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Investigating Heat Shock Protein 70 as a Binding Partner of MK-STYX, and the Role of MK-STYX in Neuronal Differentiation

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Investigating Heat Shock Protein 70 as a Binding Partner of MK-STYX, and the Role of MK-STYX in Neuronal Differentiation

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Department of Biology

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Master of Science

Lauren Elizabeth Rusnak

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ABSTRACT

Protein tyrosine phosphatases (PTPs) are important regulators of various cellular processes such as proliferation, metabolism, differentiation, and apoptosis. Intriguingly, there are members that display the structural features of PTP enzymes, but lack the critical nucleophilic cysteine in the active site for catalysis. Thus, these proteins are catalytically dead and referred to as pseudophosphatases. However, they maintain their ability to bind phosphorylated residues, thus, serving as substrate “traps”. This substrate “trap” ability is useful in understanding the functions of pseudophosphatases. A member of the dual specificity phosphatase subfamily of PTPs, MK-STYX (MAPK (mitogen-activated protein kinase) phospho-serine/threonine/tyrosine-binding protein) is a pseudophosphatase. MK-STYX is catalytically inactive due to the fact that it has a phenylalanine and serine in its signature motif instead of the expected histidine and cysteine. Our previous work utilized the substrate-trap ability and mass spectrometry for functional characterization of MK-STYX, which implicated MK-STYX in the stress response pathway. MK-STYX binds G3BP1 (GTPase activating protein (SH3 domain) binding protein 1), causing a decrease in the presence of stress granules. However, MK-STYX remains relatively uncharacterized. To further characterize MK-STYX’s function, its role in heat shock protein 70 (hsp70) signaling was investigated. Hsp70 was previously identified as a potential binding partner of MK-STYX using mass spectrometry experiments. Both MK-STYX and hsp70 have a similar function, as both proteins are capable of decreasing the presence of stress granules. In studying this interaction, hsp70 expression or the phosphorylation of heat shock transcription factor-1 (hsf-1) was not affected by MK-STYX. However, we have found that MK-STYX is a promising binding partner of hsp70. Considering the interaction between G3BP1 and MK-STYX led to understanding the pseudophosphatase’s role in the stress response pathway, further characterization of MK-STYX’s function via G3BP1’s was pursued. G3BP1 has been implicated in neuronal development. Additionally, over-expression of MK-STYX is known to induce neurite formation in PC12 cells. Here, by knocking-down MK-STYX with a short-hairpin RNA expression plasmid, we have found that the pseudophosphatase is an important regulator of neurite outgrowth. Overall, this project provides more insight into MK-STYX by further characterizing this protein’s interaction partners and cellular role.
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This M.S. is dedicated to my advisor, Dr. Shantá Hinton. Thank you so much for accepting your first graduate student, and for your continued support and mentorship.
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In order to survive, cells must carry out important cellular processes, such as communication and growth. These processes require the relay of extracellular signals to the interior of the cell to elicit the proper response. For example, neighboring cells interact through cell-surface receptors, and ligands may signal for growth. These signals are then transmitted through protein signaling cascades. Proteins can have a variety of cellular functions, such as acting as enzymes, receptors, or scaffolds. Regardless of their role, many proteins are regulated via post-translation modifications. These changes occur after a protein has been synthesized, hence post-translationally. In this process, a protein becomes tagged with another functional group or molecule. These additions can affect the localization, activity, and degradation of the protein, and thereby control cellular activity. Post-translational modifications can be either reversible, such as phosphorylation and ubiquitination, or irreversible such as proteolysis. Of the various post-translational modifications characterized, phosphorylation is the most common (Khoury et al., 2011; Walsh, 2006).

Phosphorylation is regulated by kinases which add phosphate groups, and phosphatases which remove them. As proteins become phosphorylated, a phosphate group (PO₄³⁻) is most commonly added to serine (Ser), threonine (Thr), or tyrosine (Tyr) residues. Once added, the phosphate group gives a protein the capacity to form new electrostatic interactions and hydrogen bonds.
(Johnson and Lewis, 2001). The new interactions can cause dramatic conformational changes, which in turn help regulate a protein’s function.

**Protein phosphorylation**

With about a third of mammalian proteins being regulated through phosphorylation, the balance between protein phosphorylation and dephosphorylation is an integral part of maintaining cellular homeostasis (Cohen, 1999). While phospho-Ser and phosphor-Thr residues are much more common in the cell than phosphor-Tyr (1800:200:1, respectively), 107 of the approximately 150 phosphatases are protein tyrosine phosphatases (Mann et al., 2002; Jackson and Denu, 2001; Alonso et al., 2004). The fact that over 70% of phosphatases are dedicated to dephosphorylating a small proportion of residues indicates that the reversible regulation of tyrosine phosphorylation is crucial for the cell.

**Protein tyrosine phosphatases**

The protein tyrosine phosphatase (PTP) superfamily encodes the largest group of phosphatases and is characterized by the active site signature motif HCX₅R (H: histidine, C: cysteine, X: any amino acid, R: arginine) (Tonks, 2006; Jeffrey et al., 2007). This superfamily consists of roughly 107 members and can be broken down into classes and subgroups based on flanking domains, substrate specificity and homology (Figure 1) (Alonso et al., 2004; Tonks, 2013). Although there are low sequence similarities between classes, all PTP members
maintain a similar catalytic fold and active site architecture (Stone and Dixon, 1994; Huang, 2003).

The dephosphorylation reaction catalyzed by protein tyrosine phosphatases is reliant on the active site cysteine and arginine residues. The cysteine residue is essential for catalysis, and is responsible for nucleophilic attack on the phosphate. The positively charged arginine residue facilitates this reaction by stabilizing the deprotonated cysteine at a neutral pH.

Figure 1: The protein tyrosine phosphatase superfamily. PTPs can be broken into three classes (blue boxes) based on substrate specificity and catalytic mechanism. Class I PTPs can be further divided into the classical PTPs, which only dephosphorylate phospho-Tyr residues, and the dual specificity phosphatases (DUSPs), which can dephosphorylate Ser/Thr, and Tyr residues (red boxes). Class II is comprised of the low molecular weight PTP, LMPTP. The Class III PTPs are comprised of the cell division cycle 25 (cdc25) phosphatases.
Dual specificity phosphatases

Dual specificity phosphatases (DUSPs) are members of the PTP superfamily which target phosphorylated tyrosine residues, as well as phosphorylated serine and threonine residues. In comparison to phospho-Tyr specific PTPs, these enzymes have shallower active sites which can accommodate both tyrosine and the shorter side chains of serine and threonine (Denu and Dixon, 1998; Barford et al., 1998).

