

Erythroid differentiation of human induced pluripotent stem cells is independent of donor cell type of origin

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Dorn et al. Supplementary Data

These supplementary data are comprised of a supplementary methods section with references and six supplementary figures with legends.

Supplementary Methods

Characterization of human CB CD34+ iPSCs

Immunofluorescence staining of iPSC colonies was performed using the following primary antibodies: OCT4 (Santa Cruz, Germany, 1:200), NANOG (ReproCell, Japan, 1:50), SSEA1 (1:200), SSEA4 (1:200), TRA-1-60 (1:200) and TRA-1-81 (1:200, all from Chemicon, MA, USA). Alkaline phosphatase staining was performed with the ES Cell Characterization Kit (Chemicon). For retroviral promoter silencing analysis, qRT-PCR was performed using a vector-specific primer pair (cMYC endo: CGT.GAC.CAG.ATC.CCG.GAG.TT, IRES exo: GCC.TGC.AAA.GGG.TCG.CTA.CA). Human iPSCs (1.5×10^6 cells/mice) were injected subcutaneously into the dorsal flank of severe combined immunodeficient mice. Six to eight weeks after the injection, the teratomas that formed were fixed overnight in 4% paraformaldehyde and embedded in paraffin. The sections were stained with hematoxylin and eosin dyes.

Microarray data processing

The bead intensities were mapped to the gene information using BeadStudio 3.2 (Illumina). Background correction was performed using the Affymetrix Robust Multi-array Analysis (RMA) background correction model.¹ Variance stabilization was performed using log₂ scaling, and the gene expression data were normalized using the method implemented in the Lumi package of R-Bioconductor. Data post-processing and graphing were performed with in-house developed functions in Matlab. Hierarchical clustering of genes and samples was performed with the one minus correlation metric and the unweighted average distance (UPGMA) (also known as group average) linkage method previously described.²

Global DNA methylation analysis

Genomic DNA was extracted from human iPSCs (passages ≥ 15) using the QIAamp[®] DNA Blood Mini Kit (Qiagen), and 1 μg of genomic DNA was sonicated to fragment sizes of 300-1000bp using a Vibra Cell 75022 Ultrasonic Processor. These DNA samples were then immunoprecipitated with an antibody against methylated DNA using the Diagenode's MeDIP kit. Amplification of the input and output samples was performed using the Genome Plex[®] Complete WGA Kit (Sigma Aldrich). Hybridization of 1 μg of each amplified DNA sample was performed on NimbleGen 385K RefSeq Promoter Arrays HG18 containing all known RefSeq genes (Roche). The promoter regions on these arrays are covered by 50-mer probes with approximately 100bp spacing. The hybridization procedure was performed as suggested by the manufacturer. The hybridized arrays were scanned using an Axon 4000B microarray scanner (Molecular Devices, CA, USA), and the images were analyzed with Axon GenePix software version 4.1. Image and data analysis were performed using NimbleScan version 2.5 and SignalMap version 1.9 software.

Methylation data processing

The methylation state was normalized across samples using the quantile method. Data post-processing and graphics was performed with in-house developed functions in Matlab. Hierarchical clustering of genes and samples was performed with one minus correlation metric and the unweighted average distance (UPGMA) (also known as group average) linkage method. The molecular signatures were taken from the gene set collection C2 of the version 3.0 of the Molecular Signatures Database (MSigDB).³ The significance of the gene set of the different expressed genes was analyzed using an enrichment approach based on the hypergeometric distribution. The significance (p-value) of the gene set enrichment was calculated using the hypergeometric distribution. The multitest effect influence was corrected through controlling the false discovery rate using the Benjamini-Hochberg correction at a significance level $\alpha=0.05$. For the bars plot analysis at individual loci the scaled log₂ ratios of the data were used. Each feature on the array has a corresponding scaled log₂ ratio, which

is the ratio of the input signals for the experimental (IP) and control (input) samples co-hybridized to the array. The log₂ ratio is computed and scaled to center the ratio data around zero. The centering is performed by subtracting the bi-weight mean for the log₂ ratio values for all features on the array from each log₂ ratio value.

