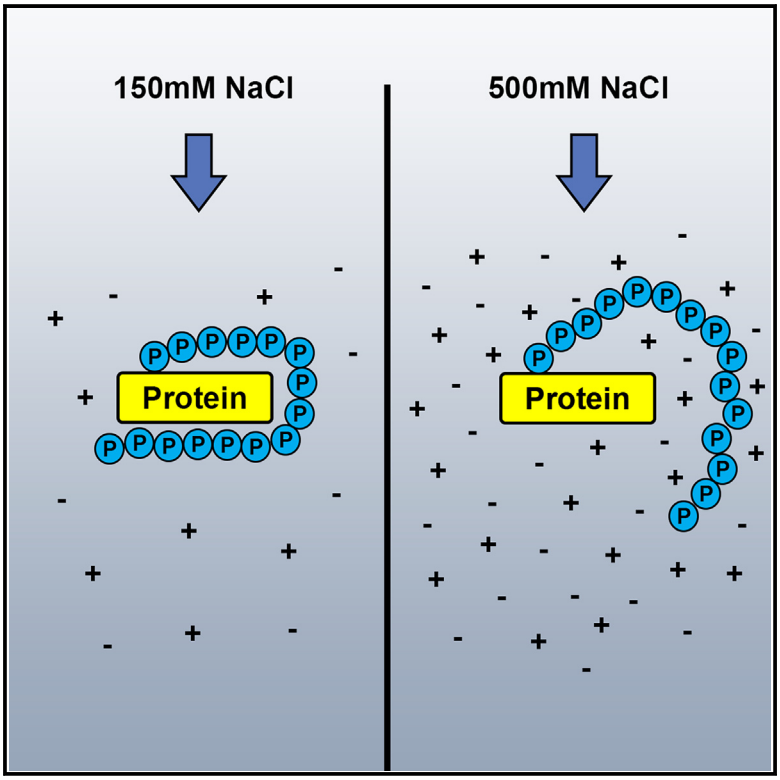
Molecular Cell

Matters Arising Response

On the covalent nature of lysine polyphosphorylation

Graphical abstract



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On the covalent nature of lysine polyphosphorylation

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SUMMARY

Post-translational modifications of proteins (PTMs) introduce an extra layer of complexity to cellular regulation. Although phosphorylation of serine, threonine, and tyrosine residues is well-known as PTMs, lysine is, in fact, the most heavily modified amino acid, with over 30 types of PTMs on lysine having been characterized. One of the most recently discovered PTMs on lysine residues is polyphosphorylation, which sees linear chains of inorganic polyphosphates (polyP) attached to lysine residues. The labile nature of phosphoramidate bonds raises the question of whether this modification is covalent in nature. Here, we used buffers with very high ionic strength, which would disrupt any non-covalent interactions, and confirmed that lysine polyphosphorylation occurs covalently on proteins containing PASK domains (polyacidic, serine-, and lysine-rich), such as the budding yeast protein nuclear signal recognition 1 (Nsr1) and the mammalian protein nucleolin. This Matters Arising Response paper addresses the Neville et al. (2024) Matters Arising paper, published concurrently in *Molecular Cell*.

INTRODUCTION

Phosphorylation is one of the most abundant types of post-translational modifications (PTM) on proteins. Phosphorylation of serine, threonine, and tyrosine residues is characterized by the formation of phosphodiester bonds (P–O), the relative stability of which is amenable to biochemical analyses, thus allowing researchers to reveal their crucial roles in a wide range of signal transduction networks. In contrast, phosphorylation of basic amino acid residues, such as arginine, histidine, and lysine, is mediated by P–N phosphoramidate bonds,[1] which are highly energetic and labile, thus posing major challenge to biochemical characterization. In Azevedo et al., we reported that inorganic polyphosphate (polyP), a linear chain of phosphate groups, is covalently attached to lysine residues.[2] Specifically, we described that nuclear signal recognition 1 (Nsr1) and its interacting partner, topoisomerase 1 (Top1), are polyphosphorylated.[2] The regions in Nsr1 and Top1 targeted by polyphosphorylation contain a high number of acidic amino acids, which we defined as the PASK domain (polyacidic, serine-, and lysine-rich).[3] Our work was further substantiated by a study from the Downey lab, which identified a great number of PASK domain-containing proteins as polyphosphorylation targets in both yeast and mammalian cells and these proteins are primarily localized within the nucleus/nucleolus.[4]

The initial cloning of Nsr1 in the early 1990s revealed that this protein does not migrate according to its predicted molecular weight of 45 kDa.[5] Our work started by observing that the huge mobility shift of Nsr1 on polyacrylamide gel electrophoresis (NuPAGE) depends on the presence of polyP. Nsr1 migrates as an ~130 kDa peptide in wild-type yeast extract but runs as a ~60 kDa protein in *vtc4*Δ extract, a mutant yeast unable to synthesize polyP.[2] Considering that a negatively charged polymer such as polyP has the intrinsic ability to bind positively charged protein domains, we carefully analyzed this previously unknown PTM using multiple approaches and concluded that the target proteins are polyphosphorylated by covalent modifications *in vivo*. Both Nsr1 and Top1 mobility was highly shifted in the presence of endogenous polyP on NuPAGE gels, which were run under denaturing conditions in the presence of lithium dodecyl sulphate (LDS). Moreover, the mobility shift of endogenously made Nsr1 remained intact after preparing the yeast extracts under strong denaturing conditions, such as in the presence of 1% sodium dodecyl sulphate (SDS) and 8 M urea. After extensive mutagenesis of the lysine residues, we abolished the Top1 mobility shift on NuPAGE. The mutagenesis data, together with the high sensitivity of this PTM to hydroxylamine, led us to conclude that polyPs attach to lysine residues by covalent interactions.

