COMPARATIVE GENOME-WIDE DNA METHYLATION ANALYSIS IN MYOCARDIAL TISSUE FROM DONORS WITH AND WITHOUT DOWN SYNDROME

Romina B. Cejas^{a, d}, Jie Wang^{b, d}, Rachael Hageman-Blair^c, Song Liu^b and Javier G. Blanco^{a, e}

Authors' affiliation:

^a Department of Pharmaceutical sciences, School of Pharmacy and Pharmaceutical Sciences, State University of New York at Buffalo, Buffalo, New York, United States of America

^b Department of Biostatistics and Bioinformatics, Roswell Park Comprehensive Cancer Center, Buffalo, New York, United States of America

^c Department of Biostatistics, School of Public Health and Health Professions, State University of New York at Buffalo, Buffalo, New York, United States of America

^d Both authors contributed equally to this work.

^e Corresponding author. jgblanco@buffalo.edu. Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, The State University of New York, 470 Pharmacy Building, Buffalo, NY 14214 – 8033, United States of America

Abstract

Down syndrome (DS, trisomy 21) is the most common major chromosomal aneuploidy compatible with life. The additional whole or partial copy of chromosome 21 results in genome-wide imbalances that drive the complex pathobiology of DS. Differential DNA methylation in the context of trisomy 21 may contribute to the variable architecture of the DS phenotype. The goal of this study was to examine the genomic DNA methylation landscape in myocardial tissue from non-fetal individuals with DS. More than 480,000 unique CpG sites were interrogated in myocardial DNA samples from individuals with (n = 12) and without DS (n = 12) using DNA methylation arrays. A total of 93 highly differentially methylated CpG sites and 16 differentially methylated regions were identified in myocardial DNA from subjects with DS. There were 18 differentially methylated CpG sites in chromosome 21, including 5 highly differentially methylated sites. A CpG site in the *RUNX1* locus was differentially methylated in DS myocardium, and linear regression suggests that donors' age, gender, DS status, and *RUNX1* methylation may contribute up to ~51% of the variability in *RUNX1* mRNA expression. In DS myocardium, only 58% of the genes overlapping with differentially methylated regions codify for proteins with known functions and 24% are non-coding RNAs. This study provides an initial snapshot on the extent of genome-wide differential methylation in myocardial tissue from persons with DS.

Keywords

Epigenetics, Myocardium, Trisomy 21, Cardiac methylation, Heart

Abbreviations

DS, Down syndrome; non-DS, non-Down syndrome; DNA, deoxyribonucleic acid; *RUNX1*, runt-related transcription factor 1; RNA, ribonucleic acid; mRNA, messenger RNA; DMR, differentially methylated region; SNP, single nucleotide polymorphism; FDR, false discovery rate; *ACTB*, actin beta; PCR, polymerase chain reaction; RT-qPCR, quantitative reverse transcription polymerase chain reaction; DSCR, Down syndrome critical region; GO, gene ontology; RRBS, reduced representation bisulfite sequencing.

1. Introduction

In humans, Down syndrome (DS, trisomy 21) is the most common major chromosomal abnormality compatible with life, with an approximate incidence of 1 in 700 to 1 in 900 live births (1). DS is caused by the presence of a full or partial extra copy of chromosome 21. Individuals with DS exhibit a range of phenotypic characteristics and distinctive physical features. Persons with DS are at increased risk for the development of various comorbidities through the lifespan. In general, the incidence of congenital heart disease, thyroid gland dysfunctions, eye and hearing disorders, pediatric leukemia, and testicular cancer is higher in persons with DS in comparison to individuals without DS (2). For example, the incidence of congenital heart disease in the DS setting is 50 times higher than in the “general population”, and the prevalence of congenital heart defects in individuals with DS ranges from 43% to 58% (3, 4).

Variable DNA methylation levels in specific regions across the genome influences the control of gene expression in different cell types and tissues. In general, relative increases in the levels of methylation in distinct CpG dinucleotides located in gene promoter regions or other regulatory regions may result in transcriptional repression (5). Differential DNA methylation in the context of trisomy 21 may contribute to the complex architecture of the DS phenotype. In this regard, the presence of differentially methylated loci has been examined in some tissues from subjects with DS. For example, Serra-Juhé et al. interrogated approximately 28,000 CpG sites in fetal heart DNA samples from donors with DS and congenital heart defects (CHD) and in samples from donors with DS and without CHD. The authors found that regions in the *GATA4* gene were hypermethylated in cardiac DNA from fetuses with DS with or without CHD, as well as in fetuses with isolated heart malformations (6).

There is a paucity of reports describing the genomic DNA methylation landscape in myocardial tissue from non-fetal individuals with DS. The goal of this pilot study was to examine genome-wide DNA methylation profiles in myocardial DNA samples from donors with and without

DS. Myocardial DNA samples were examined to interrogate > 480,000 individual CpG sites using the Illumina HumanMethylation450 BeadChip platform. Next, bioinformatic analysis was performed in order to identify differentially methylated loci in myocardial methylomes. This study contributes new data to continue to define the extent of epigenetic variability in the context of DS.

2. Materials and Methods

2.1. Human Tissue Specimens

The Institutional Review Board of the State University of New York at Buffalo (UB-IRB) approved this research. UB-IRB determined that this research is not research with human subjects. This research meets exempt criteria, 45 CFR 46.101(b)(4). Human myocardial tissue samples were provided by the National Disease Research Interchange (NDRI), the Cooperative Human Tissue Network (CHTN), and the National Institute of Child Health and Human Development Brain and Tissue Bank (NICHD-BTB). As per Federal and State regulations, tissue banks require every procurement site to obtain informed consent in compliance with all regulations governing that process in writing from any donor of human tissue (or the next of kin thereof) for the use of tissue for research. Each signed consent form is kept on file at the tissue acquisition site and information is never released to any third party. Tissue banks assign computer generated codes to each donor and do not maintain any information that could be used to identify the donor. Tissue banks provide anonymous samples coded with unique sample identification numbers. Procurement protocols for this project were reviewed and approved by NDRI, CHTN, and NICHD-BTB. Myocardium samples from left ventricles weighing 0.8 - 100 g were recovered <20 hours postmortem and snap frozen in liquid nitrogen. Upon arrival samples were stored in liquid nitrogen until further processing. Donor demographics are shown in Supplementary Table 1.