Monitoring hematopoietic and erythroid development

Flow cytometry: 1×10^5 cells were incubated for 20 minutes at 4°C with antibodies against CD41, CD43, CD45, CD34, CD71 (all from Becton Dickinson, Germany), CD11, CD14, CD15, CD36 and glycophorin A (GPA) (all from Beckman Coulter, Germany) or the corresponding isotype controls. The cells were analyzed on a Navios™ flow cytometer (Beckman Coulter). Dead cells and nuclei were excluded by 4',6-Diamidin-2-phenylindol (DAPI) staining. **Colony formation:** Colony formation was performed as described previously.⁴ Briefly, 30,000 cells from dissociated day 21 EBs were cultured in duplicate in Methocult H4434 (StemCell Technologies, CA) and scored for hematopoietic colonies after 14 days. **Cell morphology:** Cytospin preparations were stained with May-Gruenwald-Giemsa (Merck, Germany), and at least 300 cells were evaluated by microscopy (Axio Vert 200, Carl Zeiss, Germany).

Hemoglobin analysis

The percentages of tetrameric hemoglobin fractions were measured by cation exchange high performance liquid chromatography (CE-HPLC) (VWR EZ Chrom Elite, Software 3.2.1) using a Bio-Rad Variant II Hb analyzer and the β-Thalassemia kit (Bio-Rad Laboratories, CA, USA). Additionally, total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Complementary DNA synthesis was performed using M-MLV reverse transcriptase (Affymetrix) and a customized 15-bp oligo dT primer. Semiquantitative gene specific PCR for embryonic ε-chains, fetal γ-chains and adult β-chains was performed as previously described.⁵

Scanning electron microscopy

For scanning electron microscopy the cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffered saline, pH 7.4, and dehydrated stepwise in a graded ethanol series. The samples were dried with 100% ethanol via CO₂ in a critical-point apparatus (Balzers, Switzerland). The dried samples were mounted onto aluminum stubs with Leit-Tabs and coated with gold film to a thickness of 40–50 nm in a sputter coater (Leitz, Germany). The cells were viewed under a Hitachi S-530 scanning electron microscope operated in a secondary mode at 20 kV. Images were taken on 60-mm films (b/w APX-100 rollfilms, Agfa) and being scanned for digitalization.

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Supplementary Figure 1 Characterization of human CB CD34+ iPSCs

(A) Human ESC-like morphology of CD34+ iPSCs on feeder cells. **(B)** Alkaline phosphatase staining of a CD34+ iPSC colony. **(C,D)** Immunocytochemical analysis of pluripotency and surface markers. CD34-4F-iPS and CD34-2F-iPS cell colonies stained positive for OCT4, NANOG, SSEA4, TRA1-60 and TRA1-81. **(E)** Histological presentation of a teratoma formed 6 weeks after transplantation of CB derived CD34+ iPSCs into severe combined immunodeficient mice; hematoxylin and eosin (H/E) staining showing gut-like structures (endoderm), smooth muscle and chondrocytic elements (mesoderm) and neural rosettes (ectoderm) within the teratomas. **(F)** Silencing analysis of the retroviral SFFV promoter in CD34+ iPSCs.

Supplementary Figure 2 CpG methylation profiles of CB CD34+ and NSC iPSCs at hematopoietic loci I

Bars plots of the CpG methylation profiles at the CD34 region of chromosome 1, the TAL1 region of chromosome 1, the KDR region of chromosome 4 and the T region of chromosome 6.

Supplementary Figure 3 CpG methylation profiles of CB CD34+ and NSC iPSCs at hematopoietic loci II

Bars plots of the CpG methylation profiles at the EPO receptor region of chromosome 7, the GATA1 region of chromosome X and the GATA2 region of chromosome 3.

Supplementary Figure 4 CpG methylation profiles of CB CD34+ and NSC iPSCs at hematopoietic loci III

