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Role of canonical Wnt signaling/β-catenin via *Dermo1* in cranial dermal cell development

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SUMMARY

Cranial dermis develops from cephalic mesoderm and neural crest cells, but what signal(s) specifies the dermal lineage is unclear. Using genetic tools to fate map and manipulate a cranial mesenchymal progenitor population in the supraorbital region, we show that the dermal progenitor cells beneath the surface ectoderm process canonical Wnt signaling at the time of specification. We show that Wnt signaling/β-catenin is absolutely required and sufficient for *Dermo1* expression and dermal cell identity in the cranium. The absence of the Wnt signaling cue leads to formation of cartilage in craniofacial and ventral trunk regions at the expense of dermal and bone lineages. *Dermo1* can be a direct transcription target and may mediate the functional role of Wnt signaling in dermal precursors. This study reveals a lineage-specific role of canonical Wnt signaling/β-catenin in promoting dermal cell fate in distinct precursor populations.

KEY WORDS: Craniofacial, Cell fate, Cranial dermis, Cranial bone, Cartilage, Mouse

INTRODUCTION

The mammalian head, which develops from interactions of cranial neural crest and paraxial mesoderm with signals from the surface ectoderm and endoderm, is a good model with which to investigate mechanisms of cell specification of closely related cell types (Noden and Trainor, 2005). The cranial dermis develops in close proximity to the underlying distinct cranial bone lineage. An understanding of the signaling cues that guide cranial dermal cell fate induction and differentiation is a significant unresolved issue.

Beneath the epidermis, the dermis houses functional appendages such as sweat glands, nerves and blood vessels, and is important for the structural integrity of skin and wound healing. The craniofacial dermis, via crosstalk with epidermis, promotes species-specific patterning of the epidermal appendages of the skin (Eames and Schneider, 2005; Sengel and Abbott, 1963). In the mouse embryo, the dermis arises from spatially distinct locations. The dorsal and ventral trunk dermis originate from the somites (Atit et al., 2006) and the lateral plate mesoderm, respectively (Ohtola et al., 2008). By contrast, mouse craniofacial dermis originates from ectoderm-derived cranial neural crest and paraxial mesoderm (Fernandes et al., 2004; Jiang et al., 2002; Yoshida et al., 2008). However, the location and timing of craniofacial dermal cell fate selection in the embryo is not known. Whereas Wnt signaling is crucial for dermal cell determination in the mammalian trunk dermis (Atit et al., 2006; Ohtola et al., 2008), the inductive and differentiation cues that direct the craniofacial dermal lineage remain to be identified. Genetic tools to functionally manipulate only the craniofacial dermal progenitors without compromising head development are lacking.

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Cranial neural crest cells differentiate into ectomesenchymal cells to give rise to the cartilage, teeth, dentary bones, some of the cranial bones and dermal lineages (Chai et al., 2003; Jiang et al., 2002; Noden and Trainor, 2005). Similarly, the paraxial mesoderm contributes to some of the cranial bones and the overlying dermis (Yoshida et al., 2008). Given the spatial proximity and shared origins of cranial dermis and bones, gaining an understanding of the signaling cues that instruct the divergence of the dermal and cranial bone precursors requires manipulation at the time of cell specification. In this study, we have functionally manipulated the supraorbital mesenchyme and examined the effects on cranial dermal development.

Cranial neural crest cells and paraxial mesoderm migrate extensively and differentiate in response to signaling cues in the environment of their destination (Baker et al., 1997; Dorsky et al., 1998; Raible and Eisen, 1996; Shah et al., 1994; Trainor and Krumlauf, 2000). Bone morphogenetic proteins (BMP), Indian hedgehog (Ihh), transforming growth factor β (TGF-β) and Wnts are required in early stages of cranial bone development in the mouse and chick embryo (Abzhanov et al., 2007; Day et al., 2005; Hill et al., 2005; Rodda and McMahon, 2006; Seo and Serra, 2009). These studies do not address the role of signaling cues in cell fate selection of the closely related and spatially adjacent dermal cells. Here, we have tested the requirement of canonical Wnt signal transduction for dermal lineage specification in the supraorbital mesenchyme.

Wnts are secreted ligands that bind to the Frizzled receptor to activate an intracellular signal that is transduced by unphosphorylated β-catenin. In the absence of Wnts, β-catenin is phosphorylated and degraded. In the presence of canonical Wnt signaling, β-catenin is stabilized and translocates into the nucleus where it binds to Tcf/Lef transcription factors to activate the expression of Wnt-target genes (Logan and Nusse, 2004; Miller, 2002; Moon et al., 2004; Wodarz and Nusse, 1998; MacDonald et al., 2009). The canonical Wnt signaling/β-catenin pathway has multiple roles in development, including head formation and induction of craniofacial structures such as the taste papillae and teeth (Brault et al., 2001; Iwatsuki et al., 2007; Liu et al., 2007;

Chen et al., 2009; Liu et al., 2008; Wang et al., 2009). Finally, defects in Wnt signaling are linked to hypertrophic scar formation, fibrotic diseases such as scleroderma (Bayle et al., 2008; Bowley et al., 2007) and focal dermal hypoplasia, which is characterized by thinning of the dermis in some parts of the face (Goltz, 1992; Grzeschik et al., 2007; Wang et al., 2007).

In this study, we have characterized inducible genetic fate mapping tools and conducted conditional mutagenesis in the mouse embryo to: (1) identify one of the developmental origins of cranial dermal precursors in the supraorbital mesenchyme; and (2) demonstrate the necessity and sufficiency of Wnt signaling in cranial dermal cell fate selection. Unexpectedly, Wnt signaling is required to suppress a latent cartilage cell fate in craniofacial and ventral trunk dermal precursors. We have identified *Dermo1* as a tissue-specific target of Wnt signaling in dermal precursors from different embryonic origins that may mediate inhibition of Sox9 and cartilage fate specification.

MATERIALS AND METHODS

Generation and genotyping of mouse lines

En1LacZ, En1CreER$^{T1/+}$; *R26R/R26R* (Jackson Laboratories) (Sgaier et al., 2005; Hanks et al., 1995; Matise and Joyner, 1997; Soriano, 1999) mice were generated, harvested and processed for frozen sections as previously described (Atit et al., 2006). *En1Cre*-mediated β-*catenin^{lof}* mutants were obtained by crossing *En1Cre/+;* β-*catenin$^{null/+}$* males and *R26R/R26R;* β-*catenin$^{flox/flox}$* females (Brault et al., 2001; Haegel et al., 1995) [mice obtained from Alexandra Joyner (Memorial Sloan-Kettering, NY, USA) and Jackson Laboratories]. *Dermo1Cre*-mediated β-*catenin^{lof}* mutants were obtained from the cross between *Dermo1Cre/+;* β-*catenin$^{flox/+}$* males and *R26R/R26R;* β-*catenin$^{flox/flox}$* females (Yu et al., 2003) (obtained from Eric Olson, University of Texas Southwestern Medical Center, TX, USA). *En1Cre*-mediated β-*catenin^{gof}* mutants were obtained by mating *En1Cre* male mice with *R26R/R26R;* β-*catenin$^{flox3/+}$* females (obtained from Makoto M. Taketo, Kyoto University, Japan) (Harada et al., 1999). For each experiment, at least four different mutants (β-catenin$^{lof/gof}$) with littermate controls from two to four litters were analyzed. All animal procedures were approved by Case Western Reserve Institutional Animal Care and Use Committee.

In situ hybridization, immunohistochemistry and histology

Tissue preparation, histology, immunohistochemistry, X-gal staining, TUNEL, BrdU incorporation and in situ hybridization were performed as previously described (Gavrieli et al., 1992; Kanzler et al., 2000; Atit et al., 2006; Ohtola et al., 2008; Li et al., 1995). *Dermo1* probe was a gift from Eric Olson (Li et al., 1995); *Axin2* probe (Open Biosystems, clone 6827741) was obtained from C. Brian Bai (Case Western Reserve University, OH, USA). For immunohistochemistry, primary antibodies against Sox9 (rabbit anti-Sox9; 1:100; Chemicon), Runx2 (goat anti-Runx2; 1:20; R&D Biosystems), mouse anti-β-catenin (1:1000; Sigma or BD BioSciences) (Zhang et al., 2009) and species appropriate secondaries were used. Alcian Blue and Alizarin Red staining for cryosections was performed as previously described (Lev and Spicer, 1964; McGee-Russell, 1958).

Chromatin immunoprecipitation (ChIP)

In vivo ChIP assay followed by quantitative real-time PCR on trunk dermal precursors from E12.5 wild-type CD1 embryos was performed as previously published with a few modifications (Schnetz et al., 2009; Zhang et al., 2008; Zhang et al., 2009). Cells ($5.0\text{-}6.5\times10^7$) were obtained from ~100 to 120 embryos for each experiment. Either anti-Tcf4 antibody (Cell Signaling) or anti-H3K4me1 antibody (Abcam) was used. We used the following consensus sequences to identify Tcf/Lef-binding motifs: A/T A/T CAA A/T GG; CTTTG A/T A/T; GCAAAGGG (Giese et al., 1991; Spater et al., 2006; van Beest et al., 2000; van de Wetering et al., 1991). The 20 kb 5′ upstream, intronic and 20 kb 3′ downstream UTR regions were searched for these binding sites using UCSC Genome Browser and Enhancer Element Locator program (Hallikas et al., 2006; Palin et al., 2006). Relative enrichment between input DNA and immunoprecipitated DNA was determined by qRT-PCR and $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). Primers are listed in Table S1 in the supplementary material.

Luciferase plasmids

A 1.2 kb fragment upstream of *Dermo1* transcription start site was amplified from the genomic DNA of the cell line 3T3 (primers are listed in Table S1 in the supplementary material). The PCR products were cloned into *Xho*I and *Hin*dIII sites of pGL4.10 Luciferase plasmid (Promega) to make the *Dermo1*-Luc reporter plasmid and the reverse construct (*Dermo1*Rev-Luc).

Cell culture, transfection, western blotting, real-time PCR and luciferase assay

Full-length *pcDNA3-Dermo1-Flag* (from D. Sosic and E. Olson, University of Texas Southwestern Medical Center, TX, USA) and *pcDNA3-5′UT-Flag* (from V. Lefebvre, Lerner Research Institute, Cleveland Clinic Foundation, OH, USA) (Lefebvre et al., 1997) were transfected into C3H/10T1/2 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. mRNA was extracted 2 days after transfection using TRIZOL (Invitrogen) 2 days after transfection and subjected to quantitative real-time PCR for *Sox9* using SYBR Green PCR Master Mix on the ABI Prism 7300 Sequence Detection System (Applied Biosystems). All PCR reactions were carried out in triplicate and primer specificity was validated by the presence of a single peak in the dissociation stage (primers are listed in Table S1 in the supplementary material). Relative gene expression was calculated relative to housekeeping genes using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001).

For western blotting, the following antibodies were used: mouse anti-Flag (1:2000; Sigma); mouse anti-β-actin (1:4000; Novus); and goat anti-mouse-HRP conjugated (1:10000; Thermo). For luciferase assays, cells were transfected with *pCI-neo beta-catenin WT* (B. Vogelstein, Johns Hopkins University, MD, USA), luciferase plasmid (*Dermo1*-Luc, *Dermo1*Rev-Luc or pGL4.10) and renilla plasmid (Promega). One day after transfection, cells were harvested for luciferase assay following the Dual Luciferase Assay Kit protocol (Promega). Luciferase activity was normalized to protein content determined by Pierce BCA Protein Assay Kit.

