Distinct populations within *Isl1* lineages contribute to appendicular and facial skeletogenesis through the β-catenin pathway

Ryutaro Akiyama\textsuperscript{a,b}, Hiroko Kawakami\textsuperscript{a,b}, M. Mark Taketo\textsuperscript{c}, Sylvia M. Evans\textsuperscript{d}, Naoyuki Wada\textsuperscript{e}, Anna Petryka\textsuperscript{a,f,g}, Yasuhiko Kawakami\textsuperscript{a,b,g,h,*}

\textsuperscript{a} Department of Genetics, Cell Biology and Development, University of Minnesota, 321 Church Street SE, Minneapolis, MN 55455, USA
\textsuperscript{b} Stem Cell Institute, University of Minnesota, 2001 Sixth Street SE, Minneapolis, MN 55455, USA
\textsuperscript{c} Department of Pharmacology, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan
\textsuperscript{d} Skaggs School of Pharmacy, and Department of Medicine, University of California, San Diego, La Jolla, CA 92093, USA
\textsuperscript{e} Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan
\textsuperscript{f} Department of Pediatrics, University of Minnesota, 2450 Riverside Avenue, Minneapolis, MN 55455, USA
\textsuperscript{g} Developmental Biology Center, University of Minnesota, 321 Church Street SE, Minneapolis, MN 55455, USA
\textsuperscript{h} Lillehei Heart Institute, University of Minnesota, 312 Church Street SE, Minneapolis, MN 55455, USA

**A R T I C L E   I N F O**

Article history:
Received 16 October 2013
Received in revised form
27 December 2013
Accepted 3 January 2014
Available online 11 January 2014

Keywords:
*Isl1*
β-catenin
Limb
Branchial arch
Mandible

**A B S T R A C T**

*Isl1* expression marks progenitor populations in developing embryos. In this study, we investigated the contribution of *Isl1*-expressing cells that utilize the β-catenin pathway to skeletal development. Inactivation of β-catenin in *Isl1*-expressing cells caused agenesis of the hindlimb skeleton and absence of the lower jaw (agnathia). In the hindlimb, *Isl1*-lineages broadly contributed to the mesenchyme; however, deletion of β-catenin in the *Isl1*-lineage caused cell death only in a discrete posterior domain of nascent hindlimb bud mesenchyme. We found that the loss of posterior mesenchyme, which gives rise to Shh-expressing posterior organizer tissue, caused loss of posterior gene expression and failure to expand chondrogenic precursor cells, leading to severe truncation of the hindlimb. In facial tissues, *Isl1*-expressing cells broadly contributed to facial epithelium. We found reduced nuclear β-catenin accumulation and loss of Fgf8 expression in mandibular epithelium of *Isl1*\textsuperscript{-/-} embryos. Inactivating β-catenin in *Isl1*-expressing epithelium caused both loss of epithelial Fgf8 expression and death of mesenchymal cells in the mandibular arch without affecting epithelial proliferation and survival. These results suggest a *Isl1* \rightarrow β-catenin \rightarrow Fgf8 pathway that regulates mesenchymal survival and development of the lower jaw in the mandibular epithelium. By contrast, activating β-catenin signaling in *Isl1*-lineages caused activation of Fgf8 broadly in facial epithelium. Our results provide evidence that, despite its broad contribution to hindlimb mesenchyme and facial epithelium, the *Isl1*-β-catenin pathway regulates skeletal development of the hindlimb and lower jaw through discrete populations of cells that give rise to Shh-expressing posterior hindlimb mesenchyme and Fgf8-expressing mandibular epithelium.

© 2014 Elsevier Inc. All rights reserved.

**Introduction**

Mechanisms that regulate initiation and early outgrowth of the vertebrate limb bud have been extensively studied (Duboc and Logan, 2011; Rabinowitz and Vokes, 2012; Zeller et al., 2009). Limb bud mesenchymal progenitor cells in lateral plate mesoderm (LPM) maintain active proliferation, while proliferation of LPM cells in the prospective flank region declines, leading to initial budding (Searf and Janner, 1971). Directional movement of LPM cells is coupled with budding, and shapes initial limb bud morphology (Gros et al., 2010; Wyngaarden et al., 2010). Simultaneously, the fibroblast growth factor 10 (Fgf10) gene is activated in limb mesenchyme progenitor cells, which induces Fgf8 in the overlying ectoderm to establish an Fgf10 (mesenchyme)–Fgf8 (ectoderm) positive feedback loop in nascent limb buds (Min et al., 1998; Ohuchi et al., 1997; Sekine et al., 1999). Fgf8-expressing ectodermal cells are then confined to form a specialized limb bud ectodermal tissue, the apical ectodermal ridge, at the distal edge of the limb bud. Fgf8, together with other apical ectodermal ridge-derived FGFs, regulates limb bud mesenchymal cell survival and patterning (Mariani et al., 2008; Sun et al., 2002). Concomitantly, Gli3 in the anterior region and Hand2 in the posterior region of nascent limb bud pre-pattern the mesenchyme along the anterior–posterior axis (te Welscher et al., 2002a), which leads to Hand2-dependent induction of Shh expression in the posterior.
mesenchyme (Galli et al., 2010). These processes act both in the forelimb and hindlimb buds; however, recent studies have shown striking differences in upstream genetic regulation of limb bud initiation. More specifically, upstream of limb bud outgrowth and Fgf10 expression, Tbx5 and Isl1 (Isl1) are specifically required for initiation of the forelimb and hindlimb buds, respectively (Agarwal et al., 2003; Kawakami et al., 2011; Narkis et al., 2012; Rallis et al., 2003). Furthermore, retinoic acid signaling is required for initiation of forelimb but not hindlimb buds (Cunningham et al., 2013; Zhao et al., 2009).

