Developmental Biology 370 (2012) 86–97

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Multinucleated smooth muscles and mononucleated as well as multinucleated striated muscles develop during establishment of the male reproductive organs of *Drosophila melanogaster*

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ARTICLE INFO

Article history:
Received 20 January 2012
Received in revised form
10 July 2012
Accepted 11 July 2012
Available online 25 July 2012

Keywords:
Testes
Myogenesis
Sns
Duf
DMef2
Organogenesis

ABSTRACT

The adult musculature in *D. melanogaster* forms during metamorphosis. Much is known about the flight and leg musculature, but not about the muscles surrounding the male reproductive tract. The inner genitalia of males consist of the testes, which emerge from the gonads; the remaining genital organs, i.e., paragonia (or accessory glands), ejaculatory duct, sperm pump, and seminal vesicles, develop out of the genital imaginal disc. We analyzed the myoblasts forming the muscle layers of these organs. In myoblasts derived from the genital imaginal disc, the regulatory region of the transcription factor *DMef2* is active. *DMef2* is also needed for specification and differentiation of embryonic and adult myoblasts. We could discriminate three different muscle types: (i) multinucleated muscles that resemble vertebrate smooth muscles surround the testes, (ii) multinucleated muscles that resemble striated muscles comprises seminal vesicles and the sperm pump, and (iii) mononucleated striated musculature encloses the paragonia and ejaculatory duct. Members of the immunoglobulin superfamily involved in embryonic myogenesis, Dumbfounded (Duf) and Sticks and Stones (Sns), were also expressed in the genital imaginal disc, in the muscle sheath of the testes during muscle differentiation and in the secretory secondary cells, which are part of the binucleated epithelia enclosing the paragonia.

Introduction

Muscle development of the male reproductive organs of Drosophila melanogaster

The de novo assembly and rearrangement of organs and the surrounding tissues in *D. melanogaster*, including the development of the male reproductive tract, are complex processes. During early embryogenesis, germ cells are set aside as pole cells. Later, these pole cells migrate through the posterior gut primordium and join a group of mesodermal cells (gonadal mesoderm) in segment A5 and thus form the gonads (Richardson and Lehmann, 2010; for a review, see Santos and Lehmann, 2004). In the embryo, the imaginal discs are established; these discs form numerous parts of the adult body during metamorphosis. Important for gonad development is the genital imaginal disc a single disc in the posterior part of the larvae that gives rise to the somatic parts of the gonads, the genitalia, and the analia of males and females as well as the hindgut. Its development depends on homeotic genes and on the sex determination hierarchy, which define the position in the body and are responsible for establishing the somatic parts of the gonads (reviewed by Christiansen et al., 2002). In *D. melanogaster* males, the gonads give rise to the testes, whereas the genital disc gives rise to all the other organs of the genitalia, i.e., paragonia (also known as accessory glands), anal plate, ejaculatory duct, and vas deferens comprising the seminal vesicles (Estrada et al., 2003; for an overview, see Hartenstein, 1993). During metamorphosis, the gonads and the somatic parts of the reproductive tract grow towards each other and eventually join (Kozopas et al., 1998; Nanda et al., 2009; Stern, 1941).

Finally the testes, in which spermatogenesis takes place, are enclosed by a basal membrane, followed by a muscle layer, and then by the outermost layer of pigment cells. Pigment cells also surround the seminal vesicles but no other components of the male reproductive system.

Only little is known about the development and assembly of the muscle sheaths of the *D. melanogaster* male reproductive system. Twist-expressing adepithelial cells are associated with the genital imaginal disc and presumably are myoblast precursors. Upon *D*Wnt-2 signaling, the attachment between the testes and the developing vas deferens occurs. Myoblast precursors migrate over the vas deferens onto the testes to form the muscle sheaths (Kozopas et al., 1998).

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http://dx.doi.org/10.1016/j.ydbio.2012.07.022

Syncytial muscle formation has been best characterized during *D. melanogaster* embryogenesis (for a review, see Abmayr and Keller, 1998; Chen and Olson, 2004; Önel et al., 2011; Önel and Renkawitz-Pohl, 2009; Rochlin et al., 2010; Schejter and Baylies, 2010). Founder cells (FCs), expressing the cell adhesion protein Dumbfounded (Duf), define the identity of the individual muscles through a particular set of transcriptional regulators and recruit fusion-competent myoblasts (FCMs) for fusion (for an overview, see Maqbool and Jagla, 2007). On the site of the FCMs another cell adhesion molecule was identified essential for fusion: Sticks and Stones (Sns) (Bour et al., 2000).

