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Pamela Wong April 11, 2017

Determining the Effects of Myeloma-Derived Amino Acid Substitutions in the Functional Domain of Mcl-1 on Stress-Induced Apoptosis

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biology

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Abstract

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By Pamela Wong

Multiple Myeloma is a cancer of plasma cells. In 2016, there were more than 30,000 estimated new cases of multiple myeloma. Myeloma cells retain many characteristics of plasma cells, and one of which is their dependence on Mcl-1, an anti-apoptotic protein in the Bcl-2 family of apoptotic regulators. Our study investigates whether amino acid substitutions in the functional region of Mcl-1—L267V, V249L, N223S, R214Q—observed in some myeloma patients affect Mcl-1's anti-apoptotic ability. We generated FL5.12 cells that stably overexpress Wild Type(WT) Mcl-1 or mutant Mcl-1. By western blot analysis, real time, quantitative polymerase chain reaction, and proteasome inhibition, we found that the L267V mutation destabilizes the Mcl-1 protein. We then subjected FL5.12 stable cell lines to IL-3 withdrawal, treatment with chemotherapy agents (Vincristine, Etoposide, and Cisplatin), and transient overexpression of Bim. We found that overexpression of WT Mcl-1 protects FL5.12 cells from IL-3 withdrawal only transiently and does not protect against Vincristine, Etoposide or Cisplatin-induced apoptosis. We also observed that overexpression of WT Mcl-1 can protect the FL5.12 stable cell lines against transient Bim overexpression-induced apoptosis. In contrast, overexpression of Mcl-1 V249L does not protect to the extent of WT Mcl-1 overexpression; overexpression of Mcl-1 L267V cannot protect the cells at all, which is consistent with our findings that the L267V mutation destabilizes the protein. Follow up studies need to be done on why Mcl-1 can only protect FL5.12 cells from IL-3 withdrawal transiently, why it can only protect against apoptosis induced from specific methods, and the mechanism through which V249L decreases Mcl-1's anti-apoptotic ability. Our findings will provide insights into why patients with such mutations fare better, which can inform treatment.

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Acknowledgements

I would like to sincerely thank Dr. Larry Boise, my PI, for his generous support, guidance and mentorship throughout my time in the Boise Lab.

I would also like to thank every single member of the Boise Lab—Shannon Matulis, Vikas Gupta, Jason Conage-Pough, Catherine Gavile, Tyler Moser-Katz, Tony Tang, Katelyn Ponder, and David Weir—for all their help in my experiments and discussion of this project.

Finally, I would like to thank my committee members Dr. Rustom Antia and Dr. Arri Eisen for all the interesting scientific puzzles they propose to help improve my scientific thinking and for serving on my thesis committee.

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Introduction

Multiple Myeloma is a cancer of plasma cells, which are the most differentiated cells of the B-cell lineage that secret antibodies and help maintain long term immunity[1,2]. In 2016, there were more than 30,000 estimated new cases of myeloma and approximately 12,000 estimated deaths resulted from this cancer[3]. Unlike myeloma cells, the plasma cells in the bone marrow do not proliferate. Myelomagenesis is a multi-step process that requires a cancer cell to have overcame multiple checks for transformation[4]. Many different genetic alterations can contribute to the malignancy of plasma cells. The various combinations of such alterations, including chromatin addition, deletion, or gene translocations can give rise to a unique disease of myeloma in each patient[5].

Myeloma cells retain the characteristics of plasma cells which can be targeted for therapy. For instance, myeloma cells are sensitive to Bortezomib, a proteasome inhibitor, because they produce copious amounts of antibodies and can secrete those like normal plasma cells[6]. In fact, an increased presence of a monoclonal antibody or immunoglobulin light chain in the serum is observed in myeloma patients[7]. Myeloma cells are also dependent on transcription factors, such as Blimp-1, that normal myeloma cells require[8,9]. Plasma cell survival is also maintained by bone marrow microenvironmental factors like IL-6 and CD86/28 signaling[10]. These same factors are utilized by myeloma cells to survive[11]. In addition, normal B cell development and the maintenance of long-lived plasma cells require the anti-apoptotic protein Mcl-1[12]. Myeloma cells have been demonstrated to be dependent on this protein to evade apoptosis[13]. Acquiring the ability to evade apoptosis is a hallmark of cancer[4].

Apoptosis is a non-inflammatory form of programmed cell death that signals a hazardous cell, such as an infected cell or a cell with DNA damage, to die and be removed by phagocytes[14]. This process is in contrary to necrosis, which is an inflammatory form of death[15]. One of the last steps of apoptosis is the flipping of phosphotidylserine from the inner leaflet of the membrane lipid bilayer to the outside. These phosphotidylserines are an "eat me" signal that allows the apoptotic cell to be recognized. This signal promotes endocytosis by phagocytes and thus prevents an inflammatory response[16]. Apoptosis normally also plays important roles in maintaining homeostasis, such as in eliminating immune cells after an immune response during the contracting phase as well as in development[17,18]. There are two pathways for apoptosis. The first is the extrinsic pathway via death receptor, such as Fas, whose signaling is mediated by immune cells[14]. The Bcl-2 family proteins regulate the other pathway—the intrinsic, or mitochondrial, apoptosis pathway, which is induced by cell intrinsic factors such as growth factor withdrawal and DNA damage[19-21].

Members of the Bcl-2 family all have at least one Bcl-2 homology(BH) domain(BH1-BH4). The Bcl-2 family proteins can be separated into pro-apoptotic effectors, pro-apoptotic activators, pro-apoptotic sensitizers, and anti-apoptotic proteins[22]. Oligomerization of Bax and Bak, which are pro-apoptotic effectors on the mitochondrial membranes, leads to mitochondrial outer membrane permeabilization. This process results in the release of cytochrome C into the cytosol[23]. Cytochrome C in the cytosol forms the apoptosome with Apaf1 and pro-caspase 9, which then gets activated[24]. The activated caspase 9 then cleaves caspase 7 and 3 and commits a cell to apoptosis as these effector caspases cleave and activate Caspase-activated DNAse to cut up DNA[25]. (Figure 1)

BH3-only pro-apoptotic activators such as Bim can activate Bax and Bak to oligomerize. Anti-apoptotic Bcl-2 family proteins, such as Bcl-2, Bcl-X_L and Mcl-1 that are on the mitochondrial outer membrane, sequester pro-apoptotic BH3-only activators to block apoptosis[26]. Other BH3-only proteins in the Bcl-2 family, such as Noxa and Bad, are induced during cellular stress. In order to promote apoptosis, Noxa and Bad can inhibit the anti-apoptotic proteins and release the pro-apoptotic activators[27,28].

Cancer cells have been demonstrated to have a different threshold for apoptosis compared to normal cells[29]. Cancer cells can be "primed" and more sensitive to apoptosis because of the stress that they are under from their abnormal proliferation or from therapy[30]. Cellular stress upregulates BH3-only activators like Bim which occupies the anti-apoptotic proteins, and when an addition death signal is introduced, there will be fewer anti-apoptotic proteins available to sequester the additional activators induced. Myeloma cells can have most of their Bim sequestered on just Mcl-1 or have their Bim distributed on Bcl-2 and Bcl-X_L in addition to Mcl-1[31]. Furthermore, IL-6 in the bone marrow environment enforces this Mcl-1 dependence[32]. While targeting Mcl-1 is an attractive option to induce death in myeloma cells, the search of an effective Mcl-1 inhibitor has been difficult[33].

The Mcl-1 gene is located on chromosome 1 and encodes a 350 amino acid long protein[34]. The Mcl-1 protein has a PEST region that regulates its degradation, a functional domain that includes BH1, BH2, BH3 regions and is also where it binds BH3-only proteins, as well as a transmembrane domain that allows it to anchor onto the mitochondrial outer membrane[35]. Analysis of patient data from the MMRF CoMMpass study shows that Mcl-1 is the only anti-apoptotic Bcl-2 family protein that has recorded instances of being mutated in

approximately 1000 patients in the study. The CoMMpass study is a longitudinal myeloma patient study that collects genomic and transcriptomic data of myeloma patients from diagnosis. The study found that patients with Mcl-1 single nucleotide variations (SNVs) that lead to amino acid substitutions fare much better than patients without SNVs in Mcl-1 in terms of both progress-free survival and overall survival (Figure 2).

Among the non-synonymous Mcl-1 amino acid substitutions found in patients, we chose to investigate four that lie in the functional region of Mcl-1: L267V, V249L, N223S and R214Q. All of these mutations are located in or near the BH1 or BH3 region of the functional domain that forms the binding groove for BH3-only proteins. We wanted to investigate whether the four different amino acid substitutions in Mcl-1 mentioned above affect Mcl-1's anti-apoptotic ability.

Since myeloma cell lines overexpress Mcl-1 already, we resolved to using a cell line that does not overexpress Mcl-1 in order to observe the effect of overexpressing mutant versus wildtype (WT) Mcl-1 constructs. FL5.12 is a mouse liver pro-B lymphocyte cell line that is dependent on IL-3. We cloned Mcl-1 into pBABE, a retroviral vector, and generated the amino acid mutations in Mcl-1 in the vector using site-directed mutagenesis. The constructs were transfected into the virus packaging cell line Phoenix, and the virus made was used to infect FL5.12 cells. Cells that stably incorporated our constructs were selected by puromycin.

We investigated whether the overexpression of mutated Mcl-1 could confer the same amount of protection against apoptosis as overexpression of WT Mcl-1. We induced cell death through several mechanisms in our study. The first was IL-3 withdrawal because previous studies have shown that that overexpression of Bcl-X_L or Bcl-2 could protect FL5.12 cells from

IL-3 withdrawal[36]. We also treated cells with chemotherapy drugs Vincristine, Cisplatin, and Etoposide. Vincristine is a microtubule inhibitor, Etoposide is a topoisomerase II inhibitor, and Cisplatin is a DNA cross-linking agent. We also induced apoptosis by transiently overexpressing Bim. Furthermore, we performed similar experiments using mouse embryonic fibroblasts that stably overexpress our constructs.

We were also interested in determining mechanistically how these mutations affect Mcl-1's anti-apoptotic ability. Therefore, we compared Mcl-1 protein expression and mRNA expression using western blot and real time, quantitative PCR in FL 5.12 stable cell lines as well as treating the cells with Bortezomib, a proteasome inhibitor, to check if the protein expression level changes upon proteasome inhibition. Because myeloma cells are dependent on Mcl-1 for survival, we hypothesized that these mutations are hypomorphic. They cause a partial loss of function in Mcl-1 through either destabilizing the protein or decreasing its affinity to BH3-only pro-apoptotic proteins such as Bim. Our findings will provide insights into why patients with such mutations fare better, which can inform future treatment.