2.2. Nucleic Acid Extraction & Illumina Methylation Assay

Genomic DNA from myocardium was extracted and purified with AutoGen QuickGene DNA Tissue Kit S and an AutoGen QuickGene-810 Nucleic Acid Isolation System (AutoGen Inc). DNA concentrations were measured using a Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Thermo Fisher Scientific) following the manufacturer's instructions. The quality of DNA samples (260 nm/230 nm absorbance ratios) was also examined using a NanoDrop 1000

Spectrophotometer (Thermo Scientific). DS status (i.e., trisomy 21) had been previously confirmed by array comparative hybridization as described (7). Genome-wide methylation analysis was performed by interrogating 482,421 unique CpG sites using the Illumina HumanMethylation450 BeadChip platform (Illumina) at the Genomics Shared Resource, Roswell Park Comprehensive Cancer Center, Buffalo, NY (8). BeadChips were scanned using an iScan Reader (Illumina), and analysis was conducted using GenomeStudio v2011.1 (Illumina).

2.3. *Genome-Wide Methylation Analysis*

Genome-wide methylation data, in the form of intensity data (IDAT) files, were compiled in GenomeStudio v2011.1 and processed using the R package minfi following the recommended workflow (9, 10). A detection p-value was generated for every CpG site in each sample to compare the total signal for each probe to the background signal. Samples with high mean detection p-values were defined as poor quality samples and were excluded from downstream analysis. None of the 24 DNA samples were identified as poor-quality sample. Data normalization was performed using the stratified quantile normalization method in the minfi package. Then, probes with unreliable signals were filtered out before differential methylation analysis. Probes with high detection p-value ($p \geq 0.01$) in any sample were excluded. Probes located on sex chromosomes or those known to have common single nucleotide polymorphisms (SNPs) at the CpG site were filtered out and were not included in subsequent analyses. Probes showing to be cross-reactive due to multiple mapping in the genome were also filtered out (11). All remaining probes were used in the downstream analysis.

Beta values (β value) were used to define methylation, ranging from 0 - 1, for individual probes or genomic regions. Differential methylation analysis was conducted on individual CpG sites, as well as in defined genomic regions (i.e., loci). Individual CpG sites and genomic regions were identified as statistically significantly differentially methylated between DS and non-DS samples following correction for multiple testing using the Benjamini-Hochberg false discovery

rate (FDR), with a 5% FDR cut off. Highly differentially methylated CpG sites and regions were identified as those with significant p-values and an absolute methylation difference greater than 0.100 (i.e., β difference > 10.0%). Analysis of differentially methylated regions (DMR) was performed with the DMRcate package, which identifies and ranks the DMR based on a kernel smoothing of the differentially methylated CpG sites (12). Genomic location of individual CpG sites, genic regions, and corresponding gene names were obtained from the UCSC Genome Browser (hg19). Functional classification, and GO enrichment and pathway analyses were performed with the web tool PANTHER (v15.0) (13).

2.4. Quantitative Real-time Polymerase Chain Reaction

Total RNA was isolated from myocardium with Trizol reagent according to the manufacturer's instructions (Thermo Fisher). *RUNX1* mRNA expression was analyzed with specific primers: 5'-CGGTCGAAGTGAAGAGGGAA-3' (forward), 5'-ATGGCTCTGTGGTAGGTGGC-3' (reverse). Total RNA (12.5 ng) was reverse transcribed and amplified with the iTaq Universal SYBR Green One-Step Kit (Bio-Rad). *RUNX1* and *ACTB* were amplified in parallel in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) with the following cycling parameters: 50°C for 10 min (reverse transcription), 95°C for 1 min, followed by 44 cycles of 95°C for 10 s, 60.5°C for 20 s. Calibration curves were prepared to analyze linearity and PCR efficiency. qRT-PCR data were analyzed using the $\Delta\Delta C_t$ method with CFX manager Software (Bio-Rad). The ΔC_t method was utilized to determine the relative expression of *RUNX1* mRNA.

2.5. Statistical Analyses

For genome-wide methylation analysis, statistical testing was performed with *R* using Fisher tests. Statistical significance was set at FDR of 0.05. β -values were used to represent the extent of methylation at distinct CpG sites. For *RUNX1* mRNA expression analysis, the D'Agostino

& Pearson omnibus normality test was used to determine the normality of data sets. Comparisons between means were performed with the Mann-Whitney's U test.

3. Results

3.1. DNA methylation profiles in myocardial samples

Data were first preprocessed to remove unreliable probes (1,458 sites), CpG sites containing SNPs (16,928 sites), cross-reactive probes (26,519 sites), and mapping to sex chromosomes (11,295 sites). Following data preprocessing, a total of 429,312 CpG sites were retained for analysis. In general, global methylation profiles of myocardial DNA samples from donors with ($n = 12$) or without DS ($n = 12$) were similar; each myocardial DNA methylation profile showed a bimodal distribution of β values, and an average β value of 0.45 (Figure 1A). Donor's age and gender had no apparent covariate effects on global myocardial DNA methylation profiles.

3.2. Differentially methylated CpG sites in samples from donors with and without Down syndrome

A total of 192 individual CpG sites were differentially methylated between myocardial DNA samples from subjects with and without DS ($FDR < 0.05$) (Figure 1B). Of these sites, there were 143 CpG sites with increased methylation in samples with DS relative to samples without DS, whereas 49 CpG sites showed decreased methylation in samples with DS. Analysis of chromosomal locations showed that differentially methylated CpG sites on chromosome 19 were overrepresented and accounted for 15% of the total (29 sites) (Figure 1B). Of all differentially methylated CpG sites, 93 (48%) were observed to be highly differentially methylated (absolute β value difference in DS versus non-DS groups > 0.100) (Table 1). A total of 15 highly differentially methylated CpG sites were found to be less methylated in myocardial DNA samples with DS relative to samples without DS (average β difference = 0.131), whereas 78 CpG sites were more methylated in samples with DS relative to samples without DS (average β difference = 0.145). Of the 93 highly differentially methylated CpG sites, 77 (83%) were distributed in annotated genic regions (i.e., TSS200, TSS1500, 5' UTR, 1st exon, gene body, 3' UTR), and 68 (73%) were located within CpG islands (Figure 1C). Genes annotated to differentially methylated CpG sites

were functionally classified with the PANTHER Classification System (Table S2). For genes with differentially methylated CpG sites and matching protein products, the majority of the hits (25%) did not match to a specific protein class, and 22% of the listed proteins did not match to a specific molecular function (Figure 2A).

