Universitat de Lleida

Document downloaded from:

<http://hdl.handle.net/10459.1/72533>

The final publication is available at:

<https://doi.org/10.1016/j.dnarep.2017.08.001>

Copyright

cc-by-nc-nd, (c) Elsevier, 2017

Està subjecte a una llicència de [Reconeixement-NoComercial-SenseObraDerivada 4.0 de Creative Commons](https://creativecommons.org/licenses/by-nc-nd/4.0/)

Polyphosphate is a Key Factor for Cell Survival after DNA Damage in Eukaryotic Cells

Samuel Bru^a, Joan M. Martínez-Laínez^a, Sara Hernández-Ortega^a, Eva Quandt^a, Eloi Garí^b, Marta Rafel^b; Javier Torres-Torronteras J^{c,d}, Ramón Martí^{c,d}, Javier Jiménez^{a,*}
and Josep Clotet^{a,*}

a-Faculty of Medicine and Health Sciences. Universitat Internacional de Catalunya.
Barcelona, Spain

b-Institut de Recerca Biomèdica de Lleida (IRBLleida); Departament de Ciències
Mèdiques Bàsiques; Facultat de Medicina; Universitat de Lleida; Lleida, Spain

c-Research Group on Neuromuscular and Mitochondrial Disorders, Vall d'Hebron
Institut de Recerca, Universitat Autònoma de Barcelona, Barcelona, Spain

d-Biomedical Network Research Centre on Rare Diseases (CIBERER), Instituto de
Salud Carlos III, Madrid, Spain

* Corresponding authors:

jclotet@uic.cat Tel. (+34) 935042015; Fax. (+34) 935042001

jjimenez@uic.es Tel. (+34) 935042000; Fax. (+34) 935042001

Abstract

Cells require extra amounts of dNTPs to repair DNA after damage. Polyphosphate (polyP) is an evolutionary conserved linear polymer of up to several hundred inorganic phosphate (Pi) residues that is involved in many functions, including Pi storage. In the present article, we report on findings demonstrating that polyP functions as a source of Pi when required to sustain the dNTP increment essential for DNA repair after damage. We show that mutant yeast cells without polyP produce less dNTPs upon DNA damage and that their survival is compromised. In contrast, when polyP levels are ectopically increased, yeast cells become more resistant to DNA damage. More importantly, we show that when polyP is reduced in HEK293 mammalian cell line cells and in human dermal primary fibroblasts (HDFa), these cells become more sensitive to DNA damage, suggesting that the protective role of polyP against DNA damage is evolutionary conserved. In conclusion, we present polyP as a molecule involved in resistance to DNA damage and suggest that polyP may be a putative target for new approaches in cancer treatment or prevention.

Keywords

Saccharomyces, mammalian cells, human dermal fibroblasts, polyphosphate, DNA damage, repair

1. Introduction

Cells devote a substantial amount of resources to keep their genetic information unaltered. They are armed with a diverse array of mechanisms directly involved in repairing DNA when it is modified or damaged by one of many enabling factors {{670 Sancar,A. 2004; 671 Spampinato,C.P. 2016; 672 Hustedt,N. 2016}}. It has recently been reported that when DNA is damaged in yeast, the amount of triphosphate deoxynucleotides (dNTPs) present in the cells increases sharply, a response that is essential for cell survival {{346 Chabes,A. 2003}}. The molecular mechanism supporting the rise in dNTPs is derived from the downregulation of ribonucleotide reductase inhibitor Sml1 {{351 Zhao,X. 2001}} and the upregulation of *RNR* (Ribonucleotide reductase, responsible for the conversion of nucleoside diphosphate to deoxynucleoside diphosphate) genes {{400 Elledge,S.J. 1992; 401 Tsaponina,O. 2011}}. Importantly, dNTPs increase directly correlates with tolerance to DNA damage {{349 Chabes,A. 2007}} and has been associated with promoting either replication fork progression or translation {{350 Fasullo,M. 2010}}. Similarly, Ynk1 activity (the yeast nucleoside diphosphate kinase in charge of introducing the third phosphate, yielding the dNTP molecule—see Fig. 1) is also needed to support dNTP synthesis during DNA damage, as suggested by the increased mutation rate after UV treatment observed in the *ynk1* mutant {{353 Yang,M. 2009}}. In metazoan cells, the increase of dNTPs levels after DNA damage, although present, is less pronounced, probably because it is restricted to foci in which DNA damage has been produced {{354 Niida,H. 2010}}, yielding a locally high concentration that appears diluted in the overall cellular concentration.

