Massively parallel single-cell mitochondrial DNA genotyping and chromatin profiling

Caleb A. Lareau 1,2,3,16 ✉, Leif S. Ludwig 1,2,16 ✉, Christoph Muus 2,4, Satyen H. Gohil 2,5,6, Tongtong Zhao 2,7, Zachary Chiang 2,3,7, Karin Pelka 2,8,9, Jeffrey M. Verboon 1,2, Wendy Luo 1,2, Elena Christian 2,3, Daniel Rosebrock 2, Gad Getz 2,10, Genevieve M. Boland 8,11, Fei Chen 2, Jason D. Buenrostro 2,7, Nir Hacohen 2,8,9, Catherine J. Wu 2,5, Martin J. Aryee 2,10,12, Aviv Regev 2,13,14 and Vijay G. Sankaran 1,2,15 ✉

Natural mitochondrial DNA (mtDNA) mutations enable the inference of clonal relationships among cells. mtDNA can be profiled along with measures of cell state, but has not yet been combined with the massively parallel approaches needed to tackle the complexity of human tissue. Here, we introduce a high-throughput, droplet-based mitochondrial single-cell assay for transposase-accessible chromatin with sequencing (scATAC-seq), a method that combines high-confidence mtDNA mutation calling in thousands of single cells with their concomitant high-quality accessible chromatin profile. This enables the inference of mtDNA heteroplasm, clonal relationships, cell state and accessible chromatin variation in individual cells. We reveal single-cell variation in heteroplasmy of a pathologic mtDNA variant, which we associate with intra-individual chromatin variability and clonal evolution. We clonally trace thousands of cells from cancers, linking epigenomic variability to subclonal evolution, and infer cellular dynamics of differentiating hematopoietic cells in vitro and in vivo. Taken together, our approach enables the study of cellular population dynamics and clonal properties in vivo.

Mitochondria play a central role in metabolism and are unique organelles that carry their own genome, often in large copy number, encoding a subset of proteins, tRNAs and ribosomal RNAs essential to their function. Mutations in the mitochondrial genome are associated with a multitude of clinical phenotypes that are estimated to affect ~1 in 4,300 individuals, making them among the most common inherited metabolic disorders. Critically, the fraction of mitochondrial genomes carrying a specific variant, heteroplasmy, may dictate the degree of disease severity in affected patients 1–3. Furthermore, the high mutation rate (~2–10× that of nuclear DNA) leads to accumulation of somatic mtDNA mutations that may contribute to aging phenotypes 1. While genomic approaches are emerging to quantify heteroplasmy, the majority of sequencing assessments have been based on bulk cell populations, limiting detection of somatic mutations in individual cells 4,5.

Recently, we and others have shown that single-cell sequencing approaches can detect heteroplasmy or homoplasmic mutations, which we further leveraged as natural genetic markers in clone and lineage tracing of human cells, while also measuring cell state 6,7. Due to the small size of the mitochondrial genome (16.6 kb) and its higher copy number per cell, retrospective inference of cellular relationships by somatic mtDNA mutations is more cost-effective and robust compared with mutation detection in the nuclear genome by single-cell whole-genome sequencing 8. Moreover, single-cell RNA sequencing (scRNA-seq) and assay for transposase-accessible chromatin with sequencing (scATAC-seq) allow concomitant mtDNA mutation detection along with the transcriptional or accessible chromatin cell state. While this presents a powerful system for clonal/lineage tracing in humans in vivo, only modest-throughput single-cell genomic assays had sufficient coverage of mitochondrial sequences for reliable mutation detection, whereas the massively parallel methods needed to draw meaningful conclusions on many biological systems had insufficient mitochondrial coverage 9.

As recently reported droplet-based scATAC-seq techniques enable the profiling of accessible chromatin in thousands of cells per experiment 10,11, we hypothesized that with appropriate modification, they may facilitate the enrichment of transposase-accessible mtDNA 12. However, these protocols rely on processing of nuclei, thereby depleting mitochondria and resulting in only ~1% of reads mapping to mtDNA, compared with 20–50% in the original ATAC-seq protocol 11,12, a level that is inadequate for single-cell mutation calling and clonal inference. Here, we establish a mitochondrial single-cell assay for transposase-accessible chromatin with sequencing (mtscATAC-seq),...
a massively parallel protocol for high and uniform single-cell mitochondrial genome coverage that retains high-quality chromatin accessibility data, and combine it with computational methods to identify rare, clonal mtDNA mutations in healthy and diseased cells. We demonstrate the wide applicability of mtscATAC-seq to quantify single-cell mitochondrial genotypes in the context of mitochondrial disease and clonally trace thousands of human cells in vitro and in vivo. Given the multi-omic nature, we envision the broad utility and applicability of mtscATAC-seq to enhance our understanding of mtDNA genotype-phenotype correlations and reconstruct clonal dynamics across diverse areas of human health and disease.

Results

Development and validation of mtscATAC-seq. To develop mtscATAC-seq, we modified the droplet-based scATAC-seq workflow of the widely used 10X Genomics platform to improve mtDNA yield and genome coverage. As most scATAC-seq protocols use nuclei, depleting cytoplasmic mitochondria, we turned to processing whole cells to retain mtDNA. We reasoned that mild lysis or permeabilization of cells would be required for the Tn5 enzyme to integrate adapters into accessible nuclear chromatin and mtDNA. Moreover, as cells contain multiple mitochondria, which may be more readily released upon lysis or permeabilization, we reasoned that fixation should minimize mixing of mtDNA between cells. Finally, we aimed to identify conditions retaining high-quality chromatin accessibility data.

We systematically tested for conditions that satisfy these features in a mixture of two cell lines (GM11906 and TF1; Fig. 1a) by evaluating mtDNA abundance, cross-contamination, and mtDNA and chromatin fragment complexity. Because each cell line harbored private homoplasmic mutations, we sensitively detected mtDNA abundance, cell doublets and possible mtDNA crosstalk due to cell lysis/permeabilization and fragmentation that occurs in a pool. Omitting digitonin and Tween-20 in the lysis and wash buffers (‘Condition A’) yielded substantially more mtDNA fragments per single cell (median 21.5%) than the recommended protocol (1.9%; Fig. 1b, Supplementary Table 1 and Methods), consistent with earlier observations12,13. These conditions retain high-quality chromatin accessibility data: while per-cell complexity of nuclear fragments slightly decreased (Extended Data Fig. 1a), other metrics associated with scATAC-seq data quality improved (Fig. 1c and Extended Data Fig. 1b). BioAnalyzer traces confirmed an increased ratio of nucleosome-free to mononucleosome fragments, consistent with the increased recovery of mtDNA (Extended Data Fig. 1c). Based on 43 high-confidence homoplasmic mtDNA variants private to each cell line, ~8.7% of barcodes carried otherwise cell-type-specific homoplasmic variants at intermediate (60–90%) heteroplasmy, indicating contamination of mtDNA fragments between cells (Fig. 1d, Extended Data Fig. 1d and Methods). Because this contamination may occur due to the release of mitochondria during processing, we added a formaldehyde fixation step. Indeed, fixation with 0.1% or 1% formaldehyde led to a ~3× reduction in mtDNA fragment cross-contamination (Fig. 1e,f and Extended Data Fig. 1d), a 69% increase in mtDNA fragment complexity and restoration of chromatin library complexity (Extended Data Fig. 1e). After removing cell doublets, the empiric rate of contamination was 0.19% (Fig. 1f and Methods), which is consistent with the order of magnitude for short-read sequencing error14. Importantly, formaldehyde treatment did not introduce additional mtDNA mutations (Extended Data Fig. 1f).

Furthermore, we observed regions of lower coverage across the mitochondrial genome, which we determined were due to high homology (and thus low mappability) to nuclear mtDNA segments (NUMTs). We reasoned that due to the high mtDNA copy number and the high Tn5 accessibility of mtDNA, ambiguous fragments could be confidently assigned to the mitochondrial genome with a low false-positive rate. Utilizing a compendium of DNase hypersensitivity data14 and additional public scATAC-seq data, we estimated that only ~1 accessible fragment from NUMTs would be detected per cell (Methods), such that these are unlikely to be a confounding element in heteroplasmy estimation. We therefore developed a computational approach that effectively assigns reads that map to both the mitochondrial and nuclear genome strictly to mtDNA, facilitating near-uniform coverage without altering chromatin complexity (Fig. 1g and Extended Data Fig. 1g–i). Some residual variation in coverage remained after reference genome masking and was correlated with GC content of the mtDNA genome (coefficient of correlation (r) = 0.33; Extended Data Fig. 1j), likely reflecting PCR amplification and Tn5 insertion bias15.

Overall, mtscATAC-seq combines fixation, modified lysis and computational analysis of multi-mapping reads, leading to a ~20-fold increase in mean mtDNA coverage per cell (from 9.6× to 191.0×; Fig. 1g) and in fraction of mtDNA reads (median per cell from 1.9% to 36.8%; Extended Data Fig. 1h) with only modest reduction in chromatin complexity (median per cell from 87,569 to 73,864; Extended Data Fig. 1e) and in reads mapping to pre-annotated DNase hypersensitivity peaks (from 74.1% to 72.3%), retaining cell-type-specific accessible chromatin peaks (93.8% of 77,704 peaks; Extended Data Fig. 1k and Methods).

