A Soluble Pyrophosphatase Is Essential to Oogenesis and Is Required for Polyphosphate Metabolism in the Red Flour Beetle (*Tribolium castaneum*)

Klébea Carvalho 1, Lupis Ribeiro 1, Jorge Moraes 1,2, José Roberto da Silva 1,2, Evenilton P. Costa 3, Jackson Souza-Menezes 1, Carlos Logullo 2,3, Rodrigo Nunes da Fonseca 1,2,* and Eldo Campos 1,2,†,*

1 Laboratory of Integrated Biochemistry—Hatisaburo Masuda, Universidade Federal do Rio de Janeiro, Núcleo em Ecologia e Desenvolvimento Sócio Ambiental de Macaé, Avenida São José do Barreto, 764, São José do Barreto, Macaé, RJ CEP 27965-045, Brazil; E-Mails: klebeacarvalho@gmail.com (K.C.); lupisribeiro@gmail.com (L.R.); jorgemoraes@bioqmed.ufrj.br (J.M.); beto_cfrio@yahoo.com.br (J.R.S.); jacksonmenezes@gmail.com (J.S.-M.); rodrigo.nunes.da.fonseca@gmail.com (R.N.F.)

2 Nacional Institute of Science and Technology—Molecular Entomology, Rio de Janeiro, RJ CEP 21941-590, Brazil; E-Mail: logullo@uenf.br

3 Laboratory of Chemistry and Function of Proteins and Peptides and Unity of Animal Experimentation, Biotecnology and Bioscience Center, Universidade Estadual do Norte Fluminense, Avenida Alberto Lamego, 2000, Horto, Campos dos Goytacazes, RJ CEP 28015-620, Brazil; E-Mail: eveniltonpessoa@yahoo.com.br

† These authors contributed equally to this work.

* Author to whom correspondence should be addressed; E-Mail: eldocampos@macae.ufrj.br; Tel.: +55-022-3399-3967.

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**Abstract:** Polyphosphates have been found in all cell types examined to date and play diverse roles depending on the cell type. In eukaryotic organisms, polyphosphates have been mainly investigated in mammalian cells with few studies on insects. Some studies have demonstrated that a pyrophosphatase regulates polyphosphate metabolism, and most of them were performed on trypanosomatids. Here, we investigated the effects of *sPPase* gene knocked down in oogenesis and polyphosphate metabolism in the red flour beetle (*Tribolium castaneum*). A single *sPPase* gene was identified in insect genome and
is maternally provided at the mRNA level and not restricted to any embryonic or extraembryonic region during embryogenesis. After injection of Tc-sPPase dsRNA, female survival was reduced to 15% of the control (dsNeo RNA), and egg laying was completely impaired. The morphological analysis by nuclear DAPI staining of the ovarioles in Tc-sPPase dsRNA-injected females showed that the ovariole number is diminished, degenerated oocytes can be observed, and germarium is reduced. The polyphosphate level was increased in cytoplasmic and nuclear fractions in Tc-sPPase RNAi; Concomitantly, the exopolyphosphatase activity decreased in both fractions. Altogether, these data suggest a role for sPPase in the regulation on polyphosphate metabolism in insects and provide evidence that Tc-sPPase is essential to oogenesis.

**Keywords:** polyphosphate; pyrophosphatase; oogenesis; embryogenesis; insect

1. Introduction

Inorganic polyphosphates (poly P) are long chains of a few to several hundred phosphate residues linked by phosphoanhydride bonds. Taking into consideration their significance in all living organisms, inorganic polyphosphates may be separated into two groups—Namely, pyrophosphate and high-molecular-weight poly Ps—Which contain three to several hundred phosphate residues in one molecule [1]. Polyphosphates have been found in all cell types examined to date and play diverse roles depending on the cell type. The biological roles played by polyphosphates have been most extensively studied in prokaryotes and unicellular eukaryotes [2,3]. In higher eukaryotes, poly P have not been extensively investigated, although a role in the activation of Tor kinase [4], blood coagulation [5], bone tissue development [6], and apoptosis [7] have been reported.

Poly P metabolism can be specific in different cellular compartments [3,8–10]. In nuclei, for microorganisms, it has been reported that poly P control gene expression [11] is a potent inhibitor of the degradosome-dependent degradation of mRNA [12] and a promoter of ribosomal [13] and nucleoid protein degradation by activating Lon protease [14]; It also assists in the fidelity of protein translation by its interaction with ribosomes [15]. In yeast, poly P interact and inhibit poly(A) polymerase activities [16,17]. In mammalian cells, poly P accumulates and regulates myeloma proliferation [18].

