ORIGINAL RESEARCH

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DNA Methylation is Developmentally Regulated for Genes Essential for Cardiogenesis

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***Background*—**DNA methylation is a major epigenetic mechanism altering gene expression in development and disease. However, its role in the regulation of gene expression during heart development is incompletely understood. The aim of this study is to reveal DNA methylation in mouse embryonic hearts and its role in regulating gene expression during heart development.

***Methods and Results*—**We performed the genome-wide DNA methylation profiling of mouse embryonic hearts using methyl-sensitive, tiny fragment enrichment/massively parallel sequencing to determine methylation levels at ACGT sites. The results showed that while global methylation of 1.64 million ACGT sites in developing hearts remains stable between embryonic day (E) 11.5 and E14.5, a small fraction (2901) of them exhibit differential methylation. Gene Ontology analysis revealed that these sites are enriched at genes involved in heart development. Quantitative real-time PCR analysis of 350 genes with differential DNA methylation showed that the expression of 181 genes is developmentally regulated, and 79 genes have correlative changes between methylation and expression, including hyaluronan synthase 2 (*Has2*). Required for heart valve formation, *Has2* expression in the developing heart valves is downregulated at E14.5, accompanied with increased DNA methylation in its enhancer. Genetic knockout further showed that the downregulation of *Has2* expression is dependent on DNA methyltransferase 3b, which is co-expressed with *Has2* in the forming heart valve region, indicating that the DNA methylation change may contribute to the *Has2* enhancer's regulating function.

***Conclusions*—**DNA methylation is developmentally regulated for genes essential to heart development, and abnormal DNA methylation may contribute to congenital heart disease. **(*J Am Heart Assoc*. 2014;3:e000976 doi: 10.1161/JAHA.114.000976)**

Key Words: DNA methylation • DNA methyltransferase 3b • gene expression • heart development • hyaluronan synthase 2

The heart is the first organ to develop during embryogenesis. In the developing mouse heart, between embryonic day (E) 11.5 and E14.5, cardiac cells undergo differentiation, migration, and proliferation driving cardiac tissue morphogenic events including chamber septation, heart valve formation, myocardial compaction, and coronary vessel formation, all essential for proper heart development.[1–4] These processes are directed by cardiac transcriptional programs and endocardial-myocardial molecular signalings.[5–8] Both genetic and epigenetic mechanisms have been shown to control the expression of cardiac genes in a spatiotemporal manner during heart development.[9–13]

Epigenetic modifications, including DNA methylation and histone modification, regulate gene expression by changing the local chromatin structure, thus altering the interaction of chromatin and DNA-binding proteins, such as the binding of transcription activators and repressors to gene promoters and enhancers.[14–16] Different from genetic variation, epigenetic modifications regulate gene expression without altering the nucleotide sequence. In the case of DNA methylation, a methyl group is added to the carbon 5 of cytosine located at a CpG dinucleotide. It has been shown that DNA methylation is essential for gene regulation during development, especially that of tissue-specific genes, and help to maintain cell

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and tissue identity.[17–19] Notably, with the exception of imprinted genes, the mammalian genome is stripped of its epigenetic modifications in early embryogenesis and the epigenome is then re-established throughout embryonic development.[20–22]

Globally, the patterns of DNA methylation acquired during embryogenesis remain stable throughout development and adulthood.[19] However, changes in DNA methylation at individual loci do occur and can alter expression of genes with important biological functions in development and disease.[23,24] DNA methylation change also occurs in response to developmental perturbations, such as hypoxia. Altered levels of 5-methylcytosine, either genome wide or at specific gene loci, have been related to increased disease susceptibility, and dysregulation of DNA methylation has been linked to cardiovascular disease, type II diabetes, and cancer.[24–26]

Most studies on DNA methylation have focused on gametogenesis, development, disease, and stem cell function by demonstrating how it regulates gene expression and cell differentiation.[23,27–30] Few studies, however, have been devoted to understand the roles of DNA methylation in heart development. Determining the landscape of DNA methylation in this process is an essential step for understanding how DNA methylation regulates the cardiac genes essential for heart development.

Towards this end, we have applied a genome-wide approach in this study to profile developmental changes in DNA methylation in mouse embryonic hearts between E11.5 and E14.5. The morphogenic events occurring during this developmental window are less well studied than early morphogenic events such as the differentiation of cardiac cells. The results show that while the DNA methylome is stable during development, differential methylation occurs at a small subset of genes highly associated with cardiac tissue differentiation and heart development and reveal a regulatory relationship between differential DNA methylation and cardiac essential gene expression. Thus, these results provide new information on the regulation of cardiac gene expression and heart development by DNA methylation.

Methods

Animals (Mice)

ICR wild-type mice were bred in-house for timed pregnancies. Noontime on the day of first observing vaginal plugs was designated as embryonic day (E) 0.5. For *Dnmt3b* knockout studies, endocardial specific Cre mice (*Nfatc1*^{Cre})[9] were crossed with floxed *Dnmt3b* (DNA methyltransferase 3b) mice (obtained from The Jackson Laboratory) to delete *Dnmt3b* in heart valves. Conditional knockout (CKO) and control embryos were identified via PCR genotyping. All mouse experiments were performed according to the protocol approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine.

Methyl Sensitive Tiny Fragment Enrichment/ Massively Parallel Sequencing (MSFE/MPS)

Embryonic hearts from E11.5 or E14.5 were isolated from pregnant mice and non-cardiac tissues were removed. Genomic DNA was extracted from 4 groups of pooled hearts as described previously.[31] A total of 5 μg of extracted DNA from each group was used for a modified HELP-tagging assay.[32] We modified the original assay by replacing HpaII with HpyCH4IV, the restriction enzyme recognizing 5′-ACGT-3′ sites and sensitive to methylation at the CG. After HpyCH4IV digestion, the sequencing libraries were generated using the Ligation Mediated PCR Assay (LMPA).[33] The generated libraries were submitted to the Epigenomics Shared Facility at the Albert Einstein College of Medicine for massively parallel sequencing. Sequencing was performed on individual libraries prepared from 2 biological replicates for each group. The quality of the sequencing results was determined by the parameters of length and peak value of sequence reads. The raw and processed data have been submitted to GEO (accession number: GSE55141).

Luminometric Methylation Assay to Validate the Global DNA Methylation

Global DNA methylation for each biological replicate, at both E11.5 and E14.5, was confirmed using the Luminometric Methylation Assay (LUMA) as described previously.[34,35] Genomic DNA was digested for 4 hours with HpyCH4IV and EcoRI, purified and pyrosequenced at the Einstein Genomics Core.

MassArray

Loci with differential methylation, ranging from 0% to 100% determined by the massively parallel sequencing, were randomly selected and validated using Sequenom's MassArray.[36] Primers were designed using MethPrimer and T7 tags were added as per the Sequenom MassArray protocol (Table 1). Genomic DNA (0.6 μg) from 3 replicates (for technical validation) and 2 replicates (for experimental validation) was bisulfite converted using the Zymo Research EZ DNA Methylation Kit prior to amplification using the MassArray Primers. Amplified bisulfite-converted genomic DNA was then subjected to MassArray on a Sequenom machine.

Table 1. Loci and Primers (With T7 Tags, Lower Cases) for Initial Technical Validation by MassArray

ACGT Location	Forward Primer With T7 Balance (Top) Reverse Primer With T7 Tag (Bottom)
Chr4 145913471	aggaagagagAGTTTTTATGTTTTTTGTAAGGTTATTTGA cagtaatacgactcactatagggagaaggctCCAAACTCCTAATCCCAATCTAAC
Chr7 147043670	aggaagagagTTGGGGGTTTTTAATTAAGATAGTTT cagtaatacgactcactatagggagaaggctTTCCCTCTAATATATCCCATTTTACC
Chr12 62103733	aggaagagagAGTATTAGGGTTAAGTATTGAATAAATTTA cagtaatacgactcactatagggagaaggctAATCAAAATAAAAAATCAAAAAAAA
Chr11 4347732	aggaagagagTTTAGGTATATATTATTATATTTGATTTTT cagtaatacgactcactatagggagaaggctAATTACCACAAAACCTAACAC
Chr2 38354047	aggaagagagAGAGTGGTATTTGTGTTAGAGAGGA cagtaatacgactcactatagggagaaggctTTCCAAAAAAAACCAAAAAAAA
Chr6 64724205	aggaagagagTGTTTTTGTAATTTAGATAAGATTATTTTA cagtaatacgactcactatagggagaaggctAATCACATATCACTAACCAAACAATATC
Chr2 165155478	aggaagagagGGGTGATAGGAAGTTGTAGAGATTAGA cagtaatacgactcactatagggagaaggctAAAAAAACACTACCCAAACTTAAATAACA
Chr11 3036940	aggaagagagGGGTATTTGTTTAAGATATTTTTGATTTAT cagtaatacgactcactatagggagaaggctACAATAACCAAATAAAAAACACACCA
Chr12 101338922	aggaagagagTGGTGTTTTAGTTGTTAAAATGTTATAGG cagtaatacgactcactatagggagaaggctCAAAAATATTCCCCAAATATCAAAA
Chr18 55862932	aggaagagagTAGAAAAATAGGGAGAATGTGATATT cagtaatacgactcactatagggagaaggctATATCTAACTTCCCTACACCCACTAAAA
Chr1 25839243	aggaagagagTTTTAGGATTGAATAAAATTTTAAGA cagtaatacgactcactatagggagaaggctATTTAATTTACTCATTCTCTCTATATAC

Individual sites representing 0%, 25%, 50%, 75% or 100% methylation, that were consistent between the 2 assayed samples, were chosen for validation by Sequenom's MassArray to generate a standard by which to make calls on methylation levels. Primers were designed using MethPrimer and T7 tags were added as per the Sequenom guide.

Bioinformatic Analysis to Profile Genome-Wide DNA Methylation

The sequencing reads were aligned to the mouse genome (mm9) and the number of mapped reads with their 5′ ends starting at each ACGT site was recorded using the automated data analysis pipeline created by the Epigenomics Center and the Computational and Statistical Epigenomics Group at Albert Einstein College of Medicine.[32,37] The read counts at individual ACGT sites from E11.5 and E14.5 were compared and sites with significantly different counts were determined by EdgeR, a Bioconductor package designed for analysis of count based genome-wide sequencing data.[38] The resultant sites were associated with genes if they were located to promoters, gene bodies, or within 50 kb of genes.

