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Loss of Iroquois homeobox transcription factors 3 and 5 in osteoblasts disrupts cranial mineralization

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A B S T R A C T  
Cranial malformations are a significant cause of perinatal morbidity and mortality. Iroquois homeobox transcription factors (IRX) are expressed early in bone tissue formation and facilitate patterning and mineralization of the skeleton. Mice lacking Irx5 appear grossly normal, suggesting that redundancy within the Iroquois family. However, global loss of both Irx3 and Irx5 in mice leads to significant skeletal malformations and embryonic lethality from cardiac defects. Here, we study the bone-specific functions of Irx3 and Irx5 using Osx-Cre to drive osteoblast lineage-specific deletion of Irx3 in Irx5−/− mice. Although we found that the Osx-Cre transgene alone could also affect craniofacial mineralization, newborn Irx3lox/flox/Irx5−/−/Osx-Cre line mice displayed additional mineralization defects in parietal, interparietal, and frontal bones with enlarged sutures and reduced calvarial expression of osteogenic genes. Newborn endochondral long bones were largely unaffected, but we observed marked reductions in 3–4-week-old bone mineral content of Irx3lox/flox/Irx5−/−/Osx-Cre line mice. Our findings indicate that Irx3 and Irx5 can work together to regulate mineralization of specific cranial bones. Our results also provide insight into the causes of the skeletal changes and mineralization defects seen in Hamamy syndrome patients carrying mutations in Irx5.

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1. Introduction  
Craniofacial development requires tight coordination of cell migration, proliferation, and mineralization of osteogenic lineages (Willie & Morris-Kay, 2001; Franz-Odendaal, 2011). Osteoblast dysfunction is thought to be a major contributor to diseases that affect craniofacial bones and mineralization (Rice, 2008). The complex genetic and spatial interactions that occur during craniofacial development pose major challenges to understanding mineralization during the development of the skull.

Iroquois homeobox domain transcription factors (IRX) are highly conserved proteins that regulate neural, cardiac, and bone development (Kerner et al., 2009; Cavodeassi et al., 2001; Kim et al., 2012; Li et al., 2014). IRX proteins all contain two highly conserved domains. The homeodomain is postulated to regulate interactions between transcriptional regulators by binding to genomic regions to regulate target gene expression, and the IRO Box is involved in protein-protein binding (Cavodeassi et al., 2001; Hiroi et al., 2001). Six IRX transcription factors have been identified (IRX1-IRX6), of which Irx1, Irx2, and Irx4 cluster to chromosome 5 in humans (chromosome 13 in mice) and Irx3, Irx5, and Irx6 cluster to chromosome 16 (chromosome 8 in mice) (Gaborit et al., 2012; Houweling et al., 2001). Irx3 and Irx5 expression is strikingly similar in developing mouse tissues (Houweling et al., 2001).
IRX proteins are required for the formation of limbs and skeletal tissues. Irx1 proteins are required for the specification of individual placodes through BMP signaling (Glavic et al., 2004), IRX3 has been shown to bind to the Bmp10 promoter, which is important for ventricular septation, while IRX5 can bind to GATA3 and TRPS1 to regulate CXCL12 during bone progenitor migration in Xenopus embryos (Gaborit et al., 2012; Bonnard et al., 2012). Irx1 and Irx2 have been shown to regulate vertebrate digit formation, while Irx3 and Irx5 mediate early mouse limb bud specification by regulating Gli3 expression (Li et al., 2014; Becker et al., 2001; McDonald et al., 2010). Surprisingly, loss of Irx5 alone leads to a grossly normal mouse (Gaborit et al., 2012), while loss of both Irx3 and Irx5 together result in an embryonic lethal phenotype from cardiac defects and skeletal malformations (Li et al., 2014; Gaborit et al., 2012). Unfortunately, the early embryonic lethality of Irx3+/−/Irx5+/− mice contributes to our incomplete understanding of the role of Irx3 and Irx5 in osteoblast function (Li et al., 2014; Gaborit et al., 2012).

