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Intracellular Localization of the Novel PolyQ Protein FAM171B

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By

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This thesis have been examined and approved by the following members of the student's committee.

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Table of contents

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Abstract

FAM171B is a relatively uncharacterized protein that contains fourteen consecutive glutamine residues within its primary amino acid sequence; thus it is a polyglutamine (polyQ) protein. PolyQ proteins are interesting because mutations within the polyQ tract have been linked to a number of severe neurodegenerative diseases including Huntingtons Disease (HD). To date, there are minimal published data regarding FAM171B and its molecular function, such that both expression levels and intracellular localization have yet to be definitively determined. Using immunohistochemical and *in situ* hybridization analyses, our lab has recently found that FAM171B is expressed throughout the mouse brain. This current study attempts to build off this information to identify the intracellular localization of FAM171B in several different nervous system cell types including: a human glioblastoma cell line (U138), a human neuroblastoma cell line (SH-SY5Y), and mouse brain primary neurons. Using immunofluorescence, FAM171B-GFP fusion proteins, and live cell imaging (coupled with confocal microscopy) our data indicate that FAM171B has a predominantly vesicular/punctate staining within the cytoplasm of cells. Furthermore, colocalization studies using organelle specific markers shows that FAM171B localizes to both endosomes and cellular regions near the plasma membrane. Taken together, our data suggests that FAM171B may play a vesicle trafficking role within cells of the nervous system.

List of figures

Chapter 1

Introduction

Homopeptide repeats are consecutive stretches of the same amino acid within a protein's primary sequence. They are a common protein motif, and it has been suggested that at least certain homopeptide amino acid repeats may serve as protein-protein interaction domains (1). Polyglutamine (polyQ) tracts, a specific type of homopeptide repeat consisting of multiple consecutive glutamine (Q) amino acid residues are present in more than 60 human proteins (2). Indeed, some lower organisms (such as the amoeba *Dictyostellium discoideum*) possess several hundred (3). PolyQ tracts in proteins are often polymorphic in repeat copy number. However the function of polyQ tracts within proteins, and the putative consequence of this normal Q repeat copy number variation has yet to be definitively determined (2,3).

Interestingly, expansion mutation of polyQ (CAG repeats) tracts well beyond their normal polymorphic range in certain proteins leads to severe inherited neurodegenerative diseases, known collectively as polyglutamine diseases. In 1991, Fischbeck and coworkers first reported the disease associated expansion of CAG repeat in exon 1 of the androgen receptor gene in patients of spinal and bulbar muscular atrophy (SBMA) (4). Since then, similar genetic mutations of the CAG repeat expansion in the coding regions of genes other than androgen receptor gene have been found in other inherited neurodegenerative disorders. To date, a total of nine polyQ disorders have been described: six spinocerebellar ataxias (SCA) types 1, 2, 3, 6, 7, 17; huntington's disease

(HD); dentatorubral pallidoluysian atrophy (DRPLA); and spinal and bulbar muscular atrophy. In each disease, the specific translated polyQ protein leads to dysfunction and degeneration of select neuronal subpopulations (Table1)

Table 1. Summary of the nine inherited Polyglutamine disorders

Disease	**Disease Protein**	**Normal Subcellular Localization**	**Affected Brain Regions**	**CAG Repeat Length**	
				Normal	**Pathological**
Huntington's disease (HD)	Huntingtin (htt)	Cytoplasm	Striatum and cortex	6-35	36-121
Spinocerebellar ataxia 1 (SCA1)	Ataxin-1	Nuclear and cytoplasmic	Cerebellum	6-39	41-83
Spinocerebellar ataxia 2 (SCA2)	Ataxin-2	Cytoplasmic	Cerebellar Purkinje cells	14-32	34-77
Spinocerebellar ataxia 3 (SCA3)	Ataxin-3	Nuclear and cytoplasmic	Ventral pons and substantia nigra	12-40	62-86
Dentatorubral-pallidoluysian atrophy (DRPLA)	Atrophin-1	Nuclear and cytoplasmic	Cerebral cortex	3-38	49-88
Spinocerebellar ataxia 6 (SCA6)	Ataxin-6	Membrane associated	Cerebellar Purkinje cells	4-18	21-30
Spinocerebellar ataxia 7 (SCA7)	Ataxin-7	Nuclear and cytoplasmic	Cerebellar Purkinje cells, brain stem, spinal cord	7-18	38-200
Spinal and bulbar muscular atrophy (SBMA)	Androgen receptor (AR)	Nuclear and cytoplasmic	Motor neurons	6-36	38-62
Spinocerebellar ataxia 17 (SCA17)	TBP	Nuclear	Cerebellar Purkinje cells	25-43	45-63

PolyQ diseases are relatively rare, averaging 1–10 cases per 100,000 people (5). Of polyQ disorders, HD and SCA3 have the highest prevalence worldwide (6).

While the genes associated with the different polyQ diseases are structurally and functionally distinct, there are commonalities across all of these diseases. With the exception of SBMA, which is X-linked (and displays sex-limited inheritance), all other polyQ diseases are inherited in autosomal dominant pattern (7). All are neurodegenerative disorders, with disease onset typical in midlife and slowly progressive phenotypes. Certain pathologic molecular processes have been implicated in almost all of the polyQ diseases, including protein aggregation, proteolytic cleavage, transcription dysregulation, autophagy impairment, and mitochondrial dysfunction (7).

Curiously, polyQ containing proteins are ubiquitously expressed throughout the body, while the pathology is primarily restricted to neuronal tissue, displaying cell-type specificity or selective vulnerability (8). The mechanisms that are responsible for this specificity are largely unknown (7). However, the amino acid context of the polyQ proteins and their interacting protein partners may help determine the selective neuronal cell loss seen in distinct brain regions affected in polyQ diseases (9).

Importantly, the length of the polyQ repeat is critical to pathogenesis and for each disease (Table 1). There seems to be a critical threshold of polyQ expansion that must be exceeded in order to cause disease (10,11). For example, Huntington has a polyQ tract at its amino-terminus with a normal repeat length ranging from 5-35 repeats. In contrast, the polyQ tract in HD patients is abnormally expanded to greater than 40 consecutive glutamine residues and residues longer than 60 elicit juvenile forms of HD (Table1)

(11,12). Interestingly, the longer the polyQ repeat tract length- the more severe HD manifests (13). Also, expanded polyQ regions are inherently unstable and tend to expand further, resulting in an earlier age of onset and a more severe disease phenotype in successive familial generations (13). Indeed, this "genetic anticipation" is a prominent feature of all polyQ diseases (13).

The best studied pathology, HD, usually involves the loss of neurons in the striatum and layers III and V of the cortex, which affects muscle coordination and leads to cognitive impairment and psychiatric problems (5). The *IT15* gene that encodes the Huntingtin (Htt) protein is located on the short arm of chromosome four. Huntingtin is widely expressed in neuronal and non-neuronal tissues, but its level in the striatum is higher than in other organs (14). The functions of normal htt are not yet completely known, but it is suggested that non-mutated htt is involved in cellular trafficking and may function as a scaffold protein (15).

Spinocerebellar ataxia types 1–3, 6, and 7 are the most frequent among autosomal dominantly inherited cerebellar ataxia (ADCA) syndromes and account for 50-60% of all families affected by ADCA worldwide (16). Major symptoms of ataxia, such as imbalance and incoordination, are indicative of impairment of cerebellar functions, which results mainly from a massive loss of Purkinje cells. Ataxias are often accompanied by dysarthria, rigidity, and dystonia, as well as by oculomotor disorders (17).

Dentatorubral-pallidoluysian atrophy (DPRLA) is caused by a mutation in the atrophin-1 gene that results in an abnormally expanded polyQ tract in this protein (Table

1). DRPLA patients show a wide variety of progressive neurological deficits like ataxia, myoclonus, epilepsy, and dementia (5).

Spinal and bulbar muscular atrophy (SBMA), or Kennedy's disease, is characterized by slowly progressing muscle weakness and atrophy of the bulbar, facial, and limb muscles (18). Pathologically, SBMA is associated with loss of motor neurons in the spinal cord and brainstem. SBMA is caused by an abnormal expansion of CAG repeats in exon 1 of the androgen receptor (AR) gene on chromosome Xq11-12 (19).

Determination of the structure of polyQ regions has been difficult, as they appear to be unstable (1). However, abnormally expanded polyQ stretches likely undergo a conformational transition to a ß-sheet rich structure, which causes these proteins to assemble into amyloid fibril insoluble aggregates (12). Indeed, neuronal inclusion bodies composed of aggregated expanded polyQ proteins have been linked to cell death and neurodegeneration leading to behavioral and physical impairments (12). Several studies have linked these polyQ aggregates to cellular toxicity. For example, overexpression of chaperone proteins slow aggregation and decreased toxicity in both cellular and animal models (20). Both loss-of-normal-function and toxic-gain of function mechanisms have been posited to explain toxicity, but the case for latter is stronger (20). Unfortunately, to date, there is neither a cure nor prevention for these devastating diseases (9,13)

Our lab is interested in characterizing FAM171B, a novel polyQ protein that has yet to be linked to a neurodegenerative disease. Bioinformatic analysis suggests that, similar to huntingtin, FAM171B has a stretch of consecutive glutamine amino acids near its amino terminus (unpublished data). However, there is minimal information published

regarding the molecular function of this novel protein. For example, it is unknown whether the polyQ tract is polymorphic in humans. Similarly, FAM171B expression levels and subcellular localization have yet to be definitively determined.

Recent studies in our lab using *in situ* hybridization and immunohistochemistry strongly suggest that FAM171B is expressed throughout the brain- elevating its status as a candidate gene for an as yet molecularly uncharacterized neurodegenerative disease (unpublished data). Furthermore, examination of regions within the brain, indicate that FAM171B is expressed more prominently in certain brain areas compared to others. For example, the hippocampus shows very high FAM171B expression at both the mRNA and protein level. The hippocampus is a complex brain structure embedded deep into temporal lobe and it has a major role in learning and memory. The hippocampus is also important in spatial navigation, emotional behavior and regulation of hypothalamic functions (21). Many polyQ diseases such as HD, SBMA, DRPLA result in dementia and impaired memory (22) which could be due to failure in hippocampal activity. High levels of FAM171B are also found in the cerebellum. The cerebellum plays an important role in the motor system coordination. Severe abnormalities of movement are produced by pathologies of the cerebellum (23). Other non-motor functions of cerebellum includes attention, executive control, language, working memory, learning, pain, emotion, and addiction (24). In many polyQ diseases atrophic changes in the cerebellum is a prominent feature (13).

Further bioinformatic analysis suggests that FAM171B has a number of other interesting protein domains including an AT-Hook DNA binding domain, glycosylation

sites, and transmembrane regions (unpublished data). However, whether any of these putative domains actually function as predicted remains to be determined. Thus, these *in silico* analyses provide hints into molecular structure and function that need to be empirically verified via benchtop experiments.

An AT- hook is a small DNA binding motif and it was first described in high mobility group non-histone chromosome protein HMG-I (Y) (25). This motif is typically a short repeat of glycine and arginine flanked by a proline at one or both ends. The central Arg-Gly-Arg of the AT-hook inserts into the minor groove of AT-rich DNA, where there are interactions between arginine and thymidine (26). Many of the proteins with AT-hook have been shown to have an effect on the architecture of chromatin at levels beyond the action of the basic histones and also play a role in transcription regulation (25).

Transmembrane domains are regions of a protein that are generally hydrophobic, so they prefer to be inserted into cell membranes such that parts of the protein on either side of the domain are on opposite sides of the membrane (27). Integral membrane proteins have transmembrane domains that insert directly into lipid bilayers such as the plasma membrane (28).

