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Drosophila RecQ5 is involved in proper progression of early spermatogenesis

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ABSTRACT

RecQ5, a member of the conserved RecQ DNA helicase family, is required for the maintenance of genome stability. The human *RECQL5* gene is expressed ubiquitously in almost all tissues, with strong expression in the testes (Shimamoto et al., 2000). However, it remains to be elucidated in which cells RecQ5 is expressed and how RecQ5 functions in the testes. In this present study we analyzed the expression of RecQ5 in *Drosophila* testes. The RecQ5 protein was specifically expressed in germline cells in larval, pupal, and adult testes. *Drosophila* RecQ5 was localized in nuclei of male germline stem cells, spermatogonia-blasts, spermatogonia, and early spermatocytes. As growth of the early spermatocyte proceeded, the amount of RecQ5 increased in the nuclei. However, before maturation of the spermatocyte, the level of RecQ5 declined. Thus, RecQ5 expression was regulated. Furthermore, we compared *recq5* mutant testes with the wild-type ones. The most conspicuous alterations were swelling of the apical region of and an increase in the number of spermatocytes in the *recq5* testis, suggesting a relative accumulation of spermatocytes in the *recq5* mutant testes. Therefore, *Drosophila* RecQ5 may contribute to the proper progression from germline stem cells to spermatocytes for maintenance of genome stability.

1. Introduction

The RecQ proteins constitute a family of conserved DNA helicases, from bacteria to humans, that is important for maintaining genome stability [1]. The human genome encodes 5 RecQ family members: RECQL, BLM, WRN, RECQL4 and RECQL5. Mutations in 3 of these members, namely, BLM, WRN, and RECQL4, give rise to hereditary disorders associated with cancer predisposition and premature aging (e.g., Bloom's, Werner's, and Rothmund–Thomson's syndromes) [1]. Moreover, *Recql5* knockout mice exhibit elevated levels of sister-chromatid exchange and are predisposed to various types of cancer [2,3], suggesting that RECQL5 is important for maintaining genome stability.

In *Drosophila*, the frequencies of spontaneous and induced chromosomal aberrations are increased in RecQ5-mutant neuroblasts [4]. Furthermore, the loss of maternal RecQ5 leads to spontaneous mitotic defects in syncytial embryos, followed by Chk2-dependent centrosome inactivation [5]. Chk2 responds to double-stranded DNA breaks (DSBs) [6]. In addition, RecQ5 protein is localized in the interphase nuclei in syncytial embryos [7]. Therefore, RecQ5 may function in DSB repair in the interphase nuclei of syncytial *Drosophila* embryos. However, it remains unknown whether and in which cell-cycle phase RecQ5 protein functions in other tissues.

RECQL5 is unique among the RecQ family of helicase in its association with RNA polymerase II (RNAPII) [8–10]. In addition, RECQL5 controls transcript elongation and suppresses genome instability associated with transcription stress [11]. Thus, RecQ5 appears to maintain genome stability via participation in many DNA metabolic processes including DNA repair, DNA resolution, and RNA transcription processes occurring in the nucleus.

Although BLM, WRN, and RECQL4 gene expression profiles are organ-specific ones, the human *RECQL5* gene appears to be expressed ubiquitously in almost all tissues, with strong expression in the testes [12]. However, it remains to be elucidated in which testicular cells RecQ5 is expressed. In addition, the function of RecQ5 in the reproductive organs has not yet been elucidated.

As a model system for the study of germline development, *Drosophila* spermatogenesis is particularly valuable. The isolation and identification of mutations, ordering of gene activities, and molecular characterization of loci of interest have been readily accomplished [13]. *Drosophila* spermatogenesis occurs in the

Abbreviations: DSBs, double-stranded DNA breaks; RNAPII, RNA polymerase II; PBS, phosphate-buffered saline; DAPI, 4′-5′-diamino-2-phenylidole; GSC, germline stem cell; PH3, phosphorylated histone H3.

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context of a complex developmental program and is therefore likely to provide insight into the regulation of spermatogenesis in higher eukaryotes, including vertebrates. Furthermore, *Drosophila* germline cells undergoing meiosis can be identified easily and are of a size and abundance that make them well suited to the investigation of the mechanism of spermatogenesis [13].

