SUPPLEMENTAL MATERIAL

DOT1L-mediated H3K79me2 modification critically regulates gene expression during cardiomyocyte differentiation

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Detailed Materials and Methods

Purification of cardiomyocytes. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1996) and approved by the local ethical committee. The study was performed on embryos (E8.5, E10.5, E12.5, and E14.5), neonatal (1 day after birth) and adult (2-month-old) mice. Mice were housed in a controlled environment on a 12h light/dark illumination schedule and fed with a chow diet. Mice were sacrificed according to the protocol of the internal ethics committee, the hearts excised, and primary embryonic, neonatal, and adult cardiac cells were isolated as described elsewhere.[1, 2, 3]

mES cell culture and differentiation protocol. The TBV2 mouse embryonic stem (mES) cell line was used throughout this study. Cells were cultured on a feeder layer of mitotically inactivated mouse embryonic fibroblast (MEFs) in order to keep them undifferentiated in a pluripotent state. The propagation medium was high glucose DMEM supplemented with sodium pyruvate, L-glutamine, penicillin–streptomycin, 2-mercaptoethanol, 15% ES-screened FBS (Hyclone), and 10^3 U/mL LIF (Millipore). mES cells were passed twice on 0.1% gelatin-coated tissue culture dishes without MEFs before starting experiments. Differentiation of mES cells into the cardiac lineage was carried out in feeder-free conditions using standard techniques.[4] In differentiation medium (high glucose DMEM supplemented with sodium pyruvate, L-glutamine, penicillin–streptomycin, 2-mercaptoethanol, and 15% FBS (GIBCO) without LIF), embryoid bodies (EBs) were aggregated using the “hanging drop” method (300cells/drop): cells were cultured for 2 days (d0–d2) as hanging drops and then for 3 days (d2–d5) in suspension in low-attachment Petri dishes (Falcon).[4] 5-day-old EBs were plated onto 0.1% gelatin-coated tissue culture dishes in differentiation medium and harvested at different time points for mRNA analysis, ChIP, immunoblotting, and FACS.

Total RNA extraction, cDNA synthesis, and qRT-PCR gene expression analysis. RNA was extracted from mES cells and cardiac cells using TRIzol (Invitrogen). RNA was reverse-transcribed to cDNA using the Super Script VILO cDNA Synthesis Kit (Invitrogen) and amplified by real-time quantitative PCR with SYBR Green PCR master mix (Applied Biosystem) and specific primers for pluripotency and cardiac markers. Primer sequences are available upon request. Each sample was analyzed in triplicate using an ABI 7900HT (Applied Biosystems). 18s or GAPDH were used as the housekeeping gene for expression normalization.

Epigenetic enzyme TaqMan assay cards. RNA was extracted from mES cells and cardiac cells using TRIzol (Invitrogen). RNA was reverse transcribed to cDNA using Super Script VILO cDNA Synthesis Kit (Invitrogen). 1.2µg of cDNA of each sample was used to analyze the expression of 85 genes encoding important epigenetic enzymes. Custom microfluidic gene expression cards were drawn using TaqMan probes (Applied Biosystems). TaqMan probe information and card design is available upon request. Each sample was analyzed using an ABI 7900HT (Applied Biosystems). Data were analyzed with DataAssist software using the median normalization method.

Immunoblotting. Immunoblotting was carried out using standard protocols. The following primary antibodies were used overnight at 4°C: anti-KMT4 DOT1L (abcam ab64077), anti-

H3 dimethyl Lys79 (abcam ab3594), anti-H3 (abcam ab1791), anti-POU domain, class 5, transcription factor 1 (Pou5F1) (abcam ab19857), anti-myosin heavy chain (MYH) (Chemicon MAB 1552), and anti-lamin B (Santa Cruz sc6216).

Viral particle production and transduction. Two distinct short hairpin (sh)RNAs against *Dot1L* and a non-target shRNA were cloned into the plK0.1 lentiviral vector (OpenBiosystems) and tested. Viral particles were produced in 293T cells by co-transfection with pCMV-VSVG and psPAX2 plasmids.

FACS analysis. FACS was carried out using standard protocols. Cells fixed in 4% paraformaldehyde were stained with anti-troponin I (TNNI) (Millipore MAB3150) antibody. Samples acquisition was performed on FACS Canto Cell Analyzer (Becton Dickinson) and data analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Chromatin immunoprecipitation assay. Genome-wide localization of histone modifications (H3K4me3, H3K27me3, H3K79me2, and H3K9me3) for each stage was determined via chromatin immunoprecipitation followed by high-throughput sequencing with SoLiD 5500 (Life Technologies) carried out according to a standard procedure. Briefly, $5x10^6$ cells were used for each immunoprecipitation. Cells were cross-linked for 10 min at room temperature using 1% formaldehyde. Cross-linking was quenched by adding glycine to a final concentration of 0.125M. The cells were then collected, resuspended in lysis buffer (5mM PIPES pH8, 85mM KCl, 0.5% NP40, and protease inhibitors), and incubated on ice for 15 minutes before proceeding with sonication to generate 200−400bp fragments. The efficiency of sonication was assessed with agarose gel electrophoresis. Chromatin samples were pre-cleared for 1 hour with protein-G beads and then immunoprecipitated overnight at 4°C with specific antibodies: anti-H3 dimethyl Lys79 (abcam ab3594), anti-H3 trimethyl Lys4 (Active Motif 39159), anti-H3 trimethyl Lys 27 (Millipore-Upstate 07-449), anti-H3 trimethyl Lys9 (Millipore-Upstate 07-442), anti-H3 (abcam ab1791) and rabbit IgG (Millipore-Upstate 12-370). After incubation, the immunocomplexes were bound to protein-G beads for 2 hours and subsequently washed with low-salt wash buffer (0.1% SDS, 2mM EDTA, 20mM Tris HCl pH8, 1% Triton X-100, 150mM NaCl and protease inhibitors), high-salt wash buffer (0.1% SDS, 2 mM EDTA, 20mM Tris HCl pH8, 1% Triton x-100, 500mM NaCl, and protease inhibitors), and TE buffer. Immunocomplexes were eluted in elution buffer (1% SDS, 100mM $NaHCO_3$ and protease inhibitors) and the cross-linking was reverted overnight at 65°C. Samples were treated with proteinase K, extracted with phenol/chloroform, and precipitated with ethanol. Purified DNA was evaluated by qPCR on an ABI 7900HT with SYBR green PCR master mix (Applied Biosystem) using specific primers designed close to the promoter region and the gene transcription start site (TSS): A = -1000bp/-500bp from TSS; B = +500bp/+1000bp from TSS; C = +3500bp/+4000bp from TSS. Values obtained were normalized to the input and to the H3 content. The sequences of the primers used for ChIP-qPCR are available upon request.

