Twist1 mediates repression of chondrogenesis by β-catenin to promote cranial bone progenitor specification

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SUMMARY
The bones of the mammalian skull vault form through intramembranous ossification. Skull bones ossify directly, in a process regulated by β-catenin, instead of passing through a cartilage intermediate. We tested whether β-catenin is necessary for fate selection of intramembranous bone progenitors in the skull. Here, we show in mice that removal of β-catenin from skull bone progenitors results in the near complete transformation of the skull bones to cartilage, whereas constitutive β-catenin activation inhibits skull bone fate selection. β-catenin directly activated Twist1 expression in skull progenitors, conditional Twist1 deletion partially phenocopied the absence of β-catenin, and Twist1 deletion partially restored bone formation in the presence of constitutive β-catenin activation. Finally, Twist1 bound robustly to the 3’UTR of Sox9, the central initiator of chondrogenesis, suggesting that Twist1 might directly repress cartilage formation through Sox9. These findings provide insight into how β-catenin signaling via Twist1 actively suppresses the formation of cartilage and promotes intramembranous ossification in the skull.

KEY WORDS: Osteoblast, Cranial bone, Skull, Wnt, β-catenin, Twist, Cartilage, Mouse

INTRODUCTION
The mammalian skull vault, which protects the brain, comprises bones derived from dual embryonic origins. Cells from both cranial neural crest (CNC) and paraxial mesoderm (PM) form skull progenitors, and these distinct populations are already spatially segregated in rostral and caudal domains above the eye in the mouse at E11.5 (Yoshida et al., 2008). Skull bones undergo intramembranous ossification, in which osteoprogenitors are specified and subsequently differentiate into lineage-restricted osteoblasts, moving dorsally before ossifying directly as bone (Yoshida et al., 2008). In the mouse, CNC contributes to the frontal bones and the interparietal bone, and PM gives rise to the parietal bones of the posterior skull vault (Jiang et al., 2002; Yoshida et al., 2008). Bone formation occurs through two divergent mechanisms: intramembranous and endochondral ossification. The majority of the skeleton undergoes endochondral bone formation, in which a cartilage template first forms, becomes mineralized, and is replaced by osteoblast-made bone. By contrast, intramembranous bones, such as those of the skull, form by direct ossification without a cartilage intermediate. Bone-forming osteoblasts of both intramembranous and endochondral bones express the transcription factors Runx2 and Osterix (Osx; Sp7 – Mouse Genome Informatics), which are essential for initial osteoprogenitor specification and osteoblast lineage commitment, respectively (Nakashima et al., 2002; Otto et al., 1997). In these early stages of progenitor formation in endochondral bones, genetic studies have demonstrated that the BMP, Wnt, Indian hedgehog (Ihh) and Notch signaling pathways are important; however, a direct examination of their roles during the development of intramembranous bone progenitors is lacking (reviewed by Long, 2008). Several studies have revealed differences in the molecular signals required for the specification of endochondral and intramembranous bone progenitors. For example, the chondrogenic determinant Sox9 is essential for the formation of the cartilage anlage, which precedes endochondral progenitor specification. Consequently, Sox9 mutant cells from chimaeric embryos do not contribute to endochondral bone (Bi et al., 1999). One of the major roles of chondrocytes during endochondral bone formation is the paracrine secretion of Ihh that is responsible for the specification of endochondral osteoprogenitors and the initiation of Runx2 expression (St-Jacques et al., 1999). By contrast, Sox9 and chondrogenesis are dispensable in skull vault morphogenesis, consistent with the finding that intramembranous bone progenitors do not require Ihh for intramembranous osteoprogenitor specification (St-Jacques et al., 1999). Therefore, molecular differences in the initiation of osteoprogenitor cell specification between these two ossification programs are evident, and yet the signaling pathways that specify intramembranous bone progenitors remain unidentified. Since skull vault formation occurs in the absence of an intermediary cartilage, it is then plausible that the pathway initiating osteoprogenitor specification in this population might simultaneously inhibit chondrocyte formation in the skull.

Several studies have confirmed that osteoblasts and chondrocytes involved in endochondral bone formation originate from common progenitors (Akiyama et al., 2005). β-catenin-dependent signaling is crucial in determining the definitive cell fate, and it inhibits chondrocyte differentiation in endochondral osteoprogenitors (Hill et al., 2005). β-catenin transduces Wnt signaling through the Frizzled and LRP receptors by associating with members of the TCF/LEF transcription factor family in the nucleus and regulating target gene expression (Bhanot et al., 1996; Korinek et al., 1998; Liu et al., 1999; Tamai et al., 2000). β-catenin is also required for intramembranous skull vault mineralization (Day et al., 2005; Hill et al., 2005). Conditional β-catenin deletion...
in the head results in an absence of the skull vault, with replacement by cartilage. β-catenin is not, however, sufficient for bone formation as gain-of-function genetic studies in osteoprogenitors revealed a global loss of ossification, with reductions in Runx2 and Sox9 mRNA levels (Hill et al., 2005). The mechanism underlying the discrepancy between gain- and loss-of-function studies for β-catenin remains unknown.