Here we analyzed the expression of RecQ5 in the *Drosophila* testes and found that RecQ5 expression was specifically regulated during the various stages of spermatogenesis. Furthermore, we compared *recq5* mutant testes with wild-type ones. Although RecQ5 is not essential for spermatogenesis [4], its loss resulted in differences in testicular morphology and in the cell populations during the various developmental stages of spermatogenesis. These data suggest that RecQ5 contributed to the proper progression of *Drosophila* spermatogenesis for maintenance of germline cells.

2. Materials and methods

2.1. Fly stocks

The following lines were used: w^{1118} as wild type (DGRC in Kyoto), $q5^{P}$-*egfp-recq5* [7], and $recq5^{D1}/recq5^{D2}$as a *recq5* mutant [4]. Flies were grown on standard cornmeal-sugar medium, and all experiments were performed on flies grown at 25 °C.

2.2. Fixation and staining of larval, pupal, and adult testes

Newly hatched adult males were collected and cultured for 1–2 days under normal conditions without females. Larval, pupal, and adult testes were dissected in phosphate-buffered saline (PBS) and then fixed for 45 min in 4% formaldehyde in PBS. The fixed samples were washed with PBS twice for 10 min each time and then washed with $PBST_{0.5}$ (PBS containing 0.5% Triton X-100) twice for 10 min each time, followed by overnight incubation with primary antibody (1:100) at 4 °C. The samples were then washed and incubated with secondary antibody (1:100) for 2 h at room temperature. After having been washed with $PBST_{0.5}$, the samples were mounted on slides with a drop of ProlongGold with DAPI (Life Technologies). The following antibodies were used: rabbit anti-phospho histone H3 (Ser10) (Upstate), rabbit anti-cleaved caspase-3 (Asp175) (Cell Signaling), rabbit anti-GFP (Molecular Probe), mouse anti-adducin (hts), mouse anti-armadillo, mouse anti-lamin Dm0 (Developmental Studies Hybridoma Bank), Alexa 555-labeled donkey anti-mouse IgG (H + L), and fluorescein- or Alexa 488-labeled goat anti-rabbit IgG (H + L) (Molecular Probe). The stained testes were observed under a confocal laser-scanning microscope (FV-1000, OLYMPUS). Length and diameter of adult testes were measured by using ImageJ software.

2.3. Phase-contrast microscopy of adult testes

Adult testes were dissected in PBS and squashed in a drop of PBS on a slide under the weight of a cover slip [14,15]. Squashes were by phase-contrast microscopy using a Nikon DAPHOTO. Images were captured with a digital camera (Nikon NFX-35).

3. Results

3.1. The RecQ5 gene was expressed in Drosophila testes

The human *RECQL5* gene is expressed ubiquitously in almost all tissues, with strong expression in the testes [12]. However, FlyAtlas Anatomical Expression Data shows low expression of RecQ5 in the *Drosophila* testis (FlyAtlas-RNA. http://flybase.org/reports/FBgn0027375.html). So we examined whether and where RecQ5 is expressed in the *Drosophila* testes. For this purpose we generated transgenic flies expressing EGFP-tagged RecQ5 under the control of its own promoter ($q5^{P}$-*egfp-recq5*) [7]. The EGFP-RecQ5 protein was expressed in early embryos, consistent with previous findings [16]. We dissected the testes and examined the expression patterns of EGFP-RecQ5. The testes from newly eclosed males contained cells in all stages of spermatogenesis (Fig. 1A). These included transit-amplifying cells (spermatogoniablast and spermatogonia), spermatocytes, and elongated spermatids, the collective presence of which indicates ongoing spermatogenesis [13]. EGFP-RecQ5 was detected in the apical region of the adult testis (Fig. 1B). DNA staining with 4′-5′-diamino-2-phenylidole (DAPI) indicated nuclear localization of EGFP-RecQ5 (Fig. 1C). The germline stem cells (GSCs) and spermatogonia are located in a cluster at the apical end of the testis [13]. The larval and early pupal testes contain several cohorts of developing primary spermatocytes, but no cells arising from later developmental processes [14]. EGFP-RecQ5 was detected in these GSCs, spermatogonia, and early spermatocytes (Fig. 1D). In the larval testis, spermatogonia formed a zone of small cells in the apical fifth of the testis. The middle three-fifths was made up of early spermatocytes and scattered interstitial cells. EGFP-RecQ5 was localized in these zones. At the basal end of the ovoid larval testis was a cluster of somatically derived terminal cells that lacked expression of EGFP-RecQ5 (Fig. 1F). Thus RecQ5 was expressed in early stages of spermatogenesis (Fig. 1B and C).