Isl1 encodes a LIM-homeodomain protein whose expression marks progenitor populations of various organs in the mouse embryo, including the hindlimb (Yang et al., 2006). Prior to hindlimb bud outgrowth, Isl1 is expressed in posterior LPM, and its expression is confined to the posterior part of the hindlimb-forming region at E9.5 (Kawakami et al., 2011; Yang et al., 2006). A genetic lineage tracing analysis using Isl1Cre and a Rosa26-LacZ reporter (R26R) line demonstrated that Isl1-expressing cells contribute to a majority of hindlimb mesenchyme with an anterior (low)-posterior (high) gradient, suggesting heterogeneity within hindlimb mesenchyme progenitors (Yang et al., 2006). Isl1 null embryos arrest development before hindlimb bud formation (Pfaff et al., 1996), thus functional analysis of Isl1 has been performed using conditional knockout (CKO) approaches. Inactivation of Isl1 in early mesoderm using Tcre caused a complete failure to initiate hindlimb bud development (Kawakami et al., 2011; Narkis et al., 2012). Furthermore, our previous study suggested that Isl1 functions through the β-catenin pathway for hindlimb initiation (Kawakami et al., 2011). β-CATENIN is abundantly present at the plasma membrane, and its cytosolic and nuclear levels are kept low by constitutive degradation. When stabilized, β-CATENIN translocates into the nucleus and forms a complex with transcription factors, such as the members of the Lef1/TCF family. This leads to activation of downstream target genes (Nusse and Varmus, 2012). During hindlimb bud initiation, β-catenin signaling is activated in LPM. Abrogation of β-catenin broadly in LPM by Hoxb6Cre results in the failure to initiate hindlimb formation, similar to Isl1 CKO embryos (Kawakami et al., 2011). However, when the hindlimb bud begins outgrowth, ISL1-positive cells and the active β-catenin signaling domain barely overlap: ISL1-positive cells are located at the ventral-proximal domain, while the β-catenin signaling domain is detected in the distal area of the hindlimb-forming region. Thus, it remains unknown whether β-catenin signaling functions in Isl1-expressing hindlimb progenitor cells or whether Isl1 and β-catenin act in distinct populations of hindlimb progenitor cells.

β-catenin is also broadly expressed in craniofacial primordia (in both the mesenchyme and the epithelium) and is required for normal craniofacial development, as shown by conditional inactivation of β-catenin in neural crest cells by Wnt1-Cre (Brault et al., 2001) or by deleting β-catenin in facial epithelium. The latter results in severe craniofacial skeletal defects, including deformities of the nasal bone, upper jaw, lower jaw and hyoid bone with varying severity and selectivity of affected skeletal elements, depending on Cre lines used (Reid et al., 2011; Sun et al., 2012; Wang et al., 2011). While analyzing β-catenin function in Isl1-lineages during hindlimb development, we found that Isl1-lineages contribute broadly to facial epithelium, where β-catenin is known to be required for facial development. This suggested a possible relationship between Isl1 and β-catenin, similar to the process of hindlimb initiation (Kawakami et al., 2011). However, the Isl1 expression pattern in facial tissue, as well as the contribution of Isl1-lineages to the facial region, has not been studied except in branchiomeric muscle (Nathan et al., 2008). Furthermore, the relationship between Isl1-lineages and β-catenin in the development of the facial skeleton is unknown.

To test whether β-catenin functions in Isl1-expressing cells, we inactivated β-catenin in Isl1-lineages. Isl1Cre; β-catenin CKO embryos developed truncated hindlimbs with skeletal defects, in contrast to a complete lack of hindlimb buds in Hoxb6Cre; β-catenin CKO embryos. This result indicated that β-catenin functions in a subset of Isl1-lineages, which contributes to a specific subdomain within the hindlimb bud. Further analysis indicated that β-catenin functions in Isl1-lineages to maintain survival of a compartment within the posterior mesenchyme of nascent hindlimb bud. In addition, we found that the lower jaw was completely missing in the mutants. In facial tissues, we showed that, in Isl1fl/– embryos, activation of β-catenin signaling was impaired in epithelium of the mandibular component of the first branchial arch (BA1), which gives rise to Meckel’s cartilage and mandible. Although the Isl1-lineage contributes broadly to facial epithelium, a requirement for β-catenin in Isl1-lineages for facial skeletogenesis was most evident in BA1, where the epithelial β-catenin–Fgfr8 pathway regulates mesenchymal cell survival, and to a lesser extent in other tissues. Our data identify the contribution of Isl1-expressing cells to hindlimb mesenchyme and BA1 epithelium, and describe a requirement for β-catenin within subdomains of these Isl1 lineages to regulate skeletogenesis by promoting cell survival of discrete cell populations.

Materials and methods

Mouse lines

The mutant mouse alleles used in this study have been previously reported: BAT-gal (Tg(BAT-lacZ)3Mhr (Maretto et al., 2003)), conditional β-catenin knockout allele (Ctnnb1m2Rexem, Ctnnb1fl/fl), (Brault et al., 2001), conditional β-catenin activation allele (Ctnnb1tm1Mho, Ctnnb1fl/+), (Harada et al., 1999), Isl1 null allele (Itou et al., 2012), Rosa26 LacZ reporter (Gt(Rosa)26Sor1cre; R26R (Soriano, 1999) and Isl1Cre (Isl1fl/fl; Cre+/+) (Yang et al., 2006), Ctnnb1cre/+ mice were generated by germline recombination of Ctnnb1foxo2–6 mice using the CMV-Cre line (Schwenk et al., 1995). To inactivate β-catenin in the Isl1-lineage, Ctnnb1fl/+; Isl1cre/+ mice were crossed with Isl1cre/cre, Ctnnb1fl/+ mice, and Isl1cre/cre, Isl1cre/cre (hereafter, referred to as Isl1Cre; β-catenin CKO) were obtained. To constitutively activate (CA) β-catenin, Ctnnb1fl/+; Isl1cre/+; Isl1cre/cre mice were crossed with Isl1cre/cre mice, and Isl1cre/cre, Isl1cre/cre (hereafter, referred to as Isl1Cre; CA-β-catenin) were obtained. Mice were maintained on a mixed genetic background. Care and experimentation were carried out according to the approval by the Institutional Animal Care and Use Committee of the University of Minnesota.

Skeletal preparation and histology analysis

Embryonic day (E) 13.5 and 14.5 embryos were fixed with 50% ethanol, and then processed for Alcian Blue cartilage staining as previously described (Kawakami et al., 2009; McLeod, 1980). For histological analysis, embryos were dissected in 10% neutral formalin and then processed for Alcian Blue cartilage staining as described (Kawakami et al., 2009; McLeod, 1980). For histological analysis, embryos were fixed in 10% neutral formalin and processed for paraffin sectioning with 6–8 μm thickness as previously described (Petryk et al., 2004). Sections were stained with eosin–hematoxylin.

In situ hybridization, LacZ staining and immunofluorescence

Whole mount in situ hybridization and whole mount LacZ staining were performed according to previous publications (Itou et al., 2012; Kawakami et al., 2011). Section in situ hybridization was performed on 8 μm thickness paraffin sections according to a standard procedure (Itou et al., 2012). Sections were counter stained with nuclear fast red. Immunofluorescence analysis was performed on 14 μm cryosections according to a standard procedure (Itou et al., 2012). Mouse anti-β-1SL1 (39.4D5, Developmental Studies Hybridoma Bank, 4 μg/ml), rabbit anti-β-catenin (ab32572, Abcam, 1:100 dilution) and rat anti-E-cadherin (sc-59778, Santa Cruz Biotechnology, 1:200 dilution) were
used. Counter staining was done using DAPI. The fluorescent signals were detected using a Zeiss LSM710 laser scanning confocal microscope and analyzed by ZEN2009 software.

