Translational Control of Sox9 RNA by mTORC1 Contributes to Skeletogenesis

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SUMMARY

The mechanistic/mammalian target of rapamycin complex 1 (mTORC1) regulates cellular function in various cell types. Although the role of mTORC1 in skeletogenesis has been investigated previously, here we show a critical role of mTORC1/4E-BPs/Sox9 axis in regulating skeletogenesis through its expression in undifferentiated mesenchymal cells. Inactivation of Raptor, a component of mTORC1, in limb buds before mesenchymal condensations resulted in a marked loss of both cartilage and bone. Mechanistically, we demonstrated that mTORC1 selectively controls the RNA translation of Sox9, which harbors a 5′ terminal oligopyrimidine tract motif, via inhibition of the 4E-BPs. Indeed, introduction of Sox9 or a knockdown of 4E-BP1/2 in undifferentiated mesenchymal cells markedly rescued the deficiency of the condensation observed in Raptor-deficient mice. Furthermore, introduction of the Sox9 transgene rescued phenotypes of deficient skeletal growth in Raptor-deficient mice. These findings highlight a critical role of mTORC1 in mammalian skeletogenesis, at least in part, through translational control of Sox9 RNA.

INTRODUCTION

The mechanistic/mammalian target of rapamycin (mTOR) is an evolutionally conserved serine/threonine kinase in eukaryotes, and it has been implicated in various physiological processes, such as growth, proliferation, survival, autophagy, and differentiation (Saxton and Sabatini, 2017). mTOR forms two separate complexes, designated mTOR complexes 1 and 2 (mTORC1 and mTORC2), which differ in their mechanisms of activation and downstream targets as well as in their composition (Brown et al., 1994; Sabatini et al., 1994). The RAPTOR subunit is specific to the mTORC1 complex, whereas the RICTOR subunit is specific to mTORC2 (Jacinto et al., 2004; Nojima et al., 2003). Tuberous sclerosis complex 1 (Tsc1) and complex 2 (Tsc2) are critical negative regulators of mTORC1 through their GTPase-activating protein activities toward the small G-protein Ras homolog enriched in the brain (Long et al., 2005). mTORC1 stimulates translation by phosphorylating downstream targets, including the eukaryotic translation initiation factor 4E (eIF4E)-binding proteins (4E-BPs) and the ribosomal protein S6 kinases (S6ks), in response to various extracellular signals and intracellular cues. S6ks phosphorylate components of the translational machinery and associated factors, such as ribosomal protein S6, eIF4B, and programmed cell death protein 4, leading to promotion of mRNA translation initiation (Dorrello et al., 2006; Holz et al., 2005). Phosphorylation of 4E-BPs by mTORC1 leads to their dissociation from elf4E, thereby allowing association of elf4E with elf4G and assembly of the elf4E translation initiation complex at the mRNAS′ end (Brunn et al., 1997; Gingras et al., 1999).

Although global deletion of Mtor or Raptor results in embryonic lethality, cell-specific deletion strategies have revealed that mTORC1 is implicated in the pathogenesis of various diseases, including cancer, obesity, and cardiovascular disease, in addition to its physiological functions (Guertin et al., 2006; Murakami et al., 2004; Saxton and Sabatini, 2017). Genetic research recently revealed the critical role of mTORC1 in skeletogenesis through its expression in chondrocytes. This study showed that inactivation of mTORC1 signaling, by deleting either Mtor or Raptor, significantly diminished embryonic skeletal growth, causing delays in chondrocyte hypertrophy and bone formation (Chen and Long, 2014). Hyperactivation of mTORC1 by Tsc1 deletion in chondrocytes led to condrodysplasia with an uncoupling of the normal proliferation and differentiation program within the growth plate (Yan et al., 2016). Moreover, Raptor deficiency reduced the size of limb bud cells, resulting in overall diminution of the limb bud (Jiang et al., 2017). On the contrary, the precise underlying mechanisms of mTORC1 in skeletogenesis through its expression in skeletogenic progenitor cells...
(undifferentiated mesenchymal cells before chondrogenic mesenchymal condensation has occurred) remain unclear.

In our investigation of mTORC1 and its role in skeletogenesis, we found that the cartilage and bones of the appendicular skeleton were markedly diminished in Raptor-deficient mice. These mice were generated by a paired-related homeobox 1 (Prx1)-Cre-mediated recombination, which took place in undifferentiated limb bud mesenchymal cells before chondrogenic mesenchymal condensation occurred. Subsequent analyses determined that mTORC1 directly accelerates the translation of the sex-determining region Y-type high-mobility group box protein 9 (Sox9) RNA, which harbors the 5′ terminal oligopyrimidine tract (5′ TOP) motif in its 5′ UTR. This occurs through the phosphorylation of 4E-BPs, but not S6Ks in undifferentiated mesenchymal cells. Moreover, we uncovered that SOX9 is a critical mediator of mTORC1-dependent skeletogenesis and mesenchymal condensation in vivo and in vitro. Our results demonstrate that the translational control of Sox9 is an essential pathway through which the mTORC1 pathway regulates skeletal development.

RESULTS

Skeletal Development Requires the Expression of mTORC1 in Undifferentiated Mesenchymal Cells Prior to Chondrogenic Mesenchymal Condensation

To reveal the in vivo physiological role of mTOR in skeletal development, we inactivated the Raptor gene in undifferentiated limb mesenchymal cells or whole chondrocytes using Prx1-Cre transgenic mice (Logan et al., 2002) or collagen type II α1 (Col2a1)-Cre transgenic mice (Terpstra et al., 2003).

