Abstract

ANALYSIS OF MYOPODIN IN HELA CERVICAL ADENOCARCINOMA CELLS

by Melissa Harkins

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Synaptopodin-2 is a proline-rich, actin-binding protein that exists in many isoforms. Due to its large proline content, synaptopodin-2 exists in a naturally unfolded state and possesses the characteristics of a hub protein. Members of the synaptopodin-2 family have been shown to bind with actin and induce actin polymerization (Chalovich and Shroeter, 2010). It has been found that synaptopodin can regulate α-actinin, which has actin-bundling activity (Asunama 2005). This interaction is isoform specific and causes bundling and elongation of α-actinin-induced actin filaments. The significance of possessing the ability to stimulate actin and induce actin bundling lies in the fact that the remodeling of the actin polymers is important for cell migration, adhesion, division and development. Cancer cell metastasis is a multi-stage process involving invasion into surrounding tissue (intravasation) transit in the blood or lymph (extravasation) and growth at a new site. Many of these steps require cell motility, which is driven by cycles of actin polymerization, cell adhesion and acto-myosin contraction (Olsen 2008). Therefore studies have been completed to answer the role of synaptopodin-2 in cancer and contradicting results have been obtained. Interestingly, contradicting results have also been observed for other actin binding proteins: gelsolin, CapG, and α actinin (De Ganck 2009); therefore not allowing actin-binding

proteins to be classified as tumor activators or suppressors. This work provides evidence of syanptopodin-2, isoform myopodin, functions as a tumor suppressor and also suggests the existence of another isoform of synaptopodin-2 in Hela cells.

Thesis

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Under the Direction of Dr. Jean Luc Scemama and Dr. Margit Schmidt

Department of Biology

EAST CAROLINA UNIVERSITY

Requirement for the Master of Science in Molecular Biology and Biotechnology

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Most of all I would like to thank my family. Without my husband, Wolfer, none of this would have been accomplished. You are always there for me each and every day. The one person I know I could count on for any type of support I needed. You would even come into lab to wash my dishes when I would get behind and always had the encouraging words and love that I needed. I love you very much and thank you. Also to my son, Kaiden you are the best son possible. You were always able to come into lab and let me work without being a distraction. I thank you for being the best child possible.

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CHAPTER ONE: INTRODUCTION

Synaptopodin-2 belongs to the synaptopodin family of proteins. This family consists of three members: synaptopodin, synaptopodin-2, and synaptopodin 2-like proteins. Each member has numerous isoforms that arise from alternative splicing and all are in a naturally unfolded state. Members of the synaptopodin family have been shown to bind with actin, the first binding partner to be discovered for this family of proteins (Mundel et al. 1997). Figure 1 shows a model of how synaptopodin 2 induces actin polymerization (Chalovich and Shroeter, 2010). It has been found that synaptopodin 2 can be a regulator for α-actinin, which has actin-bundling activity (Asunama 2005). Asunama has shown that synaptopodin can bind with α-actinin and inhibit the branching of α-actinin-induced actin filaments. This interaction is isoform specific and causes bundling and elongation of α-actinin-induced actin filaments. The ability to induce actin bundling and therefore affecting the remodeling of the actin cytoskeleton influence cell migration, adhesion, division as well as development. These are the hallmarks of cancer, which include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan 2011). Cancer cell metastasis is a multi-stage process involving invasion into surrounding tissue (intravasation) transit in the blood or lymph (extravasation) and growth at a new site. Many of these steps require cell motility, which is driven by cycles of actin polymerization, cell adhesion and acto-myosin contraction (Olsen 2008). Looking at possible regulators of actin bundling may help answer questions on tumorigenic and metastatic processes of different human tumors.

Synaptopodin-2 is a proline rich, actin-associated protein lacking globular domains that is commonly found in brain, kidney, and skeletal muscle tissues in mammals. Due to the

abundance of proline, Synaptopodin-2 cannot form secondary and tertiary structures; therefore it exists in a naturally unfolded state that allows it to bind to many partners and is considered a hub protein. Hub proteins are known to possess binding sites for many partners and to be involved in a number of signaling cascades (Iakoucheva et al. 2002). Hub proteins are highly flexible which allow them to adapt to different binding sites or possess the ability of readily reversible binding. It is known that many cancer-associated proteins act like hub proteins (Goh 2007). BRCA1, p53, caldesmon, α-synuclein and calmodulin (Tompa 2009) have been identified as hub proteins, which have all been linked to some form of cancer. Therefore, studying synaptopodin-2 may help answer questions about the interactions and networks of cancer proteins.

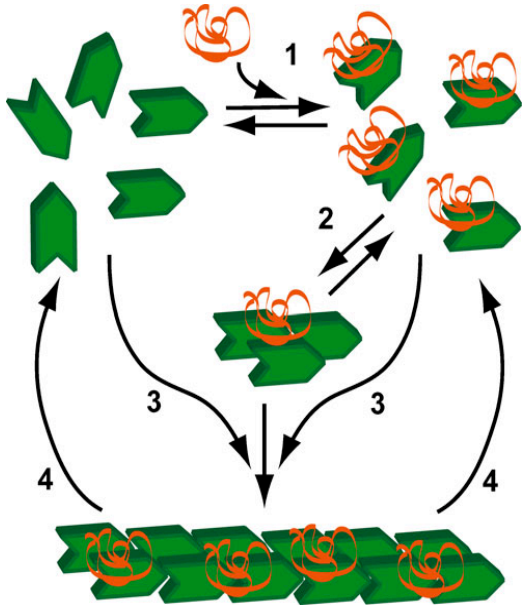

Figure 1: A representation of the proposed affect of synaptopodin-2 on actin polymerization. Actin is shown in green, while synaptopodin-2 is shown in red. (Chalovich and Schroeter, 2010).

At least five different isoforms of Synaptopodin-2 have been described: isoform A, isoform B, isoform C and myopodin. The last isoform encodes a small protein consisting of 123 amino acids that is only known as the listed NCBI number BAG65020. This unnamed protein does not share the common core region the other four isoforms share. These isoforms are the result of alternative splicing from a single gene located on chromosome 4 q 26. The gene contains seven exons: exon 1, 2, 3, 4a, 4b, 5, and 6. Exon 4a and 4b are not separated by an intron. All of these splice variants contain exon 4a but differ at the c-terminus region of the protein, except for myopodin, which is transcribed from an internal promoter and has a 395 residue N-terminal truncation. Myopodin, being the smallest in mass at 74 kDa, contains 698 amino acids. It does not contain exon 1, 2, or 3 and only has part of exon 4a. Splice variant 1 which encodes isoform A contains exon 1, 2, 3, 4a, and 6. Isoform A contains a total of 1261 amino acids and is approximately 136 kDa in mass. Isoform B differs from isoform A by its C-terminus, which is encoded by exon 4b instead of exon 6 and has a total of 1093 amino acids with a mass of 117 kDa. Isoform C's c-terminus is encoded by exon 5, has a total of 1109 amino acids and a mass of 119 kDa. Among the conserved regions of these isoforms the region encoded by exon 4a is the location of multiple actin-binding sites (Linnemann 2012). Figure 2 shows all five isoforms of synaptopodin 2.

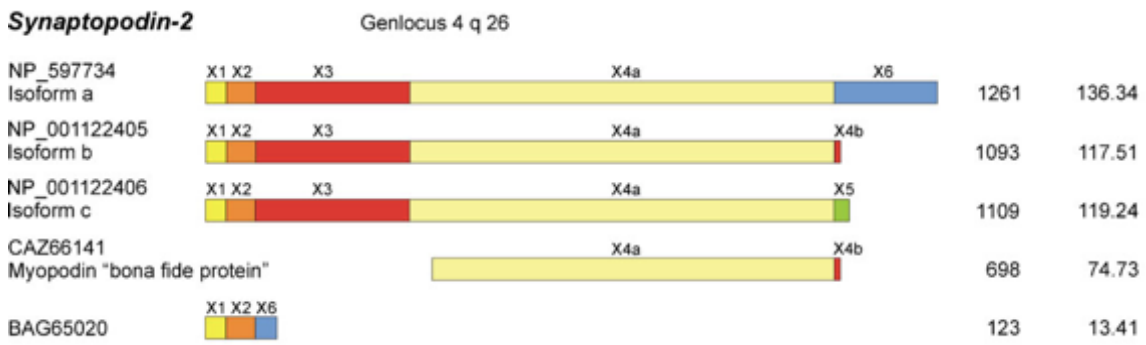

Figure 2: The four named isoforms of synaptopodin-2 formed by alternative splicing and myopodin formed by alternate promoter (Chalovich and Schroeter, 2010).

The function of these isoforms is still unclear. Kai demonstrated that truncation of the N-terminus or C-terminus of myopodin, did not alter cell migration, but when both termini were removed, migration was greatly impaired, suggesting that myopodin has a role beyond actin-binding. Also with truncation analysis Kai demonstrated that the different isoforms induced the formation of distinct types of actin bundles in the cells. When the N-termini was deleted there was no effect on actin bundle formation, but deletion of the C-termini altered the morphology and staining patterns of the actin bundles. It also alters the activation of the Rho/ROCK signaling pathway, a regulator of actin cytoskeleton, and can either promote or suppress PC3 cell migration, depending on the stimuli (Kai 2012). This is not the only study to have found myopodin to have tumor suppression activity. A genome-wide search for sequences that are deleted in aggressive prostate cancers lead to the finding that the myopodin gene is frequently deleted in advanced stages of prostate cancer (Lin 2001) and that partial or complete deletion of the gene occurred in 80% of invasive prostate cancers tested. In urothelial cancer, myopodin expression is decreased (Sanchez-Carbayo 2003) and found to suppress tumor growth and invasiveness in prostate cancer cells (Jing 2004). Jing also showed that overexpression of myopodin increased the cell doubling time in PC3 and LNCaP cells, two prostate cancer cell lines. Upon these findings, mutants with the C-terminal region deleted were constructed, and both mutants abrogated the effect of myopodin on tumor growth. The authors concluded that the tumor suppression activity of myopodin is located in the C-terminus region of the protein and more precisely between the region encoded by the most 3' part of exon 4a and exon 4b (Jing 2004).

