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# The mitochondrial DNA-polymerase promotes elimination of paternal mitochondrial genomes

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## SUMMARY

Mitochondrial DNA is typically inherited from only one parent [1–3]. In animals this is usually the mother. Maternal inheritance is often presented as the passive outcome of the difference in cytoplasmic content of egg and sperm, however active programs enforce uniparental inheritance at two levels, eliminating paternal mitochondrial genomes or destroying mitochondria delivered to the zygote by the sperm [4–14]. Both levels operate in *Drosophila* [8,12,13]. As sperm formation begins, hundreds of doomed mitochondrial genomes are visualized within the two huge mitochondria of each spermatid. These genomes abruptly disappear during spermatogenesis. Genome elimination, which is not in the interests of the restricted genomes, is directed by nuclear genes. Mutation of *EndoG*, which encodes a mitochondria-targeted endonuclease, retarded elimination [8]. Here, we show that knockdown of the nuclear-encoded mitochondrial DNA polymerase (Pol γ-α), Tamas, produces a more complete block of mtDNA elimination. Tamas is found in large particles that localize to mtDNA during genome elimination. We discount a simple possible mechanism by showing that the 3′-exonuclease function of the polymerase is not needed. While DNA elimination is a surprising function for DNA polymerase, it could provide a robust nexus for nuclear control of mitochondrial genome copy number since use of common interactions for elimination and replication might limit options for the mitochondrial genome to escape restriction. We suggest the DNA polymerase may play this role more widely and that inappropriate activation of its elimination ability might underlie association of DNA loss syndromes with mutations of the human mitochondrial DNA polymerase [15–17].

## In Brief

Yu et al., show that the mitochondrial DNA polymerase promotes the abrupt elimination of mitochondrial genomes during spermatogenesis. This surprising role for the polymerase in enforcement of uniparental inheritance might be advantageous because it targets the genome by an interaction that cannot easily be evaded.

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**Keywords**

maternal inheritance; mitochondria; mitochondrial genome; DNA polymerase; Drosophila spermatogenesis; DNA elimination; nucleoids; EndoG; replication; copy control

## RESULTS

Division of a germ-line stem cell (GSC) at the apex of the *Drosophila* testis (Figure 1A) produces a gonialblast daughter that initiates expression of *bam* and divides with incomplete cytokinesis to produce 16 interconnected pre-meiotic cells. These pre-meiotic cells grow and express genes required for subsequent spermatogenesis before meiosis produces a cyst of 64 interconnected spermatids [18]. To identify new genes required for nucleoid elimination, we used *bam-GAL4* as a spermatogenesis specific driver of express *UAS-RNAi* expression to knockdown (KD) candidate genes (Figure 1A, *bam*+). This germ line specific KD allowed us to assay phenotypes in spermatogenesis even if the candidate genes were needed earlier in development.

Just prior to the cellular reconfiguration that shapes the sperm, the numerous mitochondria fuse forming a large ball, the nebenkern, next to each spermatid nucleus of the cyst. The nebenkern subsequently unfolds into two giant mitochondria that bracket each centrosome and elongate in association with the axonemes to extend the entire 1.8 mm length of the sperm tails [18,19]. Toward the end of sperm elongation, mitochondrial nucleoids disappear in an EndoG-promoted process while the mitochondria remain intact ([8] and Figure 2B). After nucleoid elimination, a large conical actin-based structure called the individualization complex (Figure 1) travels down the axoneme, dividing the cyst into individual sperm and collecting cytoplasmic volume that is discarded in an apically extruded waste bag ([8,18], and Figure 1).

### Tam is required for paternal mtDNA elimination

The catalytic subunit of the mitochondrial DNA polymerase (Pol γ-α) of *Drosophila* is encoded by *tamas* (*tam*) [20,21]. Any of three non-overlapping transgenic RNAi hairpins targeting *tam* mRNA reduced *tam* mRNA in whole testis when expressed under *bam-GAL4* control (*bam>tam*$^{RNAi}$) and strongly suppress nucleoid elimination (Figures 1). Nucleoids are normally eliminated before spermatid individualization begins (e.g. Figure 1C). The presence of numerous nucleoids in individualizing *bam>tam*$^{RNAi}$ spermatids (Figure 1E and 2C) shows that Tam KD blocks normal nucleoid elimination. To examine how Tam and EndoG might work together to facilitate mitochondrial DNA elimination, we expressed *bam>tam*$^{RNAi}$ in the *EndoG* mutant (Figure 2). The flies with both a KD of Tam and mutation of *EndoG* exhibited a phenotype that was not obviously different from that resulting from *bam>tam*$^{RNAi}$ alone. Thus, without Tam, EndoG has no obvious ability to eliminate mitochondrial genomes.

