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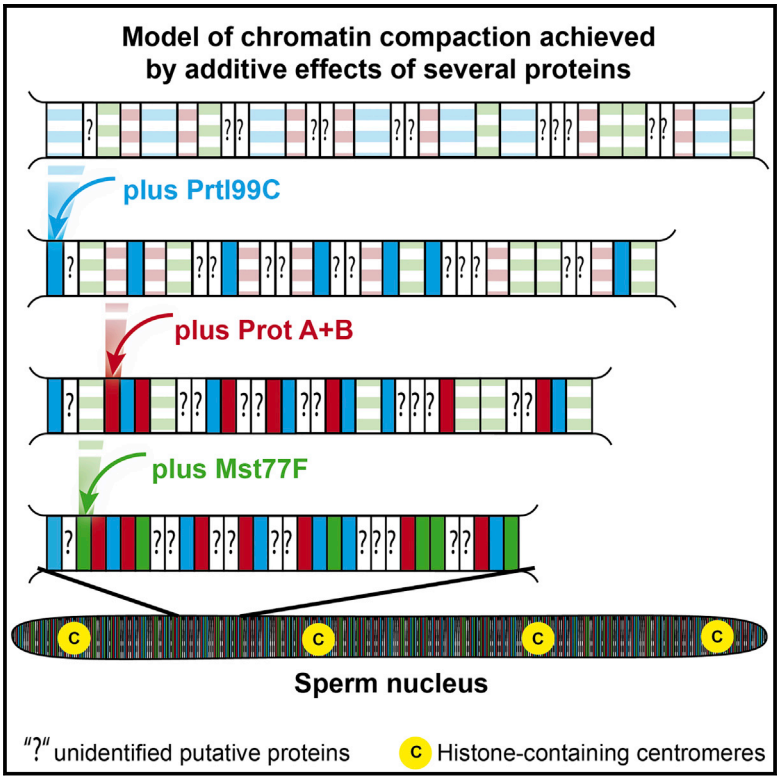
Prtl99C Acts Together with Protamines and Safeguards Male Fertility in *Drosophila*

Graphical Abstract



Highlights

- Sperm chromatin is organized by several basic proteins and not just by protamines
- Prtl99C is pivotal for sperm chromatin compaction and male fertility in *Drosophila*
- Protamines cannot be replaced by Mst77F or Prtl99C
- Prtl99C and protamines act additively to form highly compact paternal chromatin

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In Brief

Eren-Ghiani et al. find that Prtl99C acts together with protamines in *Drosophila* chromatin condensation in sperm and is pivotal for male fertility.

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Prtl99C Acts Together with Protamines and Safeguards Male Fertility in *Drosophila*

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SUMMARY

The formation of motile spermatozoa involves the highly conserved formation of protamine-rich, tightly packed chromatin. However, genetic loss of protamine function in *Drosophila* and mice does not lead to significant decompaction of sperm chromatin. This indicates that other proteins act redundantly or together with protamines. Here, we identify Prtl99C as a *Drosophila* sperm chromatin-associated protein that is essential for male fertility. Whereas the loss of protamines results in modest elongation of sperm nuclei, knockdown of *Prtl99C* has a much stronger effect on sperm nuclei. Loss of protamines and Prtl99C indicates an additive effect of these proteins on chromatin compaction, in agreement with independent loading of these factors into sperm chromatin. These data reveal that at least three chromatin-binding proteins act together in chromatin reorganization to compact the paternal chromatin.

INTRODUCTION

In many animals (e.g., humans, mice, and *Drosophila*), chromatin structure drastically changes during spermiogenesis. The nucleosomal histone-based structure is largely replaced by a protamine-based structure. The replacement of histones with protamines is gradual, as shown by live-cell imaging of *Drosophila* male germ cells (Awe and Renkawitz-Pohl, 2010). In both mammals and *Drosophila,* the loading of protamines—which in *Drosophila* depends on the chaperone subunit CAF1-p75 (Doyen et al., 2013)—is preceded by the loading of post-meiotic histone variants, multiple modifications of histones, and transition proteins (Nanassy et al., 2010; Rathke et al., 2014). During mammalian spermiogenesis, nuclear volume is reduced 20-fold during this process (Braun, 2001); in *Drosophila,* the volume is reduced 200-fold as the round spermatid matures into a sperm. This size reduction is in part due to chromatin compaction and in part caused by the removal of nucleoplasm (Fuller, 1993).

In mice, haploinsufficiency for either protamine 1 (*Prm1*) or 2 (*Prm2*) leads to male infertility, and chromatin appears heterogeneous in density, but nevertheless compact (Cho et al., 2003). In *Drosophila,* chromatin remains very compact even when both protamine-like proteins ProtA and ProtB are lost. Male flies are fertile, and only ~20% of sperm nuclei display abnormal morphology (Rathke et al., 2010; Tirmarche et al., 2014). This suggests that sperm chromatin compaction can be accomplished by factors in addition to the two cysteine-rich, basic proteins ProtA and ProtB. One possible candidate is Mst77F. A point mutation in Mst77F leads to sterility, likely because of severe nuclear shaping defects, as chromatin appears condensed in these mutants (Jayaramaiah Raja and Renkawitz-Pohl, 2005). Therefore, a dual function of Mst77F in nuclear shaping and chromatin compaction has been proposed (Rathke et al., 2010). Indeed, recent in vitro studies indicate that Mst77F on its own can cause DNA and chromatin aggregation by multimerization (Kost et al., 2015).

These data suggest that other proteins participate in chromatin condensation during spermiogenesis. Therefore, we searched for other sperm components that are involved in chromatin compaction in *Drosophila*. Here, we show that the Protamine-like 99C (Prtl99C) persists in sperm nuclei. We postulate that this very basic protein is a component of sperm chromatin and show that Prtl99C is essential for male fertility and controls chromatin packaging during spermiogenesis. We hypothesize that several proteins independently contribute to DNA compaction to form fully condensed sperm chromatin.

