Abstract

SYNAPTOPODIN-2 ISOFORM A AND D EXPRESSION IN HT-29 COLON ADENOCARCINOMA CELLS

by Kelli A. Shortt

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Director of Thesis/Dissertation: Dr. Jean-Luc Scemama & Dr. Margit Schmidt

DEPARTMENT OF BIOLOGY

Synaptopodin-2 is a largely unfolded actin-binding protein that also possesses the characteristics of a hub protein. Synaptopodin-2 has other known binding partners such as filamin and ∝-actinin (Weins, et al. 2001 and Linemann, et al. 2010), as well as zyxin and integrin-linked kinase (Yu, et al. 2006 and 2011). Previous research has shown that synaptopodin-2 promotes the rapid polymerization of actin (Chalovich and Schroeter, 2010). Cell migration, adhesion, division, and development depend on the dynamic remodeling of the actin cytoskeleton and evidence for actin-binding proteins playing tumorigenic roles in human cancers is increasingly accruing. Specifically, synaptopodin-2 has been suggested to act as a tumor suppressor in prostate and bladder cancer where loss of expression by deletion or hypermethylation leads to an increased rate of invasiveness (Lin et al., 2001 and Cebrian et al., 2008). In contrast to evidence supporting the tumor suppressor role of synaptopodin-2, a tumor activator role has also been proposed in which the over-expression of synaptopodin-2 in human endothelial kidney and mouse myoblast cells increased its invasiveness (Van Impe, et al. 2003). We hypothesize that the localization of synaptopodin-2 isoforms affects their function within HT-29 cells. As previous researchers have found synaptopodin-2 to act as a tumor suppressor when in the nucleus and a tumor activator when in the cytoplasm in prostate cancer cells, we expected to find similar results with a colon cancer cell line. Using isoform-specific GFP fusion

proteins, the role of synaptopodin-2 was studied in HT-29 human colon adenocarcinoma cells. While five isoforms of synaptopodin-2 have been reported (Chalovich and Schroeter, 2010), little is known about the function of these individual isoforms. This work provides evidence for the existence of two cytoplasmic isoforms of synaptopodin-2. We also observed that these synaptopodin-2 isoforms remain cytoplasmic after differentiation, in contrast to a nuclear isoform that translocates from the nucleus to the cytoplasm after differentiation in multiple cell lines. Ultimately, this research does not support the previous notion that synaptopodin-2 acts as a tumor suppressor in the nucleus and a tumor activator in the cytoplasm, but rather provides evidence that the effects of synaptopodin-2 are isoform dependent.

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by

Kelli A. Shortt

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Kelli A. Shortt

APPROVED BY:

DIRECTOR OF
DISSERTATION/THESIS: _____
Jean-Luc Scemama, PhD

COMMITTEE MEMBER: _____
Margit Schmidt, PhD

COMMITTEE MEMBER: _____
Joseph M. Chalovich, PhD

COMMITTEE MEMBER: _____
Mary A. Farwell, PhD

CHAIR OF THE DEPARTMENT
OF BIOLOGY: _____
Jeff McKinnon, PhD

DEAN OF THE
GRADUATE SCHOOL: _____
Paul J. Gemperline, PhD

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Chapter 1: Introduction

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. Synaptopodin-2 was first described as an actin-associated protein in kidney podocytes (Mundel et al. 1997), but has also been found in avian smooth muscle (Leinweber et al. 1999) and heart and skeletal muscle (Weins et al. 2001). Because Synaptopodin-2 is in a naturally unfolded state, it is considered a hub protein. Hub proteins bind to many partners and can affect many signaling cascades (Iakoucheva et al. 2002). Hub proteins are highly flexible which may allow them to adapt to different binding sites or possess the ability of readily reversible binding (Iakoucheva et al. 2002). Members of the synaptopodin family have been shown to bind with actin (Mundel et al. 1997). It can bind to other actin-binding proteins as well, with some of these possessing the ability to stimulate actin polymerization and induce actin bundling. Studies have found a mechanistic role for synaptopodin-2 as a regulator of the actin-bundling activity of α-actinin that may be important for the formation and dynamic reorganization of the actin cytoskeleton in highly specialized cell compartments (Asunama 2005). Many physiological processes like cell migration, adhesion, division and development are dependent on this dynamic remodeling of the actin cytoskeleton. The reversible formation of actin polymers is regulated by a set of actin binding proteins, and evidence demonstrating the multifunctional roles of actin binding proteins in tumorigenic and metastatic processes of various human tumors is steadily accruing (De Gank et al. 2008). As shown in Figure 1, synaptopodin-2 has also been found to bind to and cause rapid polymerization of G-actin; the inhibition of which is caused by Ca^{2+} calmodulin (Beall et al.; Schroeter et al). Association with α-actinin-4, an isoform of α-actinin that has been shown to shuttle from the cytoplasm to the nucleus during actin depolymerization (Honda et al., 1998;

Kumeta et al., 2009). Association with cell cytoskeleton proteins suggests synaptopodin-2 might be involved with the chromatin-remodeling complex and therefore maybe an important regulator of transcriptional activity (Castano et al., 2010). Another possible indication to the biological role of synaptopodin family is that proteins involved in cancer contain a larger than normal fraction of members with large unfolded regions (Iakoucheva et al. 2002) which would also hold true with hub proteins.

Figure 1

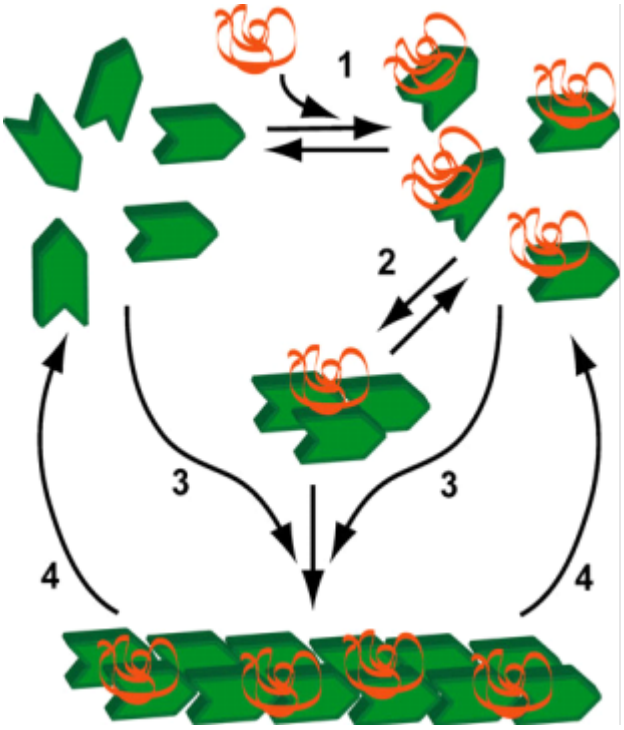

Figure 1: Schematic representation of the proposed effect of synaptopodin-2 on actin polymerization. Actin is shown in green, while synaptopodin-2 is shown in red. Binding occurs at a 1:3 ratio (Chalovich and Schroeter, 2010). Reproduced with permission from Springer Publishing.

The four known isoforms of synaptopodin-2 (a, b, c, & myopodin) are shown in Figure 2. The synaptopodin 2 gene possesses seven exons: exon 1, 2, 3, 4a, 4b, 5, and 6, with no intron separating exons 4a and 4b. All four isoforms share common exon 1-4a region. The differences between these isoforms arise from alternative splicing on the genlocus 4q26. Alternative splicing allows these isoforms to potentially have different interactions within the cell, although they are considered to be the same protein. They differ at their carboxy-terminal end with isoform A containing exon 6 and being 1261 base pairs in length, isoform B containing exon 4b and being 1093 base pairs in length, and isoform C containing exon 5 and being 1109 base pairs in length. These isoforms are all predicted to be 70-83% unfolded (Chalovich and Schroeter, 2010). Another proposed synaptopodin-2 isoform has been identified by others is referred to here as isoform D (Genbank, BAG65020). Synaptopodin 2 isoform D is significantly smaller than the other isoforms at 369 bp in length and is significant in that it does not contain the 4a exon region, but instead is made up of exons 1 and 2, and part of exon 6 (with a different reading frame than synaptopodin-2 isoform A) (Figure 2). Isoform D also contains a PDZ domain and a corepressor motif for the retinoid receptor RXR (Chalovich and Schroeter, 2010). These family members also have high proline contents, ranging from 10.6% in isoform c to 17.2% in isoform A (Chalovich and Schroeter, 2010). Synaptopodin-2 and synaptopodin-2 like proteins contain PDZ domains of unknown function that could possibly be dimerizing allowing the actin-bundling protein synaptopodin-2 to provide a platform for binding two actin filaments (De Ganck et al 2008). Synaptopodin family members also contain multiple PXXP motifs that may be involved in binding to SH3-domain-containing proteins (Asunama et al. 2007; De Ganck et al. 2008). Because Synaptopodin-2 is also a hub protein with many different binding ligands, and it can therefore affect many different protein-protein interactions. Myopodin has been found to

have a novel actin-binding site (Weins et al. 2001), while isoform A residues on its COOH terminal end (residues 752-903) contain the major interacting site for α-actinin on synaptopodin (Chalovich and Schroeter 2010). Myopodin also contains 2 lysine-rich NLS sites as well as 2 binding sites for 14-3-3, which are necessary for effective shuttling of myopodin from the Z-line in cardiac myocytes, where it is then bound to α-actinin (Faul et al. 2005). Since synaptopodin 2 contains two NLS motifs, there is the potential of a NLS/importin dependent nuclear import that can be regulated by actin-dependent movement towards the nucleus (Chalovich and Schroeter, 2010). All mouse and human synaptopodin 2 isoforms contain the α-helical LXXLL signature motif which is commonly seen in co-regulators of nuclear receptors and is believed to mediate initial contact between nuclear receptor and co-regulator to either help with or harm transcription activation (De Ganck et al. 2009).

Figure 2

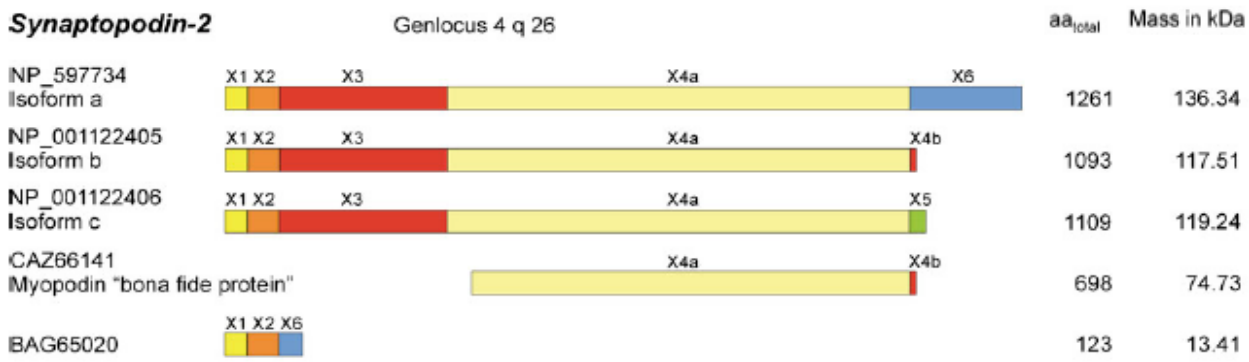

Figure 2: The three isoforms of synaptopodin-2 caused by alternative splicing, myopodin "bona fide protein", and another proposed spliceoform BAG65020 referred to in the proposal as isoform D. (Chalovich and Schroeter, 2010). Reproduced with permission from Springer Publishing.

Unpublished studies in our laboratory using immunocytochemistry showed that myopodin, a truncated version of synaptopodin-2 isoform B, is expressed in the nucleolus of multiple cell lines. These studies included both cancerous and non-cancerous cell lines: HT29, CaCo2, CV1, HeLa, and C2C12. Transcription of ribosomal genes and ribosome maturation occurs in the nucleolus and is a key cellular process tightly connected with the physiological and proliferative statuses of the cell (Philimonenko, et. al 2010). Three distinct compartments exist within the nucleolus; fibrillar centers (FCs) are the storage sites for RNA polymerase I and other essential components, the dense fibrillar components (DFCs) are where transcription occurs, and lastly, the granular compartments (GCs) are the sites of pre-ribosome maturation. (Philimonenko,et al. 2010). The fact that previous studies have shown actin to be localized within the DFC and on the FC/DFC border where transcription occurs within the nucleolus, and that synaptopodin-2 is a common binding partner of actin could indicate that synaptopodin-2 isoforms are also essential for transcription elongation and association with ribosomal genes. Synaptopodin-2 also commonly binds to calmodulin (Schroeter et al., 2004), another protein that has been shown to bind and localize to mammalian nucleoli (Thorogate and Torok 2007). Aside from being involved in ribosomal-subunit biogenesis, the nucleolus also houses proteins involved in other processes such as the regulation of cell cycle events (Catalano 2011). Other nucleolar proteins have been found to move out of the nucleoli during mitosis redistributing to areas within the nucleus before re-accumulating in the nucleoi during telophase (Catalano 2011). A similar pattern of expression has been seen in previous studies of synaptopodin-2 under differentiation conditions (Sarah Thalhamer, personal communication). Also, nucleolar localization signals have also been found to be rich in basic residues (Catalano 2011), a feature that synaptopodin-2 also shares as it is highly rich in proline. Finally, nucleolar proteins have

previously been found to be implemented in colorectal cell proliferation (Delloye-Bourgeois, et al. 2012). Interestingly enough, these nucleolar proteins were also the result of alternative splicing that gave rise to multiple splice variants of the same gene with different functions within the cell.

Previous research into the role of synaptopodin 2 in cancer has suggested that synaptopodin 2 acts as a tumor suppressor. Synaptopodin 2 first became of interest to researchers studying prostate cancer when they noticed genlocus 4q25 was deleted in greater than 50% of prostate cancers tested. They found a 54 kb minimal common deletion that contained the sequence encoding synaptopodin 2. Complete or partial deletions occurred among invasive prostate cancer cases 80% of the time (Lin et al. 2001) as well as clinical prostate cancer relapse (Sanchez-Carbayo 2003). Higher mortality rates are also seen in patients possessing the synaptopodin-2 deletion (Yu et al. 2011). Research has also been done on the role of synaptopodin-2 in invasive bladder tumors. Invasive bladder tumors were found to have decreased nuclear synaptopodin-2 expression as compared with more superficial tumors, while normal urothelium expresses synaptopodin-2 in the cytoplasm and nuclei (Sanchez-Carbayo et al. 2003). Comparative genomic hybridizations have shown that deletion in chromosome 4q may also occur in 30% of invasive bladder tumors (Sanchez-Carbayo et al. 2003). Lastly, the epigenetic silencing of synaptopodin-2 by hypermethylation was associated with gene expression being increased in vitro, and poor survival was associated with hypermethylation (Cebrian et al. 2008).

In contrast, a few studies also suggest a role for synaptopodin-2 as a tumor activator. When cells migrate, the actin cytoskeleton becomes dynamically remodeled via concerted action of different actin-binding proteins, many of which show altered expression levels in malignant

cells. Many cytoskeletal regulatory proteins such as cofilin, zyxin, vinculin, and a gelsolin-like actin filament capping protein have been found to be up-regulated in invasive cells indicating an enhanced migratory behavior for these cells (De Ganck et al. 2009). The over-expression of synaptopodin-2 in human endothelial kidney cells (HEK-293) and mouse myoblast cells (C2C12) made the cells more invasive in a collagen matrix (Van Impe et al. 2003). When synaptopodin-2 was knocked-down via RNA interference in human prostate cancer cells (PC3) and bladder cancer cells (RT4), the cells exhibited reduced invasion in both collagen type I and Matrigel, as well as slower wound healing (De Ganck et al. 2009). However, Chalovich and Schroeter (2010) noted that all of these isoforms of myopodin never entered the nucleus. To account for these opposing views, a model has been proposed by De Ganck et al. in which the localization of synaptopodin-2 could contribute to its different roles within the cell, and hence nuclear myopodin may be a tumor suppressor, while the cytoplasmic version may be a tumor activator (Figure 3). In normal urothelium, immunohistochemistry showed synaptopodin 2 to be localized both in the nucleus and the cytoplasm, while nuclear synaptopodin 2 was lost depending on tumor stage, with the higher grade tumors show lower nuclear synaptopodin 2 localization (Sanchez-Carbayo et al. 2003). In prostate cancer, the levels of cytoplasmic synaptopodin 2 were found to be notably higher than in normal cells (Yu et al. 2006). Conflicting results have also been found for other actin-binding proteins such as gelsolin, cofilin, α-actinin-4, and CapG making it difficult to classify actin-binding proteins as either tumor suppressors or tumor activators (De Ganck et al. 2009). Synaptopodin 2 expression levels may also be oscillating during tumorigenesis showing a biphasic pattern indicating that different functions of the protein may be involved in different stages of cancer progression (De Ganck et al. 2009).

