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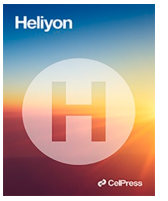

Research article

ZNF521 promotes acute myeloid leukemogenesis by suppressing the expression and acetylation of SMC3

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ABSTRACT

Zinc finger protein 521 (ZNF521) participates in the self-renewal of hematopoietic stem cells, and its abnormal expression has been implicated to promote leukemia. However, the specific role of ZNF521 in leukemia has not been fully understood. In this study, we aimed to further elucidate its role. Using acute leukemia cell line THP-1, we demonstrated that knocking down ZNF521 inhibited leukemia cell proliferation, promoted apoptosis, and induced cell arrest in G2/M phase. Interestingly, we also observed the upregulation of SMC3 expression and acetylation, as well as the downregulation of histone deacetylases 8 (HDAC8), CDK2, and CDK6. The proliferation inhibition was reversed by knocking down SMC3, suggesting the key role of SMC3 reduction in ZNF521 elevated proliferation. Conversely, ZNF521 overexpression in HL-60 cells resulted in enhanced proliferation and inhibited apoptosis. Furthermore, we discovered that ZNF521 can interact with HDAC8, which deacetylates SMC3, and the interaction promotes proliferation and suppresses apoptosis. Notably, when HDAC8 was knocked down or its activity was inhibited by a HDAC8 inhibitor, the previous observed trend was reversed. Consequently, ZNF521 plays a critical role in acute myeloid leukemogenesis by reducing the expression and acetylation of SMC3. Overall, this study sheds light on the potential for targeted treatment in highly ZNF521 expressed acute myeloid leukemia, providing a valuable clue for precise and effective therapeutic approaches.

1. Introduction

Proto-oncogene Zinc finger protein 521 (ZNF521) plays a crucial role in maintaining stem cell renewal and blocking cellular differentiation by regulating gene transcription, and it is involved in leukemogenesis process. ZNF521 was first identified as being activated by retroviral integration in mouse B-cell lymphoma [1]. It is highly expressed in acute myeloid leukemia [2,3], and is upregulated through the cooperation of HOXC13 and SPI1 [4]. Zfp521 enhances B-cell proliferation by activating cyclin D1 expression [5], maintains stem cell characteristics, and contributes to leukemogenesis [3,6]. It promotes acute myeloid leukemia (AML) in MLL-AF9 leukemia or E2A-HLF B-cell leukemia [2,7,8]. But the signal pathways involved remain unclear.

Structural maintenance of chromosome 3 (SMC3) is a subunit of cohesin complex [9]. Cohesin regulates the integration and segregation of sister chromatid during the cell cycle, with SMC3 promoting genomic integrity during DNA replication [10] and

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mediating DNA loop extrusion for chromatin remodeling in interphase cells [11]. SMC3 is also implicated in cancer progress. SMC3 downregulation promotes pancreatic cancer through inhibiting the expression of Rab27a, and its low expression suggests poor prognosis [12]. SMC3 is essential for hematopoiesis, with reduced expression impeding the maturation of myeloid cells [13]. In a Down syndrome leukemia model, Smc3 haploinsufficiency cooperates with GATA1 to enhance clonogenic potential and proliferation of acute megakaryocyte leukemia [14].

SMC3 acetylation is crucial for cell cycle regulation. It is deacetylated during G1 phase, with acetylation level increasing during S phase, and peaking in G2/M phase. SMC3 is deacetylated by histone deacetylases 8 (HDAC8) in G2/M phase to transition to the G1 phase, and the failure of the deacetylation arrests cells in G2/M phase [15,16]. Although ZNF521 interacts with HDAC3 to inhibit osteoblast maturation by suppressing Runx2 expression [17], it is not known whether ZNF521 interacts with HDAC8 to deacetylate SMC3 and regulate the cell cycle.

While studies have investigated ZNF521 and SMC3 respectively, their association remains unclear. In this study, we will explore whether ZNF521 collaborates with HDAC8 to downregulate SMC3, and examine its impact on leukemia.

