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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

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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 (Kawanami et al., 2009). The conventional null, conditional loss- and gain-of-function floxed alleles for β-catenin (*Ctnnb1*) [β-*catenin*$^{\Delta/+}$, β-*catenin*$^{fl/fl}$, β-*catenin*$^{\Delta ex3/+}$ (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 g 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 Warmann, *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 C3H10T1/2 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-myc-Twist1, 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-Renilla (Promega) as transfection efficiency controls, were used for transfection. Sox9luc, consisting of 530 bp proximal to the mouse *Sox9* transcription start site cloned into PGL4.10 (Promega), was a kind gift from Cynthia Bartels (Case Western Reserve University School of Medicine, Cleveland, OH, USA). Transient transfections were performed at 80-90% confluence using Lipofectamine 2000 (Invitrogen, for C3H10T1/2 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 separated 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-

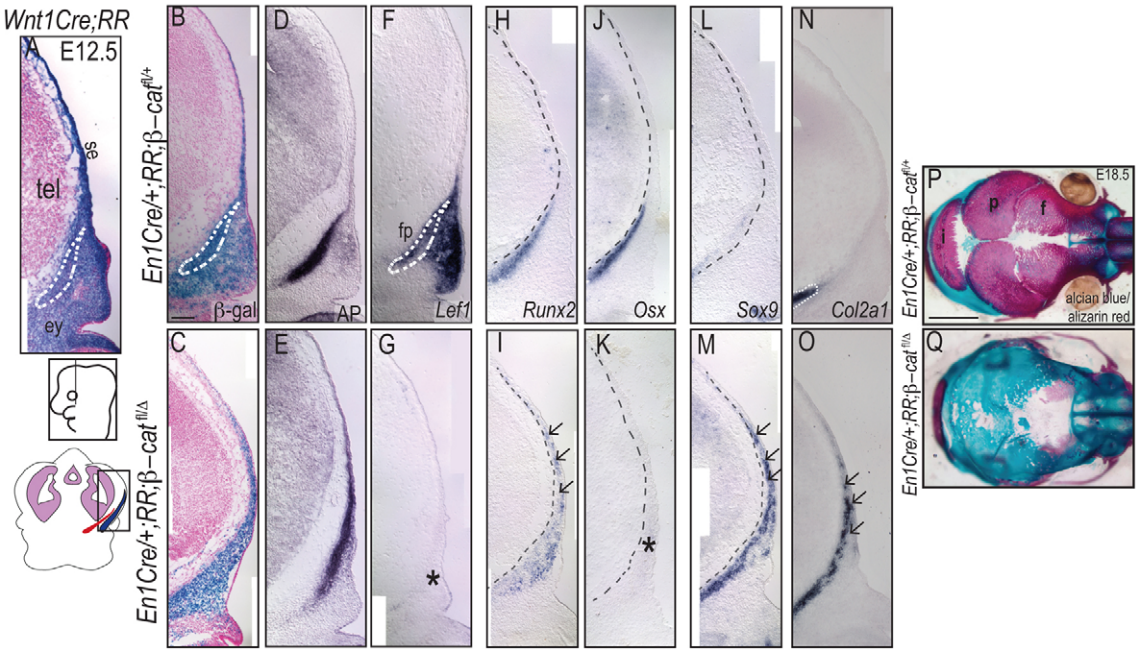

Fig. 1. β-catenin is required for cranial osteoblast lineage commitment. (**A-E**) Coronal mouse tissue sections were stained with X-Gal for β-galactosidase activity (A-C) or for alkaline phosphatase (AP) activity (D,E). The AP$^+$ domain is outlined on serial sections (A,B,F). (**F-O**) In situ hybridization of coronal mouse tissue sections. Asterisks indicate where *Lef1* and *Osx* expression was lost in cranial mesenchyme (G,K). Arrows point to expanded *Runx2*, *Sox9* and *Col2a1* domains (I,M,O). Dashed lines indicate En1Cre lineage. (**P**,**Q**) Whole-mount mouse skeletal preparations stained with Alcian Blue for cartilage and Alizarin Red for bone. Diagrams to left indicate the plane of section and region of interest (boxed). Tel, telencephalon; se, surface ectoderm; ey, eye; fp, frontal bone progenitors; l, interparietal bone; p, parietal bone; f, frontal bone. Scale bars: 100 μm in B; 25 mm in P.

