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

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Abstract

Polyphosphate exists in all life forms; however, its biological functions in metazoans are understudied. Here, we explored *Drosophila* as the first genetic model to explore polyP biology in metazoans. We first 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 then uncovered that polyP is crucial for hemolymph clotting and developmental timing. These results indicate the evolutionarily conserved role of polyP as the *ex vivo* addition of polyP 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 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 works have also implicated polyP in human diseases, e.g., 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 Alzheimer's model of *Caenorhabditis elegans* 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 yeast 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 are still 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. Function of these proteins in the regulation of polyP is yet to be tested *in vivo* in a genetically tractable metazoan model.

A major challenge in studying polyP in multicellular eukaryotes is the unavailability of a genetic model system that can allow sensitive methods for visualising, quantifying, and manipulating polyP. *Drosophila melanogaster*, the fruit fly, has been a very successful model organism to study a variety of biological processes. What makes flies unique among other models is their relatively short life span, a wealth of genetic mutants, and the availability of tools for spatio-temporal genetic manipulations. 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 an *in vivo* model to dissect the functions of polyP in metazoans.

Results:

Development of methods for extraction and quantification of polyP from flies

The polyP levels have decreased as evolution progressed (3). Since metazoans have low 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 to extract polyP from biological samples have been discussed, each with some limitations (33). 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 (34). We used the enzymatic detection method to quantify polyP extracted from fly tissues as it has higher specificity and sensitivity (35,36). This method involved the digestion of polyP by recombinant *S. cerevisiae* exopolyphosphatase 1 (*Sc*Ppx1) followed by calorimetric measurement of the released Pi by malachite green. Malachite green and ammonium molybdate, when mixed in the ratio of 3:1, form a metachromatic brown-coloured adduct that turns green upon conjugation with monophosphate (37,38) 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 (36–38). 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 is shown to specifically bind polyP *in vitro*, and detect *in situ* polyP localised in budding yeast vacuoles and mammalian mast cell granules (39,40). We created a GST-tagged PPBD (GST-PPBD) construct and purified recombinant GST-PPBD protein to label polyP in fly tissues. GST protein was used as a negative control to detect non-specific signals. We used methanol, 4% paraformaldehyde and Bouin's fixatives to fix *Drosophila* tissues and found that 4% paraformaldehyde works the best **(Supplementary Fig 2, see Materials and Methods)**. For the staining we permeabilized the fixed tissues and divided them into two parts- we incubated one part with GST-PPBD, and the other with GST (negative control) and analysed the staining pattern using an anti-GST primary antibody and fluorophore-labelled secondary antibody. We observed distinct patterns of GST-PPBD labelling in different tissue types **(Fig 2 and Supplementary Fig 3)**. We observed GST-PPBD labelling in a cytoplasmic punctae, nuclear compartments, or both in a tissue-specific manner **(Fig 2 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 2A ii, 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 2B-C)**. Further, we systematically stained polyP in larval imaginal discs (tissues that mature into organs in the adult fly) and found punctate cytoplasmic and nucleolar GST-PPBD staining in the wing and eye imaginal discs. 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 2D)**. 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 2D)**. 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 (41–43). In hemocytes (hemolymph cells, equivalent to mammalian blood cells) we also found an intense GST-PPBD labelling exhibiting polyP localization in large granular structures close to the periphery **(Fig 2E-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, this is the first comprehensive tissue staining report exhibiting the spatiotemporal

dynamics of polyP within a multicellular organism. These data also indicate tissue-specific regulation and biological function of polyP in metazoans.

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 (48). 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 (49,50). 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 3A-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 (51). 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 3C, 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 3D)**.

Further, 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 3C)**. 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 3E, See Material and Methods for sequence)**. This FLYX library, for the first time, 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 (11,44–46). To assess and quantify hemolymph clotting characteristics, we established a 'hemolymph drop assay' **(Supplementary Fig 4A-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)** (47). 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, Supplementary Fig 5)**. 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)**.

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 4A-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 4E-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 4I-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 4M-P)**. These data suggest that polyP in hemocytes is necessary for efficient hemolymph clotting.

Further, 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 4Q-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 did not find any significant difference in the weight or the size of third instar larvae, mid-pupa, or five- day old adult Cyto-FLYX and control flies **(Supplementary Fig 6A-D).** Since, pUAST vector is not efficient in driving expression in the germ line cells, we cloned Cyto-FLIX in the pUASP vector and generated a new transgenic line to deplete polyP in the germ line cells. However, we did not find any significant change in the

fecundity upon germ line expression of Cyto-FLIX using *Nos*-GAL4 driver **(Supplementary Fig 6E-F)**.

Further, we had observed polyP levels increase just before pupariation and decrease during pupal stages, so 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 5A-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) (52). 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) (53). Insulin signalling pathways via dfoxo also controls TOR signalling pathway effector genes such as, *4ebp* (54). 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 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. 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 (55) **(Fig 5C-E, Supplementary Fig 7C)**. 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 (56) **(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, for the first time, 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.

