Supplementary Material

for

Large-scale topological disruption of chromosome territories 9 and 22 is associated with nonresponse to treatment in CML

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2.2. Definition of response criteria
Complete hematologic response was defined as a white blood cell count lower than 10x10^9 cells/L, platelet counts lower than 450x10^9 cells/L, no immature cells (blasts, promyelocytes, myelocytes) in the peripheral blood, and disappearance of all signs and symptoms related to leukemia (including palpable splenomegaly). Cytogenetic responses were defined as complete (0% t(9;22)/Ph negative), partial (1% to 35% Ph positive), minor (36% to 65% Ph positive), and minimal (66% to 95% Ph positive). Cytogenetic remission was judged by standard GTG-banding cytogenetic analysis in 20 metaphases. Major molecular response was defined as a BCR-ABL1/ABL1 transcript ratio lower than 0.05% by qRT-PCR, representing a more than 3-log reduction from baseline. Complete molecular response was defined as undetectable levels of the BCR-ABL1 transcript, representing at least a 4.5-log reduction from baseline levels. Optimal treatment response was defined by complete cytogenetic response; suboptimal treatment response was defined by partial cytogenetic response, and treatment resistance was defined by minimal cytogenetic response.

2.5. Three-dimensional fluorescence in situ hybridization (3D-FISH)
FISH was performed as previously described with some modifications. CD34+ cells were placed on poly-L-lysine-coated coverslips (No. 1.5) and allowed to attach at 37°C for 10 min in a CO2 incubator. Cells were fixed in 4% paraformaldehyde (PFA) for 10 min at RT and then washed three times in 0.05% Triton X-100/1x PBS at RT (5 min each wash). Slides with fixed cells were incubated in 0.5% Triton X-100/1x PBS at RT for 20 min and then transferred to 20% glycerol/1x PBS at RT for at least 1 h. The coverslips were dipped into liquid nitrogen for 30 s and thawed. The slides were returned to 20% glycerol/1x PBS as soon as the frozen layer disappeared. The freezing/thawing step was repeated four times before washing the cells in 0.05% Triton X-100/1x PBS three times for 5 min. The cells were incubated in 0.1 N HCl for 10 min, washed in 2x SSC for 5 min and treated with RNase A at 37°C for 1 h. Finally, the cells were kept in 50% formamide/2x SSC at RT for 24 h. For hybridization, a BCR/ABL1 dual color dual fusion probe (Ref. 30-191032 Abbott Molecular, Vysis) and whole chromosome paint probe for CT9 and 22 (Ref. D-1112-125-IR; D1113-125-IR, MetaSystems) were used. The denaturalization step was performed at 80°C for 3 min in a Hybrite Slide
Stainer (Abbott Molecular) and subsequently incubated in a humidified chamber at 37°C overnight. After incubation, the slides were washed in 2xSSC for 10 min and 2xSSC/0.1% IGEPAL for 2 min at RT, 0.4xSSC/0.3% IGEPAL for 2 min at 72°C and 2xSSC/0.1% IGEPAL for 1 min at RT. The slides were counterstained with DAPI (0.1 µg/ml), incubated for 10 min followed by three 1x PBS washes and mounted with fluorescence-antifade solution (Vectashield).

