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Non-muscle myosin IIB is essential for cytokinesis during male meiotic cell divisions

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Abstract

Cytokinesis, the final stage of cell division, bisects the cytoplasm into two daughter cells. In mitotic cells, this process depends on the activity of non-muscle myosin II (NMII), a family of actin-binding motor-proteins that participate in the formation of the cleavage furrow. The relevance of NMII for meiotic cell division, however, is poorly understood. The NMII family consists of three members, NMIIA, NMIIB, and NMIIC, containing different myosin heavy chains (MYH9, MYH10, and MYH14, respectively). We find that a single non-muscle myosin II, NMIIB, is required for meiotic cytokinesis in male but not female mice. Specifically, NMIIB-deficient spermatocytes exhibit cytokinetic failure in meiosis I, resulting in bi-nucleated secondary spermatocytes. Additionally, cytokinetic failure at meiosis II gives rise to bi-nucleated or even tetra-nucleated spermatids. These multi-nucleated spermatids fail to undergo normal differentiation, leading to male infertility. In spite of the presence of multiple non-muscle myosin II isoforms, we demonstrate that a single member, NMIIB, plays an essential and non-redundant role in cytokinesis during meiotic cell divisions of the male germline.

Keywords

Spermatogenesis; Meiosis; Cytokinesis; NMIIB; MYH10; Mouse

Introduction

Cytokinesis, the final step of cell division in all animal cells, partitions the cytoplasm into two separate daughter cells. This process is regulated and executed by molecular machines that have been well characterized in eukaryotic cells (Glotzer, 2005). Attachment of a contractile ring, consisting of a network of actin and myosin filaments, to the cytoplasmic membrane induces the formation of a cleavage furrow that partitions the cytoplasm into two lobes. The assembly and constriction of the contractile ring is regulated by the RhoA GTPase, which stimulates actin polymerization and induces myosin activity. The final abscission requires cytoplasmic membrane fusion at the cleavage sites, involving the membrane vesicle trafficking system. The molecular mechanisms of cytokinesis have

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Appendix A. Supporting information

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mainly been identified from studies on mitotic cell division. Genetic screens in *Drosophila* have begun to dissect the role of various proteins for cytokinesis during meiosis, a cell division mechanism unique to germ cells (Giansanti et al., 2001,2004). However, genetic studies of cytokinesis in mammalian meiosis are lacking, possibly hampered by the developmental lethality of mutants exhibiting cytokinetic failure in somatic tissues.

Unlike somatic cells that exhibit complete abscission, dividing germ cells of most organisms undergo incomplete cytokinesis and remain interconnected by cytoplasmic connections that serve various functions. In *Drosophila*, stable ring canals allow transfer of essential factors from associated nurse cells into the transcriptionally inactive oocyte (Mahowald, 1971). In the mammalian male germ line, all germ cells derived from a single spermatogonial stem cell remain connected by intercellular cytoplasmic bridges that allow passing of factors essential for germ cell synchronization (Dym and Fawcett, 1971; Greenbaum et al., 2011; Huckins, 1971). In addition to ubiquitous cytokinesis factors of the centralspindlin complex, these intercellular bridges also contain germ-cell specific factors. TEX14 was the first of these factors found to be essential for germ cell development (Greenbaum et al., 2006). TEX14 interacts with the centrosomal protein CEP55 to block cell abscission, the final step of cytokinesis, such that stable intercellular bridges remain between germ cells (Iwamori et al., 2010). In the absence of TEX 14, the formation of intercellular bridges is disrupted, and males become sterile due to meiotic arrest before the first meiotic cell division. Other components of intercellular bridges in germ cells include KIF23 and RBM44 (Greenbaum et al., 2007, 2009; Iwamori et al., 2011). Unlike TEX14, RBM44 is neither required for the formation of intercellular bridges nor for fertility (Iwamori et al., 2011). Citron kinase (CIT), a downstream effector molecule of Rho, localizes to the cleavage furrow and the midbody (Madaule et al., 1998). Although CIT is widely expressed, its ablation in mice causes cytokinetic failure in spermatogonia (mitotic germ cells) and proliferating neurons only, but does not affect the division of other cell types (Di Cunto et al., 2000, 2002).

In mammals, meiotic cytokinesis in germ cells is sexually dimorphic in respect to timing and symmetry. In the male germ line, cytokinesis begins with puberty and results in symmetric divisions. Cytokinesis of primary spermatocytes (during meiosis I) generates two secondary spermatocytes of equal size, which again divide symmetrically, such that each primary spermatocyte ultimately gives rise to four haploid round spermatids of equal size. In females, meiosis is initiated during embryogenesis, however, primary oocytes become arrested at the prophase of meiosis I around birth and only resume meiosis I upon ovulation, with highly asymmetrical cytokinesis that produces a large secondary oocyte and a small polar body almost devoid of cytoplasm. Only the secondary oocyte proceeds with the second meiotic division and becomes arrested at the metaphase stage of meiosis II. Only upon fertilization, the secondary oocyte resumes meiosis II and cytokinesis is again highly asymmetric, producing a large ovum and a small second polar body.

