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Phosphate: from stardust to eukaryotic cell cycle control

Javier Jiménez, Samuel Bru, Mariana P.C. Ribeiro, Josep Clotet*

Department of Basic Sciences, School of Medicine and Health Sciences, Universitat Internacional de Catalunya, Barcelona, Spain

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Summary. Phosphorus is a pivotal element in all biochemical systems: it serves to store metabolic energy as ATP, it forms the backbone of genetic material such as RNA and DNA, and it separates cells from the environment as phospholipids. In addition to this "big hits", phosphorus has recently been shown to play an important role in other important processes such as cell cycle regulation. In the present review, we briefly summarize the biological processes in which phosphorus is involved in the yeast *Saccharomyces cerevisiae* before discussing our latest findings on the role of this element in the regulation of DNA replication in this eukaryotic model organism. We describe both the role of phosphorus in the regulation of G1 progression by means of the Cyclin Dependent Kinase (CDK) Pho85 and the stabilization of the cyclin Cln3, as well as the role of other molecule composed of phosphorus–the polyphosphate–in cell cycle progression, dNTP synthesis, and genome stability. Given the eminent role played by phosphorus in life, we outline the future of phosphorus in the context of one of the main challenges in human health: cancer treatment. [**Int Microbiol** 19(3):133-141 (2016)]

Keywords: *Saccharomyces cerevisiae* · Pho85 · cyclin · polyphosphate · cell cycle

Introduction

Cosmic material brought to the Earth by meteorites, often referred to as stardust, forms the basis of one of the hypotheses used to explain how phosphorus became biologically available on the early Earth. Phosphorus' career, so to speak, has thus continued at meteoric speed, making it a biological star: its major tasks include storing metabolic energy in the form of ATP, providing the backbone of genetic material, and separating cells from the environment as phospholipids. In addition, it is involved in the post-translational modification of proteins, it plays a role in enzymatic cofactors, and it takes part in cell signalling, among other processes.

Phosphorus is a mineral element that presents poor solubility, absence of a volatile phase, and low reactivity, factors that make it difficult to understand how phosphorylated molecules (ancestors of the plethora of organic molecules containing phosphorus) first started to form and become an important element in the prebiotic world [32]. The phosphorus that formed the first phosphorylated biomolecules had to have come from a mineral source, primordial molecules which evolved to produce the large variety of phosphomolecules present in modern Earth [24,32]. How did phosphorus enter into biomolecules? A hypothesis to answer this question involves a meteoritic phosphide source—the mineral schreibersite—[9,44,45]. Recent findings have shown that

*Corresponding author: J. Clotet
E-mail: jclotet@uic.cat

schreibersite could have influenced phosphorus chemistry on early Earth because it is capable of spontaneously phosphorylate organic compounds [46]. Certainly, there are other findings supporting less poetic hypotheses for the bioavailability of phosphorus, namely the "warm little pond" intuitively predicted by Charles Darwin in which a simple evaporitic environment rich in urea could promote thermodynamically favoured phosphorus solubility [10], or other environmental situations that may have been plausible on ancient Earth [18]. It should be mentioned that this review takes some literary licence with the stardust hypothesis, and not a firm position, within the context of the very interesting field of the prebiotic chemistry, field which is absolutely alien to the authors.

Whatever the case, phosphorus travelled to become biologically available and took the throne in a kingdom dominated by carbon, hydrogen, and nitrogen. Not only is phosphorus needed for nucleotides or phospholipids to exist, or for proteins to be phosphorylated, it brings about, in essence, the regulation of basically all biochemical reactions that allow cells to work and to be.

Once phosphorus is biologically available in the form of phosphate (orthophosphate, to be precise), it can be interiorised, utilised and stored by cells, from archaea to eukaryotes. Cells have developed intricate systems for phosphate intake from the environment, using systems that range from transport by transmembrane channels to scavenging by degrading molecules from the medium in which phosphate is found, or even sourcing internal reservoirs (see below). As for the other nutrients, signalling pathways are in charge of keeping the intracellular homeostasis of phosphate by impinging on intake and storage systems. All these mechanisms have been very well elucidated in the yeast *S. cerevisiae* (see [54] for an excellent review on phosphate metabolism).

