Glucose Uptake and Runx2 Synergize to Orchestrate Osteoblast Differentiation and Bone Formation

Graphical Abstract

Highlights
- Osteoblasts are addicted to glucose
- Glucose uptake is needed for osteoblast differentiation and bone formation
- Runx2 is necessary for Glut1 expression in prospective osteoblasts
- Glut1 and Runx2 crosstalk determines osteoblast differentiation and bone formation

Authors
Jianwen Wei, Junko Shimazu, Munevver P. Makinistoglu, ..., Jeffrey E. Pessin, Eiichi Hinoi, Gerard Karsenty

Correspondence
gk2172@cumc.columbia.edu

In Brief
Osteoblasts paradoxically secrete the main constituent of the bone matrix before turning on their master differentiation gene Runx2. These cells are addicted to glucose, whose uptake coordinates osteoblast differentiation and bone formation through crosstalk with Runx2, revealing a key regulatory role for metabolic demand.

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**Glucose Uptake and Runx2 Synergize to Orchestrate Osteoblast Differentiation and Bone Formation**

Jianwen Wei,1,4 Junko Shimazu,1,4 Munever P. Makinistoglu,1 Antonio Maurizi,1 Daisuke Kajimura,1 Haihong Zong,2 Takeshi Takarada,3 Takashi Iezaki,3 Jeffrey E. Pessin,2 Eiichi Hinoi,3 and Gerard Karsenty1,*

1Department of Genetics and Development, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA
2Department of Medicine and Molecular Pharmacology, The Albert Einstein College of Medicine, Bronx, New York, NY 10461, USA
3Faculty of Pharmacy, Laboratory of Molecular Pharmacology, Institute of Medical, Pharmaceutical, and Health Sciences, Kanazawa University, Kanazawa, Ishikawa 920-1192, Japan
4Co-first author
*Correspondence: gk2172@cumc.columbia.edu

**SUMMARY**

The synthesis of type I collagen, the main component of bone matrix, precedes the expression of Runx2, the earliest determinant of osteoblast differentiation. We hypothesized that the energetic needs of osteoblasts might explain this apparent paradox. We show here that glucose, the main nutrient of osteoblasts, is transported in these cells through Glut1, whose expression precedes that of Runx2. Glucose uptake favors osteoblast differentiation by suppressing the AMPK-dependent proteasomal degradation of Runx2 and promotes bone formation by inhibiting another function of AMPK. While RUNX2 cannot induce osteoblast differentiation when glucose uptake is compromised, raising blood glucose levels restores collagen synthesis in Runx2-null osteoblasts and initiates bone formation in Runx2-deficient embryos. Moreover, RUNX2 favors Glut1 expression, and this feedforward regulation between RUNX2 and Glut1 determines the onset of osteoblast differentiation during development and the extent of bone formation throughout life. These results reveal an unexpected intricacy between bone and glucose metabolism.

**INTRODUCTION**

The transcription factor RUNX2 is a master determinant of osteoblast differentiation (Long, 2012; Karsenty et al., 2009). Its expression in prospective osteoblasts precedes osteoblast differentiation; its inactivation prevents osteoblast differentiation; and its haplo-insufficiency causes a skeletal dysplasia called cleidocranial dysplasia (CCD), which is characterized by a delay in osteoblast differentiation, leading to hypoplastic clavicles and open fontanelles. Several aspects of Runx2 biology, however, remain poorly understood. For example, the nature of the molecular events leading to RUNX2 accumulation in cells of the osteoblast lineage is largely unknown. Another question is to determine if and how RUNX2 contributes to bone formation by differentiated osteoblasts. A peculiar feature of osteoblast biology raises this latter issue.

Type I collagen is by far the most abundant protein of bone extracellular matrix (ECM), and its synthesis by osteoblasts is often considered a biomarker of bone formation. Type I collagen is a heterotrimeric protein made of two α1(I) chains and one α2(I) chain, which are encoded by two different genes (Vuorio and de Crombrugghe, 1990). In vitro, RUNX2 can bind to and upregulate the activity of an α1(I) Collagen promoter fragment (Kern et al., 2001). However, in vivo type I collagen synthesis precedes Runx2 expression in prospective osteoblasts. Thus, the regulation of type I collagen synthesis in osteoblasts is not fully understood, and by extension, since bone ECM is mainly made of type I collagen, it is also unclear how bone formation by osteoblasts is regulated.

Besides being responsible for bone formation, osteoblasts are endocrine cells that secrete the hormone osteocalcin, which favors glucose homeostasis (Lee et al., 2007). Notwithstanding the molecular complexity of this emerging regulation, the identification of bone as a regulator of glucose metabolism raises a fundamental question: why would bone have this role? A prerequisite to answering this question is to define the functions of glucose in osteoblasts.

Here, we asked if the energetic needs of the osteoblast might explain how osteoblast differentiation and bone formation occurs in vivo. We found that glucose is the main nutrient of osteoblasts and it is transported in these cells in an insulin-independent manner through the facilitative Glut1 glucose transporter whose expression precedes that of Runx2 during skeletogenesis. By inhibiting one activity of AMPK, glucose is necessary for RUNX2 accumulation and osteoblast differentiation; through the inhibition of another AMPK function, glucose is necessary for collagen synthesis and bone formation. Moreover, by promoting RUNX2 accumulation, glucose uptake in osteoblasts favors Osteocalcin expression and whole-body glucose homeostasis. We further show that RUNX2 is not sufficient for timely osteoblast differentiation and proper bone formation if glucose uptake is compromised, whereas raising blood glucose levels induces collagen synthesis and bone formation in the absence of Runx2. The relationship between RUNX2 and glucose uptake is even more...
elaborate since RUNX2 is needed for Glut1 expression in osteoblasts. This crosstalk between RUNX2 and glucose uptake acts as an amplification mechanism, allowing osteoblast differentiation and bone formation to be coordinated throughout life. This study provides a bone-centric illustration of the importance of crosstalk between bone and glucose metabolism.