3.3. *Differentially methylated CpG sites on chromosome 21*

Special attention was given to differentially methylated CpG sites in chromosome 21 and to sites located in the so-called Down syndrome critical region (DSCR, 21q21.1 – q22.2) (14). Eighteen differentially methylated sites were located in chromosome 21 with a total of 6 highly differentially methylated CpG sites, and 8 differentially methylated CpG sites within the DSCR (Figure 1, and Tables 1 and 2). The mRNA expression of the highly differentially methylated gene *RUNX1* (absolute β value difference = 0.196) was assessed in samples of total myocardial RNA (Table S3). Samples with matching DNA methylation data from donors with DS ($n = 8$) exhibited higher *RUNX1* mRNA expression (4.96 ± 4.67 relative fold) compared to samples from donors without DS ($n = 12$, 1.00 ± 0.67 relative fold). The inclusion of additional myocardial mRNA samples with no matching DNA methylation data (non-DS, $n = 8$, and DS, $n = 4$) further confirmed the trend towards increased *RUNX1* mRNA expression in DS myocardium (DS: 6.73 ± 6.60 relative fold, and non-DS: 1.00 ± 0.89 relative fold) (Figure 3). Linear regression analysis was performed for the group of myocardial samples with matching *RUNX1* DNA methylation and mRNA expression data (i.e., DS, $n = 8$, and non-DS, $n = 12$). Linear regression analysis using donor's age and gender as covariates suggest that donor status (i.e., DS and non-DS) and *RUNX1* differential methylation expressed as β value contribute to a fraction of the variability observed for the expression of the *RUNX1* transcript in myocardial tissue ($R^2 = 0.61$, Adj $R^2 = 0.51$, $p = 0.005$). Hypothesis tests on regression coefficients revealed that donor status (DS and non-DS) is significant ($p = 0.004$), although *RUNX1* differential methylation was not found to be significant ($p = 0.260$), possibly due to the small sample size.

3.4. *Differentially methylated regions in DNA samples from donors with and without Down syndrome*

A total of 16 differentially methylated regions (DMR) were identified between samples from donors with and without DS (Table 3). There were no DMR in chromosome 21. DMR overlapped with 30 genes, and DMR with the highest number of CpG sites included loci in chromosome 6 (37, 32, 27 and 13 CpG sites, respectively), chromosome 22 (14 CpG sites), and chromosome 18 (10 CpG sites). A total of 20 genes mapped to the PANTHER reference list (Table 4). Genes overlapping with DMR identified by the PANTHER classification system included a relatively high proportion of hits with no specific proteins class (55%), and no assigned molecular function (45%) (Figure 2B). There was no significant enrichment for gene ontology terms (GO) and molecular pathways.

4. Discussion and Conclusions

This pilot study documents the extent of genome-wide DNA methylation in a collection of samples of myocardial tissue from individuals with and without DS. Persons with DS are at increased risk for the development of various comorbidities which may impact quality of life and mortality rate. The estimated number of people with DS living in the United States was 1 per 1,499 inhabitants in the year 2010 (15). The life expectancy for persons with DS has increased from 9 - 11 years of age in the 1900s to over 55 - 60 years of age in the 2000s, with around 10% of individuals living now up to 70 years of age (16, 17). The myocardial samples analyzed in this study have been obtained from donors representing almost the entire life span of individuals with DS (Table S1). Although donor's age and gender may potentially impact tissular DNA methylation patterns, it was not possible to fully match both sets of samples by age because the DS group included three samples from young donors (ages: 1, 1, and 10 years old) for which there were not age-matched samples from donors without DS (18-20). Nevertheless, the myocardial samples from young donors with DS were maintained for analysis due to the general scarcity of epigenetic data derived from individuals with DS.

Sailani et al. applied reduced representation bisulfite sequencing (RRBS) for the analysis of methylation patterns in DNA from skin fibroblasts from monozygotic twins with and without trisomy 21. The authors found 35 differentially methylated regions (absolute methylation differences > 25%) with enrichment in the promoter regions of genes involved in embryonic organ morphogenesis (e.g., *HOXB5*, *HOXB6*, *HOXD3*, and *HOXD10*). The authors also reported an increase in global DNA methylation in trisomic methylomes in comparison with euploid methylomes (21). RRBS of DNA from placental villi from euploid and trisomic pregnancies showed global hypermethylation in samples with trisomy 21 (22). Our comparisons revealed that global DNA methylation levels are similar in myocardial samples from subjects with and without DS (Figure 1). We identified a relatively small number of differentially methylated CpG sites and

regions in the myocardial methylomes from donors with DS (Figure 1, and Tables 1 and 3). Thus, it appears that myocardial DNA methylation profiles in the DS context exhibit subtle changes, and differences in DNA methylation levels relative to the non-DS counterpart tend to be localized in a relatively small number of loci. Differentially methylated loci showed no enrichment for GO terms or distinct pathways. In terms of relative methylation at individual CpG sites, there was a trend towards increased methylation in the DS context with 65.7% of total differentially methylated CpG sites and 83.9% of highly differentially methylated CpG sites showing increased methylation in DS samples in comparison to non-DS samples.

Our analysis identified differential methylation in CpG sites and regions overlapping genes with demonstrated roles in cardiac structure and function (i.e., *UNC45A*, *DNM2*), atrioventricular valve formation (i.e., *OLFM1*), and other cardiac processes including calcium homeostasis, muscle contraction, and cardiac conduction (i.e., *DMPK*, *RYR1*) (Table 4 and Table S2) (23-27). Previous studies have noted differential methylation in CpG sites within some of the loci identified in our analysis. For example, differential methylation in *KIAA1875*, *CELSR3*, *STK19* and *UNC45A* has been detected in DNA from epithelial cells, placenta, and fetal cortex with trisomy 21 (22, 28, 29). Likewise, we identified a number of highly differentially methylated sites located within the *KIAA1875*, *CELSR3*, *STK19*, and *UNC45A* loci (3, 5, 6, and 4 CpG sites, respectively), all of them showing increased methylation in myocardial DNA from donors with DS (Table 1).

Our study identified 8 CpG sites within the so called DSCR which were differentially methylated in DS myocardium. The DSCR included a highly differentially methylated CpG site within the *RUNX1* locus (Table 2). On average, *RUNX1* mRNA expression in trisomic myocardium was ~6-fold higher than in diploid tissue, although there was considerable interindividual variability (SD: ~6-fold) (Figure 3). RUNX family members form heterodimeric transcription factors that activate or repress gene transcription in healthy and disease states. Little is known regarding the roles of RUNX factors during cardiac physiology. A recent study in a murine model showed that

Runx1 modulates calcium ion uptake in sarcoplasmic reticulum and cardiac contractility. Of note, reduction of Runx1 function prevented adverse cardiac remodeling after myocardial infarction (30). Our simple linear regression model based on donors' age, gender, DS status, and *RUNX1* methylation suggests that these variables may contribute up to ~51% of the variability in myocardial *RUNX1* mRNA expression. Although DS status was found to be a strong predictor of *RUNX1* mRNA methylation, *RUNX1* interindividual methylation did not reach the level of significance *per se*. We hypothesize that this is due in part to the small sample size, which is a limitation for the linear regression. Elucidation of the role of differential *RUNX1* methylation during myocardial function in the context of DS deserves further investigation.

