Disruption of IκB Kinase (IKK)-mediated RelA Serine 536 Phosphorylation Sensitizes Human Multiple Myeloma Cells to Histone Deacetylase (HDAC) Inhibitors

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Post-translational modifications of RelA play an important role in regulation of NF-κB activation. We previously demonstrated that in malignant hematopoietic cells, histone deacetylase inhibitors (HDACIs) induced RelA hyperacetylation and NF-κB activation, attenuating lethality. We now present evidence that IκB kinase (IKK) β-mediated RelA Ser-536 phosphorylation plays a significant functional role in promoting RelA acetylation, inducing NF-κB activation, and limiting HDACI lethality in human multiple myeloma (MM) cells. Immunoblot profiling revealed that although basal RelA phosphorylation varied in MM cells, Ser-536 phosphorylation correlated with IKK activity. Exposure to the pan-HDACIs vorinostat or LBH-589 induced phosphorylation of IKKα/β (Ser-180/Ser-181) and RelA (Ser-536) in MM cells, including cells expressing an IκBα “super-repressor,” accompanied by increased RelA nuclear translocation, acetylation, DNA binding, and transactivation activity. These events were substantially blocked by either pan-IKK or IKKβ-selective inhibitors, resulting in marked apoptosis. Consistent with these events, inhibitory peptides targeting either the NF-κB essential modulator (NEMO) binding domain for IKK complex formation or RelA phosphorylation sites also significantly increased HDACI lethality. Moreover, IKKβ knockdown by shRNA prevented Ser-536 phosphorylation and significantly enhanced HDACI susceptibility. Finally, introduction of a nonphosphorylatable RelA mutant S536A, which failed to undergo acetylation in response to HDACIs, impaired NF-κB activation and increased cell death. These findings indicate that HDACIs induce Ser-536 phosphorylation of the NF-κB subunit RelA through an IKKβ-dependent mechanism, an action that is functionally involved in activation of the cytoprotective NF-κB signaling cascade primarily through facilitation of RelA acetylation rather than nuclear translocation.

The NF-κB family of dimeric transcription factors includes the five members RelA/p65, RelB, c-Rel, p105/p50, and p100/p52, which collectively govern diverse cellular processes, including immune response, inflammation, cell survival/proliferation/differentiation, and cancer, among others (1). Aberrant activation of NF-κB has been implicated in the pathogenesis of many cancers (2), particularly hematopoietic malignancies such as multiple myeloma (3, 4) and leukemia (5). Three NF-κB pathways have been identified (6) as follows: the canonical pathway, which is classically triggered by inflammatory cytokines such as TNFα; the alternative or noncanonical pathway, which is induced by specific cytokines such as CD40 ligand, B-cell activation factor of the TNF family, or lymphotoxin B; and the atypical pathway, which is activated in response to DNA damage, e.g. UV light. The NF-κB complex RelA-p50 dimer represents the most abundant member of the NF-κB family. Under basal conditions, RelA is sequestered in the cytoplasm, where it remains inactive, by the NF-κB-inhibitory protein IκBα. Various noxious stimuli activate the IκB kinases (IKKs),2 which form a tri-molecular complex composed of two catalytic subunits, IKKα (IKK1) and IKKβ (IKK2), and a regulatory subunit, IKKγ/NEMO. Following activation, the IKK complex phosphorylates IκBα on serine sites 32 and 36, leading to recognition by SCF(κB)/CBP and resulting polyubiquitination and degradation by the 26 S proteasome (7). Once released from IκBα binding, RelA translocates to the nucleus, binds to DNA, and promotes transcription of a large number of genes (2, 7). This process represents the primary activation mode for the canonical NF-κB signaling cascade, in which both IKKβ and NEMO are required for IκBα phosphorylation, whereas the role of IKKα in these events remains uncertain (8).

Given the broad spectrum of NF-κB biologic functions, NF-κB activity is likely to be controlled by highly regulated mechanisms. In this context, the transcriptional activity of RelA is also regulated by post-translational modifications, including phosphorylation and acetylation (6, 7). Recent studies have shown that optimal NF-κB activation is positively reg-

2 The abbreviations used are: IKK, IκB kinase; HDAC, histone deacetylase; HDACI, histone deacetylase inhibitor; MM, multiple myeloma; CBP, cAMP-response element-binding protein-binding protein; ATM, ataxia telangiectasia mutated; PI, propidium iodide; PTD, protein transduction peptide; NBD, NEMO binding domain; NEMO, NF-κB essential modulator; RLU, relative light unit; NC, negative control; HAT, histone acetyltransferase; PARP, poly(ADP-ribose) polymerase; mt, mutant.
Promote its association with newly synthesized I/Lys-221 attenuates the interaction of RelA with I/Lys-221, and Lys-310) (13, 14). Acetylation of RelA at Lys-310 and catalyzed by histone acetyltransferases (HATs, e.g., particularly IKKβ) also phosphorylate RelA at the Ser-536 site within the transactivation domain, an event facilitating nuclear import and transcriptional activity of RelA, independently of effects on IkBα (12). Moreover, RelA can be reversibly acetylated by histone acetyltransferases (HATs, e.g., p300 and CBP) at multiple lysine residues (e.g., Lys-122, Lys-123, Lys-218, Lys-221, and Lys-310) (13, 14). Acetylation of RelA at Lys-310 and Lys-221 attenuates the interaction of RelA with IkBα and enhances DNA binding/transactivation activity (15). Acetylated RelA is subsequently deacetylated by nuclear histone deacetylases (HDACs, e.g., HDAC3 (14) and SIRT1 (16)), which promote its association with newly synthesized IkBα, leading to nuclear export of RelA and thus termination of NF-κB signaling (17). It has been proposed that RelA deacetylation by HDACs represents an intracellular switch that controls the translocation and activation status of the NF-κB complex (10). Specifically, phosphorylation of RelA plays an important role in regulation of its acetylation (18, 19). For example, acetylation by p300/CBP is primarily regulated by the accessibility of its substrates (e.g., RelA) rather than by induction of acetyltransferase enzyme activity (11). The C-terminal region of unphosphorylated RelA masks its N terminus and therefore prevents access to p300/CBP, whereas phosphorylation at Ser-276 weakens the intramolecular interaction between the C and N termini, thereby permitting p300/CBP access (20). In addition, IKKβ-mediated RelA phosphorylation at Ser-536 promotes its nuclear import (21) and thus provides spatial accessibility to p300/CBP localized in the nucleus.

Histone deacetylase inhibitors (HDACIs) represent a group of structurally diverse agents that inhibit HDACs, which in conjunction with HATs reciprocally regulate histone acetylation and chromatin structure. HDACIs have been subcategorized with respect to the classes of HDACs they inhibit. For example, the benzamide HDACI MS-275 primarily inhibits class I HDACs (e.g., HDAC1–3), whereas tubacin is a specific inhibitor of the class II HDAC6 (22). In contrast, hydroxamic acid HDACs such as vorinostat and LBH-589 are pan-HDACIs that inhibit both class I and II HDACs (23, 24). The mechanism by which these and other HDACIs kill transformed cells is currently uncertain but may involve multiple processes, including induction of oxidative injury, up-regulation of death receptors, interference with the function of chaperone and DNA repair proteins, and disruption of cell cycle checkpoints, among others (25). Notably, HDACs also mediate deacetylation of many non-histone proteins, particularly in the case of transcription factors, events that might contribute to determination of HDACI lethality (26). In this context, studies have shown that treatment with HDACIs induces RelA hyperacetylation through inhibition of HDAC-mediated deacetylation and in so doing promotes its nuclear localization and transcriptional activity (17, 27), leading to expression of NF-κB-dependent pro-survival and anti-oxidative proteins (28). Conversely, blockade of these events, e.g., by IkBα “super-repressor,” prevents both RelA nuclear translocation and acetylation, thus enhancing HDACI lethality (27–29).