### Tam plays more robust roles than EndoG during mtDNA elimination

The passage of the individualization complex trims the mitochondria, collecting vesicles of mitochondrial material in the cystic bulge and eliminating them from sperm in the distal

waste bag [22,23]. This process also collects the low level of persisting mitochondrial nucleoids in *EndoG* mutants, creating sperm completely lacking mtDNA [8]. Following Tam KD, a higher density of nucleoids were present within the cystic bulge than in regions immediately in front of, or behind it, indicating that the cystic bulge retains some capacity to collect nuceloids. However, many mitochondrial nucleoids were left behind the cystic bulge in Tam KD (Figure 1E,F and 2C). The retained nucleoids imply that either Tam is required for full activity of the collection mechanism, or that collection can be overwhelmed by too many persisting nucleoids.

We next asked whether mtDNA escaping both known elimination processes persists into mature sperm. When we examined mature sperm from the seminal vesicle, the male sperm storage organ, we never observed DAPI foci in the tails of control sperm, while we frequently observed abundant nucleoids in Tam KD sperm (Figure 1G). We also used a previously described qPCR assay to detect mtDNA in mature sperm stored by females after copulation [8]. We crossed control (bam-GAL4) or Tam KD males to females carrying a small mtDNA deletion (*mt:ND2*$^{del1}$) that cannot be amplified by wild type mtDNA-specific primers. Using this assay, we detected thousands of mtDNAs in Tam KD sperm while we failed to detect any mtDNA in control sperm (Figure 1H). Thus, the mechanisms that eliminate mtDNA from developing sperm require Tamas.

The presence of mtDNA in the sperm contained in the female suggests that, upon fertilization, mitochondrial genomes will be transferred to eggs. However, a qPCR measure of the average amount of paternal mtDNA in a collection of 0–3 hour old embryos fertilized by sperm from Tam KD flies was about 0.3 copies per early embryo (Figure S1). This measured level is above the background of the assay (0.03 genomes per embryo), but it is much less than expected if each sperm where to deliver its load of multiple genomes. However, genomes initially delivered might have been eliminated in conjunction with embryonic elimination of the paternal mitochondria themselves [12,13]. Apparently, the embryonic elimination of paternal mtDNA is rapid, or the sperm are heterogeneous in their mtDNA content and only sperm with little or no remaining DNA are responsible for the majority of the fertilization.

These analyses show that Tam KD has a much stronger affect on mitochondrial genome elimination than the EndoG mutation [8], but the KD still does not override all barriers to paternal transmission of mitochondrial DNA.

## Recruitment of Tam to mitochondrial nucleoids is coordinated with nucleoid destruction

To explore how Tam might contribute to mtDNA elimination, we imaged Tam localization during spermatogenesis using a Tam antibody or a GFP-tagged Tam. Tam-GFP expressed from the Tam promoter with its upstream sequences provided complementing activity (Table S1) [24]. Both immunostaining and Tam-GFP fluorescence showed that Tam forms puncta (Figure 3 and S2). These puncta are large (about the same size as a nucleoid, ~100nm) and bright, and should be considered supra-molecular particles. We then assessed co-localization of Tam-GFP puncta with mitochondrial nucleoids during different stages of sperm development. We did not note significant co-localization of the puncta to nucleoids at most stages, including the nebenkern stage (Figure S2) when nucleotide labeling indicates that

nucleoids are replicating (Figure S3), suggesting that a less obvious form of Tam replicates the genome. In contrast, Tam-GFP puncta associated with nucleoids at the time of their disappearance (Figure 3C). Nucleoid disappearance occurs in a wave moving from the proximal end of the tail to the distal end during late elongation of the sperm tail ([8] and Figure 3). In the short tails of early elongating cysts, prior to genome elimination, nucleoids and Tam foci were abundant, but rarely co-localized (Figure 3A, D–H). We first observed frequent overlap of the DAPI and the Tam-GFP foci toward the proximal ends of sperm tails of mid-elongating cysts just prior to nucleoid loss in this region (Figure 3B, D–H). As elongation progresses, the position of co-localization moves distally in a wave just ahead of the wave of nucleoid elimination (Figure 3C, D–H). In the leading edge of the wave, Tam-associated nucleoids appeared brighter (Figure 3C3′) than in the trailing edge (Figure 3C2′), suggesting that Tam is associated with nucleoids as they disappear.