Interestingly, studies have also shown that myopodin can act as a tumor activator. These types of contradictory results have also been observed in other actin binding proteins: gelsolin, CapG, and α actinin (De Ganck 2009). Therefore, it is not allowing actin-binding proteins to be classified as tumor activators or suppressors. Overexpression of myopodin in endothelial kidney cells and mouse C2C12 myoblast cells increased their motility and invasiveness (Van Impe 2003). When using RNA inference with siRNA duplexes designed to down-regulate myopodin expression, the invasive properties of these cells were significantly reduced (De Ganck 2008). Also, PC-3 cells treated with myopodin siRNA showed a significant decrease of migration in wound healing assay and were also able to form aggregates with a larger diameter than the control suggesting that reduced myopodin expression promotes cell-cell adhesion. These studies differ from the previous one by the location of the synaptopodin 2 protein. In the previous studies, synaptopodin 2 was located within the nucleus and cytoplasm, while in the study that showed a tumor activator role the protein was located within the cytoplasm along the actin filaments. De Ganck et al. suggested that myopodin protein could be translocating during tumorigenesis, and the change in location of the protein could be associated with different functions in different stages of cancer progression (De Ganck 2009). A model has been proposed to explain opposing findings with regards to myopodin's role in cancer (Figure 3). The localization of myopodin is cell type dependent, cell differentiation dependent, and stress dependent. Studies have been completed to correlate these differences to localization within the cell. Immunohistochemistry showed that myopodin was localized both in the nucleus & cytoplasm in normal epithelium while nuclear expression of myopodin was lost with high-grade bladder tumor (Sanchez-Carbayo 2003). In bladder cancer, cytoplasmic myopodin was found to be higher in normal cells (Yu 2006). Studies of two potential nuclear localization sites within

myopodin have shown that mutations of these sites do not affect the nuclear translocation of myopodin (Weins 2001). Importin 13, a member of the importin β superfamily of nuclear transport proteins, interacts with the C-terminal peptide of full-length myopodin and mediates nuclear import of myopodin but not export. This interaction contributes to the regulation of myopodin localization in both normal development and tumor suppression (Liang 2007).

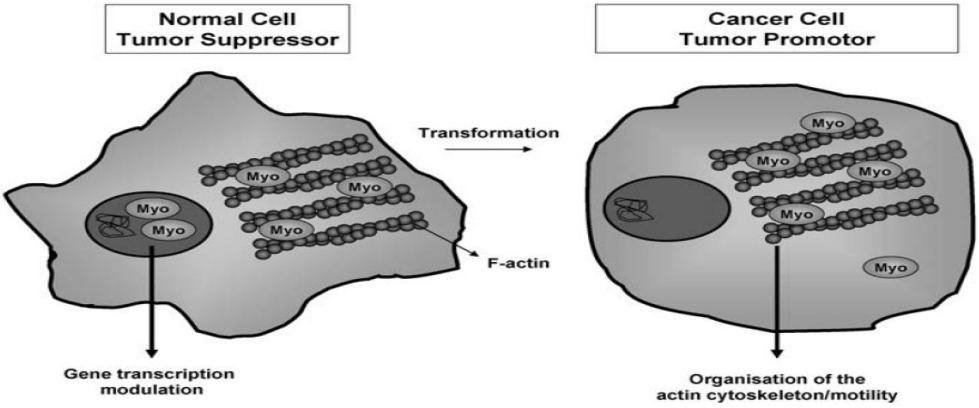

Figure 3: Model showing myopodin's localization and role in cancer. (De Ganck 2009)

Epigenetic effects within the human genome can also drive cancer. The most common epigenetic modification is DNA methylation, which can alter the expression of a gene without altering the DNA sequence. A CpG island has been identified encompassing the transcription site of myopodin. Myopodin has been found to be highly methylated in numerous bladder tumors and the methylation was associated with tumor stage and grade (Cebrian 2008).

Little is known about the function of each isoform. This study will be isoform specific due to the use of antibody NC 781, obtained from Dr. Joseph Chalovich, Brody School of Medicine, a polyclonal antibody produced from rabbits which is affinity purified against the peptide epitope that is found in exon 4b. This exon is only located within isoform B and myopodin. To further analyze isoform specific function, primers were prepared to insert myopodin into two different expression vectors. A fusion protein was developed by cloning the cDNA of myopodin into vector pEGFP-N3 that encodes a red-shifted variant of GFP for brighter fluorescence and higher expression in mammalian cells. This was used to study the localization of overexpressed myopodin within the cell. Also myopodin was cloned into an inducible vector to establish a stable cell line that would allow for controlling of the gene expression. Cell proliferation assay using the inducible vector will allow us to study the function of myopodin on proliferation. Future research can be completed using the inducible cell line, such as, wound healing assay, and invasion assays.

This thesis is written so that each chapter is independent of the others. The first chapter is an in depth background of synaptopodin 2 and followed by the second chapter which is an independent paper. The last chapter is an expansion of the materials and methods used throughout this thesis.

CHAPTER TWO

EFFECTS OF OVEREXPRESSED MYOPODIN ON CELL PROLIFERATION

Melissa Harkins,

ABSTRACT:

Synaptopodin-2 is a proline-rich, actin-binding protein that exists in several isoforms. Due to the large content of proline, syanptopodin-2 is in a naturally unfolded state and possesses the characteristics of a hub protein. Members of the synaptopodin family have been shown to bind with actin and induce actin polymerization (Chalovich and Shroeter, 2010). It has been found that synaptopodin can be a regulator for α-actinin, which has actin-bundling activity (Asunama 2005). Asunama has shown that synaptopodin can bind with α-actinin and inhibit the branching of α-actinin-induced actin filaments. This interaction is isoform specific and causes bundling and elongation of α-actinin-induced actin filaments. The significance of possessing the ability to stimulate actin and induce actin bundling lies in the fact that the remodeling of the actin cytoskeleton is important for cell migration, adhesion, division and development. Cancer cell metastasis is a multi-stage process involving invasion into surrounding tissue (intravasation) transit in the blood or lymph, extravasation, and growth at a new site. Many of these steps require cell motility, which is driven by cycles of actin polymerization, cell adhesion and acto-myosin contraction (Olsen 2008). Therefore studies have been completed to answer the role of synaptopodin-2 in cancer and contradicting results have been concluded. Interestingly, contradicting results have also been observed in other actin binding proteins: gelsolin, CapG, and

α actinin (De Ganck 2009). Therefore not allowing actin-binding proteins to be classified as tumor activators or suppressors. This work provides evidence of syanptopodin-2, isoform myopodin, function as a tumor suppressor activity and also suggests to another isoform of synaptopodin-2 existing within the nucleolus of Hela cells.

Introduction

Synaptopodin-2 is a proline rich, actin-associated protein lacking globular domains that is commonly found in kidney, and skeletal muscle tissues in mammals. Due to the abundance of proline, Synaptopodin-2 cannot form secondary and tertiary structures; therefore it exists in a naturally unfolded state that allows it to bind to many partners and is considered a hub protein. Hub proteins are known to bind to many partners and are involved in a number of signaling cascades (Iakoucheva et al. 2002). Large unfolded proteins such as hub proteins are highly flexible which allow them to adapt to different binding sites or possess the ability of readily reversible binding. It is known that many cancer-associated proteins act like hub proteins (Goh 2007). BRCA1, p53, caldesmon, α-synuclein and calmodulin (Tompa 2009) have been identified as hub proteins, which have all been linked to some form of cancer. Therefore, studying synaptopodin-2 may help answer questions about the interactions and networks of cancer proteins.

Synaptopodin-2 protein is part of the synaptopodin family of proteins. This family consists of three members: synaptopodin, synaptopodin-2, and synaptopodin 2-like proteins. Each member has numerous isoforms that arise from alternative splicing and all are in a naturally unfolded state. At least four different isoforms of Synaptopodin-2 have been described: isoform A, isoform B, isoform C and myopodin. There is also a small protein consisting of 123 amino acids that is only known as the listed NCBI number BAG65020. This unnamed protein does not share the common core region the other four isoforms share. These isoforms are the result of alternative splicing from a single gene located on chromosome 4 q 26. The gene contains seven exons: exon 1, 2, 3, 4a, 4b, 5, and 6. Between exon 4a and 4b no intron is present. All of these isoforms contain at least some part of exon 4a but differ at the c-terminus region of the protein

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because of contradicting results in studies. This is no different for myopodin. This study is aimed to answer some questions about the expression, localization and function of synaptopodin-2 isoform myopodin. We investigated the potential tumor suppression function of myopodin and indicate that there may be a new unpublished truncated isoform of myopodin.

Materials and Methods

Cell Culture

Cervical adenocarcinoma cells, Hela, were obtained from ATCC (#CCL-2) and cultured in Dulbecco's Eagle Medium (DMEM)/F12, supplemented with 10% fetal bovine penicillin 100U and streptomycin 100ug/ml. Cells were kept in a 37° C/ 5%. CO_2 incubator.

Antibiotic Sensitivity Concentration

The minimum concentration of blasticidin and zeocin was determined for the usage to establish a stable cell line after transfection. Hela cells were plated in 6 well plates to allow for 25% confluency upon the addition of antibiotics. Blasticidin concentrations were 0, 1,3,7.5, and 10 μg/ml, while zeocin concentrations were 0, 50, 125, 250, 500, and 750 μg/ml. Antibiotic supplemented media was changed every 3-4 days and the appropriate concentration of antibiotic that prevents growth within 1-2 weeks was determined. Complete cell death was observed with blasticidin at 3μg/ml and zeocin at 150 μg/ml.

