Cell-free, methylated DNA in blood samples reveals tissue-specific, cellular damage from radiation treatment

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Abstract

Radiation therapy is an effective cancer treatment although damage to healthy tissues is common. Here we establish sequencing-based, cell-type specific DNA methylation reference maps of human and mouse tissues to infer the origins of cell-free DNA fragments released from dying cells into the circulation. We find cell-type specific DNA blocks mostly hypomethylated and located within

genes intrinsic to cellular identity. In a mouse model, thoracic radiation-induced tissue damages were reflected by dose-dependent increases in lung endothelial, cardiomyocyte and hepatocyte methylated DNA in serum. The analysis of serum samples from breast cancer patients undergoing radiation treatment revealed distinct tissue-specific epithelial and endothelial responses to radiation across multiple organs. Strikingly, patients treated for right-sided breast cancers also showed increased hepatocyte and liver endothelial DNA in the circulation indicating the impact on liver tissues. Thus, cell-free methylated DNA in serum can uncover cell-type specific effects of radiation on healthy tissues and inform treatment.

Keywords: Apoptosis, Bisulfite capture-sequencing, Cell-free DNA (cfDNA), Cellular damage, DNA methylation, Liquid biopsy, Tissue-of-Origin, Radiation-induced toxicity

Introduction

Radiation therapy is an effective cancer treatment; however, surrounding normal tissues are also impacted leading to tissue damage and remodeling[1–4]. For breast cancer patients, the heart and lungs are the most common organs-at-risk for toxicity[5–7]. However, development of radiation-induced toxicities varies due to patient-specific factors and clinical symptoms may be acute or long-term, often appearing months or even years after treatment[6]. The mechanisms of radiation-induced normal tissue injury are still poorly characterized and there are few indicators of radiation-related damage. Here we address this unmet need for sensitive and tissue-specific detection of therapy-related adverse events.

Decoding the cellular origins of circulating cell-free DNA (cfDNA) from liquid biopsies is a promising approach for non-invasive monitoring of organ homeostasis, where rising levels of

cfDNA released from dying cells indicate increased tissue damage[8–13]. The majority of cfDNA fragments peak around 167 bp, corresponding to the length of DNA wrapped around a nucleosome (147 bp) plus a linker fragment (20 bp). This nucleosomal footprint in cfDNA reflects degradation by nucleases as a by-product of cell death and the tissue origins of the cfDNA fragments can be uncovered using highly cell-type specific DNA methylation patterns[8,14]. DNA methylation typically involves covalent addition of a methyl group to the 5-carbon of cytosine (5mc) with the human and mouse genomes containing 28 and 13 million CpG sites respectively[14,15]. Dynamic changes to the methylome during development and cellular differentiation lead to stable, cell-type specific patterns of DNA methylation that are conserved during DNA replication and thus provide the predominant mechanism for inherited cellular memory during cell growth[16–19].

Recent studies have demonstrated the feasibility of Tissue-Of-Origin (TOO) analysis using cell-free DNA methylation[20–24]. However, few of these studies have focused on tracking intervention-related changes over time, despite the capacity for serial sampling with liquid biopsies[25–27]. The short half-life of cfDNA (15 mins – 2 hours) is ideal for monitoring real-time changes in tissue homeostasis due to therapeutic interventions[27,28]. Also, few cfDNA analyses have taken advantage of CpG pattern analysis to increase sensitivity and specificity of cell type proportion estimates[22,23,29–31]. Since each cfDNA molecule has independent origins, pattern analysis of sequence reads allows for individual classification of each fragment as opposed to traditional methods that average the methylation status across a population of fragments aligned at single CpG sites[27,28]. Building on these advances, we present a fine-tuned approach for deconvolution of cfDNA patterns based on fragment-level CpG methylation blocks.

Here, we first report comprehensive, sequencing-based DNA methylation reference maps of healthy human and mouse cell-types and show the close relationship with cellular gene

expression. Then, we apply cell-type specific DNA methylation to trace the origins of cfDNA in serum samples. We find that hybridization capture sequencing of methylated cfDNA in serum samples reflects tissue damages in a mouse model of radiation injury. In addition, analyses of serial serum samples from breast cancer patients undergoing routine radiation treatment indicate distinct cellular damages in different organs.

Methods

Human serum sample collection

Breast cancer patients undergoing adjuvant radiation therapy were enrolled and provided signed informed consent in this IRB approved study at Medstar Georgetown University Hospital (IRB protocol # 2013-0049**)**. For serum isolation, peripheral blood (~8-12 ml) was collected in red-top venous puncture tubes and allowed to clot at room temperature for 30 minutes before centrifugation at 1500 x g for 20 min at 4ºC to separate the serum fraction. The serum was aliquoted in 0.5 mL fractions to avoid multiple freeze-thaw cycles and stored at −80ºC until use. Serial serum samples were collected from 15 breast cancer patients at Baseline (before radiation treatment), End-of-Treatment (EOT; 30 minutes after the last radiation treatment), and Recovery (one month after cessation of radiation treatment), thus allowing for a within-patient internal control and baseline. A schematic of the time series for sample collection can be found in **Figure 6**. Patients received either three-dimensional conformal RT (3D-CRT) or a combination of proton beam therapy (PBT) and 3D-CRT. Patient characteristics and treatment details including radiation dosimetry are summarized in **Supplemental Table 8.**

Mouse serum and tissue collection

C57Bl6 mice (n=18) were irradiated to the upper thorax at different doses (sham control, 3Gy, 8Gy) for 3 consecutive treatments. Serum and tissues were collected 24 hours after the last radiation dose. For serum isolation, blood was collected via cardiac puncture (~1mL) and allowed to clot at room temperature for 30 minutes before centrifugation at 1500 x g for 20 min at 4◦C to separate the serum fraction. Heart, lung, and liver tissues were harvested and sectioned to be both flash frozen and formalin fixed for subsequent analysis. Animal studies were approved by the Georgetown University IACUC (protocol #2017-0029).

Cell isolation

Reference methylomes were generated for mouse immune cell types and human endothelial cell types to augment publicly available datasets. Peripheral blood and bone marrow were isolated and spleens from healthy C57Bl6 mice were dissociated to single cells and FACS sorted using cell-type specific antibodies. Buffy coat (n=4), bone marrow (n=3), CD19+ B cell (n=1), CD4 T cell (n=1), CD8 T cell (n=1) and Gr1+ Neutrophil (n=1) methylomes were generated using the following antibodies: FITC anti-mouse CD45 (cat#103107), Alexa Fluor 647 anti-mouse CD3 (cat#100209), Brilliant Violet 711 anti-mouse CD4 (cat#100549), Brilliant Violet 421 anti-mouse CD8a (cat#100737), PE anti-mouse CD19 (cat#152407), PE/Cy7 anti-mouse Ly-6G/Ly-6C (Gr-1) (cat#108415) (all BioLegend 1:20). Cryopreserved passage 1 human liver sinusoidal endothelial cells (LSEC) were purchased from ScienCell research laboratories (SKU#5000). Purity was determined by immunofluorescence with antibodies specific to vWF/Factor VIII and CD31(PECAM). Cryopreserved passage 2 human coronary artery (HCAEC SKU#C-14022), cardiac microvascular (HCMEC SKU#C-14029), and pulmonary artery endothelial cells (HPAEC SKU#C-14024) were isolated from single donor healthy human tissues purchased from

PromoCell. All endothelial cell populations were CD31 positive and Dil-Ac-LDL uptake positive. Paired RNA-seq data was generated from the same cell-populations used for DNA methylation profiling to validate the identity of purchased cell populations through analysis of cell type expression markers.

RNA isolation, RNA-sequencing, and RT-qPCR analysis

RNA was isolated from tissues or sorted cells using the RNeasy Kit (Qiagen) following homogenization step using the MagNA Lyser (Roche) according to the manufacturer's protocol and quantified by Qubit RNA BR assay (Thermo Fisher Scientific). Total RNA samples were validated using an Agilent RNA 6000 nano assay on the 2100 Bioanalyzer TapeStation (Agilent Technologies). The resulting RNA Integrity number (RIN) of samples selected for downstream qPCR or RNAseq analysis was at least 7. Reverse transcription was done using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. Real-time quantitative RT–PCR was performed with iQ SYBR Green Supermix (Bio-Rad). Primers used for RT–qPCR were purchased from Integrated DNA Technologies, and their sequences are described in **Supplemental Table 9**. Fold change was calculated as a percentage normalized to housekeeping gene human actin (ACTB) using the delta-Ct method. All RT–qPCR assays were done in triplicate. RNA-sequencing libraries were prepared using TruSeq Total RNA library Prep Kit (Illumina) at Novogene Corporation Inc., and 150bp paired-end sequencing was performed on an Illumina HiSeq 4000 with a depth of 50 million reads per sample. A reference index was generated using GTF annotation from GENCODEv28. Raw FASTQ files were aligned and assembled to GRCh38 and GRCh37 with HISAT2 / Stringtie (V 2.1.0)[32]. The differential expression was analyzed in R with packages EdgeR (V 3.32.1) and Rsubread (V1.6.3)[33,34]. Derived counts per million and p-

value were used to create a rank ordered list, which was then used for subsequent integrative analysis. Expression levels at known cell type markers from single cell expression databases were used to validate the identity of isolated cell-type populations for methylome analysis[35].

Isolation of circulating cell-free DNA (cfDNA)

Circulating cell-free DNA was extracted from 3 to 4 mL human serum and 0.5 mL mouse serum, using the QIAamp Circulating Nucleic Acid kit (Qiagen) according to the manufacturer's instructions. Cell-free DNA was quantified via Qubit fluorometer using the dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific). Fragment size distribution of isolated cfDNA was validated by 2100 Bioanalyzer TapeStation (Agilent Technologies). Additional purification using Beckman Coulter beads was implemented to remove high-molecular weight DNA reflective of cell-lysis and leukocyte contamination as previously described[36]. Size distribution of cell-free DNA fragments were re-verified using 2100 Bioanalyzer TapeStation analysis following purification.

Isolation and fragmentation of genomic DNA

Genomic DNA from tissues was extracted with DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions and quantified via Qubit fluorometer dsDNA BR Assay Kit (Thermo Fisher Scientific). Genomic DNA was fragmented via sonification using a Covaris M220 instrument to the recommended 150-200 base pairs before library preparation. Lambda phage DNA (Promega Corporation) was also fragmented and included as a spike-in to all DNA samples at 0.5%w/w, serving as an internal unmethylated control. Bisulfite conversion efficiency was calculated through assessing the number of unconverted C's on unmethylated lambda phage DNA.

The SeqCap Epi capture pool contains probes to capture the lambda genomic region from base 4500 to 6500. The conversion rate was calculated as 1 – (sum(C_count)/sum(CT_count)) across the lambda genomic region captured.

Bisulfite capture-sequencing library preparation

Bisulfite capture-sequencing libraries were generated from either cfDNA or reference DNA inputs according to the same protocol. As a first step, WGBS libraries were generated using the Pico Methyl-Seq Library Prep Kit (Zymo Research) with the following modifications. Bisulfite-conversion was carried out using the EZ DNA Methylation Gold kit (Zymo Research) instead of the EZ DNA Methylation-Lightning Kit. For mouse samples, cfDNA from two mice in the same group was pooled as input to library preparation. An additional 2 PCR cycles were added to the recommended cycle number based on total input cfDNA amounts. WGBS libraries were eluted in 15uL 10mM Tris-HCl buffer, pH 8. Library quality control was performed with an Agilent 2100 Bioanalyzer and quantity determined via KAPA Library Quantification Kit (KAPA Biosystems).

Cell-free WGBS libraries were pooled to meet the required 1ug DNA input necessary for targeted enrichment. However, no more than four WGBS libraries were pooled in a single hybridization reaction and the 1ug input DNA was divided evenly between the libraries to be multiplexed. Hybridization capture was carried out according to the SeqCap Epi Enrichment System protocol (Roche NimbleGen, Inc.) using SeqCap Epi CpGiant probe pools for human samples and SeqCap Epi Developer probes for mouse samples with xGen Universal Blocker-TS Mix (Integrated DNA Technologies, USA) as the blocking reagent. Washing and recovering of the captured library, as well as PCR amplification and final purification, were carried out as recommended by the manufacturer. The capture library products were assessed by Agilent

Bioanalyzer DNA 1000 assays (Agilent Technologies, Inc.). Bisulfite capture-sequencing libraries with inclusion of 15-20% spike-in PhiX Control v3 library (Illumina) were clustered on an Illumina Novaseq 6000 S4 flow cell followed by 150-bp paired-end sequencing.

Bisulfite sequencing data alignment and preprocessing

Paired-end FASTQ files were trimmed using TrimGalore (V 0.6.6) with parameters "--paired -q 20 --clip_R1 10 --clip_R2 10 --three_prime_clip_R1 10 --three_prime_clip_R2 10" (https://github.com/FelixKrueger/TrimGalore). Trimmed paired-end FASTQ reads were mapped to the human genome (GRCh37/hg19 build) using Bismark (V 0.22.3)[37] with parameters "--non-directional", then converted to BAM files using Samtools (V 1.12)[38]. BAM files were sorted and indexed using Samtools (V1.12). Reads were stripped from non-CpG nucleotides and converted to BETA and PAT files using *wgbstools* (V 0.1.0), a tool suite for working with WGBS data while preserving read-specific intrinsic dependencies (https://github.com/nloyfer/wgbs_tools)[22,39].

Reference DNA methylation data from healthy tissues and cells

Controlled access to reference WGBS data from normal human tissues and cell types was requested from public consortia participating in the International Human Epigenome Consortium (IHEC)[40] and upon approval downloaded from the European Genome-Phenome Archive (EGA), Japanese Genotype-phenotype Archive (JGA), database of Genotypes and Phenotypes (dbGAP), and ENCODE portal data repositories (**Supplemental Table 1**)[41–43]. Reference mouse WGBS data from normal tissues and cells were also downloaded from selected GEO and SRA datasets (**Supplemental Table 2**)[44–57]. Downloaded FASTQs were processed and realigned in a similar manner as the locally generated bisulfite-sequencing libraries described above. However,

parameters were adjusted to account for each respective WGBS library type at both trimming and alignment steps as previously described in the Bismark User Guide (http://felixkrueger.github.io/Bismark/Docs/). WBGS libraries were deduplicated using deduplicate_bismark (V 0.22.3) Special consideration of bisulfite conversion efficiency was given to samples prepared by the µWGBS protocol and reads with a bisulfite conversion rate below 90% or with fewer than three cytosines outside a CpG context were removed[58].

Segmentation and clustering analysis

We segmented the genome into blocks of homogenous methylation as previously described in Loyfer et al 2022 using *wgbstools* (with parameters segment --max_bp 5000)[22,39]. In brief, a multi-channel Dynamic Programming segmentation algorithm was used to divide the genome into continuous genomic regions (blocks) showing homogenous methylation levels across multiple CpGs, for each sample. We applied the segmentation algorithm to 278 human reference WGBS methylomes and retained 351,395 blocks covered by our hybridization capture panel used in the analysis of cfDNA in human serum (captures 80Mb, ~20% of CpGs). Likewise, segmentation of 103 mouse WGBS datasets from healthy cell types and tissues identified 1,344,889 blocks covered by the mouse hybridization capture panel (captures 210 Mb, ~75% of CpGs). The hierarchical relationship between reference tissue and cell type WGBS datasets was visualized through creation of a tree dendrogram. The top 30,000 most variant methylation blocks containing at least three CpG sites and coverage across 90% of samples were selected. We computed the average methylation for each block and sample using wgbstools (--beta_to_table). Trees were assembled using the unweighted pair-group method with arithmetic mean (UPGMA)[59], using scipy (V 1.7.1)[60] and L1 distance, and then visualized in R with the ggtree package (V 2.4.1)[61]. The

similarity between samples was assessed by the degree of variation in distance between samples of the same cell-type (average 23,056) compared to samples between different cell-types (average 273,018). Dimensional reduction was also performed on the selected blocks using the UMAP package (V 0.2.8.2.0). Default UMAP parameters were used (15 neighbors, 2 components, Euclidean metric, and a minimum distance of 0.1).

Identification of cell-type specific methylation blocks

We reduced the original 278 human WGBS samples to a final set of 104 samples to identify differentially methylated cell-type specific blocks. We excluded samples from bulk tissues and those that did not have sufficient coverage (missing values in >50% of methylation blocks). Outlier replicates, or those clustering with fibroblasts or stromal cell types were excluded, due to possible contamination. Only immune cell methylomes that were reprocessed from raw sequencing data to PAT files were used to identify DMBs. We organized the final 104 human reference samples into groupings of 20 cell types (**Supplemental Table 1**). Similarly, the starting 103 mouse WGBS samples were reduced to a final set of 44 samples that were organized into a final grouping of 9 cell types and tissues (**Supplemental Table 2**). Tissue and cell-type specific methylation blocks were identified from the final reduced reference WGBS data using custom scripts (**Supplemental code**). We performed a one-vs-all comparison to identify differentially methylated blocks unique for each group. This was done separately for human and mouse. We first identified blocks covering a minimum of three CpG sites, with length less than 2Kb and at least 10 observations. Then, we calculated the average methylation per block/sample, as the ratio of methylated CpG observations across all sequenced reads from that block. Differential blocks were sorted by the margin of separation, termed "delta beta", defined as the minimal difference between the average

methylation in any sample from the target group vs all other samples. We selected blocks with a delta-beta ≥ 0.4 for human and ≥ 0.35 for mouse. This resulted in a variable number of cell-type specific blocks available for each tissue and cell type. Each DNA fragment was characterized as U (mostly unmethylated), M (mostly methylated) or X (mixed) based on the fraction of methylated CpG sites as previously described[22]. We used thresholds of ≤33% methylated CpGs for U reads and ≥66% methylated CpGs for M. We then calculated a methylation score for each identified cell-type specific block based on the proportion of U/X/M reads among all reads. The U proportion was used to define hypomethylated blocks and the M proportion was used to define hypermethylated blocks. Selected human and mouse blocks for cell types of interest can be found in **Supplemental Tables 3 and 4**. Heatmaps were generated using the pretty heatmap function in the RStudio Package for the R bioconductor (RStudioTeam, 2015).

