Inhibition of SUV39H1 as a potent therapeutic target in multiple myeloma

Multiple myeloma (MM) is a frequent hematologic malignancy characterized by the accumulation of malignant plasma cells within the bone marrow (BM). Genomic studies have shown substantial heterogeneity and genomic instability, a complex mutation profile, and branching patterns of clonal development.[1] Despite significant improvements in patient outcomes over the past decade through the use of immunomodulatory drugs (IMiD), proteasome inhibitors, and the introduction of immunotherapies, patients ultimately experience disease recurrence.[1]

Recent studies have identified that alongside a diverse array of genetic mutations, epigenetic changes may play significant roles in the development of MM and mechanisms of drug resistance.[2] These discoveries could shed light on new mechanisms fundamental to the origin of MM and potentially reveal innovative approaches and targets for effective treatment.

Biallelic TP53 inactivation is the most important high-risk factor associated with poor survival in MM. We previously reported that *TP53* bi-allelic events are associated with a specific H3K9me3 signature in MM cells.[2] Three proteins primarily catalyze H3K9me3 including SUV39H1, SUV39H2 and SETDB1 histone methyltransferases (HMT). SUV39H1, the HMT that creates the H3K9me3 mark, is repressed by p53.[3] Overexpression of SUV39H1 maintains high H3K9me3 at p53 target promoters, reduces p53 recruitment, and dampens p53-dependant transcription and apoptosis, whereas SUV39H1 knockdown enhances p53 target gene expression.[3] This suggests that *TP53* alterations could alter H3K9me3 levels on these promoters, thereby affecting the p53-mediated apoptotic response. Thus, we investigated if SUV39H1 and SUV39H2 HMT play a role in MM pathophysiology.

Investigating *SUV39H1* and *SUV39H2* gene expression in normal B to plasma cell (PC) differentiation, purified MM cells from newly-diagnosed patients (N=97), and human myeloma cell lines (HMCL) (N=33), we identified a significant higher expression of *SUV39H1* and *SUV39H2* in HMCL compared to primary MM cells from patients and normal PC (Figure 1A, *Online Supplementary Figure S1A*). We included a validation cohort with Affymetrix microarray data (*Online Supplementary Figure S1B, C*). We also identified a significant upregulation of SUV39H1 in plasma cell leukemia (PCL) compared to MM patients (*Online Supplementary Figure S1D*). Of interest, high expression of *SUV39H1* and *SUV39H2* is associated with a poor outcome in newly diagnosed MM patients of the CoMMPASS cohort (Figure 1B). These data were validated in two other independent cohorts of newly diagnosed MM patients and in a cohort of MM patients at relapse treated by daratumumab[4] (*Online Supplementary Figure S1E, F*). In COX analyses with high-risk cytogenetic abnormalities (HR CA) (CoMMPASS cohort), high expression of *SUV39H1* or *SUV39H2* remained independent prognostic factors when compared to del17p or 1q gain (*Online Supplementary Table S1*). Only high *SUV39H1* expression is associated with a significant poor outcome in MM patients with del17p and in patients without del17p (Figure 1C). High *SUV39H1* expression was also associated with a prognostic value in patients with 1q gain and in patients without 1q gain CA (Figure 1D). In MM, accumulation of high-risk CA worsens patients' prognosis.[5] We have assessed the effect of additional HR CA (del17p, 1q gain and t(4;14)) on the prognosis of patients with high SUV39H1 expression in the CoMMPASS cohort. Of interest, the overall survival (OS) of patients with high *SUV39H1* expression significantly decreased when associated with other HR CA (P<0.0001) (Figure 1E). *SUV39H1* expression was significantly higher in patients with del13, del17p, del1p, 1q gain, t(4;14), t(11,14), and biallelic TP53/del17p alteration CA (Figure 2A, B). Furthermore, patients with *DIS3, MAP3K1, MAX, SETD2, TP53,* and *TRRAP* mutations are associated with higher SUV39H1 expression (Figure 2C). Among the signature of genes specifically associated with H3K9me3 in TP53 double hit patients, 27% were enriched in promoter regions (*Online Supplementary Figure S1J, K*), Of interest, among these genes, we identified 5 potential tumor suppressor genes (*PHLDA3, TMEM74, COL27A1, ACKR3*, and *SPRY4*) that are specifically associated with H3K9me3 repressive mark in TP53 double hit patients in association with a poor outcome when under-expressed in MM cells and also significantly down-regulated in TP53 double hit patients and cell lines compared to wild-type TP53 status (*Online Supplementary Figure S1L, M*). Investigating the correlation between *SUV39H1* gene expression and protein expression by western blot, we identified a significant correlation (P<0.02) in a panel of 11 HMCL (*Online Supplementary Figure S1N*).

