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Etoposide-induced SENP8 confers a feed-back drug resistance on acute lymphoblastic leukemia cells

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ABSTRACT

Chemotherapy is the most common treatment for acute lymphoblastic leukemia (ALL). However, many ALL patients eventually develop relapse and treating relapsed ALL has always been challenging. Therefore, exploring the resistance mechanism of chemotherapeutic drugs and proposing feasible intervention strategies are of great significance for ALL treatment. Here, we show that *SENP8,* whose coding protein is an important deNEDDylase targeting the substrate for deNEDDylation, is highly expressed in relapsed ALL specimens. Interestingly, over-expressing SENP8 specifically reduces the chemosensitivity of ALL cells to etoposide (VP-16) and significantly alleviates the proapoptotic effect of VP-16 on ALL cells. By contrast, NEDDylation inhibition reduces the chemosensitivity of ALL cells to VP-16. Furthermore, VP-16 induces SENP8 accumulation and the instability of MDM2 as well as the stabilization of p53 in ALL cells, and SENP8 knockdown can sensitize ALL cells to VP-16. Our study reveals a novel function of SENP8 in ALL and that VP-16-induced SENP8 confers a feed-back drug resistance on ALL cells, suggesting a possibility of overcoming the chemotherapeutic resistance to VP-16 via targeting SENP8.

1. Introduction

ALL is an aggressive malignancy of lymphoid progenitor cells with a peak incidence in early childhood and in older age [1,2]. In terms of clinical treatment, multimodal chemotherapy forms the therapeutic base of ALL therapy [3]. Nowadays, the outcome of ALL patients has been substantially improved, particularly in children whose 5-year overall survival rate has exceeded 90 % [4]. However, 15–20 % of ALL patients eventually develop relapse and treating relapsed ALL has always been challenging, not only the survival rate lags well behind that observed at initial diagnosis, but also the outcome is even worse at the second or later relapse [5]. Therefore, exploring the resistance mechanism of chemotherapeutic drugs and proposing feasible intervention strategies are still of great significance for ALL treatment.

As a ubiquitin-like protein most homologous to the ubiquitin, NEDD8 is widely distributed in various tissues and activates the cullin-RING ligases (CRLs) via covalent conjugation to individual cullins, a cascade process termed NEDDylation wherein NEDD8 is activated by the NEDD8-specific E1 enzyme (a heterodimer comprising NAE1 and UBA3), then transferred to the E2 enzymes UBE2M or UBE2F, and finally conjugated to target proteins by E3 enzymes. Yet, protein NEDDylation is beyond CRLs and growing non-cullin NEDDylation substrates have also been identified over the past; NEDDylation is found throughout the cell and is essential for mammals and other lower eukaryotes [6]. On the contrary, counteracting NEDDylation is executed by the deNEDDylases. Of these, the metalloprotease COPS5, a component of the eight-subunit complex, is the major cullin deNEDDylases [7]. Unlike it, the cysteine protease SENP8 (also known as DEN1 or NEDP1) possesses distinct

Abbreviations: ALL, acute lymphoblastic leukemia; NEDD8, neural precursor cell expressed developmentally down-regulated protein 8; COPS5, COP9 signalosome complex subunit 5; VP-16, etoposide; 6-TG, 6-thioguanine; DNR, daunorubicin; VCR, vincristine; Ara-C, cytarabine; MTX, methotrexate; SDS, sodium dodecyl sulfate.

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functions in proteolytic processing of NEDD8 precursor and deconjugating primarily non-cullin NEDD8-conjugation, as well as preventing hyper NEDDylation of cullins [8]. Notably, NEDD8 and these NEDD8-specific enzymes are highly expressed in various cancers and dysregulation of NEDDylation has also been linked to many human diseases [9–12]. However, it is poorly known about the relevance of NEDDylation pathway in ALL, let alone functioning in ALL relapse. In this study, we show that the relapsed ALL specimens have significantly higher expression of *SENP8* rather than other NEDDylation pathway components compared to the diagnostic specimens. Moreover, overexpressing SENP8 specifically reduces the chemosensitivity of ALL cells to VP-16 and alleviates VP-16-induced apoptosis of ALL cells. Importantly, VP-16-induced SENP8 confers a feed-back chemotherapeutic resistance on ALL cells, suggesting a possibility of overcoming the chemotherapeutic resistance to VP-16 via targeting SENP8. Our findings have unveiled a novel role of SENP8 in conferring resistance to VP-16 on ALL cells. The study, to our knowledge, is the first to report the specific role of SENP8 in ALL cells, providing a new insight into the potential ALL targeted therapy.

2. Materials and methods

2.1. ALL specimens

The raw data of RNA sequencing for ALL specimens was kindly provided by our colleagues in Shanghai Children's Medical Center. For details, please refer to the supplemental Methods in their published article [13].