Cytokinesis involves a set of approximately 20 proteins that are evolutionarily conserved in diverse organisms including *Drosophila*, *C. elegans*, and vertebrates (Glotzer, 2005). Among these factors, non-muscle myosin II is a central component of the contractile ring that provides the motor activity for contraction (Mabuchi and Okuno, 1977; Straight et al., 2003). Non-muscle myosin II is essential for cytokinesis during mitosis. Injection of myosin II antibodies blocks cleavage of blastomeres in starfish (Mabuchi and Okuno, 1977). Blebbistatin, a specific inhibitor of non-muscle myosin II, inhibits the closure of the cleavage furrow in cultured cells by inhibiting non-muscle myosin II when it is in the detached state from actin (Kovacs et al., 2004; Straight et al., 2003). Found in all eukaryotic cells (Vicente-Manzanares et al., 2009), myosin II is a hexamer composed of a dimer of myosin heavy chains (MYH), a pair of essential light chains, and a pair of regulatory light chains. Non-muscle myosin II assembles to form bipolar myosin filaments and exert tension

on actin filaments to initiate cytokinesis (Ma et al., 2012; Zhang and Robinson, 2005). In mammals, three different genes encode the non-muscle myosin heavy chain proteins MYH9, MYH10, and MYH14, which, in combination with common light chains, form the non-muscle myosin II isoforms NMIIA, NMIIB, and NMIIC, respectively (Vicente-Manzanares et al., 2009). While MYH9 and MYH10 share 80% amino acid identity, they both share 64% amino acid identity with MYH14 (Golomb et al., 2004). These three *Myh* genes have been studied by targeted gene inactivation. While mice lacking MYH14 are viable and display no obvious abnormalities (Ma et al., 2010), inactivation of MYH9 or MYH10 causes embryonic lethality (Conti et al., 2004; Tullio et al., 1997). MYH9-deficient embryos die by E7.5 (Conti et al., 2004). Inactivation of MYH10 causes embryonic lethality relatively late during gestation (between E14.5 and birth), and leads to cytokinetic failure in cardiac myocytes (Takeda et al., 2003; Tullio et al., 1997).

In mouse meiotic germ cells, MYH10 localizes to the contractile region of testicular spermatocytes (Manandhar et al., 2000). Although both MYH9 and MYH10 are expressed in oocytes, meiotic cell divisions are unaffected by microinjection of anti-MYH9 and/or anti-MYH10 antibodies into oocytes, leaving the functional requirement of non-muscle myosin II in meiosis unknown (Simerly et al., 1998). Here, we report the functional characterization of MYH10 in mouse germ cells and demonstrate that, in male mice, MYH10 is required for cytokinesis during meiosis I and meiosis II.

Materials and methods

Mice

Mice bearing the conditional *Myh10^{f1}* allele, in which exon 2 is flanked with loxP sites, were generated previously (Ma et al., 2009) and are available from the Mutant Mouse Regional Resource Centers (Stock number: 016981-UNC; Allele symbol: *Myh10^{m7R_{sad}}*). *Ddx4-Cre* and *Hspa2-Cre* mice were obtained from the Jackson Laboratory (Stock numbers: *Ddx4-Cre*, 006954; *Hspa2-Cre*, 008870) (Gallardo et al., 2007; Inselman et al., 2010). Fig. S1 details the breeding strategy that relied on exclusively paternal transmission of the *Ddx4-Cre* allele. *Ddx4-Cre* is expressed in oocytes, such that its transmission through the female germline would cause recombination of all floxed alleles, including those from the sperm, at the zygote stage (Gallardo et al., 2007). Genotyping for *Myh10* and Cre alleles was performed separately on genomic DNA isolated from tails. Mice were maintained and used for experimentation according to the guidelines of the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Western blotting analyses

Adult testes or ovaries from 2-month-old mice were homogenized in SDS-PAGE sample buffer using a glass homogenizer. 30 µg of protein lysates were used for gel electrophoresis. Western blotting was performed with the following antibodies: anti-MYH9 (1:500; Sigma-Aldrich), anti-MYH10 (1:1000, Sigma-Aldrich), anti-MYH11 (1:500; Abcam), and anti-β-actin (1:5000; Sigma-Aldrich).

Histology, electron microscopy (EM), and immunofluorescence

For histology, testes and epididymis were fixed in Bouin's solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. EM of testes (fixed in 2.5% glutaraldehyde and 2% paraformaldehyde) was performed at the Biomedical Imaging Core facility at the University of Pennsylvania, as previously described (Yang et al., 2006). For immunofluorescence, testicular cells were fixed in 4% paraformaldehyde and stained with anti-β-tubulin (DSHB) and anti-ACRV1 antibodies (gift from P.P. Reddi, University of Virginia, Charlottesville, VA). Immunostaining with anti-ACRV1 or anti-TEX14 antibodies

(gift from M.M. Matzuk, Baylor College of Medicine, Houston, TX) was also performed on 8- μ m cryosections of testes that were fixed in 4% paraformaldehyde overnight, dehydrated in 30% sucrose solution, embedded with TBS tissue freezing medium, and frozen in ethanol/dry ice.

Measurement of DNA content

After dissection of cauda epididymides, cells were squeezed out of the tubules using forceps, fixed in 4% paraformaldehyde, adhered to glass slides, and stained with DAPI in antifade mounting medium (Vector Laboratories). Imaging was performed on an Axioskop 40 microscope (Carl Zeiss, Inc.) with a digital camera (Evolution QEi; MediaCybernetics). The DNA content in each cell was measured by quantifying the total DAPI signal using ImagePro software (Phase 3 Imaging Systems).

