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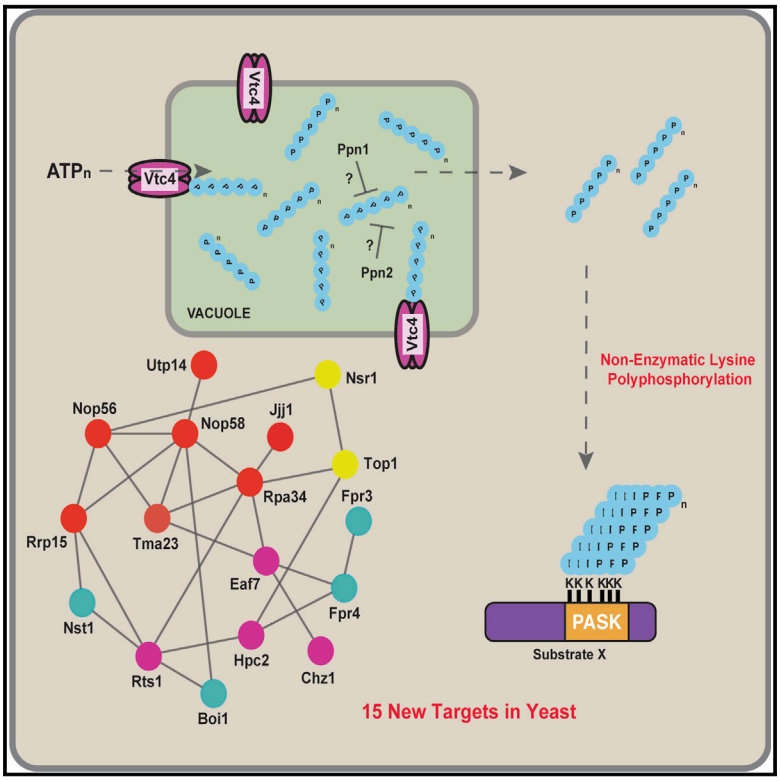
Graphical Abstract



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In Brief

Bentley-DeSousa et al. screen yeast for proteins that undergo covalent modification by polyphosphate. They describe 15 substrates enriched for functions related to ribosome biogenesis. Homologs of these and other human proteins containing certain motifs can be "polyphosphorylated" using an ectopic expression system, providing a method to explore polyphosphorylation beyond yeast.

Highlights

- A strategy for identifying targets of lysine polyphosphorylation
- 15 yeast targets include a conserved network involved in ribosome biogenesis
- Defects in polyP synthesis result in phenotypes related to translation
- Demonstration of polyphosphorylation on 6 PASK-containing human proteins

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A Screen for Candidate Targets of Lysine Polyphosphorylation Uncovers a Conserved Network Implicated in Ribosome Biogenesis

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SUMMARY

Polyphosphates (polyP) are chains of inorganic phosphates found in all cells. Previous work has implicated these chains in diverse functions, but the mechanism of action is unclear. A recent study reports that polyP can be non-enzymatically and covalently attached to lysine residues on yeast proteins Nsr1 and Top1. One question emerging from this work is whether so-called "polyphosphorylation" is unique to these proteins or instead functions as a global regulator akin to other lysine post-translational modifications. Here, we present the results of a screen for polyphosphorylated proteins in yeast. We uncovered 15 targets including a conserved network of proteins functioning in ribosome biogenesis. Multiple genes contribute to polyphosphorylation of targets by regulating polyP synthesis, and disruption of this synthesis results in translation defects as measured by polysome profiling. Finally, we identify 6 human proteins that can be modified by polyP, highlighting the therapeutic potential of manipulating polyphosphorylation *in vivo*.

INTRODUCTION

Polyphosphates (polyP) are chains of inorganic phosphates, ranging from three to thousands of moieties in length. PolyP is considered to be an 'ancient' molecule and is found in diverse cell types from bacteria to human cells. (Kornberg et al., 1999; Moreno and Docampo, 2013; Rao et al., 2009). All known synthesis of polyP chains occurs through the sequential generation of phosphoanhydride bonds by polyP polymerases, which add gamma-phosphates from ATP or other nucleoside triphosphates to growing chains. In bacteria, this is accomplished via the action of polyP kinases PPK1 or PPK2 (Akiyama et al., 1993; Tzeng and Kornberg, 2000; Zhang et al., 2002). In the budding yeast *S. cerevisiae*, polyP synthesis occurs via the action of Vtc4, which complexes to Vtc1 and either Vtc2 or Vtc3, to form the V-ATPase transporter chaperone (VTC) complex (Hothorn et al., 2009; Morrissey et al., 2012). Most VTC complexes are vacuolar bound, and the synthesis of polyP is coupled to its translocation into the vacuole (Gerasimaitė et al., 2014; Hothorn et al., 2009). Although the vast majority of polyP is constrained in the vacuole, significant levels have been detected in other cellular compartments. However, the estimated size of these non-vacuolar polyP pools varies considerably from study to study (Azevedo et al., 2015; Gerasimaitė et al., 2014; Lichko et al., 2006; Rao et al., 2009; Urech et al., 1978).

In yeast, the hydrolysis of polyP chains by exopolyphosphatase Ppx1, and endopolyphosphatases Ppn1 and Ppn2, results in the generation of free inorganic phosphates or chains of smaller length. Ppx1 is thought to be mostly cytoplasmic (Wurst et al., 1995), whereas Ppn1 and Ppn2 localize predominately to the vacuole (Gerasimaitė and Mayer, 2017; Kumble and Kornberg, 1996). In both bacteria and yeast, polyP has been proposed to function as an energy store, a phosphate reservoir, and a metal chelator (Moreno and Docampo, 2013). Phenotypically, bacteria lacking polyP show defects in biofilm formation, motility, and other processes important for infection (Rashid et al., 2000a, 2000b; Shi et al., 2004). In yeast, deletion of *VTC4* results in microautophagy defects (Uttenweiler et al., 2007) and reverses stress hypersensitivity of cells mutated for the cyclin-encoding gene *PHO80* (Huang et al., 2002).

In contrast to lower eukaryotes, mammalian polyP synthesis and degradation machineries are uncharacterized (Hooley et al., 2008). One exception is h-prune, originally characterized as a regulator of cell migration, which also acts as a short-chain exopolyphosphatase *in vitro* (Carotenuto et al., 2014; Tammenkoski et al., 2008). Whether h-prune acts on polyP chains *in vivo*

is unknown. Hence, many studies using cell lines have relied on the ectopic expression of yeast Ppx1 or the addition of synthetic polyP to culture media to alter polyP levels *in vivo* (Rao et al., 2009). In mammals, polyP chains have been implicated as regulators of the blood coagulation cascade (Smith et al., 2006, 2010; Yeon et al., 2017), neuroprotection (Cremers et al., 2016), and cell growth (Jimenez-Nuñez et al., 2012; Rao et al., 2009; Shiba et al., 2003; Wang et al., 2003). Given its diverse roles across multiple types of cells, modulation of polyP levels is suggested to have therapeutic potential (Labberton et al., 2016; Moreno and Docampo, 2013). However, the mechanisms by which polyP chains exert their molecular functions are poorly understood.

Azevedo et al. reported that polyP chains can be covalently attached to lysine residues on yeast proteins Nsr1 and Top1 as a non-enzymatic post-translational modification (PTM) (Azevedo et al., 2015). Polyphosphorylation of these proteins confers a dramatic decrease in their mobility on NuPAGE gels (Azevedo et al., 2015). The resulting electrophoretic "shift" is observed for Nsr1 and Top1 purified from wild-type cells but is absent when these proteins are purified from *vtc4*Δ mutants, which lack polyP. Modified lysine residues for both Nsr1 and Top1 proteins were mapped to poly-acidic, serine, and lysine-rich (PASK) motifs of several dozen amino acids in length (Azevedo et al., 2015). Polyphosphorylation impacts binding of Nsr1 to Top1, the *in vitro* topoisomerase activity of Top1, and the subcellular localization of both proteins (Azevedo et al., 2015). Whether additional targets of lysine polyphosphorylation exist and whether this new mechanism of protein modification is conserved in other systems are unknown.