Single-cell features of pathogenic mtDNA mutations. We used mtscATAC-seq to identify pathogenic mtDNA mutations, and gain insights into their impact. The GM11906 lymphoblastoid cells used in the mixing experiment (Fig. 1) were derived from a patient with myoclonic epilepsy with red ragged fibers (MERRF), a mitochondrial disorder that in 80–90% of cases is caused by a 8344A>G mutation that alters tRNA function (Fig. 2a). Bulk ATAC-seq analyses of these cells estimated a population heteroplasmy of 44% for the 8344A>G allele, consistent with previous reports1. We retained 818 high-quality data GM11906 cells with at least 50× single-cell mtDNA coverage and 40% reads in peaks (Fig. 2b). Interestingly, we observed a broad range of heteroplasmy (0% to 100%) for the 8344A>G allele, with a median of 38%, consistent with the bulk ATAC-seq data (Fig. 2c) and previous family studies of this mutation16. We independently replicated the distribution of heteroplasmy levels using the Fluidigm scATAC-seq platform17 and in situ genotyping18 (Fig. 2c–e, Extended Data Fig. 2a and Supplementary Table 2). Analysis of matched chromatin profiles highlighted specific loci and transcription factor (TF) activities that are associated with different levels of the 8344A>G allele. First, promoter accessibility scores19 of 32 and 94 genes were positively or negatively correlated, respectively, with single-cell 8344A>G heteroplasmy, corresponding to a <1% false-discovery rate (Fig. 2f and Methods). Binning cells into high (>60%; n = 273), intermediate (10–60%; n = 228) and low (<10%; n = 313) heteroplasmy for the pathogenic allele highlighted distinct chromatin features near the NRP2, TRMT5 and SENPS5/NCPBP2-AS2 loci (Fig. 2g–i). Notably, nearby genes have been broadly linked to mitochondria biology20–22. The accessibility profiles at other loci were virtually indistinguishable (Extended Data Fig. 2b,c), suggesting that the observed variations (Fig. 2g–i) may be a consequence of disease allele heteroplasmy. Furthermore, we identified TFs whose activity may be associated with the mutation by scoring TF binding sites from chromatin immunoprecipitation sequencing (ChIP-seq) data (Methods). In particular, MEF2A and MEF2C were strongly anticorrelated with pathogenic heteroplasmy (Extended Data Fig. 2d,e). Notably, the TF MEF2 is a target of mitochondrial apoptotic caspases, supporting a model where pathogenic allele heteroplasmy may regulate nuclear factor activity23. These analyses demonstrate the potential to study the altered cellular circuits resulting from pathogenic mtDNA variants in a heteroplasmy-dependent manner.

Notably, a second mutation, 8202T>C (bulk heteroplasmy 34%), was the most correlated mutation with the 8344A>G variant (Fig. 2j).
Fig. 1 | Optimization of a high-throughput single-cell mtDNA genotyping platform with concomitant accessible chromatin measurements. 

(a) Schematic of cell line mixing experiment between indicated two human hematopoietic cell lines. 

(b) Distribution of percentage of mtDNA reads per single cell for screened conditions. 

(c) Distribution of percentage of reads mapping to annotated DNase hypersensitivity peaks (nuclear reads only) per single cell. Each condition in panels b and c represents the top 1,000 cells (based on chromatin complexity) from one experiment. 

(d) Mitochondrial SNP mixing depiction of variants for the TF1 or GM11906 cell line for ‘Condition A’ as in b. Both axes are log_{10} transformed. 

(e) Same as d but for ‘Condition A’ with 1% formaldehyde (FA) treatment. 

(f) Summary of contamination (percentage of reads from minor cell population) for FA-treated and untreated comparison. 

Using MITOMAP19, we annotated the nonsynonymous variant (phenylalanine to serine) as a ‘probably damaging’ mutation in the cytochrome C oxidase II (MT-CO2) gene. We found that 456 of 818 GM11906 cells were positive for both mutations (>5% heteroplasmy), whereas the remaining cells showed 0% heteroplasmy for either both mutations or 8202T>C alone, but not 8344A>G alone (Fig. 2k). Of the 5,230 reads that covered both variants, 99.6% exclusively contained either both mutated or wild-type alleles (Fig. 2l). 

The co-occurrence of both mutations on the same haplotype and the presence of 8344A>G/8202T>C cells suggests the evolution of at least two subclonal populations, each spanning the complete spectrum from low to very high 8344A>G heteroplasmy (Fig. 2k,m), demonstrating how mtscATAC-seq can enhance our understanding of clonal dynamics in the context of mitochondrial disease. 

Inference of mutations for clonal lineage tracing. To facilitate clonal tracing of human cells based on reliable mtDNA variation, we developed the Mitochondrial Genome Analysis Toolkit (mgatk; Fig. 3a and Methods), a computational pipeline to identify clonal substructure in complex populations profiled using mtscATAC-seq. 

Here, we define clonal mutations as those with similar heteroplasmy that may genetically mark an individual cell and its immediate descendants to distinguish it from other more distantly related cells. 

Recent variant callers developed for single-cell genotyping were designed to separate amplicon error from true mutations17 or account for allelic dropout16, neither of which predominantly confounds heteroplasmy estimates from mtscATAC-seq (Methods). Instead, mgatk focuses specifically on clonal mtDNA variant calling in single cells, by leveraging the deep per-cell coverage from mtscATAC-seq. 

Specifically, mgatk identifies high-confidence clonal mutations by aggregating signal across cells, leveraging between-cell variability (per mutation variance mean ratio; VMR) and strand bias (Pearson correlation of counts per strand; Fig. 3a and Methods). Thus, rather than calling variants in individual cells, mgatk leverages the high-throughput nature of our data to identify between-cell properties to distinguish signal from noise. 

The resulting mutations are then used as a feature set for downstream analyses, such as the inference of clonal families.

We validated mgatk by identifying anticipated clonal substructure in the 855 TF1 cells (>50x mitochondrial genome coverage) profiled in the mixture experiment (Fig. 1). Because these cells were expanded from 30 individually sorted TF1 cells, we expected to observe multiple subclones16. We identified 48 reliable mtDNA variants by bivariate filtering of variants with a relatively high VMR and concordant heteroplasmy from both strands (Fig. 3b and Methods). Using these 48 variants as features, we determined 12 clonal cell subsets using a shared nearest neighbor clustering approach (Fig. 3c and Methods). 

Variants called by other approaches lacked sensitivity or had substantial strand bias compared with mgatk (Extended Data Fig. 3a–c and Methods). The 48 high-confidence variants enabled us to reconstruct a putative phylogenetic tree for the identified TF1 subclones (Fig. 3d). 

Though mgatk was optimized for mtscATAC-seq data, its unsupervised application performed comparably well to our previous supervised identification of multiple hematopoietic colony-specific variants from 935 cells profiled by Smart-seq210 (Extended Data Fig. 3d–h).
**Fig. 2 | Pathogenic mtDNA variability and clonal evolution in cells derived from a patient with MERRF.**

**a.** Schematic of the mitochondrial lysine tRNA secondary structure with sequence and the pathogenic single nucleotide variant (8344A>G). **b.** Quality control filtering for GM11906 single cells based on mean mtDNA genome coverage and percentage of nuclear reads in chromatin accessibility peaks. **c.** Quantification of 8344A>G heteroplasmy variability in single GM11906 cells across three technologies. Numbers (n) of cells plotted are shown. Color represents the within-assay coverage percentile. Black bars indicate the median heteroplasmy per technology; the dotted line presents the mean heteroplasmy as determined for bulk ATAC-seq. **d.** Field of view for in situ genotyped GM11906 cells (Extended Data Fig. 3i,j and Methods). Representative image selected from one of seven fields of view for one experiment. **e.** Per-gene score Spearman correlations with the 8344A>G allele heteroplasmy. The gray dots show values for a permutation. **f.** Pseudobulk chromatin accessibility track plots are shown for the NR2F2 and SENP5 loci. Pseudobulk groups were binned based on 0–10% (low), 10–60% (mid) and 60–100% (high) 8344A>G heteroplasmy. **g.** Per-mutation heteroplasmy correlation with 8344A>G allele as indicated for the pathogenic allele. **h.** Single-cell heteroplasmy for two indicated mutations. The circled population represents a double-positive population for both mutations. **i.** Single-cell heteroplasmy for two indicated mutations. The circled population represents a double-positive population for both mutations. **j.** Correlation with 8344A>G heteroplasmy (%). **k.** Coverage percentiles for 8344A>G heteroplasmy (%). **l.** Observed read distributions for 8344A>G and 8202T>C genotypes. **m.** Schematic of the mitochondrial lysine tRNA secondary structure with sequence and the pathogenic single nucleotide variant (8344A>G).

Furthermore, variants identified by mgatk substantially outperformed other unsupervised approaches in discerning cells that shared a clonal origin (Methods). However, as Smart-seq2 and other scRNA-seq methods detect a substantial number of false-positive variants, corroboration by mtDNA sequencing is highly recommended; conversely, mtscATAC-seq captures DNA directly, minimizing potential artifacts. Simulations with empirically derived parameters indicated that mtscATAC-seq has high sensitivity, high positive predictive value (PPV) and low dropout, particularly for subclonal variants of at least 5% heteroplasmy with at least ~50x coverage per cell (Extended Data Fig. 3i,j and Methods). Overall, the combination of mtscATAC-seq and mgatk provides a
Among the identified variants from mgatk, six mutations (four in Patient 1, two in Patient 2) attained homoplasmy in a subset of cells and were markedly enriched in the CD19+ nant subpopulations at single-cell resolution.

Next, we related the mtDNA clones with both their chromatin profiles and receptor clonotypes, leveraging the mtDNA coverage from 5′ scRNA-seq (Extended Data Fig. 4d,e) to link to variants identified from mtscATAC-seq. Interestingly, leukemic cells with the 14858G>A mtDNA mutation did not carry the predominant B cell receptor (BCR) clonotype, presenting a distinct subclonal population showing various differentially expressed genes (Fig. 4h,d, Extended Data Fig. 4f and Methods). Moreover, all cells in Patient 1 were positive for trisomy 12 (Methods), a common cytogenetic abnormality in CLL29, suggesting that the copy number alteration preceded the somatic mtDNA diversity detected (Fig. 4e). Performing a per-peak association with our putative subclones, we observed hundreds of loci associated with subclonal structure in these tumors (Fig. 4f and Extended Data Fig. 4g), the latter of which had been associated with chemoresistance in CLL and colorectal cancer30,31 (Fig. 4g,h). These results provide a broad basis for how mtscATAC-seq can resolve epigenetic differences in malignant subpopulations at single-cell resolution.

Among the identified variants from mgatk, six mutations (four in Patient 1, two in Patient 2) attained homoplasmy in a subset of cells and were markedly enriched in the CD19+ population (Extended Data Fig. 4h,i). Notably, the same variants were also identified in T lymphocytes, natural killer and myeloid cells (Fig. 4i–l and Extended Data Fig. 4j,k). These results point to the possible involvement of an early progenitor cell with residual multi-lineage capacity in the pathogenesis of CLL, as suggested by previous reports32–34. These results could further be corroborated in the scRNA-seq data of Patient 2 upon integration of calling somatic mutations in nuclear genes (that is, chr4:109,084,804A>C ‘LEF1’...
and chr19:36,394,730G>A ‘HCST’; identified by exome sequencing) (Extended Data Fig. 4j,k).