Two mutations in human IRX5, Ala150Pro and Asn166Lys occur in patients with Hamamy syndrome (OMIM MIM611174; Bonnard et al., 2012), who present with craniofacial dysmorphism, osteopenia, tooth formation, while loss of both Irx3 and Irx5 contribute to this decrease in survival in the neonatal stage. We observed small but statistically significant reductions in newborn total body weight and length in mice with a loss of Irx5 independent of Osx-Cre expression (Fig. 1B, C and Supplementary Fig. 2A and B). Lower limbs of Irx3lox/loxIrx5−/−/Osx-Cre− mice assessed by Dual energy X-ray absorptiometry (DEXA) showed no differences in bone mineral density (Fig. 1D and Supplementary Fig. 2C). These data indicate that global deletion of Irx5 alone was sufficient to affect body size, but that additional deletion of Irx3 in osteoblastic cells did not have further effects on body size.

During the course of our studies, it became evident that Irx3+/+ /Irx5+/+/Osx-Cre− mice had an unexpected basal phenotype with reductions in the mineralized area of the frontal bone. This was confirmed by recent reports of effects by the Osx-Cre transgene alone on craniofacial mineralization (Wang et al., 2015; Huang & Olsen, 2015). Microcomputed tomography (microCT) 3D image reconstruction showed both Irx3+/−/Irx5+/−/Osx-Cre− and Irx3lox/loxIrx5−/−/Osx-Cre− mice had reduced mineralization in the frontal, parietal, and interparietal bones (Fig. 2 and Supplementary Figs. 2D, E and 3). The long bones from both Irx3+/−Irx5+/−/Osx-Cre− and Irx3lox/loxIrx5−/−/Osx-Cre− newborn mice also appeared unaffected by microCT.

We next performed whole body skeletal staining of newborn Irx3lox/loxIrx5+/−/Osx-Cre− mice with alizarin red and alcian blue. Irx3+/flIrx5+/+/Osx-Cre− and Irx3lox/loxIrx5−/−/Osx-Cre− mice showed reduced mineralization in frontal, parietal, and interparietal bones (Fig. 3A and 3B). We measured the frontal bone total mineralized area, using the orbits as landmarks; the suture width in the same area; the length of the parietal and frontal bones along the suture; and the thickness of mineralized bone in Irx3+/+/Irx5+/−/Osx-Cre− mice, however, Irx3+/−Irx5+/−/Osx-Cre− and Irx3lox/loxIrx5−/−/Osx-Cre− mice showed an even greater reduction in frontal bone mineralization (Fig. 3D and Supplementary Fig. 2F–I). Additionally, the width of the suture was significantly greater in Irx3lox/loxIrx5−/−/Osx-Cre− mice than in Irx3+/+/Irx5+/−/Osx-Cre− and wildtype mice (Fig. 3E and F). The parietal and frontal bone total length showed a reduction caused by the Osx-Cre but not with Irx3 or Irx5 loss (Fig. 3G). We noted no significant differences between Irx3+/−/Irx5+/−/Osx-Cre− and Irx3+/−/Irx5−/−/Osx-Cre− mouse cranial measurements (Supplementary Fig. 2F–I). These data indicate that the presence of the Osx-Cre transgene alone can reduce cranial mineralization, but the absence of Irx3 and Irx5 in osteoblastic cells leads to a greater reduction in bone mineralization.

2.2. Irx3lox/loxIrx5−/−/Osx-Cre− skulls have reduced osteoblast-specific deletion of Irx3 in Irx5−/− mice.

2. Results

2.1. Body size and craniofacial bone mineralization are reduced in newborn Irx3lox/loxIrx5+/−/Osx-Cre− mice

We focused our studies on newborn Irx3lox/loxIrx5−/−/Osx-Cre− mice (Supplementary Fig. 1A) to understand the role of Irx3 and Irx5 in early bone mineralization. The allelic separation of Irx3 and Irx5 was a rare event (approximately 0.5–2%, Supplementary Fig. 1B) and thus single allele mice were excluded from further analysis. Newborn Irx3lox/loxIrx5+/−/Osx-Cre− mice appeared grossly normal but slightly smaller than control littermates (Fig. 1A). 43% of Irx3lox/loxIrx5+/−/Osx-Cre− were viable at birth, which appeared to be an effect of the Osx-Cre allele as Irx3+/+/Irx5+/−/Osx-Cre− also showed similar decreased neonatal viability (Supplementary Fig. 1C). However, Irx3lox/loxIrx5−/−/Osx-Cre− mice rarely survived to 4 weeks of age. This early lethality was not observed in 3–4-week-old Irx3lox/loxIrx5−/−/Osx-Cre− mice (Supplementary Fig. 1D) suggesting that the loss of Irx3 and Irx5 contributed to this decrease in survival in the neonatal stage. We observed small but statistically significant reductions in newborn total body weight and length in mice with a loss of Irx5 independent of Osx-Cre expression (Fig. 1B, C and Supplementary Fig. 2A and B). Lower limbs of Irx3lox/loxIrx5−/−/Osx-Cre− assessed by Dual energy X-ray absorptiometry (DEXA) showed no differences in bone mineral density (Fig. 1D and Supplementary Fig. 2C). These data indicate that global deletion of Irx5 alone was sufficient to affect body size, but that additional deletion of Irx3 in osteoblastic cells did not have further effects on body size.

