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RESEARCH ARTICLE

Cytoskeletal variations in an asymmetric cell division support diversity in nematode sperm size and sex ratios

Ethan S. Winter1,*, Anna Schwarz2,*, Gunar Fabig2,*, Jessica L. Feldman3,*, André Pires-daSilva4, Thomas Müller-Reichert2, Penny L. Sadler1,5 and Diane C. Shakes1,‡

ABSTRACT

Asymmetric partitioning is an essential component of many developmental processes. As spermatogenesis concludes, sperm are streamlined by discarding unnecessary cellular components into cellular wastebags called residual bodies (RBs). During nematode spermatogenesis, this asymmetric partitioning event occurs shortly after anaphase II, and both microtubules and actin partition into a central RB. Here, we use fluorescence and transmission electron microscopy to elucidate and compare the intermediate steps of RB formation in Caenorhabditis elegans, Rhabditis sp. SB347 (recently named Auanema rhodensis) and related nematodes. In all cases, intact microtubules reorganize and move from centrosomal to non-centrosomal sites at the RB-spERM boundary whereas actin reorganizes through cortical ring expansion and clearance from the poles. However, in species with tiny spermocytes, these cytoskeletal changes are restricted to one pole. Consequently, partitioning yields one functional sperm with the X-bearing chromosome complement and an RB with the other chromosome set. Unipolar partitioning may not require an unpaired X, as it also occurs in XX spermocytes. Instead, constraints related to spermatocyte downsizing may have contributed to the evolution of a sperm cell equivalent to female polar bodies.

KEY WORDS: Asymmetric partitioning, Spermatogenesis, X chromosome segregation, Cytoskeletal reorganization, Non-centrosomal microtubules, Caenorhabditis elegans, Spermiogenesis

INTRODUCTION

The asymmetric partitioning of cellular components along one or more axes is a crucial step in the differentiation of most cells (Nance and Zallen, 2011; Campanale et al., 2017). The resulting cell polarity is essential for proper cell function including motility in diverse cell types and the barrier function of epithelial cells; disruption of cell polarity is a hallmark of epithelial cancers (Halaoui and McCaffrey, 2015). Furthermore, cells can couple the establishment of cellular asymmetries with an oriented cell division (Halaoui and McCaffrey, 2015). Furthermore, cells can couple the establishment of cellular asymmetries with an oriented cell division (Halaoui and McCaffrey, 2015). Additionally, cells can couple the establishment of cellular asymmetries with an oriented cell division (Halaoui and McCaffrey, 2015). Furthermore, cells can couple the establishment of cellular asymmetries with an oriented cell division (Halaoui and McCaffrey, 2015).

During sperm development, asymmetric partitioning plays yet another role; it streamlines sperm for optimal motility. Mature sperm are small and motile, and thus one key step in their differentiation is the post-meiotic shedding of organelles and cytoplasmic components that are either unnecessary for or detrimental to subsequent sperm function (Fig. 1A). This shedding event involves two steps: (1) the differential partitioning of cellular components into a cellular wastebag known as a residual body (RB), and (2) the subsequent separation of this RB from the sperm (Steinhauer, 2015). In Drosophila and vertebrates, RB formation requires both actin and microtubules (Steinhauer, 2015; O’Donnell et al., 2001) and occurs as the final step of a post-meiotic cell differentiation process (spermiogenesis) that takes days to weeks and requires extensive cytoskeletal remodeling (Fabian and Brill, 2012; Clermont, 1972; Fig. 1A). In C. elegans, sperm production is accelerated by the production and pre-packaging of sperm components prior to the meiotic divisions; as a result, the highly reduced post-meiotic phase takes only minutes (Ward et al., 1981; Shakes et al., 2009; Chu and Shakes, 2013; Fig. 1B). Key to the brevity of this post-meiotic phase, RB formation occurs immediately after anaphase II and involves the replacement rather than the remodeling of cytoskeletal components (Fig. 1B; Shakes et al., 2009). Post-anaphase II, components required for sperm function, such as the fibrous body-membranous organelles (FB-MOs) partition to the haploid sperm whereas unneeded components are discarded into the RB that forms between the two sperm (Ward et al., 1981; Ward, 1986; Fig. 1D). Importantly, the discarded material includes the cell’s entire store of actin and microtubules, as nematode sperm motility is driven not by a flagellum but by the assembly/disassembly dynamics of a nematode-specific cytoskeletal protein, the major sperm protein (MSP) (Smith, 2006; Yi et al., 2009).

