Cell Reports
Report

OPEN ACCESS
CellPress

The Drug Vehicle and Solvent N-Methylpyrrolidone Is an Immunomodulator and Antimyeloma Compound

Jake Shortt,[1,2,7] Andy K. Hsu,[1,2,7] Benjamin P. Martin,[1,2] Karen Doggett,[3] Geoffrey M. Matthews,[1,2] Maria A. Doyle,[1,2] Jason Ellul,[1,2] Tina E. Jockel,[1,2] Daniel M. Andrews,[1,2] Simon J. Hogg,[1,2] Andrea Reitsma,[1,2] David Faulkner,[1,2] P. Leif Bergsagel,[4] Marta Chesi,[4] Joan K. Heath,[3] William A. Denny,[5] Philip E. Thompson,[6] Paul J. Neeson,[1,2] David S. Ritchie,[1,2] Grant A. McArthur,[1,2] and Ricky W. Johnstone[1,2,*]

[1]Research Division, Peter MacCallum Cancer Centre, Melbourne, VIC 3002, Australia
[2]Sir Peter MacCallum Department of Oncology, University of Melbourne, Melbourne, VIC 3002, Australia
[3]Walter and Eliza Hall Institute of Medical Research, Department of Medical Biology, University of Melbourne, Parkville, VIC 3052, Australia
[4]Comprehensive Cancer Center, Mayo Clinic, Scottsdale, AZ 85259, USA
[5]Auckland Cancer Society Research Centre, University of Auckland, Auckland, New Zealand
[6]Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, VIC 3052, Australia
[7]Co-first author
*Correspondence: ricky.johnstone@petermac.org
http://dx.doi.org/10.1016/j.celrep.2014.04.008
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

SUMMARY

N-methyl-2-pyrrolidone (NMP) is a common solvent and drug vehicle. We discovered unexpected antineoplastic and immunomodulatory activity of NMP in a *cMYC*-driven myeloma model. Coincident to this, NMP was identified as an acetyllysine mimetic and candidate bromodomain ligand. Accordingly, NMP-treated cells demonstrated transcriptional overlap with BET-bromodomain inhibition, including downregulation of *cMYC* and *IRF4*. NMP's immunomodulatory activity occurred at sub-BET inhibitory concentrations, and, despite phenotypic similarities to lenalidomide, its antimyeloma activity was independent of the IMiD targets cereblon and Ikaros-1/3. Thus, low-affinity yet broad-spectrum bromodomain inhibition by NMP mediates biologically potent, cereblon-independent immunomodulation and at higher doses targets malignant cells directly via BET antagonism. These data reveal that NMP is a functional acetyllysine mimetic with pleotropic antimyeloma and immunomodulatory activities. Our studies highlight the potential therapeutic benefits of NMP, the consequences of current human NMP exposures, and the need for reassessment of scientific literature where NMP was used as an "inert" drug-delivery vehicle.

INTRODUCTION

N-methyl-2-pyrrolidone (NMP) is a common solvent with low acute toxicity and no genotoxic or carcinogenic effects but is teratogenic at high exposures (Akesson, 2001; Saillenfait et al., 2007). Considered inert, NMP is used in applications with human exposure at high levels (Table S1) (Jouyban et al., 2010). Concentrations up to 60% v/v (equating to 6 M) are applied in dental barrier membranes and depot injections (Abouelfadel and Crawford, 2008; Hou et al., 2004). Transdermal patches contain up to 10% NMP (Lee et al., 2005). NMP is used to solubilize hydrophobic drugs, including kinase inhibitors in preclinical testing, with routine NMP exposures achieving millimolar plasma levels in rodents (Carnerup et al., 2005) (Table S2).

Multiple myeloma (MM) is a plasma cell malignancy that responds to immunomodulatory thalidomide analogs (IMiDs) (Shortt et al., 2013a). Malignant plasma cells rely on the mutually permissive effects of *IRF4* and *cMYC* (Delmore et al., 2011; Shaffer et al., 2008). The IMiDs bind cereblon (CRBN) (Ito et al., 2010), redirecting its E3-ligase activity toward Ikaros (IKZF)-1 and -3 resulting in IKZF degradation and downregulated *cMYC* and *IRF4* expression (Lu et al., 2014; Gandhi et al., 2014; Krönke et al., 2014).

Bromodomain (BRD)-containing proteins "read" acetyllysines. BRD and extraterminal domain (BET) family proteins are recruited to oncogenic superenhancers, augmenting aberrant *cMYC* expression (Chapuy et al., 2013). Recently developed BET inhibitors (e.g., JQ1), possess anti-MM activity due to *cMYC* downregulation (Delmore et al., 2011). Thus, although IMiDs and BET inhibitors have distinct protein targets, *cMYC* and *IRF4* represent common downstream effectors in MM.

Although testing kinase inhibitors in the Vk*MYC* MM model (Chesi et al., 2012), we noted improved survival in tumor-bearing, NMP-treated vehicle control mice compared to those treated with other drug vehicles. Further investigations revealed immunomodulatory activity and *cMYC*/*IRF4* inhibition conveyed by a potential acetyllysine mimetic pharmacophore. The transcriptome of NMP-treated MM cells was enriched for targets of the BET inhibition, but also overlapped with that of IMiD treatment. Despite immunological and transcriptional similarities to IMiDs, NMP did not alter CRBN, IKZF-1, or IKZF-3 expression, and NMP demonstrated equal activity against CRBN-deficient and IMiD-resistant MM cells. Thus, NMP's activity is mechanistically distinct from IMiDs and putatively explained by broad-spectrum BRD antagonism.

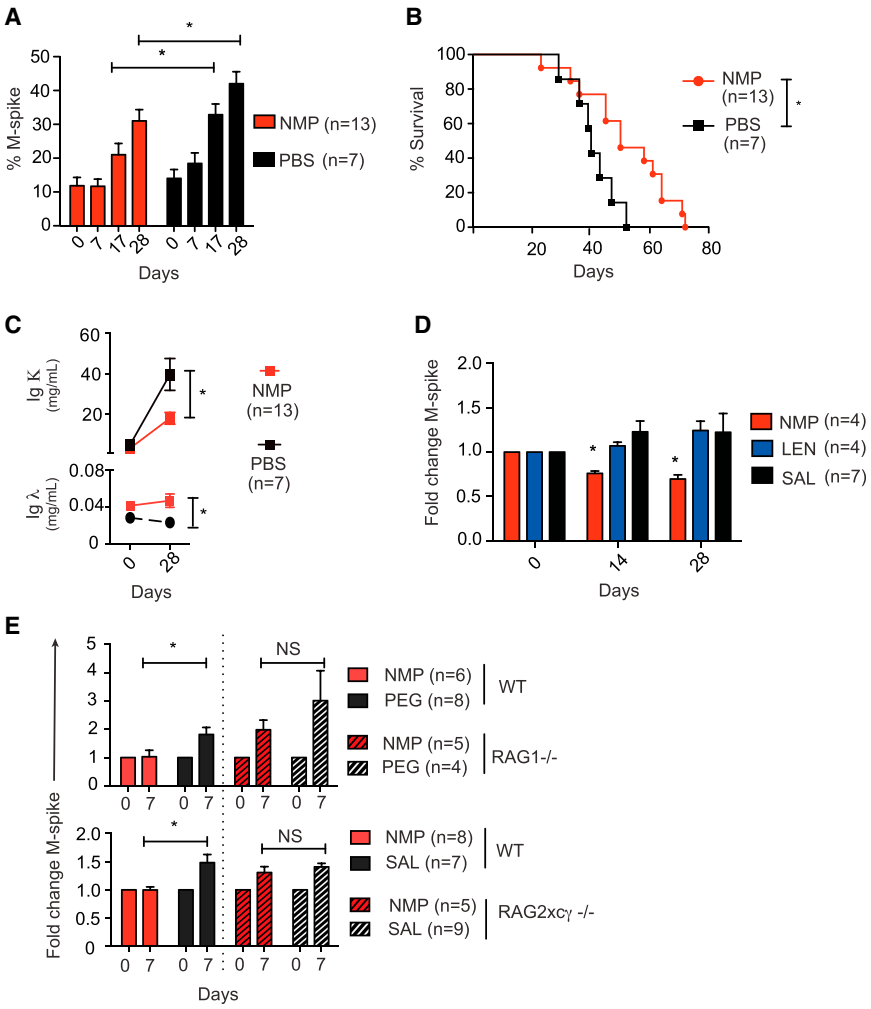


Figure 1. NMP Has Activity in Vk*MYC Myeloma that Is Abrogated in the Absence of NK and T Cells

(A) Percentage serum paraprotein (M-spike) in mice bearing aggressive transplanted Vk*MYC myeloma and treated with daily oral NMP (1 μl/g) or phosphate buffered saline (PBS).

