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The Role Of Fam129b In Cancer Metastasis

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THE ROLE OF FAM129B IN CANCER METASTASIS

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Advisor Date

DEDICATION

I would like to dedicate my thesis to my amazing family. There are will never be enough words to describe my love and appreciation for each one of you. I would not be where I am today without all your love and continued support. I could not have made it through these last few months without your continual faith in me and your constant reminder to believe in myself. You never allowed me to lose sight on my dreams and what I want out of my life, and for that I am eternally grateful.

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CHAPTER 1 INTRODUCTION

1.1 FAM129B

FAM129B, also known as MINERVA or Niban-like protein 1, is part of a small family of proteins which includes FAM129A (Niban) and FAM129C (B-cell novel protein-1). These proteins are all implicated in cancer but their comprehensive structure and function remains unknown. However, FAM129A is an endoplasmic reticulum, stress induced protein, which has been identified as a molecular marker of renal carcinogenesis and tumors with oxyphilic cytoplasms (Matsumoto, 2006). In contrast, FAM129C is a B-cell protein that is predicted to be heavily phosphorylated, by the p38MAPK kinase, suggesting that it may be involved in cancer (Patel, 2017). This, in conjunction with a study published in 2011 from our lab titled *"FAM129B/MINERVA, a Novel Adherens Junction-associated Protein, Suppresses Apoptosis in HeLa Cells"*, indicates that the FAM129 family of proteins may all partake in cancer development (Song C. , 2011).

Since the exact function of FAM129B is unknown, further analysis was conducted in our lab to examine the effects of FAM129B. In this study, FAM129B was shown to remain cytosolic in exponentially growing cells but co-localized to the cell membrane when the cells came in contact, forming adherens junctions (Song C. , 2011). This observation suggested that FAM129B may weaken the interconnections between the cells leading to an increase in cell motility. This study also showed that FAM129B suppresses the TNFα apoptotic pathway, signifying that FAM129B may be involved in cancer cell invasion (Song C. , 2011).

The correlation between FAM129B and cancer cell invasion has undergone further analysis by Haitao et al. In this study, FAM129B was shown to have a tyrosine 593 phosphorylation site, located at its carboxyl terminal end, which is phosphorylated by the

Epidermal Growth Factor Receptor (EGFR), a transmembrane receptor. Overexpression of EGFR, or a mutation, has been identified as a direct link to poorer patient outcomes (Haitao, 2016). This study further indicated that the phosphorylation by EGFR strengthens the interaction between FAM129B and Ras, to increase Ras activation, which is crucial in tumor development (Haitao, 2016).

FAM129B was first identified as a target of the MAP kinase signaling cascade in human melanoma cells (Old, 2009). When the MAP kinase pathway was active, FAM129B was found dispersed throughout the cytoplasm, however, once exposed to an MKK inhibitor, FAM129B co-localized to cell membrane (Old, 2009). This study was a breakthrough in the ongoing analyses pertaining to FAM129B and its correlation with cancer cell invasion. This group further purposed that the phosphorylation of FAM129B by B-Raf/MKK/ERK, exemplifies an important mechanism for modulating cancer cell behaviors by regulating the events which actively promote invasion (Old, 2009).

In 2012, another study indicated that the knockdown of FAM129B was in fact, involved in the subsequent delays in the wound healing process (Oishi, 2012). Since the TNFα apoptotic pathway and the pathway involved in wound healing are both dependent on an increase in cell motility, this further substantiates the perception that FAM129B may be involved in cancer cell invasion.

Another major contributor into the understanding of FAM129B's multifaceted function was a study involving its relationship with Wnt/β-catenin pathway in melanoma cells. Studies have shown that the activation of this pathway ultimately decreases tumor growth and promotes apoptosis to improve patient outcomes (Conrad, 2013). This study showed that FAM129B is a regulator of Wnt/β-catenin pathway, allowing FAM129B to link this

signaling pathway to apoptosis (Conrad, 2013). Linking Wnt/β-catenin pathway with apoptosis, enables FAM129B to regulate apoptosis through the Wnt/β-catenin pathway.

1.2 Structural Domain of FAM129B

Even though the overall functions of this family of proteins is largely unknown, there are a few structural similarities between them. For instance, FAM129B shares a 40% sequence identity with FAM129A, each of which encompass a pleckstrin homology, or PH, domain located at the amino terminus end (Old, 2009). FAM129B only shares a 27% sequence identity with FAM129C, which also contains a PH domain, located on the amino terminus end (Old, 2009).

FAM129B has a predicted molecular weight of 83 kDa, along with two distinctive structural regions; a PH, domain and proline rich domain. While the PH domain is located near the amino terminus end of the polypeptide chain, a tyrosine phosphorylation site and a proline rich domain, together, are positioned at the carboxyl terminal end, illustrated in Figure 1 (Song C. , 2011).

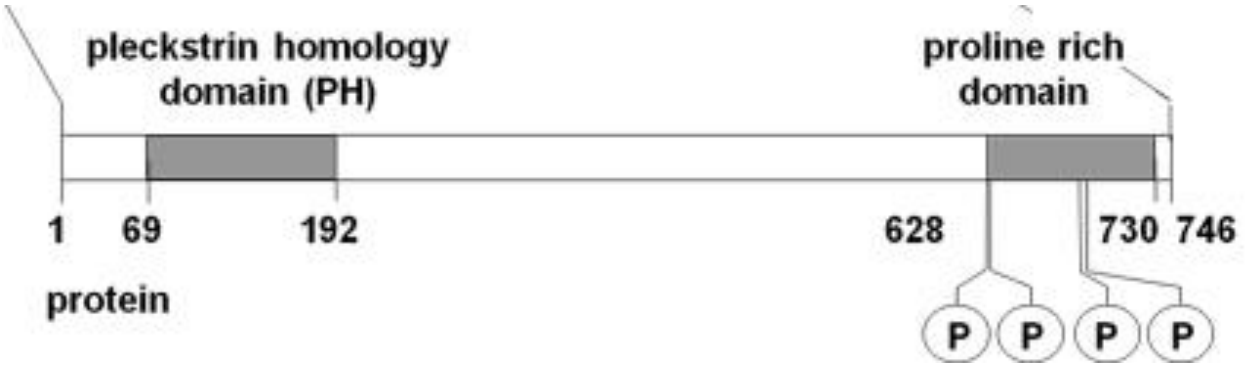

Figure 1 Structural Domain of FAM129B *(Song C. , 2011). FAM129B structural domain indicates a PH domain located on the N-terminus and a proline rich domain on the C-terminus. Also, located on the C-terminus end, is a tyrosine phosphorylation site along with six serine phosphorylation sites.*

Figure 2 demonstrates that the proline rich domain contains six serine phosphorylation sites: (ser628 (P1), ser633 (P2), ser652 (P3), ser668 (P4), ser679 (P5),

ser683 (P6)). Four of which are phosphorylated by the MAP kinase signaling cascade (Somaskharan, 2017).

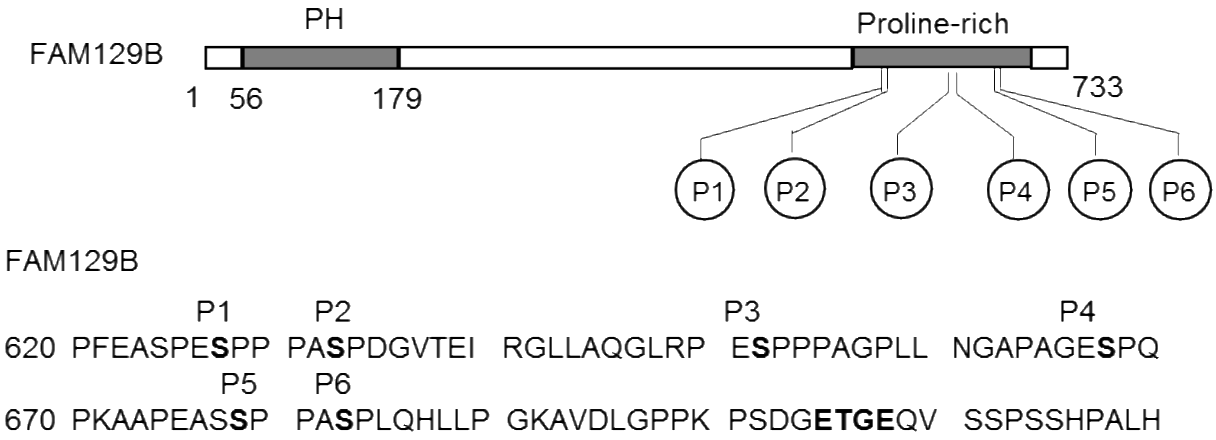

Figure 2 Six Serine Phosphorylation Sites on FAM129B *(Somaskharan, 2017)****.*** *Located on the C-terminus end are six serine phosphorylation sites.*

Per NCBI databases, in Homo Sapiens, FAM129B may exist in two isoforms in which were produced by alternative splicing. One isoform consists of 746 amino acids in length and a molecular weight of roughly 84 kDa, making this sequence its most canonical sequence. While the second isoform consists of 733 amino acids in length and a molecular weight of roughly 83 kDa. However, the sequencing between these two isoforms differ by 13 amino acids. In the canonical sequence, amino acids 1-18 are different in the non-canonical sequence: 1-18 MGDVLSTHLDDARRQHIA → MGWMG.

