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Exploring A Connection Between MK-STYX And GSK3

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COMPLIANCE PAGE

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ABSTRACT

The intriguing protein MK-STYX [MAPK (mitogen-activated protein kinase) phospho-serine/threonine/tyrosine-binding protein] has been receiving attention because recent work has implicated it in a number of biological processes. MK-STYX is a pseudophosphatase involved in cellular stress responses, neuronal differentiation, apoptosis, and several cancers. As a pseudophosphatase, it is catalytically inactive due to mutations in its enzyme active site. However, it retains structural domains that enable it to perform protein-protein interactions. It is important to consider how exactly MK-STYX may carry out its many functions. Another multifunctional protein, known as GSK3 (glycogen synthase kinase 3) has some overlapping functions with MK-STYX so it is exciting to consider that MK-STYX may impact GSK3 to exert its cellular functions. GSK3 refers to two paralogous and homologous enzymes known as GSK3α and GSK3β. These are regulated by inhibitory phosphorylation on serine 21/9 and activating phosphorylation on tyrosine 279/216 for GSK3α/β, respectively. Accordingly, biochemical studies were carried out to determine whether MK-STYX changed the phosphorylation at these sites, or indeed if MK-STYX changed the amount of GSK3. A multifunctional kinase like GSK3 has many physiological roles, so if MK-STYX changes regulatory phosphorylation sites or protein expression of GSK3, it was hypothesized that MK-STYX could affect GSK3 functions, and that additionally, MK-STYX could form a protein complex with GSK3 and then modulate it. Western blot analysis revealed that MK-STYX decreased serine 9 phosphorylation on GSK3β, suggesting that MK-STYX activates GSK3β. MK-STYX did not change serine 21 phosphorylation on GSK3α. Also, MK-STYX did not affect total expression of GSK3α/β. Further, there was no detected interaction between MK-STYX and GSK3β as indicated by coimmunoprecipitation. Overall, this thesis suggests MK-STYX may have some regulatory role over GSK3. Further biochemical work should clarify this connection and its biological significance.

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This thesis is dedicated to all the people who have guided me on my life's journey.

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Chapter 1. Introduction

Cell signaling

The past decades have witnessed truly impressive innovations in the biological sciences. In particular, the field of cell signaling, which explores how cells communicate with each other and within themselves, has come of age. Some of the most intriguing advances have occurred in protein sciences, the field which investigates how proteins, the workhorses of cells, regulate the processes that allow life to thrive and reproduce. Proteins are macromolecules made of numerous amino acid subunits, and the number and properties of their constituent amino acids contribute to their shape and function (Wüthrich, 1990). However, one especially important area in protein studies concerns protein phosphorylation. Phosphorylation is a type of post-translational modification (i.e., a feature added to proteins after their synthesis at the ribosome) that bonds a phosphate group to target proteins (Cohen 2002). Phosphate groups, which have the chemical formula PO_4^{2-}, are negatively charged and highly hydrophilic. Thus, adding or removing a phosphate from an amino acid residue on a protein changes its structure, and since a protein's function is heavily dependent on its structure, phosphorylation can dramatically alter protein activities (Ubersax and Ferrell, 2007). To this effect, kinases are enzymes that catalyze the addition of phosphate groups, or phosphorylation, while phosphatases in turn mediate the removal of phosphate groups, or dephosphorylation, of substrates (Ubersax and Ferrell, 2007). Crucially, phosphorylation can serve as the foundation for cellular

signal transduction, wherein sequential phosphorylation and dephosphorylation events can activate and deactivate relays of proteins to transmit cellular messages (Cohen 2002). Note that kinases are often thought to control the amplitude of a cellular signal, while phosphatases regulate the rate and duration.

Types of kinases and phosphatases

In eukaryotes, there are three commonly phosphorylated amino acids: serine, threonine, and tyrosine, and the phosphorylation status of these residues is carefully regulated by a bevy of enzymes (Ubersax and Ferrell, 2007). At least 60% of human proteins can be phosphorylated, and the modified amino acids phosphoserine, phosphothreonine, and phosphotyrosine account for about 86%, 12%, and 2% of total protein phosphorylation, respectively (Olsen et al., 2006). The serine/threonine-specific protein kinases carry out the phosphorylation of most serine and threonine residues, while the serine/threonine-specific phosphatases effect the dephosphorylation of most phosphoserine and phosphothreonine residues (Olsen et al., 2006). The members of the more specialized protein tyrosine kinase (PTK) superfamily catalyze the phosphorylation of tyrosine residues while their counterparts in the protein tyrosine phosphatase (PTP) superfamily handle phosphotyrosine dephosphorylation (Tonks, 2006).

The PTKs and the PTPs

The distinct structures and functions of the various PTKs and PTPs allow them to regulate many cellular processes. There are two general classes within the PTK superfamily, and these are the receptor tyrosine kinases (RTKs) and the nonreceptor tyrosine kinases (NRTKs) (Hubbard and Till, 2000). The RTKs are transmembrane proteins that are turned on by extracellular binding of their specific ligands followed by intracellular autophosphorylation. On the other hand, the NRTKs are intracellular and generally activated by signaling inputs from various receptors (Hubbard and Till, 2000). PTKs are major players in a great many signal transduction pathways regulating growth, proliferation, differentiation, and apoptosis, underscoring the need for factors to manage the careful regulation of these enzymes. Now consider the PTPs, which all share an evolutionarily conserved HCX_5R catalytic amino acid motif (Kim and Ryu, 2012). This motif enables a two-step catalytic dephosphorylation mechanism. The first step involves a nucleophilic attack from cysteine to the phosphorus atom of the target substrate. This attack forms a covalent bond between the nucleophilic cysteine and phosphorus while also breaking the bond between the phosphate group and tyrosine. The second step involves hydrolysis, wherein a water molecule breaks the cysteine-phosphorus bond, which resets the enzyme and releases the phosphate group (Kolmodin and Åqvist, 2001). The PTPs come in two varieties: the classical PTPs which dephosphorylate only phosphotyrosine residues and the dual-specificity phosphatases (DUSPs) which dephosphorylate phospho-serine/threonine/tyrosine residues (Alonso et al., 2004) (Fig. 1). Like the

PTKs, PTPs exist as transmembrane and non-transmembrane proteins (Alonso et al., 2004). Within the DUSP family, there exists the MAP kinase phosphatase (MKP) subfamily, whose members dephosphorylate and inactivate the mitogen-activated protein kinases (MAPKs) (Theodosiou and Ashworth, 2002). There are various MAPK signaling pathways with major roles in cell differentiation, proliferation, stress, and death (Morrison, 2005). Thus, MKPs are highly important proteins by merit of their ability to shut off pivotal MAPK cascades.

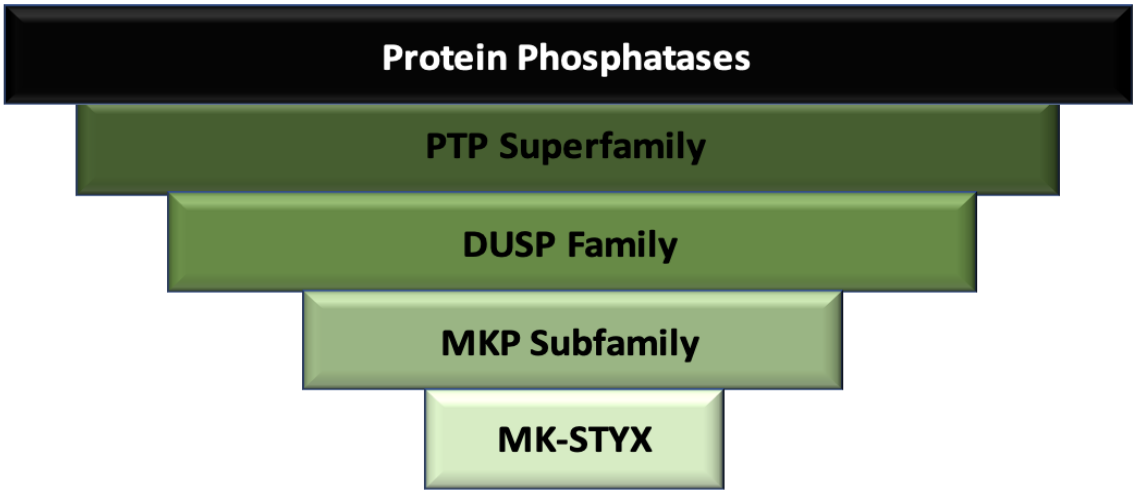


Figure 1: A representation of the phosphatases and their relationship to each other.

Pseudoenzymes

When most people think of enzymes, they picture catalytically active proteins that drive a myriad of chemical reactions. However, enzymes have catalytically inactive counterparts known as pseudoenzymes which are nevertheless prevalent throughout the genome and manifest a diverse array of biological functions (Pils and Schultz, 2004). Approximately 10% of all enzymes

are thought to be pseudoenzymes, and these are found and often evolutionarily conserved in all domains of life (Pils and Schultz, 2004). Almost every enzyme class features some pseudo-members, and the kinases and phosphatases have the most prominent of the pseudoenzymes, with a great many biological roles described (Murphy et al., 2017, Hinton 2019).

Pseudophosphatases

A riveting yet understudied part of the phosphatase family are the pseudophosphatases. These (pseudo)enzymes are evolutionarily related to the phosphatases, but they lack crucial amino acids involved in catalysis and most are rendered catalytically inactive (Tonks, 2009). However, this lack of catalytic activity does not at all equate to a lack of function, since pseudophosphatases have been found to have roles in many physiological processes and diseases (Mattei et al., 2021) (Fig. 2, Table 1). Pseudophosphatases exert these effects because they can maintain the general 3-dimensional fold of the phosphatases they descended from (Tonks, 2009). Since function follows from form in proteins, like it does in many other areas of life, pseudophosphatases can carry out some of the non-catalytic functions of their counterparts. Additionally, evolutionary changes following the initial genetic emergence of a pseudophosphatase can alter its amino acid sequence and confer new binding partners.

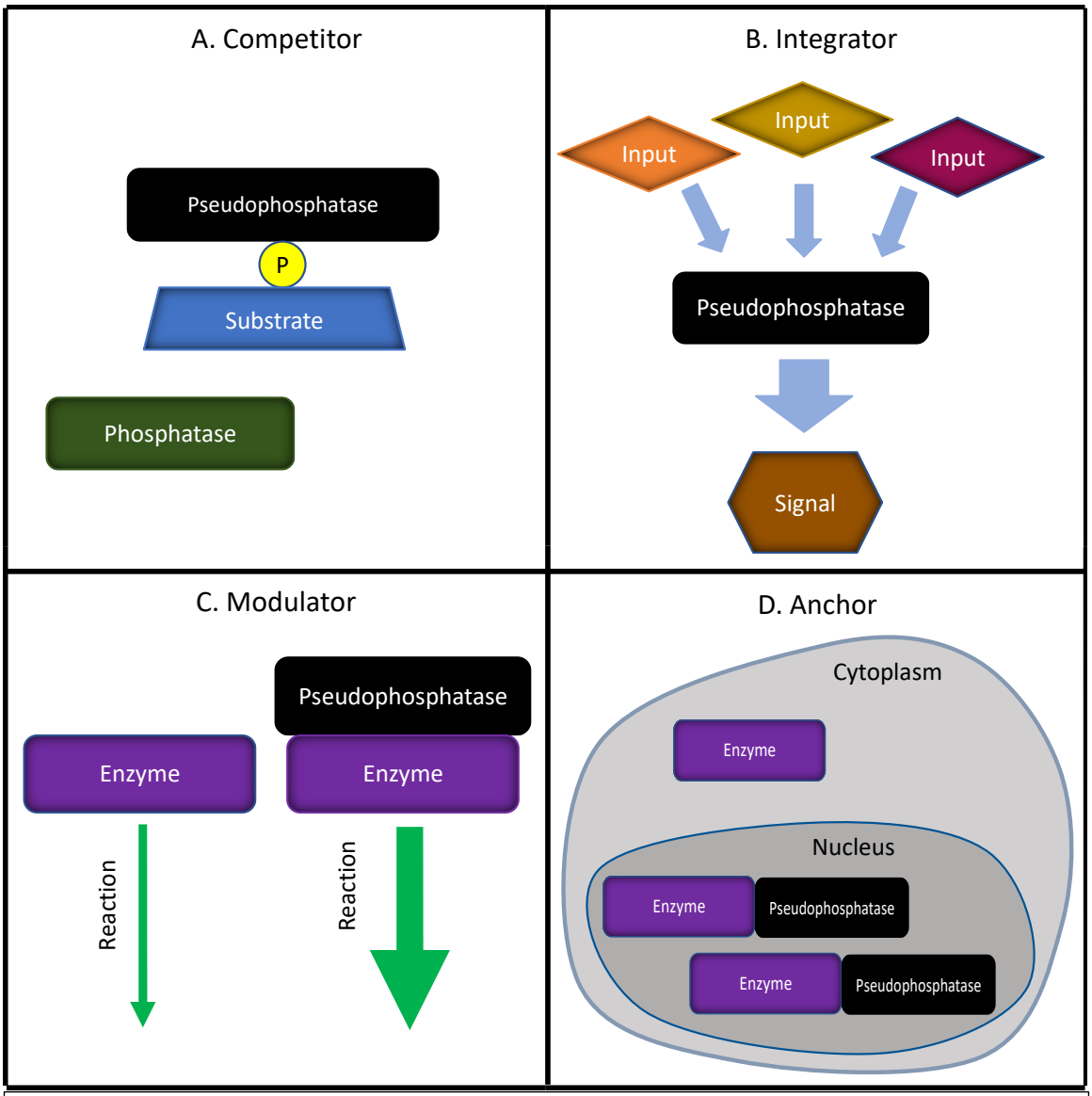


Figure 2: A diagram representing the possible functions a pseudophosphatase could carry out.
A. A pseudophosphatase competes with another protein for a substrate.
B. A pseudophosphatase integrates several cell signaling inputs to generate a unified signal output.
C. A pseudophosphatase binds and modulates the function of another protein.
D. A pseudophosphatase binds and anchors another protein in a given cellular compartment.

Table 1: A list of pseudophosphatases and their functions.
(Mattei et al., 2021).

