A broad response to intracellular long-chain polyphosphate in human cells

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Introduction

Polyphosphates (polyP) are highly anionic, linear polymers composed of long chains of inorganic phosphates linked together by phosphoanhydride bonds ranging from a few to 1000s of residues[1].

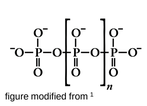
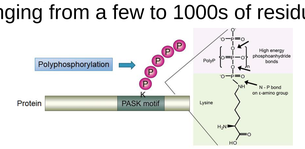


figure modified from [1]

Found in all kingdoms of life, polyP plays roles in phosphate homeostasis, cell growth, infection, and blood coagulation[1,2,3].



PolyP was shown to function as a PTM through a covalent attachment to lysine residues on proteins within a polyacidic serine lysine-rich motif[3,4].

The mechanisms for synthesizing and degrading polyP are best understood in bacteria and yeast however the enzymes responsible for this in mammals are unknown[5].

Function and regulation of polyphosphorylation/polyP remains unclear.

To study the role of polyP in mammals we developed a system to produce polyP in mammalian cells by ectopic expression of the *E. coli ppk1+* gene.

Our work provides a novel resource for studying the impact of mammalian polyP in more depth.

Hypothesis

We hypothesize that overproduction/upregulation of polyP via Ppk1 expression in HEK293Ts will allow us to uncover cellular and molecular pathways that are regulated by polyP in mammals.

Objectives

1) **Investigate the effects of upregulated internal polyP on the transcriptome in mammalian cells.**
2) **Investigate the effects of upregulating internal polyP on the proteome in mammalian cells.**
3) **Determine whether upregulating internal polyP in mammalian cells alters localization of a subset of proteins.**

Objectives

1) Transcriptome - RNA sequencing – illumia Next Generation Sequencing
2) Proteome - Mass Spectrometry - UC Davis Proteomics Core
3) Cell Fractionation and polyP extraction and visualization with DAPI TBE gels

Internal synthesis of polyP in mammalian cells:

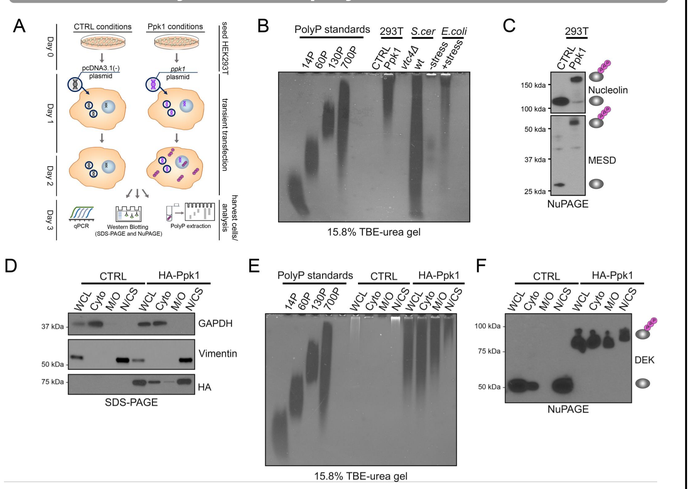


Figure 1. Characteristics of cells that accumulate internal polyP. *(A)* HEK293T were seeded on day zero and on day 2 were transfected with either empty vector (pcDNA3.1(-)) or *ppk1* expression plasmid (pcDNA3.1(-)*ppk1*) to generate control or Ppk1 conditions, respectively. After 48 hours cell lysates were harvested and analyzed with follow up experiments including, but not limited to, qPCR, western blotting (SDS-PAGE and NuPAGE), and polyP extraction. *(B)* PolyP extractions analyzed on a 15.8% DAPI urea gel from control and Ppk1 condition HEK293T cells, wt and *vtc4Δ* yeast, and *E. coli* in normal or stress conditions (amino acid starvation via growth in MOPs minimal media). PolyP standards (14P, 60P, 130P (Regentiss, Japan) and 700P (Kerafast) are presented for comparison. Image shows results from one biological replicate which is representative of N=3 *(C)* NuPAGE analysis of known polyphosphorylated proteins. Total protein was extracted using RIPA buffer prior to NuPAGE analysis via western blotting with indicated antibodies. *(D)* 48 hours after transfection HEK293T cells were fractionated using Cell Signaling Technologies Cell Fraction Kit according to manufactures protocol. SDS-PAGE analysis (4–20% Criterion™ TGX Stain-Free™ Protein Gel) of whole cell lysate (WCL) and cell fractions from control and Ppk1 conditions with antibodies known to be localized to specific fractions: Cytoplasm (Cyto) – GAPDH and Nuclear/Cytoskeleton (N/CS) – Vimentin. All proteins shown here were analyzed on separate blots. Image is representative of N≥3. *(E)* PolyP extractions analyzed on a 15.8% TBE-urea gel from cell fractionations of control and Ppk1 conditions HEK293T cells. PolyP was extracted directly from cell fraction aliquots either immediately following fractionation or from fractions stored at -80C. *(F)* NuPAGE analysis of cell fractionations from HEK293T control and Ppk1 conditions of the polyphosphorylated protein target DEK. Image is representative of N=3.