Synthesis of dNTPs also takes place every time a cell undergoes duplication, specifically, at the S phase, just before and during the duplication of DNA. It has recently been described that dNTPs synthesis produces a high consumption of Pi, which is provided, at least in part, by the degradation of polyphosphate (see below and Fig. 1) {{581 Bru,S. 2016}}.

The homeostasis of Pi is tightly controlled by cells due to the fact that Pi is a crucial structural component in extremely important molecules such as phospholipids, nucleic acids, and proteins, but it is also a regulator of biochemical pathways. For this reason, many cells are equipped with sophisticated systems to monitor, control, and respond to small variations in intracellular Pi concentration in order to maintain the balance between Pi usage and intake. Yeast use a complex system of Pi transporters responsible for intake {{411 Ogawa, N. 2000; 153 Wykoff,D.D. 2001; 487 Persson,B.L. 2003; 420 Wykoff,D.D. 2007; 286 Secco,D. 2012}}, the expression of which, in most cases, is tightly monitored and controlled by the PHO pathway {{115 Lee,Y.S. 2008}}. The PHO pathway responds to Pi internal concentration and becomes responsible for the control of Pi homeostasis (for a review on the PHO pathway see {{117 Huang,D. 2007; 318 Jimenez,J. 2013}}).

In an additional control mechanism, and probably also as a back-up system for situations of scarcity, Pi is be stored in the form of a molecule called polyphosphate (polyP) in the vacuole {{410 Kornberg,A. 1999}}. polyP is a linear chain comprised of anywhere between 3 and thousands of Pi residues present in all living organisms {{358 Kulaev,I.S. 1983}}, including yeast and mammals {{359 Kumble,K.D. 1995}}. The advantage of Pi storage in the form of polyP is avoid the osmotic effect of housing large pools of Pi in the cytoplasm, but with the extra flexibility of having access to a source of Pi when needed {{410 Kornberg,A. 1999}}, muffling any external limiting

conditions that may transiently occur {{152 Thomas,M.R. 2005}}. In yeast, polyP is a source of Pi used for sudden dNTPs demand in DNA duplication {{581 Bru,S. 2016; 610 Jimenez,J. 2016}} (see Fig 1).

In yeast, polyP is mainly synthesised and stored in the vacuoles by the vacuolar transporter chaperone (VTC) complex. The VTC complex is composed of the polyP polymerase Vtc4 and several regulatory subunits: Vtc1, Vtc2, Vtc3 {{333 Hothorn,M. 2009}}, and Vtc5 {{605 Desfougeres,Y. 2016}}. In the opposite reaction, polyP is hydrolysed to Pi by 2 polyphosphatases: Ppn1 (an endopolyphosphatase that cleaves long chains of polyP into shorter ones) and Ppx1 (an exopolyphosphatase that releases orthophosphate from the ends of polyP chains) (see Fig. 1) {{363 Andreeva,N.A. 2006}}.

In mammals, polyP is present in different tissues and in different subcellular compartments (nuclei, mitochondria, membranes and acidocalcisomes) {{359 Kumble,K.D. 1995; 673 Lorenz,B. 1997}}. A whole range of regulatory roles played by extracellular polyP in mammals is now emerging, including regulation of blood coagulation {{407 Smith,S.A. 2006; 378 Muller,F. 2009}}, bone mineralization {{379 Omelon,S. 2009}}, cell differentiation {{380 Morimoto,D. 2010}}, and gene expression {{381 Muller,W.E. 2011}}. However, only a few reports describe functions for the intracellular polyP (functionality of cardiomyocytes {{382 Seidlmayer,L.K. 2012}} and control of the RNA polymerase I {{442 Jimenez-Nunez,M.D. 2012}}), suggesting that many functions for polyP in mammalian cells remain unknown. One of the reasons for this lack of knowledge is the fact that the enzymes that control polyP metabolism in mammalian cells have not yet been identified.

In the present article, we show that polyP represents an important factor in sustaining the rise in dNTPs synthesis when DNA damage must be repaired. As our findings show, yeast mutants without available polyP produce reduced amounts of dNTPs upon DNA damage and their survival is compromised. In contrast, when polyP levels are ectopically increased, yeast cells become more resistant to DNA damage. More importantly, we demonstrate here that when polyP is artificially reduced in HEK293 mammalian cell line cells and in human dermal primarily fibroblast (HDFa), they become more sensitive to UV damage, suggesting that the protective role of polyP against DNA damage is evolutionary conserved. In conclusion, we present polyP as a molecule involved in the resistance to DNA damage.

