Comprehensive characterization of neutrophil genome topology

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Supplemental Material

Supplemental Figure Legends

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Supplemental Figure S1. Transcription signatures of progenitors and in vitro differentiated neutrophils. (*A*) Morphological changes of ECOMG derived neutrophils after 5 days of cell culture in the absence of β-Estradiol. Bars, 10 μm. (*B*) Scatter plot comparing RNA-seq profiles of ECOMG cells cultured with or without estrogen for 5 days. Colors indicate transcripts abundance in neutrophils when compared with progenitors (less abundant-blue or more abundant-red) (difference of over 2-fold for each, FPKM > 0.5).

(*C*) Scatter plot comparing differences in RNA transcripts abundance (log_2 fold) for in vitro differentiated neutrophils and progenitors plotted against the difference in RNA transcripts abundance (log_2 fold) for bone marrow derived neutrophils (BMDNs) and promyelocytes. Spearman correlation ρ = 0.605. (*D*) Top gene ontology terms (via the DAVID bioinformatics database) enriched for genes activated (*left*) or repressed (*right*) by a factor of more than 4-fold in neutrophils when compared with progenitors. (*E*) Selected group of genes that were repressed or activated during the differentiation of progenitor cells to neutrophils.

Supplemental Figure S2. The organization of topologically associating domains and PC1 continuous domains in progenitor and neutrophil genomes. (*A*) Hi-C contact heat map comparison between neutrophils (*top*) and progenitors (*bottom*) at 50-kb resolution for a 10-Mb region (Chr 3: 50,000,000-60,000,000). TADs were identified using TopDom domain calling method at 50-kb resolution using normalized matrices. The white dashed boxes indicate the shape of TADs. (*B*) Distribution of TAD size for progenitor and neutrophil genomes. TopDom resulted in 4176 TADs for progenitors, and 4136 TADs for neutrophils with a median size of 450 kb. (*C*) Distribution of PDs size across the genome, as defined by continuous regions of positive or negative PC1 values, for the A and the B compartments. (*D*) PC1 values associated with neutrophil and progenitor genomes. Signals away from the diagonal indicate PDs that switched nuclear compartments during neutrophil differentiation. (*E)* Box and whisker plot comparing the size of PDs. *P* value, Kruskal-Wallis test. (*F)* Box and whisker plot showing nuclear volume and longest axis of progenitors and neutrophils. Using Volocity 3D software, the volume and longest axis of DAPI stained nuclei of individual cells was determined by measuring the calibrated number of voxels identified within a given nucleus. Surface to volume ratios (S/V) are indicated. (*n*) Number of cells quantified for each sample. *P* value, Student's *t* test.

Supplemental Figure S3. Transcription signatures and epigenetic marks associated with neutrophil differentiation. (*A*) Scatter plot comparing the difference in nascent RNA transcripts abundance (log_2 fold;

GRO-seq) in neutrophils and progenitors versus the difference in steady-state mRNA abundance ($\log_2$ fold; RNA-seq) in neutrophils and progenitors. Colors indicate transcripts abundance in neutrophils when compared with progenitors as measured by RNA-seq (less abundant-blue or more abundant-red) (difference of over 2-fold for each, FPKM > 0.5). Spearman correlation ρ = 0.593. (*B*) Scatter plot comparing GRO-seq profiles associated with progenitors and neutrophils (difference of over 2-fold for each, RPKM > 0.5). Numbers in parenthesis indicate number of genes. Among the differentially expressed genes, 39 switched from the B to the A compartment; 26 switched from the A to the B compartment upon differentiating into neutrophils. (*C*) Scatter plot comparing GRO-seq profiles of switched PDs that were transcriptionally active (GRO-seq RPKM > 0.1). Orange indicates class II PDs that switched from the A to the B compartment in neutrophils; green refers to class IV PDs switched from the B to the A compartment in neutrophils. Numbers in parenthesis indicate number of PDs. (*D*) Average H3K27me3 ChIP-seq tag coverage in neutrophils and progenitors, plotted as a function of genomic distance from the TSS (±5 kb), gated on 1033 neutrophil activated genes or 2333 neutrophil repressed genes. (*E*) Heat map of ChIP-seq data shows the distribution of H3K4me3, H3K27me3, H3K36me3 and GRO-seq reads in progenitors and neutrophils gated on a window of 10 kb across the TSS of 1033 neutrophil activated genes. 222 TSS were associated with high levels of H3K27me3 in progenitors; 811 TSS were associated with low levels of H3K27me3 in both progenitors and neutrophils. Read density is displayed for a 10-kb window and color-scale intensities are shown in reads per million mapped reads per base pair. (*F*) Genome browser snapshots of (*left*) *Rara* locus (Chr 11: 98,756,661-98,901,380), (*middle*) *Mmp8* and *Mmp12* locus (Chr 9:6,903,918-7,803,917), (*right*) *Txndc5* locus (Chr 13:38,564,498-38,647,426), showing PC1 values for neutrophils and progenitors in a region surrounding this locus (*top* two rows), as well as read densities for nascent RNA (GRO-seq) and H3K27me3 (*bottom* four rows). Light yellow shadows highlight the gene locus.