Results and discussion

MYH10 is required for fertility in males but not females

As germline ablation (ubiquitous deletion) of *Myh10* in mice causes embryonic lethality between E14.5 and birth (Takeda et al., 2003), we evaluated the function of *Myh10* in male and female germ cells by intercrossing mice bearing a conditional *Myh10* allele (*Myh10^{fl}*) with mice expressing Cre recombinase under the control of the germ cell-specific *Ddx4* promoter through a two-generation breeding scheme (Fig. S1) (Gallardo et al., 2007; Ma et al., 2009). The conditional *Myh10^{fl}* allele contains two loxP sites flanking exon 2, such that Cre-mediated recombination results in a frame shift upon deletion of this exon (Ma et al., 2009). *Ddx4*-Cre expression is restricted to the germline lineage and begins at embryonic day 15 (E15) in both testis and ovary (Fig. 1a) (Gallardo et al., 2007). MYH10 was abundantly detected in wild type testes but only at very low levels in XX^{Y*} testes, which are completely devoid of germ cells, showing that MYH10 is predominantly expressed in germ cells (Fig. 1b). Western blot analysis confirmed efficient ablation of MYH10 in the testes of *Myh10^{fl/-}* *Ddx4*-Cre mice (Fig. 1b).

Myh10^{fl/-} *Ddx4*-Cre mice were viable without overt defects, but exhibited a sexually dimorphic fertility phenotype. *Myh10^{fl/-}* *Ddx4*-Cre males were sterile, whereas *Myh10^{fl/-}* *Ddx4*-Cre females were fertile. All 15 pups from *Myh10^{fl/-}* *Ddx4*-Cre females sired by wild type males were of *Myh10^{+/-}* genotype, suggesting that Cre-mediated ablation of *Myh10* in oocytes is complete. However, as both MYH9 and MYH10 are expressed in oocytes (Simerly et al., 1998), MYH9 may compensate for MYH10 function in the oocytes of *Myh10^{fl/-}* *Ddx4*-Cre females. In the testis, MYH9 is abundantly expressed in somatic cells, but mostly absent from germ cells, demonstrated by high levels of expression in germ-cell deficient XX^{Y*} testes, and very low levels in normal or *Myh10* mutant testes (Fig. 1b). Therefore, absence of MYH10 is probably not compensated for by MYH9 in the male germ cells of *Myh10^{fl/-}* *Ddx4*-Cre mice. In conclusion, these genetic studies demonstrate that MYH10 is required for male fertility.

MYH10 is essential for cytokinesis at meiosis I and meiosis II

Germ-cell specific inactivation of *Myh10* resulted in sharply reduced testis size. Testes from 2 to 3-mo-old *Myh10^{fl/-}* *Ddx4*-Cre mice weighed significantly less than those from age-matched *Myh10^{+/-}* mice (122.2 ± 46.2 mg/pair versus 200.0 ± 15.7 mg/pair; $n = 7$, $P < 0.0012$). Seminiferous tubules of testes from adult mice typically contain a full spectrum of spermatogenic cells including spermatogonia, spermatocytes, and spermatids (Fig. 1c). In contrast, seminiferous tubules from adult *Myh10^{fl/-}* *Ddx4*-Cre mice contained early germ cells, including spermatogonia and spermatocytes, but lacked mature spermatozoa, suggesting spermatogenic arrest (Fig. 1d and Fig. S2). In wild type seminiferous tubules,

round spermatids contain a single nucleus per cell (Fig. 1c and Fig. S2a). Remarkably, MYH10-deficient seminiferous tubules contained many multi-nucleated cells with either two or four nuclei. The size of the nuclei of binucleated cells was either comparable to that of nuclei of round spermatids, or to that of secondary spermatocytes (Fig. 1d; Fig. S2b and Fig. S3). Consistent with the testicular histology, epididymal tubules from adult *Myh10^{fl/-} Ddx4-Cre* mice lacked sperm but were filled with multi-nucleated round cells, including “tetrad” round cells with four nuclei (Fig. 1f).

To further characterize these cells, we ascertained whether “tetrad” round cells, like haploid round spermatids, would undergo spermiogenesis, including flagellum assembly, nuclear elongation, and acrosome formation (Fig. 2a). Multi-nucleated cells contained abnormal acrosomal structures (Fig. 2b and c), and each cell assembled multiple axonemes (flagella), which were arranged in a “pony tail” (Fig. 2b) or a “bouquet” fashion (Fig. 2c). Most multi-nucleated cells did not undergo nuclear elongation (Fig. 2c). We next performed ultrastructural analysis of testis sections to visualize the acrosome and the chromatoid body, a prominent electron-dense multi-lobular nuage located in the perinuclear space of wild type round spermatids (Fig. 2d) (Fawcett et al., 1970). Multi-nucleated germ cells from adult *Myh10^{fl/-} Ddx4-Cre* testes contained a large chromatoid body per cell and abnormal acrosomal structures (Fig. 2e). A single cell membrane encompassed the nuclei of multi-nucleated cells, illustrating that these cells differ from normal spermatids with surrounding membranes that are only connected by electron-dense cytoplasmic bridges (Fig. 2e). These findings suggest that the multi-nucleated round cells adopt features of spermatids, and hence result from cytokinetic failure during meiosis I of male germ cell formation.

