Glycolytic enzymes localize to ribonucleoprotein granules in *Drosophila* germ cells, bind Tudor and protect from transposable elements

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Abstract

Germ cells give rise to all cell lineages in the next-generation and are responsible for the continuity of life. In a variety of organisms, germ cells and stem cells contain large ribonucleoprotein granules. Although these particles were discovered more than 100 years ago, their assembly and functions are not well understood. Here we report that glycolytic enzymes are components of these granules in *Drosophila* germ cells and both their mRNAs and the enzymes themselves are enriched in germ cells. We show that these enzymes are specifically required for germ cell development and that they protect their genomes from transposable elements, providing the first link between metabolism and transposon silencing. We further demonstrate that in the granules, glycolytic enzymes associate with the evolutionarily conserved Tudor protein. Our biochemical and single-particle EM structural analyses of purified Tudor show a flexible molecule and suggest a mechanism for the recruitment of glycolytic enzymes to the granules. Our data indicate that germ cells, similarly to stem cells and tumor cells, might prefer to produce energy through the glycolytic pathway, thus linking a particular metabolism to pluripotency.

Keywords germ cells; glycolysis; stem cells; transposable elements; Tudor domain

Subject Categories Metabolism; Development & Differentiation; Chromatin, Epigenetics, Genomics & Functional Genomics

DOI 10.15252/embr.201439694 | Received 6 October 2014 | Revised 2 December 2014 | Accepted 9 December 2014 | Published online 19 January 2015

EMBO Reports (2014) 16: 379–386

Introduction

Glycolysis is the universally conserved and ancient metabolic pathway used for energy production and biosynthetic processes in the cell. Furthermore, glycolysis plays a crucial role in the metabolism of tumor cells in many types of cancer (the Warburg effect) [1,2] and of different classes of stem cells [3–5], thereby highlighting common metabolic features in these cells. Unlike stem cells, germ cells are highly specialized and differentiate into eggs or sperm. However, the union of egg and sperm gives rise to a zygote, which is capable of producing all cell lineages in the organism of the next generation [6,7].

Germ cells in many organisms have distinct cytoplasmic structures referred to as germ granules, which contain RNAs and proteins required for germline development [7–11]. These granules contain several important proteins including the ATP-dependent RNA helicase Vasa (Vas) [8,12–14] and Tudor (Tud), which associates with Piwi protein—small Piwi-interacting RNA (piRNA) complexes. These complexes protect germline DNA from mutations caused by retrotransposons [15–20]. It is believed that antisense piRNAs guide Piwi family proteins to retrotransposon mRNAs which are subsequently cleaved by the Piwi pathway proteins. The Tud domains of Tud proteins directly interact with symmetrically dimethylated arginines (sDMAs) of Piwi proteins in the germ granules [21]. In many organisms, Tud domain-containing proteins have multiple Tud domains, which may interact with different partners in the germ granules; in particular, *Drosophila* Tud contains 11 Tud domains [22].

Results and Discussion

Unexpectedly, we recovered two glycolytic enzymes, pyruvate kinase (PyK) and glyceraldehyde-3-phosphate dehydrogenase 2 (GAPDH2), with Tud in co-immunoprecipitations after chemical crosslinking of

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Figure 1. Glycolytic enzymes are components of Tudor protein complex and their mRNAs are enriched in germ cells.

- A** Proteins found in multiple ovarian Tud complexes. Protein complexes for both HA-full-length (FL) Tud (with 11 Tud domains) and functional mini-Tud $\Delta 3$ (lacks Tud domains 2–6) were isolated. Aub was identified in FL and mini-Tud complexes and Aub-Tud complex has been characterized [15,17,18,22,31]. The other proteins were identified in mini-Tud $\Delta 3$ complex. A likely reason for identifying the majority of proteins in the mini-Tud rather than in FL-Tud complex is the higher expression of mini-Tud $\Delta 3$ [22]. Glycolytic enzymes are highlighted. The numbers in the parentheses show the numbers of peptides sequenced for a given protein. Multiple numbers listed for a protein correspond to the numbers of this protein peptides identified in independently isolated mini-Tud $\Delta 3$ complexes.
- B** mRNAs for several glycolytic enzymes are enriched in germ cells of stage 5 embryos. Germ cells are indicated with arrows. Anterior is to the left and dorsal is up. Scale bar is 25 μ m.

