**Drosophila** Protamine-Like Mst35Ba and Mst35Bb Are Required for Proper Sperm Nuclear Morphology but Are Dispensable for Male Fertility

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**ABSTRACT** During spermiogenesis, histones are massively replaced with protamines. A previous report showed that *Drosophila* males homozygous for a genomic deletion covering several genes including the protamine-like genes Mst35Ba/b are surprisingly fertile. Here, we have precisely deleted the Mst35B locus by homologous recombination, and we confirm the dispensability of Mst35Ba/b for fertility.

Supporting information is available online at Note 1. http://www.g3journal.org/lookup/suppl/doi:10.1534/g3.114.012724/-/DC1

**KEYWORDS**

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In most animal species, the mature sperm nucleus is characterized by an extreme level of DNA compaction achieved after the massive replacement of somatic-type histones with sperm-specific nuclear basic proteins (SNBPs) (Lewis et al. 2003; Miller et al. 2010; Ward 2010; Kanippayoor et al. 2013). In mammals, the bulk of sperm chromatin is organized with two small protamines (Protamine 1 and 2) highly enriched in arginine residues (Balhorn 2007). In most animal species, the mature sperm nucleus is characterized by an extreme level of DNA compaction achieved after the massive replacement of somatic-type histones with sperm-specific nuclear basic proteins (SNBPs) (Lewis et al. 2003; Miller et al. 2010; Ward 2010; Kanippayoor et al. 2013). In mammals, the bulk of sperm chromatin is organized with two small protamines (Protamine 1 and 2) highly enriched in arginine residues (Balhorn 2007). *Drosophila* comprises at least three SNBPs: two paralogous protamine-like proteins, Mst35Ba and Mst35Bb, which are conserved among drosophilids, and the HILS1-related protein Mst77F (Russell and Kaiser 1993; Jayaramaiah Raja et al. 2013). In mammals, the bulk of sperm chromatin is organized with two small protamines (Protamine 1 and 2) highly enriched in arginine residues (Balhorn 2007). *Drosophila* comprises at least three SNBPs: two paralogous protamine-like proteins, Mst35Ba and Mst35Bb, which are conserved among drosophilids, and the HILS1-related protein Mst77F (Russell and Kaiser 1993; Jayaramaiah Raja and Renkawitz-Pohl 2005). Although the functions of *Drosophila* SNBPs remain poorly understood, Rathke et al. (2010) reported the surprising observation that *Drosophila* males homozygous for a genomic deficiency covering the Mst35B locus were fertile. This result was indeed unexpected when considering for instance the haploinsufficiency of mouse protamine genes for male fertility (Cho et al. 2001). However, according to Flybase (Flybase.org), the deficiency generated by Rathke et al. (2010) (named protΔ) is a 73.6-kb deletion that not only uncovers Mst35Ba and Mst35Bb but also removes four additional protein encoding genes (CG42682, CG15279, CG4480, CG15278) as well as three noncoding RNAs (CR43805, CR45727, CR45302). Furthermore, all these genes and noncoding RNAs are expressed in the adult testis or accessory glands, with the exception of CG15279, and transcripts of three of these genes (CG33309, CG4480, and CG15278) were detected in early spermatids (Flybase; Rathke et al. 2010). Because the simultaneous deletion of these other genetic elements could potentially interfere with a detailed functional analysis of Mst35B genes, we generated a precise deletion of the Mst35B locus by homologous recombination using the “Ends-Out” targeting technique (Gong and Golic 2003, 2004) (Figure 1A). The resulting allele, named ΔMst35B, eliminates a 5-kb genomic DNA fragment that only contains the Mst35Ba and Mst35Bb genes. To validate the elimination of these genes in the new deletion allele, we raised an antiserum against a peptide common to Mst35Ba and Mst35Bb proteins (Figure 1B). This antibody specifically stained late...
canoe stage spermatid nuclei of wild-type males but not those of ΔMst35B homozygous males (Figure 1C). At later stages of spermiogenesis, the highly compacted chromat of spermatids is no longer accessible to antibodies (Bonnefoy et al. 2007), thus explaining the absence of staining beyond the canoe stage in wild-type testes. In addition, another anti-Mst35B antibody raised against the whole Mst35Bb recombinant protein allowed us to confirm the absence of Mst35B proteins from ΔMst35B testis (Figure 1D).

