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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[13]. 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[10], or Isl1, a CPC marker[8,15]. 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[2]. Conversely, stabilization of β-catenin in the same cells led to expansion in the number of CPCs[2] *in vivo*, whereas Wnt/β-catenin signalling promoted renewal of CPCs isolated from ES cells[2,3]. Notch signalling reciprocally affects Wnt signals in many contexts[16] and is thought to inhibit cardiac differentiation[17,18], 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^{flox}* mice[19] with mice containing *Cre recombinase* in the *Isl1* locus (*Isl1^{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[2], 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

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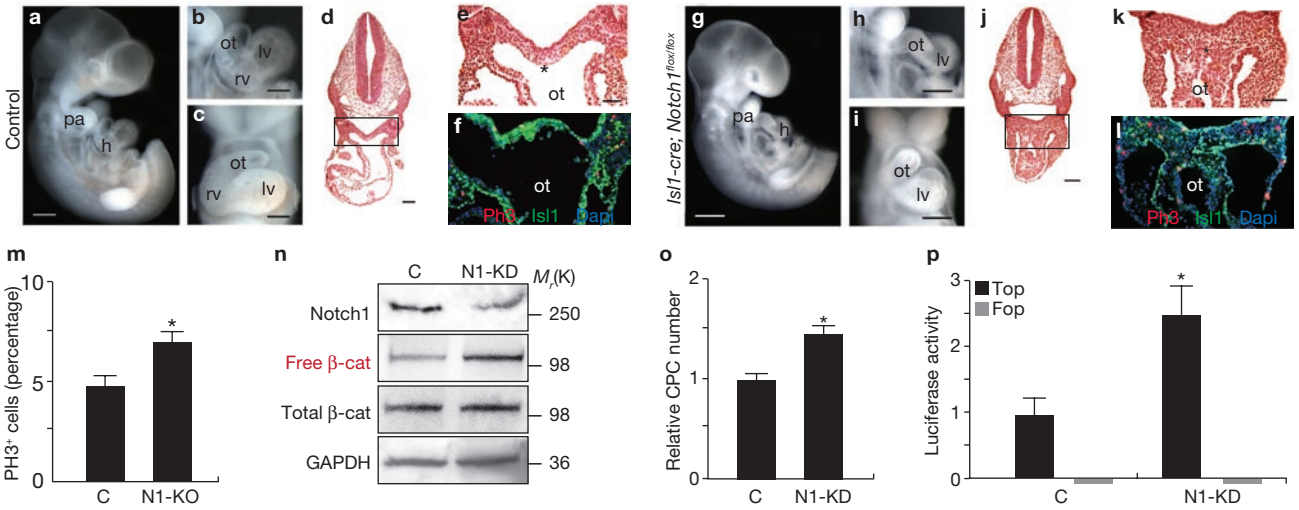

Figure 1 Notch1 loss-of-function causes CPC expansion and increases free β-catenin levels. (**a–l**) Control (**a–f**) and *Isl1*^{Cre}, *Notch1*$^{flox/flox}$ embryos (N1-KO) (**g–l**) showing lateral views of E9.5 embryos (**a**, **g**), and lateral (**b**, **h**) or frontal (**c**, **i**) views of embryos focused on cardiac regions showing absence of right ventricle (rv) in mutants. Transverse sections (haematoxylin and eosin) of embryos (**d, j**) with enlargement of boxed areas (**e, k**) show hyperplasia of precardiac progenitors (asterisk). (**f, l**) PH3 (red) and Isl1 (green) immunostaining of transverse sections through the precardiac region. To compensate for the marked downregulation of Isl1 in Notch1 mutant embryos, 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).

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[21], 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 levels of Notch1 were reduced considerably by siRNA transfection, as shown by western blot analysis (Fig. 1n). Consistent with the *in vivo* data, *Notch1* knockdown resulted in an increase in the number of CPCs (Fig. 1o). *Notch1* knockdown did not affect the levels of total β-catenin in CPCs (Fig. 1n). However, the levels of dephosphorylated (free) β-catenin, a form required to mediate Wnt/β-catenin signalling, were considerably higher in the *Notch1* knockdown CPCs (Fig. 1n). Consistent with this observation, *Notch1* knockdown CPCs showed significantly increased levels of Topflash activity, a luciferase-based reporter system for Wnt/β-catenin signalling (Fig. 1p). Increased levels of nuclear β-catenin were also observed in the cardiac mesoderm of the Notch1 mutant embryo (Supplementary Information, Fig S1b). These findings suggest that Notch1 normally represses CPC expansion and negatively regulates the active form of β-catenin.

To search for genes responsible for CPC expansion in an unbiased manner, we performed gene expression analyses of β-catenin-stabilized CPCs *in vivo*. For this analysis, we generated *Rosa*^{YFP}; *Isl1*^{Cre}; *β-catenin(ex3)loxP*^{loxP} embryos that express yellow fluorescent protein (YFP) in descendants of Isl1$^+$ progenitors in the cardiac region with stabilized β-catenin (Fig. 2a). YFP$^+$ cells from E9.0 embryos, before cardiac dysfunction, were purified by FACS (Fig. 2b) and used for mRNA expression arrays.