We next used hematoxylin and eosin staining to identify alterations to the bone architecture and mineralization in Irx3lox/loxIrx5−/−/Osx-Cre− mice. We found that bone accumulation was less in Irx3lox/loxIrx5+/−/Osx-Cre− and Irx3lox/loxIrx5−/−/Osx-Cre− mice than in Irx3+/+/Irx5+/−/Osx-Cre− and wildtype mice (Fig. 3E and F). The parietal and frontal bone total length showed a reduction caused by the Osx-Cre but not with Irx3 or Irx5 loss (Fig. 3G). We noted no significant differences between Irx3+/−Irx5+/−/Osx-Cre− and Irx3+/−Irx5−/−/Osx-Cre− mouse cranial measurements (Supplementary Fig. 2F–I). These data indicate that the presence of the Osx-Cre transgene alone can reduce cranial mineralization, but the absence of Irx3 and Irx5 in osteoblastic cells leads to a greater reduction in bone mineralization.
2.3. Irx3floxflo/Osx-Cre skulls have reduced expression of genes that regulate osteoblastic mineralization

We next examined whole calvarial expression of genes involved in osteoblast mineralization and maturation. Early markers of osteoblast lineage specification such as Runx2 (Fig. 5A) and Osx (Fig. 5B) were not significantly altered; however, the mature osteoblast markers Col1a1 (Fig. 5C) and Osteocalcin (Bglap) (Fig. 5D) were significantly reduced in Irx3floxflo/Osx-Cre calvaria, even in relation to the reductions in Bglap expression in Irx3+/+/Irx5+/+/Osx-Cre+ and Irx3floxflo/Irx5−/−/Osx-Cre+ calvaria. We compared whole calvarial expression of genes involved in osteoblast mineralization and maturation.

**Fig. 1.** Osteoblastic specific deletion of Irx3 in newborn Irx5−/− mice results in smaller mice. (A) Representative photos of newborn mice. (B) Newborn total body weight, (C) newborn body length, and (D) newborn BMD of lower right limb of indicated genotypes. Data are from n = 16 Irx3+/+/Irx5+/+/*Osx-Cre−, n = 5 Irx3+/+/*Irx5−/−/*Osx-Cre+, n = 27 Irx3floxflo/Irx5+/+/*Osx-Cre−, n = 9 Irx3floxflo/Irx5−/−/*Osx-Cre+, n = 3 Irx3floxflo/Irx5+/+/*Osx-Cre+, n = 3 Irx3floxflo/Irx5−/−/*Osx-Cre+, n = 3 Irx3floxflo/Irx5−/−/*Osx-Cre+. Statistical differences were determined by a Student’s t-test, *p < 0.05, **p < 0.01, ***p < 0.001. N.S., not significant.

**Fig. 2.** Irx3floxflo/Irx5−/−/Osx-Cre+ mice have reduced skull mineralization. Representative skull microCT imaging of newborn Irx3floxflo/Irx5−/−/Osx-Cre+ and control littermate skulls. (A) Right lateral view of the skull. (B) Posterior view of the skull. Green arrows denote the interparietal bone, red arrows denote the parietal bones, and yellow arrows denote the frontal bones. Images are representative of n = 2 of each genotype. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Osx-Cre− control calvaria. *Enam* and *Tifp11*, two genes implicated in mineralization, were not changed in expression in *Irx3flox/flox/Irx5−/−/Osx-Cre+* calvaria (data not shown).