2.7. Three-dimensional immuno-FISH

Immuno-FISH was performed on human CD34+ cells from BM and MPB adhered to poly-L lysine-coated slides, as previously described with some modifications 2. Briefly, cells were fixed as described in the previous section. After three rinses in 1x PBS, the cells were permeabilized in ice-cold 0.4% Triton-X-100/1x PBS for 20 min on ice and incubated in blocking solution for 1 h (2.5% bovine serum albumin (BSA), 10% normal goat serum, 0.1% Tween-20). The cells were incubated with the primary antibodies H3K9ac (Abcam Cat. No. ab10812) and H3K27 me3 (Abcam Cat. No. ab6002) overnight at 4°C in a humid chamber. The next day, the cells were washed with 0.2% BSA/0.1% Tween-20/1x PBS three times for 5 min at RT and subsequently incubated with the secondary antibody ATTO 647N (goat anti-rabbit, active motif cat. no. 15048) and DyLight 405 (goat anti-mouse, Thermo Fisher cat. no. 35501BID) diluted in blocking solution for 1 h at RT in a dark and humid chamber. Cell rinses were performed with 0.1% Tween-20/1x PBS three times for 5 min with shaking in the dark. Postfixation of cells was performed with 2% PFA for 10 min with three additional 1x PBS rinses. Cells were incubated with RNase A for 1 h at 37°C and washed three times with 1x PBS. A permeabilization step was performed with ice-cold 0.7% Triton-X-100/0.1 M HCl for 10 min on ice. Cells and painting probes (Ref. D-1112-125-IR; D1113-125-IR, MetaSystems) were then denatured simultaneously on a hot plate at 80°C for 3 min and hybridized overnight at 37°C in a dark and humid chamber. Final washes were performed three times in 50% formamide/2xSSC and three times in 2xSSC at 37°C for 5 min each. Finally, the cells were mounted with 10 µl of mounting medium (Vectashield) and sealed.

2.8. Microscopy and image acquisition

Three-dimensional structured illumination microscopy (3D-SIM) was performed on a Zeiss Elyra PS.1 microscope equipped with a Zeiss Plan Apochromat inverted 100x/1.46 oil immersion objective lens using an Andor EM-CCD. Diode lasers at 405, 488, 561 and 642 nm
and the standard corresponding emission filters were used. The z-stacks were acquired with five phases per image plane. The 3D-SIM image stacks were acquired at 200 nm intervals for CTs, 125 nm for immuno-FISH and 85 nm for the BCR and ABL1 genes and quadruplex FISH through each nucleus and consisted of 60-120 slices collected sequentially. A field of view was selected, and the z-stack boundaries were defined manually. TetraSpeck beads (200 nm) (T7280, Thermo Fisher Scientific) were used to calibrate alignment parameters between the different channels. The lateral pixel size, Dx and Dy, was 79 nm in the recorded images and 40 nm in the reconstructed image. Computational image reconstruction for ELYRA PS.1 was performed using theoretical optical transfer functions (OTFs) and the Zeiss algorithm (ZEN Black). Likewise, SIM data were quality controlled for effective resolution and absence of artifacts using SIMcheck, an open-source Image J plugin to assess SIM image quality via modulation contrast-to-noise ratio, spherical aberration mismatch, reconstructed Fourier plot and reconstructed intensity histogram values.

2.9. Image analysis and CT compartment definition
For all analyzed images, a background subtraction filter was used, applying a Gaussian filter to define the background at each voxel and performing a baseline subtraction of this variable background. Segmentation of CT compartments was carried out in each 3D stack representing the color channels for painted CTs and automatically identified. All voxel intensities below an automatically set threshold were set to zero. With an iterative procedure, a threshold value was estimated for each 3D data set for CT thresholding. The size and shape of the generated surface were a direct map of the intensity distribution of probe labeling within the nucleus as detected by Imaris. The segmentation procedure was monitored by merging the outline of the segmented object with the original object. Any adjustments to the surface shape were made by altering the surface size to reduce extraneous noise around the periphery during detection rather than manually shrinking or expanding the 3D model. This resulted in the best 3D masking for the nucleus, chromosomes and genes, which is critical for the quantification of the studied parameters.

The 3D reconstruction generated utilizing the abovementioned parameters was applied for compartment identification. To identify a separate compartment, we visually demonstrated the polarized arrangement of the compartments. We calculated the polarization index for compartments defined as follows:
where VA and VB are the volumes of the compartments and VS is the shared volume between them\(^3\). Only compartments with a polarization index of 1, which means a complete disconnection between them, were considered and counted as separate compartments.