RESULTS

Wnt signal transduction is required for craniofacial dermal development

To determine whether Wnt signal transduction is required for craniofacial dermal cell fate selection, we conditionally deleted β-catenin, the central transducer of the canonical Wnt signaling pathway, in an early mesenchymal progenitor cell population. To do this, we took advantage of the fact that *Dermo1/Twist2* mRNA expression is restricted to sub-ectodermal cells that will become craniofacial dermal progenitors in the developing head between E11.5-13.5 (Li et al., 1995) (data not shown). We used the *Dermo1Cre* line to genetically eliminate β-catenin and activate the lineage tracer from the *Rosa26 Reporter* (*R26R*) locus (Brault et al., 2001; Soriano, 1999; Yu et al., 2003). However, at E16.5, *Dermo1Cre* lineage-labeled cells were not only seen in the entire cranial dermis but also in the underlying frontal and parietal bones, meninges and the cartilage base of the skull in control embryos (*Dermo1Cre; R26R;* β-*catenin$^{flox/+}$*) (Fig. 1A,C). In the β-*catenin^{lof}* mutants (*Dermo1Cre; R26R;* β-*catenin$^{flox/flox}$*), cranial dermis tissue and ossified bones were both absent. Instead, we found Alcian Blue-positive cartilage tissue comprising *Dermo1Cre* lineage-marked cells and juxtaposed to the dorsal surface ectoderm of the head (Fig. 1B,B′,D,D′). This phenotype suggested a required role of Wnt signaling/β-catenin in cranial dermal and bone development. In the absence of Wnt signal transduction in the

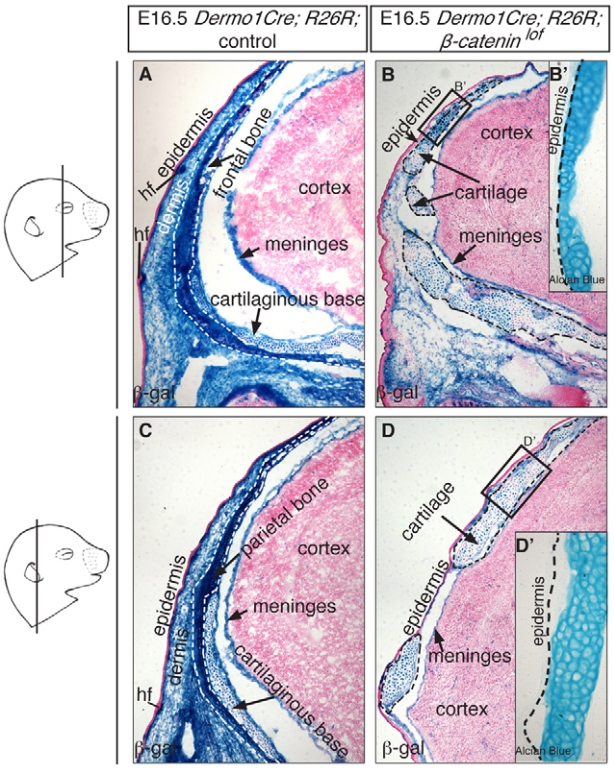

Fig. 1. Extensive contribution of *Dermo1* lineages and roles of Wnt signaling/β-catenin in cranial dermis and bones. (**A-D**) X-gal stained coronal sections of E16.5 embryos. *Dermo1Cre; R26R* marked cells contributed to the entire cranial dermis, frontal and parietal bones, and the cartilaginous base of the skull (A,C). *Dermo1Cre; R26R;* β-*catenin*$^{flox/flox}$ mutants lacked dermis and ossified cranial bones, and had ectopic cartilage tissue that was positive for Alcian Blue below the epidermis in the cranium (B,D). hf, hair follicles.

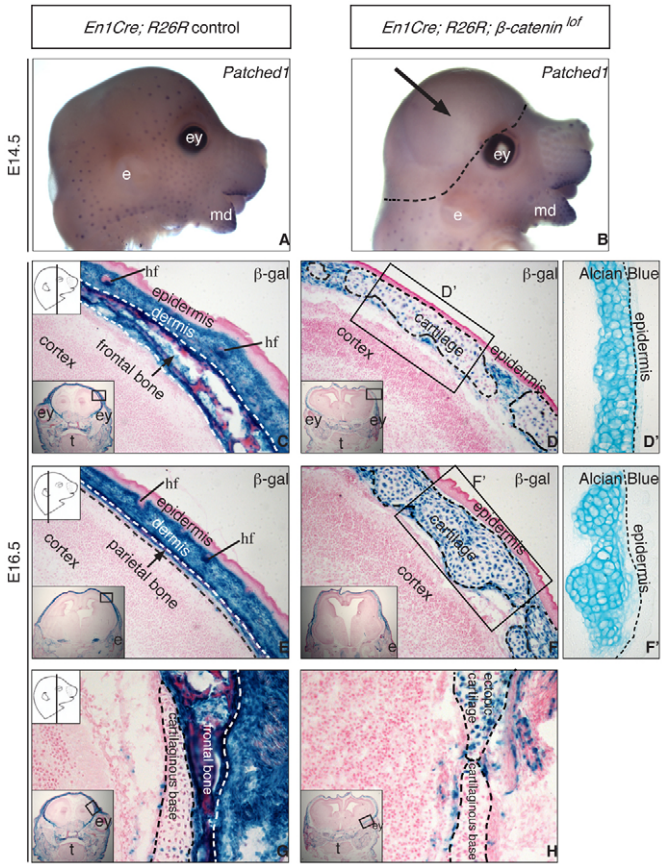

Fig. 2. Absence of cranial dermis and ectopic cartilage in the conditional *En1Cre*; β-catenin loss-of-function mutants. (**A**,**B**) Whole-mount in situ of hair follicle placode marker, patched 1 (*Ptch1*) mRNA, in E14.5 control (*En1Cre; R26R;* β-*catenin*$^{flox/+}$) and mutant embryos (*En1Cre; R26R;* β-*catenin*$^{flox/null}$). Hair follicle placodes are absent in the cranial region above the eye in the mutants (arrow in B). (**C-F**) X-gal stained E16.5 coronal sections of control and mutant embryos. In the mutants, dermis and ossified bones were absent but nodules of *En1Cre; R26R* lineage-labeled cartilage tissue were Alcian Blue positive (D,D′,F,F′). (**G**,**H**) The endogenous cartilage base was mostly β-gal^{-} cells in both controls and mutants. hf, hair follicles; ey, eye; e, ear; t, tongue; md, mandible.

Dermo1Cre-expressing cells, the meninges were thinner (Fig. 1B,D) and the facial dermis and mandible were also absent by E16.5 (data not shown). Spatio-temporal distribution of *Dermo1Cre; R26R*-marked cells in sections from E9.5 and E11.5 revealed that *Dermo1* lineage-marked cells contribute to most of the cranial neural crest and paraxial mesoderm derived lineages in the head by E11.5 (see Fig. S1 in the supplementary material). Thus, the origin of the ectopic cartilage in these Wnt signaling transduction mutants could not be determined by lineage analysis with the *Dermo1Cre* line.

Loss of Wnt signaling transduction in engrailed 1 lineage also leads to loss of cranial dermis

To delineate the requirement of Wnt signaling/β-catenin in cranial dermal development, we took advantage of the more lineage restricted engrailed 1-*Cre* (*En1Cre*) line to conditionally eliminate β-catenin (Brault et al., 2001; Kimmel et al., 2000) and also indelibly mark the mutant cells with the *R26R* reporter line (*En1Cre; R26R;* β-*catenin*^{lof}) (Soriano, 1999). To obtain a global overview of the affected areas in the skin, we performed whole-mount in situ with patched 1 (*Ptch1*) mRNA as a marker for hair follicle placodes. At E14.5, control embryos had hair follicle placodes consistently over the dorsal surface of their heads (Fig. 2A). By contrast, the *En1Cre*-mediated β-*catenin*^{lof} mutants showed a dramatic loss of hair follicle placodes on the head above the eyes (arrow in Fig. 2B). The facial region did not appear grossly different from the controls (Fig. 2A,B). The absence of an overt skin phenotype in the facial region could be because dermal specification precedes *En1Cre* activity (data not shown).

In control embryos (*En1Cre; R26R;* β-*catenin*$^{flox/+}$) at E16.5, cranial dermis was properly formed with hair follicles above ossified frontal bones (Fig. 2C) and parietal bones (Fig. 2E). By contrast, conditional β-*catenin*^{lof} (*En1Cre; R26R;* β-*catenin*$^{flox/null}$) mutants lacked cranial dermis, hair follicles and mineralized frontal bone and parietal bone (Fig. 2D,F). Instead, directly underneath the epidermis, we observed nodular tissues with cartilage cell morphology that stained positive for Alcian Blue (Fig. 2D′,F′) in all embryos that were analyzed (n=8 from 6 litters). Whole-mount skeletal preps of mutant embryos revealed cartilage tissues over both frontal and parietal plates in the *En1Cre* line (data not shown), similar to the published *Dermo1Cre* and *Prx1Cre* lines (Day et al., 2005; Hill et al., 2005). These cartilage cells were mostly β-gal^{+}, demonstrating that they were the *En1*-lineage mutant cells. They

were not derived from the cartilaginous base of the skull, which was still intact and had few *En1* lineage-marked cells (Fig. 2H, compare with 2G). The non-recombined cells in the cartilage nodules lacked β-catenin protein, but their origin could not be determined (see Figs S2 and S7 in the supplementary material). In the mutants at E16.5, we found facial dermis, grossly normal vibrissae hair follicles and ossified dentary bones in the jaw that were comparable with the control fetus (Fig. 6H, compare with 6G; data not shown). *En1Cre* was expressed in the mid-hindbrain region and significant alterations in brain morphology were also observed (Fig. 2 and data not shown). Thus, in the absence of Wnt signaling/β-catenin in the *En1* lineage, sub-ectodermal β-gal$^+$ cartilage nodules developed instead of cranial dermis and cranial bones.

However, not all the cranial mesenchyme became cartilage. The few intervening cells between the cartilage nodules continued to lack β-catenin and *Dermo1* (see Fig. S7 in the supplementary material). Next, we investigated whether these residual cells had the identity of cranial bone cells. In these β-*catenin^{lof}* mutants, Runx2$^+$ cranial bone precursors were generated and persisted in between the cartilage nodules until E15.5 (see Fig. S7A in the supplementary material; compare panels B,J,D,L with A,I,C,K) (n=7 from E13.5-E15.5 embryos). The Runx2$^+$ cells failed to differentiate into mineralized bone as assessed by Alizarin Red staining (see Fig. S7B in the supplementary material). Thus, Wnt signal transduction was not required for generating Runx2$^+$ cranial bone precursor cells for the frontal and parietal bones, but subsequently was required for cranial bone differentiation. The identity of the Runx2$^-$ cells could not be determined by additional differential marker analysis, owing to lack of cranial dermal-specific markers between E15.5 and E17.5.

The engrailed 1 domain in the supraorbital region includes some cranial bone and dermal progenitors in mouse embryos

To spatially and temporally define the cranial bone and dermal progenitor population manipulated with the *En1Cre* line, we genetically fate-mapped domains of *En1* expression. To visualize the activity of the engrailed 1 (*En1*) promoter, we analyzed *En1LacZ* knock-in embryos (Hanks et al., 1995; Matise and Joyner, 1997) by whole-mount X-gal staining (Fig. 3A). Initial expression of *En1* was restricted to the mid-hindbrain boundary from E8.5-E10.5 (Fig. 3A) (Sgaier et al., 2007; Sgaier et al., 2005). Between E10.5 and E11.5, a new expression domain of *En1LacZ* was observed in the supraorbital region (Fig. 3A,B) (Deckelbaum et al., 2006). The supraorbital region was found to contribute to the osteoprogenitors within the coronal suture, but the cranial skin was not analyzed (Deckelbaum et al., 2006; Yoshida et al., 2008). To fate map genetically all descendants of the *En1*-expressing cells between E10.75-E11.75 (Fig. 3B), we temporally restricted the activation of β-galactosidase (β-gal) expression from the *Rosa26 Reporter* (*R26R*) locus with the inducible engrailed 1 *Cre-ER^{T1}* (*En1CreER^{T1}*) line (Sgaier et al., 2005; Joyner and Zervas, 2006; Soriano, 1999). In mouse embryos treated with tamoxifen at E10.5 and analyzed at E16.5, the distribution of *En1*-lineage marked cells was restricted to the cranial dermis and frontal and parietal bones (Fig. 3B). A few labeled β-gal$^+$ cells were found in the snout but not in the endogenous cartilage skull base (data not shown). The mid-hindbrain boundary cells expressing *En1CreER^{T1}* did not migrate and were found in the brain as previously shown (Sgaier et al., 2005) (data not shown). We also labeled *En1CreER^{T1}*-expressing cells in the mid-hindbrain domain between E7.75-E8.75, but did not find labeled cells in the craniofacial dermis by E16.5 (data not shown). Previous studies have shown that the frontal bones come from cranial neural crest cells (Chai et al., 2003; Noden and Trainor, 2005), whereas the paraxial mesoderm contributes to parietal bones (Yoshida et al., 2008). Thus, the engrailed 1-expressing supraorbital mesenchyme harbors a heterogenous population of cranial bone and dermal precursors that were derived from both the cranial neural crest and paraxial mesoderm.