RESULTS

Prtl99C Encodes a Spermiogenesis-Specific Nuclear Protein

In *Drosophila* spermiogenesis, high mobility group (HMG)-box proteins are expressed in a transition-like pattern (e.g., Tpl94D; Rathke et al., 2007; and tHMG1 and tHMG2; Gärtner et al., 2015) or remain as sperm chromatin components, (e.g., ProtA and ProtB; Jayaramaiah Raja and Renkawitz-Pohl, 2005). Here, we investigated gene *CG15510*, which is specifically and highly transcribed in testes (Chintapalli et al., 2007) and encodes a basic protein with an HMG box (DROME: Q9VAF7; Mitchell et al., 2015; http://www.ebi.ac.uk/interpro/). *CG15510-eGFP* fusion genes with the putative promoter and 5′ UTR of 942 bp (containing *CG42592*) or 386 bp (lacking *CG42592*) upstream of the start codon showed an expression pattern similar to that of protamines (942 bp is shown in Figures 1 and S1A); therefore, we named it *Protamine-like 99C* (*Prtl99C*) (FlyBase: FBgn0039707) owing to its localization at 99C (St Pierre et al., 2014; http://flybase.org/). Apart from the HMG box, which is a

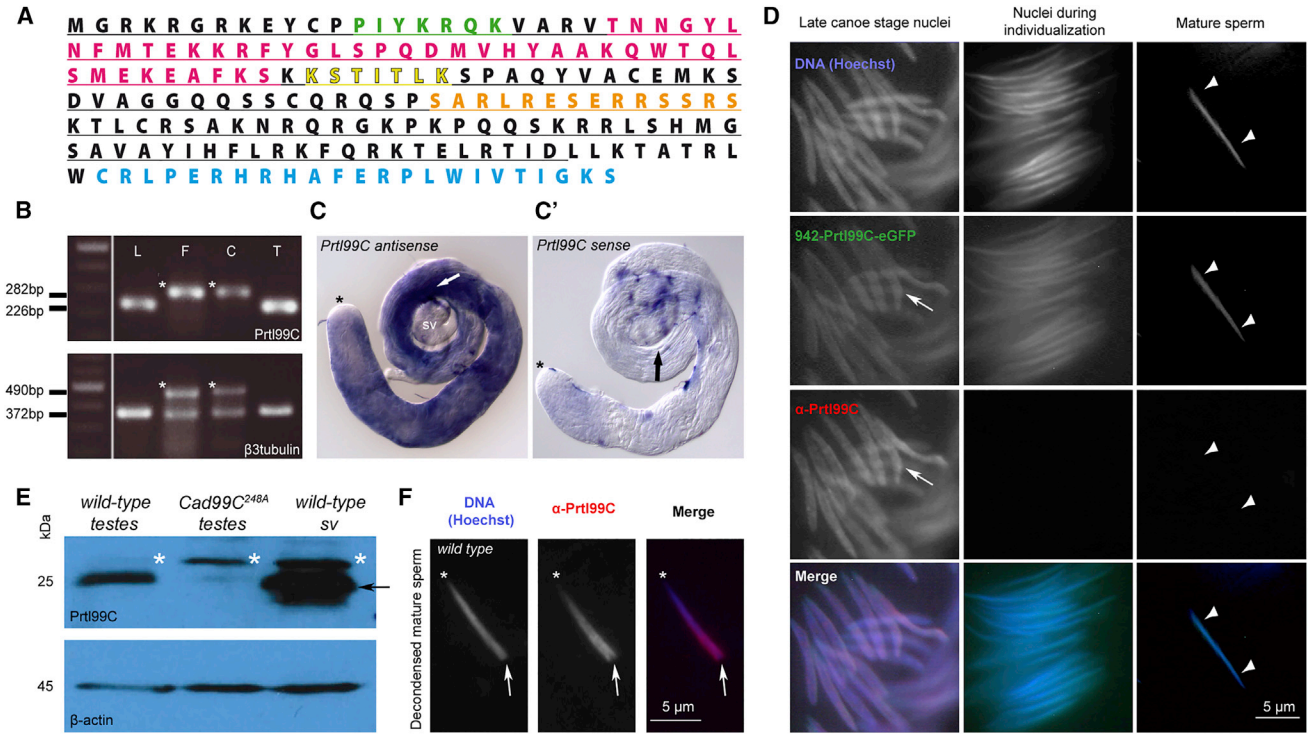


Figure 1. *Prtl99C* Is Transcribed during Spermatogenesis and Encodes a Highly Basic Chromosomal Sperm Protein

(A) Primary structure of protein Prtl99C with NLS (green), HMG box (pink), eight amino acids (yellow) only found in isoform Prtl99C-PD, and c-LCR (orange). Prtl99C antibody was raised against a C-terminal peptide (blue). The sequence (aa 1–150) maintained in mutant *Prtl99C-ΔC* is underlined.

(B) RT-PCR of *Prtl99C* from wild-type larvae (L), adult virgin females (F), carcass (C), and adult testes (T). *Prtl99C*-specific primers amplified a 226-bp cDNA fragment from the ORF in adult testes and in larvae, but not in carcass of males (adult body after removing the reproductive tract) or adult females. In carcass males and adult females, a larger, 282-bp DNA fragment (asterisks) was amplified from genomic DNA contamination because primers flank a small 56-bp intron. A 372-bp cDNA fragment of β3-*tubulin* was amplified as control in all samples; DNA contamination (490-bp fragment) occurred only with carcass males and adult females.

(C) In situ hybridization of testis with an antisense RNA probe specific for *Prtl99C* transcripts (dark staining); (C′) sense control. Arrow, post-meiotic spermatids; *, hub region; and sv, seminal vesicles.

(D) Anti-Prtl99C antibody staining of testes from transgenic flies expressing 942-Prtl99C-eGFP. Arrows, expression in late canoe stage nuclei; arrowheads, expression in mature sperm nuclei.

(E) Specificity of anti-Prtl99C in western blots. Top: anti-Prtl99C; *, non-specific protein that occasionally appeared (28 kDa). Bottom: anti-actin antibody. sv, seminal vesicles.

(F) Prtl99C detected via immunofluorescence with anti-Prtl99C antibody (arrows) in decondensed wild-type mature sperm nuclei. *, tip of the nucleus.