2. Materials and methods

2.1. Cell culture

The cell lines of THP-1, HL-60, and HEK 293T were obtained from Procell life science & technology (China). These cells had been verified to be free of mycoplasma and authenticated through STR profiling. THP-1 (RRID:CVCL_0006) cells were maintained in RMPI 1640 medium (BI, USA) with 10 % fetal bovine serum (FBS) (Thermo Fisher, USA) and 0.05 mM β-mercaptoethanol (Sigma, USA) at 37 °C under 5 % CO_2. HL-60 (RRID:CVCL_0002) cells were cultured in IMDM medium (BI, USA) with 20 % FBS at 37 °C under 5 % CO2. 293T (RRID:CVCL_0063) cells were cultured in high glucose DMEM (BI, USA), containing 10 % FBS at 37 °C and 5 % CO2.

2.2. Plasmid constructs

Plasmids of lenti-shZNF521 and lenti-ZNF521 were purchased from Origene (USA). The sequence of shSMC3 was inserted into Lenti-LV3 vector (Jima, China).

The sequence of shMSC3 is as follow:

shSMC3: CAGCGGTTGGCTTTATTGC.

2.3. Transfection

Cells (5×10^5) were seeded in a 6-well plate and cultured overnight before transfection with siRNA using Lipofectamine RNAiMAX (Thermo fisher) according to protocol. Briefly, 10 μM siRNA and 10 μl RNAiMAX were separately incubated with 100 μl optical-medium (Thermo fisher) at the temperature of 22–25 °C (Room temperature, RT) for 5 min. The solutions were then mixed and incubated at RT for additional 10 min the mixture was added to the cells, and after incubation at 37 °C 5 % CO2 for 48 h, the cells were collected for further analysis. The siHDAC8 sequence is as follow:

SiHDAC8:GCTGGGAGCUGACACAAUA.

2.4. Lentiviral production and transduction

293T cells (5×10^6) were transfected with the 15 μg backbone plasmid (lenti-shZNF521 or lenti-ZNF521), 10 μg PsPAX2 and 5 μg pMD2G using lipofectamine (Invitrogen, USA) after cultured in 10 cm dish for 24 h. The medium was harvested by centrifugation at 5,000 g for 10 min at 4 °C after additional 48 h–72 h of culture. The lentiviral supernatant was collected by centrifugation at 3,000 g for 10 min for transduction or stored at −80 °C.

THP-1 or HL-60 cells (5×10^5) were seeded in a 6-well plate and cultured overnight. They were then incubated with lentiviral supernatant and 4 μg of polybrene for 72 h before further analysis.

2.5. Quantitative RT-PCR

Total RNA was extracted using RNeasy purification kit (Qiagen, USA) after the cells were collected by centrifugation at, 90*g* for 10 min at 4 °C. cDNA was synthesized using reverse transcriptase III (Thermo fisher, USA). Quantitative PCR was performed with FastStart Essential DNA Green Master (Roche, USA). Relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method with GAPDH as a reference. The primers were as follow:

HDAC8-F:TCGCTGGTCCCGGTTTATATC
HDAC8-R:TACTGGCCCGTTTGGGGAT
SMC3-F: AACATAATGTGATTGTGGGCAGA
SMC3-R: TCCTTTTTGGCACCAATAACTCT
CDK2-F: CCAGGAGTTACTTCTATGCCTGA
CDK2-R: TTCATCCAGGGGAGGTACAAC
CDK6-F: GCTGACCAGCAGTACGAATG

CDK6-R: GCACACATCAAACAACCTGACC
GAPDH-F:GGAGCGAGATCCCTCCAAAAT
GAPDH-R:GGCTGTTGTCATACTTCTCATGG.

2.6. CCK-8 assay

To evaluate cell viability, 5×10^3 cells per well were seeded into a 96-well plate and incubated at 37 °C, 5 % CO2 for 0, 24, 48, 72, 96 and 120 h the cells subjected to a cell counting kit-8 assay by adding 10 μl of CCK-8 solution (Beyotime, China) and further incubating 3 h, the absorbance was measured at 450 nm.

2.7. Apoptotic analysis

Apoptosis was detected using the Annexin V FITC apoptosis detection kit (BD biosciences). Cells were cultured in a 6-well plate at 2×10^5 per well overnight and then transduced with target shRNA or ZNF521 overexpression lentivirus for 48 h. The cells were collected by centrifugation at 90*g* for 10 min at 4 °C, washed three times with pre-chilled PBS, and incubated with 5 μl Annexin V FITC and 5 μl propidium iodide for 15 min before analysis.

