Mutations in MCL-1 PEST Region of Breast Cancer on Binding Affinity to Bak/Bax

Deeksha Jain

BNFO 300

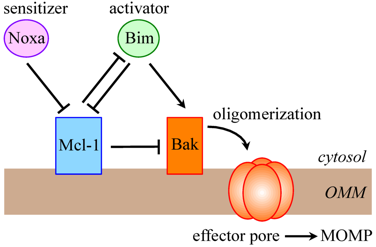
05/07/2017

1. **Introduction**

Cancer is the second leading cause of death in the United States with breast cancer being the most common type of cancer in females (Center for Disease Control). Cancer is a disease of dysregulation, resulting in the unregulated growth and division of cells with an increased propensity to form malignant tumors. A hallmark of cancer is to surpass apoptosis, the process for mediated cell death of unregulated or harmful cells. Apoptosis is vital for the removal of cancerous, damaged, and unnecessary cells within the body. The presence of cancer in the body imposes a stress onto the cells which results in apoptotic signaling. Cancer mechanisms block this signaling to allow for increased proliferation and eventually metastasis.

Myeloid Cell Luekemia-1 (MCL-1) is a protein from the BCL-2 family, which are key regulators of the apoptotic process through the induction or inhibition of apoptosis. Past research has reported amplifications of MCL-1 with increased frequency in human cancers (Wei *et al.,* 2012). Subsequent experimentation has proved the integral functioning of MCL-1 in anti-apoptotic activity with complete knockout (KO) procedures (Vick, *et al.,* 2008). Furthermore, knockout of one allele of MCL-1 has implicated the need for both functioning alleles of MCL-1 for proper anti-apoptotic functioning (Grabow *et al.,* 2016).

MCL-1 is an anti-apoptotic gene which functions by sequestering pro-apoptotic proteins Bak and Bax via hydrophobic grooves (Thomas, Lam, & Edwards, 2010). Heterodimerization of Bak or Bax by MCL-1 prevents them from oligomerizing via a conformational change. Oligomerization of Bak/Bax allows for the aggregated body to translocate to the outer mitochondrial membrane (OMM). From there, Bak/Bax work to pierce and form pores within the mitochondrial membrane to stimulate the release of cytochrome C into the cytoplasm, resulting in the initiation of apoptosis (Thomas, *et al.*, 2010). This process is called the mitochondrial outer membrane permeabilization (MOMP). Once inside the cytoplasm, cytochrome C induces the activation of caspases in what is called the caspase cascade (Thomas, *et al.*, 2010). Caspases are responsible for the macromolecular degradation of lethal cells via proteolytic activity. Additionally, MCL-1 can bind to a direct activator of Bak/Bax called Bim to prevent apoptosis (Mojsa, Lassot, & Desagher, 2014). Binding of Bak/Bax by MCL-1 prevents that process and thus the initiation of apoptosis. Noxa can bind to MCL-1 to relieve Bak/Bax and allow for apoptotic activity (Mojsa *et al.,* 2014). The pathway is elucidated in **Figure 1**.

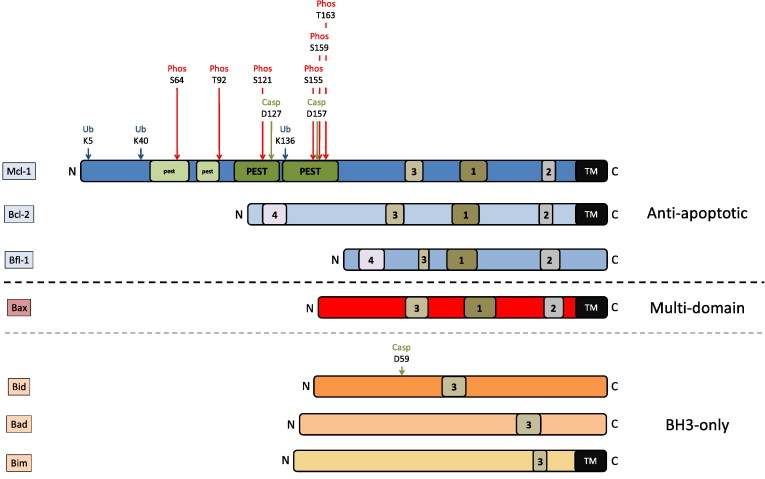


Release of cytochrome c

**Figure 1:** Protein interaction pathway of MCL-1 with Bak/Bax in apoptosis. Adapted from Figure 1 of Mojsa *et al.*

Bax

The MCL-1 gene contains, unlike other BCL-2 genes, a PEST region towards the N-terminus which is rich in proline (P), glutamic acid (E), serine (S), and threonine (T). The PEST region is responsible for the short half life of MCL-1 through enhancement of MCL-1’s degradation (Germain & Duronio, 2007). More importantly, the PEST region is the primary site of post-translational modifications, significantly including phosphorylation, caspase cleavage, and ubiquination (**Fig. 2**).