We next examined calvarial gene expression of chondrogenesis using *Sox9*, *Col2a1*, *Acan*, and *Mmp9*, since mesenchymal cells from intermembranous bones maintain chondrogenic gene expression (Aberg et al., 2005). There were no observable differences in *Sox9* (Fig. 5E) and *Col2a1* (Fig. 5F) levels, although we observed significant reductions of *Acan* (Fig. 5G) in *Irx3flox/flox/Irx5−/−/Osx-Cre+* calvaria. We observed a significant reduction in *Mmp9* expression in *Irx3flox/flox/Irx5−/−/Osx-Cre+* calvaria that was not observed in the other genotypes, even though *Irx3+/+/*Irx5+/+/*Osx-Cre+* and *Irx3flox/flox/Irx5−/−/Osx-Cre+* calvaria were modestly reduced in *Mmp9* expression (Fig. 5H). We observed no differences in expression of apoptosis related genes *Bcl2* and *Bcl-xl* in *Irx3flox/flox/Irx5−/−/Osx-Cre+* calvaria (Fig. 5I-J). This indicated that loss of *Irx3* and *Irx5* together in osteoblastic lineage cells affects later osteogenic genes more significantly than chondrocyte genes and does not result in increased apoptosis of osteogenic cells in the calvaria.

In studies using *Xenopus laevis* embryos, IRX5 interacted with GATA3 and TRPS1, forming a complex that down regulated CXCL12 production (Bonnard et al., 2012), although IRX3 and IRX5 did not directly influence Gata3 expression (data not shown). We examined the expression of *Cxcl12* and *Trps1* in order to determine if the reduced bone mineralization in *Irx3flox/flox/Irx5−/−/Osx-Cre+* mice was through downstream
mediators of Gata3, Irx3, and Irx5. Cxcl12 expression was not significantly altered in \( \text{Irx}3^{\text{flox}}/\text{Irx}5^{-/-} \text{/Osx-Cre}^- \) mice (Fig. 5K). Interestingly, there was a significant reduction in Trps1 in \( \text{Irx}3^{\text{flox}}/\text{Irx}5^{-/-} \text{/Osx-Cre}^- \text{calvaria} \) (Fig. 5L), a gene that can reduce Bglap expression in vitro and is required for proper osteoblast mineralization (Piscopo et al., 2009; Kuzynski et al., 2014). These findings suggest a role for Irx3 and Irx5 in the regulation of osteoblast mineralization gene expression and suggest that in mineralization, Irx3 and Irx5 may function through a pathway that is different from Gata3.

2.4. Older \( \text{Irx}3^{\text{flox}}/\text{Irx}5^{-/-} \text{/Osx-Cre}^- \) mice have reduced bone mineralization

We next looked at whole body and skeletal mineralization of \( \text{Irx}3^{\text{flox}}/\text{Irx}5^{-/-} \text{/Osx-Cre}^- \) mice that survived to 3–4 weeks of age to identify if there were similar reductions in bone mineralization described in Hamamy patients (Hamamy et al., 2007a), 3–4-week-old \( \text{Irx}3^{\text{flox}}/\text{Irx}5^{-/-} \text{/Osx-Cre}^- \) mice had significant reductions in body size and length (Fig. 6A–C), while body weights remained comparable in all the other genotypes through 12 weeks of age, with the exception of a significant decrease in body weight in 12 week old \( \text{Irx}3^{\text{flox}}/\text{Irx}5^{-/-} \text{/Osx-Cre}^- \) mice (Fig. 6D–E). Alizarin red and alcian blue staining of 3–4-week-old \( \text{Irx}3^{\text{flox}}/\text{Irx}5^{-/-} \text{/Osx-Cre}^- \) mice revealed an overall reduction bone mineralization with gross skeletal abnormalities and spontaneous fractures in 3 out of the 10 mice that were analyzed (Fig. 6F). Additionally, cranial bone mineralization appeared reduced in \( \text{Irx}3^{\text{flox}}/\text{Irx}5^{-/-} \text{/Osx-Cre}^- \) mice, mineralized by 3.5 weeks of age (Supplementary Fig. 6G–H). \( \text{Irx}3^{\text{flox}}/\text{Irx}5^{-/-} \text{/Osx-Cre}^- \) mice whole body bone mineral density (BMD) were unchanged compared to control littermates but there was a significant decrease in bone mineral content (BMC), consistent with the reduced bone size (Supplementary Fig. 6I–L). Unfortunately, the high lethality of \( \text{Irx}3^{\text{flox}}/\text{Irx}5^{-/-} \text{/Osx-Cre}^- \) mice limited our ability to generate sufficient numbers to adequately analyze the 4 week phenotype further. These data indicate that deletion of Irx3 and Irx5 in osteoblastic cells can influence both body size and bone mineralization in both newborn and older mice.