See also Figure S1.

common feature of spermatid-relevant nuclear proteins, Prtl99C contains a nuclear localization signal (NLS) in the N-terminal region (Kosugi et al., 2009; http://nls-mapper.iab.keio.ac.jp/) and a central low-complexity region (c-LCR) (Letunic et al., 2015; Schultz et al., 1998; http://smart.embl-heidelberg.de/) (Figure 1A). Protamines and Prtl99C share very similar HMG-box sequences (Figure S1B); however, the remaining protein regions are distinct from each other.

Prtl99C (CG15510) is predicted to encode three transcripts. However, RT-PCR of testes RNA could not amplify *Prtl99C-RB* or *C* but yielded mainly one transcript, *Prtl99C-RD* (Figure S1C, yellow letters indicate the 24 nt that are lacking in *Prtl99C-RC* transcript). *Prtl99C-RD* encodes the 23.6-kDa isoform that differs by eight internal amino acids (Figure 1A).

Prtl99C transcript was likely testis specific as shown by RT-PCRs using RNA from larvae (contain stages up to spermatocyte stage), adult females, carcass males (males without reproductive tract), or adult testes (all stages of spermatogenesis) (Figure 1B).

Due to the abundance of arginine (12.4%) and lysine (11.0%), Prtl99C is a highly basic protein (predicted pI of 11.48) and contains five cysteine (2.5%) residues; many other chromatin components of sperm are also characterized by a high pI and several cysteines. However, Prtl99C shows no sequence similarity to any known protein in mammals (http://www.ensembl.org/index.html).

In in situ hybridization of testes using a *Prtl99C*-specific probe, transcripts were richly distributed from early spermatocytes to late spermatids, but not in the hub region or seminal vesicles (Figure 1C), while *tbrd-2* transcripts, which encode a spermatocyte-specific protein, were limited to the spermatocyte stage (Figure S1D; Theofel et al., 2014). As the 942-Prtl99C-eGFP fusion protein was first detected in late canoe stage nuclei (Figure 1D), shortly before protamines appeared

(Figure S1A), we conclude that *Prtl99C* mRNAs were translationally repressed. 942-Prtl99C-eGFP remained detectable in mature sperm (Figure 1D). Prtl99C is therefore likely a component of mature sperm chromatin. To follow endogenous Prtl99C, we raised an antibody against a C-terminal peptide (Figure 1A). The antibody specifically bound to Prtl99C at the late canoe stage, but in contrast to results obtained with 942-Prtl99C-eGFP, the antibody did not detect Prtl99C at later stages or in mature sperm (Figure 1D). These results indicate that Prtl99C might not be accessible to the antibody when the chromatin is more compacted.

Prtl99C Is Expressed in Mature Sperm Chromatin

Western blotting showed the presence of Prtl99C (~25 kDa) in protein extracts of both testes and seminal vesicles from wild-type flies, which indicated that the protein persists in mature sperm (Figure 1E). By contrast, Prtl99C was not detected in Prtl99C-deficiency testes (homozygous mutant $Cad99C^{248A}$) (Figures 1E and S1E), which demonstrated the specificity of the antibody, in agreement with immunizing peptide blocking experiments (data not shown).

To visualize the localization of Prtl99C in mature sperm, we decondensed (see Experimental Procedures) mature sperm from wild-type seminal vesicles and stained them with anti-Prtl99C antibody. Prtl99C was present in a large region of the nucleus (Figure 1F). We conclude that Prtl99C is a component of mature sperm chromatin.

Deletion of *Prtl99C* and Three Neighboring Genes Leads to Male Sterility, Likely Due to the Lack of Prtl99C

To determine whether Prtl99C is required for fertility, we analyzed sperm presence in seminal vesicles, nuclear size, and fertility in a homozygous $Cad99C^{248A}$ mutant, which lacks *Prtl99C, Cad99C*, *Atg16,* and the putative gene *CG42592* (Figure 2A). Flies carrying this deletion are viable (Schlichting et al., 2006). Homozygous $Cad99C^{248A}$ males were sterile (Figure 2B), yet we occasionally observed motile sperm in their seminal vesicles in observations of living material using differential interference contrast (DIC) microscopy (Figure 2D, compare to wild-type in Figure 2C). Homozygous $Cad99C^{248A}$ male germ cells developed normally until late stages of spermiogenesis. Starting from the individualization stage, disorganized spermatid cysts with long coiled spermatid nuclei were observed (Figure 2F). In addition, the few mature sperm that entered the seminal vesicles also displayed abnormal nuclear morphology (Figure 2F), part of the nucleus had a coiled structure (Figure 3A, column 5), and unusually long (Figure 3A, column 6) or hooked (Figure 2F) nuclei were observed.

To determine whether genes neighboring *Prtl99C* (Figure 2A) are transcribed in testes, we performed RT-PCR using mRNA from adult testes (Figure S2A). *CG42592* and *Atg16*, but not *Cad99C*, were transcribed at low levels (Chintapalli et al., 2007). However, spermatid nuclei of *Atg16* mutants showed wild-type morphology (Figures S2B and S2C), and CG42592-eGFP expression was restricted to the cytoplasm of spermatocytes and during meiotic divisions (Figures S2B, S2D, and S2D′). Thus, it seemed unlikely that lack of *Atg16* or *CG42592* could cause or contribute to sterility of $Cad99C^{248A}$ owing to distortions of chromatin compaction.

To investigate whether Prtl99C is responsible for sterility and severe distortions of spermiogenesis, we performed a rescue experiment in which the *386-Prtl99C-eGFP* transgene (lacking *CG42592*) was introduced into the homozygous $Cad99C^{248A}$ mutant background (Figures 2A and 2G). Indeed, 386-Prtl99C-eGFP rescued spermiogenesis, seminal vesicles were full of motile sperm, and males were fertile (Figures 2B and 2E). We conclude that Prtl99C is responsible for fertility.