The visceral musculature is comprised of two types of muscles: the circular and the longitudinal muscles. The binucleated circular muscles arise by incomplete fusion and are interwoven with the syncytial longitudinal muscles (Klapper et al., 2001; San Martin and Bate, 2001; Schröter et al., 2006).

We investigated the muscle layers of the male reproductive tract with respect to their filament organization and the number of nuclei contained in the muscle fibers. We found that the seminal vesicles and the sperm pump contain multinucleated striated muscles; the testes are enclosed by smooth muscle filaments, whereas the paragonia and ejaculatory duct are encircled by mononucleated, striated muscle fibers. This is the first report of multinucleated smooth musculature in *D. melanogaster*. We also show that the regulatory regions of the immunoglobulin superfamily members Duf and Sns are active in the genital-disc-derived myoblasts that migrate onto the testes. RNA interference (RNAi) experiments knocking down either *sns* or *duf* in the developing testes muscles lead to a mislocalization (Mikado-phenotype) of some testicular myofilaments in adult males. Our findings indicate that the process of muscle formation of the male reproductive system during metamorphosis shows parallels to the well-characterized myogenesis that takes place in the embryo.

Materials and methods

Drosophila melanogaster stocks

D. melanogaster strains were maintained on standard medium at 25 °C. The *white*^{1118} strain (BL6326; Bloomington Drosophila Stock Center, Bloomington, IN, USA) was used as a wild-type control. The protein trap lines Sls::GFP (ZCL2144), and Fas3::GFP (YD0075) were obtained from Flytrap (http://flytrap.med.yale.edu/) to visualize the male reproductive system. Trol::GFP is a protein trap line kindly provided by Christian Klämbt (Universität Münster, Germany).

The myoblast-specific driver lines *DMef2-Gal4* (Ranganayakulu et al., 1996), *sns-Gal4* (Stute et al., 2006) and *duf-Gal4* (also known as *rp298-Gal4*; Menon and Chia, 2001) were individually crossed with UAS-GFP (BL1521, Bloomington Drosophila Stock Center) to visualize GFP expression in the genital imaginal disc and the pupal reproductive system. Flies carrying the reporter *sns-mCherryNLS* (Haralalka et al., 2011) were kindly provided by Susan Abmayr, Stowers Institute for Medical Research, Kansas City, MO, USA.

The driver line *C855a* (BL6990, Bloomington Drosophila Stock Center (Hrdlicka et al., 2002)) was used for RNAi experiments with flies carrying one of the following constructs: UAS-*duf*-RNAi (31111), UAS-*sns*-RNAi (109442), UAS-*rst*-RNAi (951, BL28672) and UAS-*hbs*-RNAi (40898) (all obtained from Vienna Drosophila RNAi Center, Austria (Dietzl et al., 2007) except for BL28672, obtained from Bloomington stock center).

Dissection of adult flies and pupae

Male flies were collected, anesthetized with ether, placed in a drop of PBS (0.13 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4) on a plastic dish, and dissected with Dumont forceps under a stereomicroscope. The male genitalia were mounted on glass slides in Fluoromount G™ (Southern Biotech, Birmingham, AL, USA) or in PBS. The specimens were observed under a fluorescence microscope.

Male pupae were collected as white pre-pupae from culture bottles, transferred to Petri dishes lined with moistened filter, and allowed to develop to the desired developmental stage at 24 °C. Pupae were dissected as described for adult flies.

Immunohistochemistry

Antibodies were used for immunohistochemical analysis of whole-mount adult testes following standard procedures (Michaud et al., 1997). Organs were embedded in Fluoromount G™ (Southern Biotech) and examined with a Zeiss Axioplan 2 fluorescence microscope or observed in a drop of PBS with a Zeiss AxioObserver Z.1 inverse microscope (Zeiss, Stuttgart, Germany).