2.2. Cell culture and transfection

ALL cell lines REH and NALM6 were cultured in RPMI1640 medium (Gibco, USA), supplemented with 10 % fetal bovine serum (Gibco, USA), 100 U/mL penicillin (Thermo Fisher Scientific, USA), 0.1 mg/mL streptomycin (Thermo, USA), and kept within the incubator at 37 °C under 5 % CO_2. All transfection employed the reagents FuGENE 6 (Promega, USA) or jetPRIME (Polyplus-transfection, France) and were manipulated with reference to the product instructions.

2.3. Stable cell lines

For overexpressing SENP8, please refer to the previous study for details [14]. Briefly, the overexpression plasmids were Flag-tagged GV303 vectors, the lentiviral infection system was employed to generate SENP8 stably-expressed REH and NALM6 cells. For knocking down SENP8, please refer to the previous study for details [15]. Briefly, the basic constructs were pLKO.1 shRNA vectors and the short-hairpin RNA sequence is as follows: *SENP8#1*, 5′-TGGCTCAATGACCATATTA-3'; *SENP8#2*, 5′CAACAAGAGAGTTGTATTT-3'. Lentivirus-infected cells were selected using puromycin (Thermo Fisher Scientific, USA) at l μg/mL. *p53−/−* NALM6 cells were kindly provided by Dr. Fan Yang [16].

2.4. Cell growth and viability

For details, please refer to the previous study [17]. For the growth curve, detection by CellTiter-Glo Luminescent Kit (Promega, USA) was once a day for 4–5 days. The relative growth rate at indicated time points was controlled by day 0. For the viability, cells were cultured for 72 h treated with serially diluted drugs and their viability was measured by CellTiter-Glo Luminescent Kit according to the instructions.

2.5. Cell apoptosis

For more details, please refer to the previous study [15]. Briefly, cells were seeded and cultured with indicated chemotherapeutic drugs for 72 h, then harvested for staining by Annexin V apoptosis detection kit (Elabscience, China), the percentage of Annexin V-positive cells by using a FACSCalibur flow cytometry (BD Biosciences, USA).

2.6. Western Blotting

Cells were lysed using 1 × SDS lysis buffer and analyzed by SDS-PAGE with the antibodies as follows: SENP8 (823,121, ZENBIO, China), MDM2 (F414, BIOWORLD, USA), p53 (ab1101, Abcam, UK), Actin (Huabio, China). Immunoblots were imaged and analyzed by using Odyssey system (LI-COR Biosciences, USA), ImageQuant LAS4000 (GE Healthcare, USA), and ChemiDoc MP (Biorad, USA). The whole uncropped images of original Western Blots in triplicate were attached to Supplemental Figures.

2.7. Real-time PCR

Total RNA was extracted using Trizol reagent (Tiangen, China). 1 μg total RNA was reversed into cDNA by PrimeScript™ RT reagent kit with cDNA Eraser (Takara, Japan). Q-PCR reactions were performed by a real-time PCR thermocycler (Agilent, USA) with SYBR green reagent (Takara, Japan). Primers were as follows: *SENP8*, *F*-TTAGGCAACAGACAGAAT, *R*-TAGCAAGTGTGGTAATGA; *GAPDH*, *F*-GAGCTGAACGGGAAGCTCACTG, *R*-TGGTGCTCAGTGTAGCCCAGGA.

2.8. Statistical analysis

Statistical data analysis and associated statistical graphics were established by GraphPad Prism software. Statistical significance was calculated using two-tailed Student's t-tests, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. Error bars represented the S.D. All the statistical details can be found in the methods and/or figure legends.

3. Results

3.1. SENP8 is highly expressed in relapsed ALL specimens

To probe into the potential effect of NEDDylation pathway in relapsed ALL, we pertinently conducted a re-analysis for RNA-Seq results of the whole transcriptomes from 61 pairs of matched diagnosis-relapse ALL specimens [13]. The results show that the transcriptional status of *NEDD8*, *NAE1*, *UBA3*, *UBE2F*, and *COPS5* has little difference in two sets of specimens, whereas another E2 enzyme UBE2M exhibits slight downregulation in relapsed ALL specimens (Fig. 1A–F). Interestingly, the average expression of *SENP8* in relapsed ALL specimens is significantly higher than that in diagnostic specimens, characterized with 39 cases of increase and 22 cases of decrease (Fig. 1G). The findings indicate that *SENP8* is highly expressed in relapsed ALL specimens, raising a worthwhile issue of whether SENP8 functions in ALL relapse. Pursuing this, exogenous SENP8 was stably expressed in human ALL cell line REH or NALM6 by lentiviral vectors to determine the impact of SENP8 on ALL cell chemosensitivity (Fig. 1H).

