

1           **Single-cell chromatin profiling reveals demethylation-dependent**  
2           **metabolic vulnerabilities of breast cancer epigenome**

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13          Word count (including abstract, but excluding methods section, references and figure legends):

14          4,436

15          Total number of figures and tables: 6 figures, 6 supplementary figures, and 6 supplementary  
16          tables

17 **Abstract**

18 Metabolic reprogramming in cancer cells not only sustains bioenergetic and biosynthetic needs  
19 but also influences transcriptional programs, yet how chromatin regulatory networks are rewired  
20 by altered metabolism remains elusive. Here we investigate genome-scale chromatin remodeling  
21 in response to 2-hydroxyglutarate (2HG) oncometabolite using single-cell assay for transposase  
22 accessible chromatin with sequencing (scATAC-seq). We find that 2HG enantiomers differentially  
23 disrupt exquisite control of epigenome integrity by limiting  $\alpha$ -ketoglutarate ( $\alpha$ KG)-dependent DNA  
24 and histone demethylation, while enhanced cell-to-cell variability in the chromatin regulatory  
25 landscape is most evident upon exposure to L2HG enantiomer. Despite the highly heterogeneous  
26 responses, 2HG largely recapitulates two prominent hallmarks of the breast cancer epigenome,  
27 i.e., global loss of 5-hydroxymethylcytosine (5hmC) and promoter hypermethylation, particularly  
28 at tumor suppressor genes involved in DNA damage repair and checkpoint control. Single-cell  
29 mass cytometry further demonstrates downregulation of BRCA1, MSH2 and MLH1 in 2HG-  
30 responsive subpopulations, along with acute reversal of chromatin remodeling upon withdrawal.  
31 Collectively, this study provides a molecular basis for metabolism-epigenome coupling and  
32 identifies metabolic vulnerabilities imposed on the breast cancer epigenome.

## 33 Introduction

34 As a dynamic system, cancer cells continuously adapt to the fluctuating microenvironment by  
35 rerouting metabolic fluxes and evolve from early initiation through progression and  
36 dissemination<sup>1,2</sup>. Metabolic reprogramming in cancer cells facilitates energy production and  
37 macromolecular synthesis to fuel cell proliferation<sup>3,4</sup>. In addition to supporting bioenergetic and  
38 biosynthetic needs, altered metabolism involves promiscuous production of non-canonical  
39 metabolic intermediates, which have been described as metabolic waste products or metabolite  
40 damage<sup>5,6</sup>. Recent studies suggest that these previously uncharacterized metabolites including  
41 oncometabolites are linked to 'non-metabolic' signaling mechanisms in cell-type-specific fate  
42 decisions<sup>7</sup>.

43 The oncometabolite 2-hydroxyglutarate (2HG) occurs as two enantiomers and  
44 accumulates up to millimolar concentrations in a broad range of hematological and solid  
45 malignancies<sup>8,9</sup>. Somatic mutations in isocitrate dehydrogenase genes, *IDH1* and *IDH2*, found in  
46 glioma and acute myeloid leukemia (AML) result in stereospecific production of the D-enantiomer  
47 (D2HG)<sup>10,11</sup>, while breast tumors frequently exhibit elevated levels of 2HG despite the lack of *IDH*  
48 mutations<sup>12,13</sup>. Recent studies indicate that the L-enantiomer (L2HG) can accumulate under  
49 acidic<sup>14,15</sup> and hypoxic conditions<sup>16,17</sup> that often coexist in the tumor microenvironment, yet the  
50 potential sources and functions of L2HG are less well established. Both enantiomers structurally  
51 resemble  $\alpha$ -ketoglutarate ( $\alpha$ KG), a key intermediate in the Krebs cycle, and potentially antagonize  
52  $\alpha$ KG-dependent dioxygenases including ten-eleven translocation (TET) DNA hydroxylases and  
53 Jumonji domain-containing histone demethylase (JHDM) enzymes<sup>18-20</sup>, which catalyze oxidative  
54 demethylation of DNA and histone proteins, respectively.

55 Promoter hypermethylation and global loss of 5-hydroxymethylcytosine (5hmC) are two  
56 prominent hallmarks of the breast cancer epigenome<sup>21-23</sup>. Genome-wide depletion of 5hmC is  
57 frequently observed in a multitude of tumor types including breast cancer and is associated with  
58 poor patient survival<sup>24,25</sup>; however, its cause and pathological consequences are largely opaque.

59 Similarly, promoter hypermethylation of tumor suppressor genes has also been considered a  
60 common and driving event in breast malignancies. Loss-of-function mutations in DNA  
61 hydroxylases (*TET1*, *TET2* and *TET3*) or overexpression of DNA methyltransferases (*DNMT1*,  
62 *DNMT3A* and *DNMT3B*)<sup>26,27</sup>, and recently tumor hypoxia<sup>28</sup> have been reported to be associated  
63 with aberrant DNA methylation, yet the molecular origin of promoter hypermethylation in breast  
64 cancer remains obscure<sup>27,29</sup>.

65 Here we show that the oncometabolite 2HG disturbs the fine-tuned spatial regulation of  
66 the mammary epithelial epigenome, and initiates global loss of 5hmC and tumor-associated  
67 promoter hypermethylation by impairing  $\alpha$ KG-dependent demethylation. The findings provide a  
68 mechanistic framework in which altered metabolism mediates two pathological hallmarks of the  
69 breast cancer epigenome, thereby priming early epigenetic events to be exploited during the  
70 development of breast cancer, in particular basal-like subtype with high 2HG accumulation. By  
71 leveraging two single-cell approaches, our study further highlights a role for 2HG oncometabolites  
72 in the dynamics of cell-to-cell variability in the epigenome of this tumor type, whereby chromatin  
73 regulatory modules are highly vulnerable to metabolic derangements.

## 74 **Results**

### 75 **2HG enantiomers progressively modulate the mammary epigenome by limiting DNA and** 76 **histone demethylation**

77 To identify the regulatory mechanism involving cellular metabolism that mediates breast cancer  
78 development, we first analyzed the intracellular levels of D2HG and L2HG using enantiomer-  
79 selective liquid chromatography-mass spectrometry (LC-MS) following chiral derivatization of the  
80 analyte (Fig. 1a,b). Relatively higher levels of D2HG were observed in both ER $\alpha$ -positive and -  
81 negative breast cancer cell lines compared to benign and primary mammary epithelial cells,  
82 whereas elevated L2HG levels were predominant in ER $\alpha$ -negative breast cancer cells (Fig. 1c,d).  
83 In line with this, ER $\alpha$ -negative cells had higher levels of total 2HG (Supplementary Fig. 1a), which

84 is supported by prior studies showing preferential accumulation of 2HG in ER $\alpha$ -negative or basal-  
85 like tumors<sup>12,13,30</sup>. We next leveraged the recently developed comprehensive Cancer Cell Line  
86 Encyclopedia (CCLE) metabolomics database<sup>31,32</sup> and found that 2HG levels were remarkably  
87 elevated in basal-like tumor cells in comparison to other tricarboxylic acid (TCA) cycle metabolites  
88 that are structurally similar to one another and are potentially involved in  $\alpha$ KG-dependent  
89 dioxygenase reactions (Fig. 1e). Of note, our investigation of somatic mutations using two  
90 independent datasets from the Sanger Catalogue of Somatic Mutations in Cancer (COSMIC) and  
91 CCLE consortia showed no evidence for gain-of-function mutations in *IDH1* or *IDH2* among 62  
92 breast cancer cell lines, further corroborating the findings of infrequent *IDH* mutations and yet  
93 high accumulation of 2HG in breast tumors (Supplementary Fig. 1b)<sup>12,13,30,33</sup>. It should be noted,  
94 however, that recurrent *IDH2* mutations are found in a rare breast cancer subtype with elevated  
95 intratumor 2HG levels<sup>34,35</sup>.

96 To determine the global impact of oncometabolites on the chromatin landscape, primary  
97 human mammary epithelial cells (HMECs), which exhibited low levels of endogenous 2HG  
98 (Supplementary Fig. 1a), were exposed for 72 hr to the cell-permeable derivatives of D2HG and  
99 L2HG. Exposure to either of the two enantiomers led to a decrease in 5-hydroxymethylcytosine  
100 (5hmC) and reciprocal increase in 5-methylcytosine (5mC) across the LINE-1 repetitive sequence  
101 elements in the genome (Supplementary Fig. 1c), indicating competitive inhibition of TET-  
102 mediated oxidation of 5mC to 5hmC during the process of active DNA demethylation<sup>36</sup> (Fig. 1f).  
103 Similarly, immunofluorescence staining using a 5hmC-specific antibody showed a dose-  
104 dependent global loss of 5hmC in response to D2HG (Fig. 1g). In contrast, global levels of  
105 H3K27me3 histone methylation were elevated in proportion to the increasing concentrations of  
106 D2HG, via inhibition of JHDM-mediated histone demethylation (Fig. 1g,h and Supplementary Fig.  
107 1d). Our observations thus indicated that short-term exposure at a relatively low range was  
108 sufficient to induce a substantial change in the mammary epithelial epigenome.

109 Unlike DNA methylation, lysine residues on histone tails can be mono-, di- and tri-  
110 methylated (Supplementary Fig. 1d) and we therefore employed a multiplexed LC-MS assay to  
111 quantify changes in histone methylation. Exposure to 2HG enantiomers led to elevated  
112 methylation levels in both repressive (H3K27me3, H3K9me3 and H4K20me3) and permissive  
113 (H3K4me3, H3K36me3 and H3K79me3) histone marks as indicated by increases in mono-, di- or  
114 tri-methylation along with a reciprocal decrease in the levels of unmethylated lysine residues (Fig.  
115 1i, Supplementary Fig. 1e and Supplementary Table 1). Consistently, repressive methylation  
116 modifications on H3K27 residues were accumulated in relation to the increasing levels of  
117 intracellular 2HG in the CCLE breast cancer cell lines (Supplementary Fig. 1f). Of note, there was  
118 no detectable alteration in cell-cycle phase distribution upon 2HG supplementation  
119 (Supplementary Fig. 1g). Together, our findings support a model that the intratumor accumulation  
120 of 2HG oncometabolites progressively modulates the mammary epigenome independent of cell  
121 cycle progression, potentially leading to extensive chromatin remodeling.

### 122 **Single-cell profiling of chromatin accessibility reveals epigenetic heterogeneity in** 123 **response to 2HG enantiomers**

124 To delineate the genome-scale dynamics of epigenetic regulatory modules in response to  
125 oncometabolite exposure, we applied single-cell assay for transposase-accessible chromatin with  
126 high-throughput sequencing (scATAC-seq)<sup>37</sup>. First, individual single cells were isolated from  
127 HMECs cultured in media supplemented with either D2HG or L2HG using integrated fluidic  
128 circuits (Fig. 2a). Following cell lysis and tagmentation of open, accessible chromatin regions by  
129 Tn5 transposase, a total of 248 single-cell libraries were independently indexed with unique  
130 barcodes and sequenced as a single pool. scATAC-seq reads exhibited the expected periodicity  
131 of ~200-bp insert size fragments corresponding to nucleosome bands<sup>37,38</sup> (Fig. 2b). Additionally,  
132 aggregate scATAC-seq profiles of 248 single cells showed high concordance with the ensemble

133 measurement of the accessibility landscape profiled by DNase-seq (Fig. 2c and Supplementary  
134 Fig. 2a;  $r = 0.76$ ).

