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Promoter hypomethylation drives *ABCB1*-mediated carfilzomib resistance in multiple myeloma

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Abstract

Background Proteasome inhibitors (PIs) are indispensable for the treatment of multiple myeloma (MM), the second most common hematologic malignancy. Although primary resistance to PIs is rare, most patients eventually relapse and develop acquired resistance, with underlying mechanisms that remain incompletely understood and appear to be drug-specific. In the case of bortezomib, resistance is often associated with *PSMB5* mutations. In contrast, resistance to carfilzomib (CFZ) is mediated by overexpression of the drug efflux transporter ABCB1. However, the regulatory mechanisms driving ABCB1 upregulation in CFZ-resistant MM remain unclear.

Methods An integrative multi-omics analysis was conducted using paired samples from a CFZ-sensitive and -resistant MM patient, alongside resistant cell line models. Whole-genome sequencing (WGS), whole-genome bisulfite sequencing (WGBS), and RNA sequencing (RNA-seq) were used to assess the genotype (structural variants, single nucleotide variants, and copy number variations), methylation status, and the expression of the *ABCB1* locus. *ABCB1* promoter methylation levels and expression levels in an independent MM subcohort were analyzed to determine clinical relevance. Functional validation was performed using dual-luciferase reporter assays, *DNMT1* knockdown, and treatment with DNA methyltransferase inhibitors (DNMTis) to evaluate methylation-dependent regulation of *ABCB1* expression.

Results Significant hypomethylation of the *ABCB1* downstream promoter region was identified (GH07J087598) in a CFZ-resistant patient sample, which correlated with elevated *ABCB1* expression. Consistent with the paired CFZ-resistant case, the independent MM subcohort showed a significant inverse association between *ABCB1* promoter methylation and *ABCB1* expression. These findings align with results obtained from CFZ-resistant MM cell line models, which demonstrated reduced promoter methylation and elevated *ABCB1* expression compared to their wild-type counterparts. Furthermore, treatment with DNA methyltransferase inhibitors as well as *DNMT1* knockdown enhanced

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ABCB1 expression while demethylating the promoter, thereby validating the functional significance of promoter hypomethylation in *ABCB1* overexpression.

Conclusions Our findings highlight *ABCB1* promoter hypomethylation as a potential epigenetic driver of CFZ resistance in MM. These results underscore the clinical relevance of epigenetic regulation in drug resistance and the potential of targeting DNA methylation as a therapeutic strategy to overcome resistance in MM.

Keywords Multiple myeloma, Multi-omics, Epigenetic regulation, Proteasome inhibitor resistance, ABCB1, DNA methylation

Introduction

Multiple myeloma (MM) is a clonal plasma cell malignancy and remains the second most common hematologic cancer [1, 2]. Proteasome inhibitors (PIs), including bortezomib (BTZ), carfilzomib (CFZ), and ixazomib, are widely used in both newly diagnosed and relapsed/refractory MM [3, 4]. Their primary mechanism of action involves the inhibition of the ubiquitin–proteasome system, leading to the accumulation of misfolded proteins, elevated cellular stress, and apoptosis [5, 6]. Owing to their proven clinical efficacy, virtually all patients with MM are exposed to PIs as part of combination therapies; however, among those who can tolerate therapy for longer timeframes, the vast majority eventually develops drug resistance [7–9]. Known mechanisms of tumor-intrinsic resistance to proteasome inhibitors involve both genetic and epigenetic alterations [9, 10]. Among genetic events, mutations in the proteasome subunit *PSMB5* are the most extensively studied. These typically occur within the β5 subunit's substrate-binding pocket and impair the interaction between PIs and the chymotrypsin-like active site, thereby reducing inhibitor affinity and proteolytic inhibition [11, 12]. In parallel, epigenetic remodeling has also been proposed as a possible resistance mechanism, mostly through altered promoter methylation of *PSMD5*, which encodes a regulatory chaperone essential for 26S proteasome assembly. Hypermethylation of the *PSMD5* promoter has been observed in a subset of PI-refractory patients and is associated with reduced gene expression, elevated proteasome biogenesis, and diminished PI sensitivity [13].

Beyond proteasomal adaptations, increased expression of *ABCB1*, a member of the ATP-binding cassette transporter family, plays a prominent role in PI resistance [14]. *ABCB1* encodes a transmembrane efflux pump that actively exports a broad range of xenobiotics, including CFZ, thereby reducing intracellular drug accumulation and attenuating therapeutic efficacy [15, 16]. Although *ABCB1* overexpression is frequently observed in PI-resistant MM, the upstream regulatory mechanisms governing its transcription remain unclear. In other malignancies, promoter hypomethylation has been implicated in *ABCB1* activation and has been linked to resistance against various chemotherapeutic agents [17, 18]. Whether such epigenetic regulation contributes to *ABCB1*-mediated PI resistance in MM has yet to be explored.

In this study, we aimed to determine whether DNA methylation contributes to *ABCB1* regulation in the context of proteasome inhibitor resistance in MM. We performed genomic, epigenomic, and transcriptomic profiling of paired samples from a patient before and after developing CFZ resistance, and validated our findings in matched PI-sensitive and -resistant cell line models. Our multi-omics approach included whole-genome sequencing (WGS), whole-genome bisulfite sequencing (WGBS) and RNA sequencing (RNA-seq) to assess potential epigenetic alterations. To assess clinical relevance, we further examined *ABCB1* promoter methylation and *ABCB1* expression in an independent MM subcohort (n = 13). Finally, functional validation was performed using a CpG-free dual-luciferase reporter assay [19], *DNMT1* knockdown (KD), and DNA methyltransferase inhibitor (DNMTi) treatment.

Methods

Patient samples

Sequential primary samples were obtained from peripheral blood of a patient with an initial diagnosis of MM who progressed to plasma cell leukemia, during routine diagnostic procedures at the Cantonal Hospital St. Gallen (Switzerland). Sample 1 was taken at relapse after a BTZ-containing regimen; sample 2 was obtained upon progression on a pomalidomide and CFZ combination therapy. In addition, bone marrow aspirates were obtained from 13 patients with MM, who were treated at the University Hospital of Wuerzburg (Germany). $CD138^+$ plasma cells were isolated using magnetic-activated cell sorting with anti-CD138 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol.

Cell culture

Human MM cell lines (AMO1, ARH77, L363; obtained from the Leibniz Institute DSMZ, Braunschweig, Germany) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, sodium pyruvate, L-glutamine, and penicillin/streptomycin. CFZ-resistant

derivatives (AMO1CFZ, ARH77CFZ, L363CFZ) were established by gradually exposing parental cells to increasing concentrations of CFZ over several months, as previously described [20], and subsequently maintained in 90 nM CFZ. The cells were routinely tested for mycoplasma contamination and short tandem repeat (STR)-typed (DSMZ) to confirm authenticity.

DNA methyltransferase inhibitor treatment

For DNA methyltransferase inhibition, CFZ-resistant ARH77 cells and the wild-type (WT) were treated with either 25 nM decitabine (Dec) or 1 µM GSK-3484862 (GSK) (MedChemExpress, Monmouth Junction, USA) for 7 days, with media replenished every 48–72 h. On day 7, 1×10^6 cells were harvested for analysis of *ABCB1* expression and promoter methylation.