Gene Expression Analysis

Custom TaqMan Array 96-Well Fast Plates (Applied Biosystems) were designed for candidate genes prioritized based on degree of differential methylation, function, and presence of multiple-associated differentially methylated ACGT sites. RNA was extracted from pooled embryonic hearts from E11.5 or E14.5 (n=3 for each stage) and atrioventricular junctions isolated from 3 wild-type or 3 CKO embryos at E11.5 and E14.5 using Trizol Reagent (Invitrogen) and reverse transcribed using the SuperScript II reverse transcription kit (Invitrogen). ΔCt values were calculated, normalizing to an endogenous control, and fold change was calculated using the $2^{-\Delta\Delta Ct}$ method.[39]

RNA In Situ Hybridization

RNA in situ hybridization for *Has2* expression in E11.5 or E14.5 hearts was carried out as described previously.[5]

Immunohistochemistry

Immunohistochemistry (IHC) was carried out to determine expression of *Dnmt3b* in the developing heart using mouse monoclonal Dnmt3b antibody (Abcam 52A1018) (1:250), according to the Vector Labs mouse-on-mouse (M.O.M.) basic kit.

Statistical Analysis

Pearson correlation was used to evaluate the overall similarity of MSFE/MPS tag counts between E11.5 and E14.5 samples.

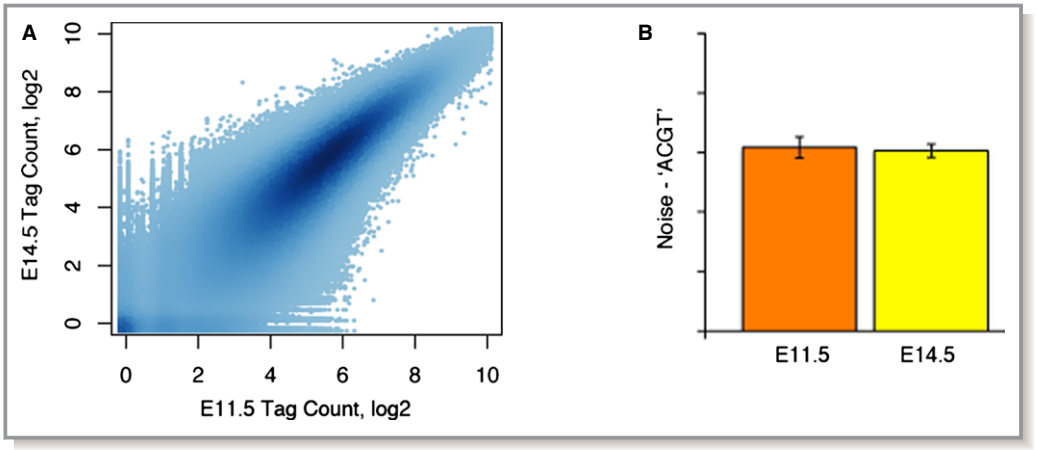


Figure 1. Limited DNA methylation changes between E11.5 and E14.5 hearts. A, The tag counts for all ACGT sites in E11.5 (*x*-axis) and E14.5 (*y*-axis) are highly correlated. The depth of the color represents the density of points in a plotting area (n=2). B, Confirmation of stable global methylation by Luminometric Methylation Assay (LUMA) (n=2). Error bars represent standard error.

A linear regression was used to fit the relationship between tag counts and DNA methylation levels at 14 selected ACGT sites. Differentially methylated (DM) sites were identified by the program EdgeR,[38] with <5% FDR. A two-sided *t* test was used to evaluate the difference of tag counts at ACGT sites located to different genomic contents, while hypergeometric test was used to evaluate the enrichment of DM sites in promoters and gene bodies. The statistical analysis of differential gene expression was performed using Microsoft Excel and the data were presented as mean±standard error (SE). Student *t* test was used for comparison between groups and *P* values <0.05 were considered as significant, Bonferonni's correction was applied to account for multiple testing in gene expression analysis. For the expression analysis of 350 genes, Mann–Whitney test was also performed (data not shown). While the significance observed for the top 15 up- and down-regulated genes in Figure 5B remained, a few other differentially expressed genes included in Figure 5A would lose statistical support.

Results

Global DNA Methylation is Stable in the Developing Heart

To study the importance of DNA methylation in heart development, we carried out a genome-wide cytosine methylation analysis of E11.5 and E14.5 mouse embryonic hearts using MSFE/MPS with HpyCH4IV, a methylation-sensitive restriction enzyme recognizing ACGT. A total of 75 278 236 and 28 496 681 sequencing reads were obtained from the 2 E11.5 replicates that were mapped to 1 522 872 and 1 447 993 ACGT sites, respectively, while 89 562 687 and 65 198 805 reads were generated from the 2 E14.5 replicates that were mapped to 1 442 766 and 1 490 463 sites, respectively. At both stages, the reads from the 2 replicates covered >83% of the total 1 756 340 ACGT sites in the mouse genome. The Pearson's correlation coefficients were 0.894 and 0.896 between the 2 replicates for E11.5 and E14.5, respectively (data not shown); indicating that the quality of the data was high. The majority of ACGT sites had highly similar and correlated tag counts between E11.5 and E14.5 (Figure 1A), suggesting at the global level no significant methylation changes occurred between the 2 developmental stages. This finding of genome-wide stable DNA methylation in the developing hearts was supported by the LUMA data (Figure 1B).

Differential DNA Methylation Occurs Locally in the Developing Heart

We then catalogued the ACGT sites into genic sites (−50 kb of transcription start sites [TSS] to +0.5 kb of transcription end sites [TES] and intergenic sites) (Figure 2A), with the former further separated into 3 types: promoter proximal sites (−5 kb to +0.5 kb of TSS), gene body sites (+0.5 kb of TSS to TES), and promoter distal or enhancer sites (Figure 2B, top panel), and also determined the distribution of ACGT tag counts across the genome by intersecting the genome-wide ACGT methylation profiles with several genomic features, including CpG islands, CTCF-binding sites, RefSeq genes, repetitive elements, and regulatory elements (Figure 2B, bottom panel). The gene annotation, CpG islands, and repeats were downloaded from the UCSC browser. Additionally, lists of regulatory elements for embryonic hearts were obtained from previous studies, including 3596 P300 binding sites

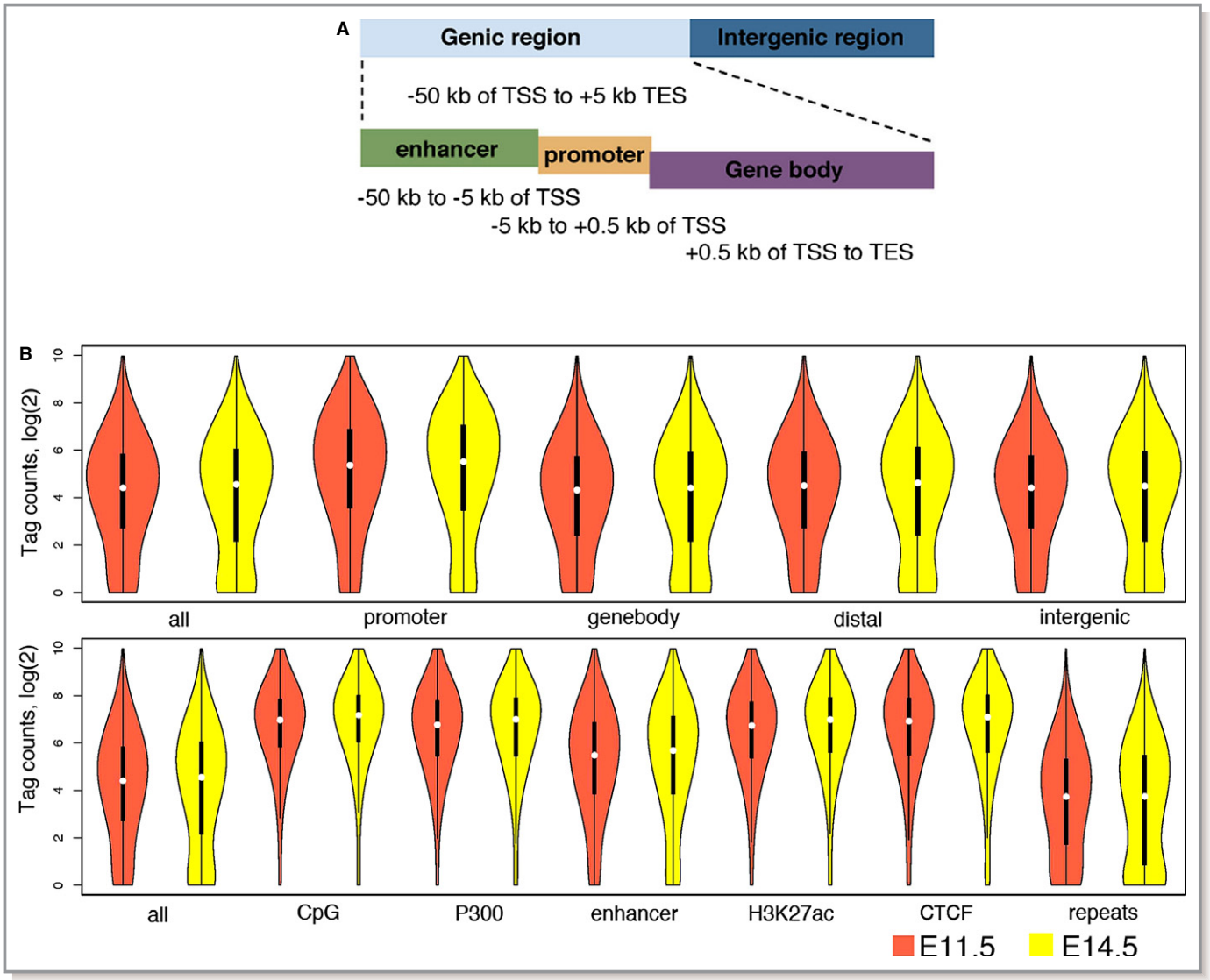


Figure 2. Distribution of DNA methylation across the mouse genome and various types of genomic elements in the developing heart. A, Cartoon depicting the genic regions (enhancer, promoter and genebody). B, Violin plot (a combination of a box plot and a kernel density plot) showing the distributions of tag counts for all ACGT (top) or differential methylated (bottom) sites in different genomic regions. As number of tag counts is inversely correlated to level of CG methylation, these plots indicate that gene promoters and regulatory regions exhibit significantly lower levels of DNA methylation than genomic background, and repetitive sequences are highly methylated. Plots in orange and yellow are for data from E11.5 and E14.5, respectively. TES indicates transcription end sites; TSS, transcription start sites.

