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Fam129b Phosphorylation And Its Effect On Membrane Localization In Confluent Hela Cells

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by

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CHAPTER 1 INTRODUCTION

1.1 Summary

1.1.1 Role of Proteins in Cancer Progression

For researchers', cancer is one of the most complex and intriguing field of study. Since 3000 BC, scientists have been revealing many properties of cancer cells that differ from normal cells. Cancer cells progress through an array of complex pathways and its survival depends on how effectively it surpasses many signaling pathways. As can be seen from the famous image by Douglas Hanahan and Weinberg, The Hallmarks of Cancer(Fig.1), cancer cells promote tumor growth by resisting cell death and promoting angiogenesis (1). The image also signifies that all cancer cells share six common traits or hallmarks that lead to the transformation of normal cells to cancer cells. They are also efficient at suppression of apoptosis and sustaining proliferative signals such as activation of (Epidermal Growth Factor Receptor) EGFR mediated Ras-Raf pathways (2,3,4). Proteins such as these play a key role in survival of cancer cells. Studies are also revealing many other proteins that interact with EGF and Ras for cancer cell invasion and metastasis. Phosphorylation and de-phosphorylation of several signaling proteins play a key role in sustaining cancer cell activity. The proteins kinases, RAF, MEK and MAPK has an active role in controlling cell growth through phosphorylation. However, dysfunction of these kinases leads to uncontrolled growth which is a necessary step for the development of all cancer (5). Overexpression and dysregulation of certain proteins in normal cells triggers pathway leading to cancer progression and invasion. One such protein that is expressed highly in many different forms of cancer is FAM129B (6,7).

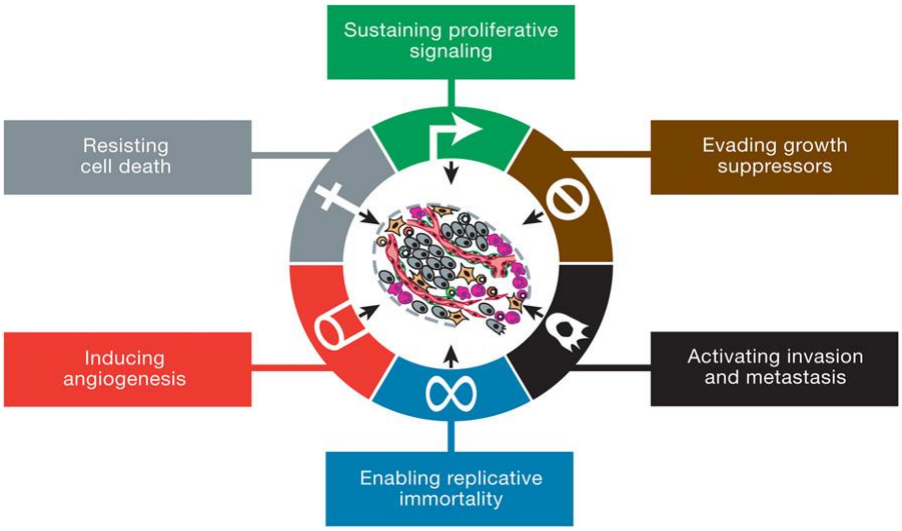


Fig 1.1. **Douglas Hanahan, Robert A. Weinberg. Hallmarks of Cancer (Cell. 2000).** The author describes the six major capabilities acquired by cells to transform to a human tumor. These six characteristics of cancer cells are common in all forms of cancer.

1.2 Significance of Protein Phosphorylation and Dephosphorylation

Once a gene is expressed and translated into a functional cellular protein, the cell is able to control the protein's fate through the use of posttranslational modification (PTMs). Phosphorylation is the most important and most thoroughly researched form of PTM. Although, proteins undergo posttranslation modification in various form such as acylation, carboxymethylation, tyrosine sulfation and glycosylation, none of these mechanisms is nearly as widespread and readily subjected to regulation by physiological stimuli as is phosphorylation. Phosphorylation occurs by covalent binding of phosphate group to certain amino acid residue (Serine, Threonine and Tyrosine) (8). This reaction is catalyzed by protein kinase. The reverse reaction of phosphorylation is dephosphorylation which is catalyzed by protein phosphatases (9).

Protein kinases and phosphatases work independently and in a balance to regulate the function of proteins (10). These two events are important switches that regulate many intracellular functions. Many pathways are connected through protein phosphorylation and dephosphorylation.

The dynamic process of phosphorylation plays key roles in the regulation of normal cells and cancer cells. When the balance of phosphorylation and dephosphorylation is disturbed, cellular activities are dysregulated leading to numerous biological problems including cancer (11). The abnormal activation of phosphorylation through kinases have been shown to drive many hallmarks of cancer biology including proliferation, cell survival, motility, angiogenesis, metabolism and evasion of antitumor immune response (12). Thus, loss of negative regulation through phosphatase activity and overexpression of kinases together play a major role in transformation of normal cell to a cancerous one.

1.3 FAM129B

FAM129B is a protein that is expressed highly in cancer cell as compared to normal cells. It is also known with different names like Meg-3, Minerva (Melanoma invasion by ERK) etc. FAM129B is upregulated in many forms of cancers including breast, kidney, large intestine, lung, endometrial cancers and hematopoietic and central nervous system tumors. It is also reported that FAM129B localizes to plasma membrane in confluent cancer cells where it is thought to interacts with adherens junction protein. In exponentially growing cells, it is exclusively in cytoplasm (17). FAM129B also plays a key role in suppression of apoptosis, thus suggesting its role in promoting cancer.

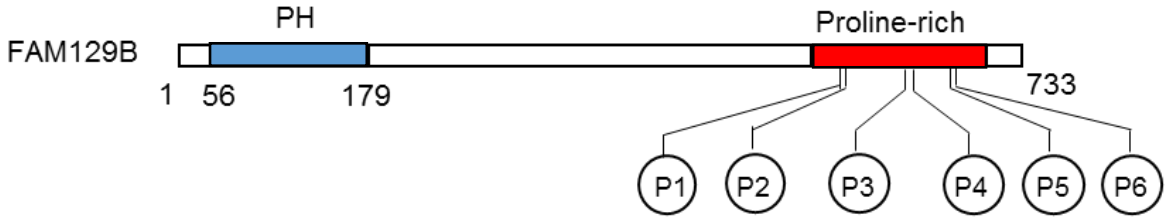
1.4 Structure of FAM129B

The protein coding gene FAM129B is a class of family with sequence similarity 129-member B. FAM129B is 746 amino acids long and approximately 84 KDa polypeptide which belongs to a protein family of unknown category and unknown basic function. Other family members are Niban (FAM129A), a stress-inducible gene found to be upregulated in renal and thyroid cancer models, and novel B-cell protein 1 (BCNP1, FAM129C), a surface membrane protein overexpressed in chronic lymphocytic leukemia (13). FAM129B and Niban/ FAM129A share 40% sequence identity, both containing a pleckstrin homology (PH) domain, while FAM129B shares 27% sequence identity with BCNP1/FAM129C. However, little else is known about the function or regulation of these proteins in any organism.

Homo sapiens FAM129B consists of a conserved Pleckstrin Homology (PH)-like domain at the N-terminal and proline rich sequence near the carboxyl end that includes a KEAP1 binding ETGE motif at the C-terminal (Fig.2). One of the main function of PH-like domain is to bind phosphatidylinositol lipids within biological membranes (14). These interactions help them recruit certain proteins to the membrane thus targeting them for other cellular functions. The isomer FAM129C also has the PH domain at the amino terminal, whereas FAM129A has only a truncated PH domain. A conserved ETGE motif in FAM129B was found to interact with KEAP1 (15). It is thought that the interaction of FAM129B with KEAP1 plays an important role in cancer cell proliferation by sequestering KEAP1 which in turn activates Nrf2 (responds to oxidative stress) and IKKβ (triggers immune response) activities. The proline rich region near ETGE motif has six serine phosphorylation sites that were first identified by a large scale phopshoproteomics

studies. All the six sites showed direct phosphorylation by ERKs (16). The six serine phosphorylation sites play an important role in FAM129B membrane localization.

Fig 1.2. FAM129B domain structure showing PH-like domain at N-terminal and ETGF motif at C-terminal. The six serine phosphorylation sites are P1 – P6. P1P2 and P5P6 are very close to each other. The sites P1, P2 and P5, P6 are phosphorylated by MAP Kinase. However, the sites



FAM129B

```
            P1      P2                    P3                P4
620 PFEASPESPP  PASPDGVTEI  RGLLAQGLRP  ESPPPAGPLL  NGAPAGESPQ
              P5    P6
670 PKAAPEASSP  PASPLQHLLP  GKAVDLGPPK  PSDGETGEQV  SSPSSHPALH
```

P3 and P4 are far apart and not as strongly phosphorylated as the other four sites

1.5 Functions of FAM129B

The significance of this protein was first mentioned by Old and colleagues through phosphoproteomic studies to selectively identify phosphopeptides (16). They found B-Raf/MKK/ERK signaling as the effective targets of FAM129B thus implicating its role in cancer cells. Their study found six serine phosphorylation site at the C-terminal of FAM129B and mutation of these sites affects the way FAM129B translocate to membrane in confluent cells. Studies have also shown that FAM129B translocate to cell membrane in confluent HeLa cells (17). However, when cells are not in contact with each other and the adherent junction is disassembled, FAM129B is mostly distributed in cytoplasm (Fig 1.3). The interaction of FAM129B

with the cell membrane and its colocalization with β-catenin, a marker for cadherin-dependent cell-cell interactions suggest that FAM129B play a key role in cancer cell invasion (17).

