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## Discovery of candidate DNA methylation cancer driver genes

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**Code availability:** The MethSig pipeline is available on GitHub at <https://github.com/HengPan2007/MethSig>.

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## Abstract

Epigenetic alterations such as promoter hypermethylation may drive cancer through tumor suppressor genes inactivation. However, we have limited ability to differentiate driver DNA methylation (DNAm) changes from passenger events. We developed DNAm driver inference – MethSig – accounting for the varying stochastic hypermethylation rate across the genome and between samples. We applied MethSig to bisulfite sequencing data of chronic lymphocytic leukemia (CLL), multiple myeloma, ductal carcinoma in situ, glioblastoma, and to methylation array data across 18 tumor types in TCGA. MethSig resulted in well-calibrated Quantile-Quantile plots and reproducible inference of likely DNAm drivers with increased sensitivity/specificity compared to benchmarked methods. CRISPR/Cas9 knockout of selected candidate CLL DNAm drivers provided a fitness advantage with and without therapeutic intervention. Notably, DNAm driver risk score was closely associated with adverse outcome in independent CLL cohorts. Collectively, MethSig represents a novel inference framework for DNAm driver discovery to chart the role of aberrant DNAm in cancer.

## Keywords

DNA methylation; Cancer drivers; Cancer epigenetics; Statistical inference framework; CLL

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## Introduction

DNA methylation (DNAm) is a central epigenetic modification of the human genome (1,2). DNAm is also thought to be an important disease-defining feature in many cancers (3-6), pointing to the cancer's cell-of-origin and predictive of the outcome. Indeed, several tumor types harbor frequent mutations in genes that encode components of the methylation machinery (2).

DNAm changes in cancer have been described along two principal axes: global hypomethylation impacting retroviral elements and genome stability, and focal hypermethylation at promoters of tumor suppressor genes (TSGs) (1,2). Promoter hypermethylation of TSGs has been surveyed across cancer in The Cancer Genome Atlas (TCGA) as well as other studies (1-3), and revealed that a plethora of cancer-related cellular pathways are disrupted by hypermethylation of TSG promoters, such as DNA repair (*MLH1*, *RBBP8*), cell cycle (*CDKN2A*, *CDKN2B*), P53 network (*CDKN2A*, *TP73*), apoptosis (*WIFI*, *SFRP1*), Ras signaling (*RASSF1*), Wnt signaling (*SOX17*) and tyrosine kinase cascades (*SOCS3*) (7-9).

While it is tempting to assume that all observed DNAm changes occur deterministically and drive the cancer phenotype, *in vitro* models and human cancers have shown that DNAm changes overwhelmingly follow a stochastic process (5,6,10,11). While these changes are stochastic, they occur at different rate across the genome, correlated with features such as low gene expression and late DNA replication (5). Thus, stochastic DNAm changes in the growing malignant population result in a cancer methylome that displays locally disordered methylation and high intra-tumoral heterogeneity (5,6). These data underscore the challenge of identifying candidate DNAm changes that are likely to be linked to the cancer phenotype among the highly abundant stochastic DNAm events across the genome, reminiscent of the challenge of distinguishing driver from passenger mutations in cancer.

However, unlike the field of cancer genomics where increasingly sophisticated tools have been developed to distinguish between driver and passenger mutations, accounting for confounding covariates (12,13), inference tools in cancer epigenomics largely rely on uniform background models. Thus, widely used statistical methods produce hundreds or thousands of candidate promoter hypermethylation sites, likely overshadowing a much smaller number of DNAm changes that impact oncogenesis (referred to here as DNAm drivers).

To address this challenge, we developed a statistical inference framework accounting for varying stochastic hypermethylation rate across the genome and between patients – MethSig, analogous to leading approaches for cancer driver gene inference (12). MethSig estimates expected tumor promoter hypermethylation with an inference model that includes biological features known to affect the stochastic rate of DNAm changes (5). We applied MethSig to reduced representation bisulfite sequencing (RRBS) (14) data across blood and solid tumor malignancies. Compared to benchmarked methods, MethSig delivers well-calibrated Quantile-Quantile (Q-Q) plots and more reproducible identification of DNAm drivers in independent cohorts. Importantly, MethSig achieved higher sensitivity and specificity in the inference of likely DNAm drivers compared to extant methods. Finally, the MethSig framework was extended to methylation array data (MethSig-array) and demonstrated the ability to identify candidate DNAm drivers, enriched in TSGs and associated with gene silencing, as well as disease outcomes. Thus, MethSig represents a novel statistical framework to infer DNAm drivers of cancer genesis and relapse, paving the way towards enhanced understanding of the role of epigenetic changes in cancer.

## Results

### MethSig infers putative DNAm drivers through the application of an optimized background model for stochastic hypermethylation

Promoter hypermethylation was measured using differentially hypermethylated cytosine ratio (DHcR), defined as the ratio of hypermethylated cytosines (HCs) to the total number of CpGs profiled in promoters (Fig. 1A; Methods). We reasoned that a large number of high DHcR promoters may result from passenger hypermethylation due to the non-uniform distribution of stochastic hypermethylation rate across the genome (5). Extant inference tools relying on uniform background models will thus lead to spuriously high number of

significantly affected promoters. To illustrate this point, we compared simplified scenarios of constant versus varying HC rate across the genome, both sharing the same average promoter HC rate (Fig. 1B). This analysis demonstrated that when the HC rate varies across the genome, the uniform background assumption leads to many of the highly hypermethylated genes being falsely determined as significantly altered.

To overcome this challenge, we devised a model to estimate promoter-specific background hypermethylation rate (Fig. 1C; Supplementary Fig. S1A-E). We included covariates known to impact hypermethylation rate such as gene expression and replication time (Fig. 1D; Supplementary Fig. S1D-E), as well as promoter proportion of discordant reads, PDR, (Fig. 1A; Supplementary Fig. S1B-C), a metric developed to characterize stochastic DNAm changes (5). Intuitively, loci and samples with high PDR (Fig. 1A, locus B) suggest lower reliability in DNAm driver identification compared to those with low PDR (Fig. 1A, locus A), akin to the role of background mutation rates in cancer driver gene inference.

To determine if a single gene promoter is significantly hypermethylated in each sample, MethSig first generates the expected promoter hypermethylation (expected DHcR) based on a beta regression model and relevant covariates (Fig. 1C, step 1). Second, MethSig tests observed promoter hypermethylation (tumor DHcR) against the expected DHcR (Fig. 1C, step 2). Of note, the beta regression model was found to deliver good fits to tumor DHcR in most genes and patients (Supplementary Fig. S1F-G). Moreover, candidate DNAm drivers were not preferentially nominated by a small subgroup of patients (Supplementary Fig. S1H). Third, hypermethylation signal is aggregated across the cohort as a stronger candidate DNAm driver is likely to affect a larger number of patients. This cross-patient aggregation procedure enables the estimation of hypermethylation enrichment at the cohort level (Fig. 1C, step 3).

To compare MethSig's performance to the currently used methods, we applied three widely used methods to identify hypermethylation in cancer: t-test, methylKit (15) and globalTest (16) (see Methods and Supplementary Data for details). These methods were applied to prospective RRBS profiling of the CLL8 cohort (17,18) (Fig. 1E; Supplementary Table S1). Notably, a comparison of top candidates across methods showed relatively limited overlap, reinforcing the need to develop better statistical models to nominate candidate DNAm drivers (Supplementary Fig. S1I; Supplementary Table S2-S3).

In a well-calibrated statistical model, p-values are uniformly distributed when the null hypothesis is true, and all other assumptions are met. We thus evaluated the performance of MethSig and benchmarked methods through Q-Q plots (19), an established method to assess the uniformity of p-value distribution in statistical genetics. As we anticipate only a small number of DNAm drivers (as compared to the much larger number of genome-wide stochastic changes), a well-calibrated Q-Q plot will mostly adhere to the diagonal, with few outliers with extreme p-values. Benchmarking methods showed inflated Q-Q plots when applied to the CLL8 dataset, which deviated from the expected line (dashed grey line) across the range of p-values (Fig. 2A, 1<sup>st</sup> row). Nearly half of gene promoters were identified as candidate DNAm drivers, likely reflecting an underlying global phenomenon such as elevated passenger DNAm alteration in CLL compared to normal B cells, rendering the

task of pinpointing candidate DNAm drivers, with biological and clinical significance, highly challenging. In contrast, MethSig exhibited a well-calibrated Q-Q plot, with a deviation factor that more closely approximated 1, and only few candidate DNAm driver p-values deviated from expected (Fig. 2A, 1<sup>st</sup> row).

Next, to test whether candidate DNAm driver nomination with MethSig is robust across datasets, we applied MethSig to an independent, previously published CLL RRBS dataset (CLL-DFCI; Fig. 1E). Similarly, MethSig resulted in a well-calibrated Q-Q plot and a deviation factor closer to 1, compared to benchmarked methods (Fig. 2A, 2<sup>nd</sup> row). To further test the generalizability of MethSig, we applied MethSig to available RRBS datasets of three additional tumor types (Fig. 1E; Supplementary Table S1). The performance of MethSig was maintained in a multiple myeloma cohort (MM-CNRS) and two solid tumor datasets (ductal carcinoma in situ, DCIS-MDACC (20); glioblastoma, GBM-MUV(21)), resulting in well-calibrated Q-Q plots (Fig. 2A, 3<sup>rd</sup> to 5<sup>th</sup> row). Here too, benchmarked methods showed inflated Q-Q plots, suggesting that these methods are challenged to distinguish oncogenic DNA hypermethylation from global DNAm changes.