These conclusions are now being challenged by the Matter Arising work of Neville et al.[6] We respond to this challenge by pointing out several key experimental differences in our respective studies. In most of our assays, we studied yeast proteins under physiological conditions, using yeast extracts and endogenously synthesized polyP. In contrast, Neville et al.[6] only used recombinant proteins produced in Escherichia coli

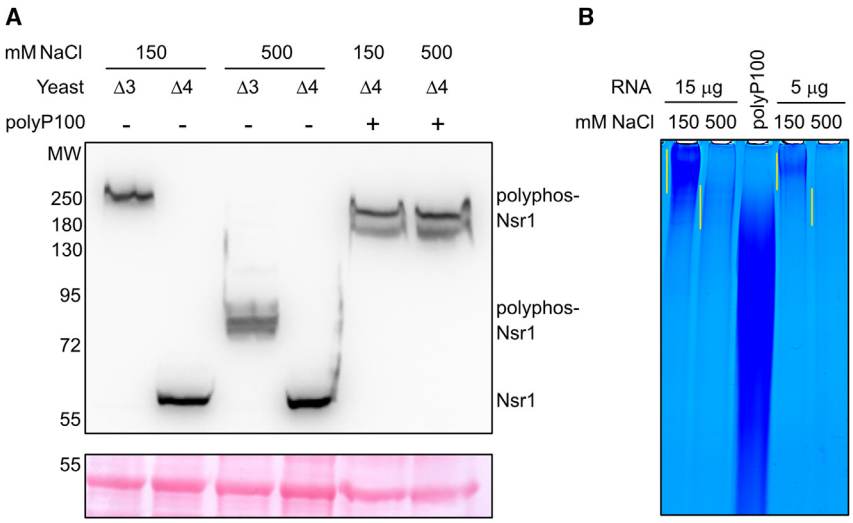

Figure 1. Polyphosphorylation of yeast Nsr1 in the presence of 500 mM NaCl

Cell lysates from logarithmically growing *ppn1*Δ *ppn1*Δ*ppx1*Δ (Δ3), a triple polyphosphatase mutant and the isogenic *vtc4*Δ mutant *ppn1*Δ*ppn1*Δ*ppx1*Δ *vtc4*Δ (Δ4), a mutant where polyP is absent, were prepared using lysis buffer containing either 150 or 500 mM NaCl and resolved on NuPAGE.

Western blot using anti-Nsr1 antibody (A) reveals a considerable mobility shift of Nsr1 in the triple polyphosphatase mutant strain (Δ3), while this mobility shift is absent in the isogenic *vtc4*Δ mutant (Δ4). Under high salt (500 mM) condition, the dehydrated shrunken vacuole[10] is not lysed, leading to a smaller Nsr1 mobility shift resulting from the modification by the less-abundant and shorter nuclear polyP.[9] An identical mobility shift is observed by adding exogenous polyP100 (+) to the Δ4 lysate, prepared with either 150 or 500 mM NaCl lysis buffer. Ponceau stain is used to verify equal loading. The data presented are representative of 2 independent experiments. PAGE analysis of polyP (B) extracted from protein lysates obtained with either 150 or 500 mM NaCl lysis buffer. Phenol-extracted polyP/RNA (5 or 15 μg RNA) were resolved on a 25% PAGE gel and visualized by toluidine blue staining. Yellow bars indicate the average length differences in the recovered polyP.

(*E. coli*), synthetic peptides, and long chain polyP produced *in vitro* (polyP700). By performing a completely different set of assays solely under *in vitro* conditions, Neville et al.[6] arrived at a different conclusion than us: that polyphosphorylation might occur non-covalently. Neville et al.[6] correctly noticed that our wash buffer contained physiological salt concentration of 150 mM NaCl and they were also correct in suggesting that we did not wash polyP away after the different treatments. However, it is fundamental to understand that our experiments were not done with recombinant proteins bound to beads and therefore a washing step could not have been included to remove endogenous polyP. Instead, we extensively used yeast genetics to modulate polyP levels. Moreover, we observed an alkaline resistance of the Top1 NuPAGE mobility shift, while Neville et al.[6] did not. In this regard, we consider it highly unlikely, as proposed by Neville et al.,[6] that detached polyP would allosterically re-associate with Top1 during the highly denaturing boiling step in the presence of LDS prior to gel loading. We also prepared yeast lysates under strong denaturing conditions, using 1% SDS or 8 M urea. Such harsh treatments instantly froze cellular biochemistry, yet Nsr1 gel mobility remained intact, confirming the covalent nature of lysine polyphosphorylation. These common yet crucial approaches were not used by Neville et al.[6] to validate their findings. Because thoughtful discussions are vitally important for scientific progress, we conducted further experiments in response to Neville et al.,[6] which supported our original argument that lysine polyphosphorylation is indeed covalent in nature.