Prx1-Cre;Raptorfl/fl embryos at 18.5 days post-coitum (E18.5) had very short limbs and a deficiency of some of the craniofacial bones, but their appearance was otherwise normal. Staining of skeletal preparations from these embryos with Alcian blue and alizarin red indicated a marked shortage of all skeletal components in forelimbs and hindlimbs (Figures 1A and 1B), recapitulating previous findings that ablation of Raptor or Mtor in limb bud progenitors leads to short limbs (Chen and Long, 2014). On the contrary, Col2a1-Cre;Raptorfl/fl embryos were characterized by a very severe and generalized chondrodysplasia. Alcian blue and alizarin red staining of skeletal preparations of E18.5 mutant embryos indicated that all the skeletal elements formed by endochondral ossification, including ribs, limbs, and vertebrae, were markedly smaller than embryos with typical growth (Figures 1A and 1C). In contrast, skeletal elements formed by intramembranous ossification lacked significant abnormalities. Histological analyses also revealed abnormalities in the long bones of both Prx1-Cre;Raptorfl/fl embryos and Col2a1-Cre;Raptorfl/fl embryos at E18.5 (Figure 1D), indicating mTORC1 inactivation by Raptor deficiency. However, cell proliferation (PCNA-positive cells) of growth plate chondrocyte was comparable between wild-type (WT) and mutant embryos (Figure S1B), which is consistent with a previous study that showed normal cell proliferation of Raptor-deficient growth plate chondrocyte by bromodeoxyuridine-labeling assay (Chen and Long, 2014).

While defects in the formation of forelimbs and hindlimbs were already observed at E16.5 in both Prx1-Cre;Raptorfl/fl and Col2a1-Cre;Raptorfl/fl embryos (Figures 1E, 1G, and 1H), these types of defects were observed previously at E14.5 only in Prx1-Cre;Raptorfl/fl embryos (Figures 1F and 1I). Col2a1-Cre;Raptorfl/fl embryos were distinguishable from WT embryos at E14.5 (Figures 1F and 1J).

Collectively, these results indicate that mTORC1 has essential roles in skeletal development both through its expression in undifferentiated mesenchymal cells prior to mesenchymal condensation, and through its expression in chondrocytes, as has been reported previously (Chen and Long, 2014; Jiang et al., 2017).

mTORC1 Controls Mesenchymal Condensation and SOX9 Protein Levels in Mesenchymal Cells

Further characterizing the precise role of mTORC1 expression in undifferentiated mesenchymal cells, scanning...
electron microscope (SEM) analysis showed that no distinct digit formation was observed in limb buds of Prx1-Cre;Raptorfl/fl embryos at E12.5 (Figure 2A). Moreover, the numbers of Alcian blue-positive cells were decreased significantly by Raptor deficiency in an in vitro high-density micromass culture of limb bud cells prepared from E12.5 embryos (Figures 2B and 2C). These results indicate no discernible chondrogenic mesenchymal condensations in limb buds of Prx1-Cre;Raptorfl/fl embryos at E12.5.

In Raptor-deficient mesenchymal cells, no significant changes were seen in the mRNA expressions of Sox5, Sox6, Sox9, or Runx2, pivotal transcription factors for skeletogenesis (Akiyama et al., 2002; Smits et al., 2001; Takarada et al., 2016), compared with expressions in WT cells.
### A

| Protein | PP242 (2.5 μM) | Rapamycin (10 nM) |
|---------|----------------|-------------------|
|         | 0   | 3   | 6   | 12  | 0   | 3   | 6   | 12  |
| p-p70S6K|     |     |     |     |     |     |     |     |
| p70S6K  |     |     |     |     |     |     |     |     |
| p-4EBP1 |     |     |     |     |     |     |     |     |
| 4EBP1   |     |     |     |     |     |     |     |     |
| SOX9    |     |     |     |     |     |     |     |     |
| RUNX2   |     |     |     |     |     |     |     |     |
| SOX6    |     |     |     |     |     |     |     |     |
| β-ACTIN |     |     |     |     |     |     |     |     |

### B

SOX9 expression (% of 0 hour)

- 0 hour
- 3 hour
- 6 hour
- 12 hour

### C

SOX9 expression (% of 0 hour)

- 0 hour
- 3 hour
- 6 hour
- 12 hour

### D

| Treatment | Control | CA-S6K | sh4EBP1/2 |
|-----------|---------|--------|------------|
| PP242     | -       | +      | -          |
| SOX9      |         |        |            |
| 4EBP1     |         |        |            |
| 4EBP2     |         |        |            |
| p-p70S6K  |         |        |            |
| β-ACTIN   |         |        |            |

### E

SOX9 expression (% of Control)

- Control
- CA-S6K
- sh4EBP1/2

### F

Control

- CA-S6K

- sh4EBP1/2

### G

Alcian blue intensity (% of Control)

- Control
- CA-S6K
- sh4EBP1/2

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In contrast, SOX9 protein expression was markedly decreased in Raptor-deficient cells, despite the lack of change in RUNX2 and SOX6 protein expression (Figure 2E), in accordance with the repression of the downstream phosphorylation of mTORC1, p70S6K, and 4E-BP1 by Raptor deficiency. Moreover, Sox9 mRNA expression in limb buds was comparable between WT and Prx1-Cre; PP242-treated cells transfected with empty vector or (G) Raptor post-hoc determined using the one-way ANOVA with the Bonferroni (Figure 2E), in accordance with the repression of the down-lack of change in RUNX2 and SOX6 protein expression in mutant embryos compared with WT embryos (Figure 2F). Moreover, the level of COL2, the transcriptional target of SOX9, also decreased in mutant limb buds (Figure S1C).

These results indicate that mTORC1 markedly controls mesenchymal condensation and preferentially regulates SOX9 protein levels, but not mRNA levels, through its expression in undifferentiated mesenchymal cells in vitro and in vivo.

