Overlapping DNA methylation changes in enhancers in clonal cytopenia of undetermined significance and myelodysplastic neoplasm patients with *TET2*, *IDH2*, or *DNMT3A* mutations

The development of myelodysplastic neoplasms (MDS) is multiphasic, and the disease shares several clinical and molecular features with the precursor entities clonal hematopoiesis of indeterminate potential (CHIP) and clonal cytopenia of undetermined significance (CCUS) and the more aggressive acute myeloid leukemia (AML). Common to these is the presence of mutations in genes regulating DNA methylation.[1-3] Notably, two of the most mutated genes in CCUS and MDS are *DNMT3A*, catalyzing *de novo* methylation, and *TET2*, catalyzing the first steps of active DNA demethylation. We have previously shown that *TET2*-mutated CHIP and CCUS are associated with a distinct pattern of DNA hypermethylation especially enriched in genes related to immune response and leukocyte function.[4] Another study found that cases of *DNMT3A*- and *TET2*-mutated CHIP have distinct and directionally opposing DNA methylation patterns but both likely promoting self-renewal of hematopoietic stem cells.[5] Furthermore, *IDH1* and *IDH2* mutations have been shown to inhibit TET2-mediated demethylation in AML and have been associated with a DNA hypermethylation profile similar to that of *TET2*-mutated AML.[6] The epigenome-wide impact of mutations in *TET2*, *IDH1*, *IDH2*, and *DNMT3A* in CCUS and MDS is, however, not yet fully elucidated nor has it been investigated whether the methylation patterns are shared across the range of clonal myeloid disorders.

We investigated whether 58 CCUS and 59 MDS patients with mutations in *TET2*, *IDH2*, or *DNMT3A* have distinct DNA methylation patterns in bone marrow mononuclear cells compared to patients without any of these mutations. Patients were recruited from hematology departments at three Danish hospitals with approval from The Danish National Committee on Health Research Ethics (ID NVK-1705391). All participants provided written informed consent prior to participation and the study was conducted in accordance with the Danish ethical regulation for work with human participants. Patients donated bone marrow aspirates at the time of diagnosis, from which mononuclear cells were isolated using density gradient separation and T-cell-depleted using the RoboSep Human CD3 Positive Selection Kit II and the RoboSep Magnetic Cell Separator (StemCell Technologies, Vancouver, Canada). DNA sequencing was performed during diagnostic work-up or using a targeted sequencing panel and Twist Libraries (Twist Bioscience, San Francisco, CA, USA; more information available upon request). Genome-wide DNA methylation levels were measured using Illumina's Infinium MethylationEPIC v1.0 BeadChips at the Genomics Core at the Van Andel Institute (Grand Rapids, MI, USA) according to the manufacturer's protocol. Data processing, quality control, and statistical analyses were done using R v4.2.0 or higher. Data were quality controlled using minfi v.1.36.0[7] and normalized using functional normalization.[8] Probes with detection *P* values >0.01, bead count <3 in at least 5% of samples, non-CpG sites, targeting to sex chromosomes, single nucleotide polymorphisms <5 bp from the target CpG, and probes which previously showed binding to multiple target CpG sites[9] were excluded using ChAMP v.2.20.1 [10] After quality control and filtering, methylation levels of 743,875 CpG sites were available for analysis. ComBat from R package sva 3.38.0 was used for sample plate correction. Differentially methylated positions (DMP) were identified by linear modeling using Limma v.3.46.0[11] with M-values as outcome and mutation group as explanatory variable adjusted for age, sex, and batch. The wild-type group was defined as patients without mutations in *TET2*, *DNMT3A*, *IDH1*, and *IDH2*. *P* values were adjusted for multiple testing using Benjamini and Hochberg's method to control the false discovery rate (FDR). If none of the DMP was significant after correcting for multiple testing, we selected the threshold for statistical significance to unadjusted *P* values <0.001. Associations between dichotomous outcomes were tested using the χ^2 test.

The patients were 41-93 years of age (CCUS 54-90 years, MDS 41-93 years), predominantly male, and most MDS patients had lower-risk disease (*Online Supplementary Table S1*).

