Precursor Pool for Murine Sinus Horn Formation

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Rationale: Canonical (β-catenin [Ctnnb1]-dependent) wingless-related MMTV integration site (Wnt) signaling plays an important role in the development of second heart field–derived structures of the heart by regulating precursor cell proliferation. The signaling pathways that regulate the most posterior elongation of the heart, that is, the addition of the systemic venous return from a Tbx18+ precursor population, have remained elusive.

Objective: To define the role of Ctnnb1-dependent Wnt signaling in the development of the cardiac venous pole.

Methods and Results: We show by in situ hybridization analysis that Wnt pathway components are expressed and canonical Wnt signaling is active in the developing sinus horns. We analyzed sinus horn (Tbx18cre-/-) -specific Ctnnb1 loss- and gain-of-function mutant embryos. In Ctnnb1-deficient embryos, the dorsal part of the sinus horns is not myocardialized but consists of cells with at least partial fibroblast identity; the sinoatrial node is unaffected. Stabilization of Ctnnb1 in this domain results in the formation of undifferentiated cell aggregates. Analysis of cellular changes revealed a role of canonical Wnt signaling in proliferation of the Tbx18+ mesenchymal progenitor cell population.

Conclusions: Wnt/β-catenin signaling maintains the Tbx18+ Nkx2–5– mesenchymal precursor pool for murine sinus horn formation. (Circ Res. 2011;109:e42-e50.)

Key Words: canonical Wnt signaling β-catenin sinus horn cardiac progenitor

The multicambered mammalian heart develops by a complex morphogenetic process from a linear tube, which is established shortly after gastrulation as a functional organ. Growth and elongation of this simple tube only partly relies on proliferation of cardiomyocytes within the tube itself but largely depends on recruitment and differentiation of mesenchymal precursor cells at the poles. In fact, myocardium of the linear heart tube, which is established at E8.25 in the mouse, will only contribute to the left ventricle. Myocardium of the outflow tract (OFT), right ventricle, and large portions of the atria of the mature heart derive from a population of splanchnic mesodermal precursor cells that is contiguous and dorsal to the heart tube. These pharyngeal mesodermal precursors, which are now termed the second heart field (SHF), express ISL1 transcription factor, LIM/homeodomain (Is1), T-box 1 (Tbx1), and the fibroblast growth factor genes Fgf8 and Fgf10. They are molecularly distinguished from the progenitors of the linear heart tube, the first heart field (FHF), which are negative for all of these markers and express (although not exclusively) NK2 transcription factor related, locus 5 (Nkx2–5), T-box 5 (Tbx5), and heart and neural crest derivatives expressed transcript 1 (Hand1). Although molecularly distinct, both precursor populations can be envisaged as a continuum that allows the spatiotemporally regulated contribution of precursors to the growing heart tube. The systemic venous return consists of the myocardial sleeves of the right superior and inferior caval veins, the sinus venarum, the coronary sinus (persisting left superior caval vein in the mouse), and the sinoatrial node (SAN). This posterior part of the heart develops after the chambers have been established at E9.5, by recruitment and myocardialization of mesenchymal precursor cells to the common atrium until E14.5. This precursor cell pool is distinguished from the FHF and SHF by presence of T-box 18 (Tbx18) and absence of Nkx2–5 expression. Loss of Tbx18 in this “third heart field” leads to a delay in myocardialization of the sinus horns and a marked reduction of the SAN. Continuous elongation of the heart tube requires a tight control of proliferation and deployment of mesenchymal progenitor cells. A number of studies identified canonical Wnt signaling as a crucial upstream regulator of SHF proliferation. Conditional inactivation of β-catenin (Ctnnb1) under control of the myocyte enhancer factor 2C (Mef2c) in the anterior aspect of the SHF resulted in reduced proliferation and subsequent truncation of the OFT and right ventricle. More recently, Tian et al showed that wingless-related MMTV integration site 2 (Wnt2) acts in the posterior portion of the SHF to stimulate proliferation of precursor cells and subsequent formation of a portion of the atrioventricular canal (AVC) and the atria. Thus, canonical Wnt signaling maintains proliferation in the SHF to allow the polar...
elongation of the heart. Notably, Wnt signaling does not play such a role in the FHF. In fact, Ctnnb1 inhibits cardiac differentiation in this region.

The signaling pathways that regulate the most posterior elongation of the heart, that is, the addition of the systemic venous return, have remained elusive. Although the SHF and sinus horn mesoderm bear different molecular signatures (Nkx2–5/Isl1 versus Nkx2–5/Tbx18), we wondered whether canonical Wnt signaling exerts a similar function in the latter context. We present data obtained from genetic loss-and gain-of-function experiments in the mouse that demonstrate a function of canonical/Ctnnb1-dependent Wnt signaling in sinus horn development.

Methods

Mice

Mice with a knock-in of the cre recombinase gene into the Tbx18 locus (Tbx18<sup>tm4cre</sup>AKis, synonym: Tbx18<sup>cre</sup>),<sup>9</sup> mice with 2 loxP sites flanking the Ctnnb1 locus from exon 2 to exon 6 (Ctnnb1<sup>tm2Kem</sup>, synonym: Ctnnb1<sup>fx</sup>),<sup>10</sup> mice with 2 loxP sites flanking exon 3 of the Ctnnb1 locus (Ctnnb1<sup>tm1Mmt</sup>, synonym: Ctnnb1<sup>Ex3</sup>fl),<sup>11</sup> and the fluorescent reporter line (Gt<sup>ROSA</sup>26Sortm4(ACTB/tdTomato, EGFP)Luo/J, synonym: R26<sup>mTmG</sup>)<sup>12</sup> were all described before. All lines were maintained on an outbred (NMRI) background. Animal care was in accordance with national and institutional guidelines.

An expanded Methods section is available in the Online Data Supplement available at http://circres.ahajournals.org.

Results

(Canonical) Wnt Signaling in Sinus Horn Development

To determine the spatiotemporal involvement of Wnt signaling in sinus horn development, the expression of genes encoding Wnt ligands (Wnt1, -2, -2b, -3, -3a, -4, -5a, -5b, -6, -7a, -7b, -8a, -8b, -9a, -9b, -10a, -10b, -11, and Wnt16), Frizzled receptors (Fzd1 to Fzd10), and Wnt antagonists of the family of secreted frizzled-related proteins (Sfrp1 to Sfrp5), was analyzed by in situ hybridization on sections of the cardiac venous pole. Because sinus horn development occurs from embryonic day (E) 9.5 to E14.5, we used E10.5, E12.5, and E14.5 embryos for this expression screen (Figure 1 and Online Figure I). Most of the tested components of the Wnt signaling pathway showed no caval vein specific gene expression. However, the Wnt ligand Wnt2 was expressed in the walls of the left and right cardinal vein that bulged into the pericardial cavity at E10.5 (Online Figure I), E12.5, and E14.5 (Figure 1A and 1E). Expression of Wnt10a was detectable in both caval veins at E14.5 (Figure 1B and 1F).