2.8. Cell cycle analysis

The cells at the density of 2×10^5 per well were cultured in a 6-well plate for 48 h. The cells were then collected by centrifugation at 90*g* for 10 min at 4 °C. The pellet was washed with pre-chilled PBS, and fixed with 70 % ethanol at 4 °C overnight. Subsequently, the cells were then incubated with 65 μg/ml propidium iodide (BD Biosciences) and 50 μg/ml RNase A (Sigma, USA) for 30 min at 4 °C and analyzed by flow cytometry.

2.9. Immunoprecipitation

THP-1 cells (1×10^7) were collected by centrifugation at 90*g* for 10 min at 4 °C. The pellet was lysed using RIPA buffer (Sigma) containing a protein inhibitor cocktail (Roche) on ice for 30 min, and the lysate was collected by centrifugation at 13,000 g for 10 min at 4 °C, and the protein concentration was determined using BCA method. For immunoprecipitation, 600 μg of protein was incubated with 20 μl protein G magnetic beads (Thermo Fisher, USA) and 2 μg anti-ZNF521 antibodies (Cat No. TA319089, Origene, USA) at 4 °C overnight. The beads were washed three times using RIPA buffer, and then subjected to western blotting.

2.10. Western blotting

Cells were lysed using RIPA buffer containing protein inhibitor cocktail, and the protein concentration was determined by the BCA method. An aliquot of lysate of 50 μg was loaded onto SDS-PAGE gel, and transferred to PVDF membrane. The membrane was blocked with 5 % skimmed milk in TBST buffer at RT for 3 h, and then incubated overnight at 4 °C with primary antibodies against ZNF521 (cat no. TA319089, Origene, USA), HDAC8 (Cat No. TA809689, Origene, USA), SMC3 (Cat No. 14185-1-AP, Proteintech, China), ac-SMC3 (Cat No. MABE1073, Sigma, USA), and GAPDH (Cat No. 60004-1-Ig, Proteintech, China). After washing with TBST 3 times, the membrane was incubated with either goat anti-mouse IgG (Cat No. SA00001-1, Proteintech, China) or goat anti-rabbit IgG (Cat No. SA00001-2, Proteintech, China) for 3 h, followed by ECL detection, and was quantified using Image J (National Institutes of Health).

2.11. Immunofluorescence

THP-1 cells in logarithmic growth (2×10^6) were harvested by centrifugation at 90*g* for 10 min at 4 °C, and washed by pre-chilled PBS. The cells were then fixed by paraformaldehyde for 10 min at RT. The cells were then incubated with PBS containing 0.5 % Triton X-100 on ice for 10 min and blocked by PBS containing 3 % BSA for 30 min at RT. The cells were then incubated overnight at 4 °C with antibodies of rabbit anti-ZNF521 (Cat No. TA319089, Origene, USA), and mouse anti-HDAC8 (Cat No. CF809689, Origene, USA) at a dilution of 1:100. After washing three times by pre-chilled PBS, the cells were then incubated with Alexa Fluor 488 donkey anti-rabbit IgG (Cat No. ab150073, Abcam, USA) and Alexa Fluor 594 donkey anti-mouse IgG (Cat No. ab150108, Abcam, USA) at RT for 60 min, stained with 100 ng/mL DAPI (Sigma) for 10 min at RT, and finally observed under laser confocal fluorescence microscopy (Zeiss, Germany).

2.12. Protein interaction prediction

The protein sequences of ZNF521 and HDAC8 were obtained from Uniport website and imported to AlphaFold 3 for prediction. In the resulting figure, blue color indicates high prediction confidence, and orange color represents low prediction confidence.

2.13. Statistical analysis

The data are presented as the mean ± SD. Statistical analysis was performed using one-way ANOVA or two-way ANOVA followed

by Bonferroni's post hoc test as appropriate.

3. Results

3.1. ZNF521 promotes proliferation and suppresses apoptosis in leukemia cells

To investigate the effect of ZNF521 on leukemogenesis, we analyzed the impact of ZNF521 knockdown on proliferation, apoptosis, and cell cycle. Folling ZNF521 knockdown in THP-1 cells (Fig. S1A), we observed a decrease in proliferation (Fig. S1B) and an increase in apoptosis (Fig. S1C), and cell cycle arrest in the G2/M phase (Fig. S1D). The results indicate that ZNF521 knockdown inhibits cell proliferation, promotes apoptosis, and causes cell cycle arrest in G2/M phase.

