Probing the function of polyP on signalling networks in mammalian systems

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ABSTRACT

Polyphosphates (PolyP) are linear chains of inorganic phosphates joined together by phosphoanhydride bonds. This polyanionic molecule has been found in all organisms and has been implicated in diverse biological processes such as cell growth and blood coagulation. Nonetheless, the mechanism of polyP's involvement in these processes and its impact at a molecular level is still unknown. In 2015, the addition of polyP chains to lysine residues was found to be a novel post-translational modification. However, a hurdle in studying the impact of polyP in the mammalian system is its low endogenous levels. In this study, I applied an ectopic expression system using *E. coli*-derived polyphosphate kinase 1 (PPK) in HEK293T cells to induce excess production of polyP. In mammalian cells, increased intracellular polyP levels lead to increased activation of both Erk1/2 and p70s6k. I also expanded our system by utilizing the yeast exopolyphosphatase, Ppx1, to deplete polyP. Overall, this work presents a novel role for polyP in mediating key signalling pathways and will help probe the function of polyP in mammalian cells.

AUTHORIZATION

Copyright

Contributions

In order to tell a complete story, I’m including Figure 7 which is modified from Charlotte Holinier’s work (Holinier, 2018) who prepared the samples and conducted the experiment. Also, Figure 8 was prepared by Dr. Emma Bondy-Chorney (Bondy-Chorney, Abramchuk, Nasser, Holinier, Denoncourt, Baijal, Mccarthy, et al., 2020) who also prepared the samples and conducted the experiment. For Figures 7 and 14, I carried out transfections and western blotting, while polyP extractions and analyses were done by Alix Denoncourt.

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
BSA	Bovine serum albumin
CTRL	Control
DAPI	4’,6-diamidino-2-phenylindole
DH5α	Doug Hanahan Strain 5
DMEM	Dulbecco’s modified eagle medium
DNA	Deoxyribonucleic acid
Ec	*Escherichia coli*
eIF5B	Eukaryotic translation initiation factor 5B
FBS	Fetal bovine serum
FGF-1	Acidic fibroblast growth factor
FGF-2	Basic fibroblast growth factor
GAPDH	Glyceraldehyde-3’-phosphate dehydrogenase
GO	Gene ontology
HEK293T	Human embryonic kidney cells, with T antigen of SV40
HGF	Hepatocyte growth factor

mTOR	Mechanistic target of rapamycin
NOP56	Human nucleolar protein 56
NHDF	Normal Human Dermal Fibroblasts
NMR	Nuclear Magnetic Resonance
PASK	Poly-acidic serine and lysine-rich
PBS	Phosphate-buffered saline
polyP	Polyphosphate
PPK	Polyphosphate kinase
PPN	Endopolyphosphatase
PPX	Exopolyphosphatase
PTM	Post-translational modification
PVDF	Polyvinylidene difluoride
qPCR	Quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation assay
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBST	Tris-buffered saline tween 20
TRP	Transient receptor potential

TWEEN 20	Polysorbate 20
VTC	Vacuolar transporter chaperone
WCE	Whole cell extract

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1. INTRODUCTION

Polyphosphates (polyP) exist in every organism and have been implicated in various biological processes in the mammalian system (Kornberg, Rao, & Ault-Riché, 1999). Furthermore, the addition of polyP chains to target proteins has recently been found to be a novel post-translational modification (PTM), called polyphosphorylation (Azevedo, Livermore, & Saiardi, 2015). However, its function and regulation are yet to be elucidated. To understand polyphosphorylation as a PTM, we must first understand the role of polyphosphate in general. **My project aims to identify whether elevated intracellular polyphosphate levels can impact signalling pathways in mammalian systems. This will help focus future efforts to follow up on polyphosphorylated proteins within key signalling pathways.**

1.1 PolyP is an ancient molecule of many functions

PolyP is a ubiquitous molecule made of long chains of inorganic phosphates that are linked by phosphoanhydride bonds **(Figure 1)** (Kornberg et al., 1999; Naranya N. Rao, Gomez-Garcia, & Kornberg, 2009). PolyP is speculated to have been present prebiotically, and currently exist in all kingdoms of: protists, bacteria, as well as all higher eukaryotic organisms (Naranya N. Rao et al., 2009). It ranges from 3 to thousands of residues in length (Kornberg et al., 1999). The enzymes responsible for the degradation of polyP in bacteria are Ppx1 and GPPA (Ppx2) **(Figure 2)** (Akiyama, Crooke, & Kornberg, 1993; Keasling, Bertscht, & Kornbergti, 1993). In bacteria, polyP is mainly synthesized by two highly conserved enzymes, PPK and Ppk2 (Ahn & Kornberg, 1990; Ishige, Zhang, & Kornberg, 2002). Ppk2 was found to be responsible for the source of polyP from *ppk* mutants of *P. aeruginosa* (Zhang, Ishige, & Kornberg, 2002). Most bacterial

species contain both enzymes. The enzyme PPK produces polyP from ATP, while Ppk2 can synthesize it from ATP as well as GTP. Interestingly, Ppk2 also often serves in the degradation of polyP, as its ability to use polyP to generate GTP is 75-fold higher than that to synthesize polyP from GTP (Ishige et al., 2002).

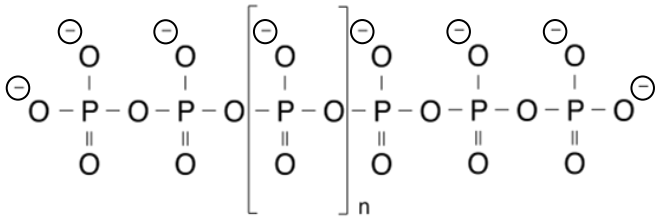

Figure 1. Linear structure of a polyP chain. Chains of polyP are joined together by phosphoanhydride bonds and range in size from 3 to 1000s of residues in length.

The concentration of polyP in bacteria typically ranges between 100-200mM (Kornberg et al., 1999). The range of polyP concentration varies between different bacterial strains. For example, in *E.* coli and *Pseudomonas aeruginosa*, the concentration of bacterial PolyP is increased by 100-fold in response to stresses like starvation (Kuroda, Murphy, Cashel, & Kornberg, 1997). In *E. coli*, increased polyP has a protective role, being linked to virulence and stress tolerance (Ault-Riché, Fraley, Tzeng, & Kornberg, 1998; Peng et al., 2016; Rashid, Rao, & Kornberg, 2000; Shiba et al., 1997). *E. coli* with genetically altered polyP concentrations experiences a growth lag after the stationary phase (Crooke, Akiyama, Rao, & Kornberg, 1994). *P. aeruginosa* with a null mutation in the *ppk* gene has impaired motility when plated on agar (Rashid et al., 2000). In *Proteus mirabilis*, *ppk* mutants are deficient for survival in oxidative, hyperosmotic, and heat stress conditions. There is also a reduction in the ability of the bacteria

to swarm and form biofilms (Peng et al., 2016). Overall, polyP is an ancient molecule which has been subject to widespread research topics.

1.1.1 PolyP in yeast – a well characterized system

A great amount of our knowledge about polyP metabolism comes from studies in *Saccharomyces cerevisiae*. In yeast, polyP is synthesized from ATP by the vacuole transporter chaperone complex (VTC complex) (Hothorn et al., 2009; Ogawa, DeRisi, & Brown, 2000). The concentration of polyP is highest in yeast compared to other organisms at >200mM (Kornberg et al., 1999). The highest concentrations of polyP accumulate inside the vacuole, making up to 20% of the cell dry weight (Brown & Kornberg, 2004; Gerasimaite & Mayer, 2016; Kornberg et al., 1999). In fact, 99% of yeast polyP is contained within the vacuole (Kornberg et al., 1999), Interestingly, this localization is crucial as accumulation of cytoplasmic polyP in yeast is toxic (Gerasimaite, Sharma, Desfougères, Schmidt, & Mayer, 2014). Yeast polyP is degraded by Ppx1, Ppn1, Ppn2 and Ddp1 **(Figure 2)** (Krishnanand D Kumble & Kornberg, 1996; Lonetti et al., 2011; Ta Gerasimaitė & Mayer, 2017; Wurst & Kornberg, 1994)**.** The exopolyphosphatase 1 (Ppx1) favourably removes terminal phosphates (Pi) from longer polyphosphate chains around 250 residues in length, but is able to process chains ranging from 3 to more than 500 Pi residues in various locales of the cell (Wurst & Kornberg, 1994). These include the cytosol, nucleus, plasma membrane, as well as the mitochondrial matrix (Lidia P. Lichko, Andreeva, Kulakovskaya, & Kulaev, 2003; Lidiya P. Lichko, Kulakovskaya, & Kulaev, 2006). Meanwhile, The diphosphoinositol polyphosphate phosphohydrolase 1 (Ddp1) endopolyhosphatase is a cytosolic and nuclear enzyme also favouring the breakdown of longer polyP chains and acting at

internal phosphoanhydride bonds (Lonetti et al., 2011). Furthermore, the endopolyphosphatases Ppn1 and Ppn2 are responsible for the breakdown of smaller chains in the vacuole (L. Lichko et al., 2006; Ta Gerasimaitė & Mayer, 2017).

PolyP chains serve as energy storage in yeast, providing a pool of phosphates upon need that can activate precursors of fatty acids, phospholipids, polypeptides, and nucleic acids (Naranya N. Rao et al., 2009). This phosphate reserve is crucial to maintain homeostasis in response to stressful conditions. In fact, deletion of *VTC4*, a member of the Vtc4 complex, results in defects in microautophagy (Uttenweiler, Schwarz, Neumann, & Mayer, 2007). Furthermore, polyP serves as a metal chelator for cations (Jen & Shelef, 1986).

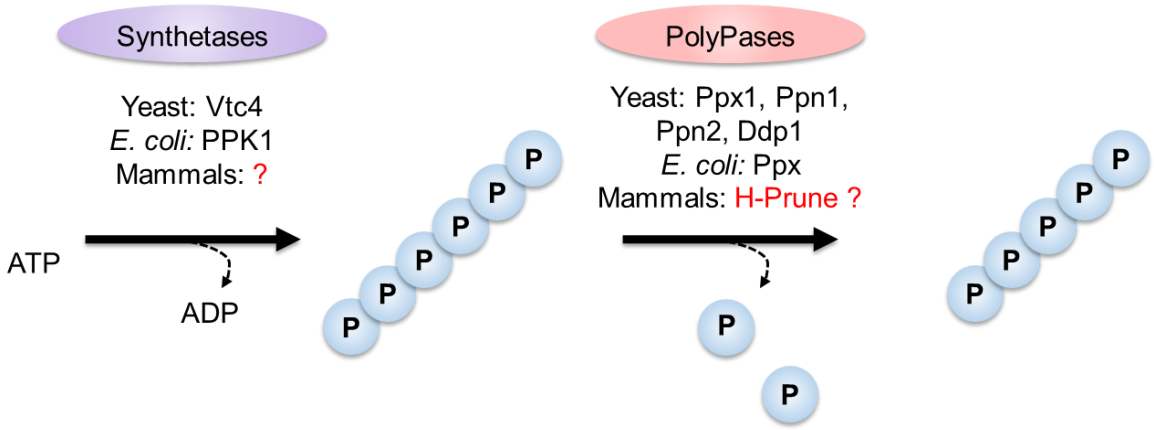

Figure 2. Metabolic enzymes of polyP. PolyP is synthesized from ATP by synthetases and degraded by polyphosphatases. The machinery required for synthesis of polyphosphates transfers terminal phosphates from ATP to polyP chains linked by phosphoanhydride bonds. Synthetases remain unknown in humans, and the activity of the proposed polyphosphatase, H-prune, has not been determined *in vivo*. In yeast and *E. coli,* however, polyP is degraded by

both endo- and exopolyphosphatases that are able to cleave chains in the middle, or remove terminal phosphates, respectively.

1.1.2 PolyP in the mammalian system – a one-sided approach

Although well studied in yeast, much about polyP in mammals remains unknown. In humans, polyP remains at a low cellular concentration of 25-120 μM (K. D. Kumble & Kornberg, 1995). Specifically, polyP in mammalian systems accumulates to its highest concentration in secretary organelles of platelets called dense granules, in mitochondria, as well as in the nucleoli (Jimenez-Nuñez et al., 2012; Ruiz, Lea, Oldfield, & Docampo, 2004). PolyP has diverse roles; in dense granules, polyP serves as a pro-coagulant by inducing clotting (Smith et al., 2006). In mitochondria, polyP has been found to play a role in mitochondrial permeability transition pore (mPTP) activation by regulating its Ca^{2+} efflux (Seidlmayer, Gomez-Garcia, Blatter, Pavlov, & Dedkova, 2012). Mice with depleted polyP levels have reduced muscular mitochondrial respiration and elevated lactic acid levels (Nakamura et al., 2018). Additionally, MCF-7 mammary cancer cells with a depletion of polyP have a deficiency in stimulating phosphorylation of the mechanistic target of rapamycin (mTOR) substrate, the initial factor 4E-binding protein (PHAS-I) (Wang, Fraley, Faridi, Kornberg, & Roth, 2003). PolyP is also able to modulate mitogenic activity in normal dermal fibroblasts (NHDF), where it was found to physically interact with the basic fibroblast growth factor (FGF-2) and enhance its ability to bind to cell surface receptors (Shiba et al., 2003). Furthermore, the covalent attachment of polyP chains on proteins has also been found to be a novel post-translational modification (PTM), termed polyphosphorylation (Azevedo et al., 2015).

1.2 Polyphosphorylation – a novel post-translational modification

In 2015, the addition of polyphosphate chains to lysine residues on proteins was found to be a novel PTM. This modification occurs non-enzymatically onto poly-acidic, serine, and lysine rich (PASK) motifs (Azevedo et al., 2015). This PTM was first discovered in the *S. cerevisiae* system, with the only method of detection being an electrophoretic shift on NuPAGE gels where polyphosphorylated proteins undergo a retardation in electrophoretic mobility **(Figure 3)**. Using a yeast mutant with impaired polyP synthesis, Azevedo *et al.* compared mobility shifts to screen for polyphosphorylated proteins relative to wild type (2015). This new PTM cannot be detected by any other method; on SDS-PAGE gels, there are no electrophoretic shifts for polyphosphorylated targets. Nonetheless, the molecular function and regulation of polyphosphorylation is poorly understood.

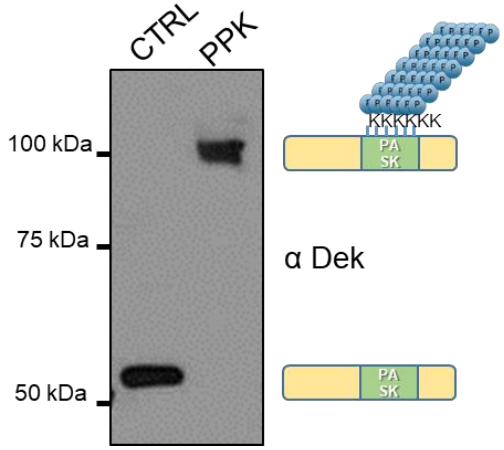

Figure 3. NuPAGE gel shift of the polyphosphorylated protein DEK in HEK293T cells. Ectopic expression of PPK in HEK293T cells reveals that DEK is polyphosphorylated. When polyP is elevated, the polyphosphorylated protein has an electrophoretic mobility shift on NuPAGE gels.