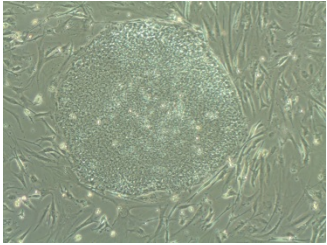
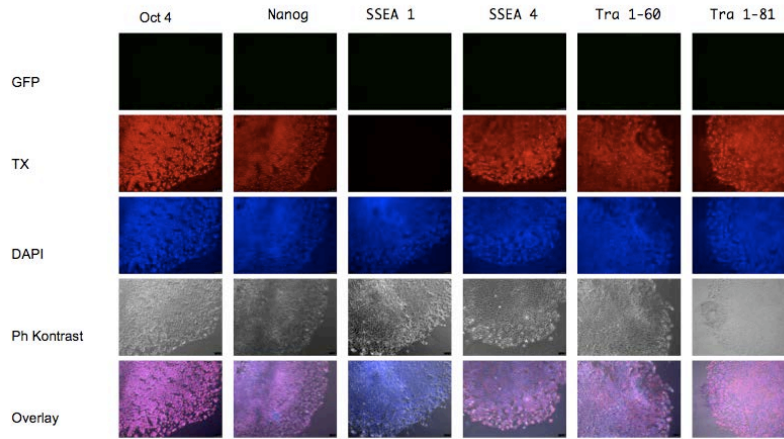
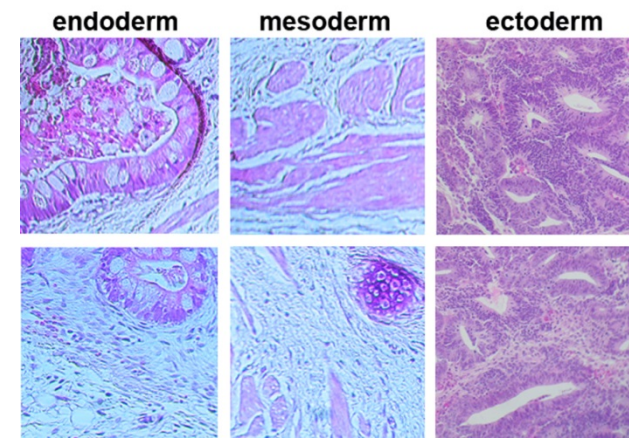
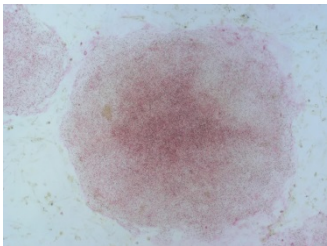
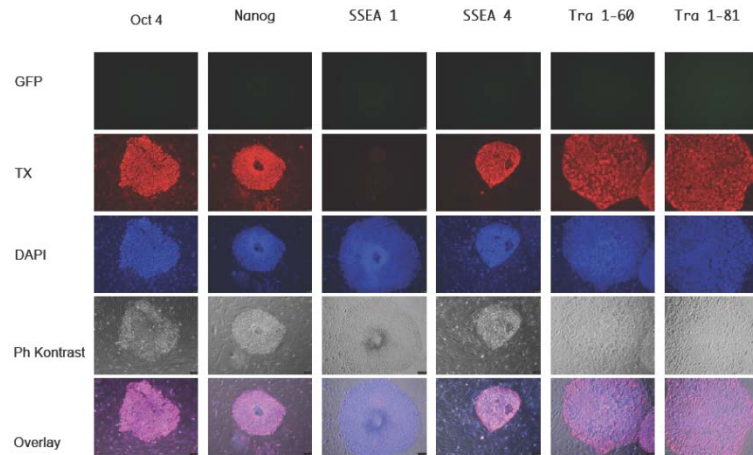
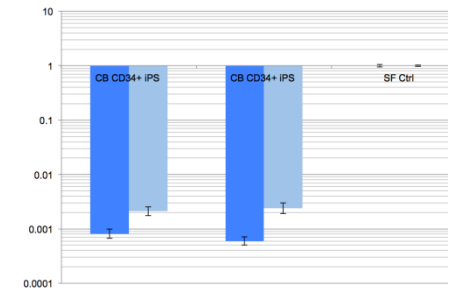
Bars plots of the CpG methylation profiles at the β -hemoglobin gene complex on chromosome 11: β -globin chain region (HBB), γ - and α -globin chain regions (HBG1 and HBG2) and ϵ -globin chain region (HBE1).

