eLife

Reviewed Preprint
v1 • March 14, 2025
Not revised

Reviewed Preprint
v2 • February 9, 2026
Revised by authors

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Competing interests: No competing interests declared

Funding: See page 19

Reviewing editor: Sonia Sen, Tata Institute for Genetics and Society, India

Probing metazoan polyphosphate biology using *Drosophila* reveals novel and conserved polyP functions

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eLife Assessment

Studying the biological roles of polyphosphates in metazoans has been a longstanding challenge to the field given that the polyP synthase has yet to be discovered in metazoans. This **important** study capitalizes on the sophisticated genetics available in the Drosophila system and uses a combination of methodologies to start to tease apart how polyphosphate participates in Drosophila development and in the clotting of Drosophila hemolymph. The data validating one of these tools (cyto-FLYX) are **solid** and well-documented and they will open up a field of research into the functional roles of polyP in a metazoan model. The other tools for tissue specific knockdown of polyP (Mito-FLYX, ER-FLYX, and Nuc-FLYX) have not yet been validated but will be invaluable to the field when they are.

https://doi.org/10.7554/eLife.104841.2.sa3

Abstract

Polyphosphate (polyP) exists in all life forms; however, its biological functions in metazoans are understudied. Here, we explored *Drosophila,* to our knowledge, as the first genetic model to explore polyP biology in metazoans. We established biochemical and *in situ* methods to detect, quantify, and visualise polyP in *Drosophila.* We then engineered a FLYX system to deplete polyP in subcellular compartments in a tissue-specific manner. Using these tools, we demonstrated a spatiotemporal and subcellular compartment-specific regulation of polyP levels in various developmental stages and tissue types. We discovered that polyP is crucial for *Drosophila* hemolymph clotting and proper developmental timing, consistent with an evolutionarily conserved role as exogenous polyP also accelerates mammalian blood clotting. Further, the transcriptomics analysis of polyP-depleted larvae demonstrates the impact of polyP on several cellular processes, including translation. These observations underscore the utility of the toolkit we developed to discover previously unknown polyP functions in metazoans.

Introduction

Inorganic polyphosphate (polyP), a linear polymer of variable chain lengths of orthophosphate (Pi) moieties linked via phosphoanhydride bonds, was first observed in the bacteria *Spirillum volutans* and was termed as volutin granules (1,2). Arthur Kornberg's works showed that polyP is present in archaea, eubacteria and mammals (3,4). Our current understanding of the biological functions and the molecular mechanisms of polyP predominantly stems from studies in prokaryotes and a few single-cell eukaryotes (e.g., *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and

Dictyostelium sp.). In bacteria, polyP is shown to serve as a phosphate reservoir, aid in biofilm formation, and help in transposon silencing (5–7). In algae, polyP acts as an antioxidant, metal chelator, and buffer against alkaline stresses (3,8–10). However, in metazoans, the biological functions of polyP have been underexplored, and thus, it used to be referred to as a molecular fossil.

Ex vivo and *in vitro* works in mammals have suggested the implications of polyP in blood coagulation, mitochondrial function, bone mineralisation, and neuronal activity (11–18). Moreover, recent works have discovered that several mammalian proteins, known to be involved in cellular processes such as transcription and translation, can bind to polyP. Binding to polyP may alter the localization, structure, or function of proteins, thereby affecting several biological processes in metazoans (19–21). Recent studies have also implicated polyP in human diseases, such as the pathogenesis of amyotrophic lateral sclerosis (ALS) (22). Further, a clinical trial has found beneficial effects of polyP in treating ulcerative colitis (23). Furthermore, feeding polyP to aβ-expressing *Caenorhabditis elegans* (Alzheimer's model) led to rescuing paralytic behaviour (5). Thus, it is emerging that polyP is not a relic; it affects several physiological processes in metazoans, including human diseases.

Efficient tools for quantification and genetic manipulation of polyP in prokaryotic models and yeast have been crucial in exploring polyP biology. Discovery of PolyP kinases (PPK1 in bacteria, VTC4 in yeasts) and Polyphosphatases (PPN2 in bacteria, ScPPX in yeasts) - enzymes to synthesise and degrade polyP in prokaryotes and yeasts allowed phenotypic studies in these organisms (24–30). In contrast, our knowledge of polyP and its roles remains limited in multicellular eukaryotes. Although recent work has reported that the FoF1 complex of ATPase in the mitochondria is required for polyP synthesis, PolyP kinases and polyphosphatases in multicellular organisms remain unknown (31). Purified human Prune is shown to have a short-chain exopolyphosphatase activity *in vitro* (32). Recently, biochemical experiments led to the discovery of endopolyphosphatase NUDT3, an enzyme known as a dinucleoside phosphatase (33). The function of these proteins in regulating polyP has yet to be tested *in vivo* in a genetically tractable metazoan model.

A significant gap in studying polyP in multicellular eukaryotes is the lack of a genetic model system that allows sensitive methods for visualising, quantifying, and manipulating polyP. *Drosophila melanogaster* provides an ideal platform to close this gap: it combines powerful genetics with short generation time and well-characterized development, enabling systematic interrogation of polyP dynamics in vivo. However, methods for sensitive quantification, visualization, and genetic perturbation of polyP have not been established in flies or any other metazoan model. Here, we report a toolkit to quantify, visualise, and genetically manipulate polyP levels in *Drosophila*. Using this toolkit, we show that polyP levels undergo temporally regulated fluctuations that can influence organismal development and physiology through possible evolutionarily conserved mechanisms. Overall, this work establishes *Drosophila* as a valuable *in vivo* model to dissect the functions of polyP in metazoans.

Results

Development of methods for the extraction and quantification of polyP from flies

The polyP levels have been shown to vary among species. Bacteria like *E.coli* maintain as low as 100uM polyP (Pi terms) which surges to nearly 50mM in stress conditions; yeasts have been reported to have a polyP content of ~100mM (Pi terms) whereas in mammalian cells, polyP levels vary in low micromolar concentrations (~10–90uM polyP in Pi terms) (3). Since metazoans have lower polyP content, to use *Drosophila* as a model system, it was essential to re-standardise a method sensitive enough to extract and quantify polyP from fly samples. Various biochemical strategies for extracting polyP from biological samples have been discussed, each with its own limitations (34). The acid-based extraction method fails to preserve the entire chain length of polyP, as polyP is unstable in strongly acidic conditions. In contrast, column purification can only

retain polyP of longer chain lengths (>60-80). PolyP extraction using saturated phenol (in citrate buffer pH 4.1) and chloroform, followed by ethanol precipitation, can efficiently pellet down polyP. However, this method also co-purifies RNA, which may interfere with polyP quantification. Thus, to extract polyP from *Drosophila* samples, we standardised the citrate-saturated phenol-chloroform-based method followed by RNase treatment (Fig 1A, Supplementary Fig 1A, See Materials and Methods).

To quantify polyP levels, several techniques based on Nuclear Magnetic Resonance (NMR), chromatography, radioactive isotope labelling, and enzymatic digestion have been used (35). We used the enzymatic detection method to quantify polyP extracted from fly tissues, as it has higher specificity and sensitivity (36,37). This method involved the digestion of polyP by recombinant *S. cerevisiae* exopolyphosphatase 1 (*Sc*PpX1), followed by colorimetric measurement of the released Pi by malachite green. Malachite green and ammonium molybdate, when mixed in a 3:1 ratio, form a metachromatic brown-coloured adduct that turns green upon conjugation with monophosphate (38,39) and can be quantified based on absorbance at 650 nm. Therefore, absorbance measurements from *Sc*PpX1 digested polyP can be interpolated to quantify the Pi released from extracted polyP samples. Thus, the intensity of the green colour is a measure of the free Pi, which in turn is a measure of the polyP levels. This assay allows the measurement of polyP content indirectly in terms of the liberated Pi (Supplementary Fig 1B, See Materials and Methods).

To standardise the polyP quantification method, we extracted polyP from fly larvae, and the extracted polyP was incubated with purified recombinant *Sc*PpX1 for 18 hours at 37°C to digest polyP completely. The other half was left untreated for 18 hours at 37°C. Malachite green was added to both samples, and the background from any preexisting Pi in the untreated sample was subtracted to quantify the Pi content. For calibration of the assay, increasing concentrations of KH_2PO_4 were used to draw a standard curve (37–39). The amount of recovered polyP was proportional to the number of larvae used for polyP extraction (Fig 1B). The polyP content in each third instar larval was found to be 419.30 ± 36.83 picomoles in Pi terms (Table 1), and the normalised polyP level is 46.8 ± 3.95 picomoles polyP (Pi terms) per milligram protein from the third instar larval lysate (Table 1).

PolyP levels are developmentally regulated

Given the various possible roles of polyP, we sought to test if polyP levels are developmentally regulated in *Drosophila*. First, we quantified polyP at every two-hour interval during the embryonic stages of development. As shown in Fig 1C and Table 2, we observed no significant change in polyP levels across various embryonic stages. We then compared polyP content at different stages of the *Drosophila* life cycle - embryos, larval stages (first instar, second instar, feeding third instar, non-feeding third instar, pupal stages (prepupae and one-day-old pupae), pharate adult, and three-days-old adults. The polyP content remains unchanged from embryos to the third instar feeding larval stages, followed by an increase in the third instar non-feeding larval stage. The polyP content reduces during metamorphosis and drops significantly in the pharate, the stage when the adults are ready to eclose from pupa, followed by a subtle increase in three-day-old flies (Fig 1D, Table 3). These observations show a temporal regulation of polyP during development, and the increased polyP levels at the late third instar larval stages could constitute a phosphate reservoir for metamorphosis during pupal stages.

In-situ labelling of polyP in *Drosophila* tissues revealed spatiotemporal polyP dynamics

To investigate the subcellular localization of polyP across different tissue types, we developed an *in situ* label of polyP using the PolyP Binding Domain (PPBD) of the *E.coli* exopolyphosphatase PPX. PPBD was initially shown to specifically bind polyP *in vitro* with a high affinity of ~45μM for longer polyP chains (35 mer and above), and it can detect polyP localized in budding yeast vacuoles, mammalian mast cell granules, and *C.elegans* (40,41) (41–43). Thus, in this study, we used recombinant GST-PPBD protein to label polyP in fly tissues (Fig 2A, Materials and Methods).

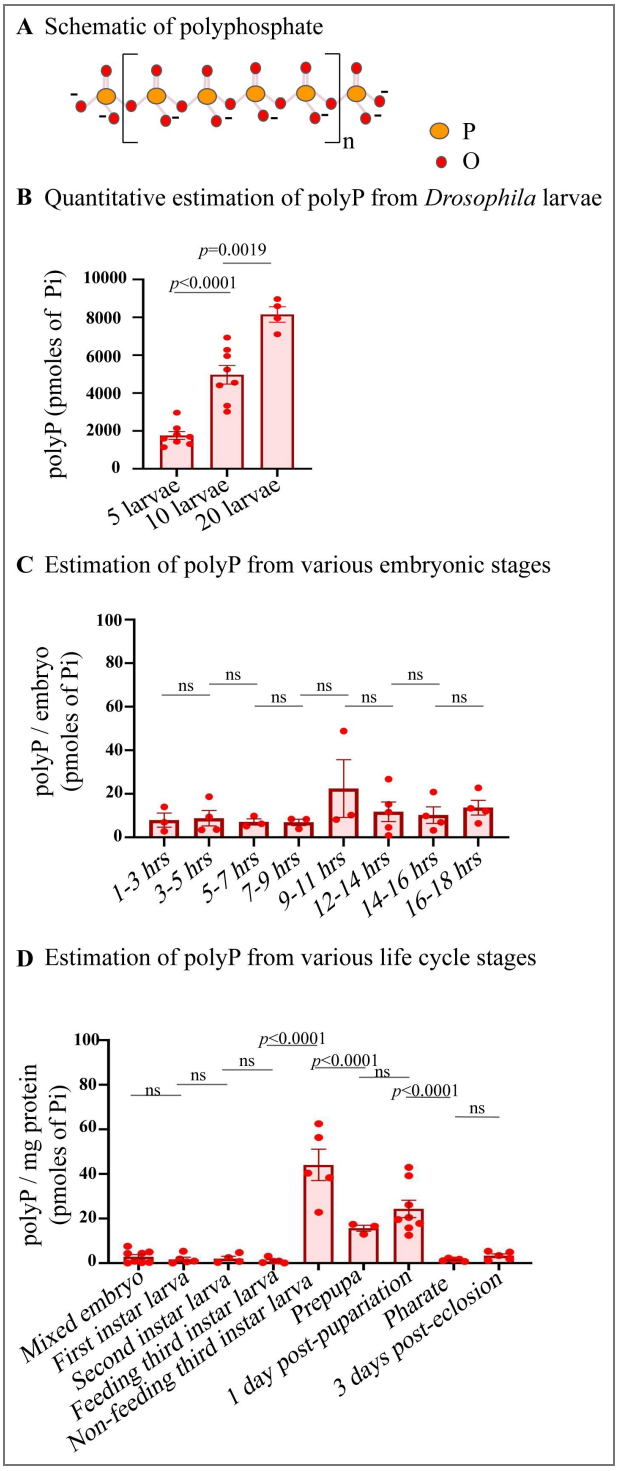

Fig 1. Polyphosphate extraction and quantification from flies.

A) Linear polyphosphate. **B)** PolyP quantification from sets of 5, 10, 20 third instar larvae (in Pi terms), N=5 in each set. Statistics: Student's *t*-test $p > 0.001$, error bar - s.e.m. *Drosophila* strain used - *CantonS*. **C)** Quantification of polyphosphate across embryonic stages. n=75 in each time point, N=3. **D)** Quantification of polyP across a fly life cycle;- embryos (n=75, N=8), first instar larvae (n=25, N=5), second instar larvae (n=25, N=5), feeding third instar larvae (n=10, N=5), non-feeding third instar larvae (n=10, N=5), prepupae (n=20, N=3), one-days-post-pupariation pupae (n=20, N=8), pharate (n=10, N=5), three-days-post-eclosion adult (n=10, N=5). Statistics: Student's *t*-test with $p > 0.001$, error bar - s.e.m. *Drosophila* strain used - *CantonS*.