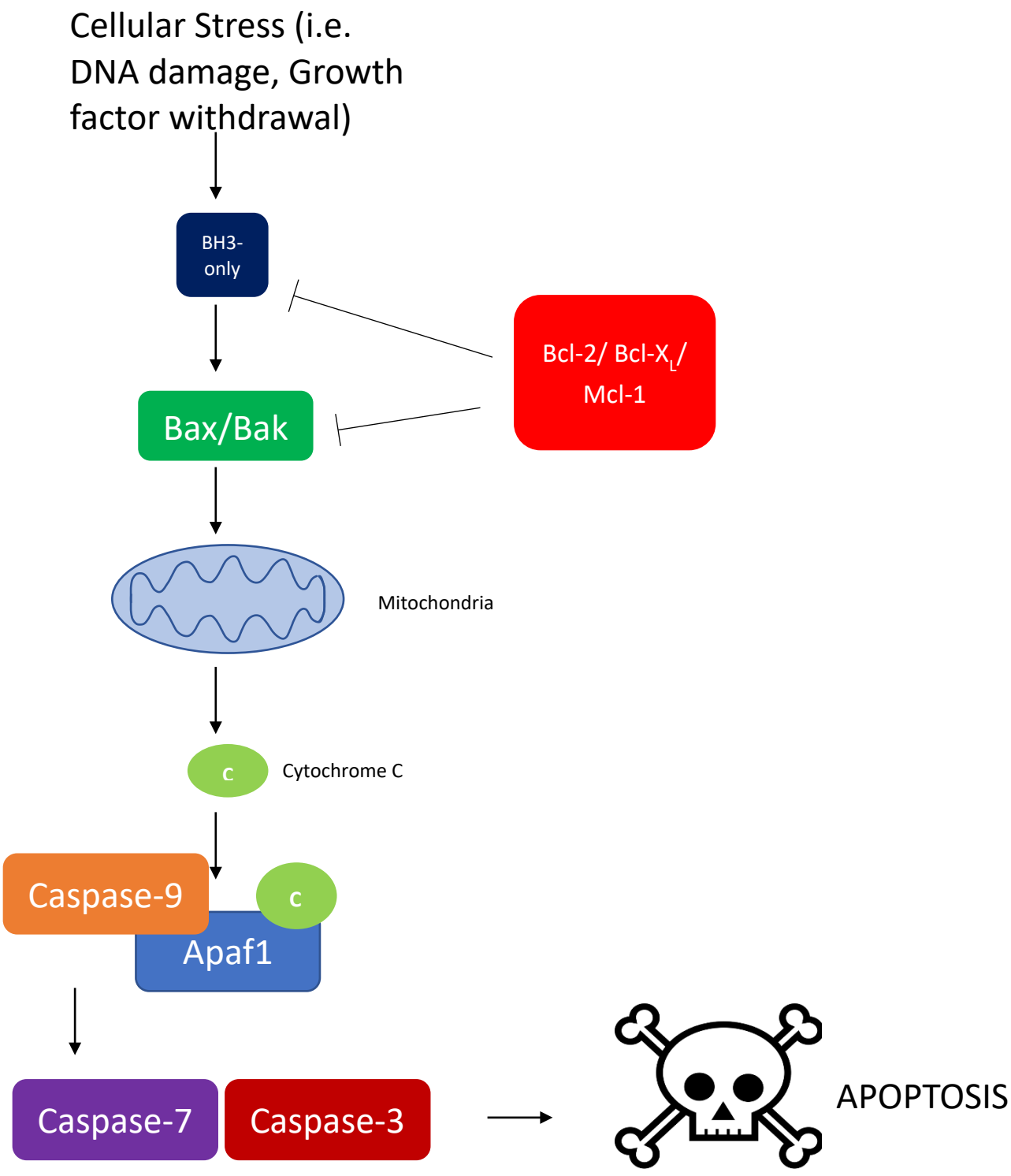
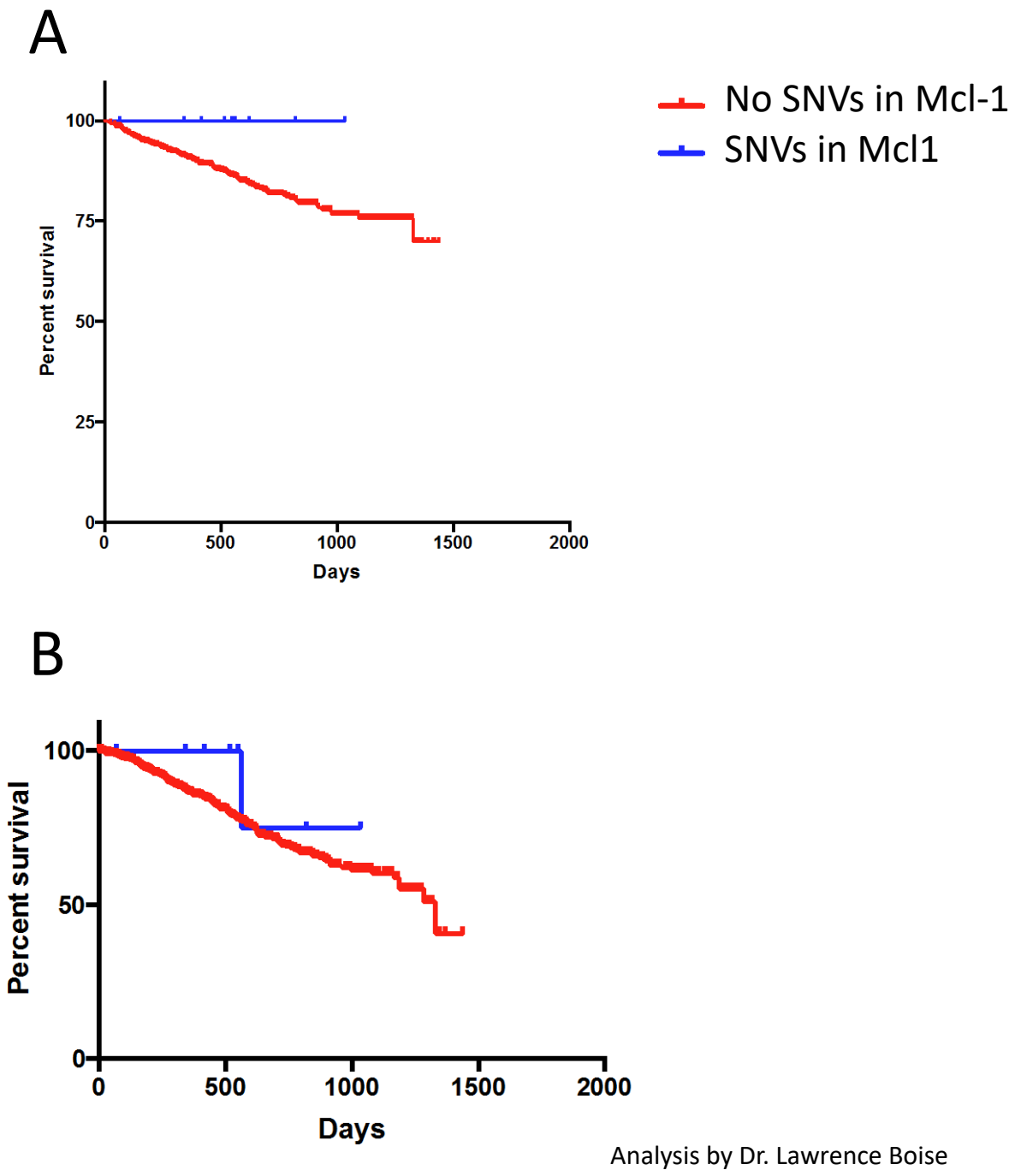


Figure 1. Simplified schematic of the intrinsic apoptosis pathway



Analysis by Dr. Lawrence Boise

Figure 2. Myeloma patients with single nucleotide variations(SNVs) in Mcl-1 fare better than patients with no SNVs in Mcl-1 in terms of (A) overall survival and (B) progress free survival.

Materials and Methods

Cell Culture

FL5.12 cells, which are IL-3-dependent murine pro-B lymphocytes, were cultured in RPMI 1640 supplemented with 10% Fetal Bovine Serum, 10% WEHI 3B-conditioned media, 100 unit/mL penicillin/streptomycin, 2 mM-glutamate, 1% HEPES buffer, and 55 µM 2-mercaptoethanol. Mouse Embryonic Fibroblasts (MEF) were cultured in Dulbecco's Modification of Eagle's Medium supplemented with 10% Fetal Bovine Serum, 100 unit/mL penicillin/streptomycin, 2 mM-glutamate, 1% non-essential amino acids, 1 mM sodium pyruvate, 1% HEPES buffer, and 55 µM 2-mercaptoethanol. Cells were cultured at 37 °C in 5% carbon dioxide. FL5.12 stable cell lines were cultured in the presence of 0.75 µg/mL puromycin, and MEF stable cell lines were cultured in the presence of 4 µg/mL puromycin.

Polymerase Chain Reaction (PCR)

PCR was used to amplify Mcl-1 coding region insert from pcDNA-Mcl-1. 1X Taq Reaction Buffer, 200 µM dNTPs, 0.2 µM forward primer, 0.2 µM reverse primer, 35 ng or 50 ng of template DNA, and 1.25 unit/50 µL Taq Polymerase were used. The forward primer had the sequence 5' GGG GAA TTC CTA TCT TAT TAG ATA TGC CAA ACC 3' and the reverse primer had the sequence 5' GGG GGA TCC CAC CAT GTT TGG CCT CAA AAG AAA C 3'. Thermocycling conditions were as follows: 1) 95°C for 30 seconds, 2)30 cycles of 95°C for 30 seconds, 59 °C for 30 seconds, 68 °C for 2 minutes and 30 seconds and 3)72 °C for 5 minutes. PCR products were visualized using gel electrophoresis on 1% agarose gel dissolved in 1X TAE buffer with 1µg/mL of ethidium bromide.

Generation of pBABE-Mcl-1 Constructs

Mcl-1 coding region sequence was cloned into pBABE-puro using the recognition sites of BamHI and EcoRI. pBABE-puro was a gift from Hartmut Land & Jay Morgenstern & Bob Weinberg (Addgene plasmid # 1764)[37]. The amino acid substitutions L267V, V249L, N223S, R214Q in Mcl-1 were generated by site-directed mutagenesis using QuikChange II XL Site-Directed Mutagenesis Kit from Agilent, following the manufacturer's protocol. Mutagenesis primers were designed using the QuikChange® Primer Design Program. The mutagenesis primers' sequences are as follows: L267V—5' ACT GGG GCA GGA TTG TGA CTG TCA TTT CTT TTG G 3' and 5' CCA AAA GAA ATG ACA GTC ACA ATC CTG CCC CAG T 3' ; V249L—5' CTG AAA ACA TGG ATC ATC AAT CGA GAC AAC GAT TTC ACA TCG 3' and 5' CGA TGT GAA ATC GTT GTC TCG ATT GAT GAT CCA TGT TTT CAG 3'; N223S—5' CCG TCT CGT GGC TGC GCT GCA CGC C 3' and 5' GGC GTG CAG CGC AGC CAC GAG ACG G 3'; R214Q—5' GCG CTG GAG ACC TTA CAA CGG GTT GGG G 3'. Site-directed mutagenesis products were sent for sequencing to confirm the incorporation of the intended mutations.

Retroviral Transduction to Generate Stable Cell Lines

Phoenix-Ecotropic (Phoenix) cell line was transfected by a plasmid (pBABE-puro, pBABE-WT Mcl-1, pBABE-Mcl-1 L267V, pBABE-Mcl-1, V249L, pBABE-Mcl-1 N223S, pBABE Mcl-1 R214Q) using lipofectamine 2000. 2,000,000 FL5.12 or 50,000 MEF cells were seeded onto a 6-well plate. Viruses generated by the Phoenix cells were used to infect FL5.12 and MEF cells. The virus-containing supernatant of the Phoenix cells was filtered through 0.45-µm syringe filters at 36, 46, and 60 hours after transfection and was applied to the FL5.12 or MEF cells with

Polybrene Infection Reagent. 24 hours after the last infection, the viral supernatant was replaced with fresh media for 24 hours. FL5.12 cells were then selected with 0.75 μg/mL puromycin while MEF cells were selected with 4 μg/mL puromycin.

In the transient infection with pBABE-Bim, no puromycin selection was performed. FL5.12 stable cell lines were infected by virus generated with pBABE-Bim-transfected Phoenix cells, following the procedures described above.

Western Blot Analysis

Cells were washed in PBS and then lysed in 20 μL of RIPA buffer supplemented with Protease Inhibitor Cocktail and PMSF protease inhibitor. A BCA Protein Assay kit was used to determine total protein concentration. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-page) was performed on 30 μg of proteins and transferred to nitrocellulose membranes. The membrane was blocked with 5% milk at room temperature for 1 hour, and then incubated with a primary antibody overnight. The membrane was then washed with 1X TBST 3 times for 5 minutes each and incubated with a secondary antibody for 1 hour. After the secondary incubation, 3 more 5 minutes TBST washes were done before the blot was developed.

Real-Time, Quantitative PCR (RT-qPCR)

Total RNA was isolated from FL5.12 stable cell lines using the Qiagen RNeasy kit. High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems was used to generate complimentary-DNA(cDNA) from 1 μg of RNA. MultiScribe™ Reverse Transcriptase and RT

random primers were used in a 20 µL reaction. The Taqman Gene Expression Master Mix, human Mcl-1 probe and murine GAPDH probes were used to amplify the cDNA, following the manufacturer's protocol. Relative Mcl-1 expression was calculated using the Ct values normalized to GAPDH samples.

Bortezomib Inhibition

FL5.12-WT Mcl-1 and FL5.12-Mcl-1 L267V cells were plated onto 6-well plates at the concentration of 250,000 cells/mL media in 3 mL media. They were then treated with 10, 30, or 100 nm of Bortezomib and harvested after 3 hours. Protein lysates were prepared and subjected to Western Blot Analysis as described above.

