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Phenotype-independent DNA methylation changes in prostate cancer

Running title: Prostate cancer specific DNA methylation

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1 *Abstract*

2 **Background:** Human prostate cancers display numerous DNA methylation changes
3 compared to normal tissue samples. However, definitive identification of features related
4 to the cells' malignant status has been compromised by the predominance of cells with
5 luminal features in prostate cancers.

6 **Methods:** We generated genome-wide DNA methylation profiles of cell subpopulations
7 with basal or luminal features isolated from matched prostate cancer and normal tissue
8 samples.

9 **Results:** Many frequent DNA methylation changes previously attributed to prostate
10 cancers are here identified as differences between luminal and basal cells in both normal
11 and cancer samples. We also identified changes unique to each of the two cancer
12 subpopulations. Those specific to cancer luminal cells were associated with regulation of
13 metabolic processes, cell proliferation and epithelial development. Within the prostate
14 cancer TCGA dataset, these changes were able to distinguish not only cancers from
15 normal samples, but also organ-confined cancers from those with extra-prostatic
16 extensions. Using changes present in both basal and luminal cancer cells, we derived a
17 new 17-CpG prostate cancer signature with high predictive power in the TCGA dataset.

18 **Conclusions:** This study demonstrates the importance of comparing phenotypically
19 matched prostate cell populations from normal and cancer tissues to unmask biologically
20 and clinically relevant DNA methylation changes.

21

22

23 ***Background***

24 Treatment-naïve prostate cancer (PCa) is characterized by an abnormal accumulation of
25 proliferative cells with a molecular phenotype similar to the luminal cells present in the
26 normal prostate^{1,2}. However, PCa samples also contain a small population of tumour cells
27 with basal features. These cells possess “cancer stem cell” features, appear to be
28 treatment-resistant, and are proposed to serve as a reservoir for tumour recurrence after
29 castration therapy³⁻⁶. DNA methylation of bulk PCa samples has been well studied⁷ and
30 aberrant methylation of promoter regions found to be a consistent feature⁸, albeit with
31 high variability both between patients and within single tumours⁹. Their frequency and
32 presence in pre-malignant tissues support a strong selective pressure for DNA
33 methylation changes during cancer development⁷. However, DNA methylation is
34 dynamically regulated during tissue development and cell differentiation¹⁰, and distinct
35 cell types possess specific DNA methylation profiles within the same tissue¹¹⁻¹³.
36 Therefore, the luminal molecular features of bulk PCa samples, in contrast to the almost
37 equal proportion of basal and luminal cells in normal prostate tissues, complicate the
38 interpretation of datasets generated on whole tissue extracts, where changes associated to
39 differences in cell types may mask the presence of malignancy-associated signatures.

40 Recent developments in tissue processing and the identification of surface
41 markers suitable for the prospective isolation of viable basal and luminal cells from
42 normal prostate tissues have enabled studies of their molecular and biological
43 characteristics¹⁴⁻¹⁷. Use of this approach has revealed that many of the genes
44 downregulated in normal luminal cells compared to basal cells are frequently
45 hypermethylated in PCa¹⁸. This data implies a functional link between DNA

46 hypermethylation and the observed expansion of cells with a luminal phenotype in PCa.
47 However, very little is known about the specific DNA methylation features of PCa cells
48 with basal and luminal phenotypes in comparison to their normal counterparts. To
49 address this issue, we generated genome-wide DNA methylation profiles of FACS-
50 purified populations of cells with basal and luminal features from a series of freshly
51 isolated patient-matched tumour and normal samples from individuals undergoing radical
52 prostatectomy. Our results show that many DNA methylation changes frequently seen in
53 PCa are characteristic differences between luminal and basal cells from both normal and
54 cancer samples. From these datasets, we were also able to identify two sets of tumour-
55 specific changes of potential clinical interest. One set consists of changes that are specific
56 to PCa luminal cells; the other set are changes shared by both basal and luminal tumour
57 but not normal prostate cells.

58

59

60 **Methods**

61 **Tissue processing:**

62 Prostate tissues were obtained from patients undergoing radical prostatectomy at Castle
63 Hill Hospital (Cottingham, UK) with informed patient consent and approval from the
64 NRES Committee Yorkshire & The Humber (LREC Number 07/H1304/121). Tissues
65 were sampled immediately after surgery. For radical prostatectomies, three core needle
66 biopsies were taken from four different sites (left base, left apex, right base, right apex)
67 and were directed by previous pathology, imaging and palpation. Tissues were
68 transported in RPMI-1640 with 5% FCS and 100U/ml antibiotic/antimitotic solution at
69 4°C, and processed immediately upon arrival. PCa diagnosis was confirmed by
70 histological examination of the whole prostate. Tissues were disaggregated as previously
71 described¹⁹, and all reagents were supplemented with 10 nM R1881 to better preserve the
72 viability of luminal cells.

73

74 **Fluorescence activated cell sorting (FACS) and characterization of cell populations:**

75 Single-cell suspensions were labelled with Lineage Cell Depletion Kit (human) and
76 CD31 MicroBead Kit (Miltenyi Biotec) and Lin⁺/CD31⁺ cells depleted twice using
77 MACS LS Columns (Miltenyi Biotec). Lin⁻/CD31⁻ cells were then labelled with EpCAM-
78 APC, CD49f-FITC and CD24-PE (Miltenyi Biotec) and DAPI and
79 EpCAM⁺/CD49f⁺/CD24⁻ and EpCAM⁺/CD49f⁺/CD24⁺ sorted at >95% purity using a
80 MoFlo (Beckman Coulter) cell sorter. Sorted populations were characterized by
81 immunofluorescence and qRT-PCR as previously described¹⁸.

82

83 **Reduced Representation Bisulphite Sequencing (RRBS):**

84 DNA was extracted from FACS-sorted populations using phenol/chloroform extraction
85 and ethanol precipitation. DNA was quantified using a NanoDrop 1000
86 Spectrophotometer (Thermo Fisher Scientific), and shipped to Zymo Research for RRBS
87 analysis. Bisulphite conversion, library preparation, sequencing, and initial
88 bioinformatics analyses were performed by Zymo Research following the Methyl-
89 MiniSeq pipeline.

90

91 **Sequence data processing and methylation calls:**

92 Fastq files were trimmed using Trim Galore! v0.4.1
93 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) with the following
94 parameters: --fastqc --illumina --paired --rrbs --non_directional. Trimmed sequences were
95 aligned to the human genome (hg19 downloaded from UCSC, 08-Mar-2009 version)
96 using bsmmap v2.90²⁰ and the following parameters: -m 0 -x 1000 -n 1 -p 8 -S 1. The
97 resulting bam files were sorted and indexed using samtools v0.1.19²¹, and methylation
98 and coverage calls for each CpG site calculated using the methratio.py script in the bsmmap
99 software (Supplementary Table 1). Methylation calls were then filtered for low (<3) and
100 high (>99.95%) read coverage and merged in non-overlapping genomic bins of 100 bp
101 using the methylKit package v0.99.2²² within R v3.3.1 to increase comparability between
102 samples. All subsequent analyses were carried out using only those genomic bins covered
103 in all samples, with the exception of the results presented in Supplementary Fig. 2 and
104 Supplementary Table 3 which were generated using single GpG information.
105

106 **Identification of differentially methylated regions (DMRs):**

107 DMRs were calculated using methylKit²²; with all pairwise comparisons between the four
108 cell populations carried out and similar populations from different donors defined as
109 biological replicates. The patient of origin was used as a categorical covariate to account
110 for the strong inter-donor variability seen. All p-values were generated using a logistic
111 regression model and corrected for multiple testing using the SLIM method²³. DMRs
112 were defined as those genomic bins with q-values <0.05 and absolute methylation
113 difference >10% in each pairwise comparison.

