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Summary

The spindle is a highly dynamic molecular machine that mediates precise chromosome segregation during cell division. Spindle size can vary dramatically, not only between species but also between different cells of the same organism. However, the reasons for spindle size variability are largely unknown. Here we show that variations in spindle size can be linked to a precise developmental requirement. *Drosophila* species have dramatically different sperm flagella that range in length from 0.3 mm in *D. persimilis* to 58.3 mm in *D. bifurca.* We found that males of different species exhibit striking variations in meiotic spindle size, which positively correlate with sperm length, with *D. bifurca* showing 30-fold larger spindles than *D. persimilis.* This suggests that primary spermatocytes of *Drosophila* species manufacture and store amounts of tubulin that are proportional to the axoneme length and use these tubulin pools for spindle assembly. These findings highlight an unsuspected plasticity of the meiotic spindle in response to the selective forces controlling sperm length.

Key words: Male meiosis, Spindle size, Sperm length, *Drosophila* species

Introduction

The spindle is a microtubule (MT)-based machine that mediates accurate chromosome segregation during both mitosis and meiosis (reviewed in Dumont and Mitchison, 2009; O'Connell and Khodjakov, 2007). Although the spindle shows little size variation between cells of the same type, spindle length can vary dramatically both within and between species (reviewed in Goshima and Scholey, 2010). It has been proposed that spindle length is affected by both extrinsic and intrinsic factors. Well-known extrinsic factors are cell size and tubulin availability (Dumont and Mitchison, 2009; Goshima and Scholey, 2010). For example, during *Xenopus laevis* embryonic development, cell size decreases from 1200 to 12 µm, and in cells smaller than 300 µm, spindle length scales linearly with cell size (Wuhr et al., 2008). This suggests that constraints imposed by cell size and, presumably, tubulin availability set the spindle length. However, in the largest *X. laevis* blastomeres spindle length has an upper limit of 60 µm, implying the existence of intrinsic factors that govern spindle size (Wuhr et al., 2008). Further evidence for spindle size regulation by intrinsic factors is provided by the observation that spindles assembled in vitro from embryonic extracts of related *Xenopus* species display different species-specific sizes (Brown et al., 2007). Another example of spindle size regulation by intrinsic mechanisms is found in the 500 µm-long *Drosophila melanogaster* syncytial embryo, which displays spindle lengths of 10 µm throughout the first 14 rounds of embryonic divisions (Kwon and Scholey, 2004; Sullivan and Theurkauf, 1995).

Studies carried out in the past few years have identified several cell-size-independent intrinsic factors that regulate spindle length. These factors include proteins that mediate MT polymerization or depolymerization, MT sliding and MT clustering (Dumont and Mitchison, 2009; Goshima and Scholey, 2010). However, perturbations of these factors result in relatively small changes in spindle length (Goshima and Scholey, 2010), suggesting that intrinsic factors are not sufficient to account for the large variations in spindle size observed in animal cells.

Although it is clear that spindle size depends on both intrinsic and extrinsic factors, the reasons for spindle size variability are largely unknown. Here we show that variations in spindle size can be linked to a precise developmental requirement. We found that meiotic spindles from males of different *Drosophila* species have extremely different sizes, and that the size of these spindles positively correlates with the length of the sperm tail. This suggests that *Drosophila* primary spermatocytes manufacture and store most of the tubulin needed for sperm tail formation and use it for meiotic spindle assembly.