Anti-GFP antibody was used to enhance the fluorescence signal of Trol::GFP in the stated cases, whereas anti-Tropomyosin (MAC 141; Abcam, Cambridge, UK; 1:1000) helped to detect the myofilaments. The primary antibodies were detected using the corresponding Cy2-conjugated secondary antibody (Dianova, Hamburg, Germany; 1:100). DAPI (Sigma-Aldrich, Steinheim, Germany; 50 μg ml^{-1}) was used to visualize chromatin, whereas F-actin was stained with Phalloidin-TRITC (Sigma-Aldrich, Steinheim, Germany; 10 μg ml^{-1}).

Scanning electron microscopy (SEM)

Adult reproductive systems were dissected and fixed in 4% glutaraldehyde overnight at room temperature, washed twice in water, and fixed again for 2 h in 1% osmium tetroxide. The samples were dehydrated twice for 20 min in 30%, 50%, 70%, 80%, and 90% ethanol and then twice for 1 h in pure ethanol. Samples were then incubated overnight in acetone and dried to the critical point (Balzers CPD 030; Bal-Tec AG, Balzers, Liechtenstein). The samples were covered with gold (SC7620 Mini Sputter Coater; Quorum Technologies, East Grinstead, U. K.) and then viewed with the scanning electron microscope Hitachi S-530 (Evex Analytical Inc, Princeton, NJ, USA).

Molecular characterization of the GFP protein trap line 311

The protein trap 311 was generated as described previously (Morin et al., 2001), using an automated sorter (Union Biometrica) to select larvae with GFP expression. To determine the genomic localization of the exon trap element, DNA from flies harboring this insertion was isolated according to standard procedures (Eggert et al., 1998). For the amplification of the 3′ end of the P-element the BDGP iPCR protocol was used with the primers ry1 and ry2 on Sau3A digested and religated DNA

(< http://www.fruitfly.org/about/methods/inverse.pcr.html).

The DNA sequence was aligned to genomic DNA using the BLAST program (Altschul et al., 1997).

Results

The testes of D. melanogaster are surrounded by smooth muscle-like myofibers, while the muscles of the other male reproductive organs are striated

We analyzed the surface of the reproductive system of adult *D. melanogaster* males in detail using scanning electron microscopy (SEM; Fig. 1A). The muscle layers of the testes and the paragonia are very thin only a few nanometers thick (Fig. 1B and C). The muscles of the paragonia appear ordered and encircle the

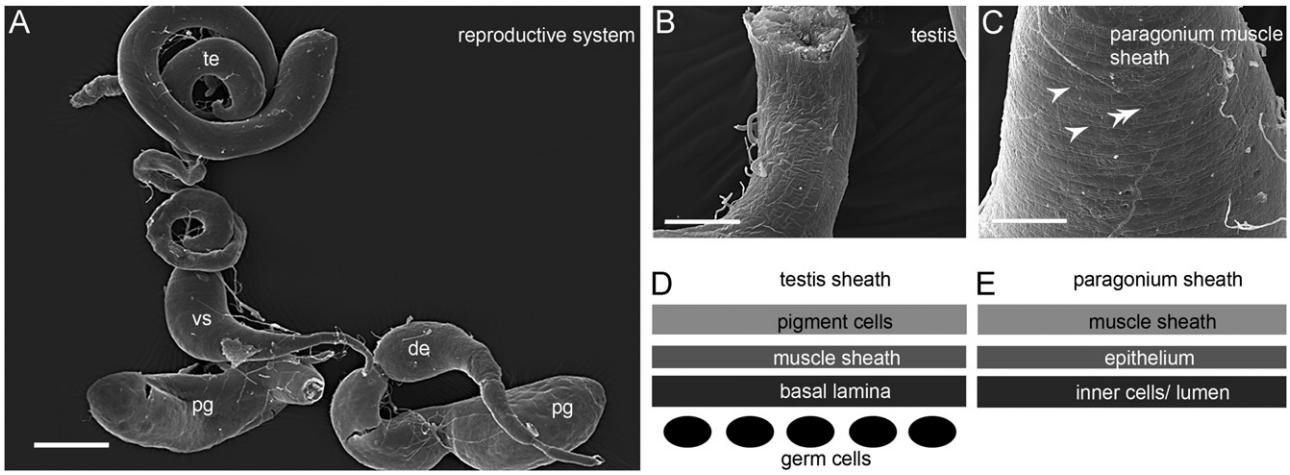

Fig. 1. Scanning electron microscopy (SEM) of the adult male reproductive system of *D. melanogaster*. (A): Overview of the male reproductive system: te: testis, pg: paragonium, de: ejaculatory duct, vs: seminal vesicle. Scale bar: 150 μm. (B): Higher magnification of an adult testis. Scale bar: 30 μm. (C): Magnification of a paragonium showing the muscle sheath. The single muscle fibers encircle the organ (arrowheads). These muscles fibers are connected to each other via thin protrusions (double arrowhead). Scale bar: 30 μm. (D) and (E): Schematic overview of (D) testis and (E) paragonium, showing the tissue layers enclosing the organs. Abbreviations are assumed from Hartenstein (1993).