114

115 **Characterization of DMRs:**

116 All genomic features were downloaded from the UCSC Table browser
117 (genome.ucsc.edu) for the hg19 genome. Gene models: “refGene” (RefSeq Genes), CpG
118 Islands: “cpgIslandExt”, Evolutionary conservation: “phastCons100way”, DNase
119 hypersensitivity sites (DHSs): “wgEncodeRegDnaseClusteredV3”, transcription factor
120 binding sites (TFBSs): “wgEncodeRegTfbsClusteredV3”, repeats: “rmsk”
121 (RepeatMasker). Overlaps and distances of DMRs to other genomic features were
122 calculated using BEDtools v2.26.0²⁴, and significance of enrichments or depletions was
123 calculated using custom R scripts. All p-values <10⁻³⁰⁰ were approximated to 10⁻³⁰⁰ to
124 avoid reaching the minimum value for a floating-point number (2.2*10⁻³⁰⁸). Average
125 conservation signals around DMRs were calculated using bwtool v1.0²⁵. P-values were
126 calculated using a bootstrapping approach comparing the average conservation of the
127 distal DMRs with the average of an equal number of randomly selected, non-overlapping,
128 distal genomic bins, 1000 times. Gene ontology (GO) analysis was performed using

129 GREAT v3.0²⁶, using all covered genomic bins as background and the default “Basal plus
130 extension” association rules. Results were filtered to include only GO categories, with a
131 Benjamini–Hochberg corrected (FDR) hypergeometric test p-value <0.05 and ≥ 3 genes
132 with associated regions. K-means clustering of GO categories (biological processes only)
133 was based on information similarity values calculated using the GOSim package within R
134 v3.3.1. Promoters frequently altered in PCa were downloaded from the review by Massie
135 et al., 2017⁷. Only promoters reported by ≥ 3 studies were considered frequently altered.
136 Genome browser plots were generated using the package Sushi within R v3.3.1 and
137 custom scripts.

138

139 **TCGA data analysis:**

140 Illumina Infinium HumanMethylation450 data generated within the The Cancer Genome
141 Atlas (TCGA) consortium²⁷ was downloaded (pre-processed Level 3 data only) from the
142 NCI Genomic Data Commons website using the provided GDC Data Transfer Tool (data
143 downloaded on 7th Dec 2016). Clinical data was downloaded from firebrowse.org (8th
144 Dec 2016). [The presence of evident batch effects was excluded by visualizing the data on TCGA
145 Batch Effects \(http://bioinformatics.mdanderson.org/tcgambatch/\)](http://bioinformatics.mdanderson.org/tcgambatch/). A data matrix containing
146 the beta values for each sample was generated using custom scripts. Probes were mapped
147 to hg19 using the positions officially reported by Illumina. Overlap of array probes with
148 DMRs was carried out using BEDtools v2.26.0. Hierarchical clustering was based on
149 Euclidean distances of unscaled beta-values. Logistic model training using least absolute
150 shrinkage and selection operator (LASSO) regression was performed using the glmnet
151 package within R v3.3.1 on a random selection of 70% of the samples. 200 lambda values

152 ranging from e^{-7} to e^{-2} were tested and 10-fold cross validation performed. The lambda
153 with the minimum mean cross-validated error was selected and resulted in 17 probes with
154 non-zero coefficients. The optimal model was then tested on the remaining 30% of
155 samples and receiver operator curve and area under the curve (AUC) calculated using the
156 ROCR package.

157

158

159 **Results**

160 **Phenotypically defined prostate cells from patient-matched normal and PCa**

161 **samples show donor-specific DNA methylation profiles**

162 Matched tumour-directed (cancer) and contralateral (normal) core needle biopsies (1 or 2
163 per site) were obtained from 4 treatment-naïve prostate cancer patients undergoing radical
164 prostatectomies. These samples were then enzymatically dissociated and labeled with
165 antibodies against EpCAM, CD49f and CD24 to enable the prospective isolation of
166 luminal (EpCAM+CD49f-CD24+) and basal (EpCAM+CD49f+CD24-) cells at >95%
167 purity (Fig. 1A). EpCAM+CD49f+CD24- cells expressed higher levels of molecular
168 markers associated with basal cells and lower levels of luminal markers compared to
169 EpCAM+CD49f-CD24+ cells from the same biopsy, both at the mRNA and protein level
170 (Supplementary Fig. 1A-B). For convenience, we named the paired subsets as follows:
171 Cancer Luminal (CL) EpCAM+CD49f-CD24+ cells purified from tumour-directed
172 biopsies; Cancer Basal (CB) EpCAM+CD49f+CD24- cells purified from tumour-directed
173 biopsies; Normal Luminal (NL) EpCAM+CD49f-CD24+ cells from contralateral
174 biopsies; Normal Basal (NB) EpCAM+CD49f+CD24- cells purified from contralateral
175 biopsies. This yielded 4 CL and CB populations, and 3 matched NL and NB populations,
176 as in one prostate the palpable tumour was extended to most of the prostate and it was not
177 possible to obtain a contralateral “normal” tissue biopsy (Supplementary Fig. 1C). DNA
178 obtained from each of these isolates was then subjected to Reduced Representation
179 Bisulphite Sequencing (RRBS). On average, this generated information on the DNA
180 methylation status of $>8.9 \times 10^6$ cytosines within CpG sites per sample (range 8×10^6 –
181 9.6×10^6 , with an average coverage of 7.5 reads, Supplementary Table 1). The data was

182 processed as described in Methods, and binned into 100 bp genomic regions to maximize
183 the comparability between samples (932,905 bins covering 4.1×10^6 CpGs in all samples).
184 Unsupervised hierarchical clustering of the top 1% most variable regions (bins) across all
185 samples showed clustering primarily according to the patient of origin, rather than the
186 subset analyzed (Fig. 1B). This indicates a high donor-determined variation in CpG
187 methylation, consistent with previous reports of similarly accrued data²⁸.

188

189 **Distinct DNA methylation profiles in basal and luminal cells**

190 We then calculated DMRs for all pairwise comparisons between the 4 sorted populations
191 (Fig. 1C, Supplementary Table 2). Among these, the comparison between CB and NB
192 cells (CB-NB comparison) produced the smallest number of DMRs. In contrast, a large
193 number of DMRs were seen when either normal or cancer luminal cells were compared
194 with either source of basal cells (i.e., NL-NB, NL-CB, CL-NB and CL-CB, Fig. 1D). Of
195 the DMRs revealed in these latter comparisons, $\sim 2/3$ were hypermethylated in luminal
196 cells, which correlates with the higher levels of DNMT3a seen in these cells¹⁸. We also
197 calculated differential methylation on single CpGs (prior the 100bp binning) with very
198 similar results (Supplementary Fig. 2 and Supplementary Table 3). Moreover, integration
199 of the DMRs identified in NL-NB proximal (± 5 kb) to annotated transcriptional start sites
200 (TSSs) with RNA-seq data of similarly purified cells¹⁵ showed the expected inverse
201 correlation (Supplementary Fig. 3A).

202 We also found an extensive overlap in the DMRs obtained from both the NL-NB and NL-
203 CB comparisons, and also from the CL-NB and CL-CB comparisons (Supplementary Fig.

204 3B-C). Accordingly, we focussed our subsequent analyses on comparisons of NL-NB and
205 CL-CB, where cells from the same biopsy could be compared directly.

206 Characterization of the genomic features of the DMRs thus identified showed that
207 >50% of them fell outside of CpG islands, shores or shelves (Fig. 1E), and >70% were >5
208 kb away from any annotated TSSs (Fig. 1F-G). These features were particularly
209 pronounced (highly significant hypergeometric test) for the hypomethylated DMRs
210 identified in the comparisons of NL-NB, CL-CB and CL-NL. Because hypermethylated
211 and hypomethylated DMRs might be anticipated to differ in their genomic context, their
212 impact on the biological properties of basal and luminal cells could also be different.

