A regulatory pathway involving Notch1/β-catenin/Isl1 determines cardiac progenitor cell fate

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Regulation of multipotent cardiac progenitor cell (CPC) expansion and subsequent differentiation into cardiomyocytes, smooth muscle or endothelial cells is a fundamental aspect of basic cardiovascular biology and cardiac regenerative medicine. However, the mechanisms governing these decisions remain unclear. Here, we show that Wnt/β-catenin signalling, which promotes expansion of CPCs\(^1-3\), is negatively regulated by Notch1-mediated control of phosphorylated β-catenin accumulation within CPCs, and that Notch1 activity in CPCs is required for their differentiation. Notch1 positively, and β-catenin negatively, regulated expression of the cardiac transcription factors, Isl1, Myocd and Smyd1. Surprisingly, disruption of Isl1, normally expressed transiently in CPCs before their differentiation\(^4\), resulted in expansion of CPCs in vivo and in an embryonic stem (ES) cell system. Furthermore, Isl1 was required for CPC differentiation into cardiomyocyte and smooth muscle cells, but not endothelial cells. These findings reveal a regulatory network controlling CPC expansion and cell fate that involves unanticipated functions of β-catenin, Notch1 and Isl1 that may be leveraged for regenerative approaches involving CPCs.

Heart malformation is the most frequent form of birth defects in humans, and heart disease remains the leading cause of adult mortality in the developed world, largely because of the limited regenerative capacity of the heart. Recent advances have provided insights into potential therapies based on multipotent CPCs. These cells can be isolated from early embryos or ES cells and cultured to differentiate into numerous cardiac cell types\(^4-12\). For example, Nkx2.5\(^+\), Flk1\(^+\) or Isl1\(^+\) CPCs purified from embryoid bodies can each give rise to cardiomyocyte, endothelial and smooth muscle cell lineages\(^7,8,10,12\).

Nkx2.5 is an ancient cardiac gene activated in CPCs of early embryos\(^11\). Nkx2.5\(^+\) cells and their progeny populate the precardiac mesoderm located dorsal to the cardiac region and the developing heart tube in vivo\(^14\). Isolated Nkx2.5\(^+\) cells differentiate spontaneously into distinct cardiac cell lineages including cardiomyocytes, smooth muscle cells and endothelial cells in vitro\(^7,12\). These cardiac cell lineages can also be generated from cells expressing Flk1, a marker of the primitive streak in early embryogenesis\(^9\), or Isl1, a CPC marker\(^8,11\). All of these CPCs show overlapping expression patterns in precardiac mesodermal cells in vivo\(^8\) and have similar differentiation potential in vitro\(^7,8,10,12\), suggesting that they comprise a similar CPC population. Although these multipotent CPCs hold great potential for cardiac repair, the mechanisms that regulate their self-renewal, expansion and differentiation remain unknown.

We and others have reported that canonical Wnt signalling is an important regulator of Nkx2.5\(^+\) and Isl1\(^+\) CPCs and is responsible for their expansion in vivo and in vitro\(^1-3\). In precardiac mesoderm, inactivation of β-catenin, the transcriptional mediator of canonical Wnt signalling, resulted in nearly complete loss of Isl1 cells that contribute to the right ventricle\(^7\). Conversely, stabilization of β-catenin in the same cells led to expansion in the number of CPCs in vivo, whereas Wnt/β-catenin signalling promoted renewal of CPCs isolated from ES cells\(^7,8\). Notch signalling reciprocally affects Wnt signals in many contexts\(^15\) and is thought to inhibit cardiac differentiation\(^13\), although its function in CPCs in vivo is unknown. Ultimately, these and other early signals must be integrated with a network of transcriptional regulators that influence CPCs.