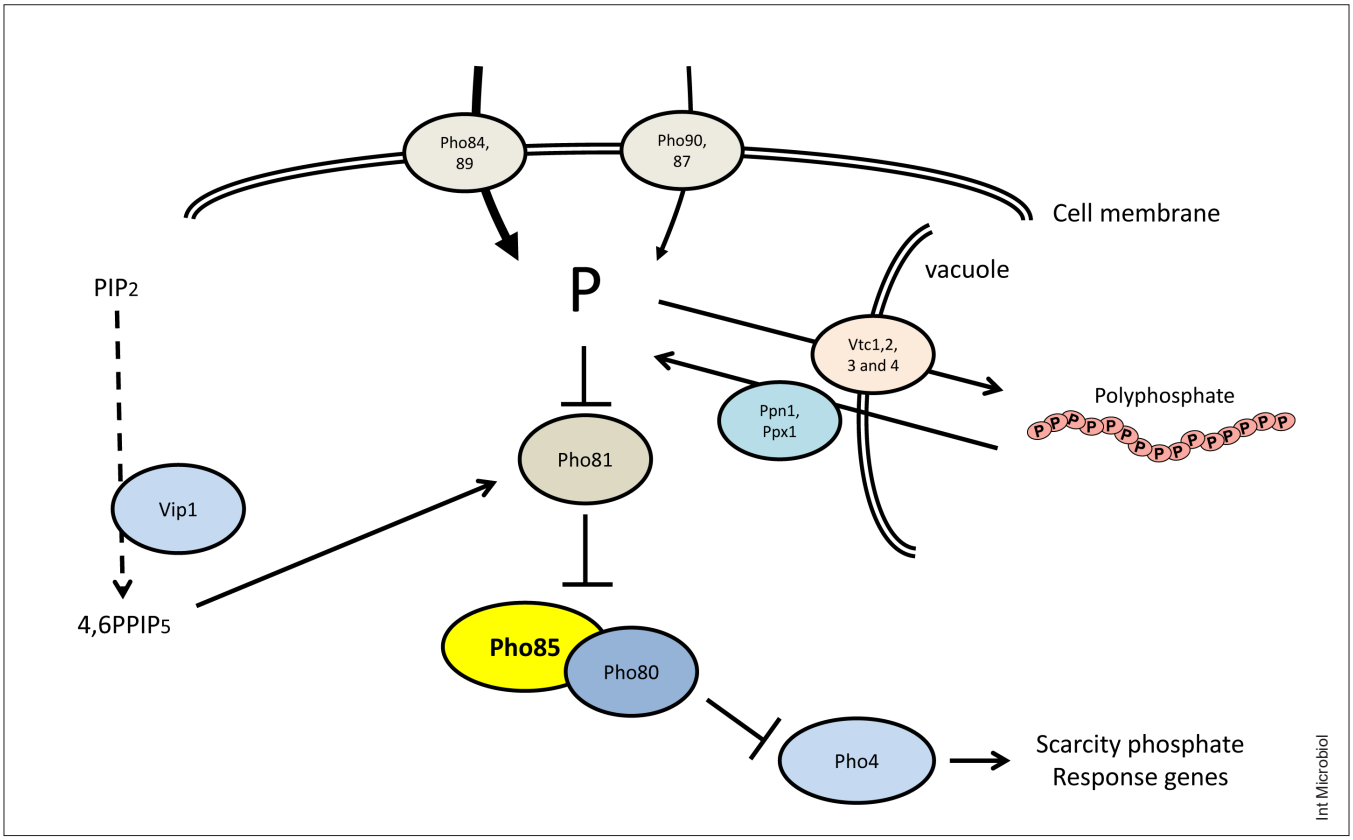

Fig. 1. The phosphate sensing and response–or Pho–pathway. The Pho pathway is led by the Cyclin Dependent Kinase (CDK) Pho85. Pho85 associates with its cyclin Pho80 and the CDK inhibitor (CDKI) Pho81, which is active when phosphate is limited, inactivating the CDK. The transcription factor Pho4 is therefore dephosphorylated and accumulates in the nucleus, inducing the gene transcription response to phosphate scarcity. Phosphate can be transported into the cell by means of 2 transporter systems: a high affinity system (Pho84-Pho89) and a low affinity system (Pho90-Pho87). Phosphate is stored in the vacuole as polyphosphate molecules by means of the Vtc2-Vtc3-Vtc4 system. The phosphate sensor involved has not yet been identified, however, a low phosphate signal is known to be transmitted via certain inositol polyphosphate species (4,6PPIP5) synthesized by Vip1, activating Pho81 and thus inhibiting Pho85.

Table 1. polyP functions

Function	Organism	Reference
A variety of biochemical reactions due to high energy bonds (analogous to those in ATP) and its properties as a polyanion	All	[34]
Buffer against alkalis	*Dunalliela salina* algae	[48]
Storage unit for Ca^{2+} and bacterial transformation	Bacteria	[11]
Detoxifier acting as a versatile metal-chelating agent	Bacteria	[31]
Antioxidative protection	Bacteria	[22]
Signalling and regulatory processes	*Saccharomyces cerevisiae*	[5]
Cell viability and proliferation	*Shigella* and *Salmonella* spp	[33]
Pathogen virulence	*Trypanosoma cruzi*	[39]
Production of poly-3-hydroxybutyrate	*Ralstonia eutropha*	[58]
Modulator of the microbial stress response	*Pseudomonas aeruginosa*	[14,23]
Structural component and chemical chaperone	*E. coli*	[23]
Cell cycle progression	*Saccharomyces cerevisiae*	[8]

Sensing, responding, and storing phosphate

Phosphate, like glucose or nitrogen, is an essential nutrient for all living organisms. Depletion of any of these molecules forces cells to enter in the quiescent G0 state [55]. Cells have thus very wisely evolved to produce sophisticated systems that monitor, control, and respond to phosphate concentration.