213

214 **Distal hypermethylated DMRs are enriched in enhancer features**

215 Given that most of the DMRs identified were outside CpG islands and far from TSSs, we
216 asked whether they might affect distal regulatory elements (enhancers). We therefore
217 examined three genomic characteristics of such elements: evolutionary conservation²⁹,
218 open chromatin shown by hypersensitivity to DNase I³⁰, and presence of TFBSs³¹. Distal
219 hypermethylated DMRs in each comparison were enriched for evolutionarily conserved
220 sequences (Fig. 2A, bootstrapped p-value) and overlapped significantly with both DHSs
221 and ChIP-seq-defined TFBSs (identified within the ENCODE project, Fig. 2B-C,
222 hypergeometric test). Distal hypomethylated DMRs generally scored lower than the
223 hypermethylated counterparts for each metric measured. DMRs hypomethylated in the
224 CL-CB and CL-NL comparisons showed the weakest enrichments. However, all distal
225 hypomethylated DMRs had high overlaps with genomic repetitive elements (Fig. 2D).

226 Specifically, LINE and LTR elements, but not SINE elements, were significantly
227 enriched in the distal CL hypomethylated regions.

228 GO enrichment analysis (Fig. 2E, Supplementary Fig. 4) showed that
229 hypermethylated DMRs in NL-NB were enriched for more than 500 terms, many of
230 which were linked to prostate development or epithelial stem cell regulation; while
231 hypomethylated DMRs in the same comparison were enriched for terms related to
232 androgen receptor signalling and response to cytokines. In the CL-CB comparison,
233 hypermethylated DMRs were also enriched for more than 500 terms, 311 of which were
234 also identified in the NL-NB comparison, suggesting a high functional overlap in
235 hypermethylated regions in luminal cells from both normal and cancer samples. In the
236 CL-NL comparison, hypermethylated DMRs were enriched in terms related to cell
237 adhesion, while hypomethylated DMRs were enriched in terms related to epithelial
238 morphogenesis. These results indicate that several pathways fundamental to the
239 establishment and maintenance of the normal prostate epithelium are altered in cancer
240 cells with a luminal phenotype.

241

242 **Phenotype-specific DMRs are shared in normal and cancerous prostate tissues**

243 As suggested by the enriched GO analyses, we found a 28% overlap in all the DMRs
244 identified from the NL-NB and the CL-CB comparisons (3852/13816, Fisher's exact test
245 p -value $< 10^{-300}$, Fig. 3A). Hierarchical clustering of all samples based on both sets of
246 DMRs separated them by phenotype (Fig. 3B), reinforcing the presence of a strong
247 phenotypic signature independent of disease state. These shared DMRs were enriched in
248 features characteristic of enhancers (Supplementary Fig. 5A-D) and linked to GO terms

249 related to prostate development, regulation of epithelial stem cells and androgen receptor
250 signalling (Supplementary Fig. 5E-F). Moreover, hypermethylated DMRs were highly
251 enriched for TFBSs of *TP63*, *TP53* and *NF1*, and hypomethylated DMRs for *FOXA1*,
252 *p65-NFkB* and *GATA3* (Fig. 3C), all well-known regulators of basal and luminal
253 epithelial cells, respectively. Interestingly, 26 of the 168 genes described as frequently
254 differentially methylated in PCa⁷, showed hyper- or hypomethylated DMRs within 5 kb
255 of their TSSs in both the NL-NB and CL-CB comparisons (Fig. 3D). These included the
256 frequently hypermethylated genes, *GSTP1* and *CCDC8* (Fig. 3E-F).

257 In summary, these analyses identified a large set of phenotype-specific and
258 disease-independent DMRs, both of which contained many binding sites for TFs with
259 known regulatory roles in the normal prostate.

260

261 **CL hypermethylate PRC2 target sites and hypomethylate repetitive elements**

262 A second group of genes frequently hypermethylated in PCa were found hypermethylated
263 in both the CL-CB and CL-NL comparisons (Fig. 4a), but not in the NL-NB comparison.
264 These might be expected to reflect a PCa-specific methylation signature. DMRs
265 identified in the CL-CB and CL-NL comparisons showed that many were shared (1472
266 DMRs, Fisher's exact test p-value < 10⁻³⁰⁰, Fig. 4B) with very few also different between
267 NL and NB cells (106 DMRs). 65% of these CL-specific hypermethylated DMRs were
268 distal to TSSs and were again highly enriched for enhancer features, but significantly
269 depleted in repetitive elements (Supplementary Fig. 6A-E). These regions were
270 associated with GO terms related to metabolic processes, cell proliferation and epithelial
271 development (Fig. 4C) and showed a high enrichment of DNA sequences potentially

272 bound by EZH2 and SUZ12, two main members of the PRC2 complex (Supplementary
273 Fig. 6F). On the other hand, distal hypomethylated DMRs were not enriched for any
274 feature of putative regulatory regions, but significantly overlapped with LINE and LTR
275 elements.

276 Since the CL subset represents the majority of the cells in untreated PCa samples,
277 we hypothesized that aberrant methylation of these DMRs would be measurable even
278 when whole tissue homogenates are analysed. We therefore interrogated the DNA
279 methylation array dataset for PCa made available by the TCGA consortium, which
280 consists of 50 PCa samples with matched normal counterparts, 452 additional PCa
281 samples without normal counterparts, and 1 metastatic PCa sample²⁷. 255 array probes
282 overlap these 1472 DMRs. Hierarchical clustering of the 50 matched normal and PCa
283 samples showed an almost perfect subdivision based on the malignancy status of the
284 samples (TPR = 0.92, TNR = 0.92, Chi-squared test p-value = 2.4×10^{-16} , Fig. 4D). The
285 same analysis carried out on all 553 samples produced similar results, with one cluster
286 highly enriched in normal samples (Chi-squared test p-value = 1.7×10^{-39} , Supplementary
287 Fig. 6G). This clustering also appeared to divide the PCa samples into two main groups,
288 according to their differences from the normal samples. Exclusive analysis of the cancer
289 samples confirmed this clustering pattern (Fig. 4E) and showed one cluster to be
290 significantly enriched for samples with extra-prostatic extensions (pT3 or pT4 in TNM
291 classification, Chi-squared test p-value < 0.005) in the absence of significant differences
292 in Gleason score (Chi-squared test p-value > 0.1).

293 Overall, these results indicate that phenotypic luminal PCa cells possess an
294 aberrant methylation signature characterized by hypermethylation of putative regulatory

295 sequences involved in tissue development, and hypomethylation of LINEs and LTRs
296 repetitive elements. This signature was also able to distinguish cancer samples from
297 normal, and organ-confined from extraprostatic disease.

298

299 **Identification of PCa-specific, phenotype-independent DMRs**

300 Comparisons of the DMRs in the CL-NL and CB-NB pairs showed a small but
301 significant overlap of both hyper- and hypomethylated DMRs in each (189 DMRs in
302 total, Fig. 5A). These common DMRs were able to cluster all samples according to their
303 disease state in a phenotype-independent manner (Supplementary Fig. 7A). Notably, they
304 included DMRs close to many genes previously implicated in prostate cancer (e.g.,
305 *NEAT1*, *MTOR*, *RHCG*, *KCNC2*, *WT1*, *HOXC12*, *KMT2B*, Fig. 5B). To determine
306 whether these DMRs would be altered in an independent dataset, we applied the same
307 analysis to the TCGA dataset, where 66 array probes overlapped these 189 DMRs.
308 Hierarchical clustering of the 50 matched normal and PCa samples produced a single
309 cluster containing 46/50 normal samples and 10/50 PCa samples (TPR = 0.8, TNR =
310 0.92, Chi-squared test p-value = 1.8×10^{-12} , Fig. 5C). Application of the same analysis to
311 all samples in the TCGA database produced similar results: one cluster was highly
312 enriched in normal samples (TPR = 0.87, TNR = 0.74, Chi-squared test p-value = 8.3×10^{-26} ,
313 Supplementary Fig. 7B), indicating that at least some of these DMRs are frequently
314 altered in PCa.

