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# The Nap family proteins, CG5017/Hanabi and Nap1, are essential for *Drosophila* spermiogenesis

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ABSTRACT

**Spermiogenesis is a dynamic process leading to alterations in cell morphology. In spermiogenesis, the roles of the histone chaperones are largely unknown. Here, I report the unexpected roles of two Nap family proteins, CG5017/Hanabi and nucleosome assembly protein 1 (Nap1) in *Drosophila*. Hanabi is mainly localized in the cytoplasm, and the *hanabi* mutant shows fully scattered nuclei and abnormality of nuclear shaping in spermatid elongation. In contrast, Nap1 is localized at the apical tip of the sperm head, and the *nap1* mutant exhibits disruption of the nuclear bundle in the later stage. These findings imply that Nap family proteins might individually sustain cytoskeleton-based morphogenesis, rather than histone biogenesis.**


## 1. Introduction

Spermatogenesis in *Drosophila* has been well studied as a representative model of the human male reproduction system and sterility [1,2]. After meiosis, 64 haploid spermatids per cyst are present. The 64 haploid spermatids then undergo dramatic changes in shape. First, there is flagellar extension. The flagellar axoneme, consisting of microtubules extends from the basal body. Along the axoneme, spherical mitochondrial derivatives elongate. In addition to these structures, cytoplasmic microtubules and F-actin cables extend along the longitudinal axis. Thus, 64 spherical spermatids clustered in one cyst elongate synchronously to generate thin long sperm tails. The next step is nuclear shaping and condensation. During the latter part of flagellar extension, the spermatid nucleus is altered from a spherical shape to a needle-like shape, and chromatin condenses as much as 200-fold. During nuclear condensation, somatic histones are replaced by highly basic proteins such as protamine. In *Drosophila*, the mammalian homologue of the protamines and transition proteins were recently identified as *Protamine A* (*ProtA*)/*Protamine B* (*ProtB*) and *Transition protein like 94D* (*Tpl94D*) [3,4]. It has been demonstrated in previous studies that a two-step replacement is also conserved; in the first step, histone is replaced by transition protein, and in the second step, transition protein is replaced by protamine in flies as well as in mammals. The next step can be termed "individualization and coiling". After the protamine associates with DNA, a conical structure composed of F-actin, called the actin cone, is formed around the nucleus. These structures synchronously move down toward the terminal end of the sperm tail, eliminating excess cytoplasm and separating the 64 connected spermatids into individualized sperm [5,6]. These separated sperm coil up, and move into the seminal vesicle.

Histone chaperones are known to be acidic proteins that interact with histones and are involved in histone trafficking, nucleosome assembly, and disassembly [7,8]. Nap1 has been particularly well studied in vitro [9,10]. In fact, Nap1 is routinely utilized for in vitro nucleosome assembly assays. In the *Drosophila* embryo, although Nap1 is mainly localized in the cytosol, it is transferred to the nucleus during S phase, suggesting its histone shuttling property [9]. However, analysis of the physiological roles of Nap family proteins, including Nap1, has not been thorough. Furthermore, in the male reproduction system the functions of Nap family proteins are largely unknown.

## 2. Materials and methods

### 2.1. Fly stocks

Flies were raised on standard cornmeal-yeast-agar medium at 25 °C. The $yw^{67c}$ line was used as the wild-type strain. The *GS10858*

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line was obtained from Kyoto Stock Center. The *His2Av-mRFP* line and *Nap1*$^{KO1}$ line were obtained from the Bloomington Stock Center. The *ProtB-eGFP* line was provided by R. Renkawitz-Pohl.

### 2.2. Generation of the hanabi null mutant

A P element was inserted about 0.5 kb upstream from the *hanabi* gene locus in the *P{GSV6}GS10858* line. Homozygous *P{GSV6}GS10858* was lethal, but homozygous viable and fertile lines were generated by meiotic recombination between chromosomes with and without a *P{GSV6}* insertion. Imprecise excision from the homozygous viable *P{GSV6}GS10858* line generated a *hanabi*$^{1}$ allele in which an approximately 1.0 kb genome region including a putative TSS of the *hanabi* gene was deleted. The range of the deletion was determined by genomic PCR followed by sequencing.

