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Jason Edward Conage-Pough Date

Characterization of Constitutive Bim Phosphorylation in Plasma Cell Malignancies

By

Jason Edward Conage-Pough
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Cancer Biology

_____
Lawrence H. Boise, Ph.D.
Advisor

_____
Jing Chen, Ph.D.
Committee Member

_____
Xingming Deng, M.D., Ph.D.
Committee Member

_____
Kelly C. Goldsmith, M.D.
Committee Member

_____
Mala Shanmugam, Ph.D.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

Characterization of Constitutive Bim Phosphorylation in Plasma Cell Malignancies

By

Jason Edward Conage-Pough
SB, Massachusetts Institute of Technology, 2009

Advisor: Lawrence H. Boise, Ph.D.

An abstract of
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Abstract

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Apoptosis plays a critical role in organismal development and cellular homeostasis. When a cell is subjected to stimuli such as growth factor withdrawal, UV damage, or aberrant proliferative cues, it typically undergoes intrinsic or mitochondrial-associated apoptosis, which culminates in mitochondrial outer membrane permeabilization (MOMP), cytochrome *c* release, and caspase activation. The Bcl-2 family of proteins serves as a crucial regulator of this pathway, with the balance between pro- and anti-apoptotic members determining whether a cell lives or dies.

The pro-apoptotic Bcl-2 protein Bim is upregulated in response to many cellular cues, and functions as a potent activator of the multi-domain effector proteins that permeabilize the mitochondria, and trigger MOMP. Because of its apoptogenic properties, Bim is extensively regulated at the transcriptional, posttranscriptional, and posttranslational levels. Notably, Bim is frequently phosphorylated, resulting in its proteasomal degradation and other alterations to its function. This occurs frequently in the context of cancer cells, where it is often necessary to suppress Bim function to facilitate oncogenic transformation. While several studies have examined Bim phosphorylation events that arise in response to various stimuli, little is currently known about the constitutive Bim phosphorylation state.

While studying the Bim phosphorylation events triggered by IL-6 stimulation of myeloma cells, we discovered that Bim was constitutively phosphorylated within these cells. Moreover, Bim was differentially phosphorylated across several myeloma cell lines and patient samples, with cells expressing anywhere from one to three or more phosphorylated forms. While we were unable to successfully utilize phospho-proteomics to identify specific sites, our use of small molecule inhibitors allowed us to partially elucidate the signaling pathways regulating Bim.

Additionally, while characterizing Bim phosphorylation within the Waldenström's macroglobulinemia cell line RPCI-WM1, we determined that the introduction of Bim phosphorylation site mutations impacted the ability of Bim to stabilize and prime Mcl-1. Our data provide evidence for the role of phosphorylation in determining the anti-apoptotic protein that Bim is bound to within a cell.

The implications of our research for cancer treatment are significant. Our data suggest that combining drugs that mobilize Bim with compounds that affect its phosphorylation state is a promising therapeutic strategy.

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I. INTRODUCTION

Apoptosis is a form of programmed cell death, that functions in several critical biological processes. In contrast with the inflammation and free release of cellular contents associated with cell death via programmed necroptosis, apoptosis is characterized by cellular shrinkage, nuclear condensation, DNA fragmentation, the formation of apoptotic bodies, and the subsequent elimination of apoptotic bodies by phagocytosis[1, 2]. Apoptosis plays a crucial role in regulating normal organismal homeostasis, with the average human losing billions of cells via apoptosis daily[3]. Notable examples of apoptosis in multicellular organisms are the removal of larval tissue as a tadpole matures into a frog, and the elimination of cells in human embryo hands and feet, resulting in separate fingers and toes[1, 4].

Apoptosis can be triggered within a cell by a myriad of stimuli, including DNA damage, serum withdrawal, and aberrant proliferative cues[5]. While there are several ways to trigger the apoptotic cascade, and multiple pathways of activation, each of these steps invariably converges on the activation of caspases—proteases that are ultimately responsible for the cleavage of cellular substrates that results in the formation of apoptotic bodies[6]. Apoptosis can be molecularly classified into two pathways: the death receptor and extracellular ligand-mediated *extrinsic* pathway, and the mitochondrial membrane associated *intrinsic* pathway of apoptosis[7].

A. Intrinsic Apoptosis and the Bcl-2 Family of Proteins

The intrinsic pathway of apoptosis translates cellular stress cues such as growth factor withdrawal into the steps necessary for a cell to undergo apoptosis[8]. Critical to this pathway is the release of cytochrome *c* through mitochondrial outer membrane permeabilization (MOMP)[9]. Traditionally known as the 'point of no return', cytochrome *c* release in most instances irreversibly leads to activation of the caspase cascade, and the downstream cleavage events that convert a dying cell into apoptotic bodies[10]. Intrinsic apoptosis is governed by the Bcl-2 family of proteins, a group of structurally related proteins that promote and antagonize cytochrome *c* release[11].

Bcl-2 family proteins are unified by the presence of one or more Bcl-2 homology (BH) domains that allow them to bind to and interact with one another[12]. The interactions between the apoptosis promoting (pro-) and anti-apoptotic Bcl-2 family members are largely what determine whether a cell lives or dies via apoptosis. Notable among the pro-apoptotic proteins are the multi-(BH)domain effectors Bax and Bak, which upon activation, homo-oligomerize and permeabilize the mitochondrial outer membrane, triggering cytochrome *c* release[13]. The remaining pro-apoptotic Bcl-2 family proteins contain only the BH3 domain, but still play a key role in activation of the apoptotic cascade[14]. These BH3-only proteins are typically upregulated when a normal cell is exposed to various pro-apoptotic stimuli, and thus serve as sentinels for the initiation of intrinsic apoptosis[15]. The direct activator BH3-only proteins Bim, Bid and occasionally Puma are capable of transiently binding to Bax and Bak, and facilitating their activation[16]. The anti-apoptotic Bcl-2 family members antagonize intrinsic apoptosis by binding to and sequestering the pro-apoptotic family members. Anti-apoptotic members

contain a hydrophobic pocket formed by BH domains 1-3, which allows them to bind the BH3 domain of pro-apoptotic Bcl-2 proteins[12, 17]. Anti-apoptotic proteins include the eponymous Bcl-2, Bcl-x_L, Mcl-1, A1, Bcl-w, and Bcl-B. The balancing act between pro- and anti-apoptotic proteins is further influenced by the presence of BH3-only 'sensitizers'—Bcl-2 family proteins such as Bad, Noxa, Bmf, and Hrk that can bind to and occupy anti-apoptotic proteins, freeing the direct activators and effectors and allowing MOMP to occur[12]. The major players and steps of intrinsic apoptosis are highlighted in Figures 1 and 2.

Bcl-2 family protein interactions are influenced by a variety of factors distinct from the BH domains, providing an additional layer of complexity and context-dependent regulation of cell death. For example, Bcl-2 proteins are extensively regulated by posttranscriptional and posttranslational events, including microRNAs, phosphorylation, and proteasomal degradation[18]. The pro-apoptotic protein Bim is subject to each of these regulatory mechanisms, impacting its ability to facilitate intrinsic apoptosis.

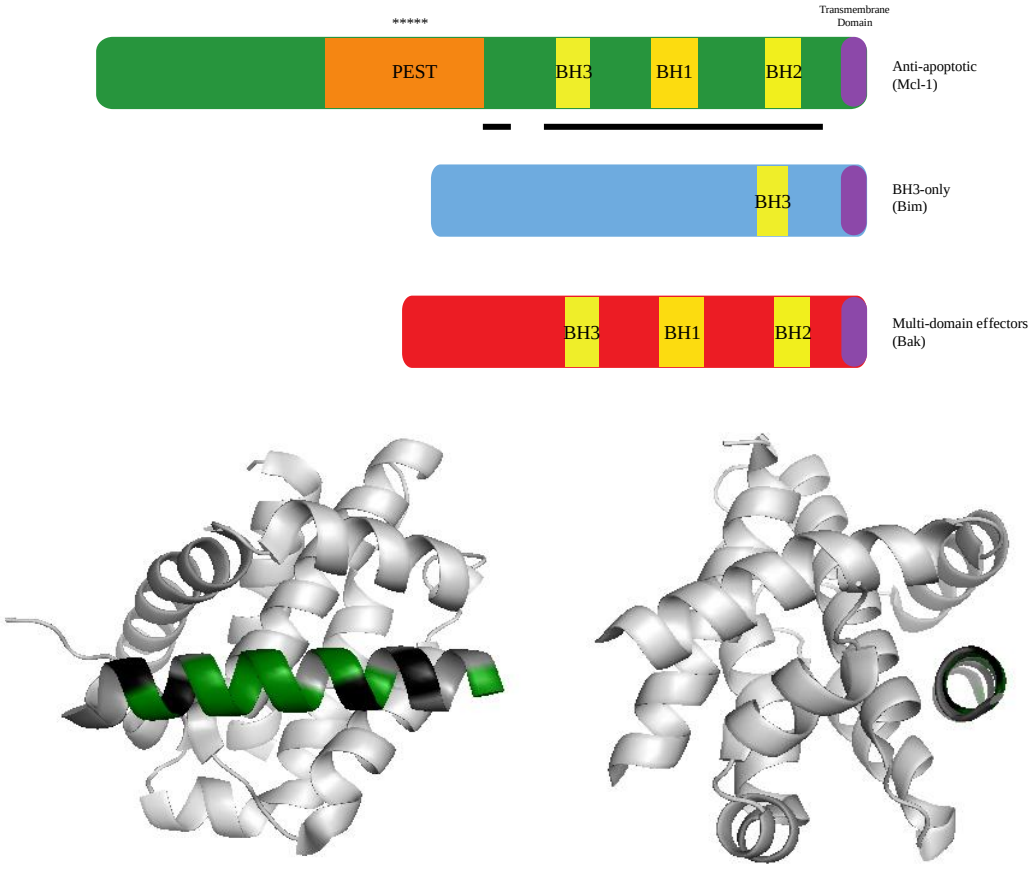


Figure I-1: The structure of Bcl-2 family members. Anti-apoptotic Bcl-2 family members are characterized by the presence of at least three Bcl-2 Homology (BH) domains. (*****) Note that Mcl-1 contains stability regulating PEST domains rather than a BH4 domain found in other anti-apoptotic family members. As the names indicate, pro-apoptotic BH3-only proteins contain the singular BH3 domain, while effector proteins such as Bax and Bak contain BH1-3. Also illustrated is the sequestering of the BH3 domain of Bim (green) by Mcl-1[19] (white). The solid black lines beneath the upper Mcl-1 schematic represent the region depicted in the ribbon diagram. The BH3 domain of Bim binds to a hydrophobic pocket within Mcl-1 consisting of BH domains 1-3.

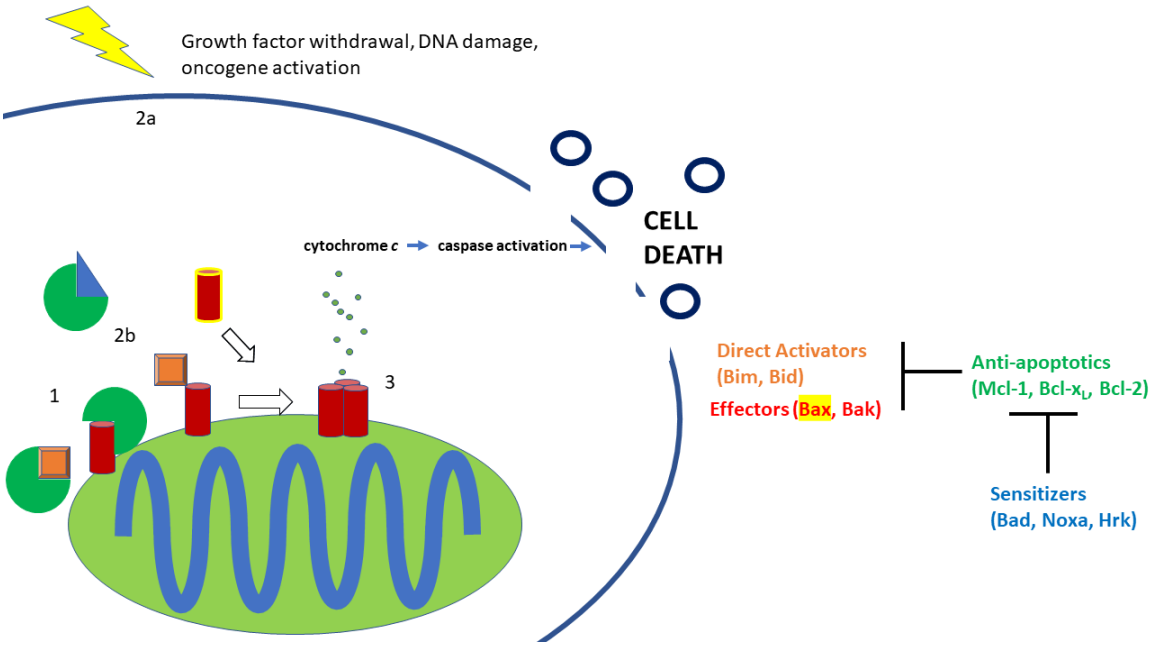


Figure I-2: The intrinsic pathway of apoptosis. Under normal conditions, anti-apoptotic proteins sequester pro-apoptotic Bcl-2 family members (1). When a cell is exposed to a pro-apoptotic stimuli (2a), sensitizers can free activators from the anti-apoptotics (2b), allowing them to transiently bind to and activate the multidomain effectors (cytosolic Bax or mitochondrial membrane bound Bak). Upon activation, the effectors homo-oligomerize and permeabilize the mitochondrial outer membrane, resulting in cytochrome *c* release (3), caspase activation and ultimately cell death.

B. Bim

Bcl-2 interacting mediator of cell death (Bim) was identified independently by two research groups screening for proteins that interacted with Bcl-2 and Mcl-1[20, 21]. Bim is coded for by the BCL2L11 gene, located on chromosome 2q. Through alternative splicing of at least 12 exons, there are currently 18 known Bim isoforms, with three designated as major isoforms: full length Bim EL (198 amino acids, ~22 kDa), Bim L (138 AA, ~16 kDa), and Bim S (108 kDa, ~13 kDa)[22] (Figure 3). While Bim EL is typically the most abundant form in mammalian tissues, Bim S is the most apoptogenic, with the length of a Bim isoform generally being inversely correlated with its apoptotic activity. Each of the three predominant isoforms contains the BH3 domain, along with a transmembrane domain, while Bim EL and Bim L contain a domain that facilitates binding to the dynein light chain component of the cytoskeleton[23] (Figure 3). In addition to its direct activating properties, Bim (along with Bid and Puma) differs from other BH3-only proteins in its ability to bind with high affinity to each of the anti-apoptotic proteins[24]. In non-apoptotic cells, Bim is nearly always sequestered, either bound to anti-apoptotic proteins in the outer mitochondrial membrane, or to the dynein light chain, where it can be mobilized in response to apoptotic stimuli[12, 25]. Bim can be induced in response to a wide array of apoptotic insults, including growth factor withdrawal, UV radiation, ER stress, and anoikis[22]. Because of its potency, Bim expression and activity must be tightly controlled through a variety of mechanisms.