Likelihood-based probabilistic model for fragment-level deconvolution

The cell type origins of cfDNA were determined using a probabilistic fragment-level deconvolution algorithm. Using this model, the likelihood of each cfDNA molecule was calculated using a 4th-order Markov Model, by which each CpG site directly depends on up to four adjacent previous sites, within each fragment. We estimated these parameters for each differential block at every tissue and cell-type, and then used Bayes' theorem to infer the posterior probability of cell-of-origin for each fragment, based on its complete methylation pattern. The model was trained on reference bisulfite-sequencing data from normal cells and tissues of known identity to learn the distribution of each marker in the target tissue/cell population of interest compared to background. Then the model was applied to predict the origins of each cfDNA molecule. The joint probability of each cfDNA molecule (methylation patterns and cellular origin) is calculated based

on the likelihood of the methylation pattern (using the parameters for that cell type) times the prior probability that a read is originating from the target cell type. A prior of 0.05 was used for all solid organ cell-types and 0.85 for the combined immune cell-type group. Finally, each fragment is assigned to the cell type of origin with the maximal posterior probability ("hard" assignment). The proportion of molecules (fragments) assigned to the tissue of interest across all cell-type specific markers was then averaged and used to determine the relative abundance of cell-free DNA derived from that tissue origins in each respective sample. We then adjusted the resulting proportions from all cell types to have a sum of 1 through imposing a normalization constraint. Predicted cell-type proportions were converted to genome equivalents and reported as Geq/mL through multiplying the concentration of cfDNA (ng/mL) by the mass of the human haploid genome 3.3×10^{-12} grams or the mouse haploid genome equivalent to 3.0×10^{-12} grams.

In-silico simulations and WGBS deconvolution

In silico mix-in simulations were performed using *wgbstools* (V 0.1.0)[39] to validate the fragment-level deconvolution algorithm at identified cell-type specific blocks included in the radiation-specific methylation atlas (**Supplemental Figures 3 and 5**). Reference data with greater than three replicates per cell type were split into independent training and testing sets, leaving at least one replicate out for testing. Since the mouse cardiomyocyte and lung endothelial reference WGBS data had only three replicates, sequenced fragments were merged across replicates for this cell type and then randomly split into training (80%) and testing (20%) sets (using wgbstools merge and then wgbstools pat_splitter). For each cell type profiled, we mixed known proportions of target fragments into a background of leukocyte fragments using wgbstools mix_pat. The leukocyte fragments were obtained from n=4 buffy coat samples in mouse and n=5 buffy coat samples in

human. We performed three replicates for each admixture ratio assessed (0.1%, 0.5%, 1%, 2%, 5%, 10%, 15%), which were analyzed as described above, and present the average predicted proportion and standard deviation across all replicates. Model accuracy was assessed through correct classification of the actual percent target mixed and relative degree of incremental change with increasing amount of target reads admixed was used to assess accuracy in estimating proportional changes across groups (mouse) and timepoints from serial samples (human). The cell-type specific blocks included in the radiation-specific methylation atlas were constructed using training set fragments only.

Analysis of serial serum samples

Longitudinal analysis was performed on serial serum samples collected from breast cancer patients. Cell-type proportions of cfDNA at the end of treatment (EOT) and at Recovery were evaluated relative to baseline levels before the start of therapy (Baseline). Fold change (FC) from baseline was used to represent the change at EOT and Recovery relative to Baseline within the same individual. A correlation analysis was performed using Pearson's Correlation Coefficient. Statistical analyses for group comparisons and correlations were performed using Prism (GraphPad Software, Inc., United States) and R (V 4.1.3).

Functional annotation and pathway analysis

Cell-type specific methylation blocks were provided as input for analysis in HOMER (V4.11.1) (http://homer.ucsd.edu/homer/)[62]. Each block was associated with its closest nearby gene and provided a genomic annotation using the annotatePeaks.pl function, with "-size given -CpG" parameters. By default, TSS (transcription start site) was defined from -1 kb to +100 bp, TTS

(transcription termination site) was defined from -100 bp to +1 kb, and CpG islands were defined as a genomic segment with GC content ≥50%, genomic length >200 bp and the ratio of observed/expected CpG number >0.6. Prediction of known and de-novo transcription factor binding motifs were also assessed by HOMER using the findMotifsGenome.pl function. The top 5 motifs based on p-value were selected from each analysis. Pathway analysis of identified tissue and cell-type specific methylation blocks was performed using Ingenuity Pathway Analysis (IPA)[63] (Qiagen) and Genomic Regions Enrichment of Annotations Tool (GREAT)[64]. GeneSetCluster was used to cluster identified gene-set pathways based on shared genes[65]. Canonical pathways/functional annotations were grouped into clusters by calculating the similarity of pathways/annotations using the relative risk (RR) of each pathway appearing based on the genes enriched within the pathway. RR scores were clustered into groups using kmeans. Over-representation analysis was implemented in the WebgestaltR (ORAperGeneSet) plugin to interpret and functionally label identified gene-set clusters[66]. Integration of methylome and transcriptome data generated from tissue-specific endothelial cells was performed using an expanded set of cell-type specific blocks (--bg.quant 0.2) compared to the more restricted set of blocks used for deconvolution analysis in the circulation (--bg.quant 0.1). The extended endothelial-specific methylation blocks can be found in **Supplemental Table 10**.

Genome Browser visualization

Endothelial methylomes and paired transcriptomes were uploaded as custom tracks for visualization on the UCSC genome browser[67]. Methylomes were converted to bigwig format using the wgbstools beta2bw. Enrichment for chromatin marks was assessed through analysis of published H3K27ac and H3K4me3 ChIP-seq data[68]. GTEx single nucleus RNAseq data was

acquired from the GTEx v9 Portal (gtexportal.org)[69] and analyzed using R (V 4.1.3). Counts per ten thousand reads (CP10K) of NOS3 were log-transformed and averaged for each specific cell type. Color represents the general cell type and intensity of color represents the number of cells expressing NOS3.

Results

DNA methylation is highly cell-type specific and reflects cell lineage specification

We obtained access to reference human and mouse WGBS datasets from publicly available databases and identified cell-type specific differential DNA methylation patterns, preferentially from primary cells isolated from healthy human and mouse tissues. Additionally, we generated cell-type specific methylomes for mouse immune cell types (CD19+ B cells, Gr1+ Neutrophils, CD4+ T cells, and CD8+ T cells) and human tissue-specific endothelial cell types (coronary artery, pulmonary artery, cardiac microvascular and liver sinusoidal endothelial cells). Due to limited cell-type specific data available for mouse, we included reference data from mouse bulk tissues if none was available from purified cell types within those tissues. This resulted in curation of methylation data from 10 different cell types and 18 tissues for mouse and over 30 distinct cell types for human (**Supplemental Tables 1 and 2**).

To better understand the epigenomic landscape of these healthy human and mouse cell types in tissues, we characterized the methylomes by first segmenting the data into homogenously methylated blocks where DNA methylation levels at adjacent CpG sites are highly correlated across different cell types[22]. Curated human WGBS datasets from healthy cell types were segmented to identify 351,395 blocks covered by our hybridization capture panel used in the analysis of cfDNA in human serum (captures 80Mb, ~20% of CpGs). Likewise, segmentation of

mouse WGBS datasets from healthy cell types and tissues identified 1,344,889 blocks covered by the mouse hybridization capture panel (captures 210 Mb, ~75% of CpGs). The human blocks had a median length of 326 bp (interquartile range, IQR = 890 bp) and 8 CpGs (IQR = 14 CpGs). Similarly, the mouse blocks had a median length of 770 bp (IQR =1,252 bp) and 7 CpGs (IQR = 7). Unsupervised hierarchical clustering analysis of the top 30,000 most variable methylation blocks amongst human cell types shows the relationship between samples and is presented as a dendrogram and UMAP projection in **Figure 1a and 1b**. The respective analysis of mouse cell types is depicted in **Figure 1c** and **Supplemental Figure 1a.** The tight relationship of methylomes of the same cell type observed from the cluster analysis reinforces the concept that methylation status is conserved at regions critical to cell identity. The variation in distance between all samples was ~12x larger than the variation in distance between samples from the same cell-type. This stability allows methylated DNA to serve as a robust biomarker of cell types across diverse populations. For the most part, cells composing distinct lineages remain closely related, including immune, epithelial, muscle, neuron, endothelial, and stromal cell types. Examples are tissue-specific endothelial and tissue-resident immune cells that cluster with endothelial or immune cells respectively, independent of the germ layer origin of their tissues of residence. Surprisingly, we observed a large epigenetic distance between immune cells of hematopoietic origin and solid organ cells from other lineages (**Figure 1a** and **1b**). This is important for the tissue-of-origin analysis of cell-free DNA in the circulation, distinguishing the solid organ origin from the hematopoietic origin. Quite unexpectedly, we also found a large number of epigenetic signatures capable of distinguishing amongst immune cells, with cell types of lymphoid and myeloid lineages forming distinct clusters. Within the immune cell cohort, we observed a clear separation of terminally differentiated cells compared to precursors, with naïve B and T cells clustering separately from

their more mature central and effector memory counterparts (**Figure 1b**). Collectively, these findings support that DNA methylation is highly cell-type specific and reflects cell lineage specification.

Differential DNA methylation distinguishes amongst cell types in healthy human and mouse tissues

Based on the above unsupervised clustering analysis, we further refined our inclusion/exclusion criteria to select a final set of reference methylomes used to identify differentially methylated cell-type specific blocks. We excluded WGBS samples from bulk tissues and samples with low coverage. This resulted in a reduction of the original 278 human WGBS data sets to a final set of 104 samples that were organized into a grouping of 20 cell types. Similarly, the starting 103 mouse WGBS samples were reduced to a final set of 44 samples that were organized into a final grouping of 9 cell types and tissues. Subsets of some related cell types were considered together to form the final groups (i.e., monocytes grouped together with macrophages and colon grouped together with small intestine). This final combination of groups was found to best represent the cell-specific epigenetic variation as a whole without overlap, using these publicly available data.

We identified cell-type specific differentially methylated blocks (DMBs) that contained a minimum of 3 CpG sites. The co-methylation status of neighboring CpG sites in these blocks were able to distinguish amongst all cell types included in the final groups. Overall, we identified 4,497 human and 7,344 mouse DMBs with a methylation difference of 0.35 for mouse and 0.4 for human. A complete summary of human and mouse cell-type specific methylation blocks is in **Supplemental Table 5**. Intriguingly, a variable number of blocks were identified for each cell-type using the same thresholds. This is likely due to the depth of coverage, purity, and degree of

separation from other tissues and cell types included in the atlas. Similar to others, we found enhanced separation of reference datasets using methylomes of purified cell types as opposed to more heterogenous mixtures from bulk tissues[24]. This is evident from the low number of DMBs identified from mouse bulk tissues as compared to mouse purified cell types (average of 310 DMBs/tissue versus 1,488 DMBs/cell type). To visualize cell-type specific DMBs, we formalized a simple methylation score that applies to both hypomethylated and hypermethylated DMBs. The score calculates the number of fully unmethylated read-pairs divided by total coverage for hypomethylated blocks (and vice versa for hypermethylated blocks). The heatmaps in **Figure 2** depicts up to 100 blocks with the highest methylation score for each cell-type group.

Differential DNA methylation is closely linked to the regulation of cell-type specific functions

We next sought to understand the role of cell-type specific methylation in shaping cellular identity and function. For this we identified genes adjacent to cell-type specific methylation blocks and performed pathway analysis of annotated genes using both Ingenuity Pathway Analysis (IPA) and GREAT[63,64]. Significantly enriched pathways were grouped into clusters based on the number of overlapping genes using GeneSetCluster, with higher similarity attributed to pathways with a larger number of shared genes[65]. Over-representation analysis (ORA) was implemented in WebGestalt to identify the cluster labels[66]. (**Figure 2c and Supplemental Figure 2**). Gene-set pathways largely clustered within independent cell-type groups, reinforcing that the identified DMBs locate adjacent to unique genes that are integral to cell-type specific functions. Important biological differences were also observed in the gene sets identified based on specific processes unique to the cell types profiled. For example, the biological function of genes associated with immune cell-type specific methylation reflects processes of leukocyte cell-cell adhesion, immune

response-regulating signaling, and hematopoietic system development (**Figure 2c**). In contrast, fatty acid metabolic process, lipid metabolism, and acute phase response signaling were identified for hepatocytes. Significantly enriched biological pathways and functions for genes associated with differential methylation in each cell type examined are provided in **Supplemental Table 12.**

Cell-type specific DNA blocks are mostly hypomethylated and enriched at intragenic regions containing developmental TF binding motifs

The majority of human and mouse cell-type specific blocks identified here were hypomethylated, consistent with other studies[14,17]. In human samples we found 86% of cell-type specific DMBs hypomethylated and only 14% hypermethylated. Strikingly, in the mouse samples, 98% of cell-type specific DMBs were hypomethylated and only 2% were hypermethylated. The schematic in **Figure 3a** depicts the location of identified human cell-type specific hypo- and hyper-methylated blocks. Interestingly, regardless of directionality the majority of cell-type specific blocks were located within intragenic regions. To assess if the genomic locations of cell-type specific blocks are distinct, we compared the locations to the captured blocks that do not vary amongst cell types (**Figure 3b, 3c and Supplemental Table 11**). We found that for both human and mouse, there was a significant enrichment of cell-type specific blocks within intragenic regions relative to other captured regions (Fisher's exact test, $p<0.05$). There was also a significant relationship between directionality and intragenic distribution, with a significantly larger proportion of cell-type specific blocks being hypermethylated in exons and hypomethylated in introns (Chi-square, df=3, $p<0.05$). The similar distribution of cell-type specific methylation blocks in human and mouse suggests a conserved biological function of these genomic regions across species.

To further explore what common purpose these identified regions may have in human and mouse development, we performed motif analysis using HOMER to see if there were commonly

enriched transcription factor binding sites (TFBS)[62]. MADS motifs bound by MEF2 transcription factors were significantly enriched in both human and mouse cell-type specific hypomethylated blocks (**Figure 3d - left)**. The MEF2 transcription factors are established developmental regulators with roles in the differentiation of many cell types from distinct lineages. In contrast, Homeobox motifs bound by several different HOX TFs were enriched in the human cell-type specific hypermethylated blocks (**Figure 3d - right**). Specifically, HOXB13 was the top TF associated with binding at sites within the human hypermethylated DMBs. Recently, HOXB13 has been found to control cell state through binding to super-enhancer regions, suggesting a novel regulatory function for cell-type specific hypermethylation[70]. In addition to the common TFBS enriched by all cell-type specific blocks, endothelial-specific TFs were found to be enriched in the endothelial-cell hypomethylated blocks, including EWS, ERG, Fli1, ETV2/4, and SOX6 (see **Figure 4e**). As a whole, this data indicate functions of these cell-type specific blocks that represent cell-specific biology still underexplored.

Methylation profiling of tissue-specific endothelial cell types reveals epigenetic heterogeneity associated with differential gene expression

Radiation-induced endothelial damage is thought to be a leading cause for development of late-onset complications from cardiovascular disease[71–73]. We thus generated tissue-specific endothelial methylomes and paired transcriptomes in order to profile damage from distinct populations of microvascular and large vessel endothelial cell types including coronary artery, pulmonary artery, cardiac microvascular, and liver sinusoidal endothelia. We also made use of publicly available liver sinusoidal endothelial[56] and umbilical vein endothelial methylomes[74] to complement our data (**Supplemental Table S1**). Previous studies support considering the heart

and lung as an integrated system in the development of radiation damage due to the shared cardiopulmonary circulation[4]. Therefore, we merged cardiac and pulmonary endothelial cell types to generate a joint cardiopulmonary endothelial signal and identified the specific methylation blocks for cardiopulmonary (CPEC, 132 blocks), liver sinusoidal endothelial (LSEC, 89 blocks), and distinct umbilical vein endothelial (HUVEC, 116 blocks) cell types. Pathway analysis of genes associated with these genomic regions confirmed endothelial cell identity based on genes involved in the regulation of vasculogenesis and angiogenesis (**Figure 4b**). In addition, unique pathways identified the tissue-specific epigenetic diversity of endothelial cell populations from different organs (**Figure 4d**). The DNA methylation status at several tissue-specific identified blocks was found to correspond with RNA expression levels of known endothelial-specific genes, confirming the identity of endothelial populations characterized (**Figure 4a and 4c, Supplemental Table 10**)[35,68,75–79]. For example, hypomethylation was associated with increased expression at several pan-endothelial genes, including NOTCH1, ACVRL1, FLT1, MMRN2, NOS3 and SOX7. Likewise, hypomethylation at cardiopulmonary- and liver-specific endothelial genes is associated with differential expression when comparing the two populations and reflects tissue-specific differences.

Development of a radiation-specific methylation atlas focusing on cell types from target organs-at-risk (OAR)

We focused our assessment of cfDNA origins in the circulation on cell types in organs at risk for radiation damage based on the target treatment area. Treatment planning for breast cancer patients receiving adjuvant radiation provides an estimate of the organ volume impacted and radiation dose level for organs-at-risk from radiation damage, including the heart and lungs (**Figure 5a**). In

addition to organs that are in close proximity with the target treatment area, the liver is another organ that may receive a substantial dose from radiation, especially in patients with right-sided breast cancer. Differential DNA methylation blocks identified from cell types composing these target organs-at-risk from radiation (lungs, heart, and liver) were selected for generation of a radiation-specific methylation atlas, separating these solid organ cell types of interest from the majority hematopoietic origins of cfDNA in the circulation (**Figure 5b and Supplemental Figure 1b**). The human and mouse DNA methylation blocks specific for these cell types can be found in **Supplemental Tables 3 and 4**. We focused on these same target organs in both human and mouse, resulting in a final curation of six groups for human (immune, lung epithelial, cardiopulmonary endothelial, cardiomyocyte, hepatocyte and liver sinusoidal endothelial) and four groups for mouse (immune, lung endothelial, cardiomyocyte and hepatocyte) based on the reference cell type data available.

Cell-free methylated DNA in blood identifies origins of radiation-induced cellular damage in tissues

Paired serum and tissue samples were collected from mice receiving thoracic radiation. In addition, serial serum samples were collected from breast cancer patients undergoing routine radiation therapy. We performed unbiased methylome-wide hybridization capture sequencing of DNA from human and mouse serum samples. Deconvolution analysis was used to trace the origins of cfDNA fragments (**Figure 6**). In comparison to previous studies using single CpG sites, the sequencing-based approach allows for fragment-level cfDNA analysis of CpG methylation patterns[80]. For this we modeled the co-methylation status of adjacent CpG sites on the same molecule implemented by a novel probabilistic deconvolution method. We applied the model using cell-type specific

blocks from the human and mouse radiation-specific methylation atlases described above. We validated the prediction accuracy of the fragment-level deconvolution through in silico mix-in simulations for each tissue and cell type of interest (**Supplemental Figures 3 and 5**).