Next, we profiled a human colorectal cancer resection (Fig. 4m). Using variance in chromatin accessibility and marker gene scores, we identified six major cell populations, including tumor-derived epithelial cells and distinct immune cell populations (Fig. 4n,o and Extended Data Fig. 4l). Using integrated calling of somatic chromosomal copy number variants (CNVs) (Fig. 4p and Methods) and mtDNA mutations (Fig. 4q), we suggest a model where copy number gains on chromosomes 6, 7, 8, 9 and 12 and a homoplastic 16147C>T variant are shared across the dominant malignant cell population (Fig. 4p–r). Multiple additional mtDNA mutations then further resolve subclonal structure within the malignant cells, as well as in nonmalignant immune cells (Extended Data Fig. 4m–o). Taken together, our results highlight the utility of the mtscATAC-seq/mgatk platform to enable the retrospective inference of cellular population dynamics in malignancies.

Linking cell state to fate in hematopoietic differentiation. The multi-modal output of mtscATAC-seq simultaneously informs about cell state and clonal relationships, allowing us to study complex physiologic processes, where genetic barcoding is not possible. We focused on human hematopoiesis, a process thought to be sustained by tens to hundreds of thousands of distinct hematopoietic stem/progenitor cells (HSPCs) under steady state16,17, potentially requiring the sampling of large cell numbers to capture the full spectrum of clonal diversity.

We first benchmarked mtscATAC-seq in an in vitro model of human hematopoiesis, where clonal contributions could be anticipated. We cultured ~500 or ~800 CD34+ HSPCs in progenitor expansion media, before induction of monocytic or erythroid differentiation. Over the course of 20 d we profiled cells from two independent cultures (two and three time points for the 500- and 800-cell inputs, respectively), yielding 18,259 high-quality mtscATAC-seq cell profiles (Fig. 5a and Methods), with a mean of 24,944 unique nuclear fragments per cell, 49.1% of which were in accessibility peaks, and a mean 74.8× mtDNA coverage per cell. Dimensionality reduction18, TF motif scoring19 and inference of pseudotime trajectories highlighted differentiation continuums from HSPCs to either the erythroid or monocytic fates (Fig. 5b–d and Methods). These findings verify that mtscATAC-seq can reconstruct cell state transitions comparable to previous scATAC-seq/mgatk studies16,18,39–41.

Fig. 4 | Clonal and functional heterogeneity in human malignancies resolved by somatic mtDNA mutations. a, Schematic of experimental design. Populations of PBMCs from two patients with CLL were separated by FACS or magnetic bead enrichment and profiled with mtscATAC-seq and 10X 5′ scRNA-seq. b, Fraction of CD19+ cells with major BCR clonotype as determined from V(D)J receptor sequencing. c, Inference of subclonal structure from somatic mtDNA mutations for Patient 1. Cells (columns) are clustered based on mitochondrial genotypes (rows). Colors at the top of the heatmap represent clusters or putative subclones. Color bar, heteroplasmy (allele frequency percentage). d, Clonotype receptors (columns) associated with somatic mtDNA mutations (rows) from Patient 1. Colors at the top of the heatmap represent BCR clonotypes. Color bar, heteroplasmy (allele frequency percentage). e, Estimated copy number of chromosome 12 across putative subclones for Patient 1. Patient-derived cells showed elevated DNA read counts of chromosome 12, consistent with a trisomy for this chromosome (see Methods). Boxplots: center line, median; box limits, first and third quartiles; whiskers, 1.5× interquartile range. f, Subclone associations with accessible chromatin. Red dots denote peaks associated at a false-discovery rate of <0.01. g,h, Examples of subclone-associated differential accessibility peaks near the TIAM1 (g) and ZNF257 (h) promoters. i, Schematic of scATAC projection framework using LSI and UMAP. A healthy PBMC reference embedding with indicated cell types is shown. j,k, Projection of cells collected from Patient 1 (j) and Patient 2 (k). Colors indicate cells positive for indicated somatic mtDNA mutations. Non-B cells are highlighted. l, Gene signature plots of PBMCs from scRNA-seq for Patient 1 corroborating mtDNA mutations in non-B cells. m, Schematic showing mtscATAC-seq profiling of a colorectal cancer resection specimen. n, Two-dimensional embedding of all quality-controlled tumor-derived cells using UMAP showing the distribution of cells based on Louvain clustering and annotation based on marker gene scores as exemplified in panel o and Extended Data Fig. 4l. o, Projection of marker gene scores for indicated genes EPCAM, PTPRC and IL1RL1. Color bar, gene score activity. p, Inferred CNV profiles for indicated cell types (x axis) and chromosomes. Arrows indicate relative increase of copy numbers in the epithelial tumor cells. Cells from the basophil-like population are shown as a control group of cells. Color bar, z-score-transformed fragment abundance. q, Inference of subclonal structure from somatic mtDNA mutations in colorectal cancer. Epithelial cells (columns) are clustered based on mitochondrial genotypes (rows). Color bar, heteroplasmy (allele frequency percentage). r, Putative model of clonal evolution of the profiled colorectal cancer specimen as suggested based on integrated analysis of nuclear CNV and somatic mtDNA mutation profiles. FSC, forward scatter.
8 cell clones that preferentially gave rise to daughter cells of erythroid or monocytic lineage by day 20, respectively (Fig. 5k and Methods). However, when restricting this analysis towards day 8 cells within the early progenitor cluster (cluster 9; Extended Data Fig. 5c), this association diminishes, though our power to detect such lineage biasing features (if present and causal for such observations) may be limited given the number of cells profiled at this stage ($n = 257$).

**Clonal tracing in human hematopoiesis in vivo.** Finally, we utilized mtscATAC-seq to gain insights into the clonal architecture of hematopoiesis in vivo. We profiled bone marrow-derived CD34+ HSPCs ($n = 7,474$ quality-controlled cells) along with PBMCs ($n = 8,591$) that were obtained after a 3-month interval from a 47-yr-old healthy donor (Fig. 6a). Using reference scATAC-seq and scRNA-seq data, we annotated cell states, revealing cell...
Fig. 5 | Clonal lineage tracing across accessible chromatin landscapes and time in an in vitro model of hematopoiesis. a, Schematic of experimental design. Approximately 800 or 500 CD34+ HSPCs were derived from the same donor, expanded and differentiated in two independent cultures over the course of 20 d as shown. Stars represent time points/populations of cells that were profiled via mscATAC-seq. b, Two-dimensional embedding of all quality-controlled cells using UMAP. Single-cell TF motif deviation scores for indicated factors are shown in color for all cells. c, Pseudotime trajectories for monocytic and erythroid trajectories are depicted. d, Identification of high-confidence variants derived from both cultures. The number of variants passing both thresholds (dotted lines) is indicated. e, Changes in heteroplasmy for 175 variants identified from the 500-input culture from day 8 to day 14. Values represent the mean over all single cells in the library. f, Increased variability in heteroplasmy shifts for the 500-cell-input culture. P value is reported from a two-sided Kolmogorov–Smirnov test comparing the observed and permuted distributions log fold-changes of heteroplasmy. g, Comparison of heteroplasmy shifts for the 800-cell-input culture. Linear regression indicates that most of the variability in heteroplasmy changes at the late time point (day 20, y axis) can be explained by the intermediate time point (day 14, x axis). Colored dots are mutations highlighted in the next panel. h, Heteroplasmy trajectories for four selected mutations from g. Values represent the mean over all single cells in the library for the indicated time point. i, Three examples of clonal populations marked by indicated mutations identified in the 800-cell-input culture that result in erythroid, monocytic or bipotent lineage outcomes. j, Systematic identification of clonal outcomes using the late time point (day 20). The y axis depicts the difference between z-score in erythroid and monocytic bias of a single clone. k, Differences in TF motif activity comparing erythroid-biased and monocytic-biased clones at the earliest sampled time point (day 8). QC, quality-controlled.
lar heterogeneity and distinct hematopoietic lineages (Fig. 6b–d and Extended Data Fig. 6a). Our high-quality chromatin accessibility (mean of 23,551 and 9,874 unique nuclear fragments for CD34⁺ cells and PBMCs, respectively) and mtDNA data enabled detailed analysis of cell states, including the inference of relatively low mtDNA copy number in plasmacytoid dendritic cells, further corroborated by analysis of bulk RNA-seq data, consistent with a previous report of mitophagy in dendritic cells (Extended Data Fig. 6b,c).

Within the HSPCs and PBMCs, mgatk called 351 and 130 high-confidence variants, respectively (HSPCs had greater mtDNA coverage than the PBMCs), 52 of which were shared among both compartments (Extended Data Fig. 6d,e). Although the 429 unique mutations were only present at low frequencies (<1%) in pseudobulk populations (Fig. 6c), allele frequencies in individual cells showed considerable homoplasy (Extended Data Fig. 6f), and the mutational signatures of identified mtDNA variants were consistent with previous reports (Fig. 6g)⁴⁴.

A community detection algorithm partitioned cells into 257 clonal groups with a median 9 and 12 cells per clone in the PBMC and HSPC compartments, respectively, noting that 92% of clones contained less than 1% of assayed cells (Fig. 6h, Extended Data Fig. 6g and Methods). Focusing on a select set of highly heteroplasmic and homoplasmic variants, we observed clonal patterns that may reflect physiologic waves of hematopoietic activity, both in terms of expansion in the HSPC compartment and in terms of contribution to the PBMC compartment (Fig. 6c–i,k). For instance, clone 008 (marked by 2788C>G) and clone 119 (12868G>A) are present in distinctive proportions in HSPCs with variable output 3 months later, as reflected in their different abundance in the PBMC compartment (Fig. 6l). By contrast, clone 032 (3209A>G) had similar prevalence in HSPCs to clone 008, but reduced output in the following months based on decreased detection in PBMCs (Fig. 6k). Overall, our results suggest relatively stable clonal output over the assessed time interval, with observed shifts in heteroplasmy in the HSPC and PBMC populations, reflecting either undersampling (Fig. 6l) or clonal succession ²⁶. These findings clearly support stable propagation of mutations present in stem and progenitor cells to the peripheral blood (Fig. 6c–i,k), and indicate that steady-state hematopoiesis is fueled by a large pool of HSPCs where the contributions of individual clones to healthy blood cell production are low (<1%), consistent with previous reports ²⁶,⁴⁴.

