Disrupting the protective signals provided by the bone marrow microenvironment will be critical for more effective combination drug therapies for acute myeloid leukemia (AML). Cells of the osteoblast lineage that reside in the endosteal niche have been implicated in promoting survival of AML cells. Here, we investigated how to prevent this protective interaction. We previously showed that SDF-1, a chemokine abundant in the bone marrow, induces apoptosis of AML cells, unless the leukemic cells receive protective signals provided by differentiating osteoblasts (8, 10). We now identify a novel signaling pathway in differentiating osteoblasts that can be manipulated to disrupt the osteoblast-mediated protection of AML cells. Treating differentiating osteoblasts with histone deacetylase inhibitors (HDACi) abrogated their ability to protect co-cultured AML cells from SDF-1-induced apoptosis. HDACi prominently up-regulated expression of the Nherf1 scaffold protein, which played a major role in preventing osteoblast-mediated protection of AML cells. Protein phosphatase-1α (PP1α) was identified as a novel Nherf1 interacting protein that acts as the downstream mediator of this response by promoting nuclear localization of the TAZ transcriptional modulator. Moreover, independent activation of either PP1α or TAZ was sufficient to prevent osteoblast-mediated protection of AML cells even in the absence of HDACi. Together, these results indicate that HDACi target the AML microenvironment by enhancing activation of the Nherf1-PP1α-TAZ pathway in osteoblasts. Selective drug targeting of this osteoblast signaling pathway may improve treatments of AML by rendering leukemic cells in the bone marrow more susceptible to apoptosis.

For decades, research into drug treatments for acute myeloid leukemia (AML)² has focused on directly targeting AML cells for destruction. Even though complete remission rates have improved, relapse remains a problem in the majority of patients with this disease. The bone marrow microenvironment has gained attention as a protective environment that promotes survival of AML stem cells, despite the killing of the majority of AML cells by standard chemotherapeutics (1–5). The endostium, the tissue between the bone marrow and ossified surface, has been particularly implicated as a protective niche because AML stem cells are localized to this region following chemotherapeutics (6, 7). Within the endosteal niche, cells of the osteoblast (bone-generating) lineage have been identified as critical mediators of AML cell survival in the bone marrow (4, 8). Transgenic mice expressing activated β-catenin specifically in osteoblasts develop myeloid malignancy, consistent with the idea that osteoblasts promote this disease (9). Unfortunately, the molecular mechanisms responsible for osteoblast-mediated protection of AML cells are incompletely understood. This lack of knowledge prevents effective therapeutic manipulation of the bone marrow microenvironment as a way to enhance targeting of AML cells within the bone marrow.

We previously reported that SDF-1, a chemokine abundantly secreted by multiple cell types within the bone marrow, induces apoptosis of AML cell lines and patient isolates that express high levels of its receptor CXCR4 (8, 10). Because AML cells thrive in the bone marrow, this apparent contradiction indicated that a cell type within the bone marrow must provide signals that protect AML cells from SDF-1-induced apoptosis. Utilizing co-culture systems, we previously demonstrated that co-culturing differentiating osteoblasts, but not earlier precursors of the osteoblast lineage, with the AML cells completely abrogated SDF-1-induced apoptosis of the AML cells (8). These results prompted us to utilize this co-culture system to identify strategies to inhibit osteoblast-mediated protection of AML cells.

HDACi are being explored as treatments for diverse malignancies. These drugs globally regulate gene expression via increasing acetylation of histones, proteins that control chromatin accessibility to transcription factors. In AML, HDACi have shown efficacy in combination with standard chemotherapeutics such as cytarabine (11–14). HDACi are known to hydroxamic acid; PP1α, protein phosphatase-1α; DMSO, dimethyl sulfoxide; qRT, quantitative RT; APC, allopurinol.
directly target cancer cells by increasing expression of many proteins including those that regulate tumor suppression, DNA repair, and cell-cycle arrest. Because HDAC inhibitors have also been shown to alter expression of many genes within cells of the osteoblast lineage (15–18), we hypothesized that HDACi might also target the leukemic microenvironment. HDAC inhibitors have been previously shown to up-regulate Nherf1 (also known as EBP-50), a scaffold protein mutated in human hypophosphatemic nephrolithiasis/osteoporosis type 2. Nherf1 has been linked with TAZ, a ubiquitously expressed transcriptional modulator, in the regulation of osteoblast differentiation (19). Localization of TAZ to the nucleus, where it increases expression of multiple genes including those controlled by the TEAD and SMAD family of transcription factors, is regulated by phosphorylation. When TAZ is phosphorylated, it is localized to the cytoplasm. Dephosphorylation by PP1α, a ubiquitously expressed Ser/Thr protein phosphatase, induces TAZ nuclear localization to mediate osteoblast differentiation (20–26). Nherf1 knock-out mice display bone defects (19, 27, 28), however, the effects of elevating Nherf1 expression and increasing TAZ localization to the nuclei of osteoblasts were previously unknown.