Figure 3

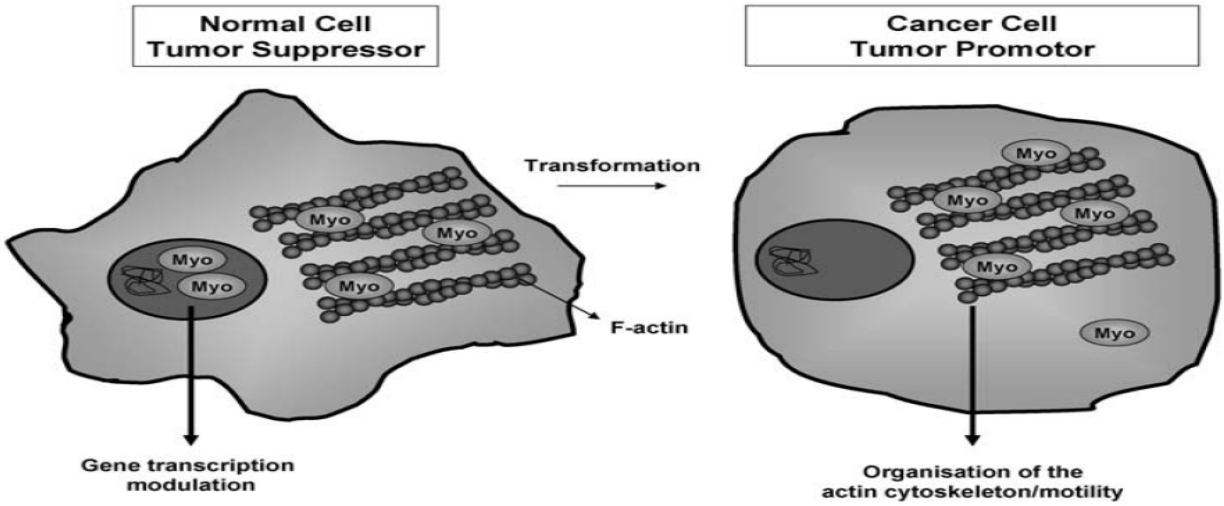

Figure 3: Proposed model showing the relationship between subcellular localization of synaptopodin-2 and its role in cancer (De Ganck et al. 2009). Reproduced with permission from Spandidos Publications.

Although Synaptopodin-2 has previously been studied in bladder cancer and prostate cancer, no research has been done on its effects in colon cancer. The majority of colorectal cancers begin with the development of polyps in the colon or rectal epithelium that progress from the innermost layers of tissue and spread outward over time. Colorectal cancer is the third most common cancer in men and women in North America and Western Europe, and the third leading cause of death from cancer. Over 148,000 people will be diagnosed with colorectal cancer each year and approximately 50,000 of these people will die from the disease. In 2011, about 101,700 new cases of colon cancer, 39,510 new cases of rectal cancer, and 49,380 deaths from colorectal cancer were reported. Adenocarcinomas make up approximately 96% of colorectal cancers, making it an important model for study ("What is colorectal cancer?" 2011). Most recently, synaptopodin-2 expression patterns were analyzed by immunohistochemistry on tissue arrays where it was found to be expressed mainly in the cytoplasm, with only a few tumors showing staining in the nucleus and nuclear membrane. Those researchers also found that synaptopodin-2 hypermethylation correlated with gene and protein expression loss, and that synaptopodin-2 was frequently methylated in colon cancer cells with a diagnostic accuracy of 83.9%. Loss of synaptopodin-2 expression was also correlated with increasing tumor stage and poor overall survival for patients supporting the role of synaptopodin-2 as a tumor suppressor.

While five isoforms of synaptopodin-2 have been reported as a result of alternative splicing from a single gene located on chromosome 4q26 (Chalovich and Schroeter, 2010), little is known about the function of these individual isoforms. Previous studies have shown that synaptopodin-2 isoform A and myopodin are expressed in HT29 cells, and that myopodin is localized in the nucleus of these cells. To further analyze the function of the different isoforms, specifically isoform A, primer pairs were designed to amplify the complete open reading frame

of each splice variant so the entire coding region can be inserted into two different expression vectors. The genes of interest were first cloned into a vector to create GFP-synaptopodin-2 isoform-specific fusion proteins to study the localization of the different isoforms within the cells by immunocytochemistry. The gene was also cloned into an inducible vector to be used for establishing stably transformed cell lines capable of over-expressing each isoform for future research. While the initial aim was to focus on synaptopodin-2 isoform A, an unnamed isoform was also identified and incorporated into this study

Chapter 2: Synaptopodin-2 Isoform A and D Expression Paper

Synaptopodin-2 Isoforms A and D Expression in HT-29 Human Colon Adenocarcinoma Cells

Kelli Shortt*, Perrine Lallemand*, Margit Schmidt*, Tamatha A. Baxley[1], Joseph M. Chalovich[1] and Jean-Luc Scemama*[2]

* Department of Biology, East Carolina University, Greenville, NC 27858

[1] Department of Biochemistry & Molecular Biology, 5E-122 Brody Building, Brody School of Medicine at East Carolina University, 600 Moye Blvd., Greenville, NC 27834, USA

Email: scemamaj@ecu.edu

Fax: 252-328-4178

Phone: 252-328-6313

Introduction

Synaptopodin-2 is a proline-rich, actin-binding protein lacking globular domains and commonly found in mammalian brain, kidney, and skeletal muscle tissues. Largely unfolded proteins like synaptopodin-2 maintain a highly flexible state allowing them to act as hub proteins, and further adapt to different binding sites (Iakoucheva et al., 2005). Synaptopodin-2 binds to actin (Mundel et al. 2007); it can also bind to calmodulin, myosin, alpha actinin, zyxin, and filamin (Schroeter et al. 2005, Pham et al., 2006). Studies also found that synaptopodin-2 plays a role in the regulation of actin-bundling by alpha actinin, and may be important for the formation and dynamic reorganization of the actin cytoskeleton in highly specialized cell compartments (Asunama et al. 2005). Further, association with alpha actinin-4, a protein that shuttles from the cytoplasm to the nucleus during actin depolymerization, has been linked with the translocation of myopodin from the cytoplasm to the nucleus, where actin-bundling is induced (Weins et al., 2001).

Upon overexpression, synaptopodin-2 is localized to cytosolic actin bundles and to nuclear actin loops, with this change in localization being stress-dependent (Weins et al., 2001). Actin-binding proteins like synaptopodin-2 regulate the reversible formation of actin polymers, and the evidence for multifunctional roles of actin-binding proteins in tumorigenic and metastatic processes of various tumor types is steadily growing (De Ganck et al., 2008). Many proteins involved in cancer contain a large number of large unfolded regions suggesting that the hub-protein nature of Synaptopodin-2 could contribute to its role in oncogenesis.

A possible role for Synaptopodin-2 in cancer was first suggested when it was observed that gene locus 4q25, which contain the synaptopodin gene was deleted in greater than 50% of prostate cancers tested (Lin et al., 2001). Comparative genomic hybridizations have also shown

that a deletion in chromosome 4q may occur in 30% of invasive bladder tumors (Sanchez-Carbayo et al., 2003). Invasive bladder tumors were found to have decreased nuclear synaptopodin-2 expression as compared with more superficial tumor, while normal urothelium expressed synaptopodin-2 in both the cytoplasm and nuclei (Sanchez-Carbayo et al. 2003). Epigenetic silencing of synaptopodin-2 by hypermethylation in bladder cancer was associated with gene expression being decreased in vitro, and associated with poor survival (Cebrian et al., 2008). The complete or partial deletion of synaptopodin-2 in invasive prostate and bladder cancers has suggested that syanptopodin-2 acts as a tumor suppressor gene. In contrast, a few studies suggest a role for synaptopodin-2 as an oncogene. For example, over-expression of synaptopodin-2 in human endothelial kidney (HEK-293) and mouse myoblast (C2C12) cells was found to increase invasiveness (Van Impe et al., 2009).

The four known isoforms of synaptopodin-2 are the product of alternative splicing. The synaptopodin-2 gene possesses 7 exons: exon 1, 2, 3, 4a, 4b, 5, and 6, with no intron separating exons 4a and 4b. Synaptopodin-2 isoforms A, B, and C differ mainly at their C-termini due to the presence of a different exon on the 3' end of each splice variant immediately after exon 4a. Isoform A contains exon 6 and is 1261 aa in length; isoform B contains exons 4b and is 1093 aa in length; and isoform C contains exon 5 and is 1109 aa in length. Myopodin is a truncation of isoform B that lacks the N-terminus encoded by exons 1, 2, 3, and part of 4a. While our previous research focused on the cellular localization of myopodin in HT-29 human adenocarcinoma cells (manuscript in progress), the present study aims to elucidate the expression, localization and function of synaptopodin-2 isoform A as well as a smaller unnamed isoform. Our results show a differential expression pattern between the isoforms, which suggests that different isoforms could have different functions and roles within the cell.

Materials and Methods

Cell Culture and Treatments: HT-29 (human colon adenocarcinoma) and CV1 (monkey kidney) cells were grown in DMEM/F12 (Life Technologies, Grand Island, NY, USA), supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin and 100 IU/ml penicillin at 37^{o}C /5% $CO_{2.}$ Differentiation of HT-29 was induced with the addition 5mM sodium butyrate for 1 week (Augeron and Laboisse, 1984).

Immunocytochemistry: Cells were grown on glass coverslips. Each of the following steps was carried out at room temperature and all reagents were added to PBS. Cells were fixed with 3.7 % formaldehyde for 8 minutes. DAPI (Life Technologies, Grand Island, NY, USA) (0.1 µg/ml) was used as nuclear stain. Cell samples were visualized using an Olympus IX2-DSU confocal microscope with a Hamamatsu EM-CCD digital camera model C9100. Images were prepared using Slidebook Version 4.2 software.

`*RT-PCR*: Human skeletal muscle cells total RNA was purchased from Ambion (AM7892), while total RNA from HT-29 was isolated using RNeasy Mini Kit (Quiagen) following the manufacturer protocol. cDNA synthesis was performed with Superscript II reverse transcriptase using an isoform A gene specific primer, 5'GTATTCACACTCACCTGTGTG3', following the manufacturer protocol (Invitrogen). Primers for PCR were designed to amplify full-length isoform A transcript, using the Expand Long Template PCR System (Roche). The same forward primer designed for isoform A and D was: 5'GAGAAGCTTAAAACATGGGCACAGGGGATTTTATCTCC3'. The reverse primers used for isoform A and D respectively were:5'GAGGGATCCTGTTTGGCGTCTCCATCCCCTTG3' and 5'GAGGGATCCGTCCATCGGTTGGTTTACTAGAG3'. All primers contained additional

restriction enzyme sites for *Bam*HI (Invitrogen) and *Hind*III (Invitrogen) for cloning into expression vectors.

Cloning of Synaptopodin-2 isoform A- and D-GFP Fusion Proteins: Human skeletal muscle cDNA was used to amplify the splice variants corresponding to isoforms A and D. The amplified products were cloned into pEGFP-N3 plasmid (Clontech) to create isoform A and isoform D-GFP fusion proteins. Sequence analyses were performed to verify the integrity of the amplicons and ensure reading frame accuracy. The plasmids were transfected into HT29 cells using the following method: The vectors were delivered into the cells using a cationic polymer Turbofect Transfection Reagent (Thermoscientific #R0531). The procedures were carried out following the manufacturer's guidelines. Briefly, the day prior transfection, cells were seeded in 4 ml of growth medium onto coverslips in 6-well plates at a density that gave 70-90% confluency. To prepare the reaction mixtures, 4 µg of DNA was diluted in 400 µl of serum-free DMEM and mixed well by vortexing. The Turbofect Reagent was briefly vortexed and 6 µl were added to each reaction tube. The solutions were incubated for 15-20 minutes at room temperature. Once complexes formed, all 400 µl of Turbofect/DNA mixture were added to each well while leaving the growth medium on the cells. The 6-well plates were gently rocked to ensure an even distribution of the complexes and stored afterwards in a CO_2 incubator at 37°C. After 24-28 hours, the transgene expression was analyzed for synaptopodin-2 A- and D-GFP fusion proteins.

Expression of Synaptopodin-2 isoform A- and D-GFP Fusion Proteins: After 24-48 hours, cells were fixed with 3.7 % formaldehyde in PBS for 8 minutes. To visualize the entire cell population, cells were counterstained with the nuclear stain DAPI (Invitrogen, 0.1 µg/ml). The transgene expression was analyzed using an Olympus IX2-DSU confocal microscope with a

Hamamatsu EM-CCD digital camera model C9100. Images were prepared using Slidebook Version 4.2 software.

Cell Fractionation: Nuclear and cytoplasmic lysates were generated with NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, 78833) according to manufacturer's specifications. All lysis buffers and reagents were supplemented with protease inhibitor cocktail (Pierce, 88661). The concentration of each lysate was determined by BCA assay using BSA as a standard.

Western Blot: Cell lysates were separated on 10% SDS-PAGE and proteins were transferred to Protran Nitrocellulose membranes (Whatman). Membranes were blocked with 5% milk in TBST (Tris Buffered Saline with 0.05% Triton X-100) for 1 hour followed by 4 hours incubation with anti-GFP antibody (Clontech, 8363-2) diluted (1:1000) in blocking buffer. Membranes were washed with TBST 5 times for 5 minutes before incubation for 1.5 hours with ECL anti-rabbit IgG HRP-linked whole antibody from Donkey (GE Healthcare, NA934V) diluted (1:10000) in blocking buffer. Membranes were washed 5 times for 5 minutes in TBST. Supersignal West Pico Chemiluminescence Substrate (Thermo) was used according to manufacturer's specification and membranes were exposed to film.

Results

As synaptopodin-2 and its isoforms are known to be expressed in skeletal muscle at high levels (De Ganck et. al., 2008), skeletal muscle was chosen as the best source for amplification of mRNA coding for these proteins. Isoform specific cDNA was prepared from human skeletal muscle total RNA a using an isoform A gene specific primer. Analysis of the PCR amplification products, with isoform A specific primers (Appendix Table 1), revealed the presence of two amplicons; a relatively intense band at approximately 800 base pairs and the expected 3783 base pair band corresponding to the full length ORF of splice variant A. The 800 base pair band was excised from the gel, sequenced, and confirmed to be a 100% match to a mRNA corresponding to a synaptopodin-2-like unnamed human protein (accession number: BAG65020). The cDNA encoding the BAG65020 protein (accession number: AK304121) was identified in a human cDNA sequencing project and mentioned in a review by Chalovich and Schroeter (2010). To date, no one has confirmed the expression of the BAG65020 protein in vitro or in vivo. Until recently, only four isoforms of synaptopodin-2 had been described. All four of these isoforms (A, B, C, and myopodin) represent the products from alternative splicing. We refer to this isoform, BAG65020, as isoform D. It is significantly smaller than the others at 123 amino acids in length, and consists of exons 1, 2, and 6.

To study synaptopodin-2 isoform A and D expression in vivo, synaptopodin-2 isoform A and D were cloned into a transient GFP expression vector. GFP-isoform A and -isoform D fusion proteins were over-expressed in HT-29 colon adenocarcinoma cells as well as CV-1 monkey kidney cells (Figure 4). Both GFP-isoform A and isoform D fusion proteins expression was restricted to the cytoplasm with more intense expression localized around the perinuclear

region. As a comparison, the GFP vector control (empty vector) was ubiquitously expressed in both nuclear and cytoplasmic compartment.

