

Wnt-promoted *Isl1* expression through a novel TCF/LEF1 binding site and H3K9 acetylation in early stages of cardiomyocyte differentiation of P19CL6 cells

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Abstract Islet 1 (ISL1), a marker of second heart field progenitors, plays a crucial role in cardiomyocyte differentiation and proliferation. However, little is known about transcriptional regulating mechanisms on *Isl1* gene expression. Recent studies have demonstrated that Wnt/ β -catenin signaling regulates *Isl1* expression during heart development. However, the detailed mechanisms still remain unclear. In the present study performed during differentiation of P19CL6 into cardiomyocytes, we explored the underlying regulating mechanisms on Wnt/ β -catenin-mediated *Isl1* expression after we first confirmed that Wnt/ β -catenin signaling promoted cardiomyocyte differentiation partly through *Isl1* activation. We found a novel TCF/LEF1 binding site that was located 2300 bp upstream of the *Isl1* ATG. Furthermore, Wnt/ β -catenin signaling upregulated histone H3K9 acetylation on TCF/

LEF1 binding sites on the *Isl1* promoter, resulting in upregulation of *Isl1* expression. This Wnt-mediated H3K9 acetylation on the *Isl1* promoter was modulated by the acetyltransferase CREB-binding protein (CBP), instead of p300, through interaction with β -catenin. Collectively, these results suggest that in early stages of cardiomyocyte differentiation Wnt/ β -catenin signaling promotes *Isl1* expression via two ways: a novel TCF/LEF1 binding site and H3K9 acetylation conducted by CBP on the *Isl1* promoter. To our knowledge, this is the first study reporting Wnt/ β -catenin-regulated H3K9 acetylation on promoters of its target genes. And this study gives new insights into transcriptional regulating mechanisms of Wnt-mediated *Isl1* expression during cardiomyocyte differentiation.

Keywords WNT/ β -catenin · ISLET 1 · Cardiomyocyte differentiation · P19CL6 cells · H3K9 acetylation

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Introduction

Islet 1 (ISL1), a LIM-homeodomain transcription factor, plays a key role in cardiac development. Fate mapping experiments with *Isl1*^{Cre} indicated that ISL1 marks second heart field (SHF) progenitors, which contribute to the formation of outflow tract, right ventricle, and much of the atria [1]. Mice homozygous null for *Isl1* die at approximately E10.5, failing to develop heart structures derived from the SHF [1]. *Isl1* mRNA is expressed in progenitors, but is downregulated as cells differentiate and enter the heart, although expression is maintained in a few subpopulations [2]. In human fetal hearts, ISL1⁺ cells are found within SHF derivatives, consistent with data in murine embryos [3]. Due to its spatial and temporal expression profile, ISL1 is regarded as a marker of cardiac progenitor

cells (CPC). In the myocardium of postnatal rats, mice, and humans, ISL1⁺ cardiac progenitors have been identified, with potential to give rise to cardiomyocytes [4]. In both mouse and human, fetal ISL1⁺ progenitors are capable of differentiating to diverse cardiovascular lineages, including cardiomyocytes, smooth muscle cells, and endothelial cells [3, 5]. Recent studies show that ISL1⁺ multipotent cardiovascular progenitors can be generated from mouse iPS cells and spontaneously differentiate into cardiovascular lineages in vivo without teratoma [6]. These findings may pave the way for new strategies to repair damaged myocardium after infarction.

In addition, ISL1 lies atop a conservative transcriptional network governing SHF development and is involved in setting in motion a cascade of downstream transcriptional events. Targets downstream of ISL1, including *Mef2c*, *Nkx2.5*, and *Fgf10*, ultimately determine cardiac development [7–10]. Some partners which interact with ISL1, including CIP and Ajuba, have been identified, and repress transcriptional activity of *Isl1* [11, 12]. However, mechanisms responsible for dynamic regulation of *Isl1* expression are still unclear. Loss of FGF8 signaling disrupts *Isl1* expression, indicating a positive role in *Isl1* expression regulation [13]. Also, *Isl1* is a direct target of Forkhead transcription factors in SHF-derived mesoderm [14]. During cardiomyocyte differentiation of the pluripotent mouse embryonic carcinoma cell line, P19CL6, the POU homeodomain protein OCT1 plays a crucial role in promoting *Isl1* expression [15]. Recently, studies have identified inhibitors of *Isl1* expression. BMP2 and BMP4 signaling downregulates *Isl1* expression via *miRNA-17-92* cluster [16]. In addition, polycomb repressive complex2 (PRC2) represses *Isl1* expression by EZH2-mediated H3K27 trimethylation of the *Isl1* promoter [17].

Wnt/ β -catenin signaling has biphasic effects on cardiac differentiation, depending on the stage of differentiation [18]. Wnt/ β -catenin signaling is required for early stages of cardiac differentiation but is decreased during terminal differentiation. In contrast, Wnt/Planar Cell Polarity (PCP) signaling is activated during differentiation. Research on the role of Wnt/ β -catenin signaling during cardiac development has been carried out in different models. In chick, forced expression of either Wnt3a or Wnt8c represses heart formation from anterior mesoderm and promotes development of primitive erythrocytes from the precardiac region. Reciprocally, Wnt inhibitors Crescent and DKK1 induce formation of heart muscle or heart-specific gene expression in posterior lateral plate mesoderm [19]. In P19CL6 cells, activation of Wnt/ β -catenin signaling promotes cardiomyocyte differentiation [20]. In ES cells, Wnt signaling is essential for mesodermal development [21]. In mice, ablation of β -catenin severely

disrupts cardiogenesis, with alterations in the proliferation and apoptosis of cardiac progenitors [22]. Similarly, stabilization of β -catenin in cardiac progenitors results in expansion of SHF progenitors and inhibition of differentiation [23–25]. Although loss of β -catenin results in decreased *Isl1* expression in SHF progenitors [22, 25], abnormally prolonged exposure to stabilized β -catenin can also cause *Isl1* downregulation in SHF progenitors [26], perhaps in a feedback loop.