β-catenin functions by regulating target gene expression. One of its targets, the Twist family, has roles in head morphogenesis, skull osteoblast differentiation and repression of cartilage growth, suggesting a role in intramembranous progenitor specification (Bialek et al., 2004; Logan and Nusse, 2004; Soo et al., 2002). β-catenin activates Twist1 in vitro and Twist2 in vivo, making these transcription factors nuclear targets of β-catenin (Howe et al., 2003; Ohtola et al., 2008; Reinhold et al., 2006; Tran et al., 2010). Twist1 is required for the early migration and survival of cranial mesenchyme, which gives rise to the skull bones (Bildsoe et al., 2009; Soo et al., 2002). Twist1-/- mice have premature intramembranous osteoblast differentiation and skull suture fusion but demonstrate no overt endochondral bone phenotype (Bialek et al., 2004; Bildsoe et al., 2009; Reinhold et al., 2006; Soo et al., 2002). Similarly, Twist2 heterozygosity can restore normal size to the clavicles, which ossify in part via the intramembranous pathway, in a mouse model of cleidocranial dysplasia (Bialek et al., 2004). Both results indicate that the Twist family could exert specific effects on osteoblasts in intramembranous bones that are not observed in endochondral bones. Furthermore, Twist1 inhibits chondrocytic differentiation in vitro, suggesting that Twist1 could serve as a chondrogenic repressor in vivo (Reinhold et al., 2006). Both Twist2Cre lineage-marked descendants and Twist1 mRNA are present in the cranial mesenchyme between E9.5 and E11.5; therefore, the spatiotemporal expression of the Twist family is consistent with a role in regulating intramembranous bone progenitor specification (Bialek et al., 2004; Tran et al., 2010; Yu et al., 2003). In spite of these data, no existing work has defined the role of Twist family members as mediators of the Wnt/β-catenin pathway during cranial bone fate selection in vivo.

Here, we tested whether β-catenin is necessary for fate selection of intramembranous bone progenitors in the mouse skull. First, we genetically deleted β-catenin in cranial bone progenitors derived from cranial mesenchyme, resulting in the near complete transformation of the skull vault into cartilage. β-catenin was required for cranial osteoblast lineage commitment and directly activated Twist1 expression. Twist1 deletion from skull progenitors also resulted in chondrocyte formation, which was limited to the posterior skull vault. Finally, we demonstrated genetic interactions of β-catenin with Twist1, and direct association of Twist1 with the 3′UTR of the central initiator of chondrogenesis, Sox9. Taken together, our data show that the Twist family comprises an essential set of effectors of β-catenin-mediated skull progenitor specification.

MATERIALS AND METHODS
Mice and genotyping
Conditional functional studies were conducted using En1Cre mice (Kimmel et al., 2000), Twist2Cre mice (Yu et al., 2003) and Prx1CreERGFP mice (Kawano et al., 2009). The conventional null, conditional loss- and gain-of-function floxed alleles for β-catenin (Cnmbl) [β-cateninlop, β-cateninRodel] (Brault et al., 2001; Haegel et al., 1995; Harada et al., 1999), the conditional floxed Twist1 allele (Chen et al., 2007) and R26R/R26R mice (Soriano 1999) were described previously. Mice and embryos were genotyped as described previously (Atit et al., 2006). For induction of CreER activity, 4-hydroxytamoxifen was dissolved in corn oil (10 mg/mL; Sigma-Aldrich) and delivered at 1 mg/40 µl bodyweight by oral gavage to pregnant mice carrying E9.5-E10.5 embryos.

For timed matings, the vaginal plug day was assigned as E0.5. At desired time points, embryos were harvested and processed for frozen sections as previously described (Atit et al., 2006). For each experiment, at least three different mutants with littermate controls were analyzed. At least two to four litters were used for each functional analysis.

Case Western Reserve Institutional Animal Care and Use Committee approved all animal procedures.

In situ hybridization, immunohistochemistry and histology
Embryos were fixed in 4% paraformaldehyde, cryopreserved, and sectioned at 8-12 µm. In situ hybridization, β-galactosidase with Eosin counterstaining, and immunohistochemistry were performed essentially as described (Atit et al., 2006; Ohtola et al., 2008). For skeletal preparations, embryos were stained in 0.03% Alcian Blue and 0.005% Alizarin Red. The in situ probe for Lef1 was a gift from Fanxin Long (Hu et al., 2005), Runx2 (Enomoto et al., 2000) was a gift from Matthew Warnmann, Sox9 (Wright et al., 1995) was obtained from Veronique Lefebvre, and Twist1 was provided by Richard R. Behringer (Baylor College of Medicine, Houston, TX, USA). Primary antibodies used for indirect immunofluorescence were: goat anti-Runx2 (1:20; R&D Biosystems); rabbit anti-Sox9 (1:100; Millipore); mouse anti-Twist1 (1:500; Santa Cruz); and rabbit anti-Lef1 (1:100; Cell Signaling). All control/mutant pairs were photographed at the same magnification. To measure immunofluorescence intensity, stained tissue sections were photographed for the DAPI (blue) and Twist1 (red) channel. Three sections were photographed per embryo and n=4 for control and mutant embryos. Cranial bone progenitor regions (see Fig. 3M,N) were selected (~400 nuclei per embryo). Nuclei were set as counting regions, and average Twist1 intensity was measured using MetaMorph software (Molecular Devices).