3.2. ZNF521 suppresses the expression and acetylation of SMC3

Given that ZNF521 knockdown results in cell cycle arrest at the G2/M phase, we investigated whether ZNF521 is involved in cell cycle regulation. We analyzed the expression of key cell cycle proteins, including CDK2, CDK6, HDAC8, and SMC3 in THP-1 cells with ZNF521 knockdown using quantitative RT-PCR. The results showed a decrease in the expression of CDK2, CDK6 and HDAC8, while SMC3 expression increased (Fig. 1A–D). We focus on SMC3 as its upregulation causes cell arrest at G2/M phase, and on HDAC8 due to its involvement in the deacetylation of SMC3 for exiting G2 phase. Further analysis by western blotting confirmed that ZNF521 knockdown led to increased expression and acetylation of SMC3, and alongside a decrease in HDAC8 expression (Fig. 1E).

3.3. SMC3 plays a key role in ZNF521 elevated proliferation

After we showed that ZNF521 knockdown increases SMC3 expression and acetylation, we considered that whether downregulating SMC3 could counteract the proliferation inhibition caused by ZNF521 knockdown. Our results demonstrated that SMC3 knockdown effectively reversed the proliferation inhibition observed in THP-1 cells with ZNF521 knockdown (Fig. 2A and B), suggesting that ZNF521 promotes proliferation by inhibiting SMC3. Further analysis revealed that SMC3 knockdown also mitigated the increase in

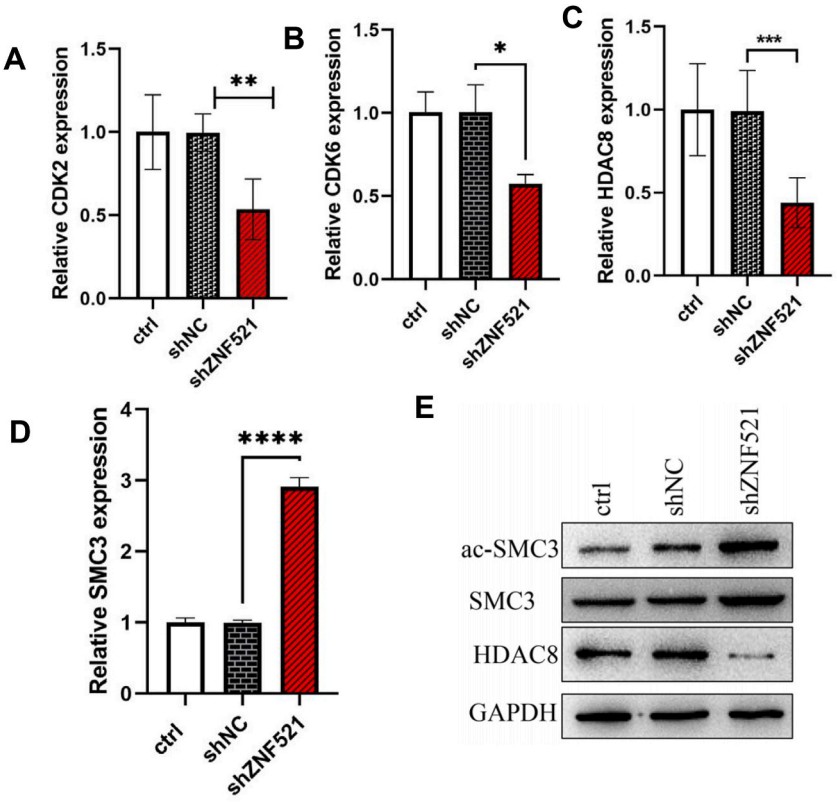

Fig. 1. ZNF521 promotes HDAC8 expression, but suppresses SMC3 expression and acetylation. A-D:Quantitative RT-PCR analysis of CDK2 (A), CDK6 (B), HDAC8 (C), and SMC3 (D) were performed after THP-1 cells were transduced with shZNF521 for 72 h. E: western blotting analysis of SMC3 expression and acetylation and HDAC8 expression after THP-1 cells were transduced with shZNF521 for 72 h. Data are presented as the mean ± SD of three independent experiments. *: $p < 0.05$, **: $p < 0.01$; ***: $p < 0.001$, ****: $p < 0.0001$.

apoptosis and G2/M arrest induced by ZNF521 knockdown (Fig. 2C and D). These findings indicate that ZNF521 indeed promotes proliferation and inhibits apoptosis through the suppression of SMC3.