Hundreds of differentially expressed genes in cells making excess polyP:

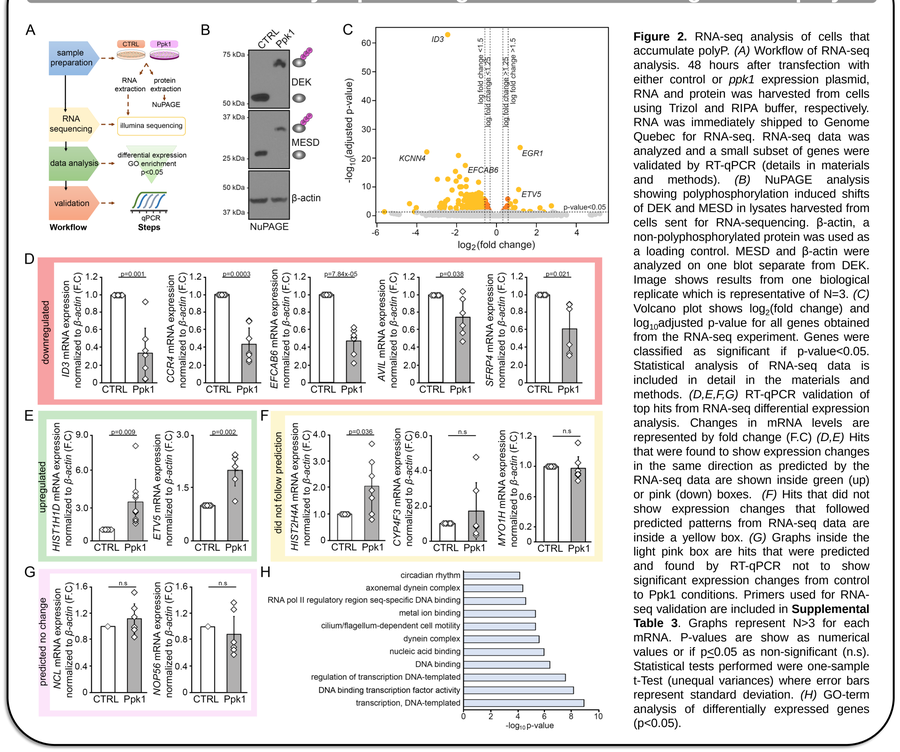


Figure 2. RNA-seq analysis of cells that accumulate polyP. *(A)* Workflow of RNA-seq analysis. 48 hours after transfection with either control or *ppk1* expression plasmid, RNA and protein was harvested from cells using Trizol and RIPA buffer, respectively. RNA was immediately shipped to Genome Quebec for RNA-seq. RNA-seq data was analyzed and a small subset of genes were validated by RT-qPCR (details in materials and methods). *(B)* NuPAGE analysis showing polyphosphorylation induced shifts of DEK and MESD in lysates harvested from cells sent for RNA-sequencing. β-actin, a non-polyphosphorylated protein was used as a loading control. MESD and β-actin were analyzed on one blot separate from DEK. Image shows results from one biological replicate which is representative of N=3. *(C)* Volcano plot shows log_2(fold change) and log_{10}adjusted p-value for all genes obtained from the RNA-seq experiment. Genes were classified as significant if p-value<0.05. Statistical analysis of RNA-seq data is included in detail in the materials and methods. *(D,E,F,G)* RT-qPCR validation of top hits from RNA-seq differential expression analysis. Changes in mRNA levels are represented by fold change (F.C) *(D,E)* Hits that were found to show expression changes in the same direction as predicted by the RNA-seq data are shown inside green (up) or pink (down) boxes. *(F)* Hits that did not show expression changes that followed predicted patterns from RNA-seq data are inside a yellow box. *(G)* Graphs inside the light pink box are hits that were predicted and found by RT-qPCR not to show significant expression changes from control to Ppk1 conditions. Primers used for RNA-seq validation are included in **Supplemental Table 3**. Graphs represent N>3 for each mRNA. P-values are show as numerical values or if p≤0.05 as non-significant (n.s). Statistical tests performed were one-sample t-Test (unequal variances) where error bars represent standard deviation. *(H)* GO-term analysis of differentially expressed genes (p<0.05).