1.3 Cancer

Cancer is a compilation of diseases where regulated cellular growth is lost and abnormal proliferation commences due to activation of oncogenes and the deactivation of tumor suppressor genes and apoptotic pathways. In 2017, cancer was the second leading cause of death.

Chronic diseases, such as diabetes, and harmful substances, such as tobacco smoke and alcohol, are some of the risk factors that increase one's risk for cancer. However,

tobacco and alcohol consumption are two of the major risk factors not only for cancer, but for many other chronic illnesses across the spectrum. In 2000, 4.9 million deaths were attributed to tobacco consumption (National Institute of Health, 2007). Based on these trends alone, 9 million people globally, will die from tobacco-related causes in the year 2020 (National Institute of Health, 2007). Nevertheless, diet and exercise may significantly reduce the risk for cancer and other chronic diseases.

Increased consumption of fruits and vegetables decreases the risk of cancer in the colon, rectum, lung, stomach, esophagus, mouth, and pharynx are due to the carbohydrates, fiber, and vitamins provided by these foods (National Institute of Health, 2007). Although manageable risk factors may increase the likelihood of developing cancer, there are many unmanageable risk factors: i.e. genetic susceptibility, ionizing radiation, and ultraviolet radiation - all of which increase the risk of cancer. Certain inheritable genes, i.e. BRCA1, increases the risk of developing breast or ovarian cancers by 70 percent over one's lifetime (National Institute of Health, 2007). Natural UV radiation, from exposure to sunlight, is the leading cause of melanoma, due to random gene mutations. There are 160,000 new cases per year, of which 80 percent are in developed regions like North America, Europe, and Australia (National Institute of Health, 2007). The only definitive way to decrease one's risk of developing cancer, is through prevention and early detection, conducted through routine screenings.

Epithelial cells line tissue surfaces and provide a protective barrier for internal organs. When these cells proliferate, they give rise to a benign neoplasm. A neoplasm is defined as an abnormal net growth of proliferative cells and are categorized as either benign, premalignant, or malignant (Sarkar, 2013). Benign neoplasms are generally confined to

a specific area within a tissue and the epithelial cells provide no evidence of invading adjacent tissues by infiltration of the basement membrane (Sarkar, 2013). Premalignant cells can change in tissue type and acquire metastatic properties, but have not yet all invaded the basement membrane (Brid M. Ryan, 2015). Malignant neoplasms, or cancer, are less differentiated and exhibit characteristics of rapidly growing cells (Lodish H, 2000).

Each stage of tumor development is influential in the overall invasiveness of the cell. As Figure 3 illustrates, the progression from normal epithelia to a malignant carcinoma occurs in multiple stages. Malignant cells are less adhesive than normal cells resulting in the reduction of epithelial markers, like E-cadherin, causing an increase in their motility (GM, 2000). Malignant neoplasms have increase cell motility enhancement receptors (Sarkar, 2013). In conjunction with the upregulation of these receptors, malignant cells additionally acquire metastatic properties that weaken cell adhesion properties (Sarkar, 2013). This overall increase in cell motility, is the major contributing factor for malignant cells to invade and metastasize.

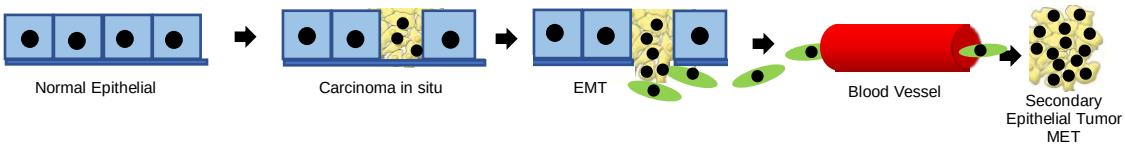

Figure 3 Cancer progression through EMT *(Sarkar, 2013)**. An illustrative progression of epithelial from normal to malignant to metastatic.***

In 2011, Douglas Hanahan and Robert A. Weirnberg published a review which stated the important hallmarks acquired by cells for the overall progression of neoplastic diseases (Hanahan, 2011). These hallmarks are biological traits that are present in most human cancers and enable the tumor to grow and metastasize.

There are six hallmarks of cancer:

1) Sustaining Proliferative Signaling
2) Evading Growth Suppressors
3) Resisting Cell Death
4) Enabling Replicative Immortality
5) Inducing Angiogenesis
6) Activating Invasion and Metastasis

Each hallmark indicates a fundamental tactic by which cancerous cells can evade the defense mechanisms that exist within the human body. Per Hanahan, sustaining proliferative signaling is arguably the most fundamental trait of cancer cells by providing them with the ability to avoid the signals to arrest in cell division, allowing them to continuously proliferate, hereby, increasing the likelihood of mutations. Hallmark two focuses on how the tumor cells evade growth suppressors to ultimately circumvent programs that will negatively regulate cell proliferation i.e. tumor suppressor genes. Hallmark three postulates that tumor cells must resist cell death to promote their own development. Cancer cells are able to avoid the physiological response elements; i.e. apoptosis, autophagy, and necrosis, and develop their own developmental processes to survive and proliferate. Hallmark four, focuses on what happens once these cells have evaded programmed cell death and acquire unlimited replicative potential. Hallmark five, Inducing Angiogenesis, emphasizes the importance of the tumor's surroundings. The environment around the tumor is vital as it uses its surroundings to grow and provide the necessary nutrients and oxygens to sustain unlimited cell division. Finally, hallmark six, concentrates on mechanisms that empower malignant cells to progress to higher pathological grades of malignancy leading to more aggressive tumor types. It has been

hypothesized that the epithelial to mesenchymal transition gives cancer cells the ability to overcome senescence and initiate metastasis (Sarkar, 2013).

1.4 Epithelial to Mesenchymal Transition

Epithelial cells are polarized cells that typically interact with the basement membrane through their basal surface while remaining bound and stagnant. (Kalluri, 2009). Essentially, they act as a protective barrier for cavities and organs that are defenseless against environmental bacteria (Ganz, 2002). For example; they form the surfaces of digestive, urinary, and reproductive tracts along with the epidermis. While mesenchymal cells, are spindle shaped cells that exert more of a supportive role among tissues, rather than a protective role. Mesenchymal cells are isolated from bone marrow and typically exhibit multipotent differentiation capabilities (Horwitz, 2012). This multipotent differentiation potential was first proposed by AJ Friendenstein in 1968, when he showed that mesenchymal stems cells have highly proliferative potential and could differentiate into bone (Horwitz, 2012) - a characteristic that is critical for tissue regeneration and the ability for cancer cells to gain migratory and invasive properties. Then in 1988, Maureen Owen proposed that mesenchymal cells are capable of maintaining the microenvironment of bone marrow tissues (Horwitz, 2012). While malignant cells are capable of transitioning from an epithelial cell into a mesenchymal cell through a process known as Epithelial to Mesenchymal Transition (EMT).

EMT is a biological process where epithelial cells lose their cell polarity and cell-cell adhesion junctions, and acquire the migratory and invasive properties of mesenchymal cells (Larue, 2005). As indicated in Figure 4, EMT is characterized by the downregulation of the cell to cell attachment receptors and the subsequent upregulation of the receptors essential for cell motility (Sarkar, 2013). This dynamic process allows

these epithelial cells to adopt the morphological characteristics of mesenchymal cells, along with their ability to differentiate into any type of smooth muscle, connective or supportive tissue, or blood cell. (Roche, 2018).