Indicators of dose-dependent radiation damage in mice

To explore the relationship between radiation-induced damage in tissues to changing proportions of cell-free DNA origins in the circulation, we used mice to model exposure from different radiation doses. Mice received upper thorax radiation of 3 Gy or 8 Gy relative to sham control, forming three groups for comparison (**Figure 6**). Tissues and serum were harvested 24 hours after the last fraction of treatment and tissues in the path of the radiation beam (heart, lungs and liver) were analyzed. H&E stained sections showed a visible, dose-dependent impact of radiation on the tissues (**Figure 7a**). The changes were most apparent in tissue sections of the lungs showing noticeable alveolar collapse with increased radiation dose. Liver tissues showed increased fibrosis with increased radiation doses and only minor changes were apparent in cardiac tissues matching with its higher resilience to radiation. Tissue effects were also assessed through qPCR analysis of established indicators of radiation effects, including expression of CDKN1A (p21), that exhibited a dose-dependent increase in expression in response to radiation in all tissues ($p<0.05$, Kruskal-Wallis Test) (**Figure 7b, Supplemental Figure 4**)[81].

To assess whether these damages of heart, lung, and liver are reflected in altered cfDNA patterns in the circulation, we used capture sequencing of CpG containing cfDNA fragments (**Figure 6**). For the data analysis we applied the above-described mouse cardiomyocyte (n=2,917), lung endothelial (n=1,546), hepatocyte (n=616) and immune (n=148) cell-type specific methylation blocks derived from the radiation atlas for target organs-at-risk. We found a

significant dose-dependent increase in lung endothelial, cardiomyocyte and combined solid organ cfDNA across all three treatment groups that correlated with radiation-induced cell death in the corresponding tissues ($p<0.05$, Kruskal-Wallis Test) (**Figure 7c, d and Supplemental Figure 6e)**. The dose-dependent increase in hepatocyte cfDNA was not statistically significant and immune cell cfDNA showed no change between treatment groups ($p\geq0.05$, Kruskal-Wallis Test) (**Figure 7e and Supplemental Figure 6d**). We conclude that changes in cfDNA fragments in the circulation can reveal the source of radiation-induced cellular damage in tissues.

Radiation treatment of patients with breast cancer

To evaluate whether changes in cfDNA patterns could indicate damages to tissues in patients after radiation, we collected serum samples from randomly selected breast cancer patients at three timepoints during their standard-of-care radiation therapy after surgery **(Figure 6)**. A baseline sample was taken for each patient before onset of radiation-therapy and a second End-Of-Treatment (EOT) sample was taken 30 minutes after the last treatment. Finally, a recovery sample was taken one month after completion of radiation-therapy. Demographic information and clinical characteristics of patients enrolled in this study are in **Supplemental Table 8.**

Radiation-induced liver damage

While liver damage is not a common radiation-induced toxicity in breast cancer patients, a substantial dose may still be administered to the liver, especially with right-sided tumors (**Figure 5a**). We used the top hepatocyte (n=200) and liver sinusoidal endothelial (n=89) DNA methylation blocks to assess the sequence data for the presence of liver-derived cfDNA. Surprisingly, in patients receiving radiation treatment of right-sided breast cancer, an increase in hepatocyte plus liver sinusoidal endothelial methylated DNA in the circulation indicated significant radiation-

induced cellular damage in the liver ($p<0.05$, Wilcoxon matched-pairs signed rank test) (**Figure 8a-f**). Elevated levels of either hepatocyte and/or liver sinusoidal endothelial cfDNA were detected in seven of the eight breast cancer patients with right-sided tumors. In contrast, there was not significant increase in hepatocyte or liver sinusoidal endothelial cfDNA in patients with left-sided breast cancer ($p\geq0.05$, Wilcoxon matched-pairs signed rank test).

Radiation-induced heart and lung damage

Due to close proximity with the target treatment area, the heart and lungs are common organs-at-risk for breast cancer patients undergoing radiation therapy. To assess therapy-induced lung damage, we examined cfDNA from serum for the presence of lung epithelial DMBs (n=69). Interestingly, we did not observe a significant increase in lung epithelial cfDNA across all patients ($p\geq0.05$, Friedman Test) (**Figure 9a**). However, a few patients showed increased lung epithelial cfDNA indicating lung damage that correlated with increasing dose and volume of the lungs targeted (**Figure 9b**). Changes in lung epithelial cfDNA after radiation correlated with the volume of the ipsilateral lung receiving 20 Gy doses (Lung V20) (Pearson's $r = 0.67$, $p <0.05$) and the total body mean dose (Pearson's $r = 0.90$, $p <0.05$). In addition to lung injury, cardiovascular disease is one of the most serious complications from radiation exposure that is associated with increased morbidity and mortality[6]. Through deconvolution using cardiopulmonary endothelial (CPEC, n=132) and cardiomyocyte-specific (n=375) DMBs, we found increased CPEC and cardiomyocyte cfDNA in the serum samples indicating significant cardiovascular cell damage across all breast cancer patients ($p<0.05$, Friedman Test) (**Figure 9d and 9g**). Surprisingly, cardiomyocyte-specific methylated DNA in the circulation correlated with the maximum radiation dose to the heart (Pearson's $r = 0.63$, $p <0.05$), but not the mean dose to the heart (Pearson's $r = -0.09$, $p\geq0.05$)

(**Figure 9h**). This suggests that radiation-induced damage of cardiomyocytes requires a threshold dose indicating the relative resilience of this cell type compared to epithelial and endothelial cells from the heart and lungs.

Distinct endothelial and epithelial damages from radiation

We observed distinct epithelial and endothelial cell-type responses to radiation across the different tissue cfDNAs profiled. Different responses to radiation were observed when comparing hepatocyte to lung epithelial damages (**Fig 8a-c vs 9a-c)**, demonstrating the ability of methylated cfDNA to distinguish between tissue-specific epithelial cell types from serum samples. Likewise, analysis for tissue-specific endothelial populations revealed differences in cardiopulmonary microvascular and liver sinusoidal endothelial responses to radiation (**Fig 8d-f vs 9d-f**). In general, there was greater magnitude of damage to the combined endothelium compared to the epithelium across different organs. The endothelium forms a layer of cells lining blood as well as lymphatic vessels and turnover of this cell type may contribute to the high amplitude of signal detected from serum[24]. This could, however, also be a result of the different sensitivities of endothelial versus epithelial cell types to radiation-induced damage. There was a five-fold higher signal from cardiopulmonary endothelial cfDNA compared to lung epithelial cfDNA. Also, in comparison to epithelial- and endothelial- derived cfDNA, sustained injury and delayed recovery is indicated by elevated cardiomyocyte cfDNA (**Figure 9c, f, i)**. This may reflect important differences in cell turnover rates leading to differential processes of regeneration and repair in these cell types. Notably, one month after completion of radiation therapy, lung epithelial and cardiopulmonary endothelial damage signatures detected from cfDNA had returned to baseline levels whereas sustained higher cfDNA from cardiomyocytes indicates lingering tissue remodeling. Taken as a

whole, these findings demonstrate applicability of this approach to uncover distinct cellular damages in different tissues during the course of treatment by the analysis of blood samples.

Discussion

This study demonstrates the feasibility of tissue-of-origin analysis of cell-free methylated DNA to monitor tissue responses to radiation therapy. The assignment of DNA fragments extracted from serum samples from patients undergoing treatment as well as from experimental animals to specific cell types required in-depth analysis of tissue- and cell-type DNA methylation patterns. We expected to at least identify a set of biomarkers from this analysis and were positively surprised by the significant association of the cell-type specific DNA methylation blocks with cell-type specific gene expression, transcription factor binding motifs and signaling pathway regulation. Cell-type specific DNA methylation is the major mechanism of passing on cell-type specific features to the next generation of cells during mitogenesis and it was reassuring to us that these covalent modifications of DNA also are linked to the control of the machinery that are inherited during cell division and define the phenotype of a given cell. We were particularly intrigued by how well-conserved cell-type specific DNA methylation appears to be across different individuals suggesting broad applicability in the monitoring of tissue damage in diverse patient populations. It appears that disease- or ageing-related changes in DNA methylation occur outside the cell-type specific blocks and thus will exert their impact without altering the features defining a particular cell type.

To enhance the quality and sensitivity of the analyses we developed a radiation-specific methylation atlas containing cell-type specific methylation patterns from target organs-at-risk including the heart, lungs, and liver. Elevated cell-type specific DNA in blood samples as an

indicator of radiation damage may be useful to identify patients who are more sensitive and thus likely to develop severe or long-term adverse effects. The analysis indicated that cardiomyocyte-specific methylated DNA in the circulation correlated with the maximum radiation dose to the heart as did lung epithelial methylated DNA with the volume of the lung receiving a 20 Gy dose. Previous studies have identified cfDNA molecules with a pattern of six unmethylated CpG sites at the FAM101A locus to be a sensitive marker of cardiomyocyte damage that is elevated in the circulation of patients with myocardial infarction and sepsis[30,82]. Taking a more comprehensive approach, we combined signals across 375 genomic loci with cardiomyocyte-specific methylation patterns for increased sensitivity to detect cardiomyocyte damage from radiation. The potential of cardiomyocyte-specific methylated cfDNA to reveal a range of cardiac pathologies, from intervention-based changes to severe pathologies could be clinically useful to complement existing biomarkers of heart disease.

We also identify endothelial cfDNA as significant indicators of damage from radiation. We generated tissue-specific endothelial methylomes to profile differing sensitivities of cardiopulmonary microvascular and liver sinusoidal endothelial cells to radiation. Our analysis indicated greater damage to the endothelium compared to epithelium in different organs. In many tissues, the vascular endothelium is among the first cell types damaged[4,83]. However, the role of radiation-induced endothelial damage in mediating acute and chronic adverse effects has yet to be fully understood. Recent studies suggest that compromised endothelial cell function also impairs wound healing by depriving tissues of signals necessary for regeneration and contributing to accelerated aging of the hematopoietic and vascular systems[84–86]. Notably, one month after completion of radiation therapy we found that epithelial and cardiopulmonary endothelial damage signatures in the circulation had returned to baseline levels whereas increased turnover of

cardiomyocytes is indicated by sustained elevation of the respective cfDNAs likely due to ongoing tissue remodeling. Exploration of these tissue- and cell-type specific differences may shed light on previously unknown mechanisms of radiation-induced cardiovascular disease and identify patients-at-risk.

The liver is not a common organ-at-risk for radiation-induced toxicity in breast cancer, and we were surprised to find an increase in hepatocyte and liver sinusoidal endothelial cell methylated cfDNA in the circulation of patients receiving radiation treatment for right-sided breast cancer. This indicates radiation-induced adverse effects to the liver associated with tumor and treatment laterality. Previous studies have not found a meaningful relationship between breast cancer RT and overt liver fibrosis, even at doses higher than 40 Gy; although, increased hepatic exposure is expected in radiation treatment of patients with right-sided breast cancer[87]. In comparison to previous methods that were unable to detect radiation-induced liver damage, our finding demonstrates the sensitivity of circulating cell-free methylated DNA to identify previously unknown cell types and tissues impacted by radiation treatment.

Comparing the origins of elevated cfDNA after radiation showed similar changes in both human and mouse serum samples. In both human and mouse, there was a significant increase in lung endothelial and cardiomyocyte cfDNA after radiation. Likewise, there was an overall increase in cfDNA derived from any solid-organ tissue post-radiation in both breast cancer patients and mice receiving radiation (**Supplemental Figure 6**). The total concentration of cfDNA was elevated in some breast cancer patients at the end of treatment (EOT) as well, suggesting an overall increase in cfDNA shortly after radiation treatment in some patients (**Supplemental Table 7**). However, this trend was not consistent for all patients. Similarly, changes in mouse cfDNA concentration with increasing radiation dose were not significant ($p \geq 0.05$, Kruskal-Wallis Test)

(**Supplemental Table 6**) as also found by others[88]. However, despite similarities, it was difficult to fully align the human and mouse results given the scarcity of deep-sequencing WGBS data from purified murine cell types. Additional methylome data generated from purified cell types will improve analyses in the future and enable toxicity monitoring of other organs that were unexplored in the current analysis such as the skin and chest wall.

There are many factors to consider when assessing the risk for adverse events, including patient characteristics, pre-existing disease, and treatment-related risk factors, such as dose, tumor location and effects of combination therapy. Cell-free DNA analyses may be used to compare these different cohorts as well as distinct toxicity profiles associated with 3D-CRT to other more recent RT techniques, such as proton beam therapy (PBT) and intensity-modulated radiation therapy (IMRT). Likewise, exploration of regional variation in tissue-specific response to radiation may offer new opportunities to reduce normal tissue side effects[89]. The mouse model and a more detailed mouse reference DNA methylation atlas will provide a means for controlled exploration of some of the variables in preclinical studies. While many factors contribute to the pathophysiology of normal tissue radiation toxicity, the ability to identify patients at elevated risk and at-risk tissues for severe adverse effects may alter treatment planning and follow-up to improve the quality of life in these patients.

Conclusion

The therapeutic benefits of radiation therapy for cancer are established, although inadvertent damage to adjacent healthy tissues is common and results in acute and long-term toxicities. However, there is still much to be learned about the sensitivity of different cell types and susceptibility to radiation damage. Here, we used cell-free methylated DNA extracted from blood

samples and uncovered radiation treatment-induced damage to distinct cell types in different tissues in patients and in a mouse model. We conclude that the minimally invasive detection of cell-free methylated DNA from serum samples can indicate organ-specific damages and reveal previously unknown cell types impacted by radiation treatment.

Declarations

Availability of data and materials: All data and code will be made available upon publication of this manuscript.

Competing interests: Georgetown University filed a patent related to some of the approaches described in this manuscript. MEB and AW are named as inventors on this application and declare that as a potential conflict of interest. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions: MEB, AJK, SMR, EB, KU, and SR facilitated collection and processing of enrolled breast cancer patient samples. EB performed chart review and organized breast cancer patient data. MEB, AJK, and SMR performed bisulfite-capture sequencing library preparation. MEB and AJK purified cell-types and performed reference methylation data generation and validation. YL and HL facilitated radiation-treatment of mice. MEB and MOS facilitated serum and tissue collection from mice. AJK performed RT-qPCR and RNA-sequencing analysis. NS performed histological analysis of mouse tissues. MEB and NL performed bioinformatics analysis of WGBS data. MEB processed data for identification of mouse and human cell-type specific differential methylation. NL and TK created *wgbstools* for bioinformatics read-specific processing of WGBS data. NL and TK generated all scripts for WGBS data processing, segmentation, and identification of cell-type specific methylation blocks. NL, SSP, and TK developed the fragment-level deconvolution model for cfDNA analysis. SJ, APM, and ADM advised on statistical and

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Graphical Abstract

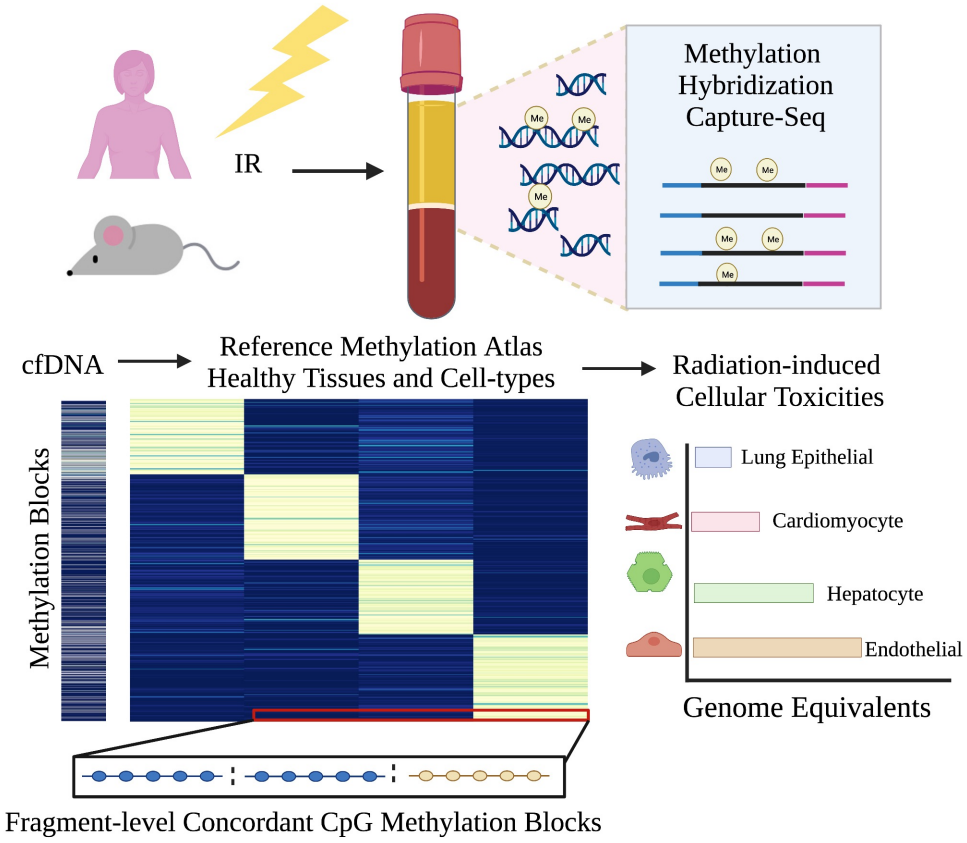


Figure 1.

