Development 136, 3969-3978 (2009) doi:10.1242/dev.039438

Dosage-dependent hedgehog signals integrated with Wnt/β-catenin signaling regulate external genitalia formation as an appendicular program

Shinichi Miyagawa[1,2], Anne Moon[3,*], Ryuma Haraguchi[2,*], Chie Inoue[4], Masayo Harada[1], Chiaki Nakahara[4], Kentaro Suzuki[1], Daisuke Matsumaru[4], Takehito Kaneko[2], Isao Matsuo[5], Lei Yang[6], Makoto M. Taketo[7], Taisen Iguchi[8], Sylvia M. Evans[9] and Gen Yamada[1,4,†]

Embryonic appendicular structures, such as the limb buds and the developing external genitalia, are suitable models with which to analyze the reciprocal interactions of growth factors in the regulation of outgrowth. Although several studies have evaluated the individual functions of different growth factors in appendicular growth, the coordinated function and integration of input from multiple signaling cascades is poorly understood. We demonstrate that a novel signaling cascade governs formation of the embryonic external genitalia [genital tubercle (GT)]. We show that the dosage of Shh signal is tightly associated with subsequent levels of Wnt/β-catenin activity and the extent of external genitalia outgrowth. In *Shh*-null mouse embryos, both expression of Wnt ligands and Wnt/β-catenin signaling activity are downregulated. β-catenin gain-of-function mutation rescues defective GT outgrowth and *Fgf8* expression in *Shh*-null embryos. These data indicate that Wnt/β-catenin signaling in the distal urethral epithelium acts downstream of Shh signaling during GT outgrowth. The current data also suggest that Wnt/β-catenin regulates *Fgf8* expression via Lef/Tcf binding sites in a 3′ conserved enhancer. Fgf8 induces phosphorylation of Erk1/2 and cell proliferation in the GT mesenchyme in vitro, yet *Fgf4/8* compound-mutant phenotypes indicate dispensable functions of *Fgf4/8* and the possibility of redundancy among multiple Fgfs in GT development. Our results provide new insights into the integration of growth factor signaling in the appendicular developmental programs that regulate external genitalia development.

KEY WORDS: External genitalia, Genetic cascade, Hedgehog, Fgf, β-catenin (Ctnnb1), Cloaca, Appendages, Mouse

INTRODUCTION

Embryonic development is controlled by a series of basic regulatory processes, including the regulation of protrusion and outgrowth. It has become clear that such developmental processes require coordinated reciprocal interactions between epithelium and the adjacent mesenchyme, frequently mediated through hedgehog, Wnt and fibroblast growth factor (Fgf) pathways. Perturbation of these pathways causes developmental abnormalities in a variety of tissues due, at least in part, to failed cross-talk. Despite the importance of this cross-talk and reciprocal interactions, our understanding of signaling pathway interactions is limited.

An embryonic bud structure (an appendage) is a representative organ that is suitable for analyzing reciprocal interactions between signaling pathways. Protruding embryonic buds are often composed of the distal epithelium accompanied by adjacent proliferating mesenchyme, which eventually gives rise to a bud structure. Significant progress has been achieved in understanding the molecular network that regulates limb development (Capdevila and Izpisua Belmonte, 2001; Chen et al., 2004; Johnson and Tabin, 1997; Kmita et al., 2005; Niswander, 2003; Yamaguchi et al., 1999; Yang et al., 2006; Zhu et al., 2008). Vertebrate limb development depends on the establishment and maintenance of the apical ectodermal ridge (AER), a specialized epithelium at the distal tip of the limb bud. Epithelial-mesenchymal interactions between the AER and its adjacent mesenchyme are essential for limb bud outgrowth. Fgf gene family members are expressed specifically in the AER. The cumulative evidence indicates that AER formation and maintenance and Fgf expression are tightly controlled by intricate interplay among several growth factors (Lewandoski et al., 2000; MacArthur et al., 1995; Mariani et al., 2008; Moon and Capecchi, 2000; Sun et al., 2000; Sun et al., 2002; Yu and Ornitz, 2008). Wnt/β-catenin signaling in the limb ectoderm regulates AER maintenance and *Fgf8* expression (Barrow et al., 2003; Soshnikova et al., 2003). Bmp signaling functions upstream of Wnt/β-catenin signaling in this process, as indicated by a failure of AER formation in mice lacking ectodermal bone morphogenetic protein receptor 1A (*Bmpr1a*) (Ahn et al., 2001). sonic hedgehog (*Shh*) is expressed in posterior limb bud mesenchyme [zone of polarizing activity (ZPA)] (Riddle et al., 1993), patterns the anterior-posterior axis of the limb, and supports expression of Fgf genes in the AER (Benazet et al., 2009; Laufer et al., 1994; Niswander et al., 1994; Riddle et al., 1993; Zuniga et al., 1999).

Another embryonic appendage, the genital tubercle (GT), is the common primordium of male and female external genitalia. GT outgrowth is the result of mesenchymal proliferation around the cloaca, accompanied by formation of the urethral plate epithelium at the ventral midline of the GT. The amniotic cavity and cloacal lumen are separated by two epithelial components: a superficial

[1]Institute of Molecular Embryology and Genetics, Global COE 'Cell Fate Regulation Research and Education Unit', and [2]Center for Animal Resources and Development, Kumamoto University, Kumamoto 860-0811, Japan. [3]Departments of Pediatrics, Neurobiology and Anatomy, and Human Genetics, University of Utah, UT 84112, USA. [4]Graduate School of Molecular and Genomic Pharmacy, Kumamoto University, Kumamoto 860-0811, Japan. [5]Osaka Medical Center and Research Institute for Maternal and Child Health, Osaka 594-1101, Japan. [6]Black Family Stem Cell Institute, Mount Sinai School of Medicine, New York, NY 10029, USA. [7]Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan. [8]National Institutes of Natural Sciences, Okazaki 444-8787, Japan. [9]Skaggs School of Pharmacy, University of California, San Diego, CA 92093, USA.

*These authors contributed equally to this work
[†]Author for correspondence (gensan@gpo.kumamoto-u.ac.jp)

Accepted 14 October 2009

DEVELOPMENT

layer of ectodermal epithelium and a thick inner endodermal cell layer. A unique developmental property of the GT is the coordinated formation of endoderm-derived tissues during its outgrowth and patterning. The endodermal epithelium expresses *Shh*, which influences gene expression in the adjacent mesenchyme (Haraguchi et al., 2001; Perriton et al., 2002). *Shh*-deficient embryos exhibit GT agenesis with loss of *Bmp4* and *Fgf10* expression (Haraguchi et al., 2001; Perriton et al., 2002), suggesting that Shh functions high up in a signaling cascade governing GT development.

As appendages, limb buds and the GT exhibit similarities in their development; for example, they undergo prominent outgrowth as an embryonic bud structure before differentiation of tissue components (Cobb and Duboule, 2005; Dolle et al., 1991; Kondo et al., 1997; Yamada et al., 2003; Yamada et al., 2006). Given the accumulating evidence of the importance of Fgf signaling (and of Fgf8 in particular) from the AER, *Fgf8* is a prime candidate for mediating signaling from the GT epithelium to promote GT mesenchymal proliferation and outgrowth. The GT epithelium, termed the distal urethral epithelium (DUE), is located at the distal tip of the endoderm-derived epithelium (Haraguchi et al., 2001; Haraguchi et al., 2000; Lin et al., 2008; Ogino et al., 2001). Surgical removal of the tip of the GT, including the DUE, results in failure of GT outgrowth (Haraguchi et al., 2000). *Fgf8* has therefore been proposed as a candidate regulator of initial GT development, although its function in GT formation is unknown.

The prospective GT region of *Shh*-deficient embryos fails to express *Fgf8* (Haraguchi et al., 2001; Perriton et al., 2002), suggesting that *Fgf8* is genetically downstream of Shh signaling. Here we show that Wnt/β-catenin signaling plays a key role in GT formation by inducing multiple factors, including *Fgf8*. The temporal requirement of Shh function and the importance of hedgehog signaling dosage are shown by analysis of conditional *Shh* mutants and of a series of Gli mouse mutants. We demonstrate that Wnt/β-catenin pathway activation by Shh signaling is responsible for subsequent GT outgrowth, and postulate that Wnt/β-catenin signaling functions in the DUE to stimulate mitogenic factors for the adjacent mesenchyme. Altogether, these data suggest a similar, and yet divergent, participation of growth factor signaling pathways, including Shh, Wnt/β-catenin and Fgf, in the development of appendicular structure, the external genitalia.

MATERIALS AND METHODS

Mouse strains and embryos

The mutant mice used were *Shh* (Chiang et al., 1996), *Gli2* (Mo et al., 1997), *Gli3*^{xt} (Hui and Joyner, 1993), *Shh*^{CreERT2} (Harfe et al., 2004), *Isl1*^{Cre} (Yang et al., 2006), *Hoxa3-Cre* (Macatee et al., 2003), β-catenin^{Ex3} (*Ctnnb1*^{Ex3}) (Harada et al., 1999), β-catenin^{lox} (*Ctnnb1*^{lox}) (Huelsken et al., 2001), *Shh*^{lox} (Dassule et al., 2000), *Fgf4*^{lox} (Moon et al., 2000), *Fgf8*^{lox} (Park et al., 2006), *R26R* (Soriano, 1999), *TopGAL* (DasGupta and Fuchs, 1999) and *BatGAL* (Nakaya et al., 2005). To increase the efficiency of production of homozygous null embryos and decrease the incidence of mosaic deletion, β-catenin-null alleles were generated by utilizing *CAGGS-Cre* mice (Araki et al., 1997), which express Cre recombinase in the germline. Noon on the day when a vaginal plug was detected was designated as E0.5. Embryos for each experiment were collected from at least three pregnant females. All procedures and protocols were approved by the Committee on Animal Research at Kumamoto University, Japan.