315 To select the probes most strongly associated with disease state (i.e., PCa vs
316 normal), we trained a logistic model using LASSO regression on 70% of the TCGA
317 samples and selected a 17-probe signature (Fig. 5D). We then tested this model on the

318 remaining 30% of the dataset. This resulted in an AUC of 0.92 (TPR = 0.9, TNR = 0.94,
319 Fisher's exact test p-value = 2.82×10^{-12} at the selected cut-off of 0.8, Fig. 5E-F,
320 Supplementary Table 4). The 17-probe signature also included sequences proximal to
321 several genes with recognized importance in PCa (e.g., *PLAGL1/HYMAI*, *HOXC12*,
322 *KCNC2*), but was completely non-overlapping with other similar signatures recently
323 developed for PCa³²⁻³⁶.

324

325 ***Discussion***

326 PCa is characterized by frequent aberrant DNA methylation of many genomic sites that
327 may contain clinically relevant signatures^{7,37}. The early establishment (presence in pre-
328 neoplastic tissues) and high prevalence of these aberrant patterns is also suggestive of
329 their direct involvement in PCA tumorigenesis. However, the normal prostate epithelium
330 is composed of similar numbers of luminal and basal cells, whereas most treatment-naïve
331 prostate cancers are largely composed of cells with many luminal features. This shift in
332 favor of a transcriptional and epigenomic program of normal luminal cells might mask or
333 complicate the identification of cancer-specific features in prostate cancer when bulk
334 analyses are performed on this type of tumour.

335 We now report a detailed comparison of genome wide methylation profiles
336 obtained separately from epithelial cells with luminal and basal phenotypes, isolated with
337 a high purity from patient-matched normal and cancer biopsy samples. From comparative
338 analyses of these profiles, we found a major proportion of the methylation differences
339 between normal basal and luminal cells were conserved in their malignant counterparts.
340 These affected many promoters frequently described as aberrantly methylated in bulk

341 PCa compared to normal tissues, consistent with the increased representation of cells with
342 a luminal phenotype in PCa, in which a higher proportion of cells carrying a methylation
343 signature of normal luminal cells might then be expected.

344 However, our study made it possible to identify, for the first time, regions
345 specifically altered in the luminal fraction of PCa. The hypermethylated DMRs in this
346 group were genes associated to genes involved in metabolic processes, cell proliferation
347 and epithelial development, all functions clearly deregulated in prostate cancer, therefore
348 potentially containing major cancer driver events. Furthermore, hypomethylated DMRs
349 were highly enriched in repetitive elements, a feature also previously reported in many
350 cancer types, where they have been thought to contribute to genomic instability and
351 aberrant gene expression³⁸⁻⁴⁰.

352 Importantly, this set of DMRs was able to discriminate not only normal and PCa
353 samples in the TCGA dataset, but also PCa samples with or without extra-prostatic
354 extensions, the former being indicative of highly aggressive, invasive cancers. Since this
355 distinction was not evident from the Gleason grades of these tumours, the epigenetic data
356 may reflect a an acquisition of specific aberrant epigenomic changes that herald disease
357 progression^{7,41-43}. Genomic regions consistently altered in both tumour phenotypes in the
358 PCa samples analyzed also have potential clinical importance. Indeed, the new logistic
359 model constructed from these regions makes use of only 17 probes to distinguish normal
360 and PCa samples with similar specificity and sensitivity to previously developed, non-
361 overlapping models^{35,36}, and may be useful in the context of the low mutagenic burdens
362 seen in most hormone-naïve prostate cancers.

363 The results reported here show that many DNA methylation changes commonly
364 associated with PCa cells are explained by a predominant luminal phenotype of the
365 treatment-naïve PCa population, and are not cancer-specific nor are likely to contain
366 driver events. Importantly however, we were able to identify two separate classes of PCa-
367 specific DNA methylation changes: those specific to cancer luminal cells that can
368 distinguish both normal from cancer samples and organ-confined cancers from those with
369 extra-prostatic extensions; those changes common to basal and luminal cancer cells that
370 are able to distinguish PCa efficiently from normal samples. These two novel sets of
371 cancer-specific changes clearly demonstrate the potential of profiling normal and cancer
372 cell subpopulations in identifying signatures that may contain previously unrecognized
373 driver events in the development and progression of PCa.

374

375 *Additional Information*

376 **Ethics approval and consent to participate**

377 Prostate tissues were obtained from patients undergoing radical prostatectomy at Castle
378 Hill Hospital (Cottingham, UK) with informed patient consent and approval from the
379 NRES Committee Yorkshire & The Humber (LREC Number 07/H1304/121).

380

381 **Availability of data and materials**

382 The methylation and coverage calls for all RRBS libraries generated are available from
383 GEO [GSE107596]. For patients' privacy reasons, raw data (FASTQ and BAM files) for
384 the RRBS libraries are not publicly available, but can be available from the
385 corresponding author on request.

386

387 **Competing Interests**

388 The authors declare no competing financial and non-financial interests.

389

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393 Columbia Cancer Agency (Strategic Priorities Fund, DP and CJE).

394

395 **Authors' Contributions**

396 DP and NJM designed the project. MSS and VMM procured the tissue samples. DP
397 processed and sorted the tissue samples, and performed all other experiments. DP, FMF
398 and ATC developed the tissue processing and sorting protocol. DP and APD conducted
399 all bioinformatic analyses. DP, CJE and NJM wrote the manuscript. All authors
400 contributed to the interpretation of the results and read and approved the manuscript.

401

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536

537 **Figure Legends**

538 **Fig. 1: Identification of DMRs between prostate cancer cell populations. (A)**

539 Representative FACS profiles of a cell suspension prepared from core needle biopsies of
540 a radical prostatectomy sample. **(B)** Heatmap showing scaled methylation values of the
541 top 1% most variable regions (100 bp bins) in the samples analyzed. Hierarchical
542 clustering is based on Euclidean distance of the unscaled values and complete linkage.
543 **(C)** Diagram showing all pairwise comparisons carried out. **(D)** Number of DMRs found
544 in each comparison. **(E)** Overlap of DMRs with CpG islands, shores (2 kb flanking
545 islands) or shelves (2 kb flanking shores). P-values from hypergeometric test against all
546 regions. E = enriched, D = depleted. **(F)** Distribution of distances of DMRs to the closest
547 TSS. Grey box indicates ± 5 kb from a TSS. Purple lines: hypermethylated DMRs, orange

548 lines: hypomethylated DMRs, gray line: all regions. **(G)** Proportion of DMRs proximal or
549 distal to TSSs. P-values from hypergeometric test against all regions. E = enriched, D =
550 depleted.

551

552 **Fig. 2: Hypermethylated distal DMRs have features of enhancers.** **(A)** Average plots
553 of evolutionary conservation scores of the distal DMRs in each set. Purple lines:
554 hypermethylated DMRs; orange lines: hypomethylated DMRs, gray line: all regions. P-
555 values are from bootstrapping analysis. **(B)** Proportion of distal DMRs overlapping with
556 DHSs (identified by ENCODE). P-values from hypergeometric test against all regions. E
557 = enriched, D = depleted. **(C)** Overlap of distal DMRs with ChIP-seq derived TFBSs
558 (identified by ENCODE). P-values are from hypergeometric tests against all regions. E =
559 enriched, D = depleted. **(D)** Overlap of each set of distal DMRs with repetitive elements
560 (UCSC repeatMask), SINEs, LINEs and LTRs. P-values from hypergeometric tests
561 against all regions. E = enriched, D = depleted. **(E)** Number of GO terms enriched by
562 each set of DMRs. GO terms identified using GREAT (FDR<0.05 and at least 3 genes in
563 the set).

