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Phosphorylation alters Bim-mediated Mcl-1 stabilization and priming

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Abstract

Mcl-1 is a highly labile protein, subject to extensive posttranslational regulation. This distinguishes Mcl-1 from other anti-apoptotic proteins and necessitates further study to better understand how interactions with pro-apoptotic Bcl-2 proteins affect its regulation. One such protein, Bim, is known to stabilize Mcl-1, and Bim phosphorylation has been associated with increased Mcl-1 binding. Consequently, we investigated the potential impact of Bim phosphorylation on Mcl-1 stability. We found that Bim stabilizes and primes Mcl-1 in RPCI-WM1 cells and is constitutively phosphorylated. Additionally, introduction of several phospho-mimetic and unphosphorylatable Bim mutations resulted in altered Mcl-1 stability and distinct Bim binding to anti-apoptotic proteins. These findings suggest Bim phosphorylation not only regulates Mcl-1 stability but also is a potential mechanism for enforcing Mcl-1 dependence.

Keywords
Mcl-1 stability; Bim phosphorylation; priming

Introduction

The Bcl-2 family of proteins plays a crucial role regulating intrinsic apoptosis [1]. When a normal, healthy cell is exposed to an apoptogenic stimulus such as DNA damage, growth factor withdrawal, or aberrant proliferation, one of the ways the cell can respond is through upregulation of the direct activator BH3-only Bcl-2 proteins Bim, Bid, and Puma [2–6]. These proteins can transiently bind to and activate the multi-domain effector proteins Bax and Bak, which upon activation, homo-oligomerize and permeabilize the mitochondrial outer membrane, resulting in cytochrome c release, caspase activation, and the subsequent processing necessary for a cell to undergo apoptosis [7]. This process is inhibited by anti-apoptotic members of the Bcl-2 family (e.g. Bcl-2, Mcl-1, Bcl-x\textsubscript{L}, Bcl-w, Bcl-B, and A1),
that bind to and sequester the pro-apoptotic family members, preventing them from facilitating activation of the apoptotic cascade [8].

While the anti-apoptotic proteins share significant structural homology and demonstrate apparent functional redundancy, there are several key differences in the structure and function of these proteins [9, 10]. Notably, they promote survival of B cells at different stages of differentiation, with Bcl-2/xL protecting more B-cell like subsets, and plasma cells being strongly dependent on Mcl-1 for survival [11–15]. Additionally, the anti-apoptotic proteins have demonstrated contrasting roles in protection of cells from a myriad of cytotoxic agents including cisplatin and taxols [16–19]. Lastly, despite Bcl-2, Bcl-xL, and Mcl-1 sharing at least three Bcl-2 Homology (BH) domains, and all containing a hydrophobic pocket for binding pro-apoptotic proteins, the crystal structure for the N-terminus of Mcl-1 remains unresolved [20]. This is likely due to the presence of several PEST domains, which result in a substantially reduced protein half-life compared to other anti-apoptotic proteins [21].

The half-life of Mcl-1 is extensively regulated through posttranslational modification of its PEST domains and through interactions of Mcl-1 with pro-apoptotic Bcl-2 family members [22–27]. The binding of these proteins to Mcl-1 can result in both the displacement of E3 ubiquitin ligases and thus Mcl-1 stabilization, or the displacement of deubiquitinases and resulting Mcl-1 degradation [28, 29]. In the former case, the direct activator Bim has been shown to displace the E3 ligase Mule (HUWE1) from Mcl-1, stabilizing Mcl-1 in a BH3-dependent manner [30].

Despite these findings, many questions remain unanswered regarding the Bim:Mcl-1 interaction. Like Mcl-1, Bim is known to be regulated through phosphorylation, particularly in the context of proteasomal degradation [31–36]. However, little is known concerning how the posttranslational regulation of Bim affects its ability to stabilize Mcl-1 within a cell. Additionally, the interplay between Bim-mediated stabilization of Mcl-1 and Bcl-2 protein dependence remains unexplored. Given our previous observation that phosphorylation of Bim is associated with increased binding to Mcl-1 [37], we hypothesized that Bim phosphorylation can result in increased or decreased stabilization of Mcl-1. Here we show that Bim stabilizes Mcl-1 in several cell lines, and that the ability of Bim to stabilize Mcl-1 is altered by introduction of various phosphorylation site mutations. We also show that phosphorylation site mutation can result in altered distribution of Bim among anti-apoptotic proteins. Together, these data suggest that Bim phosphorylation not only influences the stability of Mcl-1 but can also influence Mcl-1 priming.