It is unclear how the actin and microtubules in C. elegans spermatocytes shift from their anaphase II patterns to their final deposition within RBs or how various organelles differentially partition between the sperm and RB. In pharmacological studies, actin but not microtubule inhibitors block C. elegans sperm formation (Nelson et al., 1982). Genetic studies likewise implicate a key role for actin; mutants lacking the actin-binding protein SPE-26 fail to form RBs (Varkey et al., 1995), and loss of the unconventional myosin (myosin VI) specifically disrupts stable partitioning of actin, tubulin, mitochondria and FB-MOs (Kelleher et al., 2000). However, microtubules might also play a role as centrioles seem to specify the number and position of the sperm-RB boundaries (Peters et al., 2010). The associated transition from anaphase II to post-meiotic RB formation (Fig. 1B) is rapid and dramatic. Yet, little is known about the intermediate steps. Does nematode RB formation employ cellular mechanisms common to other asymmetric partitioning processes? Alternatively, given its unusually close juxtaposition to anaphase, has RB formation co-opted elements of the normal cytokinesis machinery?
The speed and relative simplicity of these post-meiotic events, combined with a striking degree of interspecies diversity in sperm size (Vielle et al., 2016), sperm morphology (Justine, 2002; Yushin and Malakhov, 2014) and patterns of sex chromosome segregation (Shakes et al., 2011), makes nematodes a valuable system for comparative studies. We recently described spermatogenesis in a nematode, provisionally named *Rhabditis* sp. SB347 and more recently designated *Auanema rhodensis* (Kanzaki et al., 2017), in which the unusually small spermatocytes of XO males do not form traditional RBs (Shakes et al., 2011; Fig. 1C). Instead, the asymmetric partitioning process yields functional, X-bearing sperm containing the essential sperm components and an RB containing the actin, tubulin and the non-X chromosome set. Crucial to this sex-biased gamete production, the unpaired X chromosome in the XO male spermatocytes of *R. sp. SB347* does not lag during anaphase I as in *C. elegans* male spermatocytes (Albertson and Thomson, 1993; Fig. 1B). Instead, the X splits into sister chromatids during anaphase I, and the secondary spermatocytes always have a lagging X chromatid during anaphase II (Shakes et al., 2011; Fig. 1C).

In this study, we explore the cellular mechanisms of this asymmetric partitioning process through a comparative study of spermatogenesis in *C. elegans*, *R. sp. SB347*, and additional members of the *R. sp. SB347* clade. Using a combination of fluorescence and transmission electron microscopy (TEM), we examine how sequential changes in microtubule and microfilament patterns correlate with the timing of anaphase chromosome segregation and the differential partitioning of specific organelles. We find that organelle partitioning occurs in two phases, with larger organelles partitioning early and endoplasmic reticulum (ER) partitioning only later during the final stages of sperm-RB separation. We identify the transition between anaphase II chromosome segregation and post-meiotic RB formation as the critical period when microtubules begin to reorganize and move from the centrosomes to the RB-sperm boundaries, and actin reorganizes to the RB through a combination of cortical ring expansion and clearance from the poles. In *R. sp. SB347* and near relatives with similarly small spermatocytes, we find that the conversion of a typically bipolar partitioning process becomes unipolar, through the selective inactivation of one centrosome and differential clearing of actin from that same pole. Although we previously hypothesized that unipolar partitioning in *R. sp. SB347* required an unpaired X during anaphase II (Shakes et al., 2011), we show here that both male (XO) and hermaphrodite (XX) spermatocytes divide in a unipolar fashion. The routine production during meiosis of functional and degenerate sperm during meiosis has been previously reported in rotifers (Whitney, 1918), aphids (Honda, 1921) and honeybees (Sharma et al., 1961). However, to our knowledge, this is the first example in nematodes of diminutive spermatocytes generating fewer than four functional gametes from meiosis and co-opting the process of RB formation to discard half of their genetic material into what appears to be the spermatogenesis equivalent of female polar bodies.

**RESULTS**

In *C. elegans* spermatocytes, intact microtubules reorganize and move from the centrosomes to the RB-sperm boundaries

During *C. elegans* spermatogenesis, the transition from anaphase II to post-meiotic RB formation and release includes a dramatic reorganization of the microtubule cytoskeleton from an anaphase spindle into non-spindle microtubules within the RB (Ward et al., 1981; Ward, 1986; Shakes et al., 2009). Yet the nature of this transition has been unclear. Are pre-existing microtubules reorganized or are they completely disassembled and newly reassembled within the RB? To understand the nature of this reorganization and whether it co-opts elements of the normal cytokinesis machinery, we imaged both live *C. elegans* spermatocytes using differential interference contrast (DIC) optics and fixed spermatocytes that had been co-labeled with DAPI and anti-α-tubulin antibodies (Fig. 2A). From anaphase I until early anaphase II, chromosomes segregated on typical microtubule spindles. Anaphase I spermatocytes were distinguished by the presence of a lagging X bivalent (blue arrow), which are known to...
ultimately segregate to one of the two secondary spermatocytes (Albertson and Thomson, 1993). However, as the haploid chromosome sets moved further apart and the spermatocytes elongated (partitioning phase, P), microtubules were no longer anchored at the centrosomes, and the DIC images revealed a central region lacking refractive FB-MOs. Once constrictions had formed between each sperm and the central RB (separation phase, S), microtubules had completely reorganized into two broad bands, one at each RB-sperm boundary. As the RB fully separated from the adjacent sperm products (Pr), the cortical tips of microtubules gathered into discrete foci. Newly formed RBs had two or four discrete foci, depending on whether the secondary spermatocytes had fully separated after the first meiotic division.

As microtubules reorganize and move to the RBs, their centrioles relocalized to punctate structures in the RBs, although a subpopulation remained behind with the inactive centrosomes, as has been observed in other differentiated cell types (Feldman and Priess, 2012; Zhou et al., 2009; J.L.F., unpublished data). In live metaphase II spermatocytes, GFP:TBG-1 localized exclusively to the centrosome (Fig. 2C, 0 min; Movie 1). However, as cells progressed beyond anaphase II, the centrosomes flattened and spermatocytes co-expressing GFP:γ-tubulin (TBA-1, red) and mCherry:TBA-1 (TBA-1, red; time in minutes). Anaphase I (AI), metaphase II (MII), anaphase II (AII) post-meiotic partitioning (P) and separation (S) phases, the products (Pr) that include functional sperm (red arrows) and residual bodies (white arrows), and crawling spermatozoa (Z) are indicated. Scale bars: 5 µm.

