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RESEARCH ARTICLE

# Primary cilia are necessary for Prx1-expressing cells to contribute to postnatal skeletogenesis

**Emily R. Moore*, Yuchen Yang and Christopher R. Jacobs**

## ABSTRACT

Although Prx1 (also known as PRRX1)-expressing cells and their primary cilia are critical for embryonic development, they have yet to be studied in the context of postnatal skeletogenesis owing to the lethality of mouse models. A tamoxifen-inducible Prx1 model has been developed, and we determined that expression directed by this promoter is highly restricted to the cambium layers in the periosteum and perichondrium after birth. To determine the postnatal role of these cambium layer osteochondroprogenitors (CLOPs) and their primary cilia, we developed models to track the fate of CLOPs (Prx1CreER-GFP;Rosa26$^{tdTomato}$) and selectively disrupt their cilia (Prx1CreER-GFP;Ift88$^{fl/fl}$). Our tracking studies revealed that CLOPs populate cortical and trabecular bone, the growth plate and secondary ossification centers during the normal program of postnatal skeletogenesis. Furthermore, animals lacking CLOP cilia exhibit stunted limb growth due to disruptions in endochondral and intramembranous ossification. Histological examination indicates that growth is stunted due to limited differentiation, proliferation and/or abnormal hypertrophic differentiation in the growth plate. Collectively, our results suggest that CLOPs are programmed to rapidly populate distant tissues and produce bone via a primary cilium-mediated mechanism in the postnatal skeleton.

**KEY WORDS: Primary cilia, Osteochondroprogenitors, Prx1, Periosteum, Growth plate, Postnatal skeletogenesis**

## INTRODUCTION

The periosteum and perichondrium are thin tissues surrounding bone and cartilage, respectively. They contain progenitors capable of differentiating into various cell types that populate skeletal tissues. A portion of cells in the inner cambium layer express Prx1 (also known as PRRX1), a homeobox protein whose expression is highly restricted in the postnatal appendicular skeleton (Kawanami et al., 2009). These Prx1-expressing cells are deemed osteochondroprogenitors because they preferentially commit to a chondrogenic and/or an osteogenic fate (Kawanami et al., 2009; Ouyang et al., 2014). Periosteal cells have long been known to express osteogenic markers *in vitro* (Arnsdorf et al., 2009; Bei et al., 2012; Kawanami et al., 2009; Nakahara et al., 1990a, 1991) and form cartilage and bone *in vivo* (Bilkay et al., 2008; Colnot, 2009; Leucht et al., 2008; Nakahara et al., 1990b; O'Driscoll and Fitzsimmons, 2001), but only recently have cambium-derived Prx1-expressing cells been proposed to be the main source of osteochondroprogenitors in the periosteum (Murao et al., 2013).

Prx1-expressing cells are critical for embryonic skeletal development, but have yet to be studied in the context of postnatal development. Prx1 is expressed throughout the embryonic limb bud mesenchyme and a subset of the craniofacial mesenchyme as early as 9.5 days post coitum (dpc). By 10.5 dpc, essentially all of the mesenchymal cells in the limb express Prx1 (Logan et al., 2002). These Prx1-expressing cells then contribute to formation of the growth plate and the bone collar, as well as secondary ossification centers, which subsequently direct endochondral ossification (Hall, 2015; Haycraft et al., 2007). Prx1-expressing cells have not been studied during postnatal development, when skeletal growth is most rapid (Lamon et al., 2017; Richman et al., 2001; White et al., 2010), partially due to the lethality associated with current models (Haycraft et al., 2007; Martin and Olson, 2000; Ouyang et al., 2014). Recently, Kawanami et al. developed an inducible transgenic mouse model and found that Prx1 expression becomes further restricted in the limbs after birth (Kawanami et al., 2009). This group suggests Prx1 expression is primarily in the periosteum and perichondrium, but understanding of the full postnatal expression profile is incomplete.

Periosteal progenitors populate adult skeletal tissues in response to mechanical stimulation or fracture. Progenitor cells generally demonstrate upregulation in osteogenic markers when subjected to physical stimuli *in vitro* (Chen and Jacobs, 2013; Hoey et al., 2012; Kanno et al., 2005), so it is perhaps not surprising that cambium-derived cells generate bone in response to mechanical cues *in vivo* (Colnot, 2009; Evans et al., 2013; Ferretti and Mattioli-Belmonte, 2014; Shimizu et al., 2001). Fracture studies suggest periosteal progenitors are the most important cell source for callus formation (Duchamp de Lageneste et al., 2018; Murao et al., 2013). Moreover, a considerable portion of callus cells are derived from a Prx1-expressing population that is believed to originate from the periosteum (Duchamp de Lageneste et al., 2018; Kawanami et al., 2009; Ouyang et al., 2014). Interestingly, bone marrow and periosteum both supply osteoblasts to facilitate skeletal repair, but only the periosteum provides chondrocytes. The periosteum is therefore the primary contributor to post-fracture repair, which is dominated by endochondral ossification (Colnot, 2009; Duchamp de Lageneste et al., 2018). A recent study also indicates periosteal stem cells self-renew, are primed to regenerate bone and are more proliferative *in vitro* compared to bone marrow stem cells (Duchamp de Lageneste et al., 2018). Collectively, these studies suggest the adult periosteum contains Prx1-expressing progenitors that possess an inherent, lasting osteochondrogenic nature and continue to generate skeletal tissues.

A mechanism by which osteochondroprogenitors could mediate differentiation is through their primary cilia, solitary sensory organelles that project from the surface of almost every mammalian cell. The primary cilium is an established mechanosensor and is known to be instrumental in progenitor osteochondrogenesis (Deren

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**Received 14 March 2018; Accepted 6 July 2018**

Journal of Cell Science (2018) 131, jcs217828. doi:10.1242/jcs.217828

et al., 2016; Hoey et al., 2012). Specifically, our previous work indicates that cilia are critical to osteogenic differentiation of periosteal progenitors *in vitro* (Arnsdorf et al., 2009). The primary cilium is also a potent chemosensor that directs left–right patterning and functions as a nexus for signaling pathways during embryonic vertebrate development (Anderson et al., 2008; Goetz and Anderson, 2010). Haycraft et al. disrupted primary cilia in Prx1-expressing cells, and showed that this caused severe defects in embryonic skeletal development, including dwarfism, polydactyly, an inability to form the bone collar or secondary ossification centers and abnormal growth plate structure (Haycraft et al., 2007). This knockout was so severe that mutants did not survive birth and, consequently, the role of Prx1-expressing cell primary cilia in development has yet to be examined after birth. We hypothesize that these cells and their cilia are also critical for postnatal development, considering the extent to which they contribute to embryonic skeletogenesis and adult fracture repair.

Progenitors in the cambium layers and their cilia are promising candidates for intramembranous and endochondral ossification, but are surprisingly understudied. The postnatal appendicular skeleton thickens via intramembranous ossification, whereby osteoblasts are directly recruited from the periosteum to deposit new bone. Additionally, during endochondral ossification, chondrocytes of undetermined origin populate the growth plate, proliferate, and undergo hypertrophy, resulting in lengthening of the limbs. Yang et al. proposed a model whereby periosteal and perichondrial progenitors differentiate into growth plate chondrocytes, some of which further differentiate into osteoblasts. However, studies have yet to confirm whether these progenitors are indeed a source of chondrocyte and osteoblast progeny (Yang et al., 2014). Furthermore, there are no current studies on periosteal or perichondrial primary cilia in postnatal development. Rosa Serra's group has extensively studied the role of chondrocyte cilia in the growth plate by deleting a gene critical to cilia formation and maintenance, *Ift88*. The investigators found that abrogating chondrocyte cilia and disrupting cilium-mediated signaling pathways results in decreased chondrocyte proliferation and accelerated hypertrophy (Chang and Serra, 2013; Song et al., 2007). They also determined that cilia orientation is critical to chondrocyte proliferation, and this claim is consistent with work from other groups proposing a function for primary cilia polarity (Ascenzi et al., 2007, 2011; de Andrea et al., 2010; Haycraft et al., 2007; McGlashan et al., 2006). In an *in vivo* overloading model, Rais et al. determined that chondrocyte cilia sense mechanical loading of the growth plate and accelerate chondrocyte hypertrophy in response to increased loads (Rais et al., 2015). In fact, chondrogenesis is highly dependent on chondrocyte mechanosensing, which is mediated through the cilium (Deren et al., 2016; Tummala et al., 2010). Overall, it is possible that Prx1-expressing cells reside in the cambium layers and contribute to postnatal ossification through a cilium-dependent mechanism, but this has yet to be confirmed *in vivo*.