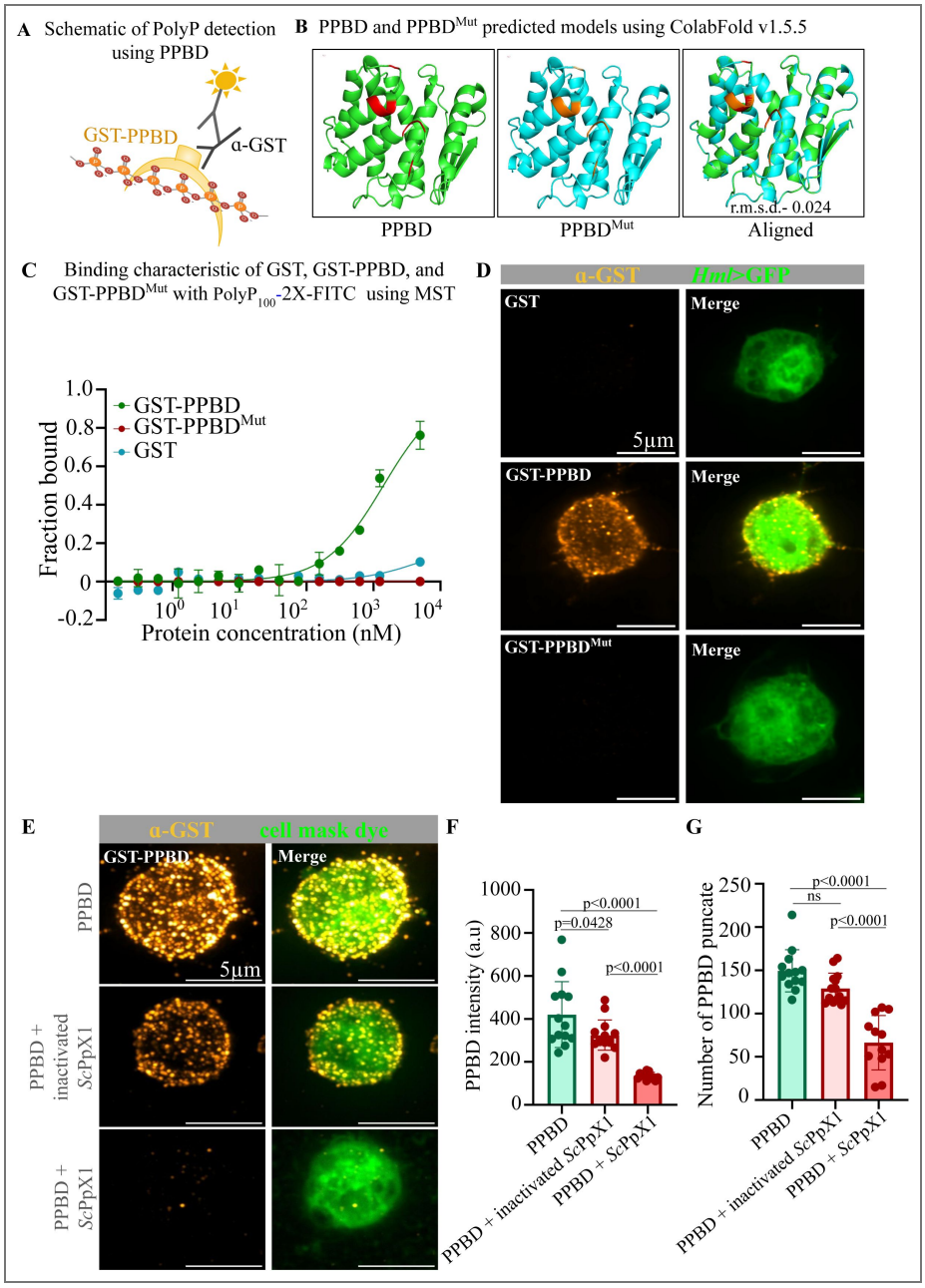

Fig 2. Validation of PPBD as a probe for polyphosphates in fly tissues

A) Schematic of PolyP detection using PPBD fused at the N-terminus with a GST tag. **B)** PPBD and PPBD^{Mut} predicted structural models using ColabFold v1.5.5: AlphaFold2 using MMseqs2 (free online). The amino residues marked in red are changed to Alanine (marked in orange). Overall alignment shows a r.m.s.d. Value of 0.024. **C)** Binding characteristic of GST, GST-PPBD, and GST-PPBD^{Mut} with polyP$_{100}$-2X-FITC using MST. Graph showing GST-PPBD fraction bound to polyP, whereas the GST-PPBD^{Mut} and GST does not bind to polyP$_{100}$-2X-FITC. **D)** Staining of hemocytes with GST, GST-PPBD, and GST-PPBD^{Mut}. Hml-GAL4>UAS GFP reporter was used to identify plasmocytes. Negative control cells incubated with GST and GST-PPBD Mutant proteins separately and stained with anti-GST antibody (GST; orange hot). For polyP staining, cells were incubated with GST-PPBD protein and stained with anti-GST antibody (GST-PPBD-polyphosphate; orange hot). Scale bar - 5µm. **E)** Staining of hemocytes with GST-PPBD in fixed and permeabilised hemocytes with 2 hour pretreatment with buffer (control) and heat inactivated *Sc*PpX1, and active *Sc*PpX1 proteins. GST-PPBD proteins were stained with anti-GST antibody (GST-PPBD-polyphosphate; orange hot), and the cell is marked by Cell Mask Membrane staining dye (green). Scale bar - 5µm. **F)** Graph showing reduced anti-GST staining intensity in hemocytes treated with *Sc*PpX1. **G)** Graph showing reduced PPBD punctae intensity in hemocytes treated with *Sc*PpX1. Statistics: One way Annova with $p > 0.001$, error bar - s.d. *Drosophila* strain used - *CantonS*.

To standardise the labelling method, we determined a tissue fixation method and tested methanol, 4% paraformaldehyde and Bouin's fixatives and found that 4% paraformaldehyde works better among the three for labeling polyP in fly tissues (Supplementary Fig 2A). To test the specificity of GST-PPBD, we used a mutant PPBD (GST-PPBD^{Mut}) and GST proteins. To create GST-PPBD^{Mut} we replaced eight basic amino acid residues with Alanine (Fig 2B, See Material and Methods for sequence). These amino acids were selected based on structural studies of *E. coli* PPX that contains PPBD domain (27,30). The predicted structure of the PPBD^{Mut} aligned with PPBD and had a r.m.s.d. value of 0.024 suggesting no major change in structure of the protein (Fig 2B). We synthesized diamine-linked polydisperse PolyP$_{100}$ and labelled both the ends with Fluorescein Isothiocyanate (FITC) to yield Bis-FITC-labeled PolyP$_{100}$ (PolyP$_{100}$-2X-FITC) following a synthetic procedure described by Fernandes-Cunha et al. (44) (see Supplementary Supplementary Text for Materials and Methods). Using Microscale Thermophoresis (MST), we found that GST-PPBD binds to PolyP$_{100}$-2X-FITC whereas GST-PPBD^{Mut} and GST do not show binding to PolyP$_{100}$-2X-FITC (Fig 2C, See Material and Methods). We then performed immunofluorescence experiments with GST, GST-PPBD, and GST-PPBD^{Mut} on hemocytes and found that unlike GST-PPBD, GST-PPBD^{Mut} or GST protein does not show any specific staining pattern (Fig 2D). Thus, GST-PPBD^{Mut} and GST protein can serve as checkpoints for the specificity of GST-PPBD based labeling of polyP. To further test the specificity, we treated fixed and permeabilised hemocytes with *Sc*PpX1 and heat inactivated *Sc*PpX1 for 2 hours at 37°C and found that both the staining intensity and the number of PPBD punctae were reduced in hemocytes treated with active *Sc*PpX1, as compared to untreated control and heat inactivated *Sc*PpX1 (Fig 2E-G, method adopted from Quarles et. al (43)).

We systematically analysed GST-PPBD labelling in several fly tissues and observed distinct patterns in different tissue types (Fig 3 and Supplementary Fig 3). We observed GST-PPBD labelling in cytoplasmic puncta, nuclear compartments, or both in a tissue-specific manner (Fig 3 and Supplementary Fig 3). In cells with larger nuclei, *e.g.* in salivary glands, we observed intense GST-PPBD labelling in subnuclear compartments devoid of DAPI staining (Fig 3Aii, iv). We suspected these compartments to be nucleoli. To test this, we co-stained GST-PPBD with an antibody against fibrillarin, a nucleolar protein, and found colocalization of these proteins, suggesting that polyP is enriched in the nucleolus (Fig 3B-C). This is consistent with recent reports which found that polyP binds to nucleolar proteins (45,46). Further, in wing and eye larval imaginal discs we found punctate cytoplasmic and nucleolar labelling of GST-PPBD. However, leg and haltere imaginal discs showed no difference in GST-PPBD staining compared to the GST control. In contrast, the larval crop and muscle showed nucleolar GST-PPBD with an intense cytoplasmic background (Supplementary Fig 3).

Interestingly, GST-PPBD labelling revealed spatial and temporal dynamics of polyP localisation during oogenesis. We observed intense GST-PPBD labelling in the nucleus and cytoplasm of the nurse and follicle cells in the early stages of ovary development (Fig 3D). However, there is a gradual reduction in the intensity of GST-PPBD labelling from stage S2 to S9 ovaries, indicating a gradual decrease in polyP levels, indicating the potential stage-specific physiological role of polyP (Fig 3D). Earlier reports have also demonstrated polyP localization in the zona pellucida of mice ovaries and in cockroach oocytes indicating a possible role of polyP during oogenesis (47–49). In hemocytes (hemolymph cells, equivalent to mammalian blood cells) we found an intense GST-PPBD labelling exhibiting polyP localization in large granular structures close to the periphery (Fig 3E-H). The hemocytes were identified by the expression of GFP reporters, driven by cell-specific promoters of *hemolectin* (*hml-GAL4>UAS-GFP*) in plasmatocytes and *lozenge* (*lz-GAL4>UAS-mCD8::GFP*) in crystal cells. Overall, to our knowledge, this is the first comprehensive tissue staining report exhibiting the spatiotemporal dynamics of polyP within a multicellular organism. Taken together, these data also indicate tissue-specific regulation and biological function of polyP in metazoans.

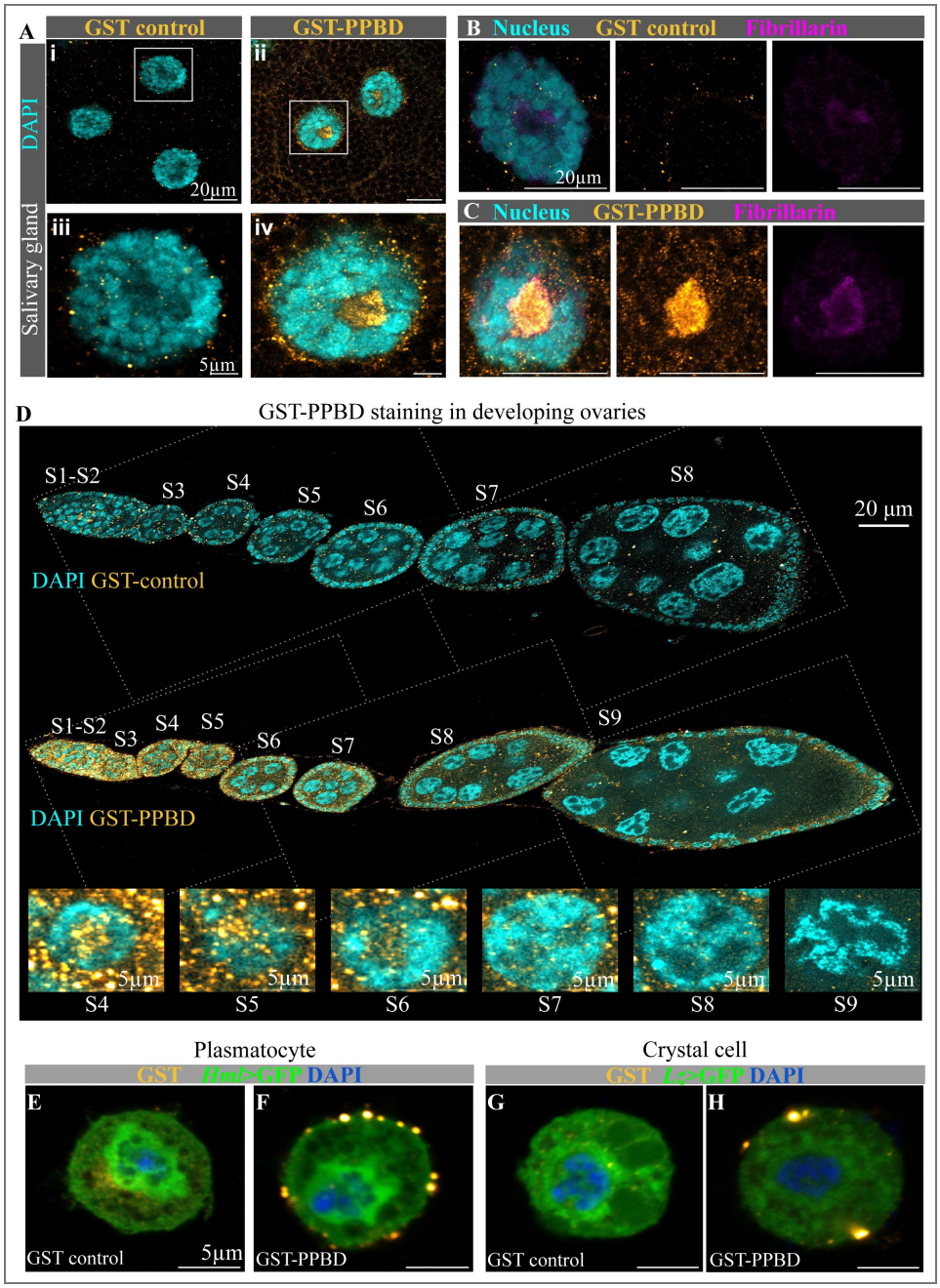

Fig 3. Polyphosphate staining of *Drosophila* tissues with PPBD.