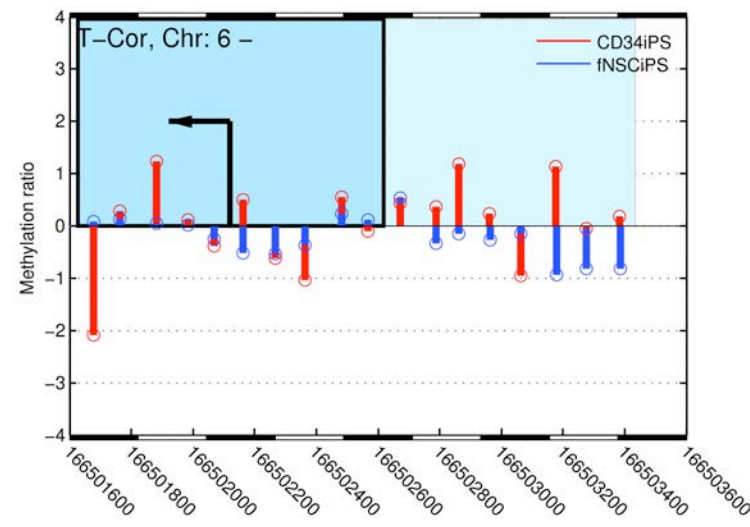
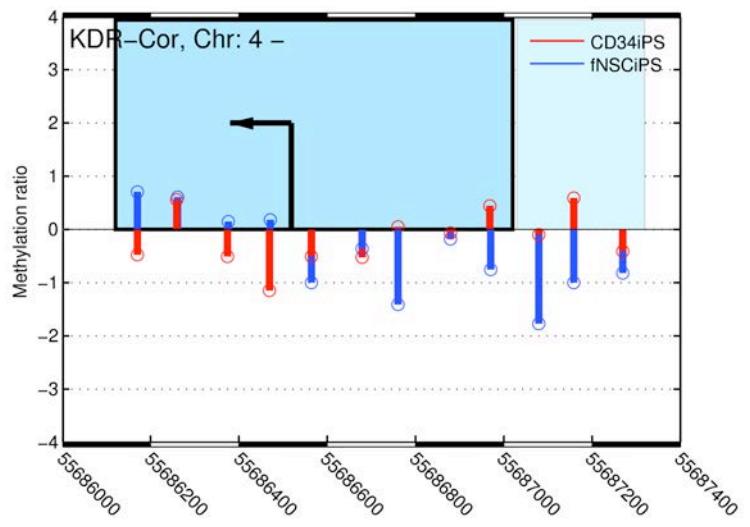
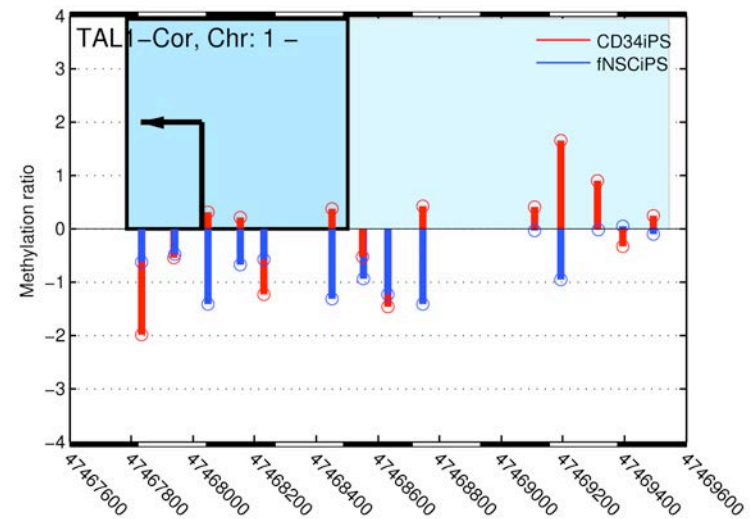
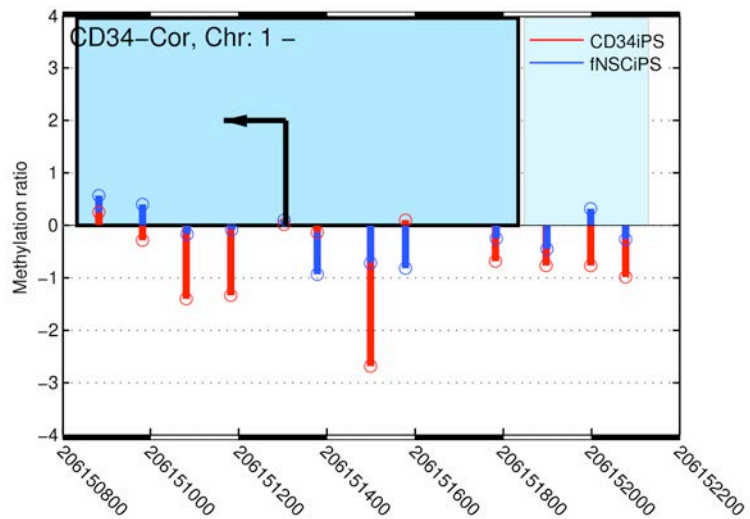
Supplementary Figure 5 CpG methylation profiles of CB CD34+ and NSC iPSCs at hematopoietic loci IV

Bars plots of the CpG methylation profiles at α -hemoglobin gene complex on chromosome 16: The α 1 chain (HBA1) region, the α 2 chain (HBA2) region and the ζ -chain region (HBZ).