Immunostaining

Coverslips were seeded with Hela cells to obtain 25-40% confluency. Coverslips were then washed with 1X PBS for five minutes and the cells were fixed with 3.7% formalin in 1X PBS for 8 minutes at room temperature. The coverslips were washed with 1X PBS for 15 minutes, three times. Cells were permeabilized with 1% triton in 1X PBS for four minutes. After an additional wash in 1X PBS, unspecific binding sites were blocked with 50 μl of 10% Fetal Bovine Serum in 1X PBS for 30 minutes. Primary antibody NC 781 diluted 1:600, was applied to the cells for 45 minutes at room temperature then washed three times in 1X PBS.

The cells were then incubated with secondary antibody, anti-rabbit conjugated HRP diluted 1:150 for 45 minutes in the dark, washed three times for 15 minutes each with 1X PBS, counter stained with DAPI, 1µg/ml, mounted with vectashield and visualized using the Olympus IX2-DSU confocal microscope with Hamamatsu EM-CCD digital camera.

Creation of Myopodin-GFP Fusion Protein

The cDNA of synaptopodin-2 isoform myopodin was cloned into the vector pEGFP-N3, which encodes a red-shifted variant of GFP for brighter fluorescence and higher expression in mammalian cells. Sequence analyses were performed to verify the integrity of the amplicons and ensure reading frame accuracy. The plasmids were transfected into HeLa cells using the following method: the highest efficiency was achieved with seeding coverslips in 6 well plates at density of 2x10^5 for each well. Incubated at 37°C with 5% CO2 for 24 hours. DNA at a concentration of 4.3µg was added to 100ul of serum free media in a micro centrifuge tube and 8µl of Lipofectamine 2000 was added to 100µl of serum free media in separate micro centrifuge tube. These were allowed to incubate for 20 minutes. Then the two tubes were combined, gently mixed and allowed to incubate for 30 minutes to allow for the complexes to form. Once the complexes had formed .8ml of serum free media was added to the tube. The coverslips were washed with 2 mls of serum free DMEM and the DNA/Lipofectamine complexes were added to the cells. The plate was gently rocked to ensure even distribution and placed in the incubator for 5 hours. After incubation transfection media was removed and fresh media containing 10% FBS was added to the cells. After 24 hours the expression of the gene can be visualized.

Whole Cell Protein Lysate Preparation

For protein lysate preparation a T75 flask with adhered cells was washed with 1X PBS and lysed with 1ml of L-Buffer and 100ul of protease inhibitor cocktail was added to the flask. The cells were scraped with a cell scraper and the solution was placed in a micro centrifuge tube. To shear the DNA and disrupt the cell membrane further, sonication was performed on ice three times for thirty seconds at an output of 1. The lysate was then centrifuged at 4° C for 10 minutes at 10,000 g. The supernatant was removed carefully not to disrupt the pellet and the pellet was resuspended in 2X Laemmli buffer. The lysate was then boiled at 95°C for ten minutes and stored at -80°C.

L-Buffer

-50mM Tris-HCL pH 6.8

-2mM EDTA

1% NP40

Bring to 10mls with dH_2O

Polymerase Chain Reaction

Plasmid containing inserts for GFP-fusion or human skeletal muscle cDNA was used as the template. A PCR core mix was prepared by combining sterile water, 10X High Fidelity buffer, 50 mM $MgCl_2$, 10 mM dNTPs, DMSO and High Fidelity Platinum Taq. The PCR core mixed was then added to each individual 0.2 ml PCR tubes that contain 1 µL or <500ng of cDNA template and 5µl of a forward and 5µl of a reverse primer. A negative control tube was prepared in which the cDNA template was omitted. These samples were placed in a thermocycler. The thermocycler was programmed for initial denature of the cDNA at 94°C for 2 minute. Thirty-five cycles of denaturing for an additional 1 minute at 94°C, annealing for 30 seconds at 60°C and

extending for 4.5 minutes at 68°C. After the thirty-five cycles, 1 extension at 68°C for 10 minutes was performed.

Primers Used

Myopodin Forward: PCR insert for GFP vector:

5'-GAGAAGCTTAAAACATGTTTAAGAAGCGTCGGAGG-3'

- PCR insert GFP vector reverse:

5'-GAGGGATCCCTCTTCCACAACAGATGG TTTCC-3'

Myopodin Forward: PCR Insert TO vector

5'-GAGAAGCTTAAAACATGTTTAAGAAGCGTCGGAGG-3'

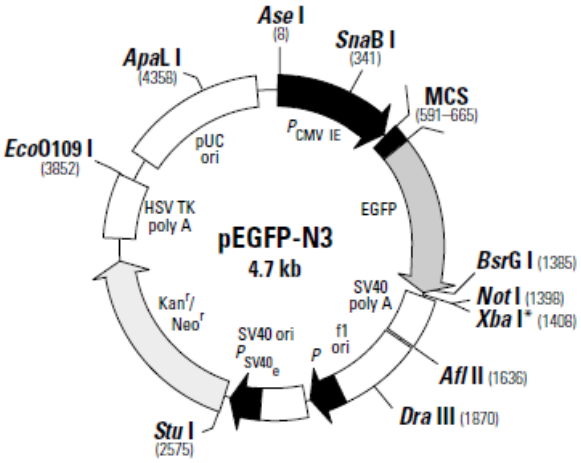
Reverse Complement Synpo Iso B BamhHI: PCR insert TO vector

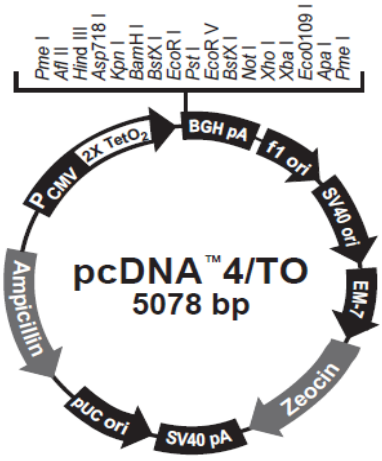
5'GAGGGATCCTTAGTGGTGATGGTGATGATGGCCCCCTCCCTCTTCCACAACAGATG GTTTCC-3'

Cloning for Transient Expression and Inducible Expression

The genes of interest were cloned into the vector pEGFP-N3 (Clontech), which encodes a red-shifted variant of GFP for brighter fluorescence and higher expression in mammalian cells. By ligating the synaptopodin-2 isoform Myopodin and isoform B to the N-terminus of EGFP, then transformed the vector into DH5α and propagated. The plasmid was used to transfect cell lines of choice.

Synaptopodin-2 isoform myopodin was also ligated into the multiple cloning site of the inducible expression vector pcDNA4/TO and propagated the same way as the GFP-myopodin plasmid.





pEGFP-N3 is a mammalian expression vector that encodes a red shift variant of GFP, which was received from the Lemasson lab at the Brody School of Medicine (left). T-REx pcDNA4/TO mammalian expression vector purchased from Invitrogen (right).

Ligation & Transformation into pcDNA4/TO vector

The DNA inserts for synaptopodin 2 isoform myopodin were ligated into the tetracycline inducible vector pcDNA4/TO using T4 DNA Ligase (Invitrogen). A reaction mixture was prepared containing 5X Ligation Buffer, a 6:1 molar ratio of insert to vector DNA, T4 DNA Ligase, and autoclaved distilled water according to the manufacturer's instructions. The reaction was incubated for 2.5 hours at room temperature. 2 µl of the reaction was used to transform into DH5α, competent cells (Invitrogen). Competent cells were thawed on ice to keep their efficiency level high; 2 µl of the reaction mixture were added and mixed gently with a pipette tip. After incubated on ice for 30 minutes, the cells were heat-shocked at 42°C for 45 second followed by incubation on ice for 2 minutes to reduce the damage to the *E. coli* cells. 1 ml of super optimal broth with catabolite repression (SOC) was added cells were incubated for 1 hour at 37°C in a

shaking incubator (225 rpm). 150 µl and 300 µl of the mixture was added to LB/ampicillin plates and allowed to grow overnight at 37°C. Colonies were selected and grown overnight in LB broth containing antibiotic ampicillin. Plasmids were purified using the Wizard Mediprep DNA purification system following the manufacturer's instructions. Plasmids were then digested using the restriction enzymes *Bam*HI (Invitrogen) and *Hin*dIII (Invitrogen) and run on a 0.8% agarose gel by gel electrophoresis for analysis. Potentially positive clones were sequenced and confirmed.

Establishment of a stable inducible cell line expressing Myopodin

To generate a HeLa cell line, that will inducible express myopodin, the transfection protocol above was performed with a few modifications to allow for a cotransfection of our Myopodin-pcDNA4/TO construct and the pcDNA6/TR and the pcDNA4/TO/lacZ control and the pcDNA6/TR. Hela cells were seeded in 6 well plates at a density of 2x10^5. Once the cells reached 70% confluency, cotransfection of the pcDNA6/TR and the inducible expression construct was conducted at a 6:1 ratio. 4.5µg of Myopodin-pcDNA4/TO plasmid and 27µg of pcDNA6/TR was diluted into 100µl of serum free media. Also 8µl of Lipofectamine 2000 was diluted in a separate tube. Both were allowed to incubate for twenty minutes at room temperature and then followed the steps described above. After the five hours incubation, the transfection mixture was removed from the cells and tetracycline-reduced FBS media was added. After twenty-four hours, the media was removed and the cells were split to achieve no more than 25% confluency within the 6 well plates. Selection media with the concentration of blasticidin and zeocin previously determined (3µg/ml of Blasticidin and 150µg/ml of Zeocin in Tet-reduced

FBS media) was added once the cells re-attach to the well. The media was changed every 3-4 days until the development of foci.