2.5. Bone density is reduced in Hamamy syndrome patients

Hamamy patient mutations in IRX5 result in craniofacial dysmorphisms and mineralization defects while loss of IRX5 in mice results in no detectable bone abnormalities (Li et al., 2014). Since the global loss of both Irx3 and Irx5 leads to cardiac phenotypes similar to those seen in Hamamy patients, we wanted to see if the decreased mineralization we found in \( \text{Irx}3^{\text{flox}}/\text{Irx}5^{-/-} \text{/Osx-Cre}^- \) mice also reflected the clinical presentation of Hamamy syndrome patients.
Patients with Hamamy syndrome at 8 and 9 years of age displayed reduced bone mineral density with spine lumbar Z-scores of −3.7 and −1.5 (Table 1). Bone mineral density improved with age in these patients to −2.7 and −1.4 at ages 19 and 20 years of age, respectively (Table 1). The femoral Z-score was determined at 9 years of age for one Hamamy patient (femoral Z-score of −2.2), but both patients femoral Z-scores remained above −1.0 at 19 and 20 years of age (Table 1). We noted dramatic reductions in bone mineral content in 3–4 week old Irx3fl/fox/Irx5−/−/Osx-Cre mice with spontaneous fractures in 3 out of 10 mice, indicating that 3–4 week old Irox3fl/fox/Irx5−/−/Osx-Cre mice have similarities to Hamamy patient bone mineralization. Furthermore, the spontaneous fractures observed in 3–4 week old Irx3fl/fox/Irx5−/−/Osx-Cre mice resembles the bone fragility reported in femora and other long bones of 8–10 year old Hamamy patients (Hamamy et al., 2007a).

3. Discussion

Proper craniofacial development requires control of bone mineralization by osteoblastic cell lineages (Mackie et al., 2008; Percival & Richtsmeier, 2013). Our studies show that osteoblast-specific loss of Irox3 and Irox5 leads to impaired mineralization in a very specific subset of cranial bones, possibly by blocking their expression of mature osteoblast mineralization genes (Fig. 7).

During the course of our study, we unexpectedly discovered that Osx-Cre mice have a newborn mineralization defect independent of the Irox3 and Irox5 mutation status. Our studies are consistent with recent reports that Osx-Cre mice alone have a newborn bone mineralization defect, specifically in intramembranous bones (Wang et al., 2015; Huang & Olsen, 2015). Interestingly, the absence of both Irox3 and Irox5 in osteoblastic cells caused an even more dramatic defect in intramembranous mineralization. Furthermore, Osx-Cre mice can survive past weaning and later stages of bone development occur normally, whereas Irox3fl/fox/Irx5−/−/Osx-Cre mice experience premature lethality around 3.5–4 weeks of age with bone fragility and spontaneous fractures. This indicates that the absence of both Irox3 and Irox5 in osteoblastic cells can influence neonatal survival at later stages of development. Our findings also emphasize the importance of using Osx-Cre littermates as controls for studies involving skeletal development. Furthermore, our results suggest that other Cre drivers, such as Runx2-Cre or Bglap-Cre mice, may be useful for future studies to confirm early skeletal mineralization phenotypes (Elefteriou & Yang, 2011).

Irx3fl/fox/Irx5−/−/Osx-Cre mice that survive to 3–4 weeks of age have smaller femora and tibiae and appeared to have signs of bone fragility, which is consistent with reports of Hamamy syndrome patients developing bone fragility and long bone fractures later in life (Hamamy et al., 2007a). Hamamy syndrome patients also had reduced BMD that was not observed in either newborn or 3–4 week old Irox3fl/fox/Irx5−/− mice. BMD measurements in mice are not particularly sensitive and more detailed analysis of Irox3fl/fox/Irx5−/−/Osx-Cre mice may be warranted. Our data demonstrate that Irx3 and Irx5 are important for both early osteoblast mineralization function and later skeletal mineralization, which also will help in understanding the bone fragility that occurs in Hamamy patients.

