Beyond "self-eating": The Role of the Pseudophosphatase MK-STYX in Regulating Autophagy

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Beyond “Self-Eating”: The Role of the Pseudophosphatase MK-STYX in Regulating Autophagy

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in the Department of Biology from The College of William and Mary

By

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Accepted for____________________

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Abstract:
Cells carry out their day to day functions through the cellular work horses, proteins. Proteins work together like bees in a hive, each having its own specific task to accomplish. This specificity creates a fine-tuned division of labor where different types of proteins work together, completing their own work for the overall functioning of the cell. This division of labor can be mapped out nicely with cellular pathways. One such pathway is the stress response pathway. One of the major “workers” in this pathway is MK-STYX [MAPK (mitogen-activated protein kinase) phosphoserine/threonine/tyrosine-binding protein]. MK-STYX is a pseudophosphatase, a member of the dual-specificity family subfamily of MAPK phosphatases (MKPs) that lack the essential nucleophilic cysteine in its signature motif required for catalytic activity. MK-STYX maintains its three-dimensional fold and ability to bind proteins; it is involved in cellular pathways such as stress response, apoptosis, and neuronal differentiation. Previously, we reported that MK-STYX interacts with G3BP-1 [Ras-GAP (GTPase-activating protein) SH3 (Src homology 3) domain binding protein-1], and inhibits stress granule formation. Stress granules, cytoplasmic storage sites for mRNA, form as a protective mechanism against stress caused by UV irradiation, hypoxia, and heat shock. Stress induces stress granules, and involves many cellular mechanisms such as post-translational modifications, protein-protein interactions, and microtubule networks. Furthermore, stress granules are targeted and cleared by autophagy, an initiated response to cellular stress. It is responsible for the degradation of cellular components. Therefore, autophagy is essential for cellular degradation. Since autophagy and MK-STYX each negatively affects stress granule assembly, we sought to determine whether MK-STYX has a role in autophagy. Pursuing the role of MK-STYX in regulating autophagy in this thesis research will enhance our understanding of MK-STYX’s mechanism in the stress response pathway. Our studies show that MK-STYX causes cytosolic TFEB (Transcription factor E-Box; the autophagy “master switch”) to localize to the perinuclear space independent of nutrient status. Whereas, MK-STYX_{active} (active mutant in which catalytic activity has been "restored"), did not show this phenotype, suggesting that the catalytic signature motif of MK-STYX may play a role in stress response. TFEB is also found to localize to the lysosomal surface, acting as negative regulator of lysosomal and autophagosomal biogenesis. MK-STYX alters lysosomal and autophagosomal dynamics; oversized lysosomes are observed in the presence of MK-STYX. Furthermore, autophagosomes localize to the distal ends of HEK/293 cellular extensions in the presence of MK-STYX. Autophagy plays a major role in major cellular processes ranging from initial developmental stages to the onset of progressive human pathologies such as neurodegenerative diseases and cancer. This thesis research suggests that MK-STYX may have an important role in autophagy, illuminating the importance of pseudoenzymes in regulating critical cellular pathways.

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Introduction

Cell Signaling

Our cells exist in an environment where the ability to quickly and efficiently respond to their environment is not only crucial, but a cornerstone of our definition of life itself. Cells respond to their environment through methods known as cell signaling. Just as our bodies do, our cells are constantly interacting with and responding to their stressed
surroundings. Cells exist in a multitude of environments ranging from the gut to the brain, with each cell fitting well into its niche through an exquisitely adapted system of gene expression fine-tuned over millions of years. These systems of external signaling and responsive gene expression are intertwined and well adapted. At the simplest level of cell signaling there is the external cue that triggers an internal response. This single response can then magnify and begin to set off cascades of signaling events (Figure 1).

A simple signal can manifest a complex cellular response with multiple interwoven levels of gene expression and protein-protein interactions (Tonks, 2013). When taken together, responses to certain stimuli, and their crucial players, can be grouped together into signaling pathways. One of these pathways is the cellular stress response. Stress can manifest itself in cells through many external triggers. They include high heat, extreme pH changes, UV radiation, lack of oxygen or hypoxia, and starvation of necessary nutrients to name a few (Anderson & Kedersha, 2006).

My project focuses on the players involved in the cellular response to stress. Cells have evolved to have a comprehensive arsenal of genes that work to protect the overall health of the cell with special attention paid to minimizing the potential for gene mutations. When stress is detected, multiple signaling genes are activated and work to turn the initial signal into a physical cellular response. My thesis research works to put the pseudophosphatase MK-STYX in context to stress signaling. This conversation will begin by discussing phosphorylation cascades and how they control cellular signaling, which will be followed by a discussion of protein tyrosine phosphatases (PTPs), and our lab’s novel protein of interest the pseudophosphatase MK-STYX. The role of MK-STYX
in cellular stress through a novel regulatory link regarding stress granule (SG) formation will be reviewed. Lastly, we will discuss autophagy, a cellular mass degradation pathway upregulated during cellular stress, and its link to SG dynamics.

Phosphorylation Cascades

Signaling cascades are controlled by a number of reversible protein post-translational modifications (PTMs). Think of these as PTMs as a biological form of modular design. A protein may contain one specific cellular task in its native conformational state, but through PTMs the cell can modify the protein; changing its structure, and therefore its function. Additionally, proteins may have several PTMs simultaneously with functional domains which can be modulated by specific PTMs. This complexity allows immense diversity of function to evolve from a smaller set of genes than would be thought of to achieve this feat (Duan, Walther, Hart, Chen-Plotkin, & Johnson, 2015).

Phosphorylation is the most common method of protein PTM used in the cell. Phosphorylation is reversible and generally is covalently added to three amino acid residues: tyrosine, serine, and threonine in eukaryotes. The phosphorylation cascade is controlled by kinases, which add phosphate groups, and phosphatases, which cleave phosphate groups from the residue. Kinases and phosphatases work in tandem to control phosphorylation status of proteins involved in carrying signals (Figure 1). Kinases act as signal amplifiers while phosphatases control rate and duration of the signal (Tonks, 2006, 2013). Regulation of dephosphorylation has become more relevant as reports are increasingly elucidating links between dysfunctional phosphatases and