Figure 4

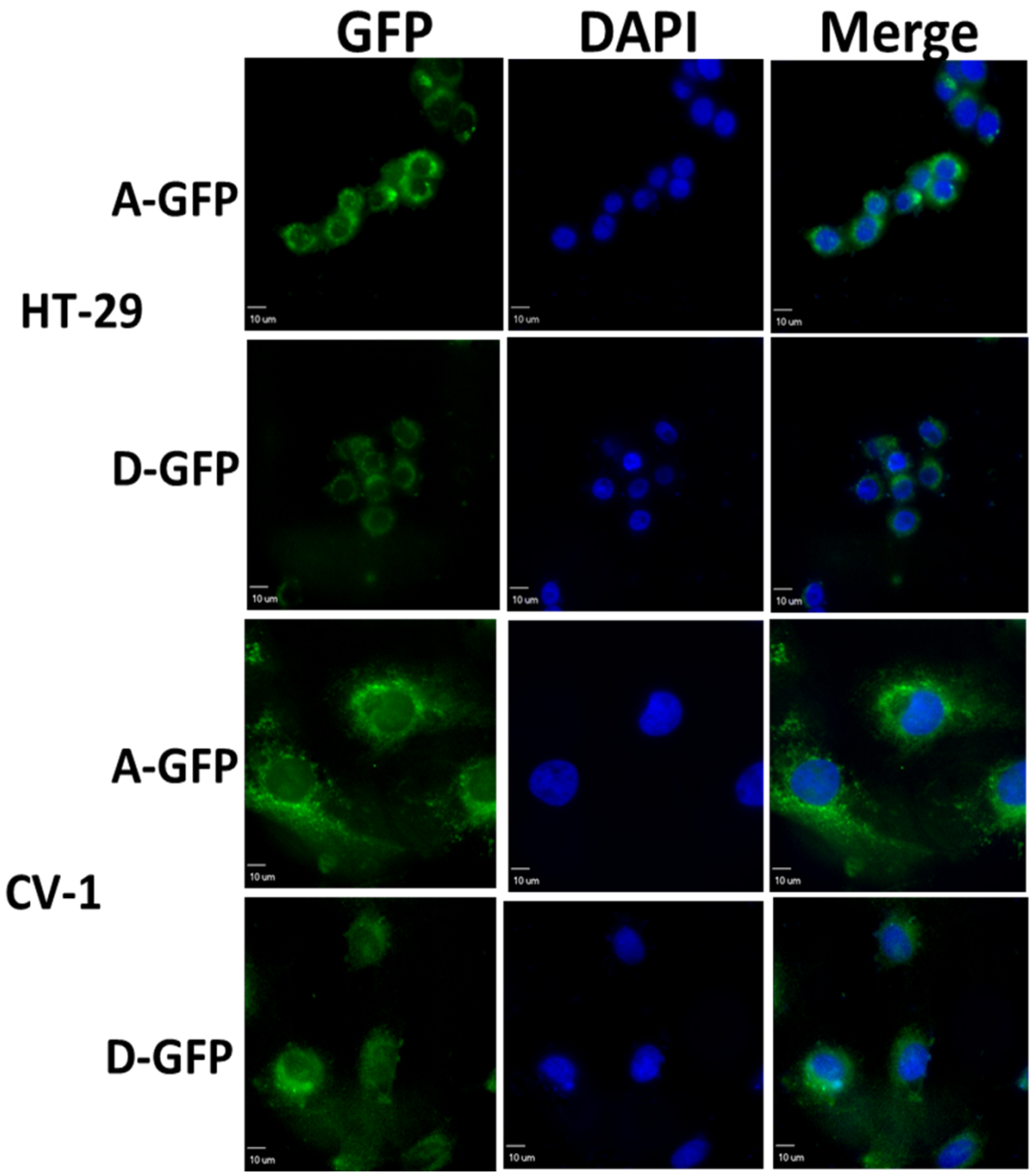

Figure 4: Expression of Synaptopodin-2 Isoform A- and D-GFP Fusion Proteins in HT-29 and CV-1 Cells. Expression of Isoform A and D as A- and D-GFP fusion proteins in HT-29 cells and CV-1 cells occurred in a mainly cytoplasmic pattern.

Interestingly, transfection with a vector expressing the myopodin-GFP fusion protein into HT-29 showed that myopodin expression is restricted to the nuclear region (Figure 5). These differences in expression between these different isoforms suggest that they also differ in their function.

Figure 5

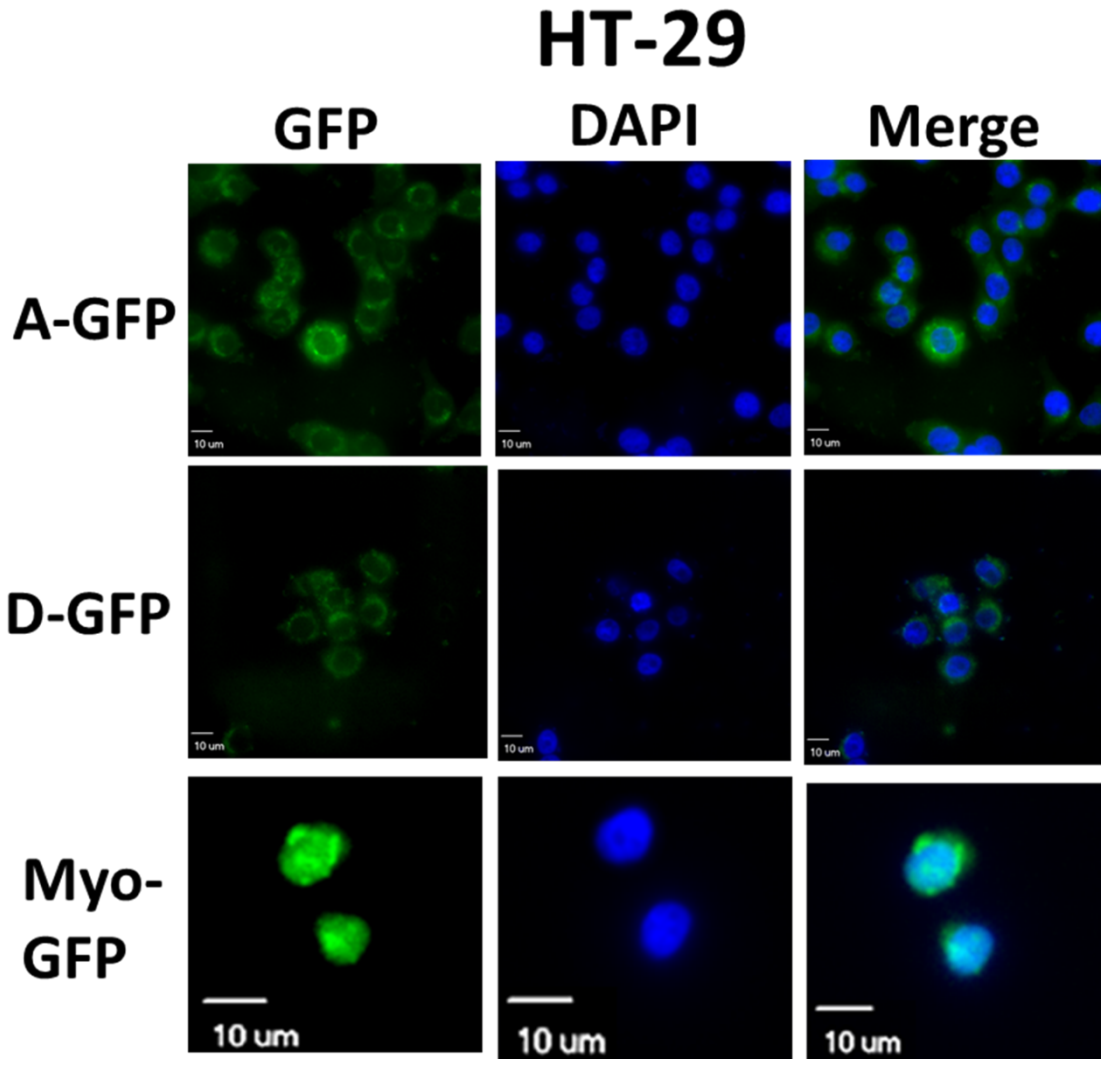

Figure 5: Expression of Synaptopodin-2 Isoform A- and D-GFP Fusion Proteins in HT-29 cells. Expression of Isoform A and D as A- and D-GFP fusion proteins in HT-29 cells occurred in a mainly cytoplasmic pattern, which was different from the strong nuclear/nucleolar staining pattern that occurs with myopodin.

To analyze if the state of differentiation of HT 29 cells influences the expression/localization of these proteins, we analyzed the expression of the different isoforms after the cells were treated for a period of 7 days with 5 mM sodium butyrate, an agent known to promote HT29 cell differentiation. Synaptopodin-2 GFP fusion proteins for isoform A and D were transfected into sodium butyrate treated HT-29 cells. The sodium butyrate treatment did not alter the localization of either Synaptopodin A nor D isoforms (Figures 6 & 7). These results differ dramatically from the previous data obtained in the lab with the myopodin isoform that shows a translocation of myopodin from the nucleus to the cytoplasm following sodium butyrate treatment (data not shown).

Figure 6

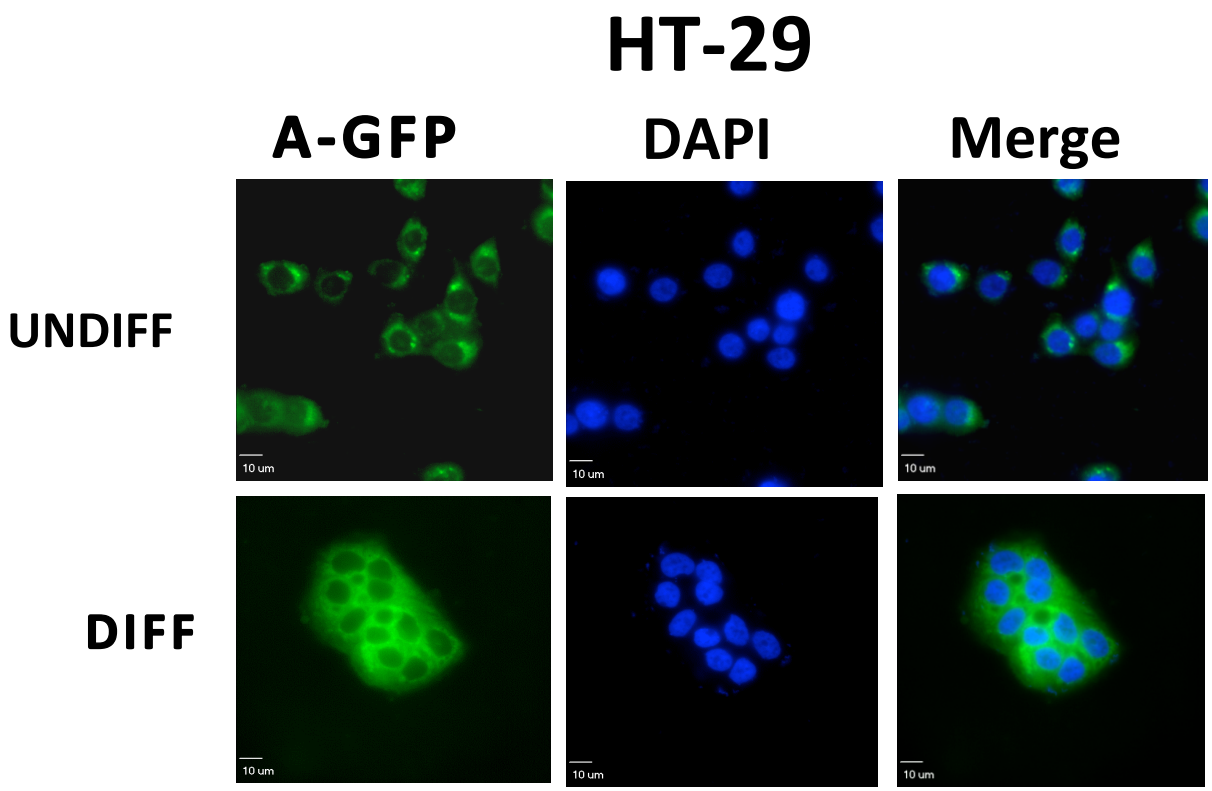

Figure 6: Expression of Synaptopodin-2 Isoform A-GFP fusion protein in undifferentiated and differentiated HT-29 cells, induced with sodium butyrate treatment. Expression of the Isoform A GFP-fusion remained cytoplasmic even after differentiation was induced.

Figure 7

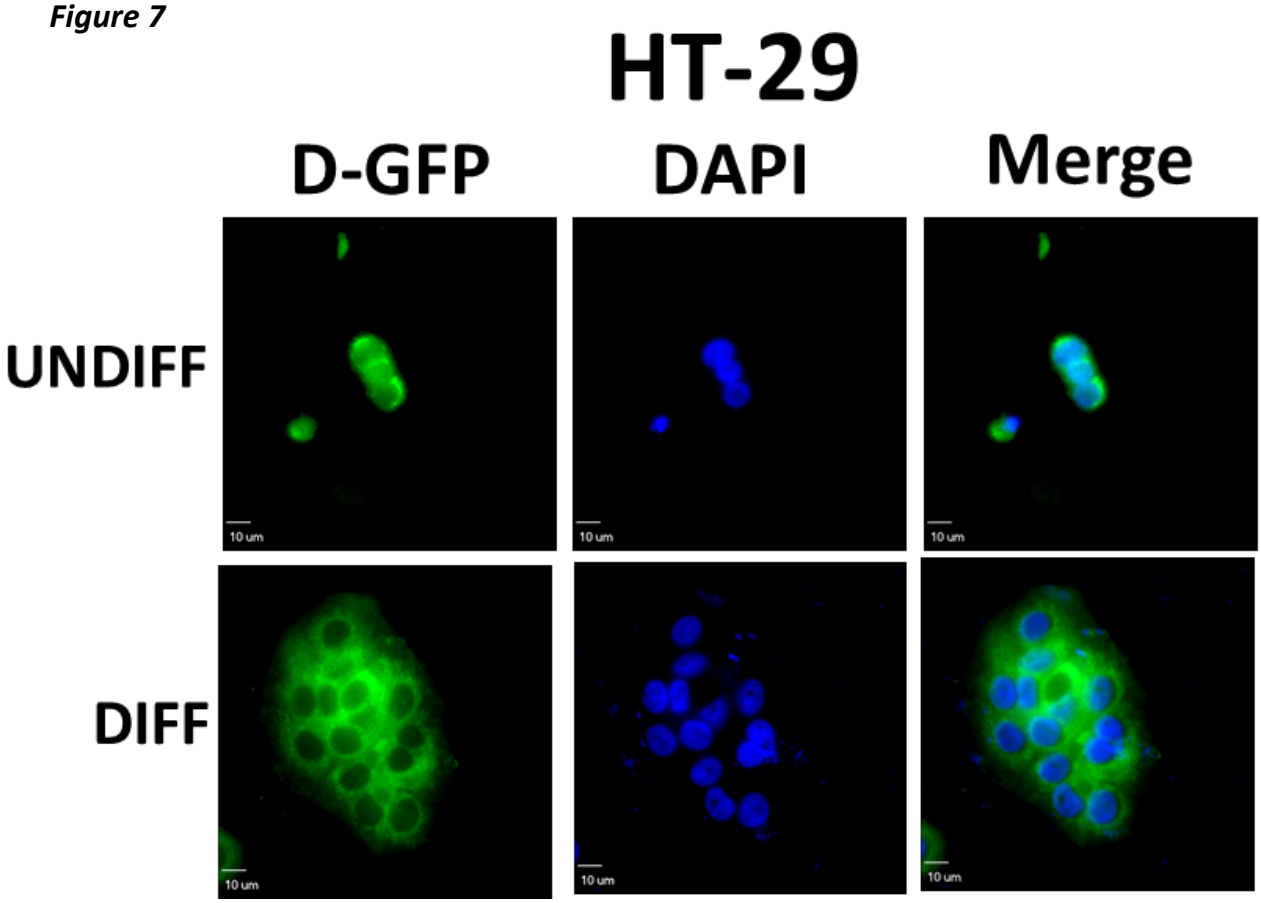

Figure 7: Expression of Synaptopodin-2 Isoform D-GFP fusion protein in undifferentiated and differentiated HT-29 cells, induced with sodium butyrate treatment. Expression of the Isoform D GFP-fusion remained cytoplasmic like the Isoform A GFP-fusion, even after differentiation was induced.

Discussion

The focus of this study was to examine synaptopodin-2 isoforms A and D and observe expression as a means to understanding the function of each. For the first time, we showed that isoforms A and D of Synaptopodin-2 are localized in the cytoplasm. While isoforms A and D remain cytoplasmic, myopodin, which was originally expressed in the nucleus, translocates into the cytoplasm upon differentiation of HT-29 cells.

