, and N).

To gain additional insights into the mechanisms of enchondroma development, we compared the transcriptional profile between control and Lkb1 mutant chondrocytes. Although broadly similar (Fig. S7B and Dataset S1), Gene Ontology (GO) analysis of differentially expressed gene highlighted significant differences among genes associated with skeletal system development (ID number GO:0001501; \( P = 1.06 \times 10^{-11} \)), regulation of cell proliferation (ID number GO:0042127; \( P = 1.71 \times 10^{-5} \)), and positive regulation of mesenchymal cell proliferation (ID number GO:0002053; \( P = 3.5 \times 10^{-4} \)). Igf1 and Igf2, which encode broad regulators of cell growth and proliferation, and Gli2, a transcriptional regulator of Hedgehog pathway targets, whose activity is linked to malignant transformation of chondrosarcomas (15), displayed an elevated transcriptional profile in Lkb1 mutant chondrocytes (Fig. S7A).

Igf1r was present at the highest levels in the zone of proliferating chondrocytes (Fig. S8). Phosphorylation of Tyr1161 on Igf1r, a site of autophosphorylation, indicated active Igf signaling in these cells (Fig. S8). This conclusion is supported further by analysis of Igf signaling dependent phosphorylation of Thr308 on Akt (Fig. S8). The extension of this domain in the skeletal elements of Lkb1 mutants is in agreement with a continued Igf signaling input with the expanded domain of proliferating, immature chondrocytes, and may contribute the maintenance of the proliferative state (Fig. S8).

To examine the potential role of Igf, we examined the effects of picropodophyllotoxin and PQ401, specific Igf pathway inhibitors, on anchorage-independent growth of Lkb1 mutant chondrocytes. Consistent with continued Igf-dependent control, both compounds partially inhibited colony formation. In contrast, GDC-0449 and XAV939, inhibitors of Hh and Wnt signaling...
mutant phenotype in vivo, and Fig. S6 mutant chondrocytes that suggest a central role for Ihh action is an uncoupling of the mutant chondrocytes in nonadherent mutant and control mutant colony. (Scale bars: 1 mm.) Bar graphs comparing cell mass through a colorimetric cell detection assay (D) and colony formation (E) in Lkb1 mutant and control chondrocyte cultures. Error bars indicate the SD of the means of three independent experiments. (F–N) Histological analysis of chondrocyte transplants recovered from NSG mice. Sections were stained with H&E to view general histology and with safranin O to highlight cartilage matrix. (Scale bars: 1 mm.) (G) Graphical plot displaying the size of recovered tissue masses (**P < 0.01, mutant vs. control populations; * *P < 0.01, indicated treatment vs. mutant population).

Fig. 4. Loss of Lkb1 results in enchondroma in postnatal skeleton. (A and B) Colony assay for anchorage-independent growth of chondrocytes. (Scale bar: 1 mm.) (C) High-magnification view of an Lkb1 mutant colony. (Scale bar: 10 μm.) Bar graphs comparing cell mass through a colorimetric cell detection assay (D) and colony formation (E) in Lkb1 mutant and control chondrocyte cultures. Error bars indicate the SD of the means of three independent experiments. (F–N) Histological analysis of chondrocyte transplants recovered from NSG mice. Sections were stained with H&E to view general histology and with safranin O to highlight cartilage matrix. (Scale bars: 1 mm.) (G) Graphical plot displaying the size of recovered tissue masses (**P < 0.01, mutant vs. control populations; * *P < 0.01, indicated treatment vs. mutant population).