To further understand the clonal contributions to the major lineages of peripheral blood, we examined the association between clonal output and inferred cell state from the mtscATAC-seq data. While we observed variability in composition of inferred clones (Fig. 6m), such a distribution is statistically consistent with random subsampling of cell states (Fig. 6n,o). These results stand in contrast to the observations of biased clonal output (Fig. 5), which may reflect conditions in an in vitro system, where fate decisions may be restricted by limited cytokine availability. Moreover, these observations may further be confounded by distinct longevity of different cell types or the averaging of rare clones not detectable from the current sample size. In this regard, additional analysis designed to discover high-confidence mtDNA mutations present in no more than three HSPCs recovered an additional 923 distinct mtDNA mutations (Extended Data Fig. 6h and Methods). Though rare, these mutations showed concordant mutational spectra and lower allele frequencies in the pseudobulk population (Extended Data Fig. 6h,i) and may mark quiescent or low-activity clones.

Taken together, our in vivo analysis demonstrates the potential, along with some of the challenges, to dissect complex physiologic systems. Our results highlight the ability of our framework to facilitate systematic studies aimed at investigating clonal population structures at single-cell resolution in vivo, which, as far as we are aware, were previously limited to model organisms or gene therapy trials ⁴⁶–⁵⁰.

Discussion

Here, we develop a high-throughput platform for measuring mtDNA mutation heteroplasmy along with accessible chromatin states in thousands of single cells. We verify data standards (Fig. 1), chart the cis- and trans- effects of pathogenic mutations (Fig. 2) and infer subclonal population structure (Fig. 3), all from a single experiment. By leveraging somatic mtDNA variation in more complex settings, our results further indicate the potential of natural genetic mtDNA barcodes to resolve clonal heterogeneity within malignancies (Fig. 4), and assess clonal dynamics in hematopoeisis (Figs. 5 and 6), while also obtaining rich information on variation in cell state. Unlike conventional high-throughput scRNA-seq approaches that suffer from uneven coverage of mitochondrial RNA or a high false-positive error rate ⁶, or require a priori knowledge of specific variants ⁴¹, our framework enables de novo discovery of variants to enable the inference of subclonal structure in complex settings, including tissue specimens directly obtained from patients. We expect that additional improvements in variant calling, clonal detection methods and heteroplasmic-specific distance functions will aid to resolve cellular hierarchies in greater detail.

Fig. 6 | Cellular population dynamics in native hematopoiesis in vivo resolved by mtDNA mutations. a, Schematic of experimental design. CD34⁺ HSPCs and PBMCs were derived from the same healthy donor at 0 and 3 months, respectively, and processed using mtscATAC-seq. b,c, Two-dimensional embedding of all quality-controlled CD34⁺ cells using UMAP colored by Louvain clustering (b) or by cell cluster annotation (c) using previously published reference data. CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte monocyte progenitor; HSC, hematopoietic stem cell; LMPP, lymphoid-primed multipotent progenitor; MEP, megakaryocyte-erythroid progenitor; MPP, multipotent progenitor; pDC, plasmacytoid dendritic cell. d, Two-dimensional embedding of all quality-controlled PBMCs using UMAP colored by the distribution of cells based on Louvain clustering and annotation using scRNA-seq data-derived label transfer (Extended Data Fig. 6a). e, Percentage heteroplasmia (log₁₀ scale) of mgatk-nominated variants and respective allele frequencies in pseudobulk CD34⁺ HSPC (x axis) and PBMC populations (y axis). Indicated select variants are further highlighted in panels i–k. f, Distribution of mgatk-nominated mutations along the mitochondrial genome averaged over both populations (pseudobulk). Inner circle, mitochondrial genome; dots, percentage heteroplasmia of each mutation; outer gray circle, genome coordinates; annotation shows color-coded mitochondrial genes. g, Substitution rate (observed over expected) of mgatk-identified heteroplasmic mutations (y axis) in each class of mononucleotide and trinucleotide change resolved by the heavy (H) and light (L) strands of the mitochondrial genome. h, Empirical cumulative distribution plots of the number of cells per clone for both HSPCs and PBMCs. The median number of cells per clone n is shown for each of the two populations. i–k, Specific mutations (top) and cell clones to which they belong (bottom) marking CD34⁺ cell and PBMC chromatin accessibility profiles (as in b–d) for mutations 2788C>A (i), 12868G>A (j) and 3209A>G (k), Numbers of cells n assigned to the respective clonal groups are shown for the CD34⁺ HSPC and PBMC cell populations. Color bar, heteroplasmia (allele frequency percentage). l, Distribution of heteroplasmia shifts in the CD34⁺ HSPCs over the PBMC population. P value: two-sided Kolmogorov–Smirnov test comparing the observed and permuted distributions log fold-changes of clonal abundances. m, Relative proportion of cells from indicated hematopoietic lineages (y axis) in each clone (x axis) identified in PBMCs. n,o, Summary statistics of CD34⁺ HSPC (n) and PBMC (o) associations between lineage (cell state) and clone. Adjusted P values (P_adj of lineage association) represent the Benjamini–Hochberg-adjusted chi-squared goodness-of-fit per clone with at least ten cells.
In addition to pathogenic mitochondrial variants, such as 8344A>G, our high-throughput platform should facilitate the examination of functional mtDNA mutations in relatively common disease settings\(^1\). Specifically, alterations in mtDNA have been associated with a variety of complex human diseases, including Alzheimer’s disease\(^5\), Parkinson’s disease\(^6\), cardiomyopathies\(^7\), pediatric cancers\(^8\) and more generally aging phenotypes\(^9\)\(^1\). As our approach facilitates rapid genotyping and concomitant chromatin profiling in thousands of cells, potential molecular consequences of mtDNA variants may now be dissected (Fig. 2), which is not otherwise possible using bulk approaches\(^1\).

Despite the relatively small size of the mitochondrial genome, the prevalence of somatic mutations, though not necessarily present in every cell, enabled inferences about cellular population dynamics in complex human tissues\(^4\)\(^5\) (Fig. 6). For future applications, we emphasize that care should be taken with respect to biological conclusions, which may require validation via orthogonal methodology across multiple donors. For example, our analyses in the context of malignancies (Fig. 4) provide a vignette of integrating nuclear point mutations, copy number alterations, immune receptor rearrangements and mtDNA variation to further resolve clonal structure and functional heterogeneity. Though the hematopoietic system was the focus of our investigations (with the exception of the colorectal cancer sample), we expect our mtscATAC-seq framework to be compatible with most human tissues\(^4\)\(^5\). Overall, the advances presented here enable avenues to study the role of cellular dynamics in human health and disease.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information,
acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41587-020-0645-6.

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Methods

Cell lines and cell culture. TF1 cells (ATCC) were maintained in RPMI 1640, 10% FBS, 2mM l-glutamine and 2mM L-glutamine recombinant human granulocyte-macrophage colony-stimulating factor (Peprotech) and incubated at 37°C and 5% CO2. GM11906 cells (Coriell) were maintained in RPMI 1640, 15% FBS and 2mM L-glutamine and incubated at 37°C and 5% CO2.

Primary cells and cell culture. CD34+ hematopoietic stem and progenitor cells were obtained from Fred Hutchinson Hematopoietic Cell Procurement and Processing Services (Seattle, USA) or StemCell Technologies. The CD34+ samples were de-identified and approval for use of these samples for research purposes was provided by the Institutional Review Board and Biosafety Committees at Boston Children’s Hospital. Healthy donor PBMCs were obtained from StemCell Technologies. CD34+ cells were thawed and cultured in StemSpan II with 1x CC100 (StemCell Technologies) at 37°C and 5% CO2. At indicated time points, these cells were seeded in media supporting the differentiation into hematopoietic and erythroid cells63,64. Briefly, cells were cultured at a density of 10^6 to 10^7 cells per milliliter in IMDM supplemented with 2% human AB plasma, 3% human AB serum, 1% penicillin/streptomycin, 31U/ml heparin, 10μg/ml insulin, 200μg/ml holo-transferrin, 1μU of erythropoietin, 10ng/ml stem cell factor (SCF) and 1ng/ml IL-3 and incubated at 37°C and 5% CO2. For mtsATAC-seq processing at indicated time points and when additional cells were to be maintained to enable sampling of cells at a later time, one-third of the cultured cells were maintained and two-thirds of the cells were forwarded to single-cell sequencing as described in the “scATAC-seq and mtsATAC-seq” section.

CLL samples. Cryopreserved PBMCs from patients with CLL consented on institutional review board approved protocols were obtained from ALLCells (Patient 1) or from Adrian Wiestler at the National Institute of Health (Patient 2). Cytogenetic analysis of Patient 1 CLL cells detected an extra copy of chromosome 12 (trisomy 12) as detected by fluorescence in situ hybridization. Cryopreserved cells were thawed and cultured by serial dilution in RPMI with 10% FBS. B lymphocytes were isolated using the negative-selection MojoSort Human Pan B Cell Isolation Kit (Biolegend, 480882) and CD19-negative immune cells were isolated from a separate aliquot using the positive-selection MojoSort Human CD19 Selection Kit (Biolegend, 480106).

Flow cytometry analysis and sorting. For flow cytometry analysis and sorting, cells were washed in FACS buffer (1% FBS in PBS) before antibody staining. For the CLL patient-derived PBMC staining, a FITC-conjugated CD19 antibody (HIB19, 302206, Biolegend) was used at 1:30 dilution. For live/dead cell discrimination, Sytox Blue was used according to the manufacturer’s instructions (HIB19, 302206, Biolegend) was used at 1:50 dilution. For live/dead cell discrimination, Sytox Blue was used according to the manufacturer’s instructions. For flow cytometry analysis and sorting, we have followed the general recommendations from 10X Genomics and observed concordant results relative to their standard protocol. As hematopoietic cell suspensions were used for protocol optimizations, additional modifications may be required to optimize results for other tissues of interest.

scRNA-seq. scRNA-seq libraries were generated using the 10X Chromium Controller and the Chromium Single Cell 5’ Library Construction Kit and human B cell and T cell V(D)J enrichment kit according to the manufacturer’s instructions. Briefly, the suspended cells were loaded on a Chromium Controller Single-Cell Instrument to generate single-cell cDNA libraries followed by reverse transcription and sample indexing using a C1000 Touch Thermal Cyclers with 96-Deep Well Reaction Module (BioRad). After the GE1s, the barcoded tagged cDNA was purified and amplified to enable sample indexing and enrichment of scRNA-seq libraries. The final libraries were quantified using a Qubit dsDNA HS Assay kit (Invitrogen) and a High Sensitivity DNA chip run on a Bioanalyzer 2100 system (Agilent).