Thus, we decided to investigate the biological functions of SUV39H1 HMT in MM biology. To investigate the functional significance of SUV39H1 overexpression in MM cells, two HMCL with a high SUV39H1 (XG7 and XG19) and LP1 with intermediate expression were transduced with a doxycycline-inducible lentivirus containing a SUV39H1 shRNA. We included one cell line without del17p and no *TP53* mutation (XG7), a cell line without del17p and with *TP53* mutation (LP1), and a cell line with del17p and *TP53* mutation (XG19). No significant correlation between SUV39H1 expression and recurrent translocation was identified (*Online Supplementary Figure S1 I*). Doxycycline treatment induced a

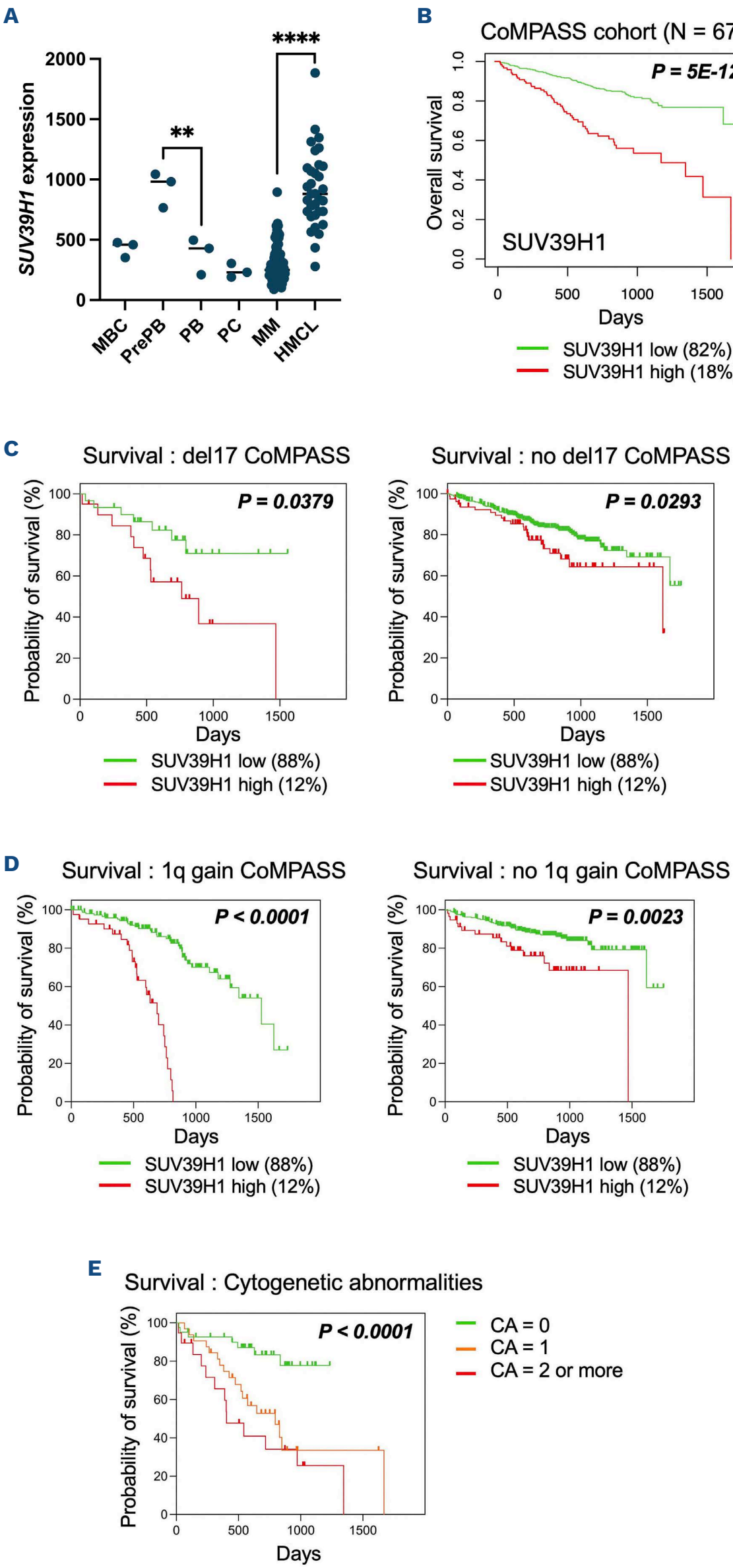


Figure 1. SUV39H1 expression is a prognostic factor in high-risk multiple myeloma patients. (A) SUV39H1 expression was quantified using RNA-sequencing in memory B cells (MBC), pre plasmablasts (PrePB), plasmablasts (PB), and plasma cells (PC) (N=3), primary multiple myeloma (MM) samples (N=97), and human myeloma cell lines (HMCL) (N=33). Wilcoxon test: ***P*<0.01, *****P*<0.0001. (B) Kaplan-Meier showing the overall survival (OS) of patients from the CoMMpass cohort (N=674) based on their SUV39H1 and SUV39H2 expression levels. High SUV39H1/2 expression was significantly associated with increased risk in MM patients. (C and D) Patients with or without del17p (C) or with or without 1q gain (D) of the CoMPASS cohort (N=674) were ranked according to SUV39H1 level and a maximum difference in OS was split patients into high or low SUV39H1 expression. High expression of SUV39H1 was significantly associated with high-risk in MM patients. (E) Kaplan-Meier showing the OS of newly diagnosed patients with MM (CoMMpass cohort) with high SUV39H1 expression according to the association with other high-risk cytogenetic abnormalities (CA) (HR CA). HR CA are defined by the presence of del(17p), t(4;14), and/or 1q gain. The green curve corresponds to patients with high SUV39H1 expression without other HR CA; the orange curve to patients with 1 CA; and the red curve to patients with ≥2 other HR CA. *P* value determined by the log-rank test comparison.