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 conditionally modulate β-catenin activity levels in postmigratory 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 cells (Soriano, 1999). 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$^+$ osteoprogenitors 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 progenitors at E11-11.5 prior to osteoprogenitor specification in the supraorbital arch (Tran et al., 2010). Compared with *En1Cre/+; RR/+* embryos, *En1Cre/+; RR/+;* β-*catenin*$^{fl/+}$ heterozygotes exhibited no demonstrable skull phenotype. Therefore, we generated *En1Cre/+; RR/+;* β-*catenin*$^{fl/\Delta}$ 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 *En1*Cre 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-*catenin*$^{fl\Delta}$ 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 chondrocytic 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-*catenin*$^{fl\Delta}$ 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

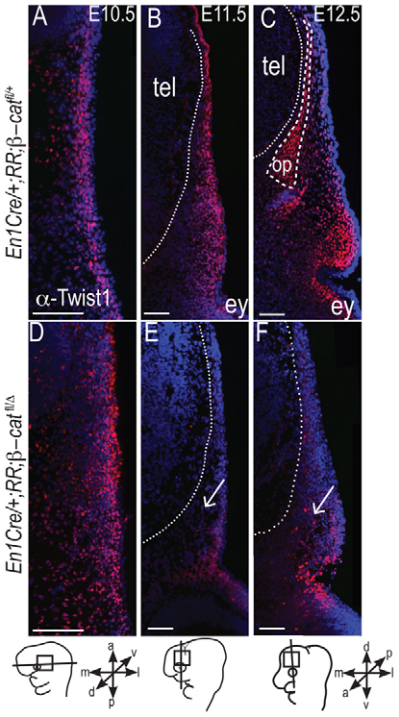

Fig. 2. Requirement for β-catenin in cranial mesenchyme for Twist1 protein expression. Indirect immunofluorescence for Twist1 on (**A**,**D**) axial or (**B**,**C**,**E**,**F**) coronal tissue sections. Nuclei were counterstained with DAPI. Ey, eye; tel, telencephalon. Dotted white lines outline the 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.

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-*catenin*$^{\Delta ex3/+}$ mutant embryos were embryonic lethal at E13.5, but were viable for analysis at E12.5 (Fig. 3A,D). *En1Cre/+; RR;* β-*catenin*$^{\Delta ex3/+}$ 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

