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# Isl1Cre reveals a common Bmp pathway in heart and limb development

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A number of human congenital disorders present with both heart and limb defects, consistent with common genetic pathways. We have recently shown that the LIM homeodomain transcription factor islet 1 (Isl1) marks a subset of cardiac progenitors. Here, we perform lineage studies with an Isl1Cre mouse line to demonstrate that Isl1 also marks a subset of limb progenitors. In both cardiac and limb progenitors, Isl1 expression is downregulated as progenitors migrate in to form either heart or limb. To investigate common heart-limb pathways in Isl1-expressing progenitors, we ablated the Type I Bmp receptor, Bmpr1a utilizing *Isl1Cre/+*. Analysis of consequent heart and limb phenotypes has revealed novel requirements for Bmp signaling. Additionally, we find that Bmp signaling in Isl1-expressing progenitors is required for expression of T-box transcription factors Tbx2 and Tbx3 in heart and limb. Tbx3 is required for heart and limb formation, and is mutated in ulnar-mammary syndrome. We provide evidence that the Tbx3 promoter is directly regulated by Bmp Smads in vivo.

**KEY WORDS: Isl1, Bmp, Tbx2, Tbx3, Heart, Hindlimb**

## INTRODUCTION

The frequency of human congenital diseases presenting with heart and limb anomalies is at minimum 1 out of 5000 live births (Wilson, 1998). A cardiomelic developmental field has been postulated based on a number of observations, including a significant positive association of limb defects with heart defects in more than 100 Mendelian disorders (Wilson, 1998). Additionally, fetal autopsy revealed that 57% of limb defects were coincident with heart defects, and that heart and limb anomalies coexisted in 81% of partial aneuploidies (Barr et al., 1994). Also of note, ten out of twelve well characterized teratogenic syndromes in humans display coincident heart and limb anomalies (Gorlin and Gorlin, 1990).

Identification of the transcription factor TBX5 as a disease gene in prototypical heart-limb syndrome I, Holt-Oram syndrome, has resulted in identification of a common genetic pathway affecting both heart and limb (Basson et al., 1997; Basson et al., 1999). A number of heart-limb syndromes, including Holt-Oram syndrome, are characterized by cardiac arrhythmias (Bell, 1951; Ruiz de la Fuente and Prieto, 1980; Silengo et al., 1990; Sinkovec et al., 2005; Temtamy and McKusick, 1978). In the case of Holt-Oram syndrome, a series of elegant experiments have demonstrated that the gap junction protein connexin40 is a direct downstream target of Tbx5 both in the heart and in the limb, accounting for conduction system anomalies in the heart and growth defects in the limb (Basson et al., 1999). Another t-box transcription factor, TBX3, is expressed in developing heart and limb, and is mutated in ulnar-mammary syndrome (UMS) (Davenport et al., 2003). Limb deformities in UMS patients have been associated with cardiac defects, including ventricular septal defects, in a subset of patients (Schinzel et al., 1987) (Craig Basson, personal communication).

Our lab has recently identified a subset of undifferentiated cardiac progenitors which is marked by expression of a LIM-homeodomain protein, islet 1 (Isl1) (Cai et al., 2003). Isl1 expression is extinguished as the progenitors migrate into the forming heart. Intriguingly, while performing fate mapping studies with an *Isl1Cre/+* mouse line generated by a knockin into the endogenous *Isl1* locus (see Materials and methods), we observed a similar paradigm for hindlimb progenitors. Isl1 mRNA is highly expressed in lateral mesoderm at the site where the hindlimb bud originates. Fate mapping with *Isl1Cre/+* and an R26R-*lacZ* reporter (Soriano, 1999), revealed that Isl1-expressing progenitors migrate into the hindlimb bud to contribute a substantial proportion of mesodermal cells to the limb bud, in a posterior to anterior gradient. Our results reveal that Isl1 marks both heart and hindlimb progenitors, suggesting potential common genetic pathways downstream of Isl1, which could be involved in heart-limb syndromes.

To investigate common pathways in heart and limb, we have examined the requirement for Bmp signaling utilizing *Isl1Cre/+* to ablate the Type1 Bmp receptor, Bmpr1a in early progenitors. Ablation of the receptor mitigates issues of ligand redundancy during heart and limb formation (Dudley and Robertson, 1997; Katagiri et al., 1998; Lyons et al., 1995; Schneider et al., 2003). Results of our analysis reveal novel requirements for Bmp signaling, and common downstream targets for Bmp signaling in heart and limb, one of which is a limb disease gene also likely to play a critical role in heart development, Tbx3.

## MATERIALS AND METHODS

### Generation of mutant mice

Floxed *Bmpr1a/Alk3* mice were kindly provided by Richard Behringer (The University of Texas, MD Anderson Cancer Center). *Isl1Cre/+* mice were generated in our lab by a *Cre* knockin into the endogenous *Isl1* locus, replacing the endogenous *Isl1* ATG. Homozygous floxed *Bmpr1a* mice were crossed with Protamine-Cre mice (O'Gorman et al., 1997) to generate *Bmpr1a* wt/null mice, which were then crossed with *Isl1Cre/+* mice to

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produce doubly heterozygous *Isl1Cre/+;Bmpr1a* floxed/null mice. These mice were then crossed to *Bmpr1a* floxed/floxed homozygous mice to obtain *Isl1Cre/+;Bmpr1a* floxed/null mutants.

#### Whole-mount RNA in situ hybridization and histological analyses

Whole-mount RNA in situ hybridization was carried out according to the protocol of Wilkinson (Wilkinson, 1992). For sectioning, mouse embryos were fixed in 4% paraformaldehyde, dehydrated in ethanol and embedded in paraffin wax. Transverse sections were cut and stained with Hematoxylin-Eosin according to standard protocols.

For bone staining in developing digits, tissues were stained with Alcian Blue according to methods described by Mcleod (McLeod, 1980).

#### Chromatin immunoprecipitation (ChIP) assay and Smad antibodies

For in vivo ChIP experiments, extracts were prepared from 10 E12.5 wild-type mouse hind limbs. Embryos were dissected in ice-cold PBS. Following gentle pipetting, tissue was crosslinked with 1% formaldehyde for 20 minutes at room temperature. Chromatin extraction and immunoprecipitations were performed using a ChIP assay kit (Upstate, 17-295) according to the manufacturer's instructions. Protein-DNA crosslinking was reversed by overnight incubation at 65°C. A PCR purification kit (Qiagen, 28106) was used to recover DNA in 50 μl $H_2O$. The following PCR primers against the 5′ Tbx3 promoter region were used: P-191 (5′-GCAGATCCGCACAAGAGAAG-3′) and P67 (5′-GGTGGCTGATCC-AGAAGAGA-3′). As control, primers against an unrelated region of Tbx3 promoter region were used: PE (5′-GAGATGGCAGGTCACACCAAG-3′) and PF (5′-GCTTTCAATGTTTCCGTGTGG-3′).

Phospho-Smad1 (Ser463/465)/Smad5 (Ser463/465)/Smad8 (Ser426/428) antibody was obtained from Cell Signaling Technology (9511s).

#### Promoter cloning and luciferase transfection assay

A 2 kb genomic DNA fragment upstream of the *Tbx3* ATG start codon was amplified with high fidelity DNA polymerase (Novagen, 71086-3) and cloned into pGL3-basic vector (Promega, E1751). Primers were: 5′ primer 5′-GCTGGGCTCAAAAGGGTCAGTA-3′, 3′ primer 5′-CCACTCCAG-ACAGGGAACCAGT-3′.

Transfections were carried out in P19 cells according to standard techniques using Lipofectamine 2000 (Invitrogen). Cells were lysed 48 hours after transfection, and luciferase and β-galactosidase activities were measured on a Luminoskan Ascent luminometer (Thermo Labsystems, Franklin, MA, USA). For luciferase reporters, CMV-β-galactosidase was used to control for transfection efficiency. Normalized luciferase activities were compared with a pGL3 control to calculate relative repression. Results shown are from one representative experiment carried out in triplicate and values are expressed as mean ± s.d. At least three independent transfection experiments were performed for each sample.

#### Cell proliferation and apoptosis assays

Mutant and wild-type embryos were collected. PFA-fixed paraffin sections were incubated with an antibody to phospho-histone H3 (Ser10) (1:100 dilution), which was obtained from Upstate (06-570) for the cell proliferation assays or an antibody to cleaved caspase 3 (Asp175) (Cell Signal, 9661s) for apoptosis assays. AP-conjugated secondary antibody and the NBT-BCIP kit (Promega) were used for detection. Sections were counterstained with Fast Red. Both assays were compared by analysis of variance and the unpaired two tailed *t*-test.