Although exposure to HDACIs has been shown to trigger IKK activation (28, 30), the possibility also exists that in addition to effects on IkBα-mediated signaling, activated IKKs might also phosphorylate RelA and thus act via a separate mechanism to regulate the cytoprotective response of transformed cells to HDACIs. This takes on added significance in the case of human multiple myeloma (MM), a hematologic malignancy associated with multiple genetic aberrations in the NF-κB signaling pathways (31–33), and which has been found to be particularly vulnerable to NF-κB inhibition, e.g., by proteasome inhibitors such as bortezomib (34, 35). To address this issue, this study was design to elucidate the role of RelA phosphorylation in the response of human MM cells to HDACIs (e.g., vorinostat and LBH-589). Here, we provide evidence that in MM cells, HDACIs induce IKK-dependent RelA phosphorylation at the Ser-536 site, resulting in increased RelA, acetylation, DNA binding, and transactivation. In contrast, prevention of HDACI-induced Ser-536 phosphorylation by either pharmacologic or genetic strategies inhibits these events and dramatically potentiates HDACI-induced apoptosis.

**Experimental Procedures**

**Human Multiple Myeloma Cell Lines—**RPMI8226, U266, and NCI-H929 cells were purchased from the ATCC (Manassas, VA). Dexamethasone-sensitive (MM.1S) and -resistant (MM.1R) cells were kindly provided by Steven T. Rosen (Northwestern University, Chicago). Cells were grown in RPMI 1640 media supplemented with 10% heat-inactivated FBS as reported previously (36). Bortezomib-resistant U266 (PS-R) cells were generated by continuously culturing cells in gradually increasing concentrations of bortezomib (beginning at 0.5 nM and increasing in stepwise increments of 0.2 nM), until a level of 15 nM, and maintained in 10% FBS/RPMI 1640 medium containing 15 nM bortezomib. IL-6-dependent aneblast (ANBL-6; 2.5 nM bortezomib, respectively. ANBL-6 cells were maintained in 10% FBS/RPMI 1640 medium containing 10 ng/ml human recombinant IL-6 (Sigma).

**Reagents—**The pan-HDAC inhibitors vorinostat (suberoylanilide hydroxamic acid) and LBH-589 (panobinostat) were obtained from Merck and Novartis (Cambridge, MA), respectively. The selective IKKβ inhibitor IKK-2 inhibitor IV (5-(p-fluorophenyl)-2-ureido[1H]thiophene-3-carboxamide) (37), the pan IKK inhibitor Bay 11-7082, and the PP1/PP2A inhibitor calyculin-A (38) were purchased from Calbiochem and Alexis (San Diego), respectively. These agents were dissolved in DMSO and stored at −80°C under light-protected conditions and subsequently diluted with serum-free RPMI medium prior to use. The cell-permeable IKK complex inhibitor NBD peptide (DRQQIKIWFQNRRMKWKKTALDWSWLDQTE), the small-se
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sequence from NEMO binding domain/NBD of IKKβ is underlined; control peptide, DRQIKIFQNRRMKWK, the sequence from the antennapedia homeodomain to make the peptide cell-permeable (39), and NF-κB p65 inhibitor PTD peptide (DRQIKIFQNRRMKWKQLRRPSDRELSE), the sequence from phosphorylation sites of RelA is underlined; control peptide, DRQIKIFQNRRMKWKQLRRPADRELSE, the underlined residues are the sites that the phosphorylation serine residues are substituted by alanine) (40) were obtained from IMGENEX (San Diego). NBD and PTD peptides were made as a stock solution of 5 mM in H2O or PBS, respectively, aliquoted, and stored at −80 °C. In all experiments, the final concentration of DMSO did not exceed 0.1%.

**Experimental Format**—All experiments were performed utilizing logaritically growing cells (4–6 × 10^6 cells/ml). Cells were treated with vorinostat or LBH-589 after 1-h pretreatment of IKK-2 inhibitor IV, Bay 11-7082, NBD, or PTD peptides, at 37 °C, 5% CO2 incubator at various intervals. After drug treatment, cells were harvested and subjected to further analysis as described below.

**Plasmids and Transfection**—The constructs encoding wild type (WT) RelA or its nonphosphorylatable (S536A) mutant in pEF6/V5-His vector were kindly provided by Dr. Carl Y. Sasaki (NIA, National Institutes of Health, Baltimore) (12). The constructs of 3×κB-luciferase reporter, which contains three copies of the NF-κB-binding site from the major histocompatibility complex class I gene upstream of the luciferase reporter gene, and its mutant form were kindly provided by Dr. Albert S. Baldwin, Jr. (Lineberger Comprehensive Cancer Center, University of North Carolina) (41). S32A/S36A double-mutated Iκβα cDNA/pUSEamp encoding a protein referred to as the Iκβα super-repressor (Iκβα SR) and mammalian siRNA expression plasmids encoding short hairpin RNA (shRNA) targeting IKKβ (GenBank™ accession number NM_001556), including two siRNA constructs (pKD-IKKβ-v2 and pKD-IKKβ-v3), or negative control sequence (pKD-Neg-Con-v1) were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). U266 and RPMI8226 cells were transfected using the Amaxa Nucleofector device (program U266, X-005; RPMI8226, G-016) with cell line-specific nucleofector kits (U266, Kit C; RPMI8226, Kit V. Amaxa GmbH, Cologne, Germany) as per the manufacturer’s instructions. For transient transfection, the transfected cells were allowed to recover for 6 h at 37 °C, 5% CO2, after which cells were treated with drugs for various intervals and subjected to further analysis. For stable expression, transfected cells were limit-diluted and continuously cultured under selection with neomycin (400 μg/ml) or blasticidin (2 μg/ml), and clones were screened for ectopic expression or down-regulation of target proteins. THE™ anti-His mAb (GenScript, Piscataway, NJ) was used to detect His-tagged WT or Ser-536 site mutant RelA.

**Flow Cytometry**—The extent of cell death/apoptosis was evaluated by flow cytometric analysis utilizing annexin V-FITC/PI or 3,3-dihexyloxacarbocyanine/7-aminoactinomycin D staining as described previously (28). Briefly, 1 × 10^6 cells were stained with annexin V-FITC (Pharmingen) and PI (Sigma) for 15 min at room temperature in the dark. Samples were then analyzed by flow cytometry within 1 h to determine the percentage of apoptotic cells displaying annexin V positivity, using FACSscan (BD Biosciences). In some cases, mitochondrial injury and cell death were assessed by double staining with 40 nM 3,3-dihexyloxacarbocyanine (Molecular Probes Inc., Eugene, OR) and 0.5 μg/ml 7-aminoactinomycin D (Sigma) at 37 °C for 20 min and then analyzed by flow cytometry.

**Western Blot Analysis**—Samples from whole cell pellets were prepared, and 30 μg/condition of proteins was subjected to Western blot analysis as described previously in detail (28). For analysis of protein phosphorylation, 1 mm each sodium vanadate and sodium pyrophosphate were added to sample buffer, and no SDS was included in the transfer buffer, and TBS was used instead of PBS throughout. The blots were probed with the appropriate dilution of primary antibody as follows: phospho-IKKα/IKKβ (Ser-180/Ser-181), phospho-IκBα/IKKβ (Ser-176/Ser-177), phospho-IκBα (Ser-32/Ser-38), phospho-NF-κB p65 (Ser-536), phospho-NF-κB p65 (Ser-468), phospho-NF-κB p65 (Ser-276), acetyl-NF-κB p65 (Lys-310), phospho-NF-κB2 p100 (Ser-866/Ser-870), NF-κB2 p100/p52, phospho-NF-κB p105 (Ser-933), Bcl-xL (Cell Signaling and Abcam, Cambridge, MA); IKKα, IKKβ, IKKγ, NF-κB p65, A1, ICAM-1, FLIPs/L, and COX2 (Santa Cruz Biotechnology, Santa Cruz, CA, and Upstate Biotechnology, Inc.); NF-κB p105/p50 and nuclear matrix protein p84 (Abcam); Bcl-2 (Dako, Carpinteria, CA); PARP (Biomol, Plymouth Meeting, PA); XIAP antibody (Transduction Laboratories); Mcl-1 (Pharmingen, San Diego); survivin and cIAP1/2 (R&D Systems, Minneapolis, MN). All blots were stripped and reprobed with β-actin (Sigma) or α-tubulin antibodies (Oncogene Inc., San Diego) as loading controls.

