Lagging X chromatids specify the orientation of asymmetric organelle partitioning in XX spermatocytes of Auanema rhodensis

Talal Al-Yazeedi 1,†, Emily L. Xu 2,†, Jasmin Kaur 1, Diane C. Shakes 1,†, Andre Pires-daSilva 1,†

1School of Life Sciences, University of Warwick, Coventry CV4 7AL, UK, 2Department of Biology, William & Mary, Williamsburg, VA 23187, USA

*Corresponding author: Department of Biology, William & Mary, Williamsburg, VA 23187, USA. Email: andre.pires@warwick.ac.uk (AP-dS)
†These authors are cofirst authors.

Abstract

The unequal partitioning of molecules and organelles during cell division results in daughter cells with different fates. An extreme example is female meiosis, in which consecutive asymmetric cell divisions give rise to 1 large oocyte and 2 small polar bodies with DNA and minimal cytoplasm. Here, we test the hypothesis that during an asymmetric cell division during spermatogenesis of the nematode Auanema rhodensis, the late segregating X chromatids orient the asymmetric partitioning of cytoplasmic components. In previous studies, the secondary spermatocytes of wild-type XO males were found to divide asymmetrically to generate functional spermatids that inherit components necessary for sperm viability and DNA-containing residual bodies that inherit components to be discarded. Here we extend that analysis to 2 novel contexts. First, the isolation and analysis of a strain of mutant XX pseudomales revealed that such animals have highly variable patterns of X-chromatid segregation. The pattern of late segregating X chromatids nevertheless predicted the orientation of organelle partitioning. Second, while wild-type XX hermaphrodites were known to produce both 1X and 2X sperm, here, we show that spermatocytes within specific spermatogonial clusters exhibit 2 different patterns of X-chromatid segregation that correlate with distinct patterns of organelle partitioning. Together this analysis suggests that A. rhodensis has coopted lagging X chromosomes during anaphase II as a mechanism for determining the orientation of organelle partitioning.

Keywords: asymmetric cell division; meiosis; Caenorhabditis elegans; Auanema; MSP; mitochondria; spermatogenesis; nematode; X chromosome

Introduction

The laws of genetics presume that meiotic chromosome segregation is both highly accurate and unbiased. Yet, nonrandom and/or unequal chromosome segregation has been well-documented in multiple species (Pardo-Manuel de Villena and Sapienza 2001; Pajpach et al. 2021; Van Goor, Shakes, et al. 2021). Such deviations from Mendelian expectations are typically associated with oocyte meiosis. During oocyte meiosis, highly asymmetric cell divisions yield a single functional gamete, while the other meiotic products are discarded as diminutive polar bodies (Dalton and Carroll 2013). This asymmetry opens a potential for biased segregation if certain chromosomes can preferentially segregate to the functional oocyte (Pardo-Manuel de Villena and Sapienza 2001; Kruger and Mueller 2021). In instances when biased chromosome segregation involves a sex chromosome, biased segregation can result in subtle or dramatically skewed sex ratios (Jaenike 2001).

Spermatocyte meiosis is not usually associated with this type of biased chromosome segregation since the meiotic divisions of individual spermatocytes typically yield 4 equal-sized gametes. As the heterogametic sex, males produce equal numbers of 2 distinct types of gametes: X- and Y-bearing sperm in the case of humans or 1X and 0X-bearing sperm in the case of most nematodes. For example, in XO Caenorhabditis elegans males, the unpaired X-chromosome lags during meiosis I and then segregates to one of the 2 secondary spermatocytes (Albertson and Thomson 1993; Shakes et al. 2009) (Fig 1a). During meiosis II, the X-bearing secondary spermatocyte divides to form 2 X-bearing sperm, whereas the non-X-bearing spermatocyte divides to form 2 non-X-bearing sperm.

All sperm differentiation programs include a postmeiotic, asymmetric partitioning step during which sperm are streamlined by discarding components that are no longer needed into a residual body (RB) (Breucker et al. 1985; Steinhauer 2015). Furthermore, the underlying cell polarization and scission events in humans (de Kretser et al. 1998), Drosophila (Noguchi et al. 2006; Steinhauer et al. 2019), and C. elegans (Ward et al. 1981; Kelleher et al. 2000; Winter et al. 2017; Hu et al. 2019) all involve common elements such as actin, myosin VI, and microtubules (Zakrzewski et al. 2021). Specific to nematode spermatogenesis, RB formation occurs surprisingly early, immediately after anaphase II (Fig 1a) (Chu and Shakes 2013). Presumably because of this juxtaposition, the partitioning
machinery coopts the anaphase II axis to establish an RB between the 2 spermatids (Fig. 1, a and d) (Winter et al. 2017).

Spermatocyte meiosis in males of the nematode Auanema rhodensis (aka, Rhabditis sp. SB347) (Kanzaki et al. 2017) deviates from this pattern in 3 key ways: (1) during meiosis I, the unpaired X chromosome splits into sister chromatids, (2) during meiosis II, the individual X chromatids segregate to the pole that will become functional sperm, and (3) in the postmeiotic phase, what is
typically a bipolar partitioning process becomes unipolar process (Shakes et al. 2011; Winter et al. 2017; Tandonnet et al. 2018) (Fig. 1, b, d, and e). Thus, meiotically dividing A. rhodensis spermatocytes yield 2 functional 1X sperm and 2 residual bodies containing the non-X-bearing DNA complement (Shakes et al. 2011; Winter et al. 2017). These deviations lead to highly skewed sex ratios in A. rhodensis (Félix 2004; Shakes et al. 2011; Tandonnet et al. 2018), as crosses between XO males and XX females produce mostly XX offspring from the union of 1X oocytes with 1X sperm.