Co-immunoprecipitation

Immunoprecipitation (IP)-antibody matrixes were formed with 3 µg mouse anti-Mcl-1 monoclonal antibody and 25 µL of Protein G, incubated on rocker at 4 °C overnight. FL5.12-Empty Vector and FL5.12-WT Mcl-1 cells were subjected to IL-3 withdrawal for 24 hours. Cells were collected and lysed in CHAPS lysis buffer (10 mM HEPES, pH 7.2, 150 mM NaCl, 2% CHAPS) supplemented with Protease Inhibitor Cocktail and PMSF protease inhibitor. Total protein concentration was measured by BCA Protein Assay Kit. Next, 100 µg of protein was incubated with 50 µL of Protein G for 1 hour on rocker at 4 °C for preclearing. The precleared lysate was then incubated with the Immunoprecipitation (IP)-antibody matrixes on rocker at 4 °C overnight. The matrix was pelleted and washed twice with PBS, and the proteins were eluted

using a reducing electrophoresis sample buffer with RIPA for western blot analysis as described above.

IL-3 Withdrawal and Serum Starvation

FL5.12 cells were washed 3 time with plain RPMI 1640, then 250,000 cells were resuspended in 1 mL of IL-3 free FL5.12 media and plated onto 24-well plates. Cells were collected at 0, 12, 24, 36, and 48 hour after IL-3 withdrawal for cell death analysis as described below.

MEF cells were washed 3 times with plain DMEM, then 250,000 cells were resuspended in 3 mL of serum-free MEF media and plated onto 6-well plates. Cells were collected at 48 hours after serum starvation for cell death analysis as described below.

Cell Death Assay

Cells were collected at indicated time points, washed with 3 mL of PBS, and stained with 2 μg/mL of Propidium Iodide in 500 μL of 1X Annexin V Binding Buffer. Flow cytometry on a BD FACSCanto II system with FACSDiva software was used to measure percent viability. FlowJo software was used to analyze the obtained data.

Drug Treatment

In 24 well-plates, 250,000 FL5.12 cells were resuspended in 1 mL of FL5.12 media and treated with 0.1 μg/mL of Vincristine, 10 μg/mL of Etoposide, or 5 μg/mL of Cisplatin. Cells were collected at 0, 24, and 48 hours after drug treatment for cell death analysis as described above.

Statistical Analysis

Statistical analysis was performed on experiments with at least 3 independent replicates. To determine the statistical significance of difference in cell death between two sets of samples, 2-sample-2-tailed-t-tests were used.

Results

In order to generate cell lines that stably overexpress Mcl-1, we started by cloning Mcl-1 that our lab has in the vector pcDNA3.1(+) into another vector pBABE-puro (pBABE). pBABE drives the expression of genes cloned into the multiple cloning site with its Moloney Murine Leukemia Virus Long Terminal Repeat. Its multiple cloning site contains recognition sequences for the restriction enzymes BamHI and EcoRI. Therefore, we flanked the Mcl-1 coding region with these sites in order to insert the Mcl-1 seequence into pBABE.

Polymerase chain reaction (PCR) was used to amplify the Mcl-1 coding region from pcDNA3.1-Mcl-l. The Mcl-1 PCR product was flanked by the BamHI recognition sequence at the start and by the EcoRI recognition sequence at the end. The forward primer includes the BamHI recognition sequence, a Kozak sequence for ribosome binding as well as the first 22 nucleotides of the Mcl-1 coding region; the reverse primer includes the EcoRI recognition sequence and the last 22 nucleotides of the Mcl-1 coding region (Figure 3A). When the PCR product was run on an agarose gel, we observed the Mcl-1 product band slightly above the 1 kilobase pairs (kb) and below the 1.2 kb ladder band, which is consistent with the expected product size of 1072 base pairs (Figure 3B).

To generate sticky ends on the Mcl-1 PCR product and pBABE for ligation, we performed a restriction digest with BamHI and EcoRI, and we visualized the result on an 1% agarose gel. The undigested pBABE, which is 5169 bp long, runs as two bands on gel electrophoresis (Figure 3C). The supercoiled form travels slower than the nicked form. After restriction digestion, the linearized plasmid runs as a single band in the digested sample, which runs slower than the supercoiled form and faster than the nicked form of the undigested plasmid. After ligation of

both the digested Mcl-1 and the digested pBABE, we confirmed the incorporation by sequencing the Mcl-1 insert with the pBABE 3' sequencing primer. We then generated pBABE-mutant Mcl-1 constructs with a single nucleotide change from pBABE-WT Mcl-1 to produce amino acid substitutions L267V, V249L, N223S, and R214Q. Sequencing results indicate that we have successfully generated pBABE-Mcl-1 constructs with the single nucleotide change that leads to the respective amino acid substitution (Figure 4).

The Phoenix-Ecotrophic (Phoenix) retrovirus-packaging cell line was utilized to package the pBABE constructs into viruses, which were then used to infect FL5.12 cells; cells that stably incorporated the constructs were selected using puromycin (Figure 5). FL5.12 parental cells did not receive any virus; FL5.12-Empty Vector cells stably express pBABE that does not have the Mcl-1 insert; FL5.12-WT Mcl-1 cells stably express pBABE-Mcl-1; FL5.12-Mcl-1 L267V, FL5.12-Mcl-1 V249L, FL5.12-Mcl-1 N223S, FL5.12-Mcl-1 R214Q cells stably express the respective pBABE-Mcl-1 mutant construct.

Mcl-1 protein expression levels of the cell lines were demonstrated using western blot analysis (Figure 6). FL5.12 parental and FL5.12-Empty Vector cells do not have detectable levels of Mcl-1 protein expression. FL5.12-WT Mcl-1, FL5.12-Mcl-1 V249L, and FL5.12-Mcl-1 R214Q have comparable levels of Mcl-1 protein expression. Interestingly, FL5.12-Mcl-1 L267V has a lower level of Mcl-1 protein expression relative to FL5.12-WT Mcl-1 and other mutants.

We then performed real time, quantitative (RT-qPCR) to study Mcl-1 mRNA expression in these cell lines. We found that all pBABE-Mcl-1-expressing cell lines express comparable levels of Mcl-1 mRNA, while FL5.12-parental and FL5.12-Empty Vector express only baseline levels of Mcl-1 mRNA (Figure 7). The RT-qPCR data along with the western blot analysis

described above suggest that the L267V mutation could be destabilizing the protein. To further investigate, we treated FL5.12-WT Mcl-1 and FL5.12 L267V with Bortezomib (Bz), a proteasome inhibitor, to block misfolded proteins degradation. Upon proteasome inhibition, Mcl-1 protein expression levels in both FL5.12-WT Mcl-1 and FL5.12 L267V Mcl-1 increase at 10, 30, and 100 nM of Bz treatment. In FL5.12-WT Mcl-1, there is an 1.6 fold increase in Mcl-1 protein expression under 30nM Bz treatment; In FL5.12-L267V, there is a 2.1 fold increase (Figure 8). In FL5.12-Mcl-1 L267V (compared to FL5.12-WT Mcl-1), the lower Mcl-1 protein expression, comparable Mcl-1 mRNA expression, and greater change in protein expression upon Bz treatment suggest that the L267V mutation destabilizes the Mcl-1 protein.

We were interested in seeing how these mutations affect Mcl-1's ability to protect FL5.12 cells from growth factor withdrawal-induced apoptosis. FL5.12 is a IL-3 dependent cell line and thus we can induce apoptosis in them by removing IL-3 in the media. We first confirmed that the human Mcl-1 expressed in FL5.12 stable cell lines can interact with mouse Bim using co-immunoprecipitation (co-IP). Whole cell lysates subjected to western blot analysis demonstrate that IL-3 withdrawal induce Bim expression (Figure 9). Co-IP of Mcl-1 subjected to western blot analysis shows that there is Bim bounded to Mcl-1 pulled down from both FL5.12-Empty Vector and FL5.12 WT Mcl-1 cells. However, there is a greater amount of Bim bounded to the Mcl-1 pulled down from FL5.12 WT Mcl-1 cells compared to Mcl-1 pulled down from FL5.12-Empty Vector cells. This difference in Bim on the Mcl-1 pulled down from FL5.12-WT Mcl-1 and FL5.12-Empty Vector cells suggests that the human Mcl-1, in addition to endogenous mouse Mcl-1, expressed by FL5.12-WT Mcl-1 can interact with mouse Bim.

Unfortunately, we could not observe protection of FL5.12 cells against IL-3 withdrawal by Mcl-1 consistently in our experiments. Even in specific replicates where we observed protection, WT Mcl-1 can only transiently protect FL5.12 cells against IL-3 withdrawal at 24 hours. We measured cell death by Propidium Iodide(PI) staining. PI is a DNA stain that can only get inside dead cells, and therefore cells that are PI+ in our flow analysis are dead cells.

At 24 hours under IL-3 withdrawal, there is a significant difference in the percentage of PI+ FL5.12-Empty Vector and PI+ FL5.12-WT Mcl-1 cells ($p=0.0012$). At 48 hours, FL5.12 parental and all FL5.12 stable cell lines are more than 90% PI+ (Figure 10). There is also a significant difference in the percentage of PI+ FL5.12-Empty Vector and PI+ FL5.12-Mcl-1 V249L, which is the cell line that has the highest average percentage of PI+ cells among all mutants ($p=0.0149$). However, larger p-value of the second pair could indicate a potentially weaker protection conferred by Mcl-1 V249L. At both 36 and 48 hours of IL-3 withdrawal, there is no significant difference in percentage of PI+ FL5.12-Empty Vector cells and PI+ cells of the FL5.12-Mcl-1 cell lines that have the lowest percentage of PI+ cells at each of those time point ($p=0.416$ and $p=0.176$, respectively).

One of our reservations about these data is the inconsistency between replicates, which is reflected by the large standard deviations. In addition, the transient protection demonstrated here is not consistent with previous studies that have shown that overexpressing a Bcl-2 family anti-apoptotic protein can protect FL5.12 from IL-3 withdrawal for more than 72 hours. Therefore, we next tried to understand the reason for this inconsistent and transient protection by overexpressing Mcl-1 in IL-3 withdrawal-induced apoptosis.

We suspected that IL-3 withdrawal induces degradation of Mcl-1 and thus FL5.12-WT Mcl-1 cells are susceptible to IL-3 withdrawal, especially after 48 hours. Using lysates collected at 0, 3, and 8 hours after IL-3 withdrawal, all cell lines upon IL-3 withdrawal have comparable Mcl-1 protein expression, demonstrated by western blot analysis (Figure 11). Mcl-1 protein expression does not decrease under IL-3 withdrawal, contrary to what we expected. Therefore, this does not explain the transiency of Mcl-1's protective effect in the IL-3 withdrawal experiments.

We then attempted to induce apoptosis by using chemotherapy drugs Vincristine, Etoposide, and Cisplatin to see if Mcl-1 can protect FL5.12 stably from death signals different than IL-3 withdrawal. Vincristine is a microtubule inhibitor, Etoposide is a Topoisomerase II inhibitor, and Cisplatin is a DNA cross-linking agent. Nevertheless, overexpressing WT Mcl-1 in FL5.12 cells cannot protect them against any of these drugs (Figure 12). Statistical significance was calculated for the difference between the average percentage of PI+ FL5.12-Empty Vector cells and the FL5.12-Mcl-1 cell line that has the lowest average percentage of PI+ cells at each time point.

At 24 hours of Vincristine treatment, there is no significant difference in the average percentage of PI+ FL5.12-Mcl-1 R214Q and FL5.12-Empty Vector cells($p=0.1135$) (Figure 12A). At 48 hours of Vincristine treatment, there is also no significant difference in the percentage of PI+ FL5.12-Empty Vector and FL5.12-Mcl-1 L267V cells($p=0.2851$). When treated with Etoposide, there also is no significant difference between the percentage of PI+ FL5.12-Empty Vector and FL5.12-Mcl-1 V249L cells at 24 and 48 hours ($p=0.2615$, $p=0.1776$, respectively) (Figure 12B). When treated with Cisplatin for 48 hours in a single experiment thus far, there is a

greater percentage of PI+ FL5.12-WT Mcl-1 cells than both PI+ FL5.12 Parental and FL5.12-Empty Vector cells (Figure 12C).

Next we thought that the Mcl-1 overexpression does not protect FL5.12 cells against IL-3 withdrawal consistently and stably because it is not overexpressed at a high enough level. Therefore, we compared the level of Mcl-1 protein expression in MM.1S, a myeloma cell line that overexpresses Mcl-1 at a high level, with FL5.12-WT Mcl-1. Western blot analysis of the MM.1S and FL5.12-WT Mcl-1 lysates shows that Mcl-1 protein expression is comparable between the two cell lines (Figure 13). We also generated mouse embryonic fibroblast (MEF) stable cell lines that overexpress our pBABE-Mcl-1 constructs because MEF cells have shown to express pBABE constructs well in past experiments in our lab. Nevertheless, overexpression of Mcl-1 in MEF cells do not protect them against serum starvation (Figure 14).

