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Hhex and *Cer1* Mediate the Sox17 Pathway for Cardiac Mesoderm Formation in Embryonic Stem Cells

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ABSTRACT

Cardiac muscle differentiation in vivo is guided by sequential growth factor signals, including endoderm-derived diffusible factors, impinging on cardiogenic genes in the developing mesoderm. Previously, by RNA interference in AB2.2 mouse embryonic stem cells (mESCs), we identified the endodermal transcription factor *Sox17* as essential for Mesp1 induction in primitive mesoderm and subsequent cardiac muscle differentiation. However, downstream effectors of *Sox17* remained to be proven functionally. In this study, we used genome-wide profiling of *Sox17*-dependent genes in AB2.2 cells, RNA interference, chromatin immunoprecipitation, and luciferase reporter genes to dissect this pathway. *Sox17* was required not only for *Hhex* (a second endodermal transcription factor) but also for *Cer1*, a growth factor inhibitor from endoderm that, like *Hhex*, controls mesoderm patterning in *Xenopus* toward a cardiac fate. Suppressing *Hhex* or *Cer1* blocked cardiac myogenesis, although at a later stage than induction of *Mesp1/2*. *Hhex* was required but not sufficient for *Cer1* expression. Over-expression of *Sox17* induced endogenous *Cer1* and sequence-specific transcription of a *Cer1* reporter gene. Forced expression of Cer1 was sufficient to rescue cardiac differentiation in *Hhex*-deficient cells. Thus, *Hhex* and *Cer1* are indispensable components of the *Sox17* pathway for cardiopoiesis in mESCs, acting at a stage downstream from *Mesp1/2*. STEM CELLS *2014;32:1515–1526*

INTRODUCTION

The developmental restriction of primitive mesoderm to a cardiac muscle fate—whether in the embryo or in pluripotent cells—depends on signals from adjacent cell types, among them the developing endoderm [1–3]. Notwithstanding differences in cardiac anatomy and even the body plan, the molecular cues responsible for cardiac induction are largely conserved across species, including bone morphogenetic proteins (BMPs), Activin and Nodal (a second branch of the BMP/transforming growth factor-β superfamily), fibroblast growth factors (FGFs), and Wnts. Previously, we demonstrated that an endoderm-associated Sry-box transcription factor, Sox17, was essential for cardiac specification in differentiating mouse embryonic stem cells (mESCs), depending on canonical Wnts and BMPs for its induction, and acting on cardiac myogenesis at least in part via cell-nonautonomous mechanisms upstream of *Mesp1/2* [4]. Mesp1 and Mesp2, two closely related helix-loop-helix proteins, are among the earliest transcription factors that direct primitive mesoderm to a cardiovascular fate [5–8]. A similar requirement for *SOX17* was shown independently in human ESCs [9]. However, the mechanism or mechanisms that communicate Sox17's effect on cardiac specification remain unproven.

A second endodermal transcription factor *Hhex* was contingent on *Sox17* and is plausible as a candidate effector, although its functional role in ESCs has not been addressed [4]. In *Xenopus*, a Wnt-activated *Hhex* pathway controls secreted signals for mesoderm patterning to a cardiac fate, acting in parallel with Nodal-dependent induction of Cerberus, a growth factor antagonist [10]. Although Xenopus Cerberus is a broad-spectrum inhibitor of BMPs, Wnts, and Nodal, the mouse ortholog Cer1 inhibits Nodal and BMPs only [11].

What are the responsible effectors for the *Sox17* pathway toward cardiac muscle differentiation in differentiating mESCs? Here, through genome-wide microarray analyses we identified *Cer1*, like *Hhex* [4], as a gene whose induction

requires *Sox17*. We then used RNA interference to prove the requirement for *Hhex* and *Cer1* in mesoderm patterning by ESCs, the requirement for *Hhex* in normal induction of *Cer1*, and the ability of ectopic *Cer1* to rescue *Hhex*-deficient cells. We further show the direct binding of Sox17 to conserved sites in the *Cer1* gene and demonstrate the induction of endogenous *Cer1* by conditional expression of *Sox17*. Thus, Sox17 contributes to *Cer1* expression both directly and, through *Hhex*, indirectly. Together, these results demonstrate an obligatory role for *Hhex* and *Cer1* in differentiating ESCs, as mediators of the *Sox17*-dependent pathway for cardiac mesoderm formation.

Materials and Methods

Cell Culture

AB2.2 ESCs [12] were provided by Allan Bradley (Baylor College of Medicine) and DE14 *Cripto*$^{-/-}$ ESCs [13] by Eileen Adamson (Sanford-Burnham Medical Research Institute). Routinely, ESCs were cultivated in serum-containing medium as hanging droplets to form embryoid bodies, as previously detailed [4]. ESCs were transduced with lentiviral vectors coexpressing enhanced green fluorescent protein (eGFP) with shRNA against the genes tested, or against firefly luciferase [4]. Transduced cells were flow-sorted based on GFP fluorescence, grown as embryoid bodies, and transferred to tissue culture plates after 4.5 days [4]. Expression profiling was performed after further culture for up to 10 days.

Where indicated, cells were plated directly as monolayers at 5×10^4 cells per milliliter using serum-free medium, containing 75% Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA; http://www.lifetechnologies.com), 25% Ham's medium F-12 (Invitrogen), 0.5× of supplements N2 and B27 (without retinoic acid) (Invitrogen), penicillin, streptomycin, 0.05% bovine serum albumin, 2 mM glutamine (Invitrogen), and 4.5×10^{-4} M 1-thioglycerol. Serum-free medium was replaced every 2 days. Recombinant Activin and Wnt3a were purchased from R&D Systems (Minneapolis, MN; http://www.rndsystems.com).

Microarray Analyses

Two independent samples were used for each condition, except where noted. Fluorescence intensities were captured with a GeneArray 2500 Scanner (Affymetrix, Santa Clara, CA; http://www.affymetrix.com). Differentially expressed genes were identified from log 2-expression data averaged over both replicates using a cross-sample variation >0.65 and a log 2-fold-change >1.2 across conditions for at least one time point. Genes were clustered according to their temporal structure following the procedure in Supporting Information Figure S1A.

RNA Interference

The modified pLL3.7 lentiviral vector (replacing the human cytomegalovirus promoter with the murine phosphoglycerate kinase promoter to drive GFP) and the *Sox17* and firefly luciferase shRNA vectors were previously described [4]. For *Hhex* and *Cer1* shRNAs, the synthetic oligonucleotides and their reverse complement were annealed and ligated into the HpaI/XhoI sites of pLL3.7pgk, downstream from the murine U6 promoter. The two *Hhex* shRNAs target 5′-GCGTCTGGCCAA GATGTTA-3′ and 5′-GGTGCCTCTTTGGATCGTT-3′ in *Hhex* mRNA. The Cer1 shRNAs target 5′-GTCCAGAACAACCTTTGCT-3′, 5′-GATGGTGATGCAAGTAGAA-3′, and 5′-GGGATGTGGAAAGCGATCA -3′ in *Cer1* mRNA.

For viral vector production, 20 μg of shRNA-encoding lentiviral backbone plasmid was transfected along with 15 μg of packaging vector psPAX2 and 10 μg of envelope vector pMD2.G (from Didier Trono, University of Geneva) into a 150 mm dish of 293T cells, using calcium phosphate. Culture supernatant containing lentiviral particles was collected 48 and 60 hours after transfection and combined. The pooled supernatant was first cleared of cell debris by centrifugation at 2,000 rpm (1,000*g*) for 5 minutes, and the subsequent supernatant subjected to ultracentrifugation at 25,000 rpm (82,705*g*) for 2 hours in a SW28 rotor (Beckman, Indianapolis, IN; https://www.beckmancoulter.com). The resulting pellet was resuspended in 136 μl phosphate-buffered saline, and the lentiviral vector stocks (>10^8 IU/ml) were stored at −80°C.

