The *Drosophila Neprilysin 4* gene is essential for sperm function following sperm transfer to females

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Sperm are modified substantially in passing through both the male and the female reproductive tracts, only thereafter becoming functionally competent to fertilize eggs. *Drosophila* sperm become motile in the seminal vesicle; after ejaculation, they interact with seminal fluid proteins and undergo biochemical changes on their surface while they are stored in the female sperm storage organs. However, the molecular mechanisms underlying these maturation processes remain largely unknown. Here, we focused on *Drosophila Neprilysin* genes, which are the fly orthologs of the mouse *Membrane metallo-endopeptidase-like 1* (*Mmel1*) gene. While *Mmel1* knockout male mice have reduced fertility without abnormality in either testis morphology or sperm motility, there are inconsistent results regarding the association of any *Neprilysin* gene with male fertility in *Drosophila*. We examined the association of the *Nep1–5* genes with male fertility by RNAi and found that *Nep4* gene function is specifically required in germline cells. To investigate this in more detail, we induced mutations in the *Nep4* gene by the CRISPR/Cas9 system and isolated two mutants, both of which were viable and female fertile, but male sterile. The mutant males had normal-looking testes and sperm; during copulation, sperm were transferred to females and stored in the seminal receptacle and paired spermathecae. However, following sperm transfer and storage, three defects were observed for *Nep4* mutant sperm. First, sperm were quickly discarded by the females; second, the proportion of eggs fertilized was significantly lower for mutant sperm than for control sperm; and third, most eggs laid did not initiate development after sperm entry. Taking these observations together, we conclude that the *Nep4* gene is essential for sperm function following sperm transfer to females.

**Key words:** male sterility, female sperm storage, *Neprilysin* genes, *Mmel1*, *Drosophila*

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**INTRODUCTION**

Mammalian sperm become functionally competent to fertilize eggs only after passing through both the male and the female reproductive tracts (Austin and Bishop, 1958; Orgebin-Crist, 1969), where they are modified considerably during processes known as epididymal maturation in males and capacitation in females. In the former process, more than 1,700 proteins are potentially added to or removed from mouse sperm during epididymal transit.
Ca²⁺ modifications to the sperm plasma membrane, increased (Skerget et al., 2015). Capacitation includes molecular modifications to the sperm plasma membrane, increased Ca²⁺ permeability, the elevation of intracellular cyclic AMP levels, hyperactivation of motility, the induction of sperm protein tyrosine phosphorylation and the acrosome reaction (Gervasi and Visconti, 2016).

Like mammals, *Drosophila* sperm are not motile in the testes but become motile in the seminal vesicle (Lefèvre and Jonsson, 1962). After ejaculation, sperm interact with seminal fluid proteins such as Sex Peptide that are transferred with them, and undergo biochemical changes on the surface while they are stored in the female sperm storage organs (Singh et al., 2018). The best-known modification is Sex Peptide binding to sperm, which is mediated by a network of other seminal fluid proteins (Ravi Ram and Wolfner, 2009; Findlay et al., 2014; Singh et al., 2018). Thus, sperm are also modified both before and after ejaculation in *Drosophila*. However, the molecular mechanisms of the maturation processes remain largely unknown.

We recently undertook an RNAi screen of 103 *Drosophila* genes for their association with male fertility and identified 63 new genes (Ibaraki et al., 2021). When downregulated by bam-GAL4-driven UAS-RNAi expression, three genes, *Neprilysin 4* (*Nep4*), *Receptor of activated protein kinase C* 1 (*Rack1*) and WASp, caused complete male sterility without obvious changes in either testis or sperm morphology. Indeed, *Rack1* was confirmed to be required in spermatogenic cells or sperm; it may play a role in sperm maturation or post-ejaculation function (Ibaraki et al., 2021). Moreover, a WASp mutant has testes filled with elongated spermatids, but they cannot be released into the seminal vesicle, although only a somatic cell requirement has been reported for WASp (Rotkopf et al., 2011). In this study, we focused on the third gene, *Nep4*, which is one of the fly orthologs of the mouse Membrane metallo-endopeptidase-like 1 (*Mmel1*) gene.