Results and Discussion

Size of *Drosophila* male meiotic spindles correlates with the length of the sperm flagellum

Although male meiotic spindles and somatic cell spindles of mammals are comparable in size (Manandhar et al., 2000), spermatocyte spindles of *D. melanogaster* are much larger than those of mitotic cells (Cenci et al., 1994). In *D. melanogaster* primary spermatocytes, metaphase chromosomes appear as a small mass at the center of a large bipolar MT assembly (Fig. 1). By contrast, in *D. melanogaster* embryonic cells, larval neuroblasts, ganglion mother cells and female meiotic cells, metaphase chromosomes occupy the entire equatorial region of the spindle or even protrude from the spindle (Bonaccorsi et al.,

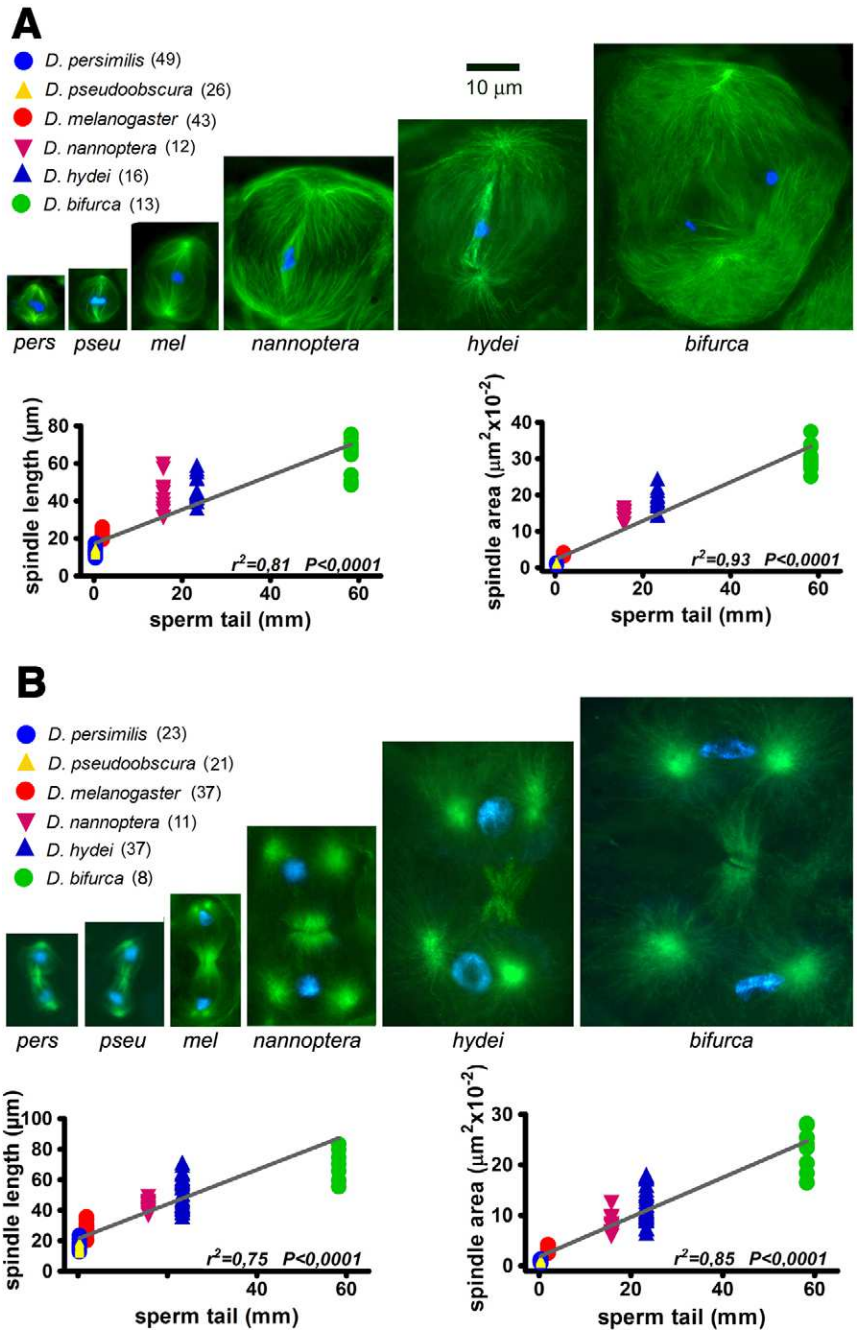

Fig. 1. Size of the male meiotic spindle of different *Drosophila* species positively correlates with the sperm tail length. (**A**,**B**) Examples of metaphase I (A) and telophase I (B) figures stained for tubulin (green) and DNA (blue); all spindles are at the same enlargement. In telophases of species with big spindles, the asters associated with the daughter nuclei often separate before completion of cytokinesis, resulting in X-shaped spindles. Graphs show the strong positive correlation between the sperm tail length and the spindle length or area. The numbers between brackets indicate the number of spindles analyzed. Note that the measures of the *D. persimilis* and *D. pseudoobscura* spindles largely overlap.

