LETTER

Pluripotency factors functionally premark cell-type-restricted enhancers in ES cells

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Enhancers for embryonic stem (ES) cell-expressed genes and lineage-determining factors are characterized by conventional marks of enhancer activation in ES cells1–3, but it remains unclear whether enhancers destined to regulate cell-type-restricted transcription units might also have distinct signatures in ES cells. Here we show that cell-type-restricted enhancers are ‘premarked’ and activated as transcription units by the binding of one or two ES cell transcription factors, although they do not exhibit traditional enhancer epigenetic marks in ES cells, thus uncovering the initial temporal origins of cell-type-restricted enhancers. This premarking is required for future cell-type-restricted enhancer activity in the differentiated cells, with the strength of the ES cell signature being functionally important for the subsequent robustness of cell-type-restricted enhancer activation. We have experimentally validated this model in macrophage-restricted enhancers and neural precursor cell (NPC)-restricted enhancers using ES cell–derived macrophages or NPCs, edited to contain specific ES cell transcription factor motif deletions. DNA hydroxyl-methylation of enhancers in ES cells, determined by ES cell transcription factors, may serve as a potential molecular memory for subsequent enhancer activation in mature macrophages. These findings suggest that the massive repertoire of cell-type-restricted enhancers are essentially hierarchically and obligatorily premarked by binding of a defining ES cell transcription factor in ES cells, dictating the robustness of enhancer activation in mature cells.

Enhancers function as critical regulatory elements that integrate genomic information for cell fate transition and cell-specific gene regulation1–3. We hypothesized that cell-type-restricted enhancers might be premarked in ES cells. To begin to explore this question, we selected macrophage enhancers, because the sequential events leading to macrophage differentiation and regulation by inflammatory signals are relatively well understood4–6. We first examined the full repertoire of macrophage-restricted enhancers in mouse ES cells and found that the majority (18,405 out of 22,684 enhancers) lacked H3K4me1 (monomethylation of histone H3 at lysine 4), H3K4me2 (dimethylation of histone H3 at lysine 4), H3K27Ac (acetylation of histone H3 at lysine 27) and H3K27me3 (trimethylation of histone H3 at lysine 27) marks (referred to as ‘unmarked’ in Extended Data Fig. 1a), although about 4,000 enhancers that were active in both macrophages and ES cells, including ‘housekeeping genes’, did exhibit H3K4me2 and H3K27Ac, generally within 200 kb of coding target genes (Fig. 1a, Extended Data Fig. 1a). Finally, a small number of enhancers (214) carried H3K27me3, which marks ‘poised’ enhancers (Fig. 1a, Extended Data Fig. 1a). We carried out an assay for transposase-accessible chromatin using sequencing (ATAC-seq) to further understand the enhancer features in ES cells. The 18,405 unmarked macrophage enhancers were in an open configuration compared to random regions (Fig. 1b), consistent with published DNase hypersensitivity analyses in ES cells (Fig. 1b), but were not as robustly accessible as enhancers that are active in ES cells (Extended Data Fig. 1b). To identify transcription factors that might bind to macrophage enhancers in open chromatin configurations, we profiled the distribution of several of the most important ES cell transcription factors—ESRRB, NANOG, OCT4 and SOX2 (ENOS)—in a −1 kb/+1 kb window, ensuring that we were exclusively analysing macrophage-restricted enhancers. Notably, 6,775 macrophage-restricted enhancers showed binding of ENOS (Fig. 1c). We established the specificity of ENOS binding in macrophage-restricted enhancers by comparing with random regions (Extended Data Fig. 1c), which revealed statistically significant binding of ESRRB (Extended Data Fig. 1d). Notably, about 80% of macrophage-restricted enhancers were bound by one only or at most two ES cell transcription factors, whereas ES cell-active enhancers were bound by all four ENOS factors (Fig. 1d, Extended Data Fig. 1e), as exemplified by genome browser images (Extended Data Fig. 1f). We also analysed 12 ES cell transcription factors from the published literature7, and found that active ES cell-restricted enhancers were characterization bound predominantly by about 4–8 of the 12 ES cell transcription factors evaluated (OCT4, SOX2, NANOG, ESRRB, SMAD1, E2F1, TCFCP2L1, ZFX, STAT3, KLF4, C-MYC and N-MYC), consistent with their reported cooperative binding7,8, while the majority of the active macrophage-restricted enhancers exhibited binding of only one or two of these factors (Fig. 1e).

To determine whether cell-type-restricted enhancers in other cell types also exhibit similar pre-marking, we examined cell-type-restricted enhancers from heart, kidney and N2A neuronal cells in a mouse model. These enhancers in ES cells again predominantly exhibited binding of a single ENOS factor and chromatin openness (Extended Data Fig. 2a–d).

Given the established role of the cohesin complex in chromatin architecture and gene regulation9–11, we investigated whether this complex has a role in premarked enhancers. Consistent with a previous report11, cohesin was colocalized with ENOS-bound regions (Fig. 2e). Therefore, we next investigated whether premarked enhancers could interact with other genomic regions. We performed circular chromatin conformation capture followed by deep sequencing (4C–seq) on a macrophage enhancer located 5′ of Il1a12. This premarked enhancer interacted specifically with other genomic regions in ES cells, including an upstream CTCF-bound site, but not with the cognate promoter. In macrophages treated with the bacterial endotoxin Kdo2-lipid A (KLA), this enhancer interacts robustly with the cognate promoter of the target coding gene (Extended Data Fig. 3a). An enhancer located 5′ of Tnfaip3 was similarly found to interact specifically with other genomic regions in ES cells, but not with its cognate promoter (Extended Data Fig. 3b). In addition, we performed RNA sequencing (RNA-seq) and examined the proximal macrophage-expressed genes of 6,775 macrophage-restricted enhancers. We found 634 differentially expressed genes (fold-change ≥ 4), which corresponded to functional categories relevant to macrophages but not ES cells (Extended Data Fig. 3c, d). These data, together with the 4C–seq results, suggest that premarked macrophage enhancers are not functional, despite their binding of ES cell transcription factors and open chromatin configurations in ES cells.