RESULTS

Protein-ligand interactions of non-covalent nature are readily disrupted by increased salt concentration. Neville et al.[6] argued that lysine polyphosphorylation is non-covalent because of its sensitivity to ionic strengths. Because in our original paper we used a wash buffer containing physiological salt concentrations of 150 mM NaCl,[7] here we assayed the Nsr1 mobility shift on NuPAGE in the presence of 500 mM NaCl, which, noteworthily, was twice the concentration used by Neville et al.[6] 500 mM NaCl is regarded as the most stringent wash condition in biochemical analyses, as it is routinely used to remove ionic interactors during GST-tagged protein purification and represents the higher end of salt gradient used to elute proteins from the ion exchange chromatography column.[8] We prepared protein extracts in the presence of either 150 or 500 mM NaCl from logarithmically growing triple polyphosphatase yeast mutant *ppn1*Δ*ppn1*Δ*ppx1*Δ and from the isogenic *ppn1*Δ*ppn1*Δ*ppx1*Δ*vtc4*Δ mutant, which is devoid of endogenous polyP[9] (see STAR Methods). The use of polyphosphatase mutants facilitates the detection of covalent lysine polyphosphorylation because polyP is rapidly degraded by polyphosphatases.[9] As expected, endogenous Nsr1 exhibited a high mobility shift in the phosphatase mutant lysates prepared in the presence of 150 mM NaCl, and the shift was absent in lysates prepared from the isogenic *vtc4*Δ strain (Figure 1A). Interestingly, when the yeast extracts were prepared in the presence of 500 mM NaCl, the Nsr1 mobility shift remained substantial, although the degree of shift was lower than that seen when using 150 mM NaCl (Figure 1A). Hence, this experiment demonstrates that polyP cannot dissociate from Nsr1, even in the presence of high salt concentration. The difference in the degrees of shift under the two salt concentrations is nonetheless curious. As elaborated below, we hypothesize that the ionic strength of the lysis buffer determines the type and amount of polyP extracted from yeast, thus affecting the degree of Nsr1 mobility shift. In fact, when we added exogenous polyP100 to the *ppn1*Δ*ppn1*Δ*ppx1*Δ*vtc4*Δ lysates extracted with the 500-mM NaCl lysis buffer, we observed a full restoration of the Nsr1 mobility shift, and the degrees of shift were identical between *ppn1*Δ*ppn1*Δ*ppx1*Δ*vtc4*Δ lysates prepared under 150 or 500 mM NaCl (Figure 1A). Hence, ionic strengths do not affect the nature of Nsr1 lysine polyphosphorylation.

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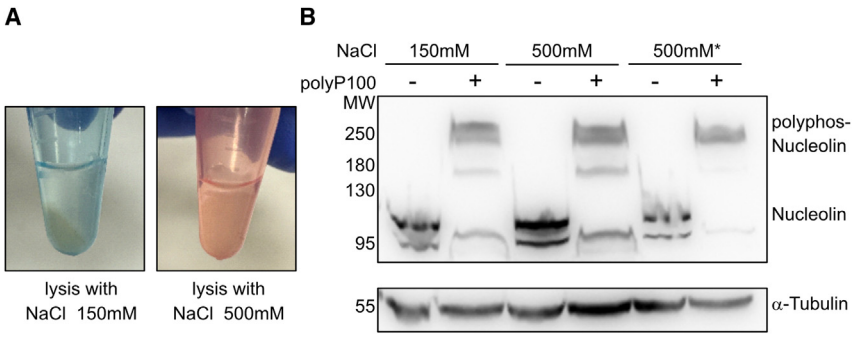

Figure 2. Polyphosphorylation of mammalian nucleolin in the presence of 500 mM NaCl

Mammalian cells (HEK293) were incubated on ice for 15 min in lysis buffers containing either 150 or 500 mM NaCl.

The high density of the 500-mM NaCl lysis buffer prevented the formation of a compacted pellet (A) after centrifugation of the lysates at 15,000g for 10 min. The cleared protein lysates were incubated for 20 min at 30°C in the presence (+) or absence (–) of polyP100. Western blot with anti-nucleolin antibody (B) revealed a considerable mobility shift of nucleolin in the samples incubated with polyP100 from both 150 and 500 mM NaCl lysates. Considering the limited extraction observed when using the lysis buffer containing 500 mM NaCl, we re-created the 500-mM salt concentration by adding extra NaCl to the 150-mM NaCl sample supernatant (500 mM*) and observed once again a dramatic nucleolin mobility shift upon polyP100 addition (+). The analysis presented is representative of three independent experiments.