The Pathway for 4E-BPs Is Implicated in the mTORC1-Dependent Control of SOX9 Protein Levels and Mesenchymal Condensation

mTORC1 is well known for controlling the translation of specific mRNAs by phosphorylating downstream substrates, including S6Ks and 4E-BPs. S6K1 phosphorylates and activates several substrates that promote mRNA translation initiation, including elf4E, which positively regulates the 5’ cap binding elf4F complex, while 4E-BPs inhibit translation by binding and sequestering elf4E to prevent assembly of the elf4F complex (Gingras et al., 1999; Holz et al., 2005).

To investigate whether mTORC1 specifically regulates the translation of Sox9 mRNA, mesenchymal cells were treated with PP242, an active-site mTOR inhibitor, or rapamycin, an allosteric mTOR inhibitor, followed by subsequent examination of protein expression. Treatment with PP242 decreased the phosphorylation of both 4E-BP1 and p70S6K, while rapamycin selectively repressed the phosphorylation of p70S6K. Under these experimental conditions, SOX9 protein level was repressed by PP242, but not by rapamycin, despite no changes in protein levels for RUNX2 and SOX6 (Figures 3A–3C).

Next, we determined genetically which pathways are associated with the regulation of SOX9 protein levels by mTORC1. To that end, mesenchymal cells were retrovirally infected with the constitutive active form of S6K (CA-S6K) or sh4E-BP1/2, and then subsequently treated with PP242, followed by determination of SOX9 expression. PP242 treatment decreased SOX9 protein levels in CA-S6K-infected cells, as was observed in control cells, but a double knockdown of 4E-BP1 and 4E-BP2, which have functional redundancy, significantly prevented the PP242-induced repression of SOX9 protein levels (Figures 3D and 3E).

In accordance with SOX9 expression, the repression of mesenchymal condensation was significantly prevented by the double knockdown of 4E-BP1 and 4E-BP2, but not by CA-S6K in Raptor-deficient cells (Figures 3F and 3G).

Altogether, these pharmacological and genetic analyses indicated that mTORC1-dependent control of Sox9 mRNA translation and mesenchymal condensation could be primarily mediated through the 4E-BPs pathway, but not the S6K pathway.

Translation of Sox9 RNA Is Controlled by mTORC1

It has been suggested recently that mTORC1 translationally controls the clear majority of mRNAs possessing a 5’ TOP and/or a pyrimidine-rich translational element (Hsieh et al., 2012; Thoreen et al., 2012). TOP mRNAs are defined as those with a cytidine immediately after the 5’ cap, followed by an uninterrupted stretch of 4–14 pyrimidine nucleotides, and they tend to encode proteins associated with translation (Hay and Sonenberg, 2004; Meyuhas, 2000). We computationally analyzed 5’ flanking regions of genes related to skeletogenesis, and then identified 5’ TOP or TOP-like motifs in the 5’ flanking region of Sox9, Sox6, and Runx2. This has been seen in eukaryotic elongation factor 2 (Eef2), an established TOP mRNA, but not in Sox5 (Figure 4A). In the Sox9 5’ flanking region, 5’ TOP or TOP-like motifs were conserved in different species (Figure 4B). To reveal whether 5’ TOP or TOP-like motifs of Sox9, Sox6, and Runx2 contribute to the mTORC1-dependent
A  Eef2  ...CACCGGTCTCTTCCGCCTGGA...
Sox9  ...TGGTTTCCTCCCTCCTCTCTGCT...
Sox6  ...AACACTTTTAAAGTTG...
Sox5  No putative sequence
Runx2  ...GGGACTCTCCCTTACAGCT...

B  Sox9 (Mouse)  ...TGTTTTTCCCTTCCTCTCTCTGCT...
Sox9 (Human)  ...TTTTTCTCCCTCTCTCTCTCTCT...
Sox9 (Rat)  ...TGTTTTTCCCTTCCTCTCTCTCT...
Sox9 (Chimpanzee)  ...TTTTTTTTTTCTCTCTCTCTCTCTCT...
Sox9 (Chiken)  ...TTTTTTTCTCTCTCTCTCTCTCT...

C  1 kb upstream of translation initiation site containing promoter and 5'UTR renilla luciferase
Sox9 WT, Sox9 GPM, Sox5, Runx2

D  Sox9 WT  ...TGTTTTTCCCTTCCTCTCTCTCT...
Sox9 GPM  ...TGTTTTTCCCTTCCTCTCTCTCT...

E  Control  PP242

F  Control  PP242

G  Control  Raptor-/-

H  Control

T  40S  60S  80S
Polysomes

I  Control  Raptor-/-

J  Control  Raptor-/-

Sox9 RNA
Runx2 RNA

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translational control of these mRNAs, reporter vectors were constructed by cloning the 1-kb fragment upstream of the translation initiation site containing the promoter and the 5′ UTR of each gene into the upstream section of the open reading frame encoding Renilla luciferase (designated as 5′ UTR-Luc) (Figure 4C). The PP242 treatment abolished reporter activities of Eef2 and Sox9, but not of Runx2 and Actb (Figures 4E and 4F). On the contrary, PP242 did not alter the reporter activity of Sox9 with the mutation in the 5′ TOP sequence (Sox9TOPM) (Figures 4D and 4E). Furthermore, Raptor deficiency impaired the reporter activities of Eef2 and Sox9, but not of Runx2 and Actb (Figure 4G).