**Figure 2:** PEST sequence and sites of post-translational modifications within MCL-1 gene in comparison to BCL-2. Adapted from Figure 2 of Thomas *et al.*

Phosphorylation is integral to the functioning of MCL-1 and its anti-apoptotic activity (Thomas, *et al.*, 2010). Phosphorylation at specific sites within these phosphoresidues has been shown to affect thestability of MCL-1 and its interactions with other proteins/pathways, notably ERK and BIM, resulting in varying degrees of apoptotic activity (Thomas, *et al.*, 2010). Deletion of this domain has been shown to impair both mitochondrial localization and anti-apoptotic activity, signifying its vital role in proper functioning of MCL-1 (Germain & Duronio, 2007). Induced mutations within this domain and the phosphoresidues have been shown to affect the anti-apoptotic activity of MCL-1 (Thomas, *et al.*, 2010).

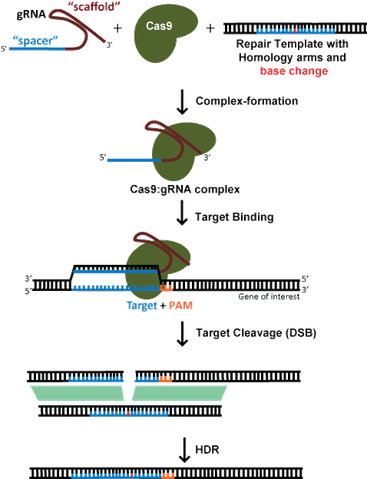
The question posed now is ***how*** are mutations within this PEST region affecting the anti-apoptotic activity of MCL-1? It is known from Thomas *et al.* that phosphorylation of and mutations at these residues affect the functioning of BIM, but it is unclear whether mutations within this region affect the binding affinity of MCL-1. The aim of this investigation is to determine whether mutations within the PEST region of MCL-1 affect its ability to bind Bak/Bax for anti-apoptotic measures.

1. **Experiment**

In order to assess whether mutations within the PEST region of MCL-1 affects its ability to bind Bak/Bax, there will need to be multiple preceding steps which will have to take place. The basic set up to test for binding affinities will include the use of previously established MCL-1 KO breast cancer cell lines and breast cancer cell lines with fully functional MCL-1 obtained externally from ABMGood per Grabow *et al*. Three cell lines will be used within the experimental set up. One cell line will remain as a complete KO cell line, whereas another will have a mutated version of MCL-1 introduced into it. The last cell line for comparison will have the fully functional MCL-1 as stated above. Then will be an incubatory period followed by a biochemical assay test to determine binding affinities.

**Mutation Creation via CRISPR/Cas9 Technology and Insertion into Cell Lines**

A mutation will be created within the PEST region of MCL-1 using the CRISPR/Cas9 genome editing technology. CRISPR/Cas9 is a site directed mutagenesis tool used to make changes to the DNA sequence of a gene within a certain location. The process is outlined in **Figure 3**. To introduce a mutation within a gene, in this case MCL-1, via CRISPR/Cas9 technology, a guide RNA (gRNA) sequence must be established (Doudna & Charpentier, 2014) (Addgene). To establish this gRNA, first the genome sequence of MCL-1 needs to be assessed to identify a unique gene sequence for modification, in this case the PEST region. This can be done via the assistance of NCBI BLAST for both identification and primer design. Once identified, a gRNA strand is designed complementary to the target sequence which will form an endonuclease by associating with Cas9. It is vital to choose a Cas9 variant that has a relatable PAM, protospacer adjacent motif, sequence (any-guanine-guanine) directly downstream the target sequence to allow for binding of the nuclease (Addgene) (Hsu, Lander, & Zhang, 2014).