(B) Kaplan-Meier survival curves for the same NMP and PBS treatment groups.

(C) Serum from baseline and after 1 month of treatment was analyzed by ELISA for Igκ and Igλ expression.

(D) Change in M-spike from mice bearing indolent Vk*MYC myeloma and treated with NMP (1 μl/g), Lenalidomide (LEN) 100 mg/kg or 0.9% saline (SAL) for 1 month. (*$p < 0.05$ for reduction from baseline).

(E) Change in serum M-spike in wild-type (WT) C57BL/6 controls compared to RAG1$^{-/-}$ (top panel) or RAG2xcγ$^{-/-}$ (lower panel) -immunodeficient recipients after 1 week of treatment with NMP (1 μl/g) or control vehicles (PEG and SAL). All results are presented as mean ± SEM. *$p < 0.05$; NS, not significant.

Our data provide insight into the immunological anticancer mechanisms of BRD inhibitors and mandate that NMP cannot be considered fit for purpose as an inert drug vehicle. Scientific data derived using NMP as a vehicle require reanalysis to determine its contribution to drug responses (Table S2). Current iatrogenic and occupational NMP exposures require examination for adverse or therapeutic effects attributable to BRD interactions. Finally, low-affinity, broad-spectrum acetyllysine mimetics should now be considered for clinical evaluation.

RESULTS

NMP Has Immunomodulatory and Antimyeloma Activity

Vk*MYC MM is an immunocompetent, syngeneic model that predicts anti-MM clinical efficacy (Chesi et al., 2012). Although using NMP as a drug vehicle in mice transplanted with Vk*MYC tumors, we consistently noted antitumor activity in "vehicle-only" NMP-treated control mice. Responses were noted in mice transplanted with multiple, independently derived myelomas demonstrated by delayed paraprotein progression and improved survival in aggressive clones (Figures 1A and 1B) and sustained regressions in more indolent disease (Figure 1D). Responses were selective for clonal kappa paraprotein, as residual polyclonal lambda immunoglobulins were maintained (Figure 1C). When the same myeloma was transplanted into immunodeficient (C57BL/6 RAG1$^{-/-}$ or C57BL/6 RAG2$^{-/-}$cγ$^{-/-}$) hosts, NMP's efficacy was attenuated (Figure 1E), indicating that host lymphocytes were required for optimal in vivo activity. We therefore postulated the therapeutic effects of NMP were mediated by immunomodulation.

NMP was then compared to standard anti-MM therapeutics in order to elucidate its mechanism of action. Direct cytotoxicity and glucocorticoid effects were excluded by reference to published literature (Akesson, 2001; Huang et al., 2011). Proteasome inhibition was excluded, as NMP treatment did not increase protein ubiquitination (data not shown). However, using the gold-standard phenotypic assay of IMiD activity (Moreira et al., 1993), NMP suppressed tumor necrosis factor α (TNF-α) release from lipopolysaccharide (LPS)-stimulated monocytes at similar concentrations to lenalidomide (Figure 2A). To characterize NMP's anti-inflammatory effects in vivo, we challenged non-tumor-bearing mice with LPS after NMP or lenalidomide prophylaxis (Figures 2B and 2C). A single "vehicle" dose of NMP prevented TNF-α release and death from endotoxic shock. Lenalidomide only weakly suppressed TNF-α and failed to improve survival. Because MM also causes dysregulated inflammatory cytokines, we assessed the sera of Vk*MYC mice before and after NMP treatment. Untreated mice bearing Vk*MYC tumors had elevated TNF-α and interleukin-6 (IL-6) (Figure S1), both of which were significantly downregulated in response to NMP (Figure 2D).

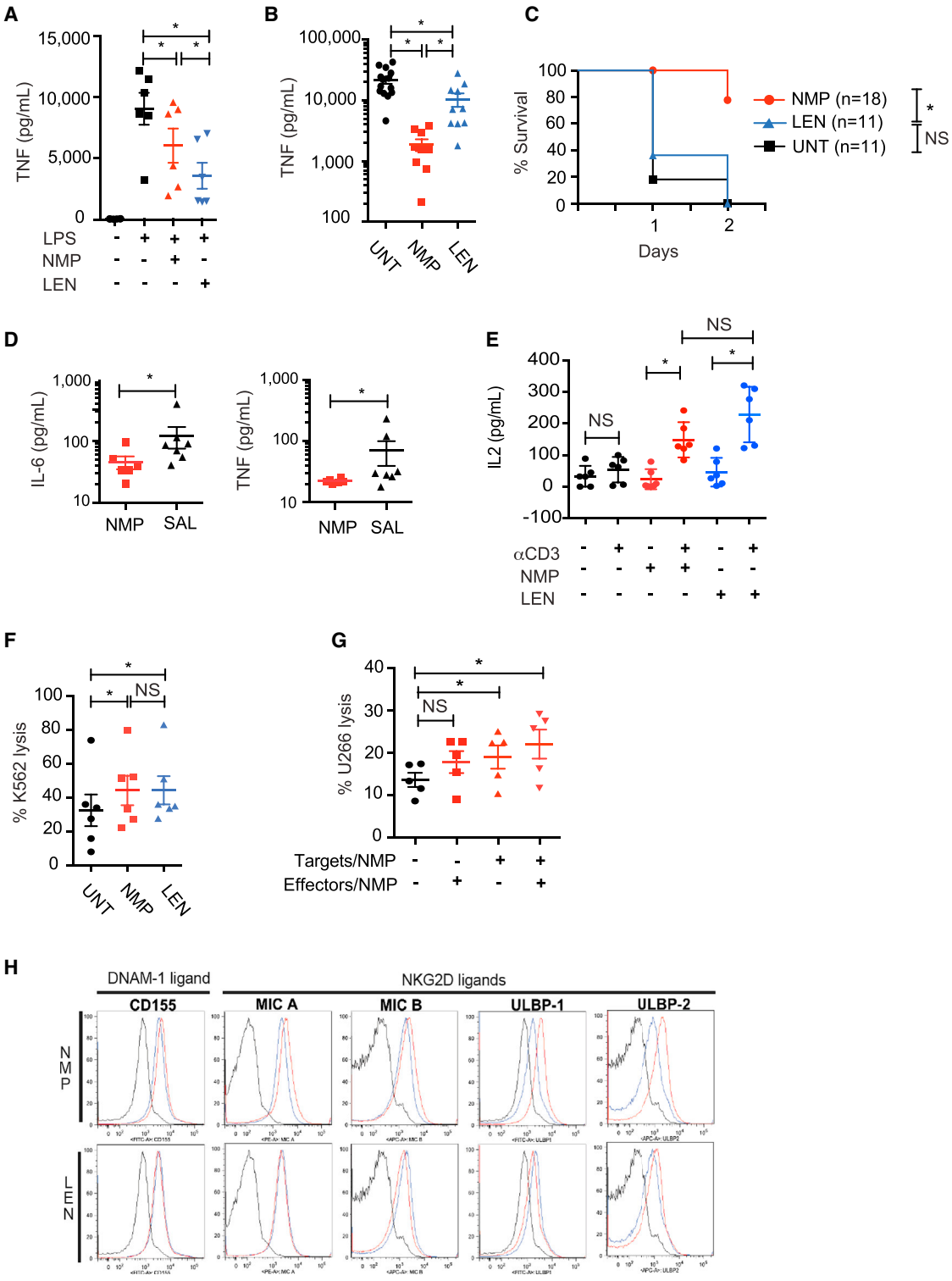


Figure 2. NMP Exhibits Biologically Potent Anti-inflammatory and Immunostimulatory Activity

(A) Human PBMC were LPS stimulated alone or with 10 μM NMP or lenalidomide (LEN) for 18 hr before measurement of TNF-α.

(B) Serum TNF-α 4 hr after LPS challenge in non-tumor-bearing mice prophylaxed with NMP (1 μl/g × 1 dose) or LEN (100 mg/kg × 2 doses) or left untreated (UNT).

(C) Survival curves for the same mice following LPS challenge.

(D) Serum IL-6 and TNF-α from mice bearing indolent Vk*MYC* MM after 1 month of treatment with NMP or saline (SAL).

