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A Role For MEK3 In the Oncogenesis of Acute Lymphocytic Leukemia: Inactivation of MAPK P38 Promotes Cell Proliferation Through Enhanced Degradation of Mutant MEK3

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Dedication

To my amazing husband:

Emmanuel

To my lovely parents:

Roberto and Iris

To my siblings:

Yamilie and Alex

To my princesses:

Alexandra and Kamila

Por su amor incondicional, oraciones, abrazos y risas. Los amo!!

(For your unconditional love, prayers, hugs, and laughter. I love you all!!)

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Abstract

In the United States, pediatric leukemia has the second-highest cancer mortality rate in Hispanic children, especially Acute Lymphocytic Leukemia (ALL). While treatment of ALL has improved overall the five-year survival rate of ~90 %, not everyone has benefited. Twenty percent of them will experience relapse, and from these, 30 – 50 % will die. Unfortunately, the cause behind these dreadful statistics is poorly understood due to the complex etiology of this disease. Thereby, it is essential to identify potential oncogenic proteins that promote ALL so that new strategies can be developed to diagnose and treat this cancer. Whole Exome Sequencing (WES) coupled with OncoMiner Pipeline sorting identified five Single Nucleotide Polymorphisms (SNP's) on the Mitogen-Activated Protein Kinase Kinase 3 (MEK3) (MAP2K3) gene in El Paso del Norte ALL cancer patient library. MAP2K3 mutations were recreated using site-directed mutagenesis and transfected into HEK293 cells to study their impact on cell function. Three mutations were located within the kinase domain and two others located to the MEK3 amino domain. Transfection of HEK293 cells revealed these variants impact protein stability by inducing increased degradation of MEK3. Data shown here further suggest that they serve to block the auto-phosphorylation of MEK3, and loss of kinase activity towards p38. MEK3 is responsible for the activation of MAPK p38 to mediate growth-inhibitory and pro-apoptotic signals. Thus, inhibition of p38 activity through enhanced degradation of MEK3 mutants renders this pathway nonfunctional contributing to tumor cell proliferation. These findings indicate MEK3 represents a therapeutic target for controlling and treating ALL.

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Chapter 1: Introduction

1.1 The Mitogen-Activated Protein Kinase (MAPK) Signaling Pathway

The Mitogen-activated Protein Kinase (MAPK) signaling pathway is comprised of a group of four serine/threonine MAPKs: Extracellular Signal-Regulated Kinases (ERK1/2), Jun-N-terminal kinases (JNK1/3), p38 (α, β, γ, δ) and ERK5 [1-3]. Each group consists of three kinases activated in a cascade, including MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK leading to several cellular activities including cell proliferation, differentiation, and apoptosis [3]. Conserved across yeast and mammals, dysregulation in the MAPK pathway are associated with neurodegenerative diseases and cancer [4].

The ERK pathway

ERKs consist of a group of five serine/threonine kinases (ERK 1, 2, 3, 5, and 6) that are localized to the cytoplasm. Once activated, they translocate to the nucleus and regulate transcription factors involved in biological processes including cellular proliferation, apoptosis, and cell cycle regulation [5]. A-Raf, c-Raf1, and B-Raf are the MAPKKKs responsible for the activation and phosphorylation of MEK1 and MEK2 (MAPKKs) [6] which then catalyze the phosphorylation of ERK1 and 2 at their Thr-Glu-Tyr motif [7]. ERK1 and 2 share 84 % of homology with a 44 and 42 kilodalton (kDa) molecular weight, respectively, and are the most studied. These enzymes are widely expressed and activated in response to mitogenic stimulation, viruses, and oncogenes [8]. ERK1/2 have the same substrate specificity; however, mice lacking ERK1 are viable in contrast to $ERK2^{-/-}$ mice. Both proteins are components of the Ras/Raf/MEK/ERK

signaling pathway involved in several diseases including cancer. Lastly, Ras kinase mutations are present in 30 % of all cancers, while only 7 % for Raf [8].

JNK pathway

The JNK pathway includes JNK1, 2, and 3, which are activated by stress and inflammatory signals, leading to the activation of transcription factors such as c-Jun, Elk-1, Activated Transcription Factor 2 (ATF2), which are involved in apoptosis, differentiation, and proliferation [7]. They are ubiquitously expressed except for JNK3 that is restricted to the testis, brain, and heart [7]. MEK4 and MEK7 are kinases responsible for phosphorylating JNKs conserved Thr-Pro-Tyr motif in their activation loop [7]. Mice deficient in JNK1 and 2 are viable; however, T-cell differentiation is affected [9].

The p38 pathway

MAPK p38 is made up of a group of four serine/threonine kinases. p38α (MAPK14) is widely expressed whereas the other three isoforms' expression is more restricted. p38β (MAPK11) is abundant in the brain, p38γ (MAPK12) in skeletal muscle, and p38δ (MAPK13) in testis, small intestine, and pancreas [10]. p38α and β are most homologous to each other, at to 75 % [11]. Mice lacking p38α are embryonically lethal while knockout mice for p38β, p38γ, or p38δ maintain normal development [10].

Downstream targets of p38 involve activation of MAPK-activated protein kinase 2 and 3 (MAPKAP-K2/3), mitogen-activated protein kinase-interacting protein MKN1/2 and MSK1/2; *transcription factors* such as the myocyte enhancing factor 2 (MEF2), C/EBP Homologous Protein (CHOP), ATF2, signal transducer and activator of transcription 1 (STAT1), p53, serum response factor accessory protein 1 (SAP1), nuclear factor of activated T-cells (NFATp), CDX3, peroxisome proliferators activated receptor γ

coactivator (PGC-1) and USF-1; and *cytoskeletal proteins* [11]. Negative regulation of p38 can occur by tyrosine specific MAPK phosphatases (TS-MKPs), serine/threonine-specific MKPs (SS-MKPs), and tyrosine/threonine dual-specificity phosphatases (DS-MSKPs) such as PTP-SL, PP2A, and MKP1, respectively [10]. Additionally, evidence indicates that microRNAs (such as the miR-17 ˜ 92 cluster, the miR-200 family, miR-196a, miR-124 and -128) play an important role to directly, or indirectly, promote negative regulation of p38 [10].

Auto-activation of p38 is possible under a non-canonical process, through T cell receptor (TCR) stimulation and binding of p38 to TAB1 (TAK1-binding protein 1) [12]. Activation of p38 by TCR is LCK/Zap70 dependent and induces phosphorylation on tyrosine residue 323, triggering autophosphorylation of p38 in its Thr180/Tyr182 motif. [13]. Still, the exact mechanisms for this regulation are not well understood.

1.2 Mitogen-Activated Protein Kinase Kinase (MAPKKs) Family

MAPK ERK kinases (MEK) are dual-specificity kinases that belong to the MAPKK family. They are responsible for the phosphorylation of serine/threonine and tyrosine residues of their specific MAPK substrate within their Thr-X-Tyr motif [3]. MEKs are very similar in structure to other kinases in that they possess an amino-terminal domain, a kinase domain and a carboxylic-terminal domain [3]. There are two domains, a docking (D) and a versatile docking domain (DVD), which are essential for binding MAPK and MAP3K, respectively [14]. The DVD domains are found downstream of their kinase domain [14]. Seven MEKs have been identified (MEK1-7) [3]. They share structural homology in their kinase domain, while their amino and carboxylic terminals are distinct [3] (Figure 1.1). MEK1 and MEK2 are specific activators of ERK1/2, MEK3, and MEK6 of

p38, MEK4, and MEK7 of JNK and MEK5 of ERK5 [1, 15, 16]. MEK4 can also activate p38 [1, 15, 16].

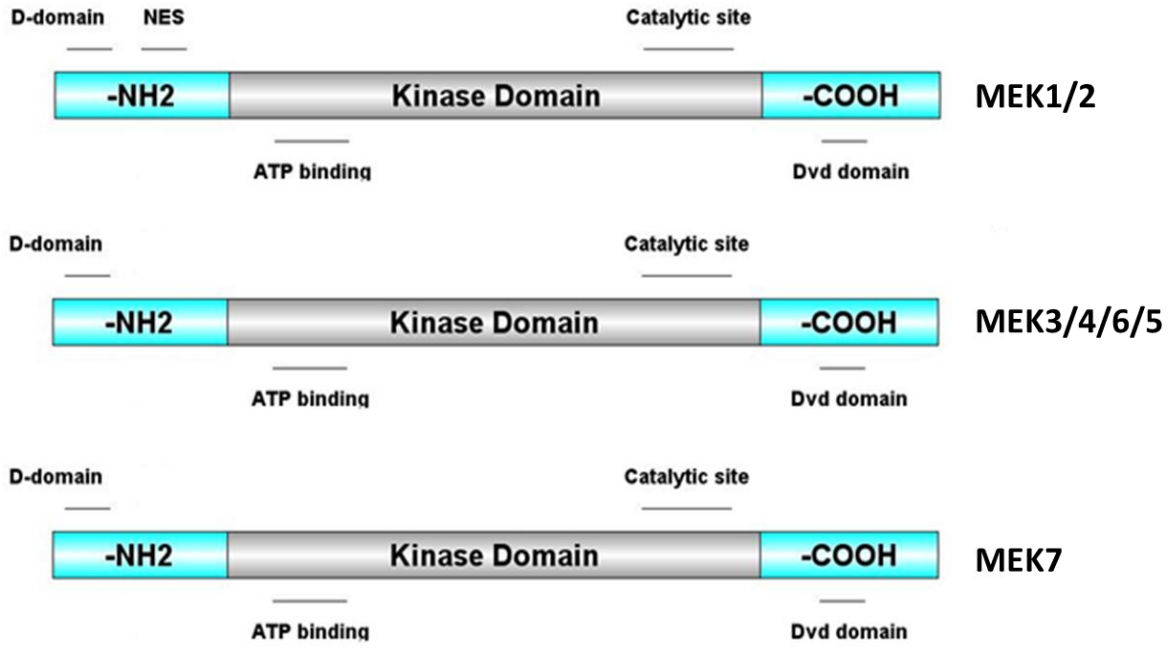

Figure 1.1: Structure of the MEK family. Schematic representation of the MEK family, which is comprised of seven members (MEK1-7). They share structural homology in their kinase domain, while their amino and carboxylic terminals are distinct. The docking (D) and the versatile docking domain (DVD) are essential for binding MAPK and MAP3K, respectively [3].

1.3 MEK3

Structure of MEK3

MEK3, also known as MAPK Kinase 3 (MAP2K3), MAPK/Kinase 3 (MKK3), and Stress-activated Protein Kinase Kinase 2 (SAPKK2 or SKK2) [17] is widely expressed and abundant in skeletal muscle [18, 19]. It is a proline-directed kinase localized in chromosome (chr) 17p11.2 with 16 exons [20]. Three isoforms differing in their amino domain are the result of alternative splicing. MEK3b (also known as variant 3) is the canonical encoding for a 347 amino acid (a.a) protein with an approximate molecular weight of 37 kDa [19]. It differs from MEK3 (isoform 1) in that it possesses 29 additional amino acids in the N-domain that are believed to play a role in p38β phosphorylation. MEK3 activation involves binding MAP3K kinases to its DVD docking site (311 - 344 a. a.) [14, 21].

The catalytic domain of MEK3 is well conserved across protein kinases. It consists of 12 subdomains which fold and create a structure of two lobes [22]. The smaller lobe, primarily β-sheet structures, comprises subdomains I-IV involved in ATP binding. In the COOH- lobe, subdomains VIA-XI participate in substrate binding and phosphotransfer. Essential residues are also conserved across the catalytic domain of kinases [23]. For instance in MEK3, the glycine-rich domain (GxGxxG) involved in ATP binding includes a.a. 42 – 48; lysine (K) 64 is the a.a. responsible for positioning the α and β phosphates of ATP; K163, located in the catalytic loop, might participate in neutralizing the negative charges of the γ phosphate of ATP; and the asparagine 179, phenylalanine 180, and glycine 181 (DFG) help position the γ phosphate for transfer (Figure 1.2).

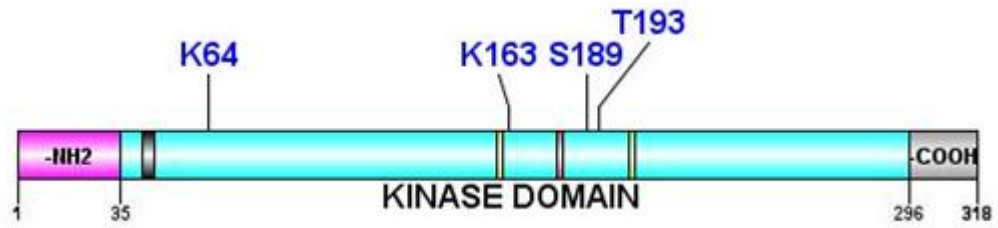

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Figure 1.2: Schematic representation of the MEK3 protein structure. The position of amino acid residues, domains, and motifs conserved across kinases are shown. Pink, blue and gray colors represent the amino-terminal domain, the kinase domain, and the carboxylic terminal domain, respectively. The black box represents the GxGxxG rich-loop essential for ATP binding; the yellow box the HRD motif; the red box the DFG motif for ATP γ phosphate orientation; and the green box representing the APE motif. Serine (S) 189 and threonine (Th) T193 are involved in MEK3 activation, lysine (K) 64 binds to the α and β phosphates of ATP, and K163 is in charge of neutralizing the negative charges of the γ phosphate, it is known as the catalytic lysine.

MEK3 signaling pathway

MEK3 signaling cascade is activated in response to numerous stimuli such as ultraviolet light (UV), osmotic shock, DNA damage, inflammatory cytokines (IL-1 α, β, and TNF-α), and growth factors (GF) [1, 24, 25]. Binding of these cytokines and GF to their receptors prompt the activation of MAPKKKs, including Mixed lineage kinase-3 (MLK3), Apoptosis signal-regulating kinase1 (Ask1), MEKK3, MEKK4, Thousand-and-one amino acid 1 and 2 (Tao1 and Tao2) along with TGF-β Activated Kinase 1 (Tak1) can activate MEK3 at Ser189 and Thr193 [1, 3, 11, 25, 26]. Activated MEK3 phosphorylates p38α, γ, δ but not β on T180 and Y182, inducing cellular activities such as cellular differentiation, migration, survival, apoptosis, and metabolism (Figure 1.3) [11, 15, 24].

One mechanism for MEK3 to participate in various cellular functions is by binding to scaffold proteins such as c-Jun NH2-terminal kinase (JNK)-interacting protein (JIP) 2, JIP4, and OSM. Activation of MEK3/p38 by Rac is possible through the interaction with the IB2/JIP2 scaffold protein. [27-29]. Moreover, the OSM/Rac/MEKK3/MEK3 is essential for p38 activation during osmotic shock and suppression of TNFα-induced cell death by which p38 depends on the RACK1/MEK3/6 complex [10].

In addition to p38 activation, MEK3 phosphorylates Mirk1 (mini-brain related kinase), a Dyrk/mini-brain family member of the dual-specificity tyrosine-regulated kinases. Mirk acts as a transcriptional regulator by activating the transcription factor HNF1α, thus inducing proliferation-related genes. Binding of p38α/β to Mirk blocks its activation in a kinase-independent manner [30, 31]. Mirk's upregulation has been observed in solid cancer and is associated with cell survival [32].