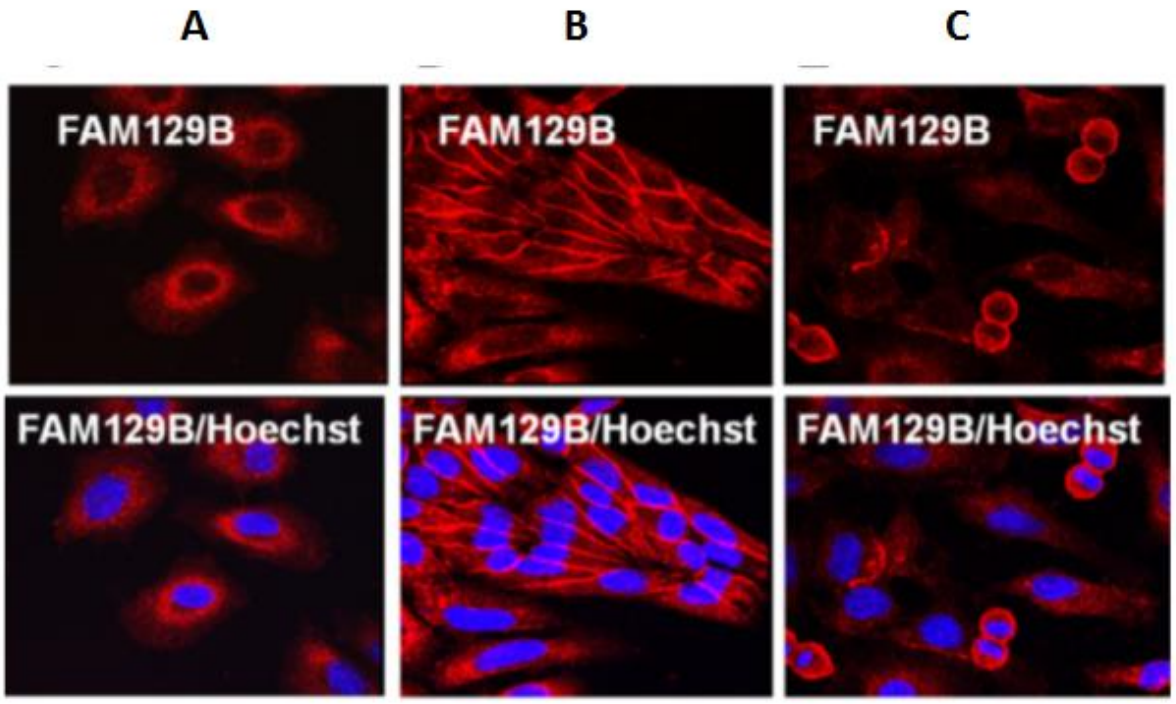


Fig 1.3 Endogenous FAM129B localization in confluent HeLa cells (Chen, Guy and Evans 2010).

(A). When cells are not in contact with each other and the membrane junction is not formed, FAM129B is mostly dispersed in cytoplasm. The image shows the cytoplasmic FAM129B (red) in exponential cells.

(B). FAM129B localizes to cell-cell adhesion junction in confluent HeLa cells. The image shows localization of FAM129B (red) to the membrane in confluent HeLa cells.

(C). FAM129B (red) in exponentially growing HeLa culture showing some mitotic cells.

FAM129B is a novel regulator of Wnt/β-catenin signal transduction in melanoma cells (18). Wnt signaling pathway are a group of signal transduction pathways made of proteins that pass signals into a cell through cell surface receptor. Wnt signaling was first identified for its role in carcinogenesis, then for its function in embryonic development. The combined

phosphoproteomics and siRNA screening study has identified FAM129B as the novel regulator of Wnt/β-catenin signaling in human melanoma. Silencing of FAM129B inhibits Wnt/β-catenin target gene expression and apoptotic response to WNT3A. Studies have also mentioned that FAM129B by itself does not have enzymatic activity or do not have enzymatic domain. However, it exhibits its cellular effects through protein-protein interaction and around 18 proteins were identified that interacts with FAM129B including KEAP1.

FAM129B deficiency has also been shown to delay cutaneous wound healing process in mice (19). This physiological effect was studied by generating gene-targeted FAM129B-mutant mice in which, the amino terminal coding exon was replaced by lacZ. It was found that homozygous mutant mice are viable and fertile and that FAM129B is considerably expressed in most of epidermal keratinocytes of both embryonic and adult mice. However, in the skin of the FAM129B-deficient mice, wound healing subsequent to skin puncturing was significantly delayed. Furthermore, overexpression of FAM129B promoted cell motility in an N-terminal pleckstrin homology domain-dependent manner. This study suggests that FAM129B is necessary for regulation of cell motility and thereby, contributes to the appropriate wound healing process.

One of the function that FAM129B is known for is the suppression of apoptosis in metastatic cancer cells. Cancer cells survive by many pathways and many factors are involved in its development as can be seen in the image by Hanahan and Weinberg. When apoptosis happens, cells shrinks, disassembling the membrane junctions followed by cell blebbing, DNA fragmentation and mRNA decay. FAM129B is shown to be present in cytosol in exponentially growing HeLa cells. However, when cells come in contact and the membrane β-catenin junction is formed, FAM129B tend to localize at the membrane. Studies done using knockdown FAM129B

in presence of apoptosis inducing factors such as TNFα/cycloheximide have shown that apoptosis was 3-4 times faster than in the presence of FAM129B (17).

A very recent study has shown that Epidermal growth factor receptors (EGFR) phosphorylated FAM129B to promote Ras activation (20). In this paper, the tyrosine residue of FAM129B (Y593) is shown to be phosphorylated by EGFR which triggers the cascade of Ras activation leading to uncontrolled cell growth and differentiation. Epidermal growth factor (EGF) is a growth factor that stimulates cell growth, differentiation and proliferation through EGFR activation. In normal cells EGF binds to EGFR and this subsequently promotes a cascade of events leading to Ras and MEK activation. However, in controlled cell growth, the activation and deactivation of Ras and MEK is maintained by Guanine exchange factors (GEFs) and GTPase activating protein (GAPs) respectively. In cancer cells, the binding of GAPs is prevented and Ras continues to be in active state which leads to uncontrolled cell differentiation and proliferation. It has been shown in this paper that in many cancer cells, EGFR phosphorylated FAM129B at Y593 residue and promotes interaction between FAM129B and H-Ras. This phosphorylation of FAM129B prevents binding of GAPs and enhances Ras activation.

1.6 FAM129B phosphorylation at its 6 serine residues.

This project mainly focusses on significance of the six serine phosphorylation sites at the C-terminal end of FAM129B, that is shown to affect localization of FAM129B to the membrane in confluent cells (16). Also, all six sites showed direct phosphorylation by ERKs. However, the four sites (Ser628, Ser633, Ser679 and Ser683) showed a strong effect by the direct phosphorylation as can be seen in the paper Old et.al where the four sites were suppressed more than 2-fold upon pathway inhibition. Hence, we focused on these four sites to find out which of these sites affects

FAM129B localization. The study was done by mutating the phosphorylation sites to alanine (A) thereby preventing any phosphorylation of these sites and see if the function is restored by the phosphomimic glutamic acid (E). Since Ser628 and Ser633 are close together, our first aim was to mutate these two together to alanine and phosphomimic glutamic acid. Similarly, Ser679 and Ser683 was mutated together to Alanine and phosphomimic Aspartic acid. Thus, four mutants (S628A/S633A, S628E/S633E, S679A/S683A and S679D/S683D) were generated for this study. The other two serine phosphorylation site (Ser652 and Ser668) were individually mutated to Alanine and Glutamic acid (S652A, S652E).

As part of this study, preliminary works were also done to see endogenous FAM129B localization in confluent and non-confluent HeLa cells. Most importantly, it was interesting to see FAM129B colocalization with N-cadherin, which is a membrane junction protein and is present only in confluent cells where cell-cell adhesion junctions are formed. Further to this, immunofluorescence studies was also done on the deletion construct that has all the six phosphorylation sites deleted. Amino acids from 1-572 was kept intact while all other C-terminal regions were deleted which includes the six serine phosphorylation sites as well. Expression of these regions also gave an important information on FAM129B localization in confluent HeLa cells.

CHAPTER 2 MATERIALS and METHODS

2.1 Polymerase Chain Reaction Site Directed Mutagenesis

2.1.1 Primer Design

Mutants for the phosphorylation studies were made using New England Biolabs (NEB) site directed mutagenesis kit. Wild type FAM129B cloned in pEGFP vector with Kanamycin resistant tag was used as a template for the PCR reactions. The whole plasmid is approximately 8000bp. The PCR Site Directed Mutagenesis was carried out on the whole plasmid. Proper primer design guidelines were used from IDT oligo analyzer tool from Integrated DNA Technologies. Primers for the PCR were generated using IDT oligo analyzer and the forward primer contained both the mutants. Both forward and reverse primers are phosphorylated at the 5-prime end for effective ligation of the linear strand formed in PCR cycle. The 5-prime phosphorylated forward and reverse primers were supplied by Thermo Fishers.

Mutants	**Forward Primer**	**Reverse Primer**
S628_633A	5’(Phos)ctgag **GCT**ccaccacctgcg**GCT**cc…	5’(Phos)ggctagcctcaaagggcagccccacc
S628_633E	5’(Phos)ctgag**GAA**ccaccacctgcg**GAA**ccgg	5’(Phos)ggctagcctcaaagggcagccccacc
S679_683A	5’(Phos) cctcc**GCT**ccgcctgcc**GCT**c	5’(Phos)cctcgggggcggccttaggct
S679_683E	5’(Phos)gcctcc**GAA**ccgcctgcc**GAA**cc	5’(Phos)ctcgggggcggccttaggct
S652A	5’(Phos)gcctgag**GCT**cccccacca	5’(Phos)cgcagaccttgggccagca
S652E	5’(Phos)gcctgag**GAA**cccccacca	5’(Phos)cgcagaccttgggccagca

Table 2.1: PCR forward and reverse primers for all the mutants. The codons highlighted in bold shows the mutation sites with the codon for serine changed to respective alanine and glutamic acid codons. The reverse primers do not contain the mutation. Both the forward and reverse primers are phosphorylated at 5’ end and is provided in a purified form using desalt technique.

2.1.2 PCR Site Directed Mutagenesis

The recombinant FAM129B expression plasmid cloned in pEGFP vector was previously made by Dr. Song Chen in our lab (17). FAM129B was also cloned into pEGFP-C3 vector to generate a fluorescent fusion protein. The whole plasmid is around 8000bp with the GFP tag at the amino end. To generate mutants, whole plasmid site directed mutagenesis was carried out with both reverse and forward primers. The forward primers carried the mutations to be incorporated. Around 30-50 ng of GFP-FAM129B was used for the reaction and mixed with Q5 reaction buffer (NEB #B9027S), dNTPS (NEB #N0447S), primers, Q5 Polymerase (NEB #M0491S) and GC enhancer (NEB #B9028A) as per manufacturer's protocol. It was made sure that polymerase was added after all other components were added except GC enhancer which was added at the last step. The final reaction volume is brought to 50µl using ultra-pure nuclease free water. PCR mix was placed in Eppendorf Master Cycler Gradient Thermal Cycler and the reaction was carried out for 18-22 cycles. The PCR product was then run through agarose gel to visualize the bands corresponding to FAM129B. The protocol is summarized in the following table.

Component	**50µl reaction**	**Final Concentration**
Q5 Reaction Buffer	10 µl	1X
10 mM dNTPs	1 µl	400 µM
10 µM Forward Primer	2.5 µl	0.5 µM
10 µM Reverse Primer	2.5 µl	0.5 µM
Template DNA	Variable	30-50 ng
Q5 High-Fidelity DNA Polymerase	1 µl	0.04 U/µl
5X Q5 GC Enhancer	10 µl	1X
Nuclease-Free Water	Up to 50 µl	

Table 2.2: Optimized PCR Reaction mix for Site Directed Mutagenesis. All mixing was done on ice in a PCR tube. The GC enhancer is optional, however since the plasmid has high GC content (60-70%), it was found to be effective.