Despite the foregoing which demonstrates the role for Wnt/ β -catenin signaling in regulating *Isl1* expression, questions still remain as to mechanisms underlying this regulation. To address this issue, we utilized pluripotent P19CL6 cells, a well-validated model for dissection of pathways determining cardiomyocyte differentiation [27]. Our data demonstrated that Wnt/ β -catenin promoted cardiomyocyte differentiation partly through *Isl1* activation. Wnt/ β -catenin signaling promotes *Isl1* expression via two ways: a novel TCF/LEF1 binding site located 2300 bp upstream of the ATG on the *Isl1* promoter and acetylation of histone H3K9 on TCF/LEF1 binding sites on the *Isl1* promoter by the acetyltransferase CREB-binding protein (CBP).

Results

The involvement of *Isl1* in Wnt-promoted cardiomyocyte differentiation of P19CL6 cells

To investigate mechanisms of Wnt-mediated *Isl1* expression during cardiomyocyte differentiation, we utilized an in-vitro cardiomyocyte differentiation model using induction of P19CL6 cells by DMSO. The cardiomyocyte differentiation of the cells was demonstrated by the expression of several cardiac-specific genes (Fig. 1a—Electronic supplementary material) and the contraction after DMSO induction for 12 days (Movie 1—Electronic supplementary material). Our results, consistent with previous reports [15, 28] indicated successful cardiomyocyte differentiation of P19CL6 cells. Also, our results revealed that the expression profile of Wnt/ β -catenin signaling molecules and transcriptional activation of Wnt/ β -catenin target genes were similar to the *Isl1* expression pattern which peaked at day 4, but attenuated at day 8 after DMSO induction (Fig. 1b–f—Electronic supplementary material). Besides, Wnt/ β -catenin signaling upregulated *Isl1* expression in early stages of P19CL6 cardiomyocyte differentiation (Fig. 2—Electronic supplementary material).

To further clarify whether Wnt/ β -catenin signaling regulated cardiac differentiation via *Isl1*, we maintained Wnt/ β -catenin signaling activity at a high level by culturing P19CL6 cells with Wnt3a from day 0 to day 4. As a

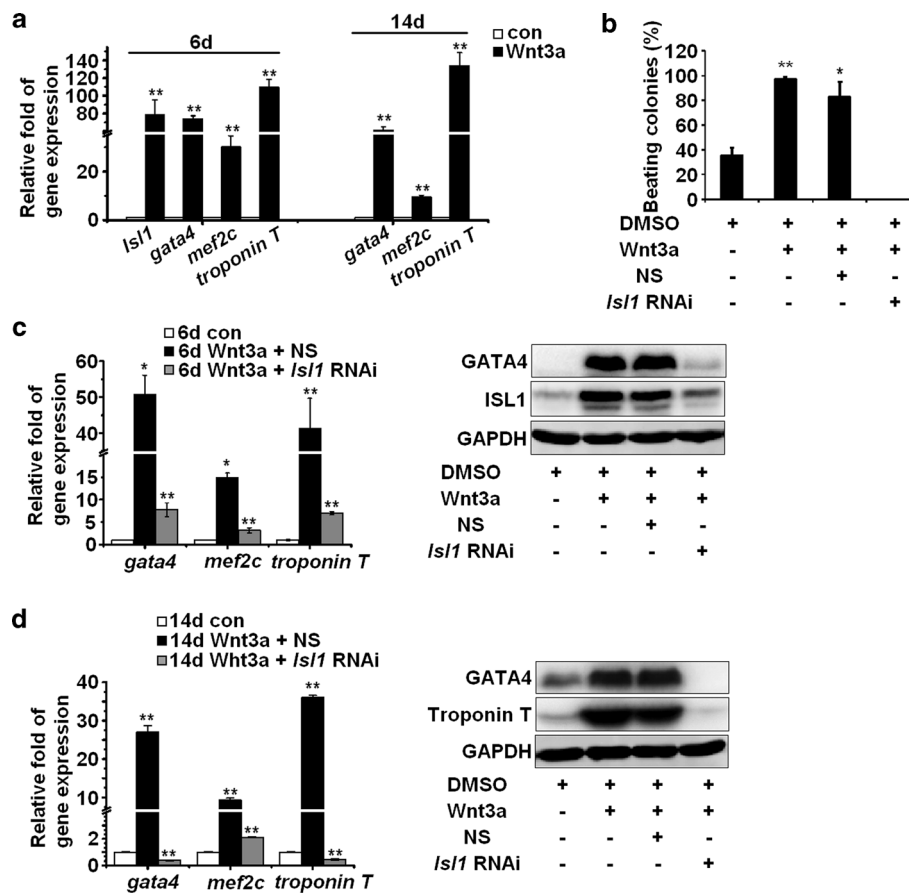


Fig. 1 The involvement of ISL1 in Wnt-promoted cardiac differentiation of P19CL6 cells. The cardiac differentiation of P19CL6 cells was induced by DMSO. **a** Real-time RT-PCR was conducted at day 6 and day 14 to evaluate the expression of *Isl1*, *gata4*, *mef2c*, and *Troponin T* in P19CL6 cells treated with Wnt3a (100 ng/ml) at day 0 and day 2. P19CL6 cells without Wnt3a treatment at day 6 or day 14 were used as controls. Data represent three independent experiments. Each bar represents mean \pm SD (** p < 0.01, vs the control). **b** Quantification of beating colonies was conducted at day 14 of culture. P19CL6 cells were treated by transient transfection with *Isl1* siRNA or non-silencer RNA (NS) at both day 0 and day 3. Wnt3a was

