PRMT5 inhibition modulates E2F1 methylation and gene regulatory networks leading to therapeutic efficacy in JAK2V617F mutant MPN

Data Supplement

Supplementary Methods

In vitro synergy experiments

100,000 SET2 cells were seeded in 96-well plates and treated with C220 and olaparib or AZD0156 for 9 days. Fresh media as well as drug was added on day 3 and 7. For synergy with ruxolitinib (SelleckChem), SET2 and BaF3 cells were treated with C220 only for 7 and 5 days respectively, followed by addition of ruxolitinib for 48 hrs. Viability was assessed using CCK-8 (WST-8) assay (Dojindo). For interpreting the value of synergy scores, we used SynergyFinder (1).

Values of synergy were defined as:

* Less than -10: we consider the interaction between two drugs is likely to be antagonism;
* From -10 to 10: we consider the interaction between two drugs is likely to be additivity;
* Larger than 10: we consider the interaction between two drugs is likely to be synergy.

Apoptosis and cell cycle assay

SET2 cells were cultured for 6 days at a density of 300,000cells/ml with increasing C220 concentrations and DMSO control. After 3 days, cells were split 1:4 and supplemented with new medium/inhibitor. 6 days after start of treatment, cells were washed twice with ice-cold PBS and apoptosis was assessed using the PE Active Caspase-3 Apoptosis kit (BD) and flow cytometry on a Fortessa2 (BD). Cell cycle status was assessed using the FITC BrdU Flow Kit (BD) and flow cytometry on a Fortessa2 (BD).

Sg design

For each gene of interest, 2 different pairs of sgRNA for CRISPR knockout were designed using the public available design tools provided by the Broad Institute (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design). All sgRNA and primers used for sequencing used are listed in Tables S1 and S2.

Plasmids and sgRNA cloning and site-directed mutagenesis

pLentiCas9-blast and pU6-sgRNA-EF1α-puro-T2A-BFP were purchased from Addgene (cat.no 52962 and 60955). For sgRNA cloning, oligos were annealed in annealing buffer (200 mM

potassium acetate, 60 mM HEPES-KOH pH 7.4, 4 mM magnesium acetate) and ligated into BstXI+BlpI digested pU6-sgRNA-EF1α-puro-T2A-BFP.

Virus production, lentiviral transduction and competition assays

HEK293FT cells were co-transfected with a construct of interest and pAX8 and pCMV-VSV using a standard calcium phosphate protocol. The viral supernatant was collected 72 hours after HEK293FT transfection and used for transduction. The cells were transduced using a RetroNectin Bound Virus Infection Method (TaKaRa) according to the manufacturer's instructions. Transduction was performed in presence of polybrene at 8 µg/ml. Antibiotic selection was added 24 hours after transduction. For the competition assays, the cells were collected 3 days after transduction and their transduction efficiency (BFP%) was measured and recorded as Day 0. The percentage of BFP% (transduced) cells was then measured on a flow cytometer (Beckman Coulter CytoFlex) at the specified timepoints. The cutting efficiency of all the sgRNAs was verified using Sanger sequencing with subsequent sequence trace decomposition using the ICE analysis online tool provided by Synthego (https://ice.synthego.com/#/) after sorting BFP+ cells 6 days after transduction. Knockout of protein was confirmed by Western Blot.

Western Blot analysis and immunoprecipitation studies

5 million cells were cultured with increasing C220 concentrations or DMSO for 6 days, replacing media and inhibitor after 3 days. After 6 days, cells were pelleted and washed twice with ice-cold PBS and resuspended in lysis buffer supplemented with Phosphatase-Inhibitor-Cocktail Set II (SIgma) and Protease arrest (Millipore Sigma). Total protein was normalized using the Bio-Rad Bradford assay and gel electrophoresis was performed using a 4%–12% Bis-Tris gel (Invitrogen). For *in vitro* studies of DNA damage repair, SET2 cells treated for 6 days with different inhibitor concentrations and DMSO control were irradiated with 10 Gy and cultivated another 6 hours afterwards in presence of the inhibitor before lysates were prepared. For *in vivo* analyses mice were sacrificed 4 hours after the last dose of inhibitor. Lysates from total BM or spleen were loaded onto the electrophoresis gel after red blood cell lysis. For immunoprecipitation studies of E2F, SET2 treated for 6 days were lysed as described above. 250-500 µg of protein lysate was incubated over night with an E2F1 antibody, followed by an incubation with 100ul of A/G protein agarose beads (sc-2003) the next morning and elution of the Antigen-Antibody complex from the beads. All primary and secondary antibodies are listed in Table S3.

