Novel tools to study polyphosphate biology in *S. cerevisiae* (yeast) and mammalian cells

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This thesis is submitted as a partial fulfillment of the M.Sc. program in Cellular and Molecular Medicine

July 16th, 2018

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AUTHORIZATION


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ABSTRACT

Polyphosphates (polyP) are long chains of phosphates residues linked via high-energy phosphoanhydride bonds. PolyP’s regulation and its functions have been studied in bacteria and in yeast, but the synthesis and degradation pathways of polyP in mammals are still unknown. This makes it challenging to study polyP biology in mammalian cells. In this thesis, the *E. coli* gene Polyphosphate kinase 1 (PPK1), which encodes for an enzyme that synthesizes polyP, was used as a tool to study polyP biology in both yeast and mammalian cells. Using yeast as a model organism, expression of *EcPPK1* led to a decrease in alpha-synuclein toxicity. Additional evidence shows the usefulness of using yeast as a model organism to study polyP’s protective effect on alpha-synuclein toxicity. In mammalian cells, transfection of *EcPPK1* allowed for the modulation of polyP concentration in the cell. This led to the identification of six human proteins that are able to be polyphosphorylated. Preliminary data from RNA sequencing also shows that polyP in HEK293T cells regulates gene expression. Overall, this work demonstrated that *EcPPK1* is a useful tool to study polyP biology in yeast and mammals.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Cdc55</td>
<td>cell division cycle 55</td>
</tr>
<tr>
<td>CTRL</td>
<td>control</td>
</tr>
<tr>
<td>Da</td>
<td>dalton</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>double-distilled water</td>
</tr>
<tr>
<td>DDP1</td>
<td>diphosphoinositol polyphosphate phosphohydrolase 1</td>
</tr>
<tr>
<td>DEK</td>
<td>DEK proto-oncogene</td>
</tr>
<tr>
<td>DH5α</td>
<td>Doug Hanahan Strain 5</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>Ec</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eiF2B5</td>
<td>eukaryotic translation initiation factor 2B subunit epsilon</td>
</tr>
<tr>
<td>eiF5B</td>
<td>eukaryotic translation initiation factor 5B</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Fpr3</td>
<td>Fk 506-sensitive proline rotamase 3</td>
</tr>
<tr>
<td>Gal</td>
<td>galactose</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3’ phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GO</td>
<td>gene ontology</td>
</tr>
<tr>
<td>GPD</td>
<td>glyceraldehyde 3-phosphate dehydrogenase (promoter)</td>
</tr>
<tr>
<td>HA</td>
<td>human influenza hemagglutinin</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HEK293T</td>
<td>human embryonic kidney cells T antigen of SV40</td>
</tr>
<tr>
<td>HeLa</td>
<td>Henrietta Lacks</td>
</tr>
<tr>
<td>hNOP56</td>
<td>human nucleolar protein 56</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HSP90AA1</td>
<td>heat shock protein 90 alpha family class A member 1</td>
</tr>
<tr>
<td>HSP90AB1</td>
<td>heat shock protein 90 alpha family class B member 1</td>
</tr>
<tr>
<td>HSP90B1</td>
<td>endoplasmin</td>
</tr>
<tr>
<td>LRP5</td>
<td>low density lipoprotein receptor-related protein 5</td>
</tr>
<tr>
<td>LRP6</td>
<td>low density lipoprotein receptor-related protein 6</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Michigan cancer foundation-7</td>
</tr>
<tr>
<td>Mesd</td>
<td>mesoderm development LRP chaperone</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NEBuilder</td>
<td>New England Biolabs builder</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>Nsr1</td>
<td>nuclear signal recognition 1</td>
</tr>
<tr>
<td>PASK</td>
<td>poly-acidic serine and lysine-rich</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s Disease</td>
</tr>
<tr>
<td>PHAS-I</td>
<td>eukaryotic translation initiation factor 4E-binding protein 1</td>
</tr>
</tbody>
</table>
PHO     phosphate-responsive
Pi      phosphate residues
polyP   polyphosphate
PPK     polyphosphate kinase
PPN     endopolyphosphate
PPX     exopolyphosphatase
PTM     post-translational modification
PVDF    polyvinylidene difluoride
qPCR    quantitative polymerase chain reaction
RIPA    radioimmunoprecipitation assay
RNA     ribonucleic acid
SaOS-2  human sarcoma osteogenic cells
SDS-PAGE sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBST    tris-buffered saline tween-20
TEM     transmission electron microscopy
Top1    topoisomerase 1
UPF3B   regulator of nonsense mediated mRNA decay
VTC     vacuolar transporter chaperone
WCE     whole cell extract
Wnt     wingless-related integration site
WT      wild-type
ACKNOWLEDGEMENTS

I would like to first thank my supervisor, Dr. Michael Downey. Thank you for giving me the opportunity to complete my Master’s degree in your lab. I have learned more than I could have ever imagined during my time in the lab. Also a big thank you to Dr. Marjorie Brand and Dr. Stephen Ferguson, my TAC members, for your help and advice during the past two years.

To all the Downey Lab members, thank you! I had the best time with you and always looked forward coming in to the lab. A special thank you to Amanda Bentley-DeSousa and Dr. Emma Bondy-Chorney, who have both listened to me rant about failed experiments probably more than I should have. Amanda, thank you for your time and help during the past two years. I truly owe you 11,000 fro-yos. Emma, thank you for being 100% supportive all of the time and spending time teaching me and mentoring me. I am forever grateful for our friendship triangle.

Thank you to my parents for giving me the opportunity to study abroad, listening to me talk science even when you didn’t understand and coming to visit when time away from home started feeling long. You inspire me everyday and I am so grateful to have you as my parents. Viktoria, thank you for making me laugh and for laughing at my lame jokes. I miss you. To my grandparents, aunt, uncles and cousins, thank you for your constant support. As I probably do not say it enough, I love you all.

I would like to thank Brad, Alex and Shane for keeping Willie busy playing Fortnite while I was writing my thesis. Thank you Susan and Chris for giving me a home away from home, and Sadie, my little bundle of joy of a dog, for putting a smile on my face when I needed it the most. Finally, thank you Willie. Thank you for handling my grumpiness in the morning, my hangry moments and my complaints; but more importantly, thank you for your support, believing in me when I doubt myself and making me laugh during the hardest days. I love you.
CHAPTER 1: INTRODUCTION

1.1 PolyP: an ancient molecule

Polyphosphates (polyP) are long chains of inorganic phosphates linked via high-energy phosphoanhydride bonds. They can range from 3 to 1000s of phosphate residues in length, and are found in all organisms (Braun, 2015; Jacobson, Halman, & Yariv, 1982; Arthur Kornberg, Rao, & Ault-Riché, 1999; Moreno & Docampo, 2013; Rao, Gómez-García, & Kornberg, 2009). This molecule was discovered in 1890 in the form of granules and was referred to as “volutin” (Kulaev, Vagabov, & Kulakovskaya, 2005; Liebermann, 1890; Meyer, 1904). In 1947, Wiame showed the granules were comprised of polyP (Wiame, 1947). In the 1950s, Kornberg became intrigued by this understudied molecule and spent several decades studying it (S. R. Kornberg, 1957; K. D. Kumble & Kornberg, 1995; H. Wurst, Shiba, & Kornberg, 1995). The mechanisms involved with polyP and its functions have been studied in bacteria and yeast, but the synthesis pathway of polyP in mammals is still unknown (Figure 1) (A. Kornberg, 1995; K. D. Kumble & Kornberg, 1995; Moreno & Docampo, 2013; H. Wurst et al., 1995).

1.1.1 PolyP in Saccharomyces cerevisiae (yeast)

1.1.1.1 PolyP biology in yeast

In yeast, Vacuolar Transporter Chaperone 4 (Vtc4) is the enzyme that catalyzes the synthesis of polyP from ATP and translocates it to the vacuole, where it is stored (Figure 1). Vtc4 is part of the Vacuolar Transporter Chaperone (VTC) complex, which has no homologous proteins in mammalian cells (Hothorn et al., 2009). The complex can
Figure 1. Synthesis and degradation of polyphosphate. A) In yeast, polyP is synthesized through the enzyme Vtc4 and is degraded by Ppx1, Ppn1, Ppn2, or Ddp1. In E. coli, polyP is produced by PPK1 and is degraded by PPX1 or PPX2. The synthesis and degradation pathway is unknown in mammals. (Prepared by Amanda Bentley-DeSousa, unpublished) B) The VTC complex in yeast synthesizes polyP and translocates it to the vacuole. (Inspired by Gerasimaite & Mayer, 2016)
be found in two forms, Vtc4/Vtc2/Vtc1 or Vtc4/Vtc3/Vtc1, with the former being more abundant on the endoplasmic reticulum (ER) and the latter on the vacuole (Hothorn et al., 2009; Uttenweiler, Schwarz, Neumann, & Mayer, 2007). All Vtc proteins possess three C-terminal helices embedded in the membrane. In addition, Vtc2, Vtc3 and Vtc4 all possess a N-terminal domain that is hydrophilic and points towards the cytosol, while Vtc1 is smaller and almost fully included in the membrane (Muller, 2003). Vtc2 and Vtc3 are thought to be accessory subunits of the complex. Vtc5 directly interacts with the VTC complex to stimulate the production and accumulation of polyP (Desfougères, Gerasimaitė, Jessen, & Mayer, 2016).

The translocation of polyP into the vacuole is important in yeast, as the accumulation of cytosolic polyP is thought to be toxic to the cell leading to abnormal morphology and impaired cell growth (Gerasimaitė, Sharma, Desfougeres, Schmidt, & Mayer, 2014). When \textit{VTC4} is not properly expressed or is deleted, polyP is no longer synthesized (Ogawa, DeRisi, & Brown, 2000). Similarly, when \textit{VTC1} is deleted, there is no synthesis of polyP (Ogawa et al., 2000), while deletion of \textit{VTC5} decreases polyP production and accumulation (Desfougères et al., 2016).

One of the enzymes that catalyzes the degradation of polyP is the exopolyphosphatase 1 (Ppx1) \textbf{(Figure 1)} (H. Wurst et al., 1995; Helmut Wurst & Kornberg, 1994). In yeast, Ppx1 cleaves the phosphate residues (Pi) at the terminal ends of a polyP chains of 3 to 500 phosphate residues, however it favours longer chains (~250 Pi) (Helmut Wurst & Kornberg, 1994). Ppx1 is mostly a cytosolic enzyme (Gerasimaitė
& Mayer, 2017; Krishnanand D. Kumble & Kornberg, 1996; H. Wurst et al., 1995), but has also been found to be located in the nucleus, at the plasma membrane, and the mitochondria matrix (Huh et al., 2003; L. Lichko, Kulakovskaya, Pestov, & Kulaev, 2006; L. P. Lichko, Pestov, Kulakovskaya, & Kulaev, 2003). Since most of the polyP is found in the vacuole, it has been suggested that the main function of Ppx1 could be to prevent the accumulation of polyP in the cytosol, where polyP can become toxic to the cell (Bentley-DeSousa et al., 2018; Gerasimaite & Mayer, 2016; Gerasimaite et al., 2014). The diphosphoinositol polyphosphate phosphohydrolase 1 (Ddp1) was identified as an endopolyphosphatase found in the cytosol and the nucleus, and has been reported to cleave long polyP chains in the middle to obtain shorter chains (Lonetti et al., 2011).

Unlike Ppx1 or Ddp1, the endopolyphosphatases Ppn1 and Ppn2 are found in the vacuole (Gerasimaité & Mayer, 2017; Krishnanand D. Kumble & Kornberg, 1996). Ppn1’s action results in smaller polyP chains of 60 Pi, 3 Pi and 1 Pi residue in length (Krishnanand D. Kumble & Kornberg, 1996). Ppn2 acts independently from Ppn1, and while it breaks down long chains of polyP into shorter chains similarly to Ppn1, it is thought to not be able to release single Pi residues (Gerasimaitė & Mayer, 2017).

In yeast, vacuolar polyP accounts for 10 to 20% of the dry weight of the cell (Indge, 1968; A. Kornberg, 1995; Urech, Dürr, Boller, Wiemken, & Schwencke, 1978). Only low amounts of polyP are found in the cytoplasm, the mitochondria and the nucleus (Arthur Kornberg et al., 1999; Saito, Ohtomo, Kuga-Uetake, Aono, & Saito, 2005; Tijssen, Beekes, & Van Steveninck, 1982; Urech et al., 1978). The overall concentration of polyP in yeast cells is around 120mM-200mM (Figure 2) (Auesukaree et al., 2004;
Arthur Kornberg et al., 1999). The exact concentration of polyP in each organelle is still unknown. However, it is thought that the vacuole contains between 90 to 99% of the total polyP in cells (Indge, 1968; Arthur Kornberg et al., 1999).

1.1.1.2 PolyP functions in yeast

In yeast, polyP acts as a source of energy and a storage of Pi residues (Arthur Kornberg et al., 1999; Moreno & Docampo, 2013). For instance, when the cells are deficient in nutrients (for example, Pi residues), polyP is able to restock the cells with Pi (Neef & Kladde, 2003). PolyP also acts as an ion chelator (Bonting, Kortstee, & Zehnder, 1993; Dunn, Gable, & Beeler, 1994; Kulaev et al., 2005), and polyP has been shown to play an important role in yeast cell survival when cells are in the stationary phase (Sethuraman, Rao, & Kornberg, 2001). The absence of polyP leads to defects in microautophagy (Uttenweiler et al., 2007), and deletion of certain genes involved in the phosphate-responsive (PHO) regulatory pathway has been shown to negatively affect the accumulation of polyP (Ogawa et al., 2000). Additionally, absence of polyP (ie. when \( VTC4 \) is deleted) decreases sensitivity to stress in \( \text{pho}80\Delta \) and \( \text{pho}85\Delta \) cells, where Pho80-Pho85 are regulators of \( VTC4 \) expression (Huang, Moffat, & Andrews, 2002).