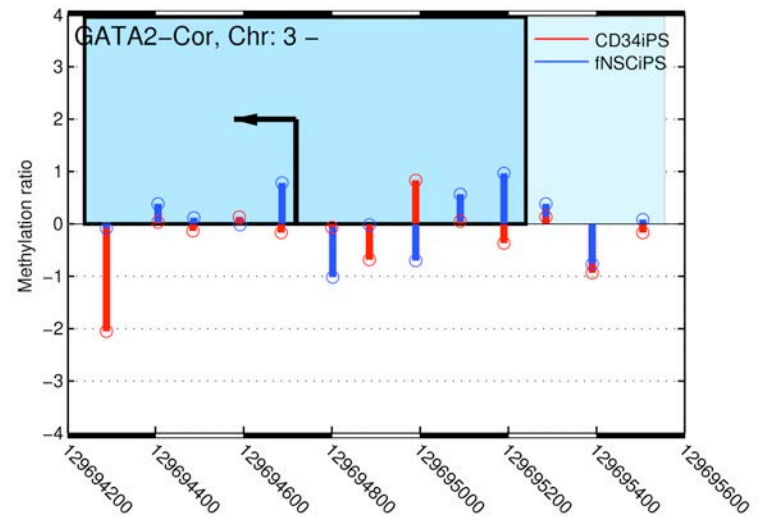
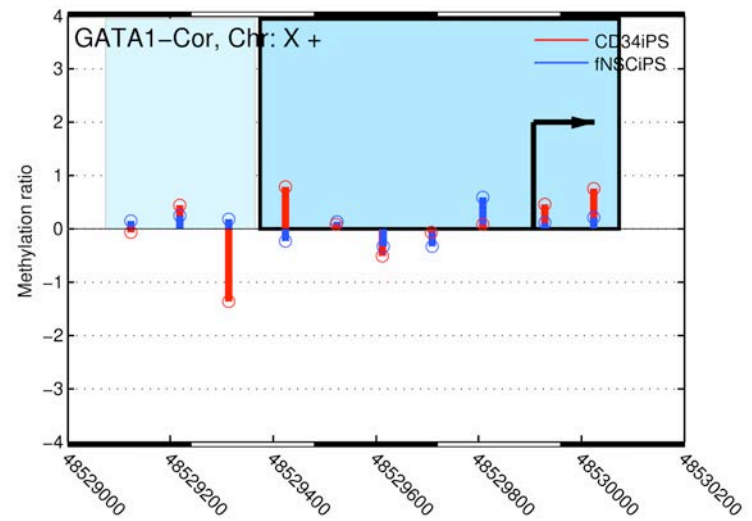
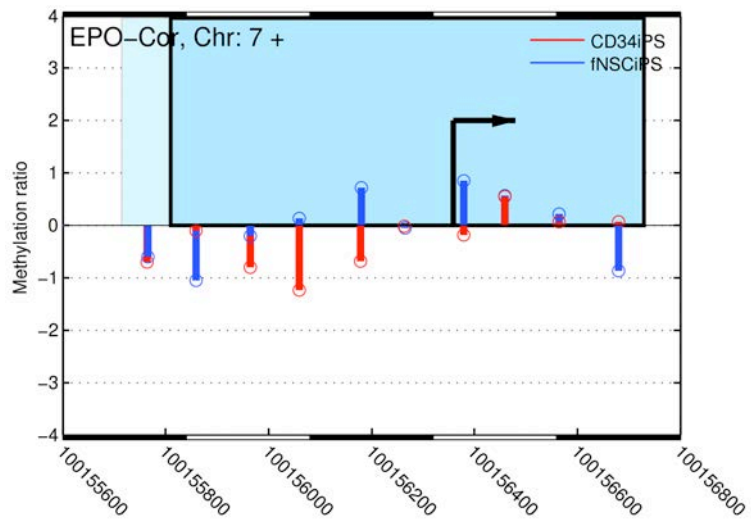
Supplementary Figure 6 CpG methylation profiles of CB CD34+ and NSC iPSCs at hematopoietic loci V

Bars plots of the CpG methylation profiles at the HOXB4 region of chromosome 17, the CDX4 region of chromosome X and RUNX1 region of chromosome 21.