ChIP-seq analysis pipeline. ChIP-seq experiments were performed as previously described.[5] Library preparation and DNA sequencing was performed at Genomnia Srl.
ChIP-seq data analysis was performed using distinct bioinformatics software. For H3K4me3, H3K27me3 and H3K9me3 modifications in mES cells, bed files were downloaded from Gene Expression Omnibus (H3K4me3: GSM307618; H3K27me3: GSM307619; H3K9me3: GSM307621). Genome coordinates were converted from mm8 to mm9 mouse reference genome using Batch Coordinate Conversion (LiftOver) created by

the UCSC Genome Bioinformatics Group. For the H3K79me2 modification in mES cells, raw sequencing reads were downloaded from Gene Expression Omnibus (GSM307150-GSM307151) and mapped to the mouse genome (version mm9) using BOWTIE (version 0.12.8).

To profile histone modifications in cardiomyocytes, sequencing reads were mapped to the mouse genome (version mm9) using BOWTIE (version 0.12.8). Uniquely mapped reads with no more than two mismatches were used for binding peak detection. To identify peaks, two peak-calling software were used: MACS (Model-based Analysis of ChIP-seq)[6] and SICER (Spatial Clustering Approach for the Identification of ChIP-Enriched Regions)[7] Both software detected binding peaks by comparing IP and input control. We used the following parameters: MACS: effective genome size = 1.87e+09, band width = 300, model fold = 5.30, p-value cutoff = 1.00e-05; SICER: windows size =200, gap size = 600, redundancy threshold = 1, FDR = 0.05. Occupancy analysis and differential binding affinity analysis were assessed with the R Bioconductor package DiffBind (v. 1.2.0).[8] The final set of binding peaks contained those that were called by both software. Genomic annotation of the peaks identified from the ChIP-seq data were performed using the R Bioconductor package ChIPpeakAnno (v. 2.4.0).[9] A summary of the genes associated with H3K79me2-, H3K4me3-, H3K27me3-, and H3K9me3-occupied regions is provided in Table S1. The complete ChIP-seq datasets are available from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nih.gov/geo/), under the accession number GSE45174.

Density profiles of chromatin marks at promoters and gene bodies were computed with HOMER v. 4.5 (Empirical Motif Optimizer).[10] Functional annotation of differentially enriched modifications was performed using GREAT.[11] Genome browser representations of H3K79me2-, H3K4me3-, H3K27me3-, and H3K9me3-enrichment profiles were obtained with IGV 2.1 visualization software.[12]

Gene expression microarray analysis pipeline. BeadChips were scanned with the Illumina iScan system. Raw data were background-subtracted and normalized using the quantile normalization method of the R Bioconductor package lumi.[13] Normalized data were filtered for genes with significant expression levels compared with negative control beads. Selection for differentially expressed genes was performed on statistical significance (adjusted p-value< 0,001) according to the Illumina t-test error model (limma R Bioconductor package).[14] The transcript with the highest median expression was selected to represent the expression of the gene if it was represented with several transcripts. Functional annotation of significant genes identified by microarray analysis was searched by the web-accessible program named Database for Annotation, Visualization and Integrated Discovery (DAVID) version 2009, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH) (david.abcc.ncifcrf.gov).[15] The complete Illumina gene expression datasets are available from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nih.gov/geo/), under the accession number GSE44829.

Correlation of ChIP-seq and gene expression data. Genes were ordered by magnitude of differential mark occupancy, and heatmaps of relative gene expression generated. Genes with higher than average expression were marked in red and those with lower than average expression were marked in green (scale in standard deviations). Scatter plots were produced to see how the expression level agreed with degree of histone modification. The Pearson correlation coefficients between histone modifications and the expression level of genes were calculated using R. Network analysis considering direct and indirect

relationship among genes was performed using Ingenuity Pathway Analysis software (v. 8.5, Ingenuity® Systems, www.ingenuity.com).

Statistical analysis. Data are presented as mean ± SD. *P*-values were determined by two-tailed t-test. P<0.05 was considered statistically significant.

