ORIGINAL ARTICLE

Deubiquitinating enzyme inhibitor alleviates cyclin A1-mediated proteasome inhibitor tolerance in mixed-lineage leukemia

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Abstract

Drug resistance is a significant obstacle to effective cancer treatment. Drug resistance develops from initially reversible drug-tolerant cancer cells, which offer therapeutic opportunities to impede cancer relapse. The mechanisms of resistance to proteasome inhibitor (PI) therapy have been investigated intensively, however the ways by which drug-tolerant cancer cells orchestrate their adaptive responses to drug challenges remain largely unknown. Here, we demonstrated that cyclin A1 suppression elicited the development of transient PI tolerance in mixed-lineage leukemia (MLL) cells. This adaptive process involved reversible downregulation of cyclin A1, which promoted PI resistance through cell-cycle arrest. PI-tolerant MLL cells acquired cyclin A1 dependency, regulated directly by MLL protein. Loss of cyclin A1 function resulted in the emergence of drug tolerance, which was associated with patient relapse and reduced survival. Combination treatment with PI and deubiquitinating enzyme (DUB) inhibitors overcame this drug resistance by restoring cyclin A1 expression through chromatin crosstalk between histone H2B monoubiquitination and MLL-mediated histone H3 lysine 4 methylation. These results reveal the importance of cyclin A1-engaged cell-cycle regulation in PI resistance in MLL cells, and suggest that cell-cycle re-entry by DUB inhibitors may represent a promising epigenetic therapeutic strategy to prevent acquired drug resistance.

KEYWORDS
cyclin A1, deubiquitinating enzyme inhibitor, drug tolerance, mixed-linage leukemia, proteasome inhibitor

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1 | INTRODUCTION

Cancer cells are highly plastic to the cytotoxic stress response during the development of drug resistance.[1,2] During rapid exposure to lethal chemotherapies, drug-sensitive cancer cells initially undergo apoptosis, leading to significant cell death and tumor regression. However, the remaining drug-tolerant cancer cells may follow different evolutionary paths and eventually acquire resistance.[3-5] Although drug resistance is widely considered to be genetically determined, emerging evidence has indicated a key role for non-mutational mechanisms in the survival of drug-tolerant cells.[6,7] These cells are thought to be capable of surviving strong apoptotic stimulation until more permanent long-term resistance mechanisms are developed.[8] This transient drug-tolerant state is usually achieved by slowing down essential cellular processes, and is thought to be the initial mechanism in the eventual acquisition of long-term resistance.[3,5,9] Targeting drug-tolerant cancer cells and reversing their tolerance therefore provide a promising therapeutic opportunity to impede tumor relapse.[5,10,11]

PIs have dramatically improved the treatment of MM and other hematological malignancies, but relapses are frequent and acquired resistance to treatment eventually emerges.[12,13] MLL, including ALL and acute myeloid leukemia (AML), is an aggressive hematologic malignancy with a poor prognosis.[14] Chromosome 11q23 translocations involving the *MLL* gene (also known as *KMT2A*) predominantly occur in pediatric patients, accounting for ~80% of infant leukemias and ~10% of adult acute leukemias.[15-17] We previously reported that the PI bortezomib was effective in mouse models and patients with pro-B MLL leukemia.[18] However, the inevitable emergence of PI resistance limits the clinical application of bortezomib.[19,20] There is therefore a need to identify the mechanism underlying PI resistance and to design novel combination strategies to overcome resistance and promote the application of PIs for MLL leukemias.

Drug tolerance is induced by therapeutic stresses through a reversible reprogramming of the cell cycle, leading to a dormant or quiescent state, in which cancer cells survive but do not proliferate.[8] The histone methyltransferase MLL orchestrates several essential cellular processes by positively regulating the *Hox* and cell-cycle genes.[21,22] Upon PI treatment, MLL leukemic cells initially enter a slow-cycling, stemness-enhanced, and reversible drug-tolerant state.[20] The remaining drug-tolerant cells eventually acquire resistance through PI-induced epigenetic reprogramming and subsequent loss of MLL protein function and cell-cycle deregulation.[19] However, little information is known about how MLL cells orchestrate their cell-cycle adaptive response under PI challenge.

Deregulation of the cell-cycle machinery is a common feature of cancer cells, and dysregulation of cell-cycle control, particularly in the G1/S phase, is thought to contribute to the development of solid tumors and hematological malignancies. Cyclins are well established regulators of the cell cycle, with indispensable roles in processes such as transcription, epigenetic regulation, tumorigenesis, and resistance to checkpoint blockade.[23] Cyclin A1 is predominantly expressed in normal testis and is highly expressed in leukemic and hematopoietic cells.[24,25] Cyclin A1 positively regulates the G1/S and S/G2 phase transitions.[26] Elevated levels of cyclin A1 in AML cells are associated with increased survival.[27] Some agents can abrogate the cell-cycle checkpoints to make cancer cells susceptible to apoptosis,[28] while the induction of cell-cycle entry can improve the efficiency of antiproliferative drugs and eliminate leukemia stem cells.[29] However, our mechanistic understanding of whether modulating cyclin A1 contributes to the progression of leukemic resistance is negligible.

In the current study, we show that cyclin A1 mediated the development of PI tolerance in MLL cells and resulted in patient relapse. The PI treatment induces remodeling of histone H2B monoubiquitination (H2Bub).[19,30] H2Bub depletion could impair the recruitment of MLL to chromatin and H3K4 methylation.[31-33] The crosstalk between H2Bub and MLL-mediated H3K4 methylation also affects the expression of cell-cycle genes. Therefore, we postulated that the altered chromatin state caused by the deubiquitinating enzyme (DUB) inhibitors in tolerant cells might overcome bortezomib resistance in tolerant cells.

2 | MATERIALS AND METHODS

2.1 | Reagents

Bortezomib (Velcade), P5091 (P005091), Degrasyn (WP1130), and etoposide were obtained from Selleck Chemicals.

2.2 | Cell culture and generation of drug-tolerant cells

Human pro-B MLL leukemia cell lines RS4;11 and SEM[34] were purchased from DSMZ. Cells were cultured in Gibco RPMI-1640 containing 10% FBS at 37°C with 5% CO_2 and were maintained between a density of 5×10^5 cells/mL and 2×10^6 cells/mL. The generation of induced drug-tolerant cells has been described previously.[20] Briefly, naïve cells were exposed to a sublethal dose of bortezomib (5 nmol/L) for 2 wk, replenishing the inhibitor every 3 d. The remaining cells after the treatment were considered as "Tolerant" cells and were collected for analysis. "Reverted" cells were generated from bortezomib-tolerant cells by culturing without bortezomib for a minimum of 4 wk. All cultured cells were tested for mycoplasma contamination before use.