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 (33,34). 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 (57–60). 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 (39). 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 (37,38). Further, to locate polyP in fly tissues, we utilised epitope-tagged PPBD, which specifically binds to polyP with a very high affinity (39,40). 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 the first comprehensive estimation and distribution of polyP in a metazoan organism **(Fig 1-2, 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 (61,62). Surprisingly, polyP-depleted flies (Cyto-FLYX) eclose faster **(Fig 5A-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 punctae. 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) (40,63) 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 (64,65). 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 (66–68). Since polyP can induce LLPS by its interactions with the nucleic acid and positively charged proteins, polyP may be involved in nucleolus organisation as the nucleolus is an LLPS organelle (69). 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, Supplementary Fig 3)**.

On a different tangent, we know that patients suffering from Type 1 Von Willebrand Disease (VWD) have decreased polyP content in plasma (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 mainly 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, this work demonstrated that polyP in plasmatocytes is crucial for hemolymph clotting **(Fig 4A-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 (63). 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 4Q-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 4H, 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 (70,71). 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. The third instar larval population usually undergoes bursts of transcription related to its development and metamorphosis. In the Gene Set Enrichment Analysis, we found gene sets enriched for translation and ribosome biogenesis. Indeed, the proteomic analysis of a Δ*ppk* mutant *E.coli*, which results in absence of polyP, also shows a similar response (72). 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.

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 FLIX 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.

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℃. 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℃. 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.

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.

HA staining:

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℃. 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 assessment of clot fibre characteristics

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 (73). 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 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 4F-J,** and **supplementary Fig 5**, 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 4B-C, and Fig 4, 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 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 25ºC. 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) (74) and imported to the R environment. Uniquely mapped reads were further filtered and normalised using the Trimmed mean of M-values (TMM) (75) method of EdgeR/limma (4.0.3) Bioconductor library (76).

Then, Gene Set Enrichment Analysis (GSEA) (77) was performed using the clusterProfiler (4.10.1) package (78). In essence, gseGO functions were used to identify the GSEA of Gene Ontology for Biological Processes (BP), Molecular Functions (MF) and Cellular Components (CC).

Code Availability

The code for data processing is available upon request.

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.

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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. JSL: Analysis of polyphosphate by gel electrophoresis. 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.

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Figure legends:

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-day post-pupariation pupae (n=20, N=8), pharate (n=10, N=5), three-day 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: 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. *Drosophila* strain used - *CantonS*.

Fig 3: 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-ScPpx1 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

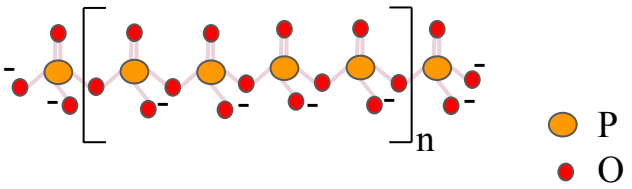
Fig 4: Genetic depletion of polyP shows clotting defects in flies. A-D) Clot phenotype analysis of *tubulin-GAL4* driven Cyto-FLYX (*tubulin*-FLYX). The control is *tubulin-GAL4>*AttP40. **A)**

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.

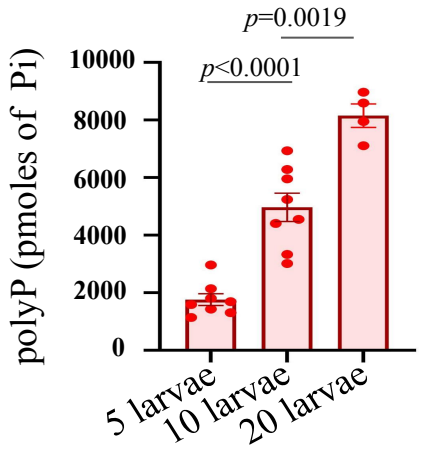
Fig 5: Genetic depletion of polyP shows accelerated eclosion. A) Cumulative percentage of eclosion of control and FLYX checked after white prepupa formation (APF), m=213 (control), 268 (FLYX), N=4 (control), N=5(FLYX). **B)** Time of 50% eclosion of the control and FLYX flies- ~134 hours APF for control 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. **(C-E)** GO analysis in Cyto-FLYX and control larvae showing Biological processes (C), Cellular components (D), and Molecular functions (E).