Supplemental Figure S4. Genome-wide RAD21 occupancy in progenitors and neutrophils. (*A*) Heat map of ChIP-seq and MeDIP-seq data showing the distribution of CTCF and RAD21 occupancy as well

as the deposition of H3K27ac, H3K4me1, H3K4me2, H3K4me3, H3K27me3, H3K9me3, 5mC, 5mhC and H3K36me3 in progenitors and neutrophils, for a window of 10-kb across RAD21-bound sites that were gated on a total of 49,391 RAD21 bound sites identified in progenitors. The data for progenitors indicate that ~26% of cohesin-occupied sites involve enhancers and promoters. Read density is displayed for a 10-kb window and color-scale intensities are shown in reads per million mapped reads per base pair. (*B*) Heat map of ChIP-seq and MeDIP-seq data showing the distribution of CTCF and RAD21 occupancy as well as the deposition of H3K27ac, H3K4me1, H3K4me2, H3K4me3, H3K27me3, H3K9me3, 5mC, 5mhC and H3K36me3 in progenitors and neutrophils, for a 10-kb window associated with 4142 progenitor-specific RAD21 peaks. The data for progenitors indicate that ~16% of RAD21-bound sites involved enhancers and promoters, ~17% of RAD21-bound sites were located close to enhancers and promoters and ~69% of bound sites were not associated with either enhancers or promoters. Read density is displayed for a 10-kb window and color-scale intensities are shown in reads per million mapped reads per base pair. (*C*) Genomic distance versus the number of significant interactions was plotted for CTCF versus CTCF interactions for pairs of CTCF-bound sites as well as pairs of progenitor specific RAD21-bound sites in progenitors and neutrophils. (*D*) Progenitor-specific RAD21 peak distribution among the four classes of switched PDs.

Supplemental Figure S5. Changes in RAD21 occupancy during neutrophil differentiation are closely associated with a neutrophil specific pattern of genomic interactions. (*A*) Circos plot showing interactions from progenitors and neutrophils, RAD21 and CTCF occupancy, deposition of H3K4me2, H3K27me3, nascent transcripts abundance (GRO-seq), cell-type specific gene, TAD and PC1 values across a 13-Mb region (Chr 11:97,000,000-110,000,000). Only significant interactions are shown: $P < 0.001$, binomial test. Thickness of the connecting lines reflects the significance of the interactions (-log P). Bin size, 50-kb. Numbers at the margins indicate genomic positions (in Mb). (*B*) Circos plot showing CTCF versus CTCF interactions for progenitors and neutrophils spanning the same genomic region as shown in (*A*). Only significant interactions with end-points falling into a 5-kb region

surrounding CTCF peaks are shown: P < 0.001, binomial test. Thickness of the connecting lines reflects the significance of the interaction (-log P). Bin size, 5-kb. Numbers at the margins indicate genomic position (in Mb). Colors indicate CTCF, RAD21, H3K4me2, H3K27me3, GRO-seq, cell-type specific genes, TADs and PC1 values (color key, *right*). Note that RAD21 depletion diminished genomic interactions between RAD21-bound sites as shown in purple dashed box.