2.3 | Cell viability and cell proliferation assays

The CellTiter 96 MTS assay (Promega) was used to determine the cytotoxicity of the relevant drugs and cell proliferation, in accordance with the manufacturer's instructions. Cell viability was measured using the MTS assay 24 h after the addition of drug with graded concentrations in triplicates.

2.4 | Apoptosis and cell-cycle assays

Apoptosis and cell cycle were measured using the Annexin V-PE Apoptosis Detection Kit and APC BrdU Flow Kit from BD Pharmingen as described by the manufacturer. The percentages of different cell-cycle phases (G1, S, and G2/M) were quantified. Cells staining with fluorochromes were acquired using flow cytometer and data were analyzed using FlowJo software.

2.5 | shRNA-mediated knockdown and qRT-PCR

Target sequences (CTCCTGAAGTAGACGAGTTTG#1 and CAC AAGAATCAGGTGTTATTC#2) against human cyclin A1, target sequences (GCCAAGCACTGTCGAAATTAC#1 and TCTA CCAACCCTAAACCCTGA#2) against human MLL C-terminus, and a control scrambled sequence (GCGCGCTTTGTAGGATTCGTT) that has no significant homology with the human genome were inserted into the pLKO.1 vector, in accordance with the manufacturer's protocol (Addgene). Generated lentivirus carrying shRNA was used to infect target cells for 2 d, and the cells were subjected to puromycin selection at 2 μg/mL.[35] Cellular RNA samples were reverse transcribed with random primers and detection was performed using 7500 Real-Time PCR Systems (Applied Biosystems). The data represent absolute mRNA copy numbers normalized to *GAPDH* used as a reference gene.[36] Relative fold expression values were determined by applying the $\Delta\Delta C_t$ method. Primers used for qRT-PCR assay were *CCNA1*: forward, AGTGGAGTTGTGCTGGCTAC, reverse, GTCAGGGAGTGCTTTCTTTC; *KMT2A* (*MLL*): forward, ACAGAAAAAAGTGGCTCCCCG, reverse, GCAAACCACCCTGGGTGTTA.

2.6 | Plasmid constructions

The wild-type (WT) *CCNA1* and mutant *CCNA1* (CCNA1mut, ctc-ctgaagtagacgagtttg to caccagaggttgatgaattcg) were inserted into the pCDH-MCS-T2A-copGFP-MSCV vector, in accordance with the manufacturer's protocol (System Biosciences). The CCNA1mut plasmid was constructed based on the shCCNA1-1 sequence, which changed the base sequence without changing the amino acids, so that it would not be affected by the shRNA. Generated lentivirus carrying the CCNA1mut was used to infect target cells.

2.7 | Immunoblots

Human CD133-PE was obtained from Miltenyi Biotec. Mouse antibody against cyclin A1 was obtained from BD Biosciences. Rabbit antibodies against MLL[C180] (D6G8N), Ubiquityl-Histone H2B (Lys120) (H2Bub), Tri-Methyl-Histone H3 (Lys4) (H3K4me3), Phospho-Histone H2A.X (Ser139) (γ-H2AX), Histone H3, and mouse anti-Histone H2B antibody were purchased from Cell Signaling Technology. Mouse anti-β-actin antibody was obtained from Sigma

Aldrich. Antibodies were detected using the enhanced chemiluminescence method (Western Lightning, PerkinElmer). Immunoblot signals were acquired with the Amersham Imager 600 (General Electric Company).

2.8 | Total RNA sequencing (RNA-Seq)

Total RNA was extracted from TRIzol in accordance with the manufacturer's instructions. The mRNA-seq library was performed using the Illumina TruSeq library construction kit. A 2 μg aliquot of total RNA was used as initiation and then prepared in accordance with the manufacturer's instructions. The mRNA-seq libraries were sequenced using BGISEQ-500 for 100-bp paired-end sequencing. Quality control of mRNA-Seq data was performed using Fatsqc, and then low-quality bases were trimmed. After quality control, clean reads were aligned to the human genome (UCSC hg19) using TopHat v.2.1.0 with a maximum of 2 mismatches for each read. After data mapping, Cufflinks software was used to analyze significant differential expression genes. GSEA was executed using public software from the Broad Institute (http://software.broad institute.org/gsea).

2.9 | Chromatin immunoprecipitation assays

ChIP assays were performed following the described protocol[37] using anti-MLL[C180] and H3K4me3 antibodies. Precipitated DNA was sequenced or analyzed using 7500 Real-Time PCR Systems.[38] Primers used for ChIP-PCR assay were located at the *CCNA1* promoter region (−503 base pairs [bp] to −348 bp relative to the transcription start site [TSS]): forward, AAGCGTAGGTGTGTGAGCCGA, reverse, AACAACCCCCTCTAACGTCTC.

2.10 | Kaplan-Meier curve analyses

We downloaded the large-scale genomic analysis of pediatric ALL data from the Therapeutically Applicable Research to Generate Effective Treatments (https://ocg.cancer.gov/programs/target) initiative, phs000464 (Phase II, TARGET, 2018). Gene expression and Kaplan-Meier curves of initially treated and relapsed patients were evaluated. The *P*-value was calculated to assess the statistical relevance.

2.11 | Statistical analysis

Student *t* test or log-rank test was used to analyze the differences between the groups. Means were illustrated using a histogram with error bars representing ± the standard deviation (SD), and statistical relevance was evaluated using the following *P* values: $P < .05$ (*), $P < .01$ (**), or $P < .001$ (***).