1.1.2 PolyP in *Escherichia coli* (bacteria)

In bacteria, PPK1 is the enzyme that produces polyP from ATP (Figure 1) (K. Ahn & Kornberg, 1990). The other polyphosphate kinase in bacteria is PPK2, which is able to synthesize polyP from either ATP or GTP (Ishige, Zhang, & Kornberg, 2002; Zhang, Ishige, & Kornberg, 2002). PolyP is hydrolyzed by PPX1, an
exopolyphosphatase, that favours the break down of longer polyP chains (Akiyama, Crooke, & Kornberg, 1993). Unlike yeast, bacteria do not have any known endopolyphosphatases (Moreno & Docampo, 2013).

Similar to yeast, polyP functions as storage for Pi residues, can be a substitute for ATP and thus, an energy source (Arthur Kornberg et al., 1999). PolyP also regulates development, stress and survival (Ault-Riché, Fraley, Tzeng, & Kornberg, 1998; Crooke, Akiyama, Rao, & Kornberg, 1994; Arthur Kornberg et al., 1999; Rao & Kornberg, 1996). During the stationary phase, ppk1 mutants show increased sensitivity to hydrogen-peroxide, menadione, heat and high salt concentration (Rao & Kornberg, 1996). Additionally, in the stationary phase, the survival of ppk1 mutants is importantly decreased (Rao & Kornberg, 1996). PolyP has also been linked to biofilm formation and virulence (Grillo-Puertas, Villegas, Rintoul, & Rapisarda, 2012; Kim, Rao, Fraley, & Kornberg, 2002; Rashid et al., 2000). At high polyP concentrations, defects in biofilm formation are observed (Grillo-Puertas et al., 2012).

When E. coli PPK1 is expressed in yeast, it is thought to produce polyP in the cytoplasm, independently from the vacuole (Gerasimaite et al., 2014). In my work, these two elements make EcPPK1 a very useful tool to manipulate the production and location of polyP chains in yeast and mammalian cells.
1.1.3 PolyP in mammals

The synthesis and degradation of polyP in mammals are not well described (Bentley-DeSousa & Downey, 2018). The only proposed human exopolypophosphatase is H-prune, which is thought to act on short polyP chains and to be inhibited by long polyP chains (Tammenkoski et al., 2008). However, H-prune’s exopolypophosphatase activity has only been shown in vitro (Tammenkoski et al., 2008). PolyP chains have been found in different cellular compartment such as the nucleus, the cytoplasm and the mitochondria (K. D. Kumble & Kornberg, 1995). PolyP concentration varies from one cell line to another, and in different tissues (K. D. Kumble & Kornberg, 1995). However, overall, the concentration of polyP in mammalian cells (<1mM) is much lower than in yeast (120-200mM), which has made studying polyP difficult in higher eukaryotes (Figure 2) (Auesukaree et al., 2004; Arthur Kornberg et al., 1999; Rao et al., 2009; Ruiz, Lea, Oldfield, & Docampo, 2004).

Exactly how polyP is regulated in mammals is unknown, but the involvement of polyP in a variety of medically related functions has been uncovered. PolyP plays a role in apoptosis (Hernandez-Ruiz, Gonzalez-Garcia, Castro, Brieva, & Ruiz, 2006). It has been shown that the addition of polyP to myeloma cell lines increases apoptosis activity compared to normal cell lines (Hernandez-Ruiz et al., 2006). Moreover, polyP is found in high concentration in the dense granules of platelets (Ruiz et al., 2004). Once released from platelets, it acts in the blood coagulation cascade (Müller et al., 2009; Smith et al., 2006; Smith et al., 2010; Smith & Morrissey, 2008). It has been reported to accelerate blood clotting and fibrinolysis and thus, is considered a procoagulant and
Figure 2. Concentration of polyP in different organisms and organelles.

Concentrations of polyP in different mammalian cells are lower than in yeast with the exception of the dense granules found in platelets (Auesukaree et al., 2004; K. D. Kumble & Kornberg, 1995; Ruiz et al., 2004). (Figure prepared by Dr. Michael Downey)
proinflammatory mediator (Müller et al., 2009; Smith et al., 2006). PolyP’s effect on the blood cascade is also dependent on chain length (Stephanie A. Smith et al., 2010). PolyP was found to play a role in certain type of cancer cells such as MCF-7 and myeloma cells (Jimenez-Nuñez et al., 2012; Wang, Fraley, Faridi, Kornberg, & Roth, 2003). One study showed that the levels of polyP were higher in myeloma plasma cells compared to normal plasma cells, and that nuclear polyP modulated RNA polymerase I activity (Jimenez-Nuñez et al., 2012). PolyP was also found to activate mTOR (Wang et al., 2003). In MCF-7 cells, in the absence of polyP, mTOR is not activated and is unable to phosphorylate PHAS-I (eukaryotic initiation factor eIF4E-binding protein), one of its substrates (Wang et al., 2003). Additionally, polyP has been reported to act as gliotransmitter by activated astrocytes, and as a neuromodulator (Holmström et al., 2013; Stotz et al., 2014). In neurons, polyP has been found to localized to the synaptic vesicles where it is thought to act as a neurotransmitter and regulate neuronal activity (Stotz et al., 2014).

1.2 PolyP and proteotoxic stress

1.2.1 Proteotoxic stress: a common regulator of neurobiological disease

Protein homeostasis, also called proteostasis, is essential for the proper functioning of a cell (Balch, Morimoto, Dillin, & Kelly, 2008; Morimoto, 2008). Protein homeostasis encompasses many mechanisms such as translation, the folding of proteins, their transportation and localization, their concentration and their degradation (Balch et al., 2008; Morimoto, 2008). If protein homeostasis is not tightly maintained and misfolded proteins are not degraded, they can aggregate and lead to disruption of critical
cell functions. This is referred to as proteotoxic stress, and is often associated with neurodegenerative diseases (Balch et al., 2008; Braun, 2015; Morimoto, 2008; Taylor, Hardy, & Fischbeck, 2002).

1.2.2 PolyP: a modifier of human protein toxicity

When proteins become misfolded and aggregate together, they form amyloid fibrils. These fibrils are insoluble and found in β-sheet conformation (Eichner & Radford, 2011). Recently, Cremers et al. (2016) investigated the role polyP plays on the amyloid fibril formation process, and more specifically of the alpha-synuclein protein. Previous work from the same group showed that in bacteria, polyP acts as a chaperone and helps stabilize proteins under stress conditions (Gray et al., 2014). Thus, they hypothesized that polyP could play a role in the stabilization and modulation of amyloid fibril formation, and more specifically of alpha-synuclein fibrils (Cremers et al., 2016).

1.2.2.1 Alpha-synuclein structure and function

The SNCA gene is found on chromosome 4q21 and encodes for the protein alpha-synuclein (Polymeropoulos et al., 1997). Alpha-synuclein is composed of 140 amino acids, and contains three domains: an amphipathic N-terminal lipid-binding α-helix, a non-amyloid component (NAC), which has been reported to be an aggregation domain, and a C-terminal acidic tail associated with protein aggregation inhibition (Clayton & George, 1998; Polymeropoulos et al., 1997; Ueda et al., 1993). Alpha-synuclein is mostly localized in the synapse of neurons found in the central nervous system (CNS), and it is thought that alpha-synuclein plays a role in the regulation of the release of dopamine,
which is a neurotransmitter controlling movement (Liang et al., 2008; Maroteaux, Campanelli, & Scheller, 1988; Venda, Cragg, Buchman, & Wade-Martins, 2010).

Alpha-synuclein pathology contributes to both familial and sporadic forms of Parkinson’s Disease (PD). PD is a neurodegenerative disease that leads to the impairment of the motor functions due to the loss of dopaminergic neurons in the substantia nigra (Lees, Hardy, & Revesz, 2009; Outeiro & Lindquist, 2003; Taylor et al., 2002). Most cases of PD are associated with the duplication or even triplication of the SNCA gene (Chartier-Harlin et al., 2004; Singleton et al., 2003). This holds true for both familial and sporadic cases (T. B. Ahn et al., 2008; Chartier-Harlin et al., 2004; Nishioka et al., 2009; Singleton et al., 2003). In some familial forms of the disease, the accumulation of alpha-synuclein in cells is due to a missense mutations of the SNCA gene (Braun, 2015). Three different missense mutations are often related to PD: A53T, A30P and E46K (Krüger et al., 1998; Polymeropoulos et al., 1997; Zarranz et al., 2004). Recently, three additional missense mutations have been identified and were reported to be affiliated with sporadic PD. They include: A18T, A29S and H50Q (Appel-Cresswell et al., 2013; Hoffman-Zacharska et al., 2013; Proukakis et al., 2013). All of these mutations are located in the N-terminal of the protein (Chai & Lim, 2013). Whether a mutation or duplication of the SNCA gene is the cause of PD, the pathology observed in patients is similar (Chai & Lim, 2013). Alpha-synuclein aggregates and forms large cytoplasmic inclusions in the brain called Lewy bodies (Outeiro & Lindquist, 2003; Taylor et al., 2002).
There is still some controversy in the field as to whether alpha-synuclein aggregation leads to toxic loss-of-function in preventing alpha-synuclein function or leads to toxic gain-of-function by allowing the cytotoxicity (Collier, Eugene Redmond, Steece-Collier, Lipton, & Manfredsson, 2016; Oikawa et al., 2016; Perez & Hastings, 2004). Overall, the mechanisms that protect normal cells from toxicity associated with aberrant expression of alpha-synuclein are currently poorly understood and need to be further studied.

### 1.2.2.2 PolyP reduces alpha-synuclein toxicity

In Lewy bodies, alpha-synuclein fibrils form aggregates. Through the process of fibril formation, intermediate oligomers of alpha-synuclein accumulate and are thought to be toxic to the cell (Bucciantini et al., 2002; Celej et al., 2012; Chiti & Dobson, 2006). These toxic oligomers are produced before the formation of mature fibrils and as a result of mature fibrils shedding (Chen et al., 2015). Cremers et al. (2016) uncovered that polyP accelerates alpha-synuclein fibril formation and reduces alpha-synuclein fibril shedding \textit{in vitro}. Moreover, they showed that polyP most likely interacts with alpha-synuclein fibril via a stable complex (Cremers et al., 2016). When observing alpha-synuclein fibrils in the presence or absence of polyP using transmission electron microscopy (TEM), they observed that alpha-synuclein fibrils with polyP were thinner, longer and straighter (Cremers et al., 2016). Using neuroblastoma cells, they showed that addition of alpha-synuclein fibrils stabilized with polyP, reduced the amount of cell death observed compared to the addition of alpha-synuclein fibrils in the absence of polyP (Cremers et al., 2016). Overall, their study provided strong evidence that polyP plays an important
role in the stabilization of alpha-synuclein fibril formation, which results in decreased cytotoxicity. However, the potential genetic and molecular interactions involved in this protective process have not been elucidated yet.

1.2.3 Yeast: a useful model system to study the impact of polyP on toxic proteins in vivo

The budding yeast system is best suited for the study of both toxic proteins and polyP biology. Yeast has been used to study human toxic proteins for almost two decades (Braun, 2015; Krobisch & Lindquist, 2000; Morimoto, 2008; Outeiro & Lindquist, 2003). Overexpression of these proteins in yeast results in cytoplasmic aggregates that cause a loss of viability, mirroring what is seen in human cells. This system has been used by the lab of Susan Lindquist and others to identify modulators of aggregation and/or toxicity (Dixon, Mathias, Zweig, Davis, & Gross, 2005; Gitler et al., 2009; Outeiro & Lindquist, 2003; Winderickx et al., 2008; Zabrocki et al., 2008). About a third of human genes that are associated to diseases share homology to yeast genes. Several genetic screens have been performed in yeast and have identified genes associated with alpha-synuclein toxicity (Cooper et al., 2006; Liang et al., 2008; Willingham, Outeiro, DeVit, Lindquist, & Muchowski, 2003), or huntingtin toxicity (Giorgini, Guidetti, Nguyen, Bennett, & Muchowski, 2005; Willingham et al., 2003). For example, Gitler et al. (2009) identified YPK9 as a gene whose overexpression reduces the toxicity of alpha-synuclein in yeast. The Ypk9 protein is the yeast homolog of parkin, which is thought to carry out a similar function in human cells.
As mentioned previously, yeast is an ideal model organism to use for the study of polyP biology as it has very high concentrations of polyP (120-200mM) compared to mammalian cells (<1mM) (Auesukaree et al., 2004; Arthur Kornberg et al., 1999; K. D. Kumble & Kornberg, 1995). In yeast, the regulators of polyP synthesis and degradation are known; whereas in mammals, the regulators have yet to be identified (Gerasimaitė & Mayer, 2017; Hothorn et al., 2009; Krishnanand D. Kumble & Kornberg, 1996; Lonetti et al., 2011; Helmut Wurst & Kornberg, 1994). The work presented in this thesis took advantage of the yeast budding system to establish this organism as a tool to investigate whether the effect of the identified yeast genes have on alpha-synuclein toxicity is occurring through polyP biology. The first step was to test whether polyP can impact alpha-synuclein toxicity within established yeast models, and use this work in yeast as a stepping-stone for mammalian work.