In this study, we sought to determine the role of Prx1-expressing progenitors in postnatal skeletogenesis as mediated by their primary cilia *in vivo*. Owing to the osteochondrogenic nature of periosteal cells and extent to which chondrocyte cilia regulate endochondral ossification, we hypothesize that Prx1-expressing cells require cilia to participate in postnatal skeletogenesis. We developed mouse models to track Prx1-expressing cells and disrupt their primary cilia *in vivo* during juvenile skeletogenesis. We anticipate that the knowledge gained from studying postnatal development will provide insights on how to recapitulate skeletogenesis *in vivo* and more effectively utilize osteochondroprogenitors and their cilia for bone regeneration therapies.

## RESULTS

### Prx1 expression is restricted to the cambium layers in the appendicular skeleton after birth

The vast majority of limb bud mesenchymal cells express Prx1 during embryonic development; however, recent studies indicate Prx1 expression is highly restricted to the calvaria and specific regions of the appendicular skeleton after birth (Kawanami et al., 2009; Ouyang et al., 2014). In order to fully characterize postnatal Prx1 expression in the forelimbs at our experimental time points, which represent distinct stages of skeletal development (Fig. 1), we injected Prx1CreER-GFP;Rosa26$^{tdTomato}$ mice with tamoxifen at post-natal day (P)7, P17 or P28. Animals were euthanized 2 days after the injection, and we visualized GFP to identify cells that expressed Prx1 at that time. We also visualized tdTomato, which denotes CreER recombination, to track cells that expressed Prx1 at the time of injection. At every time point, Prx1 expression was observed in the cambium layers of the perichondrium (Fig. 2A,B) and periosteum (Fig. 2C,D). In mice injected at P7, Prx1-expressing cells were found in the ulnar growth plate near the groove of Ranvier (Fig. 2B), where chondrocyte progenitors are recruited from the perichondrium to supply chondrocytes for appositional cartilage growth (Karlsson et al., 2009; Langenskiöld, 1998; Oni, 1997; Walzer et al., 2014). Very few Prx1-expressing cells were identified in the groove of Ranvier in mice injected at P17 and were completely absent in the P28 group. Among all of the P28 specimens examined, a mere three osteocytes located near the periosteal edge expressed Prx1. At P30, cells in the cambium layers expressed both Prx1 and recombined Cre; however, this was a rare occurrence in pups injected at P7 and examined at P9. All Prx1-expressing cells found outside the cambium layers in the growth plate and cortical bone also expressed tdTomato (Fig. 2B), indicating Cre recombination had occurred. At every time point examined, cells that solely expressed tdTomato were found in the

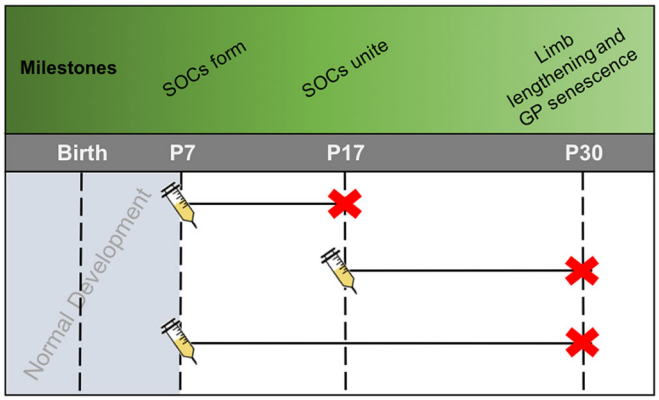


**Fig. 1. Experimental groups selected to evaluate juvenile skeletal developmental milestones.** Following normal embryonic development, postnatal transgenic mice received injections of tamoxifen to induce Cre recombination-mediated tdTomato expression and/or primary cilia deletion in Prx1-expressing cells. The earliest injections were administered at postnatal day 7 (P7) so that secondary ossification centers (SOCs) could develop normally (Cooper et al., 2013). SOCs in the ulnae generally unite by P17. Our preliminary injection trials suggested P30 was optimal to observe significant increases in limb length and growth plate (GP) senescence. The group injected at P7 and euthanized (red X) at P17 was chosen to evaluate the role of CLOPs and their cilia in SOC unification. The P17–P30 group was selected to examine their behavior after SOCs unify. The P7–P30 group was selected to observe the overall contribution of CLOPs and their cilia to juvenile skeletal development. Animals received single or daily injections until they were euthanized depending on the experiment.

Journal of Cell Science (2018) 131, jcs217828. doi:10.1242/jcs.217828

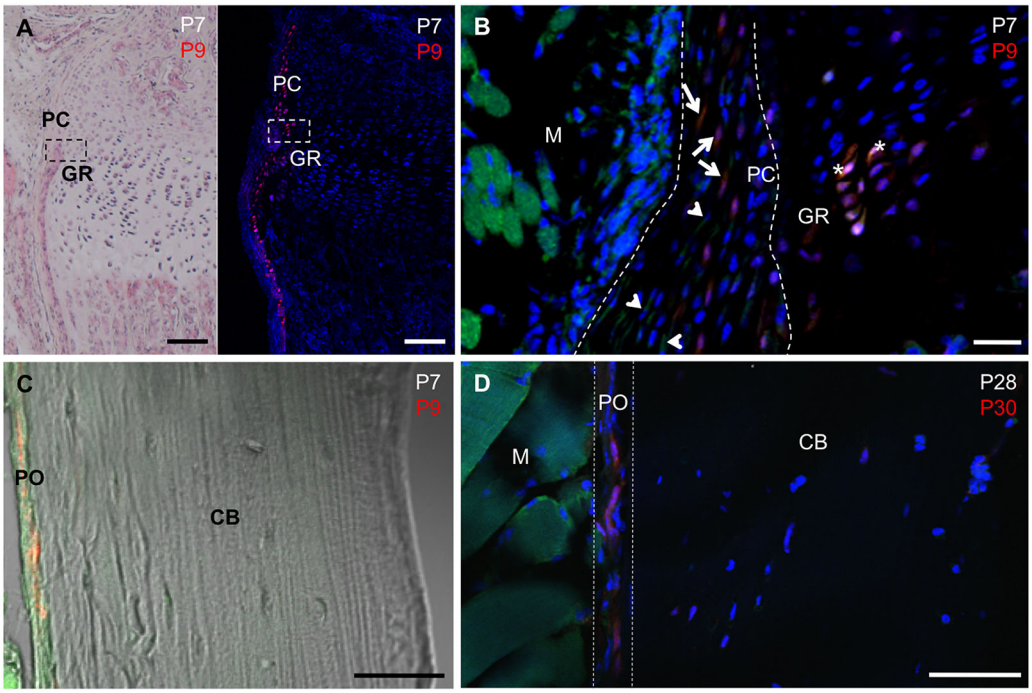


**Fig. 2. After birth, Prx1 expression is highly restricted to the cambium layers of the periosteum and perichondrium in the forelimbs.** Fluorescence microscopy was used to identify cells that expressed Prx1 at the time mice were euthanized (green), recombined cells that expressed Prx1 at the time of injection two days prior to the time at which mice were euthanized (red), and nuclei (blue) in a Prx1CreER-GFP;Rosa26$^{tdTomato}$ reporter model. (A) H&E stain of the growth plate and corresponding fluorescence image depicting recombined cells (red) at P9. (B) Magnified boxed region from A to show green Prx1-expressing cells (examples denoted by arrowheads), red recombined cells (examples denoted by arrows), and recombined cells expressing Prx1 (asterisks). (C) Fluorescence and brightfield overlay of cortical bone and periosteum at P9. (D) Fluorescence image depicting recombined cells in the periosteum at P30. Green fluorescence visualized in muscle and bone marrow was determined to be autofluorescence when compared to Ift88$^{fl/fl}$ negative controls, which lack the Prx1CreER-GFP transgene. These tissues also lacked tdTomato-expressing cells. Nuclei are displayed in blue. The injection period (white label P7 or P28) and SAC date (red label P9 or P30) are noted in the upper right corner of each image. Scale bars: 50 µm. Labels refer to cortical bone (CB), groove of Ranvier (GR), perichondrium (PC), periosteum (PO), and muscle (M). Images are representative of *n*=5 animals per group, with one limb per animal and four sections per limb.

periosteum, perichondrium, periosteal surface of cortical bone, and the groove of Ranvier in the growth plate. Cre recombination was absent from all other tissues, other than a negligible number of cells in the epiphyseal growth plate in one specimen injected at P7. In all other specimens, Cre recombination was absent from the growth plate, other than the few cells near the groove of Ranvier.

Overall, expression of our Prx1 transgene was highly restricted to the cambium layers of the periosteum and perichondrium, had a trivial presence in the growth plate and cortical bone, and was completely absent from epiphyseal centers, trabecular bone, marrow, muscle, connective tissue, and cartilage in the juvenile forelimbs. For this reason, we refer to the cell population examined in this study as cambium layer osteochondro progenitors, or CLOPs.