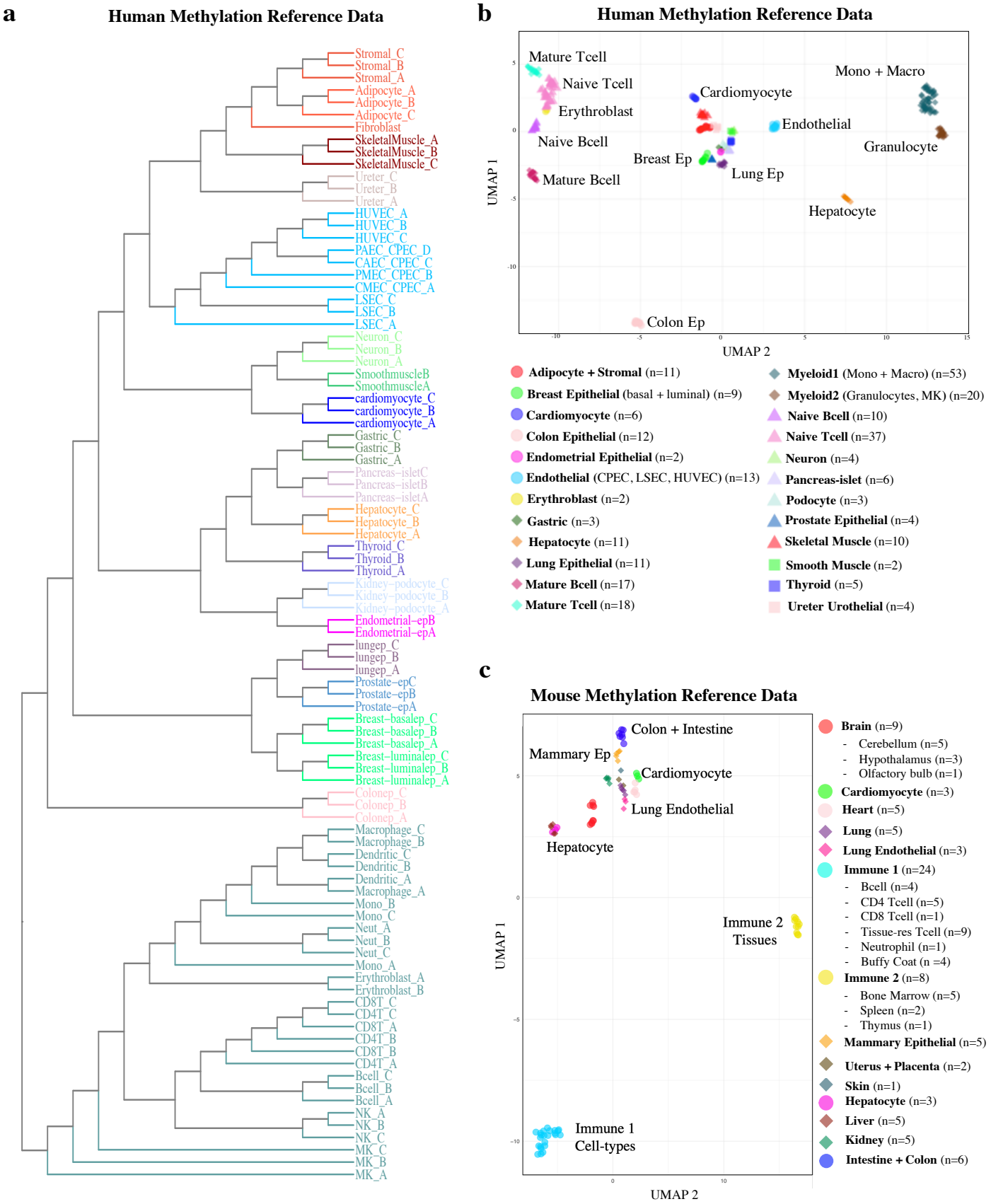

a
Human Methylation Reference Data
Stromal_C
Stromal_B
Stromal_A
Adipocyte_A
Adipocyte_B
Adipocyte_C
Fibroblast
SkeletalMuscle_A
SkeletalMuscle_B
SkeletalMuscle_C
Ureter_C
Ureter_B
Ureter_A
HUVEC_A
HUVEC_B
HUVEC_C
PAEC_CPEC_D
CAEC_CPEC_C
PMEC_CPEC_B
CMEC_CPEC_A
LSEC_C
LSEC_B
LSEC_A
Neuron_C
Neuron_B
Neuron_A
SmoothmuscleB
SmoothmuscleA
cardiomyocyte_C
cardiomyocyte_B
cardiomyocyte_A
Gastric_C
Gastric_B
Gastric_A
Pancreas–isletC
Pancreas–isletB
Pancreas–isletA
Hepatocyte_C
Hepatocyte_B
Hepatocyte_A
Thyroid_C
Thyroid_B
Thyroid_A
Kidney–podocyte_C
Kidney–podocyte_B
Kidney–podocyte_A
Endometrial–epB
Endometrial–epA
lungep_C
lungep_B
lungep_A
Prostate–epC
Prostate–epB
Prostate–epA
Breast–basalep_C
Breast–basalep_B
Breast–basalep_A
Breast–luminalep_C
Breast–luminalep_B
Breast–luminalep_A
Colonep_C
Colonep_B
Colonep_A
Macrophage_C
Macrophage_B
Dendritic_C
Dendritic_B
Dendritic_A
Macrophage_A
Mono_B
Mono_C
Neut_A
Neut_B
Neut_C
Mono_A
Erythroblast_A
Erythroblast_B
CD8T_C
CD4T_C
CD8T_A
CD4T_B
CD8T_B
CD4T_A
Bcell_C
Bcell_B
Bcell_A
NK_A
NK_B
NK_C
MK_C
MK_B
MK_A
b
Human Methylation Reference Data
Mature Tcell
Naive Tcell
Erythroblast
Naive Bcell
Mature Bcell
Cardiomyocyte
Breast Ep
Lung Ep
Endothelial
Mono + Macro
Granulocyte
Hepatocyte
Colon Ep
UMAP 1
UMAP 2
Adipocyte + Stromal (n=11)
Breast Epithelial (basal + luminal) (n=9)
Cardiomyocyte (n=6)
Colon Epithelial (n=12)
Endometrial Epithelial (n=2)
Endothelial (CPEC, LSEC, HUVEC) (n=13)
Erythroblast (n=2)
Gastric (n=3)
Hepatocyte (n=11)
Lung Epithelial (n=11)
Mature Bcell (n=17)
Mature Tcell (n=18)
Myeloid1 (Mono + Macro) (n=53)
Myeloid2 (Granulocytes, MK) (n=20)
Naive Bcell (n=10)
Naive Tcell (n=37)
Neuron (n=4)
Pancreas-islet (n=6)
Podocyte (n=3)
Prostate Epithelial (n=4)
Skeletal Muscle (n=10)
Smooth Muscle (n=2)
Thyroid (n=5)
Ureter Urothelial (n=4)
c
Mouse Methylation Reference Data
Colon + Intestine
Mammary Ep
Cardiomyocyte
Lung Endothelial
Hepatocyte
Immune 2
Tissues
Immune 1
Cell-types
UMAP 1
UMAP 2
Brain (n=9)
- Cerebellum (n=5)
- Hypothalamus (n=3)
- Olfactory bulb (n=1)
Cardiomyocyte (n=3)
Heart (n=5)
Lung (n=5)
Lung Endothelial (n=3)
Immune 1 (n=24)
- Bcell (n=4)
- CD4 Tcell (n=5)
- CD8 Tcell (n=1)
- Tissue-res Tcell (n=9)
- Neutrophil (n=1)
- Buffy Coat (n =4)
Immune 2 (n=8)
- Bone Marrow (n=5)
- Spleen (n=2)
- Thymus (n=1)
Mammary Epithelial (n=5)
Uterus + Placenta (n=2)
Skin (n=1)
Hepatocyte (n=3)
Liver (n=5)
Kidney (n=5)
Intestine + Colon (n=6)

Figure 1. Characterization of human and mouse cell-type specific reference methylation data. (a) Tree dendrogram depicting the relationship between human reference Whole Genome Bisulfite Sequencing (WGBS) datasets included in the analysis. Methylation status of the top 30,000 variable blocks was used as input for the unsupervised hierarchical clustering. Samples from cell types with greater than n=3 replicates were merged. (b) UMAP projection of human WGBS reference datasets, colored by tissue and cell-type. (c) UMAP projection of mouse WGBS reference datasets, colored by tissue and cell type. Abbreviations: CAEC = coronary artery endothelial cell, CMEC = cardiac microvascular endothelial cell, CPEC = joint cardio-pulmonary endothelial cell, HUVEV = human umbilical vein endothelial cell, LSEC = liver sinusoidal endothelial cell, MK = megakaryocyte, NK = natural killer cell, PAEC = pulmonary artery endothelial cell, PMEC = pulmonary microvascular endothelial cell.

Figure 2.

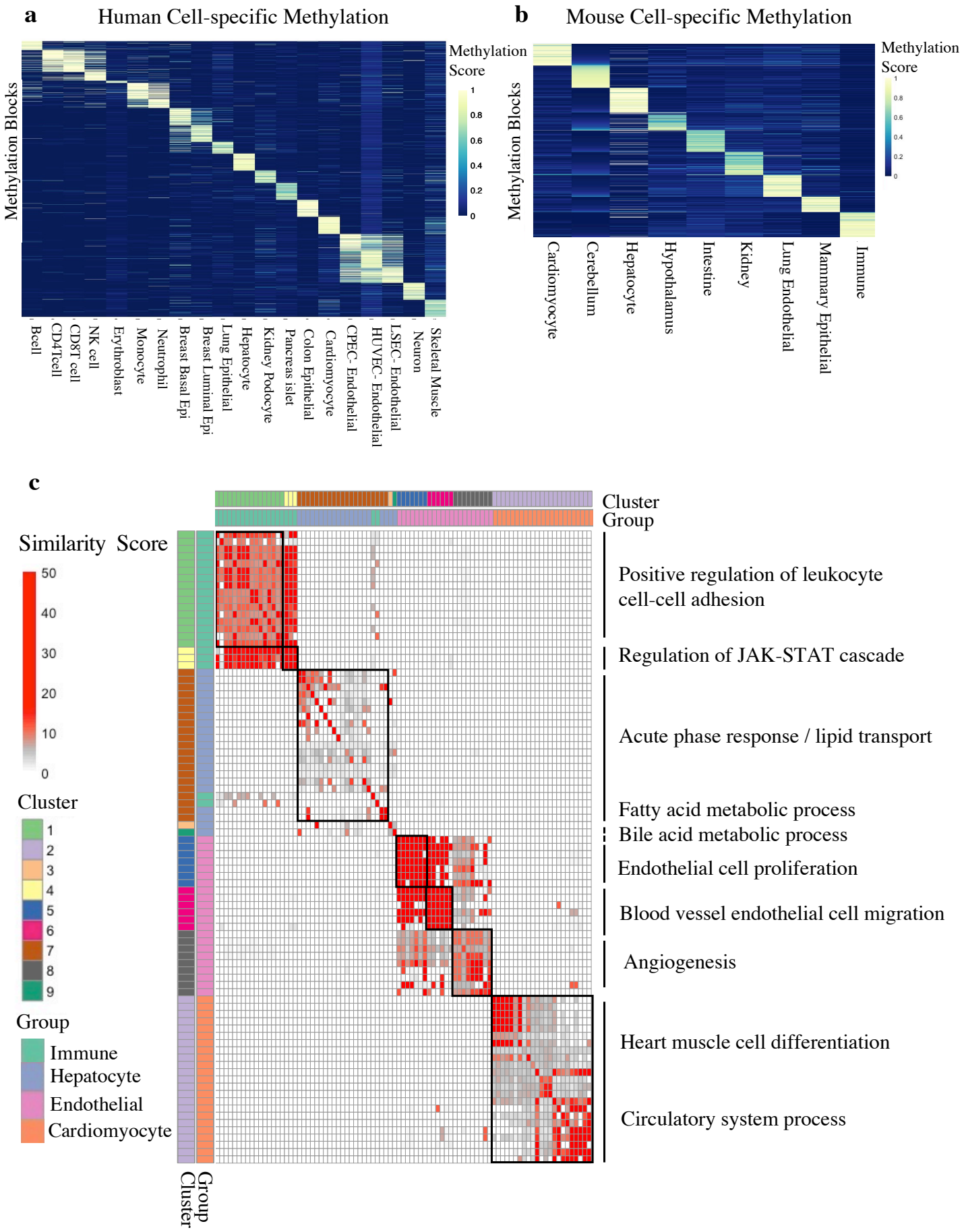
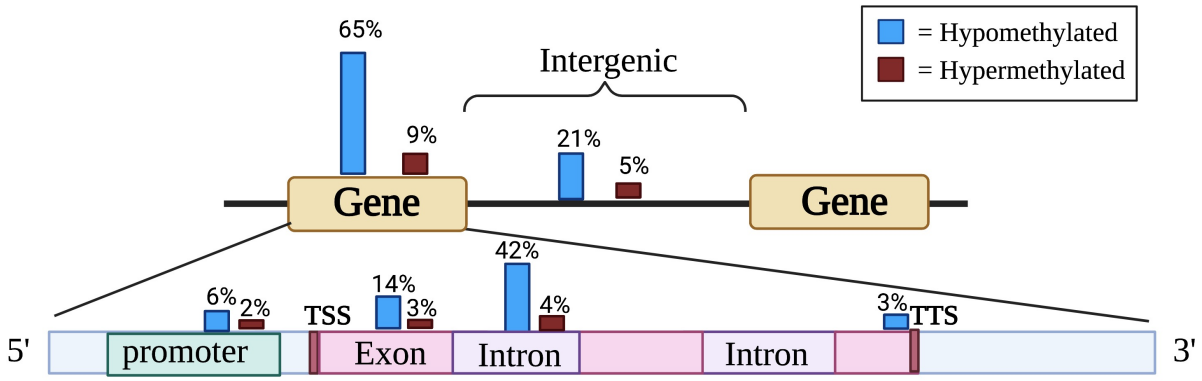

a
Human Cell-specific Methylation
Methylation Blocks
Methylation Score
1
0.8
0.6
0.4
0.2
0
Bcell
CD4Tcell
CD8T cell
NK cell
Erythroblast
Monocyte
Neutrophil
Breast Basal Epi
Breast Luminal Epi
Lung Epithelial
Hepatocyte
Kidney Podocyte
Pancreas islet
Colon Epithelial
Cardiomyocyte
CPEC- Endothelial
HUVEC- Endothelial
LSEC- Endothelial
Neuron
Skeletal Muscle
b
Mouse Cell-specific Methylation
Methylation Blocks
Methylation Score
Cardiomyocyte
Cerebellum
Hepatocyte
Hypothalamus
Intestine
Kidney
Lung Endothelial
Mammary Epithelial
Immune
c
Similarity Score
50
40
30
20
10
0
Cluster
1
2
3
4
5
6
7
8
9
Group
Immune
Hepatocyte
Endothelial
Cardiomyocyte
Cluster
Group
Group
Cluster
Positive regulation of leukocyte cell-cell adhesion
Regulation of JAK-STAT cascade
Acute phase response / lipid transport
Fatty acid metabolic process
Bile acid metabolic process
Endothelial cell proliferation
Blood vessel endothelial cell migration
Angiogenesis
Heart muscle cell differentiation
Circulatory system process

Figure 2. Identification and biological validation of cell-type specific DNA methylation blocks in human and mouse. (a-b) Heatmaps of differentially methylated cell-type specific blocks identified from reference WGBS data compiled from healthy cell types and tissues in human (a) and mouse (b). Each cell in the plot marks the methylation score of one genomic region (rows) at each of the 20 cell types in human and 9 in mouse (columns). Up to 100 blocks with the highest methylation score are shown per cell type. The methylation score represents the number of fully unmethylated or methylated read-pairs / total coverage for hypo- and hyper-methylated blocks, respectively. (c) Heatmap of distance scores between gene-set pathways identified from GeneSetCluster. Genes adjacent to human cell-type specific methylation blocks were identified using HOMER and pathway analysis was performed using both Ingenuity Pathway Analysis (IPA) and GREAT. Significantly enriched gene-set pathways ($p<0.05$) from differentially methylated blocks identified in immune, cardiomyocyte, hepatocyte, and lung epithelial cell types were analyzed using GeneSetCluster. Cluster analysis was performed to determine the distance between all identified gene-set pathways based on the degree of overlapping genes from each individual gene-set compared to all others. Over-representation analysis was implemented in the WebgestaltR (ORAperGeneSet) plugin to interpret and functionally label identified gene-set clusters. Abbreviations: CPEC = cardio-pulmonary endothelial cell, HUVEV = human umbilical vein endothelial cell, LSEC = liver sinusoidal endothelial cell, NK = natural killer cell.