Here, we describe a screen for lysine polyphosphorylation targets in yeast. We identified 15 additional targets, including an evolutionarily conserved network involved in ribosome biogenesis. Based on these results, we uncovered an unreported role for the Vtc4 polyP polymerase in promoting ribosome function. We also report that lysine polyphosphorylation is controlled by proteins involved in vacuolar biology and that bypassing this control mechanism by synthesizing polyP directly in the cytoplasm allows for target modification but renders cells sensitive to drugs that impact protein translation and cell growth. Finally, we show that human cells can be engineered to produce polyP chains that modify human PASK-containing proteins. Our work recasts polyphosphorylation of lysine as a global PTM with the potential to regulate diverse cellular processes.

RESULTS

Additional Targets of Lysine Polyphosphorylation in Yeast

To search for polyphosphorylated proteins in yeast, we generated a list of proteins that contain PASK-like sequences similar to those found in the two known targets, Nsr1 and Top1. PASK motifs in these proteins contain stretches of glutamic acid (E) and aspartic acid (D) interspersed with serine (S) and lysine (K) residues (Azevedo et al., 2015). Notably, the serine residues of Nsr1 and Top1 PASK sequences are not required for their modification with polyP (Azevedo et al., 2015). Nevertheless, we chose to retain serine as a PASK feature for our analyses, since it may be important for regulation under specific circumstances. We identified 427 proteins (out of ~6,000 total proteins in yeast) with one or more 20-amino-acid stretches made up of at least 75% D/E/S with at least one K (Figure 1A; Table S1). Gene ontology (GO)-term analysis of this set of proteins using DAVID (Sherman et al., 2007) indicated enrichment in functions related to ribosome biogenesis and rRNA processing (Figure 1B). Consistent with this, we also observed enrichment for nuclear and nucleolar subcellular localizations (Figure 1C).

We chose 90 of these PASK-containing proteins for analysis based on the length and number of PASK motifs (Table S1), with priority given to proteins with long or multiple PASK motifs. Our goal was to use NuPAGE analysis to measure the electrophoretic shift of each candidate in the presence and absence of *VTC4*, as was previously done for Nsr1 and Top1 (Azevedo et al., 2015). To do this, we took advantage of the yeast GFP-tagged collection wherein each open reading frame is expressed from its genomic locus as a fusion with GFP (Huh et al., 2003). Starting with strains pulled from this set, we used high-throughput yeast mating and selection techniques to generate isogenic *VTC4* and *vtc4*Δ strains expressing our 90 prospective targets as GFP fusions (Figure 1D, also see Experimental Procedures). For each GFP-candidate fusion, paired protein extracts from wild-type and *vtc4*Δ mutants were analyzed using NuPAGE followed by western blotting and detection of tagged candidates with an antibody against GFP (Figure 1D). Of 90 candidates, 15 fusion proteins displayed *VTC4*-dependent electrophoretic shifts characteristic of lysine polyphosphorylation (Figures 2A, 2B, and S1A–S1C). Not all GFP-fusions displayed altered mobility in *vtc4*Δ strains compared to wild-type controls, demonstrating that the GFP tag was not being polyphosphorylated (Figure S1C).

Many of the polyphosphorylated targets that we identified have functions related to ribosome biogenesis (Figure 2A). For example, Nop56, Nop58, and Utp14 are members of the small subunit processome (SSU) and are required for 18S rRNA processing and modification (Gautier et al., 1997; Lafontaine and Tollervey, 2000; Oruganti et al., 2007; Zhu et al., 2016). Rrp15 is a constituent of pre-60S ribosomal particles and is required for the processing of 27S rRNA (De Marchis et al., 2005). Rpa34 functions upstream of ribosomal assembly as a regulatory subunit of RNA pol I, which is required for transcription of 37S pre-rRNA from rDNA repeats (Werner et al., 2009). Other targets uncovered in our screen do not function directly in ribosome biogenesis but are part of a larger network connected by both genetic and physical interactions that also includes the previously identified targets Nsr1 and Top1 (Figure 2C). For example, Rts1 is a regulatory subunit of the PP2A subcomplex that has been linked to the control of cell size (Artiles et al., 2009). Targets Fpr3 and Fpr4 are prolyl isomerases and FK506 binding proteins involved in nucleosome assembly and transcriptional regulation (Benton et al., 1994; Park et al., 2014). Other chromatin regulators recovered in our screen include Eaf7, a member of the NuA4 histone-acetyltransferase complex (Mitchell et al., 2013; Rossetto et al., 2014); Chz1, a chaperone for the Htz1 histone variant (Luk et al., 2007); and Hpc2, a member of the HIR complex that contributes to nucleosome remodeling by recruiting the SWI/SNF complex to DNA (Balaji et al., 2009). Many of these targets have one or more human homologs (Figure 2A), which will

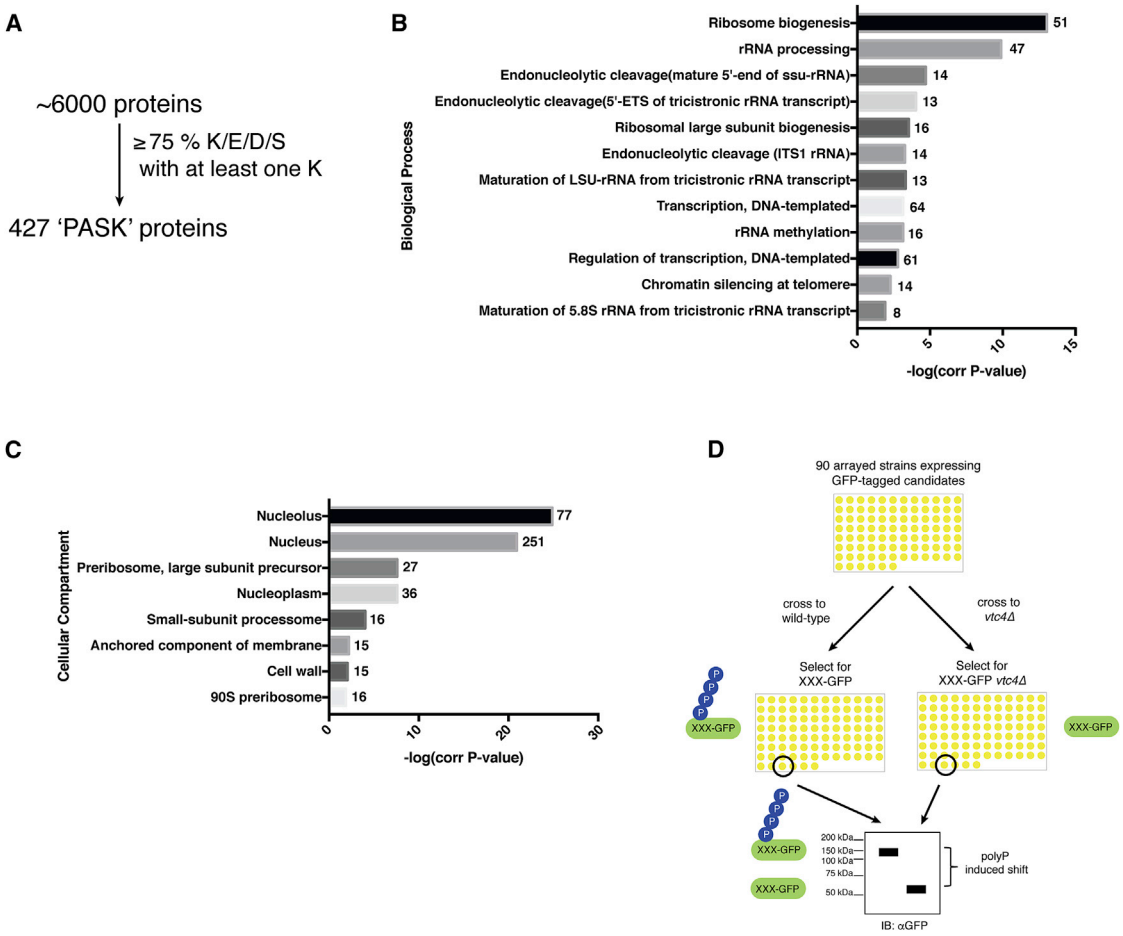

Figure 1. Analysis of PASK-Containing Proteins in Yeast

(A) Parameters used to identify PASK-like motifs in *S. cerevisiae* proteins.