We further note the following related to mtsATAC-seq optimizations: comparison of mtDNA cross-contamination between cell lines using data from Fig. 1b suggested higher levels at 0.1% formaldehyde (contamination 1.54%) compared with 2% formaldehyde fixation (contamination 1.14%). For cells that were fixed in 1% formaldehyde for 10min at room temperature. This has yielded excellent results and has been used throughout the manuscript unless indicated. Additional incubation (30 min to 12h) at 60°C to further facilitate decrosslinking before the first 72°C elongation step did not improve results (data not shown) and we recommend using the PCR conditions specified in the 10X scATAC-seq protocol. Related to 10X Chromium microfluidic chip handling, cell loading and recovery, we followed the general recommendations from 10X Genomics and observed concordant results relative to their standard protocol. As hematopoietic cell suspensions were used for protocol optimizations, additional modifications may be required to obtain optimal results for other tissues of interest.

scATAC-seq and mtsATAC-seq. scATAC-seq libraries were generated using the 10X Chromium Controller and the Chromium Single Cell 5’ Library Construction Kit and human B cell and T cell V(D)J enrichment kit according to the manufacturer’s instructions. Briefly, the suspended cells were loaded on a Chromium Controller Single-Cell Instrument to generate single-cell cDNA libraries followed by reverse transcription and sample indexing using a C1000 Touch Thermal Cyclers with 96-Deep Well Reaction Module (BioRad). After the GE1s, the barcoded complementary cDNA was purified and amplified, followed by fragmenting, A-tailing and ligation with adaptors. Finally, PCR amplification was performed to enable sample indexing and enrichment of scRNA-Seq libraries. For T cell and B cell receptor sequencing, target enrichment from cDNA was conducted according to the manufacturer’s instructions. The final libraries were quantified using a Qubit dsDNA HS Assay kit (Invitrogen) and a High Sensitivity DNA chip run on a Bioanalyzer 2100 system (Agilent).

mtsATAC-seq sequencing and preprocessing. All libraries were sequenced using NextSeq High Output Cartridge kits and a NextSeq 550 sequencer (Illumina). 10X scATAC-seq libraries were sequenced paired-end (2x 75 cycles). 10X 5’ scRNA-seq libraries were sequenced as recommended by the manufacturer. Raw sequencing data were demultiplexed using CellRanger-ATAC mkfastq. Raw sequencing reads were aligned to the regular and modified hg19 reference genome using cellranger-atac version 1.0 (for cell line mixing experiment) and version 1.2 (for all other samples).

With respect to mtsATAC-seq sequencing depth and cell numbers, we further note that for hematopoietic cells we have generally aimed to match the estimated overall library complexity of the sample; for example, sequence 100 million reads for a library with an estimated complexity of 10 million unique sequences (estimated exclusively from the nuclear genome). Furthermore, we have aimed to obtain at least 20X mitochondrial genome coverage after removal of PCR duplicated reads to enable confident mtDNA mutation calling. Mitochondrial genome coverage may improve with deeper sequencing than used here. Moreover, the content of one cell type may be dominated by mtDNA from one cell type. In another cell type that is dominated by mtDNA from another cell type, the required sequencing depth may vary and higher coverage may be readily achieved in some cell types, which would in turn enable more confident detection of low-frequent mutations.

We cannot currently specify general guidelines for the number cells to be profiled, as this will inevitably depend on the specific context (that is, the tissue
and question of interest]. Generally, this will be a function of the ‘clonality’ of each tissue and the diversity of cell types and states, the complexity of which we currently may not be able to accurately anticipate, given the relative lack of data in this area for many human tissues. All methods, when applied to a random sampling of cells, including genetic engineering approaches, are more likely to detect dominant clones, whereas the resolution of lower-frequency clones ultimately improves with an increasing number of cells sequenced. Based on our experience with data in this manuscript, we suggest that profiles from as few as ~1,000 cells can highlight subclonal structures in malignant cell populations (~1,000 cells can highlight subclonal structures in malignant cell populations) and in situ sequencing were performed as previously described20. The cell gel was stained with DAPI (ThermoFisher) and imaged on a Nikon Eclipse Ti microscope with a Yokogawa CSU-W1 confocal scanner unit and an Andor Zyla 4.2 Plus camera using a Nikon Plan Apo 60×/1.40 objective. Z stack images spanning 24 μm at 0.4-μm intervals were acquired in the following channels: 405-nm excitation with a Y okogawa CSU-W1 confocal scanner unit and an Andor Zyla 4.2 Plus the 99% threshold was not met for the major cell type. For both mtDNA and chromatin complexity estimation of the mtDNA target sequence (on the antisense strand) was made accessible for hybridization by enzymatic removal of the sense strand66: restriction digest with 0.5 μl XbaI at 37 °C for 1 h, followed by adding 0.2 U µl−1 lambda exonuclease (both New England Biolabs) at 37 °C for 30 min. The oligonucleotide probe sequences against the wild-type (5’/PHOS/ACACACCTC-TCTTTAATcaacaCAGCCACCTGCGGAACGCTGAAGAcggcTTCCTTCCGTTA AAGATATAGAGGA) and mutant (5’/PHOS/GCCAAACACCTGCTTAT CTTAATCTGAGTCTCGGGAACGCTGAAGAcggcTTCCTTCCGTTA AAGATATAGAGGA) alleles were pooled at 100 nM each in 2× SSC and 20% formamide, hybridized to the cell gels at 37 °C overnight and circulated with 6 U µl−1 RNase (Enzymatics). Real-time PCR amplification, crosslinking and in situ sequencing were performed as previously described20. The cell gel was stained with DAPI (ThermoFisher) and imaged on a Nikon Eclipse Ti microscope with a Yokogawa CSU-W1 confocal scanner unit and an Andor Zyla 4.2 Plus camera using a Nikon Plan Apo 60×/1.40 objective. Z stack images spanning 24 μm at 0.4-μm intervals were acquired in the following channels: 405-nm excitation with a Y okogawa CSU-W1 confocal scanner unit and an Andor Zyla 4.2 Plus camera using a Nikon Plan Apo 60×/1.40 objective. To best identify informative single-cell deviation scores were computed for these factors using chromVAR56.

**Variant calling and evaluation.** Overview. To best identify informative single-cell deviation scores from our mtscATAC-seq assay, we first considered existing single-cell variant-calling approaches designed for our final protocol. We adopted a framework designed for single-cell deviation scores in the mitochondrial genome. Notably, algorithms designed for genotyping typically utilize a Bayesian framework to determine the empirical probability of a certain nonreference allele being truly observed at a particular location. In this setting, the ploidy of the genome is often parameterized in the model, and the allele frequency directly influences the confidence of detecting the mutation. As mtDNA copy number per cell is variable and informative clonal mutations may occur at very low allele frequencies, we found these existing approaches to be unsuitable for our mtscATAC-seq assay. Therefore, we developed a variant calling framework to identify high-confidence heteroplasmic mutations in a manner that (1) is largely independent of the mean allele frequency; (2) is robust to variability in genome ploidy of a cell; and (3) uses the features intrinsic to the high-throughput single-cell mtscATAC-seq assay, including near uniform deep coverage, minimal dropout per cell and thousands of single cells per experiment. Our resulting variant-calling framework, mtgatk, achieves these goals. Analysis of mtscATAC-seq data from this manuscript revealed that certain positions with substantial heteroplasmic across biologically diverse sources were primarily driven by sequencing error. These ‘recurrently mutated’ loci were due in part to several low-complexity stretches in the mitochondrial genome. However, by further evaluation of these variants, we determined that the erroneous heteroplasmic was primarily driven by one strand, reflective of a photobleaching effect from surrounding ‘Gs on successive cycles’. The raw outputs of the CellRanger-ATAC count execution, specifically the barcodes passing quality control and the position–sorted .bam file, serve as inputs into the command-line interface of mtgatk. This execution produces intermediate plaintext sparse matrix files of PCR-deduplicated, per-cell, per-strand count of all alleles at all positions in the reference mitochondrial genome.

To estimate the number of accessible NUMT fragments that would be assigned to mtDNA, we considered two different approaches. First, we used a public GM12878 dataset from 10X Genomics (https://www.10xgenomics.com/solutions/single-cell-atac/) that was aligned to the standard hg19 reference and counted the number of fragments per cell overlapping our NUMT blacklisted regions, which resulted in mean 1.4 and median 1.0 fragments per cell. To estimate the number of accessible chromatin fragments overlapping NUMTs (~1 fragment) that were blacklisted. As our mtscATAC-seq assay generates ~5,000–10,000 mtDNA fragments, we conclude that our blacklist approach yields negligible NUMT contamination.

**Comparison of experimental conditions.** For all comparisons shown in the boxplots and violin plots, the top 1,000 cells/barcodes based on chromatin library complexity were plotted. The top 1,000 number was chosen to ensure the selection of real cells rather than barcode multiples51 or other barcodes associated with low counts. For the overall coverage comparison (Fig. 1g), the top 2,000 cells based on nuclear complexity were averaged (to represent the expected 2,000 cell yield from the experiment).

Cells were assigned TF1, doublet or GM11906 using the sum of alleles at homoplasmic mitochondrial SNP loci (Extended Data Fig. 1d) using a 99% threshold for assigning either major cell type. For both mtDNA and chromatin complexity estimation (Extended Data Fig. 1e), we used the number of unique and duplicate fragments as part of the CellRanger-ATAC (chromatin) and mgatk (mitochondria) output. As inputs into the ‘Lander–Waterman equation’48, which estimates the total number of unique molecules present given these counts. Complexity measures were computed per barcode passing the default cell-type exclusion.

To verify that cell-type-specific accessible peaks were retained in mtscATAC-seq, we determined 77,704 peaks present in either the TF1 or GM11906 cell lines using the mgatk approach for our final protocol. We assigned barcodes as cell doublets (Fig. 1d,e) when this 99% threshold was not met for the major cell type. For both mtDNA and chromatin complexity estimation (Extended Data Fig. 1e), we used the number of unique and duplicate fragments as part of the CellRanger-ATAC (chromatin) and mgatk (mitochondria) output. As inputs into the ‘Lander–Waterman equation’48, which estimates the total number of unique molecules present given these counts. Complexity measures were computed per barcode passing the default cell-type exclusion.

**Mitochondrial variant-calling.** We queried MITOMAP51 v1.20 and filtered for ‘Confirmed’ pathogenic base-substitution variants. Forty-six variants were annotated to alter mtDNA function, whereas 42 were annotated to alter protein-coding sequences in one or more protein-coding genes. Two additional variants were annotated to alter rRNA function.