Glycosylation is one of the most common posttranslational modifications of proteins and it plays a variety of important roles in many cellular events ranging from structural to signaling and recognition (29). Glycosylation types are classified according to the identity of the atom of the amino acid, which binds the carbohydrate chain, that is N-linked, C-linked, O-linked or S-linked (30). FAM171B has four N-linked

glycosylation sites according to bioinformatics analysis and this refers to the attachment of oligosaccharides to a nitrogen atom, usually the N4 of asparagine residues. N-glycosylation often occurs on secreted or membrane bound proteins, mainly in eukaryotes and archaea (30).

Based on the fact that FAM171B has a putative AT-Hook DNA binding domain, one may hypothesize that FAM171B may function in the nucleus and perhaps bind DNA. Alternatively, transmembrane domains suggest that FAM171B may instead localize to cellular membranes such as ER, golgi or the plasma membrane. However, whether any of these domains found in FAM171B actually function as predicted remains to be empirically determined. Indeed, this study's primary aim is to determine FAM171B's intracellular localization and shed light on whether any of these domains are in fact biologically active.

Objective

The overall objective of this project is to identify the intracellular localization of FAM171B in several different nervous system cell types- a human glioblastoma cell line (U138), a human neuroblastoma cell line (SH-SY5Y) and primary neurons harvested from mouse brain.

Prediction

Based on the aforementioned bioinformatics data, FAM171B may function either in the nucleus or as a membrane bound protein. Thus, we predicted that FAM171B would localize to the nucleus or the plasma membrane (or both) of the cell.

To test this, we utilized both FAM171B-FLAG and FAM171B-GFP expression vectors (previously constructed in the lab) to express FAM171B fusion protein in several neuronal cell types. Transfected cells were fixed and analyzed using fluorescent confocal microscopy to determine the subcellular location of FAM171B fusion proteins. We also performed immunofluorescence on fixed U138 cells, SH-SY5Y cells and primary neurons to examine endogenously expressed FAM171B using commercially available antibodies. In addition, we performed co- immunofluorescence studies using an antibody against Neu N (a marker for neurons) to ensure that at least some of the cells harvested from mouse brains were indeed primary neurons. We also used organelle specific markers to the nucleus, mitochondria, and endosomes to further establish subcellular

localization. Finally, we conducted live-cell imaging on SH-SY5Y cells transfected with FAM171B-GFP to help definitively determine its intracellular localization.

Chapter 2

Materials and Methods

2.1 Culturing U138 cells

U138 cells were grown in Eagle's Minimum Essential Medium (EMEM) (supplemented with 10% fetal bovine serum). When cells were confluent (after 6-7 days) the cells were split into a new 25 ml flask. First old media was removed from the cell plate and 2 ml of phosphate buffered saline (1x PBS) was added to rinse and remove residual media. Next, 2 ml of PBS+EDTA was added and incubated for 2 minutes to detach cells from the plate. After 2-5 minutes in the incubator the cells detach from the surface and then 2-3 drops of cells were added to a new 25 ml flask containing 6 ml of EMEM media. This process was done weekly.

2.2 Culturing primary neurons

Mouse brains were purchased from Brain Bits LLC, and cultured according to their protocol as follows.

Preparing cell dissociation solution

3 mL of hibernate solution (HE-Ca) was directly added into the 6 mg vial of papain (2 mg/mL). The vial was recapped, gently mixed and then placed in a 30 °C water

bath for 10 min to dissolve. After 10 min, the vial was removed from the water bath and placed on ice to cool for 10 min.

Cell Dispersal

Using the Pasteur pipette the brain tissue was removed with minimal hibernate (HEB) media and placed in the cell dissociation solution. The cell dissociation solution was sealed and then incubated in the 30 °C water bath for 10 minutes, gently swirling for every 5 minutes. Using the Pasteur pipet the tissue was removed with minimal cell dissociation solution and placed the tissue back into the vial containing HEB media. With the Pasteur pipette the tissue was drawn with the HEB media into the pipette and was dispensed the content into the vial immediately. This was repeated for ~1 min for 90% tissue dispersal. After this, the vial was not disturbed until undispersed pieces settle for 1 min. Next the supernatant containing dispersed cells was transferred to a sterile 15 mL tube. This was spin at 1100 rpm (200xG) for 1 min. The supernatant was discarded leaving ~ 50 μl of HEB media containing the pellet. The pellet of cells was dispersed by flicking the bottom of the tube with a finger and re-suspended in 1 ml of NbActiv1 culture medium. After re-suspending, 32 μl of the cell dispersed solution was added on to each well containing Poly-D-Lysine coated coverslips. Coverslips for primary neurons were coated with Poly-D-Lysine solution to enhance the cell attachment. The amount cell dispersed solution was increased based on the need of the cell density reducing the number of cover slips used in successive experiments.

2.3 Culturing SH-SY5Y cells

SH-SY5Y cells were growing in a media that was prepared using EMEM and F-12 (1X) nutrient mixture (50:50). When the cells are confluent, cells were split into a new T-25 Flask. First, culture media was transferred to a centrifuge tube. The flask was then rinsed with 2 ml of PBS. Next, 2 ml PBS+EDTA was added and flask was incubated at 37 °C for 3 minutes. Contents of the flask were then transferred to the respective centrifuge tube. The tube was then centrifuged for 3 minutes at 1000xg. Next the supernatant was discarded and the cell pellet was resuspended in 4 ml of fresh media. Then 1 ml of resuspended cells was added to a new flask containing 5 ml of fresh media.

2.4 Coverslip Preparation for Primary Neurons

Preparing coverslips with Poly-D-Lysine solution

Coverslips were added to each well in 6 well plates (10 plates) and sterilized overnight under the UV light. The following day 150 μL of Poly-D-Lysine solution (Brain Bits) was added onto the middle of each coverslip and incubated for 2-3 hours. After incubation, excess Poly-D-Lysine was removed and the coverslips were rinsed with tissue culture grade water (~1 ml per well). Next, the water was removed and coverslips were allowed to air-dry. Prepared coverslips were stored at 4° C.

2.5 Transfection with FAM171B-GFP

U138 cells were growing on coverslips in a 6 well plate (~5-6 drops of cells were added to each well). The following day, 5 μg of plasmid FAM171B-GFP (678 ng/μl) was added into 250 μL of opti-MEM media (for 6 wells = 30 μg /1500μL). Next, 10 μL of Lipofecatamine 2000, a transfection reagent that is used to increase the transfection efficiency of plasmid DNA, was diluted in 250 μL of opti-MEM (for 6 wells = 60 μL/1500μL). The two dilutions were let to sit for 5 minutes and then combined and incubated for another 20 minutes. After 20 minutes 500 μL of the transfection material was dripped down gently into each well containing U138 cells. This was incubated for 4-5 hours at 37 °C and media was changed into fresh EMEM. Next the transfected cells were fixed in either Methanol or Paraformaldehyde (4%).

2.6 Transfection with FLAG-FAM171B

U138 cells were growing on coverslips in a 6 well plate (~5-6 drops of cells were added to each well). The following day, 5 μg of plasmid FLAG-FAM171B (341 μg/ml) was added into 250 μL of opti-MEM media (for 6 wells = 30 μg /1500μL). Next, 10 μL of Lipofecatamine 2000, a transfection reagent that is used to increase the transfection efficiency of plasmid DNA, was diluted in 250 μL of opti-MEM (for 6 wells = 60 μL/1500μL). The two dilutions were let to sit for 5 minutes and then combined and incubated for another 20 minutes. After 20 minutes 500 μL of the transfection material

was dripped down gently into each well containing U138 cells. This was incubated for 4-5 hours and media was changed into fresh EMEM. Next, cells were fixed in methanol and immunofluorescence protocol was followed as mentioned below with primary antibody: Mouse anti FLAG (1:100) and secondary antibody Goat anti Mouse- Alexa: 568 (Novus) (1:600).

2.7 Cell Fixation

Methanol Fixation

First the media was removed and coverslips were rinsed in 1x PBS (2 ml per well). Next, 2 ml of ice-cold methanol was added onto coverslips. Coverslips were fixed for 3 min. After fixing, the coverslips were removed from methanol and allowed to air-dry. If the fixation was performed after transfection procedure, coverslips were mounted as described below. For immunofluorescence studies, below protocol was followed after air-drying.

Paraformaldehyde Fixation

Similar to above, old media was first removed and coverslips were washed gently with 1x PBS (2 ml per well) to remove old media and floating dead cells. Cells were then be fixed with 1 ml of 4% paraformaldehyde solution for 15 min. at room temperature. After fixing transfected coverslips, cells were washed 3 times with 1x PBS (2 ml per well) and mounted onto slides as described below.

When paraformaldehyde was used to fix the cells, additional steps were performed as follows to increase the cell permeability before performing the immunofluorescence procedure. After fixing coverslips with 4% paraformaldehyde for 15 min. cells were rinsed with 1x PBS 3 times (2 minutes wait time at each wash). Next, cells were permeabilized with 0.2%- Triton –x100 in PBS for 3 minutes. In this step coverslips were treated with 50 μl of this solution. Then coverslips were rinsed with 1x PBS 3 times (2 minutes wait time at each wash) and once with 1x TBS. After these steps were done, coverslips were subjected to blocking with TBS/10% milk and other steps were followed as mentioned below for immunofluorescence procedure.

Mounting coverslips onto slides after the fixation

After fixing, coverslips were rehydrated by rinsing with 1x PBS twice (2ml per well). After this, coverslips were rinsed with water. One drop of Vectashield mounting medium (Vector laboratories) was added onto a microscope slide. Excess water was removed from the coverslips by wicking, and then coverslips were slowly dropped onto the drop of vectashield on the slide (cell side down). The four sides of the coverslip were sealed using clear nail polish, and the prepared slides were analyzed using the confocal microscope.

2.8 Immunofluorescence

To perform IF experiments, fixed cells were rinsed with 2 ml of PBS (1x) three times for 2 minutes. Then the coverslips were rinsed with 2 ml of TBS (1x) once for 2 minutes. After the rinsing steps, the coverslips were blocked in TBS/10% milk (50μl) for 2 hours at the room temperature in a humidified chamber. Next, the cells were incubated with primary antibody Rabbit-FAM171B (Novus) (1:100) overnight at 4°C. Afterward the cells were washed with 2ml of TBS/Tween 6 times for 5 minutes. After the washing step, the cells were incubated with secondary antibody, Goat anti Rabbit – Alexa 488 (1:500) at the room temperature for 2 hours. Then the cells were rinsed with 2 ml of TBS/T, 6 times for 5 minutes. The last step was to rinse the cells with deionized water and then coverslips were mounted with 5 µl of Vectashield Antifade with DAPI. The coverslips were then sealed using clear nail polish and the cells were observed using confocal microscopy.

2.9 Confocal microscopy

Images were taken using the Zeiss LSM 880 confocal microscope. Per experimental coverslip, minimum of 10 cells were analyzed. For every image taken in this study, Plan-Apochromat 63x/1.4 NA oil objective lens was utilized. In Zen Blue 2.3 software, "locate" option was selected to enable the specimen to be viewed through the ocular lenses and to find the region of interest in the specimen. All cells were grown on

1.5 mm thickness coverslips specialized for this microscope and for live cell imaging a 4-well chambered coverglass was used. To set the microscope for fluorescence observation, blue/red/green emission channels were selected based on the staining we had on our cells. Once the region of interest was found and focused, "acquisition" option was selected to analyze the specimen in the computer using Zen blue 2.3 software. Confocal-4 color parameter was selected each time for image acquisition. After scanning the image, "range indicator" was activated, in which the scanned image appears in false-color representation. Red pixel indicates the saturation (maximum intensity) and blue pixels indicates zero or minimum intensity. To achieve the optimal intensity, with minimal background signal, digital gain, digital offset and laser power parameters were adjusted. The "snap" tool was used to acquire single frame (multi-channel) images. To better evaluate the images, Z –stacking strategy was utilized. It is a digital image processing method, which combines several images captured at several focal distances. This process gives rise to composite image with a bigger depth of field than any of the individual images from which the composite image is created. In acquiring Z-stack images, two ends of the specimen were set using the focus knob and the interval between each stack was set to optimal setting. Orthogonal views of the images in x, y and z planes were studied to identify FAM171B localization inside the cell. To obtain the live cell imaging, time-series option was utilized. The number of cycles and the interval between each frame were selected based on the need of the duration of the video.