Many known targets of canonical Wnt signalling, including components of the Wnt signalling pathway, were upregulated in mutants, confirming the quality of the data set (Supplementary Information, Fig S1c, Table S1). We found that expression of genes implicated in cell proliferation and differentiation (for example, *Ndrg1, Bhlhb2* and *Fgfs*) was highly upregulated (4–11 fold) in mutants (Supplementary Information, Fig S1c, Table S1). Unexpectedly, several genes essential for CPC development, including *Isl1*, *Myocd, Shh* and *Smyd1* were significantly downregulated in the mutants and this was validated by quantitative real-time PCR (qPCR) (Fig. 2c, d). Curiously, *Isl1* was downregulated when β-catenin was stabilized. In agreement with the array analyses, *Isl1* transcripts were barely detectable by *in situ* hybridization in CPCs of β-catenin-stabilized embryos (Fig. 2e, i; Supplementary Information, Fig. S1d). *Smyd1* and *Myocd* transcripts were also significantly downregulated in β-catenin-stabilized embryos, whereas *Bhlhb2* was upregulated specifically in the *Isl1*^{Cre} domain (Fig. 2f–h, j–l; Supplementary Information, Fig. S1d). Consistent with the opposing functions of Notch1 and β-catenin described above, *Isl1*, *Myocd, Shh* and *Smyd1* were significantly downregulated and *Bhlhb2* was upregulated in *Notch1* mutant embryos (Fig. 2d; Supplementary Information, Fig. S1e).

Isl1 is a homeodomain-containing transcription factor that is transiently expressed in CPCs before their migration into the heart tube, but is silenced as further migration and differentiation proceed[4]. Although

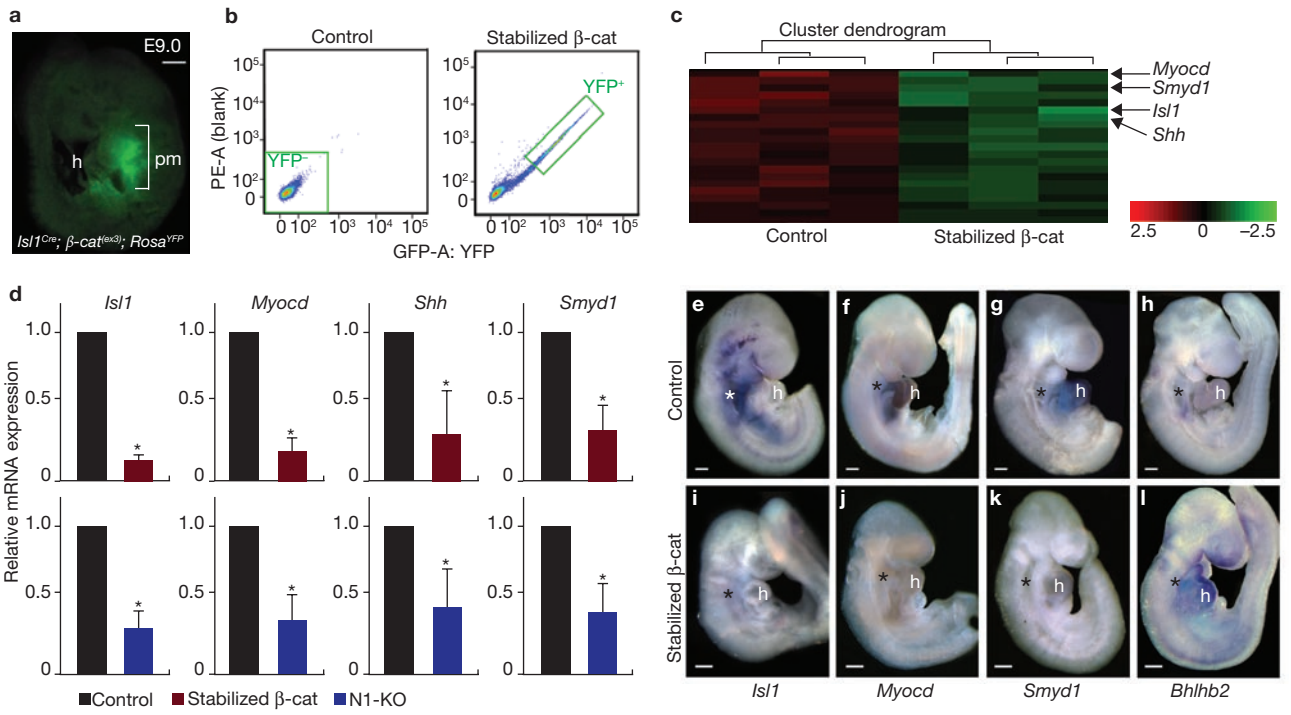

Figure 2 Identification of genes affected by stabilized β-catenin in cardiac progenitors. (**a**) Lateral view of *Rosa*^{YFP};*Isl1*^{Cre};*β-catenin(ex3)*^{loxP} embryo at E9.0 showing YFP$^+$ cells in precardiac mesoderm (pm). (**b**) Histograms of YFP$^+$ cell populations from control (*Isl1*^{Cre}, left) and stabilized β-cat (*Isl1*^{Cre};*β-catenin(ex3)*^{loxP}, right) embryos. (**c**) A heatmap of expression arrays showing downregulated cardiac genes (green) in stabilized β-catenin precardiac mesoderm cells. Colour bar indicates fold change in $\log_2$ scale. (**d**) qPCR data of downregulated genes in FACS-purified cardiac progenitors with stabilized β-catenin (top panels). These genes were similarly affected in pm of Notch1 loss-of-function embryos (bottom panels). Data are mean ± s.d.; $n = 3$; $^*P < 0.01$. (**l**) Whole-mount *in situ* hybridization of genes indicated from control (top panels) and stabilized β-catenin (bottom panels) embryos at E9.5. Asterisks indicate precardiac mesoderm (h, heart). Scale bars, 100 μm. *P* was 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*^{Cre} 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[20]. *Isl1*^{Cre} mice were bred with *Rosa*^{YFP} mice to generate *Isl1*$^{Cre/Cre}$;*Rosa*^{YFP} embryos. We quantified the number of YFP$^+$ cells at E8.0 (5 somite stage), before *Isl1*^{Cre} expression is initiated in neural cells, by FACS. Surprisingly, *Isl1*-null embryos had a significantly higher percentage of YFP$^+$ 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 mesoderm[7,8]. Knockdown of *Isl1* from ED0–3 did not change the number of Nkx2.5$^+$ 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 transfected *Nkx2.5–GFP*$^+$, FACS-purified CPCs from day 5 embryoid bodies with a stabilized β-catenin expression construct[22] 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, co-transfection 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*$^+$ 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*$^+$ CPCs from ED5 embryoid bodies and differentiated them by re-aggregation in suspension (Fig. 3g). *Nkx2.5–GFP*$^+$ CPCs are multipotent and differentiate into myocardial, smooth muscle and endothelial cell lineages[7,12]. Normal levels of endothelial gene expression (CD31, Flk1) were observed in differentiating *Isl1* knockdown CPCs (Fig. 3h). However, expression of cardiomyocyte and