identified for E11.5 hearts, 69 073 P300-marked enhancers, 14 874 CTCF sites, and 45 981 regions with the H3K27ac modification (a histone mark for active enhancer) for E14.5.[40–42]

The results showed that gene promoters and regulatory regions, represented by either CpG islands or enhancers (defined by P300 occupancy in E11.5 or H3K27ac enrichment in E14.5) had significantly lower levels of DNA methylation than genomic background, as ACGT sites within these regions had increased numbers of tag counts (P<2.2e-16, t test). Similarly, CTCF-binding sites generally have low levels of methylation. This is consistent with previous reports that CTCF is associated with hypomethylated regions.[43] In contrast, repetitive regions showed significantly higher levels of DNA methylation, as ACGT sites in these regions had decreased numbers of tag counts (P<2.2e-16, t test). Unexpectedly, the E14.5 cardiac enhancers exhibited higher DNA methylation than the elements marked by either P300 or H3K27ac at E14.5 (Figure 2B). This is probably due to the fact that those enhancers were identified based largely on H3K4me1 modifications, which is enriched in both active and poised enhancers.[44,45]

Next, we chose up to 14 ACGT sites with a range of different tag counts, representing 0%, 25%, 50%, 75%, or 100% methylation by massively parallel sequencing, and determined

their levels of methylation by MassArray. The results indicated that tag count was inversely correlated with the percentage of cytosine methylation (Figure 3A), thereby confirming the precision of the MSFE/MPS in quantifying methylation level, ie, tag counts measured accurately both the global and regional DNA methylation. We then set out to investigate how much methylation changed in the developing hearts between E11.5 and E14.5. After normalization by sequencing depths, ACGT sites with at least 1 sequencing tag in any of the 4 samples were evaluated for differential methylation using 2 complementary approaches. We used EdgeR, which modeled the tag counts by a negative binomial distribution, to determine ACGT sites that showed differential methylation. The result indicated that the majority of the ACGT sites were not differentially methylated in the developing hearts between the 2 stages, as <1% of sites were found to have different tags (nominal *P* value <0.05) (Figure 3B). Among the small fraction (2901) of the ≈1.64 million analyzed ACGT sites that were differentially methylated, 1946 (67.1%) and 955 (32.9%) sites exhibited increased and decreased methylation in the late stage hearts, respectively (FDR<0.05) (Figure 3C).[38] Of note, for the majority of these sites, the degree of difference was <50%, with no sites switching from a fully methylated to an unmethylated state.

We also compared the percentage of differentially methylated ACGT sites at various genic and intergenic regions with the percentage of the total analyzed ACGT sites located within the same defined regions. We found that the differentially methylated sites were significantly enriched in gene bodies (*P*<2.2e-16, hypergeometric test), as 51.6% of the differentially methylated sites versus 42.2% of all assayed sites were located to gene bodies (Figure 3D). On the contrary, differentially methylated ACGT sites were under-represented in promoter (4.6%) and enhancer sites (13.9%), while 6.4% and 16.7% of all ACGT sites were in promoter-proximal and enhancer regions, respectively. In total, 2032 (70%) of the 2901 sites were associated with genes, with 65.1% of them showing increased methylation at E14.5.

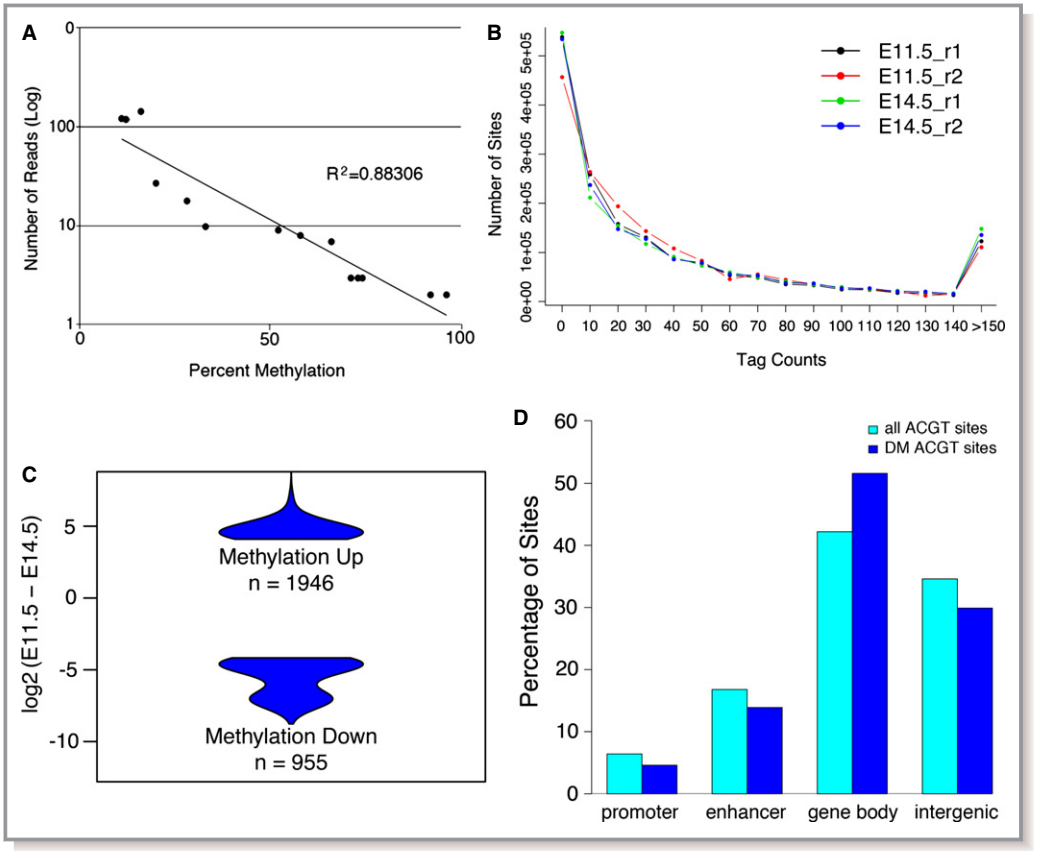


Figure 3. Analysis of the level of DNA methylation and differential methylation in the developing mouse heart. A, Methyl sensitive tiny fragment enrichment/massively parallel sequencing (MSFE/MPS) accurately detects levels of methylation as confirmed by Sequenom's MassArray of analyzed sites representing 0%, 25%, 50%, 75%, and 100% methylation determined by MSFE/MPS. B, Distribution of all ACGT sites with different numbers of tag counts from MSFE/MPS analysis. C, Violin plots showing the difference in tag counts for the 2901 ACGT sites that were significantly differentially methylated between E11.5 and E14.5. D, The distribution of all and differentially methylated (DM) ACGT sites in relation to gene annotation.

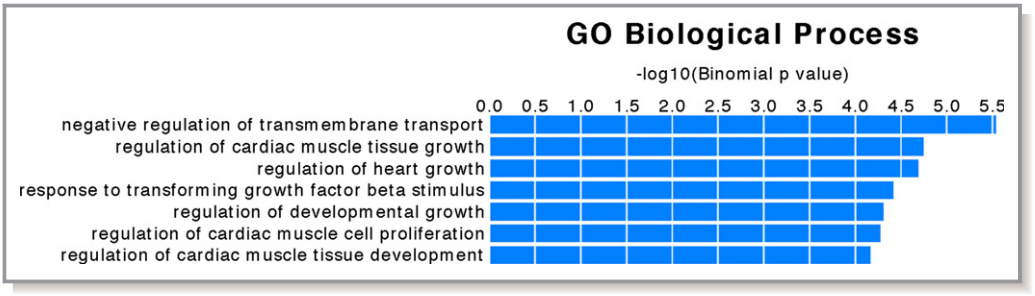


Figure 4. Gene ontology (GO) terms for the genes with differential DNA methylation during heart development. The software GREAT was used to characterize the 2901 differentially methylated sites for function. Four of the only 7 GO terms returned are involved in heart development and cardiac tissue growth.

Differential DNA Methylation in the Developing Heart Links to Heart Development

To investigate the functional importance of the small set of genes exhibiting differential methylation, we used the software GREAT to characterize the 2901 differentially methylated sites for their potential regulatory roles. Of the only 7 significantly associated gene ontology (GO) terms for biological processes returned by GREAT, 4 of them were related to heart development and cardiac tissue growth (Figure 4), indicating a significant enrichment of cardiac essential genes that have differential DNA methylation during heart development. These genes include *Erbb4*, *Gata6*, *Foxp1*, *Fgf2*, *Fgf9*, *Has2*, *Invs*, *Mef2c*, *Robo2*, and *Wnt2*. For example, *Foxp1* is important in cardiomyocyte proliferation,[42,46] while signaling from *Gata6* to *Wnt2* plays an important role in early cardiogenesis and inflow tract development,[47,48] *Mef2c* plays an essential role in heart development as a regulator of cardiac myogenesis within the right ventricle,[49,50] and *Has2* plays a role in heart valve development.[6,51] GREAT also reported that the affected genes were highly expressed in the cardiovascular system (P=5.3e-5) and they were implicated in vascular disease (P=1.4e-4) based on an analysis of Disease Ontology.[52]

Differential DNA Methylation Corresponds to Changes in Gene Expression in the Developing Heart

To directly test how the observed differential DNA methylation is related to gene expression changes in the developing heart, we picked 350 genes from the 1697 genes linked to the 2901 differentially methylated sites and performed qPCR to determine their expression levels in E11.5 and E14.5 hearts. These genes were chosen because they contained ACGT sites with a