The mitogen-activated protein kinase phosphatases (MKPs) are a DUSP subgroup (Table 1). MKPs target and dephosphorylate the mitogen-activated protein kinases (MAPKs) that have been dual-phosphorylated by MAPKKs, such as MEK. The MAPK group encompasses ERKs (extracellular signal-regulated kinases), JNKs (c-Jun N-terminal kinases), and p38s (p38 mitogen-activated protein kinases). These individual MAPK proteins interact with a vast number of downstream targets; the ERKs alone have at least 160 identified substrates. Thus, MAPKs can influence a variety of cellular pathways and outcomes (Yoon and Seger, 2006). Additionally, most cell surface receptors use at least one MAPK to transduce cellular signals (Robinson and Cobb, 1997). Without the MKPs to regulate these kinases, processes like cell growth can become unregulated and lead to cancer (Boutros et al., 2008).

The N-terminal region of MKPs is responsible for recognizing and interacting with MAPKs. This region contains a KIM (kinase-interacting motif) between two CH2 (cdc25 homology) domains and is thought to provide the
different MKPs with their specificity for the various MAPKs (Patterson et al., 2009). The C-terminal region of MKPs contains the PTP catalytic domain.

Of the eleven MKPs, ten are catalytically active, and one, MK-STYX (MAPK (mitogen-activated protein kinase) phospho-serine/threonine/tyrosine-binding protein), is catalytically inactive. MK-STYX contains a STYX domain, where catalytically essential residues are not present within the active site signature motif (Wishart and Dixon, 1998). MK-STYX was classified as a member of the MKP subgroup based on its similar domain and sequence homology.

Table 1: The mitogen-activated protein kinases phosphatase (MKP) subgroup. This PTP subgroup is composed of 10 catalytically active dual specificity phosphatases, and 1 catalytically inactive phosphatase, MK-STYX.

<table>
<thead>
<tr>
<th>Gene</th>
<th>MKP</th>
</tr>
</thead>
<tbody>
<tr>
<td>DUSP1</td>
<td>MKP-1</td>
</tr>
<tr>
<td>DUSP4</td>
<td>MKP-2</td>
</tr>
<tr>
<td>DUSP2</td>
<td>N/A</td>
</tr>
<tr>
<td>DUSP5</td>
<td>N/A</td>
</tr>
<tr>
<td>DUSP6</td>
<td>MKP-3</td>
</tr>
<tr>
<td>DUSP7</td>
<td>MKP-X</td>
</tr>
<tr>
<td>DUSP9</td>
<td>MKP-4</td>
</tr>
<tr>
<td>DUSP8</td>
<td>N/A</td>
</tr>
<tr>
<td>DUSP10</td>
<td>MKP-5</td>
</tr>
<tr>
<td>DUSP16</td>
<td>MKP-7</td>
</tr>
<tr>
<td>DUSP24</td>
<td>MK-STYX</td>
</tr>
</tbody>
</table>
Pseudophosphatases

Pseudophosphatases are proteins which lack essential amino acids needed for catalysis, rendering them catalytically inactive. Approximately 16% of proteins in the PTP superfamily lack the essential catalytic cysteine residue found in the HCX$_5$R signature motif (Wishart, 2007). Without this amino acid, pseudophosphatases do not have the ability to remove phosphate groups. The domain architecture of these catalytically dead enzymes is very similar to that of the catalytically active PTPs. Point mutations replacing the catalytic cysteine restore enzymatic activity, showing pseudophosphatases still maintain the overall active site fold to accommodate dephosphorylation reactions (Wishart et al., 1995; Hinton et al., 2010).

These catalytically dead enzymes were previously thought to act solely as dominant negatives, where they bind and protect a phosphorylated protein from being dephosphorylated by active phosphatases. However, it is now known that pseudophosphatases can have other functions in the cell, besides merely protecting phosphorylated residues.

The MTM family is composed of 14 proteins, 6 of which are catalytically inactive and lack the essential cysteine residue within the PTP catalytic motif (Laporte et al., 2003). This family of PTPs dispels the notion that pseudophosphatases act solely as dominant negatives. It has been shown that heterodimers form between the coiled-coil domains of the active and inactive MTMs (Kim et al., 2003). The inactive partner relocates and increases the activity of the catalytic MTM. In Charcot-Marie-Tooth disease type 4B (CMT4B), loss of
either the active or the inactive member of the MTM heterodimer are associated with this neuropathic disorder (Bolino et al., 2000; Senderek et al., 2003).

Besides MTMs, other pseudophosphatases in the PTP superfamily have bona fide regulatory roles, such as the EGG-4 pseudophosphatase in C. elegans. EGG-4 interferes with MBK-2 (minibrain kinase 2), which is in the same dual-specificity tyrosine-regulated kinase (DYRK) family as the MAPKs. MBK-2 becomes active following the phosphorylation of the two tyrosine residues found in the YXY motif of the activation loop, similar to the TXY motif present in the MAPKs. As a pseudophosphatase, EGG-4 functions by binding the tyrosine residues found in the YXY motif, altering the substrate binding abilities of MBK-2. Interestingly, EGG-4 can associate with MBK-2 when the tyrosine residues in the YXY motif are not phosphorylated (Cheng et al., 2009). This supports the notion that pseudophosphatases have roles in the cell other than acting as dominant negatives protecting phosphorylated residues from dephosphorylation.

**MK-STYX**

MK-STYX lacks the essential cysteine and histidine amino acids within the PTP active site signature motif, rendering it a catalytically inactive phosphatase. However, replacing the ‘FS’ sequence to ‘HC’ through site-directed mutagenesis generates an active mutant form of MK-STYX by restoring the PTP motif (Hinton et al., 2010).
Role of MK-STYX in disease

MK-STYX is over-expressed in Ewing’s sarcoma family tumors (ESFT) due to a transcription factor gene fusion (Siligan et al., 2005). In 85% of ESFTs, a chromosomal translocation between chromosomes 11 and 22 creates a fusion EWS-FLI1 gene, resulting in a misregulated transcription factor (Siligan et al., 2005; Delattre et al., 1994). The resulting chimeric protein directly promotes the expression of MK-STYX, causing MK-STYX to be consistently over-expressed in tumors with the EWS-FLI1 fusion gene. Furthermore, EWS cells show decreased survival when MK-STYX is knocked-down (Arora et al., 2010).

While over-expression of MK-STYX has been linked to cancer, its under-expression can also negatively affect cells. We have found that a modified U2OS cell line (RDG3) derived from an osteosarcoma tumor has no detectable level of MK-STYX protein (Figure 2). Other reports have shown that knockdown of MK-STYX in HeLa cells show almost complete resistance to pro-apoptotic chemotherapeutics agents, indicating that the loss of MK-STYX can promote cancer cell survival (Niemi et al., 2011).