**Immunoprecipitation**—The status of RelA acetylation was evaluated by immunoprecipitation/Western blot analysis as described previously (28). Briefly, 200 μg of protein per condition was incubated under continuous shaking with 1 μg of NF-κB p65 antibody (mouse monoclonal, Santa Cruz Biotechnology) overnight at 4 °C. 20 μl/condition of Dynabeads (goat anti-mouse IgG, Dynal, Oslo, Norway) were added and incubated for an additional 4 h. After washing three times with RIPA buffer, the bead-bound protein was eluted by vortexing and boiling in 20 μl of 1× sample buffer. The samples were separated by SDS-PAGE and subjected to Western blot analysis as described above. Acetylated lysine antibody (Upstate Biotechnology, Inc.) was used as primary antibodies.

**Nuclear Extraction**—Nuclear fractions were prepared by using the nuclear extraction kit (Active Motif, Carlsbad, CA). Briefly, after drug treatment, cells were pelleted and lysed by vigorous vortex in Hypotonic Buffer. The samples were then centrifuged at 14,000 × g for 30 s. After washing twice, the insoluble pellets were further lysed in Complete Lysis Buffer, and nuclear extracts (supernatant) were collected after a 10-min centrifugation at 14,000 × g. The amount of protein was quantified, and 10 μg of protein per condition were subjected to Western blot analysis.

**NF-κB Activity Assays**—Specific RelA-DNA binding activity was measured by using nuclear extract kit and TransAM™ NF-κB p65 Chemi Kit (Active Motif) as per the manufacturer’s instructions. For each cell type, WT and mutated consensus oligonucleotides were used as competitors for RelA-DNA binding to monitor assay specificity. Data were normalized to total
nuclear protein. For NF-κB reporter assays, cells were transiently (U266) or stably (RPMI8226) transfected with 3×κB or mutant 3×κB luciferase reporter, and luciferase activity assays were performed using a luciferase reporter assay kit (Clontech). Relative luciferase activities were normalized to total protein. For both assays, activity was expressed as relative light units (RLU) or as fold increase relative to untreated controls. In some cases, electrophoretic mobility shift assay (EMSA) was also performed to monitor NF-κB activity as described previously (28).

For in vivo analysis of NF-κB activity, female athymic NCr-nu/nu mice (The Jackson Laboratory) were inoculated subcutaneously at the flank with 5×10⁶ RPMI8226 cells stably transfected with 3×κB luciferase reporter. Analysis was performed when basal luciferase signal was detectable (10 days). NF-κB luciferase reporter activity was monitored after intraperitoneal injection of D-luciferin (150 mg/kg) by sequential scanning using an IVIS 200 Imaging System (Xenogen, Hopkinton, MA). Signal intensities from regions of interest were defined manually using Living-Image software (Xenogen), and data are expressed as photon flux (photons/s/cm²/steradian). Animal studies were approved by the American Association for Accreditation of Laboratory Animal Care and performed in accordance with current regulations and standards of the United States Department of Health and Human Services, and the National Institutes of Health.

Statistical Analysis—For analysis of cell death/apoptosis, RelA-DNA binding, and NF-κB luciferase reporter assay, values represent the means ± S.D. for at least three separate experiments performed in triplicate. The significance of differences between experimental variables was determined using the suitable Student’s t test or one-way analysis of variance with Tukey-Kramer multiple comparisons test. The significance of p values was < 0.05 (*), < 0.01 (**), or < 0.001 (***). Analysis of synergy was performed according to median dose effect analysis using the software program CalcuSyn (Biosoft, Ferguson, MO).

RESULTS

Profilng Post-translational Modifications of the NF-κB Pathways in Human Multiple Myeloma Cell Lines—Human MM cells typically exhibit high levels of basal NF-κB activity because of multiple genetic aberrations in both the canonical and noncanonical NF-κB signaling pathways (31–33), thereby providing a target for agents such as the proteasome inhibitor bortezomib (34, 35). In this context, protein levels and post-translational modifications of the core components involved in these two pathways were first examined in various untreated human MM cell lines. As shown in Fig. 1, virtually all tested lines displayed high protein levels of most examined NF-κB pathway components, including IKKs (IKKα, -β, and -γ) and IκBα (Fig. 1A), NF-κB1 (p50 and its precursor p105, Fig. 1B), NF-κB2 (p52 and its precursor p100, Fig. 1C), and RelA (Fig. 1D). In most unstimulated MM cell lines (except H929, Fig. 1C, lane 2), the precursor p100 was largely processed into the active form p52, which binds RelB to form a heterodimeric form of the transcription factor, a hallmark of activation of the noncanonical pathway (42). Correspondingly, low basal phosphorylation of p100 at Ser-866/870, which is required for recognition by SCFβγ/CP and resulting polyubiquitination and proteasome-mediated protein processing into p52 (43), was noted. Similar phenomena were observed in the case of phosphorylation (Ser-933) and processing of p105 into p50 (Fig. 1B) (44), which binds to RelA and forms a heterodimeric transcription factor of the canonical pathway. Whereas the noncanonical pathway primarily relies on protein processing of its components (e.g. p100 versus p52) for activation, activation of the canonical pathway is predominantly reflected by post-translational modifications (particularly phosphorylation) of involved signaling molecules (1). In contrast to the high and consistent IKKα and IKKγ protein levels in virtually all tested lines, expression of IκKBβ differed markedly between cell lines (Fig. 1A and supplemental Fig. S1A). Similarly, expression of total RelA (p65) protein clearly varied between cell lines (Fig. 1D and supplemental Fig. S1A), whereas levels of its partner p50 were relatively high and consistent (Fig. 1B).

Interestingly, a majority of the tested MM cell lines displayed low basal phosphorylation of IKKα/β at Ser-α180/Ser-β181 (Fig. 1A) and Ser-α176/Ser-β177 (supplemental Fig. S1A), as well as its downstream target IκBα (Ser-32/Ser-36, Fig. 1A). This was accompanied by high levels of nonphosphorylated IκBα, which binds to and sequesters RelA/p50 in the cytoplasm, the primary mode of silencing the canonical pathway (1). However, few MM cell lines (e.g. U266 and its bortezomib-resistant counterpart PS-R, Fig. 1A, lanes 7 and 8, respectively) exhibited high basal phosphorylation of both IKKα/β at Ser-α180/Ser-181 (Fig. 1A) and RelA at Ser-536 but not Ser-468 (Fig. 1D) nor Ser-276 (data not shown). Notably, Ser-536 phosphorylation appeared to correlate with IKKα/β phosphorylation (Ser-180/Ser-181) in unstimulated MM cells. The one exception (Fig. 1D, lane 1) was the IL-6-dependent MM cell line ANBL-6, in which RelA was highly phosphorylated (Ser-536), whereas IKKα/β (Ser-180/Ser-181) phosphorylation was only modest. Conversely, the basal status of RelA Lys-310 acetylation may be related to not only Ser-536 phosphorylation, which promotes acetylation (18, 19), but also to expression of HDAC3, which deacetylates RelA (17). These profiling data were validated both by using different antibodies from alternative sources (supplemental Fig. S1A) and by using TNFα exposure as a positive control (supplemental Fig. S1B).