Protein Tyrosine Phosphatases

The main job of a protein phosphatase is to catalyze the hydrolysis of a phosphate group from an amino acid residue. Therefore, it was originally proposed that phosphatases were simply housekeeping enzymes that functioned to silence signals initiated by kinases (Tonks, 2006, 2013). Over the years, research has been chipping away at this simplistic view of phosphatases with more attention being paid to the combinatory role of phosphatases and kinases in controlling cell signaling (Tonks, 2006, 2013). Evolutionary analysis has characterized phosphatases as structurally and mechanistically distinct between different families. We will focus on the protein tyrosine phosphatase (PTP) superfamily. The PTP family contains 100 genes in humans with the
Classical PTPs are cysteine-dependent enzymes that specifically bind and remove phosphate groups from phosphotyrosine residues, while dual-specificity phosphatases (DUSPs), which can bind to any phospho-site residue for hydrolysis of phospho-serine/threonine, and tyrosine (Patterson, Brummer, O'brien, & Daly, 2009). The first PTP to be successfully purified and crystalized was PTP1B (protein phosphatase 1B) (Barford, Flint, & Tonks, 1994). PTP1B was a turning point for the field, providing the first validated mechanistic data of PTP substrate recognition and catalysis (Tonks, 2003).
Classical PTPs

The active site of all PTPs contains the signature motif HCX₅R allowing the protein to recognize the negatively charged phosphate group via the positively charged arginine. Hydrolysis of the phosphate group requires two steps. First, the cysteine provides the essential amino acid for catalysis, with a sulfur atom of the thiolate side chain acting as the nucleophile for nucleophilic attack and transfer of the phosphate group. The cysteine nucleophilic attack is aided by
neighboring arginine and histidine, which contribute to lowering the $pK_a$ of the cysteine for efficient catalysis. Arginine acts to stabilize the phosphocysteine intermediate. Finally, a water molecule acts as the second nucleophile to dissociate the phosphocysteine intermediate, releasing the phosphate group (Z.-Y. Zhang, 1998; Z. Y. Zhang & Dixon, 1994). Diversity of the PTP family allows for precise substrate specificity. Substrate recognition between DUSPs and classical phosphotyrosine PTPs is determined by active site cleft "depth" (Z.-Y. Zhang & Dixon, 2006).

DUSPs
DUSPs are overall more complex than classical PTPs. Of the approximately 65 genes that encode DUSPs they are far less conserved and contain high levels of sequence diversity between genes (Figure 2). Being part of the PTP superfamily, they contain the conserved cysteine signature motif active site and share the same catalytic mechanism of action. The defining feature of DUSPs is their ability to bind and hydrolyze phosphoserine/phosphothreonine, and phosphotyrosine residues. The shallower structure of the active site, compared to classical PTPs, is what allows DUSPs to have this range of residue recognition by allowing the shorter phosphoserine/phosphothreonine residues access to the catalytic site (Yuvaniyama, Denu, Dixon, & Saper, 1996). DUSPs can be further divided into multiple subgroups each containing genes controlling a diverse range of cellular functions, once again highlighting the importance of PTPs in context of the cell. The best characterized subgroup are the MKPs (mitogen-activated protein kinase phosphatases) which are responsible for dephosphorylating MAPKs (mitogen-activated protein kinases) at both phosphoserine/phosphothreonine in the kinase activation loop (Tonks, 2006). MAPKs are crucial regulators of cell growth, hormone response, cytokine signaling, and stress response. MKPs play an important role in regulating MAPK signaling at many different levels, indicating that dysregulation of MKPs may have detrimental effects on downstream signaling cascades.

PTP Pseudophosphatases

Within the diverse superfamily of PTPs, there also exist several genes which contain conserved domains and folds found in all PTPs, but lack the critical cysteine residue
needed for catalysis. These PTPs are called pseudophosphatases, literally translating to “fake” phosphatase. Without the catalytic residue, pseudophosphatases cannot remove phosphate groups and were originally thought to have little to no signaling input. Conversely, these catalytically “dead” phosphatases have been found to be anything but “dead” when it comes to signal regulation. In fact, current data show that approximately 8% of all phosphatases are pseudophosphatases indicating a wide range of cellular functions (Reiterer, Eyers, & Farhan, 2014). Phosphatases have a wide range of substrates, indicating that pseudophosphatases may be implicated in a diverse range of signaling pathways. The prototypic pseudophosphatase STYX (phosphoserine, threonine, tyrosine interacting protein) was first described around two decades ago by Jack Dixon’s lab (Wishart & Dixon, 1998). STYX contains a DUSP PTP 3D fold but contains a mutation in the signature motif with glycine replacing cysteine, abolishing catalytic activity. Highlighting the high level of conservation of the PTP fold, a point mutation in the active site back to a cysteine restores catalytic activity (Wishart, Denu, Williams, & Dixon, 1995). This suggests that pseudophosphatases retain the ability to bind to their specific phospho-residues in a similar manner to catalytically active DUSPs. This “grab and hold-on” function allows STYX to act as a naturally occurring “substrate-trap.” This function has even been confirmed in point-mutation studies with PTP1B, where the cysteine was replaced with a glycine. This catalytic mutant could trap high affinity substrates in a stable complex (Flint, Tiganis, Barford, & Tonks, 1997). Substrate-trapping studies like these acted as a primary line of evidence for more complex functions of pseudophosphatases in cell signaling. In recent years, many other pseudophosphatases, and pseudokinases, have been discovered.
The Myotubularins and MAPKs

Due to high levels of conservation of the PTP structure, many pseudophosphatases are able to interact with substrates beyond their phospho-site. In fact, some pseudophosphatases interact through direct protein-protein interactions (Tonks, 2009). Several members of the myotubularian (MTM) family of pseudophosphatases engage with their target proteins in this manner. For example, a pseudophosphatase MTM-13 forms a complex with the catalytically active MTM-2, directing its subcellular localization (Robinson & Dixon, 2005). Additionally, some pseudophosphatases target and form complexes with kinases. As with MAPKs, the dual-specificity tyrosine-regulated kinase minibrain kinase homologue-2 (MBK-2) requires phosphorylation of an activation loop for kinase activity. The pseudophosphatases EGG-4 and EGG-5 are able to inhibit MBK-2 activation by binding directly to this activation loop independent of phosphorylation status (Cheng, Klancer, Singson, & Seydoux, 2009; Tonks, 2009).