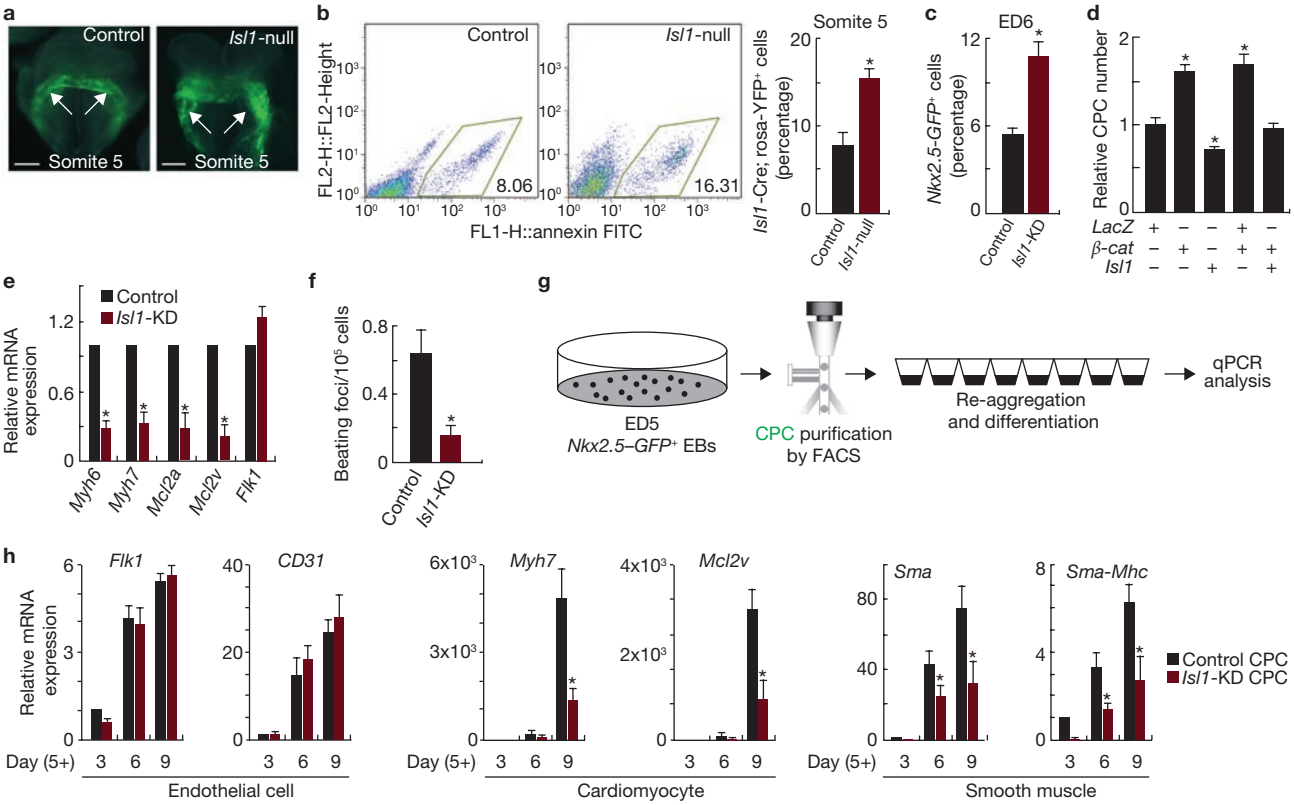

Figure 3 *Isl1* loss-of-function results in expansion of CPCs and suppression of their myocardial and smooth muscle lineages. (**a**) *YFP* expression in control (*Rosa*^{YFP}, *Isl1*$^{Cre/+}$, left) and *Isl1*-null (*Rosa*^{YFP}, *Isl1*$^{Cre/Cre}$, right) embryos at the 5-somite stage. Arrows indicate 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 *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. d.; $n = 4$; $^*P < 0.01$). (**f**) Number of beating foci per 10^5 cells in control or *Isl1* knockdown embryoid bodies at ED12. (**g**) Schematic diagram of isolating CPCs from ES cells and their differentiation (EB, embryoid bodies). (**h**) Relative mRNA expression of endothelial (*Flk1, CD31*), cardiomyocyte (*Myh7, Mlc2v*) or smooth muscle (*Sma, Sm-mhc*) genes during CPC differentiation, 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).

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.