1.3 Polyphosphorylation: a novel post-translational modification

Signaling pathways are varied, complex and essential for proper regulation in cells. Post-translational modifications (PTM) play a major role in cell signaling and include protein modifications such as ubiquitination, phosphorylation, and SUMOylation. In 2015, Azevedo et al. introduced a novel PTM called polyphosphorylation. It consists of the covalent attachment of polyP chains to lysine residues found in a poly-acidic serine and lysine-rich (PASK) motifs (Figure 3a). This modification is non-enzymatic and to date, can only be detected by electrophoretic mobility shift on NuPAGE gels (Figure 3b) (Azevedo, Livermore, & Saiardi, 2015; Bentley-DeSousa et al., 2018).
Figure 3. Yeast proteins can be polyphosphorylated. A) yeast proteins containing a PASK motif can be polyphosphorylated on lysine (K) residues by polyP chains and B) yeast WCE extract from wild-type or vtc4Δ background strains with TAP-tagged TOP1 were run on 4-12% Bis-Tris NuPAGE gel, transferred to PVDF membrane prior to immunoblotting with an antibody against the TAP tag to observe the electrophoretic mobility shift due to polyphosphorylation. (Figure prepared by Amanda Bentley-DeSousa)
1.3.1 Top1 and Nsr1 are polyphosphorylated in yeast

The first two targets identified as polyphosphorylated by Azevedo et al. (2015) were the yeast proteins Nsr1 (Nuclear signal recognition 1) and Top1 (Topoisomerase 1). Polyphosphorylation of Nsr1 was observed by electrophoretic mobility shift on a Bis-Tris NuPAGE gel. Nsr1’s predicted molecular weight is ~45 kDa; however, it was detected in WT yeast at ~130 kDa. When VTC4 was deleted or when the WT protein extract was treated with Ppx1, an exopolyphosphatase, the electrophoretic shift collapsed (Azevedo et al., 2015). A similar mobility shift was seen for Top1. Through several assays, the authors demonstrated that polyphosphorylation occurs covalently and non-enzymatically on lysine residues found in a PASK motif. The size of the shift observed for polyphosphorylation seems to correlate to the length of the polyP chain (Azevedo et al., 2015). Finally, polyphosphorylation negatively alters Nsr1-Top1 protein interaction and protein localization, and decreases Top1 activity (Azevedo et al., 2015).

1.3.2 15 new yeast polyphosphorylated targets identified

Following the Azevedo et al. (2015) study, Bentley-DeSousa et al. (2018) performed a screen to identify additional polyphosphorylated targets in yeast. From this screen, 15 new polyphosphorylated substrates were uncovered (Table 1). The screen focused on 90 of 427 PASK proteins, for which the PASK motif was described as a 20 amino acid stretch containing a minimum of 75% glutamic acid (E), aspartic acid (D), serine (S) and at least one lysine (K). WT and vtc4Δ strains fused with the GFP-tagged proteins of interest were ran on NuPAGE gels to observe which ones displayed an electrophoretic mobility shift indicative of polyphosphorylation (Bentley-DeSousa et al., 2018).
Table 1. 17 polyphosphorylated yeast substrates and their human homologs. This table lists all 17 yeast substrates that have been found to be polyphosphorylated as of today, it describes their function and indicates their human homologs. The shaded hits represents the ones found in Azevedo et al. (2015) and the unshaded ones were identified in Bentley-DeSousa et al. (2018). (Adapted from Azevedo et al., 2015; Bentley-DeSousa et al., 2018)

<table>
<thead>
<tr>
<th>Yeast protein</th>
<th>Function</th>
<th>Human homolog</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nsr1</td>
<td>mRNA localization</td>
<td>NCL</td>
</tr>
<tr>
<td>Top1</td>
<td>DNA topoisomerase</td>
<td>TOP1</td>
</tr>
<tr>
<td>Nop56</td>
<td>rRNA maturation</td>
<td>hNOP56</td>
</tr>
<tr>
<td>Nop58</td>
<td>rRNA maturation</td>
<td>hNOP58</td>
</tr>
<tr>
<td>Rts1</td>
<td>PP2A phosphatase</td>
<td>PPP2R5A-E</td>
</tr>
<tr>
<td>Fpr3</td>
<td>Propyl isomerase</td>
<td>N/A</td>
</tr>
<tr>
<td>Fpr4</td>
<td>Propyl isomerase</td>
<td>N/A</td>
</tr>
<tr>
<td>Boi1</td>
<td>Polar growth</td>
<td>NEB/NEBL</td>
</tr>
<tr>
<td>Rpa34</td>
<td>RNA polymerase I subunit</td>
<td>CD3EAP</td>
</tr>
<tr>
<td>Rrp15</td>
<td>rRNA processing</td>
<td>RRP15</td>
</tr>
<tr>
<td>Jij1</td>
<td>Chaperone</td>
<td>N/A</td>
</tr>
<tr>
<td>Chz1</td>
<td>Htz1 chaperone</td>
<td>N/A</td>
</tr>
<tr>
<td>Tma23</td>
<td>Ribosome biogenesis</td>
<td>N/A</td>
</tr>
<tr>
<td>Eaf7</td>
<td>NuA4 HAT subunit</td>
<td>MRGBP</td>
</tr>
<tr>
<td>Nst1</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Utp14</td>
<td>Ribosome assembly</td>
<td>UTP14A/C</td>
</tr>
<tr>
<td>Hpc2</td>
<td>Nucleosome assembly</td>
<td>FAM21C</td>
</tr>
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</table>
The study found that the 17 yeast proteins identified as being polyphosphorylated, including Nsr1 and Top1, are part of a large network involved in ribosome biogenesis. Moreover, polysome profiles between WT and \textit{vtc4}\textDelta yeast strains showed defects in polysome assembly, suggesting that Vtc4 plays a role in ribosome function (Bentley-DeSousa et al., 2018).

Bentley-DeSousa et al. (2018) showed that the electrophoretic mobility shifts observed on polyphosphorylated proteins collapses on SDS-PAGE gels. The Bis-Tris NuPAGE gels used for polyphosphorylation assays sold by Thermo Fisher are not polymerized with tetramethylethylenediamine (TEMED) unlike SDS-PAGE gels. Therefore, they tested their own Bis-Tris gels polymerizing them with or without TEMED and concluded that the TEMED contributes to the collapse of the shifts on SDS-PAGE gels (Bentley-DeSousa et al., 2018). Therefore, as of today, polyphosphorylation assays are only performed on Bis-Tris NuPAGE gels. This is the technique used in this thesis to identify polyphosphorylated proteins.

One of the points addressed in Bentley-DeSousa et al. (2018), a publication I am second author on, is whether human proteins containing a PASK motif can also be polyphosphorylated. This is the first step in investigating whether some functions of polyP in human cells might be mediated by its covalent attachment to proteins. The answers to this question are part of this thesis and are discussed in detail in Chapters 3 and 4.
1.4 Rationale

Although extensive research has been carried out on polyP in the last two decades, polyP biology is still an understudied field (Bentley-DeSousa & Downey, 2018). Recently, polyP has been shown to be involved in functions relevant for health research and could be used as a potential therapeutic target (Jimenez-Nuñez et al., 2012; Müller et al., 2009; Wang et al., 2003). In order to evaluate the therapeutic potential, polyP synthesis and regulation in mammals needs to be elucidated. Investigating the genetic and molecular interactions involved with polyP cytoprotective effect on alpha-synuclein may provide insight on polyP’s effect on amyloid-related diseases. The development of tools to study polyP biology in mammalian cells is necessary to help identify polyP regulators and gain a deeper understand of polyP function in mammals.

1.5 Hypotheses and Objectives

Hypothesis 1:
PolyP is a modifier of proteotoxic stress. In order to test this hypothesis, my objectives are to:

- Develop yeast as novel genetic model for polyP’s role in regulating folding of toxic proteins involved in neurobiological diseases
- Uncover the molecular impact of polyP on toxic proteins
Hypothesis 2:

Human proteins containing PASK motifs can be polyphosphorylated, and this PTM modulates the function of its target proteins. In order to test this hypothesis, my objectives are to:

- Identify which human proteins can be polyphosphorylated
- Uncover the involvement of polyphosphorylation in the function of relevant pathway
CHAPTER 2: MATERIAL AND METHODS

2.1 Investigating polyP’s effect on alpha-synuclein toxicity in yeast

2.1.1 Yeast strains and plasmids

Yeast strains and plasmids used can be found in Table 2 and Table 3 respectively.

Plasmids were transformed using DH5α competent cells and following the “Transforming Bacteria” protocol from Promega Subcloning Notebook. LB plates containing either ampicillin or kanamycin were used to grow the bacteria. After overnight incubation at 37°C, a streak was taken from the plate and the bacteria was grown for a minimum of 8 hours in 3ml LB media with either 3µl of ampicillin or kanamycin at 37°C. All plasmid stocks were prepared following the “Purification of plasmid DNA” protocol using the EZ-10 Spin column plasmid DNA MiniPreps Kit (Bio Basics Inc BS614-250).

A VTC4 knockout cassette was generated by PCR using the primers

GCTAAACAATCAAATCGGCCAATAAAAGAGCATAACAAGGCAGGAACAGCTC
AGCTGAAGCTTCGTACGC AND
TTACTTTAATTACAGTAAAAAAAACACGCTGTGTATTCAGCATAAGGCACACT
AGTGGATCTCG. Yeast strains, as indicated in Table 2, were then transformed with the knockout cassette in order to delete VTC4.
### Table 2. Yeast strains used for spot tests and Western blot analysis

<table>
<thead>
<tr>
<th>Name</th>
<th>Strain #</th>
<th>Strain genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>YMD964</td>
<td>MATα leu2d0 ura3d0 his3-1 MET15 lys2d0</td>
<td>Rudner Lab</td>
</tr>
<tr>
<td>vtc4Δ</td>
<td>YMD1577</td>
<td>MATα leu2d0 ura3d0 his3-1 MET15 lys2d0 vtc4::NATMX</td>
<td>(Bentley-DeSousa et al., 2018)</td>
</tr>
<tr>
<td>LowTox</td>
<td>YMD1761</td>
<td>MATα URA3 ura3::pRS306-GAL-aSyn-GFP</td>
<td>Lindquist Lab</td>
</tr>
<tr>
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<td>YMD1760</td>
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</tr>
<tr>
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<td>YMD1756</td>
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</tr>
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<td>YMD2111</td>
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<td>This study</td>
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<td>\textit{vtc4}\textDelta HA-\textit{Ec}PPK1-NLS SNCA\textsuperscript{WT}(EV)</td>
<td>YMD2107</td>
<td>MAT\textalpha\ leu2d0 ura3d0 his3-1 MET15 lys2d0 vtc4::NATMX his3::p121-GAL-HA-PPK1-NLS-HIS3 [pRS426-GAL1-GFP]</td>
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Table 3. Plasmids used for yeast transformation

<table>
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<tr>
<td>SNCA&lt;sup&gt;WT&lt;/sup&gt;</td>
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<td>pRS426-GAL1-GFP-URA3</td>
<td>Braus Lab PMID 22722939</td>
</tr>
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</table>
2.1.2 Yeast transformation

Yeast cells were grown overnight in YPD media with 5% dextrose at 30°C. The next day, they were diluted to 0.2 OD_{600} and grown till 0.8 OD_{600}. The cells were spun down at 3,000 rpm for 5 minutes. The cell pellet was washed in water and spun down again. The cells were transferred to an eppendorf tube using 1 mL of 0.1M LiAc and spun at 10,000 rpm for 10 seconds. The cell pellet was resuspended to the 0.5 mL mark on the Eppendorf tube with 0.1M LiAc. 50 µl of the solution was transferred to a new tube, spun down and the supernatant was discarded. The following components were added to the cell pellet: 240 µl of 50% PEG, 36 µl of 1M LiAc, 10 µl of ssDNA (10 mg/mL stock), ~1 µg of DNA and ~73 µl of water. The mix was vortexed for 1 minute and incubated for 30 minutes at 30°C. The sample was then heat shocked for 15 minutes at 42°C. The cells were spun down at 6,000 rpm for 2 minutes. A quick wash of the pellet with water was done, prior to resuspending the pellet in 200 µl of water. The solution was then plated onto either a SC-selective plate or a YPD plate followed by a drug selection plate. Finally, the plate is incubated at 30°C to allow colonies to grow.

2.1.3 Spot tests

Yeast cells were grown fresh on agar plates and were resuspended in water. The cells were then plated in five-fold dilutions on the indicated plates and left to grow at 30°C. Pictures were taken after 24-72 hours of incubation.
2.1.4 TCA protein preparation

Yeast cells were grown overnight in YPD or SC selective media. The next day, the cultures were diluted to OD$_{600}$ = 0.8 and grown for ~1 hour at 30°C. Cells were centrifuged at 3,000 rpm for 5 minutes, washed with cold water and centrifuged again. Cells were resuspended in water to be transferred into a screw cap tube and was pelleted. The pellet was resuspended in 300 µl of 20% TCA and about 200 µl of glass beads were added. To be lysed, the cells were bead beat twice 3 minutes while being kept on ice in between. The supernatant was transferred to a new tube. 300 µl of 5% TCA was added to the beads, vortexed and the supernatant was added to the initial transferred supernatant. The samples were then spun at 12,500 rpm for 4 minutes at 4°C and the supernatant was discarded. 100 µl of SDS-PAGE sample buffer was added to each pellet and the samples were vortexed prior to being boiled at 100°C for 5-10 minutes. Finally, the samples were centrifuged at 17,000 g for 4 minutes at room temperature, and the supernatant was transferred to a new tube. The samples were then run on either a 10% SDS-PAGE gel at 120V or a 4-12% Bis-Tris NuPAGE gel (Thermo Fisher NP0336BOX) at 200V. The gel was transferred to a PVDF membrane before immunoblotting.