Figure 3.

a



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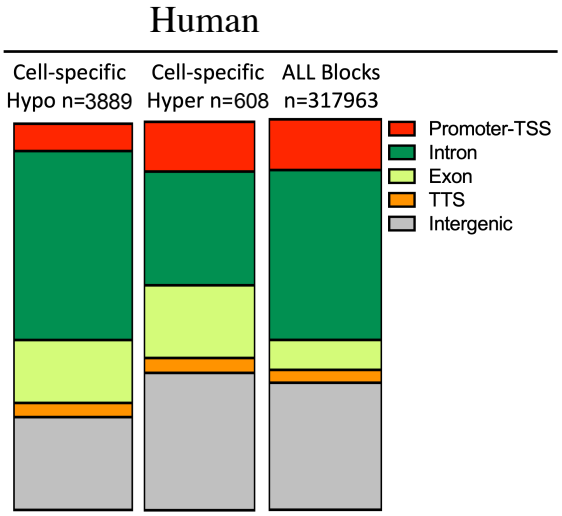
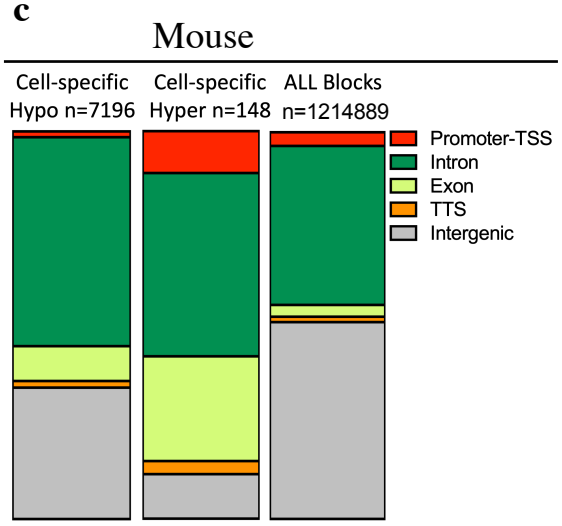


c



d

Cell-specific Hypomethylation

Human - 3889

Motif	Known	p-value
	Mef2c(MADS)	1e-20
	Mef2a(MADS)	1e-9
	Mef2b(MADS)	1e-17
	Foxf1(Forkhead)	1e-14
	Cdx2(Homeobox)	1e-13

Mouse -7196

Motif	Known	p-value
	Mef2c(MADS)	1e-151
	Mef2d(MADS)	1e-138
	Mef2b(MADS)	1e-125
	Mef2a(MADS)	1e-121
	Gata4(Zf)	1e-43

Cell-specific Hypermethylation

Human - 608

Motif	Known	p-value
	HOXB13(Homeobox)	1e-8
	Cdx2(Homeobox)	1e-6
	Hoxa11(Homeobox)	1e-5
	Hoxc9(Homeobox)	1e-5
	Oct4(Homeobox)	1e-5

Mouse -148

Motif	Known	p-value
	TEAD2(TEA)	1e-6
	NF1(CTF)	1e-5
	TEAD1(TEAD)	1e-4
	TEAD(TEA)	1e-4
	TEAD3(TEA)	1e-3

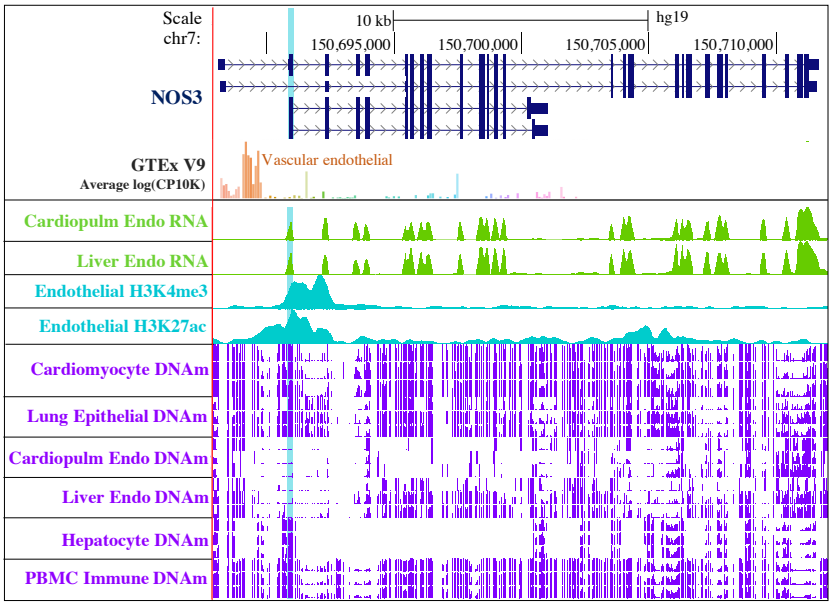
Figure 3. Cell-type specific DNA methylation is mostly hypomethylated and enriched at intragenic regions and developmental transcription factor (TF) binding motifs. (a) Schematic diagram depicting location of human cell-type specific hypo- and hyper-methylated blocks. Genomic annotations of cell-type specific methylation blocks were determined by analysis using HOMER. (b-c) Distribution of human (left) and mouse (right) cell-type specific methylation blocks relative to genomic regions used in the hybridization capture probes. Captured blocks with less than 5% variance across cell types represent blocks without cell-type specificity and were used as background. (d) Top 5 TF binding sites enriched among identified cell-type specific hypo- and hyper-methylated blocks in human (top) and mouse (bottom), using HOMER motif analysis. As above, captured blocks with less than 5% variance across cell types were used as background.

Figure 4.

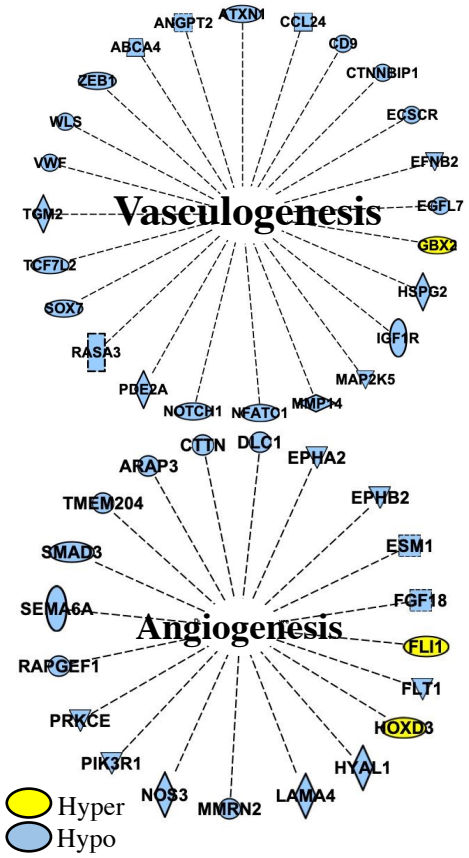
a

Endothelial marker, NOS3 intragenic

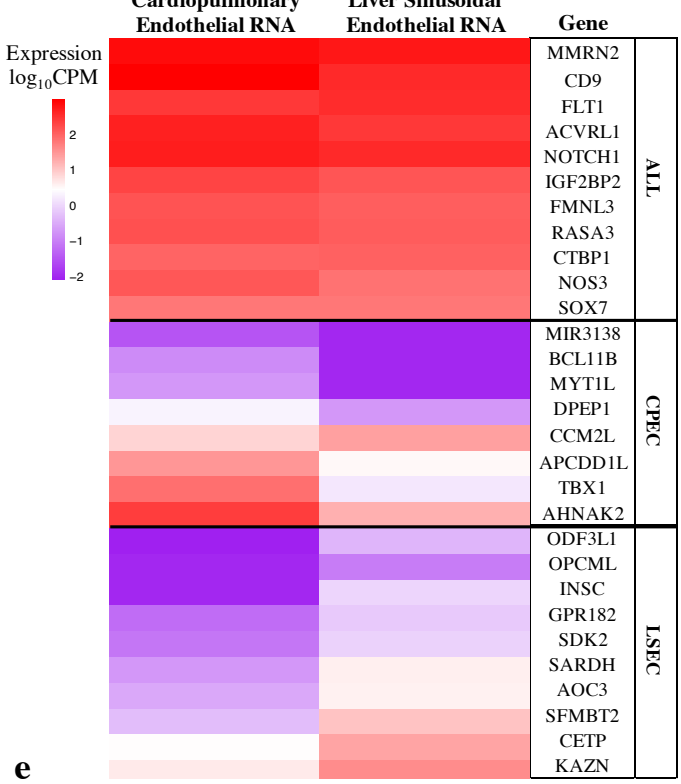
Chr7:150690881-150691038 (157bp, 7 CpGs)



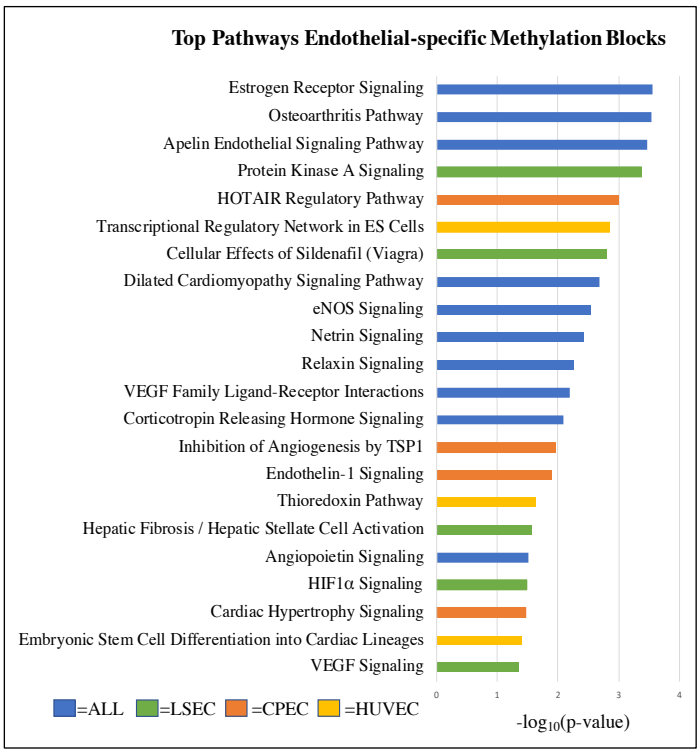
b



c



d



e

Motif	*de novo*	p-value	Motif	Known	p-value
	EWS:ERG(ETS)	1.00E-31		ERG(ETS)	1.00E-22
	AP-1(bZIP)	1.00E-22		EWS:ERG(ETS)	1.00E-20
	Sox6	1.00E-16		Etv2(ETS)	1.00E-17
	ETV4	1.00E-12		Fos(bZIP)	1.00E-15
	PB0181.1(SPDEF)	1.00E-12		Fli1(ETS)	1.00E-15

Figure 4. Methylation profiling of tissue-specific endothelial cell types reveals epigenetic heterogeneity associated with differential gene expression and biological functions. (a) Example of the NOS3 locus specifically unmethylated in endothelial cells. This endothelial-specific, differentially methylated block (DMB; highlighted in light blue) is 157bp long (7 CpGs), and is located within the NOS3 gene, an endothelial-specific gene (upregulated in paired RNA-sequencing data as well as in vascular endothelial cells, GTEx inset). The alignment from the UCSC genome browser (top) provides the genomic locus organization and is aligned with the average methylation (purple tracks) across cardiomyocyte, lung epithelial, liver sinusoidal endothelial (LSEC), cardiopulmonary endothelial (CPEC), hepatocyte, and immune (PBMC) samples (n=3 / cell-type group). Results from RNA-sequencing generated from paired cell-types are depicted (green tracks) as well as peak intensity from H3K27ac and H3K4me3 published ChIP-seq data generated in endothelial cells (blue tracks). (b) Significant functions of genes adjacent to endothelial-specific methylation blocks (all $p<0.05$). Blue color indicates nearby hypomethylated regulatory blocks. Yellow color indicates nearby hypermethylated regulatory blocks. (c) Expression levels of genes adjacent to tissue-specific endothelial methylation blocks. Expression data were generated from paired RNA-sequencing of the same cardiopulmonary endothelial (CPEC) and liver sinusoidal endothelial (LSEC) cell populations used to generate methylation reference data. Pan-endothelial genes upregulated in both populations (ALL) are identified as common endothelial-specific methylation blocks to both LSEC and CPEC tissue-specific endothelial populations. (d) Pathways supporting the biological significance of endothelial-specific methylation blocks (all $p<0.05$). Unique pathways for distinct tissue-specific endothelial cells are highlighted in distinct colors. (e) Top 5 transcription factor binding sites enriched among endothelial-specific hypomethylated blocks, using HOMER de novo and known motif analysis. The background for the HOMER analysis was composed of 3,574 non-endothelial cell-type specific hypomethylated blocks. Abbreviations: CPEC = cardio-pulmonary endothelial cell, HUVEV = human umbilical vein endothelial cell, LSEC = liver sinusoidal endothelial cell.

Figure 5.

a
i
ii
iii
iv
H
F
106.0 %
106.6 %
105.3 %

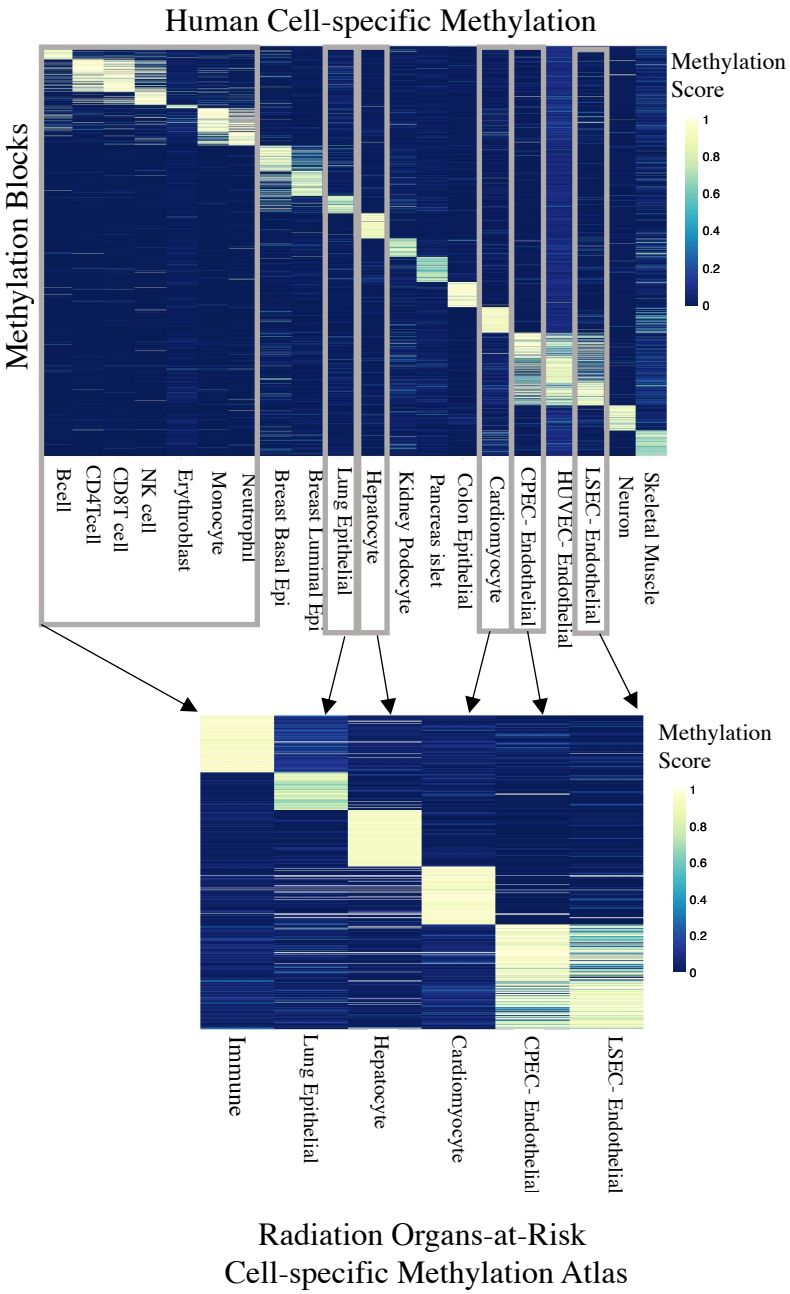

b
Human Cell-specific Methylation
Methylation Blocks
Methylation Score
1
0.8
0.6
0.4
0.2
0
Bcell
CD4Tcell
CD8T cell
NK cell
Erythroblast
Monocyte
Neutrophil
Breast Basal Epi
Breast Luminal Epi
Lung Epithelial
Hepatocyte
Kidney Podocyte
Pancreas islet
Colon Epithelial
Cardiomyocyte
CPEC- Endothelial
HUVEC- Endothelial
LSEC- Endothelial
Neuron
Skeletal Muscle
Immune
Lung Epithelial
Hepatocyte
Cardiomyocyte
CPEC- Endothelial
LSEC- Endothelial
Radiation Organs-at-Risk
Cell-specific Methylation Atlas

Figure 5. Development of a radiation-specific methylation atlas focusing on cell types from target organs-at-risk (OAR). (a) Representative 3D-CRT treatment planning for patients with right-sided (i and ii) and left-sided (iii and iv) breast cancer, respectively. Computed tomography simulation of coronal and sagittal images depicting the anatomic position of the target volume in relation to nearby organs. The color map represents different radiation dose levels or isodose lines (Green: 95% of prescription dose, yellow: 90% isodose line, cyan: 80% isodose line, orange: 70% isodose line, brown: 50% isodose line). (b) Heatmaps of differentially methylated cell-type specific blocks identified from all reference WGBS data compiled from healthy human cell-types and tissues. Each cell in the plot marks the methylation score of one genomic region (rows) at each of the 20 human cell types (columns). Up to 100 blocks with the highest methylation score are shown per cell type. Differential blocks identified from cell types comprising the target organs-at-risk from radiation (lungs, heart, and liver) were selected for generation of a radiation-specific methylation atlas, separating these solid organ cell types from all other immune cell types. Abbreviations: CAEC = coronary artery endothelial cell, CMEC = cardiac microvascular endothelial cell, CPEC = joint cardio-pulmonary endothelial cell, HUVEC = human umbilical vein endothelial cell, LSEC = liver sinusoidal endothelial cell, MK = megakaryocyte, NK = natural killer cell, PAEC = pulmonary artery endothelial cell, PMEC = pulmonary microvascular endothelial cell.

Figure 6.