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* expression construct (30 ng per 10^5 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*-overexpressed embryoid bodies (Fig. 4d; Supplementary Information, Fig. S3b). This suggests that Isl1 can promote myocardial differentiation of CPCs in an instructive manner.

In addition to *Isl1*, *Myocd* and *Smyd1* are important genes for cardiogenesis[23–27] 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 muscle[24] and cardiac gene expression[27]. Smyd1 is a muscle-restricted histone methyltransferase essential for cardiomyocyte differentiation *in vivo*[23,25]. 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

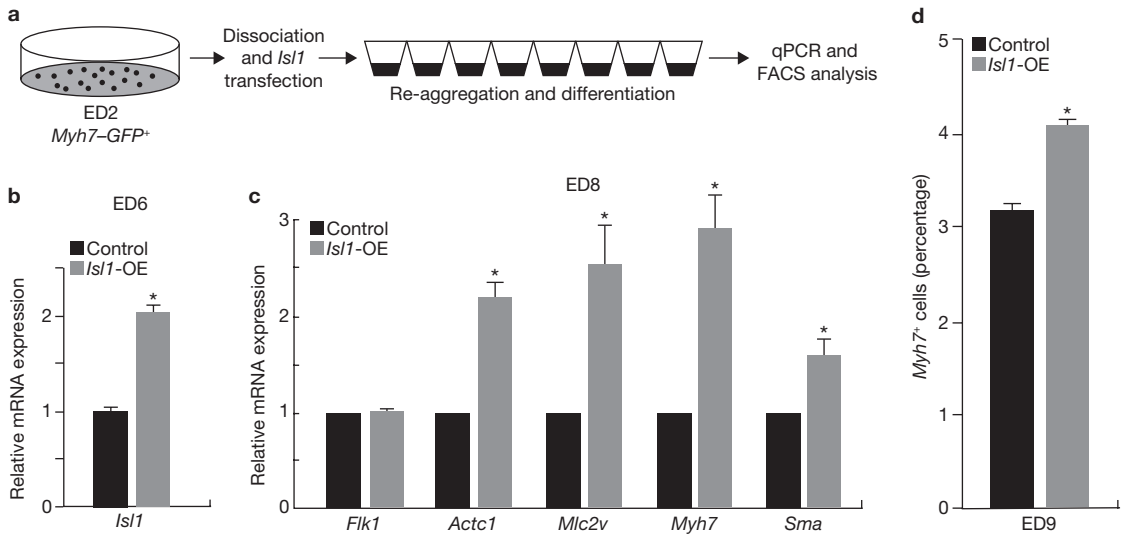

Figure 4 Increased levels of *Isl1* promote myocardial differentiation. (**a**) Schematic diagram of differentiation of *Myh7–GFP* ES cells with *Isl1* overexpression (*Isl1*-OE). (**b**, **c**) Relative expression levels of *Isl1* on ED6 embryoid bodies (**b**), and endothelial (*Flk1*), cardiac sarcomeric (*Actc1, Mlc2v, 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).

into embryoid bodies at ED6–8 (when endogenous Isl1 is enriched and biologically functional) (Fig. 5k). However, luciferase activity was significantly reduced when the Isl1 site was mutated (Fig. 5k). In addition, excessive Isl1 further increased luciferase activity when the Isl1 site was intact but not when it was mutated (Fig. 5k). Chromatin immunoprecipitation (ChIP) with anti-Isl1 antibodies in ED8 embryoid bodies revealed that the site was associated with Isl1 protein (Fig. 5l). This association was further confirmed by electrophoretic mobility shift analyses, which showed specific binding of Isl1 to the site (Fig. 5m). Together, these data suggest that Isl1 may directly regulate *Myocd* expression.

As Isl1 did not affect *Smyd1* expression, we hypothesized that β-catenin might activate a transcriptional repressor to downregulate *Smyd1* expression. Among the transcriptional repressors affected by β-catenin in our array, *Bhlhb2* was the most highly upregulated. Bhlhb2 is a basic helix-loop-helix (bHLH)-containing, DNA-binding repressor that is involved in many biological processes, including proliferation, differentiation and regulation of circadian rhythm[28–30]. Quantitative PCR confirmed that *Bhlhb2* was highly upregulated in embryos with stabilized β-catenin (Fig. 5n), consistent with upregulation shown by *in situ* hybridization in the cardiac area and other domains of $Isl1^{Cre}$ activity (Fig. 2h, l). Overexpression of *Bhlhb2* in *Nkx2.5–GFP*$^+$ CPCs mimicked Smyd1 repression observed with β-catenin stabilization (Fig. 5o). *Isl1* expression was not affected by Bhlhb2, providing an important control (Fig. 5o). We identified four conserved Lef/Tcf consensus sites in the 5′ and 3′ UTRs of *Bhlhb2* (Fig. 5p) and tested whether any were directly bound by β-catenin. ChIP with anti-β-catenin antibodies in ED8 embryoid bodies revealed that two of the four sites (A and D) were indeed associated with β-catenin (Fig. 5q). To determine which site can mediate Wnt/β-catenin signalling, conserved elements encompassing the Lef/Tcf sites were individually inserted upstream of a luciferase reporter and luciferase activity was examined in ED8 embryoid bodies. We found that the construct-containing site D, but not A, resulted in a significant increase in luciferase activity on stimulation with β-catenin or 6-bromoindirubin-3′-oxime (BIO), a Wnt/β-catenin signalling activator (Fig. 5r). This increase was, however, not observed in cells transfected with a mutant construct (Fig. 5r). These data suggest that Bhlhb2 may be a direct target of the Wnt signal.

