Supplemental Information

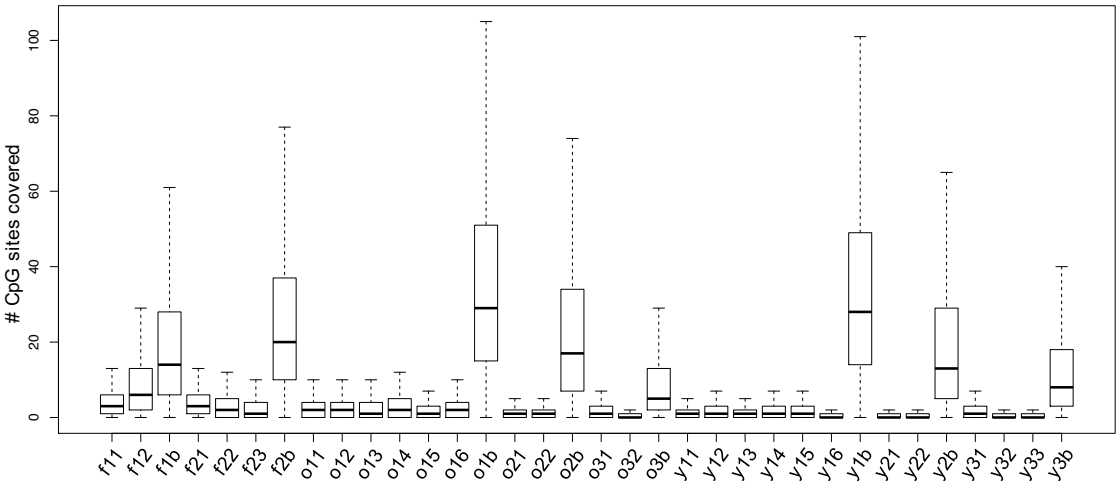

Figure S1. Sequencing coverage on CpGs in 3kb sliding windows. Single fibroblasts - f11, f12, f21, f22, f23; fibroblast bulks - f1b, f2b; hepatocytes old - o11 to o16, o21, o22, o31, o32; bulk hepatocyte old - o1b, o2b, o3b; hepatocytes young - y11 to y16, y21, y22, y31, y32, y33; bulk hepatocytes young - y1b, y2b, y3b.

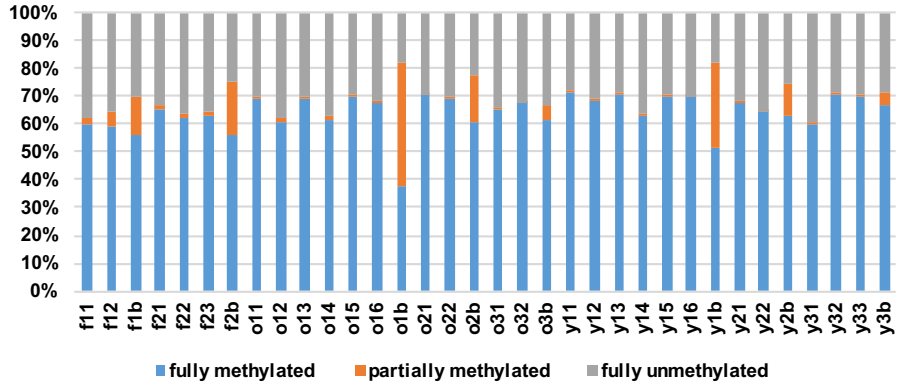

Figure S2. Distribution of single CpG methylation status in single cells and bulks