2.10 Co-localization with a Mitochondrial marker

Immunofluorescence protocol was followed as mentioned above. Primary antibody: Mouse anti Mitochondria (1:50) and secondary antibody: Goat anti Mouse: Alexa 568 (1:500) were used with the primary and secondary antibodies of FAM171B.

2.11 Live cell

Live cell imaging was performed using confocal microscope on SH-SY5Y cells after transfecting with FAM171B-GFP. First the cells were split in to a chambered coverglass, specifically designed for live cell imaging (4 wells). The SH-SY5Y cell pellet after centrifuging was resuspended in 2 ml of the media (refer to the SH-SY5Y culture protocol above). Next 0.5 ml of resuspended pellet was added to each well in the chambered coverglass. The following day, 1.25 μg of plasmid pEGFP-FAM171B (0.928 μg/ml) + 2.5 μL of P3000 reagent were added into 62.5 μL of opti-MEM media (for 4 wells = 5 μg+10 μL /250μL). Next, 2.5 μL of Lipofecatamine 3000, a transfection reagent that is used to increase the transfection efficiency of plasmid DNA, was diluted in 62.5 μL of opti-MEM (for 4 wells = 10 μL/250μL). The two dilutions were then combined and incubated for another 15 minutes at room temperature. After 15 minutes 125 μL of the transfection material was dripped down gently into each well containing SH-SY5Y cells. Finally 1 μL of Hoechst nuclear stain was added to each well prior to the imaging.

2.12 Co-localization with early endosome marker in live cells

When performing live cell imaging with early endosome marker and FAM171B-GFP, 2 μl of CellLight Early Endosome- RFP marker was dripped down to cell wells 30 seconds prior to adding the transfection reagents. Images were taken in between cycles of 20-300 with intervals ranging from 1.5 seconds to 2 seconds. Cells were maintained at 5% CO_2, 37°C conditions. For each well 5-8 respective images were obtained using the confocal microscope.

Chapter 3

Results

3.1 Immunofluorescence using two different antibodies suggests that FAM171B localizes to punctate vesicles in the cytoplasm of Primary neurons, SH-SY5Y Neuroblastoma cells and U138 Glioblastoma cells.

We performed immunofluorescent experiments using two commercially available primary polyclonal antibodies (Novus 93847) and (Novus 93846) to assay FAM171B's intracellular localization in several different neuronal cell lines (Figures 1 and 2). The Novus 93847 Ab showed that FAM171B localizes predominantly to the cytoplasm of cells in discrete punctate dots reminiscent of small endomembrane system vesicles (Figure 1). Occasionally it appeared that FAM171B may be in/near the nucleus of certain cells (co-stained with DAPI); however, Z-stack images taken using the confocal microscope suggested that FAM171B predominantly localized to the cytoplasm (Figures 3-5). Interestingly, all three cell lines (glioblastoma, neuroblastoma and primary neurons) showed very similar staining patterns (Figure 1) suggesting that this is likely FAM171B's intracellular distribution in many/most nervous system cell types. Importantly, we confirmed these results using a second primary antibody raised against a different epitope of FAM171B (Figure 2). Indeed, Novus 93846 primary Ab showed very similar staining patterns with punctate/vesicular type staining of FAM171B throughout the cytoplasm of all three cell lines (Figure 2).

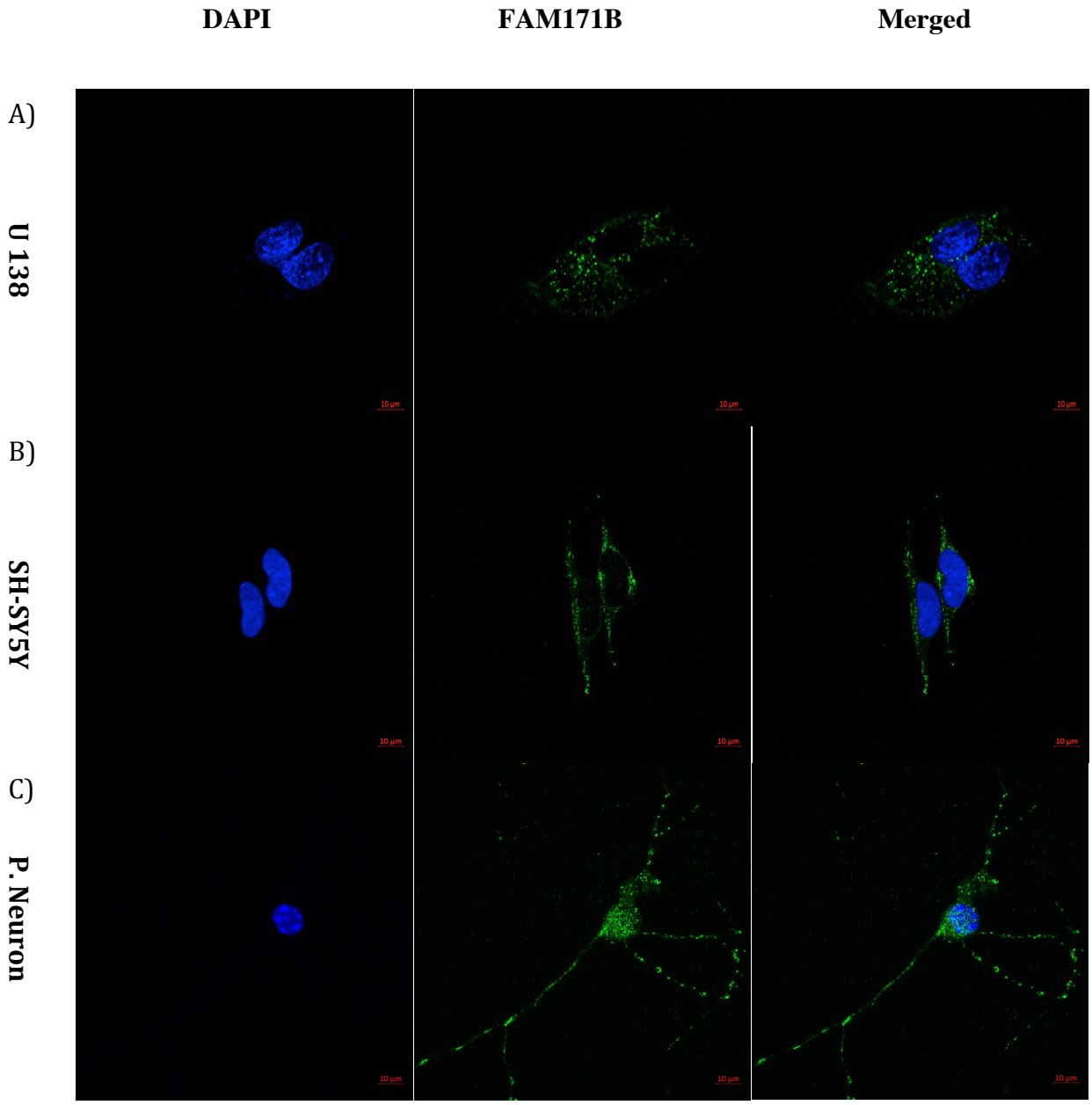


Figure 1: FAM171B displays a punctate cytoplasmic staining in primary neurons, SH-SY5Y cells and U138 cells (Immunofluorescence, Novus 93847 rabbit polyclonal Ab). Blue indicates DAPI nuclear stain and green indicates the expression of FAM171B. Methanol was used for fixation in each cell line.

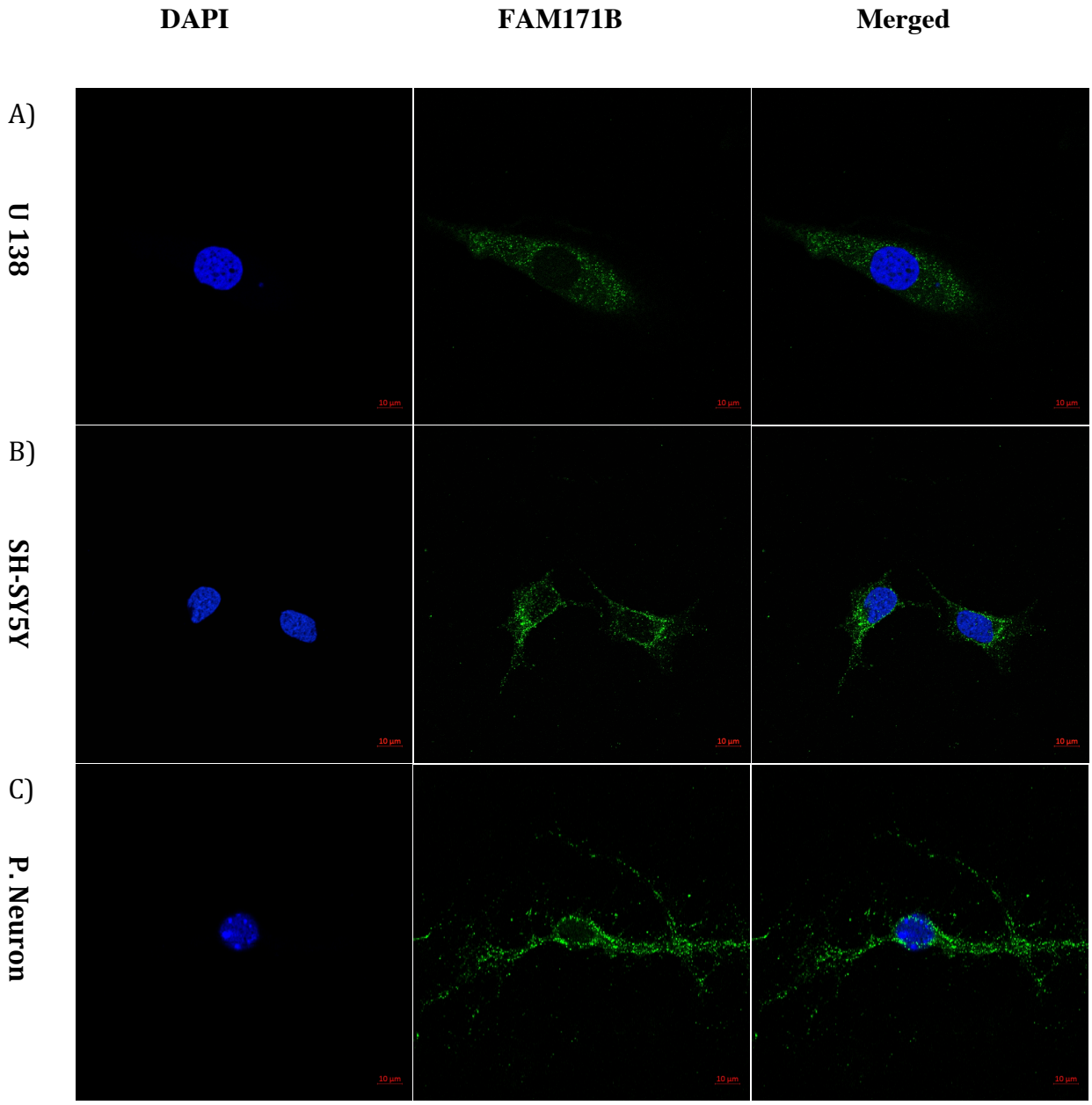


Figure 2: FAM171B displays punctate cytoplasmic staining in primary neurons, SH-SY5Y cells and U138 cells. (Immunofluorescence, Novus 93846 rabbit polyclonal Ab). Blue indicates DAPI nuclear stain and green indicates the expression of FAM171B. Methanol was used for fixation in each cell line.

