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# The role of the lysine histone methylase KMT2D in chronic myeloid leukemia

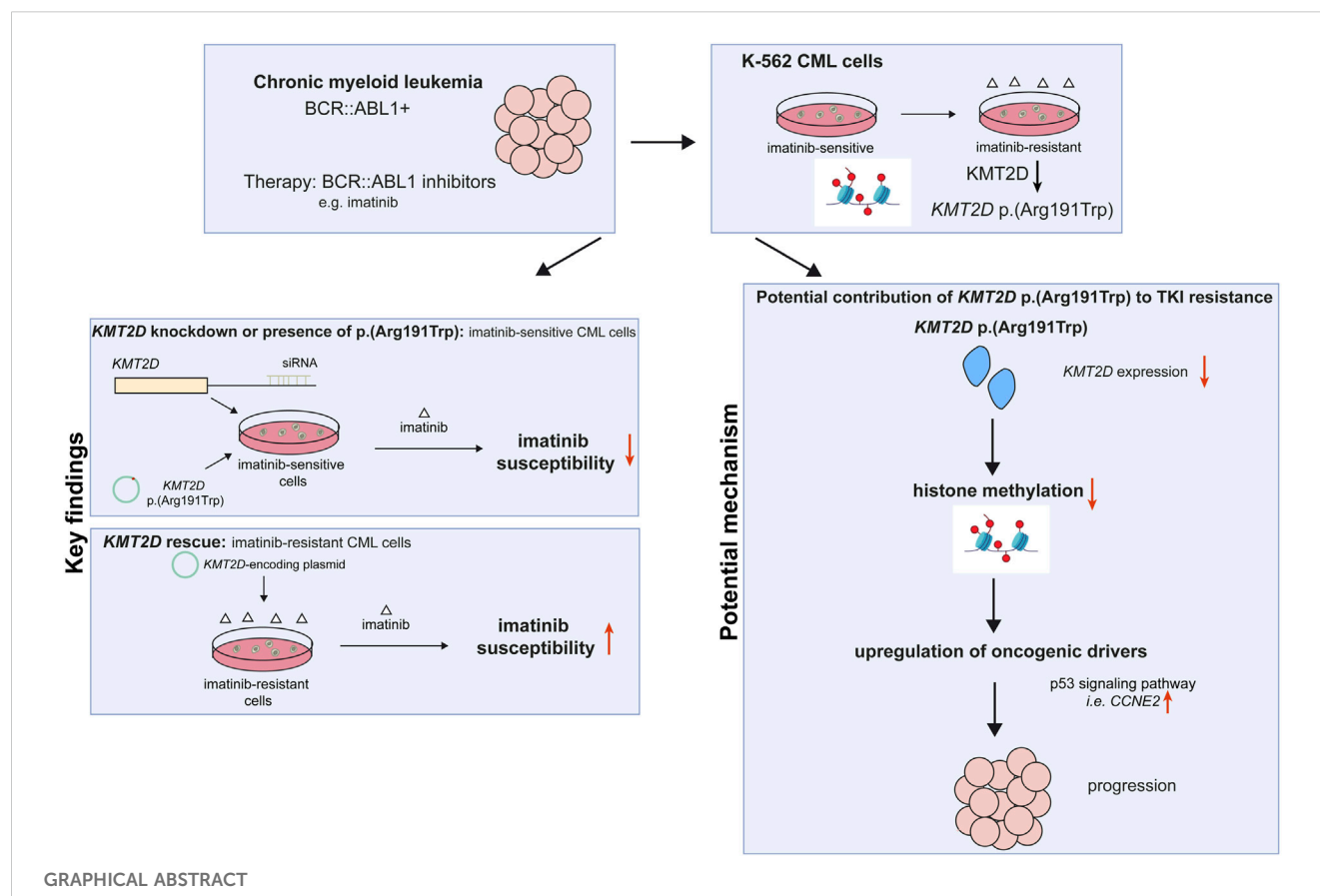
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Chronic myeloid leukemia (CML) can be effectively treated inhibiting the disease-causing BCR::ABL1 kinase by tyrosine kinase inhibitors (TKIs). Although therapy is initially tremendously successful, resistance may occur in up to 25% of CML patients. Besides aberrations in the BCR::ABL1 kinase domain, a variety of resistance mechanisms are currently discussed, among them epigenetic reprogramming. The histone-modifying enzyme lysine methyltransferase 2D (KMT2D/MLL2) belongs to the most frequently mutated genes in cancer and is also known for its association with hereditary Kabuki syndrome. However, its role in CML is widely unknown. In the present study, we analyzed the role of the *KMT2D* p. (Arg191Trp) variant in imatinib-resistant CML, which was recurrently acquired in imatinib resistance *in vitro*. siRNA-mediated *KMT2D* knockdown, but also introduction of the p. (Arg191Trp) variant into treatment-naïve K-562 cells led to impaired imatinib susceptibility visible by increased cell numbers, proliferation rates and metabolic activities under imatinib exposure ( $p < 0.001$ ). The effect of *KMT2D* p. (Arg191Trp) could be overcome by inhibiting histone demethylation with the demethylase inhibitor LSD1. In addition, rescue of *KMT2D* expression in imatinib-resistant cells reinstated the response to imatinib treatment. Furthermore, gene expression analysis revealed upregulation of *CCNE2* in cells harboring *KMT2D* p. (Arg191Trp) potentially explaining increase in cell proliferation under imatinib exposure. Overall, our findings demonstrate that the loss of the tumor suppressor *KMT2D* promotes TKI resistance in CML. Thus, *KMT2D* status could serve as an additional biomarker for TKI resistance, while restoration of its expression might be a therapeutic option to overcome this resistance.

## KEYWORDS

chronic myeloid leukemia, drug resistance, imatinib, KMT2D, epigenetics, histone modification



## 1 Introduction

Chronic myeloid leukemia (CML) is a rare hematopoietic neoplasm predominantly caused by reciprocal translocation t (9; 22) (q34; q11), resulting in the formation of the BCR::ABL1 fusion gene, which is considered as the hallmark of the disease (Nowell and Hungerford, 1960; Rowley, 1973). Since the development of tyrosine kinase inhibitors (TKIs), which inhibit the disease-causing BCR::ABL1 kinase and prevent downstream target phosphorylation, CML can be effectively treated (Druker et al., 1996). With an overall survival rate of 83%, the use of TKIs in CML became a role model for successful targeted therapy regimens (Hochhaus et al., 2017).

Nevertheless, up to 25% of patients undergoing TKI therapy suffer from TKI failure due to the development of TKI resistances within 5 years after therapy onset (Milojkovic and Apperley, 2009). Besides mutations in the BCR::ABL1 kinase, in particular in the kinase domain, TKI resistance can be caused by alternative signaling pathway activation, persistent leukemic stem cells or drug transporters (Bixby and Talpaz, 2011; Kaehler and Cascorbi, 2023). In addition, secondary driver gene mutations or epigenetic factors might lead to disease progression and/or drug resistance (Minciacchi et al., 2021).

There is increasing evidence that epigenetic modifiers play a role in TKI-resistant CML. For instance, inhibitors of histone deacetylases (HDAC), an enzyme class responsible for the removal of acetyl groups from histones, were considered to eradicate CML leukemic stem cells (Minciacchi et al., 2021). In

addition, differences in the methylation pattern and the expression of lysine methyltransferases, e.g. *EHMT1* or *EHMT2*, were observed in CML (Loh et al., 2014). Besides, histone-modifiers of the KMT2 (histone-lysine N-methyltransferase 2) family are also frequently associated with the development of cancer, in particular KMT2A/MML1 (mixed lineage leukemia 1), which rearrangement leads to an oncogenic fusion protein in acute lymphoblastic leukemia (Ford and Dingwall, 2015).