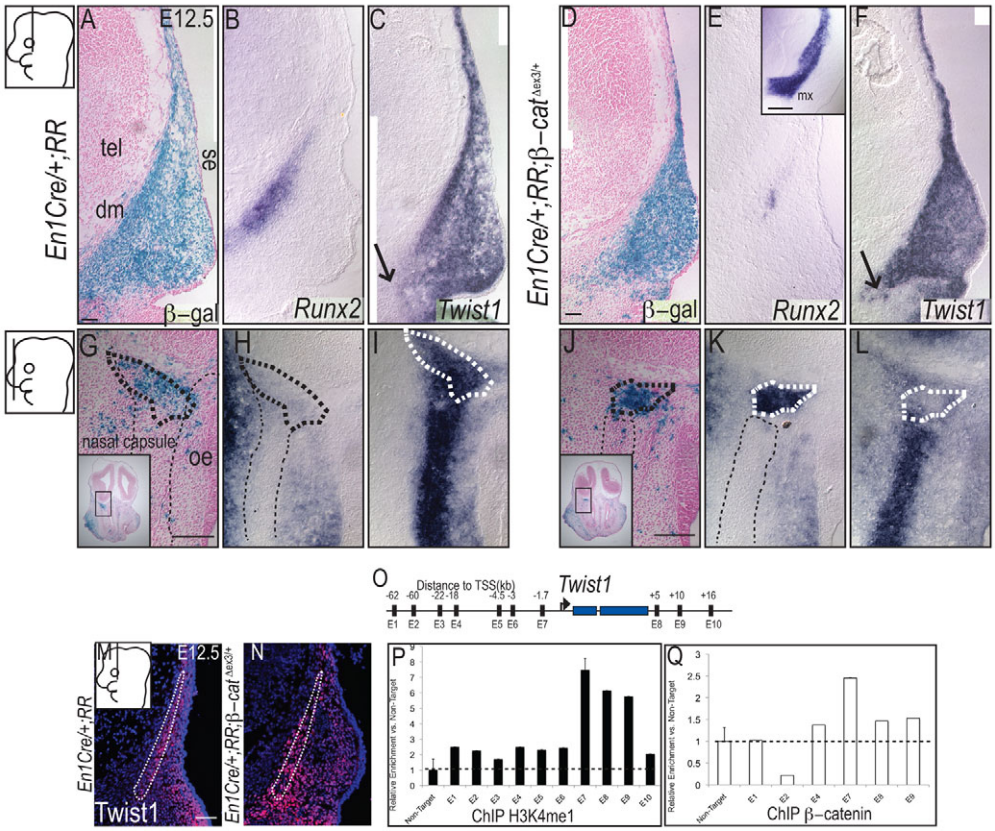

Fig. 3. β-catenin activates *Twist1* expression. (**A-L**) Shown are coronal sections stained with X-Gal (A,D,G,J) and in situ hybridization on coronal tissue sections through cranial bone progenitors (B,C,E,F) or facial cartilage (H,I,K,L). Inset in E is positive control staining on maxilla on the same section. Insets (G,J) show low magnification for orientation; and diagrams to left indicate plane of section. Arrows indicate expansion of the *Twist1* domain (C,F). Thick dashed lines indicate *En1Cre* lineage cells in nasal capsule, and thin dashed black lines detail nasal capsule primordia marked by *Sox9* expression (G-L). (**M**,**N**) Indirect immunofluorescence for Twist1 was performed on coronal sections. The average intensity of relative Twist1 immunofluorescence in cranial bone progenitors outlined in M,N was quantified (see Results). (**O**) Mouse *Twist1* locus showing distance of Lef1 binding sites (E1-E10) from the transcription start site (TSS). (**P**,**Q**) Quantitative real-time PCR was performed on immunoprecipitated chromatin from E12.5 wild-type mouse cranial mesenchyme. H3K4me1, monomethylated lysine 4 of histone H3. Negative controls for both immunoprecipitation experiments were non-target sequences characterized by low conservation and lack of a Lef1 consensus motif near the *Twist1* locus. Dashed lines indicate approximate threshold set by non-target sites. Error bars indicate s.d. Tel, telencephalon; se, surface ectoderm; dm, dura mater; mx, maxilla; Oe, olfactory epithelium. See also supplementary material Fig. S3. 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