### 2.10 Definition and analysis of nuclear topological features

#### 2.10.1 CT volume

The absolute CT volume was computed by adding the number of voxels within the 3D segmented image of each CT. The normalized chromosome volume was computed by dividing the absolute chromosome volume by the absolute nuclear volume (see also Supplementary Figure 3Ai-ii).

#### 2.10.2 Estimation of intermingling volume

To estimate the intermingling volume, we considered the detected volumes of the two CTs, \( V_{CT9} \) and \( V_{CT22} \), from the segmented 3D images. Therefore, the intermingling region was defined as the ratio of the intersection to the union of both volumes:

\[
\frac{V_{CT9} \cap V_{CT22}}{V_{CT9} \cup V_{CT22}}.
\]

This intermingling volume is divided by the volume of the nucleus to obtain the normalized intermingling volume (see also Supplementary Figure 2Aiii).

#### 2.10.3. Chromatin decompaction factor (CDF)

To estimate the chromosome decompaction factor, we obtained the sequence length for all the painted chromosomes from the NCBI database (https://www.ncbi.nlm.nih.gov/genome/?term=txid9606[orgn]). The chromosome decompaction factor was defined as follows:

\[
\frac{\text{Norm}_V \cdot V_{CT}}{L_{CT} \text{ (Mbp)} \times 10^4},
\]

where Norm\( _V\) \(\text{CT} \) is the normalized chromosome volume and \( L_{CT} \) is chromosome length in Mbp\(^31\). See also Figure 3B.

#### 2.10.4. Quantification of histone PTM occupancy on CTs and the whole nucleus
Serial SIM images from 3D immuno-FISH were used for 3D reconstructions and histone PTM quantifications. For estimation of the threshold, for every image stack for each channel, a separate small 3D volume from the area outside the cell was selected. The average intensity of this ‘background substack’ was calculated and used as a baseline to calculate the threshold. Quantification of mean voxel intensity was measured with Imaris software by selecting the area of the CT using the Surfaces tool and extracting the fluorescence information. The analysis of signal intensity vs three-dimensional CT was performed by measuring the mean gray value in a region of interest (ROI) in every stack. For quantification of the relative overlapping signals, a colocalization channel was created, and the analysis in all z optical series was performed. Relative levels of H3K9ac and H3K27me3 were calculated as a ratio of the intensity sum in the antibody channel over that in the DNA staining channel, for both CT9 (green channel) and CT22 (orange channel). For nuclear measurements, the nuclear volume was derived from the statistics of the contours drawn in the Imaris software.

2.10.5. Minimum distance definition
CT gravity centers of signals were automatically determined with Imaris software, and these centers were used to estimate distances between each CT compartment and locus signal of the genes. The shortest distances were defined after all the distances between the identified compartments and allele locus were measured. Only the minimum distances between heterologous CTs and between BCR and ABL1 genes were reported. Note that distances between genes forming the BA fusion were considered. See also Figure 4A-B.

2.10.6. Relative radial position
The relative radial position was measured from the center of mass of the nucleus, which was defined as $0$, and the distance between $0$ and each allele signal and CT gravity centers was defined as $r_A$. The nuclear ratio ($r_B$) was defined as the distance between $0$ and the nuclear periphery ($1$) passing through the signal center. The relative radial position ($r$) with respect to the nuclear center was calculated as a fraction of $r_B$ ($r = r_A / r_B$). See also Figure 4E-F.