**Figure 3:** Cas9 endonuclease formation and functionality. Adapted from Figure 4 of Addgene*.*

To design a gRNA, the sequence of interest from within the target gene needs to be identified and isolated via NCBI BLAST. Amino acids 85-176 of the MCL-1 gene are suspected to contain high levels of PEST sequences ie nucleotides ~250-535 (Domina, Vrana, Gregory, Hann, & Craig, 2004). For this reason, the aforementioned range from MCL-1 will be used to identify a PEST residue and mutate it, to confer a non-residue specific, albeit site specific, mutation within the PEST region. Specifically, a gRNA will be designed unique to that region. Sequence was located using UniProt and finding the MCL-1 protein with a PEST-like region and locating the compatible NCBI nucleotide sequence (accession ID BC017197). Nucleotides ~251-540 were obtained from the sequence.

**Target Sequence**:

5’CGTCCACCCTCACGCCAGACTCCCGGAGGGTCGCGCGGCCGCCGCCCATTGGCGCCGAGGTCCCCGACGTCACCGCGACCCCCGCGAGGCTGCTTTTCTTCGCGCCCACCCGCCGCGCGGCGCCGCTTGAGGAGATGGAAGCCCCGGCCGCTGACGCCATCATGTCGCCCGAAGAGGAGCTGGACGGGTACGAGCCGGAGCCTCTCGGGAAGCGGCCGGCTGTCCTGCCGCTGCTGGAGTTGGTCGGGGAATCTGGTAATAACACCAGTACGGACGGGTCACTACCCTCG3’

Once the target region’s sequence is isolated, it is inserted into the CRISPR design online tool by Massachusetts Institute of Technology to obtain a gRNA (MIT). The score given assesses the quality of the guide in regards to inverse likelihood of off-target binding. The derived gRNA sequence with the highest score of 96 and PAM of 5’CGG3’ is as depicted below:

**Guide RNA**: 5’ GTAATAACACCAGTACGGAC 3’

Following creation of a gRNA, a repair template has to be assembled containing the desired sequence with the mutation(s) to be introduced *without* a PAM sequence region to ensure the mutation isn’t cleaved by the nuclease. The PAM sequence needs to be downstream of the induced mutation. A mutation will be induced upstream of the PAM sequence within the PEST region where the nucleotides are 5’AGT3’, translating to 5’UCA3’ which is serine, a residue found in rich amounts within the PEST region. The middle G will be mutated into an A, reading 5’AAT3’, resulting in serine (UCA) being coded to leucine (UUA). Leucine has not been indicated to specifically assist in interacting with MCL-1 stability or anti-apoptotic activity unlike threonine (Domina *et al.*, 2005). The repair template constructed is as below:

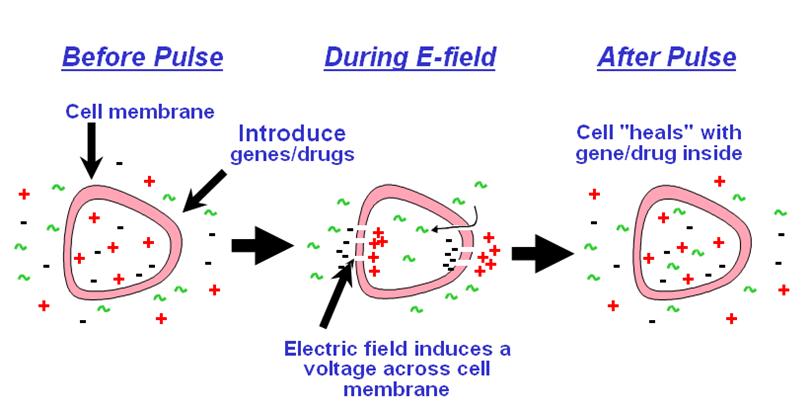
**Repair Template**: 5’ TGCCGCTGCTGGA**A**TTGGTCG’

The target sequence is shown below with both the gRNA and repair template for visualization of alignment. The repair template is highlighted in yellow, with the mutation region in red. The gRNA is highlighted in green and the PAM sequence is labeled in fuchsia. The overlap between the repair and the gRNA is highlighted in sky blue. Forward primer, process describe below, is highlighted in gray. Region in between is rich in **proline**, glutamic acid, serine, threonine.