Name of Pseudophosphatase	Functions
MTMR5 (Myotubularin-related protein 5) [A.K.A. SBF1]	-Binds and modulates MTMR2. Boosts its lipid phosphatase activity (Kim et al., 2003) -Mutations in MTMR5 lead to Charcot-Marie-Tooth disease type 4B3 (CMT4B3) (Pareyson et al., 2019). The CMT diseases are peripheral neuropathies which include symptoms like myelin folding issues, reduced nerve conduction, and peripheral axon degeneration (Pareyson et al., 2019)
MTMR9 (Myotubularin-related protein 9)	-Binds and modulates MTMR6, MTMR7, and MTMR8. Boosts their lipid phosphatase activity (Zhou et al., 2012) -MTMR9 is also an inhibitor of autophagy and apoptosis (Zou et al., 2012) -MTMR9 mutations are positively linked to obesity (Hotta et al., 2011) and epilepsy (Baulac et al., 2008)
MTMR10 (Myotubularin-related protein 10)	-The expression of MTMR10 is reduced in the esophageal mucosa of patients afflicted with esophageal achalasia (EA) In EA, patients have difficulty swallowing (Shoji et al., 2017)
MTMR11 (Myotubularin-related protein 11)	-The expression of MTMR11 is lowered in acute myeloid leukemia (Kabir et al., 2013) -MTMR11 expression reduced in HER2+ breast cancer cells (Lucci et al., 2010)
MTMR12 (Myotubularin-related protein 12) [A.K.A. 3-PAP]	-MTMR12 binds and stabilizes MTM1 in zebrafish (Gupta et al., 2013) -MTMR12 interacts with MTMR2 (Nandurkar et al., 2003)
MTMR13 (Myotubularin-related protein 13) [A.K.A. SBF2]	-Binds MTMR2 and raises its lipid phosphatase activity (Robinson et al., 2005) -Also, MTMR13 mutations lead to CMT4B2 (Laššuthová et al., 2018)
STYX (Serine/threonine/tyrosine-interacting protein)	-Supports spermatogenesis in mice (Wishart and Dixon, 2002) -STYX anchors ERK1/2 in the nucleus (Reiterer et al., 2013)

	-Promotes tumor growth in colorectal cancer (He et al., 2019) -Inhibits FBXW7 to promote endometrial cancer (Liu et al., 2020)
MK-STYX (Mitogen-activated protein kinase phosphoserine/threonine/tyrosine-binding protein) [A.K.A. STYXL1 or DUSP24]	-Inhibitor of stress granules (Hinton et al., 2010), (Barr et al., 2013) -Promoter of neuronal differentiation (Flowers et al., 2014), (Banks et al., 2017) -Also upregulates apoptosis (Niemi et al., 2011), (Niemi et al., 2014) -Alters HDAC6 localization and phosphorylation (Cao et al., 2019) -Drives hepatocellular carcinoma (Wu et al., 2020) -Roles in glioma pathogenesis (Tomar et al., 2019)
STYXL2 (Serine/threonine/tyrosine-interacting-like protein 2)	-It is expressed in muscle tissue -STYXL2 mutation impairs muscle function, suggesting it is needed for normal muscle activity (Fero et al., 2014)
TAB1 (TGF-beta-activated kinase 1 and MAP3K7-binding protein 1) [A.K.A. MAP3K7IP1]	-Promotes TGF-beta signaling by turning on TAK1 kinase (Shibuya et al., 1996) -Lowered expression of TAB1 in ovarian cancer (Zhu et al., 2013)
Tensin 1 [A.K.A. TNS1]	-Acts as an adaptor protein that connects integrin receptors and actin filaments within focal adhesions (Lo et al., 2004) -Tensin 1 expression increases cell migration (Chen et al., 2002)

MK-STYX

The protein MK-STYX [MAPK (mitogen-activated protein kinase) phospho-serine/threonine/tyrosine-binding protein] stands out as a particularly fascinating pseudophosphatase. As the name suggests, MK-STYX is a catalytically inert member of the MKP subfamily, thought it does not appear to affect MAPK signaling directly like canonical MKPs do (Hinton, 2020). Its functionality is likely

explained by its somewhat eccentric structure. MK-STYX has an N-terminal cdc 25 homology 2 (CH2) domain and a C-terminal dual-specificity phosphatase domain (DUSP) (Fig. 3). The CH2 domain, sometimes called a rhodanese domain, is catalytically inactive though in other MKPs it mediates substrate recognition. To this end, the CH2 domain contains a kinase interacting-domain (KIM), though in MK-STYX this domain has mutations that may impact what it binds (Hinton, 2020). The DUSP domain in classical MKPs carries out substrate dephosphorylation; however, in MK-STYX the canonical HCX_5R catalytic motif is mutated to FSX_5R, explaining MK-STYX's lack of phosphatase activity. MK-STYX has documented functions in neuronal differentiation, stress granule dynamics in the cellular stress response, HDAC6 modulation, and apoptosis. Preliminary work also suggests that MK-STYX has impacts on autophagy (Christian, 2016, unpublished). Additionally, MK-STYX has roles in several diseases, including glioblastoma, hepatocellular carcinoma, and Ewing sarcoma (Mattei et al., 2021) (Fig. 4).

Figure 3: The structure of MK-STYX
On the N-terminus, MK-STYX has a CH2 domain with a KIM domain nested inside.
On the C-terminus, MK-STYX has a DUSP domain.

The role of MK-STYX in neuronal differentiation

MK-STYX plays several roles in the promotion of neuronal differentiation. During neuronal differentiation, cells gradually adopt a neuronal phenotype characterized by cytoskeletal rearrangements that support the formation of relatively large cellular extensions known as neurites. The overexpression of MK-STYX leads to neurite outgrowths in PC-12 (rat pheochromocytoma) cells (Flowers et al., 2014). Additionally, MK-STYX has been found to augment the impacts of NGF (nerve growth factor), leading to longer neurites in MK-STYX and NGF treated cells relative to control and NGF treated cells. Further, inhibition of MK-STYX via shRNA knockdown showed that MK-STYX is necessary for full neurite extension following NGF treatment. Later work showed that MK-STYX can trigger neurite growth without the MAPK/ERK pathway, as neurites were still induced with a MEK inhibitor present (Flowers et al., 2014). Since MAPK/ERK is not responsible for MK-STYX's neurite induction, another pathway must fulfill this role. Previous research has found that Rho inactivation is needed to trigger PC-12 neuronal differentiation (Sebök et al., 1999). Intriguingly, MK-STYX overexpression was shown to inactivate RhoA, implying that this is part of the mechanism through which MK-STYX induces neurons. To further elucidate this neurogenic pathway, cofilin was considered since RhoA regulates this protein. The phosphorylation of cofilin is decreased by MK-STYX in normal culture media and conversely increased by MK-STYX in NGF-stimulated media (Flowers et al., 2014).

A further study by the Hinton lab sought to expand on these neuronal findings and demonstrate that the cellular extensions produced by MK-STYX were indeed *bona fide* neurites. MK-STYX overexpression was found to increase the number of primary neurites (i.e. extensions ≥ 20 μm) visible on PC-12 cells. (Banks et al., 2017). Further work was done to determine if MK-STYX induces a legitimate neuronal morphology. The neurites induced by MK-STYX contained both actin and β-tubulin, which would be expected of *bona fide* neurites. Another important consideration in neuronal differentiation are growth cones, which are locations in neurites that aid in the formation of synapses. Thus, it is significant that MK-STYX increases the number of growth cones per neurite in the presence of NGF. Intriguingly, PC-12 cells transfected with MK-STYX with and without NGF developed synapse-like structures revealed by transmission electron microscopy (TEM). It was also decided to consider MK-STYX's effects on primary hippocampal neurons, since these provide a model that more closely recreates *in vivo* conditions (Banks et al., 2017). Transfection of MK-STYX into these cells led to a major increase in the number of neurons with more than five neurites, while also increasing the total neurite outgrowth length per neuron. These myriad neurological findings demonstrate that MK-STYX is a driver of neuronal differentiation (Banks et al., 2017).

The role of MK-STYX in stress granules

Exciting work from the Hinton lab has explored the functions of MK-STYX in stress granule dynamics. Previous efforts have established that stress

granules are subcellular regions of mRNA and mRNP (messenger ribonucleoprotein) processing (Protter and Parker, 2016). As the name suggests, stress granules form in response to stressors, some of which are heat, cold, infection, and oxidative stress (Panas et al., 2016). Stress granules, which are membraneless organelles, play roles in translational regulation, viral infection, neurodegenerative disease, and the cellular stress response. A variety of molecular interactions underlie the formation of stress granules. These include RNA-RNA, RNA-protein, and protein-protein interactions (Van Treeck et al., 2018). The result of these heterotypic interactions is a liquid-liquid phase separation, wherein the stress granule forms a unique aqueous environment within the liquid milieu of the cytoplasm.

Hinton lab research has found that MK-STYX lowers stress granule formation. Specifically, in cells stressed by arsenite, which is an oxidative stressor, 60% of control cells showed stress granules while only 18% of MK-STYX-transfected cells did (Hinton et al., 2010). MK-STYX was also found to coimmunoprecipitates (CoIP) with G3BP1 [Ras-GAP (GTPase-activating protein) SH3 (Src Homology 3) domain-binding protein-1], a protein which has a major role in promoting stress granule formation. Thus, based on this interaction, it is possible that MK-STYX impairs G3BP1's stress granule promoting functions to effect its stress granule reduction. Also of interest, overexpression of G3BP1 in cells can itself induce stress granule formation. Intriguingly, about 50% of control cells transfected with G3BP1 show stress granules while only 20% of cells transfected with both MK-STYX and G3BP1 form these granules (Hinton et al.,

2010). Thus, MK-STYX can impair stress granules in two different molecular contexts.

Later Hinton lab research also endeavored to explain how exactly MK-STYX achieves stress granule reduction. The serine 149 site has been controversially found to regulate stress granule formation (Panas et al., 2019), with phosphorylation at this site reportedly blocking stress granules and dephosphorylation promoting them (Tourrière et al., 2003). Therefore, studies were conducted to see if MK-STYX would prevent the formation of stress granules induced by serine 149 to alanine mutant G3BP1 (S149A G3BP1) overexpression, since this variant is nonphosphorylatable at the relevant serine site (Barr et al., 2013). In a fascinating turn of events, cells co-transfected with MK-STYX and S149A G3BP1 developed stress granules 20% of the time while controls transfected with only S149A G3BP1 developed stress granules 60% of the time. This showed that MK-STYX does not depend on promoting the phosphorylation of serine 149 of G3BP1 to antagonize stress granules. A complementary experiment from the same study found that MK-STYX overexpression prevents the formation of stress granules following heat shock (Barr et al., 2013). The aforementioned work establishes MK-STYX as a regulator of stress granules, but the mechanism by which MK-STYX reduces these membraneless organelles still needs exploration.

MK-STYX as a regulator of apoptosis

The pseudophosphatase MK-STYX has significant upregulatory effects on the programmed cell death system known as apoptosis. To this effect, inhibition of MK-STYX by means of RNA interference (RNAi) prevents cells from carrying out mitochondrial-dependent intrinsic apoptosis (Niemi et al., 2011). This also means that cells with MK-STYX inhibition will not release cytochrome c following proapoptotic signaling, a major finding since cytochrome c is an essential part of the apoptosome that is required for intrinsic apoptosis to occur. MK-STYX also localizes to the mitochondria, a finding that inspired further investigation into how MK-STYX is promoting the mitochondria's pro-apoptotic machinery. A follow-up study found that MK-STYX strongly interacts with (i.e., forms a complex with) PTPMT1 (PTP localized to the mitochondrion 1) (Niemi et al., 2014). The phosphatase PTPMT1 inhibits intrinsic apoptosis, likely through its production of cardiolipin which prevents cytochrome c release. Additional work showed that MK-STYX expression inhibits the activity of PTPMT1, meaning that PTPMT1's blockade of apoptosis can be lifted by MK-STYX (Niemi et al., 2014).

The role of MK-STYX in HDAC6 dynamics

The Hinton Lab has also explored the impact of MK-STYX on histone deacetylase isoform 6, or HDAC6. The enzyme HDAC6 is a promoter of stress granule formation (Kwon et al., 2007), so it made sense to investigate if MK-STYX was affecting HDAC6 to impair stress granule formation. Additionally, G3BP1 and HDAC6 form a complex, so it is feasible that MK-STYX modulates HDAC6 owing to its complexing with G3BP1. Though it is evolutionarily related to

histone deacetylases, HDAC6 is a primarily cytoplasmic protein, due to a nuclear export sequence and SE14 cytoplasmic anchoring domain. This means its main substrates are proteins located in the cytoplasm (Zheng et al., 2017). Like all histone deacetylases (HDACs), HDAC6 removes acetyl groups from target proteins and in so doing alters their structure and thus function. In contrast, histone acetyltransferases add acetyl groups to substrates (Hubbert et al., 2002).

Research has shown that MK-STYX overexpression causes HDAC6 to adopt a more whole cell distribution (present in nucleus and cytoplasm) as opposed to a more cytosolic one in the control (Cao et al., 2019). This means MK-STYX may cause HDAC6 to deacetylate different substrates owing to the enzyme's altered subcellular localization. Additionally, MK-STYX reduces the number of HDAC6 aggregates, an important finding since these aggregates may correspond to stress granules which contain HDAC6. Also of interest, the phosphorylation of HDAC6 on serine 22 promotes deacetylase activity, and deacetylase activity promotes stress granule formation. Therefore, experiments were carried out to examine how MK-STYX may affect this phosphorylation site. In normal conditions, MK-STYX decreases HDAC6 serine 22 phosphorylation, while in serum starved stress conditions MK-STYX increases it (Cao et al., 2019). Further work also considered if MK-STYX would disrupt the cell's microtubule network, which is crucial for stress granule formation and so could explain how MK-STYX reduces stress granules. These studies found that MK-STYX did not alter microtubule stability, suggesting that MK-STYX reduces stress granules through another mechanism. It is still feasible that MK-STYX

influences other microtubule properties to impair stress granules. Dynein is an important promoter of stress granule formation, and detyrosinated tubulin promotes the movement of dynein. Studies were conducted to determine whether MK-STYX would reduce tubulin detyrosination and in so doing antagonize stress granules. Interestingly, MK-STYX was found to boost tubulin detyrosination, implying that this pseudophosphatase reduces stress granules in another fashion while still modulating this post-translational modification. A further microtubule modification, acetylation, increases dynein mobility and so it is possible that an increase here would promote stress granule formation. Intriguingly, acetylated tubulin expression is increased in the presence of MK-STYX, which broaches the possibility that MK-STYX blocks HDAC6's ability to modify microtubules (Cao et al., 2019). Overall, these results establish that MK-STYX exerts effects on HDAC6, and they suggest that further exploration of the MK-STYX and HDAC6 connection could yield interesting insights.

MK-STYX in Ewing Sarcoma

In the pediatric bone cancer known as Ewing sarcoma, MK-STYX expression is promoted (Siligan et al., 2005). Specifically, the EWS-FLI1 fusion protein which is a hallmark of Ewing Sarcoma facilitates the expression of MK-STYX by binding an intronic region. The significance of this interaction is unknown, but it is especially noteworthy that high MK-STYX expression was detected in all 17 Ewing sarcoma family tumor samples examined in this study (Siligan et al., 2005).

MK-STYX's role in glioma

An exciting recent study describes how MK-STYX is necessary and sufficient for full glioma pathogenesis. This work found that MK-STYX is upregulated in many cases of the brain cancer known as glioma or glioblastoma multiforme. Importantly, *in vitro* studies showed that MK-STYX promotes proliferation, colony formation, migration, and invasion of glioma cells (Tomar et al., 2019). Also of interest, MK-STYX drove the metastasis-enabling anchorage-independent growth of glioma cells, while also showing a marked ability to transform precancerous astrocytes, a type of brain cell, into glioma cells. With regards to *in vivo* work, MK-STYX was found to significantly increase transplanted glioma tumor growth in mice (Tomar et al., 2019).

MK-STYX's role in hepatocellular carcinoma

Promising work has recently been published describing how MK-STYX plays roles in the pathogenesis of hepatocellular carcinoma (HCC), a prevalent type of liver cancer (Wu et al., 2020). Specifically, qPCR analysis showed that MK-STYX expression is elevated in HCC, and that MK-STYX expression is positively correlated with HCC severity. Supporting work from the same study showed that inhibition of MK-STYX impaired HCC cell replication, while also reducing the activity of the anabolic PI3K/AKT pathway. This suggests that MK-STYX drives HCC proliferation at least in part by upregulating pro-growth PI3K/AKT cell signaling. Further experiments found that MK-STYX expression

reduced the expression of the RNA-binding protein CELF2, and that low CELF2 expression predicts poor HCC prognosis. CELF2 has other documented tumor suppressing roles, so it is reasonable to think that in HCC MK-STYX inhibits this protein to drive cancer progression (Wu et al., 2020).

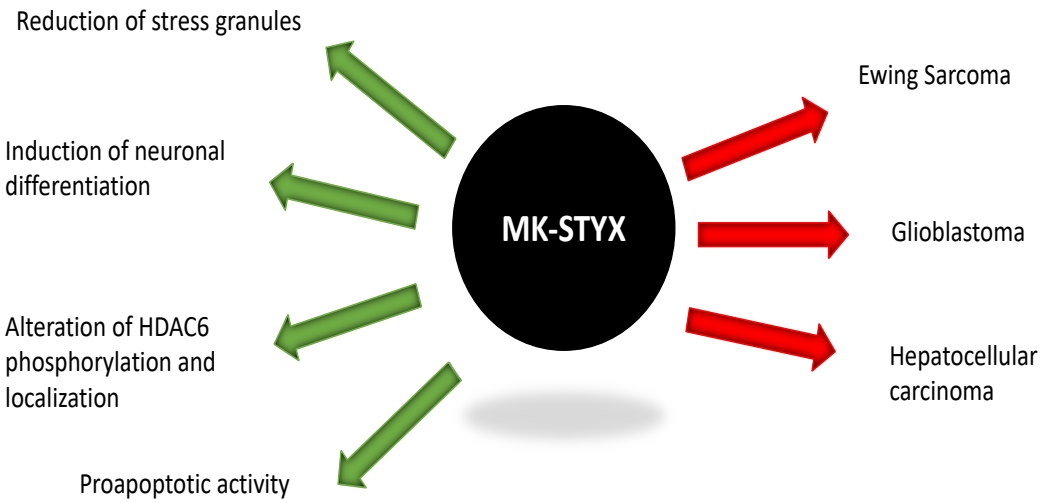


Figure 4: Major functions of MK-STYX
Green arrows indicate normal cellular functions that MK-STYX contributes to. Red arrows indicate diseases that MK-STYX is linked to.