Frizzled receptors (Fzd7 and Sfrp1) were expressed in the intrapericardial component of the cardinal veins at all tested embryonic stages (Online Figure I; Figure 1C, 1D, 1G, and 1H). Therefore, genes...
expression of the sinus horns at E12.5 and E14.5 (Figure 1J and 1N).

and 1M). Importantly, \textit{Axin2}, a bona fide target of \textit{Ctnnb1} expression marks the sinus horn lineage from E8.25 onward, allowing the specific genetic lineage of this subregion. The specific inactivation of the canonical Wnt signaling pathway in the \textit{Tbx18} $^{\text{cre}}$-expressing sinus horns was confirmed by specific absence of \textit{Ctnnb1} (Online Figure II) and of \textit{Axin2} expression in the sinus horns at E12.5 and E14.5 (Figure 1J and 1N). Expression of \textit{Tbx18} was unaffected in \textit{Ctnnb1}-deficient sinus horns, excluding that the \textit{cre} driver activity depends on \textit{Ctnnb1} function (Figure 1K, 1L, 1O, and 1P).

\textit{Tbx18} $^{\text{cre}^{-}}$; \textit{Ctnnb1} $^{\text{fx/fx}}$ mice survived embryogenesis but skeletal malformations led to death shortly after birth. At E18.5, the mutant heart had a less pronounced apex but appeared otherwise normal on a gross morphological level. Histological analysis revealed that chambers, valves, and septa were unaffected (Online Figure III). Conditional deletion of \textit{Ctnnb1} from the proepicardium/epicardium using a \textit{Gata5cre} line resulted in lethality between E15.5 and E18.5, a smaller heart with a thinned myocardial compact layer, and epicardial defects. Although we have not yet analyzed epicardial development in our mutants, we assume that phenotypic differences between the 2 mouse lines do not arise from differences in epicardial recombination but from ectopic recombination by \textit{Gata5cre} in the early ventricular myocardium and cushion tissue.

In \textit{Tbx18} $^{\text{cre}^{-}}$; \textit{Ctnnb1} $^{\text{fx/fx}}$ mice, sinus horns were positioned next to the midline and within the pericardial cavity as in control embryos (Figure 2A and 2B). However, in situ hybridization analysis for cardiomyocyte-specific troponin I cardiac 3 (\textit{Tnni3}, also known as \textit{cnfn}) revealed a specific lack of myocardium in the dorsal part of the sinus horns next to the pleuropericardial membrane in mutant hearts (Figure 2C and 2D). Serial section analysis and subsequent 3-dimensional reconstruction of representative embryos confirmed the regional specificity of the defect, as myocardialization of atria, the pulmonary vein, and ventral sinus horns was normal (Figure 2E and 2F).

To determine the onset of sinus horn defects in \textit{Tbx18} $^{\text{cre}^{-}}$; \textit{Ctnnb1} $^{\text{fx/fx}}$ hearts, we analyzed embryos at earlier developmental stages. Histological analysis at E14.5, E12.5 (Figure 2) and at E10.5 (data not shown) revealed that sinus horns were released from the pericardial wall into the pericardial cavity and positioned medially in mutant hearts similar to control embryos.
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(Figure 2G through 2J). In the latter, Tnni3 expression, and therefore myocardialization, was restricted to the most ventral aspect of the sinus horn at E10.5 (Online Figure IX). At E12.5, the Tnni3+ domain in the control embryos expanded dorsally and ensheathed the sinus horns at E14.5. In Tbx18<sup>cre<sup>+</sup></sup>;Ctnnb1<sup>fl<sup>f<sub>x</sub></sub>x</sup> embryos, myocardialization remained restricted to the ventral aspect of the sinus horn at both stages (Figure 2K through 2R). Hence, myocardialization defects arise shortly after E10.5 when Tbx18<sup>+</sup> is lost in the sinus horn lineage. 

Canonical Wnt Signaling Is Dispensable for SAN Development

Because Tbx18 is also expressed in the developing SAN of control and conditional Ctnnb1-mutant embryos (Figure 3A and 3B),<sup>5</sup> we analyzed the consequence of Ctnnb1 deletion for the integrity of this tissue. Axin2 was expressed at low levels, rarely above background, in both control and Ctnnb1-deficient SAN (Figure 3C and 3D), indicating if present at all, low levels of canonical Wnt signaling in this tissue. In E18.5 control embryos, the SAN expressed T-box 3 (Tbx3) and hyperpolarization-activated, cyclic nucleotide-gated K<sup>+</sup> 4 (Hcn4). Expression of gap junction protein, alpha 5 (Gja5), also known as Cx40) was excluded from the SAN myocardium but was detectable in the Tnni3+ myocardialized dorsal part of the right sinus horn, the endothelial cells of the SAN artery, and the atria. Tbx3 and Hcn4 expression was unaltered whereas Tnni3 and Gja5 expression was almost completely absent in the dorsal part of the right sinus horn in Tbx18<sup>cre<sup>+</sup></sup>;Ctnnb1<sup>fl<sup>f<sub>x</sub></sub>x</sup> hearts at E18.5 (Online Figure IV) and E17.5 (Figure 3E through 3H). To determine whether the shape and volume of the SAN and right caval vein is altered in mutant embryos, we additionally performed serial sectioning and subsequent 3-dimensional reconstruction analysis of the Hcn4<sup>+</sup> SAN and the Gja5<sup>+</sup> region of the right caval vein (Figure 3I through 3K). We detected a 93% decrease of the Gja5<sup>+</sup> domain in Tbx18<sup>cre<sup>+</sup></sup>;Ctnnb1<sup>fl<sup>f<sub>x</sub></sub>x</sup> mice. The lumen of the right caval vein and the volume of the SAN were only slightly decreased. The elongated comma-like structure of the SAN at the entrance to the right atrium was preserved (Figure 3J). We conclude that Ctnnb1 and canonical Wnt signaling are dispensable for SAN development but strongly affect the myocardialization of the dorsal aspect of the sinus horns.