2. Material and methods

2.1. Yeast growth conditions and strains

Yeast cells were grown in either YPD medium (1% yeast extract, 2% peptone, and 2% glucose) or complete synthetic dextrose (SD) medium (0.67% yeast nitrogen base, 0.5% NH_4SO_4 , and 2% glucose) supplemented with amino acids for auxotrophic requirements. The genetic background of the yeast in all experiments was BY4741 {{628 Brachmann,C.B. 1998}}. Mutant strains were constructed using the toolbox system {{548 Janke,C. 2004}}: YSB075 (*vtc4::kanMX*), YSB098 (*ppn1::KanMX*, *ppx1::LEU2*), YSB083 (*dun1::KanMX*), YSB088 (*adk1::KanMX*), YSB89 (*ynk1::KanMX*) and YSB074 (*vtc1::KanMX*).

2.2. Cell cycle synchronization and flow cytometry analysis

To synchronize yeast cells, cultures were grown exponentially in YPD at a density of 1×10^7 cells/ml. Cells were treated with α -factor (Biomedal) to a final concentration of 20 $\mu\text{g/ml}$. After 2 h of treatment, cells were collected by centrifugation, washed with pre-warmed fresh media, and released to resume cell cycle progression. Approximately 1×10^7 cells were collected and processed as described previously {{254 Haase,S.B. 2002}}. DNA was stained with SYBR green and subsequently analysed in a FACS Calibur cytometer (Becton Dickinson) {{42 Clotet,J. 2006}}.

2.3. Immunoblot analysis

The primary antibodies used were anti tubulin 1:200 (Sigma T5168), anti FLAG 1:500 (Sigma F3165), anti Rad53 1:700 (Santa Cruz, sc-6749), and anti-G6PDH 1:500 (Sigma, A9521). The secondary antibodies used were sheep anti-mouse HRP 1:30.000, donkey anti-goat-HRP 1:20.000, and goat anti-rabbit-HRP 1:25.000 (all from Jackson

laboratories). Immunoblots were developed using Luminata Forte Western HRP Substrate (Millipore) and images were taken with GeneSnap (Syngene) and quantified with Image Studio Lite (Li-Cor).

2.4. Quantification of polyP content

PolyP extraction and purification was performed as previously described {{676 Bru, S. Jimenez, J. Canadell, D. Arino, J. Clotet, J. 2017}}. Briefly, 1×10^7 ($0.7 \text{ OD}_{\lambda 660}$) cells were pelleted and suspended in 40 μl of SDS (20%) and 300 μl of phenol, boiled at 65°C for 10 min, and, subsequently, 300 μl of chloroform was added. After centrifugation, the supernatant was treated with a DNase/RNase solution for 1h at 37°C and applied to Qiagen PCR purification columns. After washing, polyP was eluted in 50 μl of MQ H₂O and quantified as previously described {{384 Werner,T.P. 2005}}. A microplate spectrophotometer (Synergy HT, Bio Tek Instruments) was used at 600 nm and compared with phosphate standards (0-500 $\mu\text{M P}_i$).

2.5. Measurement of dNTPs levels

Deoxynucleotides extraction was performed as previously described {{346 Chabes,A. 2003}}. Amounts were measured by means of a polymerase-based method as previously described {{383 Gonzalez-Vioque,E. 2011}}.

2.6. PolyP overproduction

Intracellular polyP content was increased in wild-type cells by 2 different methods. First, transforming the cells with a pRS426 episomal vector in which the *VTC4* yeast gene had been previously cloned. Second, wild-type cells were submitted to overloading conditions {{384 Werner,T.P. 2005}}. Cells were grown in SD low-phosphate media for 6 h, then collected by centrifugation and suspended in SD 2 mM

phosphate content for 15 min (1X), 30 min (2X) and 1 h (3X). After the incubation, polyP was measured to assess the content.

2.7. Measurement of UV DNA damage sensitivity in yeast

To measure UV sensitivity, 0.5×10^3 cells were spread on YPD plates and irradiated with the indicated doses. Cells were grown at 30°C in darkness for 48 h. Colonies were counted automatically using the GeneTools (Syngene) software. Wild-type viability was considered to be 100% survival for the experiment and was used to normalize the survival percentage.