Immunofluorescence

200 000 SET2 cells were treated with increasing concentrations of C220 and DMSO control for 6 days, followed by irradiation with 10Gy and another 6 hours of cell culture, were cytospun onto a glass slide (10'500rpm), dried for 1 hour at RT and fixed with 4% PFA for 15 minutes. Cells were incubated with blocking solution (Goat serum 1:80, 0.1% TritonX in PBS) for 15 min at RT, followed by incubation with the primary antibody Phospho-Histone H2A.X (CST 9718) in a 1:400 dilution in a solution of 1:100 goat serum in washing buffer (0.1% TritonX in PBS) for 1 hour at 37 degree Celsius. After 3 washes each 5 min with washing buffer, slides were incubated with Alexa Fluor 488 goat anti rabbit secondary antibodies (Invitrogen, A21206) in a 1:500 dilution in washing buffer for 1 hour in the dark, washed again 3 times and Vectashield/DAPI was applied onto the slides. Slides were air dried for 5 min, edges were secured with nail polish. The slides were digitized using Panoramic Flash 250 (3DHistech, Budapest Hungary) with Zeiss 40x/0.95NA objective. Images of cells were exported to tiff images using CaseViewer (3DHistech) and analyzed using custom macro written in ImageJ/FIJI (NIH). Briefly, each nucleus was segmented using the DAPI channel, and the number of pH2AX foci was counting using the Find Maxima algorithm.

Comet assay

The neutral Comet assay was performed using Comet slides and reagents provided in the Trevigen kit according to the manufacturer's instructions. Slides were stained with SYBR Green and comets imaged using using Zeiss ObserverZ1 with 20x/0.5NA objective. Comet analysis was conducted using custom ImageJ/FIJI macro which segmented each comet and its head and tail. Intensity and length of both were measured and tail moment were calculated according to the formula: Tail Moment = (Tail Intensity/(Head Intensity+Tail Intensity))* Tail width*100

Quantitative RT-PCR

Total RNA was isolated from SET2 cells using the RNeasy Micro kit (Qiagen) according to the manufacturer's instructions and treated with RNase-free DNase for removal of contaminating genomic DNA. RNA was transcribed into cDNA using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific). RT-PCR was performed using the VeriQuest Fast SYBR Green qPCR Master Mix (Affymetrix) and the QuantStudio 7 Flex System (Applied Biosystems). Delta CT Ratio with normalization for gene expression of house keeping genes was assessed for analysis.
All qPCR primers are listed in Table S4.

RNA-seq of Murine Bone marrows megakaryocyte-erythroid progenitor cells (MEP)

Sorted murine MEP cells underwent amplification (14 cycles) using the SMART-seq V4 (Clonetech) ultra-low input RNA kit for sequencing. Illumina hiseq libraries were prepared using 10 ng of amplified cDNA and the Kapa DNA library preparation chemistry (Kapa Biosystems) using 8 cycles of PCR. Samples were barcoded and run on a Hiseq 2500 1T in a 50 bp–50 bp paired-end run, using the TruSeq SBS Kit v.3 (Illumina). An average of 50 million paired reads was generated per sample. FASTQ files were mapped to the mouse genome mm10 using the Star aligner that maps reads genomically and resolves reads across splice junctions (2). We used several QC metrics for the RNA-seq library, including intron–exon ratio, intragenic reads fraction and GC bias. We quantified exon and gene expression using Salmon against the Mus musculus transcriptome GRCh38 (3). Differential expression tests were performed using the DESEQ2 algorithm (4). We considered genes that had a FDR < 0.05 to be significantly different between genotypes and ranked significant genes by fold change with a cutoff of one. Gene set enrichment analysis on KEGG pathway, GO term and Reactome gene set collection was done using the over-representation test with a hypergeometric model to assess whether the number of selected genes associated with disease is larger than expected (5). GSEA Software was used for Gene set enrichment analysis of the Molecular Signature Database with a FDR adjusted P-value cut-off of 0.05 (6).