![Figure 2: MK-STYX protein undetectable in RDG3 cells.](image) Cells were either not transfected (NT) or received a MK-STYX (M) or pMT2 (P) plasmid. Antibody for endogenous MK-STYX (Sigma) only detected protein in sample transfected with the flag-tagged MK-STYX plasmid. This shows that without transfection, there is no detectable MK-STYX protein in RDG3 cells. β-tubulin served as a loading control.
Cellular functions of MK-STYX

Although MK-STYX was first identified and described in 1998, there was little information about the cellular role of this protein until 2010 (Wishart and Dixon, 1998; Hinton et al., 2010). MK-STYX has the ability to form protein:protein interactions via its inactive PTP catalytic domain and CH2 domains. Thus, understanding its protein interactions provided insight into the function of MK-STYX.

Previously, mass spectrometry was used to identify potential binding partners of MK-STYX and the active mutant, F1 (Hinton et al., 2010). These experiments ranked the potential binding partners using expectation values (e-values). The e-value is a measure of statistical confidence, with a lower value corresponds to a more reliable reading. From the resulting readings, proteins which gave a lower e-value for MK-STYX over F1 were selected. This could indicate that the peptide was a potential binding partner for only the pseudophosphatase. Additionally, the preferential binding of MK-STYX over F1 could show that the pseudophosphatase has its own set of unique interaction partners separate from the active mutant phosphatase, implicating very specific roles for MK-STYX.

Using this approach, G3BP1 (GTPase activating protein (SH3 domain) binding protein 1) was identified as a binding partner of MK-STYX (Hinton et al., 2010; Barr et al., 2013). G3BP1 is a marker of stress granules (SGs), which are cytoplasmic mRNA-containing complexes that form upon cellular stress. These granules are composed of stalled pre-initiation complexes where translation has
halted. Other mRNA binding proteins, such as TIA-1 (TIA1 cytotoxic granule-associated RNA binding protein), contain RNA recognition motifs (RRM) which allow them to recognize and bind the untranslated mRNA. Another important domain present in these proteins is the prion-related domain (PRD) which promotes their self-aggregation and formation of SGs.

Previously, it was shown that MK-STYX reduces the presence of SGs (Hinton et al., 2010; Barr et al., 2013). Notably, another protein identified in the mass spectrometry data, heat shock protein 70 (hsp70), shares this role, and was therefore studied as a possible binding partner of MK-STYX (Table 2) (Gilks et al., 2004; Kedersha and Anderson, 2002).

Table 2: Potential binding partners of MK-STYX. Mass spectrometry was used to identify peptides which were immunoprecipitated with MK-STYX, and its active form, F1. The lower expectation value (e-value) seen in the MK-STYX column versus those for F1 suggested G3BP1 as a novel MK-STYX binding partner, which was validated through further study (blue) (Hinton et al., 2010; Barr et al., 2013). Using the same technique, mass spectrometry detected heat shock protein 70 (hsp70).

<table>
<thead>
<tr>
<th>Mass spec readings</th>
<th>MK-STYX</th>
<th>F1</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3BP1</td>
<td>$1.3 \times 10^{-23}$</td>
<td>$3.2 \times 10^{-3}$</td>
</tr>
<tr>
<td>HSP70</td>
<td>$5.4 \times 10^{-49}$</td>
<td>$1.7 \times 10^{-29}$</td>
</tr>
</tbody>
</table>
In addition to its role in SGs, G3BP1 also has been implicated in other cellular processes, such as neuronal differentiation. Studies have shown that G3BP1 deficiencies during fetal development cause lethal wide-spread neuronal cell death in mice (Zekri et al., 2005). Because of this G3BP1 link, an aim of this thesis research was to investigate whether MK-STYX had any effect on neuronal cells.

**Thesis objectives**

Although MK-STYX decreases SGs, its molecular mechanism still remains unclear. Studying the interaction between G3BP1 and MK-STYX provided insight into the role of the pseudophosphatase. Thus, clarifying the interaction between MK-STYX and other binding partners, such as hsp70, may provide further understanding of MK-STYX’s function. The goals of this thesis are to confirm whether another binding partner (hsp70) identified by mass spectrometry truly interacts with MK-STYX, and to investigate the role of MK-STYX in neuronal differentiation. The specific aims were as follows:

1. To determine if MK-STYX interacts with and alters hsp70 expression
2. To clarify MK-STYX’s mode of action in PC12 neuronal differentiation
Chapter 1: MK-STYX and Heat Shock Protein 70

Preliminary mass spectrometry data identified hsp70 as a potential binding partner of MK-STYX (unpublished data). Furthermore, hsp70 has been implicated in the SG lifecycle, which consists of assembly, persistence, and disassembly stages. This chaperone protein is capable of both blocking SG assembly and promoting SG disassembly, thereby reducing the number of SGs present in a cell (Gilks et al., 2004; Mazroui et al., 2007; Thomas et al., 2011). Similarly, when MK-STYX is over-expressed, cells have fewer stress granules (Hinton et al., 2010; Barr et al., 2013). The fact that both MK-STYX and hsp70 are capable of decreasing SGs, and the mass spectrometry showing hsp70 as a possible binding partner for MK-STYX, provided a significant foundation to pursue this interaction further.

Chaperones and heat shock proteins

Proteins are often called the “workers of the cell”. They are responsible for carrying out cellular processes, from receiving extracellular signals to energy production. However, these workers cannot perform their functions correctly unless they are in the proper conformation. Within every protein, the primary amino acid sequence of the polypeptide contains the instructions, or blueprint, for proper protein folding. As a polypeptide is being synthesized, the resulting chain emerges from the ribosome as each amino acid is added. Yet a protein cannot adopt a fold until an entire domain has been synthesized. This may initially leave
amino acids which may have hydrophobic, or “water fearing” qualities, exposed to the aqueous environment of the cell.

As a protein adopts its native structure, hydrophobic residues become buried inside the three-dimensional conformation, whereas hydrophilic, or “water loving”, amino acids are on the exterior of the protein and interact with water molecules. Without hydrophilic amino acids to shield the hydrophobic ones from water, hydrophobic residues tend to aggregate with other polypeptides which have similar characteristics. This in turn would interfere with the correct folding of the protein into its functional conformation. Thus, proper protein folding is important to carry out cellular processes, and requires mediators such as molecular chaperones.