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To further explore any potential effect of ENOS binding in ES cells on the subsequent function of enhancers bound by PU.1 (also known as SP1), a key macrophage transcription factor, we assessed the physical locations of ENOS binding in premarked enhancers. The locations of ENOS binding with respect to PU.1 binding sites varied in the 6,775 macrophage enhancers, corresponding to the locations of cognate binding sites (Fig. 2a).

Because enhancer RNA (eRNA) expression is a mark of enhancer activity, we investigated whether transcription units were present in ENOS-bound premarked enhancers. Because global run-on sequencing (GRO-seq) data on these enhancer regions were insufficiently robust to draw clear conclusions, we performed precision nuclear run-on sequencing for RNA polymerase II initiation sites (PRO-cap). We identified 2,336 significant RNA polymerase II initiation sites (cap sites) in 1 kb/±1 kb windows in 6,775 premarked macrophage enhancers and 28,450 active ES cell-restricted enhancers.

While cap sites were found to be located close to PU.1 binding sites in macrophages, corresponding to the locations of cognate binding sites (Fig. 2a), we investigated whether transcription units were present in the ENOS-bound enhancers. To address any concerns regarding serum culture conditions, we performed chromatin immunoprecipitation with sequencing (ChIP-seq) with H3K4me2 and H3K27Ac, and ATAC-seq under two different culture conditions: with inhibitors of MEK and GSK3 (2i) and with serum. H3K4me2 and H3K27Ac were not observed in macrophage enhancers in either 2i- or serum-cultured ES cells, and the ATAC-seq signal was equivalently detected in both conditions (Extended Data Fig. 4a, b), indicating that culturing ES cells in serum did not affect our observations. Indeed, most pluripotency-associated genes were transcribed at similar levels in serum- and 2i-cultured ES cells and only 8% of serum-treated ES cells were heterogeneous.

To investigate the potential function of premarking, we examined the correlation between ENOS factors and the ultimate activity of the enhancers in mature macrophages. ENOS-bound macrophage enhancers with the highest or lowest approximately 20% levels of ENOS transcription units were selected to test their ultimate activity in mature macrophages. ENOS-bound macrophage enhancers were plotted relative to PU.1 binding sites in enhancers. Last panel shows corresponding binding sites of ENOS (ENOS motif) to the actual ENOS binding. Heat map of 2,345 PRO-cap peaks identified in macrophages or ES cells in 1 kb/±1 kb window from 6,775 premarked enhancers centred on PU.1. Locations of PRO-cap peaks sites from PU.1 in macrophages or from ENOS in ES cells is calculated in 638 premarked macrophage enhancers, with PRO-cap peaks in a −500 bp/+500 bp window from PU.1 binding sites. ENOS binding and ATAC-seq signal in highest versus lowest (high and low, respectively) approximately 40% of macrophage enhancers selected on the basis of RAD21 binding intensity in ES cells. In the box plots, line shows mean and box shows 25th and 75th percentiles. P values calculated using Welch’s two-sided t-test.

Indeed, ATAC-seq revealed chromatin openness in the region where ENOS bound to the enhancers (Fig. 2d). We investigated the functional correlation between cohesin and ENOS-bound enhancers, and found that high RAD21 binding was associated with high ENOS binding and a more open configuration (Fig. 2f).

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To investigate the potential function of premarking, we examined the correlation between ENOS factors and the ultimate activity of the enhancers in mature macrophages. ENOS-bound macrophage enhancers with the highest or lowest approximately 20% levels of ENOS transcription units were selected to test their ultimate activation in macrophages. The highest group of enhancers exhibited higher activation, as determined by binding of PU.1, PRO-cap signal, eRNA transcription, levels of H3K4me2 and H3K27Ac, and binding of cohesin in macrophages (Fig. 3a–d). In addition, when the highest or lowest approximately 20% of active macrophage enhancers were extracted using GRO-seq signal in macrophages, ENOS binding and ATAC-seq signals in ES cells were higher in the most active macrophage enhancers than in the least active macrophage enhancers (Fig. 3e, f).
Indeed, putative active macrophage enhancers, defined by eRNA transcription, were found much more frequently in the 6,775 ENOS-bound enhancers than in the 11,630 macrophage enhancers that do not bind ENOS in a $-1\,kb + 1\, kb$ window (Fig. 3g). The correlation between premarking and enhancer robustness was confirmed in other tissues: spleen, lung, cortex and bone marrow (Extended Data Fig. 5a–d).

We next investigated whether the binding of these factors in ES cells had a direct role in the ultimate activation of cell-type-restricted enhancers in mature macrophages, choosing premarked macrophage-restricted enhancers linked to coding target genes that would not be predicted to influence macrophage development. We first selected a putative enhancer near the 

\[ \text{Th}1 \] locus, and then used CRISPR–Cas9 technology in ES cells to selectively delete the ESRRB binding site, followed by differentiation\(^\text{16}\) (Extended Data Fig. 6b). Mature macrophages were selected on the basis of their ability to adhere to the non-adherent culture plates, as confirmed by expression of CD11B and F4/80 (Extended Data Fig. 6a). This permitted only a limited harvest of mature macrophages, thus precluding global genomic analyses. Notably, three independently derived individual clonal lines (#3, #10 and #14) were sequence-confirmed to harbour a 8-bp deletion of the ESRRB site in the 

\[ \text{Th}1 \] enhancer (Extended Data Fig. 6c, d). Consistent with the confirmed deletion, ESRRB binding in ES cells was decreased in these mutant clones compared to wild-type clones (Fig. 4a). The mutant clonal cells were differentiated into macrophages with an equivalent efficiency to that of wild-type cells; qualitative PCR with ChIP (ChIP–qPCR) for PU.1 and H3K4me2 was performed, and eRNA transcription was measured in the ES cell-derived macrophages (ESDMs). These analyses revealed that PU.1 binding was inhibited, that eRNA transcription was lost and that there was a consistent decrease in the level of H3K4me2 after deletion of the ESRRB site (Fig. 4b).