Nsr1 is a protein that shuttles between the cytosol and the nucleus and, physiologically, its polyphopshorylation is likely to occur within the nucleus.[2] However, the bulk of polyP resides in the yeast vacuole and, in comparison, the concentration of polyP in the nucleus is considerably lower. During cell lysis, polyP released from the vacuole can polyphosphorylate nuclear-localized Nsr1, exacerbating its mobility shift.[9] This in-extraction issue was resolved when we specifically expressed exopolyphosphatasePPX1 in the vacuoles (vt-PPX1), demonstrating that despite eliminating all vacuole-stored polyP, Nsr1 still showed a high mobility shift.[9] Osmoregulation in yeast is a well-understood topic. The vacuole participates in an immediate osmoregulatory process, allowing the synthesis and accumulation of the osmolyte glycerol to occur over time.[11] The high osmolarity of a 500-mM NaCl lysis buffer results in water being drawn from the yeast vacuole (containing the bulk of polyP) into the cytoplasm, leading to a shrinkage of the yeast cells.[10] Therefore, it is highly likely that under high salt conditions, the shrunken—and hence highly compact vacuole—is not sufficiently lysed to release the vacuole-derived polyP. As a result, the observed Nsr1 mobility shift in the high salt buffer is solely generated by the less-abundant and shorter cytosolic/nuclear polyP.[9] To test this hypothesis, we purified RNA/polyP by phenol extraction from *ppn1*Δ*ppn1*Δ*ppx1*Δ protein lysates prepared in the presence of 150 or 500 mM NaCl. As hypothesized, PAGE analysis of the extracted RNA/polyP demonstrates that high salt recovered a smaller amount of polyP with shorter lengths (Figure 1B), explaining the difference in the degree of the Nsr1 mobility shift by endogenous polyP in the presence of 500 mM NaCl (Figure 1A). In fact, the shrinkage of the vacuole by high salt allowed us to detect physiological Nsr1 lysine polyphosphorylation driven by the smaller and shorter cytosolic/nuclear pool of polyP, as we observed when expressing vt-PPX1.[9]

We next tested whether the polyP-induced mobility shift of nucleolin, the mammalian homolog of Nsr1, is affected by ionic strengths in the lysis buffer. Cell lysates from human embryonic kidney (HEK)293T cell line were prepared in the presence of 150 or 500 mM NaCl. We observed that high salt lysis buffer prevented the formation of compacted nuclei and cell debris pellets after standard centrifugation procedures (Figure 2A). Nevertheless, the modest amount of clear supernatant recovered from the 500-mM NaCl lysis buffer was sufficient to perform the mobility shift assay. The cell lysates were incubated with polyP100, and western blot analysis revealed an identical and dramatic nucleolin mobility shift using lysates from both low and high salt concentrations (Figure 2B). Hence, mobility shift of a mammalian protein induced by lysine polyphosphorylation is also insensitive to the ionic strengths in the lysis buffer, thus confirming the covalent nature of this PTM. Because the lysis of mammalian cells in the presence of 500 mM NaCl recovered only a small quantity of lysate (Figure 2A), which might result in unexpected technical issues, we performed an additional experiment. We lysed the cells in a buffer containing 150 mM NaCl and only increased salt concentration to 500 mM NaCl after extraction, just prior to performing the mobility shift assay by adding polyP100. We again observed a dramatic mobility shift of nucleolin, demonstrating that the observed mobility shift is insensitive to high ionic strength and thus reliant on covalent modifications.

DISCUSSION

Comparing our findings and those of Neville et al.,[6] the fact that they only used *E. coli* recombinant peptides whereas we used endogenously synthesized yeast proteins could explain the differences in our results. It is widely accepted that proteins produced in prokaryotes and eukaryotes carry different PTMs and might fold differently, and, therefore, the correct choice of organism is vitally important for meaningful biochemical studies. In the case of the well-studied Nsr1 protein, which is 45 kDa in size, it runs on NuPAGE as a 60 kDa protein in *vtc4*Δ yeast mutant that lacks lysine polyphosphorylation, suggesting that it might be subject to additional PTMs that could influence lysine polyphosphorylation. The biophysical/biochemical properties of recombinant versus endogenously synthesized Nsr1 and Top1 were likely to be very different in our respective studies. Notably, it is known that Nsr1 and Top1 are phosphorylated on various serine residues in yeast (http://dbpaf.biocuckoo.org.). In fact, it is documented that the source of recombinant Nsr1 determines whether Nsr1 is serine pyrophosphorylated, a PTM driven by inositol pyrophosphates (PP-InsPs).[12,13] Recombinant Nsr1 produced in *E.coli* did not get pyrophosphorylated by radiolabeled PP-InsP, while yeast Nsr1 did, because PP-InsPs can only transfer the radiolabeled β-phosphate to a pre-phosphorylated serine[14]. We speculate that lysine polyphosphorylation may be similarly

regulated by pre-existing PTMs, hence explaining the difference in results between ours and those of Neville et al.[6]