**Fig. 2. Microtubule dynamics and organelle partitioning during spermatogenesis in C. elegans and R. sp. SB347.** (A-F) Live and/or fixed cells from C. elegans males (A-D), R. sp. SB347 males (E) and R. sp. hermaphrodites (F). (A,E,F) Fixed cells in which the DNA is labeled with DAPI (blue), the microtubules with anti-α-tubulin antibodies (green), and the fibrous bodies (FBs) with anti-MSP antibodies (red). Light blue arrows in DAPI columns show an unpaired X chromosome lagging during anaphase I in C. elegans male spermatocytes (A) and an unpaired X chromatid lagging during anaphase II in R. sp. SB347 male spermatocytes (E). Green arrows show new secondary microtubule foci (green). Left column in A and E shows same stage live cells imaged under DIC optics. (B) Fixed male gonad from C. elegans. DNA is labeled with DAPI (blue), endogenous GIP-1 with anti-GIP-1 antibodies (green), and centrioles with anti-IFA antibodies (red). Top image shows a developmental progression of spermatocytes in meiotic prophase on the left (distal) side and intermixed RBs (arrows) and sperm on the proximal side. Single-channel images of the boxed region are shown below. (C) Images of a live metaphase II spermatocyte transitioning to the separation phase showing the dynamics of γ-tubulin (GFP:TBG-1, green), chromosomes (histone:mCherry, red) and the cell membrane [mCherry:PH(PLC1δ1), green]. Small arrows indicate residual centrosomes. Arrowheads show non-centrosomal γ-tubulin. (D) Anaphase II to separation in a live spermatocyte expressing GFP:γ-tubulin (TBA-1, green) and mCherry:α-tubulin (TBA-1, red; time in minutes). Anaphase I (AI), metaphase II (MII), anaphase II (AII) post-meiotic partitioning (P) and separation (S) phases, the products (Pr) that include functional sperm (red arrows) and residual bodies (white arrows), and crawling spermatozoa (Z) are indicated. Scale bars: 5 µm.

In live metaphase II spermatocytes, GFP:TBG-1 localized exclusively to the centrosome (Fig. 2C, 0 min; Movie 1). However, as cells progressed beyond anaphase II, the centrosomes flattened and GFP:TBG-1 spread along the cortex (Fig. 2C, arrowheads). As spermatocytes elongated, some GFP:TBG-1 remained with the inactivated centrosome, whereas the non-centrosomal fraction of GFP:TBG-1 moved towards the RB, eventually concentrating at the RB-sperm boundary during the separation phase (40′). To assess microtubule reorganization directly, we also observed live spermatocytes co-expressing GFP:TBG-1 and mCherry:TBA-1 (α-tubulin, a core subunit of microtubules) (Fig. 2D, Movie 2). Localization of GFP:TBG-1 to the cortical tips of microtubules (arrowheads, 20′) suggests that microtubules remain intact and associated with their γ-TuRCs as they move to the RBs, and orient with their minus ends specifically abutting the RB-sperm boundaries.

In R. sp. SB347 male spermatocytes, major shifts in microtubule patterns are confined to the single, X-bearing pole

In R. sp. SB347 spermatocytes, microtubule organization was initially similar to that in C. elegans (Fig. 2E). However, by early anaphase II [All(e)] when microtubule asters were already at the two poles, the spermatocytes were only slightly elongated, and the X...
that FB partitioning in R. reorganized throughout the partitioning phase. The unpaired X in the process of FB partitioning is presumably rapid as we failed to find the pole with the larger, X-bearing chromatin mass (Fig. 2E, P). The X then moved into an anaphase plate. In 33/37 spermatocytes in which a distinct X was observed, the X was either centrally (11/11) or loosely associated with one pole (32/32) [Fig. 2E, AII(l)]. This model is consistent with earlier proposals that asymmetric spindle partitioning coincides with post-meiotic events such as sperm differentiation (spermogonogenesis) rather than part of the meiotic divisions (Shakes et al., 2009; Chu and Shakes, 2013).

Unipolar partitioning also occurs during spermatogenesis in XX R. sp. SB347 hermaphrodites
In R. sp. SB347 males, the partitioning of sperm essential components specifically to the X-bearing sperm suggested that the unpaired X chromatin might physically cue the asymmetry (Shakes et al., 2011). If so, the production of functional and non-functional sperm should be an exclusive property of XO males, because only XO secondary spermatocytes are predicted to have an unpaired X. We hypothesized that, in the absence of an unpaired X, spermatocytes from XX hermaphrodites would undergo bipolar partitioning to produce four functional sperm and an RB without DNA. Contrary to our expectations, hermaphrodite spermatogenesis yielded a mixture of DNA-containing (MSP negative, tubulin positive, white arrow) RBs and functional (MSP positive, tubulin negative, red arrow) sperm (Fig. 2F, P; 100% of >30 hermaphrodites scored at this stage). Furthermore, although they were difficult to capture, all observed post-meiotic intermediates (8/8 cells) exhibited unipolar partitioning (Fig. 2F, P). Thus, the unipolar division that generates one functional sperm and one DNA-containing RB during R. sp. SB347 spermatogenesis occurs in both XX and XO germlines.