(legend continued on next page)

In addition to anti-inflammatory activity, the IMiDs costimulate T cells (Haslett et al., 1998) to augment NK cell cytotoxicity (Davies et al., 2001). Because NMP's anti-MM activity was reduced in T- and NK-deficient mice (Figure 1E), we evaluated its capacity to costimulate T cells and activate NK cells in vitro. Like lenalidomide, NMP increased IL-2 release from CD3 stimulated peripheral blood mononuclear cells (PBMCs; Figure 2E) and augmented NK cell-mediated cytolysis against K562 and MM targets (Figures 2F and 2G). Pretreatment of MM targets with NMP also upregulated the DNAM1 and NKG2D ligands that are required for NK cell-mediated killing (Figure 2H). Therefore, NMP has anti-inflammatory and immunostimulatory activity that correlates with attenuation of in vivo anti-MM activity in immunodeficient hosts.

NMP Is an Acetylated Lysine Mimetic and Bromodomain Ligand

The results presented in Figure 2 indicated the immunological effects of NMP overlapped with lenalidomide. However, during our experiments CRBN was identified as the IMiD binding protein responsible for anti-MM and immunostimulatory effects (Ito et al., 2010; Lopez-Girona et al., 2012; Zhu et al., 2011). Because NMP lacks a glutarimide ring, it is not predicted to bind CRBN. However, NMP was recently identified as an acetyllysine mimetic with similar affinity for BET-BRDs as their endogenous ligands (Figure 3A) (Philpott et al., 2011). Despite weak biochemical affinity, NMP's very low molecular weight conveyed ligand efficiency comparable to that of larger rationally developed compounds such as JQ1 (Hewings et al., 2011; Philpott et al., 2011). Subsequent characterization of BET-selective inhibitors revealed anti-inflammatory activity in the context of LPS challenge, similar to our findings with NMP (Figures 2A and 2C) (Belkina et al., 2013; Nicodeme et al., 2010). Consistent with prior studies, JQ1 suppressed TNF-α secretion in response to LPS challenge to the same extent as NMP (Figure S2). However, unlike NMP and lenalidomide, low-dose BET inhibition with JQ1 did not costimulate IL-2 secretion from human PBMCs (Figure S2).

We therefore evaluated the role of BRD inhibition in NMP and IMiD responses. Consistent with published data (Philpott et al., 2011), NMP demonstrated competitive binding with a range of bromodomain containing proteins when tested in vitro (Table S3). In contrast to JQ1, NMP bound non-BET targets including CREBBP and TAF1 (Table S3). Importantly, lenalidomide did not demonstrate significant bromodomain interactions (Table S3).

As *cMYC*-dysregulation predicts responses to BRD inhibition (Delmore et al., 2011), we next treated human *cMYC*-translocated MM cell lines with NMP and JQ1. BET-inhibitory NMP concentrations were minimally toxic to human PBMCs (Figure 3B) but induced cytostasis and apoptosis in *cMYC*-positive, lenalidomide-resistant RPMI-8226 myeloma cells (Figure 3C) and the lenalidomide-sensitive Namalwa Burkitt line (Figure 3D). NMP responses correlated with rapid downregulation of *cMYC* mRNA and protein (Figures 3E and 3F), phenocopying JQ1's previously reported effects (Delmore et al., 2011). In contrast, prolonged lenalidomide exposure was required to downregulate *cMYC* in IMiD-sensitive OPM2 MM cells (Figures 3G and 3H). Thus, NMP and JQ1 share direct anti-MM effects that correlate with BRD inhibition and acute *cMYC* suppression. Lenalidomide lacks BRD-inhibitory activity and downregulates *cMYC* only after prolonged treatment.

NMP, JQ1, and Lenalidomide Share Common Molecular Targets, Including cMYC and IRF4

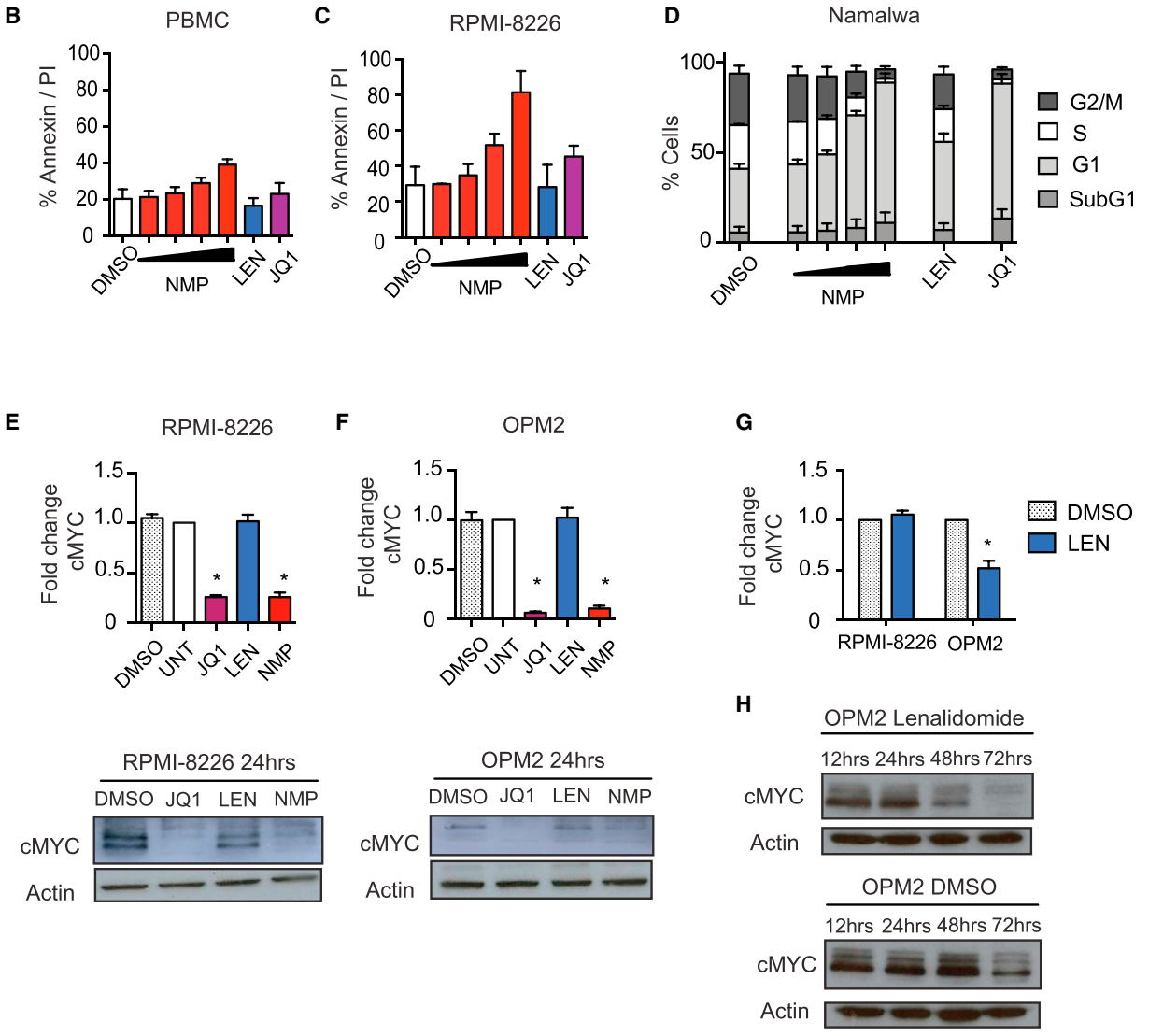
To identify potentially shared molecular targets of NMP, JQ1, and lenalidomide, we compared the transcriptional profile of NMP treatment to published lenalidomide (Zhu et al., 2011) and JQ1 (Puissant et al., 2013) signatures. NMP differentially regulated a vast array of genes, further highlighting its previously unappreciated molecular activity (Figure 4A). Consistent with data shown in Figure 3, the most downregulated gene at 4 hr was *cMYC.* Competitive gene set testing was performed using CAMERA (Wu and Smyth, 2012), to test for enrichment of lenalidomide and JQ1 signatures in the RNA sequencing (RNA-seq) data from NMP-treated cells (Figures 4B and 4C). The profile of lenalidomide-treated MM cells and a consensus JQ1 signature were both highly enriched in the NMP-treated cells (Figures 4B and 4C). Direct comparison of JQ1 with lenalidomide also yielded a highly significant correlation (Figure 4D). To clarify the specificity of this observation, we also compared with 5′ azacitidine, a DNA-methyltransferase inhibitor that does not inhibit BRDs (Matthews et al., 2013). In contrast to the overlap between JQ1, NMP, and lenalidomide, 5′ azacitidine yielded a distinct profile with no enrichment for BRD inhibitor or lenalidomide signatures (Figures 4E and 4F).