Interestingly, basal levels of phosphorylated (Ser-536) RelA also varied between primary CD138⁺ MM samples but were low in CD138⁻ bone marrow cells for both tested samples (supplemental Fig. 2A). Together, these results indicate that whereas the noncanonical NF-κB pathway is constitutively activated in most tested human MM cell lines, basal activation status of the canonical NF-κB pathway is quite heterogeneous. They also suggest that basal RelA phosphorylation (particularly Ser-536) may be associated with basal IKKα/β activity in human MM cells.

HDACIs Induce Phosphorylation of IKKα/β (Ser-180/Ser-181) and RelA (Ser-536) and NF-κB Activation in Human MM Cells Independently of Their Basal Status—To determine the impact of HDACIs on the NF-κB signaling cascades in human MM cells displaying variable basal activation status of the canonical NF-κB pathway, the effects of vorinostat and LBH-589, both of which act as pan-HDACis (supplemental Fig. S2B), on NF-κB activity were examined in multiple MM cell lines.
These lines displayed relatively low (RPMI8226, lane 5, MM.1S, lane 3, and MM.1R, lane 4) or high (U266, lane 7) basal levels of canonical NF-κB activation (Fig. 1A) as well as related RelA Ser-536 phosphorylation (Fig. 1D), respectively. Studies were first performed in RPMI8226 cells stably transfected with a WT or an inactive mutant (mt) NF-κB luciferase reporter. As shown in Fig. 2A, exposure (16 h) to the HDACI vorinostat (1.0–2.5 μM) or LBH-589 (10–30 nM) markedly increased luciferase reporter activity in WT but not mt NF-κB, cells. Similar results were observed in U266 cells transiently transfected with luciferase reporters (supplemental Fig. S2D). Consistent with these results, EMSAs revealed that vorinostat and LBH-589 increased DNA binding activity of NF-κB (Fig. 2B). Furthermore, an ELISA-based DNA binding assay specific for RelA confirmed that both vorinostat and LBH-589 significantly induced RelA-DNA binding activity in U266, RPMI8226, MM.1S, and MM.1R cells (p < 0.01 or p < 0.05 compared with untreated controls, Fig. 2C). In each case, addition of excess unlabeled specific WT, but not mt, oligonucleotide competitively blocked basal RelA-DNA binding activity in untreated cells (Fig. 2C), documenting the specificity of this assay. Moreover, luciferase signal was discernibly increased in subcutaneously implanted tumors of RPMI8226 cells bearing WT NF-κB luciferase reporter at 6 and 24 h after administration of 100 mg/kg vorinostat (Fig. 2D). Together, these findings indicate that HDACIs induce canonical NF-κB activation in MM cells regardless of the basal status of this pathway and also suggest that HDACIs (e.g. vorinostat) may trigger NF-κB activation in vivo in a murine model.

The effects of HDACIs on expression and phosphorylation of the major components of both the canonical and noncanonical NF-κB pathways were then examined. As shown in Fig. 2E and F, exposure of to either vorinostat (1.0–1.5 μM) or LBH-589 (5–20 nM) resulted in increased phosphorylation of IKKα/β (Ser-180/Ser-181) and IKKβ (Ser-276 or Ser-468) sites. A modest reduction in total protein levels of IKKβ or RelA was observed with HDACI treatments, a dose-dependent phenomenon most likely reflecting induction of apoptosis (e.g. caspase activation) by increasing HDACI concentrations (supplemental Fig. S2C). In contrast, exposure to either HDACI had little effect on p100 phosphorylation (Ser-866/Ser-870) or p65 phosphorylation (Ser-536/Ser-468) of the NF-κB p50/RelA heterodimer.
processing of the precursor p100 to p52. Both vorinostat and LBH-589 also clearly increased nuclear translocation of RelA and, to a lesser extent, its Ser-536-phosphorylated form (supplemental Fig. S2E). Together, these findings indicate that the HDACIs vorinostat and LBH-589 induce RelA phosphorylation on Ser-536, but not Ser-276 or Ser-468, in association with increased IKKα/β activation. They also suggest that in these cells, HDACIs primarily activate the canonical rather than the noncanonical NF-κB pathway.

**IKK Inhibitors Block Ser-536 Phosphorylation, Nuclear Translocation, and Acetylation of RelA, Diminishing NF-κB Activation in MM Cells Exposed to HDACIs**—To assess the effects of IKK inhibition on HDACI-induced RelA phosphorylation, the IKK inhibitors Bay 11-7082 and IKK-2 inhibitor IV were employed. Notably, subtoxic concentrations (e.g. 5 μM) of the IKKβ-selective inhibitor IV (37) did not clearly modify basal Ser-536 phosphorylation of RelA (Fig. 3A), whereas marginally toxic concentrations (1–3.5 μM) of the pan-IKK inhibitor Bay 11-7082 modestly reduced Ser-536 phosphorylation in some MM cell lines (Fig. 3B). Importantly, pretreatment (1 h) with either IKK-2 inhibitor IV (Fig. 3A) or Bay 11-7082 (Fig. 3B) prior to incubation with vorinostat or LBH-589 markedly inhibited HDACI-induced RelA Ser-536 phosphorylation in various MM cell types. Consistent with these results, IKK-2 inhibitor IV (Fig. 3C) or Bay 11-7082 (supplemental Fig. S2F) substantially blocked RelA-DNA binding activity (p < 0.01 or
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In view of the evidence that RelA phosphorylation promotes its nuclear translocation (21) and acetylation (18, 19), effects of IKK inhibitors on the latter events were then examined in MM cells exposed to HDACIs. As shown in Fig. 4, the capacity of vorinostat and LBH-589 to increase nuclear localization of RelA (Fig. 4A) and its Ser-536-phosphorylated form (Fig. 4B, upper panels) was markedly diminished by coadministration of either IKK-2 inhibitor IV or Bay 11-7082 (data not shown). Furthermore, the IKK-2 inhibitor IV (Fig. 4B, lower panel) or Bay 11-7082 (Fig. 4C) substantially attenuated vorinostat- and LBH-589-induced increases in RelA acetylation. Together, these findings suggest that inhibition of IKKs, and more specifically IKKβ, blocks HDACI-induced RelA Ser-536 phosphorylation, in association with diminished nuclear translocation, acetylation, and DNA binding/transcriptional activity in human MM cells.