The tamoxifen (TM)-inducible Cre recombinase system removes the floxed sequence from the target genome (Feil et al., 1997). TM (Sigma, St Louis, MO, USA) was dissolved in sesame oil at 10 mg/ml. Four milligrams (*Ctnnb1*^{lox} mice) or 2 mg (*Ctnnb1*^{Ex3} and *Shh*^{lox} mice) of TM per 40 g body weight was used to treat the pregnant mice. Under these conditions, no overt teratologic effects on the urogenital organs are observed (Haraguchi et al., 2007).

Histology

Hematoxylin and Eosin staining, X-Gal staining, immunohistochemistry and in situ hybridization for gene expression were performed by standard procedures as previously described (Haraguchi et al., 2007). Immunohistochemistry employed the following primary antibodies: Cd44, β-catenin, E-cadherin (BD Biosciences, Franklin Lakes, NJ, USA) and phospho-Erk1/2 (Cell Signaling, Danvers, MA, USA). For in situ hybridization, the following riboprobe templates were used: *Tcf1* (H. Clevers, University Medical Center Utrecht, The Netherlands); *Pitx1* (Y. Chen, Tulane University, New Orleans, LA, USA); *Fgf3* (C. Dickson, Imperial Cancer Research Fund, London, UK); *Fgf4* (G. Martin, University of California, San Francisco, CA, USA); *Wnt3*, *Wnt4*, *Wnt5a*, *Wnt7a*, *Wnt9b* (S. Takada, National Institutes of Natural Sciences, Okazaki, Japan); and *Fgf4*, *Fgf8*, *Fgf10*, *Bmp4*, *Gli1*, *Ptch1*, *Shh*, *Dlx5* (Haraguchi et al., 2007; Suzuki et al., 2008). The template of *Axin2* was obtained by standard RT-PCR procedures using primers 5′-CCACTTCAAGGAGCAGCTCAGCA-3′ and 5′-TACCCAGGCTCCTGGAGACTGA-3′.

Cell proliferation and death analyses

Pregnant females were injected with 100 mg BrdU (Sigma) per kg body weight. One hour after injection, embryos were collected. For cell culture experiments, BrdU (1 μg/ml) was added to the medium for 30 minutes and BrdU incorporation detected with anti-BrdU antibody (Roche, Mannheim, Germany). TUNEL assay for the detection of apoptotic cells was performed with the In Situ Apoptosis Detection Kit (Takara, Ohtsu, Japan).

Organ culture

Filter-supported organ cultures for murine GTs isolated from embryos of ICR strain (E11.5; CLEA, Tokyo, Japan) were as previously described (Haraguchi et al., 2000). The tissues were cultured for 24 hours after bead application and processed for histological analysis. Heparin acrylic beads were soaked overnight with recombinant mouse Fgf8b protein at 1.0 mg/ml in PBS (Haraguchi et al., 2000). Control beads were treated with PBS containing 0.1% BSA.

Plasmid DNA and luciferase assay

The conserved region 3′ of the *Fgf8* locus was described previously (Beermann et al., 2006). Genomic sequences of the 5′ region and conserved region 3 (CR3) of *Fgf8* loci, obtained from Ensemble (www.ensembl.org), were submitted for analysis by rVISTA (genome.lbl.gov/vista). DNA fragments of CR3 of the murine *Fgf8* locus, obtained from a BAC clone (RPCI23-98F2) by PCR, were inserted into the pGL4.24 vector (Promega, Madison, WI, USA). The mouse β-catenin expression vector was kindly provided by Dr S. Kume (Takahashi et al., 2000).

The HaCat cell line was maintained in DMEM supplemented with 10% FBS. Cells were transfected with expression and reporter plasmids using FuGENE HD (Roche) according to the manufacturer's instruction. Twenty-four to thirty hours post-transfection, luciferase activity was measured by chemiluminescence employing the Dual-Luciferase Reporter Assay System (Promega). The values were normalized against *Renilla* luciferase activity. At least three independent experiments were performed. Statistical analysis was performed using Student's *t*-test or Welch's *t*-test followed by F-test ($P<0.05$ considered significant).

Chromatin immunoprecipitation (ChIP) assay

The ChIP Assay Kit (Upstate, Lake Placid, NY, USA) was used. The distal GT region containing the DUE, and the distal tip of the hindlimb containing the AER, were dissected from embryos at E12.5 and E10.5, respectively. β-catenin (Santa Cruz) and acetyl-histone H3 (Upstate) antibodies (2 μg) were used. For mock control, rabbit or mouse immunoglobulin (Dako, Carpinteria, CA, USA) was used. More than three independent experiments were performed. PCR was performed with the following primers: 5′ flanking region, 5′-CAGAGAGAGCCGTTTGTGTTGG-3′ and 5′-TCAAAGCCCCGTAATTACAATTGC-3′; CR3, 5′-CTGGCTGAAA-GCCACAGACG-3′ and 5′-GCTGGGTCTCTGCTGGTAACC-3′.

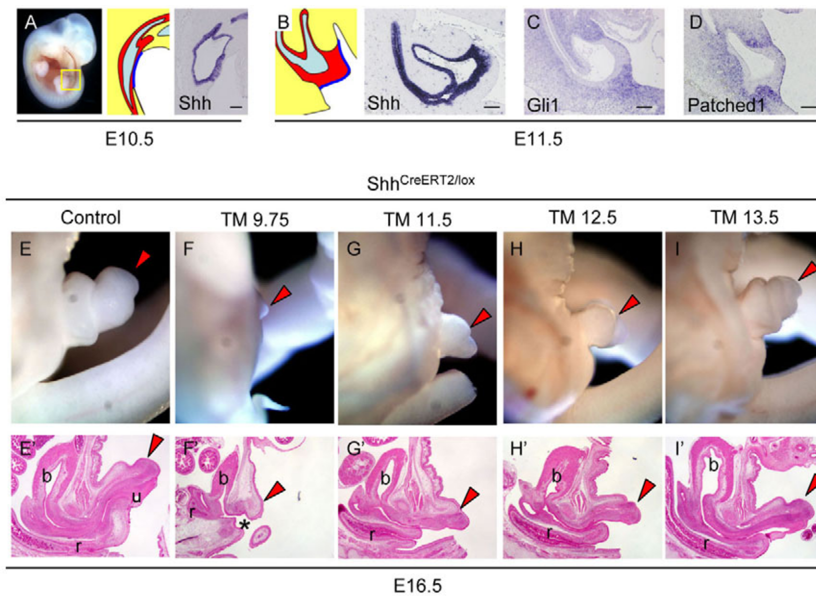

Fig. 1. Temporal requirements for hedgehog signaling during GT initiation and outgrowth. (**A**,**B**) Murine GT development and *Shh* expression at E10.5 (A) and E11.5 (B). Red regions indicate *Shh*-expressing endoderm-derived epithelia. The cloacal region includes the thick endodermal epithelia (in red, B). The dark-blue line indicates adjacent ectoderm-derived epithelium. Cloacal cavity is in light blue. (**C**,**D**) *Gli1* (C) and patched 1 (D) expression in GT mesenchyme and ectoderm at E11.5. (**E-I′**) Phenotypes after temporally staggered ablation of *Shh*. GT phenotypes of $Shh^{CreERT2/lox}$ embryos treated with TM at E9.75 (F), E11.5 (G), E12.5 (H) and E13.5 (I) versus the control GT (no TM administration, E). TM administration at E9.75 induced severe GT aplasia with a persistent cloaca (asterisk). Arrowheads indicate GTs or GT rudiments. b, bladder; r, rectum; u, urethra. Scale bars: 100 μm.

RESULTS

Progressive GT formation revealed by conditional *Shh* mutation

The murine GT begins to visibly protrude as a bud structure at E10.5-11.5. *Shh* is expressed in the endodermal cloacal epithelium at this time (Fig. 1A,B) and the hedgehog-responsive genes *Gli1* and patched 1 (*Ptch1*) are expressed in the adjacent mesenchyme and ectoderm (Fig. 1C,D). *Shh* knockout (KO; $Shh^{-/-}$) embryos exhibit a complete failure of GT outgrowth (Haraguchi et al., 2001). We analyzed the temporal dependence of GT development on Shh genetically by crossing $Shh^{CreERT2}$ and Shh^{lox} alleles, which permits inducible Cre function and *Shh* inactivation in the cloacal endodermal cells and in cells of urethral plate epithelium (UPE) upon TM administration (Harfe et al., 2004). In control embryos, a prominent GT protrusion was observed, the bladder developed normally, and the urethral and rectal orifices were completely separated (Fig. 1E). In $Shh^{CreERT2/lox}$ mutants, administration of TM at E9.75 caused severe GT hypoplasia, accompanied by a persistent cloaca; the urethral and rectal ends were shared and opened at a common orifice in the rudimentary GT (Fig. 1F). Expression of the distal marker genes *Wnt5a* and *Dlx5* was not detected in the mutants, although expression of *Pitx1* as a dorsal marker was still observed (see Fig. S1 in the supplementary material). TM administration at sequentially later stages elicited progressively milder GT phenotypes (Fig. 1G-I), such that administration of TM at E13.5 resulted in morphologically normal GTs.

Decreased expression of Wnt ligand genes in *Shh* mutant GT

Shh elicits mesenchymal and epithelial cellular responses and regulates the expression of several downstream genes during GT development (Haraguchi et al., 2001). However, the signaling pathways downstream of Shh that are required for GT development have not been identified. Because of the importance of Fgf/Shh interactions in limb AER and mesenchyme, and given that Wnt ligands can regulate *Fgf8* expression and limb outgrowth (Barrow et al., 2003; Kawakami et al., 2001; Kengaku et al., 1998), we examined the expression of Wnt ligand genes in the GT. Several Wnt ligand genes were expressed in the GT (as assessed by RT-PCR, data not shown). Among them, *Wnt3*, *Wnt4*, *Wnt7a* and *Wnt9b* were downregulated in the GT of *Shh* KO mutant embryos (Fig. 2A-D), particularly in the GT ectoderm (Fig. 2E-H).