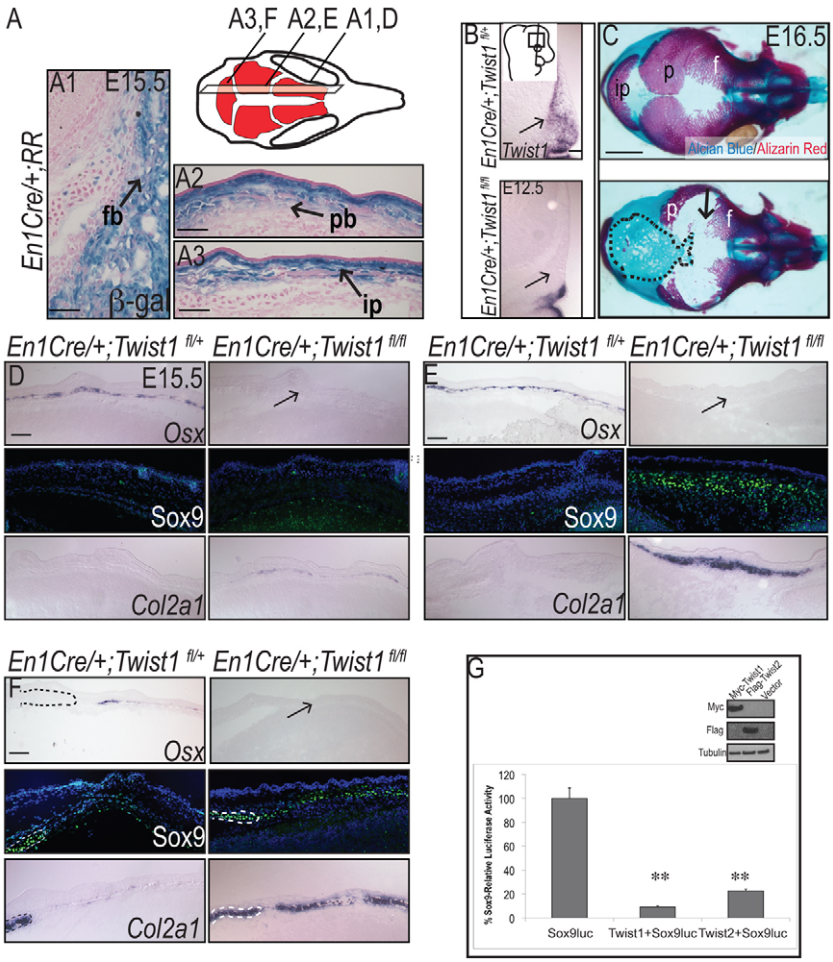

Fig. 4. Loss of Twist1 is sufficient for chondrogenesis in cranial mesenchyme. (**A**) Sagittal sections stained with X-Gal. Diagram indicates orientation for subsequent panels. (**B**) In situ hybridization for *Twist1* on coronal sections. Inset indicates plane of section. (**C**) Whole-mount skeletal preparations. (**D-F**) Indirect immunofluorescence and in situ hybridization performed on sagittal sections. Dashed line (F) outlines endogenous cartilage base. Arrows point to absent *Osx* expression domains in mutants. (**G**) Relative luciferase activity of Sox9luc in C3H10T1/2 cells in transient transfections for 24 hours. Data were normalized to negative control (empty PCI-neo vector) and are reported as a percentage. $^{**}P<0.01$ by Student's *t*-test; error bars indicate s.d. The inset shows western blots to confirm overexpression in C3H10T1/2 cells. Fb or f, frontal bone; pb or p, parietal bone; ip, interparietal bone. Scale bars: 50 μm in A,B,D-F; 25 mm in C.

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 *Twist1* mutant frontal bone progenitors. Notably, we did not detect any cell death of cranial bone progenitors at E12.5 by 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-