DNMT1 siRNA knockdown

ARH77 cells (2×10^6 per reaction) were washed with PBS, resuspended in NeonNxt™ Resuspension R Buffer (Thermo Fisher Scientific GmbH, Dreieich, Germany), and mixed with siRNA targeting DNMT1 (Thermo Fisher Scientific, #4390824) or a non-targeting control (Thermo Fisher Scientific, #4390843) to a final concentration of 2 µM. Electroporation was performed with the NeonNxt™ Transfection System at 1450 V, 10 ms, 3 pulses. Immediately after pulsing, cells were transferred to pre-warmed, antibiotic-free RPMI-1640 complete medium in 6-well plates and incubated at 37 °C with 5% CO_2. The downstream effects of DNMT1 KD were assessed at days 3, 5, and 7 post-transfection. *DNMT1* and *ABCB1* expression were quantified by RT-qPCR and CFZ sensitivity was measured using the alamarBlue assay.

CFZ sensitivity

CFZ sensitivity was assessed using the alamarBlue assay. Cells (1.8×10^4 per well) were seeded in 96-well flat-bottom plates in RPMI-1640 complete medium and treated for 72 h with CFZ (MedChemExpress, Monmouth Junction, USA) at the indicated concentrations. After 72 h, alamarBlue reagent was added at 10% of the well volume and the absorbance was measured at 570 and 600 nm using a Tecan Infinite 200 Pro plate reader (Tecan Deutschland GmbH, Crailsheim, Germany). IC_{50} values were calculated by nonlinear regression in GraphPad Prism software (v.10.1.2).

Multi-omics analysis

Whole-genome sequencing (WGS) libraries were prepared using the TruSeq PCR-free library prep kit (Illumina) and 2×150 bp paired-end reads were sequenced on a NovaSeq 6000 (Illumina) with a median coverage of 81–99×. The RNA-seq libraries were prepared with the Novogene NGS RNA Library Prep Set (PT042), and bisulfite-treated DNA libraries of whole-genome bisulfite sequencing (WGBS) were sequenced on a NovaSeq X Plus. Sequencing data were processed and analyzed using standard pipelines. Detailed protocols, including library preparation and data analysis workflows, are provided in Additional file 2: Supplementary Methods.

ABCB1 expression analysis

Total RNA was isolated from cell pellets using the AllPrep DNA/RNA kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. After quantification, 1 µg of RNA was reverse transcribed with SuperScript IV VILO Mastermix (Thermo Fisher Scientific GmbH, Dreieich, Germany) and quantitative PCR (qPCR) was performed on a StepOnePlus using TaqMan™ Gene Expression Master Mix, Hs00184500_m1 for *ABCB1* and Hs00939627_m1 for endogenous control *GUSB*. The $2^{(-\Delta\Delta CT)}$ approach was applied to compute relative quantification.

For protein quantification, frozen cell pellets were lysed in ice-cold RIPA buffer containing Halt protease inhibitor (Thermo Fisher Scientific GmbH, Dreieich, Germany). Lysates were denatured in Laemmli buffer, subjected to SDS-PAGE, and finally transferred to nitrocellulose membranes. Membranes were incubated overnight at 4 °C with primary antibodies against ABCB1 (Cell Signaling Technology #13987, 1:10,000) and GAPDH (Cell Signaling Technology #2118, 1:5,000), and subsequently incubated with HRP-conjugated secondary antibody (Cell Signaling Technology #7074, 1:10,000). Detection was performed using ECL substrate (Bio-Rad Laboratories GmbH, Feldkirchen, Germany), and images were acquired with a ChemiDoc Imaging system (Bio-Rad Laboratories GmbH, Feldkirchen, Germany).

Targeted bisulfite sequencing

500 ng of genomic DNA was bisulfite-converted using the EpiTect® Bisulfite Kit (Qiagen GmbH, Hilden, Germany). The *ABCB1* promoter region (GRCh38:7:87600603–87600971; strand: – 1) was PCR-amplified using the forward (5′-TGAGGTTGATTGGTTGGGTAGGAATA) and reverse (5′-TAACCCCACTTAATCCCCATAAACTTACC) primers. PCR products were gel-purified and subjected to Sanger sequencing on an ABI DNA Analyzer. CpG site methylation was analyzed using SnapGene software (v.5.0.8) [21].

Dual-luciferase reporter assay

The *ABCB1* downstream promoter region containing the transcription start site (GRCh38:7:87600603–87600971:-1) was cloned into the CpG-free pCpGL vector [19], and transformed into PIR1 One-Shot Competent Cells (Thermo Fisher Scientific GmbH, Dreieich, Germany). Following colony PCR confirmation, positive clones

were cultured overnight, and plasmid DNA was purified and *in vitro* methylated using M.SssI methyltransferase (New England Biolabs GmbH, Frankfurt am Main, Germany). Methylated or unmethylated pCpGL-*ABCB1* constructs were co-transfected with Renilla and CD4 vectors into AMO1 or SH-SY5Y cells. CD4-positive cells were selected via magnetic bead separation, and luciferase activity was measured using a Tecan Infinite 200 Pro plate reader (Tecan Deutschland GmbH, Crailsheim, Germany).

Statistical analysis

Statistical comparisons between two groups were performed using either the unpaired t-test (for normally distributed data) or the Mann-Whitney U test (for non-parametric data). Associations between *ABCB1* promoter methylation and *ABCB1* expression were evaluated by Spearman correlation. p values < 0.05 were considered statistically significant. All statistical analyses were conducted using GraphPad Prism software (v.10.1.2).

Results

WGS identifies proteasome-related alterations but no genomic changes at the *ABCB1* locus in CFZ-resistant myeloma

To investigate whether genomic alterations underlie *ABCB1* upregulation or reveal additional contributors to resistance, we performed whole-genome sequencing (WGS) on paired samples from a patient previously described by Besse et al. [14]. This patient exhibited a marked increase in *ABCB1* expression upon developing CFZ resistance. We compared genomic profiles obtained before treatment (CFZ sensitive) and after the development of resistance (CFZ resistant) using WGS data (Fig. 1A, B, circos plot). Two large deletions were acquired in the resistant sample: an ~ 18.8 Mb deletion in the 1p21.2–p12 region (chr1:101684001–120532000) affecting the *PSMA5* gene (mean $\log_2$ ratio: – 0.70), and a ~ 13.4 Mb deletion in the 2q36.3–q37.3 region (chr2:229817001–243185000) encompassing the *PSMD1* gene (mean $\log_2$ ratio: – 0.70). Of note, the identified 2q deletion also includes the genes *COPS7B* and *COPS8*, both belonging to the COP9 signalosome family for which an association with resistance to immunomodulatory drugs (IMiDs) has been described [22]. Beyond these deletions, additional copy number variations (CNVs) were observed on chromosome 10q comprising a ~ 7.6 Mb gain at 10q22.1–q22.3 (mean $\log_2$ ratio: + 0.35) adjacent to a large deletion of ~ 56.8 Mb at 10q22.3–q26.3 (mean $\log_2$ ratio: – 0.73) encompassing the *NFKB2* gene (Additional file 1: CNV). At the single nucleotide level, the resistant sample acquired two new mutations: a Tier 1 pathogenic mutation in the tumor suppressor gene *CYLD*, previously linked to unfavorable outcomes in MM, and a Tier 2 likely pathogenic mutation in *RASA2* (Additional file 1: SNV). Structural variation (SV) analysis showed consistent interchromosomal alterations in both samples including the translocation t(11;14)(q13;q32), but their variant allele frequencies were increased in the resistant sample (Additional file 1: SV), indicating clonal expansion. Importantly, despite genomic changes, our focused analysis of known proteasome inhibitor/CFZ resistance-associated genes (including *ABCB1*, *ABCG2*, *PSMB5*, *PSMB8*, *NFKB2*, and *XBP1* [23]) revealed no biallelic events at these loci. This suggests that while resistant cells undergo genomic rearrangements involving proteasome-related genes such as *PSMA5*, *PSMD1*, *NFKB2,* and *XBP1*, the substantial increase in *ABCB1* expression is likely regulated by non-genomic mechanisms.