## RESULTS

### Isl1 is expressed in a specific subset of hindlimb progenitors and is downregulated as they migrate into the limb

Previous lineage studies with an *Isl1Cre/+* mouse line generated by insertion of an IRES-Cre cassette into the endogenous *Isl1* locus (Srinivas et al., 2001) had revealed that descendants of *Isl1*-expressing cells contributed a majority of cells to the developing heart (Cai et al., 2003). As Cre activity in cardiac progenitors was somewhat variable with this mouse line, we generated a direct *Cre* knockin into the endogenous *Isl1* locus (see Materials and methods). To characterize Cre-mediated excision with this new line, fate mapping was performed using a R26R-*lacZ* reporter line (Soriano, 1999). Examination of hearts in fate-mapped embryos yielded results consistent with previous data (Cai et al., 2003). Descendants of cells expressing Isl1 were observed uniformly in the outflow tract and right ventricle, in a majority of atrial cells and in part of the left ventricle, similar to results previously reported. Cre-mediated excision with this new mouse line, however, was more efficient and consistent.

During lineage analysis with the new *Isl1Cre/+*, we observed that Isl1-expressing cells contributed a majority of cells to the hindlimb, but few, if any, cells to the forelimb (Fig. 1). A comparison of *lacZ* expression in hindlimbs of fate-mapped embryos to Isl1 mRNA expression in hindlimbs (Fig. 1K-U versus V-G′) demonstrated that *lacZ* was more widely expressed in developing limb buds than Isl1 mRNA. Isl1 mRNA is observed in the lateral plate mesoderm adjacent to the future hindlimb bud by E9.0, and is expressed in lateral mesoderm adjacent to the nascent bud, in a posterior to anterior gradient, by E9.5 (Fig. 1V-G′). In contrast, *Isl1Cre/+*;R26R-*lacZ* embryos express *lacZ* in adjacent lateral mesoderm and throughout the nascent limb bud itself, in a posterior to anterior gradient (posterior exhibiting higher expression) reflecting the gradient observed with Isl1 mRNA expression (Fig. 1K-U). These observations demonstrated that Isl1 mRNA is downregulated as Isl1-expressing hindlimb progenitors migrate in to contribute to the limb bud, comparable to the situation in heart. The anterior posterior gradient of Isl1 also demonstrated early anterior posterior patterning of these progenitors. Isl1-expressing progenitors contributed to mesoderm, but not ectoderm of the limb (Fig. 1S-U).

### Investigation of the requirement for Bmpr1a in Isl1-expressing progenitors of heart and hindlimb

Downstream targets of Isl1 in cardiac progenitors included bone morphogenetic protein (Bmps) Bmp4 and Bmp7 (Cai et al., 2003). To investigate the impact of decreasing Bmp signaling downstream of Isl1, we crossed mice that were floxed for the Type1 Bmp receptor, Bmpr1a/Alk3 (Mishina et al., 2002) to *Isl1Cre/+* mice. Mice of *Isl1Cre/+;Bmpr1a* floxed/null genotype were recovered at Mendelian frequencies until E10.5, but were progressively lost until E14.5 when no embryos of that genotype were recovered (Fig. 2A). Overall embryo size and gross morphology was relatively normal until E13.5 (Fig. 2B-K). Aberrant heart morphology was evident by E8.5, and alterations in hindlimb bud size and morphology evident by early limb bud stages (Fig. 2B-K,L-U).

There are several Type I Bmp receptors capable of transducing Bmp signaling in concert with the Type II Bmp receptor. To investigate the manner in which ablation of Bmpr1a in Isl1-expressing cells affected Bmp signaling, we compared expression of a Bmp indicator *lacZ* transgene (Monteiro et al., 2004) in wild-type and *Isl1Cre/+;Bmpr1a* mutant backgrounds. In wild-type embryos, Bmp signaling was observed at high levels in developing heart (Monteiro et al., 2004) (Fig. 2L,M,Q,R). Comparison with *Isl1Cre/+;Bmpr1a* mutants demonstrated that ablation of Bmpr1a severely decreased Bmp signaling in outflow tract and right ventricle.

In wild-type hindlimb, Bmp signaling was observed at low levels throughout the limb bud, with high levels at posterior and anterior margins (Fig. 2N-P). In keeping with the gradient of Isl1 hindlimb progenitors, posterior Bmp signaling was severely reduced in *Isl1Cre/+;Bmpr1a* mutant hindlimbs, both dorsally (Fig. 2S-U) and

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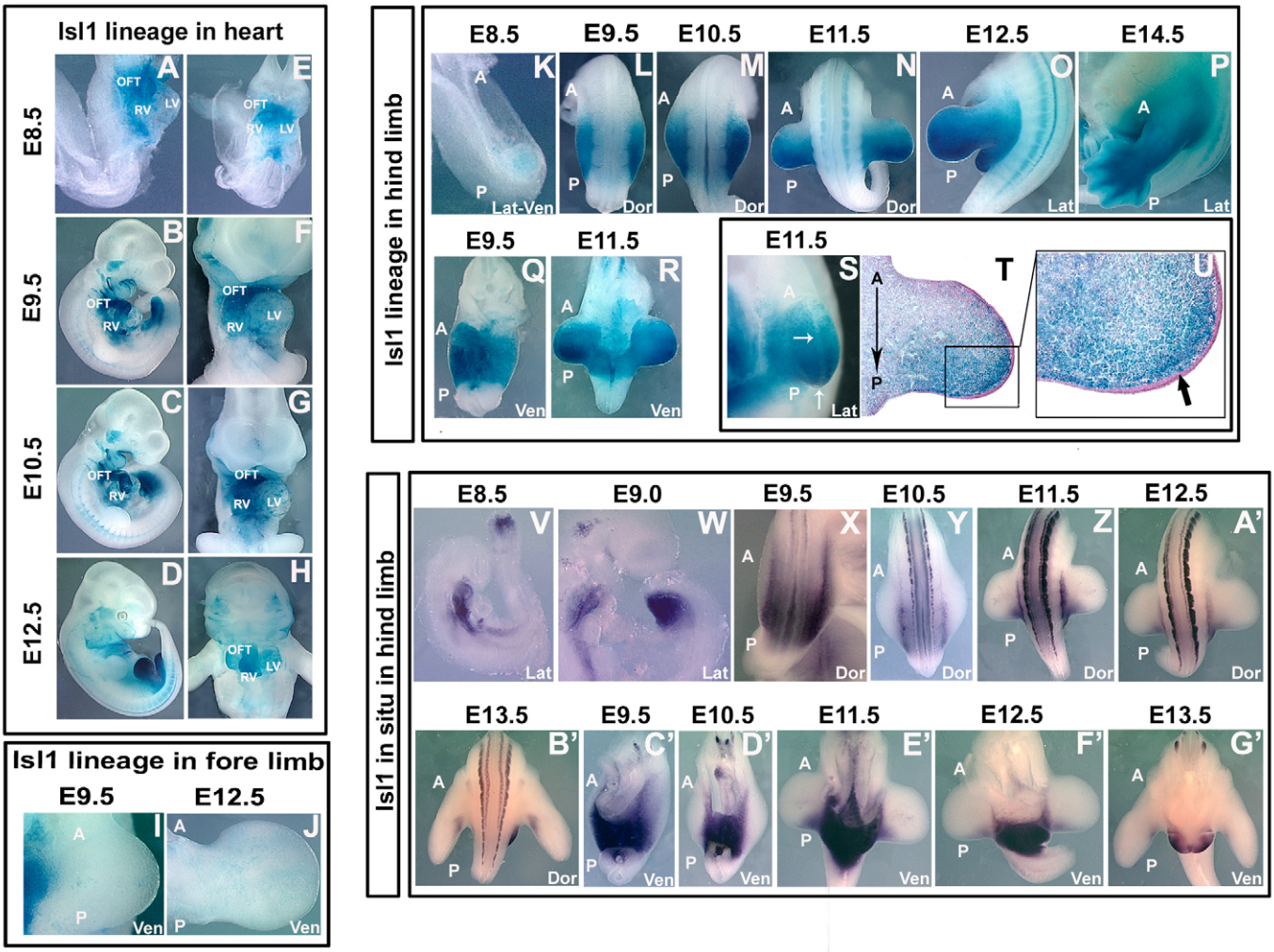