MK-STYX and GSK3

After exploring the many functions of MK-STYX, it is natural to seek to elucidate the mechanisms MK-STYX uses to achieve these effects. One enticing possibility is that MK-STYX modulates the multi-role enzyme known as glycogen synthase kinase 3 (GSK3) to carry out its varied subcellular actions. This is a reasonable hypothesis since GSK3 exerts effects in all the pathways MK-STYX is implicated in, as will be made clear after the following overview of GSK3.

Regulation of GSK3

First, it must be explained that GSK3 refers to two homologous and paralogous kinases, the slightly larger GSK3α and GSK3β. Since these enzymes have such extensive structural and functional overlap, it is generally sufficient to refer to them collectively as GSK3. GSK3 is a prominent serine/threonine kinase that shows a preference for pre-phosphorylated protein substrates and those with proline nearby (i.e. it is proline-directed) (Beurel et al., 2015; Sutherland, 2011). The fact that GSK3 target sites often require pre-phosphorylation and proline proximity helps ensure GSK3 specificity. Specificity is especially important in the case of GSK3, as it phosphorylates at least 100 different proteins in many different cellular signaling contexts (Sutherland, 2011). Crucial to GSK3's phosphorylation ability are its two major domains, an N-terminal substrate recruitment domain and a C-terminal kinase domain. These domains are in fact intimately linked with GSK3's regulation. The N-terminal substrate recruitment domains of GSK3α and GSK3β can be phosphorylated on serine 21 and 9, respectively. Phosphorylation here reduces the kinase activity of the enzyme. The anabolic kinase AKT/PKB carries out serine 21 or 9 phosphorylation, along with protein kinase A and protein kinase C, to inhibit GSK3, while protein phosphatase 1 and protein phosphatase 2A (PP2A) dephosphorylate this site to activate GSK3 (Cohen and Frame, 2001; Beurel et al., 2015). On the other hand, the C-terminal kinase domains of GSK3α and GSK3β are typically phosphorylated on tyrosines 279 and 216 (Cohen and Frame, 2001). This modification is in fact a co-translational autophosphorylation event, and it boosts

kinase activity (Cole et al., 2004). There is some evidence that PTPs may dephosphorylate the tyrosine 279 and 216 sites to inhibit GSK3 (Cole et al., 2004) (Fig. 5).

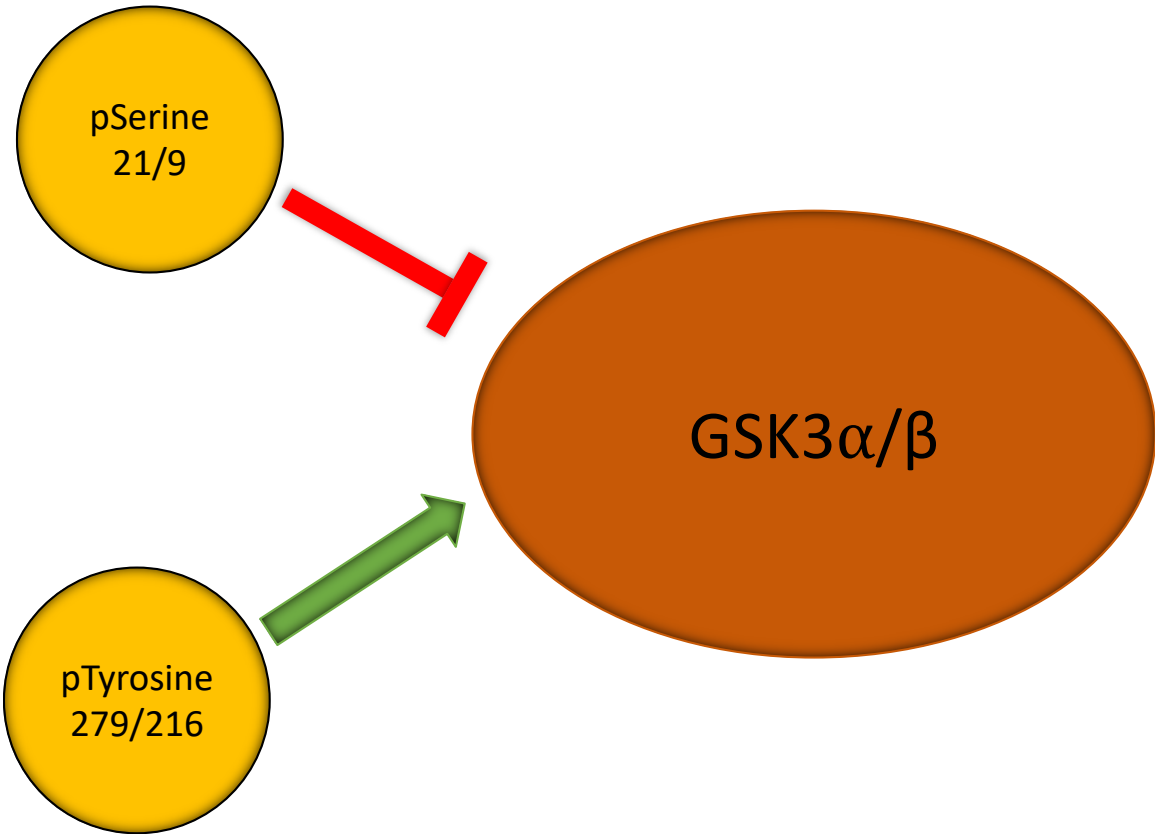


Figure 5: The regulation of GSK3
The red flat arrow indicates that phosphorylation on serine 21 of GSK3α and serine 9 of GSK3β function as inhibitors of kinase activity.
The green arrow indicates that phosphorylation on tyrosine 279 of GSK3α and tyrosine 216 of GSK3β activate kinase activity.

GSK3 functions

As the name suggests, GSK3 was first identified as an enzyme that phosphorylates and inhibits the major anabolic enzyme glycogen synthase (Embi

et al., 1980). However, dozens of other GSK3 substrates have since been identified, and it is clear that this kinase has nuanced roles in many signaling pathways. A shining example of this nuance can be found in GSK3's impacts on apoptosis. GSK3 is both an activator and a suppressor of apoptosis since it has different effects on the two main apoptotic pathways, the intrinsic and extrinsic pathways (Beurel and Jope, 2006). Specifically, GSK3 boosts intrinsic apoptotic activity by promoting cytochrome c release. Conversely, this kinase blocks extrinsic apoptosis by preventing the activation of the cellular self-destructive proteases known as caspases in response to extracellular death-inducing cues. GSK3 also has an activating role in autophagy, wherein GSK3 phosphorylates and activates the acetyltransferase TIP60 which in turn acetylates and activates the major autophagy promoting unc 51-like kinase (ULK1) (Lin et al., 2012). On another note, GSK3 is an inhibitor of the Wnt cell signaling pathway, which has important roles in cellular proliferation, migration, and differentiation. To be precise, GSK3 phosphorylates β-catenin which promotes its polyubiquitination and degradation, ultimately impairing Wnt signal transduction (Wu et al., 2010).

GSK3 and stress granules

The kinase GSK3 also has links to stress granule dynamics. For example, GSK3 inhibits stress granule formation by phosphorylating and downregulating the stress granule promoting protein Gle1 (Mason et al., 2019). Specifically, GSK3 phosphorylation of Gle1 blocks its intrinsically disordered regions from concentrating stress granule components, which impairs the eventual formation

of stress granules. Other interesting work has shown that GSK3β interacts with the major stress granule activator G3BP1. This work showed that GSK3β coimmunoprecipitates with G3BP1 and that their interaction raises levels of β-catenin in human breast cancer cells (Zhang et al., 2021).

GSK3 and cancer

GSK3 also has nuanced roles in cancer pathogenesis. In some oncogenic contexts, anabolic kinases like AKT and ERK1/2 phosphorylate and inhibit GSK3, which lifts GSK3's inhibition of the anabolic mammalian target of rapamycin complex 1 (mTORC1). Active mTORC1 can then increase lipid, nucleotide, and protein synthesis and downregulate autophagy so as to create a cellular environment that favors proliferation (Duda et al., 2020; Hermida et al., 2017). In other cancerous contexts, mutations can disrupt the destruction complex that targets protooncogenic β-catenin, and so prevent GSK3 from phosphorylating β-catenin and thus contributing to β-catenin's destruction at the proteosome (Stamos and Weis, 2013). One particularly important example of this effect concerns colorectal cancer. Here, mutations in the adenomatous polyposis coli (APC) scaffold protein prevent it from supporting the destruction complex. As a result, the protein inhibitors of β-catenin are impeded, and β-catenin accumulates and activates the transcription of cancer-promoting target genes (Shang et al., 2017). Somewhat paradoxically, GSK3 can also promote cancer. This can be achieved by GSK3's phosphorylation-mediated activation of the NF-κB essential modifier (NEMO). The activation of NEMO in turn supports the activation and

nuclear translocation of NF-κB (Medunjanin et al., 2016). The transcription factor NF-κB can then activate genes which support tumor-promoting inflammation, invasion, and metastasis.

GSK3 and neuronal differentiation

The dynamic GSK3 also has roles in neuronal differentiation. In general, GSK3 activity impairs axon growth, and inhibition of GSK3 promotes axon extension. Similarly, GSK3 predominantly impairs dendrite arborization or branching (Salcedo-Tello et al., 2011). Some of GSK3's neurite inhibition is accounted for by its ability to phosphorylate tau (Wagner et al., 1996). In a healthy neurite, tau associates with microtubules to promote extension. However, hyperphosphorylation of tau causes it to detach from microtubules and no longer support them. Additionally, GSK3 promotes the expression of amyloid precursor protein (APP), and the formation of a proteolytic cleavage product of APP known as Aβ42 (Ryder et al., 2003). Aβ42 prevents synaptic transmission and weakens neurites. These findings align with the fact that GSK3 impairs long term potentiation (LTP) and increases long term depression (LTD) of synapses. After all, in LTD, synapses are weakened and sometimes removed, which is what one would expect following inhibition of axons and dendrites by GSK3 (Peineau et al., 2008).

GSK3 and neurological disease

Since GSK3 carries out many nervous system-related actions, it is hardly surprising that this enzyme influences neurological disease as well. One such disease is bipolar disorder (BD), previously known as manic depression. Bipolar disorder is characterized by alternating moods of manic highs and intensely depressive lows that inflict great distress on sufferers (Muneer, 2017). Thus, it is of scientific and medical interest that mice with GSK3 hyperactivation show BD-like symptoms while humans with GSK3 polymorphisms develop BD earlier in life. Perhaps most importantly, the drug lithium, which is commonly used as a mood stabilizing treatment in BD, directly inhibits GSK3 by competing for magnesium binding sites in GSK3's protein structure (Freland and Beaulieu, 2012). Another major neurological disorder to which GSK3 is connected is the devastating and increasingly common neurodegenerative pathology known as Alzheimer's disease (AD). In AD, neurofibrillary tangles (NFTs) made of hyperphosphorylated tau and amyloid plaque buildups consisting largely of Aβ42 are strongly associated with disease onset and severity (Hooper et al., 2007; Oakley et al., 2006). Thus, it is extremely interesting that GSK3 phosphorylates tau and promotes the production of Aβ42 (Hooper et al., 2007). Also, BD patients treated with lithium are less likely to eventually develop AD, which offers support for the notion that GSK3 activity is involved in AD pathogenesis (Nunes et al., 2007). Further, a recent randomized controlled trial found that lithium administration to patients showing mild cognitive impairment, an early stage of

dementia, performed better on cognitive tests and attention tasks, and showed lower amounts of hyperphosphorylated tau in their cerebrospinal fluid (Orestes et al., 2018).

Elaborating the MK-STYX and GSK3 connection

As alluded to previously, the many functions of MK-STYX could in part be explained by a connection to GSK3 (Fig. 6). Specifically, it was thought possible that MK-STYX modulated GSK3 activity to induce some of the cellular changes it can produce. For example, MK-STYX and GSK3 both play inhibitory roles in stress granule dynamics, and intriguingly both of these proteins interact with the stress granule inducer G3BP1. Perhaps MK-STYX changed the phosphorylation of GSK3 to activate it, which would involve reducing inhibitory serine 21/9 phosphorylation and/or increasing activating tyrosine 279/216 phosphorylation. Also, MK-STYX could increase the expression of GSK3 by acting directly or indirectly at transcriptional, translational, and posttranslational levels. Additionally, MK-STYX could alter GSK3's actions by forming a complex with it. A direct or indirect complexing interaction could help effect changes on GSK3 itself or modulate GSK3's ability to bind and phosphorylate substrates. All three of these effects on GSK3 could help explain MK-STYX's stress granule inhibition.

As previously discussed, MK-STYX and GSK3 can both promote intrinsic apoptosis. While some of MK-STYX's proapoptotic mechanism is known, it is conceivable that MK-STYX could alter GSK3's phosphorylation, expression, or complexing to help exert this role. Such upregulation of GSK3 would allow this

kinase to phosphorylate and consequently activate apoptosis-promoting proteins while phosphorylating and deactivating apoptosis-antagonizing proteins.

Additionally, we have seen that both MK-STYX and GSK3 affect neuronal morphology, with MK-STYX promoting cellular changes associated with neuronal differentiation and GSK3 generally preventing these. It was explored if MK-STYX could downregulate GSK3 at the level of phosphorylation, expression, or protein-protein interaction and thus promote neuronal differentiation by inhibiting an inhibitor of neuronal differentiation. It is also important to mention here that GSK3 exists in several cellular pools that are largely functionally distinct (Ding et al., 2000). It is possible that MK-STYX could upregulate one pool while downregulating another to achieve seemingly paradoxical effects all by means of GSK3 regulation.

Lastly, MK-STYX and GSK3 are both proteins that impact HDAC6. In particular, MK-STYX reduces the phosphorylation of HDAC6 on serine 22 in regular cell culture conditions while increasing HDAC6 serine 22 phosphorylation in serum starved conditions. For its part, GSK3 positively regulates HDAC6 phosphorylation at serine 22 (Chen et al., 2010). Thus, it was hypothesized that MK-STYX would downregulate GSK3 in normal serum conditions while upregulating it in serum starved conditions. There are important functional implications of such an effect, since serine 22 phosphorylation increases HDAC6's deacetylase activity. It is hopefully clear now that the exploration of a link between MK-STYX and GSK3, which is described in this thesis, has had some important ramifications for determining the mechanisms of MK-STYX.

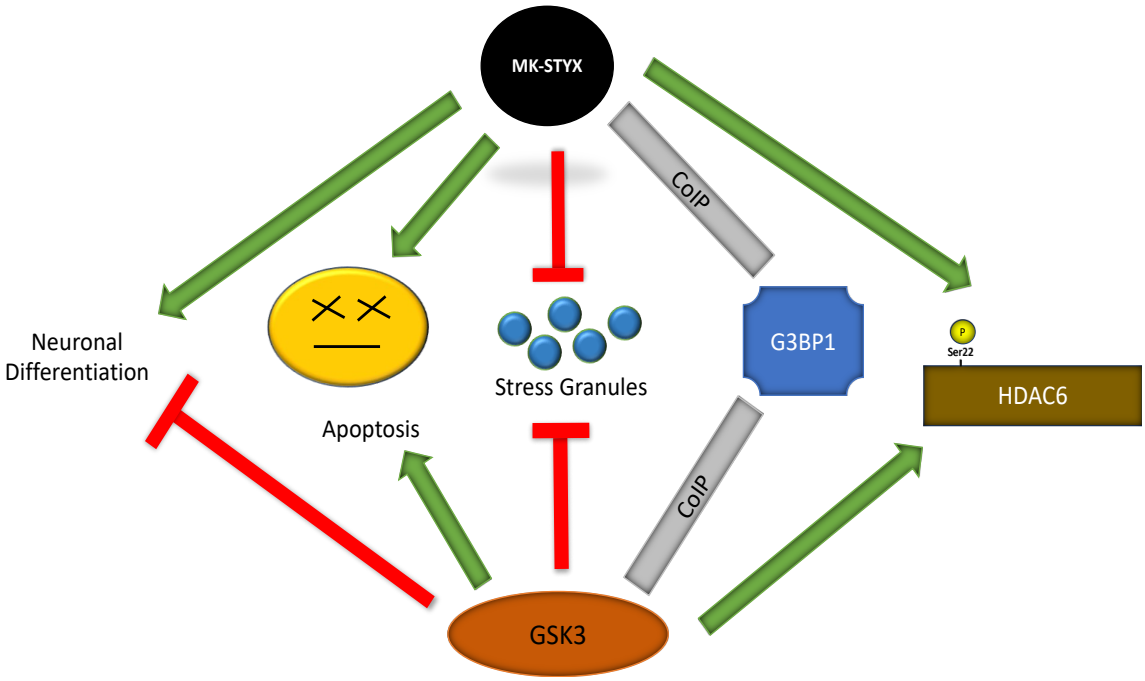


Figure 6: The hypothetical MK-STYX and GSK3 overlapping pathways Green arrows indicate functions promoted by the given protein, while red indicate functions inhibited by the given protein. The grey box labeled CoIP indicates an interaction. This figure demonstrates how MK-STYX and GSK3 have related roles in the cell.