3.2. Overexpressing SENP8 induces specific resistance of ALL cells to VP-16

We first observed that no significant difference in cell growth between SENP8-overexpressed REH (henceforth referred to "SENP8-OE") cells and REH control ("REH-Ctrl" in text) cells (Fig. 2A). Furthermore, the viability of SENP8-OE cells also presents minimal difference from the REH-Ctrl cells when treated with several common chemotherapeutic drugs for clinical ALL therapy, including 6-thioguanine (6-TG), daunorubicin (DNR), vincristine (VCR), cytarabine (Ara-C), and methotrexate (MTX) (Fig. 2B–F). Surprisingly, when treated with VP-16, the viability of SENP8-OE cells has evidently increased compared to REH-Ctrl cells (Fig. 2G). Likewise, a similar result from NALM6 cells has also testified to the reduced chemosensitivity of ALL cells to VP-16 by overexpressing

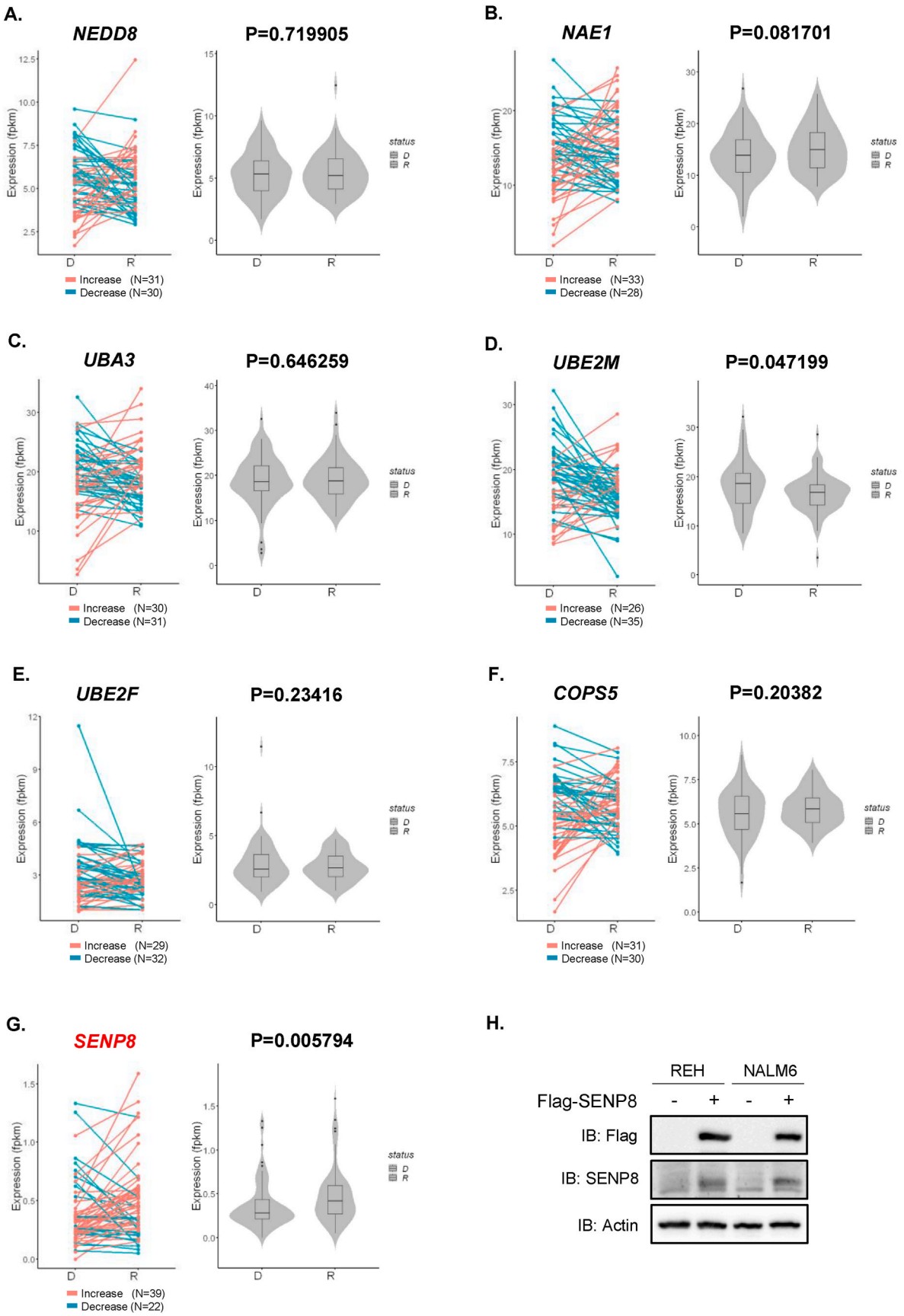

Fig. 1. *SENP8* is highly expressed in relapsed ALL specimens. (A-G) Gene expressions of *NEDD8*, *NAE1*, *UBA3*, *UBE2M*, *UBE2F*, *COPS5*, and *SENP8* from the RNA-seq data of 61 paired diagnosis-relapse ALL specimens. Data between the two groups were compared by using two-tailed Student's *t*-test. (H) Overexpression of Flag-tagged SENP8 in REH or NALM6 cells analyzed by Western Blotting.