To determine whether MYH10 plays a role also during cytokinesis of male meiosis II, we characterized germ cells of *Myh10^{fl/-} Ddx4-Cre* mice that had been sloughed off the testicular epithelium. Based on nuclear morphology, number of nuclei, and DNA content, the epididymal cells of MYH10-deficient testes could be grouped into eight categories (Fig. 3a). The majority of epididymal cells were germ cells, with the exception of cells with one large nucleus (Category F, Fig. 3a), which could also represent epididymal somatic cells. Consistent with our histological data (Fig. 1d and Fig. S3), a subset of cells contained four nuclei (Category A, Fig. 3a; 6% of all cells). We also detected cells with two large nuclei (category C) and cells with two small nuclei (category D). Cells with two large nuclei (category C) contained twice as much DNA as cells with two small nuclei (Fig. 3b), suggesting that category C cells contain two secondary spermatocyte nuclei as a result of cytokinetic failure in meiosis I (Fig. 1a). The DNA content of cells with two small nuclei (category D, Fig. 3b) was twice that of round spermatids (category G), indicating failure of cytokinesis during meiosis II in the absence of MYH10 (Fig. 1a). Intriguingly, a significant fraction (12%) of cells contained three nuclei (category B, Fig. 3a), suggesting that cytokinetic failure during meiosis I may often be followed by loss of one haploid nucleus during meiosis II. Such tri-nucleated spermatids have also been observed in *Drosophila* male meiotic mutants (Giansanti et al., 2004). Taken together, our results demonstrate that ablation of MYH 10 in male germ cells severely impairs cytokinesis during both meiosis I and meiosis II.

MYH10 is not required for cytokinesis of mitotic male germ cells (spermatogonia)

Spermatogenesis in *Myh10^{fl/-} Ddx4-Cre* mice advanced to a late stage of meiosis, and seminiferous tubules did not exhibit detectable depletion of early germ cells, suggesting that cytokinesis during the preceding mitotic stages of germ cell development may not be impaired. To examine whether cytokinesis in mitotically dividing male germ cells occurred normally in the absence of MYH10, we visualized cytoplasmic bridges that connect dividing spermatogonia by immunostaining with anti-TEX14 antibodies (Fig. S4). TEX14 localizes to the intercellular cytoplasmic bridges in wild type germ cells (Fig. S4a) and is required for

their formation (Greenbaum et al., 2006). In sections of postnatal day 10 testes and adult testes from *Myh10^{fl/-} Ddx4-Cre* mice, cytoplasmic bridges were present between type A spermatogonia (Fig. S4b), between type B spermatogonia (Fig. S4c), and between preleptotene spermatocytes (Fig. S4d). Normal formation of cytoplasmic bridges between early spermatocytes and the absence of multi-nucleated spermatogonia in testes from *Myh10^{fl/-} Ddx4-Cre* mice indicate that MYH10 is not essential for cytokinesis during mitotic divisions of spermatogonia.

Meiosis-specific ablation of MYH10 causes cytokinetic failures in spermatocytes

In *Myh10^{fl/-} Ddx4-Cre* male mice, disruption of *Myh10* begins in embryonic germ cells, prior to meiosis (Fig. 1a). We next examined the consequence of inactivation of *Myh10* specifically during meiosis, by intercrossing *Myh10^{fl/fl}* mice with *Myh10^{fl/+} Hspa2-Cre* mice, in which the onset of Cre expression occurs in leptotene/zygotene spermatocytes (Fig. 1a and Fig. S1) (Inselman et al., 2010). In testes from adult *Myh10^{fl/-} Hspa2-Cre* mice, MYH10 protein was severely depleted but remained detectable at a low level (Fig. 4a). Testes from adult *Myh10^{fl/-} Hspa2-Cre* mice weighed 19% less than those from controls (Fig. 4b), and sperm count in the cauda epididymides of mutant mice was reduced by nearly 50% (Fig. 4c). Seminiferous tubules of *Myh10^{fl/-} Hspa2-Cre* mice (Fig. 4d) contained many bi-nucleated germ cells, including germ cells with two large nuclei, which may be equivalent to secondary spermatocytes, and germ cells with two small nuclei, which may be equivalent to round spermatids. In wild type seminiferous tubules, bi-nucleated spermatocytes or round spermatids were rarely observed. *Myh10^{fl/-} Hspa2-Cre* testes also contained a substantial proportion of mono-nucleated secondary spermatocytes and round spermatids with normal appearance (Fig. 4d). Presumably, NMIIB from early germ cells is relatively stable, such that residual amounts of NMIIB protein (Fig. 4a) may be sufficient for cytokinesis in a subset of spermatocytes. Despite apparent defects in meiotic cytokinesis, *Myh10^{fl/-} Hspa2-Cre* males were fertile. This was not caused by insufficient Cre activity, because all 34 offspring from wild type females sired by *Myh10^{fl/-} Hspa2-Cre* males were *Myh10^{+/-}* (tail DNA genotype) and none harbored the un-recombined floxed allele, demonstrating efficient *Hspa2-Cre*-mediated recombination. In conclusion, eliminating NMIIB specifically during meiosis, but not at earlier stages of germ cell development, confirms a specific role of NMIIB in male germline development restricted to the regulation of cytokinesis during meiosis.