To understand the relationship between poly P metabolism and its cellular distribution, studies on poly P composition and on the enzymes required for its metabolism were performed [10,19]. Soluble inorganic pyrophosphatases (sPPases, EC 3.6.1.1) catalyze the hydrolysis of inorganic pyrophosphate (PPI), which is formed mainly as a product of many biosynthetic pathways, including oligosaccharide and fatty acid synthesis, tRNA charging/amino acid activation, and polynucleotide synthesis [20]. In nuclei, pyrophosphate is generated as a metabolite of replication, transcription, and DNA repair. During nucleic acid polymerization, the incorporation of a nucleoside in the growing chain with the concomitant liberation of pyrophosphate is a readily reversible reaction, and the hydrolysis of pyrophosphate by sPPase eliminates metabolite inhibition, therefore, shifting the reaction equilibrium in favor of polymerization [21]. Polyphosphate hydrolysis is catalyzed by exo- and endopolyphosphatases [3].
Exopolyphosphatases (PPX—Polyphosphate-phosphohydrolases; EC 3.6.1.11) splits P_i off the end of a poly P chain and are considered as the central regulatory enzymes in poly P metabolism [9,22].

To our knowledge, a few studies have demonstrated that a pyrophosphatase regulates polyphosphate metabolism, and most of them were performed on trypanosomatids [19,22,23]. Here, we investigated the polyphosphate metabolism in the red flour beetle, Tribolium castaneum, which is a common pest that has emerged as an excellent model system for studying development and metabolism in insects [24–26]. This beetle is amenable to gene knockdown by RNA interference (RNAi). Any gene can be knocked down at any stage in all tissues upon injection of double-stranded RNA (dsRNA) [27,28], have its genome sequenced [29], and have developed mutants [30].

In the present work, we analyzed the effects of the sPPase gene knocked down in oogenesis and polyphosphate metabolism. Our results show that PPase is essential to oogenesis and plays a role in poly P metabolism in cytoplasmic and nuclei fractions.

2. Results and Discussion

Thus far, poly P metabolism has been mainly investigated in mammalian cells [5,31–33], with few studies in insects [34–36]. Since all previous analyses of pyrophosphatase regulating polyphosphate metabolism were performed in trypanosomatids, we sought to investigate the enzymes involved in the red flour beetle (Tribolium castaneum).

2.1. A Single Soluble Inorganic Pyrophosphatases (sPPases) Can Be Identified in Tribolium castaneum and Other Insect Genomes

BLAST searches using the previously reported nuclear, cytosolic, or acidocalcisomal sPPases [22] against the Tribolium castaneum genome lead to a single ortholog, the Tc-004566 gene. Other insect genomes also display a single locus belonging to sPPases (Figure 1), suggesting that the previously reported paralogs with distinct functions in trypanosomatids or humans [22] probably arose due to independent duplication events.