In the yeast *S. cerevisiae*, intracellular phosphate concentration is monitored and homeostatically controlled by the Pho pathway (Fig. 1), a pathway that has been extensively studied and elucidated, mainly by the O'Shea group [36]. Using the Pho signal, transduction pathway cells are able to sense and respond to variations in environmental phosphate. This process is led by the cyclin-dependent kinase (CDK) Pho85 which, like other CDKs, must interact with a cyclin (a protein showing a cyclic expression profile) to be active. Pho85 can bind with 10 different cyclins for its many functions in the biology of *S. cerevisiae* (for reviews see [28,29]).

In the case of phosphate homeostasis, the cyclin associated with Pho85 is Pho80. Pho85-Pho80 kinase activity is regulated in response to phosphate levels by the CDK inhibitor (CDKI) Pho81, which is constitutively bound to the CDK-cyclin complex, forming a ternary CDK-cyclin-CDKI complex [50]. When phosphate is limiting, the kinase activity of Pho85-Pho80 is faded by Pho81, permitting the dephosphorylation and activation of the transcription factor Pho4 and causing the transcription of genes involved in the survival response to phosphate starvation. Among these genes are those codifying for the high-affinity phosphate transporters Pho84 and Pho89 [42,47,51] responsible for external phosphate intake and the acid phosphatases Pho5 (external), Pho3 (in the periplasmic space), and Pho11 and Pho12 located in the cell wall [47], which scavenge for all the available forms of phosphate. Yeast also contains a set of proteins that function as low-affinity transporters (Pho87 and Pho90) and are present in the plasma membrane when phosphate in the medium is abundant [67]. Homologues to the yeast Pho pathway have been described, namely, the Pho regulon in bacteria [64] and *pef1*$^+$ in *S. pombe* [56].

