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Acetylation stabilizes the signaling protein WISP2 by preventing its degradation to suppress the progression of acute myeloid leukemia

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Acute myeloid leukemia (AML) is challenging to treat due to its heterogeneity, prompting a deep understanding of its pathogenesis mechanisms, diagnosis, and treatment. Here, we found reduced expression and acetylation levels of WISP2 in bone marrow mononuclear cells from AML patients and that AML patients with lower WISP2 expression tended to have reduced survival. At the functional level, overexpression of WISP2 in leukemia cells (HL-60 and Kasumi-1) suppressed cell proliferation, induced cell apoptosis, and exerted antileukemic effects in an *in vivo* model of AML. Our mechanistic investigation demonstrated that WISP2 deacetylation was regulated by the deacetylase histone deacetylase (HDAC)3. In addition, we determined that crosstalk between acetylation and ubiquitination was involved in the modulation of WISP2 expression in AML. Deacetylation of WISP2 decreased the stability of the WISP2 protein by boosting its ubiquitination mediated by NEDD4 and proteasomal degradation. Moreover, pan-HDAC inhibitors (valproic acid and trichostatin A) and an HDAC3-specific inhibitor (RGFP966) induced WISP2 acetylation at lysine K6 and prevented WISP2 degradation. This regulation led to inhibition of proliferation and induction of apoptosis in AML cells. In summary, our study revealed that WISP2 contributes to tumor suppression in AML, which provided an experimental framework for WISP2 as a candidate for gene therapy of AML.

Acute myeloid leukemia (AML) is a heterogeneous group of hematological malignancies characterized by excessive proliferation of myeloid progenitor cells that have halted differentiation, ultimately resulting in bone marrow failure (1, 2). It is the most common acute leukemia in adults and a common pediatric cancer. The usual treatment strategies for AML include chemotherapy, radiation therapy, stem cell transplants, and targeted immune therapy. However, these traditional therapies fail to achieve satisfactory therapeutic effects, and disease relapse may occur after less successful treatment (3). Only 30 to 40% of patients have long-term disease-free survival after treatment, and the 3-years survival of relapsed/refractory AML is less than 10% (4). Therefore, further identification of new molecular lesions in AML is needed to generate potential treatment interventions that hold promise for improving the outcome of AML treatment.

Wnt-1-induced signaling protein-2 (WISP2, also known as CCN5) is a member of the connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed family, mapped to human chromosome 20q12–20q13 (5, 6). Connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed proteins are important for many different biological processes, including mitogenesis, adhesion, apoptosis, extracellular matrix production, growth arrest, and migration (7). The expression and role of WISP2 are different in diverse human cancers. It has been proven that the expression of WISP2 is downregulated in pancreatic cancer and leiomyomas (8, 9). Loss of WISP2 contributes to the proliferation (9) and epithelial-mesenchymal transition (8) of cancer cells. Ji *et al.* demonstrated that WISP2 is highly expressed in gastric cancer tissues, but its overexpression is associated with good prognosis of patients (10). Conversely, WISP2 was validated as having an oncogenic role in breast cancer; it was highly expressed in cancerous tissues of breast cancer patients with metastasis (11), and its overexpression is required for breast tumor cell proliferation (12). However, the role of WISP2 in leukemia, particularly in AML, has not yet been elucidated.

Genetic and epigenetic lesions are prevalent phenomena in AML cells. Histone deacetylases (HDACs), which reverse lysine acetylation, are pivotal epigenetic regulators of gene expression and protein activity (13). In AML, aberrant recruitment of HDACs results in gene silencing and drives leukemogenesis (14). Recently, histone deacetylase inhibitors (HDACis) have emerged as promising anticancer agents. A previous study reported that the HDACi LAQ824 triggers growth arrest and cell death in AML cells (15). Golay *et al.* found that the HDACi ITF2357 not only has antileukemic activity *in vitro* and *in vivo* but also suppresses the production of growth and angiogenic factors by bone marrow stromal cells (16). A clinical trial reported that the HDACi panobinostat

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combined with intensive induction chemotherapy for older patients with AML is well tolerated, proposing that panobinostat may augment the activity of induction therapy to improve response rates for older patients (17). Although several HDACis have entered the clinic, the mechanisms underlying their tumor selectivity are poorly understood. This is because different HDACs may regulate specific oncogenes or tumor suppressors in different cancers. Therefore, it is of great importance to identify the specific HDAC subtype that mediates the antileukemia effect of HDACi and to investigate the specific gene's biological function in AML. Interestingly, we found a protein physical interaction between HDAC1/3 and WISP2 by searching the HitPredict database. This result suggested that HDAC1/3-specific inhibitors may exert their antineoplastic effects in AML by targeting WISP2 and inducing its acetylation.

In this study, we found that the expression and acetylation levels of WISP2 were reduced in AML patients, and low WISP2 expression predicted reduced survival. Further functional and mechanical studies indicated that WISP2 was deacetylated by HDAC3 and that this interplay promoted cell proliferation and inhibited apoptosis in AML. Moreover, acetylation of WISP2 at the lysine K6 residue increased the stability of the WISP2 protein by decreasing its ubiquitination and degradation.

Results

Low WISP2 expression predicts reduced survival of AML patients

By evaluating the survival probability of 163 AML patients *via* the *UALCAN* cancer database (based on TCGA data), we found that AML patients with high WISP2 expression tended to have a better survival probability (Fig. 1*A*). Expression of a gene with a transcript per million (TPM) value less than 1 is considered low according to *UALCAN*. Although the TPM of WISP2 showed that WISP2 expression hardly varied among FAB M0-M7 subtypes, the TPM value of each AML subtype was approximately 1, which suggested low WISP2 expression in all AML samples (Fig. 1*B*). The expression of WISP2 in bone marrow mononuclear cell samples from six healthy donors and 16 AML patients enrolled in our hospital was analyzed *via* Western blot analysis. Downregulation of WISP2 was confirmed in bone marrow mononuclear cells derived from AML patients (Fig. 1*C*). At the same time, we examined the acetylation level of WISP2 in bone marrow mononuclear cells from healthy donors and AML patients. The results demonstrated that the acetylation level of WISP2 was decreased in bone marrow mononuclear cells from AML patients (Fig. 1*D*). Furthermore, the expression and acetylation levels of WISP2 in normal $CD34^+$ cells derived from healthy donors and AML cell lines (HL-60, Kasumi-1, and KG-1) were investigated. Compared with that in normal $CD34^+$ cells, WISP2 expression was downregulated in AML cells, and the acetylation level of WISP2 in AML cells was reduced (Fig. 1*E*). The above findings together implied low expression and decreased acetylation of WISP2 as a contributor to leukemogenesis in AML patients.

WISP2 overexpression inhibits proliferation and promotes apoptosis of AML cells

Next, we investigated the role of WISP2 in regulating the malignant phenotype of AML. To simulate overexpression of WISP2 *in vitro*, Kasumi and HL-60 AML cells were infected with lentivirus carrying the complete coding sequence region of WISP2. Data from the CCK-8 assay showed that the proliferation of these two AML cell lines was significantly decreased when WISP2 was overexpressed (Fig. 2, *A* and *B*). As shown in Figure 2, *C* and *D*, the percentage of apoptotic cells was robustly increased in WISP2-overexpressing HL-60 cells or Kasumi-1 cells. In addition, we stained AML cells with JC-1 dye to examine the loss of mitochondrial membrane potential (MMP), which is common in the early stages of apoptosis. WISP2 overexpression caused the loss of MMP, as evidenced by an increase in the percentage of cells emitting green fluorescence (Fig. 2, *E* and *F*). Moreover, the levels of apoptosis-related markers in WISP2-overexpressing HL-60 cells or Kasumi-1 cells were determined by Western blot analysis. The levels of proapoptotic protein (Bax), caspase cascade proteins (cleaved caspase-3, cleaved caspase-9, and cleaved PARP), and mitochondrial apoptotic protein (Bak) were increased, whereas the expression of antiapoptotic protein (Bcl-2) was downregulated in AML cells upon WISP2 overexpression. The release of mitochondrial cytochrome c into the cytosol was increased after WISP2 overexpression (Fig. 2, *G* and *H*). These results suggested that WISP2 overexpression inhibited the proliferation and promoted the apoptosis of AML cells.