**Cell proliferation and apoptosis analysis**

Cell proliferation and apoptosis assays on 14 μm cryosections were simultaneously performed by using rabbit anti-phospho Histone H3 (Ser 10) (pHis3, Millipore, #06-570; 1:500 dilution) and the In Situ Cell Death Detection Kit (Roche diagnostics) according to the manufacturer’s instruction. Alexa488 anti-Fluorescein/Oregon green (1:2000 dilution) and Alexa594 anti-rabbit IgG (Molecular Probes, 1:1000 dilution) were used as secondary antibodies. For quantitative analysis of cell proliferation and cell death in nascent hindlimb bud, pH3-3-, TUNEL- and DAPI-positive cells in the LPM were counted from two transverse sections from anterior, middle and posterior parts of each embryo. In the case of the mandibular component of the branchial arch, three consecutive transverse sections obtained at the same plane of sectioning through the medial region of the arch were examined from each embryo. Statistical significance between control and CKO embryo was analyzed by the independent Student’s t-test, and shown as average ± standard deviation. p Values are indicated within each panel.

**Results**

**Inactivation of β-catenin in the Isl1-lineage causes skeletal dysplasia in hindlimb**

Isl1 acts upstream of β-catenin during hindlimb bud initiation in mice (Kawakami et al., 2011). However, it remains unknown whether Isl1 and β-catenin function in the same cells. To examine the requirement of β-catenin in Isl1-lineages, we inactivated β-catenin using Isl1Cre; Isl1Cre; β-catenin CKO embryos died at E12.5–E14.5, likely due to cardiovascular defects (Lin et al., 2007). Isl1Cre; β-catenin CKO embryos exhibited severe hindlimb hypoplasia. Alcian blue staining revealed that mutant embryos developed normal forelimb skeletons, consistent with a lack of Isl1 expression in forelimb progenitor cells and forelimb bud (Kawakami et al., 2011; Yang et al., 2006). In contrast, the hindlimb exhibited a short femur, truncated zeugopodal cartilage elements, absence of the autopod, and absence of the posterior region of the pelvic girdle (Fig. 1A–C, and F–H, n = 8 at E13.5 or E14.5). These hindlimb defects are distinct from the complete lack of the hindlimb bud observed in Hoxb6Cre-mediated inactivation of β-catenin in broad regions of LPM (Kawakami et al., 2011). Formation of the hindlimb with skeletal defects in Isl1Cre; β-catenin CKO embryos suggested that Isl1Cre-mediated inactivation of β-catenin occurred only in a select subpopulation of hindlimb mesenchyme progenitors.

The Isl1-lineage contributes broadly to hindlimb mesenchyme, but β-catenin function in Isl1-lineages is required in a discrete posterior region

The genetic lineage analysis study demonstrated that Isl1-lineages contributed to a broad region of hindlimb mesenchyme (Yang et al., 2006). Consistent with this, Isl1-lineages (visualized as LacZ signals in Isl1Cre; R26R embryos) occupied the majority of nascent hindlimb bud immediately after initiation of outgrowth, except for a small domain in the anterior part (Fig. S1B, (Yang et al., 2006)). Previous reports have shown that Isl1 mRNA expression at E9.0, prior to hindlimb bud development, is broadly detected in

![Fig. 1. Defects in the hindlimb and lower jaw skeletons in Isl1Cre; β-catenin CKO embryos. Alcian Blue-stained skeletons of wild-type control (A–E) and Isl1Cre; β-catenin CKO (F–J) embryos at E14.5. Lateral views of whole skeletons of control (A) and mutant (F) embryos reveal the absence of the lower jaw cartilage (asterisk) and severe hypoplasia in the hindlimb skeleton (arrow) in mutant embryos. (B and G) Forelimb skeleton developed similarly in control (B) and mutant (G). The scapula (sc), humerus (h), radius (r) and ulna (u) are indicated. The autopod (auto) is indicated with a solid line. (C and H) The hindlimb in mutants contained a hypoplastic pelvic girdle (pg) and femur (fe), and truncated zeugopodal elements. The tr可怜b (t) and fibula (fl) in the wild type are indicated. (D, E, I, and J) Lateral views (D and I) and bottom views (E and J) of craniofacial skeletons. The image in (E) was taken after removing Meckel’s cartilage. The mutant completely lacks Meckel’s cartilage (asterisk, I). Other cartilaginous elements are formed, although smaller than those in control embryos. The insets in (D) and (I) show the hyoid bone primordia (hy). Cartilage elements are labeled only in control for simplicity: ala temporalis (at), Meckel’s cartilage (mc), nasal cartilage (nc), nasal septum (ns), otic capsule (oc), parachordal plate (pchp), pilapostoptica (ppso), and trabecular basal plate (tbp).
LPM (Kawakami et al., 2011). In nascent limb buds, the pattern of the \( \text{Isl1Cre} \)-mediated recombination likely occurred in hindlimb progenitor cells in LPM prior to the onset of hindlimb bud outgrowth (Yang et al., 2006).

To characterize \( \beta\)-catenin function in \( \text{Isl1}-\)lineages, we monitored activation of the \( \beta\)-catenin pathway using a BAT-gal transgene that reports activation of Lef1/TCF-\( \beta\)-catenin signaling (Maretto et al., 2003). BAT-gal signal was detected in nascent hindlimb bud at E9.75 wild-type embryos, but was downregulated in the posterior region in \( \text{Isl1Cre} \)-\( \beta\)-catenin CKO embryos (Fig. S1A and D). To constitutively activate \( \beta\)-catenin in \( \text{Isl1} \) lineages, we excised exon 3 of the \( Ctnmb1 \) gene utilizing \( \text{Isl1Cre} \), which causes stabilization of \( \beta\)-catenin, and hence, constitutive activation of the \( \beta\)-catenin pathway (Harada et al., 1999). BAT-gal signal in \( \text{Isl1Cre} \)-CA-\( \beta\)-catenin embryos was stronger in the hindlimb bud than BAT-gal signal in controls (Fig. S1E). Thus, \( \beta\)-catenin signaling was regulated in nascent hindlimb bud utilizing \( \text{Isl1Cre} \)-mediated recombination to drive loss- or gain-of-function of \( \beta\)-catenin.