Fig. 1

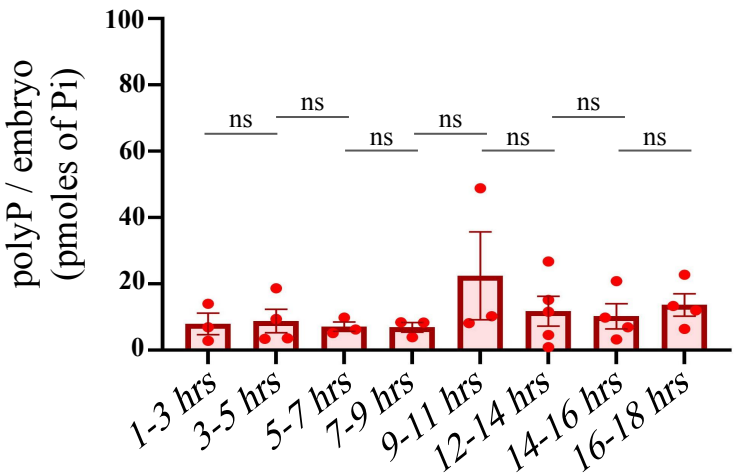
A Schematic of polyphosphate



B Quantitative estimation of polyP from *Drosophila* larvae



C Estimation of polyP from various embryonic stages



D Estimation of polyP from various life cycle stages

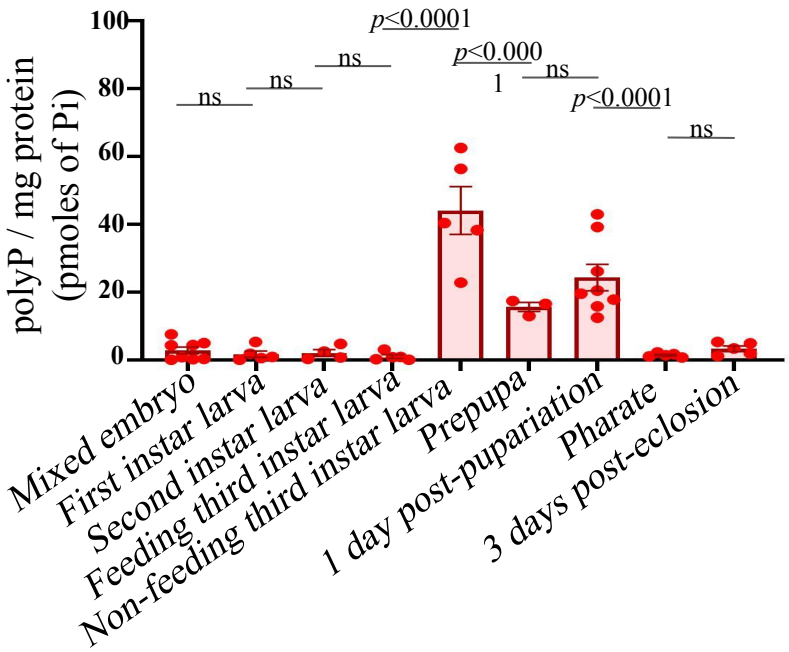
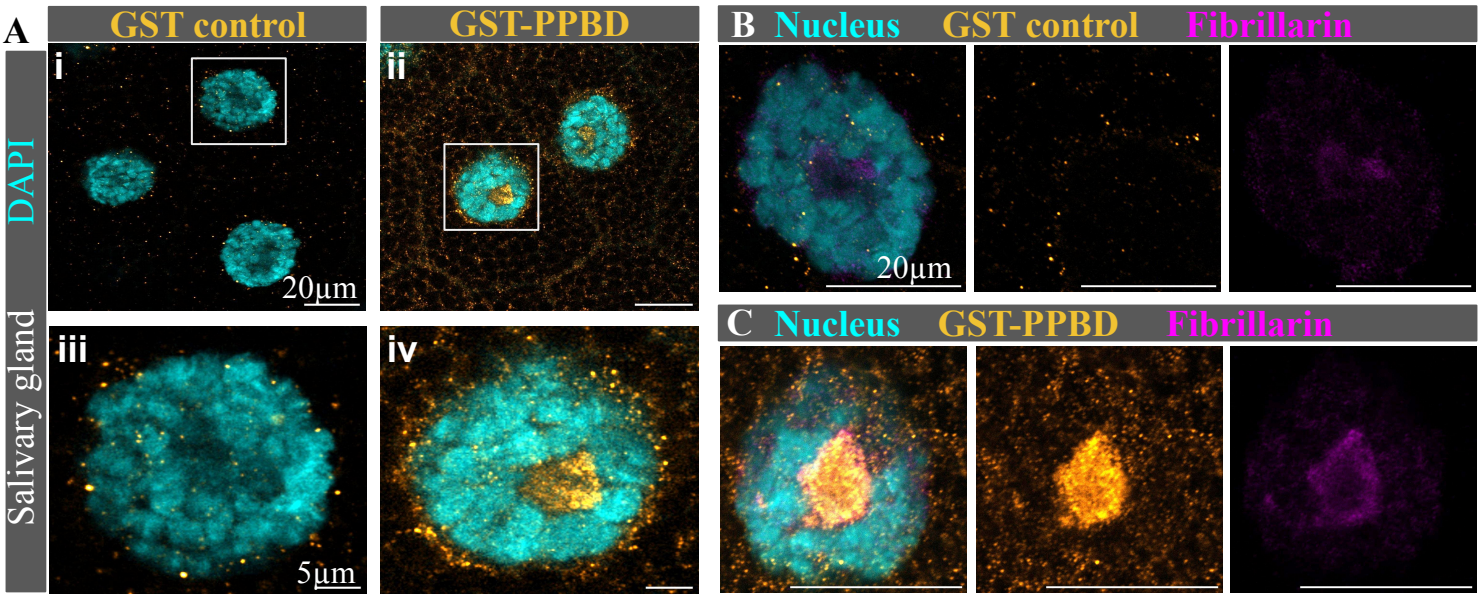