A-C) Staining of larval salivary glands with DAPI (nucleus; cyan) and anti-GST antibody (GST; orange hot). **A i, iii and B)** Samples incubated with GST (negative control). **A ii, iv and C)** Samples incubated with GST-PPBD. **B-C)** Salivary gland stained with DAPI (cyan) anti-fibrillarin antibody (magenta) and anti-GST antibody (orange hot) to detect polyphosphate colocalization within the nucleolus. Scale bar for (A i,ii,B-C)- 20µm, scale bar for (A iii,iv) - 5µm. *Drosophila* strain used - *CantonS*. **D)** Staining of fly ovaries. Negative control: Incubated with GST and stained with DAPI (nucleus; cyan) and anti-GST antibody (GST; orange hot). Stages covered - S1-S8. Incubated with GST-PPBD and stained with DAPI (nucleus; cyan) and anti-GST antibody (GST-PPBD-polyphosphate; orange hot). Stages covered - S1-S9. Yellow arrow- follicle cells, Orange arrow- nurse cells. Scale bar - 20µm. Insets reveal the nucleus and part of the cytoplasm across stages S4-S9, showing reduced polyphosphate signals. Scale bar - 5µm. *Drosophila* strain used - *CantonS*. **E-H)** GST-PPBD staining of hemocytes. *Hml-GAL4>UAS GFP* reporter was used to identify plasmocytes (E-F). *Lz-GAL4>UAS mCD8::GFP* reporter was used to identify crystal cells (G-H). Negative control (E and G) - cells incubated with GST protein and stained with DAPI (nucleus; blue) and anti-GST antibody (GST; orange hot). For polyP staining (F and H), cells were incubated with GST-PPBD protein and stained with DAPI (nucleus; cyan) and anti-GST antibody (GST-PPBD-polyphosphate; orange hot). Scale bar of (A-D) - 5µm.

A heterologous system to deplete polyP in flies: FLYX- Fly expressing *Sc*PpX1

Since metazoan polyP kinases and polyphosphatases are still elusive, direct genetic manipulation of polyP levels can not be done to probe its function in flies. Heterologous expression of budding yeast exopolyphosphatase enzyme (*Sc*PpX1) can deplete polyP in mammalian cells (50). Therefore, to investigate the biological function of polyP, we developed transgenic fly lines that allow tissue-specific and subcellular depletion of polyP by heterologous expression of *ScPpX1*. These flies that will express *Sc*PpX1 constitute the 'FLYX' system.

To develop the FLYX system, we codon optimised and synthesised N-terminal HA-tagged *ScPpX1* (HA-*ScPpX1*) (material and methods for sequence). For the tissue-specific expression of HA-*Sc*PpX1 in the FLYX system, we used the UAS-GAL4 system (51,52). The transcription activator GAL4 is expressed under a tissue-specific promoter in this system. When GAL4 binds to the upstream activator sequence (UAS) preceding the cDNA sequence of interest, the protein is expressed in the specific tissue, thereby providing a handle for spatial regulation of polyP (Fig 4A-B). We cloned codon-optimised HA-*ScPpX1* into fly transgenesis vector pUAST-AttB and pUASp, which allows site-specific integration of a construct in the fly genome via ϕC31 Integrase system (53). The construct (pUAST-HA-*ScPpX1*-AttB) was injected into embryos for its integration in the AttP40 docking site in the second chromosome, and we recovered transgenic FLYX lines. We refer to these lines as Cyto-FLYX. We then validated the expression and localisation of HA-*Sc*PpX1 (driven by *da-GAL4*) using anti-HA antibody staining and found *Sc*PpX1 localisation throughout the cytoplasm (Fig 4C, E). To test the polyP levels in FLYX, we used a *tubulin*-GAL4 driver, which allows ubiquitous expression of *HA-Sc*PpX1. We observed significantly decreased polyP content in Cyto-FLYX larvae compared with the control, suggesting that HA-*Sc*PpX1 can deplete polyP in flies (Fig 4D).

To expand the *Drosophila* polyP depletion toolkit library, we engineered HA-*ScPpX1* constructs to facilitate subcellular compartment-specific depletion of polyP. We created FLYX lines that can target *Sc*PpX1 to the nucleus (Nuc-FLYX), endoplasmic reticulum (ER-FLYX), and the mitochondria (Mito-FLYX) (Fig 4C). We tested the localisation of this FLYX in larval muscles using anti-HA antibody staining. We found that Nuc-FLYX intensely localises in the nuclear region devoid of DAPI (DNA) signal, suggesting probable nucleolar localisation; ER-FLYX co-localized with Calnexin, an ER protein; and Mito-FLYX co-localized with ComplexV subunit A, a mitochondrial protein (Fig 4E, see Material and Methods for sequence). This FLYX library provides a handle for spatial polyP depletion in an organism to explore various physiological functions.

PolyP is crucial for hemolymph clotting

Since hemocytes contribute to hemolymph clotting and *ex vivo* studies in humans have shown exogenous addition of polyP into blood plasma accelerates blood clotting, we reasoned that polyP may accelerate hemolymph clotting in flies (11,54–56). To assess and quantify hemolymph clotting characteristics, we established a 'hemolymph drop assay' (Supplementary Fig 5A-E, see Material and Methods). The clotting hemolymph drop in this assay displays characteristic inward fibre-like structures perpendicular to the edge of the drop. These fibres are significantly reduced in length and number in the *hemolectin* (*hml*), a mutant known to exhibit hemolymph clotting defects (Supplementary Fig 4A-E) (57). Therefore, we used fibre number and characteristics to assess hemolymph clotting phenotypes. To test the effect of polyP on hemolymph clotting, we compared the clot characteristics of hemolymph mixed with water (blank), Pi (1.6 nmol Pi, negative control) or polyP of different average chain lengths (1.6 nmol polyP in Pi terms, equivalent to the total polyP content from two larvae). Upon exogenous addition of polyP, we found increased clot fibre numbers along the edge of the hemolymph drop (Supplementary Fig 4F-J). Similar results were also observed when we reduced the concentration by half (125μM polyP (Pi terms) as has been used in human clotting studies) (Supplementary Fig 5F-I) (11,14). We found that the increase in the clot number density is dependent on the polyP chain length; $polyP_{14}$ did not increase the fibre numbers and length, while $PolyP_{65}$ and $PolyP_{130}$ led to a subtle and significant increase compared to blank and negative control samples (Supplementary Fig 4K-V).

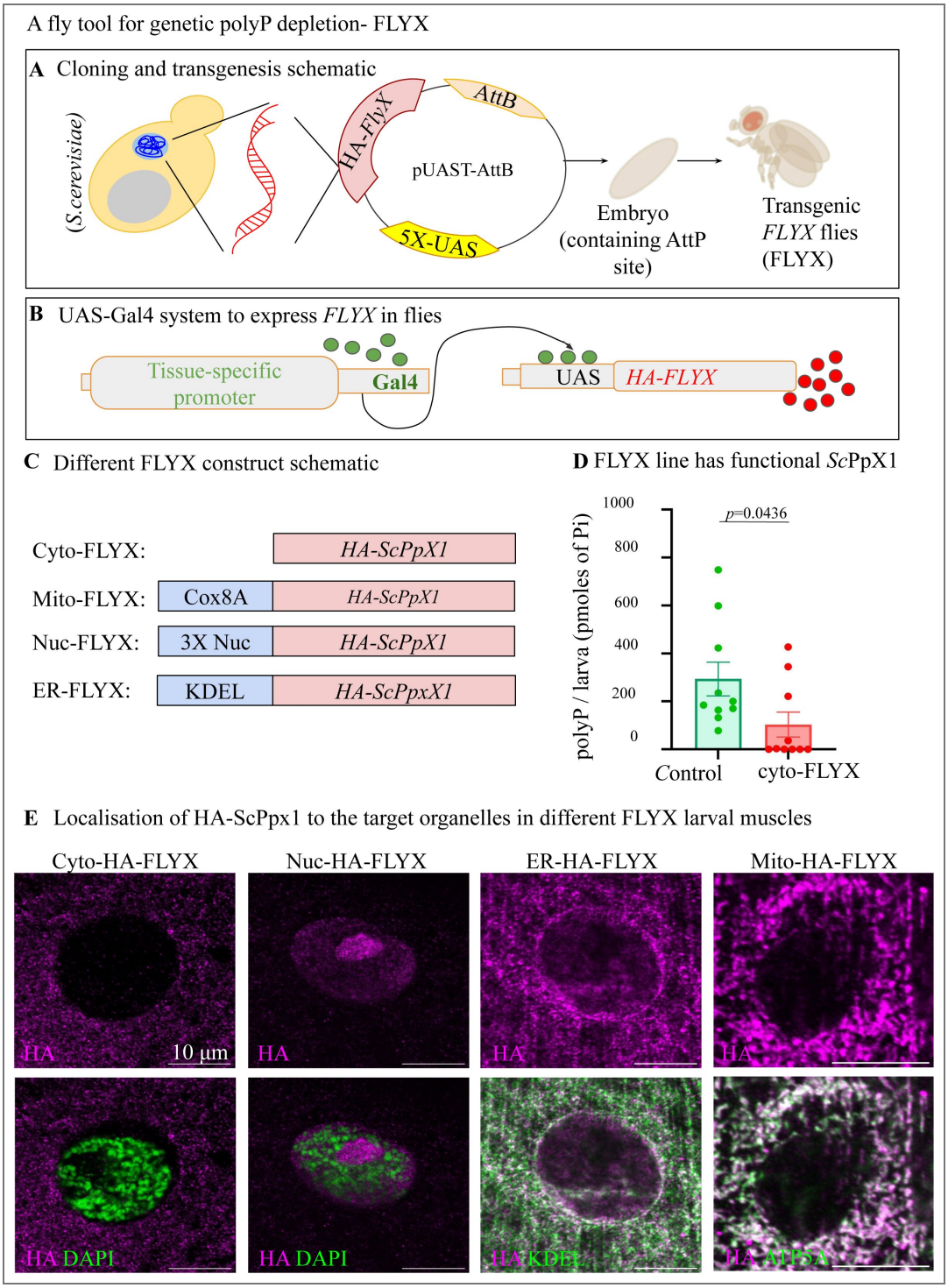

Fig 4. FLYX- Transgenic fly lines with polyP depletion expressing *Sc*PpX1.

A) Schematic of creation of FLYX by cloning *S.cerevisiae ScPpX1* cDNA into pUAST-attB vector suitable for expression in flies, followed by injection into embryos. **B)** Schematic of *Drosophila* UAS-GAL4-based protein expression system. **C)** Schematic of different FLYX lines of the FLYX library- CytoFLYX, Nuc-FLYX, Mito-FLYX, and ER-FLYX. **D)** PolyP quantification from third instar larvae of *tubulin-GAL4* driven control (AttP40) and Cyto-FLYX, N=10. Statistics: Student's *t*-test with $p > 0.001$, error bar - s.e.m. **E)** Localisation of HA-*Sc*PpX1 to the target organelles in different FLYX larval muscles- *DaGAL4>CytoFLYX*,stained for HA in magenta and nucleus (DAPI in green); *DaGAL4>Nuc-FLYX*, stained for HA in magenta and nucleus (DAPI in green); *DaGAL4>ER-FLYX*, stained for HA in magenta and ER (calnexin in green); *Mef2GAL4>Mito-FLYX*, stained for HA in magenta and mitochondria (ATP5A in green). Scale bar - 10µm

The observations that polyP can promote hemolymph clotting *ex vivo* and polyP is localised to large granular structures in hemocytes prompted us to test whether polyP is crucial for hemolymph clotting *in vivo*. Thus, we tested the effect of polyP depletion by ubiquitous expression of *Sc*PpX1 on hemolymph clotting. We observed significantly lesser clot fibre numbers, branching points and clot fibre length in the Cyto-FLYX larvae (*tubulin*>Cyto-FLYX) than the control (Fig 5A-D). These observations suggest that polyP is crucial for efficient hemolymph clotting.

We then sought to identify the cell type that contributes polyP for hemolymph clotting. Therefore, we decided to deplete polyP in a cell-specific manner using the FLYX system under three different drivers. We expressed Cyto-FLYX in fat bodies and all hemocytes using *cg*-GAL4 (*cg*>Cyto-FLYX) and observed reduced fibre length, clot fibre numbers and branching (Fig 5E-H). We then used *hml*-GAL4 (*hml*>Cyto-FLYX), which mainly expresses in the plasmatocytes, and observed significantly reduced clot fibre number and branching densities. The clot fibre length, however, was comparable to the control (Fig 5I-L). We did not observe any significant change in clot fibre numbers and branching when we expressed Cyto-FLYX only in crystal cells using *lz*-GAL4 (*lz*>Cyto-FLYX). The lengths of the clot fibres were also comparable to the control (Fig 5M-P). These data suggest that polyP in hemocytes is necessary for efficient hemolymph clotting. Next, we tested the effect of exogenous addition of polyP in the clotting of hemolymph from *tubulin*>Cyto-FLYX flies. We divided hemolymph from *tubulin*>Cyto-FLYX flies into two parts and incubated one half with Pi (negative control) while the other half was incubated with $polyP_{65}$. On $polyP_{65}$ addition, we observed no significant rescue of the relative clot fibre numbers in *tubulin*>Cyto-FLYX compared to the control (Fig 5Q-S). This suggests that intracellular polyP in the hemocytes might have a physiological role in the clot protein secretion. Taken together, the similarities in *ex vivo* clotting studies in flies and humans and the *in vivo* studies in flies suggest that the cellular and molecular function of polyP in hemolymph/blood clotting are conserved between insects and mammals.