Here, we show that HDACi inhibit the protective functions of the bone marrow microenvironment in AML by targeting osteoblasts to increase expression of Nherf1, and that Nherf1 up-regulation prevented osteoblast-mediated protection of AML cells in co-cultures. We also show that increased Nherf1 activates a novel signaling pathway in osteoblasts by binding PP1α, which promotes TAZ nuclear localization and inhibits the ability of osteoblasts to protect AML cells from apoptosis while having little effect on osteoblast differentiation. Together, these results identify several members of a novel molecular signaling pathway within osteoblasts that could be targeted in AML to ameliorate the leukemic cell protective effects of the endosteal niche.

**Experimental Procedures**

*Materials—Reagents were obtained from the following suppliers: ascorbic acid, β-glycerophosphate, dimethyl sulfoxide (DMSO), and the probe inhibitor mixture (Sigma); SDF-1 (R&D Systems), SAHA (Cancer Therapy Evaluation Program, National Cancer Institute); LBH-589 (Selleckchem.com); and live/dead viability assay and Prolong Gold anti-fade with DAPI (Invitrogen).*  
Antibodies were obtained from the following suppliers: rabbit polyclonal anti-acetylated H3 and anti-total H3 as well as S-protein agarose (Millipore); murine monoclonal anti-actin (Novus Biologicals); rabbit anti-Nherf1 (anti-EBP50); murine monoclonal anti-PP1α and agarose-conjugated rabbit-anti-PP1α (Santa Cruz Biotechnology); murine monoclonal anti-TAZ and APC-conjugated annexin-V (BD Biosciences); APC-conjugated murine monoclonal CXCR4 (R&D Systems); rabbit anti-lamin A and C (Genscript); murine monoclonal anti-α-tubulin (Sigma); and Alexa Fluor 647-conjugated anti-mouse IgG (Invitrogen). Murine monoclonal anti-S peptide antibody was generated as previously described (29).

*Cells—After informed consent was obtained on an IRB approved protocol, samples of bone marrow were harvested from AML patients prior to chemotherapy and utilized in co-cultures as described (8). MC3T3 sc4 murine calvarial osteoblasts (30) (ATCC) were cultured and differentiated as described (8). Briefly, MC3T3 cells were maintained in Medium B (α-MEM without ascorbic acid (Invitrogen), 10% FCS, 1% penicillin/streptomycin). To induce differentiation (denoted as day 0), the culture medium of confluent MC3T3 cells was replaced with osteogenic medium (α-MEM, 50 μg/ml of ascorbic acid, 4 mM β-glycerophosphate). The human bone marrow-derived tert-immortalized bone marrow stromal cell line (t-BMSC) (31) was a gift from Dario Campana (St. Jude, Memphis, TN) and was maintained as described (8). The CXCR4-expressing KG1a cells (KG1a-CXCR4) were generated via transient transfection of a plasmid encoding a CXCR4-YFP fluorescent fusion protein (32) into the AML cell line KG1a (ATCC), as previously described (10).*  

**siRNA, Plasmid Constructs, Transfection, and Subcellular Fractionation of MC3T3 Cells—ON-TARGET Plus Control siRNA pool and Nherf1-specific siRNA pool were from GE Healthcare (Dharmacon). Nherf1-specific siRNA #2 (77422) was obtained from Ambion. eYFP-N1 was from Clontech. pcDNA murine Nherf1 (33) was from Edward Weinman (Addgene, 32705). GFP-Nherf1 was generated by subcloning murine Nherf1 into pEGFP-C1 (Clontech). Nherf1 tagged with S peptide at its N terminus (S-Nherf1) was generated by subcloning murine Nherf1 into pSPN (29). Using a plasmid encoding GFP-PP1α (34) from Angus Lamond and Laura Trinkle-Mulcahy (Addgene, 44224), GFP-PP1α-T320A was generated by site-directed mutagenesis using a QuikChange II XL site-directed mutagenesis kit (Agilent Technologies). S peptide-PP1α (S-PP1α) was generated by subcloning murine PP1α into pSPN (29). TAZWT and TAZS89A plasmids (24) were from Michael Yaffe (Addgene, 19025, 19026). S peptide-TAZWT (S-TAZWT) and S peptide-TAZS89A (S-TAZS89A) were generated by subcloning into pSPN (29).

MC3T3 cells were transfected on day (−1) prior to differentiation as follows: a confluent layer of undifferentiated MC3T3 cells was detached from plates via trypsin/EDTA, washed in Medium B, combined with the indicated siRNAs or plasmids, electroporated using a BTX square wave electroporator at 315 V for 10 ms, and then replated. Six hours later, the medium was removed and replaced with fresh Medium B. The following day (day 0), osteoblast differentiation was initiated by replacing the culture medium with osteogenic medium (40–50%). Where indicated, MC3T3 were treated or transfected, and on day 2 of differentiation the MC3T3 cells were washed with PBS and subcellular fractionation was performed as described (26).