Transcriptomic profiling reveals *ABCB1* overexpression alongside epigenetic regulator upregulation in CFZ-resistant myeloma

To explore potential non-genomic mechanisms underlying *ABCB1* upregulation, we performed RNA-seq analysis on the paired CFZ-sensitive and CFZ-resistant patient samples. *ABCB1* expression was significantly higher in the CFZ-resistant (mean expression: 1142.4) compared to the CFZ-sensitive patient sample (mean expression: 519.9), representing a 2.2-fold increase (adjusted p-value = 4.67×10^{-43}). Beyond *ABCB1*, the most upregulated genes included *FBXL7* and *INSRR* ($\log_2$FC ranging from 8.8 to 9.6), whereas the most downregulated included *IGLV3-1*, *CD24*, and *IGKV1-39* ($\log_2$FC ranging from – 9.7 to – 9.3) (Fig. 2A). Among proteasome inhibitor resistance–associated genes, we observed significant downregulation of *PSMB5*, *PSMB8*, and *XBP1* ($\log_2$FC between – 1.2 and – 1.7), suggesting a diminished reliance on proteasome function in the resistant sample (Fig. 2B). Notably, key modulators of the epigenetic machinery were robustly induced, with DNA methyltransferases (*DNMT1*, *DNMT3A*, *DNMT3B*) showing $\log_2$FC values between 1.9 and 3.1, and DNA demethylating enzymes (*TET1*, *TET2*, *TET3*) exhibiting $\log_2$FC values between 1.9 and 2.6 (Fig. 2C). Taken together, while *ABCB1* upregulation occurs in the absence of detectable genomic alterations, coordinated expression of epigenetic regulators suggests active remodeling of the epigenetic landscape, which likely contributes to the transcriptional upregulation of *ABCB1* and the overall resistant phenotype.

Epigenetic profiling reveals reduced *ABCB1* promoter methylation after developing CFZ resistance

To elucidate the potential epigenetic mechanisms underlying this upregulation, WGBS was conducted, and subsequent analyses focused on the canonical *ABCB1* promoter region (GRCh38:7:87598323–87601474). This focused evaluation demonstrated pronounced CpG

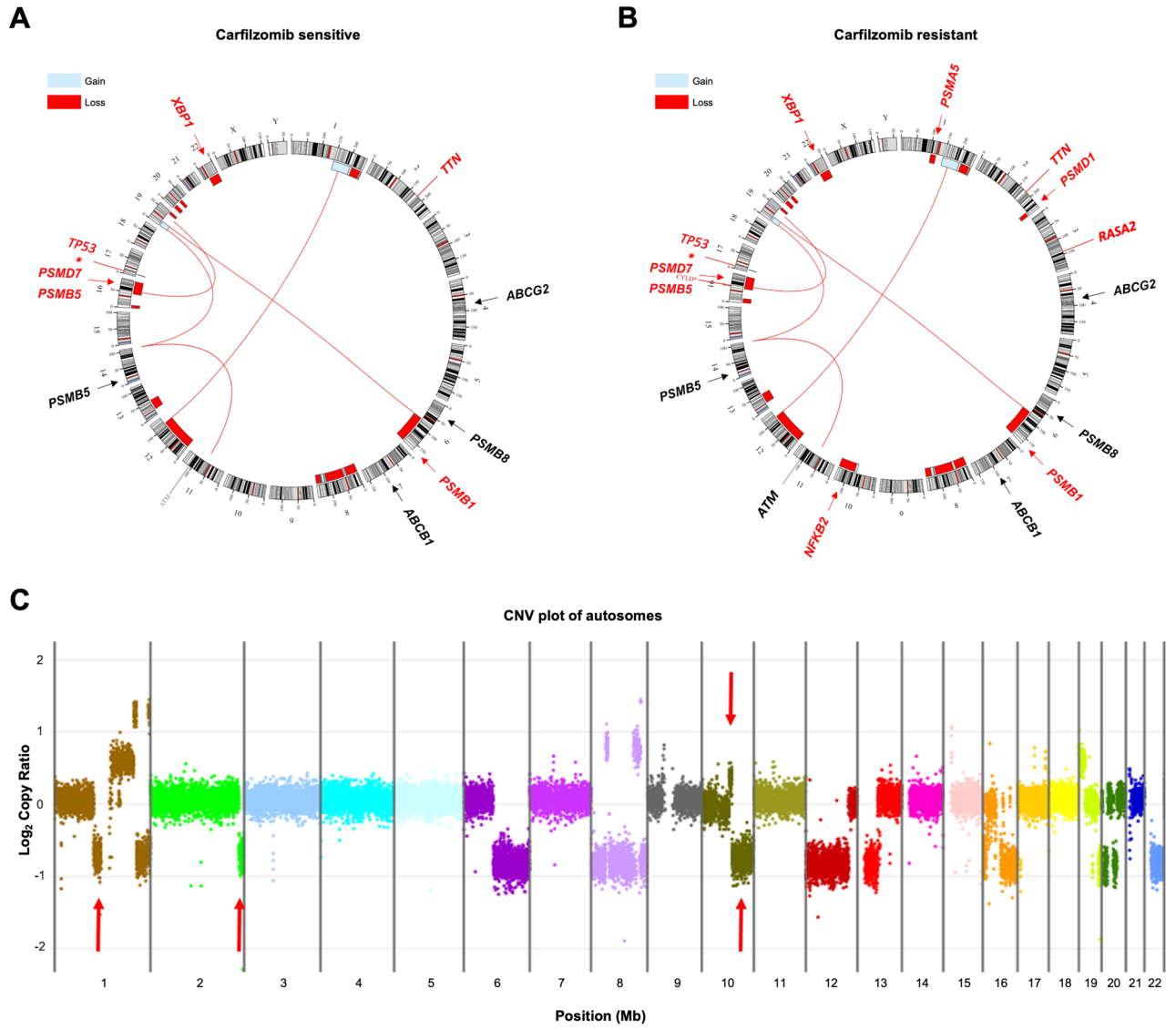


Fig. 1 Whole-genome sequencing of paired CFZ-sensitive and CFZ-resistant patient samples. **A**, **B** Circos plots with copy number variations (CNVs), interchromosomal structural variations (SVs), and single-nucleotide variants (SNVs) based on WGS data before therapy with carfilzomib and at the time of resistance to carfilzomib. Outer track runs clockwise from chromosome 1 to Y. Inner track shows CNVs (gains in light blue and losses in red). Red lines inside the circle represent interchromosomal SVs. Outside of the circle, genes with an SNV (Tier 1 or Tier 2) are depicted in red and genes with a variant of unknown significance (VUS) are shown in gray. On the very outside, the chromosomal localization of specific genes that could be relevant for carfilzomib resistance is indicated by arrows (in red in the case of a deletion). **C** CNV plot of autosomes 1–22. Red arrows highlight gains and losses identified in the carfilzomib-resistant sample compared to WGS data of a sample prior to carfilzomib treatment