(B) PASK-containing proteins function in ribosome biology. The number of proteins in each category is shown.

(C) PASK-containing proteins are enriched for nucleolar localization. The number of proteins in each category is shown. Bioinformatics analyses were carried out using DAVID with the default parameters.

(D) A screen for polyP targets. See Experimental Procedures for details. Also see Table S1.

be discussed later. Our ability to identify 15 targets from a prioritized pool of just 90 proteins (Table S1) suggests that polyphosphorylation is likely to be a widespread PTM.

Molecular Characterization of Polyphosphorylation

In support of non-enzymatic polyphosphorylation being responsible for the shifts observed in our experiments, we were able to largely restore shifts to targets Rts1 and Fpr3 with the addition of synthetic polyP to boiled *vtc4*Δ extracts (Figures 2D and 2E). In these experiments, Rts1 and Fpr3 are detected with antibodies directed against native proteins. Our inability to completely restore shifts may be due to differences in chain length between polyP attached *in vivo* versus the 75-unit synthetic polyP used in our experiments. Indeed, a similar experiment using synthetic polyP of different lengths showed that electrophoretic shift is chain-length dependent for these targets (Figures S1D and S1E). To gain further support for polyP being the agent responsible for electrophoretic shifts, we carried out *in vitro* polyphosphorylation assays with bacterially purified GST-Rts1(amino acids 1–150), which contains the 2 predicted PASK sequences for Rts1. GST-Rts1(1–150), but not GST alone, shifted on NuPAGE gels following incubation with a range of synthetic polyP concentrations (Figure 2F). In support of the idea that polyP was directly responsible for the change in electrophoretic mobility, synthetic biotinylated polyP was observed to co-migrate with the purified GST-Rts1(1–150) (Figure 2G). Finally, the GST-Rts1(1–150) shift was retained when excess polyP was washed away following capture of the fusion protein on glutathione beads (Figure S1F). We conclude that polyP can directly modify Rts1 and suggest that this is also true for our other targets.

Polyphosphorylation of Targets Requires PASK Motifs

In order to test the contribution of the PASK motifs to target polyphosphorylation, we focused on 5 targets with C-terminal PASK motifs (Nop56, Nop58, Tma23, Rpa34, and Chz1) that could be

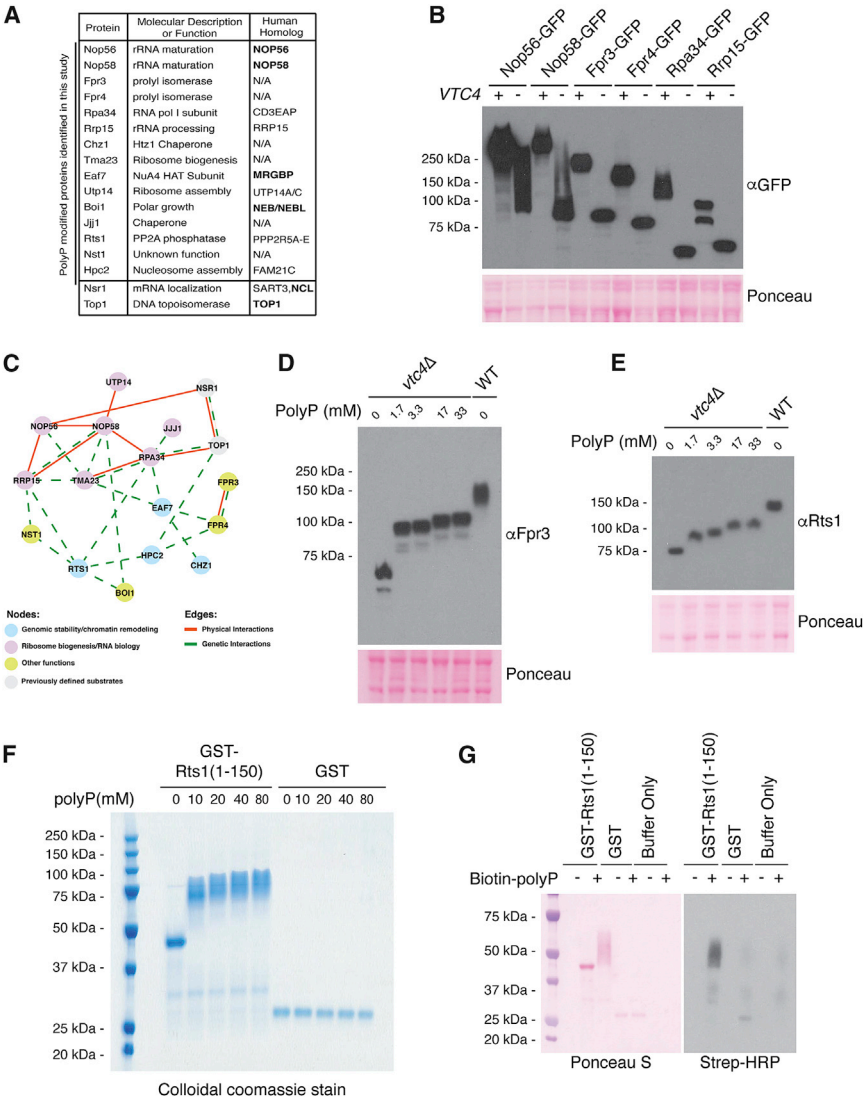

Figure 2. Identification of Polyphosphorylated Proteins

(A) 15 additional polyphosphorylated proteins were uncovered. Indicated in this table are known polyphosphorylated proteins in yeast. For human homologs, bolded text indicates conservation of PASK-like motifs. See Experimental Procedures.

(B) Polyphosphorylation causes electrophoretic shifts on NuPAGE gels. Extracts from *VTC4* or *vtc4*Δ strains expressing the indicated GFP-fusion proteins were run on NuPAGE gels and transferred to PVDF membrane. Proteins were detected using an antibody against the GFP epitope.

(C) PolyP targets are physically and genetically connected. Interactions were determined using the Genemania tool (http://genemania.org/) (Zuberi et al., 2013).

(D and E) Modification of targets with synthetic polyP. Extracts from the indicated strains were incubated with increasing concentrations of synthetic polyP with a modal length of 75 residues. Fpr3 (D) or Rts1 (E) were detected with antibodies against native proteins following NuPAGE analysis and western blotting.

(F) *In vitro* polyphosphorylation of a bacterial-purified fragment of Rts1 fused to GST.

(G) GST-Rts1(1–150), GST, or buffer was incubated with biotinylated polyP. Biotinylated polyP was detected with Strep-horseradish peroxidase (HRP) after NuPAGE and western blotting.

Also see Figure S1.

easily deleted using homologous recombination in yeast. Deletion of PASK motifs from Nop56, Nop58, Tma23, and Rpa34 resulted in collapse of the *VTC4*-dependent shift observed with NuPAGE analysis (Figures 3A–3C). These data are consistent with the PASK motif being the site of modification for these four proteins. For Rpa34, we found that mutation of PASK lysine residues to arginines largely prevented electrophoretic mobility shifts observed in wild-type strains (Figures 3D and 3E) while leaving Rts1 polyphosphorylation unaffected (Figure S2A). Moreover, a synthetic construct wherein Rpa34 lacking its PASK motif was fused to the PASK from Nop56 retained wild-type levels of polyphosphorylation, confirming that PASK motifs are portable (Figure 3F). In contrast to other deletion mutants analyzed, the net change in electrophoretic mobility for Chz1-GFP (wild-type versus *vtc4*Δ) was not significantly impacted in the absence of its C-terminal PASK when analyzed via NuPAGE (Figures S2B and S2C). Notably, Chz1 has a second PASK motif in its N terminus that could serve as the site of modification (Figure S2B). To test this idea, we expressed a series of 3HA-tagged Chz1 constructs from the GAL promoter in wild-type and *vtc4*Δ cells (Figure S2D). Consistent with our observations with Chz1-GFP, NuPAGE analysis revealed electrophoretic shifts for 3HA-Chz1 in wild-type cells, and this shift was maintained in constructs lacking the C-terminal PASK (Figure S2E). In contrast 3HA-Chz1 lacking the N-terminal PASK motif or both PASK motifs migrated identically in extracts prepared from both wild-type and *vtc4*Δ strains (Figure S2E). Altogether, these experiments indicate that Chz1 is polyphosphorylated in its N terminus.