Regulation of Wnt/β-catenin activity by Shh signaling

To examine whether the decreased expression of ectodermal Wnt ligands results in decreased Wnt/β-catenin signaling in *Shh* KO mutants, we assayed β-galactosidase (β-gal) activity from the *TopGAL* allele (DasGupta and Fuchs, 1999). During normal GT development, weak β-gal activity is detected in the cloacal region at E10.5 (Fig. 3A) (Lin et al., 2008). At E11.5, increased β-gal activity was observed in the distal region of the endodermal epithelium (Fig. 3B-D), which overlapped, in part, with the DUE as defined by *Fgf8*

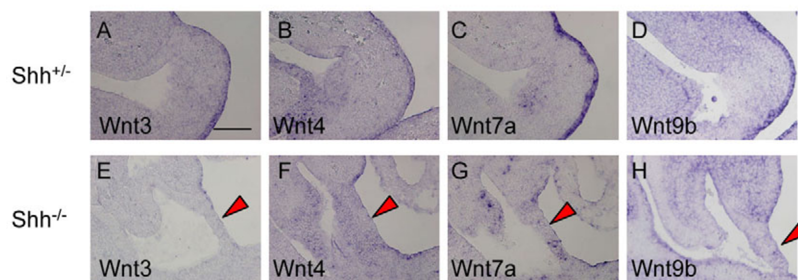

Fig. 2. Expression of Wnt ligands in control and *Shh* KO mouse embryos at E11.5. (**A-H**) Ectodermal expression (arrowheads) of *Wnt3* (A,E), *Wnt4* (B,F), *Wnt7a* (C,G) and *Wnt9b* (D,H) is decreased in *Shh* KO embryos. Scale bar: 100 μm.

DEVELOPMENT

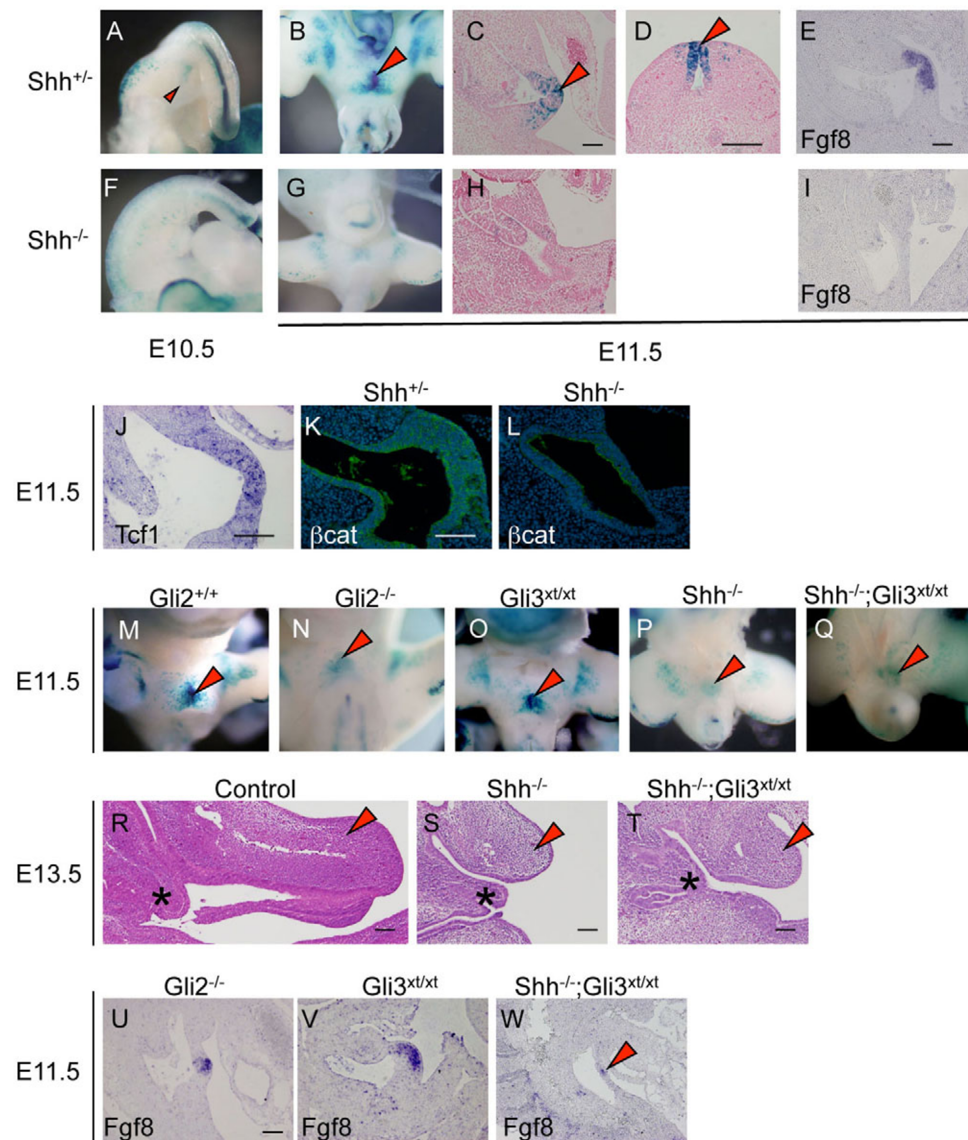

Fig. 3. Wnt/β-catenin signaling is responsive to hedgehog signaling dosage in the mouse GT. (**A-E**) TopGAL activity (arrowheads) is detectable in the distal region of the GT, including the DUE at E10.5 (A) and E11.5 (B-D). *lacZ* staining (C,D, arrowheads) overlaps with the *Fgf8* expression domain (E). (**F-I**) TopGAL activity (F-H) and *Fgf8* expression (I) are not detected in *Shh* KO embryos. (**J**) *Tcf1* is expressed in the distal GT. (**K**,**L**) β-catenin protein localizes predominantly to the endodermal epithelium (K, green) and decreases in *Shh* KO embryos (L). (**M-Q**) *Gli2* KO embryos have decreased TopGAL reporter activity (N) relative to control (M) and *Gli3$^{xt/xt}$* embryos (O). *Shh$^{–/–}$;Gli3$^{xt/xt}$* double mutants exhibit weak, but detectable, TopGAL activity (Q, arrowhead) as compared with *Shh* KO embryos (P). (**R-T**) Sections of control (R), *Shh* KO (S) and *Shh$^{–/–}$;Gli3$^{xt/xt}$* (T) embryos. GT outgrowth is restored in the *Shh$^{–/–}$;Gli3$^{xt/xt}$* double mutants at E13.5 (arrowhead). Asterisk indicates the distal end of the urorectal septum. (**U-W**) GT outgrowth and *Fgf8* expression in hedgehog pathway mutants at E11.5. Decreased *Fgf8* expression and GT hypoplasia in *Gli2* KO embryos (U, compare with E). *Gli3$^{xt/xt}$* embryos have normal GT protrusion and *Fgf8* expression (V). *Shh$^{–/–}$;Gli3$^{xt/xt}$* embryos exhibit a small bud structure with some *Fgf8* expression (W, arrowhead). Scale bars: 100 μm.

expression (Fig. 3E). This β-gal activity in the DUE persisted throughout multiple stages of GT development (see Fig. S2 in the supplementary material). In *Shh* KO embryos, TopGAL activity was almost undetectable in the cloaca at E10.5 and E11.5 (Fig. 3F-H), and was accompanied by a loss of *Fgf8* expression in the DUE (Fig. 3I). We obtained similar results with the *BatGAL* allele (see Fig. S3 in the supplementary material), another Wnt/β-catenin signaling indicator mouse line (Nakaya et al., 2005). β-catenin (*Ctnnb1*) and *Tcf1* (*Tcf7* – Mouse Genome Informatics) encode essential components of the Wnt signaling pathway and are expressed in the cloacal epithelium in an overlapping manner (Fig. 3J,K). β-catenin immunoreactivity in the cloacal epithelium and in the distal endodermal epithelium (inclusive of the DUE) was reduced in *Shh* KO embryos relative to controls at E11.5 (Fig. 3L). In *Shh$^{CreERT2/lox}$* mutants, E-cadherin (cadherin 1) expression in the cloacal membrane was normal, whereas β-catenin expression was decreased (see Fig. S4 in the supplementary material). These results suggest that decreased Wnt activity in the *Shh* mutants is likely to be induced by the defective signaling cascade.

We then assessed TopGAL activity in mouse mutants of various hedgehog signaling components. In *Gli2* KO embryos, β-gal activity was decreased and the GT hypoplasic, consistent with the general role of Gli2 as a positive effector of Shh signaling (Fig. 3M,N). Gli3 generally functions as a transcriptional repressor for hedgehog signaling (Ingham and McMahon, 2001). Although *Gli3* mutants (*Gli3$^{xt/xt}$*) do not have significant defects in GT outgrowth, *Shh;Gli3* double-mutant embryos (*Shh$^{–/–}$;Gli3$^{xt/xt}$*) restored TopGAL activity in the GT relative to that in *Shh* mutants, consistent with the dosage sensitivity of this structure to *Shh* (Fig. 3O-Q). GT outgrowth was detectable at E13.5 in *Shh;Gli3* double mutants, indicating partial rescue of the effect of *Shh* loss-of-function on outgrowth (Fig. 3R-T). These results are the first demonstration of the integrated effects of Gli2 and Gli3 in mediating hedgehog-dependent GT development, and of the dependence of Wnt/β-catenin activity during GT formation on Shh signaling. The activity of both signaling pathways correlates with the extent of GT outgrowth and *Fgf8* expression (Fig. 3U-W).

