

1 **Modular dynamics of DNA co-methylation networks**
2 **exposes the functional organization of colon cancer**
3 **cells' genome**

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20 **Summary**

21 DNA methylation dynamics is intrinsically interconnected with processes underlying the
22 malignant properties of cancer cells. By applying network-based approaches in two series of
23 colorectal cancers we dissected the long-range co-methylation structure finding consistent
24 patterns of compartmentalization in both normal and tumor tissues. Large transchromosomal
25 modules showed unique regulatory signatures and coalesced into a structured network and
26 allowing simple patient stratification. Normal-tumor comparison revealed substantial
27 remodeling of specific modules and migration of subsets of co-methylating sites denoted by
28 functional aggregates, pointing out potential sources of epigenetic and phenotypic variability.
29 We conclude that DNA methylation dynamics architecture embodies interpretable
30 information that can be used as a proxy of the drivers and the phenotypes of malignant
31 transformation.

32 **Significance**

33 DNA methylation is a key epigenetic mark directly involved in genome organization and
34 regulation. DNA methylation profiles are variable and are extensively altered in most cancers.
35 We show that DNA methylation variability follows a transchromosomal modular dynamics in
36 both normal and colon cancer cells. The reshaping of the DNA methylation variability
37 network in tumorigenesis exposes genomic and functional associations and points out both the
38 mechanisms and the phenotypes of individual tumors. This information may be used for
39 patient stratification and identification of disrupted pathways and therapeutic targets.

40 **Highlights**

- 41 • DNA methylation variability displays a modular architecture in normal and cancer.
- 42 • Coordinated transchromosomal variations supersede regional DNA methylation
43 dynamics.
- 44 • Co-methylation network modularity evinces functional and structural features.
- 45 • Epigenetic rewiring can be used as patient stratifier.

46 **Keywords**

47 DNA methylation, co-methylation, epigenetics, networks, colon, colorectal, cancer

- 48 **Running title**
- 49 DNA co-methylation architecture in colon cancer
- 50

51 **Introduction**

52 Cancer cell functional reprogramming involves gene expression dysregulation driven by
53 genetic and epigenetic changes. The contribution of epigenetic mechanisms to malignant
54 phenotypes has been thoroughly studied and includes extensive DNA methylation alterations
55 as prominent features of most cancers types (Feinberg et al., 2016; Portela and Esteller, 2010).
56 DNA methylation mainly occurs in the cytosine of the CpG dinucleotide and is usually
57 associated with a repressed chromatin state. Changes in DNA methylation have multiple
58 effects in genome regulation and have been directly associated with gene overexpression and
59 silencing, chromatin remodeling and chromosomal instability (Eden et al., 2003; Feinberg et
60 al., 2016; Jones, 2012; Rodriguez et al., 2006; Schubeler, 2015). Direct comparison of the
61 DNA methylation profiles in the tumor versus the paired normal tissue reveals both losses
62 (hypomethylation) and gains (hypermethylation) of the epigenetic mark. The extent of the
63 change may range from discrete sites and promoters to large regions (Feinberg et al., 2016;
64 Frigola et al., 2006; Hansen et al., 2011; Jones, 2012; Portela and Esteller, 2010).

65 Neighboring CpGs have a higher chance of being similarly methylated (Barrera and Peinado,
66 2012; Eckhardt et al., 2006; Libertini et al., 2016; Shoemaker et al., 2010); nonetheless, the
67 actual extent of this vicinity effect is disputed, with reports of complete to weak or very low
68 decay of co-methylation as the genomic distance increases in different cell types and tissues
69 (Akulenko and Helms, 2013; Fortin and Hansen, 2015; Li et al., 2010; Salhab et al., 2018).
70 Most studies about the functional impact of DNA methylation changes have focused the
71 analysis on local effects on neighboring genes (Jones, 2012; Schubeler, 2015). More recently,
72 taking advantage of the availability of genome-scale DNA methylation data from large
73 datasets, the study of DNA co-methylation profiles has been addressed from different points
74 of view, including the analysis of long range correlations (Akulenko and Helms, 2013; Fortin
75 and Hansen, 2015; Zhang and Huang, 2017), gene centered analyses (Gao and Teschendorff,
76 2017; Li et al., 2014) and modeling of DNA methylation variation (Jenkinson et al., 2017;
77 Libertini et al., 2018; Rulands et al., 2018; Teschendorff and Relton, 2018).

78 We hypothesize that epigenetic phenotypes exposed by DNA methylation co-variation reveal
79 the functional organization of human cancer cell's genome. To get insights into the structure,
80 functional determinants and underlying mechanisms of DNA methylation dynamics, we
81 examined the DNA methylomes of colon cancer patients by a novel network-based synthetic
82 analysis. Recent leading studies have proposed network based elucidation of molecular
83 determinants of disease (Creixell et al., 2015; Chen et al., 2014; Liu et al., 2016). Our

84 rationale is that cells subjected to complex physiopathological processes (e.g. tumor initiation
85 and progression), albeit being highly heterogeneous, share common driver and passenger
86 events from which biologically relevant phenotypic traits arise. Concomitantly, the linkage
87 between the events and the emergence of relevant traits alters the epigenome. The
88 heterogeneity of the samples, which might be classified in a wide assortment of states and
89 transitions, challenges the final state-focused differential methylation analysis (the one
90 resulting in regional hyper- or hypomethylations), whilst favoring more process-oriented,
91 flexible co-variation analysis, which also unfolds variability. Here, we have scrutinized over
92 300,000 individual CpGs in two colon cancer datasets to extract and characterize the highly
93 connected co-methylation modules. The structural and functional insights of epigenomic
94 modules are dissected providing a framework to disentangle cancer cell's genome functional
95 reorganization.

96 **Results and Discussion**

97 **Distant CpGs co-methylate in colon cancer samples**

98 We retrieved DNA methylation data as measured by Infinium HumanMethylation450 Array β
99 values from 90 tumor and 90 adjacent normal tissues from the Colonomics cohort (Closa et
100 al., 2014; Cordero et al., 2014; Sanz-Pamplona et al., 2015; Sole et al., 2014) to feed the co-
101 methylation analysis (Figure 1). Quality check consisted in three steps (Table S1). First, we
102 excluded probes non uniquely mapping to a genomic location, being polymorphic or located
103 in sex chromosomes (Price et al., 2013). Second, probes with low variability (standard
104 deviation $s_{\beta} < 0.05$) were filtered out to get rid of correlations led by outliers or presumably
105 non significant. Finally, probes with missing data in any sample were eliminated (Table S1).
106 No detectable batch effects were found (Figure S1). Next we sequentially calculated the bulk
107 pairwise Spearman's correlations between any possible pair of probes adjusting for multiple
108 testing (Table S2).

109 Co-methylations were detectable even at long distances (Figure 2A) and did not depend on
110 local probe density (Figure S2A). Correlation coefficients ρ (a measure of association ranging
111 from -1 to 1, in which 0 means full independence) were bell-shape distributed, thus indicating
112 that the majority of correlations lied on the non-significant range, as expected (Figure 2),
113 independently of the CpG location in open or closed chromatin compartments (Figure S3).
114 The ρ distribution was not centered to 0 but shifted towards positive values, thus indicating a

115 trend towards co-methylation changes. That is, the detected changes in DNA methylation
116 correspond to either the increase or decrease of the scrutinized CpGs altogether, and not in
117 opposite directions (e.g. inverse associations). The trend to co-methylate was noticeably
118 increased in cis, being close CpGs' distribution negatively skewed: co-methylations were
119 enriched at short distances, whereas anti-methylations (negative correlations) were not (Figure
120 2A).

121 To underpin the biological relevance of the findings and rule out the co-methylation structure
122 arising due to technical noise, we evaluated five possible sources of artifacts: multiple testing,
123 batch effects, leading outliers, tumor purity and chip design (see Supplemental Methods) and
124 none of them appeared to have a significant effect on the results.