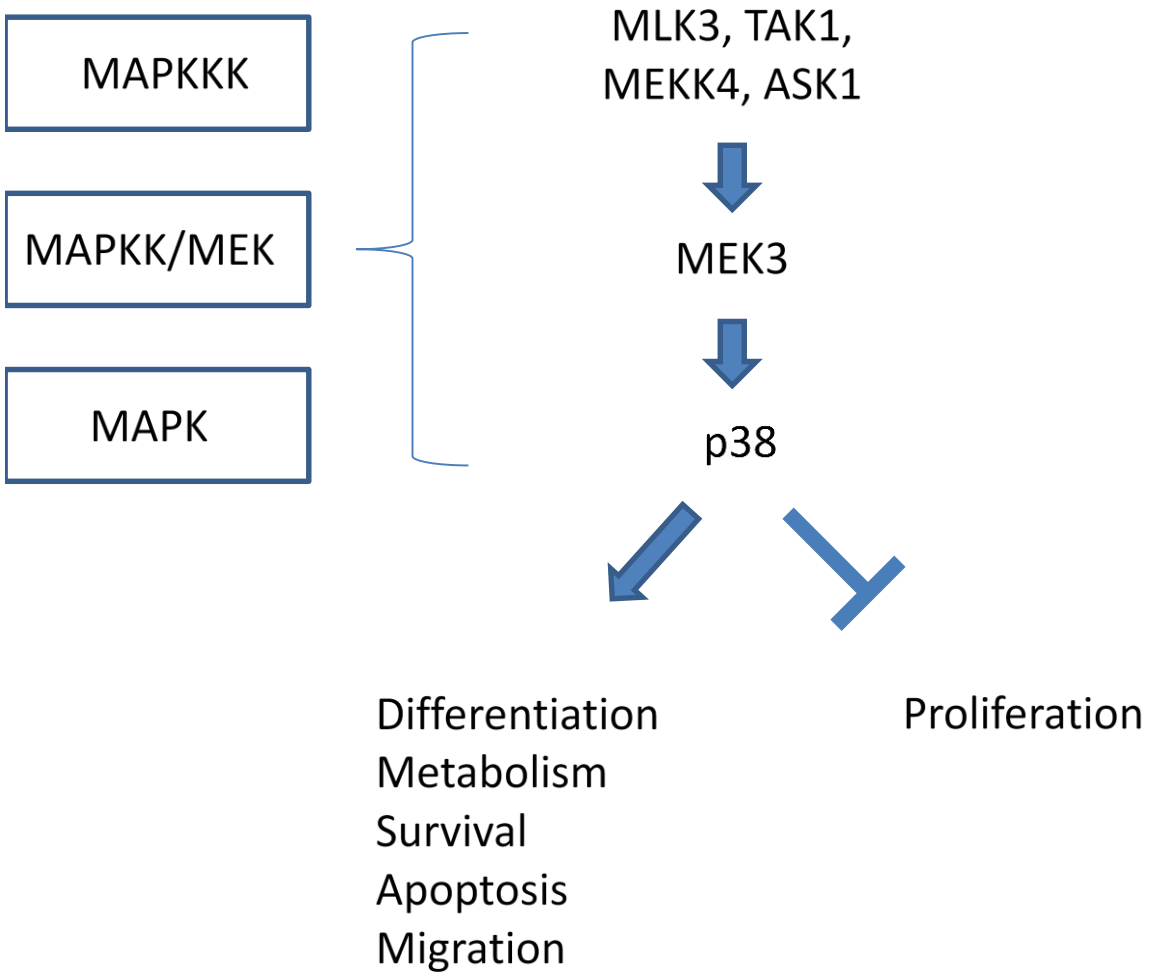


Figure 1.3: Schematic representation of mammalian p38 signaling cascade. The classical MAPK pathway is composed of MAPKKKs, MAPKKs, and MAPKs each one is activated after the other. p38 activation is induced by inflammatory cytokines such as interleukin (IL) 1 and tumor necrosis factor (TNF) α, but mainly by stress. Several MAPKK, including MEK3, MEK6, and MEK4, are responsible for p38 activation to mediate numerous cell activities.

Negative regulation of MEK3

Negative regulation of MEK3 involves the dephosphorylation of Thr193 or acetylation of S189 and T193 in its activation loop [33, 34]. Dephosphorylation of MEK3 occurs when phosphorylated MEK3 binds to alpha-4 and Protein Phosphatase 2 (PP2A). This interaction allows for the specific dephosphorylation of T193 [33]. Acetylation of MEK3 is executed by YopJ, an effector protein of *Yersinia,* which acts as an acetyltransferase using acetyl-coenzyme A (Co-A) to modify S189 and T193 in the activation loop of MEK3 [34]. Acetylation inhibits phosphorylation of these residues, thus regulating the MEK3 activity [34].

Disruption of MEK3

Disruption of the MEK3 gene in fibroblasts affects the ability of TNF- α to induce expression of IL-6 and IL-1 cytokines [35]. Mice lacking MEK3 are viable and fertile, but the production of IL-12 and INF-γ is affected, addressing the essential role of MEK3/p38 activation in innate immunity and T cell-mediated immunity, respectively [36].

1.4 Biological Functions of MEK3

Role of MEK3 in cell cycle regulation

Regulation of the cell cycle by MEK3 could be either positively or negatively impacted. For instance, MEK3 induces growth arrest by upregulating cyclin-dependent kinases (CDKs) inhibitors, downregulating cyclin D1 protein, and the polycomb protein Bim1 [37-39]. Also, overexpression of PDGFR prevents proliferation in melanoma cells via the upregulation of p38γ and MEK3, thus suggesting a negative role of MEK3 in cell cycle regulation [40]. Conversely, p38 mediates hematopoietic cell proliferation in response to G-CSF [41].

Role of MEK3 in apoptosis

Apoptosis eliminates undesirable cells, such as those that are damaged or aged, as a means of maintaining homeostasis. Inactivation of the MAPK p38 signaling pathway renders cardiomyocytes and fibroblasts resistivity against apoptosis by reducing Bax and Fas proteins [42]. However, negative regulation through the binding of mirR-214 to the 3′UTRs of MEK3 suppresses cervical cancer growth [43, 44], while by alpha-4, abrogates TNF-α-induced apoptosis and caspase 3/7 expression [45]. In contrast, activation of Mirk by MEK3 leads to antiapoptotic signals driving the survival of pancreatic cancer [31]. Still, MEK3 mediates transforming growth factor-β (TGF-β) induced apoptosis in prostate cancer cells via the TAK1/MEK3/p38 signaling pathway [46].

Role of MEK3 in differentiation and cytokine production

MEK3 plays a role in muscle [47] and osteoclast [48] differentiation. Constitutively activation of MEK3 increases the production of eosinophils, monocytes, and erythrocytes [49]. Considering, neutrophil differentiation is affected, this model suggests that MEK3/p38 is essential for myelogenous lineage differentiation of hematopoietic stem cells [50]. Moreover, MEK3 regulates TNFα-induced cytokine expression (IL-1α, IL-1β, IL-6, and TNFα), LPS-induced IL-12, and IFNγ production [35, 36, 51]. IL-12 and IFNγ regulate Th1 differentiation, suggesting a role for the MEK3/MEK6/p38 signaling pathway in $CD4^+$ differentiation [52].

1.5 Role of MEK3 in Cancer

The tumor suppressor p53 is commonly mutated in cancer, ranging from 15 - 50 % in solid cancers and 12 % in hematopoietic malignancies [53]. Loss-of-function mutations inhibit the ability of p53 to suppress growth, while gain-of-function (GOF)

mutations confer drug resistance [54]. Mutp53 regulates the expression of MAP2K3; thereby, the proliferation of tumor cells harboring mutp53 is suppressed by the downregulation of MEK3. Ultimately, this improves response to chemotherapy and further suggests a contribution to mutp53 GOF [25, 55]. MEK3 overexpression has also been recognized in the invasion and poor prognosis of glioma cell lines, implicating an oncogenic role [56, 57]. In colon cancer, MEK3 positively or negatively influences the treatment outcome depending on the p38 isotype activated [58]. Mice lacking MEK3 and MEK6 are more likely to develop cancer than their counterparts [59]. Similarly, MEK3 gene expression has been downregulated in breast, lung, colon, liver, and thyroid cancer, denoting a possible suppressive role in cancer [60]. Expression of MEK3 in hepatocellular carcinoma inhibits cell proliferation with CDK inhibitors [37]. This data suggests a role for MEK3 in cancer depends on the cell type and stage of cancer.

MEK3 in hematopoietic malignancies

Previous studies demonstrated the importance of IFNα antitumor effect in chronic myelogenous leukemia (CML). Activation of p38 is essential for the antileukemic effect of IFNα [61] and IFN-stimulated genes (ISGs) via the IFN-stimulated response element (ISRE) or the IFNγ activated site (GAS) element, which is MEK3 and MEK6 dependent [62, 63]. Constitutive activation of MEK3-6/p38 is responsible for the production of matrix metalloproteinase-9 (MMP-9), which is involved in tumor angiogenesis and survival of primary B-chronic lymphocytic leukemia (B-CLL) cells [64]. Also, p38 inhibition increases apoptosis of B-CLL treated with rituximab and sensitizes acute promyelocytic leukemia (APL) cells to all-*trans*-retinoic acid or arsenic trioxide [65-67].

MEK3 in cancer therapy

The downstream target of MEK3 (p38) is involved in genotoxic resistance to cisplatin [68], 5-fluorouracil (5-FU) [69], cytarabine, and gemcitabine [70, 71]. MEK3 and MEK6 are the kinases responsible for the activation of p38 as it mediates the growth-inhibitory response against these chemotherapeutic agents. Consequently, pharmacological inhibition of p38 is associated with drug resistance, reduction of p53-induced apoptosis, and an increase of autophagy [72].

p38 is a downstream target of BCR/ABL through phosphorylation and action of MEK3/6 [73]. Cotransfection experiments support that p38 activation by MEK3 is mostly regulated by BCR, while p38 activation by MEK6 is controlled by c-Abl [73]. Cytosine arabinoside (ara-C) is a treatment used for an array of leukemias since it causes apoptosis through c-ABL activation of p38 [74, 75]. Cell lines harboring the BCR/ABL translocation are resistant to cytarabine treatment by blocking p38 activation. In a similar manner, pharmacological inhibition of p38 makes cells resistant to ara-C treatment [73] as well as reversed growth inhibition by IFNα treatment [61]. In part, the latter might be due to the ability of IFNα to regulate BCR/ABL expression [76], not being able now to inactivate p38.

Imatinib was the first tyrosine kinase inhibitor (TKI) approved to treat leukemias positive for the Philadelphia chromosome translocation (BCR/ABL). Notwithstanding, resistance to this medication emerged from mutations in this protein; thus, Dasatinib is a second-generation TKI used to treat Imatinib-resistant strains. Activation of MEK3-6/p38 plays a crucial role in the antileukemic effect in response to these TKI treatments [77, 78]. The absence of p38 reverses apoptosis, cell cycle arrest, and growth inhibition [77].

1.5 SIGNIFICANCE

Leukemia is the most common forms of cancer in children, in 2020 an estimated 60,530 new cases in the US is projected. It is responsible for causing more deaths than any other childhood cancer and has a higher incidence rate among the Hispanic population [79-81]. From these cases, ten percent will present as acute lymphocytic leukemia (ALL), which is the most prevalent subtype in children [82]. In spite of the medical advancements and overall improvements in survival (five-year survival rate of approximately 80 %), the primary cause of treatment failure is relapse [83, 84]. Correspondingly, Hispanics have the highest rate of relapse and the worst prognosis [85]. Genomic studies associate genetic variants to these high rates [86]. However, due to the complex etiology of this disease, the cause behind it is poorly understood. To identify novel therapies that will target this disease, it is imperative that we understand the molecular mechanisms implicated in its development.

Genetic alterations have been linked to ALL. More specifically, mutations in Interleukin 2 Receptor β (IL2Rβ), Janus Kinase (JAK) 1, Interleukin 7 Receptor (IL7R), Fms Like Tyrosine Kinase 3 (FLT3), JAK3, Lymphocyte Adapter Protein (LNK), and RAS genes account for 15-20 % of Ph-like ALL cases. Likewise, chromosomal rearrangements in Colony Stimulation Factor 1A (CSF1A), Abelson Murine Leukemia 1 ABL1 and ABL2, and Platelet-Derived Growth Factor Receptor (PDGFR) comprise another 15 % of the Ph-like phenotype [87-90]. Higher expression levels of IKAROS family zinc finger 1 (IKZF1) and Cytokine Receptor-Like Factor 2 (CRLF2) are also widespread in these patients [91].

The objective of this dissertation is to determine the role of MEK3 in oncogenesis of ALL. Considering that genetic alterations and kinase-activating mutations have been implicated in ALL's pathogenesis, this observation has become the premise of the proposed studies. We **hypothesize that MEK3 plays a role in the oncogenesis of ALL malignancies**. To identify mutations involved in promoting cancer in this regional population, we performed Whole Exome Sequencing (WES) and coupled it with the OncoMiner Pipeline developed at UTEP. We identified five Single Nucleotide Polymorphisms (SNP's) in MEK3 which occurred at a high frequency in this library. Moreover, the Protein Variation Effect Analyzer (PROVEAN) scoring predicts that these mutations have a deleterious effect on the protein function which leads us to speculate that these alterations have the potential to be pathogenic, but to also represent new targets for therapeutic interventions to be developed.

Chapter 2: Identification of MEK3 Mutants in ALL Patient Samples

2.1 INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common type of leukemia affecting children [92]. It has a 5-year survival rate of 90 %, and thus, it is deemed a treatable disease; however, not everyone benefits from this statistic [93]. While it is well known about the disproportioned burden of ALL among Hispanics [94], the cause behind this remains unknown. Higher risks have been associated with environmental and lifestyle factors, as well as genetic variations [95, 96]. The highest incidence of ALL in Texas is located along the US Mexico border, where about 80 % of the El Paso population is Hispanic. Hence, El Paso del Norte region provides us with a unique population structure to identify the oncogenic promoters of ALL in this minority group. Polymorphisms have been found to play a role in ALL relapse, drug resistance and worse outcomes [97-99]. For these reasons, we first sought to identify the catalog of mutation in a small sample from El Paso del Norte region. To identify mutations that might promote ALL, the DNA from, nine ALL cancer patient samples and seven healthy donors were isolated and subjected to whole-exome sequencing (WES).

WES is a technique used to sequence coding regions of the DNA (exomes). Researchers use this technique to identify possible genes responsible for many diseases. Our lab, in collaboration with Dr. Ming-Ying Leung, developed the OncoMiner Pipeline web-server. The program is linked to different databases allowing one to identify the variants' genomic location, available information of the gene and the SNP [100]. The program also assigns a Protein Variation Effect Analyzer (PROVEAN) score, which predicts the mutational effect on the variant on protein

function; the prediction is based on the principle that conserved amino acids are essential for function [100, 101]. Comparing it to the control group, determines whether the presence or absence of a mutation in a disease group is statistically significant. Using the OncoMiner Pipeline, we were able to identify five SNP's on the MAP2K3 gene. Three mutations were located in the kinase domain, while the remaining two were identified in the amino-terminal of MAP2K3.

2.2 Materials and Methods

Whole-exome sequencing (WES)

In collaboration with local hospitals, 14 cancer patient samples from cancer patients were collected. Due to our geographic location, these samples were most likely extracted from patients of Hispanic ethnicity. The genomic DNA of seven control samples, nine Acute Lymphocytic Leukemia (ALL) samples, one T cell lymphoma, three brain samples, and one bone sample was isolated using Puregene Core Kit A (Qiagen), purified and sent to Otogenetics for Whole exome sequencing (WES**).**

OncoMiner pipeline

To identify possible mutations driving cancer in this population, the information provided by Otogenetics was uploaded to the OncoMiner Pipeline web-server. OncoMiner identified over 70,000 variants and separated those into three groups: kinases, phosphatases, and cancer-related genes. The work for this dissertation was focused on the kinase group with 2,150 variants. Rather than analyzing the entire kinase group, we focused on those mutations that prevailed across the disease group. Moreover, selected variants in which their emergence between the disease and control groups was significant. To narrow down the search, we concentrated on a shortlist of exonic variants

and selected those with a PROVEAN score of -2.5 or less [101]. Genes were selected with multiple variants, variants located in the kinase domain and variants in conserved amino acids across numerous species. The final selection criteria for the mutations was that not been previously characterized or less thoroughly studied.

Recurrently mutated genes and molecular function analysis

Variants that were present in the control group were removed from this analysis. The remaining list was filtered based on the PROVEAN score. A threshold of -2.5 or less was used to select variants with a predicted deleterious effect [101]. The frequency of distinct mutations was determined using the R program [102]. To investigate whether there is a difference in the mutation burden in El Paso del Norte region population, genes were analyzed for their frequency using the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) program (https://ocg.cancer.gov/programs/target) initiative, phs000464, and phs000218. The data used for this analysis is available at https://portal.gdc.cancer.gov/projects**.** Lastly, to further characterize their molecular function, gene ontology (Go) term classification system was employed [103].