Since the T. castaneum genome displays a single sPPase ortholog, we sought to analyze its expression by in situ hybridization during embryogenesis. Tc-sPPase (Tc-004566) is maternally provided at the mRNA level and not restricted to any embryonic or extraembryonic region during embryogenesis (Figure 2A), probably due to the requirement of energy sources and phosphates for early proliferation stages of embryogenesis. During germ band elongation, when the embryo can be readily identified, the expression can be detected in the embryo and in the extraembryonic tissue (Figure 2B). In contrast, the Tc-zen gene is specifically expressed at the extraembryonic serosal region (Figure 2C), as previously described [37].
Figure 1. A single sPPase is present in insect genomes. Gene bank accession numbers are as follows: XP 558852.4 Anopheles gambiae (Agam-sPPase), ABF18311.1 Aedes aegypti (Aaeg-sPPase), EEZ98942.1 TC004566 Tribolium castaneum (Tcas-sPPase), AAC97112.1 NURF-38 Drosophila melanogaster (Dmel-sPPase-NURF-38), XP 001976623.1 Drosophila erecta (Derc-sPPase), XP 00813751.1 Acyrthosiphon pisum (Apis-sPPase), XP 001604166.1 Nasonia vitripennis (Nvit-sPPase), XP 003249382.1 Apis mellifera (Amel-sPPase), P19117 Schizosaccharomyces pombe (Spom-sPPase), Q6MVH7 Neurospora crassa (Ncra-sPPase), O13505 Komagataella pastoris (Kpas-sPPase), P13998 Kluyveromyces lactis (Klac-sPPase), P00817 Saccharomyces cerevisiae (Scer-sPPase), Q9C0T9 Zygosaccharomyces bailii (Zbai-sPPase), Q9H2U2 Homo sapiens (Hsap-sPPase), Q91VM9 Mus musculus (Mmus-sPPase), Q15181 Homo sapiens (Hsap2-sPPase), Q9D819 Mus musculus (Mmus2-sPPase), Q6CPN0 Kluyveromyces lactis (Klac2-sPPase), Q6BLR8 Debaryomyces hansenii (Dhan-sPPase), Q9LXC9 Arabidopsis thaliana (Atha-sPPase), Q93Y52 Chlamydomonas reinhardtii (Chei-sPPase), Q7Z031 Leishmania amazonensis (Lama-sPPase), O48556 Zea mays (Zmay-sPPase), O23979 Hordeum vulgare (Hvul-sPPase), Q9SWI0 Populus tremula (Tret-sPPase), Q0DYB1 Oryza sativa (Osat-sPPase), O49949 Solanum tuberosum (Stub-sPPase), P38576 Thermus thermophiles (Tier-sPPase), O34955 Legionella pneumophila (Lpne-sPPase), P51064 Bartonella bacilliformis (Bbac-sPPase), O67501 Aquifex aeolicus (Aaeo-sPPase), O05545 Gluconobacter oxydans (Goxy-sPPase), Q9ZCW5 Rickettsia prowazekii (Rpro-sPPase). Substitution model used LG + G + I with 100 bootstraps.
Figure 2. Tc-pyrophosphatase (Tc-sPPase) is maternally provided and expressed during embryogenesis. (A) Freshly laid Tribolium castaneum egg showing Tc-sPPase expression; (B) Tc-sPPase expression during germ band elongation; (C) Tc-zen expression at the serosa during blastoderm differentiation; and (D) In situ hybridization using a Tc-sPPase probe sense control. Scale bar = 200 µm.

2.2. pRNAi Analysis Shows that Tc-sPPase Is Essential to Oogenesis and Regulates Poly P Metabolism

To investigate if Tc-sPPase is important for oogenesis and how its absence would affect poly P metabolism, parental RNAi (pRNAi) was performed as previously described for several other genes in this species [38,39]. In all experiments, we injected the unrelated dsRNA neomycin as a negative control in a separate batch of females. These neomycin dsRNA females laid the normal amount of eggs, which hatched as larvae, indicating that the injection of unrelated dsRNA had no effect on T. castaneum development.

We analyzed Tc-sPPase expression in control (dsNeo) and Tc-sPPase RNAi by real-time PCR. Injection of Tc-sPPase dsRNA (1 µg/µL) almost completely inhibited its expression when compared to the control (Figure 3A), confirming that Tc-sPPase transcription was affected. After injection of Tc-sPPase dsRNA, female survival was also reduced to 15% of the control (Figure 3B), and egg laying was completely impaired (Figure 3C), suggesting that Tc-sPPase is essential to oogenesis.
Figure 3. (A) Comparison of Tc-sPPase in control (dsNeo RNA) and sPPase dsRNA injected adults. Normalized levels to Tc-Rps3 expression as previously described [26]; (B) Adult survival in control and sPPase dsRNA injected beetles (%); and (C) Egg laying of control and sPPase dsRNA–injected beetles (%). Asterisk indicates that the difference between the two groups is statistically significant (p < 0.05).

These results stimulated the analysis of the morphology of Tc-sPPase RNAi ovaries. The morphological analysis by nuclear DAPI staining of the ovarioles of control and Tc-sPPase dsRNA–injected females showed clear differences (Figure 4). T. castaneum control ovaries display several tubelike projections, the ovarioles (e.g., [40]), which contains oocytes in different stages of maturation. In control ovaries, larger eggs are present in the distal part of the ovariole (Figure 4A,B). However, after Tc-sPPase RNAi injection, the ovariole number is diminished, degenerated oocytes can be observed, and germarium is reduced (Figure 4C,D). In strong Tc-sPPase RNAi (20%), a complete degradation of the ovarioles was observed (Figure 4E,F). This result reinforces the essential role of Tc-sPPase in oogenesis.