1. Transcriptional control of Bim

One of the most commonly described transcriptional regulators of Bim is the Forkhead Box (FoxO) family of transcription factors. Particularly, FoxO3 can be

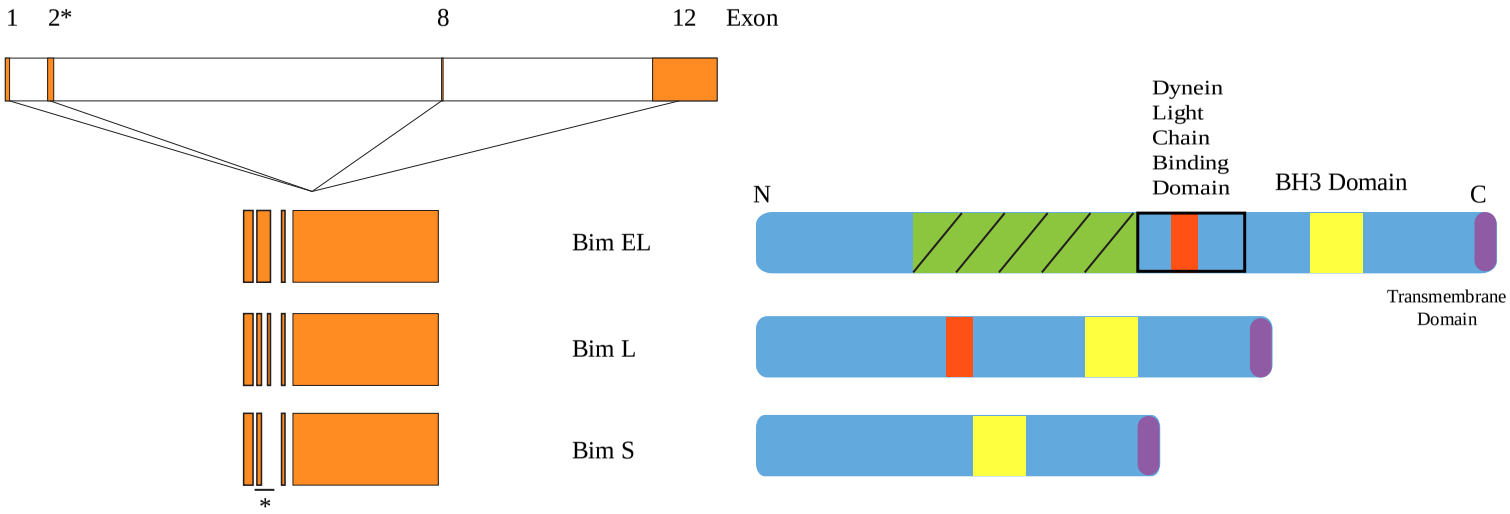


Figure I-3: Schematic of transcripts and protein domains for major isoforms of Bim. (Left) Bim mRNA for each of the major Bim isoforms is generated by exons one, two, eight, and twelve. (*) indicates alternative splicing of exon two results in mRNAs and ultimately proteins of different lengths. (Right) Each major isoform contains the BH3 domain (yellow) and a transmembrane domain (purple). Only Bim EL and Bim L contain the dynein light chain binding domain (red). The differences in exon 2 splicing result in Bim EL containing the additional amino acids represented by the green rectangle, and the amino acids in the black rectangle are present in Bim EL and L, but not Bim S.

activated upon serum withdrawal, resulting in increased Bim expression[26]. An additional layer of regulation is added by the influence of the PI3K/Akt signaling pathway over FoxO3. Upon Akt phosphorylation, FoxO3 is sequestered by the phosphorylation binding protein 14-3-3, confining it to the cytoplasm and preventing the nuclear localization required for Bim transcription[27]. Additional Akt phosphorylation events have been shown to increase nuclear FoxO3, and thus increase Bim transcription[28]. ERK phosphorylation is capable of regulating FoxO3 in a comparable manner[29]. Other transcription factors influence Bim message levels in response to distinct pro-apoptotic stimuli. For example, c-Myc is known to function as a potent transcriptional driver of cell proliferation, capable of triggering aberrant proliferation[30]. Seminal studies by Gerard Evan and others demonstrate that in order to prevent uncontrolled cell division in normal cells, c-Myc drives activation of the apoptotic program[31]. Similarly, E2F1 is known to induce Bim transcription in response to the cell cycle program[32]. In addition to being transcriptionally activated, Bim is known to be transcriptionally repressed by Trim33, HIF-1α, YY-1 and several other transcription factors[33].

2. Posttranscriptional regulation of Bim

When a cell is exposed to both pro-apoptotic and pro-survival stimuli, Bim mRNA stability and translation can be directly impacted. Several heat shock proteins, including Hsc70 and Hsp27 can bind to the 3'UTR of Bim mRNA, promoting or preventing Bim upregulation in response to pro-apoptotic stimuli[34, 35]. Additionally, microRNAs regulate Bim levels through direct and indirect mechanisms. Several microRNA clusters directly bind to the 3'UTR of Bim, preventing translation, while other

microRNAs prevent expression of transcription factors and E3 ligases, that positively or negatively impact Bim message and protein levels respectively[36, 37].

3. Posttranslational regulation of Bim

A substantial body of scientific literature has elucidated the role of posttranslational modification in regulating Bim. PTMs have been linked to the stability, apoptotic activity, cellular localization, and interactions of Bim with other Bcl-2 family members[38]. Phosphorylation and ubiquitination in particular are known to play an extensive role in modifying Bim function.

a. Regulation of the Bim phosphorylation state

The earliest reports of Bim phosphorylation showed that phosphorylation in the dynein light chain binding domain affected the pro-apoptotic activity of Bim. NGF stimulation of rat PC12 cells resulted in MEK-dependent Bim phosphorylation and decreased apoptosis, while Lei and Davis showed that UV radiation triggered JNK-mediated phosphorylation of human Bim at threonine 116, releasing it from dynein light chain, and increasing intrinsic apoptosis[25, 39].

Subsequent studies indicated that many Bim phosphorylation events are context-dependent. As an example, serine 69 phosphorylation has been attributed to JNK in rat neurons, and p38 in PC12 cells, with both modifications increasing apoptotic activity[40, 41]. Additional work has shown that ERK phosphorylation of Bim at serine residues 59, 69, and 77 results in proteasomal degradation in several cell types[42, 43]. Serine 69 phosphorylation was found to trigger additional Aurora kinase or RSK-dependent phosphorylation events at serine 93, 94, and 98, that facilitated βTrCP

binding and degradation of Bim[44, 45]. Conversely, ER stress has been linked to dephosphorylation of Bim by PP2A, increasing its stability[46].

Phosphorylation has also been implicated in affecting the interactions of Bim with other anti-apoptotic proteins and binding partners. When mouse Ba/F3 cells were stimulated with IL-3, Bim was phosphorylated at serine 87 by Akt, increasing its binding to 14-3-3, and decreasing its pro-apoptotic activity[47]. A contemporaneous study showed Protein Kinase A was also responsible for phosphorylation at serine 87 in MEFs, increasing Bim stability[48]. Serine 87 Bim phosphorylation was later identified when human lung cancer cells were subjected to nutrient deprivation, although its specific role was not determined[49]. Phosphorylation at serine 59, resulted in decreased apoptosis via anoikis and sequestration of Bim by Beclin and dynein light chain LC8 in breast cancer cells[50]. Additional work in mouse FL5.12 cells suggests ERK-mediated phosphorylation of Bim influences binding to Bax and Bcl-2[51]. Recent work has expanded upon these initial findings, showing that ERK-mediated Bim phosphorylation events such as serine 69 and serine 77 can favor preferential binding to Mcl-1[52].

The interplay between Bim phosphorylation and mitosis has also been preliminarily explored. Cyclin B1/Cdk1 is capable of phosphorylating Bim at serine 104 in response to various microtubule poisons, and data suggest this phosphorylation results in mitotic arrest and cell death in K562 chronic myeloid leukemia cells[53].

Lastly, although most publications have pointed to Bim being serine/threonine phosphorylated, evidence has emerged that Bim can be tyrosine phosphorylated in

cancer cells, inhibiting its function[54]. A summary of currently known Bim phosphorylation events is displayed in Figure 4.

b. Ubiquitination and proteasomal degradation

Bim is targeted for proteasomal degradation as a means of diminishing its pro-apoptotic activity. Bim contains two lysine residues (K3 and K112) that are potential targets for its ubiquitination, however previous work has shown that Bim can be degraded by the proteasome in a ubiquitin-independent manner, likely because it is an intrinsically disordered protein[55]. βTrCP and APC^{Cdc20} are the E3 ubiquitin ligases currently known to facilitate the degradation of Bim[44, 56]. Conversely, the deubiquitinase Usp27x stabilizes Bim, increasing apoptosis alone and in combination with ERK inhibition[57].

As a highly regulated, central player in the activation of apoptosis, Bim has proven to be an attractive therapeutic target. Several molecules have been found to perturb the transcription, stability, and binding of Bim, affecting the susceptibility of target cells to apoptosis. Notable examples include the steroid dexamethasone driving glucocorticoid-mediated transcription of Bim, small molecule inhibitors targeting receptor tyrosine kinases and inhibiting phosphorylation events that result in Bim degradation, and proteasome inhibitors preventing the degradation of Bim[58-60]. These findings have proven crucial in the effort to understand how apoptosis is dysregulated in diseases such as cancer.

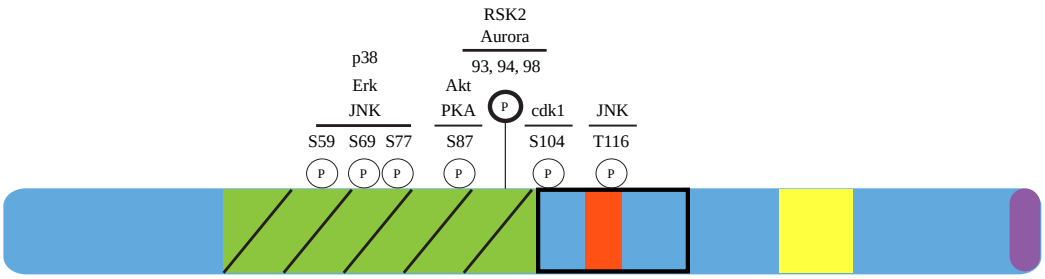


Figure I-4: Serine/Threonine phosphorylation sites of Bim. Bim phosphorylation events have been implicated in prior literature in response to several stimuli. Note serine 59, 69, 77, 87, 93, 94, and 98 are unique to Bim EL (green region), illustrating that alternative splicing affects regulation of Bim by phosphorylation.

C. Dysregulation of the Bcl-2 Family in Cancer and Mitochondrial Priming

One of the hallmarks of a cancer cell is the ability to evade pro-apoptotic signals[61]. Typically, when a normal, non-transformed cell is subjected to aberrant proliferative cues or oncogenic signaling, it responds by upregulating BH3-only proteins such as the direct activator Bim, leading to apoptosis[15]. Cancer cells, however, can escape this fate through inhibition of pro-apoptotic proteins. Mechanisms for achieving this inhibition include targeting for proteasomal destruction, altering cellular localization, and sequestration[62]. Additionally, anti-apoptotic protein expression is frequently increased in cancer cells, providing a means of tolerating the increased pro-apoptotic load by binding and sequestering pro-apoptotic proteins (Figure 5). This increase in the anti-apoptotic capacity of cancer cells is a double-edged sword—the cancer cell can survive; however, it is dependent upon specific anti-apoptotic proteins for survival, and immediately becomes susceptible to agents that can disrupt the interaction between these proteins and sequestered pro-apoptotic direct activators. These cancer cells are thus considered 'primed' for cell death, a characteristic that distinguishes them from the majority of non-malignant cell types[63].

Mitochondrial priming serves as the basis for how chemotherapy and other drugs can effectively kill cancer cells, while having a comparatively minimal effect on normal tissues[64]. Because cancer cells suppress increased pro-apoptotic protein expression, they have a significantly diminished apoptotic threshold compared to normal cells. While normal cells can generally tolerate the apoptotic insult of chemotherapy, the already burdened cancer cells cannot handle the additional load and undergo intrinsic apoptosis[65]. Similarly, noncancerous cells that demonstrate sensitivity to chemotherapy such as

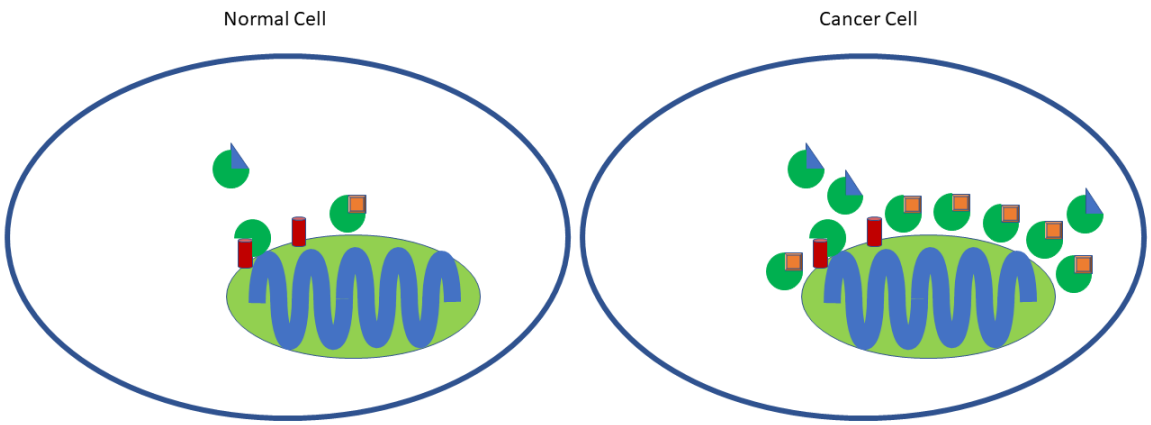


Figure I-5: Evasion of apoptosis primes cancer cells for death. Under homeostatic conditions, cells have a minimal apoptotic load. When a cell is subjected to aberrant proliferative cues, it typically responds by upregulating pro-apoptotic BH3-only proteins such as Bim. Cancer cells are able to tolerate this increase in pro-apoptotic proteins through upregulation of anti-apoptotic Bcl-2 family members that can sequester Bim. The cancer cell can survive but is rendered sensitive to compounds that can release Bim.

hematopoietic cells have also been shown to be highly primed, providing a potential explanation for why these cells are so impacted by cancer treatment[66].

In addition to facilitating oncogenic transformation, Bcl-2 family dynamics play a key role in mediating therapeutic resistance[67]. Over the course of cancer treatment, as sensitive cancer cells are eliminated, resistance can be achieved via selection for residual cancer cells deficient in various pro-apoptotic proteins (e.g. Bax/Bak, Bim, and Bid). Crucially, resistance can also develop through changes in priming[68]. A population of cancer cells may initially have the majority of their Bim bound to Bcl-x_L or Bcl-2 (Bcl-x_L/2 primed and dependent) and respond to drugs that target those specific interactions. However, successful treatment could result in an enrichment of cancer cells that are Mcl-1 dependent, and thus resistant to drugs that only target Bcl-2 or Bcl-x_L. These considerations have significantly informed the development of drugs designed to target several anti-apoptotic members of the Bcl-2 family.

D. Bcl-2 Family Antagonist Therapies

Since the discovery of the critical role of the Bcl-2 family in facilitating oncogenic transformation, significant research effort has been committed to the development of compounds that can tip the balance of Bcl-2 family dynamics in favor of apoptosis (Table 1). Early attempts to target Bcl-2 began with Oblimersen sodium, an antisense oligonucleotide designed to bind to Bcl-2 mRNA and prevent its translation via RNase H-mediated cleavage[65]. This approach failed to demonstrate appreciable clinical efficacy, and endeavors to target the Bcl-2 family largely shifted to targeting the binding interactions between pro- and anti-apoptotic Bcl-2 family members.