2.8. MMS sensitivity in yeast

Exponentially growing yeast cells ($OD_{\lambda 660}$ 0.2) were exposed to 0.033% MMS (Methyl Methane Sulfonate) from Sigma for 4 h and plated in YPD plates. The control was performed using the same cell exposed to the vehicle (DMSO) in which MMS was dissolved. Cells were grown at 30°C in darkness for 48 h. Resulting colonies were automatically counted using the GeneTools (Syngene) software. Wild-type viability was considered to be 100% and used to normalize the survival percentage.

2.9. 4-NQO treatment in yeast

4-NQO is a molecule generally used when the DNA damage experiments are performed in liquid media {{663 Tallec,B.L. 2012}} to mimic UV damage. Exponentially growing cells ($OD_{\lambda 660}$ 0.4) were exposed to 4-NQO (4-Nitroquinoline) 0.4 mg/l from Sigma. Aliquots were taken at the desired time to analyse the amount of dNTPs, polyP, or some proteins, as described in other parts of this section.

2.10. Measurement of DNA damage sensitivity in HEK293 cells

Human embryonic kidney HEK293 cells were grown in DMEM (Sigma) supplemented with 10% heat-inactivated FBS (Gibco), 2-mM L-glutamine (Sigma), 4 units/ml of penicillin, and 20 mg/ml of streptomycin (Gibco). Cells were transfected with 20 µg of the desire construct using the calcium phosphate method. At 16 h after transfection, the medium was replaced with the initial culture medium. The efficiency of overexpression of the different genes was assessed by immunoblot analysis, which demonstrated maximal effects at 48 h.

Transfected HEK293 cells carrying the pIRES2-eGFP empty vector, *scPPX1* or NLS-*scPPX1*, were seeded after 48 h of transfection on a 48-well plate at 7.5×10^4 cells/well by six-fold. At 24 h, the medium was removed, cells were irradiated with 8 mJ UV, and the medium was replaced. At 23 h, MTT at a final concentration of 0.2 mg/ml was added to each well and 1 h later the medium was removed and cells were lysed in the presence of DMSO. Absorbance was determined at 570 nm.

2.11. Lentiviral transduction

Lentiviral transduction was carried out following the manufacturer's instructions (Invitrogen). Briefly, HEK293T cells were used for virus production. HEK293T cells (5×10^6) were transfected with lentivirus and packaging (gag-pol, vsvg, rev) plasmids (Addgene) using the calcium phosphate method. Viruses were harvested 72 h post-transfection and concentrated using ultracentrifugation.

2.12. Measurement of DNA damage sensitivity in HDFa

HDFa cells were grown in M106 (Life technologies) supplemented with Low Serum Growth Supplement (Gibco), 4 units/ml of penicillin, 20 mg/ml of streptomycin (Gibco), and 10 µg/ml of gentamicin (Gibco). Cells were infected with the desired

lentivirus: pLenti6/V5-DEST, pLenti6/V5-DEST-scPPX1, or pLenti6/V5-DEST-NLS-scPPX1 with 8 µg/ml of polybrene. At 24 h after infection, medium was replaced with fresh culture medium. The efficiency of overexpression of the different genes was assessed by immunoblot assay, which demonstrated maximal effects at 48 h.

Infected HDFa cells carrying the pLenti6/V5-DEST empty vector, scPPX1, or NLS-scPPX1 were seeded after 48 h of infection on a 12-well plate at 46×10^3 cells/well by four-fold. At 24 h, the medium was removed, cells were irradiated with 6 mJ UV, and the medium was replaced. At 23 h, MTT at a final concentration of 0.2 mg/ml was added to each well and 1 h later the medium was removed and cells were lysed with DMSO. Absorbance was determined at 570 nm.

2.13. Statistical analysis

At least 3 independent experiments were performed. Data were expressed as mean value $\pm$ standard error of the mean (SEM). Statistical significance was determined using the Mann-Whitney U test. $P < 0.05$ was considered significant.

3. Results

3.1. *polyP is degraded after DNA damage*

Among its many functions, polyP is a source of Pi for biosynthesis purposes. Recently, we showed that polyP is also involved in providing Pi for the massive synthesis of dNTPs that takes place when a yeast genome is about to be replicated {{581 Bru,S. 2016; 610 Jimenez,J. 2016}} and Fig. 2A.