For the stable cell line that inducible expresses lacZ, 3.5μg of pcDNA4/TO/lacZ and 19μg of the pcDNA6/TR was used and same cotransfection protocol was followed as above.

Expansion of foci

After foci developed, a P-1000 was used to transfer individual foci into single wells of a 48 well plate. At all times the cells were maintained in selection media. The individual clones were expanded until numerous T-75 flasks were established.

Inducing of Myopodin Production

To induce the protein of interest, tetracycline was added to the selection media at a final concentration of 1μg/ml and incubated with the cells for at least 24 hours.

Western Blot

Preparation of SDS-Page Gels

An 8% resolving gel was prepared according to manufacturers' instruction (BioRAD) and poured in-between two BioRad Mini-Protein Tetra plates. Water was added on top to provide a barrier from oxygen and allowed to polymerize for at least 1 hour at room temperature. After polymerization, the water was removed and a 6% stacking gel was poured over it, a comb was inserted, and allowed to polymerize for at least 30 minutes at room temperature. The gels were either stored at 4° C with buffer for up to 1 week or used immediately.

Loading samples and running the gel

The gels are placed in the BioRAD Mini Protein Tetra apparatus and filled with Electrode buffer. The comb was removed and the wells were cleaned of all residues. 10-20 µl of prepared samples were loaded into the wells and electrophoresed at a constant 95V. The resolving gel was placed in transfer buffer while preparing for transfer.

Transfer

A semi-dry transfer was used to transfer proteins onto PVDF membrane (positively-charged nylon). PVDF membrane paper was cut according to the size of the gel pretreated with 100% methanol for 15 seconds, then transferred to distilled water for two minutes and equilibrated with cold transfer buffer for 5 minutes. Six piece of Whatman paper #1, were soaked in transfer buffer. Three pieces were placed onto the anode electrode plate, then the membrane, followed by the gel and additional 3 layers of Whatman papers. The cathode plate was placed on and the transfer was ran for 75 minutes at 250 mA. The membrane was blocked at room temperature under constant agitation in 5% milk, diluted in 1X TBST buffer for one hour. Primary antibodies were applied in appropriate dilutions and incubated under constant agitation overnight at 4°C:

Primary Antibodies used

NC-781 diluted 1:2000 in 5% milk/TBST solution

Monoclonal Anti-Beta Actin, Sigma, diluted 1:2000 in 5% milk/TBST solution

Anti 6X His-tag, Aviva Systems Biology, diluted 1:2000 in 5% milk/TBST solution

After primary antibody incubation, the membrane was washed numerous times with 1X TBST and then incubated with secondary antibody under constant agitation for one hour at room temperature.

Secondary Antibodies used

Goat anti rabbit conjugated with HRP, Pierce ImmunoPure, diluted 1:5000 in 5% milk/TBST solution

Goat anti mouse conjugated with HRP, ThermoScientific ImmunoPure, diluted 1:5000 in 5% milk/TBST solution

The membrane was thoroughly washed multiple times with 1X TBST before visualization of the protein.

Chemiluminescence

For detection of the protein, Advansta WesternBright ECL kit was used per manufactures' protocol and visualized with Chemidoc XRS System.

Cell Proliferation Assay

To study the effects of myopodin expression on the proliferation of HeLa cells, a cell growth curve was completed using the inducible myopodin stable cell line. Thirty 35mm petri dishes were seeded at a density of 150K cells per dish. Fifteen of the petri dishes were induced using tetracycline at a final concentration of 1μg/ml in the selection media and the other 15 petri dishes were maintained in selection media without tetracycline. The cells were kept at 37°C with 5% CO_2 for five days. Every day for five days a cell count was completed on three of the induced cells and three of the non-induced cells and the average was calculated for each. The media was changed every 2 days.

Results

This study was performed to investigate the expression of synaptopodin-2 isoform B or myopodin in Hela cells. Whole cell lysates were prepared and analyzed on an 8% resolving SDS-Page gel. After transfer to PVDF membrane the western blot was analyzed for the presence of synaptopodin 2 isoform B or myopodin using the primary antibody, NC 781. Neither myopodin nor isoform B was detected in HeLa cell whole cell lysate. We detected an immuno-reactive protein migrating with an apparent size between 50 and 70 kDa (Figure 4).

These results were similar to previous findings in our lab where we showed that myopodin is expressed in a human colon adenocarcinoma cells (HT29) (Baxley et al., submitted) along with a 62 kDa immunoreactive protein. That 62 kDA protein was present in the nucleolar fraction of HT29 cells but absent from the nuclear and cytoplasmic fractions. These data suggested the presence of a smaller truncated version, or a new isoform of myopodin.

To study the localization of this immuno-reactive protein, immunostaining of Hela cells with NC 781 antibody, was completed. Using DAPI, a fluorescent stain that binds to double stranded DNA, to label nuclear DNA, the protein was found to be strongly expressed within the nucleolar region of Hela cells. (Figure 5)

To confirm that myopodin expression within Hela cells was not due to an artifact of fixation, we cloned the myopodin cDNA sequence into a GFP expression vector, peGFP-N3 to form a fusion protein with GFP sequence located at the c-terminus of myopodin. The construct was transfected into Hela cells and the GPF expression followed using an epi-fluorescence microscope. (Figure 6) Overexpression of myopodin-GFP fusion protein was restricted to the nucleus of the cell with no expression being found in the cytoplasm. In contrast, the empty GFP vector used as a control, showed expression in both the nucleus and the cytoplasm.

We analyzed the kinetic of expression of the fusion protein into Hela cells at 12, 24, and 48 hours. At 12 hours, we were able to observe that myopodin-GFP expression was located within both the nucleus and cytoplasm. By 24 hours the expression of the fusion protein was mainly localized in the nucleus where it remained throughout the 48-hour time point. (Figure 7)

To study the effects of overexpression of myopodin on cell functions such as proliferation, we engineered a stable cell line containing an inducible vector expressing myopodin. The cDNA of myopodin with the addition of a polyhistidine-tag was PCR amplified and cloned into the inducible expression vector pcDNA4/TO. Tetracycline regulation in the system is based on the binding of tetracycline to the Tet repressor and derepression of the promoter controlling expression of the gene of interest. In the absence of tetracycline, the Tet repressor, pcDNA6/TR, binds to the promoter repressing the gene of interest. Once tetracycline is added, it binds to the repressor causing a conformational change rendering it unable to bind to the promoter. Therefore, allowing expression of the gene of interest. Western Blot analysis using primary antibody NC 781/his-tag, was used to confirm that we were able to induce the expression of myopodin in the presence of tetracycline. We were able to detect a band corresponding to the endogenous myopodin at around 62 kDa in size in both the induced cells and non-induced cells. In the induced cells, a protein was observed at 100 kDa in size. This was not observed in the non-induced cells. (Figure 8) Since this was not the size expected, a control stable cell line containing a LacZ cloned into the pcDNA/TO vector was used to verify that the 100 kDa protein was not an artifact due to the vector itself. The western blot analysis confirmed that the 100 kDa size band was not a product of the vector itself (Figure 9). The final confirmation that the induced protein was indeed the product of cloned myopodin, a western blot

was performed with an anti 6X His-tag antibody. A band corresponding to 100 kDa in size was detected in the induced cells, which was not present in the uninduced. (Figure 10)

To investigate the possible tumor suppression activity of myopodin, we performed a cell proliferation assay using the inducible stable cell line. The results showed that the cells overexpressing myopodin grew significantly slower than the control (non-induced) (Figure 11). These data suggest that overexpression myopodin can act as tumor suppressor in Hela cells.

Figure 4

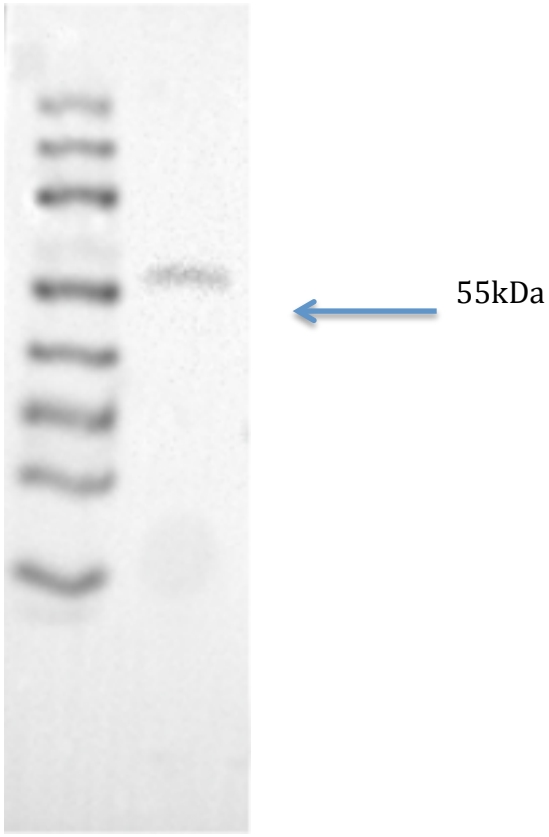


Figure 4: **Western Blot** Endogenous expression in Hela, human cervical adenocarcinoma cells recognized by antibody NC781. Whole cell lysate from Hela cells. Band found at approximately 60kDa in size.