3.2. RecQ5 was localized in nuclei of male GSCs, spermatogonia, and early spermatocytes

To identify the GSCs in the adult testis, we stained hub cells with anti-armadillo antibody. Male GSCs divide asymmetrically to yield spermatogoniablasts. EGFP-RecQ5-expressing cells surrounded the hub cells (Fig. 2A), suggesting that RecQ5 was localized in GSCs and goniablasts. Goniablasts undergo incomplete mitosis, resulting in spermatogonia that are encapsulated within a cyst. The GSCs, spermatogonia, and spermatocytes were distinguished by having a fusome morphology. The testicular fusome appeared spherical in GSCs and goniablasts, and became branched in larger cysts of spermatogonia. Testes were immunostained for fusomes with monoclonal antibody against adducin (hts) protein (Fig. 2B). EGFP-RecQ5 was expressed in GSCs, goniablasts, and spermatogonial cells (Fig. 2A and B). The level of EGFP-RecQ5 increased as early spermatocytes (primary spermatocytes) grew (Fig. 2C) and reached a maximum in polar spermatocytes (Fig. 2E) before the appearance of apolar spermatocytes (Fig. 2F), with the expression gradually fading away in mature primary spermatocytes (Fig. 2G) and differentiating spermatids. In apolar spermatocytes, typical features include a central nucleus, incompleteness of the 2nd nuclear membrane, and a variable distance between the 1st and 2nd nuclear membranes (Fig. 2F′) [14]. The nucleus of the apolar spermatocyte is much larger than that of the polar one, has as undulating surface, and becomes surrounded by a second nuclear membrane. Thus, RecQ5 expression was regulated during *Drosophila* spermatogenesis.

3.3. recq5-defective Drosophila exhibited a swelling of the apical testicular region

Though the *recq5*-mutants are fertile [4], we compared the whole *recq5* mutant testis with that of the wild-type by immunostaining for mitosis-specific phosphorylation of histone H3 (PH3). Each spermatogonium undergoes mitosis to produce primary spermatocytes, each of which expands in volume and divides meiotically to form 2 secondary spermatocytes that subsequently undergo meiosis to form 4 spermatids that, in turn, mature and