In A. rhodensis, deviations from Mendelian X-chromosome patterns extend beyond male spermatogenesis. XX females follow Mendelian predictions and produce mostly 1X oocytes; however, XX hermaphrodites produce 0X oocytes and mostly 2X sperm (Fig. 1c) (Tandonnet et al. 2018). Importantly, these unusual X-chromosome segregation patterns during hermaphrodite oogenesis and spermatogenesis correlate with an absence of X-chromosome recombination.

Variant modes of X-chromosome segregation may be necessary for XO males to produce exclusively 1X sperm and XX hermaphrodites to produce mostly 2X sperm, but they are not sufficient (Edwards 1910; Winter et al. 2017). Also, critical is the conversion of a bipolar partitioning process into a unipolar process such that half of the genetic material is discarded in residual bodies. We suspect that the diminutive size of A. rhodensis spermatocytes may necessitate halving the number of functional sperm that can be produced with sufficient mitochondria and motility proteins (Winter et al. 2017). Importantly, the almost exclusive production of X-bearing sperm also requires that materials required for sperm function are consistently partitioned to the side with X-bearing chromatin mass.

Based on our previous work, we hypothesized that the late segregating X chromatid(s) in A. rhodensis acts during the second meiotic division to dictate the direction of the asymmetric partitioning process. In this study, we critically tested this hypothesis by examining patterns of X-chromosome behavior and organelle partitioning under 2 exceptional circumstances. First, we isolated males required for sperm function are consistently partitioned to the side with X-bearing chromatin mass.

To isolate virgin APS6 females for crosses, L4 larvae from the first 12–24 h of a hermaphrodite brood were picked to individual plates and allowed to self-fertilize. To simplify screening for mutants that generate high rates of (pseudo)males, we transferred the individual F1s to new plates as 3-day-old adults, to identify individuals who produced large numbers of F2 males. We adopted this procedure because A. rhodensis hermaphrodites of this age produce fewer XO self-offspring (~3%) than younger hermaphrodites (~8%) (Chaudhuri et al. 2015). From late brood plates scored as having potential pseudomales, 10–15 sibling hermaphrodites were isolated to single plates to maintain the mutation as a heterozygous strain (Supplementary Fig. 1). Heterozygous hermaphrodites were selected based on the production of excess male offspring, which is consistent with the anticipated production of 25% male offspring. The mutant strain was backcrossed with the wild-type APS4 strain for 3 generations to remove background mutations generated during the mutagenesis. The A. rhodensis masculinizer was named Arh-mas-1 (brz-3), following the nomenclature described in Wormbase (www.wormbase.org).

**Crosses and brood counts**

To isolate virgin APS6 females for crosses, L4 larvae from the first 12–24 h of a hermaphrodite brood were picked to individual plates. Females were distinguished from their hermaphrodite siblings due to their faster sexual maturation and lack of self-fertilization (Kanzaki et al. 2017).

To determine whether a potential mas-1 pseudomale had 2 X chromosomes, individual APS4-derived pseudomales were crossed to individual APS6 females for 24 h at 20°C. Heterozygous (mas-1/+; XAPS4/XAPS6 F1) hermaphrodites from this cross were allowed to self-fertilize. The resulting F2 male offspring (either XO males or mas-1/ma-1 pseudomales) were analyzed by single worm PCR to determine the genotype of their X chromosome(s).

To quantify the broods of mas-1 males, individual putative APS4 mas-1 males were allowed to mate with individual APS6 verified females for 24 h. After 24 h, the mas-1 male was removed from the plate and dissected to confirm that the males were mas-1/ma-1 pseudomales through the cytological analysis of its gonad (see Immunocytology). Individual mated APS6 females were daily transferred to new plates to assess the nature of their offspring (male, feminine, or Dpy).

**Materials and methods**

For materials, see Table 1.

**Nematodes strains and cultures**

The A. rhodensis inbred strains APS4 and APS6 (Tandonnet et al. 2018) were maintained according to standard conditions for C. elegans, at 20°C (Stiernagle 2006). Nematodes were cultured on NGM plates seeded with either the Escherichia coli strain OP50 or the streptomycin-resistant strain OP50-1.

**Mutagenesis**

A. rhodensis APS4 was mutagenized with the chemical mutagen ethyl methanesulfonate, as previously described (Pires-daSilva and Sommer 2004; Chaudhuri et al. 2011). To screen for a masculinizing phenotype (XX pseudomales), 521 F1 (dauer) hermaphrodites from mutagenized F0s were individually transferred to single plates and allowed to self-fertilize. To simplify screening for mutants that generate high rates of (pseudo)males, we transferred the individual F1s to new plates as 3-day-old adults, to identify individuals who produced large numbers of F2 males. We adopted this procedure because A. rhodensis hermaphrodites of this age produce fewer XO self-offspring (~3%) than younger hermaphrodites (~8%) (Chaudhuri et al. 2015). From late brood plates scored as having potential pseudomales, 10–15 sibling hermaphrodites were isolated to single plates to maintain the mutation as a heterozygous strain (Supplementary Fig. 1). Heterozygous hermaphrodites were selected based on the production of excess male offspring, which is consistent with the anticipated production of 25% male offspring. The mutant strain was backcrossed with the wild-type APS4 strain for 3 generations to remove background mutations generated during the mutagenesis. The A. rhodensis masculinizer was named Arh-mas-1 (brz-3), following the nomenclature described in Wormbase (www.wormbase.org).