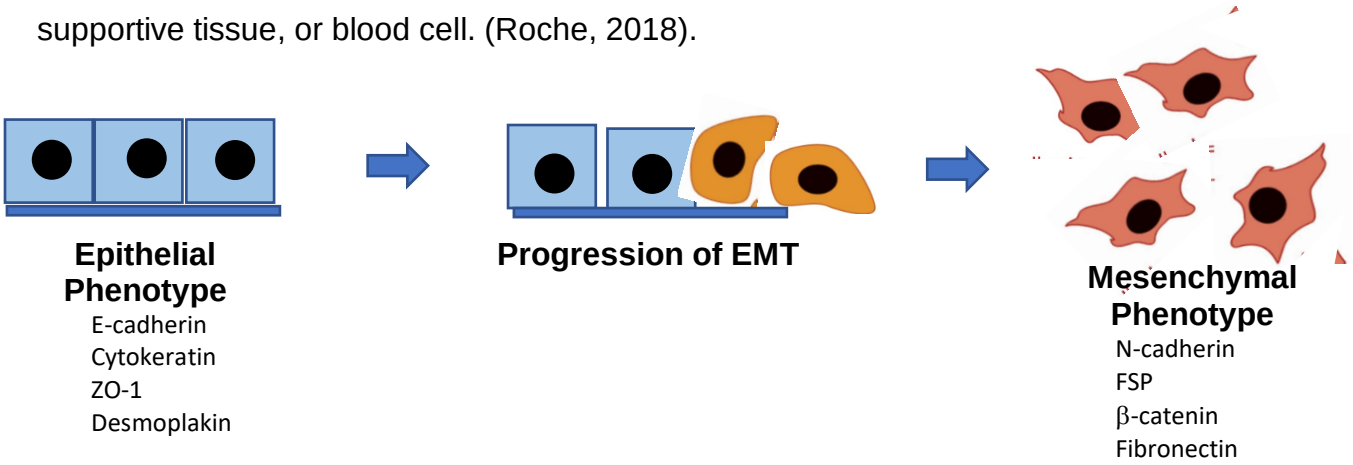

Figure 4 Epithelial to Mesenchymal Transition. The characteristics and progressive properties that occur within cells undergoing complete epithelial to mesenchymal transition and some respective biomarkers.

EMT is essential in a plethora of processes, ranging from embryonic development to tissue fibrosis to cancer metastasis (Kalluri, 2009). It increases the mortality rate for cancer patients by inducing immunosuppression, drug resistance, and increasing tumorigenic and proliferative potential. When malignant cells undergo EMT, they acquire the necessary properties to facilitate their metastatic potential and gain stem cell-like properties. Once malignant cells gain stem-cell properties, these cells now become multipotent stromal cells and can differentiate into a variety of cell types, including some that exhibit the drug resistance seen in most metastatic cancers (Roche, 2018).

EMT can be classified into three different subtypes; centered around the cells phenotypic output:

1) Type 1 is epithelial to mesenchymal transition
2) Type 2 is epithelial to fibroblast transition
3) Type 3 is cancer to mesenchymal transition

Type 1 epithelial to mesenchymal transition is an essential part of gastrulation and implantation along with the development of migrating neural crest cells in embryonic development (Zeisberg, 2009). However, Type 2, the epithelial to fibroblast transition, is induced during persistent inflammatory responses to allow secondary epithelial cells to transition into adjacent tissue fibroblasts and destroy any affected organs (Kalluri, 2009). Finally, Type 3, cancer to mesenchymal transition is the central transitioning process in which metastatic tumor cells migrate through the blood stream to a distal site (Zeisberg, 2009). Epithelial cells maintain their polarity and remain stagnant by expressing higher levels of E-cadherin, an epithelial marker, whilst expressing lower levels of mesenchymal markers. By contrast, mesenchymal cells lose their cell adhesion junctions due to lower levels of E-cadherin, while expressing higher levels of mesenchymal markers, including N-cadherin. This subsequent downregulation of E-cadherin, is the principal hallmark of type 1 and type 2 EMT, indicating complete transitions occurred (Lamouille, 2014).

However, Type 3 EMT, metastasis, is not a complete transitional process. Malignant cells remain in multiple transition states while mutually expressing both epithelial and mesenchymal biomarkers forming a hybridization state. It is imperative to observe the fundamental cell surface biomarkers to accurately determine the stage of EMT progression. (Roche, 2018). This hybridization state allows malignant cells to become more aggressive, acquire an upsurge in cell motility, and the mesenchymal capabilities necessary to degrade the extracellular matrix sanctioning metastasis (Lamouille, 2014).

EMT is initiated by the activation of transcription factors and the production of extracellular matrix degrading enzymes. In the beginning stages of EMT, cell-cell

adhesion junctions disassemble and the cells begin to lose their polarity (Lamouille, 2014). This loss of polarity and changes in actin architecture, trigger a change in cell shape.

The Transforming Growth Factor Beta (TGF-β) signaling pathway is one of the cell signaling pathways involved in the initiation of this biological process. Depedning of its physiological state, TGF-β acts as both a suppressor and a promoter (Larue, 2005). In malignant cells, TGF-β takes on the role of a tumor promotor to promote EMT by activating the expression of SNAIL, which regulates EMT by directly acting on E-cadherin. In normal cells, TGF-β acts as a tumor suppressor by arresting the cell cycle in G2/M phase to inhibit proliferation and promote apoptosis (Song J. , 2007). As tumorigenic cells undergo EMT, the tumor suppressor abilities of TGFβ are inactivated and it acquires its tumor promotor abilities; i.e. inhibiting apoptosis and increasing cell proliferation. Allowing these cells to increase proliferation causing immunosuppression and angiogenesis, ultimately making the cells more invasive. However, the exact molecular mechanism responsible for determining which TGFβ activity is expressed remains largely unknown.

So, essentially malignant cells lose these cell-cell adhesion junctions, break through the basement membrane to enter bloodstream, leading to metastasis. EMT's involvement in the metastatic spread of malignant cells is one of the hallmarks of cancer.

CHAPTER 2 MATERIALS AND METHODS

2.1 Cell Culture

Mus musculus mammary tissue cells (NMuMg) were gifted from Dr. Karen Beningo in Department of Biological Sciences at Wayne State University. Cells were maintained in 75 cm^2 non-pyrogenic sterile polystyrene flask with 12ml of complete media containing Dulbecco's Modified Eagles Media (DMEM) 1X with 4.5 g/L D-glucose, L-Glutamine, 110 mg/L Sodium Pyruvate (Gibco 11995065) with 10% Fetal Bovine Serum, 1% Penicillin-Streptomycin, 10 mcg/ml insulin (Sigma-Aldrich I0516-5ml), in 5% CO_2 at 37°C. Media was renewed two to three times per week and the cells were passaged when the confluency of the flask reached 80-90%. For cell passaging, complete media was aspirated and 3 ml of 0.25% trypsin (Gibco 25200056) was added to the flask and incubated in 5% CO_2 at 37°C for three minutes to allow cells to detach. Once detached, cells were then added to a 15-ml falcon tube with 9 ml of complete media and centrifuged at 1000 rpm for 5 minutes. The media was removed and another 12 ml of fresh, complete media was added to the 15-ml falcon tube. Cells were re-suspended, then transferred to a new 75 cm^2 non-pyrogenic sterile polystyrene flask and incubated in 5% CO_2 at 37°C until next media change. For cell freezing, complete media was aspirated and 3 ml of 0.25% trypsin (Gibco 25200056) was added to the flask and incubated in 5% CO_2 at 37°C for three minutes to allow cells to detach. Once detached, cells were then added to a 15-ml falcon tube with 9 ml of complete media and centrifuged at 1000 rpm for 5 minutes. The media was then removed and the cells were re-suspended in 1 ml of freezing media and transferred into cryogenic vials. Freezing media consisted of 90% complete DMEM media and 10% dimethyl sulfoxide Hybri-MAX® (DMSO Sigma-Aldrich D2650). Cells were stored in -80°C for 24 hours before being transferred into liquid nitrogen. For cell

thawing, vials were removed from liquid nitrogen and placed in an ice bucket while being transported to a sterile 37°C water bath. Cells were placed inside the water bath and gently swirled, while keeping the O-ring above water to avoid contamination, until partially thawed. Once partially thawed, cryogenic vials were placed within a sterilized biohazardous hood and added to a 15-ml falcon tube with 9 ml of complete media and centrifuged at 1000 rpm for 5 minutes. The media was removed and the cells were re-suspended in 12 ml complete DMEM media and dispensed into a 75 cm^2 non-pyrogenic sterile polystyrene flask and incubated in incubated in 5% CO_2 at 37°C until next media change.