To directly investigate if mTORC1 controls the translation of Sox9 mRNA, polysomes from WT cells and Raptor-deficient cells were sedimented in sucrose density gradients to efficiently separate 40S, 60S, 80S, and polysomes, followed by a determination of the distribution of RNA contents. As shown in Figure 4H, Raptor-deficient cells showed a decrease in polysome content compared with WT cells. Next, we assessed the effects of Raptor deficiency on the distribution of RNA contents in Sox9, Runx2, Sox5, Sox6, Actb, and Eef2. Raptor deficiency caused a significant decrease in Sox9 RNA contents in polysome fractions, but led to an increase in the 40S-80S fractions (Figure 4I), corresponding to the alteration of RNA distribution in Eef2 (Figure S2D). Showing consistency with the results of no changes in 5′ UTR-Luc activities in Runx2 and Sox6, and no putative 5′ TOP or TOP-like motifs in Sox5 (Figures 4A, 4F, and 4G), Raptor deficiency failed to shift the distribution of RNA contents in Runx2 (Figure 4J), Sox5, Sox6, in addition to Actb mRNA (Figures S2A–S2C). These results indicate that mTORC1 preferentially controls the translation of Sox9 mRNA, but not of Runx2, Sox6, and Sox5 mRNA.

SOX9 Contributes to mTORC1-Dependent Mesenchymal Condensation and Skeletal Development

Given that SOX9 is essential for mesenchymal condensation and skeletal development (Akiyama et al., 2002), and that translational control of Sox9 mRNA occurs through mTORC1 (Figure 4), we next determined whether the control of Sox9 translation underlines the effects of the mTORC1 pathway on mesenchymal condensation and skeletal development. To that end, we used a Sox9 cDNA lacking the 5′ UTR that harbors 5′ TOP elements. Consistent with previous findings, Sox9 overexpression increased the intensity of Alcian blue in WT cells, but, more importantly, Sox9 overexpression significantly attenuated the repression of mesenchymal condensation by Raptor deficiency (Figures SA and S5B).

Finally, we examined whether this is genetically viable in vivo. To that end, the Cre-inducible Sox9 transgene with the human cytomegalovirus enhancer (hereafter referred to as Sox9OE), which lacks the 5′ TOP elements of Sox9, was introduced into Prx1-Cre;Raptorfl/fl embryos. As is evident in Figures 5C–5E, axial skeletal abnormalities in Prx1-Cre;Raptorfl/fl embryos were markedly rescued by the forced conditional expression of Sox9 (Prx1-Cre;Raptorfl/fl; Sox9OE/OE embryos) at E18.5. Moreover, SEM analysis of limb buds at E12.5 showed a distinct digit formation in the limbs of Prx1-Cre;Raptorfl/fl; Sox9OE/OE embryos, as was seen in control embryos (Figure 5G), but no distinct digit formation was seen in the limb buds of Prx1-Cre;Raptorfl/fl embryos.

Taken together, these data provide genetic evidence that SOX9 can contribute to mTORC1-dependent mesenchymal condensation and skeletal development in vitro and in vivo.

DISCUSSION

Although previous genetic and pharmacological studies have revealed the crucial role of mTORC1 in skeletal development (Chen and Long, 2014; Jiang et al., 2017; Phornphutkul et al., 2008, 2009; Yan et al., 2016), here we provide evidence demonstrating the critical role of the mTORC1/4E-BPs/SOX9 axis in skeletogenesis. Although...
it is well-established that Sox9/Sox9 is the subject of many types of regulatory mechanisms, including epigenetic and transcriptional regulation of its gene, post-transcriptional regulation of its RNA, and post-translational modifications of its protein (Akiyama and Lefebvre, 2011; Kozhemyakina et al., 2015; Lefebvre and Dvir-Ginzberg, 2017), no previous literature has reported the translational control of Sox9 RNA thus far. Herein, using a battery of well-established biochemical and functional assays, several lines of evidence indicate that the mTORC1/4E-BPs cascade regulates the translation of Sox9 RNA in undifferentiated mesenchymal cells. First, Raptor deficiency markedly abolished the Sox9 protein, but not Sox9 mRNA, in both mesenchymal cells and limb buds (Figure 2). Second, an active-site mTOR inhibitor, PP242, suppressed the reporter activity of 5′ UTR-Sox9-1uc, which harbors its promoter and the 5′ UTR, including the 5′ TOP motif (Figure 4). Third, and most importantly, Raptor deficiency abolished the Sox9 RNA contents in polysome fractions (Figure 4). However, given that mTORC1 controls protein catabolism, most notably by autophagy, and protein turnover by a ubiquitin-proteasome system (Kim et al., 2011a; Martina et al., 2012; Zhao et al., 2015), the possibility that Raptor deficiency may affect catabolism and/or turnover of the Sox9 protein in mesenchymal cells should not be excluded.

Prx1-Cre;Raptorfl/fl embryos showed a marked loss of all skeletal components in both fore- and hindlimbs (Figure 1), resembling the mutant mice that developed from the inactivation of Sox9 in undifferentiated limb bud mesenchymal cells (Prx1-Cre;Sox90/0 embryos) (Akiyama et al., 2002). However, the phenotype of the Raptor-deficient embryos was milder than that caused by the Sox9 deficiency in terms of axial skeletal abnormalities. In addition, in vivo introduction of Sox9 did not completely rescue the loss of skeletal components in Prx1-Cre;Raptorfl/fl embryos. We, therefore, cannot exclude the possibility that another pathway also may contribute to the regulation of mTORC1-dependent skeletogenesis. Indeed, it has been reported that mTORC1 controls bone development through the S6K1/RUNX2 axis in pre-osteoblasts (Dai et al., 2017) or the S6K1/Gli2 axis in chondrocytes (Yan et al., 2016), despite no significant changes in the expression of Runx2 in Raptor-deficient mesenchymal cells (Figure 2), and no significant rescue of mesenchymal condensation by introducing CA-S6K into Raptor-deficient cells (Figure 3) in this study.