3.4. ZNF521 interacts with HDAC8 to inhibit SMC3

We have showed that HDAC8 was downregulated following ZNF521 knockdown (Fig. 1C and E). Since previous studies have reported interaction between ZNF521 and HDAC3 [17,18], we investigated whether ZNF521 also interacts with HDAC8. Immunoprecipitation assays confirmed that ZNF521 interacted with HDAC8 (Fig. 3A) and immunofluorescence microscopy further revealed their colocalization (Fig. 3B). Additionally, we utilized AlphaFold 3, an artificial intelligence tool for predicting protein interaction [19], which corroborated the interaction between ZNF51 and HDAC8 (Fig. 3C).

Given that ZNF521 interacts with HDAC8, we examined that whether HDAC8 is involved in the downregulation of SMC3. HL-60 cells were infected with ZNF521 overexpression lentivirus and treated with either siHDAC8 or HDAC8 inhibitor PCI-34051. Our results showed that ZNF521 overexpression inhibited SMC3 expression and acetylation, but these effects were reversed by HDAC8 knockdown (siHDCA8) or PCI-34051 (HDCA8i) (Fig. 4A). This suggests that ZNF521 collaborates with HDAC8 to suppress SMC3 expression and acetylation.

Furthermore, we analyzed the impact of HDAC8 on ZNF521-mediated proliferation and apoptosis. ZNF521 overexpression enhanced the proliferation of HL-60 cells, and reduced apoptosis. However, these effects were reversed by siHDAC8 or HDAC8i (Fig. 4B–E). SMC3 knockdown also restored the reversal caused by siHDAC8 or PCI-34051. These findings indicate that HDAC8 plays a