added (100 ng/ml) at day 0 and day 2. Data represent three independent experiments. Each bar represents mean \pm SD (* p < 0.05, ** p < 0.01, vs the cells at day 14). **c, d** Real-time RT-PCR and Western blot were performed at day 6 (**c**) and day 14 (**d**) to detect the expression of *Isl1*, *gata4*, *mef2c*, and *Troponin T* in P19CL6 cells. Cells were treated as mentioned above (**b**). Data represent three independent experiments, each in triplicate. P19CL6 cells at day 6 or day 14 were used as the control. Each bar represents mean \pm SD (* p < 0.05, ** p < 0.01, vs the control). GAPDH was used as an internal control for Western blot analysis

result, expression of three cardiac-specific marker genes (*gata4*, *mef2c* and *troponin T*) was significantly elevated at day 6 and day 14 (Fig. 1a). However, this elevation was attenuated when P19CL6 cells were transfected with *Isl1* siRNA from day 0 to day 6 (Fig. 1c, d). The percentage of beating colonies was also counted at day 14. Wnt3a treatment from day 0 to day 4 promoted formation of beating cardiomyocytes. However, no beating clones were observed when P19CL6 cells were exposed to *Isl1* siRNA from day 0 to day 6 even when treated with Wnt3a prior to day 4 (Fig. 1b). Therefore, these results indicate that in early stages of cardiomyocyte differentiation, Wnt/ β -catenin signaling upregulates *Isl1* expression. Additionally, knockdown of *Isl1* attenuates Wnt/ β -catenin-promoted cardiac differentiation.

A novel β -catenin/LEF1 binding site on the *Isl1* promoter

As demonstrated above, Wnt/ β -catenin signaling increased *Isl1* expression in early stages of differentiation. To detect novel binding sites of TCF/LEF1 on the *Isl1* promoter, bioinformatics analysis was performed. MatInspector software revealed a conserved TCF/LEF1 binding site 2300 bp upstream of the ATG translation start site on the *Isl1* promoter (Fig. 2a). The sequence covering the TCF/LEF1 binding site was used as a probe (the *Isl1* probe) for subsequent electrophoretic mobility shift assays (EMSA). Results showed that a specific complex was formed with the *Isl1* probe. Complex specificity was confirmed by incubating the nuclear extract with a 100-fold molar excess

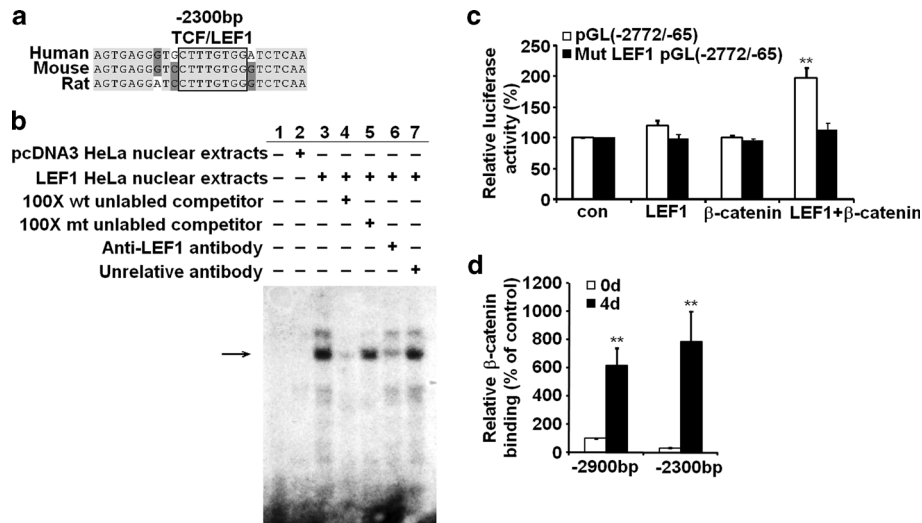


Fig. 2 The direct recruitment of β -catenin/LEF1 on the *Isll* promoter. **a** The putative TCF/LEF1 binding site at 2300 bp upstream of *Isll* ATG was identified using professional MatInspector database and indicated by a box. Alignment of *Isll* 5'UTR was performed with vector NTI10.0 software among human, mouse, and rat species, and the conserved sequences between all the species are shaded light gray. **b** EMSA was performed with HeLa cell nuclear extracts (transfected with pcDNA3.1 [28] or pCG-LEF1) for the β -catenin/LEF1 proteins binding ability to the 32 P-labeled oligonucleotides containing the putative LEF1 binding site on the *Isll* promoter. Lane 1 free probe, Lane 2 HeLa nuclear extracts transfected with pcDNA3.1 construct, Lane 3 HeLa nuclear extracts transfected with LEF1 expression construct, Lane 4 100 \times wild-type (wt) unlabeled oligonucleotide competition, Lane 5 100 \times mutant (mt) unlabeled oligonucleotide competition, Lane 6 anti-LEF1 antibody, Lane 7 unrelated antibody: goat normal IgG. **c** Luciferase assay was