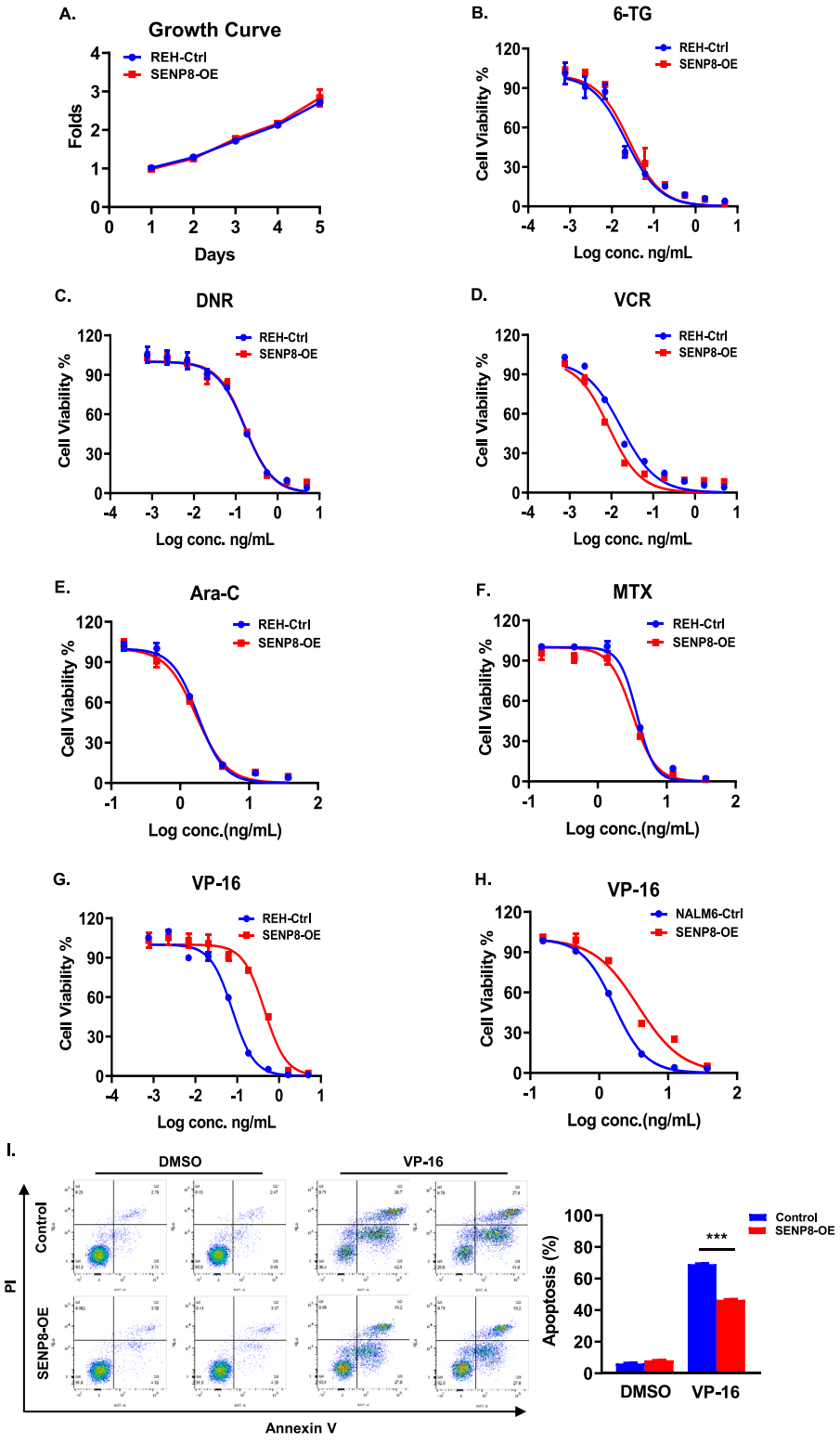

Fig. 2. Overexpressing SENP8 induces specific resistance of ALL cells to VP-16. (A) Growth curves of REH-Ctrl cells and SENP8-OE cells. Data were presented as mean ± SD. (B–G) Screening on the chemosensitivities of REH-Ctrl cells and SENP8-OE cells to the common chemotherapeutic drugs in ALL treatment analyzed by cell viability assay. (H) Chemosensitivity of NALM6 cells harboring SENP8-OE to VP-16 analyzed by cell viability assay. (I) Analysis of VP-16-induced (1 μM) apoptosis rates of REH-Ctrl cells or SENP8-OE cells by flow cytometry. The quantitative bar graph is shown at right. Data were presented as mean ± SD, ***$P <$ 0.001, each group was compared with the control.