We next compared the expression profile of lenalidomide treatment (Zhu et al., 2011) to the 50 most up- and downregulated genes across three JQ1-treated, *cMYC*-translocated cell lines (Delmore et al., 2011). Of these highly JQ1-responsive genes, 28% (14/50) and 40% (20/50), respectively, were concordant with changes observed with lenalidomide (Figure 4G). Moreover, these shared genes were concordantly regulated by NMP, with high statistical significance after 24 hr treatment (Figure 4H). Notably, *cMYC* downregulation was common to JQ1, NMP, and lenalidomide. The MSigDB compute overlaps tool (http://www.broadinstitute.org/gsea/msigdb/annotate.jsp) was used to identify known gene sets overlapping this "JQ1-lenalidomide-NMP common" signature. The gene sets identified were enriched for pathways regulated by *cMYC* and *IRF4* (Table S4).

(E) IL-2 secretion from human PBMCs after stimulation with anti-CD3 in presence 10 μM NMP, LEN, or DMSO vehicle for 72 hr.
(F) PBMCs were stimulated with 10 μM NMP or LEN prior to culture with K562 targets at a 50:1 ratio and assessed for cytolysis by chromium release.
(G) PBMC effectors were treated with 10 μM NMP or left untreated prior to washing and coculture with U266 targets (either untreated or pretreated with 10 μM NMP) at a ratio of 50:1 and assessment of cytolysis by chromium release.
(H) Expression of NKG2D (MIC-A, MIC-B, ULBP-1, ULBP-2) and DNAM-1 (CD155) ligands on U266 MM cells after treatment for 48 hr with 10 μM NMP or LEN. Black line, unstained control; blue line, untreated cells; red line, post drug treatment. Results are represented as mean ± SEM of least three independent experiments. *$p < 0.05$; NS, not significant.

A

	Acetyllysine	NMP	JQ1	DMSO	Lenalidomide
BRD4 IC50 (μM)	7,210	6,000	0.077	281,000	>1,250
CREBBP IC50 (μM)	1,180	1,900	12.94	39,500	>1,250



(legend on next page)

IRF4 itself was downregulated by NMP, which we validated on a second MM cell line (Figures 4I–4J). These data confirm that NMP is a functional BRD inhibitor and demonstrate that BRD inhibitors and IMiDs share common molecular targets. Importantly, in the context of robust anti-MM activity, pathways modulated by *cMYC* and *IRF4* were significantly and coordinately altered.

NMP Has CRBN-Independent Activity in Lenalidomide-Resistant Multiple Myeloma Cells

Because the thalidomide-binding protein CRBN mediates IMiD effects, we investigated the role of the CRBN-IKZF1/3 axis in the anti-MM activity of NMP. We again utilized published data comparing the expression profile of MM cells following shRNA *CRBN* knockdown or lenalidomide treatment (Zhu et al., 2011). The transcriptional overlap between JQ1, NMP, and lenalidomide was virtually entirely mutually exclusive of genes shared between lenalidomide and *CRBN* knockdown (Figure 5A). Interestingly, a greater proportion of genes were shared between JQ1 and lenalidomide (34% concordance) than lenalidomide and *CRBN* knockdown itself (10% concordance) (Figure 5A). Notably, and in contrast to lenalidomide treatment, *cMYC* was not represented in the *CRBN*-knockdown data (of genes with at least 1 log fold-change reduction). Consistent with these observations, CAMERA analysis did not show enrichment of the *CRBN*-knockdown signature in NMP-treated cells (Figure 5B). NMP and JQ1 did not downregulate *CRBN* transcription at time points corresponding to maximal *cMYC* suppression (Figure 5C). Because *CRBN* has multiple isoforms (Lodé et al., 2013), we inspected read mapping of the *CRBN* locus for evidence of differential isoform expression in RNA-seq data (Figure S3). Again, there was no evidence of differential *CRBN* mRNA splicing in response to NMP treatment. Western blot for CRBN in OPM2 cells showed multiple bands representing these putative splice variants. Although a dominant band running at ≈45 kDa reduced in response to NMP and JQ1 treatment, this could be rescued by addition a pan-caspase inhibitor, indicating degradation downstream of apoptosis induction (Figure 5D). Ikaros-1 and -3 are targets of lenalidomide-bound CRBN that are ubiquitinylated and degraded in response to IMiDs (Lu et al., 2014; Gandhi et al., 2014; Krönke et al., 2014). Unlike lenalidomide, NMP and JQ1 did not affect IKZF-1 or -3 expression (Figure 5E). Together these data indicate that the transcriptional and immunological similarities between lenalidomide and NMP are not explained by convergence on CRBN or the IKZF transcription factors.

Secondary lenalidomide resistance is associated with mutation and/or downregulated expression of *CRBN* (Zhu et al., 2011). We therefore investigated the potential *CRBN*-independent activity of NMP in the setting of acquired IMiD resistance due to *CRBN* knockdown or prolonged lenalidomide exposure (Figures 5F–5I). The phenotype of acquired lenalidomide resistance was validated by demonstrating attenuated reductions in IKZF-1 and -3 after lenalidomide exposure in *CRBN* knockdown or chronically lenalidomide-treated cells (Figure S4). Consistent with *CRBN*-independent activity, NMP induced similar growth arrest in MM cells with either *CRBN*-knockdown or acquired IMiD resistance (Figures 5G–5I).

DISCUSSION

MM is an incurable disease that initially responds to IMiDs, proteasome inhibitors, and emerging epigenetic therapies (Shortt et al., 2013a). Here, we report the serendipitous discovery that NMP, a drug-vehicle previously considered biologically inert, has immunomodulatory and anti-MM activity. A key discovery of our work was that NMP's biological effects overlapped with both BET inhibition and the IMiDs. At a molecular level, NMP functions as a nonselective BRD inhibitor, whereas lenalidomide does not bind BRDs. However, MM cells treated with NMP, JQ1, or lenalidomide shared highly convergent gene expression signatures, indicating that these chemically diverse agents may mediate antitumor responses through common mechanisms.

However, clear mechanistic distinctions set lenalidomide apart from NMP and JQ1. Lenalidomide acts at a post-translational level, binding CRBN to induce IKZF1/3 degradation upstream of *cMYC* and *IRF4*. BRD inhibition (by NMP or JQ1) directly antagonizes *cMYC* and *IRF4* transcription, independent of *CRBN* or IKZF1/3. Accordingly, NMP's activity was preserved in CRBN-deficient and lenalidomide-resistant MM cells. This indicates that BRD inhibitors are likely to have utility in MM with secondary IMiD resistance due to perturbations of the CRBN-IKZF1/3 axis.

The low mM NMP concentrations required to antagonize BET proteins coincide with direct cytostatic effects on *cMYC*-translocated cells. In this regard, NMP is a biochemically weak BET inhibitor compared to JQ1. However, the routine use of 10% NMP (1 M or 1 g/kg) to deliver kinase inhibitors to rodents readily achieves mM plasma concentrations. For example, a 500 mg/kg NMP dose results in median peak plasma concentrations of 7 mM in rats (Carnerup et al., 2005). Therefore, systemic BET-inhibitory NMP concentrations must be assumed in most

Figure 3. NMP Is an Acetyllysine Mimetic Bromodomain Ligand

(A) Chemical structures of acetyllysine, NMP, JQ1, DMSO, and lenalidomide (LEN) with bromodomain interacting moiety highlighted in red and cereblon binding glutarimide structure colored blue. IC_{50} for BRD4 and CREBBP as reported in an alpha-screen by Philpott et al. (2011) or extrapolated from BromoMax screen are tabulated below.

(B and C) (B) Percentage Annexin-V/propidium iodide (PI) positive PBMCs or (C) RPMI-8226 myeloma cells after 48 hr treatment with NMP (1–20 mM), LEN (10 μM), or JQ1 (0.5 μM).

(D) Cell-cycle analysis (nuclear DNA content [PI] staining) for Namalwa Burkitt cells after 48 hr treatment with NMP (1–20 mM), LEN (10 μM), or JQ1 (0.5 μM).

(E and F) (E) RPMI-8226 and (F) OPM2 myeloma cells were treated with NMP 10 mM, LEN 10 μM, JQ1 0.5 μM, or DMSO vehicle for 2 hr prior to real-time PCR (upper panel) or 24 hr prior to western blotting (lower panel) for cMYC expression.