Drosophila ovarian extracts (this study) and also in Tud- and Vas-containing complexes isolated from embryos [23] (Fig 1A). The ovarian Tud complexes also contained the Piwi protein Aubergine (Aub) [15], the DEAD-box ATP-dependent RNA helicase eIF4A, and α - and β -tubulins (Fig 1A). Importantly, all the proteins of Tud complex were recovered repeatedly from independent complex isolations and were never found in control ovarian GFP immunoprecipitations performed under the same conditions as Tud immunoprecipitations as analyzed by mass spectrometry. The presence of two glycolytic enzymes in germline protein complexes suggested that the glycolytic pathway itself (Fig 5), rather than its individual components, may play a specific role in germ granules. Therefore, we analyzed the distribution of glycolytic enzymes in the germline in more detail.

First, we performed a comprehensive set of RNA *in situ* experiments to determine the distribution of mRNAs encoding almost all glycolytic enzymes during embryogenesis. We analyzed the distribution of mRNAs for nine of ten glycolytic enzymes (all except for phosphoglucose isomerase). We found that all these glycolytic mRNAs were uniformly distributed in preblastoderm embryos before germ cell formation. However, *pyk*, *gapdh2* and *enolase* (*eno*) mRNAs became specifically enriched in germ cells in blastoderm stage embryos (Fig 1B).

Figure 2. Glycolytic enzymes are polar granule components and segregate to germ cells.

- A** Live imaging of PyK-GFP distribution at the embryo posterior pole during germ cell formation stage. Scale bar is 50 μ m.
- B** PyK colocalizes with germ cell marker protein Vasa in germ cells. Wild-type embryos were co-stained with goat anti-PyK antibody (green channel) and rabbit anti-Vasa antibody (red channel). Overlay image is shown. 50 μ m scale bar is shown in the first image.
- C, D** Immuno-EM images of the embryo germ plasm (stages 1–2) stained with anti-PyK (C) or anti-PGK antibody (D) show enrichment of these glycolytic enzymes in polar granules (indicated with arrows). m, mitochondria. 0.5 μ m scale bar in (C) is the same for (D).

Next, we asked whether glycolytic proteins are enriched in the germline. PyK was indeed enriched in germ cells as determined in live (Fig 2A) and fixed (Fig 2B) embryos. In addition, before germ cell formation, PyK was mainly localized to germ granules (polar granules) in the embryonic germ plasm (specialized cytoplasm in posterior which is necessary and sufficient for germ cell formation [24]) as shown by immunoelectron microscopy (EM) (Fig 2C and Supplementary Fig S1). We counted gold particles, which correspond to the location of PyK on the EM sections, and found that polar granules contain 5.5 times more PyK than would be expected from the same cytoplasmic area if PyK were distributed uniformly (1,079 gold particles counted).

PyK is one of the two enzymes that produce ATP during glycolysis, and polar granules contain ATP-dependent RNA helicases, including Vas and eIF4A (Supplementary Fig S1), which may benefit from the immediate source of glycolytic ATP. The other glycolytic enzyme that produces ATP is phosphoglycerate kinase (PGK). Therefore, we tested whether, similarly to PyK, PGK is also localized to polar granules. We found that PGK was indeed highly enriched in polar granules (Fig 2D), which contain 5.3 times more PGK compared to the expected amount if PGK distribution were uniform (603 gold particles counted).

The enrichment level of both PyK and PGK in polar granules is essentially the same as that for an established polar granule component, a DEAD-box ATP-dependent RNA helicase eIF4A (polar

Figure 3. Mutations in genes encoding glycolytic enzymes cause defects in germ cell formation and transposon silencing mechanisms.