To examine the CPC-autonomous role of Notch1 signalling in vivo, we deleted Notch1 in precardiac mesodermal progenitors by crossing Notch1\(^\text{loxP}\) mice\(^19\) with mice containing Cre recombinase in the Isl1 locus (Isl1\(^\text{Cre}\))\(^20\), resulting in Cre-mediated recombination in early CPCs by embryonic (E) day 7.75. The resulting Notch1-null embryos failed to populate the developing right ventricle segment, which is derived from Isl1\(^+\) CPCs (Fig. 1a–c, g–i). Strikingly, the affected Isl1\(^+\) CPC pool dorsal to the developing heart was expanded with an increase in the percentage of proliferating cells, marked by an anti-phospho-histone H3 (PH3) antibody (Fig. 1d–f, j–m). Accumulation and proliferation of CPCs behind the developing heart was similar to the effect of stabilized β-catenin on CPCs\(^8\), although in the latter, CPCs also migrated into the heart.

The striking similarity between Notch1 loss-of-function and β-catenin gain-of-function mutants in CPCs led us to hypothesize that Notch and β-catenin signalling intersect during CPC fate or expansion decisions. No significant changes in the expression of genes involved in the Notch-
signal transduction pathway were observed in β-catenin-stabilized mice (data not shown), suggesting that it is unlikely that β-catenin regulates Notch signalling in CPCs. Using an ES cell line with a bacterial artificial chromosome (BAC) containing green fluorescent protein (GFP) in the Nkx2.5 locus, we isolated Nkx2.5-GFP+ cells by fluorescence-activated cell sorting (FACS). The Nkx2.5-GFP+ cells expressed high levels of Isl1 (Supplementary Information, Fig S1a), consistent with these cells representing CPCs. We knocked down Notch1 with short interfering RNAs (siRNAs) in Nkx2.5-GFP+ CPCs cultured in a monolayer. Endogenous Isl1 signals were amplified with the TSA system. DAPI (blue) was used to counterstain the nuclei. (m) The percentage of PH3-positive cells in the precardiac mesoderm region shown in e and k (mean ± s. d.; n = 4; *P < 0.01). (n) Western blot analyses of FACS-purified CPCs transfected with control siRNA (C) or Notch1 siRNA (N1-KD) using Notch1, free or total β-catenin antibodies. Free β-catenin antibodies detect dephosphorylated β-catenin, the effector molecule of the Wnt/β-catenin signalling pathway. GAPDH antibody was used as a control. (o) Relative number of cells on the second day after transfecting CPCs with control or Notch1 siRNA (mean ± s. d.; n = 6; *P < 0.01). (p) Top/Fop flash activity in CPCs transfected with indicated siRNA. Top flash is a luciferase reporter with Tcf binding sites to read Wnt/β-catenin signalling activity. Fop flash contains mutated Tcf binding sites. Luciferase values were normalized to Renilla activity (mean ± s. d.; n = 3; *P < 0.01). Abbreviations: h, heart; pa, pharyngeal arch; ot, outflow tract; lv, left ventricle. Scale bars, 250 μm (a, g) or 100 μm (b–e, h–k). P values were determined using two-tailed Student’s t-test, type II (see Methods).
on the basis of its temporal expression, Isl1 is intuitively thought to promote CPC expansion, we investigated whether Isl1 downregulation mediates expansion of CPCs observed in embryos with stabilized β-catenin. To test this possibility, we used the Isl1<sup>Cre</sup> line described above, which contains an IRES-Cre cassette inserted into the exon encoding the second LIM domain of Isl1, resulting in an Isl1-null allele<sup>20</sup>. Isl1<sup>Cre</sup> mice were bred with Rosa<sup>T%</sup> mice to generate Isl1<sup>Cre/Cre</sup>; Rosa<sup>T%</sup> embryos. We quantified the number of YFP<sup>+</sup> cells at E8.0 (5 somite stage), before Isl1<sup>Cre</sup> expression is initiated in neural cells, by FACS. Surprisingly, Isl1-null embryos had a significantly higher percentage of YFP<sup>+</sup> cells than control embryos (Fig. 3a, b). The results suggest that Isl1 negatively regulates the number of CPCs in vivo. The significant increase is unlikely to be attributable to higher Cre expression in Isl1-null embryos, as heterozygous Cre mice mediate recombination as efficiently as homozygous Cre mice.