Global changes in protein levels in polyP producing cells:

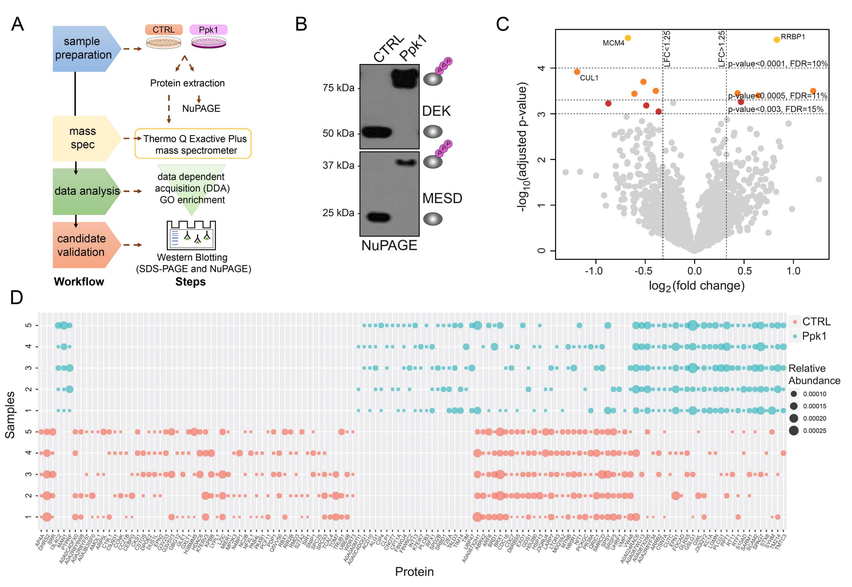


Figure 3. Global changes in protein levels in cells that accumulate polyP. *(A)* Workflow of mass spectrometry (MS) analysis. 48 hours after transfection with either control or *ppk1* expression plasmid, protein was harvested from cells and a small portion of sample was used to confirm polyphosphorylation of targets by NuPAGE in all samples. The remaining portion of each sample was immediate shipped to UCDavis for MS (details included in materials and methods). *(B)* NuPAGE analysis showing polyphosphorylation induced shifts of DEK and MESD in lysates harvested from cells sent for MS. DEK and MESD were analyzed on different blots. Image shows results from one biological replicate which is representative of N=5. *(C)* Volcano plot shows log_2(fold change) and -log_{10}adjusted p-value for all proteins obtained from the MS experiment. Statistical analysis of MS data is included in detail in the materials and methods. *(D)* "Singletons" from MS data. Categories shown in both control (pink) and Ppk1 (blue) conditions include "all or nothing" (spectra in 5/5 of one condition and 0/5 of the other), 2) "mostly all or none" (spectra in (3,4)/5 of one condition and 0/5 of the other), and 3) "all or mostly none" (spectra in 5/5 of one condition and (3,4)/5 of the other). Size of bubbles reflect relative abundance of the protein within each biological replicate (samples 1-5) for each condition.

Internal synthesis of polyP downregulates of DHRS2:

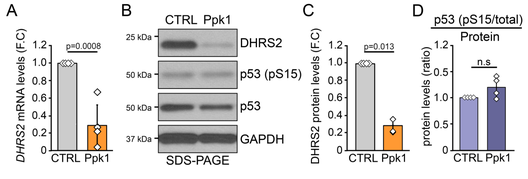


Figure 4. Accumulation of polyP leads to upregulation of DHRS2. RNA and protein were harvested from HEK293T cells following a 48-hour transient transfection of either a control or *ppk1* expression plasmid. *(A)* RT-qPCR analysis of *DHRS2* mRNA in control and Ppk1 conditions. Changes in mRNA levels are represented by fold change (F.C) *(B)* Western blot analysis with SDS-PAGE (4–20% Criterion™ TGX Stain-Free™ Protein Gel) of proteins in control and Ppk1 conditions using antibodies against DHRS2, p53, and phosphorylated p53 (Ser-15). GAPDH was used as a loading control. All proteins shown were analyzed on separate blots. Image shows results from one biological replicate which is representative of N≥3 *(C,D)* Semi-quantitative analysis of protein levels shown in panel *B*. P-values are show as numerical values or if p≤0.05 as non-significant (n.s). Statistical tests performed were one-sample t-Test (unequal variances) where error bars represent standard deviation. All experiments shown here are representative of N≥3.