RESULTS

Insulin-Independent Glucose Uptake in Osteoblasts
To determine the main nutrient(s) used by osteoblasts, we measured the oxygen consumption rate (OCR) of osteoblasts when incubated with individual nutrients. Like neurons and unlike myoblasts, osteoblasts had the highest OCR when cultured in the presence of glucose and the lowest when cultured in the presence of a representative fatty acid (Figure 1A). These results prompted us to measure, through euglycemic hyperinsulinemic clamps, the amount of glucose taken up by bone and the mechanism by which it occurs in 3-month-old wild-type (WT) mice.

In the conditions of this assay, bone takes up one-fifth of the quantity of glucose taken up by skeletal muscle, the tissue taking up the majority of glucose in the mouse (Ferrannini et al., 1988), and one-half of what is taken up by white adipose tissue (WAT) (Figure 1B). Unlike uptake for skeletal muscle and WAT, glucose uptake in bone is not enhanced by insulin (Figure 1B). We also compared the uptake of 2-[U-14C] deoxyglucose (2-DG) in osteoblasts and osteoclasts to that in myoblasts. Both bone cell types take up approximately one-third of the quantity of 2-DG taken up by myoblasts and do so in an insulin-independent manner (Figure 1C). Consistent with these observations, Glut1, which transports glucose in an insulin-independent manner, is expressed two orders of magnitude higher than any other class I glucose transporter in bone cells (Figure 1D). The rest of this study focuses on the functions of glucose in osteoblasts.

To establish the biological importance of Glut1 expression in these cells, we analyzed osteoblasts lacking or overexpressing modestly (1.75-fold) Glut1 (Figures S1A and S1B). Glut1–/– osteoblasts took up 75% less 2-DG, and a1(I)Col-Glut1 osteoblasts took up 20% more 2-DG than did control osteoblasts (Figure 1E). Consequently, glycogen content was decreased in Glut1–/– and increased 40% in a1(I)Col-Glut1 osteoblasts compared to controls (Figure S1C). Thus, Glut1 is responsible for the majority of glucose uptake in osteoblasts.

Glucose Uptake Is Necessary for Osteoblast Differentiation during Development
Next, we analyzed the spatial and temporal pattern of Glut1 expression during skeletogenesis by in situ hybridization and compared it to that of a1(I) Collagen and Runx2, two marker genes of mesenchymal cells and osteoblasts, of a1(I) Collagen, a marker of non-hypertrophic chondrocytes, and of a1(X) collagen, a marker of hypertrophic chondrocytes.

At E10.5 Glut1 is highly expressed in a1(I) Collagen-expressing mesenchymal cells of the developing hindlimbs (Figures 1Fa–1Ff), Runx2 is expressed in a1(I) Collagen/Glut1-expressing cells at E12.5 and is expressed in these cells throughout development (Figures 1Fk–1Fo). Prior to E12.5, another Runx gene, Runx1, is expressed in a1(I) Collagen/Glut1-expressing cells (Figures 1Fp–1Ft). The third Runx gene, Runx3, is not expressed before E13.5, and its expression is predominant in a1(II) Collagen-expressing chondrocytes (Figures 1Fu–1Fy and 1Fz–1Fb1). Glut1 is virtually not expressed in chondrocytes (Figures 1Fa–1Fe and 1Fz–1Fe1). In view of this pattern of expression, we analyzed the function of Glut1 during osteoblast differentiation by crossing mice harboring a floxed allele of Glut1 (Figure S2A) with mice expressing the Cre recombinase under the control of Derma1 (Yu et al., 2003) (Glut1dermo1+/−/−), which deletes genes starting at E12.5, or of Osterix regulatory elements (Rodda and McMahon, 2006) (Glut1osx+/−/−), which deletes genes starting at E14.5. We verified in each case that Glut1 was efficiently deleted in the targeted cells, but not in other cell types or tissues, and that expression of other Gluts was not affected by the Glut1 deletion (Figures S2B–S2E).

Using alcin blue/alizarin red staining of skeletal preparations to distinguish non-mineralized (blue) from mineralized ECM (red), Glut1dermo1+/−/− and control embryos were indistinguishable until E13.5 (Figure S2F). E14.0 was the first time point at which a delay in ECM mineralization in long bones and the jaws was seen in Glut1dermo1+/−/− embryos (Figure 2A). This difference in ECM mineralization between control and Glut1dermo1+/−/− embryos was verified histologically (Figure 2B). At E14.5, ECM mineralization was still absent in the mandibles of Glut1dermo1+/−/− embryos (Figure 2A). Beyond E14.5, we also studied Glut1osx+/−/− embryos. At E15.5, a large area of mineralized ECM was present in the axial skeleton of controls, but not in that of Glut1osx+/−/− and Glut1dermo1+/−/− embryos, except in long bones (Figures 2C and 2F). Von Kossa staining of histological sections detected extensive ECM mineralization in skeletal elements of control embryos, while this mineralization was more restricted in Glut1osx+/−/− embryos; alcin blue staining of these sections showed that ECM in Glut1osx+/−/− skeletal elements was mostly of cartilaginous nature (Figure 2D). Consistent with this observation, a1(X) Collagen-expressing hypertrophic chondrocytes covered a larger area in Glut1osx+/−/− than in control skeletal elements, and expression of Osteocalcin was undetectable in Glut1osx+/−/− skeletal elements (Figure 2E), indicating that osteoblast differentiation was delayed in E15.5 Glut1osx+/−/− embryos. Remarkably, despite these delays in osteoblast differentiation and bone formation, Runx2 and a1(I) Collagen were normally expressed in E15.5 Glut1osx+/−/− skeletal elements (Figure 2E).