Rapamycin, an allosteric mTOR inhibitor, potently prevents the phosphorylation of S6K1, while it has modest or little inhibitory effect on the phosphorylation of 4E-BP1. On the contrary, PP242, an active-site mTOR inhibitor, inhibits the phosphorylation of both S6K1 and 4E-BP1, indicating that PP242 could impair the translation of RNA to a much greater degree than could rapamycin, largely owing to their inhibition of rapamycin-resistant functions of mTORC1 (Choo et al., 2008; Thoreen et al., 2009).

It has previously been shown that discrete Sox9 mRNA expression domain demarcating digit primordia do not appear in Raptor-deficient embryos at E11.75 (Jiang et al., 2017), whereas we demonstrated that Sox9 mRNA expression in limb buds was comparable between WT and Prx1-Cre;Raptorfl/fl embryos at E11.5 (Figure 2). The discrepancy may be probably explained by the different embryonic stages analyzed.

In conclusion, the mTORC1/4E-BPs pathway is critical for skeletogenesis in vivo by modulating the translation of Sox9 mRNA through its expression in undifferentiated mesenchymal cells. Although further studies should be performed to determine how mTORC1 activity is regulated in undifferentiated mesenchymal cells, BMP and FGF signals could be candidates among multiple signals to modulate mTORC1 activity, in addition to hormonal and nutrient signals (Lin et al., 2011; Wang et al., 2017). Our findings may contribute to an improved understanding of the molecular mechanisms underlying skeletal development, as well as opportunities for the development of drugs targeting cartilage diseases associated with the abnormal expression or functioning of SOX9 in humans, such as campomelic dysplasia (Wagner et al., 1994).
SOX9 as well as mTORC1, is associated with tumorigenesis in several cancers (Grabiner et al., 2014; Jo et al., 2014; Pritchett et al., 2011; Sato et al., 2010), identifying the mTORC1/4E-BPs/SOX9 cascade as a prominent candidate for developing drugs targeting various human diseases.

**EXPERIMENTAL PROCEDURES**

**Mice**

Raptorfl/fl and Sox9fl/fl mice were crossed with either Prx1-Cre or Col2a1-Cre mice (Kim et al., 2011b; Logan et al., 2002; Terpstra et al., 2003). These mutant mice were backcrossed for more than five generations with C57BL/6j. Mice were bred under standard animal housing conditions at 23°C ± 1°C with humidity of 55% and a light/dark cycle of 12 hr, with free access to food and water. Genotyping was performed by PCR using tail genomic DNA. The study protocol meets the guidelines of the Japanese Pharmacological Society and was approved by the Committee for Ethical Use of Experimental Animals at Kanazawa University, Kanazawa, Japan. The numbers of animals used per experiment are stated in the figure legends.

**Skeletal Preparation and Histological Analyses**

Embryos were eviscerated and the skins were removed, and subsequent fixation in 95% ethanol overnight. Embryos were then immersed in Alcian blue solution overnight, and were transferred in a solution of 2% KOH, followed by staining in alizarin red solution overnight. Finally, the skeletons were cleaned with 1% KOH/20% glycerol and then stored in 50% ethanol/50% glycerol (Iezaki et al., 2016).

Mouse tibiae were fixed with 10% formalin neutral buffer solution, embedded in paraffin, and then sectioned at a thickness of 5 μm. Sections were stained with safranin O and von Kossa (Wang et al., 2005). Sections were incubated in a blocking buffer for 1 hr, followed by incubation with anti-phospho-4EBP1 antibody (no. 2855) or anti-phospho-p70S6K1 antibody (no. 9205), anti-phospho-4EBP1 (no. 2855), anti-4EBP1 (no. 9644), and anti-4EBP2 (no. 2845) (Cell Signaling Technologies), anti-4EBP1 (no. 9644), and anti-4EBP2 (no. 2845) (Cell Signaling Technologies), anti-β-actin (no. sc-47778) and anti-SOX6 (no. sc-17332) (Santa Cruz Biotechnology), anti-SOX9 (no. AB5535) (EMD Millipore).

**Generation of Retroviral Vectors and Infection**

pMX-CA-S6K and pMX-Sox9 vectors were generated by subcloning into pMX vector from pRK7-HA-S6K1-F5A-E389-R3A (no. 8991) and pWPXL-Sox9 (no. 36979) vectors, respectively, which were obtained from Addgene. The oligonucleotides for 4E-BP1 short hairpin RNA (shRNA) and 4E-BP2 shRNA were synthesized (Table S1), annealed, and inserted into the RNAi-Ready pSIREN-RetroQ vector. These vectors were then transfected into PLAT-E cells using the calcium carbonate method. Virus supernatant was collected 48 hr after transfection and cells were then infected with this viral supernatant in the presence of 4 μg/mL polybrene (Fukasawa et al., 2016).

**Micromass Culture System**

Limbs bud mesenchymal cells from E12.5 were treated with 0.1% collagenase and 0.1% dispase for 1.5 hr at 37°C, and subsequently resuspended in DMEM/F12 medium containing 10% fetal bovine serum (FBS) at 1.5 × 10^7 cells/mL. Droplets were carefully placed in each well interior of a 4-well plate. Cells were allowed to adhere at 37°C for 1.5 hr, followed by the addition of chondrogenic medium (DMEM/F12 medium containing 10% FBS and 50 μg/mL ascorbic acid). Medium was changed every 2 days, and Alcian blue stain was performed on day 6.

**Real-Time qPCR**

Total RNA was extracted from cells, followed by the synthesis of cDNA using reverse transcriptase and oligo-dT primer. The cDNA samples were then used as templates for real-time PCR analysis, which was performed on an MX3005P qPCR System (Agilent Technologies) using specific primers for each gene (Table S2). Expression levels of the genes examined were normalized using Actb expression as an internal control for each sample.