While trying to amplify isoform A for future cloning through RT-PCR using human skeletal muscle cDNA, an intense band at approximately 800 base pairs was frequently observed along with the expected 3783 base pair band for isoform A, which we later determined to be another smaller isoform of synaptopodin-2. This protein is 123 amino acids in length and contains a PDZ signaling domain at the amino acid residues 7-85. Synaptopodin-2 isoform A and D both share the complete exons 1 and 2, and exon 6. Isoform D lacks exons 3 and 4a; and there also is a shift in the reading frame of isoform D, which results in an early termination and in a different sequence in the C-terminus of the putative protein. It is important to note that there is no evidence that this protein is expressed.

Over-expressed isoform A and D were mainly cytoplasmic in HT-29 and CV-1, while the over-expressed myopodin-GFP fusion was mainly localized in the nuclear and nucleolar regions (Figure 5). Different isoforms appear to have different localizations within the cells suggesting that individual isoforms may have different functional roles as well. Interestingly, although synaptopodin-2 isoform A and D differ in their exons and drastically in their size, both are still localized to the cytoplasmic compartment of the cell indicating that exons 1 and 2 may contain some significant factor that causes them to be found in the cytoplasmic region as opposed to the

nuclear region. Many actin-binding proteins like severin and FrgP are commonly predominantly cytoplasmic (Van Impe et al., 2003). Actin-binding proteins are involved in a multitude of functions within the cell. In the cytoplasm, actin forms the filamentous network involved in cellular motility, cell shape, and movement of organelles (Castano et al. 2010). Actin-binding proteins help to anchor the actin network to the plasma membrane, and aid in the polymerization and de-polymerization of the growing actin filaments. These proteins are also implemented in the chromatin-remodeling complex and have been suggested not only to move or replace nucleosomes on DNA strands, but also help maintain the overall chromatin structure (Lee et al. 2007). During DNA replication/repair, actin-binding proteins even recruit factors and scaffolding on filamentous actin. Nuclear spectrin has been found to act as a scaffold, recruiting repair proteins to the site of DNA damage (Sridharan et al., 2003). The fact that synaptopodin-2 isoform D lacks the region corresponding to exon 4a may indicate that isoform D has a quite different function than isoform A. Based on previous studies, a number of the binding sites for synaptopodin-2 interactions with alpha-actinin, actin, Zyxin, 14-3-3 are located in the region corresponding to exon 4a (for review, see Chalovich et al., 2010).

Earlier studies in the lab have also focused on the synaptopodin-2 isoform myopodin. During these studies, myopodin was found to be localized in the nucleus and nucleolar region of multiple cell lines using an isoform specific antibody. The NC_781 antibody recognizes an epitope in exon 4b, so theoretically it will detect myopodin as well as isoform B. Western blot analysis using cytoplasmic, nuclear, and nucleolar lysates confirmed that myopodin was expressed only in the nucleus and not in the nucleolus. This nuclear/nucleolar localization was later confirmed by using a myopodin-GFP fusion protein. The size and number of nucleoli in human cells varies according to the cell type and proliferation rate of the cell (Grisendi et al.

2006). The function of the nucleolus is closely connected to cell proliferation, division and growth; and many cancer cells have enlarged nucleoli and several nucleolar proteins have been linked to tumorigenesis. For example, a nucleolar protein called nucleophosmin has been found to be mutated or overexpressed in various tumors and malignancies (Grisendi et al., 2006). Similarly, the nucleolar localization of an isoform of netrin-1 has been demonstrated to enhance tumor cell proliferation in several different cancers (Delloye-Bourgeois et al. 2008). CapG, an actin-capping protein, has also recently been said to exist in the nucleolus (Hubert et al. 2008).

After differentiation, the expression of the synaptopodin-2 isoform A and D GFP fusion proteins remained cytoplasmic. The differentiated phenotype of HT-29 cell is defined in part by the presence of tight junctions between adjacent cells and an increased brush borders on the apical cell surface (Anderson et al. 1993, Schneeberger and Lynch, 1992). Dome formation has also been observed in polarized cells (Gout et al, 2004). The brush borders contain bundled actin filaments that are further associated with other actin binding proteins like villin and fimbrin (Cohen et al., 1999). Similarly to the localization we have seen for isoform A and D, villin has been found have a diffuse and uniform distribution throughout the cytoplasm in undifferentiated cells, with strong apical labeling as well as a cytoplasmic localization in differentiated cells (Gout et al. 2004).

Chapter 3: Supplementary Results

The purpose of my research was to analyze the function of synaptopodin-2 isoform A. A reasonable first step in understanding the function of a particular gene or in this case splice variant is to clone the cDNA corresponding to the splice variant of interest in order to overexpress the protein. Synaptopodin-2 was originally isolated from chicken gizzards (Leinweiber et al., 1999) and rabbit smooth muscle tissues (Schroeter and Chalovich, 2005), while others have identified it in heart and skeletal muscle (Weins et al., 2001). As synaptopodin-2 and its isoforms are known to be expressed in skeletal muscle at high levels (De Ganck et. al., 2008), skeletal muscle was chosen as the best source for amplification of the cDNA. Using human skeletal muscle total RNA, isoform specific cDNA was prepared using an isoform A specific primer (Appendix). Subsequent PCR amplification using isoform A specific primers gave rise to two products; an intense band at approximately 800 base pairs was frequently observed along with the expected 3783 base pair band for isoform A (Figure 8). The 800 base pair band was excised from the gel, purified, and cloned into the pcDNA4/TO vector (Invitrogen). Once cloned, the construct was sequenced and found to be a 100% match to a 1330 bp human cDNA, moderetaly similar to synaptopodin-2 (accession number: AK304121). The cDNA encodes an unnamed human protein product, BAG65020, referred to here as isoform D. This protein was classified on Genbank as being moderately similar to full-length synaptopodin-2 and was previously mentioned in a review by Chalovich and Schroeter (Chalovich and Schroeter, 2010). Originally identified in trachea tissue, the protein is 123 amino acids in length when translated and contains a PDZ signaling domain at the amino acid residues 7-85 (Isogai and Yamamoto, 2007; Wakamatsu et al, 2008). The protein product was submitted to NCBI by the researchers Isogai and Yamamoto in 2007 as well as by the group Wakamatsu et al. as part of

a human cDNA-sequencing projects focusing on mRNA splice variants, but both manuscripts were never published. The mRNA transcript contained the full-length sequence of exons 1, 2, and 6 like isoform A, but lacks exons 3 and 4a (Figure 9). However, when translated, a shift in the reading frame occurs due to alternative splicing which gives rise to a different amino acid sequence for the last 38 residues after exon 2. The frame shift results in an early stop codon making the actual coding sequence only 369 bp. Using primers designed for its coding sequence, synaptopodin-2 isoform D was amplified and cloned into two separate vectors for over-expression studies (see Materials and Methods).

Figure 8

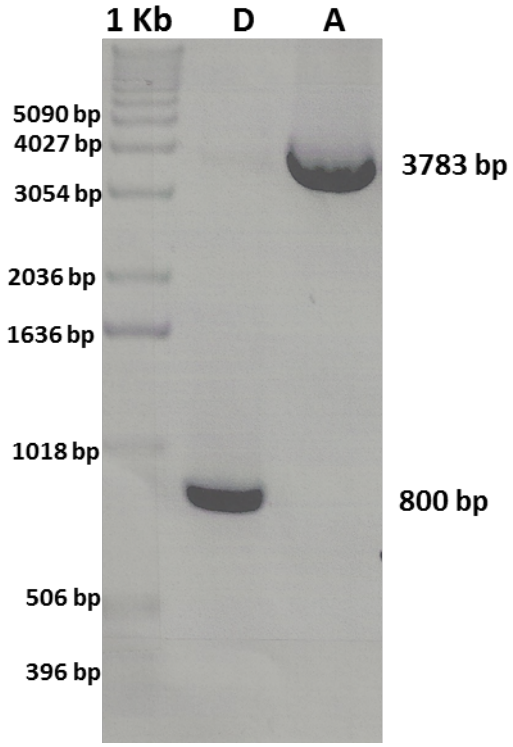

Figure 8: RT-PCR for Synaptopodin-2 isoforms A and D using isoform A specific cDNA prepared from human skeletal muscle total RNA. Samples were run on 0.8% agarose gel with TAE buffer. As expected, a 3783 bp band was seen for isoform A; however, there also was an intense 800 bp band present. This band was excised, sequenced, and determined to match the sequence for a homo sapien cDNA (GenBank: AK304121.1); and when translated was a 100% match to an 123 amino acid unnamed protein product (GenBank: BAG65020), referred to here as isoform D. Once confirmed to be Synaptopodin-2 isoforms, both A and D were cloned into the transient GFP vector pEGFP-N3 (Clontech) and the inducible vector pcDNA4/TO (Invitrogen) for the creation of stable cell lines.

Figure 9

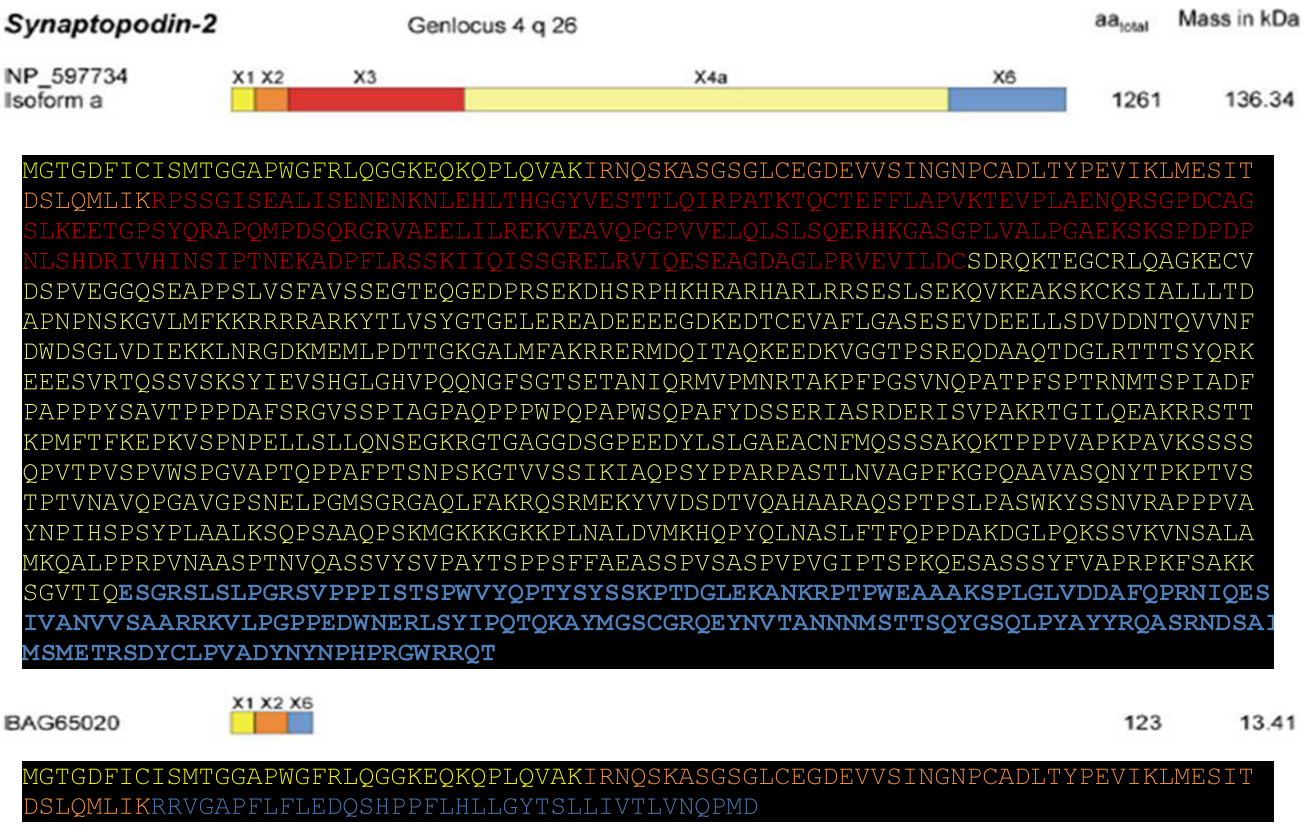

Figure 9: A comparative analysis between synaptopodin-2 isoforms A and D (BAG65020). The protein structure is shown for the two splice forms. Isoform A and D both share the complete exons 1, 2, and 6. Isoform D lacks exons 3 and 4a; there is also a shift in the reading frame of isoform D which results in 38 different amino acids after exon 2.

To confirm that synaptopodin-2 isoform A was endogenously expressed in HT-29 cells, total RNA was isolated from HT-29 cells and used to prepare isoform A specific cDNA. RT-PCR was carried out using specific primers for synaptopodin-2 isoform A and D with A specific cDNA. As isoform A and D both contain exons 1, 2, and the beginning of 6, isoform A specific cDNA should have allowed the amplification of both of these isoforms if present in our cells. Previously studies in the lab have shown that synaptopodin-2 isoform A and myopodin, but not isoforms B and C are normally naturally in HT-29 cells (Sarah Thalhamer, personal communication). This could be due to low endogenous levels of the protein as it has been reported that some cells express synaptopodin-2 more than others (De Ganck et al., 2008).

Once both synaptopodin-2 isoform A and D transcripts were amplified from human skeletal muscle, they were each cloned into two separate over-expression vectors. We used pEGFP-N3, a vector used for transient transfection containing the eGFP gene that allows for visualization of the fusion protein product in live cells, and pcDNA4/TO, a tetracycline inducible vector that will be used to create stable transfection. Isoform specific cDNA prepared from human skeletal muscle RNA was used to amplify isoforms A and D. The PCR products were ligated into the vector and the resulting plasmid constructs were then transformed into E. coli using the XL-2 competent cells (Invitrogen). After 24 hours at 37°C, colonies were chosen, cultures grown up and plasmids were purified. The purified products were then sent to be sequenced using specific sequencing primers (Appendix). Both synaptopodin-2 isoform A and D sequences were confirmed using the DNASTAR software (DNASTAR, Inc.) for sequence analysis.

We tested the GFP- fusion constructs for fluorescence via confocal microscopy. After transfection with the cationic polymer solution Turbofect Transfection Reagent (Thermo

Scientific), the localization of the over-expressed isoform A- and D-GFP fusion proteins were mainly cytoplasmic in HT-29 cells (Figure 4). This cytoplasmic pattern of expression was also observed in the monkey kidney cell line CV-1 (Figure 4). The empty GFP vector was used as a control and showed expression in both the nucleus and the cytoplasm (Figure 10). This result is interesting and very different from the expression observed with the myopodin-GPF construct, which is mainly expressed in the nucleus (Figure 5).

Figure 10

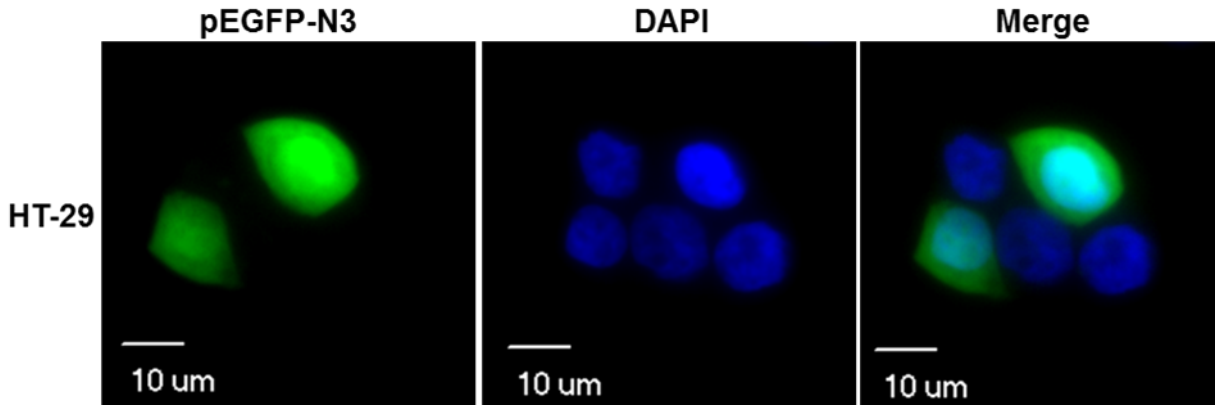

Figure 10: Expression of the pEGFP-N3 vector (Clontech) alone in HT-29 cells. Expression of GFP was seen in both nuclear and cytoplasmic compartments.