Requirement for β-catenin during GT development

Wnt/β-catenin signaling is required for caudal body formation; *Wnt3a$^{–/–}$*, *Lef1$^{–/–}$;Tcf1$^{–/–}$* and *Tcf1$^{–/–}$;Tcf4$^{–/–}$* embryos exhibit severe caudal truncation with GT agenesis (Dunty et al., 2008; Galceran et al., 1999; Gregorieff et al., 2004; Takada et al., 1994). To focus on the role of β-catenin specifically during GT formation, β-catenin

DEVELOPMENT

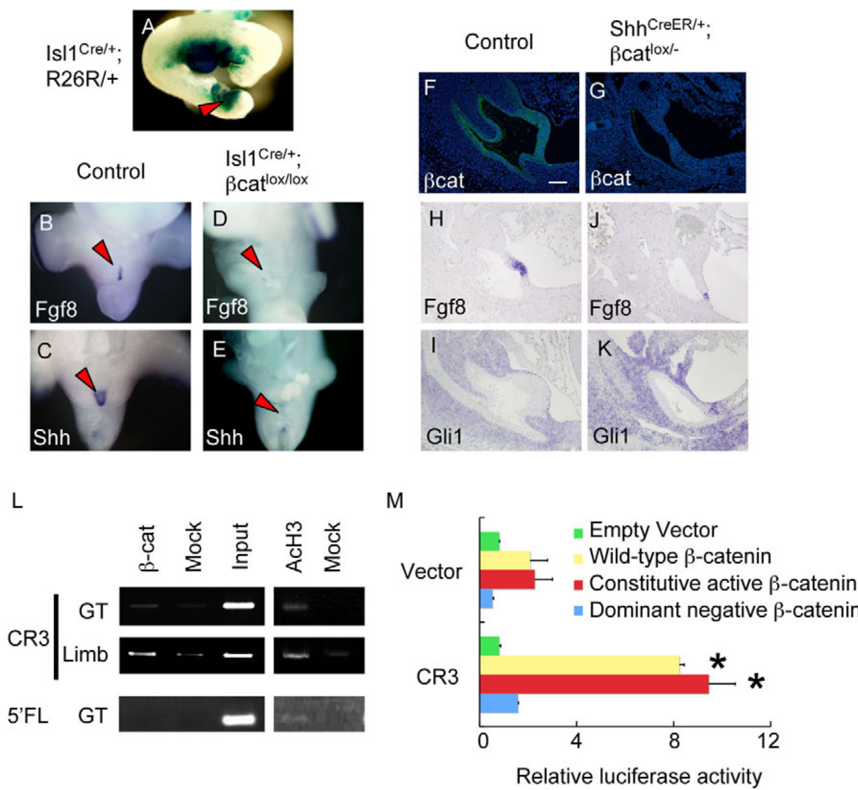

Fig. 4. Gene expression in β-catenin loss-of-function mutants. (**A**) *Isl1-Cre*-mediated *lacZ* expression in *R26R* mice. Cre is active in the caudal E9.0 embryo (arrowhead). (**B-E**) E11.5 control embryos (B,C). *Fgf8* (D) and *Shh* (E) expression (arrowheads) in $Isl^{Cre/+}$;$Ctnnb1^{lox/lox}$ embryos. (**F-K**) Failed GT outgrowth in E11.5 endoderm-specific β-catenin (βcat) conditional mutants (F,G). Green, immunofluorescence for β-catenin; blue, Hoechst counterstain. *Fgf8* expression is abnormal (H,J), whereas *Gli1* is normal (I,K), at E11.5. (**L**) ChIP/PCR on GT and limb reveals that β-catenin can bind to the murine CR3 region but not to the 5′ flanking region. Acetylated histone H3 is bound to both regions. (**M**) The CR3 putative enhancer activates expression of a luciferase reporter in response to overexpressed wild-type or constitutively active β-catenin. *, $P<0.05$ versus empty vector control. Scale bar: 100 μm.

conditional mutant mice were analyzed. We employed the $Isl1^{Cre}$ line as it drives Cre activity in the caudal region of embryos as early as E9.0 (Fig. 4A). Thus, $Isl1^{Cre}$ is useful for analyzing the developmental processes in the prospective GT region before GT outgrowth. In $Isl1^{Cre/+}$;$Ctnnb1^{lox/lox}$ mutants, the GT failed to protrude and expression of *Fgf8* and *Shh* was absent in the DUE at E11.5 (Fig. 4B-E). This raises the intriguing possibility that an early phase of β-catenin activity is necessary for cloacal *Shh* expression.

By contrast, the temporally inducible $Shh^{CreERT2}$ line is suitable for analyzing gene function during later stages of GT development, as Shh-dependent outgrowth proceeds. In $Shh^{CreERT2/+}$;$Ctnnb1^{lox/–}$ embryos, the levels of β-catenin protein in the GT region (including the DUE) were reduced at E11.5 and GT outgrowth failed (Fig. 4F,G). *Fgf8* expression was markedly reduced in the mutants (Fig. 4H,J). *Gli1* was expressed normally at these stages (Fig. 4I,K), indicating that Shh signaling is not dependent on Wnt/β-catenin signaling at this later stage.

Potential regulation of *Fgf8* expression by β-catenin

To examine the possibility that *Fgf8* expression is directly regulated by β-catenin, chromatin immunoprecipitation (ChIP) and reporter assays were performed for a region 3′ of the *Fgf8* locus that contains an evolutionarily highly conserved putative *Fgf8* enhancer. Conserved region 3 [CR3 (Beermann et al., 2006)] is a candidate enhancer that regulates reporter expression in the AER, and we considered whether it might also function as a DUE enhancer given the similarities between the DUE and the AER as a transient distal signaling epithelium. Using rVISTA, we found that CR3 contains several Lef/Tcf binding sites (see Fig. S5 in the supplementary material). We performed a ChIP assay followed by PCR using primers that amplify a CR3 genomic fragment that includes these binding sites. β-catenin-specific enrichment was observed in the extracts from the GT and limb bud (Fig. 4L). PCR amplification of a putative enhancer 5′ of *Fgf8* (Hu et al., 2004), which is also highly conserved in vertebrates but lacks Lef/Tcf binding sites, yielded no enrichment (Fig. 4L). Both regions were enriched in chromatin immunoprecipitated with anti-acetylated histone H3, as a positive control. Furthermore, overexpression of wild-type or constitutively active β-catenin activated transcription of a CR3 enhancer/luciferase reporter in HaCat cells, whereas a dominant-negative form did not (Fig. 4M). Taken together, these results suggest that Wnt/β-catenin signaling participates in the regulation of *Fgf8* expression via CR3.

Fgf8 can induce phosphorylation of Erk1/2 and cell proliferation in GT mesenchyme, but Fgf4 and Fgf8 are dispensable for GT outgrowth

In the limb, AER-derived Fgf induces phosphorylated (p) Erk1/2 (Mapk3/1 – Mouse Genome informatics) activity in the adjacent mesenchyme (Kawakami et al., 2003). Organ culture experiments with explanted GTs revealed that GT mesenchyme adjacent to Fgf8b-soaked beads has a higher level of pErk1/2 than that with a contralaterally implanted control bead (Fig. 5A). GT mesenchymal cell proliferation was also consistently increased by Fgf8b treatment of cultured GTs (Fig. 5B).

To investigate Fgf functions during GT development in vivo, we conditionally inactivated *Fgf8* in the GT with a *Hoxa3-Cre* line, which exhibits strong Cre activity throughout the caudal embryo, including the presumptive cloacal region, GT primordium and DUE (see Fig. S6A in the supplementary material) (Macatee et al., 2003). In situ hybridization using an *Fgf8* antisense riboprobe specific for the floxed exon 5 (Moon and Capecchi, 2000) confirmed the absence of *Fgf8* expression in the *Hoxa3-Cre;Fgf8* mutants (see Fig. S6B,C in the supplementary material). Notably, we found that although *Fgf4* expression was barely detected in the normal GT, ablation of *Fgf8* resulted in increased *Fgf4* expression in the DUE

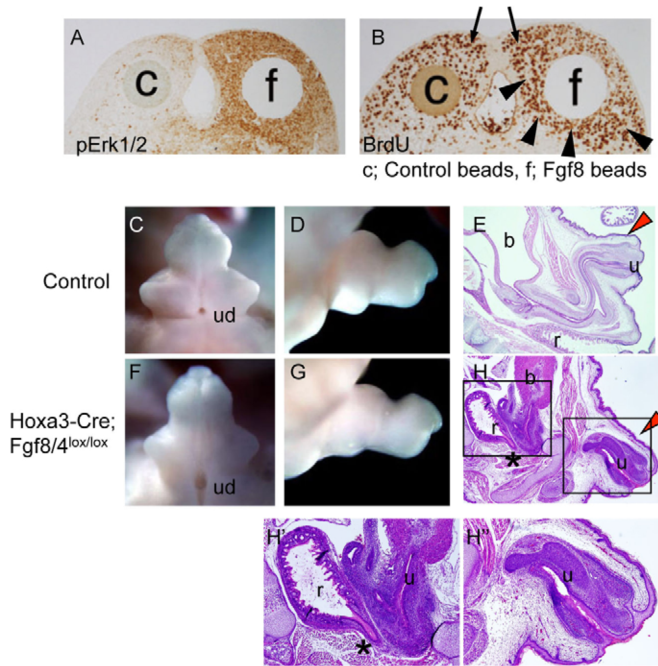

Fig. 5. *Fgf8* and *Fgf4* are dispensable for mouse GT development. (**A**,**B**) Anti-pErk1/2 and BrdU immunostaining in GT explants. pErk1/2 activity is restricted to distal GT with the control (c) bead (A). pErk1/2 (A) and BrdU incorporation (B) are augmented in regions adjacent to Fgf8 (f) beads. Arrowheads and arrows indicate augmented and endogenous cell proliferation, respectively. (**C-H″**) GT phenotype of control (C-E) and *Hoxa3-Cre;Fgf8/4$^{lox/lox}$* mutant embryos (F-H″) at E14.5 (C,D,F,G) and E18.5 (E,H-H″). GT outgrowth appears normal in mutants (arrowheads), but the urogenital duct (ud) is wide open and anal stenosis (asterisk) and urethral narrowing with proximal atresia are also present. The distal urethra appears normal. b, bladder; r, rectum; u, urethra.