125 To account for the iterative nature of the analysis, consisting in exhaustively computing any
126 pairwise correlation between the Infinium probes with variable DNA methylation, we set an
127 astringent effect size cut-off of the Spearman's correlation coefficient $\rho \geq 0.8$, which is close
128 to the conservative Bonferroni p-adjustment for the datasets used (optimization against the
129 asymptotic p-values as calculated by the Fisher Z transform, Table S2) (Fisher, 1915;
130 Shakhbazov et al., 2016). The absence of notorious clustering of DNA methylation values
131 (Figure S1) indicates that batch effects are unlikely drivers of co-methylation (Leek et al.,
132 2010). To attenuate the leading effect of DNA methylation outliers we filtered in probes with
133 sufficient variation in DNA methylation (setting a standard deviation threshold). To reinforce
134 this, nonparametric Spearman correlations were run, which rely on DNA methylation ranks
135 rather than values and are therefore more resistant to outliers (Croux and Dehon, 2010). As
136 for the Infinium array design, neither the probes GC content (Figure S4) nor the dye channel
137 (Figure S5) drove the correlations structure; probes mapping to multiple locations or
138 overlapping to SNPs were filtered out (Price et al., 2013).

139 **Anatomy of the co-methylating network in colorectal cancers**

140 We selected the top-scoring correlations ($\rho \geq 0.8$) and assembled a network in which loci and
141 correlations are represented by nodes (vertex) and links (edges) (Figure 1B). For the sake of
142 simplicity, we only considered edges with positive correlations. The Colonomics tumor series
143 network resulted in 63,130 nodes and 26 million connections (Table S3). The distribution of
144 each CpG degree (amount of connectivity, number of co-methylating neighbors) showed a
145 heavy-tail shape with the vast majority of nodes being linked to few counterparts, whereas a
146 few nodes displayed a fairly abundant connectivity (Figure S6). The degree distribution did

147 not resemble power-law, lognormal nor exponential (goodness of fit, Kolmogorov Smirnov
148 tests, $p \geq 0.05$; Figure S7), as there was not linear dependency between the cumulative
149 frequencies and the connectivities, as in other biological quantities spanning several orders of
150 magnitude and heavily skewed to the right (Newman, 2005). This structure was unaffected by
151 loci features such as chromatin state (Figure S8) and genomic category (Figure S9); but lost
152 when filtering out trans interactions, as probes placed at any distance in cis showed power-law
153 compatible distributions (Figure S10). Interestingly, the 99th percentile of the most connected
154 trans-comethylators presented homogeneous intermediate DNA methylation levels in normal
155 samples (Figures S11 and S12), with an important enrichment of imprinted loci ($n=53$, 11%).
156 Interestingly, partially methylated domains (PMDs) have been reported as loci with
157 intermediate DNA methylation values and high variability (Lister et al., 2009), which is
158 consistent to the top connected co-methylated probes; however, we did not find an enrichment
159 in them: 27% (132 CpGs) of the rich probes overlapped PMDs, similarly to the 33% (21,065
160 CpGs) of the probes with at least a significant co-methylation and what is expected from the
161 background of the whole set of Infinium probes, with an overlap of the 31% (147,257 CpGs).
162 To test the reproducibility of the network, the analysis was repeated using an independent
163 dataset, the COAD cohort from TCGA, consisting of 256 primary colon adenocarcinomas. In
164 TCGA dataset, the DNA methylation β value calling procedure differs from Colonomics', and
165 therefore reduces the chance of covariation artifacts arising due to the data processing bias.
166 No batch effects were detected (Figure S1).

167 Near a quarter million probes fulfilled the variability criteria in TCGA colon tumors (Tables
168 S6 and S7). The overall correlations distribution and the co-methylation decay with distance
169 matched that of Colonomics' (Figure 2A and Figure S2B). Next we evaluated whether the
170 correlation value for each pair of probes was conserved, including the non-significant pairs.
171 To do so, we computed exhaustive pairwise correlations of CpGs located at the chromosome
172 10 against itself and plotted the Colonomics' ρ values of each CpG pair against TCGA's. The
173 linearity of both landscapes (Figure 2B) indicated a high concordance of the overall co-
174 methylation levels.

175 In a similar vein and to compare the structure of both networks, we checked whether the
176 correlating nodes present in both cohorts displayed the same connectivity to other nodes. The
177 influence score of each node (e.g. based in the number of links arising from it) was estimated
178 using the PageRank score (Page et al., 1999) in both datasets. Both Colonomics and TCGA

179 tumor co-methylation datasets showed a reproducible distribution of nodes' PageRanks
180 mostly composed by lowly influential CpGs (Figure S13).

181 Next, we modeled the results as a network keeping the ρ cut-off at 0.8 even though the
182 multiple testing adjusted significance cut-off at TCGA cohort admitted lowering it due to the
183 larger sample size (Table S2). The network comprised 37 thousand nodes and around eight
184 million edges (Table S3). As in the Colonomics cohort, the TCGA network degree
185 distribution showed a long tail, indicating vast differences in connectedness (Figures S6 and
186 S7).

187 **Coalescent embedding of normal co-methylation networks in the tumor** 188 **networks**

189 We wondered whether some of the co-methylations found in cancers were already detectable
190 in adjacent normal colonic mucosa, and to which extent the co-methylome structure differed
191 from the tumor's one. The normal colon co-methylome network was built using 90 non-tumor
192 tissues from the Colonomics cohort. Given the equivalent sample sizes, we maintained the ρ
193 cutoff unaltered (Table S2). We found that both the number of probes fulfilling the variance
194 prerequisite ($n=99,346$) and the number of total correlations ($7,430,741$) decreased to 39%
195 and 22% of the tumor's ones, respectively. This result was consistent with the higher DNA
196 methylation variability in tumors. As expected, a predominance of positive correlations was
197 observed, being more intense for close probes (Figure 2A). Strikingly, negative correlations
198 (cut-off $\rho < -0.8$) showed a $>1,000$ -fold reduction and dropped from 7.6 million in tumor to
199 less than 7 thousands in normal tissue (Table S7). In agreement with the associations found in
200 tumors, co-methylations were underrepresented in active promoters (Table S8, Figure S14)
201 and the co-methylation network's connectivities were not power-law distributed (Figures S6
202 and S7). Probes pairs correlation values showed partial agreement between the normal and
203 tumor datasets (Figure 2B), with differences being more conspicuous at the node influence
204 level, pointing to changes in network connectivity (Figure S13).

205 We repeated the analysis with TCGA normal colon samples. It should be noted that this
206 dataset only includes 38 normal samples (Table S2), and as the correlation significance
207 depends on the sample size (Fisher, 1915), keeping the same cut-offs is likely to boost the
208 number of false positives. On the other hand, increasing the ρ cut-off to an equivalent
209 detection threshold ($\rho = 0.96$, Table S2) produced a very small network whose properties
210 might be out of scale with the previous analysis. With this cautionary note in mind, keeping

211 the $\rho = 0.8$ cut-off the network confirmed the distinctive distribution of pairwise correlations
212 (Figure S4 and Table S9), whose differences are especially conspicuous at short ranges
213 (Figure 2A) and, importantly, in nodes connectivity.

214 **Co-methylating networks display a modular structure in normal and tumor** 215 **tissue**

216 Colonomics tumor network had two noticeable giant components (Figure 3), that were not
217 present in the normal tissue, indicating a major restructuration of co-methylation architecture
218 associated with malignant transformation, as we will discuss below. TCGA tumor and normal
219 co-methylation networks replicated Colonomics overall networks structure (Figure 3).

220 In order to dig into the network preferential attachment, we explored whether the network had
221 highly connected subnetworks (also known as modules or communities). Modules consist of
222 clusters of nodes heavily interconnected as compared to the rest of the network (Fortunato,
223 2010; Newman and Girvan, 2004). Modularity is quantified as the fraction of edges
224 connecting nodes of the same type minus what it is expected in a randomly wired network.
225 Scores of 0 indicate no modularity and networks with modular structure typically range from
226 0.3 to 0.7 (Newman and Girvan, 2004). The tumor co-methylation network was found to be
227 modular (modularity = 0.47) (Table S3), and using the Clauset's fast greedy method (Clauset
228 et al., 2004) we partitioned it into 3,270 modules ranging from two to 18,727 nodes.

229 Interestingly, the normal tissue network exhibited a higher co-methylome modularity (0.62)
230 (Table S3) and network segmentation resulted in 1,265 modules ranging from two to 17,758
231 nodes. The co-methylation modules retained tight correlation structure after subtracting purity
232 effects (Zheng et al., 2017) (Figure S16).