Cell lines, plasmids, transfection and luciferase assays
Mouse C3H1OT1/2 cells were cultured in DMEM GlutaMAX-I (Gibco) with 10% fetal bovine serum (FBS) and 50 mg/ml gentamicin (Gibco). ATDC5-TwistER cells were infected with TwistER virus as described (Yang et al., 2004), then selected with 10 mg/ml blasticidin (Invitrogen) in 1:1 DMEM:F12, 5% FBS (Gibco). Individual clones were selected for chromatin immunoprecipitation (ChIP) according to Twist1 expression as determined by western blot. Transfections for the luciferase assay were performed in a 12-well plate seeded with 2.5×10^5 cells per well in the absence of antibiotics the day before transfection. The plasmids pcDNA3-myctwist1, pcDNA3-flag-Twist2 (gifts from D. Sosic, UT-Southwestern Medical Center, Dallas, TX, USA) and PCINeo (Promega), as a negative control, or PEGFP (Clontech) and P-TK-Remilla (Promega) as transfection efficiency controls, were used for transfection. Sox9HC, consisting of 530 bp proximal to the mouse Sox9 transcription start site cloned into pGDL4.10 (Promega), was a kind gift from Cynthia Barles (Case Western Reserve University School of Medicine, Cleveland, OH, USA). Transient transfections were performed at 80-90% confluence using Lipofectamine 2000 (Invitrogen, for C3H1OT1/2 cells), according to the manufacturer's instructions. Cells were harvested 24 hours after transfection for western blot, luciferase assay and quantitative real-time PCR. Total protein (25 µg) was prepared by SDS-PAGE, and western blots were performed with the following antibodies: mouse anti-Flag (1:1000; Sigma), anti-c-Myc (1:200; Santa Cruz), mouse anti-Twist1 (1:500; Santa Cruz), rabbit anti-tubulin (1:5000; ICN BioMedicals) and HRP-conjugated goat anti-mouse or anti-rabbit (1:10,000; Thermo Scientific). Twenty-four hours after transfection, a luciferase assay (Promega) was performed as described (Tran et al., 2010). Light units were normalized to protein content (see Bialek et al., 2004; Sosiv et al., 2003) determined by the BCA Protein Assay Kit (Pierce). A paired, single-tail Student’s t-test was performed using Microsoft Excel.

ChIP and real-time PCR
E12.5 cranial mesenchyme was harvested and ChIP protocols were modified from previously published procedures (Schnetz et al., 2009; Zhang et al., 2009). ATDC5 TwistER cells were treated with 20 nM 4-
hydroxytamoxifen (Sigma) for 24 hours before ChIP. Cells from tissues, as well as from C3H10T1/2 and ATDC5 cell lines were dissociated with 0.05% trypsin-EDTA (Invitrogen) followed by DMEM with 10% FBS. The basic ChIP protocol was carried out as previously described (Schnetz et al., 2009), and target or non-target control sites were PCR amplified from immunoprecipitated samples and total genomic DNA (input) in triplicate. Quantitative real-time PCR was performed using SYBR Green chemistry (ABI) as described (Tran et al., 2010; Livak and Schmittgen, 2001). Primer sequences are listed in supplementary material Table S1. Results for target sequences were normalized to non-target values and are reported as fold enrichment versus non-target sequences.

RESULTS

β-catenin is required for cranial osteoblast lineage commitment

First, we characterized Wnt signal transduction via β-catenin in membranous progenitors derived from cranial neural crest (CNC) and from paraxial mesoderm (PM). We used an Engrailed1Cre (En1Cre) knock-in line to performantly modulate β-catenin activity levels in postmigration CNC- and PM-derived skull progenitors in the supraorbital arch (Kimmel et al., 2000). To distinguish CNC-derived versus PM-derived membranous progenitors, we utilized the Wnt1Cre transgenic line (Jiang et al., 2002) to activate expression of the Rosa26 lacZ reporter (RR) specifically in premigratory CNC- and PM-derived skull progenitors in the supernumerary arch (Kimmel et al., 2000). Compared with E12.5 Wnt1Cre; RR lineage-labeled CNC derivatives (Fig. 1A), the En1Cre lineage contributed to CNC-derived frontal and interparietal bone primordia (Fig. 1C, Fig. 4A) and to PM-derived parietal bone primordia (supplementary material Fig. S1A). En1Cre lineage-marked cells were not present in the dura mater, the surface ectoderm, or the cartilage base of the skull (supplementary material Fig. S2A,E). Alkaline phosphatase (AP) staining on serial sections at E12.5 localized intramembranous osteoprogenitors of both embryonic origins to the deepest subset of the En1Cre lineage (Fig. 1D; supplementary material Fig. S1D). Expression of the Wnt/β-catenin target gene Lef1 (Hovanes et al., 2001) broadly localized to En1Cre lineage cranial mesenchyme (Fig. 1F; supplementary material Fig S1B,F), including AP+ osteoprogenitor cells at E12.5. These data suggest that a large portion of En1Cre lineage-marked cranial mesenchyme was transducing β-catenin-dependent Wnt signaling at the onset of skull morphogenesis.