(G) RPMI-8226 and OPM2 cells were treated with LEN 10 μM or DMSO for 48 hr before quantitation of *cMYC* by real-time PCR.

(H) OPM2 cells were treated with LEN 10 μM (upper blot) or DMSO control (lower blot) and harvested at the indicated time points and western blotting for cMYC.

All results are mean ± SEM of least three independent experiments; *$p < 0.05$.

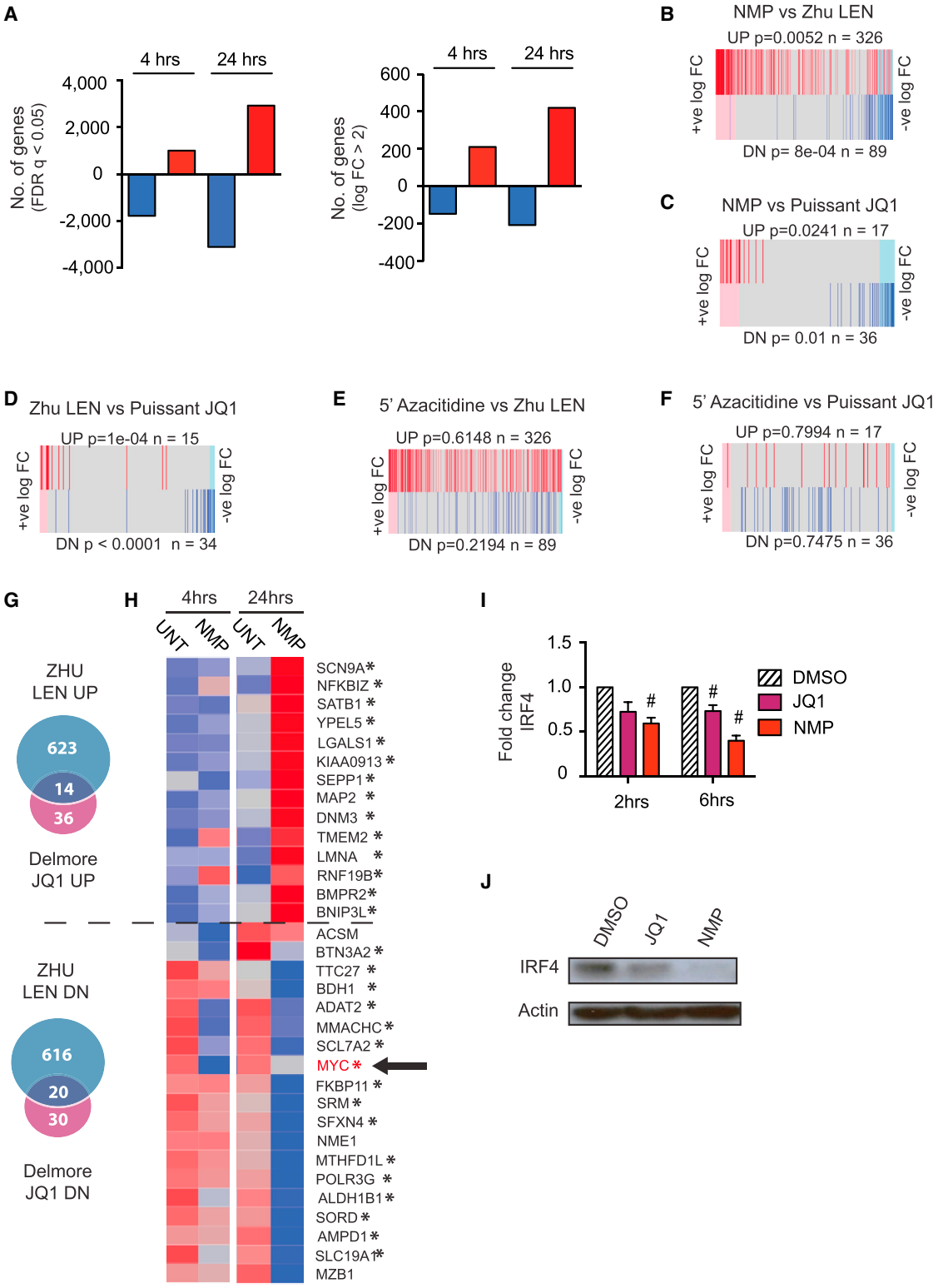


Figure 4. NMP Shares Overlapping Molecular Targets with Lenalidomide and JQ1

(A) The number of differentially regulated genes in JJN3 MM cells treated with 10 mM NMP. Red and blue bars indicate up and downregulated genes, respectively.

(B) Gene set testing represented as barcode graph showing rankings of the "Zhu" lenalidomide (LEN) signature in the expression data from NMP-treated JJN3 cells.

(legend continued on next page)

preclinical rodent studies. Given our data, all studies using NMP to deliver small molecules (Table S2) need to be scrutinized and may require reinterpretation.

NMP's immunomodulatory effects were evident at 10 μM and approximated that of the clinical lead IMiD, lenalidomide. This activity at concentrations 1,000 × lower than its biochemical IC_{50} for BET-BRDs requires further explanation, particularly because BET inhibition by JQ1 was not immunostimulatory. The BET family represents a minor proportion (4/41) of human BRD proteins that NMP potentially interacts with (Filippakopoulos et al., 2010). Indeed, many non-BET BRD targets are "less druggable" than BET proteins due to a shallow BRD pocket that may still be occupied by NMP (Vidler et al., 2012). NMP's very low molecular weight may also contribute to greater intracellular bioavailability and activity than would be predicted on the basis of binding affinities identified through biochemical assays. For example, the reported ligand efficiency of NMP for CREBBP is 0.53, this being superior to that of JQ1 for BRD4 (0.32) despite IC_{50}s of 2.4 mM and 77 nM, respectively (Hewings et al., 2011).

Although the relative affinity of NMP for the complete BRD-protein family is yet to be mapped, our limited screen identified selectivity for CREBBP and TAF1 over BET proteins, and others have reported the binding of NMP to SMARCA4 and PB1 (Filippakopoulos et al., 2012). PB1, TAF1, and SMARCA4 are all considered to have difficult "druggability" compared to BET proteins (Vidler et al., 2012) and may represent important immunomodulatory NMP targets. Indeed, SMARCA4 has demonstrated roles in inflammation (Ramirez-Carrozzi et al., 2006) and regulatory T cell function (Chaiyachati et al., 2013). The possible effects of NMP-mediated inhibition of non-BET-BRD proteins remain the focus of future studies.

The human significance of NMP's biological activity is immediate. Iatrogenic and occupational NMP exposures are common and occur at high concentrations. Because neat NMP is 10 M, concentrations proximal to depot injections and dental barrier membranes will extend into the molar (and BET inhibitory) concentration range. We also provide data indicating a one-million-fold dilution of NMP has immunomodulatory effects with similarities to lenalidomide. Single-dose transdermal delivery of 300 mg NMP to healthy volunteers resulted in peak plasma concentrations up to 44 μM (Akesson et al., 2004). Therefore, systemic immunomodulation by NMP with existing common human exposures is plausible.

NMP's capacity to combine CRBN-independent immunomodulatory activity with broad-spectrum BRD antagonism provides a unique therapeutic modality with predicted activity in the setting of IMiD-resistant MM. Additional therapeutic benefit may already be evident, because NMP (a potential component of orthopedic cement) was reported to augment bone hardening through activation of BMP signaling (Miguel et al., 2009). Because osteoporosis causes significant morbidity in MM, an agent that conveys dual antineoplastic and pro-osteoblastic activity is highly desirable. Potent BET-selective inhibitors have now entered clinical development, but experience with these agents is limited. In contrast, good manufacturing practice grade NMP is inexpensive, readily available, nontoxic, and already tested in human volunteers. Repurposing NMP as a clinical therapeutic is imminently feasible and will be pursued in an Australian phase I study in 2014.

EXPERIMENTAL PROCEDURES

In Vitro Cell Culture, Reagents, and Assays

Cell lines were all from ATCC except JJN3s (a gift from Prof. Andrew Spencer) and lenalidomide-resistant U266 cells (a gift from Prof. Keith Stewart); these were cultured in RPMI-1640 with 10% fetal calf serum and penicillin/streptomycin. Apoptosis and cell-cycle analyses were performed as previously described (Shortt et al., 2013b).

NMP was purchased from Sigma-Aldrich. Lenalidomide and 5-azacitidine were provided by Celgene. JQ1 was provided by Prof. James Bradner. qVD was purchased from Calbiochem.