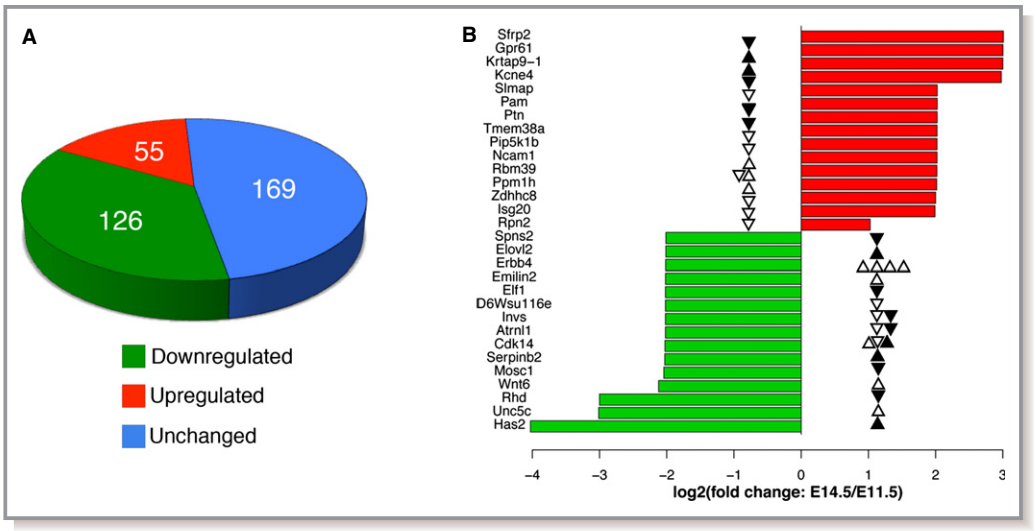


Figure 5. Expression changes for selected genes with differentially methylated sites. A, Gene expression analysis (n=3) of 350 differentially methylated genes between E11.5 and E14.5 showing that 181 genes are differentially expressed and 79 of those genes show consistent changes in DNA methylation. B, The top 15 upregulated genes (red) and downregulated genes (green) in E14.5. The solid and open triangles mark differentially methylated sites in regulatory regions (promoter proximal or distal) and gene bodies, respectively. The up and down directions of triangles indicate increased and decreased methylation, respectively.

Table 2. List of Genes With Differential Expression and Methylation

Gene Name	ACGT Chr Location	Region	% DM	Fold Change	Function
AI661453	Chr17 47582589	Genebody	**74%****	−2.02	Cellular component
Acaca	Chr11 84088161	Genebody	73%**	−2.01	Long chain fatty acid biogenesis
Abi1	Chr2 22839713	Genebody	73%**	−2.03	Negative regulation of cell growth and transformation, Ras signaling, Cardiovascular and placental development
Aff2	ChrX 66755393	Genebody	70%*	−2.02	RNA-binding protein
Atf6	Chr1 172773438	Genebody	**79%***	−2.00	Unfolded protein response during ER stress
Arhgef12	Chr9 42820787	Genebody	74%*	−2.02	Acts as a guanine nucleotide exchange factor
Ank	Chr15 27482616	Genebody	59%**	−2.03	Osteoblast/osteoclast differentiation, hypoxia responsive/regulated by Hif1α
Ank	Chr15 27513341	Genebody	78%**	−2.03	
Ank	Chr15 27427229	Genebody	**21%***	−2.03	
Ank	Chr15 27430132	Genebody	**52%**	−2.03	
Atrnl1	Chr19 58000444	Genebody	84%**	−4.06	G-protein coupled receptor signaling, may regulate energy homeostasis
Atrnl1	Chr19 57683177	Promoter	**29%**	−4.06	
Atrnl1	Chr19 58054451	Genebody	**35%**	−4.06	
Atxn1	Chr13 45770455	Genebody	67%**	−2.02	Chromatin binding factor that represses Notch signaling.
Brdt	Chr5 107807029	Genebody	**75%****	−2.00	Chromocenter organization, Spermatogenesis
Cacna2d3	Chr14 30485237	Genebody	74%**	−2.03	Voltage-gated calcium channel activity, Regulated by promoter methylation, contractility of ventricular myocytes
Cacna2d3	Chr14 30303225	Genebody	**22%**	−2.03	
Cd180	Chr13 103491174	Genebody	**66%***	−2.00	Innate immune response, life/death decision of B-cells
Cdk14	Chr5 4822015	Genebody	**76%****	−4.07	Cell cycle regulation
Cdk14	Chr5 5428041	Enhancer	**23%**	−4.07	
Casz1	Chr4 148329448	Enhancer	80%**	−2.00	Blood vessel development and lumen morphogenesis, differentially methylated in a tissue specific manner
Cdh4	Chr2 179440423	Genebody	75%**	−2.01	Calcium dependent cell adhesion, may play a role in retinal development, Regulated by methylation
Cdh4	Chr2 179292446	Genebody	**22%**	−2.01	
Cdh4	Chr2 179316146	Genebody	**21%**	−2.01	
Cdh4	Chr2 179385054	Genebody	**19%***	−2.01	
Clasp1	Chr1 120296717	Genebody	75%	−2.03	Regulation of microtubule dynamics, mitosis
Chrna9	Chr5 66367625	Genebody	71%**	−1.94	Ion transport, cochlea hair development
Chrna9	Chr5 66348791	Enhancer	20%**	−1.94	
Col9a1	Chr1 24203890	Genebody	63%**	−2.02	Expressed in developing heart, differentially methylated in cancer
Clasp2	Chr9 113641090	Enhancer	69%**	−2.02	Microtubule stabilization, mitosis
Creb1	Chr1 64569233	Enhancer	81%**	−2.02	Gene transcription, HIF-1-alpha transcription factor network
D6Wsu116e	Chr6 116164345	Genebody	76%**	−4.05	N/A
Csde1	Chr3 102840451	Genebody	59%**	−2.00	RNA binding and transcriptionally coupled mRNA turnover
Disc1	Chr8 127751886	Genebody	78%**	−2.02	Multiple roles in embryonic and adult neurogenesis. Associated with schizophrenia
Dach2	ChrX 110423200	Genebody	65%**	−2.04	Eye and limb development and sex determination
Dgkb	Chr12 38744479	Genebody	73%**	−1.88	Brain development
Dnajc11	Chr4 151311759	Genebody	78%**	−2.03	Heat shock binding protein
Dlx5	Chr6 6859827	Enhancer	77%*	−2.03	Transcriptional activator during bone development, Promotes cell proliferation, osteoblast differentiation. Cell type specific expression regulated by methylation
Dnajc2	Chr5 21291210	Promoter	**69%****	2.00	DNA replication

Continued

Table 2. Continued

Gene Name	ACGT Chr Location	Region	% DM	Fold Change	Function
Dlg2	Chr7 99358525	Genebody	60%**	−2.00	Regulation of synaptic stability, Chronic pain perception
Eif2ak4	Chr2 118271831	Genebody	86%*	−2.01	Hypoxia response
Emilin2	Chr17 71603514	Genebody	**78%****	−4.05	Extracellular matrix component, regulates methylation in breast cancer
Emilin2	Chr17 71606328	Genebody	**74%****	−4.05	
Fam73a	Chr3 151956508	Genebody	81%**	−2.01	Integral to membrane
Fam111a	Chr19 12661528	Genebody	78%**	−2.02	Simian virus 40 (SV40) host range restriction factor
Fbxw7	Chr3 84630582	Genebody	77**	−2.00	Mediates ubiquitination and subsequent proteasomal degradation of target proteins including NOTCH1, inactivated by promoter hypermethylation
Fancl	Chr11 26311200	Genebody	73%*	−2.04	Mediates monoubiquitination, may play a role in primordial germ cell proliferation
Fancl	Chr11 26368061	Genebody	96%*	−2.04	
Fbxw8	Chr5 118558780	Genebody	92%*	−2.02	Mediates ubiquitination and subsequent proteasomal degradation of target proteins, placental development
Ftsjd2	Chr17 29820177	Genebody	67%**	−2.01	Methyltransferase that mediates mRNA cap1
Fscn1	Chr5 143721993	Promoter	67%*	−2.03	Cell migration, motility, adhesion and cellular interactions
Has2	Chr15 56549727	Enhancer	**65%***	−16.31	Heart valve development
Grik4	Chr9 42754379	Promoter	79%*	−2.04	Glutamate receptor in CNS
Invs	Chr4 48429137	Genebody	67%**	−4.05	Embryonic heart tube left development and right pattern formation
Invs	Chr4 48288832	Promoter	24%*	−4.05	
Il6st	Chr13 113256622	Genebody	77%*	−2.01	Signal transduction. Plays a role in embryonic development, vascular endothelial growth
Hipk2	Chr6 38796532	Genebody	61%*	−2.00	Angiogenesis, marked for degradation by hif1-a in cancer
Itgb1	Chr8 131241575	Genebody	78%**	−2.03	Promotes endothelial cell motility and angiogenesis, Hif1 regulated in wound healing
Itsn1	Chr16 91786820	Genebody	79%*	−2.02	Adaptor protein linking endocytic membrane traffic and actin assembly machinery
Isg20	Chr7 86061520	Genebody	62%**	3.97	Viral response
Itga1	Chr13 115765151	Genebody	66%**	−2.04	Integrin and Collagen Binding, rapid methylation leading to initiation of megakaryocyte differentiation
Itga1	Chr13 115800600	Genebody	**20%****	−2.04	
Kcne4	Chr1 78770239	Enhancer	77%**	7.87	Potassium voltage channel, cardiac function (cardiomyopathy)
Kif26b	Chr1 180479937	Genebody	81%**	−2.00	Embryonic kidney development, plays a role in compact adhesion between mesenchymal cells
Klhl2	Chr8 67366115	Genebody	76%*	−2.02	Mediate ubiquitination of target proteins, Plays a role in the reorganization of actin cytoskeleton
Ksr1	Chr11 79001767	Enhancer	82%**	−2.04	Promotes MEK and RAF phosphorylation and activity
Ksr1	Chr11 78904857	Genebody	**20%**	−2.04	
Limk1	Chr5 135156130	Genebody	**80%***	−2.04	Regulation of actin filament dynamics, cell motility, cell cycle progression and differentiation
Lmf1	Chr17 25771254	Genebody	82%**	−2.04	Maturation and transport of lipoprotein lipase through the secretory pathway
Mkrn2	Chr6 115567892	Genebody	62%*	2.01	Neurogenesis
Ncoa7	Chr10 30373619	Genebody	75%**	2.02	Co-activation of several nuclear receptors
Ncam1	Chr9 49570879	Genebody	**61%***	4.06	Neural adhesion, pathological angiogenesis in oxygen induced retinopathy, ventricular wall thickening in hypertension, cardiac protection
Odz3	Chr8 49397492	Genebody	73%**	−2.00	Signal transduction, neuronal growth and tumorigenesis