2.1.5 Immunoblotting

The membranes were blocked with 5% milk in 1xTBST or 5% BSA in 1xTBST for 30 minutes. They were then incubated with the primary antibody for 1 hour at room temperature or overnight at 4°C. After 3 x 5 minute-washes with 1xTBST, the membranes were incubated with the secondary antibodies for 30 minutes at room temperature, and were then washed again 3 x 5 minutes. After incubation with ECL
reagent, the blots were developed using imaging films. The antibodies used are listed in Table 4.

2.2 Investigating polyphosphorylation in mammalian cells

2.2.1 Mammalian cell culture

HEK293T cell lines were obtained from ATCC (ATCC CRL-3216). HEK293T cells were cultured in DMEM (Wisent 319-015-CL) with 10% FBS (Wisent 080-150), 1 mM sodium pyruvate (Sigma S8636-100ML) and Penicillin-Streptomycin-Amphotericin B solution. Cells were grown in 10 cm or 6-well polystyrene dishes at 37°C and 5% CO₂. Our lab regularly monitors the cell cultures for mycoplasma contamination using a Mycoplasma Detection Kit (ABM G238).

2.2.2 PolyP WCE preparation

WT (ppn1Δ) and vtc4Δ (ppn1Δvtc4Δ) strains were grown overnight in 10 ml YEP media with dextrose. The following day, the cells were diluted to 0.8 OD in 40 ml of YEP media with dextrose and incubated for ~1 hour at 30°C. 25 OD worth of cells were transferred into 15ml falcon tube and centrifuge at 3,000 rpm for 5 minutes. The cells were transferred to screw cap tubes and washed once with 500 µl of cold water. The cells were centrifuged at 13,500 rpm for 1 minute at 4°C, and the supernatant was discarded. The pellets were resuspended in 300 µl of cold polyP lysis buffer. 100 µl of glass beads were added to each tube and using the miniblender, the samples were beat twice for 30 seconds with an ice incubation in between. The supernatant was transferred to new
<table>
<thead>
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microcentrifuge tubes and centrifuge at 13,500 rpm for 5 minutes at 4°C. The supernatant was again transferred to new microcentrifuge tubes.

2.2.3 *In vitro* polyphosphorylation assay using HEK293T cells

HEK293T were grown to ~80% confluency in a 10 cm dish. Cells were washed with 1xPBS before being scraped and collected in an Eppendorf tube. Cells were spun down for 5 minutes at 5,000 rpm in the cold centrifuge and the PBS was discarded. 1ml of mammalian polyP lysis buffer was added, cells were vortexed for 30 seconds and incubated on ice for 15 minutes. A 21-gauge needle was used to break up the cells, before being centrifuged for 15 minutes at 13,500 rpm in the cold centrifuge. The supernatant was transferred to a new Eppendorf tube and kept on ice. Using this prepared whole cell extract, 4 reactions were prepared. In tube #1, 45 µl of mammalian polyP lysis buffer was mixed with 5 µl of HEK293T WCE. In tube #2, 45 µl of WT yeast lysate (see polyP WCE preparation) was added to 5 µl of HEK293T WCE. In tube #3, 45 µl of *vtc4*Δ yeast lysate was mixed with 5 µl of HEK293T WCE. In tube #4, 45 µl of synthetic polyphosphate (final concentration of 0.5 mM) was mixed with 5 µl of HEK293T WCE. All 4 tubes were incubated for 1 hour at 4°C with end-over-end rotation. NuPAGE loading buffer was added in a 1:1 ration to each tube before being boiled. The samples were then ran on a 4-12% Bis-Tris NuPAGE gel (Thermo Fisher NP0336BOX).

2.2.4 Plasmids used

All plasmid stocks were prepared using the HiPure Plasmid Maxiprep Kit (Invitrogen K210006).
pcDNA3.1(-)EcPPK1

*E. coli* PPK1 was amplified from a yeast plasmid gifted by Andreas Mayer. The 5’ and 3’ primers used were respectively

TCCACCACACTGGGACTAGTGATGGGTCAGGAAAAGCTATAC and
TGATCAGCGGTTTTAACTTATTATTCAGGTTGTTCGAGTG designed with NEB Builder. The empty vector pcDNA3.1(-) was cut at *BamHI* and *HindIII* restriction sites, and the *EcPPK1* insert was introduced in the cut backbone using Gibson assembly (NEB E5510S). The pcDNA3.1(-) backbone used in this study contains a bleocin resistance marker.

pcDNA3.1(-)HA-EcPPK1

HA-*EcPPK1* was amplified from a yeast plasmid previously made in our lab. The 5’ and 3’ primers used were respectively

TCCACCACACTGGGACTAGTGATGTCACCATACGATGTTC and
TGATCAGCGGTTTTAACTTATTATTCAGGTTGTTCGAG designed with NEB Builder. The empty vector pcDNA3.1(-) was cut at *BamHI* and *HindIII* restriction sites, and the HA-*EcPPK1* insert was introduced in the cut vector using Gibson assembly (NEB E5510S). The pcDNA3.1(-) backbone used in this study contains a bleocin resistance marker.

2.2.5 Transfection of mammalian cells

Cells were grown to 70-80% confluency in a 6-well polystyrene dish and were transfected using Lipo2000 with 2 µg of pcDNA3.1(-)EcPPK1, pcDNA3.1(-)HA-
EcPPK1, OLLAS-EcPPK1 or MYC-LRP5, in parallel as its empty vector. The transfected cells were left to grow for 48 hours in OptiMeM (Fisher 31985-070).

2.2.6 Protein preparation for mammalian cells

Post-transfection, the cells were washed one with cold 1xPBS and then collected in 1ml of 1xPBS with the help of a scraper. The cells were spun down for 5 minutes at 5,000 rpm in the cold centrifuge. The 1xPBS was discarded and ~200 µl of RIPA Lysis Buffer was added. Cells were vortexed for 30 seconds and incubated on ice for ~20 minutes before being centrifuged for ~20 minutes at 13,500 rpm in the cold centrifuge. The supernatant was transferred to a new Eppendorf tube and 5x Laemmli Sample Buffer was added. The samples were boiled before loading of the gel.

2.2.7 Western blotting for mammalian protein extract

The samples were run on 4-12% Bis-Tris NuPAGE gels (Thermo Fisher NP0336BOX) at 200V using 1xMOPS Running Buffer. The gels were transferred to PVDF membranes at 85V for ~1 hour using 1xNuPAGE Transfer Buffer. The membranes were blocked with 5% milk in 1xTBST or 5% BSA (VWR CA97061-416) in 1xTBST for 30 minutes. They were then incubated with the primary antibody for 1 hour at room temperature or overnight at 4°C. After 3 x 5 minute-washes with 1xTBST, the membranes were incubated with the secondary antibodies for 30 minutes at room temperature, and were then washed again 3 x 5 minutes. After incubation with ECL reagent (Fisher WBLUF0500), the blots were developed using imaging films. The antibodies used are listed in Table 4.
2.2.8 PolyP extraction in HEK293T cells

After HEK293T cells were transfected with CTRL or EcPPK1 plasmids for 48 hours, the cells were washed with cold 1xPBS and collected with 1ml PBS. The cells were spun down for 5 minutes at 5,000 rpm in the cold centrifuge. The PBS was discarded and the cell pellets were resuspended in 250 µl TELS buffer and mixed with 250 µl of acid phenol/chloroform pH 4.5 [125:25] [Phenol solution pH 4.3 (P4682), Chloroform (Sigma 472476)]. 400-600 µl of glass beads were added to the samples and they were vortexed for 5 minutes at 4°C, prior to centrifugation for 5 minutes at 1,500g in the cold centrifuge. The water phase was transferred to a new tube and subjected to chloroform extraction with an equal volume of chloroform. The water phase was transferred to a new tube and polyphosphate was precipitated by adding 2.5 volumes of ethanol and an overnight incubation at -20°C. The following day, the samples were centrifuged for 10 minutes at 10,000 g for 10 minutes and the supernatant was discarded. The pellets were resuspended in 50 µl of ddH2O. This protocol was adapted from (Adapted from K. D. Kumble & Kornberg, 1995; Seidlmayer, Gomez-Garcia, Blatter, Pavlov, & Dedkova, 2012).

2.2.9 PolyP detection

PolyP standards (Regenetiss, Japan) and extracted polyP samples were run on a 10% acrylamide TBE-Urea gel at 110V for 45 minutes. The gel was then stained for 15 minutes with 0.01% Toluidine blue (Sigma T3260-5G) and washed for 3 hours with a fixation solution [25% methanol (Fisher A412P-4), 5% of glycerol (Fisher BP229-4), ddH2O].
2.2.10 Purified hNOP56 shift assay

A master mix containing 5 µl of purified hNOP56 (OriGene TP761154), 5 µl of 5x Laemmli Sample Buffer and 40 µl of polyP Lysis Buffer without inhibitors was prepared and boiled. It was then added to 7 tubes containing different concentrations (0 to 100 mM) of synthetic polyP (Kerafast EU1005). Following a 1-hour incubation at room temperature, 5x Laemmli Sample Buffer was added in a 1:1 ratio to each sample. The samples were boiled and ran on a 4-12% Bis-Tris NuPAGE gel (Thermo Fisher NP0336BOX).

2.3 RNA sequencing sample preparation

Post-transfection with pcDNA3.1(-) or pcDNA3.1(-)EcPPK1, the cells were collected with 1 ml cold 1xPBS. 400 µl were set aside for protein preparation (cf. method above) and 600 µl were used for RNA isolation. To extract the RNA, the miRNeasy Mini Kit (Quiagen 217004) was used. The samples were then sent to Genome Quebec Innovation Centre for RNA sequencing and to Canadian Centre for Computational Genomics (C3G) for bioinformatics analysis.
CHAPTER 3: RESULTS

3.1 Yeast as a model organism to study polyP’s effect on alpha-synuclein toxicity

3.1.1 The Gal overexpression system

In order to study the expression of toxic proteins in yeast, the system commonly used is the galactose (Gal) overexpression system (Johnston, 1987). In this system, the gene encoding a toxic human protein is placed under a Gal-inducible promoter. Expression is repressed in the presence of dextrose, but can be quickly induced with galactose with which it is highly expressed (Figure 4). The constructs can be integrated into the genome or expressed from a plasmid. The impact of various mutations can then be easily be studied by comparing cell growth or cell viability on different growth media via spot test or by examining expression levels of proteins via western blot.

3.1.2 HA-EcPPK1 and HA-EcPPK1-NLS constructs rescue polyphosphorylation of Fpr3 when VTC4 is deleted (vtc4Δ)

The first part of this study aimed to uncover the molecular interactions between polyP and alpha-synuclein in order to determine whether the impact of yeast genes identified as modifiers of alpha-synuclein toxicity is occurring through polyP biology. PolyP is mostly localized in the vacuole, whereas the protein aggregations associated with alpha-synuclein toxicity are found in the cytoplasm (Outeiro & Lindquist, 2003). E. coli PPK1 (EcPPK1) synthesizes polyP independently of the vacuole, and EcPPK1 synthesized polyP accumulates in the cytoplasm (Gerasimaite et al., 2014). Thus, to engineer a system wherein polyP is synthesized in the cytoplasm, EcPPK1 was
Figure 4. The galactose overexpression system. In this system, a toxic human protein, such as alpha-synuclein, is placed under a Gal-inducible promoter. Expression of the plasmid is inhibited in the presence of dextrose, which allows cell growth. In the presence of galactose, the expression is induced, which leads to toxicity and thus, cell death.
cloned under a Gal-inducible promoter. Two constructs were obtained: HA-\textit{EcPPK1} and HA-\textit{EcPPK1-NLS}, which has a nuclear localization signal (NLS).

To determine if \textit{EcPPK1} and \textit{EcPPK1-NLS} constructs are able to produce polyP, a polyphosphorylation mobility shift assay was performed since polyphosphorylation of a protein can only be observed in a \textit{vtc4Δ} background if \textit{EcPPK1} produces polyP (Bentley-DeSousa et al., 2018). HA-\textit{EcPPK1} and HA-\textit{EcPPK1-NLS} plasmids were integrated into WT and \textit{vtc4Δ} yeast strains, and the production of polyP was checked by assessing polyphosphorylation of FK506-sensitive proline rotamase 3 (Fpr3), a previously identified yeast target of polyphosphorylation. When \textit{VTC4} is deleted, there is no production of polyP and thus, a collapse of the shift (\textbf{Figure 5}). In presence of HA-\textit{EcPPK1} or HA-\textit{EcPPK1-NLS} in the WT strain, Fpr3 migrated at a higher apparent molecular weight compared to the WT strain (\textbf{Figure 5}). In the \textit{vtc4Δ} strain, HA-\textit{EcPPK1} and HA-\textit{EcPPK1-NLS} both rescued polyphosphorylation of Fpr3, suggesting production of polyP from both plasmids (\textbf{Figure 5}). The results suggest that both HA-\textit{EcPPK1} and HA-\textit{EcPPK1-NLS} constructs are able to produce polyP and this synthesized polyP can polyphosphorylate proteins.

3.1.3 HA-\textit{EcPPK1} and HA-\textit{EcPPK1-NLS} constructs do not increase cell viability associated with alpha-synuclein toxicity

To examine the expression of alpha-synuclein in the presence of the HA-\textit{EcPPK1} and HA-\textit{EcPPK1-NLS}, an alpha-synuclein (SNCA\textsuperscript{WT}) plasmid was introduced into the WT and \textit{vtc4Δ} strains expressing cytoplasmic or nuclear-targeted \textit{EcPPK1} previously
Figure 5. HA-EcPPK1 and HA-EcPPK1-NLS rescue Fpr3 polyphosphorylation.