Many of the “genes” overlapping with differentially methylated CpG sites and DMR had unknown function and/or protein-coding potential, or codify non-protein products (Figure 2, Table 4). Only 58% of the genes overlapping with DMR codify for proteins with known functions, and around 24% codify non-coding RNAs. Differential expression of non-coding RNA species has been detected in human trisomic endothelial progenitor cells and induced pluripotent stem cells (31, 32). Many non-coding RNAs regulate complex biological functions and are involved in disease processes such as cardiovascular disease (33, 34). For example, our data suggest that *RNARP11-344E13.3* is differentially methylated in DS myocardium. *RNARP11-344E13.3* is a long non-coding RNA that interacts with microRNA targets linked to the development of cardiac hypertrophy (35). It would be of interest to further investigate the role of differential DNA methylation during the expression of non-coding RNAs in myocardium with emphasis on the DS setting.

This pilot study is limited by the small number of myocardial samples from donors with and without DS. Although our analysis identified a number of differentially methylated loci in DS myocardium, these observations need to be confirmed in larger sample sizes. Research on fundamental issues concerning the pathobiology of DS continues to be hampered by the scarcity

of good quality tissue samples from donors with DS. We believe that this study provides an initial snapshot on the extent of genome-wide differential methylation in myocardial tissue from persons with DS. This study contributes new data to further examine the contribution of differential DNA methylation to the complex pathobiology of DS.

REFERENCES

1. Oster-Granite ML, Parisi MA, Abbeduto L, Berlin DS, Bodine C, Bynum D, Capone G, Collier E, Hall D, Kaeser L, Kaufmann P, Krischer J, Livingston M, McCabe LL, Pace J, Pfenninger K, Rasmussen SA, Reeves RH, Rubinstein Y, Sherman S, Terry SF, Whitten MS, Williams S, McCabe ER, Maddox YT. Down syndrome: national conference on patient registries, research databases, and biobanks. *Mol Genet Metab*. 2011;104(1-2):13-22. Epub 2011/08/13. doi: 10.1016/j.ymgme.2011.07.005. PubMed PMID: 21835664; PMCID: PMC3171614.
2. Stoll C, Dott B, Alembik Y, Roth MP. Associated congenital anomalies among cases with Down syndrome. *Eur J Med Genet*. 2015;58(12):674-80. Epub 2015/11/19. doi: 10.1016/j.ejmg.2015.11.003. PubMed PMID: 26578241.
3. Weijerman ME, van Furth AM, van der Mooren MD, van Weissenbruch MM, Rammeloo L, Broers CJ, Gemke RJ. Prevalence of congenital heart defects and persistent pulmonary hypertension of the neonate with Down syndrome. *Eur J Pediatr*. 2010;169(10):1195-9. Epub 2010/04/23. doi: 10.1007/s00431-010-1200-0. PubMed PMID: 20411274; PMCID: PMC2926442.
4. Freeman SB, Bean LH, Allen EG, Tinker SW, Locke AE, Druschel C, Hobbs CA, Romitti PA, Royle MH, Torfs CP, Dooley KJ, Sherman SL. Ethnicity, sex, and the incidence of congenital heart defects: a report from the National Down Syndrome Project. *Genet Med*. 2008;10(3):173-80. Epub 2008/03/18. doi: 10.1097/GIM.0b013e3181634867. PubMed PMID: 18344706.
5. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet*. 2012;13(7):484-92. Epub 2012/05/30. doi: 10.1038/nrg3230
nrg3230 [pii]. PubMed PMID: 22641018.
6. Serra-Juhe C, Cusco I, Homs A, Flores R, Toran N, Perez-Jurado LA. DNA methylation abnormalities in congenital heart disease. *Epigenetics*. 2015;10(2):167-77. Epub 2015/01/15. doi: 10.1080/15592294.2014.998536. PubMed PMID: 25587870; PMCID: PMC4622722.
7. Hefti E, Quinones-Lombrana A, Redzematovic A, Hui J, Blanco JG. Analysis of mtDNA, miR-155 and BACH1 expression in hearts from donors with and without Down syndrome. *Mitochondrial DNA A DNA Mapp Seq Anal*. 2016;27(2):896-903. Epub 2014/06/19. doi: 10.3109/19401736.2014.926477. PubMed PMID: 24938108; PMCID: PMC4315749.
8. Carless MA. Determination of DNA methylation levels using Illumina HumanMethylation450 BeadChips. *Methods Mol Biol*. 2015;1288:143-92. Epub 2015/04/02. doi: 10.1007/978-1-4939-2474-5_10. PubMed PMID: 25827880.
9. Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, Irizarry RA. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. *Bioinformatics*. 2014;30(10):1363-9. Epub 2014/01/31. doi: 10.1093/bioinformatics/btu049. PubMed PMID: 24478339; PMCID: PMC4016708.