*Mmel1* encodes a single-pass type II membrane endopeptidase with an extracellular catalytic domain and is predominantly expressed in adult testis in mice (Yue et al., 2014). *Mmel1* knockout male mice have normal testis morphology and sperm motility but reduced fertility (smaller litter size), probably due to decreased egg fertilization and developmental defects of fertilized eggs (Carpentier et al., 2004). This indicates a role for *Mmel1* in sperm function after ejaculation. On the other hand, there are inconsistent results about the association of the *Drosophila* orthologs with male fertility. Sitnik et al. (2014) found no evidence that any of the *Nep1–5* genes affect male fertility except for a small effect of *Nep1* RNAi on egg laying of mated females. In contrast, as mentioned above, we found that RNAi knockdown of *Nep4* causes male sterility (Ibaraki et al., 2021), but did not study other *Neprilysin* genes. The *Mmel1* gene belongs to the M13 metallopeptidase (*Neprilysin*) family, which consists of seven mouse and 28 *Drosophila* genes (MEROPS, http://www.ebi.ac.uk/merops/; Rawlings et al., 2018; FlyBase, http://flybase.org; Larkin et al., 2021). Among these, the *Drosophila Nep1–5* genes are most closely related to mouse *Mmel1* and are all expressed in testes (Bland et al., 2008; Sitnik et al., 2014). Here, we show that among the *Nep1–5* genes, the *Nep4* gene specifically affects sperm function following sperm transfer to females.

## RESULTS

**RNAi knockdown of *Nep4*, but not *Nep1, Nep2, Nep3* or *Nep5* genes, causes male sterility** In addition to *Nep4*, we examined *Nep1, Nep2, Nep3* and *Nep5* genes for their association with male fertility in *Drosophila*. In RNAi knockdown experiments with the *nos-GAL4* driver, there was no reduction in male fertility for any of the *Nep1–5* genes, including *Nep4* (data not shown). We confirmed that *Nep4* RNAi knockdown by the *bam-GAL4* driver caused almost complete male sterility as reported by Ibaraki et al. (2021), but there was no reduction in male fertility for *Nep1, Nep2* or *Nep5* (Fig. 1). The effects of *Nep3* knockdown were inconsistent between the two RNAi strains (Fig. 1). However, *Nep3* transcript levels in testes of knockdown males did not differ between the two lines: the mean (± standard error of the mean, relative to that of control *bam>CyO* males, taken as 1.0) was 0.53 ± 0.07 (number of replicates (n) = 4) in the effective HMJ21024 and 0.42 ± 0.03 (n = 3) in the ineffective HMC05504. Therefore, the reduction of fertility in *bam>RNAi-HMJ21024* males was probably due to factors other than *Nep3*. Taken together, these data indicate that among the *Drosophila* *Neprilysin* genes, *Nep4* is likely to play a central role in sperm maturation.

We then studied the sterility phenotypes of *Nep4* RNAi knockdown males in more detail. Here, we measured hatchability of eggs laid within three days after confirming that females had copulated with *Nep4* RNAi males, and found that hatchability was again much lower (0.02 ± 0.01; the number of females studied, n = 23) than that of control males (0.87 ± 0.02, n = 17). We could not find any obvious morphological defect in testes or sperm of the *Nep4* RNAi males (data not shown). Indeed, sperm of *Nep4* RNAi males were normally delivered and stored in the two female storage organs, the seminal receptacle (SR) and paired spermathecae (SP), at 1 h after the end of copulation (AEC) (Table 1). However, 96% were discarded from the SR within 24 h AEC (Table 1), while more than one-third of sperm of the control males were retained in the SR at the same point. On the other hand, sperm of *Nep4* RNAi males in the SP were fully retained at 24 h AEC.