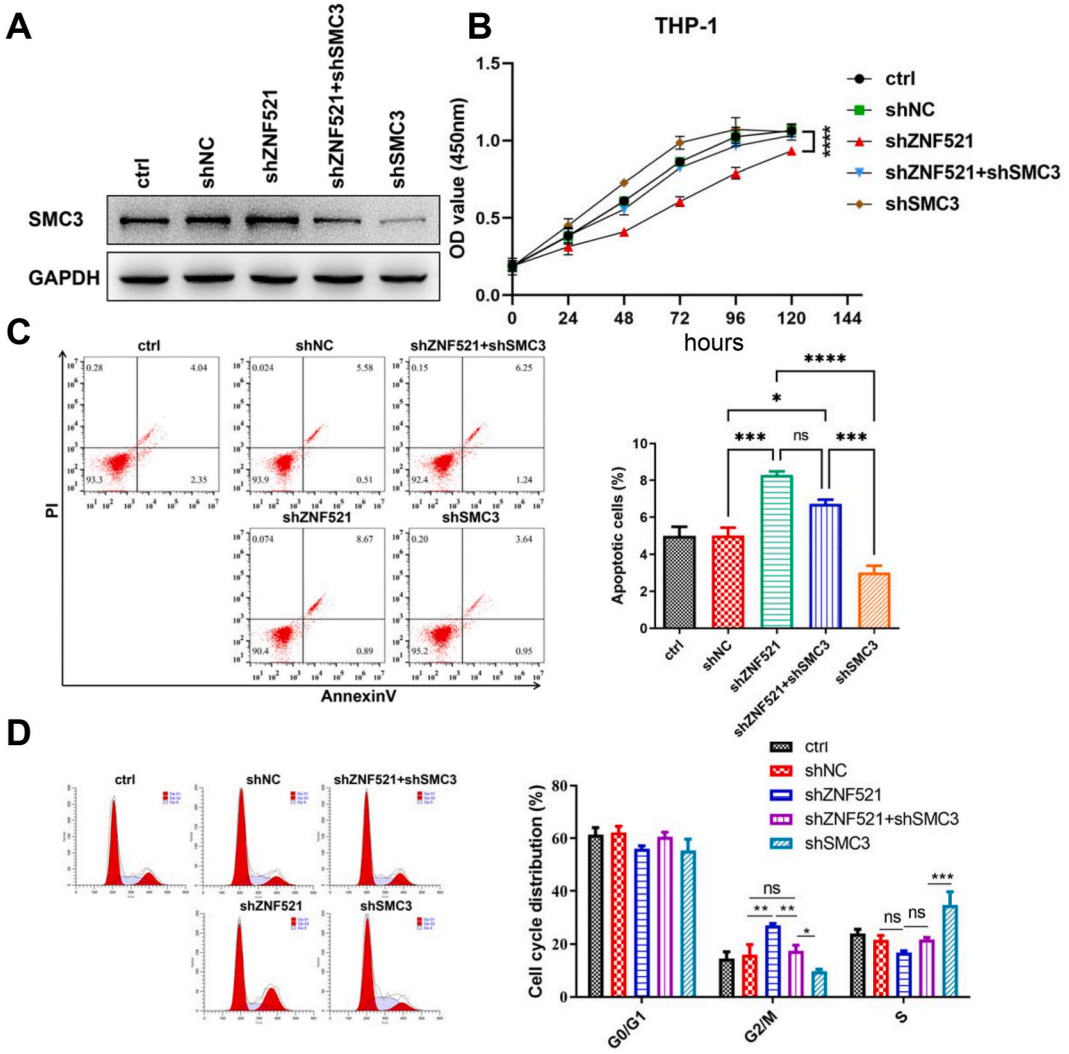

Fig. 2. SMC3 plays a key role in ZNF521 induced proliferation. A–D: SMC3 expression (A), proliferation (B), apoptosis (C), and cell cycle (D) were analyzed after THP-1 cells were transduced with shZNF521 and/or shSMC3 for 72 h. Data are presented as the mean ± SD of three independent experiments. Ns: not significant, *: $p < 0.05$, **: $p < 0.01$; ***: $p < 0.001$, ****: $p < 0.0001$.

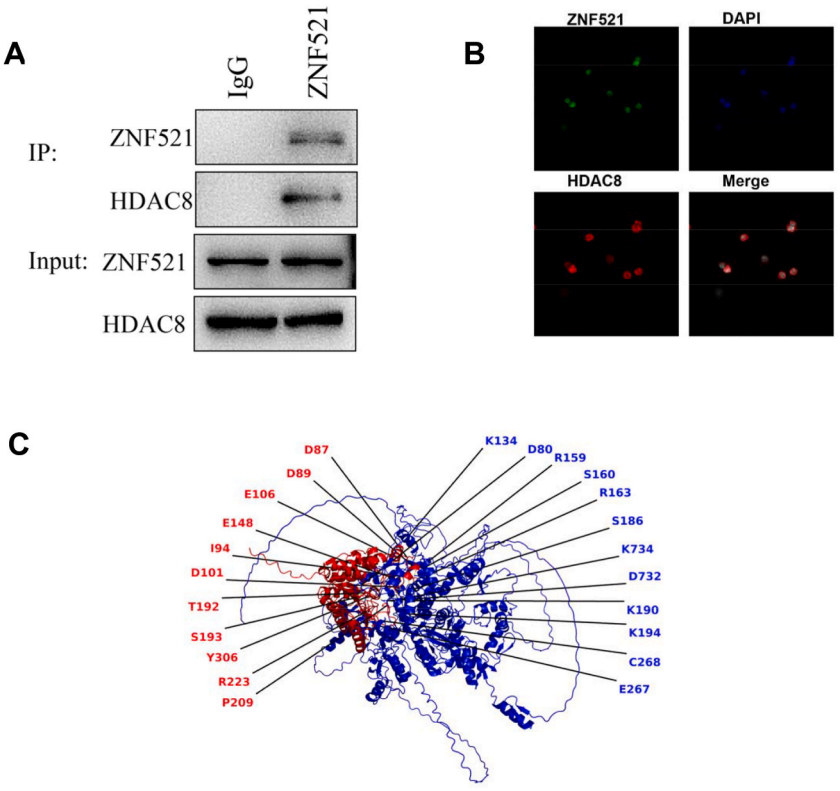

Fig. 3. ZNF521 interacts with HDAC8. A: The interaction of ZNF521 and HDAC8 by immunoprecipitation in THP-1 cells. B: immunofluorescent analysis of ZNF521 and HDAC8 in THP-1 cells. C: the interaction of ZNF521 (blue) and HDAC8 (red) predicted by AlphaFold 3.

crucial role in the proliferation of leukemia cells induced by ZNF521.