Results

**Bim-mediated stability of Mcl-1 confers Mcl-1-dependence**

We previously characterized the Bcl-2 family protein dynamics within the Waldenström’s macroglobulinemia cell line RPCI-WM1 and showed that these cells were deficient in the intrinsic apoptotic pathway effectors Bax and Bak [38]. This deficiency provided an ideal cellular system for studying the Bim:Mcl-1 interaction since Bim could be significantly overexpressed (Figure 1A). We were first interested in studying the Bcl-2 priming of this
cell line through co-immunoprecipitation studies. We observed an approximately equal
distribution of Bim among the anti-apoptotic proteins Mcl-1, Bcl-xL, and Bcl-2 in the
parental cell line (Figure 1A, 1B). Upon stable overexpression of Bim in RPCI-WM1, the
amount of Bim bound to each anti-apoptotic protein increased, however, we observed a
particularly striking increase in the preference of Bim for Mcl-1 (Figure 1A, 1B). This
binding was coupled with a robust increase in Mcl-1 expression (Figure 1A). In contrast, the
protein levels of Bcl-2 and Bcl-xL did not increase in parallel with Bim overexpression.
Complementary experiments in HEK293T cells using a BH3 domain-mutated version of
Bim confirmed this effect was mediated by direct binding of Bim to Mcl-1 (Figure 1A).
These data, together with our previous observation of Mcl-1 upregulation when RPCI-WM1
cells were treated with the proteasome inhibitor Bortezomib (Bz) [38], suggested a role for
Bim in stabilizing Mcl-1.

To determine the role Bim plays in stabilizing Mcl-1, we used siRNA to knock down Bim in
RPCI parental cells in the presence or absence of Bz (Figure 1C). Upon knockdown of Bim,
we noticed an equivalent decrease in Mcl-1 protein. This decrease was negated by Bz
treatment. We observed comparable results in three multiple myeloma cell lines,
demonstrating this phenomenon isn’t simply an artifact of the RPCI-WM1 cell line (Figure
1C). Importantly, when RPCI-WM1 cells were treated with Bz, the heat shock
transcriptional response was induced as previously described [39], however Bz alone had no
impact on Mcl-1 mRNA levels, nor did siRNA mediated knockdown of Bim, further
supporting a role for Bim in regulating the posttranslational stability of Mcl-1 (Figure 1D).
Previous literature has shown that Bim stabilizes Mcl-1 by competitively preventing the
binding of the E3 ligase Mule [30]. We immunoprecipitated Mcl-1 from RPCI parental and
the WT Bim stable cell line, to determine if there were differences in the amount of Mule
bound to Mcl-1 in the absence or presence of bortezomib (Figure 1E). While Bz treatment
appeared to moderately increase the amount of Mule in cells, we observed a slight increase
in the amount of Mule bound to Mcl-1 in the presence of proteasome inhibitor.

Bim is constitutively phosphorylated at multiple sites in RPCI-WM1

Both Bim and Mcl-1 are known to be extensively regulated by posttranslational
modification, particularly phosphorylation. Consequently, we were interested in determining
the constitutive phosphorylation status of Bim, and if it plays any role in mediating the
stability of Mcl-1. Using PhosTag™ gel electrophoresis, we showed that Bim is
constitutively phosphorylated, as evidenced by the presence of multiple lambda protein
phosphatase (λPP)-sensitive bands (Figure 2A). This was true for both the endogenous Bim
in RPCI-WM1, and the stably overexpressed Bim. Importantly, the pattern of
phosphorylation was similar between endogenous Bim and the overexpressed Bim,
indicating this is not an artifact of overexpression. Based on previous literature and kinase
prediction software, there are several putative Bim phosphorylation sites (Figure 2B) [31,
37, 40–44]. To determine which sites are phosphorylated and the functional consequences of
Bim phosphorylation in RPCI-WM1, we utilized site-directed mutagenesis to create
phospho-mimetic and unphosphorylateable mutants of several likely candidates. When we
ran lysates from stable cell lines expressing each mutant on a PhosTag™ gel, we again
detected several phosphorylated forms of Bim (Figure 2C). Of note, no single mutation
resulted in the complete removal of Bim phosphorylation, providing evidence for the phosphorylation of Bim at multiple sites within these cells.