Figure 5:

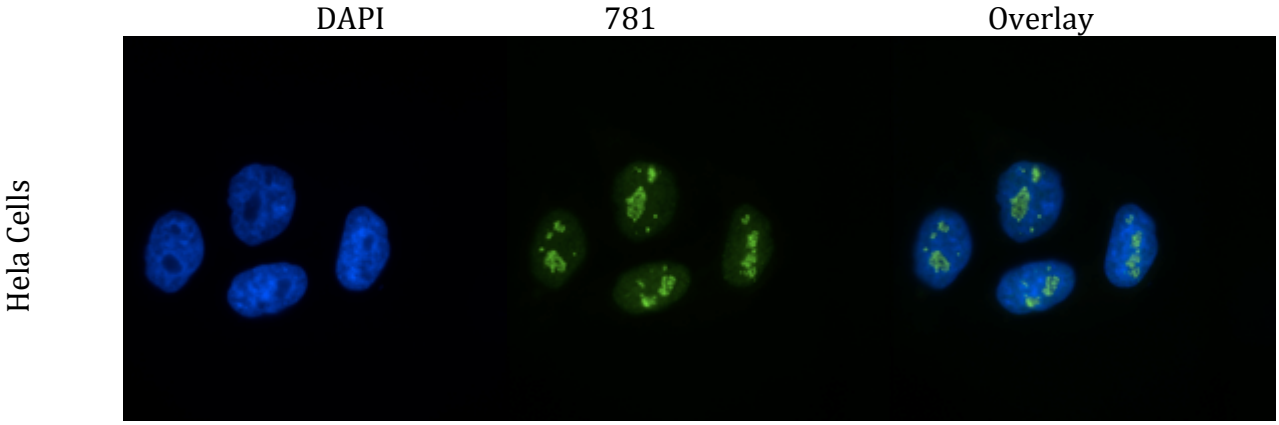


Figure 5: Endogenous expression recognized by antibody NC 781. Strong Expression occurred in the nucleolus of Hela cells.

Figure 6

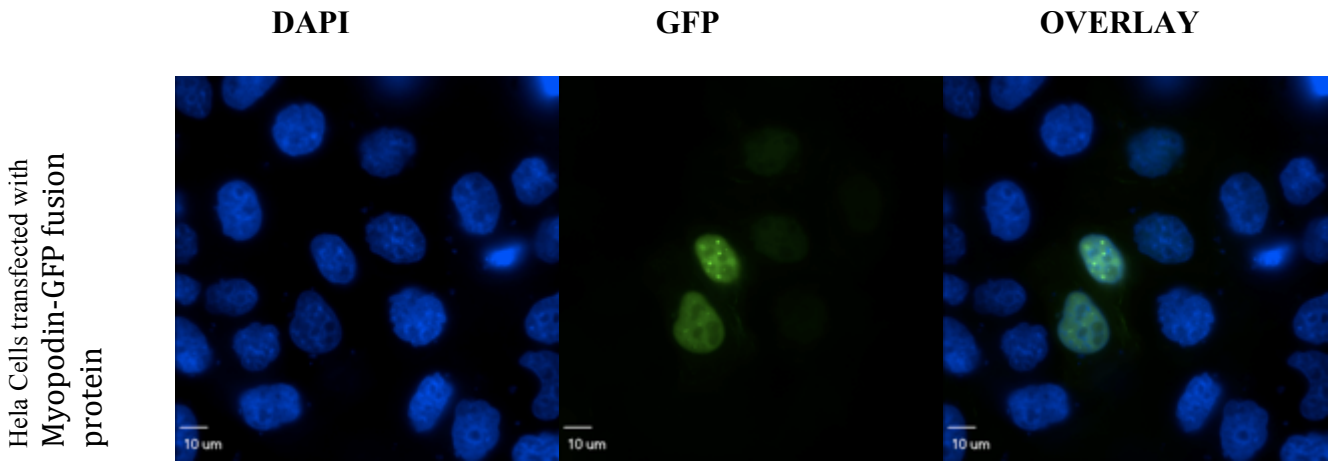


Figure 6: Expression of Myopodin-GFP fusion protein in Hela cells. Expression was localized with in the nucleus of the cell.

Figure 7:

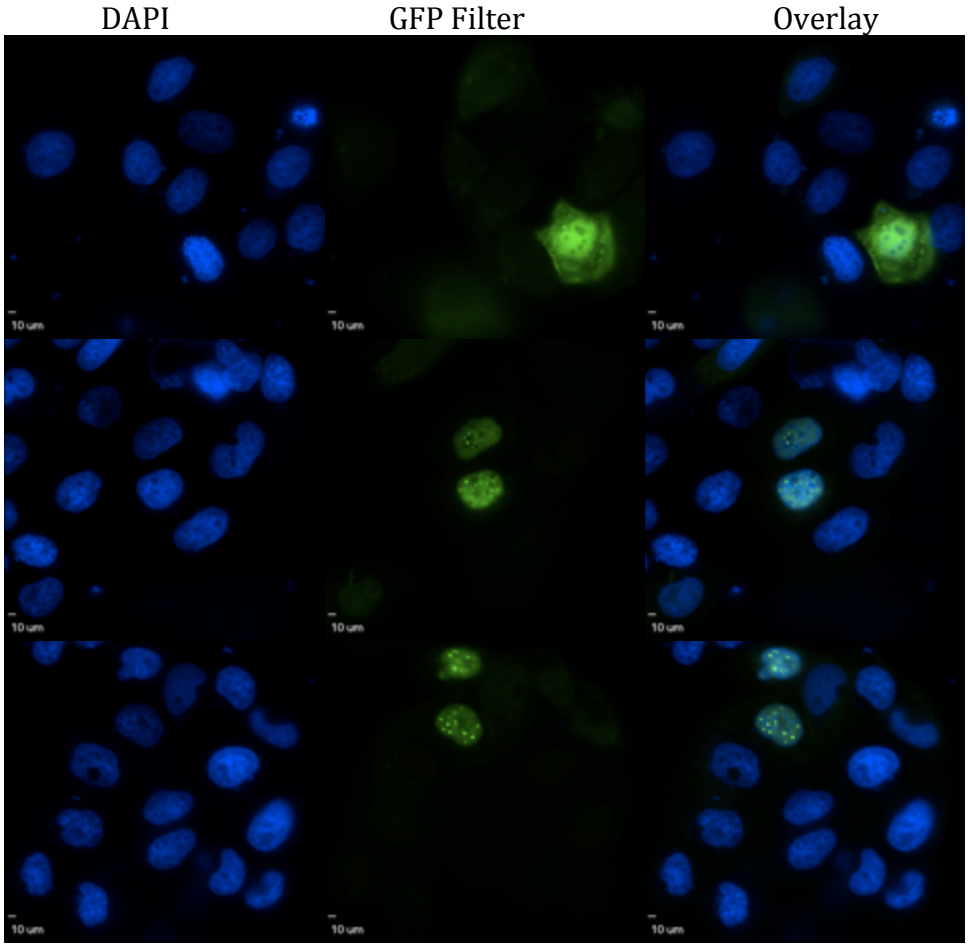


Figure 7: Translocation of isoform myopodin-GFP fusion protein in Hela cells over time course. Cytoplasmic and nuclear expression at 12 hour time point. By 24 hours after transfection expression was only localized with in the nucleus.

Figure 8

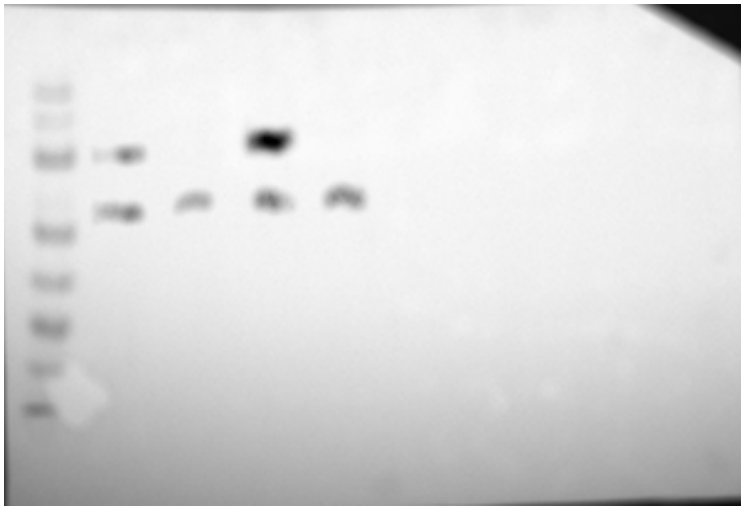


Figure 8: Western Blot. Lane 1: Colorburst Ladder. Lane 2: MYO/TO Stable clone 1, tetracycline treated induced. Lane 3: MYO/TO Stable clone 1, non-induced. Lane 4: MYO/TO Stable clone 3, tetracycline treated induced. Lane 5: MYO/TO Stable clone 3 non-induced. Antibody NC 781

Figure 9

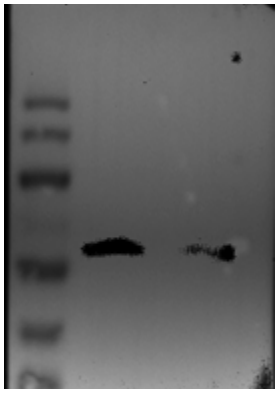


Figure 9: Western Blot. Lane 1: Colorburst ladder. Lane 2: LacZ/TO clone, tetracycline treated induced. Lane 3: LacZ/TO clone non-induced. Antibody NC 781

Figure 10

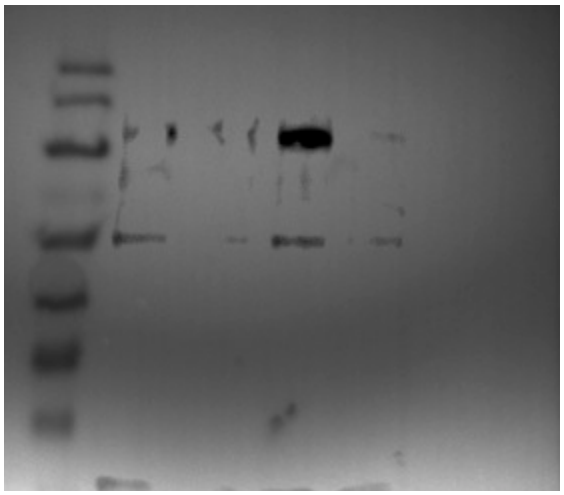


Figure 10: Western Blot. Lane 1: Colorburst Ladder Lane 2: MYO/TO Stable clone 1 induced. Lane 3: MYO/TO Stable clone 1 non-induced. Lane 4: MYO/TO Stable clone 3 induced. Lane 5: LacZ/TO stable induced. Lane 6: LacZ/TO non-induced
Antibody 6-His Tag