organs (arrowheads in Fig. 1C, and compare Fig. 2C). The muscle fibers of the paragonia appear to be interconnected with each other (double arrowhead in Fig. 1C, arrowheads in 2C), but we never observed an interwoven muscle pattern like that of the longitudinal and circular muscles of the visceral musculature of *D. melanogaster* embryos (Schröter et al., 2006). The outer layer of the paragonium is a muscle sheath (Fig. 1C, E); in contrast, the outer layer of the testis has been shown to consist of pigment cells, which lie on a muscle sheath (Fig. 1B, D).

To analyze the filament arrangement of the musculature of the adult male reproductive system further, we used the GFP protein trap line ZCL2144 to visualize the Z-discs (Fig. 2). In these flies, two splice variants of the *sallimus* (*sls*) gene, Sls and Kettin, are fused with GFP (Hudson et al., 2008). These proteins serve as a linker between the thick and thin filaments of the muscle fibers (Burkart et al., 2007). Moreover, we used Phalloidin to detect the actin filaments of the muscle cells. Thus, we not only were able to detect the muscle layers, but also were able to visualize the sarcomere arrangement of the muscles surrounding the male reproductive system by fluorescence microscopy.

The entire adult reproductive system is surrounded by muscle sheaths (Fig. 2). The muscles of the seminal vesicles, paragonia, ejaculatory duct, and the sperm pump (Fig. 2B–E) show the distribution known for sarcomeres of striated muscles. However, the muscle layers of the testes (Fig. 2A) show a different Sls/Kettin and F-actin distribution. For the testes, we could not recognize the Z-disc structure, we found for the striated muscles of the other organs (compare Fig. 2A to B–E) but a very extended Sls/Kettin region. The musculature of the testis has a chevron pattern (Fig. 2A). Similar distributions of Titin have been observed in the smooth musculature of vertebrates (Chi et al., 2008). Compared to the linear muscles forming the sperm pump (Fig. 2E), the muscle fibers of the remaining parts of the male reproductive system are smaller and thinner (Fig. 2A–D). For the muscle sheath of the paragonia, we observed again the interconnections of different muscles (arrowheads in Fig. 2C), known from the SEM analysis of the paragonia surface.

Our observations concerning the different muscle filament arrangement indicate that the muscles of the testes resemble the smooth muscles of vertebrates (Chi et al., 2008; Herrera et al., 2005) more than any other muscle type described so far for *D. melanogaster*.

Description of the morphological development of the male genital imaginal disc during metamorphosis

We aimed to gain insights into the development of the male genital imaginal disc during pupal stages. We dissected pupae 10, 20, 24, 28 and 40 h after puparium formation (APF) and determined the growth of the genital disc at these periods.

The fate map of the male genital imaginal disc of a third instar larvae shows that the posterior part of the disc develops into the anal plate region, whereas the inner genitalia arise from the dorsal and anterior parts of the disc (Fig. 3A drawn after Bryant, 1978). The vas deferens has the same origin in the genital disc as the paragonia (Fig. 3A). As metamorphosis proceeds, the pairwise vas deferens grow out of the genital disc and differentiate into two distinct parts: (i) the bulb-like seminal vesicles, where mature sperm are stored, and (ii) a short duct, which in the adult males releases the sperm into the ejaculatory duct.

Already 10 h APF the small buds that develop into the paragonia and the vas deferens, are visible (Fig. 3B, arrowheads) as well as the ejaculatory duct (Fig. 3B, de). The parts of the disc developing into the penis apparatus, the claspers, the genital arch, the anal and lateral plates are unfolding, giving vision onto the lumen (Fig. 3B and compare to Fig. 3A). After further 10 h the buds becoming the paragonia and the vas deferens have grown (Fig. 3C, arrowheads) and the rest of the genital disc has become a more prominent three-dimensional structure (Fig. 3C). At 24 h APF the paragonia and vas deferens could be discriminated from each other and the ejaculatory duct started to elongate (Fig. 3D). From the depicted dorsal view only the lumen was visible (Fig. 3D). Shortly before the vas deferens and the testes attached to each other, the paragonia and vas deferens had formed distinct tissues. The epithelia enclosing the lumen had formed as well (Fig. 3E) and mesodermal cells were migrating over these epithelial sheaths (Fig. 3E).