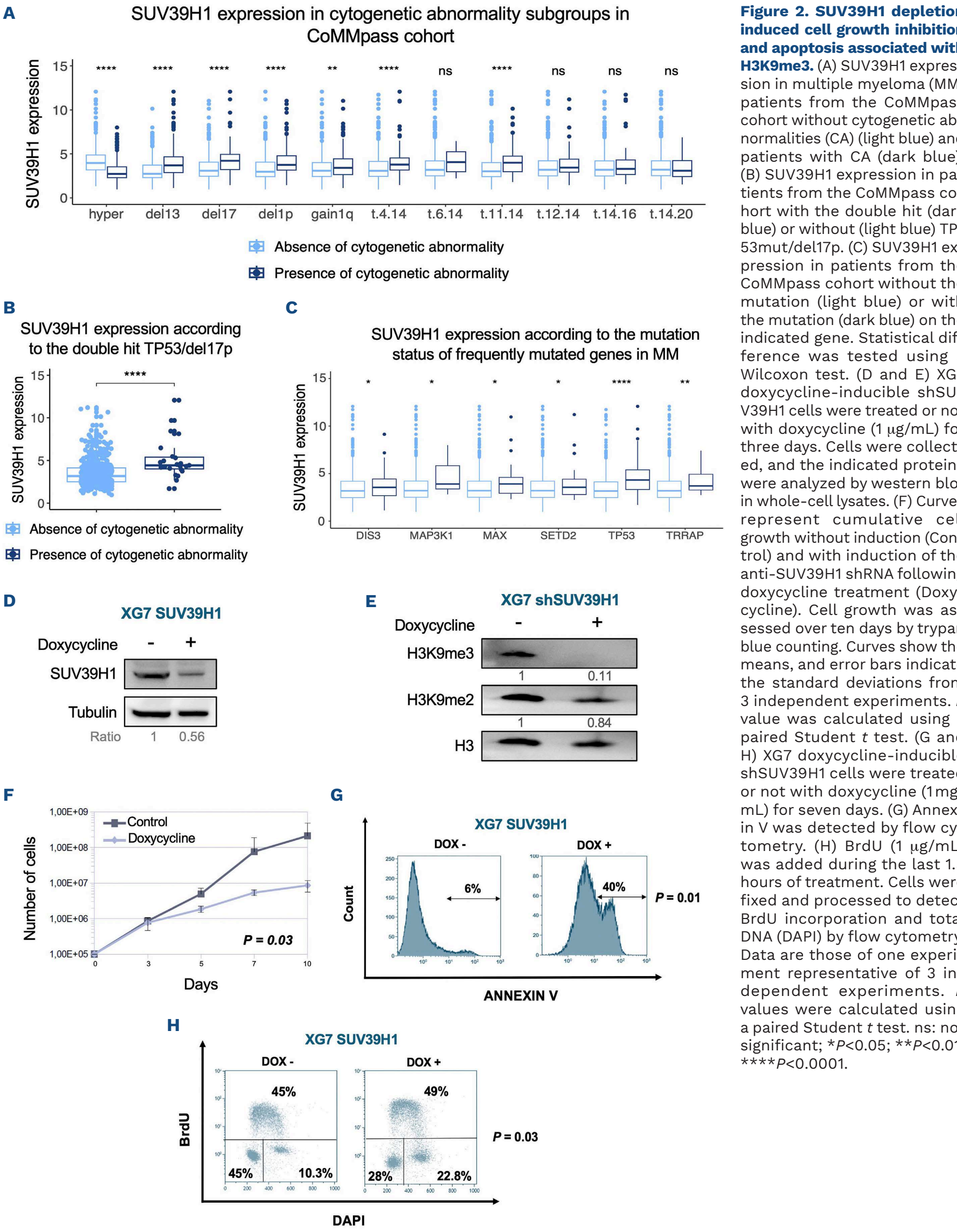
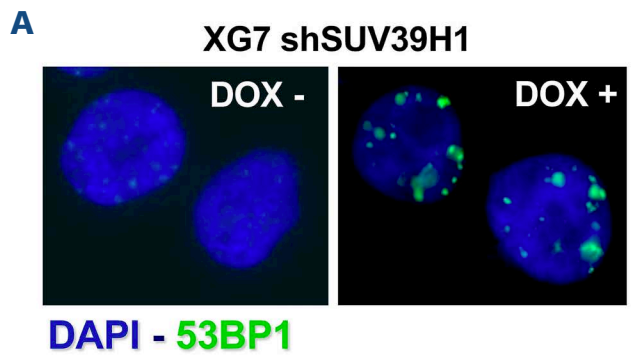
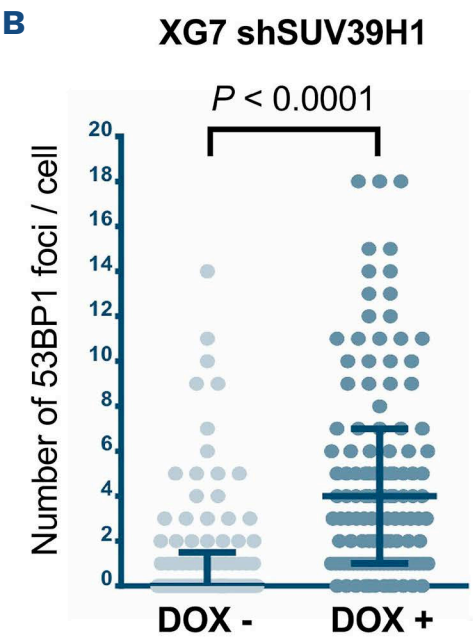