We performed extensive model optimization to ensure the robustness of DNAm driver inference by MethSig, confirming that MethSig included informative covariates, parameters and methodology (Supplementary Fig. S2A-H and S3A-G; see Supplementary Data for details). Notably, model optimization was also performed for benchmarked methods (e.g., using over-dispersion correction option in methylKit), however improvements of Q-Q plots were subtle (Supplementary Fig. S3H).

### **MethSig provides reproducible and transcription-relevant candidate DNAm drivers, enriched in genes dysregulated across cancer types**

Unlike passenger changes, DNAm drivers are anticipated to affect a large proportion of tumors and associated with silenced gene expression. Thus, we hypothesized that accurate inference of DNAm drivers can be assessed through reproducibility across independent patient cohorts, and association with gene silencing.

Considering varied numbers of candidate DNAm drivers identified by different methods using identical p-value cutoff, we compared an equal number of top ranking DNAm drivers to test the reproducibility of DNAm drivers nominated by different methods across the two CLL cohorts. MethSig resulted in a significantly higher overlap across the two cohorts, compared to benchmarked methods (Fig. 2B; Supplementary Fig. S4A).

Next, we tested whether DNAm drivers nominated by MethSig are more frequently linked to gene silencing compared to other methods (see Supplementary Data for details). Area under the receiver operating characteristic (AUROC) showed that MethSig achieved higher performance compared to benchmarked methods in identifying DNAm drivers associated with gene silencing (Fig. 2C; Supplementary Fig. S4B). Indeed, candidate DNAm drivers identified by MethSig were significantly more enriched in silenced genes compared to benchmarked methods or to randomly selected genes (Fig. 2C). Similar findings were observed in the DCIS and GBM cohorts, where matched DNAm and RNA-seq data are available (Supplementary Fig. S4C-D).

Integrating data across the two CLL cohorts, MethSig nominated 189 candidate DNAm drivers out of 9,661 promoters captured by RRBS and with available input covariates (Supplementary Table S2; see Supplementary Fig. S5A-B, Supplementary Table S4 and Supplementary Data for additional analyses to rule out confounders including CpG density, B cell subtype specific epigenetic profiles, copy number changes and driver mutations). Samples where candidate DNAm drivers were found to be hypermethylated have a higher fraction of highly methylated promoters (DHcR > 0.75) compared to samples without hypermethylation (Supplementary Fig. S5C), suggesting high clonality level consistent with positive selection (see Supplementary Fig. S5D-F and Supplementary Data for further characterization of DNAm drivers). While known transcription factor (TF) binding motifs did not show enrichment in DNAm drivers, we observed significantly higher H3K27me3 signal at putative driver loci compared with non-driver loci, suggesting that MethSig candidates may in part conform to the model of promoting cancer development due to locking-in of repression by H3K27me3 (Supplementary Fig. S5G; Supplementary Data).

To interrogate their biological significance, we performed a pathway enrichment analysis of candidate DNAm drivers. Candidate CLL, MM, DCIS and GBM DNAm drivers were enriched in genes hypermethylated or silenced across tumor types, and associated with poor clinical outcome (22) (Supplementary Table S5; Benjamini-Hochberg false discovery rate, BH-FDR  $Q < 0.25$ ). CLL and DCIS DNAm drivers were also enriched in genes downregulated by Myc and genes upregulated by p53 (22). Specifically, DCIS DNAm drivers were enriched in genes silenced in breast ductal carcinoma versus normal ductal breast cells (22), consistent with DNAm drivers-mediated repression of corresponding genes.

### **Candidate CLL DNAm drivers include established TSGs, and were functionally validated to enhance cancer cell fitness**

Candidate CLL DNAm drivers included a well-established TSG, *DUSP22*, whose function as a TSG is silenced through promoter hypermethylation, as demonstrated previously in CLL (23). In addition to *DUSP22*, MethSig also identified other TSGs as putative CLL DNAm drivers such as *RPRM* and *SASH1*. *RPRM* is known to cooperate with p53 leading to cell cycle arrest at G2 phase and has been reported to be hypermethylated or inactivated in carcinomas (24,25). *SASH1* encodes a scaffold protein involved in the TLR4 signaling pathway (26), which has been demonstrated to be a key signaling pathway in CLL (27).

To functionally validate candidate DNAm drivers identified with MethSig, given the limitations of demethylation agents and current dCas9 guided DNAm modification (Supplementary Data), we generated CRISPR/Cas9 mediated knockout (KO) to mimic gene silencing via promoter hypermethylation. We generated KO of three candidate DNAm drivers – *DUSP22*, *RPRM* and *SASH1* (see Supplementary Data for selection criteria). Of note, these candidates were suitable for functional validation given baseline gene expression, and minimal promoter methylation in the HG3 cell line (Supplementary Fig. S6A-F).

After transduction with Cas9 and locus-specific targeting sgRNAs, HG3 cells were cultured with three leading CLL therapeutic agents: ibrutinib (a targeted BTK inhibitor), fludarabine (a key chemotherapy backbone in CLL chemoimmunotherapy regimens), and venetoclax (a

BH3 mimetic) (17,28,29). HG3 cells transduced with a non-targeting sgRNA (HG3-mock) were used as control. After 11 days (~7 doubling times) of ibrutinib treatment, we observed higher fitness in cells with sgRNAs targeting all three candidate DNAm drivers (Fig. 3A). In contrast, only the *DUSP22* KO cells showed higher proliferation after fludarabine treatment (Fig. 3B), and none of the KO led to greater proliferation with venetoclax, suggesting that DNAm drivers may have context-specific effects (Fig. 3C).

HG3 cells are known to show clonal diversity (30), which may impact bulk CRISPR/Cas9 KO. Furthermore, the *DUSP22* locus is present in only one copy in HG3 cells due to a partial loss of the chromosome 6p (30), which may contribute to the greater effect in *DUSP22* KO with fludarabine compared to *RPRM* (Fig. 3B). We therefore further generated stable KO HG3 clones of *RPRM* and *DUSP22* through single cell cloning (Fig. 3D; Methods; Supplementary Data). For *RPRM*, we identified a clone with bi-allelic frameshift inducing indels and a second clone with mono-allelic frameshift deletion (Fig. 3E). As *DUSP22* locus is present in only one copy in the HG3 cell line, we generated two separate clones with complete gene KO by introducing frameshift indels in the remaining allele (Fig. 3F). A single cell derived clone with a non-targeting sgRNA was used as a control (mock cell line). After culturing all clones without treatment for 7 days, we observed faster growth for the *RPRM* KO clones with a gene dose effect, compared to controls (Fig. 3G). Similarly, a KO clone for *DUSP22* showed a significantly higher proliferation (Fig. 3G). These data are consistent with a fitness advantage in the absence of treatment, and the enrichment of these DNAm drivers in the previously untreated CLL8 cohort.

In agreement with our above results showing that candidate DNAm driver disruption confers resistance to treatment with leading CLL agents, we observed greater survival for the *RPRM* KO clones under ibrutinib and fludarabine with a gene dose effect (Fig. 3H; Supplementary Fig. S6G). Supporting the role of *DUSP22* as a candidate DNAm driver, both KO clones for *DUSP22* showed improved survival under ibrutinib and fludarabine treatment (Fig. 3I; Supplementary Fig. S6H). However, similar to the bulk transduction experiments, no fitness differences were observed with venetoclax treatment across KO clones (Supplementary Fig. S6G-H).

### **MethSig-nominated CLL DNAm drivers provide independent prognostic information, and are associated with adverse outcome**

We next sought to test the clinical significance of candidate DNAm drivers in the well-annotated CLL cohorts. Promoters whose hypermethylation is associated with failure-free survival (FFS) were defined as true positives (see Supplementary Data for details) while other promoters were defined as true negatives. In CLL8, MethSig resulted in highest AUROC compared to benchmarked methods, and DNAm drivers identified by MethSig were enriched in genes associated with outcome compared to the benchmarked methods or randomly selected genes (Fig. 4A). We further validated this association with clinical outcome in the independent CLL-DFCI cohort (Supplementary Fig. S7A). Notably, MethSig also achieved higher AUROC compared to other methods when we combined two key features that are likely to be associated with DNAm drivers (i.e., either silenced by promoter hypermethylation or associated with FFS; Supplementary Fig. S7B). These results



confirm that MethSig provides a non-incremental advance in the ability to identify likely DNAm drivers with high sensitivity and specificity.

Taking advantage of the large sample size in CLL8 cohort, we sought to further triage the list of DNAm drivers by evaluating the clustering of methylated CpG positions. Intuitively, promoters with a non-random distribution of methylated CpGs are more likely to exert repression on corresponding genes and result in a substantial phenotypic impact (Fig. 4B). We used the maximum number of consecutive methylated CpGs to quantify the clustering degree of methylated CpGs (see Supplementary Fig. S7C and Supplementary Data for details). Indeed, we observed higher clustering of methylated positions in samples where the gene was predicted to be hypermethylated compared to other samples (Fig. 4B), and therefore applied an additional criterion of higher level of clustering, decreasing the number of nominated CLL DNAm drivers to 122 (Supplementary Table S2).

To examine the prognostic value of DNAm drivers, we developed a clinical prediction score based on candidate DNAm drivers ( $n = 122$ ). Elastic net regression (31) with a Cox proportional hazards model was used (see Supplementary Fig. S7D and Supplementary Data for model selection) to assign weights (coefficients) in terms of their contribution to the prediction of FFS to each candidate DNAm driver. To safeguard against overfitting and poor generalizability, CLL8 was used as the training set to select candidate DNAm driver coefficients, while CLL-DFCI was designated as an independent, test cohort not used in the training process.