After 40 h APF the attachment of the testes and the vas deferens already occurred. The ejaculatory duct elongated (Fig. 3F) and the vas deferens and paragonia had separated lumens of their own, which disembogue into the ejaculatory duct (Fig. 3F). Even though the testes and the vas deferens were attached to each other, the lumens were still separated (Fig. 3F, arrowhead). At this stage the anal and lateral plates were recognizable (Fig. 3F, arrow) and the hindgut and anal bulb (Fig. 3F, double arrows) had already grown out of the genital disc.

Mono- and multinucleated myofibers form the musculature of the male reproductive system

We used the binary UAS-Gal4 expression system (Brand and Perrimon, 1993) to follow the fate of the myoblasts and analyzed the expression of the myogenic regulatory factors Twist (Twi) and *Drosophila* Myocyte enhancer factor 2 (DMef2). Twi is needed for the specification of the mesoderm and patterning of the somatic musculature during embryonic development. DMef2 is essential for the differentiation of the myoblasts specified by Twi (reviewed in Baylies and Michelson, 2001).

We dissected the genital discs of males during pupal stages and analyzed Twist and DMef2 expression by the presence of enhanced GFP (EGFP) and GFP signals, respectively. In the case of *twi-Gal4*, we neither detected EGFP expression in genital discs at the relevant stages at approximately 30 h APF nor in the primary myotubes of the testes after the genital imaginal disc and the testes join each other (data not shown). Therefore, we decided to investigate *DMef2* activity, as this transcription factor acts in later stages during muscles differentiation.

We observed a GFP signal in the round myoblasts migrating over the vas deferens in males expressing GFP under control of the *DMef2* regulatory region (Fig. 4A and C and higher magnifications in the upper right corner). DAPI staining of these putative myoblasts revealed that these cells are multinucleated with up to five nuclei (Fig. 4B and C, arrowheads and corresponding magnifications); we therefore refer to them as primary myotubes. The *DMef2*-driven GFP signal was still detected in primary myotubes that reached the testes at about 30 h APF (Fig. 4D and F, arrowheads), as well as in the stretching muscle fibers encircling the testes at 46 h APF (Fig. 4G and I). The vast majority of the forming myotubes had two to three nuclei (Fig. 4D–F, and compare to higher magnifications in the upper right corner). In later stages, the primary myotubes are spread all over the testis surface and form long, thin, multinucleated muscle fibers (Fig. 4G–I, arrowheads).

During embryogenesis, DMef2 plays a vital role in myoblast differentiation (reviewed in Baylies and Michelson, 2001). From our findings, this transcription factor is an amenable marker to follow the cell fate of the mono- and multinucleated myoblasts forming the testes musculature and the musculature of the residual organs of the reproductive tract.

Since the primary myotubes of the testes were multinucleated (Fig. 4D–I), we determined the number of nuclei in the muscles enclosing the other organs of the male reproductive system using GFP protein trap line to visualize the individual muscles (protein trap line 311 see 'Material and methods'). Our molecular analysis by PCR revealed that the GFP is inserted as an exon at position 27305 in the gene region of *terribly reduced optic lobes* (*trol*) thus we named the encoded protein Trol::GFP. *trol*, also referred to as *perlecan* (*pcan*), encodes an extracellular matrix protein, which is involved in the maintenance of epithelial cell polarity (Friedrich et al., 2000; Mirouse et al., 2009; Schneider et al., 2006). As Trol::GFP visualizes the extracellular matrix between the muscles (Fig. 5), it is well suitable to identify an individual myotube. We therefore dissected the reproductive tracts of adult Trol::GFP males and stained them with DAPI to visualize the nuclei (Fig. 5). With this approach we could show that the muscles of the testes, seminal vesicles, and sperm pump are multinucleated