Through use of mouse genetics and the ES cell system, we have shown that Wnt/β-catenin signalling functions as a central regulator of CPCs by integrating signals from the Notch pathway and regulating a cascade of downstream transcriptional events involving Isl1, Myocd and Smyd1 (Fig. 5s). We found that Notch1 activity within CPCs was required for their exit from the expansive state into the differentiated state, providing the first evidence for Notch signalling requirement within multipotent CPCs *in vivo*. Consistent with negative regulation of active β-catenin by Notch1, Notch1 loss-of-function and β-catenin gain-of-function had similar effects on expression of the cardiac transcription factors, *Isl1*, *Myocd, Smyd1* and *Bhlhb2*. Our finding that CPCs *in vivo* and *in vitro* had greater expansion on disruption of Isl1 and that Isl1 could promote differentiation suggests that despite its very transient expression, Isl1 triggers further development of CPCs into cardiac cells rather than promoting its renewal state. Strikingly, Isl1 downregulation induced by β-catenin was necessary for Wnt/β-catenin-induced expansion of CPCs. Manipulation of the cascade described here may be useful in guiding the expansion and directed differentiation of CPCs for regenerative therapies and other uses of stem cell-derived cardiomyocytes. □

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturecellbiology/

Note: Supplementary Information is available on the Nature Cell Biology website.

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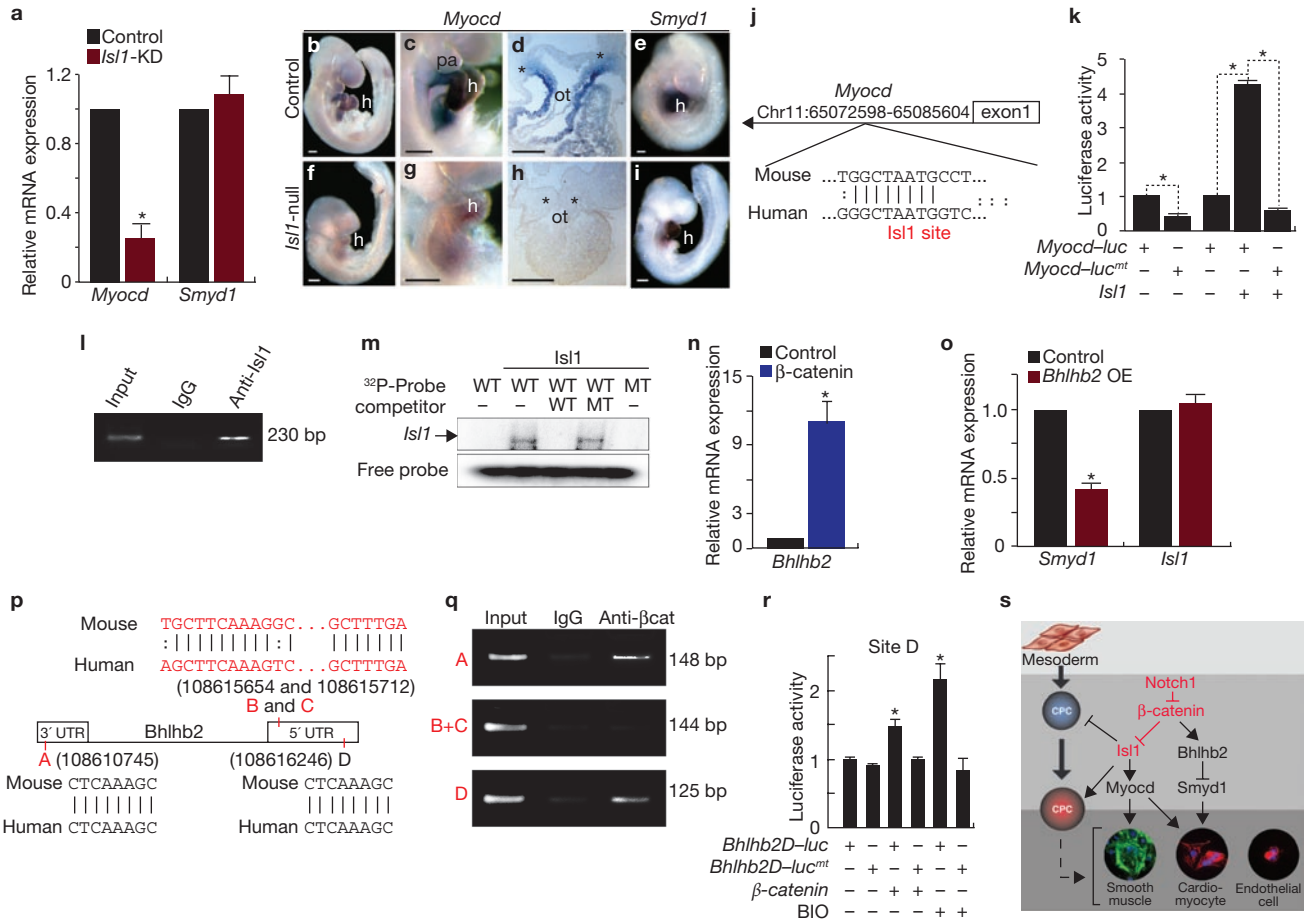