Canonical Wnt signaling is actively processed in early cranial dermal progenitors during specification

At E11.5, the sub-ectodermal cells of the *En1Cre; R26R* supraorbital domain were processing Wnt signaling as visualized by *Axin2* mRNA expression (Fig. 4A,D,G), nuclear β-catenin localization (see Fig. S2-C in the supplementary material) and Wnt signaling reporter activity (Mani et al., 2009). Simultaneously, the onset of the earliest dermal progenitor marker expression, *Dermo1* (Atit et al., 2006; Li et al., 1995; Ohtola et al., 2008), was also observed in the *En1* lineage-marked cells and *Axin2*-expressing supraorbital mesenchymal cells (Fig. 4A,D,G,J compare white brackets; see Fig. S3 in the supplementary material). At E11.5, the cranial bone progenitors were expressing low levels of the bone precursor marker *Runx2* below the *Dermo1* and *Axin2* expression domain as previous studies suggested (Hill et al., 2005; ten Berge et al., 2008). Thus, the onset of cranial dermal precursor marker expression coincides with the timing of canonical Wnt signal transduction.

Canonical Wnt signaling activity is necessary and sufficient for expression of cranial dermal progenitor marker

To test functionally the role of Wnt signal transduction during the specification of the cranial dermal lineage, we used the *En1Cre* line to conditionally manipulate the Wnt signaling activity levels in supraorbital mesenchyme (Brault et al., 2001; Harada et al., 1999; Kimmel et al., 2000). The low efficiency of the inducible *En1Cre-ER^{T1}* line was not suitable for marker analysis. *En1Cre* efficiently recombined the loss- and gain-of-function floxed alleles of β-catenin in the supraorbital region prior to E11.5, as assayed by *Axin2* mRNA expression and β-catenin protein expression (Fig. 4H,I, compare with 4G; see Fig. S2 in the supplementary material, compare S2D with S2C). In comparison with the *En1Cre; R26R;* β-*catenin$^{flox/+}$* littermate control (Fig. 4G), *Axin2* mRNA and *Dermo1* mRNA expression were completely absent in the *En1* lineage-marked domain in some conditional β-*catenin^{lof}* embryos by E11.5 (Fig. 4B,E,H,K) (n=6/9). However, as the *En1* promoter became active in the supraorbital mesenchyme at E10.5 (Fig. 3B), a few β-*catenin^{lof}* mutant embryos had incomplete recombination of *R26R* at E11.5, and we observed the onset of *Dermo1* expression by whole-mount in situ hybridization (n=3/9, white arrow in Fig. S3D in the supplementary material). By E12.5, all embryos consistently lacked *Dermo1* expression in the supraorbital region (Fig. 5B,F). The facial region has *Dermo1* expression in the control and β-*catenin^{lof}* mutant embryos from E10.5-E14.5 (see Fig. S3 in the supplementary material) and developed dermis by E16.5 (Fig. 6H). At E15.5, Runx2$^+$ cranial bone precursors were still present without Wnt signal transduction (see Fig. S7J,L in the supplementary material). These data demonstrate the lineage-specific requirement of Wnt signal transduction for the *Dermo1* dermal progenitor but not for *Runx2* bone progenitor marker expression.

We also investigated whether the loss of *Dermo1* expression in the conditional β-*catenin*^{lof} mutant could be due to a reduction in cell survival and proliferation prior to E12.5. From TUNEL analysis of both whole mount and sections, there was no perceptible increase in apoptosis in the *En1Cre*; β-*catenin*^{lof} mutant heads and supraorbital mesenchyme from E10.5-E12.5 (see Fig. S4 in the supplementary material). We observed a small but significant decrease in the proliferation of dermal precursors in the supraorbital mesenchyme at E11.5 (see Fig. S5 in the supplementary material). The proliferation index of the control was 41.02±7.15 in comparison with the mutants of 34.01±9.74 (P=0.01). Loss of dermal marker expression in E11.5-E12.5 mutant embryos was probably not due to a small decrease in proliferation. Our data strongly imply a role for Wnt signaling in cranial dermal cell fate specification.

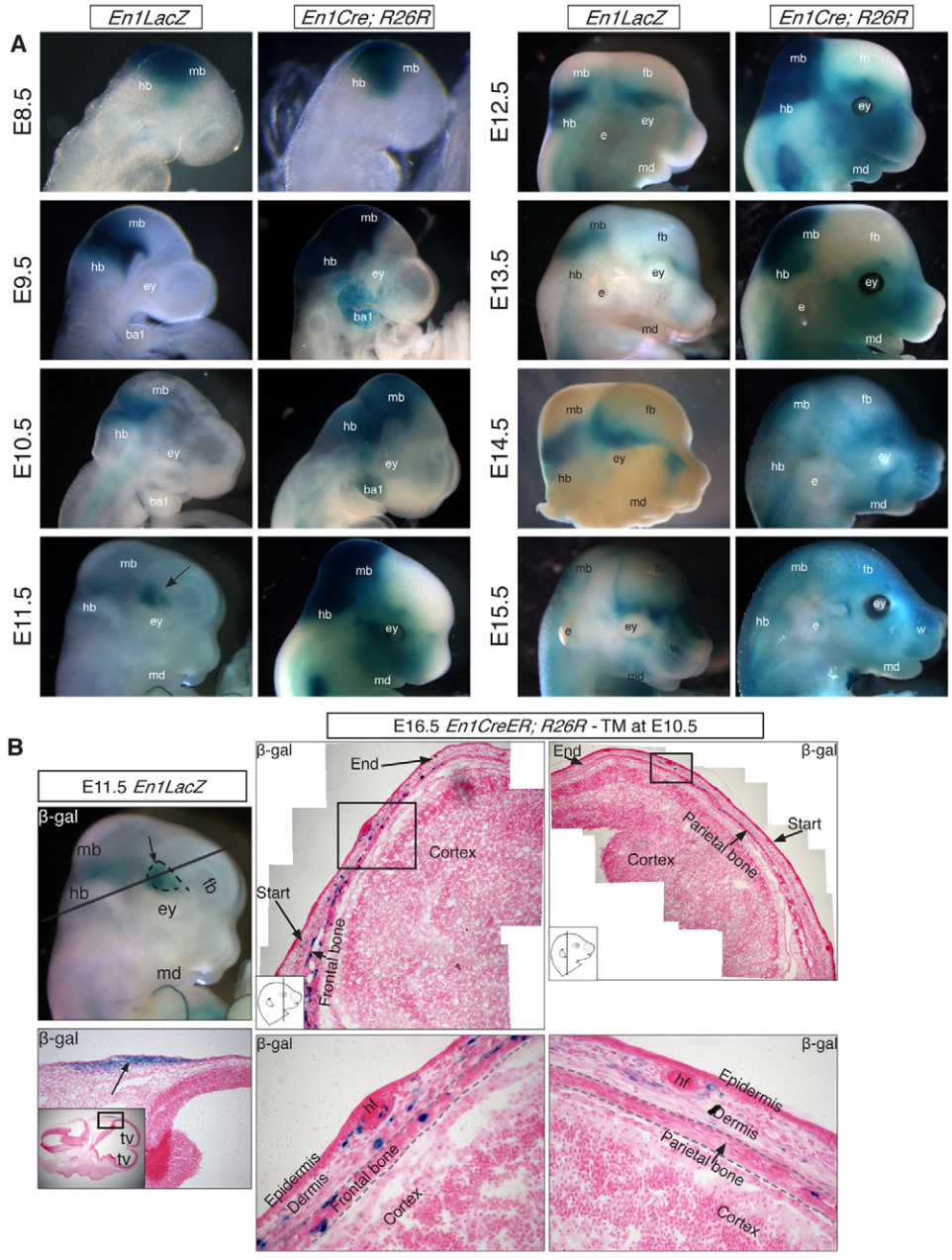

Fig. 3. Expression of engrailed 1 during mouse craniofacial development. (**A**) Endogenous and cumulative expression of engrailed 1 lineage. Whole-mount X-gal stained engrailed 1 *LacZ* (*En1LacZ*) and *En1Cre; R26R* embryos between E9.5-E15.5. *En1LacZ* was visible in the neuroepithelium of the mid-hindbrain region from E8.5-E14.5. At E9.5, *En1Cre* lineage-marked cells from the mid-hindbrain region entered the first branchial arch (ba1). By E11.5, *En1LacZ* expression was seen in the supraorbital region (black arrow) and from E12.5, *En1LacZ* was expressed in the snout. By E15.5, cumulative expression of *En1Cre; R26R* was seen in the whole head. ba, branchial arches; e, ear; ey, eye; fb, forebrain; hb, hindbrain; mb, midbrain; md, mandible; w, whisker. (**B**) Engrailed 1 (*En1*) domain in supraorbital region contributed to cranial dermis and bones of mouse embryos. (Left) X-gal stained whole mount and transverse section of E9.5-E11.5 *En1LacZ* showed that the supraorbital region was labeled by *En1* from E10.5-E11.5 (middle and right). *En1Cre-ER; R26R* lineage-labeled cells from E10.75-E11.75 contributed to the cranial dermis and underlying frontal (middle) and parietal (right) bones at E16.5. TM, tamoxifen; tv, telencephalic vesicle.