2000; Kwon and Scholey, 2004; Sullivan and Theurkauf, 1995; Theurkauf and Hawley, 1992) (Fig. 2). These differences in the basic shape of male meiotic spindles with respect to those in other fly cells are intriguing because there is no evidence that male meiotic spindles mediate chromosome segregation by any but normal mechanisms.

What is then the reason for the large size of the meiotic spindle in *D. melanogaster* males? We hypothesized that *Drosophila* spermatocytes accumulate a substantial fraction of the tubulin needed for sperm tail assembly and use it for meiotic spindle formation. This hypothesis is testable, because various *Drosophila* species have dramatically different sperm flagella. *D. persimilis*, *D. pseudoobscura*, *D. melanogaster*, *D. nannoptera* and *D. hydei* sperm tails are 0.32, 0.36, 1.91, 15.74 and 23.32 mm long, respectively; the *D. bifurca* flagellum is more than 30-fold longer than its *D. melanogaster* counterpart, with the extraordinary length of 58.29 mm (Pitnick et al., 1995a; Pitnick et al., 1995b). We examined male meiosis in these six *Drosophila* species to determine whether a correlation exists between sperm length and the size of the meiotic spindle. Dividing spermatocytes obtained from pupae were squashed under mild pressure and then stained for tubulin and DNA. To assess the spindle size, we measured both the length and the area of the spindle (see supplementary material Fig. S1). We did not measure the fluorescence of the spindle MTs to estimate the amount of tubulin, because the commercial anti-tubulin antibody we used (raised against chicken α-tubulin) might react to the tubulins of distant *Drosophila* species with varying efficiencies.

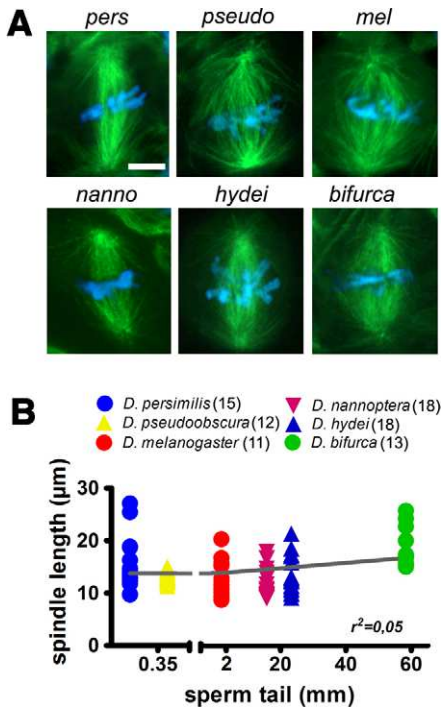

Fig. 2. *Drosophila* species exhibit mitotic spindles of comparable sizes. (**A**) Larval neuroblast metaphases of different *Drosophila* species stained for tubulin (green) and DNA (blue). (**B**) The neuroblast spindles length does not correlate with the sperm tail length. The numbers between brackets indicate the number of spindles analyzed. Scale bar: 5 µm.

The results of our analysis unequivocally showed that the six *Drosophila* species differ dramatically in the length and the area of the spindles of the first meiotic division (Fig. 1 and supplementary material Table S1). Similar differences in size were observed in spindles of the second meiotic division (supplementary material Fig. S2 and Table S1). In all cases, we found a highly significant positive correlation between the spindle size of the meiotic figures and the length of the sperm flagellum (Fig. 1 and supplementary material Fig. S2). These observations support the hypothesis that primary spermatocytes of *Drosophila* species store amounts of tubulin that are proportional to the sperm tail length, and use a substantial part of this tubulin to assemble the meiotic spindle.