performed to evaluate the transcriptional activity of the *Isll* promoter. 293T cells were transiently transfected with *Isll* promoter-luciferase reporter constructs in combination with either pcDNA3.1/ β -gal, LEF1, or β -catenin expression constructs. pGL(-2772/-65), wild-type *Isll* promoter-luciferase construct; Mut LEF1 pGL(-2772/-65), *Isll* promoter-luciferase construct with mutated LEF1 consensus sites. Data are presented as fold activity over basal promoter activity (relative activity). Each bar represents mean \pm SD from three independent experiments, each in triplicate (** $p < 0.01$, vs the control). **d** ChIP assay was performed with antibodies against β -catenin at day 0 or day 4. The immunoprecipitated DNA fragments were amplified by real-time PCR for the *Isll* promoter region from -2940 to -2630 (-2900 bp left panel) or -2341 to -2203 (-2300 bp right panel), respectively. Input represents 10 % of the total input chromatin

of unlabeled *Isll* probe prior to addition of the labeled probe. A control *Isll* probe carrying a mutation of the TCF/LEF1 binding site failed to compete for complex formation. Although the addition of unrelated antibody did not affect formation of the protein-DNA complex, formation of the complex was significantly attenuated in the presence of anti-LEF1 antibody, attesting to the specificity of the protein-DNA complex (Fig. 2b).

To further characterize the functional significance of LEF1 binding to the *Isll* promoter, luciferase assays were performed with a -2772/-65 bp *Isll*-promoter-luciferase reporter and expression vectors for LEF1 or β -catenin. Results demonstrated activation of the *Isll*-promoter by recruitment of LEF1 and activated β -catenin. This activation was disrupted by a mutation of the LEF1 binding site located 2300 bp upstream of *Isll* ATG (Fig. 2c). To further evaluate recruitment of β -catenin to the *Isll* promoter, including a previously reported TCF/LEF1 binding site located 2900 bp upstream of the ATG translation start site on the *Isll* promoter [22], ChIP assays were conducted in P19CL6 cells at day 0 and day 4. Our data showed that recruitment of β -catenin onto two TCF/LEF1 binding sites

on the *Isll* promoter was augmented after DMSO induction for 4 days (Fig. 2d). In summary, our data reveal that during cardiomyocyte differentiation of P19CL6 cells, Wnt-promoted *Isll* expression is partially dependent on a novel TCF/LEF1 binding site located 2300 bp upstream of *Isll* ATG.

Wnt-promoted H3K9 acetylation on TCF/LEF1 binding sites on the *Isll* promoter during cardiomyocyte differentiation of P19CL6 cells

Increasing evidence shows that histone modifications take part in Wnt-responsive gene expression [29]. To investigate whether histone modification is involved in Wnt-promoted *Isll* expression, a series of ChIP assays were conducted. Our data showed that mono- and tri-methylation of histone H3K4 and trimethylation of H3K9 in the two *Isll* promoter regions containing TCF/LEF1 binding sites were not changed at day4 of DMSO induction. Acetylation of H3 also remained unchanged following treatment of P19CL6 with Wnt3a or LiCl (data not shown). However, acetylation of H3K9 on the *Isll* promoter was

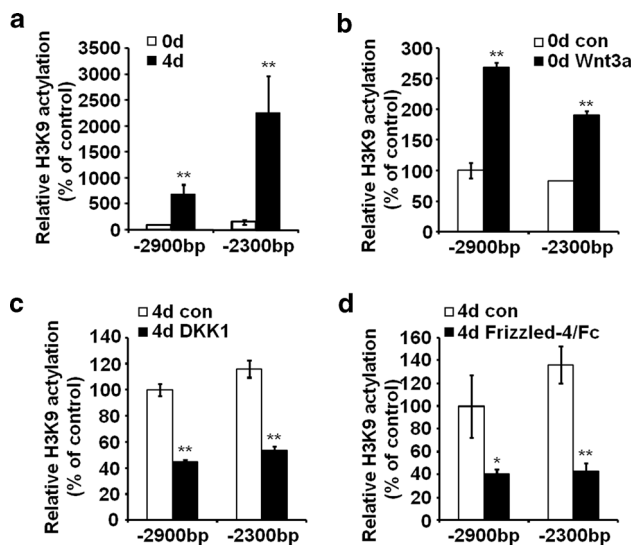


Fig. 3 Wnt-promoted H3K9 acetylation on the *Isll* promoter during cardiomyocyte differentiation of P19CL6 cells. **a** ChIP assay was conducted in P19CL6 cells at day 0 and day 4, with an antibody against acetylated H3K9. **b–d** ChIP assay was performed to detect H3K9 acetylation in response to treatment with Wnt3a (100 ng/ml, 24 h) at day 0 (**b**), and DKK1 (100 ng/ml) (**c**) or Frizzled-4/Fc (100 ng/ml) (**d**) through day 0–4 during P19CL6 cells differentiation into cardiomyocytes. Immunoprecipitated DNA fragments were amplified by real-time PCR for two *Isll* promoter regions containing TCF/LEF1 binding sites (–2900 bp: from –2940 to –2630; –2300 bp: from –2341 to –2203). The data are normalized on the basis of the corresponding input. The data for the 2900 bp binding site from P19CL6 cells uninduced or induced at day 4 without any treatment were used as a control. Each bar represents mean \pm SD from three independent experiments, each in triplicate (* $p < 0.05$, ** $p < 0.01$, vs the control)

significantly increased at day 4 (Fig. 3a), relative to day 0. Stimulation with Wnt3a also resulted in increased H3K9 acetylation on the *Isll* promoter at day 0 (Fig. 3b). Consistently, the application of DKK1 and Frizzled-4/Fc showed inhibitory effect on H3K9 acetylation on the *Isll* promoter (Fig. 3c, d). These results suggest that Wnt/ β -catenin signaling modulates H3K9 acetylation on the *Isll* promoter from day 0 to day 4 during P19CL6 cardiomyocyte differentiation.