At E18.5, Glut1osx+/−/− embryos’ skulls, which ossify mostly through an intramembranous process, were poorly mineralized, suggesting that osteoblast differentiation was delayed (Figure 2F). Von Kossa staining of histological sections showed numerous long, thick trabeculae in controls, but not in Glut1osx+/−/−, long bones, and an alcin blue staining showed many more cartilaginous remnants in Glut1osx+/−/− than in control skeletal elements (Figure 2G). Accordingly, the area occupied by a1(X) Collagen-expressing hypertrophic chondrocytes remained larger in Glut1osx+/−/− than in control skeletal elements, and expression of Osteocalcin was still barely detectable (Figures 2H–2J). This incomplete osteoblast differentiation in Glut1osx+/−/− bones and osteoblasts was further illustrated by the decreased expression of Bsp (Figures 2I and 2J). This delay in osteoblast differentiation explains why E18.5 Glut1osx+/−/− embryos had open fontanelles and hypoplastic clavicles (Figure 2F). Even though these two
Figure 1. Insulin-Independent Glucose Uptake in Osteoblasts

(A) Oxygen consumption rate (OCR) of osteoblasts, C2C12 myoblasts, or hippocampal neurons incubated with vehicle, 10 mM glucose, 2 mM glutamine, or 300 μM palmitate in 1 x Krebs-Henseleit buffer for 2 hr (n = 8).

(B) Glucose uptake measured by euglycemic hyperinsulinemic clamps in femurs, white adipose tissue, and gastrocnemius muscle of WT mice before or after insulin infusion (2.5 mU/kg/min) (n = 4).

(C) Uptake rate of 2-DG in osteoblasts (Osb), osteoclasts (Ocl), and myoblasts (n = 3).

(D) Expression of class I Gluts in osteoblasts and osteoclasts assayed by qPCR.

(E) Uptake rate of 2-DG in Glut1fl/fl, Glut1−/−, WT, and α1(I)Col-Glut1 osteoblasts (n = 6–8).

(F) In situ hybridization analysis of Glut1 (a–e), α1(I) Collagen (f–j), Runx2 (k–o) Runx1 (p–t), Runx3 (u–v), α1(II) Collagen (z–b1), and α1(X) Collagen (c1–e1) in hindlimbs during embryonic development.

All error bars represent SEM.
See also Figure S1.
Figure 2. Glucose Uptake Is Necessary for Osteoblast Differentiation during Development

(A) Alcian blue/alizarin red staining of skeletal preparations of E14.0 and 14.5 Glut1_{dermo1}^{-}\/- and Glut1_{fl/fl} embryos.
(B) Von Kossa staining of skull sections of E14.5 Glut1_{dermo1}^{-}\/- and Glut1_{fl/fl} embryos.
(C) Alcian blue/alizarin red staining of skeletal preparations of E15.5 Glut1_{osx}^{-}\/-, Glut1_{fl/fl}, and Osx-cre embryos.
(D) Von Kossa or alcian blue staining of femur from E15.5 Glut1_{osx}^{-}\/-, Glut1_{fl/fl}, and Osx-cre embryos.
(E) In situ hybridization analysis of Osteocalcin, α1(II) Collagen, and Runx2 expression in E15.5 Glut1_{osx}^{-}\/- and Glut1_{fl/fl} femurs.

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features are characteristic of CCD, a disease caused by the decreased function of RUNX2 (Mundlos et al., 1997; Lee et al., 1997). Runx2 expression was normal in E18.5 Glut1<sup>osx</sup>−/− bones. The same was true for α1(I) Collagen expression (Figures 2H–2J).

Accounting for this delay in osteoblast differentiation and CCD phenotype, there was a 70% decrease in RUNX2 accumulation in E18.5 Glut1<sup>osx</sup>−/− bones and osteoblasts (Figure 2K). This decrease in RUNX2 accumulation was not observed for other transcription factors (Figure S2G). There was also a 70% decrease in type I collagen accumulation in E18.5 Glut1<sup>−/−</sup> bones and osteoblasts because of a 60% decrease in the rate of collagen synthesis in Glut1<sup>−/−</sup> embryos. Of note, Chrebp, a transcriptional mediator of glucose signaling in other cell types (Yamashita et al., 2001), is not expressed and does not regulate glucose uptake in osteoblasts (Figures S2H–S2K).

**Glucose Uptake in Osteoblasts Is Necessary for Bone Formation and Whole-Body Glucose Homeostasis Post-natally**

To study Glut1 functions in osteoblasts post-natally, we crossed Glut1<sup>fl/fl</sup> mice with Osteocalcin-Cre mice (Glut1<sup>ocn</sup>−/−) that do not initiate gene deletion before E18.5 (Zhang et al., 2002) and performed an inducible deletion of Glut1 in osteoblasts in 6-week-old Osx-Cre;Glut1<sup>fl/fl</sup> mice (Glut1<sup>Osx</sup>−/−). After verifying the specificity and efficiency of the gene deletions (Figures S3A and S3B), we examined both models of Glut1<sup>−/−</sup> mice at 3 months of age and included in this analysis α1(I)Col-Glut1 mice that modestly overexpress (1.75-fold) Glut1 in osteoblasts (Figure S2D).

Glut1<sup>ocn</sup>−/− and Glut1<sup>Osx</sup>−/− mice of either sex presented with a low bone mass in all bones analyzed, whereas α1(I)Col-Glut1 mice displayed a high bone mass. In these mouse models, only the parameters of bone formation, i.e., circulating levels of PINP, mineral apposition rate, bone formation rate, and number of osteoblasts, were affected (Figures 3A–3C and S3C–S3E). PINP, mineral apposition rate, bone formation rate, and number of osteoblasts, were affected (Figures 3A–3C and S3C–S3E). This latter finding explained the decreased bone formation in Glut1<sup>−/−</sup> bones and osteoblasts (Figure 2K). This decrease in RUNX2 accumulation was not observed for other transcription factors (Figure S2G). There was also a 70% decrease in type I collagen accumulation in E18.5 Glut1<sup>−/−</sup> bones and osteoblasts because of a 60% decrease in the rate of collagen synthesis in Glut1<sup>−/−</sup> embryos. Of note, Chrebp, a transcriptional mediator of glucose signaling in other cell types (Yamashita et al., 2001), is not expressed and does not regulate glucose uptake in osteoblasts (Figures S2H–S2K).