Thermocycler conditions

Step	**Temperature**	**Time**
Initial Denaturation	98°C	2 minutes
18-22 Cycles	98°C	30 seconds
	*Ta	1 minute
	72°C	30 seconds/Kb
Final Extension	72°C	8 minutes
Hold	4°C	

*****Annealing temperature is obtained from primer design software. Usually temperature 1-2°C below that the mentioned is used for the thermocycler.

Table 2.3: Thermocycler conditions for FAM129B Site Directed Mutagenesis. Initial denaturation temperature can vary depending on the amount of template used. For low template concentration, initial denaturation temperature should be from 1 to 1 1/2 minutes.

2.1.3 Gel Electrophoresis

The Easy Cast DNA electrophoresis apparatus from Owl Separation Systems was used for electrophoresis of DNA samples. 0.8% agarose gel was made by dissolving 0.8g UltraPure Agarose (Invitrogen) in 100 ml 1XTAE buffer (40mM Tris base, 0.1% acetic acid, 1mM EDTA, pH8.0) and heated for 1-2 min in a microwave until boiling. The boiled agarose was cooled down to about 50°C and 10µl of SYBR Safe DNA gel stain (S33102- 24 Invitrogen) was added. The agarose solution was then poured into a gel casting apparatus and a 1.5mm 10-well comb was inserted for formation of the loading well. The gel was then allowed to solidify at room temperature and placed in the tank filled with TAE buffer. Samples were prepared by mixing the DNA to loading dye (Invitrogen #R0611) at a ratio 1:5. The samples were loaded in the gel, along with a DNA

standard, 1Kb (Invitrogen #15615-016). The agarose gel was run at 90-100V for 45 minutes, or until the bromophenol blue dye ran approximately ¾ of the length of the gel. The DNA fragments were then viewed and photographed using a UV transilluminator.

2.1.4 PCR Purification

The PCR reaction generates linear plasmids which needs to be ligated to form a circular plasmid for efficient transformation. Before proceeding to the ligation reaction, the PCR product was cleaned up using Qiagen PCR Purification Kit (Cat# 28104). The purification was carried out as per the manufacturer's protocol and 30µl elution buffer was used to elute the DNA in more concentrated form. PCR purification was carried out using PureLink Gel Extraction Kit (Invitrogen # K2100-12) and PureLink PCR Purification Kit (Invitrogen # K3100-01). However, a better yield and purity of the purification was obtained from QIAquick PCR Purification Kit (Qiagen).

2.1.5 Ligation

After PCR was carried out at the specified conditions, the linear mutated strands were ligated using Blunt/TA Ligase Master Mix (NEB #M0367S). The PCR reaction generated blunt end linear strands, hence this ligase was much specific and efficient. Around 50 ng of purified PCR product was treated with ligase master mix. Before starting the reaction, the components in the master mix tube were mixed by finger-flicking and transferred to the sample to be ligated. The reaction mix was made by pipetting up and down 7 times slowly. The volume of master mix used was approximately 50% of the total reaction volume. The reaction was carried out for 15-20 minutes at room temperature (25°C). After this the reaction, the mixture was kept on ice for 3 minutes to inactivate the ligase.

2.2 **Gene Sequencing and Analysis**

The plasmids were sent for sequencing to Genscript and Genewiz sequencing service. The samples were premixed with the sequencing primer (5' CGAGGAAGTACAGCAACA 3') as per the companies' protocol. The sequencing primer was chosen in such a way that it is 100bp upstream of the mutation sites. Also, GC content of the sequencing primer was limited to 55% and its annealing temperature in the range of 55 - 60°C. The sequencing results were analyzed using NCBI blast or ClustalW for pairwise alignment. The trace files, chromatogram that show peaks for respective nucleotides, were also analyzed in BioEdit Sequence Alignment Editor.

2.3 Transformation

One Shot Top10 competent cells were provided by Invitrogen (C4040-10). Cells were thawed on ice and around 20ng of ligated plasmids were added to 10µl of Top10 cells. The mixture was incubated for 30 minutes on ice and followed by heat shock for 30 seconds at 42°C and then placed on ice for 3 minutes. The cells were incubated in recovery media or SOC media for 1 hour at 37°C with shaking. The outgrowth is then spread onto kanamycin resistant agar plates and incubated overnight at 37°C. The colonies formed on the plates were incubated overnight at 37°C with shaking at 218 rpm in 5ml LB media to extract plasmid. These were sent for sequencing to confirm the mutations.

2.4 Top10 Competent cells

Top10 competent cells were also made in lab using the lab protocol. TSS method was used for transformation purpose and for long-term storage of Top10 cells. Before starting, the work bench, gloves and all pipettes to be used were sterilized. This is done to prevent any contamination because, in the whole process of making Top10 cells, no antibiotics were used or

added to cell culture. LB agar plates with no antibiotics were streaked with Top10 and incubated overnight at 37°C. A single colony was picked from this and cultured in 5ml LB by overnight incubation at 37°C with shaking at 280 rpm. 1 ml of this overnight culture was then inoculated in 100ml autoclaved LB in large flask with no antibiotics. This is grown at 37°C, 280 rpm till the optical density @600 nm reaches 0.3-0.4. Once the OD reached the required value, the flask was kept on ice for 15 minutes and then centrifuged in an autoclaved falcon tube at 3000rpm for 20 minutes at 4°C. After this it was kept on ice and all subsequent steps were carried on ice. After discarding the supernatant, the cell pellet was re-suspended in 10ml ice-cold TSS buffer and mixed thoroughly by pipetting the suspension up and down. This was then spin down at 3000 rpm for 20 minutes at 4°C. The supernatant was discarded and the cell pellet was re-suspended in 0.9 ml ice-cold TSS and 0.1 ml ice cold 80% glycerol. The cells were then aliquoted into pre-cooled 1.7ml centrifuge tubes on ice and stored at -70°C.

2.5 Plasmid Purification

For plasmid extraction, PureLink Quick Plasmid Miniprep kit form Invitrogen (Invitrogen #K210010) was used. Around 3-5 ml culture was centrifuged at 4000 rpm for 6 minutes. The supernatant was discarded and the cell pellet was suspended using resuspension buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA 20 mg/ml RNase). This was treated with lysis buffer (200 mM NaOh, 1% w/v SDS) and incubated for 5 minutes. After this, precipitation buffer was added and the solution is centrifuged for 10 minutes at 12000 rpm. The supernatant is loaded in the column and centrifuged at 12000 rpm for 1 minute. The column was eluted with wash buffer and centrifuged twice to remove any addition wash buffer. In the final step, plasmid is eluted using elution buffer under centrifugation at 12000 rpm for 2 minutes. The concentration of DNA was measured using

NanoDrop. For transfection and immunofluorescence studies, plasmid purification was carried out using PureLink HiPure Plasmid Miniprep Kit (Invitrogen #K210002). Plasmids of high purity and high concentration is obtained from this kit which increase the efficiency of transfection in cells.

2.6 Cell Culture (HeLa Cells)

HeLa cells are maintained in Dulbecco's Modified Eagle Media (DMEM) with 10% Fetal Bovine Serum (FBS). To avoid any contamination at initial thawing, 1% antibiotics are also added to DMEM. Cells are passaged when the confluency reaches around 80%. For cell splitting, all media is aspirated and treated with 3.5 ml trypsin. The flask is then incubated for 2-3 minutes until all the cells settle out of suspension. To this 5 ml DMEM with 10% FBS is added to inactivate trypsin by the serum. The whole suspension is then centrifuged at 2500 rpm for 5 minutes at 4°C. The media is removed and the cells are suspended in 2 ml freezing media (DMEM + 5% DMSO). These are then transferred to cryogenic vials and kept at -80 for 24 hours before storing in liquid nitrogen. For thawing, the frozen vial is taken from liquid nitrogen and immediately warmed by rapidly shaking in water bath at 37°C. Once the cells are thawed, it is transferred to T-75 flask with 5-7 ml media with antibiotic.

2.7 Transfection

Transfection was carried out using Lipofectamine 2000 reagent from Invitrogen (Cat # 11668027). HeLa cells were plated in 6-well plates a day before transfection. On the day of transfection, the cells reached a confluency of 60 –80%. DNA-Lipofectamine transfection complex were made at a ratio of 1:2 in Opti-MEM Reduced Serum Media and incubated for 15 minutes before adding to HeLa cells in the culture. The complex was added directly to cells in culture

medium with serum and no antibiotics. After 4 hours, the media was changed to fresh DMEM media with 10% FBS.

2.8 Immunofluorescence

Cells were grown on glass coverslips to 80% confluence and fixed in 1% paraformaldehyde for 15 min and permeabilized in 0.2% triton X-100 for another 15 min. After three 5 min washes in phosphate buffered saline (PBS), the coverslips were blocked in 1% BSA for 1 hour and 30 min. Coverslips were then incubated with primary antibodies, Rabbit anti-GFP polyclonal antibody (Genscript #A01388-40) for 2 hours, washed 3× 5 min with PBS, and incubated in secondary antibody goat anti-rabbit IgG antibody, alexa fluor 488 (Invitrogen #A11034) for 1 hour. For study of endogenous FAM129B colocalization with N-cadherin, imaging was done using primary rabbit FAM129B antibody (Cell Signaling #5122S) and mouse N-cadherin antibody (Cell Signaling #14215) were used. However, the secondary antibody was goat anti-rabbit IgG alexa fluor 488 for endogenous FAM129B and goat anti-mouse IgG Alexa Fluor 594 (Invitrogen # A-11032) for N-cadherin. Coverslips were washed 3× 5 min in PBS and mounted in ProLong Gold antifade reagent with DAPI (Invitrogen #P36931). The slides were dried overnight by properly covering with aluminum foil and placing in dark area. Images were collected using 40X oil immersion lens under Nikon Eclipse E800.

CHAPTER 3 RESULTS

3.1 PCR Cloning to generate FAM129B mutants

A previous study has shown that the six serine phosphorylation sites of FAM129B plays an important role in its localization to membrane the junctions (16). The sites Ser628 and Ser633 are located close to each other and hence both were mutated to Alanine together. To make the phosphomimic, these two sites were mutated to glutamic acid using the PCR site directed mutagenesis technique. Sites Ser679 and Ser683 were also mutated together to Alanine and Aspartic acid residues. When following the manufacturer's protocol, a faint or no band specific for FAM129B was observed (Fig.3.1A). A few parameters were then changed to see how they affect the PCR efficiency. When tried with different initial denaturation time, it was seen that long denaturation time tend to cause the bands to smear in gel, which may be due to multiple PCR products (Fig.3.1B). An optimal time of 1-2 minutes for initial denaturation was found to be effective and the gel showed much less smearing (Fig.3.1C). The protocol was optimized by employing different conditions and changing numerous parameters to generate a good PCR product. This is seen as a strong band in gel electrophoresis that corresponds to the size of FAM129B which is around 8Kb (Fig3.1D). The final mutant plasmids purified from plasmid miniprep kit shows 8Kb FAM129B in the gel (fig 3.1E).