In addition, tissue-specific CRISPR/Cas9-mediated
Fig. 1. Reduced fertility by bam-GAL4-driven Nep4 RNAi knockdown, but no effect of Nep1, Nep2 or Nep5 RNAi knockdown. Mean ± SEM of hatchability of eggs (A) and the number of offspring (B) produced by single females mated with treated males. The measurements were done in two sets of experiments and the results are therefore shown in separate plots. Statistical comparisons were performed separately for each set. Egg hatchability of bam > RNAi-KK100189 (Nep4) and bam > RNAi-HMJ21024 (Nep3) males was significantly lower than that of the controls (yw or w[1118]) (P < 0.001 for Nep4 in both sets and P < 0.005 for Nep3 in one-tailed Mann–Whitney U-test). The number of offspring of bam > RNAi-KK100189 (Nep4) and bam > RNAi-HMJ21024 (Nep3) males was significantly smaller than that of the control (P < 0.001 for Nep4 in both sets and P < 0.025 for Nep3 in one-tailed Mann–Whitney U-test). See text for a possible explanation for the contradictory results for the Nep3 gene.
Nep4 mutants generated by the CRISPR/Cas9 system. Three transcript isoforms (RA, RB and RC) of the *Nep4* gene, which encode two proteins with or without the N-terminal transmembrane domain (FlyBase, http://flybase.org; Larkin et al., 2021), are shown in (A), where coding and non-coding exons are indicated by filled and open boxes, respectively, and introns by interconnecting lines. The changes in the two mutants (*c1* and *c2*) are shown in (B). *c1* allele has a 1-bp (T) deletion at the 5′ splice site in the second intron and a 4-bp (TACG) deletion in the third exon. The two 20-bp sgRNA guide and protospacer adjacent motif (PAM) sequences (only coding strand sequences) are shown with arrows for the strand orientations, from 5′ to 3′ direction, and the deleted nucleotides are underlined. *c2* has a 1,767-bp deletion spanning the second exon through the third exon, which is shown as a dotted line.

**Table 1.** Sperm storage by females that had copulated with Nep4 RNAi males

| Males          | n  | Uterus Mean ± SEM | SR Mean ± SEM | SP Mean ± SEM | Total Mean ± SEM |
|---------------|----|------------------|--------------|---------------|-----------------|
| 1 h AEC       |    |                  |              |               |                 |
| Control       | 8  | 1,399.5 ± 161.6  | 624.4 ± 19.5 | 295.3 ± 9.7   | 2,319.1 ± 156.2 |
| *Nep4* RNAi   | 22 | 1,538.2 ± 78.8   | 542.5 ± 27.0 | 303.4 ± 12.9  | 2,384.0 ± 89.9  |
| 5 h AEC       |    |                  |              |               |                 |
| Control       | 7  | 0 ± 0            | 400.9 ± 16.6 | 257.6 ± 29.1  | 658.4 ± 44.5    |
| *Nep4* RNAi   | 21 | 0 ± 0            | 173.7 ± 16.6 | 262.1 ± 11.2  | 435.8 ± 20.3*** |
| 24 h AEC      |    |                  |              |               |                 |
| Control       | 11 | 0 ± 0            | 218.8 ± 23.5 | 232.3 ± 18.6  | 452.0 ± 34.4    |
| *Nep4* RNAi   | 22 | 0 ± 0            | 22.4 ± 4.8***| 283.1 ± 10.4  | 305.5 ± 9.9***   |
| 72 h AEC      |    |                  |              |               |                 |
| Control       | 17 | 0 ± 0            | 193.5 ± 27.5 | 156.1 ± 18.4  | 349.6 ± 37.4    |
| *Nep4* RNAi   | 15 | 0 ± 0            | 29.3 ± 8.1***| 150.1 ± 27.5  | 179.3 ± 26.3*** |

Control and *Nep4* RNAi are *w, bam-GAL4/Y; ProtB-EGFP.M/+* and *w, bam-GAL4/Y; KK100189/+; ProtB-EGFP.M/+* males, respectively. Differences in the number of sperm between control and *Nep4* RNAi males were evaluated by *t*-test; **P < 0.01 and ***P < 0.001 without multiple test correction. SR: seminal receptacle; SP: paired spermathecae; AEC: after the end of copulation; n: number of females studied (replications).

**Fig. 2.** *Nep4* mutants generated by the CRISPR/Cas9 system. Three transcript isoforms (RA, RB and RC) of the *Nep4* gene, which encode two proteins with or without the N-terminal transmembrane domain (FlyBase, http://flybase.org; Larkin et al., 2021), are shown in (A), where coding and non-coding exons are indicated by filled and open boxes, respectively, and introns by interconnecting lines. The changes in the two mutants (*c1* and *c2*) are shown in (B). *c1* allele has a 1-bp (T) deletion at the 5′ splice site in the second intron and a 4-bp (TACG) deletion in the third exon. The two 20-bp sgRNA guide and protospacer adjacent motif (PAM) sequences (only coding strand sequences) are shown with arrows for the strand orientations, from 5′ to 3′ direction, and the deleted nucleotides are underlined. *c2* has a 1,767-bp deletion spanning the second exon through the third exon, which is shown as a dotted line.