Post-meiotic, asymmetric partitioning occurs in two discrete phases
Whereas FB-MOs and mitochondria partition to the sperm, other organelles such as the ER partition to the RB (Ward et al., 1981; Fig. 1D). To assess the relative timing of ER partitioning, we examined ER in fixed spermatocytes using an antibody against the ER-specific cytochrome P450 marker, CYP-33E1 (Hadwiger et al., 2010). In C. elegans meiotic spermatocytes, CYP33-E1 labeled both a diffuse cytoplasmic component and discrete, elongated tube-like structures that were distributed throughout the cell (Fig. 3A). During the post-meiotic stages (P, S), the diffuse cytoplasmic component localized to the expanding RB, whereas the tubular structures remained uniformly distributed throughout most of the separation phase before ultimately partitioning to the RBs (white arrow). During R. sp. SB347 spermatogenesis, CYP33-E1 exhibited an analogous pattern (Fig. 3B). The diffuse component partitioned away from the X-bearing sperm at the beginning of the post-meiotic stage, whereas the faintly labeled tubular structures partitioned to the RB (white arrow) only later. The molecular forces involved in partitioning these late-segregating components remains unclear.

Transmission electron micrographs of R. sp. SB347 spermatocytes
Because the small size of the R. sp. SB347 spermatocytes made it challenging to visualize details of the partitioning process, we further investigated the relative timing of these events using thin section electron microscopy. In cells in which the X chromatin (marked in orange) was positioned in between the autosomes (marked in blue), the mitochondria and FB-MO complexes seemed to be equally distributed (Fig. 3C). In cells in which the X chromatin had fully incorporated into an anaphase plate, FB-MOs and mitochondria were differentially partitioned to the X-bearing pole (Fig. 3D). During these early stages, tubular and membranous structures as well as ribosomes remained evenly distributed.

In both C. elegans and R. sp. SB347, asymmetric FB-MO partitioning coincides with post-meiotic events
The major sperm protein (MSP) is a cytoskeletal protein that ultimately drives nematode sperm motility; however, when MSP is first synthesized, it is packaged in the form of paracrystals within discrete fibrous bodies (FBs) (Smith, 2006). To determine whether asymmetric FB partitioning coincides with either chromosome segregation or microtubule reorganization, FB patterns were examined in co-labeled spermatocytes. In fixed C. elegans spermatocytes, FBs were uniformly distributed until the completion of anaphase II, at which point the FBs became clearly visible (Fig. 2A). In spermatocytes in which microtubules were actively reorganizing and moving centrally to the RB-spindle boundary (Fig. 2E, green arrows, unipolar), microtubule asters at the non-X pole remained relatively unchanged. In summary, a process of microtubule reorganization and centrosome inactivation that is characteristic of spermatocytes from XX hermaphrodites (Fig. 2A) is unipolar in R. sp. SB347 (Fig. 2E).
In parallel studies, we analyzed serial 'semi-thick' (300 nm) sections, which enabled us to capture the entire volume of dividing spermatocytes. Analysis of 76 anaphase II and partitioning phase cells within six different individuals enabled us to quantify the asymmetric partitioning of the mitochondria and FB-MOs relative to the cell's progression through anaphase II (Fig. 3F,G). Within individual secondary spermatocytes, the number of organelles per cell was counted and assigned to one of three defined zones closer to the centrosome without the X chromosome (Z1), closer to the centrosome associated with the X chromosome (Z3), or in a zone in between (Z2) (Fig. 3F). Because individual cells differed in size and shape, we normalized the X-to-X-pole distance to the centrosome-to-centrosome distance. Plotting X chromatid position against the fraction of organelles in Z3 revealed that most FB-MOs and mitochondria partitioned only once the X approached the relative position of 0.2 and thus was mostly or fully incorporated into one of the anaphase plates (Fig. 3G).

Using electron tomography, we also fully reconstructed two cells, one in early anaphase II and one in early partitioning (Fig. 3H,I).
When the X chromatid was positioned centrally (Fig. 3H), so were the FB-MOs (light gray), mitochondria (dark gray) and, when scorable, Golgi complexes (white). When the X segregated to one pole and was nearly or fully incorporated into the chromosome cluster (and would have been scored as fully incorporated by DAPI staining), the FB-MOs and mitochondria were restricted to the X-bearing side (Fig. 3I). In contrast, Golgi complexes (white) within this same cell remained symmetrically distributed. These same three-dimensional reconstructions enabled us to obtain precise counts of organelle numbers; we counted 27 FB-MOs and 11 mitochondria within the anaphase II spermatocyte, and 34 FB-MOs and 14 mitochondria within the partitioning-stage spermatocyte.