To clarify the role of \( \beta\)-catenin in \( \text{Isl1} \)-lineages during hindlimb development, we examined expression patterns of \( \text{Msx1} \) and \( \text{Fgf10} \), which are broadly expressed in nascent hindlimb bud at E9.75. \( \text{Msx1} \) expression was significantly downregulated in posteriormost hindlimb bud in \( \text{Isl1Cre} \)-\( \beta\)-catenin CKO embryos (Fig. S2C and D). We also detected a slight reduction in \( \text{Fgf10} \) mRNA expression in \( \text{Isl1Cre} \)-\( \beta\)-catenin CKO embryos (Fig. S2F and G). Expression of \( \text{Fgf8} \), whose activation in the ectoderm requires \( \text{FGF10} \) (Min et al., 1998; Sekine et al., 1999), was significantly downregulated in the posterior part of nascent hindlimb bud (Fig. S2F and G). These results suggested that, despite a broad contribution of \( \text{Isl1} \) lineages to hindlimb bud mesenchyme, a discrete posterior region of nascent hindlimb bud was affected in \( \text{Isl1Cre} \)-\( \beta\)-catenin CKO embryos.

\( \beta\)-catenin function in the \( \text{Isl1} \)-lineage is required for mesenchymal cell survival in a discrete posterior region

Genetic experiments have demonstrated that \( \beta\)-catenin functions as a critical factor for cell proliferation and survival in many aspects (Grigoryan et al., 2008). Thus, we examined pHi3 (proliferation marker) and TUNEL-positive cells (cell death) in serial sections at E9.75. Analysis of alternate transverse sections allowed us to sequentially evaluate cell proliferation and death along the anterior–posterior axis in nascent hindlimb bud (Fig. S2). We found that cell proliferation was not affected at any level of the hindlimb bud. However, we detected a significant increase in mesenchymal cell death, only in the posterior part of \( \text{Isl1Cre} \)-\( \beta\)-catenin CKO hindlimbs (n = 3, Fig. 2D, E, F, H, I, and L). Condensed TUNEL-positive signals in nuclei of apoptotic cells were enriched in sections corresponding to approximately 1/5 of the hindlimb bud. These results indicated that \( \beta\)-catenin function in \( \text{Isl1} \)-lineages was required for mesenchymal cell survival in a spatially-restricted domain, which comprises approximately 1/5 of the posteriormost nascent hindlimb bud.

Loss of precursors of Shh-expressing cells in posterior mesenchyme in \( \text{Isl1Cre} \)-\( \beta\)-catenin CKO hindlimbs

To further investigate the impact of the loss of \( \beta\)-catenin in \( \text{Isl1} \)-lineages, and localized cell death in the posterior region of nascent limb bud on outgrowth and patterning processes, we examined gene expression in developing hindlimb buds. We first visualized limb buds utilizing antisense probes for Prx1 (n = 3), a limb mesenchyme marker (Cserjesi et al., 1992), and Pax1 (n = 2), a gene expressed in the entire hindlimb bud mesenchyme (Lancot et al., 1997; Shang et al., 1997; Szeot et al., 1996) at E10.5 (Fig. 3A, B, C, and E). The anterior–posterior length of the hindlimb bud in \( \text{Isl1Cre} \)-\( \beta\)-catenin CKO embryos were reduced by about the length of one somite. Thus, increased cell death at the onset of hindlimb bud outgrowth likely caused loss of the posterior tissue by E10.5.

The posterior mesenchyme of nascent limb bud gives rise to the Shh-expressing zone of polarizing activity (Honig and Summerbell, 1985; Riddle et al., 1993). Correlating with the loss of posterior mesenchyme, Shh (n = 3), and its transcriptional targets, \( \text{Gli1} \) (n = 3) and \( \text{Hoxd12} \) (n = 2) (Hui and Angers, 2011; Litengtun et al., 2002; te Welscher et al., 2002b), were not detected (Fig. 3C–E, and H–J). Fgf8 expression, whose maintenance requires SHH signaling-dependendent \( \text{Gremlin1} \) (Pamman et al., 2006; Verheyden and Sun, 2008), was also downregulated in the posterior apical ectodermal ridge (n = 3, Fig. 3K and O). Contrary to these observations, expression of \( \text{Abx4} \), a marker for anterior mesenchyme (Qu et al., 1997; Takahashi et al., 1998), was not altered (n = 2, Fig. 3L and P). These results suggested that precursors of Shh expressing cells were lost in nascent hindlimb bud of \( \text{Isl1Cre} \)-\( \beta\)-catenin embryos, and caused selective loss of posterior tissue and gene expression.

Fig. 2. Cell death in the posterior margin of the mesenchyme in nascent hindlimb buds in \( \text{Isl1Cre} \)-\( \beta\)-catenin CKO embryos. (A–C, and E–G) Expression patterns of \( \text{Msx1} \) (A and E), \( \text{Fgf10} \) (B and F) and \( \text{Fgf8} \) (C and G) in control (A–C) and mutant (E–G) embryos. \( \text{Msx1} \) and \( \text{Fgf8} \) expressions were missing in the posterior margin of nascent hindlimb bud in mutants. \( \text{Fgf10} \) expression was slightly downregulated in the mutant hindlimb bud. Black and blue arrowheads point to normal and reduced expression, respectively, and asterisks indicate absence of signals. (D, D’, H, H’ and I) Cell proliferation and cell death analysis in control (D and D’) and mutant (H and H’) embryos. Shown are representative transverse section images of the posterior part of nascent hindlimb bud. Colors represent pHi3 (red), TUNEL (green) and DAPI (blue) signals. (D’) and (H’) show close up of the boxed areas in (D) and (H), respectively. Dotted lines indicate boundary between the limb bud and the trunk. (I) Quantitative analysis of cell death (TUNEL) and cell proliferation (pHi3) in nascent limb buds along the anterior–posterior axis. The y axis represents percentage of TUNEL or pHi3 positive cells per DAPI positive nuclei. Ant, Med and Post represent anterior, middle and posterior regions of the limb bud, respectively.
The loss of posterior mesenchymal cells, as well as the lack of SHH signaling that is required for expansion of chondrogenic progenitors [Zhu et al., 2008], would cause reduction of Sox9-expressing chondrogenic progenitor cells in the hindlimb bud (Fig. 3M, N, Q, and R). Sox9 expression was also missing in the posterior-proximal region at E10.5 (n=3, Fig. 3M and Q), which was correlated with absence of the posterior region of the pelvic girdle (Fig. 1H). At E11.5, the Sox9 expression domain in mutant hindlimb bud looked more condensed, and did not extend along the proximal-distal axis as observed in control hindlimb bud (n=2, Fig. 3N and R). This Sox9 expression pattern correlated with the truncated, shorter cartilage elements at E14.5 (Fig. 1). Collectively, these results indicated that β-catenin deletion in the Isl1-lineage resulted in a specific loss of the posterior mesenchyme of the hindlimb bud, which resulted in failure to maintain the posterior gene expression program. Although the loss of mesenchyme was restricted to the posterior region, the absence of the posterior gene expression program and failure to expand chondrogenic progenitor cells would cause the truncated short skeletal elements in the Isl1Cre; β-catenin CKO hindlimb.