Lentiviral Vectors for Conditional Expression

To generate viral vectors for tetracycline-induced *Sox17* expression, the tet transactivator-dependent vector plasmid pNLTREpitt-EGFP-ΔU3 [14] was modified as follows. The SacII/BsrG1 fragment encoding eGFP was removed, the SacII/BsrG1 sites were blunted with T4 DNA polymerase, and a blunted BglII/NotI fragment of pIRES2-EGFP (Clontech, Mountain View, CA; http://www.clontech.com) containing IRES2-EGFP was inserted into the resulting sites. Conditional expression of this bicistronic vector (pNLTREpitt-IRES2-EGFP) is monitored by means of the Dox-induced eGFP fluorescence. A XhoI/NsiI fragment from pMSCVpuro (Clontech) was blunted and inserted into a blunted BamHI site of pNLrtTA2sM2, containing the rtTA2s-M2 tet transactivator sequence controlled by the constitutive human CMV-IE promoter; thus, cells harboring this vector, pNLrtTA2sM2-puro, are rendered subject to selection with puromycin. Wild-type *Sox17* and the N3 and C1 truncation mutants were subcloned from pcDNA6.2 into the BamHI site of the pNLTREpitt-IRES2-EGFP, and lentiviruses were prepared as described above.

Conditional Expression of Sox17

ESCs were infected with pNLrtTA2sM2-puro then selected in ES culture media containing 1 μg/ml puromycin, from 3 to 5 days after infection. Surviving cells were infected with pNLTREpitt-IRES2-EGFP-derived vectors for wild-type or truncated Sox17 with a V5 epitope tag and then treated with 1 μg/ml Dox, after 3 days.

Chromatin Immunoprecipitation

Cyber-green PCR primers were designed for predicted Sox-binding elements 5′-(A/T)(A/T)CAA(A/T)−3′ [15] within the *Cer1*, *Foxa1*, and *Foxa2* loci that are conserved in placental mammals (Supporting Information Table S3). Multispecies alignments and binding site detection were performed using Mulan and multi-TF in ECR Browser. Sox17 binding to the *Cer1*, *Foxa1*, and *Foxa2* loci was determined using the EZ-ChIP kit (Millipore Corporation, Billerica, MA; http://www.millipore.com).

Results

Genome-Wide Expression Profiling of *Sox17*-Dependent Genes

For a transcriptome-wide assessment of *Sox17*-dependent genes, ESCs expressing *Sox17* or luciferase shRNA were

differentiated for up to 10 days by the embryoid body method, then were analyzed using Affymetrix microarrays (Supporting Information Fig. S1A and Table S1). Roughly 800 genes (4% of those tested) showed significant changes both across time and in response to *Sox17* shRNA. The filtering thresholds were chosen guided by the change in five *Sox17*-dependent genes of known biological importance, which we had earlier identified by a candidate gene approach [4] (*Foxa1*, *Mesp1*, *Nkx2−5*, *Mef2c*, *Myh6*; Supporting Information Fig. S1B).

For the resulting overall set of *Sox17*-regulated genes, the significant gene ontology (GO) and GenMAPP terms are discussed below. Two GenMAPP categories requiring *Sox17* were indicative of cell lineage, namely, striated muscle contraction ($p = 1E-13$) and smooth muscle contraction ($p = 3E-05$). Accordingly, many of the most affected GO Biological Process terms were related to cardiovascular development and function (Fig. 1A; 16 of the top 40 including: heart morphogenesis, $p = 3E-17$; heart development, $p = 1E-14$; vasculogenesis; $p = 2E-10$). Other highly dependent categories were related to more generic events (multicellular organismal development, $p = 4E-30$; regulation of transcription, DNA dependent, $p = 3E-16$; cell fate commitment, $p = 5E-07$), or, notably, endoderm development ($p = 9E-05$). Numerous pathways for growth factor signaling were *Sox17*-dependent (SMADs, $p = 1E-06$; Wnts, $p = 5E-06$; BMPs, $p = 7E-05$; transforming growth factor beta, $p = 1E-03$). As further steps to refine and visualize these results, the filtered genes were also clustered according to their dynamic profiles (Supporting Information Fig. S1C, S1D) and the temporal clusters then subjected to GO analysis (Supporting Information Fig. S2A). Cluster III, comprising transiently expressed genes with an onset between days 2 and 4, was notably enriched for the GO processes endoderm formation, ectoderm formation, embryonic heart tube morphogenesis, and heart looping (Supporting Information Figs. S2B, S3).

Next, the changes contingent on *Sox17* were scrutinized using a manually curated set of 445 genes relevant to cardiac myogenesis, antecedent processes, and selected relatives for multigene families (Supporting Information Table S2). Significant dysregulation was observed in 28% of the genes, that is, enriched sevenfold compared to the unbiased 22K chipset (Fig. 1B). Endogenous *Sox17* and its direct targets *Foxa1* and *Foxa2* were suppressed, as expected (preconditions for the knockdown experiment to be valid). Moreover, the genome-wide analysis and specific conditions chosen for data mining were sufficient to capture all the *Sox17*-dependent genes we had found previously by a limited candidate gene approach [4]. In addition to just the representative markers *Myh7* and *Ryr2*, the lack of *Sox17* broadly downregulated the genes for diverse cardiac thick filament proteins (*Mybpc3*, *Myh6*, *Myl2/3/4/7*, *Mylk3*, *Myom1*, and *Ttn*), thin filament proteins (*Actc1*, *Actn2*, *Tnnc1*, *Tnni1/3*, Tnnt2, and *Tpm1/2*), Z disc proteins (*Csrp3/Mlp*), *Nppa*, and regulators of Ca^{2+} homeostasis (*Atp2a2*, *Pln*, and Srl).

With regard to cardiogenic transcription factors and their coactivators, the lack of *Sox17* resulted in suppression of *Foxc1/2*, *Gata4/5/6*, *Hopx*, *Irx3/5*, *Isl1*, *Smarcd3/Baf60c*, and *Smyd1/Bop*, in addition to the three factors examined previously (*Mesp1*, *Mef2c*, and *Nkx2–5*). Unlike those mentioned above, *Hand1/2* and *Tbx20* were upregulated, concomitant with other genes for neural development, consistent with their additional roles, respectively, in neural crest and motoneurons [16]. Thus, in unbiased genome-wide testing, *Sox17* expression in ESCs was a prerequisite for the induction of highly diverse cardiogenic transcription factors and cardiac structural genes.

Demonstrated by induction of *T*, *Eomes*, *Fgf8*, Gsc, *Cdx2*, and *Mixl1* to normal or increased levels, suppressing *Sox17* did not prevent the induction of primitive mesoderm, mesendoderm, or the primitive streak (Fig. 1B, Supporting Information Fig. S3). In the case of *Eomes*, a direct activator of *Mesp1* [7, 17], this may be due to loss of a known negative feedback loop [5]. Several upregulated mesodermal genes were related to hematopoiesis (*Etv2*, *Fli1*, *Hoxa9*, *Hoxb6*, *Hoxc8*, *Hba-a1/2*, *Hba-x*, *Hbb-bh1*, and *Hbb-y*). Notably, however, key early markers of the multipotential cardiovascular progenitor cell were suppressed (*Kdr/Flk1*, *Pdgfra*) [18, 19]. Thus, taking these results together, genome-wide profiling substantiates that *Sox17* specifically affects the direction of mesoderm toward a cardiovascular fate, not mesoderm formation per se [4].