Supplemental References

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S1

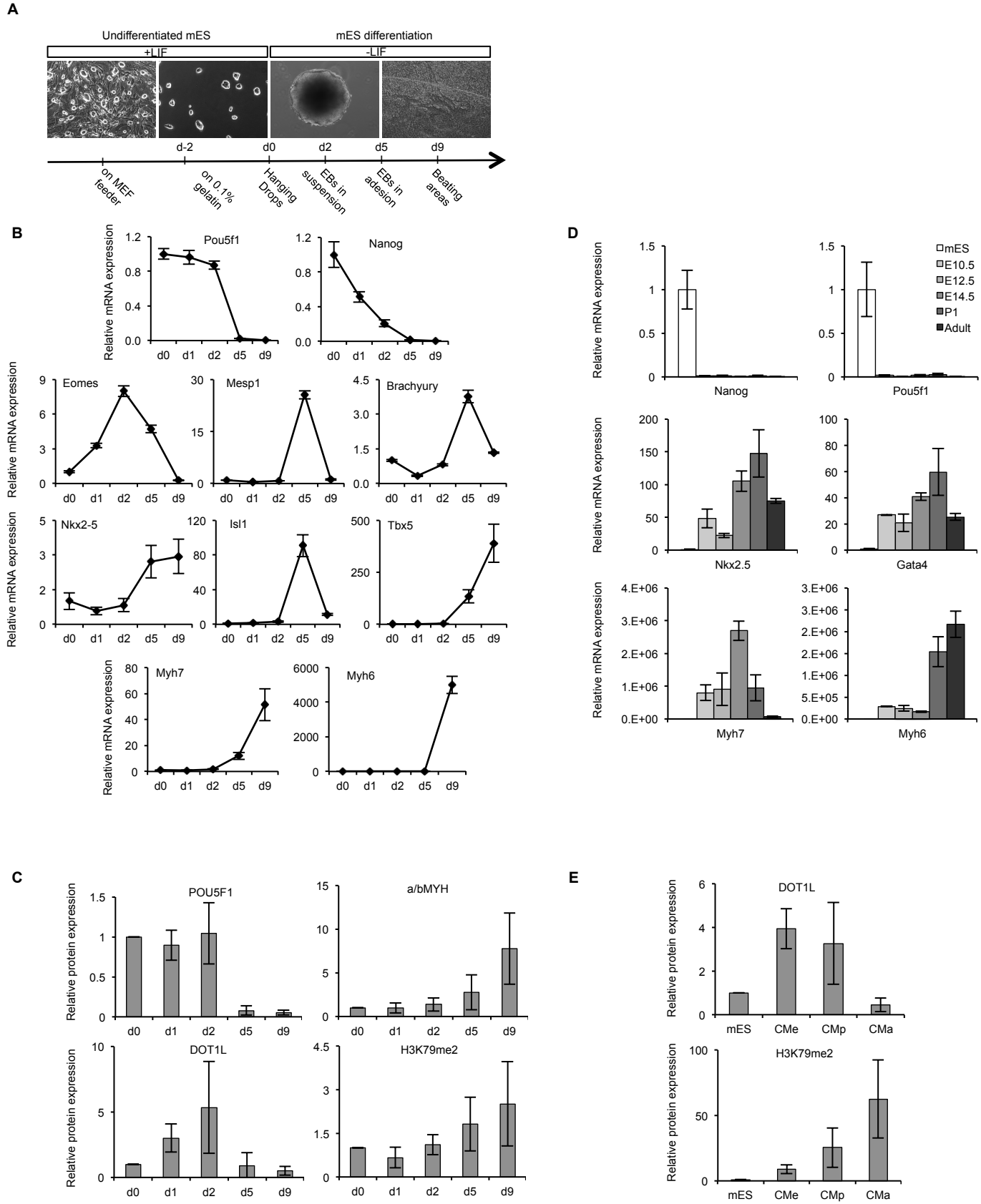
A
Undifferentiated mES
+LIF
mES differentiation
-LIF
d-2
d0
d2
d5
d9
on MEF feeder
on 0.1% gelatin
Hanging Drops
EBs in suspension
EBs in adesion
Beating areas
B
Relative mRNA expression
Pou5f1
Nanog
Eomes
Mesp1
Brachyury
Nkx2-5
Isl1
Tbx5
Myh7
Myh6
d0 d1 d2 d5 d9
D
Relative mRNA expression
mES
E10.5
E12.5
E14.5
P1
Adult
Nanog
Pou5f1
Nkx2.5
Gata4
Myh7
Myh6
C
Relative protein expression
POU5F1
a/bMYH
DOT1L
H3K79me2
d0 d1 d2 d5 d9
E
Relative protein expression
DOT1L
H3K79me2
mES
CMe
CMp
CMa

Fig. S1. A, Cardiomyogenic differentiation of mES cells. Time-course scheme and phase-contrast microscopy images of differentiation of mES cells via the hanging-drop method. **B,** Relative qRT-PCRs of differentially expressed genes during differentiation of mouse embryonic stem cells towards the cardiac lineage. *Pou5F1* and *Nanog* (pluripotency markers), *Eomes, Mesp1* and *Brachyury* (mesodermal markers), *Nkx2-5, Isl1* and *Tbx5* (cardiac progenitor markers) and *Myh7* and *Mhy6* (cardiomyocyte markers). mRNA levels are normalized to 18s, expressed as mean±SD of experimental replicates and plotted as relative mRNA expression. Three independent experiments were performed. **C**, Western blot quantification and normalization of protein expression levels in mES cells at 0, 1, 2, 5 and 9 days of differentiation. The expression levels are shown as relative to d0 and normalized to that of lamin B (LAM b) for POU5F1, α/βMYH and DOT1L, and to that of unmodified H3 for H3K79me2. Results are presented as mean±SD of three biological replicates. **D,** Gene expression profile of CM markers confirming the developmental stage used. qRT-PCR was used to assess relative mRNA expression of markers of pluripotency (*Pou5F1*, *Nanog*) and of early (*Nkx2*-5, *Gata4*, and *Myh7*) and late (*Myh6*) stages of heart development in mouse embryonic stem cells (mES) and cardiomyocytes from E10.5, E12.5, E14.5 embryos, 1-day-old pups (P1) and 2-month-old mice (Adult). Bars represent the mean±SD of three biological replicates. **E,** Western blot quantification and normalization of protein expression levels in mES and CMs from E14.5 (CMe), P1 (CMp) and adult mice (CMa). The expression levels are shown relative to mES and normalized to that of lamin B (LAM b) for DOT1L, and to that of unmodified H3 for H3K79me2. Results are presented as mean±SD of three biological replicates.

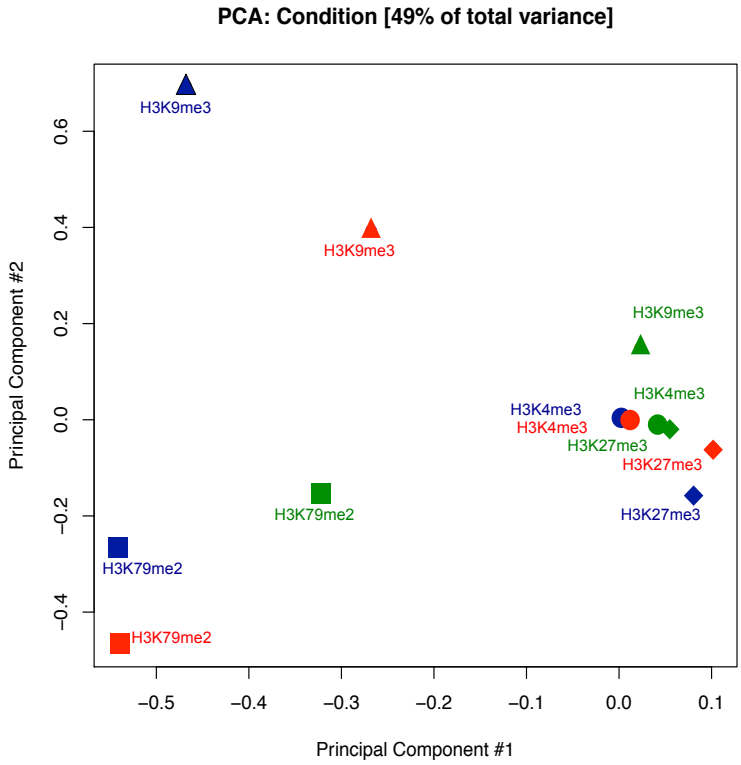

Fig. S2. Principal Component Analysis (PCA) for associations within different samples. Affinity data plotted for H3K79me2 (□), H3K4me3 (○), H3K27me3 (◇) and H3K9me3 (△) in mouse embryonic stem cells (mES, blue), cardiomyocytes from 1-day-old pups (CMp, red) and cardiomyocytes from 2-month-old mice (CMa, green). The graph displays on the x and y axes the first two principal components identified by PCA, revealing potential associations between samples and modifications.