HeLa cells were purchased from American Type Culture Collection (ATCC). Cells were maintained in 75 cm^2 non-pyrogenic sterile polystyrene flask with complete media containing Eagle's Minimum Essential Media (EMEM) 1X with balanced salt solution, non-essential amino acids, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, and 1500 mg/L sodium bicarbonate (ATC 30-2003) with 10% Fetal Bovine Serum, 1% Penicillin-Streptomycin, in 5% CO_2 at 37°C. Media was renewed every 48 hours and passaged when the confluency of the flask reached 80-90%. For cell passaging, complete media was aspirated and 3 ml of 0.25% trypsin (Gibco 25200056) was added to the flask and incubated in 5% CO_2 at 37°C for three minutes to allow cells to detach. Once detached, cells were then added to a 15-ml falcon tube with 9 ml of complete media and centrifuged at 1000 rpm for 5 minutes. The media was then removed, another 12 ml of fresh, complete media was added to the 15-ml falcon tube. Cells were re-suspended, then transferred to a new 75 cm^2 non-pyrogenic sterile polystyrene flask and incubated in 5% CO_2 at 37°C until next media change. For cell freezing, complete media was aspirated and 3 ml of

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2.2 TGFβ-induced Epithelial to Mesenchymal Transition

NMuMG cells were grown in a 6-well plate until cells reached 60-80% confluency on the day of induction. Cells were washed with DMEM media containing 1% penicillin-streptomycin and no Fetal Bovine Serum twice, then reduced serum starved in DMEM media containing 1% Fetal Bovine Serum and 1% penicillin-streptomycin for 24 hours. The media was aspirated and changed with 1.5 ml fresh DMEM media containing 1% Fetal Bovine Serum, 1% penicillin-streptomycin, and 0.1% insulin before inducing EMT with Recombinant Human TGF-β1. TGF-β1 was added directly into cell culture media to

give a final concentration of 10ng/ml (Invitrogen PHG9204) then incubated at 37°C in 5% CO_2 for 96 hours. The media was changed every 48 hours and TGF-β1 was re-added, to maintain viable cells, throughout the 96-hour induction. Cell were lysed and a protein quantification was conducted to determine the protein levels for each sample. Gel samples were made to give a final concentration of 1 ng/ml of protein per sample, then probed with N-Cadherin (Cell Signaling Technology 13A9) and E-Cadherin (Cell Signaling Technology 24E10), and analyzed via western blotting.

2.3 FAM129B Knockdown

Transfection was carried out using Lipofectamine RNAiMAX reagent from Invitrogen (Cat. #13778030). NMuMG cells were grown in a 6-well plate until cells reached 60-80% confluency on the day of transfection. Cells were then transfected with either siRNA against FAM129B (Santa Cruz Biotechnology sc-145025) or a control, that had a scrambled siRNA sequence. An siRNA-control and siRNA-FAM transfection complexes were made at a 1:3 ratio in Opti-MEM Reduced Serum Media (GIBCO 11095080). Reagent complexes were made by diluting Lipofectamine RNAiMAX in Opti-MEM Reduced Serum Media (GIBCO 11095080) at a 1:3 ratio. SiRNA complexes were made by diluting siRNA in Opti-MEM Reduced Serum Media (GIBCO 11095080) at a 1:3 ratio. Once both complexes were made, siRNA complexes were added into their respective reagent complex, then incubated for 5 minutes at room temperature, as per manufacture's protocol. The complexes were then added directly into the cell culture, then incubated at 37°C in 5% CO_2 for 72 hours, subsequently probed with anti-FAM129B markers, and analyzed by western blotting.

2.4 Protein Quantification

Total protein concentrations were measured using DC™ Protein Assay Kit II from Bio-Rad Laboratories (Cat. # 5000112) and conducted directly following the manufacturers protocol, using bovine serum albumin as the standard protein.

2.5 Cell Lysis

Cells were washed with 2ml of Dulbecco's Phosphate Buffered Saline, DPBS, no calcium and no magnesium, (ThermoFisher, 14190250) twice. Cells were then lysed using a lysis buffer containing 40 mM Tris, 120 mM NaCl, 0.5% Triton X-100, 5% glycerol, and 1% SDS brought to a pH of 7.4. The cell remnants were then scraped from the surface to the dish and lysates were collected in corresponding microcentrifuge tubes. Lysates were passed through 18 gauge needles were passed roughly 5-7 times to confirm cells were properly lysed, then spun down in a Eppendorf centrifuge for 1 min at 13,000 rpm. Gel samples were made using cell lysates and data collected from protein quantification so each sample had a final concentration of 1 ng/ml of protein. Protein samples were heated at 80°F for 6 minutes and analyzed by SDS-PAGE on a 12% gradient gel.

2.6 SDS – PAGE

Protein samples were analyzed by SDS polyacrylamide gel electrophoresis (BIO-RAD Model 200/2.0 Power Supply) on a 12% gel consisting of a separating gel and a stacking gel. The 5.5cm x 8.5cm x 1.5 cm separating gel consisted of 3.4 ml distilled water, 2.5ml of 1.5 M Tris at pH 8.8, 4ml of 30% acrylamide/Bis, 50ul of 20% SDS, 100ul of 10% APS, and 10ul of TEMED. The separating gel was immediately poured into a cassette and 1 ml of isopropanol was gently added, in a dropwise fashion, across the top of the separating gel to aid in solidification. The separating gel was allotted 17 minutes to solidify. Isopropanol was removed and a stacking gel was poured on top of the solidified

separating gel. The 2.0cm x 8.5cm x 1.5cm stacking gel consisted of 4ml of distilled water, 750ul of 1 M Tris at pH 6.8, 1ml of 30% acrylamide/Bis, 30ul of 20% SDS, 60ul of 10% APS, and 12ul of TEMED. A 1.5mm wide comb was added into the stacking gel, during solidification, to form the sample lanes. 1 mg/ml of protein were loaded into each of the 1.5 mm wide lanes and GAPDH (Santa Cruz Biotechnology #sc-25778) was used as an internal loading control, at a 1:7000 dilution. The gel was run for 60-90 minutes at 150V in a 10x running buffer consisting of 30.2g Tris, 144g Glycine,10g SDS, and up to 1000ml distilled water, which was diluted to 1X dilution. The proteins on the gel were transferred onto a 0.45um nitrocellulose membrane (Bio-Rad Laboratories #162-0115) using Mini Trans-Blot® kit from Bio-Rad Laboratories (#1703930) in 10x transfer buffer, for 65 minutes at 100V. The 10X transfer buffer containing 30.2g Tris, 144g Glycine, 10g SDS, and distilled water which was diluted to 1X. After the transfer, membranes were blocked using 5% skim milk buffer in Tris-Buffered Saline (TBST). The TBST buffer contained 20nM Tris, 150nM NaCl and 0.1% Tween 20 at pH 7.4. Membrane were then probed with primary antibodies in 5% skim milk buffer, per dilution ratios suggested by the manufacture, overnight on a shaker in a temperature control, 4°C, room.

2.7 Western Blot

Membranes were thoroughly washed using TBST wash buffer, 3 times for 7 minutes each. After washing, membranes were then probed with appropriate secondary antibodies, per dilution ratios suggested by the manufacture, for 1 hour on a light shaker at room temperature. After probing, membranes were again thoroughly washed using TBST wash buffer, 3 times for 7 minutes each and western blots were developed using Clarity™ Western ECL Blotting Substrates (Bio Rad Laboratories 1705061). Immunoblots

were then scanned using ChemiDoc-It™ Imaging System from Ultra-Violet Products Ltd. to quantify the intensities of the signals.

2.8 Immunofluorescence

HeLa cells were grown on a glass coverslip until cells reached a confluency of 70-80%. Media was then removed and cells were washed twice in 2 ml of serum starved media. Once complete, 2ml of serum starved media was added to the dish and it was incubated at 37°C in 5% CO_2 for 1 hour. After 1 hour, EGF was added to the culture media and incubated at 37°C in 5% CO_2 for another 30 minutes. Once complete, media was removed and fixed with 10% formalin and incubated at room temperature for 15 minutes. It was permeabilized with 0.2% Triton-X solution and incubated at room temperature for another 15 minutes, then washed twice with 1 ml of DPBS, that lacked calcium and magnesium (ThermoFisher 14190250). The coverslips where then blocked in 1% BSA and PBS at room temperature for 1 hour. Once blocked, the cells were washed again in 1 ml of DPBS (no calcium and no magnesium: ThermoFisher 14190250) and then incubated with the FAM129B primary antibody (Cell Signaling Technology 5122S) at room temperature for 2 hours. Once complete, it was washed three time in 1.5 ml of DPBS (ThermoFisher 14190250) three times, for 5 minutes each. Finally, it was incubated with the secondary antibody and Alexa Fluor 594 for 1 hour in a dark room, then analyzed using a fluorescent microscope (Nikon E800).