HDACI-induced RelA Ser-536 Phosphorylation Is Independent of IκBα but Dependent upon the Phosphatase PP2A for Dephosphorylation—To activate the canonical pathway, IKKs (particularly IKKβ) phosphorylate IκBα (Ser-32/Ser-36), FIGURE 3. IKK inhibitors attenuate RelA Ser-536 phosphorylation and inhibit HDACI-induced NF-κB activation. A, U266 and RPMI8226 cells were exposed to varying concentrations of vorinostat (Vor) or LBH-589 (LBH) in the presence or absence of the selective IKKβ inhibitor (IKK-2 inhibitor IV, 5 μM), for 24 or 16 h respectively. Veh, vehicle. B, multiple MM cell lines were incubated (U266, 24 h; other lines, 16 h) with vorinostat (U266 and 8226, 1.5 μM; 1S and 1R, 1 μM) or LBH-589 (U266, 15 and 1R, 10 nM; 8226, 20 nM) with or without the pan-IKK inhibitor Bay 11-7082 (Bay) (U266, 3 μM; 8226, 3.5 μM; 1S, 1 μM; 1R, 1.5 μM). A and B, after drug treatment, Western blot analysis was performed using antibodies directed against total and phosphorylated (Ser-536) RelA. C, MM cells were treated (U266, 24 h; other lines, 16 h) with vorinostat (U266 and 8226, 1.5 μM; 1S and 1R, 1 μM) or LBH-589 (U266, 15 and 1R, 10 nM; 8226, 20 nM), with or without IKK-2 inhibitor IV (U266 and 8226, 5 μM; 15 and 1R, 3 μM), after which nuclear extracts were prepared and subjected to a RelA-specific DNA binding assay. * and ** = significantly less than values for cells exposed to HDACIs in the absence of IKK-2 inhibitor IV; p < 0.05 and < 0.01, respectively. D, RPMI8226 cells stably transfected with WT 3×κB luciferase reporter were exposed to the indicated concentrations of vorinostat or LBH-589 for 16 h, after which luciferase assays were performed to monitor NF-κB activity. C and D, values are expressed as the fold-change in RLU relative to untreated controls (arbitrarily set at 1).
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FIGURE 4. HDACs induce phosphorylations of IKKα/β (Ser-180/Ser-181) and RelA (Ser-536) through an IκBα-independent but PP2A-dependent process, and IKK inhibition blocks HDAC-induced RelA nuclear translocation and acetylation. A, multiple MM cell lines were exposed (U266, 24 h; other lines, 16 h) to vorinostat (Vor) (U266 and 8226, 1.5 μM; 1S and 1R, 1 μM) or LBH-589 (LBH) (U266, 1S and 1R, 10 nM; 8226, 20 nM) in the presence or absence of IKK-2 inhibitor IV (U266 and 8226, 5 μM; 1S and 1R, 3 μM), after which nuclear (NE), cytoplasmic (CE), and whole cell extracts (WCE) were prepared and subjected to Western blot analysis to monitor expression of RelA. Veh, vehicle. B, U266 cells were treated for 24 h as described in A, after which expression of phosphorylated (Ser-536) RelA in nuclear extracts was monitored (upper panels). C, U266 and RPMI8226 cells were incubated with 1.5 μM vorinostat with or without Bay 11-7082 (U266, 3 μM; 8226, 3.5 μM) for 24 and 16 h, respectively. B (lower panels) and C, 200 μg of protein was immunoprecipitated (IP) with anti-p65 antibody. The immunoprecipitates were then subjected to Western blot analysis using anti-acetylated lysine antibody. In parallel, Western blot analysis was performed to monitor expression of total and acetylated RelA (Lys-310) in whole cell extracts. D, U266 cells were stably transfected with a Ser-32/Ser-36-mutated IκBα (designated IκBα super-repressor, IκBα-SR) construct or an empty vector (pUSE). Cells were then exposed to the indicated concentrations of vorinostat or LBH-589 for 24 h, after which they were lysed, and Western blot analysis was performed using antibodies directed against IκBα, phospho-IKKα/β (Ser-180/ Ser-181), or phospho-RelA (Ser-536). E, U266 cells were incubated (24 h) with 1.5 μM vorinostat or 10 nM LBH-589, in the presence or absence of the selective PP2A inhibitor calyculin-A (1 nM), after which Western blot analysis was performed to monitor expression of phosphorylated (Ser-536) RelA in both nuclear and cytoplasmic extracts. For Western blot analysis, β-actin (β-act) or the nuclear matrix protein p84 were reprobed as loading controls (L.C.) for whole cell or cytoplasmic extracts and for nuclear extracts, respectively.

resulting in its ubiquitination and proteasomal degradation. This unleashes RelA and permits its nuclear translocation. Collectively, these events represent the primary mode of canonical NF-κB activation (1). In this context, the effects of IκBα on HDACI-induced RelA Ser-536 phosphorylation were then examined. To this end, U266 cells were stably transfected with an IκBα super-repressor (IκBα-SR, substitution of serine 32 and 36 by alanine), which prevents IKK-mediated phosphorylation and spares IκBα from ubiquitination and proteasomal degradation (45). As shown in Fig. 4D, expression of the IκBα super-repressor modestly but clearly increased basal IKKα/β phosphorylation, accompanied by increased basal levels of RelA Ser-536 phosphorylation, possibly representing a compensatory response to interruption of the canonical NF-κB pathway. Interestingly, exposure to vorinostat or LBH-589 stimulated a marked increase in phosphorylation of IKKα/β (Ser-180/Ser-181) and RelA (Ser-536) in cells transfected with either empty vector (pUSE) or IκBα-SR (Fig. 4D), as observed in untransfected parental U266 cells (Fig. 2, E and F). In contrast, total levels of IKKβ or RelA were not modified (data not shown). Because the IκBα super-repressor binds to and traps RelA in the cytoplasm, these findings suggest that HDACI-induced RelA Ser-536 phosphorylation very likely occurs in the cytoplasm, in association with IKKα/β activation but independently of IκBα binding. However, although HDACIs induced RelA Ser-536 phosphorylation in IκBα-SR cells, exposure to HDACIs failed to prevent binding of phosphorylated RelA to IκBα or to induce NF-κB activation in these cells (supplemental Fig. S2G). Together, these results raise the possibility that although the IκBα-mediated signaling cascade plays a primary role in NF-κB activation by governing RelA nuclear translocation, Ser-536 phosphorylation of RelA may also provide an additional mechanism by which the canonical NF-κB pathway is activated in human MM cells exposed to HDACIs.

Because RelA phosphorylation (e.g. Ser-536) is negatively regulated by the phosphatase PP2A (46), the effect of PP2A on HDACI-induced Ser-536 phosphorylation was therefore examined using the selective PP2A inhibitor calyculin-A (38). As shown in Fig. 4E, administration of calyculin-A resulted in a marked increase in the Ser-536-phosphorylated form of RelA, which was predominantly localized in the nucleus. These findings are consistent with evidence that PP2A dephosphorylates...
RelA in the nucleus (12, 38). However, in the absence of calcyculin-A, HDACIs only modestly increased nuclear Ser-536-phosphorylated RelA (Fig. 4B and supplemental Fig. S2E). However, coadministration of calcyculin-A resulted in dramatic accumulation of phosphorylated RelA in the nucleus after exposure to vorinostat or LBH-589 (Fig. 4E). Together, these results suggest that HDACI-induced RelA Ser-536 phosphorylation represents a dynamic event that occurs largely in the cytoplasm independently of IκB/kinase binding, possibly promoting translocation to the nucleus where PP2A mediates its rapid dephosphorylation.

IKKβ Inhibitors Potentiate HDACI Lethality in Human MM Cells—To assess the impact of IKKβ-mediated RelA Ser-536 phosphorylation on the survival of human MM cells exposed to HDACIs, apoptosis was first assessed following exposure to minimally toxic concentrations of vorinostat or LBH-589 alone or in combination with the selective IKKβ inhibitor. As shown in Fig. 5A, although individual treatment with HDACIs or IKK-2 inhibitor IV had little effect, combined treatment resulted in a pronounced increase in the percentage of annexin V+ apoptotic cells in diverse MM cell types, including U266, RPMI8226, MM.1S, and MM.1R. Moreover, median dose effect analysis yielded combination index values less than 1.0, indicating a synergistic interaction between these agents in U266 (Fig. 5B) and other lines (data not shown). This phenomenon was further confirmed by increased PARP cleavage after coadministration of either vorinostat or LBH-589 with the IKK-2 inhibitor IV in U266 cells (Fig. 5C) and other lines (data not shown). Similar results were obtained when the pan-IKK inhibitor Bay 11-7082 was employed (Fig. 5D and E).