**Fig. 1. Isl1 marks a subset of progenitors in heart and limb.** (**A-H**) Isl1 lineages in heart. Lineage studies with a new *Isl1Cre/+* mouse line give results consistent with those observed utilizing an *Isl1-IRES-Cre* mouse line (Srinivas et al., 1999; Cai et al., 2003). Excision with the new *Isl1Cre/+* occurred more efficiently. Isl1-expressing progenitors contribute to most cells of the outflow tract, right ventricle and atria, and also to some cells in the left ventricle (Cai et al., 203). (**I,J**) Isl1 lineages in forelimb. Isl1 lineages do not contribute in any significant number to the forelimbs. (**K-U**) Isl1 lineages in hindlimb. A majority of cells in the hindlimb derive from Isl1-expressing cells. Isl1 cells contribute in an anterior-posterior gradient, with posterior domains deriving almost entirely from Isl1-expressing lineages. Isl1 lineages contribute to hindlimb mesoderm, not ectoderm (S-U). (**V-G′**) Isl1 mRNA expression in lateral plate mesoderm adjacent to and within the limb bud. A comparison of Isl1 mRNA expression to Isl1 lineage results demonstrated that Isl1 mRNA expression is downregulated as hindlimb progenitors migrate into the limb. OFT, outflow tract; RV, right ventricle; LV, left ventricle; A, anterior; P, posterior; Lat, lateral view; Dor, dorsal view; Ven, ventral view.

ventrally. Examination of ventral domains of Bmp expression revealed strong Bmp signaling within the interlimb region, which is severely reduced in *Isl1Cre/+;Bmpr1a* mutants (compare Fig. 2P and U). Isl1 mRNA and *Isl1Cre/+* fatemapping analyses demonstrated that this population of cells expressed Isl1 (Fig. 1R,E′).

## Cardiac phenotype in *Isl1Cre/+;Bmpr1a* mutants

Examination of whole mounts and histological sections of the heart in *Isl1Cre/+;Bmpr1a* mutants demonstrated aberrant morphology of outflow tract and right ventricle from E9.5 (Fig. 3A-C,J-L). However, epithelial-mesenchymal transformation of atrioventricular cushion cells appeared comparable in both wild-type and mutant embryos (Fig. 3A-C,J-L). By E11.5, ventricular muscle was thinner and less well developed (Fig. 3D-F,M-O). At E13.5, severe abnormalities of outflow tract formation were observed in *Isl1Cre/+;Bmpr1a* mutants, with evident persistent truncus arteriosus (PTA), and underdeveloped valves (Fig. 3H,Q). Ventricular and atrial septal defects were also observed in mutant hearts (Fig. 3I,R). Cardiac differentiation had occurred, as evidenced by immunostaining for myosin heavy chain expression, utilizing monoclonal antibody MF20 (data not shown).

Thinner ventricular walls and an underdeveloped ventricular septum in *Isl1Cre/+;Bmpr1a* mutants led us to examine apoptosis and proliferation, utilizing antibodies to detect activated caspase 3 and phosphorylated histone H3, respectively (see Materials and methods). Consistent findings were decreased apoptosis in outflow tract cushions and increased apoptosis atop the ventricular septum, both in cells within myocardium and at the border of myocardium and endocardial cushions (Fig. 3S-V,Z-C′,G′), suggesting lack of normal outflow tract cushion remodeling and cell loss within the ventricular septum. Proliferation of ventricular myocardium in the free wall and septum was also decreased in mutants relative to controls (Fig. 3W-Y,D′-F′,H′). Together, these observations are

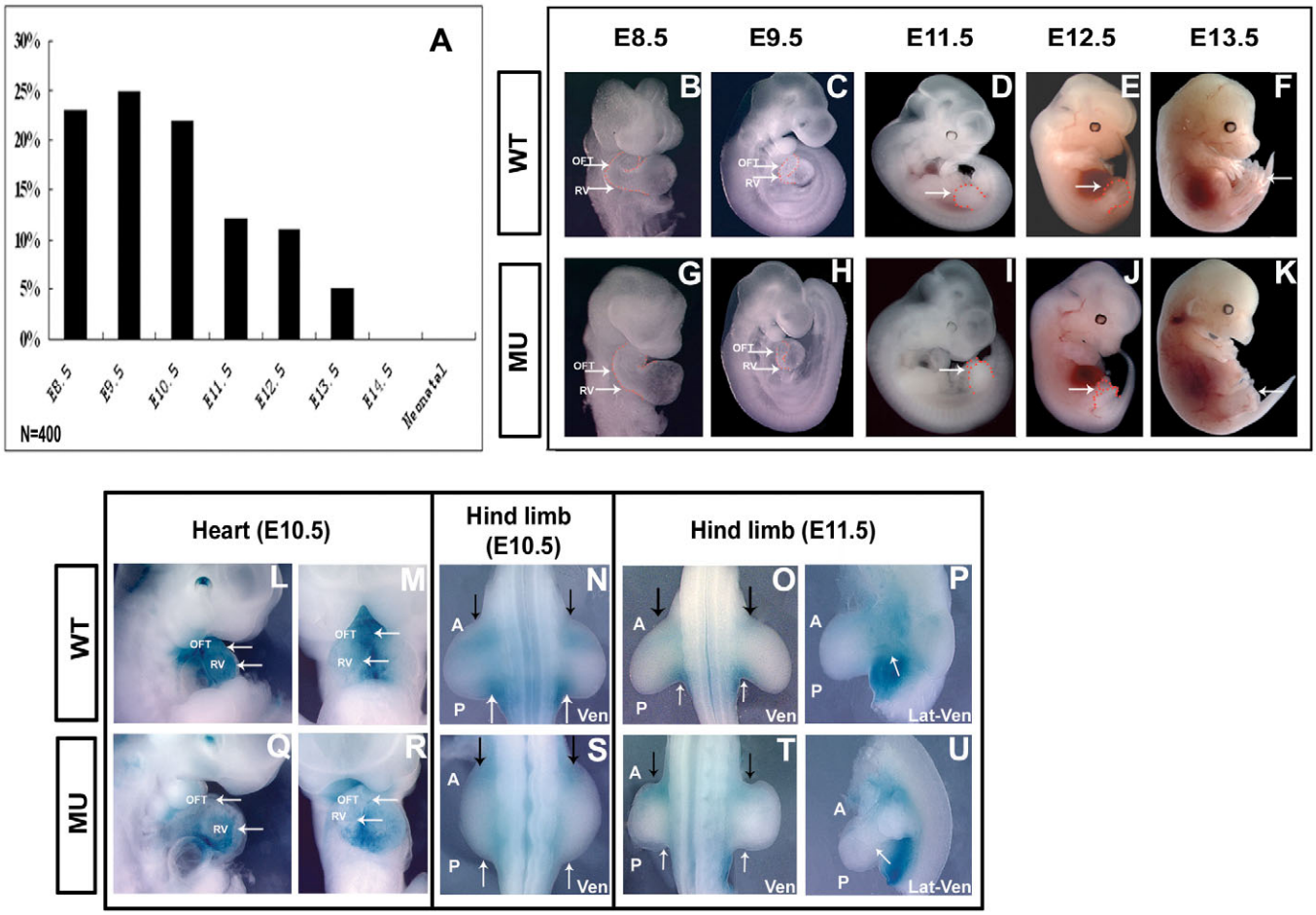


**Fig. 2. Ablation of Bmpr1a with *Isl1Cre/+* results in embryonic lethality, malformations of heart and limb, and specific reduction of Bmp signaling.** (**A**) Recovery of embryos with the *Isl1Cre/+;Bmpr1a* f/f genotype. Mendelian frequencies of *Isl1Cre/+;Bmpr1a* mutants were recovered at E10.5, but began to be lost by E11.5. No mutants were recovered at E14.5. In total 400 embryos were collected and ~50 embryos at each stage. (**B-K**) Whole-mount morphological analysis of wild-type and mutant littermates. Abnormalities of outflow tract and right ventricle were evident by E8.5 (arrows, B,C,G,H); Hindlimb abnormalities were evident by E10 (N,S; arrows D-F,I-K). (**L-U**) Bmp signaling as monitored by Bmp-*lacZ* indicator genetic background. In *Isl1Cre/+;Bmpr1a* mutants, Bmp signaling was selectively reduced in Isl1-expressing lineages. Bmp signaling was reduced in the outflow tract and right ventricle of *Isl1Cre/+;Bmpr1a* mutants relative to control littermates (L,M,Q,R). In the hindlimb, Bmp signaling was strongly downregulated in the posterior limb margins (N,O,S,T) and inter hindlimb region (P,U) in *Isl1Cre/+;Bmpr1a* mutants relative to control littermates. OFT, outflow tract; RV, right ventricle; LV, left ventricle; A, anterior; P, posterior; Lat, lateral view; Dor, dorsal view; Ven, ventral view.

consistent with and may account for aspects of observed cardiac phenotypes. No differences in proliferation of outflow tract myocardium were observed between mutant and wild-type hearts.