Our use of osteoblast specific deletion of Irox3 in Irox5−/− mice differs from previous models that have germ-line deletions of Irox3 and Irox5 or deletions of Irox1 and Irox2 in chick embryos, all of which showed severe limb defects (Li et al., 2014; Diaz-Hernandez et al., 2013). We were surprised to find that use of the Osx-Cre to delete Irox3 in Irox5−/− mice did not lead to significant limb malformations in newborn Irox3fl/fox/Irx5−/−/Osx-Cre mice; this is likely due to the fact that endochondral bone patterning is determined much earlier in development and may involve a different subset of cell types (Mackie et al., 2008; Knothe Tate et al., 2008). Furthermore, Irox3 and Irox5 germine deletion resulted in increased sonic hedgehog signaling sensitivity through upregulation of Ptc1 and Gli1, which are important for early establishment of limb...
For femur and tibia measurements, data are from Irx measurement) whole body (I) bone mineral density (BMD) and (J) bone mineral content (BMC) of Cre and control littermates. Financial (K) and tibia (L) length from 3 to 4 weeks old body weight and length old data is from n = 15 (n = 10 for length measurement) Ibx/+/Ibx5+/Ox-Cre−, n = 10 (n = 9 for length measurement) Ibx3+/Ibx5+/Ox-Cre−, n = 12 (n = 13 for length measurement) Ibx5+/Ibx5+/Ox-Cre−, n = 3 (n = 3 for length measurement) Ibx3+/Ibx5+/Ox-Cre−, and n = 4 (n = 4 for length measurement) Ibx3+/Ibx5+/Ox-Cre− mice. For femur and tibia measurements, data are from n = 6 (n = 5 for tibia) Ibx3+/Ibx5+/Ox-Cre−, n = 6 Ibx3+/Ibx5+/Ox-Cre− mice, and n = 5 Ibx3+/Ibx5+/Ox-Cre− mice, and n = 11 Ibx3+/Ibx5+/Ox-Cre− mice, pooled sexes. Statistical differences were determined by a Student’s t-test, *p < 0.05, **p < 0.01, ***p < 0.001. N.S., not significant.
formation and osteoblast proliferation, but the contribution to the expression of bone mineralization genes was minimal (Li et al., 2014; Pandol et al., 2000; Olsen, 2015). Thus, all experiments include these mice as a control in birth, consistent with recent reports (Wang et al., 2015). We obtained 43% viability of Irx3+/- mice crossed with Osx-Cre/+ mice have reduced osteoblastic mineralization indicating that IRX3 to IRX5 binding maintains an important role in Hamamy syndrome and understanding the role of IRX3 and IRX5 together will help provide insight into the roles of IRX proteins in other organs.

### 4. Methods

#### 4.1. X-ray analysis of Jordanian and Turkish patients with Hamamy Syndrome

The Jordanian patients were originally described by Hanan Hamamy at Jordan University Hospital (Hamamy et al., 2007a). The Turkish family was diagnosed by Hülya Kayserili at the Medical Genetics Department of the Istanbul Medical Faculty (Bonnard et al., 2012; Hamamy et al., 2007a). Both sets of patients provided informed consent. DEXA was used to measure area, BMD, and BMC of the lumbar spine and femoral neck, and to calculate Z-score. DEXA were performed just before puberty age (8–9 years old) and at adult age (19–20 years old).

#### 4.2. Mice

All transgenic mouse studies were approved by and performed in accordance with the Institutional Animal Care and Use Committee at the University of California, San Francisco. Irx3foxflox/Irx5+/- mice were generated as described (Gaborit et al., 2012). To create tissue specific Irx3 knockout in osteoblast-lineage cells, we crossed Irx3foxflox/Irx5+/- mice with Osx-Cre hemizygous transgenic mice (Jackson Laboratory; strain: B6-Cg-Tg(Sp7(Osx)-TA.TetO-EGFP/Cre)1Amc/J Jackson ID: 6361) to generate Irx3foxflox/Irx5+/-/Osx-Cre+ mice (Rodda & McMahon, 2006). Osx-Cre+ transgenic mice were found to have early lethality (Supplemental Fig. 1C) and a modest newborn bone phenotype that subsided by 3–4 weeks of age, which has been previously reported (Wang et al., 2015). We obtained 43% viability of Irx3foxflox/Irx5+/-/Osx-Cre+ at birth. Since the Irx3 and Irx5 loci are close together, these alleles segregate independently only at low frequency (Supplemental Fig. 1) and so these genotypes were not analyzed. In addition, we found that Osx-Cre+ mice have a skeletal mineralization deficiency at birth, consistent with recent reports (Wang et al., 2015; Huang & Olsen, 2015). Thus, all experiments include these mice as a control in our analyses. For gene expression analysis, breeding pairs of Irx3foxflox / Irx5+/- / Osx-Cre+ crossed with Irx3foxflox / Irx5+/- / Osx-Cre+ mice were used with no detectable phenotypic differences observed in Osx-Cre single and double transgenic mice. All data are from both male and female mice.