Notably, *Myh10^{fl/-} Ddx4-Cre* mice exhibited a nearly complete failure of meiotic cytokinesis (Fig. 1d), and thus a much more severe phenotype than *Myh10^{fl/-} Hspa2-Cre* mice (Fig. 4d). This difference in phenotype is likely associated with the continuous expression of *Ddx4-Cre* during germ cell development, from the embryonic germ cell through to the spermatocyte stage (Fig. 1a) (Gallardo et al., 2007). This should result in a complete depletion of MYH10 protein at the time when spermatogonia enter meiosis, rendering all spermatocytes in testes from *Myh10^{fl/-} Ddx4-Cre* mice entirely MYH10-deficient. Consistent with this hypothesis, MYH10 protein was not detectable in testes from *Myh10^{fl/-} Ddx4-Cre* mice by Western blot analysis (Fig. 1b and Fig. 4a).

Non-muscle myosin II is essential for mitotic cytokinesis in mammalian cells (Straight et al., 2003). Here, we demonstrate that of the three non-muscle myosin II isoforms in mammals (NMIIA, NMIIB, and NMIIC), a single myosin II, NMIIB, is essential for cytokinesis during mouse male meiosis. Consistent with such a role, MYH10 was previously shown to localize to the midbody in mouse spermatocytes (Manandhar et al., 2000). Conditional inactivation of *Myh10* in cardiac myocytes causes cytokinetic defects in a subset of cells, resulting in multi-nucleation (Ma et al., 2009). Therefore, specific myosin II isoforms appear to play non-redundant roles in cytokinesis in certain cell lineages and during specific divisions. Citron kinase is a previously identified regulator of cytokinesis that is essential for

mitotic division of male germ cells (Di Cunto et al., 2002). Here, we find that MYH10 is dispensable for mitotic cytokinesis in male germ cells, but required for meiotic divisions. The differences in MYH10 requirement between mitotic and meiotic cytokinesis could derive from compensation of MYH10 function by MYH9 during mitosis but not during meiosis. Alternatively, cytokinesis during male meiosis may have different MYH10-dependent requirements than during the preceding mitotic stages.

Our finding that MYH10 is not required for cytokinesis in female meiosis adds *Myh10* to a large number of mouse meiotic mutants that cause male sterility but have little or no effect on female fertility (Handel and Schimenti, 2010; Hunt and Hassold, 2002). In most cases, the underlying mechanisms of such sexual dimorphism are not clear. Interestingly, neither TEX14 nor citron kinase are required for female fertility (Di Cunto et al., 2002; Greenbaum et al., 2009). Together with the studies on TEX14 and citron kinase, our study on MYH10 has uncovered a new aspect of sexual dimorphism in meiosis – regulation of cytokinesis. The underlying cause may lie in the different expression patterns of non-muscle myosin II isoforms: *Myh10* is expressed in spermatocytes, whereas both *Myh9* and *Myh10* are expressed in oocytes and could thus be functionally redundant in female meiosis (Manandhar et al., 2000; Simerly et al., 1998). Alternatively, cytokinesis during meiosis differs in timing and symmetry between males and females, and may therefore be subject to sex-specific regulatory mechanisms and requirements that remain to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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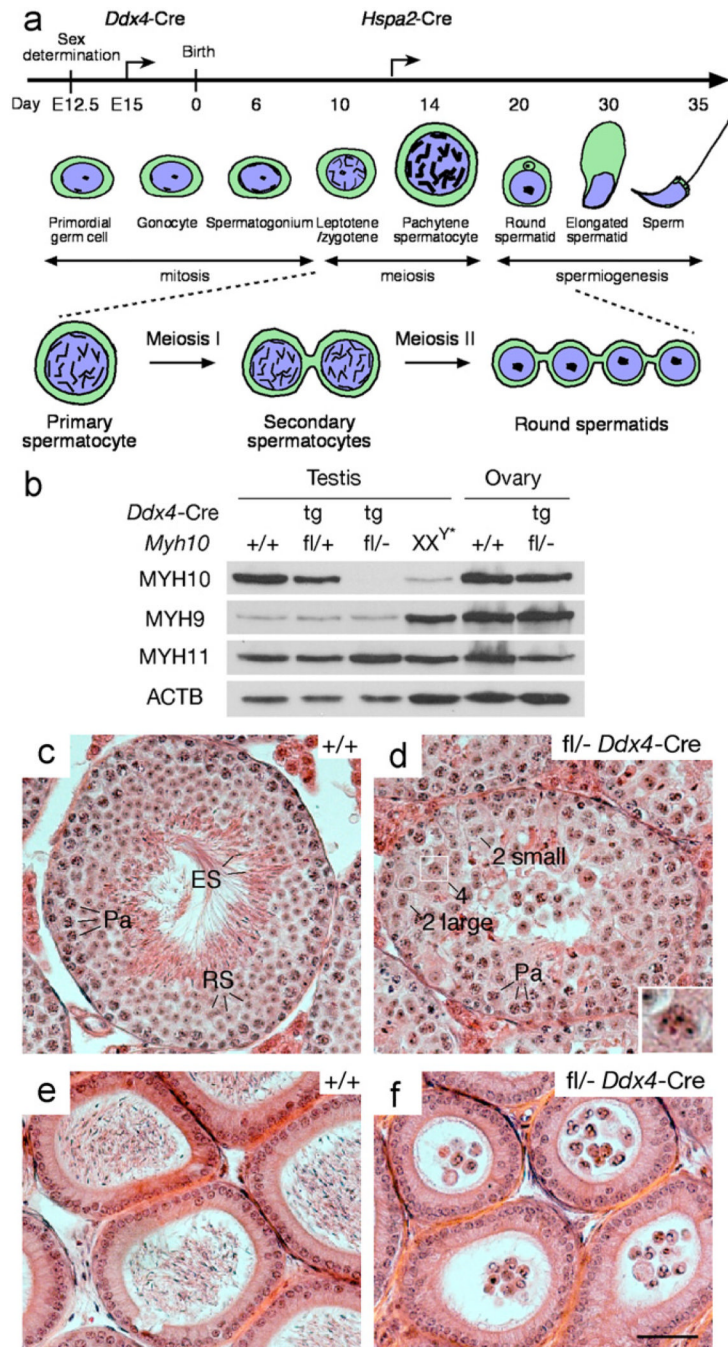