In our screen and follow-up experiments, we observed that the size of the polyP-induced shift varied dramatically depending on the target in question. The apparent molecular weight of Nop58-GFP from wild-type cells was almost 150 kDa greater than that of Nop58-GFP from *vtc4*Δ cells (Figure 2B). In contrast, the shifts measured for proteins Chz1 and Hpc2 were consistently smaller (Figures S1A and S1C). We analyzed target PASK motifs and found that the shift size for each target correlated strongly with the number of lysine residues present within these regions (Figure 3G). In contrast, we observed no correlation with the serine or acidic amino acid content (Figures S3A and S3B). Notably, an electrophoretic shift associated with canonical serine/threonine phosphorylation was not at all impacted by *vtc4*Δ mutation (Figure S4A). These data are consistent with lysine, rather than

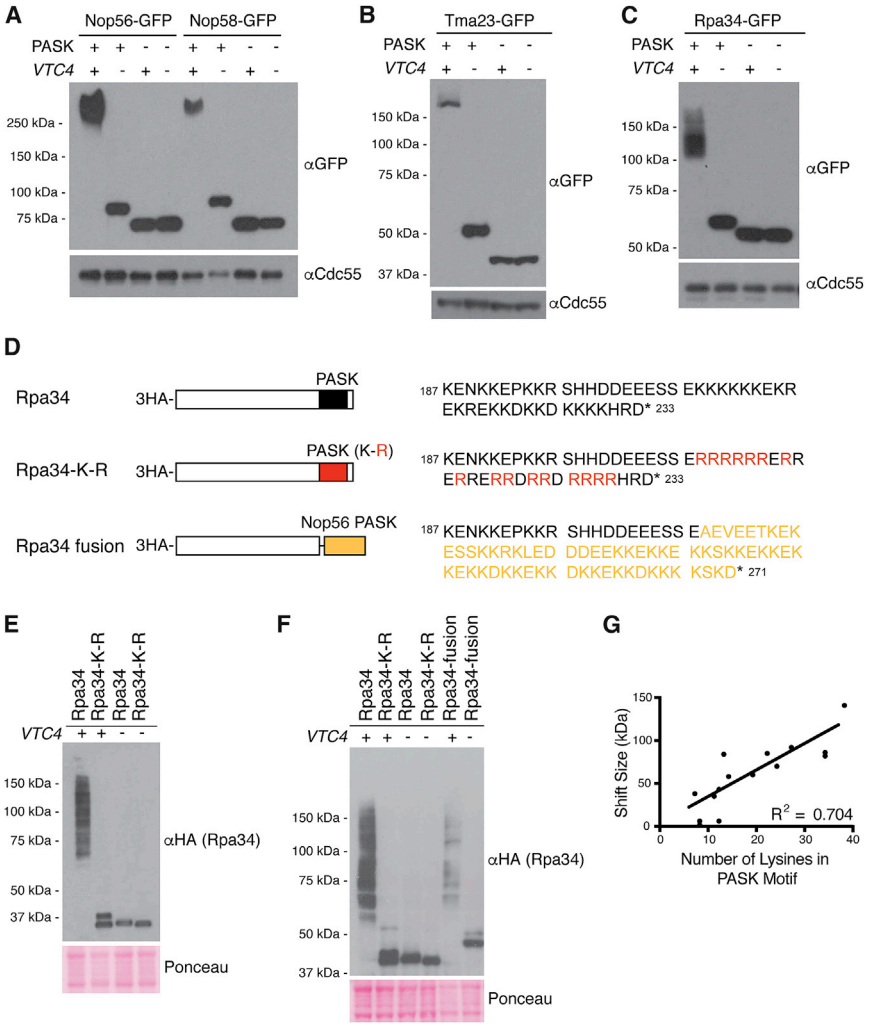

Figure 3. Molecular Characterization of Polyphosphorylation

(A–C) PASK motifs are required for target modification. GFP fusions with Nop56 and Nop58 (A), Tma23 (B), or Rpa34 (C) were detected with an antibody against GFP following NuPAGE and western blotting.

(D–F) Rpa34 constructs shown in (D) were analyzed via NuPAGE (E and F).

(G) Shift size in NuPAGE analysis correlates with lysine content of target PASK motifs.

Also see Figures S2–S4.

serine, being the site of polyP attachment. Presumably, the size of the shift is partially dependent on the number of occupied sites, with greater occupancy yielding a greater change in apparent molecular weight.

Throughout this study, we observed that in contrast to NuPAGE gels, polyP-induced shifts are not resolved on SDS-PAGE gels (Figure S4B). According to the manufacturer, NuPAGE gels, like other Bis-Tris systems, are polymerized in the absence of tetramethylethylenediamine (TEMED). To test whether the absence of TEMED was critical for resolving polyP-induced shifts, we made our own Bis-Tris gels with or without TEMED (see Experimental Procedures). We found that addition of TEMED to Bis-Tris gels largely collapsed the shift associated with polyphosphorylated GST-Rts1(1–150) (Figure S4C). While the mechanism behind this collapse is unclear, investigation of interaction between TEMED and polyP may provide insight into the stability of polyphosphorylated lysine.

Regulation of Polyphosphorylation by polyP Synthesis

PTMs play important roles in cell signaling because they are often regulated at the step of their attachment to protein targets by specific enzymes. In contrast, lysine polyphosphorylation is non-enzymatic (Azevedo et al., 2015). Therefore, we focused on the initial synthesis of polyP chains and the targeted action of polyphosphatase enzymes as mechanisms of regulation. First, Freimoser et al. identified 254 genes besides *VTC4* that contribute to the synthesis or maintenance of polyP pools in yeast (Freimoser et al., 2006). We analyzed target polyphosphorylation in 4 of these mutants that contain polyP levels 10-fold lower than wild-type cells. Deletion of *VTC1*, *VMA22*, and *VAM3* resulted in a loss of Rts1 and Nsr1 polyphosphorylation comparable to that observed in *vtc4*Δ cells (Figures 4A and S5A). A subtle but reproducible defect in the polyphosphorylation of both proteins was observed in *yol019W*Δ cells (Figures 4A and S5A). All of these genes are linked to vacuolar biology. *VTC1* encodes a member of the VTC complex required for polyP synthesis (Hothorn et al., 2009). *VMA22* and *VAM3* encode proteins involved in vacuolar protein maturation or localization (Graham et al., 1998; Srivastava and Jones, 1998). Finally, although its molecular function is unknown, *YOL019W* encodes a predicted transmembrane protein that localizes to the vacuole and outer cell membrane (Cherry et al., 2012). These data indicate a particularly important role for the vacuole in regulating polyphosphorylation through polyP synthesis and/or storage. Phenotypes shared among these and other mutants with low polyP levels may stem in part from changes to lysine polyphosphorylation.