Although a proportion of intracellular phosphate is driven to the mitochondria to enter into the energy cycle by means of the transporter Pho1 [59], the bulk of phosphate is used in the cytoplasm for several anabolic processes, such as phospholipid or ribonucleotide synthesis. However, for osmotic and biochemical reasons, the remaining phosphate cannot remain in the cytoplasm; in fact, cytoplasmic phosphate concentration in yeast is kept constant at around 20 mM [3,49,60]. To keep phosphate concentration constant, apart from using transportation, cells are able to store phosphate in the form of a molecule called polyphosphate (polyP). This function has been described in some bacterial species [17] and extrapolated to all species and cell types, and is generally accepted by the scientific community. Polyphosphate is a linear molecule made up of anywhere between a few and several hundred—or even thousands—of phosphate molecules linked by phosphoanhydride covalent bonds [34], which are present in all cell types, from archaeal to mammalian. In yeast, polyP is synthe-

sized by the Vacuole Transport Complex (VTC) comprising Vtc1, Vtc2, Vtc3, Vtc4, and Vtc5 proteins [51,16,27]. The VTC complex is located in the vacuole membrane and its main function is to fetch excess phosphate from the cytoplasm and bring it to the vacuole while includes it in the polyP polymer. In the mobilization of polyP to produce phosphate, 2 polyphosphatases have been described in yeast: Ppn1 (endo- or exo-polyphosphatase, depending on environmental conditions [2]) and Ppx1 (exo-polyphosphatase), and it is very likely that other proteins with polyphosphatase activity await discovery.

In addition to its role in storage function, polyP has been involved in many other processes, including virulence, stress response, survival, detoxification, and Ca^{2+} storage (see Table 1). The paper by Albi et al. recently provided an excellent review of polyP functions [1].

Polyphosphate plays decisive roles in mammalian cells, participating in processes such as blood clotting [40], bone mineralization [43], neurotransmission [26] and Alzheimer disease [12]. Despite the presence of polyP in mammalian cells, however, no polyP polymerase activity has been found to date [4]. With regard to polyP degradation activity, some candidates have been proposed (e.g., H-prune) [43], although the definitive main actor has not yet been identified.

Intracellular phosphate concentrations must be kept constant, regardless of demand from the different cellular processes. When phosphate is suddenly taken from the cytoplasm pool, the mobilization of stored polyP may occur as a quick-response homeostatic mechanism; if the perturbation persists, however, Pho pathway activation must take place to adapt to the new environment.

Cell cycle regulation by phosphate

It is well known that nutrients control cell cycle progression, specifically through the passage of the Start point (restriction point in mammalian cells) at the end of the G1 phase, a checkpoint that, once passed, forces cells to proceed through a new and complete round of the cell cycle [13]. Nutrients impinge on cell cycle control by activating several signalling pathways, including those of protein kinase A and Snf1, which positively regulate cell proliferation in response to glucose availability [21], and the TORC1 pathway, which controls the cell cycle according to nitrogen levels [37]. Consequently, inactivation of any of these 3 major pathways, even when other nutrients are plentiful, results in a cell cycle blockade and the production of the typical phenotypes of the G0-like growth arrest program [15]. Establishing and maintaining proper arrest in G1 is an important cellular response to nutrient deprivation; cells that fail to arrest the cell cycle at G1 during nutrient scarcity and proceed through S-phase show DNA replication stress and decreased viability [65].