In addition to *Foxa1/2*, discussed above, multiple markers of early endoderm (*Cldn6*, *Dpp4*, *Epcam*, *Foxg1*, *Nr2f1*, *Rhox5*, and *Sparcl1*), definitive endoderm (*Cd24a*, *Cxcr4*, *Foxa1/2*, and *Kitl*), and visceral endoderm (*Afp*, *Cited1*, *Dab2*, *Fxyd3*, *Hesx*, and *Ttr*) were inhibited, the latter notably including *Cer1* (Fig. 1B; Supporting Information Fig. S3, cluster III). Several of the endodermal genes cited are reportedly direct targets of *Sox17* by chromatin immunoprecipitation (ChIP) and whole-genome promoter tiling arrays [20], although whether Sox17 directly activates *Cer1* is unsubstantiated. Other *Sox17*-dependent genes included two related F box family members, *Sox18* and *Sox7*, with which *Sox17* can be redundant [21, 22]. Given these genes' sequence similarity, we confirmed that the *Sox17* shRNAs have no promiscuous effects on cotransfected *Sox18* [4] or *Sox7* (Supporting Information Fig. S4A). Thus, under the conditions tested, lack of *Sox17* downregulates the redundant family members that are required in concert with *Sox17* during embryogenesis.

Because the action of *Sox17* in mesoderm patterning is cell-nonautonomous [4], we next tested for operation of a secretory pathway, as opposed to ones requiring cell-cell contact. Doxycycline- (Dox-) dependent "inducer" ESCs bearing *Sox17* gain-of-function mutations were able to upregulate *Nkx2–5* and *Myh6* in wild-type "responder" ESCs, across a semipermeable membrane (Fig. 2A). Thus, *Sox17* is sufficient to promote cardiac muscle differentiation from ESCs via one or more soluble signals. In *Xenopus* embryos, the endodermal genes that most conclusively regulate secreted signals for cardiac specification by primitive mesoderm—the stage regulated by *Sox17*—are *Hhex* (whose relevant target is unknown) acting in parallel with *Cer1*, induced by Wnt antagonists and Nodal, respectively [10, 23]. Using the transmembrane induction assay, *Hhex*, like *Sox17*, was sufficient to upregulate *Nkx2–5* and *Myh6* in responder cells (Fig. 2A). As a prelude to more detailed investigation of these two putative effectors and their potential relationship, we substantiated our microarray finding that *Cer1*, like *Hhex*, depended on *Sox17* using quantitative real-time RT-PCR (QRT-PCR; Fig. 2B).

Hhex and *Cer1* Act in Series for Mesoderm Patterning to a Cardiac Fate in Differentiating *ESCs*

To test the requirement for *Hhex* and *Cer1* by RNA interference, preparatory studies confirmed the shRNAs' effect on

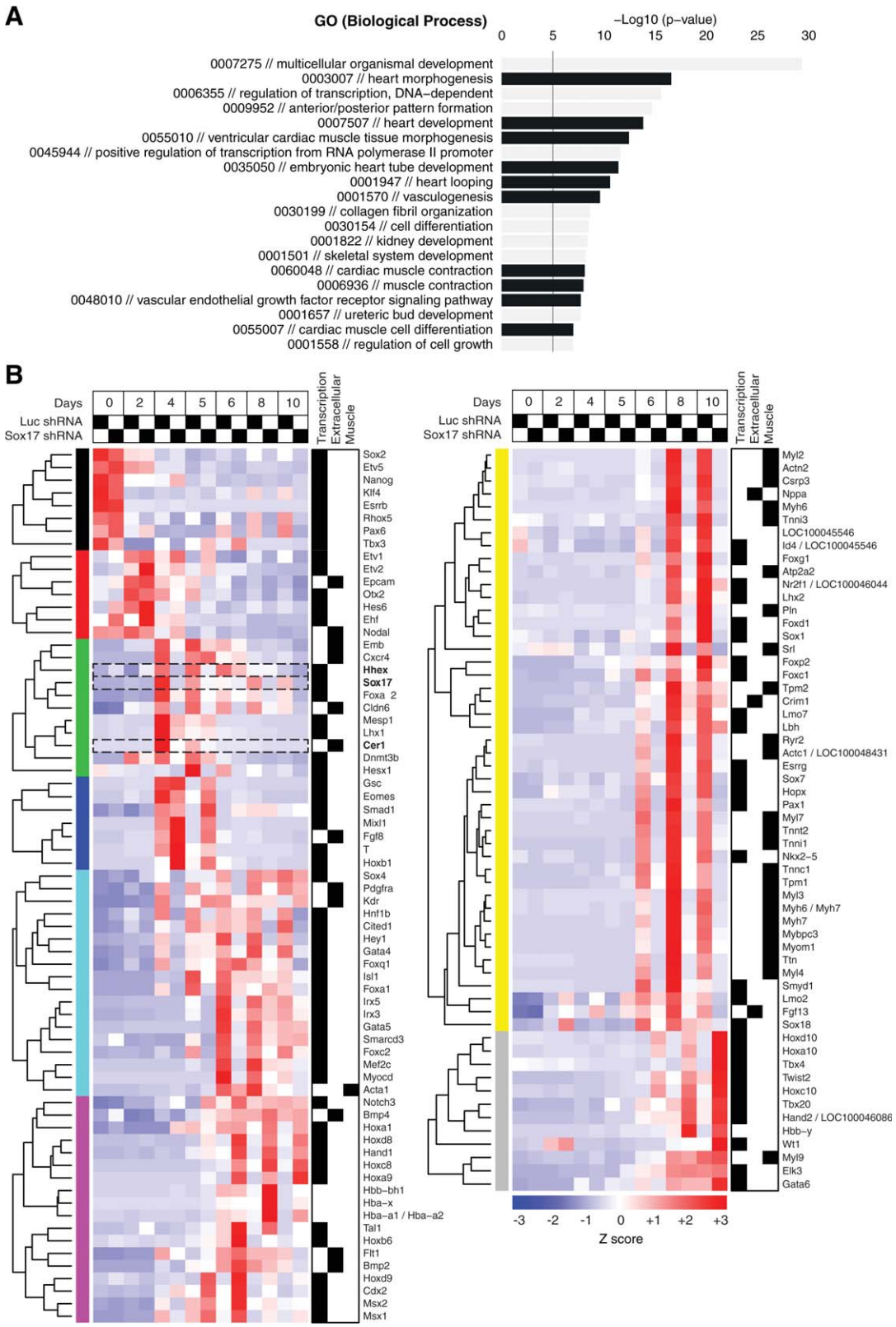

Figure 1. *Sox17*-dependent genes in differentiating ESCs.**(A)**: The top 20 GO biological process terms that were dysregulated in *Sox17*-deficient mouse ESCs. Those specific for cardiovascular development and function are highlighted (black), and a *p*-value of 1E-5 is noted for reference (Supporting Information Figs. S1, S2). **(B):** Heat map of gene expression levels for 126 *Sox17*-regulated genes from a curated gene set related to cardiac myogenesis (Supporting Information Table S2). Genes that fulfill the filtering criteria in Supporting Information Figure S1A are presented, grouped according to the temporal clusters obtained from the whole-transcriptome analysis (Supporting Information Fig. S1D). Functional annotations are shown at the right for transcription factors, extracellular/membrane proteins, and muscle-specific genes for cardiac contractility. For the complete set of affected genes refer Supporting Information Figure S3. *Sox17*, *Hhex*, and *Cer1* are highlighted. Abbreviation: GO, gene ontology.

