Disruption of spatiotemporal hypoxic signaling causes congenital heart disease in mice

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Congenital heart disease (CHD) represents the most prevalent inborn anomaly. Only a minority of CHD cases are attributed to genetic causes, suggesting a major role of environmental factors. Nonphysiological hypoxia during early pregnancy induces CHD, but the underlying reasons are unknown. Here, we have demonstrated that cells in the mouse heart tube are hypoxic, while cardiac progenitor cells (CPCs) expressing islet 1 (ISL1) in the secondary heart field (SHF) are normoxic. In ISL1+ CPCs, induction of hypoxic responses caused CHD by repressing Isil and activating NK2 homeobox 5 (Nkx2.5), resulting in decreased cell proliferation and enhanced cardiomyocyte specification. We found that HIF1α formed a complex with the Notch effector hes family bHLH transcription factor 1 (HES1) and the protein deacetylase sirtuin 1 (SIRT1) at the Isil gene. This complex repressed Isil in the hypoxic heart tube or following induction of ectopic hypoxic responses. Subsequently, reduced Isil expression abrogated ISL1-dependent recruitment of histone deacetylases HDAC1/5, inhibiting Nkx2.5 expression. Inactivation of Sirt1 in ISL1+ CPCs blocked Isil suppression via the HIF1α/HES1/SIRT1 complex and prevented CHDs induced by pathological hypoxia. Our results indicate that spatial differences in oxygenation of the developing heart serve as signals to control CPC expansion and cardiac morphogenesis. We propose that physiological hypoxia coordinates homeostasis of CPCs, providing mechanistic explanations for some nongenetic causes of CHD.

Introduction
Cardiac morphogenesis is controlled by a complex morphogenetic program driven by lineage specification, proliferation, differentiation, and migration of cardiac progenitor cells (CPCs). Disruption of the molecular pathways regulating these processes leads to cardiac malformation and congenital heart disease (CHD) (1). Numerous mutations have been identified in familiar and spontaneous forms of CHD, but the majority of CHD cases, which amount to 6 to 8 newborns in every 1,000 live births, cannot be explained by monogenetic causes (2). It is generally believed that both environmental and genetic factors based on variations in many different genes contribute to CHD. Environmental or nongenetic risk factors include diabetes mellitus, obesity, and hypoxic responses, but the molecular events driving CHD have remained enigmatic (3–6). Small observational groups and potential confounding effects have complicated analysis of the contribution of specific environmental effects in human beings. Nevertheless, studies on human populations living at high altitude have associated increased prevalence of CHD with low oxygen levels (7, 8).

Adult multipotent stem/progenitor cells frequently occupy hypoxic niches and respond to low oxygen concentrations by either proliferation or differentiation (5, 9, 10), but studies on embryonic multipotent progenitor cells are critically missing. Currently, it is not known whether CPCs utilize signals that depend on oxygen availability during development. Such processes would most likely involve induction of HIFs, which is one of the foremost cellular reactions to low concentrations of oxygen (11–13). HIF1α has profound effects on different molecules regulating the behavior of stem and/or progenitor cells. Genetic inactivation of Hif1α during early but not late developmental stages causes CHDs, suggesting a critical role of hypoxia responses for normal heart development, although the precise mechanisms of the action of HIF1α and the definition of its targets in this context have not been worked out (4).

In the heart, 2 major populations of CPCs forming the first heart field (FHF) and the second heart field (SHF) drive early cardiac morphogenesis. CPCs of the FHF generate the left ventricle (LV) and parts of the inflow tract, while the right ventricle (RV), the atria, the outflow tract, and parts of the inflow tract are mainly derived from CPCs of the SHF (14, 15). The transcription factors NK2 homeobox 5 (Nkx2.5) and islet 1 (ISL1) play key roles in the complex network, which controls fate decisions and expansion of CPCs (16–18). Isil expression marks cells in the SHF with a trilineage potential (cardiomyocytes, endothelial cells, and smooth muscle cells), but Isil is also expressed broadly in the coelomic mesoderm, which harbors precursors of both FHF and SHF (19). Nkx2.5 is expressed in the cardiac mesoderm, the adjacent endodermal cells, and cells of the FHF, which have turned off Isil expression, indicating that the difference between the FHF and SHF lineages lies primarily in the timing of differentiation (20).

Hence, Nkx2.5/ISL1+ cells in the FHF and particularly in the linear heart tube might be seen as cells that have already acquired a more mature cardiomyocyte fate, a conclusion that is also supported by the direct role of Nkx2.5 in repression of progenitor genes (e.g., Isil and Fgf10) and the persistent signature of progenitor cell gene expression in the myocardium of Nkx2.5 mutants (19, 21, 22).
IsIl transcription is dynamically regulated in CPCs and becomes silenced when CPCs are incorporated into the heart tube where oxygen concentrations are low (23, 24). Yet nothing is known about the role of hypoxia signaling for IsIl and Nkx2.5 activity, which determines CPC homeostasis and lineage specification during early cardiogenesis.

In this study, we investigated the role of hypoxia signaling for the regulation of CPCs and the potential crosstalk between IsIl and Nkx2.5 in this context. We found that IsIl+ CPCs in the SHF are less hypoxic (hereafter referred to as normoxic) compared with the primary linear heart tube. We determined that O2 availability influences the fate of IsIl+ CPCs by controlling IsIl expression and thereby IsIl1-dependent site-specific recruitment of HDAC1/5, which is required for Nkx2.5 silencing, allowing expansion of IsIl+ CPCs. Experimental induction of hypoxia responses during early heart development suppressed proliferation of IsIl+ CPCs and promoted lineage specification to cardiomyocytes. Our study decodes the molecular network that transmits hypoxia responses by identifying SIRT1 and its family bHLH transcription factor 1 (HESI) as critical components of a repressor complex mediating HIF1α-dependent silencing of IsIl. We provide insights into the molecular mechanism leading to CHDs and demonstrate that inactivation of Sirt1 in IsIl+ CPCs prevents hypoxia-induced cardiac malformations.