3 | RESULTS

3.1 | Cyclin A1 is downregulated in drug-tolerant MLL cells

We previously observed that pro-B MLL leukemia displayed selective sensitivity to the PI bortezomib, but reversible resistance inevitably emerged.[18,20] Cancer cells primarily acquire a drug-tolerant state under therapy stress, often by slowing down essential cellular processes.[5] Similar to other drug-tolerance mechanisms,[5,11] the emergence of PI tolerance is accompanied by cell-cycle arrest and growth inhibition.[20,39] We determined the reversible features of slow-cycling PI tolerance in bortezomib-tolerant SEM and RS4;11 cells by continuously treating the parental cells with sublethal doses

of bortezomib for 2 wk, and replenishing the inhibitor every 3 d. The cells remaining after the treatment were considered as PI-tolerant cells and were collected for analysis. Reverted cells were generated from bortezomib-tolerant cells by culturing without bortezomib for a minimum of 4 wk. We further characterized these PI-tolerant MLL cells by RNA sequencing (RNA-Seq). We created replicates of PI-tolerant and reverted cells to reduce the differences between samples. Hierarchical clustering of differentially expressed genes (DEGs) revealed distinct clusters of gene expression patterns (Figure 1A). Compared with PI-tolerant cells, reverted and parental cells showed similar gene expression patterns, indicating the reversibility of PI tolerance. We also performed GSEA using datasets obtained from tolerant and reverted cells, and observed an enrichment of genes involved in the cell cycle (Figure 1B,C). Notably, the *CCNA1* gene
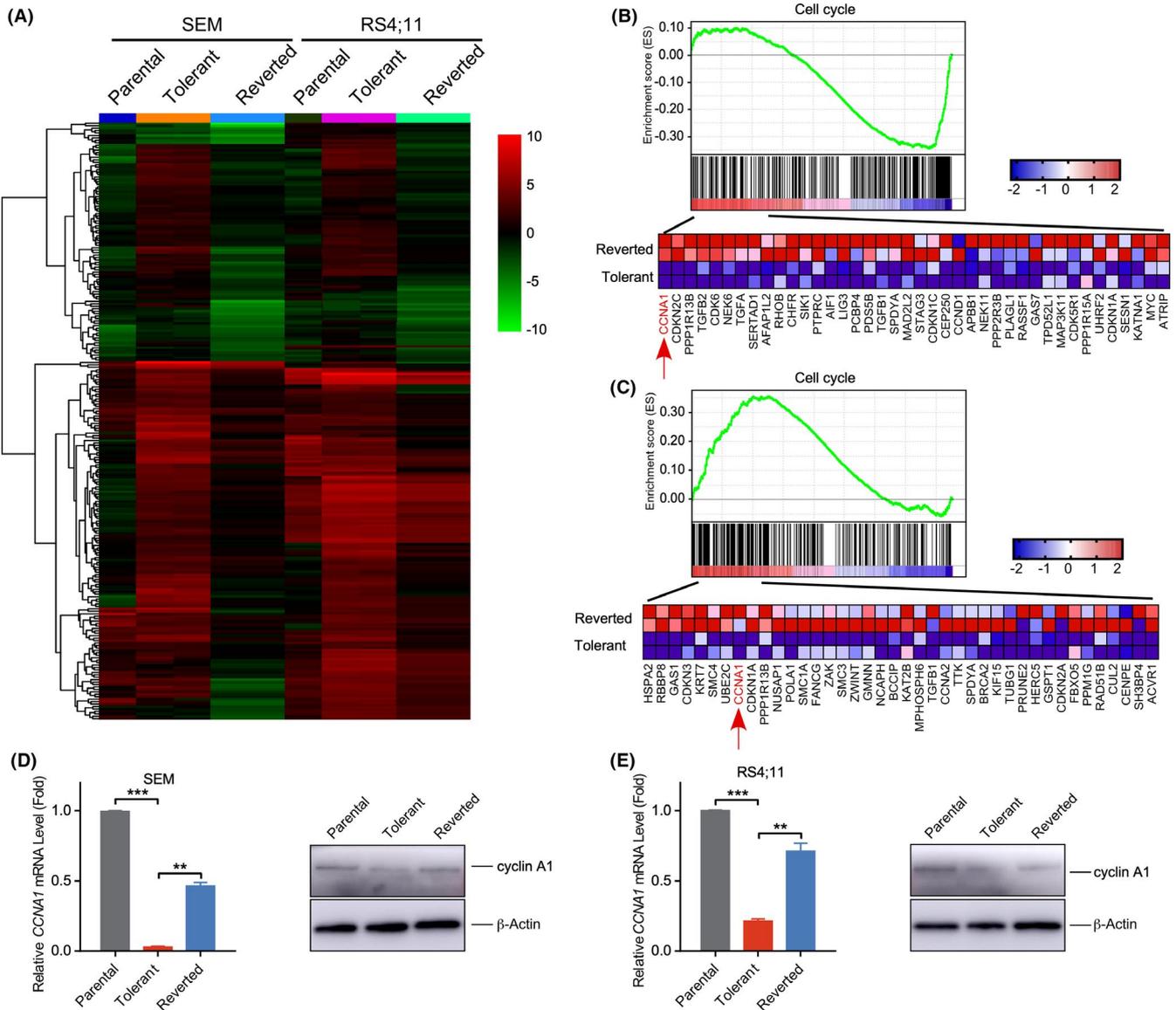
FIGURE 1 Cyclin A1 is downregulated in drug-tolerant MLL cells. A, Unsupervised hierarchical clustering heatmap of differentially expressed genes (DEGs) in parental, tolerant, and reverted SEM and RS4;11 cells. B, C, GSEA analysis of datasets obtained from the indicated SEM (B) and RS4;11 (C) cells. Heatmap showing the 40 most downregulated genes. D, E, *CCNA1* mRNA and cyclin A1 protein levels in the indicated cells. Relative mRNA expression values were normalized against *GAPDH*. β-Actin was used as a loading control in immunoblots. **$P < .01$; ***$P < .001$; two-tailed t test. Data represent the means of triplicate reactions ± SD

(encoding cyclin A1) was markedly downregulated in PI-tolerant cells (Figure 1B,C). Moreover, levels of *CCNA1* mRNA and cyclin A1 protein were significantly decreased in PI-tolerant MLL cells but recovered in the reverted cells (Figure 1D,E). These results indicate that slow-cycling PI-tolerant MLL cells are associated with reduced *CCNA1* expression.

3.2 | Cyclin A1 suppression induces drug tolerance in MLL cells

Cyclin A1 is an essential component of the cell-cycle engine that positively regulates G1/S and S/G2 phase transitions,[26] and we therefore investigated if cyclin A1 played a specific role in PI tolerance. Lentiviruses carrying short hairpin RNA (shRNA) targeting cyclin A1 (shCCNA1-1 and shCCNA1-2) were constructed and used to infect SEM cells (Figure 2A). *CCNA1* knockdown decreased the percentages of S and G2/M phase cells (Figure 2B) and suppressed the proliferation of infected SEM cells (Figure 2C), suggesting that *CCNA1*