Supplemental Figure S6. Generation of LBR-deficient ECOMG cell lines. (*A*) Top indicates schematic diagram of Cas9/sgRNA-targeting sites close to the *Lbr* exon 2 and exon 3 junction; Bottom shows the *Lbr* genomic sequence. The exon sequences are indicated in bold. The sgRNA sequences or complimentary sequences are labeled in red. The protospacer-adjacent motif (PAM) sequences are labeled in blue. The splice donor site is labeled in orange. PCR primers (F, R) used for PCR genotyping are shown as black arrows. (*B*) PCR genotyping using primers F and R produced bands with correct size in targeted ECOMG clone #1 and clone #2, indicating the deletion, but not in Wt sample. (*C*) Sequences and partial chromatographs for four mutant alleles generated in two ECOMG clones. PCR products using primers F and R were sequenced. Sequence encompassing the targeted region confirmed the bi-allelic deletion. (*D*) Wright-Giemsa staining of Wt and *Lbr*$^{-/-}$ neutrophils. Bars, 10 μm. (*E*) Immunofluorescence staining of Wt and *Lbr*$^{-/-}$ neutrophils using antibodies directed against Lamin B1 and LBR. Representative image section presented as digitally magnified images. Original magnification, 100×. (Red) LBR; (green) Lamin B1; (blue) DAPI staining. Bars, 2 μm. Note that *Lbr*$^{-/-}$ neutrophils were associated with a substantial amount of heterochromatin that was organized into a cartwheel structure, with radial spokes extending to the periphery from a centrally located large chromocenter. LBR colocalized with Lamin B1 at the lamina in Wt neutrophils, whereas in *Lbr*$^{-/-}$ neutrophils only background dots are detected in cytoplasm.

Supplemental Figure S7. Restoring Lamin B1 expression in neutrophils. (*A*) Immunofluorescence

staining in progenitors and neutrophils using a Lamin B1 antibody. Representative image section was digitally magnified. Original magnification, 100×. (Green) Lamin B1; (blue) DAPI staining. Bars, 2 μm. White arrow indicates a nucleus of an undifferentiated progenitor. (*B*) Genome browser snapshot of *Lmnb1* locus showing read densities for RNA-seq (plus strand) for progenitors and neutrophils. Note that the first five exons of *Lmnb1* were differentially spliced in neutrophils. (*C*) Immunofluorescence staining in Lamin B1-GFP transduced neutrophils using antibodies directed against GFP and B23. Representative image section presented as digitally magnified images. Original magnification, 100×. (Red) B23; (green) Lamin B1-GFP; (blue) DAPI staining. Bars, 2 μm.

Supplemental Figure S8. Diagram depicting the changes in genome topology associated with the transition from mononuclear to polymorphonuclear cells.

Supplemental Table Legends

Supplemental Table S1 List of four classes of PDs that switched compartments during neutrophil differentiation. Fold changes are shown for PDs exhibiting significant differences in nascent transcript levels (>2-fold).

Supplemental Table S2 List of PDs that are most distal-favored during neutrophil differentiation is shown.

Supplemental Table S3 List of genes whose expression is modulated and switched compartments or changed levels of H3K27me3 during the neutrophil differentiation.

Supplemental Table S4 Summary of statistics of Hi-C experiments.

Supplemental Methods

Primary neutrophil isolation

C57BL/6J mice were housed in specific pathogen-free conditions in accordance with the Institutional Animal Care and Use Committee of University of California at San Diego. Bone marrow derived neutrophils were isolated using an EasySep^{TM} Mouse Neutrophil Enrichment Kit (Stem Cell Technologies, 19762). Bone marrow cells were harvested from femur, tibia and crista iliac. Bone marrow cells were incubated with a cocktail of biotinylated lineage specific antibodies (EasySep^{TM} Mouse Neutrophil Enrichment Cocktail), conjugated with EasySep^{TM} Biotin Selection Cocktail, labeled with EasySep^{TM} D Magnetic Particles, followed by depletion of biotin-labeled cells with EasySep™ Magnet. The enriched neutrophil purity (CD11b^{+}Ly6G^{+}) > 80% was validated by flow cytometry.