135 We next sought to globally visualize single-cell DNA accessibility profiles using principal  
136 component analysis (PCA), which indicated distinct cell subpopulations that were discernible not  
137 only between but also within the treatment groups (Fig. 2d and Supplementary Fig. 2b). Cell-to-  
138 cell variability in the chromatin landscape was likewise detected among cells receiving the same  
139 treatment by using cell similarity matrix analysis based on highly accessible genomic regions  
140 (Supplementary Fig. 2c). In contrast, replicate samples were similarly distributed despite being  
141 processed in different experiments (Supplementary Fig. 2d), further supporting the technical  
142 robustness and reproducibility of the method. Interestingly, the first principal component (PC1)  
143 broadly separated L2HG-exposed cells from control and D2HG-exposed cells that were  
144 distinguishable, albeit to a lesser extent, alongside the PC2 direction (Fig. 2d), indicating that the  
145 two enantiomers could differentially modulate the chromatin organization. Furthermore, L2HG-  
146 exposed cells were clearly separated into different subpopulations oriented in the opposite  
147 direction on the PC2 axis and showed a more variable distribution compared to unexposed control  
148 cells, suggesting a substantial increase in cell-to-cell variability following L2HG exposure.

149 Consistent with the observation that epigenetic heterogeneity was most evident among  
150 L2HG-exposed cells, model-based clustering following PCA identified three major cell subsets in  
151 L2HG-exposed cells, referred to as L1, L2 and L3, along with a total of five distinct clusters among  
152 the 248 single cells (Fig. 2e and Supplementary Fig. 2e). When scATAC-seq data were  
153 aggregated, notable differences in chromatin accessibility were observed throughout the genome  
154 between L2HG subgroups (Supplementary Fig. 2f). Moreover, inspection of genome-wide  
155 distribution of scATAC-seq signals over CpG islands ( $n = 28,690$ ) and H3K4me3 peaks ( $n =$   
156  $33,116$ ) revealed a marked difference between L1 versus L2 and L3 subsets (Fig. 2f). In  
157 agreement, Cluster 1 comprised cells from all three experimental groups that were clustered in  
158 close proximity with similar multidimensional phenotypes (Fig. 2d,e), implying L1 subset being a

159 cell subpopulation that is potentially less sensitive to oncometabolite perturbation. This was  
160 contrasted with no pronounced differences in scATAC-seq accessibility profiles either across CpG  
161 islands or H3K4me3 sites detected between the three experimental groups (Supplementary Fig.  
162 2g). Together, single-cell DNA accessibility profiling revealed that the two enantiomers could  
163 distinctly modulate the mammary epigenome and drive cell-to-cell epigenetic diversity in the  
164 chromatin regulatory landscape.

### 165 **2HG depletes promoter accessibility at highly methylated genes in breast cancer**

166 To identify disease-relevant epigenetic signatures imposed upon 2HG exposure, we further  
167 interrogated our scATAC-seq data using publicly available large-scale datasets including  
168 Encyclopedia of DNA Elements (ENCODE)<sup>39</sup>, Roadmap Epigenomics<sup>40</sup>, Cancer methylome<sup>41</sup> and  
169 The Cancer Genome Atlas (TCGA)<sup>42</sup> (Fig. 3a). We first determined the global occupancy of open,  
170 accessible chromatin regions by utilizing the 12-state chromatin segmentation defined in  
171 HMECs<sup>43</sup>. As expected, ATAC-seq signals in unexposed control cells were remarkably enriched  
172 in active/weak gene promoters and strong enhancers but were significantly underrepresented in  
173 inactive genomic regions such as heterochromatin and repetitive regions, both of which were  
174 associated with nuclear lamina<sup>40</sup> (Supplementary Fig. 3a). Following 2HG exposure, chromatin  
175 accessibility was reduced in genomic regions enriched with permissive chromatin marks  
176 (H3K4me3, H3K27ac and H3K9ac)<sup>40</sup>, such as active/weak promoters and strong/weak enhancers  
177 (Fig. 3b). In contrast, genomic regions that are largely devoid of permissive chromatin marks  
178 displayed a substantial increase in chromatin accessibility, suggesting that oncometabolites can  
179 have two opposing impacts on the mammary epigenome, i.e., selective loss of accessibility in  
180 active or poised chromatin and gain of accessibility in repressive or quiescent chromatin states.  
181 The overall alterations in the chromatin landscape dynamics were particularly prominent in  
182 response to L2HG, suggesting L2HG to be more potent in modulating the mammary epigenome.



183 This is in line with earlier biochemical studies indicating that L2HG can competitively antagonize  
184  $\alpha$ KG-dependent dioxygenases to a greater extent<sup>18-20</sup>.

185 To address the sparse nature of scATAC-seq data<sup>37,38</sup>, we investigated regulatory  
186 variation in single-cell measurements by aggregating scATAC-seq signals over transcription start  
187 sites (TSS) in the genome. As shown in Fig. 3c with each color representing DNA accessibility  
188 around TSS regions (n = 20,242) in a single cell, the chromatin occupancy pattern in untreated  
189 control cells was highly concordant whereas the peaks were found to be diffused and  
190 heterogeneous in exposed cells, suggesting enhanced epigenetic variability in regulatory  
191 modules imposed by 2HG. However, the differences between exposed and unexposed cells were  
192 not quantitatively remarkable when chromatin accessibility was assessed over all TSS regions  
193 across the entire genome. In contrast, a marked reduction in accessibility was detected when a  
194 set of highly methylated genes (n = 150) in breast tumors<sup>44</sup> was investigated (Fig. 3d and  
195 Supplementary Fig. 3b). Similar results were obtained using genes that were independently found  
196 to be hypermethylated in promoter regions<sup>28</sup> (n = 150), indicating that 2HG led to reduced  
197 chromatin accessibilities in gene promoters displaying DNA hypermethylation in breast cancer. In  
198 contrast, no significant accessibility changes were observed in sets of genes that were highly  
199 methylated in HMECs (data not shown) or other tumor types including endometrial cancer. Next,  
200 we investigated epigenetic variability among the three L2HG subpopulations and found that L2  
201 and L3 subsets exhibited a significant decrease in accessibility in comparison to L1 subset, which  
202 is consistent with earlier observations that L1 may represent a cell subpopulation that is potentially  
203 less responsive to oncometabolite perturbation (Fig. 3e). Collectively, single-cell chromatin  
204 profiling suggests that 2HG disrupts the fine-tuned spatial control of the mammary epigenome  
205 and reshapes the chromatin accessibility atlas of regulatory modules, potentially leading to DNA  
206 methylator phenotype.

207 **2HG-mediated chromatin remodeling is linked to transcriptional repression and adverse**  
208 **prognosis associated with tumor hypermethylation**

209 To assess the clinical relevance of tumor-associated hypermethylated genes that exhibited  
210 concomitant chromatin compaction in response to 2HG, we first analyzed RNA-seq whole-  
211 transcriptome profiles of 521 breast tumors and 112 adjacent uninvolved tissues from the TCGA  
212 cohort (Fig. 4a). Upon unsupervised hierarchical clustering, the majority of highly methylated  
213 genes appeared to be downregulated in all five PAM50 molecular subtypes, although no statistical  
214 significance was observed in luminal A tumors (Fig. 4a and Supplementary Fig. 4a). Strikingly,  
215 transcriptional repression was most evident in basal-like breast cancer, 84% of which displayed  
216 a triple-negative phenotype (i.e., negative for expression of estrogen, progesterone and  
217 HER2/neu receptors). These findings are in agreement with earlier results showing that 2HG  
218 levels were elevated predominantly in ER $\alpha$ -negative or basal-like breast cancer. In addition, none  
219 of the breast cancer patients investigated had 2HG-producing mutations in either the *IDH1* or  
220 *IDH2* gene (Fig. 4a).

221 We next adopted functional pathway enrichment analyses to examine the physiological  
222 role of tumor-associated DNA hypermethylation. Ingenuity pathway analysis (IPA)<sup>45</sup> revealed  
223 enrichment of genes involved in hereditary breast cancer signaling, reproductive system disease  
224 and gene expression (Fig. 4b and Supplementary Fig. 4b). In line with IPA analysis, 39% of the  
225 highly methylated genes were linked to DNA-templated transcription in Gene Ontology (GO)  
226 biological processes (Fig. 4c). In addition to DNA damage response (DDR), the most enriched  
227 GO pathways included lipid metabolic process, oxidation-reduction, fat cell differentiation and fatty  
228 acid beta-oxidation, all of which were relevant to intracellular metabolic signaling (Supplementary  
229 Fig. 4c and Supplementary Table 2), and therefore suggest an intimate entwining of metabolic  
230 derangements with DNA hypermethylation in breast cancer.

231 To investigate whether altered expression of hypermethylated genes can impact the  
232 clinical outcome in breast cancer patients, we performed survival analysis of the TCGA breast

233 cancer cohort. Approximately 10% of the genes exhibited a significant association between low  
234 expression and shorter disease-free survival (DFS) (Supplementary Fig. 4d). Strikingly, their  
235 combination displayed a steeper drop in survival (HR = 2.24; 95% CI, 1.54 to 3.24;  $P = 0.00003$ ),  
236 compared to when the genes were analyzed as single variables (Fig. 4d). This suggests that  
237 concurrent downregulation of hypermethylated genes may have an additive adverse effect on  
238 patient prognosis. We further assessed the outcome of tumor hypermethylation on patient survival  
239 and found that the patients with a concurrent hypermethylation signature suffered decreased DFS  
240 (Fig. 4e). Together, the results suggest that 2HG-mediated loss of promoter accessibility, DNA  
241 methylator phenotype and concurrent transcriptional repression may present a high competing  
242 risk of mortality in breast cancer patients.

#### 243 **2HG enantiomers initiate tumor-associated promoter hypermethylation**

244 To investigate whether transcriptional repression of hypermethylated genes is correlated with  
245 chromatin accessibility landscape, we leveraged the recently reported ATAC-seq data of 70  
246 primary breast cancers from the TCGA cohort<sup>46</sup>. We found that the hypermethylated genes with  
247 transcriptional repression (boxed in Fig. 4a) showed a decrease in chromatin accessibility in  
248 basal-like or HER2-enriched breast cancers in comparison to luminal A, luminal B and normal-  
249 like tumors (Fig. 5a,b). We next sought to investigate if tumor-associated chromatin landscape  
250 could be attributable to 2HG-mediated epigenetic remodeling. To this end, we analyzed open  
251 chromatin signals corresponding to ChromHMM-annotated active promoter regions. The  
252 scATAC-seq peaks in unexposed control cells were comparable with DNase-seq and  
253 H2AFZ/H2A.Z signals that are localized at gene promoters facilitating RNA polymerase II  
254 occupancy<sup>47</sup>, while accessibility peaks were significantly reduced in D2HG- or L2HG-exposed  
255 cells (Fig. 5c). This finding suggests that oncometabolites could induce a decrease in chromatin  
256 accessibility at promoter regions of highly methylated genes that are downregulated in breast  
257 cancer. Additionally, whole-genome bisulfite sequencing (WGBS) data indicated that accessible

258 promoter regions were essentially devoid of DNA methylation in control HMECs. These  
259 observations prompted us to investigate whether 2HG can initiate tumor-associated promoter  
260 hypermethylation accompanied by restricted chromatin accessibility evident in breast tumors.