Roles of CBP in Wnt-promoted acetylation of H3K9 on TCF/LEF1 binding sites on the *Isll* promoter

Histone acetylation is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). To find out which HATs or HDACs modulate Wnt-promoted acetylation of H3K9 on the *Isll* promoter, a series of ChIP assays were performed for the two aforementioned TCF/LEF1 binding sites on the *Isll* promoter. Our ChIP assays indicated that HDAC1 and PCAF had no significant effect on Wnt-induced H3K9 acetylation in the *Isll* promoter regions (data not shown). Although both CBP and p300 co-

immunoprecipitated with β -catenin at day 4 (Fig. 4a), no significant enrichment for p300 was observed in the two *Isll* promoter regions at the same time, compared to non-specific binding in the IgG IP samples. By contrast, CBP significantly recruited onto two TCF/LEF1 binding sites on the *Isll* promoter at day 4 (Fig. 3—Electronic supplementary material). Besides, accumulation of CBP was increased at day 4, compared with that at day 0 (Fig. 4b). Coincidentally, Wnt3a stimulation increased accumulation of CBP on the *Isll* promoter at day 0 (Fig. 4c), consistent with attenuated recruitment of CBP when P19CL6 cells were treated with DKK1 and Frizzled-4/Fc prior to day 4 (Fig. 4d, e). These results suggested that Wnt/ β -catenin signaling promoted accumulation of CBP, on the *Isll* promoter. Also, overexpression of *cbp* increased *Isll* expression (Fig. 4f). To further investigate whether CBP is involved in Wnt-promoted H3K9 acetylation on the *Isll* promoter, we overexpressed *cbp* from day 0 to day 4. ChIP assays demonstrated that CBP attenuated negative effects of Dkk1 and Frizzled on H3K9 acetylation on the *Isll* promoter (Fig. 4g, h). Therefore, our results document that Wnt-mediated acetylation of H3K9 on the *Isll* promoter is conducted by CBP which interacts with endogenous β -catenin.

Discussion

ISL1, as a marker of SHF progenitors, plays a crucial role in cardiomyocyte differentiation and proliferation [1]. ISL1⁺ CPCs give rise to cardiomyocytes, smooth muscle, and endothelial cell lineages in both mice and humans [3, 5]. *Isll* mutant mice die due to lethal cardiac defects. Studies of human populations demonstrate that genetic variants and specific haplotypes in the *ISL1* gene are associated with congenital heart disease [30, 31], partly via effecting activation of *Mef2c* [32]. Aside from roles in cardiac development, increasing evidence shows that ISL1⁺ cells give rise to adult resident cardiac stem cells. Studies of adult mice hearts find that ISL1⁺ progenitors persist into adulthood in mice and rats [33, 34]. Recent studies have been focused on the role of Isll after myocardial infarction. However, the results seem contradictory. In the spleen and left ventricle, *Isll* is re-expressed as a response to myocardial infarction, suggesting a potential role in cardiac repair [35]. Other studies show that ISL1⁺ cells are scarcely detected in the infarct zone after myocardial infarction, raising questions as to whether they contribute to cardiac repair [34]. To sum up, ISL1 is crucial in cardiac development, and may be a potential target for myocardial infarction treatment. However, many questions remain unanswered. To understand mechanisms that regulate *Isll* expression is to understand mechanisms that drive heart