**Single nematode genotyping**

Single-nucleotide polymorphisms that distinguish the X chromosomes of the APS4 and APS6 strains (markers 9686 and 12469) were used to determine the origin of the X in XO males and verify that pseudomales were XX animals. These genetic markers together with the primer sequences, restriction enzymes and fragment sizes are detailed in (Tandonnet et al. 2019) and at https://data.mendeley.com/datasets/63d7rrxx28/3#file-16ff094d-6c74-478a-a3f5-8878e89fd72f.

Specimen preparation and antibody labeling followed established protocols (Shakes et al. 2009). Individual gonads were obtained by dissection of individual males (or hermaphrodites) in 5–10 µl of
| Data type (mandatory) duplicate rows as needed. Order is flexible, but row titles must be preserved | Experimental species | Symbol/name used in publication | Source—published (stock center, company, data repository) (one of D, E, F mandatory) | Source—unpublished (description, incl. lab of origin) (one of D, E, F mandatory) | Identifiers (format as ID, source: identifier) | New reagent (mandatory for new entities) | Comments (optional) Genotypes, purpose of reagent, additional information |
|---|---|---|---|---|---|---|---|
| genetic reagent (in whole organism) | *Auanema rhodensis* | *A. rhodensis*, APS4 strain | Warwick University, Pires lab. | Kanzaki et al. (2017) | Mutagenesis was conducted on the APS4 strain to obtain *Arh-mas-1* mutant. *Arh-mas-1* pseudemale was crossed with APS4 female to quantify sex of offspring. Gonad of *Arh-mas-1* identified in the APS4 strain was used for cytological studies. APS6 strain was used as a perantial line for backcrossing experiments with *Arh-mas-1* line. *Arh-mas-1* pseudemales was crossed with APS6 females and genotyping of the progeny elucidate the gametes produced by *Arh-mas-1* pseudemales. | Immunocytology of *Arh-mas-1* sperm spread |
| antibody | NA | FITC-conjugated anti-α-tubulin (mouse) | Sigma Aldrich (DM1A) | Kosinski et al. (2005) | Immunocytology of *Arh-mas-1* sperm spread |
| antibody | NA | G3197 anti-MSP monoclonal (rabbit) | Developmental Studies Hybridoma Bank | Hadwiger et al. (2010) | Immunocytology of *Arh-mas-1* sperm spread |
| antibody | NA | Anti-CYP33E1 | Developmental Studies Hybridoma Bank | Pelisch et al. (2014) | Immunocytology of *Arh-mas-1* sperm spread |
| antibody | NA | Anti-smo-1 (sumo) (mouse) | Developmental Studies Hybridoma Bank | | Immunocytology of *Arh-mas-1* sperm spread |
| antibody | NA | 3D5 anti-ATPB (mouse) | Abcam | | Immunocytology of *Arh-mas-1* sperm spread |
| antibody | NA | Alexa Fluor Plus 555-conjugated goat anti-rabbit IgG (goat) | Invitrogen | | Immunocytology of *Arh-mas-1* sperm spread |
| antibody | NA | Alexa Fluor 488 goat-antimouse IgG (H + L) (goat) | Jackson ImmunoResearch Laboratories | AB_2338840 | | |
| chemical compound, drug | NA | DAPI | Electron Microscopy Sciences | | Immunocytology of *Arh-mas-1* sperm spread |
| Chemical compound, drug | NA | Ethyl methane sulfonate | Sigma Aldrich | Pires-daSilva and Sommer (2004) | M0880 | The chemical was used to mutagenize APS4, *A. rhodensis* strain to identify *Arh-mas-1* mutant |
Edgar’s buffer (Edgar 1995). In most cases, sperm spreads to ana-
lyze detached spermatocytes were cracked in liquid nitrogen and
were fixed overnight in −20°C methanol. However, anti-CYP33E1
samples were fixed in room temperature methanol for 60–
90 min, and anti-ATPB samples were fixed in 4% parafomalde-
hyde and posttreated with Triton-X 100. Primary antibodies in-
cluded: 1:100 FITC-conjugated anti-β-tubulin (DM1A—Sigma); 1:500
G3197 rabbit anti-major sperm protein (MSP) monoclonal
(Kosinski et al. 2005); 1:100 anti-CYP33E1 (Developmental Studies
Hybridaema Bank; Hadwiger et al. 2010); 1:100 mouse anti-SMO-1
(sumo) (Developmental Studies Hybridaema Bank 6F2; Pelisch
et al. 2014); and 1:100 3D5 mouse anti-ATPB (Abcam). All samples
were incubated with primary antibodies for 60–90 min at room
temperature. Affinity-purified secondary antibodies included:
1:400 Alexa Fluor Plus 555-conjugated goat antirabbit IgG (Invitrogen)
and 1:100 Alexa Fluor 488 goat-antimouse IgG (H + L) (Jackson ImmunoResearch Laboratories).

Final slides were mounted with DAPI containing Fluoro Gel II
mounting medium (Electron Microscopy Sciences). Single-focal
plane images were acquired under epifluorescence using an
Olympus BX60 microscope equipped with a QImaging EXi Aqua
CCD camera. Photos were taken, merged, and exported for analy-
sis using the program iVision. The levels adjust function in Adobe
Photoshop was used to spread the data containing regions of the
image across the full range of tonalities.