### CLOPs initially within the cambium layers populate a number of juvenile skeletal tissues

We then utilized our reporter model to histologically track the fate of CLOPs as they differentiate and populate tissues outside the cambium layers. Prx1CreER-GFP;Rosa26$^{tdTomato}$ mice received a single tamoxifen injection at either P7 or P17 to activate tdTomato expression in CLOPs and their progeny, and were euthanized at P17 or P30 (Fig. 1). CLOPs residing in the cambium layers at P7 were found in the growth plate, proximal ulnar head, styloid processes, trabecular and cortical bone, and the endocortical surface at P17 and P30 (Fig. 3). Although P7-activated labeled cells were absent from the endosteum and marrow at P9 (Fig. 2), we identified some labeled cells at the endosteal surface of cortical bone in our P7–P17 (Fig. 3B) and P7–P30 groups, but not in the P17–P30 group. CLOPs initially in the cambium layers at P17 (Fig. 3C) populated the same areas at P30, but were fewer in number compared to those tracked at P7 (Fig. 3D). Cells activated at P7 were no longer present in the cambium layers at P17 (Fig. 3C), and appeared to populate distant tissues instead. Some cells activated at P17, however, were still present in the periosteum and perichondrium at P30 (Fig. 3D). In animals that received a single tamoxifen injection at P7 or P17 only, activated cells were primarily found in the resting zone or trabecular bone beneath the growth plate, with few in the proliferating and hypertrophic zones. We then injected Prx1CreER-GFP;Rosa26$^{tdTomato}$ mice with tamoxifen daily to determine the overall CLOP contribution to skeletal development from P7–P17, P17–P30 and P7–P30. Labeled cells consistently populated the same regions identified with single injections, but the number of labeled cells drastically increased in each group. Specifically, CLOPs initially in the cambium layers were found in trabecular and cortical bone, growth plate cartilage, periosteum, perichondrium, and the proximal and distal ends of the ulna, but were absent from marrow and muscle (Fig. 3E). Interestingly, unlike mice injected only at P7 or P17, labeled chondrocytes were present in all of the zones. In fact, the majority of osteocytes and osteoblasts in bone originated from the cambium layers, and the styloid and coronoid processes were almost entirely composed of CLOPs and their progeny. In the growth plate, CLOP progeny were found in the proliferation and hypertrophic zones in addition to the resting zone, indicating these CLOPs participate in the normal program of endochondral ossification.

Journal of Cell Science (2018) 131, jcs217828. doi:10.1242/jcs.217828

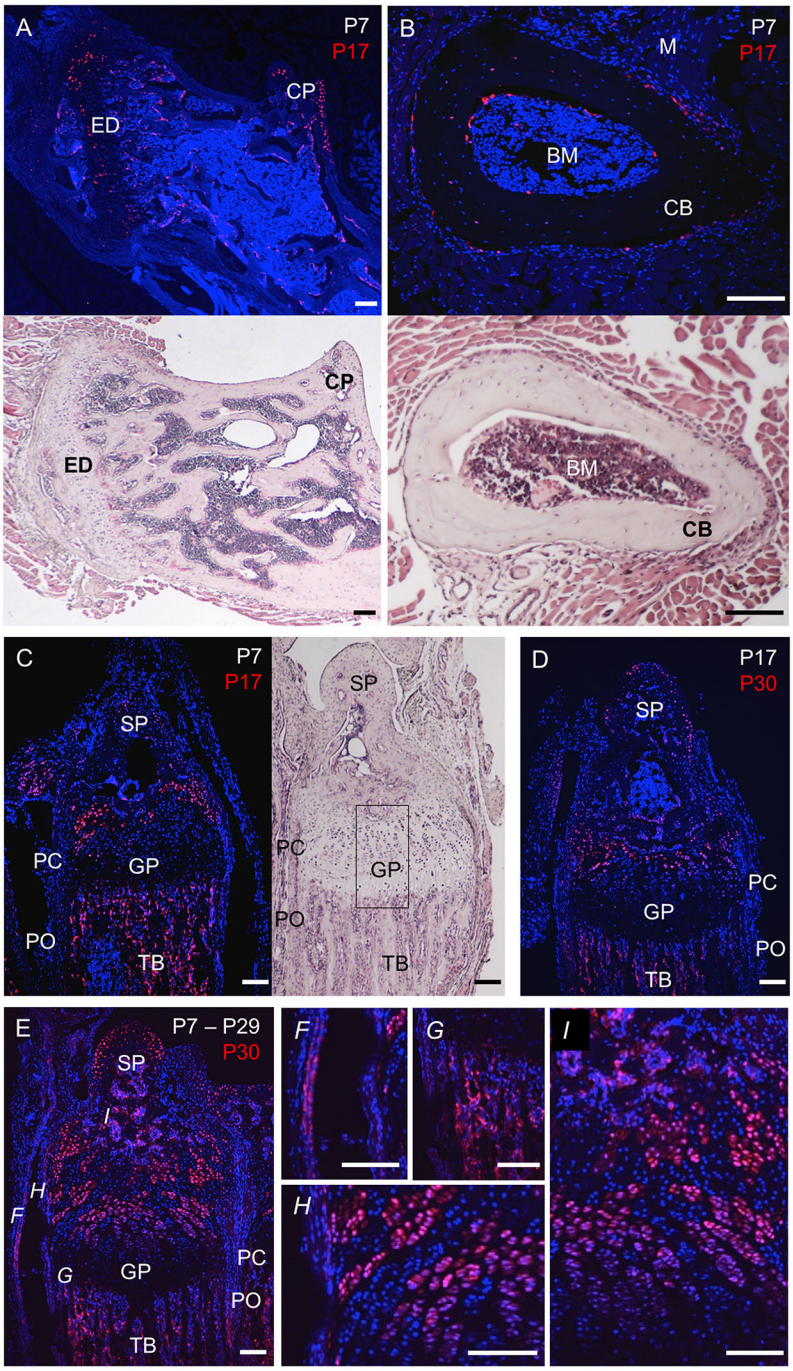


**Fig. 3. CLOPs initially in the cambium layers populate cartilage and bone tissue throughout the limb.** Prx1CreER-GFP;Rosa26$^{tdTomato}$ mice received a single (A–D) or daily (E) injection of 33 mg/kg body weight tamoxifen solution to track recombined cells (red). (A) H&E stain and corresponding fluorescence image of the proximal ulnar head at P17. (B) H&E stain and corresponding fluorescence image of a cross section of the mid diaphysis of the ulna at P17. (C) H&E stain and corresponding fluorescence image of the growth plate at P17. (D) Fluorescence image of the growth plate at P30 with a single injection of tamoxifen at P17. (E) Fluorescence image of the growth plate at P30 with daily injections beginning at P7. Regions in E were magnified to display recombined cells in the periosteum (F), trabecular bone (G), groove of Ranvier (H) and growth plate (I). Nuclei are displayed in blue. Injection period (white) and SAC date (red) are noted in the upper right corner of each image. Labels refer to bone marrow (BM), cortical bone (CB), coronoid process (CP), epiphyseal disk (ED), growth plate (GP), muscle (M), perichondrium (PC), periosteum (PO), styloid process (SP) and trabecular bone (TB). Scale bars: 100 µm. The black box in C refers to the region examined in Fig. 6. Images are representative of *n*=5 animals per group, with one limb per animal and four sections per limb.

### Primary cilia are important for CLOPs to populate the growth plate

We then generated a model to track CLOPs that lack primary cilia by eliminating a gene critical to cilia formation and maintenance (*Ift88*). Prx1CreER-GFP;Ift88$^{fl/fl}$;Rosa26$^{tdTomato}$ experimental and Prx1CreER-GFP;Rosa26$^{tdTomato}$ control mice were injected with a single dose of tamoxifen at P15 and euthanized at P17. Primary cilia were present in CLOPs in control animals, but mutants lacked cilia in nearly all recombined cells (Fig. 4). In controls, recombined cells were found in the resting and proliferation zones of the growth plate, in addition to in the groove of Ranvier (Fig. 4A). However, recombined cells were primarily located near the groove of Ranvier or the perichondrium in mice lacking CLOP primary cilia (Fig. 4B).