Figure 11

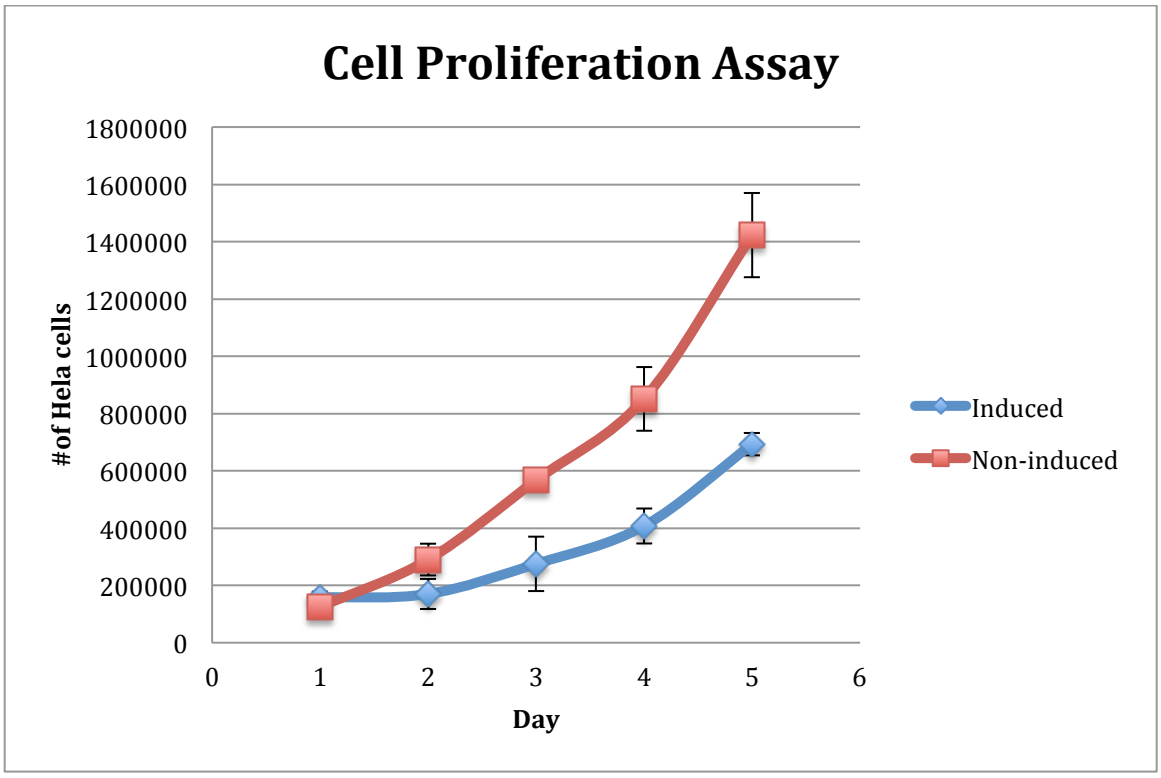


Figure 11: Tumor suppression activity of myopodin. Cell proliferation of overexpressed myopodin compared to non-induced Myopodin/TO stable cell line. Retardation shown in cell proliferation of cells overexpressing myopodin.

Discussion

Synaptopodin-2 protein belongs to the synaptopodin family. This family consists of three members: synaptopodin, synaptopodin-2, and synaptopodin 2-like proteins. Each member has numerous isoforms that arise from alternative splicing, and all are in a naturally unfolded state. Members of the synaptopodin family have been shown to bind with actin. Actin-binding proteins have failed to be classified as tumor activators or tumor suppressors because of contradicting results in studies. This is no different for myopodin, an isoform of synaptopdin-2. In this study, we have demonstrated the tumor suppression properties of the protein using a cell line with an inducible protein construct. Upon overexpression of myopodin there is approximately a 2-fold reduction in cell proliferation. Previous studies have shown that multiple isoforms of myopodin are simultaneously transcribed in cancers. Our previous studies have located a protein approximately 62kDa in size within the nucleoli extracts of HT-29 cells and myopodin located within the nucleus. Immunostaining of HT-29 cells showed a nuclear expression with pronounced staining in the nucleolus. This study has demonstrated a protein that is immuno-reactive with the antibody, NC-781 is found endogenously in Hela cells approximately 62 kDa in size. Interestingly immunostaining of Hela cells, we found that the endogenous expression lies within the nucleolar region of the cell with low expression in the nucleus. This suggests another synaptopodin-2 isoform that has not been published so far exists within the nucleolus. This is not uncommon for proteins to have a truncated version. A recent study has shown that the protein Netrin-1 has a truncated version that is located within the nucleolus, and it has been associated with cancer cell proliferation (Delloye-Bourgeois 2012). Further studies involving purification and sequencing of this unknown protein has to be completed. Affinity chromatography with the

use of antibody NC 781 should be used on Hela cells since they appear not to endogenously express any other immune-reactive protein unlike HT-29.

Analysis of synaptopodin-2 isoform myopodin-GFP fusion protein within Hela cells confirms a nuclear localization but not a nucleolus expression. This could be that processing of myopodin to the possible truncation form is necessary for translocation into the nucleolus. However the GFP moiety could be blocking the site needed for nucleolar translocation. Western blot analysis corresponded to the full myopodin construct and a band to the endogenous 62kDa protein. Time point analysis of the translocation of myopodin was completed to conclude that nucleolar translocation wasn't occurring at a slow rate. Using a vector that has the GFP location to the N-terminus of the myopodin may show if the construct is blocking the site needed for translocation. Also, overexpression of myopodin may have resulted in nuclear location only.

Western blot of the inducible myopodin/TO cell line shows the endogenous possible truncated myopodin isoform, but after treatment with tetracycline to overexpress myopodin, a larger 100kDa band appears. This was not the size expected for 74 kDa myopodin. Further investigation need to be conducted to conclude the integrity of the protein being expressed. A possible mutation could have occurred causing a loss of the stop codon, yielding a protein being expressed larger than myopodin, but sequencing of the plasmid confirmed that such mutation had not occurred. another explanation could be that the wrong plasmid was transfected into the cell line. Synaptopodin-2 isoform B is approximately 100 kDa in size and would be recognized by the NC781 antibody as well. Future studies using, PCR amplify with primers from the vector flanking the insert to confirm the construct in the stable cell line.. Isolation and purification using affinity chromatography for the polyhistidine tag will be performed; the pure protein will be digested with trypsin and the resulting peptides will be sequenced and analyzed.

Analysis of localization of the induced protein during the cell proliferation assay should be completed as well. With the use of the anti-his tag antibody in conjunction with the proliferation assay cells should be seeded on coverslips and imaged when the cell count is performed.

This study has demonstrated that there are other isoforms of synaptopodin-2 being expressed within cancer cells that are not previously published. The functions and expression of different isoforms may be more complex than previously described.

Chapter 3

Materials and Methods

Cell Culture

Cervical adenocarcinoma cells, Hela, were obtained from ATCC (#CCL-2) and cultured in Dulbecco's Eagle Medium (DMEM)/F12, supplemented with 10% fetal bovine penicillin 100U and streptomycin 100ug/ml. Cells were kept in a 37° C/ 5%. CO_2 incubator.

Antibiotic Sensitivity Concentration

The minimum concentration of blasticidin and zeocin was determined for the usage to establish a stable cell line after transfection. Hela cells were plated in 6 well plates to allow for 25% confluency upon the addition of antibiotics. Blasticidin concentrations were 0, 1,3,7.5, and 10 µg/ml, while zeocin concentrations were 0, 50, 125, 250, 500, and 750 µg/ml. Antibiotic supplemented media was changed every 3-4 days and the appropriate concentration of antibiotic that prevents growth within 1-2 weeks was determined. Complete cell death was observed with blasticidin at 3µg/ml and zeocin at 150 µg/ml.

Immunostaining

Coverslips were seeded with Hela cells to obtain 25-40% confluency. Coverslips were then washed with 1X PBS for five minutes and the cells were fixed with 3.7% formalin in 1X PBS for 8 minutes at room temperature. The coverslips were washed with 1X PBS for 15 minutes, three times. Cells were permeabilized with 1% triton in 1X PBS for four minutes. After an additional wash in 1X PBS, unspecific binding sites were blocked with 50 µl of 10% Fetal Bovine Serum in 1X PBS for 30 minutes. Primary antibody NC 781 diluted 1:600, was

applied to the cells for 45 minutes at room temperature then washed three times in 1X PBS. The cells were then incubated with secondary antibody, anti-rabbit conjugated HRP diluted 1:150 for 45 minutes in the dark, washed three times for 15 minutes each with 1X PBS, counter stained with DAPI, 1µg/ml, mounted with vectashield and visualized using the Olympus IX2-DSU confocal microscope with Hamamatsu EM-CCD digital camera.