HA-EcPPK1 and HA-EcPPK1-NLS, both under a Gal promoter, were introduced in WT and vtc4Δ yeast strains. The strains were grown in YEP media containing galactose, which drives the expression of the EcPPK1 constructs, or containing raffinose, which does not allow expression of the constructs. A TCA protein preparation was used to obtain WCE and the samples were run on a 4-12% Bis-Tris NuPAGE gel. The gel was transferred on a PVDF membrane prior to immunoblotting with an Fpr3 antibody. Cdc55 was used as a loading control.
generated (Figure 5). The cell viability of each strain was assessed through spot test assay, using a dextrose plate to inhibit HA-EcPPK1, HA-EcPPK1-NLS and alpha-synuclein (SNCA<sup>WT</sup>), and a galactose plate to drive the expression of all three constructs (Figure 6). Cremers et al. (2016) showed that polyP could decrease alpha-synuclein toxicity. Thus, I expected to observe increased cell viability of the WT and vtc4Δ strains expressing alpha-synuclein in presence of cytosolic polyP (HA-EcPPK1). Alpha-synuclein (SNCA<sup>WT</sup>) expression alone led to decreased cell viability, while HA-EcPPK1 and HA-EcPPK1-NLS impacted cell viability minimally (Figure 6). HA-EcPPK1 and HA-EcPPK1-NLS expression appeared to decrease alpha-synuclein’s cell viability phenotype in the WT background, but to increase the viability in the vtc4Δ background (Figure 6). Overall, through several trials, the spot test assays showed inconsistent results. Therefore, another approach was used for the following assays, and the constitutive promoter GPD was used to express EcPPK1 (see section 3.1.6).

### 3.1.4 Alpha-synuclein expression in yeast

We were kindly gifted by Susan Lindquist’s lab three sets of yeast strains expressing alpha-synuclein under a Gal promoter with all constructs being integrated in the genome (Outeiro & Lindquist, 2003). The first set comprises the LowTox strain that expresses one construct of alpha-synuclein with a GFP tag and the LowTox EV that has the empty vector. The second set contains the HiTox strain that expresses two different alpha-synuclein constructs both GFP tagged, and the HiTox EV strain that has the two empty vector constructs. The third set consists of the InTox strain that also expresses two alpha-synuclein constructs but that differ from the HiTox strain and that are YFP tagged,
Figure 6. HA-EcPPK1 and HA-EcPPK1-NLS do not increase cell viability in the presence of alpha-synuclein. An empty vector (EV) or an alpha-synuclein (SNCA\(^{WT}\)) plasmids were introduced into the WT HA-EcPPK1, WT HA-EcPPK1-NLS, \(vtc4\Delta\) HA-EcPPK1 and \(vtc4\Delta\) HA-EcPPK1-NLS yeast strains. The strains were plated on a dextrose plate (left) and a galactose plate (right), which drives expression of the constructs. The strains were grown overnight on SC-URA plates containing raffinose. The next day, they were resuspended in water and plated in 5-fold dilutions and then incubated at 30°C for three days before pictures were taken to assess cell viability.
and the InTox EV that contains the two empty vectors. The detailed genotype of each strain can be found in Table 2. Outeiro and Lindquist (2003) showed that when two copies of alpha-synuclein are integrated, the protein localizes to the cytoplasm in inclusions, while one integrated copy leads to protein localization to the plasma membrane.

In order to observe the phenotype of each yeast strain described above, a spot test assay was performed, using a dextrose plate to inhibit alpha-synuclein and a galactose plate to drive alpha-synuclein expression. As shown in Figure 7, the LowTox (+) strain shows no differences in cell viability as compared to its control LowTox EV. Oppositely, the InTox (++) and HiTox (+++) strains show almost complete loss of cell viability when alpha-synuclein is expressed in the presence of galactose as compared to the InTox EV and HiTox EV strains respectively (Figure 7). Altogether, this suggests that high amounts of alpha-synuclein lead to cell death, which is consistent with observations described by Outeiro and Lindquist (2003) when assessing the same yeast strains.

3.1.5 vtc4Δ does not affect cell viability in the presence of alpha-synuclein

In order to investigate whether the presence or absence of polyP affects the cell viability of the LowTox and HiTox yeast strains, VTC4 was deleted (vtc4Δ) in these two strains and a spot test was performed. Our expectation was that by deleting VTC4, hence stopping the production of polyP, we would observe increased cell death. No differences in cell viability were observed between the LowTox and the LowTox vtc4Δ strains, and the HiTox and the HiTox vtc4Δ strains (Figure 8). Since low expression level of alpha-
Figure 7. High levels of alpha-synuclein are toxic in yeast. Three sets of yeast strains expressing alpha-synuclein at expected low (+), intermediate (+++) and high (+++++) toxicity levels were plated on a dextrose plate (left) and a galactose plate (right), which drives expression of the alpha-synuclein constructs. The strains were grown overnight on YPD plates containing raffinose. The next day, they were plated in 5-fold dilutions and then incubated at 30°C for three days before pictures were taken to assess cell viability. The constructs integrated in each strain are indicated to the right of the figure. Strains names: (+) LowTox, (++) InTox, (+++) HiTox.
**Figure 8.** *vtc4Δ does not affect cell viability in the presence of alpha-synuclein.* *VTC4* was deleted in the LowTox and the HiTox. The LowTox EV, LowTox and LowTox *vtc4Δ* strains, as well as the HiTox EV, HiTox and HiTox *vtc4Δ* strains were plated on a dextrose plate (left) and a galactose plate (right), which drives the expression of the alpha-synuclein constructs. The strains were grown overnight on YPD plates containing raffinose. The next day, they were plated in 5-fold dilutions and then incubated at 30°C for three days before pictures were taken to assess cell viability.
synuclein does not appear to be toxic to the cell (Figure 7), it could explain why in the absence of polyP (vtc4Δ), the cell viability is not impacted. Moreover, the alpha-synuclein expression in the HiTox strain leads to almost complete loss of cell viability. Therefore, the detrimental effect of the absence of polyP would not be observed. In the section below, I investigated the impact of exogenous polyP in the InTox strain through the use of EcPPK1.

3.1.6 Expression of EcPPK1 appears to rescue the cell viability of the InTox strain

In order for polyP to be synthesized in the cytoplasm, EcPPK1 under the GPD promoter was introduced in the InTox yeast strain. In order to assess the cell viability of the InTox strain in the presence and absence of EcPPK1-synthesized polyP a spot test was performed. Two isolates of the InTox EcPPK1 control and the InTox EcPPK1 strains were plated on dextrose and galactose plates. Both InTox EcPPK1 isolates show an increase in cell viability when compared to the InTox EcPPK1 control isolates, which exhibit complete cell death (Figure 9).

Moreover, the expression of EcPPK1 when alpha-synuclein expression is inhibited on the dextrose plate shows some decrease in cell viability (Figure 9). This observation is not surprising as toxicity associated with the expression of EcPPK1 has been reported in the literature previously (Gerasimaite et al., 2014). Altogether, these results show that additional polyP increases cell viability in the presence of toxic expression of alpha-synuclein, which suggests that polyP is able to reduce alpha-
Figure 9. Expression of *EcPPK1* appears to rescue the cell viability of the InTox strain. An empty vector (EV) or *EcPPK1* was expressed in the InTox yeast strain. Two isolates (1) and (2) of each strain were on a dextrose plate (left) or a galactose plate (right), which drives expression of the alpha-synuclein construct. The strains were grown overnight on SC-URA plates containing raffinose. The next day, they were plated in 5-fold dilutions and then incubated at 30°C for three days before pictures were taken to assess cell viability.
synuclein toxicity. This is consistent with polyP having a protective effect on amyloid cytotoxicity (Cremers et al., 2016).

3.1.7 Alpha-synuclein is not polyphosphorylated

Next, I aimed to determine whether the mechanism through which polyP stabilized alpha-synuclein fibrils as reported by Cremers et al. (2016) was through polyphosphorylation. Therefore, the InTox EcPPK1 and its control, the HiTox, HiTox vtc4Δ, LowTox, LowTox vtc4Δ strains were grown in media containing galactose to induce the expression of alpha-synuclein and the expression levels of alpha-synuclein were analyzed by NuPAGE western blotting (Figure 10). As mentioned above, all constructs expressing alpha-synuclein are GFP or YFP tagged and thus, the blot was probed with GFP (Outeiro & Lindquist, 2003). Alpha-synuclein does not appear to be polyphosphorylated as no shifts were observed between the LowTox and LowTox vtc4Δ strains or the HiTox and HiTox vtc4Δ strains (Figure 10). These results suggest that polyP’s effect on alpha-synuclein does not occur through polyphosphorylation.

Altogether, the results show that EcPPK1 expression is a useful tool to study polyP biology. This preliminary data is the first step in investigating the genetic and molecular interactions involved with polyP and alpha-synuclein toxicity. Expression of EcPPK1 was taken advantage of to study polyphosphorylation and, more generally, polyP biology in mammalian cells, which is addressed in the rest of this study.
Figure 10. Alpha-synuclein is not polyphosphorylated. The LowTox and HiTox yeast strains and their matching \textit{vtc4Δ} strains, as well as the InTox \textit{EcPPK1} control and the InTox \textit{EcPPK1} yeast strains were grown in media containing raffinose or galactose, which drives the expression of the alpha-synuclein constructs. A TCA protein preparation was used to obtain WCE. The samples were run on a 4-12\% Bis-Tris NuPAGE gel and the gel was transferred on a PVDF membrane prior to immunoblotting with a GFP antibody to detect alpha-synuclein. Cdc55 was used as a loading control.
3.2 Can human proteins undergo polyphosphorylation?

Azevedo et al. (2015) were the first ones to introduce polyphosphorylation. They identified two substrates in yeast and Bentley-DeSousa et al. (2018) identified an additional 15. In this study, I determined whether human proteins could also be polyphosphorylated. Studying polyP in mammals is a complex task as none of the polyP regulators are known and polyP levels are low (K. D. Kumble & Kornberg, 1995). However, the work previously done in yeast using EcPPK1 expression to produce exogenous polyP gave us a great tool to work with in human cells.

With the collaboration of Dr. Norman Davey (UC Dublin), bioinformatics analysis identified 1134 human proteins containing one or more poly-acidic serine and lysine rich (PASK) motifs. Polyphosphorylation occurs on those PASK motifs that were previously described as acidic stretches of amino acids containing serine (S), aspartic acid (D), glutamic acid (E) and lysine (K) residues (Azevedo et al., 2015). Therefore, the PASK motif for the analysis was set as 75% S/D/E with at least one K. Within that list, the homologs of polyphosphorylated yeast proteins such as nucleolin (homolog of yeast Nsr1) and proteins with a high number of PASK motifs were prioritized for the following assays.

3.2.1 Nucleolin is polyphosphorylated in vitro

Nsr1 was one of the first yeast proteins identified as being polyphosphorylated (Azevedo et al., 2015). Thus, I sought to assess whether its human homolog nucleolin could also be polyphosphorylated. Since none of the polyP regulators are known in
mammalian cells, an initial synthetic assay was performed wherein polyP was added to HEK293T cell extract in vitro using WT yeast extract and synthetic polyP. Two samples were also prepared without exogenous polyP by adding \( \text{vtc4} \Delta \) yeast extract or buffer (Figure 11). These samples were run on a 4-12% Bis-Tris NuPAGE gel to observe the electrophoretic mobility shift indicative of polyphosphorylation. In the presence of polyP (WT yeast extract and 0.5 mM polyP), nucleolin migrated at \( \sim 150 \) kDa (Figure 11), whereas it ran at its expected molecular weight (\( \sim 100 \) kDa) in the control (buffer) and in the absence of yeast polyP (\( \text{vtc4} \Delta \)) (Figure 11). These results suggest that polyP chains can polyphosphorylate a human protein such as nucleolin. This is the first demonstration of polyphosphorylation occurring on human proteins.

3.2.2 EcPPK1 is expressed and polyP is produced in HEK293T cells

Since the regulators of polyP in mammalian cells are unknown, we were unable to control polyP levels in the cell. However, the expression of EcPPK1 in \( \text{vtc4} \Delta \) yeast strains is able to produce polyP and rescue polyphosphorylation as shown previously in yeast (Figure 9). Thus, to modulate the levels of polyP in vivo in order to assess mammalian protein polyphosphorylation, HA-EcPPK1 was cloned into a mammalian vector. To confirm its expression, the plasmid was transfected into HEK293T cells. The protein extracts were run on a 4-12% Bis-Tris NuPAGE gel to assess protein polyphosphorylation, as well as a SDS gel to identify the tagged HA-EcPPK1 (Figure 12). DEK proto-oncogene (DEK) contains several PASK motifs, which makes it a good candidate for polyphosphorylation. The results showed that DEK was
Figure 11. Nucleolin is polyphosphorylated in vitro. HEK293T cells were grown to ~70% confluency prior to protein extraction with RIPA buffer. The WCE was then incubated with buffer, WT yeast extract, \( vtc4\Delta \) yeast extract or 0.5 mM of synthetic polyP. The samples were run on a 4-12% Bis-Tris NuPAGE gel, transferred to PVDF membrane prior to immunoblotting with a nucleolin antibody. GAPDH was used as a loading control. (Adapted from Bentley-DeSousa et al., 2018)
polyphosphorylated in the presence of EcPPK1 and HA-EcPPK1 (Figure 12 a), and that HA-EcPPK1 was indeed expressed (Figure 12 b).