**Unipolar partitioning occurs in other trioecious species of the R. sp. SB347 clade but not in the male/female species Rhabditella axei**

To determine whether the unipolar partitioning process that yields one functional sperm and one DNA-containing RB from each R. sp. SB347 secondary spermatocyte represents an evolutionary oddity or a characteristic feature of this clade (Fig. 4A), we investigated male spermatogenesis in closely related species that, like R. sp. SB347, are both trioecious (males/females/hermaphrodites) and have small sperm. *Rhabditis* sp. SB372 males have sperm (4.6±0.9 µm² cross-sectional area) that are slightly smaller than those of R. sp. SB347 males (6.7±1.6 µm²) and much smaller than those of *C. elegans* (15.2±2.5 µm²). R. sp. SB372 spermatocytes (Fig. 4B,C) shared many similarities with those of R. sp. SB347. Primary spermatocytes divided symmetrically, and we found no evidence of lagging X chromosomes during anaphase I. Although difficult to see in these smaller spermatocytes, we routinely observed a central, lagging X chromatid during meiosis II (Fig. 4B, yellow arrow) and were able to distinguish the X-bearing pole at later stages by its larger chromatin mass. The meiotic spindle became asymmetric as the X chromatid moved to one pole (Fig. 4B, AII,P). Microtubules from the X-bearing pole subsequently shifted to the RB-sperm boundary during separation (Fig. 4B, S). FB partitioning began in late anaphase II and continued through partitioning (Fig. 4C, P). Ultimately, the microtubules partitioned to the RBs (green arrow) whereas the FBs partitioned to the X-bearing sperm (Fig. 4C, white arrow). We observed similar patterns in the even smaller sperm (4.3±0.6 µm²) of R. sp. JU1783 males (Fig. 4D,E). However, the functional sperm of R. sp. JU1783 males often retained small amounts of α-tubulin, presumably associated with the centrosome (Fig. 4D, Pr), and some males produced a mix of tubulin-enriched cytoplasm both with and without chromatin (green arrows), suggesting the production of some ‘traditional’ RBs without chromatin.

To determine whether unipolar partitioning was characteristic of this entire clade or restricted to trioecious relatives, we also examined spermatocyte partitioning in *Rhabditella axei*, the closest known male/female relative of R. sp. SB347 (Kiontke and Fitch, 2005). As in other male/female nematodes, *R. axei* males have much larger sperm (60.1±7.3 µm²). Furthermore, their spermatocyte divisions yield four functional sperm (Shakes et al., 2011). Immunostained preparations of *R. axei* spermatocytes revealed patterns both similar to and distinct from those in either *C. elegans* or R. sp. SB347 (Fig. 4F). As previously reported (Shakes et al., 2011), the male spermatocytes in *R. axei* exhibit the same X chromosome segregation patterns as in R. sp. SB347 and thus have lagging X chromatids during anaphase II (Fig. 4F, yellow arrow and full-sized DAPI images on right). Yet unlike those in R. sp. SB347, *R. axei* meiotic spindles remained symmetric throughout anaphase II. During the meiotic divisions, FBs distributed uniformly throughout the spermatocytes. FBs began clearing (purple arrow) from the central region, after the completion of anaphase II (P). By the time the chromosome sets had compacted into tight single masses (orange arrow), microtubules had fully reorganized and moved to the RB-sperm boundaries. A unique feature of *R. axei* spermatogenesis is that, although we observed pairs of sperm separating from a central RB (S**, offset DIC image at the bottom of Fig. 4F), the meiosis II cleavage furrow often proceeded to completion, generating two large, polarized sperm that each subsequently generated their own RB (Fig. 4F, S*,Pr). Despite this altered cleavage pattern, the relative timing of polarization events in *R. axei* spermatocytes is the same as in *C. elegans* and R. sp. SB347. Furthermore, these studies confirm that, despite having...
an X chromosome segregation pattern like R. sp. SB347, microtubule reorganization and FB partitioning in these much larger R. axei spermatocytes is bipolar as in C. elegans.

Actin microfilaments reorganize through a combination of cortical ring broadening and clearing from one or both poles
In all of these species, FB-MOs asymmetrically partition postmeiotically as the cells elongate and the microtubules reorganize and move to the RB-sperm boundary. But what forces establish this polarity and direct the movement of these organelles? In R. sp. SB347 male spermatocytes, the late anaphase II spindle asymmetry may help establish the initial polarity, but FB-MO partitioning occurs only later as the microtubules are reorganizing at the X-pole. Furthermore, when we assessed the proximity of mitochondria and FB-MOs to adjacent microtubules in our TEM studies, the distances were too great to be bridged by microtubule motors (data not shown). Alternatively, a key role for actin would be consistent with earlier pharmacological and genetic studies in C. elegans (Nelson et al., 1982; Kelleher et al., 2000). However, few details were known about the step-wise changes in the actin cytoskeleton as nematode spermatocytes progress from anaphase II and through the early post-meiotic partitioning events.

In fixed C. elegans spermatocytes, microfilaments were present around the entire cortex during the meiotic divisions, but an enhanced cortical ring developed during anaphase I and II (Fig. 5A, white arrows). During anaphase II, a defined ring could only be observed when the chromosomes were still fairly close together. As the spermatocytes elongated and transitioned to the post-meiotic partitioning phase (P), the central ring widened into a band (white asterisk). At the same time or shortly thereafter, microfilaments progressively cleared from the poles (orange arrows). By the separation phase, microfilaments were completely restricted to the RB, both at the cortex and within the RB cytoplasm. Separated RBs exhibited actin patches at what we assume are the former sperm attachment sites (green arrows) suggesting a potential role for actin in RB-sperm abscission.

In the larger R. axei spermatocytes, actin patterns were similar but more exaggerated (Fig. 5B). During anaphase II, microfilaments were initially present both at the cortex and in a central cortical ring (white arrows). As the spermatocyte elongated, the central actin ring expanded in the form of a gradient (P), and microfilaments progressively cleared from the poles (orange arrows). As the microfilaments continued to clear from the poles, those within the RB were no longer confined to the cortex but broadly distributed throughout.