We first investigated the association between *TET2* mutations and genome-wide methylation levels in CCUS and MDS separately. Patients with mutations in *DNMT3A*, *IDH1*, or *IDH2* were removed from the analyses resulting in 23 CCUS and 20 MDS cases with, and 20 CCUS and 26 MDS cases without, *TET2* mutations (data on 20 CCUS patients from each group have been published previously)[4] (*Online Supplementary Figure S1A, B*). After correcting for multiple testing, we identified 24,515 DMP in *TET2*-mutated CCUS compared to wild-type cases (Figure 1A). Almost all DMP (24,365 DMP, 99%) were hypermethylated. Similarly, we

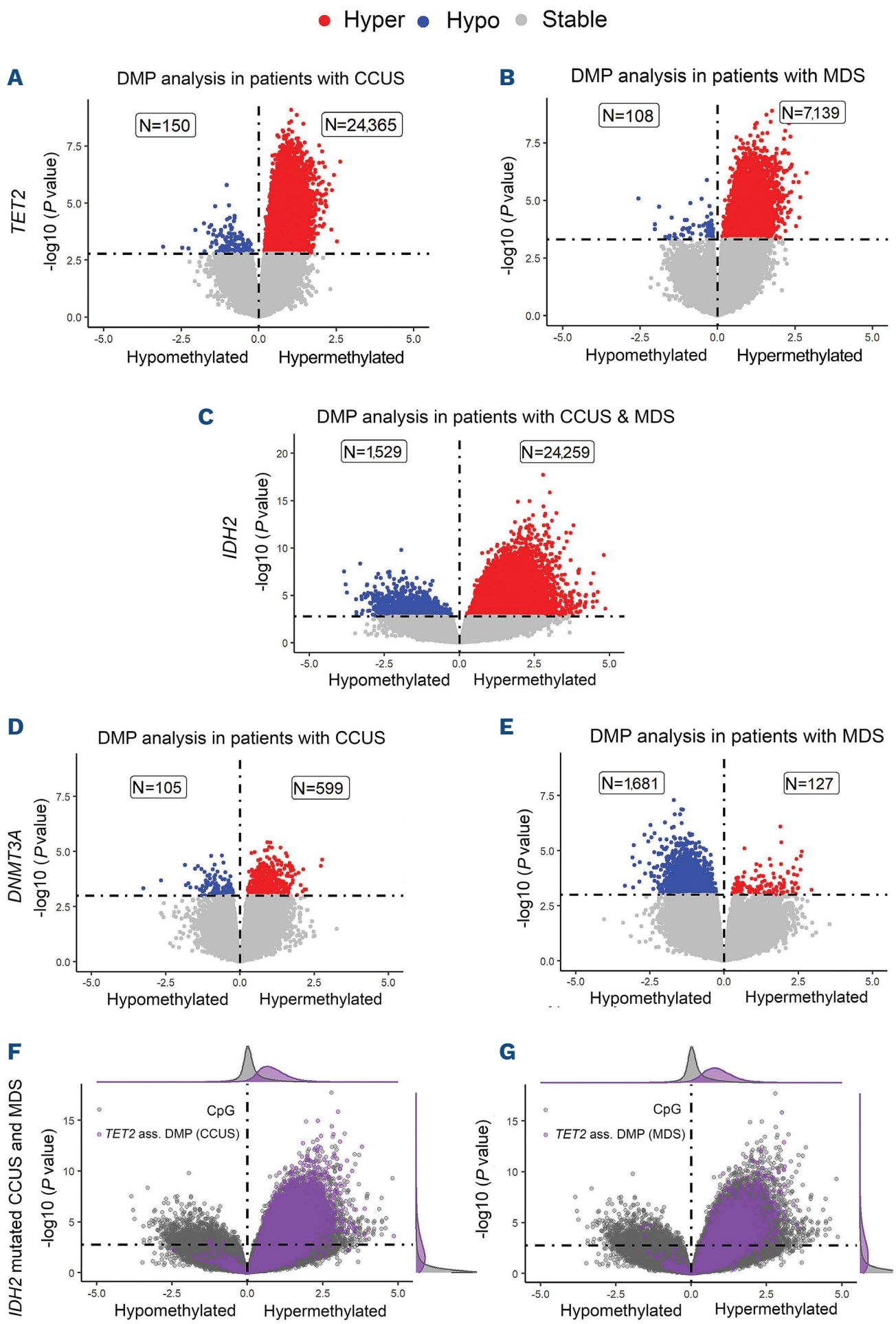

Figure 1. Differential methylation associated with *TET2*, *IDH2*, and *DNMT3A* mutations. Volcano plots of results from analysis of differentially methylated positions (DMP). (A) Twenty-three cases of *TET2*-mutated clonal cytopenia of undetermined significance (CCUS) compared to 20 cases of *TET2* wild-type CCUS. (B) Twenty cases of *TET2*-mutated myelodysplastic neoplasms (MDS) compared to 26 cases of *TET2* wild-type MDS. (C) Four cases of *IDH2*-mutant compared to 46 cases of *IDH2* wild-type CCUS and MDS. (D) Eight cases of *DNMT3A*-mutant compared to 20 cases of *DNMT3A* wildtype CCUS. (E) Five cases of *DNMT3A*-mutated MDS compared to 26 cases of *DNMT3A* wild-type MDS. Red and blue dots represent hyper- and hypomethylated CpG sites, respectively, below the significance threshold. Horizontal dashed lines represent a false discovery rate of 0.05 in (A-C) and $P<0.001$ in (D-E). (F, G) *IDH2*-mutated CCUS and MDS (same as in Figure 1C) with purple dots representing the 24,365 CpG sites showing *TET2* mutation-associated (ass.) significant hypermethylation in CCUS (F) and the 7,139 CpG sites showing *TET2* mutation-associated significant hypermethylation in MDS (G). All *IDH2*-mutated cases had co-occurring mutations in *SRSF2*. *P* values and effect size estimates in all panels were derived using a linear regression model. All *P* values are two-sided and not adjusted for multiple comparisons.

detected 7,242 DMP in *TET2*-mutated MDS compared to wild-type cases with 98% (7,139) DMP being hypermethylated (Figure 1B).