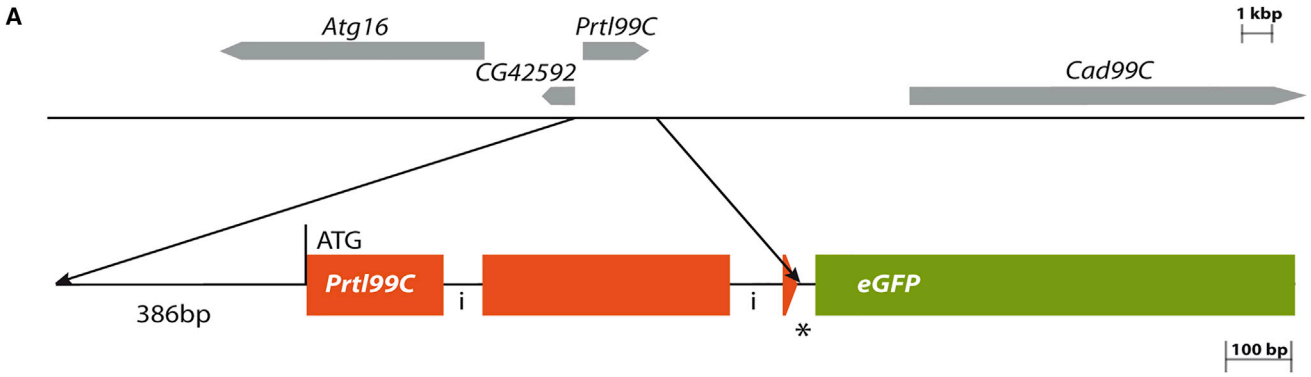
Knockdown of *Prtl99C* Leads to Severely Reduced Male Fertility and an Elongated Chromatin Region in Sperm

Given that homozygous $Cad99C^{248A}$ males showed hardly any individualized sperm, we performed RNAi-mediated knockdown of *Prtl99C* with the binary UAS/GAL4 system (for a review, see Southall and Brand, 2008), using the *bam-Gal4* driver line to drive expression of the RNAi hairpin construct in late spermatogonia up to early spermatocytes. qPCR and western blots showed that the *Prtl99C* transcript was strongly diminished and the protein was not detected in *bam-Gal4* > $Prtl99C^{RNAi}$ testes, in contrast to controls (Figures 3B and 3C), which indicated an efficient RNAi knockdown. *bam-Gal4* > $Prtl99C^{RNAi}$ males displayed strongly reduced fertility (Figure 2B). In contrast to homozygous $Cad99C^{248A}$, *bam-Gal4* > $Prtl99C^{RNAi}$ stained with Hoechst and anti-Mst77F revealed that spermiogenesis was fairly normal (Figure S3A); however, *bam-Gal4* > $Prtl99C^{RNAi}$ nuclei were longer (Figure 3A, column 3) than those of non-driven $Prtl99C^{RNAi}$ controls (Figure 3A, column 2). Thus, Prtl99C is likely essential for fertility. In addition, the nuclei were still needle shaped (Figure 3A), which suggests that Prtl99C plays a role in chromatin compaction, but not in nuclear shaping.

Prtl99C-ΔC Mutation Causes Elongation of the Chromatin Region in Sperm Nuclei, and Mutants Have Reduced Fertility

We then analyzed strain $Mi\{MIC\}CG15510^{MI10224}$, in which a transposon is inserted in *Prtl99C* (St Pierre et al., 2014; http://flybase.org/). This insertion leads to an early stop codon, which most likely results in a truncated Prtl99C-ΔC protein of 169 amino acids, containing all major domains but lacking the 32 C-terminal amino acids (Figures 1A and S3Ba). *Prtl99C-ΔC* transcripts were formed and stable (Figure S3Bb), and a corresponding *Prtl99C-ΔC-eGFP* fusion gene showed the same expression pattern as full-length 942- or 386-Prtl99C-eGFP (Figures S3Bc and S3Bd).

In *Prtl99C-ΔC* heterozygotes (Prtl99C-ΔC/+), the nucleus was slightly elongated (11.3 μm) compared to wild-type (10.5 μm); this might indicate a slight dominant-negative effect; nevertheless, these males were fertile. Interestingly, homozygous *Prtl99C-ΔC* males had reduced fertility (Figure 2B). The nucleus length (14.1 μm) of these mature sperm was increased (Figure 3A, column 4) compared to wild-type (Figure 3A, column 1). As no full-length Prtl99C protein is present in homozygous *Prtl99C-ΔC* males, we considered that the phenotype is due to the lack of the C-terminal domain. We conclude that the C terminus of Prtl99C is essential for correct chromatin compaction and affects male fertility.



B

Strain	Fertility
wild type	Fertile (all matings had progeny)
Cad99C^{248A}	Sterile (no matings had progeny)
Rescued Cad99C^{248A}	Relatively fertile (55% of matings had progeny)
Prtl99C-ΔC	Reduced (50% of matings had progeny)
bam-Gal4>Prtl99C^{RNAi}	Strongly reduced (5% of matings had few progeny)
Prtl99C-ΔC/Cad99C^{248A}	Reduced (27% of matings had progeny)
ProtΔ;Cad99C^{248A}	Sterile (no matings had progeny)