Results
Experimental induction of hypoxia responses leads to multiple cardiac defects. Physiological hypoxia (oxygen concentration <2%) and activation of HIF1α play critical roles for cardiac morphogenesis and function, and but is not clear whether hypoxia signaling is equally important for all parts of the developing heart (25). We therefore monitored the spatial distribution of pimonidazole, a nitroimidazole derivative that incorporates into hypoxic cells when oxygen concentration is below 10 mmHg (<2%) in embryonic mouse hearts between E8.0 and E9.5. Hypoxic cells were mainly localized in the myocardium of the looping heart tube as described previously (25). Surprisingly, however, CPCs in the cardiac mesoderm and the outflow tract, which we defined by expression of IsIl, showed negligible incorporation of pimonidazole, indicating that IsIl+ CPCs are maintained in a nonhypoxic (normoxic) environment (Figure 1A). The spatially distinct distribution of hypoxic cells in the developing heart should invoke hypoxic signaling indicated by HIF1α stabilization in the heart tube, but not in IsIl+ CPCs located in the cardiac mesoderm and the outflow tract. Hence, we separated the cardiac mesoderm and the outflow tract from the heart tube. Western blot analysis revealed lower levels of HIF1α in cardiac mesoderm, including the adjacent outflow/inflow tracts compared with the heart tube, confirming our hypothesis (Figure 1B).

To analyze whether induction of hypoxia responses in physiologically normoxic IsIl+ cells affects cardiac morphogenesis, we treated pregnant mice carrying E7.5 embryos with cobalt chloride (CoCl2), which elicits hypoxia-like responses (Figure 1, C and D). Morphological analysis of E15.5 embryos treated with CoCl2 (n = 22) at 15 mg/kg i.p. revealed multiple cardiac defects, such as thinner compact myocardium, ventricle septum defects (VSD), overriding aorta (OA), and RV dilation. Increased concentration of CoCl2 (30 mg/kg i.p.) resulted in more severe defects, such as double-outlet RV, persistent truncus arteriosus, and RV hypoplasia (Figure 1E), although such malformations were not seen in all embryos (n = 22) (Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/JCI88725DS1). We concluded that IsIl+ cells require a normoxic niche to contribute normally to cardiogenesis.

Hypoxia responses alter IsIl+ cell homeostatic control and lead to cardiac malformation. To investigate whether hypoxia responses have a direct impact on the expression of critical CPC regulators, we first performed a quantitative reverse transcription PCR (RT-qPCR) analysis of E9.0 hearts (including the adjacent mesoderm) after CoCl2 treatment. Interestingly, we observed a decrease of IsIl expression, the master regulator of the SHF. In contrast, expression of several factors characteristic for the FHF increased as, e.g., Hand1 and Nkx2.5, which are expressed in the FHF and at lower levels in the SHF (Figure 2A). To validate these findings and to identify local changes in expression levels, we performed in situ hybridization as well as Western blot analysis of dissected cardiac mesoderm after induction of hypoxia responses. Expression of IsIl was reduced in the foregut endoderm and cardiac mesoderm of E9.5 embryos after CoCl2 treatment (Figure 2, B and C, and Supplemental Figure 1A). In contrast, expression of Nkx2.5 was upregulated in the cardiac mesoderm (Figure 2, B and C), indicating that experimental induction of hypoxia responses inhibits IsIl but increases Nkx2.5 expression in the cardiac mesoderm. Additional antibody staining of whole-mount preparations followed by cryosections further confirmed the downregulation of IsIl, but upregulation of Nkx2.5, in the cardiac mesoderm after induction of hypoxia responses (Supplemental Figure 1B).

To exclude any potential artifacts due to chemical induction of hypoxia responses, we repeated the experiments with E7.5 embryos from pregnant mice housed in a hypoxia chamber at 10% oxygen and with isolated CPCs kept at 1% O2 (Supplemental Figure 1, C and F). Analysis of IsIl and Nkx2.5 mRNA levels by whole-mount in situ hybridization or RT-qPCR yielded results similar to those after CoCl2 treatment, confirming that hypoxia responses repress IsIl, but promote Nkx2.5, transcription (Supplemental Figure 1, D–G).

Since O2 levels have a profound effect on stem/progenitor cell niches and modulate cell-fate decisions, we analyzed proliferation and apoptosis of IsIl+ cells in CoCl2-treated embryos at E9.5. Induction of hypoxia responses significantly reduced the numbers of IsIl1/pH3 double-positive CPCs, but not of Nkx2.5/pH3 double-positive cells, relative to mock-treated embryos (Figure 2D and Supplemental Figure 1H), whereas no differences in apoptotic cells were observed (Figure 2E and Supplemental Figure 1I). Similarly, ex vivo culture of FACS-isolated IsIl+ CPCs from E8.0 IsIl1<sup>CreERT2</sup> embryos under 1% O2 (Supplemental Figure 2, A–E) reduced the number of IsIl1/Ki67 double-positive cells (Supplemental Figure 2F).

The observation that hypoxia attenuates IsIl but enhances Nkx2.5 expression in cardiac mesoderm raised the intriguing possibility that induction of hypoxia inhibits proliferation of IsIl+ cells at the expense of enhanced cardiomyocyte specification. Indeed, ex vivo culture of freshly isolated IsIl+ cells revealed a striking increase of IsIl1 Nkx2.5<sup>+</sup> cells under hypoxia (63% of the cell population) compared with normoxia conditions (19% of the cell pop-
In contrast, the relative amounts of ISL1+NKX2.5– and ISL1+NKX2.5+ cells declined from 62% under normoxia to 35% under hypoxia and from 19% under normoxia to 2% under hypoxia, respectively, indicating increased commitment of ISL1+ CPCs to the cardiomyocyte lineage (Figure 2F and Supplemental Figure 2G).

We could rule out that the higher numbers of NKX2.5+ cells were caused by augmented proliferation of already committed CPCs, since Nkx2.5 expression was nearly absent in freshly isolated ISL1+ CPCs (Supplemental Figure 2E). Instead, high expression of Nkx2.5 was detected in CPCs that had turned off Isl1 expression, suggesting that Nkx2.5 expression increases after inhibition of Isl1 (Supplemental Figure 2E).