## Potential downstream effector targets of Bmp signaling in heart

We have previously demonstrated that Tbx20 is required for proliferation of myocardium (Cai et al., 2005). As myocardial proliferation was affected in *Isl1Cre/+;Bmpr1a* mutants, we examined expression of Tbx20 in these mutants and their wild-type littermates. We found that expression of Tbx20 was decreased in Bmp mutants (Fig. 4A,G), demonstrating that expression of Tbx20 is dependent on Bmp signaling through Bmpr1a, and that decreased Tbx20 could account at least in part for observed proliferative defects.

Previously, we have shown that Tbx20 is required to downregulate expression of Isl1 as Isl1-expressing progenitors enter the heart (Cai et al., 2005). Decreased Tbx20 might therefore result in increased Isl1 expression. Examination of Isl1 expression in *Isl1Cre/+;Bmpr1a* mutants demonstrated an increased domain of Isl1 expression throughout the length of the outflow tract in mutants relative to wild-type littermates (Fig. 4B,C,H,I).

Tbx2 is required for atrioventricular canal patterning and outflow tract septation (Christoffels et al., 2004; Harrelson et al., 2004). Tbx2 expression in developing chick heart is reduced in response to noggin, an inhibitor of Bmp signaling (Yamada et al., 2000). These observations suggested Tbx2 is a potential effector target of Bmpr1a in developing heart. Tbx2 expression was downregulated in *Isl1Cre/+;Bmpr1a* mutants relative to expression in wild-type littermate controls (Fig. 4D-F,J-L).

Tbx3, highly homologous to Tbx2, is coexpressed with Tbx2 in myocardium of the atrioventricular canal, where they may be functionally redundant, as both proteins act as repressors, and can repress the same target genes in other contexts (Christoffels et al., 2004; Hoogaars et al., 2004; Lingbeek et al., 2002). We found that Tbx3 expression was also downregulated in myocardium of the atrioventricular canal in *Isl1Cre/+;Bmpr1a* mutants (Fig. 4M-O,T-V). Tbx2 and Tbx3 are coexpressed in the developing cardiac conduction

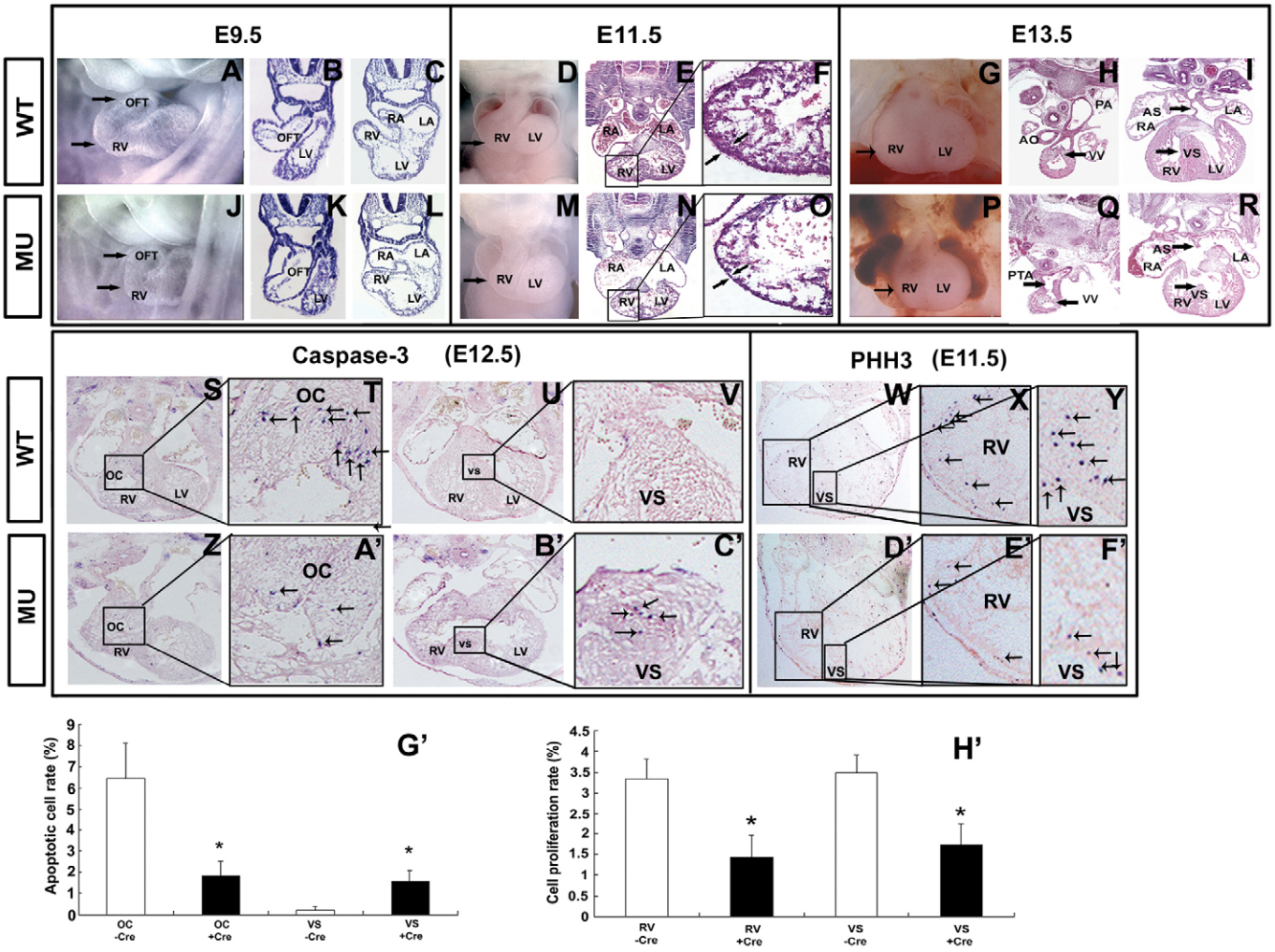


**Fig. 3. Aberrant cardiac morphology, apoptosis, and proliferation in *Isl1Cre/+;Bmpr1a* mutants.** (**A-R**) Whole mount and section analysis of cardiac morphology. *Isl1Cre/+* mutants exhibited abnormal looping of the outflow tract at E9.5 (A-C,J-L), thinning of the ventricular wall by E11.5 (D-F,M-O), and outflow tract, ventricular, and atrial septal defects by E13.5 (G-I,P-R). (**S-V**,**Z-C′**,**G′**) Analysis of apoptosis. Staining with antibody to activated caspase-3 revealed less cell death in the outflow tract cushions and increased cell death in the ventricular septum in *Isl1Cre/+*:Bmpr1a mutants relative to wild-type littermates. Arrows in T,V,A′ and C′ indicate apoptotic cells. **P*<0.05. (**W-Y**,**D′-F′**,**H′**) Analysis of cell proliferation. Staining with antibody to phosphorylated histone H3 (PHH3) demonstrated decreased proliferation in ventricular myocardium, including the septum in *Isl1Cre/+;Bmpr1a* mutants relative to wild-type control littermates. Arrows in X,Y,E′ and F′ indicate proliferating cells. **P*<0.05. OFT, outflow tract; RV, right ventricle; LA, left atrium; RA, right atrium; AO, aorta; VS, ventricular septum; AS, atrial septum; PTA, persistent truncus arteriosus; OC, outflow tract cushions; VV, valve; –Cre, control embryos; +Cre, mutant embryos. Arrows in F and O, indicate the ventricular wall.

system (Hoogaars et al., 2004). To verify that observed decreases in Tbx2 and Tbx3 expression in atrioventricular canal myocardium was consequent to specific downregulation of these genes, and not a loss of conduction system cells, we crossed *Isl1Cre/+;Bmpr1a* mutants with a mouse line containing a *lacZ* indicator for the cardiac conduction system, CCS-*lacZ* (Rentschler et al., 2001). Results of this analysis demonstrated that conduction system cells were still present in the atrioventricular canal (Fig. 4P-S,W-Z), suggesting specific downregulation of Tbx2 and Tbx3 expression.