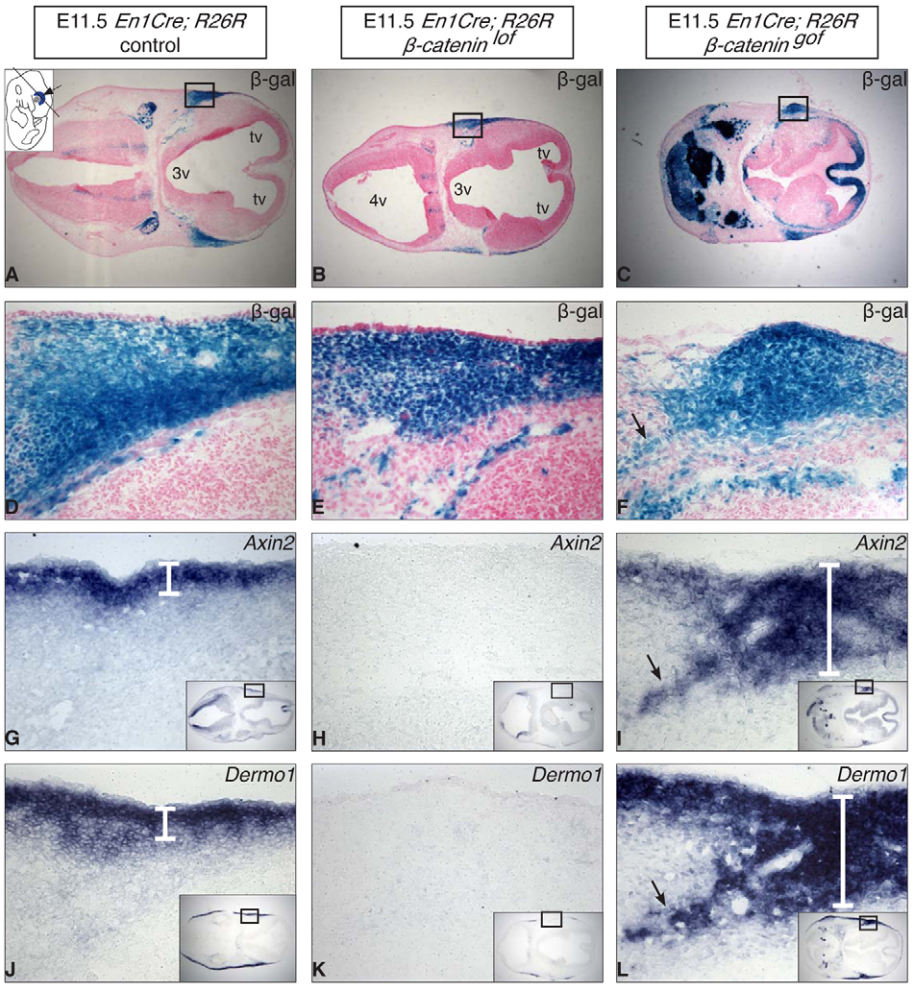

Fig. 4. Wnt signaling is necessary and sufficient for dermal specification of cranial dermis at E11.5. Comparison between *En1Cre; R26R* control (β-*catenin*$^{flox/+}$), loss-of-function (β-*catenin*$^{flox/null}$) and gain-of-function (β-*catenin*$^{flox3/+}$) mutant embryos. (**A-F**) X-gal stained transverse sections of the supraorbital region at E11.5 (**G-I**) Section in situ hybridization of Wnt-responsive *Axin2* mRNA and (**J-L**) the dermal progenitor marker (*Dermo1*) mRNA expression. Wnt signaling activity levels affect *Axin2* and *Dermo1* mRNA expression. In the gain-of-function mutants, there were both ectopic (arrows in F, I, L) and expanded domains of *En1CreRR*, *Axin2* and *Dermo1*$^{+}$ cells (compare white brackets in G and J with I and L). 3v, third ventricle; 4v, fourth ventricle; tv, telencephalic vesicle; lof, loss-of-function; gof, gain-of-function.

We next tested whether forced activation of Wnt signal transduction in the *En1* lineage was sufficient to promote dermal cell fate identity in the supraorbital region. A constitutively active form of β-catenin was conditionally generated by *En1Cre*-mediated recombination for the complementary β-catenin gain-of-function (β-*catenin*^{gof}) analysis in the embryo (Harada et al., 1999). Whole-mount in situ showed ectopic expression of *Dermo1* as early as E10.5 in the β-*catenin*^{gof} mutants in the mid-hindbrain region (red arrows in Fig. S3 in the supplementary material). In the supraorbital region of conditional β-*catenin*^{gof} mutants, β-gal^{+} cells were all processing Wnt signaling activity, resulting in a much broader expression domain of *Axin2* and *Dermo1* (Fig. 4, compare white brackets in F,I,L with those in D,G,J). Unfortunately, owing to embryonic lethality at E12.5, the β-*catenin*^{gof} mutants were not available for subsequent analysis of dermal development. Thus, conditional activation of Wnt signal transduction was sufficient for *Dermo1* marker expression in supraorbital mesenchyme and ectopically in other *En1Cre*-expressing cells.

Absence of Wnt signaling leads to loss of cranial dermal cell fate specification and gain of cartilage cell fate

Next, we determined whether the β-*catenin*^{lof} mutant cells in the supraorbital region became specified to the cartilage fate in the absence of the Wnt signaling. Sox9 is the earliest cartilage cell fate marker (Zhao et al., 1997; Lefebvre et al., 1997). In control embryos at E12.5, we found endogenous Sox9^{+} cells below the *Dermo1* domain and away from the surface ectoderm (Fig. 5E,G). Compared with the control, in the conditional β-*catenin*^{lof} mutant, we found β-gal^{+} cells with altered morphology (Fig. 5D, compare with 5C) that expressed Sox9 below the ectoderm (Fig. 5H, arrow). Similar to E11.5, *Dermo1* expression was absent in the sub-ectodermal cells of the supraorbital region at E12.5 (Fig. 4K, Fig. 5B,F).

We also examined the facial region that had mosaic distribution of *En1Cre* lineage-labeled cells under the surface ectoderm that was not adjacent to any endogenous population of bone and cartilage progenitors. In the control embryos, Sox9 expression or cartilage morphology tissue was absent under the facial surface ectoderm at E12.5 and E16.5 (Fig. 6A,C,E,G,I). Consistent with the mutant phenotype in the supraorbital region, we found *En1Cre* lineage-marked ectopic Sox9^{+} clusters of cells that were beneath the surface ectoderm of the mandible and maxilla at E12.5 (Fig. 6B,D,F). By E16.5, we found morphologically clear cartilage nodules with *En1* lineage-marked cells in the mutant embryos (Fig. 6H,J).

At E10.5, *En1Cre* is also expressed in the ventral body wall mesenchyme that gives rise to ventral dermis. Absence of Wnt signal transduction in the ventral body wall mesenchyme resulted in loss of dermis and expanded sternum (Ohtola et al., 2008). Here, we found that E11.5 β-*catenin*^{lof} mutant cells under the ventral trunk ectoderm also became Sox9^{+} in the absence of dermal specification (see Fig. S6 in the supplementary material). Thus, in

different populations of dermal progenitors in the embryo, Wnt signaling was required at the appropriate time to induce dermal cell fate and repress a cartilage cell fate program.

Dermo1 represses *Sox9* in undifferentiated mesenchymal cells

Dermo1 can function as a transcriptional repressor (Gong and Li, 2002; Lee et al., 2003), suggesting it is a candidate target factor mediating the repression of *Sox9* in cranial and trunk dermal precursors. To test this directly, we transfected an expression vector for mouse *Dermo1* in the C3H/10T1/2 mesenchymal cell line under non-differentiating conditions. *Dermo1* suppressed the expression of *Sox9* mRNA as assessed by quantitative real-time PCR relative to mock-transfected cells (P=0.03) (Fig. 5I). Thus, *Dermo1* may mediate some of the effects of Wnt signaling/β-catenin in suppressing the cartilage cell fate in skull and trunk dermal precursors.

Potential enhancers upstream of *Dermo1* are bound by Tcf4

The mechanism by which *Dermo1* is regulated in undifferentiated mesenchyme or dermal precursors is unknown. *Dermo1* mRNA expression correlates with β-catenin activity levels in cranial and trunk dermal precursors (Fig. 4) (Atit et al., 2006; Ohtola et al., 2008). Several Tcf/Lef-binding motifs are located near or within *Dermo1* (Fig. 7A,B, see Table S1 in the supplementary material, data not shown), raising the possibility that β-catenin directly regulates transcription of *Dermo1*. Additionally, several Tcf4 enhancers at the *Dermo1* locus were predicted using Enhancer Element Locator program (Hallikas et al., 2006; Palin et al., 2006). We tested whether β-catenin or Tcf4 associated with the *Dermo1* locus in vivo using chromatin immunoprecipitation (ChIP) on E12.5 mouse trunk mesenchyme. We found some sites with modest enrichment in β-catenin ChIP (data not shown), and the same sites were tested in Tcf4 ChIP. Substantial enrichment of Tcf4 was detected at the predicted Tcf/Lef-binding motifs located 1.2 kb upstream of *Dermo1* (Fig. 7B, left). The Tcf4 motif in the non-conserved –2.2 kb region showed less enrichment than these –1.2 kb sites. We next performed ChIP at the *Dermo1* locus using antibodies to mono-methylated lysine 4 of histone H3 (H3K4me1), a histone modification that is well known to coincide with the location of tissue-specific gene enhancer elements (Benko et al., 2009; Heintzman et al., 2009; Heintzman et al., 2007; Kim et al., 2010; Schnetz et al., 2009; Won et al., 2008). The highest enrichment was detectable at the highly conserved Tcf/Lef sites in the –1.2 kb region (Fig. 7B, right). In vitro luciferase reporter assay with the promoter containing the –1.2 kb Tcf/Lef motifs confirmed the β-catenin responsiveness of this putative enhancer (Fig. 7C). Addition of the non-conserved –2.2 kb site did not increase the Wnt responsive activity (data not shown).

Together with the in silico enhancer predictions, the ChIP and luciferase assay results suggest that the region located 1.2 kb upstream of *Dermo1* is a β-catenin responsive enhancer element in dermal precursors. However, further studies are clearly required to assess the regulatory potential of this region and to determine whether this directly controls *Dermo1* expression by itself, or in combination with other Tcf sites, such as the –2.2kb site [which was also enriched in H3K4me1 ChIP (Fig. 7B)].

DISCUSSION

During vertebrate head development, different cell lineages arise in close proximity from mutipotential cranial neural crest and paraxial mesoderm precursors. Here, we use genetic lineage analysis and functional studies to dissect the spatio-temporal role of Wnt signal transduction in the formation of the cranial dermal lineage from a heterogenous population of progenitors from mixed origins. We find that Wnt/β-catenin signaling has an instructive role in cranial dermal cell fate selection. This function is conserved in

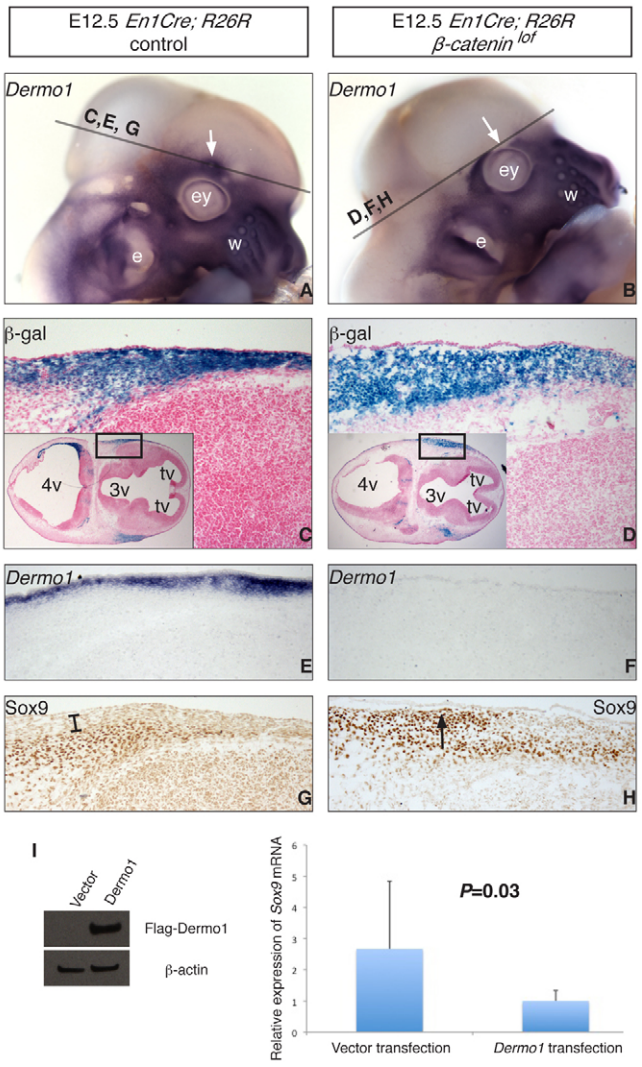

Fig. 5. Changes of early marker expression in sub-ectodermal cells in the absence of Wnt signaling/β-catenin at E12.5. (**A**,**B**) Whole-mount in situ of *Dermo1* mRNA. (**E**,**F**) Section in situ of *Dermo1* mRNA and (**G**,**H**) cartilage cell marker Sox9 expression on transverse sections. (**C**,**D**) X-gal stained alternate sections of E,F to visualize the *En1*-lineage-labeled cells in control (*En1Cre; R26R;* β-*catenin*$^{flox/+}$) and mutant (*En1Cre; R26R;* β-*catenin*$^{flox/null}$). (F) *Dermo1* mRNA expression was absent in the sub-ectodermal cells of the mutant. Sox9 expression was seen directly underneath the surface ectoderm of the mutants (compare H with G). β-Gal positive cells have different morphology in the mutants (D) compared with the controls (C). (**I**) (Left) Western blot with anti-Flag antibody showed expression of the Flag-Dermo1 protein (~18 kDa) in transfection with *pcDNA3-Dermo1-Flag* only but not with the mock vector *pcDNA3-5′UT-Flag*. (Right) Transient overexpression of *Dermo1* in C3H/10T1/2 cells led to a 2.6-fold decrease in *Sox9* expression (P=0.03 using Student's *t*-test analysis). Results were from four independent experiments, each performed in triplicate. *Sox9* mRNA level was normalized to β-*actin* mRNA level in each sample. 3v, third ventricle; 4v, fourth ventricle; e, ear; ey, eye; tv, telencephalic vesicle; w, whisker.

specifying the trunk dermis from somatic and lateral plate mesoderm. Our results reveal that without the Wnt signaling cue, a cartilage cell fate is prompted at the expense of dermal and bone lineages. We have identified *Dermo1* as a mediator of Wnt signaling in specifying dermal cell fate and suppressing the cartilage cell fate.