Previous studies left the role of β-catenin undefined in cranial bone progenitors (Brault et al., 2001; Day et al., 2005; Hill et al., 2005). Lineage analysis of En1Cre; RR embryos demonstrated labeling of presumptive intramembranous osteoprogenitors at E11-11.5 prior to osteoprogenitor specification in the supraorbital arch (Tran et al., 2010). Compared with En1Cre+/−; RR+/− embryos, En1Cre+/−; RR+/−; β-cateninfl/fl heterozygotes exhibited no demonstrable skull phenotype. Therefore, we generated En1Cre+/−; RR+/−; β-cateninfl/fl embryos to delete β-catenin in the cranial bone progenitors to pinpoint the role of β-catenin in intramembranous osteoprogenitor fate selection. β-galactosidase and AP activity staining of serial sections showed that En1Cre lineage cells contained AP+ cranial osteoprogenitor cells, in controls and mutants (Fig. 1B-E). In conditional β-catenin mutant embryos, the AP domain of CNC-derived osteoprogenitors expanded beneath the surface ectoderm (Fig.1D,E) and there were fewer AP+ osteoprogenitor cells of PM origin in the En1Cre lineage of mutants than in controls (supplementary material Fig. S1C,E). Expression of the β-catenin target gene Lef1 was completely abrogated in both CNC-derived
and PM-derived osteoprogenitor cells of mutants (Fig. 1G; supplementary material Fig. S1G), suggesting efficient deletion of β-catenin by En1Cre.

Next, we analyzed the expression of the earliest markers of osteoprogenitor fate in β-catenin-deleted conditional mutants. The osteoprogenitor marker Runx2 was expressed at E12.5 in En1Cre lineage-marked mesenchyme (Fig. 1H; supplementary material Fig. S1H) at the onset of cranial bone specification. In mutant CNC-derived mesenchyme, Runx2 expression was present at E12.5, but the domain expanded directly beneath the surface ectoderm and dorsally (Fig. 1I). In mutant PM-derived mesenchyme, however, Runx2 expression was absent by E12.5 (supplementary material Fig. S1H,I). In control embryos, Runx2+ osteoprogenitors had differentiated further, also expressing the osteoblast lineage commitment marker Osx (Nakashima et al., 2002) at E12.5 (Fig. 1J). However, none of the Runx2+ mutant cells in the En1Cre lineage expressed Osx (Fig. 1K). Expression of the earliest chondrocyte marker, Sox9 (Bell et al., 1997; Bi et al., 1999), was confined to chondrocyte progenitors, which form the skull base, outside of the En1Cre lineage-marked domain, and was not detected in intramembranous osteoprogenitor cells of controls at E12.5 and E13.5 (Fig. 1L; supplementary material Fig. S1J,Fig. S2A,B,E,F). In mutant embryos, Sox9 was still expressed in skull base progenitors outside the En1Cre lineage at E12.5 and E13.5 (supplementary material Fig. S2C,D,G,H), but we additionally detected Sox9 mRNA within the En1Cre lineage (Fig. 1M; supplementary material Fig. S1K, Fig. S2C,D,G,H). Next, we tested whether Sox9-expressing cells in mutants were also positive for other chondrocyte markers. At E12.5, Col2a1 was expressed in the skull base of controls, but not in the En1Cre lineage (Fig. 1N). However, in mutants we detected Col2a1 in the En1Cre lineage in a similar expression pattern to Sox9 (Fig. 1O). By E12.5, En1Cre/+; RR/+; β-cateninfl/fl cranial osteoprogenitors expressed Runx2 but failed to express Osx or differentiate further along the osteoblast lineage, instead expressing Sox9 and Col2a1. These results suggest that β-catenin activity is crucial during osteoblast lineage commitment for preventing induction of chondrocyte fate during cranial intramembranous bone development as early as E12.5 in the mouse embryo.

To determine whether ectopic chondrocytes form cartilage in conditional β-catenin-deficient embryos, we compared their intact cranial skeletons with those of controls to determine the affected areas in the skull. At E18.5, the skull bones of control embryonic heads stained positive for bone (Alizarin Red) but negative for cartilage (Alcian Blue) (Fig. 1P). In En1Cre/+; RR/+; β-cateninfl/fl mutant embryos, the paired frontal and parietal bones, as well as the interparietal bone, were absent, and there was an almost uniform replacement of calvarial bone with Alcian Blue-stained cartilage at E18.5 (Fig. 1Q; 8/8 embryos). These data indicate that β-catenin is crucial for intramembranous osteoprogenitor specification.

β-catenin activity is necessary for the expression of Twist1 in cranial mesenchyme

Next, we sought to determine how β-catenin regulates cranial osteoprogenitor cell fate specification. Activation of Wnt signal transduction induces expression of the transcription factor Twist1 in vitro, which, as an inhibitor of chondrogenic differentiation, could serve as a mediator of β-catenin in skull progenitor fate selection (Reinhold et al., 2006). We tested whether Twist1 expression during cranial progenitor specification (E11.5-12.5) requires β-catenin activity. At E10.5-11.5, Twist1 protein localized to cranial mesenchyme beneath the surface ectoderm above the eye (Fig. 2A,B), and at E12.5 we detected Twist1 protein within cranial bone progenitors (Fig. 2C). Previously, we showed that En1Cre was inconsistently active at E10.5 in the cranial mesenchyme, with robust activity by E11.5 (Tran et al., 2010). In β-catenin conditional null mutants, Twist1 protein expression was present in cranial mesenchyme at comparable levels to controls at E10.5, but was subsequently absent in β-catenin null cranial bone progenitors at E11.5 and E12.5 (Fig. 2D-F). β-catenin was also required for Twist1 mRNA expression at E11.5 (data not shown). Therefore, β-catenin is required for the expression of Twist1 in cranial bone progenitors.