5’CGTCCACCCTCACGCCAGACTCCCGGAGGGTCGCGCGGCCGCCGCCCATTGGCGCCGAGGTCCCCGACGTCACCGCGACCCCCGCGAGGCTGCTTTTCTTCGCGCCCACCCGCCGCGCGGCGCCGCTTGAGGAGATGGAAGCCCCGGCCGCTGACGCCATCATGTCGCCCGAAGAGGAGCTGGACG**GGT**ACGAGCCGGAGCCTCTCG**GGA**AGC**GGC**CGGCTGTCCTGCCGCTGCTGGA**G**TTGGTCGGGGAATCTGGTAATAACACCAGTACGGACGGGTCACTACCCTCG3’

Once designed, the gRNA oligos will be developed and cloned into a plasmid using standard restriction-ligation cloning (Addgene) (Doudna & Charpentier, 2014).

The gRNA/Cas9 complex and repair template will be delivered to the cells via electroporation*.* Electroporation is a transfection method compatible for infection into transformed cell lines and stem cells. Electroporation is a technique which uses electrical pulses to create temporary pores within the cell membrane from which foreign DNA can enter (Potter, 2003). The pores are conductive hence charged molecules, such as gRNA and Cas9, can cross the membrane and enter the cell successfully. The general process for conducting electroporation includes suspending the target cell solution into a cuvette containing two aluminum electrodes on the side. Following that, the plasmid to be transformed is mixed with the cell solution and the cuvette is placed within an electroporator (Potter, 2003). Once a defined voltage setting has been established following optimization of minimizing cell death, an electric current is induced throughout the cuvette ends, and therefore the solution. Immediately following completion of the current, liquid growth medium is added to the solution, the solution is incubated for cell recovery and expression within the plasmid, and a culture is spread to confirm transformation (PoFortter, 2003). Of note, during this process, it is ideal to dissolve nucleic acids in a buffer or water solution as high salt content and proteins can lower the efficiency of the procedure through electrical discharge. The technique is elucidated in **Figure 4**.



**Figure 4**: Basic electroporation technique. Adapted from Figure 1 of *Harvard Bioscience.*

Once transfection is successful, the gRNA/Cas9 complex will then move forward to seek out the DNA sequences that are complementary to the gRNA to facilitate in a double strand break. Following the cleavage of the target sequence, the repair template will be included into the gene via a homology directed repair or HDR (Addgene) (Hsu, Lander, & Zhang, 2014).

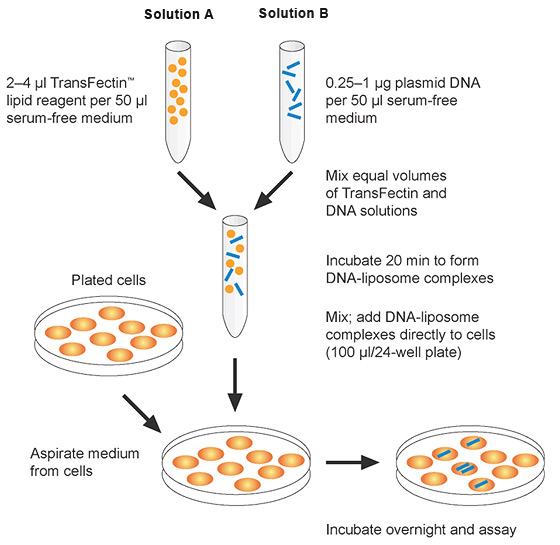
Once the the aforementioned templates have been delivered and mutation has been induced, it is necessary to validate that the genome editing worked. Validation of mutation creation will occur via PCR amplification the plasmid followed by gel electrophoresis for confirmation of necessary amplicon (Addgene) (Doudna & Charpentier, 2014). Primer sequences were designed using NCBI BLAST and Primer3. First ~200 nucleotides of the MCL-1 gene were inputted into the primer design software. Primers were analyzed and ensured to begin before the induced mutation. Primer sequences specific to the aforementioned target region is listed below:

**Forward Primer**: 5’ CATCATGTCGCCCGAAGAGG 3’

**Reverse Primer**: 3’ CCCGTCCGTACTGGTGTTATT 5’

For more accurate validation results, Sanger or next generation sequencing (NGS) can be used, which would give additional insight into off target results of the procedure (Addgene) (Hsu, Lander, & Zhang, 2014).