A–C Wild-type and indicated mutant embryos (stage 5) generated by germline clone technique were stained with anti-Vasa antibody to label germ cells. Scale bar is 25 μ m.
 D Scatterplot compares the expression levels of transposons in *ald* mutant with those in the wild-type. Multiple transposons overexpressed in *ald* mutant are plotted above the diagonal. Transposons overexpressed twofold and above are indicated in red. Consistent with the RNA-seq data, independent qPCR experiments showed overexpression of *blood*, *Burdock* and *copia* transposons.
 E Scatterplot of 9,130 non-transposon genes' expression levels in *ald* mutant versus wild-type ($R^2 = 0.94$).
 F, G Scatterplots of precursor piRNA cluster transcript levels in *ald* (F) and *eno* (G) mutants versus wild-type showing accumulation of the transcripts in the mutants.
 H Levels of piRNAs from multiple clusters are reduced in glycolytic mutants. Expression levels of piRNAs from representative clusters normalized to those from the wild-type are shown for indicated glycolytic mutants. piRNA levels are reduced in all mutants for all clusters shown except for *flam* cluster which is exclusively expressed in the ovarian soma.

Data information: In panels D–H, data are based on the sequencing results from two RNA libraries: the transcriptome library (D–G, $n = 1$) and the small RNA library (H, $n = 1$) for a given mutant or wild-type control.

granules were shown to be 5.1-fold enriched for this helicase [23]). Importantly, we found eIF4A in the same ovarian Tud complex with glycolytic enzymes, including ATP-producing PyK (Fig 1A), and showed directly in a double-labeling immunogold experiment that PyK and Vas are localized next to each other in the same polar granule (Supplementary Fig S1C), indicating the proximity of all glycolytic ATP-generating enzymes and ATP-dependent RNA helicases in the granules.

Since the glycolytic enzyme genes are essential for viability, we generated homozygous mutant germline clones which allowed us to

test for possible specific defects of mutations in *eno*, *pyk* and *aldolase* (*ald*) during germline development (Supplementary Fig S2). This technique allows one to generate homozygous mutant ovaries in an otherwise heterozygous fly [25]. Females with *ald* mutant ovaries laid very few eggs (Supplementary Fig S2A), and the embryos that formed had reduced numbers of germ cells (the average number of germ cells was 5.4 ± 1.8 (s.e.m.), 10 embryos counted) (Fig 3C). In contrast, wild-type germline clone control embryos formed on average 22.4 germ cells ± 0.7 (s.e.m.), 27 embryos counted (Fig 3A). *eno* and *pyk* mutant germline clone

ovaries developed normally and the mutant females laid wild-type numbers of eggs (Supplementary Fig S2). However, embryos generated by the *eno* mutant females showed more than a twofold reduction in germ cell number with an average of $8.9 \text{ germ cells} \pm 1.3$ (s.e.m.), 24 embryos counted (Fig 3B). Unpaired two-tailed *t*-test analyses confirmed that the reductions of germ cells in both *eno* and *ald* mutants compared with the wild-type control are statistically very significant ($P = 6.2 \times 10^{-11}$ and $P = 2 \times 10^{-6}$ for *eno* and *ald* mutants, respectively). Similar results have been observed in embryos produced by females that expressed an *eno* knockdown RNAi in the germline (N. Liu, P. L., unpublished data).