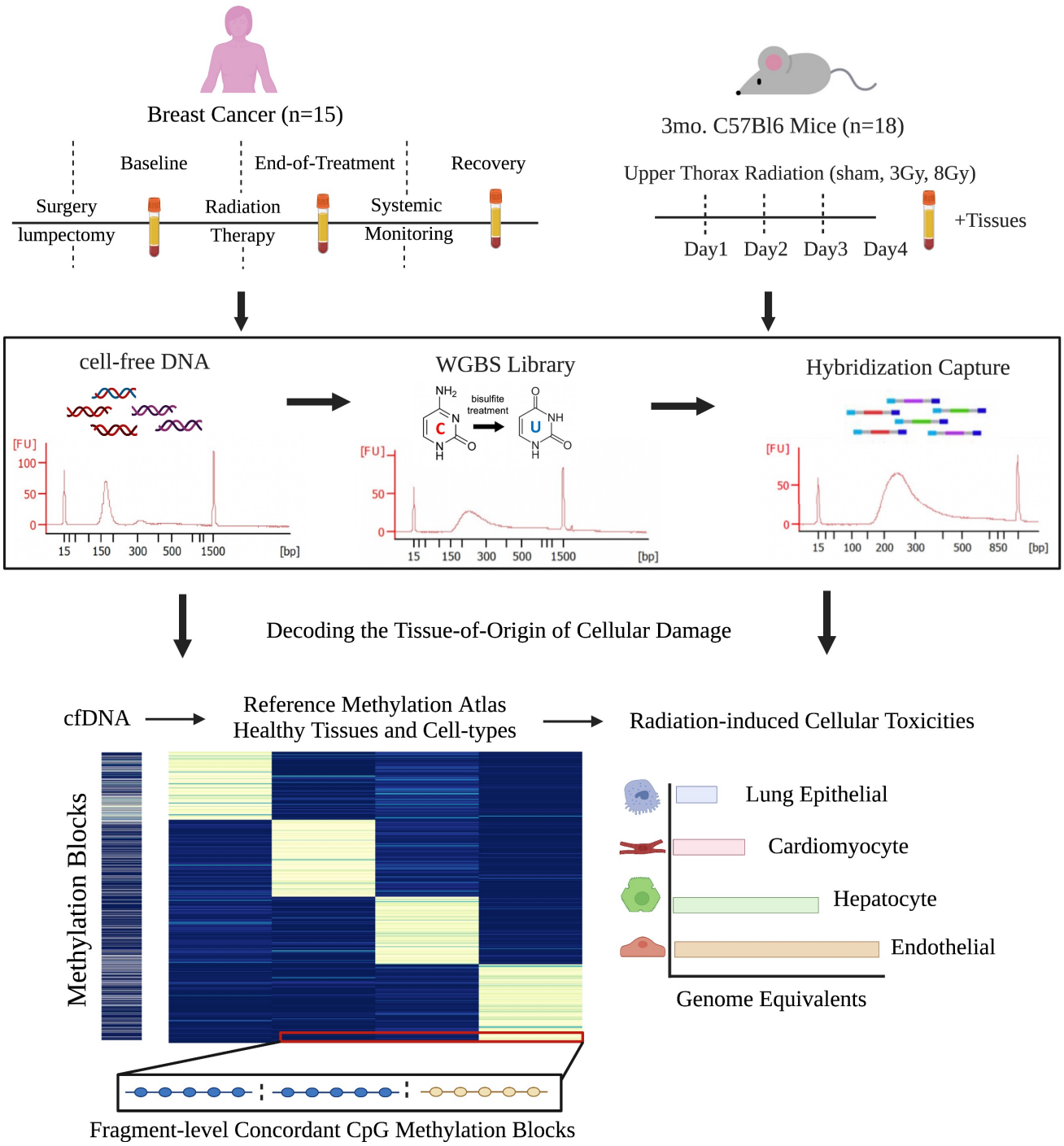

Breast Cancer (n=15)
Baseline
End-of-Treatment
Recovery
Surgery
lumpectomy
Radiation
Therapy
Systemic
Monitoring
3mo. C57Bl6 Mice (n=18)
Upper Thorax Radiation (sham, 3Gy, 8Gy)
Day1
Day2
Day3
Day4
+Tissues
cell-free DNA
WGBS Library
bisulfite treatment
Hybridization Capture
Decoding the Tissue-of-Origin of Cellular Damage
cfDNA
Reference Methylation Atlas
Healthy Tissues and Cell-types
Radiation-induced Cellular Toxicities
Methylation Blocks
Lung Epithelial
Cardiomyocyte
Hepatocyte
Endothelial
Genome Equivalents
Fragment-level Concordant CpG Methylation Blocks

Figure 6. Overview of the analysis of cell-free methylated DNA in blood to identify origins of radiation-induced cellular damage. Serial serum samples were collected from breast cancer patients treated with radiation. In parallel, paired serum and tissue samples were collected from mice receiving radiation at 3Gy or 8Gy doses compared to sham control. Methylome profiling of liquid biopsy samples was performed using a bisulfite capture-sequencing methodology optimized for cell-free DNA (cfDNA) inputs. Differential cell-type specific methylation blocks were identified from reference WGBS data compiled from healthy cell types and tissues in human and mouse. Methylation atlases were generated emphasizing cell types composing target organs-at-risk from radiation, including the lungs, heart, and liver. Deconvolution analysis of cfDNA using fragment-level CpG methylation patterns at these identified cell-type specific blocks was used to decode the origins of radiation-induced cellular injury.

Figure 7.

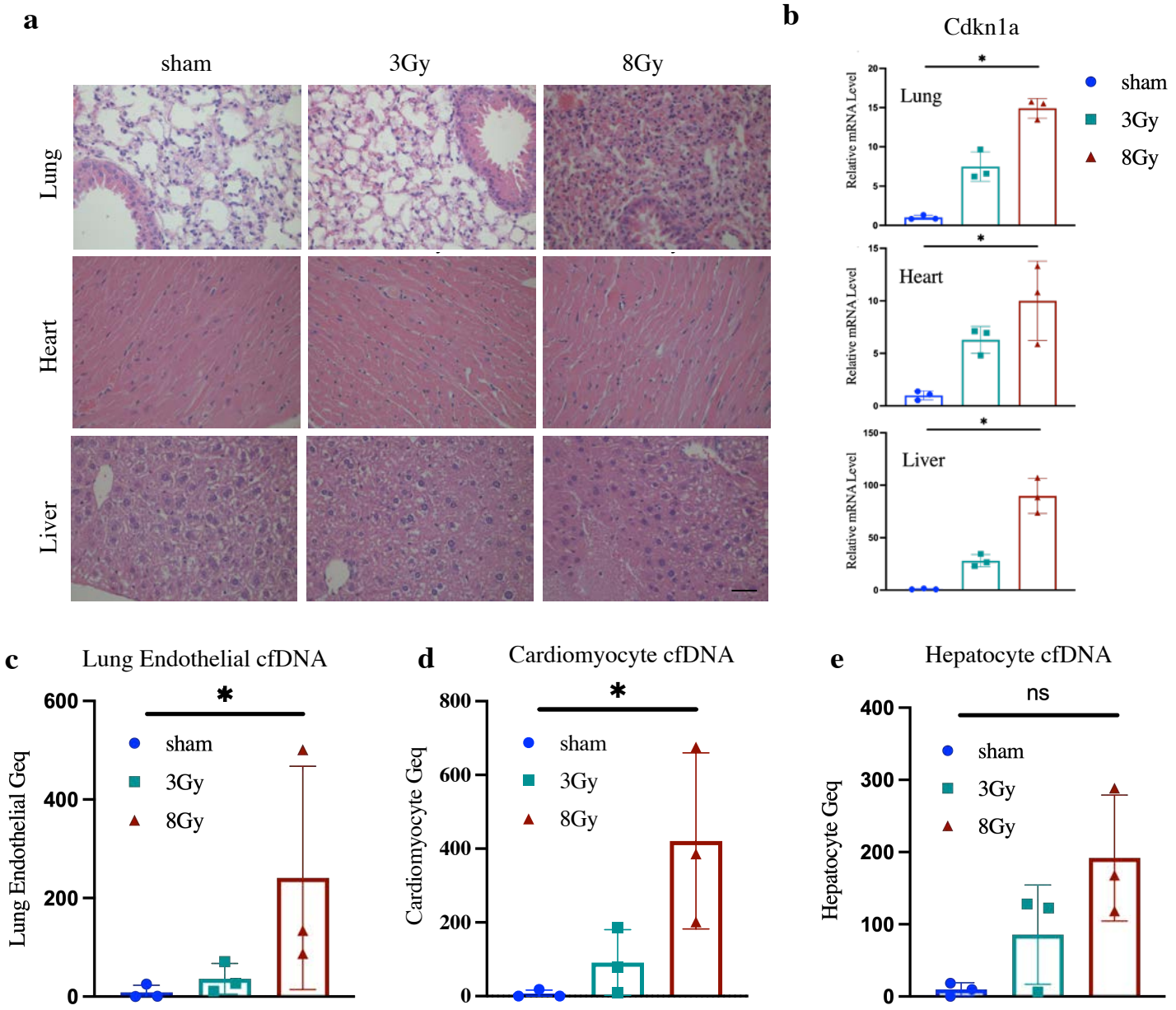

a
sham
3Gy
8Gy
Lung
Heart
Liver
b
Cdkn1a
Lung
Heart
Liver
Relative mRNA Level
sham
3Gy
8Gy
c
Lung Endothelial cfDNA
Lung Endothelial Geq
d
Cardiomyocyte cfDNA
Cardiomyocyte Geq
e
Hepatocyte cfDNA
Hepatocyte Geq
ns

Figure 7. Dose-dependent radiation damage in mouse tissues correlates with the origins of methylated cfDNA in the circulation. (a) Representative H&E staining of lung, heart, and liver tissues from mice treated with 3Gy or 8Gy radiation compared to sham control. Scale bar, 200 μm. (b) qPCR analysis of CDKN1A (p21) mRNA. The expression in each sample was normalized to ACTB and is shown relative to the expression in the sham control. Mean ± SD; N = 3. Kruskal-Wallis test was used for comparisons amongst groups; lung tissue p=0.004, heart tissue p=0.025, liver tissue p=0.004. (c-e) Lung endothelial, cardiomyocyte and hepatocyte methylated cfDNA in the circulation of mice treated with 3Gy and 8Gy radiation compared to sham control expressed in Genome Equivalents (Geq). CfDNA was extracted from 18 mice (n=6 in each group) with cfDNA from 2 mice pooled in each methylome preparation. Mean ± SD; N = 3 independent methylome preparations. Kruskal-Wallis test was used for comparisons amongst groups. ns, $P \geq 0.05$; *, $P < 0.05$; lung endothelial p=0.01, cardiomyocyte p=0.01, hepatocyte p=0.13.

Figure 8.

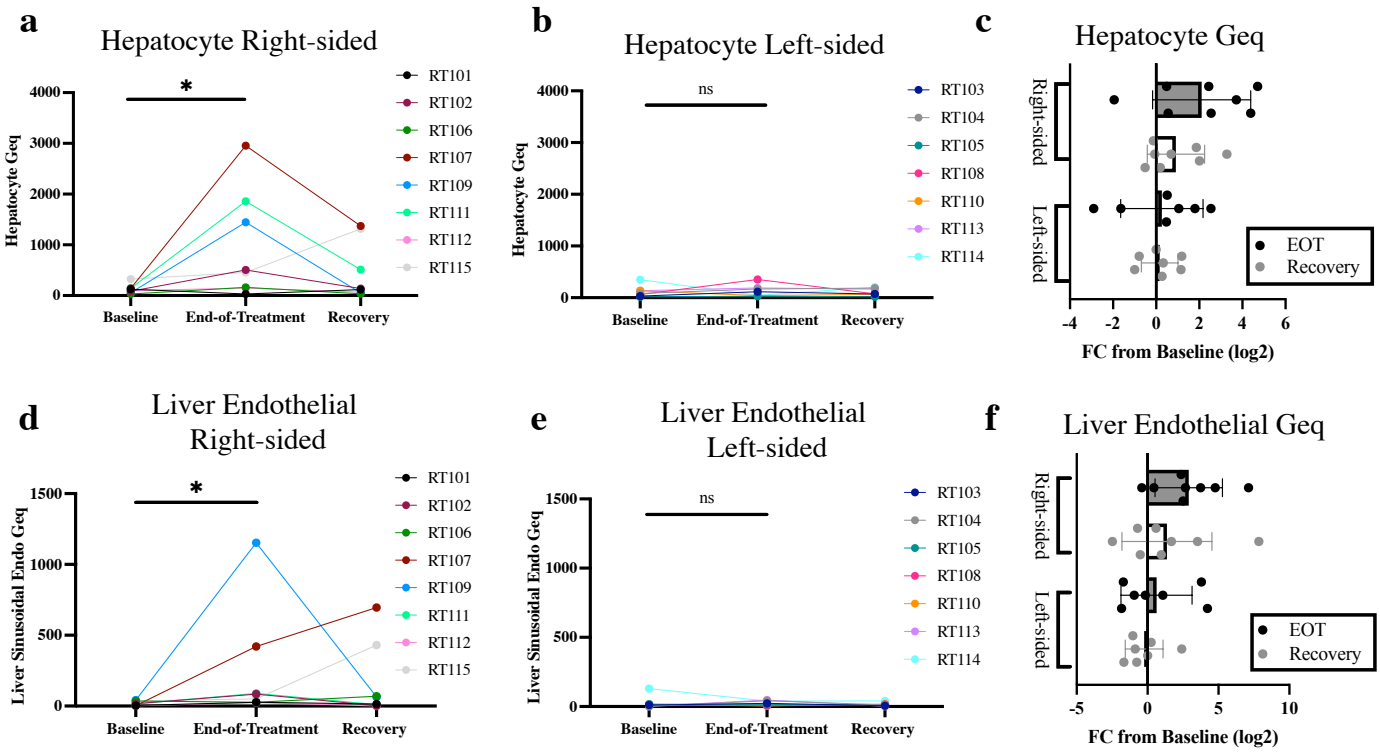

a
Hepatocyte Right-sided
*
Hepatocyte Geq
4000
3000
2000
1000
0
Baseline
End-of-Treatment
Recovery
RT101
RT102
RT106
RT107
RT109
RT111
RT112
RT115
b
Hepatocyte Left-sided
ns
Hepatocyte Geq
4000
3000
2000
1000
0
Baseline
End-of-Treatment
Recovery
RT103
RT104
RT105
RT108
RT110
RT113
RT114
c
Hepatocyte Geq
Right-sided
Left-sided
EOT
Recovery
-4
-2
0
2
4
6
FC from Baseline (log2)
d
Liver Endothelial
Right-sided
*
Liver Sinusoidal Endo Geq
1500
1000
500
0
Baseline
End-of-Treatment
Recovery
RT101
RT102
RT106
RT107
RT109
RT111
RT112
RT115
e
Liver Endothelial
Left-sided
ns
Liver Sinusoidal Endo Geq
1500
1000
500
0
Baseline
End-of-Treatment
Recovery
RT103
RT104
RT105
RT108
RT110
RT113
RT114
f
Liver Endothelial Geq
Right-sided
Left-sided
EOT
Recovery
-5
0
5
10
FC from Baseline (log2)

Figure 8. Radiation therapy-induced hepatocyte and liver endothelial cfDNAs in patient with right- versus left-sided breast cancer. (a, b) Hepatocyte cfDNA (in Geq/mL) in serum samples collected at different times. Fragment-level deconvolution used hepatocyte specific methylation blocks (n=200). Wilcoxon matched pairs signed rank test was used for comparison amongst groups and results were considered significant when *$P < 0.05$; ns, $P \geq 0.05$; right-sided p= 0.02, left-sided p=0.81. (c) Fold change in hepatocyte cfDNA after treatment (EOT) and at recovery relative to baseline. Mean ± SD; N = 8 right-sided, N = 7 left-sided. (d, e) Liver sinusoidal endothelial (LSEC) cfDNA (in Geq/mL) in the same serum samples. Fragment-level deconvolution used LSEC specific methylation blocks (n=89). Wilcoxon matched pairs signed rank test was performed between groups and results were considered significant when *$P < 0.05$; ns, $P \geq 0.05$; right-sided p=0.02, left-sided p=0.93. (f) Fold change in LSEC cfDNA Geq at EOT and recovery relative to baseline levels. Mean ± SD; N = 8 right-sided, N = 7 left-sided.

Figure 9.

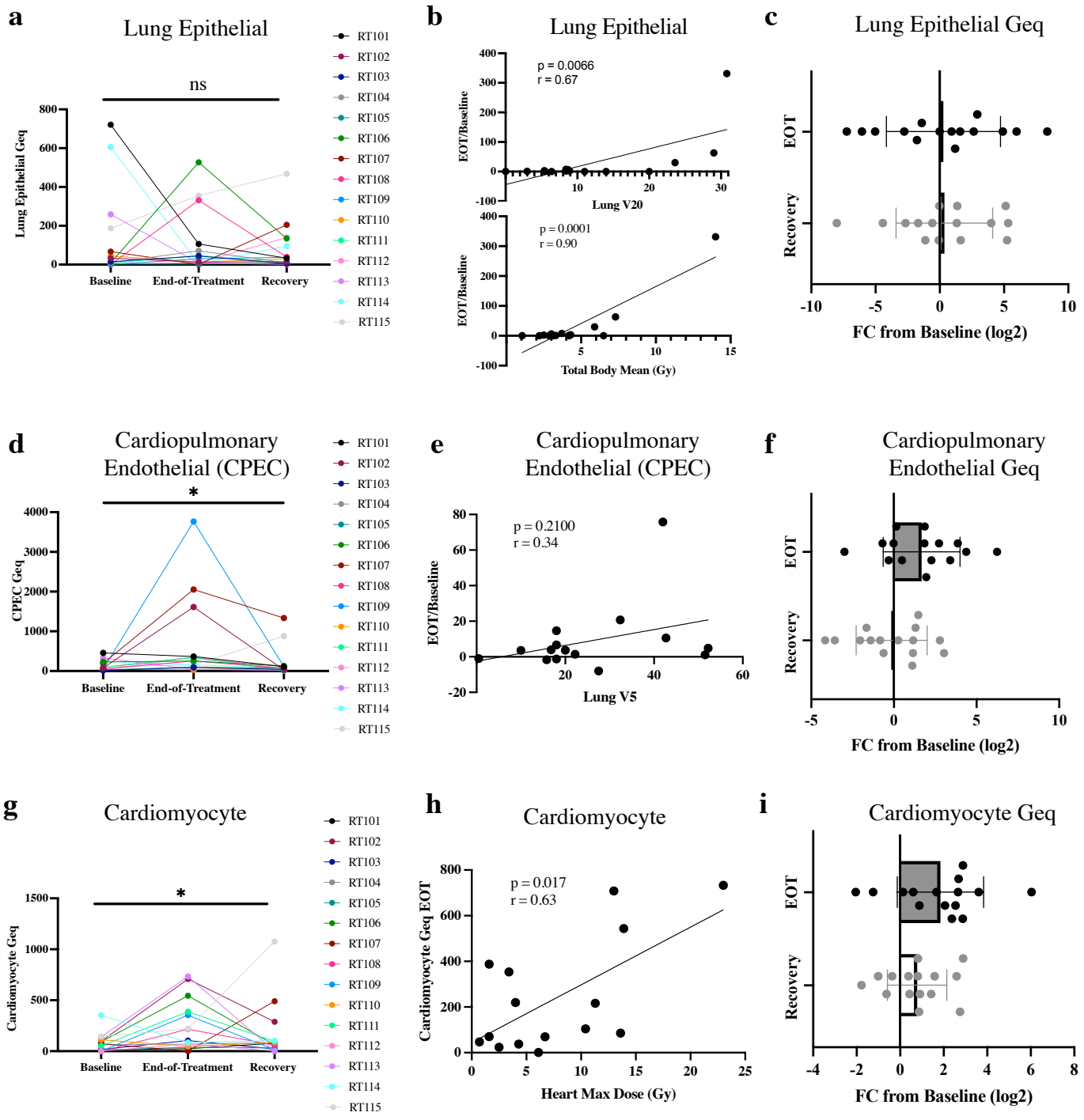
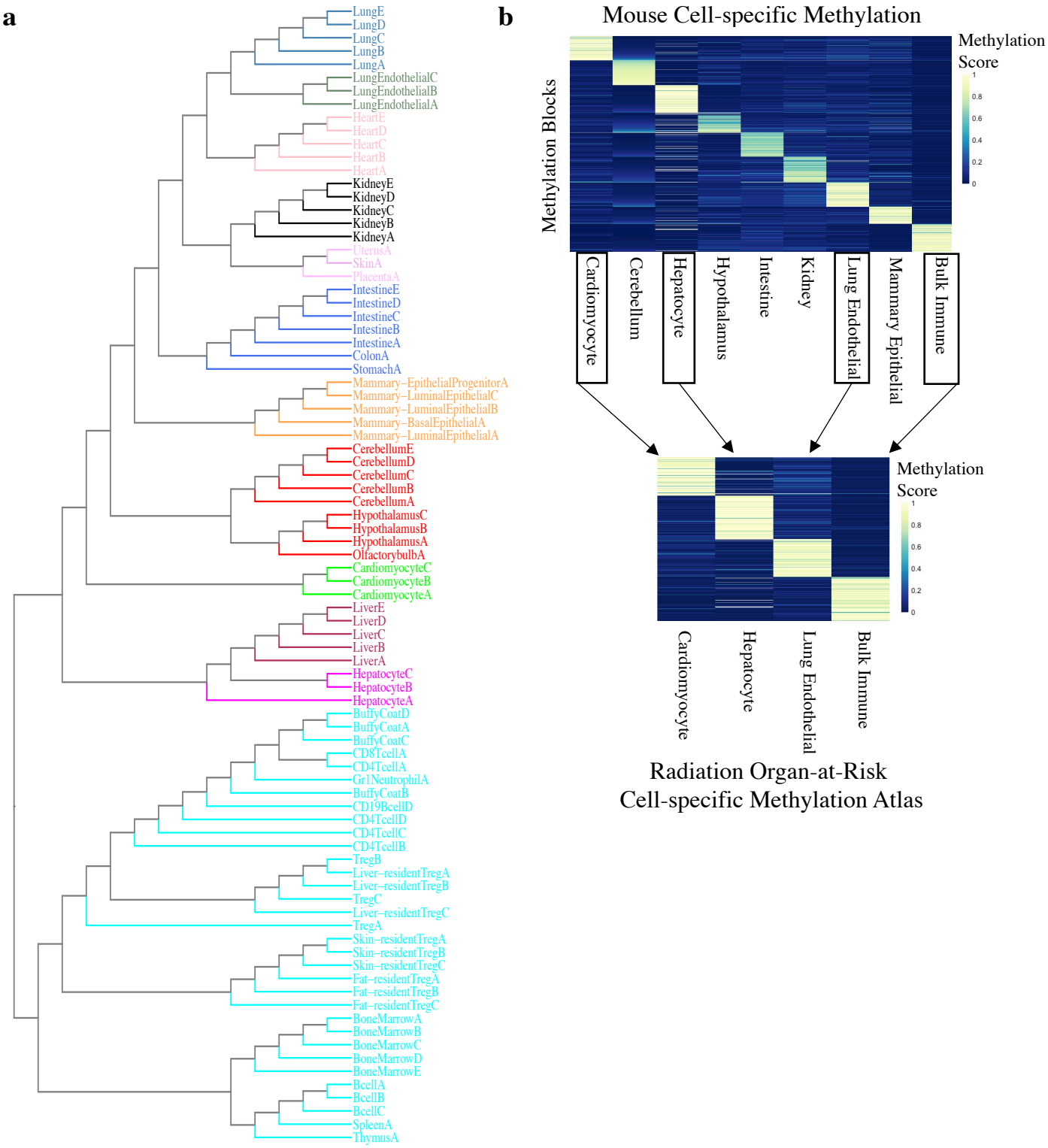