MK-STYX [MAPK (mitogen-activated protein kinase) phospho-serine/threonine/tyrosine-binding protein] provides an example of a pseudophosphatase that is homologous to MKPs. MK-STYX is inactive due to two mutations in the signature active site, replacing the nucleophilic cysteine with a serine and the adjacent histidine, important for PTP phospho-intermediate stability, with a phenylalanine (Hinton, Myers, Roggero, Allison, & Tonks, 2010). Mutations in the active site of MKPs provide evidence of in vivo functions of MK-STYX. For example, mutations in MKP-1 (MAPK phosphatase-1) lead to higher
levels of MAPK activity through prolonged MAPK activation loop phosphorylation. In studies done on catalytically inactive MKP-1, similar levels of MAPK activation and phosphorylation were noticed, indicating a "substrate-trapping" function of inactive MKP-1 (Sun, Charles, Lau, & Tonks, 1993). Substrate-trapping analysis, along with protein-protein interaction analysis, helped elucidate mechanisms of action of pseudophosphatases in vivo. More importantly, these studies provide solid evidence of cellular function for pseudophosphatases. These analyses force us to focus on viewing pseudophosphatases beyond their inactive domain and to reimagine what “activity” means for a non-catalytic proteins.

Pseudophosphatase MK-STYX

MK-STYX contains a N-terminal CH2 domain, shares the same motifs, and is homologous with several MKPs. However, MK-STYX is a pseudophosphatase due to two unique mutations within the signature active motif. Similarly to STYX, MK-STYX may also have catalytic activity restored by two point mutations (Hinton et al., 2010; Wishart & Dixon, 1998). This suggests that MK-STYX may also play the role of “substrate-trap” under certain circumstances. Due to the sequence similarity in the N-terminal CH2 domain found in both MK-STYX and MKPs, we can predict that MK-STYX may contain similar binding patterns as some MKPs (Figure 3). This brings to light possible protein-protein interactions with MAPKs, suggesting a role for MK-STYX in regulating MAPK signaling (Tonks, 2013). MK-STYX has been shown to be an important regulator of a diverse range of cellular processes. In developing neurons, the growth of axons and dendrites is closely controlled by internal and external signaling with the Ras-ERK (extracellular signal-regulated kinases)/MAPK pathway as an
important regulator. As stated earlier, MK-STYX contains high homology to MKPs, which act to dephosphorylate MAPKs. MK-STYX was shown to induce growth of neuronal extensions, but through a mechanism independent of MAPK signaling. Additionally, MK-STYX was shown to cause a shift in RhoA (Ras homolog gene family, member A), a Ras signaling protein responsible for controlling neurite extension signaling by decreasing RhoA activation (Sebök et al., 1999).

Figure 3: Schematic view of MK-STYX protein

From the N-terminus, MK-STYX contains a CH2 domain homologous to MKPs CH2 domain. MK-STYX contains a DUSP domain toward the C-terminus containing the signature motif. MK-STYX is catalytically inactive, with phenylalanine and serine replacing histidine and cysteine. The thiol group of cysteine contains a sulfur, which is more electronegative than the oxygen of the alcohol side group of serine. Higher electronegativity is why sulfur is a better nucleophile than oxygen, and therefore; why lack of cysteine makes MK-STYX catalytically inactive.
MK-STYX and G3BP-1

MK-STYX has also been found to directly bind to another Ras signaling regulatory protein, G3BP-1 (Ras-GTPase activating protein SH3 domain binding protein-1) (Hinton et al., 2010). G3BP-1 is expressed ubiquitously and has been found to be involved in several important cellular functions (Gallouzi et al., 1998). G3BP-1 contains several functional domains; including an RNA Recognition Motif (RRM) which G3BP-1 uses to bind to untranslated mRNAs. G3BP-1 also contains a Nuclear Transport Factor 2 (NTF-2)-like domain which explains why G3BP-1 can enter the nucleus while being primarily cytosolic (Parker et al., 1996). G3BP-1 is also implicated in the cellular stress response. During the stress response G3BP-1, through its RRM, is able to bind untranslated mRNA as a protective mechanism. This G3BP-1-mRNA complex can then nucleate and assemble cytosolic inclusions of mRNA called stress granules (SG) (Tourrière et al., 2003). MK-STYX binds G3BP-1 and has been found to decrease the formation of these SGs (Hinton et al., 2010).

MK-STYX and Stress Granule Formation
SGs form in response to a range of cellular stressors as a cytoprotective measure. Some examples of stressors that induce SG formation include: high heat, oxidative conditions, UV exposure, and nutrient deprivation (Buchan, Kolaitis, Taylor, & Parker, 2013; Thomas, Loschi, Desbats, & Boccaccio, 2011). In response to these stressors, the cell responds by halting large scale protein production in the cell (N. Kedersha & Anderson, 2002). Phosphorylation comes into play as the major inhibitor of translation, eIF2α is phosphorylated halting translation, and untranslated mRNAs build up in the cell. These mRNAs can then be bound to RNA-binding proteins, G3BP-1 for example. The RNA-Protein complex is shuttled to sites of nucleation via several linker proteins bound to kinesin and dynein (Buchan & Parker, 2009). The process of assembling SGs is a dynamic process that requires the coordination of many interconnected proteins. For this reason, SGs are complex heterogeneous cellular components that can contain many different types of proteins (Nancy Kedersha et al., 2005). Assays for SG formation must take the heterogeneous nature of SGs into account when using markers for SGs. G3BP-1 has been characterized as a nucleator of SGs and is therefore a good marker of SGs (Anderson & Kedersha, 2006). Studies with G3BP-1 mutants have outlined important aspects of how G3BP-1 regulates SG formation. For instance, phosphorylation of the serine 149 (S149) residue of G3BP-1 inhibits SG formation. There are several potential sites of phosphorylation on G3BP-1, but mutations that form a non-phosphorylatable version of G3BP-1 at S149 form SGs independent of stress (Tourrière et al., 2003). MK-STYX is able to bind to this S149 phospho-site, in fact, a catalytically active mutant of MK-STYX (MK-STYXactive) or F1 is able to dephosphorylate S149, inducing SG formation. MK-STYX co-expressed with G3BP-1 showed decreased
SG formation (Hinton et al., 2010), but this decrease was independent of G3BP-1 phosphorylation status (Barr, Munyikwa, Frazier, & Hinton, 2013). MK-STYX was originally thought to be a competitive inhibitor of G3BP-1 kinases at this phospho-site, yet these data point to MK-STYX acting on SG formation farther upstream. Additionally, results point to MK-STYX having a broader role in the stress response.