**Identification of subclonal variants with mgatk.** The raw outputs of the CellRanger-ATAC count execution, specifically the barcodes passing quality control and the position–sorted .bam file, serve as inputs into the command-line interface of mtgatk. This execution produces intermediate plaintext sparse matrix files of PCR-deduplicated, per-cell, per-strand count of all alleles at all positions in the reference mitochondrial genome.

**Epigenomic correlates with pathogenic heteroplasmy.** To identify chromatin accessibility features associated with pathogenic heteroplasmia in the GM11906 cell line, we considered two approaches that complemented our estimation of heteroplasmty at the single-cell level. First, to assess cis-associations, we computed single-cell gene scores as previously described9,10 and computed per-gene associations with heteroplasmia using Spearman correlation (Fig. 2f). To establish a background distribution, we permuted heteroplasmty per cell and recomputed the per-gene association statistic. We reported the number of gene scores correlated with heteroplasmia if the magnitude of the Spearman correlation exceeded 0.2. However, we note that a 1% false-positive rate from the permutation testing would be a threshold of 0.087, resulting in 752 positively and 1,992 negatively correlated gene scores. We reported the more conservative results after examination of the accessible chromatin tracks where loci exceeding a magnitude 0.2 correlation revealed more robust peak differences. Second, to assess trans-associations, we downloaded a compendium of 78 high-quality ChIP-seq sets from lymphoblastoid cell lines from the ENCODE project15. Per-single-cell deviation scores were computed for these factors using chromVAR56. To verify that cell-type-specific accessible peaks were retained in mtscATAC-seq, we determined 77,704 peaks present in either the TF1 or GM11906 cell lines using the mgatk approach for our final protocol. We assigned barcodes as cell doublets (Fig. 1d,e) when this 99% threshold was not met for the major cell type. For both mtDNA and chromatin complexity estimation (Extended Data Fig. 1e), we used the number of unique and duplicate fragments as part of the CellRanger-ATAC (chromatin) and mgatk (mitochondria) output. As inputs into the ‘Lander–Waterman equation’48, which estimates the total number of unique molecules present given these counts. Complexity measures were computed per barcode passing the default cell-type exclusion.

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To model the residual variation in mtDNA coverage (Fig. 1g), we computed rolling averages of GC content and mean coverage after masked alignment in 50-bp bins with a 25-bp step size (Extended Data Fig. 1j). To model the residual variation in mtDNA coverage (Fig. 1g), we computed rolling averages of GC content and mean coverage after masked alignment in 50-bp bins with a 25-bp step size (Extended Data Fig. 1j).

To verify that cell-type-specific accessible peaks were retained in mtscATAC-seq, we determined 77,704 peaks present in either the TF1 or GM11906 cell lines using the mgatk approach for our final protocol. We assigned barcodes as cell doublets (Fig. 1d,e) when this 99% threshold was not met for the major cell type. For both mtDNA and chromatin complexity estimation (Extended Data Fig. 1e), we used the number of unique and duplicate fragments as part of the CellRanger-ATAC (chromatin) and mgatk (mitochondria) output. As inputs into the ‘Lander–Waterman equation’48, which estimates the total number of unique molecules present given these counts. Complexity measures were computed per barcode passing the default cell-type exclusion.
To determine high-quality variants to infer clonal cell populations, mgatk then computes per-variant summary statistics that are used to define high-quality variants. First, it computes a Pearson correlation coefficient between allele counts for all cells that have at least one count observed for the alternate allele (that is, removing 0.0 points from the calculation). Intuitively, a high correlation captures the agreement of heteroplasm in the two strands and mitigates a widespread technical bias of sequencer photobleaching (Extended Data Fig. 3c). Explicitly, the Pearson correlation coefficient is the ‘strand concordance’ value in Figs. 3b and 5d and Extended Data Figs. 3d.e, 4b and 6d. For all applications in this paper, we used a threshold of 0.65. Next, we compute a per-variant VMR (y axis of the same figures) and subsequently filter out variants with a VMR < 0.01 (Figs. 3b and 5d and Extended Data Figs. 3d.e, 4b and 6d). Default values for these two thresholds were based on performance in the hematopoietic clone data (Extended Data Fig. 3). Finally, mgatk reports the number of cells where the variant was confidently detected, defined as the mutation being detected in at least two fragments aligned to both strands. Here, we require the variant to be confidently detected in at least five cells for downstream analyses (which minimizes the inclusion of mutations that would not be associated with subclonal structure). While the workflow enables custom user-defined thresholds, we consistently applied these stated thresholds across the datasets in this study.

When visualizing variants in heatmaps, we have utilized different dynamic ranges (such as up to 10 or up to 100% heteroplasm) to help display mutations in the relevant context of each figure. In general, we recommend visualizing variant by cell heatmaps at a variety of dynamic ranges to ensure best results. Specifically, the mutation frequencies are shown in Fig. 3c. We select for those variants that are of low subclonal groups of cells. Conversely, variant shown in Fig. 4d are highly heteroplasmic or homoplasmic, which would not be conveyed when keeping an upper threshold of 10% heteroplasm for visualization.

Finally, while our approach works for mtscATAC-seq and full-length scRNA-seq methods (for example, Smart-seq2; Extended Data Fig. 3d-h), our approach is not appropriate for 3’ scRNA-seq methods (as data from such platforms are typically only derived from sequencing one strand).

Comparisons with other approaches. To compare our proposed variant-calling approach with other tools, we analyzed the 855 TF1 single cells (Fig. 3) profiled in this manuscript. First, our execution of monovar failed as the genotype likelihood model is a function of a factorial of the maximum depth, which cannot be stored for the extremely deep coverage that results from our protocol. We then evaluated samtools/bcf tools and FreeBayes, treating each of the 855 cells as individual samples. To compare with mgatk (Extended Data Fig. 3a,b), the resulting vcf files from each of these tools were filtered to remove clear homoplasmic and variants that had a variant quality 0.1. While our analyses indicated that mgatk had greater sensitivity in resolving heteroplasm variants informative for subclonal structure, relaxing this variant quality threshold did not improve detection of these informative variants and instead resulted in far more variants with strand discordance (Extended Data Fig. 3c). Finally, we acknowledge that other variant-calling tools, such as GATK, utilize a Fisher’s exact test to flag variants with high strand discordance that can be removed in downstream processing. We found this approach to be unsuitable for these data due to the high copy number, resulting in extremely small P values for all variants, including those that clearly correlated with subclonal structure.

Simulations. We estimated the sensitivity and PPV of mtscATAC-seq using a simulation where we varied mutation heteroplasmy and mutation coverage (Extended Data Fig. 3i). For each of 10,000 iterations per condition, we simulated data for 1,000 cells such that 100 cells contained the subclonal mutation (denoted by the set 1). For heteroplasmy p (p ∈ [0.02, 0.05, 0.15, 0.25, 0.35, 0.45]) and coverage n (n ∈ [20, 50, 100]), we simulated the variant allele frequency (AF) for cell ∈ 1 using a random binomial distribution (dbinom):

\[
AF = \text{dbinom}(p, n) / n
\]

The simulated allele frequencies for the 900 cells that lacked the mutation (denoted by the set 2) were computed in an analogous manner instead using a value q, corresponding to the contamination (or noise) of mtscATAC-seq. From our experiments in Fig. 1, we empirically derived q = 0.19. Thus, for cell ∈ 2:

\[
AF = \text{dbinom}(q, n) / n
\]

For detection, we required the cell to have at least half of the simulated heteroplasmy (p/2). Sensitivity and PPV were reported using AUC as set of true positives and 1 − AUC as set of true negatives by the mean of the 10,000 iterations per condition.

To estimate the dropout rate of a mutation, defined by zero observations of the alternate allele, we simulated m = 10,000 observations for each value (indexed by k) of n and p and computed the ratio of draws of a binomial distribution that were identically zero to the total number of draws:

\[
\text{dropout}(n, p, m) = \frac{\text{dbinom}(0, m)}{m}
\]

All code to reproduce all simulations is contained in the online resources.

Evaluation of mgatk with Smart-seq2 data. To further benchmark our variant-calling algorithm, we reanalyzed 895 high-quality cells from poly-clonal hematopoietic cells carrying somatic mtDNA mutations identified from Smart-seq2 scRNA-seq data. We performed 895 single nucleotide mutations with mgatk for each donor, and variant calling mirrored the parameters established in the TF1 example (that is, strand concordance ≥ 0.65; −log10(VMR) ≥ 2; see Extended Data Fig. 3h). From these samples, we had previously identified 78 variants showing subclonal structure using a supervised approach (that is, the per-cell colony annotated variants were used in the identification of the variants). This set of 78 variants represents a ‘silver standard’ as variants showed disproportionate heteroplasm in a particular clone based on a Mann–Whitney U test previously described.

Overall, mgatk identified 103 variants across the two donors. This set replicated 64 of the 76 (84.2%) previously identified subclonal variants. The variants that were previously identified were associated at least one count observed for the alternate allele (P = 0.00045; Wilcoxon rank-sum test; Extended Data Fig. 3h). While we generally believe the mgatk variant-calling approach to be sensitive to low-frequency variants, we note that this supervised variant-calling procedure (when clonal annotations are known) is theoretically better-powered to detect low-frequency mutations. However, we note that one previously identified variant identified in the TF1 platform is only monozero heteroplasmy on one strand, strongly suggestive of an artificial variant that was nonetheless identified by our previous supervised approach.

To evaluate the efficacy of variant identification approaches for inferring clones, we tested their ability to correctly classify true-positive pairs of cells that belong to different subclonal groups (Extended Data Fig. 4a). We performed tree reconstruction using neighbor-joining on the cosine distance similarity metric, using mutations identified by three unsupervised approaches (bcf tools and FreeBayes), as well as our previous supervised approach for each donor. Area under the receiver operating curve (Extended Data Fig. 3g,h) was computed and can be interpreted as the efficacy of classifying pairs of cells from the same clone based on sets of mtDNA variants.

TF1 analyses. To identify putative subclones, we used the square root of the heteroplasmity matrix as inputs into the FindNeighbors/FindClusters functions from Seurat with slight modifications for these functions (cosine distance metric, k.param = 10; resolution = 1.0). In principle, this approach identifies communities of cells whose overall mutations are similar (using a shared nearest neighbors approach), and subclones are identified using a modularity optimization. Finally, we performed tree reconstruction using neighbor-joining on the cosine distance between the average heteroplasm of cells per clone using hierarchical clustering.