**Mcl-1 is differentially expressed and stabilized in Bim phosphorylation mutant cell lines**

Having determined that Bim is constitutively phosphorylated, we then tested the impact of the phosphorylation state of Bim on Mcl-1 stabilization. Although Bim was successfully overexpressed in each of our stable cell lines, we saw disparities in the level of Mcl-1 protein (Figure 3A). Importantly, these differences were independent of the minor differences in observed Bim levels. As an example, the T116E Bim mutant overexpresser had the lowest expression of Bim of the stably-infected cell lines, but the highest level of Mcl-1 protein. Additionally, despite the unphosphorylateable Bim mutants overexpressing Bim protein at comparable levels, the S87A, S94A, and S104A transduced cells expressed significantly lower Mcl-1 protein levels. To confirm that this effect was posttranscriptional, we performed qRT-PCR on each stable cell line. While the Bim mRNA levels largely correlated with observed protein levels, the Mcl-1 mRNA levels, and the levels for Bcl-2 and Bcl-xL in each stable cell line remained unchanged compared to the parental RPCI-WM1 (Figure 3B, data not shown).

Although we qualitatively established that Mcl-1 is differentially expressed across the phosphorylation mutant stable cell lines, we were interested in quantifying how much the Mcl-1 protein levels differed from what we would expect given the level of Bim expression, and thus determining if mutation of individual phosphorylation sites increases or decreases the stability of Mcl-1. We performed SDS-PAGE and immunoblotting on three independently generated lysates from each stable cell line and calculated the densitometry values for Mcl-1 and Bim, normalized to β-actin. We utilized the three OD value pairs (Bim, Mcl-1) for the parental cell line, the empty vector stable cell line, and the WT Bim overexpresser to generate a line representing what we would predict the OD Mcl-1 value to be for a cell line given its OD Bim value (Figure 3C). Across three experiments, the average OD Mcl-1 value for several cell lines differed significantly from what the model predicted (Table 1). Stable cell lines that expressed the phospho-mimetic mutants S59E, S94E and T116E and the unphosphorylateable mutants S87A, S94A, S104A and T116A all expressed Mcl-1 levels outside the 95% confidence limits of the predicted expression. The S59E, S87A, and S104A stables each expressed Mcl-1 at a significantly lower level than predicted. Interestingly, mutation of S94 and T116 to either a glutamate or an alanine resulted in decreased or increased stabilization of Mcl-1 respectively. Our model was further validated by our studies of the S69E mutation. We previously reported that phosphorylation of Bim at S69 in multiple myeloma cells was associated with increased Bim binding to Mcl-1 [37]. When we applied our densitometry-based analysis to RPCI-WM1 cells overexpressing the S69E form of Bim, we observed a strong stabilization of Mcl-1 despite relatively low Bim overexpression (Figure 3D), suggesting that Bim phosphorylation-mediated stabilization of Mcl-1 is a surrogate for priming.

To demonstrate that these observations are not unique to the RPCI-WM1 cell line, we transiently transfected 293T cells with each of our glutamate constructs. While we observed significantly less Mcl-1 stabilization in 293Ts compared to RPCI-WM1, we still identified
several glutamate mutations that altered the ability of Bim to stabilize Mcl-1 (Figure 4). Consistent with observations in RPCI-WM1, the S59E and T116E mutations decreased and increased respectively, the levels of Mcl-1. We also observed that several mutations had a different phenotype in the 293T cells compared to RPCI-WM1. This likely reflects differences in the endogenous phosphorylation patterns in these two cells.

**Mutation of Bim phosphorylation sites alters Mcl-1 priming**

We speculated that certain Bim phosphorylation events could result in increased or decreased binding to Mcl-1 compared to Bcl-2 and Bcl-xL. We tested this hypothesis by performing co-immunoprecipitation experiments using the phospho-mimetic stable cell lines, which allow us to simulate the functional impact of phosphorylation. Additionally, given the results of our densitometry analysis, we focused on the S59E and S69E Bim stable cell lines, which had Mcl-1 protein levels that differed significantly from predictions. When compared to the stable cell line overexpressing WT Bim, the S59E stable cell line had substantially more Bim bound to Bcl-xL (Figure 5A). Interestingly, cells overexpressing S69E Bim demonstrated a binding pattern similar to WT (Figure 5B), suggesting that phosphorylation on this residue may enhance Mcl-1 binding in a different manner than simply increasing the preference of Bim for Mcl-1 at the expense of Bcl-2/xL.

To determine if the observed differences in Bim binding were due to changes in the affinity of Bim for Mcl-1, we treated the WT and S59E stable cells with the Mcl-1 inhibitor S63845 [45] and performed co-immunoprecipitation experiments (Figure 5C). Treatment with the inhibitor released a moderate amount of WT Bim from Mcl-1, while releasing more of the S59E Bim in a dose-dependent fashion. Taken together, our data strongly support the potential for phosphorylation to influence the ability of Bim to prime Bcl-2 proteins and stabilize Mcl-1.