RNA-seq of human SET2 cell derived mouse xenografts (CDX)

RNA was extracted using the Qiagen all-prep DNA/RNA mini kit according to the manufacturer's instructions and used for ribogreen quantification and quality control on Agilent BioAnalyzer. Subsequently, 500 ng of total RNA was used for polyA selection and Truseq library preparation according to the instructions provided by Illumina (TruSeq RNA Sample Prep Kit v.2), with 8 cycles of PCR. Samples were barcoded and run on a Hiseq 4000 in a 125 bp–125 bp paired-end run, using the TruSeq SBS Kit v.3 (Illumina). An average of 75 million paired reads was generated per sample. FASTQ files were mapped to the human genome GRCh37 using Star aligner (2). We used several QC metrics for the RNA-seq library, including intron–exon ratio, intragenic reads fraction and GC bias. We quantified exon and gene expression using Salmon against the Homo sapiens transcriptome GRCh37 (3). Differential expression tests, statistical tests and pathway analysis was performed as described above for mouse RNA-seq.

For comparison with published human data on CD34+ MPN patients, GSEA was performed for SET2 cells treated with PRMT5i vs vehicle, and human CD34+ BM cells from JAK2 positive MPN (PV, ET and PMF) versus healthy human CD34+ BM cell control. An enrichment score was

derived as the difference of the GSEA normalized enrichment score (NES) in SET2 cell minus the normalized enrichment score in human CD34+ cells. This enrichment score was ranked and plotted in a scatterplot, were the X axis represent the relative rank and the Y axis the difference in NES between SET2 cell and CD34+ BM cells.

ATAC sequencing

ATAC-seq was performed as previously described (7). Nuclear extracts from 50,000 sorted JAK2V617F mutated MEP were incubated with 2.5 µl of transposase (Illumina)for 30 min at 37 °C in a total of 50 µl reaction. Purified fragmented DNA was amplified by PCR and high-throughput sequenced using the HiSeq 2500 platform (illumina). Fastq files were aligned to the mm10 murine genome version. Mapped ATAC-seq reads were normalized and displayed as reads counts per million mapped reads.

Serum SDMA levels in patients

Serum SDMA levels were measured using the IDEXX Catalyst SDMA Test as per manufacturer's instructions using the catalyst one chemistry analyzer (IDEXX).

Serum cytokine assay

At time of takedown of the mice after 4-6 weeks of treatment, peripheral blood was collected, incubated for 1 hours in 37 °C water bath and centrifuged at 1000 g for 10 minutes. The supernatant, containing the serum, was collected and stored at -80. Serum cytokine levels were assessed using a Luminex-based Mouse 32-Plex Cytokine Kit (catalog no. MCYTMAG- 70K-PX32, EMD Millipore) after 1:2 dilution of serum samples. Data were acquired using the FlexMAP 3D system and xPONENT software and analyzed as previously described (8).

Flow cytometry and sorting of JAK2VF+ MEP

Flow cytometry of surface markers was performed in mouse PB, BM and spleen cells. After lysis of red blood cells, cells were stained with monoclonal antibodies in PBS/1% BSA for 30-60 min on ice, washed, resuspended in single cell solutions and analyzed using an LSR Fortessa (Becton Dickinson). Data were analyzed by FlowJo software (version 10.0.8). For flow cytometry of the erythroid cells and erythroid progenitor populations, bone marrow and spleen were stained without prior lysis with antibodies for lineage markers (CD11b, Gr1, CD45R, CD3, CD4, CD8, CD19, Ter119, NK1.1), Sca-1, c-Kit, CD150, CD105, CD48, CD16/32, CD41, CD71, and Ter119 (BioLegend) as published before (9).

JAK2VF+ (=CD45.2+) MEP were flow sorted using the SONY MA900 multi-application cell sorter (Sony Biotechnology).

Pathology

All organ tissues were fixed in 4% paraformaldehyde, embedded in paraffin, sliced in 4µm slides and stained with H&E or silver impregnation (Bio-Optica) to assess reticulin fibrosis.

For each sample, degree of fibrosis in BM and spleen was evaluated in 6 HPF (0=no fibrosis, 1=focal mild fibrosis, 2=marked, but non-diffuse fibrosis, 3=diffuse fibrosis).

Supplementary References

1. Ianevski A, He L, Aittokallio T, Tang J. SynergyFinder: a web application for analyzing drug combination dose-response matrix data. Stegle O, editor. Bioinformatics. 2017;33:2413–5.

2. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013;29:15–21.

3. Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. Salmon provides fast and bias-aware quantification of transcript expression. Nat Meth. 2017;14:417–9.

4. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. BioMed Central; 2014;15:550–21.

5. Yu G, Wang L-G, Han Y, He Q-Y. clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS. 2012;16:284–7.

6. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci USA. 2005;102:15545–50.