PolyP affects developmental timing

Since we observed differential regulation of polyP during development and in various tissues, we sought to systematically explore phenotypic expression upon ubiquitous depletion of polyP (*tubulin*>Cyto-FLYX). We found no significant differences in the weight or size of Cyto-FLYX vs. control larvae, pupae, or adults (Supplementary Fig 6A-D). Since the pUAST vector is not efficient in driving expression in the germ line cells, we cloned Cyto-FLYX in the pUASP vector and generated a new transgenic line to deplete polyP in the germ line cells. However, we found no significant change in the fecundity upon germ line expression of Cyto-FLYX using *Nos*-GAL4 driver (Supplementary Fig 6E-F). Further, since we had observed polyP levels increase just before pupariation and decrease during pupal stages, we sought to test the impact of polyP depletion on the metamorphosis timing. We tested the total time taken from prepupae stages to the eclosion of adults when Cyto-FLYX is expressed ubiquitously (*tubulin*>Cyto-FLYX, Fig 6A-B). Typically metamorphosis takes ~five days, we found *tubulin*>Cyto-FLYX required ~14 hours less for the eclosion of 50% pupae as compared to control (119.2 ± 3.863 hours for *tubulin*-Cyto-FLYX and 134.5 ± 4.877 hours for control). This data uncovers yet another impact of polyP on metazonal biology.

PolyP depletion affects a wide range of biological processes

Nutrient conditions and signalling pathways, such as Ecdysone, insulin and TOR are known to impact metamorphosis timing in flies. Third instar larvae undergoes multiple pulses of ecdysone signalling mediated through, among others, *ecd* (promotes metamorphosis), *Eip74EF* (ecdysteroid target gene of metamorphosis) (58). In synergy, the insulin signalling pathway promotes ecdysone signalling, and shows characteristic changes in expression of genes such as, insulin receptors (*inR*), insulin like peptides (*dilps*), *chico* (encoder of inR substrates), and *dfoxo* (insulin sensor gene) (59). Insulin signalling pathways via dFoxo also controls TOR signalling pathway effector genes such as, *4ebp* (60). We tested if the accelerated eclosion caused by polyP depletion is a result of transcriptomic changes in any of these aforementioned genes. We however, did not find a

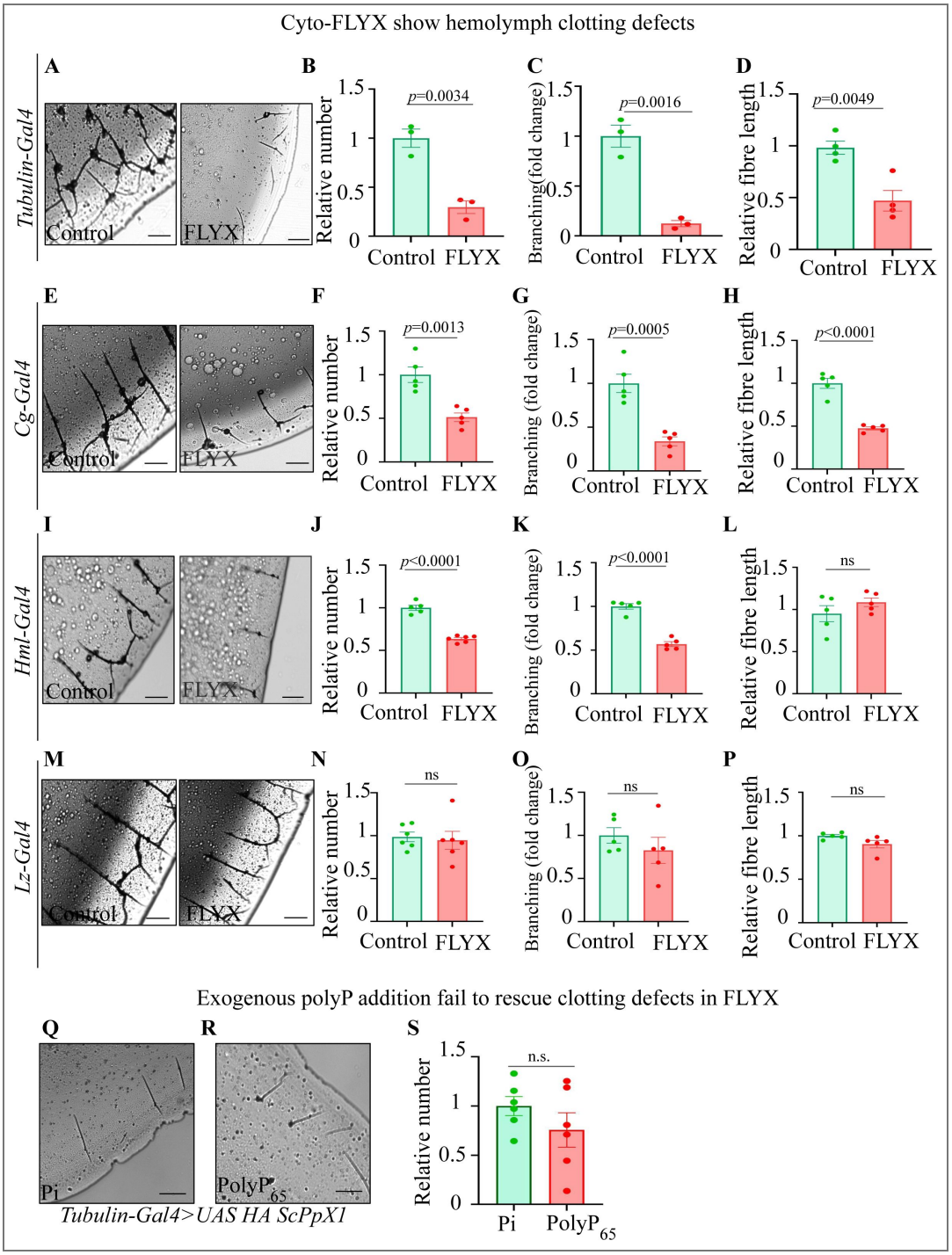

Fig 5. Genetic depletion of polyP shows clotting defects in flies.

A-D) Clot phenotype analysis of *tubulin-GAL4* driven Cyto-FLYX (*tubulin*-FLYX). The control is *tubulin-GAL4*>AttP40. **A)** clot structure of control (AttP40) and FLYX. The scale bar is 500 pixels, and the image dimensions in pixels are 2688 x 2200. **B-D)** Quantification of relative clot fibre number density N=3 (B), clot fibre branch point number density N=3 (C) and clot fibre length, N=4(D) of FLYX line with respect to control. Statistics: Student's *t*-test with $p > 0.001$, error bar - s.e.m. **E-P)** Analysis of clot fibre number, branching and length phenotype upon FLYX driven by *cg-GAL4 (E-H)*, *hml*-GAL4 (I-L) and *lz*-GAL4 (M-P) with respect to control (GAL4>AttP40). Scale bar-500 px, Image dimensions in pixels: 2688 x 2200. For quantifications - N=5. Statistics: Student's *t*-test with $p > 0.001$, error bar - s.e.m. **Q-S)** Clot phenotype of *tubulin-GAL4* driven Cyto-FLYX with exogenous Pi (Q) and $polyP_{65}$ addition (R) and quantification of fibre number density (S). Scale bar- 500 px, Image dimensions in pixels: 2688 x 2200.

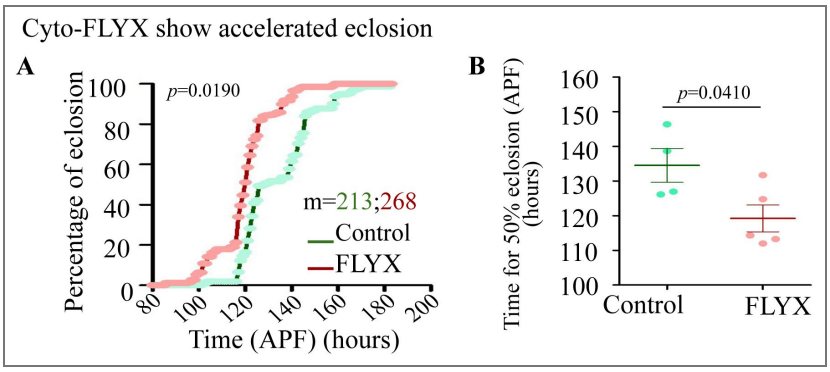

Fig 6. Genetic depletion of polyP shows accelerated eclosion.

A) Cumulative percentage of eclosion of control and FLYX checked after white prepupa formation (APF), m=213 (control), 268 (FLYX), N=4 (control), N=5(FLYX). **B)** Time of 50% eclosion of the control and FLYX flies- ~134 hours APF for control and 120 hours APF for FLYX, m=213 (control), 268 (FLYX), N=4 (control), N=5(FLYX). Statistics: Student's *t*-test with $p > 0.001$, error bar - s.e.m. RNA sequencing of *tubulin-GAL4* driven Cyto-FLYX (*tubulin*>FLYX) and *tubulin-GAL4*>AttP40 control third instar non-feeding wandering age matched larvae.

significant difference in the level of expression of genes- *ecd*, *ImpL2*, *Eip74EF*, *inR*, *chico*, *dfoxo*, *dilp2*, *tor*, *s6k*, and *4ebp* between *tubulin*>Cyto-FLYX and control third instar larvae suggesting that polyP may not directly impact Ecdysone, insulin and TOR pathways (Supplementary Fig 7A).

To understand the cellular response to polyP depletion, we performed RNA sequencing of non-feeding time-matched third instar control and *tubulin-GAL4*>Cyto-FLYX larvae. Our data covered a total of 11747 genes, and the Principal Component Analysis (PCA) segregated the two genotypes in the major axis suggesting ubiquitous depletion of polyP changes the transcriptional landscape (Supplementary Fig 7B). Using Gene Set Enrichment Analysis, with Gene Ontology annotations, we found a total of 262 sets of biological processes. We found translation and ribosome biogenesis pathways among the top ten upregulated biological processes, in the Cyto-FLYX third instar larvae. This is interesting as polyP has been proposed to bind and modulate translation factors in other systems (61,62). Possible increase in translation could be a reason for accelerated developmental progression.

Among the gene sets downregulated in Cyto-FLYX third instar larvae, we found cell-cell adhesion and synaptic transmissions among the top ten hits. With the Cellular Component functional analyses, we also found downregulation of genes linked to neuronal projections and synapses in Cyto-FLYX larvae compared to control. Interestingly, synapses and dendritic connections are known to be pruned during larval to pupal transition as flies undergo neural circuit remodelling (63) (Supplementary Fig 8A-D). In corroboration with the qPCR data, we did not find enrichment of genes linked to canonical ecdysone, TOR, and insulin pathways. Further, we also found enrichment of negative regulators of immune response in Cyto-FLYX larvae as compared to control. Humoral immune response in *Drosophila* is under ecdysone signalling influence, and it leads to downregulation of classical Toll and IMD pathways during larval to pupal transition. Thus, these Cyto-FLYX larvae might be susceptible to infections (64) (see Supplementary table for RNA sequencing analysis). Although the transcriptomic analysis from whole larvae undermines tissue specific transcriptional changes, the wide range of impact of polyP depletion on whole larvae indicates a diverse role of polyP in organismal physiology.

Discussion

Here we reported methods for quantification, visualisation, and genetic manipulation of polyP in flies. To quantify polyP levels, we streamlined a colorimetric-based protocol, and for detection of polyP in tissues, we used a GST-PPBD-based probe that specifically binds to polyP.

We investigated polyP levels in various developmental stages and tissue types and found that polyP levels are spatially, temporally, and developmentally regulated. Further, we created the FLYX system that allowed us to deplete polyP in sub-cellular and tissue-specific manner. Using FLYX we observed changes in the eclosion time of flies, suggesting the importance of the temporal polyP level regulation. We also reported that the polyP in plasmatocytes is crucial for hemolymph clotting, a process analogous to human blood clotting. With the evidence of the probable evolutionarily conserved function of polyP in flies and humans, the FLYX toolkit in flies provides a handle to explore novel functions of polyP. A possible limitation of this system is the lack of an inactive *Sc*PpX1 that can deplete polyP and thus can be used as a negative control to rule out any possible effects of overexpressing the exogenous protein.

Due to the inherent differences in polyP concentrations and chain lengths in different organisms, modifications in the polyP extraction and detection methods were required. To quantify polyP in flies we adopted the citrate-saturated phenol-chloroform-based method, which allows the purification of all chain lengths; however, with the caveat of copurification of RNA (34,35). Since RNA can interfere with polyP quantification, we incorporated an additional RNase treatment step into our extraction protocol. PolyP is often detected using DAPI, which, when bound to polyP and excited at 405 nm, emits fluorescence with a peak at 550 nm (65–68). This spectral property of DAPI-polyP has been exploited for polyP detection despite the interference from DAPI-RNA, which has a fluorescence emission maximum at 525 nm when excited at 405 nm. This interference may result in a false assessment of polyP levels, especially when polyP levels are very low and RNAse

treatment may not be efficient in digesting the complete RNA (40). Thus, for the biochemical detection, we used an enzyme-based method that involves enzymatic degradation of polyP, resulting in the release of Pi, which is then measured using Malachite Green (38,39). Further, to locate polyP in fly tissues, we utilised epitope-tagged PPBD, which specifically binds to polyP with a very high affinity (40,41). Using these tools, we assayed the polyP levels and their localization profiles across different fly developmental stages and in various tissue types, revealing spatiotemporal regulation of polyP in flies and providing a comprehensive estimation and distribution of polyP in a metazoan organism (Fig 1-3, Supplementary Fig 1-3).