Finally, while we showed earlier that human Mcl-1 can interact with mouse Bim (Figure 9), the binding may not be strong and thus the protection is not prominent. Therefore, we tried to induce apoptosis in the FL5.12 stable cell lines by transiently infecting them with pBABE-human Bim to eliminate any cross-species binding issue. Protection by WT Mcl-1 can be seen as early as 24 hours after the last of three infections when the percentage of PI+ FL5.12-Empty Vector cells is almost double of the percentage of PI+ FL5.12 -WT Mcl-1 cells (Figure 15). FL5.12-Mcl-1 L267V cells were not protected against the transient Bim infection, which was demonstrated by the greater percentage of PI+ FL5.12 Mcl-1 L267V cells than PI+ FL5.12-Empty Vector cells. This lack of protection by Mcl-1 L267V is consistent with our findings that the L267V mutation destabilizes the Mcl-1 protein. In addition, the percentage of PI+ FL5.12-Mcl-1 V249L cells is lower than that of PI+ FL5.12-Empty Vector cells but higher than that of PI+

FL5.12-WT Mcl-1 cells. This suggests that V249L mutation decreases the amount of protection that Mcl-1 provides against transient Bim overexpression. These trends are also observed 32 and 48 hours after the last infection.

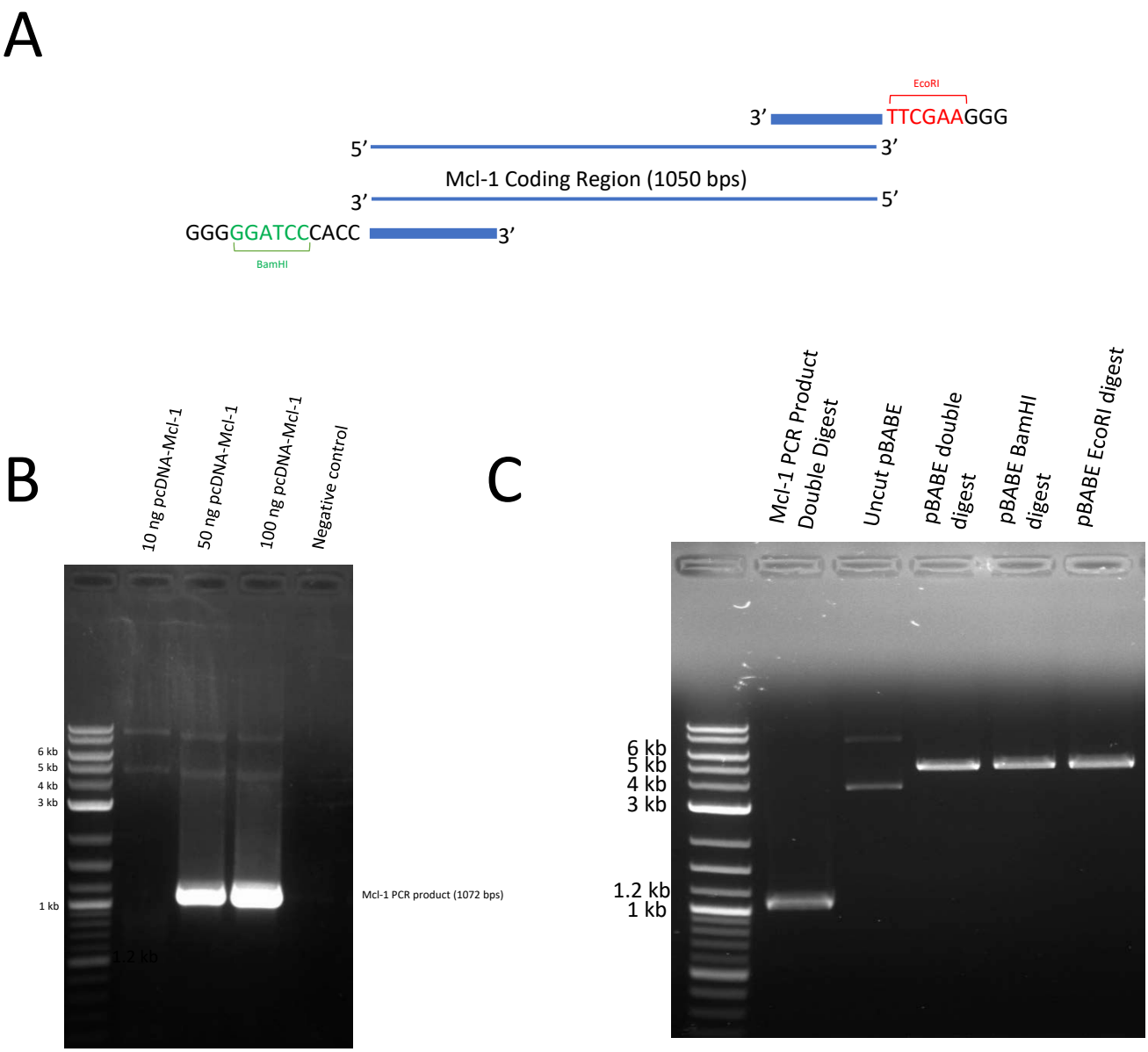
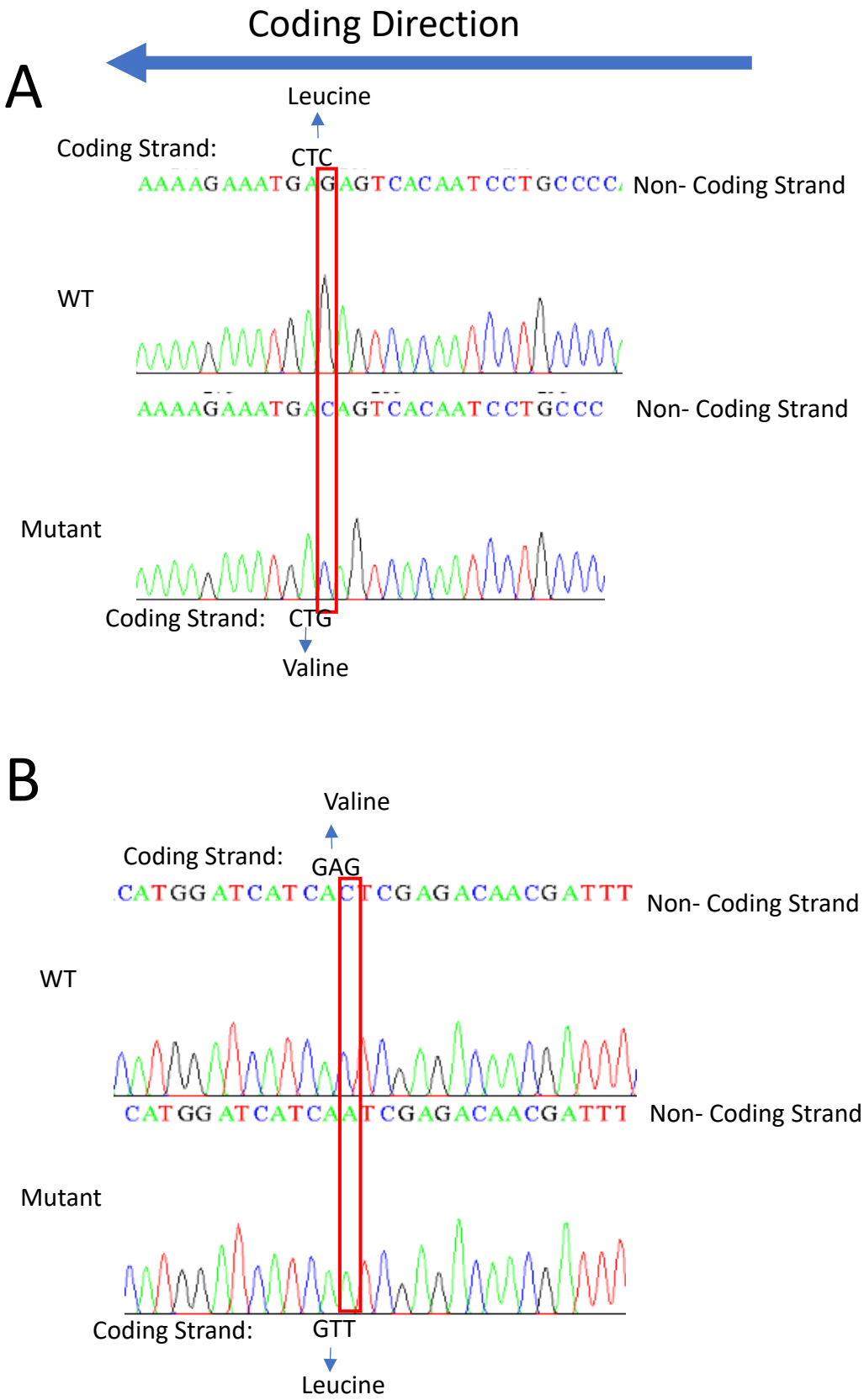


Figure 3. Mcl-1 coding region was amplified from pcDNA-Mcl-1 using PCR, followed by digestion with restriction enzyme to create sticky ends for ligation. (A) Cloning primers were flanked by BamHI and EcoRI recognition sites. Gel electrophoresis was run on 1% Agarose Gel to visualize the (B) PCR products and (C) BamHI and EcoRI digested Mcl-1 PCR products and pBABE empty vector.


Coding Direction
A
Leucine
Coding Strand:
CTC
Non- Coding Strand
WT
Non- Coding Strand
Mutant
Coding Strand:
CTG
Valine
B
Valine
Coding Strand:
GAG
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WT
Non- Coding Strand
Mutant
Coding Strand:
GTT
Leucine

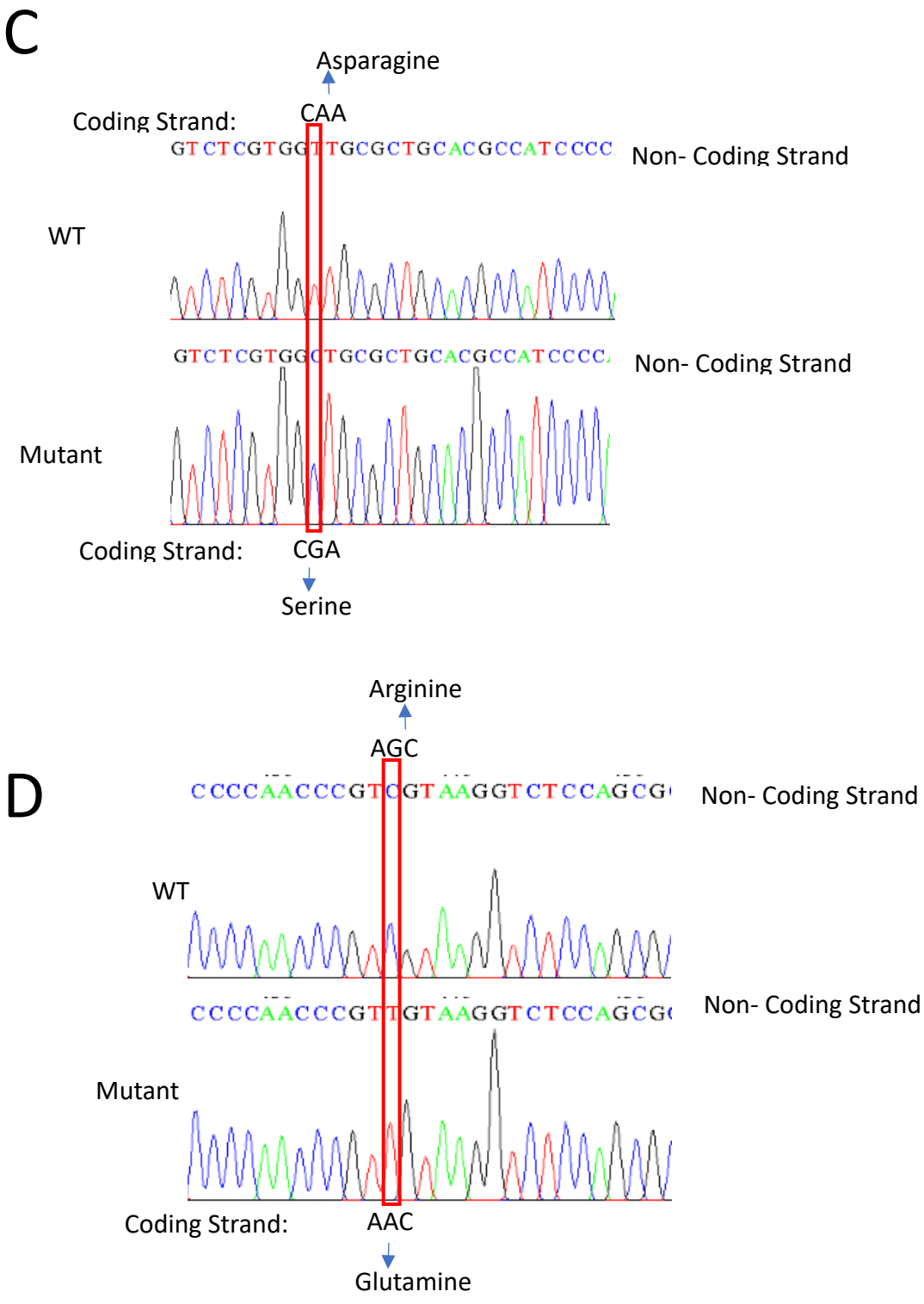


Figure 4. Successful incorporation of single-nucleotide change in pBABE-Mcl-1 constructs was observed after site-directed mutagenesis. Sequencing results show the incorporation of the

single nucleotide change in the mutant constructs: (A) pBABE-Mcl-1 L267V, (B) pBABE-Mcl-1 V249L, (C) pBABE-Mcl-1 N223S, and (D) pBABE-Mcl-1 R214Q. The 3’ pBABE sequencing primer was used to sequence 100 ng of each construct. The red box indicates the position of the nucleotide change.