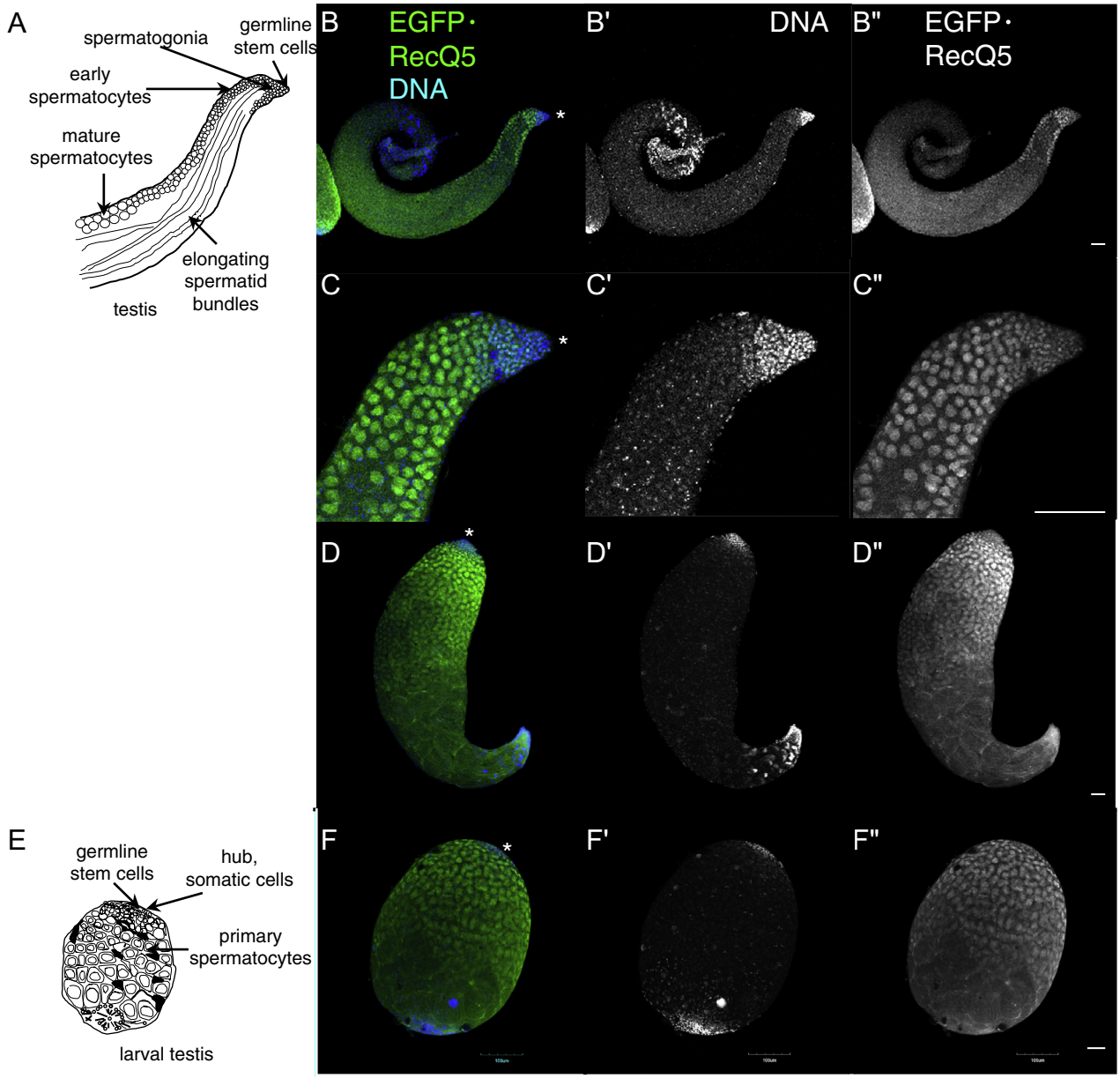

Fig. 1. *Drosophila* RecQ5 in testis. Schematic representation of adult (A) and larval testis (E). Testes of *q5^{p}-egfp-recq5* dissected from the adult (B), pupa (48 h after pupa formation, D) and 3rd instar larva (F). (C) Magnified image of "B". GFP (B–D, F) was detected as described in Section 2. DAPI (B″–D″, F″); EGFPRecQ5 (B‴–D‴, F‴); Merge (B′–D′, F′). Asterisks indicate the apical end of the testis. Scale bars: 50 μm.

elongate into spermatozoa (Fig. 1A). PH3 signals were observed in the middle region of the testes (Fig. 3A and B), corresponding to meiotic divisions. The data suggest that the meiotic divisions occurred normally in the *recq5* mutant testis. DAPI staining revealed DNA in the nuclei of GSCs, spermatogonia, spermatocytes, and spermatid heads, suggesting that mature sperm heads had formed normally in the mutant testis (Fig. 3B). The length of the *recq5* testis, from apical tip to basal end, was similar to that of the wild type (Fig. 3C). Also, the distance from the testicular tip to the meiotically dividing cells (PH3, Fig. 3A and B) was not different between the wild type and mutant. However, we did observe swelling of the apical region of the *recq5* mutant testes (Fig. 3B). To quantify this swelling, we measured the testicular diameter at 0.3 mm from the apex, because this area mainly contains early spermatocytes [17]. Thereby, we found a significant difference in the diameter of the testis at this point between the *recq5* mutant and the wild type (Fig. 3D). This increase diameter in the apical region of the testis suggests an accumulation and/or over-sizing of contents in this region.

3.4. Spermatocytes accumulated in recq5 mutants

To visualize the contents of the apical region of the testes, we mildly squashed the testes to analyze germline cell development by phase-contrast microscopy (Fig. 4A). No obvious morphological abnormality was observed in the spermatogonia, spermatocytes, spermatids or other cells in the mutant testis (Fig. 4B). Active motile sperm cells were also observed just after squashing either wild-type or *recq5* mutant testes. To analyze 16-cell cysts, we immunostained the testes for fusomes. Branched fusomes were observed in both wild-type and *recq5* mutant testes (Fig. 4C and D). These results suggest that spermatogonial stages progressed normally in the mutant testes.