Candidate DNAm drivers selected by the regression model included all three functionally validated TSGs, whose hypermethylation defined the subset with the least favorable prognosis (Fig. 4C). Higher risk score (greater than median) was significantly associated with shorter FFS in the training set (Fig. 4D, median FFS was 41.2 months in patients with high risk, and not reached in patients with low risk, hazard ratio 2.9, 95% confidence interval [CI] 2.1 to 4.0). Notably, the model was also highly significant in distinguishing patients with high versus low risk of FFS in the test set (Fig. 4E; Supplementary Fig. S7E), and a regression model including established CLL risk indicators demonstrated that DNAm drivers contribute to adverse clinical outcome independently of previously established risk factors (Fig. 4F; Supplementary Fig. S7F-H; Supplementary Data).

### **MethSig identifies relapse-specific DNAm drivers in CLL**

Our data demonstrated that MethSig is an effective tool to nominate cancer DNAm drivers through the comparison of primary malignant (T1) versus controls. We sought to extend the application of MethSig to identify DNAm drivers of relapse disease after fludarabine based chemotherapy through the comparison of relapse (T2) versus control samples (Fig. 1E; CLL8). Notably, the application of MethSig within the context of relapsed CLLs resulted in an equally well-calibrated Q-Q plot (Fig. 4G), consistent with its ability to identify the infrequent DNAm changes that likely contribute to the relapse phenotype.

We identified T2 specific ( $n = 32$ ), T1 and T2 shared ( $n = 88$ ), and T1 specific DNAm drivers ( $n = 101$ ) (Fig. 4H-I; Supplementary Table S2). In addition to previously observed DNAm drivers (e.g., T1 and T2 shared, *DUSP22*, *SASH1*), T2 specific DNAm drivers

involve additional genes with potential tumor suppressor function, such as *G0S2*. *G0S2* can promote apoptosis through *BCL2*, the therapeutic target of the BH3 mimetic venetoclax in CLL (29,32). A pathway enrichment analysis of T2 specific DNAm drivers revealed enrichment in TP53 targets and DNA damage pathway (22) while T1 and T2 shared or T1 specific DNAm drivers were not enriched in these pathways (Fig. 4I; Supplementary Table S5). The enrichment in TP53 targets and DNA damage pathway of T2 specific DNAm drivers indicates that CLL relapse after chemotherapy may follow an alternative path compared to CLL progression in the absence of therapy, offering novel insights for therapeutic strategies to address drug-resistant or relapsed cancer.

### **MethSig-array infers candidate DNAm drivers with methylation arrays**

Considering wide availability of methylation array data, we designed MethSig-array under the same statistical framework proposed by MethSig. Of note, promoter PDR cannot be estimated by array data, which does not provide read-level methylation information, and as shown in the covariate analysis, promoter PDR provides an important contribution to the model (Supplementary Fig. S3B).

Nonetheless, we applied MethSig-array to Infinium HumanMethylation450 arrays of 18 tumor types in TCGA Pan-Cancer analysis project (33) (Supplementary Table S6). As anticipated, the deviation factors of Q-Q plots derived from MethSig-array were closer to 1, compared to higher deviation factors of benchmarked methods (Fig. 5A). To further evaluate the performance of MethSig-array, AUROC was used to assess the sensitivity and specificity in the inference of likely DNAm drivers, which were defined following three key readouts: association with gene silencing, association with disease outcome, and enrichment with TSGs using different published catalogues (Supplementary Data). MethSig-array achieved higher AUROC compared to benchmarked methods in the inference of likely DNAm drivers associated with gene silencing (Fig. 5B) and clinical outcome (Fig. 5C). MethSig-array also resulted in highest AUROC compared to benchmarked methods in the inference of TSGs (Supplementary Fig. S7I, OncoKB (34) or the TCGA cancer driver study (35)). For example, *SOX17* was identified as a DNAm driver in 13 different tumor types (Supplementary Data), which encodes a TF involved in embryonic development and cell fate (9). Hypermethylation and downregulation of *SOX17* have been described in multiple cancer types, which implies the broad tumor suppression function of *SOX17* gene (9). Another important TSG is *RASSF1*, which was identified as a DNAm driver in 6 tumor types (Supplementary Data). *RASSF1* is a microtubule-associated and multitasking scaffold protein communicating with the RAS pathway, estrogen receptor signaling and Hippo pathway (36). *RASSF1* methylation is proposed as a candidate maker in many cancer types (36). Other identified important TSGs included genes in a plethora of cancer-related cellular pathways such as DNA repair (*MLH1*, *RBBP8*), apoptosis (*WIF1*, *SFRP1*) and tyrosine kinase cascades (*SOCS3*) (7,8). Collectively, these data confirm that MethSig can accurately infer likely DNAm drivers across cancer with both array and next-generation sequencing (NGS) based methylation assays.

## Discussion

Aberrant gene function due to acquired epigenetic abnormalities have been highlighted as key features of cancer over the last decade, implicated in cancer initiation, progression and treatment resistance (1,2). Although the causal role of DNAm in cancer remains to be conclusively determined (37), genome-wide DNAm analyses have provided comprehensive surveys of the cancer epigenome and tumor-associated DNAm changes, and have proposed that these changes fuel the malignant process through TSG silencing and other mechanisms (1,2).

However, in the context of steadily growing DNAm sequencing datasets, a major challenge remains: to identify the DNAm changes involved in tumor progression among the abundant stochastic DNAm changes that occur in cancer cells. This challenge is reminiscent of the challenge of distinguishing driver from passenger mutation in cancer exome or genome data. While for the latter challenge progress has been achieved through increasingly sophisticated inference tools that model the varying background mutation rate across the genome (12), inference tools in cancer epigenomics largely rely on uniform background models (38). Given the recent observation that stochastic DNAm varies widely in different genomic regions (5), statistical models relying on a uniform background assumption are anticipated to lead to spuriously high numbers of significantly affected regions.

Drawing on lessons learned in cancer genomics, we posited that robust nomination of oncogenic DNAm changes requires a rethinking of the statistical inference process to enable the differentiation of driver promoter hypermethylation changes (DNAm drivers) from the far larger number of stochastic DNAm changes without biological consequences (passenger DNAm changes). To address this challenge, we developed a statistical inference framework accounting for varying stochastic hypermethylation rate across the genome and between samples – MethSig. The model provides the expected promoter DNAm changes between tumor and control samples, allowing the identification of loci where the observed hypermethylation significantly exceeds expectation, potentially reflecting positive selection of fitness-enhancing candidate DNAm drivers.

We applied MethSig to methylation sequencing data of two CLL cohorts, including 304 CLLs from a prospective clinical trial, as well as to other malignancies with available DNAm data (MM, DCIS and GBM), and benchmarked against state-of-the-art methods. Compared with benchmarked methods, MethSig resulted in well-calibrated Q-Q plots, higher reproducibility in DNAm driver inference across independent cohorts, and increased sensitivity/specificity in the inference of likely DNAm drivers. These observations confirm that MethSig allows to separate specific cancer related DNAm drivers, which cause gene downregulation and phenotypic changes associated with tumoral progression, from stochastic passenger DNAm changes. Notably, the performance of MethSig was maintained across both hematological malignancies (CLL and MM) and solid tumors (DCIS and GBM), suggesting broad applicability for creating catalogues of candidate DNAm drivers of cancer genesis and relapse. Moreover, while MethSig was extended to array data and provided a non-incremental improvement in DNAm driver inference, we anticipate that future shift

towards NGS data will leverage the even higher performance of MethSig with read-level data.

Our data showed that MethSig can also account for broad phenomena that alter DNAm profiles in identifying gene-specific DNAm drivers. For example, DNAm of the cell-of-origin represents one of the strongest sources of variation in the cancer epigenome (4). Indeed, in CLL, DNAm has been shown to strongly encode normal B cell epigenetic reprogramming during differentiation, allowing high resolution inference of the differentiation state of the initially transformed B cell (4). It is therefore notable that MethSig candidate DNAm drivers were not significantly enriched in the most variable methylated regions identified between naïve and class-switched memory B cells (Supplementary Data).

CLL candidate DNAm drivers included TSGs inactivated through hypermethylation, such as *DUSP22*, *RPRM*, and *SASH1*, which may play important roles in the initiation and relapse of CLL. To functionally validate these candidate DNAm drivers in CLL cells, we generated single or double allele frameshift KO. While transformed cells showed superior fitness in the absence of drug selection and with therapy, DNAm drivers showed context-specific effects, which may underlay some of the heterogeneity in CLL clinical course (5). These data also show that venetoclax therapy may uniquely overcome these mechanisms, providing rationale for future DNAm driver guided trials. Of note, our data demonstrated improved risk stratifications based on candidate DNAm drivers, independent of known prognostic factors in CLL, suggesting that DNAm drivers contribute to adverse clinical outcome.

While we believe that this work presents a transformative advance in identifying DNAm drivers with higher sensitivity and specificity, further work will be needed to improve performance and reduce false positive candidates. This may be achieved through expanded datasets and future discovery of additional informative covariates, following the example of genetic driver inference where successive versions resulted in a continuous improvement in performance and reduction in false positives (12,13). Given the limitations of RRBS, including poor coverage of distal regulatory elements or other regions that are CpG poor, the future exploration of DNAm changes in other genomic areas will be greatly empowered by larger whole genome DNAm sequencing datasets. Finally, hypomethylation may also play important roles in cancer genesis by impacting genome stability. Further efforts will be needed to enable statistical inference of those epigenetic events.