261 To evaluate DNA methylation at gene promoters, we utilized oxidative bisulfite (oxBS)  
262 conversion followed by pyrosequencing. DNA methylation levels across promoter CpG sites of  
263 tumor suppressor genes including but not limited to DNA damage response (DDR) genes were  
264 elevated in 2HG-exposed cells (Fig. 5d). Specifically, a marked methylation gain was observed in  
265 the majority of genes examined including *BRCA1*, *MSH2* and *MLH1*. Promoter hypermethylation  
266 in response to 2HG was confirmed by methylated DNA immunoprecipitation followed by qPCR  
267 (MeDIP-qPCR) (Supplementary Fig. 5a). A similar, albeit less pronounced, accumulation of *de*  
268 *novo* DNA methylation was also seen in immortalized, non-transformed mammary epithelial cells  
269 (hTERT-HME1) (Supplementary Fig. 5b). To further address the disease relevance of 2HG-  
270 mediated promoter hypermethylation, we evaluated our breast cancer cohort consisted of 77  
271 primary tumors and 10 uninvolved tissue specimens<sup>48</sup>. Methyl-CpG binding domain proteins  
272 followed by sequencing (MBDCap-seq) indicated accumulation of DNA methylation at target  
273 promoter regions in tumor samples in comparison with their normal counterparts (Supplementary  
274 Fig. 5c). Collectively, these data suggest that 2HG induces loss of promoter accessibility  
275 accompanied by DNA hypermethylation of tumor suppressor genes, which is a prominent  
276 hallmark of the breast cancer epigenome.

### 277 **Single-cell mass cytometry reveals epigenetic plasticity and phenotypic heterogeneity**

278 To disentangle epigenetically heterogeneous responses to 2HG at single-cell resolution, we next  
279 performed high-dimensional mass cytometry<sup>49,50</sup> on viably cryopreserved cell suspensions from  
280 2HG-exposed or unexposed control HMECs as well as cells exposed to 2HG followed by 5-day  
281 withdrawal (Supplementary Fig. 6a,b). The panel of metal-conjugated antibodies was designed  
282 to detect both chromatin modifications and intracellular proteins including *BRCA1*, *MSH2* and

283 MLH1 that are involved in DDR and checkpoint signaling (Supplementary Fig. 6a). Consistent  
284 with mass spectrometry measurements (Fig. 1i and Supplementary Fig. 1e), mass cytometry  
285 analysis showed that 2HG exposure led to a substantial increase in all histone markers  
286 investigated (Supplementary Fig. 6c). The biaxial gating of live single cells indicated a concurrent  
287 increase in multiple distinct classes of histone methylation and the presence of cell populations  
288 that were potentially less responsive to 2HG oncometabolites (Supplementary Fig. 6c,d).

289 To further characterize cell-to-cell variability in the chromatin landscape, we next applied  
290 nonlinear dimensionality reduction using *t*-distributed stochastic neighbor embedding (*t*-SNE)  
291 analysis. The *t*-SNE visualization revealed that control and withdrawal groups had similar  
292 multidimensional phenotypes and cell density distributions, which were readily distinct from those  
293 of 2HG-perturbed cells (Fig. 6a). Accordingly, altered chromatin modifications were effectively  
294 reverted by 2HG withdrawal (Supplementary Fig. 6c,d), suggesting that chromatin remodeling  
295 imposed by 2HG is essentially reversible. Self-organizing maps generated by FlowSOM further  
296 identified six discrete clusters as shown in Fig. 6b-d. Strikingly, a large population of HMECs  
297 (Cluster 1) displayed a concomitant increase in histone markers following 2HG exposure and  
298 reciprocal decrease upon withdrawal (Fig. 6e).

299 We next asked if 2HG-induced chromatin remodeling correlated with altered expression  
300 of DDR genes displaying promoter hypermethylation in response to 2HG (Fig. 5d). As expected,  
301 2HG-responsive Cluster 1 cell subsets exhibited diminished expression of BRCA1, MSH2 and  
302 MLH1, which was restored upon withdrawal (Fig. 6f). In addition, BRCA1 downregulation was  
303 inversely correlated with H4K20 methylation (Supplementary Fig. 6e) and, to our surprise, we  
304 observed a remarkable association between the expression of the *BRCA1* and *MSH2* genes in  
305 Cluster 1 (Fig. 6g). Interestingly, this positive association was also evident in the CCLE cancer  
306 cell lines as well as in the TCGA breast cancer cohort (Fig. 6h,i). Although the precise mechanism  
307 is currently unknown, the findings may suggest that the two DDR genes are controlled by a shared  
308 set of transcriptional regulators, possibly via epigenetic modulators. Taken together, the

309 multidimensional investigation of chromatin regulators directly implicates that the epigenome  
310 reprogramming imposed by 2HG and subsequent downregulation of DDR genes associated with  
311 tumor hypermethylation could contribute to the pathogenesis of breast malignancies with high  
312 2HG accumulation.

### 313 **Discussion**

314 Breast cancer cells accumulate high levels of 2HG with preferential concentration of L2HG in  
315 basal-like subtypes. Here we show that either of the 2HG enantiomers is sufficient to broadly  
316 confer two hallmarks of the breast cancer epigenome, i.e., global loss of 5hmC and promoter  
317 hypermethylation. Following 2HG exposure, promoter regions of the tumor suppressor genes  
318 involved in DDR signaling displayed reduced chromatin accessibility accompanied by methylation  
319 gain. The associated downregulation of BRCA1, MSH2 and MLH1 in 2HG-responsive cell  
320 subpopulations was validated by mass cytometry. Dysfunctional DDR pathway is one of the  
321 inherent characteristics of 'BRCAness', which is commonly seen in basal-like breast tumors and  
322 is manifested by an enhanced mutation rate and genomic instability<sup>51</sup>. Besides 2HG-mediated  
323 inhibition of active DNA demethylation, recent studies show that 2HG can directly inhibit  $\alpha$ KG-  
324 dependent DDR signaling<sup>19,52,53</sup> or indirectly alter expression of DNA repair genes<sup>54,55</sup>, suggesting  
325 that intratumor accumulation of 2HG potentially induces DDR deficiency via multiple signaling  
326 pathways. It is of interest to note in this context that 2HG exposure has been reported to establish  
327 the BRCAness phenotype in clinically pertinent models including patient-derived glioma cell lines  
328 and primary AML bone marrow cultures<sup>55</sup>. These findings may be relevant for designing treatment  
329 strategies for breast cancer patients with high intratumor 2HG, since effector pathways such as  
330 defective DDR signaling could represent an alternate targetable vulnerability in specific tumor  
331 subtypes with a stem cell-like transcriptional signature<sup>30</sup>, other than counteracting 2HG  
332 overproduction per se, for instance by using IDH small molecule inhibitors.

333           Aside from promoter hypermethylation, we show that 2HG inhibits a multitude of histone  
334 demethylases resulting in accumulation of both repressive and permissive chromatin marks.  
335 Further, genome-scale profiling of chromatin accessibility revealed selective loss of accessibility  
336 in active or poised chromatin and gain of accessibility in repressive or quiescent chromatin states  
337 in response to 2HG. These results indicate previously uncharacterized, two opposing effects of  
338 the oncometabolites imposed on the cellular epigenome, suggesting multifaceted entwining of  
339 cancer metabolism with epigenetic regulation. Moreover, alterations in open chromatin occupancy  
340 were detected at enhancer and insulator regions. Of note, intratumor D2HG has been shown to  
341 dysregulate insulator or chromatin boundary function and promote aberrant gene-enhancer  
342 interaction, leading to constitutive activation of the glioma oncogene *PDGFRA*<sup>56</sup>. The findings  
343 therefore suggest that metabolic perturbations in breast cancer may modulate key aspects of  
344 chromatin functions including the three-dimensional (3D) genome topology.

345           Tumor heterogeneity is implicated in a wide range of neoplastic events involved in tumor  
346 evolution, dissemination, relapse or drug resistance<sup>2</sup>. Characterization of the molecular origin of  
347 intratumor diversity is thus integral to understanding and harnessing tumor heterogeneity and may  
348 provide new opportunities to define tumor subtypes or to design effective treatment. While genetic  
349 alterations have provided early insights into tumor heterogeneity, less attention has been paid to  
350 epigenetic diversity in breast cancer. In this study, scATAC-seq was applied to address cell-to-  
351 cell variability in the chromatin regulatory landscape and we found that the two enantiomers  
352 induced distinct chromatin accessibilities in the mammary epigenome. It is noted in this context  
353 that L2HG-exposed cells exhibited greater variability in comparison to D2HG-exposed cells, which  
354 is supported by the observations that hypoxic induction of L2HG could contribute to the  
355 development of epigenetic heterogeneity in glioblastoma<sup>17</sup>. Furthermore, single-cell profiling of  
356 epigenetic modifications by mass cytometry identified multiple cell subpopulations differentially  
357 responding to 2HG enantiomers. These observations suggest that metabolic dysfunction may

358 enhance epigenetic cell-to-cell variability in chromatin regulatory modules, which can functionally  
359 and dynamically contribute to intratumor heterogeneity.

360 Of note, our findings and previous studies have suggested that high 2HG breast tumors  
361 without *IDH* mutations are associated with poor survival<sup>30</sup>, whereas a rare breast cancer subtype  
362 carrying *IDH* mutations has been reported to show better prognosis<sup>34,35</sup>. Consistently, prolonged  
363 survival has been observed in glioblastoma and anaplastic astrocytoma patients with *IDH*  
364 mutations<sup>57,58</sup>. These observations imply that the pathological mechanisms of high 2HG tumors  
365 may vary in the presence or absence of *IDH* mutations, which can be partly ascribed to the  
366 different potencies of the two enantiomers, i.e., L2HG is relatively more potent than D2HG that is  
367 produced by mutated IDH enzymes<sup>18-20</sup>. Alternatively, L2HG induction has been shown to be more  
368 sensitive to metabolic disturbances and generated via promiscuous enzymatic activity under  
369 hypoxic<sup>16,17</sup> or acidic conditions<sup>14,15</sup>, which may yield cumulative adverse impacts on patient  
370 prognosis. Additionally, L2HG has been shown to promote the development of epigenetic cell-to-  
371 cell variability<sup>17</sup>. Intriguingly, more recent studies indicate that JHDM histone demethylases can  
372 directly sense cellular oxygen levels to regulate cell fate decisions<sup>59,60</sup>. In analogy, it might be  
373 plausible that  $\alpha$ KG-dependent dioxygenases including TET DNA hydroxylases and JHDM histone  
374 demethylases could serve as metabolic sensors by detecting intratumor 2HG levels, which may  
375 reflect profound metabolic disturbances accompanied by underlying acidosis and hypoxia  
376 implicated in the tumor microenvironment.

377 Collectively, the present study substantiates that 2HG imposes metabolic footprints on the  
378 mammary epithelial epigenome by impairing DNA and histone demethylation, and provides a  
379 molecular basis in which altered metabolism leads to loss of 5hmC and associated promoter  
380 hypermethylation, thereby priming early epigenetic events to be exploited during breast cancer  
381 development (Fig. 6j). This in turn suggests that defective metabolic fluxes can disrupt epigenetic  
382 homeostasis resulting in loss of chromatin integrity. Nonetheless, 2HG-induced epigenetic  
383 liabilities are found to be dynamic and essentially reverted upon withdrawal, highlighting the



384 inherent plasticity of the cellular epigenome. Finally, our findings suggest that chromatin and its  
385 regulatory modules are highly vulnerable to intracellular metabolic cues and future investigation  
386 on metabolism-epigenome coupling may lead to the identification of key molecular signatures that  
387 determine breast cancer susceptibility.