For the quantification of DNA intensity, sperm spreads were
colabeled with DAPI and anti-MSP antibodies. Spermatids were
chosen for quantification based on their DNA morphology and the
presence of MSP. NIH Imagej was used to determine inte-
grated intensity (Xu 2020). For comparisons between DNA and FB
segregation in partitioning stage mas-1 spermatocytes, asymme-
tries were calculated as the integrated intensity of 1 chromatin
mass (or FB region)/sum of the 2 integrated intensities. The Pearson
correlation coefficient (r) was determined by Excel.

Results

Arh-mas-1 has a male phenotype and XX karyotype
To isolate a masculinizing mutant that would enable us to ana-
lyze X-chromosome segregation and organelle partitioning in the
unique and unusual context of an XX male, we performed chemi-
cal mutagenesis and a genetic screen. From ethyl methanesulfo-
nate mutagenized F0s, we screened for heterozygous F1
hermaphrodites that produced significant numbers (~25%) of
male offspring in the late portion of their broods, rather than the
smaller number of males routinely produced by wild-type her-
maphrodites in the initial portion of their broods (Chaudhuri et al.
2015) (Supplementary Fig. 1). From this screen, we isolated the
sex determination mutant (Arh-mas-1) in which homozygous XX
animals exhibit a male phenotype that is almost indistinguish-
able from XO wild-type males (Fig. 2a). Unlike similar mutants in
other nematode species (Hodgkin and Brenner 1977; Pires-daSilva
and Sommer 2004; Kelleher et al. 2008), Arh-mas-1 pseudomales
do not show signs of partial feminization. Instead, Arh-mas-1
pseudomales have normal male reproductive structures, includ-
ing a single-arm gonad and morphologically normal tail (Fig. 2,
b–d). They also exhibit normal male mating behavior and are fer-
tile when crossed with wild-type females. The molecular charac-
terization of this mutant will be published somewhere else.

In the absence of genetic balancers, we propagated the
recessive mutation through heterozygous hermaphrodites
(Supplementary Fig. 1). In addition, although young adult
Arh-mas-1 XX pseudomales are indistinguishable from their XO
wild-type (or heterozygous) counterparts, older mas-1 pseudomales
display a distinct gut pigmentation pattern, which we used as a
marker to distinguish the 2 karyotypes (Fig. 2a). We do not
know if this gut phenotype is related to the mas-1 mutation or
represents a second tightly linked genetic mutation that was not
eliminated through backcrossing.

To confirm the XX karyotype of pseudomales, we crossed
Arh-mas-1 pseudomales (derived from the APS4 strain) to wild-
type females of the independently isolated APS6 strain. Using
markers for a single-nucleotide polymorphism on the X chromo-
some, we then genotyped F2 self-offspring from F1 hybrid (mas-1/+
, XAPS4/XAPS6) hermaphrodites (Fig. 2e, samples 1–6). Numerous
Arh-mas-1 pseudomales were found to be heterozygous for the
X-chromosome markers (Fig. 2e, samples 4–6), confirming that
these pseudomales have an XX karyotype and that mas-1 maps to
an autosome.

mas-1 males sire offspring using mostly 1X and
some 0X sperm
In genetic crosses with wild-type females, the composition of
offspring sired by wild-type XO males and mas-1 XX males dif-
fered (Fig. 2f). In both cases, most of the offspring were XX,
reflecting the fertilization of 1X oocytes by 1X sperm. However,
mas-1 males sired many more male offspring. As we previously
showed (Tandonnet et al. 2018), wild-type males produce exclu-
sively 1X sperm, and the small number of XO male offspring pro-
duced in male–female crosses originate from rare 0X oocytes
produced in female meiotic divisions. However, only 13/57 (23%)
of XO male offspring sired by mas-1 males inherited the paternal
X (Fig. 2e, sample 6), while the remaining 44/57 (77%) inherited
the maternal X (Fig. 2e, samples 4 and 5). These results indicate
that mas-1 XX pseudomales produce sizable numbers of func-
tional 0X sperm. mas-1 pseudomales also sired small numbers
of Dpy offspring, presumably the product of 2X sperm and analo-
gous to the Dpy XXX animals with dosage compensation defects
that have been well documented in C. elegans (Hodgkin 1979;
Vargas et al. 2017). Notably, in multiple independent brood stud-
ies of mas-1 males, dead embryos indicative of autosomal aneu-
plody were never observed, instead, chromosome variation
appears to be restricted to the X chromosome.

Cytological studies suggest Arh-mas-1 XX pseudomales
produce 0–4X sperm
The finding that XX mas-1 males sire offspring mostly using 1X
and 0X sperm was surprising, as wild-type XX A. rhodensis her-
maphrodites produce mostly 2X sperm (Tandonnet et al. 2018)
(Fig. 1c). To understand how X chromosomes were segregating
within the distinct context of XX mas-1 spermatogenesis, we ex-
amined the cytology of the meiotically dividing spermatocytes.
Although the tools for directly marking the X chromosome are
not available for A. rhodensis, meiotic spermatocytes imaged us-
ing differential interference contrast (DIC) and Hoechst revealed
highly variable patterns of lagging X chromosomes in mas-1 XX
spermatocytes (Fig. 3a). As previously reported and in contrast to
C. elegans males (Fig. 1a), we never observed lagging chromo-
somes during anaphase I in the spermatocytes of wild-type A.
rhodensis males. Instead, the X-chromatid lags during meiosis II
and remains in the center (c) before eventually segregating into
one of the 2 autosome sets (Figs. 1b and 3a) (Shakes et al. 2011).
In the spermatocytes of XX Arh-mas-1 pseudomales, we rarely ob-
served centrally positioned, lagging chromosomes during anap-
-phase I. However, examination of DAPI intensity revealed that

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while 66% ($N = 224$) of the primary spermatocytes segregated DNA symmetrically, 33% ($N = 224$) segregated unequal amounts of DNA to their daughter cells. To increase the chance of documenting a rare lagging chromosome, we imaged fixed cells labeled with an anti-sumo antibody which labels chromosomes in *C. elegans* (Pelisch et al. 2014) and identified rare examples of lagging chromosome during anaphase I (Fig. 3b).