Next, we investigated the association between *IDH2* mutations and methylation levels. After removal of patients with *TET2, DNMT3A*, or *IDH1* mutations, four cases had *IDH2* mutations (1 CCUS and 3 MDS) and 46 were *IDH2* wild-type (*Online Supplementary Figure S1C*). Like *TET2* mutations, *IDH2* mutations were associated with hypermethylation of 94% (24,259) of the 25,888 DMP identified after FDR correction (Figure 1C). Since 2-hydroxyglutarate, the metabolic product of mutated IDH2 proteins, inhibits the catalytic activity of TET2 enzymes, we investigated if the methylation profiles detected in *IDH2*-mutant cases were similar to those observed for *TET2* mutations. We found that 98% (23,807 sites) and 97% (6,917 sites) of the *TET2* mutation-associated hypermethylated sites in CCUS and MDS, respectively, were associated with increased methylation in *IDH2*-mutated cases ($P<2.2x10^{-16}$) (Figure 1F, G). *IDH1* mutations were not studied due to a low mutation count (N=1).

Eight CCUS and five MDS cases had mutations in *DNMT3A* and 20 CCUS and 26 MDS cases did not, after removal of cases with *TET2*, *IDH1*, or *IDH2* mutations (*Online Supplementary Figure S1D, E*). We did not detect any DMP in *DNMT3A*-mutated CCUS after FDR correction and only 704 DMP were detected at an unadjusted $P<0.001$ (Figure 1D). This may be due to differences in cell populations, lower clonality, and/or number of mutated cases. Similarly, only 1,808 DMP were detected in *DNMT3A*-mutated MDS at an unadjusted $P<0.001$ with 93% (1,681) being hypomethylated (Figure 1E). The larger number of DMP associated with *TET2* than *DNMT3A* mutations may be due to type and location of the mutations (*DNMT3A* mutations were mainly missense whereas *TET2* mutations were primarily frameshift and nonsense mutations, which have been associated with increased clonal selection[12]), cell type specificity, and/or power since a higher number of patients had *TET2* than *DNMT3A* mutations. In summary, mutations in *TET2* and *IDH2* are associated with a hypermethylation phenotype affecting thousands of CpG sites in both CCUS and MDS, whereas *DNMT3A* mutations are associated with a hypomethylation phenotype, which we only detected in MDS.