WISP2 overexpression exerts antileukemic effects in the in vivo *model of AML*

To extend the *in vitro* findings that WISP2 overexpression inhibited the malignant phenotype of AML, we constructed a transplant model of AML cells in NOD/SCID mice. The tumor burden in bone marrow was detected by flow cytometric analysis of CD45, a panleukocyte marker. Mice with HL-60 cell xenografts exhibited 44.99% positivity for $CD45^+$ cells in bone marrow, while mice transplanted with HL-60 cells overexpressing WISP2 showed a reduction to 21.47% in $CD45^+$ cells (Fig. 3*A*). Leishman-Wright-Giemsa staining of bone marrow confirmed the infiltration of immature cells in leukemic animals and their replacement by ring-like differentiated cells in lentivirus plasmids containing full-length WISP2 (LV-WISP2) mice (Fig. 3*B*). Thus, WISP2 overexpression led to tumor remission in a mouse model of AML.

Pan-HDAC inhibitors induce apoptosis and efficiently prevent WISP2 degradation in AML cells

Since HDAC inhibitors are capable of increasing acetylation of nonhistone proteins (18), two pan-HDAC inhibitors, valproic acid (VPA), and trichostatin A (TSA) were used to treat AML cells to determine whether WISP2 acetylation affects its protein stability. VPA and TSA suppressed proliferation (Fig. 4, *A* and *B*) and induced apoptosis of AML cells (Fig. 4, *C*–*F*). Interestingly, the Western blot results showed increased protein levels of WISP2 in AML cells treated with VPA or TSA

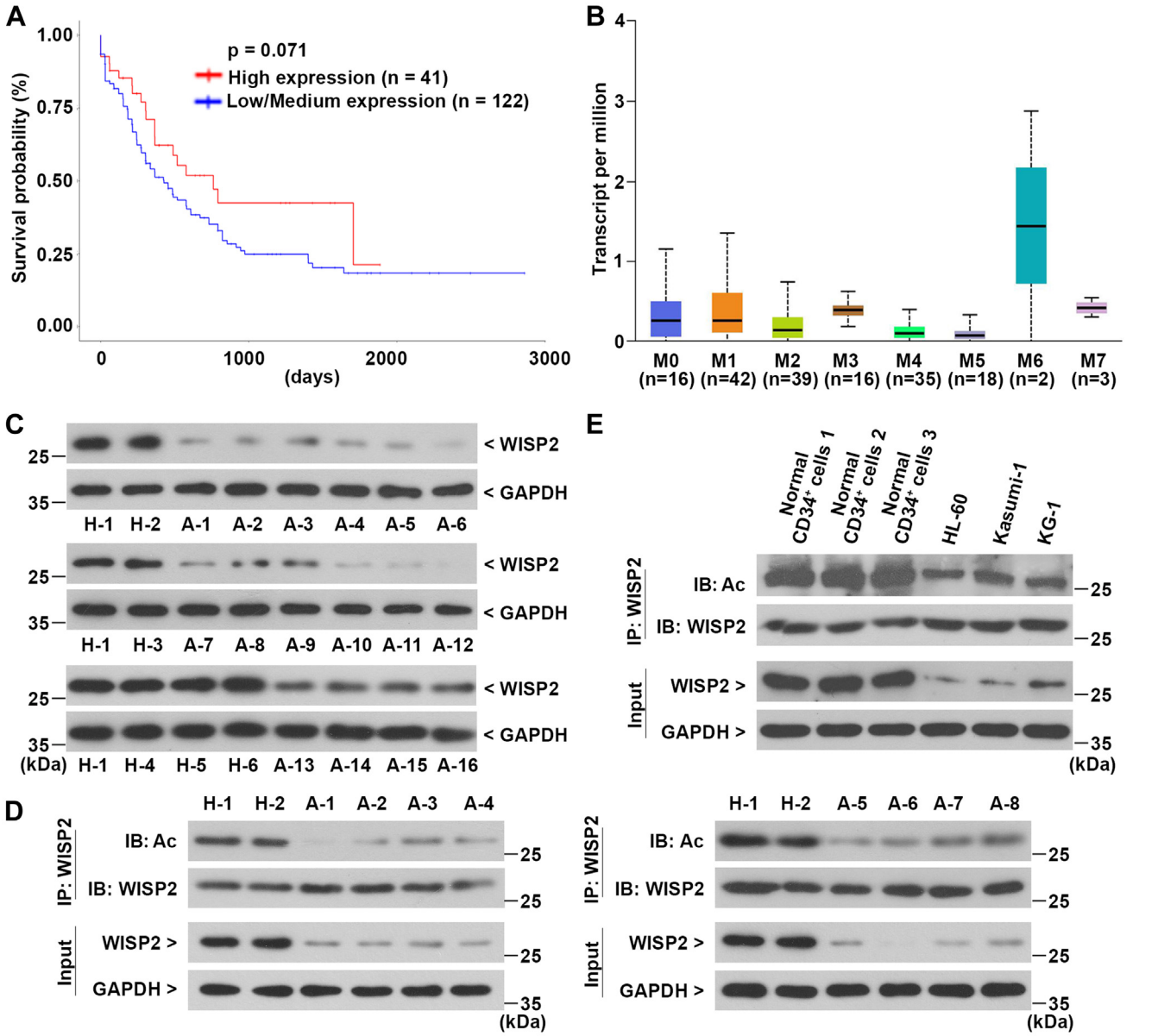

Figure 1. Low WISP2 expression predicts reduced survival of AML patients. *A*, correlation of WISP2 expression with the survival probability of AML patients *via* the *UALCAN* cancer database. *B*, analysis of WISP2 expression in AML patients with different subtypes *via* the UALCAN cancer database. *C*, Western blot analysis of WISP2 expression in bone marrow mononuclear cells from six healthy donors and 16 AML patients. *D*, Co-IP assay to detect the acetylation of WISP2 in bone marrow mononuclear cells from healthy donors and AML patients. *E*, the protein expression and acetylation level of WISP2 in normal bone marrow mononuclear cells and AML cells. Ac, acetylated lysine; AML, acute myeloid leukemia; Co-IP, co-immunoprecipitation; WISP2, Wnt-1-induced signaling protein-2.

(Fig. 4, *G* and *H*). Co-immunoprecipitation (co-IP) analysis using an anti-acetyl lysine antibody confirmed that WISP2 acetylation was enhanced post VPA or TSA treatment (Fig. 4, *I* and *J*). To explore whether WISP2 acetylation contributes to its protein accumulation within AML cells, we treated AML cells with cycloheximide to block protein synthesis and further detected WISP2 protein levels. The existing WISP2 protein began to be degraded quickly after the protein translation process was inhibited for 1 h and was hardly detected at 24 h. However, the two pan-HDAC inhibitors effectively prevented WISP2 degradation (Fig. 4, *K* and *L*). Collectively, these data suggested that acetylated WISP2 was more stable within AML cells.

HDAC3 interacts with and deacetylates WISP2 at the lysine K6 residue in AML cells

In addition to removing acetyl groups from acetylated lysine residues in histones, HDACs also induce deacetylation of nonhistone proteins (19). As illustrated in the HitPredict database (http://www.hitpredict.org/), HDAC1 and HDAC3 interact with WISP2. Hence, a co-IP assay was performed to determine the interaction between HDACs and WISP2 in AML cells. Interestingly, co-IP results confirmed that WISP2 bound to HDAC1 and HDAC3, but not to HDAC6 or HDAC7, in HL-60 and Kasumi-1 cells (Fig. 5, *A* and *B*). The online tool StarBase was then used to analyze the correlation between HDAC1 or HDAC3 and WISP2 in AML. Based on the