In addition to its role as an essential component for DNA synthesis, dNTPs are also needed for DNA repair after damage and, consequently, dNTPs concentration suddenly increase when DNA damage occurs in order to maintain cell viability {{346 Chabes,A. 2003}}. This scenario led us to hypothesise that this increment may be supported, to some extent, by the hydrolysis of polyP. To test this hypothesis, we measured polyP and dNTPs levels before and after inducing DNA damage to exponentially growing yeast cells by adding 0.4 mg/l of 4-nitroquinoline (4-NQO), a molecule that mimics the UV radiation effect {{662 Ornstein,R.L. 1980}}. As predicted, after DNA damage we observed an increase in dNTPs levels that correlated with a reduction in polyP content (Fig. 2B). To further investigate the involvement of polyP as the supplier of Pi after DNA damage, we induced DNA damage by adding 4-NQO to *vtc4Δ* cells, which do not contain polyP {{581 Bru,S. 2016}}. The results, presented in Figs. 2C and 2D, show that *vtc4Δ* cells produced significantly less dNTPs than wild-type cells. Accordingly, the DNA damage persisted for longer in the *vtc4Δ* cells as denoted by the extended phosphorylation of Rad53 (Fig. 2E), a readout generally used to show the presence of DNA damage {{664 Lee,S.J. 2003}}.

3.2. *polyP mutants are more sensitive to DNA damage*

If Pi sourced from polyP hydrolysis is involved in the rise of dNTPs after DNA damage, as described above, we deduced that polyP mutants should be more sensitive to DNA damage. To confirm or refute this, we plated the different yeast polyP mutant strains in rich medium and immediately induced DNA damage by UV irradiation. As presented in Fig. 3A, the mutants *vtc4*Δ (without polyP) and *ppn1*Δ *ppx1*Δ (unable to degrade polyP) showed a 50% reduction in viability after UV irradiation. On the basis of these findings, we reasoned that mutants in the dNTP synthesis pathway (e.g. *adk1*Δ and *ynk1*Δ) should also be more sensitive to DNA damage, and indeed this was the case (Fig. 3A). We obtained similar viability results when the damaging agent used was MMS (Fig. 3B), suggesting that the protective effect of polyP is more general and not restricted to the UV damage only.

3.3. Accumulation of polyP protects yeast cells from DNA damage

To achieve further insight on the role of polyP in cell survival after DNA damage, we performed the opposite experiment, increasing the starting quantities of polyP in wild-type cells through an overloading protocol {{384 Werner,T.P. 2005}}. Using this experimental setup, we found a statistically significant correlation between the quantity of polyP and the percentage of survival cells upon irradiation (Fig. 3C).

Accordingly, when the quantity of the protein Vtc4 was increased in cells by overexpressing the *VTC4* gene from the episomal pRS426 plasmid, leading a polyP increase of approximately 20%, we also observed greater resistance to UV radiation when compared to cells bearing the empty plasmid (Fig. 3D). Taken together, these experiments firmly suggest that polyP is somehow involved in the process of DNA repair and cell survival after DNA damage.

3.4. polyP is an important factor for mammalian cells survival after DNA damage

To further our investigation, we aimed to determine whether the protective role of polyP after DNA damage is conserved in mammalian cells. Due to the fact that the mammalian enzymes responsible for the synthesis and degradation of polyP have not yet been identified, several authors have used the overexpression of the yeast gene *PPX1* as an efficient method to deplete polyP in cultured human cells {{412 Wang,L. 2003}}. Indeed, HEK293 cells overexpressing *PPX1* present reduced amounts of polyP (Fig. 4A) and, importantly, lower viability after UV radiation (Fig. 4B). In metazoan cells, dNTPs increase during UV recovery is most likely restricted to the foci where the DNA damage took place {{354 Niida,H. 2010}}, suggesting that the important fraction of polyP during DNA damage response in metazoan cells might be nuclear. Interestingly, HEK293 cells bearing the *PPX1* gene fused to a nuclear localization signal (NLS) were still sensitive to UV radiation (Fig. 4B and C), pointing to the nuclear polyP fraction as a possible resistance factor against UV damage in cultured cell lines.