Given that the cyclin Cln3 is the most upstream control point in the cell cycle and is directly responsible for driving cells pass Start, it seems logical that it should be a sort of a hub for signals alerting to nutrient scarcity. Indeed, it has been shown that Cln3 is less stable during nitrogen deprivation [19]. Some light has recently been shed on this molecular mechanism [57]. In terms of the involvement of phosphate in cell cycle control, the Clotet group showed that the before mentioned putative master regulator Cln3 is phosphorylated by Pho85-Pho80, a phosphorylation that is essential for stabilizing Cln3, thus permitting cell cycle progression. Correspondingly, the Cln3 phosphomimetic mutant maintains high levels of Cln3 regardless of Pho85-Pho80 activity, and therefore it does not properly arrest in G1 in the absence of phosphate, dying prematurely [38,30]. Pho85 is thus a key factor in stabilizing Cln3 in rich media, allowing cell cycle progression through Start.

Mechanistically speaking, it is know that Cln3 amounts are controlled by the phosphorylation state of its destruction box, the PEST region. The responsible kinase is Cdk1, and this process determines Cln3 degradation by the proteasome [35]. When phosphate is present, Pho85-Pho80 phosphorylates 2 residues that precisely frame the PEST region, suggesting that this phosphorylation manipulates ubiquitination and subsequent destruction by proteasome. In the case of the regulation by nitrogen, Pho85 (here, bonded to the cyclins Clg1 or Pcl2) is also involved. When nitrogen is present Pho85-Pcl2 or Pho85-Clg1 cannot phosphorylate the chaperone Ssa1, event that also protects Cln3 from early destruction [57] (Fig. 2).

Cells without *cln3* are viable and show only a modest delay in G1 progression, suggesting that the regulation of Cln3 may be superfluous in ideal lab conditions (i.e., grown in YPD at 30º and agitated at 200 rpm). However, the destabilization of Cln3 appears to be a key factor in achieving correct cell arrest during nitrogen or phosphate starvation; cells that do not arrest properly are prone to entry into S-phase, rapidly losing viability [65]. Moreover, a cell that cannot sense correctly the presence of these nutrients after refeeding will have

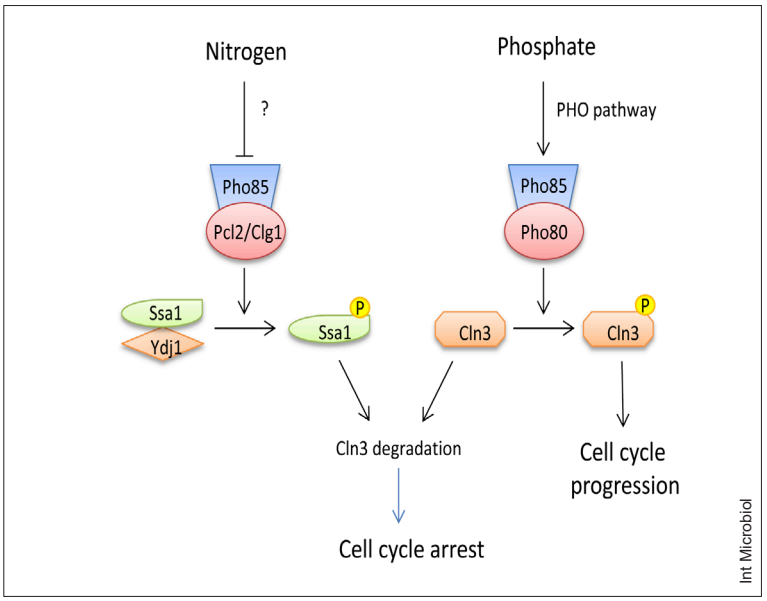

Fig. 2. The regulation of Cln3 by nutrient availability. Nitrogen scarcity activates Pho85 through an unknown mechanism, determining the phosphorylation of the chaperone Ssa1 and the degradation of Cln3. Phosphate availability activates the PHO pathway, producing direct phosphorylation and stabilization of Cln3. In both cases, nitrogen or phosphate scarcities block cell cycle progression through G_1 by Cln3 destruction.

delayed Cln3 appearance and therefore delayed restart of the cell cycle, and thus be at a competitive disadvantage (our unpublished results). Yeast in natural environments should thrive under constantly changing nutrient conditions, and in this scenario Pho85 must be fundamental in controlling the constant cell cycle stalls and restarts that a yeast cell is subjected to.