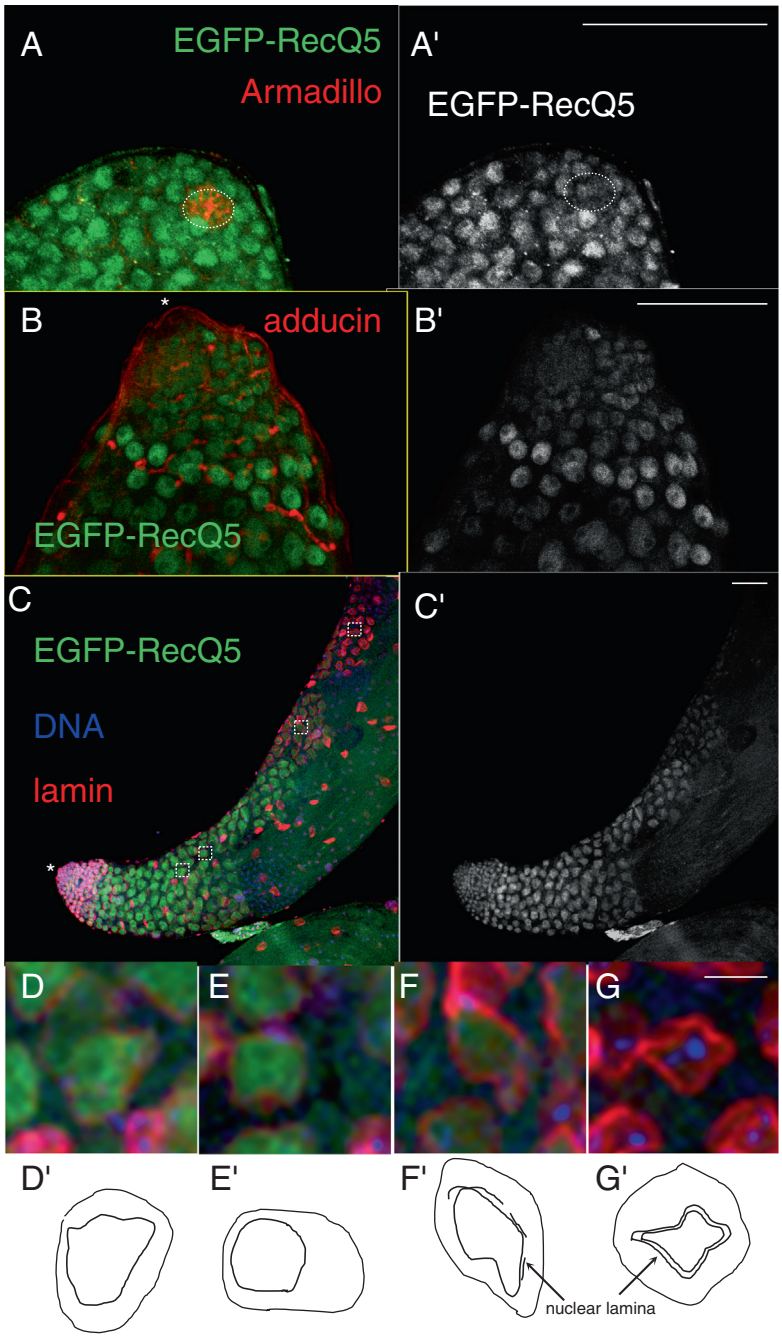

Fig. 2. *Drosophila* RecQ5 in male GSCs, goniablasts, spermatogonia, and early spermatocytes of adult flies. Immunofluorescence images of testes from adult *q5^{p}-egfp-recq5* flies. EGFP-RecQ5 (GFP; green, A–G and A′–G′), the hub (armadillo; red, outline, A), fusome (adducin [hts]; red, B), nuclear lamina (lamin; red, C–G), and DNA (DAPI; blue C–G) were stained as described in Section 2. Boxes indicate magnified portion of D–G. EGFP-RecQ5 was present in primary spermatocyte (early spermatocyte) (D), polar spermatocyte (E), and decreased in amount in apolar spermatocytes (F), but not in mature spermatocytes (G). (D′–G′) Schematic representation of spermatocyte growth phase. Scale bars: 50 μm (A–C) and 10 μm (D–G). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

During the early spermatocyte stage, the spermatocyte becomes larger, filled with irregularly shaped cavities and becomes surrounded by a second nuclear membrane. Nuclei surrounded by thick nuclear lamina were located in the apical region of the *recq5* testis, and their distribution was different from that in the wild-type ones (Fig. 4F). These data suggest that spermatocytes had relatively accumulated in the *recq5* mutant testes.