Drosophila spermatocytes deliver to the spermatids most of the tubulin and the mitochondria needed for sperm tail assembly

Spermatocytes result from four rounds of mitotic divisions of a single founding gonial cell. The 16 postmitotic spermatocytes enter a 90-hour growth phase during which undergo extensive transcription and translation accompanied by a 25-fold increase in volume (reviewed in Fuller, 1993; Schafer et al., 1995; White-Cooper, 2010). Before completion of this growth phase, spermatocytes degrade the β1-tubulin isotype and switch to the testis-specific β2 isoform. Following this switch, all MT functions are carried out by a single tubulin heterodimer composed of the α-84B and β2-tubulin isoforms (Hutchens et al., 1997; Kemphues et al., 1982). Collectively, these results indicate that spermatocytes are enriched in tubulin and other proteins needed for spermiogenesis, and that the same tubulin heterodimer is used for both meiotic spindle and sperm tail formation.

D. melanogaster male meiosis produces four spermatids, each comprising a nucleus and a mitochondrial derivative called the nebenkern. Elongating spermatids contain two classes of MTs: the MTs that comprise the axoneme, which is dispensable for sperm elongation, and the cytoplasmic MTs that drive sperm tail growth (Basto et al., 2006; Noguchi et al., 2011). The cytoplasmic MTs interact with the mitochondrial derivatives and mediate the extension of these structures, which are thought to serve as cell shape templates during spermatid elongation (Noguchi et al., 2011). Transcription is strongly reduced in *D. melanogaster* spermatids and only a few genes appear to be transcriptionally active (Barreau et al., 2008; Vibranovski et al., 2010; White-Cooper, 2010). Spermatids inherit many stabilized mRNAs from spermatocytes, including mRNAs that encode β2-tubulin, and undergo protein synthesis until late in spermiogenesis (reviewed in Fuller, 1993; White-Cooper, 2010). However, electron microscopy studies have shown that spermatids and mature sperm have a similar volume (Lindsley and Tokuyasu, 1980; Tokuyasu, 1975). This suggests that spermatocytes deliver to spermatids a substantial fraction of the material needed for sperm differentiation. It should be noted that tubulin and mitochondria delivery are closely related events. During meiotic telophase I and II, mitochondria associate with the central spindle and are equally partitioned between the two daughter cells upon execution of cytokinesis (Cenci et al., 1994; Fuller, 1993). Thus, a spindle proportionate to sperm length ensures proper transmission to the spermatids of the two major components of the sperm tail: the tubulin and the mitochondria.

Size of *Drosophila* male meiotic spindles does not correlate with the size of mitotic spindle

To control for the possibility that the differences in meiotic spindle size could be governed by species-specific factors other than the amount of tubulin needed to form the sperm tail, we examined mitotic division in larval brain squashes from all six *Drosophila* species. We observed that the spindle length in larval neuroblasts does not vary significantly between species, and that the mitotic spindle size does not correlate with the sperm length (Fig. 2). We also noted that the large differences in meiotic spindles cannot be explained by obvious differences in chromosomal complements. Although these species exhibit different karyotypes, the number of chromosomes varies only between 8 and 12 (Fig. 3), and the diploid cells of all six species contain comparable amounts of DNA (Bosco et al., 2007; see also supplementary material Table S2). We thus conclude that there are no major species-specific factors that influence mitotic spindle size, making it unlikely that meiotic spindle size is regulated by factors other than the amount of tubulin accumulated in spermatocytes.

Is the size of *Drosophila* male meiotic spindles controlled by intrinsic factors?