So far, our experiments essentially suggested that hypoxia results in a “switch” of ISL1+ cells to NKX2.5+ cells, eventually resulting in cardiac malformations. To directly address this possibility and to investigate whether untimely expression of Nkx2.5 in ISL1+ cells recapitulates hypoxia-induced cardiac defects, we inserted the Nkx2.5 cDNA into the Rosa26 locus behind a loxP-stop cassette (Supplemental Figure 3A). Activation of Nkx2.5 expression in ISL1+ cells using Isl1-Cre mice (Isl1-Cre+ Rosa26Nkx2.5 mice) (Supplemental Figure 3, B and C) caused obvious heart malformations (i.e., shortened outflow tract, cardiac looping defects) in all double-heterozygous animals at E9.5 and E10.5 (Figure 2H). The majority of Isl1-Cre+ Rosa26Nkx2.5 embryos died around E11, although a few survived until E15.5 (Figure 2G), showing cardiac malformations similar to those seen after induction of hypoxia responses, including thinner compact myocardium.
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The Journal of Clinical Investigation

Figure 2. Experimental induction of hypoxia alters the expression of Isl1 and Nkx2.5. (A) RT-qPCR analysis ofIsl1 (n = 8), Nkx2.5 (n = 8), Flik1 (n = 7), Hand1 (n = 6), Hand2 (n = 6), and Tbx5 (n = 6) expression in E9.5 embryonic hearts and the adjacent mesoderm (20–21 somites) after chemical induction of hypoxia responses. PBS-injected mice were used as control. The m3Δ44 gene was used as a reference for normalization. **P < 0.01; ***P < 0.001, t test. (B) In situ hybridization of C57BL/6 E9.25 embryos (18 somites) for either Isl1 mRNA (upper panel) or Nkx2.5 mRNA (lower panel) after chemical induction of hypoxia responses (30 mg CoCl2/kg body weight). Arrows indicate the Nkx2.5+ or Isl1+ cardiogenic region. Representative images from 2 independent experiments are shown. (C) Western blot analysis of Isl1, Nkx2.5, and sarcomeric α-actinin levels in cardiac mesoderm containing adjacent outflow tract isolated from E9.5 embryos with or without CoCl2 treatment. α-Tubulin was used as protein-loading control. Two separate experiments (3 individual litters, per experiments) were analyzed, yielding similar results. (D) Analysis ofIsl1+ cell proliferation in mock (n = 4) or CoCl2-treated (n = 4) E9.5 embryos by immunostaining for ISL1 and phospho-histone H3 (Ser10) (pH3). The percentages of pH3/ISL1 double-positive cells in cardiac mesoderm are shown. At least 6 sections from each embryo were counted. **P < 0.01, ANOVA with Dunnett’s post hoc correction. (E) TUNEL assay of ISL1+ cells (thereafter referred to as Sirt1) and Nkx2.5+ cells after FACS-based cell sorting of ISL1+ cells from E8.0 ISL1nGFP/+ embryos and 2-day cultivation of isolated cells under either normoxic or hypoxic conditions. Quantification of different cell populations was achieved by counting all immunostained cells in a multwell dish. *P < 0.05; **P < 0.001, t test (n = 3). (F) Immunofluorescence-based quantification of Isl1-Nkx2.5+, Isl1′Nkx2.5+, and Isl1′Nkx2.5− cells after FACS-based cell sorting of Isl1− cells from C57BL/6 E9.25 embryos. Arrows indicate the Nkx2.5+ or Isl1+ cardiogenic region. Scale bars: 200 μm.

SIRT1 might form a ternary complex with HIF1α to repress the Isl1 promoter in CPCs during hypoxia. Western blot and co-IP experiments revealed that hypoxia increased protein levels and promoted formation of a complex containing SIRT1, HESI, and HIF1α, as indicated by IP with either SIRT1 or HIF1α antibodies (Figure 3B and Supplemental Figure 4C). In addition, ChIP experiments indicated strongly increased binding of SIRT1 and HESI to the Isl1 proximal promoter harboring the N-box motif in CPCs under hypoxia conditions (Figure 3C and Supplemental Figure 4, D–G), which was significantly decreased under hypoxia when HIF1α was depleted by shRNAs (Figure 3D and Supplemental Figure 4H). Similarly, depletion of HES1 by shRNAs prevented binding of SIRT1 to the Isl1 promoter, suggesting that HESI recruits SIRT1 to the Isl1 promoter to repress transcription during hypoxia (Figure 3E and Supplemental Figure 5A).

To gain further evidence for the role of HES1 and SIRT1 in mediating hypoxia-dependent gene repression, we mutated the N-box within the Isl1 promoter, which increased activity of an Isl1-luciferase construct and prevented Isl1-promoter repression by hypoxia (Supplemental Figure 5B). Furthermore, inactivation of SIRT1 in proliferating CPCs either by shRNA knockdown or by treatment with the SIRT1-specific inhibitor Ex527 increased Isl1 gene expression (Figure 3F and Supplemental Figure 5, C and D) as well as the number of Isl1+ cells (Figure 3G), but suppressed other cardiac-specific genes, including Nkx2.5 and Myh7 (Figure 3F and Supplemental Figure 5, C and D). Accordingly, we found that SIRT1 binds specifically to the Isl1 but not to the Nkx2.5 promoter (Figure 3H and Supplemental Figure 4F) and that Sirt1 knockout abrogates hypoxia-mediated silencing of the Isl1 promoter (Supplemental Figure 5E). Enzymatically inactive SIRT1 (SIRT1H633Y) did not repress Isl1 promoter activity (Figure 3I), and induction of hypoxia responses decreased H3K9 and H4K16 acetylation at the Isl1 promoter in CPCs, indicating that SIRT1-mediated hypocacylation leads to Isl1 gene silencing (Figure 3J).

Interestingly, hypoxia responses did not change Sirt1 mRNA and NAD+ concentrations (Supplemental Figure 6, A–C), but caused accumulation of ROS and activation of JNK, which results in enhanced activity of SIRT1 (31) reflected by deacetylation of histone H3K9 (Supplemental Figure 6, D and E). We concluded that hypoxia-induced Isl1 gene silencing is mediated by HIF1α/HESI-dependent recruitment of SIRT1 specifically to the Isl1 promoter. Increased SIRT1 activity induced by activation of JNK might also contribute to Isl1 gene silencing after hypoxia, although we cannot exclude a mere association. This mechanism allows inverse transcriptional regulation of Isl1 and Nkx2.5 by HIF1α following differential recruitment of cofactors such as HES1 and SIRT1.