233 The vast majority of the small modules were, in fact, composed by sets of probes located at
234 close distance from each other (e.g. at CpG islands), so we discarded them and focused in
235 transchromosomal modules, with at least 10 members and located at least 1 Mbp apart or
236 placed in different chromosomes. The number of transchromosomal modules was 32 (1%) in
237 the tumor cohort and 18 (1.4%) in the normal cohort (Tables S4 and S5).

238 In agreement with Colonomics's results, TCGA tumor co-methylation network was also
239 modular (modularity score 0.41) (Table S3) and segmentation produced 3,421 modules
240 ranging from two to 8,981 nodes. The application of size and co-location filters reduced the
241 number of transchromosomal modules to 35.

242 Next, we evaluated the degree of conservation of the whole network partitioning into modules
243 across the four datasets using the adjusted Rand statistic. In this test, the distance measure can
244 be interpreted as a probability, being zero when the congruence is expected by chance and one
245 when the matching is perfect. It should be noted that the Rand statistic renders negative values
246 when finding anti-associations (Hubert and Arabie, 1985). Networks clustering on adjusted
247 Rand's distance indicates that modules memberships separate tumor's from normal's
248 networks in both datasets (Figure S15A), in line with the similarities in nodes population
249 (Figure S15B), and their spatial co-methylation patterns (Figure S2B). Overall conservation
250 of co-methylomes structure and connectivity as well as the differences between normal and
251 tumor samples was noticeable by visual inspection (Figures 3, S17 and S18). It is worth
252 noting that the use of a correlation threshold (i.e. effect size $\rho \geq 0.8$) may underestimate
253 module co-methylation maintenance when the correlations distribution gets displaced towards
254 values below, but close to, the statistical significance cut-off (Appendix 1, Figure S18).

255 Module preservation across tissue types and cohorts was also evaluated by cross-tabulation of
256 the number of shared CpGs (Table S10). Twelve Colonomics tumor modules had one or more
257 counterparts in the normal tissue network, and a similar number in TCGA tumor cohort
258 (Fisher's exact test, $p < 0.0001$) (Tables S10, S11). Strikingly, the five-top sized Colonomics
259 tumor modules partially matched to multiple TCGA's modules (Table S11). This result is in
260 concordance with the resolution limit of modularity-optimizing module detecting algorithms,
261 which tend to aggregate modules into few giant components, disregarding their inner
262 complexity (Fortunato and Barthelemy, 2007).

263 **Co-methylating module membership evinces functional signatures**

264 To test the hypothesis that co-methylation structures are directly related with functional
265 properties we investigated genomic and functional features of co-methylated CpGs. It should
266 be noted that a large subset of HumanMethylation450k probes are located in promoters and
267 promoter-related features. Thus, the specific design of the HumanMethylation450k array may
268 introduce biases as it oversamples TSS-related features, in which clusters of probes are
269 located, and disregards other genomic compartments (Bibikova et al., 2011; Sandoval et al.,
270 2011; Silva-Martinez et al., 2017). Moreover, coordinated co-methylation is expected among
271 neighboring CpGs within each one of these genomic elements (Barrera and Peinado, 2012;
272 Gaidatzis et al., 2014; Kim et al., 2008; Libertini et al., 2016; MacDonald et al., 2015; Wang

273 et al., 2016). Therefore, enrichment analysis for genomic features and compartments were
274 corrected according to the HumanMethylation450k array background.

275 The tumor modules displayed important differences in feature enrichment, including
276 chromatin states, genomic categories, CpG islands and association with known motifs (Figure
277 S19 and Appendix 1). In line with the weighted gene co-expression network analysis, in
278 which functional signatures can be told apart by mining gene co-expression (Horvath et al.,
279 2012), the module-specific co-regulation patterns denoted by distinctive abundance of
280 genomic and functional states (Figure S19; Appendix 1) of co-methylation modules pointed
281 out the existence of a latent structure. Among the multiple features analyzed, a striking global
282 enrichment of inactive promoters was observed in a large number of modules (Figure S20),
283 pointing out potential clusters of co-regulated genes.

284 Next, we explored the overlapping of the co-methylation modules with regions of DNA
285 methylation variability previously reported in colon cancer (Hansen et al., 2011).
286 Interestingly, seven out of 32 Colonomics tumor modules significantly overlies tumor
287 hypermethylated blocks (Figures S21 and S22 and Table S12). Regarding other types of DNA
288 methylation variability reported by Hansen, modules showed distinctive profiles, with
289 frequent enrichment in boundary shifts as well in loss of regulation; novel hypomethylation
290 blocks were enriched in three modules only. This complexity reinforces the individuality of
291 co-methylation modules, suggesting that they might reflect different mechanisms.

292 A comprehensive summary of structural and functional feature enrichment for each co-
293 methylation module is shown in Appendix 1 with the top associations listed in Table S13). To
294 name a few examples, multiple co-methylation modules were significantly enriched for
295 Polycomb-related marks (i.e. H3K27ME3, or SUZ12, EED and PRC2 targets; e.g. tumor
296 modules 1, 3, 5 and 598); for frequently mutated at COSMIC molecular signatures (i.e. tumor
297 modules 2, 4 and 8); and for gene expression (i.e. tumor module 8). Finally, we could also
298 confirm that co-methylation network associated features found in TCGA matched
299 Colonomics enrichment signatures, e.g., the underrepresentation of co-methylations within
300 active promoters (Figure S14).

301 To shed light into causal factors driving dynamic methylome modularity we searched for
302 enriched motifs (i.e. transcription factor binding sites) at the co-methylating loci
303 (Supplementary methods). We found that six out of the 32 Colonomics tumor modules
304 presented one or more significantly enriched motifs (Table S14). Enriched motifs included

305 ETS and RUNX families (modules 1, 3, 327), FOS family members (Fra1, Atf3, BATF,
306 Fosl2, AP-1, Jun-AP1; modules 2 and 4), FOXA1-related (FOXA1, HNF4a, FOXMA;
307 module 2), GC box (KLF5 and KLF4; module 2), C/EBP (module 3), PAX7 and MYF5
308 (module 5), homeobox (modules 5, 83), MADS (module 152), ASCL1 (module 598)
309 (Appendix 1).

310 In summary, the functional signatures of DNA co-methylation modular architecture evince
311 the putative mediators of epigenetic remodeling and signaling reprogramming in colorectal
312 cancer. We postulate that coordinated DNA methylation changes at interspersed sites (here
313 identified as belonging to the same module) regulate signaling pathways and biological
314 functions. This hypothesis is supported by recent studies demonstrating the DNA methylation
315 mediated binding of transcription factors to specific sites with a direct impact in gene
316 regulation (Kribelbauer et al., 2017; Yin et al., 2017).

317 **Modeling of module's DNA methylation variation allows categorization and** 318 **study of feature associations in new samples**

319 As shown above, modules depict shared patterns of co-methylations (network edges) which
320 emerge from structured DNA methylation levels among loci (network nodes). To dissect the
321 latter, we applied a samples stratification procedure based on the DNA methylation status of
322 their CpGs (Supplemental methods) that results in the partition of each module into two to
323 three DNA methylation profiles (Figure S23). The putative effects of tumor purity to DNA
324 methylation levels were identified and subtracted (Figure S24).

325 This methodology provides with a powerful tool to explore potential correlates of DNA
326 methylation profiles with molecular and biological features, including clinical data, and
327 importantly, enabling the model to classify new samples and to make predictions without
328 computing new correlations. An in-depth exploitation of this approach is beyond the scope of
329 this paper, but as a proof of concept, we evaluated whether module cluster membership
330 conveyed gene expression signatures to tumor samples. The pairwise differential expression
331 between samples belonging to different module clusters was computed (adjusted $p < 0.1$ cut-
332 off, Figure S25) in both cohorts of colon tumors. TCGA cohort consistently exhibited a higher
333 number of differentially expressed genes. This result may be explained by the larger size of
334 this series and the use of RNA-Seq, that has more sensitivity than the microarrays (Zhao et
335 al., 2014) applied in the Colonomics. Nevertheless, the overall gene over- and down-
336 expression trends were maintained across cohorts (Figure S26). The top 50 significant

337 differentially expressed genes in both cohorts are listed in Appendix 2 for each one of the 32
338 Colonomics tumor modules.