S3

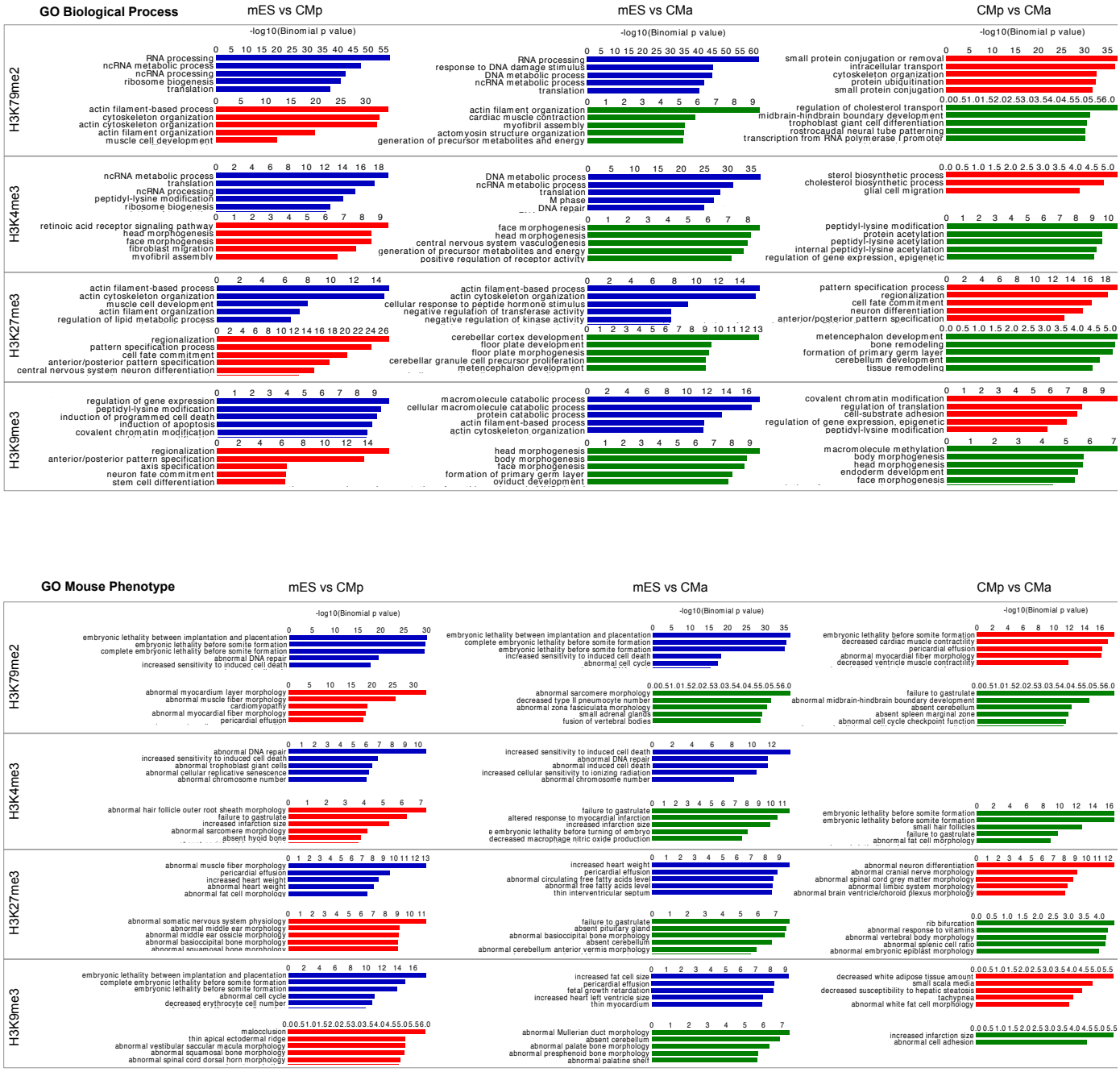

Fig. S3. Functional and molecular characterization of histone methylation marks in cardiomyocytes during differentiation. Functional annotation of differentially enriched modifications was performed using GREAT. The top 5 over-represented categories belonging to Gene Ontology (GO) Biological Process and GO Mouse Phenotype are shown. The x axis (in logarithmic scale) gives the binomial raw (uncorrelated) P-values. Results from three different comparative analyses are shown for each mark (H3K79me2, H3K4me3, H3K27me3 and H3K9me3): mES vs. CMp (left), mES vs. CMa (middle), and CMp vs. CMa (right). Blue histograms represent mES cells, red histograms represent CMp, and green histograms represent CMa.