Inactivation of Sirt1 in Isl1+ cells enhances Isl1 expression and rescues hypoxia-induced CHDs. To elucidate the physiological role of SIRT1 for silencing Isl1 expression in Isl1+ cells during hypoxia, we deleted exon 4, which encodes the conserved SIRT1 catalytic domain in mice. Consistent with a previous study (32), germline Sirt1−/− mutants showed multiple CHDs (Supplemental Figure 7, A–C). In addition, we found a strong upregulation of Isl1 mRNA in E8.0 Sirt1−/− embryos (5-somite stage) (Supplemental Figure 7D). We next specifically inactivated Sirt1 in Isl1+ cells using Isl1-Cre mice and tagged the Isl1+ lineage with a Rosa26YFP reporter (thereafter referred to as Sirt1−/−Isl1-Cre− RosaYFP+) (Supplemental Figure 7E).
Sirt1fl/– Isl1-Cre+ RosaYFP+ mice were viable and fertile, but exhibited slightly impaired RV function and reduced body weight (data not shown). In contrast with germline Sirt1 mutants, we did not detect major cardiac malformations, although we noted a thinner myocardial compact layer (Supplemental Table 2). The rather minor phenotype of Sirt1 fl/– Isl1-Cre+ compared with germline Sirt1 mutants might indicate that the loss of Sirt1 can be mostly compensated in the SHF, thereby preventing CHDs, but not in other parts of the developing heart, such as FHF or the cardiac neural crest. Importantly, inactivation of Sirt1 in ISL1+ cells increased Isl1 expression, reduced Nkx2.5 expression, and generated more ISL1+ cells (Figure 4, A and B, and Supplemental Figure 8C), validating the results of our in vitro analysis.

Since experimental hypoxia causes CHDs and SIRT1 mediates repression of Isl1 transcription in response to hypoxia, we hypothesized that reduced expression of Sirt1 might rescue the adverse effects of acute hypoxia episodes during pregnancy. Induction of hypoxia responses by injection of 15 mg/kg body
weight CoCl₂ at E7.5 caused CHDs (e.g., VSD, OA) in approximately 75% of Sirt1 fl/+ Isl1-Cre– and C57BL/6 control hearts, which increased to nearly 100% the incidence of CHD when a CoCl₂ dosage of 30 mg/kg body weight was used (Figure 4C and Supplemental Table 1). In stark contrast, mutant mice receiving the same treatment, but lacking one (Sirt1 fl/– Isl1-Cre–, Sirt1 fl/+ Isl1-Cre+) or both alleles of Sirt1 (Sirt1 fl/– Isl1-Cre+) showed no signs of CHD, such as thinner myocardium, VSD, smaller RV with shortened outflow tract, or malrotation of the outflow tract (Figure 4, C–E), suggesting that reduction of Sirt1 expression is sufficient to rescue the cardiac defects. To investigate whether the prevention of cardiac malformations caused by induction of hypoxia responses in Sirt1 fl/– Isl1-Cre–, Sirt1 fl/+ Isl1-Cre+, and Sirt1 fl/– Isl1-Cre+ embryos correlated with increased expression of Isl1, we compared Isl1 levels in WT and mutant embryos after CoCl₂ treatment. Immunofluorescence staining revealed a strong attenuation of ISL1, but not NKX2.5, expression in WT embryos after induction of hypoxia responses (Figure 5, A and B). Importantly, however, inactivation of Sirt1 in the SHF normalized expression of Isl1 (Figure 5, A and B) and restored proliferation of ISL1+ cells (Figure 5C), emphasizing the critical role of SIRT1 in mediating Isl1 suppression during hypoxia responses.

ISL1 forms a complex with HDAC1/HDAC5 and silences Nkx2.5 gene expression. Prompted by the inverse transcriptional regulation of Isl1 and Nkx2.5 in CPCs, we searched for a putative ISL1-binding site in the Nkx2.5 gene. ChIP disclosed binding of ISL1 to a site located in close proximity to the HRE motif at position −9040/−8859 (Figure 6A), which might be used by ISL1 to recruit a corepressor. To identify such a potential corepressor, we treated ISL1+ cells with inhibitors specific for class I and IIa HDACs, which dramatically increased the number of NKX2.5+ cells during differentiation, whereas treatment of CPCs with Ex527, a class III inhibitor that increases Isl1 expression, had the opposite effect (Figure 6B). Protein co-IP assays revealed that ISL1 interacts with

Figure 4. Inactivation of Sirt1 in ISL1+ cells increases the number of ISL1+ cells and rescues hypoxia-induced CHDs. (A) FACS analysis of ISL1+ cells and their derivatives in E9.5 embryonic hearts (20–24 somites) after Sirt1 inactivation. * P < 0.05, t test (n = 3). (B) Quantification of ISL1+NKX2.5+ cells in the cardiac mesoderm by immunostaining of E9.5 embryos (20–24 somites). *** P < 0.001, t test (n = 3). (C) H&E staining of E15.5 hearts of control (Sirt1 WT), Sirt1 fl/– Isl1-Cre– (Sirt1 hypomorphic), and Sirt1 fl/+ Isl1-Cre+ (Sirt1 mutant) embryos after chemical induction of hypoxia responses (15 mg CoCl₂/kg body weight) at E7.5. Note the rescue of CHDs in Sirt1 fl/– Isl1-Cre–, Sirt1 fl/+ Isl1-Cre+, and Sirt1 fl/– Isl1-Cre+ embryos compared with controls. Twenty-seven embryos from 4 litters, including 8 Sirt1 fl/– Isl1-Cre+ embryos, were analyzed. Numbers of specific CHDs are listed in Supplemental Table 2. Scale bars: 200 μm. (D) Whole-mount views of E9.5 embryos (upper panels) and E15.5 embryonic hearts (lower panels) without and with chemical induction of hypoxia responses (30 mg CoCl₂/kg body weight). For each time point, 1 litter was analyzed. Numbers of analyzed embryos for each condition are indicated in the figure. Representative images are shown. Inactivation of Sirt1 in the SHF ameliorates CHDs. OFT, outflow tract. Scale bars: 100 μm. (E) H&E staining of severe cardiac malformations in control, but not in Sirt1 fl/– Isl1-Cre+, embryos at E15.5 after chemical induction of hypoxia responses (30 mg CoCl₂/kg body weight). Arrows point to individual cardiac defects named in the figure. Scale bars: 100 μm.
activities. In fact, inhibition of SIRT1 enhanced binding of ISL1 in ChIP experiments, but prevented binding of HIF1α (Figure 6G), strongly arguing for a decisive role of the ISL1/HDAC1/HDAC5 complex in \(Nkx2.5\) gene repression. 