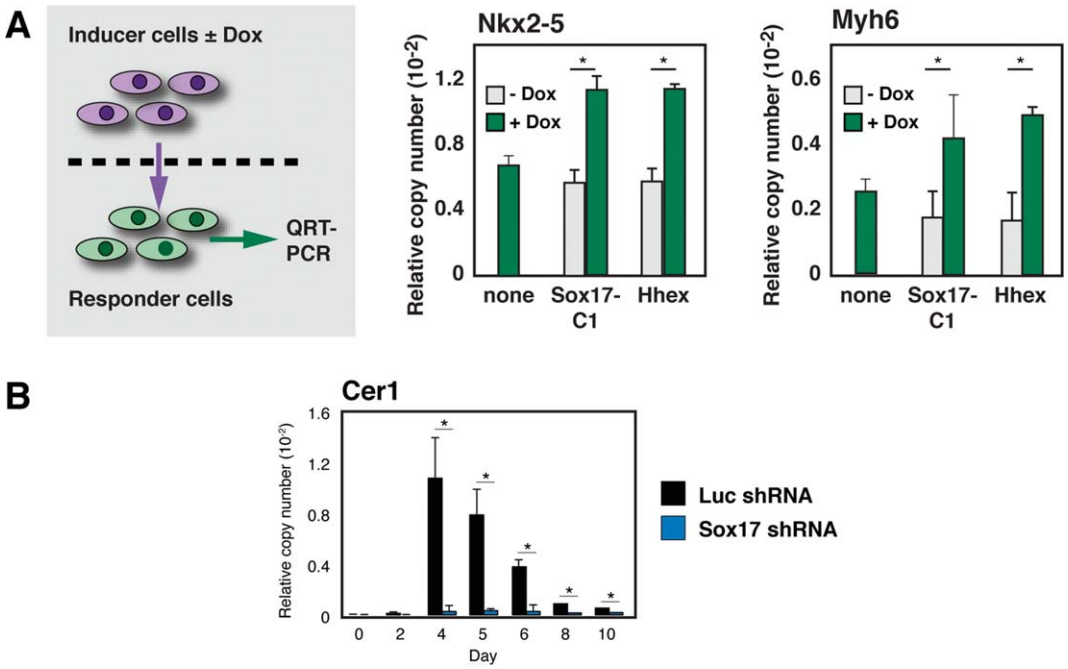

Figure 2. *Sox17* regulates secreted signals for cardiac differentiation. **(A):** (Left) Schematic cartoon of the experimental design. For details of the *Sox17* gain-of-function mutation refer Figure 4D and Supporting Information Figure S5B–S5D. (Right) QRT-PCR results for *Nkx2–5* and *Myh6*. **(B):** Corroboration by QRT-PCR of *Cer1* induction as contingent on *Sox17*. *, $p < .05$ versus control cells; $n \geq 3$. Abbreviation: QRT-PCR, quantitative real-time RT-PCR.

cotransfected *Hhex*, these sequences were retested as recombinant lentiviruses, and a block to endogenous *Hhex* was confirmed (Supporting Information Fig. S4B). The consequences of *Hhex* shRNA shown by QRT-PCR strongly resembled those of blocking *Sox17* (Fig. 3A): (a) suppression of cardiac structural genes (*Myh6* and *Ryr2*), (b) suppression of cardiogenic transcription factors (*Nkx2–5*, *Myocd*, and *Mef2c*), (c) lack of interference with downregulation of stemness factors (*Oct4* and *Sox2*), and (d) failure to inhibit *T*, implicating one or more steps later than the formation of primitive mesoderm.

Among the few differences from *Sox17*-deficient ESCs [4], *Hhex*-deficient ones showed little or no loss of *Mesp1/2*. Thus, these results suggest that *Sox17* acts on *Mesp* gene induction, whereas *Hhex* mediates a later stage. In preliminary microarray analyses, further cardiogenic transcription factors and cardiac structural genes were suppressed by *Hhex* shRNA, and, notably, like *Sox17*, *Hhex* was required for the normal induction of *Cer1* (Fig. 3B). Conversely, forced expression of *Cer1* was sufficient to rescue cardiac differentiation in *Hhex*-deficient ESCs, as shown by *Nkx2–5*, *Tbx5*, and *Myh6* expression (Fig. 3C). Thus, *Cer1* stands out among the plausible candidates to explain the impact of *Sox17*, *Hhex*, or both on cardiac myogenesis.

As done for *Hhex*, we selected shRNAs against *Cer1* and proved their efficacy in transfected 293T cells (Supporting Information Fig. S4B). After lentiviral delivery into ESCs, each shRNA inhibited endogenous *Cer1*, cardiogenic transcription factors, and cardiac structural genes (Fig. 3D), identical to the results obtained in *Hhex*-deficient cells. Like *Hhex*, *Cer1* shRNAs did not suppress the tested markers of "stemness" (*Oct4* and *Sox2*), primitive mesoderm (*T*), or precardiac mesoderm (*Mesp1/2*). Thus, *Cer1*—like *Hhex*—acts at a later stage than the conversion of primitive mesoderm to *Mesp*-expressing mesoderm and, consequently, later than *Sox17*. We saw no difference between shRNA-mediated silencing of *Hhex* versus *Cer1*, based on the candidate genes studied in both backgrounds. These results do not exclude differences that could emerge from broader surveys or genome-wide profiling. The combined knockdown of *Hhex* and *Cer1* was similar qualitatively and quantitatively to that of either alone (not shown), consistent with the ability of *Cer1* to rescue *Hhex*-deficient cells under these conditions. The lack of any additive effect suggests that *Hhex* and *Cer1* act in series, an interpretation supported strongly by the *Cer1* rescue experiment.

Sox17 Couples the Activin/Nodal Pathway to *Hhex*, *Cer1*, and Cardiac Specification

In all the experiments above, the requirement for a *Sox17-Hhex-Cer1* circuit was demonstrated in differentiating embryoid bodies after aggregation in serum-containing medium, that is, conditions that are spontaneous but biochemically undefined. To ascertain whether this *Sox17-Hhex-Cer1* circuit might be essential even if an exogenous differentiating signal were provided, we first tested for induction of these genes in serum-free monolayer culture [24] containing 25 ng/ml Activin [25] (Fig. 4A). Along with transient induction of *T* on day 4, Activin induced sustained expression of *Sox17*, *Hhex*, and *Cer1*. As expected, Activin was sufficient to provoke a cardiomyocyte phenotype, denoted by *Nkx2–5*, *Tbx5*, and *Myh6* at day 7. Thus, in addition to the later expression of cardiac markers, Activin elicited the prior induction of *Sox17*, *Hhex*, and *Cer1*.

Conversely, to assess whether the Nodal/Activin pathway was required to induce the *Sox17-Hhex-Cer1* module, we used homologous-null ESCs lacking the coreceptor Cripto. As