FIGURE 5. IKK inhibition sensitizes MM cells to HDACI lethality. A, multiple MM cell lines were treated (U266, 48 h; other lines, 24 h) as described in Fig. 3C, after which the percentage of apoptotic (annexin V+) cells was determined by flow cytometry. B, U266 cells were exposed to a range of concentrations of vorinostat (Vor) (1–2.5 μM) or LBH-589 (LBH) (10–25 nM) and IKK-2 inhibitor IV (IV) (5–12.5 μM) administered a fixed ratio (IV/vorinostat = 5:1; IV/LBH-589 = 500:1) for 48 h, after which apoptosis was monitored using annexin V/PI staining, and the fraction affected (FA) was calculated. Combination index (CI) values were then determined using commercially available software CalcuSyn. Combination index values of <1.0 indicate synergistic interactions. Two additional experiments yielded similar results. C, U266 cells were exposed to the indicated concentrations of vorinostat or LBH-589 for 48 h in the presence or absence of IKK-2 inhibitor IV (5 μM) after which Western blot analysis was performed to monitor PARP cleavage. CF = cleavage fragment. D, MM cells were incubated (U266, 48 h; other lines, 24 h) with vorinostat (U266 and 8226, 1.5 μM; 1S and 1R, 1 μM) or LBH-589 (U266, 15 and 1R, 10 nM; 8226, 20 nM) with or without Bay 11-7082 (Bay) (U266, 3 μM; 8226, 3.5 μM; 1S, 1 μM; 1R, 1.5 μM), after which apoptosis was determined as described in A. A and D, *** = significantly greater than values for cells exposed to HDACIs or IKK inhibitors alone; p < 0.001. E, Western blot analysis for PARP cleavage was performed in MM cells treated with vorinostat and Bay 11-7082 as described in D.
PCR assays revealed increased enrichment of phosphorylated (Ser-536) RelA on the cIAP2 promoter in U266 cells exposed (6 h) to 1.5 μM vorinostat (Vor) or 10 nM LBH-589 (LBH), in the presence of 100 μM NEMO binding domain NBD peptide (left panel) or 150 μM p65 phosphorylation site PTD peptide (right panel), after which flow cytometry was performed to monitor apoptosis by annexin V/PI staining and flow cytometry. In parallel, vehicle or negative control (NC) peptides were coadministered with the HDACIs as controls. ** and *** significantly greater than in the presence of NC peptides; p < 0.01 and < 0.001, respectively.

**Inhibitory Peptides Targeting IKKβ NBD or RelA Phosphorylation Sites and IKKβ shRNA Significantly Sensitizes Human MM Cells to HDACI Lethality**—To further assess the functional impact of IKK function, particularly of IKKβ, or RelA phosphorylation on the response of human MM cells to HDACIs, synthetic inhibitory peptides targeting either the C-terminal NBD of IKKβ, which prevents formation of the IKK complex and consequently blocks its activation (39), or the phosphorylation sites of RelA, a peptide linked to a PTD for cell permeabilization (40), was then employed. As shown in Fig. 6A, coadministration of vorinostat or LBH598 with either the NBD peptide (left panel) or the PTD peptide (right panel), but not negative control (NC) peptides, resulted in a significant increase in apoptosis (p < 0.01 versus NC peptides). Together, these findings indicate that inhibition of IKKs, particularly IKKβ, or disruption of RelA phosphorylation leads to a pronounced increase in HDACI lethality in human MM cells.

To validate the role of IKKβ in HDACI-induced RelA phosphorylation, IKKβ was knocked down by an shRNA (IKKβ-v2) in RPMI8226 cells (Fig. 6B, left panels). As shown in Fig. 6B (right panels), these cells displayed a marked reduction in RelA Ser-536 phosphorylation after exposure to vorinostat or LBH-589 compared with cells transfected with negative control (NC) shRNA, accompanied by a clear increase in PARP cleavage. Flow cytometric analysis confirmed that knockdown of IKKβ significantly increased the lethality of both vorinostat (Fig. 6C, upper panel) and LBH-589 (lower panel). Virtually identical results were obtained in U266 cells when an alternative shRNA (IKKβ-v3) targeting a different IKKβ sequence was employed (supplemental Fig. S4, A and B). Together, these findings support the view that IKKβ plays a significant functional role in HDACI-induced RelA Ser-536 phosphorylation. They also argue that interference with this event markedly potentiates HDACI lethality in human MM cells.

**Functional Evidence of RelA Ser-536 Phosphorylation in Regulating the Sensitivity of Human MM Cells to HDACIs**—To further assess the functional impact of RelA Ser-536 phosphorylation on the response of human MM cells to HDACIs, U266 cells were stably transfected with either His tagged WT RelA or its nonphosphorylatable mutant form S536A (serine → alanine substitution, which prevents Ser-536 phosphorylation) (Fig. 7A). As shown in Fig. 7B, nuclear localization of His-tagged WT and the S536A mutant forms of RelA were observed, a phenomenon previously described by other groups (12). As seen in parental U266 cells (Fig. 4A), exposure to vorinostat or LBH-589 clearly increased nuclear localization of WT RelA (Fig. 7B,
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**FIGURE 7.** Ser-536 phosphorylation plays a functional role in the regulation of RelA acetylation and DNA binding activity, as well as lethality in HDACI-treated MM cells. A, U266 cells were stably transfected with V5-His-tagged WT RelA or its nonphosphorylatable mutant S536A. Western blot analysis was performed to document ectopic expression of these proteins using anti-His antibody. Untransfected U266 cell lysates were loaded as a control. α-Tub, α-tubulin. B, these cells were then treated with 1.5 μM vorinostat (Vor) or 20 nm LBH-589 (LBH), in the presence or absence of IKK-2 inhibitor IV (5 μM), for 24 h, after which expression of His-tagged RelA in nuclear extracts was monitored by Western blot analysis. Veh, vehicle. C, cells were incubated with 1.5 μM vorinostat or 10 nm LBH-589 for 24 h, after which His-tagged RelA was immunoprecipitated (IP) by anti-His antibody and immunoblotted (IB) with an anti-acetylated lysine antibody. α-Tubulin/β-actin, the nuclear matrix protein p84, or IgG were shown as loading controls for whole cell or cytoplasmatic extracts, for nuclear extracts, and for IP, respectively. D, cells were exposed to the indicated concentrations of vorinostat or LBH-589 for 24 h, after which nuclear extracts were prepared and subjected to a RelA specific NF-κB-DNA binding assay. Wild type (wt) and mutant (mt) consensus oligonucleotides (pb = probe) were used as competitors for each clone untreated (U). DNA binding activity is expressed as RLU. E, cells were treated with the indicated concentrations of vorinostat or LBH-589 for 48 h, after which the extent of apoptosis was determined by annexin V/FITC staining and flow cytometry. * and ** = significantly greater than values for cells transfected with WT RelA treated with the HDACIs, p < 0.05 and < 0.01, respectively.

Ser-536 phosphorylation is unlikely to represent the primary mechanism regulating nuclear translocation of RelA in response to HDACIs, it plays a significantly functional role in RelA acetylation and NF-κB activation by HDACIs, events that contribute to attenuation of HDACI lethality in MM cells. They also provide a mechanistic basis for a strategy combining HDACIs with specific IKK (particularly IKKβ) inhibitors in MM.

**DISCUSSION**

Aberrations in the NF-κB pathways (e.g. canonical and non-canonical) have been implicated in the pathogenesis of many human malignancies, including those of hematopoietic origin, particularly multiple myeloma (3, 34, 35). NF-κB signals downstream to a variety of anti-apoptotic pathways, including those involved in cell survival, such as anti-apoptotic members of Bcl-2 family, IAP family, etc. (28). These considerations have prompted the development of strategies targeting NF-κB pathways, including the use of IKK inhibitors (33, 47), which block IκBα phosphorylation and thereby degradation, or proteasome inhibitors, which attenuate IκBα degradation more directly (32). In addition to the primary IκBα-dependent signaling cascade, NF-κB activation is also regulated by multiple post-translational modifications of NF-κB proteins, particularly RelA phosphorylation and acetylation, which exert complex effects on canonical NF-κB activation, e.g. nuclear translocation, and...
DNA binding/transcriptional activity (6, 7, 10). Although these regulatory mechanisms are now widely recognized in NF-κB activation induced by diverse physiologic stimuli (e.g. TNFα), little is known about the impact of post-translational RelA modifications on the response of transformed cells to anti-cancer agents. In this context, HDACIs represent a novel class of anti-cancer agents (48), which, in addition to effects on gene expression mediated by inhibition of histone deacetylation, also block deacylation of various non-histone proteins, including transcription factors such as NF-κB (26). In fact, hyperacetylation of RelA induced by HDACIs has been shown to play a significant role in limiting the lethal effects of these agents in both hematopoietic and non-hematopoietic tumor cells (27, 28).