**Discussion**

The effect of oxygen availability varies depending on the cell type. Stem/progenitor cell populations usually show enhanced proliferation or self-renewal and suppression of differentiation under hypoxia conditions (5, 9, 10). Here, we describe an unexpected scenario, in which ISL1 + CPCs proliferate in a nonhypoxia niche, but cease proliferation and succumb to precocious myocyte specification when exposed to a hypoxic environment. Challenge of the nonhypoxic niche by experimental hypoxia causes CHD and disrupts the inverse transcriptional control of \(I_{sl1}\) and \(Nkx2.5\), two key cardiac transcription factors that are both direct targets of HIF1α. We demonstrate that HIF1α, which is a well-known transcriptional activator, represses \(I_{sl1}\) by recruitment of SIRT1, thereby inhibit-
The temporal requirement of hypoxia signaling is interwoven with a precise spatial control determined by the existence of highly hypoxic areas within the developing embryo. We found that under physiological conditions, the SHF is considerably less hypoxic when compared with the primary heart tube, which allows proper expansion of CPCs and explains why nonphysiological shortage of oxygen at the wrong location and time causes CHDs (6). Once the contribution of the SHF has expired, transient pathological hypoxia has less severe effects. This conclusion fits well with the remarkable ability of fetal hearts to cope with hypoxia due to the adaptation of fetal cardiomyocytes to low oxygen levels (34).

The histone deacetylase SIRT1 is well known as conferring resistance to metabolic and hypoxia stress by deacetylating key signaling molecules controlling cell metabolism, survival, and proliferation (35). Lack of Sirt1 in the cardiovascular system renders Sirt1 mutants more susceptible to cell death induced by ischemia/reperfusion injury, but has only minor effects at baseline conditions (36–38). In the developing embryo, SIRT1 also mediates responses to hypoxia, but serves a different purpose, not only orchestrating stress responses, but also cellular fate decisions. The reason for this being proliferation of ISL1+ CPCs and inducing myocyte specification. Prevention of precocious myocyte specification enables self-renewal of ISL1+ cells, which requires formation of an ISL1/HDAC1/HDAC5-containing coressor complex that restricts Nkx2.5 expression. Correspondingly, directed expression of Nkx2.5 in ISL1+ cells recapitulated several aspects of nonphysiological hypoxia, including formation of CHDs, thereby validating our model (Figure 7). According to our model, Nkx2.5 is “on” in the presence of SIRT1 under pathological hypoxic conditions becauseIsl1 is “off” due to the repressive activity of the HIF1α/HES1/SIRT1 complex that prevents ISL1/HDAC-mediated inhibition of Nkx2.5. Nkx2.5 is “off” in the absence of SIRT1 under pathological hypoxic conditions, because Isl1 is “on” due to disruption of the repressive HIF1α/HES1/SIRT1 complex. In this setting, the continued expression of Isl1 allows the ISL1/HDAC1/5 complex to turn Nkx2.5 “off.”

Previous studies demonstrated differential temporal requirements of hypoxia signaling responses for normal cardiac morphogenesis (25). Notably, inactivation of Hif1α, one of the main transducers of hypoxia signaling, causes septation and conotruncal heart defects after inactivation at E10.5, but not at E13.5 (4). The temporal requirement of hypoxia signaling is interwoven with a precise spatial control determined by the existence of highly hypoxic areas within the developing embryo. We found that under physiological conditions, the SHF is considerably less hypoxic when compared with the primary heart tube, which allows proper expansion of CPCs and explains why nonphysiological shortage of oxygen at the wrong location and time causes CHDs (6). Once the contribution of the SHF has expired, transient pathological hypoxia has less severe effects. This conclusion fits well with the remarkable ability of fetal hearts to cope with hypoxia due to the adaptation of fetal cardiomyocytes to low oxygen levels (34).

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Previous studies demonstrated differential temporal requirements of hypoxia signaling responses for normal cardiac morphogenesis (25). Notably, inactivation of Hif1α, one of the main transducers of hypoxia signaling, causes septation and conotruncal heart defects after inactivation at E10.5, but not at E13.5 (4).
distinction probably lies in the requirement of physiological hypoxia for embryonic heart development, while hypoxia is an unfavorable condition in most adult organs. Interestingly, expression of Sirt1 in the heart declines dramatically between E12.5 and E13.5, when Isl1 expression is turned off and the newly formed cardiac vasculature increases oxygen supply (our unpublished data and ref. 39). The disappearance of hypoxic areas in the heart and the decline of Sirt1 expression might mark a transition of the function of SIRT1 from a morphogen to a stress-response gene that serves different functions, but employs similar mechanisms. This dual function of SIRT1 might also partially explain the phenotypic differences between germline Sirt1 and hypoxia, transcription factors, and epigenetic modifiers during early heart development. ISL1+ CPCs maintain high Isl1 expression directed by transcription activators such as β-catenin/LEF, FOXO1, and GATA4 in response to inductive signals, but low Nkx2.5 expression levels in the cardiac mesoderm, where O2 concentrations are relatively high, favoring self-renewal and expansion. ISL1 recruits HDACs to the Nkx2.5 promoter in CPCs to repress Nkx2.5 expression and prevent precocious myocyte specification. Migration of ISL1+ cells into the growing physiologically hypoxic (O2 ≤2%) heart tube results in HIF1α-SIRT1-HEs1-dependent silencing of Isl1 expression and HIF1α-p300-dependent stimulation of Nkx2.5 expression, promoting cardiomyocyte specification. In the absence of SIRT1, repression of Isl1 expression is relieved, resulting in increased Isl1 expression directed by transcription activators such as β-catenin/LEF, FOXO1, and GATA4 in response to inductive signals, but low Nkx2.5 expression levels and enhanced proliferation of CPCs. As a consequence, ISL1-mediated Nkx2.5 repression is augmented and the transition of CPCs from an ISL1+Nkx2.5+ to an ISL1–Nkx2.5+ state is hindered, compromising cardiomyocyte specification. TF, transcription factors.