### Mice lacking CLOP primary cilia have stunted intramembranous ossification

The primary cilium is critical to osteogenic differentiation of progenitors (Chen and Jacobs, 2013; Hoey et al., 2012), so we investigated the role of CLOP cilia in juvenile skeletogenesis. Progenitors in the inner cambium layer of the periosteum are known to differentiate into bone-forming osteoblasts that deposit layers of bone matrix to thicken the limbs. To determine whether CLOP primary cilia are involved in intramembranous ossification, Prx1CreER-GFP;Ift88$^{fl/fl}$ pups were injected daily with tamoxifen in order to disrupt CLOP primary cilia. We collected histological cross sections from the ulnar midshaft and quantified cortical and marrow area in mutants and controls. Indeed, mutants developed

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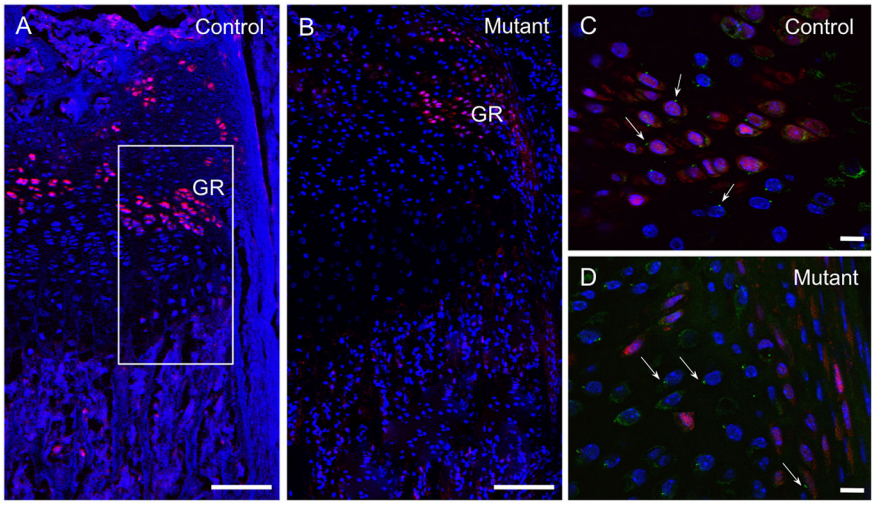


**Fig. 4. CLOPs lacking primary cilia do not readily enter the growth plate.** Prx1CreER-GFP; Rosa26$^{tdTomato}$ control and Prx1CreER-GFP;Ift88$^{fl/fl}$; Rosa26$^{tdTomato}$ experimental animals were injected with a single dose of 33 mg/kg body weight tamoxifen solution at P15 and euthanized at P17. Recombined cells (red) were visualized in the growth plates of animals with (A) and without (B) CLOP primary cilia. Magnified view of the groove of Ranvier (GR) in control (C) and experimental (D) groups. Immunohistochemistry for acetylated α-tubulin was performed to detect primary cilia (green, examples denoted by arrows). Nuclei are depicted in blue. Scale bars: 100 µm in (A,B); 10 µm (C,D). The right half of the growth plate is pictured in A and B. The white box in A refers to the region examined in Fig. 7. Images are representative of *n*=5 animals per group, with one limb per animal and four sections per limb.

significantly less cortical bone than their respective controls in all of the injection groups (Fig. 5A). Surprisingly, knockouts initiated at P7 also had wider marrow canals, but this phenotype was absent in the P17–P30 mutants (Fig. 5B).

### Endochondral ossification is attenuated in mutants

Embryos lacking primary cilia in Prx1-expressing cells have severely shortened limbs (Haycraft et al., 2007), so we also examined ulnar length in juveniles with and without CLOP primary cilia. At each time point, mutants had significantly shorter ulnae compared to their respective controls (Fig. 5C). In fact, mutants assessed at P30 had shorter ulnae than P17 controls. Mutants disrupted beginning at P7 had shorter average lengths at P30 compared to those injected at P17.

Because limb lengthening occurs via endochondral ossification, which depends on proper growth plate chondrocyte differentiation and proliferation, we investigated differences in growth plate morphology. At all stages, mutants demonstrated abnormal phenotypes but the characteristics and severity varied. Growth plate zones (Fig. 6) can be roughly determined by observing cell morphology. Chondrocytes in the resting zone are round with spherical nuclei but eventually flatten and align in vertical columns to rapidly proliferate, until they finally expand into cuboidal hypertrophic cells. The proliferating zones in P7–P17 mutants contained noticeable cartilaginous sections lacking cells (Fig. 6B). These non-cellularized regions were not present in controls (Fig. 6A), which were densely populated with cells. Chondrocytes in the P17–P30 control proliferation zones were neatly arranged in vertical columns (Fig. 6C); however, this structure was much less organized or completely absent in mutants (Fig. 6D). The lack of columnar organization and flatter morphology normally associated with proliferating cells also made it difficult to distinguish the resting and proliferation zones in P7–P17 and P17–P30 mutants. Although the growth plate zones were distinguishable in the P7–P30 group, the hypertrophic zone appeared to be much larger than in controls (Fig. 6E,F). Finally, controls evaluated at P30 exhibited ossification in the styloid processes (Fig. 6C,E), which is correlated with growth plate senescence; however, this phenomenon was drastically less pronounced in mutants (Fig. 6D,F).

In order to evaluate these observed differences in growth plate morphology, we performed immunohistochemistry for markers of growth plate chondrocyte proliferation (PCNA) and hypertrophy (Type X collagen). Indeed, mutants had fewer cells in the proliferation zone compared to controls (Fig. 5D) regardless of when progenitor primary cilia were disrupted. In all control groups (Fig. 7A,C,E), hypertrophic cells were found exclusively at the proximal end of the growth plate, where the hypertrophic zone is normally present. P7–P17 mutants generally had smaller hypertrophic zones (Fig. 7A,B) that were distinguishable from corresponding proliferation zones. In contrast, the P17–P30 (Fig. 7D) and P7–P30 (Fig. 7F) mutants did not have distinct hypertrophic and proliferation zones. Ectopic hypertrophy was present near the border between resting and proliferation zones in all mutant groups (Fig. 7B, D,F); however, these chondrocytes were primarily found near the groove of Ranvier in P7–P17 mutants but were present throughout the entire growth plate in groups evaluated at P30. We also stained our dual tracking and cilium disruption model for Type X collagen and found that the ectopic hypertrophy corresponded to recombined cells lacking cilia near or in the groove of Ranvier.

### Secondary ossification centers are underdeveloped in mutant juveniles

Prx1-expressing cells are known to form ossification centers during embryonic development (Bashur et al., 2014; Haycraft et al., 2007), and our tracking studies reveal CLOPs continue to populate ossification centers in juvenile skeletogenesis (Fig. 3). We therefore performed Toluidine Blue-O stains to examine ossification in the epiphyses and trabecular bone proximal to the ulnar growth plate. At P30, mice lacking primary cilia (Fig. 8B,D) had less bone and vasculature in the proximal head compared to what was seen in controls (Fig. 8A,C). Mutant epiphyseal disks stained a lighter purple than their respective controls, suggesting they have lower proteoglycan content. Furthermore, mutant epiphyseal disk cartilage also contained ectopic hypertrophic chondrocytes similar to those examined in the distal ulnar growth plates (Fig. 7). These phenotypic differences were slightly more severe when the knockout was initiated at P7 (Fig. 8B) compared to at P17 (Fig. 8D). In the trabecular space, controls examined at P30 (Fig. 8E) had less residual cartilage and more bone with increasing distance from the growth plate. Although mutants exhibited a similar pattern proximal to the growth plate, they contained more retained cartilage and less bone compared to controls (Fig. 8F). The phenotypic differences in the trabecular space were equally severe whether the knockout was initiated at P7 or P17. Interestingly, ossification in the aforementioned regions was comparable between mutants and controls in the P7–P17 group.

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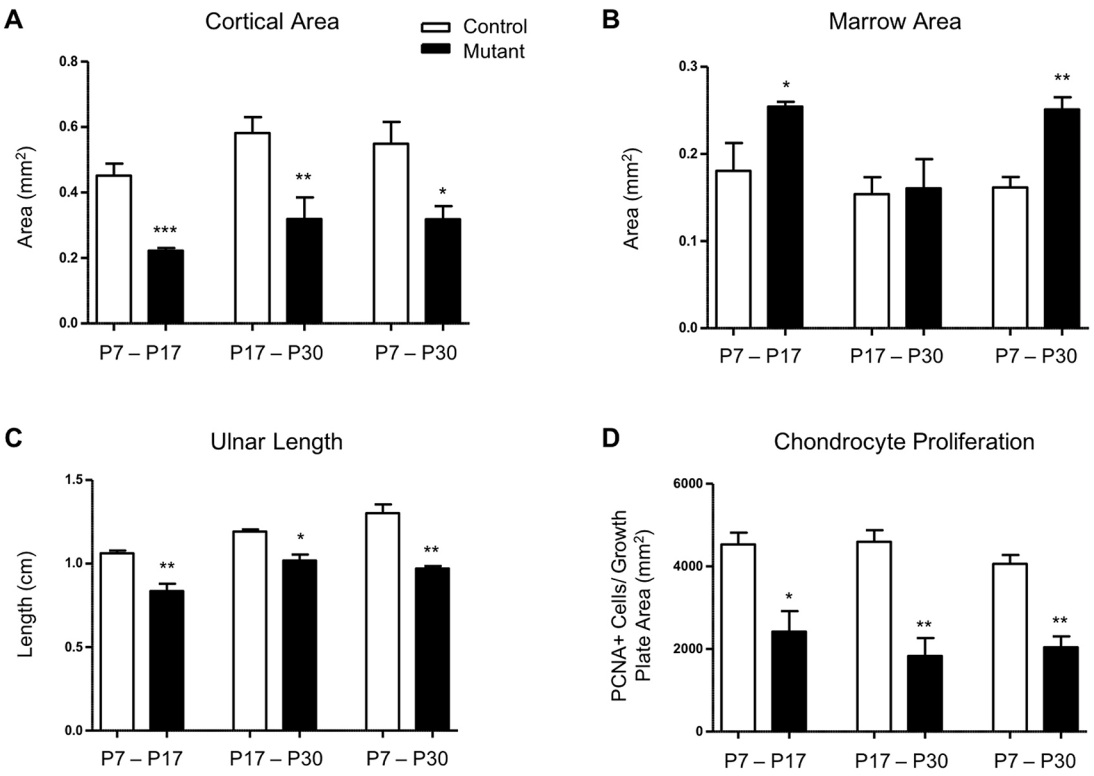