The advent of "BH3 mimetic" therapies was celebrated as a potential gamechanger in the field of cancer research. BH3 mimetics and other compounds broadly referred to as "Bcl-2 family antagonists" are designed to mimic the behavior of BH3-only sensitizer proteins, binding to the hydrophobic groove of primed anti-apoptotic proteins, releasing Bax/Bak or Bim, and triggering apoptosis in a cancer cell-specific manner[69]. However, the small molecules gossypol and obatoclax, early examples of Bcl-2 antagonists, did not demonstrate significant efficacy in pre-clinical studies and clinical trials, and even more importantly, were found to kill cancer cells independently of Bax and Bak, raising concerns of possible off-target effects and causing harm to normal tissues.

The major breakthrough in the development of a clinically effective BH3 mimetic arrived in the form of ABT-737[70]. Whereas gossypol and obatoclax were identified through functional drug screens, ABT-737 was synthesized through SAR by NMR, a structure-based approach that resulted in the fusion of two fragments bound to different

Table I-1: List of Bcl-2 antagonist therapies

Molecule	Mechanism of Action	Development Status
Oblimersen sodium	Anti-sense nucleotide	Clinically ineffective
Gossypol	Small molecule Bcl-2 family antagonist	Killed cells independent of Bax/Bak
Obatoclax	Small molecule Bcl-2 family antagonist	Killed cells independent of Bax/Bak
ABT-737	Small molecule, BH3 mimetic targeting Bcl-2, Bcl-x_L, and Bcl-w	Used largely for pre-clinical studies; triggered thrombocytopenia
ABT-263 (Navitoclax)	Orally available version of ABT-737	Dose-limiting thrombocytopenia
ABT-199 (Venetoclax)	Small molecule Bcl-2 antagonist	Clinically approved for treatment of CLL in patients with 17p deletion
S63845	Small molecule Mcl-1 antagonist	Promising pre-clinical results; nascent stages of clinical evaluation

areas within the hydrophobic groove of Bcl-x_L. The resulting compound bound with very high affinity to Bcl-2, Bcl-x_L and Bcl-w, and demonstrated efficacy in a variety of tumor types in pre-clinical *in vitro* and *in vivo* studies. However, subsequent development of ABT-737 and its clinical, orally bioavailable form ABT-263 (navitoclax) was impeded by a dose-limiting thrombocytopenia toxicity[71]. Circulating platelets were found to be strongly Bcl-x_L-dependent, and thus susceptible to treatment with these compounds. Consequently, researchers worked to create a drug that only targeted Bcl-2. The resulting orally available compound, venetoclax (ABT-199), proved even more effective at binding to Bcl-2 than navitoclax, demonstrating strong efficacy in Bcl-2 dependent cancers while sparing platelets[72]. Venetoclax received FDA approval for the treatment of chronic lymphocytic leukemia and continues to be clinically evaluated as a single agent and in combination with other treatments for a variety of malignancies.

Despite the successful development of Bcl-2 and Bcl-x_L antagonists, efforts to develop effective compounds targeting Mcl-1 have progressed at a much slower pace. Earlier iterations of these drugs have failed due to lack of efficacy and unanticipated toxicities[73]. Recently, several research groups have brought forth novel Mcl-1 inhibitors that have demonstrated promising results *in vitro*[74, 75]. While it remains to be seen whether any of these compounds proves effective in the clinical setting, the volume of interest in the development of compounds targeting Mcl-1 reflects the clinical importance of this protein in cancer development and as a mediator of therapeutic resistance.

E. Statement of Problem

Given the critical role that the Bcl-2 family plays in controlling oncogenesis and mediating therapeutic resistance, it is important that we continue to gain mechanistic insights into the factors governing Bcl-2 protein interactions. For example, despite the progress made in the development of Bcl-2 antagonists and other compounds targeting Bcl-2 proteins, we still lack a complete understanding of why these drugs work in some cell types and patients, but not others. To that end, we previously reported that although myeloma cells are strongly dependent on Mcl-1 for survival, certain subsets were sensitive to treatment with Bcl-2/x_L targeting ABT-737[76]. We found that these sensitive myelomas had a significant amount of Bim bound to Bcl-x_L and Bcl-2, indicating that sensitivity of cancer cells to compounds like ABT-737 was dictated by where Bim was bound, irrespective of the expression levels of any individual Bcl-2 protein. Subsequent work from our lab showed that dexamethasone induced transcriptional upregulation of Bim that preferentially bound to Bcl-2, independent of anti-apoptotic protein expression levels[77]. Several questions arose from these studies, most notably, what are the factors that determine where Bim binds in a cancer cell?

We speculated that the binding pattern of Bim could be dictated by either mutation of the BH3 domain of Bim or posttranslational modification. While we found no appreciable differences between the BH3 domains of myeloma cells of different dependencies (unpublished data), we identified a potential role for phosphorylation in the regulation of Bim binding. By studying the interaction between multiple myeloma cells and the bone marrow microenvironment, we discovered that when myeloma cells are stimulated with the pro-survival cytokine IL-6, the binding pattern shifts from Bcl-2/Bcl-

x_L to Mcl-1 dependence, rendering previously ABT-737/199-sensitive cells, largely insensitive[52]. Additionally, IL-6 stimulation resulted in Bim phosphorylation at serine 69 and 77 with no impact on protein stability. Together these data suggest a role for phosphorylation in determining the anti-apoptotic protein Bim is bound to.

As described in the preceding sections, Bim phosphorylation has been extensively studied in a multitude of cellular systems. However, there are several outstanding questions, particularly regarding the *constitutive* phosphorylation state of Bim in the absence of any exogenous cellular stimulation. Also, while the impact of phosphorylation on Bim stability has been well described, the alternative functions of phosphorylation and the context-dependent differences remain poorly understood. These observations, coupled with our previous findings, led us to hypothesize that constitutive Bim phosphorylation events dictate which anti-apoptotic proteins Bim associates with in a cell.

In the following chapter, efforts to identify and characterize the constitutive phosphorylation state of Bim in plasma cell dyscrasias of different Bcl-2 family dependencies will be described. We show that Bim is constitutively phosphorylated to varying degrees in both multiple myeloma and Waldenström's macroglobulinemia (WM) cells. Efforts to identify specific Bim phosphorylation events in myeloma cells were largely unsuccessful, however, as an alternate approach, we used a previously characterized Bax and Bak-deficient WM cell line, RPCI-WM1, to generate stable cell lines expressing wild type (WT) and several phospho-mimetic and unphosphorylateable Bim mutations. We determined that ERK signaling was partially responsible for the phosphorylation occurring in RPCI-WM1, and we also took advantage of the apoptotic

deficiency of these cells to further study the interaction between Bim and the anti-apoptotic proteins.

When we overexpressed wild type Bim in the RPCI-WM1 cell line, we noticed a robust increase in the level of Mcl-1, suggesting that Bim was stabilizing Mcl-1 within these cells. The labile nature of Mcl-1 compared to other anti-apoptotic proteins is well chronicled, and multiple groups have demonstrated that Bim stabilizes Mcl-1 in a BH3 domain-dependent manner[19, 78, 79]. What remains unclear is the potential interplay between Bim-mediated stability of Mcl-1 and the Bcl-2 family dependence and priming of a cell, and the impact of Bim phosphorylation on its ability to bind to and stabilize Mcl-1. Chapter three details our findings concerning the relationship between Bim phosphorylation and Mcl-1 stabilization. We determined that WT Bim overwhelmingly primes Mcl-1 in the RPCI-WM1 cell line, and introduction of Bim phosphorylation site mutations results in altered Mcl-1 stability and priming.

While we were unable to comprehensively identify constitutive Bim phosphorylation sites and the signaling cascades regulating them, we made significant strides furthering understanding of the less characterized roles for Bim phosphorylation in cancer. This research has the potential to improve Bcl-2 antagonist and other cancer therapies by providing a mechanism for influencing Bim priming in cancer cells.

II. CHARACTERIZING THE DIFFERENTIAL PHOSPHORYLATION OF PLASMA CELL DYSCRASIAS

(*Portions of this chapter originally published in Blood*[52])

Introduction

We and others have demonstrated that Bim is phosphorylated in response to a variety of cytokines, including IL-6[52]. What remains unclear is the constitutive, baseline phosphorylation state of Bim. Given the limited availability of phospho-specific Bim antibodies, and the potential association between phosphorylation and Bcl-2 family priming, we sought to determine the global phosphorylation state of Bim in cell lines of different Bcl-2 dependence.

Materials and Methods

Cell culture

The myeloma cell line U266 and the HEK293T cell line were purchased from American Type Culture Collection (ATCC). MM.1s was provided by Steven Rosen (City of Hope), KMS18 by Leif Bergsagel (Mayo Clinic), OCI-My5, Karpas 620, LP1 and KMS26 by Jonathan Keats (TGen), OPM2 by Nizar Bahlis (University of Calgary) and KMS12-PE was purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB). All myeloma cell lines were cultured as previously described[52, 77]. The RPCI-WM1 cell line was obtained from Asher Chanan-Khan and cultured as previously described[80]. Myeloma cells were isolated from patient bone marrow aspirates as previously described[52]. Briefly, plasma cells were purified from the buffy coat using the

MACS Cell Separation MS Columns and CD138 magnetic microbeads per the manufacturer's protocol (Miltenyi Biotec).

Reagents

Cells were treated with recombinant human IL-6 (R&D Systems) at a concentration of 1 ng/μl, for 30 min at 37°C. Cells were treated with 10 μM U0126 (Cell Signaling), 10 μM SB203580 (Cell Signaling), 150 nM TG02 (Tragara Pharmaceuticals), and 1 μM ibrutinib (provided by Leon Bernal-Mizrachi).

Transient and stable overexpression of Bim

Stable cell lines overexpressing wild type Bim were generated as follows: ΦNX-Amphotropic packaging cell lines (Nolan lab, Stanford University) were transfected with plasmid (N-terminal His-tagged Bim) using Lipofectamine 2000. Cells were subjected to three rounds of infection with 0.45-μm syringe filtered (Pall) viral supernatants and Polybrene Infection/Transfection Reagent (Millipore). Once cells recovered from infection they were selected with 1-2 μg/ml puromycin (Sigma). HEK293T cells were transfected with pLVX-IRES-Neo (Clontech) constructs (empty and wild type Bim) using Lipofectamine 2000. Phosphorylation mutant versions of Bim were generated using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent).

SDS-PAGE

SDS-PAGE and western blotting were performed as previously described[81]. Primary antibodies used included: rabbit polyclonal α-Bim (Millipore), rabbit polyclonal

α-Mcl-1 (Enzo), hamster monoclonal α-Bcl-2 (BD Biosciences), rabbit polyclonal α-Bcl-x_L (Cell Signaling), and mouse monoclonal β-actin (Sigma). The following secondary antibodies were used: goat α-rabbit IgG-HRP (Santa Cruz Biotechnology), mouse α-Armenian and Syrian hamster IgG-HRP (BD Biosciences), and sheep α-mouse IgG-HRP (GE Healthcare).

PhosTag™ Gel Electrophoresis

A 10% Acrylamide/Bis-acrylamide gel was cast with a final concentration of 50 μM of PhosTag (Wako), 100 μM $MnCl_2$. Electrophoresis and immunoblotting were performed as described above with the following modification: Gels were transferred to 0.2 μm PVDF membranes (Bio-Rad).

To allow for discrimination between phosphorylated and unphosphorylated forms of proteins, lysates were treated with lambda protein phosphatase (λPP) (New England Biolabs) per the manufacturer's instructions.

Results

We previously demonstrated Bim was MEK/ERK phosphorylated at residues serine 69 and 77 in response to IL-6 stimulation of myeloma cells[52]. In order to further capture the multiple phosphorylation events triggered by IL-6, we ran lysates from untreated, IL-6 stimulated, and U0126 treated MM.1s myeloma cells on a PhosTag™ gel (Figure 1). As expected, we observed multiple lambda protein phosphatase-sensitive Bim phosphorylation bands in response to IL-6, however, we also observed phosphorylation in untreated MM.1s cells, suggesting Bim is constitutively phosphorylated. This was in

contrast to the anti-apoptotic proteins which were largely unphosphorylated. We then ran whole cell lysates from several myeloma cell lines on PhosTag™ gels and confirmed that Bim is constitutively phosphorylated in each cell line, with anywhere from one to four phosphorylated forms (Figure 2A). This phenomenon was not limited to cell lines, as we observed multiple phosphorylated Bim bands when we analyzed lysates from multiple myeloma patient bone marrow aspirates using PhosTag™ gel electrophoresis (Figure 2B).

After establishing that Bim is constitutively phosphorylated in myeloma cells, we next sought to identify specific Bim phosphorylation events using phospho-proteomic approaches. Initially, this involved attempts to immunoprecipitate endogenous Bim, however the relatively low abundance of Bim protein in myeloma cells precluded the success of this approach. We subsequently attempted to overexpress Bim in a variety of myeloma cell lines, including KMS26 and LP1, two cell lines with allelic deletions of Bim. We found that viral transduction and stable selection of cells receiving the Bim constructs resulted in exonic splicing to a form of Bim that was better tolerated by the cell (Figure 3A). Even after introducing mutations preventing splicing of Bim EL to this alternate form, we were unable to express Bim to a level that would allow for mass spectrometric analysis (Figure 3B). As an alternate approach, we utilized a previously characterized Waldenström's macroglobulinemia cell line, RPCI-WM1, that is deficient in Bax and Bak. We were able to successfully overexpress His-tagged Bim, and we confirmed that Bim is phosphorylated within these cells (see chapter III). Efforts to optimize affinity chromatography protocols for phospho-proteomic analysis of Bim from these cells are ongoing.

As a parallel approach, we utilized small molecule inhibitors to elucidate the signaling cascades regulating Bim phosphorylation. We treated RPCI-WM1 cells stably overexpressing WT Bim with these inhibitors and ran the lysates on a PhosTag™ gel (Figure 4A). Using this technique, we determined that a detectable proportion of the observed constitutive Bim phosphorylation events is MEK/ERK dependent. Additionally, we utilized kinase prediction software to further narrow down the list of likely phosphorylation sites (Figure 4B)[82]. We then introduced combinations of alanine mutations at the sites with the greatest likelihood of being phosphorylated, according to the prediction software. We generated stable cell lines overexpressing S59A/T116A and S59A/T116A/S118A mutated Bim and ran lysates from these cells on PhosTag™ and standard SDS-PAGE gels (Figure 5). The S118A mutation resulted in the elimination of a large proportion of the constitutive Bim phosphorylation. In order to obtain a completely unphosphorylated form of Bim, we generated 'quadruple' alanine mutant Bim containing S59A/T116A/S118A plus an additional alanine mutation at either serine 87, 94, or 104. Strikingly, while the S94A and S104A quadruple mutants did not result in further reduction of constitutive Bim phosphorylation on PhosTag™ gels (Figure 6B), the inclusion of the S87A mutation resulted in a form of Bim that was poorly tolerated by the cells (Figure 6A). The resulting S87A stable cell line expressed very little of the Bim EL form and expressed a smaller, spliced isoform of Bim. Together our data provide evidence for the constitutive phosphorylation of Bim and provide an initial framework for identifying these phosphorylation sites in plasma cell malignancies.