Continued

Table 2. Continued

Gene Name	ACGT Chr Location	Region	% DM	Fold Change	Function
Odz3	Chr8 49336206	Genebody	**47%****	−2.00	
Odz3	Chr8 49341811	Genebody	**21%**	−2.00	
Pam	Chr1 99716878	Enhancer	79%**	4.07	Heart development and hypoxia response
Pde5a	Chr3 122538715	Genebody	79**	2.02	Signal transduction, cardiac muscle contraction and hypertrophy, hypoxia response
Pde11a	Chr2 75840713	Genebody	**70%***	1.98	Signal transduction, may play a role in vascular smooth muscle proliferation and contraction, cardiac contractility and immune cell activation
Pcgf5	Chr19 36450106	Promoter	71%*	2.02	Maintenance of transcriptional repressive state in development, including that of Hox genes
Pfkfb3	Chr2 11406131	Genebody	78%**	2.03	Induced by Hif1α
Pet112 l	Chr3 85403998	Genebody	70%*	2.03	Glutamyl-tRNA amidotransferase complex, Functions in mitochondria
Pip5k1b	Chr19 24602322	Genebody	72%**	4.06	Phosphorylation
Pnkd	Chr1 74336698	Genebody	65%**	2.00	Hydrolase activity, Plays an aggregative role in the development of cardiac hypertrophy via NF-kappa-B signaling
Pou2f1	Chr1 167866210	Promoter	70%**	−2.01	Regulates gene expression in response to stress and metabolic signals
Ppm1 h	Chr10 122245574	Genebody	67%	4.05	Phosphatase activity, drug response in cancer, associated with systemic lupus erythematosus
Ppm1 h	Chr10 122144175	Genebody	**19%****	4.05	
Prdm16	Chr4 153999781	Genebody	**68%****	2.02	Transcriptional regulation, Functions as a repressor of TGF-beta signaling
Ppwd1	Chr13 104995653	Genebody	67%**	2.02	Putative peptidylprolyl isomerase, may be involved in pre-mRNA splicing
Ptn	Chr6 36663240	Enhancer	75%**	4.07	Angiogenesis, tumorigenesis, regulation of hematopoietic stem cell self renewal, mammary gland development
Prmt8	Chr6 127665685	Genebody	79%*	−2.07	Arginine methyltransferase, embryonic and neural development, regulated by auto-methylation
Prkca	Chr11 108120704	Genebody	86%**	2.00	Regulation of transcription, cell growth, immune response, negative regulation of cell proliferation, apoptosis, differentiation, cardiac hypertrophy and angiogenesis
Ranbp3	Chr17 56833581	Genebody	78%*	2.02	Nuclear export, negative regulator of TGF-beta signaling through SMAD
Ptpro	Chr6 137362114	Genebody	71%**	2.00	Wnt-protein binding, Candidate tumor suppressor, aberrantly methylated in cancer
Ptpru	Chr4 131336827	Genebody	69%**	1.84	Cell proliferation and migration, maintenance of epithelial integrity, neural development and possible megakaryocytopoiesis
Rbfox3	Chr11 118610022	Genebody	65%*	1.97	RNA-binding, associated with neurocytoma and cerebral artery occlusion
Rhd	Chr4 134418089	Promoter	81%**	−8.00	Encodes member of Rh blood group proteins
Rbm39	Chr2 155977061	Genebody	75%	4.05	Transcriptional co-activator for steroid nuclear receptors, involved in pre-mRNA splicing
Rpia	Chr6 70726365	Genebody	67%**	2.02	Carbohydrate metabolism
Robo2	Chr16 74182881	Genebody	72%	−2.00	Heart Morphogenesis, linear hear tube formation, neuronal development
Rpn2	Chr2 157147564	Genebody	55%**	2.04	Ribosome binding, Dolichyl-diphosphooligosaccharide-protein glycotransferase activity
Rsu1	Chr2 13110936	Genebody	79%**	2.01	Ras signal transduction pathway
Rsu1	Chr2 13153759	Genebody	**50%***	2.01	
Rufy2	Chr10 62447503	Genebody	65%	2.03	Alzheimer's disease

Continued

Table 2. Continued

Gene Name	ACGT Chr Location	Region	% DM	Fold Change	Function
Sav1	Chr12 71078443	Genebody	76%**	2.03	Transcription, cell proliferation, cell death, cell migration, cell cycle exit, protein degradation and RNA splicing
Slc24a2	Chr4 86672633	Genebody	58%**	2.03	Calcium and potassium transport
Slmap	Chr14 27308224	Genebody	79%	4.07	Myoblast fusion
Slco5a1	Chr1 12939917	Genebody	61%	−1.97	Transporter activity
Sorbs2	Chr8 46655637	Genebody	79%**	2.02	Cytoskeletal adaptor activity and structural constituent of cytoskeleton
Sorbs2	Chr8 46649975	Genebody	**20%**	2.02	
Stxbp6	Chr12 46076554	Genebody	79%**	1.96	Regulates SNARE complex formation
St8sia5	Chr18 77402859	Enhancer	80%**	2.02	Synthesis of gangliosides
Srsf9	Chr5 115781199	Genebody	73%	2.03	Splicing
Tex9	Chr9 72307706	Genebody	67%**	2.02	—
Tbc1d16	Chr11 119004863	Genebody	64%**	1.99	Rab GTPase activator activity
Tbc1d16	Chr11 119017735	Genebody	**76%****	1.99	
Taf4a	Chr2 179700219	Genebody	72%**	2.01	Basal transcription
Tet2	Chr3 133187569	Genebody	85%**	2.01	DNA demethylation regulating transcription
Tmem38a	Chr8 75105110	Genebody	69%**	4.06	Potassium channel activity
Tnrc6a	Chr7 130306203	Genebody	73%**	2.02	Gene silencing by RNA and microRNA
Tmem135	Chr7 96306156	Genebody	82%*	2.02	Peroxisome organization
Tmem135	Chr7 96397839	Genebody	**29%**	2.02	
Tubg2	Chr11 101015396	Promoter	**19%**	−2.01	Major constituent of microtubules, structural molecule activity
Vmn1r73	Chr7 12307910	Enhancer	63%**	2.00	—
Wnk1	Chr6 119946851	Genebody	86%**	−1.98	Heart development, regulations of cell signaling, survival and proliferations, electrolyte homeostasis, cytoskeletal reorganization and sodium and chloride ion transport
Zbtb20	Chr16 43500263	Genebody	89%**	−2.01	Transcription factor involved in hematopoiesis, oncogenesis and immune response
Zbtb20	Chr16 43219787	Enhancer	**95%**	−2.01	
Zbtb20	Chr16 43343231	Genebody	98%	−2.01	
Zbtb20	Chr16 43455368	Genebody	**97%**	−2.01	
Wnt6	Chr1 74821915	Genebody	**65%**	−4.35	Tissue development
Zdhhc8	Chr16 18231749	Genebody	72%	4.00	Susceptibility to schizophrenia
Zfp385b	Chr2 77445243	Genebody	73%*	−2.02	Metal ion, nucleic acid, p53, and zinc ion binding, Apoptotic processes
Zfp385b	Chr2 77629407	Genebody	21%*	−2.02	
Enah	Chr1 183952945	Promoter	**25%**	−2.01	
Pkd2	Chr5 104885245	Promoter	**22%**	1.99	Tubular morphogenesis, associated with autosomal dominant polycystic kidney disease
Mylk2	Chr2 152734667	Promoter	**20%**	−1.91	Cardiac function and global muscle contraction
Ppp1r1c	Chr2 79544612	Promoter	**25%**	−1.93	Promotes cell cycle progression and increases cell susceptibility to TNF-induced apoptosis
Spns2	Chr11 72304383	Promoter	59%**	−4.03	Migration of myocardial precursors; cardiovascular, immunological and neural development
Elf1	Chr14 79879478	Promoter	31%	−4.05	Endothelial transcription factor
Ppp1r3c	Chr19 36813263	Promoter	**44%**	−2.01	Glycogen synthase, Regulated by Hif1α
Gucy1a3	Chr3 81943306	Genebody	23%**	−2.02	Cardiac function, vascular smooth muscle function
Gucy1a3	Chr3 81993497	Enhancer	**23%****	−2.02	