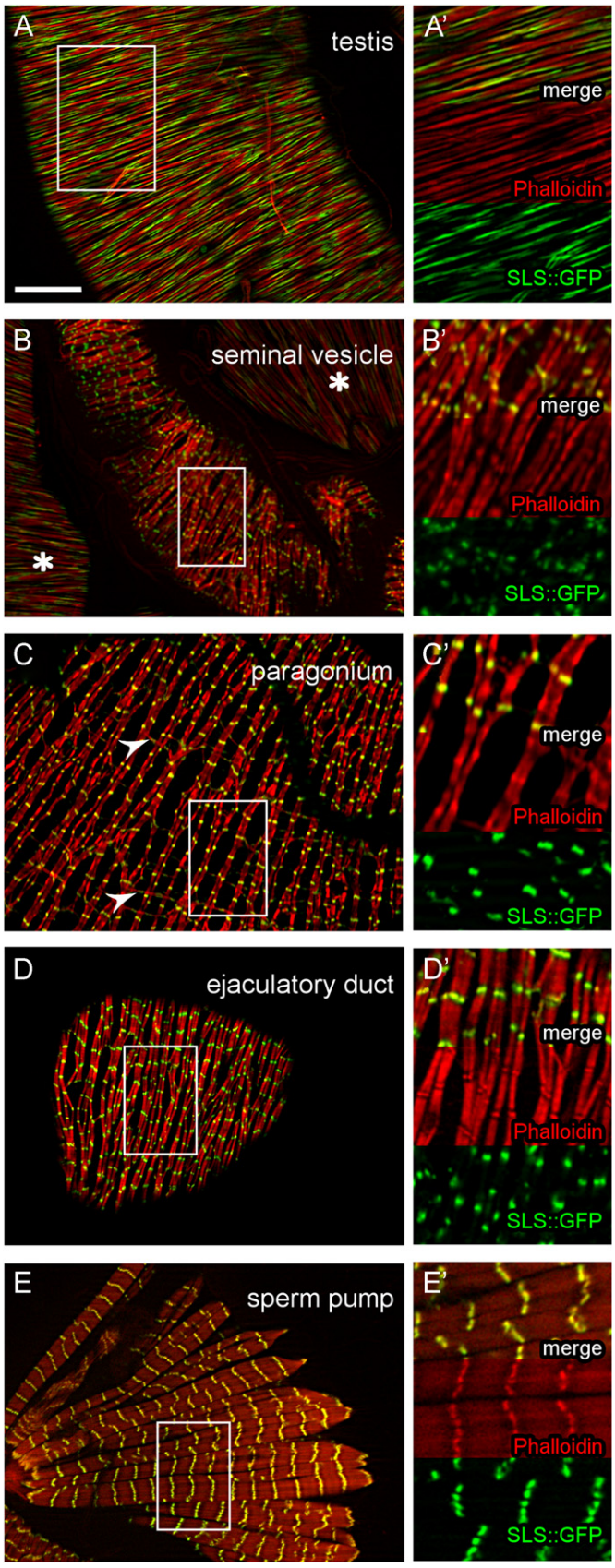

Fig. 2. The male reproductive system is surrounded by striated and smooth muscles. The adult musculature of the different organs of the male reproductive system by comparison. The muscle sheaths are visualized in optical sections with SLS::GFP for Z-discs and Phalloidin for F-actin detection. (A): Sls::GFP and F-actin are distributed in a chevron pattern throughout the whole muscle layer of the testis, resembling vertebrate smooth muscles. (B) seminal vesicle, (C) paragonium, (D) ejaculatory duct and (E) sperm pump reveal an ordered, stripe-like repetitive distribution of Sls and F-actin. This pattern clearly differs from the one, seen in testis musculature (compare to (B): testis muscles are indicated by asterisks) (C): The muscle fibers of the paragonia seem to be connected by thin protrusions (arrowheads). Boxed areas depict the higher magnifications shown on the right-hand side. Scale bar: 20 μm.