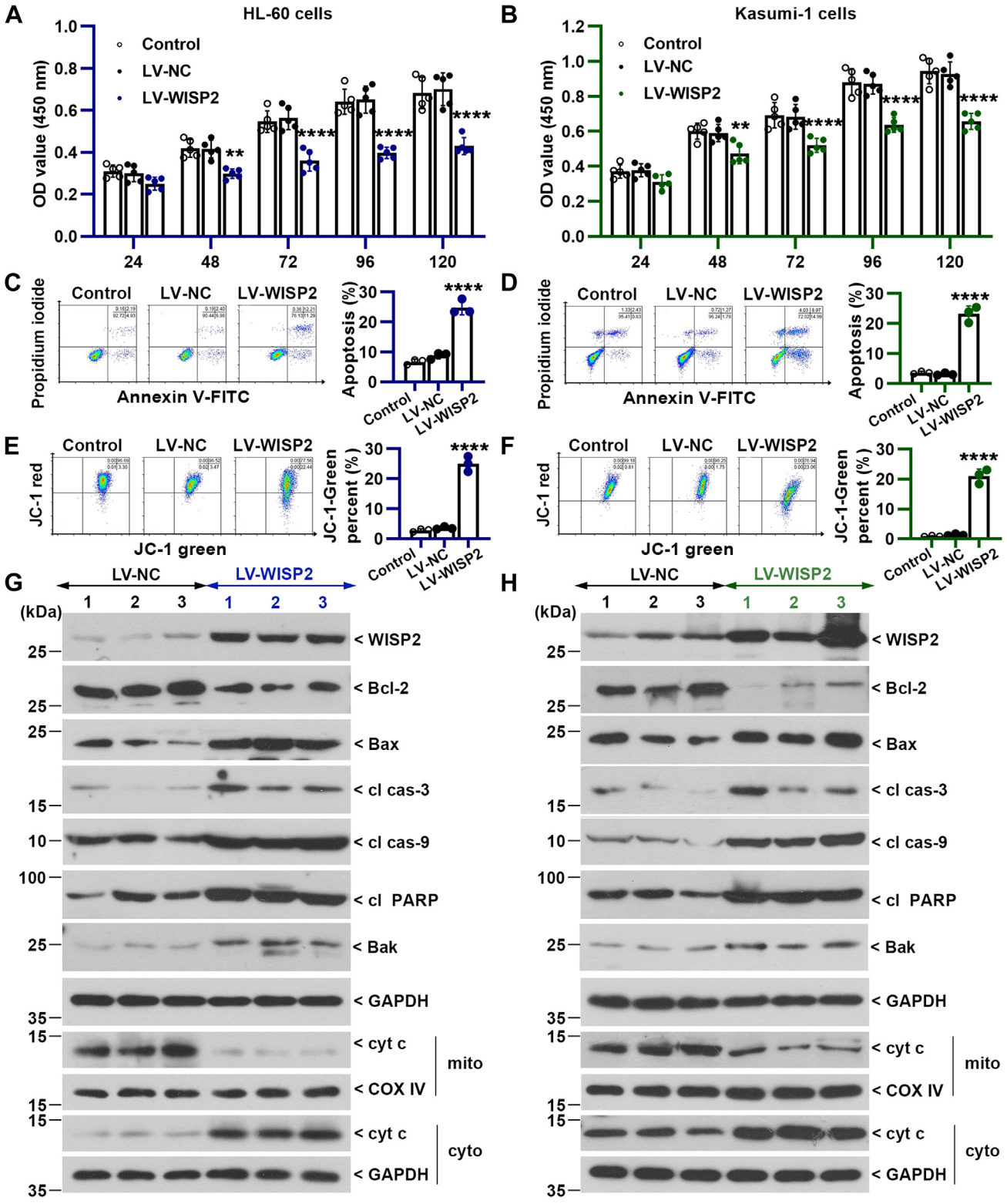

Figure 2. WISP2 overexpression inhibits the proliferation and promotes the apoptosis of AML cells. *A* and *B*, WISP2 lentivirus or negative control (NC) was infected into HL-60 cells (*A*) or Kasumi-1 cells (*B*) for 24, 48, 72 h, 96 h, and 120 h, and cell viability was determined by CCK8 assay. Data represent the mean ± SD (n = 5 for each group). *C* and *D*, apoptosis of HL-60 cells (*C*) or Kasumi-1 cells (*D*) was measured by flow cytometry after 72 h of infection with LV-WISP2 or LV-NC. Data represent the mean ± SD of three independent experiments. *E* and *F*, FACS analysis of the mitochondrial membrane potential of HL-60 cells (*E*) or Kasumi-1 cells (*F*) after 72 h of infection with lentivirus. Data represent the mean ± SD of three independent experiments. *G* and *H*, Western blot for WISP2 and apoptosis-related markers in whole cell lysates, as well as cytochrome C in mitochondrial and cytoplasmic fractions of HL-60 cells (*G*) or Kasumi-1 cells (*H*). *p* values in Panels *A* and *B* were determined with two-way ANOVA followed by Tukey's multiple comparisons test, and the *p* values in Panels *C–F* were determined with one-way ANOVA followed by Tukey's test (**$p < 0.01$, ****$p < 0.0001$). AML, acute myeloid leukemia; cl cas-3, cleaved caspase-3; cl cas-9, cleaved caspase-9; cl PARP, cleaved PARP; cyt c, cytochrome C; cyto, cytoplasmic; LV, lentivirus; LV-WISP2, lentivirus plasmids containing full-length WISP2; mito, mitochondrial; WISP2, Wnt-1-induced signaling protein-2.

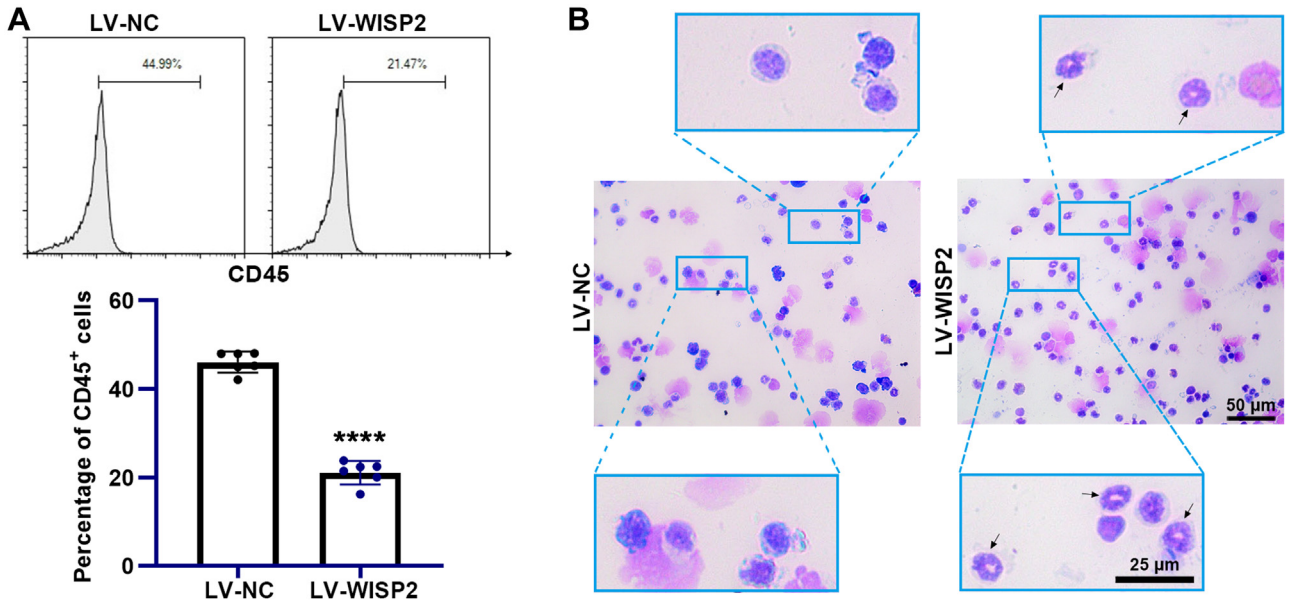

Figure 3. WISP2 overexpression exerts antileukemic effects in the *in vivo* model of AML. *A*, percentage of $CD45^+$ cells in the bone marrow of mice transplanted with HL-60 cells. *B*, Leishman–Wright–Giemsa staining of mouse bone marrow. *Black arrows* indicate ring-like differentiated cells. The *p* value in Panel *A* was determined with an unpaired *t* test (****$p < 0.0001$). AML, acute myeloid leukemia; WISP2, Wnt-1-induced signaling protein-2.

remarkable negative correlation with WISP2 (r = −0.306, p = 1.33e − 04) (Fig. 5*C*), HDAC3 was screened out for further investigation. The expression of HDAC3 in bone marrow mononuclear cells derived from AML patients was determined by Western blot analysis. The results showed an increased abundance of HDAC3 in bone marrow mononuclear cells from AML patients (Fig. 5*D*), which was contrary to the downregulation of WISP2 in bone marrow mononuclear cells from AML patients. We next treated AML cells with RGFP966 (HDAC3-specific inhibitor) for 24 h and further analyzed the expression and acetylation levels of WISP2. As shown in Figure 5, *E* and *F*, inhibition of HDAC3 increased WISP2 expression and augmented its acetylation. Additionally, exogenous overexpression of HDAC3 decreased WISP2 expression and inhibited its acetylation (Fig. 5, *G* and *H*). These results indicated that HDAC3 deacetylated WISP2 and reduced its expression in AML cells.