(see Fig. S6D,E in the supplementary material), similar to what has been reported following *Fgf8* ablation in the AER (Lewandoski et al., 2000; Moon and Capecchi, 2000). We analyzed *Fgf8;Fgf4* double-mutant embryos and found, surprisingly, that the mutant GT appeared to have normal outgrowth at E14.5 (Fig. 5C,D,F,G) and only mild abnormalities of the external genitalia at E18.5. However, these mutants did have anal stenosis and a narrowed, proximally atretic urethra, whereas the distal urethra appeared normal (Fig. 5E,H). Thus, although Fgf8 can induce Erk1/2 phosphorylation and cell proliferation in the GT in vitro, it is dispensable for GT outgrowth. In the AER and elsewhere, Fgf ligands have overlapping expression patterns and functional redundancy has been reported (Boulet et al., 2004; Ladher et al., 2005; Mariani et al., 2008; Moon and Capecchi, 2000; Sun et al., 2002). In addition to *Fgf4*, we also found increased expression of *Fgf3* in the distal GT and in the limb of *Hoxa3-Cre;Fgf8$^{lox/lox}$;Fgf4$^{lox/lox}$* (*Fgf8/4$^{lox/lox}$*) mutants (see Fig. S7 in the supplementary material).

Constitutively active β-catenin rescues GT outgrowth in *Shh* mutants

Our current findings suggest that β-catenin functions downstream of Shh to support GT outgrowth; if so, overexpression of β-catenin should at least partially rescue GT agenesis in *Shh* KO mutants. We generated *Shh$^{CreERT2/–}$;Ctnnb1$^{Ex3/+}$* mutant embryos, which express a constitutively active β-catenin (*Ctnnb1^{Ex3}*) in a *Shh*-null background. Indeed, TM treatment at E9.5 resulted in visible outgrowth of the GTs of these compound mutants at E11.5, in contrast to GT agenesis in the *Shh$^{CreERT2/–}$;Ctnnb1$^{+/+}$* (equivalent to the *Shh*-null) embryos (Fig. 6A-C). At E13.5, GT outgrowth and a preputial fold were present, although smaller than normal (Fig. 6F-H). Surviving mutant embryos at E18.5 displayed a bud structure composed of mesenchymal tissue without the urethra (Fig. 6I,J; data not shown). It is noteworthy that hedgehog signaling was not activated in the *Shh$^{CreERT2/–}$;Ctnnb1$^{Ex3/+}$* embryos, judging by *Gli1* expression (Fig. 6D,E), whereas *Fgf8* was expressed at high level in the *Shh$^{CreERT2/–}$;Ctnnb1$^{Ex3/+}$* embryos (Fig. 7A-C). The *Fgf8* expression domain coincided with the location of β-catenin augmentation, as evident by expression of *Axin2*, a downstream target of the Wnt/β-catenin pathway (Fig. 7D-F).

Cd44, another AER marker (Sherman et al., 1998), is expressed in a similar manner to *Fgf8* in the DUE (Fig. 7G). Consistent with rescued *Fgf8* expression in *Shh$^{CreERT2/–}$;Ctnnb1$^{Ex3/+}$* embryos, Cd44 expression was also restored (Fig. 7H,I). Expression of *Fgf10* and *Bmp4* in the GT mesenchyme is regulated by *Shh* and, consequently, their expression is decreased in *Shh* KO embryos (see Fig. S8A,B,D,E in the supplementary material) (Haraguchi et al., 2001; Perriton et al., 2002). *Fgf10* and *Bmp4* expression was not rescued in the GT mesenchyme of *Shh$^{CreERT2/–}$;Ctnnb1$^{Ex3/+}$* embryos (see Fig. S8C,F in the supplementary material).

In wild-type mouse embryos, pErk1/2 immunoreactivity is present mainly in the mesenchyme (Fig. 7J), and is decreased in *Shh* KO embryos (Fig. 7K). By contrast, the mesenchymal domain of pErk1/2 expression was markedly enhanced in the GT of *Shh$^{CreERT2/–}$;Ctnnb1$^{Ex3/+}$* embryos (Fig. 7L), consistent with increased Fgf8 signaling. Abundant cell proliferation was observed in control embryos (Fig. 7M), but in *Shh* KO embryos mesenchymal cell proliferation was specifically decreased (Fig. 7N). Cell death in the endodermal epithelium and mesenchyme was increased in the distal GT (Fig. 7P,Q). It has been reported that the normal DUE, as with other signaling epithelia, displays a scattered pattern of apoptosis concomitant with reduced cell proliferation (Fig. 7M,P) (Haraguchi et al., 2001; Jernvall et al., 1998). In *Shh$^{CreERT2/–}$;Ctnnb1$^{Ex3/+}$* embryos, mesenchymal cell proliferation was largely rescued (Fig. 7O), but mesenchymal cell death remained elevated above that seen in controls (Fig. 7R).

DISCUSSION

Coordinated growth factor signaling is essential for regulating morphogenesis of appendicular structures such as the limbs and external genitalia. Although previous studies significantly advanced our knowledge of individual gene contributions, understanding how these genes interact and how multiple signaling inputs are integrated during organogenesis remains a major challenge. Here we report that hedgehog signaling is essential for external genitalia development in a dosage-dependent manner, as demonstrated by analyses of different Gli compound mutants. Different levels of hedgehog signaling modulate the level of Wnt/β-catenin activity to regulate outgrowth of the embryonic external genitalia. *Shh* KO embryos display a persistent cloaca and failed GT outgrowth (Haraguchi et al., 2001; Mo et al., 2001; Perriton et al., 2002). Remarkably, we found that overexpression of constitutively active β-catenin rescues GT protrusion in the absence of *Shh* and *Fgf8* expression in the endoderm is restored as is mesenchymal proliferation. To our knowledge, this is the first demonstration of genetic rescue of embryonic appendicular agenesis.

DEVELOPMENT

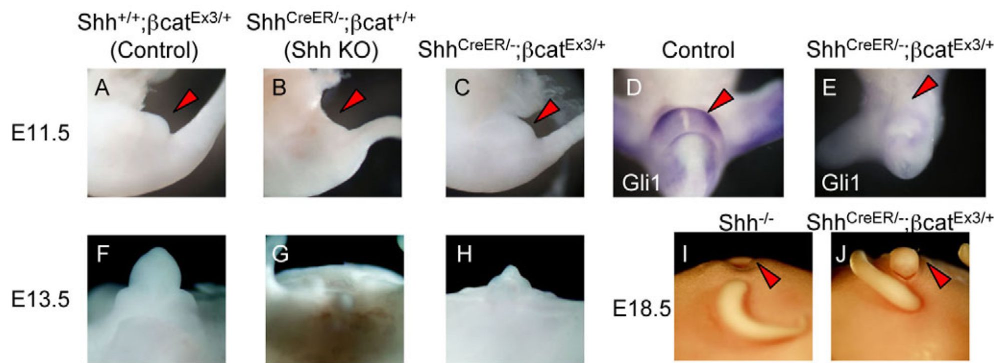

Fig. 6. Rescue of GT outgrowth in *Shh*-null mutants by constitutively active β-catenin. (**A-J**) Control GTs (A,F) and *Shh* KO mouse embryos with GT agenesis (B,G,I). $Shh^{CreERT2/-}$;$Ctnnb1^{Ex3/+}$ embryos have GT protrusion at E11.5 and continued GT outgrowth with preputial fold at E13.5 (C,H). Further outgrowth is evident by E18.5 (J). *Gli1* expression is undetectable in $Shh^{CreERT2/-}$;$Ctnnb1^{Ex3/+}$ embryos at E11.5 (D,E). Arrowheads indicate GT or prospective GT regions.

Integrated Gli and Shh signaling for GT development; hedgehog functions as a trigger for GT initiation and promotes subsequent outgrowth

Several reports have demonstrated the contribution of multiple growth factors to GT development, including hedgehog, Wnt and Fgf signaling (Haraguchi et al., 2001; Haraguchi et al., 2000; Lin et al., 2008; Morgan et al., 2003; Perriton et al., 2002; Suzuki et al., 2003; Yamaguchi et al., 1999). Shh plays a crucial role in external genitalia development (Haraguchi et al., 2001; Perriton et al., 2002). Here, stage-specific *Shh* conditional mutant analysis reveals the temporal requirements for *Shh* function during GT development. Early loss of *Shh* function induces minimal GT outgrowth and the cloaca persists, but later gene ablation elicits milder GT phenotypes. This might recapitulate early, transient requirements for *Shh* for the rapid growth of digit primordia (and digit patterning) in the limb (Zhu et al., 2008), and epithelium-derived *Shh* function in regulating tooth organ size (Dassule et al., 2000). Several pathways have been postulated to regulate proximal-distal and dorsal-ventral axis formation of the GT (Suzuki et al., 2003; Suzuki et al., 2008; Yamada et al., 2003; Yamada et al., 2006). If Shh only functions as a growth-promoting factor during early GT development, axis marker gene expression should have been normal in the early *Shh* conditional mutant GTs. However, *Dlx5* and *Wnt5a* expression was abnormal, suggesting that proximal-distal axis formation was altered by early loss of *Shh* function. However, it is necessary to consider the unique character of GT development, as its robust outgrowth from the beginning depends substantially on *Shh*. Namely, the proximal-distal patterning is tightly linked with such robust outgrowth promotion of the anlage. Hence, it is not simply possible to make a conclusion for the status of proximal-distal axis formation by the current data. By contrast, expression of *Pitx1*, as a candidate dorsal marker during GT development (our unpublished results) (Haraguchi et al., 2007), was unaffected by loss of Shh function. It has recently been suggested from lineage analysis that the dorsal side of the GT develops in association with more-anterior regions, including the peri-cloacal mesenchyme (PCM) and the lower body wall regions (Haraguchi et al., 2007). The sustained dorsal marker gene expression might reflect such a unique program of GT development and the nature as a dorsal structure is less affected by *Shh*.