Within this KMT2 family, the mixed-lineage leukemia 2/histone lysine methyltransferase 2D (*MLL2/KMT2D*) gene encodes a large 5,537 aa protein involved in mono-methylation of histone H3K4, especially in enhancer regions, thereby being involved in transcriptional activation (Rao and Dou, 2015). In Kabuki syndrome, a rare developmental disorder with craniofacial malfunctions, growth delay, impaired immune system, kidney and heart function (Boniel et al., 2024), germline missense mutations in *KMT2D* can be detected in 56%–75% of cases (Bogershausen and Wollnik, 2013). Regarding somatic mutations, *KMT2* genes, especially *KMT2C* and *KMT2D*, were found to be among the most frequently mutated genes in cancer (Kandoth et al., 2013). Nonetheless, the role of *KMT2D* in CML is still widely unknown.

In an in vitro-cell line model of TKI resistance, we detected the recurrent *KMT2D* variant c.571C>T, p. (Arg191Trp) in imatinib-resistant cells by exome sequencing (Kaehler et al., 2023). This raised the question on the role of *KMT2D* and the effect of the observed *KMT2D* variant in imatinib resistant CML. Here, we analyzed the

role of *KMT2D* and the *KMT2D* variant p. (Arg191Trp) and further epigenetic modifiers using an in vitro-imatinib resistance model providing new insights into the role of *KMT2D* in TKI-resistant CML.

## 2 Materials and methods

### 2.1 Reagents, cell lines, and generation of resistant cells

Cell experiments were performed using K-562 cells (RRID: CVCL\_0004), a cell line derived from a 53-year-old female CML patient in blast crisis (Lozzio and Lozzio, 1975) provided by the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cells were maintained as previously described (Turrini et al., 2012; Kaehler et al., 2022). Imatinib-resistant replicates were obtained by exposing treatment-naïve K-562 cells to increasing concentrations of imatinib, resulting in cells resistant to 0.5  $\mu$ M and 2  $\mu$ M imatinib.

### 2.2 RNA and DNA extraction

RNA extraction was performed using E. Z.N.A total RNA Kit I (Omega bio-tek, Norcross, Georgia, United States) following the manufacturer's instructions with the added step of centrifuging the cell lysate within QIAshredder homogenizers (Qiagen, Hilden, Germany) for 1 min at 10,000  $\times$  g after exposure to the lysis buffer to enhance RNA extraction. DNA extraction was performed using the Gentra Puregene Kit (Qiagen).

### 2.3 Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Reverse transcription of 1  $\mu$ g RNA was conducted with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Darmstadt, Germany) according to the manufacturer's protocol. RT-qPCR was performed with the QuantStudio 7 Flex (Thermo Fisher Scientific) applying default cycling conditions. Samples were examined in triplicates using the TaqMan Universal Master Mix without UNG (Thermo Fisher Scientific) and the following TaqMan assays obtained from Thermo Fisher Scientific: *KMT2D* (Hs00912419\_m1), *CDK4* (Hs00364847\_m1), *CCND3* (Hs00236949\_m1), *CCNE2* (Hs00180319\_m1), *TBP* (Hs00427620\_m1), *GAPDH* (Hs02786624\_g1), *18S* (Hs99999901\_s1). The cycle threshold (CT) value of the target genes were normalized to the housekeeping genes *TBP*, *GAPDH* and *18S* with relative mRNA expression being calculated as  $2^{-\Delta\Delta CT}$  (Livak and Schmittgen, 2001).

### 2.4 In-depth-sequencing

Amplicons of the *KMT2D* gene were generated using the AmpliTaq Gold 360 Mastermix (Thermo Fisher Scientific) and the primers 5'-GATGTCCATGGCTTTACCACTTCCCCTGC-3'

and 5'-AAAGCCATGGACATCCAGGTGAGCGG-3' (obtained from Merck, Darmstadt, Germany) with an annealing temperature of 58 °C and an elongation time of 7 min. The PCR products were purified using the GeneJET Gel extraction Kit (Thermo Fisher Scientific). Next-Generation Sequencing was performed using the Nextera XT Sequencing kit (Illumina, San Diego, California, United States) adhering to the manufacturer's protocol as previously described (Kaehler et al., 2021; Kaehler et al., 2023).

### 2.5 Cloning and plasmids

The *KMT2D*-encoding plasmid was provided by Promega (Cat# FHC12732, Madison, Wisconsin, United States) and the plasmid harboring the *KMT2D* p. (Trp191Arg) variant was obtained by mutagenesis at GenScript (Rijswijk, Netherlands). The empty pFN21A vector was obtained through restriction enzyme cloning with AsiSI and PmeI (both New England Biolabs). Plasmid DNA was isolated using PureYield Plasmid Multiprep System (Promega) or NucleoBond Xtra Midi Kit (Macherey Nagel GmbH).

### 2.6 Transient transfection

Transient transfection was performed with  $4 \times 10^6$  cells using the Amaxa Cell Line Nucleofector Kit V (Lonza, Basel, Switzerland) with the Nucleofector I device (Lonza) following the manufacturer's recommendation for K-562 cells. After respective incubation periods, cell seeding was carried out to investigate cell viability under exposure to 2  $\mu$ M imatinib in cellular fitness assays as described below. For the *KMT2D* knockdown, treatment-naïve cells were transfected with 200 nM Ambion MLL2 Silencer siRNA (Cat# AM51331) or negative control #1 siRNA (Cat# AM4611) with subsequent cell seeding after an incubation period of 6 h. Imatinib-resistant cells were transfected with 10  $\mu$ g of a *KMT2D*-encoding plasmid or empty pFN21A vector followed by cell seeding after 1 h incubation. Furthermore, treatment-naïve K-562 cells were transfected with 10  $\mu$ g of a plasmid harboring the *KMT2D* p. (Trp191Arg) variant, *KMT2D* wild-type or the empty pFN21A vector as negative control. Cells were seeded 24 h after transfection. The cells were additionally exposed to 100  $\mu$ M LSD1 inhibitor or DMSO as a solvent control.

### 2.7 Cellular fitness assays

Cells were seeded into 12-well plates with  $1 \times 10^6$  cells/mL for the Ki-67 assay as well as immunoblotting, whereas 96-well plates were used to determine cell numbers with  $2 \times 10^5$  cells/200  $\mu$ L medium and metabolic activity with  $5 \times 10^4$  cells/100  $\mu$ L medium. Cells were exposed to either 2  $\mu$ M imatinib or medium and incubated at 37 °C. To determine cell numbers, the cell suspension was mixed with trypan blue (Sigma Aldrich) to mark viable, unstained cells, which were then quantified with a Fuchs-Rosenthal cell counting chamber after 24 and 48 h. Metabolic activity was measured using the WST assay (Merck) as previously described (Kaehler et al., 2017).

Cell proliferation was determined 24 h after transient transfection using the Human Antigen Ki-67 ELISA Kit (Cat# MBS764543, MyBioSource, San Diego, California, United States) with 10 µg of protein according to the manufacturer's protocol. To determine the influence of imatinib on cell viability, the results of cells treated with imatinib were normalized to treatment-naïve cells.

## 2.8 Whole-cell lysates and immunoblotting

Cell lysis and immunoblots were performed as described elsewhere (Kaehler et al., 2017; Waetzig et al., 2019; Bruhn et al., 2020). Using 15% v/v polyacrylamide gels, 20 µg of protein were transferred onto nitrocellulose membranes and membranes were probed with the following antibodies: Histon H3: Cat# sc-517576 (Santa Cruz, Dallas, Texas, United States), RRID: AB\_2848194, 1:250; H3K4me1: Cat# 710795-20UG (Thermo Fisher Scientific), RRID: AB\_2848515, 1:1,000; HSP90: Cat# 4877 (Cell Signaling Technology, Danvers, Massachusetts, United States), RRID: AB\_2233307, 1:1,000; anti-mouse: Cat# 926-68070, RRID: AB\_10956588, Cat# 926-32210, RRID: AB\_621842; anti-rabbit: Cat# 926-68071, RRID: AB\_10956166, Cat# 926-32211, RRID: AB\_621843; all 1:10,000, LiCOR (Bad Homburg, Germany). Primary antibodies were diluted with the Intercept TBS Blocking Buffer supplemented with 0.2% v/v Tween20, whereas secondary antibodies were diluted in TBS with 0.1% v/v Tween20.