CLL scTA CaTAC analyses. For each mtscATAC-seq library, cells were processed using CellRanger-ATAC with default settings, including the ‘--force-cells 6000’ flag. Each library was further filtered such that cells had minimum 50% fragments in accessibility peaks, 1,000 unique nuclear fragments and 20x mtDNA coverage. Somatic mtDNA mutations were identified using mgatk with the default parameters for the CD19+ cells profiled with mtscATAC-seq (Extended Data Fig. 4b). Putative subclones were identified using the mutations for Patient 1 (n = 18) and Patient 2 (n = 24) separately using the FindNeighbors/FindClusters functions from Seurat with a cosine distance function on the square root of the heteroplasm matrix. We used parameters for Patient 1 (k.param = 20; resolution = 0.2; Fig. 4c) and Patient 2 (k.param = 30; resolution = 1.0; Extended Data Fig. 4c) to effectively identify subclones. For visualization of cell by mutation heteroplasmy, sets of cells from Patient 1 (2,246/6,524; Fig. 4c) and Patient 2 (3,057/5,874; Extended Data Fig. 4c) were visualized as the remaining cells had largely 0% heteroplasmy at called mutations.

To determine copy-number alterations (Fig. 4e), we first constructed mapping 10-Mb bins genome-wide using a step size of 2 Mb. Next, we overlapped the fragments tsv file from the 10x CellRanger-ATAC output with these bins to compute a bin by cell matrix for both of the CLL samples as well as a healthy control PBMC sample. Next, we computed a per-cell, per-bin z-score of the number of fragments after normalizing each cell to a consistent sequencing depth. The chromosome 12 z-score (Fig. 4e) represents the per-cell mean of the z-scores from mapping to this chromosome. To interpret the z-scores, we computed the percentage of unique autosomal reads mapping to chromosome 12 for the CLL (8.1%) and healthy PBMC samples (mean 5.3%). The 53% increase in reads mapping to chromosome 12 in CLL cells supported trisomy (rather than a higher copy number) as the chromosomal aberration.

To identify chromatin accessibility peaks associated with mtDNA mutation-derived subclones, we performed a chi squared association tests. After binarizing the chromatin accessibility count per-peak, per-cell, a contingency table of dimension n × 2 was assembled, where n is the number of subclones per tumor. The resulting chi-squared statistics were associated with P values using n − 1 degrees of freedom, and correction for multiple testing was performed using the Benjamini–Hochberg procedure. To further visualize a null association statistic, we permuted the subclonal annotations per peak to visualize a null distribution of the chi-squared statistics (see gray in Fig. 4f and Extended Data Fig. 4g). The TIAM1 and ZNF257 loci were selected based on strong association (both in the top ten most-associated peaks) and proximity to annotated transcription start sites.
To identify non-B cells with mtDNA mutations, we first embedded a healthy PBMC 5,000-cell sample from the 10X Genomics public dataset using latent Dirichlet allocation (LDA) as previously described. Using the LSI components and the projection capability of UMAP, we projected CD19+ cells from both CLL donors onto the reduced dimension space (Fig. 4i,k). Cells were annotated as positive for specific mtDNA mutations if the heteroplasmacy exceeded 20% (corresponding to at least four unique molecules containing the alternate allele; Fig. 4j,i).

**Colorectal cancer scATAC-seq analyses.** The colorectal cancer sequencing library was processed with CellRanger-ATAC with default settings. Each cell was further filtered such that it had a minimum 40% of fragments overlapping a compendium of DNase hypersensitivity peaks (integrated in the CellRanger-ATAC workflow), 1,000 unique nuclear fragments and 10x mtDNA coverage. Somatic mtDNA mutations were identified using default parameters with CellRanger 3.1.0. Mitochondrial genotyping was conducted using scRNA-seq libraries, including Variable, Diversity, and 120% of the 1,000 required peaks. The reported clones, where one mtDNA variant often corresponded to one cluster (Extended Data Fig. 6h,i), were determined using default thresholds from mgatk for each sample separately.

To define cell states for the CD34+ HSPC dataset, clustering and embedding were performed as follows. Samples were clustered at a concentration of 2 ng µl−1 and volume 100 µl (total 100 ng input) into fresh matrix tubes allowing positive barcode tracking throughout the process. Samples were sheared to yield ~180 bp size distribution. Kapa Hyperprep kits were used to construct libraries in a process optimized for somatic samples, involving end repair, adapter ligation with forked adaptors containing unique molecular indexes and addition of P5 and P7 sample barcodes via PCR. After solid phase reversible immobilization (SPRI) purification, libraries were quantified with Pico Green. Libraries were normalized and equimolar pooling was performed to prepare multiplexed sets for hybridization. Sample pools were then split and hybridized in up to eight separate reaction wells to accommodate volumes. Automated capture was performed, followed by PCR of the enriched DNA and SPRI purification. Multiplex pools were quantified with Pico Green and DNA fragment size was estimated using Bioanalyzer electrophoresis. Final libraries were quantitated by quantitative PCR and loaded across the appropriate number of Illumina flow cell lanes to achieve the target coverage. Completed exomes contained 28% (CD3+ HSPCs) or 60% (PBMCs) fragments in accessibility peaks, 1,000 unique nuclear fragments and 20x mtDNA coverage. Cutoffs were determined from the examination of the density of each parameter. Somatic mtDNA mutations were identified using default thresholds from mgatk for each sample separately.

In vivo hematopoiesis analyses. The four mtsATAC-seq libraries (2x PBMCs; 2x CD34+ HSPCs) were processed using CellRanger-ATAC-count with the ‘--force-cells 6000’ flag. Each library was further filtered such that cells had minimum 25% (CD3+ HSPCs) or 60% (PBMCs) fragments in accessibility peaks, 1,000 unique nuclear fragments and 20x mtDNA coverage. Cutoffs were determined from the examination of the density of each parameter. Somatic mtDNA mutations were identified using default thresholds from mgatk for each sample separately.

To identify the 923 additional rare variants (Extended Data Fig. 6h,i), we identified mutations that met the following criteria: (1) ‘confidently detected,’ with at least two unique fragments aligning to both the top and bottom strands (minimum two total reads) in at least two or three cells, and (2) present at an additional rare variants overlapped with the 429 clonal variants identified using the standard mgatk processing.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** Data associated with this work is available at GEO accession GSE142745.

**Code availability.** Software and documentation for mitochondrial variant calling via mgatk are available at http://github.com/caleblade/immune_cell_signatures. Custom code to reproduce all analyses and figures is available at https://github.com/caleblade/mtsATACpaper_reproducibility.

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**Author contributions**

C.A.L. and L.S.L. conceived and designed the project with guidance from A.R. and V.G.S. C.A.L. developed the software and led data analysis. L.S.L. and C.M. developed the mtscATAC-seq experimental protocol. L.S.L. led, designed and performed experiments with assistance from C.M., W.L. and E.C. S.H.G. performed experiments with assistance from C.M., W.L. and E.C. H.G. performed experiments with assistance from C.M., W.L. and E.C. S.H.G. processed CLL patient samples with assistance from C.M., W.L. and E.C. S.H.G. processed samples with assistance from C.M., W.L. and E.C. T.Z. performed the in situ genotyping experiments. C.J.W. and G.G. analyzed data. K.P. processed the colorectal cancer specimen. D.R. and G.G. aided with exome sequencing. F.C., J.D.B., M.J.A., G.M.B., N.H., C.J.W., A.R. and V.G.S. each supervised various aspects of this work. A.R. and V.G.S. provided overall project oversight and acquired funding. C.A.L., L.S.L., A.R. and V.G.S. wrote the manuscript with input from all authors.

**Competing interests**

The Broad Institute has filed for a patent related to lineage tracing using mtDNA mutations where C.A.L., L.S.L., C.M., J.D.B., A.R. and V.G.S. are named inventors. J.D.B. holds patents related to ATAC-seq. N.H. and C.J.W. are co-founders, equity holders and SAB members of Neon Therapeutics, Inc., and receive research funding from Pharmacyclics. G.G. receives research funding from IBM and Pharmacyclics. A.R. is a founder and equity holder of Celsius Therapeutics, an equity holder in Immunitas Therapeutics and an SAB member of Syros Pharmaceuticals, Neogene Therapeutics, Asimov and ThermoFisher Scientific.