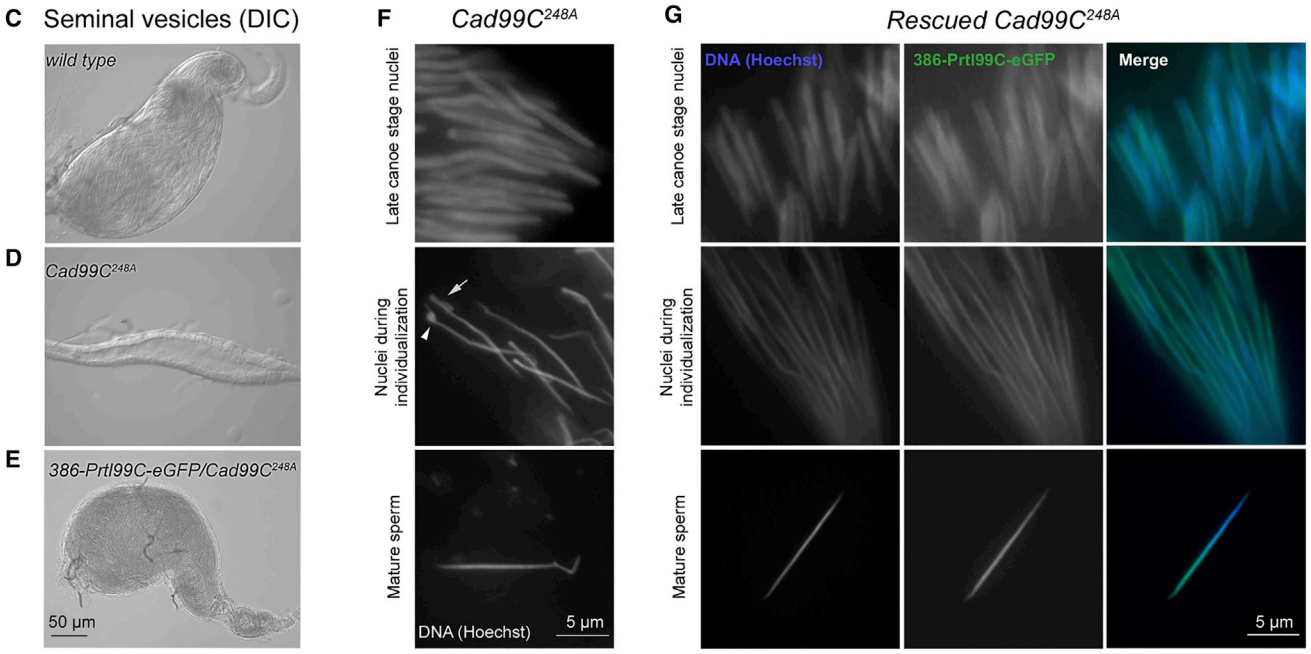


Figure 2. Prtl99C Rescues Sterility of *Cad99C^{248A}* Males

(A) Scheme of the *Cad99C^{248A}* region and *386-Prtl99C-eGFP* fusion gene used for rescue experiments. i, intron; *, part of the multiple cloning site in-frame with *Prtl99C* and *eGFP*.

(B) Male fertility of wild-type and various fly strains in which *Prtl99C* is affected.

(C–E) DIC images of seminal vesicles of 3-day-old males.

(F) Squash preparation of homozygous *Cad99C^{248A}* testis; spermatids are normal up to individualization stage. Disorganized cysts during individualization stage with abnormal nuclei (arrow) with a knob-like structure (arrowhead).

(G) Squash preparation of *Cad99C^{248A}* testis expressing 386-Prtl99C-eGFP. Spermatid nuclei and mature sperm nuclei appear normal.

See also Figure S2.

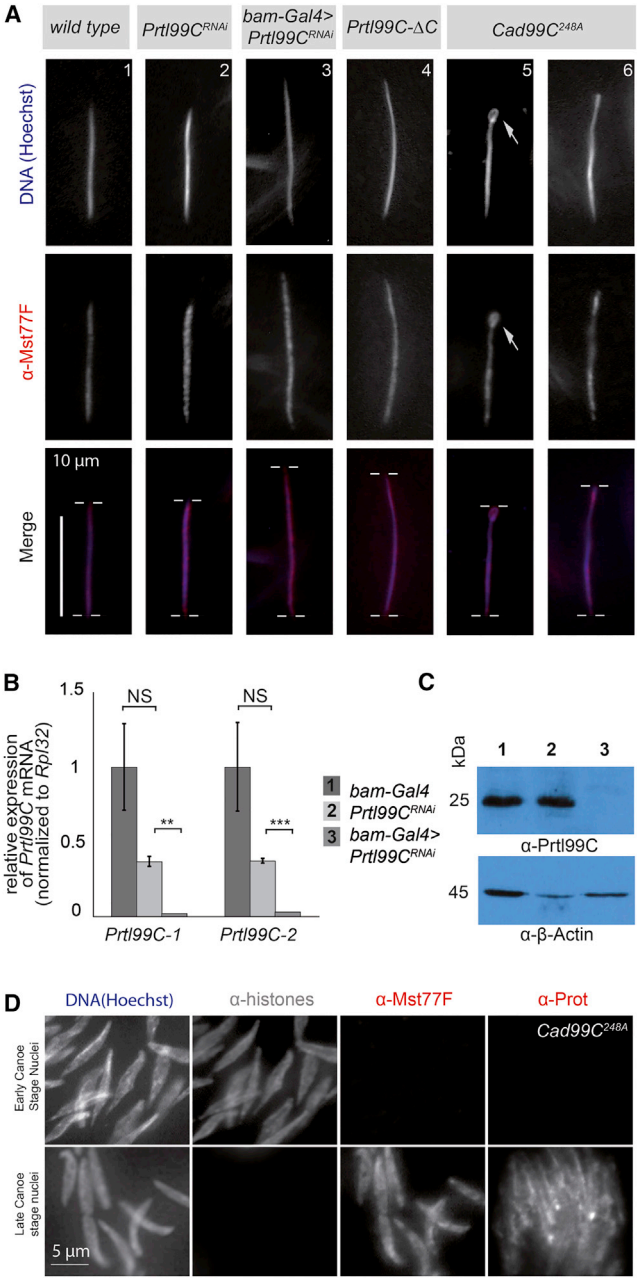


Figure 3. Both Prtl99C-ΔC and Prtl99C Knockdown via RNAi Result in Extended Length of the Chromatin Area of Sperm Heads

(A) Mst77F distribution in individual sperm heads from squashed preparations of seminal vesicles from wild-type and *Prtl99C* mutants. Dashes indicate the ends of the DNA region in the nuclei. Arrows, coiled or hooked nuclei.

(B) qPCR using cDNA of *bam-Gal4*, *Prtl99C*^{RNAi}, and *bam-Gal4* > *Prtl99C*^{RNAi} testes. *Prtl99C* cDNA was amplified using two different primer pairs: Prtl99C-1 (RTpcrMst99C-Fw and Mst99C-qRv) and Prtl99C-2 (Mst99C-qFw and CG15510-ISH-Rv) (see Supplemental Experimental Procedures for primer sequences). Significance: ***p ≤ 0.001; **p ≤ 0.01; and NS, not significant.

(C) Western blots using protein extracts from *bam-Gal4* (1), *Prtl99C*^{RNAi} (2), and *bam-Gal4* > *Prtl99C*^{RNAi} (3) testes.