Fig. 1. MYH10 is required for cytokinesis during male meiosis. (a) Developmental timeline of mouse spermatogenesis. Arrows mark the onset of expression of the *Ddx4-Cre* and *Hspa2-Cre* transgenes. Meiotic germ cells, including primary spermatocytes, secondary spermatocytes, and round spermatids are represented schematically, and intercellular bridges, resulting from incomplete cytokinesis, are shown. (b) Absence of MYH10 protein in *Myh10*^{fl/-} *Ddx4-Cre* testes. Western blot analysis was performed on testicular or ovarian protein extracts from 2 month-old adult mice. The genotype of each sample, determined by analysis of tail DNA, is indicated above each lane. MYH11, the smooth muscle myosin II heavy chain, and ACTB served as loading controls (Deng et al., 1993). (c) Testicular

seminiferous tubule from a 2-month-old wild type mouse contains a full spectrum of germ cells. (d) Tubules from 2-month-old *Myh10^{fl/-} Ddx4-Cre* mice exhibit spermiogenic arrest. Germ cells with four nuclei (Inset, enlarged view of boxed area), two small nuclei, or two large nuclei are marked. (e) Epididymal tubules from 2-month-old wild type mice are filled with sperm. (f) Epididymal tubules from 2-month-old *Myh10^{fl/-} Ddx4-Cre* mice lack sperm but contain round multi-nucleated cells. Sections (c–f) are stained with H&E. Abbreviations: Pa, pachytene spermatocytes; RS, round spermatids; ES, elongated spermatids. Scale bar, 50 μm .