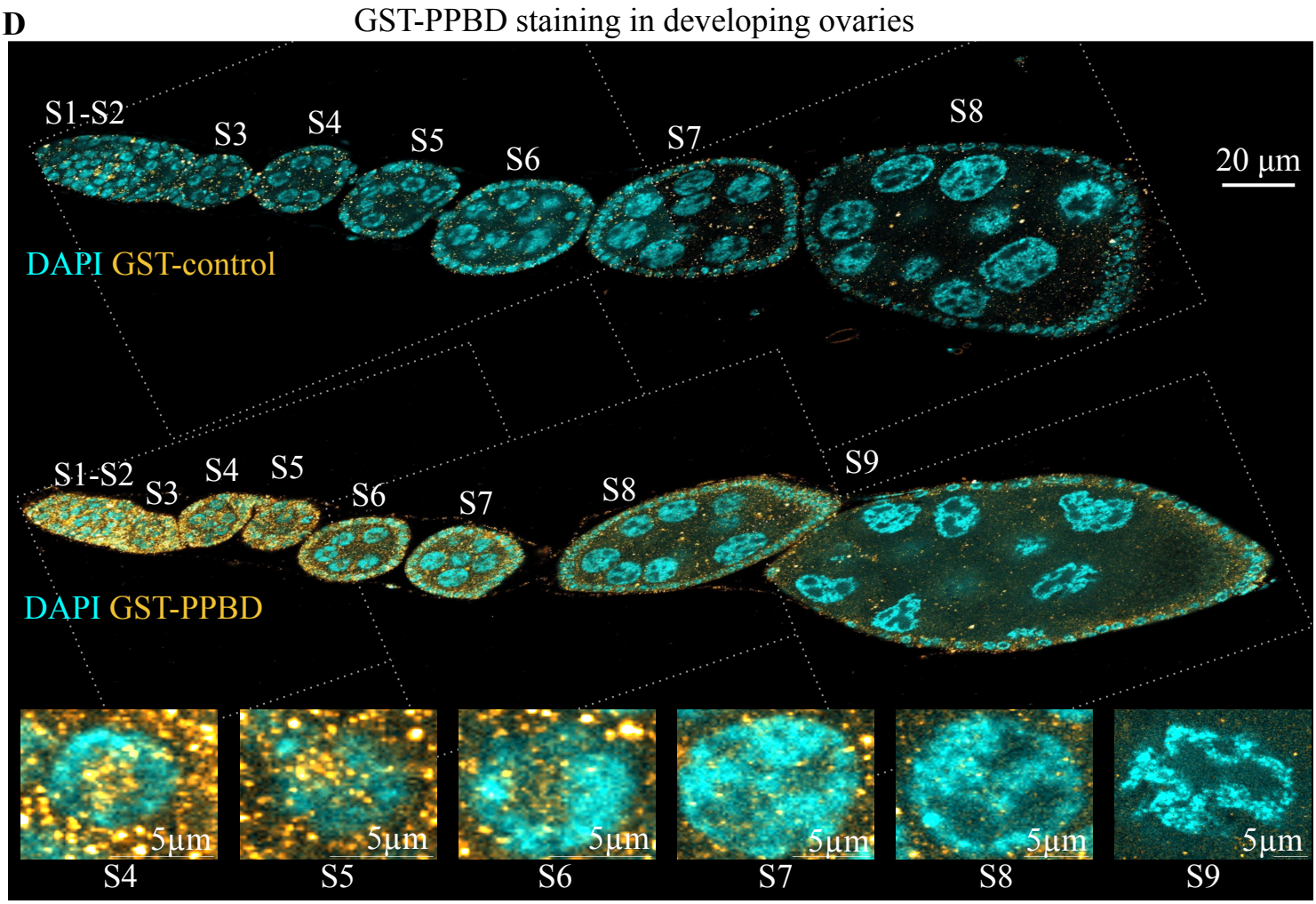


Fig 2


A
GST control
GST-PPBD
DAPI
Salivary gland
i
ii
iii
iv
20µm
5µm
B Nucleus GST control Fibrillarin
20µm
C Nucleus GST-PPBD Fibrillarin


D
GST-PPBD staining in developing ovaries
S1-S2
S3
S4
S5
S6
S7
S8
20 µm
DAPI GST-control
S1-S2
S3
S4
S5
S6
S7
S8
S9
DAPI GST-PPBD
5µm
S4
S5
S6
S7
S8
S9

