

# Epigenomic profiling of myelofibrosis reveals widespread DNA methylation changes in enhancer elements and *ZFP36L1* as a potential tumor suppressor gene that is epigenetically regulated

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## **SUPPLEMENTARY METHODS**

### **DNA Methylation profiling**

DNA methylation was assessed with Human-Methylation 450K Bead-Chip kit (Illumina, Inc., San Diego, CA, USA). The array-based assays for DNA methylation profiling were performed at the National Centre of Oncologic Investigations (CNIO, Madrid, Spain). Briefly, 500ng of genomic DNA were modified with sodium bisulfite (EZ DNA Methylation Kit, Zymo Research) and subsequently whole genome amplified following manufacturer's recommendations. Samples were then hybridized in the assay chips as previously described<sup>1</sup>. Data arising from the 450K Human-Methylation array was analyzed by Bioconductor open source software. The analytical pipeline implemented several filters to exclude technical and biological biases (i.e. sex-specific methylation or overlapping CpGs with SNPs) and taking into account the performance characteristics of Infinium I and Infinium II assays<sup>2</sup>. Differentially Methylated CpGs (DMC) were defined as previously described<sup>2,3</sup>. DNA methylation data sets are available from the Gene Expression Omnibus (GSE118241).

### **Genomic and functional annotation of CpG sites**

The hg19 version of the UCSC Genome Browser database was used to annotate raw data from the DNA methylation array. DMCs were annotated into four categories relative to CpG islands (CGI) as follows: inside CGI, CGI-shore (0-2 Kb from the CGI), CGI-shelf (>2 Kb up to 4 Kb from the CGI) and outside CGI (>4kb from the CGI). All annotations were extracted from Ensembl database (<http://www.ensembl.org>). DMCs were also annotated according to publicly available functional chromatin states of human CD34+ cells following the ChromHMM algorithm<sup>4,5</sup>. Chromatin states were categorized in six functional features (0: heterochromatin; 1-3: transcription-start sites; 4-5: enhancer regions (weak and strong); 6: promoters). This final annotation led to the identification of a group of DMC between MF and healthy controls that mapped to enhancer regions. The genes adjacent to these enhancers were then used for Gene Ontology (GO) functional enrichment analysis (GO-PANTHER) as described elsewhere<sup>6</sup>.

## **Identification of candidate genes targeted by aberrant DNA methylation in enhancers**

Data of gene expression profiling (Affymetrix gene expression array) from primary MF (n=9) and healthy peripheral blood samples (n=21) was obtained from the publicly available GEO accession bank number GSE26049<sup>7</sup>. Data was further processed using R and the open source Limma package<sup>8</sup>. Genes showing consistent and ample differences in DNA methylation between MF and the control group were included (FDR<0.01;  $\Delta\beta>0.4$ ) and then were subsequently filtered by the changes in gene expression (logFC values below 0). The final list included 31 probes, encompassing 27 coding genes. Each of these genes was subsequently explored by literature search to identify those with potential implication in the hematopoietic system.

## **Cell culture**

The SET-2 cell line (DSMZ # ACC-608; established from the peripheral blood of a patient diagnosed with essential thrombocythemia at megakaryoblastic leukemic transformation) was maintained in RPMI medium supplemented with 20% fetal bovine serum and antibiotics (100 IU/mL penicillin, 50  $\mu\text{g}/\text{mL}$  streptomycin). HEL (DSMZ # ACC-11) and HL-60 (DSMZ # ACC-3) cell lines were maintained in RPMI medium supplemented with 10% fetal bovine serum and antibiotics (100 IU/mL penicillin, 50  $\mu\text{g}/\text{mL}$  streptomycin). Cells were seeded at  $0.5 \times 10^6$  cells/ml and incubated at 37°C and 5% CO<sub>2</sub> with 95% humidity.

## **Bisulfite sequencing**

DNA methylation levels were interrogated and validated using traditional bisulfite sequencing. Briefly, after bisulfite modification (CpGenome DNA modification Kit, Merck, Darmstadt, Germany), the fragment of interest was amplified, sub-cloned into the pGEM-T easy vector system (Promega, Madison, USA) and transformed in JM109 competent cells (Promega, Madison, USA). Plasmid DNA was extracted (Nucleospin Plasmid, Macherey Nagel, Germany) and for each condition, at least 10 different CFUs (colony forming units) were sequenced by classical Sanger method using Genetic Analyzer 3130XL (Life Technologies, Carlsbad, USA). Universally methylated human DNA (Zymo research, USA) was used as a positive DNA methylation control for bisulfite modification. Primer sequences are available in **Supplementary Table 1**.