To evaluate the effect of Tc-sPPase RNAi in poly P metabolism, we determined in cytoplasmic and nuclear fractions the poly P content and PPX activity of wild-type, control (dsNeo RNA) and Tc-sPPase dsRNA injected-females. The poly P level was increased in cytoplasmic and nuclear fractions by factors of 2 and 1.5, respectively, in Tc-sPPase RNAi when compared to control (Figure 5A,B). Concomitantly, PPX activity decreased to levels of about 70% of the control in both fractions. Heparin, a PPX inhibitor, was used as control and completely abolished PPX activity (Figure 5C,D). These data suggest a role for sPPase in the regulation of poly P metabolism. Previous studies have found that a pyrophosphatase plays a key role in poly P metabolism as an alternative to exopolyphosphatase activity in eukaryotic cells, being required in poly P metabolism, metacyclogenesis, and virulence in mice [19,22]. However, both studies were performed in trypanosomatids, to the best of our knowledge; This is the first study to demonstrate that the pyrophosphatase plays a key role in poly P metabolism in an insect.
Figure 4. (A) Control dsNeoRNA ovarioles; (B) Nuclear DAPI (4',6-diamidino-2-phenylindole) staining of the ovary as in (A); (C) sPPase dsRNA–injected ovarioles; (D) Nuclear DAPI staining of the ovary in (C); (E) sPPase dsRNA–injected ovarioles; and (F) Nuclear DAPI staining of the ovary in (E).

Figure 5. Cont.
Figure 5. (A) Cytoplasm poly P fluorescence (DAPI) of wild-type, control (dsNeo RNA), and sPPase RNAi beetles; (B) Nuclear poly P fluorescence (DAPI) of wild-type, control (dsNeo RNA), and sPPase RNAi beetles; (C) Cytoplasmic PPX activity in wild-type, control (dsNeo RNA), and PPX activity in the presence and absence of heparin; and (D) Nuclear PPX activity in wild-type, control (dsNeo RNA), and PPX activity in the presence and absence of heparin. Data are the mean ± S.E. (standard error) of three independent experiments, in triplicate. * p < 0.05.

The sequence analysis of Tc-PPase revealed that it is homologue to NURF-38 from Drosophila melanogaster [21]. The Drosophila NURF is a component of several macromolecular complexes involved in nucleosome dynamics or histone metabolism, and both the NURF-38 protein and the purified NURF complex were found to possess inorganic sPPase activity [21]. We propose that sPPase gene knockdown may disturb gene expression in two ways: By NURF complex alteration and/or by nuclear poly P increase. Nuclear poly P may interact with DNA-histone binding in chromatin, and this binding has been shown to inhibit the activity of some nuclear enzymes, including topoisomerases [41]. In general, we propose that the extensive morphological effects observed during oogenesis could be caused by disturbance of gene expression required for embryo formation.

3. Experimental Section

3.1. Tribolium castaneum Strains

San Bernardino beetles are reared at 30 °C in wheat flour supplemented with 5% dried yeast. The beetles were maintained inside plastic boxes of approximately 15 cm × 15 cm with humidity between 40% and 80% as previously described [42].

3.2. Sequence Analysis

sPPases were identified in the Tribolium castaneum genome [29] using the BLAST program (NCBI). Amino acid alignment and analysis of sPPase similarity for selected species were performed using the ClustalW multiple sequence alignment program (http://www.ebi.ac.uk/clustalw). Maximum likelihood
phylogenies were generated with PhyML [43]. Trees were edited in MEGA5.05 [44]. The accession numbers of the various sequences used in that study are described in the figure legend.

3.3. Primer Design, in Situ Hybridization and RNAi

Primer sequences containing adaptor sequences (lowercase letters) were designed for in situ hybridization and dsRNA synthesis with the help of Primer3 (www.ncbi.nlm.nih.gov/tools/primer-blast/) ggcgcgggTCACGATGCCTTTTGTGG (Forward) and cccgggGACCTTTTGCGTTGCGAAT (Reverse) and the PCR product size was 736 bp. A second primer pair led to identical results (data not shown). Double-stranded RNA (dsRNA) was synthesized using T7 MEGAScript (Ambion, Austin, TX, USA), purified and injected in adult females as previously described [45]. In situ hybridization was performed using digoxigenin-labeled RNA probes and revealed with alkaline phosphatase chromogenic substrate BM Purple (Roche, Indianapolis, IN, USA). The one color in situ protocol for Tribolium was done as described [46] followed by nuclear DAPI staining (4,6-diamidino-2-phenylindole) before documentation. A sense probe was included during in situ hybridization experiments and did not show any specific staining.

3.4. Real-Time PCR

Total RNA was isolated from 100 mg of eggs using TrizolH (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. First-strand complementary DNA (cDNA) was synthesized using Superscript III reverse transcriptase (Invitrogen), and real-time PCR analysis using SYBR green-based detection was performed. For real-time PCR, the following primer pair was used: Fwd-CGCTTTTAATGGCGAAGCGA and Rev-AGGAAATGCCCTTGGCATCA leading to an amplicon of 113 bp. The reactions were carried out in triplicate, and melting curves were examined to ensure single products. The results were quantified using the “ΔΔCt” method and normalized to rps3 transcript levels and to control genotypes [47]. Data shown are averages and standard deviations from at least three independent experiments.