Phosphoramidate bonds are sensitive to hydroxylamine treatment,[15] and therefore we used this reducing agent on endogenously expressed Top1 to demonstrate the lability of the lysine polyphosphorylation. The Top1 mobility shift was affected by low millimolar range of hydroxylamine, with 100 mM hydroxylamine reducing the shift and 200 mM completely abolishing it.[2] Neville et al.[6] suggested that the reduction in mobility shift that we observed was caused by hydroxylamine-mediated polyP degradation. In the presence of 100 mM hydroxylamine, neither us nor Neville et al.[6] reported polyP degradation, refuting the idea that the loss of Top1 mobility shift was caused by polyP degradation. The results of a similar mobility shift experiment conducted by Neville et al.[6] is puzzling because they continued to observe substantial mobility shift of Top1 using 200 mM and even at 1.0 M hydroxylamine treatment, which substantially or completely degraded polyP. In Neville et al.,[6] the hydroxylamine treatment was followed by washing the recombinant Top1 immobilized on beads before processing the samples for gel electrophoresis. Here, no loss of mobility shift was observed. Maltose binding protein (MBP)-tagged proteins immobilized on amylose beads were washed without any salt, and so it is likely the loss of the polyP–N covalent bond could be compensated by the ionic interaction that occurs between the negatively charged polyP and the positively charged lysine residues.

Neville et al.[6] used nuclear magnetic resonance (NMR) to detect the P–N bond. The P-amidate (P–N) signal, like P-lysine, would resonate around 8 ppm, as Neville et al.[6] indicated. However, if another phosphate is attached, as in the case of lysine polyphosphorylation (P–P–N), then the P-anhydride (P–P) will shift the P–N signal by 10 ppm to ca. −1 or −2 (depending on pH and counterion). Therefore, if the assay has a large amount of phosphate impurity, the P–P–N bond signal could lie underneath the big phosphate peak. Using synthetic peptides, Neville et al.[6] showed that there is no phosphate contaminant in polyP-only and in peptide-only analysis. Therefore, the new peak that appears, which the authors assigned as phosphate in the peptide-plus-polyP experiment, would resonate close to the predicted signal of covalent lysine polyphosphorylation. Because the NMR results of Neville et al.[6] were confounded by high noise signals and a lack of positive controls (P-lysine and polyP-lysine peptides), they were unconvincing as an argument against the existence of P–P–N bonds.

To conclude, we believe that the arguments presented by Neville et al.[6] do not challenge our earlier conclusion that lysine residues on Nsr1 and Top1 are covalently modified by polyphosphorylation.[2] This conclusion is strengthened by the new experiments presented, in which lysine polyphosphorylation remained intact under high ionic strength. Nonetheless, it is noteworthy that Neville et al. discovered that the number and organization in lysine stretches, and as they recently demonstrated also of histidine,[16] could promote an ionic interaction with polyP sufficiently strong to induce a mobility shift on NuPAGE. However, to validate the physiological relevance of their findings, they need to re-test their ideas *in vivo* using endogenously expressed proteins and polyP. Ideally, these experiments should also take advantage of the engineered yeast strain with reduced vacuolar polyP to minimize cross-contamination during cell lysis[9] or take advantage of the higher salt lysis procedure that, by shrinking the vacuole, allowed us to purify cytosolic/nuclear polyP.

A protein array screening to define the human polyPome revealed the existence of several human proteins (Annexin A6, Annexin A9, RuvB-like AAA ATPase 1 and 2 [RUVBL1 and RUVBL2], and adaptor related protein complex 1 subunit beta 1 [AP1B1]) ionically interacting with polyP.[17] polyP does not induce a mobility shift of these proteins on NuPAGE. However, a mobility shift was observed when these types of protein-polyP interactions were analyzed by native PAGE or gel filtration experiments. The three-dimensional structure of these proteins promotes their ionic interaction with polyP.[17] Therefore, the ionic interaction we described[17] is distinct from the one proposed by Neville et al.[6] Nevertheless, their studies highlighted the multifaceted nature of lysine polyphosphorylation. Further works *in vivo* will be required to gain a full understanding of this important PTM.

Limitations of the study

Although the ionic strength of 500 mM NaCl usually represents the upper salt limit in protocols aimed at isolating proteins without contaminants or interacting partners, we could not exclude that polyP would bind so tightly to extremely positively charged proteins that would not be washed away even under these harsh conditions. However, this would not be the case for proteins containing the intrinsically negatively charged PASK domains. Additionally, we cannot exclude the possibility that what we characterized as lysine polyphosphorylation[2] could represent an intermediate state, i.e., the polyP that is initially attached to the lysine could eventually be transferred to another amino acid. In this case, the PASK domain lysines would be involved in a polyP-transfer reaction akin to phosphotransfer reactions occuring on the catalytic lysine residues of protein kinases.[18] Further work is required to test this exciting possibility.