To determine whether Isl1 also negatively regulates expansion of CPCs derived from pluripotent ES cells, we transiently knocked down Isl1 levels in the Nkx2.5-GFP ES cell line by introducing an Isl1 short hairpin RNA (shRNA) construct, which efficiently reduced Isl1 transcripts by about 75% (Supplementary Information, Fig. S1f). We then quantified the number of Nkx2.5-GFP CPCs in embryoid bodies from embryoid day (ED) 6, as cardiac progenitors begin to emerge and differentiate from primitive mesendoderm<sup>9,10</sup>. Knockdown of Isl1 from ED0–3 did not change the number of Nkx2.5<sup>+</sup> progenitors (data not shown). However, Isl1 knockdown from ED3–6, just after emergence of mesoderm, resulted in an increase in the CPC population at ED6–8 (Fig. 3c; Supplementary Information, Fig. S2a), consistent with our in vivo data.

These findings prompted us to test whether Isl1 downregulation was required for CPC expansion induced by β-catenin. We transfection Nkx2.5-GFP<sup>+</sup> embryos with stabilized β-catenin expression construct<sup>22</sup> with or without an Isl1 expression construct. As previously reported, increased CPC expansion was evident two days after transfection with stabilized β-catenin (Fig. 3d). However, cotransfection with Isl1 restored the number of CPCs to normal levels (Fig. 3d). This suggests that the decrease in Isl1 is necessary for Wnt/β-catenin signalling–mediated expansion of CPCs.

Because Isl1 seemed to be involved in repressing expansion of CPCs, we investigated whether Isl1 promotes differentiation in the ES cell system. We generated a stable Isl1 knockdown ES cell line by introducing an Isl1 shRNA construct into Nkx2.5-GFP ES cells and clonally isolating cells with effective (~80%) Isl1 knockdown (Supplementary Information, Fig. S1f). Similarly to transient Isl1 knockdown, the number of Nkx2.5-GFP<sup>+</sup> CPCs was significantly increased at ED6 (Supplementary Information, Fig. S2b). However, cells differentiated from the Isl1 knockdown ES cells showed severely reduced beating frequencies and compromised expression of cardiac sarcomeric genes (Myh6, Myh7, Mlc2a, Mlc2v) from ED9 (Fig. 3e, f). To determine the CPC-autonomous role of Isl1 during cardiac differentiation, we FACS-purified Nkx2.5-GFP<sup>+</sup> CPCs from ED5 embryoid bodies and differentiated them by re-aggregation in suspension (Fig. 3g). Nkx2.5-GFP<sup>+</sup> CPCs are multipotent and differentiate into myocardial, smooth muscle and endothelial cell lineages<sup>9,11</sup>. Normal levels of endothelial gene expression (CD31, Flk1) were observed in differentiating Isl1 knockdown CPCs (Fig. 3h). However, expression of cardiomyocyte and
After transfecting CPCs derived from embryoid bodies with Isl1 overexpression, differentiation was monitored by sarcomeric gene (for example, Actc1, Myh7) expression levels during the early phase of differentiation, companied with an increase of about twofold in cardiomyocyte number. We observed a 25% increase in Myh7+ cells in Isl1-overexpressed embryoid bodies (Fig. 3h; Supplementary Information, Fig. S3b). This suggests that Isl1 can promote myocardial differentiation of CPCs in an instructive manner.