(D) Transition from histones to Mst77F and protamines in *Cad99C*^{248A} spermatid nuclei is not affected.

See also Figure S3.

Loading of Protamines and Mst77F on Chromatin Does Not Depend on Prtl99C, and Prtl99C Loading Is Independent of Protamines

To determine whether the replacement of histones by protamines proceeds properly in *Prtl99C-ΔC* and *bam-Gal4* > *Prtl99C*^{RNAi} flies, we examined histone-to-protamine dynamics using immunofluorescence. In homozygous *Cad99C*^{248A}, *bam-Gal4* > *Prtl99C*^{RNAi}, and *Prtl99C-ΔC* males, disappearance of histones and loading of ProtA and Mst77F were not obviously altered (Figures 3D and S3A).

In addition, in *protΔ* flies, where both protamine genes are missing, Prtl99C distribution did not differ from that of wild-type testes (Figure S4A). To determine whether Prtl99C can partially replace protamines, we used rescue experiments. Expression of 942-Prtl99C-eGFP in a homozygous *protΔ* background failed to rescue misshapen sperm heads (Figure S4B).

Our results suggest that (1) ProtA, ProtB, and Mst77F likely localize to chromatin independently of Prtl99C during post-meiotic chromatin reorganization, (2) Prtl99C cannot replace protamines, and (3) protamines cannot rescue loss of Prtl99C.

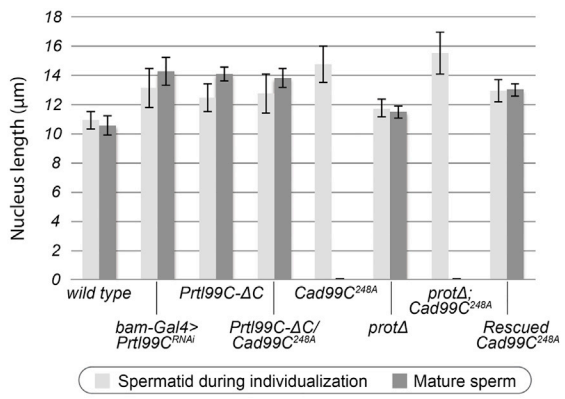
Nuclear Elongation of *Prtl99C* Mutants Is Manifested at the Individualization Stage

We measured sperm nuclei of all genotypes and calculated the average length relative to that of wild-type (Figure 4). We measured the length of the Hoechst-dye-positive area (from the beginning of the tail to the acrosome region) of 20 nuclei during individualization and of mature sperm nuclei from seminal vesicles (Figure 4), and we refer to this as nucleus length for simplicity. *bam-Gal4* > *Prtl99C*^{RNAi} and homozygous *Prtl99C-ΔC*, *Cad99C*^{248A} nuclei were longer than those of wild-type during individualization, which indicated that nuclear extension already began during individualization. The observed variation in length of wild-type nuclei is in agreement with electron microscopy data (Tokuyasu, 1974). Furthermore, we analyzed *Prtl99C-ΔC* in *trans* to *Cad99C*^{248A}, where only the C terminus of Prtl99C was missing and all other genes of the *Cad99C*^{248A} region were present in one wild-type copy. These flies had reduced fertility (Figure 2B), and their nuclei were longer, starting at the individualization stage (Figure 4A). The nuclei of mature sperm of *Prtl99C-ΔC/Cad99C*^{248A} showed the same phenotype as strains in which only Prtl99C is affected; at 13.8 μm, the nuclei were about 33% longer than the nuclei of the wild-type (Figure 4B). We conclude that the C terminus of Prtl99C is likely essential for chromatin compaction.

Nuclei of Homozygous *protΔ*; *Cad99C*^{248A} Mutant Sperm Are Longer

To examine whether the deletion of protamines has an additive effect on the length of the nuclei, we analyzed the double mutant *protΔ*; *Cad99C*^{248A}. Homozygous *protΔ*; *Cad99C*^{248A} males were sterile (Figure 2B). Loss of protamines enhanced the *Cad99C*^{248A} phenotype (for late cysts, see Figures S4Ca–S4Cf). The spermatid nuclei in *protΔ*; *Cad99C*^{248A} mutants were longer than those of *Cad99C*^{248A} mutants (15.5 μm versus 14.7 μm) (Figures 4A and 4B). These data indicate that the extension of the chromatin region is caused by loss of ProtA, ProtB, and Prtl99C in an additive manner (scheme in Figure 4C).

A



B

Strain	Mean length of nuclei (µm) ± SD	% from wild type
wild type	10.55 ± 0.33	100.0
bam-Gal4>Prtl99C^{RNAi}	14.26 ± 0.47	135.1
Prtl99C-ΔC	14.09 ± 0.26	133.6
Prtl99C-ΔC/Cad99C^{248A}	13.80 ± 0.32	130.9
*Cad99C^{248A}**	14.72 ± 0.61	139.6
protΔ	11.48 ± 0.21	108.8
protΔ;Cad99C^{248A*}	15.49 ± 0.71	146.8
Rescued Cad99C^{248A}	12.99 ± 0.22	123.1

C

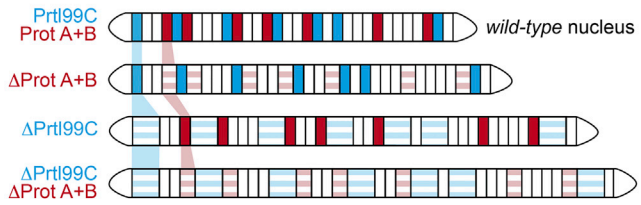


Figure 4. Deletion of Protamines in *Cad99C^{248A}* Background Enhances Nucleus Length

(A) Average length of nuclei during individualization and in mature sperm from wild-type and various fly strains in which *Prtl99C* is affected (n = 20). Only morphologically linear nuclei were measured.

(B) Mean length and SD of nuclei in mature sperm from wild-type and mutants and percentage deviation from the length of wild-type sperm (100%). *, mean length during individualization, as these strains are sterile.

(C) Simple model showing the additive effect of loss of Prtl99C (blue) and protamines (red) and other proposed unidentified proteins (white areas). Histones are not shown. The model shows how the length of a nucleus locally increases upon loss of protamines (light red horizontal stripes) or upon loss of Prtl99C (light blue horizontal stripes), and the additive effect of the combined loss of both protamines and Prtl99C.