Although polyP is found throughout the cell, including the nucleus (Azevedo et al., 2015), the majority is retained in the vacuole following its synthesis by the VTC complex (Gerasimaitė et al., 2014). How polyP is transported to other compartments is unknown. To determine whether the mechanism of polyP synthesis impacts target modification, we took advantage of a previously described system that allows for *E. coli* PPK1 (*Ec*PPK1) expression in yeast (Figure 4B) (Gerasimaitė et al., 2014). In contrast to yeast Vtc4, *Ec*PPK1 synthesizes polyP independently of vacuolar translocation and storage (Gerasimaitė

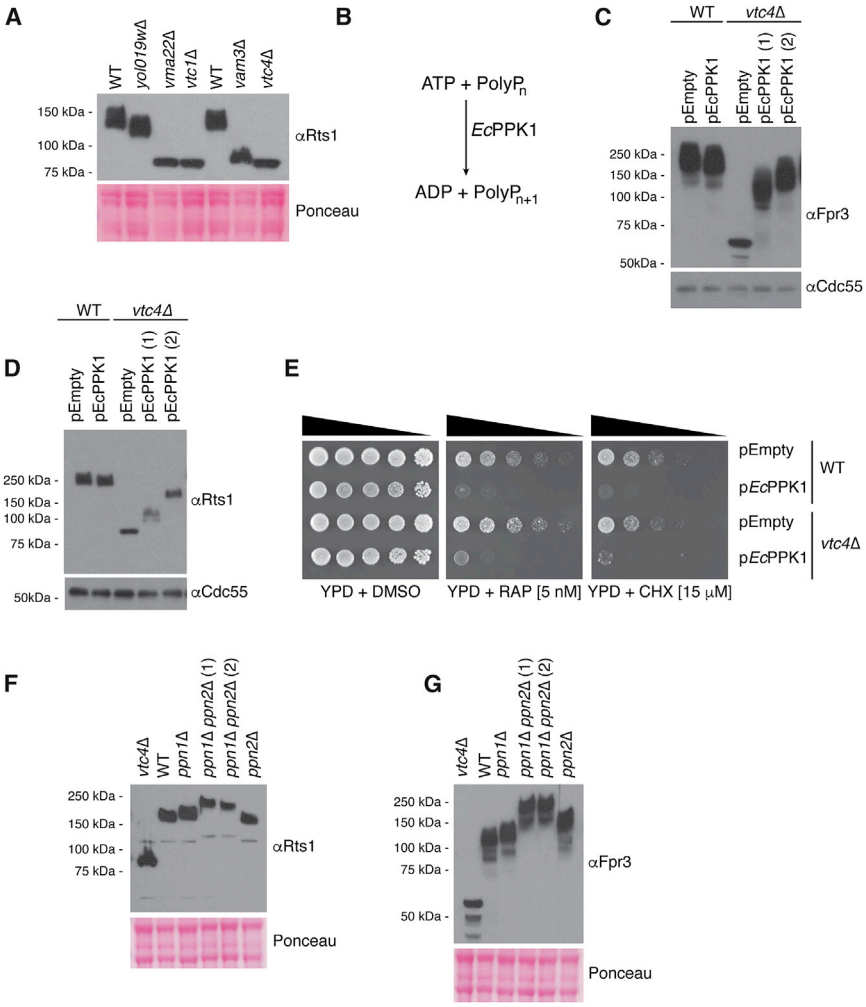

Figure 4. Regulation of Polyphosphorylation

(A) Polyphosphorylation of Rts1 is impacted by multiple genes.

(B) *Ec*PPK1 functions analogously to Vtc4 but synthesizes polyP independently of the vacuole.

(C and D) Expression of *Ec*PPK1 rescues polyphosphorylation defects of Fpr3 (C) and Rts1 (D) in *vtc4*Δ mutants. Two isolates of the *Ec*PPK1 expression in *vtc4*Δ mutants are shown.

(E) *Ec*PPK1 causes hypersensitivity to rapamycin and cycloheximide. Indicated strains were spotted in a 5-fold dilution series.

(F and G) Increased shift of targets Rts1 (F) and Fpr3 (G) in *ppn1*Δ *ppn2*Δ double mutants.

Also see Figure S5.

et al., 2014). *Ec*PPK1 expression in a *vtc4*Δ background was able to restore lysine polyphosphorylation of multiple targets (Figures 4C and 4D). These data suggest that vacuolar-coupled synthesis of polyP is not necessary for target modification. The presence of polyP, not its mechanism of synthesis, is the critical factor in target modification.

Expression of *Ec*PPK1 in yeast was previously found to result in slow growth that could be mitigated by the expression of the cytoplasmic Ppx1 polyphosphatase (Gerasimaitė et al., 2014). In an extension of this observation, we found that *Ec*PPK1-expressing cells were sensitive to cycloheximide, which directly inhibits protein translation, and rapamycin, an inhibitor of the TORC1 nutrient-sensing kinase (Figure 4E). These phenotypes held true in both wild-type and *vtc4*Δ backgrounds. In contrast, *vtc4*Δ alone did not impact sensitivity to these drugs in either spot tests (Figure 4E) or halo assays (Figures S5B and S5C). Thus, while vacuole-coupled synthesis of polyP is not required for target modification, constraining polyP in the vacuole is still important for resistance to drugs that impact important aspects of cell growth. In the Discussion, we elaborate on a previous model suggesting that *Ec*PPK1 toxicity in yeast stems from unwanted accumulation of polyP in the cytoplasm (Gerasimaitė et al., 2014).

Polyphosphatase-Dependent Regulation of Polyphosphorylation

Azevedo et al. demonstrated that the exopolyphosphatase Ppx1 and endopolyphosphatase Ddp1 are able to degrade polyP chains attached to target proteins *in vitro* (Azevedo et al., 2015). However, Ppx1 is largely cytoplasmic (Wurst et al., 1995), while most of our targets are nuclear. In order to test the function of Ppx1 more rigorously, we compared target polyphosphorylation in strains where the only copy of *PPX1* was placed under an inducible *GAL1/10* promoter. In this experimental setup, Ppx1 is not detectable when cells are grown in raffinose but is overexpressed in the presence of galactose. While target polyphosphorylation decreased with culture time in some experiments (see Discussion), no difference was observed in the presence or absence of Ppx1 overexpression (Figure S5D). These data suggest that Ppx1 may not directly reverse polyphosphorylation on target proteins. Similarly, we did not detect consistent changes in target polyphosphorylation in single deletions of polyphosphatases *PPX1* or *DDP1*, or vacuolar endopolyphosphatases *PPN1* or *PPN2*. In contrast, we observed a consistent increase in electrophoretic shifts for Rts1 and Fpr3 when NuPAGE was used to analyze protein extracts from *ppn1*Δ *ppn2*Δ double mutants (Figures 4F and 4G). Interestingly, *ppn1*Δ *ppn2*Δ mutants were previously shown to accumulate longer polyP chains in all phases growth, without an overall change to polyP levels (Gerasimaitė and Mayer, 2017). We favor a model wherein overall polyphosphorylation of targets under normal growth conditions is dictated by polyP availability rather than removal of chains by polyphosphatases. However, since Ppx1 and Ddp1 can act on polyphosphorylated targets *in vitro* (Azevedo et al., 2015), we cannot completely rule out that these enzymes have a greater impact *in vivo* under certain conditions.