SG formation, disassembly, and degradation require intricate levels of regulation to maintain proper mRNA homeostasis within cells. Several disease states that are characterized by pathological stress granules also contain mutations within the cellular degradative pathway, autophagy. SGs are a hallmark of amyotrophic lateral sclerosis (ALS) as they accumulate in ALS neurons, and a deficiency in the Atg-5 gene has been linked to ALS SG pathology. Additionally, a knockout of this gene causes formation of SGs in cells independent of stress (J.-A. Lee, 2015). Autophagy is activated by many of the same pathways that induce SG formation; therefore, it is a part of the cellular stress response (Z. Y. and D. J. Klionsky, 2013). In cells that have been stressed and formed SGs, autophagy has been found to selectively degrade SGs (Buchan et al., 2013). The mechanism behind this targeting has been associated with nutrient induced autophagy, which induces targeted autophagy rather than non-selective autophagy (Figure 4) (J.-Y. Lee et al., 2010).

Autophagy
Macroautophagy (called autophagy) is a cellular pathway responsible for the degradation of protein aggregates, damaged organelles, and stress granules in response to cellular cues to maintain energy homeostasis within the cell (Z. Y. and D. J. Klionsky, 2013). Autophagy is a conserved cellular process with links to metabolism, development, and several disease states, to name a few. Autophagy acts at a basal level in almost all eukaryotic cells, but has adaptive functions in response to many cellular stressors. The response varies depending on the stress, but usually involves upregulation of autophagic activity as a mechanism to “recycle” cellular components to provide necessary nutrients and energy during a stressful event (Deter, Baudhuin, & Duve, 1967; Mortimore & Schworer, 1977; Pfeifer, 1977). Across the various forms of autophagy exists a core set of conserved autophagy genes which are responsible for the general mechanisms that control autophagy (Z. Y. and D. J. Klionsky, 2013). Pioneering work done with mutant yeast strains helped identify the key set of genes, called the autophagy-related genes (Atg), necessary for general autophagy progression (D. J. Klionsky et al., 2003; Tsukada & Ohsumi, 1993; Xie & Klionsky, 2007).

Autophagy initiation

Autophagy induction is controlled by several protein kinases that form early complexes. Unc-51 like kinase 1 (ULK1) and vacuolar protein sorting-34 (VPS34) are both activated upon nutrient deprivation by AMP dependent protein kinase (AMPK) (Kim, Kundu, Viollet, & Guan, 2011). These are both protein kinases which phosphorylate many downstream substrates that promote autophagosomes maturation (Itakura & Mizushima, 2010; Russell et al., 2013). Active ULK1 forms a complex with Atg13 and
focal adhesion kinase family interacting protein of 200 kDa (FIP200). In complex, ULK1 autophosphorylates its partners as well as autophagy/Beclin-1 regulator 1 (AMBRA1)

Figure 4: Life cycle of stress granules

Stress granules form in response to cellular stress. Both MK-STYX overexpression and autophagy lead to stress granule clearance, but the mechanism of this reduction is not yet understood. MK-STYX may act on autophagy at the stages of stress granule targeting to autophagosomes or on the degradation step.
Phosphorylation of AMBRA1 promotes complex formation with Atg14, VSP34, and beclin1 (BECN1), which promotes translocation of the complex to the ER membrane (Russell et al., 2013). Localization of this kinase complex to the ER allows VSP34 to phosphorylate phosphatidylinositol (PI) to phosphatidylinositol-3-phosphate (PI(3)P) which allows attachment of scaffold and effector proteins. This pool of PI(3)P and recruited binding proteins form an outgrowth, which eventually envelops cytoplasmic contents into an early autophagosome (Axe et al., 2008). During elongation, ubiquitin-like modifiers (UBL) are recruited to the growing autophagosome. Microtubule-associated protein 1 light chain 3 (MAP1LC3, called LC3) is part of the Atg8 family of proteins and has been shown to play a role as both an early scaffolding protein and in promoting binding of cargo to the autophagosome during selective autophagy (Mizushima, Yoshimori, & Ohsumi, 2011). The nascent LC3 protein must be processed by cysteine proteases before entrance into the growing autophagosome. The C-terminus of the LC3 protein is cleaved by Atg4B, exposing a glycine residue and producing LC3-I (Kirisako et al., 2000). The Atg8 family of proteins are modified by several Atg enzymes which catalyze ubiquitin-like reactions to further modify LC3-I (Kirisako et al., 2000). This modified version of LC3-I is then conjugated to a phosphatidylethanolamine (PE) group within the growing autophagosomal membrane, now called LC3-II. Deconjugation of the cytosolic portion of LC3-II creates many forms of LC3 within the early autophagosome, one of which is found in high concentrations and called LC3B (Nair et al., 2012).

Long term autophagy and TFEB
An impressive aspect of autophagy is the fact that for such a complex pathway, it requires little transcriptional upregulation. A majority of autophagy genes are expressed endogenously at relatively high levels. Since autophagy’s main goal is to compensate for low levels of energy and precursor molecules by recycling cytosolic content, it would be regressive if autophagic activity required upregulation of protein synthesis to respond to stress (Cuervo, 2011). Yet, it is important to recall that autophagy has been found to last for a much longer amount of time than originally thought. While most researchers agree that there is a large enough pool of endogenous Atg proteins to last up to 8 hours into nutrient starvation, autophagy has been found to last for several days (Singh et al., 2009). Although some Atg proteins have been found to be recycled throughout the autophagic process, lysosomal proteins found within the autophagosome are degraded. Additionally, as starvation persists the rate of autophagosomal and lysosomal fusion would increase to a point where the amount of either membrane wouldn’t be able to keep up with demand (Yu et al., 2010). Uncovering this mystery leads us back to autophagic initiation. As stated earlier, active ULK1 is responsible for initiating elongation of the autophagic vesicle during starvation. Importantly, ULK1 is inactive during nutrient rich times and the control switch for ULK1 activation is the mammalian target of rapamycin complex 1 (mTORC1). mTORC1 is a complex integrated within the lysosome with kinase activity. During nutrient rich times, mTORC1 is activated by binding to a small GTP-binding protein Ras homolog enriched in brain (Rheb) (Badalà, Nouri-mahdavi, & Raoof, 2009). Active mTORC1 phosphorylates many different serine substrates including serine 758 of ULK1, inactivating ULK1 (Kim et al., 2011). Additionally, this work also lead to the discovery of another mTORC1 substrate,
transcription factor EB (TFEB) (Settembre et al., 2010). TFEB is a transcription factor originally characterized as responsible for upregulating lysosome production, but recent work has shown a broader role of autophagy upregulation along with more general lysosomal biogenesis (Settembre & Ballabio, 2011). mTORC1 phosphorylation of TFEB causes TFEB to remain cytosolic; therefore, when mTORC1 is inactive during nutrient deprivation TFEB enters the nucleus (Figure 5) (Settembre & Ballabio, 2011). Upregulation of TFEB alone has been shown to increase autophagy in several studies (Martina & Puertollano, 2013; Martini-Stoica, Xu, Ballabio, & Zheng, 2016; Settembre et al., 2012). TFEB allows the cell to not only upregulate production of lysosomes, but also increases the concentrations of autophagy proteins making TFEB a “master-regulator” of autophagy (Cuervo, 2011).