To corroborate these findings, we constructed mutant clonal cells targeting the 

\[ \text{Th}1 \] enhancer with either a 16-bp deletion (#26) or a 21-bp deletion (#45) encompassing the ESRRB binding site (Extended Data Fig. 6e–g), and differentiated them into macrophages. The reduction in PU.1 recruitment, eRNA transcription and H3K4me2 levels in mutant ESDMs correlated with inhibition of ESRRB binding in ES cells (Fig. 4c, d). As a control, we tested 

\[ \text{Th}1 \] RNA transcription in 

\[ \text{Th}1 \] mutant clones, observing no change (Extended Data Fig. 6h).

We investigated two other loci, Prdx5 and 

\[ \text{Nod}2 \], in the same way (Extended Data Fig. 7a, b, e, f) and again found that enhancer activities defined by eRNA transcription were inhibited in mutant ESDMs with loss of ESRRB binding in ES cells, but Prdx5 mutant enhancers (B11, F1, G11) exhibited the same level of 

\[ \text{Nod}2 \] eRNA transcription as in wild-type cells (Extended Data Fig. 7c, d, g). Because modifying the genomic

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**Fig. 3** ES cell transcription factor binding is predictive of future enhancer activity. a–d, Comparison of the highest or lowest approximately 20% of enhancers selected on the basis of ENOS binding and PRO-cap signal in ES cells, and the levels of H3K4me2 and H3K27Ac (a). PRO-cap and GRO-seq (b), PU.1 (c) and RAD21 (d) in mature macrophages. e, f, Comparison of the highly active or less active about 20% of macrophage enhancers selected on the basis of GRO-cap signal of premarked macrophage enhancers in macrophages, and the levels of ENOS (e) and ATAC-seq (f) in ES cells. g, Percentage of functional macrophage enhancers in 6,775 enhancers that have ENOS binding in a $-1\, kb + 1\, kb$ window (premarked enhancers) versus 11,630 enhancers that do not (non-premarked enhancers). In the box plots, line shows median and box shows 25th and 75th percentiles. P values calculated using Welch’s two-sided t-test.

**Fig. 4** Premarking in ES cells is functionally required for robust future enhancer activation. a, c, Binding of ESRRB in wild-type (WT) and mutant clones of 

\[ \text{Th}1 \] (a, #3, #10, #14) and 

\[ \text{Th}1 \] (c, #26, #45) in ES cells. One example of representative data is shown (n = 2 biological repeats). b, d, PU.1 binding, eRNA transcription, and H3K4m2 level in wild-type and mutant clones of 

\[ \text{Th}1 \] (b) and 

\[ \text{Th}1 \] (d) in ESDMs. Each dot indicates a biological experiment (n ≥ 3 biological repeats from two pooled different experiments, n = 2 biological repeats from two pooled different experiments for #45 for H3K4me2 ChIP–qPCR). e, Promoter activities in native full-length 

\[ \text{Th}1 \] enhancer response to wild-type versus ESRRB-deleted mutant in Raw264.7 cells (n = 5 biological repeats). f, Mapping of DNA methylation modification (5mC and 5hmC) and binding of TET1 in 6,775 premarked macrophage enhancers in a $-1\, kb + 1\, kb$ window centred on PU.1, g, 5-hmC in LSK (LT-HSC, ST-HSC, MPP; long term- and short term-hematopoietic stem cells, multipotent progenitors), CMP (common myeloid progenitors) and GMP (granulocyte macrophage progenitors) in 6,775 premarked enhancers in $-3\, kb + 3\, kb$ window centred on PU.1. In the box plots, line shows median, and box shows 25th and 75th percentiles. P values calculated using Welch’s two-sided t-test. Data from published sources are listed in Supplementary Table 1. Primer sets are listed in Supplementary Table 2.
locus could potentially result in different genetic events, we performed RNA-seq in Trl1 enhancer clonal cells. Normal ES cells and clonal ES cells (wild-type, #10 and #14) exhibited the same pattern of transcription in the Trl1 locus (Extended Data Fig. 6i).

Enhancers harbouring the ESRRB core site deletion were evaluated by luciferase reporter assay to test whether genomic sequence disruption had impaired enhancer function compared to wild-type enhancers in macrophages. The mutant enhancers were equally competent to increase reporter expression as the wild-type enhancer in immortalized Raw264.7 macrophages (Fig. 4e), confirming that the inhibitory effects in Trl1 mutant ESDMs resulted from inhibition of ESRRB binding in ES cells.

We further investigated this functional linkage in neural precursor cell (NPC)-restricted enhancers in the Nek1 locus and Ankrd1 locus (referred to as N4 and N8, respectively). Sox2 and Esrrb bound to N4 and Esrrb bound to N8, and we targeted the Sox2 or Esrrb binding site, in N4 or N8, respectively, for deletion (Extended Data Fig. 8a, d). Clonal ES cells were differentiated to NPCs in N2B27 medium for 6 days, with more than 70% of cells exhibiting the green signal caused by knock-in of the Sox1–GFP reporter in ES cells17. NPC differentiation was further confirmed by qRT–PCR of ES cell-specific genes (Oct4, Esrrb and Nanog) and NPC-specific genes (Sox1, Fgf5, Nestin and Pax6) (Extended Data Fig. 8g). Transcription of N4 or N8 eRNA was inhibited in N4- or N8-mutant ES cell-derived NPCs, respectively, correlating with inhibition of ESRRB binding in ES cells (Extended Data Fig. 8b, e). Sox2 binding could not be assessed because the anti-SOX2 IgG was ineffective. The specificity of N4- or N8-mutant clones on inhibition of eRNA transcription was confirmed by testing eRNA transcription in the N8 locus in N4 mutant cells, or the N4 locus in N8 mutant cells (Extended Data Fig. 8c, f). Collectively, these analyses corroborate the functional importance of ES cell factor premarking in both macrophase-restricted and NPC-restricted enhancers.

