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

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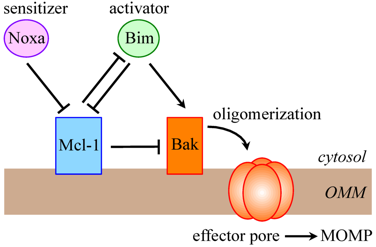
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1. **Introduction**

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 result 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 procedures (Vick, *et al.,* 2008). Furthermore, partial knockout of MCL-1 research 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 it 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). Pathway elucidated in **Figure 1**.

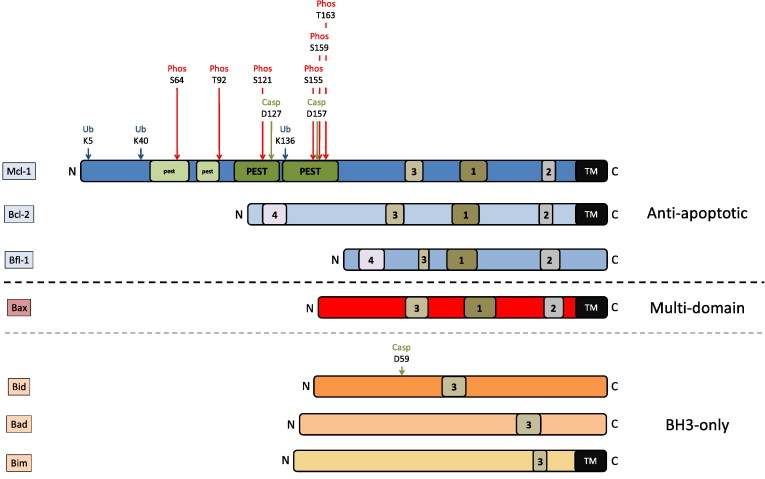


Release of cytochrome c

**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 shows to stability 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 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 shown to affect the anti-apoptotic activity of MCL-1 (Thomas, *et al.*, 2010).

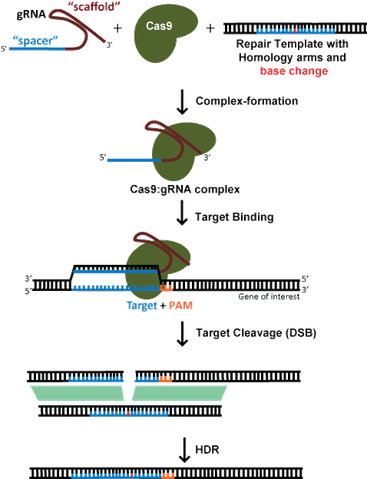
The question posed now is ***how*** are mutations within this PEST region affecting the anti-apoptotic activity of MCL-1? It is known from Thomas *et al.* that phosphorylation of and mutations at these residues affect the functioning of BIM, but it is unclear whether mutations within this region affect 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 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. These cell lines will comprise the experimental trials. One cell line will remain as a complete KO cell line, whereas another will have a mutated version of MCL-1 introduced within. 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**

Random mutations 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. To introduce a mutation within a gene, in this case MCL-1, via CRISPR/Cas9 technology, a guide RNA (gRNA) sequence must be established (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 sequence within the target sequence to allow for binding of the nuclease (Addgene). Following creation of a gRNA, a repair template has to be assembled containing the desired sequence with the mutation to be introduced without a PAM sequence region. PAM regions can be verified using online gRNA design programs.



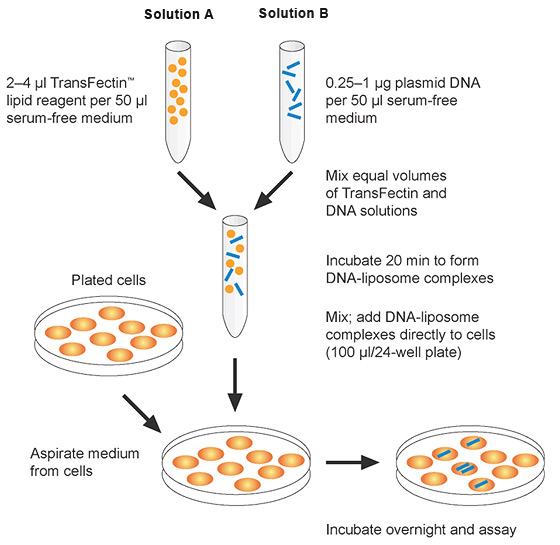
**Figure 3:** Cas9 endonuclease formation and functionality. Adapted from Figure 4 of Addgene*.*