Mutagenesis with the *nos-GAL4* driver and double sgRNAs targeting *Nep4* also drastically reduced egg hatchability (0.06 ± 0.02 of *y w; Cas9.P/ProtamineBeGFP; nos-GAL4/sgRNAs-Nep4* males (*n* = 52 females) vs. 0.82 ± 0.06 of the control *y w; Cas9.P; nos-GAL4* males (*n* = 20 females)).

*Nep4* mutants are viable and female fertile, but male sterile To further confirm the role of the *Nep4* gene during sperm maturation, we induced mutations in the *Nep4* gene by the CRISPR/Cas9 system and isolated two mutant (*c1* and *c2*) and a control (*c3*) alleles (Fig. 2). These lesions are expected to disrupt both protein isoforms in the *Nep4* gene (Meyer et al., 2009). The control strain carries the same third chromosome as the mutant strains but no mutation in the *Nep4* region. While both mutant alleles, *c1* and *c2*, were homozygous viable and female fertile, they were male sterile both in homozygotes and in the *c1 c2* heterozygotes (Table 2). They were also male sterile in heterozygotes with either *Df(3R)BSC141* or *Df(3R)BSC488* deficiency (Table 2). In conclusion, *Nep4* is essential specifically for male fertility.
Essential male fertility gene Neprilysin 4

Table 2. Hatchability of eggs laid by females that had copulated with Nep4 mutant males

| Males                  | n  | Hatchability Mean ± SEM |
|------------------------|----|-------------------------|
| Nep4[c1]               | 16 | 0.02 ± 0.01***          |
| Nep4[c2]               | 16 | 0.01 ± 0.00***          |
| Nep4[c1]/Nep4[c2]      | 19 | 0.00 ± 0.00***          |
| Nep4[c3]               | 15 | 0.64 ± 0.07             |
| Nep4[c1]/Df(3R)BSC141  | 19 | 0.02 ± 0.01***          |
| Nep4[c2]/Df(3R)BSC141  | 16 | 0.01 ± 0.00***          |
| Nep4[c3]/Df(3R)BSC141  |  9 | 0.76 ± 0.07             |
| Nep4[c1]/Df(3R)BSC488  | 16 | 0.07 ± 0.05**           |
| Nep4[c2]/Df(3R)BSC488  | 11 | 0.01 ± 0.01**           |
| Nep4[c3]/Df(3R)BSC488  | 13 | 0.44 ± 0.11             

Differences between mutants (c1 and c2) and the corresponding control (c3) were evaluated by Mann–Whitney U-test; ***P < 0.001 and **P < 0.01.

Sperm of Nep4 mutant males are quickly discarded by females and less efficient in fertilization and in initiating embryogenesis

Like the Nep4 RNAi males, the mutants also had normal-looking testes and sperm (Fig. 3). Although the total number of sperm found at 1 h AEC was one-third lower in homozygous males than in the heterozygotes, almost the same number of sperm entered the SR and SP (Table 3, Fig. 4). However, after that, three defects were observed in the sperm of the Nep4 mutant (c2). First, the sperm were quickly discarded by the females: the number of sperm stored in the SR was 9% of that of the control by 24 h AEC (Table 3, Fig. 4). Second, the proportion of fertilized eggs was significantly lower in mutant males than in control males \((755/943 = 0.801 \text{ vs. } 306/357 = 0.857, G' = 5.72, d.f. = 1, P < 0.05)\) (Table 4). Third, the majority of eggs laid by females that had copulated with Nep4 mutant males did not initiate development even after fertilization, while

Fig. 3. No obvious phenotype in testes of Nep4[c2] males. Testes of \(w; dj-GFP / +; Nep4[c2]/TM3\) (A–C) and \(w; dj-GFP / +; Nep4[c2]/TM3\) (D–F). Phase contrast images (A and D) and fluorescence images (B and E) are merged in (C) and (F), respectively.
Table 3. Sperm storage by females that had copulated with Nep4 mutant males