The wave of elimination of mitochondrial nucleoids occurs as the sperm tail extends from about 1700 to 1800 microns: a 2 hour period based on the rate of sperm tail extension [8,19]. Within this moving wave, Tam-DNA association and decline of DNA staining spatially occupies about 20% the length of the tail, and hence occurs over about 20% of the 2 hour wave propagation period (24 minutes). Disappearance of individual nucleoids could be even faster because, within the wave, Tam-DNA association is not uniform and Tam-free nucleoids appeared brighter than Tam-associated nucleoids (Figure 3C′). The coordination suggests that the interaction of the Tam-GFP puncta with the nucleoids initiates the elimination process during wave propagation.

## The exo-domain is dispensable for Tam mediated mtDNA elimination

Like many DNA polymerases, Tam has a 3′-exo-domain that provides proofreading during replication, and, uncoupled to replication, will degrade DNA from exposed 3′ ends [25–27]. We expected that this nuclease would contribute to nucleoid elimination, and envisaged a collaboration of endo- and exo-nucleolytic activities in genome elimination. The exo-domain is highly conserved and D263 is reported to be required for exonuclease activity across species [26,28]. Since homozygous exo-nuclease deficient *tam* mutants are not viable [28], we devised a test based on an assay of the ability of Tam or a mutant form of Tam to rescue the defect in paternal mtDNA elimination caused by $tam^{RNAi}$. By altering codon usage, we made a version of *tam* that is resistant to $tam^{v106955}RNAi$ (Figure 4A). We made a transgenic line expressing a wild-type version of this RNAi-resistant gene, $UASp\text{-}tam^{wt}\text{-}resistant$, and one expressing an exo-nuclease deficient version, $UASp\text{-}tam^{D263A}\text{-}resistant$ (Figure 4A). Control western blots show the effective KD of the endogenous protein and demonstrate effective and comparable expression of rescuing transgenes (Figure 4C). Expression of the control $UASp\text{-}tam^{wt}\text{-}resistant$ transgene completely rescued the defect caused by $tam^{RNAi}$. That is, mtDNA was eliminated at the late elongation stage and no mtDNA could be visualized in cystic bulges during individualization (Figure 4B, D–E). Surprisingly, we observed a similar rescue using $UASp\text{-}tam^{D263A}\text{-}resistant$ (Figure 4B, D–E). Thus, the D263A mutation does not compromise the ability of Tam to contribute to the DNA elimination program indicating that the exo-nuclease activity of Tam is not required for nucleoid elimination (Figure 4D,E).

### Is DNA elimination dependent on DNA replication function?

While seemingly the antithesis of DNA elimination, DNA replication might sensitize the DNA to destruction by incorporation of inappropriate nucleotides such as dUTP, or by producing replicative intermediates. We followed mitochondrial DNA replication using fluorescent detection of EdU incorporation (Figure S3). Replication of mitochondrial DNA occurred abundantly in the pre-meiotic gonialblasts, but then shut off during the pre-meiotic G2. A brief round of incorporation was initiated during the nebenkern stage, and was completed in the early stages of extension (Figure S3A–E). Importantly, at the time that Tam-puncta associate with nucleoids, we detected no incorporation. This argues against an involvement of significant replication at this stage.

It might be suggested that early replication is necessary to sensitize the DNA to later destruction. However, while RNAi knockdown of Tam at 29°C blocked replication in the nebenkern, incorporation was unaffected at 25°C and yet elimination was blocked at both temperatures (Figure S3F–K). The difference in sensitivity of replication and elimination to KD at 25°C might be explained by a continued decline in the Tam protein over the day and half between replication and elimination, or, alternatively, replication might require less Tam than elimination. In either case, the finding shows that elimination is blocked by Tam-RNAi under conditions (25°C) that do not alter DNA replication. We conclude that nucleotide incorporation *per se* is not involved in elimination.