However, the functional role that RelA phosphorylation might play in the response of transformed cells to HDACIs has not yet been defined. The results of this study indicate that in human MM cells displaying varied basal post-translational modifications of key signaling components of the canonical NF-κB pathway, exposure to HDACIs induces IKKβ-mediated RelA phosphorylation on serine 536, in association with RelA nuclear translocation, acetylation, and DNA binding, leading to activation of this pathway. Significantly, these events serve to limit the lethality of such agents; conversely, disruption of this process represents a mechanism for synergistic potentiation of HDACI lethality by specific IKK (particularly IKKβ) inhibitors in human MM cells.

RelA can be phosphorylated on multiple serine (e.g. Ser-276, Ser-311, Ser-468, Ser-529, Ser-535, and Ser-536) and threonine residues (e.g. Thr-254 and Thr-505) located in different functional domains. Most, if not all, such phosphorylations positively regulate the transactivation potential of RelA through disparate mechanisms, e.g. by decreasing its affinity to IκBα, promoting nuclear import, facilitating acetylation, or regulating DNA binding and oligomerization (6, 9, 10). For example, phosphorylation of Ser-276 in the Rel homology domain has primarily been associated with an enhanced ability to recruit the coactivator CBP/p300 and to displace the p50-HDAC1 complex from DNA (49). However, phosphorylation of serine 468 in the transactivation domain 2 may act to enhance inducible RelA-dependent transactivation (50) or, alternatively, to regulate NF-κB basal activity negatively (51). Phosphorylation of Ser-536 in the C terminus has been observed in response to a wide range of stimuli, including various physiologic NF-κB inducers (e.g. TNFα, LPS, T-cell costimulation, lymphotixin β, among many others) as well as chemical agents (e.g. phorbol ester/ionomycin, doxorubicin, and etoposide) (9). Notably, all reports to date illustrate the positive role that Ser-536 phosphorylation plays in NF-κB activation (6). The results of this study demonstrate, for the first time, that HDACIs share the capacity of these stimuli to induce RelA phosphorylation on serine 536, but not serine 276 or 468. Moreover, interference with this event, either by chemical or genetic disruption of IKKβ function, significantly diminished HDAC1-induced NF-κB activation and potentiated lethality. More specifically, introduction of a nonphosphorylatable RelA mutant protein S536A, in contrast to WT RelA, also prevented NF-κB activation and sensitized cells to HDACIs. Such findings argue that RelA Ser-536 phosphorylation plays a functional role in NF-κB activation in human MM cells exposed to HDACIs, and this event may represent a cytoprotective mechanism that attenuates the lethality of these agents.

In addition to phosphorylation and subsequent degradation of inhibitory molecules such as IκBα, protein kinases are also required for phosphorylation of NF-κB proteins to achieve optimal NF-κB activation (7, 10). Multiple kinases have been reported to phosphorylate RelA (9, 11, 52). Although limited phosphorylation sites within RelA protein have been identified (6), it appears that in different cell type-dependent and stimulus-dependent settings, individual sites can be phosphorylated by multiple kinases, whereas individual kinases can phosphorylate multiple sites. In the case of serine 536 site, kinases implicated in phosphorylation include IKKα, IKKβ, IKKε, TBK1, RSK1, and Akt (6). For example, IKKβ was the first and best characterized kinase responsible for RelA Ser-536 phosphorylation following stimulation by pro-inflammatory cytokines (e.g. TNFα) (53) and DNA-damaging agents (54). Another component of the tri-molecular IKK complex, IKKα, less efficiently phosphorylates this site in response to similar stimuli such as TNFα (38). Nevertheless, most stimuli failed to induce Ser-536 phosphorylation in IKKβ knock-out (55, 56), shRNA (38), or dominant-negative cells (57). Moreover, phosphorylation of IKKs (particularly IKKβ) by chemical compounds blocks Ser-536 phosphorylation induced by various stimuli (19, 56, 58). In this context, the results of this study indicate that HDACIs induce Ser-536 phosphorylation through a process dependent upon IKKs, specifically IKKβ. First, increased Ser-536 phosphorylation was closely associated with increased IKKα/β Ser-180/Ser-181 phosphorylation following exposure to HDACIs. Second, a specific IKKβ inhibitor exhibiting selectivity toward IKKβ compared with other kinases, including IKKα, for which the IC50 is ~22-fold higher than that against IKKβ in an in vitro enzyme activity assay (59), markedly blocked Ser-536 phosphorylation in cells exposed to HDACIs. Finally, knockdown of IKKβ by shRNA approaches clearly prevented HDACI-induced Ser-536 phosphorylation. In all cases, these events were associated with inhibition of NF-κB activation and potentiation of HDACI lethality. Increased lethality was also observed when inhibitory peptides targeting either the NEMO/IKKε binding domain of IKKβ or phosphorylation sites of RelA. Collectively, these findings indicate that IKKβ plays an important functional role in HDACI-induced RelA Ser-536 phosphorylation and the resulting attenuation of lethality in human MM cells. However, because the serine 536 site of RelA can be phosphorylated by multiple protein kinases, the possibility that other kinases might be involved in or contribute to these events cannot be excluded.

The mechanism(s) by which HDACIs initially activate IKKs remains the subject of ongoing debate. There are two separate signaling pathways leading to IKK-mediated RelA Ser-536 phosphorylation. Sukurai et al. (38) reported that the TRAF2/5 → TAK1/TAB1 → IKKα/β signaling cascade mediates Ser-536 phosphorylation induced by TNFα. However, Mattioli et al. (21) demonstrate that T-cell costimulation induces Ser-536 phosphorylation via the Cot (Tpl2) → NIK → IKKα/β and PKCθ → RIP → IKKα/β pathways. Although the role of these pathways in HDACI-induced IKK activation is uncertain, our
group has recently reported that in human leukemia cells, HDACI-induced IKK activation involves the atypical DNA damage-related ATM/NEMO pathway (60), similar to findings observed in the setting of DNA-damaging agents (61, 62). Specifically, HDACIs trigger generation of reactive oxygen species that induces DNA damage, leading to ATM activation and formation of an ATM-NEMO complex. Subsequent SUMOylation of NEMO allows its release of NEMO from the nucleus to the cytoplasm, binding to IKKα/β and resulting in activation of the IKK complex. Moreover, interference with this process (e.g. by transfection with SUMOylation mutants of NEMO/IKKγ) led to inhibition of RelA nuclear translocation and activation, accompanied by enhanced HDACI lethality. The present findings indicate that IKKβ inhibition or shRNA knockdown blocks HDACI-induced RelA Ser-536 phosphorylation and potentiates HDACI lethality. They further demonstrate that a peptide targeting the NEMO binding domain of IKKβ, which blocks formation of the IKK complex by preventing binding of NEMO to IKKα/β (39), acts similarly to promote HDACI-induced apoptosis. Together, these findings raise the possibility that HDACI-induced RelA Ser-536 phosphorylation may stem from ATM/NEMO-mediated activation of the tri-molecular IKK complex. Studies to test this notion are currently underway. In this context, it is interesting that exposure to doxorubicin, a DNA-damaging agent that triggers the ATM/NEMO-mediated NF-κB pathway (62), also results in RelA Ser-536 phosphorylation, while abrogation of this response by an IKKβ inhibitor induces apoptosis (58).