Finally, we challenged the idea of the protective role of polyP in a more physiological model. UV sunlight reaches the dermis in human skin and induces chemical damage in dermal fibroblasts {{398 Sander,C.S. 2002}}. We transduced primary cultures of human dermal fibroblasts (HDFa) with a lentivirus carrying the 2 previously mentioned versions of *PPX1*. Dermal fibroblasts expressing *PPX1* were more sensitive to UV radiation than control cells (Fig. 4D), suggesting that polyP may act as a protective factor against UV damage in human skin.

4. Discussion

4.1. *polyP is needed for survival after DNA damage*

polyP has a broad array of functions, both in bacteria and in eukaryotic cells. It has recently been demonstrated that yeast polyP is a source of Pi for the synthesis of nucleotides during the DNA replication process. {{581 Bru,S. 2016}}. The hypothesis explored in the present work is centred on addressing whether polyP could also be necessary to sustain the synthesis of the extra amounts of dNTPs that appear during DNA repair after damage {{346 Chabes,A. 2003}}.

This hypothesis has been very much supported, at least in prokaryotes {{680 Gon,S. 2011}}. It has been reported that polyP is degraded after UV damage in cyanobacteria {{678 Noyma,N.P. 2015}}, polyP has also been found to regulate the activity of *E. coli* Y-family polymerases {{376 Stumpf,J.D. 2005}}. Indeed, the lesion-bypass activity of these polymerases has been observed to be lost when cells are unable to synthesize polyP {{679 Foster,P.L. 2007}}. In addition, polyP has also been reported to be required for the transcriptional induction of *E. coli* RecA (a protein that regulates DNA protective mechanisms) following DNA damage {{498 Tsutsumi,K. 2000}}, indicating that polyP could perform this protective role through diverse molecular mechanisms.

In the present work, we have demonstrated that polyP in yeast is needed for cell viability after DNA damage. One of the reasons for this effect is that polyP sustains the correct increase of dNTPs levels after DNA damage. However, looking carefully at these results, it is striking that the 40% reduction in dNTPs levels in the polyP mutants during the first 80 minutes (Fig. 2C) could produce such remarkable effect in terms of survival (Figs. 3A and B), perhaps because either the reduction in dNTPs concentration

trespasses an enzymatic activity threshold, partially inactivating the DNA repair polymerase, or other molecular mechanisms may be involved in sustaining this viability. Indeed, polyP may be necessary for maintaining energy in the form of the ATP needed for repairing damaged DNA in several organisms. {{579 Seki,Y. 2014; 685 Gartner,C. 1980; 684 Seki,S. 1987}}. In metazoans, the protein Nudix5 produces ATP from ADP-ribose and pyrophosphate {{674 Wright,R.H. 2016}}, opening up the question of whether polyP may be the source of Pi for both dNTPs and the energy burst required to repair damaged DNA.

The fact that yeast polyP is needed for cell survival after DNA damage leads us to a new interpretation of some unconnected pieces of information that were not completely understood in the field of polyP. For example, the fact that the polyphosphatase Ppn1 moves from the vacuole to the cytoplasm when dNTPs synthesis is inhibited by hydroxyurea {{371 Tkach,J.M. 2012}}, and that *vtc4* mutants are also sensitive to hydroxyurea {{413 Niu, W. 2008}}. Both phenotypes may be integrated into a scenario in which polyP plays a role in DNA repair.

4.2. polyP maintains the Pi homeostasis needed for DNA damage repair

Cells growing in rich media (with Pi largely available) are equipped with low affinity Pi transporters that can easily cope with internal Pi demand {{153 Wykoff,D.D. 2001; 420 Wykoff,D.D. 2007}}. Interestingly, the role of polyP as Pi supplier in DNA repair is clearly evident, even in Pi-rich medium, suggesting that when cells face a high and unexpected demand of Pi, they prefer to obtain Pi by hydrolysing polyP rather than use the relatively slow process of expressing, synthesizing, and locating high affinity Pi transporters in the membrane. This idea is supported by numbers: in rich media, a yeast cell has an estimated Pi transport activity of between 4.6×10^6 and 7.8×10^7

molecules/min {{153 Wykoff,D.D. 2001; 369 Giots,F. 2003; 370 Zvyagilskaya,R.A. 2008}}, which is approximately the amount of phosphate needed to support the constant synthesis of RNA and phospholipids (based on our previous calculation, {{581 Bru,S. 2016}}) and subsequent extra demands must be managed differently. Since DNA damage cannot be predicted by cells, they cannot equip themselves with alternative fast response mechanisms other than polyP.