knockdown induced slow-cycling cells. We conducted cell viability assays and showed that *CCNA1* knockdown significantly reduced the sensitivity of SEM cells to bortezomib (Figure 2D). Furthermore, *CCNA1*-knockdown notably reduced the percentage of apoptotic cells induced by bortezomib (Figure 2E). We further constructed the wild-type *CCNA1*, and a mutant *CCNA1* plasmid (CCNA1mut) for shCCNA1-1, in which the base sequence was changed but the amino acid sequence remained the same, which was therefore not affected by the shRNA (Figure 2F). The results showed that overexpression of wild-type *CCNA1* and CCNA1mut has no significant effect on drug resistance, while *CCNA1* knockdown significantly reduced the sensitivity of SEM cells to bortezomib (Figure 2G). Moreover, the tolerance induced by shCCNA1 in SEM cells was notably rescued by the introduction of CCNA1mut (Figure 2G). The cell viability of shCCNA1 + CCNA1mut was different compared with the control. This discrepancy may be caused by the effect of the 2 infections on cell viability. Taken together, these results indicate that downregulation of *CCNA1* plays a causal role in the development of PI tolerance in MLL cells.
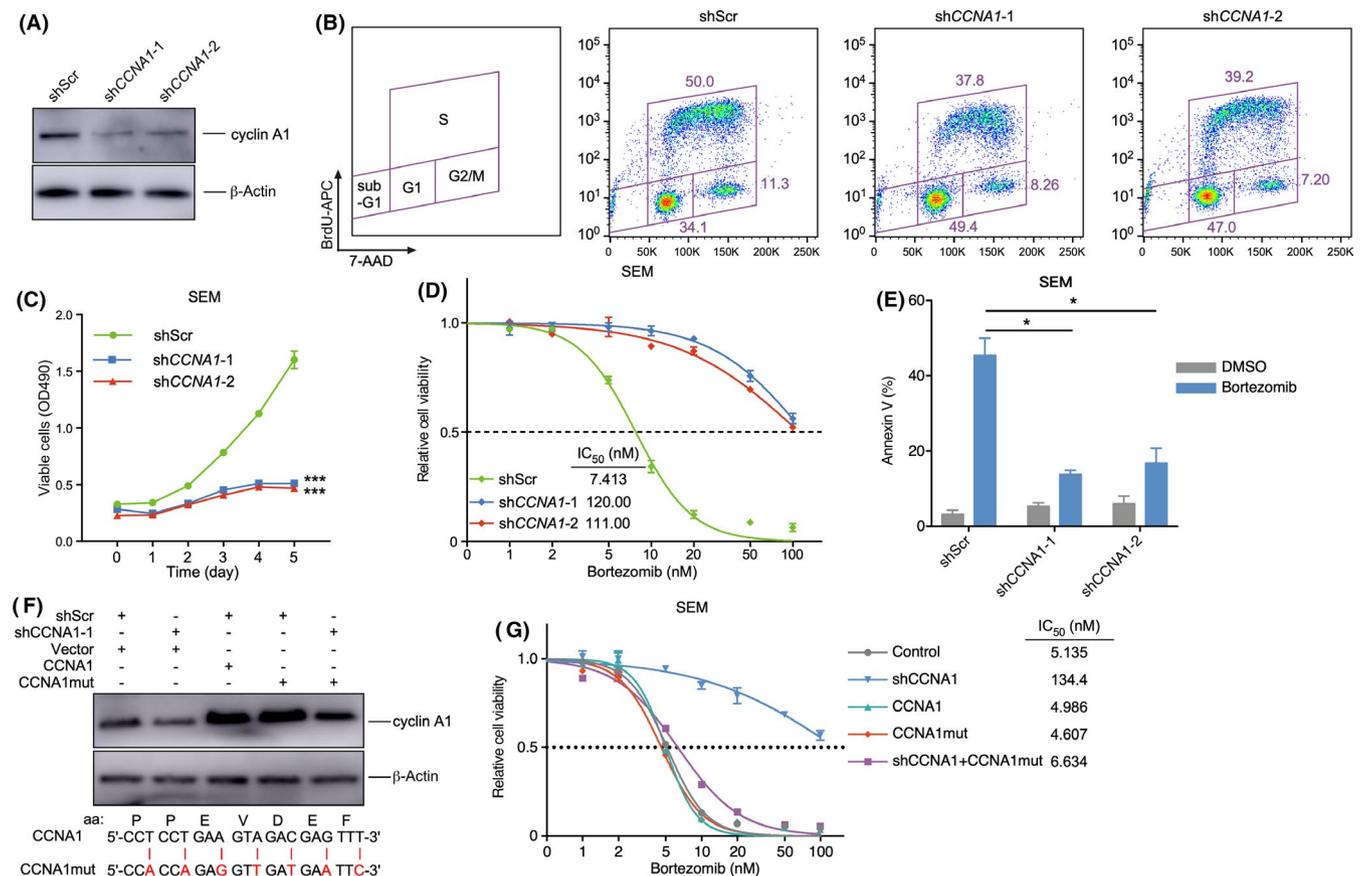
FIGURE 2 Cyclin A1 suppression induces drug tolerance in MLL cells. A, SEM cells were infected with the indicated lentiviral vectors. Immunoblots of cyclin A1 in shScr and *CCNA1*-knockdown SEM cells were analyzed. B, Cell-cycle profiling of the indicated SEM cells. One representative of 3 independent experiments is shown. C, The proliferation of SEM cells infected with indicated lentiviral vectors for 5 d. D, Cell viability of indicated SEM cells. The IC$_{50}$ of different cells was quantified. E, Apoptosis analysis of indicated SEM cells treated with dimethyl sulphoxide (DMSO) or bortezomib (5 nmol/L) for 16 h. F, G, SEM cells were infected with the indicated lentiviral vectors. Immunoblots (F) and cell viability (G) of the indicated cells were analyzed. CCNA1mut was based on the shCCNA1-1 sequence, which changed the base sequence without changing the amino acid sequence. *P < .05; ***P < .001; two-tailed *t* test. Data represent the means of triplicate reactions ± SD

3.3 | MLL directly regulates the transcriptional activity of cyclin A1

The *CCNA1* gene is transcriptionally regulated by E2F1, MYB, and pRB (RBL1 and RBL2).[26,40] To examine the mechanism of *CCNA1* reduction, we therefore evaluated the expression levels of these *CCNA1* regulators. mRNA levels of these regulators were not significantly reduced in PI-tolerant SEM and RS4;11 cells (Figure S1A), suggesting that factors other than these were responsible for suppressing *CCNA1* expression in PI-tolerant cells.