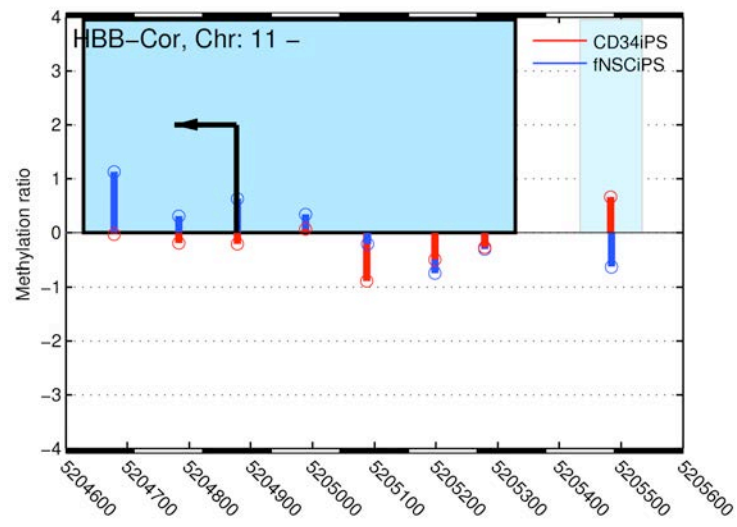
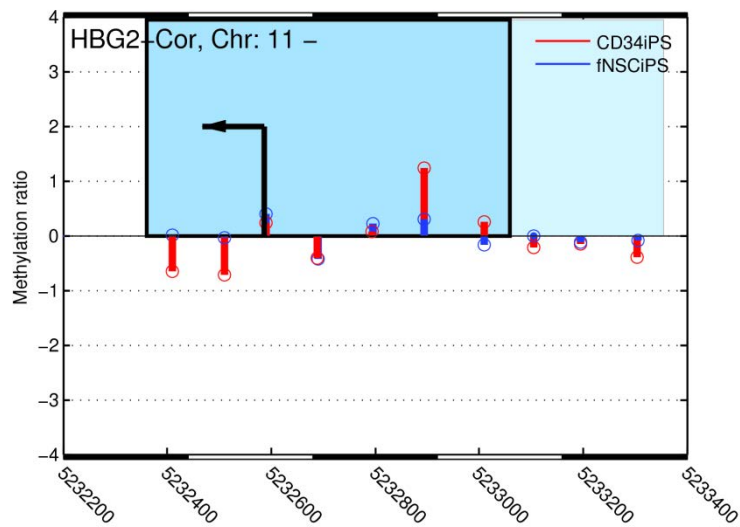
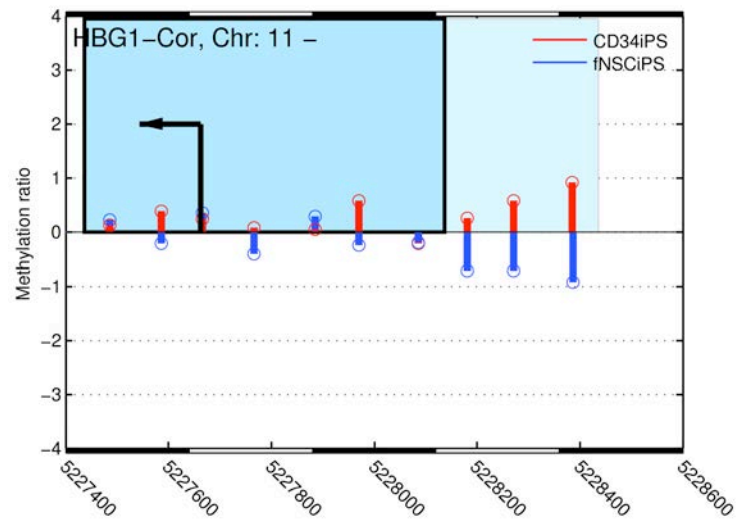
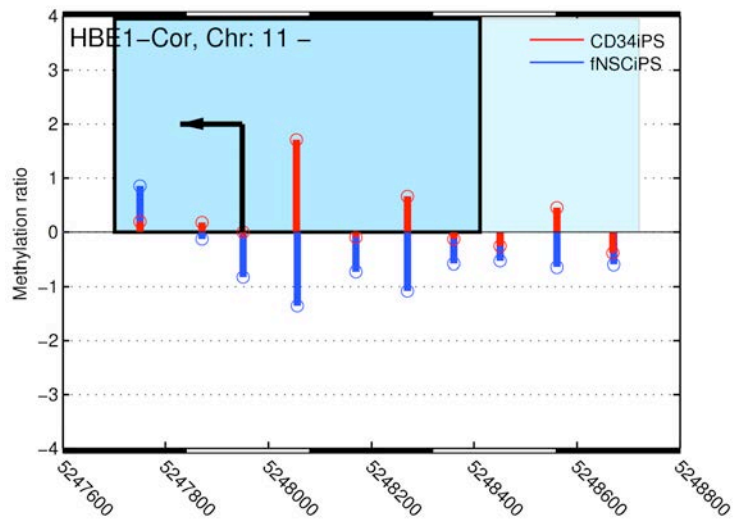
A**C****E****B****D****F**