339 **Dynamics of co-methylation modules reveals epigenetic rewiring of defined** 340 **genomic compartments in cancer**

341 As noted above, the tumor co-methylation network displayed a striking disjoint structure
342 visualized as two giant compartments (Figure 3). The emerging large compartment, not
343 present in normal tissue, spanned multiple modules (Figure 3 and Figure S27) and was
344 funneled by DNA methylation negative correlations between modules (Figure 4A). The
345 coordinated inversion of DNA methylation variation affected hundreds or even thousands of
346 sites throughout the whole genome (Figure 4B). The pervasive nature of anticorrelations
347 overcame age, gender, tumor stage and anatomical site potential effects on modules' DNA
348 methylation variation (Figure S28A). Loci with copy number alterations also conveyed the
349 module-specific DNA methylation ranks mimicking the profiles along balanced regions
350 (Figure S28B).

351 To further dissect the co-methylation dynamics we analyzed the modules preservation
352 between normal and tumor. Significant equivalences were found for most modules (Figure
353 S29): normal modules N2 and N3 largely overlapped with tumor modules T1 and T2
354 respectively (Figure S29), which suggests the preservation of module's structure and co-
355 methylation links. A large overlap of associated genes among modules was also observed
356 (Figure S29C). At the functional level, the preserved modules showed specific enrichments.
357 For instance, normal module N2 intersection with tumor module T1 ($N2 \cap T1$) showed
358 enrichment for RNA transcription and metabolic processes and DNA binding functions
359 (Figure S30) and a high proportion of probes were located in CpG islands (77%) with a clear
360 trend towards tumor hypermethylation (Figure 5B, Figure S31).

361 Next we analyzed the dynamics of CpG sites between normal and tumor modules. For the
362 sake of simplicity only probes in the four largest modules in the Colonomics normal and
363 tumor series were considered for differential module membership. A particular case was the
364 scattering of normal module N1 probes into different tumor modules (Figure 5A), including
365 the hijacking of several hundreds of sites by modules with inverse correlations, e.g.: tumor
366 modules T1 and T2 (Figure S27). The subsets of probes flowing from the normal module N1
367 to each one of the tumor modules (intersections between normal module N1 and the tumor
368 modules T1 to T4) were associated to subsets of genes with limited overlap (Figure 5C) and

369 displayed distinctive genomic features in regard to gene regulation: $N1 \cap T1$ members were
370 enriched in CpG islands, while $N1 \cap T2$ were depleted and $N1 \cap T3$ were frequently near the
371 TSS (Figure 5D). The tumor-normal DNA methylation signatures were in concordance with
372 the preferential genomic location of probes: members of the intersection $N1 \cap T1$ were
373 characterized by the prevalence of hypermethylations in the tumor, while the rest showed a
374 clear trend towards hypomethylation (Figure 5E and Figure S31).

375 As a whole, our analysis points out an overall preservation of co-methylation modules in the
376 normal-tumor transformation concomitantly with an important dispersal of subsets of sites
377 with distinctive features into tumor modules. The tumor redefined modular landscape appears
378 to have biological insights: the subsets of sites flowing from one module to another (denoted
379 here as normal-tumor module intersections) display differential enrichments in functional and
380 biological processes involved in cancer transformation (Figure 5E and Appendix 2). Some of
381 the affected signaling pathways, including polycomb regulation, chromatin binding and genes
382 defining epithelial-mesenchymal transition appear as the usual suspects contributing to the
383 epigenetic reshaping of genomic compartments and the functional reprogramming of cancer
384 cells (Appendix 2).

385 **Surfing the co-methylating networks pinpoints functional sites**

386 Beyond the remarkable functional and structural features of co-methylation modules revealed
387 by this analysis, it has not escaped our attention that the stored data provide an excellent
388 resource to carry out an insightful tracing of individual correlations. A detailed analysis of the
389 data at this level is beyond the scope of this paper, but as a simple shortcut to navigate the co-
390 methylating network and their associated functional features we developed a web tool “*corre*”
391 (available at <http://maplab.cat/corre>). To illustrate the discovery potential of this tool we
392 queried the *INHBB* gene encoding activin B, a member of the TGF-beta family, with different
393 biological activities, including a role in cell proliferation and inflammation. Epigenetic
394 silencing of *INHBB* is frequent in colorectal cancer (Frigola et al., 2006) and has been
395 proposed as indicator of poor outcome (Mayor et al., 2009). The co-methylation landscape of
396 *INHBB* exposed by the *Corre* tool showed a large number of positive correlations 20kb
397 upstream and downstream of the gene in both normal and tumor samples (Figure 6). Negative
398 correlations were only present in tumors and were enriched in poised promoters, indicating
399 the potential remodeling of bivalent states and hypermethylation (McGarvey et al., 2008;
400 Ohm et al., 2007; Rodriguez et al., 2008). Compared with the normal samples, the tumors

401 displayed an increase in the number of links for most sites, although some chromosomes,
402 especially 8 (Figure 6E), but also 13, 14, 17 and 21, showed an opposite trend with a
403 depletion of co-methylations in the tumors as compared with the normal samples (Figure
404 S32). Another interesting result was in regard to cg03699182 probe (Figure 6D, arrowhead)
405 located in the CpG island of the INHBB promoter presented 42 co-methylations ($\rho > 0.8$) in
406 the normal samples against only three in the tumors. Most of the cg03699182 co-methylations
407 affected were located in poised promoters of polycomb regulated genes (Figure S32). The
408 dynamics of the connections and the properties of the affected sites are consistent with the
409 participation of instructive mechanisms resulting in the DNA hypermethylation and long
410 range epigenetic silencing of multiple genes in colorectal cancer (Frigola et al., 2006; Keshet
411 et al., 2006; Michieletto et al., 2018).

412 **Final considerations**

413 Dissection of DNA methylation encoded information offers a far-reaching gamut of insights
414 into genome biology (Jones, 2012; Schubeler, 2015), including the inference of genome
415 architecture as demonstrated by recent studies (Fortin and Hansen, 2015; Jenkinson et al.,
416 2017; Jorda et al., 2017; Raineri et al., 2018; Zhang et al., 2017). As a new inquiry, here we
417 report a novel and robust analysis of coordinated DNA methylation dynamics in non-
418 contiguous CpGs in two cohorts of colon normal and cancer tissues. This application provides
419 a reproducible and synthetic network representation of cell's epigenome meta-structure and
420 unveils modules or genomic territories of highly connected loci. The co-methylation modules
421 comprise regions displaying common structural and functional features pointing out putative
422 drivers of variability. Despite wide overlapping between normal and tumor tissue networks,
423 striking differences in connectivity reveal specific patterns of functional rewiring and convey
424 gene expression signatures with a potential impact on cancer cell biology.

425 Our data present a remodeled epigenetic landscape of colon cancer cells outlined by
426 coordinated DNA methylation variations superseding the stochastic nature of DNA
427 methylation dynamics (Jenkinson et al., 2017; Landan et al., 2012; Pujadas and Feinberg,
428 2012). The model can be visualized by a scrambled Rubik's cube resulting from just of a few
429 flips (Figure S33A). In our case, cube's pieces correspond to the set of loci with coordinated
430 methylation, and the axes would be the mechanisms flipping one or more modules (Figure
431 S33B). This metaphor has two important corollaries with the corresponding epigenetic
432 representations:

- 433 • Pieces linked by connectors move together, which implies that any specific scrambled
434 conformation is the result of specific flips. Moreover, not all the arrangements are
435 possible unless the cube is disassembled and reassembled. Similarly, cancer cell
436 methylome dynamics is determined by sequential activation/inactivation of a limited
437 number of mechanisms affecting genomic regulation. Interestingly, chromosomal
438 rearrangements would provide an additional level of reshuffling that would be
439 equivalent to reassembling Rubik's cube.
- 440 • The scrambled cube may be solved by predictable flips that do not imply the reversal
441 of the flips that generated it. Currently we can only speculate, but this means that
442 knowing the mechanisms governing epigenetic programs, it would be possible to
443 design a strategy to reconstruct a "normal" epigenome by just turning on/off the
444 appropriate switches and in the right sequence.

445 Summing up, our approach aims to offer a contextual view of the cancer epigenetic landscape
446 to better define their nature and their eventual impact on the disease. The use of DNA co-
447 methylation architecture to portrait the complex genome regulation scenario aims to provide a
448 feasible surrogate marker that can be easily assessed in prospective clinical settings (e.g.
449 response to treatment).