Chromium release assays were performed using PBMCs as previously described (Hsu et al., 2011). TNF-α suppression assays utilized LPS-stimulated human $CD14^+$ monocytes in the presence of drug or media for 18 hr prior to supernatant collection. Costimulation assays were performed on anti-CD3 stimulated PBMCs, alone or in the presence of drugs for 72 hr prior to supernatant collection. Cytokines levels were measured by bead array (Luminex).

Bromodomain competition assays were performed by BROMO*scan* (Discoverex). Western blots were performed according to standard techniques (Shortt et al., 2013b). Primary antibodies were cMYC (Cell Signaling Technology), IRF4 (Santa Cruz Biotechnology), CRBN (Proteintech), IKZF1 (Santa Cruz), IKZF3 (Abcam), α-tubulin (Merck), and β-Actin (Sigma-Aldrich). Antibodies for NKG2D and DNAM-1 ligands were from R&D Systems.

Quantitative real-time PCR was undertaken using SYBR green nucleic acid stain (Invitrogen) and the following primers: *CRBN* F: caccctcaagaagtcagtatgg R: aaactgtgcttccctttcct; *cMYC* F: ggacgacgagaccttcatcaa R: ccagcttctctga gacgagctt; *IRF4* F: tccccacagagccaagcataaggt R: acgaggatttcccggtagta; normalized to *L32:* F: ttcctggtccacaacgtcaag R: ttgtgagcgatctcggcac in an ABI7900HT (Applied Biosystems). shCRBN and shNT lentiviral constructs were a gift from Prof. Keith Stewart (Zhu et al., 2011).

RNA Sequencing and Bioinformatics

Myeloma cells were treated for 4 or 24 hr prior to RNA extraction in three independent experiments. Paired-end reads (50 bp) were generated on an Illumina HiSeq and quality checked by FastQC and then mapped to the human reference genome (GRCh37) using Tophat2 v2.0.8b (PMID: 23618408) with maximum number of multiple hits set to 1 and first mapping to the reference transcriptome (Ensembl v69). Counts per gene were obtained using HTSeq

(C) Gene rankings of the consensus "Puissant" JQ1 signature in the expression data from NMP-treated JJN3 cells.
(D) Rankings of the "Puissant" JQ1 signature in the "Zhu" LEN expression data.
(E and F) (E) Gene rankings of the 'Zhu' LEN signature and the (F) "Puissant" JQ1 signature in expression data from 5-azacitidine (2.5 μM) -treated JJN3 cells.
(G) Venn diagrams demonstrating overlap between the 50 most up- and downregulated genes in JQ1-treated MM cell lines ("Delmore") and genes with >2-fold change in expression in OPM2 myeloma cells in response to LEN ("Zhu"). Upper panel, upregulated (UP); lower panel, downregulated (DN) genes.
(H) Heatmap showing expression of genes common to JQ1 "Delmore" and LEN "Zhu" in NMP-treated versus untreated (UNT) JJN3 myeloma cells normalized by gene (*genes with FDR $q < 0.05$).
(I) qPCR for *IRF4* expression in OPM2 cells treated for 2–6 hr with NMP (10 mM), JQ1 (0.5 μM), or vehicle (DMSO).
(J) Western blot of IRF4 expression in OPM2 cells treated for 24 hr with NMP (10 mM), JQ1 (0.5 μM), or vehicle (DMSO).
All data are representative of at least three independent experiments. #$p < 0.05$; FC, fold change; FDR, q value, statistical significance after false discovery rate correction; p values for gene set testing are from CAMERA and indicate the significance of enrichment.

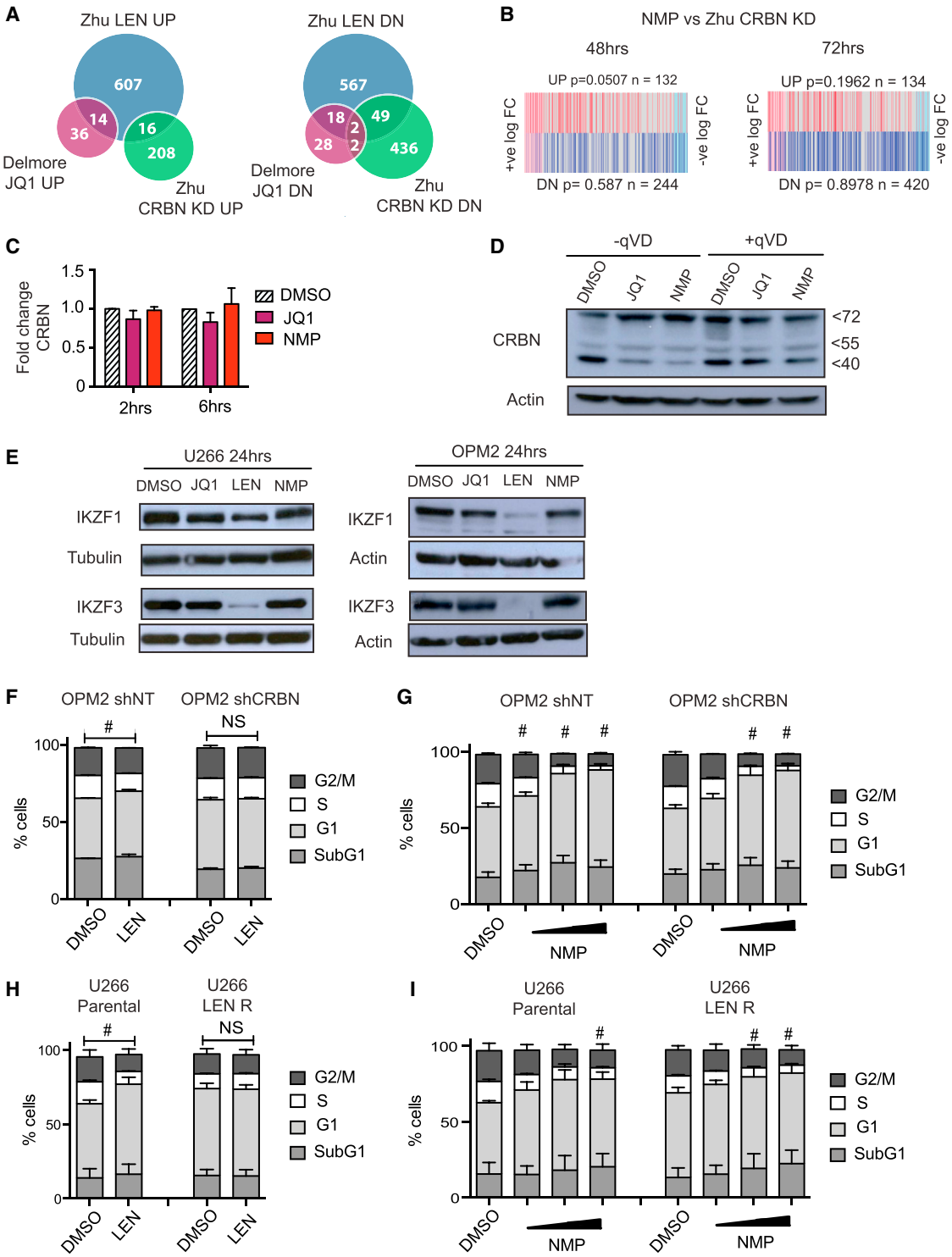


Figure 5. NMP Has CRBN-Independent Activity versus Myeloma with Acquired Lenalidomide Resistance

(A) Venn diagrams demonstrating overlap between 50 most differentially regulated genes in JQ1-treated myeloma cell lines ("Delmore JQ1") and genes with >2-fold change (FC) in expression following 48–72 hr of *CRBN* knockdown ("Zhu CRBN KD") or 48 hr lenalidomide treatment ("Zhu LEN"). Left panel, upregulated (UP) genes; right panel, downregulated (DN) genes.

(B and C) (B) Barcode plot showing the rankings of genes with >2 FC in expression following CRBN knockdown ("Zhu CRBN KD") in expression data from NMP-treated (10 mM, 24 hr) JJN3 myeloma cells (C) qPCR for *CRBN* expression in OPM2 myeloma cells treated for 2–6 hr with NMP (10 mM), JQ1 (0.5 μM), or DMSO.

(D) Western blot of CRBN in OPM2 myeloma cells treated for 24 hr with NMP (10 mM), JQ1 (0.5 μM), or DMSO with or without the pan-caspase inhibitor, qVD.

(legend continued on next page)

v0.5.3p9 with mode intersection nonempty (http://www.huber.embl.de/users/anders/HTSeq/doc/overview.html). The limma-voom method was used to identify differentially expressed genes between drug treatment and control using a false discovery rate (FDR) threshold of 0.05 (http://www.statsci.org/smyth/pubs/VoomTechReport.pdf).