Since we identified a Piwi family protein, Aub, in the Tud complex (Fig 1A), we tested whether glycolytic enzymes also contribute to transposon silencing and piRNA biogenesis. Steady-state RNA levels were determined by sequencing (RNA-seq) of the whole transcriptomes from *ald*, *eno* and *pyk* mutant germline clone ovaries and the respective wild-type controls. *ald* mutant ovaries showed significant overexpression of many transposons (6- to 30-fold increase in levels compared to wild-type; Fig 3D and Supplementary Table S1). In contrast, expression of other genes in the *ald* mutant correlated well with that in the wild-type control ($R^2 = 0.94$) (9,130 genes examined; Fig 3E). We suggest that these transposons are targeted by piRNAs since the same transposons overexpressed in *ald* mutants are also upregulated in other *bona fide* piRNA pathway mutants, notably *rhino* mutants [26]. In addition, we observed the accumulation of piRNA cluster precursor transcripts in *ald* and *eno* mutant ovaries (Fig 3F and G) indicating defective primary processing of piRNAs in the glycolytic mutants. In order to determine the piRNA levels, we deeply sequenced small RNAs from *ald*, *eno* and *pyk* germline clone mutant ovaries and found that all the mutants showed significant reduction of piRNAs generated from multiple piRNA clusters. In particular, piRNA levels from all 142 genomic clusters have been determined and piRNAs from ~20% of the clusters in each glycolytic mutant showed over twofold reduction compared to wild-type controls (Fig 3H and Supplementary Table S2).

Next, we examined possible role of glycolysis for the miRNA and siRNA pathways. Contrary to piRNA biogenesis, miRNA pathway was not affected in glycolytic mutants (Supplementary Fig S3A, C and E). Furthermore, we observed that siRNA levels are largely increased in all glycolytic mutants (Supplementary Fig S3B, D and F). Therefore, this increase in siRNA amounts cannot explain defects in silencing of transposable elements observed in our study. Interestingly, increase in siRNA levels has been described previously in *piwi* mutants, suggesting that primary defect in piRNA biogenesis may indirectly trigger an siRNA response [27]. Therefore, our data point to the specific effect of glycolysis on piRNA pathway in the germline.

Contrary to the observed primary piRNA processing defects, glycolytic mutations did not cause defects in the generation of secondary piRNAs by Ping-Pong cycle which amplifies both antisense and sense piRNAs using Piwi family proteins that cleave original piRNA precursor transcripts (producing more antisense piRNA) and transposon mRNAs (generating more sense piRNAs) (Supplementary Fig S2C) [28]. Therefore, we conclude that glycolytic mutants specifically affect primary piRNA biogenesis. We propose that glycolytic components may be similar to described known factors which affect primary piRNA biogenesis without affecting Ping-Pong cycle, for example, Vreteno and Zucchini [29].

Our data indicate that mutations in different glycolytic genes cause defects in germ cell development and transposon silencing mechanisms. Therefore, the entire glycolytic pathway, rather than individual components, might play a special role in germ cell specification and contribute to the protection of germline DNA against transposons. These data provide the first evidence for the connection between metabolism and transposon silencing.

The biochemical screen and mass spectrometry data reported here (Fig 1A) indicated that PyK is an abundant component of the crosslinked Tud complex, suggesting that Tud may bind directly to PyK within germ granules. Tud and functional PyK-GFP fusion protein [30] were purified from fly ovaries, and using these proteins, an *in vitro* binding assay was developed to test for their direct interaction. In this assay, purified PyK-GFP (or just GFP as a control) bound to agarose-conjugated anti-GFP antibody was mixed with purified full-length Tud protein (Fig 4A). Using this assay, direct binding of PyK to Tud was demonstrated (Fig 4B, lane 4).

Recent studies showed that Tud protein directly binds the Piwi family protein Aub and this binding is due to the recognition of sDMAs of Aub by Tud domains [15,17,18,31]. Therefore, we tested whether Tud domains are involved in binding to PyK. Specifically, Tud protein was preincubated with methylated Aub peptide, which corresponds to the natural target of Tud domains [18], and subsequently, preincubated Tud was added to PyK-GFP agarose. Methylated Aub peptide completely inhibited the binding of Tud to PyK (Fig 4B, lane 2), while the unrelated control peptide allowed for efficient binding (Fig 4B, lane 4). These results showed that PyK is recognized by Tud domains in Tud protein.