**Glucose Uptake Favors Osteoblast Differentiation and Bone Formation by Inhibiting AMPK**

To determine if the delay in osteoblast differentiation and the decrease in bone formation seen in Glut1<sup>osx</sup>−/− embryos and mice were due to a decrease in protein synthesis that a poor glucose uptake triggers (Jeyapalan et al., 2007; Mayer et al., 2011), we measured AMP, ADP, and ATP contents in Glut1<sup>−/−</sup> osteoblasts.

Although AMP and ATP levels were not affected, there was a 3-fold increase in ADP content, leading to a 3-fold increase in the ratio of ADP to ATP in Glut1<sup>−/−</sup> versus control osteoblasts (Figures 4A and S4A). As a result, AMPK activity, assessed by the phosphorylation of its α subunit at Thr172 and of its substrate ACC1 at Ser79 (Woods et al., 1994; Wilson et al., 1996), was increased (Figure 4B). Several lines of evidence indicated that the activity of the mTORC1 complex was decreased in Glut1<sup>−/−</sup> osteoblasts. Phosphorylation of the mTORC1 substrates p70S6K at Thr389, 4E-BP1 at Thr37, and eIF4G at Ser1108 were suppressed in Glut1<sup>−/−</sup> embryos and osteoblasts (Figure 4B). Conversely, p70S6K phosphorylation at Ser79 was increased; and mTORC1 kinase activity was decreased in Glut1<sup>−/−</sup> osteoblasts (Figures 4B and 4D). Conversely, p70S6K phosphorylation and collagen accumulation were both decreased in Raptor−/− osteoblasts, and knockdown of Tsc1 and Tsc2 not only restored mTORC1 activity as measured by p70S6K phosphorylation but also normalized collagen accumulation in Glut1<sup>−/−</sup> osteoblasts (Figures 4E and 4F).

To demonstrate that the decrease in mTORC1 activity and the phenotypes of the Glut1<sup>osx</sup>−/− mice are caused by an increase in AMPK activity in osteoblasts, we decreased the expression of Ampk in osteoblasts by generating Glut1<sup>−/−</sup> embryos or mice lacking in osteoblasts one allele of Ampkα1, the most highly expressed AMPKα subunit in these cells (Figure 4G). Phosphorylation of AMPKα1 and p70S6K, ATP contents, RUNX2, and type I collagen accumulations were similar in Glut1<sup>−/−</sup> osteoblasts (Figures 4H, S4B, and S4C); Raptor phosphorylation at Ser79 was increased; and mTORC1 kinase activity was decreased in Glut1<sup>−/−</sup> osteoblasts (Figures 4B and 4D). Conversely, p70S6K phosphorylation and collagen accumulation were both decreased in Raptor−/− osteoblasts, and knockdown of Tsc1 and Tsc2 not only restored mTORC1 activity as measured by p70S6K phosphorylation but also normalized collagen accumulation in Glut1<sup>−/−</sup> osteoblasts (Figures 4E and 4F).

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Figure 3. Glucose Uptake in Osteoblasts Is Necessary for Bone Formation and Glucose Homeostasis Post-natally

(A and B) Histomorphometric analysis of L4 vertebrae of 3-month-old Glut1fl/fl, Glut1ocn+/−, WT, and α1(I)Col-Glut1 male mice (n = 9–11).

(C) mCT analysis of proximal femurs of Glut1fl/fl and Glut1ocn+/− male mice (n = 7).

(D) Expression of Ccnd2, Ccne1, and Cdk4 in femurs of 3-month-old Glut1fl/fl, Glut1ocn+/−, WT, and α1(I)Col-Glut1 male mice (n = 7).

(E) BrdU incorporation in calvaria of P14 Glut1fl/fl, Glut1ocn+/− , WT, and α1(I)Col-Glut1 mice (n = 5–8).

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SMURF1 phosphorylation at S148 was higher in WT osteoblasts with phospho-SMURF1 antibody demonstrated AMPK phosphorylation event was needed for SMURF1-induced Runx2 destruction (Zhao et al., 2003) (Figures S5A and S5B). In vitro, AMPK phosphorylated SMURF1 at Ser148, and this phosphorylation was increased in Runx2-deficient osteoblasts. To that end, Runx2+/fl osteoblasts (Takarakda et al., 2013) were infected with a Cre-expressing adenovirus (Figure S6A); these Runx2+/fl osteoblasts were then cultured in a differentiation medium containing either 5 or 10 mM glucose.

In Runx2+/− osteoblasts cultured in the presence of 5 mM glucose, GLUT1 accumulation and glucose consumption rate were decreased, phosphorylation of AMPKα1 was high, and phosphorylation of p70S6K was low, indicating that mTORC1 signaling was inhibited. As a result, 3H-proline incorporation into collagen molecules and accumulation of type I collagen were both lower in Runx2+/− than in control osteoblasts (Figures 6A–6C). In contrast, when the glucose concentration in the culture medium of Runx2+/− cells reached 10 mM, the glucose consumption rate doubled, and phosphorylation of AMPKα1 and p70S6K, the incorporation rate of 3H-proline into collagen molecules, and the accumulation of type I collagen were normalized in Runx2+/− cells, even though type I collagen gene expression did not change (Figures 6A–6D).