564

565 **Fig. 3: Shared phenotype-specific DMRs.** **(A)** Overlap between the DMRs identified in
566 the NL-NB and CL-CB comparisons. P-values derived from Fisher's exact test. **(B)**
567 Heatmap showing scaled methylation values of the DMRs identified in the NL-NB (left)
568 or CL-CB (right) comparisons. Hierarchical clustering is based on Euclidean distances of
569 the unscaled values and complete linkage. **(C)** TFBSs enriched in the hypermethylated
570 (purple) or hypomethylated (orange) DMRs common between the NL-NB and CL-CB

571 comparisons. Left panel: analysis performed using HOMER findMotifs, p-values from
572 binomial test. Right panel: enrichment of ENCODE defined TFBSs, p-values from
573 hypergeometric test against all regions. **(D)** Frequently hyper- or hypomethylated genes
574 in PCa⁷ that were also hypermethylated (purple) or hypomethylated (orange) in the NL-
575 NB and CL-CB comparisons. **(E-F)** Genome browser plots of the promoter regions of
576 GSTP1 **(E)** and CCDC8 **(F)**. Grey squares are the bins analyzed. Lines and shaded areas
577 represent mean \pm SEM of each category (NB=light blue, NL=light red, CB=dark blue,
578 CL=dark red). DMRs are shown on top: hypermethylated=purple,
579 hypomethylated=orange.

580

581 **Fig. 4: Aberrant methylation in CL.** (A) Frequently hyper- or hypomethylated genes in
582 PCa⁷ that are also hypermethylated (purple) or hypomethylated (orange) in the CL-CB
583 and CL-NL comparisons. (B) Overlap between the DMRs identified in the CL-CB and
584 CL-NL comparisons. P-values derived from Fisher's exact test. (C) Clustering of the
585 gene ontologies (biological process) enriched in DMRs common between the CL-CB and
586 CL-NL comparisons based on information similarity. Each circle shows an individual GO
587 term enriched in regions hypermethylated (purple), hypomethylated (orange) or both
588 (green), the size of the circles is proportional to the enrichment p-value. The 2 main
589 clusters of GO terms determined by k-means are highlighted (light blue and pink), and
590 named after the most frequent terms. (D) Heatmap showing scaled methylation values (β -
591 values) of probes overlapping the DMRs common to the CL-CB and CL-NL comparisons
592 in the PCa samples (magenta) and matched normal samples (green) within the TCGA
593 dataset. Hierarchical clustering based on Euclidean distances of the unscaled values and

594 complete linkage. The dark green and gray clusters were generated by cutting the tree at
595 the first bifurcation. (E) Heatmap showing scaled methylation values (β -values) of probes
596 overlapping the DMRs common to the CL-CB and CL-NL comparisons in the PCa
597 samples (matched normal samples not included) of the TCGA dataset. Hierarchical
598 clustering based on Euclidean distance of the unscaled values and complete linkage. The
599 dark green and gray clusters are generated by cutting the tree at the first bifurcation.

600

601 **Fig. 5: PCa-specific DMRs shared between CB and CL.** (A) Overlap between the
602 DMRs identified in the CL-NL and CB-NB comparisons. P-values derived from Fisher's
603 exact test. (B) Genome browser views of KCNC2 promoter (top) and RHCG exon 2
604 (bottom). Grey squares are the bins analyzed. Lines and shaded areas represent mean
605 \pm SEM of each category (NB=light blue, NL=light red, CB=dark blue, CL=dark red).
606 DMRs are shown on top: hypermethylated=purple, hypomethylated=orange. (C)
607 Heatmap showing scaled methylation values of probes overlapping the DMRs common
608 between CL-CB and CB-NB in the matched normal and cancer samples within the TCGA
609 dataset. Hierarchical clustering based on Euclidean distances of the unscaled values and
610 complete linkage. The dark green and gray clusters were generated by cutting the tree at
611 the first 2 bifurcations. (D) Selection of a 17-probe signature distinguishing normal and
612 PCa samples applying LASSO regression on a logistic model of the training dataset (70%
613 of the TCGA samples). Lines show the changes in coefficients in relation to different
614 lambdas. The vertical dashed line shows the optimal lambda identified using cross-
615 validation. (E) Receiver-operating characteristic curve generated by applying the optimal
616 logistic model to the test dataset (30% of the TCGA samples). (F) Heatmap showing

617 scaled methylation values of the 17-probe signature in the test dataset (30% of the TCGA
618 samples). The bar plot on the left side shows the final coefficients for each probe in the
619 model, and the bar plot on top shows the logistic probability generated by for each
620 sample (Green: normal samples, magenta: cancer samples).