Collectively, our data support a novel framework for the analysis of DNAm changes in cancer to specifically identify DNAm drivers of disease progression and relapse, empowering the discovery of candidate epigenetic mechanisms that may enhance cancer cell fitness. This work addresses a central gap between cancer epigenetics and genetics, where such tools have had a transformative impact in precision oncology and cancer gene discovery. We envision that inference tools such as MethSig, coupled with novel DNAm sequencing modalities (39) and emerging tools for epigenetic editing (40), may herald a new era in cancer epigenomics in which large cohort studies will provide precision identification of oncogenic DNAm drivers for improved patient stratification and therapeutic targeting.

## Methods

### Sample acquisition:

For CLL8 cohort, blood was obtained from previously untreated patients enrolled in a prospective, randomized, open-label CLL8 trial (17,18) before the first cycle of treatment. Written informed consent for genomic sequencing of patient samples was obtained prior to the initiation of sequencing studies. For MM-CNRS cohort, bone marrow of patients presenting with previously untreated MM ( $n = 24$ ) or at relapse ( $n = 20$ ) was obtained after patients' written informed consent in accordance with the IRB and the Montpellier University Hospital Centre for Biological Resources (DC-2008-417). Genomic DNA was extracted from CLL and MM cells.

### RRBS:

RRBS libraries were generated by digesting genomic DNA with MspI to enrich for CpG-rich fragments, and then ligated to barcoded TruSeq adapters (Illumina) to allow immediate subsequent pooling. It was followed by bisulfite conversion and PCR, as previously described (14). Libraries were sequenced and aligned to the bisulfite-converted hg19 reference genome using Bismark v0.15.0 (RRID: SCR\_005604) (41).

### Promoter hypermethylation:

Promoter (defined as  $\pm 2$  kb windows centered on RefSeq transcription start site) hypermethylation was measured using DHcR, defined as the ratio of HCs to the total number of CpGs profiled in promoter. HCs of each sample were defined as CpGs at which DNAm is statistically higher than the control (FDR = 20%, Chi-squared test) (6). Only CpGs with read depth greater than 10 were included in the analysis. DHcR of each normal sample was calculated in the same way as for the tumor samples, testing against all normal sample controls. This was followed by averaging DHcR of all the normal samples as the normal DHcR.

### Promoter PDR:

If all the CpGs on a specific read are methylated or unmethylated, the read is classified as concordant. Otherwise, it is classified as discordant. At each CpG, the PDR is equal to the number of discordant reads divided by the total number of reads that cover that location. Promoter PDR is given by averaging the values of individual CpGs, as calculated for all CpGs within the promoter of interest that are covered by a minimum of 10 reads that contain at least 4 CpGs. The normal PDR was calculated by averaging PDR of all the normal samples.

### Algorithmic procedure:

The superscripts  $n$ ,  $t$ ,  $e$  are shorthand for normal, tumor and expected. The subscripts  $i$  and  $j$  represent gene  $i$  and sample  $j$ . The model was processed as following steps:

1. Estimate expected hypermethylation of tumor samples ( $DHcR^e$ ): The independent variable matrix ( $X$ ) has number of genes times number of patients rows (Equation 1). The beta regression model was implemented by R package

*betareg* (42) (Equation 2). Next, predicted distribution of  $DHcR^t$  (used as distribution of  $DHcR^e$  in the following analysis) was estimated using  $\hat{\alpha}$  and  $\hat{\beta}$  derived from the above beta regression model (Equation 3).

$$X_{i,j} = (DHcR_i^n, PDR_i^n, gexp_i^n, reptime_i, PDR_{i,j}^t, depth_{i,j}^t, ncp_g_{i,j}^t) \quad (1)$$

$$DHcR_{i,j}^t = \text{beta}(\alpha + \beta X_{i,j}) \quad (2)$$

$$DHcR_{i,j}^e = \text{beta}(\hat{\alpha} + \hat{\beta} X_{i,j}) \quad (3)$$

2. Evaluate if  $DHcR^t$  (observed) is significantly higher than  $DHcR^e$  (expected):  $DHcR^t$  was tested against the distribution of  $DHcR^e$ . The patient-specific p-value indicates the probability that observed promoter hypermethylation is significantly higher than expected (Equation 4). Only genes whose promoter hypermethylation significantly exceeded expectation will be assigned as patient-specific DNAm drivers ( $P < 0.05$ ).

$$p_{i,j} = P(DHcR_{i,j}^e > DHcR_{i,j}^t) \quad (4)$$

3. Determine if promoter hypermethylation is overrepresented in patients (DNAm driver): Wilcoxon p-value combination method was used to combine p-values from different patients to identify those frequently recurring DNAm drivers (43). To eliminate the effect of cohort size on p-value combination results, MethSig randomly sampled equal number of patients ( $K = 10$ ) iteratively ( $S = 100$ ) and used lower quartile of combined p-values to identify DNAm drivers. Wilcoxon p-value combination was performed by R package *metap* (<https://cran.r-project.org/web/packages/metap/>).

### Benchmarked methods:

In the evaluation of benchmarked methods, *t.test* of R 3.3.2, methylKit 1.0.0 (RRID: SCR\_005177) and globalTest 5.28.0 (RRID: SCR\_001256) were used.

### Pathway enrichment analysis:

Pathway enrichment analysis was limited to the chemical and genetic perturbations of the C2 gene set collection (22), which includes gene sets are more specific to cancer processes in different cancer types with or without perturbation. DNAm drivers were tested against all MethSig inferred non-drivers and only pathways with at least 10 inferred genes were included. A hypergeometric test was used to measure the enrichment of DNAm drivers in each gene set, followed by a BH-FDR procedure.

**Cell lines:**

HG3 (DSMZ #ACC-765, RRID: CVCL\_Y547), PGA1 (DSMZ #ACC-766, RRID: CVCL\_Y545) and MEC1 (DSMZ #ACC-497, RRID: CVCL\_1870) cells were provided by Leibniz Institute DSMZ in August 2018. HEK293T cells (ATCC #CRL-3216, RRID: CVCL\_0063) were provided by the American Tissue Collection Center (ATCC, Manassas, VA) in January 2017. Cells were routinely tested for mycoplasma using the MycoAlert™ Mycoplasma Detection Kit (Lonza #LT07-318). All cell lines used for described experiments came from early frozen batches between 3 and 8 passages after cell reception. HG3, PGA1 and MEC1 cells were used for RT-qPCR (Supplementary Data). HEK293T cells were used for production of lentivirus and transduction (Supplementary Data). HG3 cells were used for all the other described experiments.

**CRISPR/Cas9 design and cloning:**

sgRNA was designed using CHOPCHOP (RRID: SCR\_015723) (44), in order to minimize *in silico* predicted off target activity (1), target the first exon of the genes of interest and have a good predicted efficiency (> 56) based on the PAM sequence and the 3' nucleotide (45). Two sgRNAs have been designed for each targeted locus and the empirically defined most efficient sgRNA was then used for the CRISPR/Cas9 experiments. The sgRNAs were cloned into lentiCRISPRv2 puro (Addgene #98290) as described previously (46).

**Generation of Cas9-expressing KO HG3 clones:**

To create single and double allele KO HG3 clones, we have transduced cells with CRISPR/Cas9 and a gene-specific sgRNA, performed a 10-day puromycin [ $0.5 \mu\text{g ml}^{-1}$ ] selection, confirmed Cas9 efficiency using T7E1 – EnGen® Mutation Detection Kit (NEB), created single cell colony by cell sorting and selected single cell clones showing single or double allele KO (indels) by targeting the predicted CRISPR cutting site through NGS.

**CLL patients risk model:**

The regression model was implemented by R package *glmnet* (RRID: SCR\_015505) (47,48). When evaluating the performance of the model in the training and test set, CLL cases were divided into two subgroups based on their predicted risk scores (patients with high versus low risk, median risk score of the cohort was used as the cutoff) and the FFS difference between groups was evaluated using log-rank test.

**MethSig-array:**

Promoter DHcR and PDR cannot be estimated by array data, which does not provide read-level methylation information. MethSig-array was designed by using average promoter methylation instead of DHcR in the beta regression model and leaving out promoter PDR, under the same statistical framework proposed by MethSig.

**Statistical methods:**

Statistical analysis was performed with R version 3.3.2 (<https://www.R-project.org/>). All p-values were two-sided and considered significant at the 0.05 level unless otherwise noted.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