Continued

Table 2. Continued

Gene Name	ACGT Chr Location	Region	% DM	Fold Change	Function
Ank2	Chr3 126729907	Enhancer	**97%**	−2.01	Expression and targeting of SPTBN1 in neonatal cardiomyocytes and regulation of neonatal cardiomyocyte contraction rate
Sfrp2	Chr3 83534574	Enhancer	30%	8.05	Cell growth and differentiation, Wnt signaling, myogenesis and eye retinal development, methylation of gene is a potential marker for colorectal cancer
Erbb4	Chr1 68142043	Genebody	**23%**	−4.04	Heart development, cardiac muscle differentiation and postnatal cardiomyocyte differentiation, CNS development, neural crest cell migration, gene transcription, cell proliferation, differentiation, migration and apoptosis
Erbb4	Chr1 68846422	Genebody	**20%**	−4.04	
Erbb4	Chr1 68938673	Genebody	**38%**	−4.04	
Erbb4	Chr1 68954441	Genebody	**20%**	−4.04	
Erbb4	Chr1 69128731	Genebody	**24%**	−4.04	
Mysm1	Chr4 94660067	Enhancer	**28%****	−2.00	Histone modification and transcriptional co-activation
Foxp1	Chr6 98892345	Genebody	**19%**	−2.04	Cardiomyocyte proliferation
Foxp1	Chr6 99008217	Genebody	**22%***	−2.04	
Foxp1	Chr6 99018432	Genebody	**34%**	−2.04	
Foxp1	Chr6 99163145	Genebody	**20%**	−2.04	
Foxp1	Chr6 99197140	Genebody	23%	−2.04	
Foxp1	Chr6 99357158	Genebody	**22%**	−2.04	
Foxp1	Chr6 99391693	Enhancer	**43%***	−2.04	
Unc5c	Chr3 141321392	Genebody	**19%**	−8.07	Cell migration in neural development, axon extension and induction of apoptosis
Unc5c	Chr3 141342275	Genebody	**26%**	−8.07	
Unc5c	Chr3 141359065	Genebody	**20%**	−8.07	
Sox5	Chr6 143990289	Genebody	**20%**	−4.01	Embryonic development, cell fate determination, transcriptional regulation
Sox5	Chr6 144141969	Genebody	**19%**	−4.01	
Psd3	Chr8 70278799	Genebody	**19%***	−2.03	Guanine nucleotide exchange factor for ARF6
Psd3	Chr8 70338543	Genebody	**96%**	−2.03	
Psd3	Chr8 70459139	Genebody	**23%***	−2.03	
Dock1	Chr7 142059905	Genebody	**28%**	−2.01	Cytoskeletal rearrangements necessary for phagocytosis of apoptotic cells and in cell motility Guanine nucleotide exchange factor
Dock1	Chr7 142061686	Genebody	**19%**	−2.01	
Dock1	Chr7 142073658	Genebody	**30%**	−2.01	
Dock1	Chr7 142115224	Genebody	**24%**	−2.01	
Dock1	Chr7 142136783	Genebody	**24%**	−2.01	
Dock1	Chr7 142190260	Genebody	**53%**	−2.01	
Wwox	Chr8 117142250	Genebody	**31%**	−2.03	Apoptosis, TGFB1 signaling and TGFB1-mediated cell death. Inhibits Wnt signaling
Wwox	Chr8 117354691	Genebody	**26%**	−2.03	
Wwox	Chr8 117632214	Genebody	**22%**	−2.03	
Vegfc	Chr8 55148115	Enhancer	21%	2.02	Angiogenesis and endothelial cell growth
Vegfc	Chr8 55239360	Genebody	**18.5%***	2.02	
Vegfc	Chr8 55247617	Genebody	**22%**	2.02	
Pard3	Chr8 129591554	Genebody	**28%***	−2.00	Adaptor protein, asymmetrical cell division and cell polarization, plays a role in epithelial tight junctions
Pard3	Chr8 129640346	Genebody	**96%****	−2.00	
Pard3	Chr8 129830952	Genebody	**96%**	−2.00	
Pard3	Chr8 129845627	Genebody	21%**	−2.00	
Pard3	Chr8 129875488	Genebody	**22%**	−2.00	
Pard3	Chr8 129914177	Genebody	**24%**	−2.00	

Continued

Table 2. Continued

Gene Name	ACGT Chr Location	Region	% DM	Fold Change	Function
Thsd4	Chr9 59846658	Genebody	**19%***	−2.00	Attenuates TGFB signaling
Thsd4	Chr9 59968525	Genebody	**22%**	−2.00	
Thsd4	Chr9 60041974	Genebody	**19%***	−2.00	
Thsd4	Chr9 60098887	Genebody	**96%***	−2.00	
Rora	Chr9 68624882	Genebody	**19%****	−2.01	Regulated genes involved in lipid metabolism
Rora	Chr9 68726640	Genebody	**24%****	−2.01	
Rora	Chr9 68865723	Genebody	**19%**	−2.01	
Utrn	Chr10 12126998	Genebody	**20%***	2.02	Anchors cytoskeleton to plasma membrane
Utrn	Chr10 12213501	Genebody	**20%**	2.02	
Utrn	Chr1012395503	Genebody	**19%**	2.02	
Mef2c	Chr13 83687636	Genebody	**96%**	−1.99	Transcription activator controls cardiac morphogenesis and myogenesis, plays a role in vascular development
Mef2c	Chr13 83755977	Genebody	**30%****	−1.99	
Mef2c	Chr13 83797333	Genebody	**25%**	−1.99	
Odz2	Chr11 35940645	Genebody	**35%***	−1.98	Neural development
Odz2	Chr11 36164904	Genebody	**20%***	−1.98	
Odz2	Chr11 36400641	Genebody	32%**	−1.98	
Odz2	Chr11 36725324	Genebody	**27%****	−1.98	
Ptprk	Chr10 27825617	Genebody	**20%***	−2.01	Regulation of cell contact and adhesions, tumor invasion/metastasis, Negative regulator of EGFR signaling
Ptprk	Chr10 28084547	Genebody	**24%**	−2.01	
Ptprk	Chr10 28226602	Genebody	**24%****	−2.01	
Enox1	Chr14 77538059	Enhancer	**97%**	−2.00	Oxidoreductase activity, nucleotide binding
Enox1	Chr14 77771534	Genebody	**23%**	−2.00	
Enox1	Chr14 77876556	Genebody	**20%***	−2.00	
Enox1	Chr14 77977395	Genebody	20%**	−2.00	
Zfpm2	Chr15 40787554	Genebody	**29%**	−2.01	Transcription regulator important in heart morphogenesis and coronary vessel development from epicardium
Zfpm2	Chr15 40824705	Genebody	**95%****	−2.01	
Zfpm2	Chr15 40848971	Genebody	**19%**	−2.01	
Dach1	Chr14 98263585	Genebody	97%*	−1.99	Transcription factor important in organogenesis
Dach1	Chr14 98304412	Genebody	**26%**	−1.99	
Dach1	Chr14 98510700	Genebody	**96%**	−1.99	
Erc2	Chr14 28475844	Genebody	**97%**	−1.99	Cytomatrix organization at nerve terminal active zones regulating release of neurotransmitters
Erc2	Chr14 28668685	Genebody	**30%**	−1.99	
Erc2	Chr14 28822634	Genebody	**95%**	−1.99	
Erc2	Chr14 28845440	Genebody	**24%**	−1.99	
Vps13b	Chr15 35600466	Genebody	**23%****	−1.99	Protein sorting in post Golgi membrane traffic, may play a role in development and function of the eye, hematological system and the CNS
Vps13b	Chr15 35777972	Genebody	**19%**	−1.99	
Vps13b	Chr15 35817931	Genebody	**23%***	−1.99	
2810403A07Rik	Chr3 88506464	Genebody	84%	−2.01	RNA binding
Tle4	Chr19 14528880	Genebody	**20%***	−2.01	Transcriptional co-repressor of members in Wnt signaling
Tle4	Chr19 14621827	Genebody	**24%**	−2.01	
Prkg1	Chr19 30823222	Genebody	**19%***	−2.02	Regulates cardiac function, smooth muscle contraction, platelet activation and adhesion
Prkg1	Chr19 31528132	Genebody	**24%**	−2.02	

Continued

Table 2. Continued

Gene Name	ACGT Chr Location	Region	% DM	Fold Change	Function
Prkg1	Chr19 31595749	Genebody	**20%**	−2.02	
Hif1a	Chr12 75032797	Genebody	25%	−2.00	Hypoxia response, Master transcriptional regulator
Malt1	Chr18 65586771	Promoter	19%*	−2.00	NF-kappaB activation
Igf2r	Chr17 12965268	Promoter	**44%**	−2.02	Activation of TGF-β. Intracellular trafficking of lysosomal enzymes and degradation of IGF2, tumorigenesis, Paternally imprinted
Krtap9-1	Chr11 99731190	Promoter	**27%**	8.00	Hair shaft formation
Ccdc40	Chr11 119085948	Promoter	**20%**	−4.03	Motile cilia function. Ciliary dyskinesia type 15
Cftr	Chr6 18119186	Promoter	**20%**	−2.00	Chloride channel and enzyme binding, associated with cystic fibrosis
Cftr	Chr6 18242079	Genebody	**42%**	−2.00	
Pdzd2	Chr15 12315935	Genebody	**23%**	2.02	Prostate tumorigenesis
Pdzd2	Chr15 12342544	Genebody	**27%**	2.02	
Pdzd2	Chr15 12521967	Promoter	**19%**	2.02	
Elovl2	Chr13 41317598	Promoter	**23%**	−4.04	Atherosclerosis, protein binding and fatty acid elongase activity
Gpr18	Chr14 122316980	Promoter	**96%**	1.98	Regulation of immune system, bipolar disorder
Rpn1	Chr6 88030514	Promoter	24%	−2.01	Dolichyl-diphosphooligosaccharide-protein glycotransferase activity
Mosc1	Chr1 186637543	Promoter	19%	−4.12	—
Tmem150c	Chr5 100589921	Promoter	**25%**	−2.04	—
Shroom3	Chr5 93236380	Promoter	**27%**	−2.01	Regulation of cell shape in neuroepithelium
Calb1	Chr4 15806105	Promoter	**24%**	−1.95	Functions in purkinje cells
Gpr61	Chr3 107962781	Promoter	**22%****	8.01	G-protein coupled receptor signaling
Cd40	Chr2 164871483	Enhancer	**96%**	−2.02	Immune and inflammatory response
Ephx1	Chr1 182951005	Promoter	**23%**	2.02	Cis-stilbene-oxide hydrolase activity, epoxide hydrolase activity. Plays a role in preeclampsia
Arhgap29	Chr3 121628392	Enhancer	96%	−2.00	Rho GTPase activator activity, essential role in blood vessel tubulogenesis
Fbxl4	Chr4 22244260	Enhancer	**21%**	−2.02	Cell cycle control
Fbxl4	Chr4 22264607	Enhancer	**21%**	−2.02	
Zmat3	Chr3 32233835	Genebody	96%**	−1.96	TP53-dependent growth regulatory pathway and TP53-mediate apoptosis, inhibits tumor cell growth
Zmat3	Chr3 32278426	Enhancer	**22%**	−1.96	
Fam125b	Chr2 33790838	Enhancer	**23%****	−2.03	Vesicular trafficking
Serpinb2	Chr1 109370955	Enhancer	**31%**	−4.08	Serine-type endopeptidase inhibitor activity
Rims1	Chr1 22615396	Genebody	**97%***	−2.02	Exocytosis, maintenance of neurotransmitter release and regulation of release during short-term synaptic plasticity
Rims1	Chr1 22763512	Genebody	**96%**	−2.02	
Dst	Chr1 34249899	Genebody	**20%**	−2.01	Cytoskeletal linker protein, Regulation of keratinocyte polarity and mobility
Dst	Chr1 34315083	Genebody	**20%**	−2.01	
Dst	Chr1 34322642	Genebody	**26%**	−2.01	
Etl4	Chr2 20373784	Genebody	**23%**	2.00	Intervertebral disk development
Etl4	Chr2 20576360	Genebody	**95%***	2.00	
Esrrg	Chr1 189527991	Genebody	**46%**	−2.00	Transcriptional activator via estrogen response elements
Esrrg	Chr1 189976300	Genebody	**21%***	−2.00	
Dnm3	Chr1 163949959	Genebody	**22%**	−1.99	Megakaryocyte development, likely involved in endocytosis
Dnm3	Chr1 164108264	Genebody	**40%****	−1.99	
Rbms1	Chr2 60615270	Genebody	25%	−2.02	