2.10.7. Determination of gene location within CTs
Signal colocalization as a surrogate of the positioning of the gene within its CT was calculated. In this regard, the software quantified the number of voxels corresponding to the gene locus, which overlapped with the CT. When the level of colocalization was lower than
50%, the location between the gene locus and the border of the CT was considered to be outside of the CT. In contrast, if the level of colocalization between the gene locus and the CT was higher than 50%, the location between this gene locus and the CT was considered to be inside the territory.
## Supplementary Table 1. Demographic and baseline characteristics of CML patients, and healthy donors included in the study

| Group          | Patient ID | Gender | Age sampling (years) | Karyotype at the time of sampling | Disease stage at sampling | Age at the time of diagnosis (years) | Sokal score risk at diagnosis (¥) | TKI treatment prior to sampling | Follow-up duration after initial diagnosis (months) | Current Status |
|----------------|------------|--------|----------------------|-----------------------------------|--------------------------|--------------------------------------|-----------------------------------|----------------------------------|------------------------------------------------|-----------------|
| Healthy BM     | HBM-1      | F      | 33                   | 46,XX[20]                         |                          |                                      |                                   |                                  |                                               |                |
|                | HBM-2*     | M      | 25                   | No performed                      |                          |                                      |                                   |                                  |                                               |                |
| MPB            | MPB-1      | M      | 33                   | 46,XY[20]                         |                          |                                      |                                   |                                  |                                               |                |
|                | MPB-2      | M      | 46                   | 46,XY[20]                         |                          |                                      |                                   |                                  |                                               |                |
|                | MPB-3      | F      | 29                   | 46,XX[20]                         |                          |                                      |                                   |                                  |                                               |                |
|                | MPB-4      | M      | 27                   | 46,XY[20]                         |                          |                                      |                                   |                                  |                                               |                |
|                | MPB-5      | F      | 38                   | 46,XX[20]                         |                          |                                      |                                   |                                  |                                               |                |
| Naive-R        | CML-1      | M      | 34                   | 46,XY.t(9;22)[20]                 | CP                       | 34                                   | Low                               | 0                                | 47               | Alive          |
|                | CML-2      | M      | 25                   | 46,XY.t(9;22)[20]                 | CP                       | 25                                   | High                              | 0                                | 39               | Alive          |
|                | CML-3      | M      | 47                   | 46,XY.t(9;22)[20]                 | CP                       | 47                                   | High                              | 0                                | 36               | Alive          |
|                | CML-4      | M      | 30                   | 46,XY.t(9;22)[20]                 | CP                       | 30                                   | High                              | 0                                | 32               | Alive          |
|                | CML-5      | F      | 23                   | 46,XX.t(9;22)[20]                 | CP                       | 23                                   | High                              | 0                                | 31               | Alive          |
| Naive Non-R    | CML-6      | M      | 34                   | 46,XY.t(9;22)[20]                 | CP                       | 34                                   | High                              | 0                                | 32               | Death          |
|                | CML-7      | F      | 47                   | 46,XX.t(9;22)[20]                 | CP                       | 47                                   | High                              | 0                                | 30               | Death          |
|                | CML-8      | F      | 27                   | 46,XX.t(9;22)[20]                 | AP                       | 27                                   | High                              | 0                                | 28               | Death          |
|                | CML-9      | M      | 64                   | 46,XY.t(9;22)[20]                 | CP                       | 64                                   | High                              | 0                                | 15               | Alive          |
|                | CML-10     | M      | 44                   | 46,XY.t(9;22)[20]                 | CP                       | 44                                   | High                              | 0                                | 29               | Alive          |
| TKI-R          | CML-11     | F      | 48                   | 46,XX[20]                         | DFS                      | 36                                   | Low                               | Imatinib            | 204             | Alive          |
|                | CML-12     | M      | 26                   | 46,XY[20]                         | DFS                      | 20                                   | Low                               | Imatinib            | 132             | Alive          |
|                | CML-13     | M      | 54                   | 46,XY[15]                         | DFS                      | 41                                   | High                               | Imatinib            | 204             | Alive          |
|                | CML-14     | M      | 35                   | 46,XY[20]                         | DFS                      | 23                                   | High                               | Imatinib            | 186             | Alive          |
|                | CML-15     | F      | 49                   | 46,XX[20]                         | DFS                      | 43                                   | Low                               | Imatinib            | 108             | Alive          |
| TKI Non-R      | CML-16     | M      | 39                   | 46,XY.t(9;22)[2]/46,XY[18]        | TKI-resistant relapse    | 37                                   | Low                               | Imatinib            | 32               | Alive          |
|                | CML-17     | M      | 36                   | 46,XY.t(9;22)[5]/46,XY[10]        | TKI-resistant relapse    | 31                                   | Low                               | Imatinib            | 86               | Death          |
|                | CML-18     | M      | 35                   | 46,XY.t(9;22)[3]/46,XY[11]        | TKI-resistant relapse    | 34                                   | High                               | Imatinib            | 31               | Death          |
|                | CML-19     | M      | 38                   | 46,XY.t(9;22)[2]/46,XY[16]        | TKI-resistant relapse    | 32                                   | Low                               | Imatinib            | 96               | Alive          |
|                | CML-20     | F      | 42                   | 46,XY.t(9;22)[1]/46,XY[19]        | TKI-resistant relapse    | 41                                   | Low                               | Imatinib            | 29               | Alive          |