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## References

1. Robertson KD. DNA methylation and human disease. *Nat Rev Genet*2005;6:597–610. [PubMed: 16136652]
2. Baylin SB, Jones PA. Epigenetic determinants of cancer. *Cold Spring Harb Perspect Biol*2016;8:a019505. [PubMed: 27194046]
3. Koch A, Joosten SC, Feng Z, De Ruijter TC, Draht MX, Melotte V, et al. Analysis of DNA methylation in cancer: Location revisited. *Nat Rev Clin Oncol*2018;15:459–66. [PubMed: 29666440]
4. Oakes CC, Seifert M, Assenov Y, Gu L, Przekopowicz M, Ruppert AS, et al. DNA methylation dynamics during B cell maturation underlie a continuum of disease phenotypes in chronic lymphocytic leukemia. *Nat Genet*2016;48:253–64. [PubMed: 26780610]
5. Landau DA, Clement K, Ziller MJ, Boyle P, Fan J, Gu H, et al. Locally Disordered Methylation Forms the Basis of Intratumor Methylome Variation in Chronic Lymphocytic Leukemia. *Cancer Cell*2014;26:813–25. [PubMed: 25490447]
6. Pan H, Jiang Y, Boi M, Tabbò F, Redmond D, Nie K, et al. Epigenomic evolution in diffuse large B-cell lymphomas. *Nat Commun*2015;6:6921. [PubMed: 25891015]
7. Esteller M. Epigenetic gene silencing in cancer: The DNA hypermethylome. *Hum Mol Genet*2007;16:R50–9. [PubMed: 17613547]
8. Yu Y, Chen L, Zhao G, Li H, Guo Q, Zhu S, et al. RBBP8/CtIP suppresses P21 expression by interacting with CtBP and BRCA1 in gastric cancer. *Oncogene*2020;39:1273–89. [PubMed: 31636387]
9. Li L, Yang WT, Zheng PS, Liu XF. SOX17 restrains proliferation and tumor formation by down-regulating activity of the Wnt/ $\beta$ -catenin signaling pathway via trans-suppressing  $\beta$ -catenin in cervical cancer. *Cell Death Dis*2018;9:741. [PubMed: 29970906]
10. Landan G, Cohen NM, Mukamel Z, Bar A, Molchadsky A, Brosh R, et al. Epigenetic polymorphism and the stochastic formation of differentially methylated regions in normal and cancerous tissues. *Nat Genet*2012;44:1207–14. [PubMed: 23064413]
11. Shipony Z, Mukamel Z, Cohen NM, Landan G, Chomsky E, Zeligler SR, et al. Dynamic and static maintenance of epigenetic memory in pluripotent and somatic cells. *Nature*2014;513:115–9. [PubMed: 25043040]
12. Lawrence MS, Stojanov P, Polak P, Kryukov GV, Cibulskis K, Sivachenko A, et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature*2013;499:214–8. [PubMed: 23770567]
13. Buisson R, Langenbucher A, Bowen D, Kwan EE, Benes CH, Zou L, et al. Passenger hotspot mutations in cancer driven by APOBEC3A and mesoscale genomic features. *Science*2019;364:eaaw2872. [PubMed: 31249028]

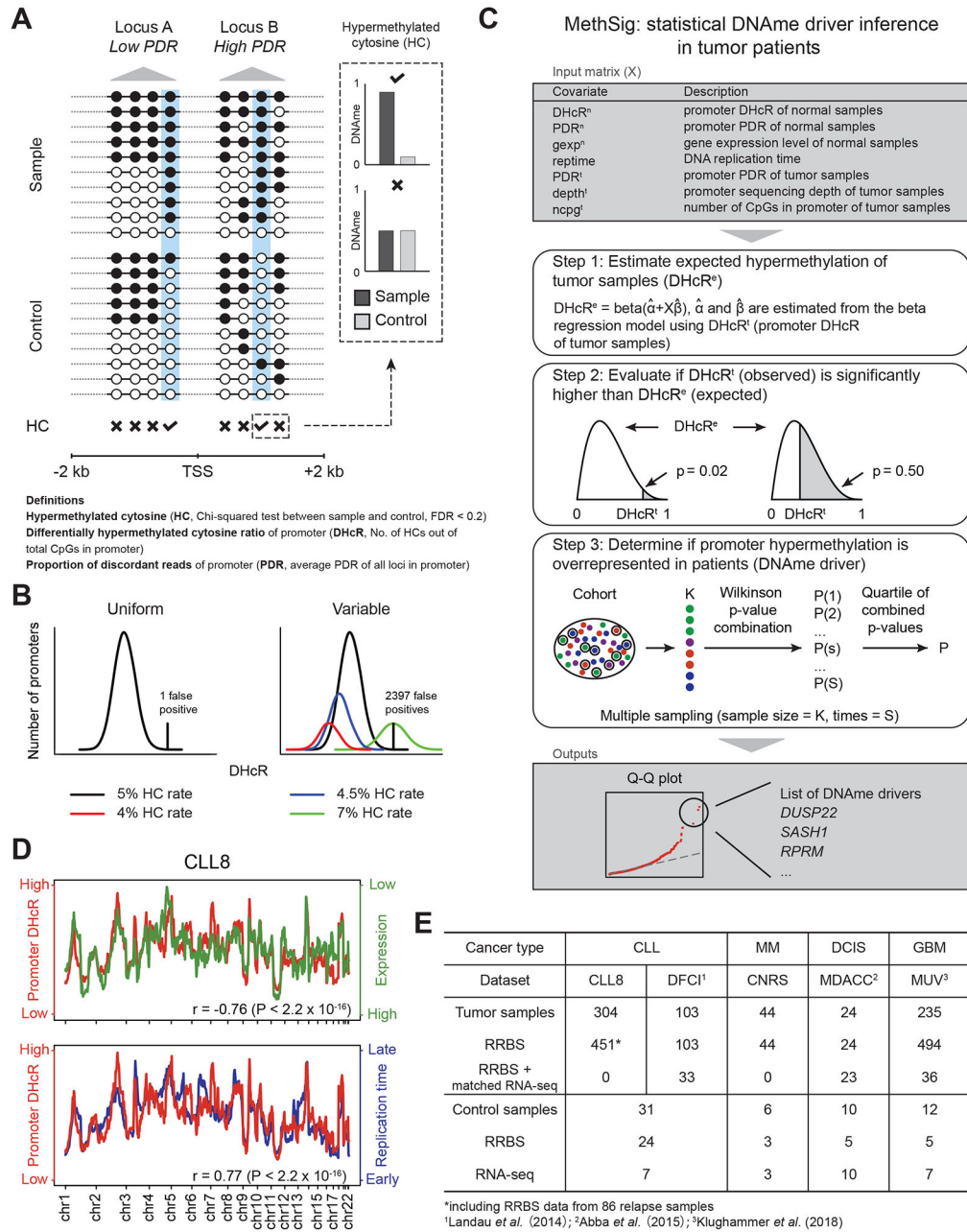


14. Boyle P, Clement K, Gu H, Smith ZD, Ziller M, Fostel JL, et al. Gel-free multiplexed reduced representation bisulfite sequencing for large-scale DNA methylation profiling. *Genome Biol* 2012;13:R92. [PubMed: 23034176]
15. Akalin A, Kormaksson M, Li S, Garrett-Bakelman FE, Figueroa ME, Melnick A, et al. methylKit: a comprehensive R package for the analysis of genome-wide DNA methylation profiles. *Genome Biol* 2012;13:R87. [PubMed: 23034086]
16. Goeman JJ, Van De Geer SA, Van Houwelingen HC. Testing against a high dimensional alternative. *J R Stat Soc Ser B Stat Methodol* 2006;68:477–93.
17. Hallek M, Fischer K, Fingerle-Rowson G, Fink AM, Busch R, Mayer J, et al. Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: A randomised, open-label, phase 3 trial. *Lancet* 2010;376:1164–74. [PubMed: 20888994]
18. Fischer K, Bahlo J, Fink AM, Goede V, Herling CD, Cramer P, et al. Long-term remissions after FCR chemoimmunotherapy in previously untreated patients with CLL: Updated results of the CLL8 trial. *Blood* 2016;127:208–15. [PubMed: 26486789]
19. Wilk MB, Gnanadesikan R. Probability Plotting Methods for the Analysis of Data. *Biometrika* 1968;55:1–17. [PubMed: 5661047]
20. Abba MC, Gong T, Lu Y, Lee J, Zhong Y, Lacunza E, et al. A molecular portrait of high-grade ductal carcinoma in situ. *Cancer Res* 2015;75:3980–90. [PubMed: 26249178]
21. Klughammer J, Kiesel B, Roetzer T, Fortelny N, Nemeš A, Nenning KH, et al. The DNA methylation landscape of glioblastoma disease progression shows extensive heterogeneity in time and space. *Nat Med* 2018;24:1611–24. [PubMed: 30150718]
22. Liberzon A, Birger C, Thorvaldsdóttir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular Signatures Database Hallmark Gene Set Collection. *Cell Syst* 2015;1:417–25. [PubMed: 26771021]
23. Arruga F, Gizdic B, Bologna C, Cignetto S, Buonincontri R, Serra S, et al. Mutations in NOTCH1 PEST domain orchestrate CCL19-driven homing of chronic lymphocytic leukemia cells by modulating the tumor suppressor gene DUSP22. *Leukemia* 2017;31:1882–93. [PubMed: 28017968]
24. Morris MR, Ricketts C, Gentle D, Abdulrahman M, Clarke N, Brown M, et al. Identification of candidate tumour suppressor genes frequently methylated in renal cell carcinoma. *Oncogene* 2010;29:2104–17. [PubMed: 20154727]
25. Xu M, Knox AJ, Michaelis KA, Kiseljak-Vassiliades K, Kleinschmidt-DeMasters BK, Lillehei KO, et al. Reprimo (RPRM) is a novel tumor suppressor in pituitary tumors and regulates survival, proliferation, and tumorigenicity. *Endocrinology* 2012;153:2963–73. [PubMed: 22562171]
26. Blonder J, Park Y-J, Hussainkhel A, Yang C, Veenstra TD, Fuller ME, et al. SASH1 Is a Scaffold Molecule in Endothelial TLR4 Signaling. *J Immunol* 2013;191:892–901. [PubMed: 23776175]
27. Dadashian EL, McAuley EM, Liu D, Shaffer AL, Young RM, Iyer JR, et al. TLR signaling is activated in lymph node-resident CLL cells and is only partially inhibited by ibrutinib. *Cancer Res* 2019;79:360–71. [PubMed: 30498085]
28. Byrd JC, Brown JR, O'Brien S, Barrientos JC, Kay NE, Reddy NM, et al. Ibrutinib versus ofatumumab in previously treated chronic lymphoid leukemia. *N Engl J Med* 2014;371:213–23. [PubMed: 24881631]
29. Fischer K, Al-Sawaf O, Bahlo J, Fink AM, Tandon M, Dixon M, et al. Venetoclax and obinutuzumab in patients with CLL and coexisting conditions. *N Engl J Med* 2019;380:2225–36. [PubMed: 31166681]
30. Quentmeier H, Pommerenke C, Ammerpohl O, Geffers R, Hauer V, MacLeod RAF, et al. Subclones in B-lymphoma cell lines: Isogenic models for the study of gene regulation. *Oncotarget* 2016;7:63456–65. [PubMed: 27566572]
31. Zou H, Hastie T. Regularization and variable selection via the elastic net. *J R Stat Soc Ser B Stat Methodol* 2005;67:301–20.
32. Welch C, Santra MK, El-Assaad W, Zhu X, Huber WE, Keys RA, et al. Identification of a protein, GOS2, that lacks Bcl-2 homology domains and interacts with and antagonizes Bcl-2. *Cancer Res* 2009;69:6782–9. [PubMed: 19706769]