S4

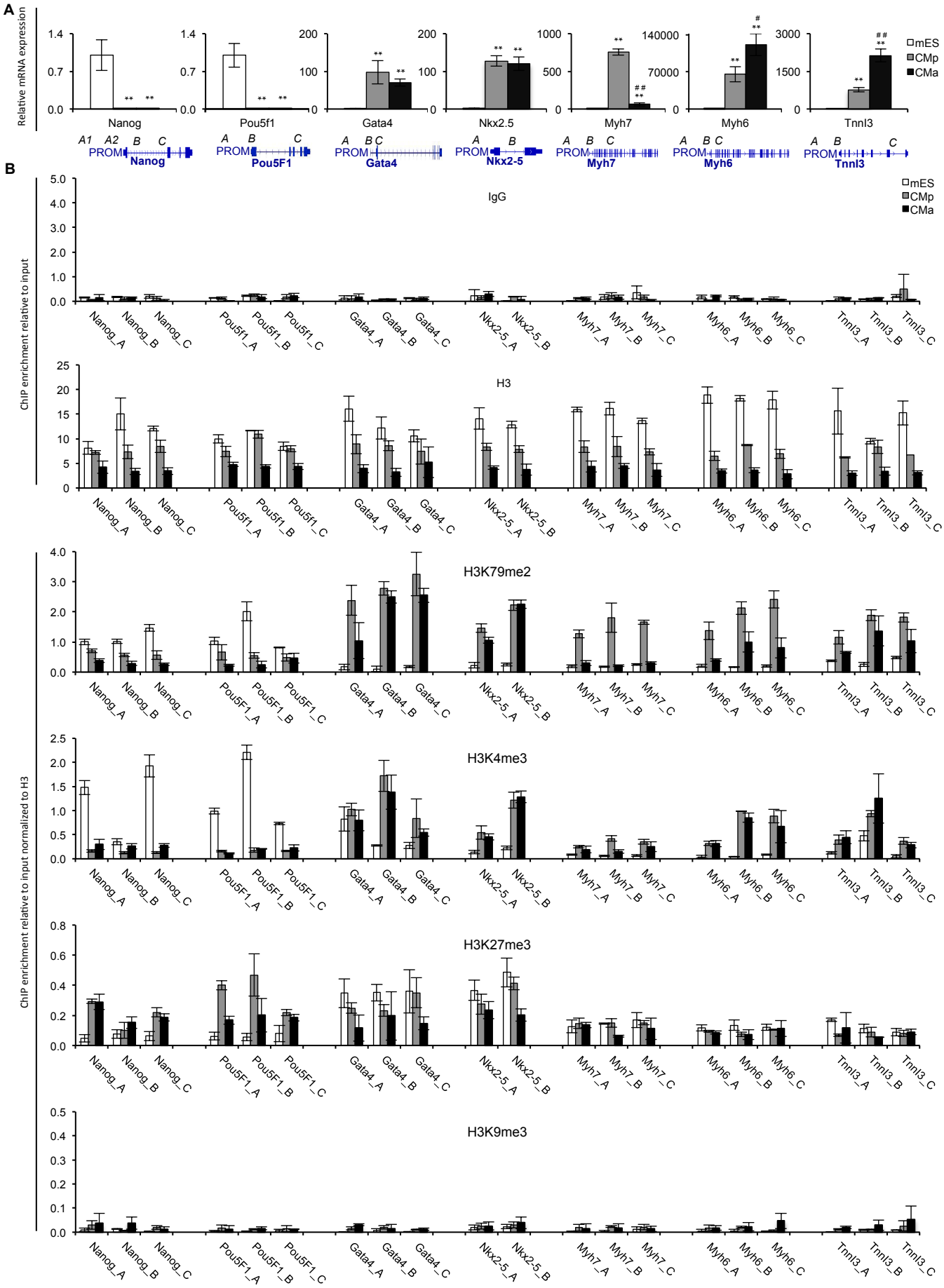
A
Relative mRNA expression
mES
CMp
CMa
Nanog
Pou5f1
Gata4
Nkx2.5
Myh7
Myh6
Tnni3
PROM
Nanog
Pou5F1
Gata4
Nkx2-5
Myh7
Myh6
Tnni3
B
ChIP enrichment relative to input
IgG
H3
ChIP enrichment relative to input normalized to H3
H3K79me2
H3K4me3
H3K27me3
H3K9me3
Nanog_A
Nanog_B
Nanog_C
Pou5f1_A
Pou5f1_B
Pou5f1_C
Gata4_A
Gata4_B
Gata4_C
Nkx2-5_A
Nkx2-5_B
Myh7_A
Myh7_B
Myh7_C
Myh6_A
Myh6_B
Myh6_C
Tnni3_A
Tnni3_B
Tnni3_C

Fig. S4. Histone methylation enrichment and mRNA expression at key cardiac differentiation genes. **A,** Relative mRNA expression was assessed through qRT-PCR in mouse embryonic stem cells (mES), post-natal cardiomyocytes (CMp) and adult cardiomyocytes (CMa). mRNA levels for *Nanog*, *Pou5F1*, *Gata4*, *Nkx2*-5, *Myh7*, *Myh6*, and *TnnI3* are normalized to 18s and expressed as the mean ±SD of three independent experiments. **, P<0.01 vs mES; ##, P<0.01 vs CMp. **B,** ChIP assay of H3K79me2, H3K4me3, H3K27me3 and H3K9me3 binding to *Nanog*, *Pou5F1*, *Gata4*, Nkx2.5, *Myh7*, *Myh6* and *TnnI3*. Levels were determined by qPCR and are expressed as fold change to the input and relative to H3. Three different regions were analysed for each gene locus: −1000bp/−500bp from TSS (_A), +500bp/+1000bp from TSS (_B), and +3500bp/+4000bp from TSS (_C). Data are the result of three independent experiments. The enrichment levels for IgG (negative control) and the unmodified H3 relative to input are given as controls.