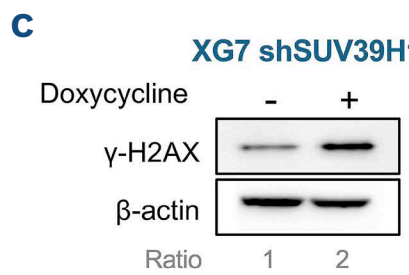
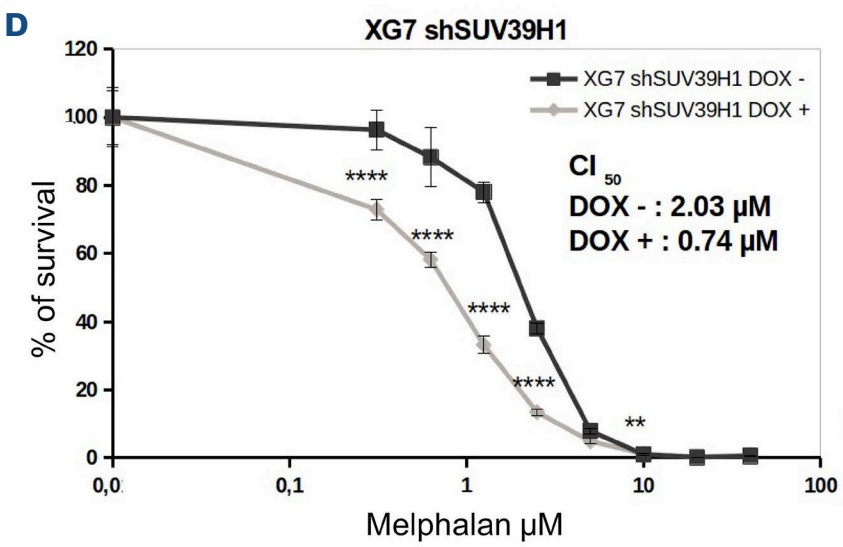