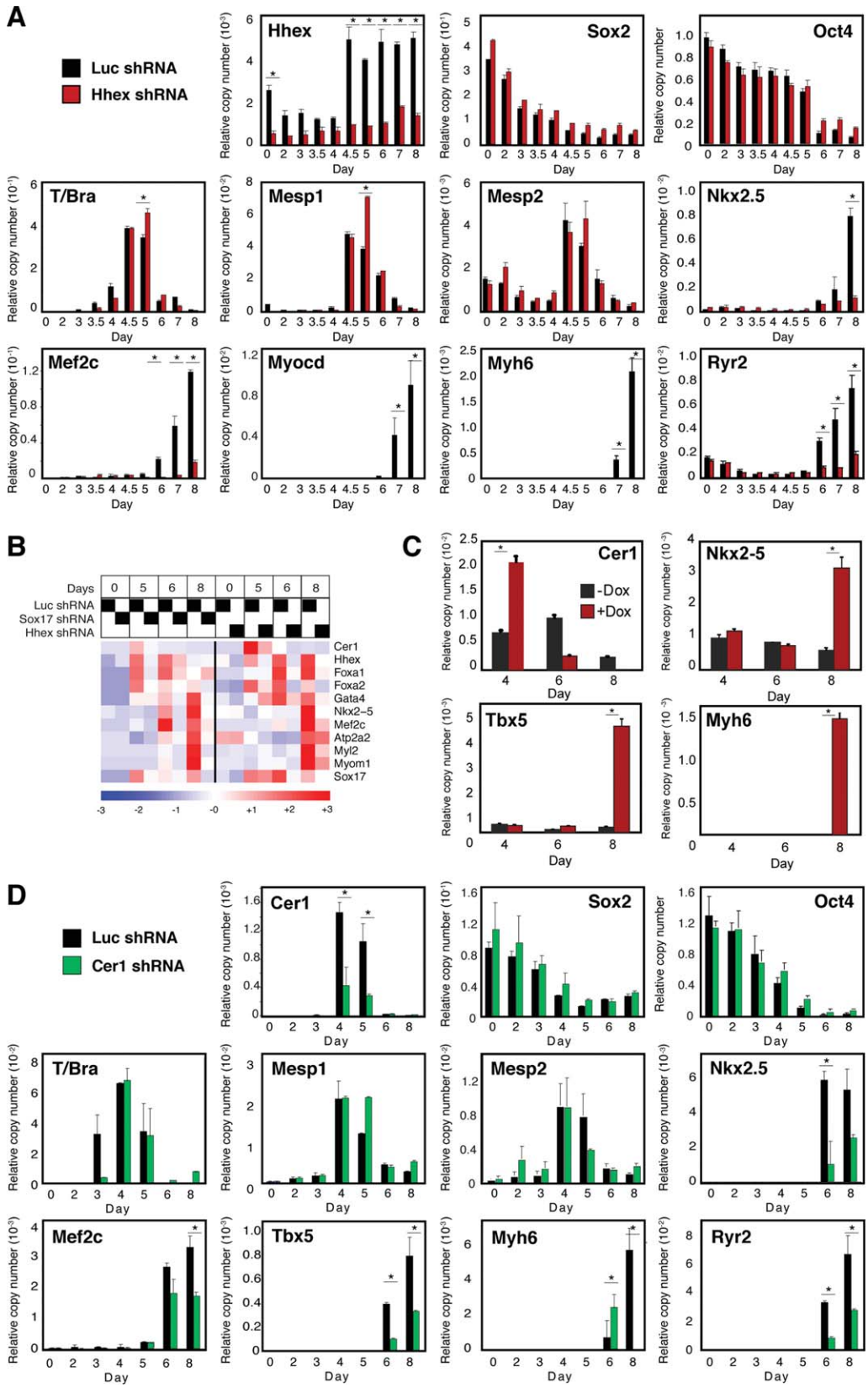

Figure 3. The *Sox17*-dependent genes *Hhex* and *Cer1* are important for cardiac myogenesis in differentiating ESCs. **(A–C):** *Hhex* and (**D**) *Cer1* shRNA suppressed the respective cognate genes in differentiating ESCs and inhibited the induction of cardiac transcription factors and structural genes, acting at a stage subsequent to induction of *Mesp1/2*. *, $p < .05$ versus control cells; $n \geq 3$. (A, D): Results are shown for the most potent of the shRNAs tested, measured by effectiveness against the endogenous transcripts. For each gene, qualitatively similar results were obtained using at least two independent shRNAs. (B): Partial comparison of the microarray findings with *Hhex* and *Sox17* shRNAs, illustrating the shared impairment of *Cer1*, cardiac transcription factors, and cardiac structural genes. In addition, a potential positive feedback loop between *Hhex* and *Sox17* is noted. $n = 2$ for *Hhex* shRNA; $n = 1$ for the *Luc* shRNA controls. (C): Ectopic Cer1 expression rescues cardiac differentiation in *Hhex*-knock down ESCs. Cer1 was encoded by a tetO-regulated lentiviral vector, and was induced on day 3 by doxycycline. Gene expression was assayed by QRT-PCR. *, $p < .05$ versus control cells; $n \geq 3$.

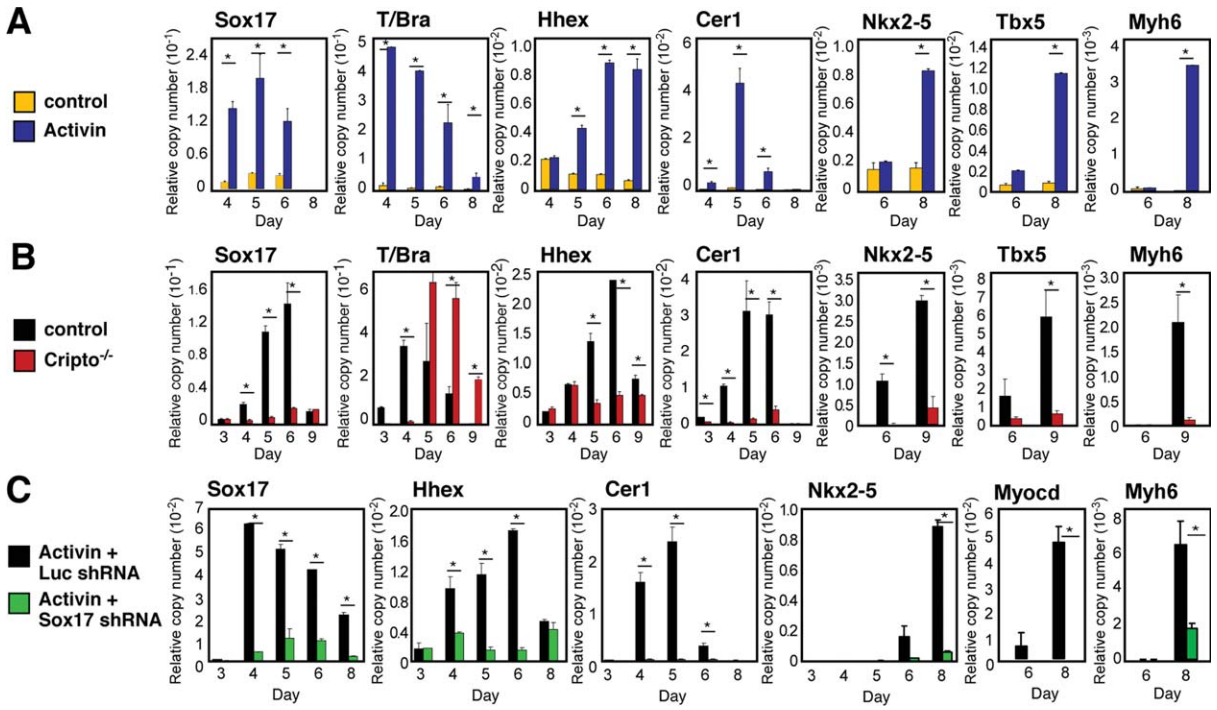

Figure 4. *Sox17* mediates the Activin/Nodal pathway for cardiac myogenesis. **(A):** Induction of *Sox17*, *Hhex*, *Cer1*, and cardiac genes in Activin-treated embryonic stem cells (ESCs). **(B):** The Nodal receptor Cripto is essential for induction of the endoderm-associated *Sox17-Hhex-Cer1* pathway. **(C):** *Sox17* shRNA recapitulates the *Cripto*-deficient phenotype in Activin-treated ESCs. Cells were grown in monolayer culture for panels (A) and (C), and as embryoid bodies for panel (B). *, $p < .05$ versus control cells; $n \geq 3$.

reported [26], cardiac transcription factors (*Nkx2–5* and *Tbx5*) and *Myh6* were not induced in the absence of Cripto (Fig. 4B). Similarly, the lack of Cripto reduced *Sox17*, *Hhex*, and *Cer1* each by 80%–90% (Fig. 4B). By contrast, *T* was expressed at levels even higher than in wild-type cells, although delayed by 1 day. Thus, in addition to canonical Wnts and BMPs [4], a third signal is essential to confer *Sox17* induction, namely the Nodal/Activin cascade.