During anaphase II, secondary spermatocytes of *mas-1* pseudomales routinely exhibited a variety of lagging chromosome patterns with 1–4X chromatids segregating either symmetrically (s) or asymmetrically (a) to the 2 autosomal sets (Fig. 3a). Symmetric segregation patterns were most common when anaphase II spermatocytes had 2 X chromatids. This analysis also revealed a subset (~20%, $N = 79$) of *mas-1* spermatocytes during the partitioning stage that formed “classic” bipolar budding figures with 2 budding spermatids flanking a central residual body (cRB). Consistent with the hypothesis that lagging X chromatids specify the orientation of the partitioning process, we found that this bipolar pattern and formation of cRBs was associated with symmetrically partitioning X chromosomes. We suspected that these bipolar figures also form when secondary spermatocytes lacked X chromatids altogether. While this morphology is the norm for most nematode spermatocytes including those of *C. elegans* (Fig. 1a), partitioning stage spermatocytes of wild-type *A. rhodensis* found that males have 6 pairs of autosomes and 1 X chromosome (Shakes et al. 2011; Tandonnet et al. 2018). However, in the spermatocytes of XX pseudomales, we observed some metaphase plates only with autosomes (0X) as well as metaphase plates with 1–4X chromatids. Finding metaphase plates with extra X chromosomes/chromatids corroborated our finding of anaphase II figures with 3 or more X chromosomes. Finding metaphase plates with 6 autosomes and no Xs explains the ability of *mas-1* pseudomales to produce 0X spermatids.

Together with our previous studies of wild-type segregation patterns (Tandonnet et al. 2018), these cytological and brood composition studies of *mas-1* pseudomales suggest potential models of the observed patterns and how sperm with variable numbers of X chromosomes might be produced (Fig. 3e) If the 2 X chromosomes pair and recombine, they should undergo normal Mendelian segregation and produce 1X sperm. We did identify partitioning secondary spermatocytes with the form of a “classic” budding figure and seemingly equal anaphase chromatin masses; however, in the absence of live imaging, we could not determine whether such figures produce 2 viable sperm or ultimately collapse to form a single function sperm (Fig. 3e). In a second scenario, we predicted the 2 X chromosomes would behave like
those previously described for wild-type A. rhodensis hermaphrodites; the X chromosomes would not pair and recombine during meiotic prophase. During meiosis I, they would separate into sister chromatids; and during meiosis II, the 2 nonsister X chromatids would segregate to 1 functional sperm. Alternatively, these X chromatids might segregate symmetrically to the sister spermatids. A third pattern would mimic that of X chromosomes during oogenesis in wild-type A. rhodensis hermaphrodites; all the X chromosomes would segregate to 1 second spermatocyte (or the first meiotic polar body of an oocyte) during meiosis I. This scenario would be a potential source ofOX sperm as well as anaphase II figures with multiple variations on segregating >2X chromatids. Lastly, mas-1 spermatocytes could plausibly segregate unequal numbers of X chromatids to the 2 secondary spermatocytes during anaphase I. This scenario could generate both the observed secondary spermatocytes with a single X chromatid and many additional patterns.

Postmeiotic sperm components cosegregate with the X chromosomes

To test our hypothesis that X chromatids were specifying the orientation of organelle segregation, we used immunocytochemistry to examine the pattern of segregation of cytoplasmic components relative to the X-chromosome segregation in fixed sperm spreads of wild-type and mas-1 males. We chose cytoplasmic components that are essential for postmeiotic sperm (MSP and mitochondria) and others that are discarded into residual bodies (endoplasmic reticulum and \( \alpha \)-tubulin). The MSP drives nematode sperm motility, but within spermatocytes, MSP is contained within organelles called fibrous bodies (FBs) (Smith 2014). Except for mitochondria, the distribution of MSP, \( \alpha \)-tubulin and endoplasmic reticulum (ER) in wild-type XO A. rhodensis spermatocytes has been previously described (Shakes et al. 2011; Winter et al. 2017).

During the first meiotic division of both wild-type XO and XX mas-1 spermatocytes; organelles partitioned equally to both secondary spermatocytes [Supplementary Fig. 2, s]. Notably, organelles segregate symmetrically during the first meiotic division of mas-1 spermatocytes, even when the chromosomes are segregated asymmetrically [Supplementary Fig. 2, a]. In wild-type XO males, secondary spermatocytes undergo a stereotypic series of cytological events which we document again here (Winter et al. 2017) [Fig. 4, a and b]. During early anaphase II, the autosomes segregate to opposite poles, the microtubule spindle remains symmetric, the X chromatid is central, and the organelles are centrally distributed. During late anaphase II, as the X moves to 1 side, the microtubules elongate specifically on that side. Once the X incorporates into the autosomal chromatin mass, organelles needed by the sperm like the MSP-containing FBs partition to the X-bearing side. Then, the centrosome on the X-bearing side deactivates while its microtubules and...
gamma–tubulin complexes move to the boundary between the sperm and the forming RB. Mitochondria (Fig. 4c) follow a similar pattern to that of the FBs. In contrast, the endoplasmic reticulum (Fig. 4d) partitions both later in the process and ultimately to the RB.