10. Assenov Y, Muller F, Lutsik P, Walter J, Lengauer T, Bock C. Comprehensive analysis of DNA methylation data with RnBeads. *Nat Methods*. 2014;11(11):1138-40. Epub 2014/09/30. doi: 10.1038/nmeth.3115. PubMed PMID: 25262207; PMCID: PMC4216143.
11. Chen YA, Lemire M, Choufani S, Butcher DT, Grafodatskaya D, Zanke BW, Gallinger S, Hudson TJ, Weksberg R. Discovery of cross-reactive probes and polymorphic CpGs in the Illumina Infinium HumanMethylation450 microarray. *Epigenetics*. 2013;8(2):203-9. Epub 2013/01/15. doi: 10.4161/epi.23470. PubMed PMID: 23314698; PMCID: PMC3592906.
12. Peters TJ, Buckley MJ, Statham AL, Pidsley R, Samaras K, R VL, Clark SJ, Molloy PL. De novo identification of differentially methylated regions in the human genome. *Epigenetics Chromatin*. 2015;8:6. Epub 2015/05/15. doi: 10.1186/1756-8935-8-6. PubMed PMID: 25972926; PMCID: PMC4429355.
13. Mi H, Muruganujan A, Ebert D, Huang X, Thomas PD. PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. *Nucleic Acids Res*. 2019;47(D1):D419-D26. Epub 2018/11/09. doi: 10.1093/nar/gky1038. PubMed PMID: 30407594; PMCID: PMC6323939.
14. Sinet PM, Theophile D, Rahmani Z, Chettouh Z, Blouin JL, Prieur M, Noel B, Delabar JM. Mapping of the Down syndrome phenotype on chromosome 21 at the molecular level. *Biomed Pharmacother*. 1994;48(5-6):247-52. Epub 1994/01/01. doi: 10.1016/0753-3322(94)90140-6. PubMed PMID: 7999986.
15. de Graaf G, Buckley F, Skotko BG. Estimation of the number of people with Down syndrome in the United States. *Genet Med*. 2017;19(4):439-47. Epub 2016/09/09. doi: 10.1038/gim.2016.127. PubMed PMID: 27608174.
16. Brown R, Taylor J, Matthews B. Quality of life--ageing and Down syndrome. *Downs Syndr Res Pract*. 2001;6(3):111-6. Epub 2001/08/15. doi: 10.3104/case-studies.101. PubMed PMID: 11501212.
17. Head E, Lott IT, Wilcock DM, Lemere CA. Aging in Down Syndrome and the Development of Alzheimer's Disease Neuropathology. *Curr Alzheimer Res*. 2016;13(1):18-29. Epub 2015/12/15. doi: 10.2174/1567205012666151020114607. PubMed PMID: 26651341; PMCID: PMC4948181.
18. Boks MP, Derks EM, Weisenberger DJ, Strengman E, Janson E, Sommer IE, Kahn RS, Ophoff RA. The relationship of DNA methylation with age, gender and genotype in twins and healthy controls. *PLoS One*. 2009;4(8):e6767. Epub 2009/09/24. doi: 10.1371/journal.pone.0006767. PubMed PMID: 19774229; PMCID: PMC2747671.
19. Illi B, Ciarapica R, Capogrossi MC. Chromatin methylation and cardiovascular aging. *J Mol Cell Cardiol*. 2015;83:21-31. Epub 2015/03/01. doi: 10.1016/j.yjmcc.2015.02.011. PubMed PMID: 25724723.
20. Zampieri M, Ciccarone F, Calabrese R, Franceschi C, Burkle A, Caiafa P. Reconfiguration of DNA methylation in aging. *Mech Ageing Dev*. 2015;151:60-70. Epub 2015/02/25. doi: 10.1016/j.mad.2015.02.002. PubMed PMID: 25708826.
21. Sailani MR, Santoni FA, Letourneau A, Borel C, Makrythanasis P, Hibaoui Y, Popadin K, Bonilla X, Guipponi M, Gehrig C, Vannier A, Carre-Pigeon F, Feki A, Nizetic D, Antonarakis SE. DNA-Methylation Patterns in Trisomy 21 Using Cells from Monozygotic Twins. *PLoS One*. 2015;10(8):e0135555. Epub 2015/09/01. doi: 10.1371/journal.pone.0135555. PubMed PMID: 26317209; PMCID: PMC4552626.
22. Jin S, Lee YK, Lim YC, Zheng Z, Lin XM, Ng DP, Holbrook JD, Law HY, Kwek KY, Yeo GS, Ding C. Global DNA hypermethylation in down syndrome placenta. *PLoS Genet*. 2013;9(6):e1003515. Epub 2013/06/12. doi: 10.1371/journal.pgen.1003515. PubMed PMID: 23754950; PMCID: PMC3675012.
23. Lee CF, Melkani GC, Bernstein SI. The UNC-45 myosin chaperone: from worms to flies to vertebrates. *Int Rev Cell Mol Biol*. 2014;313:103-44. Epub 2014/11/08. doi: 10.1016/B978-0-12-800177-6.00004-9. PubMed PMID: 25376491; PMCID: PMC4225561.
24. Li J, Zhang DS, Ye JC, Li CM, Qi M, Liang DD, Xu XR, Xu L, Liu Y, Zhang H, Zhang YY, Deng FF, Feng J, Shi D, Chen JJ, Li L, Chen G, Sun YF, Peng LY, Chen YH. Dynamin-2 mediates heart failure by modulating

- Ca²⁺-dependent cardiomyocyte apoptosis. *Int J Cardiol.* 2013;168(3):2109-19. Epub 2013/02/16. doi: 10.1016/j.ijcard.2013.01.006. PubMed PMID: 23410488.
25. Lencinas A, Chhun DC, Dan KP, Ross KD, Hoover EA, Antin PB, Runyan RB. Olfactomedin-1 activity identifies a cell invasion checkpoint during epithelial-mesenchymal transition in the chick embryonic heart. *Dis Model Mech.* 2013;6(3):632-42. Epub 2012/12/25. doi: 10.1242/dmm.010595. PubMed PMID: 23264563; PMCID: PMC3634647.
26. Kaliman P, Catalucci D, Lam JT, Kondo R, Gutierrez JC, Reddy S, Palacin M, Zorzano A, Chien KR, Ruiz-Lozano P. Myotonic dystrophy protein kinase phosphorylates phospholamban and regulates calcium uptake in cardiomyocyte sarcoplasmic reticulum. *J Biol Chem.* 2005;280(9):8016-21. Epub 2004/12/16. doi: 10.1074/jbc.M412845200. PubMed PMID: 15598648.
27. Lawal TA, Todd JJ, Meilleur KG. Ryanodine Receptor 1-Related Myopathies: Diagnostic and Therapeutic Approaches. *Neurotherapeutics.* 2018;15(4):885-99. Epub 2018/11/09. doi: 10.1007/s13311-018-00677-1. PubMed PMID: 30406384; PMCID: PMC6277304.
28. El Hajj N, Dittrich M, Bock J, Kraus TF, Nanda I, Muller T, Seidmann L, Tralau T, Galetzka D, Schneider E, Haaf T. Epigenetic dysregulation in the developing Down syndrome cortex. *Epigenetics.* 2016;11(8):563-78. Epub 2016/06/02. doi: 10.1080/15592294.2016.1192736. PubMed PMID: 27245352; PMCID: PMC4990229.
29. Jones MJ, Farre P, McEwen LM, Macisaac JL, Watt K, Neumann SM, Emberly E, Cynader MS, Virji-Babul N, Kobor MS. Distinct DNA methylation patterns of cognitive impairment and trisomy 21 in Down syndrome. *BMC Med Genomics.* 2013;6:58. Epub 2014/01/01. doi: 10.1186/1755-8794-6-58. PubMed PMID: 24373378; PMCID: PMC3879645.
30. McCarroll CS, He W, Foote K, Bradley A, McGlynn K, Vidler F, Nixon C, Nather K, Fattah C, Riddell A, Bowman P, Elliott EB, Bell M, Hawksby C, MacKenzie SM, Morrison LJ, Terry A, Blyth K, Smith GL, McBride MW, Kubin T, Braun T, Nicklin SA, Cameron ER, Loughrey CM. Runx1 Deficiency Protects Against Adverse Cardiac Remodeling After Myocardial Infarction. *Circulation.* 2018;137(1):57-70. Epub 2017/10/17. doi: 10.1161/CIRCULATIONAHA.117.028911. PubMed PMID: 29030345; PMCID: PMC5757664.
31. Costa V, Angelini C, D'Apice L, Mutarelli M, Casamassimi A, Sommese L, Gallo MA, Aprile M, Esposito R, Leone L, Donizetti A, Crispi S, Rienzo M, Sarubbi B, Calabro R, Picardi M, Salvatore P, Infante T, De Berardinis P, Napoli C, Ciccodicola A. Massive-scale RNA-Seq analysis of non ribosomal transcriptome in human trisomy 21. *PLoS One.* 2011;6(4):e18493. Epub 2011/05/03. doi: 10.1371/journal.pone.0018493. PubMed PMID: 21533138; PMCID: PMC3080369.
32. Qiu JJ, Liu YN, Ren ZR, Yan JB. Dysfunctions of mitochondria in close association with strong perturbation of long noncoding RNAs expression in down syndrome. *Int J Biochem Cell Biol.* 2017;92:115-20. Epub 2017/10/03. doi: 10.1016/j.biocel.2017.09.017. PubMed PMID: 28965985.
33. Elia L, Condorelli G. RNA (Epi)genetics in cardiovascular diseases. *J Mol Cell Cardiol.* 2015;89(Pt A):11-6. Epub 2015/07/25. doi: 10.1016/j.yjmcc.2015.07.012. PubMed PMID: 26205296.
34. Ounzain S, Burdet F, Ibberson M, Pedrazzini T. Discovery and functional characterization of cardiovascular long noncoding RNAs. *J Mol Cell Cardiol.* 2015;89(Pt A):17-26. Epub 2015/09/27. doi: 10.1016/j.yjmcc.2015.09.013. PubMed PMID: 26408097.
35. Song C, Zhang J, Liu Y, Pan H, Qi HP, Cao YG, Zhao JM, Li S, Guo J, Sun HL, Li CQ. Construction and analysis of cardiac hypertrophy-associated lncRNA-mRNA network based on competitive endogenous RNA reveal functional lncRNAs in cardiac hypertrophy. *Oncotarget.* 2016;7(10):10827-40. Epub 2016/02/13. doi: 10.18632/oncotarget.7312. PubMed PMID: 26872060; PMCID: PMC4905442.