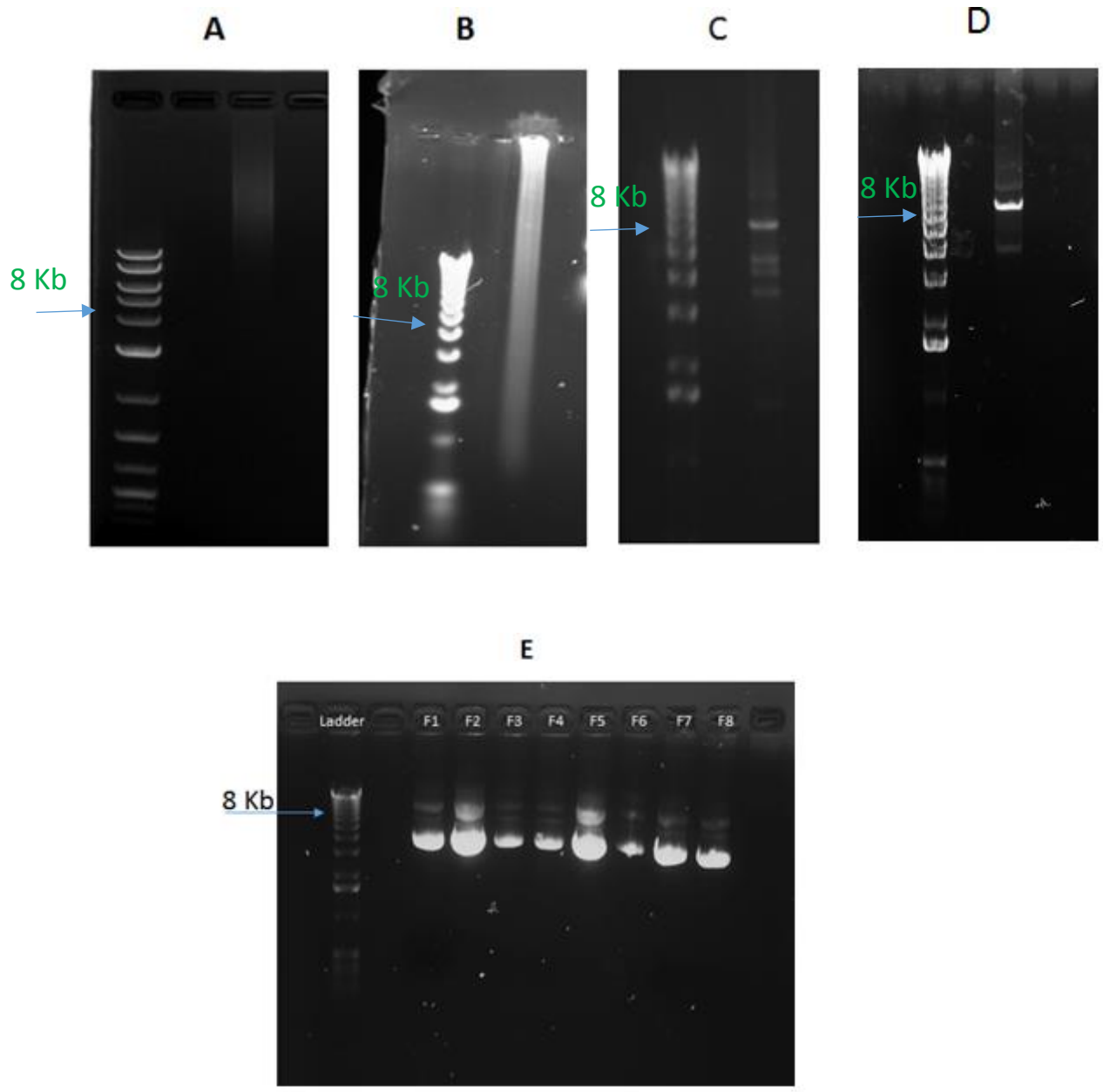


Fig 3.1. FAM129B Gel images of PCR product.

(A). No band corresponding to FAM129B was seen when used manufacturer's protocol and ran for 30 cycles.

(B). Smear showing multiple band formation due to high denaturation temperatue. Initial denaturation was given for a longer time of 5 minutes by keeping other conditions constant.

(C). Faint band with low concentration. Shows successful PCR, however, product concentration was too low (10-12 ng) for further ligation process.

(D). Optimized PCR condition shows strong band around 8Kb. By changing template and primer concentration and optimizing thermocycler conditions, a strong band for PCR product was seen and a concentration of 130ng was obtained as measured from NanoDrop.

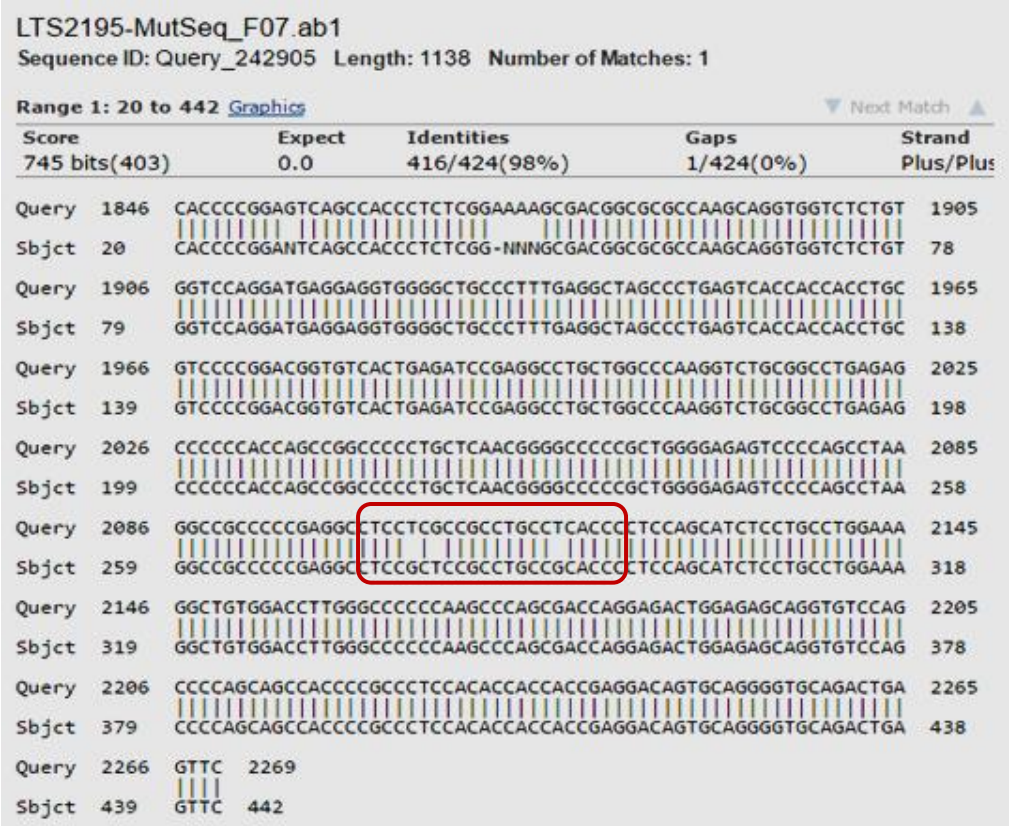
(E). Gel image of FAM129B plasmids (wild type and mutants) extracted from Top10 culture using plasmid miniprep kit. Band F1 and F2 corresponds to Wild type FAM129B and F3- F8 corresponds to the six mutants of FAM129B. The gel shows two band, one corresponding to 8Kb and other lower supercoiled band which is the native confirmation of DNA.

3.2 Confirmation of mutants using Sanger Sequencing (Genewiz and Genscript)

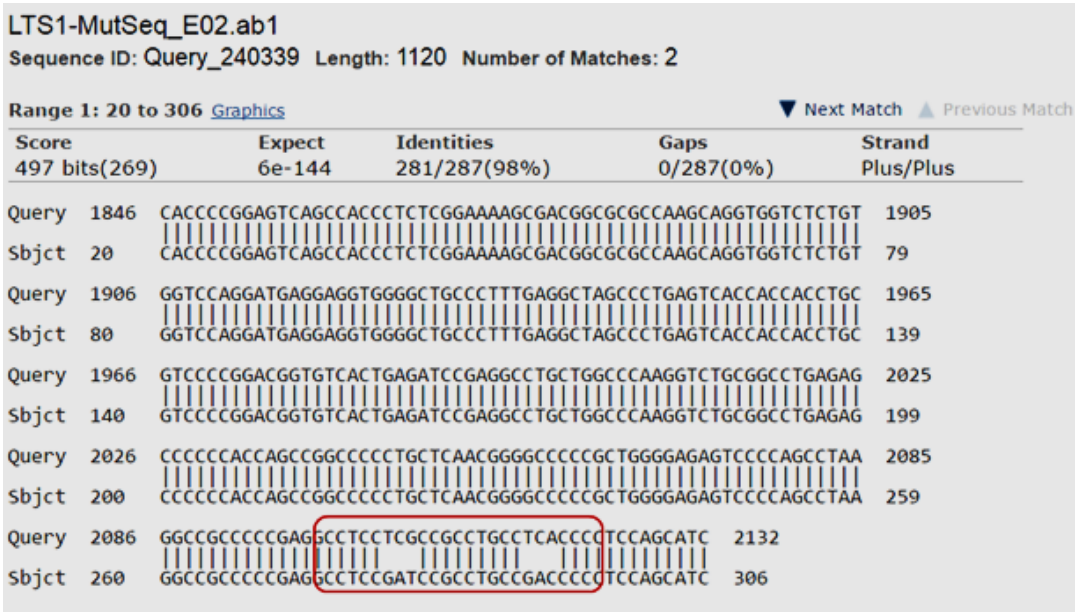
After successfully generating PCR products, it was ligated using blunt/TA ligase kit form NEB and then transformed to TOP10 E.coli cells. Very few colonies were formed and different colonies were cultured overnight in LB media. The plasmids were extracted and send for sequencing. The sequencing results were analyzed using NCBI blast tool. The mutants were compared with wild type FAM129B and as can be seen the sequence is identical except those sites where the base changes have been introduced. As can be seen in the sequence alignment, serine codons TCG and TCA were mutated to alanine codons GCT and GCA respectively (Fig 3.2A). This confirms mutation of serine to alanine at position 679 and 683 respectively. The Ser679 codon TCG was mutated to aspartic acid codon GAT and the Ser683 codon TCA was mutated to aspartic acid codon GAC (Fig 3.2B). This confirms the aspartic acid phosphomimic of Ser679Asp and Ser683Asp.