As we had previously found 2,741 hypermethylated CpG sites in *TET2*-mutated CHIP,[5] we next investigated whether the same sites were affected by *TET2* mutations across CHIP, CCUS, and MDS. We found that 2,464 CpG sites were available for evaluation after quality control, and 98% (2,458

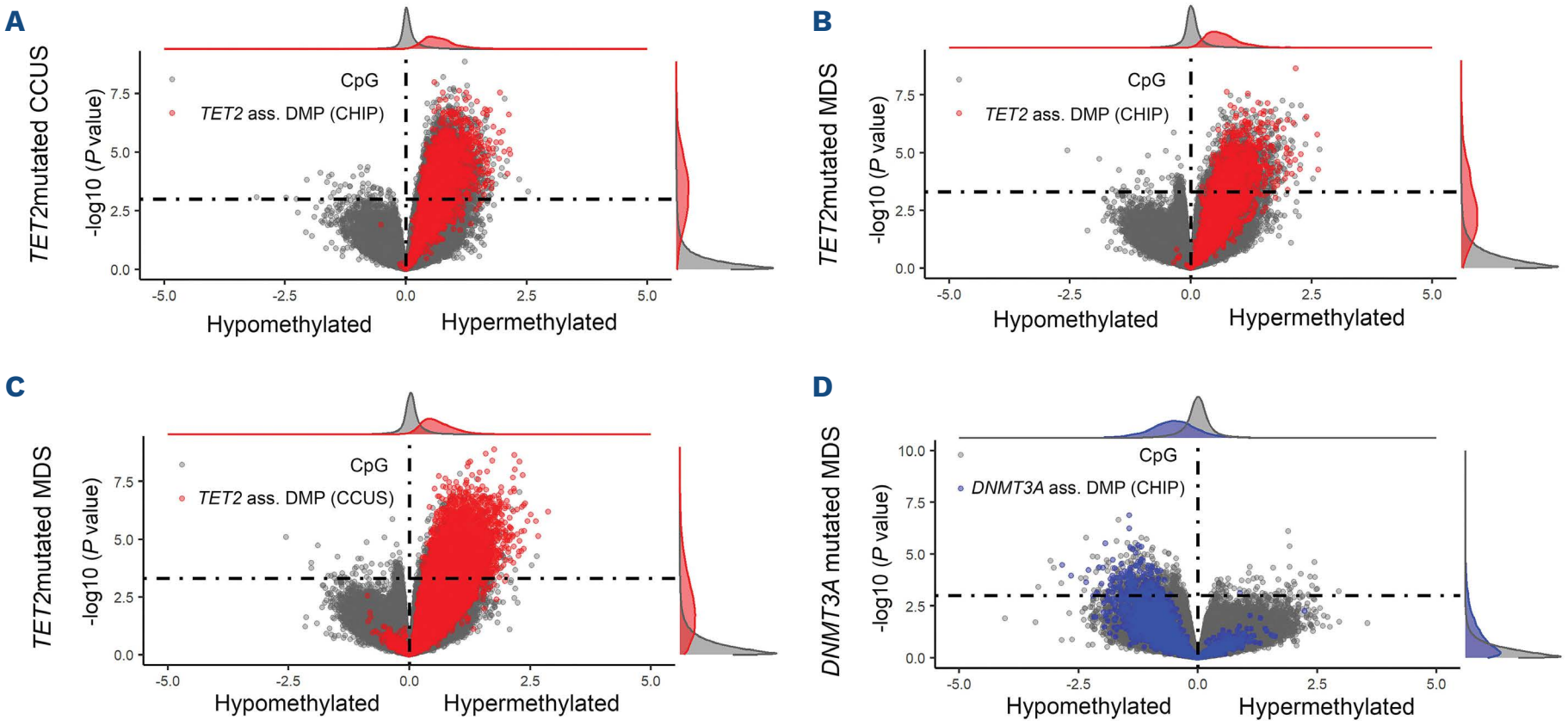

Figure 2. Comparison of results from epigenome-wide analyses of *TET2* and *DNMT3A* mutations in clonal hematopoiesis of indeterminate potential, clonal cytopenia of undetermined significance, and myelodysplastic neoplasms. Volcano plots of results of the analysis of differentially methylated positions (DMP) in: (A) *TET2*-mutated clonal cytopenia of undetermined significance (CCUS) (same as in Figure 1A) and (B) *TET2*-mutated myelodysplastic neoplasms (MDS) (same as in Figure 1B) with red dots indicating the 2,741 sites previously reported as being hypermethylated in *TET2*-mutated clonal hematopoiesis of indeterminate potential (CHIP).[4] (C) *TET2*-mutated MDS (same as in Figure 2B) with red dots representing the 24,365 CpG sites showing *TET2* mutation-associated (ass.) significant hypermethylation in CCUS. (D) *DNMT3A*-mutated MDS (same as in Figure 1E) with blue dots indicating the 3,803 CpG sites previously reported to be hypomethylated in *DNMT3A*-mutated CHIP.[5] Margins display distributions of colored sites *versus* the rest of CpG sites available after quality control (either rest of Infinium HumanMethylation450-sites in the case of CHIP or Infinium HumanMethylationEPIC-sites in the case of CCUS or MDS). *P* values and effect size estimates in all panels were derived using a linear regression model. All *P* values are two-sided and not adjusted for multiple comparisons.