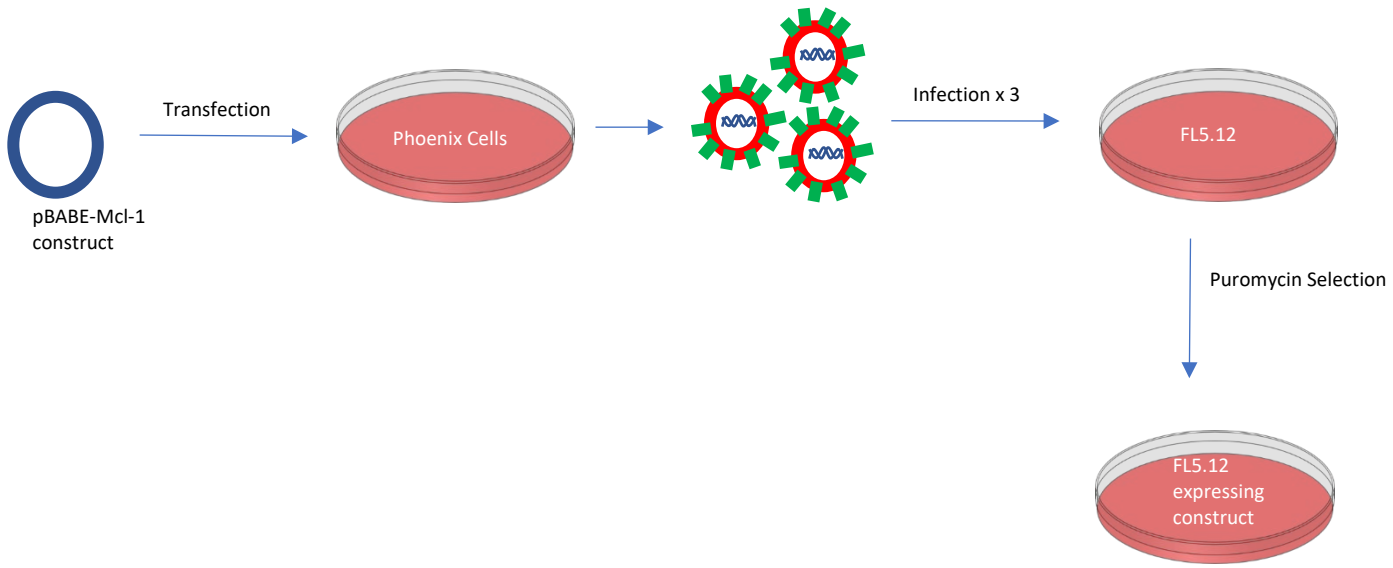


Figure 5. Schematic showing the process of generating FL5.12 cells that stably express pBABE-Mcl-1. Eight µg of pBABE construct was transfected into Phoenix cells using Lipofectamine 2000. The virus generated by the Phoenix cells were collected and used to infect FL5.12 cells at 36, 48, and 60 hours after transfection. 24 hours after final infection, cells were cultured in puromycin to select for those that incorporated the pBABE construct, which contains a puromycin resistance gene.

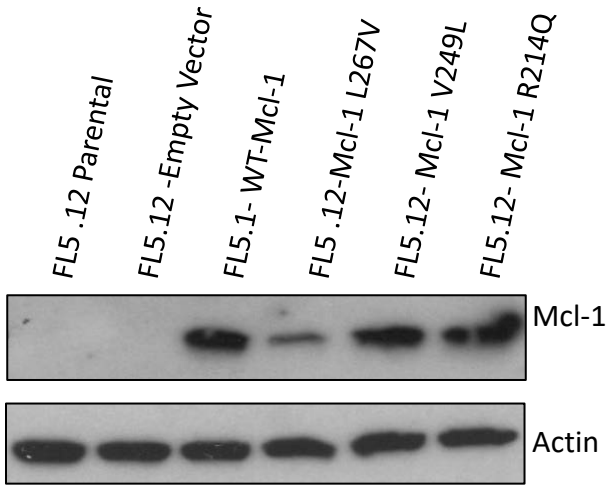
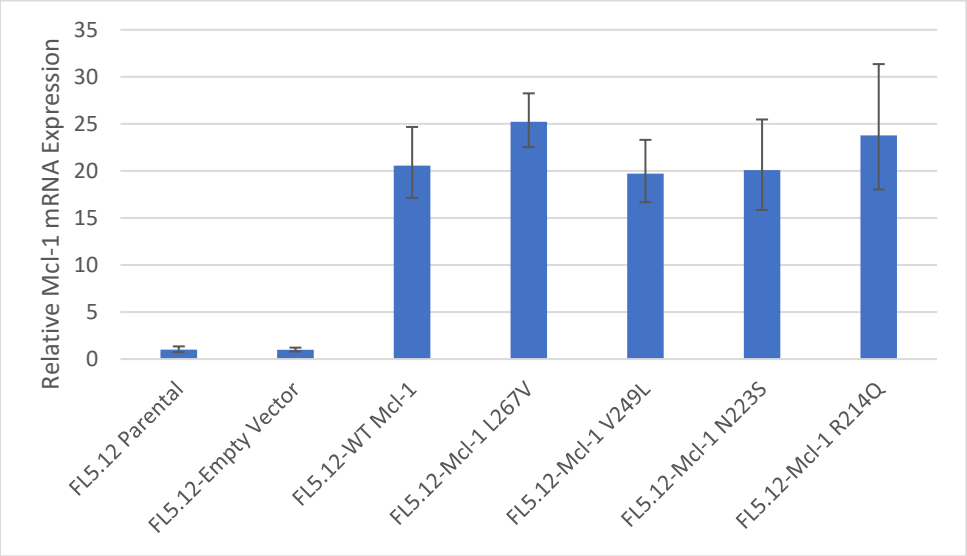


Figure 6. Mcl-1 expression differs among FL5.12 cells that stably overexpress pBABE-Mcl-1 constructs. Western blot analysis using the Mcl-1 and Actin antibodies was performed on FL5.12 cell lysates. Thirty µg of protein lysates of each cell line was used for SDS-PAGE. The blot shown is a representation of 3 independent experiments. FL5.12-Mcl-1 L267V has a lower level of Mcl-1 protein expression than the other FL5.12-Mcl-1 stable cell lines.

Replicate #1



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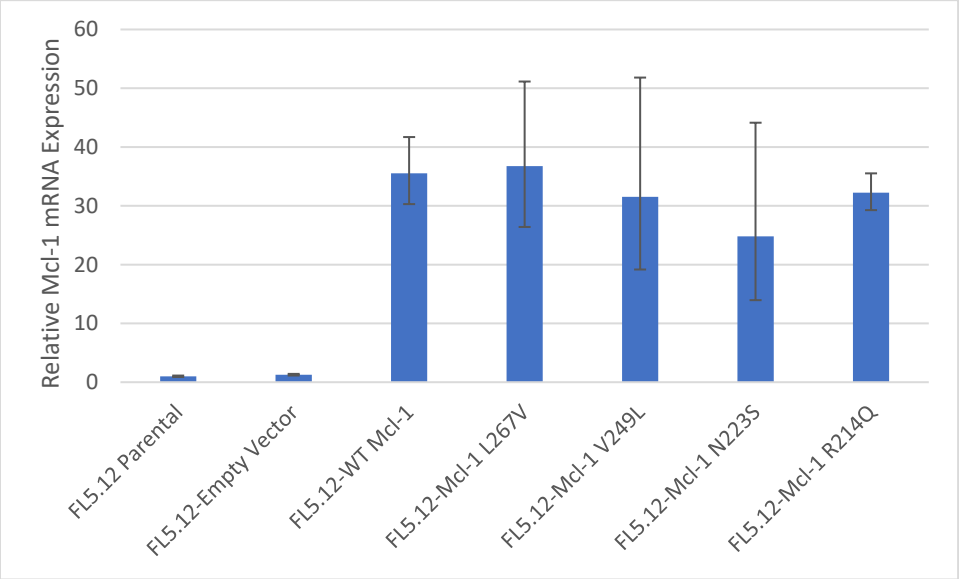


Figure 7. FL5.12 stable cell lines with incorporated pBABE-Mcl-1 constructs express Mcl-1 mRNA at comparable levels. Relative mRNA expression was calculated from Ct values obtained using RT-qPCR. The values presented are an average of duplicates in each experiment and are normalized to mouse GAPDH gene expression. All FL5.12-Mcl-1 cell lines have comparable level of Mcl-1 mRNA expression.

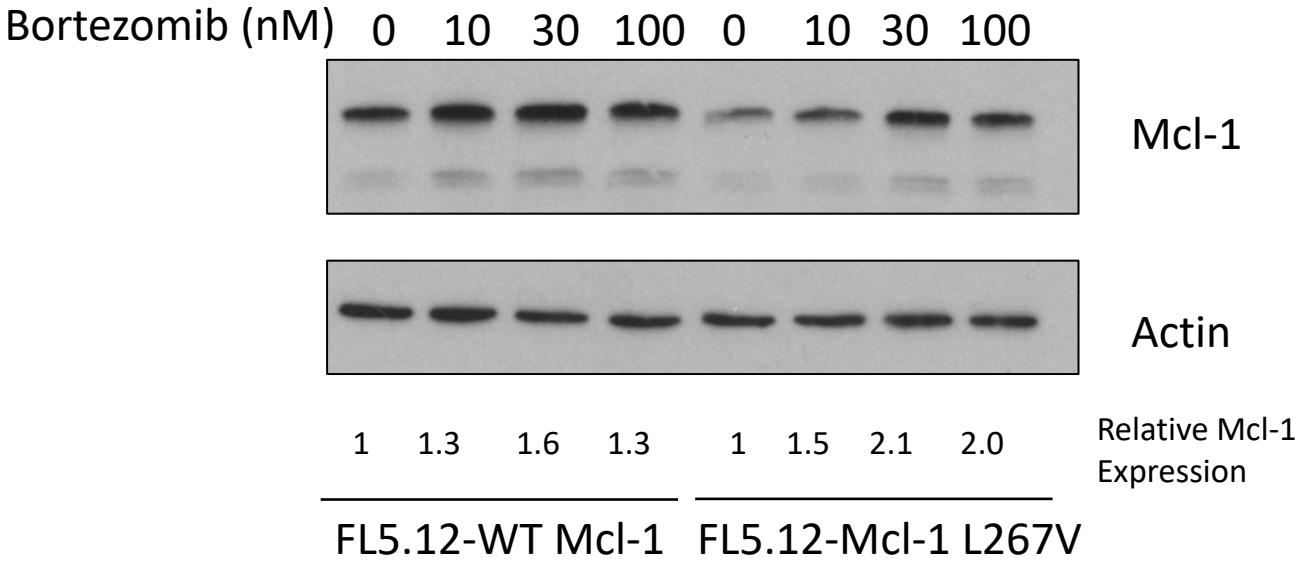


Figure 8. Proteasome inhibition increases Mcl-1 protein expression in FL5.12-WT Mcl-1 and FL5.12-Mcl-1 L267V. 750,000 cells were treated with 10, 30, 100 nM of Bortezomib for 3 hours. Cells were then collected to prepare lysates for western blot analysis with Mcl-1 and Actin antibodies. Thirty µg of protein lysate of each sample was used for SDS-PAGE. Both FL5.12-WT Mcl-1 and FL5.12-Mcl-1 L267V show an increase of Mcl-1 expression upon Bortezomib treatment. However, the fold increase is greater in FL5.12-Mcl-1 L267V cells. The blot shown is a representation of 2 independent experiments. Relative Mcl-1 protein expression was calculated using densitometry analysis of Mcl-1 expression normalized to actin expression.