3.5. Cell Fractionation

Adult female was used to obtain the cytoplasmic and nuclear fractions. The adult female were homogenized in 1 mL of an isolation buffer containing 0.5 M sucrose, 50 mM Tris-HCl (pH 7.4), 100 mM leupeptin, and 100 nM pepstatin. The homogenate was centrifuged at 500×g for five minutes to remove unbroken cell and other debris. The supernatant was carefully removed and centrifuged at 2000×g for 10 min to yield a nuclear pellet. Then, the supernatant was submitted to another centrifugation at 100,000×g for one hour to obtain the cytoplasmic fraction in the supernatant. The nuclear pellet was rinsed with cold isolation buffer and resuspended.

3.6. Exopolyphosphatase Activity

The reaction mixture consisted of 50 mM Tris-HCl buffer (pH 7.5), 5 mM MgCl2, and 5 mM polyP15, as the substrate. The reaction was carried out at 30 °C for 15 min. The activity was measured spectrophotometrically (UV Mini 1240, Shimadzu, Tokyo, Japan) by determining the rate of the Pi
formed during the reaction as described by [48]. The measurements of absorbance at 750 nm were performed after 15 min. Protein concentration was measured as described by [49], using bovine serum albumin as standard. The enzyme amount liberating 1 μmol of Pi per one minute was defined as one unit of enzyme activity (U).

3.7. Poly P Extraction and Quantification

The cytoplasmic and nuclear fractions were mixed with equal volume of acid phenol/chloroform, pH 4. The samples were vortexed for 5 min at 4 °C, followed by centrifugation at 1500× g for 5 min at 4 °C. The water phase was transferred to a new tube and subjected to chloroform extraction with the equal volume of chloroform to remove traces of organic solvents from the water phase. Poly P was precipitated from the water phase by adding 2.5 volumes of ethanol, followed by overnight incubation at −20 °C. The water-ethanol mixture was centrifuged for 10 min at 10,000× g. The resulting pellet containing poly P was resuspended in 50 μL of a buffer (0.1% SDS, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4) treated with RNase and DNase to remove nucleic acid contamination [34,35,50].

DAPI fluorescence was measured using a spectrofluorometer (Cary Eclipse, Agilent Technologies, Santa Clara, CA, USA). A Teflon stir bar was used to continuously mix the sample during fluorescence measurements. The nuclear or cytoplasmic fractions were incubated with 20 mg/mL DAPI for 30 min at room temperature. The fluorescence level at 550 nm obtained using 415 nm as the excitation wavelength was used to estimate the variation of poly P. PolyP75 (150 µg) was used to normalize the values obtained. The values of poly P were expressed as a function of the relative fluorescence signal obtained from fractions and polyP75 [35,51].

3.8. Statistical Analysis

Comparisons between groups were made by the non-paired Student’s t test and ANOVA One-way analysis of variance, using GraphPad Prism. For all tests, a difference of $p < 0.05$ was considered to be significant.