Chaperone proteins aid in non-covalent folding and unfolding, and help improve the efficiency of protein production by helping newly formed polypeptides fold correctly, while also combating protein unfolding (Ellis, 2000). Within the larger group of molecular chaperones, there is a family of proteins named the heat shock proteins (hsp). The hsp are named after their molecular weights; for example, hsp60, hsp70 and hsp90, which have respective molecular weights of 60, 70 and 90 kDa. The heat shock proteins were named based on the fact that their protein expression increased upon cellular stress, such as an increase in temperature. However, there are hsp whose protein expression is stress-inducible, as well as hsp that are expressed under normal cellular conditions. These non-inducible hsp are termed heat shock cognate proteins (hsc).
Heat shock protein 70 (hsp70)

The most well studied family of heat shock proteins is the hsp70 family, which is comprised of 11 proteins with molecular weights ranging from 66 kDa to 78 kDa (Tavaria et al., 1996). The human hsp70s share the same domain architecture of an N-terminal nucleotide-binding domain and a C-terminal substrate-binding domain. This family of proteins is capable of binding stretches of exposed hydrophobic amino acids, thereby mitigating aggregation. Within hsp70 family, the non-inducible hsc proteins interact with newly synthesized proteins. In fact, roughly 30% of synthesized proteins interact with hsp70 in eukaryotic cells (Malyshev, 2013). Hsc proteins are also important in other cellular processes like protein translocation across membranes and clathrin uncoating (Deshiaes et al., 1988; Rothman, 1989; Chappell et al., 1986). The major stress-inducible hsp70 (also named hsp72) mitigates protein unfolding and aggregation upon cellular stress. In addition, stress-inducible hsp70 antagonizes stress granules (SGs) by binding the prion-related domain (PRD) of TIA-1 (T-Cell-Restricted Intracellular Antigen-1) (Gilks et al., 2004; Anderson and Kedersha, 2002). This agrees with reports that increased hsp70 protein levels correlates with a decrease in SGs (Mazroui et al., 2007; Thomas et al., 2011).

Heat shock factor 1 (hsf-1) is a transcription factor regulating expression of genes encoding the stress-inducible member of the hsp70 family. This transcription factor is phosphorylated at twelve different sites. Some of these phosphorylation events enhance hsf-1’s transcriptional activity, while others repress it (Guettouche, 2005; Kline and Morimoto, 1997). However, the
mechanism of transcriptional repression by phosphorylation is not clear (Kline and Morimoto, 1997; Knauf et al., 1996; Chu et al., 1996). Notably, the repressive phosphorylation event at serine 307 is carried out by the mitogen-activated protein kinases (MAPKs), which are dephosphorylated by the mitogen-activated protein kinase phosphatase (MKP) subgroup (Kline and Morimoto, 1997; Chu et al., 1996; Mivechi and Giaccia, 1995; Dai et al., 2000).

**MK-STYX and hsp70**

Hsp70 was identified as a potential binding partner of MK-STYX through mass spectrometry experiments (Table 2) (unpublished data). However, heat shock proteins are often identified in large scale binding assays, as it is their cellular job to bind other proteins (Gingras et al., 2007). Thus, it was not clear if the functional significance extended beyond hsp70’s role as a chaperone. The reoccurrence of this reading in subsequent trials, and the smaller expectation value, or e-value, predicted MK-STYX as a true binding partner (unpublished data).

Along with hsp70, two members of the MKP subgroup, MKP-1 and DUSP5, show increased protein levels upon heat shock (Wong et al., 2005; Ishibashi et al., 1994). As previously mentioned, the MAPKs can repress the transcription factor hsf-1, thereby hindering hsp70 gene production. MKP-1 is capable of relieving this repression by dephosphorylating and inactivating MAPKs. As cells are stressed, there is a correlational increase in both MKP-1 and hsp70 (hsp72) (Yaglom et al., 2003; Gabai et al., 2009). This previously
characterized link between MKPs and hsp70 made the mass spectrometry data that much more intriguing, given that MK-STYX is a member of the MKP subgroup. Additionally, hsp70 has been implicated in human disease as it is over-expressed in many cancers (Zorzi and Bonvini, 2011; Leu et al., 2009; Murphy, 2013; Meng et al., 2011). In order to enhance understanding of hsp70 regulation, we set-out to determine whether MK-STYX interacts with or affects the levels of this chaperone protein.
Methods

Plasmids

MK-STYX was cloned in a pMT2 expression vector to introduce exogenous protein to cells, as previously described (Hinton et al., 2010). The MK-STYX sequence was tagged on either side by a Flag epitope, encoding a fusion protein flanked with the Flag sequence (DYKDDDDK) on both the N and C terminal ends. This tagging allows for easy detection of transfected MK-STYX, versus endogenous MK-STYX.

Additionally, an active mutant form of MK-STYX, named MK-STYX_{active}, or F1, was previously generated (Hinton et al., 2010). The F1 plasmid contains the substitution of F245 to a histidine residue, and S246 to cysteine, restoring the PTP active site signature motif and catalytic activity. This plasmid was constructed by a single point mutation of the pMT2-FLAG-MK-STYX-FLAG vector (Hinton et al., 2010). The expression vector for green fluorescent protein (GFP)-tagged hsp70 (pEGFP-C3-Hsp70) was obtained from Addgene (#15215).

Cell culture

A HeLa cell line (ATCC, #CCL-2) derived from human cervical epithelial carcinoma cells was used in these studies. Cells were maintained at 37°C with 5% CO₂ and were grown in Minimal Essential Medium (MEM) (Gibco, Invitrogen). All culture media was supplemented with 10% fetal bovine serum (Invitrogen).
To stress cells, plates were heat shocked at 45°C for 30 minutes approximately 42 hours post-seeding. Samples which were given a post-stress recovery period were transferred to a 37°C incubator immediately after heat shock. Recovery times ranged from 30 minutes to 8 hours.

**Transient transfections**

Transient transfections were performed with 2 µg of DNA, and 4 µL of Lipofectamine 2000 (Invitrogen). The plasmids and Lipofectamine were diluted into Opti-MEM I Reduced Serum Medium (Invitrogen). Opti-MEM I was removed and replaced with MEM/FBS 5 hours post-transfection.