Prediction of Acetylation on Internal Lysines indicated two potential lysine (K) residues, K6 and K20, within WISP2 that might be acetylated (Fig. 6*A*). We then mutated these two lysine residues into arginine (R) and constructed plasmids carrying the Flag tag and DNA fragments encoding wildtype WISP2 (wt-WISP2) or mutant WISP2 (WISP2_K6R and WISP2_K20R). After transfecting HEK293 cells with these plasmids, the transfection efficacy was determined by immunoblotting with an anti-Flag antibody. The results indicated that the transfection was successful (Fig. 6*B*). As shown in Figure 6*C*, the acetylation of WISP2 induced by VPA (pan-HDAC inhibitor) was disturbed when the K6 residue was mutated into arginine, whereas the mutation of K20 had little effect on it. Similarly, mutation of K6 but not K20 abolished RGFP966 (HDAC3-specific inhibitor)-induced WISP2 acetylation (Fig. 6*D*). These results provided evidence that the K6 residue was essential for WISP2 acetylation.

Acetylation of WISP2 prevents NEDD4-mediated ubiquitination

In eukaryotic cells, ubiquitin is capable of forming a polymer chain to covalently label target proteins for degradation (20). Lysine acetylation may increase protein stability by preventing its ubiquitination and proteasome-dependent degradation (21). To further explore the association between WISP2 acetylation and ubiquitination, co-IP was performed to determine WISP2 ubiquitination in HL-60 cells. The results showed that the HDAC3 inhibitor (RGFP966) impeded the ubiquitination of WISP2 in HL-60 cells, whereas overexpression of HDAC3 exhibited the opposite effects (Fig. 7, *A* and *B*). Furthermore, HL-60 cells were infected with the HDAC3 lentivirus and treated with the proteasome inhibitor MG132. HDAC3 overexpression in HL-60 cells decreased WISP2 levels, while this reduction was reversed in the presence of MG132 (Fig. 7*C*). These data indicated that HDAC3-induced deacetylation of WISP2 augmented WISP2 ubiquitination.

As shown in Figure 7*D*, NEDD4 was predicted to be the top E3 ubiquitin ligase that probably targets WISP2 for protein degradation (http://ubibrowser.ncpsb.org.cn/ubibrowser/). Therefore, a co-IP assay was performed to verify this prediction, and the results demonstrated that NEDD4 interacted with WISP2 in AML cells (Fig. 7, *E* and *F*). Next, we examined the effects of NEDD4 on the expression and ubiquitination level of WISP2. Overexpression of NEDD4 gave rise to decreased WISP2 protein levels in AML cells (Fig. 7, *G* and *H*) and augmented ubiquitination of WISP2 in MG132-treated HL-60 cells (Fig. 7*I*). To address the question of whether K6 is involved in NEDD4-modulated degradation of WISP2, we determined the ubiquitination level of WISP2 mediated by NEDD4 overexpression plasmid in the presence of wt-WISP2-flag or WISP2_K6R-flag. In the presence of transfected NEDD4, the ubiquitination level of WISP2_K6R-flag-

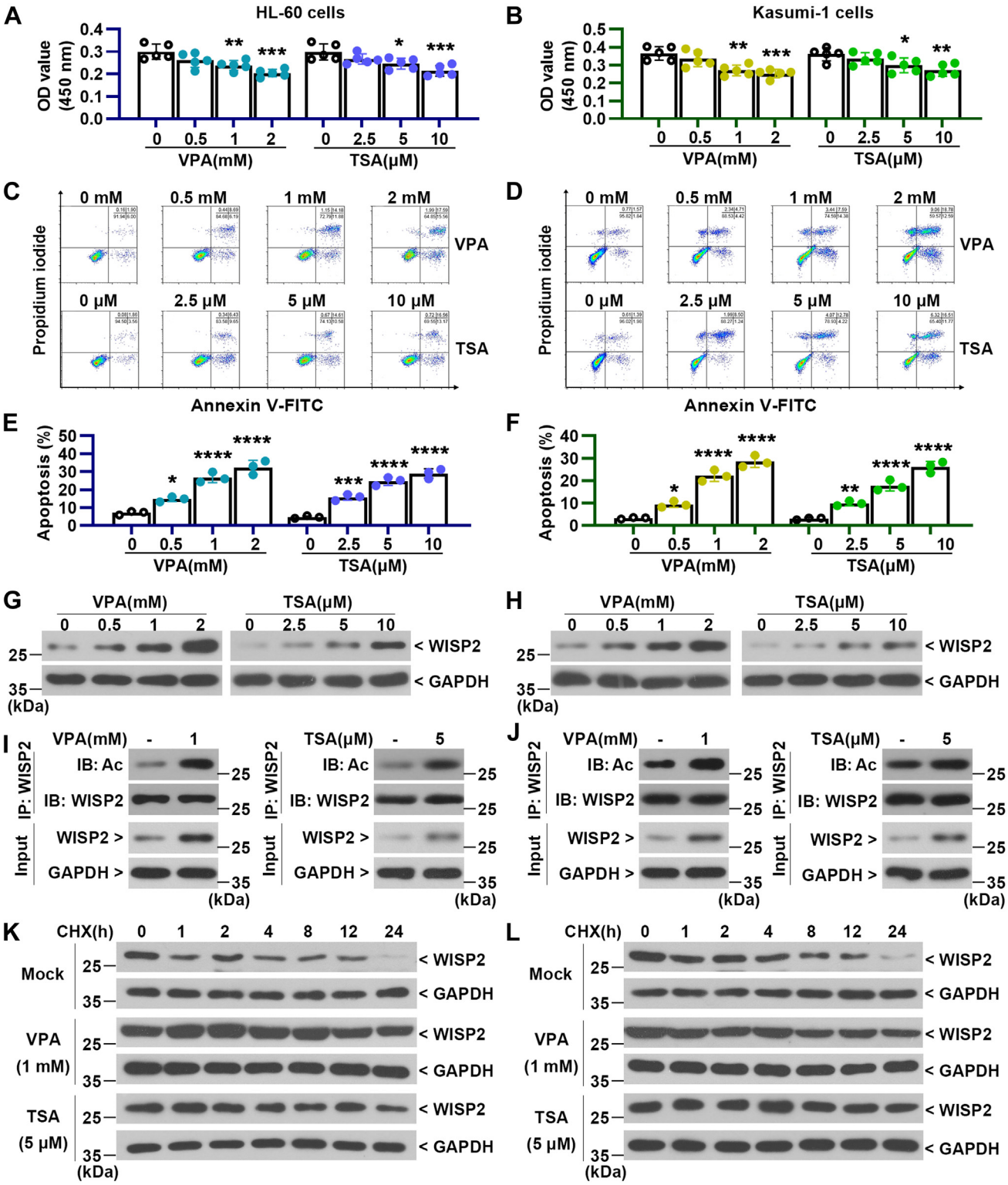

Figure 4. Pan-HDAC inhibitors induce apoptosis and efficiently prevent WISP2 degradation in AML cells. Two pan-HDAC inhibitors, VPA and TSA, were used to treat AML cells. *A* and *B*, HL-60 cells and Kasumi-1 cells were treated with VPA or TSA for 24 h and then subjected to CCK8 assay. Data represent the mean ± SD (n = 5 for each group). *C* and *D*, apoptosis of AML cells was measured by flow cytometry after 24 h of VPA or TSA treatment. Data represent the mean ± SD of three independent experiments. *E* and *F*, quantitative analysis of the percentage of apoptosis. *G* and *H*, Western blot for WISP2 in VPA- or TSA-treated AML cells. *I* and *J*, the acetylation of WISP2 in VPA- or TSA-treated AML cells was assessed by co-IP assay. *K* and *L*, AML cells were treated with VPA or TSA for 12 h and treated with CHX (25 μg/ml) for the indicated times, followed by Western blot analysis of WISP2 expression. *p* values were determined with one-way ANOVA followed by Dunnett's multiple comparisons test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). Ac, acetylated lysine; AML, acute myeloid leukemia; CHX, cycloheximide; co-IP, co-immunoprecipitation; HDAC, histone deacetylase; TSA, trichostatin A; VPA, valproic acid; WISP2, Wnt-1-induced signaling protein-2.