**Discussion**

Mcl-1 is a notoriously labile protein, with its stability differentially regulated across cell types [46, 47]. This regulation can include posttranscriptional control, posttranslational modification, and interactions with other Bcl-2 family proteins [20, 48]. While each of these areas has been extensively studied, there remain unanswered questions, particularly concerning the interplay between Mcl-1 and pro-apoptotic Bcl-2 family members.

It has been previously shown that Bim stabilizes Mcl-1 by preventing the association of Mcl-1 with Mule E3 ubiquitin ligase [30]. Our study complements these findings, showing that Bim stabilizes Mcl-1 in a BH3-dependent manner in plasma cell dyscrasias, while Bim-mediated stabilization was not observed for Bcl-2 and Bcl-xL (Figure 1A, E). Given that increased expression of Bim resulted in a higher degree of Mcl-1 priming, these observations suggest a mechanism for enforcing Mcl-1 priming in response to increased expression of pro-apoptotic Bcl-2 family members. Therefore, Bim-mediated stabilization of Mcl-1 may function as a mechanism for tolerance of oncogenic transformation and priming. When a cell is subjected to aberrant proliferative cues, it typically undergoes intrinsic apoptosis through upregulation of BH3 only proteins [49–51]. A cell with a sufficient reservoir of Mcl-1 protein would therefore be able to readily tolerate this increased pro-
apoptotic load, resulting in both stabilized Mcl-1 and a cancer cell ‘primed’ with Bim bound to Mcl-1. This could potentially explain the multitude of cell types with demonstrated dependence on Mcl-1 for survival [52–54]. Furthermore, while we previously showed that increasing the expression levels of anti-apoptotic proteins does not alter the pattern of Bim priming [52], our data suggest that this pattern can in fact be altered by increasing pro-apoptotic BH3-only expression.

We further demonstrated that Bim is constitutively phosphorylated in cancer cell lines (Figure 2A and data not shown) and hypothesized that the phosphorylation status of this protein affects its ability to stabilize Mcl-1, thus adding an additional layer of complexity to the regulation of Mcl-1. Previous studies have pointed to an association between Bim phosphorylation and altered binding among anti-apoptotic proteins [37, 55]. This observation led us to postulate that the impact of Bim phosphorylation on binding was equally relevant for Mcl-1 stabilization. Work is ongoing to identify specific Bim phosphorylation sites in different cellular contexts through phospho-proteomic approaches. As a parallel effort, we generated stable cell lines overexpressing phospho-mimetic and unphosphorylateable versions of Bim. Despite comparable Bim mutant protein levels, we saw Mcl-1 protein levels that could not be explained by differences in message level (Figure 3). We were able to quantify these differences using a densitometry-based linear regression model. Our model was able to capture cell lines where altered versions of Bim impacted the stability of Mcl-1 (Figure 3C, Figure 4). Our observations of differential Bim phosphorylation-mediated Mcl-1 stability in 293T cells, coupled with data from myeloma cell lines suggest this phenomenon is not unique to just the RPCI-WM1 cell line (Figure 1C). Our findings were further validated by the S69E Bim stable overexpresser (Figure 3D). Consistent with our previous observation that phosphorylation of Bim at serine 69 correlates with increased Mcl-1 binding, we saw increased stabilization of Mcl-1 with the S69E phospho-mimetic cell line. When we performed co-immunoprecipitation experiments, however, we did not observe increased Mcl-1 priming in the S69E Bim stable cell line relative to WT Bim (Figure 5B). One potential explanation is that in this context, the S69E mutation increases the affinity of Bim for other anti-apoptotic proteins in addition to Mcl-1. There could be free Bcl-2 or Bcl-xL available to bind Bim, and since these proteins are significantly less labile, the striking stabilizing effect is not observed. Alternatively, given that the WT Bim stable cell line is already overwhelmingly Mcl-1 primed, it may be difficult to further increase the degree of priming, particularly with the relatively low level of Bim expression in the S69E stable cell line. Serine 69 is known to target Bim for proteasomal degradation, and the lower level of Bim protein in our 69E overexpressers compared to WT supports this conclusion [56].