**Fig. 5. Mice lacking CLOP primary cilia exhibit stunted limb development and growth plate chondrocyte proliferation.** Ift88$^{fl/fl}$ control and Prx1CreER-GFP;Ift88$^{fl/fl}$ CLOP cilium knockout mice were injected with 33 mg/kg body weight tamoxifen daily beginning at either P7 or P17 until they were euthanized at either P17 or P30. (A) Cortical area, (B), marrow area and (C) length were quantified from H&E stains of control and experimental animal ulnae. (D) Proliferating growth plate chondrocytes were identified via immunohistochemistry for Proliferating Cell Nuclear Antigen (PCNA) and quantified in control experimental animals. Data are presented as mean±s.e.m. Images are representative of $n$=5 animals per group, with one limb per animal and five sections per limb. *$P$<0.05, **$P$<0.01, ***$P$<0.001. No sex-dependent differences were identified according to one-way ANOVA ($P$>0.473 for all measurements).

## DISCUSSION

Despite its ubiquity in the embryonic limb bud, Prx1 expression is highly restricted to the cambium layers after birth. When the Prx1CreER-GFP model was first developed, Kawanami et al. injected a Prx1CreER-GFP;Rosa26$^{lacZ}$ reporter at postnatal day 19 (P19) and assessed Cre recombination at P23 to report the presence of Prx1-expressing cells (Kawanami et al., 2009). Observing Cre activity 96 h later may not accurately depict Prx1 expression at the time recombination was triggered since skeletal development occurs rapidly in mice, especially in the first 3 weeks after birth (Lamon et al., 2017; Richman et al., 2001; White et al., 2010). Indeed, Kawanami et al. no longer detected recombined cells in epiphyseal chondrocytes at P26, merely 96 h after they observed them in P23 mice (Kawanami et al., 2009). Moreover, it takes only 48 h for

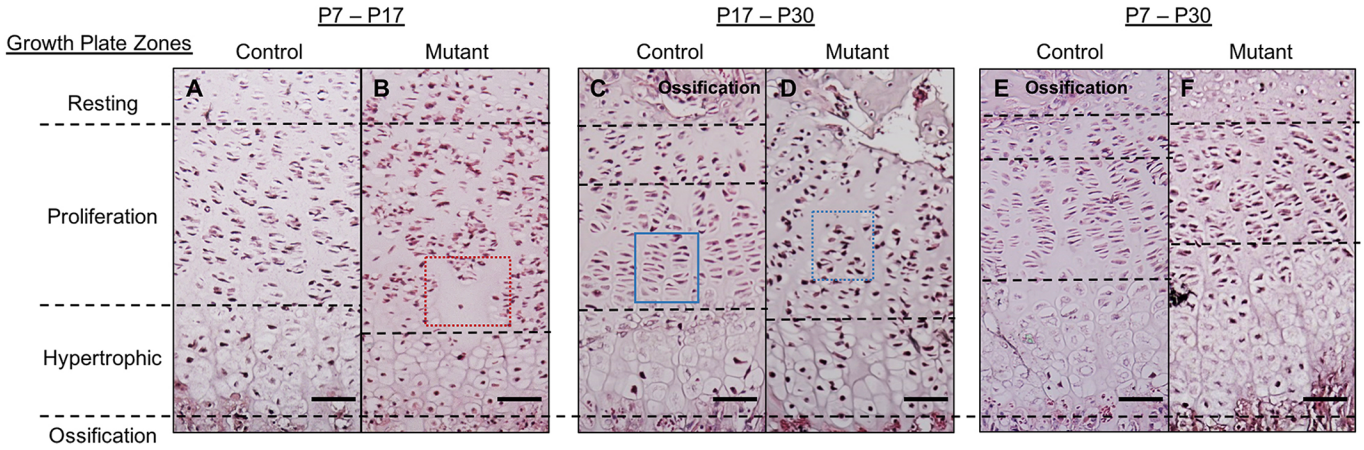


**Fig. 6. Juvenile mice lacking CLOP cilia demonstrate abnormal growth plate morphology.** Ift88$^{fl/fl}$ control and Prx1CreER-GFP;Ift88$^{fl/fl}$ CLOP cilium knockout mice were injected with 33 mg/kg body weight tamoxifen daily beginning at either P7 or P17 until they were euthanized at either P17 or P30. Growth plates were stained with H&E to discern ulnar growth plate zones (black dashed lines) in the (A,B) P7–P17, (C,D) P17–P30 and (E,F) P7–P30 groups. Scale bars: 50 µm. The region of the growth plate examined here is represented by the solid black box in Fig. 3C. Images are representative of $n$=5 animals per group, with one limb per animal and five sections per limb.

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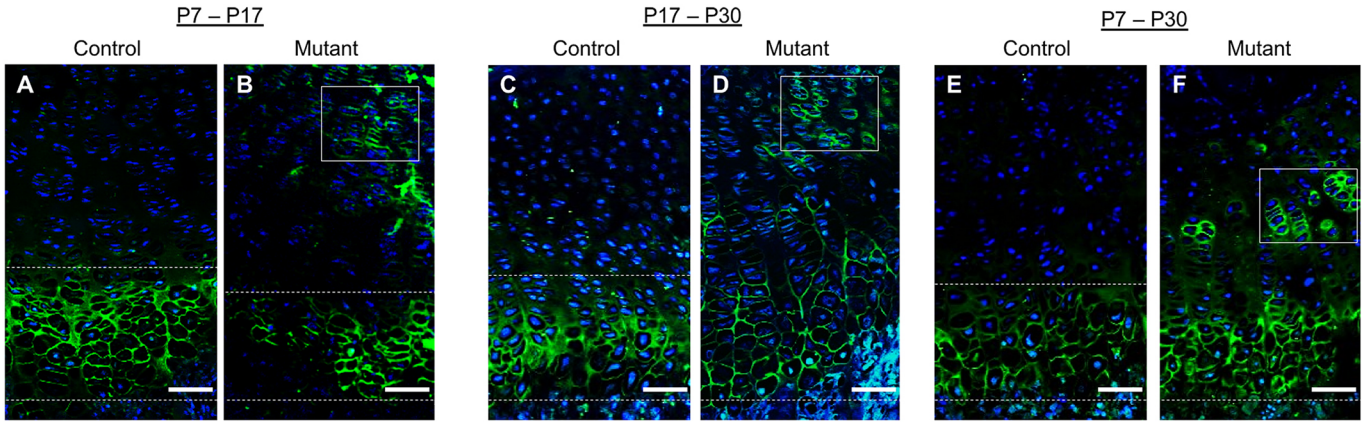


**Fig. 7. Mice lacking CLOP cilia exhibit ectopic hypertrophy in the growth plate.** Ift88$^{fl/fl}$ control and Prx1CreER-GFP;Ift88$^{fl/fl}$ CLOP cilium knockout mice were injected with 33 mg/kg body weight tamoxifen daily beginning at either P7 or P17 until they were euthanized at either P17 or P30. Immunohistochemistry for anti-Type X collagen was performed to identify hypertrophic chondrocytes (green) and the hypertrophic zone (HZ, white dashed lines) in the (A,B) P7–P17, (C,D) P17–P30 and (E,F) P7–P30 groups. Nuclei are displayed in blue. Scale bars: 50 µm. The region of the growth plate examined here is represented by the white box in Fig. 4A. Images are representative of *n*=5 animals per group, with one limb per animal and four sections per limb.

chondrocytes to transition through the hypertrophic zone and become bone-forming osteoblasts in the primary spongiosa (Yang et al., 2014). Another study of the murine forelimb suggests the entire hypertrophic zone turns over once every 24 h (Cooper et al., 2013). For this reason, it is perhaps more appropriate to evaluate the presence of GFP, which is present only when Prx1 is expressed, in addition to Cre recombination.