621

622

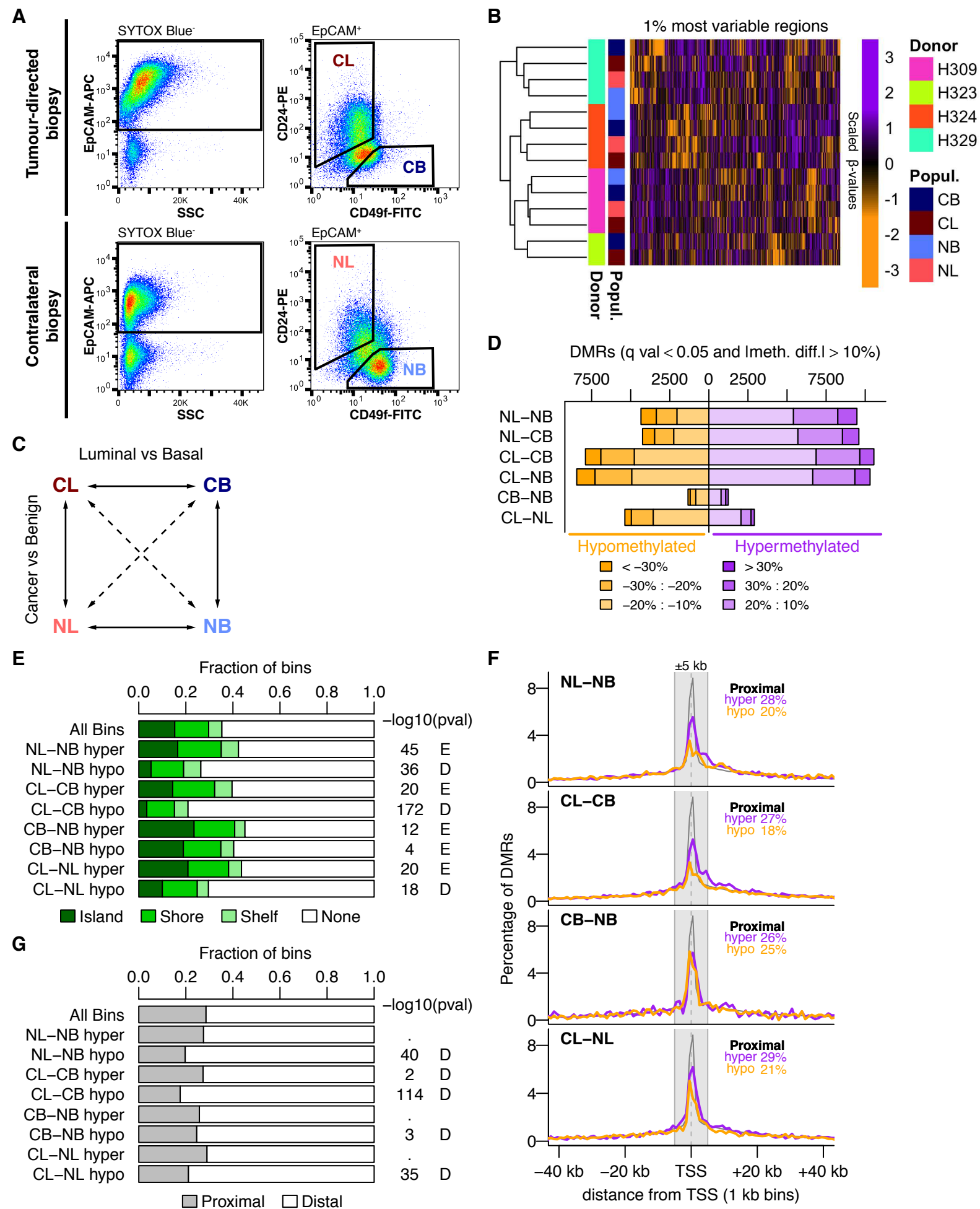
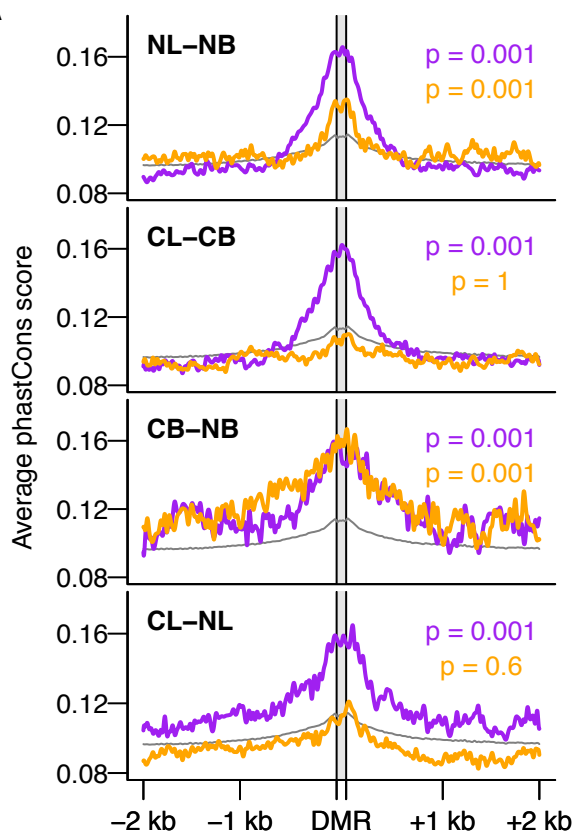
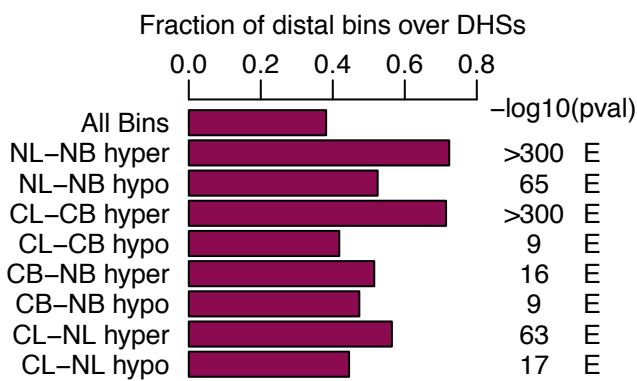
Figure 1

Figure 2

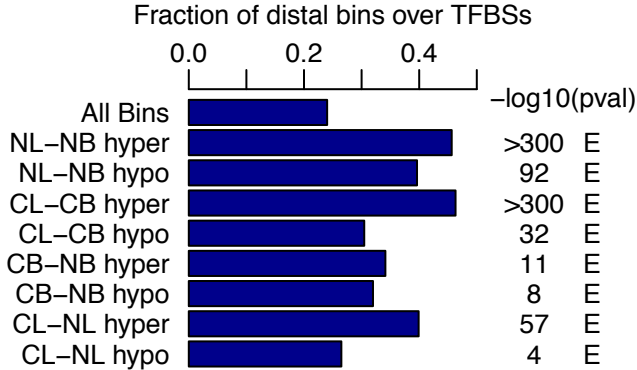
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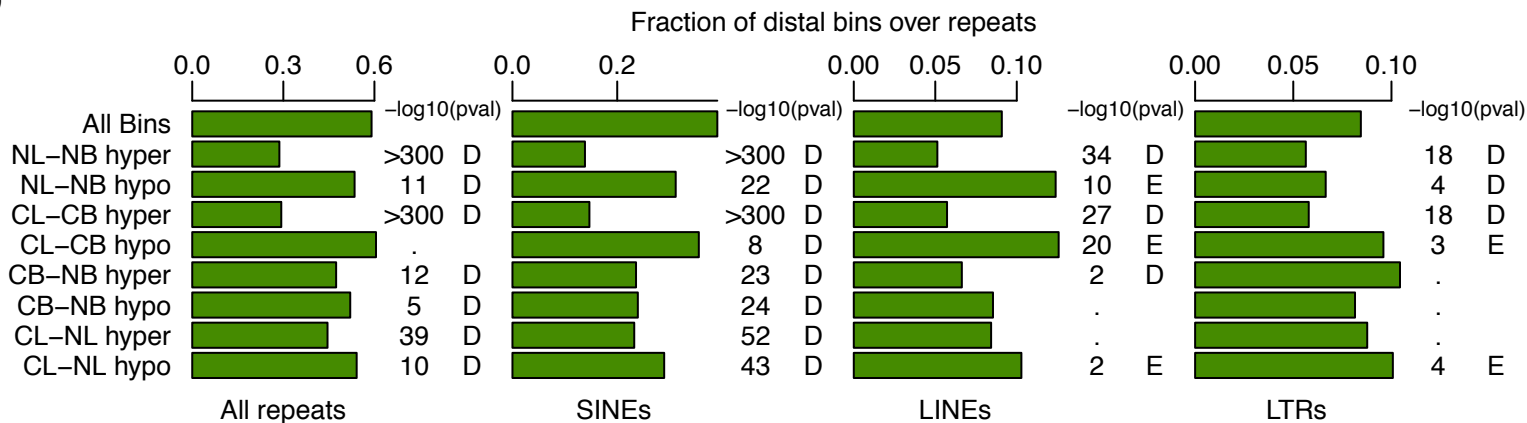
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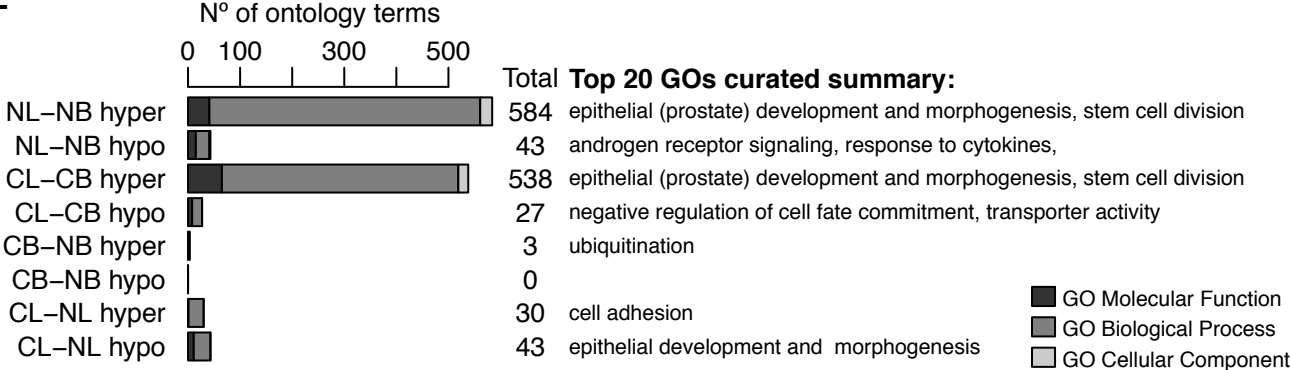
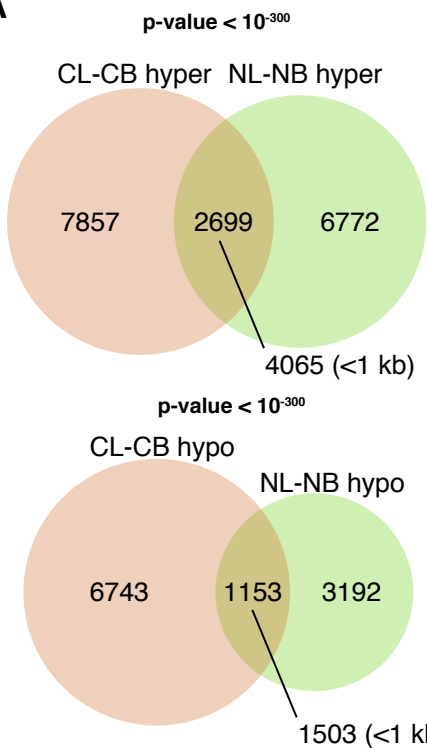
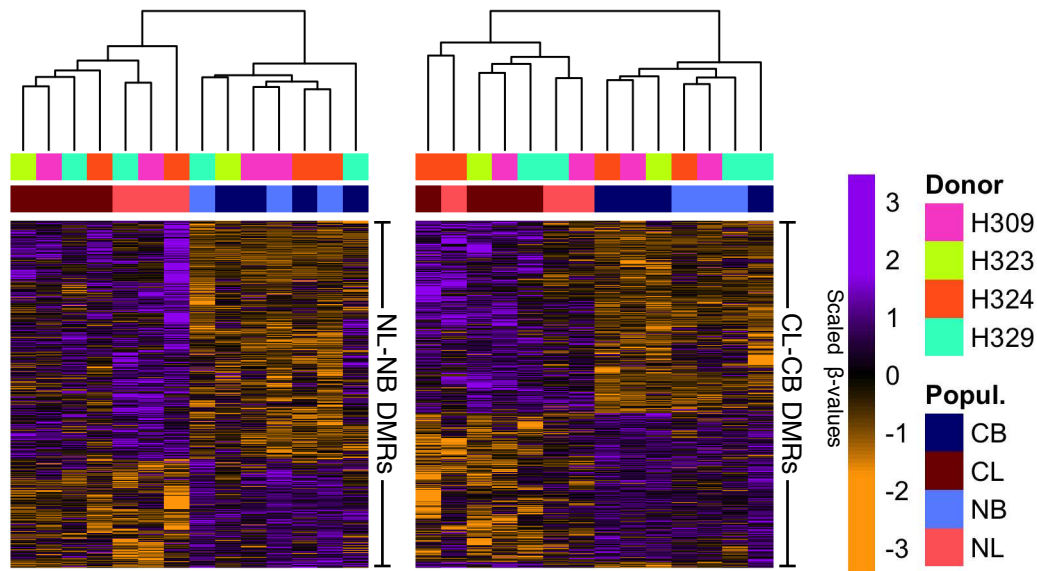