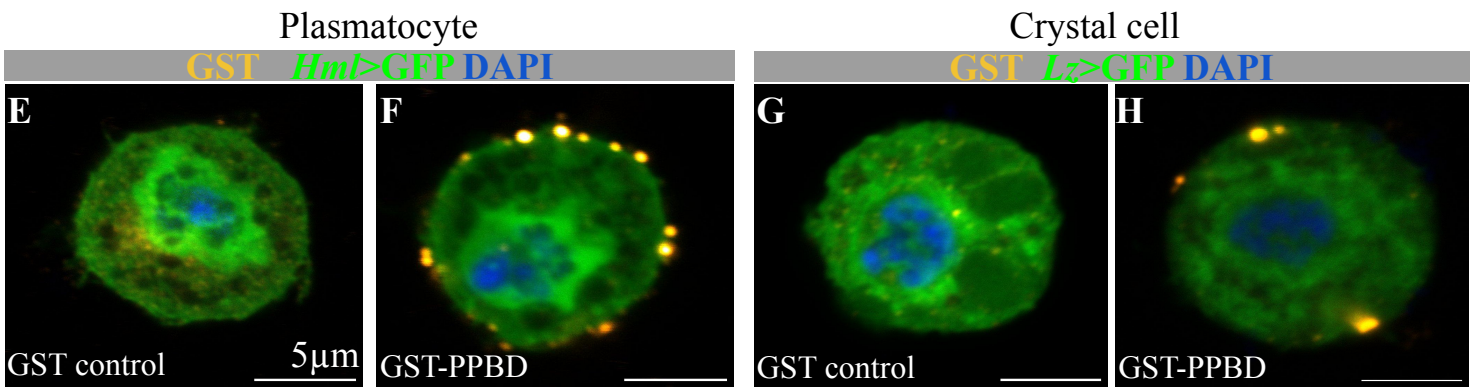
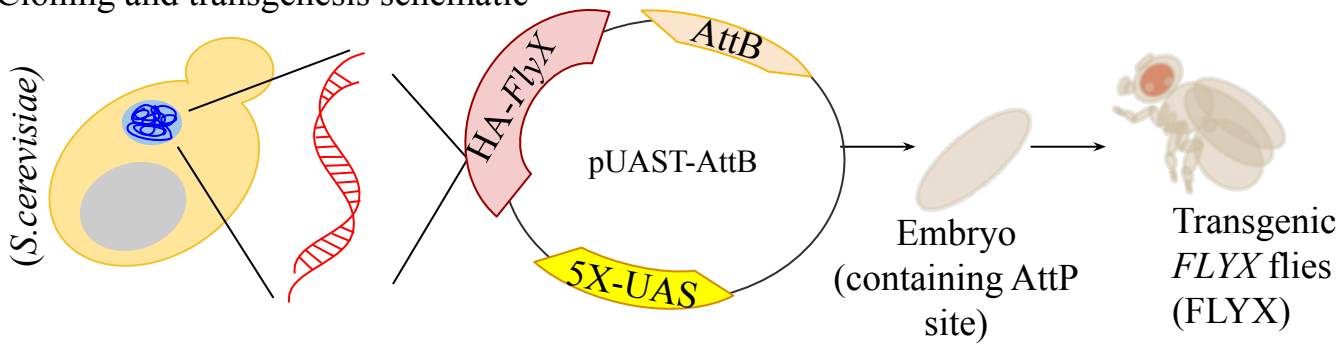

Plasmatocyte
Crystal cell
GST Hml>GFP DAPI
GST Lz>GFP DAPI
E
F
G
H
GST control
5µm
GST-PPBD
GST control
GST-PPBD