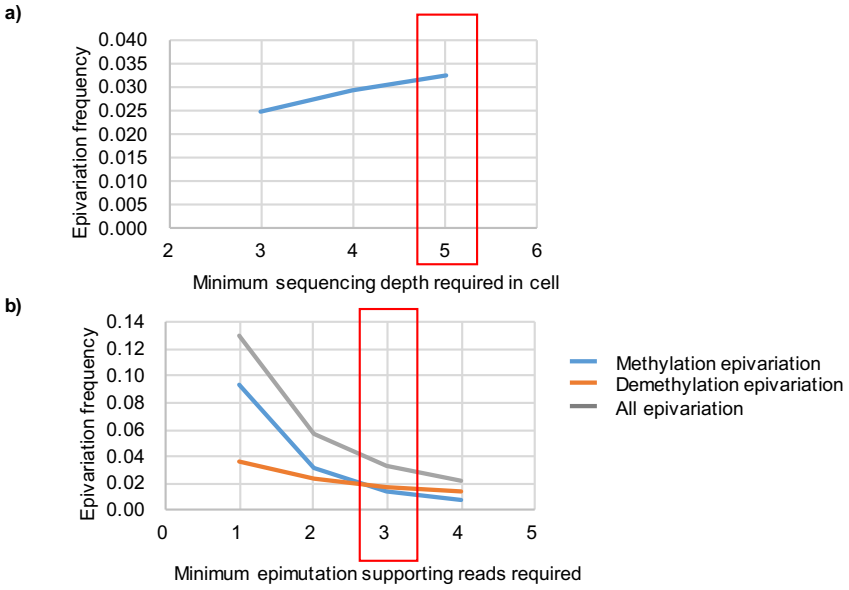

Figure S3. Criteria for epivariation calls. a) Effect of minimum sequencing depth (x-axis) on epivariation frequency estimation (y-axis). **b)** Effect of minimum epivariation supporting reads (x-axis) on epivariation frequency estimation (y-axis). Red boxes indicated final criteria.

Table S1. Bisulfite sequencing data processing summary.

Sample*	# raw reads	Mapping efficiency	Bisulfite conversion rate**	Sequence duplication level***	# CpG (depth>=1x)	Age (month)	Cell/bulk	cell type
f11	106,231,336	42.3%	98.7%	75.3%	3,785,324	-	cell	MEF
f12	110,901,598	51.1%	98.7%	56.0%	8,507,920	-	cell	MEF
f1b	132,443,151	58.0%	99.0%	16.8%	17,826,778	-	bulk	MEF
y21	51,858,580	38.6%	98.8%	92.4%	825,218	4	cell	Hepatocyte
y22	66,424,232	29.0%	97.6%	92.8%	456,387	4	cell	Hepatocyte
y2b	152,888,716	60.5%	98.9%	25.9%	19,050,595	4	bulk	Hepatocyte
y31	31,913,863	26.8%	98.6%	70.4%	1,890,962	4	cell	Hepatocyte
y32	43,059,862	13.8%	98.8%	84.3%	576,677	4	cell	Hepatocyte
y33	26,410,825	29.4%	98.9%	88.8%	461,319	4	cell	Hepatocyte
y3b	57,982,762	60.1%	99.0%	8.5%	11,740,589	4	bulk	Hepatocyte
o21	56,089,254	24.4%	98.7%	82.2%	1,425,722	26	cell	Hepatocyte
o22	50,744,157	22.5%	98.8%	79.2%	1,437,004	26	cell	Hepatocyte
o2b	156,376,935	55.9%	98.9%	32.7%	22,384,321	26	bulk	Hepatocyte
o31	58,584,746	24.0%	98.4%	81.8%	1,713,053	26	cell	Hepatocyte
o32	50,378,180	25.3%	98.6%	82.0%	831,435	26	cell	Hepatocyte
o3b	38,087,858	60.3%	98.9%	7.7%	8,869,698	26	bulk	Hepatocyte
f21	13,121,264	53.2%	99.1%	24.8%	4081273	-	cell	MEF
f22	11,189,137	49.6%	99.0%	25.6%	3307076	-	cell	MEF
f23	33,944,267	37.9%	98.9%	73.8%	2363888	-	cell	MEF
f2b	147,930,511	44.7%	99.0%	10.7%	24598670	-	bulk	MEF
o11	27,097,243	44.5%	98.7%	67.8%	2596479	26	cell	Hepatocyte
o12	41,395,901	22.3%	98.5%	58.0%	2666715	26	cell	Hepatocyte
o13	78,054,583	28.0%	97.9%	80.4%	2410838	26	cell	Hepatocyte
o14	57,368,890	46.8%	98.7%	80.1%	3337756	26	cell	Hepatocyte
o15	66,121,632	42.8%	98.5%	86.2%	2163080	26	cell	Hepatocyte
o16	28,019,317	39.2%	98.9%	64.3%	2767543	26	cell	Hepatocyte
o1b	478,304,583	56.8%	99.1%	30.1%	33654426	26	bulk	Hepatocyte
y11	41,893,787	18.3%	98.0%	69.4%	1506708	4	cell	Hepatocyte
y12	79,871,545	24.2%	97.8%	83.1%	1659713	4	cell	Hepatocyte
y13	42,767,824	25.3%	98.1%	76.1%	1590036	4	cell	Hepatocyte
y14	52,037,967	14.4%	98.0%	61.1%	2014092	4	cell	Hepatocyte
y15	56,839,977	38.3%	98.4%	83.4%	2005993	4	cell	Hepatocyte
y16	51,075,606	27.0%	98.1%	87.1%	902484	4	cell	Hepatocyte
y1b	369,426,587	55.8%	99.0%	23.4%	32332245	4	bulk	Hepatocyte

* fab/oab/yab - a indicates mouse individual a; b indicates cell id or bulk.