The metaphase spindle of *D. bifurca* primary spermatocytes with its 63 µm of length is probably the largest spindle described to date in any organism (Goshima and Scholey, 2010). The meiotic spindles of *D. hydei* and *D. nannoptera* are also very large. Our results strongly suggest that the main extrinsic factor responsible for the large size of these spindles is tubulin availability. However, we believe that the assembly of such large spindles

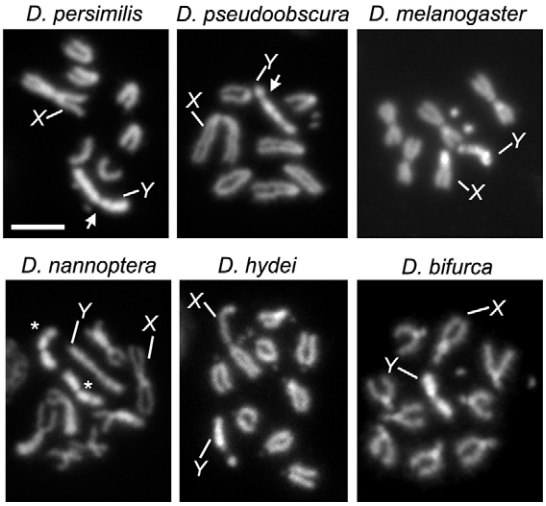

Fig. 3. DAPI-stained metaphase figures from larval brains of six *Drosophila* species. Karyotypes of *D. persimilis* and *D. pseudoobscura* are very similar and differ only for the size and morphology of the Y chromosome (the Y centromere is indicated by an arrow). *D. nannoptera* exhibits two chromosomes that appear to be entirely heterochromatic (asterisks), consistent with previous observations (Ward and Heed, 1970).

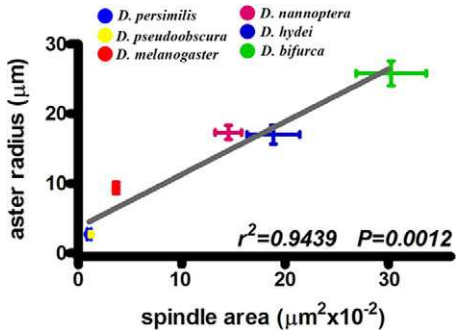

Fig. 4. Aster size of primary spermatocytes from males of different *Drosophila* species positively correlates with the spindle size. Note the strong positive correlation between the radius of late prophase–early prometaphase asters and the metaphase spindle area (the spindles areas are those in Fig. 1). Error bars indicate s.d.; the measures of the asters and spindles of *D. persimilis* and *D. pseudoobscura* largely overlap.

requires a number of intrinsic factors. One of these factors could be the centriole and/or centrosome size. In *C. elegans* embryos, the size of the centrioles and centrosomes correlates with spindle length (Greenan et al., 2010). Centriole and spindle lengths also appear to be correlated in different *D. melanogaster* cell types; the centrioles of embryonic cells, tissue culture cells and spermatocytes are 0.2, 0.2 and 2.5 μm long, respectively (Gonzalez et al., 1998; Baker et al., 2004), and the metaphase spindle lengths in the same cells are 11.8, 10.0 and 23.0 μm, respectively (Goshima and Scholey, 2010). We were unfortunately unable to test whether this correlation also held for male meiotic cells of the *Drosophila* species examined, because we found that antibodies raised against various *D. melanogaster* centrosome components failed to react either totally or partially with the centrioles of some species (we tested antibodies directed against Asterless, γ-tubulin, DSpd2 and centrosomin). However, our cytological observations indicated that species with long sperm tails and large meiotic spindles also have large asters (Fig. 1 and supplementary material Fig. S2). To assess the relationships between aster and spindle size, we measured the radius of the asters of late prophase–early prometaphase primary spermatocytes; this radius positively correlated with the area of the metaphase spindle (Fig. 4), suggesting that the centrioles and centrosomes of large spindles have a higher nucleating ability than those of small spindles. Consistent with this finding, EM studies have shown that the centrioles of *D. hydei* spermatocytes are approximately threefold longer than their *D. melanogaster* counterparts (Hennig and Kremer, 1990).