**Co-culture Assays, HDACi Treatment, and Apoptosis Analysis—MC3T3 cells were prepared for co-culture as indicated via transfection on day (−1), and differentiation was initiated on day 0. Where indicated, 0.1% DMSO (vehicle), 10 μM SAHA, or 1 μM LBH-589 was added to the culture medium for the indicated times. MC3T3 cultures were then washed to remove residual SAHA or LBH-589 and co-cultures were begun by adding 0.25 × 10⁶ cells/ml of KG1a-CXCR4 AML cells. Co-cultures were maintained at 37 °C for 1 h, then 1.3 × 10⁻⁸ M SDF-1 was added and cells were cultured for an additional 16–18 h. On day 3, KG1a-CXCR4 AML cells were removed from the
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Results

Osteoblasts Protect AML Isolates from Apoptosis in a Co-culture Model of the Bone Marrow Microenvironment—We previously demonstrated that AML patient isolates and cell lines expressing high levels of CXCR4 underwent apoptosis in response to treatment with SDF-1 (10). Preliminary studies conducted predominantly in AML cell lines also suggested that osteoblasts protect AML cells expressing high levels of CXCR4 from this SDF-1-induced apoptosis (8). Building on these studies, SDF-1-responsive patient AML isolates were co-cultured either with or without the well-characterized, rapidly mineralizing MC3T3 sc4 osteoblast cell line, and then analyzed for apoptosis via flow cytometric detection of annexin-V expression. Fig. 1, A and B, show the increased percentage of patient AML cells expressing annexin-V in response to SDF-1. In contrast, adding MC3T3 osteoblasts to co-cultures markedly inhibited apoptosis of all SDF-1-responsive patient AML isolates examined to date (Fig. 1, A–C). These results indicate that osteoblasts provide protective signals to AML cells within the bone marrow microenvironment.

Osteoblasts Treated with HDACi Fail to Protect AML Cells from SDF-1-induced Apoptosis—To identify molecular mechanisms that prevent osteoblast-mediated protection of AML cells, we utilized a more defined co-culture model of the bone marrow microenvironment (8) that allows specific alteration of the microenvironment to identify key signaling pathways that could be modulated to inhibit the protection provided to AML cells. This model (8, 10) utilizes the previously described differentiation and/or transfected as indicated on day 2 of differentiation were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris (pH 7.4), 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100), immunoprecipitated with the indicated antibodies, and analyzed via either SDS-PAGE and immunoblotting or SDS-PAGE and silver stain followed by mass spectrometry.

Immunofluorescence—Cells were imaged using a LSM780 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). ZEN software (Carl Zeiss) was utilized for acquisition of images. For live/dead assays, a 10×/0.45 M27 objective and the following wavelengths for excitation/emission were utilized: 561/626 for dead (red) cells and 488/522 for live (green) cells. The percentage of dead cells was determined by dividing the number of red, dead cells by the total number of green cells, plus the number of red, dead cells. For visualization of Nherf1, P1α, and TAZ, MC3T3 cells were fixed with 4% paraformaldehyde, permeabilized with 0.15% Triton X-100, stained with DAPI to identify nuclei, and visualized with a 100×/1.46 oil objective and laser/emission filter: 488/500–554 for GFP, 405/411–481 for DAPI, and 633/660–758 for Alexa Fluor 647. Cells were scored positive for nuclear localization if TAZ nuclear staining was stronger than TAZ cytoplasmic staining.

TABLE 1
| Gene ID | Primer Sequences |
|---------|-----------------|
| Gapdh   | ATGCCAGTGAGCTCCGCTGGTTACAG | CACCACGATCTGAGTAAGACCC |
| Act1    | TCCTGCCACAGCCGACCTGCTCAG   | CACCCAGATCTGAGTAAGACCC |
| Sep1    | CAGAGCTCCAATCGCCATGCTGCG   | CGTCACTTGTGGCGAGCTTATT |
| Hnr11   | CTGGTGAAGAGAGCCTCGGAGAG   | GTCCTGAGCTTCGTCGACAGCA |
| Ezh1    | CACACGTCAAGGACGACCATTGAA  | TCACTCGCTGATCGATGGAGGG |
| Ak1     | CTTGAGCGCTGAGAAGATCTTCT   | TCAGTATATATATATATATAT |
| Runx2   | GGTTTCTTTGGGCGAGAGATTG   | CGCTAGTATATATATATATAT |
| Sp7     | CAAAGATGGCCATTCACCAGGAC   | GCCCTATGTTTTAGCAGGCAG |
| Satb2   | CTGGTACATCCTGGAGAGCGCA   | CTGGCACTGATGGGGTTAGA |
| Ofrd    | TGCACATCCGAGAACATTGCT   | TGGCGTCACTGATGGGGTTAGA |
| Sparc   | CCCCCCGGACAGACTGAAAGTT  | ACAGGTACCCTCGTCTCCCTC |
| Ibsp    | GAATTGCGGCGTCTTCTGC    | CGGCTAATTAAAGACCCGGTT |
| Bglap   | GCAATAGGAGTGATGCAAGACATTCCCC | CCAATTAGGAGTGATGCAAGACATTCCCC |
| Alph    | CAGAGAAAGACACCTCGAGTGG   | TACAGTATAGTATAGTATAGTAT |
| Phx-1   | TCACTGCCACCCATGATGTCA   | GAAGGATTCCACCCCGCTG |
| D8-3    | CCAAGTGCGAATATACCTACGG   | TTTTCCCTCCCTCCCT |
| Twist1  | GATTGACGACCTCAACACTGCC   | AGACCCCGATAGGAGTG CCAGTATAGTATAGTATAGTAT |
| Omd     | GAACGAGCTTGGCGACTGCT   | CAGTCTGCTGATGGGGTTAGA |
| Nhef1   | CAGCGAGGAGGACAGTGGTC   | CAGCGAGGAGGAGGAGGAGGAG |

Gene expression was measured using qRT-PCR. Reactions included 25 ng of cDNA per 10 μl with QuantiTect SYBR Green PCR Kit (Qiagen) and the CFX384 Real-time System (Bio-Rad). Transcription levels were normalized to the housekeeping gene Gapdh. Gene expression levels were quantified using the 2^(-ΔΔCt) method. Gene-specific primer sequences are in Table 1.