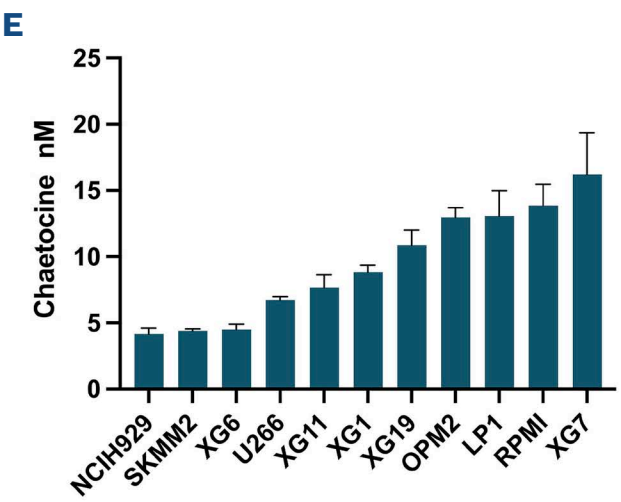
Figure 2. SUV39H1 depletion induced cell growth inhibition and apoptosis associated with H3K9me3. (A) SUV39H1 expression in multiple myeloma (MM) patients from the CoMMpass cohort without cytogenetic abnormalities (CA) (light blue) and patients with CA (dark blue). (B) SUV39H1 expression in patients from the CoMMpass cohort with the double hit (dark blue) or without (light blue) TP53mut/del17p. (C) SUV39H1 expression in patients from the CoMMpass cohort without the mutation (light blue) or with the mutation (dark blue) on the indicated gene. Statistical difference was tested using a Wilcoxon test. (D and E) XG7 doxycycline-inducible shSUV39H1 cells were treated or not with doxycycline (1 μg/mL) for three days. Cells were collected, and the indicated proteins were analyzed by western blot in whole-cell lysates. (F) Curves represent cumulative cell growth without induction (Control) and with induction of the anti-SUV39H1 shRNA following doxycycline treatment (Doxycycline). Cell growth was assessed over ten days by trypan blue counting. Curves show the means, and error bars indicate the standard deviations from 3 independent experiments. *P* value was calculated using a paired Student *t* test. (G and H) XG7 doxycycline-inducible shSUV39H1 cells were treated or not with doxycycline (1mg/mL) for seven days. (G) Annexin V was detected by flow cytometry. (H) BrdU (1 μg/mL) was added during the last 1.5 hours of treatment. Cells were fixed and processed to detect BrdU incorporation and total DNA (DAPI) by flow cytometry. Data are those of one experiment representative of 3 independent experiments. *P* values were calculated using a paired Student *t* test. ns: not significant; **P*<0.05; ***P*<0.01; *****P*<0.0001.