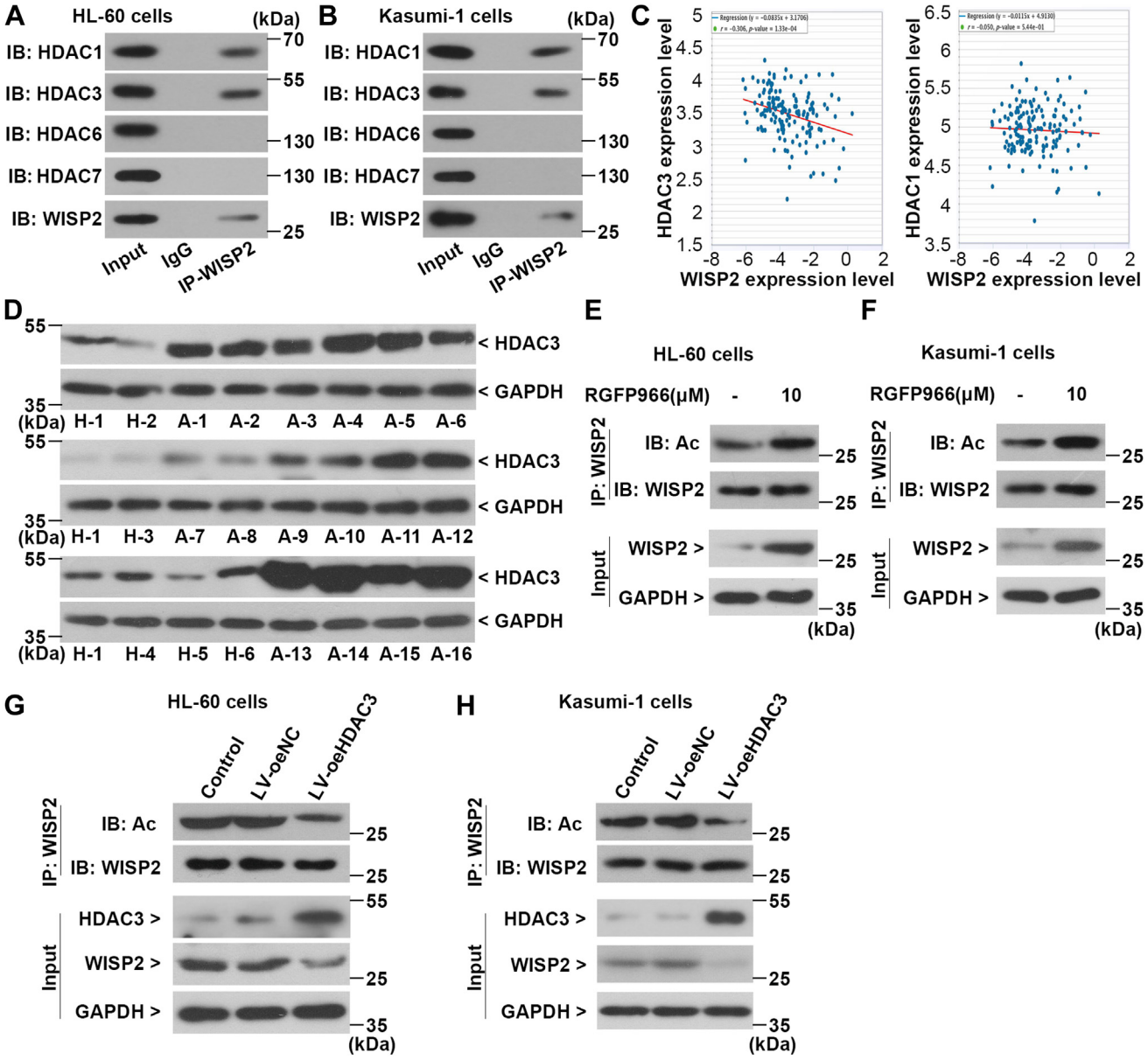

Figure 5. HDAC3 interacts with and deacetylates WISP2 in AML cells. *A* and *B*, co-IP was performed with anti-WISP2 antibody and IgG as a control, followed by immunoblotting with anti-HDAC1, anti-HDAC3, anti-HDAC6, and anti-HDAC7 antibodies in AML cells. *C*, the correlation between HDAC3 or HDAC1 and WISP2 expression levels from 151 AML samples analyzed by StarBase. *D*, Western blot analysis of HDAC3 expression in bone marrow mononuclear cells from healthy donors and AML patients. *E* and *F*, the protein and acetylation levels of WISP2 in RGFP966-treated AML cells. *G* and *H*, the protein and acetylation levels of WISP2 and the protein expression of HDAC3 in AML cells infected with HDAC3 lentivirus. Ac, acetylated lysine; AML, acute myeloid leukemia; co-IP, co-immunoprecipitation; HDAC, histone deacetylase; WISP2, Wnt-1-induced signaling protein-2.

expressing cells was similar to that of wt-WISP2-flag-expressing cells, suggesting that K6 was not the direct ubiquitination site of WISP2 (Fig. 7*J*). Several previous studies (22, 23), which showed similar or even increased ubiquitination levels in lysine mutation-expressing cells, (compared with wt-expressing cells) indicated that the acetylated lysine residues are not ubiquitination sites. Here, we also found that K6 affected the protein stability of WISP2, but it itself was not a direct ubiquitination site. Further experiments are being conducted to unravel the specific and direct sites required for ubiquitination degradation of WISP2. Moreover, knockdown of NEDD4 partly restored WISP2 protein expression in HL-60 cells overexpressing HDAC3 (Fig. 7*K*). These data implied that NEDD4 was involved in WISP2 degradation induced by HDAC3.

Discussion

AML is a complex disease originating from hematopoietic stem cell disorders. Molecular insights into leukemogenic mechanisms require further study to reduce recurrence and improve prognosis. In this study, we provided *in vitro* and *in vivo* evidence that WISP2 exerts antileukemic effects by suppressing cell proliferation and inducing cell apoptosis. We described a leukemogenic mechanism whereby HDAC3-