In mas-1 XX pseudomales, organelle partitioning within secondary spermatocytes exhibited a more variable pattern (Fig. 4, b–d). During early anaphase II, the spindles were symmetric as was the distribution of FBs (Fig. 4b) and mitochondria (Fig. 4c). However, the central X chromosomes were either stacked on top of each other as had been previously reported in the spermatocytes of wild-type hermaphrodites or spread out centrally in a novel pattern (Fig. 4b). During late anaphase, the organelles remained centrally distributed, regardless of whether the X chromatids were segregating asymmetrically (a) to 1 side or symmetrically (s) to both sides (Fig. 4, b–d). However, the microtubules mirrored the X chromosomes patterns; they became asymmetric when the X chromatids segregated asymmetrically and remained more symmetric when the X chromatids segregated symmetrically.

Fig. 4. X-chromatid segregation patterns largely predict the pattern of microtubule and organelle partitioning in mas-1 spermatocytes. a) Schematic of stage-specific spermatocytes in wild-type A. rhodensis males highlighting the chromosomes (blue), microtubules (green), and FBs (red) adapted from Winter et al. (2017). During early anaphase II (Ana II e), the X chromatid is centered, and the spindle is symmetric. During late anaphase II (Ana II l), the X chromosome segregates to 1 side, and microtubules elongate on that side. During partitioning, the FBs and mitochondria partition to the X-bearing side. The centrosome on the X-side deactivates. ER partitions to the non-X side (residual body side) which retains its original centrosome as noncentrosomal microtubules emanate from the RB-spermatid boundary. Ultimately, the spermatid detaches from the residual body. Spermatocytes from sperm spreads of XO wild-type males (boxed) and XX mas-1 pseudomales stained with DAPI (d) and labeled with antibodies against (b) α-tubulin (T) and the FB marker MSP, (c) the mitochondrial (M) β-subunit of ATP synthase, and (d) the ER-specific antibody CYP33E1 (cytochrome P450 family). Spermatocytes are indicated as having X chromatids that segregate either asymmetrically (a) or symmetrically (s). Images (b)–(d) enlarged 1.5× for visibility. Abbreviations: even (e), uneven (u), small chromatin mass (sm), and large chromatin mass (lg). Numbers under images indicate the number of examples found. Scale bar = 5 μm. e) Scatter plot of a subset of partitioning stage mas-1 spermatocytes in which FBs are partitioning to both sides. Integrated intensities of larger DAPI-stained chromatin mass/sum of the 2 masses plotted relative to the distribution MSP-labeled FBs. 0.5 indicates equal partitioning and 1 indicates partitioning to 1 side. Pearson correlation coefficient = 0.63. f) Quantification of DAPI in MSP-containing spermatids in wild-type and mas-1 males.
During the partitioning stage, we could score chromatin masses as symmetric or asymmetric. However, without the ability to specifically mark the X chromosome, we could not specifically assess how many X chromatids were segregated to each side in mas-1 spermatocytes. Most partitioning stage spermatocytes had asymmetric chromatin masses. In these cases, the FBs and mitochondria partitioned completely or mostly to the larger mass (Fig. 4, b and c). To further explore this relationship, we examined the subset of partitioning stage spermatocytes in which FBs partitioned to both sides and in which both DNA masses and the FBs were in clear focus (N = 36). For these we examined the correlation between asymmetry of the DNA masses (integrated intensity of the larger DAPI-stained chromatin mass/sum of the 2 chromatin masses) and a corresponding measurement of the MSP-labeled FBs on each side (Fig. 4e). Overall, there was a positive (r = 0.63, N = 36) between the measured asymmetries in DNA and FB (MSP) intensities. FBs generally (27/36) but not always (3/36) exhibited biased partitioning to the side with more DNA. Notably, when the DNA masses were symmetric (close to 0.5) reflecting either symmetric X-chromosome segregation or the lack of Xs from a prior anaphase I loss (6/36), FB partitioning appeared both unequal and seemingly random. In contrast to the mitochondria and FB patterns, the ER marker exhibited a variable pattern of either segregating to the smaller chromatin mass as expected or, in an unexpected pattern, to the larger chromatin mass (Fig. 4d). Also, as anticipated, some partitioning stage mas-1 spermatocytes formed bipolar figures with cRBs. In these cells, FBs and mitochondria partitioned symmetrically toward both chromatin masses (Fig. 4, b and c). ER components segregated to the cRB (Fig. 4d), and microtubule foci formed at both sperm-RB boundaries (Fig. 4b). In other cases, when organelles partitioned to both sides (Fig. 4b), the microtubule foci were distinct from either the 1 shifted foci pattern (most) or 2 shifted foci pattern (cRB).

To determine which cellular products of the partitioning stage formed spermatids rather than degrading as residual bodies, we analyzed the DNA content of spermatids that had fully detached from residual bodies and possessed both compacted DNA and MSP. In those cells, the range of DNA intensity and size of those sperms was broader than in wild-type XO males (Fig. 4f), confirming that mas-1 males make sperm with both lower and higher DNA content than wild-type males, differences which presumably reflect variable numbers of X chromatids.