## 388 **Methods**

### 389 **Cell lines, growth conditions and reagents**

390 Human breast cancer cell lines BT20, BT474, MCF7, MDA-MB-157, MDA-MB-231 and MDA-MB-  
391 361, and non-malignant immortalized epithelial cells hTERT-HME1, 184B5 and MCF12A were  
392 acquired from the American Type Culture Collection (ATCC). Unless otherwise stated, cancer cell  
393 lines were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum (Sigma-  
394 Aldrich) and 100 U/ml penicillin plus 100 µg/ml streptomycin (Gibco) as previously reported<sup>61,62</sup>.  
395 Primary HMECs were obtained from Invitrogen and maintained at low passage number (below  
396 5). HMECs, hTERT-HME1, 184B5 and MCF12A cells were cultured in mammary epithelial growth  
397 medium according to the manufacturer's instructions. Authentication of cell line genomic DNA  
398 was performed at ATCC using DNA fingerprint analysis of polymorphic, short tandem repeat  
399 sequences. Exposure to cell-permeable 2HG analogues was carried out by supplementing octyl  
400 esters of *R*-2-hydroxyglutarate or *S*-2-hydroxyglutarate (Cayman Chemical) to the culture medium  
401 at a final concentration 72 hr before harvesting. Dimethylxalylglycine (DMOG) was obtained from  
402 Sigma-Aldrich. Culture medium was replaced daily with fresh complete medium with or without  
403 oncometabolite supplementation.

### 404 **Metabolite extraction and quantification by LC-MS**

405 Following dissociation, cells were washed twice with ice-cold phosphate-buffered saline (PBS)  
406 and cell pellets were flash-frozen on dry ice. For αKG analysis, metabolites were extracted with  
407 80:20 methanol:water (-80°C) containing stable isotope-labeled internal standard [1,2,3,4-<sup>13</sup>C<sub>4</sub>]α-  
408 ketoglutaric acid (Cambridge Isotope Laboratories) and incubated at -80°C for 1 hr as described  
409 previously<sup>63,64</sup>. Extracts were then centrifuged at 13,800g for 10 min and supernatants were  
410 transferred to glass autosampler vials for high-performance liquid chromatography-electrospray  
411 ionization-mass spectrometry (HPLC-ESI-MS) measurements. For 2HG analysis, cells were  
412 processed as mentioned above except that [1,2,3,4-<sup>13</sup>C<sub>4</sub>]L-malic acid (Cambridge Isotope

413 Laboratories) was added as an internal standard and dried extracts were derivatized with diacetyl-  
414 L-tartaric anhydride (DATAN, Sigma-Aldrich). HPLC-ESI-MS detection was conducted on a  
415 ThermoFisher Q Exactive mass spectrometer with on-line separation by a ThermoFisher Dionex  
416 Ultimate 3000 HPLC. HPLC conditions for  $\alpha$ KG analysis were: column, Synergi Polar-RP, 4  $\mu$ m,  
417 2x150 mm (Phenomenex); mobile phase A, 0.1% formic acid in water; mobile phase B, 0.1%  
418 formic acid in acetonitrile; flow rate, 250  $\mu$ l/min; gradient, 1% B to 5% B over 5 minutes, 5% B to  
419 95% B over 1 min and held at 95% B for 2 min. HPLC conditions for 2HG analysis were: column,  
420 Luna NH2, 3  $\mu$ m, 2x150 mm (Phenomenex); mobile phase A, 5% acetonitrile in water containing  
421 20 mM ammonium acetate and 20 mM ammonium hydroxide, pH 9.45; mobile phase B,  
422 acetonitrile; flow rate, 300  $\mu$ l/min; gradient, 85% B to 1% B over 10 min and held at 1% B for 10  
423 min. The conditions used to selectively quantify D2HG and L2HG were: column, Kinetex C18, 2.6  
424  $\mu$ m, 2.1x100 mm (Phenomenex); mobile phase, 1% acetonitrile with 125 mg/l ammonium formate,  
425 pH 3.6; flow rate, 400  $\mu$ l/min. Full scan mass spectra were acquired in the orbitrap using negative  
426 ion detection over a range of m/z 100–800 at 70,000 resolution (m/z 300). Metabolite identification  
427 was based on accurate mass match to the library  $\pm$ 5 ppm and agreement with the HPLC retention  
428 time of authentic standards. Quantification of metabolites was carried out by integration of  
429 extracted ion chromatograms with the corresponding standard curves.

### 430 **Immunofluorescence staining**

431 Cells were plated in 8-well chamber slides (Falcon) at a density of  $1-2 \times 10^4$  cells/well at least 24  
432 hr prior to 2HG exposure. Cells were then fixed with 4% paraformaldehyde (PFA) in PBS for 10  
433 min at room temperature (RT) and permeabilized with 0.2% Triton X-100 in PBS for 10 min at RT.  
434 For 5hmC staining, permeabilized cells were treated with 2N HCl for 30 min at RT and neutralized  
435 with 100 mM Tris-HCl (pH 8.5). Nonspecific binding was blocked with 10% goat serum in 0.2%  
436 Triton X-100 and PBS for 1 hr at RT and stained with primary antibodies in PBS with 5% goat  
437 serum and 0.2% Triton X-100 overnight at 4°C. After incubation with Alexa Fluor-conjugated

438 secondary antibodies (Molecular Probes) for 1 hr at RT, nuclei were stained for 5 min with DAPI  
439 (Sigma-Aldrich). Single optical sections were acquired using a Zeiss LSM710 confocal  
440 microscope and image quantification was performed with NIH ImageJ software (version 1.52n).  
441 Primary antibodies included rabbit polyclonal anti-5hmC (1:500; 39769, Active Motif) and rabbit  
442 monoclonal anti-H3K27me3 (1:800; 9733, Cell Signaling Technologies).

#### 443 **Tet-assisted bisulfite (TAB) pyrosequencing**

444 TAB pyrosequencing was used to differentiate 5hmC from 5mC<sup>65</sup>. High molecular weight genomic  
445 DNA was extracted using Genra Puregene reagents (Qiagen), followed by an additional ethanol  
446 precipitation and resuspension in low-EDTA TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).  
447 RNase A and proteinase K digestion were included in the isolation procedure. UV absorbance  
448 was measured on a NanoDrop 2000 (ThermoFisher) and each DNA sample was routinely  
449 examined by agarose gel electrophoresis with GelRed staining to ensure the absence of  
450 contaminating RNA and degradation of genomic DNA. Isolated genomic DNA was then subjected  
451 to Tet-assisted bisulfite (TAB) treatment as we previously described<sup>66</sup>. After bisulfite conversion  
452 using EpiTect Fast Bisulfite Conversion kit (Qiagen), pyrosequencing was conducted on a  
453 PyroMark Q96 MD instrument using CpG LINE-1 assay (973043, Qiagen). To monitor bisulfite  
454 conversion efficiency, a C outside a CpG site was added within dispensation order for the  
455 sequence to be analyzed as a built-in control. The quantitative levels of 5mC and 5hmC for each  
456 CpG dinucleotide were determined using PyroMark CpG software (version 1.0, Qiagen).

#### 457 **Multiplexed chromatin profiling by mass spectrometry**

458 Nuclei were isolated from  $2 \times 10^6$  cells using Nuclear Isolation Buffer (NIB) composed of 15 mM  
459 Tris-HCl (pH 7.5), 60 mM KCl, 15 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 250 mM sucrose, 0.3%  
460 NP-40, 1 mM DTT plus 10 mM sodium butyrate added immediately prior to use, for 30 min on ice.  
461 Nuclei were pelleted at 600g for 5 min at 4°C and detergent was removed by washing twice with

462 NIB without NP-40. Histones from isolated nuclei were acid extracted with 5 volumes of 0.2 M  
463 H<sub>2</sub>SO<sub>4</sub> for 1 hr at RT. Cellular debris was removed by centrifugation at 4,000g for 5 min.  
464 Trichloroacetic acid was added to the supernatant at a final concentration of 20% (v/v) and  
465 incubated for 1 hr to precipitate histone proteins. Histones were pelleted at 10,000g for 5 min,  
466 washed once with 0.1% HCl in acetone, twice with 100% acetone followed by centrifugation at  
467 15,000g for 5 min, and then briefly air-dried. Histones were derivatized, digested and analyzed  
468 by targeted LC-MS/MS as described previously<sup>67-69</sup>.

### 469 **Flow cytometry**

470 Cell cycle phase distribution was analyzed by flow cytometry. Cells were fixed with ice-cold 70%  
471 methanol for 1 hr on ice. Following centrifugation, cells were washed with PBS and stained with  
472 10 µg/ml propidium iodide (Sigma-Aldrich) solution in PBS containing 1 µg/ml DNase-free RNase  
473 A (ThermoFisher) and incubated in the dark on ice for 1 hr. Samples were then processed on a  
474 BD FACSCalibur flow cytometer equipped with CellQuest Pro software (version 5.2.1, Becton  
475 Dickinson) and data were analyzed using FlowJo software (version 7.6.5, Tree Star).

### 476 **Single-cell ATAC-seq library preparation**

477 Single-cell ATAC-seq libraries were prepared on a Fluidigm C1 workstation using 'ATAC Seq-  
478 Cell Load and Stain Rev C' script as previously described<sup>37</sup> with modifications. Briefly, cells were  
479 passed through a 20 µm cell strainer (CellTrics, Sysmex Partac) to remove debris and remaining  
480 cell aggregates and mixed at a ratio of 7:3 with C1 suspension reagent. The resulting single-cell  
481 suspension was loaded on C1 Single-Cell Open App IFC chip (1862x, 10-17 µm, Fluidigm) at a  
482 concentration of 350 cells/µl. Captured cells were stained with 2 µM green-fluorescent calcein-  
483 AM and 4 µM red-fluorescent ethidium homodimer-1 (Molecular Probes) and visualized under an  
484 EVOS FL cell imaging station (Life Technologies) to ensure successful capture and to determine  
485 cell viability. The single-cell capture rates were typically >80% and >90% of captured single cells

486 were alive. After cell lysis and Tn5 transposition, 8 cycles of pre-amplification were run on IFC  
487 chip. Pre-amplified PCR products were transferred to 96-well plates and further amplified for an  
488 additional 13 cycles using custom Nextera dual-index primers and NEBNext High-Fidelity 2X PCR  
489 master mix (New England Biolabs). Individually barcoded libraries were pooled and purified on a  
490 single MinElute column (Qiagen). The quality and size distribution of pooled libraries were  
491 evaluated on an Agilent 2100 Bioanalyzer using High Sensitivity DNA reagents (Agilent).

### 492 **Single-cell ATAC-seq data analysis**

493 scATAC-seq libraries were sequenced on a NextSeq 500 platform with High Output reagents  
494 (Illumina) using paired-end 75-bp reads. All scATAC-seq data were preprocessed as essentially  
495 described<sup>37</sup>. In short, adapter and primer sequences were trimmed and initial quality control  
496 checks were performed using FastQC tools ([https://www.bioinformatics.babraham.ac.uk/  
497 projects/fastqc/](https://www.bioinformatics.babraham.ac.uk/projects/fastqc/)). Sequencing reads were aligned to the GRCh37/hg19 assembly of the human  
498 genome using Bowtie2 (ref. <sup>70</sup>) with the parameter '-X2000' to ensure paired reads were within 2  
499 kb of one another. PCR duplicates were eliminated using Picard tools (version 2.9.2,  
500 <http://broadinstitute.github.io/picard/>) and alignments with mapping quality less than 30 were  
501 subsequently removed by samtools. Reads mapped to the mitochondria and unmapped contigs  
502 were filtered out and excluded from further analysis. PCA projections of scATAC-seq profiles were  
503 performed using SCRAT<sup>71</sup>, and gene feature was applied to aggregate sequencing reads from  
504 each cell, in which 3,000 bp upstream to 1,000 bp downstream of TSS is regarded as the region  
505 of interest for each gene. After aggregation, the signals for each feature were normalized to adjust  
506 for library size and model-based clustering (mclust) module was utilized to identify cell  
507 subpopulations. Peak calling was performed using MACS2 with the following settings: --nomodel  
508 --nolambda --keep-dup all --call-summits. Artifact signals were excluded using ENCODE  
509 blacklist<sup>72</sup>. Circular visualization of ATAC-seq signals was carried out by employing Circos tools  
510 (version 0.69-6, <http://circos.ca>).