1.3 Overview of the function of polyP in mammalian cells

In mammalian systems, polyP is implicated in a host of biological processes ranging from blood coagulation to cell growth and division (Travers, Smith, & Morrissey, 2015). However, the synthesis and molecular function of polyP is less understood. A hurdle in studying the impact of polyP in the mammalian system is its low endogenous levels. Furthermore, its synthetic machinery is unknown. Mammalian alkaline phosphatase derived from calf intestines acts as a powerful exopolyphosphatase, but its role in polyP metabolism is yet to be elucidated (Lorenz & Schröder, 2001). The exopolyphosphatase H-Prune, the homolog of drosophila Prune, has been proposed as a polyPase, but its role has not been determined *in vivo* (Tammenkoski et al., 2008). The traditional methods of studying the effect of elevated polyP concentrations in the mammalian system are generally achieved by the addition of commercial polyP chains to culture serum (Hassanian, Dinarvand, Smith, & Rezaie, 2015). A caveat of this approach is its reliance on the internalization of polyP, along with its interaction with extracellular receptors. Alternatively, the yeast polyphosphatase Ppx1 has been used to deplete polyP. Wang *et al*. first applied this approach to reveal that polyP stimulates the activation of the mechanistic target of rapamycin (mTOR) signalling in MCF-7 human breast carcinoma cells (Wang et al., 2003). Hassanian *et al*. subsequently found that the activation of mTOR is mediated through the interaction of polyP with the receptor for advanced glycation end products (RAGE) and the $P2Y_1$ receptor (2015). This will be further discussed in the next chapter. In the mitochondria, Ppx1-mediated depletion of polyP has shown a role for polyP in the mitochondrial permeability transition pore (mPTP) activation by regulating

its Ca^{2+} efflux (Seidlmayer et al., 2012). While depleting polyP through the use of Ppx1 can probe its function in mammalian cells, elevating polyP intracellularly with *E. coli* PPK allows for studying the role of polyP in physiologically relevant scenarios, such as in response to bacterial pathogens.

1.4 PolyP and mTOR signaling

PolyP has been implicated in the activation of mTOR signalling in breast cancer cells (Wang et al., 2003). The protein kinase mTOR serves to coordinate cellular growth and proliferation though the intersection of mitogenic signals via shared effectors (Guha et al., 2001). This sets mTOR to be a master regulator which has been implicated with aberrant cell proliferation when misregulated in mammary cancer cells (Wang et al., 2003). Unlike in lower eukaryotes, which have two TOR genes, humans have only one. This serine-threonine kinase is composed of two distinct functional complexes, mTOR complex 1 and 2 (mTORC1 and mTORC2, respectively) (Schmelzle & Hall, 2000). PHAS-I is a downstream target of mTOR, which when phosphorylated, releases eIF4E (Eukaryotic Translation Initiation Factor 4E). eIF4E is an mRNA cap binding subunit that allows for the initiation of translation of mRNA encoding key cell growth and proliferation proteins (Martin & Blenis, 2002). Wang *et al*. reveal that polyP is able to stimulate phosphorylation of PHAS-I (2003). This activation was present in a chain length and concentration dependent manner, with polyP chains of 15-750 residues at 0.15-1.5 µM from western blots assessing its phosphorylation (Wang et al., 2003). Furthermore, mTOR has previously been reported to autophosphorylate when incubated with ATP alone (Scott, Brunn,

Kohn, Roth, & Lawrence, 1998). PolyP is able to increase *in vitro* autophosphorylation of mTOR in presence of ATP (Wang et al., 2003)

Hassanian *et al*. found that polyP interacts with the receptor for RAGE and the $P2Y_1$ receptor to activate both mTORC1 and mTORC2 (2015). Interestingly, when looking at the upstream extracellular signal-regulated kinases, polyP also induces phosphorylation of Erk1/2 (Hassanian et al., 2015). However, the activation of mTORC1 by polyP was found to be Erk1/2-independent (Hassanian et al., 2015). No further study was conducted to explore whether this has an effect on proteins downstream of Erk1/2 and its consequence on cell metabolism. We have assessed the effect of elevated polyP levels inside the cell on signalling cascades that work in parallel with mTOR and have shared upstream regulators, such as the Ras/MEK/Erk1/2 cascade **(Figure 4)**.

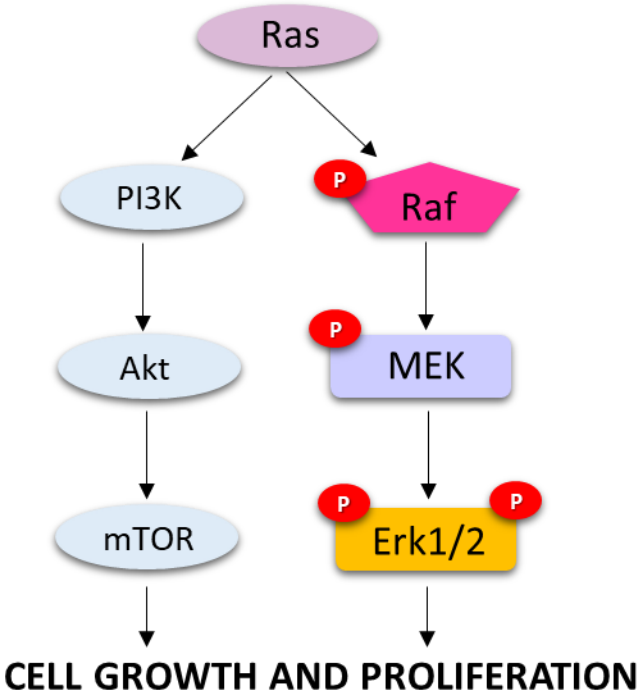

Figure 4. Interplay of the PI3K/Akt/mTOR and Ras/Raf/MEK/Erk signaling. Ras is classically activated by growth factors receptors that are induced by a variety of signals including growth factors, hormones and cytokines. Both cascades consequentially modulate effectors that regulate the growth and proliferation of cells.

1.5 Other functions of polyP

1.5.1 PolyP and ion channel activation

PolyP has also been studied in the context of ion channel activation. The transient receptor potential (TRP) family of channels is thought to be responsible for sensing cold temperatures in the peripheral nervous system (Dhaka et al., 2007). Kim and Cavanaugh investigated a role for polyP in the regulation of a member of the mammalian TRP superfamily, TRPA1 (2007). In HEK293T cells, polyP chains of 2-65 residues in length were required for preserving TRPA1 in its functional conformation as well as sensitizing it to exposure to strong chemicals (Kim & Cavanaugh, 2007). Furthermore, there appeared to be a weak interaction between TRPA1 and polyP as the channel activity was diminished with polyP removal (Kim & Cavanaugh, 2007). Zakharian *et al*. have revealed a role for polyP in the regulation of another member of the TRP superfamily, TRPM8 (2009). TRPM8 channel is activated by low temperatures, compounds such as menthol, as well as voltage, pH changes, and fatty acids (Andersson, Chase, & Bevan, 2004; Andersson, Nash, & Bevan, 2007; Behrendt, Germann, Gillen, Hatt, & Jostock, 2004; Colburn et al., 2007). Depleting polyP in HEK293T and F-11 neuronal cells inhibits TRPM8 channel activity (Zakharian et al., 2009). Furthermore, polyP was

found to associate and form a stable complex with TRPM8 (Zakharian et al., 2009). These studies highlight a role for polyP in the regulation of mammalian ion channels.

1.5.2 PolyP regulates fibroblast growth factors

Several links between polyP and mitogenic activity of fibroblast growth factors have been established. Shiba *et al*. have shown that addition of polyP alone to NHDF cells with hepatocyte growth factor (HGF) stimulates their proliferation (2003). The mitogenic activity of both the acidic fibroblast growth factor (FGF-1) as well as the basic fibroblast growth factor (FGF-2) were enhanced with the addition of polyP (Shiba et al., 2003). Furthermore, polyP was found to promote stable oligomerization of FGF-2 and enhance its binding to cell surface receptors (Shiba et al., 2003). In contrast, Han *et al.* reported that polyP inhibited mitogenic activities by inhibiting bFGF binding to its cell-surface receptor (Han et al., 2007). The effect of polyP on FGF receptor signaling is debatable but might be explained by the use of different concentrations in each study. The mechanism by which polyP is involved in the interaction between these fibroblast growth factors has yet to be elucidated.

1.5.3 PolyP may be involved in apoptosis

Hernandez-Ruiz *et al*. revealed a role for polyP in apoptosis in human plasma cells (PC) (2006). Upon addition of polyP to U266 myeloma cells, immunoglobulin (Ig) secretion was inhibited and apoptosis was stimulated (Hernandez-Ruiz et al., 2006). Addition of polyP to myeloma cells also lead to cell cycle arrest and activation of the calcium-dependent endonuclease caspase-3 (Iguchi, Miyakawa, Yamamoto, Kizaki, & Ikeda, 2003). Caspase-3 is a

key mammalian enzyme responsible for the cleavage of DNA during apoptosis (Iguchi et al., 2003). This study proposes a role for polyP in the regulation of apoptosis in mammals.

1.5.4 PolyP modulates blood clotting and inflammation

As previously discussed, mammalian polyP accumulates to the highest concentrations in calcium-rich dense granules (Ruiz et al., 2004). These specialized organelles resemble acidocalcisomes that are responsible for calcium flux into the cytosol of several organisms including trypanosomes and slime molds (Docampo, Scott, Vercesi, & Moreno, 1995; Marchesini, Ruiz, Vieira, & Docampo, 2002). Using urea-polyacrylamide gel electrophoresis, Ruiz *et al.* found that polyP ranges from 70-75 residues in length in the dense granules of human platelets (Ruiz et al., 2004). The concentration of polyP in these organelles was measured by hydrolysis with a yeast pyrophosphatase and was found to be approximately 1.1 mM (Ruiz et al., 2004).

PolyP has been found to be involved in the signalling that initiates blood clotting. Upon activation of platelets, polyP is released which activates a key pathway involved in inflammation, the contact pathway (Smith et al., 2006). This promotes the activation of factor V, which counteracts the anticoagulant protein called tissue factor pathway inhibitor (TFPI) (Smith et al., 2006). Furthermore, polyP is able to delay blood clotting by enhancing a natural antifibrinolytic agent, the thrombin-activatable fibrinolysis inhibitor (Smith et al., 2006). PolyP released from activated platelets was also found to bind and activate the plasma protease factor XII (FXII) (Müller et al., 2009). The effect of polyP on blood clotting appears to be dependent on chain length. PolyP of 500 phosphate units or greater were best at activating the

contact pathway, while shorter polymer of ~100 phosphates were optimal for factor V activation and counteraction of the tissue pathway inhibitor (Smith et al., 2010).

In endothelial cells, polyP 45, 65, and 70 units in length was found to activate NF- κB to elicit proinflammatory responses (Bae, Lee, & Rezaie, 2012). This also enhanced the adhesion of monocytic cells to polyP-stimulated endothelial cells (Bae et al., 2012). Hassanian *et al*. used inhibitors and siRNA to uncover that mTORC1 was involved in polyP-mediated NF- κB activation (2015). This presents a link between proinflammatory responses and the mTOR signalling pathway.

1.6 Novel approach to studying polyP biology in mammalian systems

To overcome the difficulties posed by the low endogenous polyP levels and the absence of known polyP synthetases in mammalian cells, our lab has designed a system to ectopically express the *E. coli* polyphosphatase kinase 1 gene (*ppk*) in human cells (Bentley-DeSousa et al., 2018). Using this method, we can induce the production of excess polyP inside the cell **(Figure 5)**. Notably, with this method, our lab was able to identify the first six mammalian targets of polyphosphorylation in 2018. These proteins are Nucleolin, NOP56, Mesd, eIF5b, UPF3B, and DEK (Bentley-DeSousa et al., 2018). Ki *et al*. presented the first report of expression of bacterial *ppk* in mammalian cells (2007). The group was able to subclone *ppk* into the mammalian expression vector pcDNA3.1(+). They then expressed *ppk* in HEK293T cells which enabled them to measure its activity via ^{31}P-magnetic resonance spectroscopy (MRS) and magnetic resonance imaging (MRI), and visualize polyP via toluidine blue staining (Ki et al., 2007). There have been no efforts to apply this system to further probe the function of polyP in mammalian cells.

Studying polyP biology in humans is a fundamental step to uncover the effects of this of PTM. Although polyP has been implicated in a diverse set of biological processes, all available literature relies on techniques that evaluate the effect of polyP when it is added extracellularly. My project will provide a novel approach to studying polyP biology by evaluating the effect of increased polyP levels inside the cell, which I can validate by probing for known mammalian targets of polyphosphorylation **(Figure 3)**. Alternatively, I can compare amounts of polyP produced with polyP extraction methods. In my work, I have taken advantage of this ectopic expression system to study the function of polyP in higher eukaryotes, with my focus being on uncovering signalling pathways that are affected by PPK expression.