We therefore crossed the Prx1CreER-GFP model with a Rosa26$^{tdTomato}$ reporter and visualized GFP to detect Prx1 expression in the forelimb, as well as tdTomato to evaluate Cre activity 48 h after recombination was induced. Kawanami et al. identified the vast majority of recombined cells in the cambium layers, with some in tendon, the epiphyses and articular cartilage, and very few at the endocortical surface and in bone marrow (Kawanami et al., 2009). In agreement with Kawanami et al., we found Prx1-expressing cells were primarily in the cambium layers of the periosteum and perichondrium and to a lesser degree in tendon. Since postnatal development is so rapid, the Prx1-expressing chondrocytes we found in the growth plates of mice injected at P7 are likely recently differentiated progenitors recruited via the groove of Ranvier, which is known to rapidly recruit cells from the perichondrium (Karlsson et al., 2009; Langenskiöld, 1998; Oni, 1997; Walzer et al., 2014). Interestingly, labeled cells were more prevalent and located further towards the center of the growth plate when activated at P15 and examined at P17 compared to the P7–P9 and P28–P30 groups, suggesting recruitment and growth perhaps occurs more rapidly at this age. Additionally, because it takes 26 h for GFP to turn over (Corish and Tyler-Smith, 1999), these cells may not express Prx1 but still contain residual GFP protein. By using our red fluorescent reporter to track cells with activated Cre, we found recombined cells in the same locations that Kawanami et al. identified, but did not detect accompanying Prx1 expression in the marrow space, articular cartilage, epiphyses or endosteum. This indicates postnatal Prx1 expression is much more restricted to the cambium layers than previously believed and that these progenitors possess a surprising ability to rapidly populate skeletal tissues.

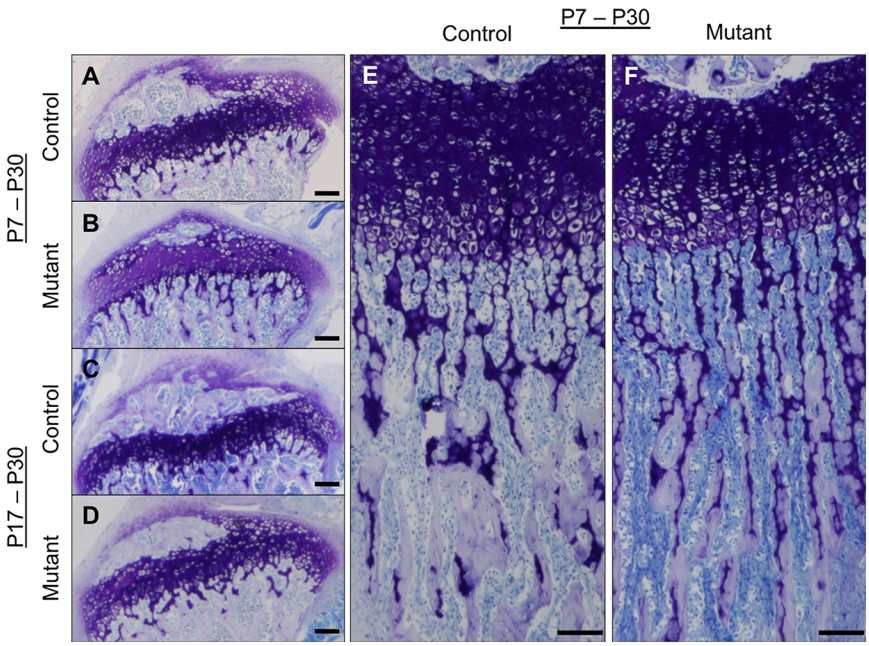


**Fig. 8. General ossification is stunted in juveniles when CLOP cilia are deleted.** Ift88$^{fl/fl}$ control and Prx1CreER-GFP;Ift88$^{fl/fl}$ CLOP cilium knockout mice were injected with 33 mg/kg body weight tamoxifen daily beginning at either P7 or P17 until they were euthanized at either P17 or P30. Toluidine Blue O stains were performed to identify cartilage (purple), bone (off-white) and marrow (light blue) in the limbs of mice with and without CLOP primary cilia. The epiphyseal disk of P7–P30 control (A) and experimental (B) mice. The epiphyseal disk of P17–P30 control (C) and experimental (D) mice. The growth plate and trabecular bone of P7–P30 control (E) and experimental (F) mice. Black scale bars represent 100 µm. Images are representative of *n*=5 animals per group, with one limb per animal and four sections per limb.

Periosteal progenitors have long been thought to participate in intramembranous ossification; however, our tracking studies show, for the first time, that CLOPs are incorporated in the postnatal growth plate and also engage in endochondral ossification. Prx1-expressing progenitor progeny are known to contribute to intramembranous and endochondral ossification during adult fracture healing (Fujii et al., 2006; Kawanami et al., 2009), but it was unknown whether these cells operate similarly in the normal program of juvenile skeletogenesis. A number of studies indicate that osteoblasts are recruited from the cambium layer of the periosteum for intramembranous ossification (Hutmacher and Sittinger, 2003), so perhaps it is not surprising that we found differentiated CLOPs embedded in cortical bone. Ono and Kronenberg determined that cells within the perichondrium and growth plate eventually become osteoblasts in the primary spongiosa (Ono et al., 2014). Furthermore, Yang et al. recently proposed that progenitors from the periosteum and perichondrium contribute to the osteogenic pool responsible for bone growth (Yang et al., 2014), and our reporter studies build on mounting evidence (Houben et al., 2016; Park et al., 2015) to support this hypothesis. Specifically, we identified that progeny from cells initially in the cambium layers make up a significant portion of cells in the growth plate and rapidly transition to produce ossified bone. Hypertrophic chondrocyte fate has been a topic of controversy since it was first suggested these cells do not simply undergo an apoptotic end (Tsang et al., 2015). We identified labeled chondrocytes in the hypertrophic zone and osteoblasts in trabecular bone beneath the growth plate, suggesting hypertrophic chondrocytes from the growth plate are later incorporated in bone formed via endochondral ossification. Chondrocyte transdifferentiation has been proposed in a number of other studies and our result is consistent with findings showing that hypertrophic growth plate chondrocytes can become bone-forming osteoblasts (Park et al., 2015; Yang et al., 2014; Zhou et al., 2014). Finally, our tracking studies importantly build on embryonic studies demonstrating that Prx1-expressing cells contribute to both forms of ossification (Haycraft et al., 2007; Takarada et al., 2016). Although Prx1 expression patterns become more restricted with age, our results indicate that the participation of this cell population in skeletogenesis continues into juvenile development.

Primary cilia are known to be critical for embryonic skeletal development, but our results suggest their participation in skeletogenesis extends beyond birth. Embryos lacking cilia in Prx1-expressing cells exhibit severely shortened limbs and loss of the bone collar (Haycraft et al., 2007). Although ossification is prevalent in juvenile growth, it was previously unknown whether primary cilia in Prx1-expressing cells play a continued role since mutants did not survive birth. We generated an inducible knockout to study a postnatal deletion of CLOP primary cilia and found that juvenile mutants had disrupted endochondral and intramembranous ossification, resulting in shorter and thinner forelimbs. This is concerning since the amount of bone accrued during postnatal development is an important factor in determining the risk for osteoporosis (Richman et al., 2001). Our immunohistochemistry suggests that the disruption in endochondral ossification is a combination of attenuated chondrocyte proliferation and abnormal hypertrophy in the growth plate. Specifically, we determined that CLOPs lacking cilia are still recruited via the groove of Ranvier, but have difficulty incorporating into the growth plate when compared to ciliated CLOPs. Moreover, our type X collagen stains indicate that CLOPs with disrupted cilia that do enter the growth plate undergo premature hypertrophy, which perhaps explains the decreased number of proliferating chondrocytes in mutants. This phenotype is similar to that in juvenile mice with a conditional chondrocyte cilia knockout (Song et al., 2007) and suggests, for the first time, that CLOPs and their progeny comprise an appreciable number of the cells present in the growth plate.

Although we observed differences in phenotype with each developmental time point, all mutants consistently displayed a delayed program of osteogenesis. Juvenile mutants had shorter ulnae, fewer proliferating cells in the growth plate, ectopic hypertrophic chondrocytes in uncharacteristic zones of the growth plate and a decreased cortical area regardless of when CLOP primary cilia were disrupted. Interestingly, P17–P30 mutants did not exhibit an increase in marrow area like the other groups, suggesting CLOPs influence development of the marrow space primarily within the first 2–3 weeks after birth. The marrow space typically widens with age as more cortical bone is laid down via intramembranous ossification and osteoclasts subsequently resorb bone at the endocortical surface. Our previous work suggests that the primary cilium mediates formation and resorption to maintain adult bone homeostasis (Malone et al., 2007), so it is possible that this disruption also creates an imbalance in juveniles. Although all mutant growth plates contained fewer proliferating cells, P7–P17 mutants suffered from a low recruitment of resting zone cells, whereas P17–P30 and P7–P30 mutants displayed poor columnar stacking and accelerated hypertrophy. Finally, our Toluidine Blue-O stains revealed that P7–P17 mutants did not display the attenuated ossification observed in the other groups. This is perhaps expected since secondary ossification centers are not formed until P7 and may not undergo detectable changes by P17. Overall, the phenotypic variation with timing of disruption suggests that the predominant role of CLOP primary cilia, or CLOPs themselves, may change with each successive stage of juvenile development.