As expected, homozygous ΔMst35B males were fully viable (not shown) and at least partially fertile (see paragraphs to follow), thus confirming the dispensability of Mst35B proteins for male fertility. Spermiogenesis (the differentiation of postmeiotic spermatids) in mutant males nevertheless appeared severely disorganized, with many elongating spermatids showing abnormal nuclear morphology (Figure 2, A–C). The spermiogenesis defects were similar in homozygous ΔMst35B and trans-heterozygous ΔMst35B/protΔ males, ruling out the possibility that the phenotypes associated with ΔMst35B were caused by a second-site mutation. In both allelic combinations, affected spermatid nuclei typically appeared bent compared with control spermatids, with the anterior tip of the nucleus sometimes folded into a hook-like structure (Figure 2, D and E). It is likely that the concentration of chromatin at one end of mutant spermatid nuclei observed by Rathke et al. (2010) actually correspond to folded nuclear extremities. A large proportion of mutant spermatids were scattered along the cysts instead of remaining tightly grouped in bundles of 64 nuclei, suggesting that they were progressively eliminated during the course of spermiogenesis (Figure 2, B and C). Accordingly, mutant males stored significantly less gametes in their seminal vesicles compared with control males (Figure 3E). Interestingly, however, we did observe morphologically aberrant mature gametes stored in the seminal vesicles of homozygous ΔMst35B and ΔMst35B/protΔ males (Figure 3, A–C), in sharp contrast to previous observations (Rathke et al. 2010). A transgene expressing Mst35Ba-EGFP rescued the abnormal nuclear shaping of ΔMst35B spermatids, thus confirming that this phenotype is actually caused by the loss of Mst35B genes (Figure 3D). However, a fraction of spermatids was still eliminated in rescued animals (Supporting Information, Figure S1), suggesting that the presence of a relatively large green fluorescent protein tag perturbs the functionality of the recombinant protein. Alternatively, both Mst35Ba and Mst35Bb proteins could be required for proper packaging of sperm DNA. In addition, we confirmed that a transgene expressing Mst77F-EGFP was normally incorporated into the chromatin of mutant gametes but failed to rescue the phenotype (Figure 3B). Finally, using a specific antibody (Figure S2), we also verified that the transition protein Tp94D (Rathke et al. 2007) was normally incorporated in mutant spermatids at the histone-to-proteamine transition (Figure 2, D and E), confirming that the nuclear defects in mutant spermatids appear after this stage.

Although the quantity and quality of gametes were affected by the loss of Mst35B genes, homozygous ΔMst35B and ΔMst35B/protΔ males were nevertheless fertile, in agreement with the study by Rathke et al. (2010). In fact, the impact of ΔMst35B on male fertility was only revealed when mutant males were allowed to mate with a large excess of virgin females (1 for 10; Figure 3F) but not with a 1:1 sex ratio (not shown). In the presence of a large excess of females, the observed reduction of fertility is likely explained by the limiting amount of sperm produced by mutant males (Figure 3E).
The organization of sperm chromatin in animals is poorly understood and most of our knowledge comes from studies on human or other mammalian species. *Drosophila* is an interesting, alternative model for the study of sperm chromatin at the functional level. The generation of a precise deletion allele of both protamine-like genes *Mst35Ba/b* provides an ideal tool for the functional study of *Drosophila* SNBPs. The fertility of *D. melanogaster* males reveals the extraordinary plasticity of the *Drosophila* sperm nucleus, which grossly maintains its architecture, motility and ability to fertilize eggs in the absence of what is considered a major component of its chromatin. It is likely that additional SNBPs compensate for the loss of the protamine-like proteins. In fact, we already know that the loss of *Mst35Ba/b* proteins does not perturb the incorporation of *Mst77F* in spermatid nuclei (this work and Rathke et al. 2010). *Mst77F*, which was originally identified in a genetic screen for β2 tubulin interactors (Fuller et al. 1989), is related to the mammalian spermatid-specific histone H1-like protein HILS1 (Iguchi et al. 2004; Yan et al. 2003). Interestingly, the *D. melanogaster* genome contains several recent copies of *Mst77F* on the Y chromosome, and eight of these *Mst77Y* genes are most likely functional (Russell and Kaiser 1993; Krsticevic et al. 2010). It has been proposed that *Mst77F* is essential for male fertility (Rathke et al. 2010), but this conclusion is based on the analysis of the antimorphic point mutation *Mst77F* (see Krsticevic et al. 2010). Future work should aim at clarifying the nuclear function of *Mst77F/Y* proteins to determine if they can indeed maintain a sperm chromatin structure compatible with male fertility in the absence of *Mst35B* proteins.

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