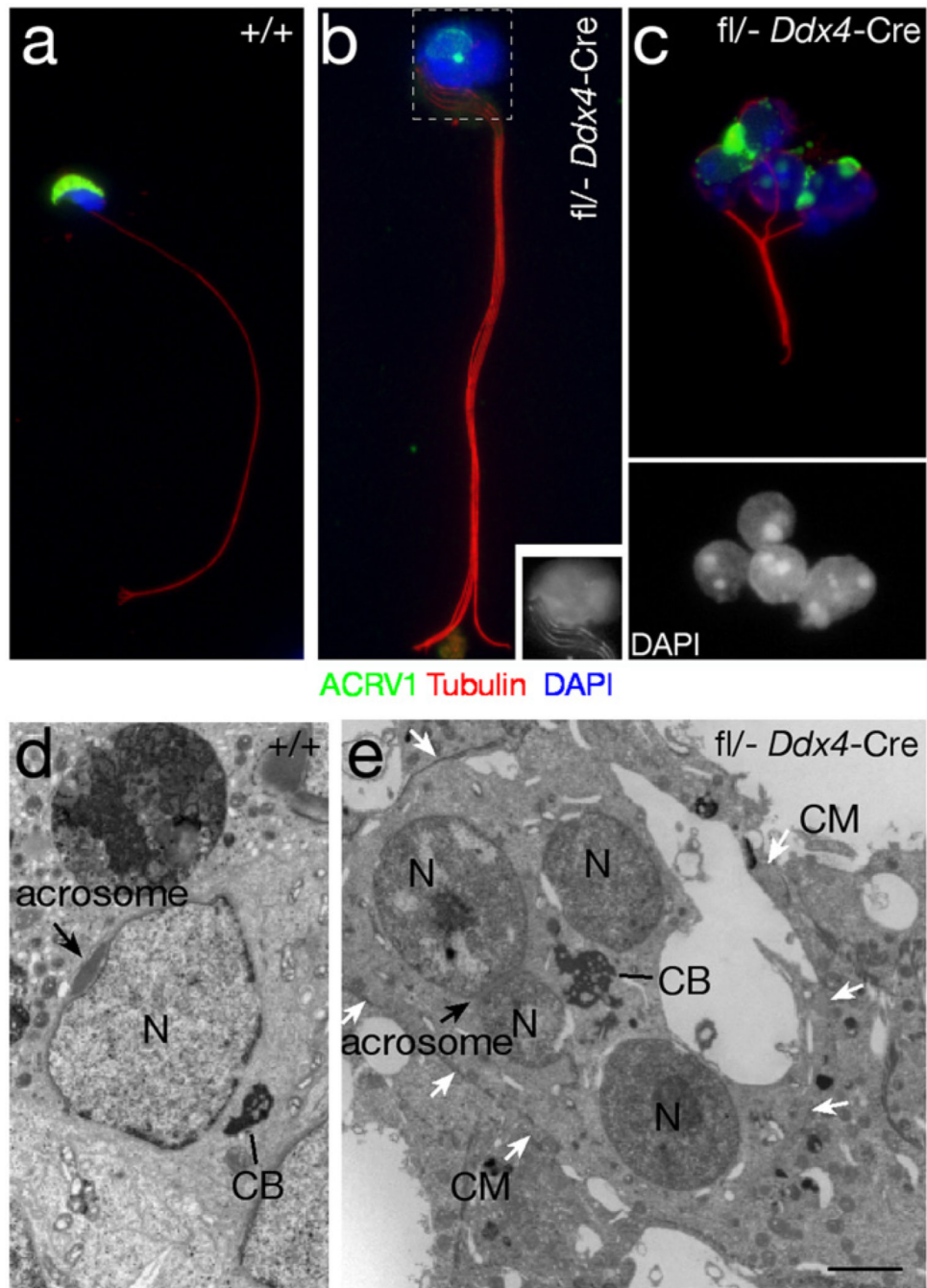


Fig. 2. Structural analysis of multi-nucleated germ cells from the testes of 2-month-old *Myh10*^{fl/-} *Ddx4-Cre* mice. (a–c) Immunofluorescence analysis of testicular germ cells, stained with an antibody against ACRV1 (green), a component of the acrosome (Reddi et al., 1995), and with an anti-tubulin antibody to visualize the axoneme (red). Nuclei are stained with DAPI (blue). (a) Wild type sperm. (b) MYH10-deficient germ cell with a bundle of four flagella. Inset: grayscale image showing the head (boxed area) with DAPI stained nuclei and four flagella (four bundles of microtubules). (c) MYH10-deficient germ cell with four nuclei (DAPI staining shown in bottom panel), bundles of microtubules, and defective acrosomes. (d and e) Ultrastructural analysis of a wild type spermatid (d), and a MYH10-deficient germ

cell with four nuclei (e). N, nucleus; CB, chromatoid body; CM, cell membrane. White arrows mark the cell membrane. Black arrow marks the acrosome. Scale bar (d and e), 2 μm .