Even if no active DNA synthesis accompanies elimination, the elimination machinery might involve contributions from other Tam-interacting replication proteins. RNAi targeting of the small subunit of the mitochondrial DNA polymerase (DNApol-γ35) disrupted early spermatogenesis, a phenotype that might be attributed to the intended KD of DNApol-γ35, or KD of the co-transcribed mitochondrial Gat-C (FLYBASE:FBgn0064115). This early disruption of spermatogenesis prevented scoring of mitochondrial genome elimination (Figure S4). Helicase (Twinkle) KD did not block nucleoid elimination (Figure S4). KD of mtSSB reduced nucleoid number and staining intensity in nebenkerns (Figure S4). Despite the obvious effectiveness of the mtSSB RNAi, the later elimination of nucleoids occurred as normal. Since KD of these replication functions did not mimic the Tam KD elimination defect, these analyses failed to support a general requirement for replication functions in mitochondrial genome elimination.

To ask whether the polymerase function of Tam is involved in elimination, we tested whether a polymerase defective mutant of Tam could rescue DNA elimination. Expression of an RNAi-resistant Tam transgene with a point mutation compromising DNA polymerase gave a partial rescue of the Tam-RNAi defect as shown by reduced nucleoid number in the proximal regions of late elongating spermatids and in individualized sperm (Figure 4D,E). However, this rescue of elimination was not complete, as evidenced by the detection of nucleoids in the cystic bulge (Figure 4D4). This ambiguous result might be due to an incomplete defect in polymerase function or because the mutation perturbed a polymerase independent aspect of Tamas function that contributes to elimination.

In sum, the results show no evidence of replication at the time elimination and offer no strong evidence favoring a role for replication.

# DISCUSSION

## How and why is DNA polymerase used to eliminate mitochondrial genomes?

Our results show a Tam requirement for nucleoid elimination during spermatogenesis. While the 3′ exo-nuclease and replication *per se* are not involved in elimination, we show a tight coupling between association of a large Tam-containing particle with nucleoids of decreasing size. We suggest that this particle includes other activities that execute the process of genome elimination, but that Tam is required for the formation and/or activity of this particle. While the composition of this particle remains to be defined, a DNA-independent particulate fraction in mammalian mitochondria included a number of repair and replication proteins including polymerase and EndoG [29].

It is interesting to consider why the mitochondrial DNA polymerase would have this pivotal but incongruous role in genome elimination. It could contribute to containment of unruly behaviors of the mitochondrial genome. Without the mechanisms that control the regimented and impartial replication and transmission of nuclear genes, mitochondrial genomes can gain advantage, one over the other, by any change that promotes replication [30–33]. Indeed, recent findings have shown that changes in regulatory sequences promote such selfish behavior [34,35]. While the nuclear genome might restrict juggernaut super-replicators by genome elimination, the mitochondrial genomes could also gain advantage by changes that evade the elimination mechanisms. Balanced and stable nuclear oversight of the mitochondrial genome could benefit by pitting the two opposing selections against each other such that changes that might be advantageous for replication would also promote elimination, and changes suppressing elimination would compromise replication. This could be achieved if both replication and elimination where controlled by common interactions with regulatory sequences. In this context, the choice of the DNA polymerase as a nexus for both processes could limit paths that the mitochondrial genome might take to achieve selfish gains. Meanwhile, the nucleus could dictate the fate of the mitochondrial genome by governing the assembly of transfactors to produce replicative or, alternatively, destructive Tam complexes.

If Tam provides a particularly robust regulatory switch to manage mitochondrial genome abundance, the Tam contribution to elimination that we describe in the fly testis might be used broadly. It may play a role in promoting uniparental inheritance in the diverse organisms using DNA elimination [5–8,36,37]. It may also have a wider role in governing mitochondrial genome levels in other tissues [38]. Inappropriate activation of its elimination potential might explain why mutations in the human mitochondrial DNA polymerase and other replication proteins give rise to a DNA loss syndrome [15–17].

# MATERIALS AND METHODS

## Antibody and Fluorescence microscopy

For immunization, DNA fragments coding for *Drosophila* Tam amino acids 30 – 653 was cloned into pET28a plasmid using *Not*I and *Nde*I sites. Recombinant plasmid was transformed into the BL21(DE3) competent *E.coli* cells and His-Tag fused protein was purified using the Ni Sepharose (Sigma-Aldrich) manufacturing instruction. The subsequent

rabbit immunization with the His-Tagged Tam was carried out by Pacific Immunology. For antibodies purification, the same DNA fragment was cloned into pET41a plasmid using SpeI and NotI sites. The resulting GST-fusion protein (Tam-GST) was expressed in bacteria, trapped on a Sepharose-GSH resin, and crosslinked to this resin using disuccinimidyl suberate (Thermo Fisher). The antibody was purified by binding to this Tam-GST column followed by elution at low pH. Eluted antibody was neutralized and diluted for staining and western blotting. Ab18251 (abcam) was used for detecting α-Tubulin as an internal control.