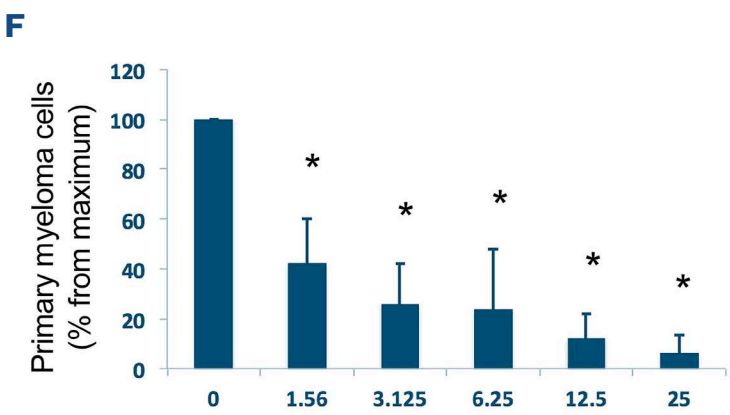


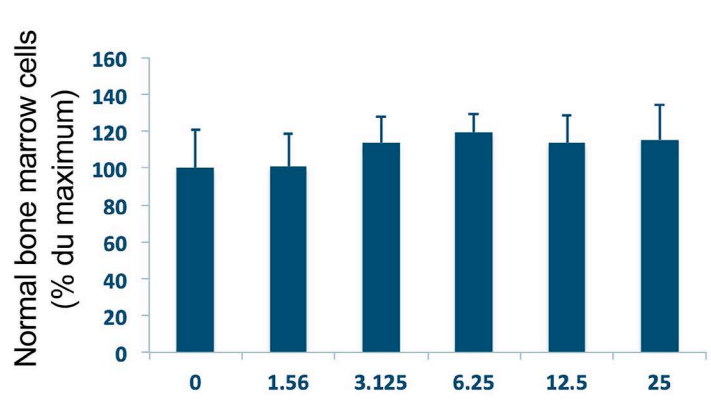


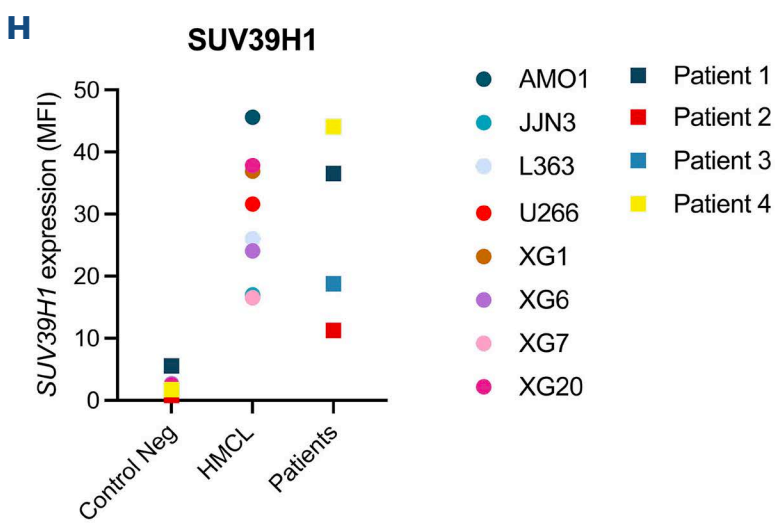












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Figure 3. SUV39H1 depletion induced DNA damage. (A) Cells were treated with doxycycline (1 μg/mL) for three days, deposited onto slides by cytospin centrifugation, fixed with 4% PFA (10 minutes) and immunofluorescence (IF) was performed to detect 53BP1 foci. DNA was stained with DAPI. (B) Dot plots showing the distribution of 53BP1 foci per cell. Statistical analysis was performed using an ImageJ macro on at least 100 nuclei. *P* value was calculated using Mann-Whitney U test. Data are those of one experiment representative of 3 independent experiments. (C) Cells were treated as indicated for three days, collected and the indicated proteins were analyzed by western blot in whole-cell lysates. Data are those of one experiment representative of 3 independent experiments. (D) XG7 doxycycline-inducible shSUV39H1 cells were pretreated or not with doxycycline (1 mg/mL) for two days. Then, treated with increasing concentrations of melphalan (0.015 to 40 μM) for four days. IC_{50} was calculated after viability assessment by CellTiter-Glo (CTG) luminescent cell viability assay. The graph shows average +/- standard deviation (SD) of 3 independent replicates. $^{**}P<0.01$; $^{****}P<0.0001$ (E) Human myeloma cell lines (HMCL) were cultured for four days with culture medium (control) and increasing concentrations of chaetocin (0.039 to 200 nM). IC_{50} was calculated after viability assessment by CTG luminescent cell viability assay. IC_{50} for each cell line was calculated using GraphPrism software. Data are mean values of 3 experiments determined on sextuplet culture wells. (F and G) Primary cells from bone marrow sample of 5 MM patients were treated with chaetocin at the indicated concentrations for four days. Myeloma cells ($CD38^+$, $CD138^+$) (F) and non-myeloma cells ($CD38^-$, $CD138^-$) (G) were analyzed by flow cytometry and expressed in % of control. Data shown are mean values of the results from samples of 5 multiple myeloma (MM) patients. Wilcoxon test: $^*P\leq0.05$. (H) SUV39H1 expression was assessed using flow cytometry in a panel of 8 HMCL and at the surface of MM cells in bone marrow samples from 4 patients. Mean fluorescence intensity data confirmed SUV39H1 protein expression in primary MM cells from patients.

51-85% reduction in *SUV39H1* expression at RNA and protein levels ($P<0.01$) (Figure 2D, *Online Supplementary Figure S2A*) and a significant reduction in H3K9me2 and H3K9me3 levels ($P<0.05$) (Figure 2E, *Online Supplementary Figure S2B*). SUV39H1 depletion induced a progressive inhibition of MM cell growth after doxycycline addition ($P=0.03$) (Figure 2F, *Online Supplementary Figure S2C*). Furthermore, SUV39H1 depletion induced apoptosis in HMCL ($P=0.01$) (Figure 2G, *Online Supplementary Figure S2D*). SUV39H1 depletion also affected the cell cycle distribution with an increase in the G2/M phase ($P<0.05$) (Figure 2H, *Online Supplementary Figure S2E*). SUV39H1 depletion was also associated with the accumulation of DNA double-strand breaks, as evidenced by the accumulation of 53BP1 foci and increased phosphorylation of the histone variant H2AX (Figure 3A-C). These results indicate that the overexpression of SUV39H1 in MMC could protect them from spontaneous DNA damage.

Since chemotherapy also induces DNA breaks, we next investigated whether SUV39H1 depletion could potentiate melphalan toxicity in MM cells. We found that the direct depletion of SUV39H1 with shRNA sensitized XG7 and XG19 MMC to melphalan (Figure 3D, *Online Supplementary Figure S2F*). Furthermore, we identified a significant correlation between *SUV39H1* basal gene expression and response to melphalan in a panel of HMCL (*Online Supplementary Figure S2G*).