SENP8 (Fig. 2H). VP-16 is a well-known DNA topoisomerase II (TOP2) inhibitor that can induce DNA double-stranded breaks (DSB) and trigger cell apoptosis, exerting anticancer effect in diverse cancers including ALL [18]. We therefore tested the VP-16-induced apoptosis of REH cells by flow cytometry and showed an obvious apoptotic alleviation in SENP8-OE cells than REH-Ctrl cells (Fig. 2I). In sum, these results suggest that SENP8 overexpression can induce specific resistance of ALL cells to VP-16.

3.3. Inhibiting NEDDylation reduces the chemosensitivity of ALL cells to VP-16

A deNEDDylase deconjugating NEDD8 from target protein, SENP8 has a significantly higher expression in relapsed ALL specimens (Fig. 1G). In contrast, UBE2M as an E2 enzyme transferring activated NEDD8 to substrate by cooperation with E3 enzyme, shows a relatively lower expression in relapsed ALL specimens (Fig. 1D). This coincident interplay between NEDD8-specific off and on enzymes has aroused our great interest to investigate whether NEDDylation inhibition affects the chemosensitivity of ALL cells to VP-16. MLN4924 (Selleck, #S7109), a specific NEDDylation inhibitor via allosteric inhibition of NAE1, has been proved to have cytotoxic effect on various tumor cells [19]. Base on this, we have chosen a lower working concentration of MLN4924 (0.05 μM) for subsequent experiments, with the aim of inhibiting protein NEDDylation without causing significant cytotoxicity (Fig. 3A and B). We found that the chemosensitivity of REH cells to 6-TG, DNR, and VCR does not significantly change when persistently treated with MLN4924 (Fig. 3C–E). However, NEDDylation inhibition by MLN4924 specifically reduces the chemosensitivity of ALL cells to VP-16 (Fig. 3F–H).

3.4. VP-16-induced SENP8 confers a feed-back drug resistance on ALL cells

We further found that VP-16 can specifically induce the transcription of *SENP8* (Fig. 4A) and the induction of SENP8 in REH cells shows time-course dependence on VP-16 treatment (Fig. 4B). It raised an interesting possibility that accumulation of SENP8 induced by VP-16 may be responsible for the reduced chemosensitivity of ALL cells to VP-16. To test this, we knocked down SENP8 in REH cells (Fig. 4C) and found that SENP8 knockdown specifically sensitizes REH cells to VP-16 without significant alteration in the normal cell growth (Fig. 4D–F). Moreover, MLN4924-reduced chemosensitivity of REH cells to VP-16 can be readily rescued by SENP8 knockdown (Fig. 4G), suggesting that VP-16-induced SENP8 confers a feed-back drug resistance on ALL cells. As for the underlying mechanism of SENP8-mediated resistance to VP-16 on ALL cells. It has been reported that SENP8 increases the p73 expression and induction of apoptosis in response to DNA damage [20], while our data have shown that overexpressing SENP8 attenuates the pro-apoptotic effect of VP-16 (Fig. 2I) and the average expression of *p73* is not directly proportional to *SENP8* in ALL specimens (Fig. 4H). Nevertheless, apart from the accumulation of SENP8, VP-16 can also induce the instability of MDM2 as well as the consequent stabilization of p53 in ALL cells (Fig. 4I). In contrast, VP-16-induced p53 is not a prerequisite for the accumulation of SENP8 (Fig. 4J), proposing a straight MDM2-p53 signal axis exerting in SENP8-mediated drug resistance to VP-16 on ALL cells (Fig. 4L).