The efficiency of the transfection was determined as a ratio of DAPI stained cells to GFP fluorescence cells, and the range was between 1-6 % depending on the experiments. A GFP time-course experiment was done in which the expression of the transient GFP was measured over time to study how long the over-expressed protein would remain observable in HT-29 cells. Results showed a visible expression for 5 days, which corresponds to approximately 5 cell cycles.

After treating HT-29 cells with sodium butyrate medium for 7 days, which results in histone hyperacetylation and induction of differentiation, cells were again transfected with synaptopodin-2 GFP fusion proteins, specifically isoforms A and D. After 48 hours, cells were mounted, counterstained with DAPI, and examined using a confocal microscope. After this treatment, the expression of the synaptopodin-2 isoform A and D GFP fusion proteins remained cytoplasmic. Therefore, synaptopodin-2 isoforms A and D remain in the cytoplasm in both the undifferentiated and sodium butyrate-treated state (Figures 6 & 7).

Previous studies in the lab have focused on the synaptopodin-2 isoform myopodin. Using the NC_781 isoform specific antibody, which recognizes an epitope in exon 4b, myopodin was determined to be localized in the nucleus and nucleolar region of multiple cell lines – HT-29, CV-1, and C2C12 cells (Figure 11). These studies used Western analysis with cytoplasmic, nuclear, and nucleolar HT-29 lysates confirmed that myopodin was expressed in the HT-29 nucleus, but not in the nucleolus (manuscript in preparation). A truncated isoform of myopodin was found being expressed in the nucleoli. This nuclear/nucleolar localization was confirmed in HT-29 by using a myopodin-GFP fusion protein (Figure 5). The nuclear/nucleolar localization of myopodin-GFP fusion protein contrasts with the cytoplasmic localization of the synaptopodin-2 isoform A and D – GFP fusion proteins.

Figure 11

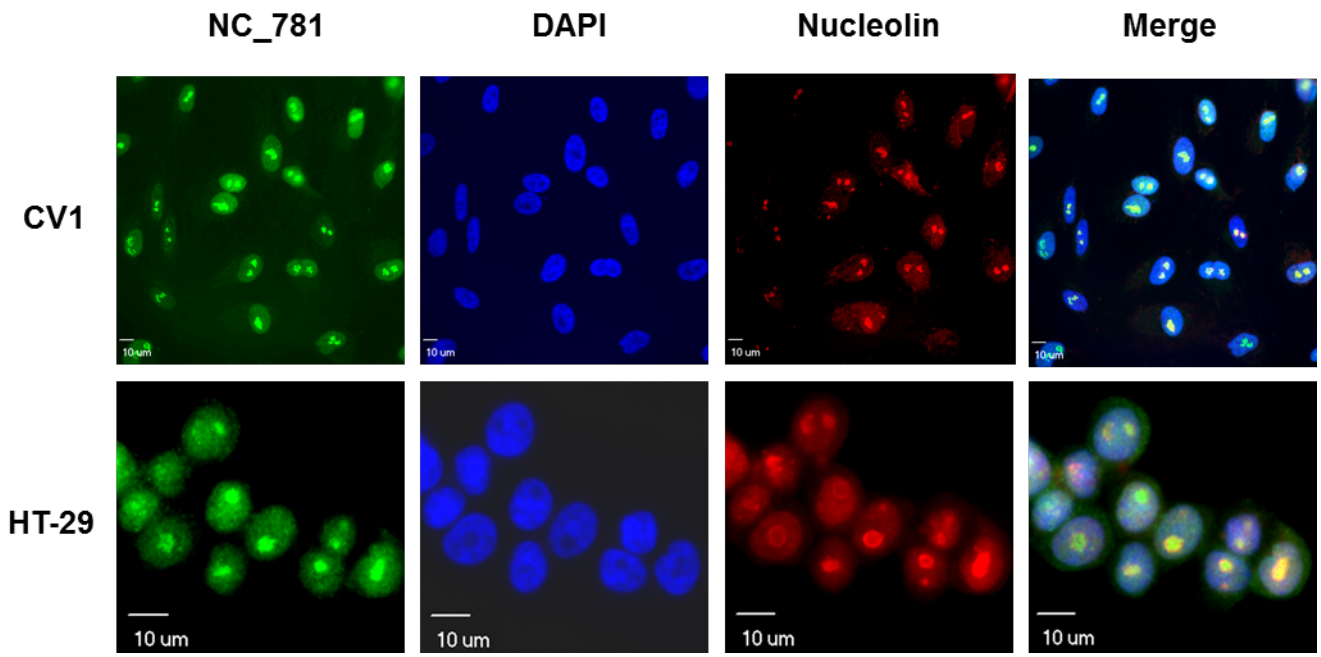

Figure 11: Immunostaining of mammalian cell lines with anti-synaptopodin-2 4b, which detects both synaptopodin-2 isoform B and myopodin. CV1 monkey kidney cells and HT-29 human colon cancer cells are shown. Notice the nuclear stain with anti-synaptopodin-2 isoform B/myopodin antibody NC781, DNA stain with DAPI and anti-nucleolin antibody demonstrate that synaptopodin-2 isoform B/myopodin reactive species are expressed in CV1 nuclei with stronger expression in the nucleoli.

C2C12, mouse myoblasts, naturally differentiate upon confluency and characteristically form multinucleate myotubes. Synaptopodin-2 isoform B/myopodin has also been shown to translocate from the nucleus to the cytoplasm during differentiation in C2C12 cells (Figure 12), which is similar to the nuclear cytoplasmic redistribution of myopodin seen in other studies of myoblast cells (Weins et al. 2001). In contrast to previously published data (Weins et al. 2001), using the NC-781 antibody, which recognizes an epitope on the C-terminus of the protein we showed that in the undifferentiated state, isoform B/myopodin is localized in the nucleus with intense staining in the nucleolar region. Weins et al. did show that in undifferentiated C2C12 cells myopodin was nuclear, but they never saw the presence of myopodin in the nucleoli. Also, in contrast to the Weins group in differentiated C2C12, myopodin is expressed in the cytoplasm of the cells within the myotubes, but remains in nucleolar region of the cells even after differentiation.

Figure 12

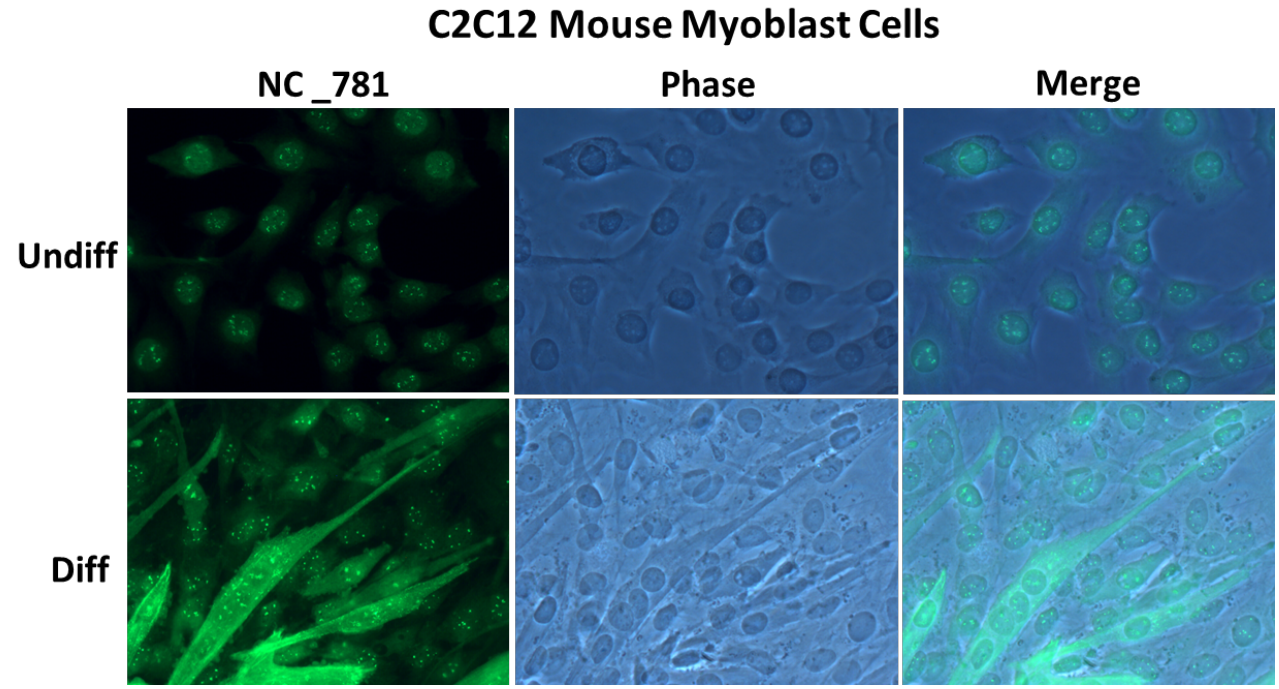

Figure 12: Immunostaining of C2C12 with anti-synaptopodin-2 4b. Both undifferentiated C2C12 (top row) and differentiated cells were stained with the anti-synaptopodin-2 isoform B/myopodin antibody NC_781. In the undifferentiated state, synaptopodin-2 isoform/myopodin was localized in the nucleus/nucleolar region of the cell. Myotube formation is characteristic of C2C12 differentiation, which does not need to be chemically induced, but rather occurs as soon as cells reach confluency. After differentiation, a translocation of isoform B/myopodin out of the nucleus/nucleolus and into the cytoplasm of the myotubes of C2C12 was observed. This is consistent to the translocation of myopodin observed by Weins et al. (2001) as well as the translocation we observed in HT-29 human colon adenocarcinoma cells. Our C2C12 data differs, however, in that we still observe nucleolar staining even after differentiation.

Similar results were obtained with HT-29 cells and antibody NC_781. After a seven-day treatment with sodium butyrate, the nuclear/nucleolar staining appeared to diffuse out of the nucleus and into the cytoplasm with nuclear/nucleolar staining no longer being observed (Figure 13).

Figure 13

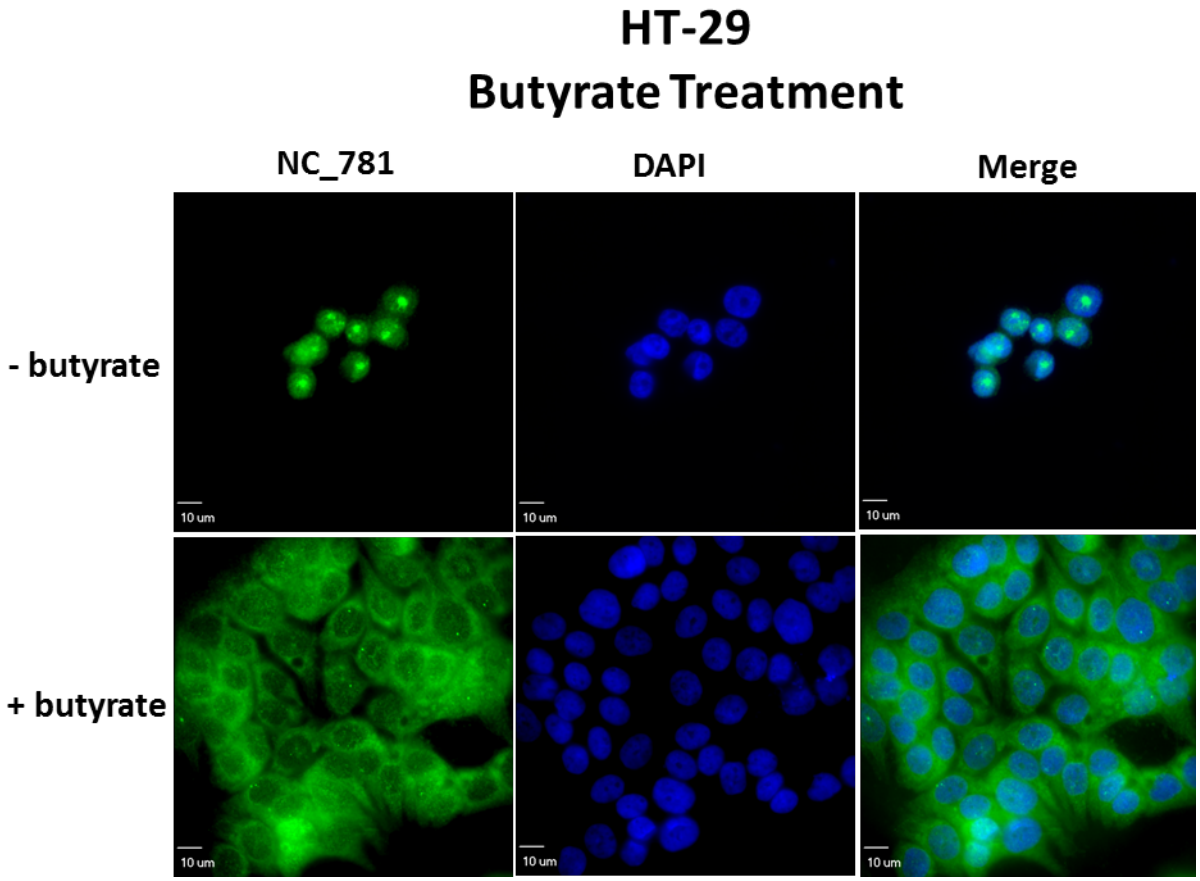

Figure 13: Immunostaining of HT-29 cells with anti-synaptopodin-2 4b. Both undifferentiated HT-29 human colon cancer cells (top row) and sodium butyrate-induced differentiated cells were stained with the anti-synaptopodin-2 isoform B/myopodin antibody NC_781. In the undifferentiated state, synaptopodin-2 isoform/myopodin was localized in the nucleus/nucleolar region of the cell. After a 7 day treatment with 5 mM sodium butyrate, a translocation of isoform B/myopodin out of the nucleus/nucleolus and into the cytoplasm was observed, with a small amount of nucleolar staining still being observed

Chapter 4: Discussion

The focus of this study was to examine the individual isoforms of synaptopodin-2 and observe expression as a means to understanding the function of each. For the first time, we showed that isoforms A and D of Synaptopodin-2 are localized in the cytoplasm. We have also observed that different isoforms of synaptopodin-2 behave differently when HT-29 cells are treated with sodium butyrate. While isoforms A and D remain cytoplasmic, myopodin, which was originally expressed in the nucleus, translocates into the cytoplasm upon differentiation of HT-29 cells.

My approach to studying synaptopodin-2 isoform A and D was to determine the localization of each in colon cancer cell lines. Studying synaptopodin-2 with a focus on the different variants has never been done before, except by De Ganck who was able to identify multiple isoforms in several cell lines through RT-PCR, but did not have an antibody that would recognize the endogenous protein (De Ganck, 2008). Commonly, commercially used antibodies are used to study synaptopodin-2, but these antibodies are made against epitope in exon 4a which is shared by all isoforms and fail to differentiate between the all the individual isoforms, except for isoform D which lacks exon 4a. Even custom made antibodies (Weins et al.) have also been designed against exon 4a and will recognize all of the isoforms. Dr. Chalovich's laboratory, however, possesses custom antibodies that are used to recognize an epitope in exon 4b, which will recognize both isoform B and myopodin. We have previously shown that HT29 cells specifically express myopodin but we have not been able to show the presence of endogenous isoform B at the mRNA or protein level. Myopodin, however, was found to be localized in the nucleus of cells in multiple cell lines – HT-29, CV-1, and C2C12. The nuclear localization of

myopodin in C2C12 was consistent with the results observed by Weins et al. (2001). Nevertheless, a related species of synaptopodin-2 was found to be expressed in the nucleolar compartment. The nucleolar species of synaptopodin-2 appeared to be a truncated form of the protein and may have been missed in previous studies that were using different antibodies.