Discussion

In the present study, we demonstrated that Bim is constitutively phosphorylated in myeloma cells. This could potentially be due to oncogenic activation of signaling cascades within these cells. As an example, the U266 cell line is known to have an activating mutation in B-Raf, which is likely responsible for the robust observed Bim phosphorylation pattern[83]. Interestingly, U266 is the only cell line we've identified with constitutive serine 69 phosphorylation, providing additional evidence mutant B-Raf is driving ERK-mediated phosphorylation of Bim in these cells. Importantly, none of these phosphorylation events was directly associated with decreasing Bim stability, further supporting additional roles for phosphorylation.

Uncovering the dysregulated signaling pathways in myeloma cell lines may provide additional insights into which kinases are regulating Bim, and the sites of constitutive phosphorylation. Using small molecule inhibitors, we showed that a slight fraction of constitutive Bim phosphorylation was MEK/ERK dependent in myeloma and Waldenström's cells (Figures 1 and 2). This stands in contrast to the IL-6 stimulated Bim phosphorylation events that were completely ablated by U0126 treatment (Figure 1). Our PhosTag™ gels indicate that the kinase(s) responsible for the majority of the constitutive Bim phosphorylation remain unidentified. While kinase prediction tools and small molecule inhibitors proved useful in identifying the contribution of ERK signaling to Bim phosphorylation, a more comprehensive approach is necessary to fully elucidate the relevant signaling cascades.

We were able to glean some insights into potential sites of constitutive Bim phosphorylation through use of site-directed mutagenesis and PhosTag™. Our triple alanine (S59A/T116A/S118A) mutant provided strong evidence that serine 118 is

constitutively phosphorylated in RPCI-WM1 (Figure 5), since this mutation resulted in significantly decreased Bim phosphorylation. Also, given that overexpression of the quadruple alanine (S59A/S87A/T116A/S118A) version of Bim was poorly tolerated by cells (Figure 6), our data suggest serine 87 phosphorylation is critical within RPCI-WM1. This observation may be influenced by the inability of Bim to interact with other binding partners. By changing threonine 116 to an alanine, Bim cannot associate with dynein light chain[25]. Mutation of serine 87 could result in an impaired ability to bind 14-3-3[47]. These restrictions, coupled with other phosphorylation site mutations that prevent the Bim:Mcl-1 interaction could prove lethal to a cell. Ultimately, our findings must be validated through phospho-proteomic analysis.

Our broader efforts to identify and characterize constitutive Bim phosphorylation sites remain incomplete. We initially faced challenges overexpressing such a potent activator of apoptosis, with myeloma cells utilizing exon splicing and limiting the level of Bim expression to survive (Figure 3). Even in the Bax/Bak-deficient RPCI-WM1 cell line Bim overexpression resulted in relatively modest protein abundance.

An additional obstacle was the determination of the appropriate protease for peptide fragmentation for mass spectrometry. While standard protocols utilized trypsin, we did not achieve sufficient peptide coverage until chymotrypsin was used. While our present efforts are focused on the use of immobilized metal affinity chromatography to isolate His-tagged Bim, a promising alternative approach would involve transient expression of Bim in 293T cells, followed by immunoprecipitation. 293T cells have proven amenable to substantial Bim overexpression and may provide a system for identifying constitutive phosphorylation sites that are relevant in other cellular contexts

(Figure 7). After identifying putative Bim phosphorylation sites, follow-up efforts would focus on determining the functional consequences of each phosphorylation through mutagenesis studies.

The data discussed in this chapter and the proposed studies seek to increase our understanding of the functional consequences of Bim phosphorylation. This knowledge could one day be used to augment the activity of Bim in cancer cells, increasing the efficacy of cancer treatments.

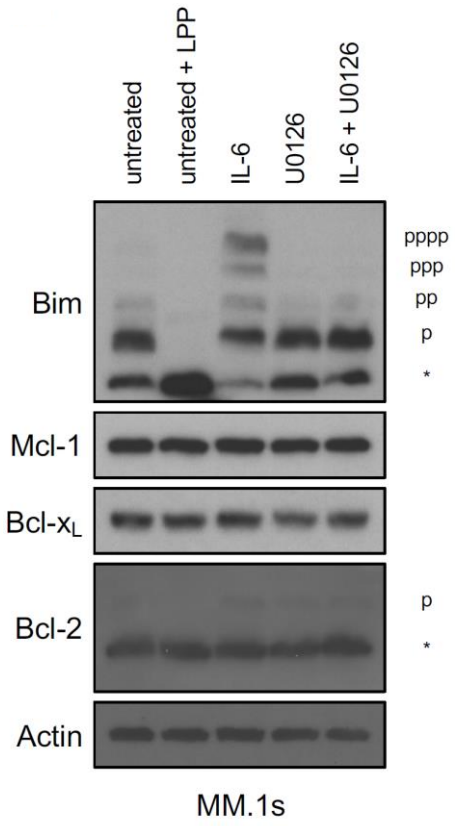


Figure II-1: Bim is constitutively phosphorylated in the absence of IL-6 stimulation. Thirty μg of whole cell lysate was collected from MM.1s myeloma cells that were untreated, lambda protein phosphatase (λPP) treated, IL-6 stimulated, and MEK inhibitor U0126 treated. These lysates were subjected to PhosTag™ gel electrophoresis, and immunoblotting. (*) represents the unphosphorylated form of the indicated protein, and (p)–(pppp) represent single through multi-phosphorylated forms.

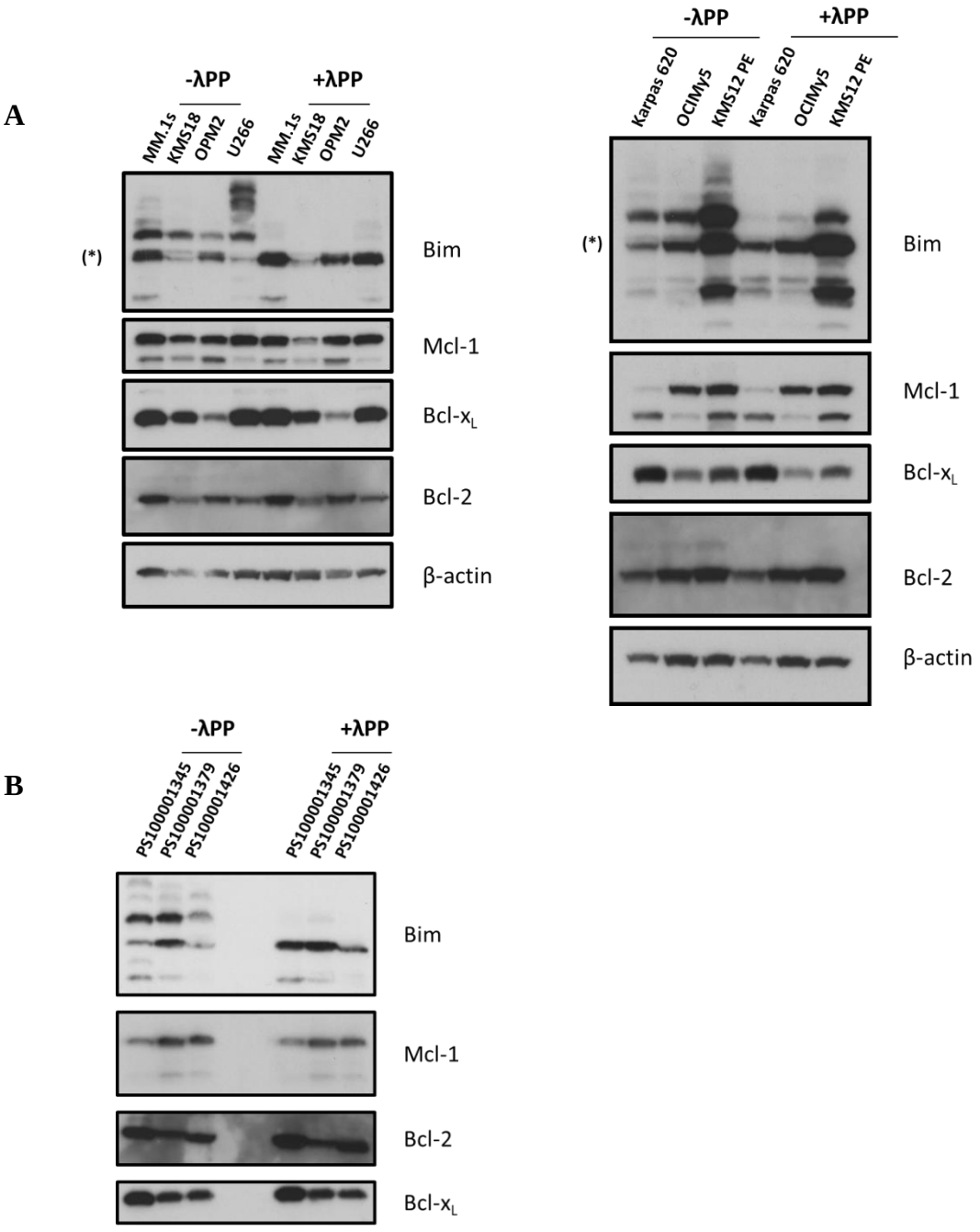


Figure II-2: Bim is differentially phosphorylated across myeloma cells. (A)Thirty μg of whole cell lysate was collected from the indicated myeloma cell lines and subjected to PhosTag™ gel electrophoresis, and immunoblotting. (B) Lysates were generated from CD138-purified myeloma cells from patient bone marrow aspirates. Thirty μg was subjected to PhosTag™ electrophoresis. Thirty μg of each lysate used was treated with lambda protein phosphatase (λPP) to allow identification of the unphosphorylated form of Bim (*).

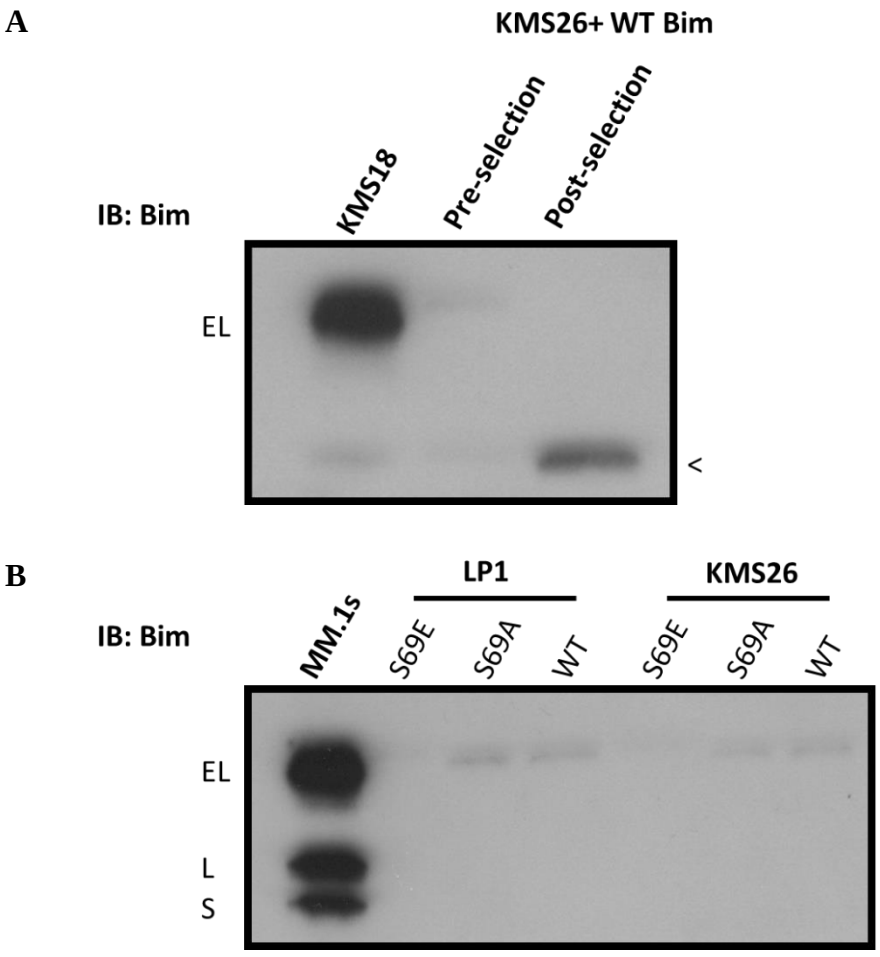
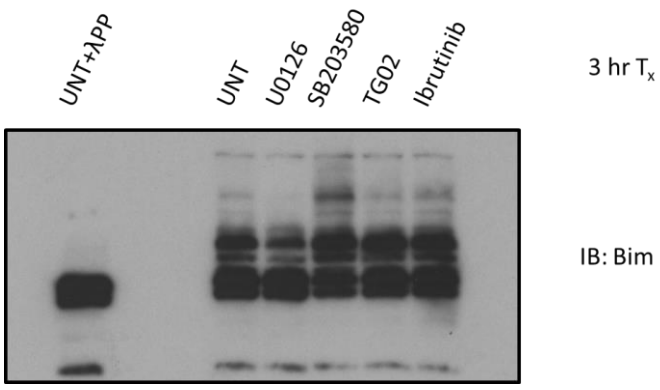


Figure II-3: Intrinsic apoptosis-capable myeloma cells poorly tolerate Bim overexpression. (A) KMS26 cells were retrovirally infected with His-tagged Bim. After infection, cells were collected before and after selection with puromycin, and lysed. Thirty μg of lysate was subjected to SDS-PAGE and immunoblotting. (<) indicates the isoform of Bim that was selected for after puromycin treatment. Whole cell lysate from the cell line KMS18 was run as a positive control for Bim EL. (B) LP1 and KMS26 myeloma cell lines were retrovirally infected with the indicated versions of Bim EL that could not be spliced and subjected to puromycin selection. Lysates from the resulting stable cell lines were subjected to SDS-PAGE. Here, MM.1s serves as a reference point for levels of endogenous Bim.

A



B

Medium Stringency

69	S	CMGC/MAPK/ERK/Erk1	GPLAPPASPGPFATR	24.743	10.326
77	S	CMGC/MAPK/ERK/Erk1	PGPFATRSPLFIFMR	14.812	10.326
104	S	CMGC/MAPK/ERK/Erk1	FSFDTDRSPAPMSCD	12.389	10.326
116	T	CMGC/MAPK/ERK/Erk1	SCDKSTQTPSPPCQA	12.806	10.326
118	S	CMGC/MAPK/ERK/Erk1	DKSTQTPSPPCQAFN	15.146	10.326
59	S	CMGC/MAPK/ERK/Erk2	GDSCPHGSPQGPLAP	13.823	4.61
69	S	CMGC/MAPK/ERK/Erk2	GPLAPPASPGPFATR	25.181	4.61
77	S	CMGC/MAPK/ERK/Erk2	PGPFATRSPLFIFMR	9.398	4.61
104	S	CMGC/MAPK/ERK/Erk2	FSFDTDRSPAPMSCD	10.044	4.61
116	T	CMGC/MAPK/ERK/Erk2	SCDKSTQTPSPPCQA	12.854	4.61
118	S	CMGC/MAPK/ERK/Erk2	DKSTQTPSPPCQAFN	13.168	4.61

High Stringency

69	S	CMGC/MAPK/ERK/Erk1	GPLAPPASPGPFATR	24.743	17.725
59	S	CMGC/MAPK/ERK/Erk2	GDSCPHGSPQGPLAP	13.823	10.451
69	S	CMGC/MAPK/ERK/Erk2	GPLAPPASPGPFATR	25.181	10.451
116	T	CMGC/MAPK/ERK/Erk2	SCDKSTQTPSPPCQA	12.854	10.451
118	S	CMGC/MAPK/ERK/Erk2	DKSTQTPSPPCQAFN	13.168	10.451

Figure II-4: MEK/ERK signaling is partially responsible for constitutive Bim phosphorylation. (A) RPCI-WM1 cells stably overexpressing WT Bim were treated with the indicated inhibitors for three hours. Cells were then collected, lysed, and 30 μg of whole cell lysate was subjected to PhosTag™ electrophoresis. (B) The Bim amino acid sequence was input into the GPS 3.0 kinase prediction software[82]. Data shown represent the likelihood that the indicated kinases (column three) phosphorylate Bim at the listed residues (columns one and two). The amino acids displayed represent sites with scores (column five) greater than the cutoff (column six).