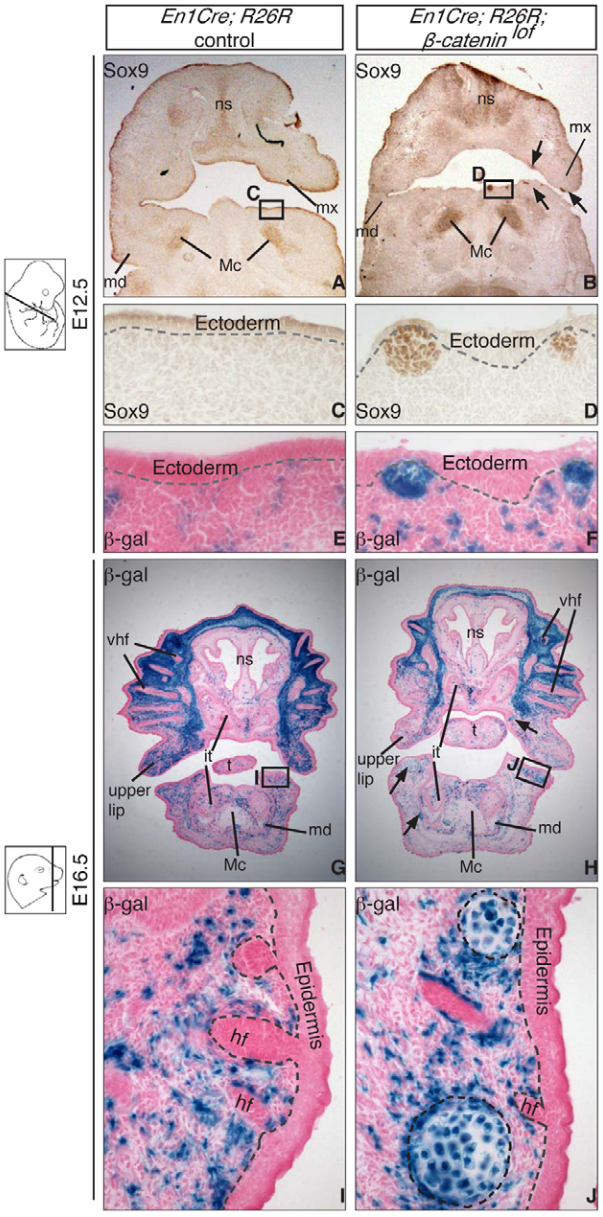

Fig. 6. Ectopic cartilage formation in the face of Wnt signaling/β-catenin loss-of-function mutants. (**A-F**) Transverse sections of E12.5 embryos stained for Sox9 (A-D) and X-gal (E,F). (**G-J**) Coronal sections of E16.5 embryos in the mandibular region stained with X-gal. At E12.5, clusters of *En1Cre* lineage-labeled cells (F) that were Sox9^{+} (D, arrows in B) were found under the ectoderm of the mandibular process in the β-*catenin*^{lof} mutants compared with the littermate control (A,C). At E16.5, morphologically distinct ectopic cartilage nodules were seen in dermal regions directly underneath the epidermis in the lower jaw (J, arrows in H). hf, hair follicle; it, incisor tooth; Mc, Markel's precartilage mass (E12.5) or cartilage (E16.5); md, mandibular process (E12.5) or mandible (E16.5); mx, maxillary process (E12.5) or maxilla (E16.5); ns, nasal septum; t, tongue; vhf, vibrissae hair follicle.

The supraorbital mesenchyme contains some cranial bone and dermal precursors

Genetic studies of craniofacial dermal development in the mammalian embryo have not been accomplished previously owing to the lack of tissue-specific reagents and markers of differentiation. In this study, we use the *En1Cre-ER*^{T1} line for genetic fate mapping and the more robust *En1Cre* line to efficiently manipulate the cranial dermal precursors (Kimmel et al., 2000; Sgaier et al., 2005). We genetically fate-map the supraorbital mesenchyme domain expressing *En1* with the inducible *En1Cre-ER*^{T1}; *R26R* mice and find contribution of β-gal^{+} cells to the cranial dermis and the underlying frontal and parietal plates. Significant contribution is seen in both cranial plates, thereby confirming that the supraorbital mesenchyme expressing *En1Cre-ER* contains cells from both the cranial neural crest and paraxial mesoderm. Our genetic fate map demonstrates that the supraorbital mesenchyme is one region containing a heterogenous population of cranial bone and dermal progenitors that migrate rostrocaudally and anteriorly to form the cranial dermis and underlying bones. However, the *En1Cre-ER*^{T1} line cannot distinguish if the supraorbital mesenchyme contains two functionally distinct lineages or a bipotential osteo-dermal precursor.

Instructive role of Wnt signaling/β-catenin in cranial dermal cell fate selection in the supraorbital mesenchyme via *Dermo1*

Our newly characterized genetic tools have enabled us to circumvent embryonic lethality and functionally manipulate the progenitors to identify an instructive role for Wnt/β-catenin signaling in early cranial dermal development (Brault et al., 2001). During cranial dermal fate selection between ~E11.0-E12.5 in the supraorbital mesenchyme, the canonical Wnt signaling activity reporter and the Wnt responsive *Axin2* mRNA are transiently expressed, which is indicative of an instructive role for Wnt signaling. Without Wnt signal transduction in these cells, the cranial dermis is absent. Instead, there are *En1* lineage-labeled cartilage cells in the place of cranial dermis and bone. By contrast, the snout and lower jaw regions have lineage-labeled cells and intact dermis because the onset of dermal specification at E10.5 precedes the timing of β-catenin recombination at ~E11.5 as monitored by R26R activity. Interestingly, a few sub-ectodermal *En1Cre* lineage-marked cells in the lower jaw region that lack β-catenin before dermal specification become Sox9^{+} and form cartilage nodules. These results are consistent with the timing of the instructive role of Wnt signaling/β-catenin for dermal specification in the dorsal trunk dermis (Atit et al., 2006). Our studies on cranial dermis demonstrate that regardless of the ectoderm or mesoderm embryonic germ layer origin, canonical Wnt signal transduction via β-catenin is a consistent requirement for dermal specification in the embryo.

We have found that *Dermo1* mRNA expression also serves as the earliest marker for cranial dermal cell fate specification in the sub-ectodermal cells of the supraorbital region. Our functional studies demonstrate that Wnt signaling/β-catenin is necessary and sufficient for *Dermo1* expression in dermal precursors (Fig. 4) (Atit et al., 2006; Ohtola et al., 2008). The identification of β-catenin responsive enhancer elements upstream of *Dermo1* strongly suggests that *Dermo1* is a transcriptional target of Wnt signaling/β-catenin at E12.5. Based on these findings, we hypothesize that the Tcf/Lef binding sites in the –1.2 kb region may be a putative 'dermal' enhancer that appears to be transcriptionally active in a

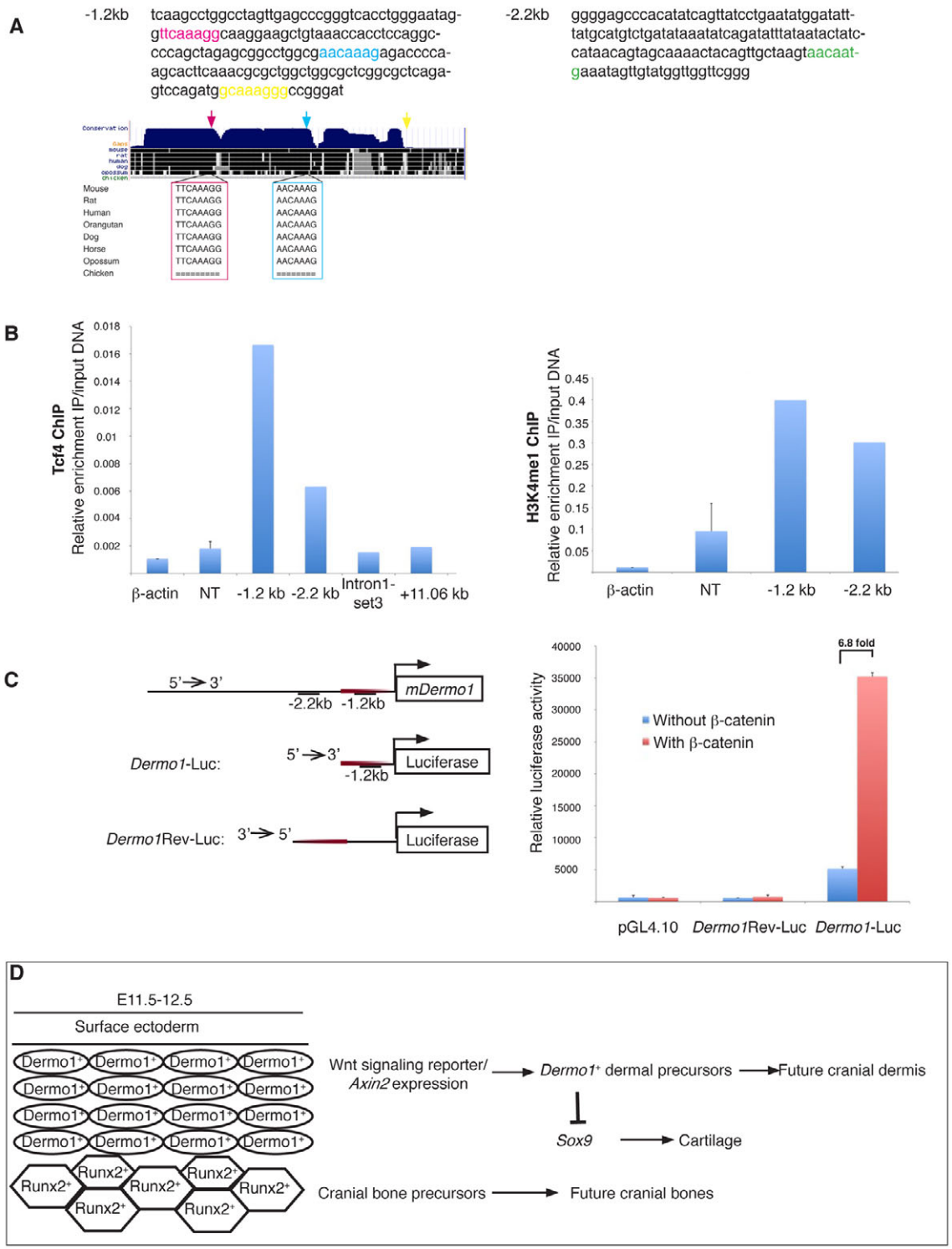

Fig. 7. *Dermo1* as a mediator of Wnt signaling/β-catenin in dermal precursors. (**A**) (Left) A highly conserved region near 1.2 kb upstream of *Dermo1* transcription start site contains a cluster of three consensus Tcf/Lef-binding sites. Two of the consensus Tcf/Lef-binding sites (magenta and blue) were conserved across mammalian species (adapted from UCSC Genome browser). (Right) The –2.2 kb site contains a putative Tcf/Lef-binding site (green) and is not conserved. (**B**) Tcf4 and H3K4me1 chromatin immunoprecipitation (ChIP) show enrichment at the –1.2 kb and –2.2 kb Tcf/Lef motifs compared with β-actin (16-fold enrichment in Tcf4 ChIP and 34-fold enrichment in H3K4me1 ChIP for –1.2 kb; 6 and 26-fold enrichment for –2.2 kb site, respectively) or nearby non-target sites (ninefold enrichment in Tcf4 ChIP and fourfold enrichment in H3K4me1 ChIP for –1.2 kb; four and threefold enrichment for –2.2 kb site, respectively). (**C**) Schematic of the luciferase reporter plasmids used in the luciferase assay. *Dermo1* promoter element with –1.2 kb Tcf/Lef sites had substantial transactivation in the presence of wild-type β-catenin. The negative controls (empty pGL4.10 vector and the reverse construct *Dermo1*Rev-Luc) showed comparable luciferase activity with and without β-catenin transactivation. (**D**) Proposed model for the role of Wnt signaling/β-catenin in the selection of cranial dermal cell fate. IP, immunoprecipitated; NT, non-target.

stage-dependant and tissue-specific manner in dermal precursors. Future studies will focus on testing the function of the –1.2 kb region and other putative enhancers by genetic deletion in transgenic mouse embryos.