## 2.9 Inhibition assays

Inhibition experiments were conducted in 96-well plates with triplicates of  $5 \times 10^4$  cells/100 µL medium supplemented with 2 µM imatinib with DMSO as solvent control. LSD1 was inhibited using 1–200 µM LSD1 Inhibitor II (S2101, Merck Millipore, United States). Furthermore, 1–100 µM of the histone-deacetylase (HDAC) inhibitor vorinostat (Cat# SML0061, Merck) and 0.1–250 µM of the DNA-methyltransferase (DNMT) inhibitor 5'-azacytidine (Hözel Diagnostika, Köln, Germany) were used. After an incubation of 48 h at 37 °C, metabolic activity was measured as described above. IC50 values were calculated by non-linear regression with variable slope (four parameters) for  $N = 3$  including at least six concentrations.

## 2.10 Meta-analyses of exome sequencing and genome-wide gene expression data

Exome sequencing data of TKI-resistant biological replicate cell lines was obtained from the European Nucleotide Archive (ENA), accession number PRJEB60565. *KMT2D* variants were identified as previously described (Kaehler et al., 2023). In silico prediction of the variant effect was performed using gnomAD (gnomad.broadinstitute.org). Genome-wide gene expression data was derived from the GEO datasets GSE227347 and GSE203342 as previously published (Kaehler et al., 2022; Kaehler et al., 2023). Comparing treatment-naïve and imatinib-resistant cell lines, genes with a fold change  $\pm 2$  and a false discovery rate (FDR)-corrected p-value  $p < 0.05$  were considered to be differentially expressed. Venn

diagrams for the comparison of these differentially expressed genes with the *KMT2D* essentiality network (Takemon et al., 2024) were obtained using the PNNL software (omics.pnl.gov (Oliveros, 2007)). KEGG pathway prediction was performed using DAVID Functional Annotation Tool (DAVID Bioinformatics Resources (Huang et al., 2009; Sherman et al., 2022)) and interaction networks using the STRING database (string-db.org, Version 12.0 with medium confidence).

## 2.11 Software and statistical analysis

Primers were designed with the NCBI primer design tool (National Center for Biotechnology Information, Bethesda, Maryland, United States). Unless indicated otherwise, statistical analyses were performed using student's t-tests or One-way ANOVA followed by Dunnett's tests to examine multiple comparisons with the GraphPad Prism software (Version 10.2.3 for Windows, San Diego California, United States). For all experiments, data from at least three replicates was analyzed. Results were considered as statistically significant with a p-value  $< 0.05$ .

## 3 Results

### 3.1 *KMT2D* expression and presence of p.Arg191Trp in imatinib-resistant CML

First, genetic variants contributing to imatinib resistance in an in vitro-K-562 CML cell line model were analyzed by exome sequencing. In two out of seven imatinib-resistant biological replicate cell lines that did not harbor BCR::ABL1 mutations, the *KMT2D* variant p. (Arg191Trp) (NM\_003482) was recurrently detected with allele frequencies of 27% and 37%, respectively ((Kaehler et al., 2023), unpublished data, Figure 1A), while this variant was not detected in treatment-naïve K-562 cells. The presence of this variant was confirmed in these two cell lines by in-depth sequencing, as it was present in 43% and 52% of cells resistant to low dose imatinib (0.5 µM) and 43% and 56% cells resistant against high dose imatinib (2 µM, Figure 1A). This raised the question on the role of this gene and this particular *KMT2D* variant in imatinib resistance. In silico prediction revealed a CADD score of 29.6 and a PolyPhen score of 0.999 indicating a detrimental effect on *KMT2D* protein function. Thus, the *KMT2D* mRNA expression was analyzed and found to be significantly downregulated in both imatinib-resistant cell lines harboring the *KMT2D* p. (Arg191Trp) variant compared to treatment-naïve cells (R1: 2 µM IM: -33.3%,  $p = 0.03$ ; R2: 0.5 µM IM: -15.1%,  $p = 0.03$ ; 2 µM IM: -33.9%,  $p < 0.001$ , Figure 1B). In imatinib-resistant cell line replicates not carrying *KMT2D* variants, *KMT2D* mRNA was not differentially reduced, but even significantly upregulated in one replicate (Supplementary Figure S1). As *KMT2D* regulates methylation of H3K4, protein levels of histon 3 (H3) and its methylated form H4K4me1 were investigated, but did not reveal significant changes in the methylation between treatment-naïve and *KMT2D*-variant imatinib-resistant cells (Figure 1C).

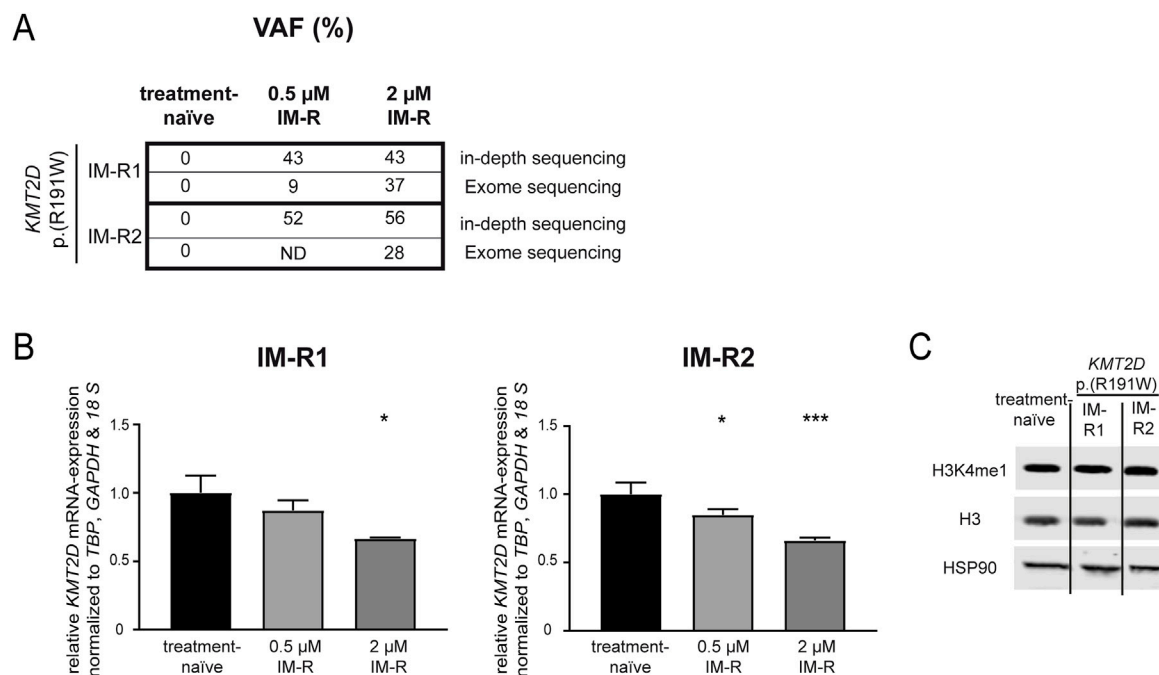


FIGURE 1

*KMT2D* p.(Arg191Trp) variant acquisition and expression in imatinib resistance. (A) Variant allele frequencies (VAF) of the *KMT2D* p.(Arg191Trp) variant in imatinib-resistant cell lines determined by exome or in-depth sequencing of K-562 cells resistant against 0.5 or 2  $\mu$ M imatinib. (B) *KMT2D* mRNA expression in imatinib-resistant cells harboring *KMT2D* p.(Arg191Trp) measured by RT-qPCR and normalized to *TBP*, *GAPDH* and *18 S* and treatment-naïve cells. (C) Protein levels of H3K4me1, Histone 3 (H3) and HSP90 in treatment-naïve and imatinib-resistant cells harboring *KMT2D* p.(Arg191Trp). Statistical analyses were performed using One-way ANOVA followed by Dunnett's tests. Error bars indicate standard deviation. N = 3. \*:  $p < 0.05$ , \*\*\*:  $p < 0.001$ . IM, imatinib; ND, no data; R, resistant.