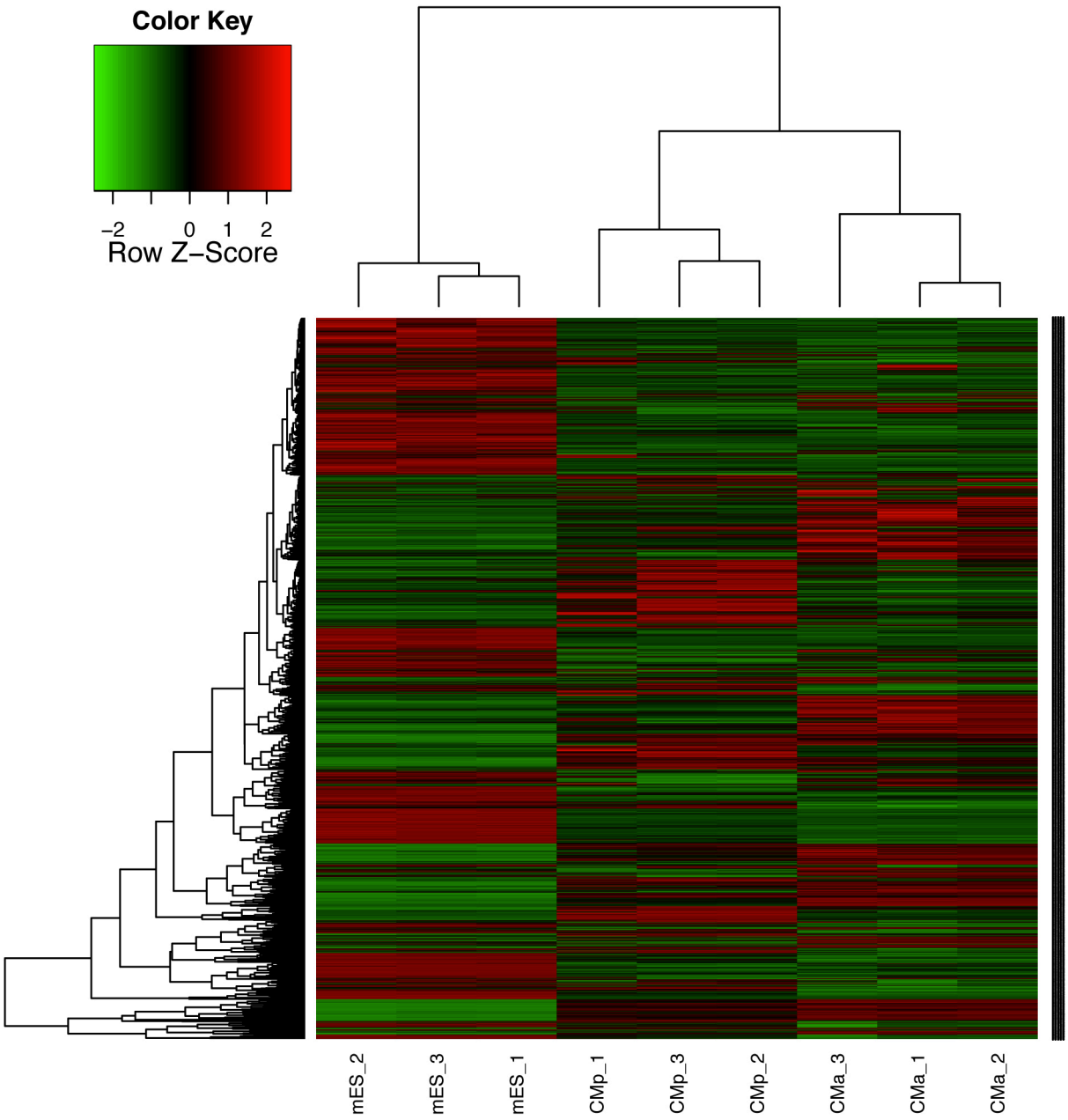

Fig. S5. Gene expression profile of cardiomyocytes at different stages of differentiation. Illumina mRNA gene expression datasets are presented for three biological replicates of mouse embryonic stem cells (mES), post-natal cardiomyocytes (CMp) and adult cardiomyocytes (CMa). Data were subjected to two-way hierarchical clustering (centred correlation distance, centroid linkage); the heatmap was created using the limma R Bioconductor package. Samples with higher than average expression are represented in red; samples with lower than average expression are represented in green.

S6

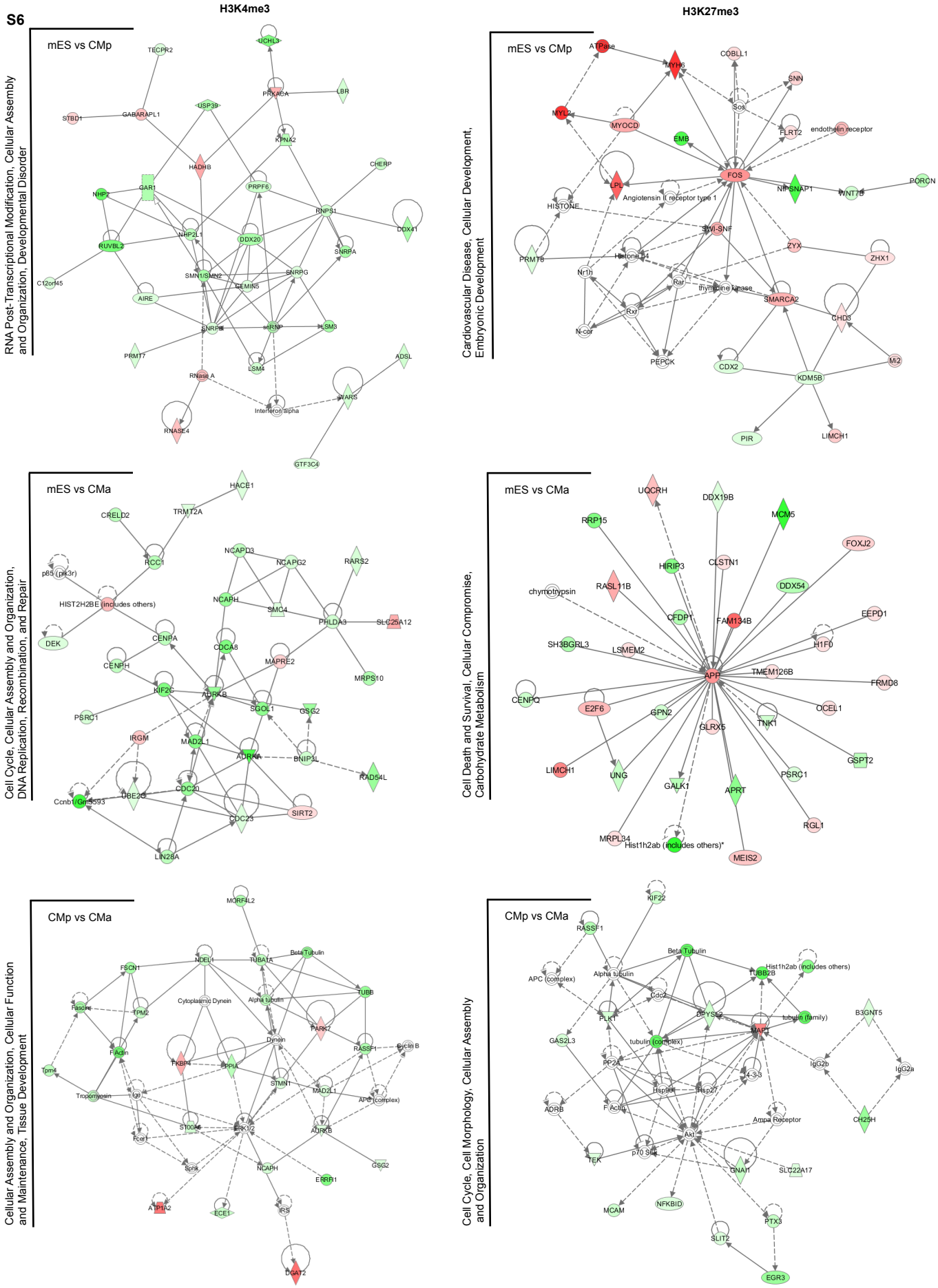
H3K4me3
mES vs CMp
RNA Post-Transcriptional Modification, Cellular Assembly and Organization, Developmental Disorder
UCHL3
TECPR2
PRKACA
LBR
USP39
STBD1
GABARAPL1
KPNA2
HADHB
CHERP
GAR1
NHP2
PRPF6
RNPS1
DDX41
NHP2L1
DDX20
RUVBL2
SNRPA
SMN1/SMN2
SNRPG
C12orf45
GEMIN5
AIRE
SNRPB
snRNP
LSM3
PRMT7
LSM4
ADSL
RNase A
IARS
Interferon alpha
RNASE4
GTF3C4
H3K27me3
mES vs CMp
Cardiovascular Disease, Cellular Development, Embryonic Development
ATPase
MYH9
COBLL1
SNN
Sos
MYL2
MYOCD
FLRT2
endothelin receptor
EMB
FOS
LPL
Angiotensin II receptor type 1
NIP-SNAP1
WNT7B
PORCN
HISTONE
SWI-SNF
ZYX
ZHX1
PRMT6
Histone H4
Nr1h
Rar
thymidine kinase
SMARCA2
Rxr
CHD3
N-cor
PEPCK
CDX2
Mi2
KDM5B
PIR
LIMCH1
mES vs CMa