33. Weinstein JN, Collisson EA, Mills GB, Shaw KRM, Ozenberger BA, Ellrott K, et al. The cancer genome atlas pan-cancer analysis project. *Nat Genet*2013;45:1113–20. [PubMed: 24071849]
34. Chakravarty D, Gao J, Phillips S, Kundra R, Zhang H, Wang J, et al. OncoKB: A Precision Oncology Knowledge Base. *JCO Precis Oncol*2017;1:00011.
35. Bailey MH, Tokheim C, Porta-Pardo E, Sengupta S, Bertrand D, Weerasinghe A, et al. Comprehensive Characterization of Cancer Driver Genes and Mutations. *Cell*2018;173:371–385.e18. [PubMed: 29625053]
36. Malpeli G, Innamorati G, Decimo I, Bencivenga M, Nwabo Kamdje AH, Perris R, et al. Methylation Dynamics of RASSF1A and Its Impact on Cancer. *Cancers (Basel)*2019;11:959.
37. Lappalainen T, Grealley JM. Associating cellular epigenetic models with human phenotypes. *Nat Rev Genet*2017;18:441–51. [PubMed: 28555657]
38. Klein HU, Hebestreit K. An evaluation of methods to test predefined genomic regions for differential methylation in bisulfite sequencing data. *Brief Bioinform*2016;17:796–807. [PubMed: 26515532]
39. Simpson JT, Workman RE, Zuzarte PC, David M, Dursi LJ, Timp W. Detecting DNA cytosine methylation using nanopore sequencing. *Nat Methods*2017;14:407–10. [PubMed: 28218898]
40. Lei Y, Huang Y-H, Goodell MA. DNA methylation and de-methylation using hybrid site-targeting proteins. *Genome Biol*2018;19:187. [PubMed: 30400938]
41. Krueger F, Andrews SR. Bismark: A flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics*2011;27:1571–2. [PubMed: 21493656]
42. Cribari-Neto F, Zeileis A. Beta regression in R. *J Stat Softw*2010;34:1–24.
43. Wilkinson BA statistical consideration in psychological research. *Psychol Bull*1951;48:156–8. [PubMed: 14834286]
44. Labun K, Montague TG, Krause M, Torres Cleuren YN, Tjeldnes H, Valen E. CHOPCHOP v3: expanding the CRISPR web toolbox beyond genome editing. *Nucleic Acids Res*2019;47:W171–4. [PubMed: 31106371]
45. Doench JG, Hartenian E, Graham DB, Tothova Z, Hegde M, Smith I, et al. Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. *Nat Biotechnol*2014;32:1262–7. [PubMed: 25184501]
46. Stringer BW, Day BW, D'Souza RCJ, Jamieson PR, Ensby KS, Bruce ZC, et al. A reference collection of patient-derived cell line and xenograft models of proneural, classical and mesenchymal glioblastoma. *Sci Rep*2019;9:4902. [PubMed: 30894629]
47. Friedman J, Hastie T, Tibshirani R. Regularization paths for generalized linear models via coordinate descent. *J Stat Softw*2010;33:1–22. [PubMed: 20808728]
48. Simon N, Friedman J, Hastie T, Tibshirani R. Regularization paths for Cox's proportional hazards model via coordinate descent. *J Stat Softw*2011;39:1–13.
49. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio Cancer Genomics Portal: An open platform for exploring multidimensional cancer genomics data. *Cancer Discov*2012;2:401–4. [PubMed: 22588877]
50. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal*2013;6:pl1. [PubMed: 23550210]

**Statement of significance**

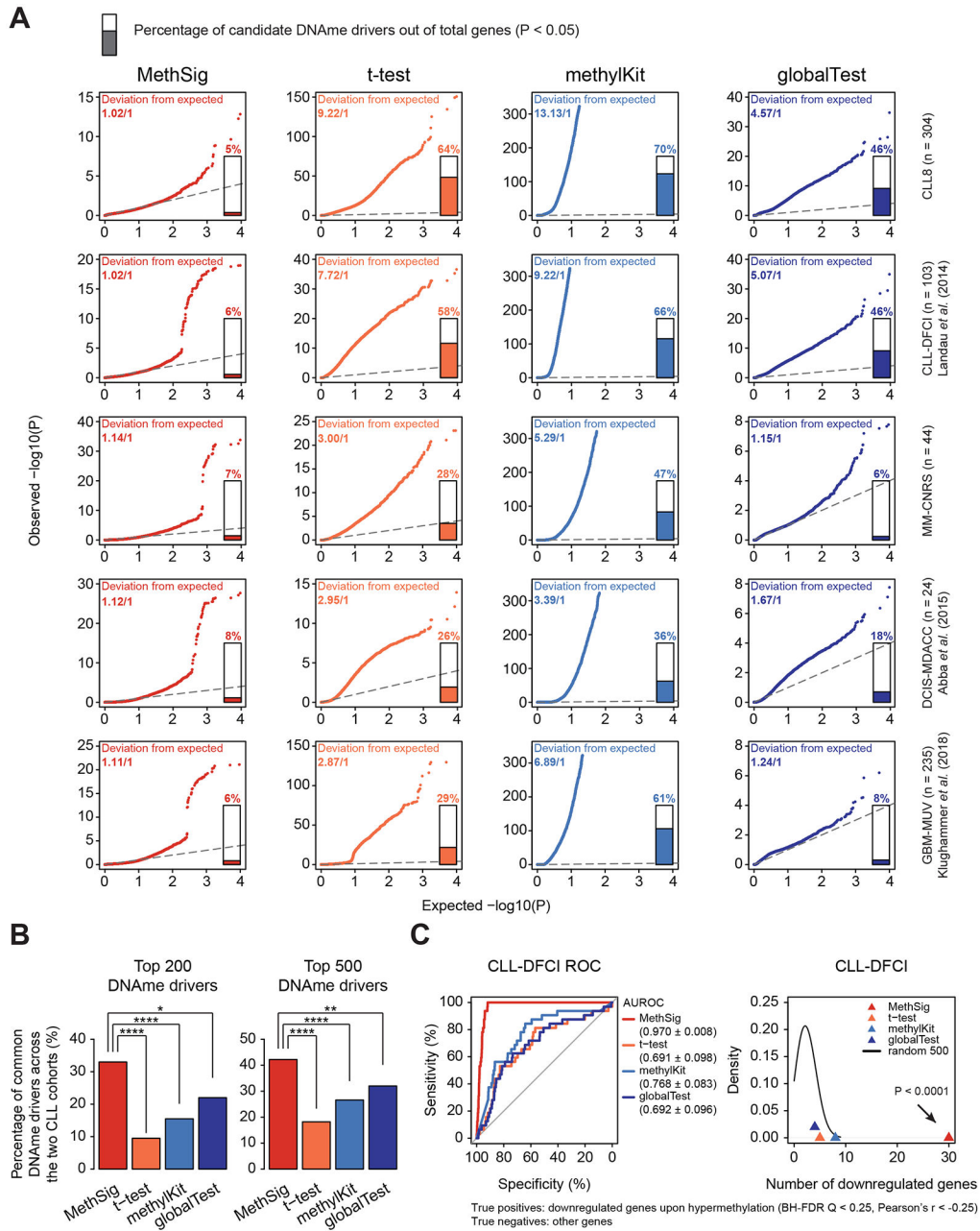
MethSig provides a novel statistical framework for the analysis of DNA methylation changes in cancer, to specifically identify candidate DNA methylation driver genes of cancer progression and relapse, empowering the discovery of epigenetic mechanisms that enhance cancer cell fitness.



**Figure 1. Overview of MethSig and datasets.**

**A**, HC, promoter DHcR and PDR were calculated as shown. Methylation patterns of sample and control are shown (black circles, methylated CpGs; white circles, unmethylated). HCs are highlighted in light blue while DNA methylation (DNAm) of sample and control are detailed inside dashed lines. **B**, Simplified illustration of the challenge on DNAm driver detection due to non-uniform hypermethylation rate across the genome. Here, we simulated a simplified methylome consisting of 20,000 promoters. The average promoter HC rate is 5%, and all hypermethylation is assumed to be due to stochastic processes (i.e., there are no functional DNAm drivers). Two variants of this scenario are compared. Left panel, uniform model whereby all promoters have a constant 5% HC rate. The plot shows a histogram of

promoters by their observed DHcR. The vertical black line indicates a significance threshold that allows a single false positive promoter, which corresponds to 7% HC rate. In contrast, right panel shows a variable model consistent with prior observation of varying HC rate across the genome. In this scenario one quarter of promoters (red) have a HC rate equal to 4%; another half of promoters (blue) have a HC rate of 4.5%; and the final quarter of promoters (green) have a HC rate of 7%. Applying the same threshold for significance (vertical black line corresponding to 7%), 2,397 promoters will be determined as having significant DNAm changes. **C**, An overview of the MethSig statistical inference model. n ~ normal; t ~ tumor; e ~ expected. **D**, Top panel, average promoter DHcR and gene expression level plotted across the entire genome in CLL8. Bottom panel, average promoter DHcR and replication time plotted across the entire genome in CLL8. The average values were calculated based on a sliding window across the human genome with 50 Mb window size and 2.5 Mb step size. Note that gene expression is reversed in the figure in order to emphasize the correlation with methylation (low gene expression at the top and high gene expression at the bottom). Statistical analysis was performed by two-sided Pearson correlation. **E**, Description of datasets.