Pooled enhancers, another class of well-studied cell-type-specific enhancer that is marked in ES cells, are often found near lineage-determining factors18,19. The 214 macrophage enhancers exhibited poised chromatin signatures (Extended Data Fig. 1a) with binding of about 2–4 ENOS factors in the enhancer cores in ES cells (Extended Data Fig. 9a, b). For example, we noted that Spi1 (which encodes PU.1) has four clustered enhancers with poised chromatin signatures in ES cells. In contrast to the two enhancers (Enh2 and Enh3) that are active in mature macrophages, the other two (Enh1 and Enh4), which are bound by ESRRB and OCT4, are not (Extended Data Fig. 9c, d). These two premarked enhancers in ES cells might participate in the early activation of Spi1, which is known to exhibit a positive feedback loop on the two PU.1-bound enhancers20.

A particularly intriguing question is how the marking of future cell-type-restricted enhancers might be ‘remembered’ for their ultimate activation later in development. Published results and analysis of the mRNA levels of the ENOS factors suggest that the temporal pattern of disappearance of these factors virtually coincides with the appearance of the first lineage-determining factors, Tal1, Gata2 and Runx1, followed by the appearance of Pu.1 and C/EBPα, which initiate macrophage enhancer activation21 (Extended Data Fig. 10a). Another potential explanation is that binding of one of the ES cell transcription factors to the future cell-type-restricted enhancers might be accompanied by a specific DNA demethylation event that serves to ensure that an enhancer remains accessible to transcription factors, the binding of which may be impaired by DNA methylation. We therefore analysed available data regarding DNA methylation in ES cells, and found the 5-hydroxymethylcytosine (5-hmC) mark and the enzyme responsible for that mark, TET1, in the 6,775 premarked macrophage enhancers (Fig. 4f), reflecting the presence of TET1 in a complex with ESRRB and OCT422. Notably, knockdown of Esrrb reduced 5-hmC levels in ESRRB-bound macrophage enhancers in ES cells (Extended Data Fig. 10b). To investigate whether 5-hmC is maintained during differentiation, such that it could serve as a marker for molecular memory, we examined 5-hmC during haematopoiesis using published data23, and found that 5-hmC was maintained during haematopoiesis (Fig. 4g, Extended Data Fig. 10c). The enhancer histone marks, H3K4me1 and H3K27Ac, were studied during haematopoiesis24, but not found in ES cells, or even in mesoderm, being gained gradually early in haematopoiesis (Extended Data Fig. 10c, d).

Investigation of the signature of cell-type-restricted enhancers in ES cells has provided an insight into the process of genomic enhancer recognition in ES cells underlying cell-type-specific transcriptional programs. Furthermore, the marking of the cell-type-restricted enhancers in ES cells license the ultimate robust activation of the cell-type-restricted enhancer in mature differentiated cells (Extended Data Fig. 10e).

Online content
Any Methods, including any statements of data availability and Nature Research reporting summaries, along with any additional references and Source Data files, are available in the online version of the paper at https://doi.org/10.1038/s41586-018-0048-8.

Received: 24 June 2016; Accepted: 5 March 2018;
Published online: 18 April 2018

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by grants from NIH to M.G.R. (5R01NS093066, DK018477, DK039949, NS093066, and GM104459).

Reviewer information Nature thanks H. Stunnenberg and the other anonymous reviewer(s) for their contribution to the peer review of this work.

Author contributions H.S.K. and M.G.R. conceived the project. H.S.K. performed most of the experiments, with particular contributions from W.M. (CRISPR-Cas9), Y.T. (PRO-cap) and E.D. (4C-seq). D.M. and Y.T. performed most of the bioinformatics analyses. T.S. performed the DNA methylation experiment. K.O. prepared samples for deep sequencing assays. Q.M. analysed 4C-seq. Additional experiments or methods and discussion were contributed by E.D., D.S.-K. and M.F. H.S.K. and M.G.R. wrote the manuscript.

Competing interests The authors declare no competing interests.

Additional information Extended data is available for this paper at https://doi.org/10.1038/s41586-018-0048-8. Supplementary information is available for this paper at https://doi.org/10.1038/s41586-018-0048-8. Reprints and permissions information is available at http://www.nature.com/reprints. Correspondence and requests for materials should be addressed to H.S.K. or M.G.R.

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Methods

Cell culture. 46c mouse ES cells were a gift from A. Smith. Cells were grown in feeder-free conditions as previously described. ES cells were maintained in serum-free medium DMEM (Invitrogen 10298-018) supplemented with 2% ES cell qualified fetal bovine serum (Omega, FB-05), 2 mM nonessential amino acids (Invitrogen 11140-050), glutamax (Invitrogen 35050-061), penicillin/ streptomycin (Invitrogen 15140122), 2-mercaptoethanol (Sigma, M7522) and 1000 U/ml LIF (ESGRO, ESG1106). ES cells in 2i medium were treated with polyethylene glycol (Enzymatics) and precipitated. The RNA was dephosphorylated with calf intestinal phosphatase (NEB) and 5'-de-capped with tobacco acid pyrophosphatase (Epicentre). The reaction was stopped and RNA was extracted with Trizol LS reagent (Invitrogen). Following DNase treatment, the RNA was fragmented. Biotin-incorporated fragmented RNA was immunoprecipitated with anti-streapavidin beads (Invitrogen). Then the RNA was treated with polyethylene glycol (Enzymatics) and precipitated. The RNA was dephosphorylated with calf intestinal phosphatase (NEB) and 5'-de-capped with tobacco acid pyrophosphatase (Epicentre). The reaction was stopped and RNA was extracted with Trizol LS, and libraries were prepared by ligating Illumina TrueSeq-compatible adapters to the RNA 3' ends and 5' ends with truncated mutan RNA ligase 2 (K227Q) and RNA ligase 1 (NEB), respectively, followed by reverse transcription, CDNA isolation and PCR amplification. Final libraries were size selected on TBE gels to 60–110 bp insert size. Pro-caps results were trimmed to remove A-stretches originating from the library preparation. Each sequence tag returned by the Illumina Pipeline was aligned to the mm9 assembly using Bowtie2 allowing up to three mismatches. Only tags that mapped uniquely to the genome were considered for further analysis. Each sequencing experiment was normalized to a total of 10^7 uniquely mapped tags by adjusting the number of tags at each position in the genome to the correct fractional amount given the total tags mapped.