Our findings concerning how Bim expression and phosphorylation regulate Mcl-1 priming and stability are summarized in Figure 6. We propose that in some cells, Bim plays a crucial role regulating the levels of Mcl-1 through binding and stabilization. When Bim is phosphorylated at certain sites, this ability to stabilize Mcl-1 is significantly altered, as is the distribution of Bim among anti-apoptotic proteins. Although we did not identify specific Bim phosphorylation events, our semi-quantitative approach provided insight into which sites are potentially phosphorylated in the RPCI-WM1 cell line (Table 1). Upon overexpression of Bim with alanine mutations at residues serine 87 or 104, the level of
Mcl-1 protein differed significantly from our model predictions (Figure 3C). Our data suggest that these sites are phosphorylated, and removal of phosphorylation results in decreased binding and destabilization of Mcl-1. In the case of the phospho-mimetic mutants, our data suggest that while serine 59 is not phosphorylated in the RPCI-WM1 cell line, upon phosphorylation this site would alter the ability of Bim to stabilize Mcl-1 (Figure 3C). Additionally, the binding pattern of S59E Bim differed significantly from WT, with a higher proportion of Bim bound to Bcl-xL (Figure 5A). Experiments with the Mcl-1 inhibitor S63845 showed that a greater proportion of S59E Bim was released from Mcl-1 than WT Bim for a given dose of inhibitor, suggesting that the mutation decreases the affinity of Bim for Mcl-1 (Figure 5C). Future studies utilizing surface plasmon resonance could more definitively determine the impact of phosphorylation site mutations on the in vitro Bim:Mcl-1 interaction. Phosphorylation of Bim at serine 59 has been previously linked to proteasomal degradation, sequestration in autophagocytic bodies, and inhibited apoptogenic function [36, 41]. Our qRT-PCR data support the potential for Bim proteasomal degradation, given the disparity between S59E Bim mRNA and protein levels (Figure 3), and if phosphorylation of Bim at S59 results in sequestration, this could result in less Bim available for binding to Mcl-1. Interestingly, mutation of serine 94 or threonine 116 to either an alanine or glutamic acid resulted in decreased or increased Mcl-1 binding respectively (Figure 3). This finding supports a role for these two amino acids in mediating protein-protein interactions, such that mutation to any other amino acid results in altered Bim binding. Previous work supports this observation, particularly in the case of T116. T116 has been implicated in mediating the binding of Bim to the dynein light chain complex [31, 57, 58]. Our model posits that mutation of this residue to anything other than a threonine, results in an inability of Bim to bind to dynein light chain, necessitating increased binding to Mcl-1 to allow the cell to survive. This is consistent with findings demonstrating that phosphorylation of this residue by JNK results in the release of Bim from dynein light chain [31].

Additional questions remain concerning how phosphorylation or the introduction of phosphorylation mutations alters the structure of Bim to affect its binding to Mcl-1. Bim binding to anti-apoptotic proteins is mediated by the insertion of its BH3 domain into the hydrophobic groove of its sequestering protein [59]. Since none of the phosphorylation sites in our analysis are located in the BH3 domain region, our data support a regulatory role for the non-BH3 domain parts of Bim. Previous work has characterized the interplay between phosphorylation and different isoforms of Bim, with data showing that Erk phosphorylation sites in Bim EL (serine 59, 69, and 77) are dispensable for Bim pro-apoptotic function in thymocytes [60]. Our work, in conjunction with previous efforts, emphasizes the importance of studying the entirety of the Bim protein.

The study of Bim:anti-apoptotic protein interactions has substantially informed the development of more effective cancer therapeutics [61]. Notable in these efforts has been the development of Bcl-2 antagonist therapies that can disrupt the interaction between Bim and anti-apoptotic proteins, allowing Bim to activate Bax/Bak and initiate cancer cell apoptosis [62–65]. This approach has resulted in the development of several promising compounds, including venetoclax, a Bcl-2 inhibitor approved for the treatment of CLL [66, 67]. Efforts to develop effective therapies for disrupting the Mcl-1:Bim interaction have been
comparatively less successful, due to unexpected toxicities, off-target effects, and differences in the structure of Mcl-1 compared to other anti-apoptotic proteins [68, 69]. Recently, multiple Mcl-1 inhibitors have demonstrated promise in the in vitro setting [45, 64, 65, 70]. A common theme among several of these compounds is the ability to impact the stability of Mcl-1. Our study provides additional rationale for understanding the regulation of Mcl-1 stability to better inform therapeutic development. We’ve shown that Bim plays a role in stabilizing Mcl-1 in myeloma cells, even in cells that were strongly dependent on Bcl-2/xL for survival (Figure 1C) [37, 52]. Mcl-1 is overexpressed in many cancers including myeloma, frequently due to chromosome 1q amplification [71]. Our data suggest that the consequences of this amplification aren’t fully realized until Mcl-1 is bound and stabilized by pro-apoptotic proteins. This may be particularly relevant when Mcl-1 binds Bim induced or released by therapeutics and mediates resistance.