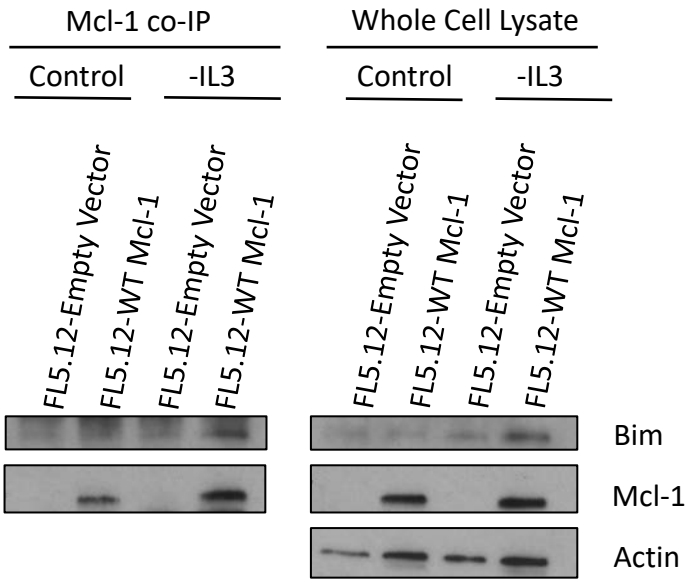


Figure 9. Human Mcl-1 interacts with mouse Bim, whose expression increases under IL-3 withdrawal. FL5.12-Empty Vector and FL5.12-WT Mcl-1 cells were subjected to IL-3 withdrawal, and protein lysates were prepared for Mcl-1 co-immunoprecipitation and for whole cell lysate western blot analysis. Ninety μg of protein lysate was incubated with Mcl-1 immunoprecipitation matrix overnight and then eluted for SDS-PAGE; 20 μg of whole cell lysate was used for SDS-PAGE. There were more Bim proteins bounded onto Mcl-1 pulled down from FL5.12-WT Mcl-1 than from FL5.12 Empty Vector cells.

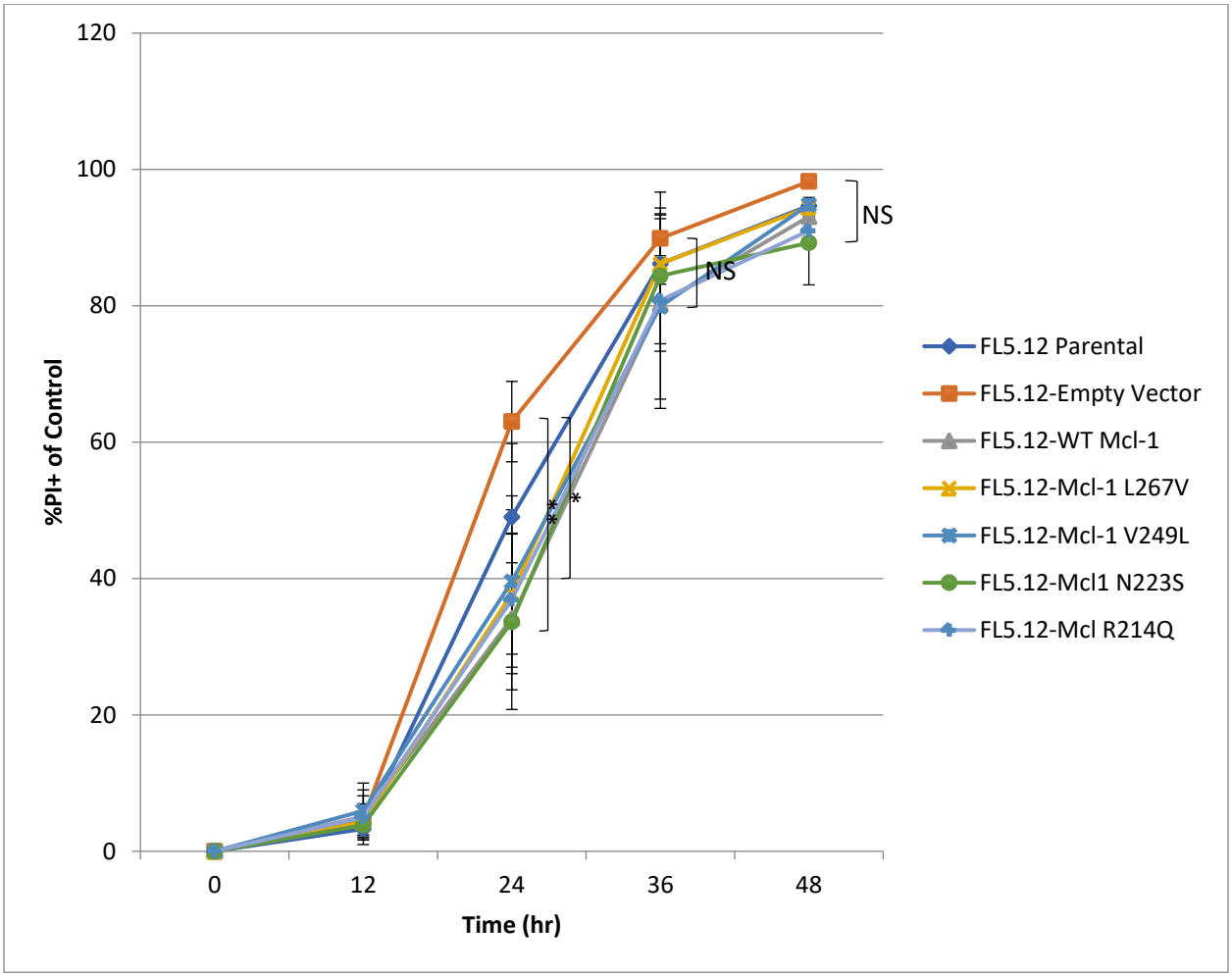


Figure 10. Overexpression of WT Mcl-1 inconsistently and transiently protects FL5.12 from IL-3 withdrawal-induced apoptosis. FL5.12 cells were subjected to IL-3 withdrawal. Cells were collected at 0, 12, 24, 36, and 48 hours after IL-3 withdrawal, and stained with Propidium Iodide(PI) to assess cell death using flow cytometry. There is significant protection by WT Mcl-1 at 24 hours compared to FL5.12-Empty Vector. However, there is no significant difference at 36 and 48 hours between FL5.12-Empty Vector and the FL5.12-Mcl-1 cell line that has the lowest percent of PI+ cells. Data shown are mean of 4 independent experiments. Statistical significance was assessed using two-tailed t-test using GraphPad (*$p<0.05$, **$p<0.01$, NS $p>0.05$).

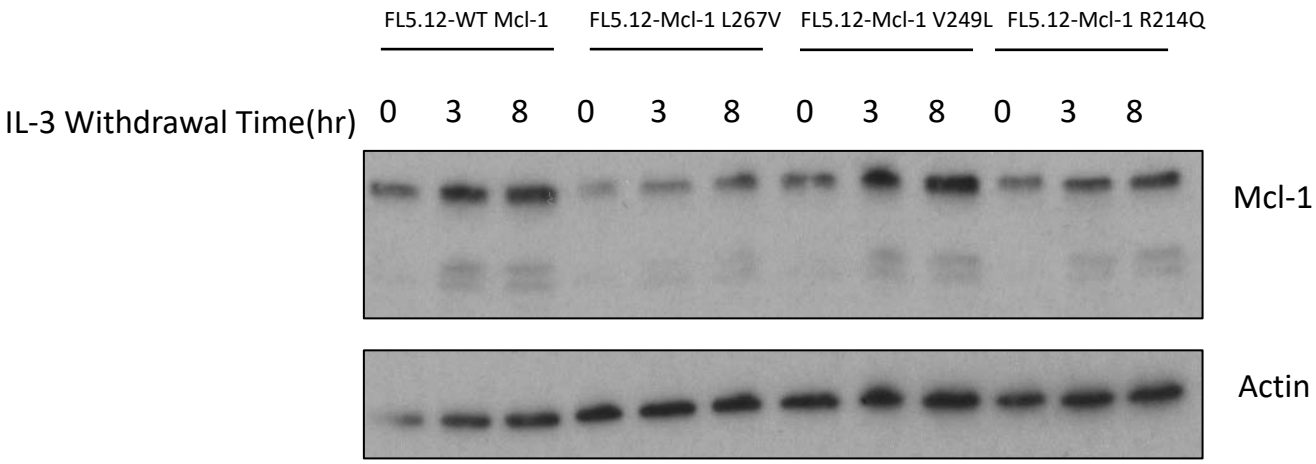
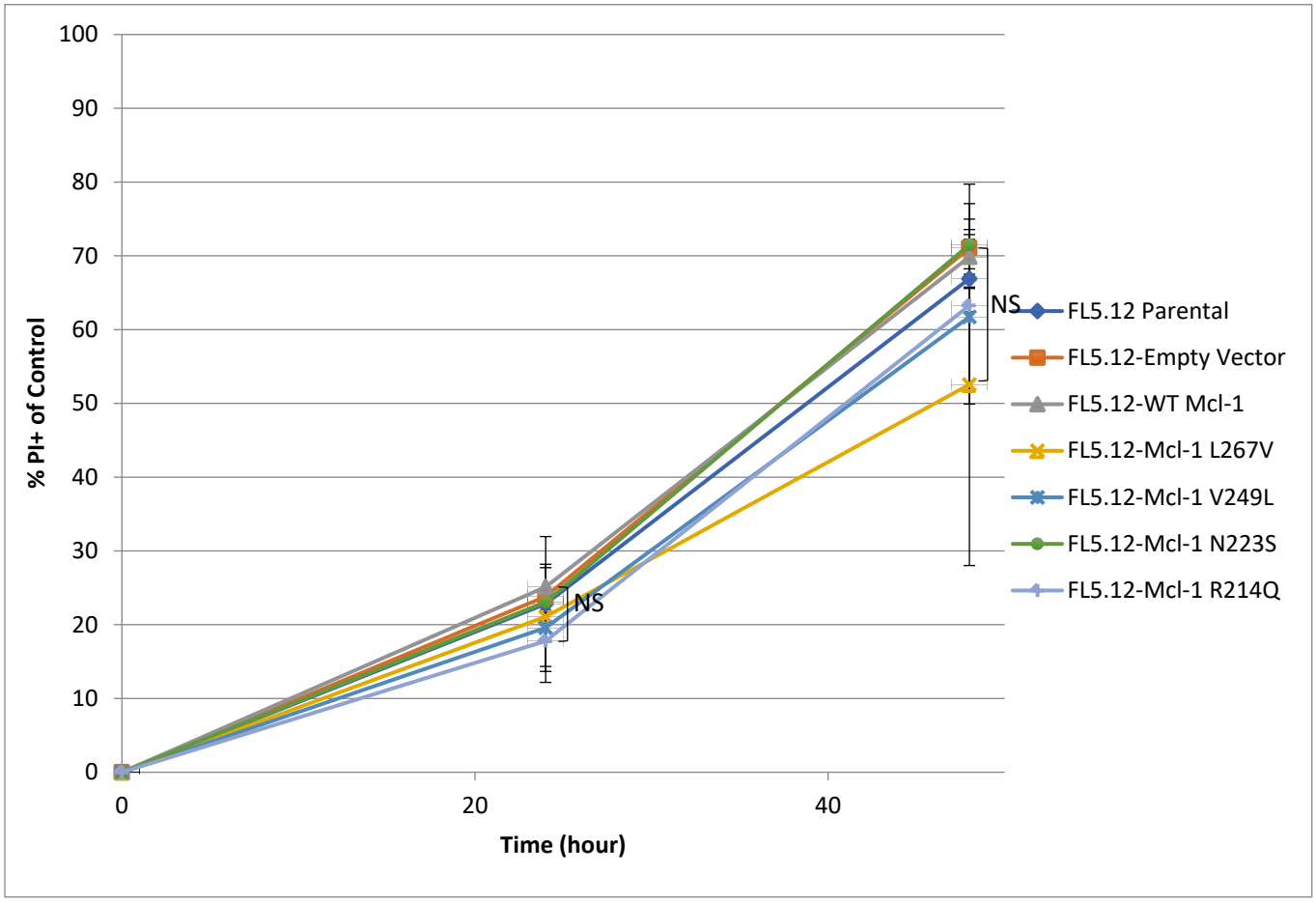
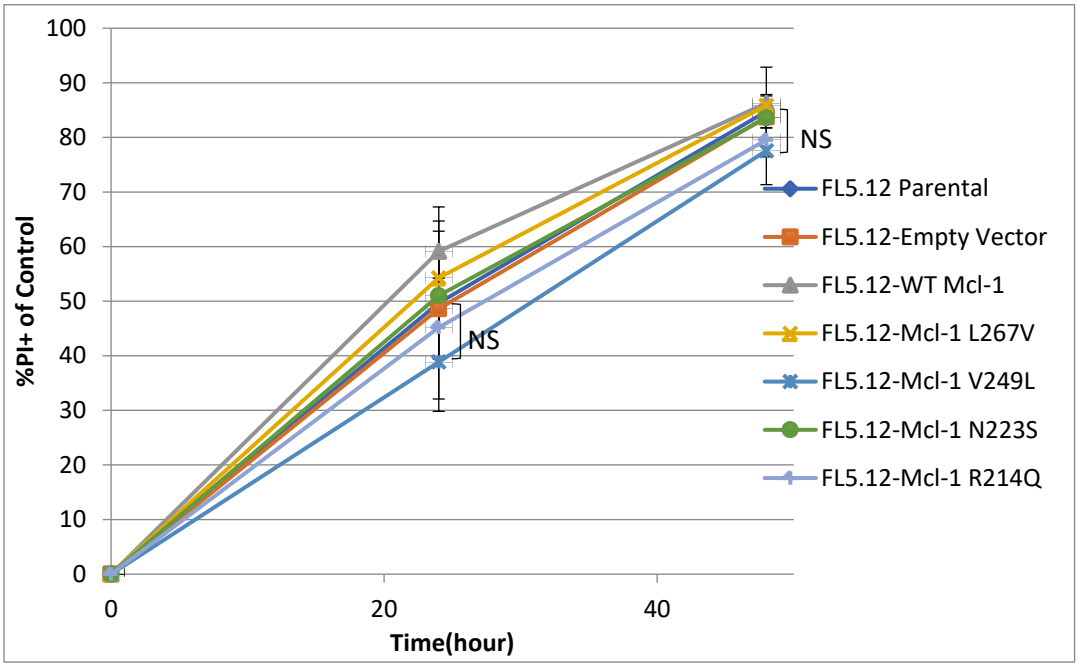