|                | Number of sperm |                |                |                |
|----------------|----------------|----------------|----------------|----------------|
|                | Males           |                | Uterus | SR   | SP   | Total               |
|                |                |                | Mean ± SEM | Mean ± SEM | Mean ± SEM | Mean ± SEM |
| 1 h AEC        | Nep4[c2]/TM6B  | 11             | 1,215.8 ± 179.6 | 540.4 ± 21.9 | 302.7 ± 7.5 | 2,058.9 ± 197.3 |
|                | Nep4[c2]       | 11             | 606.1 ± 112.0** | 455.9 ± 35.4 | 250.5 ± 13.8** | 1,312.5 ± 15.9** |
| 5 h AEC        | Nep4[c2]/TM6B  | 14             | 0 ± 0   | 349.0 ± 15.5 | 286.3 ± 7.1 | 635.3 ± 16.3 |
|                | Nep4[c2]       | 14             | 0 ± 0   | 118.7 ± 23.5*** | 247.3 ± 22.9 | 366.0 ± 32.9*** |
| 24 h AEC       | Nep4[c2]/TM6B  | 14             | 0 ± 0   | 188.6 ± 31.6 | 243.0 ± 13.9 | 431.6 ± 29.2 |
|                | Nep4[c2]       | 14             | 0 ± 0   | 17.1 ± 6.0*** | 210.9 ± 27.0 | 227.9 ± 28.1*** |

Genotypes of heterozygous and homozygous males are w; ProtamineB-eGFP/+; Nep4[c2]/TM6B and w; ProtamineB-eGFP/+; Nep4[c2], respectively. Differences in the number of sperm between mutant heterozygote and homozygote males were evaluated by t-test; **P < 0.01 and ***P < 0.001 without multiple test correction. SR: seminal receptacle; SP: paired spermathecae; AEC: after the end of copulation; n: number of females studied (replications).

Fig. 4. Sperm in female sperm storage organs, seminal receptacle and spermatheca. The seminal receptacle and spermathecae were dissected together with the uterus at 1 h, 5 h and 24 h after the end of copulation and observed under a fluorescence microscope. Images are storage organs of females mated with control w; ProtamineB-eGFP/+; Nep4[c2]/TM6B males and those mated with w; ProtamineB-eGFP/+; Nep4[c2] males. GFP-labeled sperm heads are shown in green, with auto-fluorescence under UV illumination in magenta.
Table 4. Smaller proportion of eggs fertilized by Nep4 mutant male sperm and failure to initiate embryonic development

| Male genotype                  | Number of eggs examined | Number of fertilized eggs (b/a × 100) | Number of eggs with needle-shaped sperm nucleus | Number of developing embryos (c/b × 100) |
|--------------------------------|-------------------------|---------------------------------------|-----------------------------------------------|----------------------------------------|
| w; dj-GFP/+; Nep4[c2]          | 129                     | 943                                   | 755 (80.1)                                    | 736 (3)                                |
| w; dj-GFP/+; Nep4[c2]/TM3      | 83                      | 357                                   | 306 (85.7)                                    | 6 (299)                                |

Frequencies of fertilized eggs and developing embryos were tested between the two genotypes by the G-test of independence, where the differences were statistically significant (frequency of fertilized eggs: G' with Williams’s correction = 5.72, degrees of freedom = 1, P < 0.025; frequency of developing embryos: G' with Williams’s correction = 1,158.79, degrees of freedom = 1, P < 0.001). 

DISCUSSION

We here examined the association of the Drosophila Nep1–5 genes with male fertility and found that the Nep4 gene is specifically required for male fertility in germ cells. While mouse Mmel1 has been shown to be involved in male fertility (Carpentier et al., 2004), a previous study did not find evidence for a strong association of any of its orthologous Drosophila Neprilysin genes with male fertility. Sitnik et al. (2014) tested RNAi knockdown effects of these genes, finding that only Nep1 knockdown affects egg laying of the mated females. Although we do not know the exact reason, methodological differences could explain the discrepancy between their and our Nep4 results. Sitnik et al. (2014) used tubulin-GAL80ts in addition to the tubulin-GAL4 driver to avoid lethality caused by this ubiquitous driver and experimental flies were exposed to a non-permissive temperature for short periods before and after eclosion. The RNAi knockdown in their experiments might have been too weak to produce a consistent phenotype; alternatively, transcription during the permissive temperature might have been sufficient to retain normal fertility. Irrespective of the reason, the consistent results of RNAi knockdown and mutants in this study strongly suggest an essential role for Nep4 in male fertility. This does not rule out the involvement of other Neprilysin genes, especially in somatic cells, because we only used germline drivers. For example, Nep2 is expressed in the somatic cyst cells (Thomas et al., 2005), in which it may be required. In any case, a question, then, is how is Nep4 special? Nep4 is the most abundantly expressed gene in adult testes among the Neprilysin family genes; for example, the Nep3 mRNA expression level is more than 30 times lower (Brown et al., 2014). However, an important question remains as to whether there may be factors, such as enzymatic speci-

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most eggs fertilized with sperm of the control males did so (Table 4, Fig. 5).
ficity, other than the expression level that determine the importance of Nep4. This will be investigated in future work. In addition, Nep4 has two isoforms (Meyer et al., 2009); one is authentic membrane-tethered and the other lacks the membrane domain. It will be interesting to see whether one or both of these isoforms are vital for fertility.