Constitutive activation of β-catenin signaling in the Isl1-lineage impairs the Hand2–Shh pathway in the hindlimb through upregulation of Gli3

To further examine β-catenin function in Isl1-lineages, we examined developmental consequences of constitutive activation of the β-catenin pathway. Isl1Cre; CA-β-catenin embryos died around E10.5–E11.0, likely due to cardiovascular defects (Kwon et al., 2007). We detected comparable expression of Fgf10 (n=3) and Hand2 (n=3) in nascent hindlimb bud at E9.75 (Fig. 4A, B, G, and H), suggesting that hindlimb progenitor cells in LPM were not affected by Isl1Cre-mediated activation of β-catenin signaling. However, at E10.0 (30–31 somite stages), we detected posterior expansion of Gli3, normally excluded from the posterior region of nascent limb bud in wild-type embryos (n=3, Fig. 4C and I) (te Welscher et al., 2002a). Consistent with the mutual antagonism between anterior Gli3 and posterior Hand2, we observed increased downregulation of Hand2 in posterior mesenchyme at E10.0 in Isl1Cre; CA-β-catenin mutants (n=2, Fig. 4D and J, 32–33 somite stages). In agreement with the known role of Hand2 in inducing Shh in the limb bud (Galli et al., 2010), expression of Shh (n=3) and Gli1 (n=2) was significantly downregulated in Isl1Cre; CA-β-catenin hindlimb buds at E10.5 (Fig. 4E, F, K, and L). These results suggested that proper levels of β-catenin signaling were critical for normal activation of the Hand2–Shh pathway in posterior mesenchyme. Our results have indicated that loss- and gain-of β-catenin function in Isl1-lineages caused loss or downregulation of Shh in hindlimb buds by distinct mechanisms, namely loss of precursor cells (Isl1Cre; Ctnnb1 CKO) and dysregulation of Hand2–Gli3 antagonism (Isl1Cre; CA-β-catenin). Thus, maintaining proper levels of β-catenin function in Isl1-lineages is crucial for Shh expression in limb buds.
The Isl1-lineage through β-catenin contributes to craniofacial development

In addition to hindlimb defects, Isl1Cre; β-catenin CKO embryos exhibited defects in craniofacial development (Figs. 1A and F and S3). Mutant embryos exhibited agnathia, a complete lack of the lower jaw, a loss of tongue, and hypoplasia of nasal and maxillary processes (Fig. S3). Alcian blue staining demonstrated that mutants lacked Meckel’s cartilage, while other cartilaginous elements, such as hyoid bone primordia, were slightly reduced in size (Fig. 1D, E, I, and J, n = 8). Previous studies have shown that deletion of β-catenin causes severe skeletal defects in the craniofacial region (Hu and Ornitz, 2010; Joeng et al., 2011; Reid et al., 2011; Sun et al., 2012; Wang et al., 2011). The complete loss of the lower jaw, which is derived from the mandibular prominence of BA1 (Depew and Simpson, 2006; Minoux and Rijli, 2010) in Isl1Cre; β-catenin CKO embryos indicated that β-catenin function in Isl1-lineages contributed to a substantial degree to BA1-derived craniofacial structures.

Expression of Isl1 in BA1 epithelium and broad contribution of Isl1-lineages to facial epithelium

The Isl1 lineage has been shown to contribute to subpopulations of head muscle (Nathan et al., 2008); however, Isl1 expression in other craniofacial tissue has not been characterized. Thus, we examined Isl1 mRNA and protein expression, as well as Isl1-lineages during development of BA1. Isl1 expression was detected as early as E8.5 in the BA1 prominence (Fig. 5A). Immunoreactive Isl1 signals were predominantly detected in the epithelium, in addition to some scattered mesenchymal signals (Fig. 5B and C). At E9.0, Isl1 signals in BA1 (as well as BA2) were broadly detected in the epithelium, and the scattered mesenchymal signals, which likely represent branchiomeric muscle precursors, became more prominent (Fig. 5D–F). Transverse sections at E9.5 demonstrated that ISL1 signals were in both ectodermal and endodermal components of the mandibular epithelium, in addition to the branchiomeric muscle primordia in the core of the mesenchyme (Fig. 5G–J). The epithelial ISL1 signal continued to be detected, but became weaker at E10.5 and 11.5 (Fig. 5K–P).

The recombination in Isl1Cre; R26R embryos were consistent with the expression pattern of Isl1, and LacZ staining was detected in BA1 at E8.5 and E9.0 (Fig. S4A and B), indicating early and efficient recombination in this tissue. At E9.5, Isl1–lineages were detected broadly in the maxillary and mandibular components of BA1, as well as BA2 (hyoid arch) (Fig. S4C and D). Transverse and sagittal sections indicate that Isl1-lineages were present in epithelium of ectoderm and endoderm, consistent with the ISL1 signal (Fig. S4E–G). Isl1–lineages were also detected in medial and lateral nasal processes at E10.5 (Fig. S4H and I). At E13.5, Isl1–lineages were specifically detected in epithelia of the nasopharyngeal process, lower jaw and the distal tip of the tongue (Fig. S4J and K). These results demonstrated highly localized Isl1 expression in facial epithelium and efficient recombination by Isl1Cre in a broad region of facial epithelium.