4. Discussion

SENP8 is an important deNEDDylase and its cellular function is usually depicted by its specific substrates. In this regard, SENP8 shows more activities in deNEDDylating hyper-NEDDylated cullins or NEDD8-specific E2 enzyme UBE2M to limit aberrant NEDDylation of NEDD8 pathway components, maintaining proper NEEDylation levels for CRL-dependent proteostasis [6,8,21]. However, growing non-cullin NEDDylation substrates have been continuously identified over the past. For instance, SENP8-mediated BCA3 (breast cancer–associated protein 3) deNEDDylation enhances NF-κB-dependent transcription and inhibits TNFα-induced apoptosis of breast cancer cells [22]. By contrast, DNA damage-induced SENP8 promotes E2F1-mediated transcription of *p73* by deNEDDylating E2F1, which triggers the induction of apoptosis [20]. In addition, the chemotherapeutic drugs doxorubincin (DOX) and neocarzinostatin (NCS) can increase cellular SENP8 to deNEDDylate MDM2, causing the instability of MDM2 concomitant with p53 activation [23]. Nevertheless, our present data have not shown apparent difference in the average expression of *p73* between relapsed and diagnostic ALL specimens whereas *SENP8* is highly expressed in relapsed ALL specimens (Fig. 4H *vs* Fig. 1G). On the other hand, we did find that VP-16 significantly enhances the expression of SENP8 in REH cells (Fig. 4A and B) along with the parallel stabilization of p53 and decreased MDM2 (Fig. 4I), and the accumulation of SENP8 is independent of the concomitant VP-16-mediated p53 activation (Fig. 4J). Meanwhile, SENP8 knockdown sensitizes ALL cells to VP-16 (Fig. 4F and G) and ectopic expression of SENP8 in ALL cells induces the drug resistance to VP-16 to alleviate its proapoptotic effect (Fig. 2G–I). This can be explained by such a celebrated thesis that the tumor suppressor p53 acts as a critical brake in tumor development [24,25] and become another footnote to the research of our colleagues [16]. However, the regulatory mechanism govering SENP8-mediated resistance of ALL cells to VP-16 remains unclear, albeit the nonnegligible change of cellular NEDDylation levels by modulating SENP8 expression (Fig. 4K).

ALL accounts for more than 25 % of all childhood cancers, several new targeted therapies and molecularly targeted agents have been developed in the past [3,4,26]. Nevertheless, chemotherapy is the most effective treatment for ALL, though relapse is still the leading cause of mortality and drug resistance is always very tricky. Our present results have shown that the relapsed ALL specimens have significantly higher expression of SENP8 compared to the diagnostic specimens. Overexpressing SENP8 specifically reduces the chemosensitivity of ALL cells to VP-16 and alleviates VP-16-induced apoptosis of ALL cells. Perhaps more interestingly, VP-16-induced SENP8 confers a feed-back chemotherapeutic resistance on ALL cells, providing such a possibility of overcoming the chemotherapeutic resistance to VP-16 via targeting SENP8. However, we are very aware of our shortcoming on the exploration of mechanism by which SENP8 contributes to this, and an in-deep investigation based on the existing clues and proposed hypotheses (Fig. 4I–L) remains to be sought after. Overall, our findings have revealed a novel role of SENP8 in conferring resistance to VP-16 on ALL cells and the present study, to our knowledge, is the first to unveil the novel function of SENP8 in ALL cells, and theoretically, suggested new avenues for ALL targeted therapy.

Data accessibility

Data will be made available on request.

CRediT authorship contribution statement

Shuzhang Sun: Conceptualization, Data curation, Formal analysis, Investigation, Methodology. **Yixuan Cheng:** Data curation, Formal analysis, Investigation, Validation. **Wanxin Hou:** Data curation, Formal analysis, Investigation, Validation. **Yinjie Yan:** Formal analysis, Investigation, Methodology. **Tian Meng:** Formal analysis, Investigation. **Hegen Li:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision. **Ning Xiao:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial

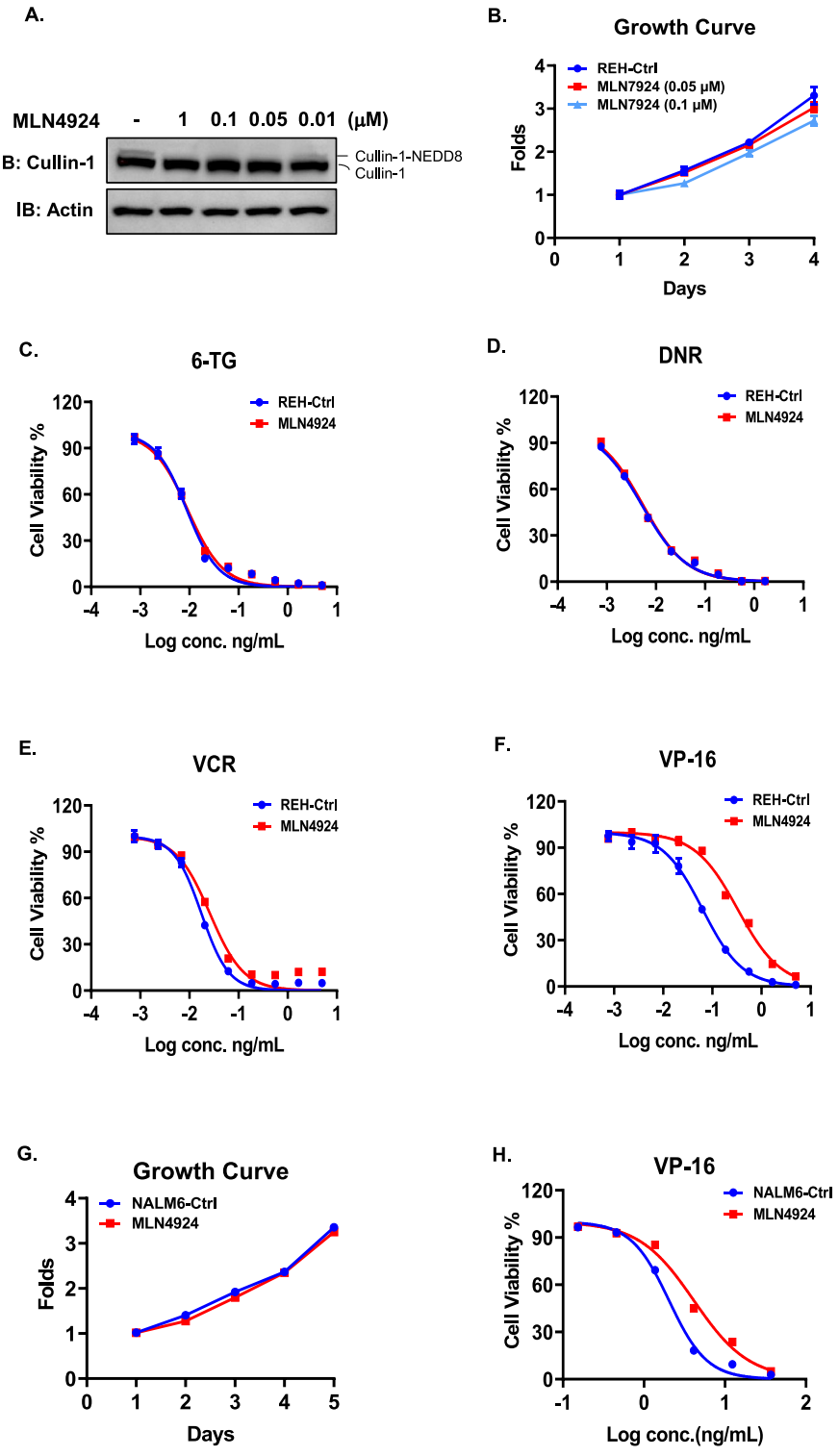

Fig. 3. Inhibiting NEDDylation reduces the chemosensitivity of ALL cells to VP-16. (A) Efficacy of NEDDylation inhibition in REH cells treated with different concentrations of MLN4924 analyzed by Western Blotting. (B) Growth curves of REH-Ctrl cells and REH cells treated with lower concentrations of MLN4924 (0.1 μM or 0.05 μM). Data were presented as mean ± SD. (*C*–F) Screening on the chemosensitivities of REH-Ctrl cells and SENP8-OE cells to the common chemotherapeutic drugs in ALL treatment, REH cells were persistently treated with MLN4924 (0.05 μM). (G) Growth curve of NALM6 cells persistently treated with MLN4924 (0.05 μM). Data were presented as mean ± SD. (H) Chemosensitivity of NALM6 cells persistently treated with MLN492 (0.05 μM) to VP-16 analyzed by cell viability assay. Date were presented as mean ± SD.