hypomethylation in the CFZ-resistant sample relative to its sensitive counterpart. Site-specific methylation analysis revealed substantial decreases at the majority of the CpG sites, with methylation levels decreasing from 25.9 to 3.3% at position 87598419 and from 64 to 6.5% at position 87598445, representing absolute differences of 22.6 and 57.6%, respectively (Fig. 3A). Notably, across the promoter region, the sensitive sample exhibited a mean methylation level of 11.9% (± 2.4%; range: 0–64.0%), whereas the resistant sample displayed only 4.0% (± 1.3%; range: 0–34.6%) (Fig. 3B). An inverse relationship was observed between ABCB1 promoter methylation (11.9% in sensitive vs. 4.0% in resistant) and its gene expression (4.2 FPKM in sensitive vs. 9.2 FPKM in resistant) (Fig. 3C). To extend clinical relevance, an independent MM subcohort ($n = 13$) was analyzed for *ABCB1* promoter methylation and *ABCB1* expression. An inverse association was observed (Spearman ρ = − 0.59, $p = 0.036$), with promoter methylation ranging from 0.3 to 34.8% and *ABCB1* expression of 0.06–7.49 ($\log_2$[TPM + 1]). Notably, the highest expression was only observed in samples with < 1% promoter methylation

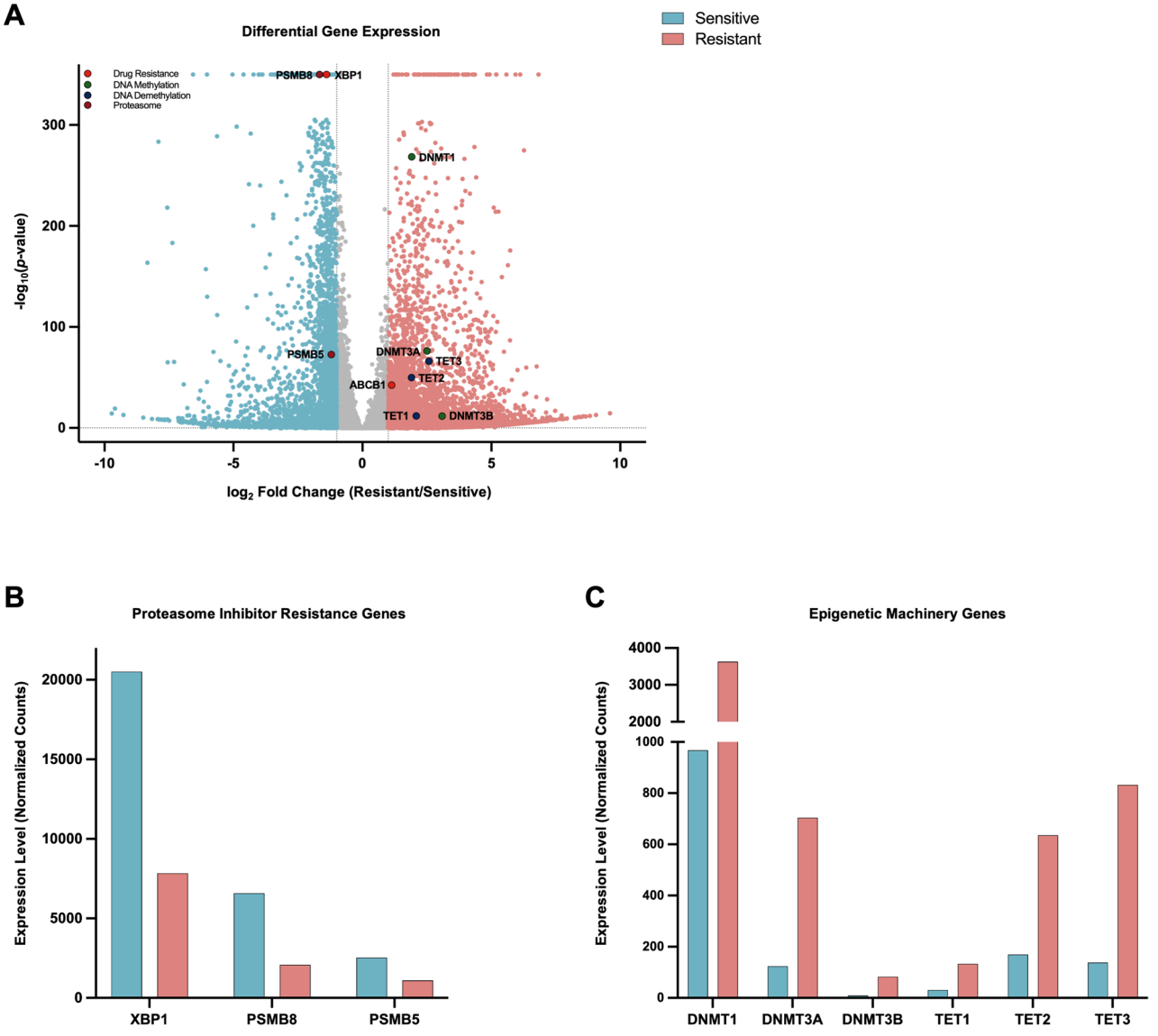


Fig. 2 RNA-seq of paired CFZ-sensitive and CFZ-resistant patient samples. **A** Volcano plot displaying differential gene expression between carfilzomib-resistant and -sensitive patient samples. X-axis represents log2 fold change and Y-axis the -log10(adjusted p-value). Significantly differentially expressed genes are highlighted in colors based on functional categories: DNA methylation (green), drug resistance (orange), DNA demethylation (blue), and proteasome subunits (red). **B** Bar plot comparing expression levels of PI resistance–associated genes between carfilzomib-sensitive (blue) and -resistant (red) samples. **C** Bar plot showing expression of epigenetic regulator genes (DNA methyltransferases and DNA demethylating enzymes) in carfilzomib-sensitive (blue) and -resistant (red) samples.

(Additional file 2: Table S1 and Fig. S1). Together, these data indicate that the methylation of the *ABCB1* promoter regulates the transcriptional activity of *ABCB1*, enabling enhanced CFZ efflux and subsequent resistance in MM.

Validation of epigenetic reprogramming-driven ABCB1-mediated carfilzomib resistance in MM cell line models

To extend our patient-derived findings and further dissect the mechanisms of carfilzomib resistance, three independent CFZ-resistant MM cell line models (AMO1CFZ, L363CFZ, and ARH77CFZ) were generated through long-term exposure to escalating drug concentrations. Characterization via alamarBlue assays revealed that all CFZ-resistant cell lines exhibited significantly elevated IC_{50} values relative to their parental counterparts. AMO1CFZ cells displayed a 62.1-fold increase in IC_{50} (from 3.2 nM in WT to 195.7 nM in the resistant derivative). Similarly, L363CFZ cells showed a 43.8-fold increase (from 5.8 to 253.6 nM), while ARH77CFZ cells demonstrated a 41.5-fold increase (from 3.5 to 114.4 nM) (Fig. 4A).