Following validation of successful genome editing, this new entry vector containing the DNA of interest needs to be transferred over to a destination vector, i.e. the established cell lines. This plasmid transfection will be done via lipofection as described in Demelesh *et al.* Lipofetion is a technique used to inject genetic material into a cell by the means of liposomes, or vesicles which merge with the cell membrane easily as they both contain a phospholipid bilayer. The Lipofectamine 2000 (Life Technologies) kit will be used as described by the manufacturer to open pores within the cell membranes. Cell lines in poly-L-lysine coated glass coverslips would be transfected with the mutated MCL-1 containing plasmid. The medium would be changed in 24 hour and cells would be incubated for 48 hours prior to verifying transgene expression. Stable transfectants would be selected with geneticin. Basic process as outlined in **Figure 5**. To verify transgene



**Figure 5**: Basic Lipofection process. Adapted from Figure 1 of BioRad.

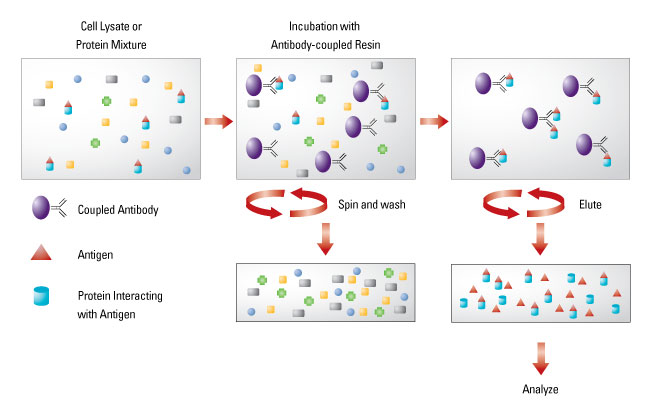
expression, PCR would be conducted to amplify significant portions of the target sequence. PCR in this step will be conducted with primers containing six histidine tags located towards the N-terminus needed in later steps for protein purification. These results can be analyzed via Western Blot following lysis of samples within cell line. Additionally, Sanger sequencing or NGS could be run again for more accurate results.

**Binding Affinity via Biochemical Assay**

Following confirmation of mutant MCL-1 gene within breast cancer cell lines and a short incubatory period, the experiment can move forward with testing the binding affinity of MCL-1 with Bak/Bax. MCL-1 firstly has to be isolated with lysing of the cells with 2% CHAPS lysis solution. MCL-1 isolation will occur using poly-histidine tags (sequences amplified in PCR above) and the QIAGEN Ni-NTA fast start protein purification kit as described by the manufacturer. Polyhistidine tags work by allowing for purification by elution of solely those proteins which express the 6x histidine sequence (Bornhorst & Falke, 2011). Following lysis of cells, the mixture is incubated with a nickel resin which has a high affinity for, and binds proteins marked with a polyhistidine tag, in this case the mutated MCL-1 gene (Bornhorst & Falke, 2011). The resin is washed with a phosphate buffer to remove all unbound contaminants and then the bound proteins are eluted with imidazole from the buffer, conferring purification (Bornhorst & Falke, 2011).

Once the protein is purified, MCL-Bak/Bax binding affinity will be tested via a co-immunoprecipitation assay as described in Vartak *et al.* and Sharma *et al.* Co-immunoprecipitation is a technique of precipitating a protein antigen out of a solution using an resin-antibody complex that binds to that specific protein, and eluting the antibody, and all bound components, including the protein of interest, and all components that interacted with the protein (Lee, 2007). In this case, the antibody/resin would bind to MCL-1, unbound components would be washed out, and the antibody eluted. When the antibody is eluted, it would pull MCL-1 out of the mixture, alongside Bak/Bax given they interacted with MCL-1 and were bound (Lee, 2007). This later allows for binding affinity testing between the aforementioned via SDS-PAGE. Co-immunoprecipitation explicated in **Figure 6** below.

**Figure 6**: Co-immunoprecipitation methodology as described above. Adapted from *ThermoFisher Scientific*.



Antibodies directed against MCL-1 needed will be purchased externally. MCL-1 has previously been purified indicating no need for a preclearing purification step within the assay itself. To move forward with the co-immunoprecipitation assay, a bound resin bead and primary polyclonal antibody raised against MCL-1 would be incubated with MCL-1 lysate (Sharma *et al.,* 2015*)* (Vartak *et al.,* 2017*)*. The beads will be washed, incubated in a buffer, and finally eluted. The protein will be resolved on SDS-PAGE. Following electrophoresis, proteins will be transferred to a PVDF membrane and re-probed with the appropriate primary antibodies for MCL-1(Sharma *et al.,* 2015*)* (Vartak *et al.,* 2017. Tubulin will be used as an internal loading control. Immunoblotting will evaluate the extent of coimmunoprecipitation and scanned by a gel documentation system for analysis. A Multi Guage (V3.0) software will be used for quantification of bands and binding affinities (Sharma *et al.,* 2015*)* (Vartak *et al.,* 2017*)*.