The Dorsal Aspect of Ctnnb1-Deficient Sinus Horns Is Made Up of Cells With at Least Partial Fibroblast Identity

It is known that Tbx18<sup>+</sup> precursors are the source for the sinus horn myocardium in wild-type embryos.<sup>3</sup> To determine the fate of Tbx18<sup>+</sup> precursors in Ctnnb1-deficient sinus horns, we introduced an R26<sup>mTmG</sup> reporter allele into the mutant background. In Tbx18<sup>cre<sup>+</sup></sup>;R26<sup>mTmG</sup>/H11545;Ctnnb1<sup>fl<sup>f<sub>x</sub></sub>x</sup> mice, Tbx18<sup>cre<sup>+</sup></sup>-expressing cells and their descendants became irreversibly labeled by expression of a bright membrane-bound green fluorescent protein (GFP). Double immunofluorescence analysis for GFP and the cardiomyocyte marker proteins Tnni3 and troponin T2, cardiac (Tnnt2) at E18.5 confirmed the derivation of the sinus horn myocardium from the Tbx18<sup>+</sup> mesenchyme of the caval veins in control (Tbx18<sup>cre<sup>+</sup></sup>;R26<sup>mTmG</sup>/H11001;Ctnnb1<sup>fl<sub>x</sub></sup>fl<sub>x</sub>) embryos (Figure 4A and 4E and Online Figures V and VI). In Tbx18<sup>cre<sup>+</sup></sup>;R26<sup>mTmG</sup>/H11545;Ctnnb1<sup>fl<sub>x</sub></sup>fl<sub>x</sub> mutant embryos, both the myocardium of the SAN and the dorsal nonmyocardIALIZED region was GFP<sup>+</sup>, that is, derived from Tbx18<sup>+</sup> precursors. GFP expression in the dorsal sinus horn region appeared lower due to the small number of these mesenchymal cells (Figure 4E through 4H and Online Figures V and VI). To more precisely define the cellular character of this nonmyocardialized part of the sinus horns, we expanded our immunohistochemical analysis. In control hearts, the extracellular matrix protein fibronectin 1 (Fn1) was detected in few cells intermingled in the Tbx18-derived sinus horn myocardium, in the pleuropericardial membrane, and in the adjacent tissue next to the dorsal mesocardium (Figure 4B). The GFP<sup>+</sup> region of the dorsal aspect of the right sinus horn in Tbx18<sup>cre<sup>+</sup></sup>;R26<sup>mTmG</sup>/H11545;Ctnnb1<sup>fl<sub>x</sub></sup>fl<sub>x</sub> embryos stained for Fn1 as well as for peristin, another extracellular matrix protein commonly present in fibroblasts. 

Figure 3. Morphology of the SAN in Tbx18<sup>cre<sup>+</sup></sup>;Ctnnb1<sup>fl<sub>x</sub></sub>x hearts. A through H, In situ hybridization analysis of transverse sections through the SAN of control and Tbx18<sup>cre<sup>+</sup></sup>;Ctnnb1<sup>fl<sub>x</sub></sub>x hearts for Tbx18 (A and B), the canonical Wnt signaling target Axin2 (C and D), the SAN marker Hcn4 (E and F), and the atrial marker Gja5 (G and H). I through K, 3-dimensional reconstructions of the lumen of the right superior caval vein (orange), the Hcn4<sup>+</sup> SAN (blue), and the Gja5<sup>+</sup> myocardium of the right sinus horn (red) (I and J), and volume determination of the respective region using Amira software (n=1) (K). Note the reduction of the Gja5<sup>+</sup> domain. Arrowheads point to the dorsal domain of the right sinus horn. Genotypes and stages are as indicated. cv indicates caval vein; rsh, right sinus horn; rsbv, right superior caval vein; rsh, right sinus horn; and SAN, sinoatrial node.
associated with fibrocytes (Figure 4F and 4G and Online Figure V). In the wild-type control, actin, alpha 2, smooth muscle, aorta (Acta2) was expressed in vascular smooth muscle cells and in a thin cell layer underneath the endothelial lining of the dorsal aspect of the sinus horns. In the mutant, this expression was conserved (Figure 4D and 4H and Online Figure V). Moreover, we verified the nearly complete loss of Gja5 in Tbx18cre\(^{+/+}\);R26\(^{mTmG/}\) mutant right sinus horns. rsh indicates right sinus horn; SAN, sinoaatrial node.

**Molecular Analysis of Ctnnb1-Deficient Sinus Horns**

We next analyzed by in situ hybridization analysis whether the observed defects in the Ctnnb1-deficient sinus horns are accompanied by altered expression of factors or pathways associated with regionalization and differentiation of the posterior cardiac pole. Expression of marker genes for the FHF (Nkx2–5) and the SHF (Tbx5, Isl1, and Fgf10) was absent from wild-type and mutant sinus horns at E12.5. Tbx5 was expressed in the atria and sinus horns of both wild-type and Ctnnb1-deficient mice (Online Figure VII). The Fgf signaling pathway promotes proliferation in the anterior SHF and is important for the development of the OFT, but Fgf8 and Fgf10 as well as its target genes ets variant gene 4 (Etv4, also known as Pea3) or Etv5 (also known as Erm) were not detectable in control or Tbx18\(^{cre/+}\);Ctnnb1\(^{lox/lox}\) sinus horns (Online Figure VII, and data not shown). Because the bone morphogenetic protein (Bmp) signaling pathway is crucial for the differentiation of cardiomyocytes in the OFT, we additionally analyzed the expression of Bmp2 and Bmp4, as well as its target genes homeobox, msh-like 1 (Mshx1) and Mshx2, in the developing sinus horns at E12.5. Bmp2 and Bmp4 expression was detectable in the SAN tail, which is located in the sinus venosus, in both control and Ctnnb1-deficient mice (Online Figure VII, and data not shown). Loss of sonic hedgehog (Shh) signaling in Isl1\(^+\) cardiac progenitors leads to OFT defects. Expression of Shh and its target gene patched homolog 1 (Ptc1) was not found in the intrapericardial domain of wild-type and mutant sinus horns at E12.5 (Online Figure VII, and data not shown). A feed-forward loop between the Wnt ligand Wnt2 and the GATA binding protein 6 (Gata6) is important for proliferation of SHF progenitors of the heart and hence for the development of the atria and the AVC. We detected expression of Wnt2 and Gata6 in the developing sinus horns at E14.5 in both wild-type and mutant embryos (Online Figure VIII). Similarly, Sfrp1 expression was unchanged, excluding a self-regulation of Wnt/Ctnnb1 signaling in this region (Online Figure VIII). Furthermore, loss of Ctnnb1 did not affect expression of markers of the pleuropéricardial membrane and its underlying mesenchyme including Sfrp2, Wt1, and aldehyde dehydrogenase family 1, subfamily A2 (Aldh1a2) (Online Figure VIII). In conclusion, these results again demonstrate the distinct molecular signature of the developing sinus horns and the SAN. We could not reveal a role of Bmp, Fgf, and Shh signaling during sinus horn development. Loss of Ctnnb1 does not affect regionalization and precursor contribution at the posterior cardiac pole.