Consistent with the patient data, expression analysis using qPCR demonstrated significant upregulation of

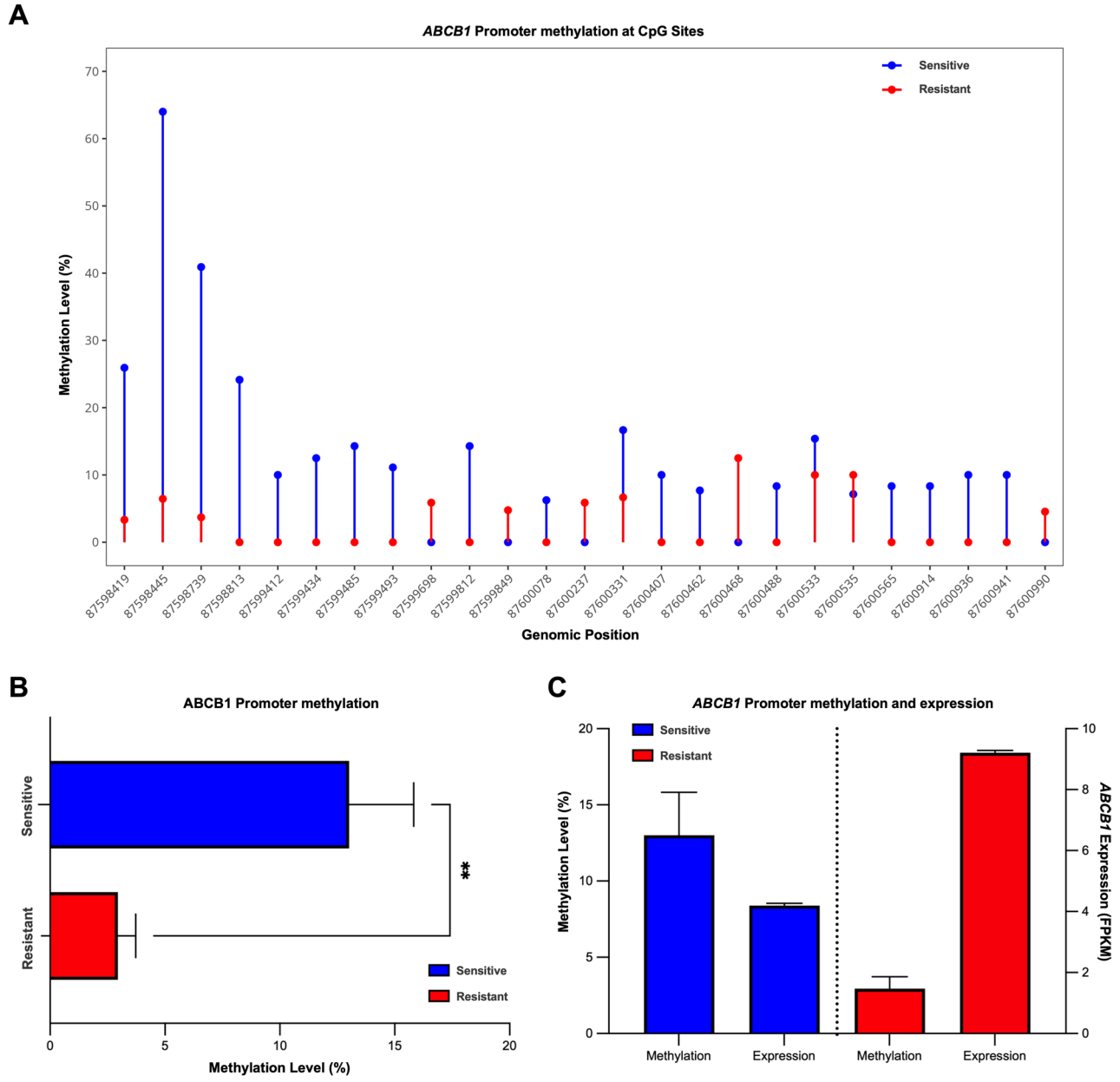


Fig. 3 *ABCB1* promoter methylation and gene expression in paired CFZ-sensitive and CFZ-resistant samples. **A** Methylation profile of the canonical *ABCB1* promoter region (GRCh38:7:87598323–87601474) comparing carfilzomib-sensitive (blue) and -resistant (red) samples from the index patient. The plot displays site-specific CpG methylation levels across the promoter region. **B** Bar plot showing mean methylation levels across the entire *ABCB1* promoter region in carfilzomib-sensitive (blue) and -resistant (red) samples. **C** Comparison of *ABCB1* expression levels (FPKM) between carfilzomib-sensitive (blue) and -resistant (red) samples, demonstrating the negative association between promoter methylation and gene expression

ABCB1 mRNA across all three CFZ-resistant cell lines. Quantitative analysis by the $2^{(-\Delta CT)}$ method revealed that all WT cell lines exhibited low baseline *ABCB1* expression, with AMO1 and ARH77 showing modest levels ($2^{(-\Delta CT)}$ values of 0.13 and 0.1, respectively) and L363 displaying near-undetectable transcript levels ($2^{(-\Delta CT)} = 6.3 \times 10^{-6}$). In contrast, all three CFZ-resistant derivatives demonstrated substantially elevated absolute expression levels (AMO1CFZ: 4.6; ARH77CFZ: 3.7; L363CFZ: 5.3). The comparison of these absolute $2^{(-\Delta CT)}$ values clearly demonstrated a significant and consistent upregulation of *ABCB1* expression upon the development of carfilzomib resistance, with the L363 model highlighting the remarkable dynamic range of this regulatory mechanism (Fig. 4B). In parallel, Western blot analysis confirmed the transcriptomic findings at the protein level, demonstrating corresponding increases in ABCB1 protein expression compared to their WT counterparts (Fig. 4C), with