ChiP-seq. Cells were fixed with 2 mM disuccinimidyl glutarate (DSG) (protocol) for 45 min and 1% formaldehyde for 10 min, and quenched using, glycerol for 5 min. Nucleus lyses were prepared using sonication buffer with 1% SDS, and immunoprecipitation was performed with several different antibodies. After overnight incubation with antibodies, beads were added for another 3 h, and washing was performed. Reverse-crosslinking was done overnight at 56°C and DNA was purified using QIAquick Spin column (Qiagen). For ChiP-seq, extracted DNA was ligated to adaptors and deep sequencing was performed with Illumina's HiSeq 2000, 2500, or 4000 system according to the manufacturer's instructions. The first 48 bp of each sequence tag returned by the Illumina Pipeline was aligned to the mm9 assembly using BFAST or Bowtie2. Only uniquely mapped tags were selected for further analysis. The data were visualized by preparing custom tracks on the UCSC genome browser using HOMER. Genomic binding peaks for transcription factors were identified using the findPeaks.pl command from HOMER with eightfold enrichment over the input sample, fourfold enrichment over local background, a minimal tag number of 16, and normalization to 10^7 mapped reads per experiment. For histone marks, initial seed regions of 500 bp were considered to calculate enriched reads.

Regions of maximal density exceeding a given threshold with FDR < 0.001 were called as peaks. Peaks within ± 1,000 bp of from the RefSeq gene transcription start site were considered to be promoters, and to focus the analysis on enhancers, peaks within 3 kb of a gene promoter were filtered out.

4C-seq. Chromosome confirmation capture was performed as previously described. In brief, 10 million cells were cross-linked with 1% formaldehyde for 10 min and quenched with glycerol for 5 min on ice. Soluble chromatin was incubated with 400 U HindIII (NEB) overnight at 37°C, and then intramolecular ligations were performed using 1,000 U T4 DNA ligase (NEB) for 4 h at 16°C under dilution. Chromatin was inactivated at 65°C and then treated with RNase to remove DNA before purification using several phenol and phenol–chloroform extractions and ethanol precipitation. The second restriction digestion was also performed overnight at 37°C, using 50 U DpnII (NEB). DNA was ligated overnight and purified as before and ultimately using Qiagen columns and subjected to inverse PCR (Expand Long-Range PCR system; Roche Diagnostics) using a first primer designed on the viewpoint near a HindIII site and a second outer primer designed beside the DpnII site. Both primers contained Illumina sequencing adapter sequences and barcodes for multiplexing. PCR samples were purified using a Roche kit and quantified using a Qubit.

We analysed data using a previously described bioinformatics pipeline. Genomic DNA for hMeDIP was isolated from cells using Qiagen's DNeasy Blood and Tissue Kit. Isolated genomic DNA was then fragmented to 100–300 bp through sonication using Diagnode's Bioruptor platform and the size distribution was confirmed through gel electrophoresis. Barcoded adaptors for Illumina sequencing were added to 1 μg of fragmented genomic DNA per experiment, using the NEBNext Ultra II DNA Library Prep Kit for Illumina, following the manufacturer's instructions. This protocol was stopped after adapter ligation and cleanup (and before any amplification steps), and the adapter ligated fragmented DNA was then used for hydroxyl-methylated DNA pulldown. Denaturing, immunoprecipitation, washing, and purification of hydroxyl–methylated DNA were performed overnight. The following modifications were performed: 50 μl microtitre of 5-hmC antibody-containing serum was used per immunoprecipitation reaction. Washing was done five times, with each wash for 15 min at 4°C. Next, DNA was eluted from beads using 200 μl digestion buffer, incubated overnight and precipitated. Gene ontology analysis and genetic association analysis were performed using Metascape (http://metascape.org).

Pro-caps. Pro-cap and library preparation for sequencing have previously been described. Nuclei were prepared from ~40 million cells for run-on assay. Run-on reactions were stopped and RNA was extracted with Trizol LS reagent (Invitrogen). Following DNase treatment, the RNA was fragmented. Biotin-incorporated fragmented RNA was immunoprecipitated with anti-streapavidin beads (Invitrogen). Then the RNA was treated with polyethylene glycol (Enzymatics) and precipitated. The RNA was dephosphorylated with calf intestinal phosphatase (NEB) and 5'-de-capped with tobacco acid pyrophosphatase (Epicentre). The reaction was stopped and RNA was extracted with Trizol LS, and libraries were prepared by ligating Illumina TrueSeq-compatible adapters to the RNA 3' ends and 5' ends with truncated mutan RNA ligase 2 (K227Q) and RNA ligase 1 (NEB), respectively, followed by reverse transcription, CDNA isolation and PCR amplification. Final libraries were size selected on TBE gels to 60–110 bp insert size. Pro-caps results were trimmed to remove A-stretches originating from the library preparation. Each sequence tag returned by the illumina Pipeline was aligned to the mm9 assembly using Bowtie2 allowing up to three mismatches. Only tags that mapped uniquely to the genome were considered for further analysis. Each sequencing experiment was normalized to a total of 10^7 uniquely mapped tags by adjusting the number of tags at each position in the genome to the correct fractional amount given the total tags mapped.
with Proteinase K at 50 °C, and purified using Qiagen's QIAquick PCR Purification Kit. Purified hydroxy-methylated genomic DNA was then further processed with NEBNext Ultra II DNA Library Prep Kit for Illumina at the step of PCR enrichment of adaptor-ligated DNA, per the manufacturer's instructions, continuing with the entire protocol to prepare libraries for Illumina sequencing. Data were mapped to mm9 using Bowtie2 with standard settings, and peaks were found by using MACS with default parameters.