**Ectopic Activation of Canonical Wnt Signaling in Developing Sinus Horns**

To further clarify the role of Ctnnb1-dependent Wnt signaling during sinus horn development, we used a gain-of-function approach with conditional (Tbx18\(^{cre+}\)-mediated) overexpression of a stabilized form of Ctnnb1 (Ctnnb1\(^{E33Q}\)). Tbx18\(^{cre/+}\);Ctnnb1\(^{E33Q}\)/\(^{lox/lox}\)/H11001;R26\(^{mTmG/}\) embryos died during midgestation due to cardiovascular insufficiencies (to be described in full detail somewhere else). Histological inspection of surviving embryos at E12.5...
revealed the presence of large cell aggregates in the developing sinus horns of mutant embryos (Figure 5A and 5B). Strong Axin2 expression confirmed activation of canonical Wnt signaling in these cell clusters (Figure 5C and 5D). Expression of the regional marker genes Tbx18, Tbx3, and short stature homeobox 2 (Shox2) was present, whereas cardiomyocyte marker expression (Tnni3) and the SAN marker gene Hcn4 were absent in these cell aggregates that were derived from Tbx18+ precursors as shown by coexpression of GFP in Tbx18cre/+;R26mTmG+/−, Ctnnb1(Ex3)fl/+ embryos (Figure 5E through 5P). Inspection of earlier developmental stages revealed presence of small cell aggregates from E10.5 onward. Expression of Ev5, a target gene of the Fgf signaling pathway, which is known to promote proliferation in SHF progenitors, was found in the outer layers of these clusters. Also the expression of cyclinD1 (Ccnd1), a direct Wnt target gene and positive regulator of cell cycle progression, was detectable in these cell clusters (Online Figures IX and X). We conclude that sinus horn–specific expression of a stabilized form of Ctnnb1 results in formation of aggregates composed of mesenchymal precursor cells, which are not able to differentiate into cardiomyocytes.

**Cellular Changes in the Tbx18-Positive Region of the Developing Sinus Horns**

We next investigated whether changes in proliferation and apoptosis may underlie the observed lack of cardiomyocytes in the dorsal aspect of Tbx18cre/++;Ctnnb1fl/+ sinus horns. The BrdU incorporation assay revealed decreased proliferation of Tbx18 expressing sinus horn precursor cells between E10.5 and E14.5 (Figure 6D and Online Figure XI), reaching significant values at E10.5 (wild-type 0.274±0.0183 versus mutant 0.217±0.0341; P=0.042) and E14.5 (wild-type 0.173±0.0197 versus mutant 0.100±0.0254; P=0.018) but not at E12.5 (wild-type 0.208±0.0252 versus mutant 0.181±0.0116; P=0.175). Apoptosis was not increased in the sinus horns in any of these stages (data not shown), suggesting that decreased proliferation contributes to the observed defects in mutant embryos.

Because we noted an increasing size of the cell clusters in the Tbx18cre/+;Ctnnb1(Ex3)fl/+ embryos from E10.5 to E12.5, we also analyzed cell proliferation and apoptosis in these mutant mice. At E10.5, the proliferation in the Tbx18+ cell aggregates was highly and significantly increased (control, 0.167±0.0390 versus mutant, 0.437±0.0275; P=0.002). At E11.5, proliferation rates decreased in control and mutant clusters but were maintained at a significantly higher level in the mutant (control 0.190±0.0300 versus mutant 0.235±0.0675; P<0.001) (Figure 6E and Online Figure XII). At E10.5, low levels of apoptosis was observed in Tbx18+ cell aggregates but not in the cardiac veins of both control and gain-of-function embryos (we analyzed 3 control and 3 Tbx18cre/++;Ctnnb1(Ex3)fl/+–mutant embryos). At E11.5 and E12.5, the whole center of these cell aggregates became largely apoptotic (we analyzed 2 embryos of each genotype), whereas apoptosis was not detectable in control embryos (Online Figure XIII). Hence, canonical Wnt signaling is required and sufficient to maintain proliferation of mesenchymal precursor cells of the sinus horn myocardium.

**Discussion**

The myocardial sleeves of the superior and inferior caval veins and the SAN myocardium derive from a distinct Tbx18*Nkx2–5– mesenchymal precursor cell population. Here, we have shown that canonical Wnt signaling is required for the myocardialization of the dorsal domain of the developing sinus horns but not of the SAN. Forced Wnt signaling is sufficient to induce high proliferation and prevent the differentiation into cardiomyocytes in this region (Figure 6F). We suggest that Ctnnb1-dependent Wnt signaling in the intrapericardial domain of the developing caval veins is part of a
signaling system that balances proliferation and cardiomyocyte differentiation of these mesenchymal precursor cells.