Mutants of Ser652 were also analyzed by sequencing. The serine codon AGC is mutated to alanine codon GCT using site directed mutagenesis technique (Fig 3.2C). To generate S652E, the serine codon AGC is mutated to glutamic acid codon GAA. Confirmation of the mutations Ser652Ala and Ser652Glu were shown in Fig 3.2D.

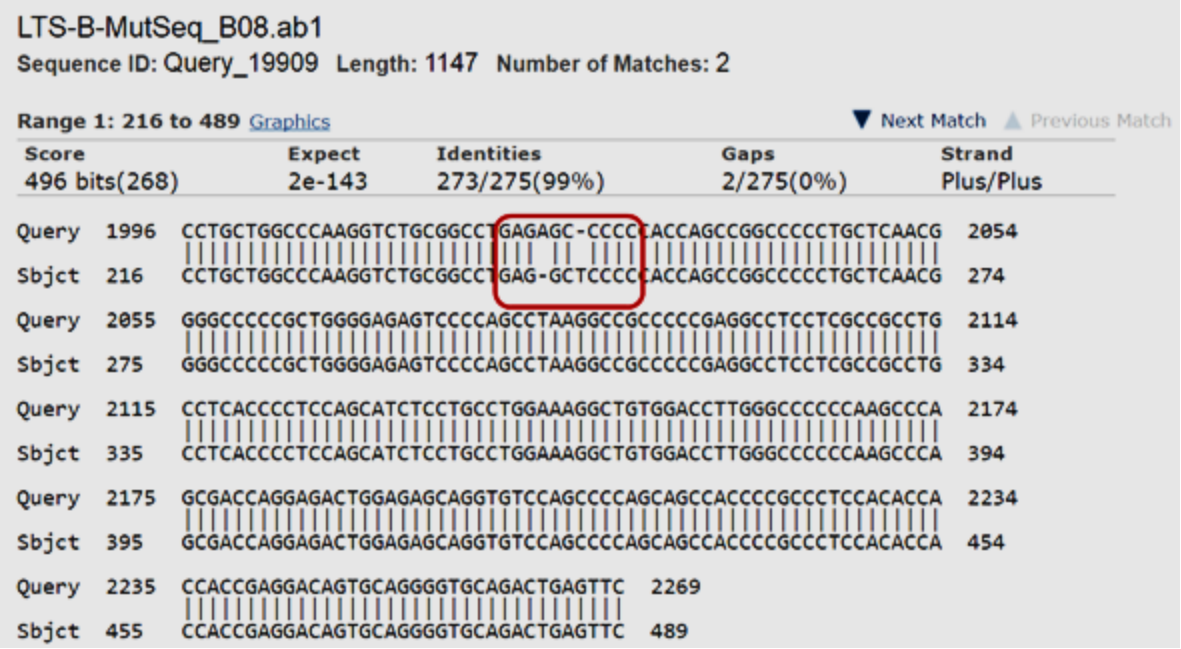
A. TCG/TCA ⟶ GCT/GCA Ser678Ala and Ser683Ala



B. TCG/TCA ⟶ GAT/GAC Ser678Asp and Ser683Asp



C. AGC ⟶ GCT Ser652Ala



D. AGC ⟶ GAA Ser652Glu

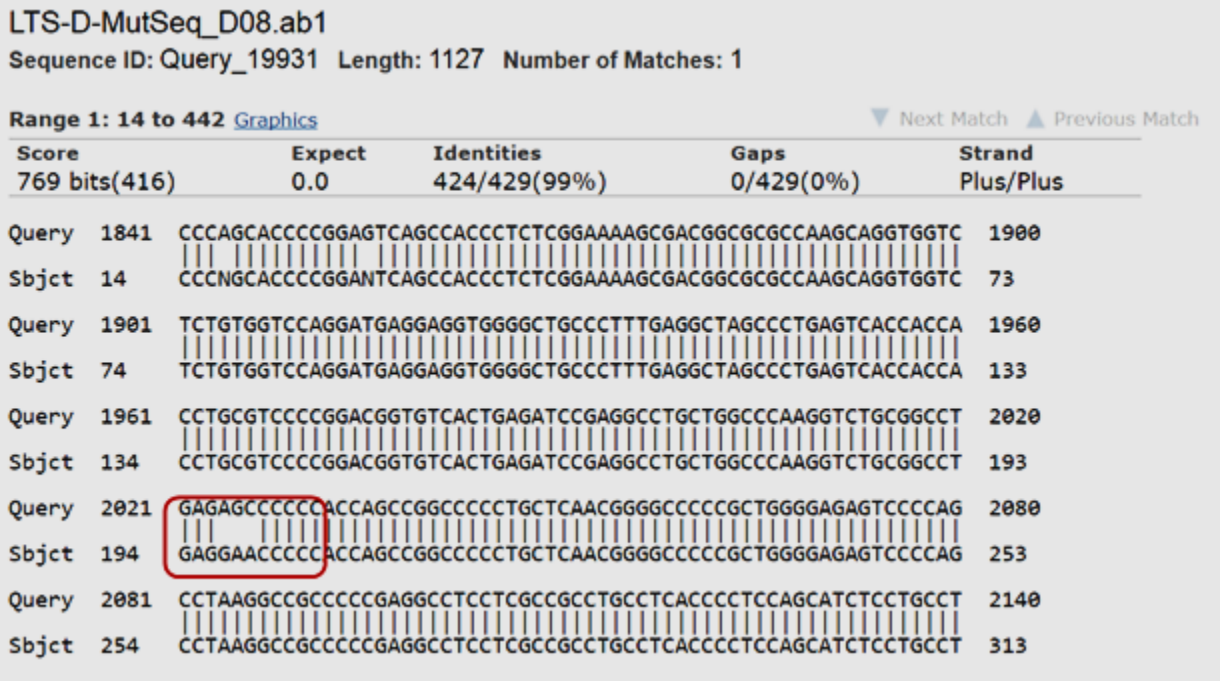


Fig. 3.2. Confirmation of mutations through Genewiz sequencing. The sequencing results were compared with the wild type FAM129B using multiple sequence alignment tool https://blast.ncbi.nlm.nih.gov

(A) Blast sequence alignment shows the serine codons (TCG/TCA) of wild type FAM129B is mutated to alanine codon (GCT/GCA). The alanine mutant act as a non-phosphorylation site and prevents FAM129B phosphorylation at position 679 and 683.

(B) Sequence alignment shows mutation at 679 and 683 where serine is codon (TCG/TCA) is converter to the phosphomimic aspartic acid (GAT/GAC). This keeps these two sites always in phosphorylated state.

(C) The serine codon (AGC) at position 652 is mutated to alanine (GCT) that preventing phosphorylation of this site.

(D) Serine codon (AGC) at 652 is mutated to glutamic acid (GAA) which mimics phosphorylation.

3.3 Immunofluorescence studies to check the effect of mutants on FAM129B localization.

3.3.1 GFP-FAM129B membrane colocalization with N-cadherin.

Previous studies have shown that FAM129B is overexpressed in cancer cells and it localizes to membrane in confluent cells (17). To study this, HeLa cell lines were chosen to see the localization of endogenous FAM129B using immunofluorescence technique. Cells were also transfected with GFP-FAM129B (wild type) and checked for localization. Localization of GFP-FAM129B was compared with the membrane protein N-cadherin. Cadherin is a transmembrane protein that plays an important role in cell adhesion, forming adherent junctions to bind cells with tissues together. There are different types of cadherin. N-cadherin is found in HeLa cells, whereas these are absent in A549 lung cancer cells. When membrane junctions are not formed, N-cadherin is not seen at the membrane of HeLa cells. However, in confluent HeLa cells, N-cadherin is seen in the membrane junctions. As can be seen in Fig 3.3, endogenous FAM129B (green) colocalizes with N-cadherin (red) at the membrane. In cells that are not in contact with each other FAM129B is not at the membrane as there is no membrane junction formed as can be seen by the absence of N-cadherin. Experiments were also done by transfecting GFP-FAM129B

and observe its localization in confluent and non-confluent HeLa cells (Fig. 3.4). GFP-FAM129B was treated with Rabbit anti-GFP polyclonal antibody (Genscript #A01388-40) that specifically shows signal for transfected FAM129B.

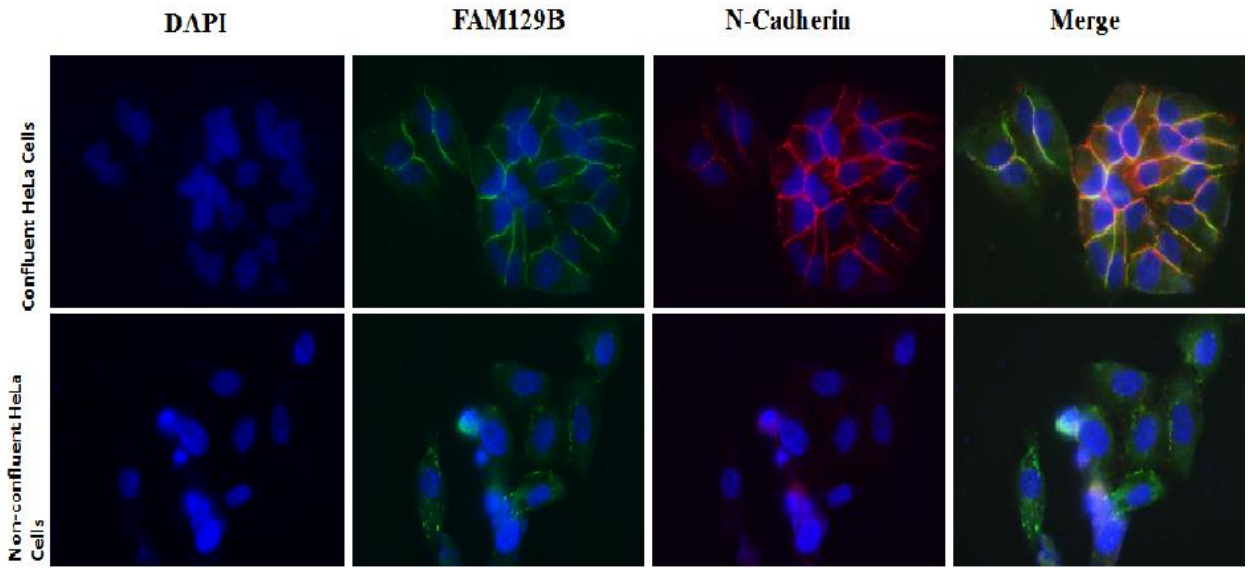


Fig 3.3 Endogenous FAM129B localization in confluent HeLa cells. Cells were plated on coverslips at different confluency. The cells were treated with primary antibody FAM129B rabbit antibody (Cell signaling #5122S) and N-cadherin mouse mAb (Cell signaling #14215S) for 2 hours. The secondary antibody for FAM129B (green) is goat anti-rabbit IgG Alexa Fluor 488 (Invitrogen #A11034) and for N-cadherin (red) is goat anti-mouse IgG Alexa Fluor 594 (Invitrogen #A11032) for 1 hour. The immunofluorescence protocol is as followed in materials and methods.