Morphological evaluation

ECOMG progenitors, in vitro differentiated neutrophils and BMDNs were gently centrifuged (1000 rpm, 1 min) onto coverslips by Cytospin. Morphological evaluation was performed with Wright-Giemsa stain (Sigma, WS16, GS500).

CRISPR/Cas9 mediated genome editing

Genome editing was performed using CRISPR/Cas9 essentially as described (Cong et al. 2013). Briefly, target-specific oligonucleotides were cloned into a plasmid carrying a codon-optimized version of Cas9. sgRNA sequences were cloned into the BbsI recognition sites as described (http://www.genome-engineering.org/crispr/). The sequences of guide RNAs and PCR primers are listed below. sgRNAs targeting *Lbr* exons 2 and 3 were transfected into ECOMG progenitors. Transfection was carried out with the Neon kit (Invitrogen) according to the manufacturer's instructions. Two days after transfections, cells were plated at clonal density. Individual colonies were picked, expanded, and genotyped by PCR for deletion. The edited alleles were cloned and verified by Sanger sequencing.

sgRNA #1 top: CACCGATACAAAGATGGCACCGAAC

sgRNA #1 bottom: AAACGTTCGGTGCCATCTTTGTATC

sgRNA #2 top: CACCGCGGCTGCCACGGCGTCGAGA

sgRNA #2 bottom: AAACTCTCGACGCCGTGGCAGCCGC

PCR primer F: TGCCAAGTAGGAAGTTTGTTGAGG

PCR primer R: CCTCATGGGAAGCAGAGACGGATC

RNA-seq

ECOMG RNA was isolated and sequenced as previously described (Bossen et al. 2015). mRNA was purified from total RNA with a Dynabeads mRNA purification kit (Life Technologies). Libraries were prepared with the TruSeq primer set and were selected by size by 8% PAGE and sequenced for 50 cycles on Illumina HiSeq 2000. Reads were aligned to the mm9 reference genome using Tophat2. FPKMs for each RefFlat gene were calculated from aligned reads using Cufflink. For a gene to be considered expressed, the cutoff of FPKM is 0.5.

GRO-seq

GRO-seq was performed as previously described (Lin et al. 2012). Duplicates of GRO-seq experiments were performed on progenitors and neutrophils. Libraries were prepared with custom GRO-seq PCR primers and were selected by size by 8% PAGE and sequenced for 50 cycles on Illumina HiSeq 2000. Reads were aligned to mm9 with Bowtie2 and reads non-uniquely mapped were discarded. Data were analyzed with HOMER (Lin et al. 2012).

ChIP-seq

Chromatin was immunoprecipitated as previously described (Lin et al. 2012). Antibodies used in these experiments were as follows: anti-H3K27ac (ab4729; Abcam), anti-H3K4me1 (ab8895; Abcam), anti-H3K4me2 (ab7766; Abcam), anti-H3K4me3 (ab8580; Abcam), anti-H3K27me3 (07-449; Millipore), anti-

H3K9me3 (ab8898; Abcam), anti-H3K36me3 (ab9050; Abcam), anti-CTCF (07-729; Millipore) and anti-RAD21 (ab992; Abcam). Libraries were prepared with the NEBNext primer set and were selected by size by 8% PAGE and sequenced for 50 cycles on Illumina HiSeq 2000 or 2500. Reads were aligned to mm9 with Bowtie2 and reads non-uniquely mapped were discarded. Data were analyzed using HOMER. The position of each tag 3′ of its position was adjusted by 150 bp. One tag from each unique position was examined to eliminate peaks generated by clonal amplification. CTCF or RAD21 peaks were identified by searching for groups of tags located in a sliding 200-bp window. Adjacent peaks were required to be separated from each other by at least 500 bp. The threshold for the number of tags that generate a peak was selected for a FDR of 0.001. Additionally, required peaks were to have at least 4-fold more tags (normalized versus total number) than input samples. They were also required to have 4-fold more tags relative to the local background region in a 10-kb region to avoid identification of DNA segments containing genomic duplications or nonlocalized occupancy. Peaks were associated with gene products by identification of the nearest TSS. Variable sizes of H3K27me3-marked regions were identified by searching for groups of tags located in a sliding 10-kb window. Regions were required to contain at least 1.5-fold more tags (normalized versus total number) than input samples. Adjacent regions were required to be separated from each other by at least 50 kb. Clustering of data was done using Cluster3. Heat maps of clusters were generated using Java Tree View.