A Regionalized Requirement for Canonical Wnt Signaling in Sinus Horn Development

The general significance of canonical Wnt signaling in heart development has been shown in previous studies.1,2,4 Conditional loss of Ctnnb1 in the early mesodermal progenitors of both the FHF and SHF revealed that Ctnnb1-dependent Wnt signaling does not affect cardiac crescent formation, which derives from the FHF but is required for the development of SHF derived structures and cardiac looping.6 Wnt2 has been identified as a canonical ligand acting in the Nkx2.5+ Isl1+ inflow tract mesoderm to regulate addition of atria and AVC to the posterior pole of the growing heart tube.8,25

Our analysis of the expression of Wnt pathway components and the function of Ctnnb1 expands the role of canonical Wnt signaling to sinus horn formation. Deletion of Ctnnb1 from the sinus venous lineage results in a specific lack of myocardialization in the dorsal aspects of the sinus horns. However, loss of Ctnnb1 does not affect formation of the SAN that also derives from a Tbx18+ cell lineage. Although the SAN considerably expands between E10.5 and E14.5, increase in size may not rely on recruitment and myocardialization of mesenchymal precursor cells but may occur by division of primitive cardiomyocytes that are specified early to a SAN sublineage. Evidence is provided by the distinct molecular character of the developing SAN such as the expression of Tbx3, Shox2, Hcn4, and Bmp signaling components. Although Tbx3 was discussed as a target of canonical Wnt signaling in cancer,26 unaltered expression in the Tbx18Cre+;Ctnnb1fl/fl mutant systemic venous return including the ectopic cell aggregates are P=0.2637 (ns) at E9.5, P=0.4976 (ns) at E10.5, P=0.018 (**) at E12.5, and P=0.175 (not significant) at E14.5. E, The probability values in the Ctnnb1-deficient sinus horns are P=0.042 (*) at E10.5, P=0.175 (not significant) at E12.5, and P=0.018 (**) at E14.5. E, The probability values in the Tbx18Cre+;Ctnnb1fl/fl mutant systemic venous return including the ectopic cell aggregates are P=0.2637 (ns) at E9.5, P=0.4976 (ns) at E10.5, P=0.018 (**) at E12.5, and P=0.175 (not significant) at E14.5.

Support for a role of canonical Wnt signaling in the developing sinus horns is provided by expression of Wnt ligands and receptors, and most importantly by expression of Axin2 that has been described as a bona fide target of this pathway. Loss of expression of Axin2 from the intrapericardial domain of the sinus horns after Tbx18Cre-mediated deletion of Ctnnb1 function and strong induction of Axin2 expression after stabilization of Ctnnb1 in this domain confirms the character of Axin2 as a target of canonical Wnt signaling for the development of the dorsal aspect of the sinus horns, and proves the efficiency of our genetic approach to manipulate canonical Wnt signaling. Our Wnt expression screen argues that canonical Wnt signaling in the developing sinus horns is mediated by Wnt2 and Wnt10a ligands and the Fzd7 receptor.
Wnt signaling may be modulated (diminished) by sFRP1 that binds the ligands and prevents activation of the pathway.

In the posterior SHF, Wnt2 works in a feed-forward transcriptional loop with Gata6 to regulate atria and AVC development.4 Wnt2 as well as Gata6 expression were unchanged in the Tbx18\textsuperscript{cre\mbox{-}/-}Ctnnb1\textsuperscript{fx/fx} sinus horns, suggesting that Wnt signaling in the “third heart field” uses different downstream mediators compared with the SHF, and that Gata6 acts upstream or in parallel with Wnt signaling in this region. The analysis of additional signaling pathways further emphasizes the distinct character of the Tbx18\textsuperscript{Nkx2–5\mbox{-}cre} precursor pool. The Bmp, Fgf, and Shh signaling pathways are important for the development of SHF-derived structures, especially the OFT region of the murine heart.1 None of the analyzed components or target genes of these pathways were detectable in the developing sinus horns, nor were they derepressed in Ctnnb1-deficient sinus horns. However, we cannot rule out that these pathways use different modes of signal transduction and activate a different set of target genes in this region.

Our results also suggest that Tbx18 and canonical Wnt signaling act independently or act onto different subpools of the sinus horn mesenchyme. Tbx18-deficient mice exhibit only a transient delay in sinus horn myocardialization but show a severe reduction of the SAN.4,5 In contrast, the conditional Ctnnb1 mutants presented in this study have a normal SAN and a severe lack of sinus horn myocardium. Furthermore, Tbx18\textsuperscript{cre\mbox{-}/-}Ctnnb1\textsuperscript{fx/fx} hearts were indistinguishable from wild-type hearts again arguing against genetic interaction between Tbx18 and canonical Wnt signaling in sinus horn development.

Canonical Wnt Signaling Maintains Proliferation of Mesenchymal Precursor Cells

We have recently shown that the myocardialized proximal aspects of the superior and inferior caval veins form only after heart looping by recruitment and subsequent myocardial differentiation of Tbx18\textsuperscript{+} pericardial precursors.4 The lack of myocardium in the dorsal aspect of the sinus horns of Ctnnb1-deficient embryos can therefore be explained in at least 2 ways. Canonical Wnt signaling may promote the expansion of the mesenchymal precursor pool or additionally/alternatively direct the differentiation of these progenitors into cardiomyocytes.

In Ctnnb1-deficient sinus horns, cell proliferation was significantly reduced as early as E10.5, suggesting that the progenitor pool was strongly and quickly diminished. Our lineage analysis suggests that these few cells remained mesenchymal and differentiated into fibroblast like cells. Furthermore, forced activation of canonical Wnt signaling by a stabilized form of Ctnnb1 leads to hyperproliferative cell aggregates, which express regional marker genes including Tbx18, Tbx3, and Shox2. Absence of Tnni3 and Hcn4 expression indicates that enhancement or maintenance of canonical Wnt signaling interferes with terminal differentiation into SAN and sinus horn myocardium.

Together, these findings strongly argue that canonical Wnt signaling maintains the sinus horn precursor pool. A similar mode of action was described for Ctnnb1-dependent Wnt signaling in the entire SHF\textsuperscript{1} and Wnt2 function in the posterior SHF and goes along the general line of Wnt signaling as a positive regulator of the cell cycle.24,29 Interestingly, pharmacological or genetic activation of canonical Wnt signaling in the SHF led to increased proliferation but not to the formation of large cell aggregates.1 This may simply reflect a higher sensitivity to levels of Wnt signaling in the Tbx18\textsuperscript{+} precursors compared to SHF cells. Alternatively, it may be linked to the different topology or different microenvironment of this progenitor pool.

Not surprisingly, we found activation of Ccnd1 expression, a direct Wnt target gene and positive regulator of cell cycle progression, in the cell aggregates expressing stabilized Ctnnb1 in the sinus horns. Increased levels of canonical Wnt/Ctnnb1 signaling were also found to be sufficient to induce Fgf signaling that has been associated with increased cell proliferation in other contexts including SHF proliferation.30 It remains unclear whether Fgf signaling represents a nonphysiological response to enhanced Wnt signaling because we did not detect expression of the Fgf target gene Ev5 in wild-type sinus horns.