Recurrently ALL-driving genes

The frequency of mutated genes implicated in ALL's pathogenesis was determined using the catalog of somatic mutations in cancer (COSMIC) cancer gene census (CGC). CGC provides information on genes with a causal impact on the progression of a wide variety of cancers [104]. Next 80 ALL-driving genes and their frequency in the case group was determined using the R program [102] and compared with their frequency in the TARGET program.

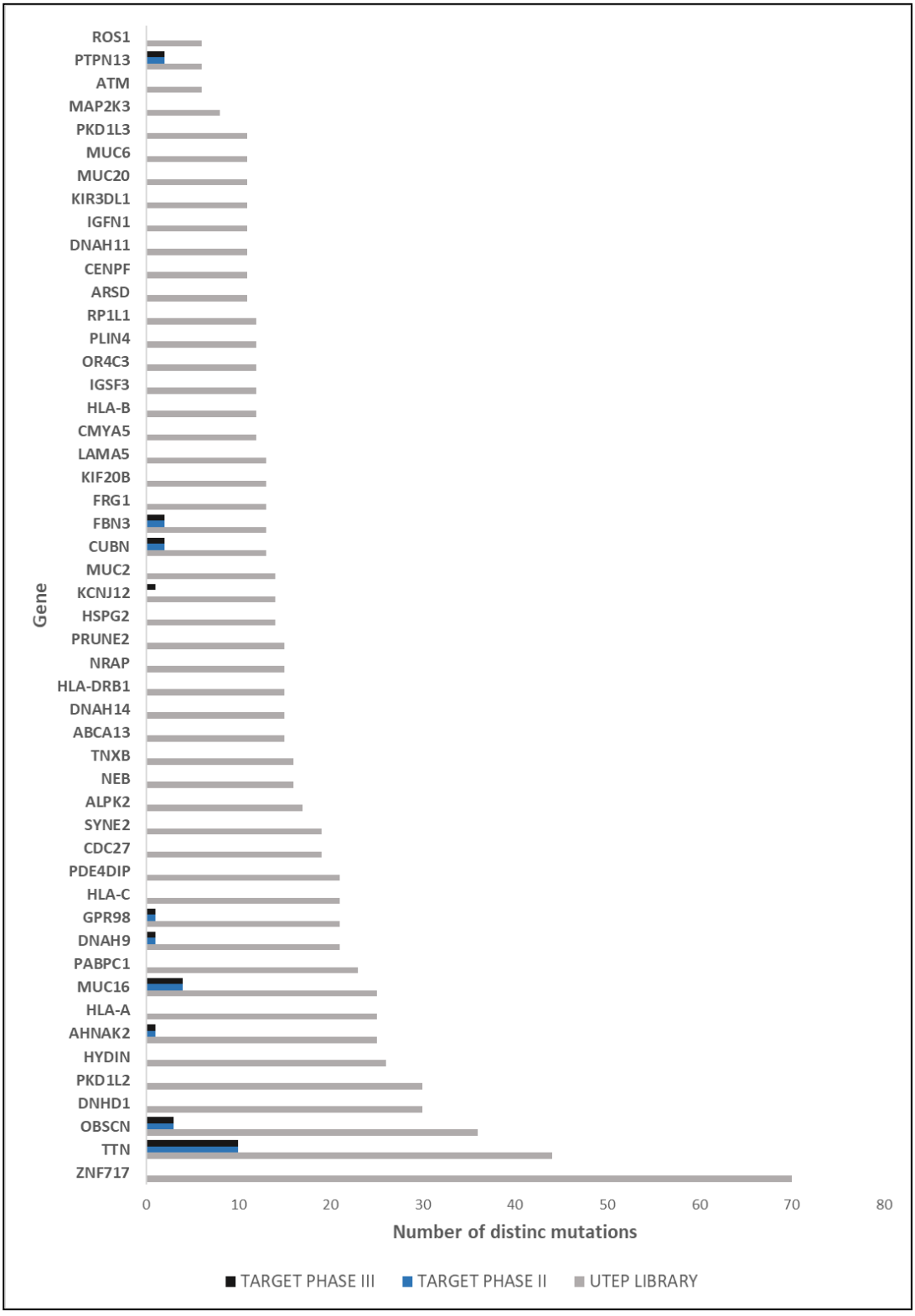
2.3 RESULTS

Frequently mutated genes are absent or present with a low frequency of mutations in the TARGET program.

To investigate the landscape of mutations in this region, the frequency of recurrently mutated genes was determined. Nine ALL samples with 41,158 SNPs were studied. The categorization of these variants into germline and somatic was not possible due to the absence of matched healthy tissues. Instead, variants were compared to normal samples from donors. Of these 41,158 variants, 23,829 were identified as potentially pathogenic (PROVEAN < -2.5) over 14,121 genes. The most altered gene was ZNF717, harboring 71 variants followed by OBSCN with 48 and 44 for TTN. Subsequently, their mutation frequency in TARGET was analyzed with most of these genes either absent or present with a shallow frequency of mutations in the TARGET program (Figure 2.1). To further characterize the mutated genes, the Go term classification system was used. Only 23 were classified within ten categories in which the majority (10 genes) were identified as structural molecules (Figure 2.1).

ZNF717 encodes for the zinc finger protein 717 which belongs to a family of transcriptional regulators involved in proliferation, apoptosis, and differentiation [105]. Its role in cancer is not clear. However, it is highly mutated in hepatocellular carcinoma and gastric cancer [106, 107]. OBSCN and TTN are both components of the muscular system and are frequently mutated in all types of cancer in COSMIC, together with MUC16, NEB, DNAH9, DNAH11, and ATM, being TTN the second-ranked [108].

(A)


ROS1
PTPN13
ATM
MAP2K3
PKD1L3
MUC6
MUC20
KIR3DL1
IGFN1
DNAH11
CENPF
ARSD
RP1L1
PLIN4
OR4C3
IGSF3
HLA-B
CMYA5
LAMA5
KIF20B
FRG1
FBN3
CUBN
MUC2
KCNJ12
HSPG2
PRUNE2
NRAP
HLA-DRB1
DNAH14
ABCA13
TNXB
NEB
ALPK2
SYNE2
CDC27
PDE4DIP
HLA-C
GPR98
DNAH9
PABPC1
MUC16
HLA-A
AHNAK2
HYDIN
PKD1L2
DNHD1
OBSCN
TTN
ZNF717
Gene
0
10
20
30
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50
60
70
80
Number of distinc mutations
TARGET PHASE III
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UTEP LIBRARY

(B)

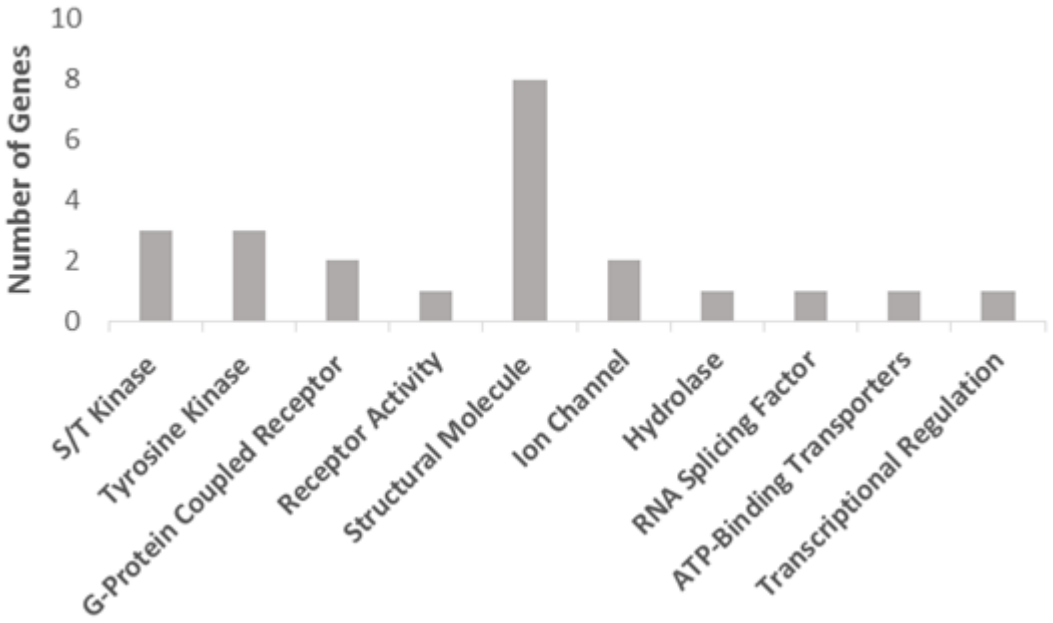


Figure 2.1: Frequently mutated genes are absent or present with a low frequency of mutations in the TARGET program. (A) Using the program R, the variants' frequency on mutated genes was determined and compared with the TARGET database. (B) Genes were classified according to their molecular function using the Go-term classification system [103].

Variants in known ALL-driving genes

To investigate whether ALL-driving genes were recurrently mutated, the frequency of eighty genes was determined and compared with TARGET. 252 variants were identified in 41 different genes. Within the most altered genes, BCR (29 variants), AFF3 (12 variants), IL7R (21 variants), JAK1 (8 variants), LCK (2 variants), PML (15 variants), and STIL (14 variants) were identified, yet they were absent in TARGET (Figure 2.2).

Identification of MEK3 mutants in ALL patient samples

Greater than 70,000 variants were identified within fourteen samples using OncoMiner. Focusing on the kinase group, 93 variants were present in the majority of the case group as opposed to the control; however, only 23 were statistically significant. From these, eight variants in four different genes (MAP2K3, MYLK, GUCY2C, and ERBB2) were identified as being potentially pathogenic (Figure 2.3). Interestingly, none of them were found in known ALL-driving genes.

MAP2K3 was determined to harbor five different SNPs with PROVEAN scores less than -2.5. All of them were absent in the control group (Table 2.1). Two were located in the amino domain (R26T and P11T), while the other three in the kinase domain (Figure 2.4). Of these, four of them have been previously reported in ClinVar P11T, R26T, R65L, and R67W (dbSNP:rs33911218, dbSNP:rs36047035, dbSNP:rs56067280, and dbSNP:rs56216806, respectively). However, there are no publications associated with their characterization. Eight patients had combination of the four missense with the nonsense mutation, translating into a truncated protein lacking 245 a.a. of its carboxylic domain. Only one patient had the full-length MEK3 with a combination of the four missense SNPs in conserved residues near the ATP binding site and the substrate-

binding site (Figure 2.4). Sequence alignment showed high conservation of mutated amino acids across multiple species (Table 2.2). The other variants were located in MYLK, GUCY2C, and ERBB2 genes. Still, these variants were confined to the amino (MYLK and GUCY2C) or carboxylic domain (ERBB2) and have been previously reported as either natural or sequence conflict variants. As a result they were excluded from further analysis.

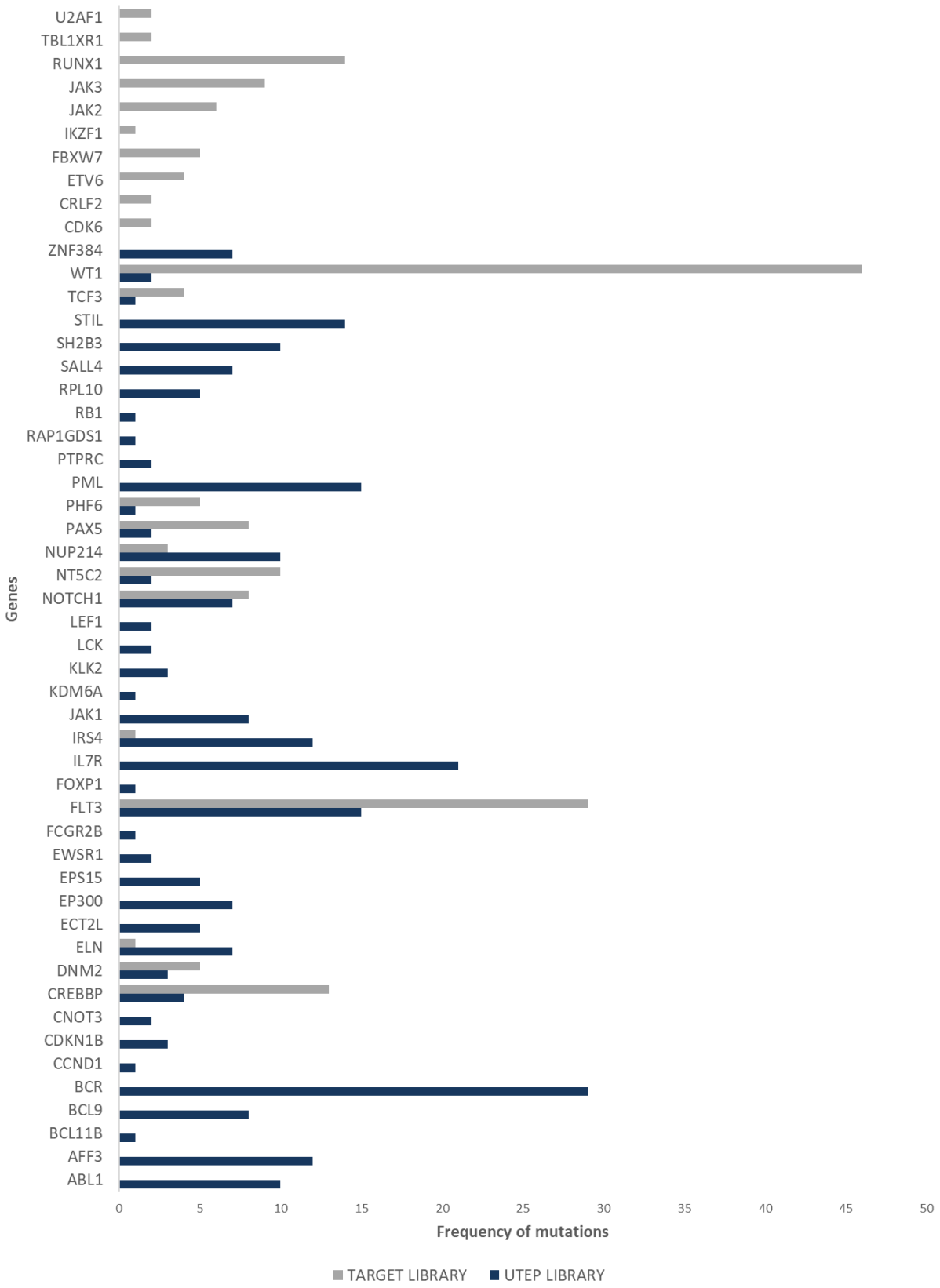

U2AF1
TBL1XR1
RUNX1
JAK3
JAK2
IKZF1
FBXW7
ETV6
CRLF2
CDK6
ZNF384
WT1
TCF3
STIL
SH2B3
SALL4
RPL10
RB1
RAP1GDS1
PTPRC
PML
PHF6
PAX5
NUP214
NT5C2
NOTCH1
LEF1
LCK
KLK2
KDM6A
JAK1
IRS4
IL7R
FOXP1
FLT3
FCGR2B
EWSR1
EPS15
EP300
ECT2L
ELN
DNM2
CREBBP
CNOT3
CDKN1B
CCND1
BCR
BCL9
BCL11B
AFF3
ABL1
Genes
0
5
10
15
20
25
30
35
40
45
50
Frequency of mutations
TARGET LIBRARY
UTEP LIBRARY

Figure 2.2: Frequency of variants in ALL-driving genes. Using COSMIC CGC, 80 ALL-driving genes were identified. Their mutation frequency in the case group was determined using the R program and compared with the TARGET program (https://portal.gdc.cancer.gov/projects).