## Hindlimb phenotype in *Isl1Cre/+;Bmpr1a* mutants

Abnormal hindlimb morphology was evident from early limb bud stages in *Isl1Cre/+;Bmpr1a* mutants (Fig. 2N-P,S-U,D-F,I-K and Fig. 5A,B,F,G). At E10, hindlimb buds were smaller, and by E11.5, ectopic outgrowths were observed on the ventral surface of the limb bud (Fig. 5A,F). By E13.5, hindlimb formation was severely abnormal (Fig. 5B,G).

Analysis of proliferation and apoptosis in developing hindlimb buds demonstrated that apoptosis was not increased (Fig. 5D,I), whereas proliferation was severely decreased in *Isl1Cre/+;Bmpr1a* mutants (Fig. 5E,J), suggesting that observed decreased size of hindlimb buds in mutants could be attributed to reduced cell proliferation rather than increased cell death.

## Potential downstream effector targets of Bmp signaling in hindlimb

Ectopic outgrowths from the ventral surface of the limb have been reported in mice which are null for the homeodomain transcription factor engrailed-1 (En1) (Loomis et al., 1996). Engrailed marks and is required for ventral limb identity and normal apical ectodermal ridge (AER) formation. Therefore, we examined expression of En1 in *Isl1Cre/+;Bmpr1a* mutant hindlimbs and those of wild-type littermate controls. Examination of En1 expression (Fig. 6A-D,F-I) revealed that expression of En1 in both endogenous and ectopic

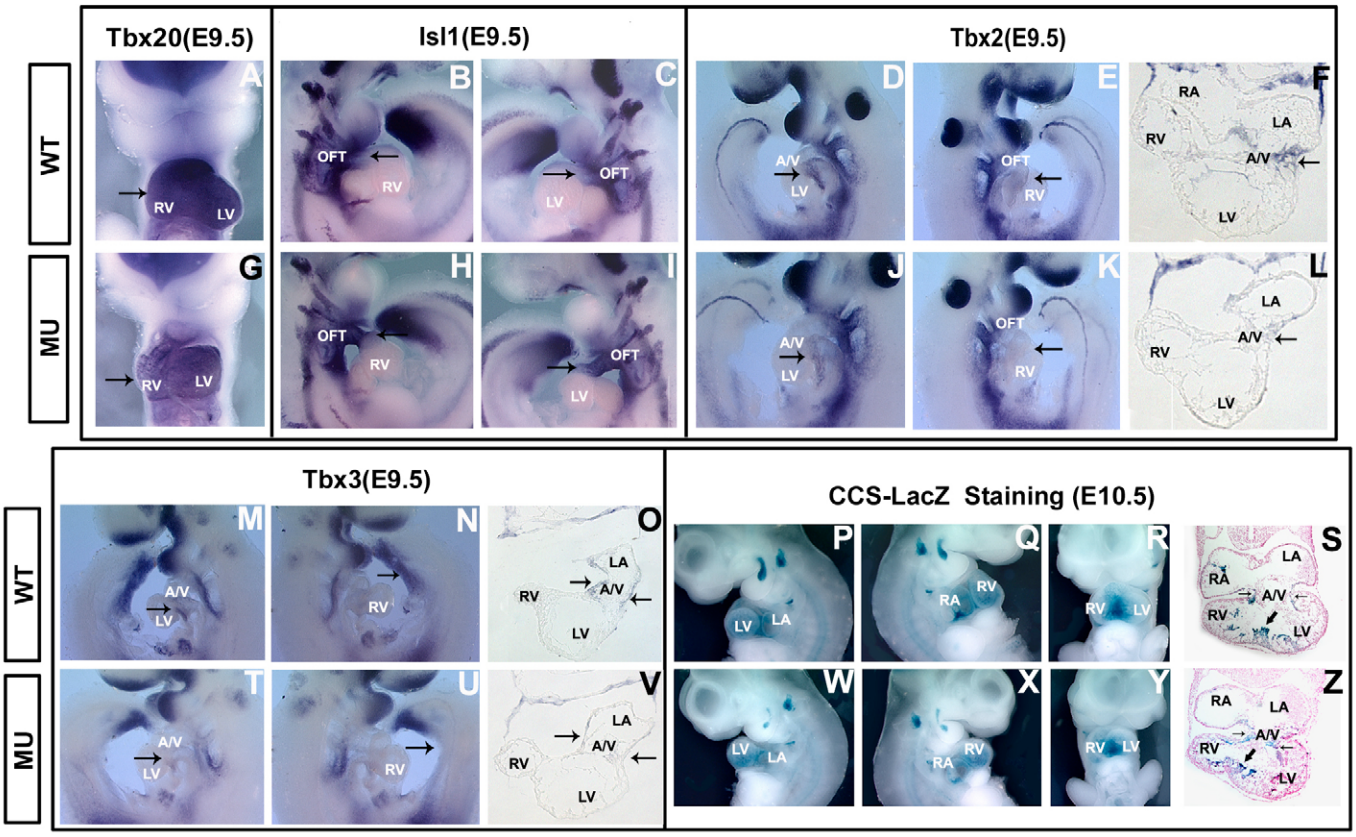


**Fig. 4. Analysis of potential downstream effector targets of Bmp signaling in *Isl1Cre/+;Bmpr1a* mutants and wild-type littermate controls.** (**A-O**,**T**,**U**,**V**) Whole-mount and section RNA in situ analysis of transcription factors required for cardiac development. In *Isl1Cre/+;Bmpr1a* mutants, expression of Tbx20 is down (A,G), Isl1 is upregulated in outflow tract (B,C,H,I); Tbx2 is downregulated in the heart and Tbx3 is downregulated both in heart and hindlimb bud (D-F,J-L,M-O,T-V), relative to somite-matched littermate controls. (**P-S**,**W-Z**) X-gal staining of a conduction system marker, CCS-*lacZ* in wild-type and *Isl1Cre/+;Bmpr1a* mutant backgrounds. Conduction system cells are formed and present in *Isl1Cre/+;Bmpr1a* mutants, suggesting that severe reduction of Tbx3 staining does not reflect absence of Tbx3-expressing cells. OFT, outflow tract; RV, right ventricle; LV, left ventricle; A/V, atrioventricular canal; LA, left atrium; RA, right atrium. Arrows in A,G indicate the heart; in B,C,E,H,I,K indicate the OFT; in D,F,J,L,M,O,T,V indicate A/V; and in N,U indicate the hind limb bud. In S and Z, thin arrows indicate A/V, thick arrows indicate ventricular septum.

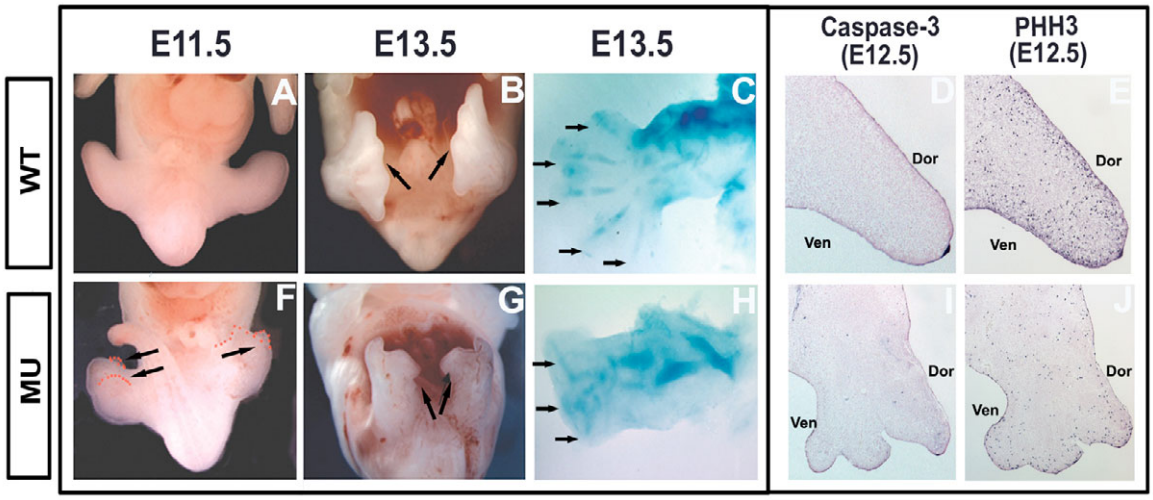


**Fig. 5. Aberrant hindlimb morphology and proliferation in *Isl1Cre/+;Bmpr1a* mutants.** (**A**,**B**,**F**,**G**) Whole-mount views of hindlimbs of wild-type and mutant embryos. Mutant limbs had ectopic outgrowths on ventral surface at right angles to endogenous limb (arrows, F,G). (**C**,**H**) Alcian Blue staining for cartilage. Cartilage structures were severely abnormal in mutant hindlimbs, with a reduced number of digit elements compared with wild type (arrows C,H). (**D**,**I**) Apoptosis analysis. No increases in apoptosis were evident in mutant relative to wild-type hindlimb. (**E**,**J**) Cell proliferation analysis. Staining for phosphorylated histone H3 (PHH3) revealed reduced rates of proliferation in mutant hindlimbs relative to controls. Arrows in B indicate the hind limbs.