β-catenin activity promotes expression of Twist1 in cranial mesenchyme

We next tested whether constitutive activation of β-catenin signaling in the En1Cre lineage was sufficient to induce Twist1 expression in cranial mesenchyme. En1Cre/+; RR; β-cateninfl/+ mutant embryos were embryonic lethal at E13.5, but were viable for analysis at E12.5 (Fig. 3A,D). En1Cre/+; RR; β-cateninfl/+ embryos revealed a complete absence of Runx2 in cranial bone progenitors (Fig. 3B,E). Twist1 was expressed in E12.5 cranial dermal and osteoprogenitor domains of En1Cre lineage-marked cells of control embryos (Fig. 3C). Upon constitutive activation of β-catenin signaling, the Twist1 expression domain expanded into the entire En1Cre lineage (Fig. 3C,F, compare arrow). Constitutive β-catenin activation also resulted in an ~60% increase in relative

Fig. 2. Requirement for β-catenin in cranial mesenchyme for Twist1 protein expression. Indirect immunofluorescence for Twist1 on (A,D) axial or (B,C,E,F) coronal tissue sections. Nuclei were counterstained with DAPI. Ey, eye; tel, telencephalon. Dotted white lines outline the telencephalon (B,C,E,F). Dashed lines indicate osteoprogenitors (op, C). Arrows indicate loss of Twist1 expression (E,F). Diagrams beneath indicate plane of section, region of interest and embryonic axes. Scale bars: 100 μm.
Twist1 protein immunofluorescence (Fig. 3M,N). Therefore, β-catenin signaling promotes Twist1 expression in the cranial mesenchyme. In further support of this conclusion, in the mutant we also identified sites of ectopic Twist1 expression in the trigeminal ganglia (supplementary material Fig. S3) and in the nasal capsule (Fig. 3H,K), which were both restricted to En1Cre lineage-marked cells with forced activation of β-catenin signaling (Fig. 3G,J), and, in chondroprogenitors, corresponded with a reduction in endogenous Sox9 expression (Fig. 3I,L). Collectively, these experiments demonstrate that β-catenin activity is required and sufficient for Twist1 expression.

β-catenin forms a molecular complex that acts on the Twist1 promoter
Next, we determined whether Twist1 is a direct transcriptional target of β-catenin in vivo. We identified consensus TCF/LEF binding motifs in nine regions at the Twist1 locus in addition to one previously identified in the minimal Twist1 promoter (Fig. 3O) (Howe et al., 2003). Chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) for monomethylated lysine 4 of histone H3 (H3K4me1), which is predictive of active or poised enhancer elements, revealed significant enrichment (greater than 6-fold) for three TCF/LEF consensus sequences (Fig. 3P) compared with non-target sites in the Twist1 locus in E12.5 cranial mesenchyme (Creyghton et al., 2010; Heintzman et al., 2007).

Next we tested by ChIP-qPCR on E12.5 cranial mesenchyme whether a transcription complex containing β-catenin directly binds to H3K4me1-marked Twist1 regulatory elements in vivo. β-catenin binding was enriched 2.5-fold at the TCF/LEF consensus site marked by H3K4me1 ~1.7 kb upstream of the transcription start site (Fig. 3Q). These data indicate that β-catenin binds to a putative 5′ enhancer element upstream of the promoter of Twist1 in cranial mesenchyme, which is consistent with the requirement and sufficiency of β-catenin activity for Twist1 expression. However, we cannot rule out the possibility that β-catenin acts on enhancers located more distal to those tested here, or even in trans.

Deletion of Twist1 is sufficient to induce chondrogenesis in cranial mesenchyme
β-catenin positively regulates Twist1 expression and negatively regulates chondrogenesis in cranial bone progenitors. We tested the
hypothesis that Twist1 is a key mediator of β-catenin and a negative regulator of chondrogenesis in vivo. We used the En1Cre line and the Twist1 conditional null allele to delete Twist1 from cranial mesenchyme prior to fate specification (Chen et al., 2007). β-galactosidase staining on En1Cre; RR embryos at E15.5 revealed En1Cre lineage cells in the paired frontal and parietal bones as well as in the interparietal bone (Fig. 4A). The lack of Twist1 mRNA confirmed that deletion was effective in Twist1 mutant cranial bone progenitors by E12.5 (Fig. 4B). Conditional Twist1 deletion resulted in Alcian Blue-stained cartilage encompassing the posterior half of the skull from the interparietal bones laterally to the parietal bones, and anteriorly to the coronal suture (Fig. 4C; n=6/6). Twist1 conditional mutants were also missing a posterior section of the frontal bones, an anterior portion of the parietal bones, and a significant amount of the interparietal bone as judged by Alizarin Red staining at E16.5 (Fig. 4C).

In Twist1 mutants, the frontal bone lacked Osx expression (Fig. 4D). However, there was no evidence of Sox9 expression in conditional Twist1 mutants, even though we detected subtle domains of Col2a1 expression in E12.5 from immunofluorescence for cleaved caspase 3 (data not shown). The parietal and interparietal bones of Twist1 conditional mutants did not express Osx (Fig. 4E,F). In place of osteoprogenitors we identified the presence of chondrocytes by ectopic Sox9 and Col2a1 expression (Fig. 4E,F). Therefore, loss of Twist1 in cranial bone progenitors resulted in replacement of posterior parts of the calvarium with cartilage, partially resembling the transformation of the skull bones to chondrocytes induced by conditional β-catenin deletion.