In view of these results, we asked whether chronic hyperglycemia would improve bone formation in Runx2+/− embryos. This was achieved by injecting streptozotocin (STZ; 150 mg/kg) into Runx2+/− female mice as soon as a vaginal plug was seen. By decreasing the number of β cells and circulating insulin levels, this manipulation caused a severe hyperglycemia in E18.5 embryos (Figure 6E). We focused our analysis on bones forming through intramembranous ossification because they are the ones in which osteoblast differentiation is hampered by Runx2

Runx2 Cannot Induce Osteoblast Differentiation when Glucose Uptake Is Hampered

In the course of these experiments, we noticed that Runx2 knockout resulted when GLUT1 in control, Glut1+/− osteoblasts (Figure 5A). Normal Runx2 knockout ubiquitination in Glut1+/−, Ampkα1+/− and Glut1+/−/Ampkα1+/− osteoblasts (Figure 5B) implicated AMPK in Runx2 polyubiquitination. Mass spectrometry and bioinformatics analyses identified a possible AMPK recognition site in SMURF1, an E3 ubiquitin ligase involved in Runx2 degradation (Zhao et al., 2003) (Figures S5A and S5B).

Two experiments showed that increasing AMPK activity in osteoblasts is deleterious for bone. Treating mouse osteoblasts with AICAR, an AMPK agonist, profoundly decreased type I collagen and RUNX2 accumulations in these cells, and WT mice treated with AICAR from 6 to 14 weeks of age showed a significant decrease in bone formation parameters and bone mass (Figures 4N and 4O). Accordingly, and as is the case for Glut1+/−–osteoblasts, WT osteoblasts deprived of glucose had higher levels of P-AMPK and lower levels of P-P70S6K and type I collagen than if cultured in the presence of glucose (Figure S4D).

Glucose Can Initiate Bone Formation in Runx2-Deficient Embryos

Next, we asked if conversely, raising the extracellular concentration of glucose was sufficient to initiate type I collagen synthesis in Runx2-deficient osteoblasts. To that end, Runx2/fl/fl osteoblasts (Takarakda et al., 2013) were infected with a Cre-expressing adenovirus (Figure S6A); these Runx2+/− osteoblasts were then cultured in a differentiation medium containing either 5 or 10 mM glucose.

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Expression of osteoblast marker genes in the femurs of 3-month-old Glut1/fl/fl, Glut1+/−−/−, WT, and α1(fl)Col-Glut1 mice (n = 8). (G) α1(fl) Collagen and Runx2 accumulations in the femurs of 3-month-old Glut1/fl/fl, Glut1+/−−/−, WT, and α1(fl)Col-Glut1 mice. (H and I) Glucose-stimulated insulin secretion in 3-month-old Glut1/fl/fl, Glut1+/−−/−, WT, and α1(fl)Col-Glut1 mice (n = 10–11). (J and K) Glucose tolerance test in 3-month-old Glut1/fl/fl, Glut1+/−−/−, WT, and α1(fl)Col-Glut1 mice (n = 5–10). (L) ITT in 3-month-old Glut1/fl/fl, Glut1+/−−/−, and WT mice (n = 8–10). (M) Glucose infusion rate in 3-month-old Glut1/fl/fl and Glut1+/−−/− mice (n = 6). (N) ITT in 3-month-old WT and α1(fl)Col-Glut1 mice (n = 8–10). All error bars represent SEM. See also Figure S3.
haplo-insufficiency. While no difference was seen in WT embryos carried by STZ-treated mothers (Figure S6B), alcian blue/alizarin red staining of skeletal preparations showed that clavicles of E18.5 Runx2+/− embryos carried by STZ-treated mothers were twice as long as those of Runx2+/- embryos carried by vehicle-treated mothers (1.78 ± 0.08 mm and 0.85 ± 0.05 mm). Interparietal bones were also 2-fold larger in Runx2+/- embryos carried by STZ-treated mothers (2.09 ± 0.12 mm2 and 1.01 ± 0.13 mm2) than in those carried by control mothers, and the fontanelles of these embryos were less open than were those of Runx2+/- embryos carried by vehicle-treated mothers (Figures 6F and 6G). Histological analyses showed the presence of mineralized bone trabeculae in the clavicles of E18.5 Runx2+/- embryos carried by STZ-treated mothers (Figures 6H and 6I). An immunohistochemistry analysis showed the presence of type I collagen molecules in the clavicles of Runx2+/- embryos carried by STZ-treated mothers, whereas only type X collagen molecules were present in those of Runx2+/- embryos carried by vehicle-treated mothers (Figure 6J). Accumulation of type I collagen was also increased in the long bones of Runx2+/- embryos carried by STZ-treated mothers (Figures 6K and 6L). To further link the rescue of bone phenotype in Runx2-deficient embryos to the increase in blood glucose levels, we repeated this experiment using lower doses of STZ (50 or 100 mg/kg). One hundred milligram per kilogram of STZ was toxic for embryos carried by STZ-treated mothers (Figure S6B), alcian blue/alizarin red staining of skeletal preparations failed to show any ECM mineralization of skeletal preparations as in the skeletons of E18.5 embryos.

**Figure 4. Glucose Uptake Favors Osteoblast Differentiation and Bone Formation by Inhibiting AMPK**

(A) ADP content and ADP-to-ATP ratio in WT and Glut1−/− osteoblasts (n = 6).

(B) AMPK, ACC1, Raptor (B), p70S6K, 4E-BP1, and eIF4G phosphorylation in WT and Glut1−/− osteoblasts.

(D) Kinase assay of immune-precipitated mTORC1 complex in WT and Glut1−/− osteoblasts (n = 3).

(E) Raptor, p70S6K phosphorylation, and α1(I)Collagen accumulations in WT and Raptor−/− osteoblasts.

(F) TSC1, TSC2, and p70S6K phosphorylation, α1(I)Collagen, and RUNX2 accumulations in WT and Glut1−/− osteoblasts transfected with siRNAs targeting Tsc1 and Tsc2 or scrambled siRNA.

(G) Expression of various AMPK subunits in osteoblasts.