Another way to assess the production of polyP with the expression of EcPPK1 is by extracting polyP from HEK293T cells transfected with the control plasmids or EcPPK1. An acid phenol/chloroform extraction was performed and the samples, along side polyP standards, were run on a 10% TBE-UREA gel prior to toluidine blue staining. As shown in Figure 13, a large amount of polyP was extracted from the cells transfected with EcPPK1 compared to the control cells containing low endogenous levels of polyP. This confirms the production of exogenous polyP in HEK293T when transfected with EcPPK1.

3.2.3 Six human proteins can be polyphosphorylated when EcPPK1 is expressed

After confirming that EcPPK1 is expressed (Figure 12) and polyP is produced (Figure 13), this system was used to manipulate polyP levels in HEK293T cells in order to perform a small screen to identify substrates of polyphosphorylation. Cells were transfected with an empty vector or EcPPK1, and protein extracts were run a 4-12% Bis-Tris NuPAGE gel. The western blot was probed with different antibodies. Nucleolin was shifted in the presence of EcPPK1 (Figure 14 a), which is consistent with the in vitro assay results shown above (Figure 11). Using this method, 5 other human proteins were identified as capable of being polyphosphorylated: nucleolar protein 56 (hNOP56), mesoderm development LPR chaperone (Mesd), DEK proto-oncogene (DEK), eukaryotic
**Figure 12. HA-*EcPPK1* is expressed in HEK293T and leads to polyphosphorylation of DEK.** An empty vector and HA-*EcPPK1* cloned into a mammalian vector were transfected into HEK293T cells and grown for 48 hours prior to protein extraction with RIPA buffer. The protein extracts were then ran on a 4-12% Bis-Tris NuPAGE gel (A) and a SDS-PAGE gel (B). The gels were transferred to PVDF membrane prior to immunoblotting with the indicated antibodies (DEK and HA). GAPDH was used as a loading control.
Figure 13. PolyP is present in EcPPK1 transfected HEK293T cells. An empty vector and EcPPK1 was transfected into HEK293T cells and grown for 48 hours prior to polyP extraction by acid phenol-chloroform extraction. PolyP standards were used as controls: short chain (14 Pi units), medium chain (60 Pi units), and long chain (130 Pi units). The samples were run on a 10% TBE-UREA gel prior to staining with toluidine blue for 15 minutes and washing with a fixation solution for 3 hours.
Figure 14. Expression of EcPPK1 in HEK293T cells results in polyphosphorylation of 6 human proteins. An empty vector and EcPPK1 cloned into a mammalian vector were transfected into HEK293T cells and grown for 48 hours prior to protein extraction with RIPA buffer. The protein extracts were then ran on a 4-12% Bis-Tris NuPAGE gel and transferred to PVDF membrane prior to immunoblotting with the indicated antibodies. (Adapted from Bentley-DeSousa et al., 2018)
translation initiation factor 5B (eIF5B) and regulator of nonsense mediated mRNA decay (UPF3B) (Figure 14 b-f). Overall, 37 antibodies were tested. 6 human proteins were confirmed to be polyphosphorylated (Figure 14), 16 human proteins were found not to be polyphosphorylated (data not shown) and 15 were inconclusive. Expression of EcPPK1 in HEK293T was a key element in this screen and allowed us to show for the first time that human proteins can be polyphosphorylated.

3.2.4 Polyphosphorylation shift is dependent on polyP concentration

In yeast, it has been observed previously that the shift size (in relative molecular weight) of a polyphosphorylated target seems to depend on the concentration of polyP and its chain length (Azevedo et al., 2015; Bentley-DeSousa et al., 2018). In order to test if this was also occurring with mammalian proteins, purified hNOP56 was incubated with different concentrations of polyP (75 Pi units). This in vitro assay showed that the hNOP56 polyphosphorylation shift observed was dependent on the polyP concentration (Figure 15).

3.3 EcPPK1 as a tool to study polyP biology using RNA sequencing

To investigate on a larger scale the effect polyP has in human cells, I decided to study the gene expression changes in the presence of exogenous polyP in HEK293T cells. Three biological replicates of HEK293T cells were transfected with an empty vector and EcPPK1. After 48 hours, cells were collected, and divided for protein and RNA extractions. Using the protein extract, the samples were run on a 4-12% Bis-Tris NuPAGE gel and probed with a DEK antibody (Figure 16). In the presence of EcPPK1,
Figure 15. hNOP56 polyphosphorylated mobility shift size depends on polyP concentration. Purified human NOP56 was incubated in vitro with increasing concentrations of synthetic polyP of medium chain (75 Pi units). The samples were then ran on a 4-12% Bis-Tris NuPAGE gel and transferred to PVDF membrane prior to immunoblotting with a hNOP56 antibody. (Adapted from Bentley-DeSousa et al., 2018)
Figure 16. DEK is polyphosphorylated in the RNA sequencing samples. Three biological replicates of HEK293T cells were transfected with an empty vector and *EcPPK1*. The cells and grown for 48 hours prior to protein extraction with RIPA buffer. The protein extracts were then ran on a 4-12% Bis-Tris NuPAGE gel and transferred to PVDF membrane prior to immunoblotting with a DEK antibody. GAPDH was used as a loading control.
DEK is shifted in all three biological replicates (Figure 16). This western blot suggests that EcPPK1 is producing polyP in the cells.

The RNA extraction was performed using the miRNeasy Mini Kit (Quiagen) and the samples were sent to Genome Quebec Innovation Centre for RNA sequencing and to the Canadian Centre for Computational Genomics (C3G) for bioinformatics analysis. Overall, 112 genes were found to be up- or down-regulated in presence of polyP with a p value lower than 0.05 and a fold change equal or greater than 2 (Figure 17). There is a greater number of genes being down-regulated (104) compared to up-regulated (8). The top 30 genes, whose expression is altered in the presence of polyP, are listed in Table 5. Finally, a Gene Ontology (GO) term analysis identified enrichment in nuclear and nucleolar localizations (Figure 18). The analysis also showed enrichment in DNA related processes and functions, as well as a strong enrichment in metal ion binding function (Figure 18). This data is the first step towards gaining a better understanding of polyP biology in mammals.
Figure 17. Genes up- or down-regulated in the presence of polyP. Three biological replicates of HEK293T cells were transfected with an empty vector and EcPPK1. RNA was collected and RNA sequencing was performed. The volcano plot represents the entire data set.
Table 5. Top 30 up- and down-regulated genes in the presence of polyP. Three biological replicates of HEK293T cells were transfected with an empty vector and EcPPK1. RNA was collected and RNA sequencing was performed. The data was sorted based on a p value < 0.05 and a fold change > 2. The top 30 up- and down-regulated genes in the presence of polyP are included in the table below.
<table>
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<tr>
<th>Gene</th>
<th>Log2_FC</th>
<th>Adjusted p value</th>
<th>Function</th>
</tr>
</thead>
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<td></td>
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</tr>
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<td>Subunit of a voltage-independent potassium channel</td>
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<tr>
<td>LTA</td>
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<td>1.80E-03</td>
<td>Cytokine involved in the inflammation response and development of lymphoid organs</td>
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Three biological replicates of HEK293T cells were transfected with an empty vector and EcPPK1. RNA was collected and RNA sequencing was performed. A GO enrichment analysis was performed by the Canadian Centre for Computational Genomics. For the analysis, all differentially expressed genes with an adjusted p-value under 0.1 were used.
CHAPTER 4: DISCUSSION

PolyP regulation and function have been studied in yeast and bacteria, but the synthesis and degradation of polyP in mammals have not been characterized (Hooley, Whitehead, & Brown, 2008). To study different aspects of polyP biology in both yeast and mammalian cells, EcPPK1, a bacterial enzyme that synthesizes polyP, was used to modulate polyP concentration and localization. Overall, my work demonstrates the advantage of EcPPK1 as a novel tool to study polyP biology in yeast and in mammalian cells.

4.1 Yeast as a model organism to study polyP’s effect on alpha-synuclein toxicity

First, I aimed to establish yeast as a model organism to study alpha-synuclein aggregation and to investigate the molecular interactions involved in polyP’s protective effect on alpha-synuclein toxicity. In the past two decades, researchers have established yeast as a model organism to study neurodegenerative diseases such as PD, Huntington’s disease and Alzheimer’s disease (Krobitsch & Lindquist, 2000; Outeiro & Lindquist, 2003; Winderickx et al., 2008). About 31% of the yeast protein-encoding genes have a mammalian homolog (Botstein, Chervitz, & Cherry, 1997), and about 31% of the disease-associated genes in humans have been found to share homology to yeast genes (Foury, 1997). This led to genetic screens performed in yeast to identify genes associated with huntingtin toxicity (Giorgini et al., 2005; Willingham et al., 2003), and alpha-synuclein toxicity (Cooper et al., 2006; Liang et al., 2008; Willingham et al., 2003). Major cellular processes have also been found to be conserved between yeast and
mammals, which makes work in yeast a stepping-stone in identifying the mechanisms involved with neurodegenerative diseases in humans (Winderickx et al., 2008). Additionally, polyP regulators are known in yeast. Altogether, yeast is a useful model organism to study whether the effect of the yeast genes on alpha-synuclein toxicity occurs through polyP biology.

4.1.1 Expression of EcPPK1 can rescue alpha-synuclein toxicity

Since polyP is mainly localized in the vacuole, whereas alpha-synuclein aggregates form in the cytoplasm (Indge, 1968; Outeiro & Lindquist, 2003; Urech et al., 1978), EcPPK1 was used as a tool to produce increased amounts of cytoplasmic polyP compared to a WT cell (Gerasimaite et al., 2014). Using a HA-tagged EcPPK1 construct with and without a NLS, and an alpha-synuclein plasmid, I investigated the impact of polyP on alpha-synuclein toxicity. Based on the idea that polyP protects the cell from alpha-synuclein toxicity (Cremers et al., 2016), I hypothesized that EcPPK1 would lead to an increase in cell viability. I observed an increase in cell viability from HA-EcPPK1 or HA-EcPPK1-NLS in the presence of alpha-synuclein in a vtc4Δ background but a decrease in cell viability in a WT background (Figure 6). Overall, through different trials, the results appeared to be inconsistent, which led us to take a different approach. While HA-EcPPK1 and HA-EcPPK1-NLS are functional constructs (Figure 5), microscopy work needs to be performed to confirm the localization of the polyP they produced.
Using the InTox yeast strain (two copies of YFP-tagged alpha-synuclein integrated), an EcPPK1 plasmid with a GPD promoter was introduced. A spot test assay showed that in the presence of EcPPK1, cell viability was increased suggesting that exogenous polyP reduced alpha-synuclein toxicity (Figure 9). Similarly, adding polyP to the media of HeLa cells secreting alpha-synuclein reduces the amount of cell death observed (Cremers et al., 2016). Thus, my results are consistent with their findings. Altogether, while the results regarding polyP’s effect on cell viability in the presence of alpha-synuclein varied between experimental set-ups, expression of EcPPK1 as a tool to study polyP biology allowed manipulation of polyP by increasing its overall concentration in the cell. It also allows for the possibility of localizing polyP production in different cell compartments, which is key knowing that typically ~90% of polyP in the cell is found in the vacuole (Indge, 1968; Urech et al., 1978). In the future, a tetracycline-inducible promoter, which allows to turn genes on and off in a titrable manner in the presence of the drug tetracycline, could be used to better regulate the expression of alpha-synuclein or EcPPK1 in yeast (Gossen & Bujard, 1992).

In the future, it would be interesting to investigate the impact of polyP chain length on alpha-synuclein toxicity. EcPPK1 is believed to produce polyP chains made of ~100 to ~750 Pi (Ault-Riché et al., 1998; Krishnanand D Kumble, Ahn, & Kornberg, 1996), which are longer than the ~50 Pi synthetic polyP chains used by Cremers et al. (2016) in their study. The exopolyphosphatase Ppx1 could be overexpressed along side EcPPK1 to shorten the polyP chains produced by EcPPK1. Additionally, deletion of VTC4 combined with mutations of genes previously found to affect alpha-synuclein
toxicity in yeast (Cooper et al., 2006; Liang et al., 2008; Willingham et al., 2003) could give insight on the mechanism and molecular interactions involved with polyP’s ability to increase alpha-synuclein fibril stability.

4.1.2 Absence of polyP through deletion of \textit{VTC4} does not impact alpha-synuclein toxicity

PolyP decreases alpha-synuclein toxicity by reducing the amount of toxic oligomers produced during alpha-synuclein fibril formation (Cremers et al., 2016). I aimed to determine whether alpha-synuclein toxicity increased when stopping the production of polyP in yeast by deleting \textit{VTC4}, the gene that encodes for the enzyme responsible for the synthesis of polyP. I found that the absence of polyP (\textit{vtc4}Δ) had no impact on alpha-synuclein toxicity (Figure 8). Although I expected to see decreased cell viability in the presence of alpha-synuclein and absence of polyP (\textit{vtc4}Δ), alpha-synuclein expression in the HiTox strain (two copies of GFP-tagged alpha-synuclein constructs integrated) showed almost complete loss of cell viability, thus masking observation of any potential decrease in cell viability when lacking polyP (\textit{vtc4}Δ).

4.1.3 PolyP does not polyphosphorylate alpha-synuclein

Cremers et al. (2016) suggested that polyP’s cytoprotective effect occurs through the stabilization of alpha-synuclein fibrils rather than affecting alpha-synuclein protein levels. This led to the hypothesis that polyP could polyphosphorylate alpha-synuclein to stabilize the mature fibrils. My results suggest that polyP’s effect on alpha-synuclein is not through polyphosphorylation (Figure 10), however additional research is needed to
further investigate how polyP interacts with alpha-synuclein fibrils. Since the yeast strains gifted by Dr. Lindquist’s lab express GFP- or YFP-tagged alpha-synuclein, microscopy would allow visualization of the alpha-synuclein aggregates (Outeiro & Lindquist, 2003). Using DAPI, which detects polyP at wavelength of 525-550 nm (Allan & Miller, 1980; Gomes et al., 2013), we could test whether polyP co-localizes with the aggregates or leads to the localization of the aggregates somewhere other than the cytoplasm. While spot test assays give a quick and clear phenotype on cell viability, microscopy would be more precise and allow us to visualize more subtle changes that could be happening in the cell when EcPPK1 produces polyP.