Isl1 is necessary for nuclear accumulation of β-CATENIN in BA1 epithelium

The absence of Meckel’s cartilage in Isl1Cre; β-catenin CKO embryos, as well as expression of ISL1 in facial epithelium where β-catenin is required for facial development, raised the possibility that Isl1 regulates Meckel’s cartilage development through the β-catenin pathway, similar to the pathway required for initiation of hindlimb buds (Kawakami et al., 2011). Isl1 null embryos arrest at E9.5 (Pfaff et al., 1996), excluding the possibility of direct examination of Isl1 function in the development of Meckel’s cartilage. However, visualizing BA1 by Prx1 expression at E9.0 showed hypoplasia of the mandibular component of BA1 in Isl1−/− mutants (n = 2, Fig. 6A and G), demonstrating a requirement for Isl1 in BA1 development. Fgf8 in BA1 epithelium is crucial for the development of Meckel’s cartilage (Macatee et al., 2003; Trumpf et al., 1999). Indeed, we found that Fgf8 expression in BA1 was lost in Isl1−/− embryos, while Fgf8 expression in the midbrain–hindbrain boundary and forelimb bud ectoderm was maintained (n = 2, Fig. 6B, C, H, and I). These results suggested that Isl1 regulated BA1 development through Fgf8 expression in epithelium.

It has been recently demonstrated that β-catenin signaling regulates Fgf8 expression in facial epithelium (Reid et al., 2011; Sun et al., 2012; Wang et al., 2011), suggesting that Isl1 regulates...
β-catenin function in Isl1-lineages is required for mesenchymal cell survival in BA1 through epithelial Fgf8

LacZ signals in Isl1Cre; R26R embryos demonstrated efficient recombination by Isl1Cre and a broad contribution of Isl1-lineages to facial epithelium (Fig. S5). However, in Isl1Cre; β-catenin CKO embryos, defects were more severe in Meckel’s cartilage than other skeletal elements (Fig. 1). Thus, we next investigated the activation status of β-catenin signaling by examination of BAT-gal reporter signals in facial tissue. We observed BAT-gal signals in maxillary and mandibular components of BA1 and BA2 (Fig. S6A and B), consistent with the previous report of active β-catenin signaling in these tissues (Brugmann et al., 2007). In Isl1Cre; β-catenin CKO embryos, severe downregulation of BAT-gal signals was observed in the mandibular component of BA1, while effects on the maxillary process of BA1 and BA2 seemed to be milder (Fig. S6C and D). Contrary to this, activation of β-catenin signaling in Isl1Cre; CA-β-catenin embryos resulted in stronger BAT-gal signal, which appeared in a punctate pattern and was broadly detected in BA1 and BA2 (Fig. S6E and F). These results confirmed efficient loss- and gain-of function of β-catenin by Isl1Cre in facial tissues, and further demonstrated that the requirement for β-catenin in Isl1-lineages was more significant in the mandibular component of BA1 than other craniofacial regions.

Consistent with this notion, in situ hybridization of Prrx1 at E9.5 demonstrated selective defects in the mandibular component of BA1,
while the maxillary process was comparable in control and Isl1Cre; β-catenin CKO embryos (Fig. 7A and D). Meckel’s cartilage develops from cranial neural crest cell-derived mesenchyme in BA1 (Gross and Hanken, 2008; Ito et al., 2002), while Isl1 expression and Isl1-lineage contribution are specific to the epithelium (Figs. 5 and S4). Thus, to investigate how β-catenin function in Isl1-lineages affected Meckel’s cartilage development, we examined cell proliferation and survival in the mandibular component of BA1. Surprisingly, cell proliferation and cell survival were not affected in BA1 epithelium of Isl1Cre; β-catenin CKO embryos compared to wild-type embryos (Fig. 7B, D, and E). However, we detected increased cell death with changes in cell proliferation in BA1 mesenchyme in Isl1Cre; β-catenin CKO embryos (Fig. 7B, D, and F). TUNEL signals condensed in the nuclei of apoptotic cells were clustered close to the epithelium. Thus, deletion of β-catenin in the Isl1-lineage caused cell death specifically in the mesenchyme.

Given downregulation of β-catenin signaling and loss of Fgf8 expression in epithelium of the mandibular component of BA1 in Isl1−/− embryos (Fig. 6), we examined how Fgf8 expression was affected in Isl1Cre; β-catenin CKO embryos. Fgf8 expression was severely downregulated in the mandibular component of BA1, while weak expression was detectable in the maxillary component and in the frontonasal process at E9.75 in Isl1Cre; β-catenin CKO embryos (Fig. 8A, B, F, and G, n = 3). We also examined expression of Barx1 and Dusp6, targets of Fgf8 signaling (Kawakami et al., 2003; Trumppe et al., 1999). In Isl1Cre; β-catenin CKO embryos, both genes were downregulated to different degrees (Dusp6 to a greater degree than Barx1), which could reflect different threshold responses to FGF8. The residual Fgf8 expression in the maxillary process at this stage (Fig. 8F and G) appeared sufficient to maintain a low level of Barx1 expression in the lateral region (Fig. 8C and H, n = 2). Conversely to this, Dusp6 expression was significantly downregulated in the entire BA1 (Fig. 8D and I, n = 2), likely because the residual Fgf8 expression was not sufficient to maintain Dusp6 expression.

In Isl1Cre; CA-β-catenin mutants, Fgf8 expression was detected broadly in BA1 and BA2 (n = 3, Fig. 8K and L). Fgf8 in situ mRNA detection on transverse and sagittal sections at E9.75 demonstrated ectopic Fgf8 expression in epithelium as well as epithelial thickening in BA1 (Fig. S7, n = 4). In contrast, no ectopic Fgf8 was induced in the mesenchyme of BA1 (Fig. S7), although Isl1Cre can recombine in the myogenic core of the mesenchyme (Fig. S4) (Nathan et al., 2008). Thus, β-catenin regulation of Fgf8 in the Isl1-lineage was specific to the epithelium.

Barx1 expression seems to be unchanged in the mandibular component of BA1, suggesting that FGF8 signaling was above a threshold for Barx1 expression in the Isl1Cre; CA-β-catenin (Fig. 8M, n = 2). However, Barx1 signals in the maxillary process were stronger than control embryos (Fig. 8M, arrowhead), likely due to upregulated Fgf8 expression in this domain. Dusp6 expression was expanded towards the medial domain, and the signals became stronger compared to control wild-type embryos (Fig. 8N, n = 2). These data further supported observed alterations of Fgf8 expression in the facial region in Isl1Cre; β-catenin CKO and Isl1Cre; CA-β-catenin embryos.

In addition to Barx1 and Dusp6, which are lateral markers of the mandibular component of BA1, a medial mandibular marker, Hand2 (Thomas et al., 1998), was also downregulated in Isl1Cre; β-catenin CKO embryos at E9.75 (Fig. 8E and J, n = 3). In Isl1Cre; CA-β-catenin mutants Hand2 expression in the mandibular component of BA1 appeared to be slightly expanded to the lateral region (Fig. 8O, n = 4).