See also Figure S4.

DISCUSSION

The sperm nucleus undergoes dramatic changes in chromatin organization from a histone-based nucleosomal structure to a mainly protamine- or protamine-like-based structure. True protamines of birds and mammals are small arginine- and lysine-rich basic proteins with several cysteines. Lower vertebrates and many invertebrates harbor protamine-like proteins that are also basic but heterogeneous and mostly larger than true protamines (Balhorn, 2007). It has been proposed that these fulfill a similar role in chromatin compaction and protection of the paternal genome (Kanippayoor et al., 2013). In mammals, protamine genes are transcribed shortly after meiosis; in *Drosophila*, the genes are transcribed in the spermatocyte stage. In both mammals and *Drosophila*, both *protamine* transcripts are translationally repressed until late spermatid stages (Rathke et al., 2014). Surprisingly, deletion of protamines in *Drosophila* leads to only a moderate extension of the chromatin area, reflected in elongated sperm nuclei (reported here), as observed also in mice with protamine haploinsufficiency (Cho et al., 2001, 2003). Although the size of the chromatin area in the sperm head of these mice is only weakly affected, acridine orange staining and transmission electron microscopy show that head shape is significantly disturbed and chromatin is less compact (Cho et al., 2001, 2003). In these mice as well as in humans, changes in the ratio of PRM1 and PRM2 correlate to male infertility (Jodar and Oliva, 2014). In homozygous *prot⊿* males of *Drosophila*, the nucleus length is slightly increased (this work), 20% of the sperm have slim and bent nuclei, and their genome is more sensitive to X-rays, indicating that the chromatin is less compact (Rathke et al., 2010). These results led us to hypothesize that other proteins in addition to protamines contribute to compaction of sperm chromatin in *Drosophila*. Indeed, we previously identified Mst77F and, here, Prtl99C as chromosomal proteins of sperm that are essential for male fertility.

Prtl99C Is an Important Chromosomal Sperm Protein with Unique Characteristics

Here, we identified Prtl99C as a basic protamine-like protein of *Drosophila* containing an HMG-box, a feature shared with ProtA and ProtB, and a c-LCR; by contrast, Mst77F contains a C-terminal domain conferring DNA binding and a coiled-coil domain facilitating multimerization (Kost et al., 2015). Prtl99C has not been identified in the sperm proteome (Dorus et al., 2006); however, chromatin components might be underrepresented in whole-sperm proteomics, as the sperm head, with a length of ~10–12 µm, is tiny compared to the sperm tail with a length of nearly 2 mm.

Prtl99C Is Required for Male Fertility, and Its 32 C-Terminal Amino Acids Contribute to Fertility

The efficient rescue of fertility of the *Cad99C^{248A}* deletion by *386-Prtl99C-eGFP* provided evidence that male sterility of *Cad99C^{248A}* mutants was caused by the loss of Prtl99C. Furthermore, depletion of Prtl99C or deletion of the 32 C-terminal amino acids of Prtl99C in *Prtl99C-⊿C* led to reduced male fertility. These data indicate that Prtl99C-ΔC, which still contains the N-terminal HMG-box domain and c-LCR, is not fully functional. Taken together, these genetic data strongly indicate that Prtl99C is required for male fertility and its C terminus contributes significantly to fertility.

Chromatin of Sperm Lacking Prtl99C Is Less Compact

The nuclei of all analyzed *Prtl99C* mutants were still needle shaped; thus, reduced fertility of these mutants due to faulty nuclear shaping is unlikely. The sperm chromatin regions of *Prtl99C* mutants were considerably longer than those of wild-type (Figures 4A and 4B). In *Prtl99C* mutants, elongation of the nucleus beyond the length of the wild-type nucleus was first

observed during the individualization stage, which indicated an early role of Prtl99C in chromatin packaging. The elongated chromatin region in the nuclei could be an explanation for the strongly reduced fertility.

Histone Removal and Mst77F and Protamine Deposition Are Independent of Prtl99C

Histone removal and deposition of Mst77F and protamines were independent of Prtl99C, and Prtl99C was deposited in homozygous *prot⊿* males. This indicates that these processes are independent. Moreover, 942-Prtl99C-eGFP, like Mst77F-eGFP, did not rescue the morphological sperm-head defect of *prot⊿* males (Rathke et al., 2010). We propose that Prtl99C, protamines, and Mst77F are deposited independently and have distinct individual functions during chromatin reorganization.

A Model for Additive Chromatin Condensation by Several Proteins in *Drosophila* Sperm

As has been observed in mammals (reviewed by Miller et al., 2010), proteomics data suggest that even mature *Drosophila* sperm contain residual histones (Dorus et al., 2006). In mammals, sperm contain histone variants at telomeres and centromeres (Miller et al., 2010), as is also known for centromeres in *Drosophila* (Raychaudhuri et al., 2012). Based on previous data and our results here, at least four proteins in addition to histones participate in sperm chromatin organization: ProtA, ProtB, Mst77F, and Prtl99C.

Sperm nuclei of *Prtl99C* mutants were up to 40% longer than those in wild-type testes. Those of *prot⊿* mutants were moderately elongated, at 109% of the length of wild-type testes (Figure 4B). This small elongation in *prot⊿* mutants is noteworthy, as these sperm have an increased sensitivity to X-rays. Sperm nuclei deficient in both Prtl99C and protamines were 147% of the wild-type length, indicating that the elongation effects are additive (Figure 4B). A possible additive effect of Mst77F could not be assayed in vivo because of its compaction-unrelated function in nuclear shaping. Recent in vitro assays have shown that Mst77F on its own is able to condense naked DNA and chromatin (DNA-based oligonucleosomal arrays) (Kost et al., 2015). Thus, we propose that loss of Mst77F would lead to an even further elongation of the nucleus length in sperm.

Based on our results, we hypothesize that chromatin in *Drosophila* is condensed by several proteins with different domains that act in parallel, i.e., loss of one type of protein leads to elongation of sperm nuclei, and an additional loss of another type of protein leads to an additive elongation of sperm nuclei (see Figure 4C). Different proteins (Mst77F, Prtl99C, protamine A and B, and perhaps other unidentified proteins) may package different genomic regions in the sperm. The temporal order of deposition remains to be clarified.