Immunoprecipitation and Mass Spectrometry—MC3T3 cells were treated and/or transfected as indicated on day 2 of differentiation were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris (pH 7.4), 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100), immunoprecipitated with the indicated antibodies, and analyzed via either SDS-PAGE and immunoblotting or SDS-PAGE and silver stain followed by mass spectrometry.

Immunofluorescence—Cells were imaged using a LSM780 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). ZEN software (Carl Zeiss) was utilized for acquisition of images. For live/dead assays, a 10×/0.45 M27 objective and the following wavelengths for excitation/emission were utilized: 561/626 for dead (red) cells and 488/522 for live (green) cells. The percentage of dead cells was determined by dividing the number of red, dead cells by the total number of live, green cells plus the number of red, dead cells. For visualization of Nherf1, P1α, and TAZ, MC3T3 cells were fixed with 4% paraformaldehyde, permeabilized with 0.15% Triton X-100, stained with DAPI to identify nuclei, and visualized with a 100×/1.46 oil objective and laser/emission filter: 488/500–554 for GFP, 405/411–481 for DAPI, and 633/660–758 for Alexa Fluor 647. Cells were scored positive for nuclear localization if TAZ nuclear staining was stronger than TAZ cytoplasmic staining.
treatment with the HDACi SAHA (suberoylanilide hydroxamic acid, also known as vorinostat) alters the ability of osteoblasts to provide protective signals to AML cells. First, we initiated osteogenic differentiation of MC3T3 cells on day 0. Beginning 24 h later we added DMSO or 10 μM SAHA for 30 h. On day 2 of MC3T3 differentiation (a time point we previously showed is sufficient for these differentiating osteoblast cells to provide maximal AML cell protection in this co-culture system (8)), we washed the MC3T3 to remove residual SAHA, added KG1a-CXCR4 AML cells, and added SDF-1 prior to incubation for an additional 16–18 h. SAHA treatment of osteoblasts inhibited histone deacetylation within differentiating MC3T3 osteoblasts as expected, shown by increased acetylation of histone-3 (Fig. 2B). On day 3, KG1a-CXCR4 cells were harvested from the co-cultures and analyzed for apoptosis via annexin-V, cleaved poly(ADP-ribose) polymerase, or cleaved Caspase-3 staining and flow cytometric analysis of gated YFP cells. Fig. 2C shows representative results, whereas Fig. 2, D–F, summarizes results of multiple experiments performed on different days. As previously reported (8, 10), KG1a-CXCR4 cells cultured in the absence of osteoblasts responded to SDF-1 treatment by significantly increasing their apoptosis, whereas KG1a-CXCR4 cells co-cultured with day 2 differentiated MC3T3 osteoblast cells were protected (Fig. 2, C–F). Interestingly, SAHA pretreatment of the differentiating MC3T3 cells prior to adding the KG1a-CXCR4 AML cells significantly prevented their ability to protect the AML cells from apoptosis both in the presence and absence of SDF-1 (Fig. 2, C–F). To ensure that SAHA was not simply killing the differentiating osteoblasts, we performed a live/dead assay to check for cell viability. SAHA did slightly increase the percent of dead MC3T3 cells from 1% in DMSO-treated cells to 6% in SAHA-treated cells. However, the SAHA-treated MC3T3 osteoblast cultures still formed a confluent monolayer of predominantly live (green) cells (Fig. 2G).

Extending the time of SAHA treatment of MC3T3 cells also inhibited the osteoblasts from protecting AML cells (Fig. 3A). Even MC3T3 cells maintained in differentiation medium for an extended time (14 days) and permitted to accumulate extracellular matrix and retain associated secreted factors still responded to a brief SAHA treatment by significantly decreasing their ability to protect AML cells (Fig. 3B).

To ensure that inhibition of osteoblast-mediated protection of AML cells was not unique to a single HDACi, we tested an additional HDACi, LBH-589 (also known as Panobinostat), in co-cultures performed as in Fig. 4, A–C. As expected, LBH-589 treatment increased acetylation of Histone-3 in differentiating MC3T3 osteoblast cells (Fig. 4A), whereas causing only minimal death of these cells that permitted the formation of a confluent layer of predominantly live (green) cells (Fig. 4G). Extending the time of SAHA treatment of MC3T3 osteoblasts also inhibited the osteoblasts from protecting AML cells (Fig. 3A). Even MC3T3 cells maintained in differentiation medium for an extended time (14 days) and permitted to accumulate extracellular matrix and retain associated secreted factors still responded to a brief SAHA treatment by significantly decreasing their ability to protect AML cells (Fig. 3B).