7. Buenrostro JD, Giresi PG, Zaba LC, Chang HY, Greenleaf WJ. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. Nat Meth. Nature Publishing Group; 2013;10:1213–8.

8. Kleppe M, Kwak M, Koppikar P, Riester M, Keller M, Bastian L, et al. JAK-STAT pathway activation in malignant and nonmalignant cells contributes to MPN pathogenesis and therapeutic response. Cancer Discov. 2015;5:316–31.

9. Pronk CJH, Rossi DJ, Månsson R, Attema JL, Norddahl GL, Chan CKF, et al. Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy. Cell Stem Cell. 2007;1:428–42.

10. Rampal R, Al-Shahrour F, Abdel-Wahab O, Patel JP, Brunel J-P, Mermel CH, et al. Integrated genomic analysis illustrates the central role of JAK-STAT pathway activation in myeloproliferative neoplasm pathogenesis. Blood. American Society of Hematology; 2014;123:e123–33.

11. Zini R, Guglielmelli P, Pietra D, Rumi E, Rossi C, Rontauroli S, et al. CALR mutational status identifies different disease subtypes of essential thrombocythemia showing distinct expression profiles. Blood Cancer J. Nature Publishing Group; 2017;7:638–13.

12. Calura E, Pizzini S, Bisognin A, Coppe A, Sales G, Gaffo E, et al. A data-driven network model of primary myelofibrosis: transcriptional and post-transcriptional alterations in CD34+ cells. Blood Cancer J. Nature Publishing Group; 2016;6:e439–9.

Supplementary Tables

Table S1: List of sg used for competition assay	
name	**sequence**
E2F1_1_fwd	TTGCACAGGTGTGAAATCCCCGGGTTTAAGAGC
E2F1_1_rev	TTAGCTCTTAAACCCGGGGATTTCACACCTGTGCAACAAG
E2F1_2_fwd	TTGAAGGTCCTGACACGTCACGTGTTTAAGAGC
E2F1_2_rev	TTAGCTCTTAAACACGTGACGTGTCAGGACCTTCAACAAG
E2F2_1_fwd	TTGTATATCTCAAGAGCACCCAAGTTTAAGAGC
E2F2_1_rev	TTAGCTCTTAAACTTGGGTGCTCTTGAGATATACAACAAG
E2F2_2_fwd	TTGGGAGCCGGACAGTCCTTCCGGTTTAAGAGC
E2F2_2_rev	TTAGCTCTTAAACCGGAAGGACTGTCCGGCTCCCAACAAG
E2F3_1_fwd	TTGGGACCTCAAACTGTTAACCGGTTTAAGAGC
E2F3_1_rev	TTAGCTCTTAAACCGGTTAACAGTTTGAGGTCCCAACAAG
E2F3_2_fwd	TTGGGAGGGAGGGTATTACCGGAGTTTAAGAGC
E2F3_2_rev	TTAGCTCTTAAACTCCGGTAATACCCTCCCTCCCAACAAG
PRMT5_fwd	TTGGCGGATAAAGCTGTATGCTG GTTTAAGAGC
PRMT5_rev	TTAGCTCTTAAAC CAGCATACAGCTTTATCCGCCAACAAG
MCM2_fwd	TTG TAGGCCAGCACGTGCTCCCGTTTAAGAGC
MCM2_rev	TTAGCTCTTAAAC GGGAGCACGTGCTGGCCTACAACAAG

Table S2: List of primers used for PCR and sequencing of CRISPR cell lines	
name	**sequence**
E2F1_gDNA_fwd	TAAACTGAGGCCCAGGTGAC
E2F1_gDNA_rev	TTGAGTGGGTAGGAGGTTGG
E2F2_gDNA_fwd	AAACTAAGGCCGGTCTCTCC
E2F2_gDNA_rev	TAGGCCAGATGGAGTTGGTC
E2F3_gDNA_fwd	TGCAGTCTGTCTGAGGATGG
E2F3_gDNA_rev	ACACGTGCACACACACACAC
PRMT5_gDNA_fwd	TTCCCCATTCTTCAAACTGC
PRMT5_gDNA_rev	AGTGAGTGCTTTCCCCTCAA
MCM2_gDNA_fwd	GTGCCCCCTTCTAGAGAACC
MCM2_gDNA_rev	ATCACACTCCTGTGCAACCA