MeDIP-seq

Methylated DNA IP (MeDIP) was performed as previously described (Pomraning et al. 2009) with anti-5-Methylcytosine (5-mC) (A-1014-050, epigentek) and anti-5-Hydroxymethylcytosine (5-hmC) (39770, active motif). Two replicates of experiments were performed on progenitors and neutrophils. Libraries were prepared with the NEBNext primer set and were selected by size by 8% PAGE and sequenced for 50 cycles on Illumina HiSeq 2500. Reads were aligned to mm9 with Bowtie2 and reads non-uniquely mapped were discarded. Data were analyzed using HOMER.

In situ Hi-C

Cells were harvested under three different biological conditions: undifferentiated ECOMG cells, in vitro differentiated neutrophils, and neutrophils isolated from murine bone marrow. For in vitro condition, 7 independent experiments were performed starting with 5 million cells and for BMDNs, 2 independent experiments were performed starting with 1 million cells. A total of 16 independent harvests were generated processed into 19 libraries (including PCR duplicates for each BMDN harvest). In situ Hi-C was performed essentially as previously described (Rao et al. 2014). Libraries were prepared with the NEBNext primer set and were selected by size by 6% PAGE and sequenced for 50 cycles on Illumina HiSeq 2500. Reads were aligned to mm9 with Bowtie2 and reads non-uniquely mapped were discarded. The raw reads were filtered and analyzed using HOMER. Hi-C statistics was processed using HiCUP (Wingett et al. 2015) (Supplemental Table S4). After stringent data filtering, including removal of PCR duplicates, self-ligation, restriction ends, continuous genomic fragments and re-ligation events, a total of 243, 261 and 31 million unique pair of contacts were obtained from progenitors, neutrophils and BMDNs combined replicate Hi-C libraries, respectively. The resulting neutrophil data set had more unique pair of contacts than the progenitor data set. To allow direct comparisons across progenitors and neutrophils, we resampled the neutrophil data set such that an equal number of unique pair of contacts contributed to each Hi-C data matrix. Normalized contact matrices were obtained by assuming that each genomic bin interacts with other bins with equal chances and that such interaction depends on their linear distance along the chromosome. The normalized contact probability heat maps were generated by Java Tree View or R. PCA analysis, applied to the normalized interaction matrix, to define sub-nuclear compartments and PC1 values for each 25-kb bin with positive and negative values, corresponding to A and B compartments, respectively. We consider the difference in PC1 value (>20) and the PC1 value change from positive to negative or vice versa to identify genomic bins that switch compartments. For data analysis in Supplemental Fig. S3B, we defined that a gene is associated with a compartment flip event if the gene promoter or gene body is overlapped with or is located within a genomic bin with such a compartment flip. Significantly interacting regions were identified as those regions that showed a

markedly higher probability than expected based on a binomial test ($P < 0.001$). The circular format plots were generated using Circos. The TAD coordinates were identified by TopDom (Shin et al. 2015). Interchromosomal contact probability index (ICP) was calculated as the sum of a region's interchromosomal contact frequencies divided by the sum of its inter- and intrachromosomal contact frequencies (Kalhor et al. 2012). The distal ratio was calculated using SeqMonk (http://www.bioinformatics.bbsrc.ac.uk/projects/seqmonk/).