** Bisulfite conversion rate is estimated as the ratio between the number of non-CpG methylations and total non-CpGs

*** Duplicated sequences, mostly a result of PCR, were marked by bismark and removed.

Table S2. Comparision between our and previous single cell whole-genome bisulfite sequencing methods.

Method	Species	Single end or paired end	Analyzable reads	Bisulfite conversion rate	Unique CpGs Covered
Gravina	mouse	single-end	4,132,144	98.51%	2,203,258
Farlik*	human	paired-end	687,423	>99%	1,261,269
Farlik*	mouse	paired-end	719,999	>99%	1,028,520
Smallwood**	mouse	paired-end	3,393,033	97.53%	3,891,832

*Smallwood SA, Lee HJ, Angermueller C, Krueger F, Saadeh H, Peat J, et al. Nat Methods. 2014 Aug;11(8):817-20.

**Farlik M, Sheffield NC, Nuzzo A, Datlinger P, Schönegger A, Klughammer J, Bock C. Cell Rep. 2015 Mar 3;10(8):1386-97.

Table S3. Resource of genome annotations.

Annotation	Counts	Resource	Defined by or as
CpG island (repeat masked version)	16,027	UCSC	Irizarry, RA. Et al., *Nat Genet* , 2009
CpG island shore	32,053	-	2kb flanking CpG island
Protein coding gene transctiptionsites	76,835	Ensembl Biomart	Ensembl Biomart
Protein coding gene exon	383,868	Ensembl Biomart	Ensembl Biomart
Protein coding gene intron (consensus)	207,353	Based on gene and exon from Ensembl Biomart	-
Protein coding gene 5' utr	61,497	Ensembl Biomart	Ensembl Biomart
Protein coding gene 3' utr	56,117	Ensembl Biomart	Ensembl Biomart
Promoter	76,835	-	TSS to its 2kb upstream
Repeat DNA transposon	158,329	repeatMask	repeatMask
Repeat LINE	969,721	repeatMask	repeatMask
Repeat LTR	854,046	repeatMask	repeatMask
Repeat SINE	1,520,027	repeatMask	repeatMask
Repeat simple repeat	1,062,130	repeatMask	repeatMask
Repeat other	433,821	repeatMask	repeatMask
H3K27ac	38,492	ENCODE, ID:ENCSR000CDH	ENCODE
H3K27me3	33,402	ENCODE, ID:ENCSR000CEN	ENCODE
H3K36me3	88,353	ENCODE, ID:ENCSR000CEO	ENCODE
H3K4me1	77,192	ENCODE, ID:ENCSR000CAO	ENCODE
H3K4me3	16,888	ENCODE, ID:ENCSR000CAP	ENCODE
H3K79me2	68,593	ENCODE, ID:ENCSR000CEP	ENCODE
H3K9ac	29,230	ENCODE, ID:ENCSR000CEQ	ENCODE
Liver specific genes	58	Lin, S. et al., *PNAS* , 2014	Lin, S. et al., *PNAS* , 2014

A. Modeling methylation frequency using sliding windows

To quantify methylation frequency, we subdivided the genome using sliding windows of 3 kb in size and 600 bp in step size. Methylation counts of single CpGs within a window were pooled together. Windows with at least 5 CpGs covered are used in following analysis. Methylation frequency of a window i in sample (single cell or bulk) j was modeled as a binomial distribution. So methylation frequency is given as using Laplacian smoothing to avoid unreasonable 0 or 1 estimations when methylation count in a window is small,

$$\hat{m}_{i,j} = \frac{c^{+}_{i,j} + 1}{c^{+}_{i,j} + c^{-}_{i,j} + 2} \quad (1)$$

Where $c^{+}_{i,j}$ and $c^{-}_{i,j}$ are methylated counts and unmethylated counts separately. And the standard error is given as,

$$se_{i,j} = \sqrt{\frac{\hat{m}_{i,j} \cdot (1 - \hat{m}_{i,j})}{n_{i,j}}} \quad (2)$$

Where $n_{i,j}$ is the sum of $c^{+}_{i,j}$ and $c^{-}_{i,j}$.

B. Estimating heterogeneity level

To quantify heterogeneity level, we first paired each cell with its bulk (in the following text we refer it to "pairs"), and then used variance value and made two estimations – 1) global difference across all windows for one cell and bulk pair to estimate the difference between the cell and its corresponding bulk; 2) local variance for one window across all cells to estimate the heterogeneity level within the window. Both estimations were based on the following.