4. Discussion

ZNF521 plays a pivotal role in promoting leukemia development. Retroviral insertion-induced overexpression of ZNF521 has been linked to the emergence of lymphomas in mice [20]. Furthermore, the protein has been implicated in various malignancies including ovarian cancer [21], gastric cancer [22], and acute myeloid leukemia [7]. Despite extensive research, the precise mechanism underlying ZNF521's role in acute leukemia remains enigmatic. Our study reveals that ZNF521 not only fuels the proliferation of leukemia cells but also suppresses apoptosis. We discovered that knocking down ZNF521 leads to cell cycle arrest in G2/M phase in leukemia cells. This arrest is accompanied by an elevation in both the expression and acetylation of SMC3. It is indeed very interesting, given that failure to deacetylate SMC3 leads to cell cycle arrest in G2/M phase.

Our findings underscore that ZNF521 promotes the proliferation of acute leukemia cells by downregulating its expression and acetylation. SMC3, a component of the cohesin complex, is a critical player in the cell cycle regulation [16]. Its acetylation is essential for cells to progress into the G2 phase, while deacetylation is necessary for their exit from G2/M phase. Our study demonstrated that when ZNF521 is knocked down, cells are halted in the G2/M phase (Fig. S1D), and this halt is accompanied by an increase in both the expression and acetylation of SMC3 (Fig. 1D and E). Notably, the expression of HDAC8, an enzyme responsible for deacetylating SMC3 [15], is reduced following ZNF521 knockdown. This observation is particularly intriguing given the established link between low SMC3 expression and both hematopoiesis and leukemia progression. AML patients exhibiting low SMC3 expression often face a poorer prognosis, while other cohesin proteins do not exhibit such a correlation [23]. Smc3 haploinsufficiency has been shown to impede germinal center differentiation and foster lymphomagenesis [24], further implicating low SMC3 level in leukemia. But how ZNF521 inhibits SMC3 is to be elucidated.

Considering the involvement of HDAC8 in SMC3 deacetylation [15], we investigated its role in ZNF521-mediated SMC3 downregulation. Our results reveal a direct interaction between ZNF521 and HDAC8 (Fig. 3A and B). The interaction appears to be crucial, as the ZNF521-induced reduction in SMC3 expression and acetylation is abrogated by either by siRNA-mediated silence of HDAC8 or pharmacological inhibition of HDAC8 (Fig. 4A). HDAC8 has been implicated in acute leukemia, sustains the leukemia status by inhibiting p53 acetylation [25,26]. Notably, HDAC8 inhibition has been shown to halt leukemia progression [26,27]. Given that HDAC8 deacetylates SMC3 during the late G2 phase [15], and that ZNF521 knockdown leads to cell cycle arrest in the G2/M phase (Fig. S1D), it is conceivable that ZNF521 and HDAC8 collaborate to regulate SMC3 deacetylation. Further, our findings indicate that