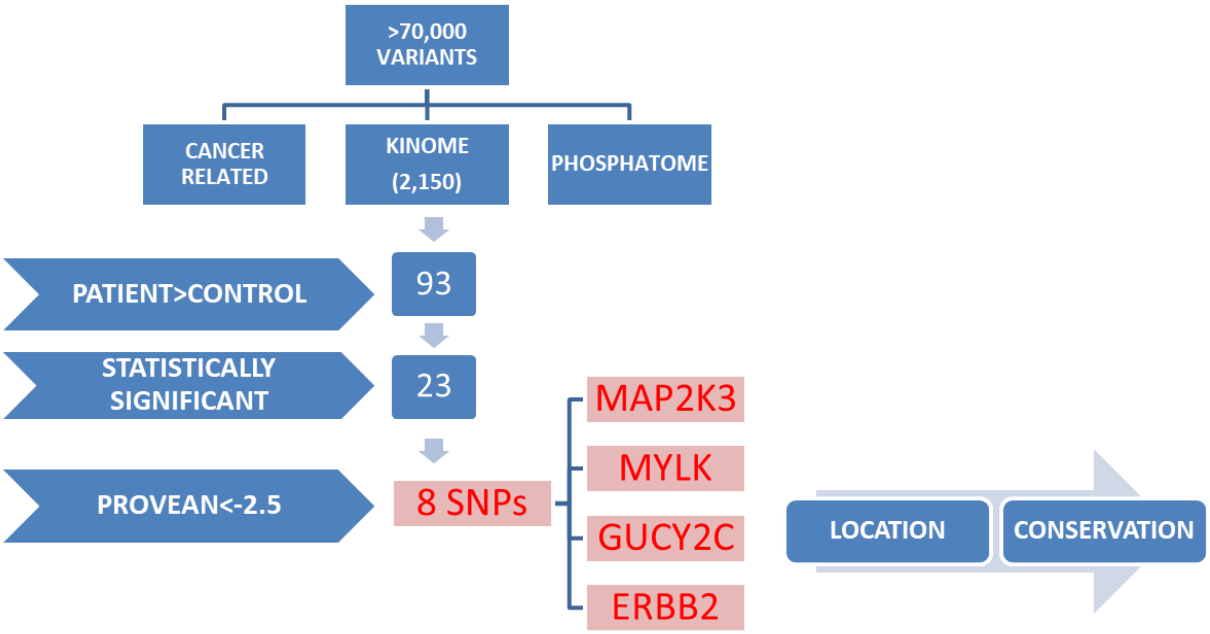


Figure 2.3: Schematic representation of selection criteria for identifying Hispanic ALL causing genes. Schematic diagram showing the criteria used to end up with five variants in MEK3 with a PROVEAN score <-2.5 and no previous publications on the SNP's.

Table 2.1 Identification of SNPs in the MAP2K3 gene using the OncoMiner Pipeline

Gene	*Provean*	*A.A from*	*Location*	*A.A to*	*Patients*	*Control*
MAP2K3	-6.557	R	65	L	9	0
	-5.539	P	11	T	9	0
	-5.085	R	67	W	9	0
	-2.605	R	26	T	9	0
	-7.739	Q	73	*	8	0

Table 2.2. MAP2K3 residues are conserved across multiple species

					11			
Homo sapiens	P	N	P	T	**P**	P	R	N
Mandrillus	P	N	P	T	**P**	P	R	N
Gorilla	P	N	P	T	**P**	P	R	N
Loxodonta Africana	P	N	P	T	**P**	P	R	N
Saimiri	P	N	P	T	**P**	P	R	N
Rattus	S	N	P	T	**P**	P	R	N
Mus musculus	S	N	P	T	**P**	P	R	N

				26
T	I	G	D	**R**
T	I	G	D	**R**
T	I	G	D	**R**
T	I	G	D	**R**
T	I	G	D	**R**
T	I	G	D	**R**
T	I	G	D	**R**

65		67						73
R	I	**R**	A	T	V	N	S	**Q**
R	I	**R**	A	T	V	N	S	**Q**
R	I	**R**	A	T	V	N	S	**Q**
R	I	**R**	A	T	V	N	S	**Q**
R	I	**R**	A	T	V	N	S	**Q**
R	I	**R**	A	T	V	N	S	**Q**
R	I	**R**	A	T	V	N	T	**Q**

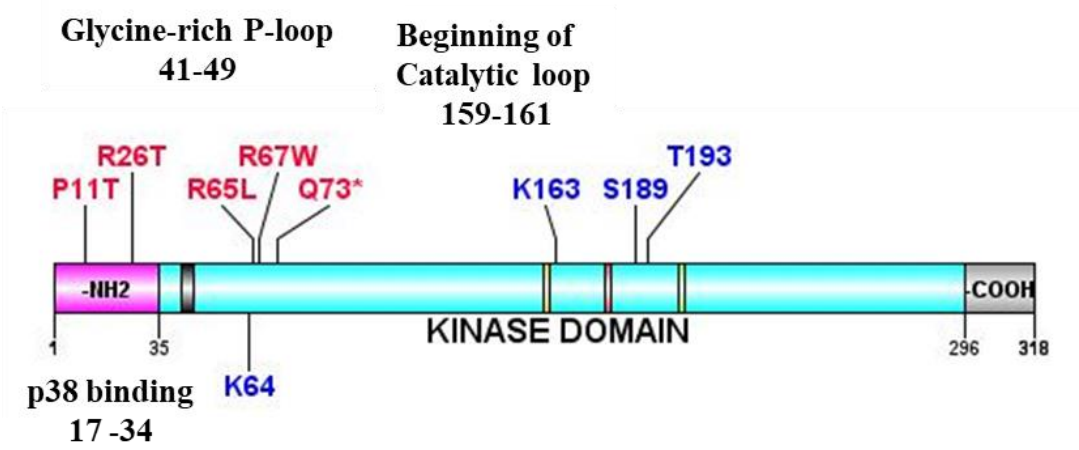


Figure 2.4: Schematic location of amino acids mutated in MEK3. Diagram showing the location of mutated amino acids (in red) found in a human cancer patient library in relation to structural domains.

2.4 DISCUSSION

WES was performed from a predominantly Hispanic patient library. Using this library, coupled with OncoMiner Pipeline, more than 70,000 SNPs were identified. Differences in the nature and frequency of recurrently mutated genes and ALL-driving genes compared to the TARGET program cluster, which might reflect that less than 1 % of samples in genome-wide association studies are from Hispanic [109]. However, because Hispanic data are rare, and the genetic distance between our ALL samples and the TARGET cohort is unknown, it is unclear whether these differences might be implicated in ALL's oncogenesis in this region. Subsequently, the search was narrowed to identify eight potentially pathogenic variants in MAP2K3, MYLK, GUCY2C, and ERBB2 genes.

MAP2K3 was highly mutated in ALL patient samples from our local area compared with normal controls suggesting that these mutations might be involved in this disease pathogenesis. Three mutations were located in the kinase domain (R65L, R67W, and Q73stop), and two (P11T and R26T) were positioned in the amino-terminal domain of MEK3. These sites sequence alignment showed high homology across multiple species, further suggesting that these amino acids might be essential for protein function. Also, wild-type (WT) amino acid properties are often lost during mutations. For instance, in R67W, the mutated residue size is bigger, and there is an observable difference in hydrophobicity, which might impact folding or interactions. Future analysis of these mutations is necessary to determine their transforming potential and establish whether they could be used as biomarkers or therapeutic targets for the treatment of ALL.

Chapter 3: MEK3 Mutants Protein Expression is Unstable

3.1 Introduction

Protein abundance is determined by a balance between protein synthesis and degradation. Missense mutations have been associated with disruption of this equilibrium, causing either accumulation or accelerated degradation of mutated proteins [110]. During cancer, tumor suppressors are a constant target for inactivation [111]. MEK3/p38 signaling pathway plays a crucial role in the inhibition of proliferation and induction of apoptosis [12]. Therefore, inactivation of this signaling pathway or altered expression of molecules involved in its regulation would result in cancer progression.

To further elucidate the role of MEK3 mutants in protein stability, we first sought to evaluate WT and mutant MEK3 proteins' expression levels expressed in HEK293 cells. The strategy occupied in this chapter exploited the use of the protein synthesis inhibitor cycloheximide (CHX) and the synthetic proteasome inhibitor MG132. The evidence provided here demonstrates that MEK3 mutant protein expression is unstable. Moreover, it shows an accelerated rate of degradation compared with WT.

3.2 Materials and Methods

Cell culture

The human embryonic kidney, HEK293 cell line was maintained in RPMI 1640 medium containing 10 % fetal bovine serum (FBS; Atlanta Biologicals), 2 mM L-glutamine (Corning), and 1 % penicillin-streptomycin (Corning).

Plasmid and site-directed mutagenesis

The RC218101 plasmid for MAP2K3 was purchased from OriGene. The primers were designed using the Agilent mutagenesis primer design tool. Primers used were

R65L (forward 5'-tggccgtgaagctgatccgggccac-3' and reverse 5'-gtggcccggatcagcttcacggcca-3'); P11T (forward 5'-gcacccaaccccacaaccccccgg-3' and reverse 5'-ccgggggggttgtggggttgggtgc-3'); R67W (forward 5'-cgtgaagcggatctgggccaccgtgaa-3' and reverse 5'-ttcacggtggcccagatccgcttcacg-3'); R26T (forward 5'-ttcatcaccattggagacactaactttgaggtggaggctg-3' and reverse 5'-cagcctccacctcaaagttagtgtctccaatggtgatgaa-3'); Q73stop (forward 5'-ccgggccaccgtgaactcataggagcagaa-3' and reverse 5'-ttctgctcctatgagttcacggtggcccgg-3'). MEK3 mutants were made using the Quickchange II XL Site-directed Mutagenesis kit (Agilent Technologies) according to the manufacturer's instructions. All mutations were verified by DNA sequencing at the Genomic Analysis Core Facility of the Border Biomedical Research Center at The University of Texas at El Paso.

Transfection, lysis of cells, immunoprecipitation, and Western blot

HEK293 cells were seeded in 6 well plates and transiently transfected when they reached 90-100 % confluency. Wild-type (WT) or mutants MEK3 plasmid were transfected (3 µg) using 5 µl of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 24 hours post-transfection cells were pelleted, lysed in Triton lysis buffer (10 mM Tris-HCl [pH 7.6], 5 mM EDTA [pH 8.0], 50 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM Na_3VO_4, 1 % Triton X-100) in the presence of 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 2 µg/ml leupeptin, and 1 µg/ml pepstatin A proteases inhibitors for 1 hour at 4 °C and cleared by centrifugation (20,800 xg, 15 min, 4 °C). Total protein concentration was determined using the bicinchoninic acid method (Pierce). For MEK3 immunoprecipitation, samples were incubated with 2 µg of cMyc antibody (sc-40) for 2 hours at 4 °C. The complex was washed three times with cold lysis

buffer, eluted using 2x sample buffer, separated in a 4 - 20 % SDS-PAGE, and transferred to a PVDF membrane. Membranes were blocked with 1 % BSA for 1 hour at room temperature and blotted overnight at 4 °C using a phospho-specific antibody against MEK3 T222 (OriGene), anti-cMyc antibody (sc-40) to assess MEK3 expression or anti-MEubiquitin (A104R) to detect protein ubiquitination. Membranes were developed using an HRP goat anti-mouse antibody and visualized by enhanced chemiluminescence using LICOR and Image Studio Lite software. Membranes were placed in stripping buffer (62.5 mM Tris (pH 6.7), 2 % SDS, and 100 mM β-mercaptoethanol) for 30 min at 55 °C, blocked and reprobed with anti-GAPDH or anti-cMyc for 1 hour at room temperature.

MEK3 mutant proteins turnover

For analysis of protein turnover, HEK293 cell lines were plated in 6-well plates and transfected as previously indicated. Twenty-four hours post-transfection, cells were treated with the translational inhibitor cycloheximide (CHX) at a final concentration of 100 µg/mL, cells were collected after incubation at 0, 12, 24, and 48 hours. To investigate protein turnover in the presence of the proteasome inhibitor MG132, cells were treated as mentioned before, except that MG132 at a final concentration of 10 µM was added simultaneously.

3.3 RESULTS

N-Terminal and kinase domain variants blocked auto-phosphorylation of MEK3

For the purpose of studying the role of single mutants in the activation of MEK3 protein, HEK293 cells were transfected with WT or mutant plasmids, and the phosphorylation of T222 was assessed 24 hours post-transfection. Cells were lysed, pelleted and MEK3 immunoprecipitated using cMyc. To determine these mutants' effect,

activation of MEK3 was assessed using a phospho-specific antibody for T222. Data indicate that of the five mutations, only R67W, P11T, and R65L, decreased phosphorylation of MEK3 at T222 compared to the WT. Also, R26T exhibited no change in the phosphorylation of T222 (Figure 3.1). The impact of single variants in MEK3 protein expression was further analyzed by stripping membranes and reprobing them with anti-cMyc. As a result, P11T and R26T showed a strong signal comparable with WT; however, the expression of R65L and R67W variants diminished with respect to WT. As predicted, no band was observed around 37 kDa for the Q73* variant (Figure 3.1).

Quadruple and truncated MEK3 are unstable

To investigate the phenotype observed in these patients, quadruple (4M-MEK3) and truncated (Δ-MEK3) mutants were generated. Their effect on protein expression was explored in HEK293 cells transfected with either WT or mutant MEK3 constructs. Twenty-four hours post-transfection, cells were harvested and whole-cell lysates were probed for MEK3 using anti-cMyc. The quadruple mutant displayed significantly low levels of protein expression compared with WT. No band was observed for the truncated mutant in the predicted size (~15 kDa) (Figure 3.1).

Quadruple MEK3 mutant is degraded at an accelerated rate compared with the wild-type

We analyzed the half-life of the quadruple MEK3 mutant in the presence of the translational inhibitor CHX. The binding of CHX to the ribosome inhibits the elongation step mediated by the eukaryotic elongation factor 2 (eEF2) [112]. Quadruple mutant has a faster turnover rate compared with WT MEK3, with a half-life shorter than 12 hours compared with over 24 hours, respectively (Figure 3.2).