1. **Discussion**

The importance of PEST regions with MCL-1 and its functioning were stressed earlier. If all goes as directed, at the end, binding affinities of mutated MCL-1 for Bak/Bax will be indicated to have a decreased affinity for binding to Bak/Bax given mutations within the PEST sequences. PEST sequences are responsible for assisting MCL-1 with it’s localization and anti-apoptotic action. Given mutations in such an integral part of the gene, it can affect how the protein binds, disable to protein’s actions, or even further decrease the gene’s half life, resulting in decreased binding of MCL-1 to Bak/Bax. It is a possibility that the binding affinity of both proteins are not affected, or even that the affinity is increased for increased anti-apoptotic activity on MCL-1’s part.

A potential concern within the design of this experiment is that the experiment does not fully address past research in regards to mutations within the PEST residues and their after affects on apoptotic activity. While the region specific mutation was chosen at random, the experiment could have worked to look into the phosphoresidues and choose mutations within specific ones. The currently induced mutation additionally may have been an integral residue unknowingly. Studying the cause, effects, and past history of those would contribute to increased awareness and stricter parameters within the experiment for more meaningful results.

Another potential concern that this experiment does not address completely is the formation of insertion/deletion mutations (InDels) in off-target locations within the gene which may indirectly affecting binding abilities. While HDR is precise in its process of involving a repair template into the target region, a portion of the DNA damage, the DSB, can be repaired with non-homologous end joining, or NHEJ (Doudna & Charpentier, 2014). This can occur further away from the site of mutagenesis. NHEJ has been indicated to cause increased InDels within target sequence. While a small InDel could be harmless, it could just as much be harmful to the functionality of the gene itself.

Furthermore, future experimentations can take into account the aforementioned concerns and work to circumvent them to increase the accuracy of results given a relationship might be defined in the current study described above.

Despite the potential setbacks, decreased binding affinity for Bak/Bax translates to a major step as it reduces the anti-apoptotic activity of the gene, allowing for apoptosis to occur, and for cancer to be combatted. This can assist with mediated drug transfers and treatments within patients, or even as a preventative measure for those in remission.

**References**

Active Motif. Nuclear Complex Co-IP Kit. (n.d.). Retrieved April 2017, from

<https://www.activemotif.com/catalog/25/nuclear-complex-co-ip-kit>

Addgene. CRISPR/Cas9 Guide. Retrieved April 2017, from

<https://www.addgene.org/crispr/guide/>

Aubrey, B., Kelly, G., Kueh, A., Brennan, M., O’Connor, L., Milla, L., . . . Herold, M. (2015).

An Inducible Lentiviral Guide RNA Platform Enables the Identification of Tumor-Essential Genes and Tumor-Promoting Mutations In Vivo. *Cell Reports,10*(8), 1422-1432. doi:10.1016/j.celrep.2015.02.002

BioRad. Chemical- and Viral-Based Transfection Methods. (n.d.). Retrieved April 2017, from

<http://www.bio-rad.com/en-us/applications-technologies/chemical-viral-based->transfection-methods

Bornhorst, B. J., & Falke, J. J. (2011). Reprint of: Purification of Proteins Using Polyhistidine

Affinity Tags. *Protein Expression and Purification*. doi:10.1016/j.pep.2011.08.022

Center for Disease Control. Statistics for Different Kinds of Cancer. (2016, August 16).

Retrieved April 2017, from <https://www.cdc.gov/cancer/dcpc/data/types.htm>

Demelash, A., Pfannenstiel, L. W., Tannenbaum, C. S., Li, X., Kalady, M. F., Devecchio, J., &

Gastman, B. R. (2015). Structure-Function Analysis of the Mcl-1 Protein Identifies a Novel Senescence-regulating Domain. *Journal of Biological Chemistry,290*(36), 21962-21975. doi:10.1074/jbc.m115.663898

Domina, A. M., Vrana, J. A., Gregory, M. A., Hann, S. R., & Craig, R. W. (2004). MCL1 is

phosphorylated in the PEST region and stabilized upon ERK activation in viable cells,

and at additional sites with cytotoxic okadaic acid or taxol. *Oncogene,23*(31), 5301-5315.