Transfection Efficiency

To maximize our cell transfection efficiency, Hela cells were transfected with Myopodin GFP vector and the transient expression was visualized using the Olympus IX2-DSU confocal microscope. Three different variables were manipulated to achieve the highest transfection efficiency: seeding density, amount of DNA, and amount of transfection reagent. The highest efficiency was achieved with seeding coverslips in 6 well plates at density of $2x10^5$ for each well. Incubated at 37°C with 5% CO_2 for 24 hours. DNA at a concentration of 4.3µg is added to 100µl of serum free media in a micro centrifuge tube and 8µl of Lipofectamine 2000 was added to 100µl of serum free media in separate micro centrifuge tube. This was completed for each well to be transfected. These were allowed to incubate for 20 minutes. Then the two tubes were combined, gently mixed and allowed to incubate for 30 minutes to allow for the complexes to form. Once the complexes had formed .8ml of serum free media was added to the tube. The coverslips were washed with 2 mls of serum free DMEM and the total volume of DNA/Lipofectamine complexes were added to the cells. The plate was gently rocked for 30 seconds to ensure even distribution and placed in the incubator for 5 hours. After incubation, transfection media was removed

and fresh media containing 10% FBS was added to the cells. After 24 hours, the expression of the gene was visualized.

Counting to determine efficiency

Coverslips with adhered Hela cells were washed with 1X phosphate buffer saline (PBS) for five minutes at room temperature and cells fixed with 3.7% formalin in 1X PBS for 8 minutes. To remove fixing solution, cells were washed 3 times with PBS for 5 min and cells were then counterstained with 1µg/ml DAPI for 5 minutes and rinsed once with 1X PBS. The coverslips were then mounted cell side down onto slides using Vectashield and observed using Olympus IX2-DSU confocal microscope with Hamamatsu EM-CCD digital camera. To determine efficiency triplicates of coverslips were used and 4 random locations on each coverslip were analyzed. The number of cells expressing transient GFP were counted and divided by the number of cells stained by DAPI.

Creation of Myopodin-GFP Fusion Protein

The cDNA of synaptopodin-2 isoform myopodin was cloned into the vector pEGFP-N3, which encodes a red-shifted variant of GFP for brighter fluorescence and higher expression in mammalian cells. Sequence analyses were performed to verify the integrity of the amplicons and ensure reading frame accuracy. The plasmids were transfected into HeLa cells using the following method: the highest efficiency was achieved with seeding coverslips in 6 well plates at density of 2x10^5 for each well. Incubated at 37°C with 5% CO2 for 24 hours. DNA at a concentration of 4.3µg was added to 100ul of serum free media in a micro centrifuge tube and 8µl of Lipofectamine 2000 was added to 100µl of serum free media in separate

micro centrifuge tube. These were allowed to incubate for 20 minutes. Then the two tubes were combined, gently mixed and allowed to incubate for 30 minutes to allow for the complexes to form. Once the complexes had formed .8ml of serum free media was added to the tube. The coverslips were washed with 2 mls of serum free DMEM and the DNA/Lipofectamine complexes were added to the cells. The plate was gently rocked to ensure even distribution and placed in the incubator for 5 hours. After incubation transfection media was removed and fresh media containing 10% FBS was added to the cells. After 24 hours the expression of the gene can be visualized.

Whole Cell Protein Lysate Preparation

For protein lysate preparation a T75 flask with adhered cells was washed with 1X PBS and lysed with 1ml of L-Buffer and 100ul of protease inhibitor cocktail was added to the flask. The cells were scraped with a cell scraper and the solution was placed in a micro centrifuge tube. To shear the DNA and disrupt the cell membrane further, sonication was performed on ice three times for thirty seconds at an output of 1. The lysate was then centrifuged at 4° C for 10 minutes at 10,000 g. The supernatant was removed carefully not to disrupt the pellet and the pellet was resuspended in 2X Laemmli buffer. The lysate was then boiled at 95°C for ten minutes and stored at -80°C.

L-Buffer

-50mM Tris-HCL pH 6.8

-2mM EDTA

1% NP40

Bring to 10mls with dH_2O

Polymerase Chain Reaction

Plasmid containing inserts for GFP-fusion or human skeletal muscle cDNA was used as the template. A PCR core mix was prepared by combining sterile water, 10X High Fidelity buffer, 50 mM $MgCl_2$, 10 mM dNTPs, DMSO and High Fidelity Platinum Taq. The PCR core mixed was then added to each individual 0.2 ml PCR tubes that contain 1 μL or <500ng of cDNA template and 5μl of a forward and 5μl of a reverse primer. A negative control tube was prepared in which the cDNA template was omitted. These samples were placed in a thermocycler. The thermocycler was programmed for initial denature of the cDNA at 94°C for 2 minute. Thirty-five cycles of denaturing for an additional 1 minute at 94°C, annealing for 30 seconds at 60°C and extending for 4.5 minutes at 68°C. After the thirty-five cycles, 1 extension at 68°C for 10 minutes was performed.

Primers Used

Myopodin Forward: PCR insert for GFP vector:

5'-GAGAAGCTTAAAACATGTTTAAGAAGCGTCGGAGG-3'

- PCR insert GFP vector reverse:

5'-GAGGGATCCCTCTTCCACAACAGATGG TTTCC-3'

Myopodin Forward: PCR Insert TO vector

5'-GAGAAGCTTAAAACATGTTTAAGAAGCGTCGGAGG-3'

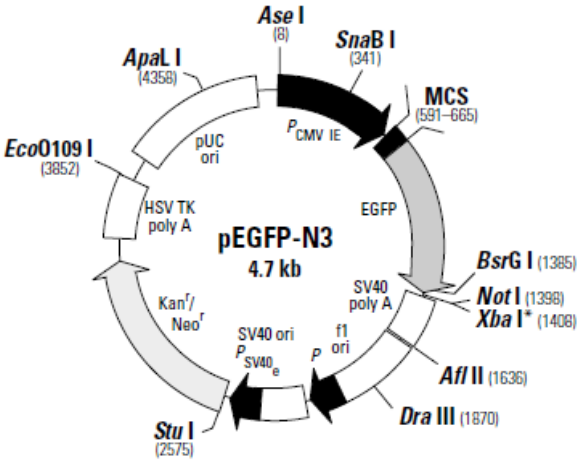
Reverse Complement Synpo Iso B BamhHI: PCR insert TO vector

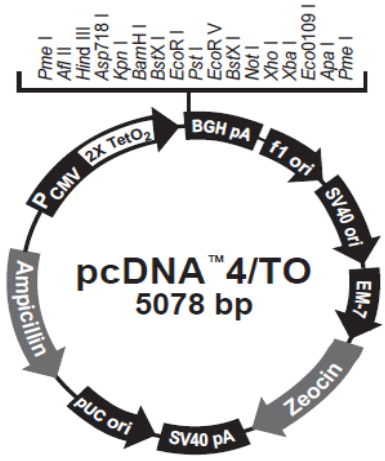
5'GAGGGATCCTTAGTGGTGATGGTGATGATGGCCCCCTCCCTCTTCCACAACAGATG GTTTCC-3'

Cloning for Transient Expression and Inducible Expression

The genes of interest were cloned into the vector pEGFP-N3 (Clontech), which encodes a red-shifted variant of GFP for brighter fluorescence and higher expression in mammalian cells. By ligating the synaptopodin-2 isoform Myopodin and isoform B to the N-terminus of EGFP, then transforming the vector into DH5α and propagating the plasmid was used to transfect cell lines of choice.

Synaptopodin-2 isoform myopodin was also ligated into the multiple cloning site of the inducible expression vector pcDNA4/TO and propagated the same way as the GFP-myopodin plasmid





pEGFP-N3 is a mammalian expression vector that encodes a red shift variant of GFP, which was received from the Lemasson lab at the Brody School of Medicine (left). T-REx pcDNA4/TO mammalian expression vector purchased from Invitrogen (right).

Ligation & Transformation into pcDNA4/TO vector

The DNA inserts for synaptopodin 2 isoform myopodin were ligated into the tetracycline inducible vector pcDNA4/TO using T4 DNA Ligase (Invitrogen). A reaction mixture was prepared containing 5X Ligation Buffer, a 6:1 molar ratio of insert to vector DNA, T4 DNA Ligase, and autoclaved distilled water according to the manufacturer's instructions. The reaction

was incubated for 2.5 hours at room temperature. 2 μl of the reaction was used to transform into DH5α, competent cells (Invitrogen). Competent cells were thawed on ice to keep their efficiency level high; 2 μl of the reaction mixture were added and mixed gently with a pipette tip. After incubated on ice for 30 minutes, the cells were heat-shocked at 42°C for 45 second followed by incubation on ice for 2 minutes to reduce the damage to the *E. coli* cells. 1 ml of super optimal broth with catabolite repression (SOC) was added cells were incubated for 1 hour at 37°C in a shaking incubator (225 rpm). 150 μl and 300 μl of the mixture was added to LB/ampicillin plates and allowed to grow overnight at 37°C. Colonies were selected and grown overnight in LB broth containing antibiotic ampicillin. Plasmids were purified using the Wizard Mediprep DNA purification system following the manufacturer's instructions. Plasmids were then digested using the restriction enzymes *Bam*HI (Invitrogen) and *Hin*dIII (Invitrogen) and run on a 0.8% agarose gel by gel electrophoresis for analysis. Potentially positive clones were sequenced and confirmed.

Establishment of a stable inducible cell line expressing Myopodin

To generate a HeLa cell line, that will inducible express myopodin, the transfection protocol above was performed with a few modifications to allow for a cotransfection of our Myopodin-pcDNA4/TO construct and the pcDNA6/TR and the pcDNA4/TO/lacZ control and the pcDNA6/TR. HeLla cells were seeded in 6 well plates at a density of 2x10^5. Once the cells reached 70% confluency, cotransfection of the pcDNA6/TR and the inducible expression construct was conducted at a 6:1 ratio. 4.5μg of Myopodin-pcDNA4/TO plasmid and 27μg of pcDNA6/TR was diluted into 100μl of serum free media. Also 8μl of Lipofectamine 2000 was diluted in a separate tube. Both were allowed to incubate for twenty minutes at room temperature

and then followed the steps described above. After the five hours incubation, the transfection mixture was removed from the cells and tetracycline-reduced FBS media was added. After twenty-four hours, the media was removed and the cells were split to achieve no more than 25% confluency within the 6 well plates. Selection media with the concentration of blasticidin and zeocin previously determined (3μg/ml of Blasticidin and 150μg/ml of Zeocin in Tet-reduced FBS media) was added once the cells re-attach to the well. The media was changed every 3-4 days until the development of foci.