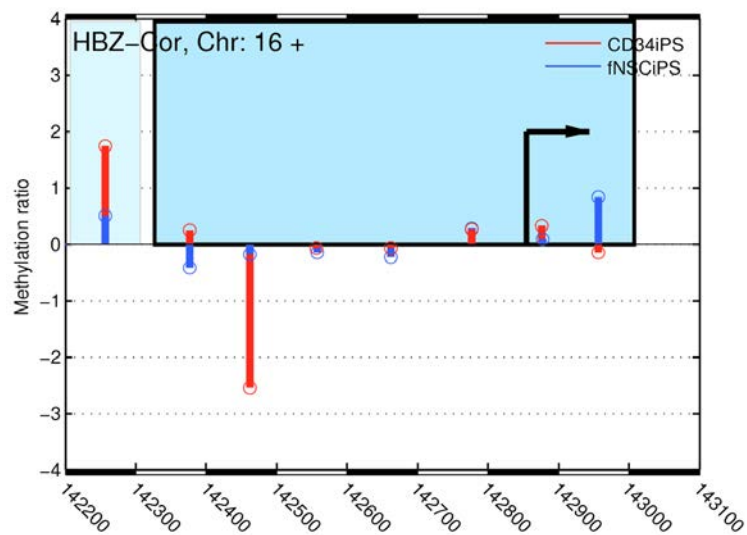
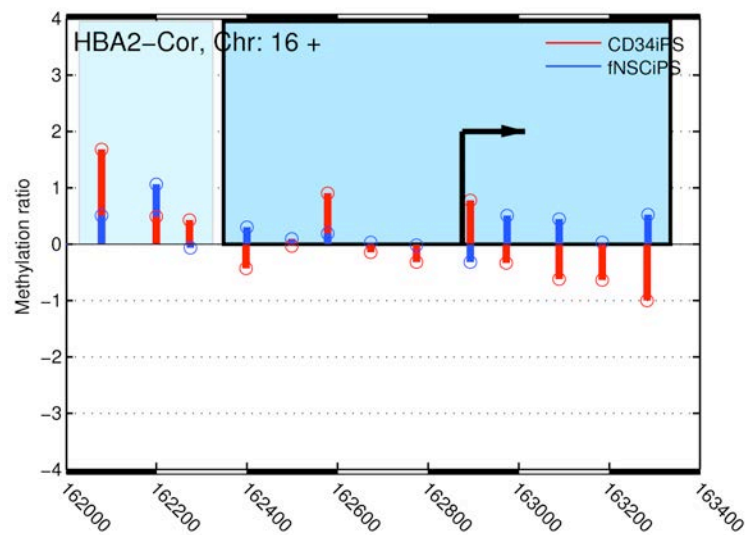
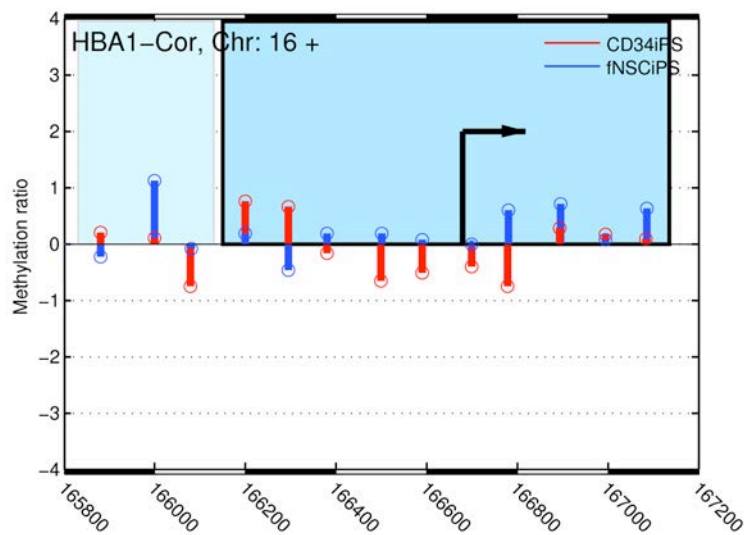
Suppl. Figure 2