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A. Day 0: Add different media to osteoblasts. Day 1: Add DMSO or SAHA. Day 2: Wash osteoblasts to remove SAHA. Add fresh media. Day 3: Harvest KG1a-CXCR4 cells for apoptosis assay.

B. SAHA: Ac-H3, Total H3.

C. KG1a-CXCR4.

D. E. F. G.
sis in both the absence and presence of SDF-1 (Fig. 4B). Together, Figs. 2–4 indicate that differentiating MC3T3 osteoblasts are capable of protecting KG1a-CXCR4 AML cells from apoptosis and that this protection can be significantly inhibited by pretreating the differentiating osteoblasts with HDACi drugs.

The HDACi-induced Up-regulation of Nherf1 Inhibits Osteoblast-mediated Protection of AML Cells in Response to SDF-1—Because HDACi cause global changes in gene expression patterns, we sought to identify the specific molecular mechanism responsible for the effects of osteoblasts on AML cells. We previously showed that multiple HDACi drugs, including SAHA, significantly increase expression of the Nherf1 scaffold protein in osteoblasts (15, 16, 18). HDACi treatment of differentiating MC3T3 cells in our co-culture system similarly increased expression of both Nherf1 mRNA and protein (Fig. 5, A and B). We therefore explored the role of Nherf1 in the mechanism by which osteoblasts protect AML cells. MC3T3 cells were transfected either with a Nherf1 siRNA pool or a control siRNA pool on day (−1). The MC3T3 cells were then differentiated on day 0 and used in co-cultures as in Fig. 2, A–D. Nherf1 siRNA transfection successfully prevented Nherf1 protein up-regulation in differentiating MC3T3 cells treated with SAHA and also significantly reversed the effects on co-cultures of pretreating these MC3T3 cells with SAHA (Fig. 5C). Similar results were observed with a second Nherf1-specific siRNA (Nherf1 siRNA #2) (Fig. 5D). These results indicate that Nherf1 up-regulation is required for SAHA to inhibit osteoblast-mediated protection of AML cells.

We next addressed whether increased Nherf1 expression in osteoblasts would be sufficient for inhibiting osteoblast-mediated protection of AML cells, independent of HDACi treatment. MC3T3 cells were transfected on day (−1) prior to differentiation with either a control plasmid vector or a plasmid encoding Nherf1. The MC3T3 cells were then differentiated on day 0 and used in co-cultures as in Fig. 2, A–D, except that SAHA was not used. Remarkably, overexpression of Nherf1, but not empty vector, significantly reversed the inhibitory effect of the differentiating osteoblasts on SDF-1-induced apoptosis of KG1a-CXCR4 cells (Fig. 5E). This result was not due to Nherf1 overexpression causing osteoblast death (Fig. 5F), dead cells: less than 1% for empty vector and Nherf1). Together, these results indicate that HDACi-induced Nherf1 up-regulation makes an important contribution to the modulatory effects of HDACi on osteoblast-mediated protection of AML cells from SDF-1-induced apoptosis.

Increased Nherf1 Expression Does Not Inhibit Osteoblast Differentiation—We previously demonstrated that precursors of osteoblasts, bone marrow-derived mesenchymal stromal cells and osteoprogenitors, are unable to protect AML cells mediated protection of AML cells from SDF-1-induced apoptosis. A, a confluent layer of MC3T3 cells were differentiated on day 0, treated with DMSO or SAHA on day 1, rinsed on day 4, and co-cultured with KG1a-CXCR4 cells for 1 h prior to the addition of SDF-1 for 16–18 h. On day 5, apoptosis of KG1a-CXCR4 cells was analyzed as described in the legend to Fig. 2C, n = 3. B, MC3T3 cells were differentiated on day 0, and osteogenic medium was changed every 2–3 days. On day 14, DMSO or SAHA was added and 30 h later the cells were rinsed and co-cultured with KG1a-CXCR4 cells for 1 h prior to addition of SDF-1 for 16–18 h. On day 16, apoptosis of KG1a-CXCR4 cells was analyzed as described in the legend to Fig. 2C, n = 3. * in panels A and B indicates p < 0.05.

FIGURE 2. Osteoblasts treated with the HDACi SAHA fail to protect AML cells from SDF-1-induced apoptosis. A, schematic of co-culture design. On day 0, osteogenic differentiation media is added to a confluent layer of MC3T3 cells. 24 h later, 0.1% DMSO or 10 μM SAHA were added to the culture for 30 h. On day 2, the MC3T3 cells were rinsed with PBS to remove residual SAHA and receive fresh RPMI medium supplemented with 10% FCS. Then KG1a-CXCR4 cells were added to the co-culture, 1 h later 1.3 × 10^5 or SDF-1 was added, and the cells were then cultured for 16–18 h prior to harvest of the KG1a-CXCR4 cells and analysis of apoptosis. B, MC3T3 cells were cultured as described in A and harvested on day 2 for analysis of acetylation of Histone-3 (Ac-H3) via immunoblotting. The same membrane was stripped and re-blotted for total Histone-3 as a control, n = 3. C–F, the co-culture assay was prepared as in A, and on day 3, KG1a-CXCR4 cells co-cultured alone or with DMSO- or SAHA-treated MC3T3 cells were assayed for apoptosis by staining with APC-conjugated annexin-V, phycoerythrin-conjugated antibody to cleaved poly(ADP-ribose) polymerase (PARP), or phycoerythrin-conjugated antibody to cleaved Casp-3 followed by flow microfluorimetry. Gating as shown was used to measure apoptosis only of KG1a cells expressing similar high levels of CXCR4-YFP (see “Experimental Procedures”). In panel C the results of one representative experiment are shown. Panels D–F summarize three independent experiments performed as in C. Each bar denotes the mean % of YFP− cells that stained positive for annexin-V, cleaved poly(ADP-ribose) polymerase, or cleaved Casp-3. Error bars, ± S.E. *, indicates p < 0.05. G, MC3T3 cells were treated as in A, except on day 2 cells were rinsed, stained with the live/dead viability dye, and analyzed via confocal microscopy for confluence of live (green) cells and for the percentage of dead (red) cells. Images were acquired on 3 separate days for a total of 14–30 images per condition.
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A.