It is possible that also in mammals other sperm chromatin components are required for chromatin compaction and that protamines are not the only stabilizers of chromatin. The identification of more chromatin components will help to elucidate the molecular basis of male infertility in *Drosophila* and mammals.

EXPERIMENTAL PROCEDURES

Fly Strains and Culture

Drosophila melanogaster strains were maintained on standard medium at 25°C (30°C for RNAi crosses). w^{1118} was used as the wild-type strain. For RNAi against *Prtl99C* (v106856), we used KK lines from the Vienna *Drosophila* Resource Centre (Dietzl et al., 2007) driven by Bam-Gal4-VP16 (Caporilli et al., 2013; Chen and McKearin, 2003). *bam-Gal4* (*Bam-Gal4/Bam-Gal4; Sp/CyO;-Bam-Gal4/MKRS*) female virgins were crossed with homozygous RNAi transgenic males. *Cad99C* (Schlichting et al., 2006) and *prot⊿* deletion mutants (Rathke et al., 2010) have been described. *Mi{MIC}CG15510*^{MI10224} (53232) was obtained from the Bloomington Stock Center. Our strains were balanced over the TM6B balancer.

In Situ Hybridization

Whole-mount in situ hybridization of adult testis was performed as described previously (Morris et al., 2009). DIG-labeled *Prtl99C*-specific RNA probes were generated using a 367-bp fragment of the Prtl99C open reading frame (ORF) amplified by PCR from genomic DNA and cloned into pCRII-TOPO Vector (Invitrogen).

RT-PCR of *Prtl99C*

For RT-PCR of *Prtl99C*, total RNA was extracted from wild-type male and female larvae, adult virgin females, carcass males, and wild-type testes using TRIzol (Invitrogen). We used the OneStep RT-PCR Kit (QIAGEN) to amplify a 226-bp cDNA fragment of *Prtl99C* (282 bp from DNA). *β3-tubulin* primers were used as controls (372-bp cDNA product from RNA and 490-bp product from DNA).

cDNA Synthesis and qPCR

qPCR were performed with cDNA synthesized from total RNA (Gärtner et al., 2015) extracted from 100 testes of *bam-Gal4* (control), *Prtl99C*^{RNAi} (undriven control), and *bam-Gal4 > Prtl99C*^{RNAi} flies.

Cloning of the *942-Prtl99C-eGFP* and *386-Prtl99C-eGFP* Constructs

To generate the *942-Prtl99C-eGFP* construct, the ORF together with 942 bp upstream of the translational start codon was PCR amplified and fused to *eGFP*; transgenic flies were established according to Rathke et al. (2007). To generate the *386-Prtl99C-eGFP* construct, the *Prtl99C* gene together with 386 bp upstream of the translational start codon was PCR amplified using genomic DNA and primers with linked *EcoRI* and *SpeI* restriction sites. The PCR fragment was inserted into the *Drosophila* P element vector *pChab⊿sal⊿lacZ*-eGFP (Theofel et al., 2014) modified according to Thummel et al. (1988). Transgenic fly strains were established in a w^{1118} background.

Immunofluorescence Staining

Hoechst staining was used to visualize chromatin. Squashed testes were immunofluorescence stained as described in Hime et al. (1996) and Rathke et al. (2007). A peptide antibody (aa 179–201) against Prtl99C raised in rabbit was affinity purified and applied at a dilution 1:500 (Pineda-Antibody-Service; http://www.pineda-abservice.de). Other antibodies were used at the following dilutions: anti-histone antibody 1:1,200 (Millipore MABE71) used to detect core histones, anti-Mst77F 1:500 (Rathke et al., 2010), and anti-ProtA/B 1:500 (Doyen et al., 2013). Cy3-conjugated anti-rabbit 1:100 (Dianova), Cy3-conjugated anti-guinea pig 1:100 (Dianova), and Cy5-conjugated anti-mouse 1:100 (Dianova) were used as secondary antibodies. All signals were examined using a Zeiss microscope (AxioPlan2). Images were individually recorded and processed with Adobe Photoshop 7.0.

Decondensation of Sperm Nuclei

Squashed seminal vesicles were treated with 10 mM DTT, 0.2% Triton X-100, and 400 U heparin in PBS for 30 min (modified after Li et al., 2008; D. Miller, personal communication). The slides were then washed quickly in PBS before immunofluorescence staining (see above).

Fertility Test

Each adult male (0–1 day old) was placed with three wild-type virgin females in a vial at 25°C (n = 20). After 5 days, the parental generation was removed.

After 2 weeks, the number of matings with and without progeny was counted.

DIC Microscopy

Dissected seminal vesicles in PBS from adult males were placed on slides and squashed in a drop of PBS and observed under DIC using a Zeiss microscope (Axiophot).

Western Blotting

Western blotting followed standard methods. Proteins were extracted from both wild-type and homozygous *Cad99C*^{248A} mutant testes (20 each) or seminal vesicles (80 each). Protein extracts (Leser et al., 2012) were applied to an SDS-polyacrylamide (12.5%) gel. Anti-Prtl99C (1:100 dilution in 2.5% dry milk in 1 × TBS) and anti-Pan-Actin (Cell Signaling #4968; 1:1,000 dilution in 5% dry milk in 1 × TBS) were used. Horseradish-peroxidase-conjugated anti-rabbit and anti-mouse antibodies (Jackson Immunology) were subsequently applied at a dilution of 1:5,000. Enhanced chemiluminescence reagents (Invitrogen) were used according to the manufacturer's recommendation.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.11.023.

AUTHOR CONTRIBUTIONS

Z.E.-G. performed 90% of the experiments, prepared figures, and wrote the first draft of the manuscript. C.R. designed genetic experiments, generated and analyzed the first transgenic flies (942-Prtl99C-eGFP), performed some in situ hybridizations, and supervised the project. I.T. performed qPCR and western blots. R.R.-P. designed and supervised the project and interpreted the results. Z.E.-G. and R.R.-P. wrote the manuscript; all other authors agreed with the statements.

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