Although we had antibodies against isoform B and myopodin, our lab did not have any way to study the expression of isoforms A and C at the protein levels. Previous RT-PCR confirmed that only synaptopodin-2 isoforms A, B, and myopodin are expressed endogenously in HT-29 cells (Sarah Thalhamer, personal communication). In order to study the localization of the different isoforms within the cells using fluorescence analysis, primer pairs were designed to amplify the open reading frame of each splice variant so that the entire coding region could be inserted into a vector to create GFP-synaptopodin-2 isoform-specific fusion proteins. While trying to amplify isoform A through RT-PCR using human skeletal muscle cDNA, an intense band at approximately 800 base pairs was frequently observed along with the expected 3783 base pair band for isoform A. The 800 base pair band was excised, cloned, and sequenced. The band was found to be a 100% match to an unnamed human protein product called BAG65020 in the National Center for Biotechnology Information (NCBI) database. Originally discovered in tracheal tissue, the protein, referred to here as isoform D, is 123 amino acids in length and contains a PDZ signaling domain at the amino acid residues 7-85. The protein product was submitted to NCBI by the researchers Isogai and Yamamto in 2007 as part of a human cDNA-sequencing project focusing on splice variants of mRNA, but it was never published. BAG65020 was classified as being moderately similar to synaptopodin-2 and was mentioned in a review by Chalovich and Schroeter. Synaptopodin-2 isoform A and D both share the complete exons 1 and 2, and exon 6. Isoform D lacks exons 3 and 4a; and there also is a shift in the

reading frame of isoform D, which results in an early termination and in a different sequence in the C-terminus of the putative protein. It is important to note that there is no evidence that this protein is expressed.

Primers were designed to specifically amplify the open reading frame of isoform D, and it was cloned along with isoform A into a GFP vector for transient expression studies in HT-29. Another cell line CV-1, green monkey kidney, was also used to confirm the localization of these isoforms. Transfections were performed using a cationic polymer reagent, which allowed for gentler and more efficient transfections. Other methods of transfection such as electroporation, lipid-based reagents, and nucleofection systems were also tested to see which would provide us with the highest transfection efficiency. These methods only yielded a 1% efficiency of transfection in HT-29 as compared with the cationic polymer solution, which gives an efficiency of up to 6%. Only the Amaxa nucleofection system was comparable.

Over-expressed isoform A and D were mainly cytoplasmic in HT-29 and CV-1, while the over-expressed myopodin-GFP fusion was mainly localized in the nuclear and nucleolar regions (Figure 5). Different isoforms appear to have different localizations within the cells suggesting that individual isoforms may have different functional roles as well. The empty GFP vector was used as a control and showed expression in both the nucleus and the cytoplasm (Figure 10). Interestingly, although synaptopodin-2 isoform A and D differ in their exons and drastically in their size, both are still localized to the cytoplasmic compartment of the cell indicating that exons 1 and 2 may contain some significant factor that causes them to be found in the cytoplasmic region as opposed to the nuclear region. Many actin-binding proteins like severin and FrgP are commonly predominantly cytoplasmic (Van Impe et al., 2003). Actin-binding proteins are involved in a multitude of functions within the cell. In the cytoplasm, actin forms the

filamentous network involved in cellular motility, cell shape, and movement of organelles (Castano et al. 2010). Actin-binding proteins help to anchor the actin network to the plasma membrane, and aid in the polymerization and de-polymerization of the growing actin filaments. These proteins are also implemented in the chromatin-remodeling complex and have been suggested not only to move or replace nucleosomes on DNA strands, but also help maintain the overall chromatin structure (Lee et al. 2007). During DNA replication/repair, actin-binding proteins even recruit factors and scaffolding on filamentous actin. Nuclear spectrin has been found to act as a scaffold, recruiting repair proteins to the site of DNA damage (Sridharan et al., 2003). The fact that synaptopodin-2 isoform D lacks the region corresponding to exon 4a may indicate that isoform D has a quite different function than isoform A. Based on previous studies, a number of the binding sites for synaptopodin-2 interactions with alpha-actinin, actin, Zyxin, 14-3-3 are located in the region corresponding to exon 4a (for review, see Chalovich et al., 2010).

As previously stated, earlier studies in the lab have also focused on the synaptopodin-2 isoform myopodin. During these studies, myopodin was found to be localized in the nucleus and nucleolar region of multiple cell lines using an isoform specific antibody. The NC_781 antibody recognizes an epitope in exon 4b, so theoretically it will detect myopodin as well as isoform B. Western blot analysis using cytoplasmic, nuclear, and nucleolar lysates confirmed that myopodin was expressed only in the nucleus and not in the nucleolus. This nuclear/nucleolar localization was later confirmed by using a myopodin-GFP fusion protein. The size and number of nucleoli in human cells varies according to the cell type and proliferation rate of the cell (Grisendi et al. 2006). The function of the nucleolus is closely connected to cell proliferation, division and growth; and many cancer cells have enlarged nucleoli and several nucleolar proteins have been

linked to tumorigenesis. For example, a nucleolar protein called nucleophosmin has been found to be mutated or overexpressed in various tumors and malignancies (Grisendi et al., 2006). Similarly, the nucleolar localization of an isoform of netrin-1 has been demonstrated to enhance tumor cell proliferation in several different cancers (Delloye-Bourgeois et al. 2008). CapG, an actin-capping protein, has also recently been said to exist in the nucleolus (Hubert et al. 2008).

A useful characteristic of HT-29 adenocarcinoma cells is their ability to differentiate in culture. When grown under standard conditions, the cells are multilayered and non-polarized. However, under certain conditions such as treatment with sodium butyrate as we have done, the morphology of HT-29 can be modulated to show a more polarized phenotype. After treating our HT-29 cells with sodium butyrate media for 7 days, which results in histone hyperacetylation and induction of differentiation, cells were again transfected with our synaptopodin-2 GFP fusion proteins, specifically isoforms A and D. After 48 hours, cells were mounted, counterstained with DAPI, and examined using a confocal microscope. After differentiation, the expression of the synaptopodin-2 isoform A and D GFP fusion proteins remained cytoplasmic. The differentiated phenotype of HT-29 cell is defined in part by the presence of tight junctions between adjacent cells and an increased brush borders on the apical cell surface (Anderson et al. 1993, Schneeberger and Lynch, 1992). Dome formation has also been observed in polarized cells (Gout et al, 2004). The brush borders contain bundled actin filaments that are further associated with other actin binding proteins like villin and fimbrin (Cohen et al., 1999). Similarly to the localization we have seen for isoform A and D, villin has been found have a diffuse and uniform distribution throughout the cytoplasm in undifferentiated cells, with strong apical labeling as well as a cytoplasmic localization in differentiated cells (Gout et al. 2004). Meanwhile, the actin cytoskeleton is dynamic, adjusting the positioning of microtubules within

the cells. Notably, the specific organization of the microtubules is different between polarized and non-polarized cells. In polarized epithelial cells specifically, microtubules have been found to aid in vesicle trafficking to and from the Golgi and plasma membrane (Grindstaff et al. 1998). Differentiation of HT-29 cells is also characterized by E-cadherin and F-actin redistribution. The assembly of brush borders is further accompanied by the protein synthesis of some actin-binding proteins and redistribution of specific molecules to the apical domain. These synthesized actin-binding proteins, including villin, fimbrin, ezrin, and fodrin, are produced before brush border membrane assembly and then concentrate sequentially at the apical surface (Cohen et al., 1999). Differentiation of HT-29 cells is also characterized by E-cadherin and F-actin redistribution to the lateral membranes, without any increase the level of over-all expression. Through experimentation, the activation of E-cadherin and its association with the cytoskeleton are responsible for the gain in cell adhesion observed in polarized cells (Gout et al., 2004).

While synaptopodin-2 isoform A and D were expressed in the cytoplasm in both the undifferentiated and differentiated states, the synaptopodin-2 isoform myopodin was expressed in the nucleus/nucleolus in the undifferentiated state and the cytoplasm after differentiation. The translocation of myopodin from the nucleus to the cytoplasm was shown by our lab in two different cell lines: HT-29 and C2C12 mouse myoblasts. Our results are consistent with previous observations by other labs in which myopodin was determined to shuttle between the nucleus and cytoplasm in a developmentally regulated manner (Weins et al., 2001). Supervillin, another actin-bundling protein and component of focal adhesions, has four predicted nuclear localization signals and is predicted to shuttle in and out of the nucleus in prostate cancer cells (Sampson et al., 2001). CapG, an F-actin barbed end capping protein controlling actin microfilament turnover in cells, can reside in both the nucleus and cytoplasm with its localization

being controlled by the phosphorylation of the protein (Van Impe et al., 2003; Gettemans et al. 2005). Supervillin is a nuclear/cytoplasmic actin-bundling protein that acts as coactivator of androgen receptor, and enhances the activity of hormones receptors like the glucocorticoid receptor and estrogen receptor (Ting et al. 2002). Recent work has confirmed actin's role in nucleocytoplasmic transport. Some heterogenous nuclear ribonucleoproteins bind actin and some of them have even been found to shuttle between the nucleus and cytoplasm (Percipalle et al., 2002, 2003). Paxillin also shuttles between focal adhesions and the nucleus to aid other proteins such as Ab and STAT3 in translocation from the nucleus to focal adhesions (Silver et al, 2004). Actin-binding proteins are also involved in the structure of the nuclear architecture. Lamins are especially important as they make up the nuclear lamina and make up the nuclear matrix. They can also link the cytoskeleton with the nucleoskeleton. Nesprins interact with F-actin and connect cytoplasmic actin filaments to actin microfilaments to SuN1 and SuN2 on the inner nuclear membrane, which binds to lamin A on the nuclear scaffold (Ostlund et al., 2009). Other actin-binding proteins can contribute to the structural properties of the nucleus by regulating the polymerization state of nuclear actin. Plastins may exist in the nucleus to crosslink nuclear actin and organize the actin filaments into bundles (Loomis et al., 2003). Myopodin is also known to bundle actin and exist in the nucleus of differentiating myoblasts (Weins et al. 2003).

Different effects of synaptopodin-2 on cancer could also be explained by changes in localization (De Ganck et al., 2009). For example, prohibitin (PHB) is expressed in the nucleoli of leukemic cell lines and have been found to possess both anti-tumorigenic and pro-tumorigenic functions, depending on its subcellular localization (Theiss and Sitaraman, 2011). An increase of PHB on the plasma membrane may facilitate tumerogenesis, while an increase of PHB in the

nucleus is associated with tumor suppression (Theiss and Sitaraman, 2011). The nuclear and cytoplasmic localization of synaptopodin-2 is differentiation and stress dependent (Weins et al., 2001). Cytoplasmic localization has been proposed to play a protective role in prostate cancer, while nuclear synaptopodin-2 is a cancer promoter (De Ganck et al., 2009). A few studies have shown a tumor suppressor role for synaptopodin-2 (De Ganck et al., 2009; Lin et al., 2001; Cebrian 2008). The role of myopodin as a tumor suppressor was described when synaptopodin-2 expression was found to be decreased in urothelial cancer (Sanchez-Carbayo et al. 2003). Also, a lower level of invasiveness and growth was seen in prostate cancer cells possessing synaptopodin-2 (Jing et al. 2003). Others, however, have shown that synaptopodin-2 overexpression and/or downregulation leads to an increase or decrease in invasiveness in myoblast and prostate cancer cells respectively (Weins et al., 2001; De Ganck et al., 2009).

In this study, our original hypothesis was that the localization of synaptopodin-2 isoforms affects their function within HT-29 cells. As previous researchers have found synaptopodin-2 to act as a tumor suppressor when in the nucleus and a tumor activator when in the cytoplasm in prostate cancer cells, we expected to find similar results with our HT-29 colon cancer cell line. One of our findings was evidence for the existence of two cytoplasmic isoforms of synaptopodin-2, isoforms A and D. We also observed that these synaptopodin-2 isoforms remain cytoplasmic after differentiation, in contrast to the nuclear isoform, myopodin, that has previously been found to translocate from the nucleus to the cytoplasm after differentiation in multiple cell lines. Ultimately, this research does not support the previous notion that synaptopodin-2 acts as a tumor suppressor in the nucleus and a tumor activator in the cytoplasm, but rather provides evidence that the effects of synaptopodin-2 are isoform dependent. This research also presents the idea that there are some isoforms of synaptopodin-2 that do not

translocate to-and-from different compartments of the cells at all. Again, HT-29 makes an interesting model for study because the undifferentiated, proliferating cell state is more comparable to tumorigenic cells, while the differentiated, polarized cells are comparable to cells in their normal state. Whereas De Ganck et al. found that the presence of myopodin in the nucleus in prostate cancer cells led to reduced motility and invasion, we found that myopodin is present in the nucleus and nucleolar regions only in proliferating HT-29 cells. Also, when our cells were differentiated and hence more like normal epithelial colon cells, myopodin translocated from the nucleus/nucleoli to the cytoplasm of the cells. In our case, synaptopodin-2 was cytoplasmic in what can be compared to be normal colon cells. Our results were again confirmed using the mouse myoblast cell line, C2C12. Again, we saw a translocation of myopodin from the nucleus/nucleolar region into the cytoplasm after cell differentiation. Interestingly, we saw that in C2C12 the nucleolar staining of myopodin remained even after differentiation. This research indicates that cell type also must be accounted for when studying synaptopodin-2. Therefore, it is my new hypothesis that the role of synaptopodin-2 in colon cancer is not necessarily dependent on where synaptopodin-2 is localized within cells, but rather is dependent on the different synaptopodin-2 isoforms that are present at any given time within specific cancer cell types.

As previously state, conflicting views of whether synaptopodin-2 is a tumor suppressor or activator could be due to the observation of different synaptopodin-2 isoforms expressed in the same cell (De Ganck et al., 2008). Major forms share the same N-terminus and all isoforms contain an actin-binding site, except for isoform D. All of the isoforms of synaptopodin-2 except myopodin possess a PDZ signaling domain in exon 1 (De Ganck et al. 2008), which could help anchor transmembrane proteins to the cytoplasm and act as a scaffold to hold together signaling

complexes (Bezproxvanny et al. 2001). PDZ domains are known to recognize C-terminal polypeptides as well as internal sequences of similar structure to the C-terminus, and are therefore able to bind to many different proteins oligomerize into branched protein networks (Harris and Lim, 2001). It is possible that these PDZ domains are dimerizing, allowing synaptopodin-2 to serve as a platform for two actin filaments to bind (De Ganck et al. 2008). Another feature of synaptopodin-2 family members is their multiple PXXP motifs, which may be involved in binding to SH3-domain-containing proteins (Chalovich and Schroeter, 2010). SH3 domains recognize and bind to these proline-rich segments, and are often present in transduction and cytoskeletal proteins. As proline-rich PXXP domains are highly exposed, their rates of binding are extremely fast allowing them to easily and quickly bring proteins together in a way that the following protein interactions are more probable (Kay et al. 2000). Myopodin also possess 2 lysine-rich nuclear localization sites (NLS) as well as 2 binding sites for the 14-3-3 domain, which have been found to be necessary for shuttling myopodin away from the Z-line in cardiac myocytes, where it then binds to α-actinin (Faul et al. 2005). The 14-3-3 domain has been implemented in many different subcellular compartments and has been found to regulate many different protein types including cytoskeletal proteins, signaling molecules, and tumor suppressors. Furthermore, binding with the 14-3-3 domain can influence the stability, activity, localization or phosphorylation state of the target protein (Dougherty and Morrison, 2004). Lastly, synaptopodin-2 isoforms contain the alpha helical LXXLL motif that either helps or hinders transcription activation by acting as a mediator for the initial contact between nuclear receptors and their co-regulators (De Ganck et al. 2009). As mentioned previously, Synaptopodin-2 has been shown to stimulate actin polymerization and bundle actin, and myopodin has been found to possess a novel actin binding site (Weins et al. 2001).

After examining just a few of the many possibilities for the role of actin-binding proteins, the biological function of synaptopodin-2 appears to be highly multidimensional. The role of Synaptopodin-2 could be easily affected by expression of its individual isoforms, the cellular compartment the protein is localized to, or the translocation of the protein to different locations within the cells. While there is much to speculate about synaptopodin-2, it is evident that more research must be done before its role can be fully elucidated. One possible future tool for study has already been considered and prepared by our lab. Synaptopodin-2 isoforms A and D have also been cloned into a tetracycline-inducible vector, pcDNA4/TO, for the purpose of creating stable cell lines. Through these constructs, isoforms A and D will be over-expressed and then forced to undergo antibiotic selection so that only cells over-expressing synaptopodin-2 will be passaged into future generations. These stable lines can then be used for migration and invasion assays to further study the role of the individual isoforms of synaptopodin-2 as either a tumor promoter or suppressor. If the synaptopodin-2 isoforms could be expressed as protein, the co-localization studies could be done by pulling down the protein with HIS-TAG. Future experiments may also involve truncation experiments similar to those performed with the nucleolar protein netrin in which researchers were able to identify a novel nucleolar localization signal by studying the localization of truncated-versions of netrin-1 inserted into a GFP fusion vector (Delloye-Bourgeois et al. 2008). Co-localization studies could also be performed using other cytoplasmic and nuclear/nucleolar proteins in order to gain a better understanding of the potential roles of the isoforms of Synaptopododin-2 in colon cancer.