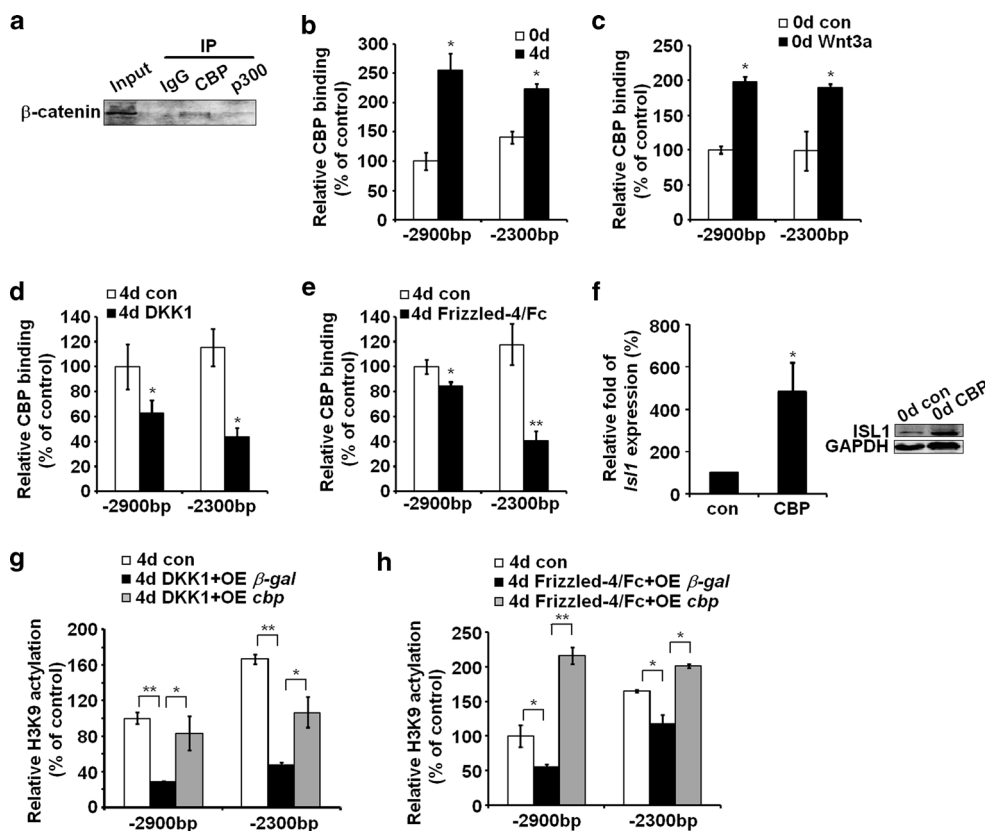


Fig. 4 The roles of CBP in Wnt-promoted acetylation of H3K9 on the *Isl1* promoter. **a** CoIP assay was performed with cell lysates from P19CL6 cells at day 4. Immunocomplex was precipitated with anti-CBP or p300 antibodies and then detected by anti- β -catenin antibody. 10 % of total cell lysates were used as an input. Normal IgG was used as a control antibody. **b–e** ChIP assay was carried out to detect CBP recruitment on two TCF/LEF1 binding sites on the *Isl1* promoter in P19CL6 cells at day 0 and day 4 (**b**), or treated with Wnt3a (100 ng/ml, 24 h) at day 0 (**c**), or exposure to DKK1 (100 ng/ml) (**d**) and Frizzled-4/Fc (100 ng/ml) (**e**) through day 0–4. The data for the 2900 bp binding site from P19CL6 cells uninduced or induced at day 4 without any treatment were used as controls (* $p < 0.05$, ** $p < 0.01$, vs the control). **f** Real-time RT-PCR and Western blot were performed to evaluate the expression of *Isl1* in P19CL6 cells transfected with CBP plasmids (* $p < 0.05$, vs the cells transfected with pcDNA3.1/ β -gal).

GAPDH was used as an internal control. **g–h** ChIP assay was conducted to analyze the H3K9 acetylation on two TCF/LEF1 binding sites on the *Isl1* promoter after treated with the inhibitors of canonical Wnt/ β -catenin signaling. P19CL6 cells overexpressing *cbp* were incubated with DKK1 (100 ng/ml) (**g**) or Frizzled-4/Fc (100 ng/ml) (**h**) through day 0–4. The cells were harvested at day 4. The DNA extractions were amplified using the primers that covered two TCF/LEF1 binding sites on the *Isl1* promoter (–2900 bp: from –2940 to –2630; –2300 bp: from –2341 to –2203) by real-time PCR. The data are normalized on the basis of the corresponding input control. The data for the 2900 bp binding site from P19CL6 cells induced at day 4 without any treatment were used as controls. Each bar represents mean \pm SD from three independent experiments, each in triplicate (* $p < 0.05$, ** $p < 0.01$, vs the cells transfected with pcDNA3.1/ β -gal).

development as well as to understand potential strategies for heart regeneration.

Wnt/ β -catenin signaling exerts developmental stage-specific effects on cardiac development by promoting cardiogenesis but inhibiting terminal cardiomyocyte differentiation [36]. Several transcription factors are regulated by Wnt/ β -catenin signaling during cardiac development, including BRY, NKX2.5, and GATA6 [37–39]. Aside from selective TCF/LEF1 occupancy, chromatin around TCF-bound states in Wnt-inducible promoters shows active histone marks, including DNA hypomethylation, H3 hyperacetylation, and H3K4 trimethylation [29]. Several epigenetic modifiers recruited by TCF/LEF1 have been identified to be involved in Wnt-regulated gene expressions.

For example, when Wnt/ β -catenin signaling is poised for activation, HDAC1 dissociates from LEF1 to facilitate binding of β -catenin to LEF1, indicating that repression by LEF1 requires HDAC activity [40]. In addition, recruitment of β -catenin with different acetyltransferases, including p300, CBP, and PCAF has been reported [41, 42].

In this study, we investigated mechanisms involved in Wnt-mediated *Isl1* expression during P19CL6 cardiomyocyte differentiation. We first confirmed that Wnt/ β -catenin molecules and *Isl1* exhibited similar expression pattern and Wnt-upregulated *Isl1* expression in early stages of cardiomyocyte differentiation. In addition, we found that expression of three cardiac-specific markers, *gata4*, *mef2c*, and *Troponin T*, which mark early, middle, and late stages

of cardiac differentiation, respectively, increased significantly following incubation of P19CL6 cells with Wnt3a. However, knocking down *Isl1* inhibited expression of all three markers to varying degrees, even when Wnt3a was added to P19CL6 cells prior to day 4. Addition of Wnt3a significantly increased the percentage of beating clones. However, in *Isl1*-deficient P19CL6 cells, no beating cardiomyocytes were observed. These results suggested that activation of Wnt/ β -catenin signaling in early stages promoted the generation of cardiomyocytes partly through *Isl1*.