## 511 **Breast cancer cohorts, resources and data analysis**

512 Level 3 TCGA Breast Invasive Carcinoma (BRCA) data of tumor and normal samples were  
513 accessed from the Broad GDAC Firehose (<http://gdac.broadinstitute.org>) and RSEM-normalized  
514 RNA-seq values were log<sub>2</sub> transformed before analysis. Unsupervised hierarchical clustering was  
515 utilized to distinguish mRNA expression profiles among different genes and heat maps were  
516 generated using heatmap.2 function implemented in gplots package of R statistical program.  
517 Clinical data including PAM50 intrinsic subtypes, ER/PR/HER2 expression and IDH mutation  
518 status were retrieved using the Cancer Genomics cBioPortal<sup>73</sup> and were integrated into RNA-seq  
519 heat map. TCGA DNA methylation data generated using Infinium Human Methylation 450K  
520 (HM450K) BeadChip array were retrieved from the cBioPortal database. Normalized methylation  
521 scores at each CpG dinucleotide are expressed as  $\beta$  values, representing a continuous  
522 measurement from 0 (completely unmethylated) to 1 (completely methylated). In the event of  
523 multiple CpG probes per gene, the most negatively correlated with mRNA expression was  
524 selected. Chromatin accessibility data of TCGA primary tumor tissue samples were extracted from  
525 the UCSC Xena browser (<https://xenabrowser.net/>). After z-scale normalization of ATAC-seq  
526 signals, open chromatin occupancies at promoter regions were correlated with PAM50 gene  
527 signature to evaluate DNA accessibility profiles across breast cancer subtypes. Our breast cancer  
528 methylome data generated using MBDCap-seq are available at The Cancer Methylome System  
529 (<http://cbbiweb.uthscsa.edu/KMethylomes/>). Global chromatin profiling and metabolomics  
530 datasets were retrieved from the Broad Institute CCLE (<https://portals.broadinstitute.org/ccle>).

## 531 **Oxidative bisulfite (oxBS) pyrosequencing**

532 To selectively detect 5mC modification, genomic DNA was subjected to oxBS conversion<sup>74</sup> using  
533 TrueMethyl oxBS module (NuGEN Technologies) as per the manufacturer's recommendations.  
534 In short, genomic DNA was affinity-purified using 80% acetonitrile (Fisher Scientific) and  
535 TrueMethyl magnetic beads to eliminate potential contaminating compounds. After the

536 denaturation step, genomic DNA was oxidized to convert 5-hydroxymethylcytosine to 5-  
537 formylcytosine. Bisulfite treatment was then carried out to convert 5-formylcytosine to uracil,  
538 leaving 5-methylcytosine intact. Following desulfonation and purification, converted DNA was  
539 quantified using Qubit ssDNA assay (Invitrogen). PCR amplification of oxBS converted DNA was  
540 carried out with biotin-labeled primers. Primer design was carried out using PyroMark Assay  
541 Design software (version 2.0, Qiagen). Pyrosequencing of biotinylated PCR products was  
542 performed using PyroMark Q48 Advanced CpG reagents (Qiagen) on a Pyromark Q48 Autoprep  
543 apparatus (Qiagen) following the manufacturer's protocol. 5mC levels at CpG sites were  
544 determined using PyroMark Q48 Autoprep software (version 2.4.2, Qiagen) in CpG Assay mode.  
545 All samples were prepared, amplified and sequenced in triplicates. PCR and pyrosequencing  
546 primers are listed in Supplementary Tables 3 and 4.

#### 547 **Methylated DNA precipitation PCR (MeDIP-qPCR)**

548 Prior to the 5mC immune-capture procedure, genomic DNA was fragmented to an average length  
549 of 200-600 bp using a Covaris 220 system. MeDIP was performed using MeDIP reagents (Active  
550 Motif) as per the manufacturer's instructions. In brief, fragmented DNA was heat-denatured and  
551 immunoprecipitated with anti-5mC antibody (39649, Active Motif). An additional quantity of  
552 fragmented DNA equivalent to 10% of DNA being used in the immunoprecipitation reaction was  
553 also denatured and saved as input DNA. Immunoprecipitated DNA and input DNA were then  
554 purified with phenol/chloroform extraction and amplified using GenoMatrix Whole Genome  
555 Amplification kit (Active Motif). Quantitative PCR was performed using PowerUP SYBR Green  
556 master mix on an ABI StepOnePlus real-time PCR instrument (Applied Biosystems). All PCR  
557 reactions were run in triplicates. The relative enrichment of target sequences after MeDIP was  
558 evaluated by calculating the ratios of the signals in immunoprecipitated DNA versus input DNA.  
559 Locus-specific primers were designed with NCBI Primer-BLAST and synthesized by Integrated  
560 DNA Technologies. Primer sequences are provided in Supplementary Table 5.



## 561 **Panel design and heavy-metal labeling of antibodies**

562 Prior to antibody conjugation, the antibody panel was designed by allocating targets to specific  
563 heavy-metal isotopes depending on the sensitivity of the mass cytometer, e.g., assigning low  
564 abundance targets to high sensitivity channels in order to minimize potential spectral overlap<sup>75</sup>.  
565 Subsequently, in-house conjugation of antibodies was performed using Maxpar X8 antibody  
566 labeling reagents (Fluidigm) as previously described<sup>76</sup> with some modifications. Briefly, up to 100  
567 µg of carrier-free IgG antibody was subjected to buffer exchange by washing with R-buffer using  
568 a 50 kDa Amicon filter (Millipore) that was pre-soaked with R-buffer. Antibodies were then partially  
569 reduced with 4mM TCEP (ThermoFisher) for 30 min at RT followed by washing with C-buffer. In  
570 parallel, metal chelation was carried out by adding lanthanide metal solutions (Fluidigm) to  
571 chelating polymers (Fluidigm) in L-buffer. Metal-loaded polymers were then washed with L-buffer  
572 and concentrated on a 3 kDa Amicon filter (Millipore). Partially reduced antibodies were incubated  
573 with metal-loaded polymers for 90 min at RT followed by washing with W-buffer. Following  
574 conjugation, antibody concentration was determined by spectrometry with a NanoDrop 2000  
575 (ThermoFisher). Metal-conjugated antibodies were stored in antibody stabilization solution  
576 (Candor Bioscience) supplemented with 0.05% sodium azide at 4°C. The panel of metal-  
577 conjugated antibodies is provided in Supplementary Table 6.

## 578 **Multidimensional chromatin profiling by mass cytometry**

579 Cell suspensions were prepared at a concentration of  $1 \times 10^7$  cells/ml in serum-free, protein-free  
580 medium and stained with 1 µM cisplatin (195Pt) for 5 min at RT to determine cell viability. After  
581 quenching with CyTOF buffer composed of PBS with 1% BSA (Invitrogen), 2mM EDTA (Ambion)  
582 and 0.05% sodium azide (Teknova), staining with lanthanide-conjugated antibodies was  
583 performed as previously described<sup>50</sup>, but with the following modifications. In brief, following  
584 extracellular marker staining, cells were fixed with 1.6% PFA (Electron Microscopy Sciences) for  
585 15 min at RT and permeabilized with ice-cold methanol (Fisher Scientific) for 30 min at 4°C. After

586 adding Fc receptor blocker (BioLegend), cells were labeled overnight at 4°C with a cocktail of  
587 antibodies recognizing chromatin modifications or intracellular components. On the next day,  
588 excess of antibodies were washed off with CyTOF buffer and cells were stained with 250 nM  
589 191/193Ir-containing DNA intercalator (Fluidigm) in PBS with 1.6% PFA for 30 min at RT. After  
590 resuspending in double-deionized water, samples were kept on ice. Immediately prior to  
591 acquisition, cells were prepared at a concentration of 0.2-1.0x10<sup>6</sup> cells/ml in 0.1X EQ bead  
592 solution containing four element calibration beads (Fluidigm) and filtered through a 20 µm cell  
593 strainer (CellTrics, Sysmex Partac) to remove any potential aggregates. Cells were then acquired  
594 at a rate of 300-500 events/s using a Helios mass cytometer (Fluidigm) and CyTOF software  
595 (version 6.7) with noise reduction, a lower convolution threshold of 400, event length limits of 10-  
596 150 pushes, a sigma value of 3 and a flow rate of 0.030 ml/min.

### 597 **Mass cytometry data analysis**

598 Data analysis was conducted using the cloud-based platform Cytobank<sup>77</sup> and the statistical  
599 programming environment R. Following data acquisition, mass cytometry data were normalized  
600 using EQ calibration beads as previously described<sup>78</sup>. Bead-normalized data were then uploaded  
601 onto Cytobank platform to carry out initial gating and population identification using the indicated  
602 gating schemes (Supplementary Fig. 6b). For downstream analysis, live single cells were  
603 identified based on 140Ce bead, event length, DNA content (191Ir) and live/dead (195Pt)  
604 channels. Histograms and two-dimensional contour plots were generated to assess the global  
605 levels of chromatin modifications across the samples. Using an equal number of randomly  
606 selected live singlets from each sample, dimensionality reduction was implemented by *t*-SNE  
607 analysis with the following settings: perplexity = 60, theta = 0.5, iteration = 1,000. FlowSOM  
608 clustering was carried out on the same data using the standard parameters to quantify changes  
609 in cell subsets in an unbiased manner. The 2D coordinates of the *t*-SNE map were fed to  
610 FlowSOM analysis for population identification based on hierarchical consensus clustering.

611 Comparisons of chromatin modifications among the samples in each cluster were performed by  
612 generating heat maps in R using gplots package and median signal intensities extracted from  
613 Cytobank.

#### 614 **Statistical analysis**

615 Pairwise comparisons were carried out with a two-tailed unpaired Student's *t*-test and multiple  
616 comparisons were assessed using a one-way ANOVA followed by Dunnett's multiple comparison  
617 post-hoc test unless otherwise indicated in the figure legends. For Kaplan-Meier survival analysis,  
618 expression or methylation values were classified as high or low by using the median as a cutoff  
619 value and disease-free survival data was used to measure prognosis. Log-rank (Mantel-Cox) test  
620 was used to evaluate statistical differences and hazard ratio was reported with 95% confidence  
621 interval. Statistical analyses were performed using GraphPad Prism program (version 8.1). For  
622 all statistical analyses, differences of  $P < 0.05$  were considered statistically significant. All  
623 quantitative data are presented as mean  $\pm$  s.e.m. unless specified otherwise.

#### 624 **Data availability**

625 The raw and processed single-cell ATAC-seq data have been deposited in the National Center  
626 for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) and are available under  
627 accession GSE135412. The R code used in the study is available upon request from the authors.  
628 All other data described, analyzed and represented in the figures that support the findings of this  
629 study are available from the corresponding authors upon request.

#### 630 **Acknowledgements**

631 We thank all laboratory members for helpful discussions and technical assistance. We are grateful  
632 to the BioAnalytics and Single-Cell Core (BASiC) for single-cell analysis, Mass Spectrometry Core  
633 for metabolite mass spectrometry, Optical Imaging Facility for confocal imaging, Genome

634 Sequencing Facility for next-generation sequencing and Flow Cytometry Shared Resource  
635 Facility at the University of Texas Health Science Center at San Antonio for flow cytometry,  
636 Northwestern Proteomics Core Facility for proteomics analyses. This study was supported by NIH  
637 grants U54CA217297, P30CA054174 and CPRIT grant RP150600. J.R. acknowledges funding  
638 from the US National Science Foundation (ABI 1565076). M.K. is a recipient of the CPRIT  
639 predoctoral fellowship (RP170345).