DAPI or PicoGreen/Phalloidin and antibody staining and imaging of fixed spermatid cysts and individualized sperm from seminal vesicle were performed as previously described [8]. Images were acquired using a spinning disc confocal microscope, processed with Volocity 6 Software. The lengths of spermatid cysts were measured using Volocity 6 Measurements module. To illustrate the recruitment of Tam to mitochondrial nucleoids during sperm development (Figure 3), the Volocity 3D opacity module was used to generate a perspective image from a collapsed z-stack.

### Quantitative image analysis to measure expression level and spatial overlapping

To calculate the number of Tam-GFP, DAPI or dual staining pixels, at a given position along each analyzed spermatid bundle three nearby 6 × 6μm squares were chosen for each data point. Within each area, a full stack of images representing the depth of the sperm tail bundle was analyzed using MATLAB. The program identified and enumerated pixels having a signal above threshold in either or both DAPI and GFP channels. The numbers of such pixels for each of the three areas analyzed at each position were averaged and plotted versus position along the bundles as shown in Figure 3D–3H. The source code will be provided on request.

### qPCR measurement of mtDNA copy number in sperm and paternal mtDNA copy number in embryos

*mt:ND*$^{del1}$ females were mated to *mt:ND2*, *bam-GAL4* (control) or *mt:ND2*, *bam-GAL4/UAS-tam*$^{RNAi}$ males. *mt:ND2* (male-derived) copy number in *mt:ND2*$^{del1}$ female sperm storage organs or 0–3 hour old embryos was measured as previously described [8].

### Measurement of Tam mRNA in whole testis

For each experiment, 20 testes were dissected and pooled in PBS. Total RNA from testes of the stated genotype was isolated using Trizol reagent (Invitrogen) and further cleaned with TURBO DNase (Ambion). cDNA was prepared using the SensiFast cDNA Synthesis Kit (BIOLINE). Quantitative PCR (qPCR) was performed on a real-time detector machine (STRATAGENE-MX300p) and results were analyzed and graphed with the software GraphPad Prism6. All qPCR values are the mean of three independent experiments after normalization. PCR primers and other methodological details are given in supplemental materials.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Highlights

- DNA is eliminated from mitochondria during *Drosophila* spermatogenesis
- Genome elimination requires the mitochondrial DNA polymerase (Pol γ-α/Tamas)
- A non-replicative Tamas particle associates with nucleoids during elimination
- Genome elimination is independent of Tamas 3′-exonuclease function or replication