![Diagram](image.png)

**FIGURE 4.** LBH-589 inhibits osteoblast-mediated protection of AML cells from SDF-1-induced apoptosis. A, MC3T3 cells were differentiated on day 0, received DMSO or 1 μM LBH-589 on day 1, and 30 h later were harvested for immunoblotting of Ac-H3 and total H3. n = 3. B, MC3T3 cells were co-cultured with KG1a or KG1a CXCR4 cells for 48 h, added SDF-1 on day 2, and harvested for immunoblotting of Actin and total H3. n = 3. C, KG1a-CXCR4 cells were co-cultured with KG1a-CXCR4 cells for 48 h, added SDF-1 on day 2, and harvested for immunoblotting of Actin and total H3. n = 3. These results indicate that Nherf1 and PP1α likely interact in the cytoplasm.

B.

![Graph](image.png)

**DAY 0**

MC3T3 cells were differentiated on day 0, treated with DMSO or LBH-589 on day 1, and assayed for viability as described in the legend to Fig. 2G, in 3 independent experiments for a total of 28 images per condition.

C.

![Images](image.png)

**DIC Live Dead Inverted dead**

**DMSO**

**LBH-589**

Nherf1 interacts with PP1α and the Constitutively Active Form of PP1α Inhibits Osteoblast-mediated Protection of AML Cells—To address the mechanism by which increased Nherf1 expression inhibits osteoblast-mediated protection of AML cells, we utilized mass spectrometry to identify proteins that copurify with Nherf1. MC3T3 cells were differentiated and treated with SAHA as in Fig. 2A, then lysed so that endogenously expressed Nherf1 could be isolated via immunoprecipitation. Interestingly, mass spectrometry identified multiple peptides of the α, β, and γ subunits of PP1 co-purifying with immunoprecipitated Nherf1 in these cells (Fig. 7A). To confirm this previously unidentified interaction between Nherf1 and PP1α, we transfected MC3T3 cells on day (−1) with a plasmid encoding GFP-PP1α with or without a plasmid encoding GFP-Nherf1, added differentiation medium on day 0, and harvested the cells on day 2. GFP-Nherf1 was present in PP1α immune complexes from cells co-expressing both GFP-Nherf1 and GFP-PP1α but not from cells expressing GFP-PP1α alone (Fig. 7B). Similarly, immunoprecipitation with a Nherf1-specific antiserum resulted in co-immunoprecipitation of GFP-PP1α (Fig. 7B). To ensure that these results were not simply a consequence of GFP dimerization, the interaction was further confirmed by pulling down S peptide-tagged versions of Nherf1 and PP1α on S protein-agarose and examining the recovery of the GFP-tagged partner protein in the pulldowns (Fig. 7C). Collectively, results in Fig. 7, B and C, indicate Nherf1 and PP1α associate upon increased expression of Nherf1 in differentiating osteoblasts. Examining the subcellular localization of GFP-PP1α and GFP-Nherf1 revealed that GFP-Nherf1 was largely cytoplasmic and excluded from nuclei, whereas GFP-PP1α localized to both compartments in differentiating MC3T3 osteoblasts (Fig. 7D). These results indicate that Nherf1 and PP1α likely interact in the cytoplasm.

We next addressed whether PP1α might play a role in the mechanism by which Nherf1 overexpression inhibits the osteoblast-mediated protection of AML cells. Inhibiting PP1α is not expected to be informative because PP1α is required for osteoblast differentiation (20, 21), and we previously showed that MC3T3 differentiation is required for protection of KG1a-CXCR4 AML cells (8). Thus, we utilized the approach of overexpressing either wild-type (PP1αWT) or constitutively active PP1α (PP1αT320A) in MC3T3 cells and examining the effects on the AML cells. Indeed, overexpression of PP1αT320A in differentiating MC3T3 cells inhibited the ability of these cells to protect the KG1a-CXCR4 cells from SDF-1-induced apoptosis (Fig. 7E). In contrast, PP1αWT-expressing MC3T3 cells protected AML cells to a similar level as control cells transfected with the vector alone (Fig. 7E). Overexpression of either PP1αWT or PP1αT320A was not toxic to the MC3T3 cells (Fig. 7F, dead cells: less than 1% for PP1αWT and PP1αT320A). Together, the results in Fig. 7 suggest that the elevated Nherf1 induced by SAHA treatment of osteoblasts interacts with PP1α and stimulates PP1α to inhibit osteoblast-mediated protection of AML cells.