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Pthrp signaling is also a critical determinant of the transition point between mitotic and postmitotic chondrocyte programs whereby Ihh governs Pthrp levels coupling chondrocyte proliferation (i.e., direct Ihh action) with chondrocyte differentiation (i.e., indirect action through Ihh control of Pthrp) (21). As with loss of Lkb1 activity, enhanced Pthrp signaling leads to a marked extension of the proliferative zone of immature chondrocytes at the expense of hypertrophic chondrocyte development. We failed to observe any change in the Ihh pathway aside from the apopto- sitional activation of Ihh reflecting the marked delay in formation of postmitotic prehypertrophic chondrocytes. Further, the failure of a Hh pathway antagonist to block chondrocyte proliferation specifically in Lkb1 mutants suggests that loss of Lkb1 removes the dependence on Ihh signaling for normal proliferative control of chondrocytes. The absence of a direct readout of Pthrp signaling precludes an assessment of Pthrp signaling within the Lkb1 mutant model; however, the enchondroma-like end state observed in Lkb1 mutant mice is distinct from the skeletal pheno- type observed on constitutive activation of Pthrp signaling in chondrocytes (22).

Lkb1 is a multifunctional kinase: by activating different AMP kinase family members, Lkb1 regulates cellular polarity and coordinates cell growth and proliferation with the energy state of the cell (4, 5). Our data show elevated levels of mTORC1 activity in Lkb1 mutant chondrocytes that suggest a central role for Lkb1 in suppression of mTORC1 action in the transition between mitotic and postmitotic hypertrophic cell states. Consistent with this view, high mTORC1 activity, as measured by phosphorylation of 4e-bp1 and rpS6, normally associates with proliferative and hypertrophic chondrocytes. Further, rapamycin-mediated inhibition of mTORC1 normalizes the Lkb1 mutant phenotype in vivo, and inhibits expansion of Lkb1 mutant chondrocytes in nonadherent culture, and on transplant into mice. Interestingly, the Igf pathway is a critical regulator of mTORC1 action (23), and inhibition of Igf pathway activity inhibits proliferation of Lkb1 mutant chondrocytes suggesting that the observed phenotype is dependent on an upstream Igf input.

The avascular growth plate has limited nutrient and oxygen supply, which may affect the energy balance of chondrocytes constraining the zone of active chondrocyte proliferation and potentially contributing to apoptosis of mature hypertrophic cells. At P5, we observed a core of apoptotic cells within the extended growth plate surrounded by cells exhibiting molecular signatures of hypoxia. Likely, extreme hypoxia within the core underlies the observed apoptotic phenotype (Fig. S6). Despite the change in environment, ultimately proliferating chondrocytes transition to a hypertrophic cell fate. Thus, Lkb1 is not essential for making the hypertrophic switch, but coordinates the timing and position of this critical cellular transition within the normal growth plate.

In conclusion, the coordination of chondrocyte proliferation and hypertrophic differentiation is crucial to the longitudinal growth, cellular organization, and appropriate mineralization of the developing endochondral skeleton. Our work demonstrates that Lkb1 is critical for the normal function and organization of the growth plate, suggesting a link between the integration of basic pathways of energy balance and growth control in a key developmental decision-making process, and raising the possibility that Lkb1/mTORC1 deregulation may contribute to cartilaginous tumor formation in men.

Materials and Methods

Animal Breeding and Procedures. To generate the Lkb1 conditional KO (Col2a1-Cre; Lkb1fl/fl), Col2a1-Cre mice were mated with Lkb1fl/fl mice to obtain Col2a1-Cre; Lkb1fl/fl mice, which were then mated with Lkb1fl/w mice. Rapamycin was injected into the peritoneum of pregnant mice to block mTORC1 activity from E16.5 to E18.5. GDC-0449 was delivered by gavage 4 d before the mouse was euthanized on P30. All experiments and procedures were approved by the animal and...
We thank Dr. R. A. DePino for sharing the Il2rg
Prkdc
mouse (female, age 6–8 wk). Rapamycin treatment (delivered through drinking water; 5 mg/kg body weight/d) of Nsg mice started 1 d after injection. Three months after transplantation, tumors were dissected, fixed, sectioned, and stained as described earlier.

Statistical Analyses. One-way ANOVA with post hoc Bonferroni test was performed unless otherwise specified. Microarray data were normalized and analyzed with d-Chip, as well as the Database for Annotation, Visualization and Integrated Discovery software tools (25, 26).

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