In this context it must be noted that the polyP function in tackling the unexpected demands of phosphate has also been found in a different scenario: the switch from low glucose to high glucose media {{357 van Heerden,J.H. 2014; 414 van Heerden,J.H. 2014}}. This transition implies a sudden activation of glycolysis, resulting in a massive Pi consumption in a very short period of time. polyP is therefore essential in muffling sudden internal variations due to periodic situations such as glycolysis onset {{357 van Heerden,J.H. 2014; 414 van Heerden,J.H. 2014}}, DNA replication {{581 Bru,S. 2016; 610 Jimenez,J. 2016}}, and DNA repair, the subject of the present work.

4.3. polyP, a new UV protection factor in mammalian cells

The results presented here point to polyP as an important factor in the recovery from DNA damage in HEK293 renal cells and, more importantly, also in primary cultures of human dermal fibroblasts. Dermal fibroblast protection by polyP is especially relevant because these cells are components of the skin and thus they are the most exposed and vulnerable cells to UV insults in the human body {{398 Sander,C.S. 2002}}. Although our results indicate that polyP plays a protective role by sustaining dNTPs synthesis, the underlying molecular mechanism remains unknown.

DNA damage and the correct levels of dNTPs are important factors that are directly related to cancer {{450 Reichard,P. 1988; 681 Kunz,B.A. 1994; 683

Kohnken,R. 2015; 373 Bester,A.C. 2011}}. Here we present results supporting the hypothesis of the protective role of polyP against malignant transformation. Consequently, polyP and the molecular machinery involved in its metabolism, which remains unknown, have the potential to become targets for cancer treatment or prevention, like other repair mechanisms or pathways {{665 Helleday,T. 2008; 666 Kelley,M.R. 2014}}. However, further advancements in this field are needed to identify and describe the biochemical machinery involved in mammalian polyP metabolism, a project that our laboratory is fully engaged in.

5. Conclusions

- 1- polyP in yeast is consumed during DNA repair after damage
- 2- polyP is involved in providing Pi for the extra synthesis of the dNTPs needed for DNA repair in yeast.
- 3- Yeast and mammalian cells without polyP are more sensitive to DNA damage.

Acknowledgments

We would like to thank all the members of our group (E. Bállega, O. Mirallas, M. Ribeiro, A. Sánchez, B. Semper, and R. Carballar) for day-to-day talks, and Marta Pérez for technical assistance. This work was supported by funding from the Spanish Government, with a MINECO grant (Ref: BFU 2013-44189-P) awarded to J. Clotet and a MICINN grant (Ref: BFU 2010-20293) awarded to E. Garí. J.M.M. was the recipient of a post-graduate Junior Faculty Fellowship from the UIC and l'Obra Social la Caixa.

Figure Legends

Figure 1: The dNTPs synthesis pathway. Inorganic phosphate (Pi) is required for the synthesis of triphosphate deoxy-Nucleotides (dNTPs), both in the conversion from Nucleotide MonoPhosphate (NMP) to Nucleotide DiPhosphate (NDP) and in the conversion from deoxy-Nucleotide DiPhosphate (dNDP) to deoxy-Nucleotide TriPhosphate (dNTP). The enzymatic activities involved in these conversions in yeast are shown. Phosphate is stored in the cell in the form of polyP, which can be turned back into phosphate by the polyphosphates Ppn1 and Ppx1, and vice versa by the VTC complex.

Figure 2: dNTPs and polyP levels change upon DNA damage. A) Correlation between polyP and dNTP levels during the yeast cell cycle. Wild-type yeast cells were synchronized in G₁ with α -factor and released into fresh YPD medium. At the indicated times, samples were taken to measure polyP and dNTP. For polyP, data are presented as mean $\pm$ SEM from 4 independent experiments. The dNTP data was obtained from a single experiment, and the values represent the sum of the levels of the 4 dNTPs (dATP, dTTP, dCTP, and dGTP). The indicated length of each phase was determined by flow cytometry analysis. B) Correlation between polyP and dNTP levels upon DNA damage. 4-NQO at a final concentration of 0.4 mg/l was added to exponentially and non-synchronically growing wild-type yeast cells. At the indicated times, samples were taken to measure polyP and dNTP levels. For polyP, data are presented as mean $\pm$ SEM from 4 independent experiments. The dNTP data was obtained from a single experiment, and the values represent the sum of the levels of the 4 dNTPs. C) Relative dNTPs content in cells without polyP after DNA damage. The same experiment was performed as in B, but it included a *vtc4* Δ mutant strain that was unable to synthesize