Cell Cycle, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair
HACE1
CRELD2
TRMT2A
RCC1
p85 (pik3r)
NCAPD3
NCAPG2
RARS2
HIST2H2BE (includes others)
NCAPH
SMC4
PHLDA3
SLC25A12
DEK
CENPA
CDCA8
MAPRE2
CENPH
MRPS10
KIF2C
ADRKB
PSRC1
SGOL1
GSG2
IRGM
MAD2L1
ADRKA
BNIP3L
RAD54L
Ccnb1/Gm5593
UBE2S
CDC20
SIRT2
CDC25
LIN28A
mES vs CMa
Cell Death and Survival, Cellular Compromise, Carbohydrate Metabolism
UQCRH
DDX19B
MCM5
RRP15
FOXJ2
HIRIP3
CLSTN1
chymotrypsin
RASL11B
DDX54
CFDP1
FAM134B
EEPD1
SH3BGRL3
LSMEM2
H1F0
APP
TMEM126B
FRMD8
CENPQ
E2F6
GPN2
GLRX5
OCEL1
TNK1
LIMCH1
UNG
GSPT2
PSRC1
GALK1
APRT
RGL1
MRPL34
Hist1h2ab (includes others)*
MEIS2
CMp vs CMa
Cellular Assembly and Organization, Cellular Function and Maintenance, Tissue Development
MORF4L2
FSCN1
NDEL1
TUBA1A
Beta Tubulin
Cytoplasmic Dynein
Alpha tubulin
TUBB
Fascin
TPM2
PARK7
Dynein
RASSF1
Cyclin B
F Actin
FKBP4
PPP1A
Tpm4
STMN1
MAD2L1
Tropomyosin
Up
APC (complex)
S100A6
ERK1/2
ADRKB
Fcer1
Sphk
NCAPH
GSG2
ERRFI1
ATP1A2
ECE1
IRS
DGAT2
CMp vs CMa
Cell Cycle, Cell Morphology, Cellular Assembly and Organization
KIF22
RASSF1
Beta Tubulin
Hist1h2ab (includes others)
APC (complex)
Alpha tubulin
TUBB2B
Cdc2
PLK1
DPYSL2
tubulin (family)
B3GNT5
MAP1
GAS2L3
tubulin (complex)
PP2A
IgG2b
IgG2a
14-3-3
Hsp90
Hsp27
ADRB
F Actin
Ampa Receptor
CH25H
Akt
TEK
p70 S6k
GNAI1
SLC22A17
MCAM
NFKBID
PTX3
SLIT2
EGR3