Continued

Table 2. Continued

Gene Name	ACGT Chr Location	Region	% DM	Fold Change	Function
Rbms1	Chr2 60789032	Genebody	**19%**	−2.02	Cell cycle progression, apoptosis, DNA replication and gene transcription.
Lrba	Chr3 86163560	Genebody	**23%**	−2.01	Signal transduction and vesicle trafficking
Lrba	Chr3 86267521	Genebody	**21%**	−2.01	
Plcb1	Chr2 134819839	Genebody	**23%**	−1.99	Intracellular transduction of extracellular signals
Plcb1	Chr2 135145732	Genebody	**25%**	−1.99	
Meis2	Chr2 115688950	Genebody	**96%**	−2.01	Transcriptional regulation
Meis2	Chr2 115750462	Genebody	**96%**	−2.01	
Bach2	Chr4 32560314	Genebody	**21%***	−2.00	Transcriptional regulation
Bach2	Chr4 32629036	Genebody	**21%**	−2.00	
Kcnd3	Chr3 105447527	Genebody	**16%***	−1.84	Smooth muscle contraction, heart rate, insulin secretion, neuronal excitability and cell volume
Fam19a1	Chr6 96068370	Genebody	**31%**	−1.91	Regulators of immune cells and cells of the nervous system
Fam19a1	Chr6 96235296	Genebody	**19%****	−1.91	
Drosha	Chr15 12715566	Enhancer	**22%**	−2.03	Cleaves ds-RNA in micro RNA processing
Drosha	Chr15 12817558	Genebody	**27%**	−2.03	
Gsk3b	Chr16 38062422	Enhancer	**20%**	−2.01	Negative regulator in hormonal control of glucose homeostasis, Wnt signaling and the regulation of transcription factors and microtubules. Regulates NFatc1 expression. Mediates development of insulin resistance
Gsk3b	Chr16 38138300	Genebody	**20%****	−2.01	
Gsk3b	Chr16 38218276	Genebody	**96%**	−2.01	
Pmm2	Chr16 8627532	Enhancer	**33%**	−2.02	Glycoprotein biosynthesis
Osta	Chr16 32515415	Enhancer	31%	−2.00	Transporter activity
Krt8	Chr15 101867475	Enhancer	**23%**	−4.02	Signal transduction and cellular differentiation
Slc38a4	Chr15 96905406	Enhancer	**96%**	−2.00	Sodium-dependent amino acid transporter
Slc38a2	Chr15 96516885	Enhancer	20%*	−2.03	Supply of maternal nutrients to fetus through placenta, transport of amino acids at blood-brain barrier
Adra2a	Chr19 54118496	Promoter	**26%**	1.99	Mediates the catecholamine-induced inhibition of adenylate cyclase

Table provides differentially expressed genes with corresponding differential methylation (DM) sites. Bolded DM values indicate decreased methylation at E14.5 whilst unbolded DM values indicates increased methylation at E14.5.
*Notes $P<0.05$ in methylation changes and **notes $P<0.01$ in methylation changes. Gene functions are summarized.

≥50% change in methylation between the 2 stages, their known function (such as roles in embryonic development preferentially heart development), and/or presence of multiple differentially methylated sites. Change in mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method and genes were ranked based on fold change.[39] Of the 350 genes assayed in the gene expression analysis, 181 (51.7%) genes, including *Erbb4*, *Has2*, *Invs*, *Robo2*, and *Vegfc*, were differentially expressed between E11.5 and E14.5 (>1.2-fold change and $P<0.05$; adjusted for multiple testing), and among these, expression of 55 genes was upregulated whereas expression of 126 genes was downregulated (Figure 5A, Table 2).

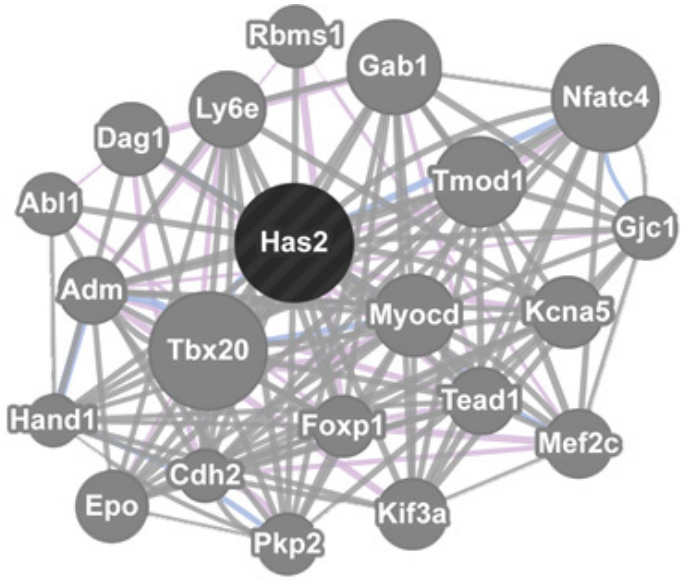
Recent studies of the correlation between DNA methylation and gene expression have found that increased promoter and enhancer methylation often lead to gene silencing while DNA methylation at gene bodies corresponds with gene activation.[53] We therefore examined the functional correlation between differential gene expression and changes in DNA methylation. We found that among the top 15 downregulated genes at E14.5, 4 of them contained an increase in methylation of sites located within promoter or enhancer regions and an additional 4 showed decreased methylation in their gene bodies (Figure 5B). Of the top 15 upregulated genes, 4 had decreased methylation in their enhancers and 3 exhibited increased methylation in their gene bodies. We found that, while 12.7% (23) of the 181 differentially expressed genes contained differentially methylated sites within the promoter region, methylation of only 60.8% (14) of those genes was predictive of their expression difference between E11.5 and E14.5, and overall 43.6% (79) of genes had differentially methylated sites predictive of expression change. The findings suggest that not all DNA methylation is

functional; most genes are regulated independent of methylation.[54] Nevertheless, the observed correlations between gene expression and DNA methylation during heart development do support that DNA methylation regulates expression of a subset of genes during heart development.

Increased DNA Methylation at Enhancers is Associated With Decreased Expression of the Cardiac-Essential Gene *Has2* in the Developing Heart

These correlations suggest a regulatory relationship between DNA methylation and cardiac-important genes. Notably, *Has2* is essential for endocardial to mesenchymal transformation and heart valve formation.[6,51,55,56] Gene network analysis using the Genemania open freeware (http://www.genemania.org) further revealed potential genetic and/or physical interactions among genes or pathways involved in heart development. The top 20 genes that were identified to interact (either genetically or physically) with Has2 by the network analysis were significantly enriched with functions involved in heart development. These genes included *Cdh2*, *Epo*, *Kcna5*, *Myocd*, *Tbx20*, *Hand1*, *Mef2c*, and *Nfatc4* (Figure 6).[49,57–59] Regulation of these genes by DNA methylation to influence their expression will ultimately affect their

A



B

Function	FDR	Genes in network	Genes in genome
cardiac muscle tissue development	7.66E-11	9	150
cardiac ventricle development	2.20E-08	7	99
cardiac chamber development	6.16E-08	7	121
cardiac ventricle morphogenesis	1.31E-07	6	68
heart morphogenesis	8.63E-07	7	189
cardiac chamber morphogenesis	1.41E-06	6	107
cardiac muscle tissue morphogenesis	5.61E-06	5	60
muscle tissue morphogenesis	8.01E-06	5	66
muscle organ morphogenesis	1.28E-05	5	74
muscle cell development	1.34E-05	6	169
organ growth	5.06E-05	5	101
ventricular cardiac muscle tissue morphogenesis	1.27E-04	4	45
regulation of cardiac muscle tissue development	1.53E-04	4	48
ventricular cardiac muscle tissue development	1.54E-04	4	49
cardiac muscle tissue growth	1.83E-04	4	52
smooth muscle cell differentiation	1.89E-04	4	54
cardiac chamber formation	1.89E-04	3	12
embryonic heart tube morphogenesis	2.07E-04	4	56
heart growth	2.26E-04	4	58
regulation of cardiac muscle cell differentiation	3.14E-04	3	15
embryonic heart tube development	4.39E-04	4	70
vasculogenesis	7.19E-04	4	80
determination of left/right symmetry	8.78E-04	4	85

Figure 6. Network analysis for DNA methylation-regulated Has2. Top: Genemania analysis reveals multiple relationships between Has2 and multiple cardiac genes including Myocd, Kcna5, Mef2c, Hand1, Tbx20 and Nfatc4. Bottom: Top functions of genes in the Has2 network.

pathway and downstream functions, which are essential for heart development.