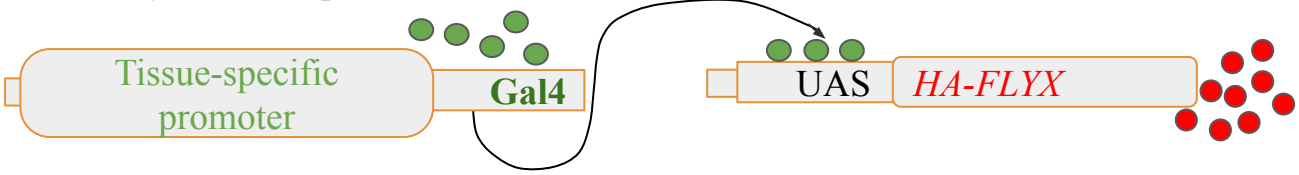
Fig 3

A fly tool for genetic polyP depletion- FLYX

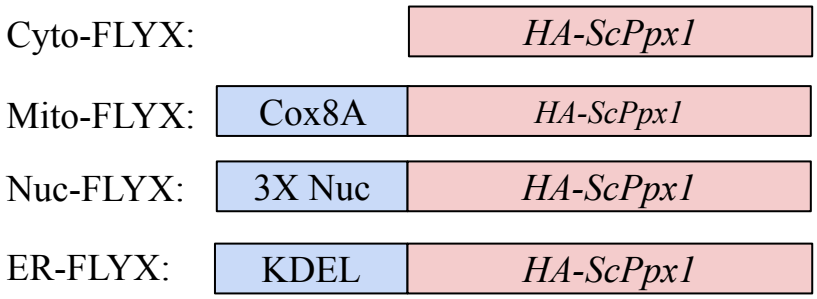
A Cloning and transgenesis schematic



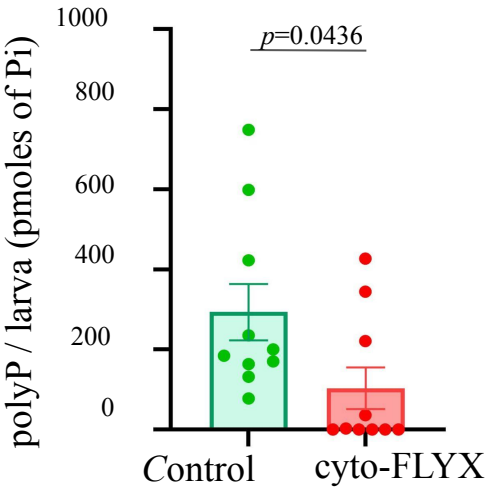
B UAS-Gal4 system to express *FLYX* in flies



C Different FLYX construct schematic



D FLYX line has functional *Sc*Ppx1



E Localisation of HA-ScPpx1 to the target organelles in different FLYX larval muscles