**PP1α-T320A Overexpression, SAHA Pretreatment, or Up-regulation of Nherf1 in Osteoblasts Induces the Nuclear Localization of TAZ, Which Inhibits Osteoblast-mediated Protection of AML Cells from SDF-1—We next determined whether PP1α inhibits the osteoblast-mediated protection of AML cells via a mechanism involving TAZ, a transcriptional modulator previously identified as a target of PP1α in osteoblasts. Upon dephosphorylation of Ser-89 by PP1α, TAZ localizes to the nucleus where it drives expression of a large number of genes, including
many required for osteoblast differentiation (20–26). To determine whether there is an association between PP1 and TAZ that is regulated by Nherf1, we transfected MC3T3 cells on day 1 with plasmids encoding S-TAZWT and GFP-PP1WT with or without a plasmid encoding Nherf1, differentiated the cells on day 0, and isolated S-TAZWT complexes on day 2. Indeed, GFP-PP1 was pulled down on S protein-agarose with S-TAZWT, and this association was enhanced with increased expression of Nherf1 (Fig. 8A). Consistent with PP1α acting via TAZ to inhibit osteoblast protection of AML cells, overexpression of PP1αT320A, but not PP1αWT, in differentiated MC3T3 cells not only inhibited osteoblast protection of AML cells (Fig. 7E) but also significantly increased nuclear localization of TAZ (Fig. 8, B and H) as detected by immunofluorescence. Additionally, we utilized subcellular fractionation to confirm an increase in nuclear localization of
TAZ with expression of PP1αT320A compared with PP1αWT (Fig. 8C). In these experiments, TAZ in the nuclear fraction consisted of multiple bands, likely reflecting posttranslational modifications, including phosphorylation at multiple residues, which occurs within the nucleus (20, 26, 35). As shown below (see Fig. 9C), the upper band comigrates with TAZS89A, a form of TAZ that is localized in nuclei and mimics dephosphorylation of TAZ by PP1α. The lower band comigrates with TAZ found in the cytoplasm, likely indicating that this form of TAZ will be exported to the cytoplasm. Fig. 8C shows an increase of the band corresponding to TAZS89A in the nuclear fraction with expression of PP1αT320A compared with PP1αWT, suggesting that constitutively active PP1αT320A enhances nuclear localization of TAZ. Similarly enhanced TAZ nuclear localization was seen in differentiating MC3T3 osteoblasts following either SAHA-induced Nherf1 up-regulation (Fig. 8, D, E, and H) or overexpression of Nherf1 (Fig. 8, F–H), consistent with TAZ acting downstream of both SAHA and Nherf1. These results suggest that Nherf1 up-regulation enhances the ability of PP1α to drive nuclear localization of TAZ.

Because PP1αT320A overexpression, SAHA pretreatment, and overexpressed Nherf1 all inhibit osteoblast-mediated protection of AML cells, while also driving nuclear localization of TAZ, we finally asked if localizing TAZ to the nucleus of osteoblasts is sufficient to inhibit the osteoblast-mediated protection of AML cells. For this purpose we utilized TAZS89A, a form of
FIGURE 7. Nherf-1 interacts with PP1α and the constitutively active form of PP1α inhibits osteoblast-mediated protection of AML cells. A, MC3T3 cells were differentiated and treated with SAHA as described in the legend to Fig. 2A. On day 2, cells were harvested for immunoprecipitation with a Nherf1 antibody and analyzed via SDS-PAGE and silver stain. A 36-kDa band was isolated for mass spectrometry, and the indicated peptides were identified as PP1α, PP1β, and PP1γ. The green and yellow highlighting indicates common sequences between the three different catalytic subunits, whereas the peptides in white indicate unique sequences to each subunit. B and C, MC3T3 cells were transfected with the indicated plasmids on day 0. The cells were differentiated on day 0 and harvested on day 2 for immunoprecipitation for PP1α, Nherf1, or the S peptide tag and immunoblotted to reveal copurifying proteins. D, MC3T3 cells were transfected and cultured as in B and E. Cells were differentiated on day 0, and then on day 2 were harvested for immunoblotting for PP1α (inset), washed and co-cultured with KG1a-CXCR4 cells for analysis of apoptosis of KG1a-CXCR4 (graph) as in Fig. 2C, n = 3, or (F) analyzed for viability as described in the legend to Fig. 2G, in 3 independent experiments for a total of 14 images per condition.
TAZ that cannot be phosphorylated at Ser-89 and therefore constitutively localizes to the nucleus (20–25) as confirmed in Fig. 9, A–C. MC3T3 cells expressing TAZS89A were significantly impaired in their ability to protect AML cells from SDF-1-induced apoptosis as compared with MC3T3 cells expressing either the vector or wild-type TAZ (TAZWT) (Fig. 9D). Overexpression of either TAZWT or TAZS89A did not induce death of MC3T3 cells (Fig. 9E), dead cells: less than 1% for TAZWT and TAZS89A). Together, the results in this article support the model shown in Fig. 10 in which HDACi treatment of osteoblasts increases their expression of Nherf1, thereby activating a Nherf1-PP1α-TAZ signaling pathway that impairs osteoblast-mediated protection of AML cells.