Hedgehog signal transduction comprises a series of complex intracellular events. Shh signaling modulates the function of Gli transcription factors. In mammals, Gli2 is a major positive transcriptional effector of hedgehog signal transduction. In the absence of Shh, Gli2 is completely degraded, and Gli3 is converted into a transcriptional repressor, Gli3R (Ingham and McMahon, 2001). By titrating the relative amounts of these factors, we found that both the extent of GT outgrowth and the level of Wnt/β-catenin activity are responsive to the effective level of hedgehog signaling. In the wild-type situation, Shh activates Wnt/β-catenin signaling and GT outgrowth, but in *Shh* KO embryos the net effect of the absence of Gli2 activator and presence of Gli3R leads to the most severe phenotype: GT agenesis. The contribution of Gli3R to the *Shh* KO phenotype is indicated by the restoration of TopGAL activity and moderate GT outgrowth in *Shh;Gli3* double mutants ($Shh^{-/-}$;$Gli3^{xt/xt}$). Shh activity during GT outgrowth is likely to prevent formation of Gli3R. In *Gli2* KO embryos, Shh prevents formation of Gli3R, but loss of Gli2 activator causes a modest decrease in downstream Wnt/β-catenin activity and the phenotypic outcome is GT hypoplasia. Thus, normal GT development requires the Shh pathway as balanced by the activation of Gli2 and Gli3. Integrated Gli function has been thoroughly explored with regard to anterior-posterior patterning of the limb, in which a major role of Shh is to prevent formation of Gli3R (Nieuwenhuis and Hui, 2005). In the GT, Gli2 activation downstream of Shh is essential for outgrowth, and inhibition of Gli3R production appears less important.

Context-dependent cross-talk between Wnt/β-catenin and Shh signaling

Shh KO embryos exhibit failed GT outgrowth and loss of *Fgf8* expression in the DUE (Haraguchi et al., 2001; Perriton et al., 2002). In addition to our finding that hedgehog signaling regulates the level of Wnt/β-catenin activity in a dosage-dependent manner, we found that Wnt/β-catenin in turn regulates *Fgf8* expression. Surgical removal of the ectodermal epithelium results in GT truncation, indicating an inductive role of the ectodermal epithelium in GT development (Murakami and Mizuno, 1986). We found that the expression of several ectodermal Wnt ligand genes was reduced in *Shh* KO embryos. Indeed, Wnt/β-catenin activity in the cloacal membrane and later DUE was affected by the level of hedgehog signaling. Ectoderm-derived Wnt ligands are likely candidates for induction of Wnt/β-catenin activity in the distal GT, including the DUE. In the limb, ablation of *Wnt3* from the limb ectoderm results in agenesis as the most severe outcome, suggesting a similarly essential role for ectodermal Wnt ligands in appendicular outgrowth (Barrow et al., 2003).

DEVELOPMENT

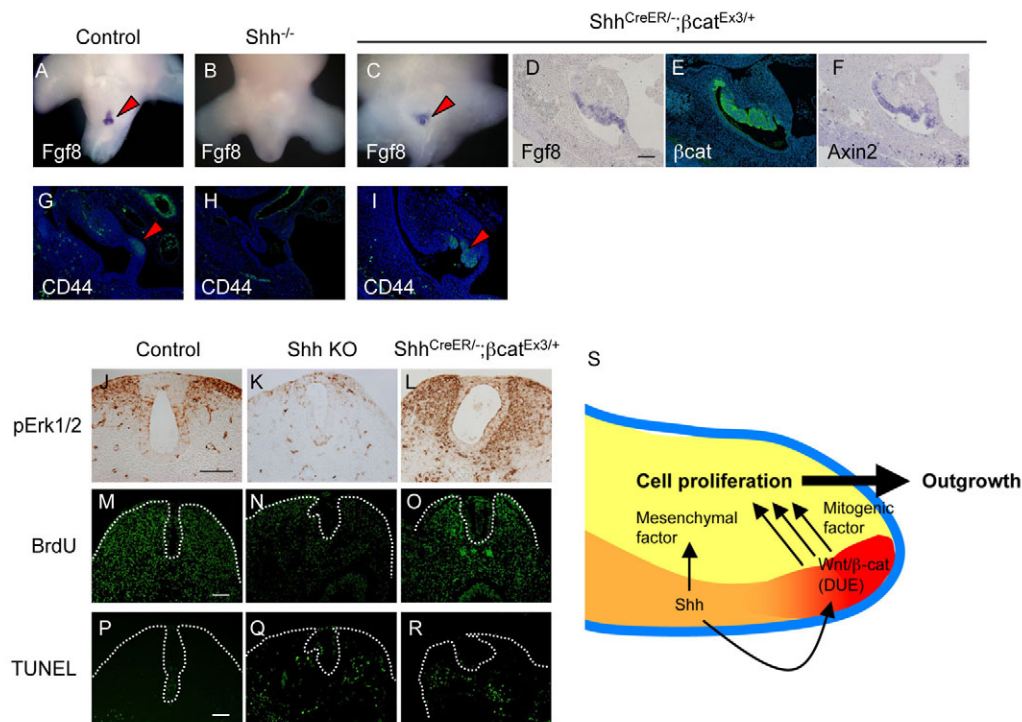

Fig. 7. Regulation of *Fgf8* expression by Shh and β-catenin. (**A-I**) *Fgf8* (A,B) and Cd44 (G,H) expression (arrowheads) in control (A,G) versus *Shh* KO (B,H) DUE. Expression is recovered in $Shh^{CreERT2/-};Ctnnb1^{Ex3/+}$ embryos (arrows in C,I). *Fgf8* and Cd44 expression overlap with regions of augmented β-catenin and *Axin2* (D-F,I). (**J-L**) pErk1/2 staining in control (J) versus *Shh* KO (K) and $Shh^{CreERT2/-};Ctnnb1^{Ex3/+}$ (L) embryos. (**M-R**) BrdU labeling (M-O) and TUNEL assays (P-R). *Shh* KO embryos show significantly decreased cell proliferation (N). By contrast, expression of stabilized β-catenin in *Shh* KO mutants restores cell proliferation (O). Mesenchymal apoptosis was unchanged in $Shh^{CreERT2/-};Ctnnb1^{Ex3/+}$ embryos as compared with *Shh* KO embryos (Q,R). Dotted lines in the midline GT indicate the basal layer of the endodermal and ectodermal epithelia. (**S**) Proposed signaling cascade for GT outgrowth. Shh signaling is required for GT initiation and subsequent outgrowth. *Shh* is expressed moderately in the DUE (red) and strongly in urethral epithelium (orange). Wnt ligands are expressed in the distal GT ectoderm, and Wnt/β-catenin activity is detected in the distal region of the GT, including the DUE. Wnt/β-catenin activity depends on Shh signaling. This Wnt/β-catenin activity, and possibly urethral plate-derived Shh, induce production of mitogenic factors leading to GT outgrowth. Yellow, mesenchyme; blue line, ectoderm-derived epithelium. Scale bars: 100 µm.

Given the strong Wnt/β-catenin activity in the endoderm-derived DUE, we used temporally inducible $Shh^{CreERT2}$ to analyze the effects of β-catenin loss-of function in the endoderm during GT outgrowth. $Shh^{CreERT2/+};Ctnnb1^{lox/-}$ embryos treated with TM at E9.5 exhibited markedly reduced *Fgf8* expression. However, *Gli1* expression was normal at E11.5, indicating that Shh signaling was not affected by disrupting Wnt/β-catenin signaling at this later stage. Taken together with the results of our β-catenin gain-of-function studies (see below), Wnt/β-catenin signaling appears to be downstream of hedgehog signaling during GT outgrowth and upstream of *Fgf8* expression.

Wnt/β-catenin signaling is required during early embryogenesis (E9.0) for the formation of the caudal embryo and $Wnt3a^{-/-}$, $Lef1^{-/-};Tcf1^{-/-}$ and $Tcf1^{-/-};Tcf4^{-/-}$ embryos exhibit severe caudal truncation of the embryonic axis and GT agenesis (Dunty et al., 2008; Galceran et al., 1999; Gregorieff et al., 2004; Takada et al., 1994). Owing to the wide range of Wnt/β-catenin functions, the epistatic relationships between hedgehog and Wnt/β-catenin signaling are likely to vary in different developmental contexts. To investigate such a possibility with regard to GT development, we conditionally ablated β-catenin with $Isl1^{Cre}$, in which Cre is active in the prospective GT region at the stage required for caudal body formation (~E9.0). $Isl1^{Cre/+};Ctnnb1^{lox/lox}$ embryos exhibited a loss of *Shh* expression at this early stage, consistent with the proposal that *Shh* lies downstream of β-catenin in the endoderm (Lin et al., 2008). The importance of Wnt/β-catenin activity during formation of the caudal embryo suggests that Wnt/β-catenin activity might contribute to cloaca formation, a possibility that merits further study.

Possible regulation of *Fgf8* expression by β-catenin and functional redundancy among Fgfs during GT outgrowth

The CR3 enhancer (located 24 kb 3′ of the *Fgf8* coding region) contributes to *Fgf8* expression in the AER (Beermann et al., 2006). *Fgf8* expression is augmented in the limb by overexpression of stabilized β-catenin in the ectoderm (Soshnikova et al., 2003). We questioned whether similar regulatory actions of β-catenin on *Fgf8* expression operate in the DUE via CR3. We found that β-catenin is bound to a region of CR3 in nuclear extracts from GTs and that this region confers a transcriptional response to exogenous β-catenin in luciferase assays. *Fgf8* expression is decreased in both GT and limb bud in β-catenin loss-of-function mutants (this study) (Lin et al., 2008; Soshnikova et al., 2003). These results suggest β-catenin as a candidate upstream regulator of *Fgf8* expression in the DUE. Regulation of *Fgf8* by β-catenin might be a shared characteristic of the AER and DUE. Such regulation through another candidate enhancer in intron 3 of *Fgf8* was recently reported for tooth development (Wang et al., 2009).

DEVELOPMENT

Although we found that *Fgf4/8* are largely dispensable for GT development, it is likely that compensation by other Fgf ligands occurs, as has been described in the limb, tooth, inner ear and brain (Boulet et al., 2004; Mariani et al., 2008; Moon and Capecchi, 2000). In the current study, a specific Fgf can be (also ectopically) induced upon mutation of another Fgf. Our data suggest that a feedback mechanism operates to maintain the amount of Fgf signaling emanating from the DUE, and that this mechanism can induce expression of Fgf ligand-encoding genes that are otherwise often undetectable. We have made similar observations in the pharyngeal tissues of *Fgf8* mutants (A.M., unpublished) (Moon et al, 2000). The redundancy of Fgf function can also be analyzed in Fgf receptor (Fgfr) conditional mutants. Our preliminary analyses of conditional Fgfr mutants reveal GT defects (our unpublished results), and *Fgfr1*-null mutants have caudal agenesis (Deng et al., 1994; Yamaguchi et al., 1994). Dispensable *Fgf8* function was also recently demonstrated (Seifert et al., 2009).