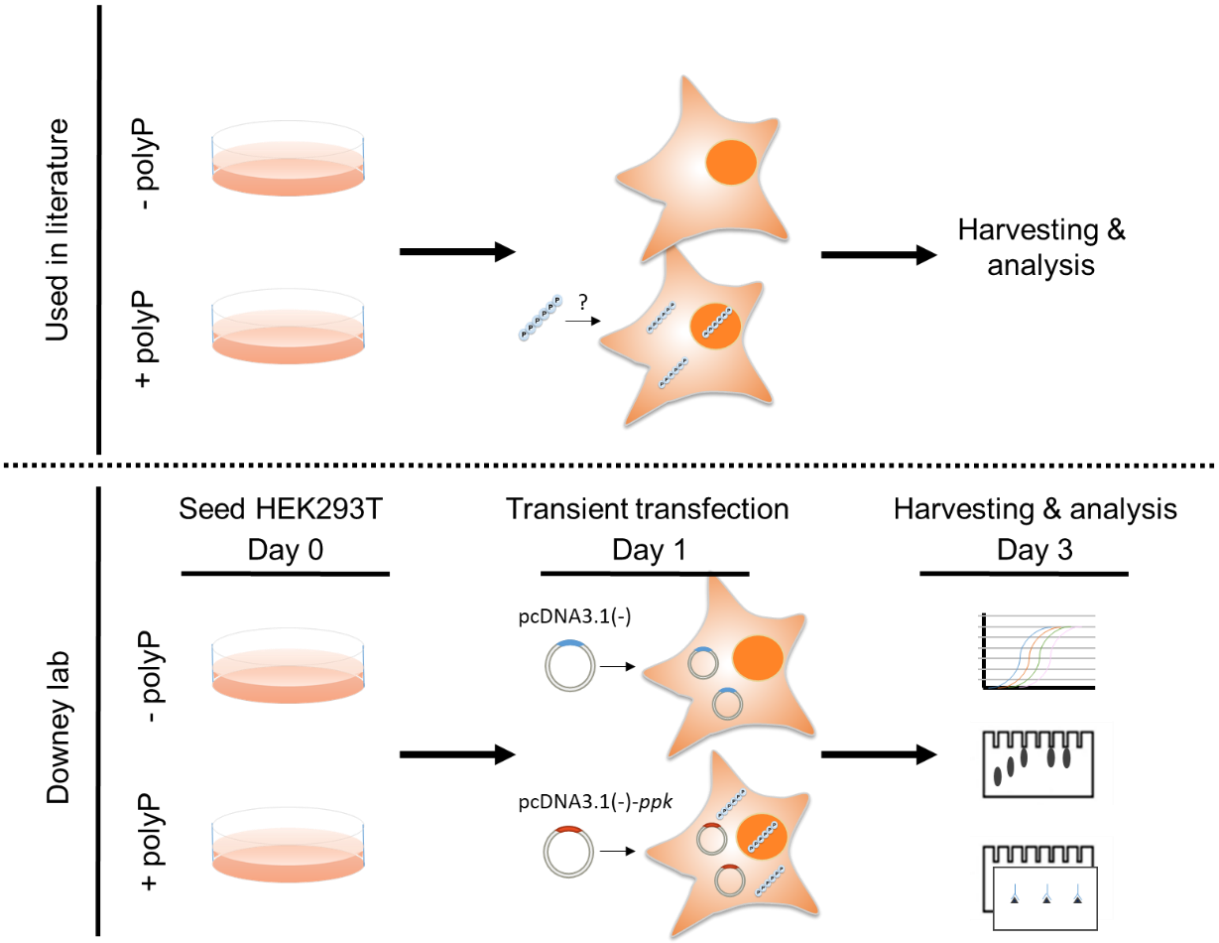

Figure 5. Comparison of available methods to increase the concentration of polyP in mammalian cells. While traditionally various length chains of polyP are added to culture media to increase polyP concentrations in mammalian cells, I have employed a novel method to increase polyP levels inside cells. To do so, HEK293T cells are transfected with an empty vector or a *ppk* expression plasmid. Cells are then harvested 48 hours after transfection and analyzed using western blotting with SDS-PAGE and NuPAGE gels, qPCR, and polyP extractions.

1.7 RNA sequencing reveals differential expression of transcriptional regulators

To explore the role of intracellularly-expressed polyP in human cell lines, we used our novel system to induce ectopic expression of PPK and uncover genes differentially regulated by increased polyP levels. To do this, we used RNA sequencing as an unbiased large-scale method to uncover any changes in gene expression in HEK293T cells when polyP levels are elevated intracellularly.

Among these differentially regulated genes, the Early growth response protein 1 (EGR1) transcriptional regulator was up-regulated in the presence of excess polyP (Holinier, 2018). EGR1 expression relies on interaction with the Elk-1 transcription regulator which is driven by the serum response element (SRE) (Mayer, Willars, Nishida, & Thiel, 2008). The mechanism by which polyP may drive the increase of EGR1 gene expression is yet to be explored. PolyP may be involved in the regulation of EGR1 expression at several levels. For example, PolyP could increase EGR1 expression by interacting directly with the EGR1 promoter, indirectly via signalling molecules that regulate EGR1 transcription, or a combination of both. Uncovering

how polyP increases EGR1 expression will help in our understanding of the role of polyP in mediating signalling networks that have diverse biological impacts in humans.

1.8 Project Rationale

PolyP has been implicated in a host of biological processes. The molecular function of polyP in these processes has yet to be determined. Current literature has relied on the extracellular addition of polyP to study its role in the mammalian system (Hassanian, Ardeshirylajimi, Dinarvand, & Rezaie, 2016; Wang et al., 2003). In contrast, our system looks at the effect of increased polyP within the cell. I take advantage of our novel system to study whether polyP made inside the cell can regulate signalling proteins as previously reported with extracellular polyP.

1.9 Hypothesis and Objectives

Hypothesis: I hypothesize that PPK expression will effect mammalian signaling pathways that have previously been reported to be regulated by the addition of polyP outside of the cell, For example: Erk1/2.

I have two objectives:

Objective 1: Determine whether polyP regulates Erk1/2 signaling.

Rationale: Although exogenous polyP has been reported to increase phosphorylation of Erk1/2, our system is unique because it is the first to express PPK inside cells. This system may elucidate polyP's role in cell growth, as well as explain the mechanism of interaction of polyP with proteins within the Erk1/2 signalling pathway that have previously been observed to be regulated by polyP.

Objective 2: Design a method to modulate the levels of polyP produced.

Rationale: Our lab has applied a novel system to elevate intracellular levels of polyP in mammalian systems. However, no efforts of expanding this system have been made. Improvement of this system would enable us to further understand the molecular function of polyP in mammalian cells.

2. MATERIALS AND METHODS

2.1 Determining whether polyP regulates Erk1/2 signalling

2.1.1 RNA extractions

To isolate RNA, cells were harvested in 1 X PBS then 1 mL TRIzol reagent was added (Life Technologies, 15596026). 200 µL Chloroform was then added, followed with a spin at 12000 RCF at 4 °C. 500 µL of isopropanol was then added to the aqueous phase and the sample was incubated at -20 °C. The samples were then spun at 12000 RCF to remove the pipette and ethanol was added to the pellet. The samples were again spun at 7600 RCF at 4 °C. Finally, the pellet was dried and resuspended in 25 µL water.

2.1.2 RNA sequencing preparation

Three biological replicates of HEK293T transiently transfected with either pcDNA3.1(-) or pcDNA3.1(-)-*ppk* were used for the RNA-Seq. 600 µL of cells were harvested 48hrs after transfection. The harvested cells in PBS were extracted with the miRNAeasy Mini Kit (Qiagen 217004). Protein was also isolated from these samples for analysis on NuPAGE gels to confirm polyphosphorylation shifts for known targets, DEK and MESD. RNA pellets were then stored at -80 °C and shipped on dry ice to Genome Quebec Innovation Centre where the collected RNA was sequenced. The Canadian Centre for Computational Genomics conducted the bioinformatics analysis. A detailed protocol of the RNA sequencing steps as well as computational analysis is available in Bondey-Chorney *et al.* (2020).

2.1.3 qPCR prep for RNA-seq validation

RNA was combined with 5 X All-In-One RT Master Mix (Applied Biological Materials, G490) for cDNA synthesis. The conditions for reverse transcription were 25 °C for 10 minutes, 42 °C for 50 minutes, and 85 °C for 5 minutes. cDNA was used immedietly or stored at -20 °C. qPCR Primers were designed using the NCBI Primer-BLAST program with the following parameters: product size between 80-200 bps, Tm=~60 °C (±3 °C), GC content >50%. For qPCR reactions, cDNA was combined with SYBR Green Supermix (Bio-rad, 1708880**)**, as well as primers designed for corresponding genes. The conditions used were 95 °C 30 seconds, 60 °C 30 seconds, 72 °C 45 seconds)×40 cycles, 72 °C 10 minutes. For standard curves, cDNA was diluted by a factor of ten for 3 technical replicates and normalized with a control gene.

2.1.4 Cell culture

HEK293T cell lines (ATCC CRL-3216) used were female. The cells were cultured in DMEM (Wisent, 319-015-CL) supplemented with 10% Fetal Bovine Serum (Wisent, 085-150), 1% 1 mM sodium pyruvate (Thermo Fisher, 11360), and 1% Penicillin-Streptomycin-Amphotericin B solution(Wisent 450-201-EL). Cells were incubated at 37 °C and 5 % CO_2 in 10 cm polystyrene petri dishes and transferred to 6-well polystyrene upon seeding.

2.1.5 Transfection and drug treatment

Cells were transiently transfected at ~75% confluency with 2 μg of the control plasmid (pcDNA3.1(-)), *ppk* expression plasmid (pcDNA-3.1(-)-*ppk*), the codon optimized *ppk* expression plasmid (pcDNA3.1(-)-co*ppk*), or the *PPX1* expression plasmid (pcDNA3.1(-)-*PPX1*). The

transfections were conducted using Lipofectamine 2000 (Thermo Fisher, 11668). The media was changed to Opti-MEM reduced Serum Medium for 48 hours before harvesting (Thermo Fisher, 319850070). For drug treatments, 0.5 µM SCH772984 (Cedarlane, HY-50846) or 10 µL DMSO (vehicle) were added to cells for 24 hours before collection. For the MEK inhibitor experiments, 1 µM of U0126 (Selleck Chemicals, S1102) was added to cells for 2 hours before collection.

2.1.6 Whole cell extractions

Cells were harvested 48 hours after transfection in ice-cold 1 X PBS. Cells were then centrifuged at 5000 RPM for 5 min. 150 µL of RIPA Lysis buffer (50 mL ice cold lysis buffer [10 mM Tris-HCl pH 7.4, Hydrochloric acid (Fisher Scientific, A144 212), 100 mM NaCl (Fisher Scientific, AM9759), 1 mM EDTA (Sigma-Aldrich, 03690-100), 1% IGEPAL (Sigma-Aldrich, I8896), 0.5% Sodium Deoxycholate (Sigma-Aldrich, D6750), 0.1% SDS (Thermo Fisher Scientific, 15525-017)), protease inhibitor cocktail (Sigma-Aldrich, 4693159001) and sodium fluoride (Sigma fluoride, 201154) were then added to the cell pellets followed by incubation on ice for 25 minutes to lyse the cells. Cells were then centrifuged at 13500 RPM for 25 min at 4 °C. The supernatant was combined with 5 X Laemmli Sample Buffer and boiled at 100 °C. The samples were then either used immediately or stored at -20 °C.

2.1.7 Western blotting & immunoblotting

Cells extracts were run on % SDS-PAGE or 4-12 % Bis-Tris NuPAGE gels. For SDS-PAGE, the samples were separated on 13% SDS-PAGE gels run at 100 V for the stacking layer of the

gel, and then 160 V for the resolving portion of the gel. The SDS-PAGE Running buffer used for the run was comprised of 100 mL of 10 X 1L stock [30.2 g Tris Base (Fisher Scientific, BP152-5), 188 g glycine (Sigma-Aldrich, G7126), 10 g SDS (Thermo Fisher Scientific, 15525-017), filled to 1L with ddH_2O] and 900 mL of ddH_2O. The gel was then transferred onto a polyvinylidene fluoride membrane (Bio-Rad, 162-0177) at 85 V for 1 hour and 10 minutes in SDS-PAGE transfer buffer comprised of 100 mL of 10 X 1L stock [30.275 g Tris Base (Fisher Scientific, BP152-5), 166.175 g glycine (Sigma-Aldrich, G7126)], 200 mL methanol (Fisher Scientific, A412P-4), filled to 1L with ddH_2O) and 700 mL ddH_2O.

For NuPAGE gels, samples were separated on a 4-12% Bis-Tris NuPAGE gels (Thermo Fisher Scientific, NP0336BOX) at 200 V with 1 X NuPAGE running buffer comprised of 50 mL of 20 X 1L stock [209.2 g MOPS (Sigma-Aldrich, M1254), 121.1 g Bis-Tris (SigmaAldrich, B9754), 20 g SDS (Thermo Fisher Scientific, 15525-017), 12 g EDTA (Sigma-Aldrich, 03690-100ml), filled to 1L with ddH_2O] and 950 mL ddH_2O. The gel was then transferred onto a polyvinylidene fluoride membrane (Bio-Rad, 162-0177) at 85 V for 1 hour and 10 minutes with a 1 X NuPAGE transfer buffer comprised of 50 mL of 20 X 1L stock [81.6 g bicine (Sigma-Aldrich, B3876), 104.8 g Bis-Tris (Sigma-Aldrich, B9754), 6 g EDTA (Sigma-Aldrich, 03690-100ml), filled to 1L with ddH_2O], 200 mL methanol (Fisher Scientific, A412P-4) and 750 mL ddH_2O.

For both types of gels, the PVDF membrane were blocked on a shaker at room temperature in 5 % BSA (VWR, 97061-416) in 1 X TBST [100 mL of 10 X 1 L TBST stock (90 g Tris base (Fisher Scientific, BP152-5), 88 g NaCl (Fisher Scientific, AM9759), 2 g KCl (Sigma-Aldrich 746436), adjusted pH with HCl to 7.5, filled to 1 L ddH_2O), 10 mL of 10% Tween 20 (Fisher

Scientific BP337500) and 850 mL ddH2O]. The membranes were then incubated on a shaker with primary antibodies diluted in 5% BSA in 1 X TBST overnight at 4 °C or for 1 hour at room temperature. The membrane was then washed with 1 X TBST at room temperature three times, 10 minutes for each wash. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 25 minutes. Next, the membranes were again washed with 1 X TBST at room temperature three times, 10 minutes for each wash. Finally the membranes were incubated with Luminata Forte Western HRP Substrate solution (Fisher Scientific, WBLUF0500). The membrane were then exposed to chemiluminescent autoradiography film (Harvard Apparatus Canada, DV-E3018). The list of antibodies used and their sources is listed in **table 2**.

2.1.8 Statistical Analysis

Western blots were quantified using the Image J software. The statistical details of each experiment are presented in the respective figure legend. Overall, figures quantified are representative images for biological replicates of $N \geq 3$. For quantification, N numbers 3-8 were used. The Student's t-test was used in Excel. To assess differential protein expression, a two-tailed, two-sample, student's t test assuming unequal variance was used.