Mmel1 knockout mice are viable but have reduced fertility (smaller litter size) probably due to decreased egg fertilization and developmental defects of fertilized eggs (Carpentier et al., 2004). Disruption of the non-essential Nep4 gene also caused a decrease of the fertilization rate and an early developmental defect, suggesting conservation of function between mouse Mmel1 and fly Nep4. Sperm storage and release directly affect female fecundity in Drosophila and are affected not only by female factors, but also by male factors (Neubaum and Wolfrner, 1999). Indeed, male genes, mostly encoding seminal fluid proteins, that are involved in sperm entry into the storage organs, maintenance in the organs and exit from the organs have been identified (Bloch Qazi et al., 2003; Schnakenberg et al., 2012). For instance, the females mated with Acp36DE-deficient males store few sperm in the storage organs (Neubaum and Wolfrner, 1999; Bloch Qazi and Wolfrner, 2003); conversely, females mated with CG1652, CG1656, CG17575 and CG9997 RNAi knockdown males store more sperm in these organs, implying a failure of sperm release (Ravi Ram and Wolfrner, 2007). The protein products of the latter class of genes as well as antares and Seminase mediate sperm binding of another seminal fluid protein, Sex Peptide (also known as Acp70A) (Singh et al., 2018); Sex Peptide from the male and its receptor Sex peptide receptor in the female are then required for the normal release of sperm (Avila et al., 2010, 2015). On the other hand, females mated with wasted (wst) mutant males release more sperm at each ovulation and thus waste sperm (Ohsako and Yamamoto, 2011). wst mutant males also show decreased fertilization rate and failure of formation of the male pronucleus. Although we did not examine pronucleus formation, the Nep4 phenotypes are similar to those described for the wst mutant. Incomplete sperm maturation may have caused all phenotypes, namely sperm storage failure, reduced fertilization and failure to initiate embryonic development. However, we should also note that the significantly reduced fertilization rate of the Nep4 mutant was not simply due to the shortage of sperm in female storage organs because the reduction was observed in females within 8 h after copulation (Table 4), suggesting that, at least, the effect on fertilization is separable from sperm storage. These phenotypes may be related to distinct biological functions of NEP4 protein or proteins. By contrast, Nep4 is not involved directly or indirectly in sperm transport. Many interesting questions remain unanswered: what changes happen on the surface of sperm during maturation, what molecules are targets for the NEP4 peptidase, and how many factors are involved in this process. To obtain a better understanding of the sperm maturation process, further molecular and functional studies of the protein products will be needed.

It is interesting to note that 13 mouse genes required for binding of sperm to oocyte zona pellucida such as Adam2, Adam3 and calmegin are also required for passage of sperm through the uterotubal junction (Nakanishi et al., 2004; Okabe, 2015), which constitutes a barrier to sperm migration in the female reproductive tract and the entrance to and part of the functional sperm reservoir together with the lower isthmus (Suarez, 1987). There are likely epitopes on the surface of sperm that allow them to interact with and to pass through the junction (Suarez and Pacey, 2006). A common mechanism may underlie sperm storage in the reproductive tract and sperm–oocyte binding (fertilization). This continues to be the subject of intense research.

**MATERIALS AND METHODS**

**Drosophila strains** To drive expression of UAS constructs in testes, we used two germline GAL4 drivers: nos-GAL4 (w; nos-GAL4, KYOTO Stock Center, DGRG 107955) and bam-GAL4 (w, bam-Gal4, originally provided by Dennis M. McKearin, DGRG 118175). We used multiple available RNAi strains and they are listed in Supplementary Table S1. We also used two UAS-Cas9 strains to apply the CRISPR/Cas9 system, hsFLP, y w; UAS-Cas9.P (II) (Bloomington Drosophila Stock Center, BDSC 54594) and hsFLP, y w; UAS-Cas9.P (III)/TM6B, Tb (BDSC 54592). The ProtamineB-GFP strains w; ProtamineBeGFP/CyO (KYOTO Stock Center, DGRG 109173) and w; ProtB-EGFP,M (KYOTO Stock Center, DGRG 109643), and the dj-GFP strain w; P[w[+mC]=dj-GFP.S]/AS1/Cyo, P[ry+t.2]=sevRas1.V12/FK1 (KYOTO Stock Center, DGRG 108217), were used to visualize sperm. We used w[1118] (BDSC 6326) and the highly inbred y w (TT16, Tanaka et al., 2014) stocks as controls. For the complementation test of Nep4 mutants, we used two deficiency strains, Df(3R)BSC141/TM6B (BDSC 9501) and Df(3R)BSC488/TM2 (BDSC 24992).