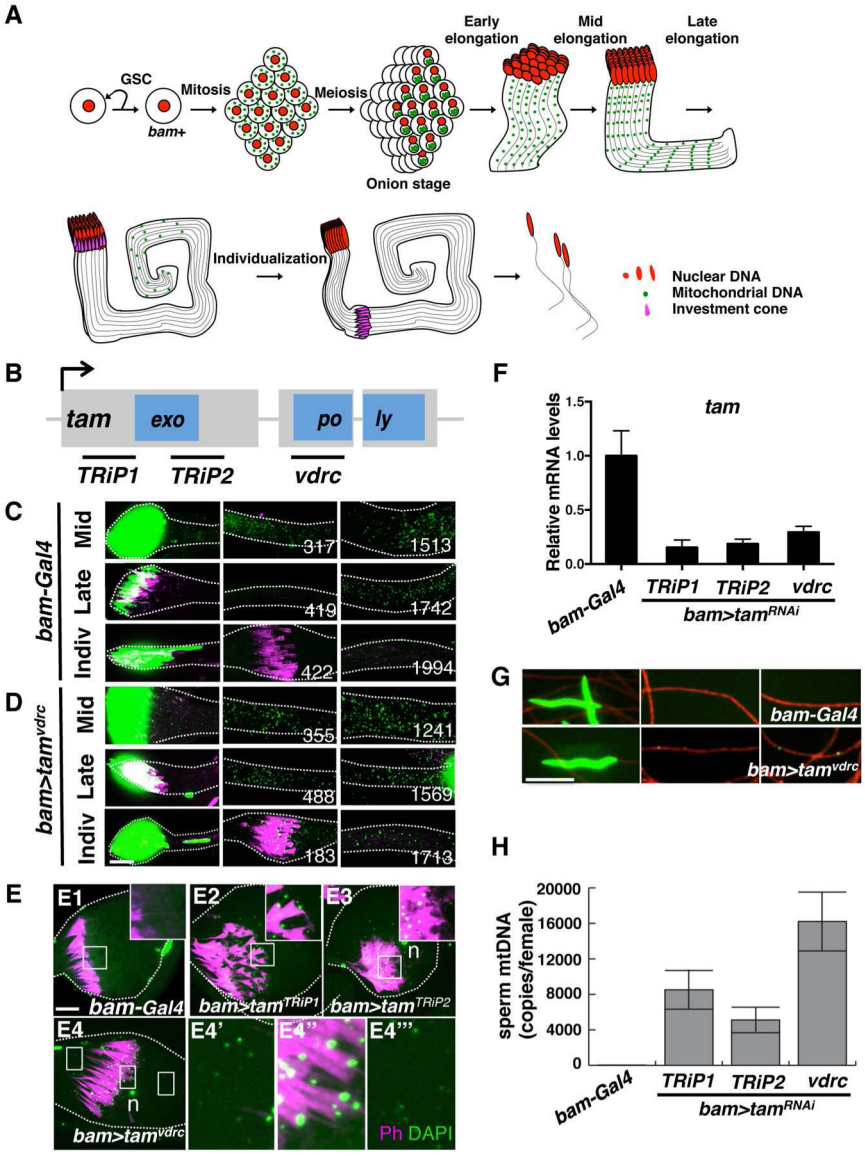


**Figure 1. Mitochondrial DNA polymerase knockdown prevent mtDNA elimination in developing spermatids**

**A)** Schematic showing a single synchronously developing spermatid cyst with nuclei in red, mitochondrial DNA in green and the investment complex in magenta. Early, Mid and Late stages of elongation (10–1200μm, 1200–1700μm and >1700 μm, respectively) are staged by measuring tail length and the location of the cystic bulge stages individualization (Indiv). **(B)** Schematic of the *tam* gene with exonuclease and polymerase encoding regions in blue, and positions targeted by 3 non-overlapping RNAi hairpins. **(C–E)** Spermatid bundles stained for DNA with DAPI (green) and actin with Phalloidin (Ph: magenta). Numbers indicate position of the image along the bundle of tails in microns. **(C)** *bam-GAL4* control. **(D)** *bam>tam*$^{v106955}$. **(E)** Cystic bulges show nucleoid elimination in control (*bam-GAL4*) but not Tam KD spermatids. Enlarged subpanels show persistence of nucleoids behind the investment cones, which move distally (to the right). n indicates nuclear DNA fragments.

Scale bars are 10μm. **(F)** qPCR measure of *tam* mRNA in *bam>tam*$^{RNAi}$ adult testis relative to control. Standard deviation for three independent experiments. **(G,H**) DAPI staining (G) and PCR (H) show persistence of mtDNA in Tam KD but not control mature sperm. (G) Mature sperm collected from seminal vesicles. Images from three positions show DNA (DAPI stain in green) and mitochondria (mtSSB-RFP in red) (see Materials and Methods). **(H)** qPCR quantification of sperm mitochondrial genomes (*mt:ND2*) found in the sperm storage organs of *mt:ND2*$^{del1}$ females mated to control (*bam-Gal4*) or Tam KD males. See also Figure S1.