RelA phosphorylation on Ser-536 optimizes NF-κB activation through multiple mechanisms, including diminishing affinity for IkBα, increasing nuclear import, and promoting acetylation and transactivation (9). For example, Sasaki et al. (12) reported that RelA Ser-536 phosphorylation and nuclear translocation in cells exposed to phorbol esters or TNFα involved an IkBα-independent process, representing an alternative to the classical IkBα-dependent pathway. This notion was further supported by evidence that transfection with the phosphomimetic (S536D) form of RelA led to induction of NF-κB transcriptional activity following stimulation, even in the presence of dominant-negative IkBα. However, Ser-536 phosphorylation induced by T-cell costimulation involves an intact NF-κB-IkBα complex and requires prior phosphorylation of IkBα at serine 32 and 36 (21). In contrast to these stimulus-specific phenomena, it is noteworthy that Ser-536 phosphorylation consistently occurs in the cytoplasm of cells exposed to various stimuli (38, 63, 64). In this study, HDACIs effectively induced Ser-536 phosphorylation in cells transfected with an IkBα super-repressor, in contrast to previous results involving T-cell costimulation. Such results suggest that HDACI-induced Ser-536 phosphorylation in MM cells may involve a process independent of IkBα binding. In this model, IKKβ phosphorylates Ser-536 of RelA in the cytoplasm after exposure to HDACIs, which then shuttles to the nucleus where it is rapidly dephosphorylated by protein phosphatases such as PP2A (38, 63). Consistent with this model, coadministration of the PP2A inhibitor calyculin-A with HDACIs resulted in striking accumulation of Ser-536-phosphorylated RelA in the nucleus. This raises the possibility that although HDACI-induced Ser-536 phosphorylation might promote RelA nuclear import, sustained nuclear localization of Ser-536-phosphorylated RelA may be predominantly regulated by PP2A-mediated dephosphorylation.

Interestingly, differences in nuclear localization between WT and the nonphosphorylatable mutant (S536A) forms of RelA were not discernible, as observed by others (12, 19), suggesting that Ser-536 phosphorylation may not represent a primary mechanism controlling RelA nuclear translocation or that this phenomenon may be cell type- or stimulus-specific. The former possibility was further supported by the findings that IκBα-SR was able to bind to phosphorylated (Ser-536) RelA and thereby to prevent NF-κB activation even in the presence of HDACIs. In contrast, Ser-536 phosphorylation markedly increased RelA acetylation in human MM cells exposed to HDACIs. In this context, it is known that Ser-536 phosphorylation positively regulates acetylation. For example, it has been shown that both Ser-276 (PKAc- or MSK1-mediated) and Ser-536 (IKKα/β-mediated) phosphorylation promote Lys-310 acetylation by increasing RelA association with the HAT p300 (18). In addition, it has also been reported that IKKα/mediated Ser-536 phosphorylation occurs prior to Lys-310 acetylation and displaces the corepressor SMRT-HDAC3, allowing p300 to acetylate RelA (19). In human MM cells, Ser-536 phosphorylation induced by HDACIs was closely associated with increased RelA acetylation, whereas both of these events were blocked by IKK inhibitors and particularly by a selective IKKβ inhibitor. More specifically, HDACIs failed to induce acetylation of the nonphosphorylatable mutant S536A. Collectively, these results suggest that HDACI-induced RelA Ser-536 phosphorylation represents a dynamic event, which is initiated by activation of IKKs in the cytoplasm independently of IkBα binding, possibly promoting translocation to the nucleus. In the nucleus, Ser-536-phosphorylated RelA is subjected to acetylation by HATs (e.g. p300) and dephosphorylation by nuclear phosphatase (e.g. PP2A). Because acetylated RelA is not able to be bound by IkBα and, as a consequence, is not exported to the cytoplasm (15), it remains in the nucleus after exposure to HDACIs, which inhibit its deacetylation. However, the nuclear appearance of Ser-536-phosphorylated RelA represents a transient event because of its rapid dephosphorylation by PP2A. In this case, RelA Ser-536 phosphorylation is most likely involved in initiation of HDACI-triggered NF-κB activation, whereas further acetylation is responsible for prolongation of NF-κB activation (17, 28). These findings also suggest that HDACI-induced RelA Ser-536 phosphorylation contributes functionally to NF-κB activation primarily by facilitating its acetylation, whereas IkBα-mediated signaling acts as the predominant mechanism for RelA nuclear import. In addition, they strongly support the notions that RelA nuclear translocation, via IKK-mediated IkBα phosphorylation and subsequent degradation, is not the sole regulatory mechanism for NF-κB-dependent transcription and that further activating and inactivating processes, including RelA post-translational modifications (e.g. phosphorylation and acetylation), also play important roles (63).

In view of its pleiotropic actions, the mechanism(s) by which interruption of IKKs promote(s) HDACI lethality are likely to be multifactorial. For example, RelA acetylation on various
lysine residues (e.g. Lys-221 and Lys-310), a phenomenon regulated by nuclear HDACs (e.g. HDAC3) acting in conjunction with HATs, has been invoked to explain the more sustained induction of NF-κB observed in cells exposed to HDACIs versus TNFα. In the latter case, re-synthesis of IκBα results in binding to and nuclear export of RelA, leading to termination of the NF-κB signal (17). However, exposure to HDACIs results in RelA hyperacetylation by blocking its deacetylation (e.g. via inhibition of HDAC3), which diminishes its binding to IκBα and nuclear export, leading to sustained DNA binding/transactivation (28). Conversely, by preventing IκBα phosphorylation and proteasomal degradation, IKK inhibitors allow IκBα to trap RelA in the cytoplasm, thereby attenuating both nuclear translocation and acetylation (19, 28). Furthermore, the present findings indicate that IKKβ-mediated Ser-536 phosphorylation also plays a significant functional role in RelA acetylation and activation in MM cells exposed to HDACIs. Thus, interference with the function of IKKs may block HDACI-induced canonical NF-κB activation by at least two distinct mechanisms, i.e. inhibition of the classic IκBα-dependent signaling cascade and disruption of IκBα-independent RelA Ser-536 phosphorylation.

The significance of the present findings is that interruption of the IKK-mediated pathway may represent a logical strategy to enhance the activity of HDACIs, particularly in the setting of human MM and related hematologic malignancies. In this context, recent gene profiling studies have identified and validated NF-κB as an important target for treatment of human MM. The rationale for developing the present strategy is based on multiple considerations. First, MM cells are known to be dependent on an intact NF-κB pathway for survival (65). Second, activating mutations in both the canonical and noncanonical NF-κB pathways occur frequently in MM (31–33), and the presence of such abnormalities predicts for susceptibility to pharmacologic NF-κB inhibitors, particularly IKK inhibitors (33, 47). Third, preclinical models also suggest a potential role for HDACIs in the treatment of MM (66). Fourth, the results of this study indicate that IKKβ activation is responsible for HDACI-induced RelA Ser-536 phosphorylation, which in turn functionally regulates its acetylation and activation. Consequently, interruption of this process, i.e. by IKKβ-specific inhibitors, blocks NF-κB-dependent cytoprotective responses of MM cells to HDACIs and significantly potentiates lethality. Fifth, direct interruption of RelA Ser-536 phosphorylation by the RelA-binding protein LZAP (67), a phosphorylation site-targeted inhibitory peptide (68), or induction of nonphosphorylatable mutant S536A (in the present study) blocks RelA activation and thus sensitizes cells to apoptosis. This evidence supports the notion that RelA Ser-536 phosphorylation plays an important functional role in the survival of transformed cells, including human MM cells, exposed to agents such as HDACIs. Finally, these findings, together with results of recent studies, indicate the following: (a) HDACI-induced NF-κB activation is initiated via the ATM/NEMO-mediated IKK activation pathway induced by oxidative DNA damage (60), and (b) sustained HDACI-induced NF-κB activation can be blocked by diminishing RelA post-translational modifications (i.e. phosphorylation and acetylation) and provide a theoretical foundation for a new strategy involving concomitant HDAC and IKK inhibition for multiple myeloma and potentially other hematologic malignancies.

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