CHAPTER 3 RESULTS

3.1 The Role of FAM129B in Membrane Localization

Preliminary data shows that FAM129B is overexpressed in HeLa cells and is distributed throughout the cytosol (Song C. , 2011). However, once two cells become in contact with each other, adhesion junctions formed, and FAM129B begins to localize at the plasma membrane (Song C. , 2011). To further confirm this analysis, HeLa cells were used to study the localization of endogenous FAM129B using indirect immunofluorescence microscopy, as verified in Figure 5.

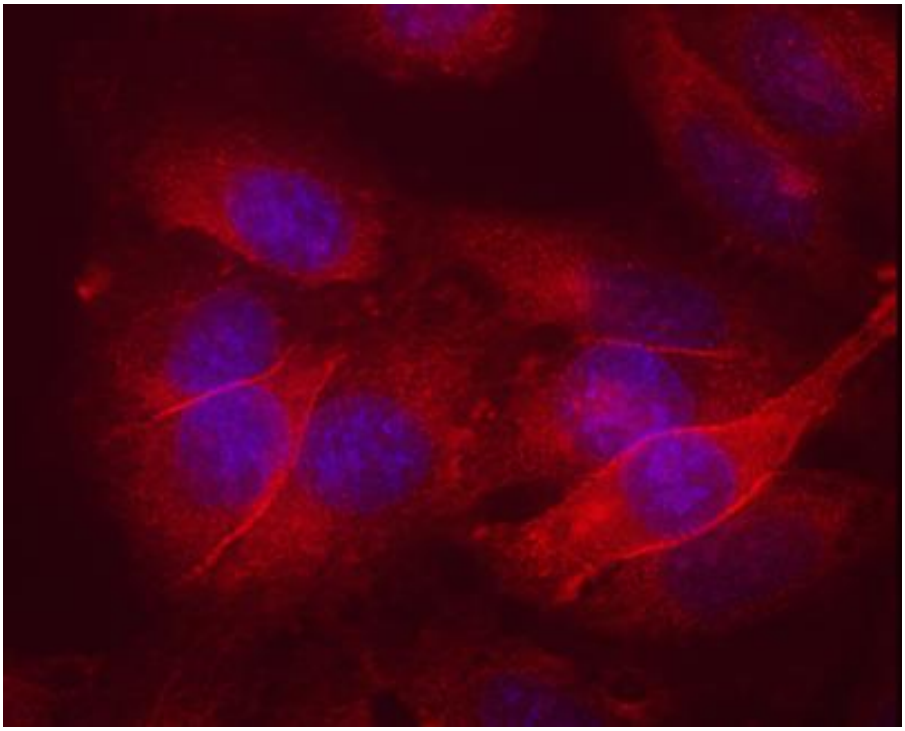

Figure 5 FAM129B co-localizes at Plasma Membrane in Confluent HeLa Cells.
Indirect immunofluorescence imaging confirmed FAM129B co-localizing at the plasma membrane in confluent HeLa cells.

3.2 The Role of FAM129B in Suppressing Apoptosis

FAM129B has been implicated in suppressing apoptosis in HeLa cells (Song C. , 2011). Apoptosis, or programmed cell death, is a highly regulated, normal, biological process experienced by all cells and is characterized by changes in cell morphology.

During apoptosis, a cell undergoes the protrusion of cell membrane and nuclear chromatin condensation (pyknosis).

Our hypothesis suggests that FAM129B suppresses apoptosis by binding to Keap1. Keap1 is a cytosolic protein involved in ubiquitination and protein degradation by the proteasome. Consistently, Figure 11 confirms that FAM129B exhibits the same binding motif, DLG and ETGE, required to successfully bind to Keap1 (Yoichiro Mitsuishi, 2012). Since FAM129B is cytosolic under normal conditions, we predict that FAM129B is capable of binding with Keap1 through that same 'hinge and latch' model shown in Figure 10. We propose that it is through this Keap1-FAM129B complex, that FAM129B suppresses apoptosis. Once the FAM129B-Keap1 complex is formed, we then can predict how FAM129B further suppresses apoptosis through its downstream effects on the TNFα pathway. As illustrated in Figure 12, if FAM129B binds to Keap1, it will prevent Keap1 from binding to IKKβ, an oncogenic kinase. If this interaction is prevented, Keap1 will be unable to aid in ubiquitination and apoptosis will not occur.

To further confirm this analysis, HeLa cell line were grown on a 35-mm dish until cells reached 70-80% confluent, as shown in Figure 6a. HeLa cell lysates were analyzed via western blot analysis using antibodies against FAM129B and Keap1. In Figure 10b, A549 cell line were also grown on a 35-mm dish until cells reached 70-80% confluent. EMT was induced by TGF-β1 for 48h in A549 cell line. A549 cell lysates were analyzed via western blot analysis using antibodies against FAM129B and Keap1.

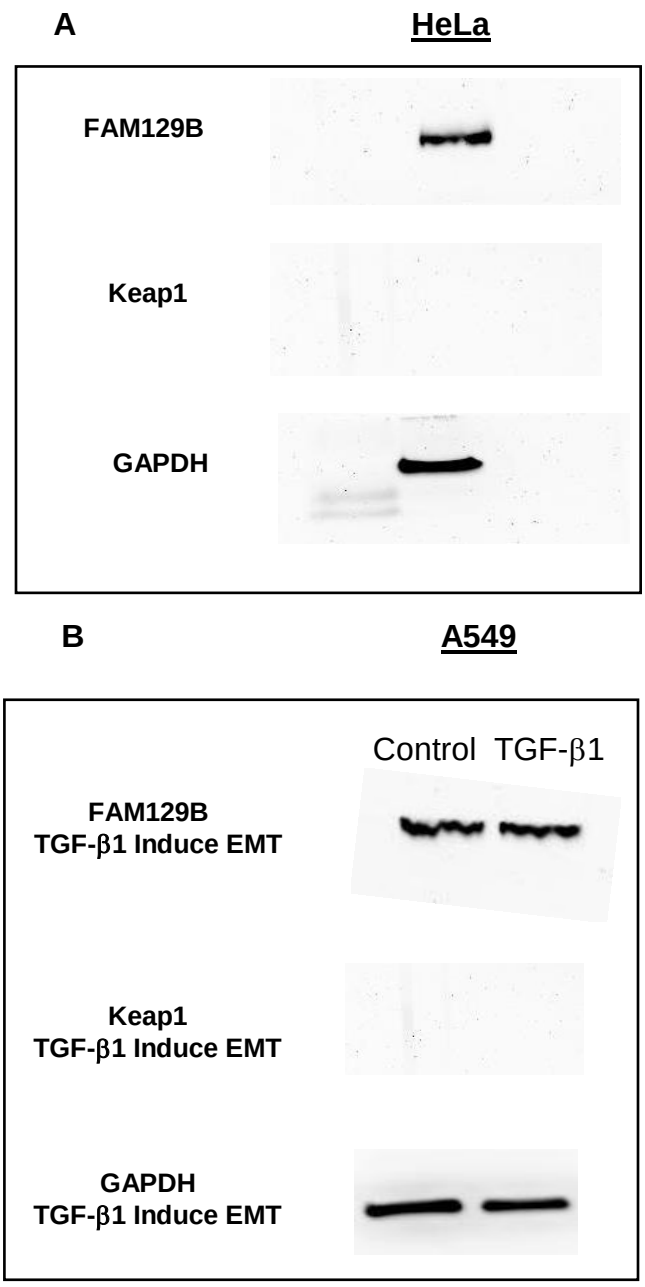

Figure 6 Endogenous FAM129B and Keap1 Levels in HeLa and A549. *(A) Endogenous Fam129B and Keap1 levels in confluent HeLa cells. (B) FAM129B and Keap1 levels in confluent A549 cells.*

As illustrated in Figure 6, both HeLa and A549 cells contained undetectable levels of Keap1, therefore we were unable to test our hypothesis of whether FAM129B suppresses TNFα apoptotic pathway by forming the FAM129B-Keap1 complex in either of these cell lines.

3.3 The Role of FAM129B in Epithelial to Mesenchymal Transition

Epithelial to mesenchymal transition is one of the major hallmarks of cancer. As it is through this transition; malignant cells gain the migratory and invasive properties necessary to become more invasive and metastasize. Mus musculus mammary tissue (NMuMg) cell line were used as a model to elucidate the role of FAM129B in epithelial to mesenchymal transition. These immortalized cells are widely used to study TGF-β induced responses and their induction response time is quicker than in human cancer cell lines, rendering the NMuMG cell line the best model for elucidating TGF-β induced EMT.