Funding

This study was supported by the National Cancer Institute (award R21CA245067) and the National Institute of General Medical Sciences (award R01GM073646).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Figure legends

Figure 1. Differential methylation in myocardial DNA from donors with DS. **A)** Density plots of DNA methylation levels (β values) in myocardial DNA from donors with and without DS (DS, $n = 12$, and non-DS, $n = 12$). **B)** P values ($-\log_{10}$) of differentially methylated CpG sites stratified by chromosomal location. The blue line depicts the threshold for statistical significance (FDR = 0.05). **C)** Location of differentially methylated sites in relation to functional subregions (left panel) and CpG islands (right panel).

Figure 2. PANTHER functional classification. **A)** Classification of genes mapped to differentially methylated CpG sites. **B)** Classification of genes mapped to differentially methylated regions (DMR). Top panels: protein class, middle panels: molecular function, and bottom panels: biological processes. Genes without hits are listed as non-classified.

Figure 3. Relative *RUNX1* mRNA fold expression in myocardial samples from donors without and with DS (non-DS, $n = 16$, and DS, $n = 16$). Black dots represent additional myocardial mRNA samples from donors without (8 mRNA samples) and with DS (4 mRNA samples) with no matching DNA methylation data. Each point represents the mean from three separate measurements performed in triplicates. Horizontal bars show the mean $\pm$ SD. *** $P < 0.001$, Mann Whitney test.

Table 1. Highly differentially methylated CpG sites in myocardial DNA from subjects with DS.

Table 2. Differentially methylated sites within the DSCR in myocardial DNA from subjects with DS.

Table 3. Differentially methylated regions in myocardial DNA from subjects with DS.

Table 4. Genes showing overlap with DMR in myocardial DNA from subjects with DS. Functional classification according to the PANTHER classification system.

A **C** **B**

Table 1. Highly differentially methylated CpG sites in myocardial DNA from subjects with DS.

ID	Chromosome	Relation to Island	UCSC Reference Gene Name	UCSC Reference Gene Group	adj. P.Value	β -value NDS	β -value DS	$\Delta \beta$ -value
cg04094791	chr01	Island	HSPA7	Body	6.24E-03	0.59	0.73	-0.135
cg05304531	chr01	N_Shore	DHDDS;HMGN2	3'UTR;TSS1500	3.35E-02	0.54	0.64	-0.105
cg18139641	chr01	OpenSea	CSRP1	5'UTR	3.67E-02	0.71	0.60	0.114
cg06940110	chr02	N_Shore			3.69E-02	0.40	0.57	-0.163
cg14515364	chr02	Island			7.29E-04	0.64	0.78	-0.140
cg03223076	chr03	Island	CELSR3	1stExon	1.08E-02	0.48	0.69	-0.209
cg02222844	chr03	Island	CELSR3	Body	4.21E-02	0.48	0.65	-0.178
cg02773588	chr03	Island	CELSR3	1stExon	2.04E-03	0.62	0.79	-0.162
cg08640901	chr03	Island	CELSR3	Body	4.32E-03	0.47	0.59	-0.116
cg15142694	chr03	Island	CELSR3	Body	4.85E-02	0.64	0.74	-0.103
cg12477903	chr05	Island	F12	Body	8.30E-05	0.18	0.31	-0.127
cg04149916	chr06	N_Shore	STK19;STK19;DOM3Z	TSS1500;TSS1500;Body	2.58E-04	0.32	0.48	-0.162
cg20194050	chr06	N_Shore	STK19;STK19;DOM3Z	TSS1500;TSS1500;Body	9.99E-03	0.54	0.69	-0.150
cg13069441	chr06	N_Shore	STK19;STK19;DOM3Z	TSS1500;TSS1500;Body	8.85E-03	0.37	0.52	-0.147
cg23906067	chr06	N_Shore	STK19;STK19;DOM3Z	TSS1500;TSS1500;Body	1.12E-02	0.38	0.52	-0.141
cg18319852	chr06	S_Shore	KIF25	Body	3.56E-02	0.34	0.48	-0.138
cg26077005	chr06	OpenSea			4.21E-02	0.50	0.62	-0.118
cg10914467	chr06	N_Shore	STK19;STK19;DOM3Z	TSS1500;TSS1500;Body	1.92E-02	0.32	0.44	-0.117
cg26968378	chr06	OpenSea			4.45E-02	0.28	0.39	-0.117
cg16190718	chr06	N_Shore	STK19;STK19;DOM3Z	TSS1500;TSS1500;Body	3.94E-02	0.38	0.48	-0.103
cg10052687	chr07	N_Shelf	CLIP2	Body	4.32E-03	0.53	0.43	0.102
cg21243198	chr07	S_Shelf	HEATR2	Body	5.21E-03	0.79	0.66	0.128
cg25468618	chr08	Island	FAM83H	Body	4.87E-06	0.54	0.77	-0.223
cg13735469	chr08	Island	FAM83H	Body	7.42E-04	0.50	0.70	-0.193
cg26272248	chr08	N_Shore	KIAA1875	Body	3.75E-02	0.45	0.63	-0.178
cg01399317	chr08	Island	FAM83H	Body	2.54E-04	0.59	0.77	-0.171
cg07962934	chr08	Island	FAM83H	Body	2.49E-05	0.67	0.84	-0.163
cg01612292	chr08	Island	FAM83H	Body	1.89E-12	0.77	0.92	-0.145
cg24063962	chr08	Island	LOC100130274	TSS200	1.26E-02	0.42	0.55	-0.129
cg02601489	chr08	N_Shore	TDH	Body	1.33E-02	0.41	0.54	-0.128
cg18925846	chr08	Island	KIAA1875	Body	1.26E-02	0.69	0.81	-0.121
cg21429107	chr08	Island	LOC100130274	TSS200	9.99E-03	0.34	0.46	-0.119
cg01423393	chr08	Island	KIAA1875	Body	4.53E-03	0.49	0.61	-0.117
cg05358723	chr08	Island	LRRRC24;LRRC14	Body;3'UTR	1.06E-02	0.41	0.51	-0.101
cg09381737	chr09	Island	C9orf167	Body	4.10E-02	0.55	0.68	-0.129
cg04471485	chr09	Island	CLIC3	Body	3.63E-03	0.32	0.44	-0.116
cg14147184	chr09	Island	OLFM1	Body	1.45E-02	0.56	0.44	0.120
cg19511338	chr10	N_Shore			4.81E-02	0.56	0.66	-0.107
cg02493211	chr11	Island	BCL9L	TSS200	4.43E-02	0.38	0.58	-0.196
cg01425746	chr11	Island	CHST1	Body	9.94E-03	0.66	0.79	-0.131
cg24985772	chr11	Island			4.08E-02	0.36	0.49	-0.129
cg06938878	chr11	N_Shore	CALCB	TSS1500	6.24E-03	0.62	0.72	-0.102
cg10287786	chr11	N_Shore	DSCAML1	Body	1.69E-02	0.75	0.61	0.140
cg03434624	chr12	Island	VPS37B	TSS200	3.10E-02	0.19	0.32	-0.131
cg17566325	chr12	N_Shore			3.16E-02	0.40	0.28	0.114