**Immunoblotting Analysis**

Cells were solubilized in lysis buffer containing 1% Nonidet P-40. Samples were then subjected to SDS-PAGE, followed by transfer to polyvinylidene difluoride membranes and subsequent immunoblotting. The primary antibodies used were, anti-RUNX2 (no. 12556), anti-RAPTOR (no. 2280), anti-phospho-p70S6K1 (no. 9205), anti-p70S6K1 (no. 2708), anti-phospho-4EBP1 (no. 2855), anti-4EBP1 (no. 9644), and anti-4EBP2 (no. 2845) (Cell Signaling Technologies), anti-β-actin (no. sc-47778) and anti-SOX6 (no. sc-17332) (Santa Cruz Biotechnology), anti-SOX9 (no. AB5535) (EMD Millipore).

**Luciferase Assay**

Plasmids pIS1 (no. 12179), pIS1-Eef25UTR-renilla (no. 38235), and pIS1-Actb5UTR-renilla (no. 38234) were obtained from Addgene, and plasmids 5′ UTR-Runx2-luc, 5′ UTR-Sox6-luc and 5′ UTR-Sox9-luc were generated by PCR-based cloning with mouse genomic DNA. To generate 5′ UTR-Sox9TOPM-luc, site-directed mutagenesis was performed using the PrimeSTAR Max Mutagenesis Basal Kit (Takara Bio) with specific primers (Table S3). For the luciferase assay, cells were transfected with reporter vectors using the lipofection method as previously described (Hinoi et al., 2014), followed by the preparation of cell lysates and subsequent determination of luciferase activity using specific substrates in a luminometer (ATTO). Transfection efficiency was normalized by determining the LacZ activity.

**Polysome Fraction**

Cells were incubated with 100 μg/mL cycloheximide for 5 min at 37°C, and subsequently solubilized in lysis buffer (20 mM HEPES-NaOH [pH 7.5], 100 mM KCl, 10 mM MgCl2, 1 mM DTT, 0.25% NP-40, 100 μg/mL cycloheximide) containing protease inhibitor cocktail and RNase inhibitor. The lysate was clarified by centrifugation at 14,000 rpm for 10 min, and the supernatant was then loaded onto ultracentrifuge tubes containing sucrose gradient buffer (10%–40% sucrose) and centrifuged at 40,000 rpm for 2.5 hr with an SW41 rotor. Fraction samples were collected and RNA was extracted with TRI Reagent LS (Sigma). The RNA samples were used for real-time PCR analysis using KAPA SYBR Universal One-Step RT-qPCR Kit (KAPA Biosystems) with specific primers for each gene (Table S4).
**SEM Observation with Ionic Liquid**

Ionic liquid (IL), HILEM IL1000 (Hitachi High-Technologies) was used as coating reagent as described in previous papers (Ishigaki et al., 2011; Tsuda et al., 2011). In brief, this IL was mixed with pure water prior to use. The mixture was then pre-warmed at 40°C in a block heater to decrease its viscosity. This IL-water mixture was dropped onto the fixed samples and left for 1 min at room temperature. Excess IL was absorbed by paper and blowing away by a hairdryer. Then, the sample was evenly pasted onto an electrical conductive tape (Nisshin EM) that was put on an aluminum sample stub for SEM. A Hitachi S3400N SEM (Hitachi High-Technologies) was used in this study.

**Whole-Mount In situ Hybridization and Immunohistochemistry**

For whole-mount in situ hybridization, limb buds were fixed with 4% paraformaldehyde, dehydrated in methanol, rehydrated by a series of methanol/PBS gradient, and treated with 0.2 M HCl and 10 μg/mL proteinase K. Limb buds were then subjected to acetylation in 0.1 M triethanolamine with 0.25% acetic anhydride. After prehybridization, limb buds were incubated with digoxigenin-labeled cRNA probe at 60°C for 16 hr and subsequently treated with 4 μg/mL RNase A. Limb buds were further incubated with anti-digoxigenin-ALP Fab fragments for 16 hr, followed by treatment with NBT/BCIP.

For whole-mount immunohistochemistry, limb buds were fixed with methanol:DMSO (4:1) overnight, and washed three times with methanol:DMSO (4:1) overnight, and washed three times with 1% Triton X-100/PBS. After treatment with blocking buffer (PBS containing 1% Triton X-100 and 5% normal goat serum) for 1 hr, limb buds were incubated with anti-SOX9 antibody (no. AB5535) (EMD Millipore) or anti-COL2 antibody (no. ab34712) (Abcam) in blocking buffer for 24 hr. Limb buds were further incubated with Alexa Fluor 488 anti-Rabbit IgG (no. A11008) (Invitrogen) in blocking buffer for 48 hr.

**Data Analysis**

All results are expressed as the mean ± SEM, and statistical significance was determined using the two-tailed, unpaired Student’s t test or one-way ANOVA with the Bonferroni post-hoc test.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes two figures and four tables and can be found with this article online at [https://doi.org/10.1016/j.stemcr.2018.05.020](https://doi.org/10.1016/j.stemcr.2018.05.020).

**AUTHOR CONTRIBUTIONS**

T.I. and E.H. conceived and designed the study. T.I., T.H., K.F., Y.N., G.P., Y.O., and K.O. performed in vivo experiments. T.I., T.H., K.F., M.K., T.K., M.H., and Y.K. performed in vitro experiments. K.K., T.M., Y.I., and M.O. discussed the results, conceived some experiments, and provided critical reagents and comments. T.I. and E.H. wrote the manuscript.