To elucidate FAM129B's role, normal levels of FAM129B and the EMT markers, N-cadherin and E-cadherin were measured in untreated NMuMG cell lines. The NMuMG cell line was grown on 35-mm dish until they reached 70-80% confluency. Cells lysates where then analyzed via western blot analysis using antibodies against FAM129B, E-cadherin, and N-cadherin. Figure 7 shows that FAM129B and EMT biomarkers are present in NMuMG cell lines. Since, the EMT biomarkers are present, and this epithelial cell line is frequently used to study TGF-β induced responses, we proceeded to study the role of FAM129B in epithelial to mesenchymal transition.

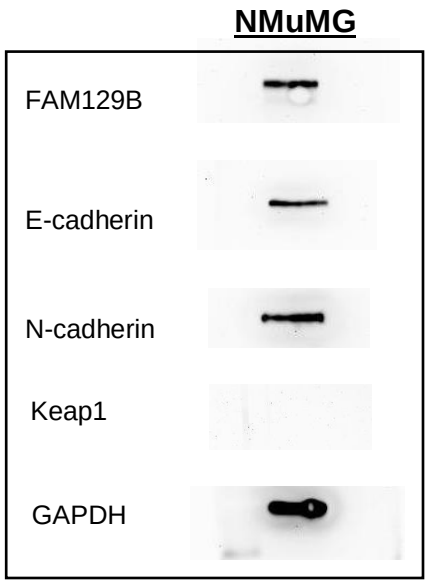

Figure 7 Levels of FAM129B, Keap1, and EMT Markers in Non-treated NMuMG cells. *Cell lysates were analyzed via western blot analysis using antibodies against FAM129B, N-cadherin, and E-cadherin to determine their existing levels.*

The FAM129B gene expression levels were knocked down using siRNA technology, validated in Figure 8. The objective behind gene knockdown, is to determine whether we induce EMT with TGF-β1 in the absence of FAM129B. At this point, if EMT does not proceed, then we can conclude that FAM129B induces EMT. If EMT continues to proceed, then we can conclude that FAM129B is not required for EMT.

NMuMG cell lines were plated in a 6-well plate to allow the cells to reach 60-80% confluency on the day of transfection. Cells were then transfected with siRNA against FAM129B and a control for siRNA, which had a similar length and nucleotide composition, however, the sequence was scrambled. Figure 8 confirms that the expression of the FAM129B gene was silenced after 72 hours, whilst GAPDH served as loading control, demonstrating that 1mg/ml of protein was used in each sample.

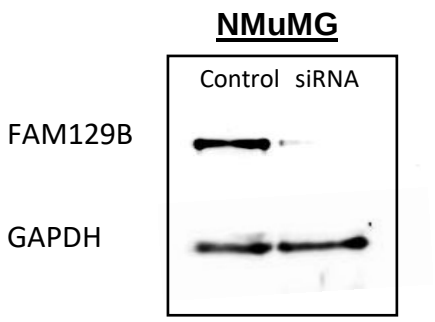

***Figure 8 siRNA Knockdown of FAM129B Expression**. FAM129B gene expression was knocked down after 72 hours. Cell lysates were analyzed via western blot analysis using antibodies against FAM129B to confirm gene silencing.*

The FAM129B gene expression levels were knocked down using siRNA technology. Once FAM129B gene expression was silenced, epithelial to mesenchymal transition was induced by TGF-β1, as shown in Figure 9, to determine whether EMT will be induced without FAM129B present. EMT biomarkers were measured to evaluate the hypothesis.

NMuMG cell lines were plated in a 6-well plate to allow the cells to reach a confluency of roughly 20% on the day of transfection. FAM129B gene was knocked down using the same 1:3 ratio of siRNA-control and siRNA-FAM129B complexes, as stated in Figure 12 and in Experimental Procedures, for 72 hours. Once the FAM129B was knocked down, media was changed before inducing EMT with Recombinant Human TGF-β1. TGF-β1 was added directly into cell culture media to give a final concentration of 10 ng/ml, then incubated at 37°C in 5% CO_2 for an additional 96 hours. NMuMG cell lysates were that analyzed via western blot analysis using antibodies against FAM129B, N-cadherin, E-cadherin, and GAPDH, which served as an internal loading control to demonstrate that 1 mg/ml of protein was used in each sample.

Figure 9, verifies that when FAM129B gene was silenced and EMT induced with 10 ng/ml TGF-β1, still proceeded. The observation that EMT still progressed in the

absence of FAM129B, strongly suggests that FAM129B is not required for epithelial to mesenchymal transition.

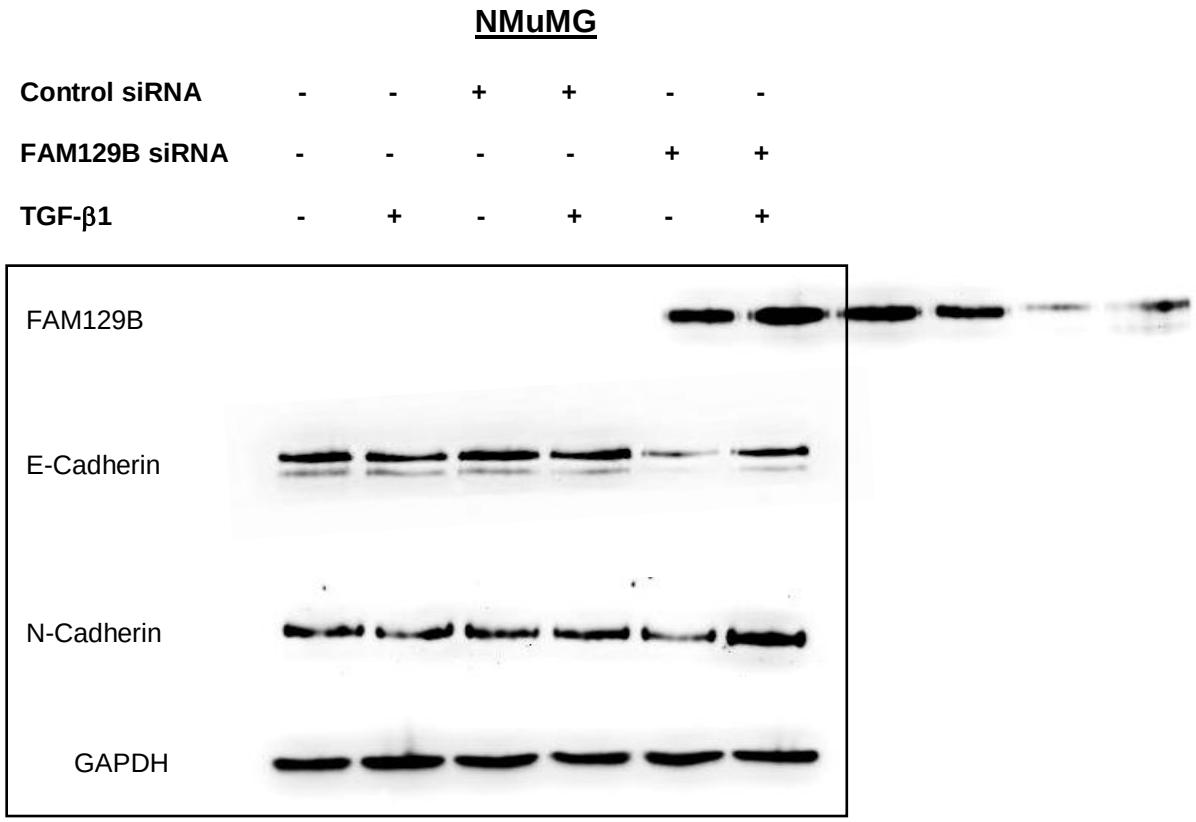

Figure 9 TGF-β1 Induced Epithelial to Mesenchymal Transition with FAM129B Knocked Down. *FAM129B was knocked down using siRNA technology, while the control siRNA had similar length and composition but different sequence, for 72 hours. Epithelial to mesenchymal transition was induced by 10 ng/ml TGF-β1 for an additional 96 hours. Cells lysates were analyzed via western blot analysis using antibodies against FAM129B and EMT markers. GAPDH served as an internal loading control.*

CHAPTER 4

4.1 Discussion

The structural domains of FAM129B (Figure 11a) show fundamental similarities with the structural domains of Nrf2, a transcription activator. In cytoplasm, under normal conditions, Nrf2 binds to Keap1 resulting in degradation by the proteasome. Keap1 has been identified as a cytoplasmic Nrf2-interacting protein, regulating Nrf2 activity through ubiquitination of the lysine residues and tagging proteins for degradation at the proteasome. This Keap1-Nrf2 system is important in cancer, as cancer cells ultimately commandeer this system and acquire malignant properties (Yoichiro Mitsuishi, 2012).