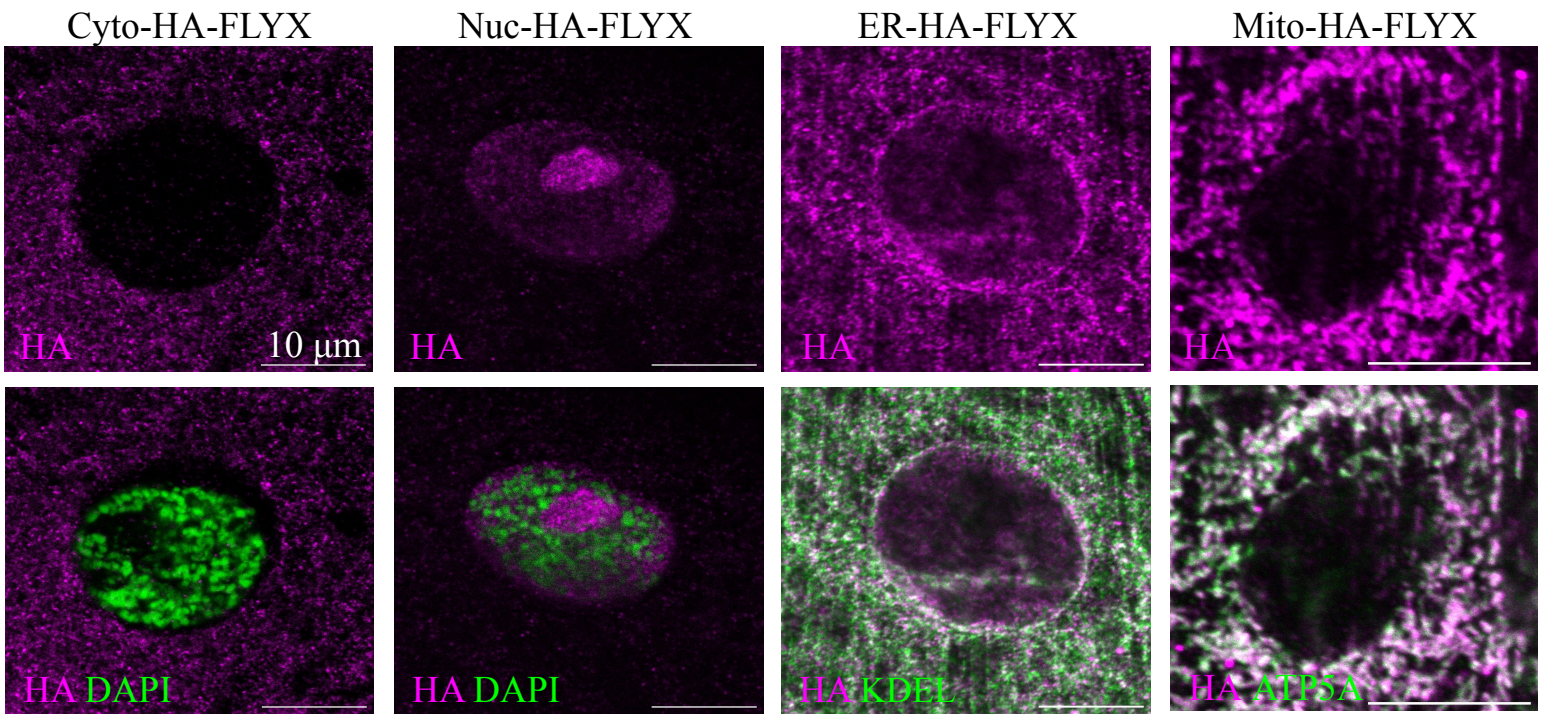
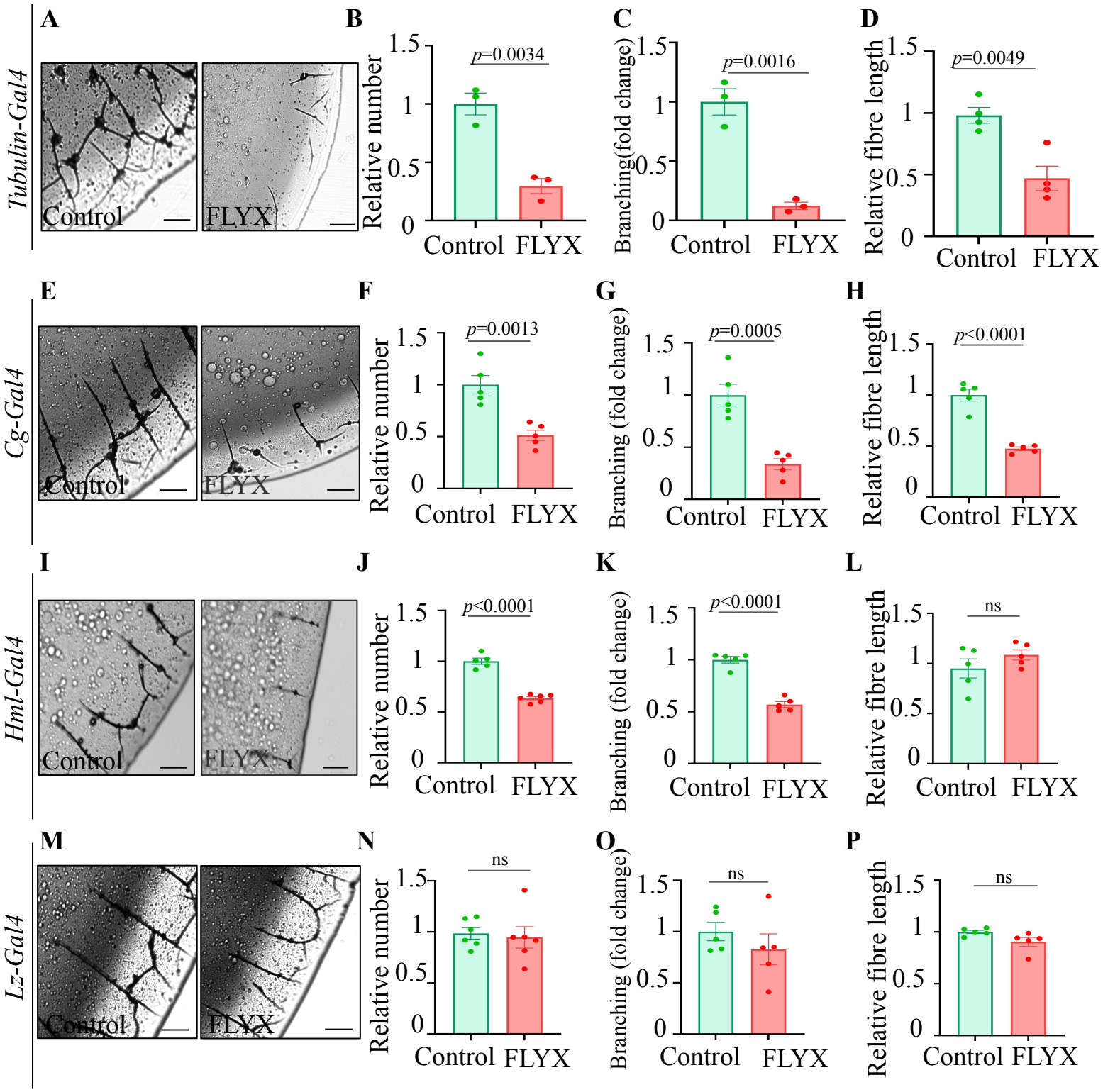