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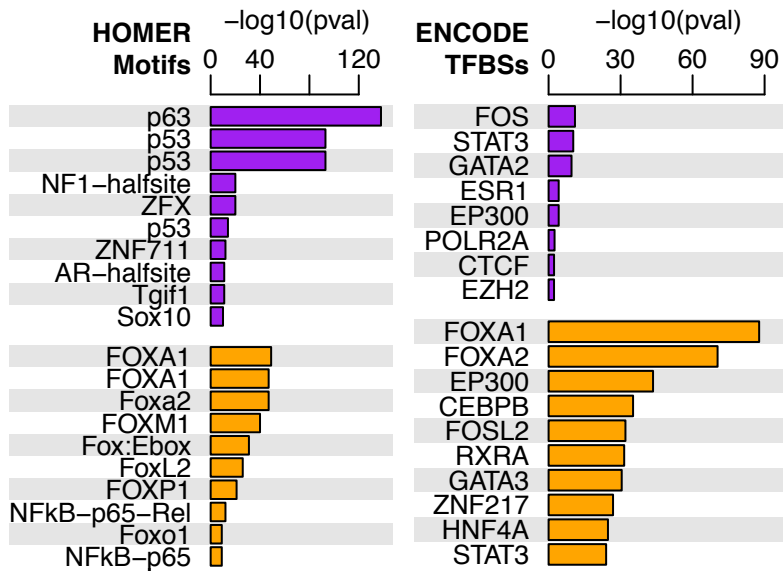
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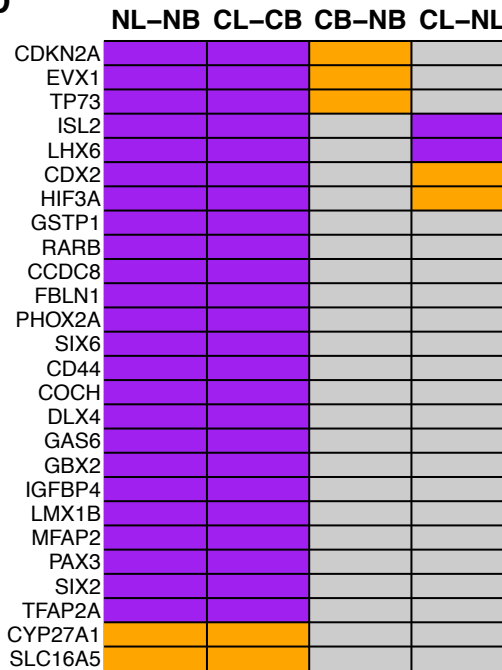
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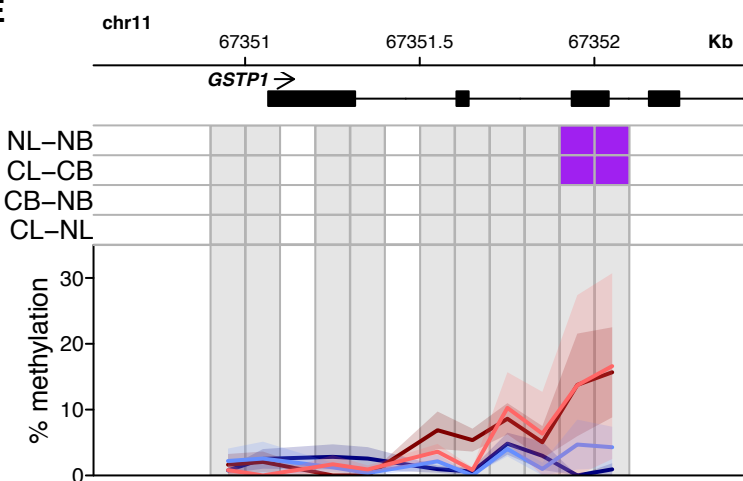
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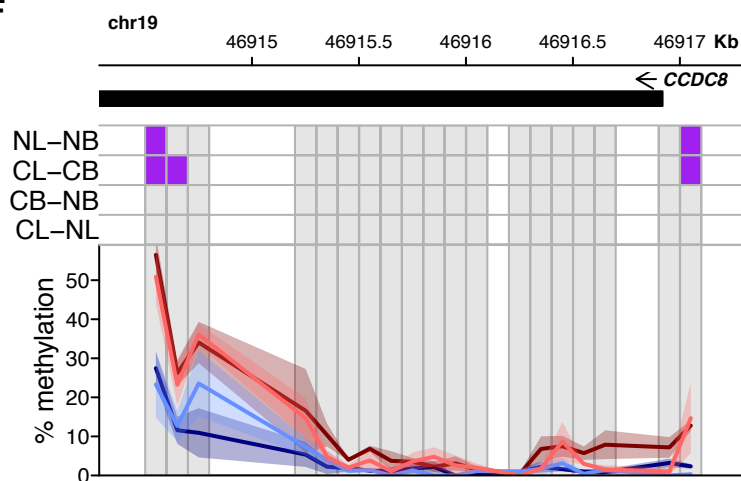