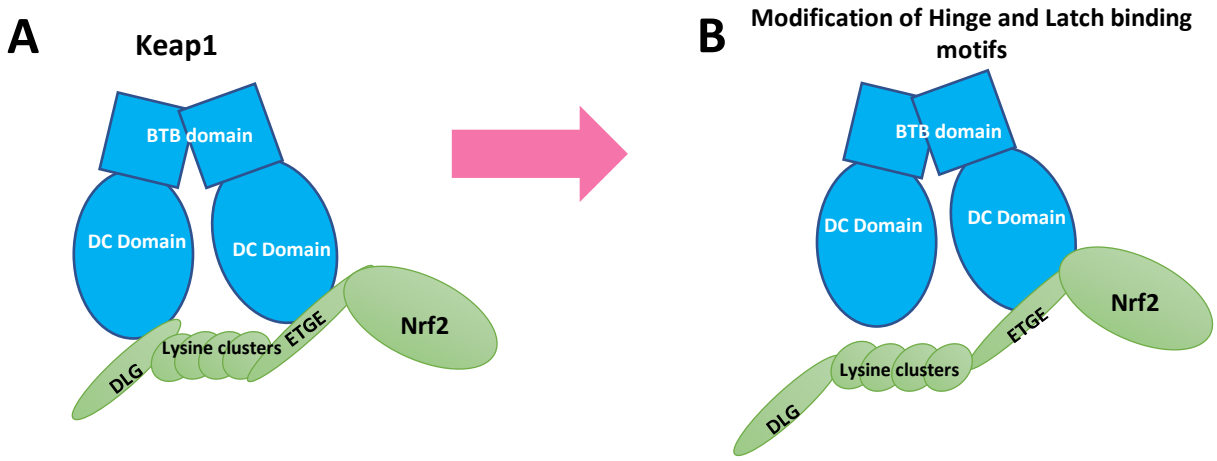

Figure 10 Keap1-Nrf2 Complex: Binding Models.

Figure 10 shows the formation of the Keap1-Nrf2 complex. Under high concentrations of electrophiles, two molecules of Keap1 dimerize using their BTB domains at the N-terminal terminus (Yoichiro Mitsuishi, 2012). While the two DC domains, located on the C-terminal end of the homodimer, associate with one molecule of Nrf2, as shown in Figure 10a (Yoichiro Mitsuishi, 2012). It is the N-terminal region on Nrf2, that bridges these two DC domains together through the DLG and ETGE motifs (Yoichiro Mitsuishi, 2012). The binding of these two motifs is based off the 'hinge and latch' model, where the DLG motif acts as the 'latch' and ETGE motif acts as a 'hinge'. The modification

of cysteine residues generates a conformational change in the Keap1 homodimer, leading to the dissociation of the DLG motif as shown in Figure 10b (Yoichiro Mitsuishi, 2012).

For further clarification, Figure 11 presents a visual representation of the Keap1 comparative binding sites between FAM129B (Figure 11a) and Nrf2 (Figure 11b).

A

FAM129B

601 ekrrrakqvv svvqdeevql pfeaspespp paspdgvtei rgllaqqlrp espppagpll
661 ngapagespq plaapeassp pasplghllp gkavdlgppk psdqetgeqv sspsshpalh
721 tttedsagvq tef

B

Nrf2

20 ILWRQDIDLG AGREVFDLSY RQKKVQLQRQ KELEEEKRQQ IVREQEKALL
70 AQLQLDEETG EFVPRLTPTN NTLTQANTVP AEITQNVDFT EENGDAMSFD
120 ECMQLLAETF PLVEPAVEHA PPCLDPSIPS CTDNSQLMMP GEIPMLTQNP

Figure 11 Keap1 Binding Motifs in Nrf2 and FAM129B. *(A) FAM129B contains the DLG (latch) motif and ETGE (hinge) motif. (B) Nrf2 contains the DLG (latch) motif and ETGE (hinge) motif responsible for the interaction with Keap1.*

Our hypothesis suggests that FAM129B suppresses apoptosis by binding to Keap1. Subsequently, Figure 11a confirms that FAM129B contains a DLG and ETGE binding motifs, as well as NrF2 (Figure 11b). It is these binding motifs that form the 'hinge and latch' model, which is responsible for its interaction with Keap1 (Yoichiro Mitsuishi, 2012). Since, FAM129B also contains these binding sites, we predict that FAM129B may be capable of binding with Keap1 through that same 'hinge and latch' model. Leading us to propose that it is through this Keap1-FAM129B complex, how FAM129B suppresses apoptosis.

Once FAM129B-Keap1 complex is formed, we can then predict how FAM129B suppresses apoptosis through its downstream effects on the TNFα pathway, as illustrated by our lab in Figure 12.

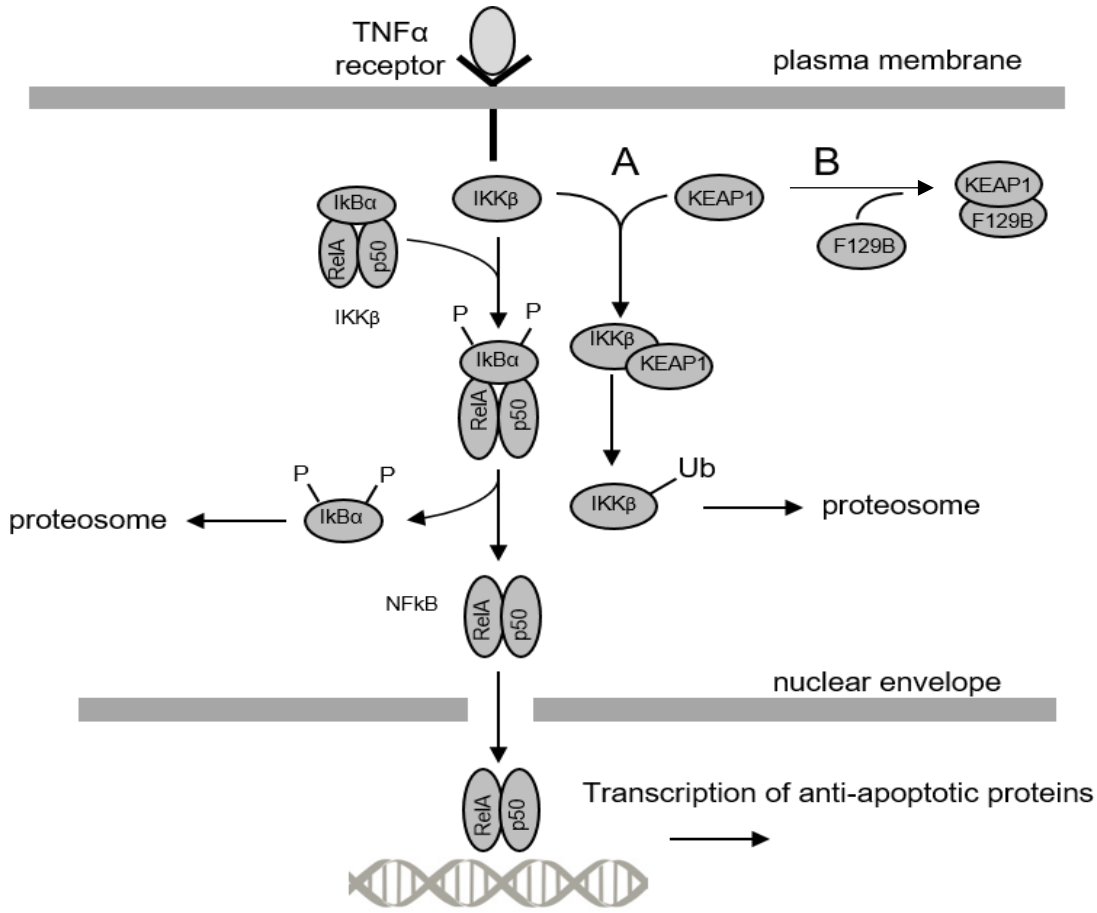

Figure 12 Schematic model of FAM129B binding to Keap1. *TNFα binds to its receptor and induces apoptosis (A) IKKβ binds to Keap1, which is involved in ubiquitination, tags it to be sent to the proteasome for degradation. (B) FAM129B binds to Keap1, blocking Keap1 from binding to IKKβ and suppressing it downstream and apoptosis will not occur.*

As shown in Figure 5, FAM129B localizes at the plasma membrane in confluent HeLa cells. However, FAM129B is a cytosolic protein under normal conditions, where it may potentially interact with KEAP1. If FAM129B binds to Keap1, it will prevent Keap1 from binding to IKKβ, an oncogenic kinase that aids Keap1 in ubiquitination, illustrated in Figure 12. This Fam129B-Keap1 complex will then disrupt the normal interaction between IKKβ and KEAP1, and IKKβ will be protected from degradation by the proteasome. This will lead to an upregulation of NF-κβ, suppression of downstream anti-apoptotic proteins, and apoptosis will not occur.