Table S3: list of antibodies used for Western Blot, IP or IF		
name	**source**	**identifier**
β- Actin	Cell Signaling	4970
Akt	Cell Signaling	4691
Alexa Fluor 488 goat anti-rabbit IgG	Invitrogen	A21206
Anti-mouse IgG, HRP-linked	Cell Signaling	7076
Anti-rabbit IgG, HRP-linked	Cell Signaling	7074
E2F1	Abcam	179445
E2F1	Cell Signaling	3742S
E2F2	LifeSpan Biosciences	LS-C352136-100
E2F2	Abcam	ab138515
E2F3	Invitrogen	MA1-25333
Flag-tag M2	Sigma	F1804
GAPDH	Abcam	ab181602
γH2A.X	Cell Signaling	9718
JAK2	Cell Signaling	3280
MAPK	Cell Signaling	9102
MCM2	Abcam	ab133325
PARP	Cell Signaling	9542
Phospho AKT Ser473	Cell Signaling	4060
Phospho-Histone H2A.X Ser139	Cell Signaling	9718
Phospho-JAK2 Tyr1007/1008	Cell Signaling	3776
Phospho-p44/42 MAPK	Cell Signaling	9101
Phospho STAT5 Tyr694	Cell Signaling	9351
PRMT5	Cell Signaling	79998
Rb (D20)	Cell Signaling	9313
SDMA	Cell Signaling	13222
STAT5	Cell Signaling	9363

Table S4: List of primers used for qPCR	
name	**sequence**
human beta-Actin forward	AGGCACCAGGGCGTGAT
human beta-Actin reverse	GCCCACATAGGAATCCTTCTGAC
human p53 forward	GCCCAACAACACCAGCTCCT
human p53 reverse	CCTGGGCATCCTTGAGTTCC

Table S5: Overlap of downregulated GSEA signatures by single Ruxolitinib and single C220			
Enrichment FDR	**Genes in list**	**Total genes**	**Functional Category**
1.10E-12	13	198	INTERFERON GAMMA RESPONSE
1.77E-07	7	94	INTERFERON ALPHA RESPONSE
0.000210386	6	195	IL2 STAT5 SIGNALING
0.000703759	4	87	IL6 JAK STAT3 SIGNALING
0.001152077	5	197	INFLAMMATORY RESPONSE
0.001152077	5	197	HEME METABOLISM
0.008557642	4	198	TNFA SIGNALING VIA NFKB
0.037094413	3	194	HYPOXIA
0.037094413	3	197	MTORC1 SIGNALING
0.037094413	3	196	P53 PATHWAY
0.037094413	3	199	ALLOGRAFT REJECTION

Table S6: Overlap of upregulated GSEA signatures by single Ruxolitinib that are downregulated by single C220			
Enrichment FDR	**Genes in list**	**Total genes**	**Functional Category**
1.12E-18	41	196	E2F TARGETS
1.12E-14	36	197	MTORC1 SIGNALING
1.34E-09	29	197	MYC TARGETS V1
3.82E-07	15	73	CHOLESTEROL HOMEOSTASIS
1.68E-06	21	157	FATTY ACID METABOLISM
1.39E-05	22	195	G2M CHECKPOINT
1.55E-05	21	183	OXIDATIVE PHOSPHORYLATION
2.94E-05	11	58	MYC TARGETS V2
0.00019272	13	97	ANDROGEN RESPONSE
0.00222172	12	109	UNFOLDED PROTEIN RESPONSE
0.00520614	16	190	ADIPOGENESIS
0.00588009	13	141	DNA REPAIR
0.00674282	16	198	GLYCOLYSIS
0.01160074	10	103	PEROXISOME

Supplementary Table legends

Table S1: sgRNA designed to target E2F1, E2F2 and E2F3 genes used in the competition assay in Cas9 transduced SET2 cells. fwd=forward, rev=reverse

Table S2: primers used for PCR amplification and Sanger sequencing of gDNA derived from BFP+ sorted SET2-Cas9 cells 6 days after transduction.

Table S3: antibodies used for western blot, immunoprecipitation (IP) and immunofluorescence (IF) studies.

Table S4: primers used for qPCR.

Table S5: GSEA signatures of the overlapping genes that are down-regulated by single ruxolitinib and single C220 derived from SET2 xenografts treated for 2 weeks are depicted.

Table S6: GSEA signatures of the overlapping genes that are up-regulated by single ruxolitinib and down-regulated by single C220 derived from SET2 xenografts treated for 2 weeks are depicted.