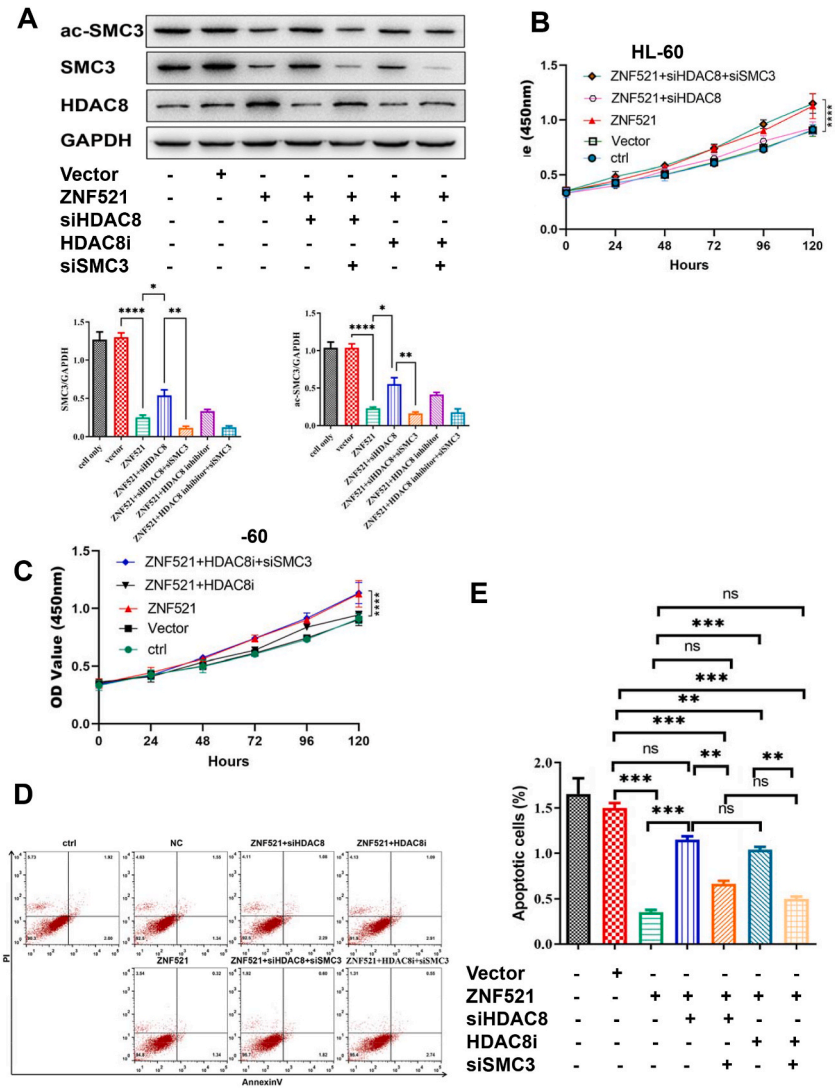

Fig. 4. The interaction of ZNF521 and HDAC8 promotes proliferation and inhibits apoptosis by suppressing SMC3. A: SMC3 expression and acetylation is inhibited by ZNF521 and HDAC8. Upper panel: the WB and down panel: graphic analysis of WB. B–E: proliferation (B and C) and apoptosis (D–E) were analyzed after HL-60 cells were transduced with ZNF521 overexpression plasmid, and treated with siHDAC8 or HDAC8 inhibitor, and siSMC3 for 72 h. Data are presented as the mean ± SD of three independent experiments. Ns: not significant, **: $p < 0.01$; ***: $p < 0.001$, ****: $p < 0.0001$.

while HDAC8 inhibition reduces leukemia cell proliferation and induces apoptosis, these effects are reversed by SMC3 knockdown (Fig. 4D–F). This suggests that HDAC8 plays a pivotal role in both ZNF521-mediated SMC3 deacetylation and leukemia development.

In summary, our study established that ZNF521 promotes the development of hematological malignancies by interacting with HDAC8 to downregulate the expression and acetylation of SMC3 (Fig. 5).

Data availability

All data generated or analyzed during this study are included in this published article.

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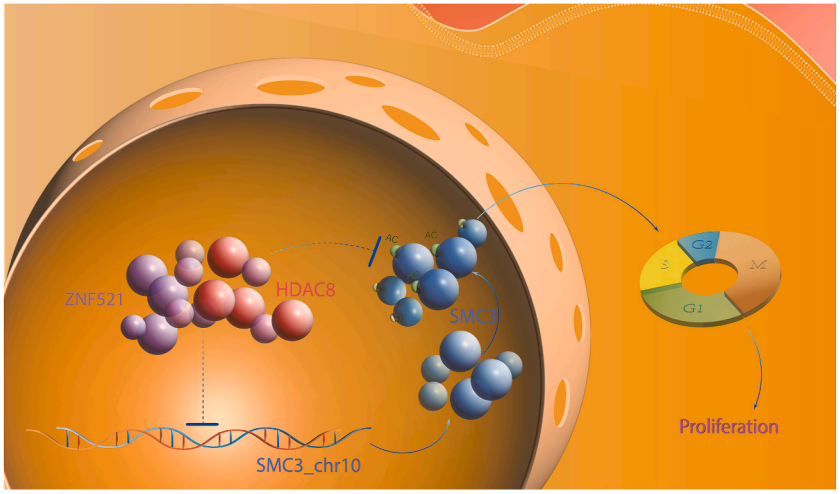

Fig. 5. The model of ZNF521interacting with HDAC8 to suppress the expression and acetylation of SMC3.

CRediT authorship contribution statement

Rong Qin: Formal analysis, Data curation. **Tongshuo Yang:** Formal analysis. **Hongchao Jiang:** Investigation, Funding acquisition. **Ming Yu:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37528.

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