**Autophagy and stress granules**

SG formation and selective autophagy require a large amount of signal integration, which as specified above, involves phosphorylation at several points. SG degradation through autophagy requires the integration of interplaying stress signaling molecules. As SGs become pathologic in several disease states, the increased autophagy needed to degrade these SGs would require a constant supply of autophagosomes and lysosomes, along with their associated proteins. Several studies

![Diagram](image-url)
have found evidence to suggest selective degradation of SGs through autophagy as a method for SG clearance (Buchan et al., 2013). Additionally, in several disease states, such as ALS, there is a correlation between both autophagic dysregulation and SG formation (Shewmaker, 2016). MK-STYX has been found to be a master regulator of
cellular stress, decreasing SGs in response to stress (Hinton et al., 2010), yet the mechanism behind this decrease, to date, remains unclear. Autophagy has been shown to decrease SGs in response to cellular stress (Buchan et al., 2013), but the mechanism behind this decrease remains elusive.

Hypothesis

Autophagy and MK-STYX both work in response to stress signaling. The missing link between these two cellular processes is the lack of mechanistic evidence behind the decrease in SGs. Therefore, it is hypothesized that MK-STYX regulates autophagy
in response to stress. To investigate this link, several biological questions will be asked to elucidate the regulatory link of MK-STYX on autophagy:

1. Does MK-STYX cause a localization change in TFEB, the master regulator of autophagy and lysosomal biogenesis?
2. Does MK-STYX cause a change in number of autophagosomes?
3. Does MK-STYX cause a change in the number of lysosomes?

Methods

Plasmids

The pMT2-FLAG-MK-STYX-FLAG, pMT2-FLAG-MK-STYX-FLAG, and pMT2 constructs were generated as previously described (Hinton et al., 2010). pEGFP-MK-STYX constructs were generated by Vinny Roggero. The integrity of all constructs
derived from PCR was confirmed by sequencing. The pEGFP expression vector has been previously described (Hinton et al., 2010).

**Cell Culture**

HEK293 cells (Human Embryonic Kidney cells 293; American Tissue Culture Collection) were grown and maintained at 37°C and 5% CO₂ in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco), supplemented with 10% fetal bovine serum (FBS) (Invitrogen). Cells were maintained with 15 ml per flask (Thermo Scientific).

**Transfections**

For transient transfection assays, 2.5 x 10⁵ HEK293 cells were seeded on glass cover slips (Fisher) placed in each well of a 6-well plate. Approximately 24hr later, depending on the experimental conditions, different plasmid combinations were introduced using either Lipofectamine 3000 or Lipofectamine 2000 according to the manufacturer’s specifications (Invitrogen). Cells were incubated with transfection reagents for 4-6hr before the cell medium was replaced. The next day, three out of six wells underwent serum starvation with DMEM lacking fetal bovine serum (DMEM 0% FBS) overnight. Cells were imaged approximately 48hrs post transfection.

**Fixation and immunofluorescence**

Approximately 48 hr post-transfection, cells were fixed with fresh 3.7% formaldehyde, made from diluted 37% formaldehyde stock, for 10 min and permeabilized for 5 min with 0.2% Triton-X-100 (Fisher) for antibody staining. The antibodies were used as follows: anti-TFEB at 1:250 (TFEB marker, Cell Signaling Technology), anti-LC3B at 1:250 (Autophagosomal marker, Cell Signaling Technology), anti-LAMP1 at 1:250 (Lysosomal
marker, Cell Signaling Technology) anti-FLAG at 1:250 (FLAG-MK-STYX marker); Cy3-goat anti-mouse or Cy3-goat anti-rabbit at 1:250 (Zymed Laboratories). After staining, cell coverslips were mounted onto microscope slides in Fluoro-Gel II (Electron Microscopy Sciences) containing the DNA counter stain DAPI (4,6-diamino-2-phenylindole dihydrochloride) (Sigma).

Fluorescence microscopy and cell scoring

Prepared cell sides were analyzed with bright field phase and fluorescence microscopy using a Nikon Eclipse Ti inverted fluorescence microscope. At least 3 replicate experiments were performed, with a minimum of 100 cells scored per replicate. All images were taken at 40x magnification. Additionally, for some experiments, cells were scored using a Cell Profiler program graciously developed in part by Dr. William Buchser. NIS Elements software was used to analyze TFEB localization.

Results

**MK-STYX causes a perinuclear shift in TFEB**

TFEB localization controls whether autophagy genes will be upregulated during long term nutrient deprivation, or not. To understand if MK-STYX plays a role in regulating autophagy, it is therefore important to visualize TFEB localization in the presence of MK-STYX. To investigate TFEB localization, we transfected HEK293 cells with expression vectors containing MK-STYX, MK-STYX \text{active}, or pMT2. Additionally, to
investigate the effect of MK-STYX on localization during periods of long-term nutrient deprivation, HEK293 cells with expression vectors containing MK-STYX, MK-STYX_{active}, or pMT2 were exposed to nutrient starvation overnight. These cells were then fixed and immunostained for TFEB localization using anti-TFEB antibody (Figure 6). In nutrient rich cells, in which the MK-STYX vector was introduced, a perinuclear pattern of TFEB localization emerged with 44% (SEM=12.99) of cells containing perinuclear TFEB. In the control cells, only 9% (SEM=5.21) of cells contained perinuclear TFEB along with 3% (SEM=1.00) of MK-STYX_{active} cells (Figure 7). The trend of a significant increased (ANOVA, p-value=0.03) perinuclear localization of TFEB in MK-STYX cells persisted during nutrient deprivation, with around 30% (SEM=10.48) of cells exhibiting perinuclear TFEB. In contrast, control cells had 3% (SEM=1.2) cells exhibiting perinuclear localization and 4% (SEM=0.88) of MK-STYX_{active} cells exhibiting this phenotype (Figure 8). This result indicates that MK-STYX causes a significant (ANOVA, p-value=0.02) increase in cells exhibiting perinuclear localized TFEB, independent of nutrient conditions. Interestingly, the MK-STYX catalytically active mutant showed a significantly lower percentage of cells exhibiting perinuclear TFEB, indicating that being “pseudo” drives this phenotype.
Figure 6: MK-STYX causes a perinuclear shift of TFEB