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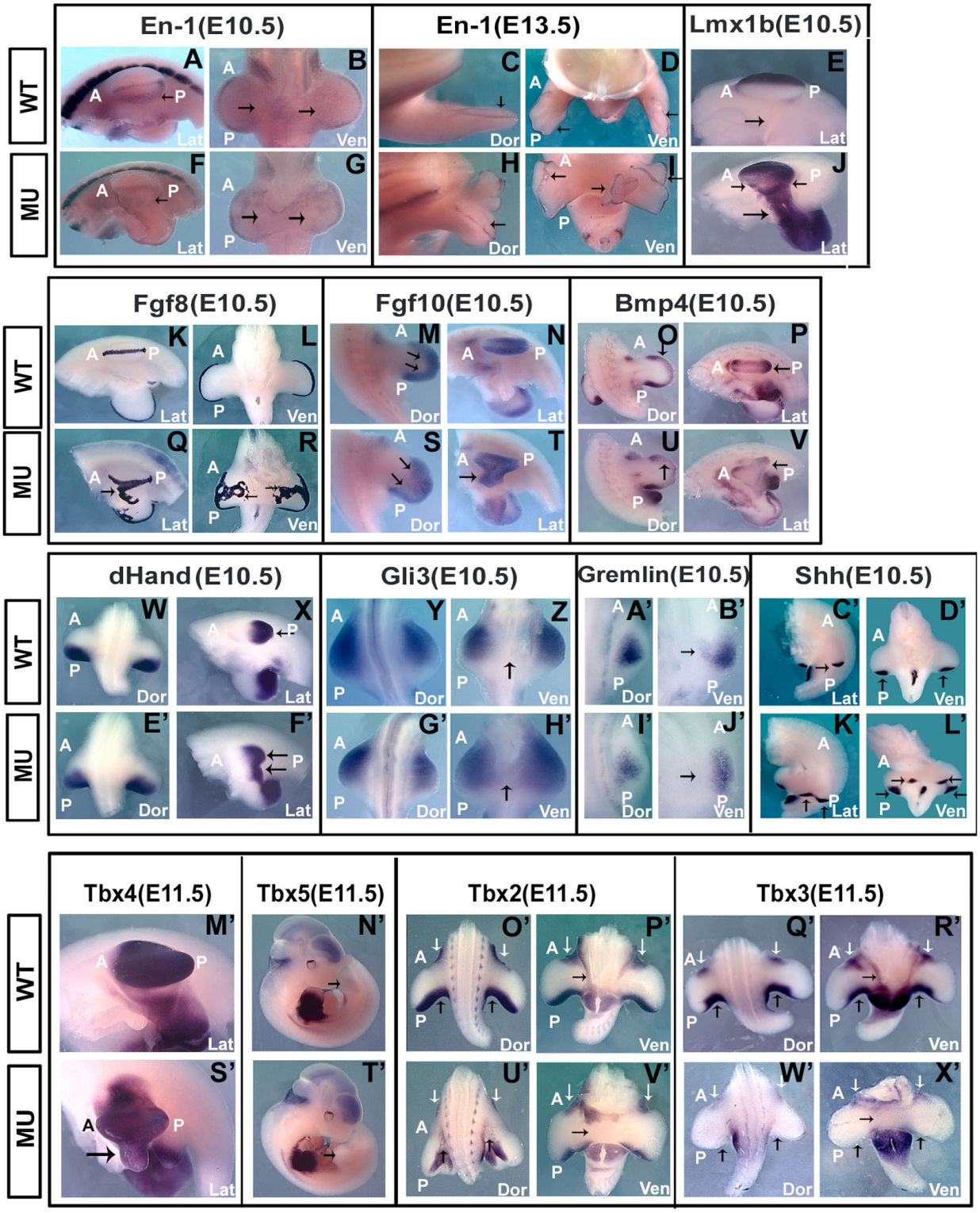


**Fig. 6. Gene expression analysis in hindlimbs of *Isl1Cre/+;Bmpr1a* mutants and wild-type littermate controls.** Aberrant dorsal ventral patterning in mutants is evidenced by decreased expression of En1 in ventral ectoderm (**A-D**,**F-I**), and ventral expression of Lmx1b (**E**,**J**). En1 continues to be expressed in AER of mutants, but AER expression is sometimes disrupted or split (arrows H,I). Fgf8 expression in AER is broadened and ectopically expressed in mutant hindlimbs (**K**,**L**,**Q**,**R**). Fgf10 expression appears normal (**M**,**N**,**S**,**T**), whereas Bmp4 expression is decreased in mutants (**O**,**P**,**U**,**V**). Anterior-posterior patterning and the zone of polarizing activity appear normal in both endogenous and ectopic hindlimb structures, as evidenced by expression of Hand2 (dHand), Gli3, gremlin and Shh (**W-L'**). In *Isl1Cre/+;Bmpr1a* mutants, expression of Tbx4 and Tbx5 is unaffected (**M'**,**N'**,**S'**,**T'**); Tbx2 expression is somewhat reduced in limb and interlimb regions (arrows **O'**,**P'**,**U'**,**V'**); and Tbx3 expression is severely reduced throughout the limb (arrows **Q'**,**R'**,**W'**,**X'**). A, anterior; P, posterior; Lat, lateral view; Ven, ventral view; Dor, dorsal view. Arrows in E, Z and H′ indicate tissue between the two hind limbs.

AERs was present in mutant hindlimbs, although there were sporadic interruptions in expression in the AER in mutant limbs (arrows Fig. 6H,I). Expression of En1 in ventral ectoderm, however, was disrupted in *Isl1Cre/+;Bmpr1a* mutants, preferentially in posterior ventral ectoderm, in keeping with fate mapping results of *Isl1Cre/+* limb progenitors, which demonstrated a preferential contribution to posterior mesoderm (arrows Fig. 6F,G).

En1 is required to maintain dorsal-ventral (DV) identity of the limb, and to maintain the apical ectodermal ridge (Kimmel et al., 2000). En1 specifies ventral identity through repression of Wnt7a in ventral ectoderm. Wnt7a in dorsal ectoderm induces expression of the LIM-homeodomain transcription factor Lmx1b in underlying dorsal mesenchyme to specify dorsal identity (Niswander, 2003; Tickle, 2003). In *En1* mutants, Lmx1b is ectopically expressed in ventral mesoderm, resulting in dorsalization (Kimmel et al., 2000; Loomis et al., 1996; Loomis et al., 1998). Examination of Lmx1b expression in *Isl1Cre/+;Bmpr1a* mutants revealed expression in ventral mesoderm, consistent with decreased En1 expression in posterior ventral ectoderm, demonstrating ectopic dorsal identity within the ventral domain (Fig. 6E,J). Lmx1b expression extended throughout the interlimb region, where ectopic growth of tissue was observed.

In En1 mutants, the ventral domain of the AER is expanded, and the AER is broader (Loomis et al., 1996). The AER marker Fgf8 is also ventrally expanded and broader in *Isl1Cre/+*: Bmpr1a mutants (Fig. 6K,L,Q,R). Examination of Fgf8 expression also demonstrated striking ectopic domains, consistent with ectopic AERs being present in *Isl1Cre/+;Bmpr1a* mutants. Fgf10 expression appeared similar in mutants and wild-type littermates (Fig. 6M,N,S,T). Expression of Bmp4 in the region of the AER was diminished in *Isl1Cre/+;Bmpr1a* mutants relative to wild-type littermate controls (Fig. 6O,P,U,V).