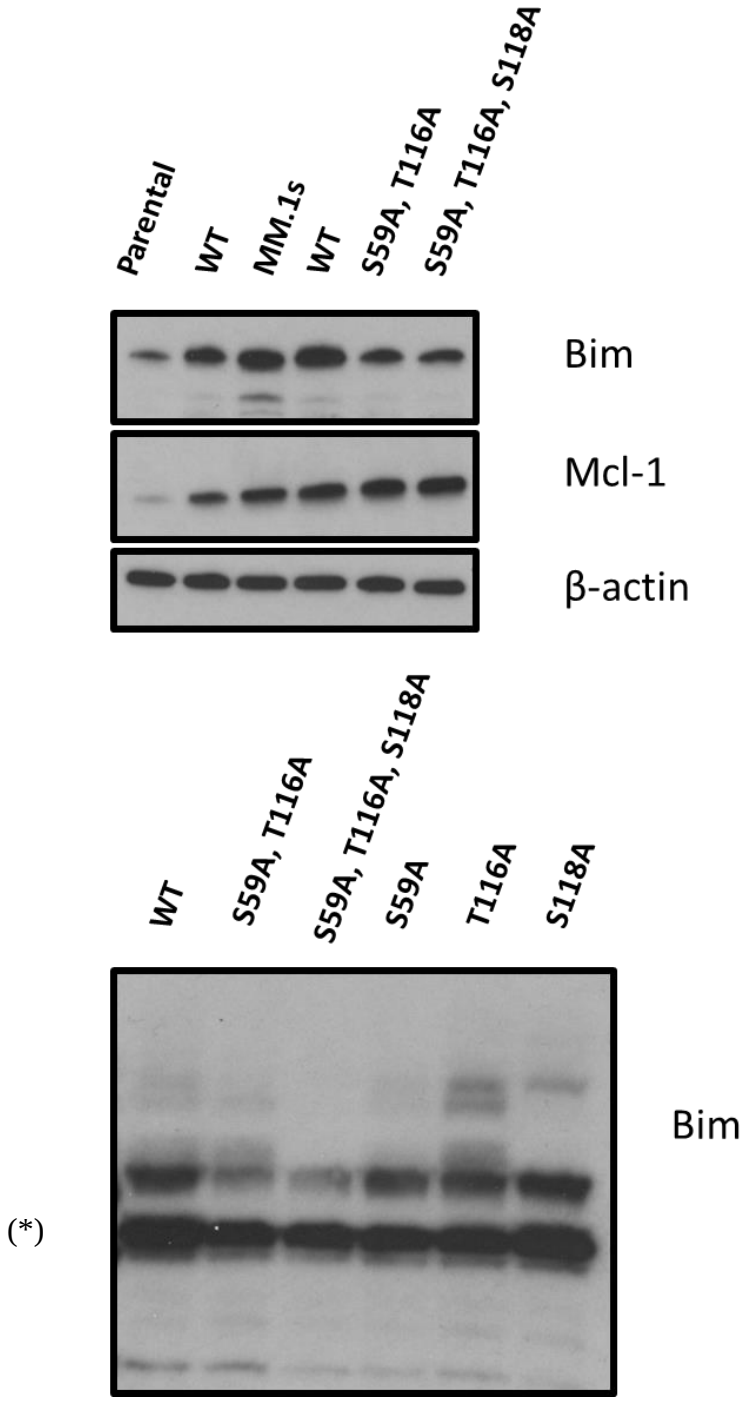


Figure II-5: Introduction of alanine mutations results in partial ablation of constitutive Bim phosphorylation. Thirty μg of lysate from the indicated RPCI-WM1 stable cell lines was subjected to standard (top) and PhosTag™ gel electrophoresis (bottom), and immunoblotting. Site-directed mutagenesis was used to introduce alanine mutations in Bim at the indicated phosphorylation sites. In the upper blot, the myeloma cell line MM.1s serves as a comparison for endogenous Bim expression. (*) indicates where unphosphorylated Bim migrates

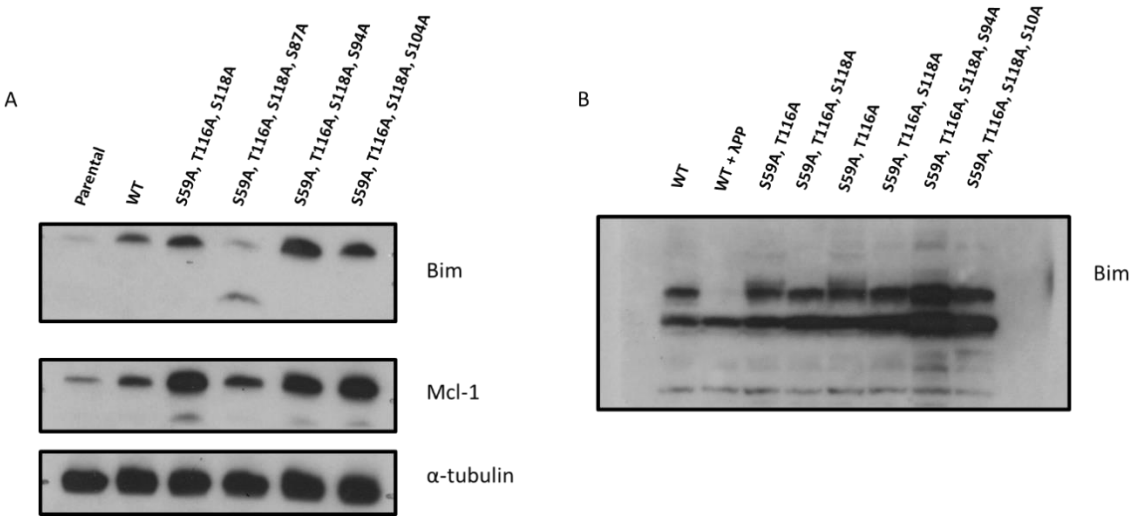


Figure II-6: Quadruple alanine mutants have varying effects in the RPCI-WM1 cell line. (A) Thirty μg of lysate from the indicated cell lines was subjected to SDS-PAGE and immunoblotting. Site-directed mutagenesis was utilized to introduce four alanine mutations at potential Bim phosphorylation sites. (B) 30 μg of lysate was also utilized for PhosTag™ gel electrophoresis.

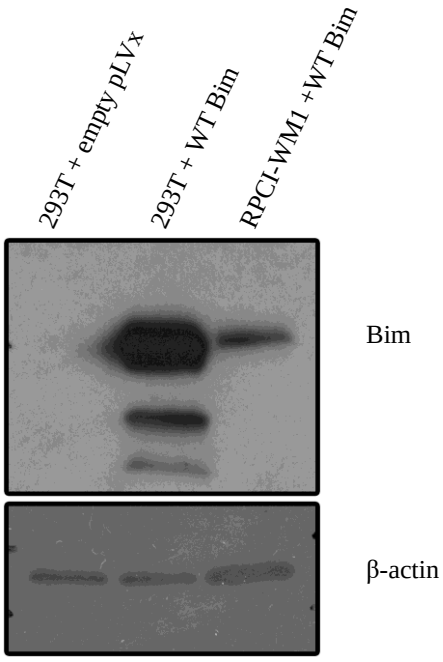


Figure II-7: Transient Bim expression in 293T cells may provide a pathway for phospho-proteomic analysis. 293T cells were transfected with empty vector and wild type Bim. After 48 hours, cells were collected and lysed, and 30 μg of the resulting lysates was subjected to SDS-PAGE alongside 30 μg of lysate from a stable cell line overexpressing WT Bim (RPCI-WM1).

III. DETERMINING THE IMPACT OF BIM PHOSPHORYLATION ON BINDING TO MCL-1

(*Originally submitted and revised for the FEBS Journal*)

Phosphorylation alters Bim-mediated Mcl-1 stabilization and priming

Jason E. Conage-Pough[1,2] and Lawrence H. Boise[2]

[1]Cancer Biology Graduate Program and [2]Department of Hematology and Medical Oncology, Winship Cancer Institute, Emory University, Atlanta, GA

Correspondence: Lawrence H. Boise, Winship Cancer Institute, Emory University, 1365C Clifton Road NE, Suite C4012, Atlanta, GA 30322, USA. Ph: (+1)404-778-4724. Fax: (+1)404-778-5530 E-mail: lboise@emory.edu

Running Title: Bim phosphorylation alters Mcl-1 stability & priming

Keywords: Mcl-1 stability, Bim phosphorylation, priming

Conflicts of Interest: The authors declare no conflict of interest.

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 unphosphosphorylateable 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.

Introduction

The Bcl-2 family of proteins plays a crucial role regulating intrinsic apoptosis [12]. 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 [84-88]. 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 [89]. This process is inhibited by anti-apoptotic members of the Bcl-2 family (e.g. Bcl-2, Mcl-1, Bcl-x_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 [90].

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 [91, 92]. Notably, they promote survival of B cells at different stages of differentiation, with Bcl-2/x_L protecting more B-cell like subsets, and plasma cells being strongly dependent on Mcl-1 for survival [93-97]. Additionally, the anti-apoptotic proteins have demonstrated contrasting roles in protection of cells from a myriad of cytotoxic agents including cisplatin and taxols [98-101]. Lastly, despite Bcl-2, Bcl-x_L, 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 [102]. 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 [103].

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 [79, 104-108]. 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 [19, 109]. 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 [78].

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 [25, 43-45, 51, 110]. 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 [52], 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 [80]. 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-x_L, 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-x_L 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) [80], 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 [111], 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 [78]. 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) [25, 47, 50, 52, 82, 112, 113]. 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-x_L 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 [52]. 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-x_L. 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-x_L (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/x_L.

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 [75] 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 [114, 115]. This regulation can include posttranscriptional control, posttranslational modification, and interactions with other Bcl-2 family proteins [102, 116]. 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 [78]. 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-x_L (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 [31, 117, 118]. 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 [76, 119, 120]. Furthermore, while we previously showed that increasing the expression levels of anti-

apoptotic proteins does not alter the pattern of Bim priming [76], 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 [52, 121]. 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-x_L 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 [42].

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-x_L (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 [50, 51]. 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 [23, 25, 122]. 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 [25].

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 [123]. 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 [124]. 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 [125]. 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 [70, 71, 74, 126]. This approach has resulted in the development of several promising compounds, including venetoclax, a Bcl-2 inhibitor approved for the treatment of CLL [72, 127]. 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 [128, 129]. Recently, multiple Mcl-1 inhibitors have demonstrated promise in the *in vitro* setting [74, 75, 126, 130]. 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/x_L for survival (Figure 1C) [52, 76]. Mcl-1 is overexpressed in many cancers including myeloma, frequently due to chromosome 1q amplification [131]. 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 [80, 132]. 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 [81].

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'[78]) using Lipofectamine 2000 (Thermo Fisher, Waltham, MA, USA), according to the manufacturer's instructions. Stable cell lines were generated as previously described [133]. 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 [81]. Primary antibodies used included: rabbit polyclonal α-Bim (Millipore), rabbit polyclonal α-Mcl-1 (Enzo, Farmingdale, NY, USA), hamster monoclonal α-Bcl-2 (BD Biosciences, San Jose, CA, USA), rabbit polyclonal α-Bcl-x_L (Cell Signaling, Danvers, MA, USA), rabbit polyclonal α-Lasu1 (Bethyl Laboratories, Montgomery, TX, USA), and mouse monoclonal β-actin (Sigma). The following secondary antibodies were used: goat α-rabbit IgG-HRP (Santa Cruz Biotechnology, Dallas, TX, USA), mouse α-Armenian and Syrian hamster IgG-HRP (BD Biosciences), and sheep α-mouse IgG-HRP (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

PhosTag™ Gel Electrophoresis

PhosTag™ Gel Electrophoresis was performed as previously described [111, 134]. Briefly, a 10% Acrylamide/Bis-acrylamide gel was cast with a final concentration of 50 μM of PhosTag (Wako, Osaka, Japan), 100 μM $MnCl_2$. 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 [111].

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-x_L [135]. 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 [52, 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) [81]. The following probes were used: Bim (BCL2L11) Hs00708019_s1, Mcl-1 Hs01050896_m1, Bcl-2 Hs00608023_m1, Bcl-x_L (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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Author Contributions

JEC-P and LHB conceived the study, designed the experiments, and wrote and revised the manuscript. JEC-P performed the experiments and LHB supervised the study.

Phosphorylation Site	Phospho-mimetic (Glutamate)	Unphosphorylateable (Alanine)
T116	↑ Mcl-1 Stabilization	↑ Mcl-1 Stabilization
S59	↓ Mcl-1 Stabilization	--
S87	--	↓ Mcl-1 Stabilization
S94	↓ Mcl-1 Stabilization	↓ Mcl-1 Stabilization
S104	--	↓ Mcl-1 Stabilization

Table 1. Summary of Bim phosphorylation site mutations and their impact on Mcl-1 stability in RPCI-WM1 cells

Figure Legends

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-x_L, and Bcl-2 in the 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^{TM} 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 unphosphorylateable (S/T→A) forms of Bim indicated. WCLs were subject to PhosTag^{TM} 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); unphosphorylateable, (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 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.