**Cell lysis and sample collection**

All samples were collected with lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 10 mM NaF, 1% Nonidet P-40 alternative, 1 mM sodium orthovanadate). For each 10 mL of lysis buffer, one ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail tablet was used (Roche). After collecting lysates, sonication was performed to break cellular membranes, release proteins, and to shear cellular DNA. Following centrifugation at 4°C and 20,800 x g for 10 minutes, protein concentration was determined by NanoDrop quantification.
Western blots

Samples were prepared with 5X sodium dodecyl sulfate (SDS) sample loading buffer (250 mM Tris pH 6.8, 10% SDS, 30% glycerol, 0.02% bromophenol blue) and dithiothreitol (DTT) (Fisher Scientific). Following three minutes at 100°C and 1 minute centrifugation at 17,000 x g, samples were loaded and resolved on 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) gels. A protein ladder was used to determine molecular weights of protein bands (BioRad, Kaleidoscope Pre-stained ladder). Proteins were transferred onto a polyvinyl difluoride (PVDF) membrane using semidry transfer at 14 volts for 30 minutes in transfer buffer (48 mM Tris base, 39 mM glycine, 1.3 mM SDS, 20% methanol, final volume of 1 L). Membranes were then blocked in 5% milk for 40 minutes (Carnation). Probing for β-tubulin (1:1000, secondary anti-rabbit 1:3000, Thermo Scientific) served as a loading control to ensure that the same amount of sample was added to each well. To detect inducible hsp70, an antibody specific for hsp72 was used (1:1000, Enzo Life Sciences). To detect transfected MK-STYX, anti-Flag (1:1000) was used (Sigma). For both hsp72 and Flag secondary antibodies against mouse were used (1:2500, Thermo Scientific). Hsf-1 antibody was used in a 1:1000 ratio (Enzo Life Sciences) with secondary antibodies against rabbit (1:2500, Thermo Scientific). Enhanced chemiluminescence (ECL Prime, GE Healthcare) was used to visualize protein bands.
**Immunoprecipitation**

Following lysis, a small aliquot of lysate was removed and stored for total protein and western blot analysis. The remaining sample was incubated on ice with 20 µL of IgG beads (GE) for 30 minutes. This pre-clearing removed any nonspecific binding of background proteins. The supernatant was then transferred to a new tube containing 5 µL of the appropriate antibody, and was put on ice for one hour. The incubated lysates were purified with 30 µL of IgG beads by rocking at 4°C for 1-2 hours. Centrifugation (4°C, 15 minutes, 20,800 x g) separated the unbound proteins from the beads, and supernatant was aspirated. Remaining lysis buffer was used to wash the beads 3 times (1 mL washes, centrifuged at 17,000 x g for 1 minute at room temperature following aspiration). Bound proteins were eluted using 40 µL of 5X SDS-sample loading buffer with 2 µL DTT, and were analyzed by western blotting.
Results

In order to determine the optimal conditions for heat shock, HeLa cells were exposed to an elevated temperature of 45°C for 30 minutes. Following heat stress, samples were given a range of recovery periods from 30 minutes to 8 hours at 37°C. Hsp70 protein levels were highest 6 hours post-stress (Figure 3).

Following preliminary characterization of hsp70 expression upon stress, we next wanted to determine if MK-STYX had an effect on hsp70 protein levels. Cells were transiently transfected with either the empty pMT2 vector, MK-STYX, or the active mutant, F1. Western blot analysis was used to compare the cellular levels of hsp70 in cells grown under normal unstressed conditions, and those exposed to heat stress. Following heat shock, there was no change in hsp70 protein levels between the empty vector control and MK-STYX transfected samples. Thus, we conclude that MK-STYX had no effect of hsp70 protein expression (Figure 4A).

Cell lysates from transfected cells were also used to investigate the effect of MK-STYX and the active mutant on the phosphorylation pattern of heat shock factor 1 (hsf-1), the transcription factor for hsp70. Phosphorylation sites in hsf-1 can either enhance or repress transcriptional activity. To determine whether MK-STYX or the active mutant, F1, altered the phosphorylation pattern of hsf-1, western blot analysis for hsf-1 was performed. Multiple bands reflect hsf-1’s complex phosphorylation pattern, with the lower bands being the least phosphorylated and the upper bands representing hyperphosphorylated hsf-1.
(Figure 4B) (Guettouche, 2005). There was no striking change in the phosphorylation pattern of hsf-1 in the presence of MK-STYX or F1. This result was consistent with the finding that MK-STYX and F1 had no effect on altering hsp70 protein levels. Thus, it was not warranted to further investigate MK-STYX effects on hsf-1 phosphorylation at specific sites.

However, the question still remained whether either MK-STYX or F1 interacted with hsp70. Therefore, immunoprecipitation experiments were performed to determine whether hsp70 interacted with either MK-STYX or F1. To ensure that any interaction with hsp70 was present under normal cellular conditions, and was not due to hsp70 performing its chaperone function, cells and cell extracts were handled under unstressed conditions. However, this posed a challenge, as cellular levels of hsp70 are low in the absence of stress (Figure 5A). To remedy this, a second immunoprecipitation experiment was performed using a GFP-tagged hsp70 expression vector was transiently transfected into cells, allowing for over-expression under unstressed conditions and increasing the odds of capturing potential interactions. This tagging caused a molecular weight shift in the GFP-hsp70 versus endogenous hsp70, since GFP is roughly 27 kDa (Figure 5B). Our results indicated that the active mutant, more so than MK-STYX, complexes with hsp70 (Figure 5). This result is interesting, as it suggests the pseudophosphatase has different binding partner specificities from the active mutant. If MK-STYX were solely functioning as a dominant negative, we would expect for both proteins to interact with hsp70 with the same intensity. However, we observe the active mutant being a stronger hsp70 interaction
partner than the wild type MK-STYX (Figure 5A). Taken together, these data support the role of MK-STYX as more than a dominant negative or substrate-trap for the cell.
Discussion

Although the pseudophosphatase MK-STYX was first reported in 1998, only one interaction partner has been identified, G3BP1, which allowed some insight into the functional role of MK-STYX (Wishart and Dixon, 1998; Hinton et al., 2010; Barr et al., 2013). However, understanding of the overall molecular mechanism of MK-STYX still remains unclear. Thus, identifying other binding partners of this pseudophosphatase is imperative.

Here, we investigated hsp70 as a potential interaction partner of MK-STYX. Hsp70 was detected using the same proteomics approach which successfully identified G3BP1. Preliminary studies indicate that hsp70 is a binding partner for both the active mutant and MK-STYX. The interaction between hsp70 and F1 appears to be stronger than seen with MK-STYX. However, the first immunoprecipitation experiment had low levels of hsp70 protein (Figure 5A), while the second trial had decreased transfection efficiency of the MK-STYX expression plasmid (Figure 5B). The fact that hsp70 and MK-STYX were never equally over-expressed in the same trial could explain why capturing any potential interaction was more elusive. Further trials in samples over-expressing both proteins of interest could help validate these preliminary findings.