Next, we tested the effect of Twist1 protein on Sox9 proximal promoter activity. Although the regulatory elements that drive tissue-specific expression of Sox9 remain elusive, a 530 bp sequence has been utilized as a proximal promoter in most transgenic assays (Bagheri-Fam et al., 2006; Wunderle et al., 1998). Previous studies indicate that the proximal promoter is crucial in driving basal Sox9 expression. A luciferase reporter driven by the Sox9 promoter was active in undifferentiated C3H10T1/2 immortalized mouse mesenchymal cells. However, in the presence of overexpressed Twist1 or its ortholog Twist2 (Fig. 4G), Sox9-luciferase activity was significantly downregulated (Fig. 4G; P<0.01). The Twist transcription factor family may therefore negatively regulate Sox9 transcription. Overall, our findings indicate that Twist1 negatively regulates cartilage formation in the skull bones in vivo, at least in part by repressing the chondrogenic initiation program.

### β-catenin and the Twist family interact in cranial mesenchyme in vivo

Since Twist1 conditional mutant skull bones partially phenocopied those of β-catenin conditional mutants, and both family members exhibit similar repressive functions in vitro, we tested whether Twist2 could also function downstream of the Wnt/β-catenin pathway in cranial bone primordia. The Twist2Cre+/+; RR+/+; β-
catenin\[\beta\] mutant, which is heterozygous for Twist2 (Yu et al., 2003), formed ectopic chondrocytes in the skull at E18.5 (Day et al., 2005). Genetic lineage analysis of Twist2\[Cre\]/; RR\[+/\] embryos revealed expression in cranial mesenchyme at E9.5 and E10.5 (Tran et al., 2010). Consistently, we detected expression in cranial mesenchyme at E9.5 and E10.5 (al., 2005). Genetic lineage analysis of as efficiently as the En1Cre line, as \(\beta\)-catenin conditional mutants from both lines lacked Lef1 expression in cranial osteoprogenitors by E12.5 (Fig. 5B,D, Fig. 1F,G). At E12.5, En1Cre/\(+\); RR; \(\beta\)-catenin\[\[\Delta\[\text{ex3/4}\]\]] mutants resulted in substantially more Sox9 expression within the cranial osteoprogenitors (Fig. 5E–H; n=3/3). Therefore, deletion of a single allele of Twist2 in CNC cells exacerbated the conversion of Runx2\[+\] osteoprogenitors to Sox9\[+\] chondroprogenitors in the absence of \(\beta\)-catenin.

To further characterize the genetic interaction between \(\beta\)-catenin and the Twist family we tested whether Twist1 loss-of-function could rescue the inhibition of skull osteoprogenitor fates induced by \(\beta\)-catenin gain-of-function (Fig. 3E) (Hill et al., 2005). The En1Cre; \(\beta\)-catenin\[\Delta\[\text{ex3/4}\]\] mutants died by E12.5, so we used an inducible Prx1CreER driver (Kawanami et al., 2009) (Prx1 is also known as Prx1 – Mouse Genome Informatics). In the head, the Prx1Cre transgene labelled cranial mesenchyme at E11 (Hill et al., 2005), and induction of Prx1CreER at E9.5 and E10.5 resulted in Prx1CreER lineage cells contributing to the frontal, parietal and interparietal bones by E16.5 (supplementary material Fig. S4A). Constitutive \(\beta\)-catenin activation induced expression of Lef1 but not Sox9 in the frontal bone (supplementary material Fig. S4B–G). Prx1CreER; \(\beta\)-catenin\[\Delta\[\text{ex3/4}\]\] embryos had an almost complete loss of the posterior frontal bones and greatly reduced parietal bones at E16.5 compared with controls (Fig. 5I, J). At E18.5, Prx1CreER; Twist1\[\Delta\[\text{ex3/4}\]\] embryos exhibited decreased bone mineralization in the posterior frontal bone and anterior parietal bones compared with controls (Fig. 5K, L). At E18.5, Prx1CreER; \(\beta\)-catenin\[\Delta\[\text{ex3/4}\]\] Twist1\[\Delta\[\text{ex3/4}\]\] mutants rescued a substantial amount of mineralization in the frontal and parietal bones compared with Prx1CreER; \(\beta\)-catenin\[\Delta\[\text{ex3/4}\]\] mutants (Fig. 5M). Prx1CreER; \(\beta\)-catenin\[\Delta\[\text{ex3/4}\]\] Twist1\[\Delta\[\text{ex3/4}\]\] mutants had AP activity indicative of ossification in both anterior and posterior portions of the frontal bone, which was missing from Prx1CreER; \(\beta\)-catenin\[\Delta\[\text{ex3/4}\]\] mutants (supplementary material Fig. S4K; n=3/3). AP expression in the frontal bone of Prx1CreER; \(\beta\)-catenin\[\Delta\[\text{ex3/4}\]\]; Twist1\[\Delta\[\text{ex3/4}\]\] mutants coincided with Lef1-expressing \(\beta\)-catenin-stabilized cells (supplementary material Fig. S4H–J), whereas neither Lef1 nor Sox9 was detected in the frontal bones of controls with wild-type \(\beta\)-catenin levels (supplementary material Fig. S4C, D; data not shown). Therefore, deletion of Twist1 restored the bone formation inhibited by constitutively active \(\beta\)-catenin. Our data suggest that an exquisitely dose-sensitive genetic interaction in vivo between \(\beta\)-catenin and Twist1 is required to ensure proper osteoprogenitor fate in intramembranous bones of the skull.