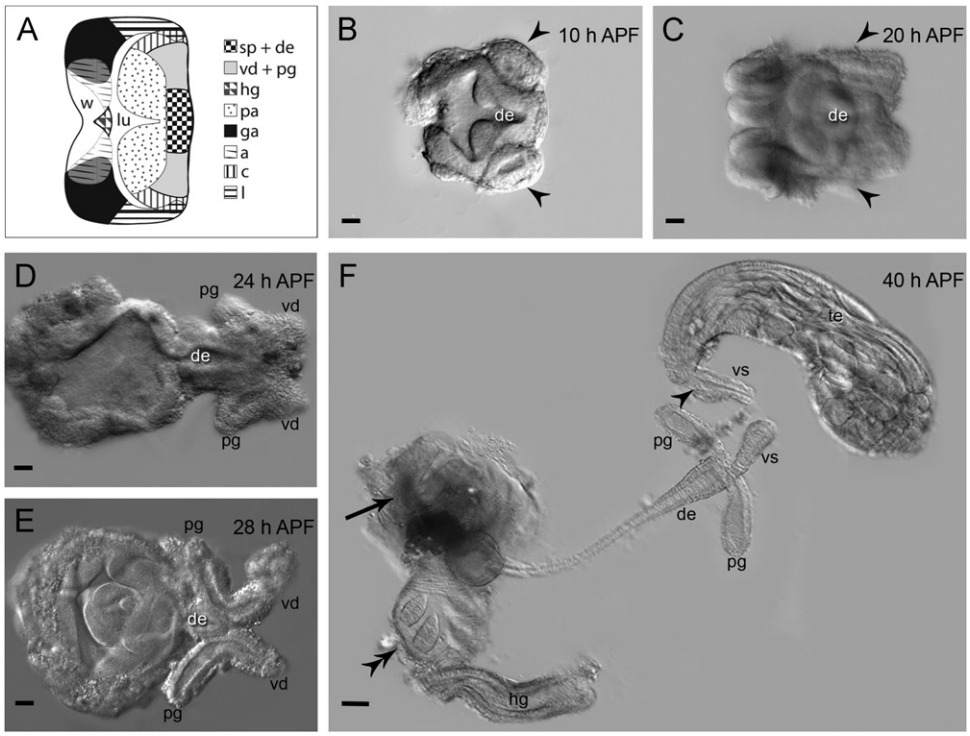

Fig. 3. Distinct developmental stages of the male genital disc during metamorphosis. (A): Schematic fate map of the larval male genital disc posterior view (modified after Bryant, 1978). (B): Ventral view of a genital disc 10 h APF. (C): Ventral view of a genital disc 20 h APF. Arrowheads in B and C show the buds, which develop to vas deferens and paragonia. (D): Dorsal view of a genital disc 24 h APF. (E): ventral view of a genital disc 28 h APF. (F): Male reproductive system 40 h APF. One testis was lost during dissection. Abbreviations: sp: sperm pump; de: ejaculatory duct; vd: vas deferens; pg: paragonia; hg: hindgut; pa: penis apparatus; ga: genital arch; a: anal plates; c: claspers; l: lateral plates; lu: lumen; w: cellular material without known imaginal equivalent; te: testis. Scale bars in (B)–(E): 20 μm. Scale bar in (F): 40 μm.