As previous researchers have found synaptopodin-2 to act as a tumor suppressor when in the nucleus and a tumor activator when in the cytoplasm in prostate cancer cells, we expected to find similar results with our HT-29 colon cancer cell line. Ultimately, this research does not

support the previous notion that synaptopodin-2 acts as a tumor suppressor in the nucleus and a tumor activator in the cytoplasm, but rather provides evidence that the effects of synaptopodin-2 are isoform dependent. We also present the idea that there are some isoforms of synaptopodin-2 that do not translocate to-and-from different compartments of the cells at all. The role of synaptopodin-2 in colon cancer therefore may not necessarily dependent on where synaptopodin-2 is localized within cells, but rather may be dependent on the different synaptopodin-2 isoforms that are present at any given time within specific cancer cell types.

Chapter 5: Materials and Methods

Cell Culture -

HT-29 colon cancer cell-line was obtained from ATCC (Cat number: HTB38) and was used as the model for this research project. HT-29 was cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 (Invitrogen), supplemented with 10 % fetal bovine serum (FBS), penicillin and streptomycin (100 U and 100 μg/ml, respectively). For differentiation of HT-29 cells, cells were treated with normal media supplemented with 5 mM sodium butyrate (Sigma) for a minimum of seven days. Cells were kept at 37°C in a humidified CO_2 incubator (5 % CO_2).

RNA Preparation –

Total RNA was extracted from HT-29 cells using the RNeasy Plus Mini Kit from Qiagen. Approximately 1 x 10^7 cells were harvested as a cell pellet prior to lysis. After washing several times with PBS, the cells were disrupted using the appropriate volume 600 μl of Buffer RLT Plus and gently mixed by flicking the tube. To homogenize, the lysate was passed through a 20-guage needle fitted to an RNase-free syringe at least five times. One volume of 70% ethanol was added to the lysate and mixed well by pipetting. 700 μl of the sample, including any precipitate that may have formed, were transferred to an RNeasy spin column placed in a 2 ml collection tube, centrifuged for 15 seconds at ≥ 8000 x g, and the flow-through discarded. This step was repeated until the whole sample was applied to the column. Then 500 μl of Buffer RPE were added to the spin column and centrifuged for 15 seconds at ≥ 8000 x g to wash the spin column membrane. This step was repeated with a 5 minute centrifugation. The column bound with RNA was then transferred to a new tube and centrifuged for 1 minute at ≥ 8000 x g to remove

residual RPE buffer or flow-through on the outside of the column. Then, the RNeasy spin column was transferred to a new 1.5 ml collection tube and 50 µl of RNA-free water was added directly to the spin column membrane. RNA was eluted in RNA-free water by centrifugation for 1 minute at ≥ 8000 x g. DNase treatment was used to remove any genomic DNA (Ambion, AM1907). In brief, to a 5 µg solution of RNA sample, 0.1 volume of 10X TURBO DNase buffer and 0.5 µl TURBO DNase (2 U/µl) was added, and mixed gently. The samples were incubated at 37°C for 30 minutes; then an additional 0.5 µl of TURBO DNase was added and the samples were incubated at 37°C for an additional 30 minutes before adding 0.1 volume of DNase Inactivation Reagent. Samples were incubated at room temperature for 5 minutes, mixing periodically, then centrifuged at 10,000 x g for 1.5 minutes, and the supernatant transferred into a new test tube. Five µg of total purified RNA were aliquoted into 0.5 ml and stored at -80°C.

cDNA Synthesis –

Total RNA isolated from HT-29 cells as well as purchased total RNA from human skeletal muscle (Clontech) was used to synthesize cDNA. First, 1 µl of 50 µM oligo(dT) primers or 2 pmoles of isoform A specific primer (Table #), and 1 µl of 10 mM dNTP that are mixed well were added to 5 µg RNA. The final volume was adjusted to 12 µl with sterile RNase free water. Samples were incubated at 65°C for 5 minutes, and then transferred to ice for 1 minute. To each RNA/primer mix sample, was added 4 µl of 5X First-Strand Buffer, 2 µl of 0.1 M DTT, and 1 µl of RNase OUT (40 U/µl). The contents of the tube were gently mixed, incubated for 2 minutes at 42°C, and 1 µl of Superscript II RT (200 U/µl) was added. The samples were then incubated for 50 minutes at 42°C, and then inactivated by heating at 70°C for 15 minutes. Finally, 1 µL of *E. coli* RNase H was added and the sample incubated for 20 minutes at 37°C. The resulting cDNA was stored at -20°C.

Cloning

Primer Design –

Primers were designed to clone the full-length cDNA of synapotopdin-2 isoform A and D into different expression vectors, allowing a GFP fusion protein expression and inducible expression with the TREX system (Invitrogen). A 6xHIS-tag was added to the reverse primer for the inducible system. Restriction enzyme sites were added to the forward primer for (*Hin*dIII) as well as a Kozak sequence and reverse primer with a *Bam*HI sequence (See Primer Table). The first full length coding sequence was amplified through long range PCR and cloned into the pcDNA4/TO vector. After amplification and selection in bacteria, the cloned sequence was checked for accuracy by sequencing using specially designed primers for synaptopodin-2 (See Primer Table). The plasmid was then used to transfect HT-29 cells. The plasmids were used as a template to amplify the full length of synaptopodin-2 isoforms A and D to allow the entire genes to be inserted into a transient expression vector encoding a variant of GFP for fluorescence. Restriction enzyme sites were also added to the forward (*Bam*HI) and reverse (*Hin*dIII) primers to allow the PCR product to be ligated into the vector.

Long Range Polymerase Chain Reaction-

An Expand Long Template PCR system amplification kit was purchased from Roche. Following the manufacturer's instruction, cDNA from human skeletal muscle, HT29 cells, or plasmid containing inserts for GFP-fusion was used as the template. A PCR core mix was prepared by combining sterile water, 10X Expand Long Template buffer 1, 50 mM $MgCl_2$, 10 mM dNTPs, and Expand Long Template Enzyme mix for each individual test tube. The PCR core mixed was then added to each individual 0.2 ml PCR tubes that contain 1 µL of cDNA template and 2.5 µl

(30 nMol) of a forward and 2.5 µl (30 nMol) 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 optimal annealing temperatures for each primer set was determined by running a temperature gradient ranging from 55-65°C. The thermocycler is programmed to denature the cDNA at 92°C for 1 minute, then 9 cycles: 92°C for 10 seconds, the specified annealing temperature for the primer set for 15 seconds, and 68°C for 4.5 minutes for elongation. Following the previous 9 cycles, another 25 cycles: 92°C for 10 seconds, 60°C for 15 seconds, and 68°C for 4 minutes and 50 seconds. After all 34 cycles have been completed, samples will be at held at 68°C for 5 minutes and then held at 15°C.

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 fusing the genes for synaptopodin-2 isoform A and isoform D to the N-terminus of EGFP, the localization of the target gene was visualized through the fusion protein. The recombinant vector was transfected into HT-29 cells (Figure 14).

Next, transforming into a tetracycline inducible expression vector allowed us to induce overexpression isoforms A and D, and analyze their effects on cell proliferation and migration when compared to the normal cells. The cDNA of synaptopodin-2 isoform A and isoform D was be cloned into *Hind*III and *Bam*HI of the multiple cloning site of the inducible expression vector pcDNA4/TO (Figure 14).

Figure 14

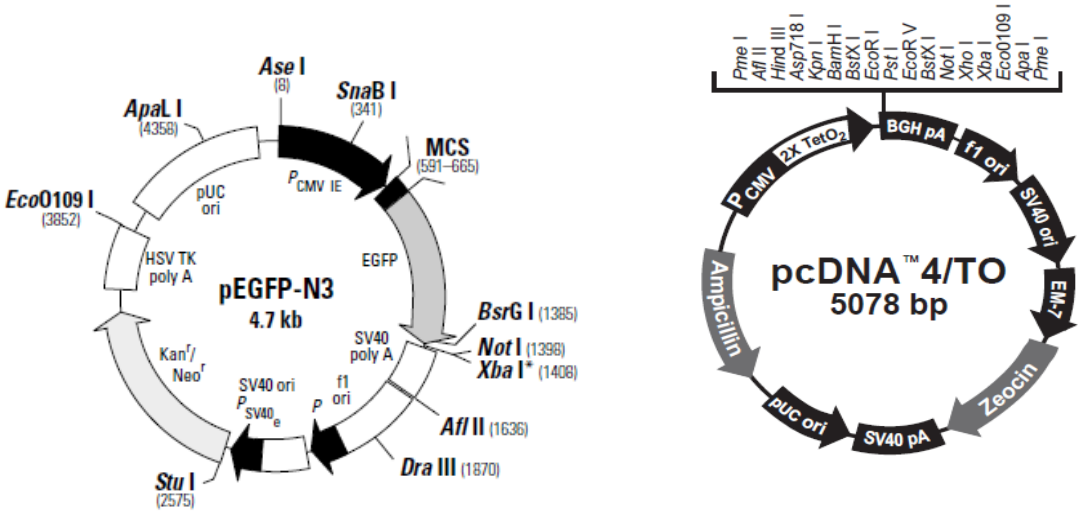

Figure 14: pEGFP-N3 is a mammalian expression vector that encodes a red shift variant of GFP, which was received from the Lemasson lab at the Brody School of Medicine (left). T-REx pcDNA4/TO mammalian expression vector purchased from Invitrogen (right). HindIII and BamHI restriction enzyme sites will be used to insert the amplified PCR product into the vectors.

Ligation & Transformation into XL-2/TOP10 Competent Cells

To produce circular recombinant molecules, the DNA inserts for synaptopodin 2 isoform A and D were ligated into the tetracycline inducible vector pcDNA4/TO and into the GFP-tagged p-EGFP-N3 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 5 minutes at room temperature. Next, 2 μl of the reaction was used to transform into XL-2 Blue Ultracompetent cells (Invitrogen). First, microcentrifuge tubes were chilled on ice. The competent cells were removed from -80°C freezer and thawed on ice to keep their efficiency level high. When thawed, 2 μl of the reaction mixture was added to 50 μl of XL-2 cells and mixed gently with a pipette tip. The reaction mixture and the cells were then incubated on ice for 30 minutes. The mixture was 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. Following the heat shock and incubation on ice, 1 ml of super optimal broth with catabolite repression (SOC) was added and tubes are incubated for 1 hour at 37°C in a shaking incubator. After an hour, 150 μl and 300 μl of the SOC mixture was added to LB plates and allowed to grow overnight at 37°C. Colonies were picked 16 hours later and grown overnight in LB broth containing antibiotics (either kanamycin or ampicillin depending on the antibiotic resistance gene present in the plasmid). Plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen) 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. Clones were sequenced and confirmed using DNASTAR molecular biology software (DNASTAR, Inc.).

Transfection into HT-29 Cells

All vectors will be delivered into the cells using a cationic polymer Turbofect Transfection Reagent (Thermoscientific #R0531). The polymer is able to form positively charged complexes with DNA that protect it from degradation and allow easier gene delivery in eukaryotic cells. The procedure was carried out using manufacturer's guidelines. Briefly, the day prior transfection, cells are seeded in 6 ml of growth medium in a 60 mm petry dish at a density that gives a 70-90% confluency. To prepare the reaction mixture, 6 ug of DNA is diluted in 600 µl per well of serum-free DMEM and mixed well by vortexing. The Turbofect Reagent will be briefly vortexed and 12 µl will be added to the to each reaction tube. The solutions are then be incubated for 15-20 minutes at room temperature. Once complexes have formed, all 600 µl of Turbofect/DNA mixture is added to each dish while leaving the growth medium on the cells. The dish is gently rocked to ensure an even distribution of the complexes and stored afterwards in a CO_2 incubator at 37°C. After 24-48 hours, the transgene expression can be analyzed for isoforms A and D.

Antibiotic Concentration Determination

To generate a stable cell line expressing the Tet repressor and the protein of interest, the minimum concentration of blasticidin and Zeocin needed to kill the untransfected HT29 cells was first determined. First, HT29 cells were seeded in triplicate in a 12-well plate at 25% confluency 24 hours prior to the first antibiotic treatment. For blasticidin selection, concentrations of 0, 1, 3, 5, 6, 7.5, and 10 µg/ml blasticidin were tested, while for Zeocin selection, concentrations of 0, 50, 125, 250, 500, 600, 800, and 100 µg/ml of Zeocin were tested. Every 3-4 days, the selective media was changed. After 2 weeks, the number of viable cells was

determined by counting. To perform a cell count, cells were collected through trypsinization and centrifugation, and were resuspended in 1 ml of complete DMEM/F12 media. From that cell solution, 50 µl were transferred into a microcentrifuge tube and are stained with 50 µl of 0.4% trypan blue. Approximately 20 µl of the stained cells were transferred to a Neubauer hemocytometer gridded slide to allow for cell counting. cell counting, established the minimum concentration to be blasticidin 6 µg/ml, while the minimum concentration of Zeocin needed was 600 µg/ml.

Immunocytochemistry -

Immunocytochemistry was used to analyze the localization of the isoform A- and D-GFP fusion proteins within HT-29 cells. Coverslips with adhered HT-29 cells were washed 3 times with 1X phosphate buffered saline (PBS) and fixed in 3.7% formalin in 1X PBS for 8 minutes at room temperature. After fixation, coverslips were washed several times to remove traces of fixing solution. In order to easier visualize the expression of the pEGFP-N3/synaptopodin-2 fusion proteins under the microscope, cells were counterstained with the nuclear stain DAPI (Sigma) at a concentration of 1 µg/ml in 1X PBS. Approximately 50 µl of DAPI was added to each coverslip and allowed to incubate for 10 minutes at room temperature before being visualized. After the final rinse in 1X PBS, the coverslips were mounted with Vectashield (Vector Laboratories, Inc.) and were observed using an Olympus IX2-DSU confocal microscope with a Hamamatsu EM-CCD digital camera model C9100. Images were prepared using Slidebook Version 4.2 software.

Nuclear and Cytoplasmic Protein Cell Extract Preparation -

To analyze the location of specific proteins for either the cytoplasm or the nucleus, a nuclear and cytoplasmic extraction was performed using the NE-PER kit (Thermo, 78833). All lysis buffers and reagents were supplemented with protease inhibitor cocktail (Pierce, 88661). Following the manufacturer's instructions, adherent HT-29 cells were harvested with 0.05% trypsin-EDTA, centrifuged at 500 x g for 5 minutes, and washed by resuspension in 1X PBS. Approximately 2 x 10^6 cells were transferred to a 1.5 ml microcentrifuge tube and pelleted by centrifugation at 500 x g for 2-3 minutes. The supernatant was carefully removed and discarded leaving only the dry pellet. Next, 20 µl of ice-cold CER I were added to the cell pellet. The tube was vortexed at a maximum speed of ~16,000 x g for 15 seconds to fully suspend the cell pellet and incubated on ice for 10 minutes. Then 22 µl ice-cold CER II were added to the tube and vortexed on high for 5 seconds. The tube was then incubated on ice for 1 minute. Again, the tube was vortexed on high for 5 seconds and centrifuged at maximum speed of in a microcentrifuge. The supernatant (cytoplasmic extract) was immediately transferred to a clean pre-chilled tube and kept on ice. Next, the insoluble fraction containing the nuclei was resuspended in 100 µl of ice-cold NER reagent. Cells were vortexed on the highest setting for 15 seconds, placed on ice and vortexed for 15 seconds every 10 minutes for a total of 40 minutes. Following a 40 minute incubation on ice, the tube was centrifuged at a maximum speed of ~16,000 x g in a microcentrifuge for 10 minutes. Immediately, the supernatant fraction (nuclear) was added to a clean pre-chilled tube and placed on ice. A small amount was aliquoted for quantification by Pierce 660 nm protein assay (Thermo Scientific) following the manufacturer's instructions. The lysates were then stored at -80°C or immediately prepped for SDS-polyacrylamide gel electrophoresis.