Previous knockout studies in mice have shown that *Has2* is essential for development of cardiac valves and septa.[51,56] Here, our DNA methylation analysis indicated that an ACGT site located within 1 kb of a previously determined enhancer of *Has2*, marked by enriched H3K27ac at E14.5,[44] exhibited an increase in methylation as confirmed by Sequenom's MassArray (Figure 7A). Based on what is known about DNA methylation, we expected to see a decrease in *Has2* expression at E14.5, with confirmation by qPCR analysis (Figure 7B). To further characterize its expression in the developing hearts, we carried out RNA in situ hybridization. The results showed that *Has2* expres-

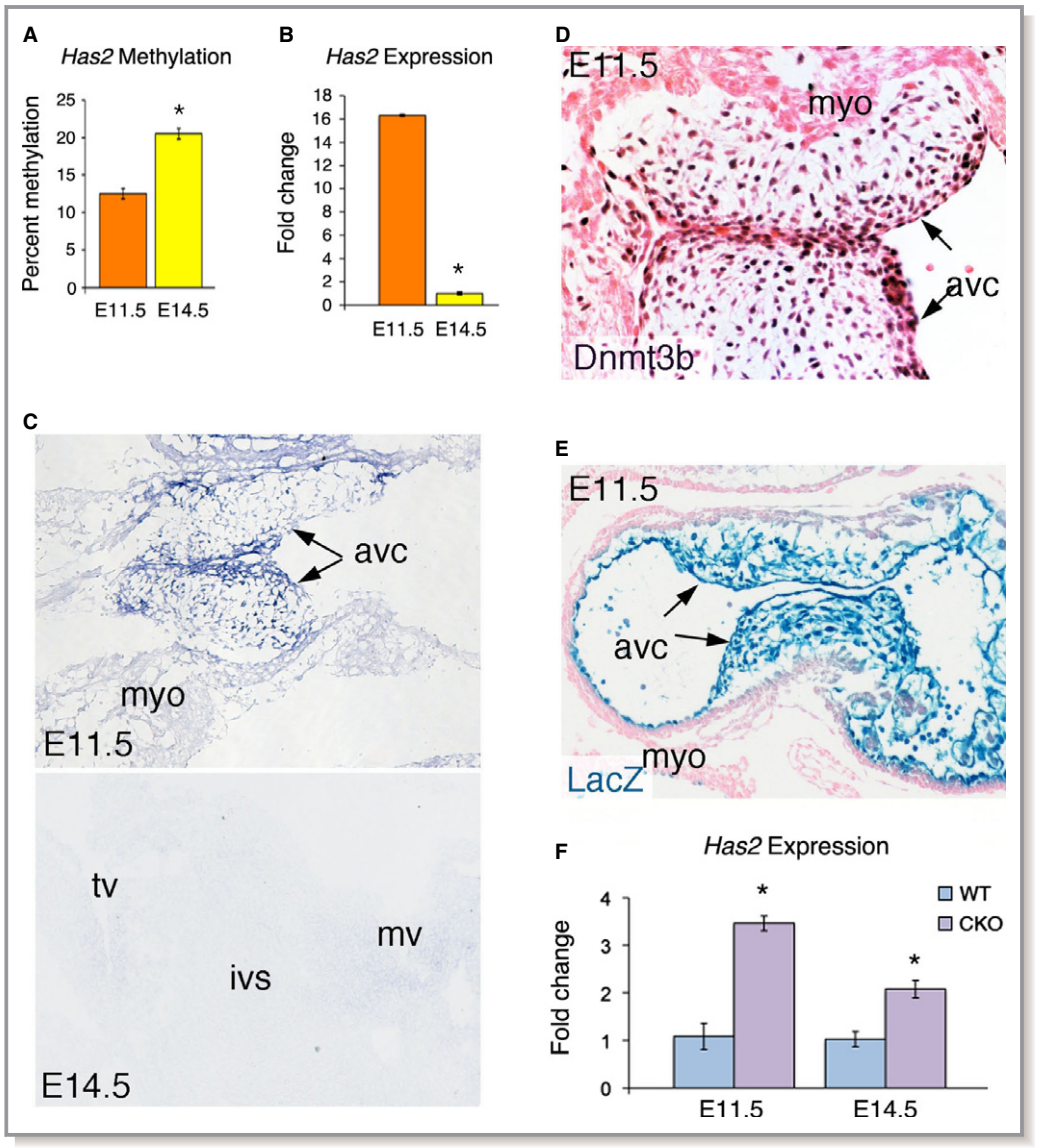


Figure 7. *Has2* expression is regulated by Dnmt3b. A, MassArray showing increased *Has2* enhancer methylation at E14.5 (n=2). B, RT-qPCR showing decreased *Has2* expression at E14.5 (n=3). C, RNA in situ hybridization showing that *Has2* expression is predominantly in the atrioventricular canal (avc), with less expression in the myocardium (myo) at E11.5; and the expression is diminished by E14.5. mv/tv, mitral/tricuspid valve; ivs, interventricular septum. D, IHC showing Dnmt3b is predominantly expressed in AVC at E11.5. E, X-gal staining showing the Nfatc1-Cre mediated LacZ expression in the AVC at E11.5. F, RT-qPCR showing that deletion of *Dnmt3b* resulted in increased *Has2* expression at E11.5 and E14.5 (n=3). Error bars represent standard error. *Marks statistical significance ($P<0.001$, 2 factor ANOVA in [F]). ANOVA indicates analysis of variance; CKO, conditional knockout; IHC, immunohistochemistry; RT-qPCR, quantitative real-time polymerase chain reaction; WT, wild type.

sion was predominately expressed in the endocardial cells and their mesenchymal progeny cells that form the primitive heart valves at E11.5, but its expression markedly diminished by E14.5 (Figure 7C), consistent with its function in endocardial to mesenchymal transformation around E11.5 for heart valve development.

Dnmt3b Suppresses Expression of the Cardiac-Essential Gene *Has2* in the Developing Heart

We next chose to determine experimentally the function of DNA methylation in *Has2* expression in the developing heart valves. To overcome the difficulty of not being able to directly assay the in vivo role of methylation of the *Has2* enhancer on the expression of the gene in the developing heart, we inactivated the activity of DNA methyltransferase 3b (*Dnmt3b*), which is responsible for de novo methylation during embryonic development.[22] First, we showed that, like *Has2*, *Dnmt3b* was expressed predominately in the endocardial cells, precursor cells for the heart valves (Figure 7D). The expression pattern of *Dnmt3b* suggests that it has a role in DNA methylation in the endocardial cell lineages and may therefore regulate *Has2* expression in the developing heart valves. Indeed, deletion of *Dnmt3b* in the endocardial cells and their valve progeny, using the endocardial-specific *Nfatc1*^{Cre} mice (Figure 7E),[9] resulted in significantly increased *Has2* expression in the developing heart valves at E11.5 and E14.5 (Figure 7F). The results support that DNA methylation of the *Has2* enhancer plays a role in repressing its expression during heart development.

Discussion

In this study, we generated a developmental profile of DNA methylation using methyl sensitive tiny fragment enrichment coupled with massively parallel sequencing (MSFE/MPS). Greater coverage and increased sensitivity for the detection of methylation are achieved using this method compared with microarray-based techniques. The method also provides more detailed information specific for ACGT sites and is capable of detecting intermediate levels of methylation, allowing for detection of modest changes in methylation.[60]

Our technique was adapted from the HpaII tiny fragment enrichment by ligation mediated PCR (HELP)-tagging assay developed by Suzuki et al[32,33] In the original HELP-tagging assay a methylation sensitive restriction enzyme, HpaII, is used to assess methylation of CpG dinucleotides located within its recognition site 5′-CCGG-3′. We modified this technique by using a different methylation-sensitive restriction enzyme, HpyCH4IV, whose recognition site is 5′-ACGT-3′ even though it lacks a methylation-insensitive isoschizomer. Previous studies have confirmed that sequencing reads/tags from a single methylation sensitive restriction enzyme without its isoschizomer are highly correlated with methylation status.[32,61] Furthermore, we used independent validation methods, such as LUMA and MassArray to confirm methylation levels determined by the MSFE/MPS.

HpyCH4IV provides comparable genome coverage to HpaII, having 1.7 million recognition sites located throughout the genome. Future studies using both enzymes will not only double the coverage but also examine and compare DNA methylation in both CG rich and non-CG rich regions. In addition, the use of HpyCH4IV will, in future studies, allow us to directly examine, in a genome-wide manner, the effect of methylation on transcription factor binding sites such as Hif1α, whose consensus binding sequence is 5′-ACGTG-3′ and has been shown to be regulated by DNA methylation.[62]

The original analytical pipeline was also modified to analyze our MPS data generated by using HpyCH4IV.[37] Within the HELP-tagging protocol an internal experimental control is used in which contaminating fragments are recognized based on the absence of the digested restriction site.[60] Additionally, to better identify differentially methylated sites between developmental stages, we generated a threshold to determine levels of methylation at individual loci. We employed these modifications to generate a genome wide developmental profile of methylation at ACGT sites in the developing heart. The results showed no significant global change in methylation over mid-stage heart development, although our study did not include the repetitive regions of the genome as they could not be aligned and thus discarded in the pipeline. We further confirmed that there is no significant change in global methylation patterns using LUMA.

Although drastic changes in global DNA methylation are not present during heart development, differential methylation was detected at a small subset of individual loci throughout the genome in this study. Furthermore, we detected a number of differentially methylated sites in which the change in methylation between E11.5 and E14.5 corresponds with the observed change in expression of the nearby gene, suggesting that there is a regulatory relationship between DNA methylation and the expression of cardiac-important genes including *Has2*.

Has2 has previously been identified to be essential for heart development, playing a role in epicardial cell differentiation, heart valve development, and septation.[51,55] A DNA methylation-regulated cardiac gene program was generated by performing a network analysis for Has2 using Genemania. Network analysis revealed multiple relationships between Has2 and other cardiac-important gene products, including previously mentioned Tbx20, involved in endocardial cushion formation and heart valve remodeling,[57] Hand 1 involved in ventricle morphogenesis,[58] and Nfatc4, a member of the

nuclear factor of activated T-cell family that are known to be essential for heart development.[59] Additional connections have been identified between Has2 and Gjc1 (Connexin45), known to play an important role in cardiac morphogenesis and conduction,[63] and Cdh2, Epo, Kcna5, Mef2c, and Myocd, which are essential for heart development and function.

We further studied methylation of *Has2* and its expression in the developing heart in detail, as it has an increase in enhancer methylation that corresponds with a decrease in its expression over mid-stage heart development in the developing heart valves. We showed by qPCR analysis, RNA in situ hybridization, and genetic knockout that Dnmt3b regulates *Has2* expression, possibly through its enhancer methylation.

In this study we were able to assay 1.64 million ACGT sites for potential changes in DNA methylation during mid-stage cardiac development, identifying 2901 differentially methylated sites, and determined a number of developmentally important cardiac genes that are likely to be regulated by DNA methylation. However, current methods for studying functionality of DNA methylation at specific sites in the developing heart are limited. We circumvented this limitation by revealing the dependence of *Has2* expression on *Dnmt3b* expression in the developing heart valves. Our results are mainly discovery and by necessity preliminary, and will require a much larger sample size to detect more sites with more subtle DNA methylation changes between the 2 developmental stages.

In conclusion, our results support an essential role for DNA methylation in the regulation of cardiac essential genes during heart development and suggest abnormal DNA methylation may contribute to the pathogenesis of congenital heart disease. Using this study as a starting point, we plan to investigate further candidate genes as well as the role of additional epigenetic modifications that may play a role in heart development and disease.

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Disclosures

None.

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