TFEB is localized in the cytosol in nutrient rich conditions and moves to the nucleus in response to nutrient deprivation (serum starvation). HEK 293 cells were transfected with MK-STYX, MK-STYXactive, or pMT2 and stained for both TFEB and FLAG (FLAG is conjugated to both MK-STYX and MK-STYXactive). In cells transfected with MK-STYX, TFEB localized to the perinuclear space in nutrient rich and deprived conditions.
Figure 7: MK-STYX causes an increase in perinuclear TFEB in nutrient rich cells

HEK293 cells were scored for the presence of perinuclear TFEB in nutrient rich cells. Three replicate experiments were performed (n=100, per trial); error bars indicate +/- SEM. ANOVA analysis showed that MK-STYX caused a significant increase (*p-value < 0.05) in TFEB in nutrient rich cells.
MK-STYX does not affect the number of autophagosomes produced, but changes localization in response to starvation

As longer periods of nutrient deprivation progress, TFEB will increase the expression of autophagy and lysosomal proteins via translocation to the nucleus. This upregulation of autophagy and lysosomal genes during long-term autophagy is important for replenishing proteins that cannot be recycled efficiently by the autophagic process. Many autophagosome related proteins are degraded as the autophagosome “matures,” meaning it increases in intervascular pH. As stated previously, LC3 is a crucial autophagosome related protein. The various forms of the LC3 gene contribute to its diverse range of responsibilities within autophagy. For example, LC3B-II contributes to

Figure 8: MK-STYX causes an increase in perinuclear TFEB in nutrient deprived cells

HEK293 cells were scored for the presence of perinuclear TFEB in nutrient deprived (serum starved, or SS) cells. Three replicate experiments were performed (n=100); error bars indicate +/- SEM. ANOVA analysis showed that MK-STYX caused a significant increase (*p-value < 0.05) in TFEB in nutrient deprived cells.
early autophagosomal membrane elongation along with the fusion of autophagosomes with lysosomes (Weidberg et al., 2011).

LC3B is a critical autophagy protein, and is used as marker for autophagosomes and autophagy activity more broadly (Barth, Glick, & Macleod, 2010). TFEB has been found to upregulate the expression of LC3B (Kilpatrick, Zeng, Hancock, & Segatori, 2015). As we noticed the effect on the localization of TFEB by MK-STYX, we wanted to see if this caused a change in autophagosomal dynamics. To understand the role of MK-STYX in regulating autophagosomes, we stained for LC3B positive vesicles in the presence of MK-STYX. We cotransfected HEK293 cells with expression vectors containing MK-STYX-GFP, or pEGFP. Additionally, to investigate the effect of MK-STYX on autophagosomes during periods of long-term nutrient deprivation, HEK293 cells with expression vectors containing MK-STYX-GFP, or pEGFP were exposed to nutrient starvation overnight. These cells were then fixed and immunostained for autophagosomes using anti-LC3B antibody (Figure 9). Additionally, these cells were scored for number of LC3B puncta/cell using Cell Profiler, as these puncta indicate autophagosomes. Cells in nutrient rich conditions that were transfected with MK-STYX-GFP had an average of 2.6 (SEM=0.35) LC3B puncta/cell. This is lower than the average for GFP transfected cells, which had 3.4 (SEM=0.93) LC3B puncta/cell on average in nutrient rich conditions (Figure 10). In response to overnight nutrient deprivation, MK-STYX-GFP cells showed an increase in the average number of LC3B puncta/cell with 5.3 (SEM=1.03) per cell. The GFP transfected cells also exhibited an increase in the average number of LC3B puncta/cell with 5.1 (SEM=1.34) per cell.
(Figure 11). These results do not show a statistically significant change in the number of LC3B puncta/cell in the presence of MK-STYX. This indicates that TFEB perinuclear localization in response to MK-STYX doesn’t seem to be affecting either nutrient deprived or nutrient rich cells production of autophagosomes. Interestingly, MK-STYX cells did cause a shift in autophagosome localization, with higher concentrations of autophagosomes localizing to the ends of cellular extensions.
HEK293 cells were transfected with GFP-MKSTYX or GFP and stained for LC3B. In cells transfected with GFP-MK-STYX and GFP in nutrient rich conditions, autophagosomes were found throughout the cytosol. In cells transfected with GFP-MK-STYX in nutrient deprived (serum starved) conditions, LC3B was found localized to cellular extensions (blue arrows).
**Figure 10: MK-STYX does not affect autophagosome production in nutrient rich cells**

HEK293 cells were scored for LC3B puncta (indicate autophagosome) with cell profiler. Three replicate experiments were performed; error bars indicate +/- SEM(n=100, per trial). Cells transfected with GFP-MK-STYX (MK) produced less autophagosomes per cell on average (not significant) than cells transfected with GFP.
MK-STYX does not affect autophagosome production in nutrient deprived cells

HEK293 cells were scored for LC3B puncta (indicate autophagosome) with cell profiler. Three replicate experiments were performed; error bars indicate +/- SEM (n=100, per trial). Cells transfected with GFP-MK-STYX (MK) and GFP both produced the same amount of autophagosomes on average.
While autophagosomes are responsible for collecting cytosolic cargo for degradation, that degradation cannot happen without fusion of the lysosome with the autophagosome. The lysosome is a crucial component of autophagic flux, the full cycle of autophagy as it is required for breakdown of autophagosomal targets for reuse. As is the case for some autophagy proteins, many proteins responsible for lysosomal function are not recycled from one lysosome to another. Therefore, upregulation of lysosomal proteins during long periods of autophagy is necessary for proper autophagy function.