polyP. The asterisk represents statistically significant differences ($p<0.05$). Values were relative to time 0. D) polyP content of wild-type and *vtc4Δ* cells after DNA damage. The same experiment was performed as in B, but it included a *vtc4Δ* mutant strain that was unable to synthesize polyP. The asterisk represents statistically significant differences ($p<0.05$). The values of polyP obtained in the *vtc4Δ* strain were virtually 0, the columns and bars only indicate that the experiments were performed. E) DNA damage lasts longer in *vtc4Δ* cells. The same experiment was performed as in B, with samples taken for analysis of electrophoretic migration of the protein Rad53 by Western Blot using specific antibodies. G6PDH was used as a loading control.

Figure 3: polyP is involved in the survival of yeast cells after DNA damage. A)

Strains that cannot use (or do not have) polyP are more sensitive to UV stress. Exponentially growing wild-type, *vtc4Δ*, and *ppn1Δ/ppx1Δ* cells were plated on YPD plates and UV irradiated (60J /m²). The plates were incubated at 30°C for 48 h and the resulting colonies were automatically counted. Strains with defects in dNTP synthesis were included in the experiment (*ynk1*, *dun1*, and *adk*). Mean +/- SEM from 4 independent experiments is shown. The asterisk indicates statistically significant differences ($p<0.05$) and the double asterisk indicates $p<0.01$. B) *vtc4Δ* cells with low levels of polyP strain are more sensitive to MMS stress. Wild-type and *vtc4Δ* exponentially growing cells in synthetic medium were incubated with 0.033% MMS during 4 h, then plated on YPD plates. The plates were incubated at 30°C for 48 h and the resulting colonies were automatically counted. Mean +/- SEM from 3 independent experiments is shown. The asterisk indicates statistically significant differences ($p<0.05$). C) In wild-type cells, a higher level of polyP correlates with greater survival after UV stress. Wild-type cells were overloaded with polyP by changing the growing conditions (see Materials and Methods). Under these special conditions, overloaded

wild-type cells present 3 times more polyP than wild-type cells growing under normal conditions (not shown). Both cultures were plated in YPD and irradiated with 40J /m². The plates were incubated at 30°C for 48 h and the resulting colonies were automatically counted. Mean +/- SEM from 3 independent experiments is shown. The asterisk indicates statistically significant differences ($p<0.05$). D) Overexpression of *VTC4* confers resistance to UV stress. *VTC4* was cloned in a multicopy pRS426 vector and transformed in a wild-type strain. Cells with an empty vector or with pRS426-*VTC4* were plated in YPD and irradiated with 60J /m². The plates were incubated at 30°C for 48 h and the resulting colonies were automatically counted. Mean +/- SEM from 3 independent experiments is shown. The asterisk indicates statistically significant differences ($p<0.05$).

Figure 4: polyP depletion reduces survival rates of mammalian cells after DNA damage. A) *PPX1* and *NLS-PPX1* overexpression produces polyP depletion in HEK293 cells. *PPX1* and a version in which a Nuclear Localization Signal (NLS) was added to *PPX1* were cloned in a pIRES vector and transfected in HEK293 cells before measuring the amount of polyP. Mean +/- SEM from 3 independent experiments is shown. The asterisks indicate statistically significant differences ($p<0.05$), while the double asterisk indicates $p<0.01$. B) Low levels of polyP decrease resistance to UV stress in mammalian cell line cultures. HEK293 cells were transfected with the same plasmid as in A). Cultured cells were irradiated with 80J /m² and viability was assessed by MTT assay. Mean +/- SEM from 3 independent experiments is shown. The asterisks indicate statistically significant differences ($p<0.05$) and the double asterisk indicates $p<0.01$. C) Ppx1 and NLS-Ppx1 are expressed in transfected HEK293 cells. Cells transfected with the noted constructs were processed and analyzed by Western blot, with tubulin used as loading control. D) Low levels of polyP decrease the resistance to UV stress in primary

HDFa cells. A primary culture of Human Dermal Fibroblasts (HDFa) transfected with the indicated lentivirus was irradiated with 60J /m². Viability was assessed by the MTT method after 24 h. Mean +/- SEM from 5 independent experiments is shown. The asterisk indicates statistically significant differences ($p<0.05$).

Figure 1

Figure 2

Figure 3

Figure 4