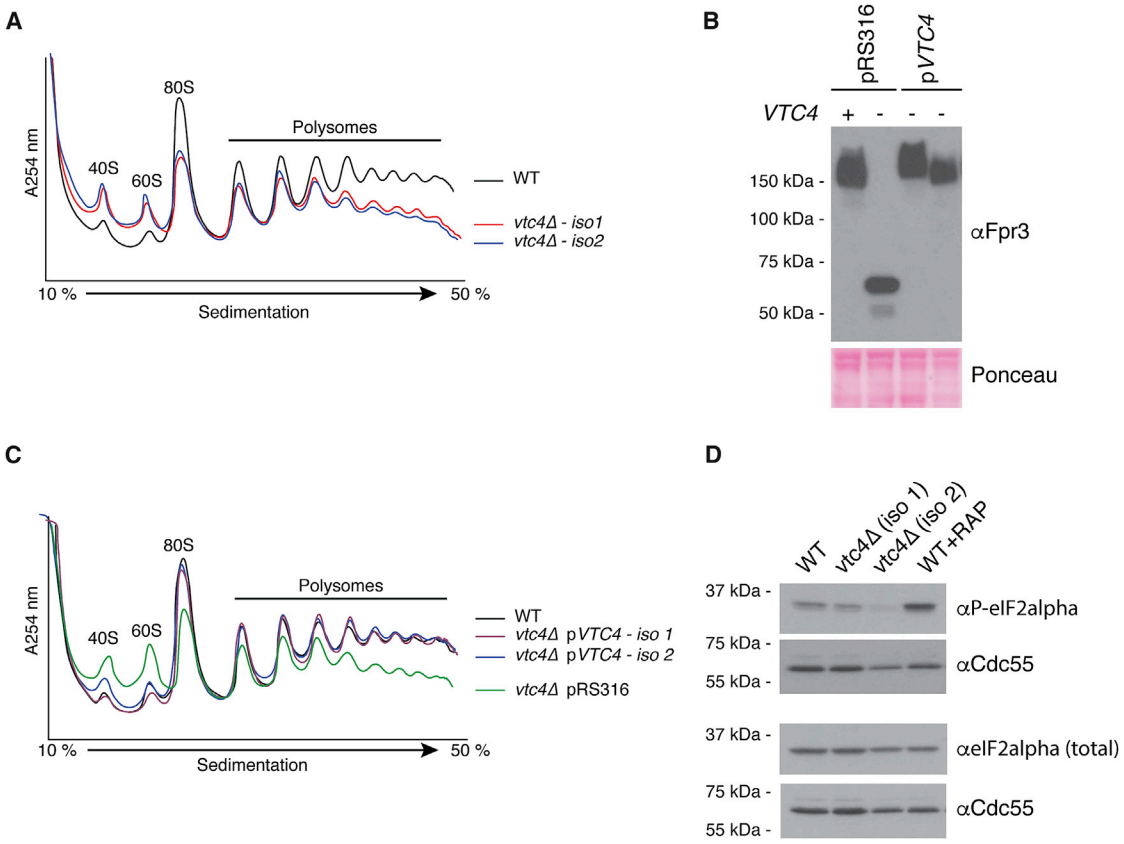

Figure 5. Vtc4 Impacts Ribosome Function

(A) Polysome profiles of cycloheximide-treated wild-type and *vtc4*Δ mutant strains (see Experimental Procedures for details).

(B and C) p*VTC4* complements polyphosphorylation (B) and polysome defects (C) in a *vtc4*Δ mutant strain. Two isolates of the *VTC4* rescue are shown.

(D) eIF2alpha is not hyperphosphorylated in *vtc4*Δ mutants.

PolyP Regulates Ribosome Function

The 17 known polyphosphorylated proteins (our 15 targets plus Nsr1 and Top1) show preferential localization to the nucleolus, and a subset of these form a conserved network of interacting proteins implicated in ribosome biogenesis (Figure 2C). Since the regulation of ribosome biogenesis is tightly coupled to a cell's capacity to support translation and cell growth, we predicted that *vtc4*Δ cells, in which polyP is absent, would also show defects in ribosome function. To test this, we performed polysome profiling to examine ribosome assembly and translation elongation along mRNA. Here, *vtc4*Δ mutants showed a strong defect in 80S monosome and polysome assembly compared to wild-type, with a concomitant increase in free 40S and 60S subunits (Figure 5A). To confirm that polysome defects observed in *vtc4*Δ cells were due to loss of the *VTC4* gene, we re-introduced the open reading frame (ORF) on a low copy plasmid under its own promoter. Expression of this construct in *vtc4*Δ mutant strains rescued target polyphosphorylation (Figure 5B) and reversed defects in polysome assembly (Figure 5C). The polysome profiles of *vtc4*Δ mutant cells are somewhat complex but are most similar to mutants having defects in translation initiation, consistent with a role for polyP in ribosome function (Li et al., 2009). Notably, cells lacking *VTC4* do not show elevated phosphorylation of eIF2α (Figure 5D), an event associated with inhibition of translation following diverse cellular stresses (Dever, 2002). Therefore, it is unlikely that the polysome profiles stem from a general stress response triggered by the absence of Vtc4 or polyP. Since we have not yet been able to identify a PASK mutant that shares this phenotype, we favor the hypothesis that this phenotype represents the combined effects of polyphosphorylation changes to multiple targets.

Polyphosphorylation of Human Homologs

The core network of yeast polyphosphorylation targets implicated in ribosome biogenesis is conserved in human cells (Figure 6A). This led us to consider whether polyphosphorylation of lysine also occurs in higher eukaryotes. In order to understand the potential scope of lysine polyphosphorylation in mammalian cells, we screened the human proteome for proteins containing PASK motifs (75% S/D/E with at least one K). 1,134 proteins fit these criteria, and this set was enriched for nuclear/nucleolar factors with diverse functions (Figures 6B and 6C). Critically, included within this list (Table S2) were 6 homologs of polyphosphorylated yeast proteins (bolded in Figure 2A), including nucleolin, a functional homolog of Nsr1 that functions in various aspects of ribosome biogenesis. Nucleolin was previously

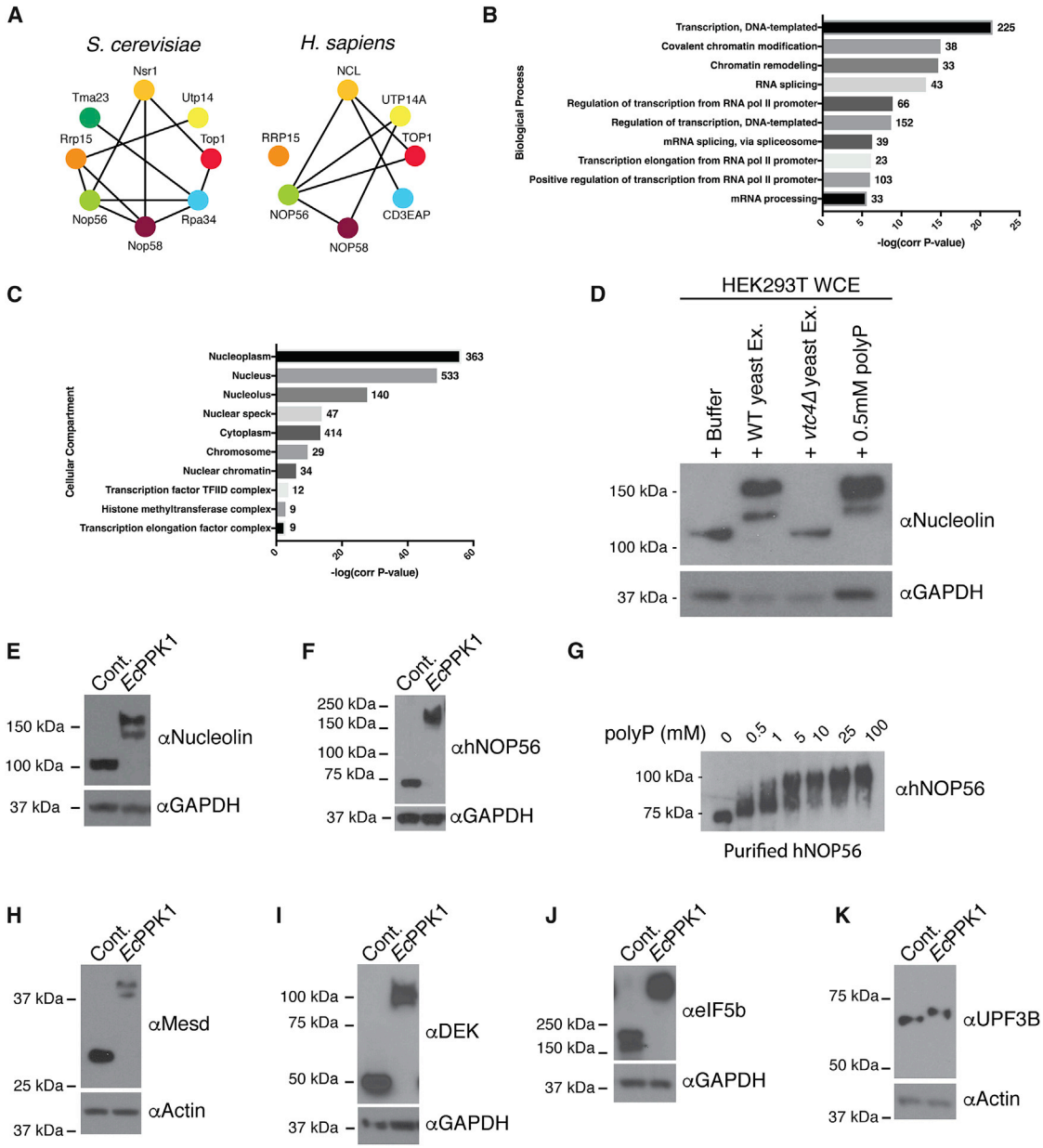

Figure 6. Polyphosphorylation of Human Proteins

(A) A network of physically interacting polyphosphorylated yeast targets is conserved in human cells. Network connections determined using Genemania.

(B and C) Analysis of PASK-containing proteins in human cells using DAVID with default settings. The number of proteins in each category is shown for biological process (C) and cellular component (D).