To verify whether *Sox17* is essential for the induction of *Hhex* and *Cer1* by exogenous Activin, we next compared control and knockdown ESCs in the serum-free monolayer cultures (Fig. 4C). Corresponding to the requirement for *Sox17* in embryoid bodies (Fig. 1B; Supporting Information Fig. S3, cluster III), suppressing *Sox17* likewise prevented the induction of *Hhex* and *Cer1* by recombinant Activin. Despite forced stimulation of the Activin/Nodal pathway, suppressing *Sox17* resulted in the failure of cardiac myocyte differentiation, measured here by *Nkx2–5*, *Myocd*, and *Myh6* (Fig. 4C). Together, these complementary results clearly position *Sox17* upstream from both *Hhex* and *Cer1*, mammalian counterparts of the endodermal signals for cardiac myogenesis in *Xenopus*, and likely explain the absence of *Cer1* seen in Cripto-deficient ESCs [27].

Sox17 Binds and Activates Endogenous Cer1

To distinguish whether *Sox17* confers expression of *Cer1* only indirectly, via *Hhex*, or also acts on *Cer1* directly, we performed Sox17 ChIP assays, using (A/T)(A/T)CAA(A/T) sites conserved across the mouse, canine, rhesus, and human *Cer1* genes [28], guided by the (A/T)(A/T)CAA(A/T)G consensus binding site for the Sox family [15] and the ATTGT core site for Sox17 itself [20] (Fig. 5A; Supporting Information Table S3). Epitope-tagged *Sox17* was transduced into ESCs using a tetracycline-inducible lentiviral system, to obviate potential confounding effects of constitutive expression. Negative controls were randomly selected regions lacking this motif, remote from predicted binding sites. Predicted Sox sites from the *Foxa1* and *Foxa2* loci (Fig. 6A) also were assayed, as these genes are proven direct targets of *Sox17* [20]. In concordance with identification of *Cer1* as a potential Sox17 target by ChIP-chip, albeit along with 1,800 other genes [20], we specifically confirmed Sox17 binding at each of the predicted sites we tested from the upstream 6 kbp, with enrichment at least equal to that for the sites in *Foxa1/2* (Fig. 5B). Thus, predicted binding sites in the upstream region bind Sox17 efficiently.

To test whether exogenous Sox17 suffices to induce *Cer1*, we first mapped the transactivation domain of mouse Sox17, based on the structural organization of XSox17β and other Sox proteins [29] (Fig. 6A, 6B). Each construct was cotransfected into 293T cells with the Sox-dependent luciferase reporter SOP, containing a concatamer of CTTTGTT (an inverse of AACAAAG) [30] (Fig. 6C). Activation was obtained only with wild-type Sox17 or an N-terminal truncation that retains both the DNA binding domain (HMG box) and C-terminus ("C1"). C1 was 14-fold more potent than wild-type Sox17, suggesting the presence of auto-inhibitory elements in the N terminus. None of the constructs activated the inactive control reporter, NOP.

To confirm the putative function of this C-terminal activation domain, we constructed chimeric expression vectors encoding the Sox17 truncations in frame with the Gal4 DNA-binding domain (DBD; Fig. 6D, 6E). Tested using a Gal4-dependent luciferase reporter gene, four of the five fusion proteins—all those preserving the distal C-terminus—were at

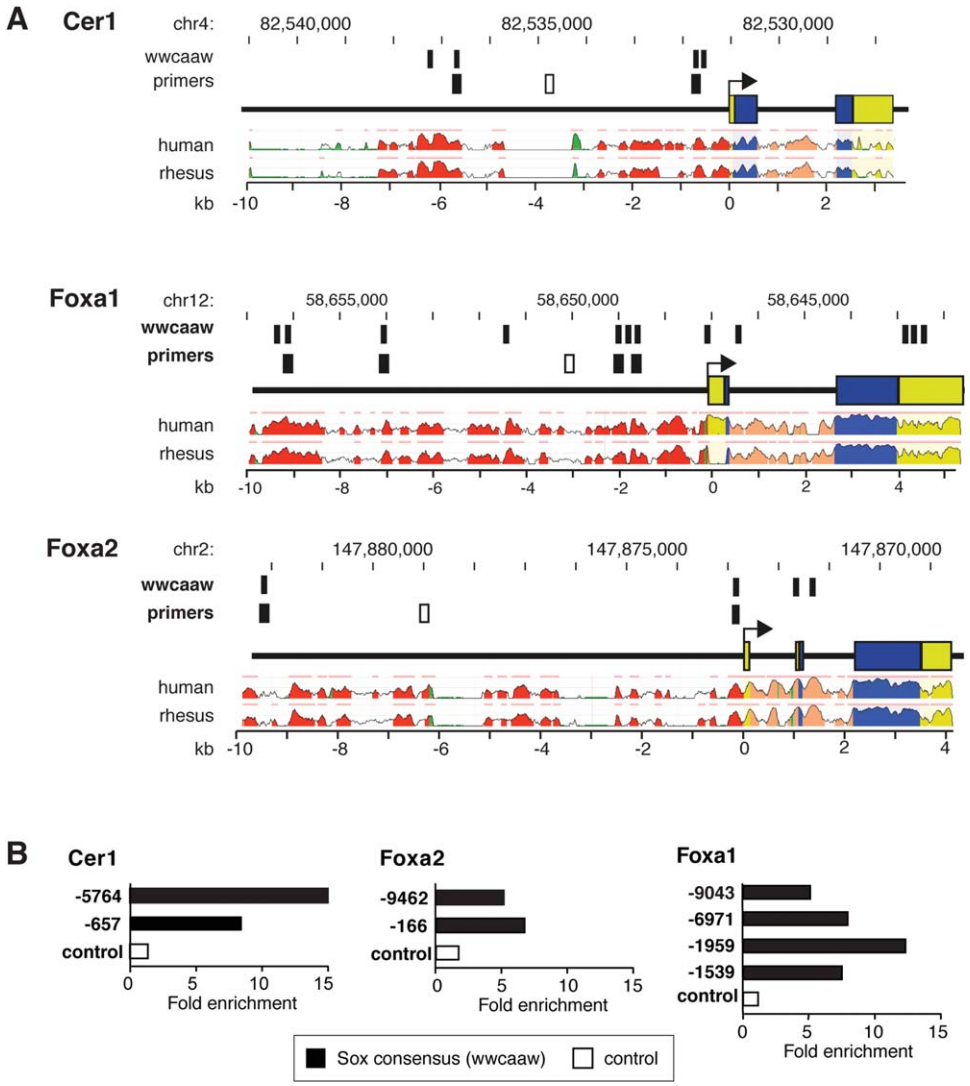

Figure 5. Sox17 binds to evolutionarily conserved Sox sites in the *Cer1* upstream region. **(A):** Predicted Sox17 binding sites in the *Cer1*, *Foxa1*, and *Foxa2* loci. Primers corresponding to predicted binding sites versus irrelevant control regions are indicated in black and white, respectively. Conservation profiles are shown for the human and rhesus orthologs (range, 50%–100%). Pink bars above the profiles denote regions of conservation with the mouse genome; blue, coding exons; yellow, untranslated regions; salmon, introns; red, intergenic regions; green, transposons and simple repeats. **(B):** Chromatin immunoprecipitation, assayed by quantitative PCR, shown as the fold enrichment for the indicated regions (V5 antibody, normalized for nonspecific precipitation by nonimmune IgG). Black, predicted Sox binding sites; white, irrelevant regions. For *Foxa1* and *Foxa2* refer Figure 6A.

least as potent as the Gal4-VP16 control, but the C-terminal residues 129–299 were inactive, similar to the Gal4DBD alone (Fig. 6F). Thus, the Sox17 trans-activation domain is located in the distal C-terminus.