The development of an embryonic bud structure requires an expansion of mesenchymal tissue during outgrowth (Sun et al., 2002). We found that constitutively active β-catenin in the DUE induced Erk1/2 phosphorylation and cell proliferation in the adjacent GT mesenchyme. This suggests that Wnt/β-catenin stimulates the distal GT, including the DUE, to secrete mitogenic factors required for GT mesenchymal growth (see Fig. S7 in the supplementary material). However, the nature of these factors is still unclear (see discussion of Fgfs above). We have shown that Bmp7 from the DUE has some role in GT outgrowth, suggesting Bmp signaling as an essential growth regulator of the GT (Suzuki et al., 2008). *Bmp4* is induced by *Shh* and our previous data also suggest roles for Bmp signaling in the DUE and distal ectoderm in GT outgrowth (Suzuki et al., 2003).

Our work reveals both conserved and divergent features of the developmental programs that trigger GT and limb bud formation and how they interact to control subsequent outgrowth. Initiation and outgrowth of the GT primordia also critically influence anorectal/urogenital organ development (Hynes and Fraher, 2004), as revealed by the fact that *Shh* KO mutants display not only GT agenesis, but also a persistent cloaca (Mo et al., 2001). *Gli2* and some conditional *Shh* mutant embryos show a severe form of defects for UPE formation in the GT. Overall, an improved understanding of the molecular mechanisms of genital development might shed light on the mechanisms that underlie congenital abnormalities of multiple organ systems, in addition to those affecting the external genitalia and anorectal/urogenital organs.

Acknowledgements

We thank Drs A. Joyner, C. C. Hui, P. Chambon, A. McMahon, W. Birchmeier, G. Martin, M. Lewandoski, T. P. Yamaguchi, B. Hogan, J. Partanen, L. Ma, T. Tsukiyama, Y. Yamaguchi, K. Araki, K. Yamamura, D. Ornitz, N. Nakagata, S. Kume and S. Takada for their invaluable support; Ms S. Miyaji for assistance; and UCSD and Bristol-Myers Squibb for mouse distribution. This study was supported by Grant-in-Aid for Young Scientists B, Grant-in-Aid for Scientific Research B, and for Scientific Research on Priority Areas; by Mechanisms of Sex Differentiation and General Promotion of Cancer Research in Japan; by the global COE program 'Cell Fate Regulation Research and Education Unit'; by a Grant for Child Health and Development, and a Health Sciences Research Grant from the Ministry of Health, Labour and Welfare. This study was also supported by NIH grant (R01ES016597-01A1). The content is solely the responsibility of the authors and does not necessary represent the official views of the NIH. Deposited in PMC for release after 12 months.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/23/3969/DC1

References

Ahn, K., Mishina, Y., Hanks, M. C., Behringer, R. R. and Crenshaw, E. B., 3rd (2001). BMPR-IA signaling is required for the formation of the apical ectodermal ridge and dorsal-ventral patterning of the limb. *Development* **128**, 4449-4461.

Araki, K., Imaizumi, T., Okuyama, K., Oike, Y. and Yamamura, K. (1997). Efficiency of recombination by Cre transient expression in embryonic stem cells: comparison of various promoters. *J. Biochem.* **122**, 977-982.

Barrow, J. R., Thomas, K. R., Boussadia-Zahui, O., Moore, R., Kemler, R., Capecchi, M. R. and McMahon, A. P. (2003). Ectodermal Wnt3/beta-catenin signaling is required for the establishment and maintenance of the apical ectodermal ridge. *Genes Dev.* **17**, 394-409.

Beermann, F., Kaloulis, K., Hofmann, D., Murisier, F., Bucher, P. and Trumpp, A. (2006). Identification of evolutionarily conserved regulatory elements in the mouse Fgf8 locus. *Genesis* **44**, 1-6.

Benazet, J. D., Bischofberger, M., Tiecke, E., Goncalves, A., Martin, J. F., Zuniga, A., Naef, F. and Zeller, R. (2009). A self-regulatory system of interlinked signaling feedback loops controls mouse limb patterning. *Science* **323**, 1050-1053.

Boulet, A. M., Moon, A. M., Arenkiel, B. R. and Capecchi, M. R. (2004). The roles of Fgf4 and Fgf8 in limb bud initiation and outgrowth. *Dev. Biol.* **273**, 361-372.

Capdevila, J. and Izpisua Belmonte, J. C. (2001). Patterning mechanisms controlling vertebrate limb development. *Annu. Rev. Cell Dev. Biol.* **17**, 87-132.

Chen, Y., Knezevic, V., Ervin, V., Hutson, R., Ward, Y. and Mackem, S. (2004). Direct interaction with Hoxd proteins reverses Gli3-repressor function to promote digit formation downstream of Shh. *Development* **131**, 2339-2347.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**, 407-413.

Cobb, J. and Duboule, D. (2005). Comparative analysis of genes downstream of the Hoxd cluster in developing digits and external genitalia. *Development* **132**, 3055-3067.

DasGupta, R. and Fuchs, E. (1999). Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. *Development* **126**, 4557-4568.

Dassule, H. R., Lewis, P., Bei, M., Maas, R. and McMahon, A. P. (2000). Sonic hedgehog regulates growth and morphogenesis of the tooth. *Development* **127**, 4775-4785.

Deng, C. X., Wynshaw-Boris, A., Shen, M. M., Daugherty, C., Ornitz, D. M. and Leder, P. (1994). Murine FGFR-1 is required for early postimplantation growth and axial organization. *Genes Dev.* **8**, 3045-3057.

Dolle, P., Izpisua-Belmonte, J. C., Brown, J. M., Tickle, C. and Duboule, D. (1991). HOX-4 genes and the morphogenesis of mammalian genitalia. *Genes Dev.* **5**, 1767-1776.

Dunty, W. C., Jr, Biris, K. K., Chalamalasetty, R. B., Taketo, M. M., Lewandoski, M. and Yamaguchi, T. P. (2008). Wnt3a/beta-catenin signaling controls posterior body development by coordinating mesoderm formation and segmentation. *Development* **135**, 85-94.

Feil, R., Wagner, J., Metzger, D. and Chambon, P. (1997). Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. *Biochem. Biophys. Res. Commun.* **237**, 752-757.

Galceran, J., Farinas, I., Depew, M. J., Clevers, H. and Grosschedl, R. (1999). Wnt3a(–/–)-like phenotype and limb deficiency in Lef1(–/–)Tcf1(–/–) mice. *Genes Dev.* **13**, 709-717.

Gregorieff, A., Grosschedl, R. and Clevers, H. (2004). Hindgut defects and transformation of the gastro-intestinal tract in Tcf4(–/–)/Tcf1(–/–) embryos. *EMBO J.* **23**, 1825-1833.

Harada, N., Tamai, Y., Ishikawa, T., Sauer, B., Takaku, K., Oshima, M. and Taketo, M. M. (1999). Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. *EMBO J.* **18**, 5931-5942.

Haraguchi, R., Suzuki, K., Murakami, R., Sakai, M., Kamikawa, M., Kengaku, M., Sekine, K., Kawano, H., Kato, S., Ueno, N. et al. (2000). Molecular analysis of external genitalia formation: the role of fibroblast growth factor (Fgf) genes during genital tubercle formation. *Development* **127**, 2471-2479.

Haraguchi, R., Mo, R., Hui, C., Motoyama, J., Makino, S., Shiroishi, T., Gaffield, W. and Yamada, G. (2001). Unique functions of Sonic hedgehog signaling during external genitalia development. *Development* **128**, 4241-4250.

Haraguchi, R., Motoyama, J., Sasaki, H., Satoh, Y., Miyagawa, S., Nakagata, N., Moon, A. and Yamada, G. (2007). Molecular analysis of coordinated bladder and urogenital organ formation by Hedgehog signaling. *Development* **134**, 525-533.

Harfe, B. D., Scherz, P. J., Nissim, S., Tian, H., McMahon, A. P. and Tabin, C. J. (2004). Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. *Cell* **118**, 517-528.

Hu, T., Yamagishi, H., Maeda, J., McAnally, J., Yamagishi, C. and Srivastava, D. (2004). Tbx1 regulates fibroblast growth factors in the anterior heart field through a reinforcing autoregulatory loop involving forkhead transcription factors. *Development* **131**, 5491-5502.