**Discussion**

The AML stem cells that survive chemotherapeutics reside in the endosteal region of the bone marrow microenvironment, suggesting that cells of the osteoblast lineage provide protective signals that promote the relapse of this disease (6, 7). Much research has therefore focused on identifying and targeting the protective cellular and molecular mechanisms of this cancer microenvironment (2, 4–7, 36). Osteoblast-secreted factors are
linked to worse prognosis of AML (37) and provide chemoresistance to AML cells (38). Genetic manipulation of cells of the osteoblast lineage in vivo promotes the development of AML (9, 39). Furthermore, we previously demonstrated that differentiating osteoblasts potently protect AML cells from SDF-1-induced apoptosis, a chemokine abundantly expressed in the bone marrow (8). These findings indicate that osteoblasts provide protective signals to AML cells and that disruption of these protective signals in combination with standard chemotherapeutics may be required to more completely eliminate AML cells residing in the bone marrow.

Here, we show that osteoblast-mediated protection of AML cells can be inhibited by HDACi and identify the molecular mechanisms responsible for this effect. In our co-culture model, SDF-1 induces apoptosis of AML cells expressing high levels of CXCR4 unless these cells are co-cultured with differentiating osteoblasts, as previously described (8, 10). HDACi abrogated this osteoblast-mediated protection of AML cells via up-regulation of Nherf1, which inhibited protection. We additionally identified PP1α as a novel Nherf1 interacting protein in differentiating osteoblasts. We showed that enhancing either Nherf1 expression or PP1α activity in osteoblasts inhibits the osteoblast-mediated protection of co-cultured AML cells by promoting the nuclear localization of the transcriptional regulator TAZ. Moreover, we showed that TAZ nuclear localization was sufficient to inhibit osteoblast-mediated protection of AML cells. Thus, we here describe a novel molecular signaling pathway that is capable of inhibiting the osteoblast-mediated protection of AML cells (Fig. 10).

Our results indicate that HDACi target the bone marrow microenvironment in addition to directly killing AML cells, which may help explain their efficacy in AML combination therapy (11, 13, 14). Disordered epigenetics within AML cells originally suggested that HDACi may be useful for treating AML (40, 41). HDACi kill AML cell lines and patient samples in vitro (42–44). Yet, HDACi drugs have displayed only limited efficacy (12, 45, 46). In contrast, combining an HDACi (SAHA) with traditional chemotherapeutics (cytarabine and idarubicin)
followed by maintenance therapy with SAHA produced more favorable outcomes (40). Our results here suggest that SAHA may be directly acting on the osteoblasts to inhibit their protection of AML stem cells during induction and maintenance therapy.

Although promising, HDACi alter expression of thousands of proteins and the functions of many cellular signaling pathways. Interestingly, we found that increased expression of Nherf1 alone caused inhibition of osteoblast-mediated protection of AML cells similar to that of HDACi treatment. Additionally, depletion of Nherf1 limited the HDACi-mediated inhibition of protection. Thus, regulation of Nherf1 expression appears to play a major role in osteoblast-mediated protection of AML cells.

Our further studies investigated how Nherf1 affects SDF-1 sensitivity. Nherf1 is a scaffold protein that exerts its effects by interacting with numerous proteins (47, 48). Here, we show for the first time that Nherf1 interacts with PP1α. PP1 specificity and phosphatase activity are regulated by formation of holoenzymes with over 200 regulatory proteins that modulate both PP1α interactions with target proteins and activity (49). The interaction between Nherf1 and PP1α may alter PP1α binding to negative or positive regulatory subunits and thereby facilitate PP1α-mediated TAZ dephosphorylation.

Our results implicating TAZ in the process by which the bone marrow microenvironment mediates protection of leukemic cells suggest that other pathways regulating TAZ might also be useful drug targets in AML. We show here that nuclear localization of TAZ is sufficient to inhibit osteoblast-mediated protection of AML cells. TAZ activity is tightly regulated to maintain development and homeostasis because TAZ regulates the proliferation, differentiation, adhesion, and apoptosis of diverse cell types (50, 51). Interestingly, TAZ phosphorylation on Ser-89 by the Hippo pathway kinase LATS promotes formation of a complex between TAZ and 14-3-3 that retains TAZ in the cytoplasm (20, 26, 52). Thus, LATs kinase inhibitor drugs, currently under development (35, 49–51, 53) or natural compounds (54) shown to promote dephosphorylation of TAZ may, like HDACi, be useful for AML combination therapy. Together, these results indicate novel molecular mechanisms that could potentially be targeted in conjunction with standard chemotherapeutics to improve AML therapy via inhibiting osteoblast-mediated protection of AML cells.

**Author Contributions**—K. N. K. designed, performed, and analyzed experiments, and wrote the manuscript with critical input from A. D., S. H. K., J. J. W., A. J. W., and K. E. H. A. D. performed experiments. A. D. H., B. D. S., and J. E. K. obtained patient samples for these studies and provided critical input on the manuscript.

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