Rationale of research aims

Based on the aforementioned information, the following general hypotheses were put forth. First, it was hypothesized that MK-STYX alters GSK3 phosphorylation on serine 21 or 9 of GSK3α or GSK3β, respectively (abbreviated as phosphoserine21/9GSK3α/β), which would affect the inhibition of GSK3. Second, it was also hypothesized that MK-STYX alters GSK3 phosphorylation on tyrosine 279 or 216 of GSK3α or GSK3β, respectively (abbreviated as phosphotyrosine279/216 GSK3α/β), which would impact the activation of GSK3. The third hypothesis was that MK-STYX changes the amount of GSK3α or

GSK3β, with the functional consequences of this being more GSK3 presumably leading to more GSK3 activity and less GSK3 leading to less GSK3 activity. Lastly, the fourth hypothesis was that MK-STYX interacts with GSK3 by directly or indirectly binding it in a complex. By interacting with GSK3, MK-STYX could theoretically alter GSK3's post-translational modification by bringing kinases, phosphatases, or ubiquitin ligases into proximity. Also, MK-STYX binding of GSK3 could change GSK3's conformation and/or its affinity for substrates.

Research Questions

1. Does MK-STYX overexpression cause a change in phosphorylation on serine 21 or 9 of GSK3α or GSK3β?

2. Does MK-STYX overexpression cause a change in phosphorylation on tyrosine 279 or 216 of GSK3α or GSK3β?

3. Does MK-STYX overexpression cause a change in expression of GSK3α or GSK3β?

4. Does MK-STYX or the catalytically active MK-STYX$_{\text{active mutant}}$ interact with GSK3β?

Chapter 2: Materials and Methods

Tissue culture

In all the experiments described in this thesis, human embryonic kidney 293 (HEK-293) cells were used. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). They were kept in an incubator at 37 °C with 5% CO_2 to simulate conditions in the human body.

Plate seeding

The HEK-293 cells were grown to approximately 40% to 100% confluency and then trypsinized to get cells off plates. To seed cells, 1 mL from the flask was added to 10 cm plates.

Transfection

Transfection was used to insert plasmids encoding the genes of interest into HEK-293 cells. These plasmids consisted of a pMT2 control vector, MK-STYX, and MK-STYX$_{\text{active mutant}}$. Note that MK-STYX$_{\text{active mutant}}$ is a catalytically active version of MK-STYX that was generated by site-directed mutagenesis (Hinton et al., 2010). In MK-STYX$_{\text{active mutant}}$, the phenylalanine and serine in MK-STYX's atypical active site motif are changed to catalytically competent histidine and cysteine, respectively. By including the MK-STYX$_{\text{active mutant}}$ variant in experiments, researchers can directly see how MK-STYX's catalytic inactivity confers unique functionality relative to a catalytically active enzyme. Additionally, MK-STYX and MK-STYX$_{\text{active mutant}}$ constructs encode a FLAG tag epitope to

simplify their detection. The plasmid constructs were set up as follows: pMT2, pMT2-FLAG-MK-STYX-FLAG, pMT2-FLAG-MK-STYX$_{\text{active mutant}}$-FLAG. The transfection agent was Lipofectamine 2000. Transfection was carried out approximately 24 hours after plate seeding.

Stressor

Cells were stressed by means of serum starvation. This consisted of placing them in DMEM supplemented with only 0.1% FBS for approximately 12 hours. Stress exposure is useful for examining if MK-STYX's cellular effects are altered when cells are exposed to adverse conditions.

Lysate preparation

After serum starvation, cells were lysed in lysis buffer and lysates were prepared to analyze cellular constituents. The lysis buffer had the following formulation: 50mM of the buffer HEPES, 150mM NaCl, 10mM of NaF as a serine/threonine phosphatase inhibitor, 10% glycerol to increase sample solubility, 1% NP-40 as a detergent, pH set to 7.5 and phosphatase and protease inhibitor tablets were added immediately before use. The phosphatase inhibitor tablets were Roche's PhosSTOP, which inhibit acid and alkaline phosphatases, serine/threonine phosphatases, and protein tyrosine phosphatases. The protease inhibitor tablets were Roche's cOmplete EDTA-free, which inhibit serine and cysteine proteases. Lysates were sonicated and subjected to centrifugation. Sonication was carried out in 10 bursts of 1 second each, at an amplitude setting

of 30. Centrifugation occurred at 14,000 x gravity at 4°C for 15 minutes. The protein concentration of lysates was determined using Pierce's Detergent Compatible Bradford Assay.

Protein expression analysis

Lysate sample preparation

Lysate samples were prepared to enable downstream analysis of changes in protein expression levels with Western blot analysis. To do this, 50 µg of protein sample was added to sample tubes, which were also brought to a 1X concentration of sample loading buffer to visualize proteins as they resolved through the gel. Also, 100 mM of dithiothreitol (DTT) was included to promote the proteins' run through the gel, and ddH_2O was added to bring samples to their final volume.

SDS-PAGE

To enable separation of proteins by apparent mass, a necessary step for their identification, acrylamide gels were prepared. The gels contained 10% acrylamide to create pores through which the proteins would run and separate. For the resolving portion of the gel, an additional component was 1.5M Tris Buffer solution at pH 8.8. Also, a 10% sodium dodecyl sulfate (SDS) solution was included to keep proteins denatured as they ran through the gel. Then, 10% ammonium persulfate (APS) was used to catalyze gel polymerization. Additionally, TEMED (N, N, N′, N′-tetramethylethane-1,2-diamine) was used to catalyze polymerization as well. For the stacking portion of the gel, a 1M Tris

Buffer solution set to pH 6.8 was used instead of the 1.5M Tris Buffer solution set to pH 8.8.

Samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to separate proteins by apparent mass, in which the heaviest proteins would move the least down the gel and the lightest would move the most.

The proteins contained in the gels were transferred to PVDF [poly (vinylidene fluoride)] membranes to enable Western blot analysis. Transfer was performed with Bio-Rad's Transblot system.

No-Stain application

To enable total protein quantification and normalization, the No-Stain reagent from Invitrogen (A44717) was applied to the membranes after transfer.

Immunoblotting

After No-Staining, PVDF membranes were blocked with 5% non-fat dry milk (NFDM) diluted in 1X tris-buffered saline tween (TBST).

To detect possible changes in phosphoserine21/9GSK3α/β, an antibody that recognizes these two epitopes was used at a 1:500 concentration (Abcam, ab 226778). For observing possible changes in GSK3α/β, an antibody that picks up epitopes on these whole proteins was used at a 1:1000 concentration (Abcam, ab 62368). To prove transfection with MK-STYX and MK-STYX$_{\text{active mutant}}$ overexpression constructs, an antibody that finds the FLAG epitope was used at a 1:1000 concentration (Sigma-Aldrich).

To detect possible changes in phosphotyrosine279/216GSK3α/β, an antibody against these two epitopes was used at a 1:1000 concentration (Abcam, ab 75745). Following this, membranes were stripped based on Abcam's Mild Stripping Protocol.

To detect β-tubulin, which served as a loading control, a cognate antibody was used at a 1:1000 concentration (Invitrogen).

An anti-rabbit-HRP (horse radish peroxidase) secondary antibody was used to detect the primary antibodies that recognized phosphotyrosine279/216GSK3α/β, phosphoserine21/9GSK3α/β, GSK3α/β and β-tubulin. For the FLAG antibodies, an anti-mouse-HRP secondary antibody was used to visualize them. All secondary antibodies were used at a 1:1000 concentration. The HRP conjugated to the secondary antibodies reacts with an enhanced chemiluminescence (ECL) substrate to generate a detectable light signal proportional to the amount of protein of interest present.

Western blot signal quantification

The Western blot signals generated were quantified with an iBright 1500 imaging system from Thermo Fisher Scientific. Specifically, the iBright has a charge-coupled device (CCD) camera that picked up the signals and a supporting software suite that produced corresponding densitometry data. Additionally, the No-Stain reagent generated a fluorescent signal with the Western blot that was used to quantify total protein levels and then normalize between lanes.

Coimmunoprecipitation

To determine possible protein-protein interactions, coimmunoprecipitation or CoIP was used. To begin, lysate samples containing 500 μg of protein were diluted to a final volume of 1 mL in lysis buffer in separate tubes. Then, 20 μL of Protein G Sepharose beads (cytiva) were added to samples and then put on a nutating mixer and centrifuged at 14,000 x gravity at 4°C for 5 minutes to preclear samples. This was followed by adding 5 μL of anti-FLAG antibody to sample tubes and then the nutating mixer was used. After this, 25μL of Protein G Sepharose beads were added followed by time on the nutating mixer. Subsequently, samples were centrifuged at 14,000 x gravity at 4°C for 15 minutes to pull down FLAG-containing proteins and their interactors. Supernatant was then aspirated off. Then, samples were washed 4 times with lysis buffer. Each wash involved adding 1 mL of lysis buffer, pipetting samples up and down to mix, and centrifugation at 17,000 x gravity for 1 minute. After the last wash, supernatant was aspirated off and 50 μL of 6X sample loading buffer followed by 5 μL of 1M DTT was added for a final DTT concentration of about 100mM. Samples were then centrifuged at 17,000 x gravity for 1 minute, boiled for 3-5 minutes at 100°C, and centrifuged at 17,000 x gravity for 1 minute again. Then, 40 μL of the eluent from each sample was loaded into an SDS-PAGE gel as described above. Whole lysate gels were included to show that the input samples contained the proteins necessary for a valid CoIP. These were prepared in an identical manner to the “Lysate sample preparation” procedure described above.

Chapter 3: Results

Research question 1: Does MK-STYX overexpression cause a change in phosphorylation on serine 21 or 9 of GSK3α or GSK3β?

To test the hypothesis that MK-STYX affected phosphorylation on serine 21 or 9 of GSK3α or GSK3β, respectively, Western blots of protein lysates from MK-STYX-expressing HEK-293 cells were carried out, using antibodies that recognized these serine phosphorylation sites (Fig. 7). Since phosphorylation at these sites inhibits GSK3 kinase activity, it was reasoned that analyzing MK-STYX's impacts on these sites would help determine if MK-STYX is a regulator of GSK3.

First, it was considered if MK-STYX overexpression would affect the phosphorylation of serine 21 of GSK3α. Quantification of replicate Western blots by densitometry showed that MK-STYX did not significantly change phosphorylation at this site (Figs. 7 and 8). Since serine 21 of GSK3α is an inhibitory site, it can be concluded that MK-STYX does not affect GSK3 kinase activity through this site.

It was also hypothesized that MK-STYX would affect serine 9 phosphorylation of GSK3β. Intriguingly, Western blot densitometry showed that MK-STYX overexpression leads to a statistically significant reduction in

phosphorylation at this site (Figs. 7 and 9). Specifically, MK-STYX-transfected cells demonstrated about 40% less phosphoserine9GSK3β than pMT2-transfected controls in normal serum conditions. The observed reduction in phosphorylation at this inhibitory phosphorylation site can be expected to increase GSK3β kinase activity. This would lead to higher phosphorylation on GSK3β substrates.

To obtain a more complete picture of MK-STYX's impact on GSK3's inhibitory phosphorylation, serine 21 and serine 9 phosphorylation of GSK3α and GSK3β were considered together (i.e. total serine 21/9 phosphorylation). It is clear from the results of Western blot densitometry that MK-STYX did not significantly change total inhibitory serine phosphorylation (Figs. 7 and 10). This argues that MK-STYX does not increase or decrease total GSK3 kinase activity.

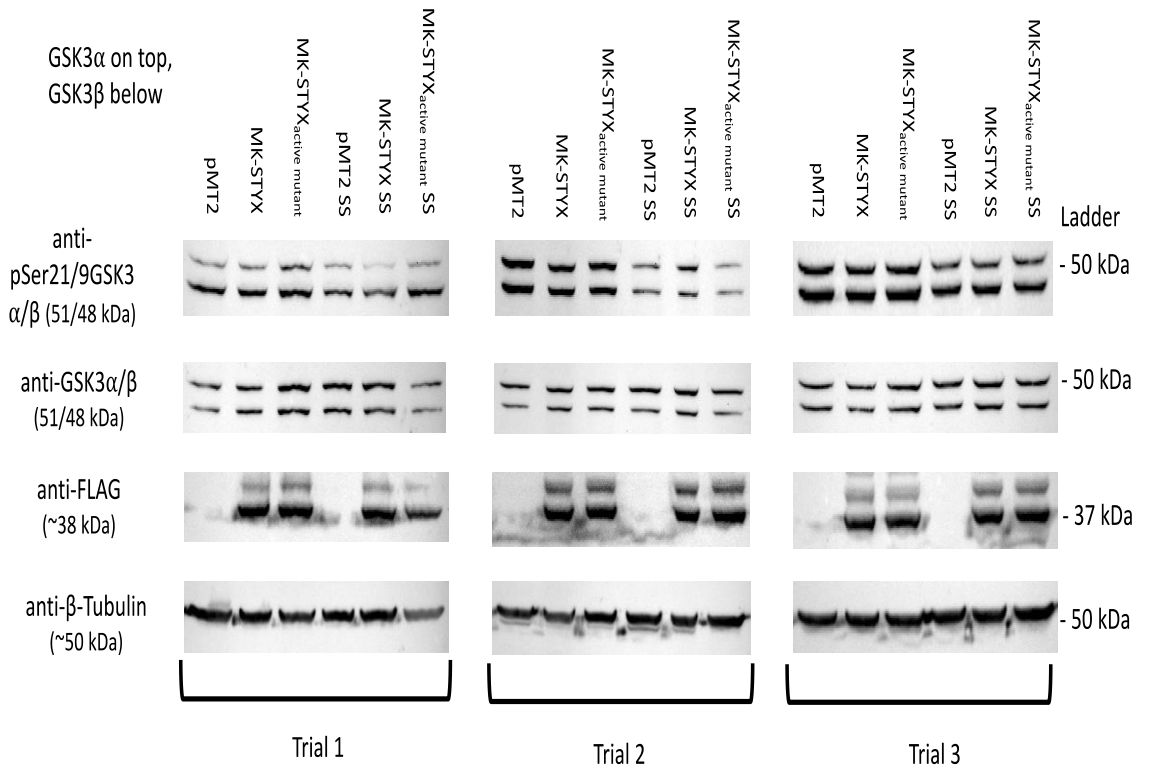


Figure 7: The impact of MK-STYX overexpression on serine 21 or 9 phosphorylation of GSK3α or GSK3β. Western blots for trials 1, 2, and 3. This figure shows the Western blots which were used to determine changes in phosphorylation of serine 21 or 9 of GSK3α or GSK3β, respectively. HEK-293 cells were grown in 6 separate tissue culture plates. Then, 2 plates were transfected with pMT2 plasmids, 2 with pMT2-FLAG-MK-STYX-FLAG plasmids, and 2 with pMT2-FLAG-MK-STYX$_{\text{active mutant}}$-FLAG plasmids. Following this, 1 plate from each transfection treatment type was subjected to stress by means of serum starvation (SS). Lysates were prepared from each plate and appropriate amounts of lysate samples were used in Western blotting as described earlier. First, blotting membranes were probed with an antibody against phosphoserine 21 and 9 GSK3α and GSK3β. Membranes were stripped. Then they were probed for GSK3α and GSK3β. Membranes were stripped again. Then they were probed for FLAG. Membranes were stripped again. Finally, they were probed for β-tubulin as a loading control.