CCNA1 repression in quiescent cells is associated with chromatin modification of its promoter.[41] Moreover, the reversibility of PI tolerance is indicative of an epigenetic rather than a genetic mechanism,[3,42] and transcription of the *CCNA1* gene can be regulated by

MLL-mediated histone H3 lysine 4 trimethylation (H3K4me3) on its promoter.[21,22,41] We therefore examined if the tolerant state was associated with changes in MLL and H3K4me3 modification of the *CCNA1* promoter.[43] To confirm the association between MLL reduction and *CCNA1* suppression, we knocked down the *MLL* gene in SEM cells and observed that MLL depletion significantly reduced *CCNA1* gene expression (Figure 3A). In ALL, downregulation of MLL is accompanied by a decrease in cell sensitivity.[19,44] To confirm the association of MLL expression with PI sensitivity in MLL leukemia, we knocked down the *MLL* gene in SEM cells and observed that MLL depletion significantly decreased the sensitivity of SEM cells to bortezomib and suppressed the apoptosis induced by bortezomib (Figure 3B,C). We further performed chromatin immunoprecipitation sequencing (ChIP-seq) analysis using MLL-CT antibody
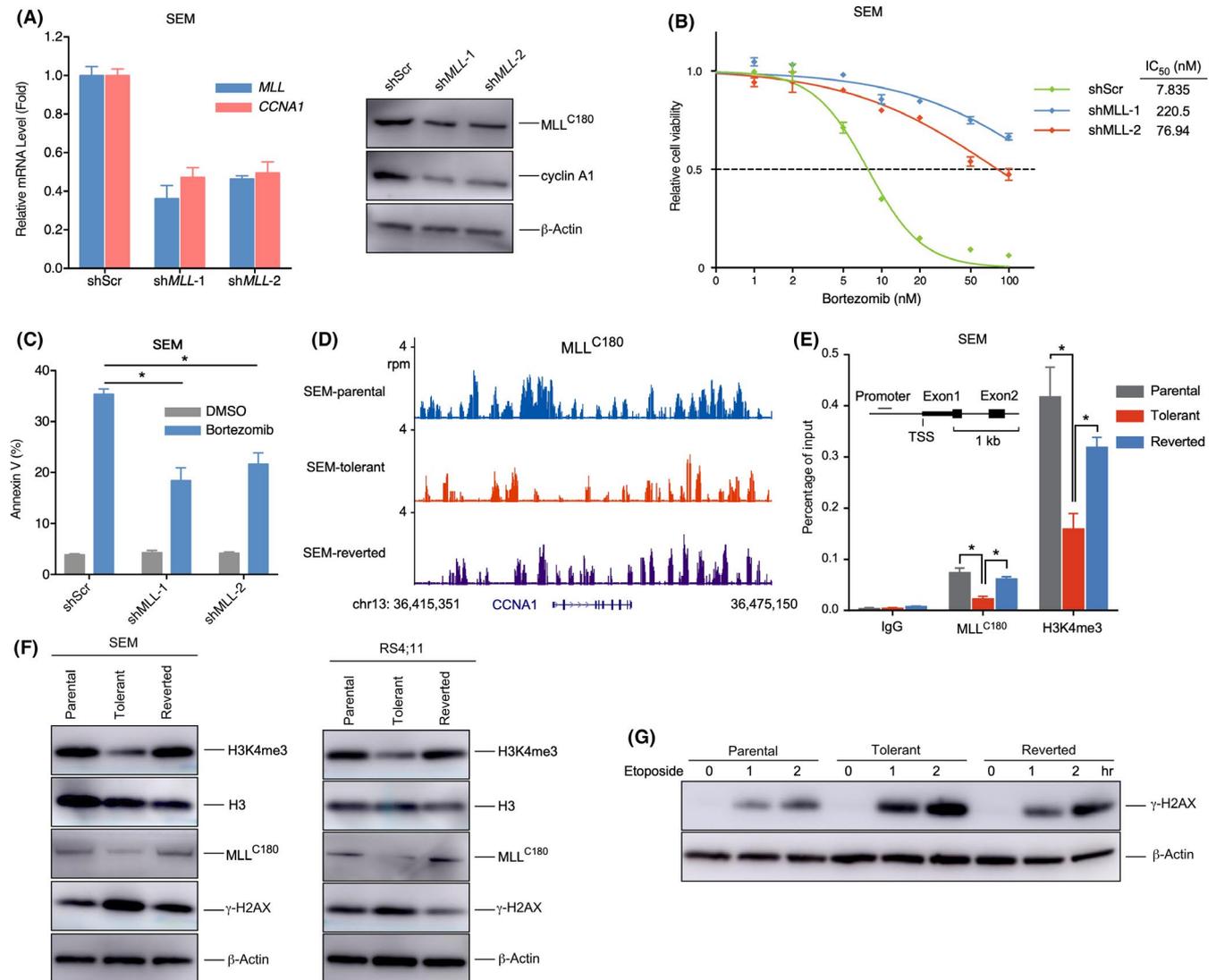
FIGURE 3 MLL directly regulates the transcriptional activity of cyclin A1. A, Immunoblots and mRNA levels of SEM cells infected with the indicated lentiviral vectors. B, Cell viability of SEM cells infected with the indicated lentiviral vectors. The IC$_{50}$ of different cells was quantified. C, Apoptosis analysis of indicated SEM cells treated with DMSO or bortezomib (5 nmol/L) for 16 h. D, Genome browser tracks of MLL^{C180} ChIP-seq at *CCNA1* loci in parental, tolerant, and reverted SEM cells. E, ChIP analyses at the promoter region of the *CCNA1* locus in the indicated SEM cells. TSS, transcription start site. F, Immunoblots of endogenous wild-type MLL (MLL^{C180}), histone, and histone modifications in the indicated cells. G, Indicated SEM cells were treated with etoposide (25 μmol/L) and then analyzed by anti-γH2AX immunoblots. *$P < .05$; two-tailed *t* test. Data represent the means of triplicate reactions ± SD

(A)