Given that Isl1 loss-of-function suppressed cardiomyocyte differentiation, we sought to determine whether Isl1 has an instructive role in myocardial lineage formation. Isl1 expression levels were upregulated in embryoid bodies from ED4–5 (Supplementary Information, Fig. S3a). To prematurely increase Isl1 expression levels in a temporally and physiologically relevant way, we transiently transfected an Isl1-expressing embryoid body construct (30 ng per 10⁵ cells) into dissociated ED2 embryoid body cells and re-aggregated them for further differentiation (Fig. 4a). This resulted in an increase of about twofold in Isl1 levels at ED6 (Fig. 4b). Myocardial differentiation was monitored by sarcomeric gene (for example, Myh7, Mlc2v, Actc1) expression over the course of embryoid body differentiation. Sarcomeric gene expression levels did not change during the early phase of CPC differentiation (data not shown). However, by ED8, Isl1-transfected embryoid bodies expressed higher levels of cardiac muscle genes than control embryoid bodies (Fig. 4c). To determine the effect of excess Isl1 on the number of cardiomyocytes, we used the Myh7-GFP ES cell line to quantify cardiomyocyte number. We observed a 25% increase in Myh7+ cells in Isl1 (mean ± s. d.; n = 6; *P < 0.01). (e) Relative mRNA expression of indicated genes in control or Isl1-KD embryoid bodies at ED9, determined by qPCR (mean ± s. e. m.; n = 4; *P < 0.05). P values were determined using two-tailed Student’s t-test, type II (see Methods).

Given that Isl1 loss-of-function suppressed cardiomyocyte differentiation, we sought to determine whether Isl1 has an instructive role in myocardial and smooth muscle lineages. Isl1 overexpression in control (Rosa26, Isl1lox/lox, left) and Isl1-null (Rosa26, Isl1lox/lox, right) embryos at the 5-somite stage. Arrows indicate YFP+ CPCs. Scale bars, 50 μm. (b) Histograms of YFP+ cells (left panels) and quantification of YFP+ cells in indicated embryos at somite 5 (mean ± s. d.; n = 3; *P < 0.01). (c) Quantification of GFP+ cells in ED6 Nkx2.5-GFP embryoid bodies with or without Isl1 knockdown (Isl1-KD; mean ± s. d.; n = 3; *P < 0.01). (d) Relative number of cells on the second day after transfecting CPCs derived from embryoid bodies with lacZ, β-catenin or smooth muscle cell genes was markedly downregulated (Fig. 3h). This suggests that Isl1 not only represses expansion of CPCs, but is also necessary for proper differentiation of CPCs into myocardial and smooth muscle, but not endothelial, cell lineages.

In addition to Isl1, Myocd and Smyd1 are important genes for cardiogenesis12–17 that were downregulated in CPCs with increased β-catenin (Fig. 2c–g, i–k). Myocd is a potent co-activator of serum response factor regulation of smooth muscle24 and cardiac gene expression27. Smyd1 is a muscle-restricted histone methyltransferase essential for cardiomyocyte differentiation in vivo28,29. To determine whether Isl1 regulates these genes in CPCs, we used Nkx2.5-GFP+ CPCs purified from the stable Isl1 knockdown ES cell line. Smyd1 levels did not change, but Myocd levels were significantly reduced in the Isl1 knockdown CPCs (Fig. 5a). To determine whether this is also the case in vivo, we performed in situ hybridization for Myocd transcripts in Isl1-null embryos. In agreement with in vitro data, Myocd levels were severely compromised in Isl1-null embryos, whereas Smyd1 levels did not change (Fig. 5b–i). This suggests that Isl1 is required for normal Myocd expression.

Through bioinformatic searches, we identified an Isl1 consensus site in an evolutionarily conserved island (555 bp) located in the first intron of the Myocd locus (Fig. 5j). We observed robust transactivation of luciferase when the element was linked to luciferase reporter and introduced...
Figure 4 Increased levels of Isl1 promote myocardial differentiation. (a) Schematic diagram of differentiation of Myh7-GFP ES cells with Isl1 overexpression (Isl1-OE). (b, c) Relative expression levels of Isl1 on ED6 embryoid bodies (b), and endothelial (Flik1), cardiac sarcomeric (Actc1, Mic2v, Myh7) and smooth muscle (Sma) genes on day 8 embryoid bodies (c), determined by qPCR. (d) FACS analyses on ED9 embryoid bodies to determine the percentage of cells entering the myocardial lineage. Data are mean ± s. e. m.; n = 3; * P < 0.005, P was determined using two-tailed Student’s t-test, type II (see Methods).