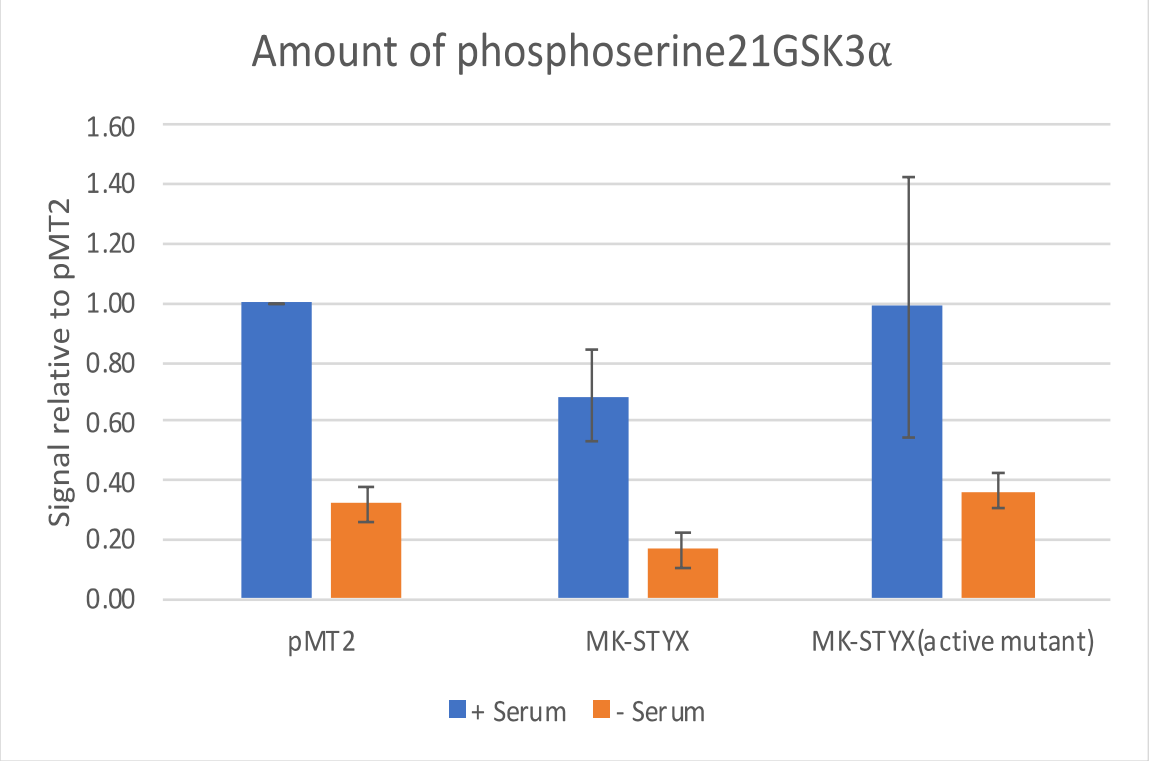


Figure 8: There is no significant change in phosphoserine21GSK3α in the presence of MK-STYX constructs.
Plates containing HEK-293 cells were transfected with pMT2, pMT2-FLAG-MK-STYX-FLAG, or pMT2-FLAG-MK-STYX$_{\text{active mutant}}$-FLAG and either maintained in normal serum media (+ Serum) or subjected to serum starvation (- Serum). Western blotting (See Fig.7) coupled with normalization was used to generate densitometry results. First, sample signals were subject to No-Stain total protein normalization. Then, signals were normalized relative to pMT2 in each trial to enable comparison between trials. Error bars are +/- SEM and 3 biological replicates were performed. A two-tailed paired t-test was used to test for significant differences between transfection treatments. The threshold for significance was set at $p < .05$. The p-value comparing pMT2 and MK-STYX in normal serum conditions was 0.1762.

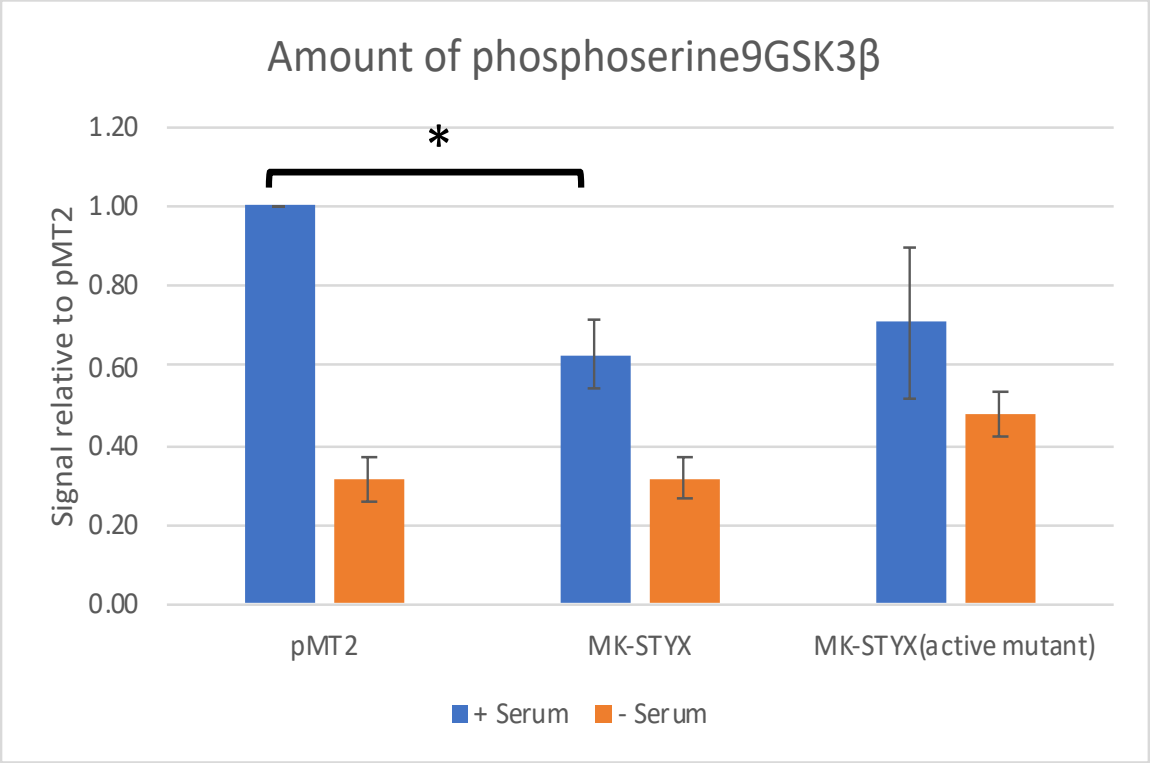


Figure 9: MK-STYX significantly decreases the amount of phosphoserine9GSK3β.
Plates containing HEK-293 cells were transfected with pMT2, pMT2-FLAG-MK-STYX-FLAG, or pMT2-FLAG-MK-STYX$_{\text{active mutant}}$-FLAG and either maintained in normal serum media (+ Serum) or subjected to serum starvation (- Serum). Western blotting (See Fig. 7) coupled with normalization was used to generate densitometry results. First, sample signals were subject to No-Stain total protein normalization. Then, signals were normalized relative to pMT2 in each trial to enable comparison between trials. Error bars are +/-SEM and 3 biological replicates were performed. A two-tailed paired t-test was used to test for significant differences between transfection treatments. The threshold for significance was set at $p < .05$. Brackets identify the groups being compared and asterisks show significance. The p-value comparing pMT2 and MK-STYX in normal serum conditions was 0.0497.

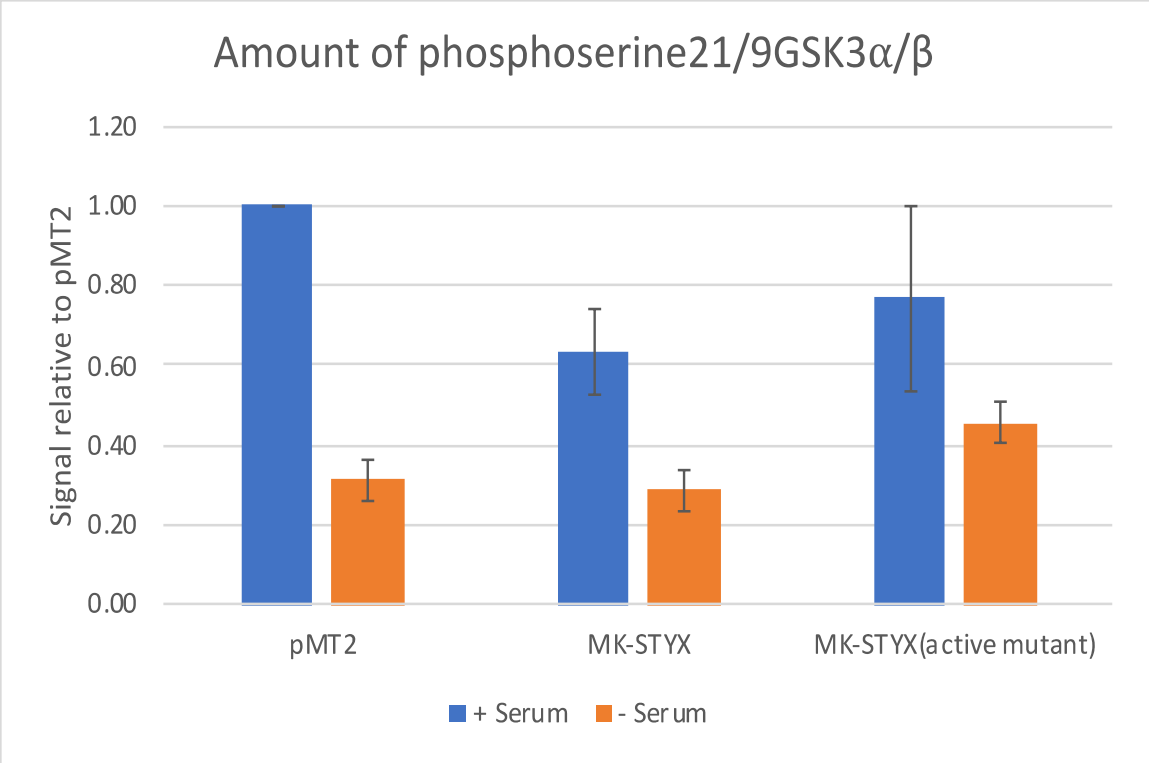


Figure 10: There is no significant change in phosphoserine21/9GSK3α/β in the presence of MK-STYX constructs.
Plates containing HEK-293 cells were transfected with pMT2, pMT2-FLAG-MK-STYX-FLAG, or pMT2-FLAG-MK-STYX$_{\text{active mutant}}$-FLAG and either maintained in normal serum media (+ Serum) or subjected to serum starvation (- Serum). Western blotting coupled with normalization was used to generate densitometry results. First, sample signals were subject to No-Stain total protein normalization. Then, signals were normalized relative to pMT2 in each trial to enable comparison between trials. Error bars are +/- SEM and 3 biological replicates were performed. A two-tailed paired t-test was used to test for significant differences between transfection treatments. The threshold for significance was set at $p < .05$. The p-value comparing pMT2 and MK-STYX in normal serum conditions was 0.073.

Research question 2: Does MK-STYX overexpression cause a change in phosphorylation on tyrosine 279 or 216 of GSK3α or GSK3β?

The second hypothesis, that MK-STYX would change phosphorylation on the kinase-activating sites tyrosine 279 or 216 of GSK3α or GSK3β, respectively, was tested by means of Western blotting of protein lysates from MK-STYX-expressing HEK-293 cells. Antibodies that recognize these tyrosine phosphorylation sites were used (Fig. 11). Because phosphorylation at these sites increases GSK3 kinase activity, analyzing the impacts of MK-STYX here also helped determine if MK-STYX regulates GSK3.

It was first considered if MK-STYX overexpression changed phosphorylation on tyrosine 279 of GSK3α. Western blot densitometry results made clear that MK-STYX overexpression did not change the amount of phosphorylation on tyrosine 279 of GSK3α (Figs. 11. and 12). This means that MK-STYX did not change GSK3α kinase activity by modulating this kinase-activating site.

It was then considered if MK-STYX overexpression would affect phosphorylation on tyrosine 216 of GSK3β (Figs. 11 and 13). Western blot densitometry showed that MK-STYX did not significantly change phosphorylation

here, which demonstrates that MK-STYX does not activate or deactivate GSK3β by regulating this kinase-activating amino acid residue.

In order to visualize MK-STYX's overall impact on activating tyrosine phosphorylation of GSK3, tyrosine 279 of GSK3α and tyrosine 216 of GSK3β were examined together. Western blot densitometry showed that overexpression of MK-STYX did not significantly change phosphorylation on these sites (Figs. 11 and 14). Such a finding implies that MK-STYX does not change total GSK3 kinase activity.

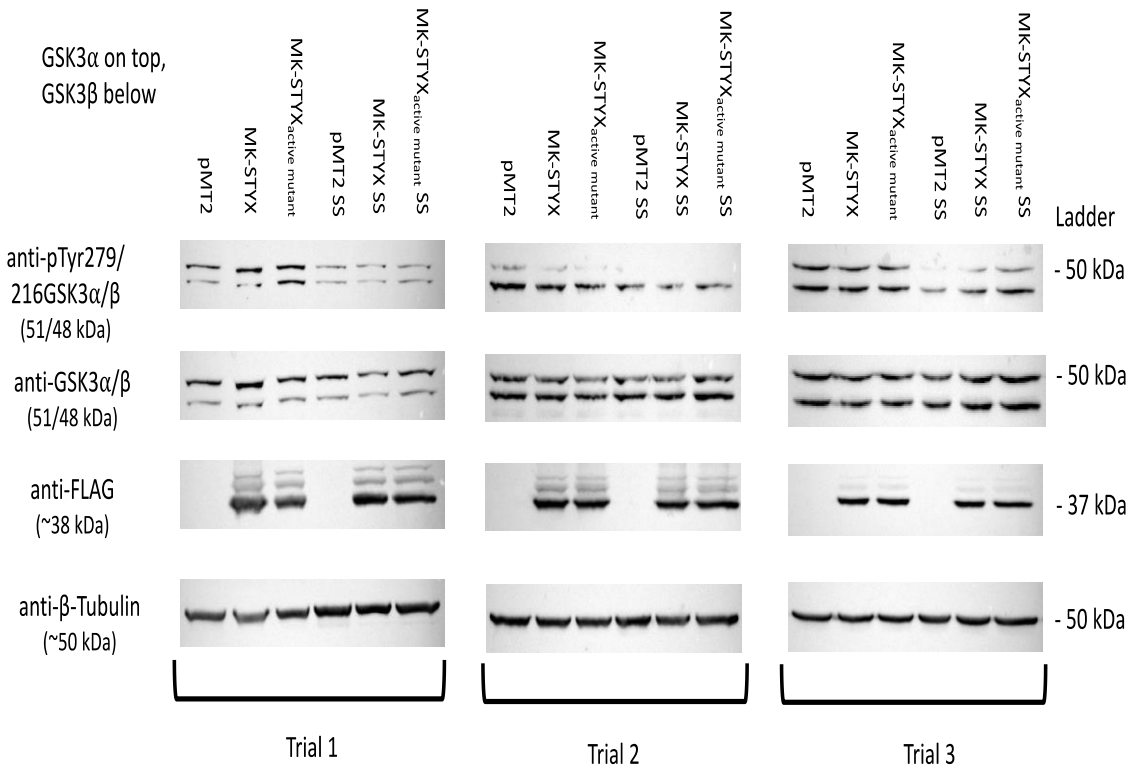


Figure 11: The impact of MK-STYX overexpression on tyrosine 279 or 216 phosphorylation of GSK3α or GSK3β. Also, the impact of MK-STYX overexpression on the amount of GSK3α or GSK3β. Western blots for trials 1, 2, and 3.

This figure shows the Western blots which were used to determine changes in phosphorylation of tyrosine 279 or 216 of GSK3α or GSK3β as well as changes in the total amount of GSK3α and GSK3β. HEK-293 cells were grown in 6 separate tissue culture plates. Then, 2 plates were transfected with pMT2 plasmids, 2 with pMT2-FLAG-MK-STYX-FLAG plasmids, and 2 with pMT2-FLAG-MK-STYX$_{\text{active mutant}}$-FLAG plasmids. Following this, 1 plate from each transfection treatment type was subjected to stress by means of serum starvation (SS). Lysates were prepared from each plate and appropriate amounts of lysate samples were used in Western blotting as described earlier. First, blotting membranes were probed with an antibody against phosphoserine 21 and 9 GSK3α and GSK3β. Membranes were stripped. Then they were probed for GSK3α and GSK3β. Membranes were stripped again. Then they were probed for FLAG. Membranes were stripped again. Finally, they were probed for β-tubulin as a loading control.