(H) AMPK and p70S6K phosphorylation, RUNX2, and α1(I)Collagen accumulations in Glut1fl/fl, Glut1ox/o−/−, and Glut1ox/o−/−;Ampka1ox+/- osteoblasts. (I and J) Alcian blue/alizarin red staining of skeletal preparations of E18.5 (I) and 18.5 (J) Glut1fl/fl, Glut1ox/o−/−, Glut1ox/o−/−;Ampka1ox+/-, and Glut1ox/o−/−;Ampka1ox+/-/embryos.

(K) Alcian blue staining of histological sections of femurs of E18.5 Glut1fl/fl, Glut1ox/o−/−, Glut1ox/o−/−;Ampka1ox+/-, and Glut1ox/o−/−;Ampka1ox+/-/embryos.

(L) In situ hybridization analysis of Osteocalcin, α1(I), α1(II), and α1(X) Collagen expression in femurs of E18.5 Glut1fl/fl, Glut1ox/o−/−, Glut1ox/o−/−;Ampka1ox+/-, and Glut1ox/o−/−;Ampka1ox+/-/embryos.

(M) Histomorphometric analysis of L4 vertebrae of 3-month-old of Glut1fl/fl, Glut1ox/o−/−, Glut1ox/o−/−;Ampka1ox+/-, and Glut1ox/o−/−;Ampka1ox+/- male mice (n = 7–10).

(N) Osteocalcin phosphorylation, RUNX2, and α1(I)Collagen accumulations in osteoblasts treated with vehicle or AICAR (0.1 mM) for 16 hr.

(O) Histomorphometric analysis of L4 vertebrae of 3-month-old of WT mice treated with vehicle or AICAR (250 mg/kg/day) for 8 weeks (n = 6).

All error bars represent SEM.

See also Figure S4.
of histological sections of long bones showed an enlargement of the area of hypertrophic chondrocytes and a cartilaginous ECM in E18.5 Glut1osx+/--;Runx2+/-- embryos (Figure 7J). Osteocalcin expression was undetectable in E18.5 Glut1osx+/--;Runx2+/-- skeletal elements, while it was present in single heterozygous embryos. Conversely, the area covered by α1(X) Collagen-expressing cells was greatly enlarged in E18.5 Glut1osx+/--;Runx2+/-- embryos (Figure 7K). The extent of the delay in osteoblast differentiation in Glut1osx+/--;Runx2+/-- embryos and mice explained why they all died peri-natally. This crossregulation between Runx2 and Glut1 also determined the extent of bone formation since 3-month-old Glut1osx+/--;Runx2+/-- mice had a low bone mass not seen in either Glut1osx+/+ or Runx2+/+ mice (Figure 7L).

Accounting for this severe phenotype, and in agreement with the feedforward loop between glucose uptake in osteoblasts and RUNX2, accumulation of GLUT1, type I collagen, and RUNX2 was decreased more than 80% and phosphorylation of AMPK and SMURF1 was increased in Glut1osx+/--;Runx2+/-- compared to single heterozygous embryos (Figure 7M). Hence, crossregulation of RUNX2 and Glut1 is an amplification mechanism that determines the onset of osteoblast differentiation and the extent of bone formation throughout life. We also treated Runx2+/-- mothers with STZ (150 mg/kg) as soon as a vaginal plug was observed. This dose of STZ raised blood glucose levels in mothers and embryos (Figure S7C) and increased bone formation in skeletal elements of the skull and in clavicles in Glut1osx+/--;Runx2+/-- embryos (Figure 7N).

**DISCUSSION**

This study identifies glucose uptake in prospective osteoblasts as the earliest determinant of osteoblast differentiation and bone formation. It also shows that the coordination of osteoblast differentiation and the extent of bone formation throughout life is maintained by a feedforward regulation between glucose uptake in osteoblasts and RUNX2 accumulation. In addition, glucose uptake in osteoblasts is necessary for whole-body glucose homeostasis.

**The Bases of the Coordination between Osteoblast Differentiation and Bone Formation**

A striking feature of bone biology that has never been explained is that the synthesis of the main constituent of bone ECM, type I collagen, precedes the expression of, and is not regulated by, RUNX2, the earliest transcriptional determinant of osteoblast differentiation. This apparent disconnect between osteoblast differentiation and type I collagen synthesis in the bone could be explained by a single mechanism controlling both aspects of osteoblast biology and acting upstream of RUNX2. Our results suggest that this is the case and that this mechanism is glucose uptake in osteoblast progenitor cells. Glucose is the main nutrient of osteoblasts and is taken up by these cells in an insulin-independent manner through Glut1, whose expression precedes that of Runx2. Glucose uptake in osteoblasts favors osteoblast differentiation and bone formation through two distinct mechanisms. First, by inhibiting the activity of AMPK, glucose uptake enhances the activity of the mTORC1 pathway and therefore protein synthesis. Second, and unexpectedly, glucose uptake inhibits another function of AMPK documented here: its ability to favor RUNX2 ubiquitination in part via SMURF1. The two distinct functions of AMPK in osteoblasts revealed here explain why agonists of AMPK activity exert a deleterious influence on bone formation in vivo.

The Glut1-RUNX2 pathway described here could have clinical relevance. For instance, Glut1osx+/--;Runx2+ mouse embryos develop CCD, a disease most often caused by a decrease in RUNX2 expression. However, a significant number of CCD patients do not have any detectable mutations in Runx2 (Puppin et al., 2005; Tessa et al., 2003). Conceivably, in some of these patients, CCD may be caused by a decrease in glucose uptake or utilization in osteoblasts. If this were the case, it would further support the notion that skeletal dysplasia may have a nutritional basis (Elefteriou et al., 2006). Moreover, the predilection of osteoblasts for glucose demonstrated here provides a plausible explanation for the high prevalence of glucose uptake in osteoblasts.
(legend on next page)
for why children chronically fed a ketogenic diet experience poor longitudinal growth (Groesbeck et al., 2006).