Discussion

Isl1 lineages and heterogeneity in nascent hindlimb bud mesenchyme and facial epithelium

In this study, we demonstrated that Isl1-lineages contributed to skeletogenesis of the hindlimb and lower jaw through β-catenin signaling. While abrogating β-catenin has been shown to cause severe defects in the development of the hindlimb and facial tissue (Kawakami et al., 2011; Reid et al., 2011; Sun et al., 2012; Wang et al., 2011), deletion of β-catenin in Isl1-lineages caused severe defects in more restricted tissues.

Our previous study showed that Isl1 acts upstream of the β-catenin pathway during hindlimb initiation (Kawakami et al., 2011). However, ISL1-positive cells and nuclear β-catenin-positive cells barely overlap just prior to hindlimb initiation. Sensitivity of antibodies in our previous study hampered further examination of the possibility of β-catenin signaling in Isl1-lineages at earlier stages.

Fig. 7. Inactivation of β-catenin in the Isl1-lineage causes cell death in the mesenchyme not in the epithelium of BA1. (A, C) Expression pattern of Prx1 in wild-type (A) and Isl1Cre; β-catenin CKO (C) embryos at E9.5. Arrowheads and arrows point to the maxillary and mandibular components of BA1, respectively. The blue arrow in (C) indicates hypoplastic mandibular component of BA1. (B, D) Confocal images of phH3 (red) and TUNEL (green) staining on transverse sections of the mandibular component of BA1 at E9.75. Medial–lateral and proximal–distal axes are indicated with arrows. Massive cell death is visualized by condensed TUNEL signals (arrows, D). (E and F) Quantitative analysis of cell death and cell proliferation in the epithelium (E) and mesenchyme (F) of BA1. Blue bars and red bars represent control and Isl1Cre; β-catenin CKO embryos, respectively. The y axis represents percent positive cells, compared to DAPI-positive nuclei. Asterisk indicates statistical significance. Increased cell death in the BA1 mesenchyme showed significant difference.
stages. A genetic approach in this study using Isl1Cre to inactivate β-catenin provided evidence that β-catenin was required in Isl1-lineages, but this requirement was limited to a portion of the hindlimb bud mesenchyme progenitors, which contributes to the posterior region of nascent hindlimb buds. This is evident by the observations that localized cell death in nascent hindlimb buds was restricted to posterior one somite level, and the anterior–posterior length of hindlimb buds was reduced by approximately one somite length in mutants (Figs. 2 and 3). The contribution of Isl1-lineages to a large portion, but not the entire hindlimb mesenchyme, as well as the requirement of β-catenin in Isl1-lineages, indicated that the seemingly homogenous nascent limb bud mesenchyme is in fact heterogeneous from the onset of hindlimb development.

In facial tissue, Isl1-lineages broadly contributed to facial epithelium, including the epithelium of BA1 and BA2 (Fig. S4). Similar to hindlimbs, inactivating β-catenin in Isl1-lineages exhibited severe skeletal defects in a localized manner. More specifically, the mandibular component of BA1 was most severely affected, leading to the absence of Meckel’s cartilage and lower jaw (Figs. 1 and S3). By contrast, the upper jaw, which is largely derived from the maxillary process and the frontonasal process, formed, but was slightly smaller. Similarly, the hyoid bone primordium that is derived from BA2 was present, but hypoplastic. Thus, the functional significance of β-catenin also appeared to differ within Isl1-lineages in facial tissue.

Relationship between Isl1 and β-catenin in limb development

The relationship between Isl1 and β-catenin function during embryonic development has been extensively studied in the heart, where β-catenin positively regulates Isl1 expression in cardiac progenitor cells in the second heart field (Ai et al., 2007; Cohen et al., 2012; Klaus et al., 2012; Klaus et al., 2007; Kwon et al., 2007; Lin et al., 2007; Qyang et al., 2007). These studies indicate that β-catenin acts upstream of Isl1 expression and/or Isl1-lineage development. In contrast, our current findings and previous study (Kawakami et al., 2011) suggest that Isl1 functions upstream of β-catenin in hindlimb and BA1. Contrary to the heart where β-catenin regulates proliferative expansion of cardiac progenitors, our analysis in nascent hindlimb buds indicated that a loss of β-catenin did not cause defects in proliferation in Isl1-lineages (Fig. 2). Instead, our analysis highlighted the function of β-catenin in the survival of a portion of Isl1-lineages. Cell survival seems to

Fig. 8. Altered Fgf8 expression and FGF8 signaling in loss and gain of β-catenin signaling in Isl1-lineages in the facial region. Expression pattern of Fgf8 (A, B, F, G, K, and L), Barx1 (C, H, and M), Dusp6 (D, I, and N) and Hand2 (E, J, and O) in control (A–E), Isl1Cre; β-catenin CKO (F–J) and Isl1Cre; CA-β-catenin (K–O) embryos at E9.75. Arrowheads and arrows point to expression in the maxillary and mandibular components of BA1, respectively. In (F–J), asterisks and blue arrows/arrowheads indicate absence of and reduced expression, respectively. In (K–O), red arrows and arrowheads indicate upregulated expression. Small arrows in (K) and (L) point to ectopic Fgf8 expression. For simplicity, not all ectopic signals are labeled. Shown are frontal images, except for (A), (F), and (K), which are lateral images. For (B–E), (G–J) and (L–O), close up of the mandibular component of BA1 is shown under each panel.
be a common target of mesenchymal β-catenin signaling during different steps of limb development. For instance, early inactivation of β-catenin in LPM prior to initiation of hindlimb bud outgrowth by Hoxb6Cre caused cell death broadly in hindlimb progenitor cells as well as the complete failure to activate the Fgf10–Fgf8 feedback loop (Kawakami et al., 2011). In the case of inactivating β-catenin with Prx1Cre in the developing limb bud mesenchyme, a failure to maintain the apical ectodermal ridge and apoptosis of the proximal mesenchyme was detected during limb bud elongation (Hill et al., 2006). Cell death in proximal mesenchyme is likely to be secondary to reduced secretion of FGFs from the apical ectodermal ridge, whose loss is known to cause proximal cell death in developing limb buds (Mariani et al., 2008; Sun et al., 2002). The present study also found a requirement for β-catenin in cell survival in Isl1-lineages. However, unlike previous reports, only a part of Isl1-lineages located in posteriormost nascent hindlimb buds was affected. Morphological and gene expression analyses in Isl1Cre; β-catenin CKO hindlimb buds suggested that apoptotic cells in posteriormost hindlimb included precursors of Shh-expressing cells (Fig. 3), which are located at the posterior margin of the developing limb bud (Riddle et al., 1993). This idea is in agreement with our recent study, which demonstrated Isl1 regulation of the Hand2–Shh morpho-regulatory pathway in the posterior mesenchyme, specifically in hindlimb buds (Itou et al., 2012).