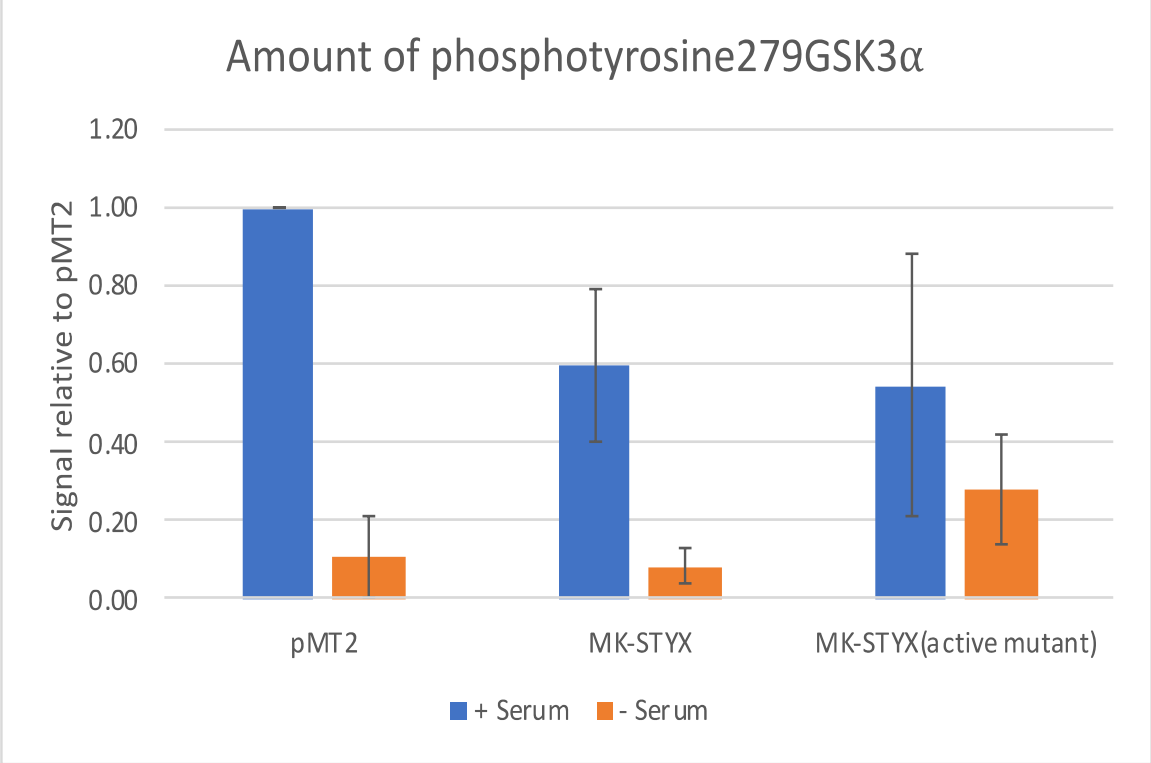


Figure 12: There is no significant change in phosphotyrosine279GSK3α in the presence of MK-STYX constructs.
Plates containing HEK-293 cells were transfected with pMT2, pMT2-FLAG-MK-STYX-FLAG, or pMT2-FLAG-MK-STYX$_{\text{active mutant}}$-FLAG and either maintained in normal serum media (+ Serum) or subjected to serum starvation (- Serum). Western blotting coupled with normalization was used to generate densitometry results. First, sample signals were subject to No-Stain total protein normalization. Then, signals were normalized relative to pMT2 in each trial to enable comparison between trials. Error bars are +/- SEM and 3 biological replicates were performed. A two-tailed paired t-test was used to test for significant differences between transfection treatments. The threshold for significance was set at $p < .05$. The p-value comparing pMT2 and MK-STYX in normal serum conditions was 0.1784.

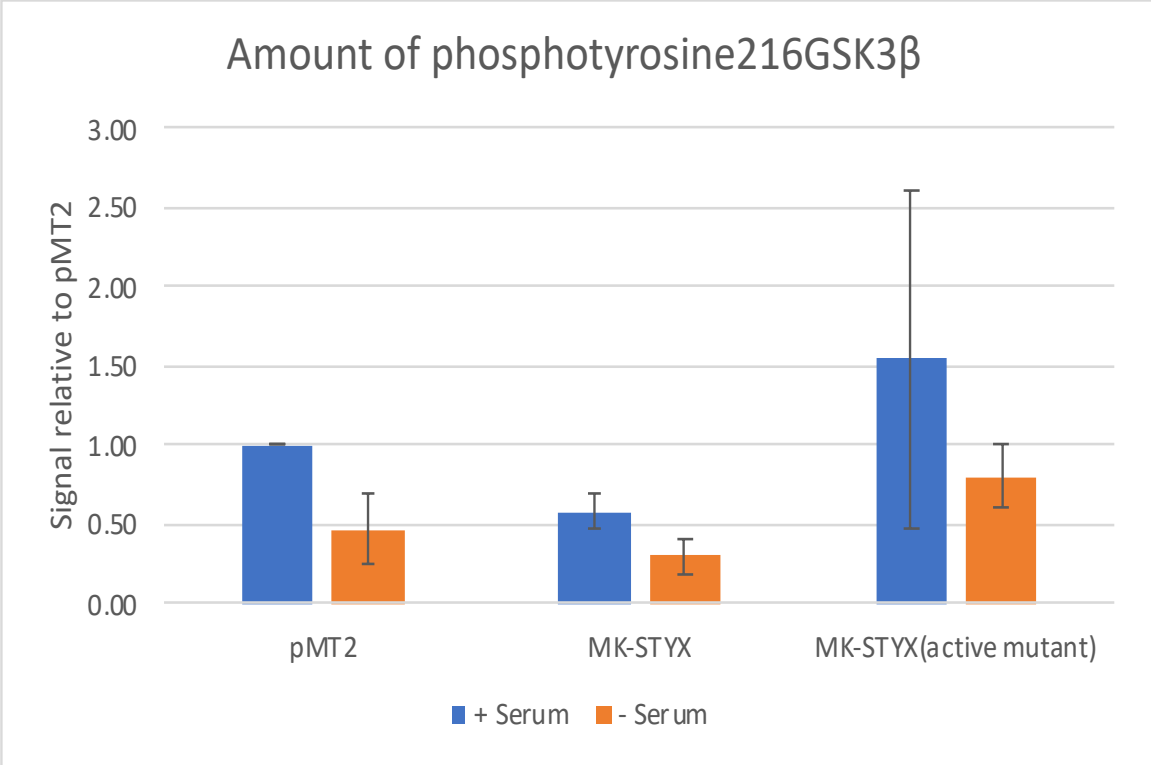


Figure 13: There is no significant change in phosphotyrosine216GSK3β in the presence of MK-STYX constructs
Plates containing HEK-293 cells were transfected with pMT2, pMT2-FLAG-MK-STYX-FLAG, or pMT2-FLAG-MK-STYX$_{\text{active mutant}}$-FLAG and either maintained in normal serum media (+ Serum) or subjected to serum starvation (- Serum). Western blotting coupled with normalization was used to generate densitometry results. First, sample signals were subject to No-Stain total protein normalization. Then, signals were normalized relative to pMT2 in each trial to enable comparison between trials. Error bars are +/- SEM and 3 biological replicates were performed. A two-tailed paired t-test was used to test for significant differences between transfection treatments. The threshold for statistical significance was set at $p < .05$. The p-value comparing pMT2 and MK-STYX in normal serum conditions was 0.058.

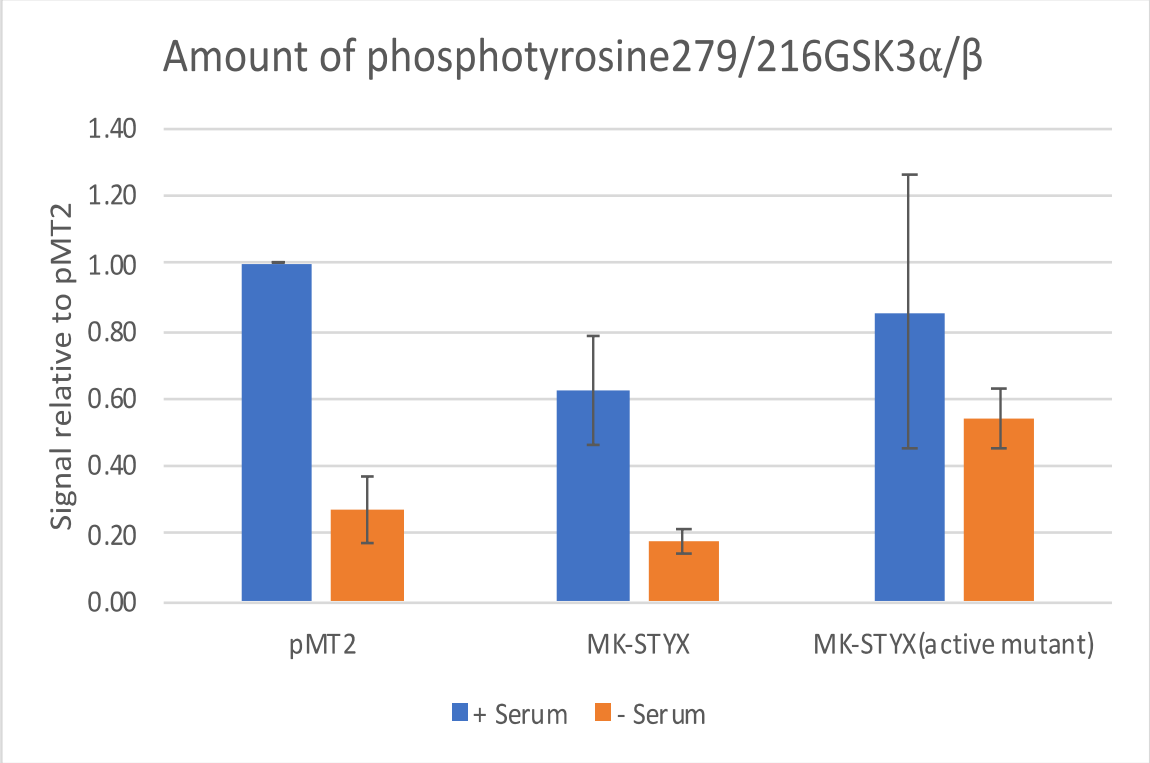


Figure 14: There is no significant change in phosphotyrosine279/216GSK3α/β in the presence of MK-STYX constructs.
Plates containing HEK-293 cells were transfected with pMT2, pMT2-FLAG-MK-STYX-FLAG, or pMT2-FLAG-MK-STYX$_{\text{active mutant}}$-FLAG and either maintained in normal serum media (+ Serum) or subjected to serum starvation (- Serum). Western blotting coupled with normalization was used to generate densitometry results. First, sample signals were subject to No-Stain total protein normalization. Then, signals were normalized relative to pMT2 in each trial to enable comparison between trials. Error bars are +/- SEM and 3 biological replicates were performed. A two-tailed paired t-test was used to test for significant differences between transfection treatments. The threshold for statistical significance was set at $p < .05$. The p-value comparing pMT2 and MK-STYX in normal serum conditions was 0.1449.

Research question 3: Does MK-STYX overexpression cause a change in expression of GSK3α or GSK3β?

The third hypothesis of this thesis considered if MK-STYX overexpression would change the expression of GSK3α or GSK3β. To test this, Western blots using antibodies that recognized GSK3α or GSK3β were performed (Fig. 11). These were the same Western blot membranes used to detect tyrosine 279 and 216 phosphorylation (Figs. 11-14), only the membranes were stripped and then probed for GSK3α and GSK3β.

First, the total expression of GSK3α was examined. Interestingly, it was found using Western blot densitometry that overexpression of MK-$STYX_{active\ mutant}$ decreased GSK3α expression (Figs. 11 and 15). This suggests that MK-$STYX_{active\ mutant}$ reduces the amount of phosphorylation of GSK3α substrates by reducing GSK3α itself.

The next part of this hypothesis investigated how MK-STYX overexpression changed the amount of GSK3β. The overexpression of MK-$STYX_{active\ mutant}$ was found to increase the amount of GSK3β in serum starved conditions (Figs. 11 and 16). This implies that in the presence of stress, MK-$STYX_{active\ mutant}$ increases phosphorylation of GSK3β targets by boosting the levels of GSK3β.

The last part of this hypothesis called for the determination of MK-STYX's impact on the expression of GSK3α and GSK3β combined, i.e., whole GSK3.

Excitingly, Western blot densitometry showed that overexpression of MK-STYX$_{\text{active mutant}}$ led to a decrease in total GSK3 (Figs. 11 and 17). One could infer that MK-STYX$_{\text{active mutant}}$ decreases phosphorylation of GSK3 substrates by reducing the amount of GSK3, the kinase carrying out the phosphorylation.

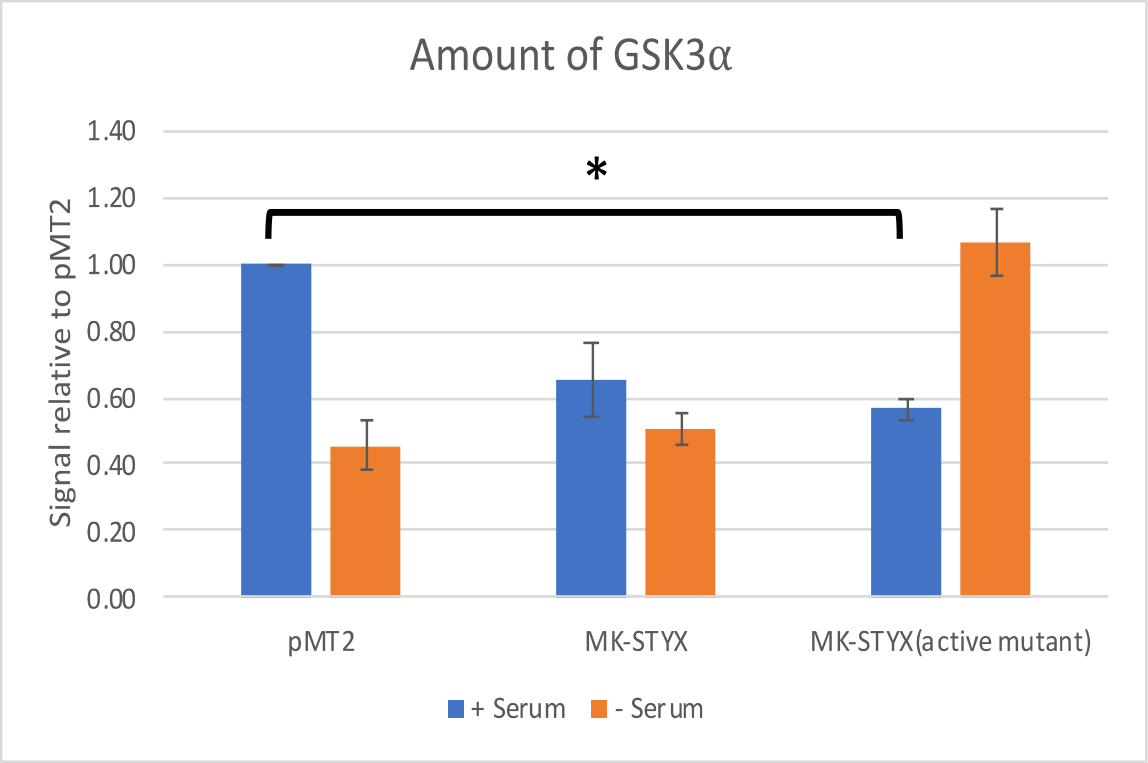


Figure 15: There is significantly less GSK3α with MK-STYX$_{\text{active mutant}}$ overexpressed.
Plates containing HEK-293 cells were transfected with pMT2, pMT2-FLAG-MK-STYX-FLAG, or pMT2-FLAG-MK-STYX$_{\text{active mutant}}$-FLAG and either maintained in normal serum media (+ Serum) or subjected to serum starvation (- Serum). Western blotting coupled with normalization was used to generate densitometry results. First, sample signals were subject to No-Stain total protein normalization. Then, signals were normalized relative to pMT2 in each trial to enable comparison between trials. Error bars are +/- SEM and 3 biological replicates were performed. A two-tailed paired t-test was used to test for significant differences between transfection treatments. The threshold for statistical significance was set at $p < .05$. Brackets identify the groups being compared and asterisks show statistical significance. The p-value comparing pMT2 and MK-STYX$_{\text{active mutant}}$ in normal serum conditions was 0.0057. Also, the p-value comparing pMT2 and MK-STYX in normal serum conditions was 0.0915.