Western Analysis

SDS-Page Gels-

Cell lysates were loaded onto a SDS-PAGE using a BioRad Mini-PROTEAN Tetra Cell. A 10% resolving gel and 4% stacking gel were prepared according to the manufacturer's instructions (Invitrogen). After allowing the resolving gel to polymerize for 1 hour, the 4% stacking gel was poured over the resolving gel and allowed to polymerize for at least 30 minutes at room temperature. Once the stacking gel had polymerized, the combs were removed, and the apparatus was filled with Electrode Buffer (25 mM Tris-HCl, 192mM glycine, 0.1% SDS – pH 8.3). Next, 30 μg of protein was combined with an equal volume of 2X Laemmli Buffer (0.125M Tris-HCl pH 6.8, 10% 2-mercaptoethanol, 20% glycerol, 0.0004% bromophenol blue, 4% SDS). The samples were then incubated for 5 minutes at 95°C. After a brief centrifugation, samples were loaded along with 7 μL of Novex Sharp pre-stained protein standard (Invitrogen). The samples were run at 75V until they reached the bottom of the resolving gel. To check for the total amount of transferred protein, the membrane was stained for 1 minute with a Ponceau S. solution (0.1% w/v in 5% acetic acid). To determine if the HT-29 cell lysate contains proteins of specific interest and whether or not those proteins are expressed in the cell extracts, a wet transfer was used to transfer the protein from the SDS-PAGE to a Protran nitrocellulose membrane (Whatman). The membrane and 3 mm whatman paper were cut to the dimensions of the gel. To prepare the wet transfer apparatus, the clear base is laid down followed by a spacer, the cathode, and another spacer until it is fully covered with transfer buffer. Four sponges were prepared in a separate dish by complete saturation with transfer buffer. Two of these sponges were then be laid on top of the spacer, followed by a sheet of chromatography paper, and the gel containing the protein. After wetting the nitrocellulose membrane, the membrane was then be

laid over the gel and then covered by another sheet of whatman paper. The final two sponges were added as well as another spacer, the platinum anode, and the final spacer. The plastic lid was then added and everything was be placed inside the outer portion of the contraption and placed upright. Transfer buffer was added to the upright device so that it was completely covering the gel. The entire apparatus was packed on ice in a cooler to reduce the amount of heat generated. The transfer was run for the first 20 minutes at 12V and then for another hour at 24 V.

Blocking and Antibody Incubation-

Once the protein transfer is complete, unspecific protein-protein interactions are blocked by incubating the membrane under constant agitation in 5% milk, diluted in TBST buffer (Tris Buffered Saline with 0.05% Triton X-100) for 30 minutes. After blocking, the membrane was washed 5 times for 1.5 minutes with TBST, and then incubated under constant agitation for 4 hours at room temperature with an anti-GFP primary antibody (Clontech, 8363-2) diluted (1:1000) in blocking buffer. The membrane was washed again with TBST five times for 1.5 minutes, and then incubated under constant agitation for 1 hour at room temperature with the horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody in goat (Sigma, A0545) diluted (1:20000) in blocking buffer. The membrane was thoroughly washed multiple times with TBST before proceeding to chemiluminescence.

Chemiluminescence-

In order to visualize the proteins from the western blot, Supersignal West Pico Chemiluminescence Substrate (Thermo) was used. For a working solution of substrate, equal volumes of the luminol/enhancer and the stable peroxide buffer were mixed together. The

chemiluminescent working substrate was incubated on the membrane for 5 minutes at room temperature, excess substrate was removed, and the membrane was exposed to film.

REFERENCES

Anderson JM, Balda MS, Fanning AS. (1993). The structure and regulation of tight junctions. Curr Opin Cell Biol. 5, 772-778.

Asanuma K, Kim K, Oh J, Giardino L, Chabanis S, Faul C, Reiser J, Mundel (2005) Synaptopodin regulates the actin-bundling activity of α-actinin in an isoform specific manner. J Clinical Investigation 155:1188-1198.

Bezprozvanny I, Maximov A (2001). PDZ Domains: More than just a glue. PNAS 98: 787-789.

Castano E, Philimonenko VV, Kahle M, Fukalova J, Kalendova A, Yildirim S, Dzijak R, Dingova-Krasna H, Hozak P (2010) Actin complexes in the cell nucleus: new stones in an old field. Histochem Cell Biol 133:607–626.

Catalano A, O'Day DH (2011) Nucleolar localization and identification of nuclear/nucleolar localization signals of the calmodulin-binding protein nucleomorphin during growth and mitosis in dictyostelium. Histochem Cell Biol: 136 (6): 677-88.

Cebrian V, Alvarez M, Aleman A, Palou J, Bellmunt J, Gonzalez-Peramato P, Cordón-Cardo, Garcia J, Piulats J.M., Sanchez-Carbayo M (2008) Discovery of myopodin methylation in bladder cancer. J Pathol 216: 111-119.

Cohen, Esther, Ophir, Ilana, Yehuda, Ben Shaul (1999) Induced differentiation in HT29, a human colon adenocarcinoma cell line. J. of Cell Science 112: 2657-2666.

Chalovich J, Schroeter M (2010) Synaptopodin family of natively unfolded actin binding proteins: physical properties and potential biological functions. Biophys Rev 2: 181-189.

De Ganck A, De Corte V, Staes A, Gevaert K, Vanderkerckhove J, Gettemans J (2008) Multiple isoforms of the tumor suppressor myopodin are simultaneously transcribed in cancer cells. Biochem Biophys Res Commun 370: 269-273.

De Ganck A, De Corte V, Bruyneel E, Bracke M, Vandekerckhove, Gettemans J (2009). Down-regulation of myopodin expression reduces invasion and motility of PC-3 prostate cancer cells. Int J Oncol 34(5):1403-1409.

Delloye-Bourgeois, Celine, et al. (2012), Nucleolar Localization of a Netrin-1 Isoform Enhances Tumor Cell Proliferation. Science Signaling 5 (236): 1-14.

Dougherty MK, Morrison DK (2004). Unlocking the code of 14-3-3. J of Cell Sci. 117:1875-1884.

Eichinger L, Bruyneel E, Mareel M, Vandekerckhove J, Gettemans J (2003) The Nucleo-cytoplasmic actin-binding protein CapG lacks a nuclear export sequence present in structurally related proteins. J Biol Chem 278:17945– 17952.

Faul C, Dhume A, Alison D, Mundel P. (2005). Protein Kinase A, Ca2+/Calmodulin-Dependent Kinase II, and Calcineurin Regulate the Intracellular Trafficking of Myopodin between the Z-Disc and the Nucleus of Cardiac Myocytes. Molecular and Cellular Biology 27 (23): 8215-8227.

Gettemans J, Van Impe K, Delanote V, Hubert, Vandekerckhove J, De Corte V (2005). Nuclear actin-binding proteins as modulators of gene transcription. Traffic 6: 847-857.

Gout S, Marie C, Laine M, Tavernier G, Block MR, Jacquier-Sarlin M. (2004). Early enterocytic differentiation of HT-29 cells: biochemical changes and strength increases of adherens junctions. Exp Cell Research. 299, 498-510.

Grindstaff KK, Bacallao RL, Nelson WJ. (1998) Apiconuclear organization of microtubules does not specify protein delivery from the trans-Golgi network to different membrane domains in polarized epithelial cells. Mol. Biol. Cell. 9, 685-699.

Harris BZ, Lim WA (2001). Mechanism and role of PDZ domains in signaling complex assembly. J. Cell Sci. 114: 3219-3231.

Honda K., T. Yamada, R. Endo, Y. Ino, M. Gotoh, H. Tsuda, Y. Yamada, H. Chiba, and S. Hirohashi. (1998). Actinin-4, a novel actin-bundling protein associated with cell motility and cancer invasion. J. Cell Biol. 140, 1383–1393.

Hubert T, Van Impe K, Vandekerckhove J, GEttemans J (2008). The F-actin filament capping protein CapG is a bona fide nucleolar protein. Biochem Biophys Res Commun 377: 699-704.

Iakoucheva LM, Brown CJ, Lawson JD, Obradovic Z, Dunker AK (2002) Intrinsic disorder in cell-signaling and cancer-associated proteins. J Mol Biol 323:573–584.

Isogai T, Yamamoto J. (2007). Human cDNA sequencing project focused on splicing variants of mRNA in NEDO functional analysis of protein and research application project supported by Ministry of Economy, Trade and Industry Japan. Retrieved from NCBI. (Genbank BAG65020.1)

Jing L, Liu L, Yu YP, Dhir R, Acquafondada M, Landsittel D, Cieply K, Wells A, Luo J-H (2004). Expression of myopodin induces suppression of tumor growth and metastasis. Am. J. Pathol. 164:1799-1806.

Kay BK, Williamson MP. Sudol M (2000). The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains. The FASEB J. 14: 231-241.

Kumeta M, Yoshimura SH, Harata M, and Takeyasu K (2009). Molecular mechanisms underlying nucleocytoplasmic shuttling of actinin-4. J. Cell Sci. 123, 1020-1030.

Lee K, Shim JH, Kang MJ, Kim JH, Ahn JS, Yoo SJ, Kim Kwon Y, Kwon H (2007). Association of BAF53 with mitotic chromosomes. Mol Cells 24:288-293.

Leinweber BD, Fredricksen RS, Hoffman DR, Chalovich JM (1999). Fesselin: a novel synaptopodin-like actin binding protein from muscle tissue. J Muscle Res Cell Motil 20:539-545.

Lin F, Yu YP, Woods J, Cieply K, Gooding B, Finklestein P, Dhir R, Krill D, Becich M.J., Michalopoulos G, Finklestein S, Luo JH (2001) Myopodin, a synaptopodin homologue, is frequently deleted in invasive prostate cancers, Am J Pathol 159: 1603-1612.

Loomis PA, Zheng L, Sekerkova G, Changyaleket B, Mugnaini E, Bartles JR (2003). Espin cross-links cause the elongations of microvillus-type parallel actin bundles in vivo. J. Cell Biol. 163:1045-1055.

Mundel P, Heid HW, Mundel TM, Krüger M, Reiser J, Kriz W (1997) Synaptopodin: an actin-associated protein in telencephalic dendrites and renal podocytes. J Cell Biol 139:193–

204.

Ostlund C, Folker ES, Choi JC, Gomes ER, Gundersen GG, Worman HJ (2009). Dynamics and molecular interactions of linker of nucleoskeleton and cytoskeleton (LINC) complex proteins. J. Cell Sci. 122: 4099-4108.

Percipalle P, Jonsson A, Nashchekin D, Karlsson C, Bergman T, Guialis A, Danehold B (2002). Nuclear actin is associated with a specific subset of hnRNP A/B-type proteins. Nucleic Acids Res. 30:1725-1734.

Philimonenko VV, Janacek, J, Harata M, Hozak P (2010) Transcription-dependent rearrangements of actin and nuclear myosin I in the nucleolus. Histochem Cell Biol 134:243-249.

Sampson ER, Yeh SY, Chang HC, Tsai MY, Wang X, Ting HJ, Chang C. (2001). Identification and characterization of androgen receptor associated coregulators in prostate cancer cells. J. Biol. Regul. Homeost. Agents. 15, 123-129.

Sanchez-Carbayo M, Schwarz K, Charytonowicz E, Cordón-Cardo C, Mundel P (2003) Tumor suppressor role for myopodin in bladder cancer: loss of nuclear expression of myopodin is cell cycle dependent and predicts clinical outcome, Oncogene 22: 5298-5305.

Schroeter MM, Chalovich JM (2004). Ca^{2+} -calmodulin regulates fesselin-induced actin polymerization. Biochemistry 43:13875-13882.

Schroeter MM, Chalovich JM (2005). Fesselin binds to actin and myosin and inhibits actin

activated ATPase activity. J Muscle Res Cell Motil 26:183-189.

Schneeberger EE and Lynch RD. (1992). Structure, function and regulation of cellular tight junctions. Am. J. Physol. 273, G253-G257.

Silver DL, Naora H, Liu J, Cheng W, Montell DJ (2004). Activated signal transducer and activator of transcription (STAT 3): localization in focal adhesions and function in ovarian cancer cell motility. Cancer Res. 64:3550-3558.

Ting HJ, Yeh S, Nishimura K, Chang C (2002). Supervillin associates with androgen receptor and modulates its transcriptional activity. Proc. Natl. Acad. Sci. USA 99:661-666.

Van Impe K, De Corte V, Eichinger L, Bruyneel E, Mareel M, Vandekerckhove J, Gettemans J. (2003). The Nucleo-cytoplasmic actin-binding protein CapG lacks a nuclear export sequence present in structurally related proteins. J Biol Chem 278:17945– 17957

Wakamatsu A, Yamamoto J, Kimura K, Ishii S, Watanabe K, Sugiyama A, Murakawa K, Kaida T, Tsuchiya K, Fukuzumi Y, Kumagai A, Oishi Y, Yamamoto S, Ono Y, Komori Y, Yamazaki M, Kisu Y, Nishikawa T, Sugano S, Nomura N, Isogai T. (2008). NEDO human cDNA sequencing project focused on splicing variants. Retrieved from NCBI. (Genbank BAG65020.1)

Weins A, Schwarz K, Faul C, Barisoni L, Linke W.A., Mundel P (2001) Differentiation and stress-dependent nuclear cytoplasmic redistribution of myopodin, a novel actin-bundling protein, J Cell Biol 155: 393-404.

“What is Colorectal Cancer?” Cancer.org. The American Cancer Society. 8 March 2011. Web. 1 April 2011.

Yu YP, Luo JH (2006) Myopodin-mediated suppression of prostate cancer cell migration involves interaction with zyxin. Cancer Res 66:7414–7419.

Yu YP. Luo JH (2011) Phosphorylation and integration of myopodin by integrin-link kinase lead to suppression of cell growth and motility in prostate cancer cell. Oncogene 30: 4855+.

APPENDIX

Table 1

Synaptopodin-2 Primers		
Primer Name	**Sequence 5' to 3'**	**Purpose**
RTSynpo2IsoA	GTATTCACACTCACCTGTGTG	RT-PCR
Synapto_1F	ATCAGCCTCATCATCTTATTTTGT	PCR
Synapto_1R	CCACTAGACCGAGAGGAGACTTTG	PCR
Synpo2ForwardK	AAAACATGGGCACAGGGGATTTTATCTGC	PCR with intenti on to clone
Synpo2StartF	GAGAAGCTTAAAACATGGGCACAGGGGATTTTATCTGC	PCR with intenti on to clone
RevComplementSynpoIsoABamHI	GAGGGATCCTCAGTGGTGATGGGTGATGATGCCCGCCCCCTGTTTGGCGTCTCCACCCT	PCR with intenti on to clone
GFP vector variant A	GAGGGATCCTGTTTGGCGTCGTCTCCATCCCCTTG	PCR with intenti on to clone
GFP vector variant D	GAGGGATCCGTCCATCGGTTGGTTACTAGAG	PCR with intenti on to clone
Sequencing 1F	AGTGCACAGAATTCTTCC	Sequencing
Sequencing 1R	AGAAGAGCTGATCTTAAG	Sequencing
Sequencing 2F	AGTGATCCAGGAAAGTGA	Sequencing
Sequencing 2R	AAGATCCACGCTCGGAAA	Sequencing
Sequencing 3F	AGTTAGCTACGGTACTGG	Sequencing
Sequencing 3R	AGTTGTGAACTTTGACTG	Sequencing
Sequencing 4F	TGTGAGCAAAAGCTACAT	Sequencing
Sequencing 4R	AACCCGAAACATGACGAGTC	Sequencing
Sequencing 5F	GAGAAGCACGACAAAACC	Sequencing
Sequencing 5R	TGTTTGCTCCAAAACCTGCAGT	Sequencing
Sequencing 6F	TTCAAAGGACCACAAGCA	Sequencing
Sequencing 6R	AGTCGAGAATGGAGAAGT	Sequencing
Sequencing 7F	TCAGCTCAATGCATCCTT	Sequencing
Sequencing 7R	TTCGTCAGTGTACTCGGT	Sequencing
Sequencing 8F	CAAGTCTCCTCTCGGTCTAGTGG	Sequencing
Sequencing 8R	AATGTGGTTTCAGCAGCT	Sequencing

Table 1: List of all synaptopodin-2 primers used in this study.

Table 2

Antibodies			
Primary	**Dilution**	**Secondary**	**Dilution**
NC_781 (Schroeter).	1:500 IF	FITC-Conj Rabbit IgG (Sigma)	1:150 IF
NC_781 (Schroeter)	1:1000 WB	HP-Conjugated Rabbit IgG (ECL)	1:20000 WB
anti-nucleolin (Pierce)	1:500 IF	Cy3-conjugated Mouse IgG (Sigma)	1:500 IF
anti-GFP (Clontech)	1:1000 WB	HP-Conjugated Rabbit IgG (Sigma)	1:20000 WB

Table 2: List of antibodies used in this study.