Interestingly, the immunoprecipitation results do not agree with the initial mass spectrometry data (Table 2), which suggested that MK-STYX would have greater affinity for hsp70 over F1. A potential reason for this disagreement could
be due to a difference in cell lines used for each assay. The initial mass
spectrometry data was performed in COS-1 cells, which are African green
monkey fibroblast-like cells (Hinton et al., 2010). Alternatively, the
immunoprecipitation experiments utilized the HeLa cell line, derived from human
cervical epithelial carcinoma cells.

The fact that MK-STYX and the active mutant appear to have different and
unique affinities for a shared substrate supports the idea that
pseudophosphatases have specific binding partners from their active homologs.
Furthermore, it provides evidence that pseudophosphatases, such as MK-STYX,
function beyond a dominant negative role of protecting phosphorylated residues
from phosphatases.

Historically, catalytically inactive enzymes were thought solely to act as
substrate traps, or dominant negatives. These proteins would effectively protect
target substrate from interacting with other catalytically active enzymes (Hunter,
1998). Other catalytically inactive PTPs, or pseudophosphatases, have been
found to have regulatory roles in cell, such as the catalytically inactive MTM
proteins which regulate their active MTM binding partners (Laporte et al., 2003;
Begley and Dixon, 2005). If MK-STYX were acting as a dominant negative, it
would be predicted to have similar binding affinities as the active mutant
phosphatase.

Additionally, we found that MK-STYX does not alter the protein levels of
hsp70. Simultaneous experiments were performed to determine whether MK-
STYX and F1 were altering the phosphorylation pattern of the hsp70 transcription
factor, hsf-1. We found no apparent changes in hsf-1 phosphorylation caused by the presence of either MK-STYX or F1, indicating that MK-STYX and F1 were not altering the repressive phosphorylation of hsp70’s transcription factor. This result is consistent with the finding that MK-STYX did not alter hsp70 protein levels.

Although MK-STYX and hsp70 may not be strong interaction partners, it would be interesting to investigate their combined effect on decreasing SGs. MK-STYX is known to interact with G3BP1, a nucleator of SG formation. On the other hand, hsp70 is able to promote SG disassembly though binding the PRDs of proteins like TIA-1. If MK-STYX was blocking SG formation, and hsp70 was promoting disassembly, the combined effect of both of these proteins could cause a drastic decrease in SGs, which could have important therapeutic applications.

Many neurodegenerative disorders, such as Parkinson's and Alzheimer’s disease, are caused by an increase in amyloidosis, or insoluble protein aggregates. Hsp70 has a role in disassembling aggregates, and has been identified by many groups as a potential therapeutic target (Cummings et al., 2001; Evans et al., 2010). Additionally, it has been suggested that a high incidence of SGs is linked to and promotes these diseases.

While stress granules are primarily composed of mRNA, some of the RNA-binding proteins responsible for promoting SG formation, like TDP-43 (TAR-DNA–binding protein) and FUS (fused in sarcoma), are also components of neurodegenerative protein aggregates (Li et al., 2013; Wolozin, 2012). Because of this association, SGs are thought to serve as initiators, or “seeds” for protein
aggregates (Bentmann et al., 2013). Notably, both TDP-43 and FUS contain prion-related domains, and it is through interactions with this domain that hsp70 disassembles SGs (Gilks et al., 2004).

By 2050, it is predicted that one person will develop Alzheimer’s disease every 33 seconds in the U.S. (Alzheimer’s Association, 2012). Thus, there will be a national crisis for development of therapeutics that will combat Alzheimer’s and other neurodegenerative diseases. Furthering our understanding as to how both MK-STYX and hsp70 decrease the presence of SGs may be insightful to drug development. The data presented in this chapter show MK-STYX has no effect on hsp70 protein expression or hsf-1 phosphorylation. However, the preliminary results suggesting differential binding of wild-type MK-STYX and the active mutant (F1) with hsp70 could be a significant finding and warrants further investigation. In addition, it will be interesting to determine whether MK-STYX and hsp70 can synergistically combat cellular aggregates which are major culprits of neurologic disorders.
Figure 3: Time course of inducible hsp70 protein levels in HeLa cells. All samples were grown and cultured at 37°C. (A) Stressed samples were exposed to heat shock (45°C for 30 minutes) and had varied post-stress recovery times at 37°C. (B) ImageJ was used to normalize hsp70 protein levels to β-tubulin.
**Figure 4: Effects of MK-STYX and F1 on hsp70 protein levels and hsf-1 phosphorylation.** HeLa cells were either not stressed, or heat shocked (HS) at 45°C for 30 minutes with a 6 hour recovery period at 37°C. P: pMT2, M: MK-STYX, F1: active mutant (n=3). (A) In the absence of stress, all samples had low levels of inducible hsp70. Following heat shock, there was no change between the empty vector control and experimental samples. (B) The same samples showed no obvious changes in the phosphorylation pattern of hsf-1 in the presence of MK-STYX or the active mutant, as seen in the multiple bands ranging from 80-95 kDa representing phosphorylation events.

### Table A

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Figure 5: Heat shock protein 70 immunoprecipitation. M: MK-STYX, F1: active mutant, P: pMT2, H: hsp70, G: GFP. (A) Endogenous hsp70 was used to detect potential interactions with MK-STYX or the active mutant. HeLa cells were transfected with either pMT2, MK-STYX, or F1. This result agrees with panel A showing the active mutant as a hsp70 interaction partner; however, it also shows MK-STYX interacting with hsp70, but to a lesser extent. (B) HeLa cells were co-transfected with expression plasmids for proteins of interest (Flag-tagged MK-STYX, Flag-tagged F1, pMT2, hsp70, pEGFP). The top panel shows F1 interacting with hsp70. The bottom panel shows the presence of transfected and endogenous hsp70.
Chapter 2: MK-STYX and Neuronal Differentiation

As neuronal cells receive signals such as neurotransmitters and neurotrophins, they respond with biochemical and morphological changes. This process can be seen in the commonly used rat PC12 cell line. In the absence of nerve growth factor (NGF), PC12 cells have a rounded morphology. Upon NGF stimulation, they undergo dramatic morphological changes with the extension of neuronal outgrowths, or neurites (Greene and Tischler, 1976). Additionally, these cells are capable of differentiating into sympathetic neurons and can form specialized neuronal structures, such as synapses (Fujita et al., 1989; Schubert et al., 1977). Previously, the Hinton lab found that over-expression of MK-STYX is sufficient to neurite outgrowth in PC12 cells. Here, we show MK-STYX as a critical regulator of neuronal differentiation, as knocking-down MK-STYX expression results in a decrease in neurite outgrowth.