Table 1. Antibodies used for western blotting for SDS-PAGE and NuPAGE gels

Target	Dilution used in western blots	Vendor	Catalogue number
Beta-Actin HRP	1:40,000	Santa Cruz Biotechnology	Sc-47778
Dek	1:1,000	Santa Cruz Biotechnology	136222
EGR1	1:500	New England Biolabs	4154S
eIF5B	1:200	Santa Cruz Biotechnology	393564
GAPDH HRP	1:40,000	VWR	85760
Goat anti-Mouse	1:10,000	Biorad	172-1011
Goat anti-Rabbit	1:10,000	Biorad	170-6515
MESD	1:1,000	New England Biolabs	2763S
NOP56	1:1,000	Cedarlane Labs	A302-718A
Nucleolin	1:5,000	Abcam	22758
Phospho p-44/42	1:1,000	Cell Signaling Technologies	9101S
Ppx1	1:10,000	Abcam	225684
Total p44/42	1:1,000	Cell Signaling Technologies	4695S
Phospho p70s6k	1:1,000	Cell Signaling Technologies	9234S
Total p70s6k	1:1,000	Cell Signaling Technologies	2708S
Vinculin	1:10,000	Abcam	Ab129002

2.2 Expanding the current ectopic expression system to modulate polyP concentrations

2.2.1 Plasmids

The subcloning of the *E. coli ppk* into the mammalian expression vector pcDNA3.1(-) containing a bleomycin resistance marker is described in detail in Bentley-DeSousa *et. al* (2018). This sequence is available online through Addgene (Plasmid #108850). The optimized sequence of *E. coli ppk* was subcloned from the pUC57 backbone into pcDNA3.1(-). Gibson assembly was used to insert the codon-optimized *ppk* gene into pcDNA3.1(-) with the restriction enzymes *Bam*HI (NEB R3136S) and *Hind*III (NEB R3104S). The primers used were TCCACCACACTGGACTAGTGATGGGGCAGGAAAAACTGTAT and TGATCAGCGGTTTAAACTTATTATTCGGGCTGTTCCAGGC. For pcDNA-3.1(-)-*PPX1*, *Eco*RI (NEB R3101T) and *Bam*HI (NEB R3136S) were used with the primers ACTGTGCTGGATATCTGCAGATGTCGCCTTTGAGAAAG and TAAGCTTGGTACCGAGCTCGTCACTCTTCCAGGTTTGAG. All sequences were confirmed by Sanger Sequencing (Génome Québec Innovation Centre).

2.2.2 Codon optimization

The sequence of *E. coli ppk* sequence was sent to GenScript for codon optimization. The final codon optimized synthesized gene was cloned into the pUC57 vector containing a kanamycin resistance marker. The codon usage bias in humans was changed by upgrading the codon adaptation index from 0.72 to 0.95. The GC content of the sequence was increased to

prolong half-life of the mRNA. Negative cis-acting sites (splice and destabilizing elements) were modified. Also, Stem-Loop structures which affect the stability of mRNA were broken.

2.2.3 Plasmid transformation

Ready for use DH5α competent cells were used for all transformations (Thermo Fisher Scientific, 18263012). 2 ng of DNA was added to the thawed competent cells and incubated on ice for 30 minutes. Cells were then heat shocked at 42 °C before 250 µL of super optimal broth medium was added (New England Biolabs, B9020S). The cells were then incubated at 37 °C while shaking at 225 rpm for 60 minutes before being spread and grown on LB plates with either ampicillin or kanamycin for a final concentration of 100 µg/mL (Wisent, 400-110-IG and 400-145-IG). After overnight incubation at 37 °C, colonies were resuspended in 3 mL of LB with 3 µL ampicillin or kanamycin. After overnight incubation at 37 °C, plasmids were isolated from bacterial cultures using the QIAprep Spin Miniprep kit protocol (Qiagen 27104). For higher yield, colonies were resuspendend in 300 mL of LB with 30 µL ampicillin or kanamycin, followed by maxiprep using the Purelink HiPure plasmid maxiprep kit protocol (Thermo Fisher Scientific).

2.2.4 PolyP extraction

Harvested cell pellets were suspended in 400 µL LETS buffer at 4 °C (100 mM LiCl, 10 mM EDTA, 10 mM Tris-HCl pH 7.4, 0.2 % SDS). The cells were then transferred into a mix of 600 µL neutral phenol pH 8 and 150µL RNAse free water and then vortexed for 20 seconds. After this step, the samples were heated for 5 minutes at 65 °C, then cooled down on ice. 600 µL of chloroform was then added and the cells were vortexed for 20 seconds. The samples were then

centrifuged at 13,000 RCF for 2 minutes at room temperature. The water phase was then moved to a new tube where an additional 600 µL of chloroform was added and then vortexed again for 20 seconds before beinge centrifuged again at 13,000 RCF at room temperature. The supernatant was then incubated for 1 hour with 2 µL of 10 mg/mL RNAse A and 2 µL of 10 mg/mL DNAse I at 37 °C. The mixture was then transferred to pre-cooled tubes containing 1 mL of 100 % ethanol and 40 µL of 3M sodium acetate pH 5.3 were added and incubated at -20 °C for 3 hours to allow for the polyP to precipitate. The mixture was then centrifuged at 13,000 RCF at 4 °C. The supernatant was then discarded and the pellet was washed with 500 µL of 70% ethanol and centrifuged again for 5 minutes. The supernatant was again discarded and the pellet was air-dried. The pellet was then suspended in 20 µL RNAse free water and stored at -80 °C.

2.2.5 PolyP detection and negative DAPI staining

Extracted polyP, along with loading dye (10 mM Tris-HCl pH 7, 1 mM EDTA, 30% glycerol, bromophenol blue) was run on a 15.8 % acrylamide TBE-Urea gel (5.8 M Urea - 5.25 g Urea, 7.9 mL 30% acrylamide, 3 mL 5 X TBE, 150 µL 10% APS, 15 µL TEMED) with 1 X TBE running buffer at 100 V at room temperature for 1 hour and 45 minutes. After completion of the run, the gel was incubated in fixative solution (25 % methanol, 5 % glycerol, and 50 mM Tris Base pH 10.5) containing 2 µg/mL DAPI (Sigma-Aldrich D9542-5MG) at room temperature for 30 min. The gel was then destained in fresh fixative solution twice after 30 minutes each. The gel was then exposed on a 365 nm UV trans-illuminator to photobleach the polyP-bound DAPI. Lastly, the gel was imaged at 312 nm with a Chemi Doc.

3. RESULTS

3.1 PPK is a novel and unique tool that enables for the study of polyP in human cell lines

3.1.1 Ectopic expression of PPK *elevates polyP levels in HEK293T*

To increase polyP levels inside human cells, an *E. coli ppk* expression plasmid (pcDNA3.1(-)-*ppk*) was transfected into HEK293T cells. To check if polyP is increased following PPK protein expression, I assessed whether the polyP produced is able to modify proteins previously reported to be polyphosphorylated. As discussed previously, polyphosphorylation results in electrophoretic mobility shifts that can only be detected on NuPAGE gels. To do this, I looked for electrophoretic shifts on NuPAGE gels for known mammalian targets of polyphosphorylation in samples with polyP accumulation (Bentley-DeSousa et al., 2018).

Although the focus of my project is not on polyphosphorylation, shifts were checked in all experiments that rely on the increase of elevated polyP upon PPK expression **(Figure 6A)**. Electrophoretic mobility shifts on Bis-Tris NuPAGE gels presented in this thesis have previously been used to identify human targets of polyphosphorylation (Bentley-DeSousa et al., 2018). On this gel, transfection with the empty vector does not affect the molecular weight of Nucleolin, a protein known to be polyphosphorylated. Meanwhile, the electrophoretic mobility of Nucleolin is retarded when cells are transfected with a plasmid expressing the *E. coli ppk* gene. Polyphosphorylation shifts were used throughout this study to check for increased polyP accumulation in PPK conditions. This data shows that following PPK expression, mammalian polyP levels can be elevated.

In addition, I used the extraction and detection of polyP to qualitatively assess the amount of polyP produced. This method complimented the validation of our ectopic expression system as we can visualise accumulated polyP. In samples where PPK is expressed, long chain polyP (>130 residues) is detected while in samples with the empty vector, pcDNA3.1(-), no polyP is present **(Figure 6B)**. This result validates the use of PPK to increase polyP in human cells.

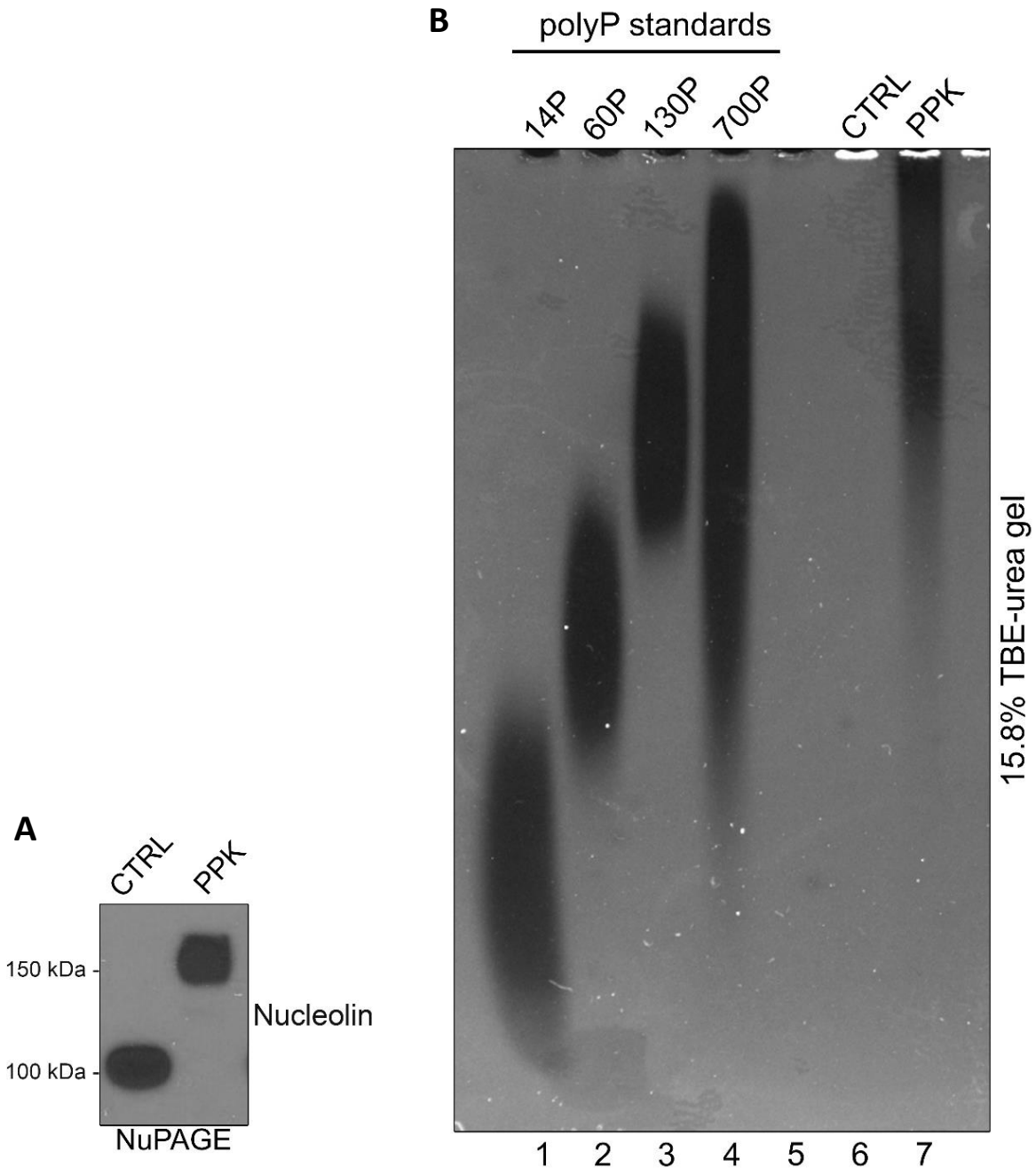

Figure 6. Expression of PPK elevates levels of polyP in mammalian cells. HEK293T cells were transfected with either an empty vector (pcDNA3.1(-)) or *Ec-ppk* expression plasmid (pcDNA3.1(-)-*ppk*) 48 hours prior to harvesting to generate control and PPK conditions. **(A)**

NuPAGE analysis of Nucleolin, a known target of polyphosphorylation. Whole cell lysates were collected with RIPA lysis buffer and then analyzed on a 4-12% Bis-Tris NuPAGE gel, transferred onto PVDF, then probed with an antibody that recognizes Nucleolin. **(B)** PolyP extractions from samples with control or PPK. PolyP was extracted using phenol-chloroform extraction and then analyzed on a 15.8% acrylamide TBE-urea gel and stained with DAPI. N=3.

3.1.2 PPK *expression leads to differential regulation of several transcription factors*

As a starting point to understanding the role of mammalian polyP, RNA sequencing was used to uncover genes differentially regulated by elevated polyP levels in HEK293T cells. PolyP production in the samples transfected with the *E. coli ppk* gene was confirmed via NuPAGE analysis for known polyphosphorylated proteins. Using RNA-seq, 112 genes were found to be differentially regulated when polyP levels is elevated **(Figure 7)**. Genes were then classified as significant if they followed the parameters of a p-value < 0.05 and a fold change > 2. The top ranked protein coding genes from both up- and downregulated genes that were significant based on the stringent parameters assigned were selected as candidate targets predicted to have differential expression. To validate these 12 top ranked targets, RT-qPCR was carried out. 9 out of the 12 targets showed statistically significant differences consistent with the predicted direction of regulation by RNA-seq and are presented in detail in Bondy-Chorney *et al.* (2020).

From these genes, EGR1 was identified as a top target to follow up on. EGR1, which is part of the Early Growth Response proteins of zinc finger transcription factors, is required for normal progression through mitosis and is a target of Erk1/2 (Gregg & Fraizer, 2011). The expression of EGR1 relies on the interaction of phosphorylated Elk-1, a positive transcription regulator driven by the serum response element (SRE) (Mayer et al., 2008). Erk1/2 has been reported to phosphorylate Elk-1, which highlights its role in cell growth and proliferation control (Guha et al., 2001; Rolli, Kotlyarov, Sakamoto, Gaestel, & Neininger, 1999). To follow up on the RNA sequencing, western blotting was also used to verify that this effect is applicable on

the protein levels. Indeed, EGR1 protein levels were also increased upon PPK expression **(Figure 8)**.

To follow up on the report by Gregg & Fraizer *et al.* which linked EGR1 expression to Erk1/2 signalling (2011), the role of elevated intracellular polyP levels on Erk1/2 activation was investigated. Commercial antibodies were used to determine whether PPK expression regulates Erk1/2. A phospho-Erk1/2 antibody which recognizes endogenous Erk 1 and 2 when dually phosphorylated at threonine 202 and tyrosine 204 or Erk 1, or singly phosphorylated at threonine 202 was used. When levels of polyP inside the cell are increased, phospho-Erk1/2 is increased, while total Erk1/2 levels remain unchanged **(Figure 9)**. This result is consistent with reports that addition of polyP to cell culture media mediates Erk1/2 phosphorylation (Hassanian et al., 2015). These results suggest a role for polyP in the regulation of the MEK/Erk signalling pathway.