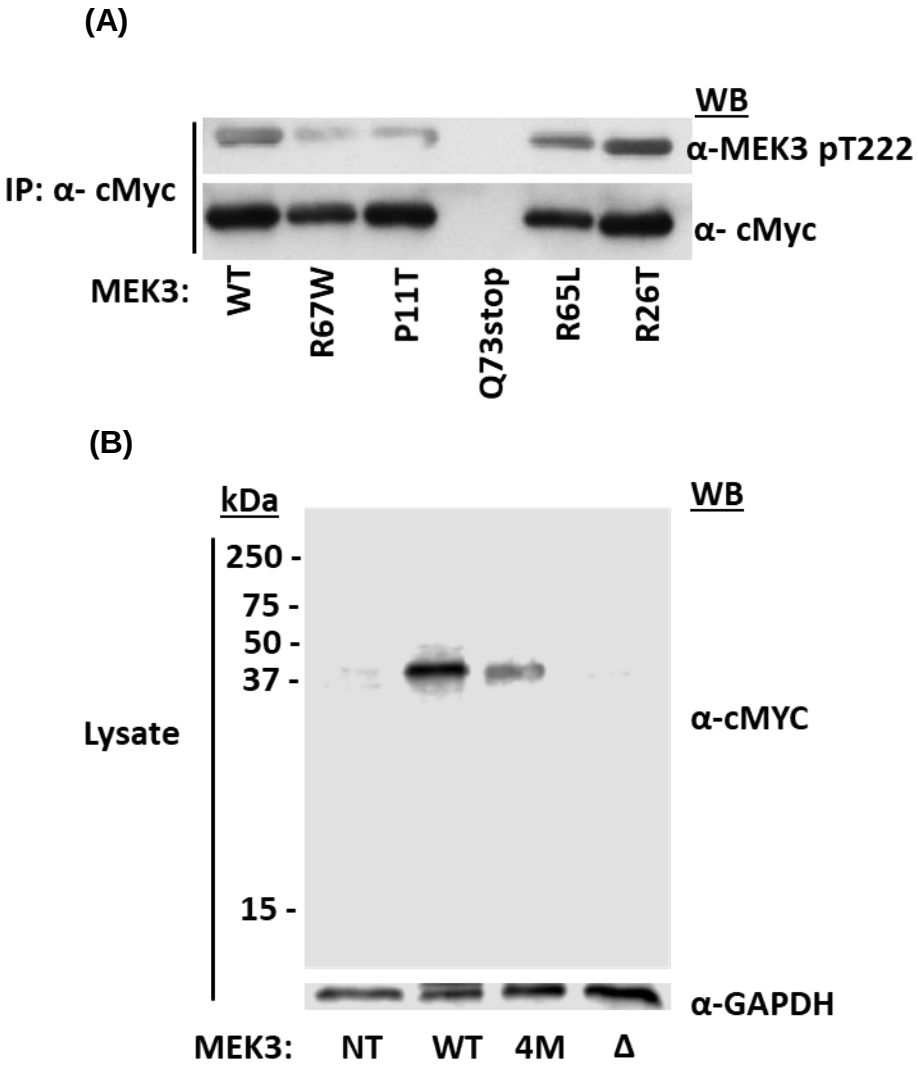

(A)
WB
α-MEK3 pT222
IP: α- cMyc
α- cMyc
MEK3:
WT
R67W
P11T
Q73stop
R65L
R26T
(B)
kDa
WB
250 -
75 -
50 -
37 -
Lysate
α-cMYC
15 -
α-GAPDH
MEK3:
NT
WT
4M
Δ
(C)
1.2
1
0.8
0.6
0.4
0.2
0
MEK3 Normalized fold expression
*
NT
WT
4M
Δ

Figure 3.1: MEK3 mutant proteins are unstable. HEK293 cells were non-transfected (NT) or transiently transfected with WT-cMyc or MEK3-cMyc mutant constructs. Cell lysates or immunoprecipitated (IP) cMyc tag were separated by SDS-PAGE and immunoblotted (WB) as indicated. **(A)** Overexpression of WT, N-terminal domain (P11T, and R26T) or kinase domain (R65L, R67W, and Q73*) single variants. **(B)** Overexpression of quadruple (4M) and truncated (Δ) MEK3 mutants. **(C)** MEK3 protein expression band intensities were normalized to GAPDH using densitometric analysis. Quantification of MEK3 mutant protein expression relative to WT. Data are presented as mean ± SEM (n=3). The two-tailed Student's paired *t*-test was used to determine statistical significance. *, $p < 0.05$.

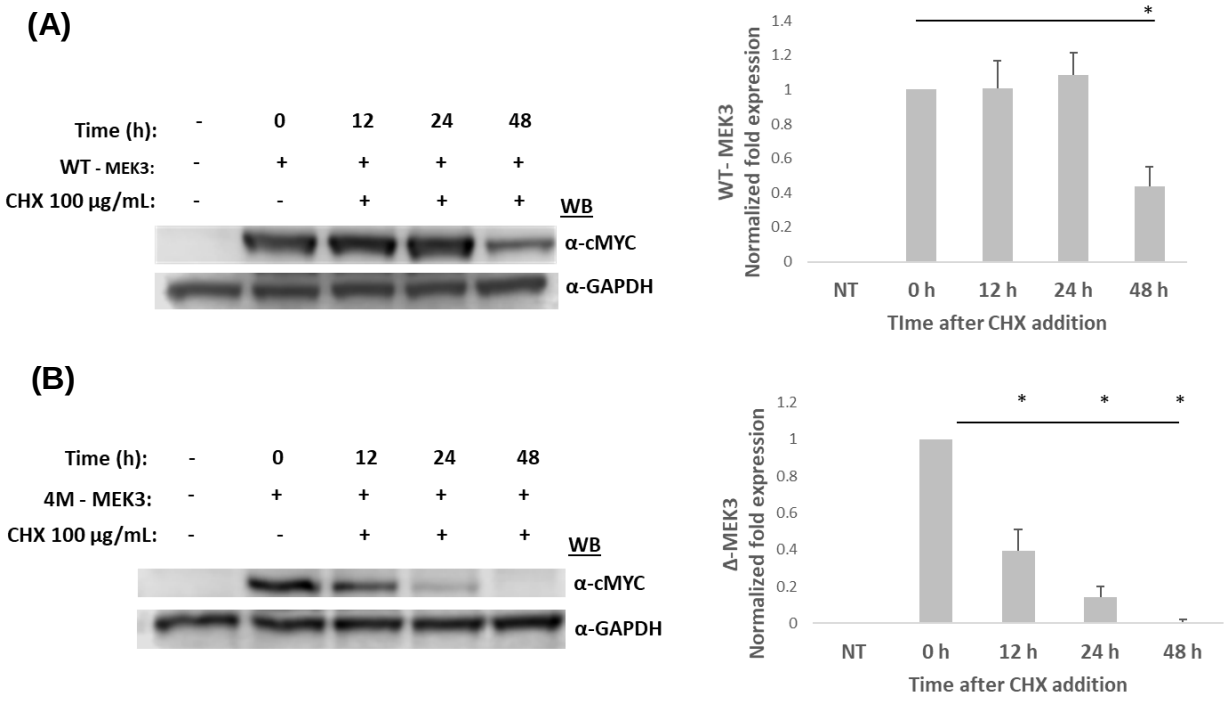


Figure 3.2: Quadruple MEK3 mutant is degraded at an accelerated rate compared to wild-type protein. HEK293 cells were non-transfected (-) or transiently transfected with WT-cMyc **(A)** or 4M-MEK3-cMyc mutant **(B)** constructs. Twenty-four hours post-transfection, cells were treated with DMSO (-) or CHX for the indicated time points. Cell lysates were collected and separated by SDS-PAGE and immunoblotted (WB), as indicated. MEK3 protein expression band intensities were normalized to GAPDH using densitometric analysis. Graphs represent WT or 4M MEK3 protein expression quantification relative to time 0. Data are presented as mean ± SEM (n=3). The two-tailed Student's paired *t*-test was used to determine statistical significance. *, $p < 0.05$.

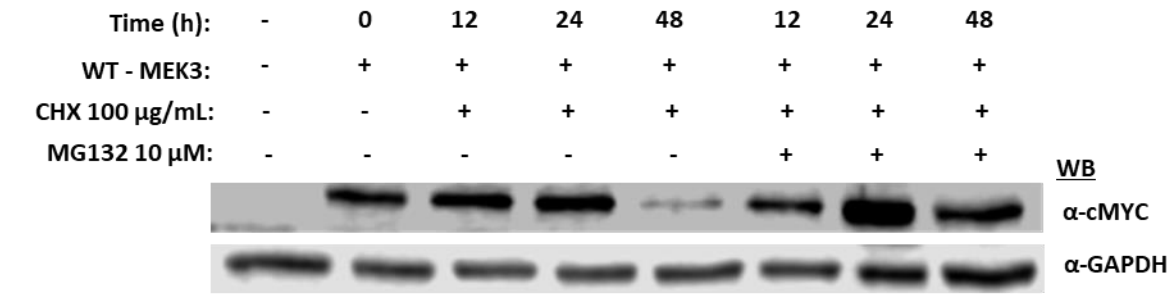
MG132 reduced the turnover of the wild-type protein but had no effect on the quadruple MEK3 mutant

Several mechanisms and pathways mediate protein degradation. However, the majority of proteins in the cell are targeted for proteasomal degradation [113]. The proteasome is comprised of two subcomplexes, the 19S and 20S. Together, they form what is known as the 26S complex. It is a cylindrical-shaped structure with the 19S cap at the end [114]. The 19S structure recognizes target proteins, unfolds them, and creates conformational changes that allow for the 20S pore opening. Once inside the 20S core, proteins are degraded [114]. To investigate whether proteasome inhibition stabilizes the quadruple mutant expression, HEK293 cells transiently transfected to express WT, or quadruple mutants MEK3 plasmids were treated with MG132. As a result, WT protein expression was stabilized by proteasomal inhibition, whereas no effect was observed in the quadruple MEK3 mutant (Figure 3.3).

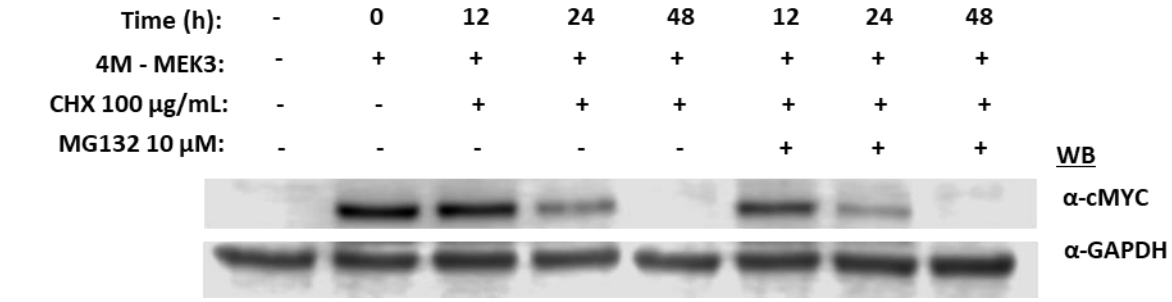
Truncated MEK3 mutant proteasomal degradation is ubiquitin independent

To explore whether the truncated mutant was either not expressed or rapidly degraded, we treated HEK293 cells transfected with WT or mutants MEK3 plasmids with MG132. Stable WT and quadruple MEK3 expression levels were observed for 8 hours, while truncated MEK3 mutant protein expression was rescued by the proteasome inhibitor MG132 after four hours of treatment (Figure 3.4). Proteasome degradation typically involves substrate modification by the addition of ubiquitin to lysine residues. Hence, to assess MEK3 ubiquitination, lysates from transfected cells were immunoprecipitated for MEK3 using anti-cMyc antibodies, and membranes probed for ubiquitin. No evidence for ubiquitination was observed (Figure 3.5).

(A)



(B)



(C)

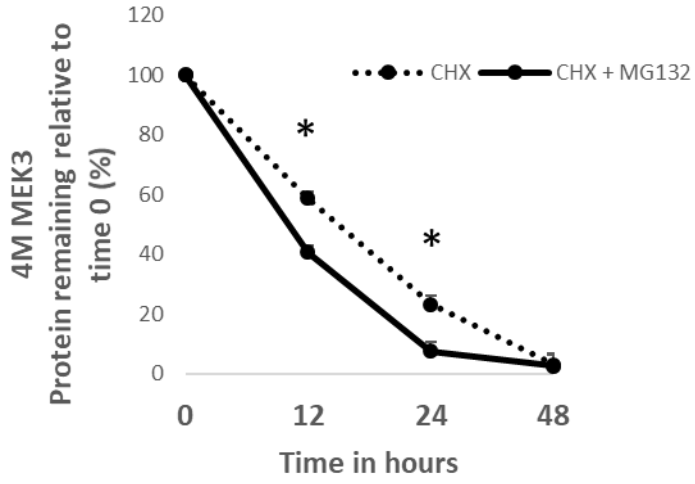


Figure 3.3: MG132 reduced wild-type protein turnover but had no effect on the quadruple MEK3 mutant. HEK293 cells were non-transfected (-) or transiently transfected with WT-cMyc **(A)** or 4M-MEK3-cMyc mutant **(B)** constructs. Twenty-four hours post-transfection, cells were treated with DMSO (-), CHX, or a combination of CHX

plus MG132 for the indicated time points. Cell lysates were separated by SDS-PAGE and immunoblotted (WB), as indicated. MEK3 protein expression band intensities were normalized to GAPDH using densitometric analysis. **(C)** The graph represents 4M-MEK3 protein expression quantification relative to time 0. Data are presented as mean ± SEM (n=3). The two-tailed Student's paired *t*-test was used to determine statistical significance. *, $p < 0.05$.

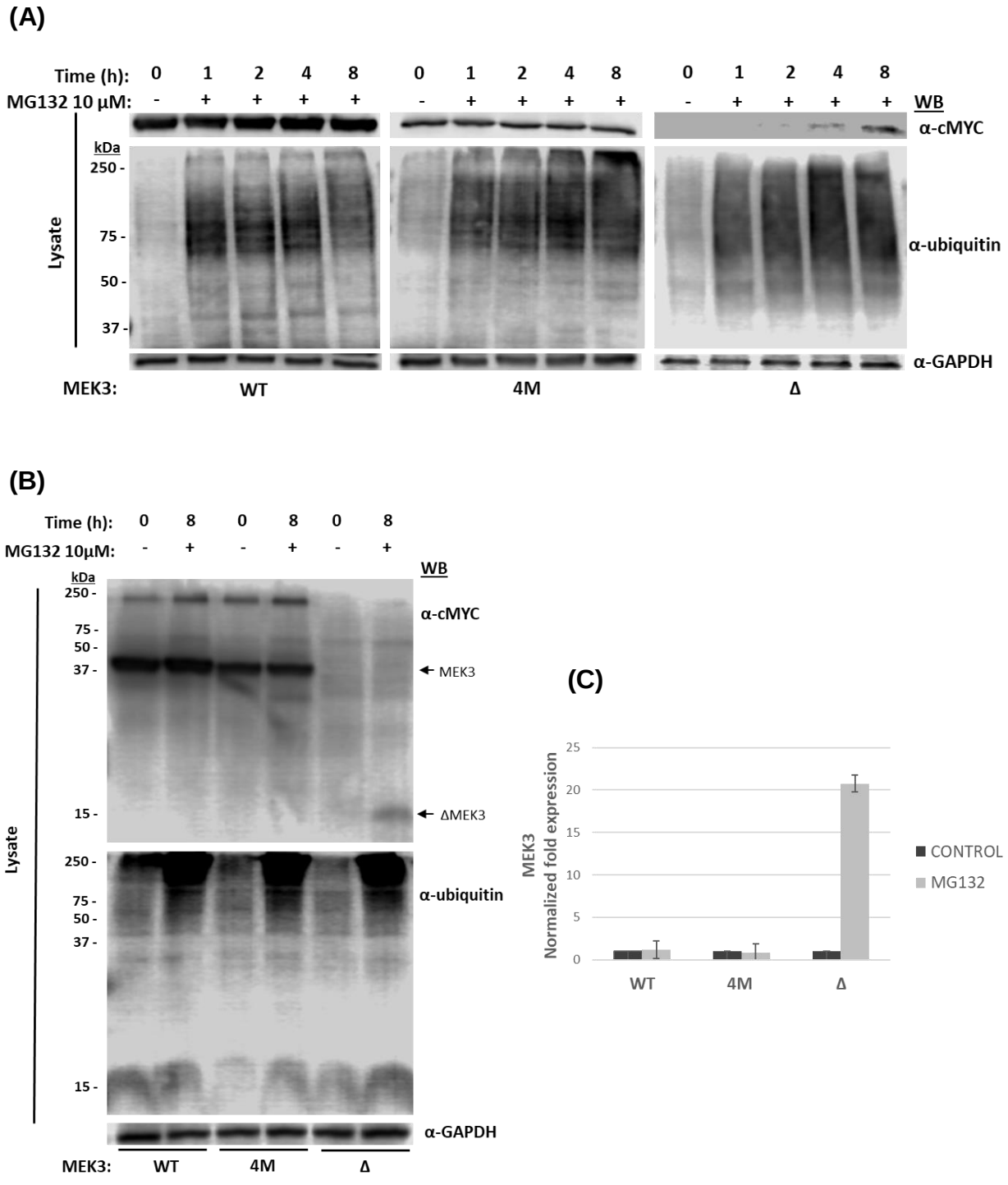


Figure 3.4: Truncated MEK3 mutant protein expression is rescued by the proteasome inhibitor MG132. HEK293 cells were transiently transfected with WT-cMyc or MEK3-cMyc mutant (4M-MEK3 and Δ-MEK3) constructs. Twenty-four hours post-

transfection, cells were treated with DMSO (-) or MG132 for the indicated time points. Cell lysates were separated by SDS-PAGE and immunoblotted (WB), as indicated. **(C)** Quantification of WT or MEK3 mutants protein expression relative to time 0. Band intensities were normalized to GAPDH using densitometric analysis. Data are presented as mean ± SEM (n=3). The two-tailed Student's paired *t*-test was used to determine statistical significance. *, $p < 0.05$.