Interestingly, the P7–P30 mutant growth plates regained some columnar structure, and the boundary between the resting and proliferation zones was more distinct compared to the phenotype in P17–P30 mutants. This recovery implies CLOP cilium disruption delays rather than prevents endochondral ossification in juveniles. One possible explanation is that mutant cells are outcompeted by other cells in the growth plate over time. An alternative is that catch-up growth occurs in mutants in the later stages of juvenile development. Catch-up growth refers to the extremely rapid development at a future age because the growth plate continues to operate in the absence of senescence (Lui et al., 2011). Growth plate senescence is dictated by the progress of growth, rather than age itself (Gafni et al., 2001; Lui et al., 2011; Marino et al., 2008). Since mutants have appreciably less developmental progress and delayed senescence, it is possible that this mechanism functions to compensate for the lost progress. Regardless of whether catch-up growth does occur, we did not find evidence that the negative effects of CLOP cilium disruption on ossification are fully recoverable at the examined time points. In order to fully appreciate the observed potential recovery of growth plate structure, ulnar length and cortical area must be quantified nearer to skeletal maturity to determine whether growth is stunted or delayed in our experimental animals. Additionally, it is critical to know whether CLOP cilium deletion disrupts signaling in the perichondrium – which is essential to mediate growth plate chondrocyte proliferation and hypertrophy – in order to answer these questions.

The phenotypic abnormalities in mutants are potentially due to disruptions in ciliary signaling pathways that are known to coordinate intramembranous and endochondral ossification (i.e. pathways mediated by Ihh, PTHrP, TGFβ and BMP). Perhaps the

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most recognized signaling pathway in the growth plate is the interplay between Indian hedgehog (Ihh) and parathyroid hormone-related protein (PTHrP), which coordinate with the perichondrium to control the timing and rate of hypertrophy. The primary cilium has long been known to mediate hedgehog signaling during development (Huangfu et al., 2003), and juvenile mutants lacking chondrocyte primary cilia exhibit disrupted Ihh signaling (Chang and Serra, 2013) and accelerated hypertrophic differentiation (Song et al., 2007). Although transforming growth factor β (TGFβ) and bone morphogenic protein (BMP) have established roles in skeletal development, only recent studies show that the TGFβ/BMP signaling pathway is directly influenced by the primary cilium (Clement et al., 2013; Lindbæk et al., 2015). Prx1Cre embryos containing a knockout of TGFβ receptor 2 exhibit dwarfism and severely attenuated intramembranous ossification (Seo and Serra, 2007). The deficiencies in endochondral ossification are perhaps not surprising since the Ihh/PTHrP pathway is believed to be intimately connected with TGFβ2 and BMP signaling to influence chondrocyte hypertrophy (Alvarez et al., 2002; Minina et al., 2001). We speculate, from these prior studies, that our CLOP cilia knockouts display attenuated intramembranous ossification in part due to disrupted BMP signaling and exhibit ectopic and accelerated hypertrophy as a result of interrupted Ihh, PTHrP, TGFβ and BMP signaling. However, further studies are required to determine the exact mechanisms and distinguish the role of CLOP cilia in the crosstalk between growth plate chondrocytes and the perichondrium.

Chondrocyte proliferation is also limited when primary cilia and their associated signaling pathways are disrupted. Chondrocytes in the proliferation zone are able to rapidly proliferate by dividing and rotating to stack themselves in tight vertical columns, a process known as chondrocyte rotation. Primary cilia are normally oriented along the long axis of the growth plate in the proliferation zone and this directionality is required for rotation to occur (Ascenzi et al., 2011; de Andrea et al., 2010; Song et al., 2007). For example, transgenic mice containing a chondrocyte-specific primary cilia knockout exhibit randomly oriented cilia in clusters of chondrocytes that fail to vertically align, resulting in severely attenuated proliferation (Song et al., 2007). It has been suggested that the cilium mediates this process through noncanonical Wnt signaling (Chang and Serra, 2013; Song et al., 2007) since chondrocyte rotation is tightly regulated by the Wnt/planar cell polarity (Wnt/PCP) pathway (Li and Dudley, 2009). In addition to their roles in chondrocyte hypertrophy, Ihh and BMP signaling also enhance chondrocyte proliferation. In fact, overexpression of *Ihh* alone is sufficient to enhance proliferation (Long et al., 2001). Another form of Hedgehog signaling, mediated by Sonic hedgehog (Shh), and BMP signaling are believed to trigger resting zone chondrocytes to enter the proliferation zone (Wu et al., 2002; Yoon et al., 2006). We speculate that our primary cilium knockouts cannot adequately recruit cells from the resting zone, and those cells that enter the proliferation zone prematurely undergo hypertrophy or fail to engage in chondrocyte rotation due to disruptions in ciliary signaling pathways. Again, further studies are required to identify the specific mechanisms and signaling pathways affected by Prx1-driven cilium deletion.

One limitation in our study is that we infrequently detected labeled cells that did not fit within the expression patterns displayed in our initial short-term tracking studies. Specifically, we observed tdTomato+ cells at the endosteal surface of cortical bone in mice activated at P7 (Fig. 3B). In juvenile limb development, periosteal cells differentiate into osteoblasts that lay down appositional layers of bone at the periosteal surface and osteoclasts resorb the endosteal surface to widen the marrow canal, resulting in mature cortical bone. The labeled cells found at the endosteal surface are perhaps periosteal cells that became embedded osteocytes and were exposed after osteoclast resorption. Alternatively, this finding might mean that (1) CLOPs somehow enter the vasculature or (2) Prx1 expression is perhaps not as restricted to the cambium layers as our initial studies suggest. More perplexing is the observation of labeled cells in trabecular bone 48 h after tamoxifen was injected (Fig. 4A). Although postnatal growth is very rapid and Prx1-expressing cells are known to migrate quickly in response to wound healing (Duchamp de Lageneste et al., 2018), it is unlikely that the osteoblasts present in Fig. 4A originated from the perichondrium. These cells did not express GFP, but perhaps contained residual CreER and were subsequently responsive to tamoxifen. Cre expression and recombination is typically less specific in females and we did, in fact, observe these uncommon labeled cells specifically in female juveniles. We never observed tdTomato+ cells without introducing tamoxifen. Regardless, these cells were infrequent and the vast majority initially resided in the periosteum and perichondrium. We conclude that this Prx1CreER-GFP model is a valuable tool for studying osteochondroprogenitors in the periosteum and perichondrium, but recommend investigators design appropriate breeding strategies and characterize Prx1 expression and Cre recombination before proceeding with any Prx1 model.

Our results suggest that CLOPs have greater regenerative potential than previously envisioned and are perhaps a suitable source for recapitulating bone regeneration. Mesenchymal cells are the leading cell type for regenerative applications since they self-renew, differentiate into a vast array of cell types and can be extracted from many different tissues (Ferretti and Mattioli-Belmonte, 2014). Bone marrow is a commonly used source since it contains osteogenic precursors; however, the periosteum has also been identified as a clinically useful progenitor source (Chang and Knothe Tate, 2012). Prx1-expressing cells are critical to embryonic skeletogenesis (Haycraft et al., 2007) and the majority of cells in the adult fracture callus are derived from periosteal progenitors (Colnot, 2009; Duchamp de Lageneste et al., 2018). Additionally, periosteum-derived progenitors demonstrate high proliferation rates and regenerative performance does not vary with age (Duchamp de Lageneste et al., 2018; Ferretti and Mattioli-Belmonte, 2014). We demonstrated that Prx1-expressing cells differentiate into osteoblasts and chondrocytes to populate a surprising number of nearby and distant skeletal tissues during standard juvenile development in mice. Because of their continued existence after birth, regenerative potential, and pre-programmed fate towards a pro-osteogenic lineage, Prx1-expressing cells in the periosteum may prove to be a valuable tool for regenerating skeletal tissues. Furthermore, we have shown that disrupting the primary cilium alone results in significant skeletal abnormalities, implicating primary cilium-mediated sensing as a potential mechanism to manipulate CLOPs for regenerative strategies.