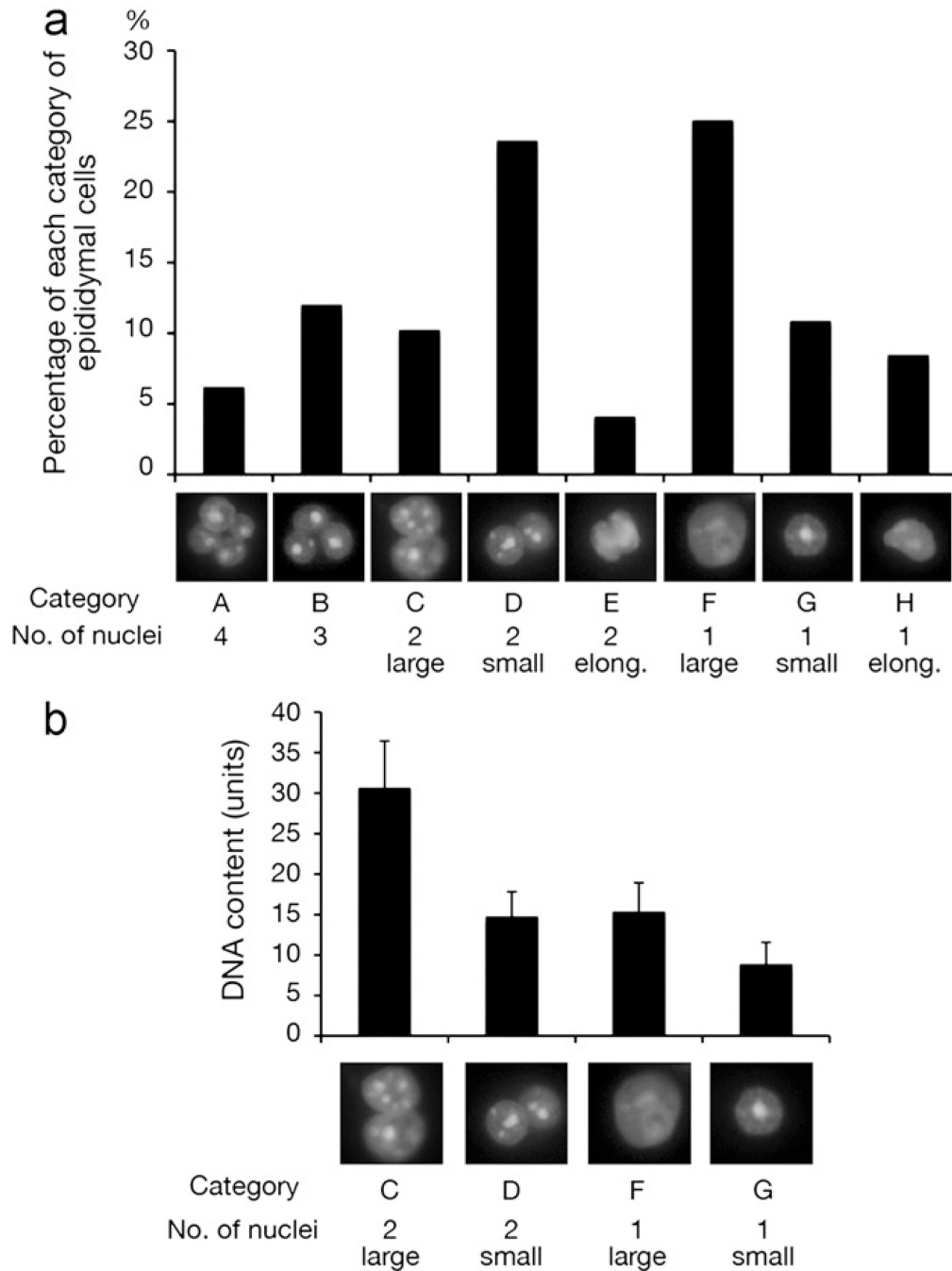


Fig. 3. Nuclear morphology and DNA contents of MYH10-deficient epididymal germ cells. (a) Epididymal cells from 2-month-old *Myh10*^{fl/-} *Ddx4*-Cre mice form eight distinct categories based on the number and size of nuclei. One representative nuclear image (DAPI) is presented for each category, and percentages refer to the total number of epididymal cells analyzed ($n = 620$). (b) Quantification of DNA content. The DNA content (shown in arbitrary units) was determined by quantification of the DAPI signal of 43 cells per category. Values shown represent the mean \pm SD.

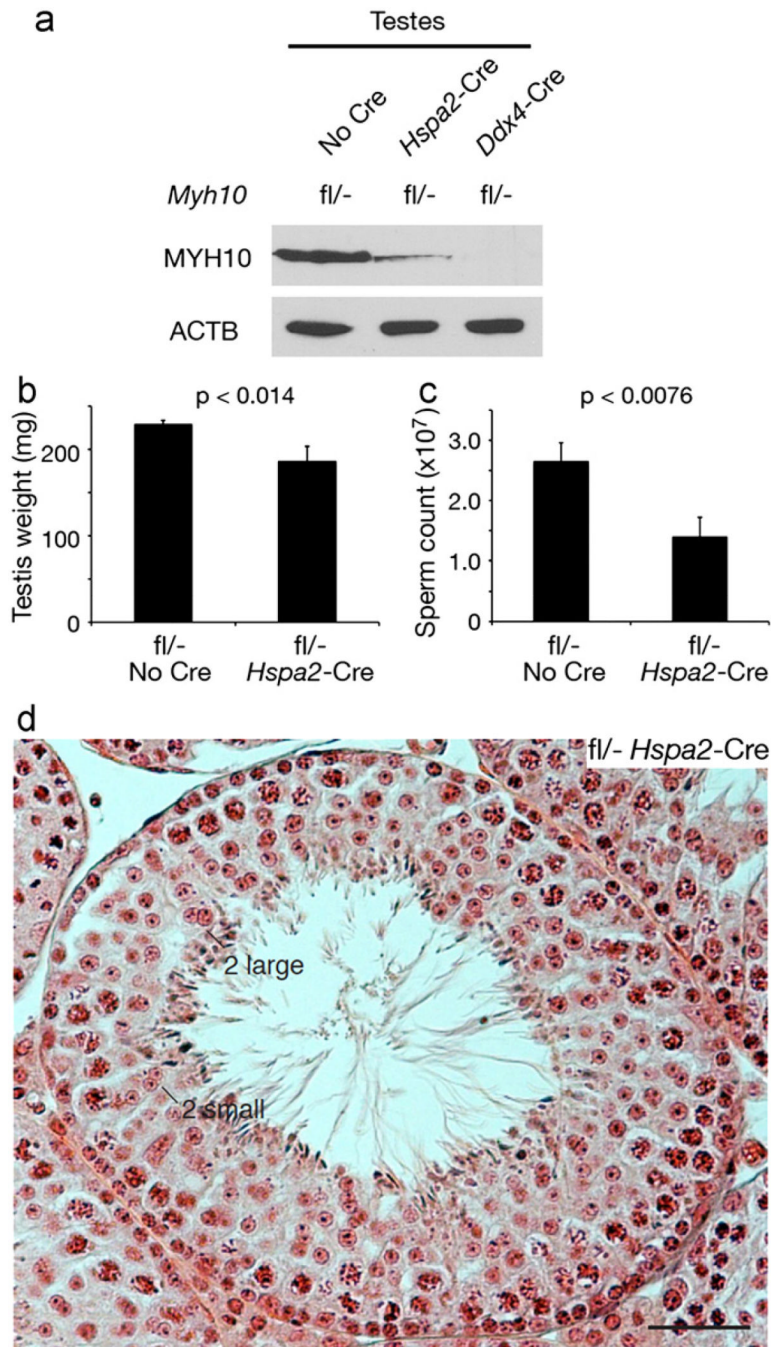


Fig. 4. Impaired meiotic cytokinesis in male germ cells of adult *Myh10*^{fl/-} *Hspa2*-Cre mice. (a) Western blot analysis of MYH10 in testes from 2-month-old *Myh10* mutant mice. ACTB served as a loading control. (b and c) Significant reduction in testis weight and sperm count in *Myh10*^{fl/-} *Hspa2*-Cre mice at 2–3 months of age. Values are statistically significant by Student's *t*-test. (d) Histological analysis (H&E) of testes from 2-month-old *Myh10* mutant mice. Bi-nucleated germ cells with large and small nuclei are marked. Scale bar, 50 μ m.