### 3.2 Knockdown of *KMT2D* expression impairs the response to imatinib

In a next step, the effect of *KMT2D* downregulation on imatinib susceptibility was analyzed by a siRNA-mediated knockdown. After successful knockdown of *KMT2D* ( $p = 0.02$ , Figure 2A), the cells were exposed to imatinib and cellular fitness was investigated. A significant increase in the cell number (90.6%,  $p < 0.001$ ), metabolic activity (23.9%,  $p < 0.001$ ) and proliferation rates (Ki-67-expression: 2.0-fold,  $p = 0.01$ ) in the *KMT2D* knockdown was observed compared to negative control-transfected cells (Figures 2B–D). In addition, methylation of H3K4 was analyzed after silencing of *KMT2D*, revealing a slight decrease in H3K4me1 compared to Histone 3 and HSP90 levels (Figure 2E). This indicates that CML cells benefit from the loss of *KMT2D* expression under imatinib exposure, while histone methylation is reduced.

### 3.3 Rescue of *KMT2D* expression in imatinib-resistant CML cells restores imatinib susceptibility

As *KMT2D* expression was significantly downregulated in imatinib-resistant cell lines harboring the p.(Arg191Trp) variant, we were interested whether restoration of its expression by transfection of a *KMT2D*-encoding plasmid in these cell lines would increase imatinib-sensitivity (both:  $p < 0.001$ , Figure 3A). After *KMT2D* rescue, exposure to imatinib led to a reduction of cell

numbers (IM-R1:  $-46.6\%$ ,  $p < 0.001$ , IM-R2:  $-41.2\%$ ,  $p < 0.001$ ) and metabolic activities (IM-R1:  $-12.9\%$ ,  $p = 0.02$ , IM-R2:  $-15.7\%$ ,  $p = 0.02$ ) compared to the empty vector control transfection. These findings indicate a restored susceptibility towards imatinib in both resistant cell lines (Figure 3B,C). However, proliferation rates were only significantly reduced in IM-R1 ( $-29.7\%$ ,  $p = 0.03$ , Figure 3D). In addition, analysis of H3K4 methylation did not reveal any changes (Figure 3E).

### 3.4 *KMT2D* p.(Arg191Trp) impairs the response to imatinib

Our previous findings suggested that the absence of *KMT2D* would be favorable for the development of imatinib resistance. In addition, it also pointed to a detrimental effect of the p.(Arg191Trp) variant on the *KMT2D* protein function. However, as the *KMT2D* variant's effect on the protein function was still unclear, transfection experiments were performed to compare H3K4-methylation, cell numbers, proliferation and metabolic activity of either *KMT2D* wild-type or p.(Arg191Trp) in treatment-naïve K-562 cells (WT:  $p < 0.001$ ; p.(R191W):  $p = 0.003$ , Figure 4A). In the presence of the *KMT2D* variant, methylation of H3K4 was slightly decreased compared to the *KMT2D* wild-type (Figure 4B). Under imatinib treatment, the presence of *KMT2D* p.(Arg191Trp) led to a significant increase in cell number compared to wild-type *KMT2D* (93%,  $p < 0.001$ , Figure 4C).



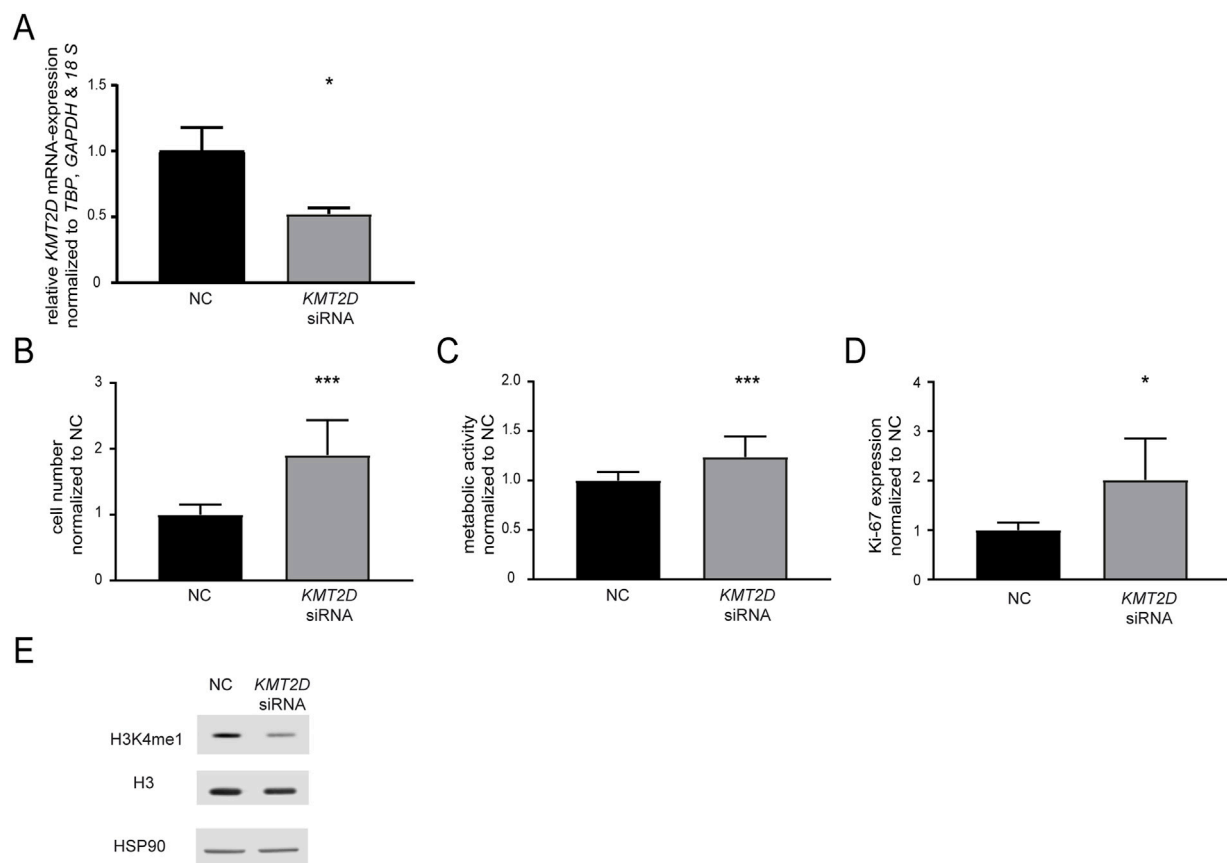


FIGURE 2

Knockdown of *KMT2D* hampers the response to imatinib treatment. (A) *KMT2D* mRNA expression after transfection with an *KMT2D*-targeting siRNA or siRNA negative control (NC) analyzed by RT-qPCR and normalized to *TBP*, *GAPDH*, *18 S* and NC. (B–D) Cell fitness after *KMT2D* knockdown under treatment with 2  $\mu$ M imatinib analyzed on the level of (B) cell numbers (C) metabolic activity and (D) Ki-67 expression. (E) Protein levels of H3K4me1, histone H3 and HSP90 after knockdown of *KMT2D*. N = 3. Statistical analyses were performed using student's tests. Error bars indicate standard deviation. N = 3. \*:  $p < 0.05$ , \*\*\*:  $p < 0.001$ .

In addition, metabolic activities (23%,  $p < 0.001$ ) and proliferation rates (19.7%,  $p = 0.04$ ), were also significantly increased under imatinib treatment (Figures 4D,E). These data confirm that the *KMT2D* p. (Arg191Trp) variant augments the development of imatinib resistance in CML.