sites) and 99.6% (2,455 sites) of those were positively associated with *TET2* mutations in CCUS and MDS, respectively ($P<2.2x10^{-16}$) (Figure 2A, B). Similarly, 98% (23,973 sites) of the 24,365 sites hypermethylated in *TET2*-mutated CCUS were hypermethylated in *TET2*-mutated MDS ($P<2.2x10^{-16}$) (Figure 2C). This indicates that methylation changes associated with *TET2* mutations are affecting many of the same loci irrespective of disease state.

Next, we investigated whether *DNMT3A* mutations affect DNA methylation similarly in MDS and CHIP. We found that 85% (2,924 sites) of the CpG sites previously reported to be hypomethylated in *DNMT3A*-mutated CHIP[5] (3,433 of 3,803 sites available for analysis) were associated with lower methylation in *DNMT3A*-mutated MDS ($P<2.2x10^{-16}$) (Figure 2D). In summary, the large overlap of mutation-associated DMP across different hematopoietic contexts indicates that methylation phenotypes associated with *TET2* and *DNMT3A* mutations are remarkably specific, which is in line with previous findings in CHIP and AML.[4-6,13] Due to the large overlap in CCUS and CHIP, it is unlikely that mutation-associated DMP in MDS are influenced by blast count or Revised International Prognostic Scoring System score. Although mutation-specific DMP were distributed across the entire genome, they were significantly enriched at enhancer regions and depleted at CpG islands for both hypermethylated sites associated with *TET2* and *IDH2* mutations and hypomethylated sites associated with *DNMT3A* mutations. Indeed, 11% of hypermethylated sites *versus*