**In wild-type XX hermaphrodites, rare 1X sperm originate from specific spermatogonial clusters and distinctive patterns of X chromosome and organelle partitioning**

Self-fertilizing wild-type *A. rhodensis* hermaphrodites produce XO male offspring in their early brood (Chaudhuri et al. 2015; Tandonnet et al. 2018; Supplementary Fig. 1), but the cytological underpinnings of how rare 1X hermaphrodite sperm form has not been previously documented. We hypothesized that the formation of rare 1X sperm would be associated with distinct spermatogonial clusters that enable *A. rhodensis* hermaphrodites to produce sperm throughout adulthood (McCaig et al. 2017). This mode of gonad organization means that the same stage, same spermatogonial cluster-derived spermatocytes would be physically clustered together unless separated during the process of gonad dissection (Fig. 5A—early anaphase II). By analyzing hermaphrodites that were either young adults or in the preceding molting stage, we maximized our chance of capturing at least some anaphase II or partitioning stage spermatocytes that were fated to generate 1X rather than 2X sperm. In fixed samples co-labeled with DAPI, antitubulin, and anti-MSP, anaphase I and early anaphase II patterns were as previously reported (Fig 5A). Chromosome segregation during anaphase I was symmetric and lagging chromosomes were not observed. During early anaphase II, 2 X chromatids typically lagged in the center and appeared to be stacked on top of each other, suggesting they were independently attached to the spindle. We also observed rare patterns of chromosome segregation in late anaphase II in which the Xs segregated symmetrically (s) to the 2 poles rather than the typical pattern of segregating asymmetrically (a) to 1 pole. During the partitioning stage, rare spermatocytes with symmetric (s) chromosome segregation exhibited bipolar budding figures and organelles partitioning toward both poles; although within the same spermatogonial cluster, we often observed cells that appeared to have resolved into a unipolar pattern as proposed in Fig. 3d. In spermatocytes with the asymmetric (a) pattern of both X chromatids segregating to 1 pole, organelles segregated completely (ai) or mostly (aii) to the 2X side. Parallel studies using anti-sperm antibodies to label chromosome and actin-patterns (Fig. S8), confirmed these results. Spermatocytes from symmetric clusters (a, b) have X chromatids segregating to both poles and equal-sized chromatin masses (c, d) during the partitioning stage, whereas in asymmetric clusters (f, g), both X chromatids segregate to 1 pole and the partitioning stage chromatin masses are uneven. These results not only reveal how variations in anaphase II chromosome segregation patterns enable XX hermaphrodites to produce either 1X or 2X sperm, but they also show that organelle partitioning orients toward the late segregating X chromosomes.

**Discussion**

The initial observation of a few male offspring derived from *Auaena* male and female crosses revealed male spermatocytes undergo a modified spermatogenesis resulting in functional X-bearing sperm and residual bodies with the non-X genetic complement (Shakes et al. 2011; Winter et al. 2017). This meiotic variation generates biased sex ratios, which provides adaptive advantages in certain ecological circumstances (Van Goor, Herre, et al. 2022). In the present study, we examined X-chromosome behavior and organelle partitioning in 2 exceptional conditions: XX mas-1 pseudomales and young wild-type hermaphrodites which can make both 1X and 2X sperm. Our study resulted in 3 key findings: (1) in the abnormal context of XX male spermatogenesis which has not been subjected to evolutionarily adaptation, patterns of X-chromosome segregation were even more variable, (2) during wild-type hermaphrodite spermatogenesis, 1X and 2X sperm are produced in distinct spermatogonial clusters and arise from 2 different patterns of X-chromatid segregation during anaphase II, and (3) even with the additional variations, the patterns of lagging X-chromatid segregation during anaphase II largely predicted the orientation of organelle partitioning. However, when X chromatids segregated symmetrically or were potentially absent, FBs often partitioned to both sides in less predictable patterns. Both these findings are consistent with our hypothesis that X chromatids serve a directive role in orienting the direction of organelle partitioning.

Identification and isolation of the sex determination mutant mas-1 allowed us to study patterns of X-chromosome segregation in the nonadapted context of an XX male. Since the pseudomales were fertile, analysis of the sired offspring enabled us to assess the composition of the functional/competitive sperm as ¼ 1X, ¼ 2X.
0X, and a small number of 2X sperm (Fig. 2f). However, our cyto-
logical analysis of both spermatocytes and mature spermatids
from isolated pseudomale gonads indicated a much higher frac-
tion of 2–4X sperm were being produced. This result suggested
that many of these sperm were either nonfunctional or noncom-
petitive and thus failed to contribute to the production of either
viable offspring or dead embryos. Some of this discrepancy can
be explained by the likely overrepresentation in fixed sperm
spreads of late anaphase figures with multiple X chromatids,
since the presence of extra and odd numbers of chromatids pre-
sumably prolongs anaphase II. Conversely, secondary spermato-
cytes without X chromatids were underrepresented as the
absence of lagging X presumably shortens anaphase II.

Prior to this study, we knew that, in A. rhodensis, spermatocyte
meiosis yielded 2 rather than 4 functional sperm and that organ-
elles required for sperm function partitioned to X-bearing chro-
matin side; the side with the single X chromatid in male
spermatocytes or the 2 nonsister X chromatids in hermaphrodite
spermatocytes (Shakes et al. 2011; Tandonnet et al. 2018). The par-
titioning process itself is a universal part of RB formation, and its
relationship to the anaphase II axis is a feature of nematode sper-
matogenesis. However, the conversion of a bipolar to a unipolar
process is unique either to Auanema or potentially to nematode
species with highly diminutive spermatocytes. This study
addressed whether, as we studied additional variations in ana-
phase II X-chromatid segregation patterns, would we continue to
see a correlation between the pattern of lagging X chromatids
and the orientation of organelle partitioning. With the important
limitations that we lacked the tools to either directly mark the X
chromosome or live image organelle partitioning through time,
essential organelles partitioned toward the X chromatids in both
the spermatocytes of XX pseudomales and in early spermato-
cytes of wild-type hermaphrodites. When all the Xs segregated to
1 side, FBs and mitochondria typically did as well. When Xs segre-
gated symmetrically or may not have been present at all, FBs and
mitochondria are often partitioned to both sides but unequally