Polyphosphate involvement in the cell cycle

Polyphosphate is the other form in which phosphate is present in cells, appearing to play a role in the storage of phosphate. However, a number of recent reports, especially on prokaryotic cells, have suggested that the amount of polyP is related in some way with the cell cycle stage. In the bacteria *Caulobacter crescentus*, the biogenesis and localization of polyP is controlled as a function of the cell cycle, ensuring regular partitioning of polyP granules between mother and daughter cells [25]. When polyP production is impaired, cells improperly initiate chromosome replication [7]. In the cyanobacteria *Synechococcus elongatus* the average size of polyP bodies increases gradually during the dark period, without a significant change in number or distribution. However, during the light period, the number of polyP bodies increases while the size of each polyP body decreases, with cells elongating until the end of this light period, when most cells divide. The regular coordinated changes of polyP bodies and DNA shape during the cell division cycle, together with an intimate physical interaction, suggest that polyP bodies play a role in supplying material for DNA [53]. Other findings that support the correlation of the cell cycle and polyP have been reported in *Chlamydomonas reinhardtii* [66]: polyP amounts peak during late cytokinesis and a slight fluctuation of polyP occurs during the cell cycle. The overexpression of an exopolyphosphatase in *Pseudomonas* sp. produces decreased levels of polyP, among other phenotypes, bringing about a cellular division malfunction [61]. In *Synechococcus* sp. from microbial mats, the levels of enzymes involved in the metabolism of polyP are differentially accumulated during the diel cycle; the levels of polyphosphate kinase peak at night, while polyP levels are highest during the early morning hours [20]. Finally, polyP and cell cycle have also been reported to correlate in mammalian cells. The proliferation of normal human fibroblast cells is enhanced by the addition

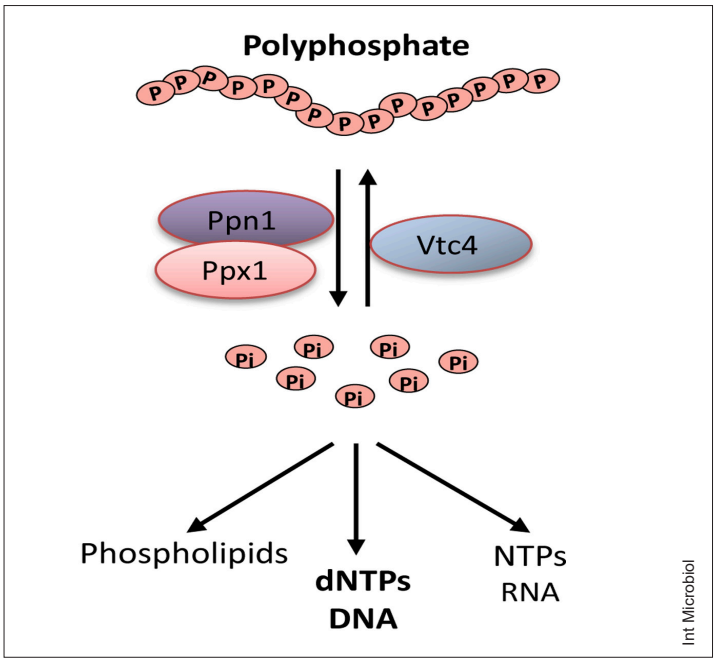

Fig. 3. The many roles played by polyP. In *S. cerevisiae*, the polyphosphatases Ppx1 and Ppn1 degrade polyP chains previously synthesised by Vtc4 and stored in the vacuole. The resultant inorganic phosphate (Pi) could be used for steady synthesis of RNA and phospholipids, and for the dNTPs synthesis that takes place every time a cell passes through G1 and S phases.

of inorganic polyP into culture media [52], suggesting that polyP plays a role in promoting the cell cycle, although it might be mediated through the interaction with cell-membrane receptors.