450 **Materials and methods**

451 Two colon cancer datasets were used. Colonomics (<http://www.colonomics.org>) series
452 included 90 paired primary tumors (stage IIA and IIB) and their adjacent normal tissue. Of the
453 90 patients, 67 were males and 23 females, aged 43-86 years (mean: 70.37), and 20 developed
454 metastasis. All tumors were microsatellite-stable. Samples were evaluated for DNA
455 methylation (Illumina Infinium HumanMethylation450 BeadChip Array), gene expression
456 (Affymetrix Human Genome U219), and somatic mutations (exome sequencing) (Closa et al.,
457 2014; Cordero et al., 2014; Sanz-Pamplona et al., 2015; Sole et al., 2014).

458 The Cancer Genome Atlas (TCGA) series was composed by 256 primary tumor and 38
459 adjacent non-tumor samples from the colon adenocarcinoma (COAD) cohort (Zhu et al.,
460 2014). Patients were aged 31 to 90 years at diagnosis (mean 65.61), and included 141 males,
461 144 females and one unassigned. Pathologic stages included Stage I (40), Stage II (97), Stage
462 III (75), Stage IV (32); 11 were not available or discrepant. Regarding microsatellite
463 instability, 10 were positive, 65 negative and 181 were either not tested or had an unknown

464 status. Samples readouts included DNA methylation by Illumina Infinium Array, gene
465 expression by RNA-Seq counts and somatic mutations by exome sequencing.

466 A scheme summarizing data processing and workflow is depicted in figure 1. Briefly, DNA
467 methylation beta values were subjected to serial pairwise correlation analysis for the
468 Colonomics tumor (primary dataset) and normal adjacent tissue, as well and both the TCGA
469 normal samples and tumors (external datasets). Strong associations (effect size Spearman's ρ
470 ≥ 0.8) were stored. Co-methylation networks were built upon the correlations data using
471 previously described approaches (Clauset et al., 2009; Csardi and Nepusz, 2006; Cullen and
472 Frey, 1999; Delignette-Muller and Dutang, 2015; Gillespie, 2014; Saha et al., 2017; Zhang
473 and Horvath, 2005), from which highly connected modules according to the fast greedy
474 community detection algorithm were isolated (Clauset et al., 2004; Csardi and Nepusz, 2006).
475 Module members were further classified into major DNA methylation clusters using kNN
476 (Venables and Ripley, 2002) taking into account not only the co-methylation but the purity-
477 corrected DNA methylation status (Aran et al., 2015; Zheng et al., 2017) of their members
478 (i.e. consistently lowly or highly methylated) (Chang et al., 2010; Wang et al., 2007). Next,
479 modules and/or profiles were functionally annotated according to public datasets (Aryee et al.,
480 2014; Fortin and Hansen, 2015; Hansen et al., 2011; Lister et al., 2009), molecular features
481 databases (Heinz et al., 2010; Liberzon et al., 2011) and expression signatures (Gel et al.,
482 2016; Love et al., 2014; Quinlan and Hall, 2010; Smyth, 2005). Modules characterization,
483 including reproducibility assessment, consisted in mutual profiles comparison and differential
484 expression analysis among different cohorts (Akdemir and Chin, 2015; Hubert and Arabie,
485 1985; Krzywinski et al., 2009; Langfelder et al., 2011; Shannon et al., 2003).

486 A Web application "Corre" has been implemented to facilitate browsing the DNA co-
487 methylation events of investigator's favorite locus or gene in the both the Colonomics and
488 TCGA COAD datasets. The tool allows candidate queries either by gene symbol or Illumina
489 Infinium probename, providing the annotated co-methylations full list. Apart of downloadable
490 spreadsheets, Corre renders interactive plots to evaluate zonal (chromosome) and functional
491 (chromatin color) enrichments (Conway et al., 2016; Ernst et al., 2011; Gesmann and de
492 Castillo, 2011; Zhang et al., 2013). Source code is available at
493 <https://bitbucket.org/imallona/corre> under the GPL terms. Corre can be accessed freely and
494 without registration at <http://www.maplab.cat/corre>.

495 Extended methods are available in supplementary material.

496 **Acknowledgements**

497 We thank Iñaki Martínez de Ilarduya for his excellent technical support and Francisco Chen
498 for help parallelizing the sequential correlations. The data presented here are in part based
499 upon data generated by the TCGA Research Network: <http://cancergenome.nih.gov/>.

500 **Declarations**

501 **Authors' contributions**

502 IM, VM and MAP conceived the study. IM developed the method and wrote the software.
503 IM, SA, ADV and VM analyzed data. IM and MAP wrote the manuscript. All authors read
504 and approved the manuscript.

505 **Samples, Ethics approval and consent to participate**

506 Colonomics samples were collected at the Bellvitge Hospital. The Clinical Research Ethics
507 Committee (CEIC) of the Bellvitge Hospital approved the study protocol, and all individuals
508 provided written informed consent to participate and for genetic analyses to be done on their
509 samples. The approval number is PR178/11. Additional information about the study and
510 patient samples can be found at <http://www.colonomics.org>. TCGA data were obtained at
511 <http://cancergenome.nih.gov/>.

512 **Availability of data and material**

513 The code is available at: <https://bitbucket.org/imallona/correlations> under the GPL v3 terms.
514 The *Corre* Web tool can be freely accessed at <http://maplab.cat/corre>; its source code is
515 available at <https://bitbucket.org/imallona/corre> under the GPL v3 terms. Colomics data may
516 be accessed at <http://colonomics.org>. TCGA data were obtained at
517 <http://cancergenome.nih.gov/>.

518 **Competing interests**

519 MAP is cofounder and equity holder of Aniling, a biotech company with no interests in this
520 paper. The rest of the authors declare no conflict of interest.

521 **Funding**

522 This work was funded by the Spanish Ministry of Science, Innovation and Universities
523 (FEDER, SAF2015-64521-R to MAP), the Agency for Management of University and
524 Research Grants (AGAUR) of the Catalan Government grants 2017SGR723 and
525 2017SGR529. The funding agency had no role in the design of the study, collection, analysis,
526 interpretation of data nor manuscript writing.

527

528 **Figure Legends**

529 **Figure 1.** Co-methylation analysis framework. **A**, CpGs co-methylation occurs at close (cis)
530 and long distances (trans). **B**, DNA co-methylation networks display a modular structure in
531 normal and tumor samples. **C**, Co-methylation modules display differential genomic and
532 functional signatures. **D**, Analysis of normal-tumor co-methylation dynamics points out
533 cancer pathways and mechanisms.

534 **Figure 2.** **A**, Correlation distribution in normal and tumor samples among probes located in
535 chromosome 10. The bell-shaped distribution and shifted towards positive values at the tumor
536 cohort (whole chromosome 10); the trend to co-methylate is noticeably increased in cis (chr10
537 probes located at less than 10 kbp). Red: negative correlations ($\rho < -0.5$); green: positive
538 correlations ($\rho \geq 0.5$). **B**, Correlations replication across cohorts. For each cohort, a pairwise
539 correlation analysis was conducted for any probe with $sd \geq 0.05$. The correlation coefficient
540 rho (X and Y axes) for each probe pair was plotted to check whether the co-methylation
541 landscape was reproduced. Analysis was restricted to chromosome 10 Infinium450K probes.

542 **Figure 3.** Modular structure of colorectal tumor and normal tissue co-methylation networks.
543 The networks were built independently for each dataset, but the nodes (CpGs) are colored
544 using the Colonomics Tumor module membership. Nodes with no cross-representation are
545 shown in black. Graphs are limited to a random sample of 5,000 nodes and solitary nodes are
546 not plotted; network layout was calculated by $1 - \rho$ (edges) weighted springs.

547 **Figure 4.** The two largest tumor modules show opposed DNA methylation dynamics. **A**,
548 Correlation of three randomly picked CpGs from modules 1 and 2 in 92 Colonomics tumor
549 samples. **B**, DNA methylation ranks of modules 1 and 2 in four Colonomics tumor samples.
550 Patients labeled with color codes as depicted in panel A.