Comparative polyP levels across different developmental stages and tissue types in metazoans have yet to be assessed, and it is tempting to predict that differential regulation of polyP levels might be conserved during the development of all multicellular organisms due to complex and differential metabolic regulation. Intriguingly, though polyP level does not change significantly during the *Drosophila* embryonic development, we observed a significant increase in polyP level in the late larval stage, just before the pupariation, followed by a gradual decrease during metamorphosis (Fig 1C-D). We suspect that phosphates are synthesised and stored as a reservoir in the form of polyP during the late larval stage, which is then used during metamorphosis. An assessment of polyP levels during the development of *Dictyostelium discoideum*, a model that has been used to study the origin of multicellularity, revealed a 100-fold increase in polyP levels during the transition from a single cellular vegetative state to a multicellular fruiting body. This increase in polyP is necessary for metabolic regulation during the development of *Dictyostelium*, linking the necessity of polyP to the origin of multicellularity (69). Surprisingly, polyP-depleted flies (Cyto-FLYX) eclose faster (Fig 6A-B), uncovering a yet unknown function of polyP during metamorphosis. Intriguingly, this faster eclosion did not affect the size and weight of larvae and adults (Supplementary Fig 6A-D). Although it is unclear why polyP depletion leads to shorter metamorphosis time, these observations could prompt several lines of phenotypic investigations to explore polyP functions.

Through our investigations, using PPBD probe, we found differential localisation of polyP in various subcellular compartments in a tissue specific manner. In most tissues, we found polyP localisation in the form of cytosolic puncta. While we do not know the identity of these structures, similar localization has been observed in platelet (dense granules) and mast cells (serotonin containing granules) (41,70) Strikingly, we observed intense PPBD staining in the nucleolus of salivary glands, muscle, crop, and wing disc cells. We also found nuclear targeted HA-*Sc*PpX1(Nuc-FLYX) localises in the nucleoli of the nucleus of muscle cells. These data indicate a nucleolar function of polyP in some cell types. The nucleolar polyP has also been previously reported in myeloma cells and cisplatin-treated HeLa cells (46,71). Nucleolar polyP has also been observed upon forced delivery of polyP into mammalian cells or by overexpression of polyP kinase in plant and mammalian cells (44,45,72). Since polyP can induce LLPS by its interactions with the nucleic acid and positively charged proteins, polyP may be involved in the organisation of the nucleolus, which is an LLPS organelle (45,73).

Overall, the polyP localization profile using GST-PPBD staining, which uncovered spatial and temporal regulation of polyP across various *Drosophila* tissues, would accelerate the discovery of various tissue-specific functions of polyP, particularly with the use of sophisticated fly genetic tools *in vivo* (Fig 2-3, Supplementary Fig 3). That the fly gut microbiota can also potentially contribute to the polyP content in fly tissues is one important consideration. In this article we allude to several observations that argue polyP is synthesized by fly tissues: (i) polyP levels remain very low during feeding stages but build up in wandering third instar larvae after feeding ceases (Fig 1D); (ii) PPBD staining is absent from the gut except the crop (Fig. S3O–P); and (iii) Recent work on *C.elegans* have reported polyP presence in the worm intestines, whereas in flies, polyP is present only in the crop with the rest of the gut containing undetectable amounts. Moreover, in *C. elegans*, intestinal polyP was unaffected when worms were fed polyP-deficient bacteria (43).

Recently, decreased polyP levels were found in the plasma of patients suffering from Type 1 Von Willebrand Disease (VWD) (12). Moreover, mice lacking the inositol pyrophosphate synthesising enzyme IP6K1 are reported to have reduced polyP levels and show blood clotting defects (13).

Despite blood clotting being the best-known function of polyP in metazoans, studies on polyP-mediated blood clotting in mammals have been done by the *ex vivo* addition of polyP due to the limitation of direct genetic manipulations of polyP synthesis in platelets. Using FLYX- a transgenic fly system of polyP depletion, we show that polyP in plasmatocytes is crucial for hemolymph clotting (Fig 5A-S). Studies in humans revealed that polyP localises inside granules in platelets, which, over the past two decades, have been shown to aid in mammalian blood clotting (70). Given our observation of the peripheral arrangement of polyP granules, we propose that polyP is either required for secretion, modification of clotting factors in plasmatocytes or contributes to the localised increase in polyP levels at the clotting site (Fig 5Q-S). Our data also indicates the polyP in fat bodies also contributes to hemolymph clotting - notably, the length of clot fibres becomes significantly shorter when polyP is depleted in all hemocytes as well as fat bodies together but not when depleted only in plasmatocytes (Fig 5H, L). As fat bodies are known to secrete hemolymph clotting factors, including proteins like Fondue, which gets incorporated in the clots, polyP may modify or promote the secretion or activation of Fondue or any other factor crucial for fibre lengthening (74,75). Given conserved mechanisms of hemolymph and blood clotting, modelling polyP studies in flies can help understand the regulation, release and function of polyP during hemolymph and blood clotting.

The change in the whole larval transcriptome of Cyto-FLYX reiterates the myriad possibilities of polyP functions in metazoans. We performed GSEA due to the intra-sample variation (PC2 = 19.10%, Supplementary Fig 7B) as well as our aim to identify major differences in cellular responses/pathways between Cyto-FLYX and Control (76). The third instar larval population usually undergoes bursts of transcription related to its development and metamorphosis. Through GSEA, we found gene sets enriched for translation and ribosome biogenesis (Supplementary Fig 8A-D). Indeed, the proteomic analysis of a Δ*ppk* mutant *E.coli*, which results in absence of polyP, also shows a similar response (77). The reason for the increase in such biological processes in the absence of polyP (either due to no polyP synthesis or polyP depletion) remains to be investigated. Earlier polyP binding assays have shown that polyP can bind to proteins associated with translation. By linking polyP depletion to both increased translational gene expression and accelerated developmental progression, we are tempted to hypothesize a mechanistic axis in which polyP restrains protein synthesis to coordinate the timing of metamorphosis. Overall, by cytoplasmic depletion of polyP with the FLYX toolkit, we demonstrated the conservation of polyP function in hemolymph/blood clotting and the importance of polyP regulation during metamorphosis.

Through transcriptomics, followed by Gene Set Enrichment Analysis, we found the impact of cytoplasmic polyP in several biological processes including translation and ribosome biogenesis processes during late larval stage, reiterating the myriad possibilities of polyP functions in metazoans. An unbiased phenotypic analysis following polyP depletion in various tissues and subcellular compartments using FLYX lines developed in this work would facilitate the discovery of many unknown functions of polyP. Further, the use of *Drosophila* genetic epistatic studies, by combining spatial and temporal regulation of polyP with structure and function analysis would be useful in assigning biological meaning to several metazoan polyphosphorylated proteins that have been identified in recent studies (19–21). Finally, given the recent implications of polyP in human diseases, the fly model would be a valuable and unique *in vivo* system to uncover the pathogenic mechanisms of polyP-linked diseases.

In summary, this study establishes *Drosophila* as a much-needed metazoan model for polyphosphate biology, revealing conserved roles in clotting and development. The tools and findings presented here will pave the ways for new investigations to uncover functions of this ancient polymer in animal biology and its implications in human diseases.

Materials and methods

Polyphosphate quantification using malachite green Assay

Malachite green (MG) detects monophosphates released from polyP pools due to the addition of purified yeast exopolyphosphatase (*Sc*PpX1). Samples with extracted polyP were divided into two parts; one part was treated with 5ug/ml *Sc*PpX1 for 18 hours at 37°C while the other half acted as the untreated control. MG reagent was prepared by mixing MG (0.045% in water) and Ammonium molybdate (4.2% in 4N HCl) in 3:1(vol/vol) ratio and filtered through a Whatman grade 1 paper. K_2HPO_4 (0.1 – 20 nmoles) was used as a phosphate standard. Samples or standards (50 µl) were loaded into 96 healthy plates, and 200 µl of MG reagent was added per sample, followed by incubation in the dark for 15 minutes at room temperature. Absorbance was measured at 650 nm using the Omega PolarStar Plate Reader. PolyP content of the samples (in Pi terms) was determined by interpolation from a linear regression analysis of the K_2HPO_4 standard.

GST-PPBD staining

Tissues were dissected in 1X TBS (Tris Buffered Saline - 50mM Tris-HCl pH 7.6, 150mM NaCl), and fixed in 4% paraformaldehyde for 20 minutes. They were then subjected to three 1X TBST (TBS with 0.2% Triton X-100) washes for ten minutes each. 5% NGS (nascent goat serum) was added to the tissues as a blocking agent, and incubated for one hour at room temperature. The tissues were next incubated with 25ng/µl GST or GST-PPBD (purification process mentioned in GST and GST-PPBD Protein Purification section of methods) for one hour at room temperature followed by three 1X TBST washes for ten minutes each. The tissues were then incubated in mouse anti-GST antibodies overnight at 4°C. The next day, the tissues were washed thrice with 1X TBST and incubated with goat anti-mouse antibody conjugated with Alexa555 dye at room temperature for two hours. Following incubation, the tissues were washed thrice with 1X TBST and incubated with 100µM DAPI to stain nuclei for 15 minutes at room temperature. DAPI staining was followed by a final step of three washes with 1X TBST before mounting the dissected tissues on slides for imaging. Images were acquired with a Leica STELLARIS 5 confocal microscope.

For colocalisation with nucleolus, Rabbit anti-Fibrillarin antibody was incubated with the primary Mouse anti-GST antibodies overnight at 4°C. The next day, the tissues were washed thrice with 1X TBST and incubated with goat anti-mouse antibody conjugated with Alexa555, and goat anti-rabbit antibody conjugated with Alexa488 dye at room temperature for two hours. This was followed by a final step of three washes with 1X TBST before mounting the dissected tissues on slides for imaging. Images were acquired with a Leica STELLARIS 5 confocal microscope. Figures of Supplementary 3A-B were imaged with Nikon microscope CSU-W1.

Creation of FLYX - transgenic *Sc*PpX1 flies

The *ScPpX1* gene sequence from pTrc-HisB-*ScPpX1* was codon optimised for *Drosophila melanogaster* in open access platform Benchling. The codon-optimised *ScPpX1* sequence (see Supplementary material) was tagged with HA epitope (TATCCGTATGATGTTCCGGATTATGCA) in the N-terminal region, and flanked by restriction sites EcoRI and XbaI. The entire fragment was synthesised by GeneScript. The HA-*ScPpX1* was cloned out from the carrier vector using restriction sites EcoR1 and Xba1 into fly expression vector pUAST-attB. The plasmid was then injected into embryos for site-directed transgenesis in the second chromosome using the *y; AttP40* flies. Two independent lines (Cyto-FLYX) were generated, and one was used in the experiments of this study.

For targeted *Sc*PpX1 expression, the codon-optimised *ScPpX1* sequence (see Supplementary material) was tagged with HA epitope (TATCCGTATGATGTTCCGGATTATGCA) in the N-terminal region following the 3XNLS (nuclear localisation signal) for Nuc-FLYX, Cox8A signal sequence for Mito-FLYX, and the KDEL sequence for ER-FLYX. The gene inserts (see Supplementary material) were all flanked by restriction sites EcoRI and XbaI. The entire fragment was synthesised by GeneScript. The HA-*ScPpX1* was cloned out from the carrier vector using restriction sites EcoR1

and Xba1 into fly expression vector pUAST-attB. The plasmid was then injected into embryos for site-directed transgenesis in the second chromosome using the *y; AttP40* flies. Two independent lines were generated, and one was used in the experiments of this study.

For expression in the gonads, the Cyto-FLYX construct was subcloned into pUASp vector, and three independent lines based on p-element insertions in the second chromosome were identified and retrieved.

Immunohistochemistry

Larval filae were prepared for staining muscle cells for HA expression in GAL4 driven FLYX lines in 1X TBS (Tris Buffered Saline - 50mM Tris-HCl pH 7.6, 150mM NaCl), and fixed in 4% paraformaldehyde for 20 minutes. They were then subjected to three 1X TBST (TBS with 0.2% Triton X-100) washes for ten minutes each. 5% NGS (nascent goat serum) was added to the tissues as a blocking agent, and incubated for one hour at room temperature. For Cyto-FLYX and Nuc-FLYX, to stain HA, we used mouse anti-HA primary antibody whereas, for ER-FLYX and Mito-FLYX, to stain HA, we used rabbit anti-HA primary antibody, mouse anti-KDEL primary antibody (ER-F:YX) and mouse anti-ATP5A (Mito-FLYX) primary antibody. The tissues were incubated overnight at 4°C. The next day, the tissues were washed thrice with 1X TBST and incubated with goat anti-mouse antibody conjugated with Alexa555, and goat anti-rabbit antibody conjugated with Alexa488 dye at room temperature for two hours. The tissues were washed thrice with 1X TBST buffer. For Cyto-FLYX and Nuc-FLYX, after the secondary antibody incubation and washing, tissues were incubated with 100µM DAPI to stain nuclei for 15 minutes at room temperature. This was followed by a final step of three washes with 1X TBST before mounting the dissected tissues on slides for imaging. Images were acquired with a Leica STELLARIS 5 confocal microscope.

Hemocyte adherence and immunostaining

For adherence, the hemolymph was collected from 10 larvae in 15µl TBS and allowed to adhere on a glass slide at room temperature for 30 minutes - the method adopted by Hankde et.al. 2013 (78). The supernatant was taken out carefully, and the adhered cells were fixed using 4% PFA for 20 minutes. This was followed by immunostaining with GST-PPBD using the GST-PPBD staining procedure mentioned above.