For the stable cell line that inducible expresses lacZ, 3.5μg of pcDNA4/TO/lacZ and 19μg of the pcDNA6/TR was used and same cotransfection protocol was followed as above.

Expansion of foci

After foci developed a P-1000 was used to transfer individual foci into 48 single wells of a 48 well plate. At all times the cells were maintained in selection media. The individual clones were expanded until numerous T-75 flasks.

Inducing of Myopodin Production

To induce the protein of interest, tetracycline was added to the selection media at a final concentration of 1μg/ml and incubated with the cells for at least 24 hours.

Western Blot

Preparation of SDS-Page Gels

An 8% resolving gel was prepared according to manufacturers' instruction (BioRAD) and poured in-between two BioRad Mini-Protein Tetra plates. Water was added on top to provide a barrier from oxygen and allowed to polymerize for at least 1 hour at room

temperature. After polymerization, the water was removed and a 6% stacking gel was poured over it, a comb was inserted, and allowed to polymerize for at least 30 minutes at room temperature. The gels were either stored at 4° C with buffer for up to 1 week or used immediately.

Loading samples and running the gel

The gels are placed in the BioRAD Mini Protein Tetra apparatus and filled with Electrode buffer. The comb was removed and the wells were cleaned of all residues. 10-20µl of prepared samples were loaded into the wells and electrophoresed at a constant 95V. The resolving gel was placed in transfer buffer while preparing for transfer.

Transfer

A semi-dry transfer was used to transfer proteins onto PVDF membrane (positively-charged nylon). PVDF membrane paper was cut according to the size of the gel pretreated with 100% methanol for 15 seconds, then transferred to distilled water for two minutes and equilibrated with cold transfer buffer for 5 minutes. Six piece of Whatman paper # 1 were soaked in transfer buffer. Three pieces were placed onto the anode electrode plate, then the membrane, followed by the gel and additional 3 layers of Whatman papers. The cathode plate was placed on and the transfer was run for 75 minutes at 250 mA. The membrane was blocked at room temperature under constant agitation in 5% milk, diluted in 1X TBST buffer for one hour. Primary antibodies were applied in appropriate dilutions and incubated under constant agitation overnight at 4°C:

Primary Antibodies used

NC-781 diluted 1:2000 in 5% milk/TBST solution

Monoclonal Anti-Beta Actin, Sigma, diluted 1:2000 in 5% milk/TBST solution

Anti 6X His-tag, Aviva Systems Biology, diluted 1:2000 in 5% milk/TBST solution

After primary antibody incubation, the membrane was washed numerous times with 1X TBST and then incubated with secondary antibody under constant agitation for one hour at room temperature.

Secondary Antibodies used

Goat anti rabbit conjugated with HRP, Pierce ImmunoPure, diluted 1:5000 in 5% milk/TBST solution

Goat anti mouse conjugated with HRP, ThermoScientific ImmunoPure, diluted 1:5000 in 5% milk/TBST solution

The membrane was thoroughly washed multiple times with 1X TBST before visualization of the protein.

Chemiluminescence

For detection of the protein, Advansta WesternBright ECL kit was used per manufactures' protocol and visualized with Chemidoc XRS System.

Cell Proliferation Assay

To study the effects of myopodin expression on the proliferation of Hela cells, a cell growth curve was completed using the inducible myopodin stable cell line. Thirty 35mm petri dishes were seeded at a density of 150K cells per dish. Fifteen of the petri dishes were induced using tetracycline at a final concentration of 1μg/ml in the selection media and the other 15 petri dishes were maintained in selection media without tetracycline. The cells were kept at 37°C with 5% CO_2 for five days. Every day for five days a cell count was completed on three of the induced

cells and three of the non-induced cells and the average was calculated for each. The media was changed every 2 days.

Supplemental Data

Figure 12

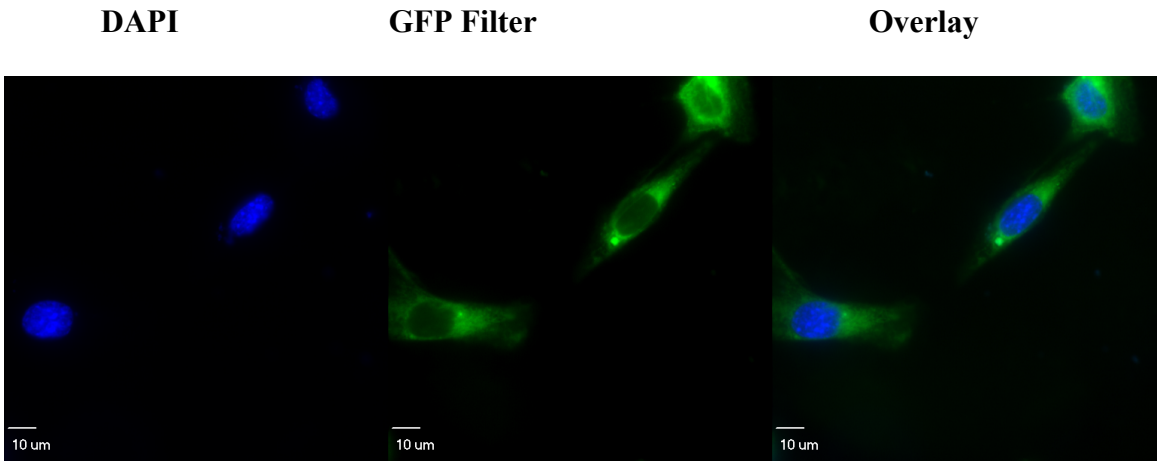


Figure 12: Myopodin-GFP fusion protein expression in C2C12 cells shows cytoplasmic expression.

Figure 13

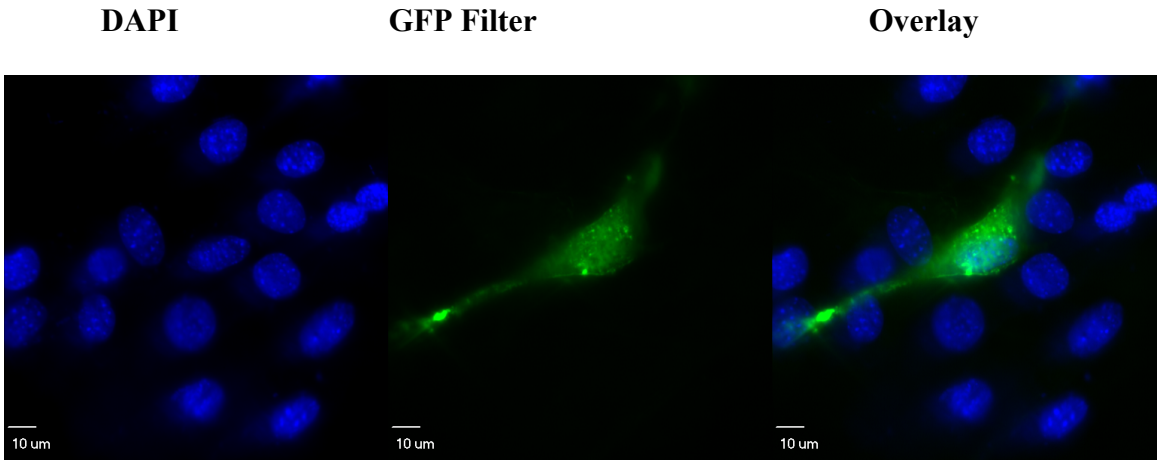


Figure 13: Synaptopodin-2 B-GFP fusion protein expression in C2C12 cells shows cytoplasmic expression.

Figure 14

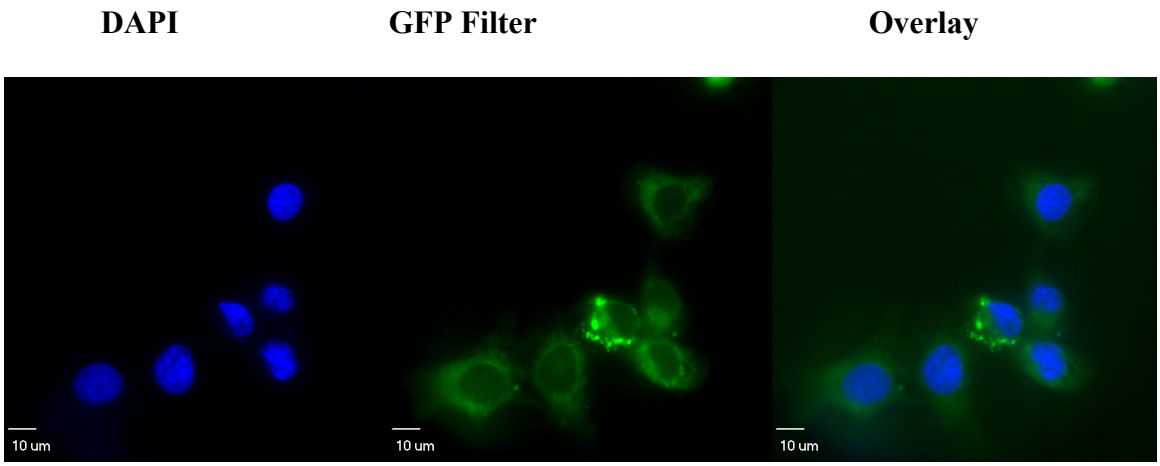


Figure 14: Synaptopodin-2 B-GFP fusion protein expression in HT-29 cells shows cytoplasmic expression.

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