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AUTHOR CONTRIBUTIONS
C.K. designed, performed, supervised in vivo and in vitro work and wrote the manuscript; L.O. performed flow cytometry and EMSA, and contributed in luciferase assays; P.C. designed and performed Isl1 gain-of-function studies and contributed in ChIP and luciferase assays; V.N. performed β-catenin western and Top/Fop flash assays; J.A. contributed to ChIP assays; D.S. designed and supervised this work and wrote the manuscript.

COMPETING INTERESTS
P.S. serves on the scientific advisory board of iPierian.

(n) Relative expression levels of Bhlhb2 in CPCs with stabilized β-catenin, determined by qPCR (mean ± s. d.; n = 3; *P < 0.005). (o) Relative expression levels of Smyd1 and Isl1 after transfecting FACS-purified CPCs with Bhlhb2 and differentiating them for 3 days (mean ± s. d.; n = 3; *P < 0.005). (p) The Bhlhb2 locus showing four conserved Lef/Tcf binding sites. (q) ChIP assays performed with Lef/Tcf consensus sites shown in p. β-catenin forms complexes with sites A and D as revealed by amplification of those sites. (r) Luciferase activity determined with luciferase reporters containing the intact Lef/Tcf site D (Bhlhb2D-luc) or with a mutant Lef/Tcf site D (Bhlhb2D–lucmt) in the presence or absence of Isl1 (mean ± s. d.; n = 3; *P < 0.005). (s) A molecular cascade involving Notch1→β-catenin/Isl1 during CPC fate determination. Notch1 functions to negatively regulate accumulation of free β-catenin, which regulates Myocd and Smyd1 through Isl1 and Bhlhb2, respectively, to determine CPC fates. Relationships indicated may be direct or indirect. P values were determined using two-tailed Student’s t-test, type II (see Methods).

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METHODS

Mouse genetics and CPC and ES cell culture. Control (Rosa<sup>TOM</sup>; Isl1<sup>Cre</sup>) or mutant (Rosa<sup>TOM</sup>; Isl1<sup>Cre</sup>; β-catenin<sup>(ex3)loxPloxP</sup>) embryos were obtained by crossing Rosa<sup>TOM</sup>; β-catenin<sup>(ex3)loxPloxP</sup> with Isl1<sup>Cre+</sup> mice. YFP+ cells from the resulting embryos were purified by FACS and used for gene expression analyses. To quantify embryonic CPCs, Rosa<sup>TOM</sup>; Isl1<sup>Cre+</sup> were crossed with Isl1<sup>Cre+</sup> mice, and YFP+ cells from the resulting embryos were counted by FACS. To generate Isl1<sup>Cre+</sup>; Notch1<sup>(loxP)</sup> mice, Isl1<sup>Cre+</sup>; Notch1<sup>loxP</sup> mice were crossed with Notch1<sup>loxP/loxP</sup> mice. Genotyping was performed as described previously. To identify Isl1-het (Isl1<sup>Cre+</sup>) or null (Isl1<sup>NoCre</sup>) embryos, DNA was isolated from individual embryos, and qPCR was performed using SYBR Green (Applied Biosystems). Control embryos, DNA was isolated from individual embryos, and qPCR was performed amplified with the ABI Prism system (Applied Biosystems) was used to transiently transfect embryoid bodies and to generate Isl1 protein. All EMSA probes are listed in Supplementary Information, Table S2. For luciferase assays, Renilla was used as an internal normalization control.