Gene set testing was performed using CAMERA (Wu and Smyth, 2012) and the MSigDB v3.1 C2 curated gene set collection (Subramanian et al., 2005). The Zhu expression data were downloaded from Gene Expression Omnibus (GEO) accession number GSE31421. These are expression files for lenalidomide-treated or CRBN shRNA-infected OPM2 cells. The cel files were loaded into R and normalized with gcrma. Because the data contained no replication, a t-statistic could not be generated to calculate p values, so fold-change cutoffs were used to construct gene signatures. For CRBN knockdown, we selected genes exhibiting a 2-fold change at 48 and 72 hr. For lenalidomide treatment, we used minimum fold change of 3 at 48 hr and 4 at 72 hr to ensure sufficient gene numbers for interrogation of other data sets.

In Vivo Experiments

Mouse experiments were performed in accordance with Peter Mac Animal Experimental Ethics Committee guidelines. All strains were on a C57BL/6 background and purchased from the Walter & Eliza Hall Institute (Parkville, Australia) or bred in-house. In vivo studies were performed on mice bearing transplanted Vk*MYC myeloma as previously described (Chesi et al., 2012). To prevent adoptive transfer of immune cells with grafts into immunodeficient mice, we transplanted fluorescence-activated cell-sorted CD138$^+$ cells. Prior to treatment, the onset of transplanted disease was confirmed by serum protein electrophoresis. For quantitative immunoglobulin estimates, serum was analyzed by ELISA using Igλ or IgK capture antibodies (Southern Biotech).

Mice were gavaged with NMP diluted in PEG300 (Sigma-Aldrich) according to standard practice for drug administration using NMP/PEG as a vehicle. JQ1 was administered by intraperitoneal injection in 10% DMSO/9% HPBCD/81% sterile water. Lenalidomide was gavaged in 0.9% saline suspension. Mice were culled with the development of overt myeloma, including hind-limb paralysis and disseminated extramedullary disease.

For LPS challenges, mice were administered LPS (*E. coli* 0127:B8, gamma-irradiated; Sigma) 1 mg/30 g mouse weight by i.p. injection, and serum was obtained 4 hr after injection. For cytokine assessment in myeloma-bearing mice, serum was collected 4 hr after the last dose of treatment.

Statistical Analysis

One-way ANOVA was used to compare means of multiple groups; t tests and Mann-Whitney tests were used to compare the means of two groups. Statistical comparisons and log-rank (Mantel-Cox) survival analyses were calculated using GraphPad Prism version 5.0b at the $\alpha = 0.05$ level of significance.

ACCESSION NUMBERS

The GEO accession number for the RNA sequencing data reported in this paper is GSE56623.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.04.008.

AUTHOR CONTRIBUTIONS

J.S. and A.K.H. and designed and performed experiments, analyzed data, and wrote the manuscript. B.P.M., G.M.M., S.J.H., T.E.J., K.D., A.R., D.M.A., D.F., and J.K.H. designed or performed experiments. P.L.B. and M.C. provided essential reagents and expertise in Vk*MYC MM. P.E.T. and W.A.D. interpreted data and provided expertise in medicinal chemistry. M.A.D. and J.E. performed bioinformatics analyses. G.A.M., D.S.R., and P.J.N. designed experiments and interpreted data. R.W.J. designed experiments, interpreted data, and wrote the manuscript. All coauthors reviewed the manuscript prior to submission.

ACKNOWLEDGMENTS

The authors thank Prof. James Bradner and Prof. Constantine Mitsiades for advice and critical evaluation of experimental data. Dr. Chris Ott and Dr. Gang Lu provided assistance with IKZF assays. J.S. is supported by the Leukaemia Foundation of Australia and the Co-operative Research Centre for Biomedical Imaging Development. T.E.J. is supported by Deutsche Krebshilfe. R.W.J. is a Principal Research Fellow of the National Health and Medical Research Council (NHMRC) of Australia and supported by NHMRC Program and Project Grants, Cancer Council Victoria, the Leukaemia Foundation of Australia, and the Victorian Cancer Agency.

Received: January 12, 2014
Revised: March 12, 2014
Accepted: April 7, 2014
Published: May 8, 2014

REFERENCES

Abouelfadel, Z., and Crawford, E.D. (2008). Leuprorelin depot injection: patient considerations in the management of prostatic cancer. Ther. Clin. Risk Manag. *4*, 513–526.

Akesson, B. (2001). N-Methyl-2-pyrrolidone. Concise international chemical assessment document 35, N-Methyl-2-pyrrolidone. International Programme on Chemical Safety and Inter-Organization Programme for the Sound Management of Chemicals (Geneva: World Health Organisation).

Akesson, B., Carnerup, M.A., and Jönsson, B.A. (2004). Evaluation of exposure biomarkers from percutaneous absorption of N-methyl-2-pyrrolidone. Scand. J. Work Environ. Health *30*, 306–312.

Belkina, A.C., Nikolajczyk, B.S., and Denis, G.V. (2013). BET protein function is required for inflammation: Brd2 genetic disruption and BET inhibitor JQ1 impair mouse macrophage inflammatory responses. J. Immunol. *190*, 3670–3678.

Carnerup, M.A., Saillenfait, A.M., and Jönsson, B.A. (2005). Concentrations of N-methyl-2-pyrrolidone (NMP) and its metabolites in plasma and urine following oral administration of NMP to rats. Food Chem. Toxicol. *43*, 1441–1447.

Chaiyachati, B.H., Jani, A., Wan, Y., Huang, H., Flavell, R., and Chi, T. (2013). BRG1-mediated immune tolerance: facilitation of Treg activation and partial independence of chromatin remodelling. EMBO J. *32*, 395–408.

Chapuy, B., McKeown, M.R., Lin, C.Y., Monti, S., Roemer, M.G., Qi, J., Rahl, P.B., Sun, H.H., Yeda, K.T., Doench, J.G., et al. (2013). Discovery and

(E) Western blot of IKZF-1 and -3 expression in U266 and OPM2 MM cells treated for 24 hr with JQ1 (0.5 μM), LEN (10 μM), NMP (10 mM), or DMSO vehicle.
(F) Cell-cycle analysis of OPM2 cells expressing shCRBN or shNT control after 48 hr LEN treatment (10 μM).
(G) Cell-cycle analysis of OPM2 cells expressing shCRBN or shNT control after 48 hr NMP treatment (5–20 mM).
(H) Cell-cycle analysis of parental and LEN-resistant U266 myeloma cells after 48 hr LEN treatment (10 μM).
(I) Cell-cycle analysis of parental and LEN-resistant U266 myeloma cells after 48 hr NMP treatment (5–20 mM).
All data are representative of at least three independent experiments. p values for gene set testing are from CAMERA and indicate the significance of enrichment. #p < 0.05 for a reduction in S phase.

characterization of super-enhancer-associated dependencies in diffuse large B cell lymphoma. Cancer Cell *24*, 777–790.

Chesi, M., Matthews, G.M., Garbitt, V.M., Palmer, S.E., Shortt, J., Lefebure, M., Stewart, A.K., Johnstone, R.W., and Bergsagel, P.L. (2012). Drug response in a genetically engineered mouse model of multiple myeloma is predictive of clinical efficacy. Blood *120*, 376–385.

Davies, F.E., Raje, N., Hideshima, T., Lentzsch, S., Young, G., Tai, Y.T., Lin, B., Podar, K., Gupta, D., Chauhan, D., et al. (2001). Thalidomide and immunomodulatory derivatives augment natural killer cell cytotoxicity in multiple myeloma. Blood *98*, 210–216.

Delmore, J.E., Issa, G.C., Lemieux, M.E., Rahl, P.B., Shi, J., Jacobs, H.M., Kastritis, E., Gilpatrick, T., Paranal, R.M., Qi, J., et al. (2011). BET bromodomain inhibition as a therapeutic strategy to target c-Myc. Cell *146*, 904–917.

Filippakopoulos, P., Qi, J., Picaud, S., Shen, Y., Smith, W.B., Fedorov, O., Morse, E.M., Keates, T., Hickman, T.T., Felletar, I., et al. (2010). Selective inhibition of BET bromodomains. Nature *468*, 1067–1073.

Filippakopoulos, P., Picaud, S., Mangos, M., Keates, T., Lambert, J.P., Barsyte-Lovejoy, D., Felletar, I., Volkmer, R., Müller, S., Pawson, T., et al. (2012). Histone recognition and large-scale structural analysis of the human bromodomain family. Cell *149*, 214–231.