(B)
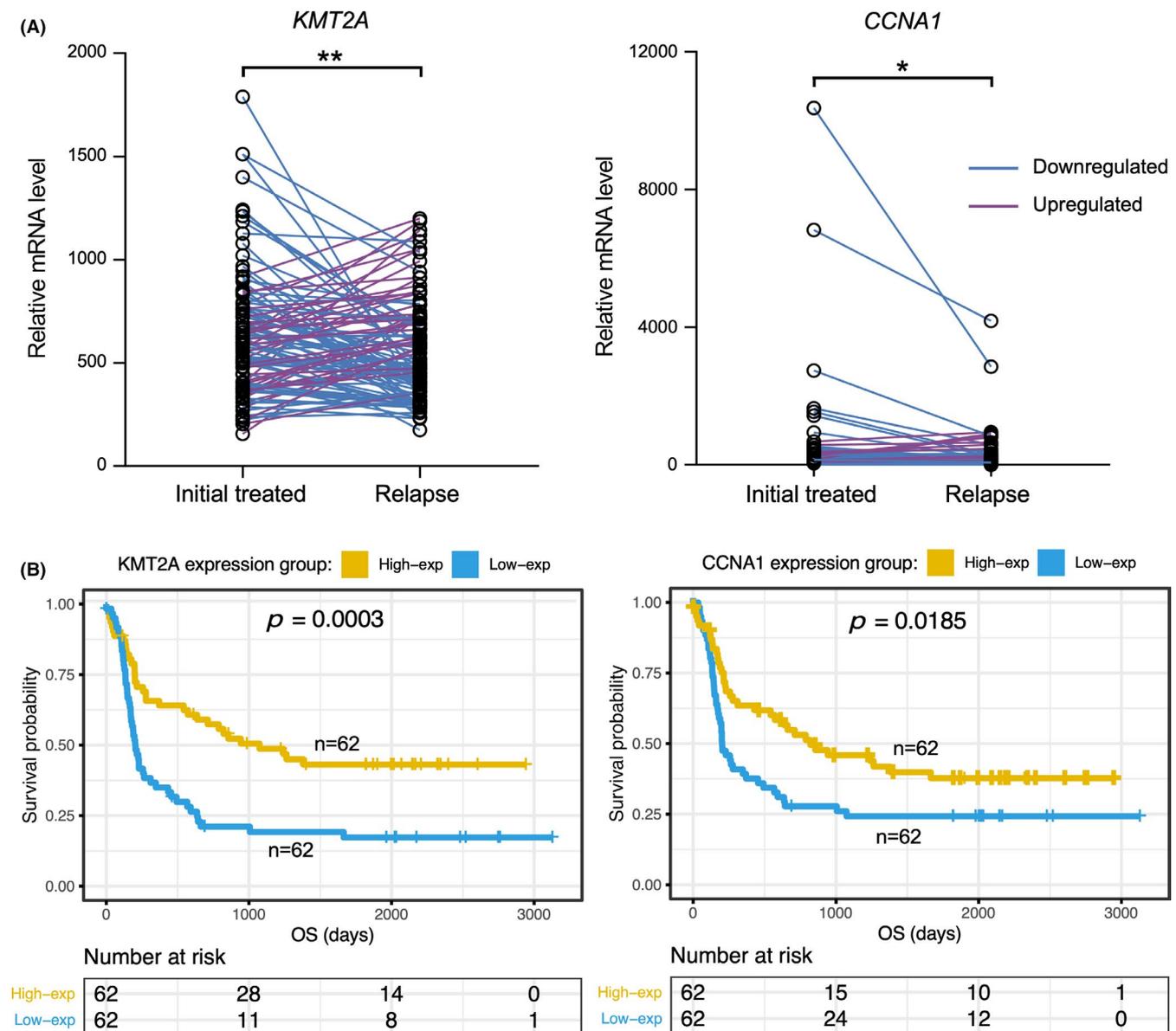
FIGURE 4 Downregulation of MLL and cyclin A1 is correlated with the short survival in ALL patients. A, Line plots of mRNA levels in initial treated or relapsed ALL patients (pediatric ALL—Phase II, TARGET, 2018). Blue lines, downregulated genes; purple lines, upregulated genes. B, Kaplan-Meier survival curve of ALL patients from the pediatric ALL—Phase II (TARGET, 2018) dataset (low expression: 50%, n = 62; high expression: 50%, n = 62). *P < .05; **P < .01; by two-tailed t test or log-rank test for significance. Data represent the means of triplicate reactions ± SD

(MLL^C180) in parental, tolerant, and reverted SEM cells. ChIP assays showed that the MLL antibody had decreased *CCNA1* promoter occupancy in SEM tolerant cells (Figure 3D). Moreover, compared with parental and reverted cells, PI-tolerant cells showed significantly decreased levels of both global and *CCNA1* promoter-bound MLL and H3K4me3 (Figure 3E,F). Overall, these results favor a positive correlation between MLL and the acquisition of PI resistance in MLL leukemia cells, indicating that cyclin A1 is regulated by MLL and mediates PI tolerance in MLL cells.

In addition, consistent with a critical role in the DNA-damage responses,[22] reduction of MLL in PI-tolerant cells was associated with an increase in histone γ-H2AX (Figure 3F). Furthermore, etoposide-induced γ-H2AX levels were notably increased in PI-tolerant cells

compared with parental and reverted cells (Figure 3G). These results indicate that PI-tolerant cells are prone to DNA damage, potentially resulting in secondary mutations and hardwired drug resistance, which could partly explain why the reverted cells failed to regain full sensitivity to PI compared with the parental cells.

3.4 | Prognostic relevance of MLL and cyclin A1 expression in ALL patients

To clarify the clinical significance of MLL and cyclin A1 expression, we examined the relationships between *KMT2A* (encoding MLL) and *CCNA1* gene expression levels and patient survival in ALL patients

from a pediatric ALL dataset (Phase II, TARGET, 2018). Interestingly, compared with initially treated patients, patients who relapsed following chemotherapy showed significantly reduced expression levels of *KMT2A* and *CCNA1* (Figure 4A), consistent with our findings in drug-tolerant cells. Furthermore, Kaplan-Meier analysis with a 50% cut-off value revealed that ALL patients with low *KMT2A* and *CCNA1* expression levels had significantly lower overall survival compared with those with high expression levels (*P* = .0003 and *P* = .0185, respectively; Figure 4B). Taken together, these results indicate that *KMT2A* and *CCNA1* expression may have prognostic relevance in terms of patient survival and chemotherapy relapse, with downregulation of MLL and cyclin A1 being correlated with shorter survival, and high expression of MLL and cyclin A1 as a prognostic marker of higher overall survival in patients with ALL. These results are consistent with our data that reduction of MLL and cyclin A1 plays an important role in the acquisition of PI resistance in MLL cells.