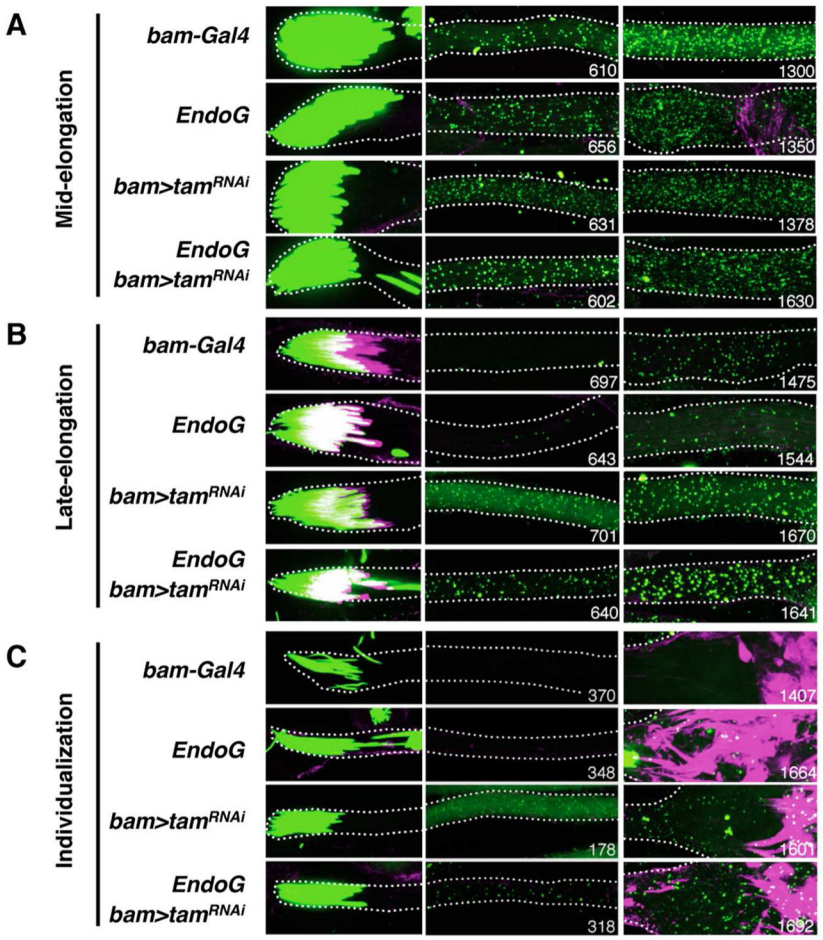


**Figure 2. Genetic interactions between *tam* and *EndoG***

**(A–C)** Spermatid bundles of the indicated stage and genotype stained for DNA (PicoGreen: green) and actin (Phalloidin: magenta). Numbers indicate the distance of each image (μm) from basal tip of the spermatid.