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Author Contributions

Conceived and designed the experiments: Eldo Campos, Rodrigo Nunes da Fonseca; Performed the experiments: Klébea Carvalho, Lupis Ribeiro; Analyzed the data: Jorge Moraes, Jackson Souza-Menezes, José Roberto da Silva, Evenilton P. Costa, Carlos Logullo, Jorge Moraes, Rodrigo Nunes da Fonseca;
Contributed reagents/materials/ analysis tools: Carlos Logullo, Rodrigo Nunes da Fonseca, Eldo Campos; and Wrote the paper: Eldo Campos, Rodrigo Nunes da Fonseca.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Kulaev, I.S.; Vagabov, V.M. Polyphosphate metabolism in microorganisms. Adv. Microb. Physiol. 1983, 24, 83–171.
2. Kornberg, A.; Rao, N.N.; Ault-Riche, D. Inorganic polyphosphate: A molecule of many functions. Annu. Rev. Biochem. 1999, 68, 89–125.
3. Kulaev, I.; Kulakovskaya, T. Polyphosphate and phosphate pump. Annu. Rev. Microbiol. 2000, 54, 709–734.
4. Wang, L.; Fraley, C.D.; Faridi, J.; Kornberg, A.; Roth, R.A. Inorganic polyphosphate stimulates mammalian TOR, a kinase involved in the proliferation of mammary cancer cells. Proc. Natl. Acad. Sci. USA 2003, 100, 11249–11254.
5. Smith, S.A.; Mutch, N.J.; Baskar, D.; Rohloff, P.; Docampo, R.; Morrissey, J.H. Polyphosphate modulates blood coagulation and fibrinolysis. Proc. Natl. Acad. Sci. USA 2006, 103, 903–908.
6. Doi, K.; Kubo, T.; Takeshita, R.; Kajihara, S.; Kato, S.; Kawazoe, Y.; Shiba, T.; Akagawa, Y. Inorganic polyphosphate adsorbed onto hydroxyapatite for guided bone regeneration: An animal study. Dent. Mater. J. 2014, 33, 179–186.
7. Kawano, M.M. Inorganic polyphosphate induces apoptosis specifically in human plasma cells. Haematologica 2006, 91, 1154A.
8. Campos, E.; Facanha, A.R.; Costa, E.P.; da Silva Vaz, I., Jr.; Masuda, A.; Logullo, C. Exopolypophosphatases in nuclear and mitochondrial fractions during embryogenesis of the hard tick Rhipicephalus (Boophilus) microplus. Comp. Biochem. Physiol. Part B 2008, 151, 311–316.
9. Kulaev, I.S.; Vagabov, V.M.; Kulakovskaya, T.V.; Lichko, L.P.; Andreeva, N.A.; Trilisenko, L.V. The development of A. N. Belozersky’s ideas in polyphosphate biochemistry. Biochemistry (Mosc.) 2000, 65, 271–278.
10. Kulakovskaya, T.; Kulaev, I. Enzymes of inorganic polyphosphate metabolism. Biomed. Inorg. Polym. 2013, 54, 39–63.
11. Rao, N.N.; Gomez-Garcia, M.R.; Kornberg, A. Inorganic polyphosphate: Essential for growth and survival. Annu. Rev. Biochem. 2009, 78, 605–647.
12. Blum, E.; Py, B.; Carpousis, A.J.; Higgins, C.F. Polyphosphate kinase is a component of the Escherichia coli RNA degradosome. Mol. Microbiol. 1997, 26, 387–398.
13. Kuroda, A.; Nomura, K.; Ohtomo, R.; Kato, J.; Ikeda, T.; Takiguchi, N.; Ohtake, H.; Kornberg, A. Role of inorganic polyphosphate in promoting ribosomal protein degradation by the Lon protease in E. coli. Science 2001, 293, 705–708.
14. Kuroda, A.; Nomura, K.; Takiguchi, N.; Kato, J.; Ohtake, H. Inorganic polyphosphate stimulates lon-mediated proteolysis of nucleoid proteins in Escherichia coli. Cell. Mol. Biol. 2006, 52, 23–29.
15. McInerney, P.; Mizutani, T.; Shiba, T. Inorganic polyphosphate interacts with ribosomes and promotes translation fidelity in vitro and in vivo. *Mol. Microbiol.* **2006**, *60*, 438–447.

16. Holbein, S.; Freimoser, F.M.; Werner, T.P.; Wengi, A.; Dichtl, B. Cordycepin-hypersensitive growth links elevated polyphosphate levels to inhibition of poly(A) polymerase in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **2008**, *36*, 353–363.

17. Sillero, M.A.; de Diego, A.; Silles, E.; Osorio, H.; Sillero, A. Polyphosphates strongly inhibit the tRNA dependent synthesis of poly(A) catalyzed by poly(A) polymerase from *Saccharomyces cerevisiae*. *FEBS Lett.* **2003**, *550*, 41–45.

18. Jimenez-Nunez, M.D.; Moreno-Sanchez, D.; Hernandez-Ruiz, L.; Benitez-Rondan, A.; Ramos-Amaya, A.; Rodriguez-Bayona, B.; Medina, F.; Brieva, J.A.; Ruiz, F.A. Myeloma cells contain high levels of inorganic polyphosphate which is associated with nucleolar transcription. *Haematologica* **2012**, *97*, 1264–1271.

19. Lemercier, G.; Espiau, B.; Ruiz, F.A.; Vieira, M.; Luo, S.; Baltz, T.; Docampo, R.; Bakalara, N. A pyrophosphatase regulating polyphosphate metabolism in acidocalcisomes is essential for *Trypanosoma brucei* virulence in mice. *J. Biol. Chem.* **2004**, *279*, 3420–3425.

20. Baykov, A.A.; Cooperman, B.S.; Goldman, A.; Lahti, R. Cytoplasmic inorganic pyrophosphatase. *Inorg. Polyphosphates* **1999**, *23*, 127–150.