By contrast, constitutive activation of β-catenin in Isl1-lineages caused expansion of Gli3 expression into the posterior margin of nascent hindlimb buds (Fig. 4). It has been demonstrated that Gli3 in the anterior part and Hand2 in the posterior part of nascent limb buds are mutually antagonistic (te Welscher et al., 2002a), and Hand2, in combination with Hox genes, induces Shh expression in posterior limb bud mesenchyme (Galli et al., 2010; Kimita et al., 2005; Tarchini et al., 2006). The expansion of Gli3 expression toward the posterior margin and increased downregulation of Hand2 in the posterior mesenchyme of nascent hindlimb buds correlate with downregulation of Shh in Isl1Cre; CA-β-catenin mutant hindlimb buds (Fig. 4). Thus, constitutive activation of β-catenin signaling likely impacts the balance between Gli3 and Hand2. Phenotypes resulting from either up- or down-regulating β-catenin functions in Isl1-lineages suggested that levels of β-catenin signaling need to be properly regulated to establish posterior gene expression and cell survival for proper development of the hindlimb.

**IsI1 and function of β-catenin in the craniofacial region**

In this study, we found that Isl1 is expressed in the epithelium of BA1 and that Isl1 is necessary for nuclear accumulation of β-CATENIN (Figs. 5 and 6), similar to hindlimb bud progenitors (Kawakami et al., 2011). Absence of Fgf8 expression in BA1 in Isl1−/− embryos (Fig. 6) as well as the requirement of β-catenin for Fgf8 expression (Reid et al., 2011; Sun et al., 2012; Wang et al., 2011), strongly suggested a pathway (Isl1→β-catenin→Fgf8) to regulate BA1 development. This idea was supported by selective cell death in mesenchyme of BA1 in Isl1Cre; β-catenin CKO embryos (Fig. 7), even though ISL1 is detected in the epithelium.

The Isl1-lineage also contributes to branchiomatic muscle (Nathan et al., 2008). However, lack of ectopic Fgf8 expression in mesenchyme of Isl1Cre; CA-β-catenin embryos indicated that the Isl1→β-catenin→Fgf8 pathway is specific to epithelium of the branchial arch (Fig. S7). Given that epithelial Fgf8 is essential for survival of BA1 mesenchyme that will give rise to Meckel’s cartilage (Macatee et al., 2003; Trumpp et al., 1999), it is unlikely that β-catenin function in branchiomatic muscle contributes to Meckel’s cartilage development. Our data support the idea that loss of Meckel’s cartilage in Isl1Cre; β-catenin CKO is caused by disrupting an epithelial Isl1→β-catenin→Fgf8 pathway. Therefore, our study identified a novel role of Isl1 as a regulator of β-catenin→Fgf8 pathway during craniofacial skeletogenesis.

Analysis of Lef1/Tcf-β-catenin reporters has shown that β-catenin signaling is broadly activated in the craniofacial region (Brugmann et al., 2007) and Fig. S4). In addition, a functional analysis of epithelial β-catenin suggested differential requirements for β-catenin in the upper and lower jaws, implying that high levels of epithelial β-catenin signaling support lower jaw development (Sun et al., 2012). Given that ISL1 is necessary for nuclear accumulation of β-catenin (Fig. 6), Isl1 might function in creating higher β-catenin levels in the epithelium of BA1 to promote normal development of the lower jaw.

An evolutionarily conserved β-catenin–Fgf8 pathway in branchial arch and limb bud, and implications for evolutionary origins of a genetic module

The present study and previous studies highlight a common role for the β-catenin–Fgf8 pathway in the epithelium of the limb bud and BA1. In the limb bud, high levels of β-catenin signaling are necessary for Fgf8 expression in the apical ectodermal ridge (Barrow et al., 2003; Kawakami et al., 2001; Kengaku et al., 1998; Soshnikova et al., 2003). Moreover, ectopic activation of β-catenin signaling in limb ectoderm can induce ectopic Fgf8 expression in a punctate manner, which was associated with ectoderm thickening that resembles the pseudostratified apical ectodermal ridge (Barrow et al., 2003; Kawakami et al., 2001, 2004; Kengaku et al., 1998; Soshnikova et al., 2003). The β-catenin–Fgf8 pathway is activated during early limb development both in forelimb and hindlimb bud. However, upstream genetic regulation differs in forelimbs and hindlimbs. Specifically, mesenchymal Isl1 is genetically upstream of the epithelial β-catenin–Fgf8 pathway in the hindlimb bud (Kawakami et al., 2011), while forelimb buds use another pathway, likely through Tbx5 (Agarwal et al., 2003; Rallis et al., 2003).

Similar to the limb bud epithelium, the present study and recent studies demonstrated β-catenin regulation of Fgf8 in the epithelium of BA1 (Reid et al., 2011; Sun et al., 2012; Wang et al., 2011). Furthermore, ectopic activation of the β-catenin pathway in the facial epithelium was associated with surface thickening (Fig. S7). The common epithelial β-catenin–Fgf8 pathway in limb buds and BA1 supports the idea of deep homology between the pharyngeal arch and limb bud (Schneider et al., 1999; Shubin et al., 1997, 2009).

Preservation of the molecular machinery of the epithelial β-catenin–Fgf8 pathway in vertebrate limb and jaw development is also important from an evolutionary standpoint. More specifically, analysis of gene expression and patterning in the chondrichthyan gill arch and fin, as well as chick limb buds, suggest that developmental genetic modules controlling limb development may have been co-opted from modules functioning in gill arch development (Gillis et al., 2009). The epithelial β-catenin–Fgf8 pathway might be an example of such a shared genetic module between limbs and gill arches.

**Acknowledgments**

We are grateful to Dr. Juan Carlos Izpisúa Belmonte for in situ probes, and Dr. Yasushi Nakagawa and Dr. Michael O’Connor for the use of their equipment. We thank Thu Quach, Elizabeth West, Jenna Matson, Julia Wong and Brian Schmidt for their excellent technical support, and Austin Johnson for editorial assistance. This work was supported by the National Institute of Dental and Craniofacial Research of NIH to A.P. (DE016601) and by
the National Institute of Arthritis and Musculoskeletal and Skin Diseases of NIH to Y.K. (RO1AR064195).

Appendix. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.01.001.

References