Figure 5 Isl1 targets *Myocd* and β-catenin regulates *Bhlhb2* to repress *Smyd1*. (**a**) Relative expression levels of *Myocd* and *Smyd1* in FACS-purified control and *Isl1* knockdown (*Isl1*-KD) CPCs, determined by qPCR (mean ± s. d.; $n = 4$; $^*P < 0.005$). (**b–i**) Control (**b–e**) and *Isl1-null* (**f–i**) embryos at E9.5 after *in situ* hybridization with *Myocd* (**b–d**, **f–h**) or *Smyd1* (**e, i**) riboprobes. (**c**, **g**) Lateral views focused on heart (h) and pharyngeal arch (pa) regions. (**d**, **h**) Transverse section through the outflow tract. Asterisks indicate pre-cardiac mesoderm. Scale bars, 100 μm. (**j**) Location of the conserved island containing Isl1 binding site (red) in the *Myocd* locus. (**k**) Relative luciferase activity determined with luciferase reporters linked to the conserved island with the intact Isl1 site (*Myocd–luc*) or with a mutant Isl1 site (*Myocd–luc*^{mt}) in the presence or absence of Isl1 (mean ± s. d.; $n = 3$; $^*P < 0.005$). (**l**) ChIP assay shows specific PCR amplification of the *Isl1* consensus site shown in **j**, representing association with Isl1 protein. (**m**) Electophoretic mobility shift assay with *in vitro* synthesized Isl1 protein and radiolabelled probes (Probe) spanning the Isl1 site shown in **j**. Unlabelled probes were used as competitors. WT, wild-type; MT, mutant. (**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**) Relative luciferase activity determined with luciferase reporters containing the intact Lef/Tcf site D (Bhlhb2D-luc) or with a mutant Lef/Tcf site D (Bhlhb2D–luc^{mt}) in the presence or absence of β-catenin or BIO (2 μM). Data are 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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AUTHOR CONTRIBUTIONS

C.K. designed, performed, supervised *in vivo* and *in vitro* work and wrote the manuscript; L.Q. 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.

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METHODS

Mouse genetics and CPC and ES cell culture. Control (*Rosa*$^{YFP/+}$; *Isl1*$^{Cre/+}$) or mutant (*Rosa*$^{YFP/+}$; *Isl1*$^{Cre/+}$; *β-catenin(ex3)loxP*$^{loxP/+}$) embryos were obtained by crossing *Rosa*$^{YFP/+}$; *β-catenin(ex3)loxP*$^{loxP/+}$ with *Isl1*$^{Cre/+}$ mice[20,31]. YFP$^+$ cells from the resulting embryos were purified by FACS and used for gene expression analyses. To quantify embryonic CPCs, *Rosa*$^{YFP/+}$; *Isl1*$^{Cre/+}$ were crossed with *Isl1*$^{Cre/+}$ mice, and YFP$^+$ cells from the resulting embryos were counted by FACS. To generate *Isl1*$^{Cre/+}$; *Notch1*$^{loxP/loxP}$, *Isl1*$^{Cre/+}$; *Notch1*$^{loxP/+}$ mice were crossed with *Notch1*$^{loxP/loxP}$ mice[19]. Genotyping was performed as described previously[2]. To identify *Isl1-het* (*Isl1*$^{Cre/+}$) or null (*Isl1*$^{Cre/Cre}$) 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[2]. For CPC differentiation, FACS-purified GFP$^+$ cells were re-aggregated in suspension (10^5 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, UCSF. 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* shRNA 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-lacZ* (Invitrogen) was used as a control. For *Notch1* knockdown studies, Block-iT Alexa Fluor Red (46-5,318, Invitrogen) or *Notch1* siRNA (M-041,110-00-0005, Dharmacon) was used at concentration of 50 or 100 nM. *Myocd–luc* was generated by cloning their corresponding regions into the *pGL3* luciferase vector (Promega). *Myocd–luc*^{mt} was generated using QuikChange Site-Directed Mutagenesis Kit (Stratagene). For *Bhlhb2D–luc* and *Bhlhb2D–luc*^{mt} generation, oligonucleotides containing the Tcf/Lef site were cloned into the *pGL3* vector. All the oligonucleotide sets are listed 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[32,33]. For EMSAs, the pCITE–ISL1[34] 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 probes[4,23,26]. *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 uM) or transfected with *Isl1* or *β-catenin* constructs[22] (100 ng 10^{-5} 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).