cg26352652	chr12	N_Shore			4.08E-02	0.49	0.35	0.147
cg01863042	chr13	OpenSea			7.00E-03	0.46	0.58	-0.128
cg07980777	chr13	OpenSea	N6AMT2	3'UTR	3.78E-03	0.71	0.59	0.121
cg01351822	chr15	Island	UNC45A;UNC45A	5'UTR;1stExon	8.85E-03	0.12	0.34	-0.223
cg13778201	chr15	Island	UNC45A	TSS200	4.21E-02	0.28	0.46	-0.177
cg08551047	chr15	Island	UNC45A	5'UTR	8.85E-03	0.21	0.33	-0.124
cg16414568	chr15	Island	UNC45A	5'UTR	3.48E-03	0.10	0.20	-0.107
cg02156899	chr16	Island	NOXO1	Body	8.30E-05	0.42	0.63	-0.211
cg02649063	chr16	Island	CES4	Body	3.22E-02	0.39	0.60	-0.202
cg04599341	chr16	Island	NOXO1	Body	3.45E-06	0.40	0.56	-0.163
cg09465703	chr16	Island	JMJD8	Body	6.19E-03	0.14	0.29	-0.151
cg17300750	chr16	Island	DNASE1L2	Body	1.88E-02	0.27	0.39	-0.122
cg00029825	chr16	Island	SLC5A2	Body	2.65E-03	0.60	0.71	-0.111
cg05673293	chr16	N_Shore			1.26E-02	0.49	0.59	-0.108
cg14560110	chr17	Island	CCDC144NL	TSS200	7.42E-04	0.51	0.74	-0.237
cg22570042	chr17	Island	CCDC144NL	TSS200	1.24E-02	0.57	0.73	-0.160
cg06809326	chr17	Island	CCDC144NL	TSS200	3.63E-03	0.59	0.74	-0.152
cg21980100	chr17	Island	CCDC144NL	TSS200	3.52E-02	0.32	0.42	-0.103
cg13704680	chr18	Island	LOC100130522	TSS1500	4.21E-02	0.34	0.18	0.152
cg05251669	chr18	Island	LOC100130522	TSS1500	4.45E-02	0.44	0.28	0.162
cg02452966	chr19	Island	RYR1	Body	3.10E-02	0.32	0.60	-0.278
cg02749105	chr19	Island	ZNF837	3'UTR	7.76E-03	0.34	0.56	-0.212
cg24052338	chr19	Island	ZNF837	3'UTR	3.69E-02	0.47	0.67	-0.193
cg09594291	chr19	Island	ZNF837	3'UTR	2.16E-02	0.26	0.45	-0.192
cg05317207	chr19	Island	ZNF497	Body	3.97E-03	0.22	0.39	-0.174
cg00879218	chr19	Island			2.04E-03	0.47	0.63	-0.167
cg26659805	chr19	Island	AMH	Body	6.19E-03	0.19	0.34	-0.147
cg02452418	chr19	Island	RYR1	Body	4.21E-02	0.16	0.31	-0.146
cg08504662	chr19	Island	ZNF497	Body	4.45E-02	0.21	0.35	-0.143
cg03598159	chr19	Island	RYR1	Body	3.68E-02	0.17	0.30	-0.130
cg11707067	chr19	Island	MZF1;LOC100131691	3'UTR;Body	3.65E-02	0.65	0.78	-0.129
cg08095654	chr19	Island			1.12E-02	0.44	0.55	-0.110
cg08084812	chr19	Island	ZNF837	Body	1.33E-02	0.67	0.78	-0.110
cg22914897	chr19	Island	MZF1;LOC100131691	3'UTR;Body	7.29E-04	0.69	0.79	-0.106
cg18154954	chr19	Island	RYR1	Body	1.83E-02	0.64	0.75	-0.105
cg08620751	chr19	Island			3.78E-03	0.46	0.56	-0.103
cg20285546	chr19	Island			4.77E-02	0.41	0.51	-0.101
cg13382072	chr19	Island			3.68E-02	0.36	0.46	-0.101
cg15820033	chr19	Island	PTPRS	Body	8.72E-03	0.38	0.26	0.117
cg23796967	chr19	OpenSea	GP6	1stExon	7.29E-04	0.60	0.47	0.127
cg05267394	chr20	Island	ASIP	Body	3.22E-02	0.38	0.23	0.152
cg00994804	chr21	Island	RUNX1;RUNX1	1stExon;Body	5.89E-03	0.12	0.32	-0.196
cg00949980	chr21	Island	SUMO3	Body	2.54E-02	0.53	0.68	-0.148
cg07397296	chr21	Island	ABCG1	Body	3.65E-02	0.14	0.25	-0.102
cg03419014	chr21	Island	HLCS	TSS1500	1.21E-03	0.58	0.45	0.121
cg03543861	chr21	Island			1.83E-02	0.71	0.56	0.153
cg10384919	chr22	Island	MEI1	TSS200	9.94E-03	0.14	0.28	-0.137

Table 2. Differentially methylated sites within the DSCR in myocardial DNA from subjects with DS.