To restore the reduced methylation of H3K4 caused by the potential loss-of-function *KMT2D* p. (Arg191Trp) variant, the lysine-specific histone demethylase (LSD1) counteracting *KMT2D* function was inhibited. As expected, methylation of H3K4 was slightly increased under treatment with the LSD1 inhibitor in cells carrying the *KMT2D* variant (Figure 5A). Subsequently, the effect on imatinib susceptibility in these cells was analyzed. LSD1 inhibition led to a significant decrease in cell number (–26.7%,  $p < 0.001$ ), metabolic activity (–58.4%,  $p = 0.007$ ) and proliferation (–52.2%,  $p < 0.001$ , Figure 5B). Moreover, to assess whether LSD1 inhibition alters imatinib sensitivity in the context of the *KMT2D* variant, we compared the IC<sub>50</sub> values of imatinib in the presence of the LSD1 inhibitor. Cells harboring the *KMT2D* variant exhibited a significantly higher IC<sub>50</sub> compared to wild-type (imatinib IC<sub>50</sub>: 60.7  $\mu$ M vs. 72.4  $\mu$ M, 19%,  $p = 0.004$ , Figure 5C) indicating a reduced imatinib susceptibility in the presence of the *KMT2D* variant.

### 3.5 Epigenetic modulators in imatinib resistance and the response to imatinib

To get a deeper insight into mechanisms underlying the loss of imatinib-susceptibility in presence of *KMT2D* p. (Arg191Trp), genome-wide expression data from imatinib-resistant cells harboring *KMT2D* wild-type or p. (Arg191Trp) were obtained and compared to imatinib-sensitive K-562 cells ((Kaehler et al., 2022; Kaehler et al., 2023); GSE227347, GSE203342). These expression profiles were compared with the *KMT2D* essentiality network, a list of 1954 genes from Takemon et al. (Takemon et al., 2024), to identify potential genes targeted by *KMT2D*. In *KMT2D* wild-type imatinib-resistant cells, 51 genes were detected, while in *KMT2D* variant cells 197 genes were found (Figure 6A). By subsequent KEGG pathway cluster analysis, an enrichment with genes involved in the p53 signaling pathway was detected (Figure 6B), also showing an interaction of the respective genes in the STRING annotation (Figure 6C). These upregulated genes were the cyclins *CCND3* (3.2-fold) and *CCNE2* (4.3-fold) and the cyclin-dependent kinase *CDK4* (2.2-fold enriched in the imatinib-resistant *KMT2D* variant cells). As these genes are putative indirect interaction partners of *KMT2D* via the chromatin-remodeling

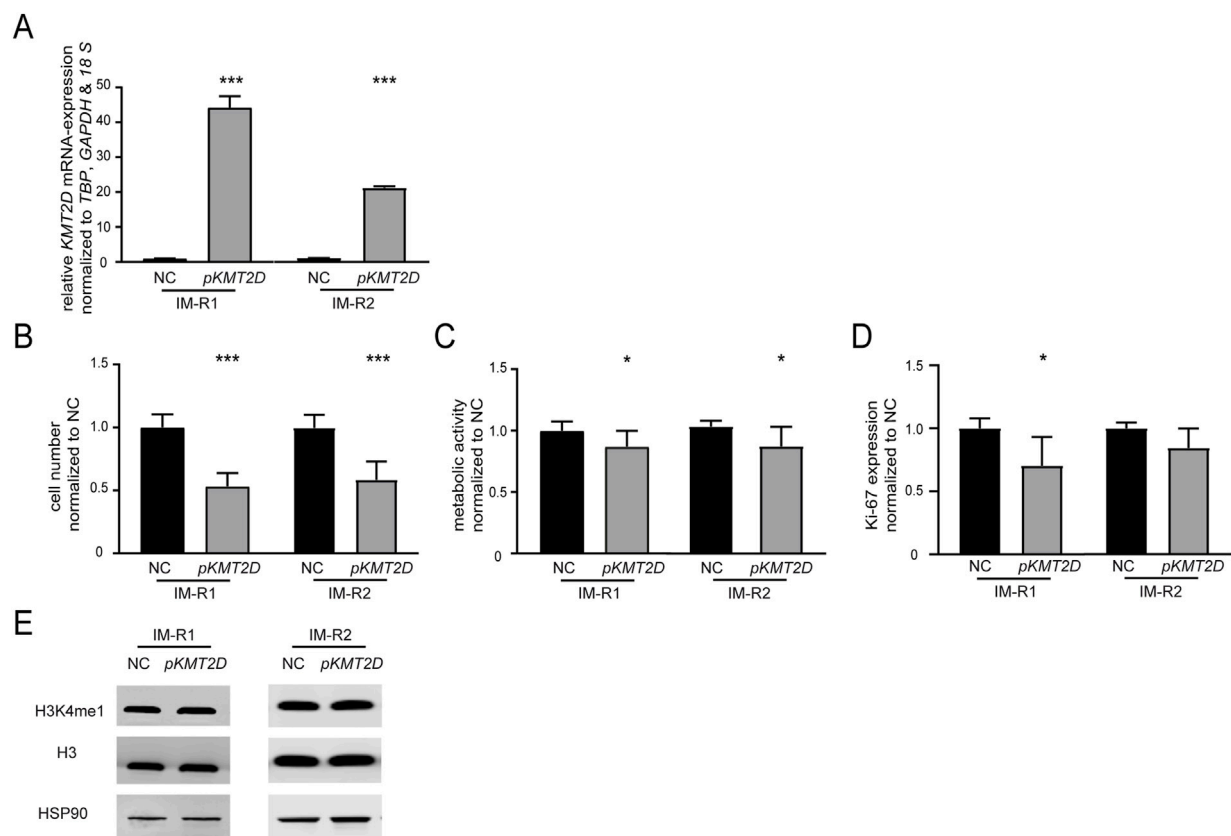


FIGURE 3

Restoration of *KMT2D* expression improves the response to imatinib in imatinib-resistant cells. (A) *KMT2D* mRNA expression after rescue of *KMT2D* in two imatinib-resistant cell lines harboring *KMT2D* p. (Arg191Trp) analyzed by RT-qPCR. Data was normalized to *TBP*, *GAPDH*, *18 S* and the respective empty vector negative control transfection (NC). (B–D) Cell fitness after rescue of *KMT2D* expression analyzed by (B) cell numbers, (C) metabolic activity and (D) Ki-67 expression after imatinib exposure. (E) Protein levels of H3K4me1 and histone H3 compared to HSP90 after restoration of *KMT2D* expression. Statistical analyses were performed using student's tests. Error bars indicate standard deviation. N = 3. \*:  $p < 0.05$ , \*\*\*:  $p < 0.001$ . NC: negative control.

complex protein ARID1A or the co-activator of transcription CREBBP, the question arose if their expression is influenced by the *KMT2D* variant. In subsequent analysis of mRNA expression levels, upregulation of *CCNE2* (2.2-fold,  $p < 0.001$ ) in imatinib-resistant cells harboring the *KMT2D* variant compared to treatment-naïve cells was confirmed, while expression of *CCND3* and *CDK4* was not altered (Figure 6D). These findings indicate that proliferation of cells harboring the *KMT2D* variant could be mediated by upregulation of *CCNE2*.

Besides *KMT2D*, deregulation of other epigenetic factors in imatinib-resistant CML was analyzed. Thus, genome-wide gene expression from imatinib-resistant and treatment-naïve K-562 biological replicate cell lines harboring *KMT2D* wild-type or p. (Arg191Trp) derived from the GSE203342 and GSE227347 datasets were compared and filtered for significant deregulation of genes encoding epigenetic modulators and histones. The number of differentially expressed genes varied between four and 54 between *KMT2D* wild-type and variant (Figure 7A). As *DNMT* or *HDAC* genes were differentially deregulated between *KMT2D* wild-type or variant imatinib-resistant cells, this raised the question on the efficiency of epigenetic modulation in the presence and absence of the

*KMT2D* variant. Thus, the cell lines were exposed to the *DNMT* inhibitor 5'-azacytidine or the *HDAC* inhibitor vorinostat. Under treatment with 5'-azacytidine, the imatinib IC<sub>50</sub> was significantly reduced in *KMT2D* p. (Arg191Trp) cells compared to wild-type cells (~60%,  $p = 0.04$ , Figure 7B), while the *HDAC* inhibitor vorinostat did not alter the response to imatinib (Figure 7C).