**Abbreviations:** HBM, healthy bone marrow; MPB, mobilized peripheral blood; CML, chronic myeloid leukemia; TKI, Tyrosine Kinase Inhibitor; R, responders; Non-R, no responders; F, female; M, male; CP, chronic phase; AP, accelerated phase; DFS, disease-free survival.

* Fresh healthy BM sample were purchased from Lonza (1M-105, Lonza).

¥ The Sokal score is based on the patient’s age, spleen size during clinical examination, platelet count, and percentage of blasts in the peripheral blood.

§ Nilotinib and dasatinib are second generation TKIs.
Supplementary Figure 1

(A) Healthy BM
46,XX

(B) MPB
46,XY

(C) Naïve Responder
46,XY,t(9;22)

(D) Naïve Non-Responder
46,XY,t(9;22)

(E) TKI Responder
46,XY

(F) TKI Non-Responder
46,XY,t(9;22)
Supplementary Figure 1. Representative G-banding karyotypes from the bone marrow of patients included in the study.

(A) Normal female karyotype (46, XX) in the BM from a healthy donor.

(B) Normal male karyotype (46,XY) in the MPB from a healthy donor.

(C) Abnormal karyotype 46,XY,t(9;22) from the BM of a naïve CML patient at the time of diagnosis. Derivative chromosomes are indicated with an arrowhead. This patient subsequently responded to TKI first-line treatment.

(D) Abnormal karyotype 46,XY,t(9;22) from the BM of a naïve CML patient at the time of diagnosis. Derivative chromosomes are indicated with an arrowhead. This patient did not subsequently respond to TKI first-line treatment.

(E) Normal male karyotype (46,XY) from the BM of a CML patient with complete cytogenetic response that no longer presents chromosome translocation t(9;22) after receiving TKI treatment.

(F) Abnormal karyotype 46,XY,t(9;22) in the BM of a CML patient who, after receiving TKI first-line treatment, did not present a complete cytogenetic response. Derivative chromosomes are indicated with an arrowhead.
Supplementary Figure 2. Representative BM metaphase spreads showing whole chromosome painting (WCP) for chromosomes 9 and 22.

(A) Localization of normal FISH signals on chromosomes 9 (green signal) and 22 (orange signal) in a BM metaphase spread from a healthy donor.

(B) Localization of normal FISH signals on chromosomes 9 (green signal) and 22 (orange signal) in a MPB metaphase spread from a healthy donor.

(C) Localization of FISH signals on derivative chromosomes 9 and 22 originated from t(9;22) translocation (indicated with arrowheads) in the BM of a patient naïve to treatment. This patient subsequently responded to TKI first-line treatment.

(D) Localization of FISH signals on derivative chromosomes 9 and 22 originated from t(9;22) translocation (indicated with arrowheads) in the BM of a patient naïve to treatment. This patient did not subsequently respond to TKI first-line treatment.