Role of Wnt signaling/β-catenin in suppressing cartilage cell fate of cranial bone and dermal progenitors

Sox9 and β-catenin proteins may reciprocally inhibit each other to regulate in vivo cartilage differentiation during long bone development (Akiyama et al., 2004; Hartmann, 2007). Previous studies conditionally eliminated Wnt signaling early in the cranial skeletal mesenchyme and reported loss of cranial bone ossification with an ectopic cartilage phenotype (Day et al., 2005; Hill et al., 2005). However, these studies did not analyze the mutant embryos at the time of cranial bone or dermal fate selection. The molecular mechanism of how Wnt signaling functionally mediates the repression of *Sox9* and cartilage cell fate in vivo remains unknown.

Our data from the conditional loss of Wnt/β-catenin signaling mutant demonstrate an absence of the dermal precursor marker expression and an expansion of the cartilage domain between E11.5-E12.5 in the head, face and ventral trunk regions. The *En1Cre* conditional β-*catenin*^{lof} mutant cells in the supraorbital mesenchyme fail to express the dermal progenitor marker *Dermo1* between E11.5 and E12.5. As early as E11.5, these sub-ectodermal cells are morphologically different and expressing the cartilage marker Sox9. We also find ectopic Sox9 expression in a small population of lineage-labeled conditional β-*catenin*^{lof} mutant cells under the ectoderm away from endogenous cartilage tissue in the jaw and ventral trunk region. Thus, in the absence of the Wnt signaling cue, the sub-ectodermal cells fail to express *Dermo1* and instead express *Sox9* directly in distinct regions of the embryo. We also find that *Dermo1* expression leads to decrease in *Sox9* mRNA expression in undifferentiated mesenchymal cells. These data suggest *Dermo1* may mediate Wnt signaling to directly or indirectly decrease *Sox9* mRNA expression and inhibit the cartilage cell fate in dermal precursors.

A model of cranial dermal cell fate determination from a population of cranial mesenchyme

Our data lead to a model whereby Wnt signaling has a key role in determining the fate of supraorbital mesenchymal cells (Fig. 7D). In normal development, the sub-ectodermal cells in this region express *Dermo1* and become cranial dermal cells, whereas deeper cells express Runx2 and become osteo-progenitors. The sub-ectodermal cells process Wnt signaling between E11.5 and E12.5. High levels of Wnt signaling via *Dermo1* suppress *Sox9* expression and any cartilage cell differentiation in these cranial dermal progenitor cells. In the absence of Wnt signaling/β-catenin at the time of cell specification, *Dermo1* expression is lost and repression of *Sox9* is removed in cells that were originally dermal precursor cells. These cells subsequently execute the cartilage program to replace the dermis layer under the surface ectoderm. By contrast, early bone precursors do not seem to require Wnt signaling/β-catenin for *Runx2* expression until E15.5 and the lack of mineralized bone suggests a later role for differentiation into functional osteoblasts.