Our molecular analysis in the myocardium of the sinus horns did not detect Bmp signaling, a known promoter of cardiomyocyte differentiation. It therefore remains to be deciphered what other signaling pathway(s) counteract Wnt signaling to promote myocardial differentiation during sinus horns development.

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Disclosures

None.

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Wnt/β-Catenin Signaling Maintains the Mesenchymal Precursor Pool for Murine Sinus Horn Formation
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**Wnt/beta-catenin signaling maintains the mesenchymal precursor pool for murine sinus horn formation**

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Supplemental Online Materials and Methods

Mice and genotyping
Mice with a knock-in of the cre recombinase gene into the Tbx18 locus (Tbx18\textsuperscript{m4D(cre)Aki} \textsuperscript{3}), synonym: Tbx18\textsuperscript{cre},\textsuperscript{1} mice with two loxP sites flanking the Ctnnb1 locus from exon 2 to exon 6 (Ctnnb1\textsuperscript{tm2Kem} \textsuperscript{3}, synonym: Ctnnb1\textsuperscript{fx},\textsuperscript{2} mice with two loxP sites flanking exon 3 of the Ctnnb1 locus (Ctnnb1\textsuperscript{tm1Mmt} \textsuperscript{3}, synonym: Ctnnb1\textsuperscript{E1} \textsuperscript{(Ex3)}\textsuperscript{fl} \textsuperscript{4}) and the fluorescent reporter line (Gt\textsc{(ROSA})26Sor\textsuperscript{m4D(ACtB-tdTomato,-EGFP)Liu/J} \textsuperscript{5}, synonym: R26\textsuperscript{mTmG}\textsuperscript{4} were all described before. All lines were maintained on an outbred (NMRI) background. The sinuses horns specific Ctnnb1 loss-of-function mutants (Tbx18\textsuperscript{cre+/tm2}), Ctnnb1\textsuperscript{fx/} and Tbx18\textsuperscript{cre+/tm2}, Ctnnb1\textsuperscript{fx/} were obtained from matings of Tbx18\textsuperscript{cre+/} and Ctnnb1\textsuperscript{fx/} females. Sinus horns specific Ctnnb1 gain-of-function mice (Tbx18\textsuperscript{cre+/tm1Mmt} \textsuperscript{2} females. Sinus horns specific Ctnnb1 of mice (Tbx18\textsuperscript{cre/+}, Ctnnb1\textsuperscript{E1} \textsuperscript{(Ex3)}\textsuperscript{fl/} \textsuperscript{4} and Tbx18\textsuperscript{cre/+}, Ctnnb1\textsuperscript{E1} \textsuperscript{(Ex3)}\textsuperscript{fl/} \textsuperscript{4} were derived from matings of Tbx18\textsuperscript{cre/+} and Tbx18\textsuperscript{cre/+}, Ctnnb1\textsuperscript{E1} \textsuperscript{(Ex3)}\textsuperscript{fl/} \textsuperscript{4} males, respectively, with Ctnnb1\textsuperscript{fx/} females. Vaginal plugs were checked in the morning after mating, for timed pregnancies now was taken as embryonic day (E) 0.5. After harvesting the embryos in PBS, they were fixed in 4% paraformaldehyde overnight and stored in 100% methanol at –20°C before further use. For the analysis of the conditional Ctnnb1 loss-of-function phenotype, heterozygous animals (Tbx18\textsuperscript{cre/+}, Ctnnb1\textsuperscript{fx/}) were used as control embryos. For the Ctnnb1 gain-of-function analysis Tbx18\textsuperscript{cre/+} Ctnnb1\textsuperscript{E1} \textsuperscript{(Ex3)}\textsuperscript{fl/+} embryos were used as control. Genomic DNA prepared from yolk sacs or tail biopsies was used for genotyping by PCR (protocols upon request). H. Hedrich, state head of the animal facility, approved the care of animals and experiments at Medizinische Hochschule Hannover.

Histological analysis
For histological stainings embryos were fixed overnight in 4% paraformaldehyde, paraffin embedded, and sectioned to 10-µm. Sections were stained with haematoxylin and eosin, following standard procedures. At least two embryos of each genotype were used for each analysis.

In situ hybridization analysis
Nonradioactive in situ hybridization analysis with digoxigenin-labeled antisense riboprobes was performed as described.\textsuperscript{5} At least two embryos of each genotype were used for each analysis. Details of used probes upon request.

Documentation
Sections were photographed using a Leica DM5000 microscope with Leica DFC300FX digital camera. Images were processed in Adobe Photoshop CS3.

Three-dimensional reconstruction
For three-dimensional visualization and geometry reconstruction 10-µm serial sections of the whole heart of representative embryos of each genotype were used for in situ hybridization and documented as described. Further analysis was performed using the software “Amira” (Version 4.1.1, Mercury Computer Systems Inc) as described previously.\textsuperscript{6} After documentation, the pictures were aligned, and regions for reconstruction were labeled. A three-dimensional model was obtained after surface conversion. Based on this reconstruction the volume of the different labels was calculated by the software “Amira”. 

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**Immunohistochemistry**

For immunofluorescence analysis, mouse monoclonal antibody against GFP (1:250, 11814460001, Roche), mouse monoclonal antibody against Fn-1 (1:300, F7387, Sigma), rabbit polyclonal antibody against periostrin (Postn) (1:300, ab14041, Abcam), mouse monoclonal antibody against Acta2, FITC conjugate (1:250, F3777, Sigma), goat polyclonal antibody against Tbx3 (1:200, sc-31656, Santa Cruz), rabbit polyclonal antibody against Gja5 (1:50, AB1726, Millipore), goat monoclonal antibody against Tnni3 (1:300, 4T21/2, HyTest), mouse monoclonal antibody against troponin T2, cardiac (Tnt2) (1:500, MS-295-P1, Thermo Scientific), rabbit polyclonal antibody against Ctnnb1 (1:400, C2206, Sigma-Aldrich) and rabbit polyclonal antibody against WT1 (1:400, C-19, Santa Cruz Biotechnology) were used as primary antibodies. Alexa488 goat-anti-rabbit (1:250, Invitrogen), Alexa488 donkey-anti-mouse (1:250, Invitrogen), biotinylated goat-anti-rabbit (1:250, 111-065-003, Dianova) and biotinylated donkey-anti-goat (1:250, 705-065-003, Dianova) were used as secondary antibodies. Nuclei were stained with 4′,6-diamidino-2-phenylindol (DAPI) (Roche). The immunofluorescence analysis against Ctnnb1 was performed on cryosections. These were fixed overnight in 4% paraformaldehyde, embedded in tissue freezing medium (Jung), and sectioned to 8-µm. The other immunofluorescence analysis was performed on 5-µm paraffin sections. All sections were pressure cooked for 3 min in antigen unmasking solution (H-3300, Vector Laboratories Inc). Endogenous peroxidase activity was blocked by 3% H2O2. The signal was amplified using either the Tyramide Signal Amplification (TSA) system from Perkin-Elmer (NEL702001KT, Perkin Elmer LAS) or the Mouse-on-mouse (M.O.M.) immunodetection kit from Vector Laboratories (PK-2200, Vector Labs). The anti-GFP staining was performed in a second experiment directly after the first immunodetection was finished and followed the published protocols.