551 **Figure 5.** **A**, Sankey diagram depicting balanced probes overlap between normal and tumor
552 modules in the Colonomics datasets. Only intersections with >300 probes are annotated, and
553 the number of associated genes is indicated. **B**, Distribution of probes according to the mean
554 DNA methylation values in normal tissue (Y-axis) against the tumor-normal delta value (X-
555 axis). Only probes overlapping in normal and tumor modules (intersections) are represented.
556 A decomposed version of this figure is shown in Figure S31. **C**, Circos representation of
557 modules associated genes overlap (purple connectors) and enriched terms sharing (blue
558 connectors). **D**, Genomic context enrichment of normal module 1 intersections with tumor

559 modules. **E**, Gene set functional enrichment of normal module 1 intersections with the four
560 largest tumor modules.

561 **Figure 6:** Illustrative example of the *Corre* web tool usage. This application queries a user
562 selected Infinium array probe (i.e.: cg25924274) or a set of gene associated probes (i.e.:
563 *INHBB*) and renders graphs displaying the feature distribution of the anchor (preselected site)
564 and correlating CpGs, including DNA methylation levels, genomic element category, HMM
565 chromatin states, etc. In addition, tables containing genetic and functional information on the
566 correlating sites may be downloaded for further analysis. **A**, UCSC genome browser
567 representation of the region encompassing the preselected *INHBB* gene. **B**, The tool renders
568 graphs showing relevant features (see legends) for each one of the gene associated probes
569 (anchor CpGs). **C**, Distribution of DNA methylation levels in anchor CpGs and the
570 correlating sites. **D**, Sum of correlating sites in normal and tumor tissues for each anchor CpG
571 represented by chromatin state frequency. Positive (+) and negative (-) correlating sites show
572 distinct chromatin state profiles in the tumors. **E**, Genomic distribution of cg11513884 co-
573 methylating CpGs located in chromosomes 2 (red), 7 and 8 (blue).

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Figure 1

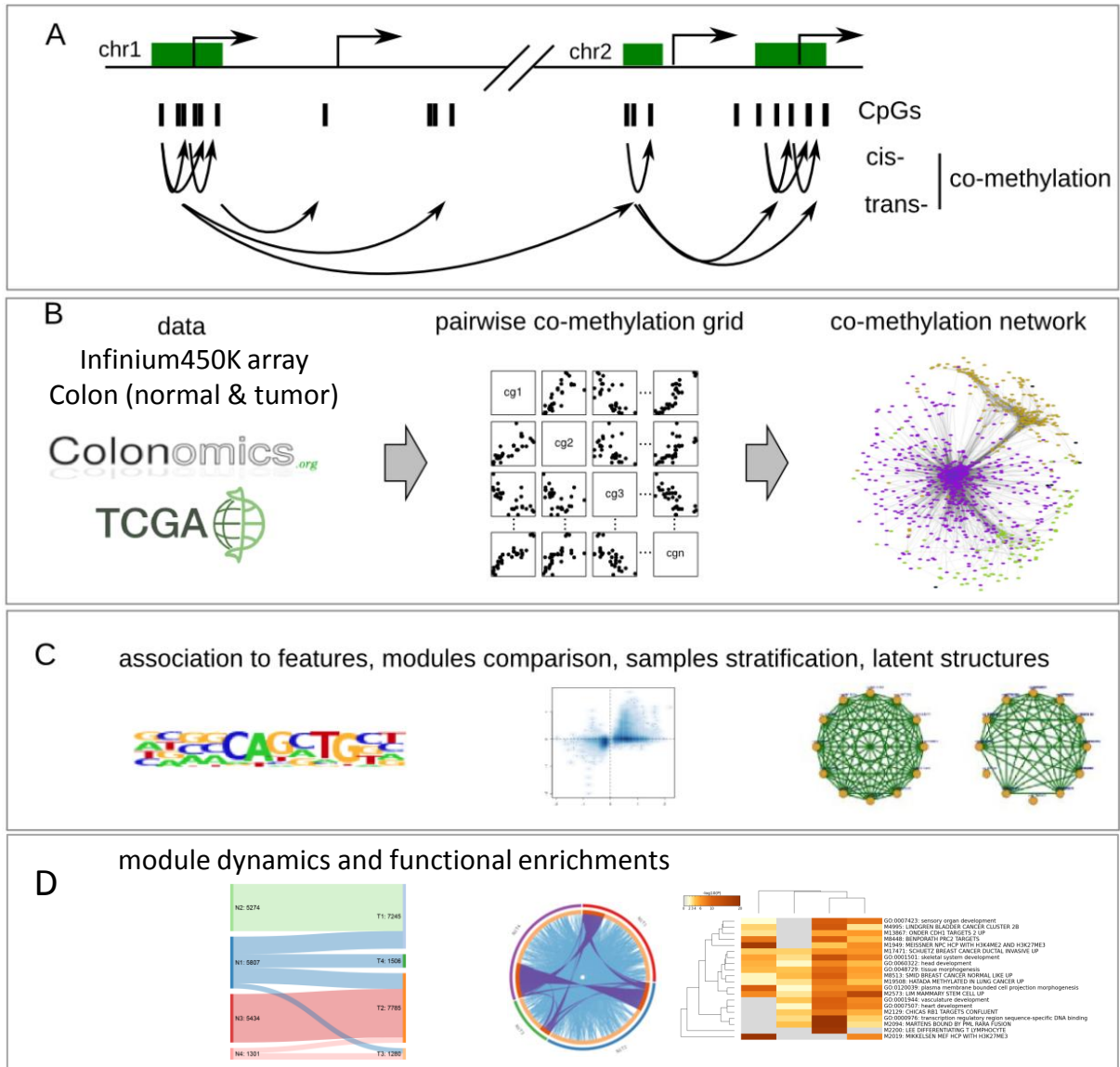


Figure 2

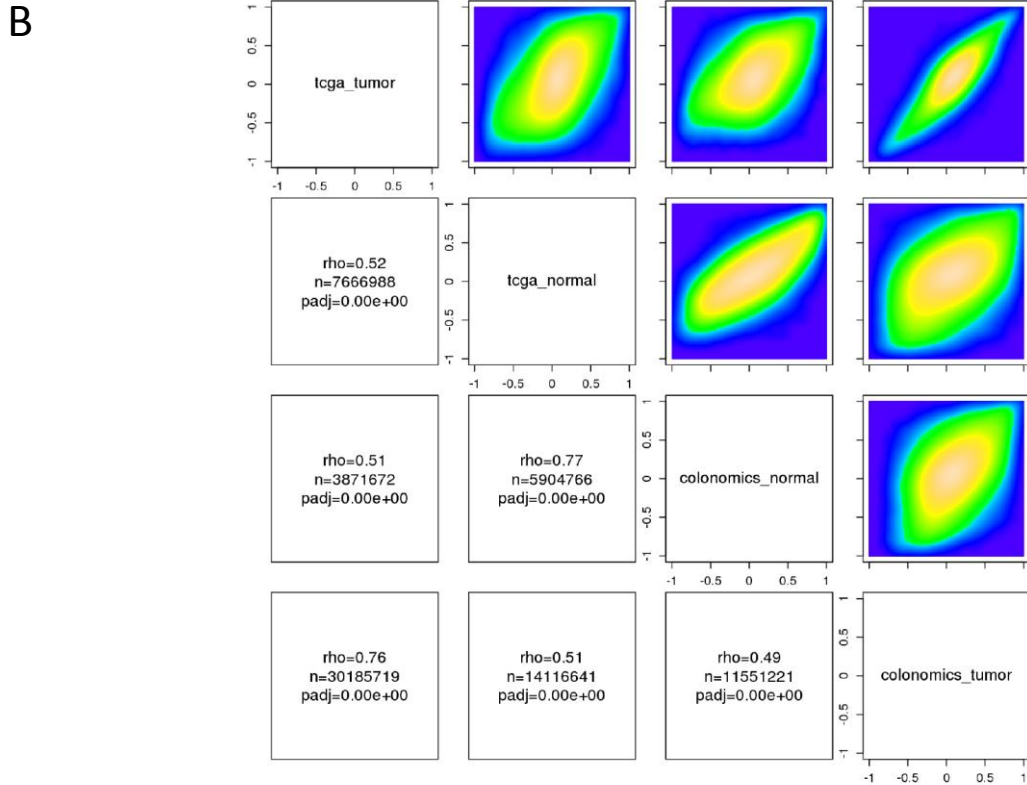
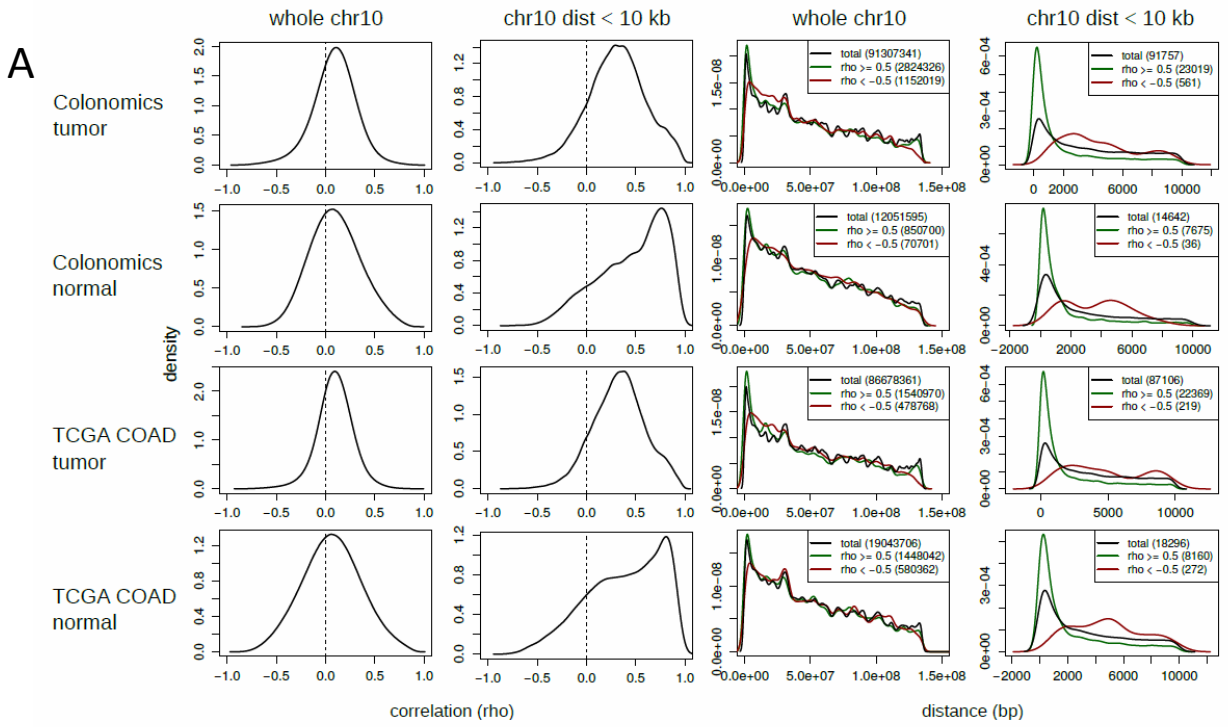