3.5 | DUB inhibitors show potential efficacy in PI-tolerant MLL cells

PI-induced H2Bub depletion affects MLL function and the expression of cell-cycle genes.[19] We hypothesized that DUB inhibitors could overcome bortezomib resistance in tolerant cells by promoting the elevation of H2Bub. Furthermore, the DUB inhibitors P5091 and WP1130 are currently being tested in combination with various anticancer therapies.[45,46] Notably, H2Bub and H3K4me3 were significantly induced by P5091 and WP1130, leading to increased cyclin A1 expression (Figure 5A). Consistently, *CCNA1* mRNA was significantly induced by DUB inhibitors in PI-tolerant cells (Figure 5B). ChIP assays demonstrated that MLL and H3K4me3 had increased *CCNA1* promoter occupancy in SEM tolerant cells (Figure 5C). Interestingly, although MLL was not significantly upregulated by DUB inhibitors in PI-tolerant cells (Figure 5A), ChIP assays revealed incremental loading of MLL following DUB inhibitor treatment (Figure 5C), indicating that DUB inhibitors induce H2Bub and H2Bub-dependent H3K4me3 modification by crosstalk between H2Bub and MLL-mediated H3K4 methylation, promoting *CCNA1* expression and potentially restoring cell sensitivity. We further examined the effects of DUB inhibitors on cell-cycle progression of PI-tolerant cells. The results showed that the S-phase cells that significantly suppressed in PI-tolerant cells were markedly restored by P5091 and WP1130 (Figure 5D). Taken together, these findings indicate that the DUB inhibitors P5091 and WP1130 can reverse cyclin A1 expression and induce cell-cycle re-entry in PI-tolerant cells.

We further investigated whether cell-cycle re-entry induced by DUB inhibitors could reverse cell sensitivity and therefore have a therapeutic potency in PI-tolerant cells. The DUB inhibitors P5091 and WP1130 could significantly induce apoptosis in both naïve and PI-tolerant cells to varying degrees (Figure S2A). We then examined the effect of DUB inhibitors in combination with bortezomib. The results demonstrated that when the DUB inhibitor P5091 or WP1130 was combined with bortezomib, apoptosis in naïve cells was not significantly increased (Figure S2B). However, co-treatment of bortezomib with P5091 or WP1130 led to a strong reduction in cell viability (Figure 5E,F) and induced notable apoptosis in PI-tolerant cells (Figure 5G,H). Collectively, these findings suggest that DUB inhibitors have a significant synergistic effect with bortezomib in PI-tolerant cells, and a dual strategy using PIs and DUB inhibitors might be effective in preventing PI tolerance.

4 | DISCUSSION

Drug tolerance is induced by therapeutic stress through reversible reprogramming of the cell cycle, leading to a dormant or quiescent state, in which cancer cells survive but do not proliferate.[8] These drug-tolerant cells subsequently accumulate gene mutations or epigenetic changes that facilitate the emergence of drug resistance, associated with cell-cycle exit and concomitant unresponsiveness to applied therapies.[47] The acquired cell-cycle defects in tolerance cells are associated with specific vulnerabilities that distinguish them from their normal counterparts and provide a window of opportunity for therapeutic strategies.[28] Here, we demonstrated that cyclin A1 was significantly reduced in PI-tolerant MLL cells, and that restoring cyclin A1 was effective in overcoming PI tolerance. These results highlight the importance of preventing the emergence of drug-tolerant cancer cells in cancer therapy. Conceivably, attenuating the acquisition of drug tolerance by modulating deregulation of the cell-cycle process might be an effective means of preventing treatment failure and relapse.

Cyclins are generally implicated as oncogenes when cancer cells undergo cell-cycle deregulation, and some cyclin-dependent kinase inhibitors are thought to act as cancer suppressors in a variety of malignancies.[48,49] From the treatment perspective, drug-tolerance-mediated resistance provides an operational framework for developing therapeutic strategies.[47] One approach, known as a "sleeping strategy," aims to keep cancer cells dormant,[50] while the "awakening strategy" stimulates cancer cells to re-enter the cell cycle to improve anti-tumor efficiency.[29] The third method involves the induction of

FIGURE 5 DUB inhibitors have potential efficacy against PI tolerance. A, B, SEM and RS4;11 tolerant cells were treated with DMSO, P5091, or WP1130 for 20 h. Immunoblots of the indicated antibodies (A) and *CCNA1* mRNA levels (B) were analyzed. C, ChIP analyses at the promoter region of the *CCNA1* locus in SEM tolerant cells after the treatment with DMSO, P5091 (5 μmol/L), or WP1130 (1 μmol/L). Assays were performed with the indicated antibodies and immunoprecipitates were subjected to quantitative PCR analyses. D, Cell-cycle profiling of the indicated cells treated with DMSO, P5091 (1 μmol/L), or WP1130 (1 μmol/L) for 20 h. One representative of 3 independent experiments is shown. E, F, Cell viability of SEM and RS4;11 tolerant cells treated with 5 μmol/L P5091 (E) or 1 μmol/L WP1130 (F). G, H, Apoptosis analysis of SEM and RS4;11 tolerant cells treated with the combination of bortezomib with P5091 (G) or WP1130 (H) for 20 h. n.s., not significant; *P* < .05; **P* < .01; by two-tailed *t* test. Data represent the means of triplicate reactions ± SD
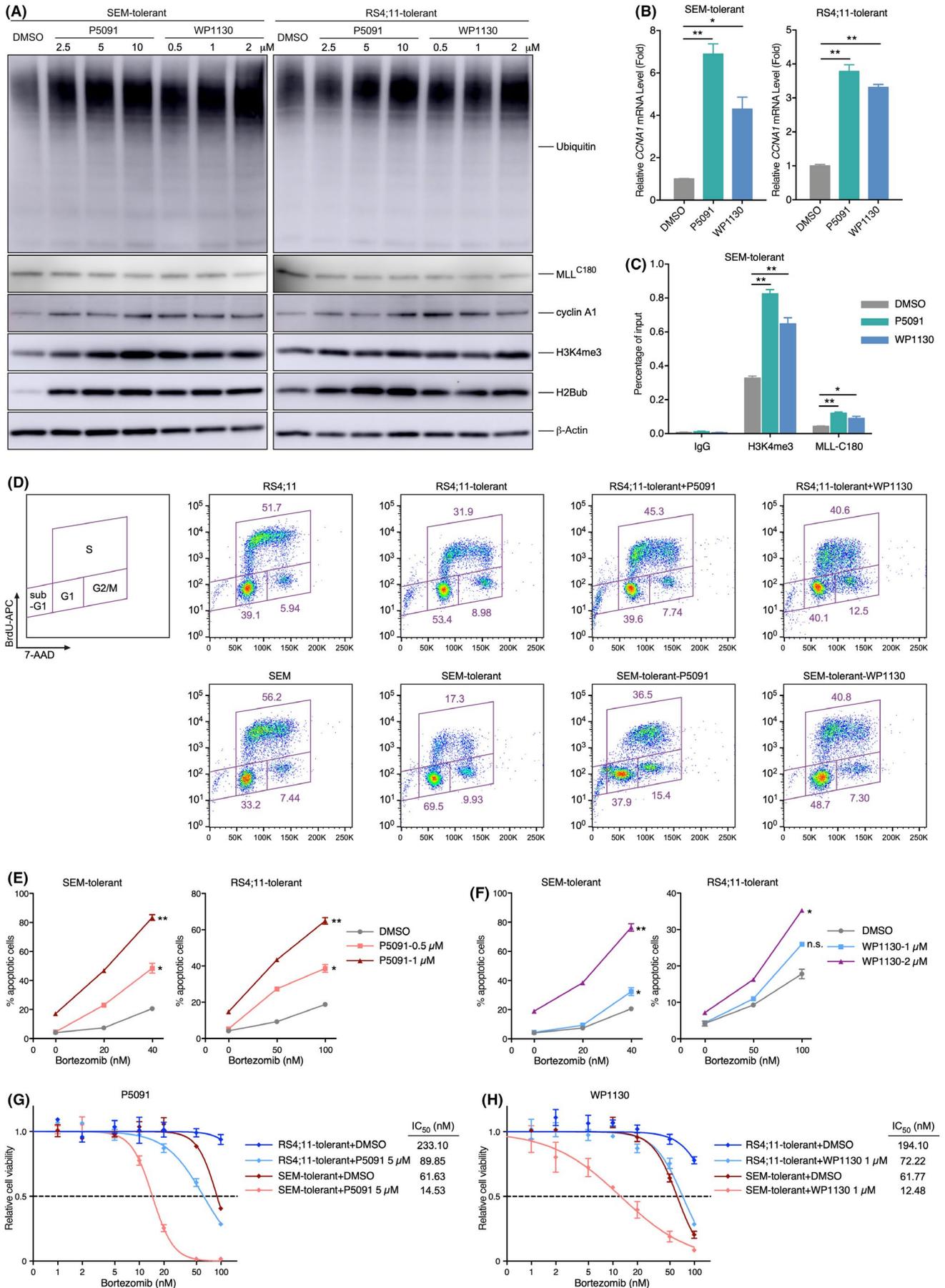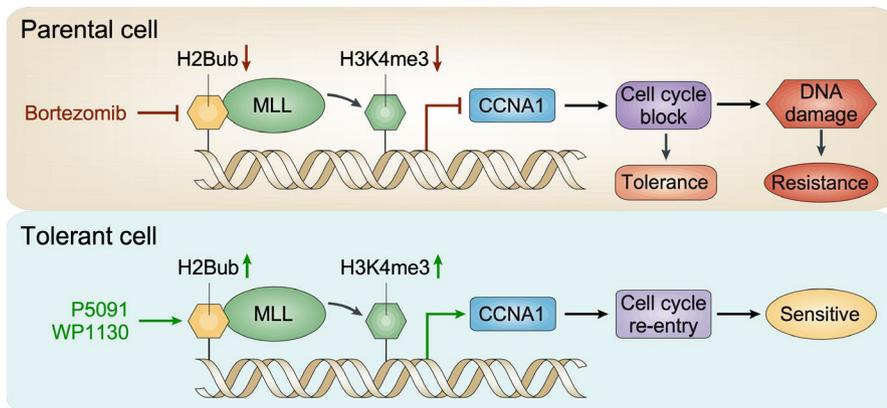
FIGURE 6 Schematic model. The illustration depicts how PI-induced epigenetic reprogramming elicits tolerance in MLL cells and the therapeutic function of DUB inhibitors in MLL