Considering the single cell – bulk data structure, we first gave weight to each single cell – bulk pair p at window i,

$$se_{i,p} = \sqrt{se^{2}_{i,c} + se^{2}_{i,b}} \quad (3)$$

$$w_{i,p} = se^{-2}_{i,p} \quad (4)$$

Where c refers to a single cell and b refers to its corresponding bulk.

In the first estimation, difference between one cell and its bulk is,

$$\hat{v}_c = \frac{\sum_i w_{i,p}}{(\sum_i w_{i,p})^2 - \sum_i w_{i,p}^2} \times \sum_i \left(w_{i,p} \times \left(\hat{m}_{i,pc} - \hat{m}_{i,pb} \right)^2 \right) \quad (5)$$

Where $\hat{m}_{i,pc}$ and $\hat{m}_{i,pb}$ refers to the estimated methylation frequency from the single cell c and the bulk b of the pair p separately. By this definition (equation 5), we quantified the difference between one cell to its bulk over all the genome.

To rule out a concern that $\hat{v}_c$, which we detected were caused by artifacts of difference in sequencing depth between a single cell and its bulk, we estimated the artificial heterogeneity level $\hat{v}_{noise}$ caused only by the above concern using a downsampling as the following. We first downsampled the bulk data to the cell level. Specifically, we randomly selected x number of methylation counts over the whole genome from bulk, where x refers to the number of methylation counts we obtained from a single cell in whole genome. Then, we calculated $\hat{v}_{noise}$ using equation (5) and the downsampling were performed 20 times for each cell. **Figure 3a** shows that $\hat{v}_c$ is significantly higher than $\hat{v}_{noise}$ for all cells. This indicates that difference between cell and bulk we observed is not due to the above technical concerns.

Similar to 1), cell-to-cell variance in window i is,

$$\hat{v}_i = \frac{\sum_p w_{i,p}}{(\sum_p w_{i,p})^2 - \sum_p w_{i,p}^2} \times \sum_p \left(w_{i,p} \times \left(\hat{m}_{i,pc} - \hat{m}_{i,pb} \right)^2 \right) \quad (6)$$

To minimize potential bias caused by sequencing depth and coverage, in $\hat{v}_i$ estimation, we downsampled methylation counts to a same coverage level that for each window and each sample we have 5 counts in a single cell and 20 in the corresponding bulk. Variance value are approximate as the variance values from the downsampling. Additionally, considering the data structure – 21 single hepatocytes-bulk pairs, we included windows with at least 10 pairs in each group passed criteria for the variance estimation (283,726 windows, about 11% of the genome), and the estimation is based on the top 10 best covered pairs in each group (**Figure 3c**). By this definition (equation 6), we quantified the variations of a specific region (window) among a group of single cells.

C. Defining differentially methylated windows

We used the following test statistic to identify differentially methylated windows, i, between a single cell and its corresponding bulk,

$$s_{i,p} = \frac{\hat{m}_{i,c} - \hat{m}_{i,b}}{se_{i,p}} \quad (7)$$

where $se_{i,p}$ is the pooled standard error of a single cell – bulk pair defined by (3). For windows passing all of the following criteria,

$$\hat{m}_{i,c} \cdot n_{i,c} > 5 \quad (8)$$

$$(1 - \hat{m}_{i,c}) \cdot n_{i,c} > 5 \quad (9)$$

$$\hat{m}_{i,\mathrm{b}} \cdot n_{i,\mathrm{b}} > 5 \quad (10)$$

$$(1 - \hat{m}_{i,\mathrm{b}}) \cdot n_{i,\mathrm{b}} > 5 \quad (11)$$

we applied two-sided z-test, and for windows don't pass the criteria, t-test. Thus, p values and confidence intervals (CIs) for windows were obtained. Windows with p value less than 0.0001 and the absolute value of two bonds confidence interval larger than 0.1, were defined as differentially methylated windows.

D. Estimating epivariation frequency

We define epivariation as methylation difference between a single cell and its bulk at a single CpG site, specifically, mostly (≥90%) methylated or unmethylated in bulk and multiple supporting reads (≥3) in a cell supporting the alternative methylation statues (in both cell and bulk requiring sequencing depth ≥ 5). Results under different depth and supporting reads criteria were presented in **Figure S3**. Thus epivariation frequency was calculated as a ratio between number of epivariation and CpG sites passed the depth criteria.

E. Others

Circos plot (**Figure 1a**) was generated using R package OmicCircos (Hu et al., 2014).

Supplemental References

Hu, Y., Yan, C., Hsu, C.H., Chen, Q.R., Niu, K., Komatsoulis, G.A., and Meerzaman, D. (2014). OmicCircos: A Simple-to-Use R Package for the Circular Visualization of Multidimensional Omics Data. Cancer informatics *13*, 13-20.