**ATAC-seq.** ATAC-seq was performed as previously described 27. Nuclei were prepared from 50,000 cells and the transposition reaction was performed for 30 min at 37 °C. DNA fragments were amplified by PCR and purified, and deep sequencing was performed with Illumina's HiSeq 2000, 2500, or 4000 system according to the manufacturer's instructions. ATAC-seq data were mapped to mm9 using Bowtie2 with standard settings. Tag directories with reads mapped to the mitochondrial chromosome filtered out were created. ATAC-seq peaks were identified using findPeaks.pl in HOMER with the settings: -style histone -size 75 -minDist 75 -minTagThreshold 6 -L 8 -F 8. BED files were created from Tag directories using the HOMER package.

**Deep sequencing.** For all ChIP–seq, RNA-seq, 4C–seq, ATAC-seq, MeDIP–seq and PRO-cap experiments, the DNA libraries were sequenced for 50 cycles on Illumina’s HiSeq 200, 2500 or 4000 system according to the manufacturer's instructions. Sequencing experiments were visualized by preparing custom tracks for the UCSC Genome browser.

**Bioinformatic characterization of enhancers.** The criteria for identifying PU.1-H3K4me2 co-bound enhancer regions was that the distance from the centre of a PU.1 peak to the H3K4me2 peak-occupied region was ≤ 1 kb. ES cell factor-bound macrophage enhancers were defined by calculating the distance ≤ 1 kb between ES cell factor peak-spanning regions and PU.1 bound macrophage enhancers. The active ES cell enhancers or active macrophage enhancers were defined using H3K27Ac (over 100 tags) to examine binding of 12 different ES cell factors. The functional macrophage enhancers were defined using GRO-seq (over 20 tags) in macrophages, and these enhancers were used to count the number of functional enhancers in ENOS-bound enhancer or non-bound enhancer using a −1 kb/+1 kb window. Highly active or less active macrophage enhancers were created after excluding non-active macrophage enhancers using GRO-seq (less than 5 tags) in macrophages. The comparison of tag intensity of ChIP–seq, ATAC-seq, PRO-cap and GRO-seq or distances between different categories are presented as boxplots by using normal scales. P values were calculated using Welch's two-sided t-test. To profile the distribution of ES cell factors surrounding PU.1-H3K4me2 co-bound enhancer regions, ChIP–seq signals surrounding PU.1 peak centres were separated into 40 bins, and then were sorted by the tag numbers based on the distance to the PU.1 peak centre.

**Reporting summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

**GEO data.** The GEO datasets used in this study are listed in Supplementary Table 1.

**Data availability.** All deep sequencing data have been deposited in GEO under accession number GSE81681.

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Extended Data Fig. 1 | Features of macrophage-restricted enhancers in ES cells. a, PU.1+ H3K4me2+ macrophage enhancer profiles in mouse ES cells. b, Normalized tag counts of ATAC-seq and DNase-seq in 18,405 random regions versus 18,405 macrophage enhancers versus 28,450 ES cell enhancers. c, Percentage of macrophage enhancers or random regions bound by ENOS in a −1 kb/+1 kb window. d, ESRRB binding in random regions versus macrophage enhancers versus ES cell enhancers in ES cells. e, ENOS binding profiles in 6,775 macrophage enhancers.

f, UCSC genome browser screen shots show ENOS binding with chromatin openness but no enhancer marks, such as H3K4me2 and H3K27Ac, in macrophage-restricted enhancers in ES cells. The Ifgl gene locus has two different macrophage-restricted enhancers bound by ES cell transcription factors. The blue bar indicates macrophage-restricted enhancers. In the box plots, line shows median, and box shows 25th and 75th percentiles. P values calculated using Welch's two-sided t-test.
Extended Data Fig. 2 | ES cell transcription factors bind to heart-, kidney- and N2A cell-restricted enhancers in ES cells. a, Profiles of cell-type-restricted enhancers in heart, kidney, and N2A neuronal cells, with percentages of enhancers that are unmarked, poised and active in ES cells. b, Target percentage of ENOS-bound heart-, kidney- and N2A cell-restricted enhancers in ES cells. c, Heat map of ESRBB, NANOG, OCT4 and SOX2 binding within a −1 kb/+ 1 kb window centred on each transcription factor in 17,561 heart-, 14,242 kidney- and 12,027 N2A cell-restricted enhancers in ES cells. Data from published sources are listed in Supplementary Table 1.
Extended Data Fig. 3 | Premarked macrophage-restricted enhancers do not interact with cognate target coding genes of macrophages in ES cells. **a**, **4C–seq in ES cells and macrophages.** Primer sets for 4C are listed in Supplementary Table 2. **a**, 4C–seq analysis of the *Il1a* gene locus from the enhancer viewpoint in KLA-treated peritoneal macrophages or ES cells. Black arrows represent interaction frequency based on the trunc mean in a 400-kb window. The statistic of trunc mean calculated standard mean but with a truncated high and low value. Red box shows position of macrophage putative enhancer 5′ of *Il1a*. **b**, 4C–seq analysis of the *Tnfaip3* gene locus from the enhancer viewpoint in KLA-treated Raw 264.7 macrophages or in ES cells. Black arrows indicate interactions based on linear mean in 1-Mb window. Red box indicates position of putative *Tnfaip3* enhancer and blue circle indicates *Tnfaip3* promoter. **c**, The 634 differentially expressed genes (fold-change ≥ 4, over 20 tags in macrophage) among the proximal macrophage-expressed genes of 6,775 macrophage-restricted enhancers were visualized by heat map in ES cells and macrophages. Colour indicates normalized tag counts with log2 transform. **d**, Gene ontology (GO) term analysis of 634 differentially expressed genes.
Extended Data Fig. 4 | Features of 6,775 macrophage enhancers in ES cells are not affected by ES cell culture medium (2i or serum).