STAR★METHODS

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AUTHOR CONTRIBUTIONS

C.A. conceived, designed, and directed the study and wrote the manuscript; F.B. conducted the experiments, analyzed the data, and prepared the figures; X.B.S. generated reagent and wrote the manuscript; A.S. conceived, designed, and directed the study and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

1. Hauser, A., Poulou, E., Müller, F., Schmieder, P., and Hackenberger, C.P.R. (2021). Synthesis and Evaluation of Non-Hydrolyzable Phospho-Lysine Peptide Mimics. Chemistry *27*, 2326–2331. https://doi.org/10.1002/chem.202003947.
2. Azevedo, C., Livermore, T., and Saiardi, A. (2015). Protein polyphosphorylation of lysine residues by inorganic polyphosphate. Mol. Cell *58*, 71–82. https://doi.org/10.1016/j.molcel.2015.02.010.
3. Azevedo, C., and Saiardi, A. (2016). Why always lysine? The ongoing tale of one of the most modified amino acids. Adv. Biol. Regul. *60*, 144–150. https://doi.org/10.1016/j.jbior.2015.09.008.
4. Bentley-DeSousa, A., Holinier, C., Moteshareie, H., Tseng, Y.C., Kajjo, S., Nwosu, C., Amodeo, G.F., Bondy-Chorney, E., Sai, Y., Rudner, A., et al. (2018). A Screen for Candidate Targets of Lysine Polyphosphorylation Uncovers a Conserved Network Implicated in Ribosome Biogenesis. Cell Rep. *22*, 3427–3439. https://doi.org/10.1016/j.celrep.2018.02.104.
5. Lee, W.C., Xue, Z.X., and Mélèse, T. (1991). The NSR1 gene encodes a protein that specifically binds nuclear localization sequences and has two RNA recognition motifs. J. Cell Biol. *113*, 1–12. https://doi.org/10.1083/jcb.113.1.1.
6. Neville, N., Lehotsky, K., Klupt, K.A., Downey, M., and Jia, Z. (2024). Polyphosphate attachment to lysine repeats is a non-covalent protein modification. Molecular Cell *84*, https://doi.org/10.1016/j.molcel.2024.03.028.
7. Schmidt-Nielsen, B. (1975). Comparative physiology of cellular ion and volume regulation. J. Exp. Zool. *194*, 207–219. https://doi.org/10.1002/jez.1401940114.
8. Gentiluomo, L., Schneider, V., Roessner, D., and Frieß, W. (2019). Coupling Multi-Angle Light Scattering to Reverse-Phase Ultra-High-Pressure Chromatography (RP-UPLC-MALS) for the characterization monoclonal antibodies. Sci. Rep. *9*, 14965. https://doi.org/10.1038/s41598-019-51233-6.
9. Azevedo, C., Desfougères, Y., Jiramongkol, Y., Partington, H., Trakansuebkul, S., Singh, J., Steck, N., Jessen, H.J., and Saiardi, A. (2020). Development of a yeast model to study the contribution of vacuolar polyphosphate metabolism to lysine polyphosphorylation. J. Biol. Chem. *295*, 1439–1451. https://doi.org/10.1074/jbc.RA119.011680.
10. Mager, W.H., and Siderius, M. (2002). Novel insights into the osmotic stress response of yeast. FEMS Yeast Res. *2*, 251–257. https://doi.org/10.1016/S1567-1356(02)00116-2.
11. Latterich, M., and Watson, M.D. (1993). Evidence for a dual osmoregulatory mechanism in the yeast Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. *191*, 1111–1117. https://doi.org/10.1006/bbrc.1993.1331.
12. Morgan, J.A.M., Singh, A., Kurz, L., Nadler-Holly, M., Penkert, M., Krause, E., Liu, F., Bhandari, R., and Fiedler, D. (2022). Pyrophosphoproteomics: extensive protein pyrophosphorylation revealed in human cell lines. Preprint at bioRxiv. https://doi.org/10.1101/2022.11.11.516170.
13. Ganguli, S., Shah, A., Hamid, A., Singh, A., Palakurti, R., and Bhandari, R. (2020). A high energy phosphate jump - From pyrophospho-inositol to pyrophospho-serine. Adv. Biol. Regul. *75*, 100662. https://doi.org/10.1016/j.jbior.2019.100662.