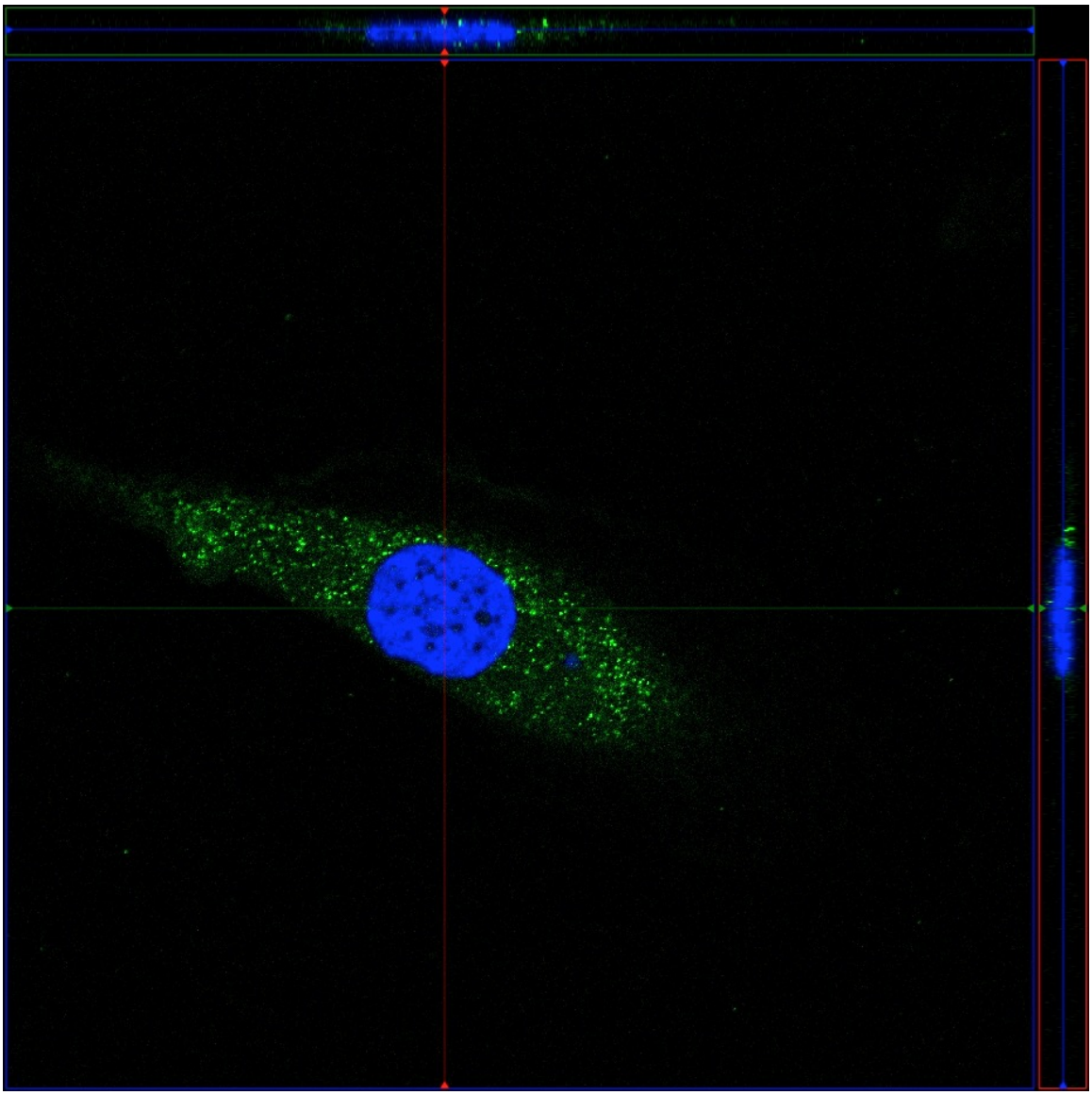


Figure 3: Orthogonal view of the merged image of U138 (Immunofluorescence, Novus 93846 rabbit polyclonal Ab) indicates FAM171B predominantly localizes to the cytoplasm of the cell. Blue indicates DAPI nuclear stain and green indicates the expression of FAM171B.

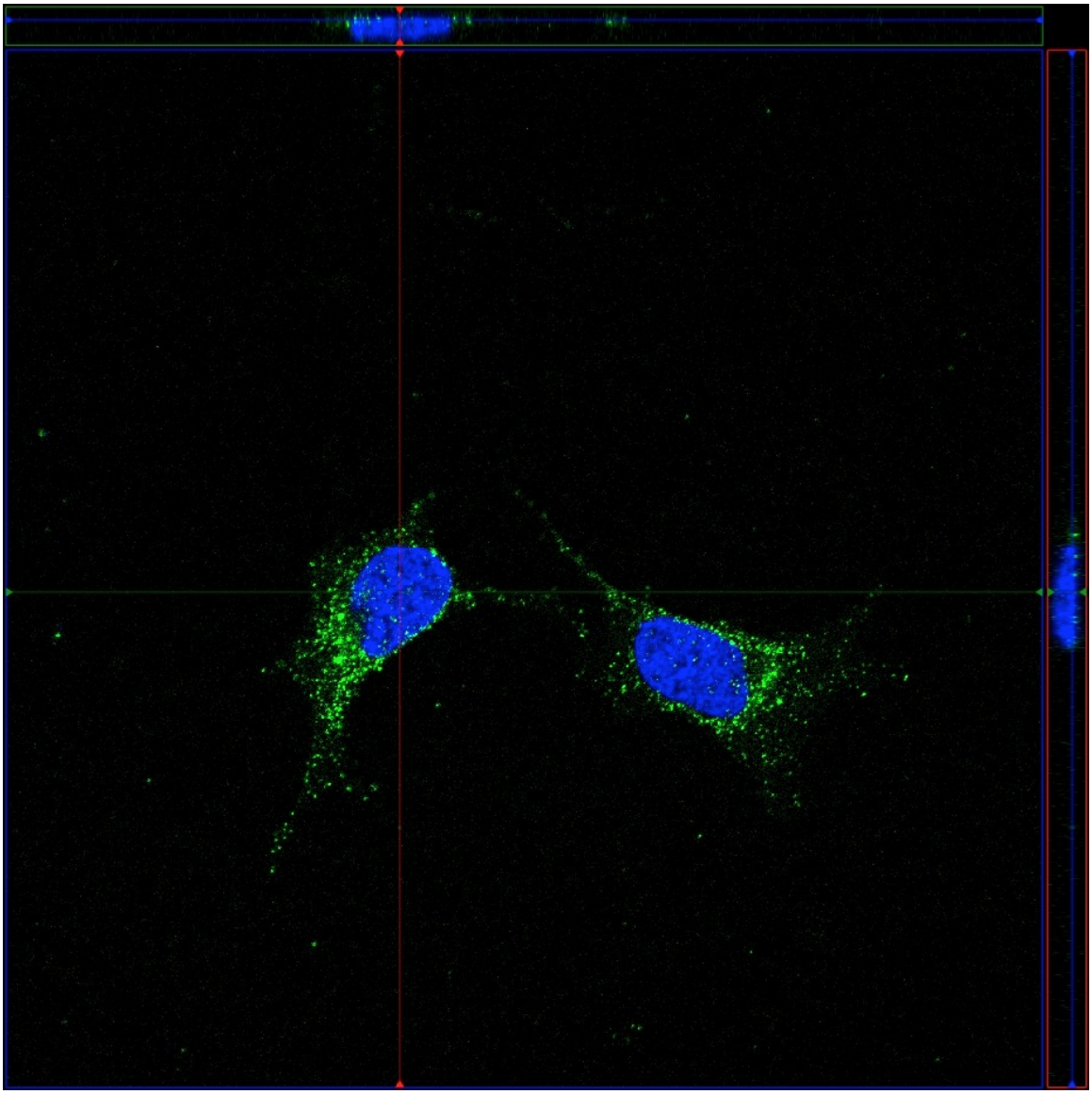


Figure 4: Orthogonal view of the merged image of SH-SH5Y (Immunofluorescence, Novus 93846 rabbit polyclonal Ab) indicates FAM171B predominantly localizes to the cytoplasm of the cell. Blue indicates DAPI nuclear stain and green indicates the expression of FAM171B.

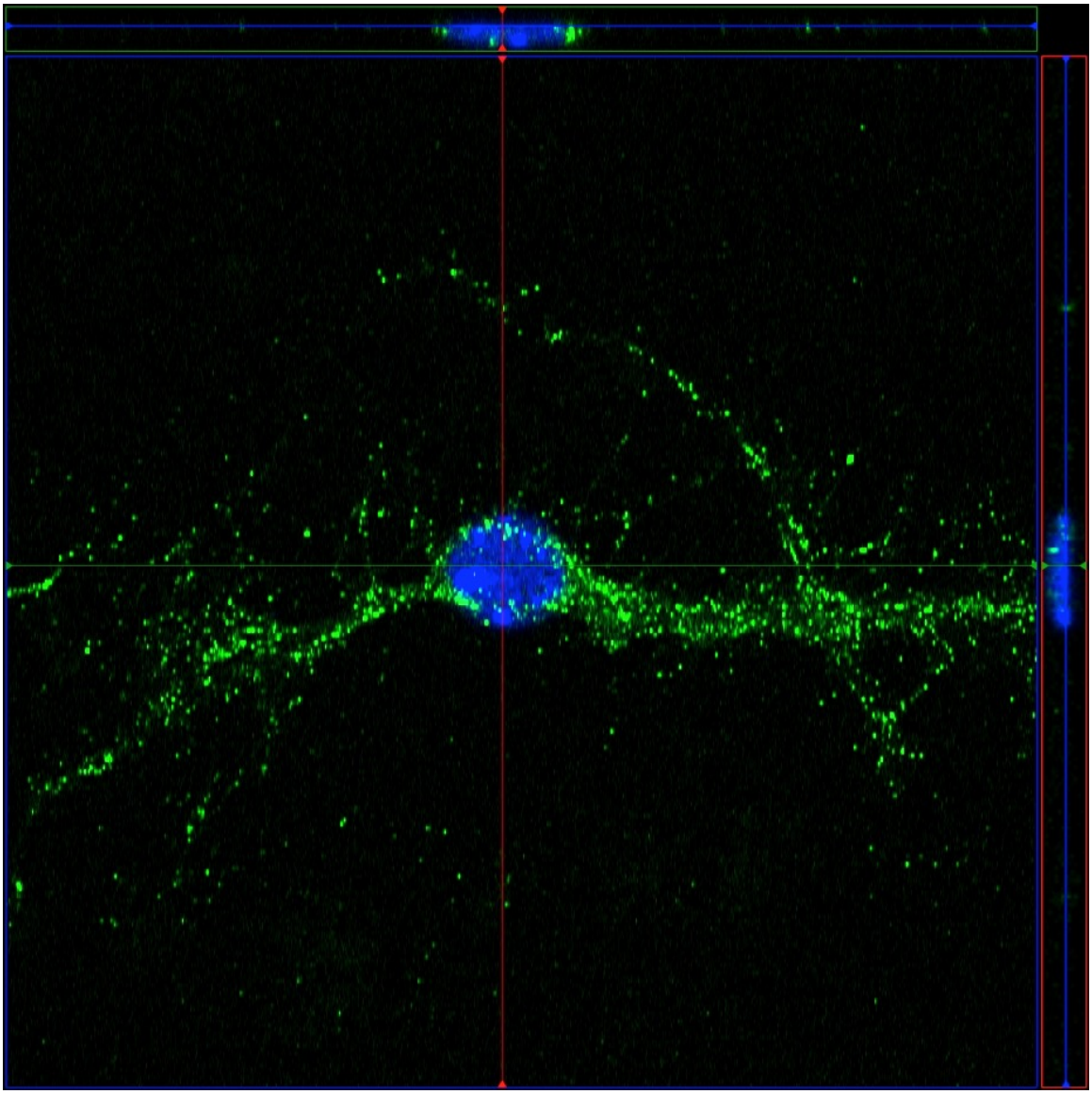


Figure 5: Orthogonal view of the merged image of primary neuron (Immunofluorescence, Novus 93846 rabbit polyclonal Ab) indicates FAM171B predominantly localizes to the cytoplasm of the cell. Blue indicates DAPI nuclear stain and green indicates the expression of FAM171B.