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**REFERENCES**

Akiyama, H., and Lefebvre, V. (2011). Unraveling the transcriptional regulatory machinery in chondrogenesis. J. Bone Miner. Metab. 29, 390–395.

Akiyama, H., Chaboissier, M.C., Martin, J.F., Schedl, A., and de Crombrugghe, B. (2002). The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev. 16, 2813–2828.

Brown, E.J., Albers, M.W., Shin, T.B., Ichikawa, K., Keith, C.T., Lane, W.S., and Schreiber, S.L. (1994). A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature 369, 756–758.

Brunn, G.J., Hudson, C.C., Sekulic, A., Williams, J.M., Hosoi, H., Houghton, P.J., Lawrence, J.C., Jr., and Abraham, R.T. (1997). Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. Science 277, 99–101.

Chen, J., and Long, F. (2014). mTORC1 signaling controls mammalian skeletal growth through stimulation of protein synthesis. Development 141, 2848–2854.

Choo, A.Y., Yoon, S.O., Kim, S.G., Roux, P.P., and Blenis, J. (2008). Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation. Proc. Natl. Acad. Sci. USA 105, 17414–17419.

Dai, Q., Xu, Z., Ma, X., Niu, N., Zhou, S., Xie, F., Jiang, L., Wang, J., and Zou, W. (2017). mTOR/Raptor signaling is critical for skeletogenesis in mice through the regulation of Runx2 expression. Cell Death Differ. 24, 1886–1899.

Dorrello, N.V., Peschiaroli, A., Guardavaccaro, D., Colburn, N.H., Sherman, N.E., and Pagano, M. (2006). Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. Science 277, 99–101.

Fukasawa, K., Park, G., Iezaki, T., Horie, T., Kanayama, T., Ozaki, K., Onishi, Y., Takahata, Y., Yoneda, Y., Takarada, T., et al. (2016). ATF3 controls proliferation of osteoclast precursor and bone remodeling. Sci. Rep. 6, 30918.

Grabiner, B.C., Nardi, V., Birsoy, K., Possemato, R., Shen, K., Sinha, S., Jordan, A., Beck, A.H., and Sabatini, D.M. (2014). A diverse array of cancer-associated MTOR mutations are hyperactivating and can predict rapamycin sensitivity. Cancer Discov. 4, 554–563.
Guertin, D.A., Stevens, D.M., Thoreen, C.C., Burds, A.A., Kalaany, N.Y., Moffat, J., Brown, M., Fitzgerald, K.J., and Sabatini, D.M. (2006). Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt–FOXO and PKCalpha, but not S6K1. Dev. Cell 11, 859–871.

Hay, N., and Sonenberg, N. (2004). Upstream and downstream of mTOR. Genes Dev. 18, 1926–1945.

Hinoi, E., Nakamura, Y., Takada, S., Fujita, H., Iezaki, T., Hishizume, S., Takahashi, S., Odaka, Y., Watanabe, T., and Yoneda, Y. (2014). Growth differentiation factor-5 promotes brown adipogenesis in systemic energy expenditure. Diabetes 63, 162–175.

Holz, M.K., Ballif, B.A., Gygi, S.P., and Blenis, J. (2005). mTOR and the translational landscape of mTOR signalling steers cancer initiation and metastasis. Nature 435, 55–61.

Iezaki, T., Ozaki, K., Fukasawa, K., Inoue, M., Kitajima, S., Muneta, T., Takeda, S., Fujita, H., Onishi, Y., Horie, T., et al. (2016). ATF3 deficiency in chondrocytes alleviates osteoarthritic development. J. Pathol. 239, 426–437.

Ishigaki, Y., Nakamura, Y., Takehara, T., Nemoto, N., Kurihara, T., Koga, H., Nakagawa, H., Takegami, T., Tomosugi, N., Miyazawa, S., et al. (2011). Ionic liquid enables simple and rapid sample preparation of human culturing cells for scanning electron microscope analysis. Microsc. Res. Tech. 74, 415–420.

Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Ruegg, M.A., Hall, A., and Hall, M.N. (2004). Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat. Cell Biol. 6, 1122–1128.

Jiang, M., Fu, X., Yang, H., Long, F., and Chen, J. (2017). mTORC1 signaling promotes limb bud cell growth and chondrogenesis. J. Cell Biochem. 118, 748–753.

Jo, A., Denduluri, S., Zhang, B., Wang, Z., Yin, L., Yan, Z., Kang, R., Shi, L.L., Mok, J., Lee, M.J., et al. (2014). The versatile functions of Sox9 in development, stem cells, and human diseases. Genes Dis. 1, 149–161.

Kim, J., Kundu, M., Violeet, B., and Guan, K.L. (2011a). AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat. Cell Biol. 13, 132–141.

Kim, Y., Murao, H., Yamamoto, K., Deng, J.M., Behringer, R.R., Nakamura, T., and Akiyama, H. (2011b). Generation of transgenic mice for conditional overexpression of Sox9. J. Bone Miner. Res. 29, 123–129.

Kozhemyakina, E., Lassar, A.B., and Zelzer, E. (2015). A pathway to bone: signaling molecules and transcription factors involved in chondrocyte development and maturation. Development 142, 817–831.

Lefebvre, V., and Dvir-Ginzberg, M. (2017). Sox9 and the many facets of its regulation in the chondrocyte lineage. Connect. Tissue Res. 58, 2–14.

Lin, X., Zhang, Y., Liu, L., McKeehan, W.L., Shen, Y., Song, S., and Wang, F. (2011). FRS2alpha is essential for the fibroblast growth factor to regulate the mTOR pathway and autophagy in mouse embryonic fibroblasts. Int. J. Biol. Sci. 7, 1114–1121.