**Additional information**

Extended data is available for this paper at https://doi.org/10.1038/s41587-020-0645-6. Supplementary information is available for this paper at https://doi.org/10.1038/s41587-020-0645-6. Correspondence and requests for materials should be addressed to C.A.L., L.S.L., A.R. or V.G.S. Reprints and permissions information is available at www.nature.com/reprints.
Extended Data Fig. 1 | Additional validation of biotechnological and computational basis for single-cell mtDNA genotyping. (a) Comparison of chromatin library complexity (estimated number of unique fragments) across screened lysis conditions as shown in Fig. 1. (b) The same variable lysis conditions showing the TSS rate per cell. (c) BioAnalyzer traces of mtscATAC-seq library fragment size distribution for regular conditions and mtDNA-enriched conditions. (d) Heteroplasmy heatmap of single cells (columns) for 43 private homoplasmic mutations (rows) in the TF1 or GM11906 cell lines with (left) and without (right) FA treatment. Color bar, heteroplasmy (% allele frequency). (e) Comparison of mtDNA fragment complexity and chromatin complexity between the original regular 10x scATAC protocol and modified lysis conditions with and without formaldehyde (FA) treatment. (f) Heteroplasmy of sum of single-cell ATAC-seq libraries with variable FA treatment. (g) Schematic, method, and results of improving mtDNA genome coverage via hard-masking the reference genome (Methods). (h) Comparison of % reads mapping to mtDNA and (i) chromatin complexity with (red) and without (blue) the hard masking. (j) Comparison of average coverage of mtscATAC-seq (y axis) and GC content (x axis) at each 50 bp bin (dot) in the mtDNA genome. (k) Accessible chromatin landscapes aggregated from single cells near the ETV2 locus for both cell lines as assayed via regular scATAC-seq and mtscATAC-seq. For boxplots in (a, b, e, h, i), each condition represents the top 1,000 cells (based on chromatin complexity) for one experiment. Boxplots: center line, median; box limits, first and third quartiles; whiskers, 1.5x interquartile range.
Extended Data Fig. 2 | Further inferences in analysis of the GM11906 (MERRF) lymphoblastoid cell line. (a) Alternative field of view for GM11906 in situ genotyping imaging experiment. Representative image selected from one of seven fields of view for one experiment. Pseudo bulk accessibility track plots are shown for the (b) ETV2 and (c) CD19 loci. Pseudo-bulk groups represent 0-10% (low), 10-60% (mid), and 60-100% (high) m.8344 A > G heteroplasmy. (d) Spearman correlation of heteroplasmy against the ChIP-seq deviation scores computed via chromVAR. Each bar is a single transcription factor with selected factors highlighted. (e) Depiction of MEF2C deviation scores from chromVAR for m.8344 A > G heteroplasmy bins, corresponding to 0-10% (Low), 10-60% (Mid), and 60-100% (High). Boxplots: center line, median; box limits, first and third quartiles; whiskers, 1.5x interquartile range. Bins contain single cells collected over one experiment where bins correspond to high (>60%; n = 273), intermediate (10-60%; n = 228), and low (<10%; n = 313) heteroplasmy (see Fig. 2c).
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Supporting information for somatic mtDNA mutation calling via mgatk. (a) Venn diagrams depicting comparisons of heteroplasmic mutations identified by mgatk, samtools/ bctools, and (b) FreeBayes. (c) Comparison of heteroplasmy estimated from reads aligned to either strand. The top row are three variants called specifically by mgatk; 3549 C > A was identified only by FreeBayes. 7399 C > G and 546 A > C were called specifically by bctools. (d) Identification of 67 and (e) 36 heteroplasmic variants from previously published Smart-seq2 hematopoietic colony data. Blue variants represent known RNA-editing events. (f) Comparison of population heteroplasmy values for variants replicated by mgatk from a previous supervised approach. Boxplots: center line, median; box limits, first and third quartiles; whiskers, 1.5x interquartile range. Statistical test: two-sided Mann-Whitney U Test. (g) Concordance between discerning cells sharing a clonal origin based on colony-specific mtDNA mutations and their unsupervised identification using indicated algorithms (mgatk, bctools, FreeBayes) and previously described supervised approach6. Receiver operating characteristic (ROC) using the per cell pair mtDNA similarity metric to identify pairs of cells sharing a clonal origin based on sets of mtDNA variants. The number of variants in each set is also depicted. (h) Area under the ROC (AUROC) is denoted for each donor group and indicated variant caller as depicted in (g). Each bar represents the statistic from one evaluation per donor per tool. (i) Estimated sensitivity (y axis, left), positive predictive value (y axis, right), and (j) estimated % dropout (y axis) for mtcATAC-seq at different simulated levels of heteroplasmy (x axis; Methods). Vertical line: 5% heteroplasmy for a subclonal mutation. The in-graph numbers indicate the values from the curve at a single-cell heteroplasmy of 5% with colors corresponding to different per-cell coverage values in the simulation.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Supporting information for clonal and functional heterogeneity in malignant populations revealed by mtDNA mutations. (a) Flow cytometry gating strategy of CLL patient derived PBMCs showing expansion of CD19+ cells. (b) Identification of high-confidence variants for Patient 1 (top) and Patient 2 (bottom). The number of variants n is indicated. (c) Inference of subclonal structure from somatic mtDNA mutations for patient 2. Cells (columns) are clustered based on mitochondrial genotypes (rows). Colors at the top of the heatmap represent clusters or putative subclones. Color bar, heteroplasmy (% allele frequency). (d) Dot plots showing the mitochondrial genome coverage (log10; y-axis) for the top 500 cells per technology for four indicated scRNA-seq technologies. (e) The mean per-position mitochondrial genome coverage for the same 500 cells as in (d). (f) Volcano plot showing differential gene expression analysis from major and minor clonotypes defined by BCR sequence. Immunoglobulin (IG) genes are shown in purple; all other genes with an FDR < 0.05 are shown in blue. (g) Results for per-peak chi-squared association with sub-clonal group. Each dot is a peak rank-sorted by the chi-squared statistic. (h) Heteroplasmy from the sum of single-cells in the CD19+ and CD19- mscATAC-seq experiments for indicated mutations and patients. (i) Histograms showing the distribution of heteroplasmy across the profiled population of cells for six selected variants, four from Patient 1 (left) and two from Patient 2 (right). The number of variants in the top heteroplasmy bin (>90%) are shown in red. (j) Allele frequency from the sum of single cells from the 5’ CD19+ and CD19- scRNA-seq libraries for two indicated variants - chr4:109,084,804A > C (‘LEF1’) and chr19:36,394,730G > A (‘HSCT’). (k) Corroboration of T cells based on gene expression signatures and carrying indicated somatic nuclear and mtDNA mutations (Patient 2). (l) Gene activity scores supporting cell type annotations in Fig. 4n. Arrows: cluster enriched for respective gene score. (m) All mtDNA mutations (rows) by cells (columns) observed in the CRC tumor. Columns are colored by defined chromatin cell state defined as in Fig. 4n. (n,o) Chromatin-derived UMAP with cells marked by select mtDNA mutations enriched in (n) epithelial and (o) immune cells. Color bar: heteroplasmy (% allele frequency).
Extended Data Fig. 5 | Supporting information for clonal lineage tracing across accessible chromatin landscapes and time in an in vitro model of hematopoiesis. (a) Depiction of single-cell UMAP embedding showing the original distribution of cells for each library/time point, (b) relative cell density, (c) Louvain cluster, and (d) mitochondrial DNA coverage per single cell. (e) Overlap of variants called for each of the two datasets. (f) Comparison of log2 fold change in heteroplasmy from day 14 to day 8 for 19 overlapping variants. The p-value shown is for the beta 1 coefficient of the depicted linear regression model. (g) Proportion of cells (%) at day 8 of the 500 cell (x axis) and 800 cell (y axis) input culture carrying shared mtDNA variants as derived from panel (e) suggests limited clonal overlap. (h) Known pathogenic mtDNA mutations detected from a healthy donor. Each dot is a cell separated by the sampled library. All cells with a heteroplasmy of at least 2% are shown. (i) Depiction of unsupervised clustering of groups of cells based on shared somatic mtDNA mutations (y-axis) with corresponding individual mtDNA mutations (x-axis) associated with each cluster for the 500 cell input and (j) 800 cell input culture. Color bar, heteroplasmy (% allele frequency). (k) Fraction of cells (y-axis) carrying number of somatic mtDNA variants (x-axis) above indicated thresholds (≥1%, ≥5%, ≥10% heteroplasmy; red, black, and blue lines, respectively) for indicated cultures.
Extended Data Fig. 6 | Support information for cellular population dynamics in native hematopoiesis in vivo resolved by mtDNA based tracing.
(a) Assignment probabilities (%, colorbar) of scRNA-seq data derived transfer labels (rows) across mtscATAC-seq derived Louvian data clusters (columns) as identified in Fig. 6d. (b) Distribution of percent mitochondrial reads derived from mtscATAC-seq data (y axis) across PBMC populations (x axis). (c) Percent mitochondrial counts (y axis) in FACS sorted populations (x axis) from bulk RNA-seq data. (d) Identification of high confidence variants from CD34+ HSPC and PBMC cell populations. Number of variants passing both thresholds (dotted lines) is indicated. A Venn diagram depicts the overlap of shared mutations. (e) Percent duplicates of sequenced mtDNA fragments, mean mtDNA coverage and percent mitochondrial reads for CD34+ HSPC and PBMC cell populations as derived from mtscATAC-seq data. Boxplots: center line, median; box limits, first and third quartiles; whiskers, 1.5x interquartile range. (f) Distribution of maximum level of heteroplasmy of mgatk derived variants from (d) in individual cells. (g) Unsupervised clustering of groups of cells based on shared somatic mtDNA mutations (y-axis) with corresponding individual mtDNA mutations (x-axis) associated with each cluster/clone. (h) Fold-change (observed over expected) of identified rare mutations (y axis) in each class of mononucleotide and trinucleotide change from the CD34+ HSPC data. (i) Comparison of pseudobulk allele frequencies from mgatk identified variants (blue) and rare variants (green). Boxplots for (b,c,e): center line, median; box limits, first and third quartiles; whiskers, 1.5x interquartile range. Bounds are contained within the data range shown. Sample sizes exceed 100 single cells from one experiment.
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Life sciences study design

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| Sample size | No sample size calculations were performed a priori. Analyses involved hundreds if not thousands of cells per comparison, providing a robust sample size in-line with similar high-throughput scRNA-seq comparisons and technologies. |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | Common scATAC-seq quality control cutoffs were applied to identify true cells. Barcodes failing to meet analysis-specific thresholds [outlined in Supplementary Table 3] were excluded. While the metrics used for exclusion [FRIP; # fragments] were determined ahead of time, the exact thresholds were determined empirically using the density of all single cells to determine appropriate, dataset-specific thresholds. |
| Replication | We replicated the enriched mtDNA content of our mtscATAC-seq assay [developed on cell lines] across a range of primary-cell samples over approximately 10 independent experiments. All attempts were successful. Verification of a successful library varied depending on the cell input materials but generally consisted of a pseudobulk TSS score > 5 and an average mtDNA content exceeding 15%. |
| Randomization | There were no variables or interventions to randomize in this study. |
| Blinding | Blinding is not relevant to our study, as our tools are not dependent on blinding. Investigators could not be blinded during data collection or analysis as there was no intervention. Further, analyses were performed in an exploratory manner where blinding is not possible. |

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| ☒ Palaeontology                  | ☒ Flow cytometry |
| ☒ Animals and other organisms    | ☒ MRI-based neuroimaging |
| ☒ Human research participants    |         |
| ☒ Clinical data                  |         |

Antibodies

Antibodies used

FITC-conjugated CD19 antibody (HIB19, 302206, Biolegend) at 1:50 dilution

Validation

Each lot of this antibody is quality control tested by immunofluorescent staining with flow cytometric analysis from Biolegend.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

Cornell: GM11906 cell line, [https://www.coriell.org/0/Sections/Search/SMaple_Detail.aspx?Ref=GM11906; ATCC: TF1 cell line, [https://www.atcc.org/products/all/CRL-2003.aspx.](https://www.atcc.org/products/all/CRL-2003.aspx)

Authentication

The GM11906 cell line was authenticated via analysis of the m.8344 variant. The TF1 cell line was not authenticated.

Mycoplasma contamination

Cell lines are routinely tested for mycoplasma contamination. Results were consistently negative.

Commonly misidentified lines

(See [ITCC register](http://www.coriell.org/0/Sections/Search/SMaple_Detail.aspx?Ref=ITCC).)

No commonly misidentified lines were used as part of this study.