Individualization complexes, which consist of 64 cones of actin that assemble around the sperm nuclei, move to the basal end of the tails, forming a characteristic cystic bulge that contains an accumulation of cytoplasm, syncytial membrane, and vesicles [17]. The advancing cystic bulge collects the spermatids' cytoplasm and most of the organelles and eventually is pinched off from the base of the cyst and discarded as a waste bag. These waste bag-like structures were observed more frequently in the mutant testes (46% of the testes, $n = 89$; Fig. 4B) than in the wild-type ones (19% of the testes, $n = 59$; Fig. 4A). The *recq5* mutant testes were stained to visualize active effector caspases (anti-cleaved caspase 3), and the spermatids' nuclei (DAPI). Elongated spermatids undergoing individualization, as well as cystic bulges and waste bags, were stained with anti-active caspase 3 in both the wild-type and *recq5* mutant testes (Fig. 4G and H) [18]. These data suggest

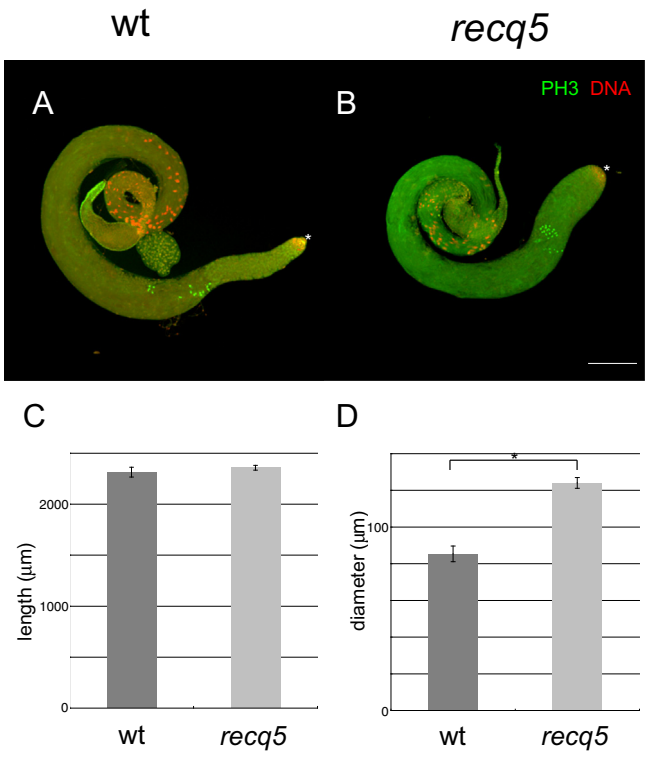

Fig. 3. Morphological difference in *Drosophila recq5* mutant testis. (A and B) Immunofluorescence images of testes from adult flies. The meiotic cells (PH3; green) and DNA (DAPI; red) were stained as described in Section 2. Scale bars: 200 μm. (C–D) Graph of the average length (C) and diameter at 300 μm from the apex (D) of testes from wild-type ($n = 25$) and *recq5* mutant ($n = 59$). Error bars represent standard error. Bracket with * indicates a statistically significant difference $p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