**Twist1 binds to the Sox9 3’ UTR in vivo in cranial mesenchyme.**

Next, we tested whether Twist1, which is induced in response to \(\beta\)-catenin, could bind to the Sox9 locus in vivo. In *Drosophila*, Twist
consensus sites differ substantially from canonical E-box sites (Ozdemir et al., 2011). We took an unbiased approach to identifying binding sites for Twist1 in the Sox9 locus and mined ChIP-seq (ChIP followed by sequencing) data from a human mammary epithelial cell line (Casas et al., 2011). Twist1 was enriched by 9-fold at a site within the 3'UTR of SOX9 (Fig. 6A; chr17:67633050-67633250, hg18 assembly). Next, we tested whether Twist1 bound to the orthologous region of mouse Sox9 in skeletogenic and mesenchymal murine cell lines (Fig. 6B,C). We observed Twist1 enrichment at the same site in Sox9 3'UTR in three different cell contexts: in vivo in the cranial mesenchyme of wild-type E12.5 embryos, in C3H10T1/2 cells transiently overexpressing Twist1, and in an ATDC5 chondrosarcoma cell line stably expressing a tamoxifen-inducible form of Twist1 (Yang et al., 2004). Notably, we did not detect enrichment for Twist1 at the Sox9 promoter (Fig. 6B). Therefore, Twist1 binds robustly in vivo to a genomic element downstream of the Sox9 coding region, suggesting that Twist1 might regulate Sox9 in skull progenitor cells.

In summary, we identified requirements for β-catenin in skull osteoblast lineage commitment and a mechanism for suppression of chondrogenesis. We identified Twist1 as a target of β-catenin, and that Twist1 deletion both phenocopies and genetically compensates for β-catenin function in determining cell fate in the skull. In early cranial bone development, direct differentiation of cranial bone progenitors (Runx2⁺) into osteoblasts (Osx⁺) occurs (Fig. 6D). In our model, β-catenin activates and functions through Twist1 to promote a bypass of chondrocyte fate (Sox9⁺) (Fig. 6D). Our data suggest that β-catenin directly activates Twist1 transcription (Fig. 6E). Although the precise mode of repression of chondrocyte fate is less clear, genetic deletion of β-catenin or Twist1 results in ectopic expression of Sox9. Since Twist1 can associate with Sox9 chromatin, it is tempting to speculate that Twist1 mediates negative regulation of Sox9 transcription by β-catenin (Fig. 6E).

DISCUSSION

Previous studies have implicated a role for β-catenin in intramembranous bone formation-related osteoblast differentiation. We used multiple Cre lines to functionally manipulate mouse cranial bone progenitors contributing to the frontal, parietal and interparietal bones (Fig. 1B, Fig. 4A, Fig. 5A; supplementary material Fig. S2I). Our data provide mechanistic insight into the molecular basis of cranial bone progenitor specification during intramembranous bone formation in the skull.

By combining genetic lineage analysis with a spatially restricted En1Cre line, this study provides key in vivo evidence that β-catenin-deficient skull bone progenitors become cartilage cells
early at the onset of osteoprogenitor fate selection at E11.5-12.5 (Fig. 1). Deletion of β-catenin from skull bone osteoprogenitors results in the near complete replacement of the bony skull plates with cartilage arising from the En1Cre lineage (Fig. 1Q). Currently, no single or compound Wnt ligand knockout mouse phenocopies the β-catenin conditional knockout; although the Wnt9a−/− mouse has a small amount of ectopic cartilage, skull ossification is mostly intact (Spiteri et al., 2006). Therefore, future studies should identify the specific ligands required to prevent chondrogenesis in the skull. Although Sox9 expression localized to β-catenin conditional mutant skull osteoprogenitors (Fig. 1), the cranial En1Cre lineage comprises not only osteogenic mesenchyme but also non-osteogenic dermal mesenchyme. The dermal progenitors form first, and the osteogenic mesenchyme grows dorsally through the non-osteogenic layer (Roybal et al., 2010). Therefore, the more dorsal population of Sox9- or Runx2-expressing cells in β-catenin mutants may be converted dermal fibroblasts (Fig. 11,M). We previously demonstrated that cranial dermal progenitors convert to cartilage upon deletion of β-catenin, and others have shown that non-osteogenic mesenchyme progenitors can ossify (Roybal et al., 2010; Tran et al., 2010). Multiple cranial lineages, therefore, may form chondrocytes upon ectopic activation of Sox9, which can be sufficient for chondrogenesis (Eames et al., 2004).

We also demonstrate that constitutive activation of β-catenin resulted in an absence of Runx2 expression in the skull in vivo, and a previous study demonstrated similar results in limb bud culture (Fig. 2) (Hill et al., 2005). The incongruous results between loss- and gain-of-function studies for β-catenin could be explained by a sequential, biphasic role for β-catenin in regulating osteoprogenitor fate that is too subtle for resolution by existing Cre lines. Alternatively, it has been speculated that, at high levels, β-catenin represses Runx2 as an extension of the general mechanism through which it inhibits Sox9 in skull progenitors (Hill et al., 2005). The mechanism by which β-catenin controls cranial osteoprogenitor fate remains incompletely defined, but we show that β-catenin directly activates Twist1, which results in increased Twist1 protein levels (Fig. 3O). Since Twist1 is an inhibitor of differentiation of both the osteoblast and chondrocyte lineages (Bialek et al., 2004; Gu et al., 2012), constitutive activation of β-catenin may inhibit differentiation through high levels of Twist proteins.