(E) Localization of normal FISH signals on chromosomes 9 and 22 in the BM of a CML patient with a complete cytogenetic response that no longer presents chromosome translocation t(9;22) after TKI treatment.

(F) Localization of FISH signals on derivative chromosomes 9 and 22 originated from t(9;22) translocation (indicated with arrowheads) in the BM of a CML patient who, after receiving TKI first-line treatment, did not present a complete cytogenetic response and still presents chromosome translocation t(9;22) after TKI treatment.

The DAPI counterstain has been converted into grayscale to simulate a G-like banding pattern.
Supplementary Figure 3. CT overlap and occupancy of histone marks reveal remarkable differences between naïve-R and naïve non-R patients.

(A) Representative composite image showing the 3D reconstruction of a CD34+ cell and its respective CTs from which volume calculations and subsequent normalizations were performed. i) Three-dimensional reconstruction of a cell showing two recognizable compartments for CT9 (green, CT9 (a) and CT9(b)) and two recognizable compartments for CT22 (orange, CT22 (a) and CT22(b)). Note the intermingling between two heterologous CTs (CT9(a) and CT22(b)). ii) Schematics showing how CT9 and CT22 volumes were normalized. The volume of the recognizable CT (CT9 or CT22) compartments was divided by the nuclear volume of the cell and multiplied by 100. iii) Schematic 3D reconstruction showing the intermingling region between two heterologous chromosomes (yellow). Normalized intermingled volume was defined as the total volume where the heterologous CTs overlapped and subsequently normalized to nuclear volume and multiplied by 100. iv) Proportion of CTs overlapping was defined as the percentage of a CT volume occupied by another heterologous CT. For calculation of this proportion, the intermingled volume (iii) was divided by the total volume of each CT (CT9 and CT22 separately) and multiplied by 100.

(B) Box plot showing the dispersion of nuclear volume in µm³ among groups.

(C) The proportion of CT9 overlapping with CT22 expressed as a percentage (%).

(D) Proportion of CT22 overlapping with CT9 expressed as a percentage (%).

(E) Bubble chart displaying the chromatin decompaction factor of the nuclear volume average for each group.

(F) Comparison of the relative fluorescence intensity (FI) of H3K9ac on CT9 among groups. Bar charts show the average H3K9ac/CT9 signal ratio.

(G) Comparison of the relative fluorescence intensity (FI) of H3K27me3 on CT9 among groups. Bar charts show the average H3K27me3/CT9 signal ratio.

Data in B, C, D, F and G are presented as boxplots. Data in E are presented as bubble chart. p values of 0.01 to 0.05 were considered significant (*), p values of 0.001 to 0.01 were considered very significant (**) and p values < 0.001 were considered extremely significant (***, ****).
Supplementary Figure 4

(A) Healthy BM, Naïve R, TKI R, MPB, Naïve Non-R, TKI Non-R

(B) Relative H3K9ac FI on Nucleus

(C) Relative H3K27me3 FI on Nucleus
Supplementary Figure 4. Nuclear histone marks occupancy shows remarkable differences between naïve-R and naïve non-R CML patients.

(A) Representative 3D-SIM images of CD34+ cells showing histone marks H3K9ac (magenta) and H3K27me3 (cyan) in a single plane. Scale bar: 1 µm.

(B) Relative fluorescence intensity (FI) of H3K9ac in the entire nucleus of CD34+ cells compared among groups. Bar charts show the average H3K9ac/nucleus signal ratio.

(C) Relative fluorescence intensity (FI) of H3K27me3 in the entire nucleus of CD34+ cells compared among groups. Bar charts show the average H3K27me3/nucleus signal ratio.

Data in B and C are presented as boxplots. p values of 0.01 to 0.05 were considered significant (*), p values of 0.001 to 0.01 were considered very significant (**), and p values < 0.001 were considered extremely significant (***, ****).

Supplementary References

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