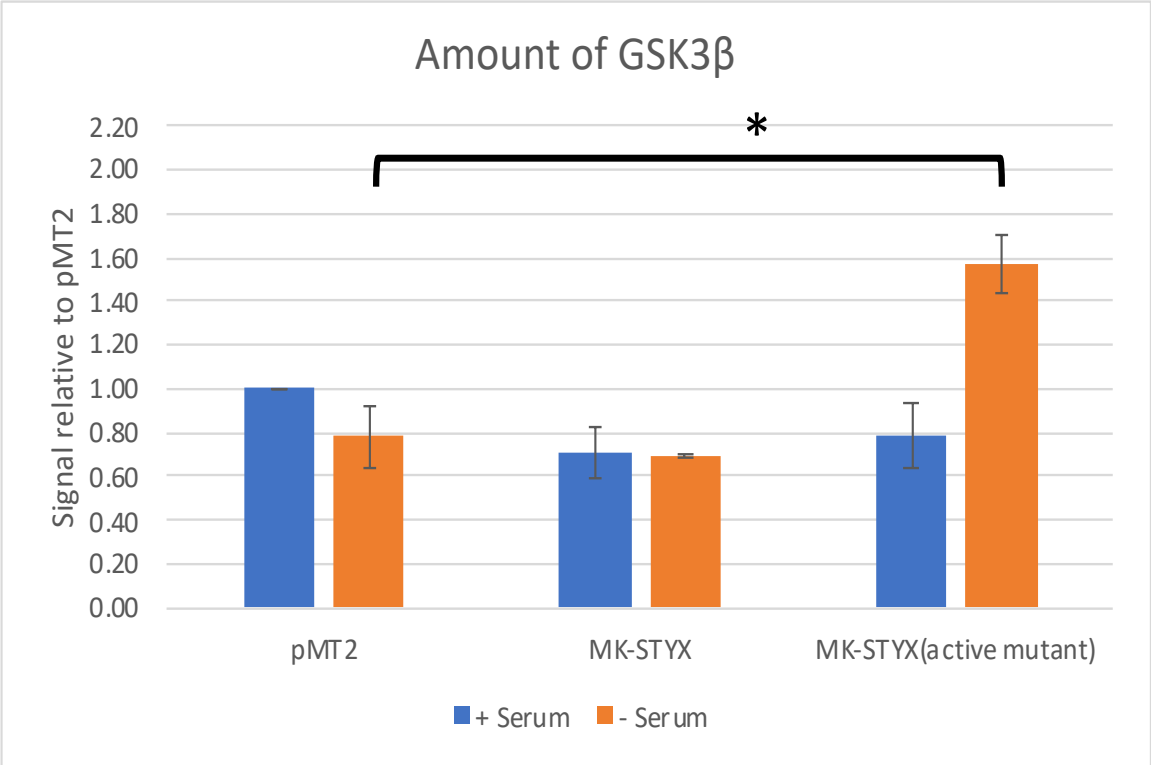


Figure 16: There is significantly more GSK3β with MK-STYX$_{\text{active mutant}}$ overexpressed in serum starved conditions.
Plates containing HEK-293 cells were transfected with pMT2, pMT2-FLAG-MK-STYX-FLAG, or pMT2-FLAG-MK-STYX$_{\text{active mutant}}$-FLAG and either maintained in normal serum media (+ Serum) or subjected to serum starvation (- Serum). Western blotting coupled with normalization was used to generate densitometry results. First, sample signals were subject to No-Stain total protein normalization. Then, signals were normalized relative to pMT2 in each trial to enable comparison between trials. Error bars are +/- SEM and 3 biological replicates were performed. A two-tailed paired t-test was used to test for significant differences between transfection treatments. The threshold for statistical significance was set at $p < .05$. Brackets identify the groups being compared and asterisks show statistical significance. The p-value comparing pMT2 and MK-STYX$_{\text{active mutant}}$ in serum starved conditions was 0.0134. Also, the p-value comparing pMT2 and MK-STYX in normal serum conditions was 0.141.

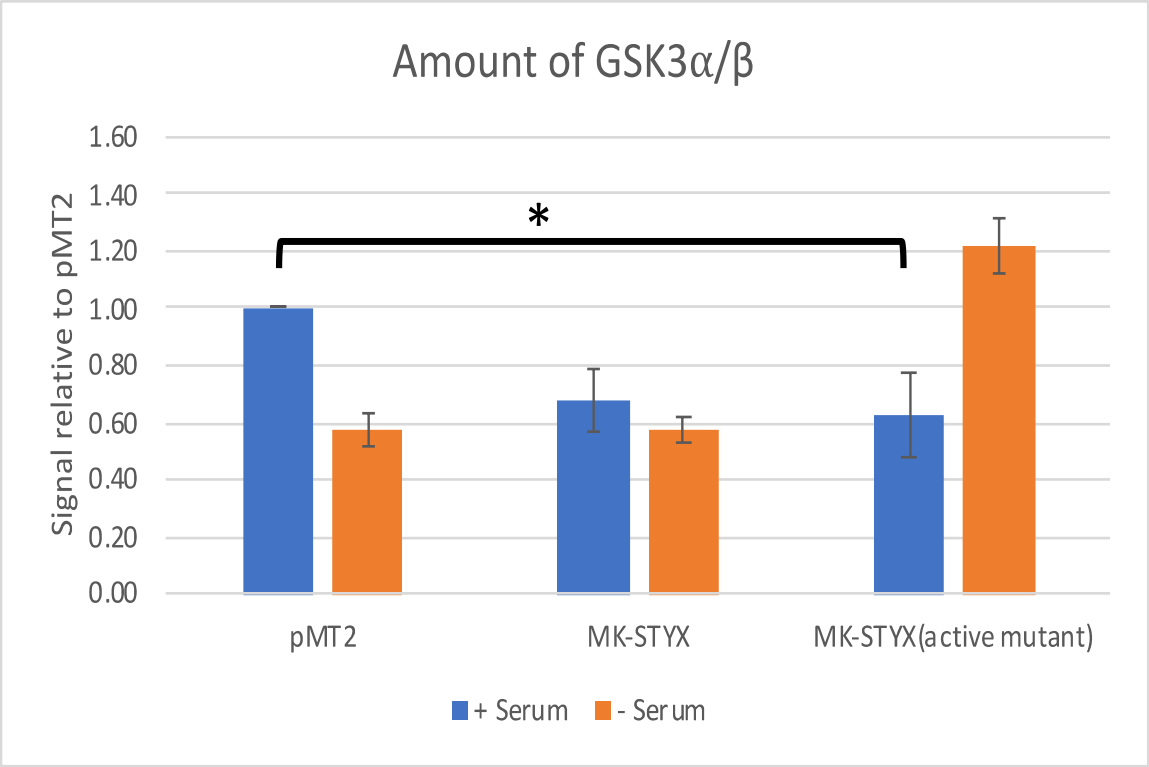


Figure 17: There is significantly less GSK3α/β with MK-STYX$_{\text{active mutant}}$ overexpressed.
Plates containing HEK-293 cells were transfected with pMT2, pMT2-FLAG-MK-STYX-FLAG, or pMT2-FLAG-MK-STYX$_{\text{active mutant}}$-FLAG and either maintained in normal serum media (+ Serum) or subjected to serum starvation (- Serum). Western blotting coupled with normalization was used to generate densitometry results. First, sample signals were subject to No-Stain total protein normalization. Then, signals were normalized relative to pMT2 in each trial to enable comparison between trials. Error bars are +/- SEM and 3 biological replicates were performed. A two-tailed paired t-test was used to test for significant differences between transfection treatments. The threshold for statistical significance was set at $p < .05$. Brackets identify the groups being compared and asterisks show statistical significance. Also, the p-value comparing pMT2 and MK-STYX$_{\text{active mutant}}$ in normal serum conditions was 0.0034. Also, the p-value comparing pMT2 and MK-STYX in normal serum conditions was 0.0947.

Research question 4: Does MK-STYX or the catalytically active MK-STYX$_{\text{active mutant}}$ interact with GSK3β?

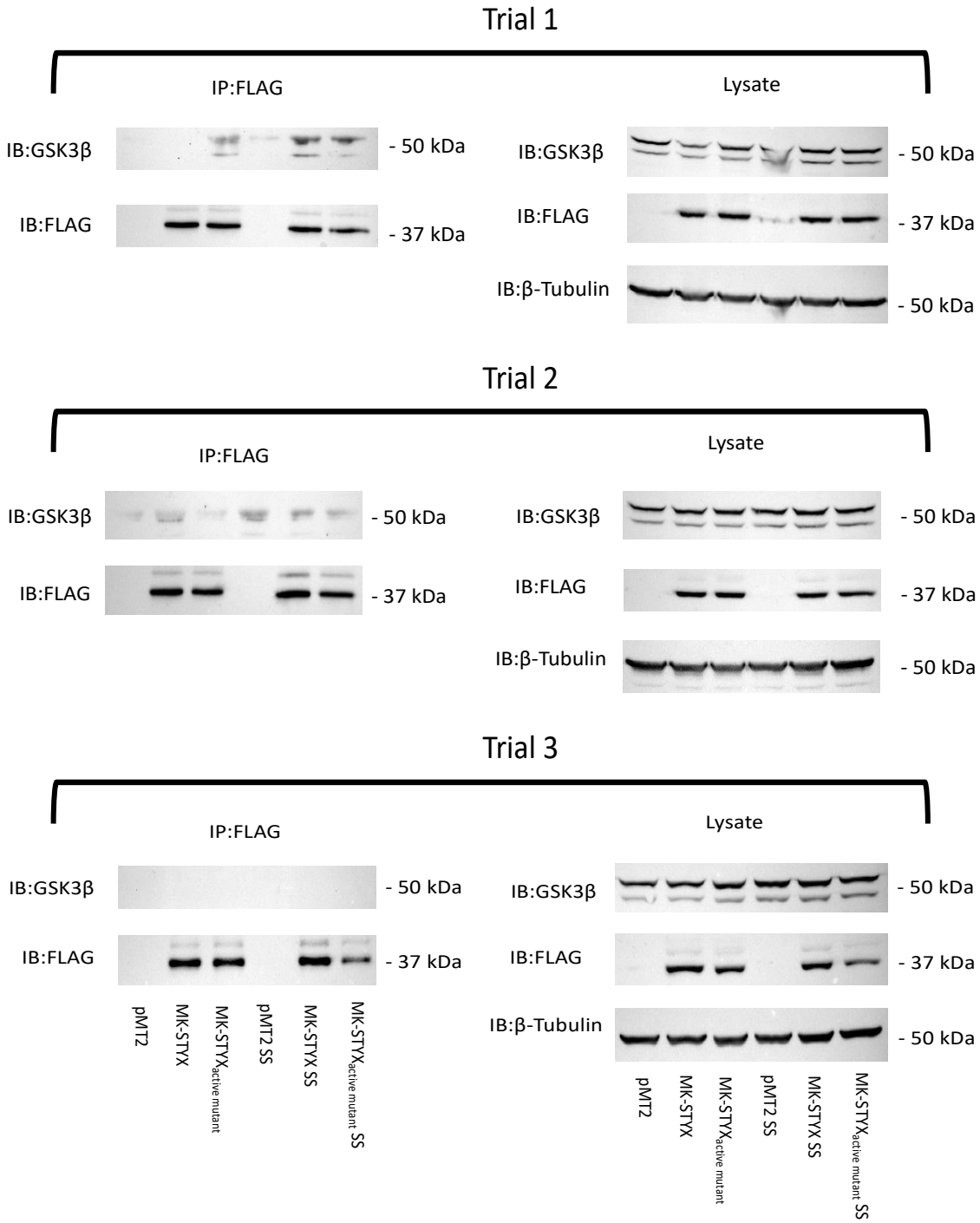
To test the hypothesis that either MK-STYX or MK-STYX$_{\text{active mutant}}$ interacted with GSK3β, coimmunoprecipitation followed by Western blot, referred to as immunoblot (IB) here, was utilized. Specifically, an anti-FLAG antibody was used to immunoprecipitate (IP) samples, while an anti-GSK3β antibody tested if GSK3β was pulled down via immunoblot (IB) (Fig. 18).

The three trials overall demonstrated that neither MK-STYX nor MK-STYX$_{\text{active mutant}}$ coimmunoprecipitated with GSK3β. This supports the notion that MK-STYX does not interact with GSK3β through a protein-protein bond. Furthermore, it is unlikely that MK-STYX will directly modulate GSK3β by blocking or enhancing its access to substrates since MK-STYX does not form a complex with this kinase.

On a technical note, trials 1 and 2 showed some equivocal evidence of protein-protein interactions while trial 3 clearly showed none. It is important to note that the washing steps in trial 3 were the most thorough of all the trials. Additionally, trials 1 and 2 both improbably show an interaction in pMT2 negative control lanes. These facts argue that trial 3 is the most representative, and that putative protein-protein interactions in trials 1 and 2 are non-specific.

CoIP of MK-STYX or MK-STYX$_{\text{active mutant}}$ and GSK3β

IP:FLAG
IB:GSK3β



(Figure legend on next page)

Figure 18: There is no interaction between MK-STYX or MK-STYX$_{\text{active mutant}}$ and GSK3β.
Plates containing HEK-293 cells were transfected with pMT2, pMT2-FLAG-MK-STYX-FLAG, or pMT2-FLAG-MK-STYX$_{\text{active mutant}}$-FLAG and either maintained in normal serum media or subjected to serum starvation (SS). On the left, coimmunoprecipitated samples are shown, and on the right, input from whole lysate is shown. The FLAG tag was used to immunoprecipitate protein complexes. These were then subject to immunoblotting against GSK3β to determine if GSK3β formed a complex with FLAG-tagged proteins. The membrane was then stripped and probed for FLAG to show that FLAG-tagged proteins are indeed in the immunoprecipitated pull-down. On the right for the raw lysate, GSK3β was probed for. The membrane was then stripped and FLAG was probed for. Finally, membranes were stripped and probed again for β-tubulin. It was important to look for GSK3β, FLAG, and β-tubulin in the lysate input to demonstrate the validity and relevance of the CoIP. Three biological replicates were performed, represented as trials 1, 2, and 3 above.

Chapter 4: Discussion and Future Directions

This thesis set out to explore the pseudophosphatase MK-STYX's connection to the kinase GSK3. This work examined how MK-STYX could alter regulatory phosphorylation of GSK3, total expression of GSK3, and if MK-STYX interacted with GSK3 by directly or indirectly binding to it. Results described here demonstrate that MK-STYX and the MK-STYX$_{\text{active mutant}}$ exert some intriguing effects on GSK3. Some major findings are highlighted below (Fig. 19).

MK-STYX overexpression leads to a statistically significant decrease in phosphorylation on serine 9 of GSK3β (Fig. 19). Since serine 9 phosphorylation is an inhibitory modification, this means that MK-STYX inhibits GSK3β's inhibition, or in other words MK-STYX activates GSK3β. As previously mentioned, it is reasonable to think that this would lead to an increase in phosphorylation of GSK3β substrates (Fig. 20). Some important GSK3β substrates are tau and β-catenin (Wagner et al., 1996; Wu et al., 2010) (Fig. 20). A basic level of tau phosphorylation is consistent with healthy cellular functioning, but hyperphosphorylation impairs tau function and is associated with Alzheimer's disease (Wagner et al., 1996). If MK-STYX increases GSK3β activity, then the result could well be increased tau phosphorylation or even tau hyperphosphorylation. An important first step in assessing this speculative link would be a GSK3β kinase activity assay. This would directly show if MK-STYX is upregulating GSK3β activity as the serine 9 phosphorylation decrease indicates.

If the kinase activity assay showed GSK3β activation, it would then be important to test for possible tau hyperphosphorylation using Western blotting.