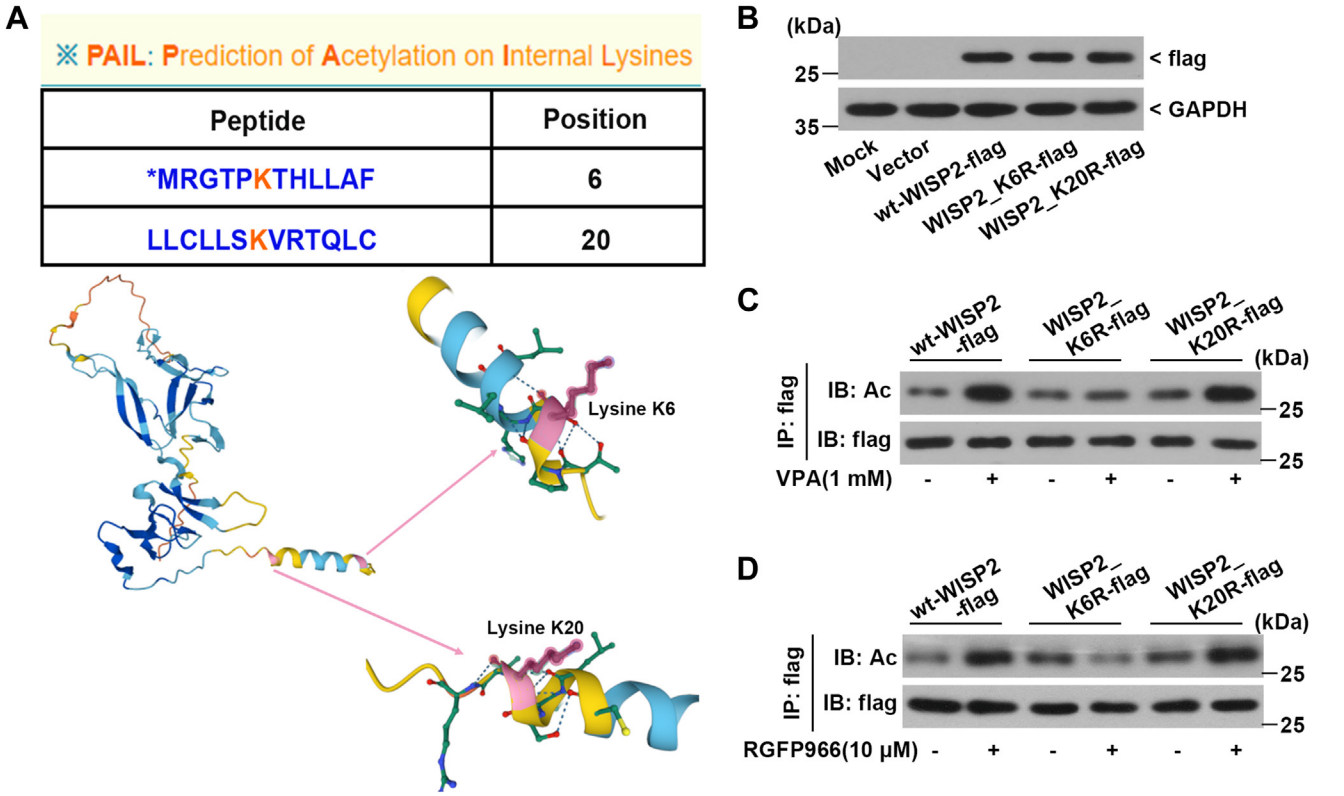

Figure 6. The lysine K6 residue is essential for WISP2 acetylation. *A*, prediction of acetylation on internal lysine residues indicated two potential acetylated lysine (K) residues, K6 and K20, within WISP2. *B*, HEK293 cells were transfected with wildtype WISP2 (wt-WISP2-flag), mutant WISP2_K6R (WISP2_K6R-flag), or mutant WISP2_K20R (WISP2_K20R-flag) for 48 h, and the transfection efficiency was determined by immunoblotting with an anti-Flag antibody. *C* and *D*, HEK293 cells were transfected with wt-WISP2-flag, WISP2_K6R-flag, or WISP2_K20R-flag for 48 h and then treated with VPA or RGFP966 for 24 h. Whole-cell lysates were subjected to detection with anti-acetylated-lysine antibody. Ac, acetylated lysine; VPA, valproic acid; WISP2, Wnt-1-induced signaling protein-2.

induced deacetylation disrupts WISP2 stability by augmenting NEDD4-mediated ubiquitination. The blockade of deacetylation by HDAC inhibitors restores WISP2 expression in AML cells and results in decreased leukemia cell proliferation and increased apoptosis (Fig. 7*L*). Considering the association of WISP2 with differentiation in gallbladder adenocarcinoma (24) and the regulation of WISP2 on the differentiated phenotype in breast cancer cells (25), we reasoned that WISP2 may be involved in modulating the differentiation of AML cells. This inference remains to be explored in future studies.

The expression of WISP2 is regulated at both the transcriptional and posttranscriptional levels. Epidermal growth factor, estrogen receptor, hypoxia-inducible factor 2α, and dimerization of FOXP1/2/4 (Forkhead box P1/2/4) were identified to be involved in WISP2 transcriptional activation or repression (26–29). MiR-450a-5p and miR-449 were reported to posttranscriptionally repress the expression of WISP2 mRNA transcripts (30, 31). Until recently, whether WISP2 is involved in posttranslational modification remains unclear and awaits further investigation. A considerable number of studies have shown that posttranslational modifications, such as phosphorylation, methylation, acetylation, and ubiquitination, have a major role in the development of leukemia (32–35). Protein acetylation, a posttranslational modification that has a potent impact on protein expression and function, is balanced by acetylases and deacetylases. In this study, we found that the acetylation level of WISP2 was decreased in bone marrow mononuclear cells from AML patients, and HDAC3 was the regulator of WISP2 deacetylation. Furthermore, we revealed that WISP2 acetylation was induced by HDAC3 inhibition at lysine residue K6. WISP2 acetylation increased its protein stability, thereby inhibiting cell proliferation and inducing apoptosis. The current study broadened our understanding of the posttranslational modification of WISP2.

The HDAC superfamily has 18 HDAC proteins that are divided into four classes: Class I (HDAC1, 2, 3, and 8), Class II (HDAC4, 5, 6, 7, 9, and 10), Class III (SIRT1, 2, 3, 4, 5, 6, and 7), and Class IV (HDAC11 only) (13). It was corroborated that changes in the expression and/or activity of HDACs were observed in solid tumors and leukemia (36). A large and growing body of literature has shown that the aberrant recruitment of HDACs plays a key role in leukemogenesis (37–40). For example, HDAC3 has a prooncogenic function in acute promyelocytic leukemia, and it is required for leukemia initiation (41). Similarly, an increased abundance of HDAC3 in bone marrow mononuclear cells from AML patients was proven by our results. Due to their potent anticancer activities, much attention has been focused on HDACis, which have been proven to possess promising therapeutic potential for hematological malignancies, such as AML (42), resistant acute promyelocytic leukemia (43), and multiple myeloma (44). The common mechanisms of the antineoplastic role of HDACis are