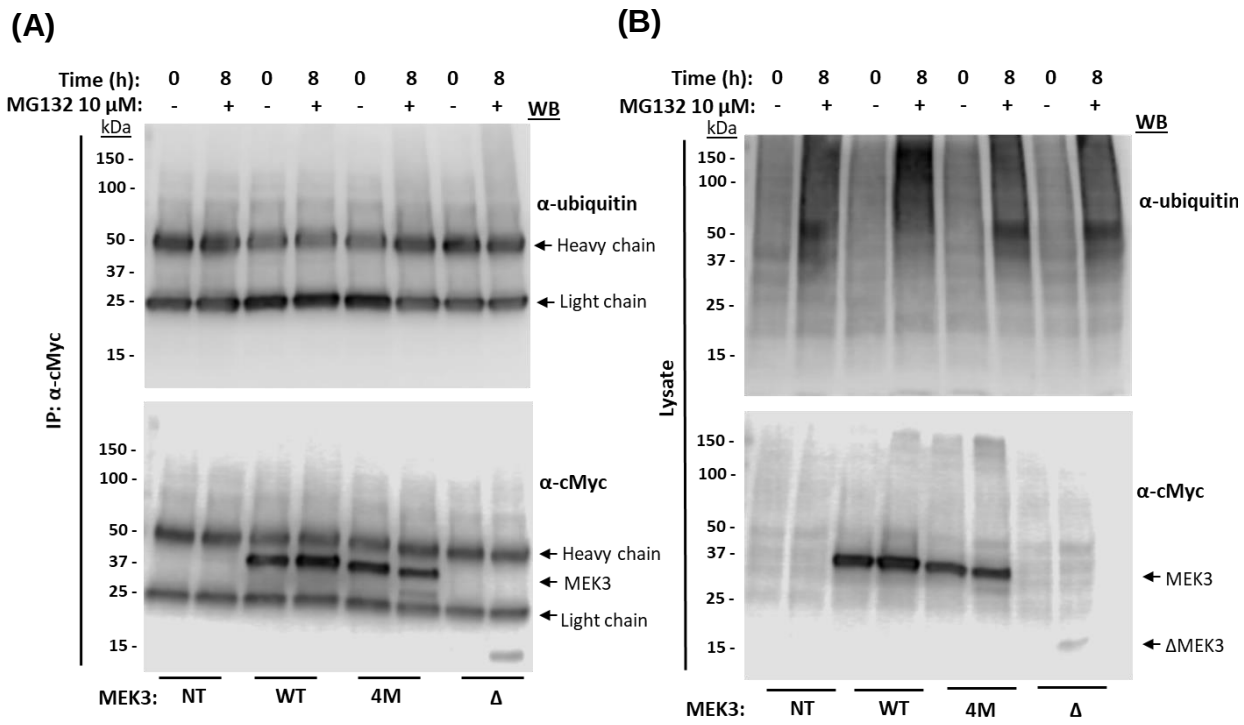


Figure 3.5: Truncated MEK3 mutant proteasomal degradation is ubiquitin independent. HEK293 cells were non-transfected (NT) or transiently transfected with WT-cMyc or MEK3-cMyc mutant (4M-MEK3 and Δ-MEK3) constructs. Twenty-four hours post-transfection, cells were treated with DMSO (-) or MG132 per 8 hours. Immunoprecipitated (IP) cMyc **(A)** or cell lysates **(B)** were separated by SDS-PAGE and immunoblotted (WB) as indicated.

3.4 DISCUSSION

Kinase activating mutations have gained attention because of their ability to drive cancers, thus becoming targets for drug development. However, loss-of-function mutations are also of great importance, considering they can impact protein production by decreasing stability or affecting folding kinetics. This can instigated protein unfolding and creating small quantities of incorrectly folded proteins [115]. To investigate whether MEK3 mutants affect protein stability, cell lysates from HEK293 cells transfected with WT or MEK3 mutant constructs were probed for MEK3 using anti-cMyc. Unsurprisingly, we discovered that single mutants R65L and R67W affect not only the auto-phosphorylation of MEK3 but also its protein expression levels.

K64 is essential for kinase activity following binding to the α and β phosphates of ATP [116]. Moreover, K64, R65, and R67 are located in αE-helix, which, together with AH1, the β3-, β4-, and β5-strands, comprise the large hydrophobic cluster [116]. Hence, we speculated that R65L and R67W create physico-chemical distortions by replacement of polar to hydrophobic residues. The impact of single MEK3 mutants in the autophosphorylation of MEK3 was investigated, finding that R67W, P11T, and R65L decreased phosphorylation of MEK3 at T222 compared to WT. Since S218 and T222 are involved in MEK3 protein activation, this data indicates that these mutations may block MEK3 auto-phosphorylation.

The quadruple mutant has a shorter half-life, and its degradation is proteasome-independent. Previous studies have shown that the degree of folding in a protein can influence its rate of degradation [117]. Therefore, it is likely that the quadruple mutant is partially folded compared to WT, thereby inducing an accelerated rate of degradation.

Perhaps, a disruption in protein structure in such a way can facilitate modifications (which in return will enhance degradation) or sequences involved in degradation become exposed [115, 118]. These mutations are also likely to create hydrophobicity changes and thermal stabilities. Indeed, structural features for protein stabilization have been found in the amino domain [119]. The molecular mechanisms implicated in WT MEK3 turnover are yet to be characterized; however, the fact that the quadruple mutant's degradation did not involve the proteasome suggests that another mechanism might be responsible. Certainly, this is plausible given that multiple mechanisms can act on the degradation of a single protein [117].

Furthermore, the data presented in this chapter indicates that the lack of expression of the truncated MEK3 mutant is due to enhanced proteasomal degradation, which explains its lack of detection. Additionally, the truncated mutant is more susceptible to degradation than the WT and quadruple mutant. Taken together, this data suggests the degradation of MEK3 mutants is likely to result in loss of MEK3 WT function. It seems plausible to expect that the MEK3 mutant results in impaired p38 activation and purpose of continuing studies described in Chapter 4.

Chapter 4: MEK3 Mutants Promote Cell Proliferation Through MAPK p38 Inactivation

4.1 Introduction

MEK3 is activated by cytokines and growth factors but mainly by stress. Once activated, it binds to MAPK p38 and induces its activation triggering pleiotropic effects. It is well known the dual role for p38 in cellular proliferation. However, the molecular mechanism that favors cell proliferation versus cell cycle arrest is not understood [12]. p38 can negatively regulate proliferation via cell cycle arrest and through senescence or apoptosis [12]. Indeed, mice lacking MEK3 and MEK6 are susceptible to tumorigenesis, and decreased levels of MEK3 have been observed in different types of cancer [59, 60]. Additionally, p38 inhibits oncogenic Ras-dependent transformation and initiates apoptosis during ROS production [120, 121]. Alternatively, it has been shown to enhance survival and metastasis [122]. Despite these models, p38 is not a target of genetic instability [123].

The focus of this chapter is to determine whether P11T, R26T, Q73stop, R65L, and R67W have oncogenic potential. To further elucidate the role of MEK3 mutants in auto-activation and catalytic activity, HEK293 cells were transiently transfected with WT or mutant MEK3 plasmids. We assessed their auto-activation and capacity to activate the downstream effector p38 in comparison to the WT MEK3. Here we provide evidence that quadruple and truncated mutants enzymatic activity is impacted. Consequently, the absence of p38 activation enhances HEK293 cells proliferation.

4.2 Materials and Methods

siRNA-mediated silencing of MAP2K3

siRNA-mediated silencing of MAP2K3 (SMARTpool catalog number M-003592-03-0010) and control non-targeting (siGENOME Non-Targeting siRNA Pool #1 catalog number D-001206-13-20) siRNA were purchased from Dharmacon. HEK293 were transfected with either non-targeting control siRNA (100 nM) or MEK3 specific siRNA using 8 µl of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Transfection, lysis of cells, immunoprecipitation, and Western blot

HEK293 cells were seeded in 6 well plates and transiently transfected when they reached 90-100 % confluency. WT or mutants MEK3 plasmids were transfected (3 µg DNA) using 5 µl of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 24 hours post-transfection cells were pelleted, lysed in Triton lysis buffer (10 mM Tris-HCl [pH 7.6], 5 mM EDTA [pH 8.0], 50 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM Na_3VO_4, 1 % Triton X-100) in the presence of 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 2 µg/ml leupeptin, and 1 µg/ml pepstatin A proteases inhibitors for 1 hour at 4 °C and cleared by centrifugation (20,800 xg, 15 min, 4°C). Immunoprecipitated MEK3 samples were incubated with 2 µg of cMyc antibody (sc-40) for 2 hours at 4 °C. The complex was washed three times with cold lysis buffer, eluted using 2x sample buffer. Immunoprecipitated MEK3 and whole-cell lysates were separated in a 4-20 % SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked with 1 % BSA for 1 hour at room temperature and blotted with a phospho-specific antibody for T222 on MEK3 (oriGene TA326175) or a phospho-specific antibody for T180/Y182 on p38 (cell signaling 4115S). Membranes were developed using an HRP goat anti-rabbit and visualized by enhanced chemiluminescence using LICOR and Image Studio Lite software. After developing membranes, they were placed in stripping buffer (62.5 mM Tris

(pH6.7), 2 % SDS, and 100 mM β-mercaptoethanol) for 30 min at 55 °C, blocked and re-probed with anti-cMyc (sc-40) or anti-p38 (sc-728) for 1 hour at room temperature.

Cell viability assay

To examine whether these MEK3 mutants impact proliferation, HEK293 cells were transfected in 6 well plates with 3 µg of WT or MEK3 mutant plasmids. Twenty-four hours post-transfection, cells were harvested and plated in 96-well plates (10,000 cells per well) and incubated overnight at 37 °C to allow cell attachment. To measure cell viability, 20 µl of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3- carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reagent (Promega) was added to each well, and the cells were incubated for an additional 1 hour at 37 °C. Absorbance was measured at 490 nm using a microplate reader. Data are the mean of three independent experiments with their corresponding standard deviations and are presented as percentages of cell viability.

Proteome Profiler Human Cell Stress Array

HEK29 cells were transfected with WT or Mutant MEK3 constructs. Protein concentration was determined by the bicinchoninic acid method (Pierce Biotech). Equal concentrations of protein were analyzed using the proteome profiler human cell stress array (R&D Systems #ARY018) according to the manufacturer's protocol. Membranes were visualized by enhanced chemiluminescence and X-ray film (Phenix). Densitometric analysis was performed using the software Image Studio Lite Ver 5.2.

4.3 RESULTS

MEK3 mutants blocked auto-phosphorylation of MEK3 at T222 and reduced phosphorylation of p38 MAPK at Thr180/Tyr182

To assess whether previously identified MEK3 mutations regulate the function of p38, HEK293 cells were transfected with WT or mutant MEK3 plasmids. Twenty-four hours post-transfection, cells were lysed, pelleted, and MEK3 immunoprecipitated using cMyc. MEK3 autophosphorylation was assessed using a phospho-specific antibody for T222. Whole-cell lysates were collected, and activation of p38 was investigated using a phospho-specific antibody for MAPK p38 Thr180/Tyr182 (cell signaling #4511). Densitometric analysis was performed and data were normalized to cMyc or p38. Quadruple and truncated MEK3 mutants displayed an inability to autophosphorylated compared to the WT. Furthermore, activation of p38 was inhibited (Figure 4.1).

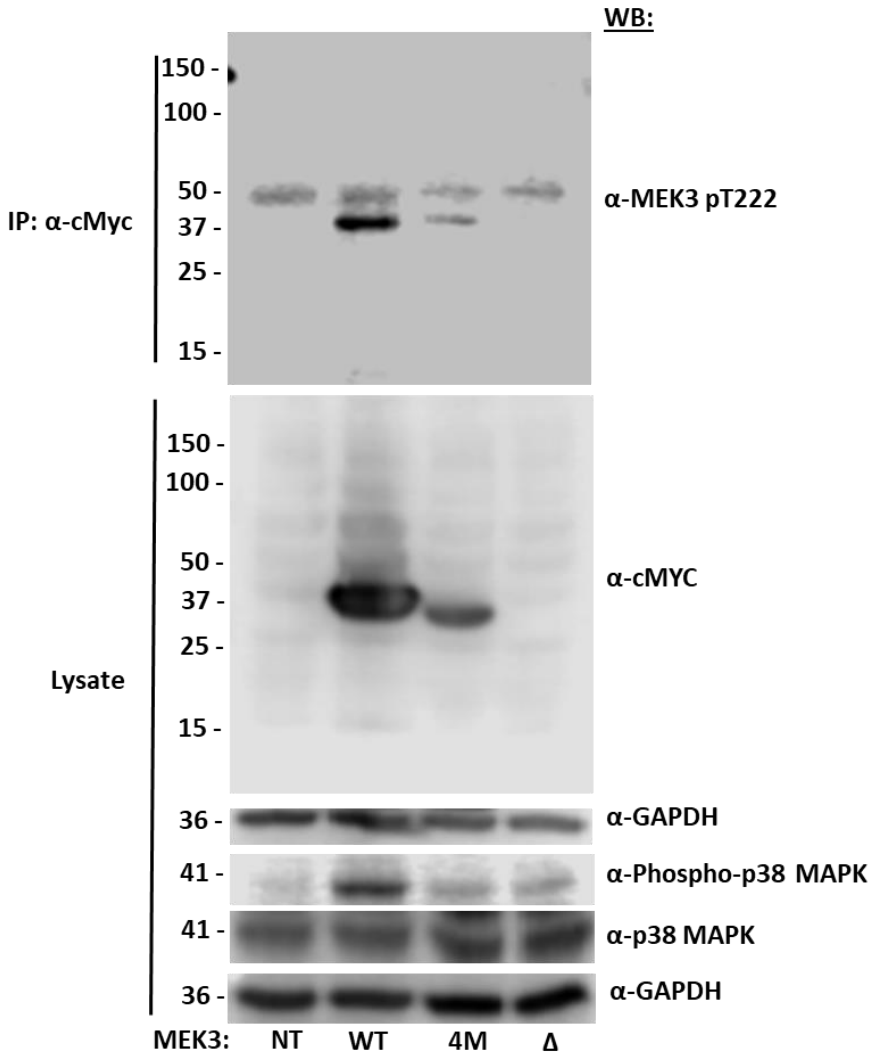
MEK3 mutants promote the viability of HEK293 cell line

Previous studies show an essential role for the MEK3/p38 signaling pathway in proliferation. MEK3 mutants' ability to induce proliferation was measured in HEK293 cells transfected with WT and mutant MEK3 plasmids using MTS. Transfected cells were seeded in 96-well plates, and viability was measured at 24, 48, and 72 hours. Data are presented as percent viability. Compared with WT, the quadruple and truncated mutants result in a significant increase cell proliferation in 24 hours. No significant increase was seen at 48 or 72 hours (Figure 4.2).

MEK3 mutants enhance HIF-1α and CITED-2 protein expression

The MEK3/p38 signaling pathway is activated by cytokines and growth factors, but also by stress. To investigate whether MEK3 mutants impact the expression of 26 stress-related proteins, whole-cell lysates from HEK293 transfected with WT or mutants MEK3 plasmids were mixed with a cocktail of biotinylated antibodies and then incubated overnight with membranes containing capture antibodies. Compared with WT, quadruple,

and truncated MEK3 mutants, enhanced expression of the hypoxia-inducible factor 1 α (HIF-1α) and the cAMP-responsive element-binding protein (CBP)/p300-interacting transactivator with ED (glutamic acid and aspartic acid)-rich tail 2 (CITED2) (Figure 4.3).