#### 640 **Author contributions**

641 T.H.M.H., K.M. and M.K. jointly conceived the project, designed the experiments, interpreted the  
642 results and prepared the manuscript with contributions from all co-authors. M.K. carried out the  
643 majority of experiments and data analysis. J.R., M.Z. and C.L.L assisted with scATAC-seq data  
644 analysis, computational modeling and statistical methods. K.M. and M.K. established the methods  
645 for chromatin mass cytometry. C.M.W, N.D.L and N.K assisted with validation of metal-tagged  
646 antibodies. All authors read and approved the manuscript.

#### 647 **Competing interests**

648 T.H.M.H holds stock options and is on the medical advisory board of LiSen Imprinting Diagnostics  
649 Wuxi Co., Ltd. All other authors have no competing interests.

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818 **Figure legends**

819 **Fig. 1 | 2HG imposes a global loss in 5hmC and progressive gain in histone methylation**  
820 **on the mammary epithelial epigenome. a**, Schematic of reductive conversion of  $\alpha$ KG to the  
821 enantiomers of 2HG. 2HG is stereospecifically produced from  $\alpha$ KG by mutant IDH proteins  
822 (mIDH1/2) or by promiscuous enzymatic reactions. **b**, Extracted-ion chromatogram showing chiral  
823 derivatives of 2HG detected in breast cancer cells. **c,d**, Chiral LC-MS analysis of the two  
824 enantiomers D2HG (**c**) and L2HG (**d**) in breast cancer cell lines, benign and normal mammary  
825 epithelial cells (n = 3 independent replicates). **e**, Intratumor levels of  $\alpha$ KG, 2HG, succinate and  
826 fumarate in the CCLE breast cancer cell lines.  $\alpha$ KG, 2HG, succinate and fumarate are structurally  
827 similar to one another and are potentially involved in DNA and histone demethylation processes.  
828 \* $P < 0.05$  versus basal-like tumor cells by one-way ANOVA with Dunnett's multiple comparison  
829 test. **f**, Schematic of 2HG-mediated inhibition of  $\alpha$ KG-dependent DNA demethylation by the TET  
830 family of DNA hydroxylases. TET enzymes catalyze oxidation of 5mC to 5hmC with oxygen and  
831  $\text{Fe}^{2+}$  as cofactors. **g**, Global levels of 5hmC and H3K27me3 following 72-hr exposure to D2HG.  
832 Line plot is shown for the mean intensity of 5hmC (blue) and H3K27me3 (red) assayed by  
833 immunofluorescence staining. \*\*\* $P < 0.001$  versus control cells by one-way ANOVA with  
834 Dunnett's multiple comparison test (n = 8 images per condition). ns, not significant. At least 60  
835 nuclei were examined and signal intensities were normalized to DAPI nuclear counterstain. **h**,  
836 Representative immunofluorescence images of HMECs after 72-hr exposure to D2HG at the  
837 indicated concentrations. Scale bar, 10  $\mu\text{m}$ . **i**, Fold change in different types of histone methylation  
838 upon 2HG exposure. Following 72-hr exposure to 100  $\mu\text{M}$  of either D2HG or L2HG, global levels  
839 of methylated/unmethylated lysine residues were assayed by multiplexed mass spectrometry.  
840 While two major histone H3 variants H3.1 and H3.3 are highly similar in their amino acid  
841 composition, H3.1 is predominantly localized at heterochromatin, which is in contrast to the  
842 preferential H3.3 accumulation in intragenic regions of transcribed genes. \* $P < 0.05$ , \*\* $P < 0.01$ ,  
843 \*\*\* $P < 0.001$  versus control unexposed HMECs by two-tailed unpaired Student's *t*-test with Holm-

844 Sidak correction for multiple comparisons (n = 3 independent replicates). Error bars in column  
845 charts or line plot represent s.e.m.  $\alpha$ KG,  $\alpha$ -ketoglutarate; 2HG, 2-hydroxyglutarate; IDH, isocitrate  
846 dehydrogenase; ER, estrogen receptor; LC-MS, Liquid chromatography-mass spectrometry;  
847 CCLE, Cancer Cell Line Encyclopedia; HER2, human epidermal growth factor receptor 2; 5hmC,  
848 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; TET, ten-eleven translocation; me0,  
849 unmethylated; me1, monomethylated; me2, dimethylated; me3, trimethylated histone lysine  
850 residues.

851 **Fig. 2 | Single-cell ATAC-seq identifies epigenetically heterogeneous responses to 2HG**  
852 **exposure. a**, Schematic view of the scATAC-seq protocol. Culture medium was replaced daily  
853 by freshly prepared media with or without oncometabolite supplementation (arrowheads). **b**, Insert  
854 size distribution of ATAC-seq fragments from a single cell displaying characteristic nucleosome-  
855 associated periodicity. Diagnostic insert sizes for mononucleosome, dinucleosome and  
856 trinucleosome are labeled. **c**, Representative genome browser tracks showing open chromatin  
857 regions in HMECs detected by scATAC-seq, which are highly consistent with DNase  
858 hypersensitive sites (DHS) detected by bulk DNaseI-seq (GSE29692). Single cell profiles are  
859 shown below the aggregated profiles. **d**, Principal component analysis (PCA) of 248 single cells  
860 to identify cell subpopulations based on the chromatin accessibility landscape. Ctrl (n = 83),  
861 D2HG- (n = 90) and L2HG-exposed (n = 75) cells are colored by sample type. Each data point  
862 represents a single cell. **e**, Proportions of cell subpopulations identified by model-based  
863 clustering. Cell clusters are color-coded as indicated. **f**, Whole-genome heat maps showing  
864 enrichment of scATAC-seq signals in L2HG cell subsets over a 20-kb region centered on CpG  
865 islands and H3K4me3 peak summits. Each row represents one individual genomic locus and is  
866 sorted by decreasing scATAC-seq signal. The color represents the intensity of chromatin  
867 accessibility. Intervals flanking indicated feature are shown in kilobases. ATAC, assay for  
868 transposase accessible chromatin; HMECs, human mammary epithelial cells.

869 **Fig. 3 | 2HG decreases chromatin accessibility at genes that are highly methylated in breast**  
870 **cancer patients. a**, Workflow of the integration of scATAC-seq data with high-throughput omics  
871 datasets to characterize clinically relevant epigenetic signatures imposed upon 2HG exposure. **b**,  
872 Distribution of ATAC-seq signals across ENCODE-ChromHMM functionally annotated regions in  
873 response to 2HG perturbation. **c**, Metagene analysis of chromatin accessibility assayed by  
874 scATAC-seq. Epigenomic accessibility landscape is centered on TSS across the genome ( $n =$   
875 20,242 genes). Colors represent individual cells. Solid lines indicate mean values and semi-  
876 transparent shade around the mean curve shows s.e.m. across the region. While a sharp and  
877 abrupt peak is evident over TSS in unexposed control cells, accessibility peaks are distorted in  
878 2HG-exposed cells (arrowheads). **d**, Chromatin accessibility profiles for highly methylated genes  
879 in the TCGA cancer cohorts. scATAC-seq profiles in 2HG-exposed and unexposed cells are  
880 centered on TSS of highly methylated genes ( $n = 150$  genes) in breast cancer (two independent  
881 datasets) and endometrial cancer patients. Colors represent single cells (left) and average  
882 accessibility profiles in each experimental condition (right). **e**, Chromatin accessibility profiles in  
883 L2HG subpopulations across TSS of highly methylated genes ( $n = 150$  genes) in the TCGA  
884 cancer cohorts.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$  versus unexposed control cells by one-way  
885 ANOVA followed by Dunnett's multiple comparison test. ENCODE, Encyclopedia of DNA  
886 Elements; ChromHMM, chromatin Hidden Markov Modeling; TSS, transcriptional start site;  
887 TCGA, The Cancer Genome Atlas; Txn transition, transcriptional transition; Txn elongation,  
888 transcriptional elongation; Weak Txn, weak transcribed; CNV, copy number variation.

889 **Fig. 4 | Tumor-associated DNA hypermethylation is linked to transcriptional repression**  
890 **and poor patient prognosis in basal-like breast cancer. a**, Heat map depicting mRNA  
891 expression of highly methylated genes whose promoter regions showed diminished chromatin  
892 accessibility following 2HG exposure. The column dendrogram indicates unsupervised  
893 hierarchical clustering of 150 genes that are highly methylated in the TCGA breast cancer cohort.

894 Rectangle outlined in white represent genes downregulated in basal-like tumors. Vertical sidebars  
895 indicate the category of each patient with regard to *IDH1/2* mutations (m*IDH1/2*), ER, PR, HER2  
896 and PAM50 status. **b**, Overrepresented canonical signaling and metabolic pathways from IPA  
897 profiling of highly methylated genes in the TCGA breast cancer cohort. Colored bars (top x axis)  
898 represent  $-\log_{10} P$  values obtained by Fisher's exact test whereas the black line (bottom x axis)  
899 indicates the ratio between the number of genes compared to the total number of genes in a given  
900 pathway. **c**, Pie chart indicating GO biological processes enriched in genes associated with tumor  
901 hypermethylation. See also Supplementary Table 2. **d,e**, Prognostic significance of concurrent  
902 transcriptional repression (**d**) and hypermethylation (**e**) of genes shown in Supplementary Figure  
903 4d. Disease-free survival (DFS) was investigated using Kaplan-Meier analysis, and log-rank  
904 (Mantel-Cox)  $P$  values and hazard ratios (HR) are shown (see Methods for further details). TCGA,  
905 The Cancer Genome Atlas; ER, estrogen receptor; PR, progesterone receptor; HER2, human  
906 epidermal growth factor receptor 2; PAM50, prediction analysis of microarray 50; IPA, Ingenuity  
907 Pathways Analysis; GO, Gene Ontology.

908 **Fig. 5 | 2HG induces DNA hypermethylation of tumor suppressor genes accompanied by**  
909 **loss of promoter accessibility. a**, Heat map showing a decrease in chromatin accessibility of  
910 hypermethylated genes ( $n = 150$ ) in basal-like and HER2-enriched breast cancer subtypes.  
911 Rectangle outlined in white represents genes downregulated in basal-like tumors as indicated in  
912 Fig. 4a. Vertical sidebar indicates five different molecular subtypes. **b**, Quantitative comparison  
913 of chromatin accessibility of hypermethylated genes across the TCGA breast cancer subtypes.  
914 The upper and lower whiskers indicate the minimum and maximum values of the data, center  
915 lines indicate the median, and the first and third quartiles are indicated by the bottom and top  
916 edges of the boxes respectively.  $***P < 0.001$  versus basal-like tumors by one-way ANOVA with  
917 Dunnett's multiple comparison test: basal-like ( $n = 15$ ), HER2-enriched ( $n = 11$ ), luminal A ( $n =$   
918 18), luminal B ( $n = 25$ ) and normal-like ( $n = 1$ ) subtypes. **c**, IGV genome browser tracks showing

919 diminished chromatin accessibility at gene promoters following 2HG exposure. ChromHMM-  
920 defined chromatin states (GSE38163), DNaseI-seq (GSE29692), H2AFZ/H2A.Z ChIP-seq  
921 (GSE29611), WGBS profiles (GSE86732) from HMECs are shown. Arrows indicate TSS and  
922 direction of transcription initiation, and y-axes show read coverage. Histone variant H2A.Z is  
923 enriched around TSS. **d**, Promoter methylation measured by oxBS pyrosequencing.  $*P < 0.05$ ,  
924  $**P < 0.01$ ,  $***P < 0.001$  versus control by one-way ANOVA with Dunnett's multiple comparison  
925 test ( $n \geq 3$  individual replicates). Error bars denote s.e.m. Validation by MeDIP-qPCR was also  
926 done in the same samples and promoter hypermethylation following 2HG exposure was  
927 confirmed using hTERT-HME1 cells (see Supplementary Figure 5a,b). IGV, Integrative Genomics  
928 Viewer; ChromHMM, chromatin Hidden Markov Modeling; WGBS, whole-genome bisulfite  
929 sequencing; TSS, transcriptional start site; oxBS, oxidative bisulfite; MeDIP, methylated DNA  
930 immunoprecipitation.