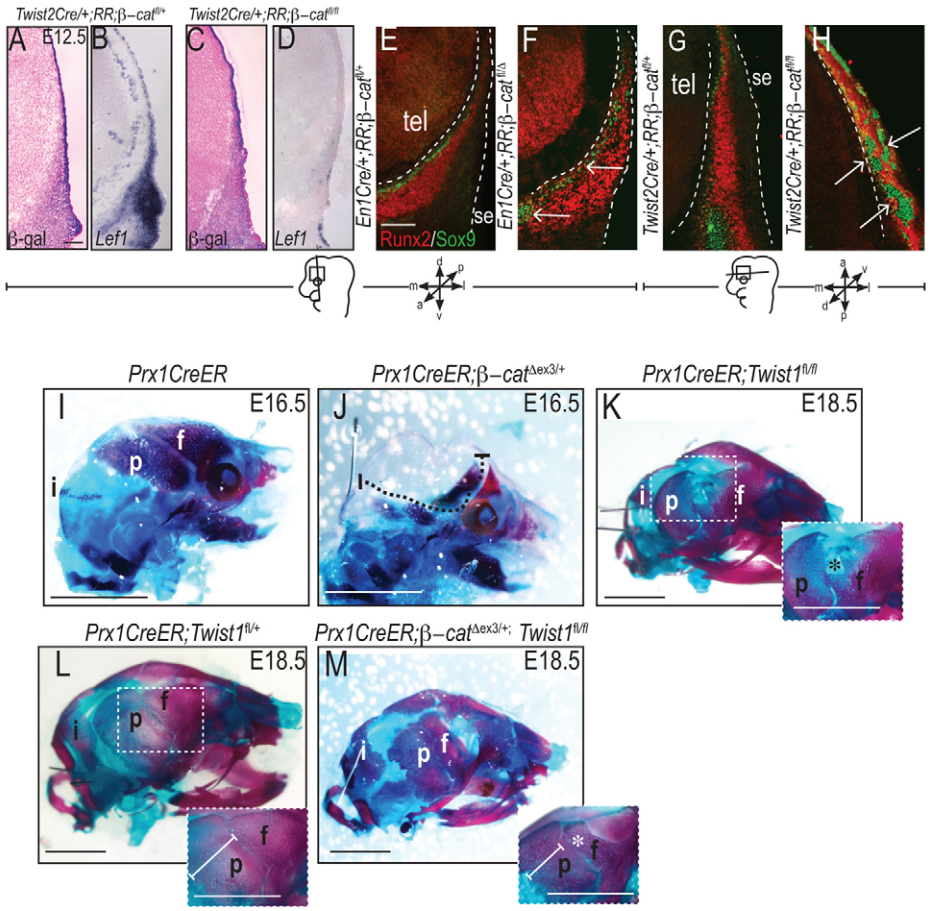

Fig. 5. β-catenin and the Twist family interact in cranial mesenchyme in vivo. (**A-D**) X-Gal staining (A,C) and in situ hybridization (B,D) on coronal mouse tissue sections. (**E-H**) Double immunohistochemistry on coronal (E,F) or axial (G,H) sections. The white dashed lines outline surface ectoderm and telencephalon and arrows indicate Sox9 expression in the Runx2$^+$ domain. (A-H) Diagrams beneath indicate plane of section, region of interest and embryonic axes. (**I-M**) Whole-mount cranial skeletal preparations; lateral views. White bracket (L,M) indicates parietal bone width. Black dotted line (J) indicates missing portions of frontal and parietal bones. Black asterisk indicates that the notch of the posterior frontal bone is missing (K, inset), and white asterisk indicates posterior frontal ossification (M, inset). All control/mutant pairs were photographed at the same magnification. Embryonic axes are indicated. Tel, telencephalon; se, surface ectoderm; f, frontal bone; p, parietal bone; i, interparietal bone. Scale bars: 100 μm for A-H; 25 mm for I-M.

catenin$^{fl/fl}$ 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 *Twist2Cre/+; RR/+* embryos revealed expression in cranial mesenchyme at E9.5 and E10.5 (Tran et al., 2010). Consistently, we detected *Twist2Cre* lineage-marked osteoprogenitor cells at E12.5 in controls and *Twist2Cre/+; RR;* β-*catenin$^{fl/fl}$* mutants (Fig. 5A,C). *Twist2Cre* deleted β-catenin as efficiently as the *En1Cre* line, as β-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;* β-*catenin$^{fl/\Delta}$* osteoprogenitors produced both Runx2 and Sox9 protein, whereas heterozygosity for *Twist2* in *Twist2Cre/+; RR;* β-catenin$^{fl/fl}$ 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 β-catenin.

To further characterize the genetic interaction between β-catenin and the Twist family we tested whether *Twist1* loss-of-function could rescue the inhibition of skull osteoprogenitor fate induced by β-catenin gain-of-function (Fig. 3E) (Hill et al., 2005). The *En1Cre;* β-*catenin$^{\Delta ex3/+}$* mutants died by E12.5, so we used an inducible *Prx1CreER* driver (Kawanami et al., 2009) (*Prx1* is also known as *Prrx1* – 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 β-catenin activation induced expression of Lef1 but not Sox9 in the frontal bone (supplementary material Fig. S4B-G). *Prx1CreER;* β-*catenin$^{\Delta ex3/+}$* embryos had an almost complete loss of the posterior frontal bones and greatly reduced parietal bones at E16.5 compared with controls (Fig.5 I,J). At E18.5, *Prx1CreER; Twist1$^{fl/fl}$* embryos exhibited decreased bone mineralization in the posterior frontal bone and anterior parietal bones compared with controls (Fig. 5K,L). At E18.5, *Prx1CreER;* β-*catenin$^{\Delta ex3/+}$; Twist1$^{fl/fl}$* mutants rescued a substantial amount of mineralization in the frontal and parietal bones compared with *Prx1CreER;* β-*catenin$^{\Delta ex3/+}$* mutants (Fig. 5M). *Prx1CreER;* β-*catenin$^{\Delta ex3/+}$; Twist1$^{fl/fl}$* mutants had AP activity indicative of ossification in both anterior and posterior portions of the frontal bone, which was missing from *Prx1CreER;* β-*catenin$^{\Delta ex3/+}$* mutants (supplementary material Fig. S4K; *n*=3/5). AP expression in the frontal bone of *Prx1CreER;* β-*catenin$^{\Delta ex3/+}$; Twist1$^{fl/fl}$* mutants coincided with Lef1-expressing β-catenin-stabilized cells (supplementary material Fig. S4H-J), whereas neither Lef1 nor Sox9 was detected in the frontal bones of controls with wild-type β-catenin levels (supplementary material Fig. S4C,D; data not shown). Therefore, deletion of *Twist1* restored the bone formation inhibited by constitutively active β-catenin. Our data suggest that an exquisitely dose-sensitive genetic interaction in vivo between β-catenin and *Twist1/2* 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 β-catenin, could bind to the *Sox9* locus in vivo. In *Drosophila*, Twist