Chaetocin has been shown to be an inhibitor of SUV39H HMT.[6] Chaetocin was also shown to affect the protein stability and expression of SUV39H1 at low concentration.[7] With higher concentration, chaetocin was also reported to inhibit another HMT, G9a, which affects H3K9me2/3 levels and has been implicated in MM pathobiology.[8] We tested the effects of chaetocin on MM cell growth using 11 different MM cell lines. We identified that chaetocin has a significant anti-myeloma effect at low doses, with an IC_{50} of between 4 and 17 nM in the 11 HMCL tested (Figure 3E). A high SUV39H1 expression was correlated with higher sensitivity to chaetocin ($r = -0.55$, $P=0.05$). As reported with *SUV39H1* depletion, chaetocin treatment inhibits H3K9 trimethylation in LP1 cells (*Online Supplementary Figure S2H*). Chaetocin treatment induced a significant increase in apoptotic cell death, accompanied by a marked reduction of the cells in S phase (*Online Supplementary Figure S2 I, J*). We, therefore, aimed to validate these results using primary MM cells from patients co-cultured with their BM microenvironment and exogeneous IL-6 (2 ng/mL). Primary BM samples were collected with the approval of the institutional board of Montpellier University Hospital (DC2008-417) after patients written informed consent. We identified a specific toxicity of chaetocin at nanomolar concentrations on primary MM cells from patients (N=5 MM patients; $P<0.05$) (Figure 3F) whereas no significant toxicity was observed on normal BM cells in the co-culture (Figure 3G). SUV39H1 protein expression in HMCL and primary MM cells from patients was validated by flow cytometry (Figure 3H). Altogether, our data suggest that targeting of SUV39H1 may represent a promising therapeutic approach in MM.

To analyze the transcriptional programs regulated by SUV39H1 in MM cells, XG19 and XG7 transduced with a doxycycline-inducible lentivirus containing a SUV39H1 shRNA were treated with doxycycline for 48 hours and gene expression profiles were analyzed using Affymetrix microarrays. LP1, XG19, and XG7 HMCL were also treated with chaetocin and the transcriptomic profiles were analyzed. A total of 215 genes were significantly up-regulated in the treated cells and SUV39H1 depleted cells compared with control (fold change ≥2; false-discovery rate <0.05) (*Online Supplementary Figure S2K*). No significantly down-regulated genes were identified. Gene set enrichment analysis revealed a significant enrichment in TP53 and RB1 target genes, polycomb PRC2 target genes, genes associated with DNA methylation, mature plasma cell genes, and genes involved in ferroptosis and inflammatory response. We previously reported the presence of H3K9me3 and H3K27me3 co-localization in gene promoters of tumor suppressor genes in MM cells.[2] Our data highlight the deregulation of genes related to TP53 pathway and associated with epigenetic

regulation reported to participate in the pathophysiology of the MM.[2,9-12]

SUV39H1 has a significant impact on cancer development and progression across multiple types of cancer. Specifically, the H3K9me3 modification catalyzed by SUV39H1 can suppress the tumor suppressor gene *p16INK4a*, which leads to unrestricted cell proliferation in both acute myeloid leukemia (AML) and lung cancer cells.[13,14] Cheatocin was reported to induce differentiation of AML cells and synergistic toxicities when combined with other epigenetic drugs.[7] Recently, Lopez-Cobo *et al*. demonstrated that SUV39H1 ablation enhances the long-term function of chimeric antigen receptor T-cell therapy in solid tumors,[15] underlining a potential common interest in targeting MM cells and improving immune-based therapies.

Treatment outcomes of patients with MM have significantly improved. However, a subpopulation of MM patients with high-risk CA still experiences early death due to disease progression. We believe our study may provide new therapeutic avenues towards the development of targeted therapies based on molecular pathology that may help to improve the outcome of patients with high-risk MM.

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Disclosures

No conflicts of interest to disclose.

Contributions

LA, SO and JD carried out the research and helped write the paper. MT, GR and NR helped carry out the research. GC, LV, and CH performed clinical data analysis and helped write the paper. AK and MA performed bio-informatic analyses. AMM and GC helped carry out the research and helped write the paper. JM and CB supervised the research and preparation of the paper.

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Data-sharing statement

For original data, please contact c-bret@chu-montpellier.fr and jerome.moreaux@igh.cnrs.fr. Gene expression data have been deposited in the ArrayExpress public database under accession numbers E-TABM-937, E-MTAB-372 and E-TABM-1088.

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