LAMP1 (Lysosomal-associated membrane protein 1) is an important lysosomal protein. While its precise physiological role has been hard to pin down, LAMP1 has been shown to be an important regulator of lysosomal integrity and pH (Eskelinen, 2006). Due to the abundance of LAMP1 on the surface of lysosomes, it has been found to be a good marker for lysosomes. TFEB has been shown to upregulate LAMP1 during long-term nutrient deprivation and autophagy activity. (Porter, Nallathambi, Lin, & Liton, 2013). To understand the role of MK-STYX in regulating lysosomes, we stained for LAMP1 positive vesicles in the presence of MK-STYX. We transfected HEK293 cells with expression vectors containing MK-STYX-GFP, or pEGFP. Additionally, to investigate the effect of MK-STYX on lysosomes during periods of long-term nutrient deprivation, HEK293 cells with expression vectors containing MK-STYX-GFP, or pEGFP were exposed to nutrient starvation overnight. These cells were then fixed and immunostained for lysosomes using anti-LAMP1 antibody (Figure 12). Additionally, these cells were scored for number of LAMP1 puncta/cell using Cell Profiler. Cells in nutrient rich conditions transfected with MK-STYX-GFP showed an average of 3.0
LAMP1 puncta/cell. Similarly, GFP transfected cells in nutrient rich conditions showed an average of 2.9 (SEM=0.68) LAMP1 puncta/cell (Figure 13). This indicates that MK-STYX’s effect on TFEB localization doesn’t affect lysosomal biogenesis in nutrient rich cells. However, in nutrient deprived cells, MK-STYX-GFP transfected cells showed a decrease in the number of LAMP1 puncta per cell to an average of 2.61 (SEM=0.46). The GFP transfected cells continued to follow the normal response to long-term nutrient deprivation by increasing the number of LAMP1 puncta/cell to an average of 4.5 (SEM=1.37) (Figure 14). This data indicates that the effects of MK-STYX on TFEB localization is also accompanied with decreased lysosome production after nutrient deprivation. Additionally, MK-STYX is causing LAMP1 puncta to localize at the ends of cellular extensions and LAMP1 to the plasma membrane during nutrient deprivation.
Figure 12: MK-STYX causes LAMP1 to localize to the plasma membrane and lysosomes to clump at the ends of cellular extensions in nutrient deprived cells.

HEK 293 cells were transfected with GFP-MK-STYX or GFP and stained for LAMP1. In cells transfected with GFP-MK-STYX and GFP in nutrient rich conditions, LAMP1 was found throughout the cytosol. In cells transfected with GFP, LAMP1 localized cytosolically in nutrient deprived cells, while cells transfected with GFP-MK-STYX, some LAMP1 localized toward the plasma membrane (red arrow). LAMP1 puncta indicate lysosomes, and GFP-MK-STYX caused lysosomes to localize and clump to cellular extensions (blue arrows).
Figure 13: MK-STYX does not affect lysosomal production in nutrient rich cells

HEK293 cells were scored for LAMP1 puncta (indicate lysosome) with cell profiler. Three replicate experiments were performed; error bars indicate +/- SEM (n=100, per trial). Cells transfected with GFP-MK-STYX (MK) showed no significant difference in the average number of lysosomes produced compared to GFP.
Figure 14: MK-STYX causes a decrease in the number of lysosomes in nutrient deprived cells

HEK293 cells were scored for LAMP1 puncta (indicate lysosome) with cell profiler. Three replicate experiments were performed; error bars indicate +/- SEM (n=100, per trial). Cells transfected with GFP-MK-STYX (MK) showed a decrease in the average number of lysosomes (not significant) compared to GFP.

Discussion
A role for MK-STYX in regulating autophagy signaling

The cellular stress response pathway works to protect cells after a period of cellular stress. Several families of kinases and phosphatases work within the stress response pathway to regulate its activity. Within the phosphatase families that work in response to stress, there also exist several pseudophosphatases. MK-STYX is a pseudophosphatase part of the PTP superfamily. Up until recently, pseudophosphatases have been classically ignored when it comes to regulating cell signaling pathways. Pioneering work in our lab has laid the ground work in determining that catalytic “inactivity” doesn’t necessarily equate with physiological “inactivity” with pseudophosphatases. The major route this investigation began with was characterizing the role of MK-STYX in regulating stress through SG dynamics. SGs are known to accumulate in several neurodegenerative diseases (Shewmaker, 2016), and as such, research into ways to clear these accumulations is important for future therapies. A major player in nucleating SGs is G3BP-1 which interacts with MK-STYX (Hinton et al., 2010). Expression of the MK-STYX gene causes a decrease in cellular SGs independent of G3BP-1 activation, meaning this effect was occurring through other players (Barr et al., 2013). The mechanism behind this decrease is poorly characterized and as such we chose to investigate the current mechanisms known to drive SG clearance. One such pathway is autophagy, which can selectively degrade SGs through a process termed granulophagy (Buchan et al., 2013). We chose to investigate whether MK-STYX was working to regulate autophagic SG clearance. An MK-STYX dependent regulatory effect on autophagy was therefore investigated first.
TFEB localization acts a “master switch” for long term autophagy

Initial autophagy activity can be efficiently activated without transcriptional upregulation. This is due to high levels of endogenously expressed Atgs and other autophagy related proteins (Cuervo, 2011). During periods of long-term autophagy, several critical genes become scarce due to their inability to be efficiently recycled. A transcription factor, TFEB, works to upregulate autophagy and lysosomal genes during long-term autophagy. TFEB acts as the second line of defense to replenish autophagy and lysosomal genes when they are needed most. Under nutrient rich circumstances, TFEB is kept in the cytoplasm through phosphorylation and 14-3-3 binding. When nutrients are removed from the system, TFEB is no longer phosphorylated and localizes to the nucleus (Settembre et al., 2012). This pattern of localization determines TFEB’s activity and therefore autophagy activation. A change in localization could therefore have many downstream consequences for upregulating certain autophagy genes. TFEB is also a crucial component of the lysosomal nutrient sensing pathway, and localizes to lysosomes when nutrients are plentiful. Expression of MK-STYX caused a drastic perinuclear shift of cytosolic TFEB in cells containing nutrient rich media. Upon nutrient deprivation, the perinuclear shift was still apparent, accompanied with an increase in nuclear TFEB. This perinuclear shift indicates that MK-STYX plays a role in TFEB regulated autophagy signaling. The MK-STYX active protein had no such effect on the localization. This provides evidence that this effect is being driven by the “pseudo” MK-STYX, as the only difference between the active mutant and MK-STYX is two amino acids in the active motif. Additionally, as there is more TFEB localized away from the cell periphery; lysosomes, which are localized to the periphery when cells are nutrient
rich for more efficient signaling, may have a lesser effect on nutrient signaling upon MK-STYX expression.