**Proliferation and apoptosis assays**

Cell proliferation in E9.5 to E14.5 embryos was investigated by detection of incorporated 5-bromo-2-deoxyuridine (BrdU) on 5-µm sections of paraffin-embedded specimens similar to previously published protocols.7 Wildtype littermates were used as controls for each genotype and stage. Both the left cardinal vein and right cardinal veins were counted. For analysis of Tbx18cre/+,Ctnnb1fl/fl hearts, five sections each of four embryos of each genotype at E10.5, and five sections each of three embryos of each genotype at E12.5 and E14.5 were used for quantification. The quantification of proliferation in Tbx18cre/+;Ctnnb1(Ex3)fl/+ and control hearts was performed with six sections each of four embryos of each genotype at E9.5, and six sections each of three embryos of each genotype and stage at E10.5 and E11.5. The BrdU-labeling index was defined as the number of BrdU-positive nuclei relative to the total number of nuclei, as detected by DAPI counterstain, in the Tbx18 expressing region of the left and right cardinal veins. Statistical analyses were performed using the 2-tailed Student’s t-test. Data were expressed as mean ± SEM. Differences were considered not significant when the P-value was higher than 0.05, significant (*) when the P-value was below 0.05, highly significant (**) when the P-value was below 0.01, and extremely significant (***) if P<0.001.

Detection of apoptotic cells on 10-µm paraffin sections of wildtype and Tbx18cre/+;Ctnnb1(Ex3)fl/+ mutant embryos was based on modification of genomic DNA utilizing terminal deoxynucleotidyl transferase (TUNEL assay) and indirect detection of positive cells by fluorescein-conjugated anti-digoxigenin antibody. The procedure followed the recommendation of the manufacturer (Serologicals Corp.) of the ApopTag kit used. Four sections each of three embryos of each genotype at E10.5, E11.5, and E12.5 were analyzed.
References


Supplemental Online Figures

**Online Figure I. Wnt signaling in sinus horn development.** *In situ* hybridization analysis on transverse sections through the venous pole region for expression of Wnt pathway components *Wnt2, Wnt10a, Fzd7* and *Sfrp1* (**A through D**), and of *Axin2* and *Tbx18* (**E and F**) in wildtype embryos at E10.5. Arrowheads point to the intrapericardial components of the left and right cardinal vein. Probes are as indicated. lcv, left caval vein; ra, right atrium; rcv, right caval vein.
Online Figure II. Absence of Ctnnb1 in the Tbx18^{cre/+};R26^{mTmG/+};Ctnnb1^{fx/fx} mutant sinus horns. Immunofluorescence analysis of Ctnnb1 in the cellular derivate of the Tbx18^{+} domain of the right sinus horn, marked by GFP expression, in control (genotype: Tbx18^{cre/+};R26^{mTmG/+};Ctnnb1^{fx/+}) and Tbx18^{cre/+};R26^{mTmG/+};Ctnnb1^{fx/fx} mutant sinus venosus region at E14.5. Genotypes are as indicated, nuclei are counterstained with DAPI. Arrows point to the GFP^{+}Ctnnb1^{+} sinus horn myocardium in control embryos, arrowheads mark the GFP^{+}Ctnnb1^{+} domain of the Tbx18^{cre/+};R26^{mTmG/+};Ctnnb1^{fx/fx} mutant right sinus horns. Note the dotted pattern of Ctnnb1 expression. rsh; right sinus horn.
Online Figure III. Morphology of $Tbx18^{cre/+};Ctnnb1^{fx/fx}$ hearts. A through D, Morphology of control (genotype: $Tbx18^{cre/+};Ctnnb1^{fx/+}$) and $Ctnnb1$-deficient (genotype: $Tbx18^{cre/+};Ctnnb1^{fx/fx}$) whole hearts (A, B) and histological stainings of mid-transverse sections (C, D) at E18.5. Genotypes are as indicated. IVS, interventricular septum; la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle.
Online Figure IV. Absence of the Gja5⁺ myocardium in the dorsal domain of the right sinus horn in Tbx18cre/+;Ctnnb1fx/fx hearts. A through H, In situ hybridization analysis for SAN expression of Tbx3, Hcn4, cardiomyocyte specific Tnni3, and Gja5 in E18.5 hearts of control (left column) and Tbx18cre/+;Ctnnb1fx/fx (right column) embryos on transverse sections through the base of the caval vein. Arrowheads point to the Tnni3⁺ domain of the right sinus horn. rsh, right sinus horn; SAN, sinoatrial node.
Online Figure V. Cellular contributions to the Ctnnb1-deficient systemic venous return of the heart. A through X, Comparative immunofluorescence analysis of cellular derivates of the Tbx18° domain of the sinus horns, marked by GFP expression, and cardiomyocyte specific Tnni3 (A through F), the fibroblast markers Fn1 (G through H) and Postn (M through R), or the smooth muscle marker Acta2 (S through X) in control (genotype: Tbx18°cre/°;R26°mTmG/°;Ctnnb1±/°) and Tbx18°cre/°;R26°mTmG/°;Ctnnb1fx/fx mutant sinus venosus region at E18.5. Antibodies and genotypes are as indicated, nuclei are counterstained with DAPI. Arrowheads mark the Tnni3 part of the Tbx18°cre/°;R26°mTmG/°;Ctnnb1fx/fx mutant right sinus horns. rsh; right sinus horn; SAN, sinoatrial node.
Online Figure VI. Cellular contribution to the systemic venous return in E18.5 embryos with conditional deletion of Ctnnb1. A through X, Comparative immunofluorescence analysis on transverse sections through the sinus venosus region of control (genotype: Tbx18<sup>cre/+;R26<sup>mTmG/</sup></sup>;<sup>Ctnnb1<sup>f/+</sup></sup>) and Tbx18<sup>cre/+;R26<sup>mTmG/</sup></sup>;<sup>Ctnnb1<sup>f/f</sup></sup> mutant hearts. Cellular derivates of the Tbx18<sup>+</sup> domain of the sinus horns are marked by GFP expression and compared to the cardiomyocyte marker Tnnt2 (A through F), the gap junction protein Gja5 (G through L), the conduction system marker Tbx3 (M through R) and the mesothelial marker Wt1 (S through X). Arrowheads mark the Tnni3<sup>+</sup> part of the Tbx18<sup>cre/+;R26<sup>mTmG/</sup></sup>;<sup>Ctnnb1<sup>f/f</sup></sup> mutant right sinus horns. Genotypes are as indicated.