WB:
IP: α-cMyc
150 -
100 -
50 -
37 -
25 -
15 -
α-MEK3 pT222
Lysate
150 -
100 -
50 -
37 -
25 -
15 -
α-cMYC
36 -
α-GAPDH
41 -
α-Phospho-p38 MAPK
41 -
α-p38 MAPK
36 -
α-GAPDH
MEK3: NT WT 4M Δ

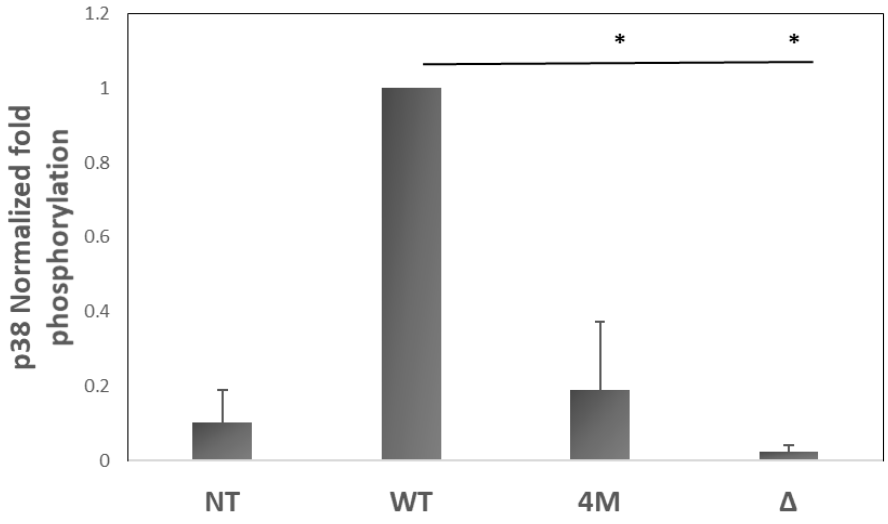

p38 Normalized fold phosphorylation
1.2
1
0.8
0.6
0.4
0.2
0
*
*
NT
WT
4M
Δ

Figure 4.1: MEK3 mutants blocked auto-phosphorylation at T222 and reduced phosphorylation of p38 MAPK at Thr180/Tyr182. HEK293 cells were non-transfected (NT) or transiently transfected with WT-cMyc or MEK3-cMyc mutant (4M-MEK3 and Δ-MEK3) constructs. Cell lysates or immunoprecipitated (IP) cMyc tag were separated by SDS-PAGE and immunoblotted (WB) as indicated. Phospho-p38 band intensities were normalized to total p38 using densitometric analysis. Quantification of phospho-p38 relative to WT. Representative data for n=3 are presented as mean ± SEM. The two-tailed Student's paired *t*-test was used to determine statistical significance. *, $p < 0.05$.

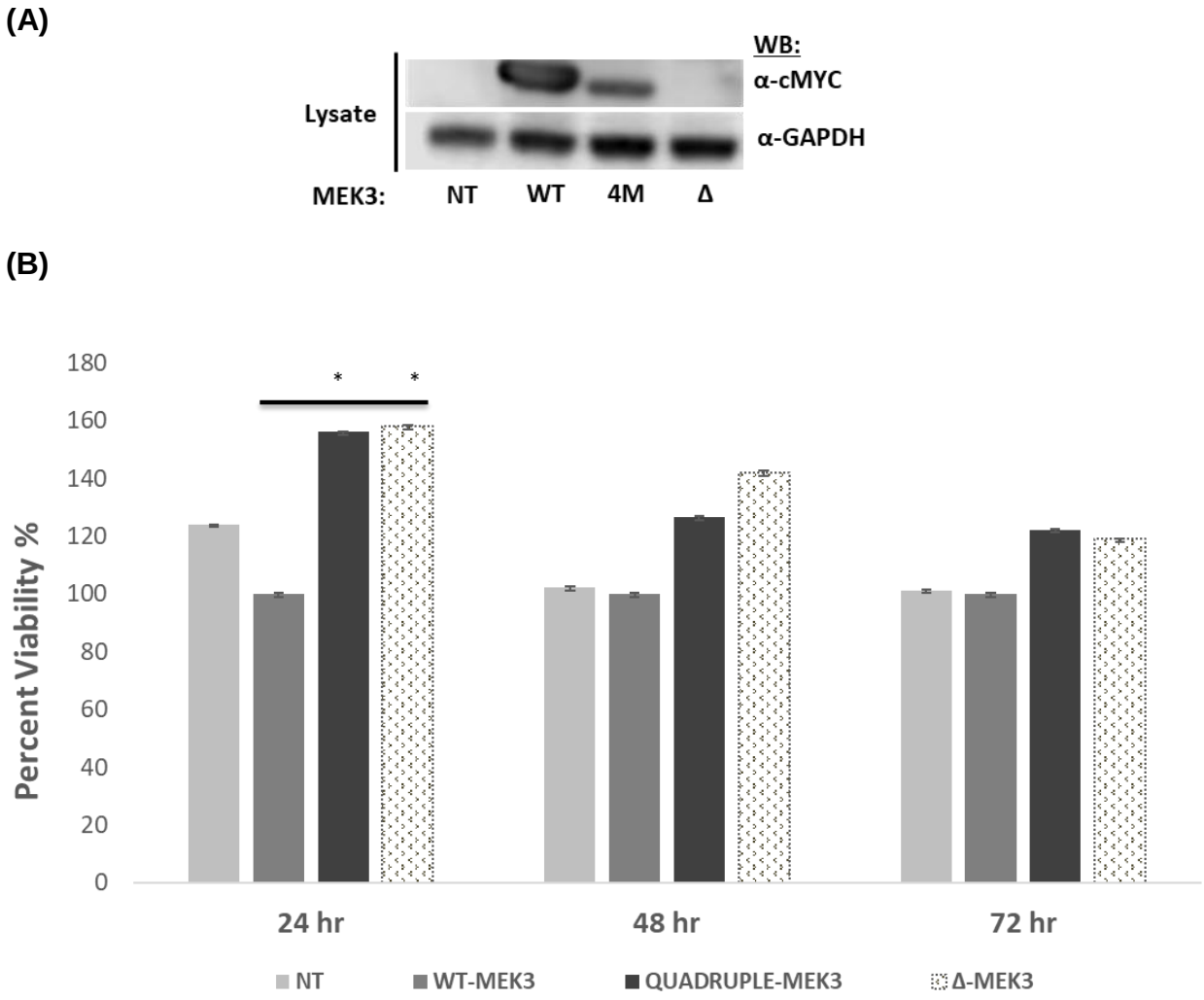
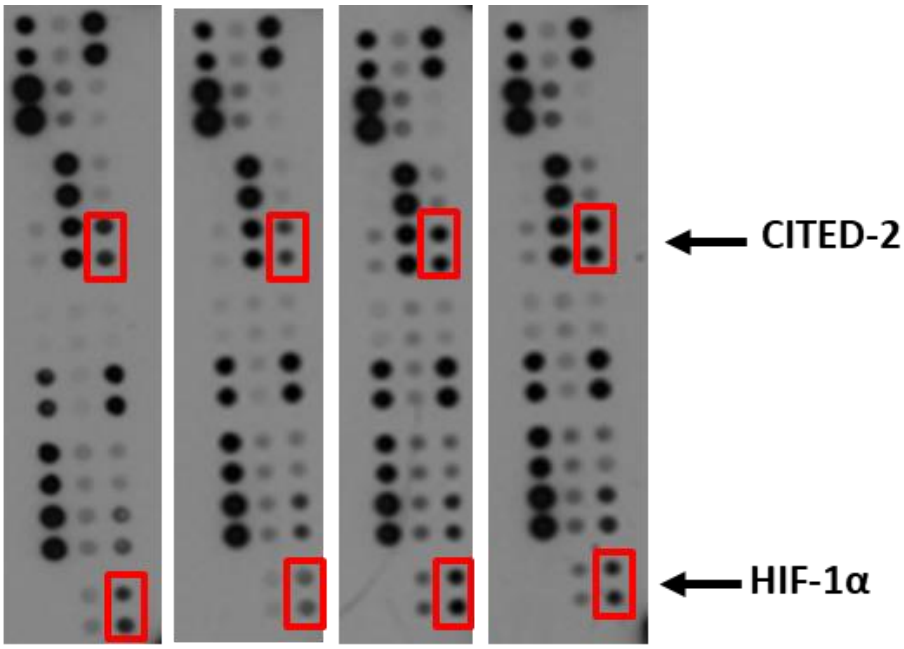
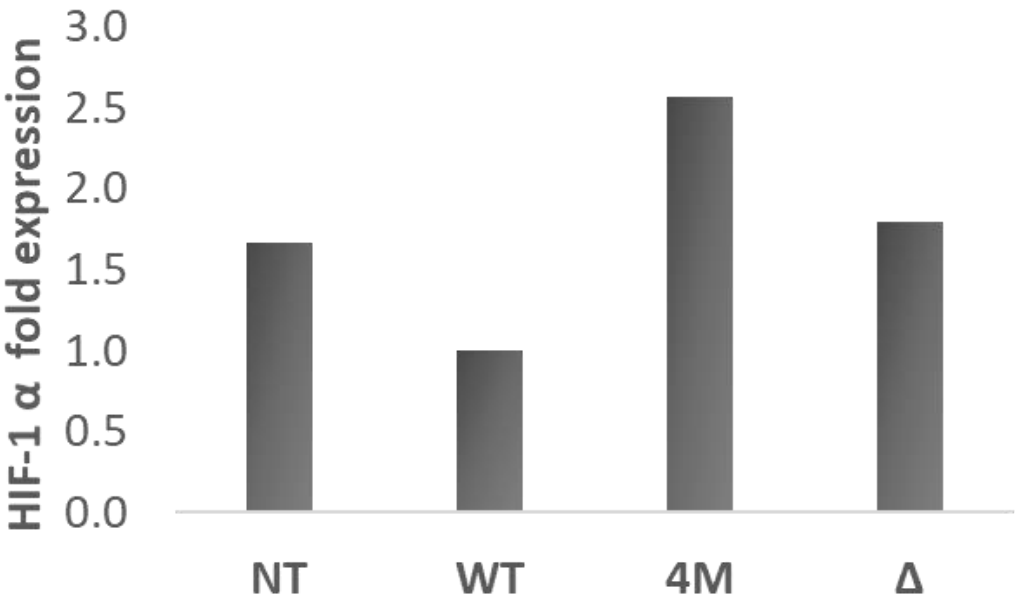


Figure 4.2: MEK3 mutants promote the viability of the HEK293 cell line. HEK293 cells were non-transfected (NT) or transiently transfected with WT-cMyc or MEK3-cMyc mutant (4M-MEK3 and Δ-MEK3) constructs. (A) Cell lysates from HEK293 transfected with WT or mutant plasmids were separated by SDS-PAGE and immunoblotted (WB) as indicated. (B) Cell viability was measured at 24, 48, and 72 hours using MTS. Values from three independent experiments are presented as the mean ± SEM percent viability. The two-tailed Student's paired t-test was used to determine statistical significance. *, $p < 0.05$.

(A)



(B)



MEK3: NT WT 4M Δ

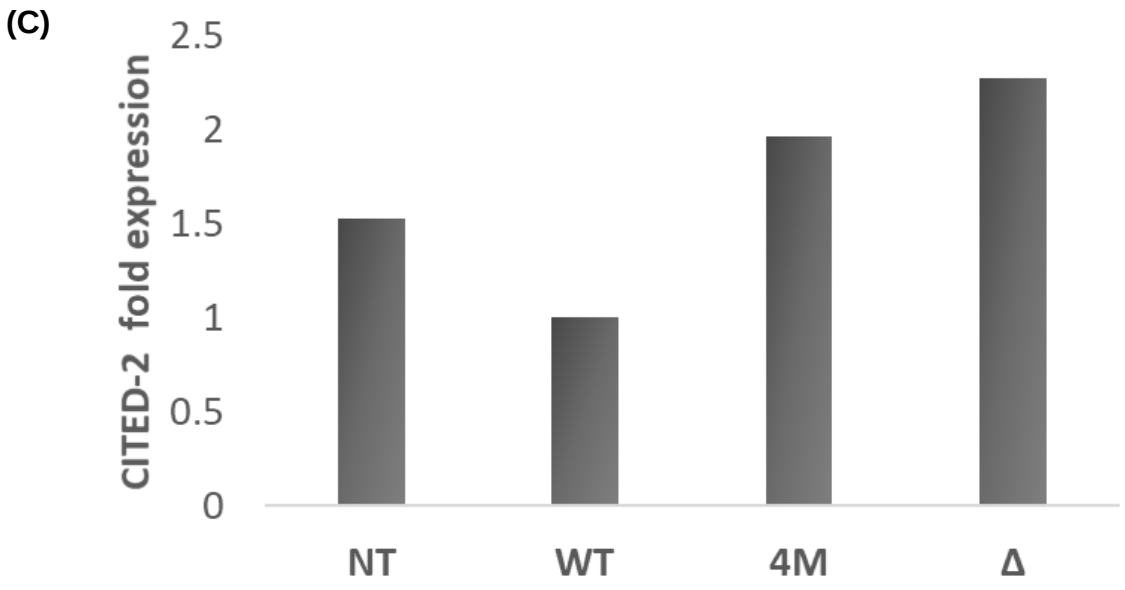


Figure 4.3: MEK3 mutants enhance HIF-1α and CITED-2 protein expression. HEK293 cells were transfected with either non-targeting control siRNA (100 nM) (NT) or MEK3 specific siRNA (100 nM) (WT, 4M, Δ). Forty-eight hours after the knockout, cells were non-transfected (NT) or transiently transfected with WT-cMyc or MEK3-cMyc mutant (4M-MEK3 and Δ-MEK3) constructs. Twenty-four post-transfection cells were harvested. **(A)** Cell lysates were mixed with a cocktail of biotinylated antibodies for 1 hour. Then these sample/antibody complexes were incubated overnight with membranes spotted with capture antibodies. **(B)** HIF-1α **(C)** CITED-2 protein quantification is relative to WT. (n=1)

4.4 DISCUSSION

The structural features of MEK3 have not been characterized. Structural data related to the MEK family are only available for MEK1/2 and MEK6 [124]. However, MEK3 shares ~ 80 % of homology with MEK6 [125]; their significant differences are located in the amino domain. Therefore, based on amino acid and domain conservation within the p38 activators (MEK3 and MEK6), assumptions regarding the location and function of the five residues are assumed.

Kinase autophosphorylation occurs when its enzymatic domain catalyzes the reaction, usually via dimerization, when another enzyme of the same type provides the active site. Previous structural studies suggest that MEK6 adopts dimerization in an anti-parallel configuration through its amino domain [124]. Dimerization allows the autoinhibition of MEK6. Additionally, within this interphase, an arginine stack involving R65 is positioned [124]. It is thought to act by stabilizing binding interactions, which support the concept that these mutations (R65L and R67W) might impair dimer formation. Consequently, MEK3 enzymatic activity is affected, leading to the disruption p38 cellular function. Since activation of p38 is required for metabolism, differentiation, migration, apoptosis, survival and in blocking proliferation [41,42], these alterations uncouple the MEK3/p38 pathway contributing to tumor cell proliferation by enhancing expression of stress-related proteins such as HIF-1α and CITED-2. Expression of HIF-1α and CITED-2 is known to be upregulated in leukemia, and their inhibition induces apoptosis [126-128]. Moreover, pharmacological inhibition of HIF-1α is associated with enhanced susceptibility to chemotherapy [126].

Chapter 5: Overview and Future Directions

5.1 Overview

This dissertation's objective was to identify potential oncogenes involved in ALL's oncogenesis in this region. Using WES coupled with bioinformatics, **we identified over 70,000 SNPs from a regional, mostly Hispanic cancer patient library**. Interestingly, most recurrently mutated genes in our samples were either absent or present with a low frequency of mutations in the TARGET program. **The strategy employed recognized MEK3 as a potential oncogene**. Five SNPs were identified, and sequence alignment of these residues revealed high homology across multiple species.