Furthermore, we investigated mechanisms of Wnt-mediated *Isl1* expression and found a novel TCF/LEF1-binding site located 2300 bp upstream of the *Isl1* ATG. EMSA and luciferase assays showed that Wnt/ β -catenin signaling positively regulated transcription of *Isl1* by direct binding of β -catenin to the promoter. Before our study, another TCF/LEF1 binding site located 2900 bp upstream of the *Isl1* ATG had been reported [22]. Our ChIP assays of these two TCF/LEF1 binding sites revealed β -catenin binding during DMSO-induced P19CL6 cardiomyocyte differentiation. In addition, our data showed that Wnt/ β -catenin signaling promoted histone H3K9 acetylation on two TCF/LEF1 binding sites on the *Isl1* promoter, but no significant changes in H3K4 de- or tri-methylation, H3K9 trimethylation or H3 acetylation.

Two possible mechanisms might be involved in Wnt-mediated H3K9 acetylation. Wnt/ β -catenin signaling may activate HAT or facilitate HAT binding to target chromatin regions. Alternatively, Wnt/ β -catenin signaling may inhibit HDAC activity or the binding of HDAC proteins to chromatin. At first, we were inclined toward the second possibility, because Wnt/ β -catenin signaling promotes *Nkx2-5* expression via HDAC1 [38]. However, we failed to observe any changes in accumulation of HDAC1 on the *Isl1* promoter after P19CL6 cells were treated with Wnt3a, none did an acetyltransferase PCAF. Previous study reported interaction of β -catenin with two acetyltransferases coactivators, p300 and CBP, with diverse functions in murine embryonic stem cells [43]. In our study, we observed interaction between β -catenin and CBP and p300 at day 4. However, we failed to observe significant recruitment of p300 to the *Isl1* promoter at the same time. In contrast, CBP was significantly recruited to the two TCF/LEF1 binding sites on the *Isl1* promoter in P19CL6 induced at day 4. Furthermore, Wnt/ β -catenin signaling regulated recruitment of CBP to the *Isl1* promoter when P19CL6 cells treated with a Wnt activator or inhibitors. Overexpression of *cbp* promoted expression of *Isl1*, indicating the regulation of CBP to *Isl1*. These data suggested that Wnt/ β -catenin signaling promoted the recruitment of CBP to the *Isl1* promoter, as well as CBP-promoted H3K9 acetylation in the two TCF/LEF1 binding sites on the *Isl1* promoter. Additionally, overexpression of *cbp* partially rescued H3K9 hypoacetylation on

the *Isl1* promoter caused by DKK1 or Frizzled-4/Fc, indicating that Wnt/ β -catenin signaling promoted H3K9 acetylation on the *Isl1* promoter partly via CBP. Based on a previous study showing that interaction between β -catenin and CBP induces ES cell proliferation and interaction between β -catenin and p300 initiates a differentiation program [43], our data indicated that ISL1 might play a role in CPCs proliferation, which is consistent with the previous study [1]. Interestingly, we found that at day 4, on the 2300 bp TCF/LEF1 binding site, in most situations, both H3K9 acetylation and CBP recruitment are more than that on the 2900 bp binding site, indicating the possible preference between these two binding sites.

In conclusion, we found that Wnt/ β -catenin signaling regulates cardiomyocyte differentiation partially via *Isl1*. Wnt/ β -catenin signaling regulates *Isl1* expression through a novel TCF/LEF1 binding site and H3K9 acetylation on TCF/LEF1 binding sites on the *Isl1* promoter modulated by CBP. This study first reports that Wnt/ β -catenin-regulated H3K9 acetylation on promoters of its target genes and provides new insights into mechanisms of Wnt-mediated *Isl1* expression during cardiomyocyte differentiation.

Experimental procedures

Cell culture, induction of differentiation and reagents

P19CL6 cells were cultured and induced to cardiomyocytes by DMSO as described previously [38]. After induction for 14 days, the spontaneously contracting colonies were counted, and the percentage of beating colonies was calculated. At least, three independent experiments were performed. As activators of Wnt pathway, recombinant mouse Wnt3a (100 ng/ml, 24 h) (catalog no. 1324-WN) and LiCl (10 mM, 12 h) were purchased from R&D Systems, Inc., (Minneapolis, MN, USA) and Sigma-Aldrich Corporation (St. Louis, MO, USA). Frizzled-4/Fc (100 ng/ml) (catalog no. 194-fz) and DKK1 (100 ng/ml) (catalog no. 5897-DK/CF), as inhibitors of Wnt pathway, were purchased from R&D Systems, Inc., (Minneapolis, MN, USA). The concentrations of these reagents were optimized in our pilot studies (data not shown) and previous reports [28, 38].

RT-PCR and real-time PCR

Total RNA extraction from P19CL6 cells and real-time RT-PCR were performed as previously described [15]. To quantify each experiment, the C_t value was relatively quantified using *Gapdh* as an internal reference control. Transcript levels were normalized to the average expression levels of the controls or undifferentiated samples. Each value represented the average of at least three independent

Table 1 Primers used in real-time RT-PCR

Genes	Primer sequence (5'–3')	Product size (bp)	Annealing (°C)
<i>Isl1</i>	F: CTGCTTTTCAGCAACTGGTCA R: TAGGACTGGCTACCATGCTGT	128	60
<i>Gapdh</i>	F: GCACCACCAACTGCTTAGC R: TCTTCTGGGTGGCAGTGATG	105	60
<i>Mef2c</i>	F: CTGAGCGTGCTGTGCGACTGT R: GCTCTCGTGCGGCTCGTTGTA	132	60
<i>Gata4</i>	F: CACCCCAATCTCGATATGTTTGA R: GGTTGATGCCGTTTCATCTTGT	151	60
<i>Troponin T</i>	F: GGCAGAACCGCTGGCTGAA R: CTGCCACAGCTCCTTGGCCT	109	60

experiments, each in triplicate. The primers are listed in Table 1.