PolyP synthesis activates ERK1/2 signaling:

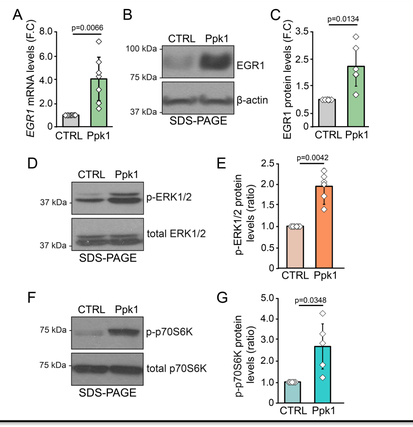


Figure 5. Activation of ERK1/2 signaling by production of polyP. RNA and protein were harvested from HEK293T cells following a 48-hour transient transfection of either a control or *ppk1* expression plasmid. *(A)* RT-qPCR of *EGR1* mRNA levels in control and Ppk1 conditions. Changes in mRNA levels are represented by fold change (F.C) *(B)* western blotting analysis by SDS-PAGE (4–20% Criterion™ TGX Stain-Free™ Protein Gel) in control and Ppk1 conditions cells using the EGR1 antibody with β-actin as a loading control. EGR1 and β-actin were analyzed on different blots. Image shows results from one biological replicate which is representative of N≥3. *(C)* Semi-quantitative analysis of protein levels shown in panel B. *(D)* Western blotting analysis by SDS-PAGE in control and Ppk1 conditions cells using antibodies against total Erk1/2 and phosphorylated Erk1/2. Total Erk1/2 and phosphorylated Erk1/2 were analyzed on separate blots. Image shows results from one biological replicate which is representative of N≥3. *(E)* Semi-quantitative analysis of protein levels shown in panel D. *(F)* Western blotting analysis by SDS-PAGE in control and Ppk1 conditions cells using antibodies against total p70S6K and phosphorylated p70SK6. Total p70S6K and phosphorylated p70SK6 were analyzed on separate blots. Image shows results from one biological replicate which is representative of N≥3. *(G)* Semi-quantitative analysis of protein levels shown in panel G. P-values are show as numerical values or if p≤0.05 as non-significant (n.s). Statistical tests performed were one-sample t-Test (unequal variances) where error bars represent standard deviation.

Relocalization of nuclear proteins in cells accumulating polyP:

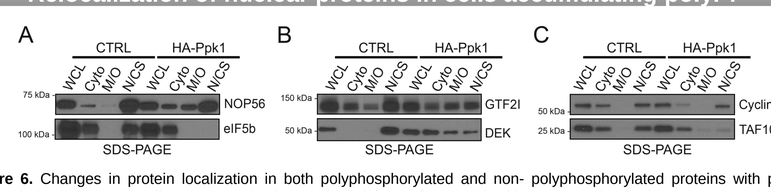


Figure 6. Changes in protein localization in both polyphosphorylated and non- polyphosphorylated proteins with polyP accumulation. HEK293T cells were transiently transfected for 48 hours with either a control or *ppk1* expression plasmid. Fractionation of cells was performed using Cell Signaling Technologies Cell Fraction Kit (9038) according to manufactures protocol. SDS-PAGE analysis (4–20% Criterion™ TGX Stain-Free™ Protein Gel) of whole cell lysate (WCL) and cell fractions from control and Ppk1 conditions with antibodies against known polyphosphorylated proteins NOP56, eIF5b, GTF2I, and DEK and non-polyphosphorylated proteins, Cyclin B1 and TAF10 were also assessed using antibodies against indicated proteins. All proteins were analyzed on separate blots. Amount of cell material used to generate each fraction was consistent between all biological replicates tested. Images in each panel show results from one biological replicate which is representative of N≥3.

Conclusions and Discussion

1. Expression of *E. coli* Ppk1 protein in mammalian cells upregulates internal polyP which is localized to all compartments of the cells → mostly cytoplasmic and allows for polyphosphorylation of target proteins.
2. Upregulation of internal polyP causes changes in the mammalian transcriptome and proteome.
3. Upon internal upregulation of polyP of nuclear proteins DEK, ,eIF5b, TAF10, and GTF2I showed changes in their localization.

Future directions

Understanding the molecular mechanistic details of how upregulation of internal polyP in mammalian cells is resulting in changes of:

- RNA expression → targeting transcription factors, promoter regions?
- Protein levels → transcription based? degradation pathways?
- Protein localization → polyP binding to target proteins? Generation of PASK mutants

References

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Acknowledgments

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