For treatment of hemocytes with *Sc*PpX1 and heat inactivated *Sc*PpX1, hemocytes were subjected to three 1X TBST (TBS with 0.2% Triton X-100) washes for ten minutes each, and then incubated with 5ug/ml of *Sc*PpX1 in 1X TBS or heat inactivated *Sc*PpX1 in 1X TBS (incubated protein at 65°C for 30minutes) at 37°C for 2 hours. The control untreated hemocytes were also given the same incubation at 37°C for 2 hours. This was followed by three washes and immunostaining with GST-PPBD using the GST-PPBD staining procedure mentioned above.

Assessment of clot fibre characteristics

For assessment of the clot fibre parameters - number, branching, and length- 20 larvae were collected, washed in 1X TBS and dried on tissue paper. The dorsal side mouth hook region was gently torn to let only the hemolymph ooze out. For Supplementary Fig 5F-J, and Supplementary Fig 6, the hemolymph was collected and divided into two sets of 2µl drops. 2µl hemolymph was mixed with either 1µl water or 1µl Pi (0.8 nmole) as control, and 1µl PolyP ($PolyP_{14}$, $PolyP_{65}$, $PolyP_{130,}$ each containing 0.8 nmole in Pi terms) as test samples. For Supplementary Fig 5B-C, and Fig 5, the hemolymph was collected, and 2µl was mixed with 1µl of water in each of the cases tested. The slide was incubated at room temperature (24°C) for 25-30 minutes to allow evaporation. Post-incubation, clot fibres were imaged using an Olympus BX53 upright microscope with a 10X objective lens fitted with a Retiga-R6 camera. Post imaging, the number of fibres, fibre branch points, fibre length, and circumference of the clot were measured manually using ImageJ analysis software. The number of fibres, branch points, and length of fibres were then normalised to the circumference of the respective clot. The mean of the normalised data sets of the control was analysed. Every data set is represented as relative to the mean of the normalised data of the control.

Eclosion kinetic study

To study pupal eclosion kinetics, flies were allowed to lay eggs for 12 hours, after which 150 first instar larvae were collected in a span of 30 minutes to have a synchronous culture and grown at 25°C. White prepupa were collected after 6 days and staged again. The time of collection of white prepupa was set to T=0. The time of eclosion of flies was noted. A total of 213 control flies, and 268 FLYX were scored for eclosion in three independent sets. Only plates from where at least 25 flies eclosed in a day were considered for the study.

RNA Transcriptome analysis

Sets of five larvae were subjected to RNA isolation using TRIzol (Ambion life tech—15596018) method. The RNA library was prepared using NEBNext® Ultra™ II Directional RNA Library Prep with Sample Purification Beads. Downstream analyses were done in the condo (23.7.4) and R (4.3.2) environments. Briefly, raw reads were pair-end pseudo-aligned to the annotated *Drosophila melanogaster* genome (BDGP6.32) dataset using Kallisto (0.48.0) (79) and imported to the R environment. Uniquely mapped reads were further filtered and normalised using the Trimmed mean of M-values (TMM) (80) method of EdgeR/limma (4.0.3) Bioconductor library (81).

Then, Gene Set Enrichment Analysis (GSEA) (82) was performed using the clusterProfiler (4.10.1) package (83). In essence, gseGO functions were used to identify the GSEA of Gene Ontology for Biological Processes (BP), Molecular Functions (MF) and Cellular Components (CC).

Graph and statistical analysis

All the graphs and statistical analysis were done using GraphPad Prism software. The statistical tests used are mentioned in the respective figure legends.

Acknowledgements

We thank Adolfo Saiardi for his kind gift of plasmid pTrc-HisB-*ScPpX1*. We also thank T. Shiba for generously sharing polyP of different chain lengths -polyP ($PolyP_{14}$, $PolyP_{65}$, $PolyP_{130}$). We thank Shubham Kumar Agrawal for help with the purification of *Sc*PpX1 and Manisha Mallick for assisting PPX and PPBD binding experiments. We acknowledge Debaditya De, Aditya Rane, and Manasa Chanduri for the construction of the *ScPpX1* and GST-PPBD expression plasmids. We thank Rakesh Mahato for assisting in the MST experiments and Dr. Pankaj Suman for sharing the MST set up at the National Institute of Animal Biotechnology, India. We thank Lolitika Mandal for sharing *HmlGAL4>UAS GFP* fly lines with us. We thank Aprotim Mazumder for sharing anti-Fibrillarin antibodies. We thank Vipin Agarwal for sharing the SEC setup for protein purification. We thank Dr. Deepti Trivedi and NCBS fly facility for injections and Dr. Awadhesh Pandit and his team at NCBS for RNA library preparation and RNA sequencing. We thank Kalyaneswar Mandal, Sonal Nagarkar Jaiswal and members of the Laboratory of Cell Signalling CDFD and MJ lab TIFRH for discussions and valuable feedback. We thank Anand T Vaidya, Vinay Bulusu and Oguz Kanca for the critical reading, comments and valuable suggestions on the manuscript.

Additional information

Data availability

Transcription is provided in an XL sheet

Code Availability

The code is available in the supplementary text.

Funding

This work is funded by DBT-DFG grant IC12025(11)/2/2020-ICD-DBT to MJ and RB. MJ is supported by the Department of Atomic Energy (Project Identification No. RTI4007), Department of Science and Technology, SERB (CRG/2020/003275), Department of Biotechnology (BT/PR32873/BRB/10/1850/2020), Government of India. MJ was a Ramalingaswami fellow of the Department of Biotechnology, Government of India, under project number BT/RLF/Re-entry/06/2016. R.B. acknowledges support from the Department of Biotechnology, Ministry of Science and Technology, Govt. of India. (BT/PR29960/BRB/10/1762/2019); Science and Engineering Research Board, Department of Science and Technology, Govt. of India (CRG/2019/002597); and CDFD core funds. HJ acknowledges funding for this project by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation, project number 445698446) in collaboration with the Indian Department of Biotechnology (DBT). Moreover, this work was supported by the Deutsche Forschungsgemeinschaft (DFG) under Germany's Excellence Strategy (CIBSS-EXC-2189-Project ID 390939984, to HJJ). SS is an Infosys fellow (Leading Edge TG/(R-11)/09/). A Shyama Prasad Mukherjee Fellowship from the Council of Scientific and Industrial Research, Government of India, supports JSL.

Author contributions

SS, RB and MJ have conceptualised the study. SS: Designed the methods, validated, and performed most experiments and analysed the data. HS: GST and GST-PPBD labelling, hemolymph clotting experiments along with SS. SM: Synthesis of PolyP-100-2X-FITC. JSL: Analysis of polyphosphate by gel electrophoresis, and assistance in initial polyP characterisation in flies. SKYH: RNA transcriptome analysis and fecundity experiment. DB: RNA extraction and qRT-PCR. SRK: Size Exclusion Chromatography for *Sc*PpX1 purification. SS and MJ wrote the original draft. Everyone has reviewed and edited the manuscript. MJ and RB supervised the study. MJ and RB were responsible for the project administration and funding acquisition.

Classification: Biological Sciences/Physiology

Funding

Funder	Grant reference number	Author
Department of Biotechnology, Ministry of Science and Technology, India (DBT)	IC12025(11)/2/2020-ICD-DBT	Manish Jaiswal Rashna Bhandari
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Additional files

Supplementary Text

Supplementary Figures

Supplementary RNA seq datafile

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Peer reviews

Reviewer #2 (Public review):

Summary:

The authors of this paper note that although polyphosphate (polyP) is found throughout biology, the biological roles of polyP have been under-explored, especially in multicellular organisms. The authors created transgenic Drosophila that expressed a yeast enzyme that degrades polyP, targeting the enzyme to different subcellular compartments (cytosol, mitochondria, ER, and nucleus, terming these altered flies Cyto-FLYX, Mito-FLYX, etc.). The authors show the localization of polyP in various wild-type fruit fly cell types and demonstrate that the targeting vectors did indeed result in expression of the polyP degrading enzyme in the cells of the flies. They then go on to examine the effects of polyP depletion using just one of these targeting systems (the Cyto-FLYX). The primary findings from depletion of cytosolic polyP levels in these flies is that it accelerates eclosion and also appears to participate in hemolymph clotting. Perhaps surprisingly, the flies seemed otherwise healthy and appeared to have little other noticeable defects. The authors use transcriptomics to try to identify pathways altered by the cyto-FLYX construct degrading cytosolic polyP, and it seems likely that their findings in this regard will provide avenues for future investigation. And finally, although the authors found that eclosion is accelerated in pupae of Drosophila expressing the Cyto-FLYX construct, the reason why this happens remains unexplained.

Strengths:

The authors capitalize on the work of other investigators who had previously shown that expression of recombinant yeast exopolyphosphatase could be targeted to specific subcellular compartments to locally deplete polyP, and they also use a recombinant polyP binding protein (PPBD) developed by others to localize polyP. They combine this with the considerable power of Drosophila genetics to explore the roles of polyP by depleting it in specific compartments and cell types to tease out novel biological roles for polyP in a whole organism. This is a substantial advance.

Weaknesses:

Page 4 of Results (paragraph 1): I'm a bit concerned about the specificity of PPBD as a probe for polyP. The authors show that the fusion partner (GST) isn't responsible for the signal, but I don't think they directly demonstrate that PPBD is binding only to polyP. Could it also bind to other anionic substances? A useful control might be to digest the permeabilized cells and tissues with polyphosphatase prior to PPBD staining, and show that the staining is lost.

In the hemolymph clotting experiments, the authors collected 2 ul of hemolymph and then added 1 ul of their test substance (water or a polyP solution). They state that they added either 0.8 or 1.6 nmol polyP in these experiments (the description in the Results differs from that of the Methods). I calculate this will give a polyP concentration of 0.3 or 0.6 mM. This is an extraordinarily high polyP concentration, and is much in excess of the polyP concentrations used in most of the experiments testing the effects of polyP on clotting of mammalian plasma. Why did the authors choose this high polyP concentration? Did they try lower concentrations? It seems possible that too high a polyP concentration would actually have less clotting activity than the optimal polyP concentration.

In the revised version of the manuscript, the authors have productively responded to the previous criticisms. Their new data show stronger controls regarding the specificity of PPBD with regard to its interaction with polyP. The authors also have repeated their hemolymph clotting experiments with lower polyP concentrations, which are likely to be more physiological.

https://doi.org/10.7554/eLife.104841.2.sa2

Reviewer #3 (Public review):

Summary:

Sarkar, Bhandari, Jaiswal and colleagues establish a suite of quantitative and genetic tools to use Drosophila melanogaster as a model metazoan organism to study polyphosphate (polyP) biology. By adapting biochemical approaches for use in D. melanogaster, they identify a window of increased polyP levels during development. Using genetic tools, they find that depleting polyP from the cytoplasm alters the timing of metamorphosis, accelerationg eclosion. By adapting subcellular imaging approaches for D. melanogaster, they observe polyP in the nucleolus of several cell types. They further demonstrate that polyP localizes to cytoplasmic puncta in hemocytes, and further that depleting polyP from the cytoplasm of hemocytes impairs hemolymph clotting. Together, these findings establish D. melanogaster as a tractable system for advancing our understanding of polyP in metazoans.

Strengths:

• The FLYX system, combining cell type and compartment-specific expression of ScPpx1, provides a powerful tool for the polyP community.

• The finding that cytoplasmic polyP levels change during development and affect the timing of metamorphosis is an exciting first step in understanding the role of polyP in metazoan development, and possible polyP-related diseases.

• Given the significant existing body of work implicating polyP in the human blood clotting cascade, this study provides compelling evidence that polyP has an ancient role in clotting in metazoans.

Limitations:

• While the authors demonstrate that HA-ScPpx1 protein localizes to the target organelles in the various FLYX constructs, the capacity of these constructs to deplete polyP from the different cellular compartments is not shown. This is an important control to both demonstrate that the GTS-PPBD labeling protocol works, and also to establish the efficacy of compartment-specific depletion. While not necessary to do for all the constructs, it would be helpful to do this for the cyto-FLYX and nuc-FLYX.

• The cell biological data in this study clearly indicates that polyP is enriched in the nucleolus in multiple cell types, consistent with recent findings from other labs, and also that polyP affects gene expression during development. Given that the authors also generate the Nuc-FLYX construct to deplete polyP from the nucleus, it is surprising that they test how depleting cytoplasmic but not nuclear polyP affects development. However, providing these tools is a service to the community, and testing the phenotypic consequences of all the FLYX constructs may arguably be beyond the scope of this first study.

Editors' note: The authors have satisfactorily responded to our most major concerns related to the specificity of PPDB and the physiological levels of polyPs in the clotting experiments.

We also recognise the limitations related to the depletion of polyP in other tissues and hope that these data will be made available soon.

https://doi.org/10.7554/eLife.104841.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Polymers of orthophosphate of varying lengths are abundant in prokaryotes and some eukaryotes, where they regulate many cellular functions. Though they exist in metazoans, few tools exist to study their function. This study documents the development of tools to extract, measure, and deplete inorganic polyphosphates in *Drosophila*. Using these tools, the authors show:*
>
> *(1) That polyP levels are negligible in embryos and larvae of all stages while they are feeding. They remain high in pupae but their levels drop in adults.*
>
> *(2) That many cells in tissues such as the salivary glands, oocytes, haemocytes, imaginal discs, optic lobe, muscle, and crop, have polyP that is either cytoplasmic or nuclear (within the nucleolus).*
>
> *(3) That polyP is necessary in plasmatocytes for blood clotting in Drosophila.*
>
> *(4) That ployP controls the timing of eclosion.*
>
> *The tools developed in the study are innovative, well-designed, tested, and well-documented. I enjoyed reading about them and I appreciate that the authors have gone looking for the functional role of polyP in flies, which hasn't been demonstrated before. The documentation of polyP in cells is convincing as its role in plasmatocytes in clotting.*

We sincerely thank the reviewer for their encouraging assessment and for recognizing both the innovation of the FLYX toolkit and the functional insights it enables. Their remarks underscore the importance of establishing *Drosophila* as a tractable model for polyP biology, and we are grateful for their constructive feedback, which further strengthened the manuscript.