Overall, my work demonstrates that EcPPK1 expression in yeast is a useful tool to manipulate polyP concentrations and localization in the cell. This preliminary data provides a starting point for future analyses of the genetic and molecular interactions involved with polyP and alpha-synuclein toxicity in yeast. This work could be extended to study other amyloid proteins such as amyloid-β and Tau that have also been investigated in yeast. The work in yeast is the first step in gaining a deeper understanding of alpha-synuclein toxicity and translating the findings to mammalian models.

4.2 EcPPK1 as a tool to study polyphosphorylation in mammalian cells

Polyphosphorylation, as a PTM, was first discovered in yeast (Azevedo et al., 2015). As of today, 17 yeast proteins have been found to be polyphosphorylated (Azevedo et al., 2015; Bentley-DeSousa et al., 2018). Azevedo et al. (2015) and Bentley-DeSousa et al. (2018) brought up the question of whether human proteins could also be
polyphosphorylated. A bioinformatics analysis conducted in collaboration with Dr. Davey (UC Dublin) showed that 1134 human proteins contain a PASK motif (75% S/D/E with at least one K), but whether the proteins can actually be polyphosphorylated was unknown. The challenges in assessing polyphosphorylation of human proteins are the low polyP concentrations found in most mammalian cells (K. D. Kumble & Kornberg, 1995), and the unknown polyP regulators in mammals, which make it difficult to modulate polyP levels in the cell and visualize quantitative polyphosphorylation via electrophoretic mobility shift on Bis-Tris NuPAGE gels. Here, I investigated whether polyphosphorylation is conserved in higher eukaryotes by exploiting the expression of the EcPPK1 to produce exogenous polyP in HEK293T cells – hence increasing polyP concentration in the cell.

4.2.1 EcPPK1 expression in HEK293T cells produces exogenous polyP

EcPPK1 was transfected in HEK293T and its expression was confirmed via western blot (Figure 12), as well as polyP extraction (Figure 13). Interestingly, when comparing the polyP extracted from the EcPPK1 transfected cells to the polyP standards, it appears that the polyP chain lengths produce by EcPPK1 are longer than 130 Pi residues. The long smear observed suggests that the polyP produced might vary greatly in chain lengths. It has been reported that EcPPK1 can synthesize polyP chains of ~100 Pi to ~750 Pi residues (Ault-Riché et al., 1998; Krishnanand D Kumble et al., 1996). My results suggest long chains of polyP can be produced by EcPPK1 in HEK293T cells.
4.2.2 Human proteins can be polyphosphorylated

Expressing EcPPK1 in HEK293T cells increased polyP concentration and allowed me to carry out a small screen to identify human proteins able to be polyphosphorylated. Six human proteins were identified as able to be polyphosphorylated: nucleolin, hNOP56, Mesd, DEK, eIF5B, and UPF3B (Figure 14). Nucleolin, the homolog of yeast substrate Nsr1, is a protein found in the nucleolus and is known to play a role in the synthesis and maturation of ribosomes (Ginisty, Sicard, Roger, & Bouvet, 1999). hNOP56, the homolog of yeast substrate Nop56, is also located in the nucleolus and plays a role in ribosome biogenesis (Gautier, Bergès, Tollervey, & Hurt, 1997; Hayano et al., 2003). Both Mesd and DEK are proteins without yeast homologs. Mesd is found in the endoplasmic reticulum (ER) and plays an important role in the localization of the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5 and LRP6) at the cell surface (Hsieh et al., 2003). DEK is a nuclear phosphoprotein that binds to chromatin (Waldmann, Scholten, Kappes, Hu, & Knippers, 2004). eIF5B is one of the initiation factors required for initiation of translation (Pestova et al., 2001). Finally, UPF3B is a protein found in the nucleus and acts as a shuttling protein for the nonsense-mediated mRNA decay pathway between the nucleus and the cytoplasm (Serin, Gersappe, Black, Aronoff, & Maquat, 2001). The non-enzymatic property of the PTM brings up a caveat of this polyphosphorylation assay. Indeed, it is difficult to assess whether the polyP chains produced by EcPPK1 bind covalently to the protein fully in vivo or also partially when lysing the cells for protein extraction in vitro. Thus, while the EcPPK1 system allows for the identification of human proteins that can be
polyphosphorylated, whether polyphosphorylation detected is occurring in vivo or in vitro, is unknown.

One of the limits of this polyphosphorylation screen is the lack of specific definition of the PASK motif. In order to prioritize which proteins to examine, the bioinformatics analysis done in collaboration with Dr. Davey gave a list of 1134 human proteins that possessed one or more amino acid stretch comprised of 75% S/D/E with at least one K. While all the proteins we identified as polyphosphorylated were in this list of proteins containing a PASK motif, others I tested in the screen based on the bioinformatics analysis were not polyphosphorylated. I also cannot exclude the possibility that proteins not included in this list can be polyphosphorylated. Unfortunately, looking at the PASK motifs of identified polyphosphorylated targets in yeast and humans has not been able to narrow down the amino acids required in the PASK motif. Further research should focus on determining what constitutes the PASK motif, which would help gain a better understanding of polyphosphorylation (Bentley-DeSousa & Downey, 2018).

A recent study used a different approach to identify human polyphosphorylated proteins (Azevedo et al., 2018). A human proteome array using an 8-Pi residue polyP molecule was used to identify human proteins binding to polyP. The human proteome array slide used included ~15 000 human proteins (Azevedo et al., 2018). From their two screens, 75 proteins appeared to be consistent candidates for polyphosphorylation. Electrophoretic mobility shift assays were then performed to determine if the proteins
found to bind polyP were polyphosphorylated (Azevedo et al., 2018). The authors confirmed six human proteins as polyphosphorylation targets: tetratricopeptide repeat domain 27 (TTC27), endoplasmic (HSP90B1), eukaryotic translation initiation factor 2B subunit epsilon (eIF2B5), general transcription factor IIl (GTF2I), gelsolin (GSN), and EYA transcriptional coactivator and phosphatase 1 (EYA1) (Azevedo et al., 2018). While HSP90α/β (HSP90AA1 and HSP90AB1) did not come up in their screen through NuPAGE analysis, they showed it was also able to be polyphosphorylated (Azevedo et al., 2018).

HSP90B1, HSP90AA1, HSP90AB1 and eIF2B5 are proteins containing a PASK motif based on our group’s bioinformatics analysis and as suggested by Azevedo et al. (2018). Interestingly, Azevedo et al. (2018) found all three isoforms of the Hsp90 protein to be polyphosphorylated in vitro, while HSP90AA1 (Bentley-DeSousa et al., 2018) and HSP90α/β (data not shown) were found not polyphosphorylated in my work. While I used the EcPPK1 system to produce exogenous polyP in HEK293T cells, Azevedo et al. (2018) incubated HeLa cells with synthetic polyP of ~100 Pi. As mentioned previously, EcPPK1 has been reported to produce polyP chain length from 100 Pi to 750 Pi (Ault-Riché et al., 1998; Krishnanand D Kumble et al., 1996). Thus, the variations in cell lines used and polyP chain lengths could explain the differences observed regarding polyphosphorylation of HSP90AA1 and HSP90AB1. It would be interesting to further cross-reference Azevedo et al. (2018) 75 preliminary hits with our group’s bioinformatics analysis to determine how many of their hits are considered to have a PASK motif. Confirming additional hits as able to be polyphosphorylated would also help determine
more specifically the composition of the PASK motif, as well as gaining more insight on polyphosphorylation.

In yeast, changes in the electrophoretic mobility of polyphosphorylation substrates correlates with the number of lysines, but not with the number of serines or acidic amino acids contained in their PASK motifs (Bentley-DeSousa et al., 2018). Performing the same analysis with all 12 human proteins found to be polyphosphorylated and with additional confirmed targets from Azevedo et al. (2018) screen would further expand our knowledge on the PASK motif and its role in polyphosphorylation.

One of the main questions in the field that still has not been demonstrated, in yeast and in mammalian cells, is whether polyphosphorylation occurs *in vivo*. Electrophoretic mobility shift on NuPAGE gels is currently the only way of detecting polyphosphorylation (Azevedo et al., 2015, 2018; Bentley-DeSousa et al., 2018). In HEK293T cells, the polyP concentration is much lower than in yeast (K. D. Kumble & Kornberg, 1995), which might prevent detection of the mobility shift of polyphosphorylated proteins on NuPAGE gels (Bentley-DeSousa et al., 2018). Increasing polyP concentration in the cell may exaggerate the polyphosphorylation shift but still be representative of what occurs *in vivo*. Moreover, in yeast, the shift of a polyphosphorylated protein collapses when *VTC4* is deleted (Azevedo et al., 2015; Bentley-DeSousa et al., 2018). Since we do not know what synthesizes polyP or what degrades it in mammals, endogenous polyP levels cannot be modulated and the collapse of the shift observed upon stopping polyP synthesis cannot be reproduced. Uncovering
polyP regulators in mammals could be the key to detecting polyphosphorylation in vivo. The size of the polyP chains in vivo may also differ from the long polyP chains produced by EcPPK1. Shorter polyP chains may not confer visible polyphosphorylation by mobility shift assay. Developing additional assays to detect polyphosphorylation would be an asset to study polyphosphorylation in vivo.

4.2.3 Polyphosphorylation mobility shift depends on polyP concentration

I found that polyphosphorylation of purified hNOP56 shift increases with increasing polyP concentration (Figure 15). These results are consistent with similar in vitro assays performed in yeast (Azevedo et al., 2015; Bentley-DeSousa et al., 2018). My results suggest polyphosphorylation does not occur or cannot be detected at polyP concentrations lower than 0.5 mM. While polyphosphorylation might not be detectable in certain mammalian cells due to low polyP concentration (K. D. Kumble & Kornberg, 1995) compared to yeast (Auesukaree et al., 2004), some cells or organelles do have higher endogenous polyP concentrations, which could allow for quantitative polyphosphorylation in vivo. For example, the dense granules in platelets contain ~130 mM of polyP (Ruiz et al., 2004). In platelets, activation of the coagulation cascade leads to the release of the polyP found in the dense granules (Ruiz et al., 2004). PolyP then induces clot formation while inhibiting fibrinolysis (Ruiz et al., 2004; S. A. Smith et al., 2006). Additionally, polyP chains were suggested to bind to K residues on thrombin (Choi, Smith, & Morrissey, 2011; Mutch, Myles, Leung, & Morrissey, 2010). Therefore, it would be interesting to investigate whether polyP acts on the blood cascade through polyphosphorylation (Bentley-DeSousa & Downey, 2018).
4.2.4 Finding a function for polyphosphorylation in mammals

What still has not been demonstrated in mammals is the function of polyphosphorylation. A protein of interest to investigate polyphosphorylation function is the Mesd chaperone. The PASK motif of Mesd is found within the C-terminal of the protein, which is also an important region for LRP5 and LRP6 proteins with the chaperone (Figure 19) (J. Chen et al., 2011; Hsieh et al., 2003; Lin et al., 2013). Mesd is found in the ER and transports LRP5/6 to the membrane, where they act as Wnt co-receptors with Frizzled receptor (Bhanot et al., 1996; Hsieh et al., 2003; Pinson, Brennan, Monkley, Avery, & Skarnes, 2000; Wehrli et al., 2000). If LRP5 and LRP6 are not localized to the cell membrane, defects in Wnt signaling are observed (Hsieh et al., 2003). Previously, Azevedo et al. (2015) demonstrated that polyphosphorylation could impact protein-protein interaction, protein localization, and protein function. Thus, polyphosphorylation could play a role in the interaction of LRP5/6 with Mesd, which could also impact localization and function of the two receptors (Bentley-DeSousa et al., 2018). Preliminary data from our lab show that polyphosphorylation is preserved during immunoprecipitation of Mesd using our EcPPK1 system in HEK293T cells. A co-immunoprecipitation with EcPPK1 transfected cells would be a first step to investigate whether polyphosphorylation affects Mesd interaction with LRP5 or LRP6. Microscopy work could also be performed to assess the localization of Mesd, LRP5 and LRP6 in low and high concentrations of polyP.
Figure 19. The PASK motif of Mesd is found in a region interacting with LRP5/6.

(Adapted from Bentley-DeSousa et al., 2018)
4.3 *EcPPK1* as a tool to study polyP biology in mammalian cells

4.3.1 PolyP up- and down-regulates gene expression in HEK293T cells

To gain better insight on polyP biology and function in mammalian cells, RNA sequencing was performed in order to study gene expression changes related to the presence of exogenous polyP in HEK293T. I hypothesized that that data would indicate changes in expression of genes involved in specific pathways. The results showed that a total of 112 genes are up- or down-regulated in the presence of ectopic polyP with cut-offs of p value < 0.05 and fold change > 2 (Figure 17). Out of those 112 genes, 104 are down-regulated while only 8 are up-regulated.