Flow cytometry and gene expression analysis. A Becton Dickinson FACS DIVA flow cytometer and cell sorter were used for quantifying and purifying Flow cytometry and gene expression analysis. To quantify embryonic CPCs, Rosa<sup>TOM</sup>; Isl1<sup>Cre+</sup> were crossed with Isl1<sup>Cre+</sup> mice, and YFP+ cells from the resulting embryos were counted by FACS. To generate Isl1<sup>Cre+</sup>; Notch1<sup>(loxP)</sup> mice, Isl1<sup>Cre+</sup>; Notch1<sup>loxP</sup> mice were crossed with Notch1<sup>loxP/loxP</sup> mice. Genotyping was performed as described previously. To identify Isl1-het (Isl1<sup>Cre+</sup>) or null (Isl1<sup>NoCre</sup>) embryos, DNA was isolated from individual embryos, and qPCR was performed using SYBR Green (Applied Biosystems). Control Isl1 and Cre primers are shown in Supplementary Information, Table S2. ES cells and purified Nkx2.5-GFP CPCs were propagated undifferentiated or differentiated as described previously. For CPC differentiation, FACS-purified GFP+ cells were re-aggregated in suspension (10<sup>6</sup> cells per well) in ultra-low-attachment 24-well plates (Corning).

Flow cytometry and gene expression analysis. A Becton Dickinson FACS DIVA flow cytometer and cell sorter were used for quantifying and purifying Nkx2.5-GFP or Myh7-GFP cells. For the microarray analysis and qPCR, total RNA was amplified with the WT-Ovation Pico RNA Amplification System, fragmented and labelled with the FL-Ovation CDNA Biotin Module V2 (Nugen). Hybridization, staining and scanning of the Affymetrix GeneChips were performed in the Gladstone Genomics Core Lab. Raw data generated from at least three independent experiments were further analysed by the group of Ru-Fang Yeh at the Center for Informatics and Molecular Biostatistics, UCSD. To quantify gene expression in Notch1 mutant embryos, total RNA was isolated from hearts and pharyngeal arches from E10.0 embryos, qPCR was performed with the ABI Prism system (7900HT, Applied Biosystems). TaqMan primers used in this study are listed in Supplementary Information, Table S2. All samples were run at least in triplicate. Real-time PCR data were normalized and standardized with SDS2.2 software.

 Constructs, siRNA, transfection, EMSA and luciferase assays. For Isl1 knockdown experiments, an Isl1 siRNA construct set (RMM4534-NM, 021,459, Open Biosystems) was used to transiently transfect embryoid bodies and to generate stable knockdown ES cell lines. For Isl1 or Bhlhb2 overexpression studies, their full-length cDNAs (Open Biosystems) were amplified and cloned into the pEF-DEST51 vector (pDEST51-Isl1 or Bhlhb2) through the pENTR vector (pENTR-Isl1 or Bhlhb2) using the Gateway system (Invitrogen). pEF-pLuc (Invitrogen) was used as a control. For Notch1 knockout studies, Block-IT Alexa Fluor Red (46-5,318, Invitrogen) or Notch1 siRNA (M-041,110-00-0006, Dharmacon) was used at concentration of 50 or 100 nM. Myocd–luc<sup>mt</sup> was generated by cloning their corresponding regions into the pGL3 luciferase vector (Promega). Myocd–luc<sup>mt</sup> was generated using QuickChange Site-Directed Mutagenesis Kit (Stratagen). For Bhlhb2D–luc and Bhlhb2D–luc<sup>mt</sup> generation, oligonucleotides containing the Tcf/Lef binding sites in the Isl1 locus were shown in Supplementary Information, Table S2. Stabilized β-catenin and Top/Fop-flash luciferase constructs were provided by A. Barth (Stanford University, CA) and the laboratory of R. Moon (University of Washington, WA), respectively. ES cells, embryoid bodies or CPCs were transfected with the indicated constructs or siRNA using Lipofectamine 2000 (Invitrogen) after generating single-cell suspensions with Accutase (Chemicon). EMSAs and luciferase assays were performed as described previously. For EMSAs, the pCITE–ISL1<sup>34</sup> construct containing the truncated Isl1 cDNA with the homeodomain was provided by B. Black (University of California, San Francisco) and used to generate Isl1 protein. All EMSA probes are listed in Supplementary Information, Table S2. For luciferase assays, Renilla was used as an internal normalization control.