The mechanism behind polyP's regulation of EGR1 levels remained unclear. To determine where polyP may be acting in the signalling cascade to upregulate EGR1, siRNA or inhibitors of effectors in the signalling network were used. The goal of this is to monitor whether the polyP-dependent up-regulation of EGR1 is maintained by inactivation of these effectors. As a starting point, because the Erk1/2 pathway was previously reported to regulate EGR1 mRNA transcription (Gregg & Fraizer, 2011), inhibitors were used to supress the activity of Erk1/2. Antibodies recognizing both endogenous phosphorylated and total Erk1/2 were used to check for efficient inhibition of Erk1/2. In this preliminary work, when cells were treated with the Erk1/2 inhibitor SCH772984, Erk1/2 phosphorylation decreased in both the control and PPK

conditions while total Erk1/2 levels remain unchanged. EGR1 protein levels also appeared to decrease in both the control and PPK conditions **(Figure 10)**. However, this result is preliminary as SCH772984 was inconsistent in the inhibition of Erk1/2 phosphorylation between the 4 biological replicates tested. This experiment is an example of future work that would elucidate the role of PPK expression in mediating the Erk1/2-EGR1 cascade and requires optimization of the inhibiter for conclusive results. This results suggests that increased Erk1/2 regulates EGR1 protein levels, as shown previously (Gregg & Fraizer, 2011), and that PPK expression increased EGR1 protein levels, likely via Erk1/2.

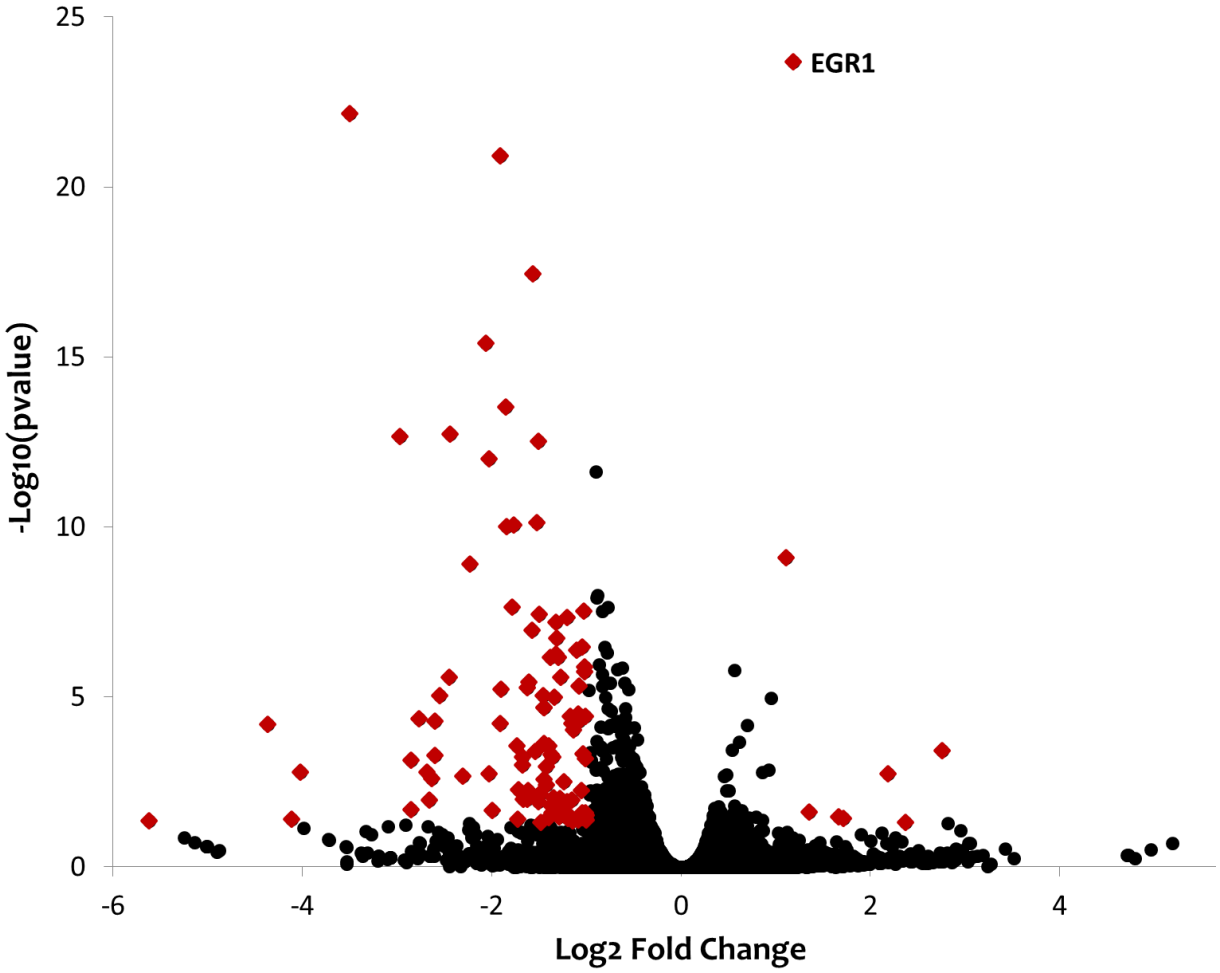

Figure 7. Differential expression of mammalian genes in the presence of excess intracellular polyP production. HEK293T cells were transfected with either an empty vector (pcDNA3.1(-)) or *E. coli ppk* expression plasmid (pcDNA3.1(-)-*ppk*) 48 hours prior to harvesting to generate control and PPK conditions. RNA was extracted using the miRNEasy Mini kit and sent to Genome Quebec Innovation Centre for sequencing and to the Canadian Centre for computational Genomics for bioinformatics analysis. This volcano plot shows $\log_2$ (fold change) and $-\text{Log}_{10}$ (p-value) of genes detected by RNA-seq. Genes coloured in red are classed as significant (p value <0.05, $\log_2$ fold change >2).

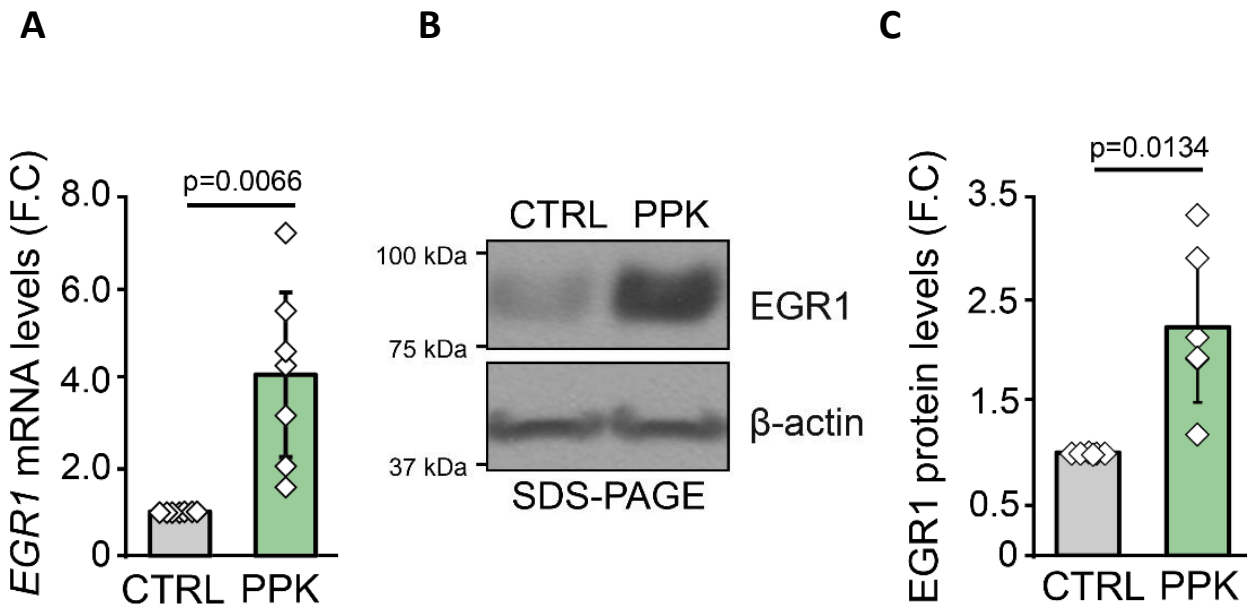

Figure 8. PPK expression upregulates EGR1 mRNA and protein levels. HEK293T cells were transfected with either an empty vector (pcDNA3.1(-)) or *E. coli ppk* expression plasmid (pcDNA3.1(-)-*ppk*) 48 hours prior to harvesting to generate control and PPK conditions. **(A**) RT-qPCR analysis of EGR1 mRNA levels represented by fold change for both the control and PPK samples. β-actin was used as a loading control. N=7 **(B)** Whole cell lysates were collected with RIPA lysis buffer and analyzed on a 4–20% Criterion TGX Stain-Free Protein Gel. Proteins were transferred onto PVDF and probed with an EGR1 antibody, as well as a β-actin antibody as a loading control. Western blot shown is a representative image of of N=6. **(C)** Semi-quantitative analysis of EGR1 protein levels shown in B normalized to β-actin control. One-sample t-test (unequal variances) were used where error bars represent standard deviation. (Figure prepared by Dr. Emma Bondy-Chorney)

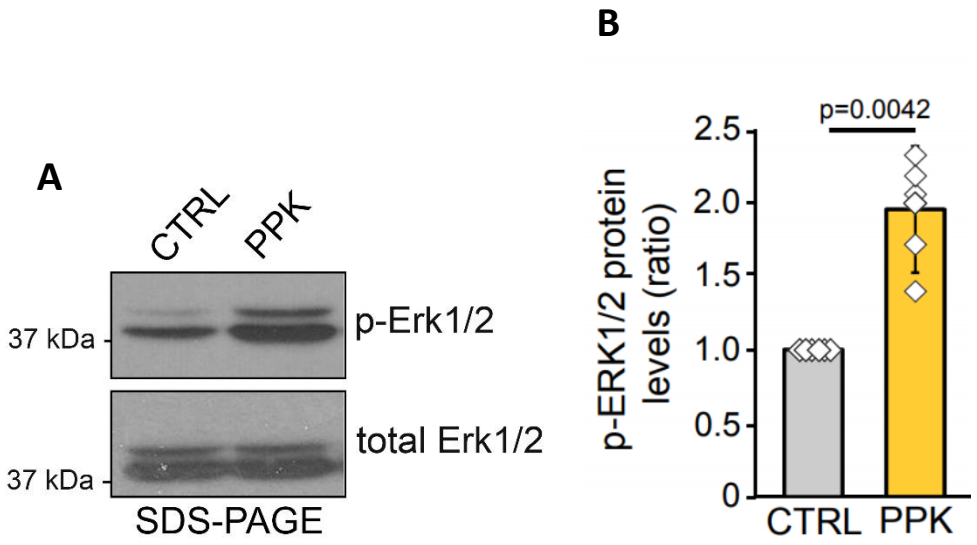

Figure 9. Phospho-Erk1/2 is upregulated with PPK expression. HEK293T cells were transfected with either an empty vector (pcDNA3.1(-)) or *E. coli ppk* expression plasmid (pcDNA3.1(-)-*ppk*) 48 hours prior to harvesting to generate control and PPK conditions. **(A)** Whole cell lysates were collected with RIPA lysis buffer and analyzed on a 10% SDS Gel. Proteins were transferred onto PVDF and probed with antibodies for both phospho- and total Erk1/2. Western blot shown is a representative image of N=6. **(B)** Semi-quantitative analysis of protein levels shown in A. One-sample t-test (unequal variances) were used where error bars represent standard deviation.

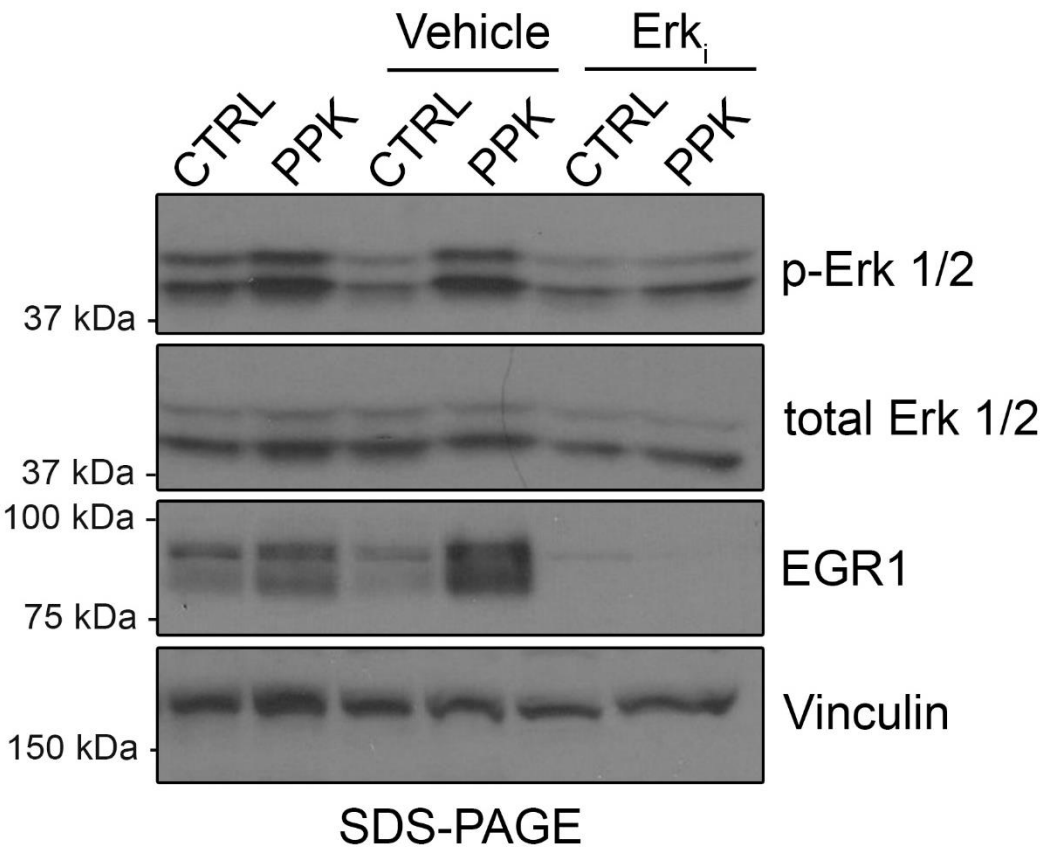

Figure 10. Erk1/2 is involved in mediating EGR1 protein levels**.** HEK293T cells were transfected with either an empty vector (pcDNA3.1(-)) or *E. coli ppk* expression plasmid (pcDNA3.1(-)-*ppk*) 48 hours prior to harvesting to generate control and PPK conditions. Cells were treated with DMSO as a *Vehicle*, and *Erk$_i$* conditions were treated with 1 µM of SCH772984. Whole cell lysates were collected with RIPA lysis buffer and analyzed on a 10% SDS-PAGE Gel. Proteins were transferred onto PVDF and probed with antibodies for phospho- and total Erk1/2, EGR1, as well as vinculin as a loading control. Vinculin was used as a loading control. N=4, refer to results section for more details on inconsistency between replicates.

3.2 Elevated polyP levels inside the cell activate the Erk1/2 signalling cascade

The addition of extracellular polyP has been reported to increase activation of mTOR, with this activation being independent of the phosphorylation of Erk1/2 (Hassanian et al., 2015; Wang et al., 2003). To follow up on this and investigate whether increasing intracellular concentrations of polyP would have the same effect, western blotting with commercial antibodies was used.