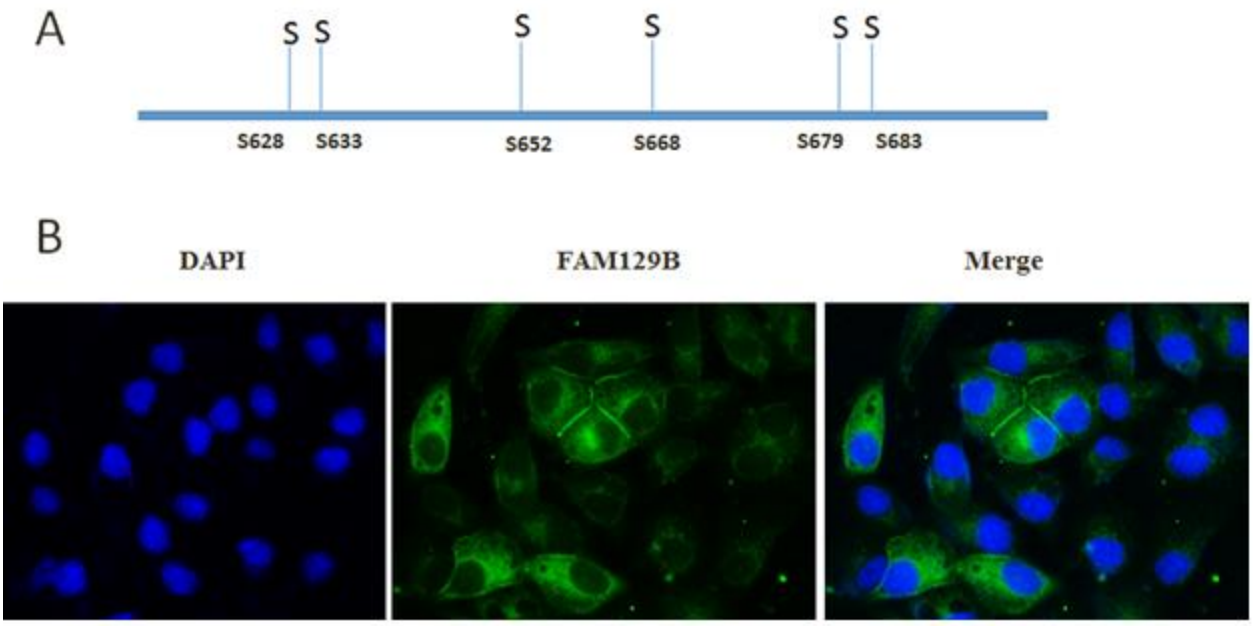


Fig 3.4 GFP-FAM129B transfected in HeLa cells showing membrane localization in confluent and non-confluent cells. Transfection was carried out with lipofectamine at a ratio of 1:2 DNA to lipofectamine. Transfected cells were treated with rabbit anti-GFP primary antibody (Genscript #A01388-40) for 2 hours and incubated in secondary antibody goat anti-rabbit IgG antibody, alexa fluor 488 (Invitrogen #A11034) for 1 hour.

(A) Diagram showing positions of six serine phosphorylation sites of wild type GFP-FAM129B.

(B) Immunofluorescence image of wild type FAM129B sowing localization to membrane in confluent HeLa cells. Whereas, cells that are not in contact, FAM129B is shown to be dispersed in cytoplasm.

3.3.2 Membrane localization studies using C-terminal deletion construct.

C-terminal deleted region that has all the six serine phosphorylation sites removed of FAM129B was made by a previous student, Dr. Song Chen, in our lab. This deletion construct with GFP tag at the amino end was transfected in HeLa cells and protein expression at the membrane was studied after 48 hours through immunofluorescence using rabbit anti-GFP primary antibody and goat anti-rabbit IgG secondary antibody. Amazingly the immunofluorescence images show that FAM129B does not localize to membrane in confluent HeLa cell. They are mostly distributed

in cytoplasm just like the non-confluent cells (Fig. 3.5). This suggests that the six serine phosphorylation sites play an important role in FAM129B localization in confluent HeLa cells.

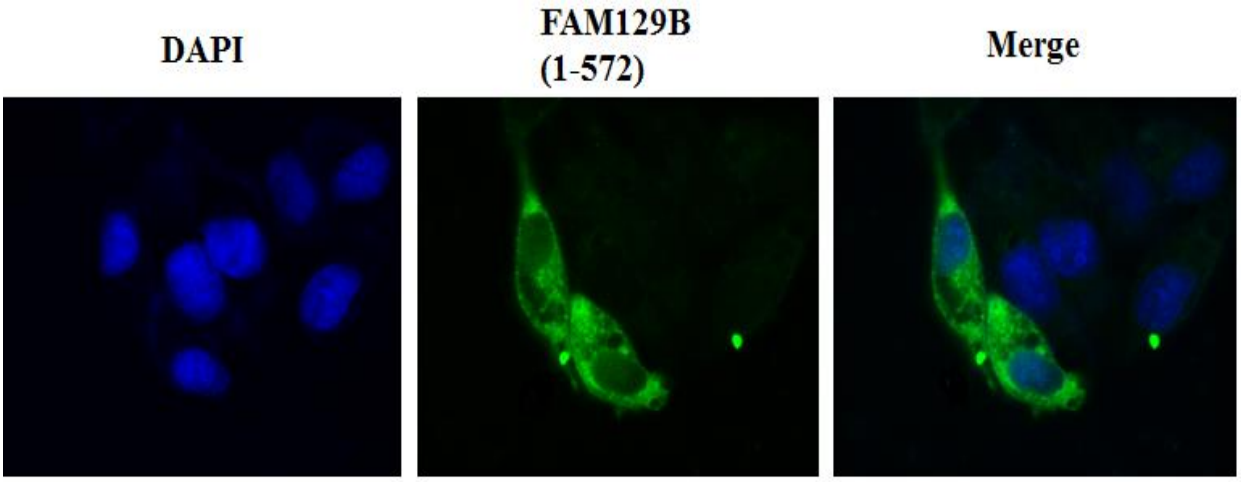


Fig 3.5 FAM129B C-terminal deletion construct lacks all phosphorylation sites and is not localized to membrane in confluent HeLa cells.

3.3.3 Membrane localization studies with FAM129B mutant, S628A and S633A and S628E and S633E.

In this experiment, two of the serine phosphorylation sites were mutated to alanine and glutamic acid and its localization was studied. Ser628 and Ser633 were mutated together to alanine to study its effect on FAM129B membrane localization. The immunofluorescence study using anti GFP antibodies shows that FAM129B Ser628Ala/Ser633Ala mutant localizes to membrane in confluent HeLa cells (Fig. 3.6). Since serine phosphorylation is mediated by EGF, these cells were treated with EGF for 30 minutes in serum free media and checked for membrane localization. It is seen that FAM129B Ser628Ala/Ser633Ala localizes to membrane in a similar fashion as without EGF or cells grown in 10%FBS DMEM. The phosphomimics Ser628Glu/Ser633Glu were studied in similar manner and its seen that the phosphomimics

localize to membrane in confluent HeLa cells (Fig 3.6). This suggests that phosphorylation and de-phosphorylation of these two sites does not play an important role in FAM129B localization.

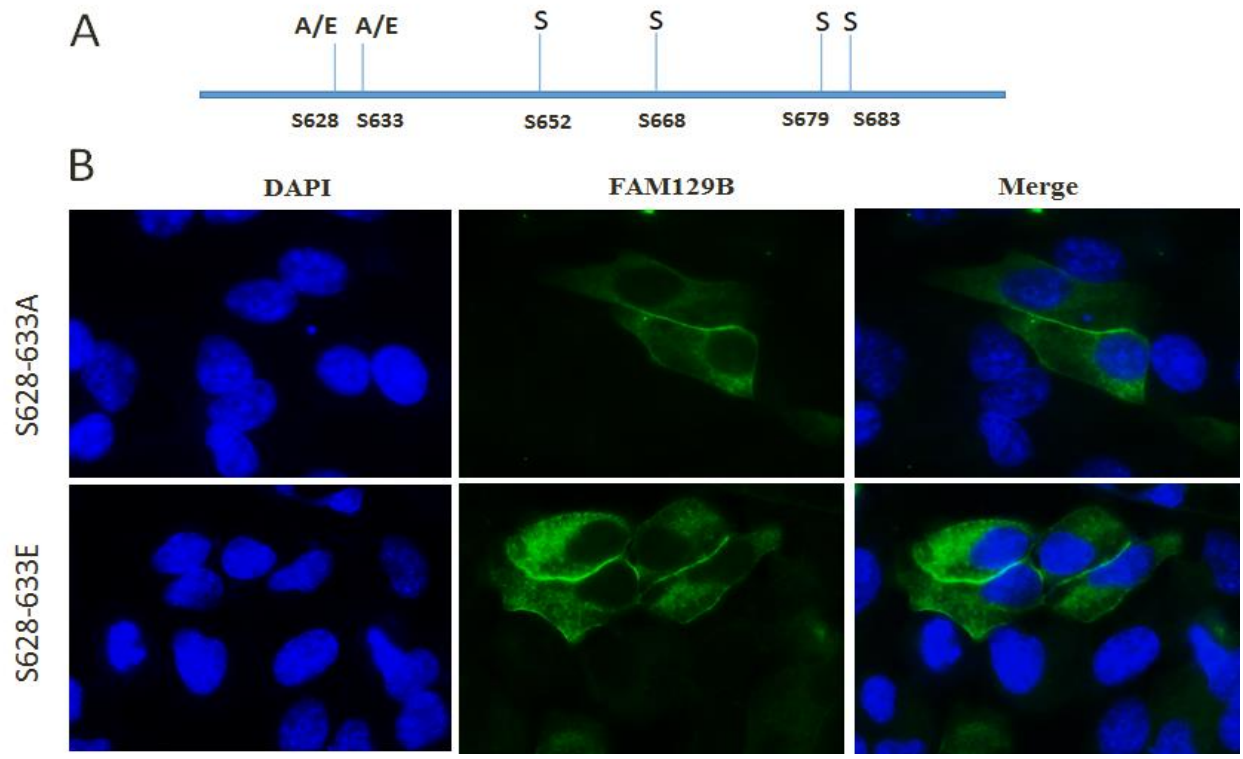


Fig 3.6. Localization of FAM129B mutant S628E and S633E.