During heart development, SIRT1 closely interacts with HIF1α to control expansion of ISL1+ CPCs. So far, SIRT1 has been described as an amplifier of HIF1α activity during hypoxia (40–42), deacetylating and stabilizing HIF1α, thereby leading to enhanced hypoxia responses. Here, we demonstrate that SIRT1 also functions as a negative coregulator of HIF1α, which silences Isl1 expression of hypoxia deacetylation of histones. In addition, SIRT1 might also deacetylate HIF1α, thereby boosting the effects of hypoxia on Isl1 gene silencing, although this remains to be shown. HIF1α itself has been mainly described as a transcriptional activator forming a complex with the HAT p300. Hence, it was intriguing that interaction of HIF1α with the HES1/SIRT1 complex converted HIF1α from an activator into a repressor. This scenario resembles increased binding of SIRT1 to MASH1 in adult neuronal stem cells during oxidative stress and hence might indicate a general principle (29). Repression of Isl1 expression might not rely solely on hypoxia responses, since inactivation of Nkx2.5 causes persistent expression of Isl1 in the forming heart tube (19). Furthermore, a recent finding demonstrated direct repression of Isl1 by Nkx2.5 for proper development of the ventricular myocardium (21). However, we assume that hypoxia is the decisive initial step to silencing Isl1 expression in SHF CPCs migrating into the linear heart tube, which relieves suppression of Nkx2.5 by Isl1, subsequently enabling repression of Isl1 by Nkx2.5.

Various combinations of cardiac transcription factors drive development of the SHF (14, 15), in which ISL1 plays a central role. Similarly to what occurs in Nkx2.5, mutations in the human Isl1 gene are associated with a diverse range of cardiac malformations (43–45). Therefore, it is not surprising that a complex net-
work rules the activity of Isl1 (46, 47), to which we added a decisive component. We demonstrated that Isl1 is repressed by hypoxia responses through HES1/HIF1α-dependent recruitment of SIRT1 to the Isl1 gene and by activation of Sirt1. Inhibition of Isl1 expression occurs when ISL1+ CPCs migrate from the normoxic cardiac mesoderm to the hypoxic heart tube, where Isl1 gene expression is low, but Nkx2.5 expression is high.

The presence of an ISL1-binding site in the Nkx2.5 gene close to the HRE establishes another regulatory layer. The high level of Isl1 expression in normoxic conditions attenuates Nkx2.5 gene expression, which is required for preventing precocious myocyte specification of CPCs. Suppression of Nkx2.5 is best achieved by a factor that defines the SHF, such as Isl1. Since Sirt1 does not get activated in the normoxic SHF and the regulatory region of the Nkx2.5 lacks an N-box next to the HRE element, which is required for recruitment of SIRT1, other HDACs, such as HDAC1/HDAC5, are necessary to suppress Nkx2.5 gene expression. The direct regulation of Nkx2.5 by ISL1 and HDAC1/HDAC5 represents what we believe to be a novel facet of SHF development and reflects the necessity of a proper balance of Isl1 and Nkx2.5 levels to control proliferation and differentiation of CPCs (22). Exacerbation of hypoxic responses under pathological or experimental conditions disrupts this balance and results in a “switch” of ISL1 to Nkx2.5 cells due to SIRT1-dependent downregulation of Isl1, which relieves repression of Nkx2.5. To prove the validity of this model, we expressed Nkx2.5 specifically in ISL1+ cells, which essentially recapitulated many hypoxia-induced heart defects. However, it should be mentioned that not all severe heart defects seen in hypoxia-treated embryos were apparent in Isl1-Cre+ Rosa26^{Nkx2.5} embryos at E15.5. We assume that this phenomenon is caused by early embryonic lethality, since the number of Isl1-Cre+ Rosa26^{Nkx2.5} embryos at E15.5 was substantially lower than the expected Mendelian ratio.

Taken together, our data support a model in which hypoxia signaling–induced crosstalk between transcription factors and epigenetic modulation determines the fate of ISL1+ cells during early cardiogenesis and prevents CHD. We demonstrate that hypoxia and SIRT1 tie Isl1 and Nkx2.5 into a negative regulatory loop that coordinates expansion of SHF CPCs and ensures proper acquisition of myocyte subtype identity, thereby shaping the heart.

Methods

**Animals.** Sirt1-floxed mice were generated by flanking exon 4 with loxP sites (Supplemental Figure 6A). Sirt1-floxed mice were crossed to the CMV-Cre deleter strain (The Jackson Laboratories) to obtain heterozygous Sirt1 mutant mice (Sirt1<sup>+/−</sup>). Rosa26YFP mice were obtained from Frank Constantini (Columbia University, New York, New York, USA) (48). Rosa26<sup>Nkx2.5</sup> mice were generated by insertion of an HA-NKX2.5cDNA-V5 PCR fragment into the Rosa26 locus by homologous recombination in V6.5 embryonic stem (ES) cells obtained from Rudolph Jaenisch (Whitehead Institute for Biomedical Research, Cambridge, Massachusetts, USA) (49) (Supplemental Figure 3A). Isl1-Cre transgenic mice were provided by Sylvia Evans (UCSD, San Diego, California, USA) (50). Isl1<sup>loxP/loxP</sup> mice (Supplemental Figure 2A) were generated by the dRMEC method using Isl1<sup>loxP/loxP</sup> ES (RI ES cell line) cells obtained from EUCOMM (51). All mouse strains were backcrossed and maintained on a C57BL/6 genetic background. C57BL/6 mice were obtained from Charles River Laboratories. Primers used for genotyping are listed in Supplemental Table 3. To induce hypoxic responses, pregnant females were placed in their home cages in a hypoxia chamber with 10% O<sub>2</sub>/90% N<sub>2</sub>. Oxygen in the chamber was measured using an oxygen analyzer (Engineered Systems and Designs). During hypoxia, humidity and temperature were monitored in accordance with Max Planck Institute Institutional Animal Care and Use Committee procedures. Gas flow was maintained at 0.1-0.21 per minute. For chemical induction of hypoxic responses, CoCl<sub>2</sub> (Sigma-Aldrich) was administered intraperitoneally at 15 or 30 mg/kg body weight per injection. Pimodidazole was administered intraperitoneally at 60 mg/kg body weight per injection 90 minutes before analysis. Each time point or treatment modality was covered by analysis of at least 2 randomly selected embryos from at least 2 litters.