Fig S6. Network analysis of direct relationships among genes acquiring H3K4me3 and indirect relationships among genes acquiring H3K27me3 with transcriptional potential. The analyses were performed using Ingenuity Pathway Analysis software (v. 8.5, Ingenuity® Systems, www.ingenuity.com). Networks are displayed graphically as nodes and edges. Distinct shapes indicate distinct gene functions. Results from three different comparative analyses are shown: mES vs. CMp (top), mES vs. CMa (middle) and CMp vs. CMa (bottom). Green indicates gene downregulation and red indicates gene upregulation in CMp (mES vs. CMp), CMa (mES vs. CMa) and CMa (CMp vs. CMa), with a more intense shade indicating a greater change.

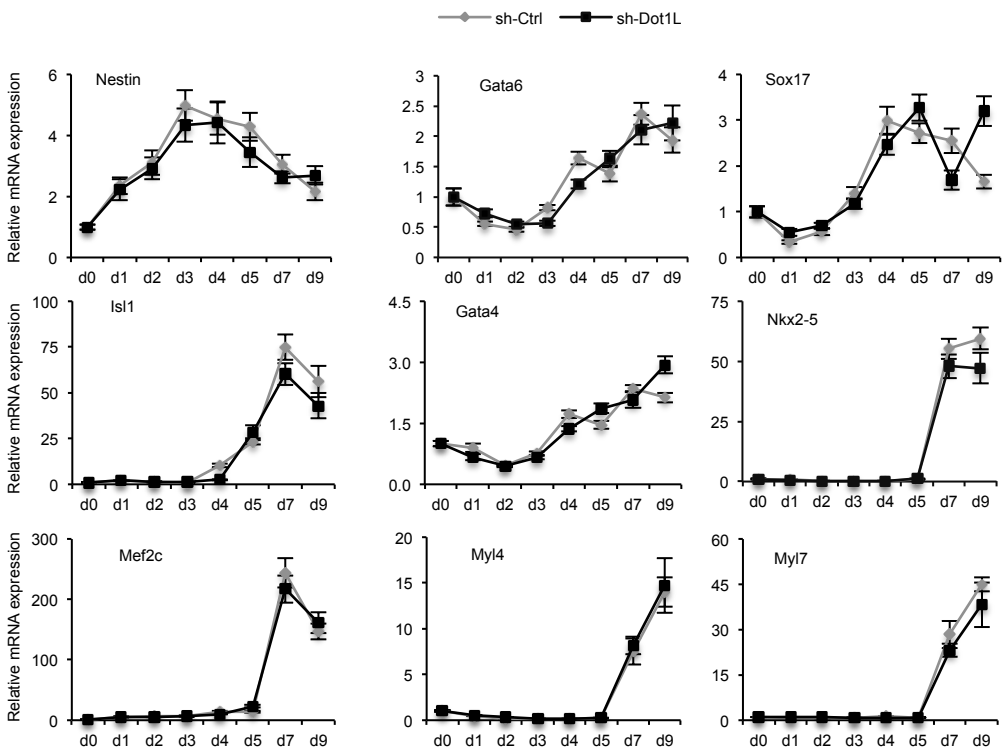

Fig S7. mRNA levels of *Nestin, Gata6, Sox17, Isl1, Gata4, Nkx2-5, Mef2C, Myl4 and Myl7* in undifferentiated (day 0) mouse embryonic stem cells and after days 1, 2, 3, 4, 5, 7 and 9 of differentiation in control (sh-Ctrl, grey line) and *Dot1L*-knockdown (sh-Dot1L, black line) cells. Levels are normalized to 18s or GAPDH, expressed as the mean±SD of experimental replicates and plotted as relative mRNA expression. Three independent experiments were performed.

SUPPLEMENTAL FIGURE LEGENDS

Fig. S1. A, Cardiomyogenic differentiation of mES cells. Time-course scheme and phase-contrast microscopy images of differentiation of mES cells via the hanging-drop method. **B,** Relative qRT-PCRs of differentially expressed genes during differentiation of mouse embryonic stem cells towards the cardiac lineage. *Pou5F1* and *Nanog* (pluripotency markers), *Eomes, Mesp1* and *Brachyury* (mesodermal markers), *Nkx2-5, Isl1*, and *Tbx5* (cardiac progenitor markers), and *Myh7* and *Mhy6* (cardiomyocyte markers). mRNA levels are normalized to 18s, expressed as mean±SD of experimental replicates, and plotted as relative mRNA expression. Three independent experiments were performed. **C**, Western blot quantification and normalization of protein expression levels in mES cells at 0, 1, 2, 5, and 9 days of differentiation. The expression levels are shown as relative to day 0 and normalized to that of lamin B (LAM b) for POU5F1, α/βMYH, and DOT1L, and to that of unmodified H3 for H3K79me2. Results are presented as mean±SD of three biological replicates. **D,** Gene expression profile of CM markers confirming the developmental stage used. qRT-PCR was used to assess relative mRNA expression of markers of pluripotency (*Pou5F1*, *Nanog*) and of early (*Nkx2*-5, *Gata4*, and *Myh7*) and late (*Myh6*) stages of heart development in mouse embryonic stem cells (mES) and cardiomyocytes from E10.5, E12.5, and E14.5 embryos, 1-day-old pups (P1), and 2-month-old mice (Adult). Bars represent the mean±SD of three biological replicates. **E,** Western blot quantification and normalization of protein expression levels in mES and CMs from E14.5 (CMe), P1 (CMp), and adult (CMa) mice. The expression levels are shown relative to mES and normalized to that of lamin B (LAM b) for DOT1L, and to that of unmodified H3 for H3K79me2. Results are presented as mean±SD of three biological replicates.

Fig. S2. Principal Component Analysis (PCA) for associations within different samples. Affinity data plotted for H3K79me2 (□), H3K4me3 (○), H3K27me3 (◇), and H3K9me3 (△) in mouse embryonic stem cells (mES, blue), cardiomyocytes from 1-day-old pups (CMp, red), and cardiomyocytes from 2-month-old mice (CMa, green). The graph displays on the x and y axes the first two principal components identified by PCA, revealing potential associations between samples and modifications.

Fig. S3. Functional and molecular characterization of histone methylation marks in cardiomyocytes during differentiation. Functional annotation of differentially enriched modifications was performed using GREAT. The top 5 over-represented categories belonging to Gene Ontology (GO) Biological Process and GO Mouse Phenotype are shown. The x axis (in logarithmic scale) gives the binomial raw (uncorrelated) P-values. Results from three different comparative analyses are shown for each mark (H3K79me2, H3K4me3, H3K27me3, and H3K9me3): mES vs. CMp (left), mES vs. CMa (middle), and CMp vs. CMa (right). Blue histograms represent mES cells, red histograms represent CMp, and green histograms represent CMa.

Fig. S4. Histone methylation enrichment and mRNA expression at key cardiac differentiation genes. **A,** Relative mRNA expression was assessed through qRT-PCR in mouse embryonic stem cells (mES), post-natal cardiomyocytes (CMp), and adult cardiomyocytes (CMa). mRNA levels for *Nanog*, *Pou5F1*, *Gata4*, *Nkx2*-5, *Myh7*, *Myh6*, and *TnnI3* are normalized to 18s and expressed as the mean±SD of three independent experiments. **, P <0.01 vs mES; $^{\#\#}$, P <0.01 vs CMp. **B,** ChIP assay of H3K79me2, H3K4me3, H3K27me3, and H3K9me3 binding to *Nanog*, *Pou5F1*, *Gata4*, Nkx2.5, *Myh7*,

Myh6, and *TnnI3*. Levels were determined by qPCR and are expressed as fold change to the input and relative to H3. Three different regions were analyzed for each gene locus: −1000bp/−500bp from TSS (_A), +500bp/+1000bp from TSS (_B), and +3500bp/+4000bp from TSS (_C). Data are the result of three independent experiments. The enrichment levels for IgG (negative control) and the unmodified H3 relative to input are given as controls.

Fig. S5. Gene expression profile of cardiomyocytes at different stages of differentiation. Illumina mRNA gene expression datasets are presented for three biological replicates of mouse embryonic stem cells (mES), post-natal cardiomyocytes (CMp), and adult cardiomyocytes (CMa). Data were subjected to two-way hierarchical clustering (centered correlation distance, centroid linkage); the heatmap was created using the limma R Bioconductor package. Samples with higher than average expression are represented in red; samples with lower than average expression are represented in green.

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SUPPLEMENTARY TABLE LEGENDS

Table 1. Differential histone modification sites. Differential histone modification sites were found comparing mES vs. CMp, mES vs. CMa, and CMp vs. CMa. mES, mouse embryonic stem cells; CMp, cardiomyocytes from 1-day-old mouse pups; CMa, cardiomyocytes from 2-month-old adult mice.

Table 2. Significantly modulated genes. Illumina microarray was used to assess differential gene expression between mES, CMp and CMa. mES, mouse embryonic stem cells; CMp, cardiomyocytes from 1-day-old mouse pups; CMa, cardiomyocytes from 2-month-old adult mice.