doi:10.1038/sj.onc.1207692

Doudna, J. A., & Charpentier, E. (2014). The new frontier of genome engineering with CRISPR-

Cas9. *Science,346*(6213), 1258096-1258096. doi:10.1126/science.1258096

European Bioinformatics InstituteProtein Information ResourceSIB Swiss Institute of

Bioinformatics. (n.d.). European Bioinformatics Institute. Retrieved May 2017, from http://www.uniprot.org/

Grabow, S., Delbridge, A., Aubrey, B., Vandenberg, C., & Strasser, A. (2016). Loss of a Single

Mcl-1 Allele Inhibits MYC-Driven Lymphomagenesis by Sensitizing Pro-B Cells to Apoptosis. *Cell Reports,14*(10), 2337-2347. doi:10.1016/j.celrep.2016.02.039

Germain, M., & Duronio, V. (2007). The N Terminus of the Anti-apoptotic BCL-2 Homologue

MCL-1 Regulates Its Localization and Function. *Journal of Biological Chemistry,282*(44), 32233-32242. doi:10.1074/jbc.m706408200

Harvard Bioscience. Electroporation: Frequently Asked Questions. Retrieved May, 2017, from

<http://www.btxonline.com/technical-resources/faq.html>

Hsu, P., Lander, E., & Zhang, F. (2014). Development and Applications of CRISPR-Cas9 for

Genome Engineering. *Cell,157*(6), 1262-1278. doi:10.1016/j.cell.2014.05.010

Lee, C. (2007). Coimmunoprecipitation Assay. *Methods in Molecular Biology Circadian*

*Rhythms,* 401-406. doi:10.1007/978-1-59745-257-1\_31

Massachusetts Institute of Technology (MIT). Optimized CRISPR Design. Retrieved May, 2017,

from <http://crispr.mit.edu:8079/>

Mojsa, B., Lassot, I., & Desagher, S. (2014). Mcl-1 Ubiquitination: Unique Regulation of an

Essential Survival Protein. *Cells,3*(2), 418-437. doi:10.3390/cells3020418

NCBI Primer designing tool. Retrieved May 2017, from

https://www.ncbi.nlm.nih.gov/tools/primer-blast/

Nucleotide - NCBI. Retrieved May 2017, from https://www.ncbi.nlm.nih.gov/nucleotide

Potter, H. (2003). Transfection by Electroporation. *Current Protocols in Molecular Biology*.

doi:10.1002/0471142727.mb0903s62

Sharma, S., Javadekar, S. M., Pandey, M., Srivastava, M., Kumari, R., & Raghavan, S. C.

(2015). Homology and enzymatic requirements of microhomology-dependent alternative end joining. *Cell Death and Disease,6*(3). doi:10.1038/cddis.2015.58

ThermoFisher Scientific. Co-Immunoprecipitation (Co-IP). Retrieved May 2017, from

<https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/co-immunoprecipitation-co-ip.html>

Thomas, L. W., Lam, C., & Edwards, S. W. (2010). Mcl-1; the molecular regulation of protein

function. *FEBS Letters,584*(14), 2981-2989. doi:10.1016/j.febslet.2010.05.061

Vartak, S. V., Iyer, D., Santhoshkumar, T., Sharma, S., Mishra, A., Goldsmith, G., . . .

Raghavan, S. C. (2017). Novel BCL2 inhibitor, Disarib induces apoptosis by disruption of BCL2-BAK interaction. *Biochemical Pharmacology,131*, 16-28. doi:10.1016/j.bcp.2017.02.015

Vick, B., Weber, A., Urbanik, T., Maass, T., Teufel, A., Krammer, P. H., . . . Schulze-

Bergkamen, H. (2008). Knockout of myeloid cell leukemia-1 induces liver damage and increases apoptosis susceptibility of murine hepatocytes. *Hepatology,49*(2), 627-636. doi:10.1002/hep.22664

Wei, G., Margolin, A., Haery, L., Brown, E., Cucolo, L., Julian, B., . . . Golub, T. (2012).

Chemical Genomics Identifies Small-Molecule MCL1 Repressors and BCL-xL as a Predictor of MCL1 Dependency. *Cancer Cell,21*(4), 547-562. doi:10.1016/j.ccr.2012.02.028