## MATERIALS AND METHODS

### Animal models

All mouse models are on a C57BL6 background. Prx1CreER-GFP and Ift88$^{fl/fl}$ mice are previously described (Haycraft et al., 2007; Kawanami et al., 2009). These animals and Rosa26$^{tdTomato}$ mice are available through Jackson Laboratory (Table S1). Prx1CreER-GFP mice were bred with Ift88$^{fl/fl}$ or Rosa26$^{tdTomato}$ mice to generate Prx1CreER-GFP;Ift88$^{fl/fl}$ and Prx1CreER-GFP;Rosa26$^{tdTomato}$ offspring, respectively. These offspring were then bred to generate Prx1CreER-GFP;Ift88$^{fl/fl}$;Rosa26$^{tdTomato}$ offspring. Ift88$^{fl/fl}$ females were bred with Prx1CreER-GFP;Ift88$^{fl/fl}$ males to generate Prx1CreER-GFP;Ift88$^{fl/fl}$ experimental pups and Ift88$^{fl/fl}$ littermate

controls that received tamoxifen injections (Fig. 1). To avoid embryonic recombination, mothers were isolated for 1 month after any potential exposure to tamoxifen before breeding again. Genotype was determined using standard PCR and agarose gel electrophoresis. Primer sequences are provided in Table S1. Animals were housed, maintained and evaluated for health complications in accordance with IACUC standards. All experiments were approved by the Institute of Comparative Medicine at Columbia University.

### Tamoxifen injections

All animals were injected with tamoxifen solution, but primary cilia remained intact in knockout controls (Ift88$^{fl/fl}$) and tdTomato expression was not activated in reporter controls (Rosa26$^{tdTomato}$). Tamoxifen (Sigma Aldrich, T5648) was dissolved in corn oil (Sigma Aldrich, C8267) in a shaking incubator at 37°C to create a 20 mg ml$^{-1}$ stock solution stored at 4°C and protected from light. Injection solution was prepared fresh daily by diluting the stock solution to 10 mg ml$^{-1}$ and adding 10% ethanol to prevent infection. Prx1CreER-GFP; Ift88$^{fl/fl}$ experimental pups and Ift88$^{fl/fl}$ littermate controls received daily intraperitoneal injections of 33 mg per kg body weight tamoxifen solution until they were euthanized (Fig. 1). Prx1CreER-GFP; Rosa26$^{tdTomato}$ pups either received a single dose of 100 mg per kg body weight or daily injections of 33 mg per kg body weight tamoxifen solution until they were euthanized. Prx1CreER-GFP;Ift88$^{fl/fl}$;Rosa26$^{tdTomato}$ pups and Prx1CreER-GFP;Ift88$^{fl/fl}$; Rosa26$^{tdTomato}$ controls received a single dose of 33 mg kg$^{-1}$ at P15 and were euthanized at P17. Pups were injected as early as postnatal day 7 (P7) depending on the group to which they were randomly assigned (Fig. 1).

### Histology

After mice were euthanized, ulnae were dissected and fixed overnight at 4°C. Prx1CreER-GFP;Ift88$^{fl/fl}$ and control specimens were fixed in 10% formalin (Sigma Aldrich, HT5011), decalcified, embedded in paraffin and sectioned in 5 µm increments. Prx1CreER-GFP;Rosa26$^{tdTomato}$ ulnae were fixed in 4% paraformaldehyde (Sigma Aldrich, P6148), decalcified overnight at room temperature in RDO Gold solution (Apex Engineering Products Corporation, RDO gold 01), and cryosectioned in 5 µm increments. Prior to decalcification, the midpoint along the length of each ulna was measured and labeled with tissue marking dye (Davidson, 13-10132) to ensure transverse sections were collected at the same region.

### Staining and immunohistochemistry

Cryosections were placed in distilled water upon removal from the freezer, incubated in mounting medium containing a nuclear stain (Electron Microscopy Sciences) for 5 min, washed with PBS, mounted, and sealed. Paraffin embedded specimens were deparaffinized and rehydrated in a tissue processor (Leica ASP300S). Toluidine solution was prepared by diluting 0.05% w/v Toluidine Blue-O (Sigma Aldrich, T3260) in 100 mM sodium acetate buffer, pH 6. Slides were submerged in Toluidine solution for 10 min and submerged in Hematoxylin solution (Sigma Aldrich, MHS1) for 10 min followed by 30 s in Eosin solution (Sigma Aldrich, HT110216). To identify proliferating cells in the growth plate, slides were incubated in 2 mg ml$^{-1}$ hyaluronidase (Sigma Aldrich, H3884) for 1 h at 37°C, blocked with 15% goat serum (Abcam, ab7481) for 20 min at room temperature, and incubated in an anti-PCNA primary antibody (1:10,000; Abcam, ab29) in 15% goat serum for 1 h at 37°C, followed by overnight incubation at 4°C. This protocol was repeated to detect primary cilia but with an anti-acetylated α-tubulin primary antibody from C3B9 hybridoma media cultured in our laboratory (1:100; Sigma Aldrich). To detect hypertrophic cells in the growth plate, slides were incubated in 2 mg ml$^{-1}$ hyaluronidase for 1 h at 37°C, blocked with 15% goat serum and 0.3% Triton-X 100 (Sigma Aldrich, T9284) in PBS, and incubated with anti-type X collagen primary antibody (1:250; abcam, ab58632) in 3% goat serum and 0.3% Triton-X 100 overnight at 4°C. PCNA, primary cilia and Type X collagen slides were incubated in a fluorescent secondary antibody conjugated to Alexa Fluor 488 (1:500; Life Technologies, A11029 and ab150077) in PBS, for 1 h at room temperature. All slides were washed with PBS then distilled water, mounted and sealed. Toluidine and H&E stains were visualized on an Olympus CKX41 inverted microscope and micrographs were captured with a Canon EOS60D 18.0 MP digital SLR camera. All fluorescence images were collected using an Olympus Fluoview FV1000 confocal microscope.

### Quantifying length, area and cell count

Ulnar length and area were quantified from H&E images using ImageJ software (National Institutes of Health). Proliferating cells were counted and normalized to growth plate area from PCNA immunohistochemistry slides using ImageJ. At least five consecutive sections per specimen were analyzed and averaged when assessing differences in phenotype and quantifying ulnar length, cortical and marrow area, and the number of proliferating cells. Investigators were blinded to the groups during the experiment, specimen generation, image acquisition and post-imaging analysis steps. Quantifications were performed by two separate investigators to confirm repeatability and ensure at least 95% accuracy of reported results between the two investigators.

### Statistics

No sex-dependent morphological differences were identified according to visual inspection or a one-way ANOVA ($n$=3 for each gender) for qualitative observations and quantitative measurements, respectively. Males and females were therefore grouped together for analysis. The data satisfied conditions of normality according to the Shapiro–Wilk test and were analyzed with a two-tailed Student's $t$-test. Values are reported as mean± s.e.m., with $P<0.05$ considered statistically significant. Sample size was determined in order to achieve a power of at least 80%.

#### Acknowledgements

We would like to thank Dr Shunichi Murakami and his group formerly at Case Western Reserve University in Cleveland, Ohio for the generous gift of the Prx1CreER-GFP mice and technical discussions regarding this mouse model. We would also like to thank the Molecular Pathology Shared Resource in the Herbert Irving Comprehensive Cancer Center core facilities at Columbia University for their expertise and assistance in producing histological specimens, Dr Thorsten Kirsch at New York University in New York, NY for his consultation on growth plate morphology and staining, and Andrea G. Schwartz for her consultation on staining and imaging. Finally, we thank Michael Duffy, Milos Spasic, Michael Sutton, and Julia Chen in the Cell and Molecular Biomechanics Lab for technical discussions and manuscript revisions.

E.R.M. wishes to dedicate this manuscript to Dr Chris Jacobs, who sadly passed away days before it was accepted for publication. Chris was particularly excited about this work because it was a departure from his traditional expertise, and he would have been very proud to see it published. E.R.M. would like to thank Chris for always supporting her 'overly bold' project ideas, even when he didn't agree with them. Chris was an excellent researcher, mentor, and friend who left an impressive legacy and will be truly missed.

#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: E.R.M., C.R.J.; Methodology: E.R.M., Y.Y., C.R.J.; Validation: E.R.M., Y.Y.; Formal analysis: E.R.M., Y.Y., C.R.J.; Investigation: E.R.M., Y.Y.; Resources: C.R.J.; Writing - original draft: E.R.M.; Writing - review & editing: E.R.M., C.R.J.; Visualization: E.R.M.; Supervision: E.R.M., C.R.J.; Project administration: E.R.M., C.R.J.; Funding acquisition: C.R.J.

#### Funding

This work was supported by National Institute of Arthritis and Musculoskeletal and Skin Diseases at the National Institutes of Health (grant R01-AR062177) and National Institutes of Health training grant T32-AR59038. Deposited in PMC for release after 12 months.

#### Supplementary information

Supplementary information available online at
http://jcs.biologists.org/lookup/doi/10.1242/jcs.217828.supplemental

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