Logan, M., Martin, J.F., Nagy, A., Lobe, C., Olson, E.N., and Tabin, C.J. (2002). Expression of Cre recombinase in the developing mouse limb bud driven by a Prxl enhancer. Genesis 33, 77–80.

Long, X., Lin, Y., Ortiz-Vega, S., Yonezawa, K., and Avruch, J. (2005). Rheb binds and regulates the mTOR kinase. Curr. Biol. 15, 702–713.

Martina, J.A., Chen, Y., Gucek, M., and Puertollano, R. (2012). mTORC1 functions as a transcriptional regulator of autophagy by preventing nuclear transport of TFE2. Autophagy 8, 903–914.

Meyuhas, O. (2000). Synthesis of the translational apparatus is regulated at the translational level. Eur. J. Biochem. 267, 6321–6330.

Murakami, M., Ichisaka, T., Maeda, M., Oshiro, N., Hara, K., Edenshofer, F., Kiyama, H., Yonezawa, K., and Yamanaka, S. (2004). mTOR is essential for growth and proliferation in early mouse embryos and embryonic stem cells. Mol. Cell. Biol. 24, 6710–6718.

Nojima, H., Tokunaga, C., Eguchi, S., Oshiro, N., Hidayat, S., Yoshino, K., Hara, K., Tanaka, N., Avruch, J., and Yonezawa, K. (2003). The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. J. Biol. Chem. 278, 15461–15464.

Phornphutkul, C., Wu, K.Y., Auyeung, V., Chen, Q., and Gruppuso, P.A. (2008). mTOR signaling contributes to chondrocyte differentiation. Dev. Dyn. 237, 702–712.

Phornphutkul, C., Lee, M., Voigt, C., Wu, K.Y., Ehrlich, M.G., Gruppuso, P.A., and Chen, Q. (2009). The effect of rapamycin on bone growth in rabbits. J. Orthop. Res. 27, 1157–1161.

Pritchett, J., Athwal, V., Roberts, N., Hanley, N.A., and Hanley, K.P. (2011). Understanding the role of SOX9 in acquired diseases: lessons from development. Trends. Mol. Med. 17, 166–174.

Sabatini, D.M., Erdjument-Bromage, H., Lui, M., Tempst, P., and Snyder, S.H. (1994). RAFT1: a mammalian protein that binds to yeast TORs. Cell 78, 35–43.

Sato, T., Nakashima, A., Guo, L., Coffman, K., and Tamaoki, F. (2010). Single amino-acid changes that confer constitutive activation of mTOR are discovered in human cancer. Oncogene 29, 2746–2752.

Saxton, R.A., and Sabatini, D.M. (2017). mTOR signaling in growth, metabolism, and disease. Cell 168, 960–976.

Smits, P., Li, P., Mandel, J., Zhang, Z., Deng, J.M., Behringer, R.R., de Crombrugghe, B., and Lefebvre, V. (2001). The transcription factors L-Sox5 and Sox6 are essential for cartilage formation. Dev. Cell 1, 277–290.

Takarada, T., Nakazato, R., Tsuchikane, A., Fujikawa, K., Iezaki, T., Yoneda, Y., and Hinoi, E. (2016). Genetic analysis of Rumi2 function during intramembranous ossification. Development 143, 211–218.

Terpstra, L., Prud’homme, J., Arabian, A., Takeda, S., Karsenty, G., Dedhar, S., and St-Arnaud, R. (2003). Reduced chondrocyte proliferation and chondrodysplasia in mice lacking the integrin-linked kinase in chondrocytes. J. Cell Biol. 162, 139–148.
Thoreen, C.C., Kang, S.A., Chang, J.W., Liu, Q., Zhang, J., Gao, Y., Reichling, L.J., Sim, T., Sabatini, D.M., and Gray, N.S. (2009). An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. J. Biol. Chem. 284, 8023–8032.

Thoreen, C.C., Chantranupong, L., Keys, H.R., Wang, T., Gray, N.S., and Sabatini, D.M. (2012). A unifying model for mTORC1-mediated regulation of mRNA translation. Nature 485, 109–113.

Tsuda, T., Nemoto, N., Kawakami, K., Mochizuki, E., Kishida, S., Tajiri, T., Kushibiki, T., and Kuwabata, S. (2011). SEM observation of wet biological specimens pretreated with room-temperature ionic liquid. Chembiochem 12, 2547–2550.

Wagner, T., Wirth, J., Meyer, J., Zabel, B., Held, M., Zimmer, J., Pasantes, J., Bricarelli, F.D., Keutel, J., Hustert, E., et al. (1994). Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. Cell 79, 1111–1120.

Wang, L., Hinoi, E., Takemori, A., Takarada, T., and Yoneda, Y. (2005). Abolition of chondral mineralization by group III metabotropic glutamate receptors expressed in rodent cartilage. Br. J. Pharmacol. 146, 732–743.

Wang, M.H., Zhou, X.M., Zhang, M.Y., Shi, L., Xiao, R.W., Zeng, L.S., Yang, X.Z., Zheng, X.F.S., Wang, H.Y., and Mai, S.J. (2017). BMP2 promotes proliferation and invasion of nasopharyngeal carcinoma cells via mTORC1 pathway. Aging 9, 1326–1340.

Yan, B., Zhang, Z., Jin, D., Cai, C., Jia, C., Liu, W., Wang, T., Li, S., Zhang, H., Huang, B., et al. (2016). mTORC1 regulates PTHrP to coordinate chondrocyte growth, proliferation and differentiation. Nat. Commun. 7, 11151.

Zhao, J., Zhai, B., Gygi, S.P., and Goldberg, A.L. (2015). mTOR inhibition activates overall protein degradation by the ubiquitin proteasome system as well as by autophagy. Proc. Natl. Acad. Sci. USA 112, 15790–15797.