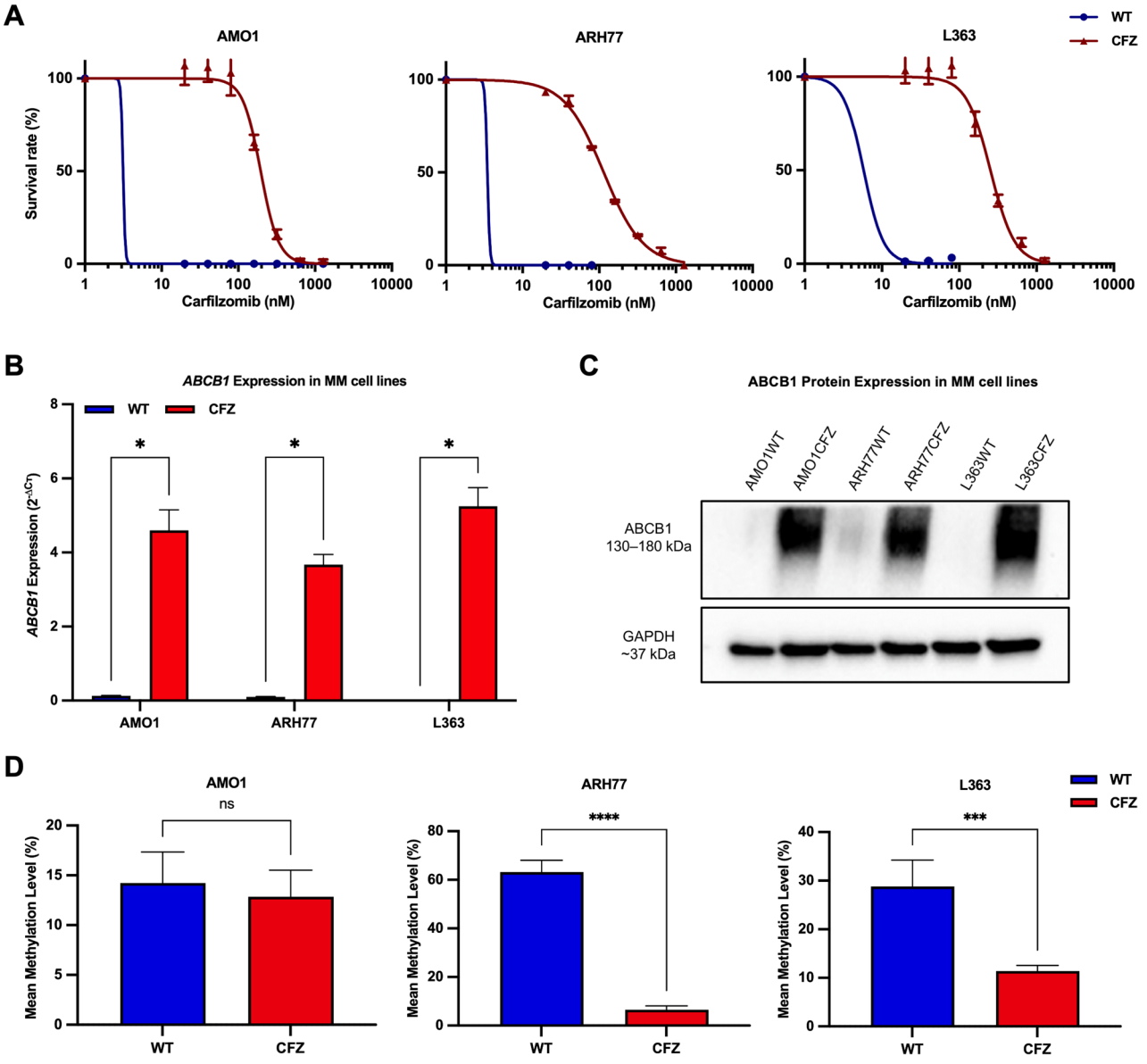


Fig. 4 Functional validation of ABCB1 upregulation and promoter hypomethylation in CFZ-resistant MM cell lines. **A** Dose-response curves showing carfilzomib cytotoxicity in WT and resistant MM cell lines (AMO1, L363, and ARH77), with corresponding IC_{50} values demonstrating significant resistance in CFZ-resistant derivatives. **B** Bar plot comparing *ABCB1* mRNA expression levels ($2^{(-\Delta CT)}$) between WT and CFZ-resistant cell lines, showing consistent upregulation across all three models. **C** Western blot analysis of ABCB1 protein expression in WT and CFZ-resistant cell lines, confirming transcriptional findings at the protein level. **D** Bar plot displaying *ABCB1* promoter methylation levels (GRCh38:7:87598323–87601474) in WT and CFZ-resistant cell lines, demonstrating significant hypomethylation in L363CFZ and ARH77CFZ models

strong concordance between mRNA and protein levels, indicating that the observed upregulation is primarily driven by transcriptional activation rather than post-translational mechanisms.

To further explore the regulatory underpinnings of *ABCB1* overexpression, WGBS with 30× coverage was performed on paired parental and CFZ-resistant cell lines, focusing specifically on the downstream promoter GH07J087598 (GRCh38:7:87598323–87601474). While the AMO1 models showed only marginal changes (14.2% vs. 12.9%), in the L363 and ARH77 models, the *ABCB1* downstream promoter exhibited methylation levels of 28.8 and 63.2% in WT cells, which decreased significantly to 11.4% ($p = 0.0007$) and 6.5% ($p < 0.0001$) in their resistant derivatives, respectively (Fig. 4D). Notably, a negative correlation was observed between promoter methylation and *ABCB1* expression in the L363 and ARH77 models, wherein substantial decreases in methylation corresponded with pronounced increases in *ABCB1* expression. This pattern, which closely mirrors

the epigenetic alterations observed in patient specimens, supports a conserved mechanism driving *ABCB1* overexpression under CFZ-induced selective pressure.

Functional validation confirms methylation-dependent regulation of *ABCB1* expression

To investigate whether promoter methylation regulates *ABCB1* expression, we conducted a dual-luciferase reporter assay to assess promoter activity, and subsequently treated cells with DNA methyltransferase inhibitors (DNMTis) to evaluate effects on endogenous gene expression and methylation. First, the impact of methylation on the *ABCB1* downstream promoter (GH07J087598) was evaluated using a CpG-free vector system (pCpGL). In AMO1 cells, the normalized luciferase activity (Firefly/Renilla) of the unmethylated pCpGL-*ABCB1* construct was 26.8-fold higher than that of the empty vector, confirming the promoter functionality of the cloned *ABCB1* core region. Methylation of the construct reduced luciferase activity by 8.1-fold, clearly demonstrating methylation-dependent transcriptional repression. Similarly, in SH-SY5Y cells, methylation of the *ABCB1* promoter construct led to a 129.3-fold decrease in activity relative to the unmethylated version, further supporting that hypermethylation significantly impairs the transcriptional capacity of the *ABCB1* downstream promoter (Fig. 5A). These results confirm that hypermethylation of the promoter directly impairs its activity.

To further examine this relationship in a cellular context, ARH77WT cells, characterized by high baseline *ABCB1* promoter methylation (63.2%), were treated with two DNMTis (25 nM decitabine and 1 μM GSK-3484862). After seven days of treatment, site-specific analysis revealed that both DNMTi treatments consistently reduced methylation across all individual CpG sites within the *ABCB1* core promoter region compared to the control, demonstrating a comprehensive demethylation effect (Additional file 2: Fig. S2A). This pattern was reflected in overall promoter methylation levels: DMSO-treated control cells maintained high methylation (69.9%), while decitabine reduced methylation to 38%, and GSK-3484862 further decreased it to 19.7% (Additional file 2: Fig. S2B). Notably, *ABCB1* expression (measured by $2^{(-\Delta\Delta CT)}$) increased significantly with a 21.7-fold upregulation following decitabine treatment and a 35.7-fold increase with GSK-3484862 (Additional file 2: Fig. S2C), which inversely correlated with the observed promoter hypomethylation. However, DNMTi exposure decreased cell viability over time (Additional file 2: Fig. S2D) and co-treatment with sub-cytotoxic GSK concentrations (500 nM, 1 μM) and CFZ (72 h) showed no detectable shift in the CFZ IC_{50} relative to DMSO (Additional file 2: Fig. S2E).

To avoid DNMTi-related cytotoxicity, we next performed DNMT1 KD using siRNAs. ARH77WT cells were electroporated with a DNMT1 siRNA or a non-targeting control siRNA (Ctrl-siRNA). The mRNA level of *DNMT1* was lowest at day 3 and partially recovered over time (Fig. 5B), whereas *ABCB1* expression level peaked at day 5 and declined by day 7 (Fig. 5C). In parallel, *ABCB1* promoter methylation was significantly reduced under DNMT1 KD at day 5 (44.8%) compared with both controls (59.3–60.1%) (Fig. 5D). In the following CFZ sensitivity test at day 5, DNMT1 KD cells revealed a right-shift in the CFZ IC_{50} (15.12 nM) relative to the CFZ IC_{50} from electroporation control (12.39 nM) and Ctrl-siRNA (12.94 nM) (Fig. 5E), indicating reduced CFZ sensitivity.