R. sp. SB347 spermatocytes exhibited a unipolar version of these same events (Fig. 5C). In metaphase spermatocytes, microfilaments distributed uniformly around the cortex (data not shown), but during anaphase II, they differentially accumulated in a central cortical ring (white arrow). Once the lagging X chromatid fully incorporated into an anaphase plate, microfilaments specifically cleared from the cortex of the X-bearing pole (orange arrows). Throughout the partitioning phase, microfilaments remained at the RB cortex and established a concentrated central band (purple arrows) adjacent to the RB-sperm boundary. In detached RBs, microfilaments distributed throughout the cytoplasm. Although these observations do not directly test whether actin functions in FB partitioning, they are consistent with either actin or actomyosin forces functioning to physically exclude larger organelles from the RB.

DISCUSSION
How nematode spermatocytes generate haploid sperm lacking both actin and tubulin has always been an intriguing cellular phenomenon,
Fig. 6. Conserved and divergent aspects of cytoskeletal reorganization in diverse nematode spermatocytes. Comparative schematic of the differential partitioning events during residual body formation in C. elegans, R. sp. SB347 and R. axei spermatocytes. Actin microfilaments (red); centrosomal (c) or non-centrosomal (nc) microtubules (MT) (green); chromatin (blue); and large organelles (purple). X chromatids have heavy black outline.

second switch is restricted to one pole. In male spermatocytes, the loss of centrosomal MTOC function occurs specifically at the X-pole as the lagging X incorporates into the anaphase plate; yet the same unipolar switch occurs in hermaphrodite spermatocytes which presumably lack a lagging X. In both C. elegans and R. sp. SB347, the centrosomal to non-centrosomal switch correlates with anaphase completion and a key step in sperm differentiation, the remodeling of chromosomes into a single tight chromatin mass. In R. axei, where these events occur sequentially, MTOC reassignment correlates with the later event of chromatin remodeling. In other developmental contexts, asymmetry in centrosome behavior is linked to cell fate. For example, asymmetric MTOC function at the centrosome allows for the selective retention of the daughter centrosome in Drosophila neuroblasts and of the mother centrosome (or spindle pole body) in Drosophila male germ line stem cells, mouse radial glial cells and Saccharomyces cerevisiae bud cells (Yamashita et al., 2007; Wang et al., 2009; Conduit et al., 2010; Januschke et al., 2011; Pereira and Schiebel, 2001). We have yet to determine whether the non-X pole in R. sp. SB347 spermatocytes stereotypically associates with the mother or daughter centrosome, but the maintenance of an active centrosome MTOC within the developing RB suggests a similar link between centrosome asymmetry and cell fate.

Our actin results, showing (1) cortical ring broadening throughout anaphase and (2) actin clearing from the poles as spermatoocytes elongate, also have parallels in other cell types. Efficient metaphase spindle assembly requires uniform cortical rigidity (Matthews et al., 2012) whereas mid-anaphase cell elongation requires relaxation at the poles through the localized loss or remodeling of actin microfilaments (Roubinet et al., 2011; Kunda et al., 2012; Rodrigues et al., 2015). Typically, this remodeling includes a minor reduction in actin microfilaments at the poles and localized deactivation of the actin-plasma membrane linker moesin. In nematode spermatocytes, the clearing of actin from one or both poles coincides with spermocyte elongation and post-anaphase partitioning, but in its exaggerated form, it also provides a mechanism for clearing actin from the sperm.

In other systems, differential clearing of myosin from one pole creates an asymmetry in actin forces that shifts the cleavage furrow and generates an asymmetric cell division (Ou et al., 2010; Connell et al., 2011). In C. elegans spermatoocytes, the combination of clearing of actin from both poles and accumulating actin centrally might create asymmetric actin forces that bi-directionally shift cleavage furrow activity away from the center and towards the two RB-sperm boundaries. Conversely, unipolar clearing in R. sp. SB347 may account for the single, displaced cleavage furrow. At the other extreme, stability rather than regression of the central cleavage furrow may be favored in the larger R. axei spermatoocytes, such that they first cleave in two before the individual sperm secondarily separate from their RB. Future studies might show that a two-step process is typical for larger spermatoocytes. Notably, our results indicate that R. axei spermatoocytes still initiate partitioning immediately after completing anaphase II; only RB-sperm abscission is delayed.

Broadening of the actin cortical ring coupled with localized accumulation of non-cortical microfilaments may also facilitate both RB formation and separation. An expanding band of cortical actin could provide counterbalancing rigidity for spermocyte elongation at the softened poles and support rounding up of the RB into a sphere, the shape of which is largely independent of the cytoplasmically linked sperm. Furthermore, because larger organelles (e.g. FB-MOs and mitochondria) in R. sp. SB347 and R. axei do not partition in association with microtubules, perhaps non-cortical microfilaments within the expanding RBs partition them through exclusion. During Drosophila spermatogenesis, an
actin meshwork functions in this manner. During RB formation and separation, often referred to as individualization, an actin cone moves down the length of the axoneme and an actin meshwork within excludes cytoplasm and organelles from the rest of the sperm (Fabrizio et al., 1998; Noguchi et al., 2006). Our finding that nematode RB formation is associated with post-anaphase II actin remodeling confirms its value as an informative parallel to RB formation in non-nematodes.