Figure 11. IL-3 withdrawal does not decrease Mcl-1 protein expression in FL5.12 stable cell lines. FL5.12-WT Mcl-1, FL5.12-Mcl-1 L267V, FL5.12-Mcl-1 V249L, FL5.12-Mcl-1 R214Q cells were subjected to IL-3 withdrawal and were collected after 0, 3, and 8 hours. Protein lysates were prepared for western blot analysis with Mcl-1 and Actin antibodies. For each sample, 30 µg of protein lysate was used for SDS-PAGE. Three and eight hours after IL-3 withdrawal, Mcl-1 protein expression does not decrease in any of the stable cell lines.

A


100
90
80
70
60
50
40
30
20
10
0
% PI+ of Control
0
20
40
Time (hour)
NS
NS
FL5.12 Parental
FL5.12-Empty Vector
FL5.12-WT Mcl-1
FL5.12-Mcl-1 L267V
FL5.12-Mcl-1 V249L
FL5.12-Mcl-1 N223S
FL5.12-Mcl-1 R214Q

B



C

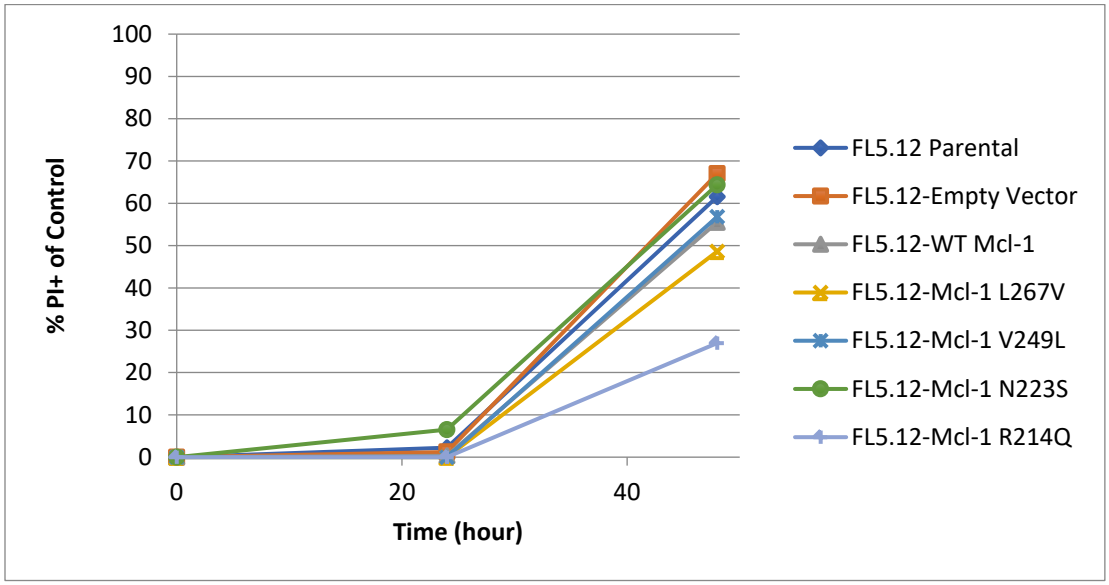


Figure 12. Overexpression of WT Mcl-1 in FL5.12 cells cannot protect against chemotherapy agents-induced apoptosis. FL5.12 cells were treated with (A) 0.1 µg/mL of Vincristine, (B) 10

μg/mL of Etoposide, and (C) 5 μg/mL of Cisplatin. Cells were collected 0, 24, and 48 hours after treatment and were stained with PI to assess cell death using flow cytometry. After 24 and 48 hours of Vincristine or Etoposide treatment, there is no significant difference between the percentage of PI+ FL5.12-Empty Vector cells and the FL5.12-Mcl-1 cell line that has the lowest percent of PI+ cells at each time point. For (A) and (B), data shown are mean of 3 independent experiments. Statistical significance was assessed using two-tailed t-test using GraphPad (NS $p>0.05$). For (C), data shown is from a single independent experiment.

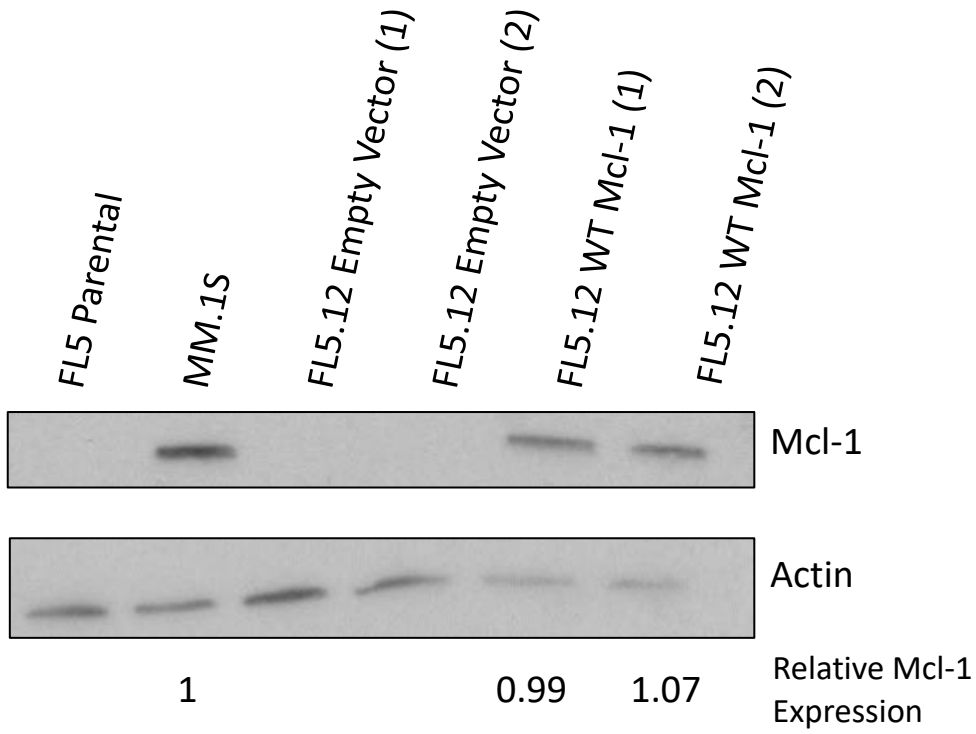
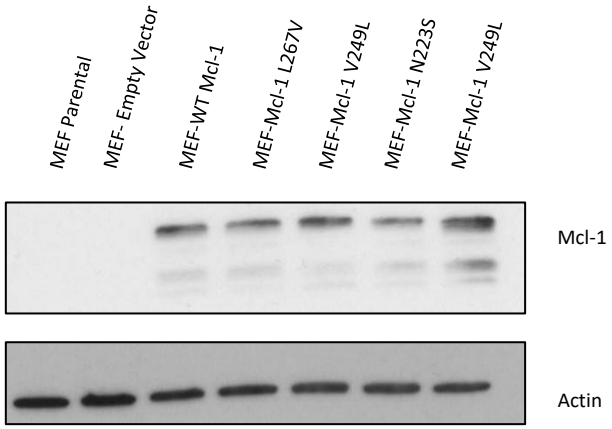


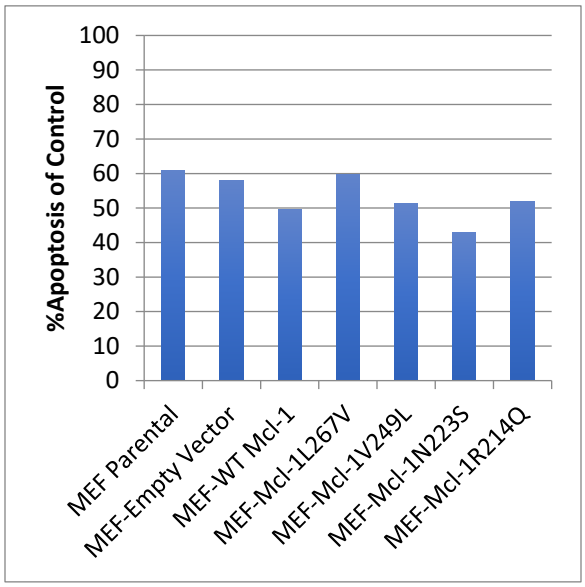
Figure 13. FL5.12-WT Mcl-1 stable cell lines express comparable level of Mcl-1 protein as myeloma cell line MM.1S. Protein lysates from MM.1S and 2 different sets of FL5.12 stable cell lines were prepared. Western blot analysis using Mcl-1 and Actin antibodies was performed on FL5.12 cells lysates. Thirty μg of protein lysates of each cell line was used for SDS-PAGE. Relative Mcl-1 protein expression was calculated using densitometry analysis of Mcl-1 expression normalized to actin expression.

A



B

Replicate #1



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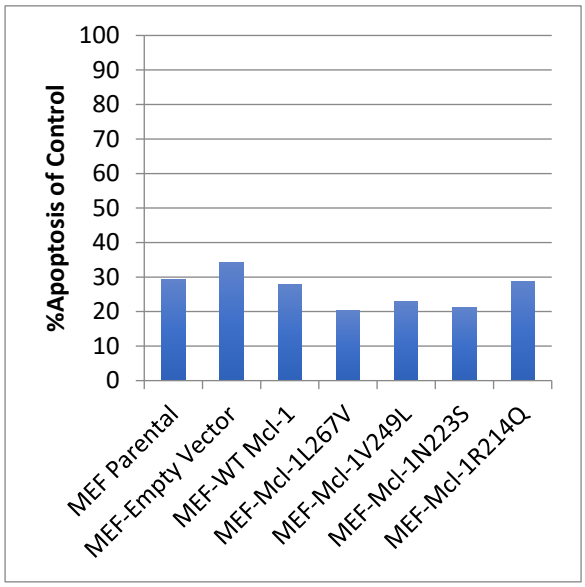

Figure 14. (A) Mouse Embryonic Fibroblast (MEF) cells that stably overexpress Mcl-1 were generated. (B) Stable overexpression of WT Mcl-1 does not protect MEF cells from serum starvation-induced apoptosis. (A) Western blot analysis using Mcl-1 and Actin antibodies was performed on MEF cells lysates. Thirty µg of protein lysates of each cell line was used for SDS-PAGE. (B) MEF cells were subjected to serum starvation for 48 hours. Cells were then harvested

and stained with Annexin V-FITC and PI to assess cell death using flow cytometry. Percentages of PI+ cells are comparable across all MEF cell lines under serum starvation.