The Respective Functions of Glucose Uptake and Runx2 in Osteoblasts

Remarkably, restoring Runx2 accumulation in GLUT1−/− cells did not translate into efficient bone formation in vivo simply because in osteoblasts unable to properly take up glucose, protein synthesis remains low regardless of the level of expression of Runx2. On the other hand, raising the extracellular concentration of glucose was sufficient to initiate bone formation even though osteoblasts were not fully differentiated in Runx2+/− embryos. Raising blood glucose levels in Runx2−/− embryos also increased collagen synthesis but that did not translate into the presence of a mineralized bone ECM because expression of Alp, a gene necessary for bone ECM mineralization, is low in the absence of Runx2. Thus, Runx2 is necessary for osteoblast differentiation and bone ECM mineralization, but not for, paradoxically, the synthesis of the main constituent of this ECM. In broader terms, the importance of glucose uptake in osteoblast differentiation described here raises the hypothesis that glucose uptake may be a more general determinant of cell differentiation during embryonic development. This may be particularly relevant for tissue like muscle that uptakes large amounts of glucose.

Independently of its regulation of bone formation, glucose uptake in osteoblasts is necessary for another cardinal function of bone: the regulation of whole-body glucose metabolism. Indeed, through its regulation of RUNX2 accumulation, glucose favors expression of the hormone osteocalcin.

The Synergistic Functions of Glucose Uptake and Runx2 in Osteoblasts

How is osteoblast differentiation and bone formation coordinated throughout life? The fact that Runx2−/− and GLUT1−/− osteoblasts had a similar metabolic profile suggested that this coordination might be explained if Glut1 was a target gene of RUNX2.

In support of this hypothesis, both molecular and genetic evidence identifies RUNX2 as a major regulator of Glut1 expression and glucose uptake in osteoblasts. As a result, Runx2−/− osteoblasts bear metabolic similarities with GLUT1−/− osteoblasts; Glut1+/−;Runx2+/− embryos are similar to Runx2−/−− embryos; and Glut1+/−;Runx2+/− mice display an osteopenia not seen in either Runx2+/− or Glut1+/−− mice. Hence, the reciprocal regulation between GLUT1-mediated glucose uptake and RUNX2 acts as an amplification chamber that determines the onset of osteoblast differentiation and the extent of bone formation throughout life. In addition, Runx1 expression in the developing skeleton suggests that this Runx protein may regulate Glut1 expression and thereby favor type I collagen accumulation in prospective osteoblasts before E12.5.

In broader terms, the results of this study cannot be separated from the recently described role of osteoblasts in maintaining glucose homeostasis in physiological and pathological situations (Lee et al., 2007; Ferron et al., 2010; Wei et al., 2014). The absolute necessity of glucose uptake for osteoblast differentiation, bone formation, and glucose homeostasis documented here illustrates, from the perspective of osteoblasts, the fundamental importance of crosstalk between bone and glucose metabolism.

EXPERIMENTAL PROCEDURES

Mice Generation

To generate Glut1+/+ mice, a targeting vector harboring two LoxP sites flanking exons 3–10 of Glut1 were electroporated into embryonic stem cells (ES cells) (CSL3,129/SvEvTac) (Figure S2A). Targeted ES cells were detected by Southern blots and injected in 129Sv/EVTG blastocysts to generate chimeric mice. Chimeric mice were crossed with Glut1+/+− mice, and Glut1+/+ mice were then crossed with Dermo1-Cre (Yu et al., 2003), Osterix-Cre (Rodda and McMahon, 2006), or Ocn-Cre mice (Zhang et al., 2002) to generate Glut1+− mice, whose progenies were intercrossed to obtain Glut1−/−, Glut1+/−, and Glut1+/−/− mice, respectively. To generate a(I)Col−/−Glut1 transgenic mice, a cDNA fragment of the mouse Glut1 was cloned into a plasmid containing a 2.3-kb a(I) collagen promoter and microinjected using standard protocols. Ampkα1+/+ mice were obtained from the Jackson Laboratory (Nakada et al., 2010). Runx2+/+ mice were generated as previously described (Takahara et al., 2013). Shn3+/− and Smurf1+/+ mice were generous gifts of Dr. L. Glimcher (Weill Cornell Medical College) and Dr. J. Wrana (University of Toronto, Canada), respectively (Jones et al., 2006; Narimatsu et al., 2009). Except for a(I)Col−Glut1− mice, which were backcrossed to a C57 background five times, all other mice analyzed were maintained on a C57/129 mixed background. Control littermates were analyzed in all experiments. All procedures involving animals were approved by CUMC IACUC and conform to the relevant regulatory standards.

Figure 6. Glucose Can Initiate Bone Formation in Runx2-Deficient Embryos

(A) Glucose consumption rate in WT and Runx2−/− osteoblasts cultured with 5 or 10 mM glucose for 16 hr. (B) Runx2, a(I)Collagen, and GLUT1 accumulations, AMPKα1, and p70S6K phosphorylation in WT and Runx2−/− osteoblasts cultured with 5 or 10 mM glucose for 14 days. (C) 3H-proline incorporation into collagen molecules of WT and Runx2−/− osteoblasts cultured with 5 or 10 mM glucose for 14 days (n = 5). (D) qPCR analysis of osteoblast marker genes in WT and Runx2−/− osteoblasts cultured with 5 or 10 mM glucose for 14 days (n = 6). (E) Blood glucose and insulin levels in STZ (50, 100, and 150 mg/kg) and vehicle-treated Runx2+/− mothers and progenies at E18.5 (n = 5–12). (F) Glut1−/− osteoblasts cultured with 5 or 10 mM glucose for 14 days (n = 5).
Cell Culture
Mouse calvaria osteoblasts were isolated and cultured as described previously (Ducy and Karsenty, 1995). Osteoclast precursors (monocytes) were isolated by culturing bone marrow cells with sMEM/10% fetal bovine serum (FBS) containing M-CSF (10 ng/ml) for 6 days and then treated with RANKL (30 ng/ml) and M-CSF (10 ng/ml) for 7 days. C2C12 myoblasts (ATCC), the mHippoP-14 embryonic mouse hippocampal hypothalamic cell line (Cellutions Biosystems), and COS-7 cells were cultured in DMEM/10% FBS. Glut1-/-, Runx2-/-, Runx2-/-;Osteocalcin-/-, Runx1-/-, and Runx1-/-;Osteocalcin-/- calvaria osteoblasts were generated by infecting Glut1fl/fl, Runx2fl/fl, Glut1fl/fl;Osteocalcin-/-, Glut1fl/fl;ApOA1afl/fl, ApOA1a-/- osteoblasts with either empty vector or Cre-expressing adenovirus (1:800 MOI) (University of Iowa). Small interfering RNAs (siRNAs) against Tsc1 and Tsc2 (Dharmacon) were transfected to primary osteoblasts, according to the manufacturer’s protocol.