The reminder of this dissertation was to determine the oncogenic potential of MEK3 mutants. The strategy of transiently transfecting HEK293 transiently with WT or mutants MEK3 constructs revealed that **mutant protein expression is unstable**. To elucidate whether MEK3 mutants were targeted for degradation, we employed the protein synthesis inhibitor cycloheximide and the proteasome inhibitor MG132. We observed that the **quadruple MEK3 protein expression is not stabilized by proteasomal inhibition**, **while truncated MEK3 is degraded by the proteasome in a ubiquitin independent manner.** Moreover, we assessed the impact of the auto-activation and enzymatic activity of the WT protein versus the mutants by Western blot analysis. We determined that **MEK3 mutants are unable to auto-phosphorylate and activate MAPK p38.** Taken together, these data suggest that these mutations block auto-phosphorylation of MEK3, and inhibit its activation and regulation of p38 activation. Since p38 is involved in cellular proliferation, we determined their impact on cellular proliferation by MTS assay, which

revealed that **MEK3 mutants increased cell proliferation of HEK293 through elevated expression of HIF-1α and CITED-2.**

In conclusion, we were able to identify five mutations in the MEK3 protein from nine ALL patient samples. MEK3 is responsible for the activation of p38 MAPK to mediate growth-inhibitory and pro-apoptotic signals. Consequently, inhibition of p38 activity through enhanced degradation of MEK3 mutant dysregulates this pathway contributing to tumor cell proliferation. Therefore, modulation of the MAPK p38 negative regulators, inhibition of downstream targets such as HIF-1α or CITED-2, might be an alternative to the absence of the p38 signaling activity (Figure 5.1).

5.2 Future Directions

The amino-terminal phosphorylation of the Retinoblastoma (RB) tumor suppressor by p38, specifically S249 and T252, delays G1 cell cycle progression [129]. Furthermore, downregulation of MKP1 positively upregulates activation of p38 and JNK pathways inducing sensitivity to genotoxic agents such as Cisplatin and Gemcitabine [130]. Thus, drugs that mimic conformational changes caused by p38 phosphorylation of downstream targets and the use of small-molecule inhibitors to target negative regulators of p38 should prove of therapeutic benefits. However, the versatility of p38 is a challenge for developing and testing effective p38 inhibitors in clinical trials. Seven clinical trials targeting p38 are being evaluated to treat ovarian cancer, glioblastoma and myeloma, but thus far, drugs that target p38 have not been approved [12]. Systemic side effects by such drugs have been the primary cause of failure. Moreover, p38 inhibition during cancer treatment might induce tumor initiation in other tissues [12]. To overcome this situation, the treatment strategy is to create a synergistic effect of classical chemotherapy with p38

inhibitors and/or targeting p38 downstream targets such as MK2 kinase might reduce their deleterious effects [12]. Additionally, it is essential to identify specific p38 targets that exert each function and how they contribute to the different stages of tumor cell proliferation. The development of novel drugs that disrupt cell proliferation but maintain the apoptotic ability of p38 or switch between activation or inactivation might yield a better understanding of this pathway signaling and oncogenic pathway.

The development of tools and novel biomarkers might be another strategy to co-support p38 inhibitors [131]. For instance, mutations identified herein could be used as markers of p38 inactivation, thereby develop p38 inhibitors for these patients. However, they might benefit from modulation of p38 activation or specific inhibition of downstream targets of p38.

Determine whether quadruple MEK3 mutant is degraded through the lysosome

Data presented herein suggests that quadruple MEK3 mutant did not undergo proteasomal-mediated degradation. It is unclear whether this mutant is degraded in the lysosome. One approach to address this question would be to explore lysosomotropic agents such as chloroquine or NH_4Cl to determine the degradation pathway of the quadruple mutant. Furthermore, determine whether this mutant has enzymatic activity towards p38 in the presence of lysosomotropic agents would be interesting. This would suggest that therapeutic agents that block its degradation could be used as a possible therapy for these patients and could be tested in cell lines or animal models for ALL and other types of cancers.

Stable knockdown of endogenous MEK3

MEK3 is ubiquitous and abundantly expressed. Therefore, endogenous MEK3 might attenuate the effect of exogenously expressed MEK3 on cellular function. Generation of stable HEK293 cells lacking MEK3 might shed light on how MEK3 mutants can affect proliferation, apoptosis, and cell cycle regulation.

Investigate cellular signaling pathways activated by MEK3 mutants through the use of transformed lymphocytic cells lines

Previous studies suggest that p38 signaling regulates cellular activities is cell type-dependent. Thus, it would be interesting to investigate whether these mutants have the same outcome observed in the HEK293 system. Moreover, the use of lymphocytic cell lines, which rely on extracellular stimuli for survival, will provide us with a system to determine whether lymphocytic cells harboring these mutations can bypass these stimuli and promote cell survival.

Determine the sensitivity of MEK3 mutants to HIF-1α inhibitors

HIF-1α protein levels are elevated in Jak2V617F, present in 90 % of polycythemia vera (PV). Pharmacological inhibition of HIF-1α suppresses growth and survival by inducing apoptosis and cell cycle arrest in cells harboring Jak2V617F [132]. Thus, it is important to determine whether HIF-1α inhibitors will effectively treat ALL caused by MEK3 mutations, similar to those addressed herein.

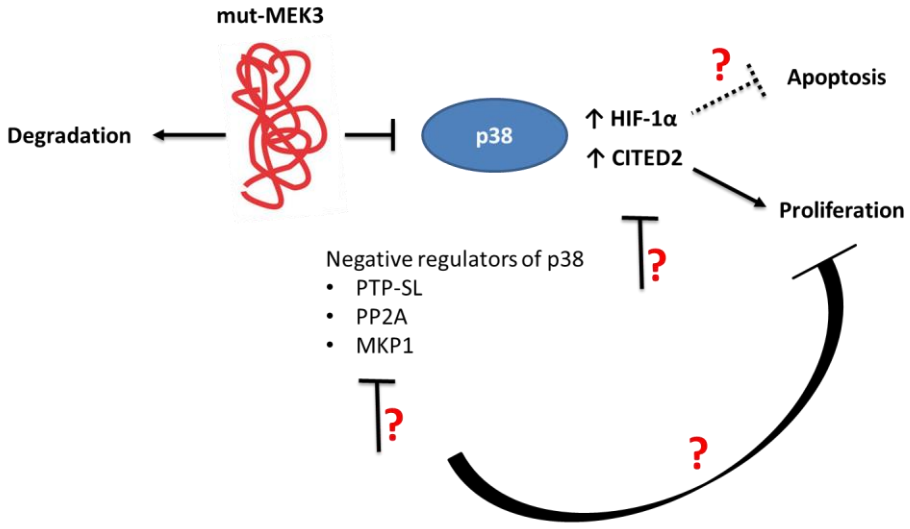


Figure 5.1: MEK3 implication in ALL. MEK3 mutants negative impact p38 activity inducing expression of HIF-1α and CITED-2 proteins and promoting proliferation. Future works involve (1) investigating these mutants' role in apoptosis, (2) and determine whether pharmacological inhibition of HIF-1α or modulation of the MAPK p38 signaling reverses the effects induced by these mutations.

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Abbreviations

MAPK, Mitogen-Activated Protein Kinase; ERK, Extracellular Signal-Regulated Kinases; JNK, Jun-N-terminal Kinases; p38, protein 38; MAPKKK, MAPK Kinase Kinase; MAPKK, MAPK Kinase; Raf, Rapidly accelerated fibrosarcoma; Thr, Threonine; Glu, Glutamic acid; Tyr, Tyrosine; kDa, kilodalton; c-Jun, proto-oncogene cJun; Elk-1 ETS transcription factor Elk-1, ATF2, Activated Transcription Factor 2; Pro, Proline; MAPKAP-K2/3, MAPK-Activated Protein Kinase 2 and 3; MKN1/2, Mitogen-Activated Protein Kinase-Interacting protein; MSK1/2, Mitogen- and Stress-Activated Protein Kinase; MEF2, Myocyte Enhancing Factor 2 (MEF2); CHOP, C/EBP Homologous Protein; STAT1, Signal Transducer and Activator of Transcription 1; p53, protein 53; SAP1, Serum Response Factor Accessory Protein 1; NFATp, Nuclear Factor of Activated T-cells, PGC-1, Peroxisome Proliferators Activated Receptor γ Coactivator; USF-1, upstream stimulator factor 1; TS-MKP, Tyrosine Specific MAPK Phosphatase; SS-MKP, Serine/Threonine-Specific MAPK Phosphatase; DS-MSKP, Tyrosine/Threonine Dual-Specificity Phosphatase; MKP1, Mitogen-Activated Protein Kinase Phosphatase 1; miR, microRNA; TCR, T Cell Receptor; TAB1, TGF-Beta Activated Kinase 1 (MAP3K7) Binding Protein 1; LCK, Lymphocyte-Specific Protein Tyrosine Kinase; Zap70, Zeta-Chain-Associated Protein Kinase 70; N-domain, amino domain; COOH-, carboxylic domain; G, glycine; K, lysine (K); IL-1, interleukin 1; TNFα, Tumor Necrosis Factor Alpha; MLK3, Mixed Lineage Kinase-3; Ask1, Apoptosis Signal-Regulating Kinase1; Tao1 and Tao2, Thousand-and-One Amino Acid 1 and 2 , Tak1, TGF-β Activated Kinase 1, Ser, serine; JIP, c-Jun NH2-terminal Kinase Interacting Protein; OSM, Oncostatin M; Mirk1 Mini-brain Related Kinase; HNF1α, Hepatocyte Nuclear Factor 1-Alpha; PP2A, Protein Phosphatase 2; IL-6,

interleukin 6; IL-12, interleukin 12; INF-γ, Interferon gamma; CDK, Cyclin-Dependent Kinases; Bmi-1, B cell-specific Moloney murine leukemia virus integration site 1 (Polycomb complex protein BMI-1); PDGFR, Platelet Derived Growth Factor Receptor; G-CSF, Granulocyte Colony Stimulating Factor; UTRs, Untranslated Regions; TGF-β, Transforming Growth Factor-β; Th1, T Helper Cell Type 1; $CD4^+$, Cluster of Differentiation 4; CML, Chronic Myelogenous Leukemia; ISGs, Interferon-Stimulated Genes; ISRE, Interferon-Stimulated Response Element (ISRE); GAS, Gamma-IFN Activated Site element; MMP-9, Matrix Metalloproteinase-9; B-CLL, B-Chronic Lymphocytic Leukemia; APL, Acute Promyelocytic Leukemia; 5-FU, 5-Fluorouracil; BCR/ABL, Breakpoint Cluster Region/Tyrosine Kinase; ara-C, cytosine arabinoside; TKI, Tyrosine Kinase Inhibitor; ALL, Acute Lymphoblastic Leukemia; IL2Rβ, Interleukin 2 Receptor β (IL2Rβ); JAK 1 and JAK3, Janus Kinase 1 and Janus Kinase 3; IL1R, Interleukin 1 Receptor; FLT3, Fms Like Tyrosine Kinase 3; LNK, Lymphocyte Adapter Protein; CSF1A, Colony Stimulation Factor 1A, ABL1 and ABL2, Abelson Murine Leukemia 1 and 2; Ph-like, Philadelphia like phenotype; IKZF1, IKAROS Family Zinc Finger 1; CRLF2, Cytokine Receptor-Like Factor 2; WES, Whole Exome Sequencing; SNPs, Single Nucleotide Polymorphisms; PROVEAN, Protein Variation Effect Analyzer; TARGET, Therapeutically Applicable Research to Generate Effective Treatments; COSMIC, Catalog of Somatic Mutations in Cancer; CGC, Cancer Gene Census; ZNF717, Zinc Finger Protein 717; OBSCN, Obscurin; TTN, Titin; MUC16, Mucin 16; NEB, Nebulin; DNAH9 and DNAH11, Dynein Heavy Chain 9 and 11; ATM, Ataxia-Telangiectasia; AFF3, AF4/FMR2 Family Member 3; STIL, SCL-Interrupting Locus Protein; MAP2K3, Mitogen-Activated Protein Kinase Kinase 3; MYLK, Myosin Light Chain Kinase; GUCY2C, Heat-stable enterotoxin receptor;

ERBB2, Receptor Tyrosine-Protein Kinase erbB-2; R, arginine; P, proline; L, leucine; T, threonine; WT, wild-type; W, tryptophan; CHX, cycloheximide; HEK293, Human Embryonic Kidney; FBS, Fetal Bovine Serum; GAPDH, Glyceraldehyde 3-phosphate Dehydrogenase; IP, immunoprecipitation; SDS, Sodium Dodecyl Sulfate; PAGE, Polyacrylamide Gel Electrophoresis; AA, amino acids; PVDF, Polyvinylidene Difluoride; eEF2, Eukaryotic Elongation Factor 2; WB, Western blot; SEM, Standard Error of the Mean; DMSO, Dimethyl Sulfoxide; ROS, Reactive Oxygen Species; HIF-1α, Hypoxia-Inducible Factor 1 α; CITED-2, cAMP-responsive element-binding protein (CBP)/p300-Interacting Transactivator with ED (glutamic acid and aspartic acid)-rich tail 2.

Appendix

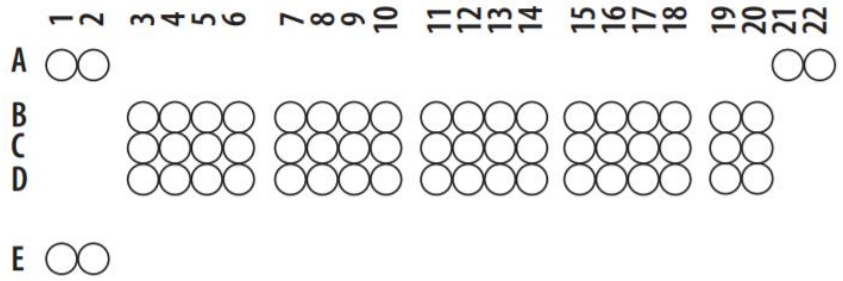

Figure 6.1: Coordinate's reference for analyte identification.

Table 6.1 Human cell stress array coordinates of the analytes

Coordinate	Analyte/Control	Alternate Nomenclature
A1, A2	Reference Spots	—
A21, A22	Reference Spots	—
B3, B4	ADAMTS1	—
B5, B6	Bcl-2	—
B7, B8	Carbonic Anhydrase IX	CA9
B9, B10	Cited-2	—
B11, B12	COX-2	—
B13, B14	Cytochrome c	—
B15, B16	Dkk-4	—
B17, B18	FABP-1	L-FABP
B19, B20	HIF-1α	—
C3, C4	HIF-2α	EPAS1
C5, C6	Phospho-HSP27 (S78/S82)	—
C7, C8	HSP60	—
C9, C10	HSP70	—
C11, C12	IDO	Indoleamine 2,3-dioxygenase
C13, C14	Phospho-JNK Pan (T183/Y185)	—
C15, C16	NFκB1	—
C17, C18	p21/CIP1	CDNK1A
C19, C20	p27	Kip1
D3, D4	Phospho-p38α (T180/Y182)	—
D5, D6	Phospho-p53 (S46)	—
D7, D8	PON1	—
D9, D10	PON2	—
D11, D12	PON3	—
D13, D14	Thioredoxin-1	—
D15, D16	SIRT2	Sirtuin 2
D17, D18	SOD2	Mn-SOD
D19, D20	Negative Control	Control (-)
E1, E2	Reference Spots	—

Vita

Yoshira M. Ayala-Marin earned her Bachelor of Science degree in Microbiology from the University of Puerto Rico at Humacao in 2009. After working as a research laboratory technician for two years, she joined the Department of Biological Science Doctoral Program at The University of Texas at El Paso (UTEP) in 2015, focusing her studies on cancer.

While pursuing her degree, Yoshira was funded by The Research Initiatives for Scientific Enhancement Graduate Scholars Program and was a recipient of the Dissertation Completion Fellowship Award. She presented her research at national and international conferences, including the Research Centers in Minority Institutions International Symposium on Health Disparities in Washington, D.C., and the Third Border Biomedical Research Center Symposium in El Paso, Texas.

Yoshira worked as a teaching assistant for two semesters and then as a research assistant in the Department of Biological Sciences. During graduate school, she had the opportunity to mentor numerous undergraduates and is co-author of several peer-reviewed scientific articles.

Findings from Yoshira's dissertation "A Role for MEK3 in Oncogenesis of Acute Lymphocytic Leukemia: Inactivation of MAPK p38 Promotes Cell Proliferation Through Enhanced Degradation of Mutant MEK3", under the supervision of Dr. Robert A. Kirken, will be submitted for publication in a peer-reviewed scientific journal.

This thesis/dissertation was typed by Yoshira M. Ayala-Marin