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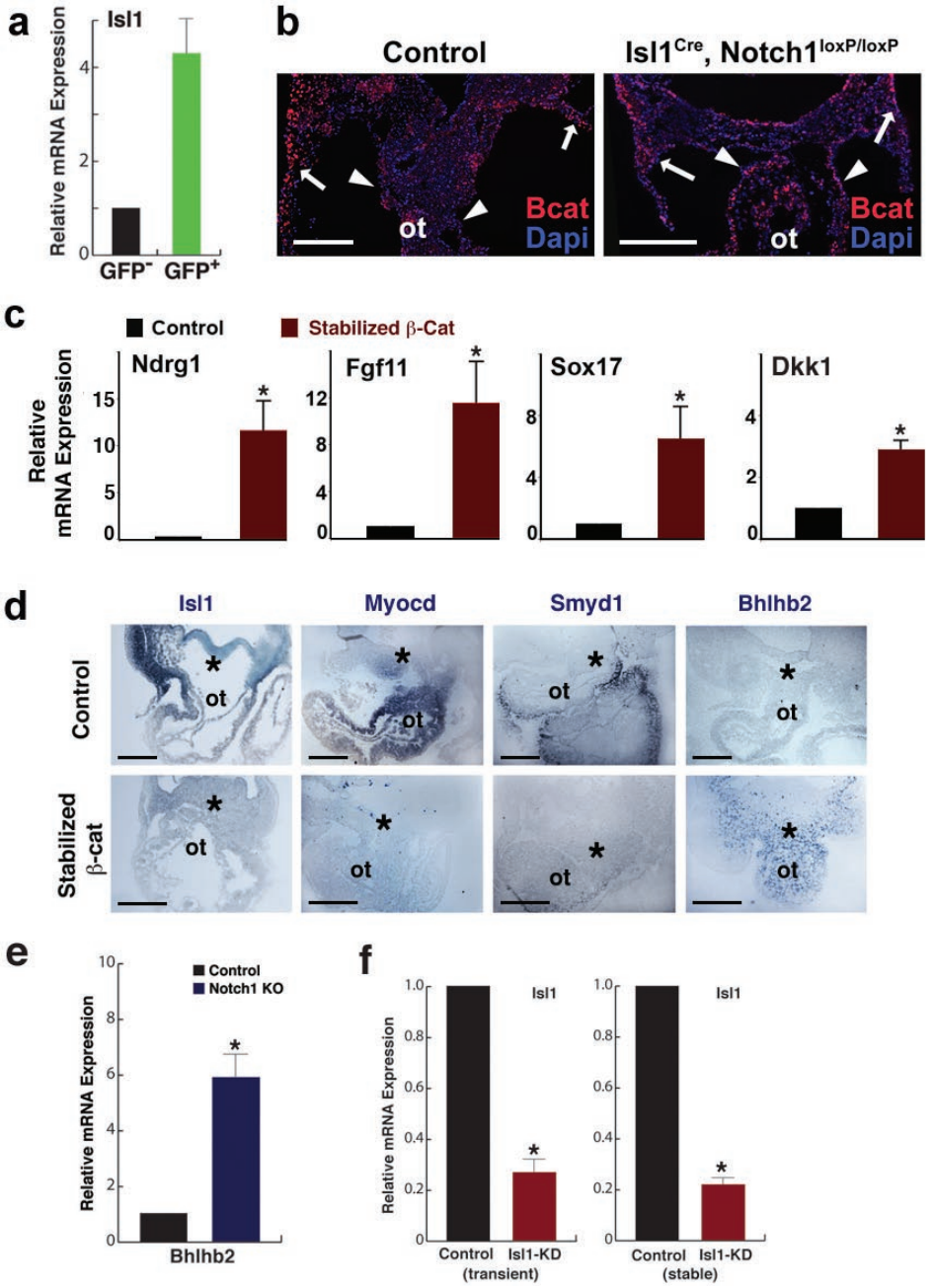

Figure S1 a, Relative *Isl1* expression levels in GFP$^-$ and GFP$^+$ cells isolated from Day 5 *Nkx2.5-GFP* EBs, determined by qPCR (mean ± s. d.; *n*=3). **b,** Immunostaining of transverse sections through the pre-cardiac mesoderm and outflow tract (ot) of indicated E 9.5 mouse embryos for nuclear 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. h, heart. **e,** Relative *Bhlhb2* expression levels in hearts and precardiac mesoderm from E10.0 control or *Isl1*^{Cre}*; Notch1*$^{flox/flox}$ 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.