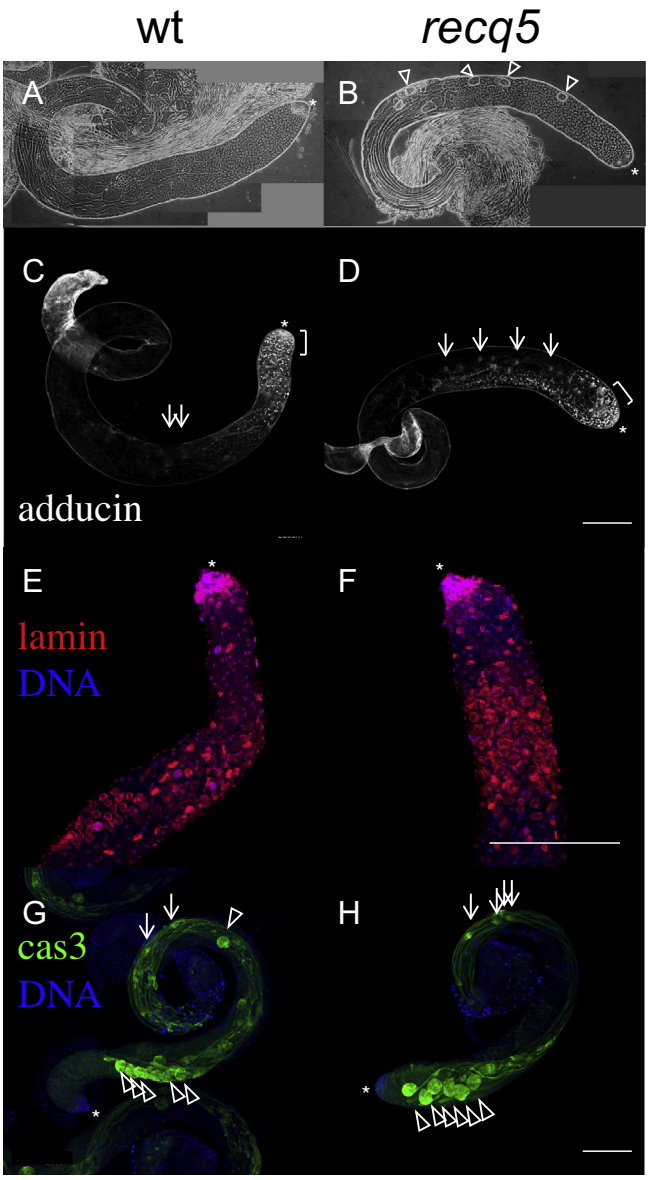

Fig. 4. Loss of *Drosophila* RecQ5 affects early spermatocytes. (A and B) Phase-contrast images of squashed testes. (A) wild-type and (B) *recq5* mutant. Asterisk marks the testicular tip. Arrowheads indicate waste bag-like structure. (C–H) Immunofluorescence images of testes. Testes from wild-type (C, E, and G) and *recq5* mutant (D, F, and H) were stained for adducin (red, C, D) and DNA (blue, E–H). Branched fusomes (brackets) and elongation cone (arrows) were observed in both wild-type (C) and *recq5* mutant (D) testes. Distribution of cells surrounded by intense nuclear lamina (red) were different between wild-type (E) and *recq5* mutant (F) testes. Active caspase 3 (green) was present in multiple elongated cysts in both wild-type (G) and *recq5* mutant (H) testes. Cystic bulges and waste bags are indicated by arrows and arrowheads, respectively. Scale bars: 200 μm (C–H). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that the individualization processes had operated normally during in *recq5* mutant spermatogenesis.

4. Discussion

The RecQ5 protein was specifically expressed in male germline cells in the *Drosophila* testis (Figs. 1 and 2). *Drosophila* RecQ5 was localized in nuclei of male GSCs, spermatogonia, and early spermatocytes. As the primary spermatocytes matured, the level of RecQ5 declined (Fig. 2). Thus, RecQ5 expression was regulated. In accordance with RecQ5 expression in the apical region of the testis, *recq5* mutant testes were different from the wild-type ones in this region: they showed (i) an increase in testicular diameter (Fig. 3) and (ii) the accumulation of spermatocytes (Fig. 4).

Drosophila RecQ5 expression in the testes is consistent with previous reports showing that human RECQL5 mRNA is expressed strongly in the testis [12]. As growth of the spermatocyte continues, RecQ5 became increasingly localized in the nuclei of early spermatocytes. At the early spermatocyte stage, the distribution of RecQ5 in the nuclei overlapped but did not coincide with that of DNA (Fig. 2D and E). After the apolar primary spermatocyte stage, the level of RecQ5 declined (Fig. 2F and G). RecQ5 was down-regulated in late spermatocytes. Therefore, *Drosophila* RecQ5 may be linked to the early stage of germline development. Interestingly, RecQ5 interacts with Rad51 [19,20], and mouse Rad 51 protein is expressed in spermatogonia and in spermatocytes during early and mid-prophase of meiosis in testis [21]. Mouse Rad51 expression is closely related to the state of cell proliferation and is presumably involved in DNA repair coupled with DNA replication, as well as in meiotic DNA recombination in spermatocytes. On the other hand, meiotic stages such as leptotene, zygotene, and pachtene are absent in *Drosophila* males [13]. In addition, RecQ5 was absent in late spermatocytes (Fig. 2G). Therefore, *Drosophila* RecQ5 is presumably involved in DNA repair coupled with DNA replication in male germline cells, as well as in early maturation processes of spermatocytes.