Together, these data demonstrate that reducing or inhibiting DNMT1 lowers *ABCB1* promoter methylation levels and restores *ABCB1* expression, supporting a methylation-dependent regulatory mechanism that contributes to CFZ resistance in MM cells.

Discussion

Genetic and epigenetic alterations in key therapeutic targets contribute significantly to drug resistance in MM. For instance, *CRBN*-coding mutations impair the binding of IMiDs [24], while hypermethylation of an intronic *CRBN* enhancer can silence its expression [25]. In the context of GPRC5D-directed immunotherapy, resistance has been linked to inactivating mutations/deletions [26, 27] and/or hypermethylation of the *GPRC5D* promoter [28]. Comparable epigenetic and genetic mechanisms have been implicated in resistance to proteasome inhibitors. Mutations in the proteasome subunit beta type-5 (*PSMB5*) alter proteasome conformation, reducing chymotrypsin-like activity and impairing PI binding. However, such mutations are rarely detected in patients and are mostly confined to cell line models, limiting their clinical relevance [29]. More compelling is the observation that altered promoter methylation of *PSMD5* is present in approximately 25% of PI-refractory patients [13].

Beyond proteasome subunit alterations, increased expression of drug efflux pumps also plays a critical role. The multidrug resistance transporter ABCB1, in particular, is upregulated in both PI-resistant patient samples and CFZ-resistant cell line models. Importantly, HIV protease inhibitors such as nelfinavir and lopinavir have been shown to inhibit ABCB1 activity, thereby restoring carfilzomib's proteasome-inhibitory function and overcoming drug resistance at clinically achievable concentrations [14]. However, the molecular mechanisms driving *ABCB1* upregulation remain largely unclear. While genetic alterations have been proposed to influence *ABCB1* expression [30], the accumulating evidence points to a significant role for epigenetic regulation in modulating *ABCB1* levels and contributing

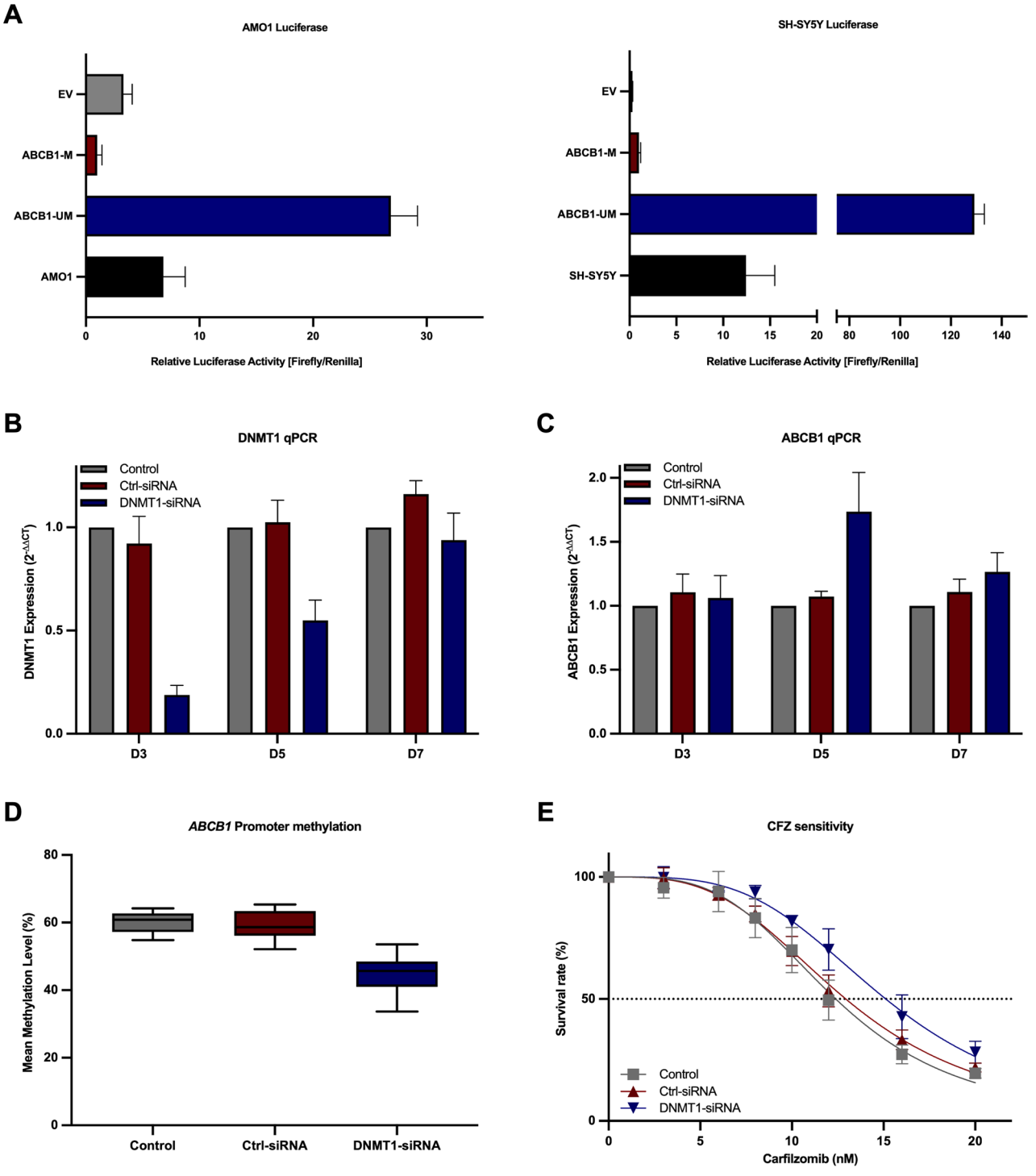


Fig. 5 Methylation-dependent regulation of *ABCB1* promoter activity and expression in *in vitro* models. **A** Dual-luciferase reporter assay showing *ABCB1* promoter activity in AMO1 and SH-SY5Y cells with unmethylated (ABCB1-UM) or methylated (ABCB1-M) pCpGL-*ABCB1* constructs, or the empty vector control (EV), demonstrating methylation-dependent transcriptional repression. **B***DNMT1* expression (qPCR) of DNMT1 siRNA (blue), non-targeting siRNA (Ctrl-siRNA, red), and electroporation control (gray). **C***ABCB1* expression (qPCR) of DNMT1 siRNA (blue), non-targeting siRNA (Ctrl-siRNA, red), and electroporation control (gray). **D***ABCB1* promoter methylation level (%) of electroporation control (gray), non-targeting siRNA (Ctrl-siRNA, red), and DNMT1 siRNA (blue) at day 5. **E** CFZ sensitivity of DNMT1 siRNA (blue), non-targeting siRNA (Ctrl-siRNA, red), and electroporation control (gray)

to treatment resistance across various cancer types [31–33]. This is further supported by the presence of a distinct downstream *ABCB1* promoter (GH07J087598; GRCh38:7:87598323–87601474), in which differential methylation can modulate its transcriptional activity directly and may also facilitate transcription factor (TF) binding [34, 35]. In this context, NF-κB, AP-1, and FOXP1 have been reported to regulate *ABCB1* expression in other malignancies [36–38].