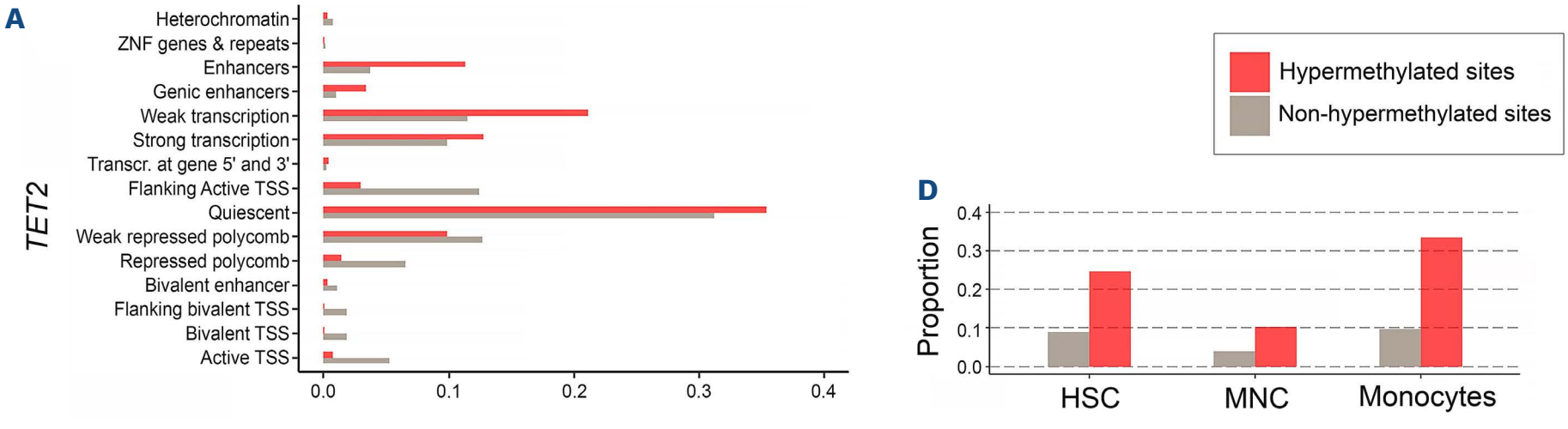

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Figure 3. Annotation of *TET2*, *IDH2*, and *DNMT3A* mutation-associated differentially methylated positions to chromatin states and cell-specific enhancers. (A-C) Annotation of interrogated CpG sites and methylation changes to chromatin states of peripheral blood mononuclear cells. (A) Proportions of the 24,365 *TET2* mutation-associated hypermethylated sites in clonal cytopenia of undetermined significance (CCUS) (red bars) and the rest of the sites (gray bars) at different chromatin states. (B) Proportions of the 24,259 *IDH2* mutation-associated hypermethylated sites in CCUS and myelodysplastic neoplasms (MDS) (red bars) and the rest of the sites (gray bars) at different chromatin states. (C) Proportions of the 1,681 *DNMT3A* mutation-associated hypomethylated sites in MDS (blue bars) and the rest of the sites (gray bars) at different chromatin states. Differentially methylated positions (DMP) were annotated to genomic regions using the annotation package for Illumina's methylation arrays and to chromatin states using the mononuclear dataset from the Roadmap Epigenomics project.[15] (D-F) Proportions of DMP and the rest of available sites interrogated by the EPIC v1.0 array located in regions annotated as enhancers in hematopoietic stem cells (HSC), mononuclear cells (MNC), or monocytes. DMP are the 7,139 CpG sites hypermethylated in *TET2*-mutated MDS (95% confidence intervals (CI): 0.32-0.35 and 0.23-0.25 for monocyte and HSC enhancers, respectively) (D), the 24,259 CpG sites hypermethylated in *IDH2*-mutated CCUS and MDS (95% CI: 0.33-0.34 and 0.31-0.32 for monocyte and HSC enhancers, respectively) (E), and the 1,681 CpG sites hypomethylated in *DNMT3A*-mutated MDS (95% CI: 0.23-0.27 and 0.40-0.45 for monocyte and HSC enhancers, respectively) (F). TSS: transcription start site.

4% of non-hypermethylated sites detected in *TET2*-mutated CCUS were in enhancer regions ($P<2.2x10^{-16}$) (Figure 3A), and only 3% of hypermethylated *TET2*-mutated sites *versus* 20% of non-hypermethylated sites were in CpG islands ($P<2.2x10^{-16}$). Strikingly similar results were observed for hypermethylated sites associated with *TET2*-mutated MDS and *IDH2*-mutated CCUS and MDS ($P<2.2x10^{-16}$) (Figure 3B). Likewise, 18% of all hypomethylated sites *versus* 4% of the non-hypomethylated sites associated with *DNMT3A* mutations in MDS were in enhancer regions ($P<2.2x10^{-16}$) (Figure 3C) and only 8% of hypomethylated sites were in CpG islands while this was the case for 19% of non-hypomethylated sites ($P<2.2x10^{-16}$). Interestingly, *TET2* mutation-associated hypermethylation was especially enriched in regions annotated as enhancers in monocytes (Figure 3D), whereas *IDH2* mutation-associated hypermethylation was equally present at monocyte and hematopoietic stem cell enhancers (Figure 3E). In contrast, *DNMT3A* mutation-associated hypomethylation was enriched in hematopoietic stem cell enhancers (Figure 3F). These findings demonstrate that the methylation changes are non-random and context-dependent, implying that the activity of TET2 and DNMT3A may be targeted to specific genomic regions in hematopoietic cells. TET2 has been suggested to have the strongest demethylation effect proximal to bound transcription factors[14] and we have previously shown a strong enrichment of ETS and C/EBP transcription factor binding motifs near *TET2* mutation-associated hypermethylated sites in CHIP and CCUS.[4]