Taken together, our data show that Bim phosphorylation can function as an additional regulator of Mcl-1 stability and a determinant of priming. While previous research efforts have been directed at developing therapies to target Mcl-1, our work may provide an impetus to consider targeting signaling cascades that regulate Bim as a complementary approach.

Materials and Methods

Cell culture

The RPCI-WM1 cell line was cultured as previously described [38, 72]. The myeloma cell line RPMI-8226 (8226) and the HEK293T cell line were purchased from American Type Culture Collection (ATCC). MM.1s was provided by Steven Rosen (City of Hope), and KMS12-PE was purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB). Myeloma cells were cultured as previously described [73].

Transient transfection and generation of stable cell lines via retroviral transduction

HEK293T cells were transfected with pBabe-puro (empty, wild type Bim, or phosphorylation mutant Bim) or pLVX-IRES-Neo (Clontech, Mountain View, CA, USA) constructs (empty, wild type Bim, or Bim with the BH3 domain mutation L152A/D157A ‘BH3mut’[30]) using Lipofectamine 2000 (Thermo Fisher, Waltham, MA, USA), according to the manufacturer’s instructions. Stable cell lines were generated as previously described [74]. Briefly, ΦNX-Amphotropic packaging cell lines (Nolan lab, Stanford University) were transfected with plasmid (empty pBabe or N-terminal His-tagged Bim) using Lipofectamine 2000. RPCI-WM1 cells were subjected to three rounds of infection with 0.45-µm syringe filtered (Pall) viral supernatants and Polybrene Infection/Transfection Reagent (Millipore, Burlington, MA, USA). Once cells recovered from infection they were selected with 2 µg/ml puromycin (Sigma, Burlington, MA, USA). Phosphorylation mutant versions of Bim and the BH3 mutant version were generated using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA, USA).

Immunoblotting

SDS-PAGE and western blotting were performed as previously described [73]. Primary antibodies used included: rabbit polyclonal α-Bim (Millipore), rabbit polyclonal α-Mcl-1
PhosTag™ Gel Electrophoresis

PhosTag™ Gel Electrophoresis was performed as previously described [39, 75]. Briefly, a 10% Acrylamide/Bis-acrylamide gel was cast with a final concentration of 50 μM of PhosTag (Wako, Osaka, Japan), 100 μM MnCl₂. Electrophoresis and immunoblotting were performed as described above with the following modification: Gels were transferred to 0.2 μm PVDF membranes (Bio-Rad, Hercules, CA, USA).

To allow for discrimination between phosphorylated and unphosphorylated forms of proteins, lysates were treated with lambda protein phosphatase (λPP) (New England Biolabs, Ipswich, MA, USA) as previously described [39].

Immunoprecipitation

Immunoprecipitation was performed using Protein G (Millipore) and the following antibodies: mouse α-Bim (Santa Cruz Biotechnology), mouse α-Mcl-1 (BD Biosciences), hamster α-Bcl-2 (BD Biosciences), and mouse α-Bcl-xL [76]. Briefly, cells were lysed in 2% CHAPS buffer, and 100 μg of lysate was incubated with antibody:bead complexes overnight. Thirty μg of whole cell lysate was used as input along with the entirety of the eluted bound fraction for SDS-PAGE and immunoblotting. Bim binding patterns in the immunoprecipitation experiments were quantified as previously described [37, 77].

siRNA

Five to six million cells were transfected with siBim or non-targeting control si (Dharmacon, Lafayette, CO, USA) using the Amaxa Nucleofector II (Lonza, Basel, Switzerland). Cells were transfected using the Nucleofector Kit V, and program ‘G-015’ (RPCI-WM1, 8226, KMS12PE) or ‘O-023’ (MM.1s).

Bortezomib treatment

RPCI-WM1 cells were treated with 5 nM Bortezomib (LC Labs, Woburn, MA, USA) for 12–20 hours. Myeloma cell lines were treated for three hours with 30 nM Bortezomib.

qRT-PCR

RNA was isolated from one million cells using the RNeasy Kit (Qiagen, Hilden, Germany). cDNA was generated as previously described using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA), and amplified using the TaqMan Gene Expression Master Mix (Life Technologies) on the 7500 Fast Real-Time PCR System following the manufacturer’s protocol (Applied Biosystems) [73]. The following probes were used: Bim (BCL2L11) Hs00708019_s1, Mcl-1 Hs01050896_m1,
Bcl-2 Hs00608023_m1, Bcl-xL (BCL2L1) Hs00236329_m1, HSPA1A Hs00359163_s1 and GAPDH 4332649.