3.2 FAM171B-GFP expression in fixed U138 cells

Exogenous expression of FAM171B was then studied by transfecting U138 cells with a FAM171B-GFP fusion protein plasmid (previously constructed in the lab). Similar to what we observed with immunofluorescence, FAM171B-GFP expression was predominantly localized to the cytoplasm of transfected cells (Figure 6). Also, FAM171B-GFP had a similar cytoplasmic vesicular staining pattern and intriguingly it could be seen more concentrated closer to the plasma membrane. These data suggest that endogenous and exogenous expression of FAM171B is very similar.

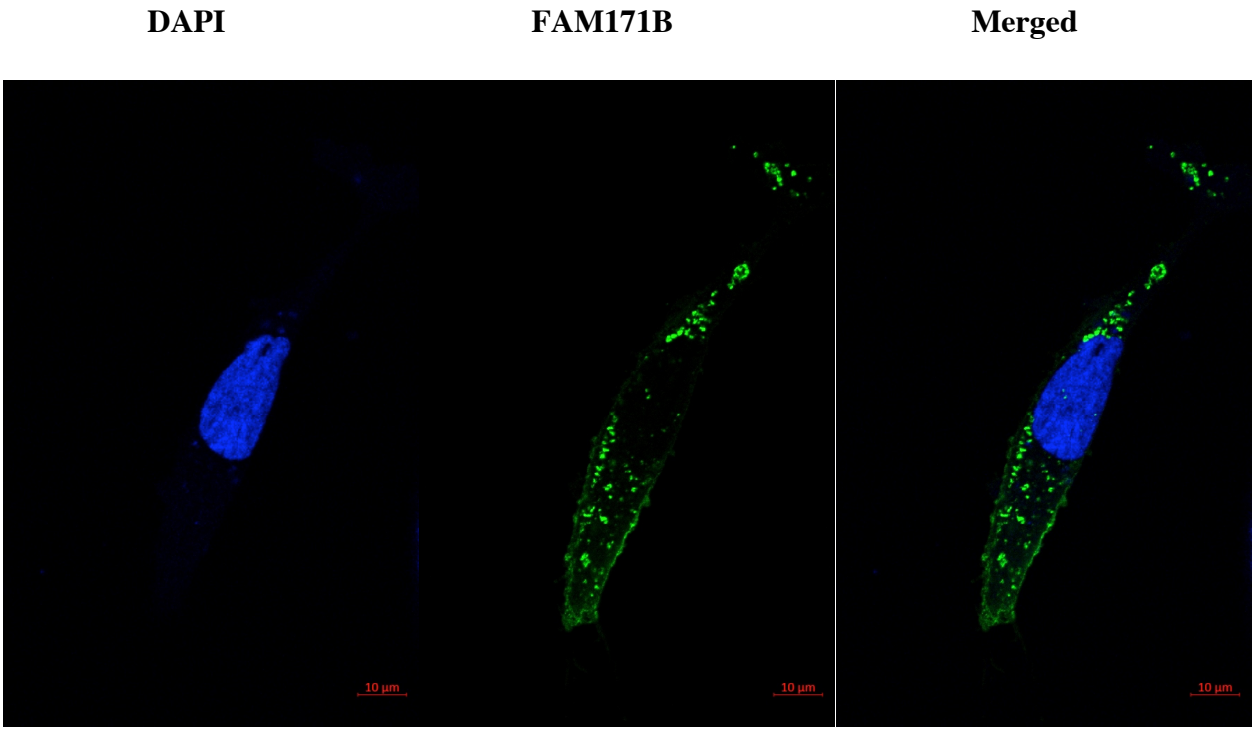


Figure 6. FAM171B-GFP displays a punctate signaling pattern in U138 cells (fixed). Blue indicates DAPI nuclear stain and green indicates FAM171B (Methanol was used for fixation and 5 μg of plasmid was used in this experiment).

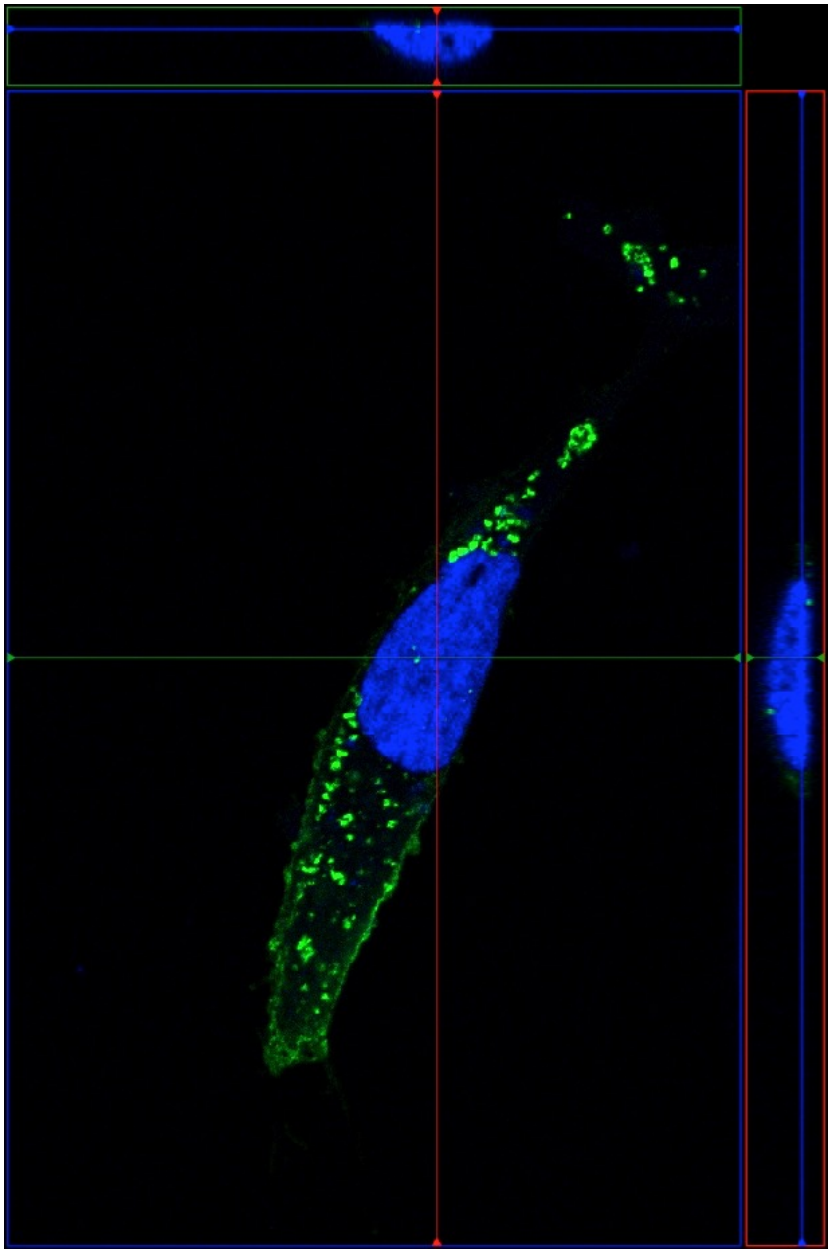


Figure 7: Orthogonal view of transfected U138 cell with FAM171B-GFP (merged image) indicates FAM171B localizes predominantly to the cytoplasm of the cell.

Blue indicates DAPI nuclear stain and green indicates FAM171B.

3.3 FAM171B-FLAG expression in fixed U138 cells

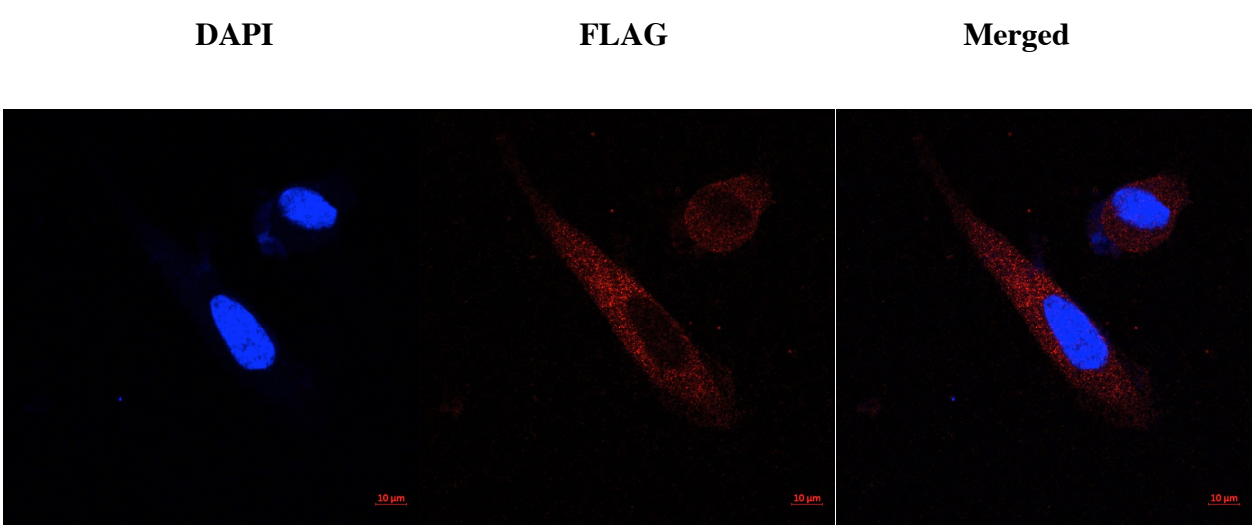


Figure 8: FAM171B-FLAG localizes to the cytoplasm of U138 cells. Red signaling indicates the expression of FAM171B and DAPI in blue indicates the nucleus. Methanol was used for fixation.

To further examine the exogenous expression of FAM171B and its intracellular localization, we utilized a second fusion protein, FAM171B-FLAG (previously constructed in our lab) to transfect U138 cells. FAM171B-FLAG also localizes to the cytoplasm of the cell and displays a vesicular/punctate-staining pattern (Figure 8), similar to what we witnessed in transfection studies with GFP fusion protein and immunofluorescence data. This further validates the similarity between the endogenous and exogenous expression of FAM171B.