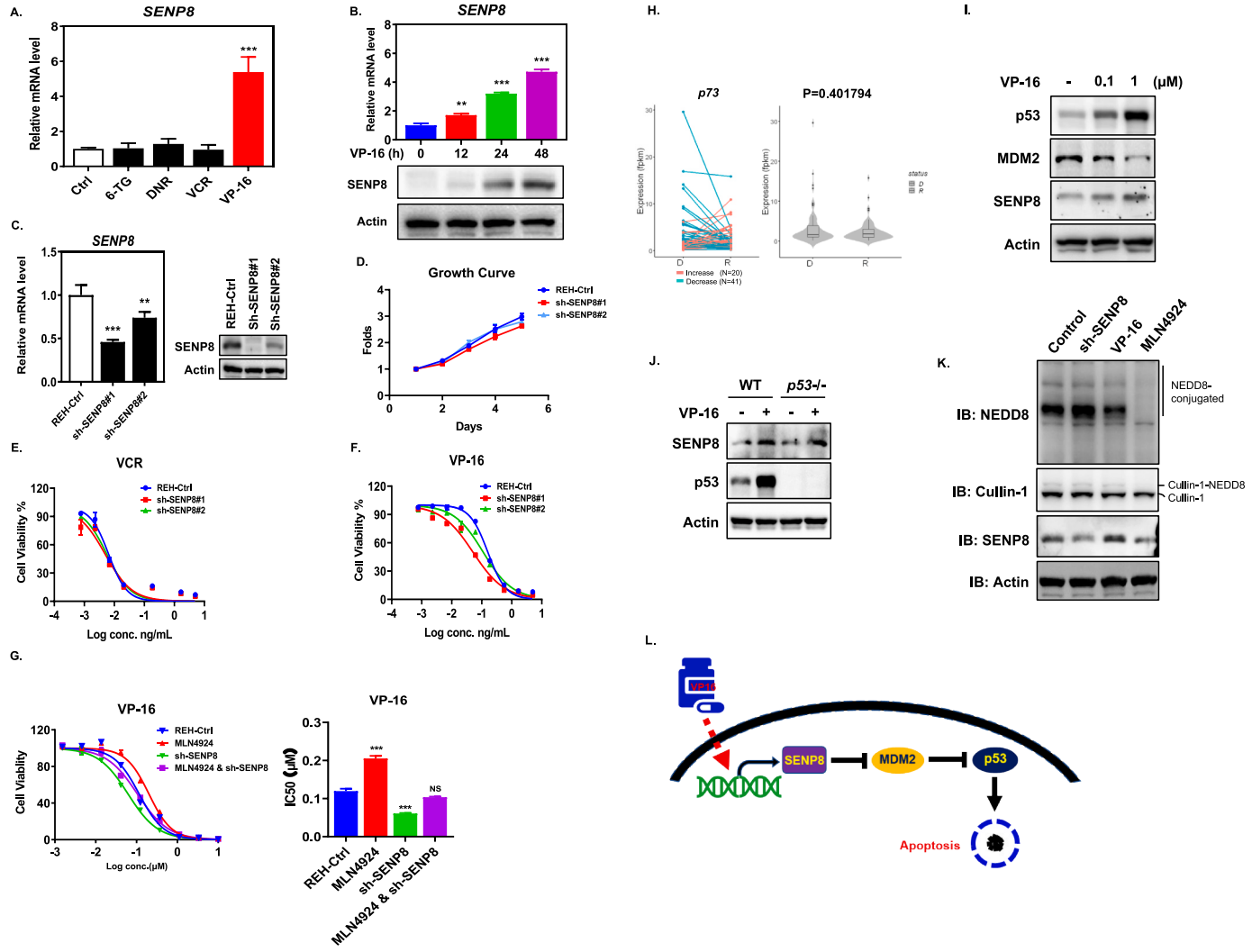

Fig. 4. VP-16-induced SENP8 confers a feed-back drug resistance on ALL cells. (A) The effect of commonly used ALL chemotherapeutic drugs on the mRNA levels of *SENP8* in REH cells analyzed by real-time quantitative PCR. ***$P < 0.001$. (B) The effect of VP-16 (1 μM) on the relative mRNA level of *SENP8* at different time points in REH cells. **$P < 0.01$; ***$P < 0.001$, two-tailed Student's t-test, each group was compared with the control. (C) Endogenous SENP8 in REH cells was knocked down by using shRNA. (D) Growth curves of REH-Ctrl cells and SENP8 knockdown cells. Data were presented as mean ± SD. (*E*–F) Chemosensitivities of REH-Ctrl cells and SENP8 knockdown cells to chemotherapeutic drugs VCR and VP-16. (G) Chemosensitivities of REH cells under SENP8 knockdown, MLN4924 treatment (0.05 μM), or combination to the chemotherapeutic drug VP-16. The 50 % inhibitory concentration (IC50) was calculated with GraphPad Prism software (La Jolla, CA, USA). ***$P < 0.001$; NS: non-specific, two-tailed Student's t-test, each group was compared with the control. (H) Gene expression of *p73* from the RNA-seq data of 61 paired diagnosis-relapse ALL specimens. Data between the two groups were compared by using two-tailed Student's *t*-test. (I) Analysis for the protein levels of p53, MDM2, and SENP8 in REH cells treated with different concentrations of VP-16 by Western Blotting. (J) Analysis for the protein levels of SENP8 and p53 in wild type (WT) or *p53*−/− NALM6 cells treated with VP-16 (1 μM) by Western Blotting. (K) Analysis for the protein levels of cellular NEDDylation, Cullin-1, and SENP8 in REH cells under SENP8 knockdown or treated with VP-16 (1 μM) or MLN4924 (0.05 μM) by Western Blotting. (L) A proposed schematic model of VP-16-induced SENP8 confers a feed-back drug resistance on ALL cells.

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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