**Densitometry and generation of linear regression models**

For three western blots from independent sets of lysates, ImageJ software was used to calculate OD values for Mcl-1, Bim, and β-actin for each cell line. The OD values for Mcl-1 and Bim were normalized by dividing each by the corresponding OD values for β-actin. The normalized values for the parental RPCI-WM1, RPCI-WM1-empty pBabe stable, and the RPCI WT Bim stable overexpresser were plotted in GraphPad Prism, and a linear regression with 95% confidence interval was generated. The average of the normalized values for the other stable cell lines (phospho-mimetic and unphosphorylateable) was then overlaid on the linear regression curves.

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**Abbreviations:**

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<th>Description</th>
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<tr>
<td>Bz</td>
<td>bortezomib</td>
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<tr>
<td>λPP</td>
<td>lambda protein phosphatase</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<td>wild type</td>
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<td>RQ</td>
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**References**


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Figure 1. Mcl-1 is stabilized and primed by Bim.
(A) (Left) One hundred μg of whole cell lysate (WCL) from the RPCI-WM1 cell line (RPCI Parental) and from RPCI cells that stably overexpress wild type (WT) Bim was subjected to immunoprecipitation (IP) with the indicated antibodies. Eluates from the IPs and 30 μg of input were used for SDS-PAGE and western blotting. (Right) Thirty μg of WCL from RPCI parental, empty vector, and WT Bim overexpressing stable cell lines or HEK293T cells transfected with the indicated constructs was subjected to SDS-PAGE and immunoblotting.

(B) Pie charts showing the proportion of Bim bound to Mcl-1, Bcl-xL, and Bcl-2 in the

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immunoprecipitation experiments in (A). Pie charts were generated by determining densitometry readings for Bim bound to each anti-apoptotic protein and dividing by the sum of these OD readings. Pie charts reflect the average proportion of Bim bound in two (Parental) or four (WT) independent experiments. (C) (Left) RPCI Parental cells were nucleofected with either off target siRNA or siRNA targeting Bim. After 24 hours, cells were collected and grown in the absence or presence of 5 nM Bortezomib (Bz) for an additional 20 hours. Cells were subsequently collected and lysed, and 30 μg of lysate per condition was used for SDS-PAGE and western blotting. The indicated myeloma cell lines were treated with 30 nM bortezomib (Bz) for three hours (middle) or nucleofected with the indicated siRNAs (right). Cells were subsequently collected, lysed, and 30 μg of WCL was subjected to SDS-PAGE and immunoblotting. (D) (Left) RPCI parental cells were grown in the absence or presence of 5 nM Bz for 20 hours. Cells were then collected for lysates and RNA isolation. qRT-PCR was performed on cDNA from the untreated and treated conditions. Mcl-1 and HSPA1A mRNA levels are displayed as relative quantities to the untreated RPCI parental cell line and normalized to GAPDH. (Right) RPCI parental cells were nucleofected with either off target siRNA or siRNA targeting Bim. After 24 hours, cells were collected for RNA isolation. qRT-PCR was performed on cDNA from the si(−) and siBim conditions. Bim and Mcl-1 mRNA levels are displayed as relative quantities to the si(−) transfected RPCI parental cell line and normalized to GAPDH. (E) The RPCI-WM1 cell line (RPCI Parental) and RPCI cells that stably overexpress wild type (WT) Bim were grown in the absence or presence of 5 nM of bortezomib for 12 hours. Cells were then collected, lysed, and one hundred μg of whole cell lysate (WCL) was subjected to immunoprecipitation (IP) with Mcl-1 antibody. Eluates from the IPs and 30 μg of input were used for SDS-PAGE and western blotting. (*) indicates where bands for Mule migrate.
Figure 2. Bim is constitutively phosphorylated in the RPCI-WM1 cell line.
(A) WCLs were generated from RPCI parental and WT Bim overexpressers, and 30 μg of untreated (−) and lambda protein phosphatase-treated (+) lysate for each cell line was subjected to PhosTag™ gel electrophoresis, and immunoblotting. (*) represents unphosphorylated Bim while the phosphorylated forms are contained within the bracket (B) Schematic illustrating the location of putative Bim phosphorylation sites within the three major splice isoforms of Bim. (C) WCLs were generated from stable cell lines overexpressing WT and the phospho-mimetic (S/T E) and unphosphorylatable (S/T A)
forms of Bim indicated. WCLs were subject to PhosTag™ gel electrophoresis and immunoblotting. (*) represents the primary Mcl-1 isoform, and ns represents a non-specific band.
Figure 3. Bim phosphorylation mutations alter its ability to stabilize Mcl-1 in RPCI-WM1.