931 **Fig 6 | Single-cell mass cytometry demonstrates downregulation of hypermethylated**  
932 **genes in 2HG-responsive cell subpopulations.** **a**, Representative *t*-SNE plots showing levels  
933 of histone modification markers in response to 2HG exposure. C, unexposed control cells; D, cells  
934 exposed to D2HG for 72 hr; L, cells exposed to L2HG for 72 hr; LW, cells exposed to L2HG for  
935 72 hr followed by 5-day withdrawal of L2HG. Each data point on the *t*-SNE maps represents an  
936 individual cell and its color corresponds to cellular levels of each marker assessed. Density plots  
937 at the bottom show cell population distribution in each experimental group (~10,000 cells per  
938 condition). **b**, *t*-SNE plot of HMECs from all experimental groups merged. Each data point is  
939 colored by condition. **c**, *t*-SNE projections of epigenetically distinct cell subsets defined by  
940 FlowSOM. Cells are colored according to the cluster they were assigned to using consensus  
941 hierarchical clustering in FlowSOM analysis. **d**, Bar chart showing changes in cell frequency  
942 distribution across different treatment groups. **e**, Heat maps depicting epigenetically  
943 heterogeneous responses upon 2HG exposure. Normalized median values of signal intensities

944 are shown for each cluster. Pie charts indicate the proportion of cells from different experimental  
945 groups in each cluster. **f**, Violin plots showing expression levels of the *BRCA1*, *MSH2* and *MLH1*  
946 genes in Cluster 1.  $**P < 0.01$ ,  $***P < 0.001$  versus control by one-way ANOVA followed by  
947 Dunnett's multiple comparison test. **g,h,i**, Scatter plots showing the correlation between the  
948 expression of *BRCA1* and *MSH2* in the mass cytometry Cluster 1 (**g**), CCLE breast cancer cell  
949 lines (**h**) and TCGA breast cancer cohort (**i**). Data in **g** are transformed to arcsinh scales with the  
950 cofactor of 5 and four experimental groups (C, D, L and LW) are indicated by different colors. In  
951 the CCLE and TCGA datasets, red and brown dots indicate cancer cell lines or tumors with  
952 mutations in the *BRCA1* and *MSH2* genes respectively. Spearman's correlation coefficient ( $r$  with  
953  $P$  value) is indicated at the bottom of the panel. **j**, A model for metabolic rewiring of the breast  
954 cancer epigenome. TCA cycle metabolite  $\alpha$ KG enhances TET and JHDM enzyme activities  
955 facilitating active DNA and histone demethylation such that promoter regions of tumor suppressor  
956 genes (TSG) remain accessible. Cancer metabolism accompanied with intratumor accumulation  
957 of 2HG oncometabolites, which antagonize DNA hydroxylases or histone demethylases, may  
958 drive global loss of 5hmC and promoter hypermethylation leading to DNA methylator phenotype.  
959 L2HG exposure could also confer enhanced cell-to-cell variability in the chromatin regulatory  
960 landscape by reversibly remodeling the breast cancer epigenome, potentially contributing to  
961 extensive intratumor heterogeneity. *t*-SNE, *t*-distributed stochastic neighbor embedding;  
962 FlowSOM, flow cytometry data analysis using self-organizing maps.