To investigate whether phosphorylation of the mTOR complex 1 substrate p70s6k is induced by polyP, we used an antibody that recognized p70s6k when phosphorylated at its threonine 389 residue. This was complemented with the use of an antibody that recognized total endogenous p70s6k levels. When PPK is expressed, p70s6k phosphorylation is increased, while total p70s6k levels remain unchanged **(Figure 11)**.

Hassanian et. al. also reported that the increase of mTOR activation by exogenous polyP was independent of Erk1/2 phosphorylation (2015). To investigate whether increasing polyP inside the cell would induce the same effect, cells were treated with the Erk1/2 inhibitor SCH772984. The decrease of Erk1/2 levels was validated with commercial antibodies. Phospho-Erk1/2 levels decreased when the inhibitor is added to cells while total Erk1/2 levels remained unchanged. In cells treated with SCH77298, phospho-p70s6k levels remained unchanged in both the control and PPK conditions **(Figure 12)**. However, there was inconsistency between the two biological replicates tested in terms of the total Erk1/2 levels. This result, if reproducible with consistent total Erk1/2 levels, suggests that elevated intracellular polyP levels

increase mTOR activity in an Erk1/2-independent manner. These results remain preliminary and require more replicates before a final conclusion can be made.

To determine the role of polyP in mediating Erk1/2 phosphorylation, the MEK inhibitor U0126 was used to assess whether increased Erk1/2 activation upon PPK expression is regulated by the upstream kinase MEK. Classically, MEK phosphorylates and activates Erk1/2 (Duncia et al., 1998). Therefore, by using the non-competitive and selective inhibitor U0126, whether the PPK-induced increase of Erk1/2 activation is reliant on this kinase can be verified. Although treatment with U0126 decreased phosphorylation of Erk1/2, it was not entirely inhibited when polyP levels are elevated **(Figure 13)**. This preliminary experiment is a first example of future work that would elucidate the involvement of the MEK kinase in the increased Erk1/2 phosphorylation upon PPK expression. While there appears to be a decrease in phospho-Erk1/2 in both control and PPK conditions for all three biological replicates (lanes 3, 4, 7, 8, 11, 12), this decrease might be attributed to the decreased total Erk1/2 levels in replicates B and C (lanes 7, 8, 11, 12). Because U0126 did not decrease Erk1/2 phosphorylation consistently, no conclusion can be made on the role of MEK in mediating the PPK-induced increase in Erk1/2 activation. Optimization of U0126 concentration and/or treatment time, as well as additional replicates, are required.

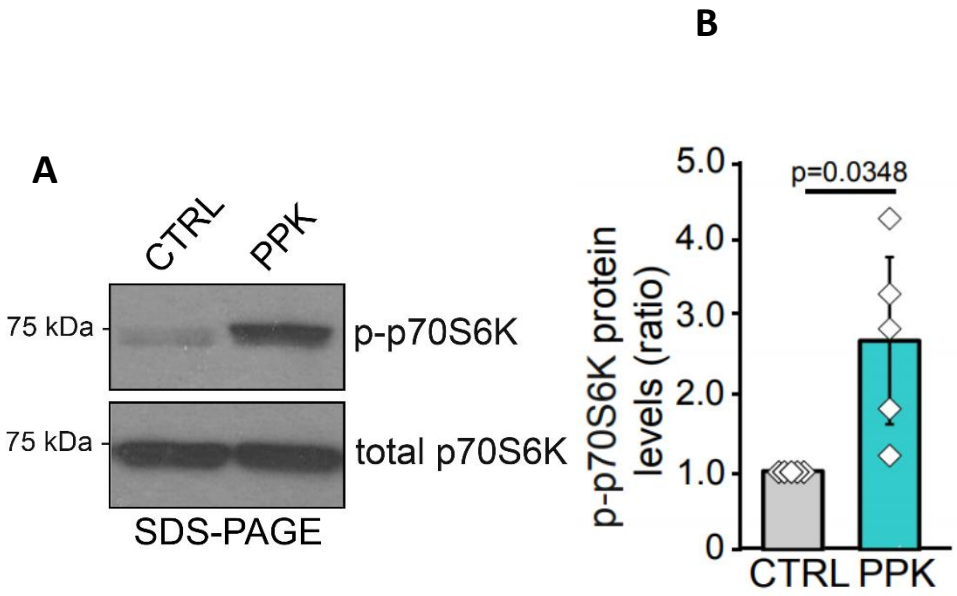

Figure 11. Phospho-p70s6k is upregulated with elevated PPK expression. HEK293T cells were transfected with either an empty vector (pcDNA3.1(-)) or *E. coli ppk* expression plasmid (pcDNA3.1(-)-*ppk*) 48 hours prior to harvesting to generate control and PPK conditions. **(A)** Whole cell lysates were collected with RIPA lysis buffer and analyzed on a 10% SDS Gel. Proteins were transferred onto PVDF and probed with antibodies for both phospho- and total p70s6k. Western blot shown is a representative image of N=5. **(B)** Semi-quantitative analysis of protein levels shown in A. One-sample t-test (unequal variances) were used where error bars represent standard deviation.

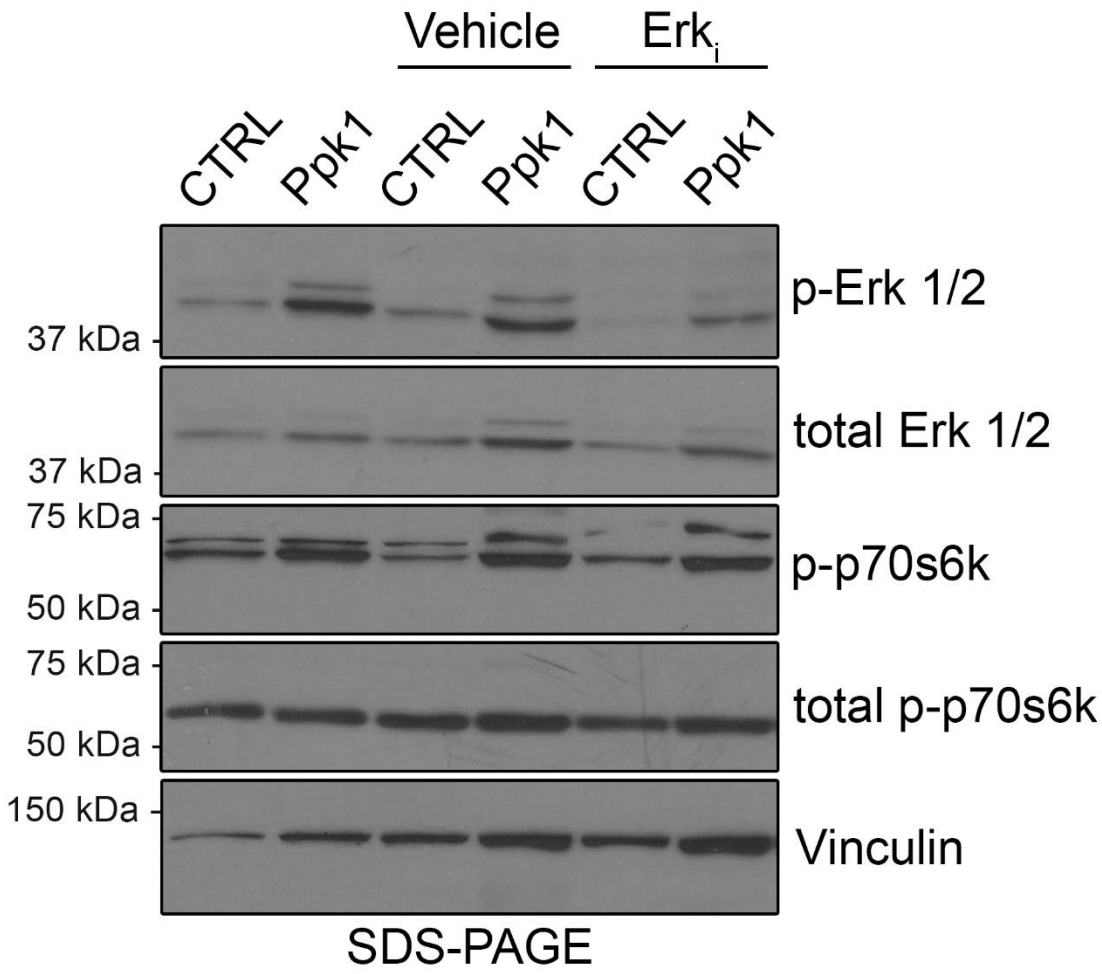

Figure 12. Increase of phospho-p70s6k with PPK expression appears independent of Erk1/2 activation . HEK293T cells were transfected with either an empty vector (pcDNA3.1(-)) or *E. coli ppk* expression plasmid (pcDNA3.1(-)-*ppk*) 48 hours prior to harvesting 24 hours before collection, cells were treated with DMSO as a *Vehicle*, and *Erk$_i$* conditions with 1 µM of SCH772984. Whole cell lysates were collected with RIPA lysis buffer and analyzed on a 10% SDS Gel. Proteins were transferred onto PVDF and probed with antibodies for both phospho- and

total p70s6k. Vinculin was used as a loading control. N=2, refer to results section for notes on inconsistency between replicates.

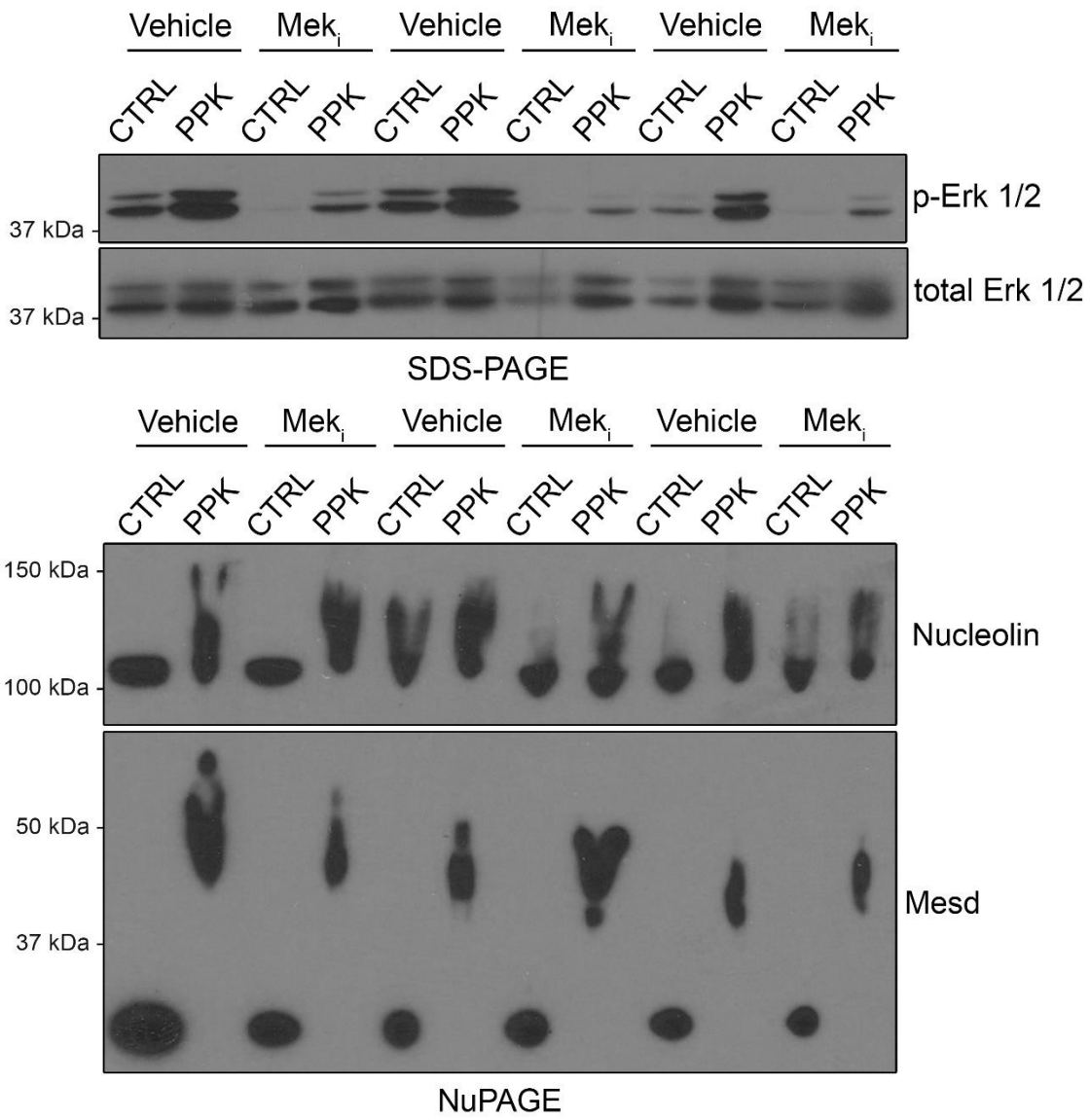

Figure 13. Dissecting the contribution of MEK in the increase of Erk1/2 phosphorylation upon PPK expression with U0126. HEK293T cells were transfected with either an empty vector (pcDNA3.1(-)) or *E. coli ppk* expression plasmid (pcDNA3.1(-)-*ppk*) 48 hours prior to harvesting to generate control and PPK conditions. 2 hours before collection, *Vehicle* conditions were treated with DMSO, and *Erk$_i$* conditions with 1 µM of U0126. Whole cell lysates were collected with RIPA lysis buffer and analyzed on a 10% SDS Gel. Proteins were transferred onto PVDF and probed with antibodies for both phospho- and total p70s6k. Western blots present are for N=3.

3.3 Expanding our existing ectopic expression system in HEK293T cells

3.3.1 Codon-optimized PPK *produces less polyP*

At this point, we have validated that our ectopic expression system can increase polyP inside HEK293T cells, evident by visualization of extracted polyP as well as electrophoretic mobility shifts of known polyphosphorylated mammalian proteins on NuPAGE gels. However, due to the unknown threshold of polyP required to induce the electrophoretic shift of these proteins, as well as no absolute quantification methods, we looked for a method to maximize the amount of polyP being produced inside the cells with PPK expression. The rationale behind this is that an even greater increase of intracellular polyP may exaggerate effects of polyP that may happen endogenously, and potentially have a clear downstream effect. Codon optimization appeared to be a good option; optimizing the sequence of the *E. coli ppk* gene would increase its expression, and consequently increase the amount of polyP produced inside the cell. Interestingly, polyP extractions revealed that codon-optimized PPK (coPPK) resulted in a lesser accumulation of polyP than its non-optimized counterpart **(Figure 14A, lanes 6 and 7)**. However, both PPK and codon-optimized PPK produced an electrophoretic shift of mammalian polyphosphorylated proteins, as shown by the example of eIF5B **(Figure 14B, lanes 2 and 3)**. These combined results show that codon-optimized PPK does not produce more polyP than its non-optimized counterpart.