(A) Thirty μg of WCL from the indicated cell lines (phospho-mimetic, (left); unphosphorylatable, (right)) was subjected to SDS-PAGE and immunoblotting. Blot is representative of three independent experiments.

(B) qRT-PCR data from the indicated cell lines. Bim and Mcl-1 mRNA levels are displayed as relative quantities to the RPCI parental cell line and normalized to GAPDH. Data are representative of three independent experiments and displayed as mean values plus standard error of the mean (SEM).

(C) Densitometry values were obtained for Bim and Mcl-1 and normalized to β-actin for

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western blots from three independent experiments (as shown above). The three densitometry readings (OD Mcl-1 vs OD Bim) for the RPCI parental, empty pBabe, and WT Bim overexpressers (points shown in blue) were used to generate a linear regression (thick middle line), with 95% confidence interval (thinner, dashed lines). Densitometry readings for the phosphorylation mutant Bim stable cell lines were plotted against the line—points shown had an average OD Mcl-1 value that differed significantly from what would be predicted based on the average OD Bim value, indicated by points falling above or below the 95% confidence interval lines. (D) (Left) Thirty μg of WCL from the indicated cell lines was utilized for SDS-PAGE and immunoblotting. Western blot shown is representative of two independent experiments. (Right) Graph was generated as described in C.
Figure 4. Bim phosphorylation site mutations impact the stability of Mcl-1 in HEK293T cells. (A) Thirty μg of WCL from HEK293T cells transfected with the indicated constructs was collected and subjected to SDS-PAGE and immunoblotting. Western blot is representative of two independent experiments (B) Graph was generated as described in Figure 3.
Figure 5. Phosphorylation site mutation alters the distribution of Bim among anti-apoptotic proteins.

(Left) One hundred μg of WCL from cells overexpressing S59E (A) or S69E Bim (B) was subjected to immunoprecipitation (IP) with the indicated antibodies. Eluates from the IPs and 30 μg of input were used for SDS-PAGE and western blotting. (>) indicates the upper band of the immunoblot that represents Bcl-xL. Immunoblots are representative of at least three independent experiments. (Right) Pie charts showing the proportion of Bim bound to Mcl-1, Bcl-xL, and Bcl-2 in the phospho-mimetic stables compared to the WT Bim.
overexpressing stable cell line (WT pie chart previously shown in 1B). Pie charts were generated as described in 1B and reflect the average proportion of Bim bound in four (WT) or three (S59E, S69E) independent experiments. (C) RPCI WT Bim and S59E Bim stable cell lines were grown in the absence or presence of the Mcl-1 inhibitor S63845 at the indicated concentrations for four hours. Cells were then collected, lysed, and one hundred μg of whole cell lysate (WCL) was subjected to immunoprecipitation (IP) with Mcl-1 antibody. Eluates from the IPs and 30 μg of input were used for SDS-PAGE and western blotting.
Figure 6. Model for the role of Bim phosphorylation in priming and stabilizing Mcl-1. Within RPCI-WM1, Mcl-1 is a frequent target for ubiquitination. When Bim is overexpressed, it preferentially binds to Mcl-1, stabilizing and preventing its proteasomal degradation. Our model suggests phosphorylation may strengthen or adversely impact the ability of Bim to stabilize and preferentially bind to Mcl-1.
Table 1.
Summary of Bim phosphorylation site mutations and their impact on Mcl-1 stability in RPCI-WM1 cells

<table>
<thead>
<tr>
<th>Phosphorylation Site</th>
<th>Phospho-mimetic (Glutamate)</th>
<th>Unphosphorylatable (Alanine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T116</td>
<td>↑ Mcl-1 Stabilization</td>
<td>↑ Mcl-1 Stabilization</td>
</tr>
<tr>
<td>S59</td>
<td>↓ Mcl-1 Stabilization</td>
<td>--</td>
</tr>
<tr>
<td>S87</td>
<td>--</td>
<td>↓ Mcl-1 Stabilization</td>
</tr>
<tr>
<td>S94</td>
<td>↓ Mcl-1 Stabilization</td>
<td>↓ Mcl-1 Stabilization</td>
</tr>
<tr>
<td>S104</td>
<td>--</td>
<td>↓ Mcl-1 Stabilization</td>
</tr>
</tbody>
</table>