Though testicular length was similar between mutant and wild-type testicle (Fig. 3), the length of the testis would be mainly determined by spermatid elongation. The spermatid elongation process may thus have been normal in the *recq5* mutant testis. The large

volume of the apical region of the testis may reflect the difference in the content of differentiating cell populations. One of the reasons that the apical region of the *recq5* testis was so large was the accumulation of early spermatocytes (Fig. 4F). Spermatogenesis is a sequential and multi-stage process proceeding from GSCs to primary spermatogonia, polar spermatocyte, apolar spermatocyte, mature spermatocyte, spermatid, and sperm. The accumulation of early spermatocytes in the *recq5* mutant testis suggests that the entry speed to the apolar spermatocyte was accelerated and/or that the exit speed from it was slowed. RecQ5 may control the primary spermatocyte pool in the *Drosophila* testis. The elevated entry speed into the apolar spermatocyte might have impaired sperm quality. However, after individualization of spermatids, the damaged sperms are eliminated by the coiling process [22]. The degeneration of abnormal sperms might be much progressed in the *recq5* mutant testis. Meanwhile, the slow down of the developmental speed from the apolar spermatocyte may have caused overgrowth of the cells. Consequently, it is possible that signals of anti-cleaved caspase 3 in the waste bags were more intense in the mutant testes (Fig. 4H).

Another reason for the enlarged apical region of the *recq5* mutant testis is that the waste bags were located near the apex in the mutant (Fig. 4H). In addition, waste bag-like structures were more frequently observed in the *recq5* mutant testes than in the wild-type ones (Fig. 4A and B). During spermatid elongation, the cytoskeleton of the fusome reorganizes into a cylindrical structure (elongation cone) at the growing ends of the individual cells [23]. In the *recq5* mutant testis, the elongation cones, recognized with antibody against adducin, were prominent (Fig. 4D). Therefore, there is a possibility that the individualization process occurs differently between the wild-type and mutant testes.

Drosophila RecQ4 is expressed only in the mitotic domain of the testis, not in the growing spermatocytes [24]. One *mus309* allele, which carries a stop codon between 2 of the helicase motifs of *Drosophila* BLM, causes partial male sterility [25]. Spermatogenesis in *Drosophila* is estimated to take approximately 250 h from birth of gonial founder cell to production of mature sperm [26]. Each GSC is thought to produce a gonial founder cell cycle every 10 h; and at any given time, the steady-state adult testis is thought to contain 25 cysts of differentiating germline cells descended from the same stem cell [26]. Therefore, it is important to maintain genome stability in GSCs. RecQ5 may contribute genome stability of germline cells, acting in concert with other RecQ members.

The bulk of primary spermatocyte development takes place during an extended G2 phase of the cell cycle corresponding to meiotic prophase [27]. Though there is no male meiotic recombination in *Drosophila* [13], RecQ5 suppresses inappropriate homologous recombination in mouse ES cells [2], chicken DT40 cells [10,28], and *Drosophila* [29]. Furthermore, RecQ5 regulates homologous recombination by disrupting RAD51 recombinase-mediated presynaptic filaments [3]. Therefore, RecQ5 may suppress homologous recombination in meiotic prophase.

As male germ cells enter the primary spermatocyte stage, they switch from a program of cell division to one of growth and gene expression [26]. The main feature of the primary spermatocyte stage is a high level of gene expression and active growth, resulting a 25-fold increase in the nuclear volume [26]. The subsequent differentiation program, including meiotic divisions and elongation, is largely mediated by genes that have been transcribed during the primary spermatocyte period [30]. During the growth phase, transcription occurs at a high level. The relatively high level of RecQ5 in early spermatocytes (Fig. 2) suggests its important role in the maintenance of the genome at this stage. RECQL5 suppresses transcription stress and its detrimental effects, and thereby prevents genome instability in the transcribed region of genes [11]. In the absence of RecQ5, RNAPII may tend to move fast, giving rise to transcriptional posing and arrest in primary spermatocytes. As a result, the immature primary spermatocytes in the *recq5* mutant testis may have grown faster than normal, leading to the increase in diameter of the testis. RecQ5 may function under active transcription during the extended G2 phase in the primary spermatocyte.

This study revealed for the first time that RecQ5 was expressed in male GSCs, spermatogonia, and early spermatocytes and that a deficiency in RecQ5 led to irregular progression of spermatogenesis. Therefore, it is possible that RecQ5 plays important roles in spermatogenesis. Further experiments are required to address the role of RecQ5 in germline maintenance against endogenous genome damage as well as exogenous stress.

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