Gandhi, A.K., Kang, J., Havens, C.G., Conklin, T., Ning, Y., Wu, L., Ito, T., Ando, H., Waldman, M.F., Thakurta, A., et al. (2014). Immunomodulatory agents lenalidomide and pomalidomide co-stimulate T cells by inducing degradation of T cell repressors Ikaros and Aiolos via modulation of the E3 ubiquitin ligase complex CRL4(CRBN.). Br. J. Haematol. *164*, 811–821.

Haslett, P.A., Corral, L.G., Albert, M., and Kaplan, G. (1998). Thalidomide costimulates primary human T lymphocytes, preferentially inducing proliferation, cytokine production, and cytotoxic responses in the CD8+ subset. J. Exp. Med. *187*, 1885–1892.

Hewings, D.S., Wang, M., Philpott, M., Fedorov, O., Uttarkar, S., Filippakopoulos, P., Picaud, S., Vuppusetty, C., Marsden, B., Knapp, S., et al. (2011). 3,5-dimethylisoxazoles act as acetyl-lysine-mimetic bromodomain ligands. J. Med. Chem. *54*, 6761–6770.

Hou, L.T., Yan, J.J., Tsai, A.Y., Lao, C.S., Lin, S.J., and Liu, C.M. (2004). Polymer-assisted regeneration therapy with Atrisorb barriers in human periodontal intrabony defects. J. Clin. Periodontol. *31*, 68–74.

Hsu, A.K., Quach, H., Tai, T., Prince, H.M., Harrison, S.J., Trapani, J.A., Smyth, M.J., Neeson, P., and Ritchie, D.S. (2011). The immunostimulatory effect of lenalidomide on NK-cell function is profoundly inhibited by concurrent dexamethasone therapy. Blood *117*, 1605–1613.

Huang, R., Xia, M., Cho, M.H., Sakamuru, S., Shinn, P., Houck, K.A., Dix, D.J., Judson, R.S., Witt, K.L., Kavlock, R.J., et al. (2011). Chemical genomics profiling of environmental chemical modulation of human nuclear receptors. Environ. Health Perspect. *119*, 1142–1148.

Ito, T., Ando, H., Suzuki, T., Ogura, T., Hotta, K., Imamura, Y., Yamaguchi, Y., and Handa, H. (2010). Identification of a primary target of thalidomide teratogenicity. Science *327*, 1345–1350.

Jouyban, A., Fakhree, M.A., and Shayanfar, A. (2010). Review of pharmaceutical applications of N-methyl-2-pyrrolidone. J. Pharm. Pharm. Sci. *13*, 524–535.

Krönke, J., Udeshi, N.D., Narla, A., Grauman, P., Hurst, S.N., McConkey, M., Svinkina, T., Heckl, D., Comer, E., Li, X., et al. (2014). Lenalidomide causes selective degradation of IKZF1 and IKZF3 in multiple myeloma cells. Science *343*, 301–305.

Lee, P.J., Langer, R., and Shastri, V.P. (2005). Role of n-methyl pyrrolidone in the enhancement of aqueous phase transdermal transport. J. Pharm. Sci. *94*, 912–917.

Lodé, L., Amiot, M., Maiga, S., Touzeau, C., Menard, A., Magrangeas, F., Minvielle, S., Pellat-Deceunynck, C., Bene, M.C., and Moreau, P. (2013). Cereblon expression in multiple myeloma: not ready for prime time. Br. J. Haematol. *163*, 282–284.

Lopez-Girona, A., Mendy, D., Ito, T., Miller, K., Gandhi, A.K., Kang, J., Karasawa, S., Carmel, G., Jackson, P., Abbasian, M., et al. (2012). Cereblon is a direct protein target for immunomodulatory and antiproliferative activities of lenalidomide and pomalidomide. Leukemia *26*, 2326–2335.

Lu, G., Middleton, R.E., Sun, H., Naniong, M., Ott, C.J., Mitsiades, C.S., Wong, K.K., Bradner, J.E., and Kaelin, W.G., Jr. (2014). The myeloma drug lenalidomide promotes the cereblon-dependent destruction of Ikaros proteins. Science *343*, 305–309.

Matthews, G.M., Lefebure, M., Doyle, M.A., Shortt, J., Chesi, M., Banks, K.M., Vidacs, E., Faulkner, D., Atadja, P., Bergsagel, P.L., and Johnstone, R.W. (2013). Preclinical screening of histone deacetylase inhibitors combined with ABT-737, rhTRAIL/MD5-1 or 5-azacytidine using syngeneic Vk*MYC multiple myeloma. Cell Death Dis. *4*, e798.

Miguel, B.S., Ghayor, C., Ehrbar, M., Jung, R.E., Zwahlen, R.A., Hortschansky, P., Schmoekel, H.G., and Weber, F.E. (2009). N-methyl pyrrolidone as a potent bone morphogenetic protein enhancer for bone tissue regeneration. Tissue Eng. Part A *15*, 2955–2963.

Moreira, A.L., Sampaio, E.P., Zmuidzinas, A., Frindt, P., Smith, K.A., and Kaplan, G. (1993). Thalidomide exerts its inhibitory action on tumor necrosis factor alpha by enhancing mRNA degradation. J. Exp. Med. *177*, 1675–1680.

Nicodeme, E., Jeffrey, K.L., Schaefer, U., Beinke, S., Dewell, S., Chung, C.W., Chandwani, R., Marazzi, I., Wilson, P., Coste, H., et al. (2010). Suppression of inflammation by a synthetic histone mimic. Nature *468*, 1119–1123.

Philpott, M., Yang, J., Tumber, T., Fedorov, O., Uttarkar, S., Filippakopoulos, P., Picaud, S., Keates, T., Felletar, I., Ciulli, A., et al. (2011). Bromodomain-peptide displacement assays for interactome mapping and inhibitor discovery. Mol. Biosyst. *7*, 2899–2908.

Puissant, A., Frumm, S.M., Alexe, G., Bassil, C.F., Qi, J., Chanthery, Y.H., Nekritz, E.A., Zeid, R., Gustafson, W.C., Greninger, P., et al. (2013). Targeting MYCN in neuroblastoma by BET bromodomain inhibition. Cancer Discov. *3*, 308–323.

Ramirez-Carrozzi, V.R., Nazarian, A.A., Li, C.C., Gore, S.L., Sridharan, R., Imbalzano, A.N., and Smale, S.T. (2006). Selective and antagonistic functions of SWI/SNF and Mi-2β nucleosome remodeling complexes during an inflammatory response. Genes Dev. *20*, 282–296.

Saillenfait, A.M., Sabaté, J.P., and Gallissot, F. (2007). Comparative developmental toxicities of the three major metabolites of N-methyl-2-pyrrolidone after oral administration in rats. J. Appl. Toxicol. *27*, 571–581.

Shaffer, A.L., Emre, N.C., Lamy, L., Ngo, V.N., Wright, G., Xiao, W., Powell, J., Dave, S., Yu, X., Zhao, H., et al. (2008). IRF4 addiction in multiple myeloma. Nature *454*, 226–231.

Shortt, J., Hsu, A.K., and Johnstone, R.W. (2013a). Thalidomide-analogue biology: immunological, molecular and epigenetic targets in cancer therapy. Oncogene *32*, 4191–4202.

Shortt, J., Martin, B.P., Newbold, A., Hannan, K.M., Devlin, J.R., Baker, A.J., Ralli, R., Cullinane, C., Schmitt, C.A., Reimann, M., et al. (2013b). Combined inhibition of PI3K-related DNA damage response kinases and mTORC1 induces apoptosis in MYC-driven B-cell lymphomas. Blood *121*, 2964–2974.

Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., and Mesirov, J.P. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. USA *102*, 15545–15550.

Vidler, L.R., Brown, N., Knapp, S., and Hoelder, S. (2012). Druggability analysis and structural classification of bromodomain acetyl-lysine binding sites. J. Med. Chem. *55*, 7346–7359.

Wu, D., and Smyth, G.K. (2012). Camera: a competitive gene set test accounting for inter-gene correlation. Nucleic Acids Res. *40*, e133.

Zhu, Y.X., Braggio, E., Shi, C.X., Bruins, L.A., Schmidt, J.E., Van Wier, S., Chang, X.B., Bjorklund, C.C., Fonseca, R., Bergsagel, P.L., et al. (2011). Cereblon expression is required for the antimyeloma activity of lenalidomide and pomalidomide. Blood *118*, 4771–4779.