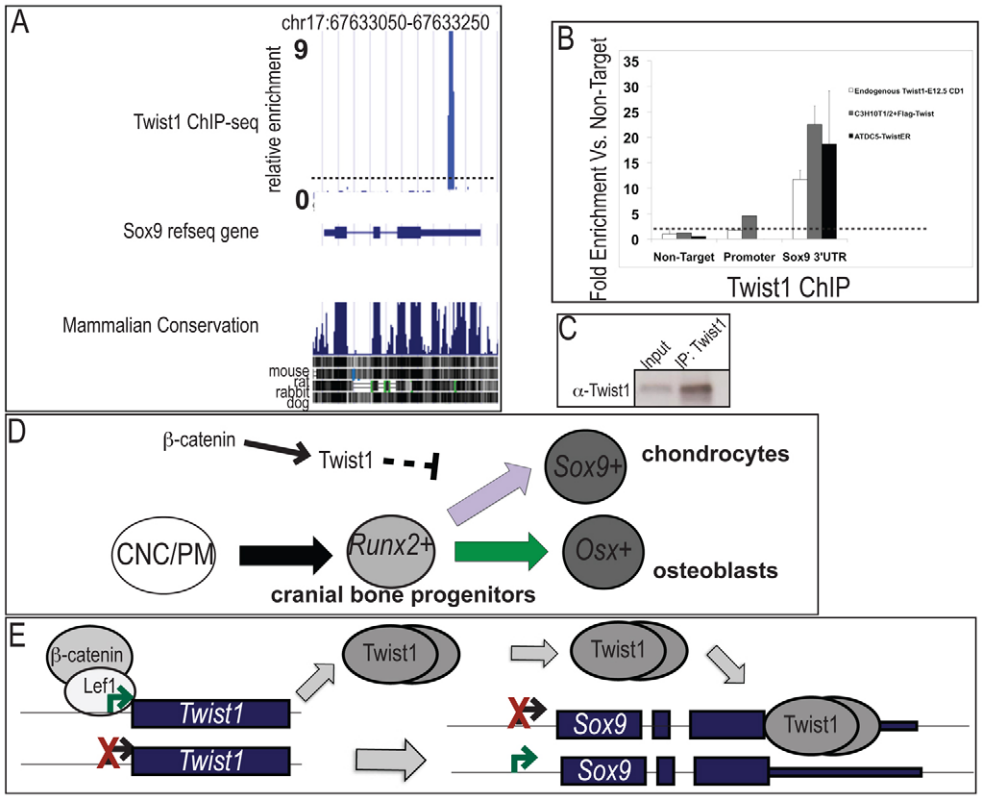

Fig. 6. Twist1 binds to the *Sox9* 3′UTR in vivo in cranial mesenchyme. (**A**) Alignment of a ChIP-seq performed on human mammary epithelial cell line expressing TwistER at the *SOX9* locus using the UCSC genome browser, 2006 assembly (Casas et al., 2011). (**B**) ChIP-qPCR was performed using anti-Flag (C3H10T1/2 cells) or anti-Twist1 (E12.5 cranial mesenchyme, ATDC5-TwistER cells) antibodies and primers for non-target sites on mouse chromosome 12, the *Sox9* promoter, or the *Sox9* 3′UTR. Dashed lines indicate the approximate threshold set by non-target sites. (**C**) Western blot for Twist1 performed on E12.5 cranial mesenchyme input or anti-Twist1 immunoprecipitate. (**D**) Model for the role of β-catenin and Twist1 in cranial bone progenitor specification. Thick black, green or purple arrows represent steps of differentiation; thin arrows denote transcriptional activation or inhibition. (**E**) Model for the direct activation of *Twist1* by β-catenin and for binding of Twist1 to *Sox9* in cranial bone progenitors. Green and black promoter arrows represent active and inactive transcription, respectively. CNC, cranial neural crest; PM, paraxial mesoderm.

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 (Später 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. 1I,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 (Creyghton 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 co-factors 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 dermatocranium to chondrocranium. The mutant phenotype resembled the skull plan of chondrichthyes 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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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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