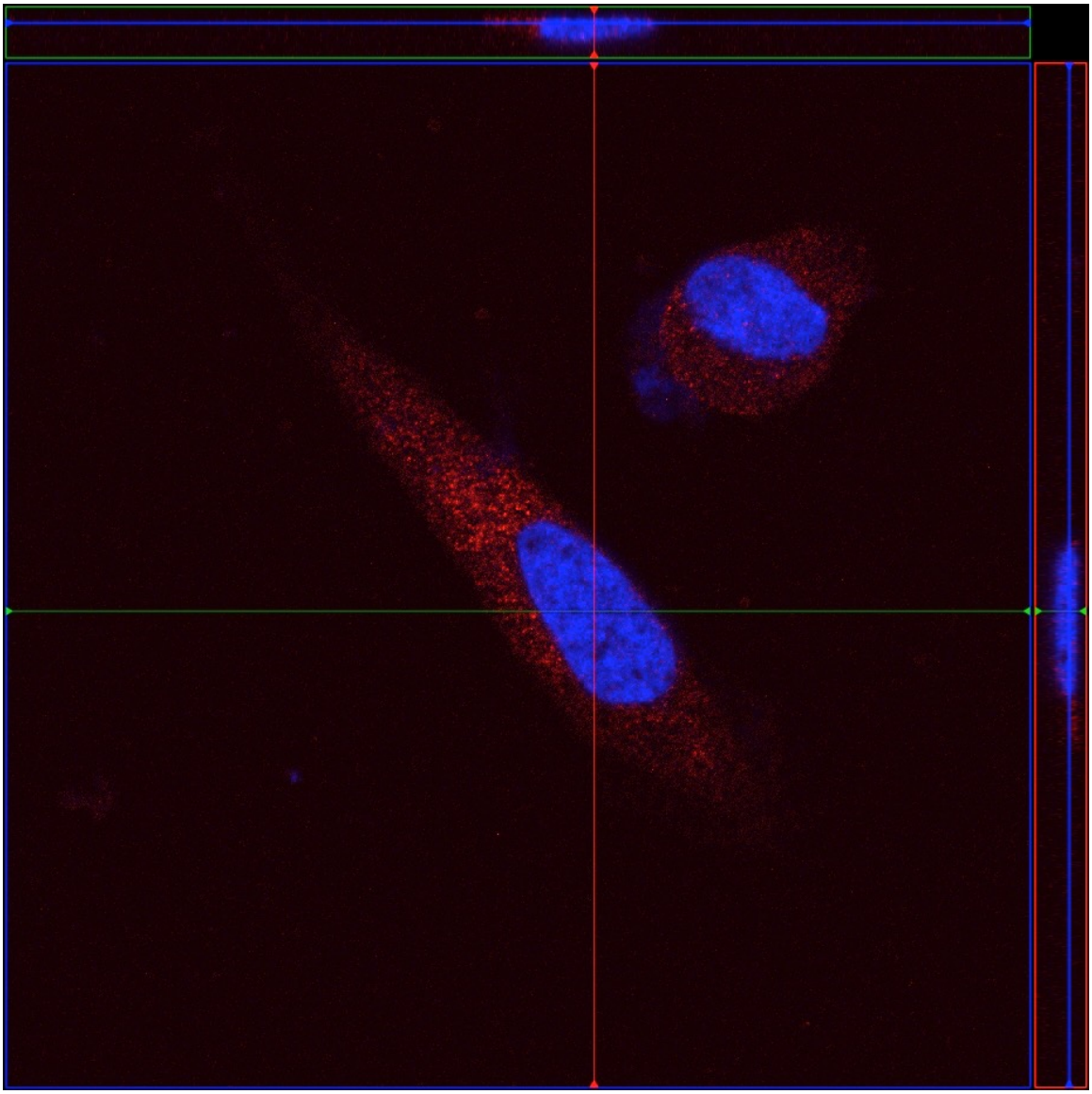


Figure 9: Orthogonal view of the merged image of U138 cell transfected with FAM171B-FLAG shows FAM171B predominantly localizes to the cytoplasm of the cell. Red signaling indicates the expression of FAM171B and DAPI in blue indicates the nucleus.

In order to analyze whether this punctate signaling pattern changes with different amounts of plasmid, U138 cells were subjected to transfection with varying amounts of FAM171B-GFP plasmid, ranging from 10 ng to 10 μg. It was observed that at each plasmid concentration, FAM171B's punctate/ vesicular signaling pattern continued to be consistent, confirming that plasmid concentration does not affect the signaling pattern of the protein (Figure 10). At lower concentrations of the plasmid, the number of transfected cells was reduced and 5 μg of plasmid was used in our successive experiments. Moreover two fixing methods were tested: paraformaldehyde and methanol. We found no differences in expression pattern between the two fixing methods and methanol was used in most experiments (data not shown). Figure 10 shows transfections with three lower concentrations of FAM171B-GFP plasmid: 10ng, 100ng, 500ng, and at each concentration, FAM171B shows the same signaling pattern.

A) **B)** **C)**

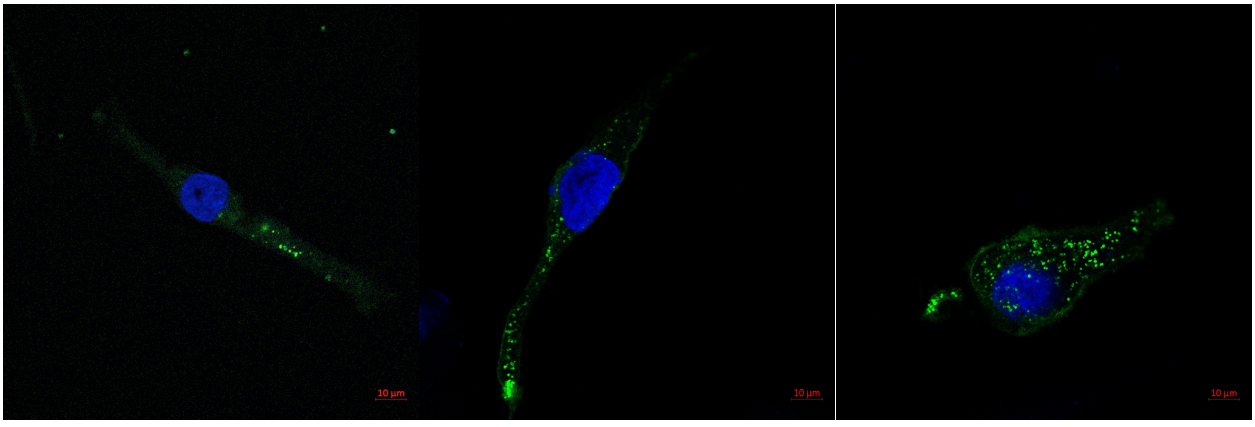
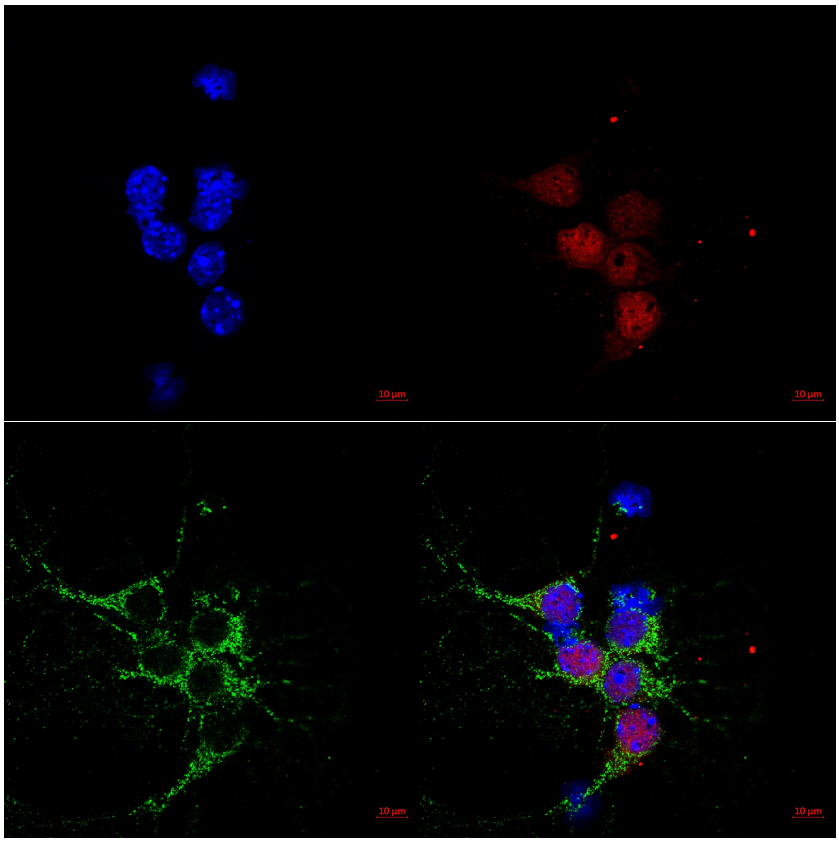


Figure 10: FAM171B-GFP exhibits similar expression pattern at different plasmid concentrations. Image A) indicates transfection with 10ng, B) with 100ng and C) with

500ng. Green signaling depicts FAM171B signaling and blue signaling depicts where the nucleus is. Methanol was used for fixation.

3.4 Co-immunofluorescence of FAM171B and NeuN in primary neurons

DAPI **NeuN**



FAM171B **Merged**

Figure 11: FAM171B is expressed in the cytoplasm of neurons. Red indicates the NeuN signaling, green indicates the FAM171B expression (Novus 93846 polyclonal antibody) and nucleus is stained in blue with DAPI. Methanol was used for fixation.

Primary neurons in our study were harvested from mouse brain cerebellum purchased from Brain Bits LLC. To confirm the cells we study are indeed neurons, NeuN, a neuronal biomarker that is used to identify neurons was utilized. Harvested primary neurons were subjected to co-immunofluorescence using antibodies against FAM171B (Novus 93846) and against NeuN (Figure 11). Red signaling indicates the staining of NeuN, which signifies the presence of primary neurons. This confirmed that the cells we were analyzing were indeed neuronal cells.

3.5 Co-immunofluorescence of FAM171B and mitochondria in primary neurons

In attempt to identify the subcellular localization of the FAM171B in the cell, different organelle markers were utilized to analyze possible co-localization. Primary neurons were subjected to co- immunofluorescence with antibodies against mitochondria in the neurons and with antibodies against FAM171B (Figure 12). Based on the acquired data it was noticed that FAM171B does not co-localize with mitochondrial organelles, as little signal overlap was detected (Figure 13). This indicated that FAM171B protein might not function in mitochondrial activity in the cell.