Our study on early cranial dermal development highlights a consistent role for Wnt/β-catenin signaling via *Dermo1* in dermal specification in different regions of the embryo. A recent published study has shown that *Dermo1* mutations are linked to the focal facial dermal dysplasia disorder (Tukel et al., 2010), which suggests future analysis on *Dermo1* as an important dermal target gene, especially for craniofacial region. Future in vivo genetic analysis of Wnt signaling responsive enhancers of genes expressed in dermal precursors will allow us to identify the transcriptional regulation of Wnt target genes. Identification of dermal-specific promoters and enhancers will enable us to define the genetic program for cranial dermis development. Altogether, these studies will have great implications for wound healing and skin tissue engineering.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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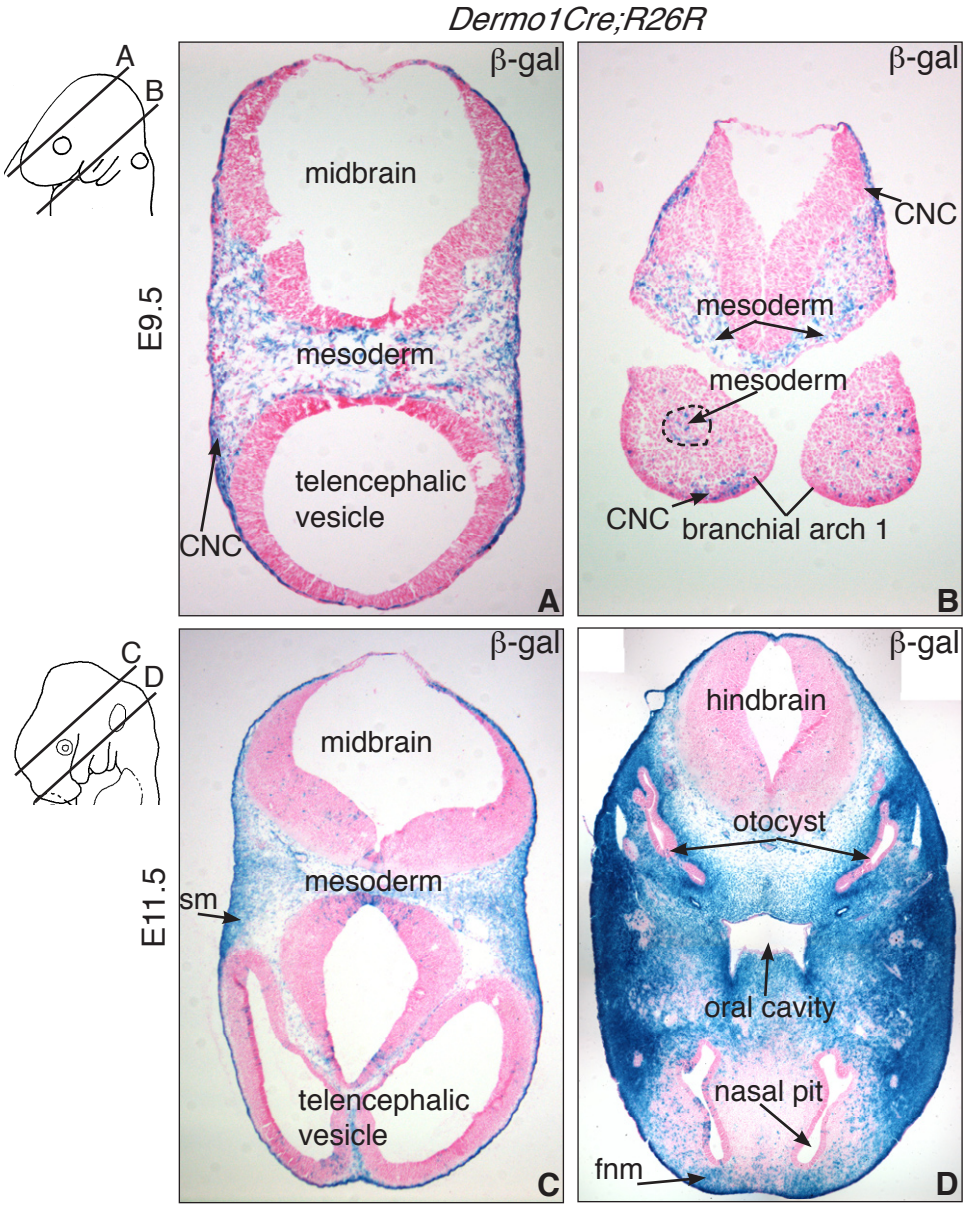
Dermo1Cre;R26R
A
B
E9.5
β-gal
midbrain
mesoderm
telencephalic vesicle
CNC
A
β-gal
CNC
mesoderm
mesoderm
CNC
branchial arch 1
B
C
D
E11.5
β-gal
midbrain
mesoderm
sm
telencephalic vesicle
C
β-gal
hindbrain
otocyst
oral cavity
nasal pit
fnm
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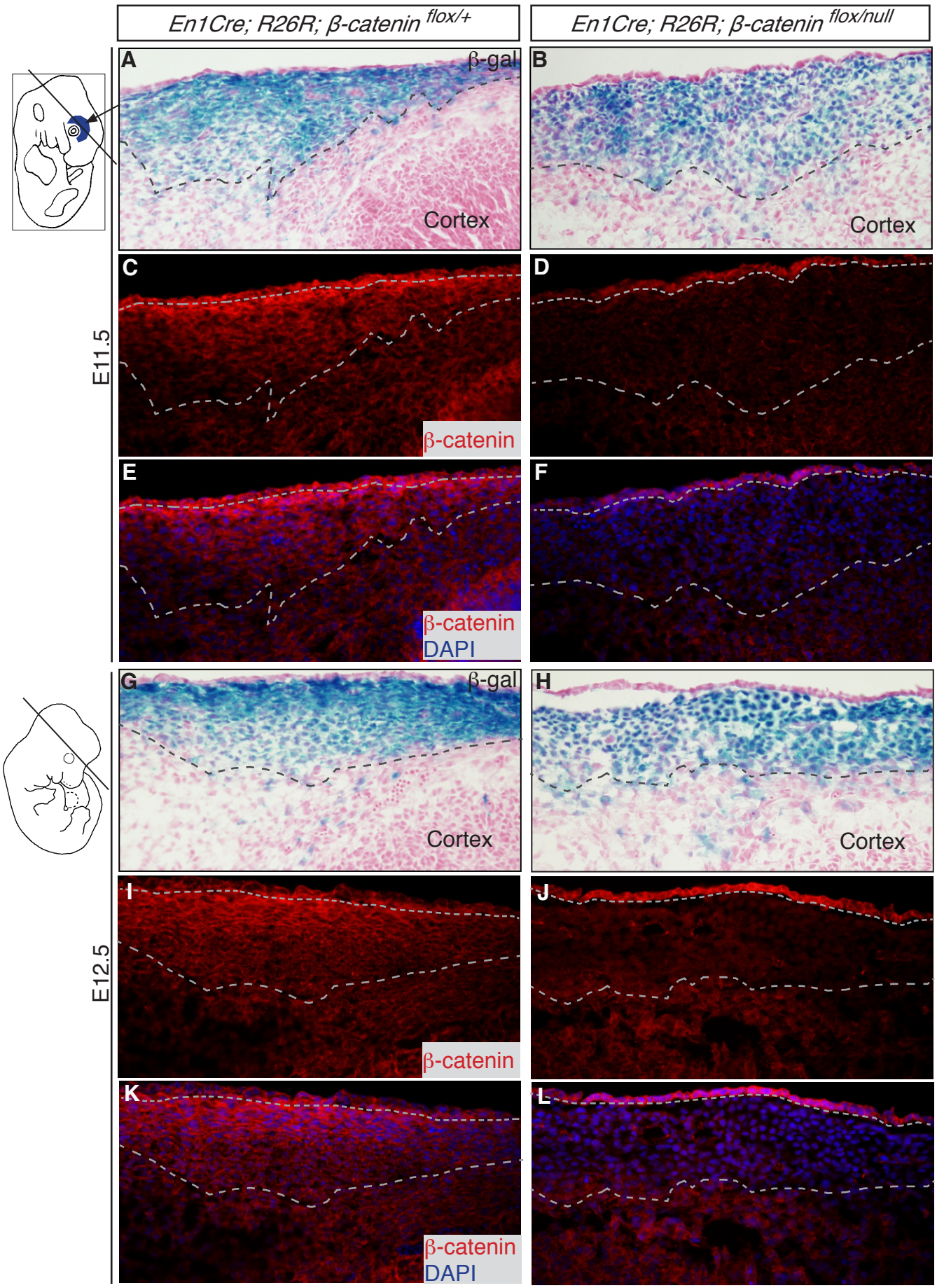
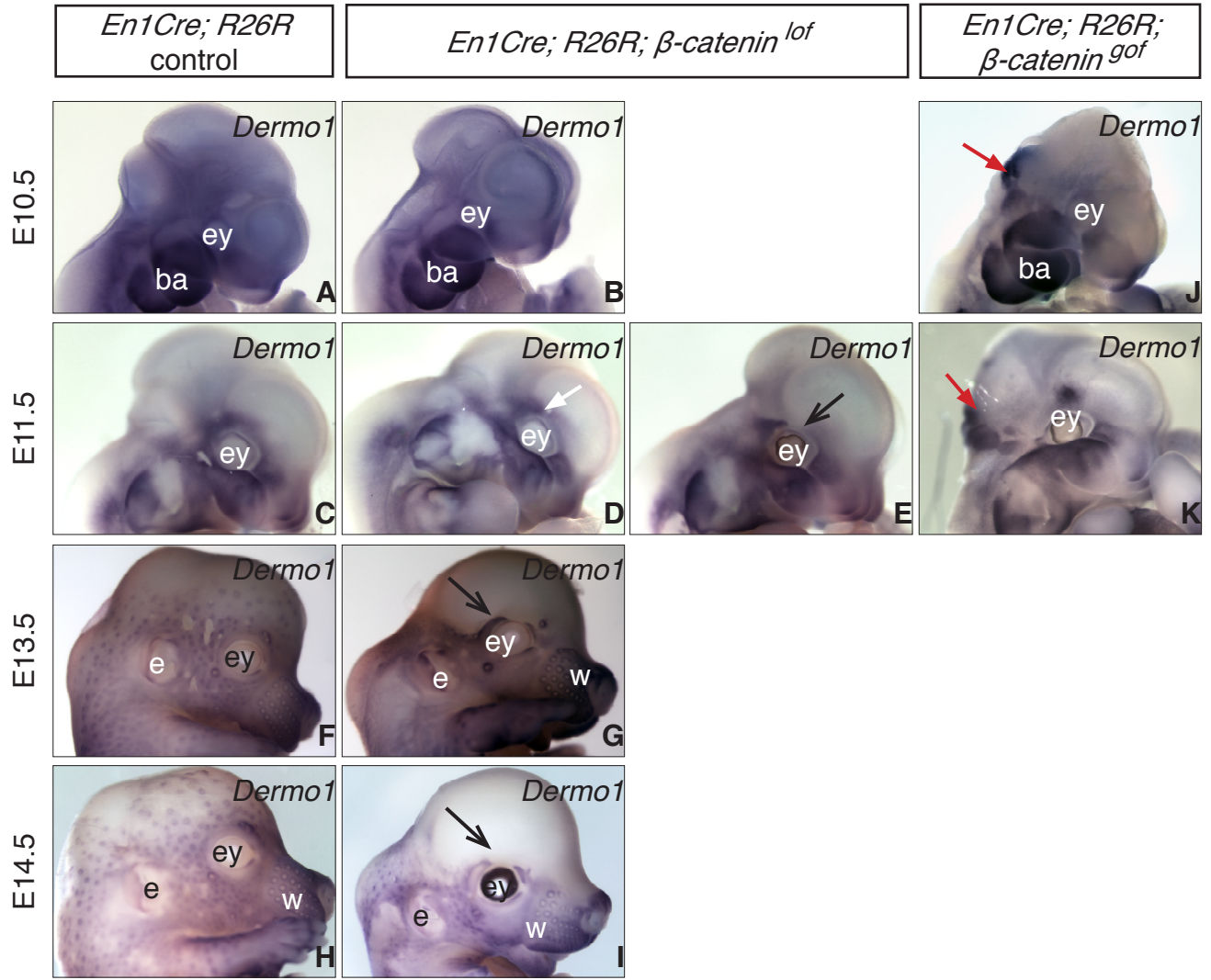
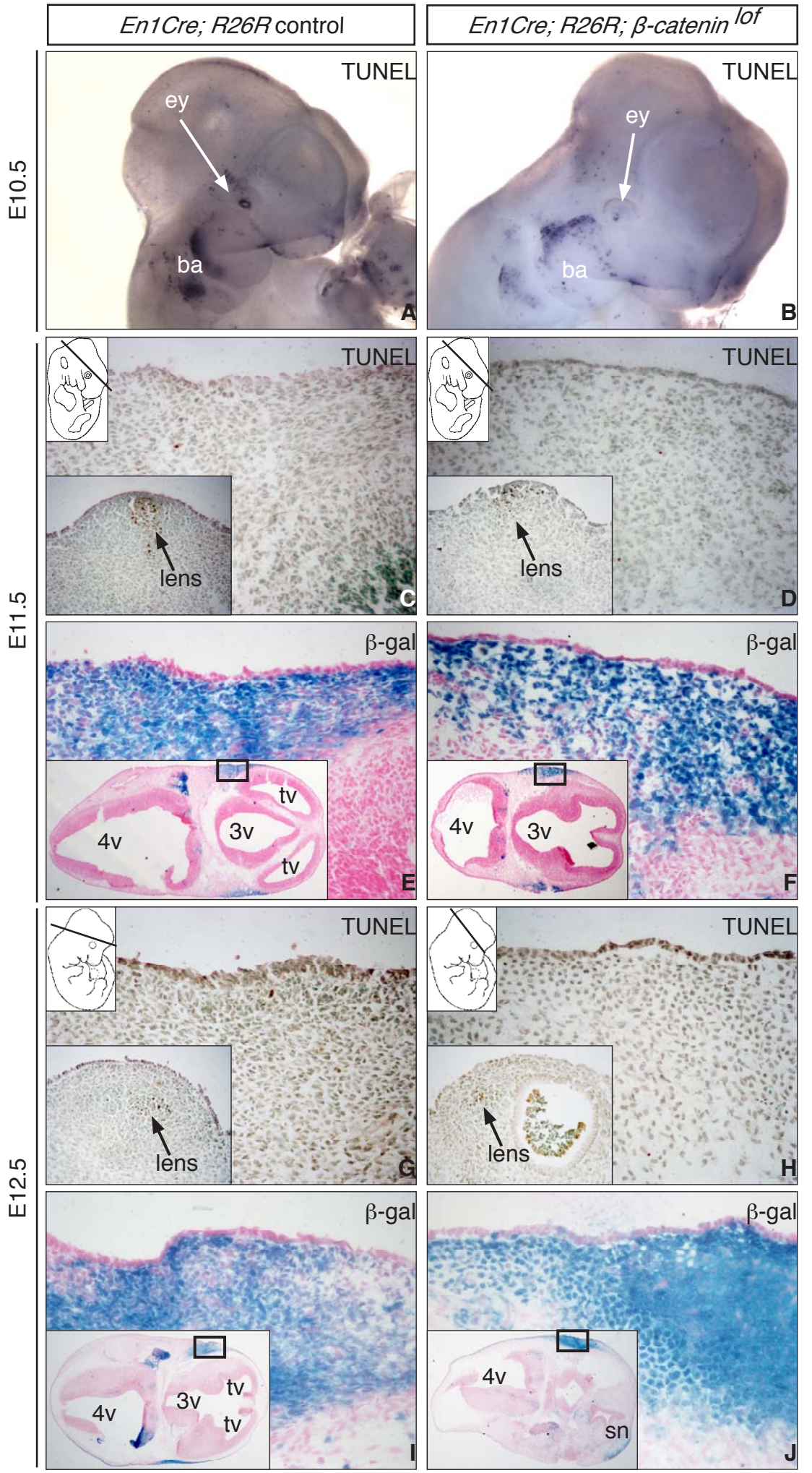

En1Cre; R26R; β-catenin flox/+
En1Cre; R26R; β-catenin flox/null
A
β-gal
Cortex
B
Cortex
C
β-catenin
D
E
β-catenin
DAPI
F
E11.5
G
β-gal
Cortex
H
Cortex
I
β-catenin
J
K
β-catenin
DAPI
L
E12.5


En1Cre; R26R
control
En1Cre; R26R; β-catenin lof
En1Cre; R26R;
β-catenin gof
E10.5
E11.5
E13.5
E14.5
Dermo1
ey
ba
e
w
A
B
C
D
E
F
G
H
I
J
K