Gene ontology term enrichment analysis of genes in the vicinity of the DMP revealed strikingly similar results for *TET2* and *IDH2* mutation-associated hypermethylated sites and *DNMT3A* mutation-associated hypomethylated sites with enrichment of genes related to immune system process and leukocyte activation (*Online Supplementary Figure S2A-D*). Similarly, genes near the 442 DMP that were common to all three mutations were involved in immune regulation and immune cell activation (*Online Supplementary Figure S2E*). These findings support a shared target pathway of *TET2*, *IDH2*, and *DNMT3A* mutation-induced methylation changes. Taken together, our results demonstrate the existence of distinct and non-random DNA methylation changes associated with mutations in *TET2*, *IDH2* and *DNMT3A* in CCUS and MDS. We observed similar mutation-induced methylation changes across a spectrum of myeloid clonal disorders, suggesting that mutation-associated methylation changes may be an early event in leukemogenesis. Moreover, mutations in *TET2*, *IDH2*, and *DNMT3A* especially induce methylation changes at enhancer regions but with differential preferences for enhancers according to cell type, suggesting that these mutations affect regulatory regions of genes at various stages of hematopoietic cell differentiation. This is in line with a previous study showing that *DNMT3A* mutations led to DNA hypomethylation in regulatory regions of genes that are related to hematopoietic stem cell activity, while *TET2* mutations led to DNA hypermethylation at regulatory regions of genes that downregulate the activity of more differentiated progenitor cells, thus both maintaining a stem cell-like state.[5] Future studies of the genes regulated by these enhancers may shed light on the potential pathogenic impact of methylation changes during leukemogenesis.

Authors

Katja Kaastrup,[1,2] Morten Tulstrup,[1,2] Jakob W. Hansen,[1,2] Claudia Schöllkopf,[1] Klas Raaschou-Jensen,[3] Andreas D. Ørskov,[4] Bo Porse,[2,5,6] Peter A. Jones,[7] Joachim Weischenfeldt,[2,6] Linn Gillberg[1,2,8#] and Kirsten Grønbæk[1,2,5#]

[1]The Epi-/Genome Lab, Department of Hematology, Copenhagen University Hospital - Rigshospitalet, Copenhagen, Denmark; [2]Biotech Research and Innovation Center (BRIC), University of Copenhagen, Copenhagen, Denmark; [3]Department of Hematology, Odense University Hospital, Odense, Denmark; [4]Department of Hematology, Zealand University Hospital, Roskilde, Denmark; [5]Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark; [6]The Finsen Laboratory, Faculty of Health Sciences, Copenhagen University Hospital - Rigshospitalet, Copenhagen, Denmark; [7]Department of Epigenetics, Van Andel Institute, Grand Rapids, MI, USA and [8]Department of Biomedical Sciences, University of

Copenhagen, Copenhagen, Denmark.

#LG and KG contributed equally as senior authors.

Correspondence:
K. GRØNBÆK - kirsten.groenbaek@regionh.dk

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Disclosures

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Contributions

KK, LG, JWH, JW and KG designed the study. CS, KR-J, ADØ, BP and KG provided study samples. KK handled T-cell depletion and DNA isolation. MT handled variant filtration. KK analyzed the data. LG, JWG, JW and KG supervised statistical analyses. KK, MT, LG, JWH, PAJ, JW and KG interpreted the results. KK wrote the first draft of the paper. All authors edited the paper and approved the final manuscript.

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Data-sharing statement

The raw and processed data generated in this study as well as other individual-level data are only available under restricted access since these data are considered sensitive personal data according to Danish Law and the European Union General Data Protection Regulation (GDPR) and thus cannot be shared with third parties without prior approval. To access data, an application must be sent to kirsten.groenbaek@regionh.dk. Access can only be granted for research purposes, and only if a data processor or data transfer agreement can be made in accordance with Danish and European law at the given time. The expected timeframe from response until access is granted is ~6 months.

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