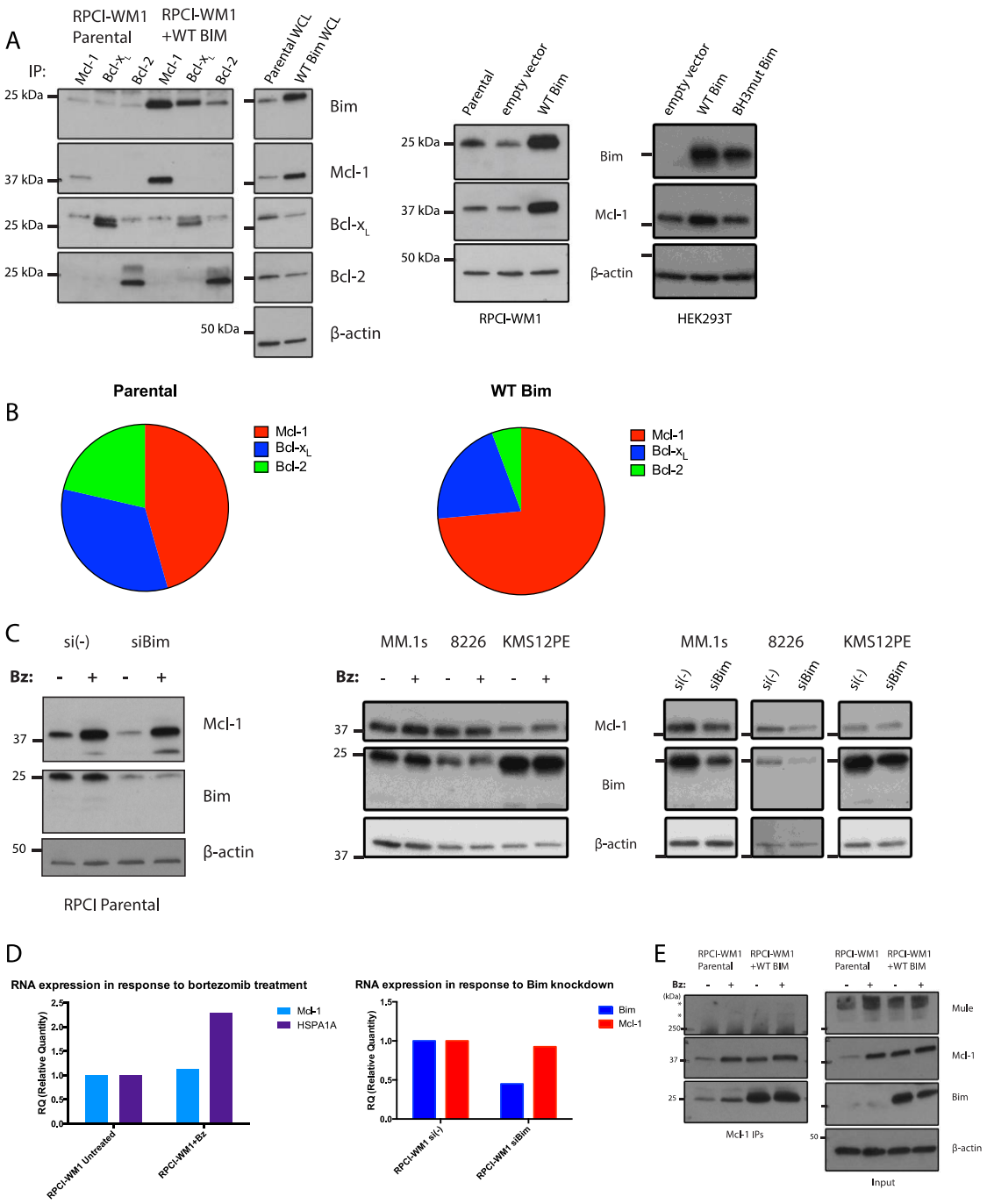
Figure 4. Bim phosphorylation site mutations impact the stability of Mcl-1 in HEK293T cells. (A) Thirty μg of WCL from HEK293T cells transfected with the

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-x_L. Immunoblots are representative of at least three independent experiments. (Right) Pie charts showing the proportion of Bim bound to Mcl-1, Bcl-x_L, 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.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RPCI-WM1 Parental
RPCI-WM1 +WT BIM
IP:
Mcl-1
Bcl-xL
Bcl-2
Mcl-1
Bcl-xL
Bcl-2
Parental WCL
WT Bim WCL
25 kDa
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25 kDa
25 kDa
50 kDa
Bim
Mcl-1
Bcl-xL
Bcl-2
β-actin
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Bim
Mcl-1
β-actin
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Mcl-1
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Mcl-1
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β-actin
D
RNA expression in response to bortezomib treatment
RQ (Relative Quantity)
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2.0
1.5
1.0
0.5
0.0
Mcl-1
HSPA1A
RPCI-WM1 Untreated
RPCI-WM1-Bz
RNA expression in response to Bim knockdown
RQ (Relative Quantity)
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1.0
0.5
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Mcl-1
RPCI-WM1 si(-)
RPCI-WM1 siBim
E
RPCI-WM1 Parental
RPCI-WM1 +WT BIM
Bz: - + - +
(kDa)
250
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25
Mcl-1 IPs
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Mule
Mcl-1
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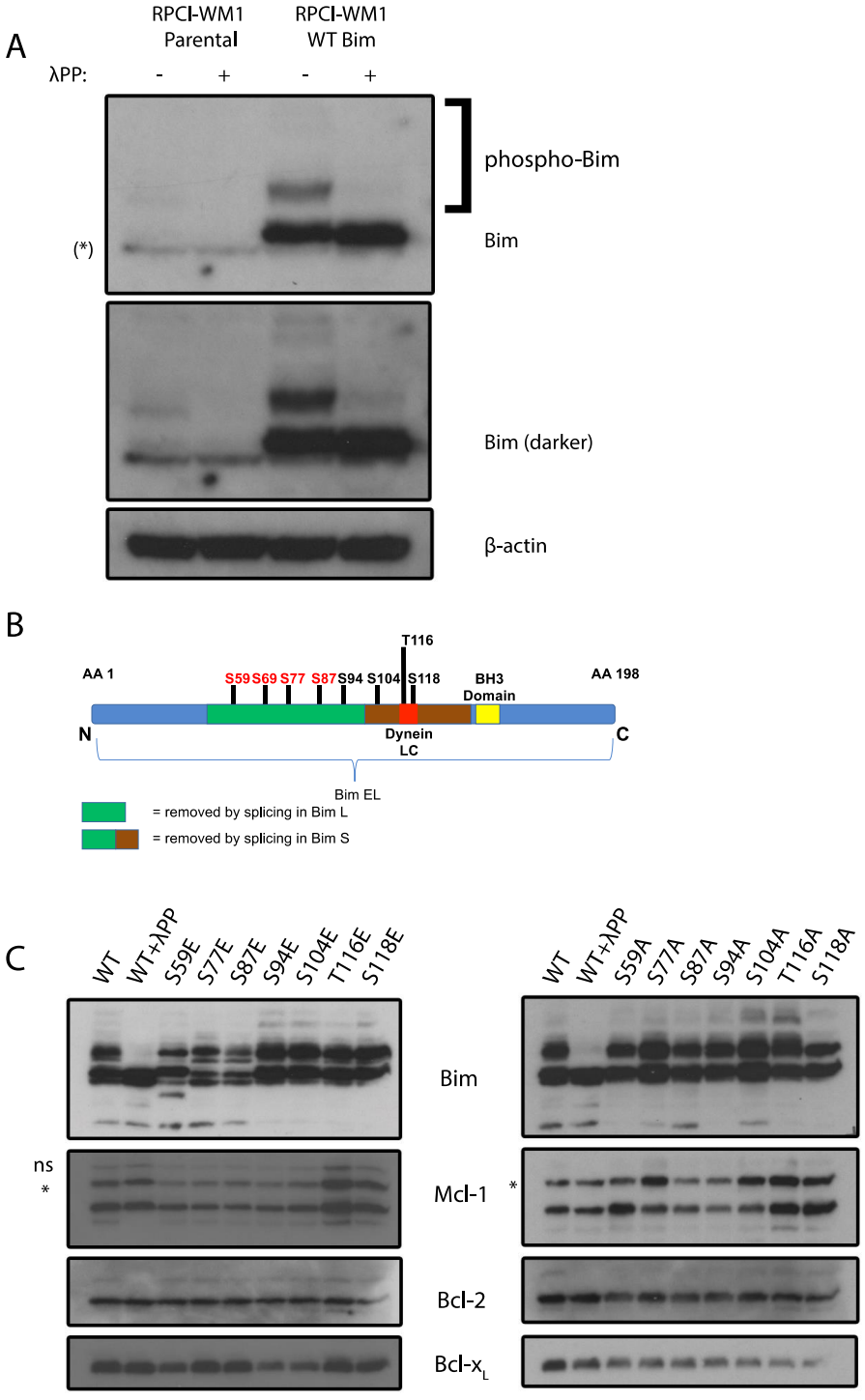
Input

Figure 1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Bim (darker)
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Dynein LC
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C
WT
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S87E
S94E
S104E
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Mcl-1
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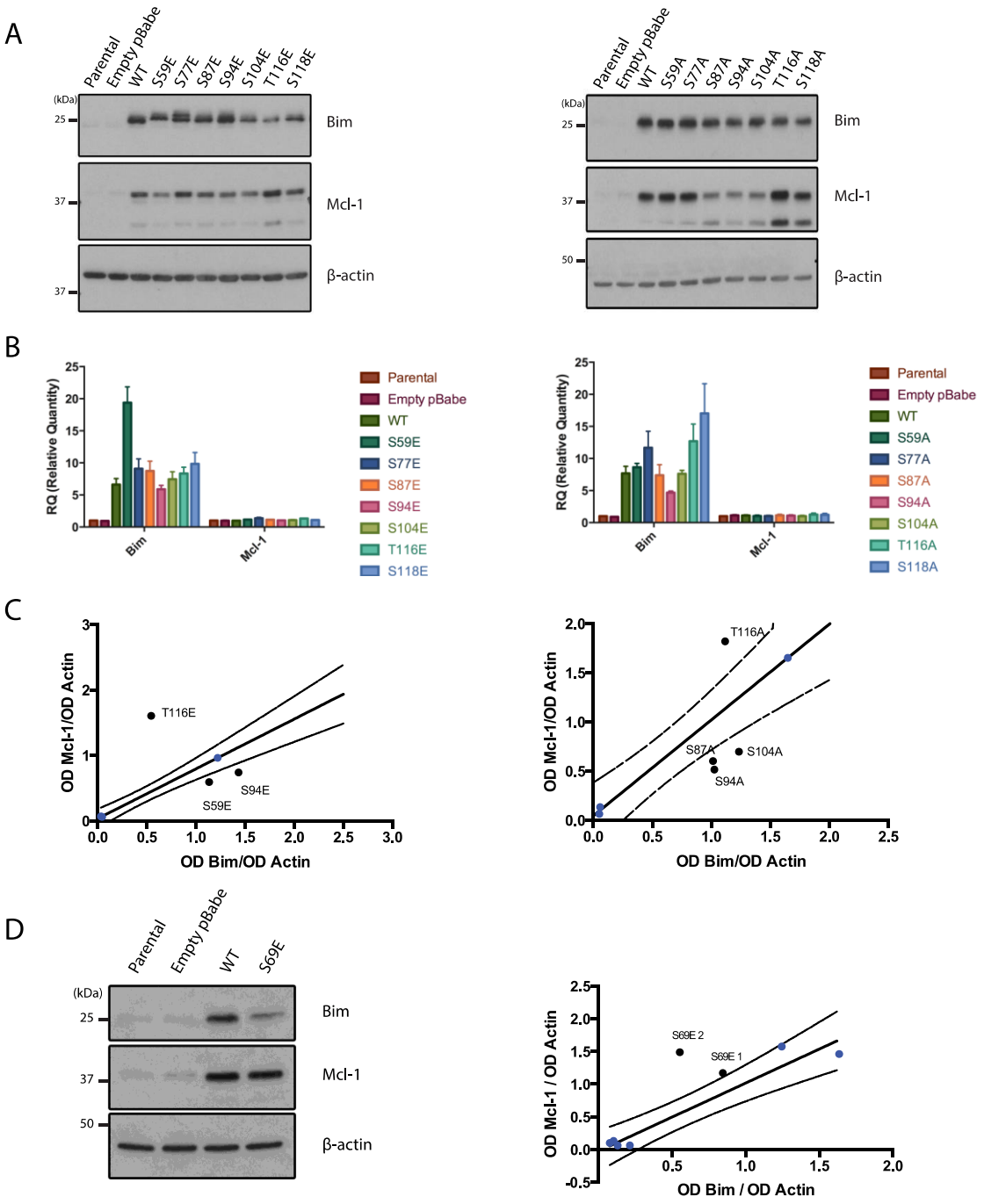
Bcl-2
Bcl-x$_L$

Figure 2


A
Parental
Empty pBabe
WT
S59E
S77E
S87E
S94E
S104E
T116E
S118E
(kDa)
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37
Bim
Mcl-1
β-actin
37
Parental
Empty pBabe
WT
S59A
S77A
S87A
S94A
S104A
T116A
S118A
(kDa)
25
37
50
Bim
Mcl-1
β-actin
B
RQ (Relative Quantity)
Bim
Mcl-1
Parental
Empty pBabe
WT
S59E
S77E
S87E
S94E
S104E
T116E
S118E
RQ (Relative Quantity)
Bim
Mcl-1
Parental
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S59A
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Parental
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β-actin
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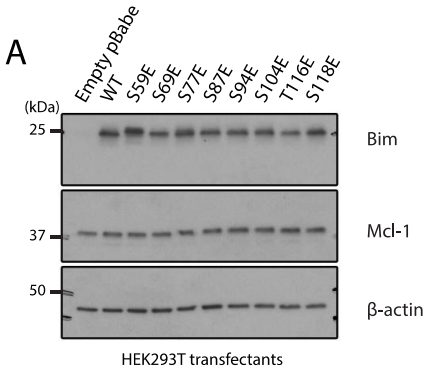
S69E 1

Figure 3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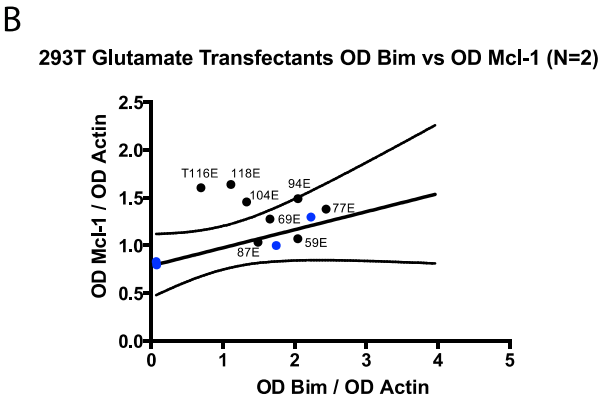
Bim
Mcl-1
β-actin
HEK293T transfectants