**Figure 2. MethSig provides statistically robust, reproducible and functionally relevant DNAm driver inference.**

**A**, Q-Q plots comparing observed  $-\log_{10}$  p-values of MethSig and benchmarked methods to expected  $-\log_{10}$  p-values. Results of CLL8, CLL-DFCI, MM-CNRS, DCIS-MDACC and GBM-MUV cohorts are listed from top to bottom. For each row, results of MethSig, t-test, methylKit, and globalTest are listed from left to right. Bar plot in each figure represents the percentage of genes with p-values less than 0.05. Deviation from expected factor was the slope value derived from linear regression through zero, modeling the relationship between observed and expected  $-\log_{10}$  p-values for each method. Expected p-values used for all the methods were sampled from uniform distribution (*runif* function in R starting from

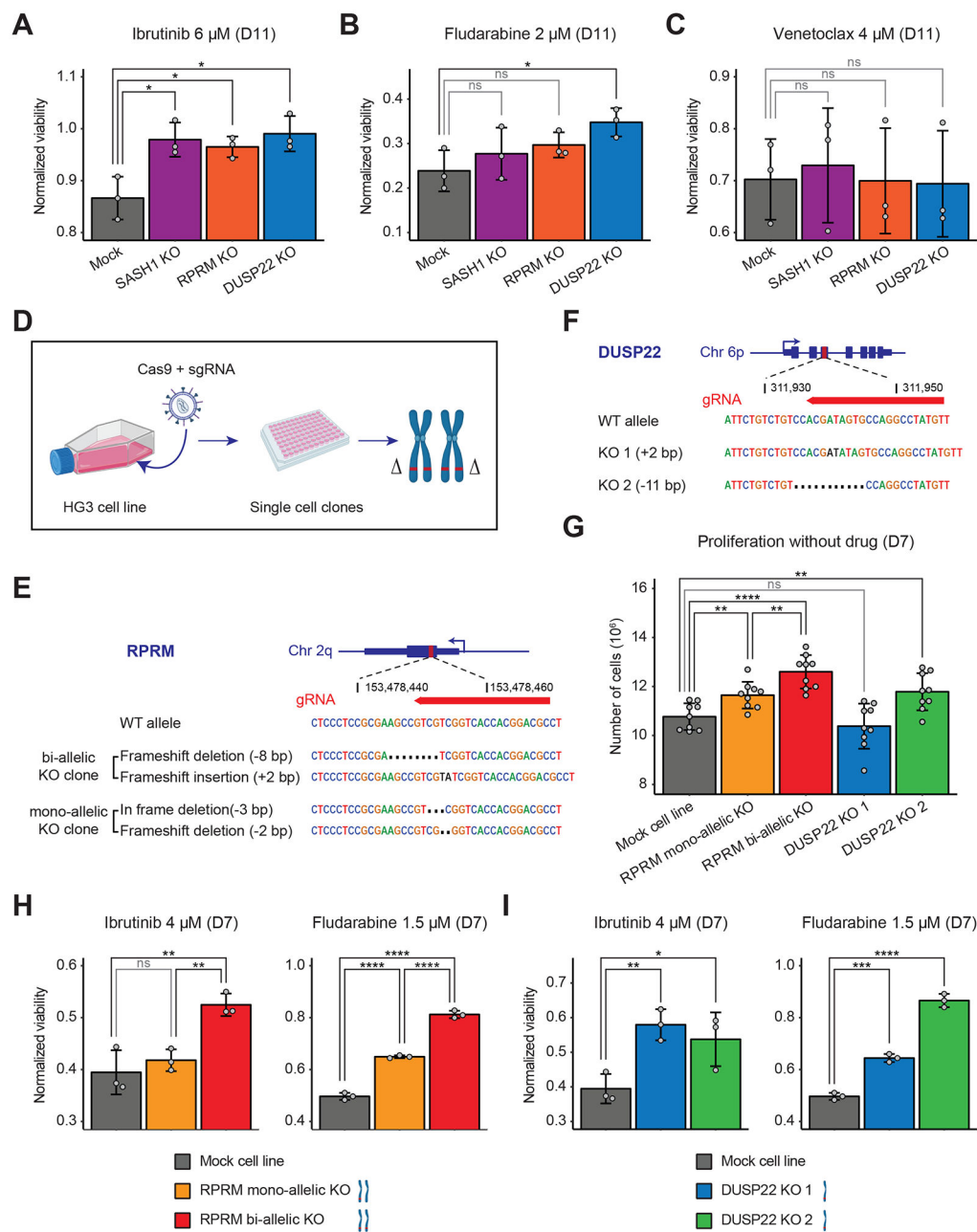
identical random seed number). **B**, Percentage of shared candidate DNAm drivers between CLL8 and CLL-DFCI cohorts. Top 200 or 500 DNAm drivers ranked by p-values of each method were used. Statistical analysis was performed by Chi-squared test: \*\*\*\*P < 0.0001; \*\*P<0.01; \*P < 0.05. **C**, Left panel, MethSig showed higher AUROC compared to benchmarked methods in the inference of likely DNAm drivers in CLL-DFCI (one-sided DeLong's test, compared to method t-test,  $P = 1 \times 10^{-8}$ ; method methylKit,  $P = 1 \times 10^{-6}$ ; method globalTest,  $P = 8 \times 10^{-9}$ ). ROC  $\pm$  95% CI are shown. The same list of genes was used among all four methods. Right panel, number of true positives in top 500 candidate DNAm drivers identified by MethSig and benchmarked methods, as well as 500 randomly selected genes in CLL-DFCI cohort. The black curve indicates the density of true positive number estimated by 10,000 times of random selection. Empirical p-value was calculated according to the probability that the number found in top MethSig candidates is greater than the number found in an equal number of randomly selected genes from all the inferred promoters.

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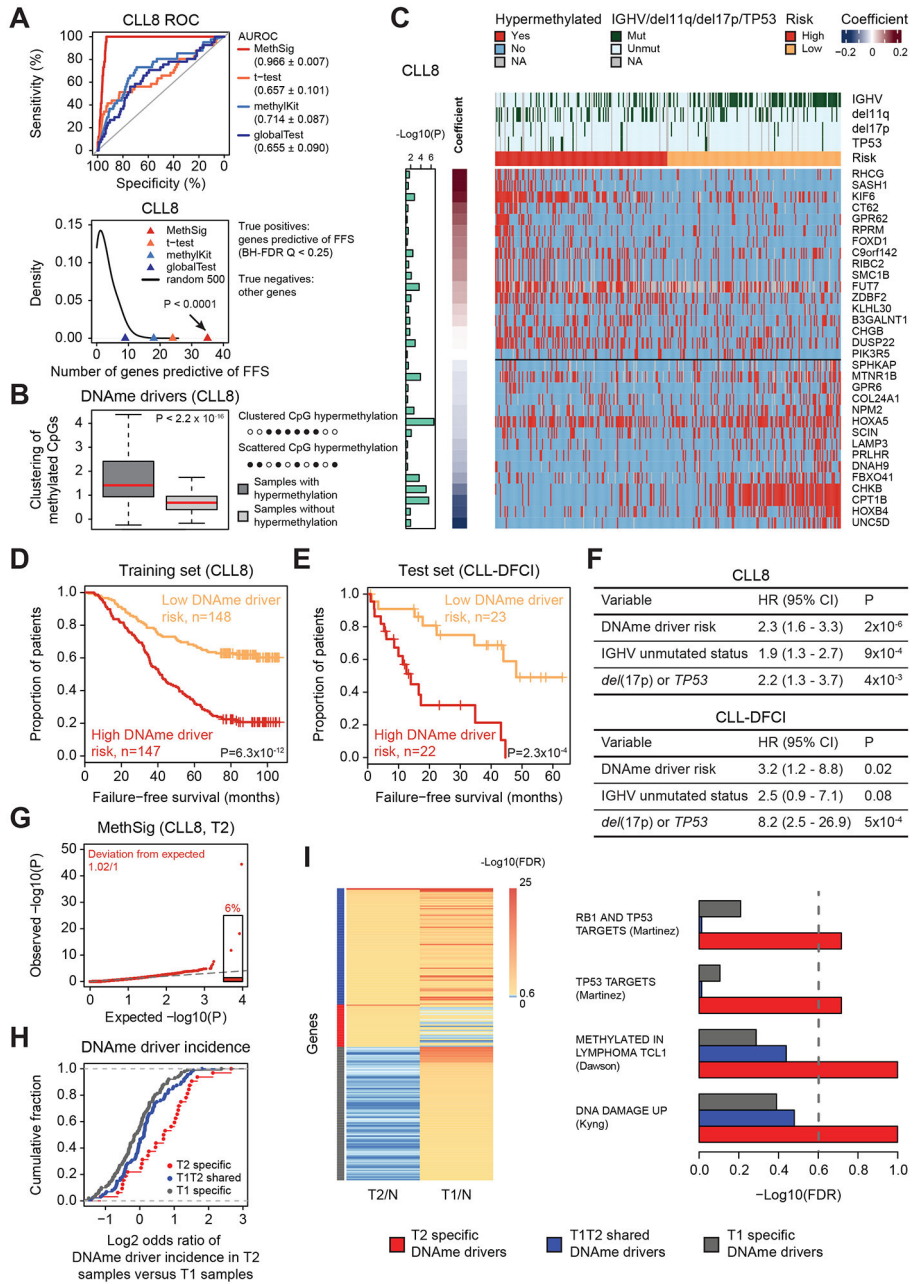