In *R. sp. SB347* males, the production of two rather than four functional products from spermatocyte meiosis combined with the irreversible segregation of the X to the functional sperm provides a convenient and evolutionarily useful mechanism for generating a femine-biased sex ratio. Yet this study suggests that an unpaired X during anaphase II is neither sufficient nor necessary for this pattern of division. Despite having an unpaired X chromatid during anaphase II, the large spermatocytes of *R. axei* males yield four functional sperm with Mendelian 50:50 sex ratios. Conversely, the tiny spermatocytes in *R. sp. SB347* XX hermaphrodites only yield two functional sperm, despite presumably having paired X chromosomes in both meiotic divisions. Tiny male spermatocytes in *R. sp. SB347* near relatives typically yield two functional sperm and two DNA-containing RBs. In *R. sp. SB372* males, these unipolar divisions also correlate with skewed sex, feminine-biased sex ratios (Kanzaki et al., 2017), but further studies of the other near relatives are needed to assess both their sex ratios and how often their RBs lack DNA. Collectively, our current data is consistent with the unpaired X in *R. sp. SB347* male spermatocytes merely following the RB-sperm asymmetry, and that the crucial, shared feature of these modified unipolar divisions is not an unpaired X during anaphase II but the diminutive size of the spermatocytes.

What possible evolutionary advantage could be gained by throwing away half of one’s potential sperm? Studies of nematode sperm size in both the genus *Caenorhabditis* and the family Rhabditidae suggest that sperm size is driven by two opposing factors. Larger sperm are more competitive (LaMunyon and Ward, 1999), and thus they are favored when sperm competition between genetically dissimilar males is high, as typically occurs in male/female species. However, the costs of producing larger sperm are that sperm production is slower and fewer sperm can be stored within the spermatheca for subsequent fertilization events (LaMunyon and Ward, 1999; Murray et al., 2011; Vielle et al., 2016). Therefore, small sperm are favored in hermaphroditic species where sperm competition is low and smaller sperm can be produced more quickly and stored in higher numbers (LaMunyon and Ward, 1999; Baldi et al., 2011). Within the family Rhabditidae, the sperm of *R. sp. SB347* and its trioecious near relatives (this study) are the smallest reported to date (LaMunyon and Ward, 1999; Vielle et al., 2016, this study). We hypothesize that evolutionary pressures to reduce sperm size in *R. sp. SB347* may have reached a cellular and development threshold. To function, the motile spermatooza require a minimal stock of mitochondria and cytoplasmic components. Already, the thin shell of cytoplasm surrounding the DNA of *R. sp. SB347* spermatooza seems barely enough to support motility. Furthermore, the developmental program of spermatogenesis requires throwing away materials that could be detrimental for subsequent sperm function. Perhaps in *R. sp. SB347* and its trioecious near relatives, the advantage of rapid sperm production outweighs the cost of throwing away haploid complements of genetic material. If so, these spermatocytes have effectively adopted a standard strategy of oocytes; producing functional sperm of the necessary size at the cost of discarding meiotic products within RBs, the spermatogenesis equivalent of oocyte polar bodies.

**MATERIALS AND METHODS**

**Maintenance and origin of strains**

All nematode strains were maintained on plates of MYOB agar (Church et al., 1995) or NGM agar (Brenner, 1974) seeded with the *Escherichia coli* uracil auxotroph mutant strain OP50. Strains were maintained at 20°C. Strains used for live imaging were J23330 [dmd[6]pie-1;GFP:TBG-1]; islts37[pie-1;hs-24:mCherry]; slts10116[his-72;hs-24:mCherry]; islts4[pie-1;mcCherry-PH (PLC181)]; and J2418 [dmd[6]pie-1;GFP:TBG-1]; zuls278[pie-1; mCherry:tha-1] (Feldman and Priess, 2012). The *C. elegans* strain CB1489 him-8(e1489) and the *Rhabditella axei* strain (DF5006) were obtained from the *Caenorhabditis* Genetics Center. *Rhabditis* sp. SB347 and *Rhabditis* sp. JU1783 were kind gifts from Marie-Anne Félix (Institut de Biologie de l’Ecole Normale Supérieure, Paris, France). *Rhabditis* sp. SB372 was from Karin Kiontke (Department of Biology, New York University, USA). *Rhabditis* sp. SB347 was isolated from a *Drosophila* wild population collected in Brittany in August 2003. JU1783 was sampled in La Réunion, in a star fruit, in Melissa domain, Saint-Benoît, in September 2009.