B
293T Glutamate Transfectants OD Bim vs OD Mcl-1 (N=2)
OD Mcl-1 / OD Actin
OD Bim / OD Actin
T116E
118E
94E
104E
77E
69E
87E
59E
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0.5
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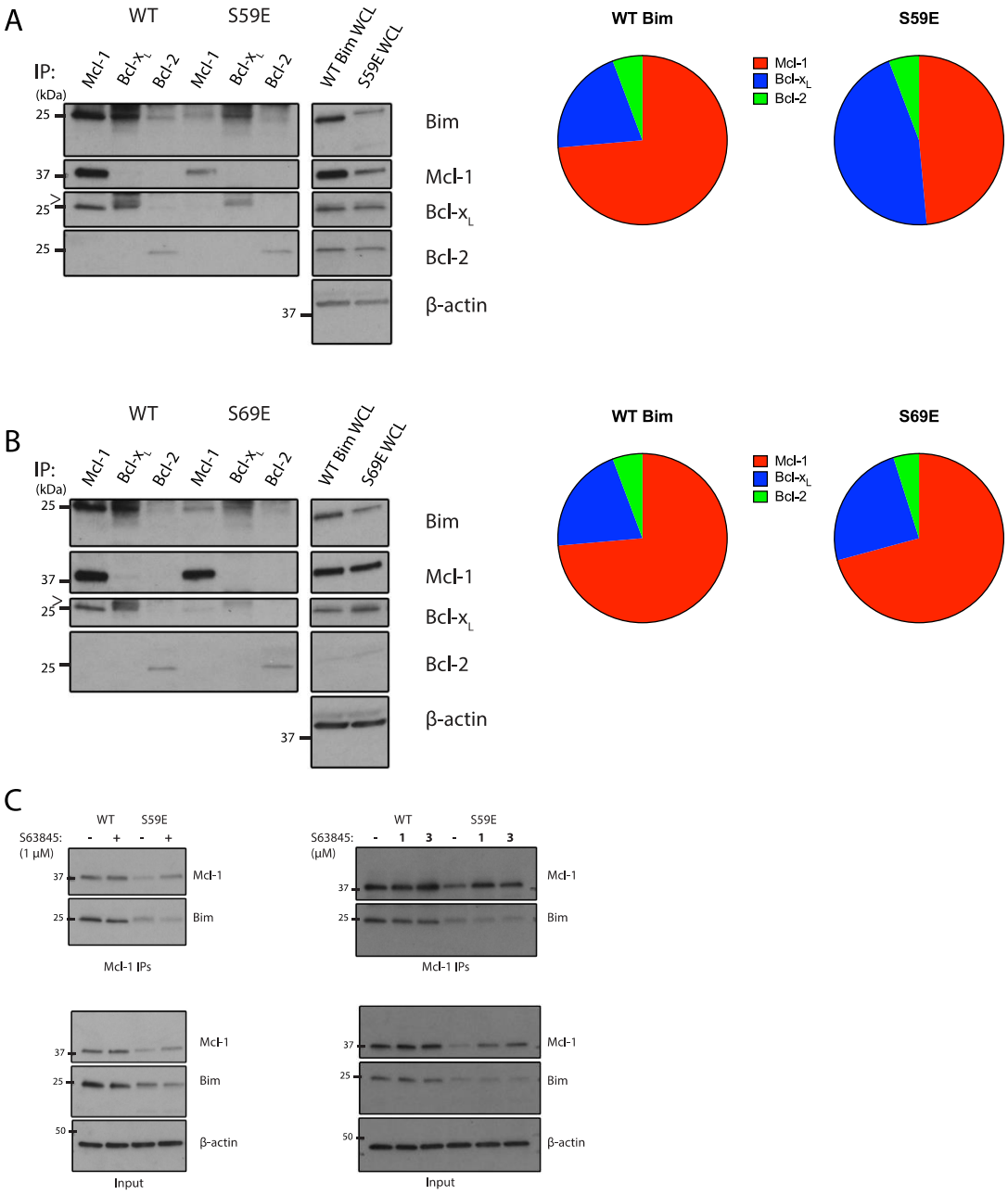
1.5
2.0
2.5
0
1
2
3
4
5

Figure 4


A
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β-actin
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Figure 5

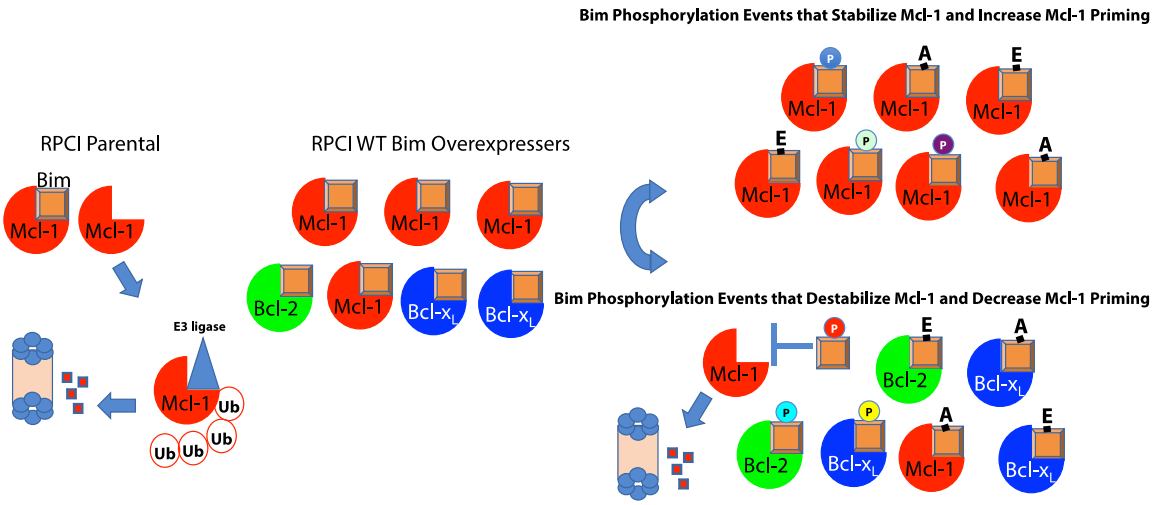
Bim Phosphorylation Events that Stabilize Mcl-1 and Increase Mcl-1 Priming
RPCI Parental
Bim
RPCI WT Bim Overexpressers
Mcl-1
Bcl-2
Bcl-x$_L$
E3 ligase
Ub
P
A
E
Bim Phosphorylation Events that Destabilize Mcl-1 and Decrease Mcl-1 Priming

Figure 6

IV. DISCUSSION

A. Implications from the characterization of constitutive Bim phosphorylation

In our studies of plasma cell dyscrasias, we demonstrated that Bim is constitutively phosphorylated. Previous efforts have largely focused on understanding Bim phosphorylation in response to stimuli such as the addition or removal of cytokines and growth factors and UV radiation[25, 47, 51]. Conversely, few studies have identified and functionally characterized constitutive Bim phosphorylation events. Our work shows that the pattern of constitutive Bim phosphorylation varies between different cell lines, with some cell lines having singular phosphorylation events, and others having as many as three or four. There was no correlation between the amount of Bim phosphorylation and the characteristics of a given cell line—neither sensitivity to ABT-737/199 nor Bim binding pattern were predictive. While we were unable to fully identify the kinases driving these phosphorylation events, our observations point to potential differences in the signaling cascades regulating Bim within these cells.

One possible explanation for constitutive Bim phosphorylation is the presence of activating mutations within putative kinases. RNA-Seq data (courtesy of Jonathan Keats) for each of the myeloma cell lines used in our studies identifies several missense mutations in key oncogenic kinases, particularly in cell lines with the most Bim phosphorylation (Table 1). Aberrant signaling via proteins such as K-Ras, EGFR, and FGFR3 could ultimately result in the observed phosphorylation, while simultaneously providing a therapeutic target to influence the Bim phosphorylation state.

Another potential driver of these Bim phosphorylation events is autocrine and paracrine signaling. Myeloma cells and other hematopoietic-derived lineages are

notoriously dependent on secreted cytokines for survival and growth[52, 136, 137]. Myeloma cells in culture could secrete factors in an autocrine or paracrine fashion, resulting in 'constitutive' phosphorylation events that are in fact cytokine induced. Similarly, purified myeloma cells from bone marrow aspirates may still be subject to residual cytokine stimulation from bone marrow stromal cells at the time of collection and lysis.

An additional point of consideration is the culturing of cells in serum containing media. Serum typically contains mitogenic factors that facilitate cell growth and could potentially result in phosphorylation of cellular proteins, including Bim. Unpublished work from our lab and additional studies suggest the contributions of serum to Bim phosphorylation are minor and would likely be associated with proteasomal degradation of Bim, which we do not observe[120]. Our model for the potential mechanisms underlying constitutive Bim phosphorylation is presented in Figure 1.

Bim is not unique among Bcl-2 family members as a frequent target of posttranslational modification. Other pro-apoptotic family members are subject to phosphorylation events that may provide insight into the functional consequences of constitutive Bim phosphorylation. Consistent with previously described Bim phosphorylation outcomes, both pro- and anti-apoptotic members of the Bcl-2 family are subject to phosphorylation-mediated degradation, direct ubiquitination, and phosphorylation events that alter function[18]. Notable among the pro-apoptotic BH3-only sensitizers, Bad can be sequestered by 14-3-3 in a phosphorylation-dependent manner and Bmf phosphorylation regulates its interaction with the dynein light chain[25, 138]. The direct activator Bid is subjected to phosphorylation events that impact its ability to be cleaved to its active form by caspase 8 and ATM-dependent phosphorylation that may

toggle the function of Bid between triggering apoptosis and causing DNA damage-induced cell cycle arrest[139, 140]. Based on these observations, constitutive Bim phosphorylation events could potentially regulate Bim protein structure or its presence in larger protein complexes. Recent studies have suggested molecules of Bim can form large aggregate complexes with dynein light chain and Mcl-1 that provide an additional layer of regulation of its function[141]. Phosphorylation could possibly play a role in governing the formation of these and other complexes within a cell.

A caveat to our findings is our exclusive focus on constitutive Bim phosphorylation events within cancer cells. While many studies have looked at Bim phosphorylation in non-transformed cells, this has typically been in the context of cytokine or growth factor stimulation. Running lysates from normal plasma cells or B cells on a PhosTag™ gel would provide an opportunity to determine if Bim is phosphorylated in normal cells and discern if constitutive Bim phosphorylation is associated exclusively with oncogenic transformation. Moreover, our data suggest an additional research direction focused on the interplay between Bim phosphorylation and the degree of differentiation of a cancer cell. Among the myeloma cell lines we analyzed via PhosTag™ electrophoresis (Chapter II, Figure 2), several retain normal B cell markers such as CD20 and CD79. Ongoing work in our lab seeks to understand the relationship between how 'plasma cell-like' myeloma cells are and their sensitivity to therapeutics. This work could be extended to examine not only how the phosphorylation state of Bim differs as plasma cell phenotype of a cancer cell is altered, but also how it evolves as a cancer cell becomes more undifferentiated.

Future studies should prioritize the identification of Bim phosphorylation sites through proteomics approaches. This effort has proven elusive, with few groups able to successfully leverage mass spectrometry for this purpose, and the overwhelming majority utilizing mutagenesis or biochemical approaches[44, 54, 124]. Even research efforts that have made use of proteomics have exclusively utilized transient overexpression systems due to the apoptogenic nature of Bim[49]. Our work has sought to build upon these studies by focusing on the isolation and phospho-proteomic analysis of endogenous, human Bim. While we faced many of the same challenges encountered by other groups—low Bim protein abundance, high apoptotic activity, and poor tolerance of exogenous Bim expression, we were able to stably overexpress Bim in an intrinsically Bax/Bak-deficient cell line. Future efforts could draw on the insights gained concerning the low Bim protein abundance and perform large scale ($> 10^8$ cells) immunoprecipitation experiments. This approach could be further refined through use of CRISPR technology to introduce an affinity tag to the endogenous Bim protein. In order to fully characterize the identified phosphorylation sites, proteomic analysis should ultimately be coupled with elucidation of the signaling pathways influencing Bim phosphorylation.

Given the number of publications focused on Bim phosphorylation, identification of specific Bim phosphorylation sites alone may provide clues as to the relevant kinases. However, the context dependence of Bim phosphorylation allows for multiple kinases to regulate a given phosphorylation site, particularly in the case of MAPK family members[40, 42, 121]. Moreover, our limited success with small molecule inhibitors (Chapter II, Figure 4) suggests a more comprehensive approach is warranted. Future experiments could utilize a phospho-kinase array to identify phosphorylated cellular targets that may

help pinpoint the kinases targeting Bim. Additionally, cross-linking of immunoprecipitated Bim may allow identification of kinases, phosphatases, and other proteins that posttranslationally regulate Bim.

Despite the significant research focus on kinases that phosphorylate Bim, we cannot dismiss the possibility of phosphatases providing an additional layer of regulation. For example, PP2A, as described in Chapter I, is known to increase Bim stability in response to ER stress[46]. Future studies could compare the expression and mutation status of PP2A across cell lines with differential Bim phosphorylation and determine if there is a causal relationship.

Similarly, while the entirety of Bim posttranslational modification literature is encompassed by phosphorylation and ubiquitination, other PTMs may be possible. As we've discussed at length, achieving comprehensive mass spectrometric analysis of Bim has been challenging. The same barriers to obtaining phospho-proteomic data may preclude the detection of other PTMs. The direct activator Bid—in its truncated form tBid—is subjected to N-myristoylation, facilitating its mitochondrial localization and increasing its pro-apoptotic activity[142]. Bim may be subjected to other modifications that will only be uncovered through further PTM mass spectrometry.

Lastly, our work necessitates exploring the interplay between cancer cell response to therapy and Bim phosphorylation. Given the critical role that phosphorylation plays in controlling Bim activity, particularly in cancer cells, and the dependency of many cancer therapeutics on perturbations of the apoptotic cascade, it is prudent to understand the potential influences of these drugs on the Bim phosphorylation state. We have preliminarily explored this question by running lysates from myeloma cells treated with

proteasome inhibitors and dexamethasone on PhosTag™ gels. While we observed no discernible changes in the Bim phosphorylation state in response to these drugs, studying the effect on Bim phosphorylation of other compounds such as IMiDs and the recently FDA-approved therapies daratumumab and elotuzimab could be worthwhile. This approach could also be expanded to other tumor types and broader classes of chemotherapy and anti-cancer agents.

B. Implications from studying the impact of Bim phosphorylation on Mcl-1 stability and priming

In chapter III, we demonstrated that the introduction of various Bim phosphorylation site mutations results in differential ability to stabilize Mcl-1 (Chapter III, Figure 4). Other groups have previously shown that the stability of Mcl-1 is influenced by interactions with BH3-only proteins, with Puma and Bim capable of increasing Mcl-1 stability, and Noxa binding resulting in decreased Mcl-1 levels[78, 108, 109]. Our work is novel in its description of a potential role for Bim phosphorylation in altering this effect. Moreover, we also observed that Bim preferentially primes Mcl-1 in the RPCI-WM1 cell line, with phosphorylation site mutation abrogating this effect (Chapter III, Figure 1 and 5). Notably, introduction of a phospho-mimetic mutation at serine 59 resulted in an increased fraction of Bim bound to Bcl-x_L. The factors that determine which anti-apoptotic protein Bim binds to remain poorly understood. Several groups have proposed Bim binding is dictated by the expression levels of anti-apoptotic proteins[143]. However, our work provides additional mechanistic insight complementing previous studies showing that aberrant signaling can drive Bcl-2 family priming and dependence (Figure 2)[52, 120]. Data from one such study show that inhibiting EGFR signaling can result in decreased association of Bim with Mcl-1, and an increase in the proportion of Bim bound to Bcl-2. Serine 59 is a canonical MAPK phosphorylation target, providing additional evidence that compounds targeting MAPK proteins or signaling pathways that converge on MAPK proteins may directly influence preferential binding of Bim[124]. Waldenström's macroglobulinemia cells are frequently characterized by activating mutations in MyD88 or CXCR4[144]. These mutations could drive aberrant signaling in the

RPCI-WM1 cell line that results in Bim phosphorylation events that drive the substantial Mcl-1 priming we observe. By introducing the S59E mutation, we would in effect be superseding the constitutive Bim phosphorylation events that result in Mcl-1 dependence in these cells.

An important shortcoming of these studies is a lack of comprehensive phospho-proteomic Bim analysis and a reliance on mutagenesis studies. The aforementioned mass spectrometry efforts should be leveraged to identify the observed constitutive Bim phosphorylation events in RPCI-WM1 (Chapter III, Figure 2). Given the adverse impact of the serine to alanine mutations on Mcl-1 stability, our data suggest that serine 87 and 104 may be two of the constitutive phosphorylation events. The poor tolerance of the serine 87 quadruple alanine mutant by RPCI-WM1 provides additional evidence that serine 87 phosphorylation plays an important role within this cellular context (Chapter II, Figure 6). The identification of serine 87 as a constitutive phosphorylation in a previous study further bolsters this hypothesis, although the function of this phosphorylation remains unclear[49]. As mentioned in chapter I, serine 87 has been linked to Akt and Protein Kinase A signaling, with phosphorylation via the former resulting in 14-3-3 sequestration, and the latter increasing Bim stability[47, 48]. While our data show that mutation of serine 87 to an alanine has no impact on Bim stability and results in decreased Mcl-1 stability, follow up studies should determine if this mutation alters Bim binding and priming within RPCI-WM1. An alternative explanation for the decreased Mcl-1 stability could be that removal of serine 87 Bim phosphorylation results in reduced binding to all Bim binding partners.