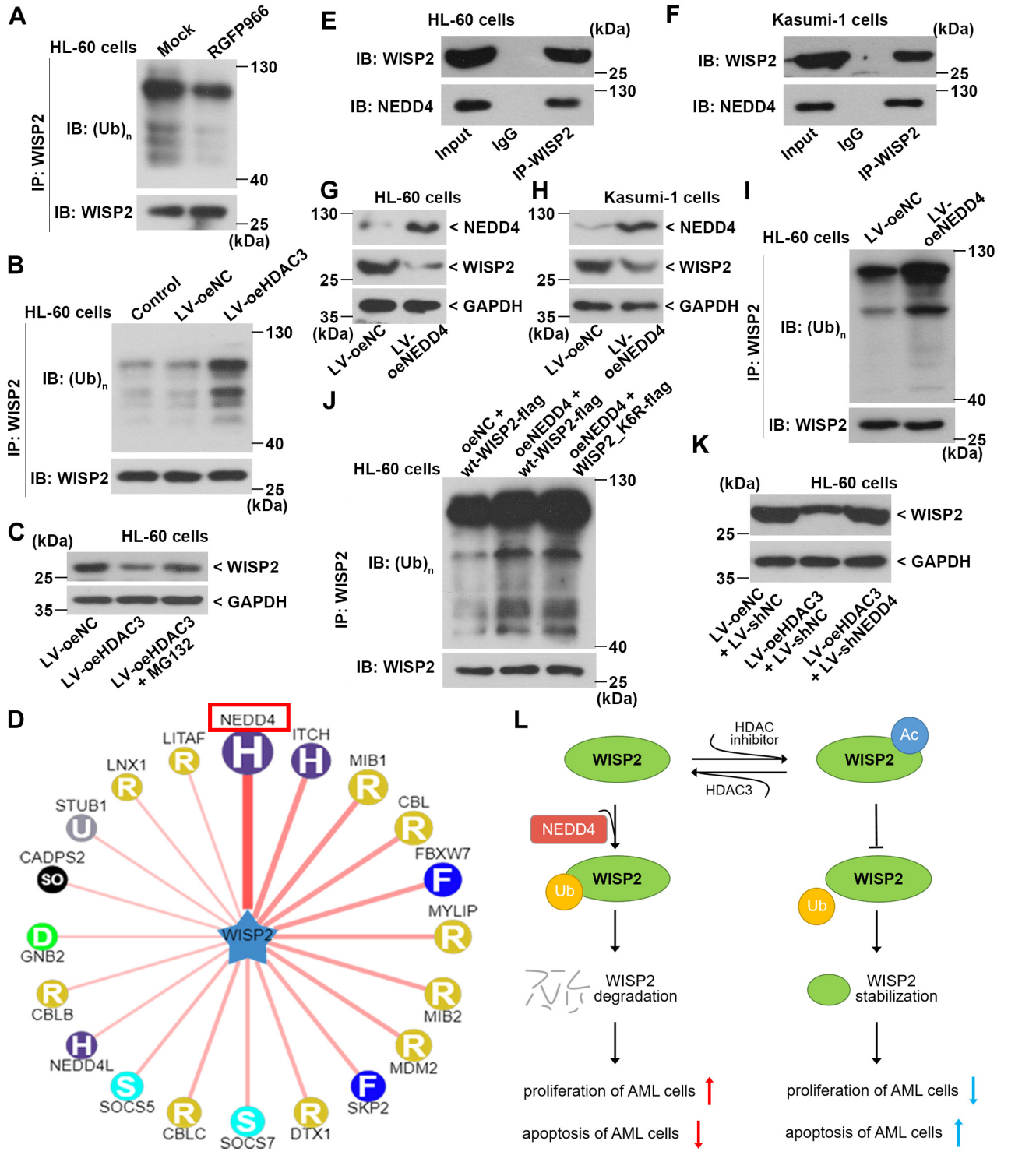

Figure 7. Acetylation of WISP2 prevents NEDD4-mediated ubiquitination. *A*, HL-60 cells were treated with or without RGFP966 for 24 h, and MG132 was added 6 h before harvesting cells. Proteins were co-IP with anti-WISP2 antibody, followed by immunoblotting with anti-ubiquitin or anti-WISP2 antibodies. *B*, HDAC3 lentivirus was infected into HL-60 cells for 72 h, and MG132 was added 6 h before harvesting cells. The ubiquitination of WISP2 was detected by co-IP with an anti-WISP2 antibody, followed by immunoblotting with the indicated antibodies. *C*, HL-60 cells were infected with HDAC3 lentivirus. After 72 h, the cells were treated with MG132 for 6 h. Total lysates were analyzed by Western blotting using an anti-WISP2 antibody. *D*, prediction of Ubibrowser indicated that NEDD4 was the top E3 ubiquitin ligase that probably targets WISP2. *E* and *F*, endogenous interaction between WISP2 and NEDD4 in HL-60 cells and Kasumi-1 cells was examined by co-IP assay. *G* and *H*, the protein levels of NEDD4 and WISP2 were confirmed by Western blotting in NEDD4-overexpressing AML cells. *I*, co-IP assay to assess WISP2 ubiquitination in HL-60 cells infected with NEDD4 lentivirus. *J*, co-IP assay to assess WISP2 ubiquitination in HL-60 cells transfected with NEDD4 overexpression plasmid and wt-WISP2-flag or WISP2_K6R-flag. *K*, HL-60 cells were infected with LV-oeHDAC3 and LV-shNEDD4 as indicated. Western blot analysis was performed to determine WISP2 expression. *L*, schematic diagram for the antileukemic effect of WISP2: HDAC3-induced deacetylation disrupts WISP2 stability by augmenting ubiquitination mediated by NEDD4, which contributes to the increase in tumor cell proliferation and the suppression of apoptosis. The blockade of histone deacetylation by HDAC inhibitors restores WISP2 expression in AML cells, thereby allowing WISP2 to exert its antileukemia effect. AML, acute myeloid leukemia; co-IP, coimmunoprecipitation; LV, lentivirus; ub, ubiquitin; WISP2, Wnt-1-induced signaling protein-2.

divided into two categories. One is that HDACis induce the acetylation of oncogenic proteins, resulting in decreased activity or expression of proteins. For instance, HDAC3 contributes to chemotherapy resistance in AML by deacetylating AKT and promoting AKT phosphorylation, while inhibition of HDAC3 downregulates AKT activity and sensitizes leukemia cells to chemotherapy drugs (45). Alternatively, HDACis induce the acetylation of tumor suppressors, leading to an increase in their activity or expression. A study conducted by Meng *et al*. showed that an HDAC6-specific inhibitor induces PTEN activation through PTEN acetylation at K163, thereby contributing to tumor suppression (46). Consistent with the study of Meng *et al*., we found that WISP2 acetylation induced by HDACi gave rise to increased WISP2 expression, which promoted the antineoplastic effect of WISP2 on AML.

Crosstalk between posttranslational modifications is common, and acetylation may modulate other modifications, such as ubiquitination. The mechanism by which acetylation potentiates protein stability is for preventing protein ubiquitination and for inhibiting subsequent proteasome-dependent degradation. For example, acetylation of AFP at lysine 194, 211, and 242 stabilizes the AFP protein by restraining its ubiquitination and proteasomal degradation (23). Conversely, acetylation also destabilizes protein stability by promoting its ubiquitination and subsequent proteasomal degradation. DNMT1 acetylation triggered by acetyltransferase Tip60 recruits ubiquitin ligase UHRF1, resulting in DNMT1 ubiquitination and proteasomal degradation (47). Our findings are consistent with this mechanism in which acetylation prevents protein ubiquitination. WISP2 is destabilized *via* HDAC3 deacetylation, and HDACi-induced acetylation of WISP2 at lysine residue K6 prevents its ubiquitination mediated by NEDD4 and proteasomal degradation. These findings revealed that the downregulation of WISP2 in AML is attributed to increased HDAC3 expression and proteasomal degradation of WISP2. The current study focused on the acetylation of WISP2 and indicated that K6 was the acetylation site of WISP2. WISP2 ubiquitination mediated by NEDD4 was preliminarily explored. Further work needs to be done to identify the specific and direct sites required for ubiquitination degradation of WISP2.

Taken together, our study indicated that WISP2 drives tumor suppression in AML. We also provided novel mechanistic insight into the downregulation of WISP2 in AML, which is that WISP2 deacetylation induced by HDAC3 augments WISP2 ubiquitination. The study proposed that increasing WISP2 acetylation, such as with HDAC3 inhibitors, may constitute a promising therapeutic strategy for AML.

Experimental procedures

Human samples and processing

Bone marrow samples were obtained from 16 AML patients (M2 subtype: 3 cases; M3 subtype: 7 cases; M4 subtype: 2 cases; M5 subtype: 4 cases) and six healthy donors enrolled in Affiliated Hospital of Jining Medical University. All individual participants signed informed consent before sample collection. All procedures in studies involving human participants were approved by the Ethics Committee of Jining Medical University and were performed in accordance with the Declaration of Helsinki and its later amendments or comparable ethical standards.

Bone marrow mononuclear cells were isolated from bone marrow samples. Bone marrow samples were diluted with phosphate-buffered saline (PBS), and the diluted samples were spread flat on the lymphoprep. After centrifugation, the buffy coat containing bone marrow mononuclear cells was collected. After bone marrow mononuclear cells were stained with CD34 antibody (Thermo Fisher Scientific; 12–0349–42), $CD34^+$ cells were sorted by flow cytometry.

Cell lines and culture

The human AML cell lines HL-60, Kasumi-1, and KG-1 were obtained from Procell Life Science & Technology Co, Ltd HL-60 and KG-1 cells were incubated in Iscove's modified Dulbecco's medium supplemented with 20% fetal bovine serum. Kasumi-1 cells were cultured in Roswell Park Memorial Institute-1640 medium supplemented with 20% fetal bovine serum. Cells were incubated at 37 °C in an atmosphere of 5% CO_2.

Lentivirus packaging and infection

LV-WISP2, HDAC3 (LV-oeHDAC3), and NEDD4 (LV-oeNEDD4) were obtained from Hunan Fenghui Biotechnology Co, Ltd. Lentivirus plasmids containing short hairpin RNA (shRNA) of NEDD4 (LV-shNEDD4) or negative control (LV-NC) were obtained from Hunan Fenghui Biotechnology Co, Ltd. HEK293 cells were transfected with the indicated plasmids using Lipofectamine 3000 (Invitrogen) following the manufacturer's instructions. After transfection for 6 h, the medium was replaced with fresh complete medium and incubated for 48 h. Afterward, the supernatant, namely lentivirus, was collected by filtering with a 0.45-μm membrane filter. Concentrated lentivirus (the viral titer was 2×10^8 TU/ml) at a multiplicity of infection of 50 was added to AML cells. The following experiments were performed 72 h after infection.

Cell treatment

HL-60 and Kasumi-1 cells were treated with the pan-HDAC inhibitor VPA (MedChemExpress; HY-10585) or TSA (Shanghai Yuanye Bio-Technology; S48702) at the indicated concentrations for 24 h and then subjected to subsequent experiments.

The HDAC3-specific inhibitor RGFP966 (Shanghai Yuanye Bio-Technology, S82976) at a concentration of 10 μM was added to HL-60 and Kasumi-1 cells and incubated for 24 h.