Anterior-posterior (AP) patterning was examined in *Isl1Cre/+;Bmpr1a* mutants. Early AP patterning results from mutual antagonism between transcription factors Gli3 in the anterior, and Hand2 (dHand) in the posterior domains, respectively (Niswander, 2003). Hand2 then induces sonic hedgehog (Shh) in the posterior zone of polarizing activity (ZPA). Expression of these AP patterning genes appears relatively normal in *Isl1Cre/+;Bmpr1a* mutants, with the exception that ectopic domains of expression are observed in ectopic outgrowths, suggesting that in these outgrowths, too, AP patterning is occurring normally (Fig. 6W-L).

Gremlin (Grem1 – Mouse Genome Informatics) is a Bmp antagonist required to maintain the AER (Michos et al., 2004). Expression of gremlin was reduced in *Isl1Cre/+;Bmpr1a* mutants, suggesting that its expression depends in part on active Bmp signaling.

Tbx5 and Tbx4 are specifically expressed in forelimb and hindlimb. We examined whether expression of either of these genes was perturbed consequent to ablation of Bmpr1a in hindlimb progenitors. No alterations were observed (Fig. 6M′,S′,N′,T′).

Experiments in chick suggest that expression of Tbx2 and Tbx3 in the limb is downstream of Bmp signaling (Suzuki et al., 2004; Tumpel et al., 2002), but whether Bmp signaling is required within mesoderm or ectoderm has not been examined. In limbs of *Isl1Cre/+;Bmpr1a* mutants Tbx2 was found to be expressed in most domains, although at reduced levels relative to controls (Fig. 6O′,P′,U′,V′), whereas Tbx3 expression was severely reduced (Fig. 4N,U; Fig. 6Q′,R′,W′,X′). Tbx2 expression was evident in ectopic outgrowths of *Isl1Cre/+;Bmpr1a* mutants (Fig. 6U). These results suggest that Bmp signaling within mesoderm is required for Tbx3 expression, and may also contribute to Tbx2 expression.

### Tbx3 is a direct target of Bmp Smads

A requirement for Bmp signaling within limb bud mesoderm for Tbx3 expression suggested that Tbx3 might be a direct target of Bmp Smads. To investigate this possibility, we performed bioinformatics analysis of upstream regions of the Tbx3 gene, and identified a T-box binding site conserved between human and mouse Tbx3 genes. Several additional conserved elements were also identified, previously demonstrated to be required for binding and regulation by Bmp Smads within the Id1 promoter (Korchynskyi et al., 2002) (Fig. 7A). We performed chromatin immunoprecipitation (ChIP) analysis utilizing genomic DNA isolated from embryonic limb buds, and an antibody that recognizes Bmp Smads (Fig. 7A; see Materials and methods). Results of this analysis demonstrated specific binding of Bmp Smads to a region of the Tbx3 promoter containing the identified conserved binding sites. Transient cotransfection analyses of a 2 kb Tbx3 promoter-luciferase reporter and expression vectors for Smad1 and Smad4 were performed in P19 cells, and demonstrated that the Tbx3 promoter was significantly activated in response to Smad1 and/or Smad4 (Fig. 7B). Together, these data provide the first evidence that Tbx3 is a direct target of Bmp Smads in vivo.

## DISCUSSION

Our data have demonstrated that Isl1 marks a progenitor population for developing hindlimb, as it does for cardiac progenitors, suggesting that Isl1 may be upstream in common heart/hindlimb pathways. Isl1 is expressed in an anterior to posterior gradient in the lateral mesoderm flanking the limb bud, suggesting that it may play a role in early AP patterning of the limb. Analysis of the contribution of Isl1-expressing cells to the hindlimb bud illustrates an AP gradient of these cells, with highest numbers posteriorly. Intriguingly, this distribution is similar to that observed for hedgehog responding cells within the limb (Ahn and Joyner, 2004). Lineage studies with *Isl1Cre/+* and R26R-*lacZ* have demonstrated that Isl1-expressing cells contribute to a majority of mesenchymal cells in the limb, consistent with previous fate mapping studies in chick and in mouse, where posterior progenitors have been demonstrated to contribute disproportionately to the limb bud (Vargesson et al., 1997; Wanek et al., 1989). Expression of other genes, including *Pitx1* and *Tbx4* is also specific to hindlimb (Logan and Tabin, 1999; Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999), and it will be of interest to investigate potential interactions of these transcription factors with Isl1.

To investigate requirements for Bmp signaling in Isl1-expressing progenitors of heart and limb, we utilized *Isl1Cre/+* to ablate Bmpr1a. Previous data utilizing an α-myosin heavy chain-specific Cre, which is first active in differentiated myocytes, demonstrated that Bmp signaling through Bmpr1a is required in differentiated myocytes for ventricular septation, atrioventricular cushion morphogenesis and myocyte survival (Gaussin et al., 2002). Outflow tract formation in *α-MHC-cre;Bmpr1a* mutants was normal. In *Isl1Cre/+;Bmpr1a* mutants, we observe a similar spectrum of defects, including defective atrial septation not previously described. In contrast to results with *α-MHC-Cre* ablation of Bmpr1a, with *Isl1Cre/+* we observe persistent truncus arteriosus (PTA). Together, these observations suggest that signaling through Bmpr1a is required for outflow tract formation in the Isl1 expression domain, but not in differentiated cardiomyocytes. It is possible that outflow tract defects in *Isl1Cre/+;Bmpr1a* mutants reflect delayed development, however mutations in *BmprII*, *Bmp4*, and *Bmp7* also result in PTA (Delot et al., 2003; Liu et al., 2004), suggesting that a Bmpr1a/BmprII receptor complex mediates signaling by Bmp4 and Bmp7 to effect outflow tract septation.