MK-STYX in neuronal processes

To date, the only published binding partner of MK-STYX is G3BP1, a well characterized marker for stress granules (Hinton et al., 2010; Barr et al., 2013; Tourriere et al., 2003). Identifying this binding partner led to an investigation of MK-STYX’s role in stress granule assembly (Hinton et al., 2010). However, G3BP1 has also been shown to play a role in neuronal processes. More specifically, mouse studies have shown that knocking-out G3BP1 lead to dysfunctional synaptic signaling and ataxia (Martin et al., 2013). These G3BP1
results inspired us to explore whether MK-STYX might also be important in neuronal differentiation. In doing so, we have found that the pseudophosphatase induces neuronal outgrowths in rat pheochromocytoma PC12 cells with and without nerve growth factor stimulation (unpublished data, Kristen Wong). These neurites have not been specifically categorized as either dendrites or axons.

**Extracellular signal-regulated kinases**

MAPKs are kinases which target phospho-Ser and phospho-Thr residues and are activated by a dual phosphorylation event in their conserved T-X-Y motif (T: threonine, X: any amino acid, Y: tyrosine). This group is composed of the ERKs (extracellular signal-regulated kinases), along with the JNKs (c-Jun N-terminal kinases) and p38s (p38 mitogen-activated protein kinases). The ERK family includes ERK1 and ERK2 (commonly referred to as ERK1/2), which share 85% of their sequences (Boulton and Cobb, 1991). Furthermore, the ERKs have been implicated in neuronal outgrowth; their sustained activation is critical for neurite extension in PC12 cells (Fukuda et al., 1995; Kao et al., 2001; Vaudry et al., 2002).

Epidermal growth factor (EGF) and nerve growth factor (NGF) are ligands which activate the small GTPase, Ras. This protein is active when it is bound to guanosine triphosphate (GTP), and becomes inactive when GTP is hydrolyzed to guanosine diphosphate (GDP). Other proteins termed GEFs (guanine nucleotide exchange factors) and GAPs (GTPase-activating proteins) help cycle GTPases between the “on” and “off” state, respectively. Once activated, Ras initiates the
MAPK signaling cascade which results in the dual phosphorylation and activation of ERK1/2 by MEKs (mitogen-activated protein kinase kinases).

Different growth factors and the duration of ERK signaling can cause two different outcomes in PC12 cells. Stimulation with epidermal growth factor (EGF) induces transient ERK signaling, leading to cell proliferation. In contrast, stimulation with nerve growth factor (NGF) results in prolonged ERK signaling, and induces neuronal outgrowth.

As MK-STYX is a member of the mitogen-activated protein kinase phosphatase (MKP) subgroup, it was of interest to determine whether the pseudophosphatase affects ERK activity in PC12 cells. We found that MK-STYX induces neuronal outgrowths without NGF stimulation, and longer neurites upon stimulation (unpublished data, Michelle Munyikwa). MK-STYX was not inducing neurite outgrowth by altering Ras activity or the downstream MAPK cascade (unpublished data, Brittany Flowers and Kristen Wong).

**Rho subfamily**

The Rho family is a subgroup of Ras-related GTPases, which also switch between “on” and “off” states based on their GTP or GDP binding state, respectively. Within this family, Rac1, Cdc42 and RhoA have been implicated in the regulation of neuronal differentiation.
RhoA and cell rounding

Whereas Rac1 and Cdc42 promote neurite outgrowth through the actin cytoskeleton (Daniels et al., 1998; Aoki et al., 2004), RhoA causes neurite retraction via cytoskeletal contraction, thereby promoting cell rounding. When PC12 cells are stimulated with NGF, RhoA becomes phosphorylated on serine 188, which selectively blocks the binding of its downstream effector ROCK (Rho-associated kinase) (Nusser et al., 2006). The serine/threonine kinase ROCK is the main effector which causes neurite retraction in PC12 cells. This is achieved through downstream signaling via increasing myosin light-chain phosphorylation and activating LIMK (LIM kinase 1). Both of the downstream effects promotes neuronal cell rounding (Meberg and Bamburg, 2000; Fujita et al., 2001).

RhoA, Ras and G3BP1

Interestingly, the GAPs for Ras and RhoA (p120 and p190 respectively) have been found to interact, thereby linking Ras and RhoA signaling (Ellis et al., 1990; Settleman et al., 1992). p120 [Ras GTPase activating protein] has an N-terminal region composed of a SH3 domain, flanked by two SH2 domains. p190 [Rho GTPase activating protein] becomes phosphorylated on two tyrosine residues which serve as docking sites for the SH2 domains of p120. This association facilitates the localization of p190 to RhoA at the cell membrane, leading to a decrease in RhoA signaling (Bradley et al., 2006). Alternatively, the GAP:GAP interaction causes a decrease in p120 activity resulting in prolonged Ras/MAPK activation (Moran et al., 1991).
As p120 associates with p190, its SH3 domain becomes more accessible for additional protein interactions (Hu and Settleman, 1997). Notably, G3BP1 is one of the few proteins known to bind the SH3 domain of p120 (Irvine et al., 2004; Pamonsinlapatham et al., 2009). This interaction between G3BP1, p120, and p190 links MK-STYX to the Ras and RhoA signaling pathways (Figure 6).

**Figure 6: Potential pathways for MK-STYX induced neurite outgrowth.** MK-STYX induces PC12 differentiation. Both the Ras and RhoA pathways are known to influence this process through their respective downstream effectors, the MAPK cascade/ERKs and ROCK. As a MKP, MK-STYX could be acting through ERK signaling. However, MEK inhibitor studies show that MK-STYX acts independently of this MAPK. Alternatively, by decreasing RhoA activity, the pseudophosphatase could block cell rounding and promote neurite extension.
MK-STYX affecting RhoA activity

Although MK-STYX is a member of the mitogen-activated protein kinase phosphatases (MKPs), MK-STYX induces neurite outgrowths independently of Ras/MAPK signaling (unpublished data, Brittany Flowers and Kristen Wong). Thus, we focused on the RhoA signaling cascade.

As RhoA activation antagonizes neurite outgrowth, an initial goal was to determine whether MK-STYX was inducing neuronal outgrowths by altering RhoA activity. Using a RhoA activity assay, we found a significant correlation between over-expressing MK-STYX and decreasing RhoA activity (unpublished data, Brittany Flowers).

While we have accumulated a significant amount of data on the role of MK-STYX in PC12 cell differentiation, experiments have relied on introducing and over-expressing exogenous protein. Thus, this thesis research sought to determine whether MK-STYX is essential for PC12 differentiation. To address this question, MK-STYX knockdown experiments in PC12 cells were performed.
Methods

Cell culture

The PC12 cell line was originally isolated from a rat pheochromocytoma tumor. These cells were obtained from the American Type Cell Culture (ATCC) (Greene and Tischler, 1976). Cells were grown at 37°C with 5% CO$_2$ in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% horse serum (HS) and 5% fetal bovine serum (FBS) (Gibco, Invitrogen).