3.3.2 Yeast Ppx1 *degrades polyP produced by mammalian cells*

An alternative approach to study mammalian polyP is the ectopic expression of the yeast exopolyphosphatase Ppx1 (Wang et al., 2003). To achieve this, yeast *PPX1* was cloned into the mammalian expression vector pcDNA3.1(-). Cells were transfected with the yeast *PPX1* gene alone, or cotransfected along with *ppk/*codon optimized *ppk*. Ppx1 expression was verified with a commercial antibody recognizing yeast Ppx1 **(Figure 14C)**.

PolyP extractions of cells transfected with *ppk* and *PPX1* showed no difference in the accumulated polyP levels relative to cells transfected with *ppk* alone **(Figure 14A, lanes 6 and 9)**. However, in cells co-transfected with codon-optimized *ppk* and *PPX1*, the long chain polyP levels appear to decrease compared to cells transfected with codon-optimized *ppk* alone **(Figure 14A, lanes 7 and 10)**. This result indicates that Ppx1 expression in human cells can counter elevated intracellular polyP levels. Furthermore, expression of Ppx1 along with PPK affected the electrophoretic mobility shift on NuPAGE gels of the polyphosphorylated protein eIF5b **(Figure 14B, lanes 2 and 5)**. However, the effect of Ppx1 appeared to be dependent on the amount of polyP in the cell; when transfected alongside codon optimized *ppk*, *PPX1* is able to cause a collapse in the electrophoretic mobility shift seen in cells transfected with codon optimized *ppk* alone **(Figure 14B, lanes 3 and 6)**. This result suggests that Ppx1 depletes polyP produced upon PPK expression, however, its measurable efficiency via NuPAGE electrophoretic shifts and polyP extractions depends on the available amount of polyP.

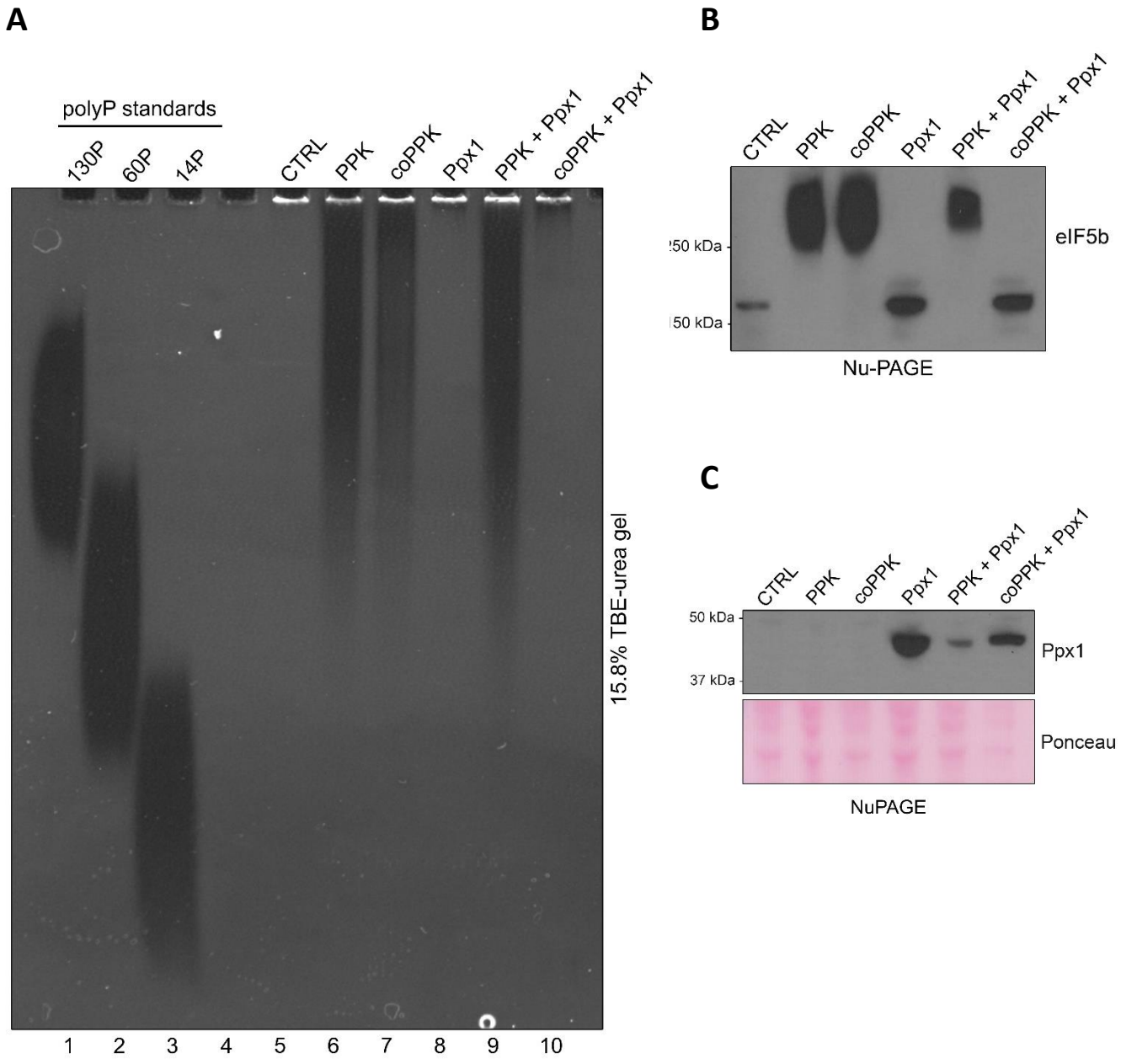

Figure 14. Codon-optimized PPK and Ppx1 modulate the levels of polyP inside HEK293T cells. HEK293T cells were transfected with either one or a combination of the following: empty vector pcDNA3.1(-), pcDNA3.1(-)-*ppk*, pcDNA3.1(-)-co*ppk*, or pcDNA3.1(-)-*PPX* 48 hours prior to harvesting. **(A)** PolyP extractions from samples with control, PPK, coPPK, Ppx, PPK and Ppx, or coPPK and Ppx. PolyP was extracted using phenol-chloroform extraction then analyzed on

15.8% acrylamide TBE-urea gel and stained with DAPI. **(B)** NuPAGE analysis of eIF5b, a known target of polyphosphorylation. Whole cell lysates were collected with RIPA lysis buffer and analyzed on a 4-12% Bis-Tris NuPAGE gel. Proteins were transferred onto PVDF and probed with an antibody that recognizes eIF5b. **(C)** Analysis of Ppx1 protein levels. Whole cell lysates were collected with RIPA lysis buffer and analyzed on a 4-12% Bis-Tris NuPAGE gel. Proteins were transferred onto PVDF and probed with an antibody that recognizes Ppx1. All images of this figure are using the same samples, and are representative of N=3.

4. DISCUSSION

The study of mammalian polyP biology has mainly depended on its addition extracellularly. The work in my thesis was focused on producing a system where polyP made inside the cell can be studied. Since the machinery for polyP synthesis in mammals remains unknown, the use of exogenous PPK expression enables us to study the function of polyP when elevated inside the cell. Meanwhile, using expression of Ppx1 allows us to validate any regulatory role that intracellular polyP may elicit. Through these systems, we are able to bypass the need for polyP to be internalized or to interact with extracellular receptors. Furthermore, this system could be used to study scenarios of bacterial infection in mammalian cells.

4.1 Ectopic expression of PPK elevates polyP levels in HEK293T

The majority of studies interested in uncovering roles or functions of mammalian polyP rely on the addition of polyP to culture media. A major caveat to this technique is the reliance on the internalization of the polyP as it presents with another variable in the study of polyP inside the cells. To address this, we developed a system to increase polyP concentrations in mammalian cells using PPK. Previous work has used *E. coli ppk* expression in mammalian cells but has solely focused on the measurement of polyP activity via MRI as well as visualization of polyP via toluidine blue staining (Ki et al., 2007). My work has expanded the applications of the bacterial polyP synthetase to investigate the role of polyP in human cells.

As a means of increasing intracellular polyP levels, I transfected HEK293T cells with the *E. coli ppk* plasmid. To verify that intracellular levels of polyP were indeed increased, we looked

at electrophoretic shifts for known targets of polyphosphorylation **(Figure 6)** (Bentley-DeSousa et al., 2018). As expected, shifts were only detected in cells transfected with the *E. coli ppk* gene. These electrophoretic mobility shifts on NuPAGE gels are the only method currently used to detect polyphosphorylation (Azevedo et al., 2015; Bentley-DeSousa et al., 2018). The basal concentration of polyP in mammalian cells is much lower than that in yeast or bacteria and therefore does not illicit electrophoretic shifts. This makes the use of PPK expression the best available method to increase polyP levels in HEK293T cells inside the cell. Although the focus of this study is not polyphosphorylation, these NuPAGE electrophoretic shifts were then utilized throughout this study to ensure transfections with *E. coli ppk* were efficient and to validate that our system was successful at elevating mammalian polyP levels.

As a secondary method of validation, polyP extractions were used to ensure that expression of PPK yields an increase of intracellular polyP. Indeed, polyP was only detected in HEK293T cells when transfected with *E. coli ppk*. In cells transfected with an empty vector, there was no visible polyP when DAPI staining was used. This lack of visible polyP could be explained by the low levels of endogenous polyP in mammalian cells (Kornberg et al., 1999). In cells transfected with *E. coli ppk*, long chains polyP of approximately 700 residues were detected. *E. coli* PPK has been reported to synthesize homogeneous polyP chain lengths of 750 ± 50 residues (Krishnanand D Kumble, Ahn, & Kornberg, 1996). The polyP length produced by PPK in HEK293T cells is consistent with the reported length of polyP produced in *E. coli* in literature (Krishnanand D Kumble et al., 1996). Moreover, while human platelets contain short chain polyP (60-100P) (Müller et al., 2009; Ruiz et al., 2004), longer chains have been reported

in other mammalian samples such as rat brain (K. D. Kumble & Kornberg, 1995). This suggests that polyP produced by PPK in HEK293T cells is representative of that produced in some mammalian cell types.

It could be argued that relying on PPK expression to study the role of polyP in mammalian cells is not physiologically relevant. This system elevates polyP levels abnormally and thus any effects seen may be an artifact of overexpression. However, this study presents a valuable tool to study cellular mechanisms *in vitro* in an artificial context and could possibly represent an *in vivo* setting. Ultimately, due to the ambiguity of polyP synthesis in mammalian cells, relying on PPK expression is a fundamental tool to understand the function of mammalian polyP.

Alternatively, the PPK expression system may be useful to study the response of mammalian cells in the context of bacterial infection. Although polyP concentrations vary among bacterial species, ranging from 100-200mM (Kornberg et al., 1999), it is much higher than the average amount found in mammalian cells (25-120 µM) (K. D. Kumble & Kornberg, 1995). Increased intracellular polyP levels via PPK expression could be used to model the effect of increased polyP concentrations produced by bacteria during infection. Recently, Dahl *et al*. used mesalamine, a drug used in the treatment of ulcerative colitis, to decrease the polyP content of bacteria found in the human gut microbiome (2017). Treatment with mesalamine reduced polyP levels in human microbiota and increased oxidative stress sensitivity of uropathogenic *E. coli*. Mesalamine treatment also mirrored the loss of *ppk* as it decreased the ability of uropathogenic *E. coli* to form biofilms and reduced the number of antibiotic resistant

cells (Dahl et al., 2017). This study suggests that PPK can be inhibited by mesalamine *in vivo*, however, the mechanism of this inhibition remains unknown. To investigate whether mesalamine could function as a PPK inhibitor in the context of bacterial infection in humans, our ectopic expression system could be applied.

Furthermore, nanoparticles have recently been used to facilitate the delivery of intracellular polyP into mammalian cells to study its function (Fernandes-Cunha et al., 2018). Ectopic expression of PPK in mammalian cells bypasses the need for nanoparticles to increase intracellular polyP concentrations.

4.2 Elevated intracellular polyP levels through expression of PPK lead to differential regulation of several transcription factors

TheRNA-seq experiment revealed several differentially regulated genes in response to increased polyP levels **(Figure 7)**. Among those, EGR1 was revealed to be upregulated with PPK expression **(Figure 8)**. Previous work by Gregg & Faizer has linked EGR1 expression to Erk1/2 phosphorylation (Gregg & Fraizer, 2011). Erk1/2 phosphorylates Elk-1 which in turn interacts with SRE to drive EGR1 expression (Gregg & Fraizer, 2011). We therefore investigated whether elevated polyP levels upregulate EGR1 expression via increased Erk1/2 phosphorylation. To understand the mechanisms by which PPK expression leads to upregulation of EGR1, inhibitors of members of the Erk1/2-mTOR signalling cascade were used.

The first inhibitor used was SCH772984, an ATP competitive selective inhibitor of Erk1/2 (Morris et al., 2013). The Erk1/2 inhibitor was validated with commercial antibodies for

phospho- and total Erk1/2. With SCH772984, Erk1/2 phosphorylation was inconsistently decreased. However, where phospho Erk1/2 is decreased, EGR1 protein levels appear to decrease as well. Both the time of treatment and amount of the inhibitor need to be optimized to make a conclusion based on this result. However, if reproducible, this result suggests that increased EGR1 protein levels upon PPK expression may be mediated by Erk1/2 phosphorylation.

This was the first use of RNA sequencing to analyze the effect of elevated intracellular polyP levels on mammalian gene expression. Previous work by Lui et al looked at the effect of extracellular polyP addition to SaOS-2 cells (Lui, Ao, Li, Khong, & Tanner, 2016). Similarly to the present study, they reported a significant upregulation of EGR1 mRNA and an increase in phosphorylated Erk1/2 (Lui et al., 2016).

4.3 Elevated intracellular polyP levels activate the Erk1/2 signalling cascade

We also followed up on work linking elevated polyP concentrations to the activation of the mTOR signalling pathway. Previously, addition of exogenous polyP was reported to activate the Erk1/2-mTOR signalling cascade via interaction with the RAGE and $P2Y_1$ receptors (Hassanian et al., 2015). With our unique system, elevated polyP inside HEK293T cells was found to activate Erk1/2 as well as the mTOR substrate p70s6k **(Figures 9 and 11)**.

As previously discussed, inhibitors for members of the Erk1/2-mTOR signalling pathway were used. Initially, they were applied to study the mechanism by which elevated intracellular polyP levels increase EGR1 expression. When cells are treated with SCH772984, Erk1/2

phosphorylation is decreased, with or without the generation of increased intracellular polyP levels. When the phosphorylation of p70s6k was assessed in the presence of the Erk1/2 inhibitor, there were inconsistencies in the total Erk1/2 levels for both biological replicates tested. However, the phosphorylation of p70s6k appears to be unaffected when Erk1/2 is inhibited. If the inconsistency in total Erk1/2 levels is addressed and p70s6k phosphorylation is seen to be unaffected with Erk1/2 inhibitors, this finding suggests that the increase of phospho Erk1/2 with elevated polyP levels is independent of the activation of mTOR. This result is expected as the addition of extracellular polyP was also seen to increase Erk1/2 activation independent of phospho p70s6k levels reported by Hassanian *et al*. (2015).