Two Tud deletion constructs have been generated in previous studies, mini-Tud $\Delta 3$ [22] (contains Tud domains 1, and 7–11) and Tud7–11 [31] (contains Tud domains 7–11). Both these constructs can rescue germ cell formation in embryos produced by *tud* null mutant females. Similarly to full-length Tud, these smaller versions of Tud were purified from ovaries (Fig 4A) and tested for direct binding to PyK *in vitro*. Both mini-Tud $\Delta 3$ and Tud7–11 were able to bind to PyK directly (Fig 4C).

Tud domains are known to interact with sDMAs of target proteins. Therefore, we tested whether PyK contains an sDMA in a Western blot experiment using SYM11 antibody, which specifically recognizes sDMAs [32]. Figure 4D shows that both ovarian PyK and Aub react with SYM11 indicating that PyK contains sDMA. Contrary to PyK and Aub, GFP expressed in the ovaries (negative control in this experiment) failed to react with SYM11 (Fig 4D). Interestingly, PyK protein sequence contains an arginine-glycine motif which is a common site of sDMA modifications [20,33].

Since Tud binds to different proteins, including Piwi family proteins and PyK, using its multiple protein–protein interaction Tud domain modules, we hypothesized that Tud might be a flexible molecular scaffold. This flexibility could allow Tud to interact with several biochemically diverse proteins simultaneously and still assemble them into germ granules, which are also known to be quite dynamic and to adopt different shapes (Fig 2C, D and Supplementary Fig S1). To test this hypothesis, we expressed and purified full-length Tud using baculovirus expression system (Fig 4E) and showed that the purified Tud is highly active in binding to methylated Aub peptide. Furthermore, using size exclusion chromatography, we determined that Tud molecule is a monomer under native conditions (Supplementary Fig S4). To

Figure 4. Tudor is a structurally flexible scaffold and interacts directly with pyruvate kinase using its Tudor domains.

- A Silver-stained gel showing full-length (FL) Tud, mini-Tud $\Delta 3$ and Tud7-11 proteins (left panel) purified from ovaries and used in *in vitro* binding assay with GFP-PyK, and their diagrams (right panel). In the diagrams, Tud domains are indicated as squares. In mini-Tud $\Delta 3$ and Tud7-11 proteins, deleted domains are shown as empty squares.
- B PyK is recognized by Tud domains of FL-Tud. Purified FL-Tud was preincubated with known target of Tud domains, Aub peptide with symmetrically dimethylated arginines (sDMAs) and with a control peptide. Subsequently, these peptide-preincubated proteins were added to the anti-GFP beads bound to PyK-GFP or to GFP as a control. Tud protein bound to PyK-GFP was detected by Western blot using anti-HA antibody. Binding of PyK-GFP to Tud was completely inhibited by Aub peptide (lane 2) and not by a control peptide (lane 4). Lanes 1 and 3 correspond to control reactions with GFP-bound beads and Tud. GFP proteins were detected with anti-GFP antibody.
- C Functional mini-Tud protein constructs bind to PyK. Purified mini-Tud $\Delta 3$ and Tud7-11 proteins were added to PyK-GFP beads and bound Tud proteins were detected with anti-HA antibody (lanes 2 and 4). Similarly, control reactions with GFP-conjugated beads and mini-Tud $\Delta 3$ (lane 1) or Tud7-11 (lane 3) were carried out.
- D Detection of an sDMA in PyK with SYM11 antibody. GFP, PyK-GFP and Aub-GFP proteins were purified from fly ovaries using anti-GFP agarose beads. Protein reactivity with SYM11 antibody, which specifically recognizes sDMAs [32], is detected for PyK and Aub (positive control) but not for GFP (negative control) (top panel). The corresponding proteins have been subsequently detected on the same membrane using anti-GFP antibody (bottom panel).
- E Silver-stained gel showing FL-Tud purified from Sf9 cells under native conditions that was used for EM structural analysis. Mass spectrometry analysis confirmed 100% purity of Tud.
- F Representative EM image of negatively stained FL-Tud. Scale bar, 50 nm.
- G Gallery of negatively stained FL-Tud particles. Side length of panels: 33.6 nm.
- H Representative class averages of FL-Tud particles obtained by reference-free classification. The number of particles included in each class is shown in the bottom left corner. Side length of panels, 33.6 nm.
- I Diagrams of polar granule from Fig 2C (orange) and its component Tud (blue) are shown at the same scale. For Tud molecules, shapes of four class averages are reproduced from Fig 4H.