Figure 4

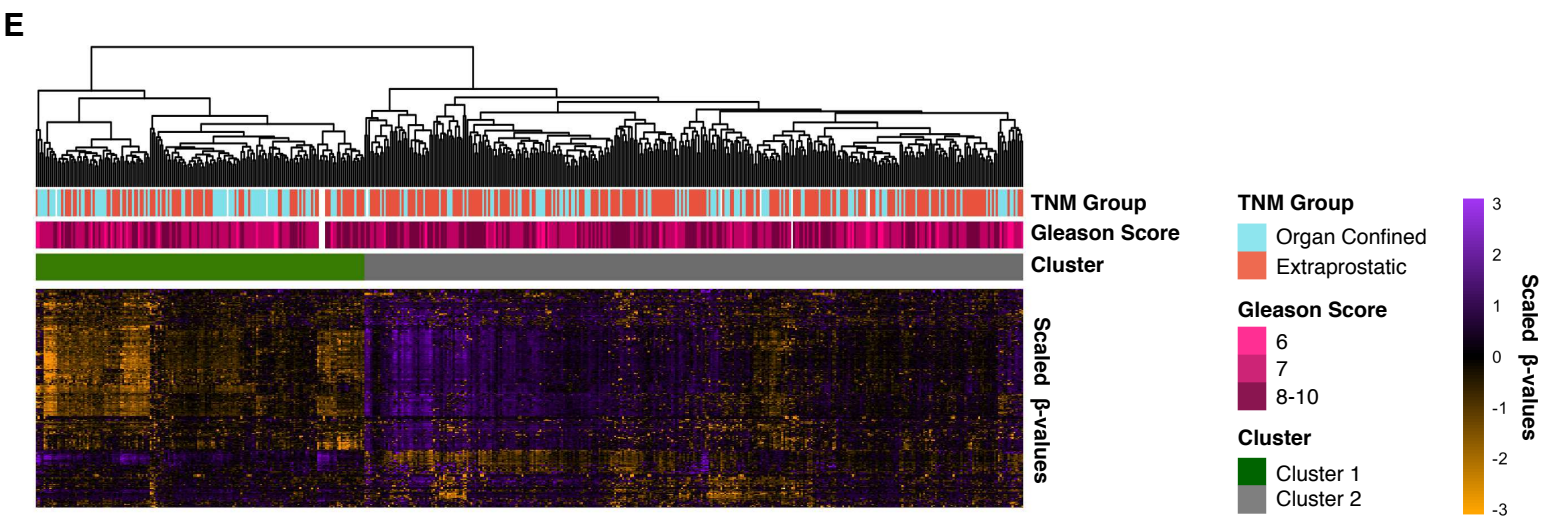
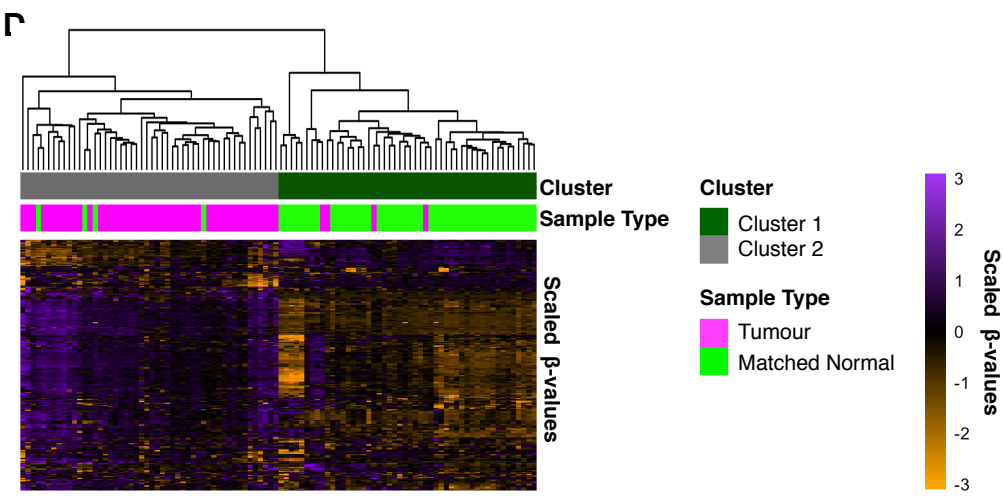
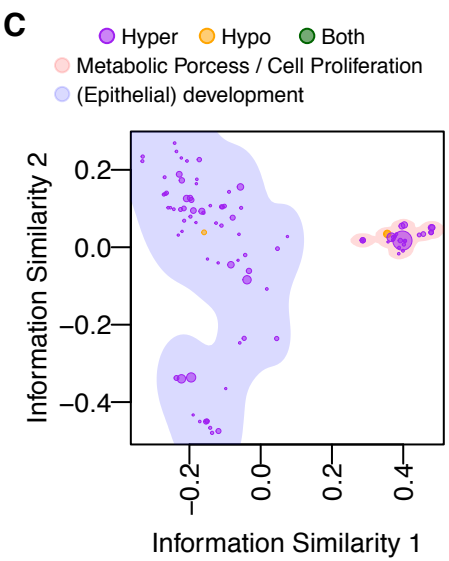
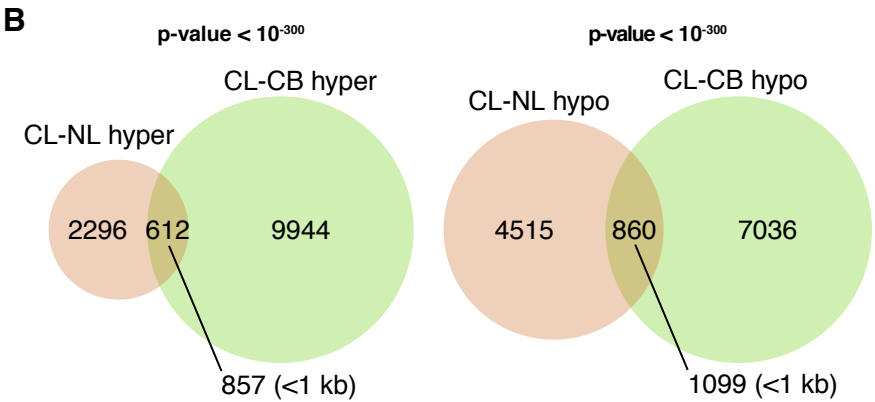
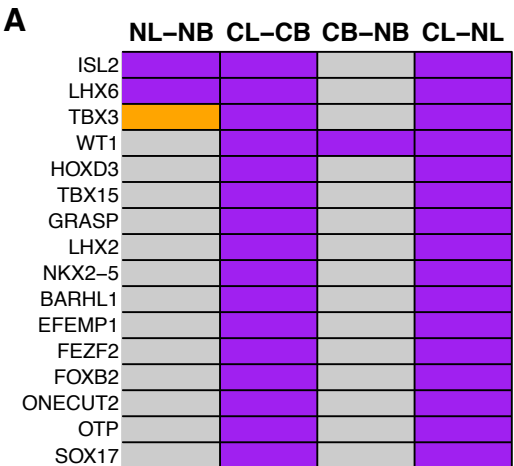


Figure 5