**Plasmids.** pcDNA3-Flag-Isl1 and pcDNA3-Myc-Isl1 were described previously (46); pcDNA3-Flag-HDAC9 was described previously (52); pcDNA-Flag-HDAC5 was purchased from Addgene (catalog 33209); pcDNA3-Myc-HDAC1, pcDNA3-Myc-HDAC4, pcDNA3-Flag-SIRT1, and pcDNA3-Flag-SIRT1H633Y have been described elsewhere (53, 54). The Isl1 promoter was cloned from WT mouse genomic DNA (C57BL/6) into pGEM-T Easy Vector (Promega) and subsequently subcloned in pTA-Luciferase plasmid (Invitrogen). The primers used for cloning are listed in Supplemental Table 3.

**Embryo isolation, cell sorting, and FACs analysis.** For isolation of embryonic ISL1+ CPCs (ISL1<sup>loxP/loxP</sup>−) and cells derived from the ISL1+ lineage (ISL1-Cre Rosa2FP), E8.0 (5 pairs of somites) and E9.0 (14 pairs of somites) embryos or individual embryonic hearts from E15.5 embryos were dissected and dissociated into single cells as described previously (55). ISL1<sup>−/−</sup> or Nkx2.5<sup>−/−</sup> CPCs were isolated from embryoid bodies (EB) by treatment with 0.05% trypsin/EDTA for 3 minutes at 37°C. Dissociated cells were filtered through a 40-μm cell strainer and fluorescence sorted using a FACSariaIII (BD Biosciences). Sorted ISL1<sup>+</sup> CPCs were further cultured within differentiation or proliferation medium on cardiac fibroblast feeder cells as described (56). For FACs analysis, cells were fixed in methanol followed by washing (PBS) and blocking steps (1% BSA in PBS) in a volume of 100 μl for 10 minutes at room temperature. Afterwads, cells were incubated with antibodies listed in Supplemental Table 5, washed in PBS, and resuspended in sorting buffer (PBS containing 25 mM Heps, pH 7.0, 2 mM EDTA, 1% PBS) for FACs analysis. Data were acquired on an LSRII flow cytometer (BD) and analyzed using FlowJo software. To induce hypoxia in CPCs, cells were grown in a humidified atmosphere at 37°C at 1% O<sub>2</sub>/5% CO<sub>2</sub> to induce hypoxic responses. Cells cultivated in a 21% O<sub>2</sub>/5% CO<sub>2</sub> incubator were used as normoxic controls.

**ES cell differentiation, NAD+/NADH measurement, and ROS measurement.** Undifferentiated ES cells (V6.5 ES cell line) were maintained on mouse embryonic fibroblast (MEF) feeder cells in DMEM supplemented with 15% FCS (Invitrogen), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol (Sigma-Aldrich), 0.1 mM nonessential amino acids (Invitrogen), 4.5 g/ml d-glucose, and 1,000 U/ml of leukemia inhibitory factors (LIH). To induce EB formation, 1 × 10<sup>4</sup> ES cells/10 cm dish were cultured in 10 ml ES medium without LIH. Intracellular NAD concentrations were determined using the NAD/NADH Quantification Kit (BioVision Inc.). Briefly, 2 × 10<sup>5</sup> cells were sonicated in the NAD/NADH extraction buffer and passed through 10-kDa cut-off filters. One half of the lysate was used to determine total NAD concentration;
the other half was heated to 60°C for 30 minutes and used to determine NADH concentration. Reactions were prepared in triplicate in 96-well plates and read at 450 nm. NAD^+ concentration was determined by subtracting the NADH from the total NAD concentration. Intracellular ROS levels were determined in freshly sorted ISL1^+ CPCs cultured for 16 hours under normoxia or hypoxia using the CellROX probe (0.5 mM) (Thermo Fisher Scientific), which was directly added for 30 minutes to the culture medium. After 3 rinses with PBS at 37°C, cells were fixed for 5 minutes in PFA and rinsed 3 times in PBS. Cells were taken up in PBS and with an LSRII FACS (BD Biosciences). Data were analyzed using FlowJo software.

**Cell culture and plasmid transfection.** HEK293T and C2C12 cells (ATCC) were grown in DMEM (Sigma-Aldrich) supplemented with 10% FCS (Sigma-Aldrich), 2 mM L-glutamine, 100 U penicillin, and 100 μg/ml streptomycin at 37°C, 5% CO2. HEK293T cells were transfected with 10 μg DNA using calcium phosphate precipitation at a density of 2 × 10^4/10 cm dish.

**Luciferase reporter assay.** A 1.0-kb genomic DNA fragment upstream of the *Isl1* transnational start site was amplified and cloned into pTA-Luc (Promega). Mutations in the conserved HES1-binding site (N-box) were introduced using the QuickChange Site-Directed Mutagenesis Kit (Agilent) according to the manufacturer’s protocol. Primers are listed in Supplemental Table 3. Firefly luciferase and renilla activities were determined using the Dual-Luciferase Reporter Assay (Promega) with a Mithras LB940 plate reader (Berthold) 48 hours after Lipofectamine 2000–based transfections (Invitrogen) into HEK293T or C2C12 cells. Firefly luciferase activities were normalized to renilla. Each transfection was done in triplicate.