Figure 1

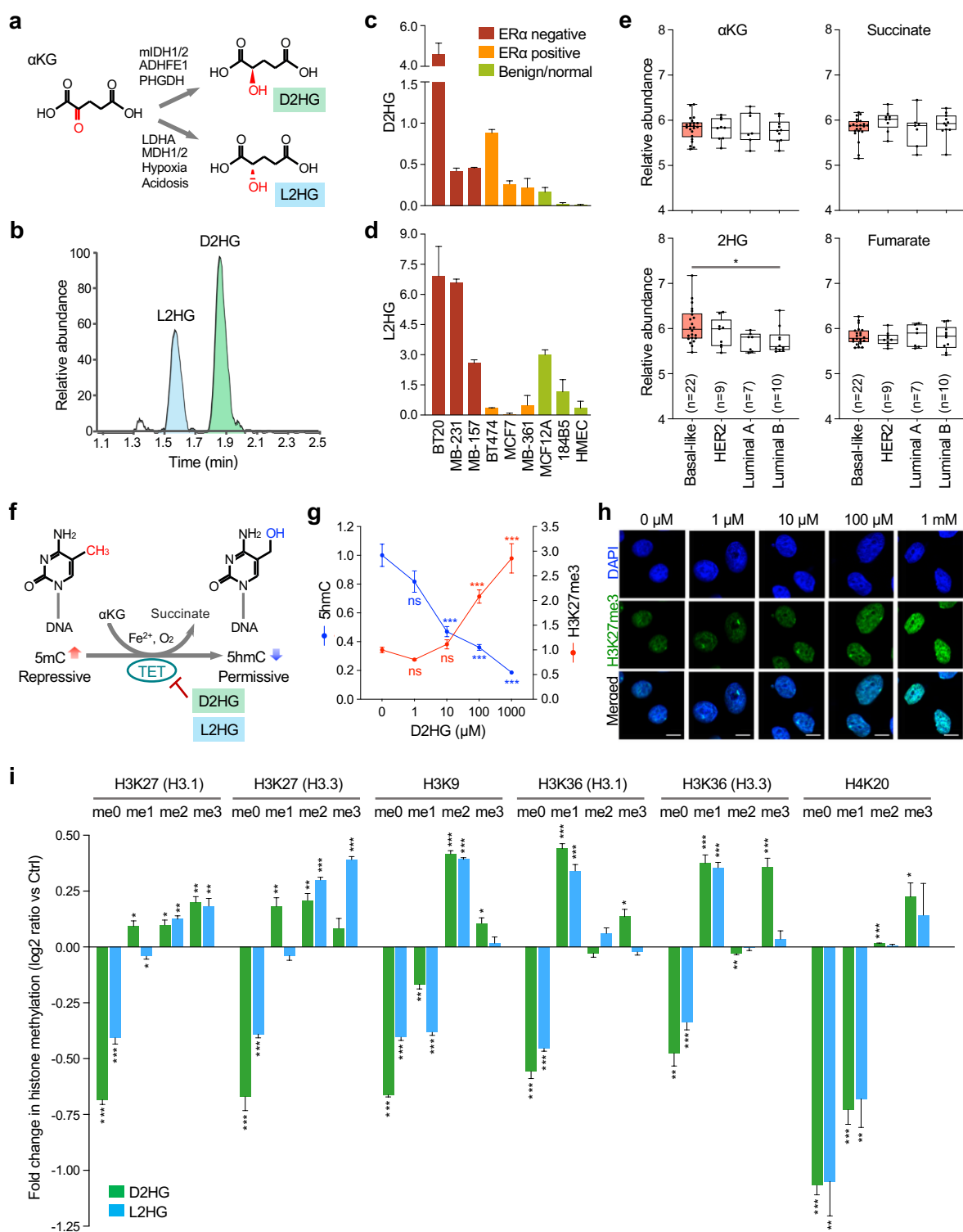




Figure 2

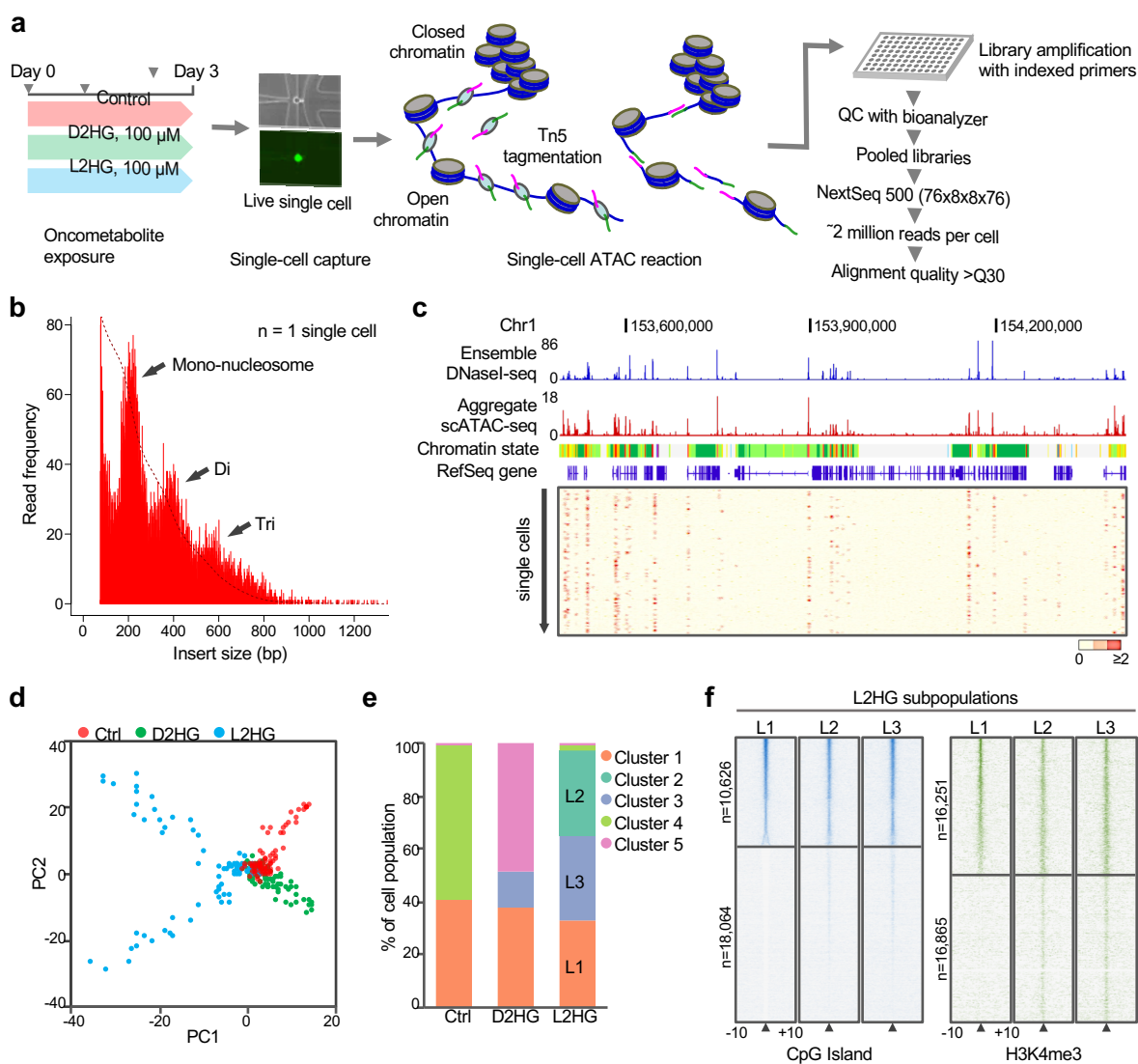


Figure 3

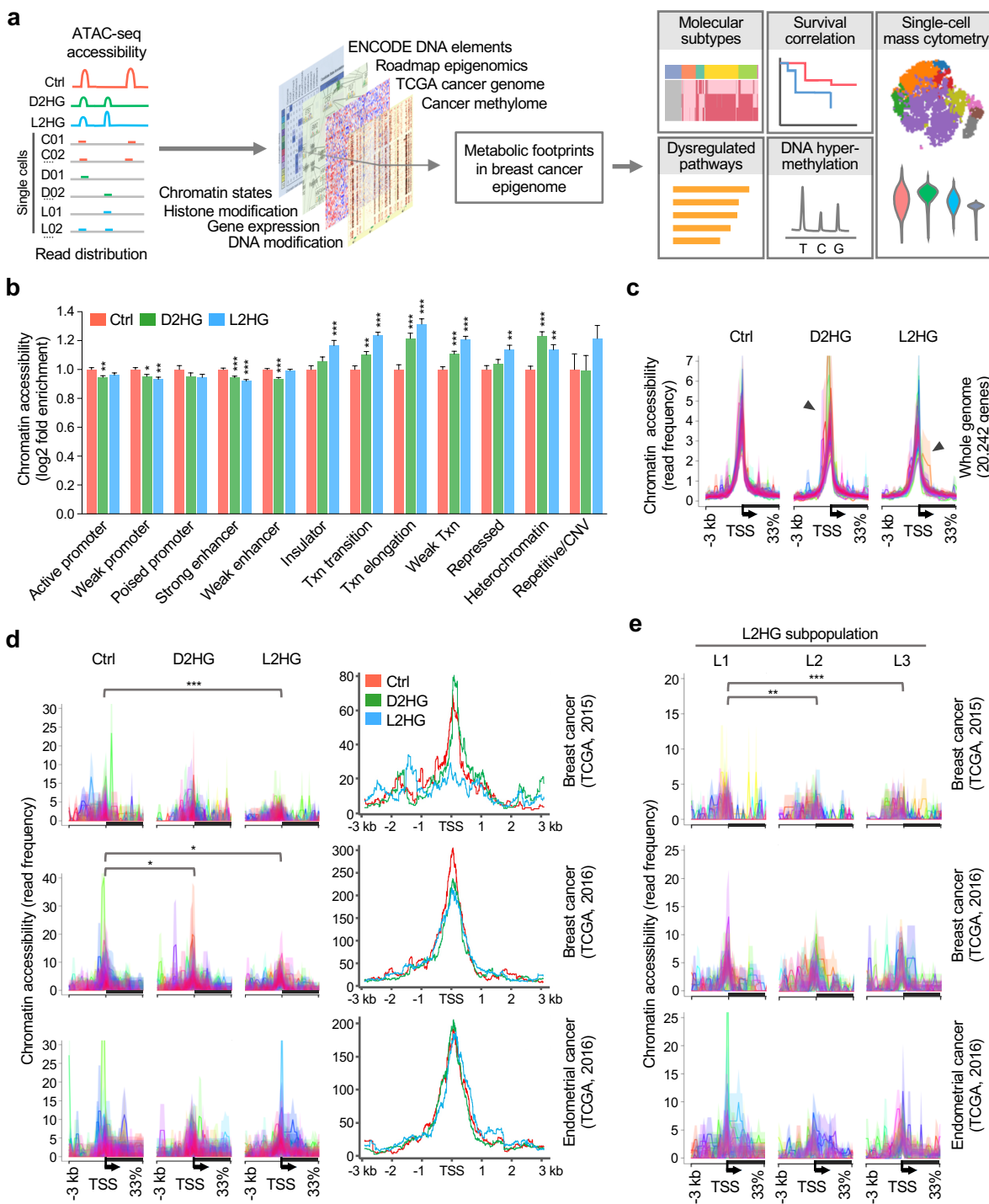


Figure 4

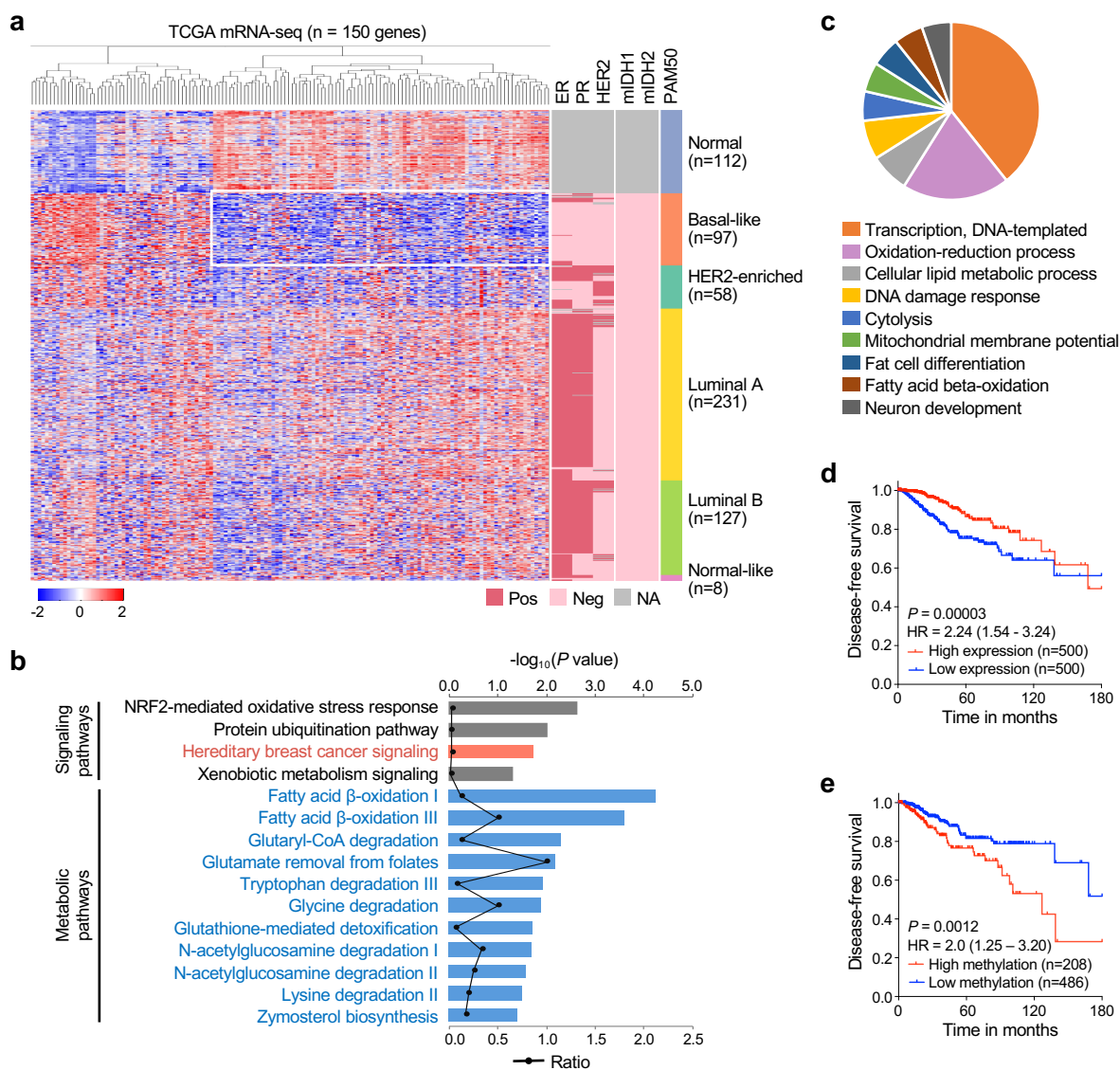


Figure 5

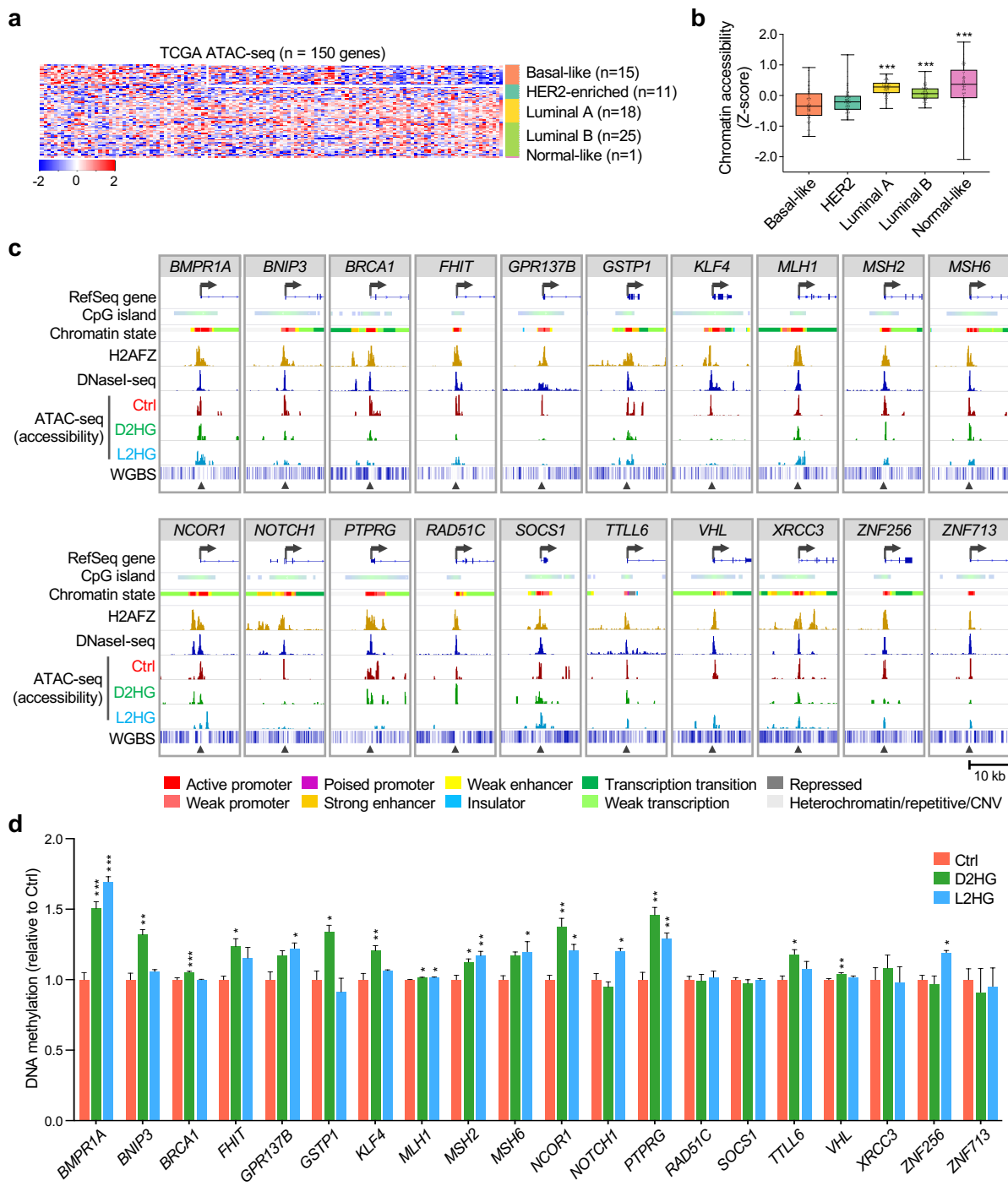


Figure 6