Western blot

Western blot was performed as previously described [15, 28]. Antibodies were used, including Islet1 (ab-109517, Abcam, Cambridge, MA, USA), Gata4 (sc-25310, Santa Cruz, CA, USA), GAPDH (ab-9484, Abcam, Hong Kong, China), β -catenin (sc-7199, Santa Cruz, CA, USA), Troponin T (ab-10214, Abcam, Cambridge, MA, USA), and horseradish peroxidase-conjugated secondary antibody from Santa Cruz.

Luciferase assay

The DNA fragment upstream the *Isl1* start codon was amplified and cloned into pGL3-basic vector (Promega). Primers were 5' primer 5'-AAGGTACCACATAGCAATCAACTAGGGGAGGAGGAGAAA-3' and 3' primer 5'-AACTCGAGAGAGGATGCTGGTGTGGCTAGG-3'. The QuikChange sited-directed mutagenesis kit (Stratagene) was used to make a point mutation in the conserved LEF1-binding site in the *Isl1* 5'-promoter region according to the manufacturer's protocol. The mutation primers were 5' primer 5'-GAAGAGTGAGGGTCAGGTGTGGTCTCAAGTC-3' and 3' primer 5'-GACTTGAGACCCACACCTGACCCTCACTCTTC-3'. Transfections were carried out in P19CL6 or HeLa cells using LipofectamineTM2000 (Invitrogen, Carlsbad, CA, USA). For luciferase reporter analysis, pcDNA3.1/ β -gal was used as a control for measuring transfection efficiency. Data are presented as fold activity over Renilla luciferase activity (relative activity). The data represent three independent experiments, each in triplicate. Each bar represents mean \pm SD from three samples.

Plasmids transfection and RNA interference

P19CL6 cells were transfected with plasmids or synthesized siRNA for gene overexpression or knockdown, respectively.

The PCG-LEF1-HA construct was provided by Dr. Klaus Wolff (Department of Experimental Dermatology at the University of Vienna, Austria). Constitutively active β -catenin was obtained from Dr. Roel Nusse (Stanford University School of Medicine, USA). CBP plasmid was kindly provided by Dr. Kendall Nettles (Department of Cancer Biology, the Scripps Research Institute Florida, USA). Non-silencer siRNA (sense: UUCUCCGAACGUGUCACGUTT; antisense: ACGUGACACGUUCGGAGAAT) and *Isl1* siRNA (sense: UCCUUCAUGAGCGCAUCUGTT; antisense: CAGAUGCGCUCAUGAAGGATT) were synthesized by Sigma. Transfection of the plasmids or siRNA was carried out by LipofectamineTM2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. After 48 h of transfection, cells transfected with the plasmids were harvested at indicated days of differentiation. For RNA interference, cells were transfected with the siRNA at day 0 and day 3, and harvested at indicated days. All the transfections were performed at least three times.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from HeLa cells. Oligonucleotides used in EMSA assay were labeled with [γ -³²P] ATP as described previously [44]. The sequences of the sense strand of these oligonucleotides were as follows. Wild-type *Isl1* probe: 5'-GAA GAG TGA GGG TCC TTT GTG GGT CTC AAG TCA C-3'; Mutant *Isl1* probe: 5'-GAA GAG TGA GGG TCA GGT GTG GGT CTC AAG TCA C-3'. Antibody analyses were performed by pre-incubation of nuclear protein extract with specific LEF1 antibody (sc-8591, Santa Cruz, CA, USA).

Chromatin immunoprecipitation (ChIP) assays

For ChIP experiments, extracts were prepared from P19CL6 cells treated as indicated, and the lysates were immunoprecipitated with nonspecific IgG, β -catenin (sc-7199, Santa Cruz, CA, USA), acetylated-H3K9 (ab-108112, Abcam,

Cambridge, MA), and CBP (sc-369, Santa Cruze, CA, USA) antibodies for 12 h at 4 °C. Immune complexes were incubated with Protein A/G-Sepharose CL-4B (Amersham Biosciences, Uppsala, Sweden) for 2 h at 4 °C. Protein-DNA cross-linking was reversed by overnight incubation at 65 °C. The precipitated DNA was amplified by real-time PCR for fragments of the *Isl1* promoter. The following PCR primers were used: primers P-2940 (5'-GCG CCA GGA ACT GTG CTC CAA-3') and P-2630 (5'-AGG GGC GAC CTC TTG TGT TCA ATG-3'); primers P-2341 (5'-GGT GAA TGC CTG TAT ATG TTT GGA-3') and P-2203 (5'-AAA TAG GGG ATC TGA ACC AAT TCT-3').

Co-immunoprecipitation (CoIP) assay

For CoIP experiments, the lysates derived from P19CL6 cells at day 4 were incubated with anti-CBP (sc-369, Santa Cruze, CA, USA) or p300 (RW-128, upstate Biotechnology, NY, USA) or normal IgG overnight at 4 °C, and then with 50 µl of Protein A/G-Sepharose CL-4B (Amersham Biosciences, Uppsala, Sweden) for 3 h at 4 °C. Immunoprecipitates were washed four times with wash buffer and subjected to SDS-PAGE electrophoresis, then analyzed by Western blot using anti-β-catenin (sc-7199, Santa Cruze, CA, USA) antibody.

Statistic analysis

The data were displayed as mean ± standard deviation (SD). Comparisons between groups were analyzed by Student's *t* test or ANOVA. The significance was analyzed with SPSS10.0 software, and a *p*-value <0.05 was considered to be statistically significant.

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Conflict of interest The authors declare no conflicts of interest.

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