**Figure 3. Candidate DNAm driver KO CLL cell lines show superior fitness in drug treatment compared with controls.**

**A-C**, CellTiter-Glo Viability assay of four different cell lines: *SASH1* KO, *RPRM* KO, *DUSP22* KO and HG3-mock as a control after 11 days of exposure to ibrutinib, fludarabine and venetoclax. Triplicates were performed for each condition. **D**, Workflow of the CRISPR KO single cell clone experiment: transduction of the HG3 cell line with a lentivirus containing CRISPR/Cas9 and a targeting sgRNA followed by puromycin selection; isolation and expansion of single cell clones; NGS assessment of KO. **E-F**, Representation of the sgRNA and the indels found in different clones compared to the wild type (WT) allele. For *RPRM* (**E**), 2 single cell clones are shown: a clone with bi-allelic frameshift inducing



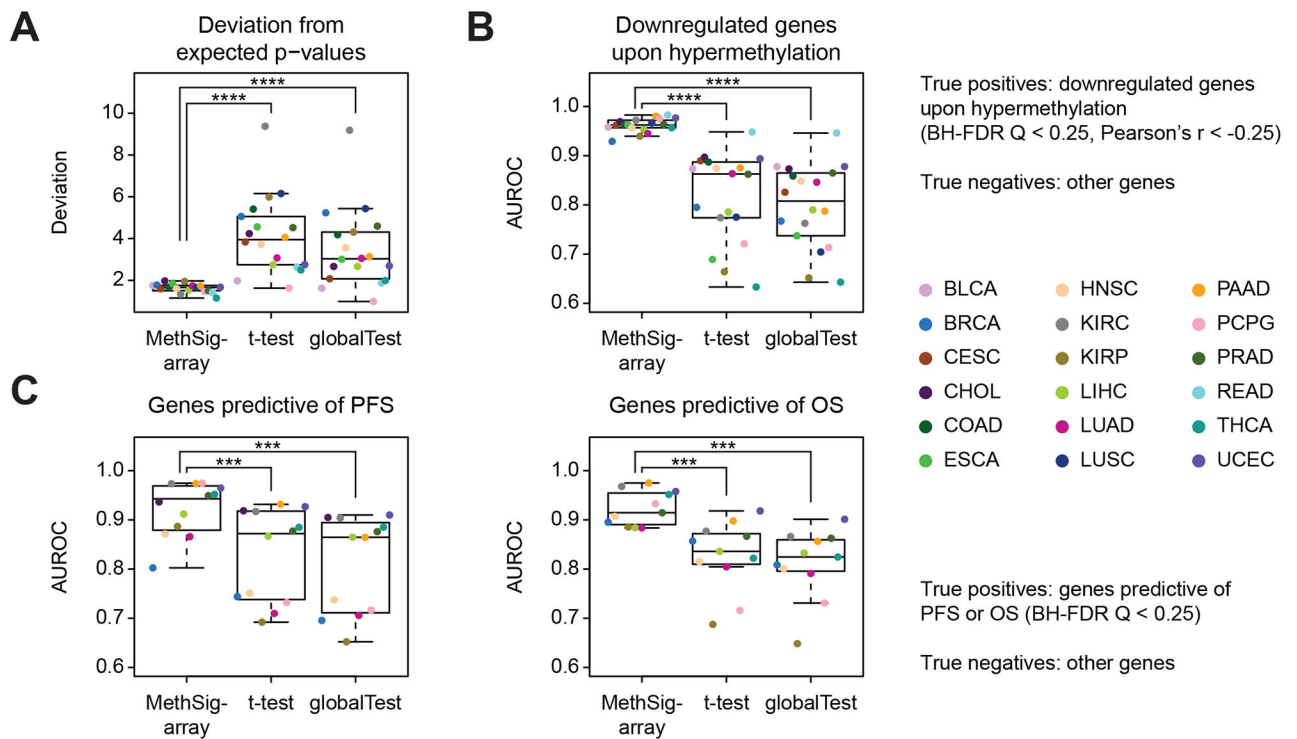
indels and a second clone with a mono-allelic frameshift deletion (2 bp deletion inducing a frameshift on the first allele and 3 bp non-frameshift deletion on the second allele). For *DUSP22* (**F**), 2 clones with indels inducing complete KO as the *DUSP22* locus in HG3 is present in only one copy as the result of a partial loss of the chromosome 6p. **G**, CellTiter-Glo Viability assay of 2 single cell *RPRM*KO clones, 2 single cell *DUSP22* KO clones and control (cf method) after 7 days of growth without any drug. Nine replicates were performed for each cell line. **H-I**, CellTiter-Glo Viability assay after 7 days of exposure to ibrutinib and fludarabine. Triplicates were performed for each condition. In **A-C** and **G-I**, data are presented as means  $\pm$  s.d. Statistical analysis was performed by one-way ANOVA: \*\*\*\*P < 0.0001; \*\*\*P < 0.001; \*\*P < 0.01; \*P < 0.05; ns, not significant.



**Figure 4. Candidate DNAm drivers provide independent prognostic information, are associated with adverse outcome in CLL, and define distinct alterations in relapsed CLL after fludarabine based therapy.**

**A**, Top panel, MethSig resulted in highest AUROC compared to benchmarked methods in the inference of likely DNAm drivers in CLL8 (one-sided DeLong’s test, compared to method t-test,  $P = 1 \times 10^{-9}$ ; method methylKit,  $P = 8 \times 10^{-9}$ ; method globalTest,  $P = 9 \times 10^{-12}$ ). ROC ± 95% CI are shown. The same list of genes was used among all four methods. Bottom panel, number of true positives in top 500 candidate DNAm drivers identified by MethSig and benchmarked methods, as well as 500 randomly selected genes in CLL8 cohort. The black curve indicates the density of true positive number estimated by 10,000

times of random selection. Empirical p-value was calculated according to the probability that the number of genes found in top MethSig candidate genes is greater than the number of genes found in an equal number of randomly selected genes from all the inferred promoters. **B**, Different clustering of methylated CpGs between samples with or without hypermethylation in DNAm drivers. The median, upper and lower quartiles are shown. Whiskers represent upper quartile + 1.5 interquartile range (IQR) and lower quartile – 1.5 IQR. Clustered or scattered CpG hypermethylation are shown (black circles, methylated CpGs; white circles, unmethylated). Statistical analysis was performed by two-sided paired Mann-Whitney *U* test. **C**, Candidate DNAm drivers selected by the model are depicted in descending order of their association (coefficients) with poor FFS. Heatmap showing which selected DNAm drivers are hypermethylated in each patient in CLL8. Bar plot showing  $-\log_{10}$  p-values in CLL8 cohort. **D**, Kaplan-Meier plot showing FFS in CLLs with high versus low risk in the training set (CLL8). **E**, Kaplan-Meier plot showing FFS in CLLs with high versus low risk in the independent test set (CLL-DFCI). In **C-D**, *alpha* equal to 0.1 was used in the elastic net regression. In **D-E**, statistical analysis was performed with log-rank test. **F**, Multivariable analyses for DNAm driver risk with the addition of well-established poor outcome predictors in CLL (IGHV unmutated status and *del*[17p] or *TP53* mutation status) in CLL8 and CLL-DFCI cohorts. **G**, Q-Q plot comparing observed  $-\log_{10}$  p-values of MethSig to expected  $-\log_{10}$  p-values. Result of relapsed (T2) patients in CLL8 cohort is presented. Bar plot represents the percentage of genes with p-values less than 0.05. Deviation from expected factor was the slope value derived from linear regression through zero, modeling the relationship between observed and expected  $-\log_{10}$  p-values. Expected p-values were sampled from uniform distribution (*runif* function in R starting from identical random seed number). **H**, Cumulative distribution function plot of three subgroups of DNAm drivers in terms of  $\log_2$  odds ratio of DNAm driver incidence in T2 over T1 samples. **I**, Left panel, heatmap of  $-\log_{10}$  (BH-FDR *Q*) derived from DNAm driver identification of T2 or T1 samples over control samples. Right panel, the enrichment of three subgroup DNAm drivers in selected pathways. The dashed grey line indicates BH-FDR *Q* = 0.25, cutoff for significant enrichment of DNAm drivers in each pathway.



**Figure 5. MethSig-array outperforms benchmarked methods in identifying candidate DNAm drivers.**

**A**, Deviation from expected factors of MethSig-array, t-test and globalTest. Deviation from expected factor was the slope value derived from linear regression through zero, modeling the relationship between observed and expected  $-\log_{10}$  p-values. **B**, AUROC of MethSig-array, t-test and globalTest in the inference of likely DNAm drivers associated with gene silencing. Matched tumoral DNAm array and RNA-seq data in TCGA Pan-Cancer analysis project (33) were used. **C**, AUROC of MethSig-array, t-test and globalTest in the inference of likely DNAm drivers associated with progression-free survival (PFS) or overall survival (OS). Clinical information obtained from cBioPortal (49,50). Statistical analysis was performed by two-sided paired Mann-Whitney  $U$  test: \*\*\*\* $P < 0.0001$ ; \*\*\* $P < 0.001$ . The median, upper and lower quartiles are shown. Whiskers represent upper quartile + 1.5 IQR and lower quartile - 1.5 IQR. In **B-C**, only tumor types with a minimum of 10 likely DNAm drivers were included into the analysis. In each AUROC analysis, the same list of genes was used among all methods. One of the benchmarked methods – methylKit – could not be applied to TCGA dataset due to lacking count-based data.