### 2.3. Generation of constructs and transgenic flies

All of the following constructs were cloned into a pW8 germline transformation vector. Transgenic flies were generated by BestGene (http://www.thebestgene.com). The *hanabi* genomic rescue plasmid was a 2.0 kb genomic fragment containing the *hanabi* transcription unit and 0.5 kb and 0.1 kb, respectively, of the 5′ and 3′ genomic flanking regions. This fragment was cloned into pW8 with restriction sites designed for KpnI and NotI. The *hanabi::mRFP-1×Flag* genomic rescue plasmid was constructed by inserting the *mRFP-1×Flag* sequence immediately upstream from the stop codon in a *hanabi* genomic rescue plasmid. The *tpl94D::mRFP-1×Flag* genomic plasmid was generated by inserting the *mRFP-1×Flag* sequence immediately upstream from the stop codon of a 2.6 kb genomic fragment which contained the *tpl94D* transcription unit and 1.0 kb and 0.5 kb, respectively, of the 5′ and 3′ genomic flanking regions. This fragment was cloned into pW8 with restriction sites designed for KpnI and NotI. The *mRFP::nap1* genomic plasmid was generated by inserting the *mRFP* sequence (except for the start and stop codons) immediately downstream of the start codon in a 3.5 kb genomic fragment which contained the *nap1* transcription unit and 1.0 kb and 0.4 kb, respectively, of the 5′ and 3′ genomic flanking regions. This fragment was cloned into pW8 with restriction sites designed for KpnI and NotI. The *nap1*$^{hanabi\ promoter}$*::mRFP-1×Flag* genomic plasmid was constructed as follows. The full length *nap1* cDNA (except for the start and stop codons) with restriction sites designed for XbaI and XhoI, was inserted in the *hanabi::mRFP-1×Flag* genomic rescue plasmid that was deleted from immediately downstream of the start codon to immediately upstream of the stop codon with restriction sites designed for XbaI and SalI. Detailed information concerning the construct schemes and PCR primer sequences are available on request.

### 2.4. Male fertility tests

0 to 1 day old adult males were placed individually with two *wild type* virgin females in separate vials for a week at 25 °C. Then the flies were removed, and the presence of F1 progeny was assessed.

### 2.5. cDNA cloning

Full length *cg5017/hanabi*, *nap1* cDNA clones were obtained from the *Drosophila* Gene Collection (DGC) (Clone ID *AT14585*, *LD21576*).

### 2.6. Antibody generation

A truncated version of Hanabi (amino acids 1–100) (to avoid the highly conserved region in common with Nap1) was cloned into both the pGEX4T-1 vector (GE Healthcare) and the pMAL-cRI vector (New England Biolabs). These vectors were transformed into BL21-CodonPlus (DE3)-RIL (Agilent Technologies). Glutathione S-transferase (GST)-fused proteins purified by glutathione Sepharose 4B (GE Healthcare) were used as an antigen to raise rabbit polyclonal antibody. Maltose-Binding Protein (MBP)-fused proteins purified with amylose resin (New England Biolabs) were used for affinity purification.

### 2.7. Testis squash and immunostaining

Dissected testes from 0 to 2 day old adult males were placed on poly-L-lysine coated slides, squashed in a drop of solution under a cover slip, inverted, pressed, and then frozen in liquid nitrogen. The cover slip was removed and put in methanol for 5 min at −20 °C followed by acetone for 2 min at −20 °C as previously described [11]. For the F-actin staining, slides were put in ethanol for 10 min at −20 °C followed by 4% paraformaldehyde in PBS for 7 min at room temperature. Primary antibodies were diluted in 3% BSA in 1× PBST (1× PBS, 0.1% Triton X-100). Anti-Hanabi antibody was used at 1:50, anti-Nap1 antibody (active motif #39577) at 1:100. Secondary antibodies coupled with Alexa-Fluor 488 (Molecular Probes) were subsequently used at 1:200. F-actin was visualized by Alexa Fluor 594 Phalloidin (Molecular Probes #A12381) at 1:50. DNA was visualized by DAPI staining in VECTORSHIELD (Vector Laboratories). For visualization of mitochondria before fixation dissected testes were cultured in Shields and Sang M3 insect medium (Sigma #S3652) with 10% fetal bovine serum and antibiotics and 50 nM Mitotracker Red (Molecular Probes #M7512) for 2 h at room temperature. A Zeiss LSM510 META confocal microscope was used to visualize the fluorescence images. To avoid non-uniform staining for each sample in Figs. S1 and S2, wild-type and each mutant testis were prepared on the same slide, and genotypes were discriminated by the phenotype of the testes.

### 2.8. Phase contrast microscopy

Dissected testes from adults male in testis buffer (183 mM KCl, 47 mM NaCl, 10 mM Tris–HCl pH 6.8, 1 mM EDTA) were placed on slides and squashed in a drop of solution under a cover slip without pressing. Live squashed testes were observed under phase contrast using an OLYMPUS IX70 microscope. For Fig. 5, fixed samples were observed.

### 2.9. Western blotting

Western blotting was carried out using standard protocols. Primary antibodies were diluted in 3% BSA in 1× TBST (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20). Anti-Hanabi antibody was used at 1:500, anti-β-Actin antibody (mouse monoclonal, Abcam #8224) at 1:1000, anti-Nap1 antibody (active motif #39577) at 1:1000, anti-Flag antibody (Sigma #F7425) at 1:1000. HRP-conjugated secondary antibodies were subsequently used at 1:5000 (Dako). Proteins were detected by an ECL reagent (GE Healthcare).

### 2.10. RT-PCR

Total RNAs were extracted from adult whole bodies or testes with TRIzol (Invitrogen). First-strand cDNA from the RNA sample was generated using oligo-dT primers with the Superscript III reverse transcriptase system (Invitrogen) following the manufacturer's instructions. As a negative control, reverse transcriptase was omitted from the reaction. The PCR reaction was conducted with rTaq DNA polymerase (TaKaRa), with 30 amplification cycles. PCR primer sequences are