Building on this knowledge, we analyzed the methylation status of this downstream *ABCB1* promoter and identified consistent hypomethylation in a primary CFZ-resistant MM patient sample. This was associated with pronounced *ABCB1* upregulation in the absence of genetic mutations or copy number alterations. In addition, transcriptomic profiling revealed elevated expression of several key epigenetic regulators, including DNMTs and TET family enzymes, suggesting broad epigenetic reprogramming during the development of resistance. Consistent with the paired CFZ-resistant patient case, an independent MM subcohort showed an inverse association between *ABCB1* promoter methylation and *ABCB1* expression. Although this cohort was not enriched for CFZ-resistant cases, the observed pattern supports the clinical relevance of *ABCB1* promoter regulation in MM.

This methylation pattern was recapitulated in our CFZ-resistant cell line models, which showed pronounced promoter hypomethylation together with *ABCB1* upregulation compared to their parental CFZ-sensitive WTs, mirroring the paired patient case. Functional validation with dual-luciferase assays confirmed that methylation of GH07J087598 directly suppresses promoter activity. Furthermore, pharmacologic inhibition of DNMT1 hypomethylated the *ABCB1* promoter, and DNMT1 KD reduced promoter methylation while inducing *ABCB1* expression. KD of DNMT1 also shifted the CFZ IC_{50} to higher concentrations, supporting a model in which promoter hypomethylation induces *ABCB1* expression, thereby increasing efflux of CFZ and reducing CFZ sensitivity.

Given that CFZ is an ABCB1 substrate, DNMTi-mediated upregulation of ABCB1 would be expected to antagonize CFZ. By contrast, decitabine has been reported to restore BTZ sensitivity [39], for which other resistance mechanisms have been described (e.g., *PSMB5* mutations, altered proteasome assembly, or *PSMD5* hypermethylation) [13, 29]. In our DNMTi experiments, concentrations required to demethylate the *ABCB1* promoter were cytotoxic. However, the CFZ IC_{50} did not shift, likely reflecting a balance between modest *ABCB1* upregulation and DNMTi cytotoxicity. This was supported by the DNMT1 KD experiment which confirmed ABCB1-mediated antagonism to CFZ.

Another layer of transcriptional regulation is provided by histone modifications. Prior studies indicate that histone acetylation at the *ABCB1* promoter can enhance transcription and promote drug resistance in non-MM cancer models [40, 41]. While our data establish promoter hypomethylation as a putative driver of ABCB1 activation in CFZ-resistant MM, the specific histone marks and transcription factors that may cooperate with DNA hypomethylation at this locus remain to be defined to fully understand the epigenetic regulation of *ABCB1*. Methodologically, our study comprises a paired CFZ-resistant case, CFZ-resistant cell-line models, and a small independent MM subcohort. Thus, larger, longitudinal CFZ-treated cohorts are required to determine the incidence and clinical outcome of *ABCB1* promoter hypomethylation in MM. Finally, while *ABCB1* overexpression is clearly an important factor, it is likely that other resistance mechanisms also contribute to the complex phenotype observed in CFZ-resistant MM. Expanding the understanding of these additional mechanisms will be crucial for developing comprehensive strategies to overcome resistance and improve treatment outcomes.

In summary, our findings identify hypomethylation of a downstream *ABCB1* promoter as a previously unrecognized but functionally important mechanism of carfilzomib resistance in MM. These results highlight DNA methylation as a potentially actionable target and support further exploration of epigenetic therapies to overcome PI resistance in MM.

Abbreviations

BTZ	Bortezomib
CFZ	Carfilzomib
CNV	Copy number variation
Dec	Decitabine
DMSO	Dimethyl sulfoxide
DNMTi	DNA methyltransferase inhibitor
FC	Fold change
FPKM	Fragments per kilobase of transcript per million mapped reads
GSK	GSK-3484862
IMiDs	Immunomodulatory drugs
IC_{50}	Half-maximal inhibitory concentration
KD	Knockdown
MM	Multiple myeloma
PI	Proteasome inhibitor
qPCR	Quantitative polymerase chain reaction
RNA-seq	RNA sequencing
SNV	Single nucleotide variant
STR	Short tandem repeat
SV	Structural variant
TPM	Transcript per million
WGBS	Whole-genome bisulfite sequencing
WGS	Whole-genome sequencing
WT	Wild-type

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-026-02115-y.

Additional file 1 (.xlsx). Whole-genome sequencing results of paired CFZ-sensitive and CFZ-resistant patient samples. CNVs (copy number varia-

tions called using GATK); SNVs (single nucleotide variants and small indels called with Strelka); SVs (structural variants called with Manta). Variants are listed after filtering against gnomAD (v.2.1.1) at >0.05% global population frequency.

Additional file 2 (.docx). Supplementary Information. Table S1 (clinical annotation for the independent MM subcohort, n = 13); Figure S1 (scatter plot of ABCB1 promoter methylation vs. expression in the independent MM subcohort); Figure S2 (DNMTi treatment results in ARH77WT cells); and Supplementary Methods.

Additional file 3 (.pdf). Uncropped Western blot images for Figure 4C showing ABCB1 and GAPDH protein expression in WT and CFZ-resistant MM cell lines.

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Author contributions
S.H., U.M., L.H., L.B., and K.M.K. conceived and designed the study. S.H., M.T., A.H., C.L., S.K., S.N., C.V., E.B., N.R., J.L., M.K., and L.B. performed the experiments and collected the data. S.H., M.T., C.V., Y.T., X.Z., T.S., T.H., L.R., A.B., L.B., J.W., and K.M.K. analyzed and interpreted the data. The manuscript was written by S.H., M.T., L.B., and K.M.K., and reviewed and approved by B.K., O.S., C.H., H.E., L.R., J.W., and C.D.

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Data availability
The anonymized methylation data from WGBS are available in the Zenodo repository with the dataset identifier (DOI: 10.5281/zenodo.18547283). The raw whole-genome sequencing (WGS), whole-genome bisulfite sequencing (WGBS), and RNA sequencing data generated from the patient samples are not publicly available in accordance with European General Data Protection Regulation (GDPR). Access to the raw genome-wide sequencing data can be provided upon reasonable request to the corresponding author, subject to ethical approval and a Data Transfer Agreement.

Declarations

Ethical approval and consent to participate
The study was approved by the independent Cantonal Ethical Committee (EKOS09/057) and by the Ethical Committee of Wuerzburg University (No. 8/21). All patients provided written informed consent.

Consent for publication
Not applicable. No identifiable patient data are presented in this study.

Competing interests
J.W. has received personal fees from GSK, Menarini-Stemline, Pfizer, Takeda, Sanofi, Oncopeptides, Janssen, and Skyline Dx, as well as research support from BMS. H.E. reports grant funding and other support from Sanofi, Takeda, Novartis, BMS, GSK, Amgen, and Janssen. C.H. is a shareholder in and employee of MLL Munich Leukemia Laboratory. L.R. has received personal fees from Sanofi, Amgen, GSK, Janssen, Pfizer, and BMS. K.M.K. reports personal fees from GSK, Takeda, AbbVie, Celgene, and BMS, and has also received both grants and personal fees from Janssen. All remaining authors declare no competing interests.

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