ID	position	strand	Relation to Island	UCSC Reference Gene Name	UCSC Reference Gene Group	adj P-Value	β -value NDS	β -value DS	$\Delta \beta$ -value
cg00994804	36259383	-	Island	RUNX1	1stExon; Body	0.006	0.123	0.318	0.196
cg05409047	37259564	-	Island			0.009	0.801	0.750	-0.051
cg13753900	37528677	+	Island			0.042	0.137	0.086	-0.050
cg23933759	37836671	-	OpenSea	CLDN14	5'UTR	0.039	0.753	0.693	-0.060
cg03419014	38362742	+	Island	HLCS	TSS1500	0.001	0.576	0.455	-0.121
cg16435782	39087399	-	OpenSea	KCNJ6	Body	0.030	0.805	0.771	-0.034
cg18539325	39493489	-	OpenSea	DSCR8; DSCR4	TSS200	0.044	0.741	0.797	0.056
cg21649569	40752157	-	Island	WRB	TSS200	0.010	0.150	0.115	-0.035

Table 3. Differentially methylated regions in myocardial DNA from subjects with DS.

DMR	Chromosome	Start	End	Width	CpGs #	Min smoothed FDR	Overlapping genes
1	chr06	33282313	33283849	1537	37	5.24E-87	SNORA38, ZBTB22, SNORA20
2	chr06	31938527	31939884	1358	32	7.08E-99	SNORA38, STK19, DXO, SNORA20
3	chr06	30038754	30039380	627	27	1.33E-32	RNF39, SNORA20
4	chr06	28829171	28829503	333	13	8.22E-33	RPL13P, XXbac-BPG308K3.6, SNORA20
5	chr08	144808965	144810854	1890	7	5.80E-203	FAM83H
6	chr08	144790187	144790410	224	5	5.70E-32	ZNF707, CCDC166
7	chr13	23309774	23310464	691	7	1.04E-35	SNORA16, SNORD37
8	chr15	91473059	91473569	511	9	2.78E-51	UNC45A
9	chr16	2029256	2030009	754	4	4.15E-59	TBL3, NOXO1
10	chr17	20799408	20799644	237	6	4.83E-43	SNORA69, RP11-344E13.3, CCDC144NL
11	chr18	77905119	77905947	829	10	2.57E-35	ADNP2, AC139100.2
12	chr19	55549414	55549842	429	8	4.79E-35	CTC-550B14.7, GP6, CTC-550B14.6
13	chr19	2251588	2252029	442	4	1.53E-35	AMH
14	chr22	45809244	45809793	550	14	4.71E-43	RIBC2, SMC1B
15	chr22	42095347	42095536	190	6	5.52E-32	MEI1
16	chr22	51016703	51016950	248	3	1.64E-30	CPT1B, CHKB-CPT1B

Table 4. Genes showing overlap with DMR in myocardial DNA from subjects with DS.

Functional classification according to the PANTHER classification system.

Gene ID	Gene name	GO database Biological Process Complete
AC139100	Partitioning defective 6 homolog gamma	centrosome cycle; establishment or maintenance of cell polarity; cell division; regulation of cellular localization; bicellular tight junction assembly
ADNP2	Activity-dependent neuroprotector homeobox protein 2	regulation of transcription by RNA polymerase II; neuron differentiation; positive regulation of cell growth; cellular response to oxidative stress; negative regulation of cell death; cellular response to retinoic acid
AMH	Muellerian-inhibiting factor	preantral ovarian follicle growth; urogenital system development; Mullerian duct regression; cell-cell signaling; gonadal mesoderm development; sex determination; sex differentiation; aging; positive regulation of gene expression; response to organic cyclic compound; BMP signaling pathway; response to drug; positive regulation of NF-kappaB transcription factor activity; negative regulation of ovarian follicle development
CCDC144NL	Putative coiled-coil domain-containing protein 144 N-terminal-like	
CCDC166	Coiled-coil domain-containing protein 166	
CHKB-CPT1B	CHKB-CPT1B readthrough (NMD candidate) (Fragment)	phosphorylation; glycerophospholipid biosynthetic process
CPT1B	Carnitine O-palmitoyltransferase 1, muscle isoform	fatty acid metabolic process; fatty acid beta-oxidation; carnitine shuttle; carnitine metabolic process; long-chain fatty acid transport
DXO	Decapping and exoribonuclease protein	mRNA catabolic process; RNA destabilization; nuclear mRNA surveillance; nucleic acid phosphodiester bond hydrolysis; NAD-cap decapping
FAM83H	Protein FAM83H	positive regulation of cell migration; biomineral tissue development; protein localization to cytoskeleton; intermediate filament cytoskeleton organization
GP6	Platelet glycoprotein VI	enzyme linked receptor protein signaling pathway; blood coagulation; platelet activation; leukocyte migration
MEI1	Meiosis inhibitor protein 1	meiosis I; male meiosis I; spermatid development; meiotic telomere clustering
NOXO1	NADPH oxidase organizer 1	superoxide metabolic process; regulation of hydrogen peroxide metabolic process; extracellular matrix disassembly; positive regulation of catalytic activity; regulation of respiratory burst
RIBC2	RIB43A-like with coiled-coils protein 2	
RNF39	RING finger protein 39	biological_process
SMC1B	Structural maintenance of chromosomes protein 1B	sister chromatid cohesion; meiotic cell cycle
STK19	Serine/threonine-protein kinase 19	protein phosphorylation; phosphorylation; positive regulation of Ras protein signal transduction
TBL3	Transducin beta-like protein 3	maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA; rRNA processing
UNC45A	Protein unc-45 homolog A	muscle organ development; cell differentiation; chaperone-mediated protein folding
ZBTB22	Zinc finger and BTB domain-containing protein 22	regulation of transcription by RNA polymerase II
ZNF707	Zinc finger protein 707	regulation of transcription, DNA-templated
CTC-550B14.6	Unable to map	
CTC-550B14.7	Unable to map	
RP11-344E13.3	Unable to map	
RPL13P	Unable to map	
SNORA16	Unable to map	
SNORA20	Unable to map	
SNORA38	Unable to map	
SNORA69	Unable to map	
SNORD37	Unable to map	