Figure 5. Enzymes of glycolytic pathway implicated in germ cell development by this study.

Glycolytic pathway with its intermediates and enzymes are shown. ATP-generating reactions are indicated. Five enzymes implicated in germ cell development by this study are shown in red, and a short description of the experimental data reported here for a given enzyme is included next to that enzyme.

examine Tud structure, we used single-particle EM and analyzed 2,366 EM images of individual Tud molecules. Consistent with our hypothesis that Tud acts as a flexible scaffold, images of full-length Tud in negative stain reveal variably shaped multi-lobed particles. A representative subset of individual particles and two-dimensional (2D) projection averages show that Tud is an elongated molecule that adopts multiple conformations (Fig 4F–H and Supplementary Fig S5).

Tud protein is a germ granule component, and it is absolutely required for granule assembly in the posterior of the oocyte and early embryos and for primordial germ cell formation [22,34,35]. While the assembly mechanism and precise structure of granules are not known, individual structures of the known components of these granules, especially as large as Tud, combined with *in vitro* biochemical granule reconstitution experiments, may provide insights into the granule formation and structure (Fig 4I).

Our study reports an unexpected discovery of glycolytic enzymes in germ granules during germline development and indicates a special role of glycolysis in germ cell biology (Fig 5). Our data show the involvement of glycolytic enzymes in protection

of germline DNA from transposable elements and provide the first evidence for the role of glycolytic metabolism in the silencing of transposons and piRNA biogenesis. Furthermore, our results support a mechanism whereby PyK is recruited to germ granules by a structurally flexible scaffold Tud protein and demonstrate that, in addition to the Piwi protein Aub, PyK is recognized by Tud domains.

Interestingly, a large genomic screen to identify germ cell-specific transcripts in flatworm planarian *Schmidtea mediterranea* revealed that five glycolytic enzyme mRNAs were enriched in germ cells, namely *gapdh*, *pyk*, *ald*, *eno* and *triose phosphate isomerase* [36]. These results are consistent with our data and may indicate a remarkable evolutionary conservation of the role of glycolytic metabolism in germ cells from distant organisms.

In addition, there is emerging evidence that different classes of stem cells depend on glycolysis as their primary energy-producing pathway. In particular, human embryonic stem cells (hESC) and mouse epiblast stem cells (EpiSC) mainly use glycolysis but not mitochondrial respiration for ATP production [5]. Similarly, hematopoietic stem cells (HSCs) generate energy primarily by glycolysis [3]. In germ cells, Tud may recruit glycolytic pathway enzymes to germ granules to generate local ATP for efficient function of several ATP-dependent RNA helicases required for both piRNA biogenesis (for example, UAP56, Vas, Armitage) [13,37,38] and primordial germ cell formation (for example, Vas and eIF4A) [23]. All these helicases are localized at or near germ granules (see also Supplementary Fig S1) and, therefore, would benefit from an immediate source of local ATP. In support of the helicase–glycolytic pathway coupling in the germ granules, we demonstrated that mutations in several glycolytic enzymes cause defects in the helicase-dependent primary piRNA processing and primordial germ cell formation.

Many types of tumor cells show enhanced glycolysis (Warburg effect) [1]. The high rate of glycolysis leads to the increased production of glycolytic intermediates used for biosynthesis of nucleotides, lipids and amino acids required for rapid proliferation of cancer cells [2]. Another important consequence of the metabolic shift to glycolysis is the reduction of the reactive oxygen species (ROS), which would be otherwise produced by mitochondrial respiration. It is beneficial to minimize the level of ROS since they are responsible for oxidative damage of proteins, lipids and cause mutations [39]. We suggest that germ cells, stem cells and cancer cells are similar in their preference for glycolysis-enhanced metabolism. Glycolysis may provide energy, biosynthetic precursors and protection against ROS, which are all crucial for the future generations produced by these cells.