Immuno 3D-FISH and imaging

DNA-FISH was done as previously described (Lin et al. 2012). The bacterial artificial chromosome (BAC) probes were RP24-322E3, RP23-333E5, RP23-21G14, RP24-258K7, RP23-6H7, RP23-139O3, RP23-28K9, RP23-61I14, RP23-57D4, RP23-331F6, RP23-21F10, RP23-382C22, RP23-349B11 for chromosome 17, and RP23-225M6 for rDNA (Grozdanov et al. 2003) from the BACPAC Resource Center at Children's Hospital Oakland Research Institute. Probes against mouse major satellite repeats were generated by PCR from mouse genomic DNA using the following primers: major satellite 5'-GCGAGAAAACTGAAAATCAC and 5'-TCAAGTCGTCAAGTGGATG; LINE-1 probe was generated from a cloned 3.6kb LINE-1 sequence using primers: LINE-1 5'- ACTCAAAGCGAGGCAACACTAGA and 5'-GTTCATAATGTTGTTCCACCT; Centromere probe 5'-ATTCGTTGGAAACGGGA was purchased from PNA Bio. In all cases, probes were labeled by nick-translation using Alexa-647 dUTP. The nuclear lamina was stained first with primary antibody to Lamin B1 (sc-6217; Santa Cruz Biotechnology), followed by secondary staining with donkey antibody to goat IgG conjugated to Alexa-488 (A11055; Invitrogen). The nucleolus was stained first with primary antibodies to B23 (ab10530; Abcam), followed by secondary staining with donkey antibody to mouse IgG conjugated to Alexa-647 (A31571; Invitrogen). To check for LBR expression and localization, cells were first stained with anti-LBR (12398-1-AP; proteintech), followed by secondary staining using a donkey antibody to rabbit IgG conjugated to Alexa-568 (A10042; Invitrogen).

RNA-FISH was performed as previously described. Cells were washed with PBS and subsequently fixed with paraformaldehyde at a final concentration of 4% for 10 minutes. After 10 minutes the cells were spun down for 2 min at 1000rpm. The fixed cells were attached to coverslips coated with 0.1% poly-l-lysine (Sigma) by cytospin. To permeabilize the cells, the cells were placed in 70% ethanol overnight. RP23-225M6 for rRNA was labeled by nick-translation using Alexa-647 dUTP, and hybridized with standard FISH hybridization buffer containing 50% formamide. For hybridization conditions 75 ng probes per 15 µl of hybridization buffer were used. The probes were hybridized for 16 h at 42°C followed by two wash steps with wash buffer containing 50% formamide and 2x SSC. The cells were counterstained with DAPI before imaging.

Images were acquired on Deltavision deconvolution microscope with a 100× objective. Optical sections (z stacks) 0.2 μm apart were obtained in the DAPI, FITC, Red and Cy5 channels. Distances between FISH foci to nuclear lamina were measured with Volocity 3D image analysis software (Perkin Elmer). To measure the nuclear volume and longest axis, nuclei were counterstaining with DAPI. Images were captured using Olympus FV1000 Spectral Deconvolution Confocal microscope with a 60× objective. Using Volocity software, the volume and longest axis of DAPI stained nuclei was determined by measuring the calibrated number of voxels identified within a given nucleus.

Statistics

P values of less than 0.05 were considered statistically significant. *P* values are reported in figures; the method of statistic analysis and the exact value of sample number (*n*) are stated in figure legends. All the tests are two-tailed and unpaired. For statistical comparison of two groups, Student’s t-test or Mann-Whitney test was performed; for multiple comparison, one-way ANOVA or Kruskal-Wallis test was performed where appropriate, using GraphPad Prism 6.0 (GraphPad Prism Software, Inc.). F test was carried to compare variabilities between the samples.

For studying the number of major satellites, centromere, rDNA foci and nucleoli, at least 100 nuclei were analyzed from each cell type. We considered that the number of observations was large

enough to use Gaussian asymptotic results and then one-way ANOVA to determine significance of differences. Correct for multiple comparisons: confidence intervals and significance. Post test: Tukey. Report multiplicity adjusted *P* value for each comparison. For measuring the volume and distance from fluorescent foci to nuclear lamina, statistical analysis involved fluorescent signals obtained from > 100 cells per group. We use Kruskal-Wallis test. Corrrect for multiple comparisons: significance without confidence intervals. Post test: Dun's. Report multiplicity adjusted *P* value for each comparison. For Hi-C interactions, the significance of the difference between observed and expected interactions was calculated using the binomial distribution based on the total number of interactions. Box and whisker plots were plotted by GrapPad Prism 6.0, where the mean value (''+''), the extreme data points (top and bottom bars), the 25th-75th percentiles (box), and the median (Center lines) are indicated. Whisker position and outlier (dots) display are according to Tukey style.

Publically available RNA-seq dataset

GSE48307 (Wong et al. 2013).

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