## 4 Discussion

In the present study, the role of *KMT2D* and its variant p. (Arg191Trp) were analyzed in imatinib-resistant CML cell lines *in vitro*. We found that the *KMT2D* variant was recurrently acquired in imatinib resistance, while its expression was reduced in the respective cell lines. Applying transfection experiments, it could be confirmed that the presence of the variant promotes imatinib resistance, which could be mimicked by siRNA-mediated knockdown of *KMT2D* expression. In addition, in resistant cells the imatinib susceptibility could be restored by rescue of *KMT2D* expression.

For our study, we used concentrations of 0.5 and 2  $\mu$ M imatinib to study the effects of *KMT2D* and its variant on imatinib

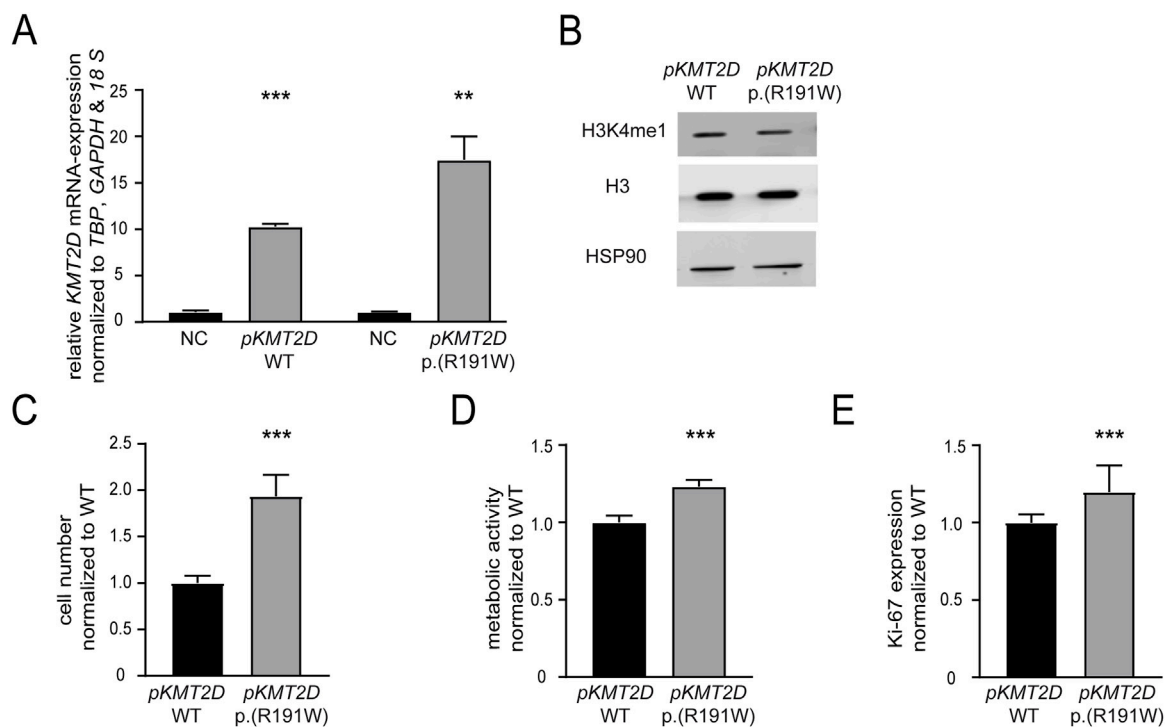


FIGURE 4

The presence of the *KMT2D* p.(Arg191Trp) variant promotes imatinib resistance. Transfection of treatment-naïve K-562 cells with *KMT2D* wild-type (WT) or p. (Arg191Trp). (A) RT-qPCR of *KMT2D* compared to *TBP*, *GAPDH*, *18 S* and normalized to the respective empty vector negative control (NC). (B) Protein levels of H3K4me1 and histone H3 compared to HSP90. (C–E) Cell fitness after transfection of *KMT2D* WT and p. (Arg191Trp) under imatinib treatment. (C) Cell numbers, (D) metabolic activity and (E) Ki-67 expression after exposure to 2  $\mu$ M imatinib. Statistical analyses were performed using student's tests. Error bars indicate standard deviation. N = 3. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ . NC: negative control, *pKMT2D*: plasmid encoding *KMT2D* wild-type or p. (Arg191Trp).

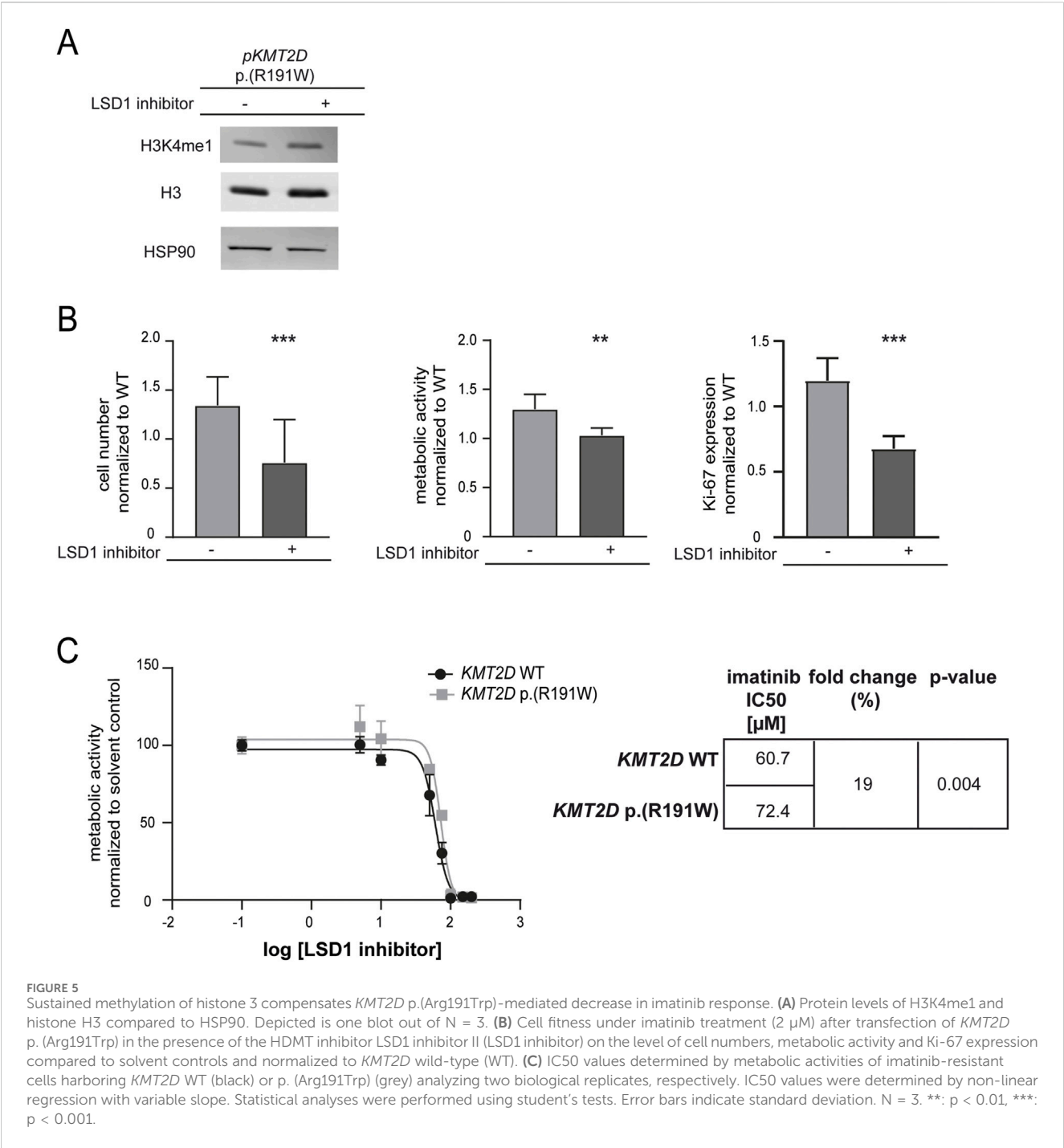
susceptibility and resistance. These concentrations reflect the range of 0.3 and 3.4  $\mu$ M determined in plasma of CML patients undergoing imatinib therapy. The ideal plasma concentration is 1  $\mu$ g/mL (1.7  $\mu$ M) (De Kogel and Schellens, 2007; Picard et al., 2007). This dose range also reflects the facts that variable biotransformation or comorbidities lead to interindividual differences in plasma concentrations and that distribution into deeper body compartments, such as the bone marrow is lower (Leveque and Maloisel, 2005; Peng et al., 2005; Cortes et al., 2009).