On this basis, wild-type Sox17, the N-terminal portion N3, and the C-terminal portion C1 were compared for their ability to induce endogenous *Cer1*, using Dox-dependent lentiviral vectors. Flow cytometry for eGFP 1 day after Dox administration demonstrated that roughly 80% of the cells were successfully induced (Fig. 6G), versus 0.5%–0.8% for cells without Dox. All three Sox17 proteins were induced efficiently, without discernible leak (Fig. 6H, upper panel). In agreement with their respective activity toward the SOP reporter (Fig. 6H, lower panel), viruses encoding wild-type Sox17 and C1 conferred Dox-dependent induction of endogenous *Cer1* (16–120-fold; Fig. 6I), with C1 (lacking the auto-inhibitory domain) being even more potent than the wild-type protein and N3 (lacking the activation domain) being altogether ineffective.

To determine whether Sox17 can specifically activate the binding sites verified in the *Cer1* locus, the inducible expression vectors were then also tested against *Cer1* luciferase reporter genes. Construction was based on the SOP reporter, containing instead a 3× multimer of the *Cer1* proximal or distal Sox17 binding site, and using each in its wild-type or mutationally inactivated form (−657, Fig. 6J; −6017, not shown). Both wild-type Sox17 and the gain-of-function mutation C1 evoked Dox-dependent, sequence-specific trans-activation via the multimerized proximal site. No induction was seen if the Sox17 binding motif was mutated, a minimal promoter was tested, or N3 was used, lacking the trans-activation domain. As the distal Sox17 site was activated by C1, but not wild-type Sox17, its activation should be interpreted more cautiously, even though sequence-

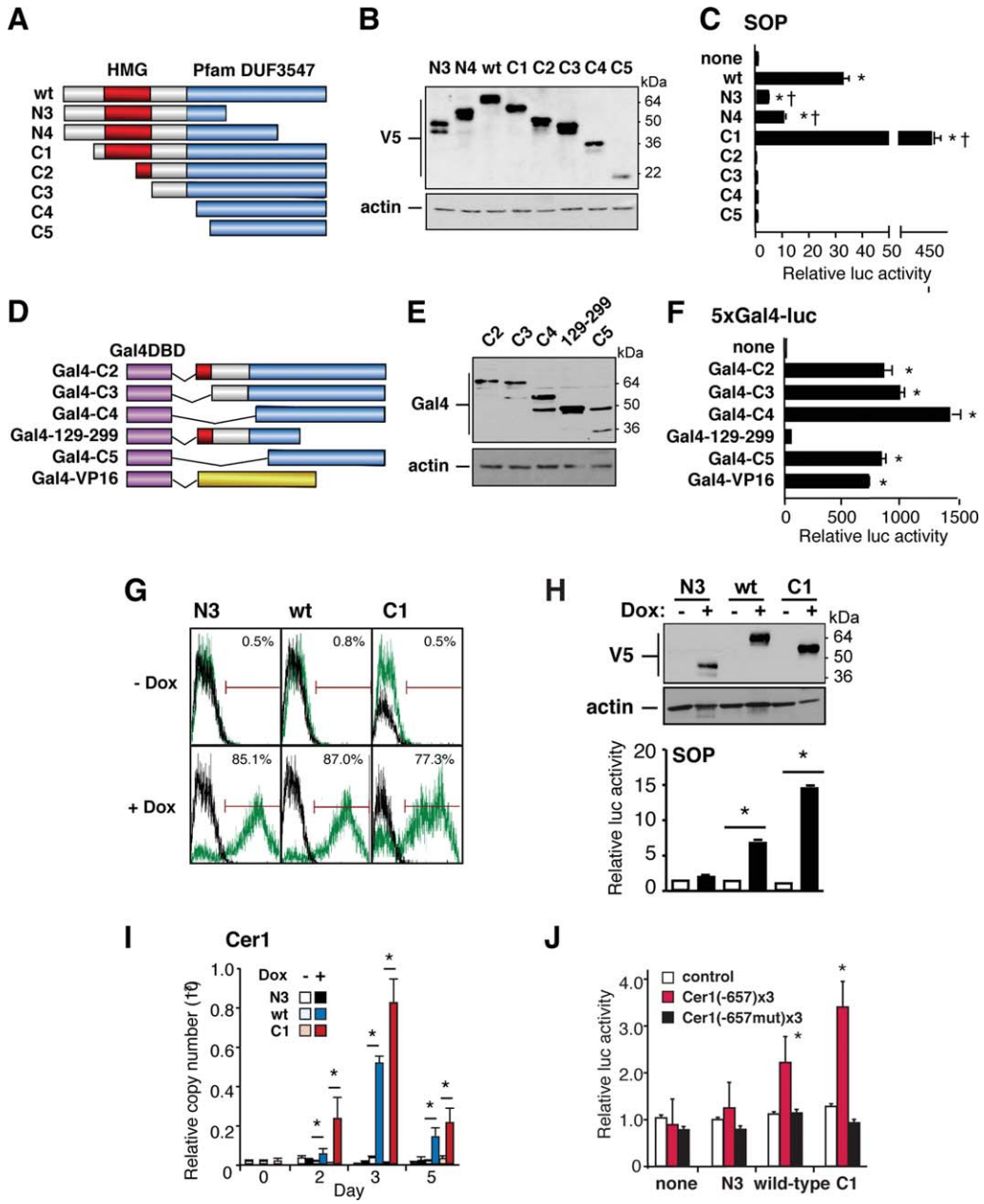

Figure 6. *Sox17* activates *Cer1*. **(A–F):** Mapping the Sox17 transactivation domain. (**A**) Schematic representation of the Sox17 deletion mutants. Domain of unknown function 3547 (Pfam 24.0) designates the conserved C-terminal region of F group Sox proteins. (B): Western blot analysis of the constructs in 293T cells. (C): Sox-dependent reporter gene activity (SOP-FLASH) in 293T cells, in the presence of cotransfected Sox17 expression vectors. Deletion of the N terminus (C1) increases the transcriptional activity. Deletions of the conserved C-terminal domain (N3, N4) attenuate transactivation. (D): Schematic representation of the GAL4DBD-Sox17 fusion proteins. (E): Western blot analysis of the constructs in 293T cells, using antibody to Gal4. (F): Reporter gene activity (5xGal4-luc) in 293T cells, induced by GAL4-Sox17 vectors. Deletion of the Sox17 C-terminal domain (GAL4 129–299) cripples transactivation. Other constructs showed activity equal to or greater than that of GAL4-VP16. **(G, H):** Doxycycline-dependence of the Sox17 vectors, measured in AB2.2 cells by flow cytometry (G), Western blotting (H, above), and transactivation of SOP (H, below). **(I):** Wild-type *Sox17* and the C1 truncation both induce endogenous *Cer1*. $n \geq 3$; *, $p < .05$ versus control embryonic stem cells. **(J):** The multimerized Sox17 site at −657 of the *Cer1* locus mediates Dox-dependent, sequence-specific trans-activation. $n = 6$; *, $p < .01$ versus the absence of Dox.

specific. Together with our evidence that Sox17 specifically binds the *Cer1* promoter, these gain-of-function studies indicate that *Sox17* may directly drive *Cer1* transcription, at least in part through the proximal Sox17 site. None of the *Sox17* expression vectors upregulated endogenous *Hhex* (data not shown), suggesting that *Sox17* is required but not sufficient for *Hhex* induction.