Figure 3

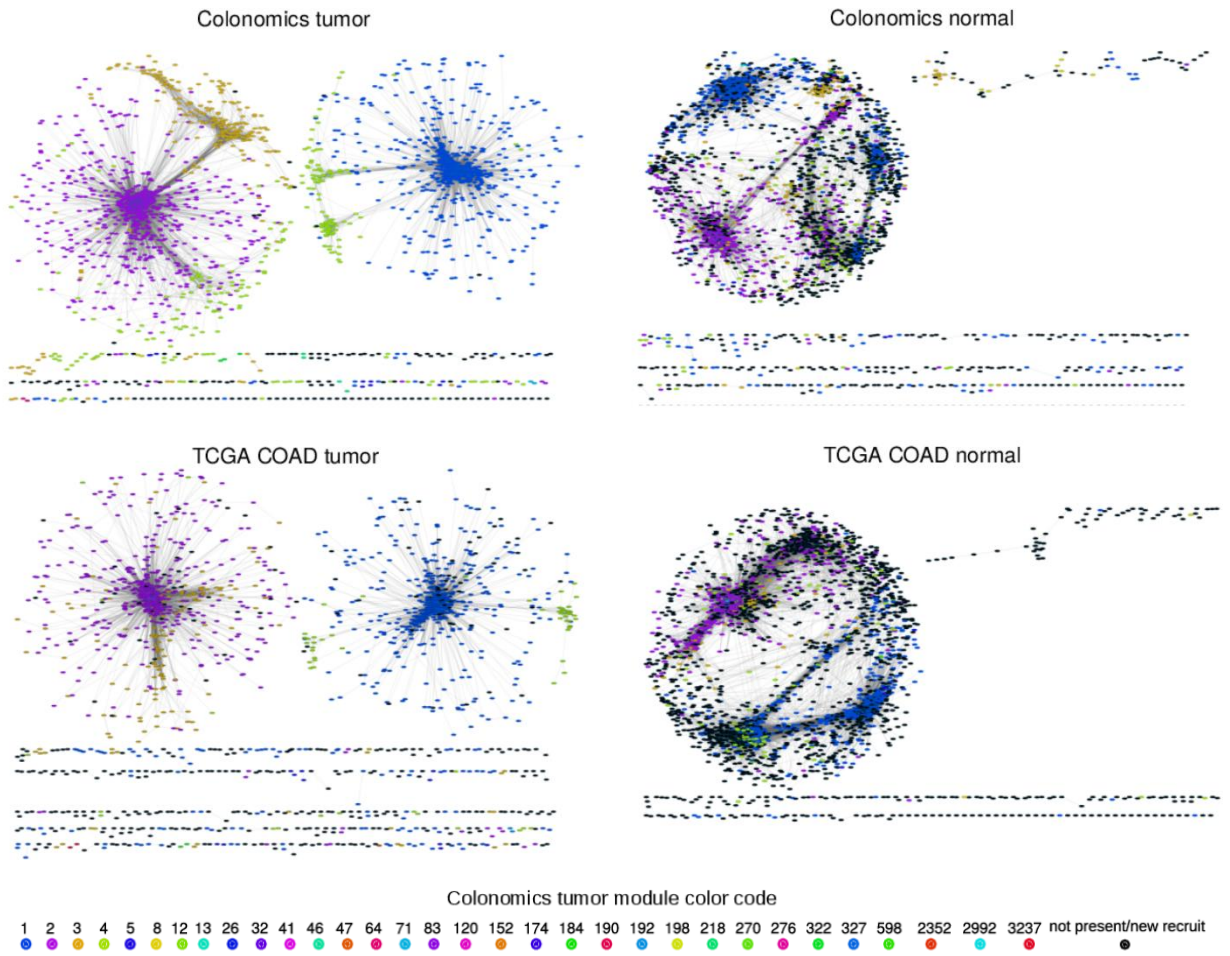


Figure 4

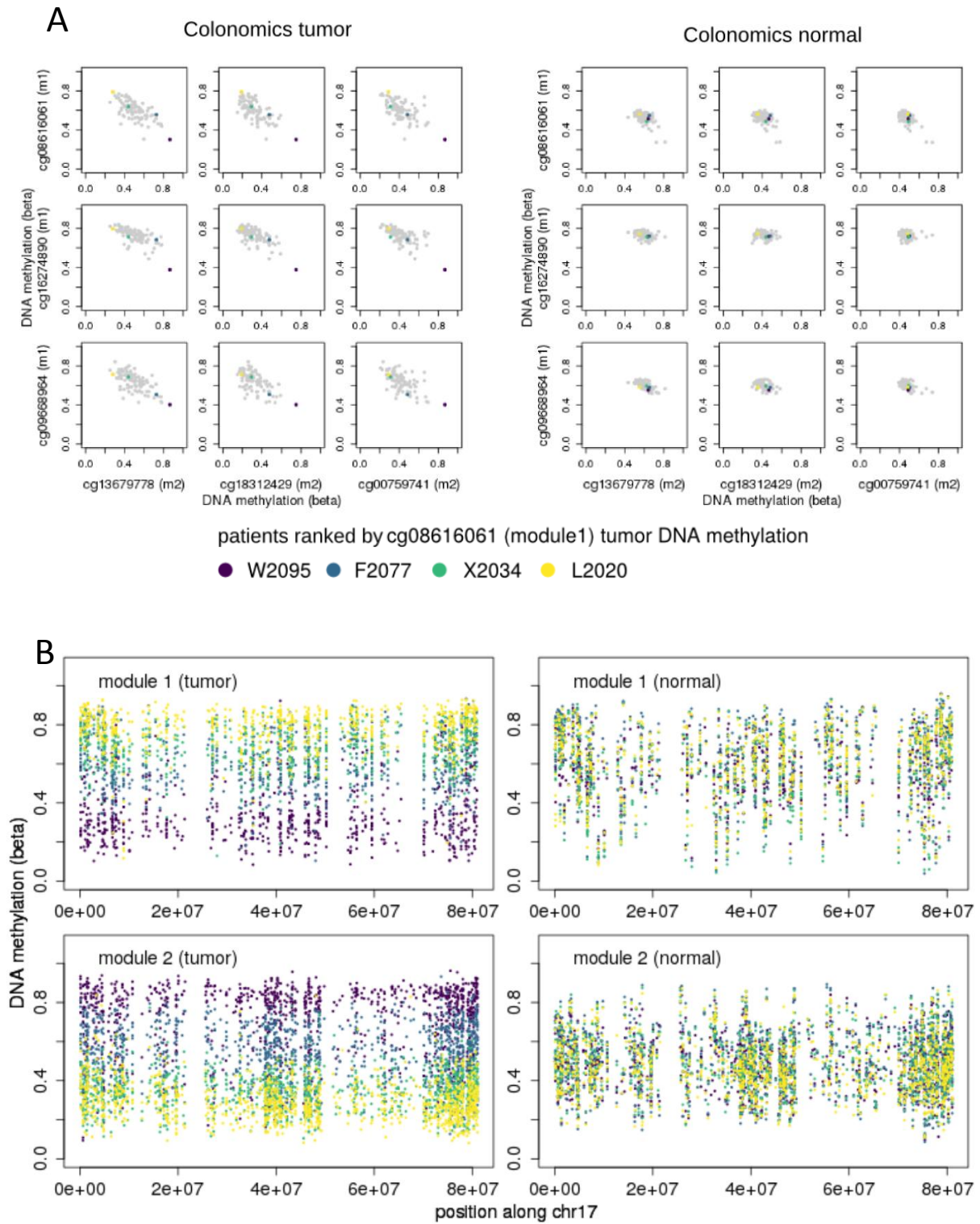


Figure 5

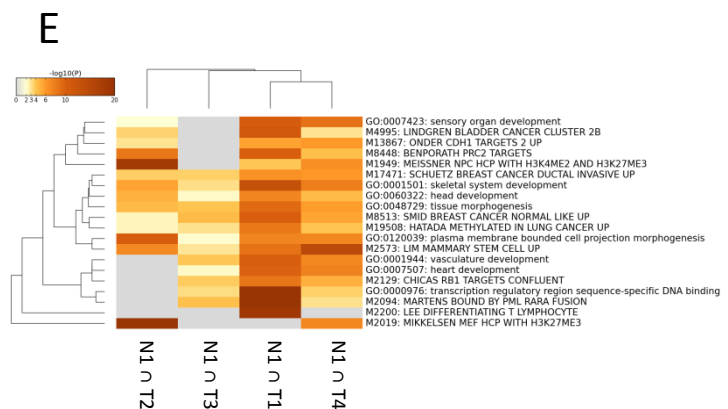