An additional caveat of these studies is our focus on singular phosphorylation site mutations, despite our data indicating that Bim is phosphorylated at several sites in the RPCI-WM1 cell line. The presence of multiple phosphorylation events complicates efforts to understand the functions and regulation of any given site. For example, several previous studies have demonstrated that phosphorylation at serine 69 is accompanied by other phosphorylation events such as serine 59 and 77 or serine 93, 94 and 98[44, 52]. What remains to be seen in many of these cases is the individual contributions of each phosphorylation and their temporal sequence. For example, if IL-6 stimulated phosphorylation of serine 69 and 77 is driving Mcl-1 dependence in myeloma cells, it's unknown if either of these phosphorylation sites is sufficient to drive this phenotype or if one has to occur first. Future studies should utilize complete phosphorylation-deficient mutants in addition to individual site alanine mutants to further elucidate the impact of multiple phosphorylations.

Given the observed effect of the S59E mutation on the distribution of Bim within the RPCI-WM1 cell line, our data suggest that portions of the Bim protein independent of the BH3 domain regulate its interactions with other Bcl-2 family members. This is somewhat surprising, given that the BH3 domain is responsible for mediating binding to anti-apoptotic proteins and activating the effectors Bax and Bak[14]. Subsequent studies should determine the potential phosphorylation-mediated changes in Bim structure that affect its interactions with anti-apoptotic proteins. The negative charge of a phosphorylation event or steric hindrance in a different part of the Bim protein could unfavorably impact the interaction of the BH3 domain with the hydrophobic pocket of an

anti-apoptotic protein. Our observations provide additional impetus for obtaining the complete crystal structure of Bim.

The potential role of phosphorylation in determining Bcl-2 family dependence raises intriguing questions concerning B cell development and plasma cell differentiation. We and others have shown that as a B-cell progresses from the pro-B cell state and ultimately undergoes differentiation into a plasma cell, the anti-apoptotic proteins that the cell depends on for survival change[93, 94, 97]. For example, early lymphoid progenitor cells, pro- and pre-B cells are dependent on Mcl-1 for survival, as are plasma cells. Additionally, we previously reported an intermediate step in plasma cell differentiation that is Bcl-x_L dependent. If our hypothesis is true, these changes in dependence could be regulated by signaling cascades resulting in Bim phosphorylation. This idea becomes more compelling when we consider recent evidence showing that the B cell receptor associated Src kinase Lyn is capable of phosphorylating Bim[54]. Future experiments could examine this potential interplay between B cell development, Bim phosphorylation, and anti-apoptotic dependence.

An additional consideration in our study of Bcl-2 family dependence is the contribution of anti-apoptotic protein phosphorylation. Bcl-2, Bcl-x_L, and Mcl-1 are all regulated by phosphorylation, with phosphorylation status influencing the stability of these proteins and their ability to sequester pro-apoptotic proteins[18]. In our extensive PhosTag™ studies, we only observed phosphorylation of Bcl-2 (Chapter II, Figure 1 and 2; Chapter III, Figure 2). Bcl-2 phosphorylation can promote or inhibit apoptosis depending on cellular context. Moreover, Bcl-2 phosphorylation was purported to substantially decrease the sensitivity of CLL cells to ABT-737, ABT-263, and ABT-199

by increasing the affinity of Bcl-2 for Bim and Bak[145]. As a point of comparison, the proportion of phosphorylated Bcl-2 observed in our cells appears to be quite low, and the degree of Bcl-2 dependence is variable. Nonetheless, the potential impact of anti-apoptotic phosphorylation should be considered in any therapeutic strategy designed to influence Bim priming within cancer cells.

We've demonstrated a potential role for constitutive Bim phosphorylation in determining the anti-apoptotic protein Bim is bound to. The long-term goal of this research is to ultimately improve the efficacy of cancer therapeutics that function through alterations of the intrinsic apoptotic pathway. As described in chapter I, Bcl-2 antagonist therapies have demonstrated strong potential in clinical and pre-clinical settings[65, 146]. As with other therapeutic modalities, significant challenges prevent greater efficacy and widespread use of these compounds, including *de novo* and acquired resistance. We propose that the effectiveness of drugs such as venetoclax can be augmented by pairing them with compounds that affect the phosphorylation state and priming of Bim (Figure 3). While there are pre-clinical efforts and some clinical trials that combine Bcl-2 family antagonists with inhibitors of signaling cascades, the inhibitors used in these studies are largely aimed at targeting proteins with specific mutations that contribute to the overall dysregulated signaling of the cancer cell[147, 148]. We believe this approach can be further refined by identifying kinases and phosphatases that regulate Bim constitutive phosphorylation. Upon determining the specific sites that influence Bim binding in a cancer cell, compounds that modulate Bim phosphorylation could be used to prime a specific anti-apoptotic protein, and the appropriate Bcl-2 family antagonist could be

deployed in conjunction. Given the relevance of priming to a myriad of tumor types, this approach has the potential to have a substantial impact on cancer treatment.

Table IV-1: List of notable signaling genes with mutations in myeloma cell lines

Cell Lines	Kinases with Missense Mutations or Codon Deletions*	Mutation Associated with Activation?
MM.1s	EGFR KRAS MEKK1*	Unknown Yes Unknown
KMS18	FGFR3 FLT4 MEKK1* ASK1	No Unknown Unknown Unknown
U266	BRAF FLT4 PIK3CG	Yes Unknown Unknown
Karpas620	KRAS ALK MEKK1*	Yes Unknown Unknown
KMS12PE	ERBB2 FGFR3	Unknown No
OPM2	FGFR3 MEKK1*	Yes Unknown
OCIMy5	ERBB3 FLT3 ALK MEKK1*	Unknown Unknown Unknown Unknown

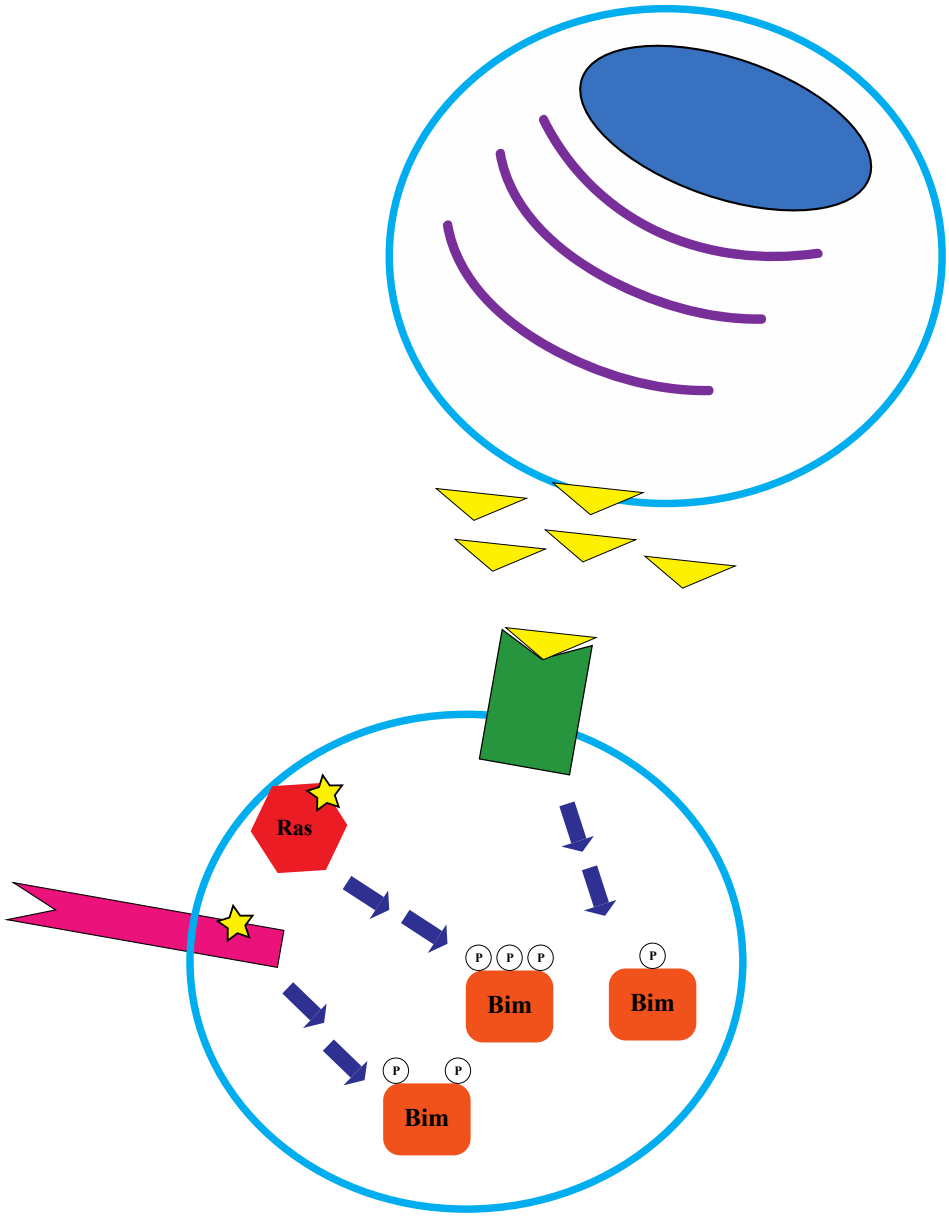

Figure IV-1: Proposed model for sources of constitutive Bim phosphorylation. Potential sources of the observed phosphorylation events are activating mutations in receptor tyrosine kinases and signaling effectors, or autocrine and paracrine signaling among myeloma cells.

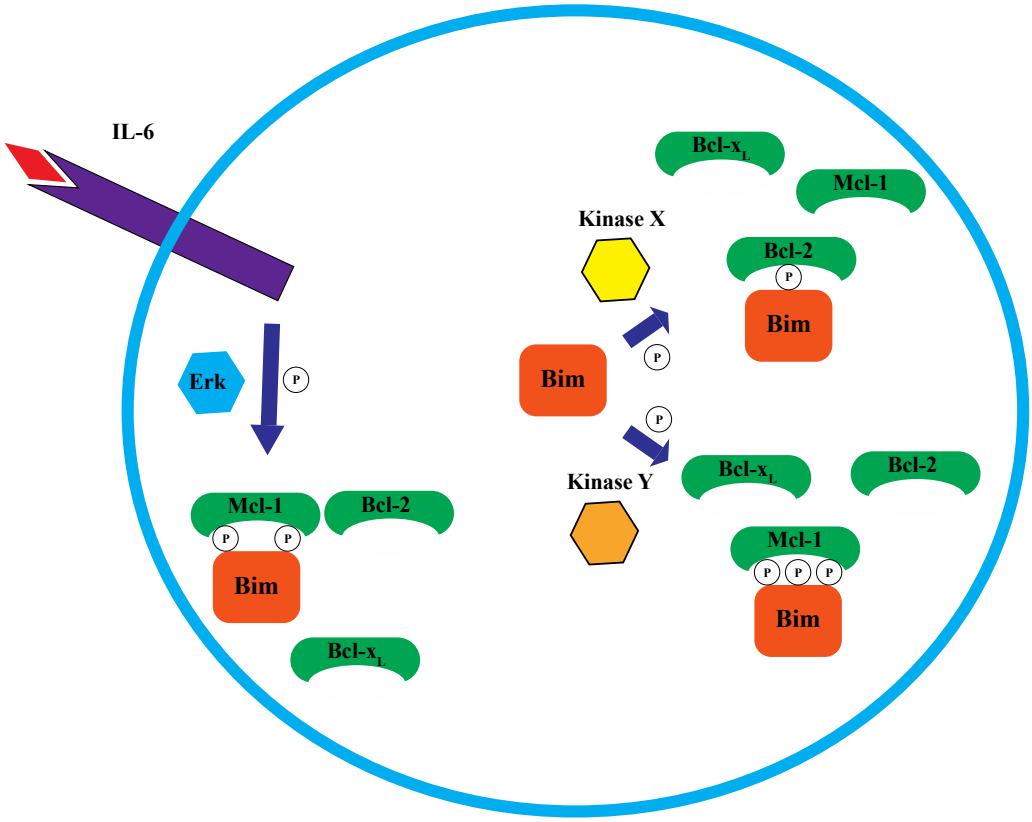

Figure IV-2: Proposed model for the impact of Bim phosphorylation on binding to anti-apoptotic proteins. As previously described, ERK phosphorylation of Bim has been linked to increased affinity for Mcl-1. We propose that constitutive phosphorylation events, mediated by currently unidentified kinases, dictate which anti-apoptotic protein Bim binds to.

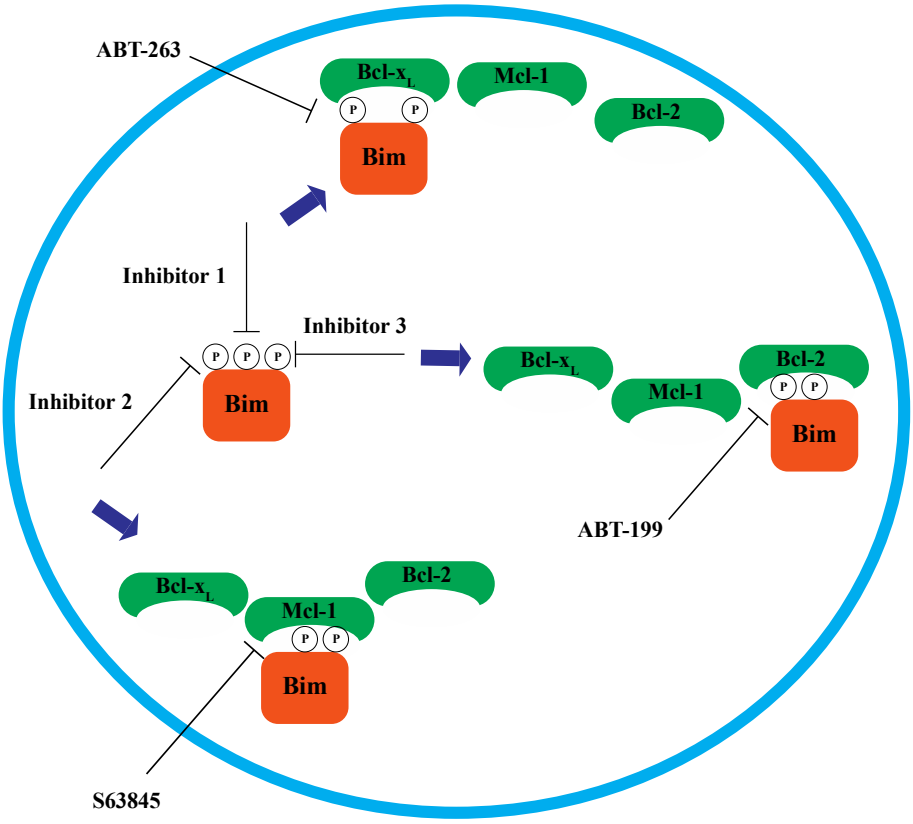

Figure IV-3: Potential therapeutic implications of Bim phosphorylation-mediated priming. By using inhibitors (1, 2, 3) that target Bim kinases, we can potentially influence the priming in a cancer cell. These inhibitors could be coupled with the appropriate Bcl-2 antagonist therapy.

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