**Real-time PCR analysis** We dissected one- to four-day-old virgin males and extracted total RNA from the testes (four males per replicate) using a NucleoSpin RNA Kit (Macherey-Nagel). We then reverse transcribed RNA into cDNA with SuperScript VILO Master Mix (Thermo Fisher Scientific) and performed qPCR using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) and the StepOne Real-Time PCR System (Applied Biosystems). Expression values were normalized to that of RpL32. Primers (Nep3.RT_F1, Nep3.RT_R1, qPCR-dRpL32-F and qPCR-dRpL32-R) used for qPCR are
shown in Supplementary Table S1.

**Construction of UAS-sgRNA clones and strains** We generated a single-guide RNAs (sgRNAs) construct as described by Ibaraki et al. (2021). Briefly, we designed sequences of sgRNAs that targeted two exon–intron boundaries, one the second exon/second intron boundary (gRNA-Nep4[1]: 5’-gctcttagagccatcacct-3’; protospacer-adjacent motif (PAM), TGG) and the other the third exon/third intron boundary (gRNA-Nep4[2]: 5’-CGAATG-GAGAGATCGCA-3’; PAM, GGG), where the exon and intron numbers refer to the isoform Nep4-RC and the exonic and intronic sequences are in uppercase and lowercase letters, respectively. These two targeted sequences were cloned into a single pCFD6 vector, resulting in the construct UAS-(DmRNA Gly)-(gRNA-Nep4[1])-(OstRNA Gly)-(gRNA-Nep4[2])-(OstRNA Gly)-terminator (Port and Bullock, 2016). We then injected this plasmid DNA into embryos of y w M[1-attP-3B]VK00033/+; P[acman]Gal4-TM6B flies to males of y w; ProtamineB-eGFP/CyO; UAS-sgRNAs-Nep4 and, using the F1 males, established three third chromosome strains, c1, c2 and c3.

**Fertility test** Male fertility was measured as the number of offspring and hatchability of eggs produced by single females mated with single mutagenized or control males as described by Ibaraki et al. (2021). Briefly, in RNAi knockdown experiments, we crossed females of the GAL4 driver strains to males of UAS-RNAi strains. Two- to three-day-old virgin F1 males were individually crossed to y w single females. The females were allowed to lay eggs in single vials for three days to count the number of offspring. To assess egg hatchability, the parental females were placed in an egg counting chamber with a 20-ml grape juice agar plate for one day and transferred to new plates twice or three times (in total, three or four plates for each female). One and two days after transfer, we counted the number of hatched and unhatched eggs. The measurements were done in two sets of experiments, which were performed on different days but under identical experimental conditions. Different controls (y w in set 1 and w[1118] in set 2) were used for each set of experiments because the hatchability of y w in set 1 was rather low. Statistical analyses were performed separately for each set.

In the second RNAi experiment, y w females that had copulated with Nep4 RNAi males for 10 min or longer were immediately placed in an egg counting chamber and then assessed for hatchability of eggs laid within three days. For CRISPR/Cas9-mediated mutagenesis, we crossed females of y w; UAS-Cas9.P/CyO; nos-GAL4/TM6B to males of y w; ProtamineB-eGFP/CyO; UAS-sgRNAs-Nep4 and assessed male fertility in the same way. All flies were raised at 25 °C.

**Sperm count and fertilization rate estimation** We counted sperm in female sperm storage organs as described by Tomaru et al. (2018). For assessment of sperm entry and fertilization, we collected females that had copulated with males in question for 10 min or longer during a 1-h period. We then transferred the females to an egg collection vial and collected eggs laid in the following 8 h. According to Ohsako et al. (2003), we fixed eggs and then observed them under a Keyence BZ-X800 microscope.

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