Once designed, the gRNA oligos will be developed

and cloned into a plasmid using standard restriction-ligation cloning (Addgene). The gRNA/Cas9 complex and repair template will be delivered via lentiviral transfection similar to as described in Aubrey *et al.* 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). Process is outlined in **Figure 3**.

Once the the aforementioned templates have been delivered, it is necessary to validate that the genome editing worked. Validation of mutation creation will occur via PCR amplification of the plasmid followed by gel electrophoresis for confirmation of necessary amplicon (Addgene). For more accurate results, Sanger or next generation sequencing (NGS) can be used, which would give additional insight into off target results of the procedure (Addgene).

Following validation of successful genome edits, 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 was 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 4**. To verify transgene

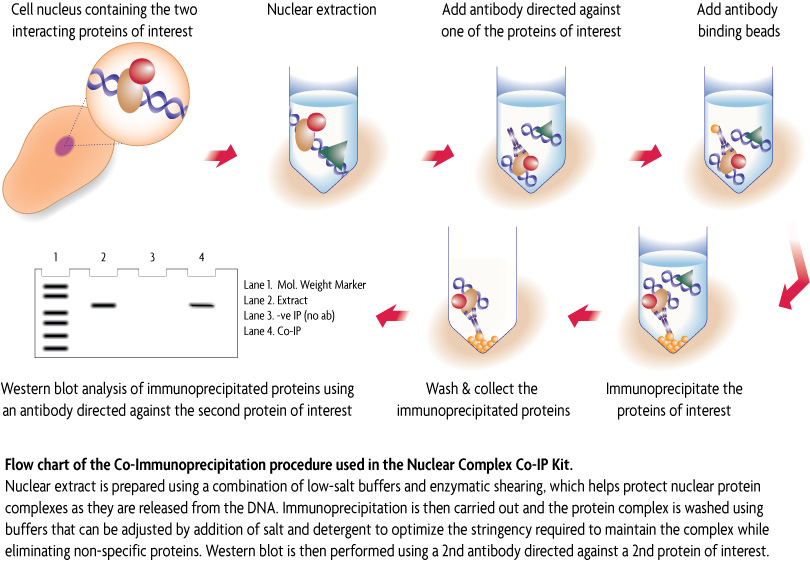


**Figure 4**: Basic Lipofection process. Adapted from Figure 1 of BioRad.

expression, PCR would be conducted to amplify significant portions of the target sequence which 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 and lysed (2% CHAPS buffer) from the cell lines using poly-histidine tags and the QIAGEN Ni-NTA fast start protein purification kit as described by the manufacturer. Once purified, MCL-Bak/Bax binding affinity will be test via a co-immunoprecipitation assay as described in Vartak *et al.* and Sharma *et al*. Antibodies needed will be purchased externally. MCL-1 lysates would be precleared via Protein G agarose beads. Primary polyclonal antibody would be incubated with MCL-1 lysate. The beads will be washed and heated in an buffer. The protein will be resolved on SDS-PAGE. Following electrophoresis, proteins will be transferred to a PVDF membrane and probed with the appropriate primary antibodies for MCL-1, and secondary antibodies as needed. 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. Generalized protocol for coimmunoprecipitation clarified in **Figure 5.**



**Figure 5**: Basic coimmunoprecipitation methodology. Adapted from Active Motif.

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 proteins actions, or even further decrease the genes half life, resulting in decreased binding of MCL-1 to Bak/Bax. It is a possibility that the binding affinity of both protein are not affected, or even that the affinity is increased for increased anti-apoptotic activity on MCL-1’s part.

A 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 (Addgene ). 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.

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.

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