### Table 1

| Hamamy Patient Z-scores. | Patient 1 | Patient 2 |
|--------------------------|-----------|-----------|
| Age (years) | 9 | 20 | 8 | 19 |
| Z-score lumbar spine | -1.5 | -1.4 | -3.7 | -2.7 |
| Z-score femoral neck | Greater than -1 | N.D. | Greater than -1 |
4.3. Alizarin red and alcian blue staining of skeletons

Newborn mice and 3–4 week old mice of both sexes were euthanized and prepared for alizarin red and alcian blue skeletal staining ([Ovcinnikov, 2009]) by fixing in 100% ethanol for 24 h. Samples were then switched to acetone (Sigma-Aldrich) for an additional 24 h. Once fixed, samples were stained with final concentration of 5% glacial acetic acid, 0.5% alizarin red S (Sigma-Aldrich), 0.9% alcian blue 8GX (Sigma-Aldrich) in ethanol for 3 h at 37 °C and then at room temperature for 24 h. Samples were then placed in 1% KOH (Amresco) for 3 h and replaced with fresh KOH until non-bone tissue was transparent. Samples were then replaced with increasing concentrations of glycerol and photographed with a Leica MZFLIII dissection microscope with Diagnostic Instruments 14.2 Color Mosaic camera for newborn samples. 3–4 week old samples were photographed with a Nikon E5200 without a microscope.

4.4. Histology

Newborn skulls were skinned and fixed in neutral buffered formalin for at least 48 h and then replaced with 70% ethanol for at least 24 h. Skull tissues were paraffin embedded and sectioned. Skulls were then cut at the midline and then stained with hematoxylin & eosin, using standard protocols (J. David Gladstone Institutes Histology Core).

4.5. Bone densitometry and microCT imaging

DEXA was used to measure mouse whole-body BMD and BMC. Mice were anesthetized with inhaled isoflurane (1.5% to 2% in oxygen) and scanned on a GE Lunar PIXimus2 (PXimus). Newborn mice that underwent whole-mouse microCT scans were sacrificed and stored in 70% ethanol before scanning. Ex vivo images were obtained on a Scanco vivoCT–40 microCT scanner (Scanco) at an X-ray energy of 55kV, with sigma 0.8/support 1/threshold 120 (103.7 mg HA/cm3), a voxel size of 76 μm, and integration times of 200 ms for whole-body images.

4.6. RNA isolation, cDNA synthesis, and qPCR

Whole calvaria or dissected calvarial tissues were placed in Trizol (Invitrogen) and homogenized using a Powergen 125 homogenizer (Fisher). RNA was isolated using chloroform extraction for whole calvaria or by Picopure RNA isolation columns for dissected calvaria (Life Technologies). Purified mRNA was then used as a template to synthesize cDNA with oligo dT primers with the Superscript III (Invitrogen) kit as described (Cain & Manilay, 2013). qPCR expression analysis was performed using TaqMan primers for qPCR reactions (Supplementary Table 1) on a Via7 real-time thermocycler (Applied Biosystems) run in 5 μl sample volumes in triplicate or preamplified using Fluidigm preamplification qPCR mix and assayed using Fluidigm dynamic array IFC qPCR plates (Fluidigm). All expression values were normalized to Gapdh levels.

4.7. Statistics

Differences between the means of biological replicates for all analyses were calculated using two tailed Student’s T-test (GraphPad Prism. La Jolla, CA). Analyses were considered statistically significant if p ≤ 0.05. Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bonr.2016.02.005.

Conflict of interest statement

Edward Hsiao receives funding from Clementia Pharmaceuticals for an unrelated clinical trial.

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