Fig 4

Cyto-FLYX show hemolymph clotting defects



Exogenous polyP addition fail to rescue clotting defects in FLYX

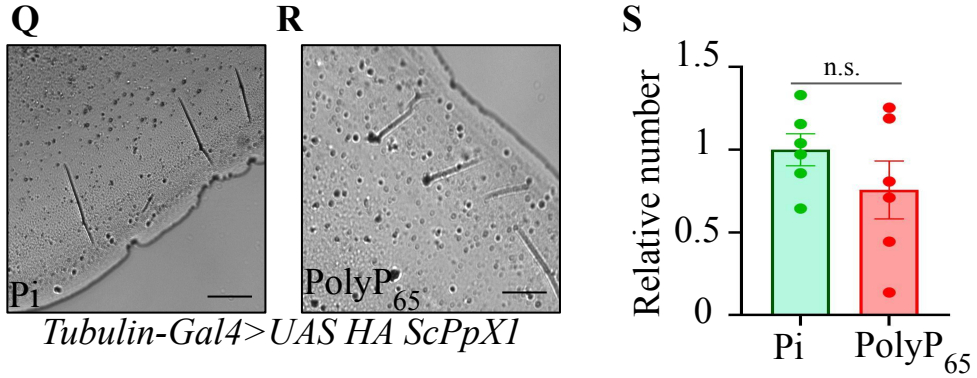
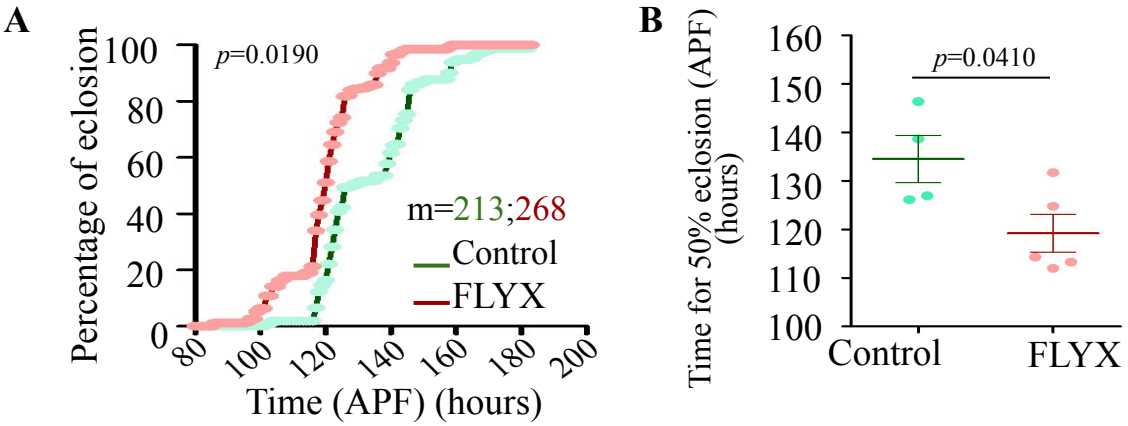


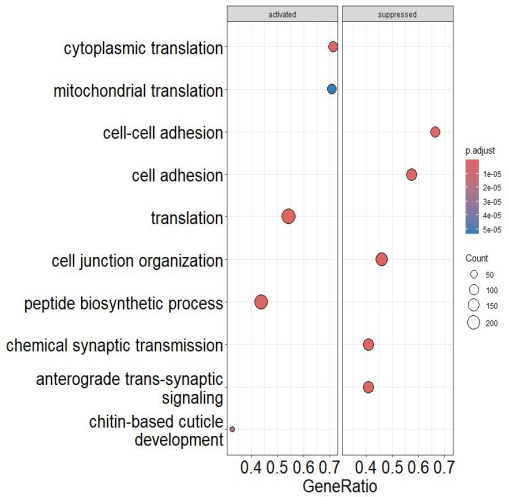
Fig 5

Cyto-FLYX show accelerated eclosion

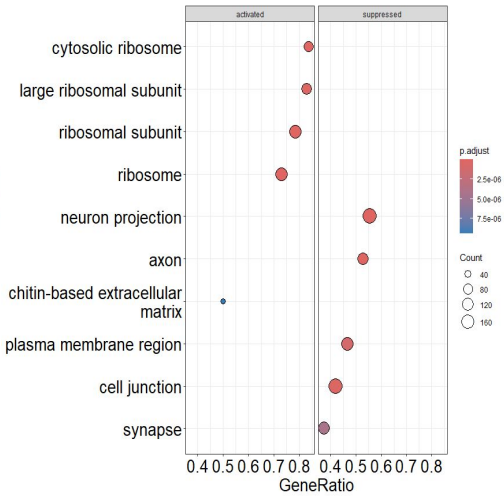
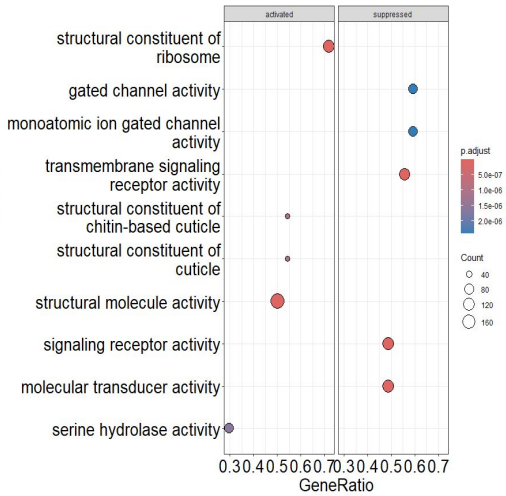


Gene Set Enrichment Analysis in Cyto-FLYX and control larvae

C Biological processes



D Cellular components



E Molecular functions