### **Luciferase reporter assays**

The CpG-free vector (pCPG-L), gently provided by Dr. Michael Rehli<sup>9</sup>, was used to clone the *ZFP36L1* enhancer region, after amplification with high-fidelity Platinum<sup>TM</sup> Taq polymerase (Invitrogen, Waltham, USA). Cloned plasmids were amplified on PIR1 competent *E. coli* cells (Invitrogen, Carlsbad, USA) and then treated with SssI CpG methyltransferase enzyme (New England Biolabs, Ipswich, USA) following manufacturer's instructions. HEK293T cells were co-transfected with 10 ng/μl of DNA methylated or unmethylated reporter plasmids and 0.5 ng/μl renilla luciferase vector (pRL-SV40 Renilla Luciferase Control Reporter Vector, Promega), using Lipofectamine- 2000 (Invitrogen, Carlsbad, CA). Forty-eight hours after transfection, luciferase/Renilla activity was analyzed using the Dual Luciferase® reporter assay system (Promega, Madison, USA) in an automatic 96-well plate reader according to manufacturer's instructions. Luciferase experiments were performed in triplicates. Primer sequences are available in **Supplementary Table 1**.

### **Gene expression by Q-PCR**

Quantitative PCRs (Q-PCR) were performed with SYBR Green Master Mix (Applied Biosystems, Foster City, USA) and QuantStudio-3 96-well real-time PCR system (Applied Biosystems, Foster City, USA). *GUSB* gene was used as housekeeping reference gene in all cases. Primer sequences are available in **Supplementary Table 1**.

### **Overexpression of *ZFP36L1***

A vector containing *ZFP36L1* open reading frame was kindly provided by Dr. Murphy and sub-cloned into a PL-SIN-GK vector<sup>10</sup>. The same vector backbone carrying an EGFP open reading frame was used as experimental control. Lentiviral particles were generated by co-transfecting HEK293T cells with *ZFP36L1*-encoding plasmid, psPAX2 (Addgene, #12260) and pMD2G (Addgene, #12259) plasmids (3:2:1 ratio) using Lipofectamine-2000 (Invitrogen, Carlsbad, CA). Viral supernatant was harvested after 72h, filtered (0.2 μm), concentrated by ultra-centrifugation (26,000 g for 2.5 hours at 4 °C) and supplemented to the cells for infection with polybrene (Sigma-Aldrich, Saint Louis, USA) at 4 μg/ml. SET-2 cell line was used as an *in vitro* model of *JAK2V617F* mutated MPN. Infection efficiency was assessed after 72h, determining EGFP positive

cells by FACScanto-II™ flow cytometer (BD Biosciences, San Jose, USA) and CellQuest software™ (Becton Dickinson, Franklin Lakes, USA).

### **Apoptosis and cell proliferation assays**

Apoptosis was assessed using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, USA) following manufacturer's protocol. Apoptosis was analyzed using FACScanto™ flow cytometer and CellQuest software™ (Becton Dickinson, Franklin Lakes, USA). Cellular proliferation was assessed with standard MTS assays using the CellTiter 96® AQueous MTS Reagent (Promega, Madison, USA). All experiments were performed in triplicates.

### **Western Blotting**

After standard protein extraction, 50µg of protein were separated by 10% SDS-PAGE electrophoresis and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, USA). Membranes were incubated with the primary antibodies as follows: polyclonal rabbit anti ZFP36L1/ZFP36L2 (#2119 Cell Signaling, Leiden, Netherlands), loading control was made with mouse anti β-actin antibody (A5441 Sigma-Aldrich, St. Louis, MO). Anti-rabbit IgG (A3687 Sigma-Aldrich, St. Louis, USA) and anti-mouse IgG (A1418 Sigma-Aldrich, St. Louis, USA) antibodies conjugated with alkaline phosphatase were used as secondary antibodies.