Previous studies suggest that Twist1 is a target of Wnt/β-catenin pathway activation in vitro, and that Twist1 inhibits the differentiation of chondrocytes in cell lines (Howe et al., 2003; Reinhold et al., 2006). In vivo, Twist1 haploinsufficiency inhibits the differentiation of cranial bone osteoblasts (Bialek et al., 2004); however, genetic evidence for the protein as a chondrogenic repressor was lacking. Here, we extend previous knowledge by first showing that β-catenin activates Twist1 expression and associates with an upstream putative enhancer element of Twist1, providing evidence that Twist1 is a direct target of the Wnt/β-catenin pathway in vivo (Fig. 3). We tested Twist1 function through conditional deletion in cranial osteoprogenitors, and detected the presence of chondrocytes in the posterior skull (Fig. 4). We did not detect chondrocytes in the anterior frontal bones. There was, however, a loss of ossification at the prospective coronal suture boundary between the frontal and parietal bones (Fig. 4). Twist1 promotes the survival of premigratory CNC cells (Bildsoe et al., 2009), although we did not detect an increase in cell death in Twist1 conditional null osteoblast progenitors. Our finding that Twist1 represses chondrogenesis in embryonic development is significant, although deletion of Twist1 from mesenchymal progenitors of the appendicular skeleton does not result in the formation of ectopic chondrocytes (Krawchuk et al., 2010; Loebel et al., 2012; Zhang et al., 2010). Our data could therefore provide insight into basic mechanistic differences between the two modes of vertebrate ossification. However, in Twist1 conditional null mutants, instead of the entire skull, it was the interparietal bone that was most completely replaced by cartilage (Fig. 4). Our data are consistent with observations that Twist1 heterozygosity in mice, and human TWIST1 mutations in Saethre-Chotzen syndrome, disproportionately affect the interparietal bone (El Ghouzzi et al., 1997). One possibility is that Twist1 shares a role with other factors during skull progenitor fate selection, and the posterior skull might represent the least intrinsic redundancy.

We provide mechanistic insight into how β-catenin regulates intramembranous bone formation and suppresses cartilage fate. Thus, if β-catenin inhibits cartilage fate in skeletal progenitors, it might do so through recruitment of Twist1 as a repressor. Here, Twist1 deletion rescued the inhibition of bone formation by constitutive β-catenin activation (Fig. 5). In addition to Twist1, Twist2 also contributes to repression of chondrocytic gene expression in the skull, which is consistent with previous reports that Twist family members can have overlapping functions (Sosic et al., 2003).

How does β-catenin promote cranial osteoblast formation? We show that β-catenin activates Twist1 in cranial osteoprogenitor cells (Figs 2, 3), which is required to inhibit chondrocyte formation (Fig. 4). Sox9 is the central regulator of chondrogenesis, but it also triggers Runx2 degradation and negatively regulates Runx2 transcription (Cheng and Genever, 2010; Yamashita et al., 2009). Further, Sox9 overexpression is sufficient at high levels to inhibit bone formation in vivo (Eames et al., 2004; Zhou et al., 2006). Therefore, the role of β-catenin and Twist might be largely permissive and represses the dominant molecular program, i.e. chondrogenesis, in order to promote osteogenesis. The exact mechanism of repression of chondrogenesis remains elusive, but Sox9 protein and mRNA were undetectable in cranial bone progenitors (Fig. 1), and deletion of β-catenin or Twist1 results in ectopic Sox9 expression (Figs 1, 4). Additionally, genome-wide mapping of Twist1 binding sites revealed that Twist1 binds immediately downstream of Sox9 in a robust fashion (Fig. 6). This is consistent with studies that showed that regulatory elements frequently reside in the 3′ region of genes and can form chromatin loops that interact with 5′ enhancers or the promoter (Creighton et al., 2010; Heintzman et al., 2007; Palmer et al., 2007; Yochum et al., 2008; Yochum et al., 2010). We found that Twist1 could bind Sox9 chromatin in non-chondrogenic cells such as human mammary epithelial cell lines (HMLEs), even in the absence of cofactors normally found in chondrogenic cells (Fig. 6). However, HMLEs also express Twist1 in response to Wnt pathway activation (Howe et al., 2003), so canonical Wnt signaling could play a role in targeting Twist1 binding to Sox9. Although future studies are clearly required, our results suggest that the mechanism of chondrogenic inhibition could involve Twist1 binding to Sox9 (a model is shown in Fig. 6E). Future experiments will test the requirement and role of Twist1 binding to Sox9 for β-catenin function in cranial bone progenitors.

The vertebrate skeleton is diversely patterned across species; indeed, the study of the phylogeny of the skeleton is one of the underpinnings of vertebrate evolutionary theory. Biologists have postulated that the phylogenetic changes that yielded various skeletal patterns and elements in chordates must have been complex in nature (DeBeer, 1937). However, our data provide proof of principle for a model in which a single cue is required to
switch between chondrogenic precursors of endochondral bone formation and the mesenchymal condensation of intramembranous bone formation. In our model, the morphogenesis of the skull remains intact in β-catenin loss-of-function mutants, but the underlying fate of the skeletal tissue transforms nearly completely from dermatochondral to chondrocranial. The mutant phenotype resembled the skull plan of chordichthyces fish, where cartilage elements constitute not only the skull base but also the skull vault. Whether different β-catenin levels account for species-specific differences in skull plans is the subject of future work. Additional studies further elucidating how the β-catenin-Twist1/2 axis interacts to prevent chondrogenesis in intramembranous ossification might also begin to explain the evolutionary basis for intramembranous bone formation.

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