HL-60 and Kasumi-1 cells were treated with VPA or TSA for 12 h and then continuously treated with 25 μg/ml cycloheximide (MedChemExpress, HY-12320) for the indicated times to determine the expression of WISP2.

Western blot analysis

For total protein extraction, cells were lysed with RIPA lysis buffer (SolarbioR0010) supplemented with 1 mM

phenylmethylsulfonyl fluoride (Solarbio, P0100). For mitochondrial protein extraction, cells were lysed with a mitochondrial protein extraction kit (Nanjing Jiancheng Bioengineering Institute; G008). For cytoplasmic protein extraction, cells were lysed with cytoplasmic lysis buffer. Protein concentration was determined using a BCA Protein Assay Kit (Solarbio, PC0020). Protein samples were separated on 5% to 15% SDS–PAGE gels and transferred onto polyvinylidene fluoride membranes (Millipore; IPVH00010). After they were blocked by 5% nonfat milk, membranes were incubated with the following primary antibodies: anti-WISP2 antibody (ABclonal; A7456), anti-Bax antibody (ABclonal, A19684), anti-Bcl-2 antibody (ABclonal, A19693), anti-cleaved caspase-3 antibody (Affinity; AF7022), anti-cleaved caspase-9 antibody (BOSTER; BA0690), anti-cleaved PARP antibody (CST; #5625), anti-Bak antibody (ABclonal, A0204), anti-cytochrome c antibody (ABclonal, A4912), anti-COX IV antibody (GeneTex; GTX49132), anti-HDAC3 antibody (ABclonal, A19537), anti-NEDD4 antibody (ABclonal, A4385), and anti-GAPDH antibody (Proteintech; 60004-1-Ig). Afterward, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Solarbio, SE134, SE131). Finally, the membranes were washed, and protein bands were detected by enhanced chemiluminescence (ECL; Solarbio, PE0010).

Coimmunoprecipitation assay

Cells were treated or transfected as indicated before harvesting. Cells were lysed with RIPA lysis buffer, and the supernatant was subjected to a BCA Protein Assay Kit to determine the protein concentration. A fraction of the cell lysate was set apart as input samples. The lysates were incubated with 1 μg of antibodies against WISP2 (Santa Cruz; sc-514070), Flag-tag (Abcam; ab125243) or IgG (Solarbio, SP031) overnight at 4 °C with gentle rotation, followed by incubation with 60 μl of Protein A Affinity Chromatography Media (Aladdin; P5362–01) for 2 h. The immunocomplex was washed, denatured, and subjected to Western blotting using primary antibodies against WISP2 (ABclonal, A7456), acetylated-Lysine (ABclonal, A2391), HDAC1 (ABclonal, A19571), HDAC3 (ABclonal, A19537), HDAC6 (ABclonal, A1732), HDAC7 (ABclonal, A13008), Flag-tag (Abcam, ab205606), and NEDD4 (ABclonal, A4385). The input samples were also subjected to Western blotting using primary antibodies against WISP2 and GAPDH. GAPDH was used as a loading control.

Ubiquitination assay

Ubiquitination assays were performed as described previously. Cells subjected to different treatments were treated with 10 μM MG132 (Aladdin, M126521) for 6 h before cell harvesting. Then, the cells were lysed and immunoprecipitated with an antibody against WISP2 followed by protein A affinity chromatography media. Then, the samples were detected by immunoblotting using ubiquitin antibody (ABclonal, A19686).

CCK-8 assay

Cells were seeded into 96-well plates (4×10^3 cells/well) and incubated in medium containing LV-NC or LV-WISP2 for 24 h, 48 h, 72 h, 96 h, and 120 h. For VPA or TSA treatment, cells were treated with VPA (0, 0.5, 1, 2 mM) or TSA (0, 2.5, 5, 10 μM) for 24 h. Then, 10 μl of CCK-8 reagent (KeyGen Biotech; KGA317) was added to each well, and the plates were incubated at 37 °C for 2 h. Absorbance values were measured at 450 nm using a microplate reader (BIOTEK; 800Ts).

Apoptosis assay

Apoptosis assays were performed with an Annexin V-FITC Apoptosis Detection Kit (KeyGen Biotech, KGA106). Cells were harvested and then washed twice with PBS. After centrifugation, the cells were resuspended in 500 μl of binding buffer. Cells were double-stained with 5 μl of Annexin V-FITC and 5 μl of propidium iodide for 15 min in the dark. The mixture was analyzed on a flow cytometer (Aceabio).

MMP analysis

A mitochondrial membrane potential assay kit with JC-1 (Beyotime; C2006) was used to detect alterations in MMP in the AML cells. Cells were collected and incubated with 0.5 ml of JC-1 dye for 20 min at 37 °C. After centrifugation at 600*g* for 3 min at room temperature, the supernatant was removed. Cells were rinsed with JC-1 washing buffer (1×) two times and then resuspended in 500 μl of washing buffer. JC-1 monomers (green fluorescence) or aggregates (red fluorescence) were analyzed by flow cytometry.

Mice and leukemia transplant model

Animal experiments were approved by the Ethics Committee of Affiliated Hospital of Jining Medical University and were performed in accordance with Guide for the Care and Use of Laboratory Animals. Female NOD/SCID mice aged 6 to 8 weeks were adapted for 1 week and were randomly divided into the LV-NC and LV-WISP2 groups (with n = 12 for each group). On the day prior to transplantation, mice received 300 cGy of total body irradiation. HL-60 cells (5×10^6) transduced with LV-NC or LV-WISP2 were inoculated into mice by tail vein injection. After 6 weeks, the mice were euthanized. Bone marrow cells were collected by flushing femurs with culture medium. Tumor burden in bone marrow was detected by flow cytometric analysis of CD45.

Leishman–Wright–Giemsa staining

Bone marrow cells were harvested from mice and prepared into smears. The Wright–Giemsa Stain Kit (Nanjing Jiancheng Bioengineering Institute, D010) was applied to conduct the experiment. The smears and sections were incubated with R1 staining solution for 1 min and then incubated with R2 buffer solution for another 7 min. The stained smears and sections were rinsed with water and differentiated in 80% ethanol for decolorization. The smears and sections were naturally dried

and observed under a microscope (OLYMPUS) at 400× magnification.

Determination of the acetylation site

Mutations of lysine residues (K6 and K20) to arginine (K6R and K20R) were generated. DNA fragments encoding wildtype WISP2 (wt-WISP2) or mutant WISP2 (WISP2_K6R and WISP2_K20R) was inserted into the region between the EcoR I and BamH I restriction sites of the p3xFLAG-CMV-10 vector. The vectors encoding wt-WISP2, WISP2_K6R or WISP2_K20R were transiently transfected into HEK293 cells. After 48 h, HEK293 cells were subjected to subsequent treatment or experiments.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8.0 Software. Significance between groups was determined by two-way ANOVA, one-way ANOVA, or unpaired *t* test. All data are presented as the mean ± standard deviation (SD). The results with p values < 0.05 were considered significant ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

Data availability

The data generated from online database are available from UALCAN cancer database (http://ualcan.path.uab.edu/index.html/), HitPredict (http://www.hitpredict.org/), StarBase (https://starbase.sysu.edu.cn/), Prediction of Acetylation on Internal Lysines (PAIL, http://bdmpail.biocuckoo.org/), and Ubibrowser (http://ubibrowser.ncpsb.org.cn/ubibrowser/). Other data that support the findings of our study are available from the corresponding author upon reasonable request.

Author contributions—H. Z. methodology; H. Z., W. S., and X. M. investigation; H. Z. and W. S. formal analysis; H. Z. writing—original draft; X. M. software; M. Y. and L. C. validation; M. Y. visualization; L. C. data curation; Y. T. conceptualization; Y. T. project administration; funding acquisition; Y. T. writing—review & editing.

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Abbreviations—The abbreviations used are: AML, acute myeloid leukemia; CHX, cycloheximide; Co-IP, co-immunoprecipitation; HDACs, histone deacetylases; HDACi, histone deacetylase inhibitors; LV-WISP2, lentivirus plasmids containing full-length WISP2; MMP, mitochondrial membrane potential; PBS, phosphate-buffered saline; TPM, transcript per million; TSA, trichostatin A; VPA, valproic acid; WISP2, Wnt-1-induced signaling protein-2.

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