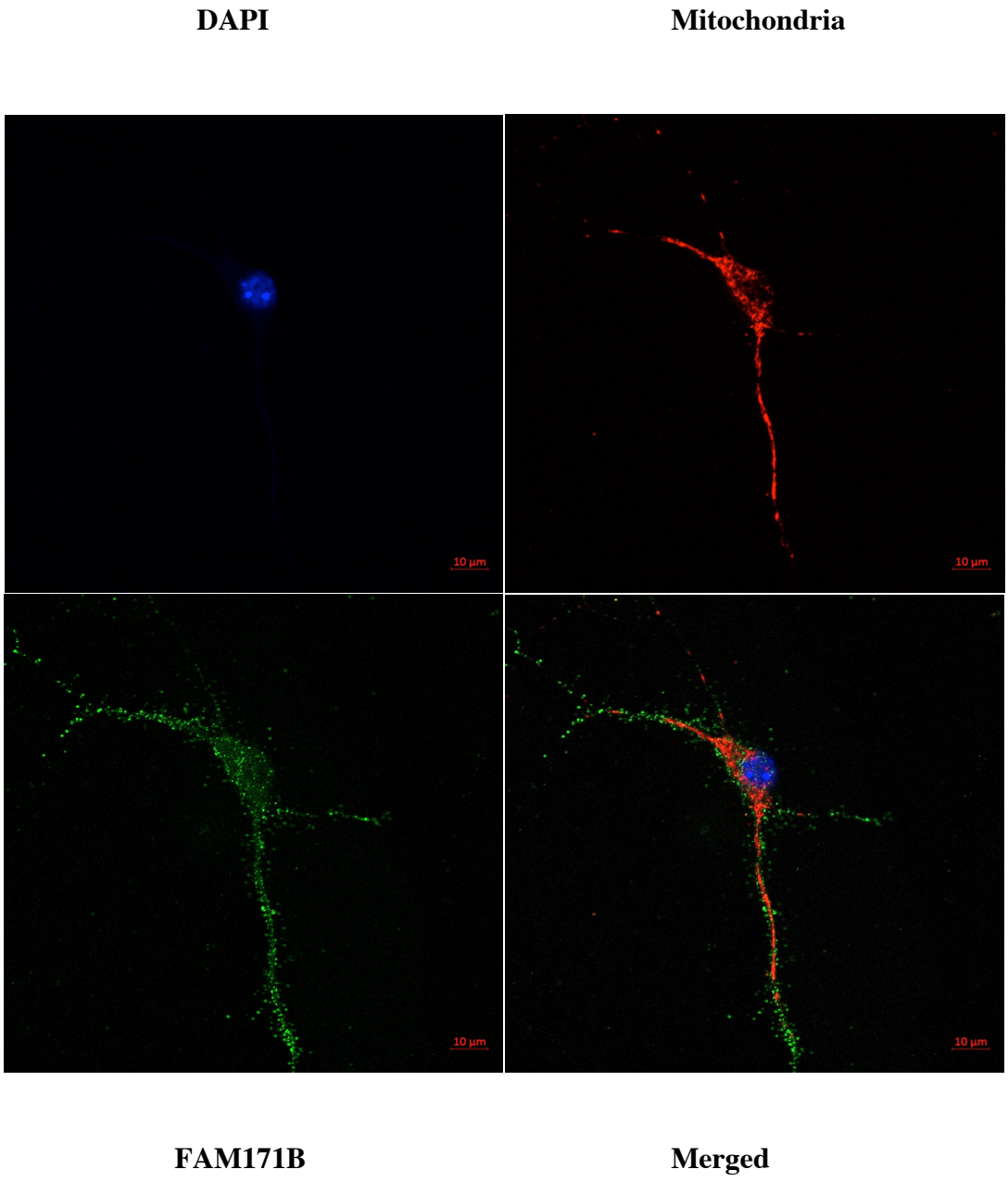


Figure 12: FAM171B does not co-localize to mitochondria in primary neurons. Red signaling indicates the mitochondria and green signaling indicates the FAM171B expression (Novus 93846 polyclonal antibody). Methanol was used for fixation.

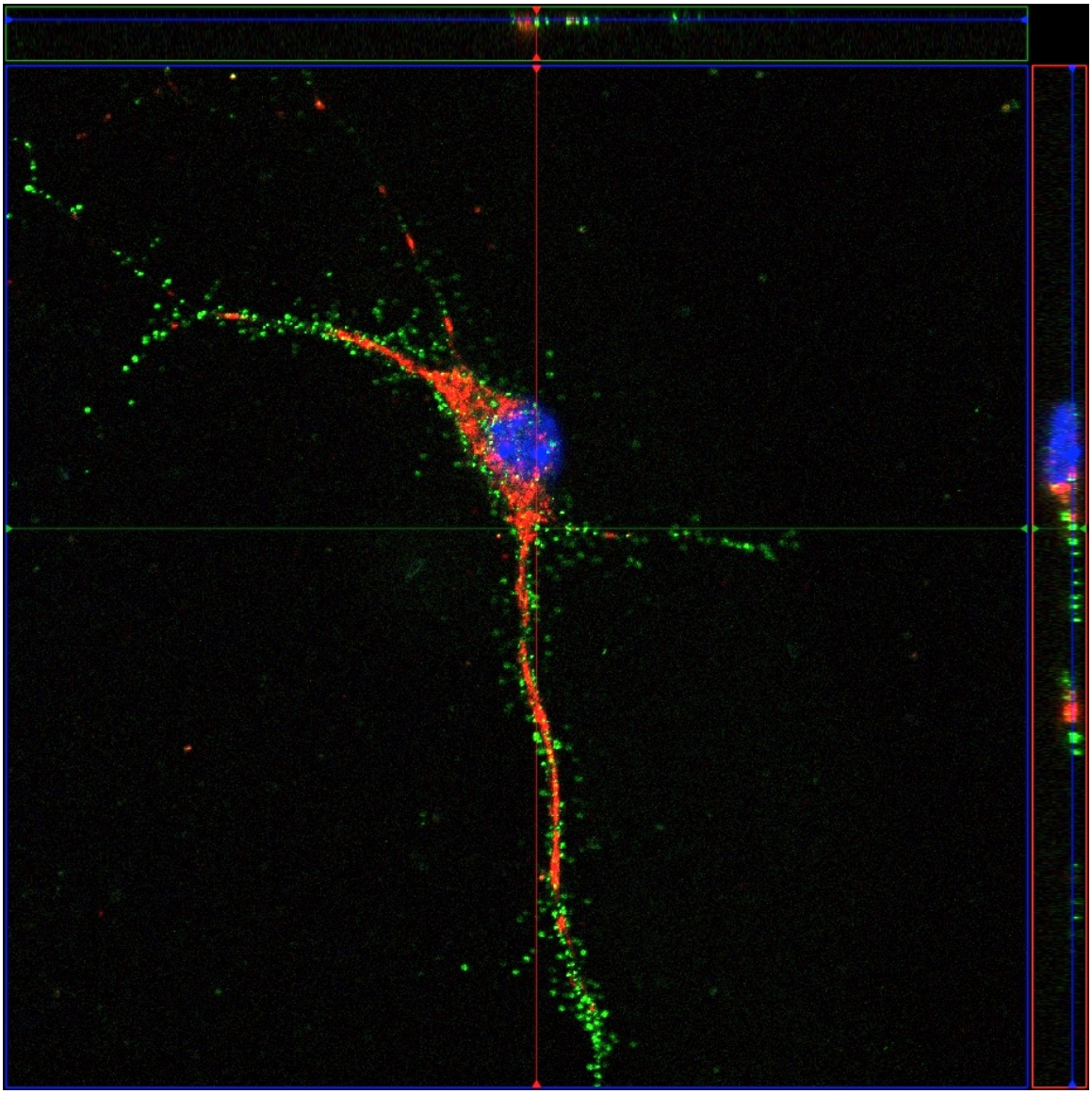


Figure 13: Orthogonal view of the merged image of primary neuron, subjected to co-immunofluorescence with antibody against FAM171B (Novus 93846) and mitochondria. FAM171B does not co-localize well with mitochondria.

3.6 Pseudopod-like protrusions.

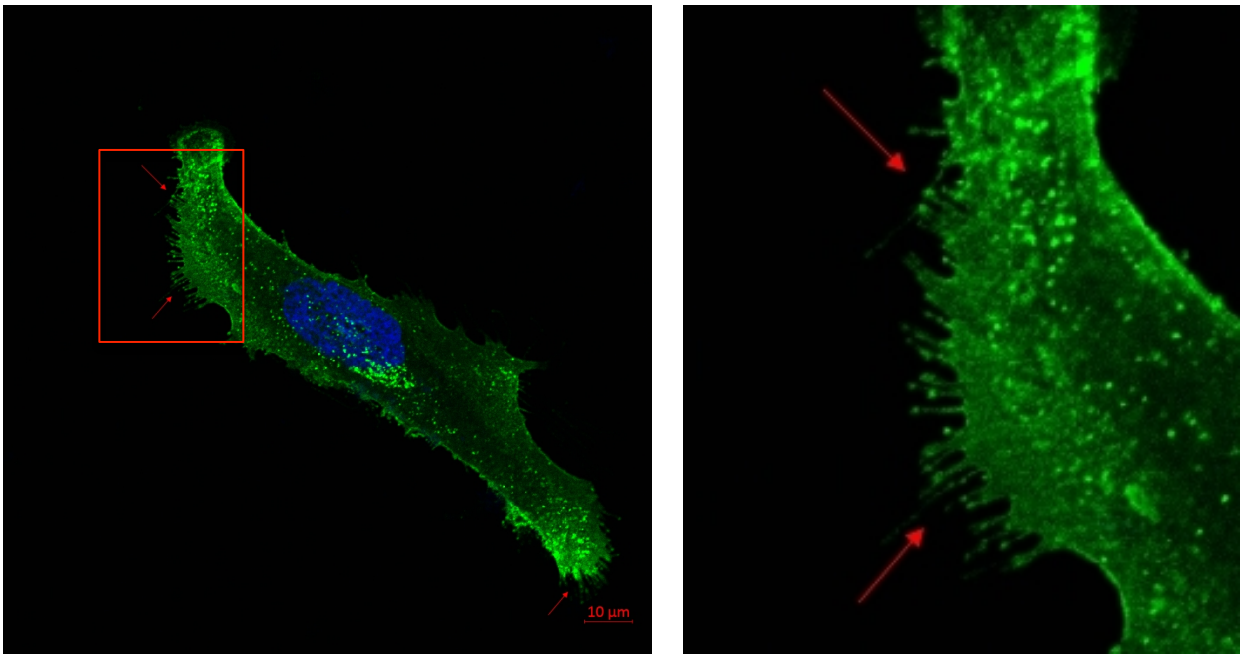


Figure 14: FAM171B localizes to pseudopod protrusions near the plasma membrane in cells. U138 cell was subjected to IF and green signaling indicates the expression of FAM171B (Novus 93847 polyclonal antibody).

We observed that FAM171B signals localizes to cellular regions near plasma membrane that resembles pseudopod-like cellular structures. Interestingly, this cellular characteristic was observed in both fixed and live cell imaging (SH-SY5Y cells). Enlarged cross sections of U138 cells depicted above (Figure 14) show pseudopod like plasma membrane protrusions and vesicular FAM171B signaling could be seen alongside the edges of these membrane protrusions. When analyzing static images, Z-stack images

were obtained (taking multiple images at different focal distances) and these membrane protrusions were noticed at certain stacks.

3.7 Live- cell imaging

To gain more insight on FAM171B's expression in real time in its cellular environment, we performed live cell imaging on SH-SY5Y cells. We first transfected the cells with FAM171B-GFP and live cell imaging showed vesicular green particles moving in the cytoplasm of the cell resembling the process of vesicular trafficking (Link 1). Also there were pseudopod like protrusions emanating from the edges of the plasma membrane of these live cells (Link 2) which was also observed in Z-stack images of the fixed transfected U138 cells and cells that were subjected to immunofluorescence. Interestingly it could be seen that these dynamic cellular structures in live cells were constantly extending and retracting from the cell membrane.

Next the live cells were co-stained with early endosome marker after transfecting the cells with FAM171B-GFP. It was observed that approximately one-half of the FAM171B-GFP signaling localizes to early endosome in live SH-SY5Y cells suggesting FAM171's possible function in endocytic processes (Link 3). Cell nuclei were stained in blue using the Hoechst DNA stain, and again FAM171B expression was typically not found in the nucleus. Figure 15 below shows a screen capture of live-cells after co-staining with early endosome.