**shRNA-mediated knockdown by lentiviral infection.** Lentivirus-mediated shRNA knockdown of *Sirt1* (Sigma-Aldrich MISSION shRNA library, SHCLNG-NM_019812.1), *HIF1α* (Sigma-Aldrich MISSION shRNA library, SHCLNG-NM_010431.1), *Isl1* (CGGCAATCAAAT-TCAGCAGCA), and *Hey1* (Sigma-Aldrich MISSION shRNA library, SHCLNG-XM_192801.2) was accomplished using previously described protocols (57). Nonconfluent cells were incubated for 24 hours in lentivirus-containing medium with 8 μg/ml polybrene, which was replaced with growth media containing 2 μg/ml puromycin for another 2 days before further analysis. Efficient knockdown of target genes was confirmed by both Western blot and RT-qPCR analysis.

**Immunoprecipitation and Western blot analysis.** Embryonic hearts or FACS-isolated CPCs were washed in cold PBS and lysed in buffer containing 10 mM Tris (pH 7.4), 200 mM NaCl, 2 mM EDTA, 2 mM EGTA, and 1% Triton X-100 with protease inhibitors. Either 2 μg antibody (listed in Supplemental Table 5) or IgG control was added to the lysate. Lysates were gently rotated for 4 hours before protein A agarose beads (Roche) were added for 2 hours at 4°C. After extensive washing with lysis buffer, beads were heated in SDS sample buffer or eluted with 3× Flag peptide (200 ng/ml) for second immunoprecipitation. Immunoprecipitated proteins were fractionated, blotted, and analyzed with different antibodies as indicated. For Western blots, tissue or cells were incubated in lysis buffer and resolved by SDS-PAGE before transfer to nitrocellulose filters. Protein expression was visualized using an enhanced chemiluminescence detection system (GE Healthcare) and quantified using a ChemiDoc gel documentation system (Bio-Rad). Antibodies are listed in Supplemental Table 5.

**ChIP.** Tissue samples or freshly sorted cells were crosslinked with 1% formaldehyde (Sigma-Aldrich), and chromatin DNA was sheared to an average size of 200–500 bp by sonication with a Bioruptor (Diagenode). Protein-DNA complexes were immunoprecipitated with control IgG or specific antibodies, followed by incubation with Protein A/G agarose beads (Roche). After washing and elution, protein-DNA complexes were purified using chelex-100 (Bio-Rad) as described (58). Immunoprecipitated chromatin was analyzed by qPCR using SYBR green with primers specific for promoter regions of *Isl1* and *Nkx2.5* (see Supplemental Tables 3 and 4). All experiments were performed at least in triplicate.

**Gene expression analyses.** Total RNA from FACS-isolated CPCs, embryonic hearts, or embryos was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA was reverse transcribed with Superscript II (Invitrogen) following standard procedures. Real-time PCR was performed with 3 technical replicates using the iCycler (Bio-Rad) and the Absolute QPCR SYBR Green Fluorescein Mix (Abgene). Relative quantitation of mRNA gene expression was performed using either a standard curve–based data processing method as described (59) or the ΔCt method. The Ct values of the target genes were normalized to the *m36b4* housekeeping gene using the equation ΔCt = Ct_target – Ct_reference, and expressed as ΔCt. Relative mRNA expressions are shown, with the average from control samples set as 0.5. Primers and PCR conditions are listed in Supplemental Table 4.

**Whole-mount and histological analysis.** Embryos or embryonic hearts of different developmental stages were isolated and immediately fixed in 4% PFA. For paraffin sections, samples were dehydrated following standard protocols, embedded into paraffin, sectioned at 10 μm, and stained with H&E (Chroma). For cryosections, fixed tissues were equilibrated in 30% sucrose/PBS and frozen on dry ice. Sections of 8 μm were mounted on Superfrost slides for immunofluorescence staining. For in vitro CPC differentiation, purified ISL1^+ CPCs were cultured and differentiated on fibronectin-coated glass chamber slides (fibronectin from BD Biosciences; glass chamber slides from Greiner) as described previously (60). HDAC inhibitors were added for 48 hours to cultured CPCs before fixation with 4% PFA and subsequent immunofluorescence staining. The following final concentrations were used: 1 μM for EX527 (Cayman), 5 μM for Ms257, 1 μM for MC1568, and 1 μM for TMP269 (Selleckchem). Antibodies for immunofluorescence are listed in Supplemental Table 5. Image acquisition and analysis were acquired with an ImageXpress microscope equipped with MetaXpress software (Molecular Devices). TUNEL assays to monitor apoptosis were carried out using the In Situ Cell Death Detection Kit (Roche) according to the manufacturer’s protocol. Whole-mount in situ hybridizations for *Isl1* mRNA and *Nkx2.5* mRNA were performed as described previously (55) using dual DIG-labeled *Isl1* or *Nkx2.5* antisense probes synthesized from ISL1- and Nkx2.5-cDNA pCR-BluntII-TOPO clones (21).

**Statistics.** For all quantitative analyses, a minimum of 3 biological replicates were analyzed. Statistical tests were selected based on the assumption that sample data come from a population following a probability distribution based on a fixed set of parameters. To determine statistical significance of differences between 2 groups, *t* tests were used, and for multiple groups, ANOVA with Dunnett’s or Tukey’s post hoc correction was used, as indicated. *P* < 0.05 was considered statistically significant. Calculations were done using the GraphPad Prism 5 software package. Error bars represent SEM. No statistical method was used to predetermine sample size.
Study approval. All animal experiments were done in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, 1996) and were reviewed and approved by the Committee for Animal Rights Protection of the State of Hessen (Regierungspräsidium Darmstadt, Darmstadt, Germany, project number B2/1010).

Author contributions

XY and TB conceived and designed experiments and wrote the manuscript. XY performed most of the experiments, analyzed the data, and prepared figures. HQ and FW performed cell culture and mouse studies. XL performed shRNA knockdown and ChIP experiments. JF and EB generated essential reagents. VZ and GD contributed to experimental design, data analysis, and manuscript writing.

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