a, b, Heat map of H3K4me2 and H3K27Ac (a) and ATAC-seq (b) in 6,775 premarked macrophage enhancers and 28,450 ES cell enhancers in 2i- or serum-cultured ES cells. Centred on ENOS and visualized in a −1 kb/+ 1 kb window.
Extended Data Fig. 5 | ENOS binding correlates with future enhancer activity. a–d, Comparison of the approximately 10% 'most' marked (high) and 'least' marked (low) enhancers selected on the basis of ENOS binding, and the level of H3K4me1 and H3K27Ac on these enhancers in mature tissues for spleen (a), lung (b), cortex (c) and bone marrow (d). In the box plots, line shows median and box shows 25th and 75th percentiles. Red dots indicate each value of H3K4me1 or H3K27Ac. P values calculated using Welch's two-sided t-test. Data from published sources are listed in Supplementary Table 1.
Extended Data Fig. 6 | Premarking in ES cells is required for robust future macrophage enhancer activation. a, Photomicrographs of macrophages differentiated from ES cells documented by F4/80 and CD11B staining. Scale bar, 50 μM. b, Screenshot of the Tlr1 locus. The blue box corresponds to the CRISPR–Cas9 target region. c, d, Putative Tlr1 enhancer mutant sequence diagram and DNA sequence documentation of several of the homozygous mutation clones, used for analysis. e, Screenshot of the Tnfaip3 locus. The blue box corresponds to the CRISPR–Cas9 target region. f, g, Putative Tnfaip3 enhancer mutant sequence diagram and DNA sequence documentation of several of the homozygous mutation clones, used for analysis. h, Tlr1 eRNA transcription was tested in wild-type and mutant clonal cells (#26, #45) of the Tnfaip3 enhancer. One representative experiment is plotted (n = 2 biological repeats). i, UCSC genome browser shot of RNA-seq in Tlr1 locus in normal ES cells and clonal mutant ES cells (wild-type, #10, #14). Primer sets are listed in Supplementary Table 2.
Extended Data Fig. 7 | Premarking in ES cells is required for robust future macrophage enhancer activation. a, b, Putative Prdx5 enhancer mutant sequence diagram and DNA sequence documentation of several of the homozygous mutation clones, used for analysis. c, Three mutant clones (B11, F1, G11) of the putative Prdx5 enhancer decreased level of Prdx5 eRNA transcription in ESDMs (right). Each dot indicates one biological experiment (n = 3 biological repeats from two pooled different experiments; n = 2 biological repeats from two pooled different experiments for B11). ESRRB binding in ES cells is a representative experiment (left) (n = 2 biological repeats). d, Nod2 eRNA transcription was tested in wild-type and mutant (B11, F1, G11) clonal cells of the Prdx5 enhancer. Data from one representative experiment are plotted (n = 2 biological repeats). e, f, Putative Nod2 enhancer mutant sequence diagram and DNA sequence documentation of several of the homozygous mutation clones, used for analysis. g, Two mutant clones (D2, D12) of the putative Nod2 enhancer decreased level of Nod2 eRNA transcription in ESDMs (right). Each dot indicates one biological experiment (n = 3 biological repeats from two pooled different experiments; n = 2 biological repeats from two pooled different experiments for D12). ESRRB binding in ES cells is a representative experiment (left) (n = 2 biological repeats). In the box plots, line shows median and box shows 25th and 75th percentiles. P values calculated using Welch’s two-sided t-test. Primer sets are listed in Supplementary Table 2.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | Premarking in ES cells is required for robust future NPC enhancer activation. **a**, The putative Nek1 enhancer, referred to as N4; mutant sequence diagram and DNA sequence documentation of several mutation clones (N4C3, N4C16), used for analysis. **b**, Two mutant clones (N4C3, N4C16) of the N4 enhancer decreased level of N4 eRNA transcription in NPCs (right). Each dot indicates one biological experiment. A representative experiment for ESRRB binding in ES cells is presented (left) (n = 3 biological repeats for N4C3, n = 2 biological repeats for N4C16). **c**, N8 eRNA transcription was tested in N4 mutant clones (N4C3, N4C16) to show specificity of N4 mutant cells on inhibition of N4 eRNA transcription. A representative experiment is presented (n = 2 biological repeats). **d**, The putative Ankrd1 enhancer, referred to as N8; mutant sequence diagram and DNA sequence documentation of several of the mutation clones (N8C6, N8C12), used for analysis. **e**, Two mutant clones (N8C6, N8C12) of the N8 enhancer decreased level of N8 eRNA transcription in NPCs (right). Each dot indicates one biological experiment. ESRRB binding in ES cells is a representative experiment showing inhibition of ESRRB binding in ESRRB-deleted N8 locus (left) (n = 3 biological repeats). **f**, N4 eRNA transcription was examined to prove the specificity of N8 mutant cells on inhibition of N8 eRNA transcription. Results of one representative experiment are presented (n = 3 biological repeats). **g**, NPC differentiation was confirmed on day 6 after differentiation by testing expression of ES cell-expressing genes and NPC-expressing genes. A representative experiment is presented (n = 2 biological repeats). In the box plots, line shows median and box shows 25th and 75th percentiles. P values calculated using Welch’s two-sided t-test. Primer sets listed in Supplementary Table 2.
Extended Data Fig. 9 | Binding of ES cell transcription factors in poised enhancer. a, Profile of 214 macrophage enhancers with poised chromatin signatures in ES cells. b, ES cell transcription factors (OCT4, SOX2, NANO6, ESRRB, E2F1, TCFCP2L1, ZFX and KLF4) were analysed in 129 macrophage enhancers to test binding of ES cell transcription factors. These 129 macrophage enhancers were chosen according to the following criteria: high H3K27me3 in ES cells and high H3K27Ac (over 20 tags) in macrophages. Data from published sources are listed in Supplementary Table 1. c, Schematic diagram of Pu.1 enhancers. d, Genome browser screenshot of the poised Pu.1 enhancers. Blue arrow indicates ESRRB binding region and red arrow indicates OCT4 binding region. Red boxes indicate four clustered putative Pu.1 enhancers.
Extended Data Fig. 10 | Establishment and maintenance of 5-hmC on premarked enhancers and proposed model. **a**, Esrrb, Oct4 and Cebp level measured at four different time points through differentiation process from ES cells to macrophages. **b**, 5-hmC level in 6,775 premarked enhancers in ES cells expressing scrambled (siCtrl) or Esrrb-targeted (siEsrrb) siRNA. In the box plots, line shows median and box shows 25th and 75th percentiles. P values calculated using Welch’s two-sided t-test. **c**, Schematic of the haematopoietic differentiation stages. **d**, H3K4me1 and H3K27Ac in 6,775 premarked enhancers were analysed during haematopoietic differentiation (ES cells, mesoderm, LT-HSC, ST-HSC, MPP, CMP, GMP and macrophages), and presented in heat map within a −3 kb/+3 kb window centred on PU.1. Data from published sources are listed in Supplementary Table 1. **e**, Model of ‘premarked’ lineage-determining and terminal-differentiation enhancer indicating that poised enhancers bind several ES cell transcription factors, whereas late-activated cell-specific enhancers are premarked by binding of a single ES cell transcription factor, causing chromatin opening, transcription of a non-coding RNA, and appearance of a 5-hmC mark in the area of the enhancer that may provide the molecular memory for what ultimately will be the PU.1–C/EBPα core from which eRNAs will be transcribed.
Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