Materials and Methods

Fly lines

Fly lines expressing HA-tagged full-length Tud and mini-Tud $\Delta 3$ have been described [22]. HA-Tud7-11 transgenic flies were generated previously [31], and *gfp-pyk* line has been described [30].

Isolation of ovarian Tud complexes

Fly lines expressing HA-tagged full-length Tud and mini-Tud $\Delta 3$ were used for the isolation of ovarian Tud complexes. Ovaries were

homogenized in lysis buffer (PBS, 10% glycerol) with protease inhibitors (Roche). Crosslinking and purification of Tud complexes were performed as described [23].

Antibodies

The following antibodies were used: rabbit anti-Vasa [40], rabbit anti-PGK [41], goat anti-PyK (ab6191) and rabbit anti-GFP (ab290) from Abcam; rat anti-HA (12013819001) from Roche; and rabbit anti-sDMA SYM11 (07-413) from Millipore.

Accession numbers

Sequence data from this publication have been submitted to the NCBI database [<http://www.ncbi.nlm.nih.gov/>] with the following accession numbers: for small RNA data, SRR1686767, SRR1686795, SRR1686809, SRR1686815, SRR1686817; and for RNA-seq transcriptome data, SRR1687994, SRR1688034, SRR1688038, SRR1688092, SRR1688160.

Supplementary information for this article is available online: <http://embor.embopress.org>

Acknowledgements

We thank R. Lehmann for help with immuno-EM and providing Tud7-11 line. We are grateful to W. Theurkauf for help with next-generation sequencing approaches and comments on the manuscript. Also, we thank G. Davis for providing PyK-GFP line and H. McDonald for mass spectrometry analysis of purified Tud. In addition, we thank J. Davis, M. Elsayed, N. Huynh, A. Kelly, S. Kim, M. Mozeleski, B. Oliver, E. Schadler, B. Turley, K. Witbrodt and J. Zheng for help at various stages of the work. We thank Bloomington Stock Center for fly stocks and Drosophila Genomics Resource Center for providing cDNAs. We also thank D. Sullivan, D. Maughan and J. Vigoreaux for anti-PGK and anti-Ald antibodies. This work was supported by NSERC Individual Research Grant RGPIN 46628-06 to P.L., NIH grant award DP2OD004483 to M.D.O., NIH grant award T32 GM08320 to S.E.C., NIH grant award U41HG007000 to Z.W., and Murray State University CISR grant, a KBRIN Investigator Development Award (IDeA) from the NIH National Institute of General Medical Sciences under grant number 8P20GM103436-14, NIH grant award R15GM087661 from the National Institute of General Medical Sciences and NSF CAREER grant award MCB-1054962 to A.L.A.

Author contributions

MG, TMC and SNL performed biochemical screen of Tud-associated proteins from ovarian extracts, purified full-length, mini-Tudor proteins and pyruvate kinase and developed *in vitro* binding assay to demonstrate direct interaction of Tud domains and pyruvate kinase with advice from ALA, MG and YL expressed and purified Tud using baculovirus system for structural analysis with advice from ALA and MDO and SEC performed EM structural analysis. TCT and ALA performed immunostaining experiments. TCT prepared wild-type embryos to be used for immuno-EM experiments and with ST and ZW performed RNA-seq and small RNA deep sequencing analysis for mutants provided and characterized by MG, SNL, PM and ALA, CAJ performed RNA *in situ* experiments. TCT and PL developed methodology of biochemical screening of Tud crosslinked complexes. MG, PM, MDO, TCT and ALA prepared the figures. ALA and MG wrote the paper with help from PL and MDO, and ALA supervised the project.

Conflict of interest

The authors declare that they have no conflict of interest.

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