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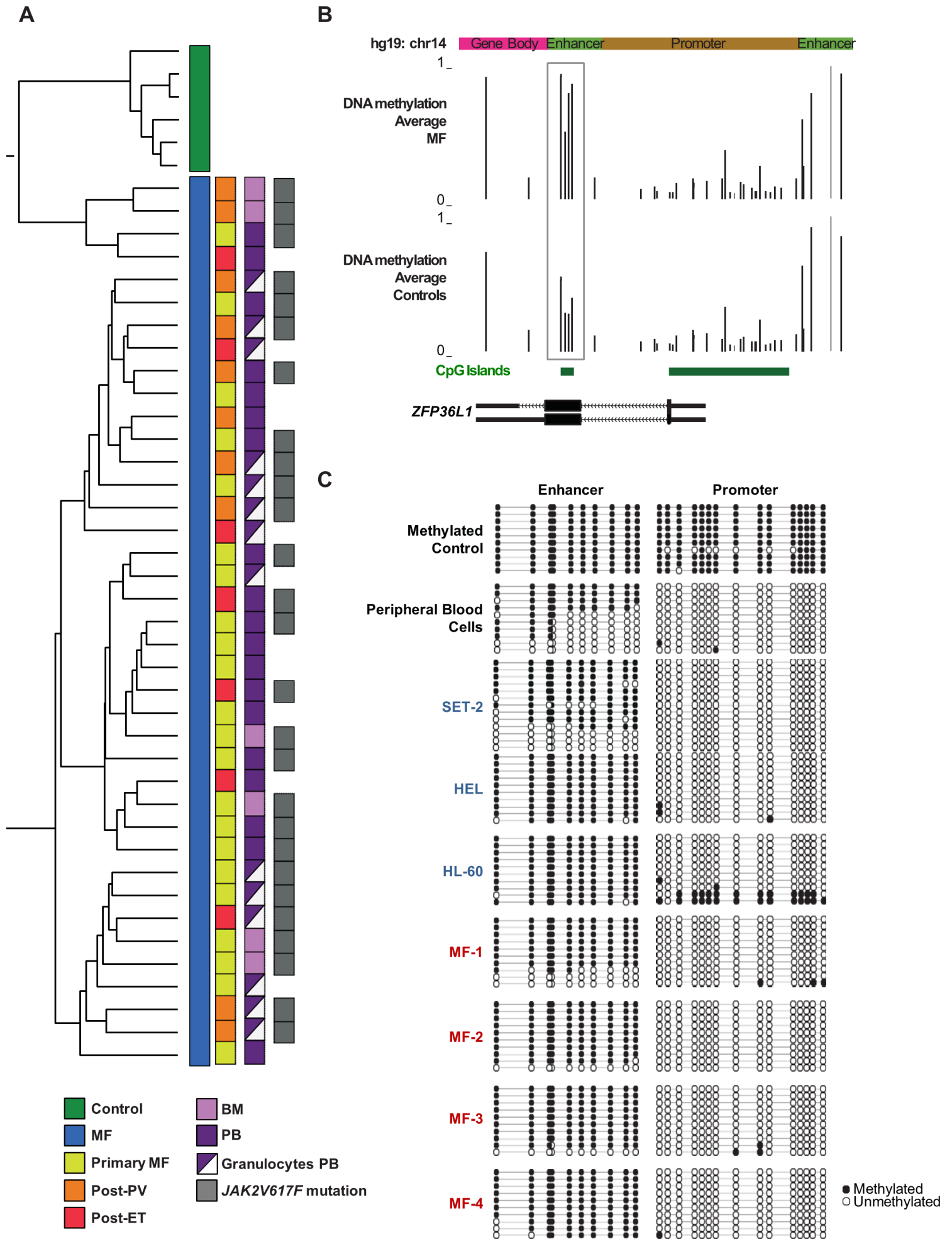
## SUPPLEMENTARY FIGURE LEGEND

**FIGURE S1. *ZFP36L1* is hypermethylated in MF** **A.** Unsupervised dendrogram of DMC between MF and control samples show a distinctive pattern of DNA methylation of MF samples and controls (and no differences between primary and secondary MF). **B)** Schematic representation of *ZFP36L1* locus and the comparative DNA methylation of all CpG dinucleotides included in the array for MF samples (upper panel) and controls samples (lower panel). Vertical bars represent normalized DNA methylation value as per the scale on the left. Chromatin state annotation is depicted on the color-coded horizontal bar on the top. Green boxes represent predicted CpG islands from UCSC genome browser. RefSeq transcript variants are shown in the bottom. **C)** Bisulfite sequencing of the enhancer and promoter region of *ZFP36L1* in peripheral blood cells, different myeloid cell lines and MF patients. Black dots represent methylated and white dots are unmethylated CpG dinucleotides.

## SUPPLEMENTARY TABLES

**Supplementary Table 1.** Lists of primers used for pyrosequencing, gene expression (qPCR) and pCPGL vector cloning.

**Supplementary Table 2.** Differentially methylated CpG mapped to enhancer regions (FDR<0.05) and expression value of most adjacent gene.



Supplementary Figure 1