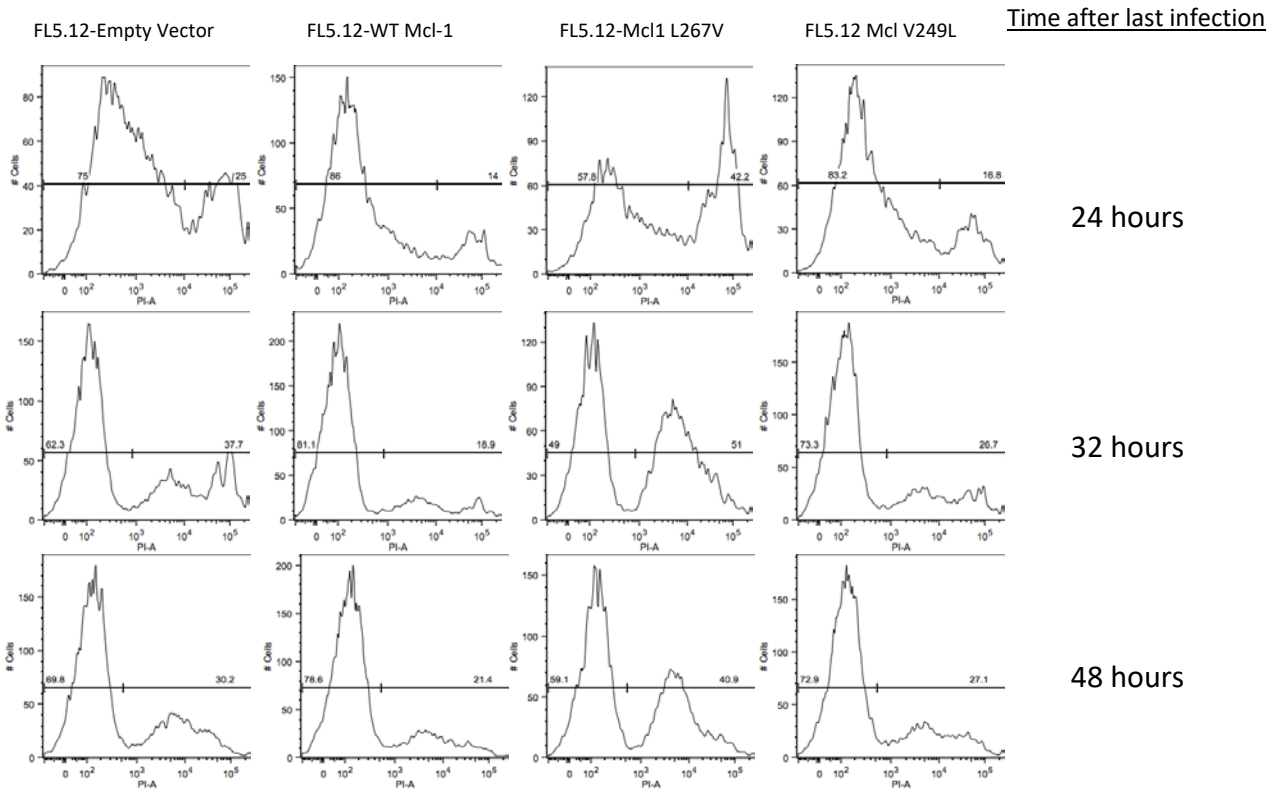

Figure 15. Overexpression of human WT Mcl-1 can protect FL5.12 against apoptosis induced by transient pBABE-human Bim infection. Eight µg of pBABE-human Bim was transfected into Phoenix cells using Lipofectamine 2000. The viruses generated by the Phoenix cells were collected and used to infect FL5.12 stable cell lines (FL5.12-Empty Vector, FL5.12-WT Mcl-1, FL5.12-Mcl-1 L267V, and FL 5.12-Mcl-1 V249L). Cells were collected 24, 32, and 48 hours after the final round of infection and stained with PI to assess cell death using flow cytometry. There is a lower percentage of PI+ FL5.12-WT Mcl-1 cells than PI+ FL5.12-Empty Vector cells at 24, 32, and 48 hours.

Discussion

Many previous studies have shown the importance of Mcl-1 expression in promoting survival and proliferation of Multiple Myeloma cells[13,38]. Therefore, studying the outcomes of myeloma patients with Mcl-1 mutations and how these specific mutations contribute to their outcomes can provide additional insight into molecular mechanisms of Mcl-1 function as an anti-apoptotic protein. This research could then provide better understanding of its role in Multiple Myeloma.

In our study, we were able to generate pBABE constructs that contain 4 of the mutations observed in patients--L267V, V249L, N223S, and R214Q. We used these constructs to generate FL5.12 cells that stably express them so that we can assess these mutations' effects on Mcl-1's stability and function. Our hypothesis that the mutations observed in patients' Mcl-1 lower the protein's anti-apoptotic function was supported in our experiments of the L267V and V249L mutants thus far. We showed that the L267V mutation destabilizes the Mcl-1 protein, which correlates with the lack of protection of FL5.12-Mcl-1 L267V against Bim-overexpression-induced apoptosis. This destabilization of the Mcl-1 protein perhaps lowers the threshold for apoptosis in cells expressing this mutant. Thus, destabilizing mutations in Mcl-1 observed in myeloma patients could mean that these patients are more susceptible to treatment.

The ribbon structure of the Mcl-1 functional domain provide potential explanations for our observations. For the L267V mutation, the change to Valine, which is smaller than Leucine, could have taken away the interaction of the original leucine side chain with the other side of the molecule, and thus this mutation destabilizes the protein (Figure 16). The V249L mutation

lies near where the BH3-domain of Bim binds Mcl-1; the change from Valine to Leucine at this position could be interfering with the binding of Bim to Mcl-1 (Figure 16).

As we demonstrated in our study, Mcl-1 is subjected to some level of degradation even when the cells are not under cellular stress. It is important to note that previous studies have shown that its rapid turnover contributes to its ability to regulate apoptosis[39]. While this could have potentially been the reason we were not able to obtain consistent and long-term protection against IL-3 withdrawal-induced apoptosis, we showed that Mcl-1 protein level does not decrease under IL-3 withdrawal.

We also observed protection by overexpression of Mcl-1 in our experiments where we transiently infected cells with pBABE-human Bim. While we showed through co-IP western blot analysis that human Mcl-1 can interact with mouse Bim, it is possible that this human-mouse interaction is weaker than same species Mcl-1 and Bim interaction. This possibility would explain the transiency or lack of protection by Mcl-1 in our other functional experiments. While mouse Bim only differs from human Bim by 3 residues[40], the differences between cross-species and same-species Mcl-1 and Bim interaction will require further investigation.

For our future directions, if the cross-species interaction is an issue, we could generate human cell lines that stably overexpress human Mcl-1. Furthermore, our experiments show that overexpression of Mcl-1 cannot protect against Vincristine, Etoposide, and Cisplatin even transiently. Therefore, it would be interesting to figure out why Mcl-1 can only protect against certain death-signals. IL-3 withdrawal affects the P13K/AKT pathway, which is the signaling pathway activated by IL-3 receptor binding[41]. Vincristine, Etoposide, and Cisplatin all have also been shown to be able to affect the AKT pathway[42-44]. Perhaps these drugs affect another

survival pathway or affect P13K/AKT pathway with different mechanisms that Mcl-1 overexpression cannot protect. We also would like to investigate how the V249L mutation makes Mcl-1 less effective at blocking apoptosis induced by transient overexpression of human Bim. The V249L mutation does not appear to be destabilizing the protein according to our results. Thus, its effect could be on the binding affinity of Mcl-1 to Bim, which could be tested by performing co-immunoprecipitation of Mcl-1 and probing for Bim in the FL5.12-Mcl-1 V249L cells. Finally, we would also like to investigate how Mcl-1 mutations observed in myeloma patients that do not fall in the functional domain affect Mcl-1's stability and function. Those that fall in the PEST region of Mcl-1 could affect the regulation of Mcl-1 degradation[45]. These proposed future directions will allow us to understand more precisely how myeloma-derived amino acid substitutions in Mcl-1 affect the protein and how they contribute to patients' responses to therapy.

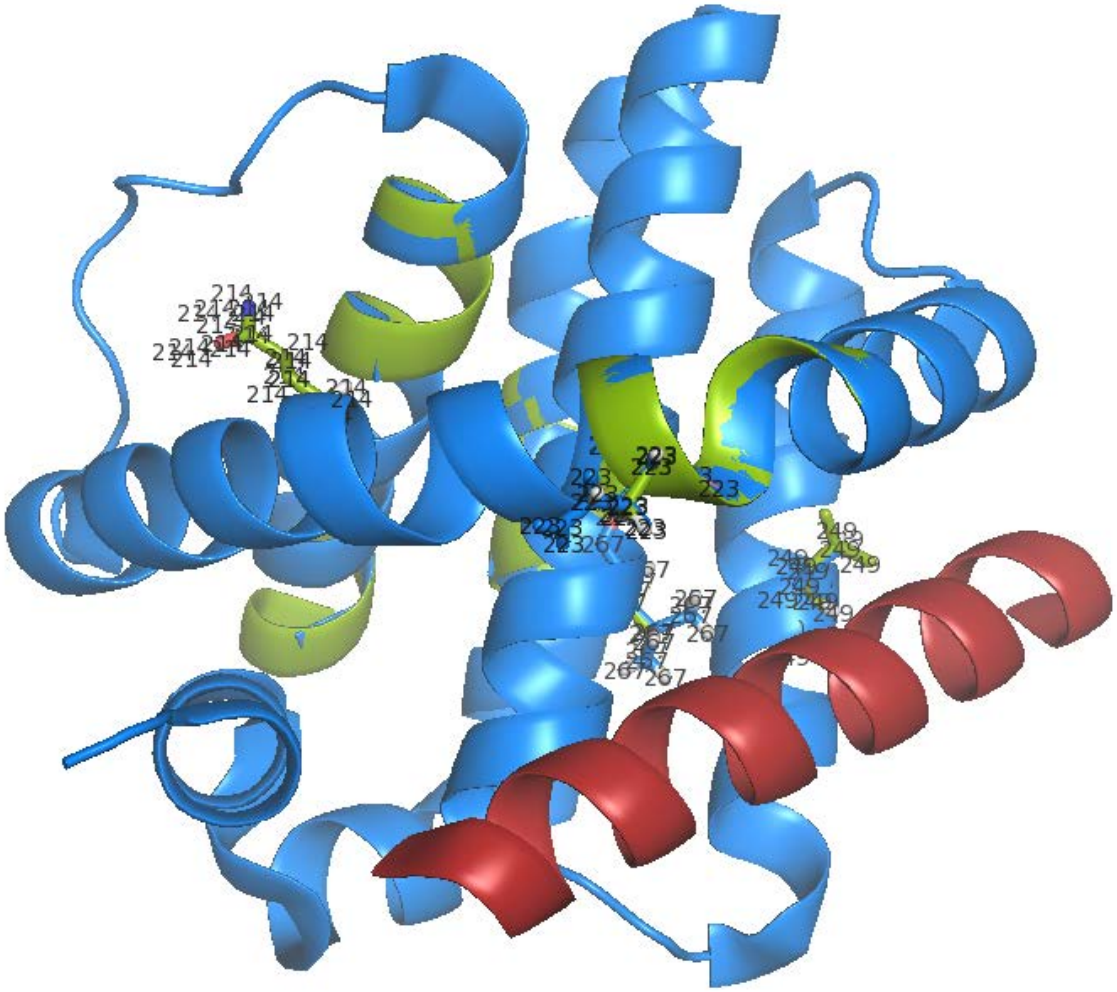

Figure by Dr. Lawrence Boise

Figure 16. Ribbon structure of Mcl-1 binding to Bim (red). Mcl-1 mutations L267V, V249L, N223S, R214Q (green) are overlaid on WT-Mcl-1 (blue). The functional domain of Mcl-1 is shown here interacting with the BH3-domain of Bim.

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