Besides *KMT2C*, *KMT2D* belongs to one of the most frequently mutated genes in cancer and is considered as a tumor suppressor gene displaying negative effects on cell growth (Kandoth et al., 2013; Lawrence et al., 2014). A study from Liu et al. revealed somatic *KMT2D* mutations in about 19% of patients suffering from diffuse large B cell lymphoma (DLBCL) promoting tumor progression (Liu Q. X. et al., 2024). In addition, in 16% of cases with childhood medulloblastoma, *KMT2D* deficiency was found with the majority being protein alternating missense mutations or truncations (Parsons et al., 2011). Also, in head and neck squamous cell carcinoma (HNSCC), *KMT2D* was identified as a tumor suppressor gene promoting cell growth through increasing glycolysis (Liu W. et al., 2024). In general, heterozygote loss of *KMT2D* has not been considered as the initial disease-causing driving force of cancer, as studies have not revealed spontaneous tumor

formation after deletion of the gene (Rao and Dou, 2015). However, loss of *KMT2D* might be linked to tumor progression or development of therapy resistance, as observed here.

The *KMT2D* protein contains of N-terminal two plant homology domain (PHD) cluster and a C-terminal SET domain (Froimchuk et al., 2017). According to Rao et al., pathogenic *KMT2D* mutations mainly affect the SET domain (37.0%) and the PHD domains (60%) and in most cases result in protein altering truncations (Rao and Dou, 2015). Regarding the *KMT2D* variant c.571C>T, p. (Arg191Trp) recurrently detected here, this particular variant (s1555198522) is considered to be associated with hereditary Kabuki syndrome (Bogershausen and Wollnik, 2013; Boniel et al., 2024). However, knowledge about this variant in oncogenic diseases is still missing. According to UniProt, the p. Arg191 residue is located in the histone-binding PHD-type 1 zinc-finger motif, which ranges from the amino acids 170 to 218 (UniProt, 2025). Thus, amino acids exchanges in this region could likely affect the function of the PHD domain and thereby lead to loss-of-function, also indicated by the respective variant prediction scores. However, for the same residue, the c.572G>A, p. (Arg191Gln) variant, which results in an amino acid exchange to glutamine (rs548930191), the effect on the *KMT2D* protein seem to be benign (Kopanos et al., 2019). In the present study, we demonstrate that *KMT2D* p. (Arg191Trp) was acquired in an

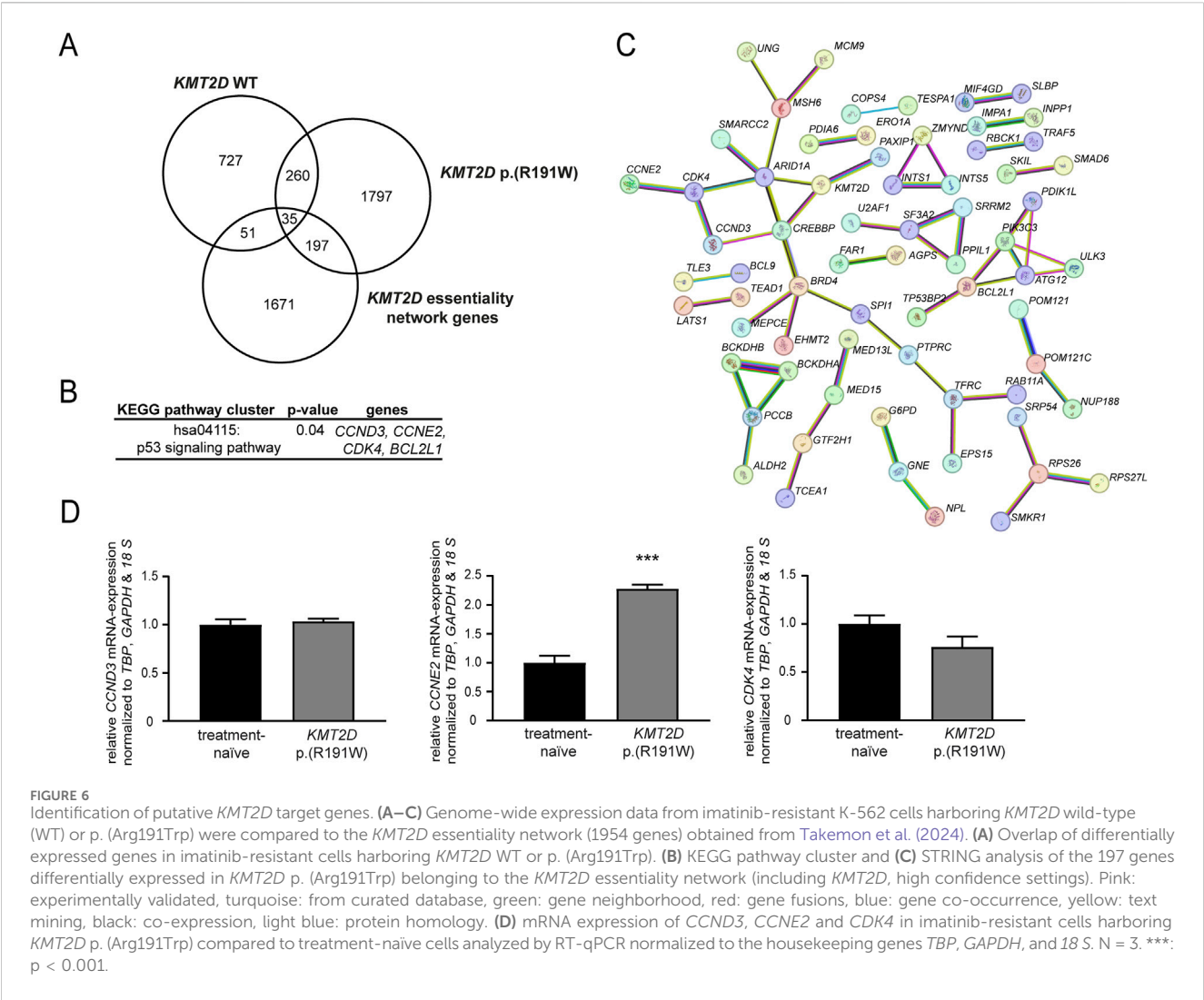




imatinib-resistant CML cell line and its introduction into treatment-naïve K-562 cells displayed similar effects as siRNA-mediated *KMT2D* downregulation impairing the response to imatinib. In addition, mono-methylation of H3K4 was reduced in both cases, while sustained methylation by inhibition of H3K4-methylation restored the response to imatinib in the presence of *KMT2D* p.(Arg191Trp). These findings indicate *KMT2D* p.(Arg191Trp) as a loss-of-function variant affecting *KMT2D* protein function. In CML, *KMT2D* (but also *KMT2C*) downregulation was found to correlate with the CML phases, and thus, disease progression, but also with the response of CML

patients (Rabello et al., 2018). These findings stand in line with our present in vitro-study, where the response to imatinib could be improved by rescue of *KMT2D* expression in imatinib-resistant cells.