(D) Nucleolin can be polyphosphorylated *in vitro*. Extracts from HEK293T cells were incubated with wild-type or *vtc4*Δ mutant yeast extracts or with synthetic polyP prior to NuPAGE analysis.

(E and F) Expression of *Ec*PPK1 in HEK293T cells confers polyphosphorylation of human targets nucleolin (E) and hNOP56 (F).

(G) *In vitro* polyphosphorylation of bacterially purified hNOP56.

(H–K) Polyphosphorylation of human proteins Mesd (H), DEK (I), eIF5b (J), and UPF3B (K) following *Ec*PPK1 expression.

Also see Table S2 and Figure S6.

reported to migrate at an apparent molecular weight of ~100 kDa on SDS-PAGE (Ginisty et al., 1999), and we found that it migrated at a similar position on NuPAGE gels (Figure 6D, lane 1). PolyP concentration in most mammalian tissues and cell lines is significantly lower than in yeast (~50 μM versus > 100 mM) (Kumble and Kornberg, 1995). We reasoned that the polyP concentration in HEK293T cells used for our experiment may be too low to cause the dramatic changes in

electrophoretic mobility observed for yeast targets. In support of this interpretation, mixture of HEK293T cell lysate with extracts from wild-type, but not *vtc4*Δ yeast, conferred a dramatic electrophoretic shift to the entire population of nucleolin protein (Figure 6D, lanes 2 and 3). A similar result was obtained by adding synthetic polyP to the extract (Figure 6D, lane 4).

The enzymes that synthesize polyP in human cells are currently unknown. Therefore, to test whether human cell lines could be engineered to produce polyP in quantities sufficient to confer lysine polyphosphorylation visible by NuPAGE analysis, we transfected cells with a plasmid expressing *Ec*PPK1. Nucleolin recovered from these cells displayed the electrophoretic shifts characteristic of lysine polyphosphorylation on NuPAGE gels (Figure 6E). This was also true for hNOP56, the homolog of yeast target Nop56 (Figure 6F). *In vitro*, synthetic polyP shifted hNOP56 purified from bacteria in a concentration-dependent manner (Figure 6G). To further demonstrate the utility of Table S2, we cross-referenced this list with commercially available antibodies and selected additional candidates for testing with NuPAGE analysis. We found evidence for *Ec*PPK1-induced polyphosphorylation of Mesd, DEK, eIF5b, and UPF3B (Figures 6H–6K). In contrast, the electrophoretic mobility of PASK-containing proteins Hsp90alpha and HIP were not impacted by *Ec*PPK1 expression (Figures S6A and S6B). Therefore, as we saw in yeast, the presence of a PASK motif alone is not enough to predict quantitative polyphophorylation. Critically, the observation that human cells can support the production of polyP in quantities allowing for NuPAGE detection of protein modification provides an avenue to study lysine polyphosphorylation in higher eukaryotes.

DISCUSSION

Polyphosphorylation as a Global Regulator

Polyphosphorylation of lysine was originally identified as a novel PTM on yeast proteins Nsr1 and Top1 (Azevedo et al., 2015). In this work, we have exploited tools unique to the budding yeast model to identify 15 previously unreported targets, including a conserved network of proteins implicated in ribosome biogenesis. Our work provides evidence of polyphosphorylation as a common modifier. Critically, our 15 targets were found in a set of only 90 prioritized candidates. There are an additional 337 proteins in yeast that contain PASK motifs, and these function in diverse pathways (Table S1). As such, lysine polyphosphorylation is likely to have a broad impact on cell function. To facilitate the use of our work as a resource, we have fully annotated Tables S1 and S2 to indicate PASK motifs in both yeast and human proteins that overlap with unique functional, structural, or regulatory features.

We centered our search for targets around the PASK motif. However, we cannot discount the possibility that sequences lacking PASK characteristics are also polyphosphorylated. As such, it will be critical to develop other methods to identify polyphosphorylated proteins. Notably, Azevedo et al. were unable to detect polyP linkage to Nsr1 using mass spectrometry (Azevedo et al., 2015). While we are revisiting this approach for other targets, the unbiased identification of new targets may be possible with other high-throughput methods. The use of protein chips (Zhu et al., 2001)—in which thousands of test proteins are immobilized on glass slides that can be analyzed using a microarray scanner—is an attractive option. Our work will serve as a means to benchmark new methods for identifying targets of polyphosphorylation in yeast and mammalian systems.

A Role for polyP in Ribosome Function

Based on our recovery of a conserved network of targets that function in ribosome biogenesis, we predicted that cells that cannot make polyP would have defects in ribosome function. Indeed, cells lacking *VTC4* accumulate free 40S and 60S subunits and have a corresponding decrease in joined 80S monosomes and polysomes (Figure 5). These data are consistent with polyP being critical for a biogenesis step that impacts late-stage assembly, although a more direct role for polyP in the regulation of translation is also possible. As yet, we have been unable to directly link polyphosphorylation of any one target to defects in ribosome biogenesis or translation. It is possible that modulating the polyphosphorylation of multiple targets is required to confer observable phenotypes (Figure 7). Alternatively, modification of single targets with polyP chains could function redundantly with other pathways.

Molecular Function of Polyphosphorylation

We envision that polyphosphorylation of targets that we uncovered could regulate protein function through diverse molecular mechanisms. First, the addition of polyP chains would alter the charge of targets, which could have dramatic consequences for their interactions with other proteins and nucleic acids. Second, polyphosphorylation could also compete directly with other global lysine modifications such as acetylation, ubiquitylation, or SUMOylation. Additionally, polyphosphorylation could crosstalk with non-lysine modifications. For example, many serine residues within PASK domains are known sites of (mono) phosphorylation (Table S1).

Finally, the phosphoanhydride bonds formed between inorganic phosphate residues in polyP chains are of very high energy (Rao et al., 2009). Polyphosphorylation of nucleolar proteins could have the secondary effect of concentrating this energy source in the nucleolus. In this scenario, polyphosphatase-mediated hydrolysis of polyP attached to protein targets could be coupled to energy-dependent events driving ribosome biogenesis.

Regulation of Polyphosphorylation

For all of our polyphosphorylated targets, the vast majority of the protein population appears to exist in the modified state as judged by NuPAGE analysis. This is in contrast to other lysine modifications such as acetylation and SUMOylation, which often occur on only a small fraction of total target protein (Weinert et al., 2014) (Sarangi and Zhao, 2015). We suggest that the near complete modification of targets by polyphosphorylation results from the very high concentration of polyP (>100 mM) in budding yeast (Auesukaree et al., 2004). Yet, because polyP is added non-enzymatically (Azevedo et al., 2015), we cannot discount the possibility that some polyphosphorylation is occurring in solution following cell lysis. *In vivo*, target modification may be sub-stoichiometric due to local variation in polyP