a
Lung Epithelial
ns
Lung Epithelial Geq
Baseline
End-of-Treatment
Recovery
RT101
RT102
RT103
RT104
RT105
RT106
RT107
RT108
RT109
RT110
RT111
RT112
RT113
RT114
RT115
b
Lung Epithelial
p = 0.0066
r = 0.67
EOT/Baseline
Lung V20
p = 0.0001
r = 0.90
Total Body Mean (Gy)
c
Lung Epithelial Geq
EOT
Recovery
FC from Baseline (log2)
d
Cardiopulmonary Endothelial (CPEC)
*
CPEC Geq
e
Cardiopulmonary Endothelial (CPEC)
p = 0.2100
r = 0.34
Lung V5
f
Cardiopulmonary Endothelial Geq
g
Cardiomyocyte
*
Cardiomyocyte Geq
h
Cardiomyocyte
p = 0.017
r = 0.63
Cardiomyocyte Geq EOT
Heart Max Dose (Gy)
i
Cardiomyocyte Geq

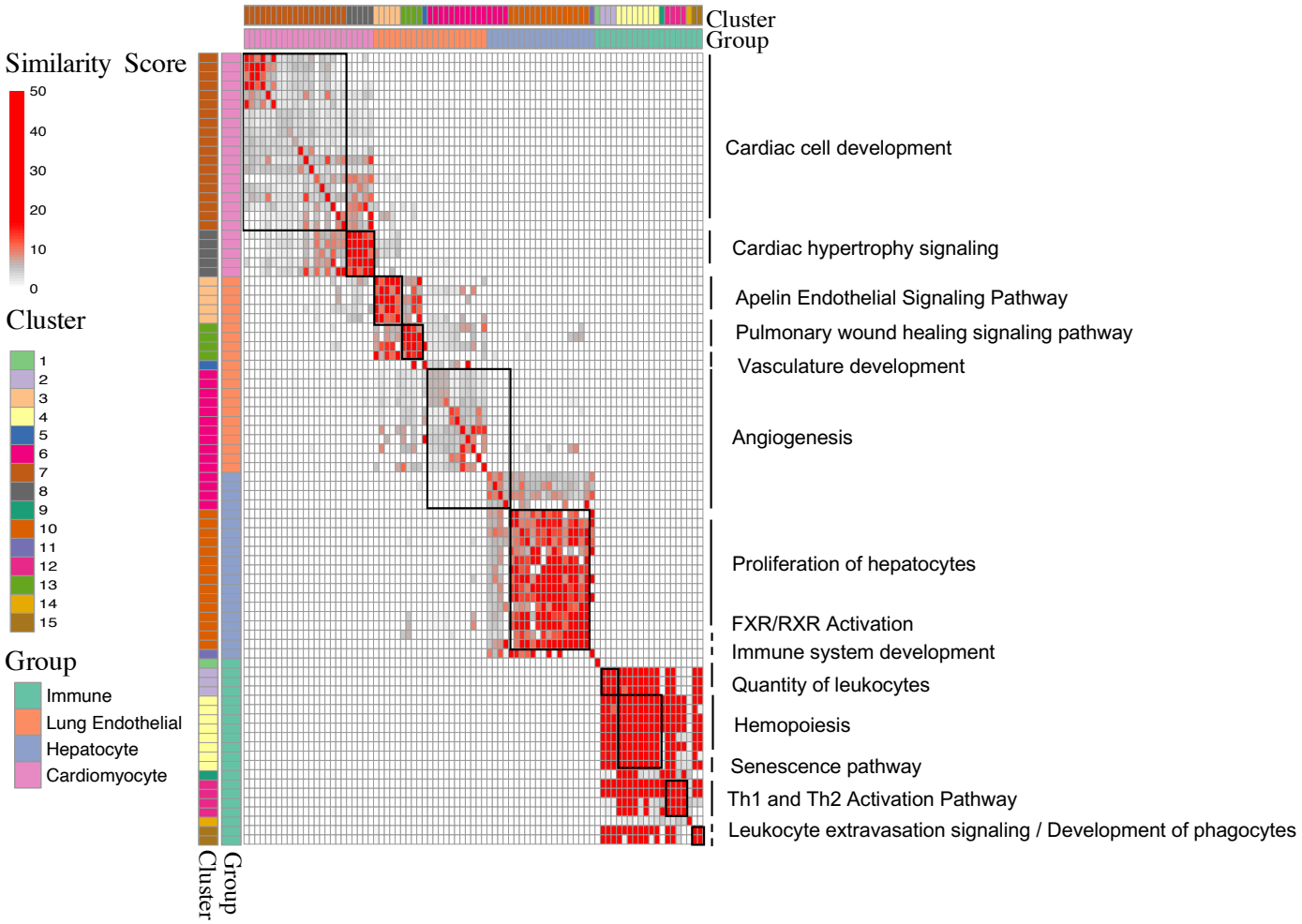
Figure 9. Radiation therapy-induced cardiopulmonary cfDNAs in patients correlates with the radiation dose and indicates sustained injury to cardiomyocytes. (a) Lung epithelial cfDNA (in Geq/mL) in serum samples collected at different times. Fragment-level deconvolution used lung epithelial specific methylation blocks (n=69). (b) Correlation of lung epithelial cfDNA with dosimetry data. EOT/Baseline represents the fraction of lung epithelial cfDNA post-radiation at end-of-treatment (EOT) relative to baseline levels. The volume of the lung receiving 20 Gy dose is represented by Lung V20 (%) and the mean dose to the total body represented by total body mean (Gy). (c) Fold change in lung epithelial cfDNA at EOT and recovery relative to baseline. (d) Cardiopulmonary endothelial (CPEC) cfDNA (in Geq/mL). Fragment-level deconvolution used CPEC specific methylation blocks (n=132). (e) Correlation of CPEC cfDNA with dosimetry data. The volume of the lung receiving 5 Gy dose is represented by Lung V5 (%). (f) Fold change in CPEC cfDNA at EOT and recovery relative to baseline levels. (g) Cardiomyocyte cfDNA (in Geq/mL). Fragment-level deconvolution used cardiomyocyte specific methylation blocks (n=375). (h) Correlation of cardiomyocyte cfDNA with the maximal heart dose (Gy). (i) Fold change in cardiomyocyte cfDNA at EOT and recovery relative to baseline. *(a, d, g) Friedman test was performed comparing paired results at baseline, EOT, and recovery timepoints. The results were considered significant when $*P < 0.05$; ns, $P \geq 0.05$; lung epithelial p=0.98, cardiopulmonary endothelial p=0.02, cardiomyocyte p= 0.03. (b, e, h) Pearson correlation r was calculated, and linear correlation was considered significant when $*P < 0.05$. (c, f, i) Data presented as mean ± SD; N = 15.

Supplemental Figure 1.



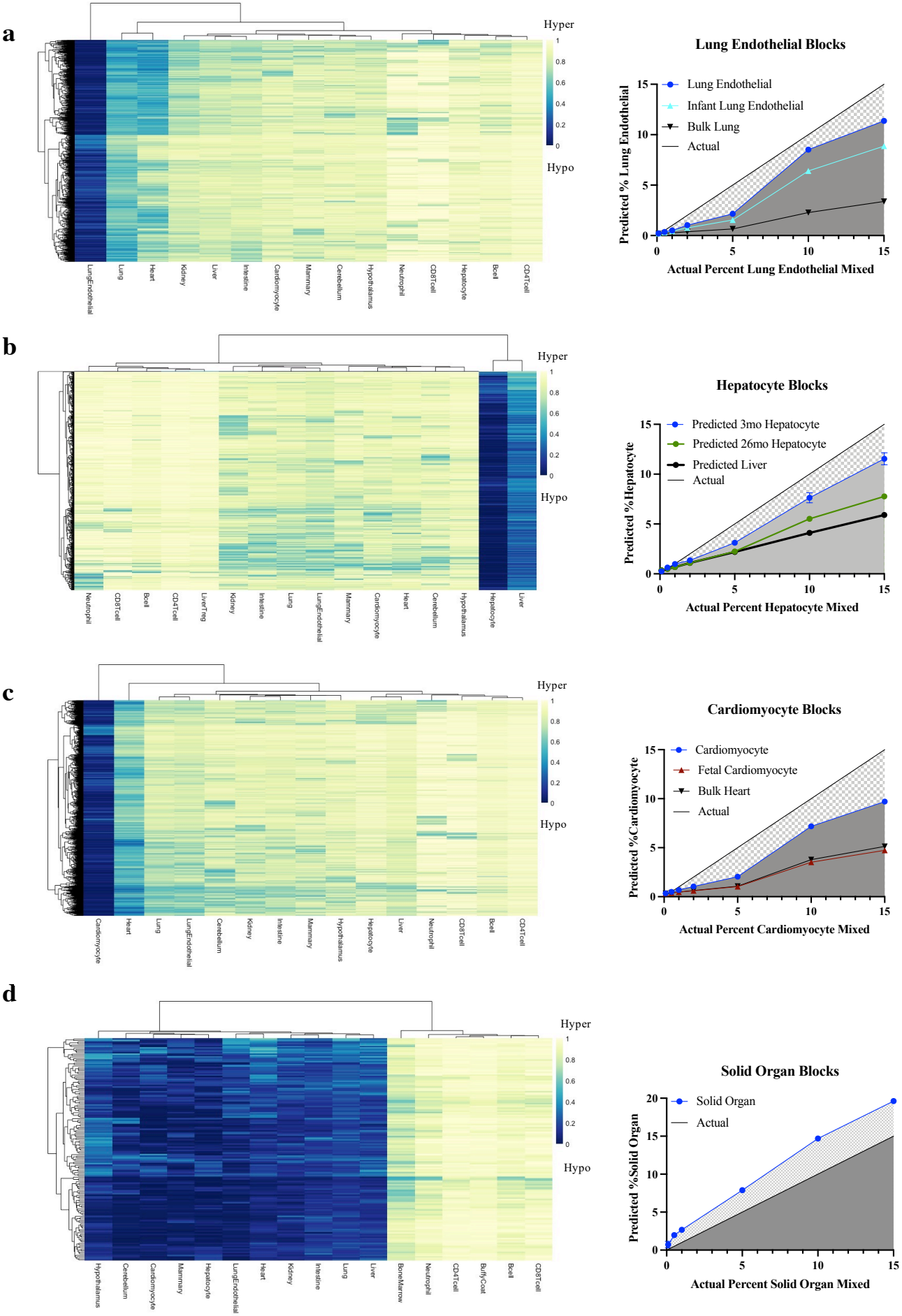
Supplemental Figure 1. Characterization of mouse cell-type specific reference methylation data. (a) Tree dendrogram depicting relationship between mouse reference Whole Genome Bisulfite Sequencing (WGBS) datasets included in the analysis. Methylation status at the top 30,000 variable blocks was used as input data for the unsupervised hierarchical clustering. (b) Heatmaps of differentially methylated cell-type specific blocks identified from reference WGBS data compiled from healthy cell types and tissues in mouse. Each cell in the plot marks the methylation score of one genomic region (row) at each of the 9 mouse tissues and cell types (columns). Up to 100 blocks with the highest methylation score are shown per cell type. Differential blocks identified from cell types comprising the target organs-at-risk from radiation (lungs, heart, and liver) were selected for generation of a radiation-specific methylation atlas, separating these solid organ cell types from all other immune cell types.

Supplemental Figure 2.



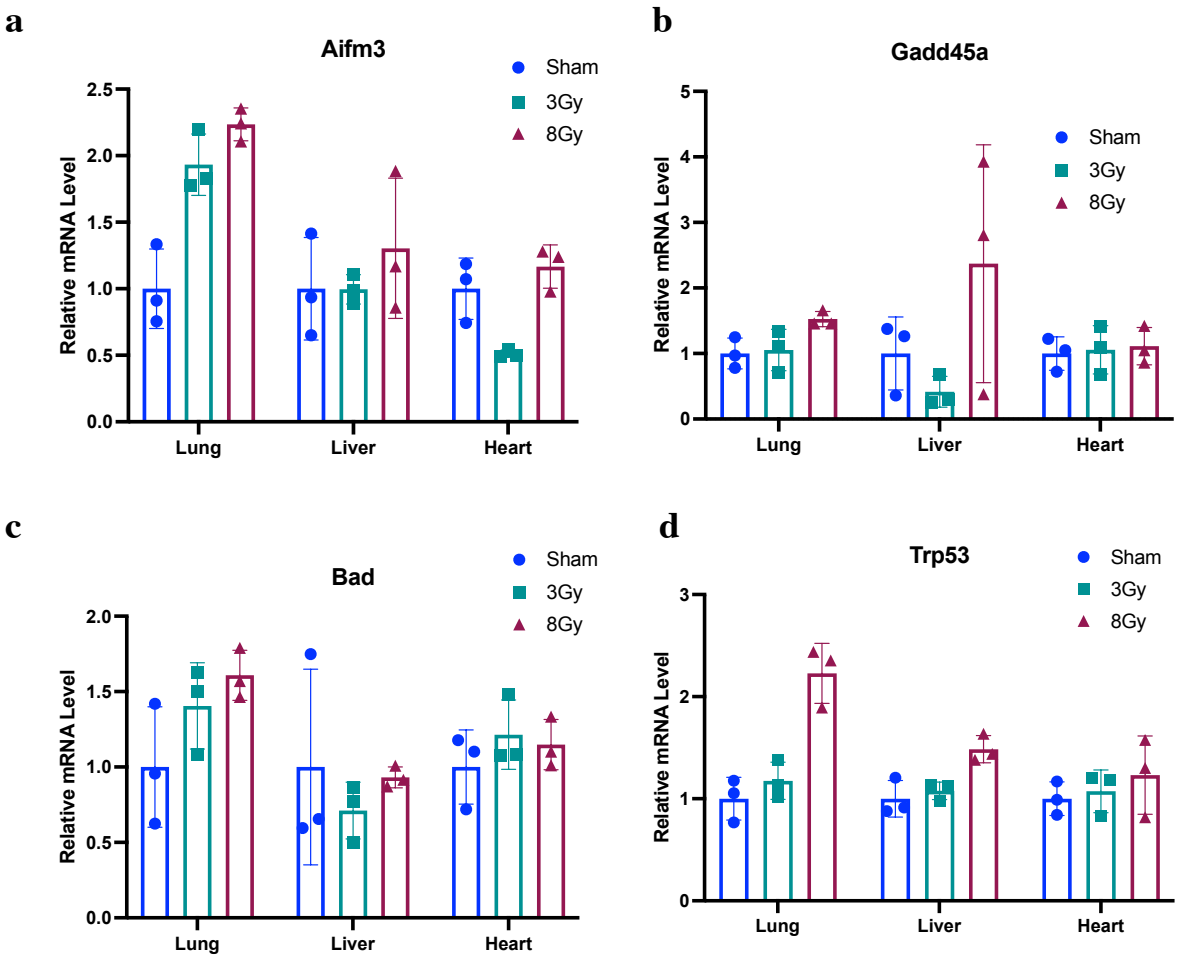
Supplemental Figure 2. Biological function of mouse cell-type specific methylation blocks. Heatmap of distance scores between gene-set pathways identified from GeneSetCluster. Genes adjacent to cell-type specific methylation blocks were identified using HOMER and pathway analysis was performed using both Ingenuity Pathway Analysis (IPA) and GREAT. Significantly enriched gene-set pathways ($p<0.05$) from differentially methylated blocks identified in immune, cardiomyocyte, hepatocyte, and lung endothelial cell types were analyzed using GeneSetCluster. Cluster analysis was performed to determine the distance between all identified gene-set pathways based on the degree of overlapping genes from each individual gene-set compared to all others. Over-representation analysis was implemented in the WebgestaltR (ORAperGeneSet) plugin to interpret and functionally label identified gene-set clusters.