The GO term analysis showed enrichment in nuclear and nucleolar localization (Figure 18). Targets of polyphosphorylation have been found to be enriched in nuclear and nucleolar localization as well (Azevedo et al., 2015; Bentley-DeSousa & Downey, 2018). Whether these two observations suggest a link between gene expression affected by polyP and proteins polyphosphorylated in the nucleus and nucleolus is yet to be determined. Additionally, the GO term analysis showed enrichment in DNA related processes and functions, as well as metal ion binding function (Figure 18). The latter appears to be consistent with reports in the literature of polyP acting as an ion chelator (Archibald & Fridovich, 1982; Bonting et al., 1993; Dunn et al., 1994; Kulaev et al., 2005). As an adjusted p-value of 0.1 was used to perform the GO term analysis, it would be useful to redo the analysis with more stringent criteria (ex. p < 0.05, 1.5 to 2 fold change) to narrow down the most enriched cellular processes and molecular function
associated with polyP. While our hits still need to be validated using qPCR, the results suggest that the presence of polyP in mammals impacts gene expression.

4.3.2 PolyP impacts gene expression in SaOS-2 cells

A recent study performed a cDNA microarray in SaOS-2 cells after treatment with polyP and found 23 genes that were up-regulated and 11 genes that were down-regulated (Lui, Ao, Li, Khong, & Tanner, 2016). Their most up-regulated gene was interleukin 11 (IL-11), while their most down-regulated gene was solute carrier family 30 member 1 (SLC30A1) (Lui et al., 2016). In my RNA sequencing data, these two genes showed no change in expression between our control and EcPPK1 transfected cells. The early growth response 1 (EGR1) gene was found to be up-regulated in both studies, while inhibitor of DNA binding 3 (ID3), inhibitor of DNA binding 1 (ID1) and maltase-glucoamylase (MGAM) where found to be up-regulated in their microarray but down-regulated in our RNA sequencing (Lui et al., 2016). The differences observed between the two studies can be explained by several differences in the experimental set up. First, different cell lines were used (HEK293T vs. SaOS-2). HEK293T is a human embryonic kidney cell line, whereas SaOS-2 is a human osteosarcoma cell line. Second, different approaches were used to introduce polyP to the cell. Lui et al. (2016) treated the SaOS-2 cells with extracellular polyP, whereas we expressed EcPPK1 in HEK293T to synthesize polyP in the cell. Third, their cells were treated for 3 hours with synthetic polyP of 65 Pi residues in chain length. For our work, cells were left to grow 48 hours post-transfection with EcPPK1, and polyP extraction from EcPPK1 transfected cells showed that the polyP chain length synthesized are longer than 130 Pi residues. The polyP chain lengths in each
experiment and the exposure time of the cells to polyP were different, which implies that the concentration of polyP in the cells varied between the two experimental set-ups. While the data by Lui et al. (2016) were validated by qPCR, the RNA sequencing results from our groups have not. This is a potential caveat when comparing the results of both studies. Nonetheless, the results do suggest that polyP could up- or down-regulate different genes from one cell line to another due to differences in endogenous polyP concentrations and polyP chain length sizes. It would be interesting to investigate further changes in gene expression from polyP presence in different cell lines using our EcPPK1 expression system.

4.4 Future directions

My work showed that EcPPK1 is a useful tool to modulate polyP in yeast and in mammalian cells. In yeast, the work done with EcPPK1 could be extended to investigate human protein aggregations. Toxic aggregates, such as alpha-synuclein inclusions, often lead to diseases that are still poorly understood. Determining how polyP protects cells against the toxicity from protein aggregates could be a stepping-stone in finding therapeutic treatments for neurodegenerative diseases, such as Parkinson’s disease or Alzheimer’s disease. Additionally, I showed that EcPPK1 could also be expressed in mammalian cells. Thus, EcPPK1 could be used as a tool in neurons to further investigate polyP’s role in neurodegenerative diseases.

In mammalian cells, as polyP’s regulators are still unknown and the concentration of polyP generally low compared to yeast, it is difficult to study polyP biology
Expression of *EcPPK1* in mammalian cells is a new approach that allows for the synthesis of exogenous polyP in the cell. Using this system, I was able to demonstrate that human proteins can be polyphosphorylated. The next step, using *EcPPK1*, is to find a function for polyphosphorylation in mammalian cells. As explained previously, Mesd appears to be a good candidate to investigate the impact polyphosphorylation has on its function. Furthermore, analysis of the PASK motifs of the human polyphosphorylated targets reported in my work and by Azevedo et al. (2018) would broaden our knowledge of the PASK motif and polyphosphorylation.

To validate the hits from the RNA sequencing, *EcPPK1* transfected cells will be used to collect RNA and primers will be designed for qPCR. A deeper analysis of the bioinformatics data from the RNA sequencing could give leads on undiscovered functions of polyP in mammals. On a broader scale, it would also be interesting to investigate polyP’s impact on gene expression in various cell lines.

As of today, the human enzymes involved in the synthesis and degradation of polyP are unknown (Hooley et al., 2008). H-prune is the only proposed human exopolyphosphatase, but its role has only been shown in vitro (Tammenkoski et al., 2008). H-prune is thought to act only on short chains of polyP and to be inhibited by long chains of polyP (Tammenkoski et al., 2008). Identifying the enzymes involved in polyP regulation in mammals would be an important stepping-stone in polyP biology research, and a key element in studying polyphosphorylation in vivo.
4.5 Conclusions

This study investigated polyP biology in different organisms. My work in yeast provided a starting point for future analyses of the genetic and molecular interactions involved with polyP and alpha-synuclein toxicity, through the use of EcPPK1. In mammals, in which polyP’s regulators are still unknown, EcPPK1 expression allowed to demonstrate for the first time that human proteins can be polyphosphorylated. Moreover, the RNA sequencing data, when comparing control and EcPPK1 transfected cells, showed that polyP up- and down-regulates gene expression. While these results still need to be validated using qPCR, they are the first step in gaining better insight into polyP biology in mammals. Altogether, I have demonstrated that expression of EcPPK1 in yeast and in mammals is a remarkable tool to study polyP biology.
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APPENDIX 1: Solutions and buffers

YEP media

For 1L: 10 g Yeast extract (BD 212750), 20 g Bactopeptone (BD 211677), 5 mL 1% Tryptophan [5 g Tryptophan (SLBD9323V), ddH₂O], 5 mL 1% Adenine [5 g Adenine (Sigma 071M1375V), 5 mL 10M NaOH (Fisher BP359-500), ddH₂O] and ddH₂O

LB media

For 1L: 10 g NaCl (Sigma 89888-5KG), 5 g Yeast extract (BD 212750), 10 g Tryptone (BD 211699) and ddH₂O

SDS-PAGE sample buffer

100 ul 1M DTT (Bio Basic DB0058) and 100 µl 1.5M Tris-HCl pH 8.8 [Tris base (Fisher BP152-5), Hydrochloric acid (Fisher A144-212)] are added to 800 µl of stock sample buffer [30% Glycerol (Fisher BP229-4), 6% SDS (Fisher BP166), 160mM Tris-HCl pH 6.8 [Tris base (Fisher BP152-5), Hydrochloric acid (Fisher A144-212)] and 0.004% Bromophenol blue (Fisher BP115-25)]

NuPAGE sample buffer

0.6 mL 4X LDS Sample Buffer (Thermo Fisher NP0007), 0.25 mL 10X Sample Reducing Buffer (Thermo Fisher NP0009) and 0.15 mL ddH₂O

5x Laemmli sample buffer

0.5M DTT (Bio Basic DB0058), 50% Glycerol (Fisher BP229-4), 250mM Tris-HCl pH 6.8 [Tris base (Fisher BP152-5), Hydrochloric acid (Fisher A144-212)], 10% SDS (Fisher BP166), 25% B-mercaptoethanol (Sigma M6250) and Bromophenol blue (Fisher BP115-25)
**TELS buffer**

0.5 mL of 1M LiCl stock (AnalaR 10374), 0.1 mL of 0.5M EDTA pH 8.0 (Sigma 03690), 0.05 mL of 1M Tris pH 8.0 [Tris base (Fisher BP1525), Hydrochloric acid (Fisher A144-212)], 0.25 mL of 10% SDS (Invitrogen 15525-017) and 4.1 mL of ddH2O

**PolyP lysis buffer**

9.7 mL ice cold lysis buffer stock solution [7 mM EDTA (Sigma ED2SS), 150 mM NaCl (Sigma S9888), 50 mM Tris-HCl pH 8.9 [Tris base (Fisher BP152-5), Hydrochloric acid (Fisher A144-212)], 20 µl 1M Imidazole (Sigma I0250), 10 µl 1M Sodium Fluoride (Sigma 201154), 11.5 µl 1M Sodium Molybdate (Sigma 243655), 50 µl 200 mM Sodium Orthovanadate (NEB P0758S), 50 µl 1M DTT (BioBasic DB0058), 10 µl 5 mM Cantharidin (Sigma C7632), 100 µl 2.5 mM (-)-p-bromotetramisole Oxalate (Sigma 190047), 100 µl Sigma protease inhibitor cocktail (Sigma P8215), 100 µl 100 mgs/mL PMSF (Sigma P7626) and 1 cOmplete protease inhibitor tablet (Roche 4693132001)

**RIPA lysis buffer**

10 mL ice cold lysis buffer stock solution [100 mM NaCl (Invitrogen AM9759), 10 mM Tris-HCl pH 7.4 [Tris base (Fisher BP152-5), Hydrochloric acid (Fisher A144-212)], 1 mM EDTA (Sigma 03690), 1% IGEPAL CA-630 (Sigma I8896), 0.5% Sodium Deoxycholate (Sigma D6750), 0.1% SDS (Invitrogen 15525-017)], 500 µl Sodium Floride (0.5M, Sigma 201154), 500µl Glycerol-2-Phosphate (1M, Sigma G9422), 500µl Nicotinamide (1M, Sigma N3376), 500µl PMSF (1mg/mL, Sigma P7626), 500µl Sodium Butyrate (1M, Sigma 303410) and 1 cOmplete protease inhibitor tablet (Roche 4693132001)
**Triton X buffer (0.01%)**

500μl 1M Tris pH 7.4 [Tris base (Fisher BP152-5) and Hydrochloric acid (Fisher A144-212)], 1.5 mL 5M NaCl (Sigma S9888), 50μl 10% Triton X stock solution [Triton X (Sigma X100) and ddH₂O], fill to 45 mL with ddH₂O, 1 cOmplete protease inhibitor tablet (Roche 4693132001), 500 μl Sodium Floride (0.5M, Sigma 201154), 500μl Glycerol-2-Phosphate (1M, Sigma G9422), 500μl Nicotinamide (1M, Sigma N3376), 500μl PMSF (1mg/mL, Sigma P7626) and 500μl Sodium Butyrate (1M, Sigma 303410)

**SDS running buffer**

100 mL of 10X 1L stock solution [188g Glycine (Fisher BP381-5), 30.2 g Tris base (Fisher BP152-5), 10 g SDS (Fisher BP166)] and 900 mL ddH₂O

**SDS transfer buffer**

100 mL of 10X 1L stock solution [166.175 g Glycine (Bio Basic GB0235), 30.275 g Tris base (Fisher BP152-5), ddH₂O], 200 mL Methanol (Fisher A412P-4) and 700 mL ddH₂O

**NuPAGE running buffer**

50 mL of 20X 1L stock solution [121.1 g Bis-Tris (Sigma B9754), 209.2 g MOPS (Sigma M1254), 20 g SDS (Fisher BP166), 12 g EDTA (Sigma ED2SS), ddH₂O] and 950 mL ddH₂O

**NuPAGE transfer buffer**

50 mL of 20X 1L stock solution [104.8 g Bis-Tris (Sigma B9754), 81.6 g Bicine (Sigma B3876), 6 g EDTA (Sigma ED2SS), ddH₂O], 200 mL Methanol and 750 mL ddH₂O

**SDS-PAGE gel**

For the resolving gel: 30% Acrylamide solution (Bio-rad 161-0158), ddH₂O, 1.5M Tris buffer pH 8.8 [Tris base (Fisher BP1525), Hydrochloric acid (Fisher A144-212)], 10%
APS [5 g Ammonium Persulfate (Sigma A3678), up to 50 mL ddH2O], 10% SDS (Invitrogen 15525-017) and TEMED (Bio Basic TB0508). For the stacking gel: 30% Acrylamide solution (Bio-rad 161-0158), ddH2O, 1M Tris buffer pH 6.8 [Tris base (Fisher BP1525), Hydrochloric acid (Fisher A144-212)], 10% APS (Sigma A3678), 10% SDS (Invitrogen 15525-017) and TEMED (Bio Basic TB0508)

5xTBE
For 1L: 54 g Tris Base (Fisher 164824), 27.5 g Boric acid (Fisher 141011), 20 mL 0.5M EDTA pH 8.0 (Sigma 03690) and ddH2O

10% TBE-Urea gel
8 mL of 5xTBE stock, 6.67 mL of 30% Acrylamide solution (Bio-rad 161-0158), 19.2 g UREA (Fisher BP169), up to 40 mL ddH2O, 400 µl of 10% APS [5 g Ammonium Persulfate (Sigma A3678), up to 50 mL ddH2O] and 40 µl of TEMED (Bio Basic TB0508)

1xTBS-T
100 mL of 10X 1L stock solution TBS pH 7.5 [30 g Tris base (Fisher BP152-5), 88 g NaCl (Sigma S9888-5KG), 2 g KCl (Sigma P9333-500G), Hydrochloric acid (Fisher A144-212)], 10 mL 10% Tween (Fisher BP337-500) and 890 mL ddH2O

20xPBS
For 2L: 320 g NaCl (Sigma S9888-5KG), 8 g KCl (Sigma P9333-500G), 57.6 g Na2HPO4-H2O (Analar B10249-34), 9.6 g KH2PO4 (Sigma P0662-500G) and ddH2O