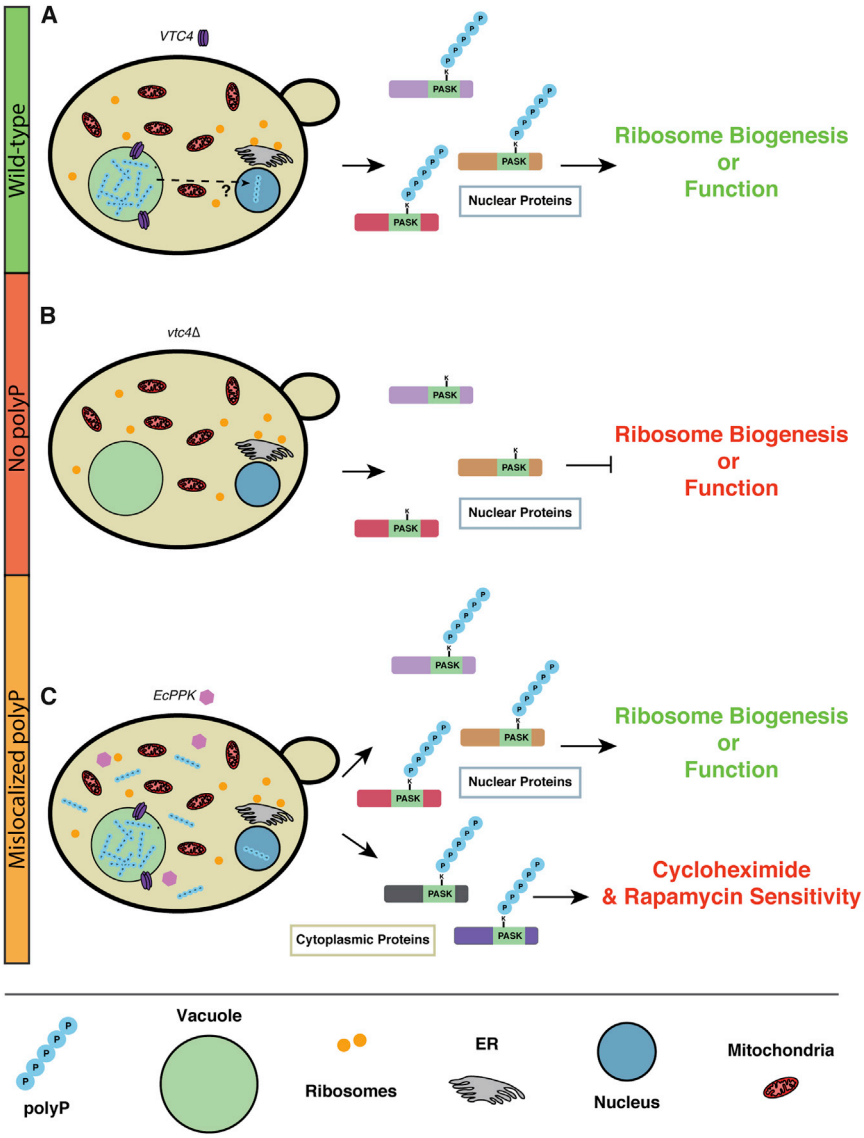

Figure 7. An Updated Model for polyP Function in Yeast

(A) In wild-type cells, polyP is synthesized by Vtc4 and is stored in the vacuole. It is transported to other areas of the cell by unknown mechanisms. In the nucleolus, polyphosphorylation of one or more ribosome biogenesis proteins is important for ribosome function.

(B) In the absence of *VTC4*, key substrates are no longer polyphosphorylated and ribosome biogenesis and/or translation are defective.

(C) Expression of *Ec*PPK1 permits extra polyP accumulation in the cytoplasm. Targets can still be polyphosphorylated, but the extra polyP disrupts important cellular functions.

concentrations, competition with other PTMs or occlusion of PASK motifs by protein-protein or protein-nucleic acid interactions.

We found that deletion of genes required for the maintenance of polyP levels impacted lysine polyphosphorylation of multiple targets (Figures 4A and S5A). This highlights that polyP synthesis is likely to be a critical regulator of target modification. In some experiments, we noticed that target polyphosphorylation diminished when cultures were grown for long periods of time (Figure S5D). However, this was somewhat inconsistent, with other trials showing persistent target polyphosphorylation even after many days at saturation. While we cannot currently explain this observation, it is possible that subtle variations in growth conditions can trigger pathways that prevent complete degradation of polyP chains.

In an elegant study, Gerasimaitė et al. showed that polyP synthesis in the cytoplasm causes slow growth and aberrant cellular morphology. The authors proposed a model wherein the obligate coupling of polyP synthesis to vacuolar storage prevents this toxicity in wild-type cells (Gerasimaitė et al., 2014). We used this same system to demonstrate that cytoplasmic polyP synthesis renders cells sensitive to the translation inhibitor cycloheximide and the TOR inhibitor rapamycin (Figure 4E). We suggest that high concentrations of cytoplasmic polyP could drive unwanted polyphosphorylation of PASK-containing proteins to interfere with important cell functions (Figure 7).

Conservation of polyP and Lysine Polyphosphorylation

Many of the targets we identified are conserved in human cells. While the human polyP polymerase is currently unknown, our finding that 6 human proteins (nucleolin, hNOP56, Mesd, DEK, eIF5b, and UPF3B) can undergo polyphosphorylation suggests that regulation of proteins by this PTM may also be conserved. Of these targets, Mesd, a molecular chaperone involved in Wnt/β-catenin signaling, is particularly notable for the location of its 22-amino-acid PASK motif. This PASK is situated within a 38-amino-acid C-terminal and vertebrate-specific region of Mesd (Figure S6C). Peptides from this region inhibit Wnt ligand binding to LRP5 and dampen downstream transcription of Wnt/β-catenin-regulated genes (Lin et al., 2013).

Notably, expression of the *Ec*PPK1 polyP kinase was required to observe quantitative polyphosphorylation of Mesd and other human proteins by NuPAGE analysis in HEK293T cells (Figures 6E–6K). The lack of quantitative polyphosphorylation in cells transfected with control plasmids likely stems from the lower concentration of polyP relative to that seen in yeast (Kumble and Kornberg, 1995). As such, polyphosphorylation could be more selective in higher eukaryotes. PolyP has previously been associated with cell growth. Decreasing polyP concentration through the ectopic expression of yeast exopolyphosphatase

Ppx1 inhibits proliferation of the MCF7 breast carcinoma cell line (Wang et al., 2003). Mechanistically, polyP activates the nutrient and energy sensing kinase mTOR by promoting its (canonical) phosphorylation (Wang et al., 2003). Our work raises the possibility that signaling through mTOR and other pathways governing cell growth and division could be mediated in part by lysine polyphosphorylation. Intriguingly, we also found that alternative isoforms of many human proteins include changes to predicted PASK sequences (Table S2). Differences in lysine polyphosphorylation could underlie variations in function observed for such isoforms. Our study provides a foundation for investigating this potential mode of regulation in higher eukaryotes.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids

S. cerevisiae strains were generated and grown using standard techniques (Goldstein and McCusker, 1999; Longtine et al., 1998). Synthetic DNA constructs and strain genotypes are listed in Tables S4 and S5.

Statistical Analyses

For halo assays (see Supplemental Experimental Procedures), n = 4 with error bars representing SE. No significant difference was found via Student's t test. Description of corrected p values for bioinformatics is contained in the Supplemental Experimental Procedures.

Western-Blotting Screen for Polyphosphorylated Proteins

From the 427 proteins with PASK domains, 90 candidates were prioritized largely based on known expression level as well as number and length of PASK sequences present in each protein. Generation of matched *ppn1*Δ and *ppn1*Δ *vtc4*Δ GFP-tagged strains was achieved using Synthetic Genetic Array Technology (Tong et al., 2001). Details are contained in the Supplemental Experimental Procedures. Protein extracts were prepared using a trichloroacetic acid (TCA)-lysis method (Rössl et al., 2016) and analyzed on Bis-Tris NuPAGE gels (Thermo Fisher Scientific NP0336BOX). *VTC4*-dependent electrophoretic shifts of potential targets were considered a "hit." See Table S3 for antibody details.

In Vitro Polyphosphorylation Assays

For analysis of Rts1 and Fpr3, synthetic polyP (Kerafast EUI005) was added to TCA lysis extracts for 1 hr at room temperature. For the chain-length experiment in Figure S1, polyP standards from Regenetiss, Japan were used. Samples were analyzed by NuPAGE.

Transfections and Protein Preps from *Ec*PPK1-Expressing HEK293T Cells

HEK293T cells cultured as described in Supplemental Experimental Procedures were grown to ~70% confluency and transfected with 2–28 μg of DNA using Lipofectamine 2000 (Invitrogen 11668-019). To isolate protein, cells were washed with ice-cold 1 × PBS, scraped, and lysed in 0.2–1 mL of RIPA Lysis buffer (see supplemental buffer recipes). Cells were incubated for 15 minutes on ice and centrifuged for 15 min at 13,500 rpm. The supernatant was collected prior to addition of Laemmli sample buffer and analysis using NuPAGE.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and five tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.02.104.

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AUTHOR CONTRIBUTIONS

A.B.-D. and M.D. devised experiments and wrote the paper. A.B.-D., H.M., Y.-C.T., Y.S., C.H., C.N., A.R., G.F.A., S.K., E.B.-C., N.E.D., and M.D. carried out experiments and/or analysis. A.G. supervised H.M. All authors reviewed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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