Supplemental Figure 3.



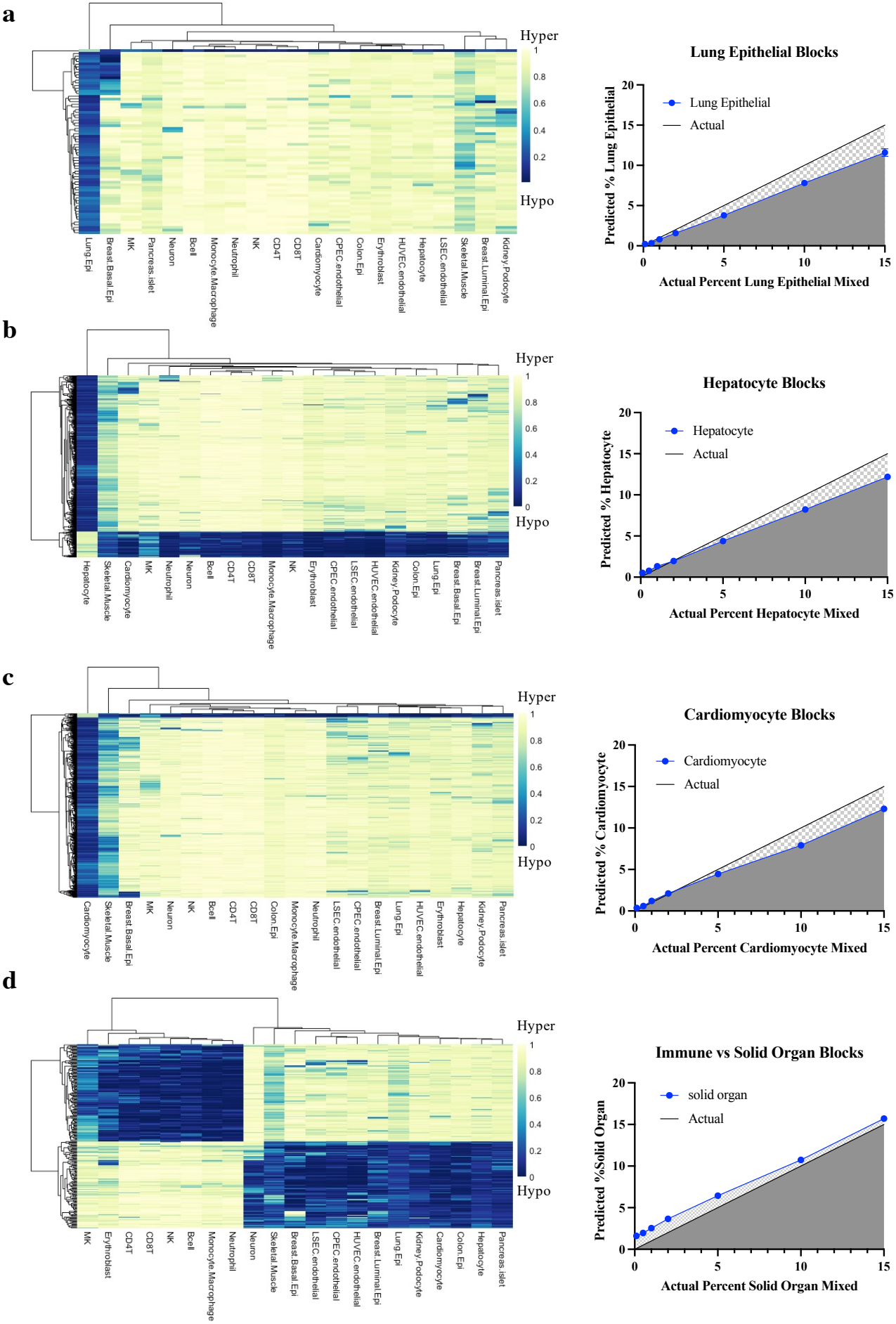
Supplemental Figure 3. Sensitivity and specificity of identified mouse cell-type specific differentially methylated blocks. (a-d) (Left) Heatmap of all cell-type specific methylation blocks selected for each target cell type. All blocks contain 3+CpG sites and have a margin of beta difference greater than or equal to 0.35 separating the target cell type from all others included in the reference maps. All identified methylation blocks for lung endothelial (n=1,546), hepatocyte (n=616), and cardiomyocyte (n=2,917) mouse cell types were hypomethylated. In contrast, all identified immune cell-specific blocks (n=148) were hypermethylated relative to other solid organ cell types in mouse. (Right) In-silico mix-in validation of fragment-level probabilistic deconvolution model. Target cell-type read-pairs were in-silico mixed into a background of lymphocyte or buffy coat read-pairs at various known percentages (0.5%, 1%, 2%, 5%, 10%, 15%). The deconvolution model was validated on these in-silico mixed samples of known cell-type proportions at the blocks selected. The average predicted %target is graphed relative to the known %mixed to assess sensitivity and specificity of the identified cell-type specific blocks and deconvolution model. Data presented as mean ± SD; N=3 replicates per proportion. Reference WGBS samples with less than 3 replicates were split into "0.8 train" to select methylation blocks and "0.2 test" to generate in-silico mixed samples. When available, in-silico mixed samples of the same cell-type derived from different aged mice were tested. In addition, bulk tissue of the respective cell type was tested as well.

Supplemental Figure 4.



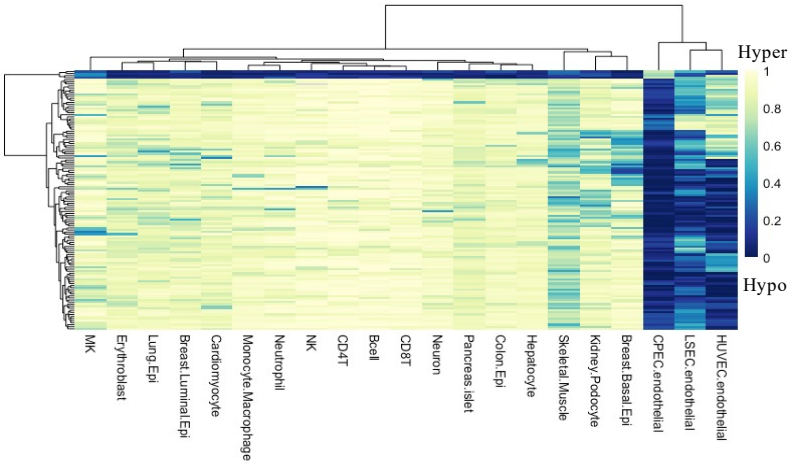
Supplemental Figure 4. Apoptotic damage from radiation in mouse tissues. qPCR analysis of markers of apoptosis (Trp53, Gadd45a, Aifm3, and Bad) in mouse Lung, Heart, and Liver tissues treated with 3Gy and 8Gy radiation compared to sham control. The expression of each sample was normalized to the expression of ACTB (beta- actin). Data presented as mean ± SD; N = 3**.**

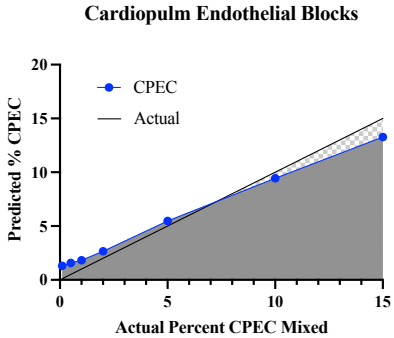
Supplemental Figure 5.



Supplemental Figure 5.

e





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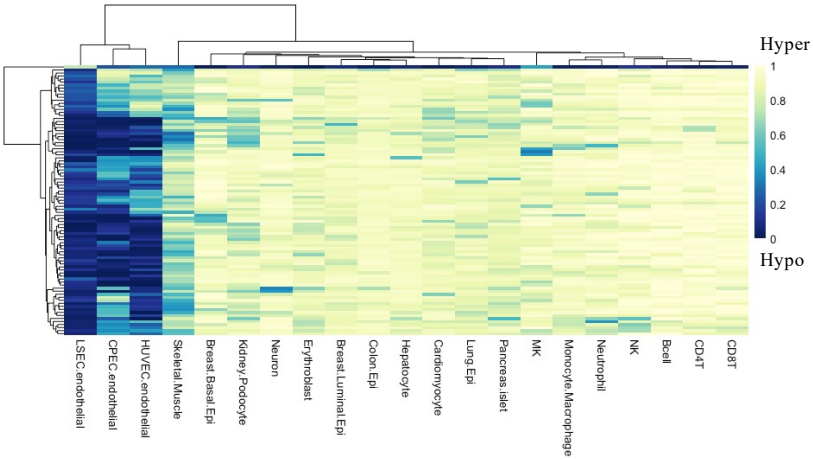
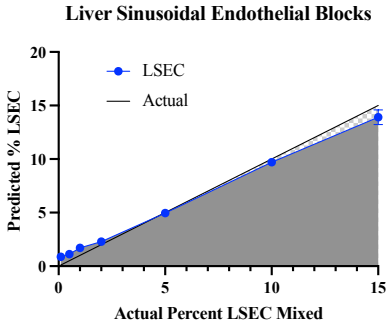
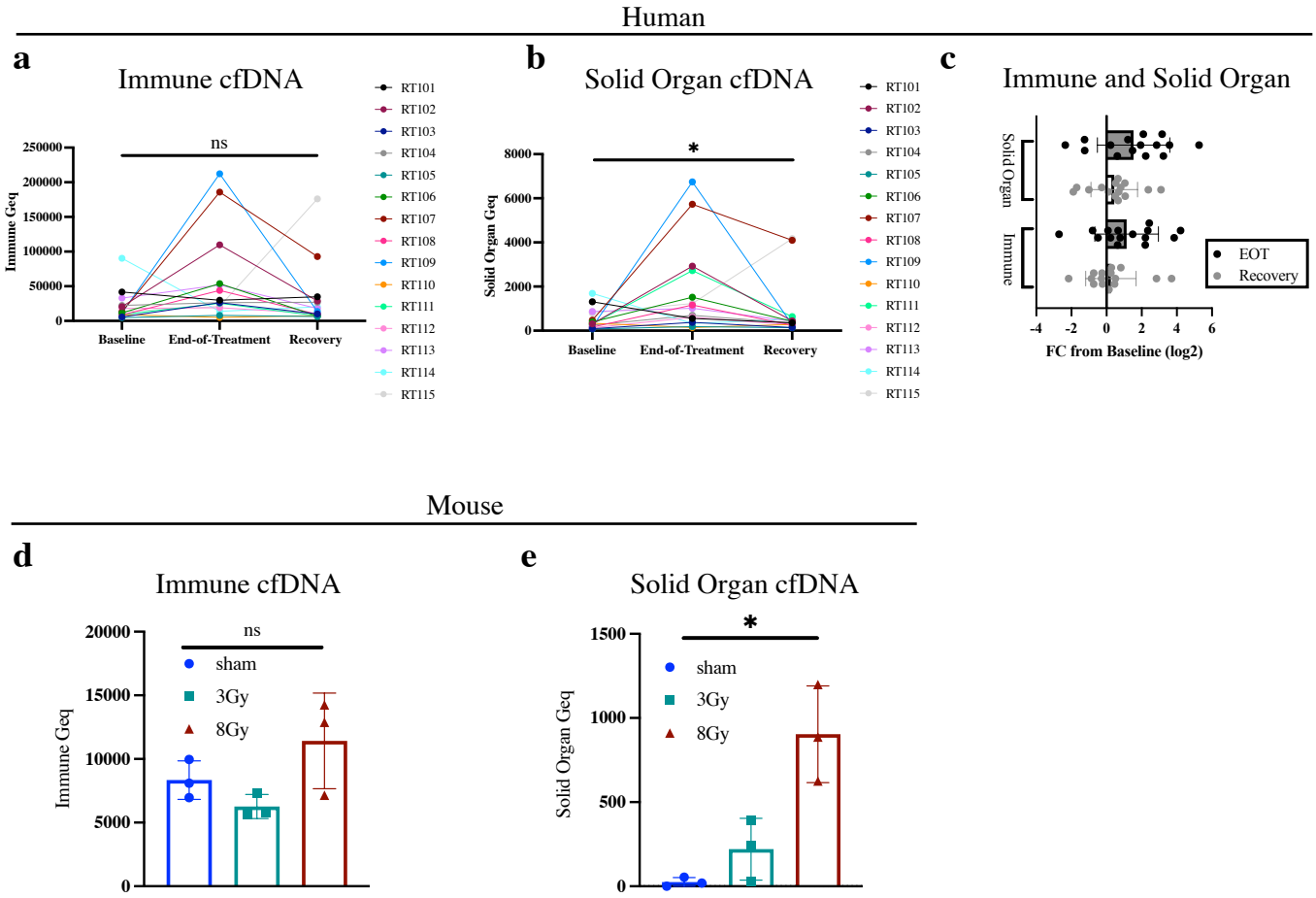




Supplemental Figure 5. Sensitivity and specificity of identified human cell-type specific differentially methylated blocks. (a-f) (Left) Heatmap of all cell-type specific methylation blocks selected for each target cell type. All blocks contain 3+CpG sites and have a margin of beta difference greater than or equal to 0.4 separating the target cell type from all others included in the reference maps. (Right) In-silico mix-in validation of fragment-level probabilistic deconvolution model. Target cell-type read-pairs were in-silico mixed into a background of lymphocyte or buffy coat read-pairs at various known percentages (0.5%, 1%, 2%, 5%, 10%, 15%). The deconvolution model was validated on these in-silico mixed samples of known cell-type proportions at the blocks selected. The average predicted %target is graphed relative to the known %mixed to assess sensitivity and specificity of the identified cell-type specific blocks and deconvolution model. Data presented as mean ± SD; N=3 replicates per proportion.

Supplemental Figure 6.



Supplemental Figure 6. Radiation-induced effects on immune and solid organ cell-free DNA in human (a-c) and mouse (d-e) samples. (a) Predicted human immune-derived cfDNA in Geq. Human Geq are calculated by multiplying the relative fraction of cell-type specific cfDNA x initial concentration cfDNA ng/mL x the weight of the haploid human genome. Immune cfDNA was assessed at n = 222 methylation blocks found to separate immune cell types from solid organ cell types. (g1 = Bcell, CD4Tcell, CD8Tcell, NK, MK, Erythroblast, Monocyte, Macrophage, Neutrophil; g2 = breast basal/luminal epi, lung epi, hepatocyte, kidney podocyte, pancreas islet, colon epi, cardiomyocyte, LSEC, CPEC, HUVEC, neuron, and skeletal muscle). (b) Predicted human solid organ-derived cfDNA in Geq where %solid organ is defined as 100-%immune using these same n=222 methylation blocks. (c) Fold change in human immune versus solid organ Geq at EOT and recovery relative to baseline. Data presented as mean ± SD; N = 15. (A, B) Friedman test was performed for comparisons amongst groups. ns, $P \geq 0.05$; *, $P < 0.05$; immune p=0.07, solid organ p=0.008. (d) Predicted mouse immune-derived cfDNA in Geq. Mouse Geq are calculated by multiplying the relative fraction of cell-type specific cfDNA x initial concentration cfDNA ng/mL x the weight of the haploid mouse genome. Immune cfDNA was assessed at n = 148 methylation blocks found to separate immune cell types from solid organ cell types. (g1 = Bcell, CD4Tcell, CD8Tcell, Neutrophil; g2 = mammary epi, cardiomyocyte, hepatocyte, lung endothelial, cerebellum, hypothalamus, colon, intestine, kidney). (e) Predicted mouse solid organ-derived cfDNA in Geq. (D-E) Mean ± SD; N = 3 independent methylome preparations. Kruskal-Wallis test was used for comparisons amongst groups. ns, $P \geq 0.05$; *, $P < 0.05$; immune p = 0.20, solid organ p = 0.01.

Legends for Supplementary Data File 1:

Supplemental Table S1. Human reference methylation data from healthy tissues and cell types.

Supplemental Table S2. Mouse reference methylation data from healthy tissues and cell types.

Supplemental Table S3. Identified human cell-type specific methylation blocks. Annotation was performed using Homer. The margin of separation represents the delta-beta (maximum higher – minimum lower) across all samples. Blocks with a (-) direction are hypomethylated and (+) direction are hypermethylated.

Supplemental Table S4. Identified mouse tissue and cell-type specific methylation blocks. Annotation was performed using Homer. The margin of separation represents the delta-beta (maximum higher – minimum lower) across all samples. Blocks with a (-) direction are hypomethylated and (+) direction are hypermethylated.

Supplemental Table S5. Summary of identified human and mouse cell-type specific methylation blocks.

Supplemental Table S6. Mouse cfDNA sample concentrations and predicted cell-type proportions from deconvolution analysis at identified cell-type specific blocks for target cell types.

Supplemental Table S7. Human cfDNA sample concentrations and predicted cell-type proportions from deconvolution analysis at identified cell-type specific blocks for target cell types.

Supplemental Table S8. Characteristics of breast cancer patients enrolled in this study.

Supplemental Table S9. Primers used for RT–qPCR in radiation-treated mouse tissues.

Supplemental Table S10. Extended endothelial-specific methylation blocks (bg.quant 0.2) used for pathway analysis and validation of cell identity through integration with paired RNA expression data.

Supplemental Table S11. Genomic annotation of identified human and mouse cell-type specific hypomethylated and hypermethylated blocks relative to all captured blocks.

Supplemental Table S12. Top 25 significantly enriched biological pathways and functions for genes associated with differential methylation in each cell-type.