1. Experimental design

   1. Sample size
      
      Describe how sample size was determined.
      
      18,405 Pu.1+, H3K4me2+ macrophage enhancers were selected and this was described in Extended Data Figure 1a and methods. 6,775 Pu.1+, H3K4me2, ENOS+ macrophage enhancers were selected and this was described in Figure 1c and main text.

   2. Data exclusions
      
      Describe any data exclusions.
      
      No data were excluded.

   3. Replication
      
      Describe whether the experimental findings were reliably reproduced.
      
      All attempts at replications were successful.

   4. Randomization
      
      Describe how samples/organisms/participants were allocated into experimental groups.
      
      This is not relevant to this study.

   5. Blinding
      
      Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
      
      All the fastq file are generated without the any knowledge of the experimental groups by the UCSD genomics core.

   Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a | Confirmed
   --- | ---
   [ ] | The exact sample size \( (n) \) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   [ ] | A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   [ ] | A statement indicating how many times each experiment was replicated
   [ ] | The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   [ ] | A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   [ ] | The test results (e.g. \( P \) values) given as exact values whenever possible and with confidence intervals noted
   [ ] | A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   [ ] | Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

R is used for statistics.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

No unique materials were used.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

H3K4me2; Millipore 07-030; Evaluated by western blotting on Hela acid extract by manufacturer.
H3K27Ac; Abcam ab4729; validated by peptide array by manufacturer.
Rad21; Abcam ab992; validated by IHC, ChIP, IF, WB and IP by manufacturer.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

46C cells (mouse embryonic stem cells) were gifted from Austin Smith.
Peritoneal macrophage was obtained from 6-8 week old female C57BL/6J mice obtained from Jackson Laboratory. Raw264.7 cells were from ATCC.

b. Describe the method of cell line authentication used.

None of the cell lines used have been authenticated.

All cell lines tested were negative for mycoplasma contamination.

No commonly misidentified cell lines were used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

6-8 weeks old female C57BL/6J mice

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

The study did not involve human research participants.
ChIP-seq Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

Data deposition

1. For all ChIP-seq data:
   a. Confirm that both raw and final processed data have been deposited in a public database such as GEO.
   b. Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

2. Provide all necessary reviewer access links.
   The entry may remain private before publication.
   https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=qufmiuuvdchdmh&acc=GSE81681

3. Provide a list of all files available in the database submission.
   H3K4me2-ESC
   Rad21-ESC
   Rad21-macrophage
   ChIP-seq H3K4me2_2i-ESC
   ChIP-seq H3K4me2_serum-ESC
   ChIP-seq H3K27Ac_2i-ESC
   ChIP-seq H3K27Ac_serum-ESC
   ChIP-seq Rad21 (#2)
   ChIP-seq Rad21 (#3)

4. If available, provide a link to an anonymized genome browser session (e.g. UCSC).

Methodological details

5. Describe the experimental replicates.
   H3K4me2 in ESCs has two biological replicates. Rad21 in ESCs has three biological replicates.

6. Describe the sequencing depth for each experiment.
   ChIP-seq H3K27Ac_serum
   66065155 reads; of these:
   66065155 (100.00%) were unpaired; of these:
   12269731 (18.57%) aligned 0 times
   41294041 (62.51%) aligned exactly 1 time
   12501383 (18.92%) aligned >1 times
   81.43% overall alignment rate

   ChIP-seq H3K27Ac_2i
   63436260 reads; of these:
   63436260 (100.00%) were unpaired; of these:
   11407698 (17.98%) aligned 0 times
   38033796 (59.96%) aligned exactly 1 time
   13994766 (22.06%) aligned >1 times
   82.02% overall alignment rate

   Rad21-ESC
   16619683 reads; of these:
   16619683 (100.00%) were unpaired; of these:
   1242462 (7.48%) aligned 0 times
   12772818 (76.85%) aligned exactly 1 time
   2604403 (15.67%) aligned >1 times
   92.52% overall alignment rate
7. Describe the antibodies used for the ChIP-seq experiments.

H3K4me2; Millipore 07-030; Evaluated by western blotting on Hela acid extract by manufacturer.
H3K27Ac; Abcam ab4729; validated by peptide array by manufacturer.
Rad21; Abcam ab992; validated by IHC, ChIP, IF, WB and IP by manufacturer.

8. Describe the peak calling parameters.

For transcription factors: -style factor -center -size 200 -minDist 500 -fdr
9. Describe the methods used to ensure data quality.

False discovery rate, default < 0.001

10. Describe the software used to collect and analyze the ChIP-seq data.

HOMER (http://homer.ucsd.edu/homer/)