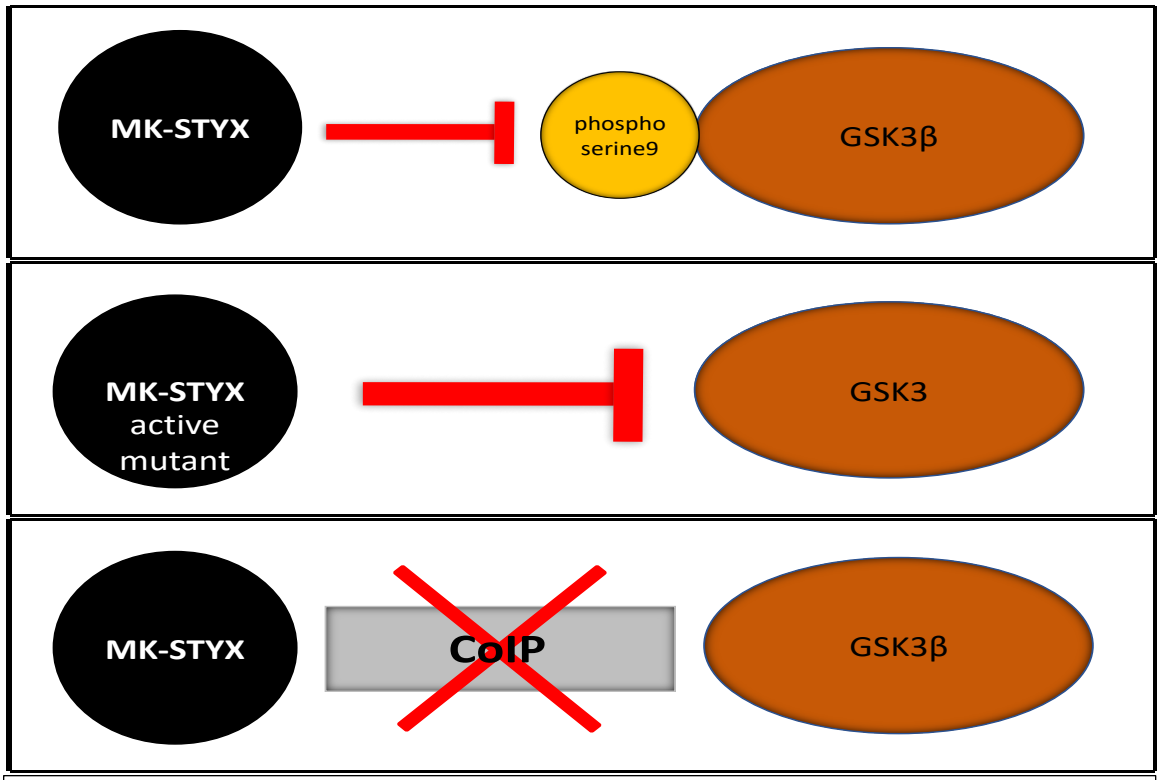


Figure 19: A schematic showing the major findings of this thesis
At the top, one can see that MK-STYX overexpression reduces phosphorylation of serine 9 on GSK3β.
In the middle, observe how MK-STYX$_{\text{active mutant}}$ lowers total GSK3 (i.e., GSK3α/β) expression.
Below, it is shown that MK-STYX does not interact with GSK3β.

β-catenin is a proto-oncogene that is central to Wnt signaling and negatively regulated by GSK3β. High β-catenin levels can drive oncogenic cellular changes like cell growth and proliferation (Shang et al., 2017). In light of MK-STYX's decrease of serine 9 phosphorylation on GSK3β, it is feasible that MK-STYX could increase GSK3β's phosphorylation of β-catenin. Increased β-

catenin phosphorylation promotes its polyubiquitination and subsequent proteasomal degradation (Stamos and Weis, 2013). Destruction of β-catenin has a general tumor suppressing role. One interesting future study would be to examine if MK-STYX overexpression decreases levels of β-catenin (Fig. 20).

It is also important to determine mechanistically how MK-STYX is lowering GSK3β serine 9 phosphorylation. Protein kinase A (PKA), protein kinase B (Akt/PKB), and protein kinase C (PKC) are known to phosphorylate serine 9 of GSK3β, while protein phosphatase 1 (PP1) and protein phosphate 2A (PP2A) dephosphorylate this site (Beurel et al., 2015). It is plausible that MK-STYX could inhibit one of these protein kinases or activate one of these phosphatases to decrease GSK3β serine 9 phosphorylation. Akt kinase activity is increased by phosphorylation on threonine 308 and serine 473 (Liao and Hung, 2010). A future series of Western blots could explore if MK-STYX overexpression reduces Akt phosphorylation on these sites. A lowering of phosphorylation at these sites would suggest that MK-STYX achieves its reduction of serine 9 phosphorylation on GSK3β by inhibiting Akt.

As previously discussed, excessive GSK3 activity is associated with bipolar disorder (Muneer, 2017). This study has shown that MK-STYX may increase GSK3β kinase activity. A strong enough increase in GSK3β activity could promote the oxidative stress, neuroinflammation, and circadian rhythm disruptions seen in bipolar disorder (Muneer, 2017; Luca et al., 2016) (Fig. 20). This possible disease link drives home the importance of carrying out a kinase activity assay to see if MK-STYX truly does augment GSK3β kinase function.

As mentioned earlier, GSK3 can inhibit stress granule formation (Mason et al., 2019). This thesis suggests that MK-STYX overexpression could activate GSK3β. Greater GSK3β activity would then be expected to further reduce stress granules. It is interesting to consider that MK-STYX's stress granule reduction mechanism may depend on activating GSK3β (Fig. 20). Such a connection is especially important because stress granules are linked with several neurodegenerative diseases, including amyotrophic lateral sclerosis, frontotemporal dementia, and Alzheimer's Disease (AD) (Wolozin and Ivanov, 2019). In the case of AD, stress granules were shown to fuse with tau aggregates, while the stress granule-promoting protein T-cell intracellular antigen 1 (TIA1) interacted with tau and TIA1 overexpression led to formation of inclusions containing phosphorylated tau (Vanderweyde et al., 2012).

Additionally, MK-STYX has been found to promote intrinsic apoptosis, and GSK3 also promotes this process (Niemi et al., 2011; Beurel and Jope, 2006). Considering this study's findings, it seems possible that MK-STYX's upregulation of intrinsic apoptosis could partly be explained by activating GSK3β through decreasing its inhibitory serine 9 phosphorylation (Fig. 20). Regulation of intrinsic apoptosis is an especially important research area since intrinsic apoptosis is a major cancer-fighting program within cells (Hanahan and Weinberg, 2011).

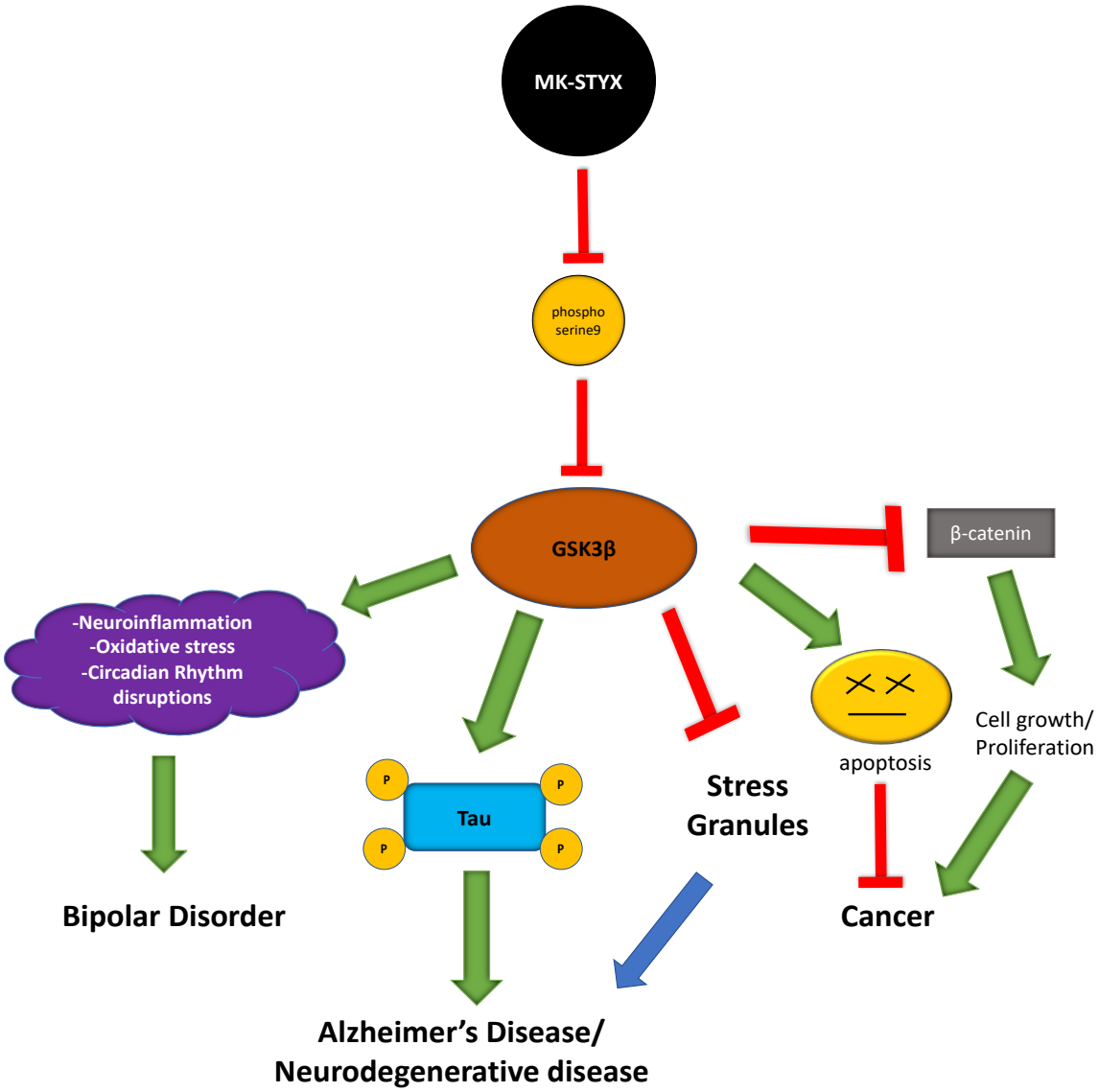


Figure 20: The potential implications of this study
By regulating GSK3β phosphorylation, MK-STYX could impact important physiological and pathophysiological pathways. This study has only demonstrated that MK-STYX reduces phosphorylation of serine 9 on GSK3β. However, all of the cellular impacts represented here could feasibly occur following this change. Future work could validate these implications experimentally.

An additional significant finding of this thesis was that MK-STYX$_{\text{active mutant}}$ overexpression significantly decreases the amount of GSK3α. One would expect that less GSK3α would lead to less phosphorylation of its substrates. An active mutant is important when studying pseudophosphatases because it enables biochemical comparisons to be drawn (Hinton et al., 2010; Reiterer et al., 2020). Such comparisons allow scientists to determine whether a pseudophosphatase has different binding partners from an active phosphatase. Pseudophosphatases resemble substrate trapping mutants used to identify the substrates of active phosphatases (Flint et al., 1997), therefore, it is important to consider how pseudophosphatases bind targets compared to their active mutants (Tonks, 2013). While MK-STYX$_{\text{active mutant}}$ is not a naturally occurring protein, these results are still interesting because they raise the possibility that MK-STYX's lack of catalytic activity helps prevent alterations in protein expression.

MK-STYX$_{\text{active mutant}}$ overexpression also significantly increased the amount of GSK3β in serum starved conditions. This suggests that MK-STYX$_{\text{active mutant}}$ could increase phosphorylation of GSK3β substrates by directly raising the amount of GSK3β in stressful cellular conditions. This finding again strengthens the claim that MK-STYX's pseudophosphatase character is an important part of its regulation and function, since MK-STYX$_{\text{active mutant}}$ exerts a sizable effect on the signaling protein GSK3β.

Further, MK-STYX$_{\text{active mutant}}$ overexpression significantly reduced the total expression of GSK3 (i.e., GSK3α/β). It is reasonable to anticipate that such an effect leads to decreased phosphorylation of GSK3 substrates overall. Once again, these findings suggest that maintaining MK-STYX's catalytic inactivity is important for safeguarding normal cellular conditions. It is also worth noting that MK-STYX overexpression lowered total GSK3 expression, but that this apparent reduction did not meet statistical significance due to high sample variation. Since MK-STYX$_{\text{active mutant}}$ is identical to MK-STYX save two amino acids, it seems possible that MK-STYX itself could reduce GSK3. Such an effect might be revealed if the statistical power of the analysis was boosted by conducting more biological replicates. It should also be pointed out that MK-STYX$_{\text{active mutant}}$ studies generally give an indication of what would happen should MK-STYX naturally mutate and reactivate. Also, these MK-STYX$_{\text{active mutant}}$ studies suggest that future work should instead include a close catalytically active relative of MK-STYX like an MKP for comparison (Hepworth and Hinton, 2021).

In this study, MK-STYX and MK-STYX$_{\text{active mutant}}$ did not interact with GSK3β according to coimmunoprecipitation. This lack of interaction held in normal serum conditions and serum starved conditions as well. It would have been interesting if MK-STYX immunoprecipitated GSK3β strongly while MK-STYX$_{\text{active mutant}}$ immunoprecipitated GSK3β weakly. Such a finding would show that MK-STYX's pseudophosphatase character allowed it to bind a substrate more strongly. It should be noted that phosphatase inhibitors were added to the

lysis buffer used to prevent activity. Clearly, coimmunoprecipitation studies showed that under these experimental conditions, MK-STYX did not interact with GSK3β, yet it should be stated that MK-STYX could still act through other mechanisms to impact GSK3β. However, it is still feasible that the interaction is especially short-lived or perhaps too weak to survive the repeated rounds of centrifugation and washing that are needed to purify CoIP samples. Another technique for exploring protein-protein interactions is bioluminescence resonance energy transfer, or BRET (Pflegler and Eidne, 2006). In BRET, a bait protein, for example MK-STYX, has a light-generating enzyme fused to it, while a prey or target, in this case GSK3β, has a fluorescent polypeptide fused to it. If the fusion proteins come into extremely close proximity, like in a protein-protein bond, a fluorescent signal will be produced as the light-generating fusion protein will transfer energy to and activate the fluorescent fusion protein (Pflegler and Eidne, 2006). An added advantage of BRET is that it offers *in vivo* information since the interaction-indicating fluorescent signal is produced in live cells, whereas CoIP depends on an intricate *in vitro* balance of buffers, antibodies, beads, washes, and centrifuging.

This thesis shows that MK-STYX and MK-STYX$_{\text{active mutant}}$ have some exciting impacts on GSK3. As previously described, future studies could strengthen and build on the connections found here. More biological replicates of each experiment, say 6 total instead of the current 3, would offer these studies greater statistical power and possibly reveal additional significant changes. For

example, in many of the Western blot densitometry analyses listed earlier, there were large mean differences between treatments that did not quite reach statistical significance. Greater statistical power through additional biological replicates would reduce the risk of possible “false negatives” or Type II errors.

The overall goal of this thesis was to determine if MK-STYX impacts GSK3, since MK-STYX and GSK3 play roles in many of the same pathways. The general answer to this question is a yes, since MK-STYX decreases serine 9 phosphorylation of GSK3β, which would be expected to activate GSK3β. To determine if MK-STYX’s cellular effects depend on GSK3, it would be interesting to see if MK-STYX is still able to induce neuronal differentiation, drive apoptosis, modulate HDAC6, and inhibit stress granules if GSK3 is antagonized. To this end, if shRNA-mediated knockdown of GSK3 abolished MK-STYX’s cellular effects, it would then be clear that GSK3 plays at least some role in these effects. A more specific case could examine if shRNA knockdown of GSK3 prevents MK-STYX from reducing stress granules. Later work could also consider if knockdown of MK-STYX reduced GSK3’s ability to phosphorylate its substrates. This would suggest that MK-STYX is necessary for normal GSK3 function.

Overall, the author hopes that this Master’s Thesis has imparted on the reader an appreciation for the array of things that a pseudophosphatase like MK-STYX can do. An ever-growing body of research describes MK-STYX’s roles in both normal physiological processes and disease. Many exciting facts about MK-STYX have been discovered, and no doubt many more biochemical revelations will follow in the future.

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