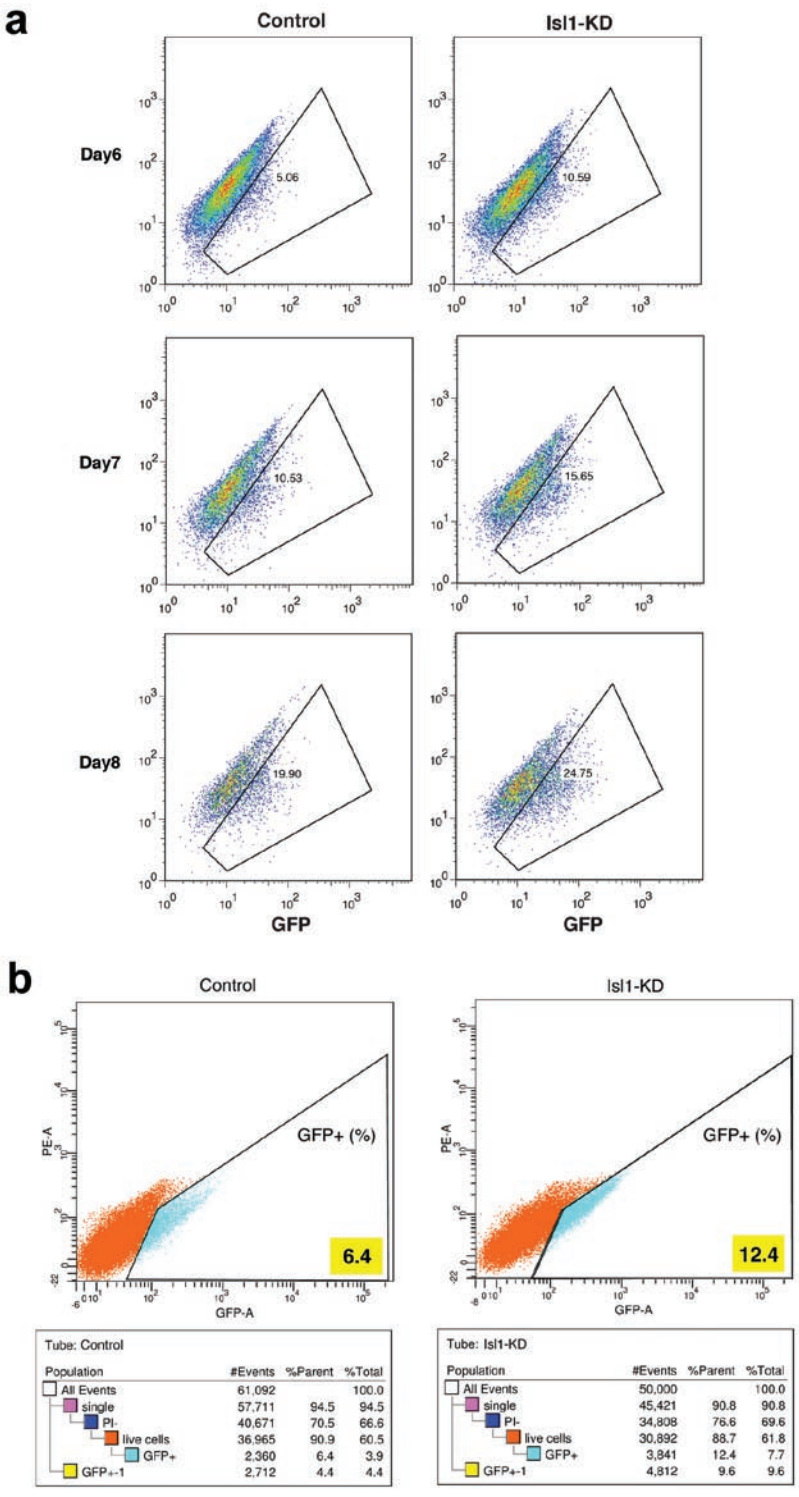

Figure S2 a, Histograms showing percentages of GFP$^+$ cells of ED6, 7, and 8 EBs after transient transfection with an *Isl1 siRNA* construct on ED3. **b,** Histograms showing percentages of GFP$^+$ cells of ED6 EBs differentiated from control and stable *Isl1-KD* lines.

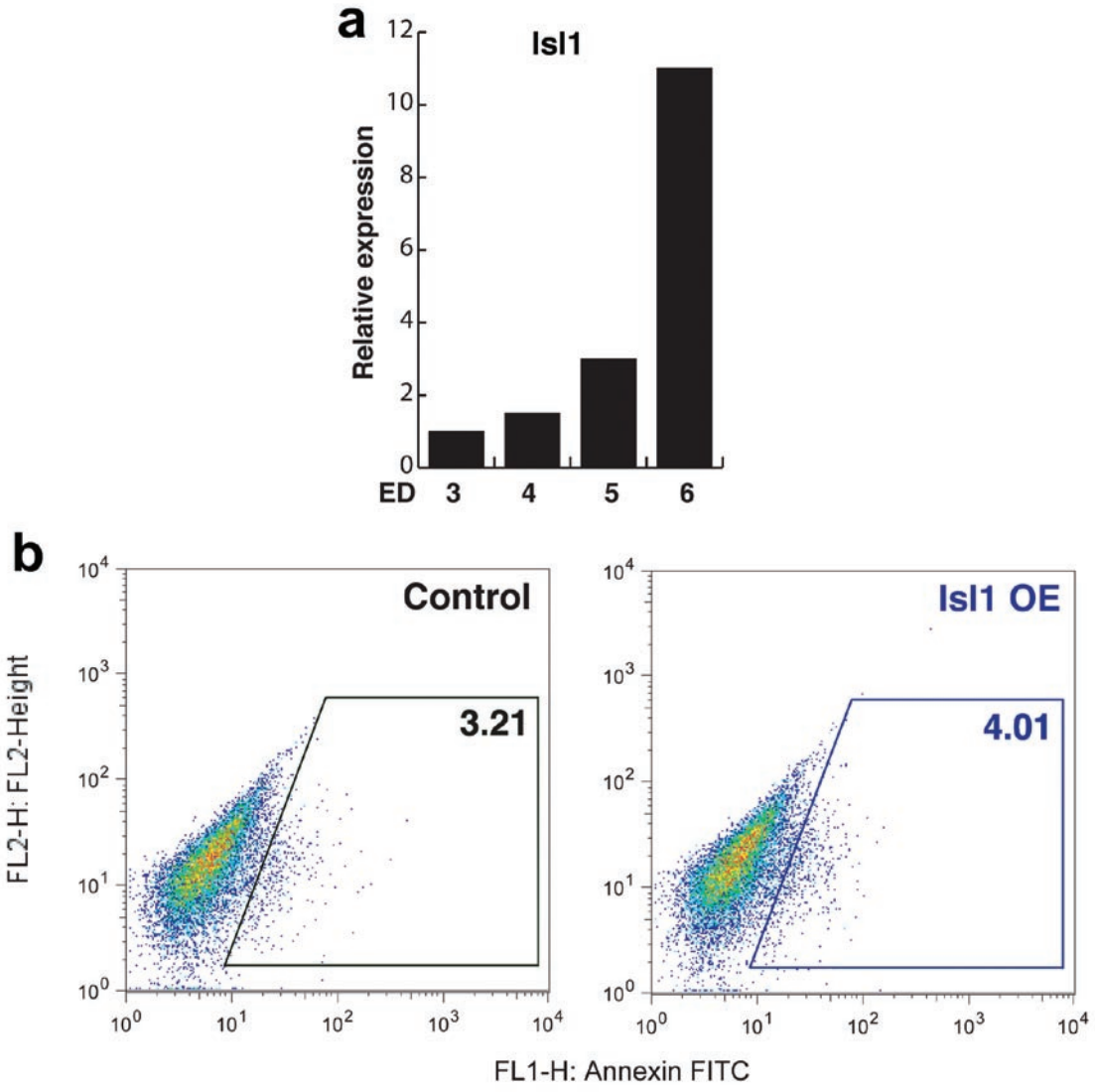

Figure S3 a, Relative *Isl1* expression levels in EBs at indicated days of differentiation (ED), determined by qPCR. **b,** Histograms showing percentages of $Myh7^+$ cells entering myocardial-lineage in ED9 EBs.

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.