14. Bhandari, R., Saiardi, A., Ahmadibeni, Y., Snowman, A.M., Resnick, A.C., Kristiansen, T.Z., Molina, H., Pandey, A., Werner, J.K., Jr., Juluri, K.R., et al. (2007). Protein pyrophosphorylation by inositol pyrophosphates is a posttranslational event. Proc. Natl. Acad. Sci. USA *104*, 15305–15310. https://doi.org/10.1073/pnas.0707338104.
15. Duclos, B., Marcandier, S., and Cozzone, A.J. (1991). Chemical properties and separation of phosphoamino acids by thin-layer chromatography and/or electrophoresis. Methods Enzymol. *201*, 10–21. https://doi.org/10.1016/0076-6879(91)01004-L.
16. Neville, N., Lehotsky, K., Yang, Z., Klupt, K.A., Denoncourt, A., Downey, M., and Jia, Z. (2023). Modification of histidine repeat proteins by inorganic polyphosphate. Cell Rep. *42*, 113082. https://doi.org/10.1016/j.celrep.2023.113082.
17. Azevedo, C., Singh, J., Steck, N., Hofer, A., Ruiz, F.A., Singh, T., Jessen, H.J., and Saiardi, A. (2018). Screening a Protein Array with Synthetic Biotinylated Inorganic Polyphosphate To Define the Human PolyP-ome. ACS Chem. Biol. *13*, 1958–1963. https://doi.org/10.1021/acschembio.8b00357.
18. Carrera, A.C., Alexandrov, K., and Roberts, T.M. (1993). The conserved lysine of the catalytic domain of protein kinases is actively involved in the phosphotransfer reaction and not required for anchoring ATP. Proc. Natl. Acad. Sci. USA *90*, 442–446. https://doi.org/10.1073/pnas.90.2.442.
19. Gueldener, U., Heinisch, J., Koehler, G.J., Voss, D., and Hegemann, J.H. (2002). A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. Nucleic Acids Res. *30*, e23. https://doi.org/10.1093/nar/30.6.e23.
20. Lonetti, A., Szijgyarto, Z., Bosch, D., Loss, O., Azevedo, C., and Saiardi, A. (2011). Identification of an evolutionarily conserved family of inorganic polyphosphate endopolyphosphatases. J. Biol. Chem. *286*, 31966–31974. https://doi.org/10.1074/jbc.M111.266320.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
α-tubulin	Biolegend	Cat#625902; RRID:AB_493416
NSR1	Abcam	Cat# ab4642; RRID:AB_304550
Nucleolin	Santa Cruz Biotechnology	Cat# sc-17826; RRID:AB_670270
Secondary horseradish peroxidase-conjugated	Sigma	Cat#GENA934; RRID:AB_2722659
Chemicals, peptides, and recombinant proteins		
500× Protease inhibitor cocktail (mammalian)	Sigma	Cat#P8340
500× Protease inhibitor cocktail (bacterial)	Sigma	Cat#P8465
Albumin	Sigma	Cat#A2153
Phenol	Sigma	Cat#P4682
Chloroform	Sigma	Cat#C2432
Glass beads	Sigma	Cat#G8772
Acrylamide	National Diagnostic	Cat#EC-849
Dulbecco's Modified Eagle Medium (DMEM)	Gibco	Cat#21969035
Fetal Bovine Serum (FBS)	Gibco	Cat#10500064
GlutaMAX™	Gibco	Cat#35050087
NuPAGE 4–12% bis-tris gels	Invitrogen	Cat#NP0321
NuPAGE LDS Sample Buffer (4X)	Invitrogen	Cat#NP0008
$polyP_{100}$	RegeneTiss Co.	N/A
PVDF membrane	Bio-Rad	Cat#1620177
Synthetic Defined Media	Formedium	Cat#CSM0210
CSM-Leu	Formedium	Cat#DCS0091
YPD broth	Formedium	Cat#CCM0210
Critical commercial assays		
Clarity Max™	Bio-Rad	Cat#1705062
DC Protein Assay	Bio-Rad	Cat#5000116
Experimental models: Cell lines		
Human embryonic kidney (HEK) 293	ATCC	Cat#CRL-1573; RRID:CVCL_0045
Yeast strains		
ppx1Δppn1Δppn2Δ	BY4742 *ppx1Δ::NATNT2 ppn1Δ::KANMX ppn2Δ::URA3*	Azevedo et al.[9]
ppx1Δppn1Δppn2Δvtc4Δ	BY4742 *ppx1Δ::NATNT2 ppn1Δ::KANMX ppn2Δ::URA3 vtc4Δ::LEU2*	This study
Oligonucleotides		
VTC4_KO_forward	GCTAACAATCAAATCGGCCAATAAA AGAGCATAACAAGGCAGGAACAGCT cagctgaagcttcgtacgc	
VTC4_KO_reverse	CTAATATGATTATTACTTAATTATA CAGTAAAAAAAACACGCTGTGTAT gcataggccactagtggatctg	
VTC4_5_forward	GCCTCTCTGCTTTCCACG	
VTC4_internal_reverse	CCAGCAGATGAGTCTCCC	
Deposited data		
https://doi.org/10.17632/vczpgbtmdp.1		