senescence in quiescent cells by inhibiting autophagy.[51] Some agents target quiescent cells through these approaches to make cancer cells susceptible to apoptosis.[28,29] The current study demonstrated that cell-cycle deregulation mediated by cyclin A1 suppression elicited the development of transient PI tolerance, while DUB inhibitors increased H2Bub-dependent H3K4 methylation and induced cyclin A1 expression. These findings revealed that induction of cell-cycle entry could improve the efficiency of eliminating drug-tolerant cells and overcoming PI tolerance, indicating that re-entry into the cell cycle may represent a therapeutic strategy to prevent acquired drug resistance.

Many mechanisms have been proposed for PI resistance, especially in MM cells. MM cells are known to have increased demands for protein synthesis and are therefore more susceptible to the cytotoxic effects of proteasome inhibition, which disrupts protein homeostasis.[52,53] Recent findings have consistently highlighted the importance of re-establishing protein homeostasis to overcome PI resistance.[12,13,53,54] However, unlike myeloma cells, pro-B MLL leukemic cells have low levels of protein synthesis, suggesting that MLL leukemic cells might achieve PI resistance by different strategies. The PI treatment induces MLL leukemic cells to acquire a reversible drug-tolerant state, which represents the initial mechanism leading to eventual drug resistance.[20] The current results demonstrate that the development of PI tolerance is dependent on cyclin A1, which is regulated directly by MLL protein. MLL dysfunction leads to the down-regulation of CCNA1 and is conducive to acquiring PI resistance. Moreover, reduction in MLL and cyclin A1 results in drug resistance and relapse, leading to reduced survival. The clinical significance of cyclin A1 expression in different leukemia types has previously been evaluated.[27,55] The present results indicate that MLL and cyclin A1 expression might have notable prognostic relevance in terms of patient survival and chemotherapy relapse, and that downregulation of MLL and cyclin A1 is correlated with short survival in ALL patients.

Our finding that PI-tolerant MLL leukemic cells were prone to DNA damage reinforced the idea that mutational and non-mutational mechanisms underlying drug resistance are not necessarily mutually exclusive.[8] Transient effects such as tolerance and persistence have been suggested to provide initial resistance, while the addition of drugs then induces epigenetic reprogramming until secondary

mutations convert the progression to relapse, and the cells become genetically hardwired.[5,56,57] The present study further suggested that this "rewiring" involving epigenetic chromatin modifications not only conferred initial fitness, but also facilitated the hardwiring process giving rise to inheritable resistance.

In conclusion, we clarified the relationship between MLL and cyclin A1 expression and the generation of drug-tolerant cells (Figure 6). The reversible and DNA-damage-prone tolerant state is associated with MLL and cyclin A1 dysfunction, which in turn promotes the eventual acquisition of PI resistance. The results also suggested that combination therapy with PIs and DUB inhibitors may be more effective in treating MLL leukemias than solo therapy, by preventing the emergence of tolerant cells. The use of DUB inhibitors to restore cyclin A1 expression also suggests that cell-cycle re-entry may be a therapeutic target for preventing acquired drug resistance, which may also extend to cancers other than MLL leukemia.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTION

MG designed and performed most of the experiments, analyzed the data, and wrote the draft manuscript; QX performed some experiments; TK, DL, RW, ZC, SX, and WW performed some experiments and analyzed the data; HL contributed grant support, designed the

entire project, and wrote the manuscript. All authors discussed the results and commented on the manuscript.

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