**Immunohistochemistry and microscopy**

Isolates and antibody labeling of dissected gonads followed established protocols (Shakes et al., 2009). Unless otherwise noted, representative images for the figures were selected from the analysis of spermatocytes from 20-150 male gonads. Primary antibodies included: FITC-conjugated anti-α-tubulin (mouse monoclonal DM1A, Sigma, 1:80), anti-MSI from David Greenstein (Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, USA) (4D5 mouse monoclonal, 1:300; G3197 rabbit polyclonal, 1:15,000), undiluted anti-cyp33-E3 mouse monoclonal (Developmental Studies Hybridoma Bank at the University of Iowa) developed by Hadwiger et al. (2010), anti-GIP-1 (rabbit polyclonal, 1:1000, provided by Anthony Hyman (Hannak et al., 2002), and anti-IFA (mouse monoclonal, 1:100 (Pruis et al., 1981)). Affinity-purified secondary antibodies (Jackson ImmunoResearch Laboratories) (1:100) included goat anti-rabbit TRITC-labeled IgG, DyLight 488-labeled goat anti-mouse IgG, and Alexa 488 anti-goat IgG. Actin microfilaments were stained with rhodamine phalloidin (Molecular Probes). Final slides were mounted with DAPI containing Fluoro Gel II mounting medium (Electron Microscopy Sciences). Images were acquired using an Olympus BX60 microscope using a QImaging EXi Aqua CCD camera. Photos were taken, merged, and exported for analysis using the program iVision. In some cases, the levels adjust function in Adobe Photoshop was used to spread the data containing regions of the image across the full range of tonalities.

Live imaging was performed on a Nikon Ti-E inverted microscope (Nikon Instruments) using a 60× Plan Achromat objective (NA=1.4) and controlled by NIS Elements software (Nikon). Images were acquired with an Andor Ixon Ultra back thinned EM-CCD camera using 491 nm or 561 nm lasers and a Yokogawa X1 confocal spinning disk head equipped with a 1.5× magnifying lens. Images were taken at a z-sampling rate of 0.3 µm and processed in NIS Elements, ImageJ or Adobe Photoshop.

**Enhancing the numbers of *R. sp. SB347* males**

Twelve to fifteen dauer larvae, which inevitably develop into hermaphrodites (Chaudhuri et al., 2011), were picked to 60 mm worm plates and allowed to produce a male-enriched early brood (first 12-24 h of egg laying) before removing the adults. Alternatively, dauers were isolated from densely populated but unsynchronized cultures by washing the worms off the plates with ddH2O, centrifuging the worms, and then treating the worm pellet with 1% w/v SDS in ddH2O for 30 min at room temperature to kill all worms except the resistant dauer stages. After two washes with ddH2O, the surviving dauers were transferred to a fresh plate and then removed after they had produced an early brood.

**High-pressure freezing, electron microscopy and quantitative image analysis**

Three to five males were placed in 1 µl of 20% (w/v) bovine serum albumin in M9 buffer in a hexadecene (Merck)-coated aluminum carrier (cavity 0.1 µm, Art. 241 & 242, Wohlwend, Sennwald, Switzerland). Animals were ultra-
rapidly frozen under high pressure using a Wohlwend HPF Compact 01 (Wohlwend). Freeze-substitution was performed over a period of 3 days at −90°C in anhydrous acetone containing 1% (w/v) OsO4 and 0.1% (w/v) uranyl acetate using an automated freeze substitution machine (EM AFS, Leica Microsystems). Epon/Agarite-infiltrated worms were flat-embedded in a thin layer of resin, polymerized for 3 days at 60°C and mounted on dummy blocks (Müller-Reichert et al., 2007). Serial thin (70 nm) and semi-thick (300 nm) sections were cut using a Reichert Ultracut S microtome (Leica Microsystems), subsequently collected on Formvar-coated copper slot grids and post-stained with 2% (w/v) uranyl acetate in 70% ethanol followed by 0.4% (w/v) lead citrate. Both sides of grids with semi-thick sections were then covered with 15 nm colloidal gold. The meiotic region within the male worms was located and individual meiotic cells within thin sections were recorded with a TEM (Morgagni 286, FEI) operated at 80 kV. Next, serial semi-thick sections were recorded at a magnification of 2156× with a TEM (EM 906, Zeiss) operated at 80 kV. Consecutive images were registered and stacked with Fiji software (Schindelin et al., 2012). Individual cells were cropped out and analyzed section by section with Fiji. For that, the coordinates of each centrosome was exported, as well as the center of each X chromosome. Then, distances between the two centrosomes and between the X chromosome and the future X-bearing pole were calculated. For quantifying organelles, mitochondria and FB-MOs were counted and assigned either to the non-X (zone Z1), the X-pole (Z2), or the region between the poles (zone Z2).

For electron tomography, dual tilt series of serial semi-thick sections were acquired from a thin layer of resin, polymerized for 3 days at 60°C and mounted on dummy blocks (Müller-Reichert et al., 2007). Serial thin (70 nm) and semi-thick (300 nm) sections were cut using a Reichert Ultracut S microtome (Leica Microsystems), subsequently collected on Formvar-coated copper slot grids and post-stained with 2% (w/v) uranyl acetate in 70% ethanol followed by 0.4% (w/v) lead citrate. Both sides of grids with semi-thick sections were then covered with 15 nm colloidal gold. The meiotic region within the male worms was located and individual meiotic cells within thin sections were recorded with a TEM (Morgagni 286, FEI) operated at 80 kV. Next, serial semi-thick sections were recorded at a magnification of 2156× with a TEM (EM 906, Zeiss) operated at 80 kV. Consecutive images were registered and stacked with Fiji software (Schindelin et al., 2012). Individual cells were cropped out and analyzed section by section with Fiji. For that, the coordinates of each centrosome was exported, as well as the center of each X chromosome. Then, distances between the two centrosomes and between the X chromosome and the future X-bearing pole were calculated. For quantifying organelles, mitochondria and FB-MOs were counted and assigned either to the non-X (zone Z1), the X-pole (Z2), or the region between the poles (zone Z2).

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