A positive correlation between the centrosome nucleating ability and spindle size implies a major role of the centrosomes in male meiotic spindle formation in Drosophilidae. This view is supported by previous studies in *D. melanogaster* that indicated that centrosome-nucleated MTs are crucial for spindle assembly in spermatocytes but not in somatic cells. Mutants in which centrosome function is inhibited form anastral but otherwise functional mitotic spindles by exploiting MTs nucleated near the chromosomes (Basto et al., 2006; Bonaccorsi et al., 2000; Giansanti et al., 2008; Megraw et al., 2001). However, although the same mutants nucleate MTs around the meiotic chromosomes, their male meiotic spindles are highly defective, leading to male sterility (Bonaccorsi et al., 1998; Giansanti et al., 2008). Consistent with these findings, *D. melanogaster* secondary spermatocytes have the peculiar ability to assemble a spindle in the complete absence of chromosomes, exploiting only MTs nucleated by the centrosomes (Bucciarelli et al., 2003). Collectively, these results support the hypothesis that the centrosome is an important intrinsic factor in spindle size determination in *Drosophila* spermatocytes.

Coevolution of giant meiotic spindles and sperm

The reason why some *Drosophila* species evolved giant sperm tails is still matter of debate. The most popular current hypothesis asserts that the gametes and reproductive structures of males and females coevolved in response to sexual selection (Bjork and Pitnick, 2006; Joly and Schiffer, 2010; Miller and Pitnick, 2002). Our results underscore the strength of the selective forces that drove sperm length increase during *Drosophila* evolution. Formation of the giant sperm tails was not simply achieved through an increase in postmeiotic translation of sperm proteins, but involved the evolution of giant spindles that utilize a substantial fraction of the tubulin that will be used after meiosis for sperm tail differentiation. The assembly of such spindles is likely to result from a series of concerted evolutionary changes in the regulation of spindle-associated proteins and in centrosome structure and function.

Materials and Methods

Drosophila species

The *D. melanogaster* wild-type stock used in this study was an Oregon R strain. The other species were all obtained from the *Drosophila* Species Stock Center of the University of California, San Diego (https://stockcenter.ucsd.edu). *D. melanogaster*, *D. persimilis*, *D. pseudoobscura* and *D. hydei* were reared at 25°C on standard yeast–sucrose–agar medium. *D. nannoptera* and *D. bifurca* were grown on banana medium supplemented with opuntia cactus powder; the recipe for the medium can be found at https://stockcenter.ucsd.edu.

Cytology

DAPI-stained metaphases and larval neuroblast spindles were prepared according Gatti et al. and Bonaccorsi et al., respectively (Gatti et al., 1994; Bonaccorsi et al.,

2000). Meiotic spindle preparations were obtained from pupal testes fixed and processed according to published methods (Cenci et al., 1994). Brain and testis preparations were incubated overnight at 4℃ with a monoclonal anti-α-tubulin antibody (diluted 1:1000 in PBS; Sigma-Aldrich), which was detected by 1 hour of incubation at room temperature with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG+IgM (diluted 1:20 in PBS; Jackson Laboratories). Immunostained preparations were mounted in Vectashield medium H-1200 (Vector Laboratories) containing the DNA dye DAPI, and examined with a Zeiss Axioplan fluorescence microscope equipped with a CCD camera (Photometrics CoolSnap HQ).

Spindle and aster measurement

Spindle and aster measurements were carried out on digital images at defined enlargement. The spindle length is the distance between the two spindle poles, as indicated in supplementary material Fig. S1. To calculate the spindle area we used the MetaMorph program (Meta Imaging Series Software from Molecular Devices). We outlined the spindle perimeter as shown in supplementary material Fig. S1 and the program calculated the spindle area.

Statistical analysis

Regression analysis was performed using the GraphPad software. The r^2 value measures the goodness-of-fit of linear regression; the P value evaluates the probability of the null hypothesis.

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Supplementary material available online at
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