En1Cre; R26R control
En1Cre; R26R; β-catenin lof
E10.5
E11.5
E12.5
TUNEL
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ba
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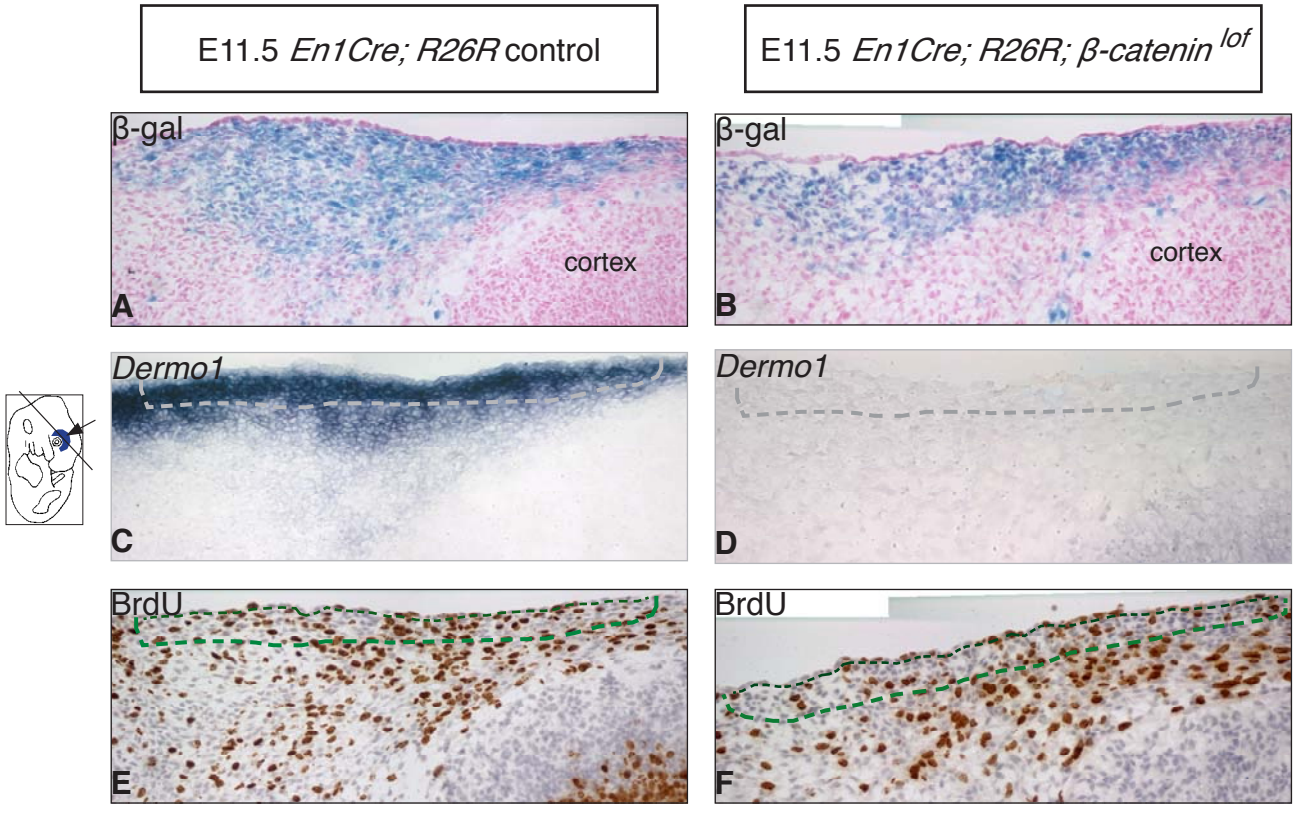
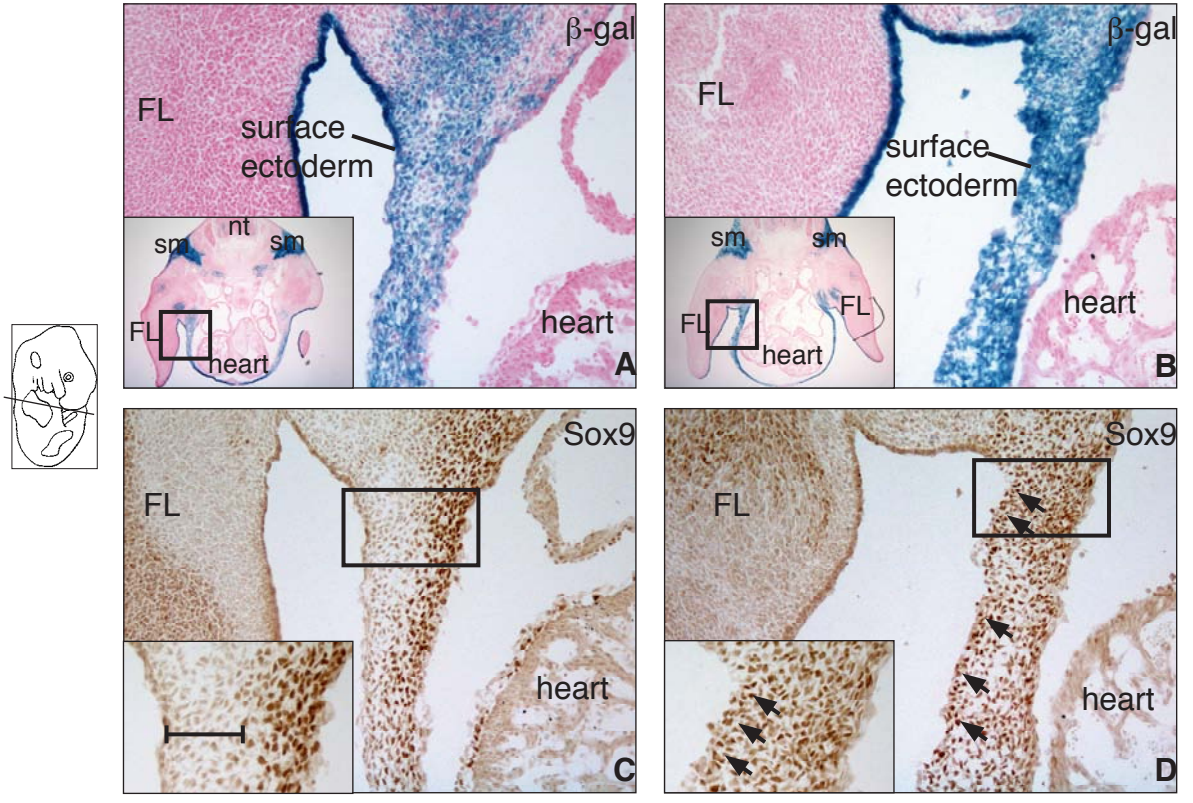
B
lens
C
D
β-gal
tv
3v
4v
E
F
G
H
I
J
sn


E11.5 *En1Cre; R26R* control
E11.5 *En1Cre; R26R; β-catenin* ^{lof}
β-gal
cortex
A
β-gal
cortex
B
Dermo1
C
Dermo1
D
BrdU
E
BrdU
F


E11.5 En1Cre; R26R
control
E11.5 En1Cre; R26R
β-catenin lof
β-gal
FL
surface
ectoderm
nt
sm
sm
FL
heart
heart
A
β-gal
FL
surface
ectoderm
sm
sm
FL
FL
heart
heart
B
Sox9
FL
heart
C
Sox9
FL
heart
D

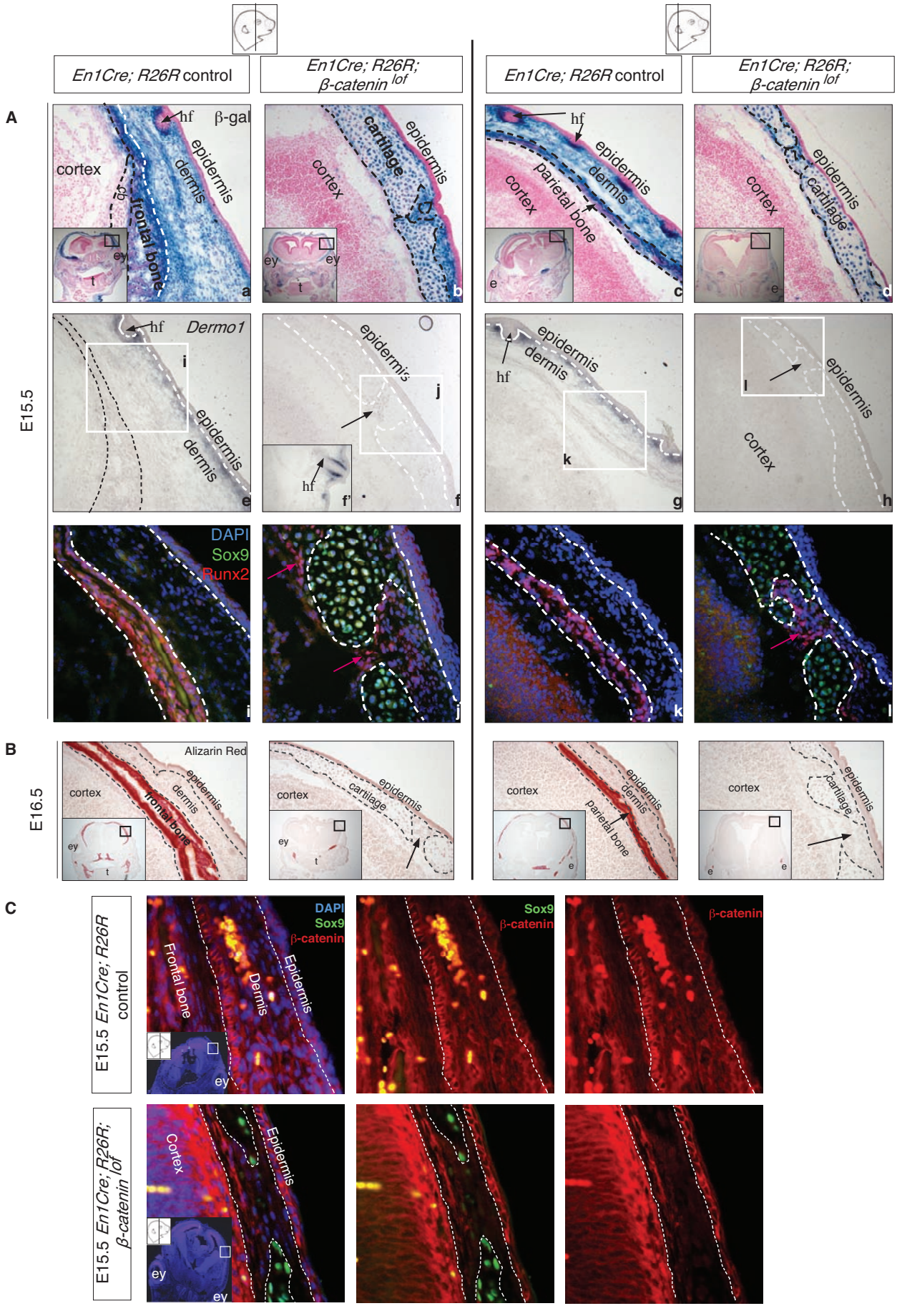

A
En1Cre; R26R control
En1Cre; R26R; β-catenin lof
En1Cre; R26R control
En1Cre; R26R; β-catenin lof
E15.5
hf
β-gal
cortex
epidermis
dermis
frontal bone
cartilage
parietal bone
Dermo1
DAPI
Sox9
Runx2
B
E16.5
Alizarin Red
C
E15.5 En1Cre; R26R control
E15.5 En1Cre; R26R; β-catenin lof
Frontal bone
Dermis
Epidermis
Cortex
β-catenin
ey
t
e

Table S1. Primer sequences

		Forward primer (5′-3′)	Reverse primer (5′-3′)
ChIP primers	β-Actin	GTACTAGCCACGAGAGAGCGAAG	GTTCCGAAAGTTGCCTTTTATG
	NT-61kb	GGGTCCAAGGTTGTTCTTCA	TAAATTCTGTGCACCCCCAT
	NT-54kb	CTTTTCTTGGGCTCTCGTTG	TCCTCATCCTCCTTGACCAC
	NT-27kb	CCCCAGCATCGTGTCTTATT	ATTGCTGTGTGTGCCTGTGT
	NT-26kb	CCATTCCTGACCATCATTCC	GAGAAACCCACCTCCATTCA
	NT-19kb	TGGATACCTCTTGGAACTGGA	AGTGCTGTCTGAGGCTGAGG
	NT-9kb	GCCATAAAAGTGGACCCTCA	TATTTGCTTGGTCCCAGGTG
	NT-4kb	GCTTGGGCATGACTCTATCC	GAAGCATATGAGCTCTGGCC
	NT-3kb	TGCTTCTTTGCCTCTCTCAA	ACAGGTAGGCTCAGTGCACC
	1.2 kb	TCAAGCCTGGCCTAGTTGAG	GCCTTGGTGAACAGACATCA
			AGGCCTTGGTGAACAGACAT
	–1.6 kb	AGGAAGCTGGGATCTGGATG	TGGGAAGGCCACATTTTATC
	–2.2 kb	GGGGAGCCCACATATCAGT	CCCGAACCAACCATACAACT
	–3.1kb	GTCCCAGCATTGAAGACCAT	TGCTCTCTTTCAGCCAATGA
	–3.6 kb	CTGCTGTGCAAATCACTGGT	AACAAAAACGGGATCTGTGG
	Intron1_set1	CTGAGGCACAGTGGGATTTT	CATGCTAGCCTCTCCAGCTT
	Intron1_set2	ATGCTCAGAAGCAGCCTAGC	ATTCTGCAACCCAAAAGCAG
	Intron1_set3	CTGAGGCACAGTGGGATTTT	TGTTTTCAAGAAGGGCTGGA
	+11.06 kb	GACTGAAGCCCTCCAGATCA	AGGGTAAGCTGGTCCATGTG
Sub-cloning primers	Dermo1-Luc	GACCTCGAGCAGTCTCTGGCCTCAGGTTC	TATAAGCTTGGCGCCCGCCCGGCGCGCGTGGGGCT
	Dermo1Rev-Luc	TATAAGCTTATCCCGGTCCTAACATAGGG	GATCTCGAGGGCGCCCGCCCGGCGCGCGTGGGGCT
qRT-PCR primers	β-Actin	AGGCCAACCGCGAAGATGACC	GAAGTCCAGGGCGACGTAGCAC
	Sox9	TCCACGAAGGGTCTCTTCTC	AGGAAGCTGGCAGACCAGTA

List of primers used in ChIP, sub-cloning and quantitative Real-time PCR. The primers for sub-cloning had restriction enzyme recognition sites (underlined) to clone the PCR products into *Xho*I-*Hind*III sites of the vector pGL4.10. ChIP, chromatin immunoprecipitation; NT, non-target.