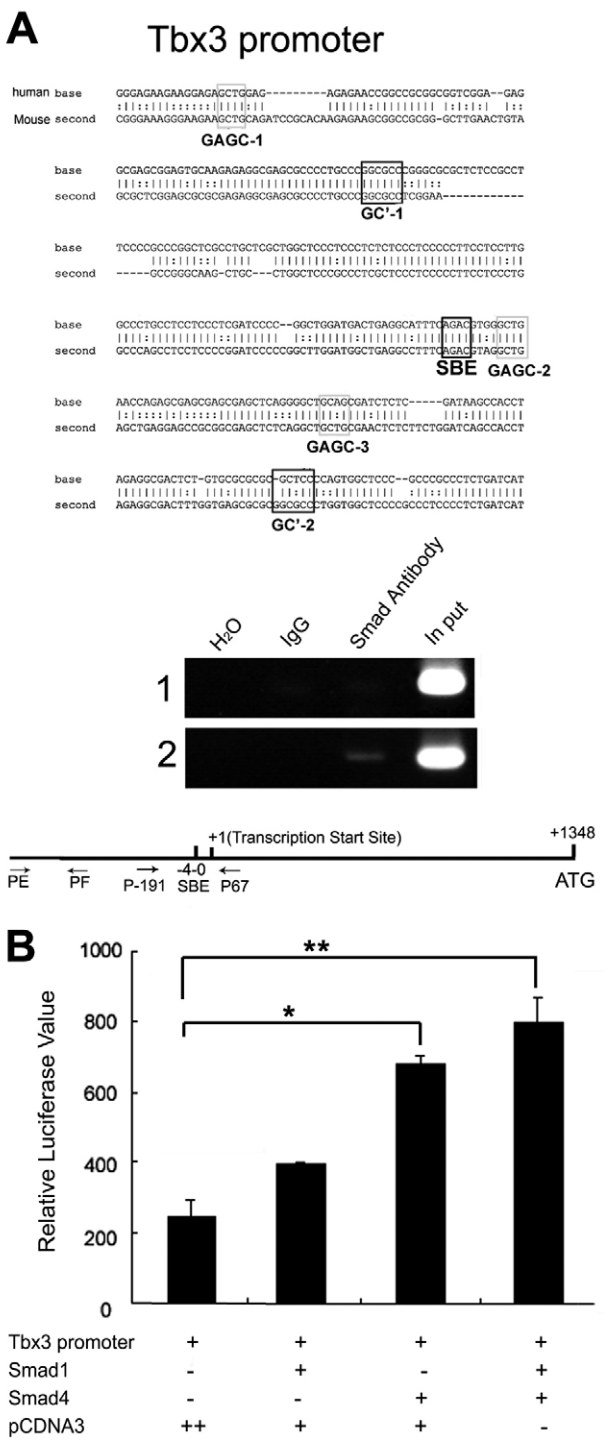


**Fig. 7. The Tbx3 promoter is directly activated by Bmp Smads.** (**A**) Bmp Smads bind to the Tbx3 promoter in vivo. (Top) A comparison of the human and mouse sequences showing a conserved consensus Smad binding site approximately 1.3 kb upstream of the ATG site within the Tbx3 promoter. Two other conserved elements (GAGC sequences and GC′ sequences), previously found to be required for Smad activation of the Id1 promoter (Korchynskyi et al., 2002), were identified within the Tbx3 promoter region. (Bottom) In vivo ChIP analysis utilizing extracts from embryonic hindlimb and an antibody specific for Bmp Smads demonstrated specific binding of Bmp Smads to a region containing the Smad binding element (SBE; 2). Control primers flanking an unrelated region demonstrated no specific binding with Bmp Smads (A; 1). No recruitment was found with IgG. (**B**) Transient transfection analysis in P19 cells. A 2 kb fragment of the Tbx3 promoter region driving luciferase expression was cotransfected into P19 cells with control vector alone (pCDNA3), or expression constructs for Smad1, Smad4, or both Smad1 and Smad4. The Tbx3 promoter exhibited a significant increase in relative luciferase activity in response to Smad1/Smad4. $^{**}P<0.01$, $^{*}P<0.05$, paired *t*-test.

Proliferative defects were found in hearts of *Isl1Cre/+;Bmpr1a* mutants. We observed decreased expression of Tbx20 in *Isl1Cre/+;Bmpr1a* mutant hearts. Tbx20 is required for cardiac proliferation, and therefore may be an effector target of Bmp for cardiac proliferation. Factors that regulate Tbx20 expression in heart have not previously been identified. Future studies will examine whether Tbx20 is a direct target of Bmp Smads. Expression of Isl1 is also highly upregulated in outflow tracts of *Isl1Cre/+;Bmpr1a* mutants. In Tbx20 mutants, expression of Isl1 is upregulated at low levels throughout the heart (Cai et al., 2005). However, in *Isl1Cre/+;Bmpr1a* mutants, Isl1 is highly upregulated, selectively in the outflow tract. These observations suggest that Bmp signaling may downregulate expression of Isl1, as Isl1 progenitors enter the heart, and may cooperate with Tbx20 in doing so, effecting the transition between proliferative modes in developing cardiac progenitors. As ablation of Bmp10 also results in proliferative defects within myocardium (Chen et al., 2004), our observations suggest that Bmp10 may be acting through Bmpr1a.

We observed decreased expression of Tbx2 and Tbx3 in *Isl1Cre/+;Bmpr1a* mutant hearts. Previous data in chick utilizing noggin-coated beads demonstrated that cardiac expression of Tbx2 was dependent on Bmp signaling (Yamada et al., 2000). Here, we demonstrate that both Tbx2 and Tbx3 are downstream of Bmp signaling in the heart. Tbx2 mutants exhibit defects in outflow tract remodeling and in atrioventricular canal development. The cardiac phenotype of Tbx3 mutants is currently being analyzed (Robert Kelly and Ginny Papaioannou, personal communication). Tbx2 and Tbx3 are coexpressed in an overlapping manner in the heart, and can function redundantly to regulate expression of target genes (Christoffels et al., 2004; Hoogaars et al., 2004; Lingbeek et al., 2002). These observations suggest that Tbx3 may also be playing a role in heart development. Additionally, a subset of ulnar-mammary patients present with cardiac defects, including ventricular septal defects (Craig Basson, personal communication). This suggests that downregulation of Tbx3 may account for ventricular septal defects observed in *Isl1Cre/+;Bmpr1a* mutants. It is possible that reducing expression of both genes simultaneously, consequent to reduced Bmp signaling, may have more severe consequences on heart development than knockout of either gene alone. This idea will be tested by analysis of cardiac phenotypes in double knockout mice.

Ablation of Bmpr1a in Isl1 progenitors resulted in a remarkable hindlimb phenotype, with ectopic outgrowths emerging from the ventral limb surface. Ectopic ventral outgrowths are also observed in *En1* mutants. In *En1* mutants, ectopic AERs are relatively unstable (Loomis et al., 1996). In contrast, in *Isl1Cre/+;Bmpr1a* mutants, ectopic AERs were quite robust, resulting at later stages in multiple outgrowths from the ventral limb surface. This difference may reflect distinct domains of En1 expression affected in the two mutants. Examination of En1 expression in *Isl1Cre/+;Bmpr1a*

mutants revealed selective downregulation of En1 in ventral limb ectoderm, whereas En1 expression in the AER was largely maintained, although disruptions were evident. En1 is required for dorsal ventral patterning and for AER maintenance (Loomis et al., 1996). Both endogenous and ectopic AERs appear to be maintained in *Isl1Cre/+;Bmpr1a* mutants, in contrast to the situation with En1 null mice. This suggests that maintenance of the AER requires En1 expression in the AER itself. Expansion of the AER, however, occurs in both En1 and *Isl1Cre/+;Bmpr1a* mutants, suggesting that ventral suppression of the AER requires expression of En1 in extra-AER ventral ectoderm. Ectopic AERs in *Isl1Cre/+;Bmpr1a* mutants were observed at the intersection of ventral En-expressing ectoderm and aberrantly dorsalized non-En-expressing ectoderm, supporting the model that AER formation occurs at dorsal-ventral interfaces (Loomis et al., 1996; Niswander, 2003).

Bmp4 is highly expressed in ectoderm and mesenchyme immediately flanking the DV midline of the AER, and may mediate restriction of the AER. Broadening of the AER has been observed consequent to blocking Bmp signaling by ectopic expression of noggin in the AER, or consequent to loss of Bmp4 in mesoderm (Selever et al., 2004; Wang et al., 2004). In *Isl1Cre/+;Bmpr1a* mutants, Bmp4 expression is diminished in the region of the AER, which may contribute to AER broadening. This pathway may be upstream of En1 in ventral ectoderm, or parallel to it.

Excision of Bmpr1a specifically in limb ectoderm (Ahn et al., 2001) results in a distinct phenotype from that observed with excision of Bmpr1a in Isl1-expressing limb mesoderm. Excision of Bmpr1a in limb ectoderm resulted in complete loss of En1 expression, defective or no AER formation, and dorsal ventral patterning defects. These results in concert with those presented here demonstrate requirements for Bmpr1a signaling in both limb mesoderm and limb ectoderm for dorsal ventral patterning.

Ablation of Bmpr1a with *Isl1Cre/+* resulted in ablation of Bmp signaling from the interlimb region. This resulted in a fusion between the two hindlimbs. These data demonstrate a requirement for Bmp signaling in maintaining a separation between the two hindlimbs, to allow normal interlimb development. Analogous effects of Bmp ablation have been observed in feathers, where ablation of Bmp signaling results in fusions between feathers (Bardot et al., 2004).

Our results provide evidence to demonstrate that Tbx3 is a direct target of Bmp Smads in vivo. In *Isl1Cre/+;Bmpr1a* mutants, Tbx3 expression was severely downregulated, beginning in lateral mesoderm adjacent to the future hindlimb bud, yet no differences in AP limb patterning were observed. This was somewhat surprising, given that in Tbx3 null mice, Shh expression in the zone of polarizing activity is severely absent or reduced (Davenport et al., 2003). This suggests that domains of Tbx3 expression not affected in the *Isl1Cre/+;Bmpr1a* mutant are required for Shh expression/ZPA formation, or that Bmp signaling may be an effector of adverse effects on ZPA formation in Tbx3 mutants.

In summary, our results have highlighted common pathways between heart and limb in development. We have discovered that the LIM homeodomain transcription factor Isl1 marks progenitors of both, and is turned off as progenitors migrate into forming heart or hindlimb. It will be of great future interest to investigate requirements for Isl1 in hindlimb formation. We have investigated the role of Bmp signaling in the Isl1 domain in both heart and limb. Ablation of Bmp signaling in limb progenitors affects specific domains of En1 expression, allowing attribution of distinct spatial requirements for En1 in limb development. We have identified novel targets of Bmp signaling in heart, including Tbx20, Isl1 and Tbx3. Additionally, we have found that Tbx3, the gene which is mutated in human syndromes that affect both limb and heart, is a direct downstream target of Bmp signaling through Bmpr1a.

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