> *Its control of eclosion timing, however, could result from non-specific effects of expressing an exogenous protein in all cells of an animal.*

We now explicitly state this limitation in the revised manuscript (p.16, l.347–349). The issue is that no catalytic-dead *Sc*PpX1 is available as a control in the field. We plan to generate such mutants through systematic structural and functional studies and will update the FLYX toolkit once they are developed and validated. Importantly, the accelerated eclosion phenotype is reproducible and correlates with endogenous polyP dynamics.

> *The RNAseq experiments and their associated analyses on polyP-depleted animals and controls have not been discussed in sufficient detail. In its current form, the data look to be extremely variable between replicates and I'm therefore unsure of how the differentially regulated genes were identified.*

We thank the reviewer for pointing out the lack of clarity. We have expanded our RNAseq analysis in the revised manuscript (p.20, l.430–434). Because of inter-sample variation (PC2 = 19.10%, Fig. S7B), we employed Gene Set Enrichment Analysis (GSEA) rather than strict DEG

cutoffs. This method is widely used when the goal is to capture pathway-level changes under variability (1). We now also highlight this limitation explicitly (p.20, l.430–432) and provide an additional table with gene-specific fold change (see Supplementary Table for RNA Sequencing Sheet 1). Please note that we have moved RNAseq data to Supplementary Fig. 7 and 8 as suggested in the review.

> *It is interesting that no kinases and phosphatases have been identified in flies. Is it possible that flies are utilising the polyP from their gut microbiota? It would be interesting to see if these signatures go away in axenic animals.*

This is an interesting possibility. Several observations argue that polyP is synthesized by fly tissues: (i) polyP levels remain very low during feeding stages but build up in wandering third instar larvae after feeding ceases; (ii) PPBD staining is absent from the gut except the crop (Fig. S3O–P); (ii) In *C. elegans*, intestinal polyP was unaffected when worms were fed polyP-deficient bacteria (2); (iv) depletion of polyP from plasmatocytes alone impairs hemolymph clotting, which would not be expected if gut-derived polyP were the major source and may have contributed to polyP in hemolymph. Nevertheless, we agree that microbiota-derived polyP may contribute, and we plan systematic testing in axenic flies in future work.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *The authors of this paper note that although polyphosphate (polyP) is found throughout biology, the biological roles of polyP have been under-explored, especially in multicellular organisms. The authors created transgenic Drosophila that expressed a yeast enzyme that degrades polyP, targeting the enzyme to different subcellular compartments (cytosol, mitochondria, ER, and nucleus, terming these altered flies Cyto-FLYX, Mito-FLYX, etc.). The authors show the localization of polyP in various wild-type fruit fly cell types and demonstrate that the targeting vectors did indeed result in the expression of the polyP degrading enzyme in the cells of the flies. They then go on to examine the effects of polyP depletion using just one of these targeting systems (the Cyto-FLYX). The primary findings from the depletion of cytosolic polyP levels in these flies are that it accelerates eclosion and also appears to participate in hemolymph clotting. Perhaps surprisingly, the flies seemed otherwise healthy and appeared to have little other noticeable defects. The authors use transcriptomics to try to identify pathways altered by the cyto-FLYX construct degrading cytosolic polyP, and it seems likely that their findings in this regard will provide avenues for future investigation. And finally, although the authors found that eclosion is accelerated in the pupae of Drosophila expressing the Cyto-FLYX construct, the reason why this happens remains unexplained.*
>
> *Strengths:*
>
> *The authors capitalize on the work of other investigators who had previously shown that expression of recombinant yeast exopolyphosphatase could be targeted to specific subcellular compartments to locally deplete polyP, and they also use a recombinant polyP-binding protein (PPBD) developed by others to localize polyP. They combine this with the considerable power of Drosophila genetics to explore the roles of polyP by depleting it in specific compartments and cell types to tease out novel biological roles for polyP in a whole organism. This is a substantial advance.*

We are grateful to the reviewer for their thorough and thoughtful evaluation. Their balanced summary of our work, recognition of the strengths of our genetic tools, and constructive suggestions have been invaluable in clarifying our experiments and strengthening the conclusions.

> *Weaknesses:*
>
> *Page 4 of the Results (paragraph 1): I'm a bit concerned about the specificity of PPBD as a probe for polyP. The authors show that the fusion partner (GST) isn't responsible for the signal, but I don't think they directly demonstrate that PPBD is binding only to polyP. Could it also bind to other anionic substances? A useful control might be to digest the permeabilized cells and tissues with polyphosphatase prior to PPBD staining and show that the staining is lost.*

To address this concern, we have done two sets of experiments:

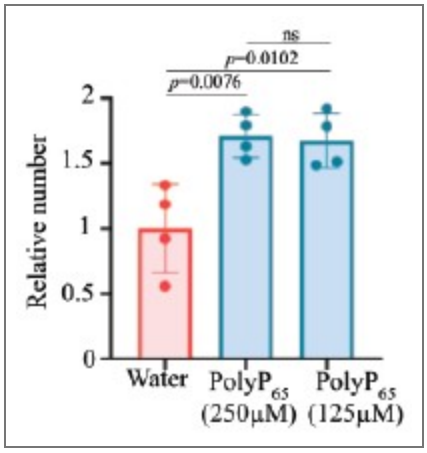
(1) We generated a PPBD mutant (GST-PPBD^{Mut}). We establish that GST-PPBD binds to polyP-2X FITC, whereas GST-PPBD^{Mut} and GST do not bind polyP$_{100}$-2X FITC using *Microscale Thermophoresis*. We found that, unlike the punctate staining pattern of GST-PPBD (wild-type), GST-PPBD^{Mut} does not stain hemocytes. This data has been added to the revised manuscript (Fig. 2B-D, p.8, l.151–165).

(2) A study in *C.elegans* by Quarles et.al has performed a similar experiment, suggested by the reviewer. In that study, treating permeabilized tissues with polyphosphatase prior to PPBD staining resulted in a decrease of PPBD-GFP signal from the tissues (2). We also performed the same experiment where we subjected hemocytes to GST-PPBD staining with prior incubation of fixed and permeabilised hemocytes with *Sc*PpX1 and heat-inactivated *Sc*PpX1 protein. We find that both staining intensity and the number of punctae are higher in hemocytes left untreated and in those treated with heat-inactivated *Sc*PpX1. The hemocytes pre-treated with *Sc*PpX1 showed reduced staining intensity and number of punctae. This data has been added to the revised manuscript (Fig. 2E-G, p.8, l.166-172).

Further, Saito et al. reported that PPBD binds to polyP in vitro, as well as in yeast and mammalian cells, with a high affinity of ~45μM for longer polyP chains (35 mer and above) (3). They also show that the affinity of PPBD with RNA and DNA is very low. Furthermore, PPBD could detect differences in polyP labeling in yeasts grown under different physiological conditions that alter polyP levels (3). Taken together, published work and our results suggest that PPBD specifically labels polyP.

> *In the hemolymph clotting experiments, the authors collected 2 ul of hemolymph and then added 1 ul of their test substance (water or a polyP solution). They state that they added either 0.8 or 1.6 nmol polyP in these experiments (the description in the Results differs from that of the Methods). I calculate this will give a polyP concentration of 0.3 or 0.6 mM. This is an extraordinarily high polyP concentration and is much in excess of the polyP concentrations used in most of the experiments testing the effects of polyP on clotting of mammalian plasma. Why did the authors choose this high polyP concentration? Did they try lower concentrations? It seems possible that too high a polyP concentration would actually have less clotting activity than the optimal polyP concentration.*

We repeated the assays using 125 μM polyP, consistent with concentrations employed in mammalian plasma studies (4,5). Even at this lower, physiologically relevant concentration, polyP significantly enhanced clot fibre formation (Included as Fig. S5F–I, p.12, l.241–243). This reconfirms the conclusion that polyP promotes hemolymph clotting.



Author response image 1.

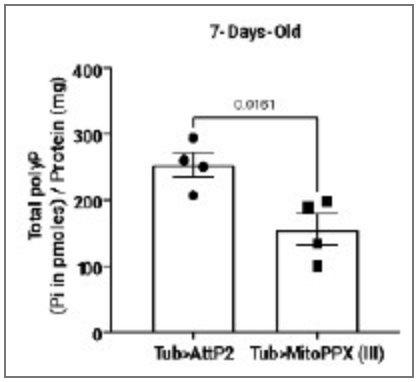
> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *Sarkar, Bhandari, Jaiswal, and colleagues establish a suite of quantitative and genetic tools to use Drosophila melanogaster as a model metazoan organism to study polyphosphate (polyP) biology. By adapting biochemical approaches for use in D. melanogaster, they identify a window of increased polyP levels during development. Using genetic tools, they find that depleting polyP from the cytoplasm alters the timing of metamorphosis, accelerating eclosion. By adapting subcellular imaging approaches for D. melanogaster, they observe polyP in the nucleolus of several cell types. They further demonstrate that polyP localizes to cytoplasmic puncta in hemocytes, and further that depleting polyP from the cytoplasm of hemocytes impairs hemolymph clotting. Together, these findings establish D. melanogaster as a tractable system for advancing our understanding of polyP in metazoans.*
>
> *Strengths:*
>
> *(1) The FLYX system, combining cell type and compartment-specific expression of ScPpx1, provides a powerful tool for the polyP community.*
>
> *(2) The finding that cytoplasmic polyP levels change during development and affect the timing of metamorphosis is an exciting first step in understanding the role of polyP in metazoan development, and possible polyP-related diseases.*
>
> *(3) Given the significant existing body of work implicating polyP in the human blood clotting cascade, this study provides compelling evidence that polyP has an ancient role in clotting in metazoans.*

We sincerely thank the reviewer for their generous and insightful comments. Their recognition of both the technical strengths of the FLYX system and the broader biological implications reinforces our confidence that this work will serve as a useful foundation for the community.

> *Limitations:*
>
> *(1) While the authors demonstrate that HA-ScPpx1 protein localizes to the target organelles in the various FLYX constructs, the capacity of these constructs to deplete polyP from the different cellular compartments is not shown. This is an important control*

> *to both demonstrate that the GTS-PPBD labeling protocol works, and also to establish the efficacy of compartment-specific depletion. While not necessary to do this for all the constructs, it would be helpful to do this for the cyto-FLYX and nuc-FLYX.*

We confirmed polyP depletion in Cyto-FLYX using the malachite green assay (Fig. 3D, p.10, l.212–214). The efficacy of *Sc*PpX1 has also been earlier demonstrated in mammalian mitochondria (6). Our preliminary data from Mito-*Sc*PpX1 expressed ubiquitously with Tubulin-Gal4 showed a reduction in polyP levels when estimated from whole flies (see Author response image 2 below, ongoing investigation). In an independent study focusing on mitochondrial polyP depletion, we are characterizing these lines in detail and plan to check the amount of polyP contributed to the cellular pool by mitochondria using subcellular fractionation. Direct phenotypic and polyP depletion analyses of Nuc-FLYX and ER-FLYX are also being carried out, but are in preliminary stages. That there is a difference in levels of polyP in various tissues and that we get a very little subscellular fraction for polyP analysis have been a few challenging issues. This analysis requires detailed, independent, and careful analysis, and thus, we refrain from adding this data to the current manuscript.



Author response image 2.

Regarding the specificity, Saito et.al. reported that PPBD binds to polyP in vitro, as well as in yeast and mammalian cells with a high affinity of ~45μM for longer polyP chains (35 mer and above) (3). They also show that the affinity of PPBD with RNA and DNA is very low. Further, PPBD could reveal differences in polyP labeling with yeasts grown in different physiological conditions that can alter polyP levels. Now in the manuscript, we included following data to show specificity of PPBD:

To address this concern we have done two sets of experiments:

We generated a PPBD mutant (GST-PPBD^{Mut}). Using Microscale Thermophoresis, we establish that GST-PPBD binds to polyP$_{100}$-2X-FITC, whereas, GST-PPBD^{Mut} and GST do not bind polyP$_{100}$-2X-FITC at all. We found that unlike the punctate staining pattern of GST-PPBD (wild-type), GST-PPBD^{Mut} does not stain hemocytes. This data has been added to the revised manuscript (Fig. 2B-D, p.8, l.151–165).

A study in *C.elegans* by Quarles et.al has performed a similar experiment suggested by the reviewer. In that study, treating permeabilized tissues with polyphosphatase prior to PPBD staining resulted in decrease of PPBD-GFP signal from the tissues (2). We also performed the same experiment where we subjected hemocytes to GST-PPBD staining with prior incubation of fixed and permeabilised hemocytes with *Sc*PpX1 and heat inactivated *Sc*PpX1 protein. We find that both intensity of staining and number of punctae are higher in hemocytes that were left untreated and the one where heat inactivated *Sc*PpX1 was added. The hemocytes pre-treated with *Sc*PpX1 showed reduced staining intensity and number of punctae. This data has been added to the revised manuscript (Fig. 2E-G, p.8, l.166-172).

> *(2) The cell biological data in this study clearly indicates that polyP is enriched in the nucleolus in multiple cell types, consistent with recent findings from other labs, and also that polyP affects gene expression during development. Given that the authors also generate the Nuc-FLYX construct to deplete polyP from the nucleus, it is surprising that they test how depleting cytoplasmic but not nuclear polyP affects development. However, providing these tools is a service to the community, and testing the phenotypic consequences of all the FLYX constructs may arguably be beyond the scope of this first study.*

We agree this is an important avenue. In this first study, we focused on establishing the toolkit and reporting phenotypes with Cyto-FLYX. We are systematically assaying phenotypes from all FLYX constructs, including Nuc-FLYX, in ongoing studies

> ***Recommendations for the authors:***
>
> ***Reviewing Editor Comment:***
>
> *The reviewers appreciated the general quality of the rigour and work presented in this manuscript. We also had a few recommendations for the authors. These are listed here and the details related to them can be found in the individual reviews below.*
>
> *(1) We suggest including an appropriate control to show that PPBD binds polyP specifically.*

We have updated the response section as follows:

(a) Highlighted previous literature that showed the specificity of PPBD.