(A) Diagram showing location of the six serine phosphorylation sites and the position of mutants. Here, the serine phosphorylation sites were mutated to alanine (S628A and S633A) and glutamic acid (S628E and S633E).

(B) Immunofluorescence image of FAM129B mutant showing localization to the membrane. The alanine mutant and the phosphomimic glutamic acid, both shows membrane localization suggesting that dephosphorylation and phosphorylation of serine at position 628 and 633 has no effect on FAM129B localization in confluent HeLa cells.

3.3.4 Membrane localization studies with FAM129B mutant, S679/683A and S679/683D.

The serine phosphorylation sites that were close to the C-terminal region of FAM129B were mutated to alanine for analyzing FAM129B membrane localization. As seen, FAM129B Ser679Ala and Ser683Ala did not localize to membrane in confluent HeLa cells. Since serine phosphorylation is mediated by EGF, FAM129B Ser679Ala/Ser683Ala transfected cells were treated with EGF for 30 minutes in serum free media and checked for membrane localization. It is seen that FAM129B Ser679Ala/Ser683Ala again did not localize to membrane in a similar fashion as without EGF (Fig 3.7B). The phosphomimics Ser679Glu/Ser683Glu were studied in similar manner and showed that the phosphomimics localize to membrane in confluent HeLa cells. This suggests that eliminating phosphorylation of S679A/S683A prevents its localization to membrane in confluent cells and the phosphorylation of these two sites play an important role in FAM129B localization (Fig. 3.7B).

Since the alanine mutant of Ser679 and Ser683 together prevented FAM129B localization to membrane in confluent HeLa cells, it was interesting to know if one had any effect on the localization. Hence, a single mutation was done in which only Ser683 was mutated to alanine and checked by immunofluorescence microscopy, whereas, all other sites were serine. This mutant was previous made in our lab by Dr. Song Chen. It was seen that a single mutation to alanine did not show the same effect as the double mutant. FAM129B would still localize to membrane in confluent cells (Fig. 3.7D). The phosphomimic Ser683Asp also localizes to membrane like Ser683Ala (Fig.3.7D). This suggests that phosphorylation and dephosphorylation of Ser683 alone does not play an important role in FAM129B localization to membrane in confluent HeLa cells.

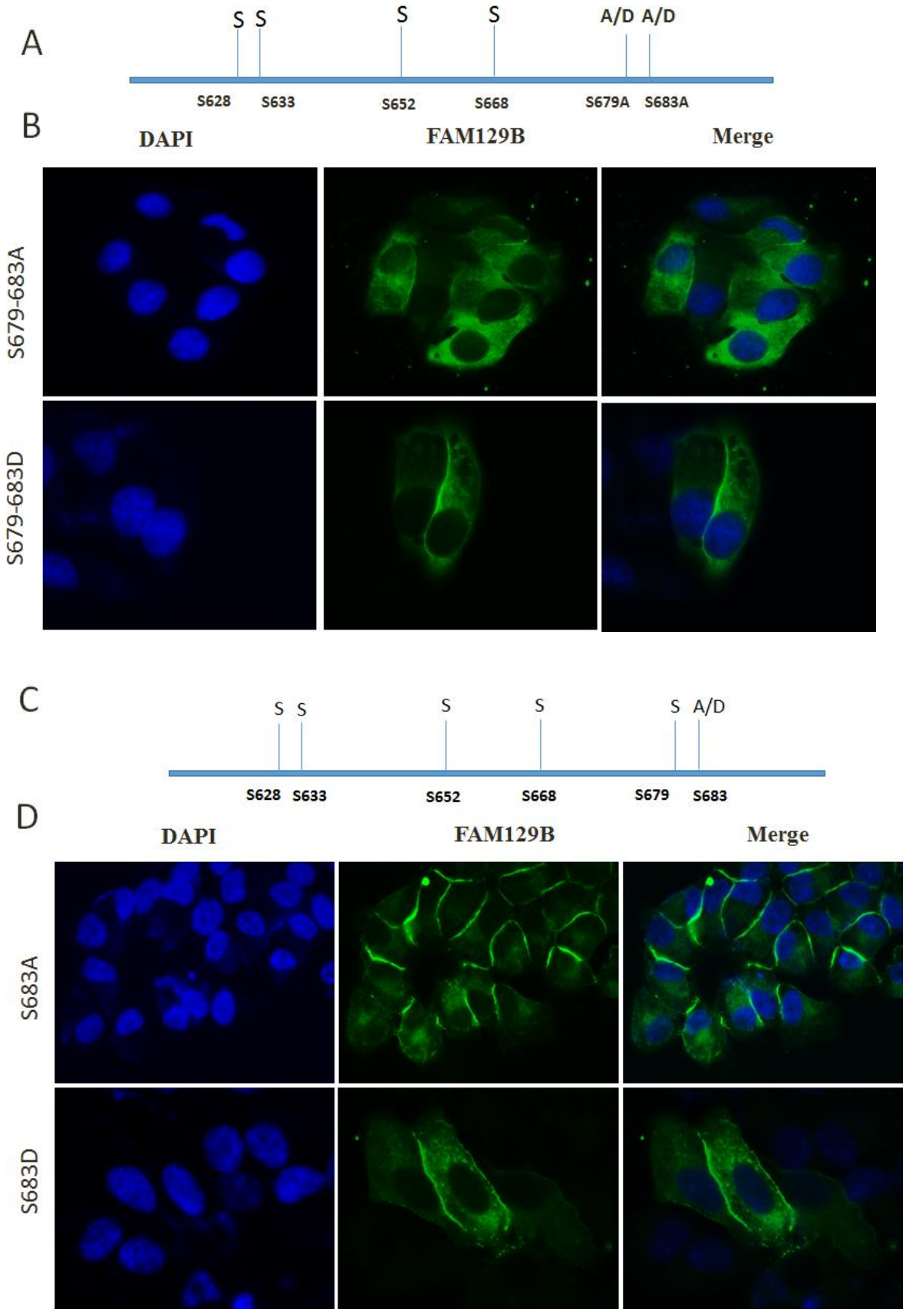

A
S S
S
S
A/D A/D
S628
S633
S652
S668
S679A
S683A
B
DAPI
FAM129B
Merge
S679-683A
S679-683D
C
S S
S
S
S A/D
S628
S633
S652
S668
S679
S683
D
DAPI
FAM129B
Merge
S683A
S683D

Fig 3.7 Effect of FAM129B phosphorylation and dephosphorylation at positions S679 and S683.

(A) Diagram showing position of serine mutant. Serine at position 679 and 683 were mutated to alanine so that it is prevented from phosphorylation at these two sites and to phosphomimic aspartic acid that keeps these two positions always phosphorylated.

(B) Immunofluorescence image shows that dephosphorylation of FAM129 at positions Ser679 and Ser683 prevents its localization to membrane. However, the phosphomimic mutant brings it to the membrane junction in confluent HeLa cells.

(C) Diagram showing position of mutant Ser683Ala and Ser683Asp.

(D) Single mutation at 683 position to alanine showed no effect on FAM129B localization. Both the alanine and phosphomimic mutant shows FAM129B localization to membrane in confluent HeLa cells.

3.3.5 Membrane localization studies with FAM129B mutant, S652A and S652E.

Ser652 and Ser668 are also serine phosphorylation sites of FAM129B. They are located very far apart from each other in contrast to the two pairs. Also, these sites are not strongly phosphorylated by MAP kinase when compared to the other four sites (16). The phosphorylation sites Ser652 was chosen to see if it plays any role in membrane localization of FAM129B. Ser652 was mutated to alanine, keeping all other phosphorylation sites intact. The alanine mutant prevents any phosphorylation at this position due to the absence of serine. The mutant is analyzed for membrane localization by immunofluorescence microscopy using antibody directed against GFP. As seen in the fig.3.8, FAM129B localizes to membrane in confluent HeLa cells. This shows that Ser652 does not play any role in FAM129B membrane localization. The phosphomimic mutant Ser652Glu showed similar results suggesting that phosphorylation and de-phosphorylation of FAM129B at Ser652 position does not affect its membrane localization (Fig. 3.8).

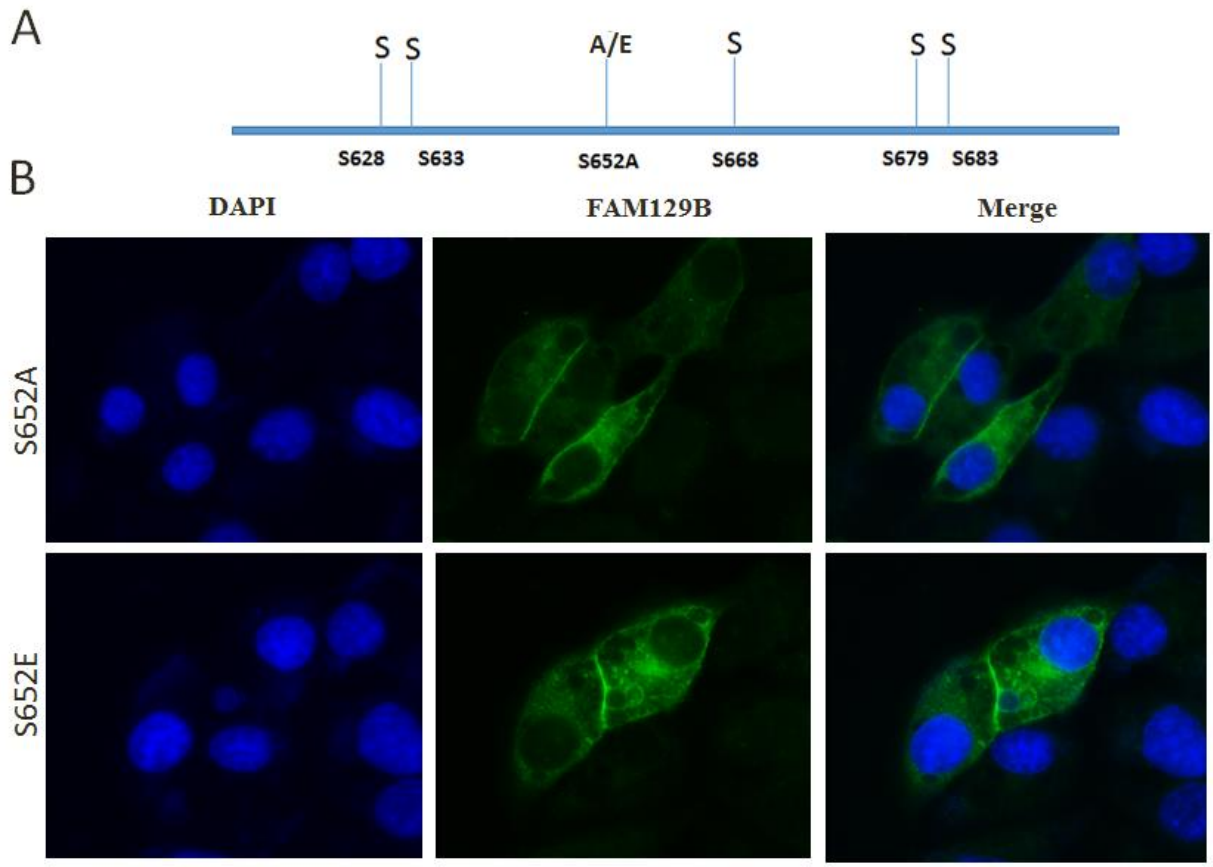


Fig 3.8. FAM129B localization studies on the mutant Ser652A/E.