To analyze the genes affected by the *KMT2D* p.(Arg191Trp) variant, genome-wide expression changes in imatinib-resistant cells harboring the variant were analyzed and compared to the *KMT2D* essentiality network (Takemon et al., 2024). Of the 197 genes detected from this network, four were enriched in the p53-signaling pathway. However, only upregulation of cyclin E2 (*CCNE2*) expression could be confirmed by RT-qPCR. As a cell

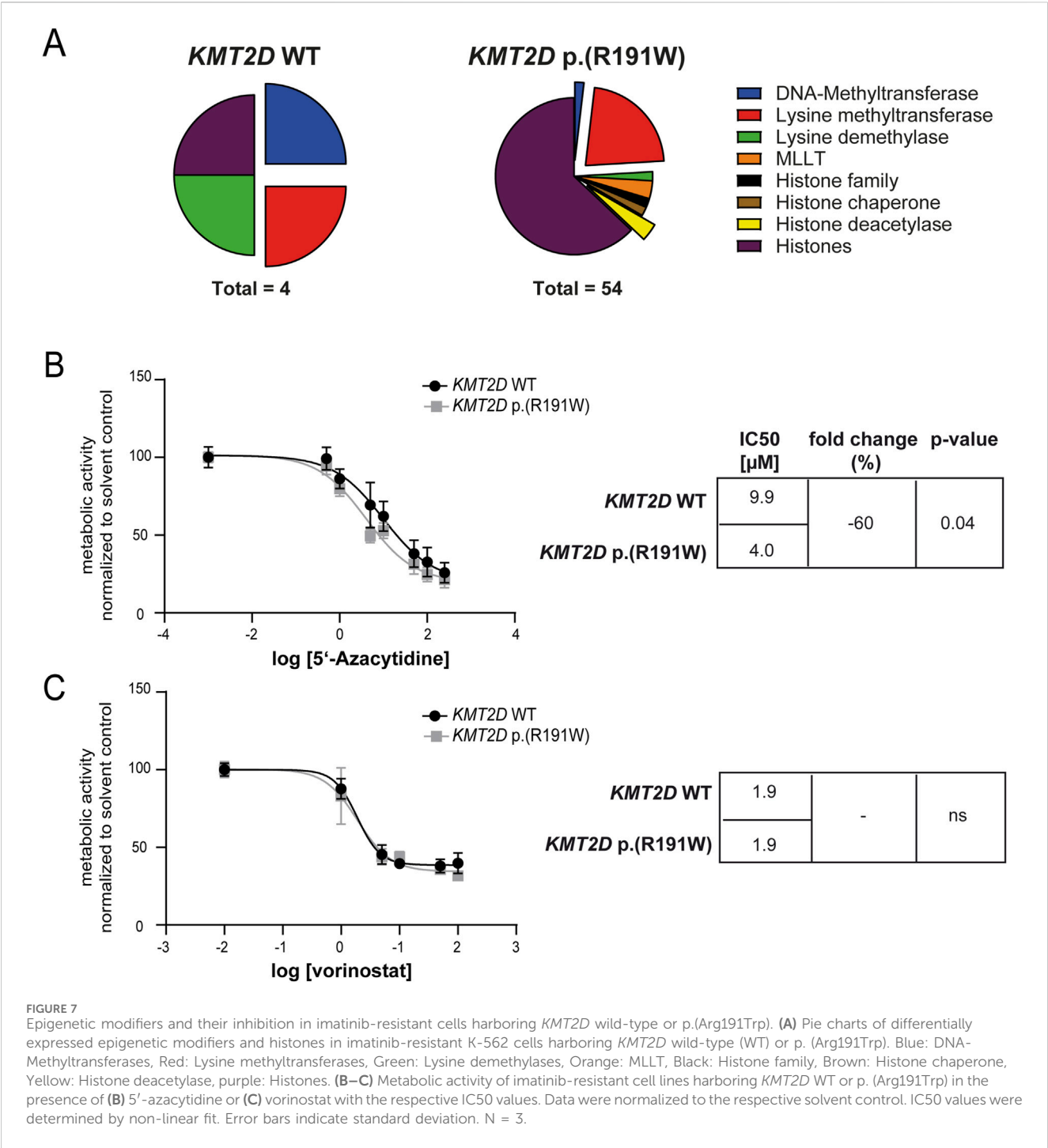


cycle progressor, *CCNE2* has been shown to promote proliferation of cancer cells, as demonstrated, e.g. in prostate or ovarian cancer (Xie et al., 2017; Liu et al., 2020; Fagundes and Teixeira, 2021). The upregulation of *CCNE2* in cells harboring *KMT2D* p. (Arg191Trp) with loss of *KMT2D* function potentially promotes the development of resistance against imatinib in CML. However, to provide more insights into this, the *KMT2D* network of treatment-naïve CML cells expressing *KMT2D* wildtype or p. (Arg191Trp) needs to be analyzed to exclude potential effects due to adaptations of gene expression in TKI resistance. Overall, our data as well as in silico-analyses indicate that the *KMT2D* p. (Arg191Trp) variant results in a loss-of-function of the protein. As a potential result, the histone methylation of target genes, among them genes of the p53 signaling pathway, is decreased. This leads to their upregulation, as demonstrated, e.g. for *CCNE2*, and subsequently, CML progression and the development of TKI resistance.

Thus, the question arises if epigenetic modulators would be beneficial to overcome TKI resistance in CML. In our study, inhibition of DNMTs by 5'-azacytidine led to a slight increase in imatinib susceptibility in cells harboring *KMT2D* p. (Arg191Trp), while inhibition of HDACs by vorinostat did

not display any effects (regardless from the presence of *KMT2D* mutations or deregulation of other epigenetic modifiers). This indicates DNMT inhibition as potential strategy to overcome TKI resistance. In a study on CML evolution, it has been demonstrated that epigenetic reprogramming and aberrant DNA methylation contributes to CML progression (Amabile et al., 2015; Bugler et al., 2019). Thus, it was shown that the concomitant use of decitabine and imatinib may result in improved TKI responses (San Jose-Eneriz et al., 2009). It has been demonstrated that low-dose decitabine can be effective in CML taking advantage of its demethylating properties, while reducing cytotoxicity (Yang et al., 2006). In addition, other TKI combinations with BCL-2- inhibitors, e.g. venetoclax, or HDAC inhibitors, e.g. panobinostat, were experimentally tested and showed promising results (Amir and Javed, 2021).

A limitation of the present study is the fact that these findings are based on an in vitro-model of imatinib-resistant CML cells. While the *KMT2D* p. (Arg191Trp) variant has been recurrently detected in biological replicates of imatinib resistance, their occurrence needs to be further evaluated in a clinical study in CML patients. Regarding the efficacy of downstream target



inhibition, e.g. CCNE2 inhibitors, to overcome imatinib resistance, further studies are necessary to address the role of *KMT2D* in therapy resistant CML. This is also the case for the potential use of epigenetic modifiers in combinatory treatment regimens in CML.

## 5 Conclusion

Overall, our data demonstrate that *KMT2D* and its variant p. (Arg191Trp), which seems to result in a protein altering loss-

of-function variant, are involved in the development of TKI resistance in CML in an in vitro-model. The variant itself does not seem to be the single driver mutation in cancer, but as *KMT2D* variants are recurrently acquired in cancer, the loss of *KMT2D* pronounced tumor progression, or as observed here, therapy resistance potentially due to increased genetic instability and epigenetic reprogramming. These findings indicate *KMT2D* as a potential target vulnerability for combinational therapy in CML, but also in other cancer entities. Further, *KMT2D* status could be a potential biomarker for the treatment of CML with TKIs.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

## Author contributions

LS: Formal Analysis, Investigation, Writing – original draft. IN: Methodology, Writing – review and editing. IV: Methodology, Writing – review and editing. IC: Methodology, Writing – review and editing. MK: Conceptualization, Formal Analysis, Writing – original draft.

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## Supplementary material

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