Knock-down experiments and transfection

The Hinton lab has shown that MK-STYX is endogenously expressed in PC12 cells (unpublished data). To reduce MK-STYX protein expression, a short hairpin RNA expression plasmid (shRNA-STYXL1, referred to as MK-STYX shRNA) (Qiagen) was transfected into PC12 cells 12 hours after seeding in 60 mm culture dishes. Additionally, a scrambled shRNA negative control was used. This scrambled shRNA is nonspecific and has no gene target once introduced into the cell. The shRNA expression plasmids contained a green fluorescent protein (GFP) tag, allowing for easy discrimination between transfected and non-transfected cells.

In order to introduce 0.4 µg of shRNA expression plasmid per sample, 0.8 µL Lipofectamine 2000 (Invitrogen) was used. The shRNA expression plasmids and Lipofectamine were diluted with Opti-MEM I Reduced Serum Medium (Invitrogen). Opti-MEM I was removed and replaced with fresh RPMI/HS/FBS 4
hours post-transfection. Real-time quantitative polymerase chain reaction (RT-qPCR) was used to ensure knock-down of MK-STYX by the MK-STYX shRNA construct (data not shown). Cells were stimulated with 100 ng/mL of nerve growth factor (NFG) 24 hours after the shRNAs were introduced.

**Cell imaging**

Live cell images were taken over the course of a week using an inverted fluorescence microscope (Nikon). Neurite outgrowth (≥ 20 μm) was measured with NIS-Elements Basic Research software (version 3.10, Nikon). Figures were formatted using Adobe Illustrator software.
Results

Over the course of seven days, NGF-stimulated PC12 containing either the scrambled shRNA or MK-STYX shRNA expression plasmids were assayed for neurite outgrowth. By day 2, cells containing scrambled shRNA exhibited neurite formation ($\geq 20 \mu m$) (Figure 7). In contrast, PC12 cells transfected with MK-STYX shRNA did not form neurites until day 3, and neurites that subsequently formed were shorter than those in the negative control cells (Figure 8).

Because neither sample showed neurite outgrowth until day 2, cells were scored for neurite outgrowth on day 3. This way, we were able to capture the initial stages of neurite growth. Additionally, as PC12 cells are grown for extended periods of time, the cells clump together (Greene and Tischler, 1976). By scoring on day 3, we were able to visualize individual cells, allowing for more precise scoring. The resulting data showed that the MK-STYX shRNA decreased the percentage of neurite outgrowth when compared to the scrambled shRNA negative control (Figure 9).

In the future, it would be interesting to assay MK-STYX protein expression along the 7 day course using western blot analysis. If the MK-STYX shRNA expression vector was not consistently expressed throughout the 7 days, it could potentially correlate to the formation and elongation of neurites in knock-down samples.
Discussion

In order to learn about the functional role of MK-STYX, potential binding partners were identified using a proteomics approach. Through this method, G3BP1 was identified as the first interaction partner of this pseudophosphatase (Hinton et al., 2010). Because of G3BP1’s role in neuronal development, we were curious whether MK-STYX would have a role in this process, and focused our attention on MK-STYX and neuronal processes. Here, we show MK-STYX is an important regulator of PC12 differentiation that is capable of inducing neurite outgrowth. Furthermore, MK-STYX knockdown data suggest that MK-STYX is important for PC12 neuronal differentiation.

Two of the important signaling pathways that regulate neuronal differentiation are controlled by the small GTPases, Ras and RhoA. The Ras/MAPK pathways causes neurite outgrowth, while the RhoA pathway promotes cell rounding. Activity assays showed MK-STYX was not altering Ras/MAPK signaling, but was decreasing RhoA activity (unpublished data, Brittany Flowers). Now that MK-STYX is known to influence RhoA activity, it will be interesting to determine how this pseudophosphatase affects downstream effectors of this GTPase.

Parkinson’s disease is a neurodegenerative disorder characterized by the loss of dopamine-producing cells. As an important neurotransmitter, the loss of dopamine impairs proper motor movement and muscle control (National Parkinson Foundation, 2013). Interestingly, when PC12 are stimulated with NGF,
the cells are capable of releasing large amounts of dopamine (Lim et al., 1995; Amino et al., 2002). Because of this, PC12 grafts have been investigated in various hosts, such as guinea pigs, and primates. In all of these studies, PC12 grafts have proven to be an effective dopamine delivery method, lasting for up to 1 year (Ono et al., 1997; Date et al., 2000; Yoshida et al., 1999). MK-STYX is an important regulator of neuronal differentiation in PC12 cells, and could potentially promote dopamine release in grafted PC12 cells. Learning more about how MK-STYX induces differentiation could potentially point to enhancing therapeutic applications for Parkinson’s disease.
Figure 7: Neuronal outgrowth in PC12 cells containing scrambled shRNA. Cells were transfected GFP-tagged scrambled shRNA, and stimulated with NGF 24 hours later. Neurite length (μm) was then measured over a 7 day course.
Figure 8: shRNA knock-down of MK-STYX reduces neurite outgrowth in PC12 cells. Cells were transfected with a GFP-tagged MK-STYX shRNA expression plasmid. Following NGF stimulation, neurite length (µm) was measured over 7 days.
Figure 9: shRNA knock-down of MK-STYX significantly decreases number of PC12 cells with neurite outgrowth. shRNA expression plasmids were transiently transfected into cells. The scrambled shRNA served as a negative control for the experimental MK-STYX shRNA. From each sample, 100 cells were scored for neurite outgrowth (≥ 20 μm) 3 days after NGF stimulation (100 ng/mL). Roughly 80% of scrambled shRNA-transfected cells had neurite formation, while cells containing the MK-STYX shRNA showed only ~25% of cells having neurites (n=3).
References


epidermal growth factor and nerve growth factor in PC12 cells. Journal of Biological Chemistry 276, 18169.


Martin, S., Zekri, L., Metz, A., Maurice, T., Chebli, K., Vignes, M. and Tazi, J. (2013). Deficiency of G3BP1, the stress granules assembly factor, results in
abnormal synaptic plasticity and calcium homeostasis in neurons. Journal of neurochemistry 125, 175.


Murphy, M.E. (2013). The HSP70 family and cancer. Carcinogenesis 34, 1181.

National Parkinson Foundation. (2013). What is Parkinson’s disease?