21. Gdula, D.A.; Sandaltzopoulos, R.; Tsukiyama, T.; Ossipow, V.; Wu, C. Inorganic pyrophosphatase is a component of the *Drosophila* nucleosome remodeling factor complex. *Genes Dev.* **1998**, *12*, 3206–3216.

22. Espiau, B.; Lemercier, G.; Ambit, A.; Bringaud, F.; Merlin, G.; Baltz, T.; Bakalara, N. A soluble pyrophosphatase, a key enzyme for polyphosphate metabolism in *Leishmania*. *J. Biol. Chem.* **2006**, *281*, 1516–1523.

23. Galizzi, M.; Bustamante, J.M.; Fang, J.; Miranda, K.; Soares Medeiros, L.C.; Tarleton, R.L.; Docampo, R. Evidence for the role of vacuolar soluble pyrophosphatase and inorganic polyphosphate in *Trypanosoma cruzi* persistence. *Mol. Microbiol.* **2013**, *90*, 699–715.

24. Brown, S.J.; Shippy, T.D.; Miller, S.; Bolognesi, R.; Beeman, R.W.; Lorenzen, M.D.; Bucher, G.; Wimmer, E.A.; Klingler, M. The red flour beetle, *Tribolium castaneum* (Coleoptera): A model for studies of development and pest biology. *Cold Spring Harb. Protoc.* **2009**, *2009*, pdb emo126.

25. Fraga, A.; Ribeiro, L.; Lobato, M.; Santos, V.; Silva, J.R.; Rezende, G.; Gomes, H.; Moraes, J.; Logullo, C.; Campos, E.; *et al.* Glycogen and glucose metabolism are essential for early embryonic development of the red flour beetle *Tribolium castaneum*. *PLoS ONE* **2013**, *8*, e65125.

26. Shippy, T.D.; Coleman, C.M.; Tomoyasu, Y.; Brown, S.J. Concurrent in situ hybridization and antibody staining in red flour beetle (*Tribolium*) embryos. *Cold Spring Harb. Protoc.* **2009**, *2009*, pdb prot5257.

27. Miller, S.C.; Miyata, K.; Brown, S.J.; Tomoyasu, Y. Dissecting systemic RNA interference in the red flour beetle *Tribolium castaneum*: Parameters affecting the efficiency of RNAi. *PLoS ONE* **2012**, *7*, e47431.

28. Posnien, N.; Schinko, J.; Grossmann, D.; Shippy, T.D.; Konopova, B.; Bucher, G. RNAi in the red flour beetle (*Tribolium*). *Cold Spring Harb. Protoc.* **2009**, *2009*, pdb prot5256.
29. Tribolium Genome Sequencing, C.; Richards, S.; Gibbs, R.A.; Weinstock, G.M.; Brown, S.J.; Denell, R.; Beeman, R.W.; Gibbs, R.; Beeman, R.W.; Brown, S.J.; et al. The genome of the model beetle and pest *Tribolium castaneum*. *Nature* **2008**, *452*, 949–955.

30. Trauner, J.; Schinko, J.; Lorenzen, M.D.; Shippy, T.D.; Wimmer, E.A.; Beeman, R.W.; Klingler, M.; Bucher, G.; Brown, S.J. Large-scale insertional mutagenesis of a coleopteran stored grain pest, the red flour beetle *Tribolium castaneum*, identifies embryonic lethal mutations and enhancer traps. *BMC Biol.* **2009**, *7*, 73.

31. Harada, K.; Shiba, T.; Doi, K.; Morita, K.; Kubo, T.; Makihara, Y.; Piattelli, A.; Akagawa, Y. Inorganic polyphosphate suppresses lipopolysaccharide-induced inducible nitric oxide synthase (iNOS) expression in macrophages. *PLoS ONE* **2013**, *8*, e74650.

32. Wang, X.; Schroder, H.C.; Diehl-Seifert, B.; Kropf, K.; Schlossmacher, U.; Wiens, M.; Muller, W.E. Dual effect of inorganic polymeric phosphate/polyphosphate on osteoblasts and osteoclasts *in vitro*. *J. Tissue Eng. Regen. Med.* **2013**, *7*, 767–776.

33. Wang, X.; Schroder, H.C.; Grebenjuk, V.; Diehl-Seifert, B.; Mailander, V.; Steffen, R.; Schlossmacher, U.; Muller, W.E. The marine sponge-derived inorganic polymers, biosilica and polyphosphate, as morphogenetically active matrices/scaffolds for the differentiation of human multipotent stromal cells: Potential application in 3D printing and distraction osteogenesis. *Mar. Drugs* **2014**, *12*, 1131–1147.