rsh, right sinus horn.
Online Figure VII. Analysis of FHF and SHF marker genes and signaling pathways in Ctnnb1-deficient sinus horns. A through T, *In situ* hybridization analysis of the FHF marker genes *Nkx2-5* and *Tbx5* (A through D), the SHF genes *Tbx1*, *Isl1* and *Fgf10* (E through J), the target of Fgf signaling *Etv5* (K and L), the Bmp signaling components *Bmp2*, *Bmp4* and its target gene *Msx1* (M through R) and the Shh signaling target gene *Ptch1* (S and T) on transverse sections through the systemic venous return of control (genotype: *Tbx18<sup>+/+</sup>;Ctnnb1<sup>+/+</sup>·Ctnnb1<sup>+/+</sup>) and *Tbx18<sup>+/+</sup>;Ctnnb1<sup>+/+</sup>·Ctnnb1<sup>+/+</sup> mutant embryos at E12.5. Probes and genotypes are as indicated. lc, left caval vein; ra, right atrium; rcv, right caval vein.
Online Figure VIII. Analysis of molecular changes in the $Tbx18^{cre/+};Ctnnb1^{fx/fx}$ venous pole region of the heart. A through L, In situ hybridization analysis of the Wnt ligand $Wnt2$ (A and B), $Gata6$ (C and D), the Wnt antagonists $Sfrp1$ (E and F) and $Sfrp2$ (G and H), and the mesothelial marker genes $Wt1$ (I and J) and $Aldh1a2$ (K and L) on transverse sections through the systemic venous return of control and $Tbx18^{cre/+};Ctnnb1^{fx/fx}$ mutant embryos at E14.5. Probes and genotypes are as indicated. Arrowheads mark gene expression in the intrapericardial domain of the caval veins. la, left atrium; Iscv, left superior caval vein; ppm, pleuro-pericardial membrane; ra, right atrium, rscv, right superior caval vein.
Online Figure IX. Ectopic cell aggregates in $Tbx18^{cre/+};Ctnnb1^{(Ex3)}fl/+\$ mutant sinus horns are detectable at E10.5. A through H. In situ hybridization analysis on transverse sections for the canonical Wnt signaling target gene $Axin2$ (A and B), the cardiomyocyte marker $Tnni3$ (C and D), the target gene of Fgf signaling $Etv5$ (E and F) and the direct Wnt target gene and positive regulator of cell cycle progression $Ccnd1$ (G and H) in the venous pole region of control (left column, genotype: $Tbx18^{+/+};Ctnnb1^{(Ex3)}fl/+\$) and $Tbx18^{cre/+};Ctnnb1^{(Ex3)}fl/+\$ mutant (right column) hearts at E10.5. The arrow points to the $Tnni3^+$ ventral domain of the right sinus horn. Arrowheads highlight the ectopic cell clusters in $Tbx18^{cre/+};Ctnnb1^{(Ex3)}fl/+\$ sinus venosus region. lcv, left cardinal vein; rcv, right cardinal vein.
Online Figure X. Developmental onset of ectopic cell aggregate formation in the Tbx18<sup>cre/+</sup>;Ctnnb1<sup>(Ex3)fl/+</sup> sinus venosus region of the heart at E11.5. A through J. In situ hybridization analysis on transverse sections for the Wnt target gene Axin2 (A and B), the sinus horn marker gene Tbx18 (C and D), the cardiomyocyte marker Tnni3 (E and F), the target gene of Fgf signaling Etv5 (G and H), and the direct Wnt target gene and positive regulator of cell cycle progression Ccnd1 (I and J) in the venous pole region in control (left column) and Tbx18<sup>cre/+</sup>;Ctnnb1<sup>(Ex3)fl/+</sup> (right column) embryos at E11.5. Arrowheads point to the ectopic cell aggregates in the mutant sinus venosus region. lcv, left cardinal vein; rcv, right cardinal vein.
Online Figure XI. Cellular changes in Ctnnb1-deficient sinus horns. A through F, Analysis of proliferation in the red encircled, Tbx18+ intrapericardial domain of the sinus horns performed on transverse sections through the posterior systemic venous return by BrdU immunohistochemistry. BrdU positive cells are labeled in green, nuclei are counterstained with DAPI. Stages and genotypes are as indicated. lcv, left cardinal vein; Ish, left sinus horn; rcv, right cardinal vein.
Online Figure XII. Increased cell proliferation in ectopic aggregates of Tbx18<sup>cre/+;Ctnnb1<sup>(Ex3)fl/+</sup> sinus horns. A through F, Analysis of cell proliferation in the red encircled, Tbx18<sup>+</sup> intrapericardial domain of the sinus horns in control hearts and the corresponding intrapericardial domain of the sinus horns in Tbx18<sup>cre/+;Ctnnb1<sup>(Ex3)fl/+</sup> mutant systemic venous return including the ectopic cell aggregates by BrdU immunohistochemistry. BrdU positive cells are labeled in green, nuclei are counterstained with DAPI. Stages and genotypes are as indicated. lu, lung; other abbreviations are as in Online Figure 8.
Online Figure XIII. Apoptosis is detectable in the ectopic cell aggregates in the sinus venosus region of Tbx18<sup>cre/+;Ctnnb1<sup>(Ex3)fl/+</sup> hearts at different embryonic stages. A through F, Analysis of apoptosis in control (A through C) and Tbx18<sup>cre/+;Ctnnb1<sup>(Ex3)fl/+</sup> (D through F) sinus horns. Stages and genotypes are as indicated. White arrows mark the mutant cell aggregates. lc, left cardinal vein; lu, lung; rcv, right cardinal vein.