Discussion

We and others have shown previously that *Sox17* is essential for cardiac mesoderm specification in ESCs, working through a cell nonautonomous mechanism for patterning the primitive mesoderm, whose effectors were suppositional [4, 9]. As summarized schematically in Figure 7, these findings establish, that

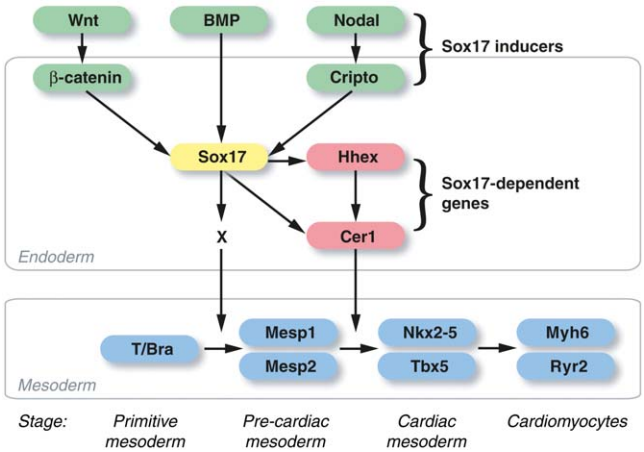

Figure 7. The *Sox17-Hhex-Cer1* pathway for heart induction in differentiating mouse embryonic stem cells (ESCs). *Sox17* expression is contingent on input from β-catenin-dependent Wnts, BMPs, and Nodal. Its induction in the endoderm is mandatory for a cell nonautonomous signal (X) that activates *Mesp1* and *Mesp2*, the essential first step directing the primitive mesoderm toward cardiac muscle specification (stages noted below the mesoderm compartment). Two *Sox17*-dependent endodermal genes, *Hhex* and *Cer1*, act in series downstream from *Mesp1/2* to trigger the induction of cardiogenic transcription factors, such as *Nkx2–5* and *Tbx5*, which denote and execute the cardiac muscle lineage decision. *Sox17* activates *Cer1* both directly, via sequence-specific binding and trans-activation, and indirectly, via *Hhex*. Forced expression of *Cer1* can reduce the lack of cardiac muscle differentiation in *Hhex*-deficient ESCs.

(a) *Sox17* integrates three convergent pathways for cardiac differentiation, requiring Nodal/Activin for its induction, beyond just Wnts and BMPs [4]; (b) the cell nonautonomous requirement for *Sox17* is mediated by secreted signals, not cell contact; (c) *Sox17* is essential for induction of an endodermal growth factor inhibitor *Cer1*, in addition to the endodermal gene *Hhex*, a *Sox17*-dependent transcription factor [4]; (d) *Sox17* is required for the induction of *Hhex*, *Cer1*, and cardiac differentiation even using exogenous Activin as a defined stimulus; (e) *Cer1* and *Hhex* are essential in ESCs for mesoderm patterning to cardiac muscle, akin to their obligatory role in *Xenopus*; (f) *Hhex*, in addition, was required for normal induction of *Cer1*; (g) exogenous *Cer1* was sufficient to rescue *Hhex*-deficient cells; (h) *Sox17* binds consensus sites in the *Cer1* locus and activates sequence-specific transcription of *Cer1* reporter genes.

Sox17 is best known for its function in the developing endoderm [29, 31–34], but, in addition, is required in fetal and neonatal hematopoietic stem cells and can convert adult hematopoietic progenitors to fetal-like hematopoietic stem cells [35]. *Sox17*, *Sox18*, and *Sox7* have nonredundant functions in hematopoiesis [36], yet seeming redundancy in cardiovascular development, which has complicated defining their contribution in vivo [21, 22]. A combined deletion of all three F box family members remains to be reported, and in zebrafish, the ortholog *sox17* itself lacks the conserved β-catenin binding site, whereas endoderm formation relies instead on a unique fourth F group gene, *casanova*/*sox32* [37, 38]. Loss of *Hhex* in mice is sufficient for severe cardiac structural malformations including hypoplasia of the right ventricle and abnormal development of compact myocardium, along with atrioventricular cushion, septal, outflow tract, and vascular defects [39].

Like our studies of *Sox17*-deficient ESCs created by RNA interference, investigations of *Sox17*-null ESCs observe phenotypes (in the latter case, an essential role in extraembryonic mesoderm) [20] that are not manifest in *Sox17*-deficient embryos [31, 40]. It was speculated that the difference reflects compensatory changes in networks controlling intrauterine development, illustrated by the persistence of *Gata4* and *Sox7*, two proven *Sox17* targets, in embryos lacking *Sox17* [20]. Another potential basis is the effect of mouse strain [21, 41]. Alternatively, either the greater spatial precision of instructive signals in the embryo than in embryoid bodies, differences in coexpressed factors [42], or the trans-placental rescue of soluble factors in heterozygous mothers, might make differentiating ESCs a more sensitized system for disruption of cell nonautonomous circuits. Illustrated by genetic modifiers as well as trans-placental rescue in the case of TGFβ1 deficiency [43, 44], two or even more of these mechanisms might coexist.

Several features of this *Sox17-Hhex-Cer1* circuit bear mention. First, although all three genes act on mesoderm pattern to a cardiac fate, *Hhex* and *Cer1* act at a later stage than *Sox17* itself and are dispensable for the induction of *Mesp1/2*. This signifies the operation of as-yet-unidentified effectors of *Sox17* beyond just *Hhex* and *Cer1* in endoderm that dictate this initial step. Indeed, factors apart from *Cer1* contribute to cardiac myogenesis in mESCs stimulated by endoderm from embryonal carcinoma cells, in part via contact-dependent signals, or by conditioned medium from extraembryonic endoderm [45]. Although we show that *Cer1* expression requires *Hhex*, and is sufficient to rescue *Hhex*-deficient cells, these results do not altogether exclude the possibility that *Hhex* might also function in parallel with *Cer1*, the epistatic relation in *Xenopus*. Potential paracrine effectors of *Sox17* shRNA at the time of *Mesp1* induction include increased expression of *Nodal* (Fig. 2B), whose upregulation can impair Mesp1 expression [46]. Interestingly, *Eomes*, a pivotal inducer of *Mesp1*, was expressed despite the lack of *Sox17*; however, its ability to drive *Mesp1* expression is known to be blocked by high levels of Activin [17].

Second, the molecular mechanisms whereby *Sox17* and *Hhex* both activate *Cer1* are expected to differ, with Sox17 binding the *Cer1* promoter directly as shown here, but *Hhex*, a homeodomain transcriptional repressor related to Antennapedia, perhaps more likely inhibiting a key inhibitor [47]. Interestingly, in addition to its role as a trans-activator, *Sox17* can function as a repressor of Wnt/β-catenin signals [4, 48],

its mode of action at later stages of heart development in an endocardial pathway for FGF expression [49].

Third, our study defines the pathway coupling *Sox17* to *Cer1* and thus complements a recent investigation elegantly mapping the effectors of *Cer1*, including inhibition of BMPs and Nodal (growth factors that are essential to the induction of *Sox17* in the endoderm, at an earlier stage), thereby leading to induction of *Baf60c*, the cardiomyogenic component of the SWItch/Sucrose NonFermentable (SWI/SNF) chromatin remodeling complex (42). Consistent with the latter report, *Baf60c* was markedly suppressed by the absence of *Sox17* (Supporting Information Fig. S2, Cluster V), as predicted from the resulting defect in *Cer1*. Taken together with the latter findings, this study represents a further advance in the genetic dissection of endodermal signals for cardiac muscle creation. Because *Sox17* is also expressed by cardiac-resident stem cells [50], it will be intriguing to assess their function in adult cardiac progenitor cells and especially the context of cardiac self-repair.

Conclusion

We report a genome-wide analysis of *Sox17*-dependent genes in differentiating ESCs, substantiate through unbiased expression profiling that *Sox17* controls cardiac myocyte creation at the stage of mesoderm patterning, and prove the requirement for two *Sox17*-dependent genes, *Hhex* and *Cer1*, acting in series.

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Author Contributions

Y.L.: conception and design, collection and assembly of data, data analysis and interpretation, and manuscript writing; R.K.: conception and design, collection and assembly of data, and data analysis and interpretation; T.W.L., R.J.S., and M.B.: data analysis and interpretation and manuscript writing; T.S.: collection and assembly of data and data analysis and interpretation; O.T.: collection and assembly of data; G.M.: provision of study material; M.D.S.: conception and design, financial support, data analysis and interpretation, manuscript writing, and final approval of manuscript. Y.L. and R.K. contributed equally to this article.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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