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RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to the lead contact, Adolfo Saiardi (a.saiardi@ucl.ac.uk).

Materials availability

All reagents generated in this study are available from the lead contact.

Data and code availability

- Any raw files or information required to reanalyse the data reported in this paper is available to download (https://doi.org/10.17632/vczpgbtmdp.1) or from the lead contact upon request.
- This paper does not report any original code.
- Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mammalian cell culture

Human embryonic kidney (HEK) 293 cells were grown and maintained in DMEM (Gibco, #21969035) supplemented with 10% fetal bovine serum (Gibco, #10500064) and 1% GlutaMAX™ (Gibco, #35050087), in a humidified cell culture incubator, under a 5% CO_2 atmosphere, at 37°C.

Yeast strains and culture

VTC4 was deleted from the triple mutant *ppx1Δppn1Δppn2Δ* strain[9] by standard yeast homologous recombination. Briefly, *vtc4Δ::LEU2* knockout fragment was amplified by PCR using plasmid pUG73[19] as template and primers *VTC4*_KO_forward, and *VTC4*_KO_reverse. *VTC4* deletion was verified by PCR using primers *VTC4*_5_forward and *VTC4*_internal_reverse and phenotypically by analysing polyP level. The *vtc4Δ::LEU2* knockout was initially selected on synthetic defined media (Formedium, #CSM0210) supplemented with CSM-Leu (Formedium #DCS0091). Yeasts were grown and maintained in standard YPD broth (Formedium, #CCM0210).

METHOD DETAILS

Protein extraction for mammalian cells

HEK293 cells from a 10 cm 80% confluent plate were washed twice with PBS, scraped, and then centrifuged for 1 minute at 2,000 × g. The PBS was removed, and the cell pellet was resuspended in 100 μl of lysis buffer (50 mM Tris-HCl, pH 8.0; 5 mM DTT; 0.5% Triton X-100) with two different NaCl concentrations: 150 mM (LB1) and 500 mM (LB2), along with 100× phosphatase inhibitor (200 mM imidazole, 100 mM sodium fluoride, 115 mM sodium molybdate, 100 mM sodium orthovanadate) and 500× protease inhibitor cocktail for mammalian cells (Sigma, #P8340). The samples were rotated for 5 minutes at 4°C and then centrifuged for 5 minutes at 15,000 × g. The supernatant was collected in a new tube for further assays. The sample LB1* was created by collecting the supernatant from LB1 and adjusting the NaCl concentration to 500 mM using a 5 M NaCl solution.

Protein extraction for yeast

Protein extraction from yeast was performed in accordance with the protocol proposed by Azevedo et al.[9] with some modifications. Yeast cells grown overnight in YPD were diluted to an OD_{600} of 0.8 and then grown for 90 minutes at 30°C in 10 ml of fresh media. The cells were pelleted through centrifugation at 2,000 × g for 2 minutes and washed once in ice-cold MilliQ water. Subsequently, proteins were extracted using the same lysis buffers as described for mammalian cells. The cells were vortexed in the presence of glass beads for 1 minute, and the homogenates were centrifuged at 15,000 × g for 10 minutes at 4°C. The supernatants were immediately boiled in 4× NuPAGE LDS sample buffer (containing 10% β-mercaptoethanol) for 5 minutes and then loaded onto the gel.

Shift-up assay

Protein concentrations were measured using the DC Protein Assay (Bio-Rad, #5000112). A portion of the supernatant collected from mammalian cells (20 μg) and yeast *ppx1Δppn1Δppn2Δvtc4Δ* lysate (15 μl) was transferred to a new tube, and 20 mM of $polyP_{100}$ concentration expressed as phosphate monomer was added. The samples were then incubated for 20 minutes at 30°C. After this incubation period, the samples, along with untreated controls, were boiled in 4× NuPAGE LDS sample buffer (containing 10% β-mercaptoethanol) for 5 minutes before being loaded onto the gel.

Western blotting

Equal amounts of protein extracts (20 μg) were resolved using NuPAGE 4–12% bis-tris gels (Invitrogen, #NP0321) and proteins were transferred to PVDF membranes (Bio-Rad, #1620177). Membranes were blocked for 1 hour using 5% non-fat milk blocking solution (100 nM NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% Tween-20) and then blotted overnight for the following primary antibodies at 1:1000 in 1% albumin (Sigma, #A2153): nucleolin (Santa Cruz Biotechnology, #sc-17826), Nsr1 (Abcam, #ab4642) and α-tubulin (Biolegend, #625902). Secondary horseradish peroxidase-conjugated antibody (Sigma, #GENA934) was diluted in 3% albumin. Immunocomplexes were detected using a luminol peroxidase chemiluminescence kit (Clarity Max™, Bio-Rad, #1705062) and acquired using the Alliance Q9, UVITEC imaging system.

Polyphosphate extraction and PAGE analysis

To the yeast protein extracts (100 μl) were added 100 μl of 2×LETS buffer (0.2 M LiCl, 20 mM EDTA, 20 mM Tris-HCl, pH 7.4 and 0.4% SDS), 200 μl of acidic phenol pH 4.3 (Sigma, #P4682), and 200 μl of glass beads (Sigma, #G8772). Extracts were vortexed for 5 minutes at 4°C and spun at 15,000 × g for 5 minutes. The water layer was transferred to a fresh tube and extracted with 200 μl of chloroform (Sigma, #C2432). After centrifugating at 15,000 × g for 5 minutes, the water layer was transferred to a fresh tube and precipitated with 2.5 volume of ethanol overnight at -20°C. Extracts were spun at 17,000 × g for 15 minutes at 4°C, supernatant aspirated and samples air-dried before being resuspended in 100 μl of sterile water. Quantification of RNA in polyP extract was determined by reading absorbance at 260 nm in the nanodrop. PolyP was analysed on a 25% polyacrylamide (National diagnostic, #EC-849) gel according to previously described protocol.[20] After electrophoresis, the gel was stained with toluidine blue staining solution as described[20] and an Epson photo scanner was used to record the result.

QUANTIFICATION AND STATISTICAL ANALYSIS

This article does not report any quantification or statistical analysis.