In yeast, polyP amount is cyclically regulated [8,41]: polyP content is reduced when cells enter into the S-phase and recover before mitosis. Interestingly, despite of the reduction in polyP amount in these phases of the cell cycle, cytoplasmic phosphate concentration remains constant [8]. This finding brings about 2 fundamental and somewhat related questions: i) what is the purpose of polyP cyclical reduction? and ii) is there a homeostatic system in place to address the cyclical variation in the demand of intracellular phosphate?

In terms of the implication of polyP in the cell cycle, the supporting evidence in the different systems and models described above that point to polyP playing a role in cell cycle progression compelled us to further analyse this possibility. Our strategy [8] was to study cell cycle progression in yeast mutants that are deficient in polyP, either because cells are unable to produce it (*vtc4*Δ) or unable to mobilize it (*ppn1*, *ppx1*Δ). In both cases, cell cycle progression was found to be impaired and was restored when external phosphate was added. The effect on the cell cycle appears to be more remarkable when cells grow in phosphate-limiting conditions, a situation that occurs normally when yeast cells are in a natural, fluctuating environment. On this basis, the high concentration of phosphate in the lab-growth media may be the main reason why some polyP functions in yeast, like those discussed here, have been so recalcitrant and escaped the scrutiny of the dedicated scientific community for so long.

Phosphate is steadily consumed in the production of essential molecules, mainly RNA and phospholipids [68]. To our understanding, RNA and phospholipids are steadily synthesized, and consequently phosphate demand resulting from this synthesis throughout the cell cycle has not been described, nor is it thought to happen. Because polyP reduction occurs concomitantly with DNA synthesis, we speculated about the role of polyP in providing phosphate for the swift production of nucleotides (dNTPs), the basic building blocks for DNA synthesis, at the end of the G1-phase. To this end, we measured the dNTPs content of yeast cells deficient in polyP and found that their ability to produce them is impaired. This evidence supports the hypothesis that polyP

consumed at the end of G1 and during the S-phase provides the phosphate necessary for DNA duplication (Fig. 3).

This hypothesis is coherent with phosphate numbers. The phosphate amount included in a yeast genome can be easily calculated (7.5×10^7 molecules) and, according to our quantification, the reduced polyP amount in a cell accounts for approximately 4.5×10^8 phosphate molecules, a sufficient amount to meet the demand produced by the duplication of a genome. This work puts forth evidence to support what was suggested by others [7,53], revealing a new role for polyP as a phosphate supplier for the synthesis of dNTPs and, in turn, DNA duplication.

DNA replication under conditions with limited amounts of dNTPs results in genomic instability [6,62]. Accordingly, polyP mutants, which have reduced amounts of dNTPs, show increased genomic instability, shown by the ability to lose plasmids and the presence of recombination events [8]. polyP is therefore relevant in cell physiology in terms of this new role in sustaining DNA replication.

Considering the data derived from our work on polyP, we suggest that polyP plays a homeostatic role in managing short-term variations in the internal concentration of phosphate, eventualities that can arise as a consequence of discrete phenomena such as DNA replication or DNA damage repair.

Concluding remarks

Phosphate has the ability to impact cell cycle progression to benefit the fate of a cell. Cells need phosphate, together with the mechanisms involved in its regulation, to proliferate in an orderly fashion. Following this line of argument, it is evident that phosphate homeostasis could be a target to treat cells that proliferate uncontrollably. In fact, insertion and expression of the yeast polyphosphatase gene *PPX1* in MCF-7 mammary cancer cells has been reported to produce a markedly deficient response to mitogens [63]. Preliminary experiments are being carried out in our lab to identify conditions in which the absence of polyP in cancer cells works together with chemotherapeutic treatment, potentially allowing for a reduction in dosage and toxic effects. This dazing meteoric story of phosphorus, and the understanding of microbial phosphate metabolism, has allowed us to envisage its future involvement in cancer treatment, one of the most relevant problems faced by modern medicine.

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Competing interests. None declared.

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