In situ hybridization, immunostaining and western analysis. Whole-mount in situ hybridization was performed as described previously, with designated antisense probe<sup>4,23</sup>. Bhlhb2 antisense riboprobe was synthesized and purified from pENTR-Bhlhb2. To detect proliferating cells in CPCs, embryo sections were stained with anti-PH3 (Upstate) and anti-Isl1 (DSHB). To visualize Isl1 protein in Notch1 mutant embryos, the TSA System (PerkinElmer) was used to amplify Isl1 signals. Nuclear β-catenin was detected with anti-PY489 antibody (DSHB). For western blotting, lysates from day 3 CPCs after transfection with indicated siRNAs were analysed using antibodies against Notch1 (DSHB), dephospho β-catenin (Calbiochem) and GAPDH (Santa Cruz Biotechnology).

Chromatin immunoprecipitation assays. For chromatin immunoprecipitation (ChIP) assay, embryoid bodies were treated with BIO (2.5 µM) or transfected with Isl1 or β-catenin constructs<sup>10</sup> (100 ng 10<sup>6</sup> cells) from ED5–7, and collected at ED8. Crosslinking of histones to DNA, chromatin extraction, immunoprecipitation and elution were performed using the ChIP assay kit (Upstate) with anti-IgG–HRP, Isl1 (Abcam) or β-catenin (Santa Cruz Biotechnology). PCR primer sets spanning the indicated Lef/Tcf binding sites in the Bhlhb2 locus are shown in Supplementary Information, Table S2.

Statistical analyses. The two-tailed Student’s t-test, type II, was used for data analyses. P < 0.05 was considered significant.

Accession number. The full microarray data performed in this study are available in NCBI Gene Expression Omnibus (GEO, accession number: GSE15232).

**Figure S1.**

**a,** Relative *Isl1* expression levels in GFP⁻ and GFP⁺ cells isolated from Day 5 *Nkx2.5-GFP* EBs, determined by qPCR (mean ± s. d.; *n*=3).

**b,** Immunostaining of transverse sections through the pre-cardiac mesoderm and outflow tract (ot) of indicated E9.5 mouse embryos for nuclear β-Catenin. Higher levels of β-Catenin are observed in precardiac regions (arrowheads) in Notch1 mutants. Numerous Wnts are expressed in the ectodermal cells and form a gradient pattern of active β-Catenin (arrows), providing a positive control.

**c,** qPCR data of positively affected genes in cardiac progenitors with stabilized β-Catenin (mean ± s. d.; *n*=3; *P* < 0.01). **d,** Transverse sections of corresponding embryos (Fig. 2e–l), focused on precardiac mesoderm (asterisk) and outflow tract (ot) area. **e,** Relative Bhlhb2 expression levels in hearts and precardiac mesoderm from E10.0 control or *Isl1Cre; Notch1floxflox* embryos (Notch KO), determined by qPCR (mean ± s. d.; *n*=3; *P* < 0.01). **f,** Relative *Isl1* expression levels in EBs 2 days after transfection with an *Isl1 siRNA* construct (transient *Isl1-KD*, left) and in ED6 EBs differentiated from control and stable *Isl1-KD* lines (stable *Isl1-KD*, right), determined by qPCR (mean ± s. d.; *n*=3; *P* < 0.01), Scale bars, 100 µm.
Figure S2 a, Histograms showing percentages of GFP+ cells of ED6, 7, and 8 EBs after transient transfection with an *Is1* siRNA construct on ED3. b, Histograms showing percentages of GFP+ cells of ED6 EBs differentiated from control and stable *Is1-KD* lines.
Figure S3 a, Relative *Isl1* expression levels in EBs at indicated days of differentiation (ED), determined by qPCR. b, Histograms showing percentages of Myh7<sup>+</sup> cells entering myocardial-lineage in ED9 EBs.
Supplementary Table Legends

**Supplementary Table 1** List of genes significantly affected by stabilized β-Catenin in cardiac progenitors.

**Supplementary Table 2** List of qPCR primers and oligonucleotides used in this study.