Autophagosome localization affects efficiency

LC3B can be upregulated in a TFEB-dependent manner (Porter et al., 2013), indicating that a shift in TFEB localization may affect expression of LC3B. LC3B is an important marker for autophagosomes, and is used in some circumstances to measure autophagy activity (Barth et al., 2010). Within autophagy, LC3B acts as a substrate binding protein for linker protein-substrate complexes. This activity of LC3B makes it crucial in proper function of selective autophagy. Selective autophagy is the method used to degrade SGs; therefore, the role MK-STYX plays in regulating LC3B are important for our understanding. Upon long-term nutrient deprivation, autophagosomes and LC3B will be upregulated through TFEB. Interestingly, long lived pathological SGs would require long term autophagy activation for proper clearance of autophagosomes (Martini-Stoica et al., 2016). MK-STYX seemed to have little to no effect on the number of autophagosomes, and cells expressing MK-STYX also followed the general pattern of upregulation in response to nutrient deprivation. This doesn’t mean that MK-STYX didn’t play a regulatory role, as expression of MK-STYX also caused accumulations of autophagosomes along cellular extensions during nutrient starvation. As the normal localization of autophagosomes upon starvation is perinuclear (Korolchuk & Rubinsztein, 2011), this shift to cellular extensions by MK-STYX expression suggests a regulatory role for MK-STYX in autophagosomal transport. Autophagosomes will accumulate in the perinuclear region during starvation to increase efficiency of
autophagosome-lysosomal fusion. A shift to cellular extensions would go against this general paradigm and autophagic efficiency would be diminished considerably. That is, unless lysosomes can also be recruited to cellular extensions in a similar manner.

Lysosomes localization and autophagy signaling

Lysosomes and late endosomes each act to carry proteases and other degrading enzymes to autophagosomes. These enzymes work properly in the high pH environment of the mature autophagosomes to break down autophagic substrates into their respective “building block” molecules. The lysosomes and late endosomes are crucial in the proper recycling function of autophagy; cells to create new macromolecules when nutrients are scarce may use these building blocks. Lysosomal proteases and other important lysosomal proteins must be in high concentrations in the cell endogenously, as these proteins have low recycling efficiency. Lysosomes and late endosomes also carry proteins on their cytosolic side that are crucial for fusion and nutrient signaling. Important for this signaling is lysosomal localization. When a cell has all the nutrients it needs it would be wasteful to have upregulation of autophagy genes, as they are not needed. In contrast, when nutrients are not available, the cell needs to be able to upregulate certain genes for endogenous autophagy proteins to be replaced. Localization plays a key role in regulating autophagic signaling. When nutrients are being brought in by the cell, it is good for lysosomes to be localized at the cell periphery, to sense these nutrients and downregulate autophagy through TFEB phosphorylation. In contrast, when nutrients are not available lysosomes need to move towards the ER to ensure autophagosomes have lysosomes to fuse with (Dunlop & Tee, 2014).
Dysregulation of lysosomal localization, or improper lysosomal morphology each would contribute to dysregulation of autophagy signaling. MK-STYX caused a decrease in the number of lysosomes per cell in response to nutrient deprivation, while keeping the number of autophagosomes steady. While this decrease was not statistically significant, the trend of decrease in response to starvation shown in the data is noteworthy enough to follow. A lack of available lysosomes could indicate a decrease in autophagy flux, as the end of the autophagy cycle would be halted and proper recycling of autophagic substrates would not be accomplished. Or, perhaps the number of lysosomes was decreased due to an increase in lysosomal fusion with autophagosomes, which would visually show up as less lysosomes (Barth et al., 2010). At this point no definitive conclusion can be made to effects on flux as autophagic flux was not tested in this study.

Lysosomal “clumping”
MK-STYX also caused a clumping of lysosomes at the ends of cellular extensions during nutrient deprivation. Research into lysosomal clumping is lacking as not many researchers have seemed to present this phenotype. There have been studies on the lysosomal membrane protein, LAMP1, which showed lysosomal clumping when a LAMP1-GFP vector was overexpressed. Yet, this was shown to in the study to be caused by GFP-GFP anti-parallel dimerization. Upon removal of this dimerization domain, the clumping of lysosomes was abolished (Falcón-Pérez, Nazarian, Sabatti, & Dell'Angelica, 2005). Lysosomal trafficking requires protein complexes to bind to lysosomal membrane proteins in a manner similar to ER-golgi vesicle trafficking. One of
these complexes is called retromer, a protein complex responsible for lysosomal protein recycling. This method of transport is also crucial for recycling of LAMP1 back to newly formed lysosomes, and dysregulation of this system would have consequences for proper lysosomal localization and morphology. Members of the retromer complex are SNX5 and SNX6 (sortin nexin 5 and 6); SNX5 is also a potential binding candidate of MK-STYX (unpublished data) (Maruzs et al., 2015). Therefore, perhaps expression of MK-STYX in these nutrient deprived cells upregulates lysosomal transport to the ends of cellular extensions, as was the case with autophagosomes. As SNX5 and MK-STYX can bind, perhaps this interaction can also lead to formation of complexes with other lysosomal retromer complexes. In addition to this, MK-STYX expression also caused accumulations of LAMP1 to the plasma membrane, which indicates increased lysosomal fusion with the plasma membrane LAMP1 surface association has been found to be correlated to metastasis and cell-cell adhesion (Falcón-Pérez et al., 2005). This adds another level of signaling complexity that is regulated by expression of MK-STYX.

Conclusion and future directions
Through this work, we have shown that lack of catalytic activity does not mean “inactive.” The expression of MK-STYX has been found to regulate the “master-switch” of autophagy, dependent on being “inactive.” MK-STYX was found to have little effect on one side of autophagy (autophagosomes), while decreasing another (lysosomes), without any apparent compensatory mechanism. MK-STYX was also found to create
large-scale changes in localization of lysosomes and autophagosomes toward cellular extensions. This was found along with promising phenotypic observations of increased extensions in cells transfected with MK-STYX. These data require many more experiments to verify the mechanisms behind these changes, but, importantly, these data suggest that MK-STYX is acting to regulate autophagy. To bring this project back to its roots, we need to be able to connect this regulatory mechanism to SG clearance. To accomplish this, future studies would have to first determine the effect on overall autophagy activity. This study generated findings showing regulation of the players individually, but activity itself cannot be tested as these studies only can tell a story of the cells at the point they were fixed. Beyond activity, to verify what effects decreased lysosomes is having on activity, autophagic flux will also have to be tested. Finally, to verify the link between SG-MK-STYX-Autophagy, tests on ubiquitin-cargo-targeting activity would have to be assessed, as this is the hypothesized mechanism of SG targeting to autophagy.

Bibliography


Klionsky, D. J., Cregg, J. M., Dunn, W. A., Emr, S. D., Sakai, Y., Sandoval, I. V., ...


Mizushima, N., Yoshimori, T., & Ohsumi, Y. (2011). The Role of Atg Proteins in


lysosome via mTOR and TFEB. The European Molecular Biology Organization Journal, 31(5), 1095–108. https://doi.org/10.1038/emboj.2012.32