(A) Diagram showing position of mutants Ser652Ala and Ser652Glu.

(B) Phosphorylation and dephosphorylation of FAM129B at position Ser652 has no effect on FAM129B localization to membrane. The alanine mutant S652A and the phosphomimic S652E both localizes to membrane in confluent HeLa cells.

CHAPTER 4 DISCUSSION

The protein encoded by FAM129B gene, has not been widely studied. Also, the protein structure is unknown. Its purpose and function is only basically understood. However, it is known that FAM129B interacts with protein receptor EGFR and promotes a cascade of events that leads to transformation of normal cell to cancer cell (20). This makes it important to study FAM129B at the molecular level and the effect of the mutation of key residues. Future studies on FAM129B might help us understand and know if it can be used as a potential drug targeting agent to treat cancer cells. In this project, FAM129B was studied at the gene level by phosphorylation and mutations on the six serine residues and its effect on cells were observed by the localization of protein coded by this gene. It is well known that FAM129B is expressed highly in many forms of cancer and in confluent cells, it colocalizes at the membrane with β-catenin membrane junction protein (17). However, study done by Old et.al and group has seen a change in membrane localization behavior of FAM129B when all six serine phosphorylation sites were mutated. Hence, in this study the phosphorylation sites were tested pairwise and individually to point out the specific serine residue that changes the behavior of FAM129B pertaining to membrane localization.

Previous studies on FAM129B and repeated experiments have shown that FAM129B localizes to membrane in confluent HeLa cells. Immunofluorescence studies on endogenous FAM129B using FAM129B antibodies have frequently shown that FAM129B co-localizes with N-cadherin in confluent HeLa cells (Fig. 3.3). Also, these localizations of FAM129B has been shown to be affected by the six serine phosphorylation sites. Hence, when C-terminal deleted FAM129B, with all the six serine phosphorylation sites removed, is transfected in HeLa cells, the

immunofluorescence image shows more dispersed FAM129B in cytoplasm rather than the localized endogenous FAM129B in confluent HeLa cells (Fig. 3.4). This suggests that the six serine phosphorylation sites play an important role in FAM129B localization in confluent HeLa cells.

GFP-FAM129B wild type localization was studied in HeLa cells. They were transfected and the membrane localization was studied by immunofluorescence imagining after 48 hours of transfection (Fig. 3.5). It is seen that they localize to membrane in confluent HeLa cells. However, in exponential cells when cells are not in contact with each other, FAM129B is mostly dispersed in cytoplasm. Unless the cells are in physical contact the adherens junctions are not formed. This is seen by analyzing the important membrane junction protein N-cadherin that can be seen in the cell-cell adhesion junction. However, since junction are not formed in exponential cells, N-cadherin is absent and cannot be seen by immunofluorescence imaging. This behavior is similar to FAM129B localization. This suggest that FAM129B localizes to membrane only in confluent cells when the adherens junctions are formed. Since cell-cell adherens junctions are not formed in exponential cells, FAM129B is mostly dispersed in cytoplasm (Fig. 3.4).

Four of the six MAPK phosphorylation sites are located in the sequence in pairs in which the targeted serine residues are separated by only 4 or 5 amino acid residues; in one pair Ser628 and Ser633 and in the other Ser679 and Ser683. Both serine residues in each pair were mutated simultaneously. The other two sites, 652 and 665 were spaced further apart and located between the two pairs. S652 was studied to see its effect in localization. The pair S628A and S633A, that cannot be phosphorylated were still localized to membrane similar to the wild type FAM129B. The phosphomimic of these two sites S628E and S633E also showed membrane localization like the wild type FAM129B in confluent HeLa cells. Thus, phosphorylation and de-

phosphorylation of sites S628 and S633 does not play any role in FAM129B membrane localization (Fig. 3.6 B).

The pair S679/S683 were mutated together to alanine (S679A/S683A) and examined by immunofluorescence microscopy. Surprisingly, when these two sites were mutated to alanine and thus could not be phosphorylated, FAM129B does not localize to the membrane in either confluent or exponentially growing cells. They are mostly seen dispersed in cytoplasm. This suggests that blocking phosphorylation of these two sites prevented localization of FAM129B to membrane in confluent HeLa cells. The phosphomimic of these two sites however, showed membrane localization when cells are in contact with each other. This specifically suggest that dephosphorylation of S679 and S683 prevents FAM129B localization and the phosphorylation at S679 and S683 takes its to the membrane in confluent cells (Fig. 3.7 B). Further analysis was done for these two sites by studying S683 alone. Hence S683A and S683D mutants were transfected in HeLa cells and after immunofluorescence study it was seen that both the mutants behave similar to the wild type by localizing to membrane in confluent HeLa cells (Fig 3.7 D). This suggest that either the pair needs to be phosphorylated or just S679 needs to be phosphorylated to prevent FAM129B localization to membrane. These two sites thus provide us with an important information on the invasiveness of cancer cells since FAM129B localization has been shown to affect cancer cell invasion and thus metastasis.

The other two sites S652 and S668 are weakly phosphorylated by MAP kinase/ERK pathway. This is shown by studies done by Old et.al when they inhibited MEK kinase, the kinase that activates MAP kinase and found that the phosphorylation of all six serine residue was diminished. However, the activity of four sites S628, S633, S679 and S683 were suppressed twice

as compared to S652 and S668 phosphorylation sites which were very less dephosphorylated or their phosphorylation is unaffected. The site S652 was mutated to alanine and studied for FAM129B localization. It showed localization like wild type FAM129B and hence do not play any role in FAM129B membrane localization. The phosphomimic S652E also localized to membrane in confluent HeLa cells suggesting that phosphorylation and de-phosphorylation of Ser652 does not play any important role in FAM129B colocalization with N-cadherin (Fig. 3.8 B).

FAM129B	No Cell Contact	Cell Contact
Wild Type	Cytoplasm	Membrane
Deletion Mutant	**Cytoplasm**	**Cytoplasm**
S628A and S633A	Cytoplasm	Membrane
S628E and S633E	Cytoplasm	Membrane
S679A and S683A	**Cytoplasm**	**Cytoplasm**
S679D and S683D	Cytoplasm	Membrane
S683A	Cytoplasm	Membrane
S683D	Cytoplasm	Membrane
S652A	Cytoplasm	Membrane
S652E	Cytoplasm	Membrane

Table 4. Summary of FAM129B Mutant Intracellular Location. Table shows the mutants and their corresponding effect on FAM129B localization in confluent (cell contact) and non-confluent (no cell contact) HeLa cells. As seen, the deletion construct that has all the six serine phosphorylation sites deleted and the mutant pair S679A/S683A, prevents FAM129B localization to membrane in confluent HeLa cells.

Metastasis and invasion is the leading cause of high mortality rate of patients with cancer. The main reason for these characteristics of cancer cell is the disruption of adherens junction which consists of protein complexes that keep the cell to cell contact intact in epithelial and endothelial tissues. During metastasis, the adherens junctions are disrupted leading to detachment of cancer cell and its spreading to another part of the body. FAM129B is known to

be associated with cancer cell invasion in melanoma cells (16). Invasion studies done by Old et.al. shows that knockdown of endogenous FAM129B in melanoma cells blocked its invasion into a collagen matrix. Also, invasion was prevented when all the six phosphorylation sites were not phosphorylated. This signifies that FAM129B plays an important role in melanoma cell invasion and phosphorylation of the six serine residue play a key role in metastasis and invasion of cancer cells. Further research on the six serine phosphorylation residues will shed some light on how the phosphorylation affects the metastasis and invasion, thus making FAM129B as a potential drug target for cancer therapy.

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ABSTRACT

FAM129B Phosphorylation and its Effect on Membrane Localization in Confluent HeLa Cells

by

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May 2017

Advisor: Dr. David Evans

Major: Biochemistry and Molecular Biology

Degree: Master of Science

Phosphorylation and de-phosphorylation of many proteins is a key regulator of cellular life. It maintains cellular activity through an array of signaling pathways like cell division, proliferation and growth. However, Overexpression or mutations by the constitutive activation of phosphorylation machinery disrupt the balance in the cell, driving the inappropriate activation or deactivation of the cellular processes it controls. FAM129B is a protein whose activity is partly maintained by phosphorylation and dephosphorylation at the six serine residues at the C-terminal. FAM129B is expressed highly in many forms of cancer and its main function is to suppress apoptosis and enhances cancer cell invasion. In this project, FAM129B phosphorylation studies are done to see how the mutation at the serine residues affects its localization to membrane in confluent HeLa cells. We demonstrated that endogenous FAM129B colocalizes with N-cadherin at the adherens junction in confluent HeLa cells. However, deletion of the six serine residues phosphorylated by MEK, prevented its localization to membrane in confluent cells. It is seen that out of the six serine residues, the two residues Ser679 and Ser683 plays an

important role in FAM129B localization. The alanine mutant of Ser679Ala and Ser683Ala prevented FAM129B localization to membrane in confluent HeLa cells and the activity is restored by the phosphomimic glutamic acid mutant Ser679Asp and Ser683Asp shows membrane localization at the cell-cell adherens junction. Thus, blocking phosphorylation of Ser679 and Ser683 prevented FAM129B localization to membrane and its phosphorylation takes the FAM129B to the membrane in confluent cells. It is also studied that the phosphorylation and dephosphorylation of the other serine phosphorylation sites, Ser628, Ser633 and Ser652 has no effect on FAM129B membrane localization in confluent HeLa cell.