34. Gomes, F.M.; Oliveira, D.M.; Motta, L.S.; Ramos, I.B.; Miranda, K.M.; Machado, E.A. Inorganic polyphosphate inhibits an aspartic protease-like activity in the eggs of *Rhodnius prolixus* (Stahl) and impairs yolk mobilization *in vitro*. *J. Cell. Physiol.* **2010**, *222*, 606–611.

35. Gomes, F.M.; Ramos, I.B.; Motta, L.M.; Miranda, K.; Santiago, M.F.; de Souza, W.; Machado, E.A. Polyphosphate polymers during early embryogenesis of *Periplaneta americana*. *J. Insect Physiol.* **2008**, *54*, 1459–1466.

36. Ramos, I.; Gomes, F.; Koeller, C.M.; Saito, K.; Heise, N.; Masuda, H.; Docampo, R.; de Souza, W.; Machado, E.A.; Miranda, K. Acidocalcisomes as calcium- and polyphosphate-storage compartments during embryogenesis of the insect *Rhodnius prolixus* Stahl. *PLoS ONE* **2011**, *6*, e27276.

37. Van der Zee, M.; Berns, N.; Roth, S. Distinct functions of the *Tribolium zerknu llt* genes in serosa specification and dorsal closure. *Curr. Biol.* **2005**, *15*, 624–636.

38. Baumer, D.; Strohlein, N.M.; Schoppmeier, M. Opposing effects of Notch-signaling in maintaining the proliferative state of follicle cells in the telotrophic ovary of the beetle *Tribolium*. *Front. Zool.* **2012**, *9*, 15.

39. Fu, J.P.; Posnien, N.; Bolognesi, R.; Fischer, T.D.; Rayl, P.; Oberhofer, G.; Kitzmann, P.; Brown, S.J.; Bucher, G. Asymmetrically expressed *axin* required for anterior development in *Tribolium*. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 7782–7786.

40. Lynch, J.A.; Peel, A.D.; Drechsler, A.; Averof, M.; Roth, S. EGF Signaling and the origin of axial polarity among the insects. *Curr. Biol.* **2010**, *20*, 1042–1047.

41. Schroder, H.C.; Lorenz, B.; Kurz, L.; Muller, W.E. Inorganic polyphosphate in eukaryotes: Enzymes, metabolism and function. *Prog. Mol. Subcell. Biol.* **1999**, *23*, 45–81.

42. Handel, K.; Grunfelder, C.G.; Roth, S.; Sander, K. Tribolium embryogenesis: A SEM study of cell shapes and movements from blastoderm to serosal closure. *Dev. Genes Evol.* **2000**, *210*, 167–179.
43. Guindon, S.; Lethiec, F.; Duroux, P.; Gascuel, O. PHYML Online—A web server for fast maximum likelihood-based phylogenetic inference. *Nucleic Acids Res.* **2005**, *33*, W557–W559.
44. Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **2011**, *28*, 2731–2739.
45. Bucher, G.; Scholten, J.; Klingler, M. Parental RNAi in *Tribolium* (Coleoptera). *Curr. Biol.* **2002**, *12*, R85–R86.
46. Stylianopoulou, E.; Lykidis, D.; Ypsilantis, P.; Simopoulos, C.; Skavdis, G.; Grigoriou, M. A rapid and highly sensitive method of non radioactive colorimetric *in situ* hybridization for the detection of mRNA on tissue sections. *PLoS ONE* **2012**, *7*, 1.
47. Lord, J.C.; Hartzler, K.; Toutges, M.; Oppert, B. Evaluation of quantitative PCR reference genes for gene expression studies in *Tribolium castaneum* after fungal challenge. *J. Microbiol. Methods* **2010**, *80*, 219–221.
48. Fiske, C.H.; Subbarow, Y. The nature of the “Inorganic Phosphate” in voluntary muscle. *Science* **1927**, *65*, 401–403.
49. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
50. Abramov, A.Y.; Fraley, C.; Diao, C.T.; Winkfein, R.; Colicos, M.A.; Duchen, M.R.; French, R.J.; Pavlov, E. Targeted polyphosphatase expression alters mitochondrial metabolism and inhibits calcium-dependent cell death. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 18091–18096.
51. Aschar-Sobbi, R.; Abramov, A.Y.; Diao, C.; Kargacin, M.E.; Kargacin, G.J.; French, R.J.; Pavlov, E. High sensitivity, quantitative measurements of polyphosphate using a new DAPI-based approach. *J. Fluoresc.* **2008**, *18*, 859–866.

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