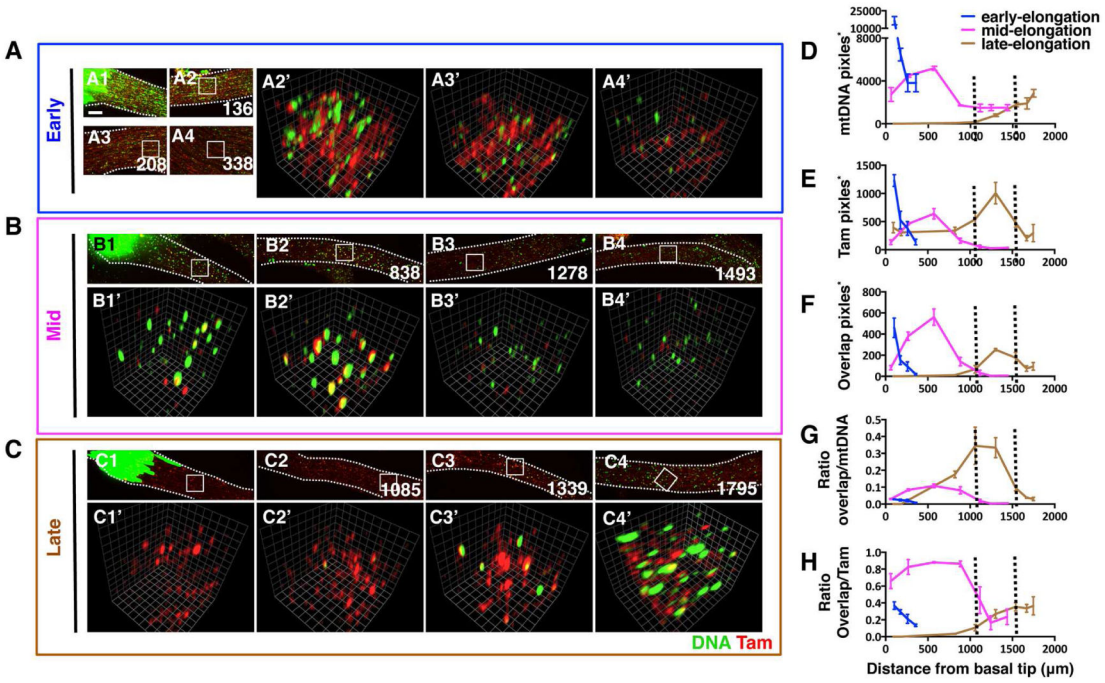


**Figure 3. Tam associated with nucleoids prior to their destruction**

**(A–C)** Images of elongating spermatid bundles (526,1570 and 1846μm in length, respectively) showing DNA (DAPI: green) and Tam-GFP (red). Numbers indicate the distance of each image (μm) from basal tip of the spermatid. Areas highlighted in white squares (6×6μm) are shown in magnified 3D opacity views. Scale bars are 5μm. **(D–E)** Pixel counts having above threshold staining for mtDNA and/or Tam within a volume beneath each of three 6×6μm areas such as those marked in A–C were averaged to give the values and SD at the positions indicated. **(F)** The overlap pixel counts represent those pixels with above threshold staining for both GFP and DAPI as calculated by MATLAB program described in Materials and Methods. **(G–H)** Ratio of overlap/mtDNA and overlap/Tam are generated by dividing the number of overlap pixels in F to mtDNA or Tam pixels along the sperm bundles. The Early-, Mid- and Late-elongation stages are indicated in blue, magenta and brown, respectively. * indicates that pixel-number is a sum from imagining planes through the depth of the tail bundle. See also Figure S2 and Table S1.

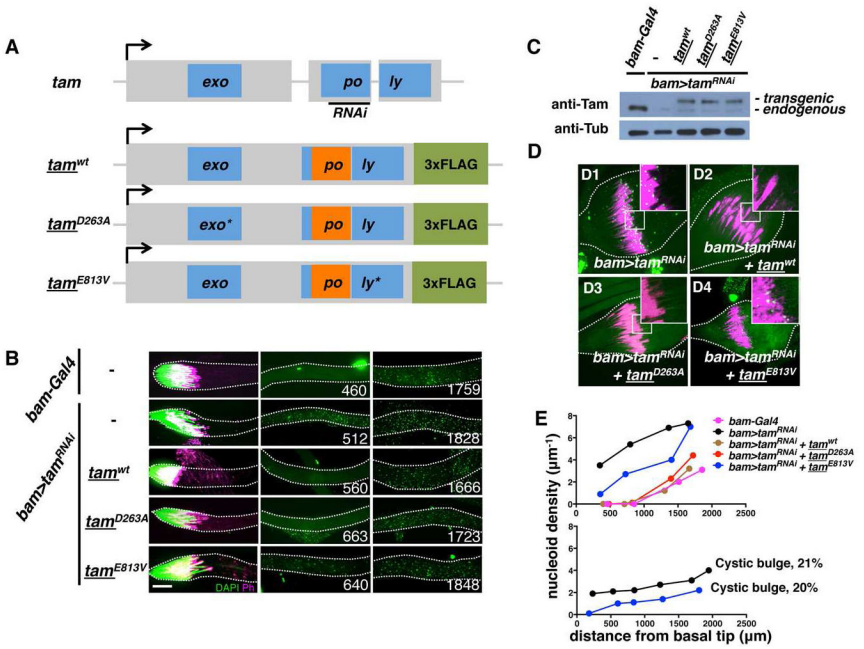


**Figure 4. Tam exo-domain is dispensable for mtDNA elimination**

**(A)** Schematic of the *tam* gene showing the region targeted by *tam*$^{v106955}$*RNAi* and *tam* rescue constructs containing an RNAi resistant sequence (orange). **(B)** Control, and *tam*-RNAi without or with *tam* rescue constructs. Late elongation spermatid bundles stained for DAPI (DNA) and Ph (Phalloidin, actin-cone). Numbers indicate the distance of each image (μm) from basal end of the spermatid. **(C)** Western analysis of testis expressing indicated transgenes in the germline. Note the efficient KD of the endogenous Tam in all RNAi expressing testes (somatic cells of testis contribute to faint residual band), and relatively similar expression of rescuing transgenes whose product is slightly larger due to the FLAG tag. **(D)** Cystic bulges of indicated genotype stained for DAPI (DNA) and Ph (Phalloidin, investment complex). The empty boxes (10×10μm) with white border are magnified and showed at the right corner. Scale bars are 10μm. **(E)** Quantifying rescue: counts of nucleoid number at positions along developing sperm tails. Rescue by wt Tam and exo-deficient Tam appears complete, while the polymerase defective Tam gave only partial rescue and still retained some nucleoids during the later individualization stage. See also Figure S3 and S4.