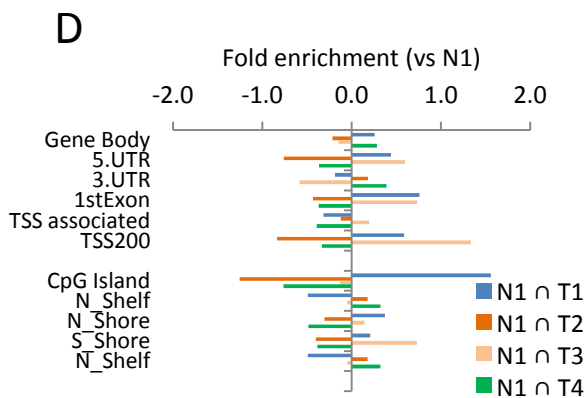
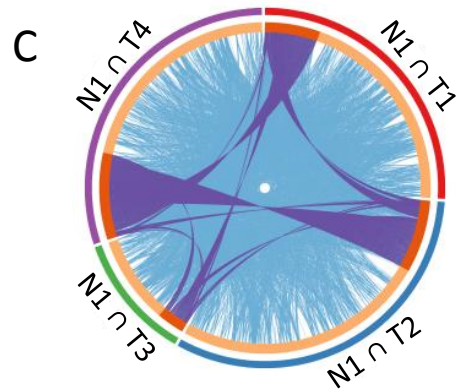
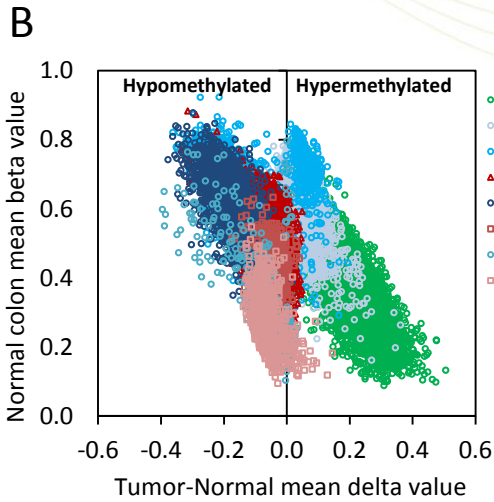
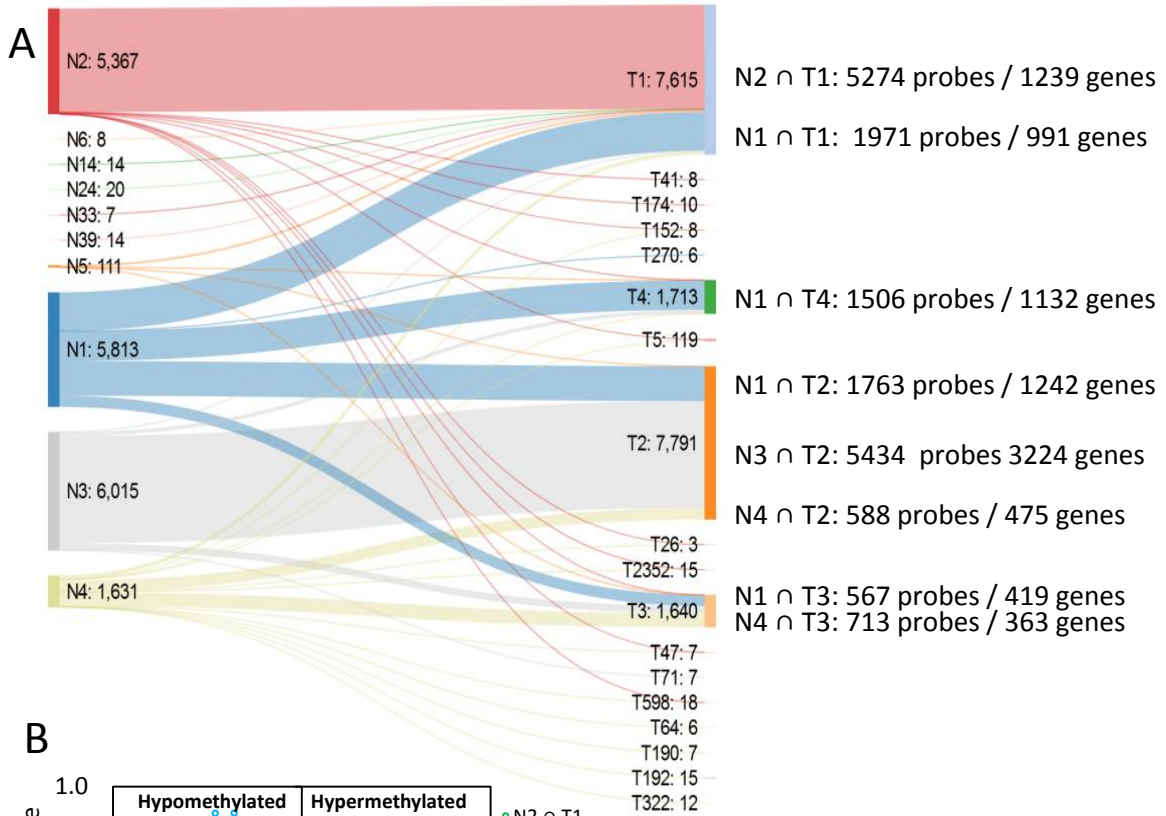


Figure 6