Molecular Biology and Biochemistry
For quantifying gene expression, RNA samples were extracted using TRIzol reagent (Invitrogen). One to two micrograms of total RNA were converted into cDNA using M-MLV reverse transcriptase (Invitrogen). qPCR analyses were performed using CFX-Connect real-time PCR system (Bio-Rad). Relative expression levels of each gene were normalized to the levels of 18S rRNA or β-actin. Western blot analyses were carried out using standard protocols. All antibodies were obtained from Cell Signaling Technology, except for the anti-Glut1 (EMD Millipore), anti-COL1A1, anti-RUNX2, anti-SMURF1 (Santa Cruz), anti-Phospho-Ser148 SMURF1 (Genescript), and anti-β-actin (Sigma). Quantification of western blots was performed using ImageJ. Protein levels were quantified and normalized to ACTIN or GAPDH levels. Relative protein levels were calculated with respect to control samples. All western blot experiments were repeated at least three times, with different samples.

Glucose Consumption and Uptake Assay
For glucose consumption measurements, following 16-hr incubation with osteoblasts, the glucose concentration in the culture medium was assayed with a Glucose Assay Kit (Biovision). Glucose uptake was determined by the uptake rate of 2-[U-14C] deoxyglucose (2-DG) in cells. Following a 1-hr fast in glucose-free Krebs-Ringer phosphate buffer (250 mM HEPES [pH 7.4], 136 mM NaCl, 4.7 mM KCl, 1.25 mM MgSO4, 1.25 mM CaCl2, and 0.1% BSA), cells were cultured in KRH buffer containing 100 μM 2-deoxyglucose and 0.5 μCi/ml 2-14C-DG (287 mCi/mmol, Perkin Elmer, NEC495A) for 1 hr. For glucose uptake in bones, 10 μCi of 2-14C-DG were immunoprecipitate injected in mice at a Ci/ml of 2-14C-DG were immunoprecipitate injected in mice at a rate of 2-[U-14C] deoxyglucose (2-DG) in cells. Following a 1-hr fast in glucose-free Krebs-Ringer phosphate buffer (250 mM HEPES [pH 7.4], 136 mM NaCl, 4.7 mM KCl, 1.25 mM MgSO4, 1.25 mM CaCl2, and 0.1% BSA), cells were cultured in KRH buffer containing 100 μM 2-deoxyglucose and 0.5 μCi/ml 2-14C-DG (287 mCi/mmol, Perkin Elmer, NEC495A) for 1 hr. For glucose uptake in bones, 10 μCi of 2-14C-DG were immunoprecipitate injected in mice at a random feed state for 1 hr, and calvariae were then collected for analysis. The amount of 2-14C-DG in total cell or bone lysates was quantified by liquid scintillation counter (WALLAC 1409) and normalized to protein content (Bio-Rad).

Skeleton Preparation, Bone Histology, and In Situ Hybridization
Skeleton preparations and alcian blue/alizarin red staining were carried out according to standard protocols (McLeod, 1985). Bone histology analyses, including Von Kossa staining and alcian blue staining, were performed with histological sections of femurs or clavicle bones using standard protocols. For all skeletal analyses, at least three litters for each embryonic stage and at least five embryos for each genotype were examined. Bone histomorphometry analyses were performed on L3 and L4 vertebrae as described previously (Chappard et al., 1987; Parfitt et al., 1987). Von Kossa/van Gieson staining, toluidine blue staining, and calcine double-labeling were performed to measure mineralized bone volume over the total tissue volume (BV/TV), osteoblast number per tissue area (N.Ob/T.Ar), mineralization apposition rate (MAR), and bone formation rate per bone surface (BFR/BS). For in situ hybridization, tissues were fixed in 4% paraformaldehyde/PBS overnight at 4°C and then embedded in paraffin after dehydration and sectioned at 5 μm. In situ hybridization was performed using 35S-labeled riboprobe as described (Ducy et al., 1997). The Runx1, Runx3, Runx2, a1(I)Col, a 1(I)Col, α 1(X)Col, Osteocalcin, and Bsp probes have been previously described (Takeda et al., 2001). The Glut1 probe is a 500-bp fragment of the Glut1 3’ UTR (see the Supplemental Experimental Procedures for sequence information). Hybridizations were performed overnight at 55°C, and washes were performed at 63°C.

Statistics
All data are presented as mean ± SEM. Statistical analyses were performed using unpaired, two-tailed Student’s t test for comparison between two groups, and an ANOVA test was used for experiments involving more than two groups. For all experiments, * denotes p ≤ 0.05, and # denotes p ≤ 0.001 compared to control.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.05.029.

AUTHOR CONTRIBUTIONS
J.W. and G.K. conceived and designed the studies; J.W. and J.S. performed most experiments; M.P.M. generated the Glut1fl/fl mice; A.M. performed some histological analysis; D.K. performed the in vivo AICAR treatment experiment; H.Z. and J.E.P. performed glucose clamp analyses; T.T., T.L., and E.H. performed experiments using Runx2-/- osteoblasts; and J.W. and G.K. wrote the paper.

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