DEVELOPMENT

Huelsken, J., Vogel, R., Erdmann, B., Cotsarelis, G. and Birchmeier, W. (2001). beta-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. *Cell* **105**, 533-545.
Hui, C. C. and Joyner, A. L. (1993). A mouse model of greig cephalopolysyndactyly syndrome: the extra-toesJ mutation contains an intragenic deletion of the Gli3 gene. *Nat. Genet.* **3**, 241-246.
Hynes, P. J. and Fraher, J. P. (2004). The development of the male genitourinary system. I. The origin of the urorectal septum and the formation of the perineum. *Br. J. Plast. Surg.* **57**, 27-36.
Ingham, P. W. and McMahon, A. P. (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* **15**, 3059-3087.
Jernvall, J., Aberg, T., Kettunen, P., Keranen, S. and Thesleff, I. (1998). The life history of an embryonic signaling center: BMP-4 induces p21 and is associated with apoptosis in the mouse tooth enamel knot. *Development* **125**, 161-169.
Johnson, R. L. and Tabin, C. J. (1997). Molecular models for vertebrate limb development. *Cell* **90**, 979-990.
Kawakami, Y., Capdevila, J., Buscher, D., Itoh, T., Rodriguez Esteban, C. and Izpisua Belmonte, J. C. (2001). WNT signals control FGF-dependent limb initiation and AER induction in the chick embryo. *Cell* **104**, 891-900.
Kawakami, Y., Rodriguez-Leon, J., Koth, C. M., Buscher, D., Itoh, T., Raya, A., Ng, J. K., Esteban, C. R., Takahashi, S., Henrique, D. et al. (2003). MKP3 mediates the cellular response to FGF8 signalling in the vertebrate limb. *Nat. Cell Biol.* **5**, 513-519.
Kengaku, M., Capdevila, J., Rodriguez-Esteban, C., De La Pena, J., Johnson, R. L., Belmonte, J. C. and Tabin, C. J. (1998). Distinct WNT pathways regulating AER formation and dorsoventral polarity in the chick limb bud. *Science* **280**, 1274-1277.
Kmita, M., Tarchini, B., Zakany, J., Logan, M., Tabin, C. J. and Duboule, D. (2005). Early developmental arrest of mammalian limbs lacking HoxA/HoxD gene function. *Nature* **435**, 1113-1116.
Kondo, T., Zakany, J., Innis, J. W. and Duboule, D. (1997). Of fingers, toes and penises. *Nature* **390**, 29.
Ladher, R. K., Wright, T. J., Moon, A. M., Mansour, S. L. and Schoenwolf, G. C. (2005). FGF8 initiates inner ear induction in chick and mouse. *Genes Dev.* **19**, 603-613.
Laufer, E., Nelson, C. E., Johnson, R. L., Morgan, B. A. and Tabin, C. (1994). Sonic hedgehog and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* **79**, 993-1003.
Lewandoski, M., Sun, X. and Martin, G. R. (2000). Fgf8 signalling from the AER is essential for normal limb development. *Nat. Genet.* **26**, 460-463.
Lin, C., Yin, Y., Long, F. and Ma, L. (2008). Tissue-specific requirements of {beta}-catenin in external genitalia development. *Development* **135**, 2815-2825.
MacArthur, C. A., Lawshe, A., Xu, J., Santos-Ocampo, S., Heikinheimo, M., Chellaiah, A. T. and Ornitz, D. M. (1995). FGF-8 isoforms activate receptor splice forms that are expressed in mesenchymal regions of mouse development. *Development* **121**, 3603-3613.
Macatee, T. L., Hammond, B. P., Arenkiel, B. R., Francis, L., Frank, D. U. and Moon, A. M. (2003). Ablation of specific expression domains reveals discrete functions of ectoderm- and endoderm-derived FGF8 during cardiovascular and pharyngeal development. *Development* **130**, 6361-6374.
Mariani, F. V., Ahn, C. P. and Martin, G. R. (2008). Genetic evidence that FGFs have an instructive role in limb proximal-distal patterning. *Nature* **453**, 401-405.
Mo, R., Freer, A. M., Zinyk, D. L., Crackower, M. A., Michaud, J., Heng, H. H., Chik, K. W., Shi, X. M., Tsui, L. C., Cheng, S. H. et al. (1997). Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. *Development* **124**, 113-123.
Mo, R., Kim, J. H., Zhang, J., Chiang, C., Hui, C. C. and Kim, P. C. (2001). Anorectal malformations caused by defects in sonic hedgehog signaling. *Am. J. Pathol.* **159**, 765-774.
Moon, A. M. and Capecchi, M. R. (2000). Fgf8 is required for outgrowth and patterning of the limbs. *Nat. Genet.* **26**, 455-459.
Moon, A. M., Boulet, A. M. and Capecchi, M. R. (2000). Normal limb development in conditional mutants of Fgf4. *Development* **127**, 989-996.
Morgan, E. A., Nguyen, S. B., Scott, V. and Stadler, H. S. (2003). Loss of Bmp7 and Fgf8 signaling in Hoxa13-mutant mice causes hypospadia. *Development* **130**, 3095-3109.
Murakami, R. and Mizuno, T. (1986). Proximal-distal sequence of development of the skeletal tissues in the penis of rat and the inductive effect of epithelium. *J. Embryol. Exp. Morphol.* **92**, 133-143.
Nakaya, M. A., Biris, K., Tsukiyama, T., Jaime, S., Rawls, J. A. and Yamaguchi, T. P. (2005). Wnt3a links left-right determination with segmentation and anteroposterior axis elongation. *Development* **132**, 5425-5436.
Nieuwenhuis, E. and Hui, C. C. (2005). Hedgehog signaling and congenital malformations. *Clin. Genet.* **67**, 193-208.
Niswander, L. (2003). Pattern formation: old models out on a limb. *Nat. Rev. Genet.* **4**, 133-143.
Niswander, L., Jeffrey, S., Martin, G. R. and Tickle, C. (1994). A positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature* **371**, 609-612.
Ogino, Y., Suzuki, K., Haraguchi, R., Satoh, Y., Dolle, P. and Yamada, G. (2001). External genitalia formation: role of fibroblast growth factor, retinoic acid signaling, and distal urethral epithelium. *Ann. N. Y. Acad. Sci.* **948**, 13-31.
Park, E. J., Ogden, L. A., Talbot, A., Evans, S., Cai, C. L., Black, B. L., Frank, D. U. and Moon, A. M. (2006). Required, tissue-specific roles for Fgf8 in outflow tract formation and remodeling. *Development* **133**, 2419-2433.
Perriton, C. L., Powles, N., Chiang, C., Maconochie, M. K. and Cohn, M. J. (2002). Sonic hedgehog signaling from the urethral epithelium controls external genital development. *Dev. Biol.* **247**, 26-46.
Riddle, R. D., Johnson, R. L., Laufer, E. and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75**, 1401-1416.
Seifert, A. W., Yamaguchi, T. and Cohn, M. J. (2009). Functional and phylogenetic analysis shows that Fgf8 is a marker of genital induction in mammals but is not required for external genital development. *Development* **136**, 2643-2651.
Sherman, L., Wainwright, D., Ponta, H. and Herrlich, P. (1998). A splice variant of CD44 expressed in the apical ectodermal ridge presents fibroblast growth factors to limb mesenchyme and is required for limb outgrowth. *Genes Dev.* **12**, 1058-1071.
Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.
Soshnikova, N., Zechner, D., Huelsken, J., Mishina, Y., Behringer, R. R., Taketo, M. M., Crenshaw, E. B., 3rd and Birchmeier, W. (2003). Genetic interaction between Wnt/beta-catenin and BMP receptor signaling during formation of the AER and the dorsal-ventral axis in the limb. *Genes Dev.* **17**, 1963-1968.
Sun, X., Lewandoski, M., Meyers, E. N., Liu, Y. H., Maxson, R. E., Jr and Martin, G. R. (2000). Conditional inactivation of Fgf4 reveals complexity of signalling during limb bud development. *Nat. Genet.* **25**, 83-86.
Sun, X., Mariani, F. V. and Martin, G. R. (2002). Functions of FGF signalling from the apical ectodermal ridge in limb development. *Nature* **418**, 501-508.
Suzuki, K., Bachiller, D., Chen, Y. P., Kamikawa, M., Ogi, H., Haraguchi, R., Ogino, Y., Minami, Y., Mishina, Y., Ahn, K. et al. (2003). Regulation of outgrowth and apoptosis for the terminal appendage: external genitalia development by concerted actions of BMP signaling. *Development* **130**, 6209-6220.
Suzuki, K., Haraguchi, R., Ogata, T., Barbieri, O., Alegria, O., Vieux-Rochas, M., Nakagata, N., Ito, M., Mills, A. A., Kurita, T. et al. (2008). Abnormal urethra formation in mouse models of Split-hand/split-foot malformation type 1 and type 4. *Eur. J. Hum. Genet.* **16**, 36-44.
Takada, S., Stark, K. L., Shea, M. J., Vassileva, G., McMahon, J. A. and McMahon, A. P. (1994). Wnt-3a regulates somite and tailbud formation in the mouse embryo. *Genes Dev.* **8**, 174-189.
Takahashi, N., Ishihara, S., Takada, S., Tsukita, S. and Nagafuchi, A. (2000). Posttranscriptional regulation of alpha-catenin expression is required for Wnt signaling in L cells. *Biochem. Biophys. Res. Commun.* **277**, 691-698.
Wang, X.-P., O'Conell, D. J., Lund, J. J., Saadi, I., Kuraguchi, M., Turbe-Doan, A., Cavallesco, R., Kim, H., Park, P. J., Harada, H. et al. (2009). Apc inhibition of Wnt signaling regulates supernumerary tooth formation during embryogenesis and throughout adulthood. *Development* **136**, 1939-1949.
Yamada, G., Satoh, Y., Baskin, L. S. and Cunha, G. R. (2003). Cellular and molecular mechanisms of development of the external genitalia. *Differentiation* **71**, 445-460.
Yamada, G., Suzuki, K., Haraguchi, R., Miyagawa, S., Satoh, Y., Kamimura, M., Nakagata, N., Kataoka, H., Kuroiwa, A. and Chen, Y. (2006). Molecular genetic cascades for external genitalia formation: an emerging organogenesis program. *Dev. Dyn.* **235**, 1738-1752.
Yamaguchi, T. P., Harpal, K., Henkemeyer, M. and Rossant, J. (1994). fgfr-1 is required for embryonic growth and mesodermal patterning during mouse gastrulation. *Genes Dev.* **8**, 3032-3044.
Yamaguchi, T. P., Bradley, A., McMahon, A. P. and Jones, S. (1999). A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. *Development* **126**, 1211-1223.
Yang, L., Cai, C. L., Lin, L., Qyang, Y., Chung, C., Monteiro, R. M., Mummery, C. L., Fishman, G. I., Cogen, A. and Evans, S. (2006). Isl1Cre reveals a common Bmp pathway in heart and limb development. *Development* **133**, 1575-1585.
Yu, K. and Ornitz, D. M. (2008). FGF signaling regulates mesenchymal differentiation and skeletal patterning along the limb bud proximodistal axis. *Development* **135**, 483-491.
Zhu, J., Nakamura, E., Nguyen, M. T., Bao, X., Akiyama, H. and Mackem, S. (2008). Uncoupling Sonic hedgehog control of pattern and expansion of the developing limb bud. *Dev. Cell* **14**, 624-632.
Zuniga, A., Haramis, A. P., McMahon, A. P. and Zeller, R. (1999). Signal relay by BMP antagonism controls the SHH/FGF4 feedback loop in vertebrate limb buds. *Nature* **401**, 598-602.

DEVELOPMENT