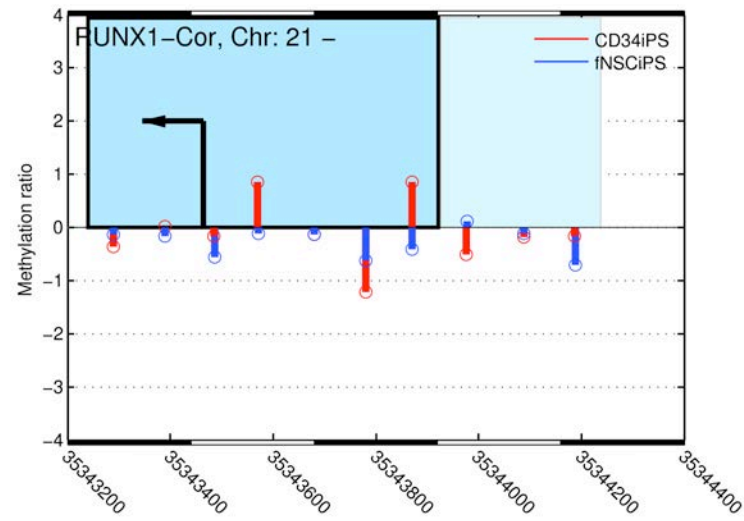
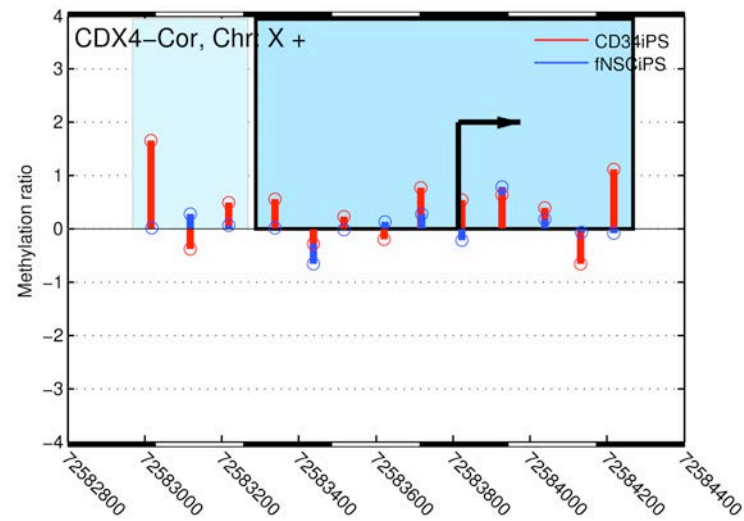
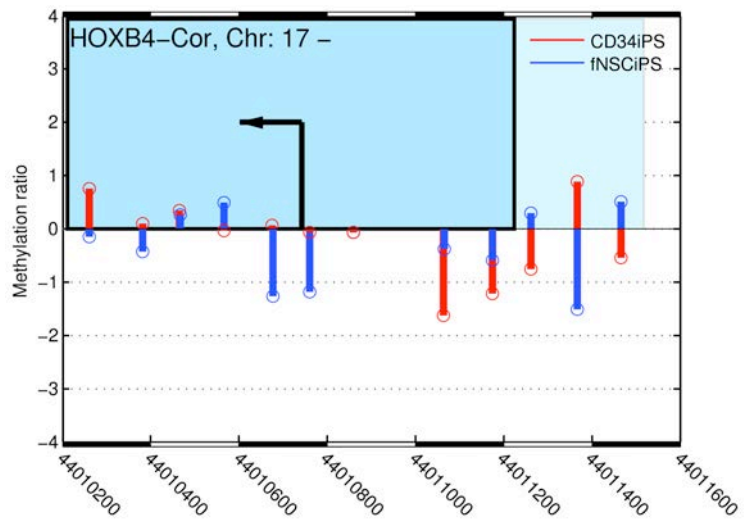
Suppl. Figure 3



Suppl. Figure 4



Suppl. Figure 5



Suppl. Figure 6