Fig. 5. Two patterns of spermatocyte divisions in wild-type hermaphrodites. A) Staged spermatocytes from wild-type XX A. rhodensis hermaphrodites
stained with DAPI (D) and labeled with antibodies against α-tubulin (T) and the FB marker MSP (M) Spermatocytes are indicated as having X chromatids
that segregate either symmetrically (s) or asymmetrically (a). In partitioning spermatocytes with asymmetric DNA masses, either all (i) or the majority
(ii) of the FBs partitioned to the larger chromatin mass. B) Spermatocytes clusters labeled with anti-sumo antibody, anaphase II spermatocytes. Scale
bar = 5 μm. In symmetric clusters (a, b) the X chromatids segregate to both poles in equal-sized chromatin masses (c, d) during the partitioning stage. In
asymmetric clusters (f, g), both X chromatids segregate to 1 pole and the partitioning stage chromatin masses are uneven.
and unpredictably. Additional technical limitations of our studies included using DAPI fluorescence as a proxy for DNA amounts when differential DNA packing could impact DAPI binding and performing the analysis of single-focal plane images, which included most but perhaps not all of the full DNA mass and likely not all of the FBs. Despite these technical limitations, these studies are the first to document bipolar budding figures in *A. rhodensis*, which were observed to accompany symmetric X-chromatid segregation in both *mas-1* spermatocytes and early wild-type hermaphrodite spermatocytes. Still unknown is whether the observed bipolar budding figures ever yield 2 functional sperm or whether they must secondarily restructure to form a single functional sperm. Presumably, sperm require a minimal number of mitochondria to power the sperm and enough MSP and cytoplasm to form a pseudopod that can support motility and traction within the female reproductive tract.

A notable exception to our finding that proportional DNA mass correlates with the direction of organelle partitioning was in 27% (*N* = 255) of partitioning stage *mas-1* spermatocytes, ER markers partitioned “incorrectly” toward the larger chromatin mass, a situation which may compromise normal sperm function. One potential explanation is that ER partitioning is known to occur late in the partitioning process and correlates with microtubules moving to the RB ([Winter et al. 2017](https://doi.org/10.1093/genetics/iyaa009)). Although we did not directly study the relationship between ER and microtubule patterns, we did document unusual microtubule patterns in partitioning stage *mas-1* spermatocytes that differ from either the unipolar or bipolar reorganization of a microtubule organizing center to the spermatid-RB boundary (Fig. 4b). Although *mas-1* pseudomales are fertile, defects in organelle partitioning may contribute to a subset of their sperm being fertilization incompetent. If inappropriately partitioned ER is a proxy for the RB material, inappropriate amounts of RB material could be both detrimental and/or trigger apoptosis ([Huang et al. 2012](https://doi.org/10.1016/j.celrep.2012.07.007)).

The finding that most *mas-1* offspring are the product of 1X and 0X sperm combined with the observed cytology suggests that spermatocytes in *mas-1* XX males possess the full range of *A. rhodensis* genetic “tricks.” As occurs in XX hermaphrodite oocyte meiosis ([Tandonnet et al. 2018](https://doi.org/10.1016/j.molcel.2018.04.008)), the 2 Xs can segregate into one of the 2 secondary spermatocytes during anaphase I, and we suspect that the resulting secondary spermatocytes without Xs are the most likely source of 0X sperm. Conversely, 1X sperm that accounts for most of the progeny sired by *mas-1* pseudomales could potentially arise from either standard Mendelian patterns as occurs in XX female oocyte meiosis or from the symmetric segregation of nonsister X chromatids during anaphase II that we documented for the first time in rare, early spermatocytes of wild-type hermaphrodites. In a novel pattern, spermatocytes of *mas-1* pseudomales exhibit diverse patterns of X-chromatid segregation during anaphase II, reflecting both differing numbers of X chromatids and differential associations (e.g. stacked X chromatids vs singlets distributed along the spindle axis). These anaphase II variations presumably reflect stochastic variations in meiotic spindle dynamics and seem nonadapted, as the numerous sperm with 2 or more X observed cytologically are not represented in the broods sired by *mas-1* males. In future studies, it will be interesting to determine exactly how these X chromatids are attached to the meiotic spindle, given the novel features of lagging X-chromosome segregation mechanisms recently described in *C. elegans* males ([Fabig et al. 2020](https://doi.org/10.1016/j.cell.2020.09.002)). In addition, now that we have determined that 1X sperm in wild-type *A. rhodensis* hermaphrodites are generated within specific spermatogonial clusters, it will be interesting to explore whether the numbers of these rare clusters increase adaptively in response to specific environmental conditions.

This study represents an important next step in the analysis of this fascinating genetic system in which noncanonical patterns of X-chromatid segregation support sex ratios that are highly biased against males. The coupling of postmeiotic organelle partitioning to the pattern of late segregating X chromatids in *A. rhodensis* then allows for the efficient conversion of a normally bipolar partitioning process into a unipolar process. At the same time, the establishment of this linkage appears to have compromised the efficiency of organelle partitioning in the context of either the *mas-1*-specific incidences of having no X chromatids or during symmetric X-chromosome segregation, which in wild-type animals is restricted to a rare subset of hermaphrodite spermatogonial clusters.

**Data availability**

Strains are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

**Supplemental material** is available at GENETICS online.

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**Conflicts of interest**

None declared.

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