In this study, I initially set out to determine if Keap1 was an important component in the mechanism of suppression of apoptosis; by forming a complex with FAM129B. However, as illustrated in Figure 6, both HeLa and A549 cells contained undetectable levels of Keap1, although Keap1 has been previously reported to be present in HeLa cell line. Therefore, we were unable to test our hypothesis of whether FAM129B suppresses TNFα apoptotic pathway by forming the FAM129B-Keap1 complex in these cell lines (Song C. , 2011).

Since FAM129B already contains the Keap1 specific binding motifs, a mutant must be created to test this interaction. These FAM129B mutants would have their Keap1 specific, ETGE, binding residues replaced by an ETAA residue. Further analysis through co-immunoprecipitation, would be necessary to identify if there is a protein-protein interaction between FAM129B and Keap1.

The structural domain of FAM129B also illustrates a great deal about how FAM129B may regulate different pathways. For instance, FAM129B contains a tyrosine 593 phosphorylation site and a proline rich domain, which contains six serine phosphorylation sites located at the carboxyl terminal end of FAM129B (Haitao, 2016). Four of the six serine phosphorylation sites play an important role once phosphorylated, as they are phosphorylated by MAP kinase pathway (Song C. , 2011). It is this phosphorylation of FAM129B, that ultimately regulates FAM129B localization, as shown in Figure 5. Previous studies completed in our lab and the immunofluorescence results in Figure 5, both confirm that FAM129B migrates and localizes at the plasma membrane in confluent HeLa cells.

FAM129B has been previously shown to be implicated in apoptosis and metastasis. Furthermore, intracellular levels of FAM129B have also been reported to be significantly higher in metastatic cell lines. These observations together, suggest the hypothesis that FAM129B may be required for epithelial to mesenchymal transition.

During EMT, malignant cells acquire the necessary antics to become more invasive and motile, allowing cancer cells to metastasize. Ultimately, this causes an increase in patient mortality rates by increasing tumorigenic and proliferative potential, making EMT a prerequisite for cancer metastasis (Roche, 2018). The changes in E-cadherin and N-cadherin expression levels are ideal biomarkers for EMT detection. During EMT, E-cadherin levels track the change in epithelial cells, while N-cadherin levels track changes in mesenchymal cells. Realizing that EMT is not a complete transitional process in malignant cells, it is pivotal to observe these fundamental biomarkers to determine the stage of progression.

The silencing of FAM129B gene expression was a crucial fragment in this study, as it helps determine whether FAM129B affects TGF-β1 induced EMT. As illustrated in Figure 8, transfection using siRNA effectively depletes FAM129B from NMuMG cell lines.

TGF-β1 is a prototypical inducer for studying EMT in many epithelial cell lines. Since TGF-β can act both as a tumor suppressor and a tumor promotor, we chose this pathway to explore the role FAM129B in EMT. The TGF-β signaling pathway induces EMT by promoting downstream the inactivation of genes translating epithelial protein and activating the genes encoding mesenchymal proteins (M-F Pang, 2016). This approach has been validated for NMuMG cells, which are the preeminent model for studying the effects of TGF-β1 induced epithelial to mesenchymal transition.

Once FAM129B knockdown was established in our NMuMG cell lines, EMT was induced with 10ng/ml of TGF-β1 with, and without, FAM129B present (Figure 9). When FAM129B was knocked down and EMT was induced by TGF-β1, E-cadherin, which characterizes epithelial cells, increased as well as N-cadherin, which characterizes mesenchymal cells, demonstrating that Type 3 EMT has been initiated in the absence of FAM129B. This change in E-cadherin and N-cadherin levels, between the FAM129B knockdown control and FAM129B knockdown induced EMT, indicates that there is a hybridization state present and epithelial to mesenchymal transition is in progress – thereby demonstrating that FAM129B is not required for EMT. Furthermore, I was able to confirm the progression of EMT documented in Figure 9 through visual analysis of the cells, but did not have the equipment to photograph the changes at the time. When these experiments are repeated by my successor, a digital microscope should be used to photograph the transitioning cells.

4.2 Future Direction

These experiments were repeated four times to allow us to suggest that FAM129B is not be required for EMT. However, further studies can be conducted to further confirm this claim, as limitations arose and need to be addressed.

Although there was indication that EMT had occurred as the cells clearly exhibited the mesenchymal morphology, the results tested with the biomarkers were ambiguous. Consequently, the time course and extent of the EMT must be carefully documented by 1) documenting the time course of the morphological changes, 2) employ many more biomarkers that have been found to identify epithelial and mesenchymal cells and 3) conduct well established *in vitro* assays that assess the expected increase in cell invasiveness, an essential characteristic of EMT.

One of the major limitations I noticed was the inability to keep FAM129B fully knocked down while inducing EMT with TGF-β1 (Figure 9). Conducting a knockdown experiment results in a transient knockdown and does not result in a complete knockdown of the gene; previous unpublished data from our lab shows that FAM129B remains knocked down for up to 5 days. While Figure 9 suggests that EMT was induced with TGF-β1 after 5 days, however, it also shows that the expression levels of FAM129B is beginning to increase in these the cells. Other publications have shown that EMT can also be induced over longer periods of time; i.e. 7-10 days. Since FAM129B returns roughly 5 days after gene knockdown, we cannot test this 7-10-day induction proposed in other studies. However, one way this could potentially be tested, would be to create a knocked-out cell line using CRISPR to mediate a successful knockout the FAM129B gene. Once FAM129B gene was knocked out, we could proceed with the 7-10 day TGF-β1 induced EMT to further confirm the ambiguity of EMT biomarkers and that FAM129B does not affect epithelial to mesenchymal transition over different periods of time.

We also hypothesized that FAM129B binds to Keap1 through two binding sites, DLG and ETGE, to form a complex which aid in suppression of apoptosis. Since we were unable to detect Keap1 in HeLa, A549 and NMuMG cells, we could not test our hypothesis that the interaction between FAM129B and KEAP1 is responsible for the suppression of apoptosis by FAM129B. However, when comparing the FAM129B sequence (Figure 11a) with Nrf2 sequence (Figure 11b), even though they both contain the same Keap1 binding motifs, they are noticeably closer together in FAM129B than in Nrf2. This may indicate that they may potentially bind in a different way; i.e. through the ETGE motif, and no aid from the DLG motif. However, this hypothesis would require further testing using

conditions that allow detection of KEAP1 and Keap1 pull-down assay, to further confirm this hypothesis. In addition to the potential effect on apoptosis, the interaction of FAM129B-Keap1 may play a role in metastasis or cancer cell invasion - making FAM129B a novel chemotherapeutic drug target.

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ABSTRACT

THE ROLE OF FAM129B IN CANCER METASTASIS

by

LAUREN MARIE IACOBELLI

May 2019

Advisor: Dr. David Evans

Major: Biochemistry and Molecular Biology

Degree: Master of Science

Epithelial to mesenchymal transition (EMT) is a natural, biological process within the human body. Under normal circumstances, it a complete transition from epithelial cells to mesenchymal cells, i.e. embryonic development and tissue fibrosis. However, under malignant metabolic processes, EMT is an incomplete transition. These cells maintain a hybridization state that sanctions malignant cells more aggressive than others, ultimately leading to metastasis. EMT's involvement in the metastatic spread of tumorigenic cells is one of the hallmarks of cancer. In this study, we analyzed the role of FAM129B/MINERVA in epithelial to mesenchymal transition. This novel protein, has been implicated in apoptosis and metastasis of melanoma cells, another hallmark of cancer. We first confirmed that this protein co-localizes to plasma membrane upon contact with other cells through adherens junctions. Through knockdown of this protein, followed by TGF-β1 induced EMT, we preliminarily concluded that FAM129B does not play a direct role in the induction of epithelial to mesenchymal transition.

AUTOBIOGRAPHCAL STATEMENT

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