To determine whether increased phosphorylation of Erk1/2 upon PPK expression is mediated by MEK, cells were treated with the MEK non-competitive inhibitor U0126 (Favata et al., 1998). The MEK inhibitor did not entirely inhibit Erk1/2 phosphorylation when polyP levels were increased. The total Erk1/2 levels were inconsistent, as well as the shifts for Nucleolin, a known mammalian target of polyphosphorylation. Therefore, this result is inconclusive and serves as an example of future work that would help elucidate the mechanism by which PPK expression increased phospho Erk1/2 levels. If the experiment is repeated without any inconsistencies in total Erk1/2 levels or polyphosphorylation shifts and phospho Erk1/2 still appears increased in the PPK conditions upon treatment with the MEK inhibitor, it would sugest a novel role for polyP in mediating Erk1/2 phosphorylation, bypassing the need for activation by the upstream MEK kinase. However, as previously discussed, this data was inconsistent across biological replicates and is thus unreliable.

In terms of mechanism, we suggest a model in which polyP interacts with upstream effectors to activate mTOR signalling. However, polyP may also be undergoing trafficking to the cell surface where it would interact with extracellular receptors.

Previously, Wang *et al*. found that polyP increases mTOR activation by direct interaction (2003). Similarly, polyP may be interacting with the upstream MEK, Raf, or Ras kinases directly to activate Erk1/2. This model is consistent with the work of Azevedo *et al*. who identified H-Ras as a candidate interactor of polyP using protein microarrays (2018).

4.4 Codon-optimized PPK produces less intracellular polyP

We previously confirmed that our system increases intracellular polyP levels using electrophoretic shifts on NuPAGE gels and polyP extractions. However, due to the ambiguity over the amount of polyP produced, I resorted to codon optimization of *ppk* to increase the expression of PPK. Codon optimization is the process of altering the assignment of codons to maximize production of a desired protein (Mauro & Chappell, 2014). Codon optimization involves several factors, for example increasing the GC content to improve mRNA stability and assessing codon usage with the codon adaptation index, a measure of relative codon usage of the gene of interest to that of common highly expressed genes (Graf, Deml, & Wagner, 2004). This also includes decreasing the amount of negative cis-regulatory elements which can affect expression levels of foreign proteins (Chung & Lee, 2012). By using bioinformatics for gene design, codon optimization allows a work around of biological limitations, for example low RNA stability and translation efficiency (Graf et al., 2004). However, there was no visible increase in

polyP production with the codon-optimized version of PPK. When compared to the polyP detected with DAPI staining after polyP extraction, there was less polyP detected on the gel **(Figure 14A)**. Furthermore, the shifts produced by codon-optimized PPK on bis-tris NuPAGE gels for polyphosphorylated proteins were in line with that produced by PPK which hints that the amount of polyP produced is relatively the same **(Figure 14B)**.

The functionality of codon optimization involves several factors that may contribute to PPK producing more polyP than codon-optimized PPK (Hanson & Coller, 2018). Indeed, with the combination of microarrays and quantitative shotgun proteomics in the Daoy medulloblastoma cell line, codon bias was revealed to correlate poorly with translation efficiency (Vogel et al., 2010). In *E. coli*, optimized codons are associated with a lower mRNA folding energy (Goodman, Church, & Kosuri, 2013). This is due to the non-optimal codons having greater AT content versus the optimized counterparts which aim for higher GC content (Goodman et al., 2013). This suggests that the reduced secondary structure of RNA must be in balance with codon optimality to maximize translation.

The initiation of translation is another step to consider in the context of translation efficiency. With codon optimization, if initiation is the rate limiting step, changes in elongation will just lead to overcrowding of mRNA with ribosomes (Hanson & Coller, 2018). This alteration will in turn decrease translation efficiency due to ribosome collisions and queues which delay protein production (Mitarai, Sneppen, & Pedersen, 2008).

Another factor that may explain why PPK expression lead to greater polyP production when compared to codon-optimized PPK is protein misfolding. To illustrate this, Komar *et al*. codon-optimized the *E. coli* chloramphenicol acetyltransferase gene (1999). Although this resulted in the overall rate of protein production being increased, it was coupled with an increase of protein misfolding (Komar et al., 1999).

The aforementioned examples of codon optimization consequences are among several factors that may explain why PPK expression lead to greater polyP production than codon-optimized PPK. The caveat to our ectopic expression is that we have not applied a method to quantify polyP concentration. Absolute quantification of polyP would present an accurate comparison between the polyP produced by PPK and codon-optimized PPK.

4.5 Yeast Ppx1 degrades polyP produced by mammalian cells

Wang *et al*. previously expressed the yeast exopolyphosphatase Ppx1 in mammalian cells to study the role of polyP in mTOR activation (2003). Due to the mammalian polyP machinery being unknown, the expression of Ppx1 has been used to deplete mammalian polyP. I applied the expression of Ppx1 as an extension of our ectopic expression system. The rationale behind this was to decrease polyP and assess whether the effects seen with PPK expression could be countered.

Co-transfection of *E. coli ppk* with *PPX1* did not appear to decrease polyP amounts compared to *ppk* alone **(Figure 14A, lines 6 and 9)**. The electrophoretic shifts of eIF5b, a polyphosphorylated proteins, was maintained on NuPAGE **(Figure 14B)**. Co-transfection with

PPX1 and codon-optimized *ppk* produced less polyP compared to transfection with *ppk* alone as detected by DAPI staining **(Figure 14A)**. Furthermore, *PPX1* was able to collapse the electrophoretic mobility shift when co-transfected with codon-optimized *ppk* **(Figure 14B)**. The decreased efficiency of codon-optimized PPK may explain the decreased amount of polyP visible on gel and the collapsed electrophoretic shifts seen with *PPX1* co-transfection. Also, although the shift produced in samples transfected with *ppk* and *PPX1* is in line in electrophoretic mobility with that produced in samples transfected with *ppk* alone, the size of the band is much smaller. The smaller size of the band may be explained by a decrease in long chain polyP as Ppx1 favors cleavage of terminal residues of ~500 Pi (Akiyama et al., 1993). This would decrease the heterogeneity of the polyP chains that induce the shift, and as such produce a smaller band detected on NuPAGE gels for eIF5b, a polyphosphorylated target. Ultimately, the amount of polyP required to induce an electrophoretic shift remains unknown. In line with this, it is unknown how much of a decrease in polyP concentration would collapse the shift. This could be addressed by quantifying polyP levels.

The transfection of *PPX1* was validated with an antibody that recognizes Ppx1, **(Figure 14C)**. Ppx1 expression is highest in cells transfected with *PPX1* alone, (lane 4), followed by co-transfection of *PPX1* and codon-optimized *ppk* (lane 6), and lastly by co-transfection of *PPX1* and *ppk* (lane 5). Notably, throughout this study, increased polyP has been seen to decrease the expression of co-expressed plasmids. This may explain why Ppx1 expression is highest when it is transfected alone. Next, Ppx1 expression is greater with codon optimized *ppk* as opposed to that with *E. coli ppk* as the expression of codon optimized *ppk* may be less efficient. This may

explain why less polyP accumulates with codon optimized PPK compared to *E. coli* PPK. Therefore it may be less demanding for the cell to express Ppx1 when transfected with codon optimized ppk. Ultimately, this experiment is an example of future work that will further expand our ectopic expression system.

4.6 Future Directions

Overall, this work presents novel tools to study the role of polyP in mammalian cells. Our findings that polyP has a role in the Erk1/2-mTOR signalling axis highlights polyP as a potential regulator for cell proliferation and growth. Furthermore, we have identified polyP as a potential mediator for the transcription factor EGR1, which is required for cell differentiation and mitogenesis (Adams et al., 2017).

With the increase of intracellular polyP by PPK expression revealing an increase in activation of effectors in the MEK-Erk-mTOR cascade. Previously, addition of exogenous polyP was found to activate NF-κB in endothelial cells (Hassanian et al., 2015). In fact, when Akt and NF-κB were both inhibited, polyP-mediated phosphorylation of their shared effector TSC1/2 was also inhibited (Hassanian et al., 2015). This work was done by the addition of polyP extracellularly and should be investigated with the use of PPK expression instead. Links between NF-κB and mTOR signalling present significant interplay between these signalling pathways with phenotypes mediating inflammation and proliferation.

Furthermore, the mechanism of polyP-mediated increase in Erk1/2 and p70s6k phosphorylation remains unknown. Aside from inhibitors of upstream effectors, siRNA could also be used to investigate this mechanism. While inhibitors target proteins of interest, siRNA could be useful to study specific isoforms of encoded proteins of interest. For example, Raf has 3 different isoforms, c-Raf, B-Raf, and A-Raf, all differentially activated by different members of the upstream Ras family (Matallanas et al., 2011). Furthermore, the ability of Raf isoforms to

phosphorylate MEK 1 and 2 varies substantially between isoforms, with B-Raf having the highest activity, followed by c-Raf and A-Raf (Marais, Light, Paterson, Mason, & Marshall, 1997). While small molecule inhibitors for Raf such as Dabrafenib exist, it is specific for B-Raf V600E wherein the valine of B-Raf is substituted for glutamic acid at the 600th amino acid (Davies et al., 2002). However, over 100 B-Raf mutations have been described, with even low-activity mutants being able to hyperstimulate the Erk1/2 signalling cascade (Wan et al., 2004). siRNA would enable for selective targeting against specific isoforms of these effectors and effectively study the role that polyP plays in he Erk1/2 pathway.

Methods for quantifying polyP concentration are a priority for future work. Although this work has shown that PPK expression increases polyP levels inside the cell, the amounts produced remain unknown. Previous work by our lab has reported the first mammalian targets of polyphosphorylation that allow us to ascertain that our ectopic expression system is increasing polyP concentrations upon PPK expression (Bentley-DeSousa et al., 2018). However, relying on the electrophoretic mobility shifts produced by PPK expression presents a problem; the threshold of polyP required to induce the shifts is unknown and assumes that any PPK sample that does not produce an electrophoretic mobility shift does not have elevated polyP levels. Furthermore, although we can visualize that Ppx1 is able to decrease polyP levels when co-transfected with codon-optimized *ppk*, we cannot ignore that it may also be decreasing polyP concentration in cells co-transfected with *E. coli ppk*. The amount of polyP being degraded may simply not be enough for a detectable difference on TBE-urea gels or to collapse polyphosphorylation shifts. To address this method for quantifying polyP levels is necessary. For

example, Kornberg *et al.* have proposed the use of NMR analysis to identify polyP in intact cells (Kornberg et al., 1999). A problem of NMR is the requirement of high concentrations as well as the lack of sensitivity to detect aggregates of polyP or intact polyP in metal complexes (N. N. Rao, Roberts, & Torriani, 1985). Radioactive labeling of polyP can be used in enzymatic assays to detect released polyP (Narayana N. Rao, Liu, & Kornberg, 1998). Alternatively, because polyP modifies the excitation emission spectra of DAPI (Aschar-Sobbi et al., 2008), polyP localization and quantification is made possible by fluorescence microscopy. Our lab is implementing the use of the malachite green assay to quantify polyP. In this colorimetric assay, under an acidic pH, released phosphates from hydrolyzed polyP form phosphomolybdate complexes which causes a detectable change in the absorbance of malachite green (Van Veldhoven & Mannaerts, 1987). Quantification of polyP would validate of our ectopic expression system in mammalian cells and eliminate variability between biological replicates.

Subcloning of *PPX1* into a mammalian expression vector allowed us to expand our ectopic expression system and represents a valuable tool to study the role of polyP in HEK293T cells. Ppx1 has been reported to localize in the cytosol and soluble mitochondrial fraction of yeast (Lidia P. Lichko et al., 2003). The localization of Ppx1 in HEK293T cells when subcloned into a mammalian expression vector is unknown and is a question to address. Furthermore, it is still unknown how polyP relocalizes to subcellular compartments. Using cell fractionation techniques, our lab has shown that PPK expression elevates intracellular polyP levels throughout the cell (Bondy-Chorney, Abramchuk, Nasser, Holinier, Denoncourt, Baijal, Mccarthy, et al., 2020). It would be interesting to investigate whether the yeast

exopolyphosphatase is acting on specific intracellular pools of polyP. Determining the localization of Ppx1 in human cells would help elucidate how its expression in mammalian cells alters subcellular polyP concentrations and consequently the impact of polyP on different proteins.

The significance of this work is that it represents tools to study the role of polyP by increasing polyP concentrations inside the cell as opposed to its addition to culture media. An obvious next step is to compare the effect of PPK expression to the addition of extracellular polyP in HEK293T. A caveat here is that the majority of the previous studies are done in cell lines other than HEK293T. For example, the study that shows that the addition of extracellular polyP increases mTOR activation was done in vascular endothelial cells (Dinarvand et al., 2014). Aside from the cell lines, while we transfect our cells with the expression vectors 72 hours prior to collection, in the study by Dinarvand *et al.*, cells were treated with synthetic polyP for 4 hours before analysis (2014). Also, while PPK produces long chain polyP comparable to that produced in bacteria, the polyP chain length used is shorter (70P in this example) (Dinarvand et al., 2014). Therefore, the expansion of our system to investigate the effect of increased intracellular polyP in different cell lines is of interest as the amounts and chain length of polyP present are different. I expect that our ectopic expression would reveal differentially regulated genes upon increasing polyP concentrations in other cell types aside from HEK293T.

Furthermore, the difference in phospho-Erk1/2 between cells that express PPK and control cells is exaggerated in conditions where DMSO is used as a solvent in their treatment, i.e. for vehicle conditions or treatment with the MEK and Erk1/2 inhibitors **(Figure 10, lanes 1-**

4). DMSO has been reported to induce epigenetic modifications and this may indicate that cell stress exacerbates the effect of polyP within this signalling pathway (Verheijen et al., 2019). This could be tested by inducing stress (e. g. oxidative treatment with hydrogen peroxide) prior to analyses. These experiments will help explain the role of polyP in mediating the activity of mTOR, a master switch within the Erk/mTOR signalling cascade and warrant a new set of RNA sequencing and proteomics analysis.

4.7 Conclusions

My work has presented the use of novel tools to investigate polyP biology in human cells. Through PPK expression, the increase of polyP inside the cell has been revealed to play a role in the regulation of the Erk/mTOR signalling pathways. To expand our system, tools such as codon optimization and subcloning of the yeast exopolyphosphatase Ppx1 into mammalian expression vectors have been utilized. By studying mammalian polyP biology, we can also expand our knowledge of polyphosphorylation as a PTM. Overall, this work is fundamental to understanding the function of polyP in higher eukaryotes.

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