(b) We show that the punctate staining observed by PPBD is not demonstrated by the mutant PPBD ($PPBD^{Mut}$) in which amino acids that are responsible for polyP binding are mutated.

(c) We show that $PPBD^{Mut}$ does not bind to polyP using Microscale Thermophoresis.

(d) We show that treatment of fixed and permeabilised hemocytes with *Sc*PpX1 reduces the PPBD staining intensity and number of punctae, as compared to tissues left untreated or treated with heat-inactivated *Sc*PpX1.

We have included these in our updated revised manuscript (Fig. 2B-G, p.8, l.151–157)

> *(2) The high concentration of PolyP in the clotting assay might be impeding clotting. The authors may want to consider lowering this in their assays.*

We have addressed this concern in our revised manuscript. We have performed the clotting assays with lower polyP concentrations (concentrations previously used in clotting experiments with human blood and polyP). Data is included in Fig. S5F–I, p.12, l.241–243.

> *(3) The RNAseq study: can the authors please describe this better and possibly mine it for the regulation of genes that affect eclosion?*

In our revised manuscript, we have included a broader discussion about the RNAseq analysis done in the article in both the 'results' and the 'discussion' sections, where we have rewritten the narrative from the perspective of accelerated eclosion. (p.15 l.310-335, p. 20, l.431-446).

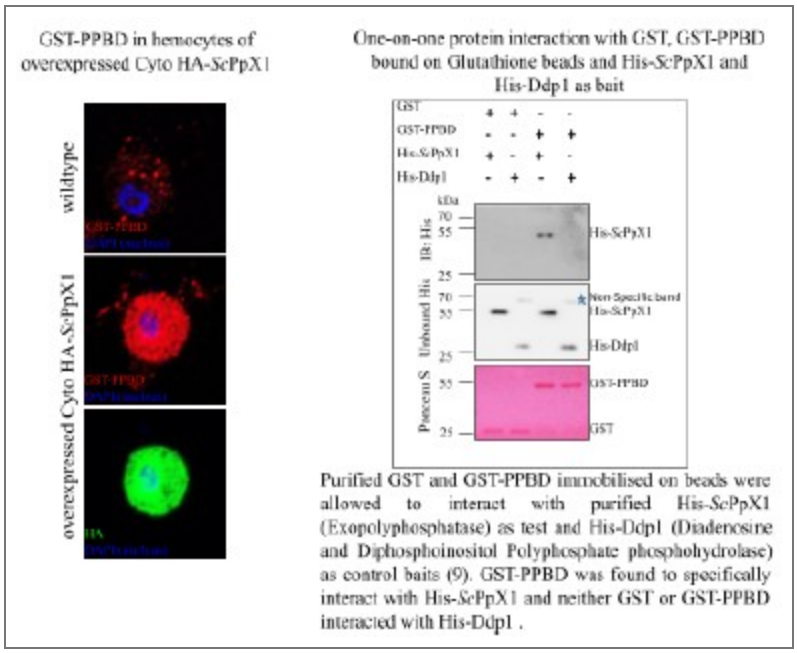
> *(4) Have the authors considered the possibility that the gut microbiota might be contributing to some of their measurements and assays? It would be good to address this upfront - either experimentally, in the discussion, or (ideally) both.*

This is an exciting possibility. Several observations argue that fly tissues synthesize polyP: (i) polyP levels remain very low during feeding stages but build up in wandering third instar larvae after feeding ceases; (ii) PPBD staining is absent from the gut except the crop (Fig. S3O–P); (iii) in *C. elegans*, intestinal polyP was unaffected when worms were fed polyP-deficient bacteria (2); (iv) depletion of polyP from plasmatocytes alone impairs hemolymph clotting, which would not be expected if gut-derived polyP were the major source and may have contributed to polyP in hemolymph. Nevertheless, microbiota-derived polyP may contribute, and we plan systematic testing in axenic flies in future work.

> ***Reviewer #1 (Recommendations for the authors):***
>
> *(1) While the authors have shown that the depletion tool results in a general reduction of polyP levels in Figure 3D, it would have been nice to show this via IHC. Particularly since the depletion depends on the strength of the Gal4, it is possible that the phenotypes are being under-estimated because the depletions are weak.*

We agree that different Gal4 lines have different strengths and will therefore affect polyP levels and the strength of the phenotype differently.

We performed PPBD staining on hemocytes expressing *Sc*PPX; however, we observed very intense, uniform staining throughout the cells, which was unexpected. It seems like PPBD is recognizing overexpressed *Sc*PpX1. Indeed, in an unpublished study by Manisha Mallick (Bhandari lab), it was found that His-*Sc*PpX1 specifically interacts with GST-PPBD in a protein interaction assay (see Author response image 3 below). Due to these issues, we refrained from IHC/PPBD-based validation.



Author response image 3.

> *(2) The subcellular tools for depletion are neat! I wonder why the authors didn't test them. For example in the salivary gland for nuclear depletion?*

We have addressed this question in the reviewer responses. We are systematically assaying phenotypes from all FLYX constructs, including Mito-FLYX, and Nuc-FLYX, in ongoing

independent investigations. As discussed in #1, a possible interaction of *ScPpX* and PPBD is making this test a bit more challenging, and hence, they each require a detailed investigation.

> *(a) Does the absence of clotting defects using Lz-gal4 suggest that PolyP is more crucial in the plasmatocytoes and for the initial clotting process? And that it is dispensible/less important in the crystal cells and for the later clotting process. Or is it that the crystal cells just don't have as much polyP? The image (2E-H) certainly looks like it.*

In hemolymph, the primary clot formation is a result of the clotting factors secreted from the fat bodies and the plasmatocytes. The crystal cells are responsible for the release of factors aiding in successfully hardening the soft clot initially formed. Reports suggest that clotting and melanization of the clot are independent of each other (7). Since Crystal cells do not contribute to clot fibre formation, the absence of clotting defects using LzGAL4-CytoFLYX is not surprising. Alternatively, PolyP may be secreted from all hemocytes and contribute to clotting; however, the crystal cells make up only 5% hemocytes, and hence polyP depletion in those cells may have a negligible effect on blood clotting.

Crystal cells do show PPBD staining. Whether polyP is significantly lower in levels in the crystal cells as compared to the plasmatocytes needs more systematic investigation. Image (2E-H) is a representative image of the presence of polyP in crystal cells and can not be considered to compare polyP levels in the crystal cells vs Plasmatocytes.

> *(b) The RNAseq analyses and data could be better presented. If the data are indeed variable and the differentially expressed genes of low confidence, I might remove that data entirely. I don't think it'll take away from the rest of the work.*

We understand this concern and, therefore, in the revised manuscript, we have included a broader discussion about the RNAseq analysis done in the article in both the ‘results’ and the ‘discussion’ sections, where we have rewritten the narrative from the perspective of accelerated eclosion. (p.15 l.310-335, p. 20, l.431-446). We have also stated the limitations of such studies.

> *(c) I would re-phrase the first sentence of the results section.*

We have re-phrased it in the revised manuscript.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *(1) The authors created several different versions of the FLYX system that would be targeted to different subcellular compartments. They mostly report on the effects of cytosolic targeting, but some of the constructs targeted the polyphosphatase to mitochondria or the nucleus.*
>
> *They report that the targeting worked, but I didn't see any results on the effects of those constructs on fly viability, development, etc.*
>
> *There is a growing literature of investigators targeting polyphosphatase to mitochondria and showing how depleting mitochondrial polyP alters mitochondrial function. What was the effect of the Nuc-FLYX and Mito-FLYX constructs on the flies?*
>
> *Also, the authors should probably cite the papers of others on the effects of depleting mitochondrial polyP in other eukaryotic cells in the context of discussing their findings in flies.*

We have addressed this question in the reviewer responses. We did not see any obvious developmental or viability defects with any of the FLYX lines, and only after careful investigation did we come across the clotting defects in the CytoFLYX. We are currently

systematically assaying phenotypes from all FLYX constructs, including Mito-FLYX and Nuc-FLYX, in independent ongoing investigations.

We have discussed the heterologous expression of mitochondrial polyphosphatase in mammalian cells to justify the need for developing Mito-FLYX (p. 10, l. 197-200). In the discussion section, we also discuss the presence and roles of polyP in the nucleus and how Nuc-FLYX can help study such phenomena (p. 19, l. 399-407).

> *(2) The authors should number the pages of their manuscript to make it easier for reviewers to refer to specific pages.*

We have numbered our lines and pages in the revised manuscript.

> *(3) Abstract: the abbreviation, "polyP", is not defined in the abstract. The first word in the abstract is "polyphosphate", so it should be defined there.*

We have corrected it in the revised version.

> *(4) The authors repeatedly use the phrase, "orange hot", to describe one of the colors in their micrographs, but I don't know how this differs from "orange".*

'OrangeHot' is the name of the LUT used in the ImageJ analysis and hence referred to as the colour

> *(5) First page of the Introduction: the phrase, "feeding polyP to αβ expression Alzheimer's model of Caenorhabditis elegans" is awkward (it literally means feeding polyP to the model instead of the worms).*

We have revised it. (p.3, l.55-57).

> *(6) Page 2 of the Introduction: The authors should cite this paper when they state that NUDT3 is a polyphosphatase: https://pubmed.ncbi.nlm.nih.gov/34788624/*

We have cited the paper in the revised version of the manuscript. (p.4, l. 68-70)

> *(7) Page 2 of Results: The authors report the polyP content in the third instar larva (misspelled as "larval") to five significant digits ("419.30"). Their data do not support more than three significant digits, though.*

We have corrected it in the revised manuscript.

> *(8) Page 3 of Results (paragraph 1): When discussing the polyP levels in various larval stages, the authors are extracting total polyP from the larvae. It seems that at least some of the polyP may come from gut microbes. This should probably be mentioned.*

This is an interesting possibility. Several observations argue that polyP is synthesized by fly tissues: (i) polyP levels remain very low during feeding stages but build up in wandering third instar larvae after feeding ceases; (ii) PPBD staining is absent from the gut except the crop (Fig. S3O–P); (ii) In *C. elegans*, intestinal polyP was unaffected when worms were fed polyP-deficient bacteria (2); (iv) depletion of polyP from plasmatocytes alone impairs hemolymph clotting, which would not be expected if gut-derived polyP were the major source and may have contributed to polyP in hemolymph. We mention this limitation in the revised manuscript (p.19-20, l. 425-433).

> *(9) Page 3 of Results (paragraph 2): stating that the 4% paraformaldehyde works "best" is imprecise. What do the authors mean by "best"?*

We have addressed this comment in the revised manuscript and corrected it as 4% paraformaldehyde being better among the three methods we used to fix tissues, which also

included methanol and Bouin's fixative (p.8, l. 152-154).

> *(10) Page 4 of Results (paragraph 2, last line of the page): The scientific literature is vast, so one can never be sure that one knows of all the papers out there, even on a topic as relatively limited as polyP. Therefore, I would recommend qualifying the statement "...this is the first comprehensive tissue staining report...". It would be more accurate (and safer) to say something like, "to our knowledge, this is the first..." There is a similar statement with the word "first" on the next page regarding the FLYX library.*

We have addressed this concern and corrected it accordingly in the revised version of the manuscript (p.9, l. 192-193)

> ***Reviewer #3 (Recommendations for the authors):***
>
> *(1) The authors should include in their discussion a comparison of cell biological observations using the polyP binding domain of E. coli Ppx (GST-PPBD) to fluorescently label polyP in cells and tissues with recent work using a similar approach in C. elegans (Quarles et al., PMID:39413779).*

In the revised manuscript, we have cited the work of Quarles et al. and have added a comparison of observations (p.19,l.408-410). In the discussion, we have also focused on multiple other studies about how polyP presence in different subcellular compartments, like the nucleus, can be assayed and studied with the tools developed in this study.

> *(2) The gene expression studies of time-matched Cyto-FLYX vs WT larvae is very intriguing. Given the authors' findings that non-feeding third instar Cyto-FLYX larvae are developmentally ahead of WT larvae, can the observed trends be explained by known changes in gene expression that occur during eclosion? This is mentioned in the results section in the context of genes linked to neurons, but a broader discussion of which pathway changes observed can be explained by the developmental stage difference between the WT and FLYX larvae would be helpful in the discussion.*

We have included a broader discussion about the RNAseq analysis done in the article in both the 'results' and the 'discussion' sections, where we have rewritten the narrative from the perspective of accelerated eclosion. (p.15 l.310-335, p. 20, l.431-446). We have also stated the limitations of such studies.

> *(3) The sentence describing NUDT3 is not referenced.*

We have addressed this comment and have cited the paper of NUDT3 in the revised version of the manuscript.(p.4, l. 68-70)

> *(4) In the first sentence of the results section, the meaning/validity of the statement "The polyP levels have decreased as evolution progressed" is not clear. It might be more straightforward to give an estimate of the total pmoles polyP/mg protein difference between bacteria/yeast and metazoans.*

In the revised manuscript, we have given an estimate of the polyP content across various species across evolution to uphold the statement that polyP levels have decreased as evolution progressed (p. 5, l. 87-91).

> *(5) The description of the malachite green assay in the results section describes it as "calorimetric" but this should read "colorimetric?"*

We have corrected it in the revised manuscript.

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https://doi.org/10.7554/eLife.104841.2.sa0