FAM171B **E. Endosome** **Merged**

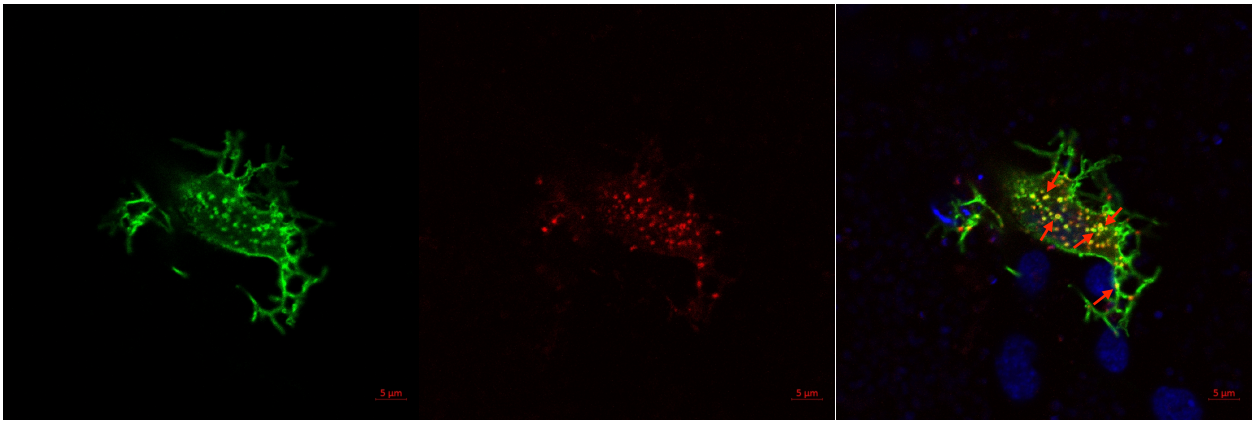


Figure 15: FAM171B co-localizes with early endosomes in live SH-SY5Y cells. Green signaling: expression of FAM171B and red: expression of early endosome in the cell. Blue signaling indicates the nucleus of the cell and yellow signaling indicates the co-localization of FAM171B and early endosomes (red arrows).

Link 1:

SH-SY5Y Neuroblastoma cells : 33 hours post-transfection: This live-cell video shows the punctate vesicular signaling of FAM171B in SH-SY5Y cells, moving in the cytoplasm (60 seconds).

Link 2:

SH-SY5Y Neuroblastoma cells: 31 hours post-transfection: This video shows an enriched staining of FAM171B near the plasma membrane and highlights the dynamic cellular protrusions of the live cells similar to what was observed in fixed cells (6 seconds).

Link 3:

SH-SY5Y Neuroblastoma cells: Co-localization with early endosomes: 72 hours post-transfection: This video shows co-localization of FAM171B with early endosomes. Yellow signaling specifies the overlap of FAM171B with early endosomes (4 seconds). Static images of this video are illustrated above.

Chapter 4

Discussion

FAM171B is a novel polyQ protein with 14 glutamine repeats in its amino acid sequence. There is minimal published data regarding this protein and its location and the function are yet to be determined. Based on characteristics it shares with other neurodegenerative diseases caused by abnormal expansions in the polyQ regions, FAM171B could also be linked to another molecularly uncharacterized neurodegenerative disease. Previous research conducted in our lab found that this novel protein is expressed widely throughout the developing and adult mouse brain through two different approaches, *in situ* hybridization and immunohistochemistry analysis. Moreover it was found that expression levels are more prominent in some brain regions compared to others.

The purpose of the current research was to further expand the knowledge of this protein by looking into the intracellular localization. Proteins are responsible for a multitude of diverse vital tasks in all living organisms. Given that a protein's function and role are strongly related to its subcellular location, protein location prediction is an important research area (31). It can provide useful insights about their functions, particularly, one of the fundamental goals in cell biology and proteomics is to identify the functions of proteins in the context of compartments that organize them in the cellular environment (32). It can indicate how and in what kind of cellular environments the proteins interact with each other and with other molecules, this is especially important for

the in-depth study of protein-protein interaction (PPI). Also it can help our understanding of the intricate pathways that regulate biological processes at the cellular level and hence it is crucial for many studies in system biology (33). Furthermore it is very useful for identifying and prioritizing drug targets during the process of drug development (32).

To obtain intracellular localization data of FAM171B, three different cell lines were used in this study: human glioblastoma cells (U138), human neuroblastoma cells (SH-SY5Y) and mouse primary neurons. Furthermore, two distinct approaches were utilized- immunofluorescence (with two separate antibodies) and fusion protein expression using both FLAG and green fluorescent protein (GFP) tags. Cells were also fixed using two different approaches (methanol and paraformaldehyde). In addition, colocalization with subcellular markers (nucleus, mitochondria, and endosomes) was performed. Finally, time-lapse imaging of SH-SY5Y live cells expressing FAM171B-GFP was performed to further understand the dynamic nature of FAM171B movement within cells.

Immunofluorescence (IF) was studied in all three cell lines and our data showed that FAM171B displayed a consistent punctate/vesicular type expression pattern (Figures 1,2). This signaling predominantly localized to the cytoplasm of the cell and this characteristic was observed in every cell line and fixation method used. Z-stack images of the cells were taken to create 3D images and those images were used to confirm that FAM171B is expressed in the cytoplasm (Figures 3-5).

Next, cells were transfected with plasmids created in our lab to confirm whether the endogenous and exogenous expression of the FAM171B is similar (Figures 6,8).

Cells were first transfected with FAM171B-GFP, and we found that FAM171B-GFP signaling localizes to the cytoplasm of the cell, and displayed a punctate/vesicular type signaling in the cytoplasm similar to IF data for FAM171B (Figure 6). Different quantities of plasmid were used with varying amounts of Lipofectamine 2000 (transfection reagent) to transfect the cells to analyze if the signaling pattern changes based on the plasmid/transfection reagent concentration (Figure 10). The signaling pattern continued to display the same cytoplasmic punctate/vesicular stain, although we observed that at lower plasmid concentrations only few cells were transfected and at high concentration more cells were transfected (Figure 10).

Next, cells were transfected with a different FAM171B fusion protein constructed in our lab. This fusion protein has a tag called FLAG, consisting of eight amino acids (AspTyrLysAspAspAspAspLys) (34) and immunofluorescence was performed with antibodies against FLAG peptide sequence. FLAG-FAM171B signaling, similar to the GFP transfection and IF data, was localized to the cytoplasm of cells further validating our data (Figure 8).

After analyzing the IF and transfection data it was noted that FAM171B is a cytoplasmic protein and next we tried to utilize organelle specific markers to find the subcellular location of the protein. First, primary neurons were stained with a mitochondrial marker and our data indicated that FAM171B and mitochondria do not co-localize well as little signal overlap was observed (Figures 12,13). We are confident that FAM171B is present in the cytoplasm of neurons as a number of the primary cultured

cells derived from the mouse brain also stained for the neuronal marker NeuN (Figure 11).

The punctate/vesicular expression suggests that FAM171B could be a protein associated with vesicular transport. To further explore this possibility, we utilized an early endosomal marker in conjunction with FAM171B-GFP and found a strong colocalization between the two (Figure 15). Indeed, when subjected to live cell imaging these two vesicle compartments moved in tandem in the same direction (Figure 15, Link 3). These results strongly suggest that FAM171B is at least occasionally present in endosomes. Thus, FAM171B may play a role within vesicular trafficking system (specifically endosomes) or it may itself be trafficked as cargo either in or out of the cell. Transport vesicles play a central role in the traffic of molecules between different membrane-enclosed compartments of the secretory pathway and endocytic pathway which is important for organelle biogenesis, constitutive and regulated secretion and endocytosis (35,36).

Efficient sorting of the material internalized by endocytosis is essential for key cellular functions and represents a major trafficking pathway in mammalian cells (37). Incoming material such as solutes, receptors and cargos, lipids and even pathogenic agents are routed to various destinations within mammalian cells at two major sorting stations: the early and late endosome (37). Sorting events initiated at early endosomes determine the subsequent fate of internalized proteins and lipids, destining them either for recycling to the plasma membrane, degradation in lysosomes or delivery to the trans-golgi network (38). The late endosome provides a central hub for incoming traffic from

the endocytic, biosynthetic and autophagic pathways and outgoing traffic to the lysosomes, the golgi complex or the plasma membrane (37). Our data suggest that FAM171B could be a protein functioning in cellular endocytosis since approximately half of our GFP-FAM171B signaling was co-localizing with early endosome markers in live-cell imaging and due to the punctate/ vesicular type expression in live and fixed cells. Moreover these vesicular structures were denser in the peripheral area of the cell where early endosomes usually reside in the cell.

Furthermore it was observed that these cells (fixed and live) show pseudopod-like protrusions around the edges of the cell membrane (Figure 14, Link 2). In fixed cells it was detected at different Z-stacks. In these protrusions FAM171B vesicles could be detected. These protrusions were prominent in live-cell imaging, and could be seen growing and retracting over the course of minutes (Link 2). The ability of cells to extend cell membranes is central to numerous biological processes, including cell migration, axon pathfinding, cadherin-mediated junction formation and phagocytosis (39). Moreover plasma membrane regions that form new protrusions could arise from the flow of membrane components from other regions of the cell, including vesicles such as endosomes (40).

Basquin *et al* (41), revealed a new mechanism that they call protrusion-based endocytosis, which is used to internalize receptors into small vesicles. In their work, they showed that interleukin-2 receptor (IL-2R) endocytosis requires its recruitment at the base of protrusions via its association with the WAVE complex (41). The WAVE complex is a nucleation-promoting factor activating the actin-related proteins 2 and 3

complex, inducing actin polymerization and membrane protrusion (41). These findings suggest that the cellular protrusion we observed in our cells could be playing a role in endocytosis further supporting FAM171B's possible function in the process.

One more possibility of these cellular protrusions observed in static and live cells is that this could be functioning in axon path finding or synapse formation (synaptogenesis). A common theme regarding the synaptogenesis is that growing axons explore their pathways and target regions by elaborating many dynamic, often transient protrusive filopodia and branchlets (42). Filopodia are long and slender cellular protrusions, which are one of the types of pseudopoda, have roles spanning the initiation and guidance of neuronal processes, axons and dendrites (43). This highly protrusive behavior of filopoda appears to be essential to the axon's ability to pathfind and locate appropriate target cells (42). Extensions and retractions of cellular protrusions was a prominent feature in our live cell imaging of SH-SY5Y cells.

In order to further evaluate this position, future studies could include studying co-localization of FAM171B with other organelle markers such as late endosomes. Also if this protein is functioning in endosome activity and if the expression of this protein is inhibited by gene knock out, complications in endocytosis process would be expected to be seen. Moreover pseudopod like protrusions might also be inhibited if the gene for FAM171B is deleted.

Conclusion

FAM171B is a novel polyglutamine protein with 14 glutamine repeats in its amino acid sequence. There is minimal publish data regarding this protein such that its function and its intracellular location have yet to be discovered. Based on the homology this protein shares with other polyQ neurodegenerative diseases, this could also be linked to another unknown polyQ disease. Previous research done in our lab found out that this protein is widely expressed throughout the mice brain using two different approaches: *in-situ* hybridization and immunohistochemical identification. This current research was undertaken to figure out the intracellular location of this protein using immunofluorescence and transfection studies. Our data indicate that FAM171B predominantly localizes to the cytoplasm of the cell. This was consistent between every cell line we analyzed. Expression pattern displayed a punctate/vesicular signaling and these signaling was more pronounced closer to the plasma membrane of the cell. Live-cell imaging data revealed that FAM171B-GFP localized to early endosomes. Moreover pseudopods like cellular cell membrane protrusions were observed in static and live cells. Taken together, these data suggest that FAM171B may play a role in cellular activities near the plasma membrane such as endocytosis and/or axon pathfinding. If FAM171B is linked to an unknown neurodegenerative disease, it could be contributing to the pathogenesis by disrupting the endosome function or by inhibiting the formation of neural circuits.

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