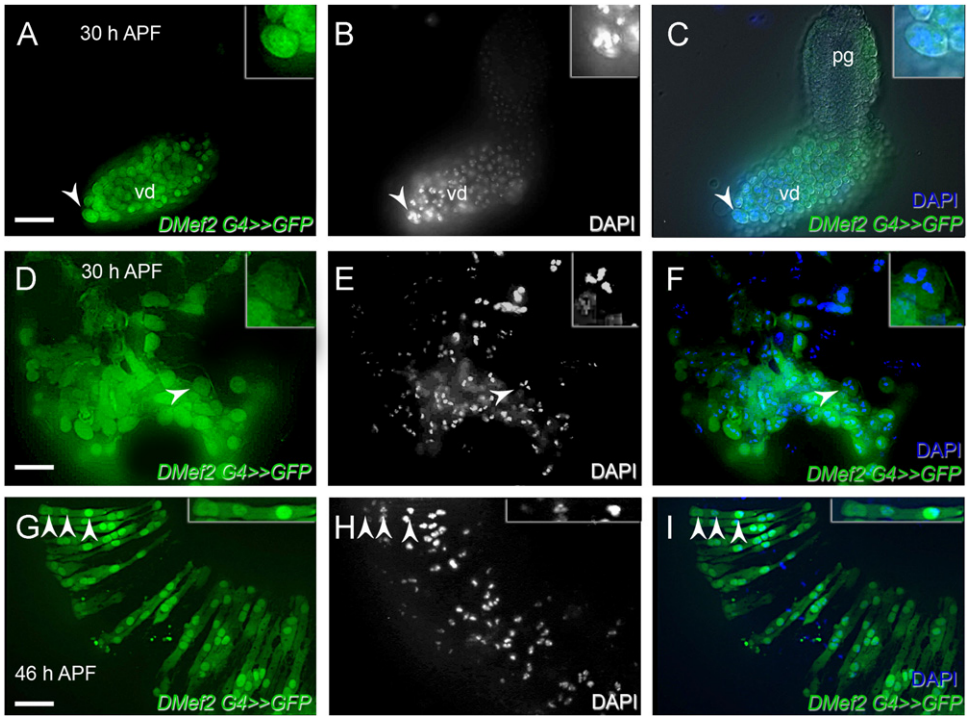

Fig. 4. The myoblasts forming the muscle layer of the testes are multinucleated as they migrate onto the testes. (A), (D) and (G): GFP signal in (A) pupal vas deferens and (D and G) pupal testes. (B), (E) and (H): DAPI staining of same structures as in (A), (D) and (G), respectively, to visualize the nuclei. (C), (F) and (I): Merged GFP and DAPI images. (A)–(C): Dissected pupal paragonia (pg) and vas deferens (vd). (D)–(F): Pupal testis: multinucleated round-shaped muscle cells are located at the distal tip of the testis and start to migrate over the testis. Arrowheads in (A)–(F): multinucleated cells. (G)–(I): Optical section of a pupal testis at about 46 h APF. The myotubes spread all over the testis and form fibers surrounding it. The boxes in the upper right corner depict higher magnifications of the myoblasts marked by arrowheads. Scale bars: 20 μm.

(Fig. 5A, B, and E). In contrast, the muscle layers of the paragonia and ejaculatory duct are made up of mononucleated myofibers (Fig. 5C and D). We noticed that the nascent myotubes at the pupal testes are already multinucleated (Fig. 4). Thus, we asked if fusion-relevant proteins known from embryonic myogenesis are expressed in adepithelial cells of the genital disc.

The genital disc contains a large population of myoblasts with duf and sns activity

We analyzed Sns by the *sns-mCherryNLS* reporter construct (Haralalka et al., 2011) and Duf using *duf*-driven GFP expression as a reporter on the genital disc. In these early stages the two cell adhesion molecules might, like during embryogenesis, be involved in myoblast fusion events. We dissected the genital discs of pupae before attaching to the testes. *duf*-driven GFP expression is detectable in myoblasts occupying the parts of the genital imaginal disc that will develop into the outer genitalia, sperm pump and hind gut (Fig. 6A–C, arrowheads). Additionally, *duf*-driven GFP expression was found in cells migrating over the developing paragonia and vas deferens (Fig. 6A and C). Interestingly, *duf*-driven GFP expression was also found for the epithelia at the tip of the paragonia presumptively leading to the secondary cells (Fig. 6A and C, asterisks compare to Fig. 7H and I for *duf* and *sns* activity in secondary cells). Furthermore, Sns-mCherryNLS expression is visible in the nuclei of myoblasts, which localize over regions of the genital disc that will differentiate into the outer genitalia, sperm pump and hind gut (Fig. 6D–F, arrowheads), as it was observed for *duf*. Additionally, we found *sns-mCherryNLS* expression in the cells migrating over the vas deferens and paragonia (Fig. 6D and F).

We aimed to get a first entry into the question where and when fusion is likely to happen to form the to the testes migrating primary myotubes. Therefore we established transgenic lines with the genetic background *duf-Gal4* ≫ UAS-GFP, *sns-mCherryNLS*. As we observed multinucleated primary myotubes over the vas deference primordia (Fig. 4C), we focused our analysis to this region. Indeed, we observed many cells positive for *duf-Gal4* ≫ UAS-GFP over the vas deference primordia of genital discs of these pupae (Fig. 6G) and some expressing Sns-mCherryNLS (Fig. 6H). We found a colocalization of *duf*-driven GFP and *sns-mCherryNLS* expression in a few cells migrating over the vas deference primordium (Fig. 6G–I, arrowheads).

Sns and duf are active during the migration of primary myotubes over the testes

Our results indicate that multinucleated muscles surround the testes, and these arise from primary myotubes that migrate over the vas deferens towards the testes (compare Fig. 4 and Kozopas et al., 1998). Extensive morphogenesis of these primary myotubes is required to create a monolayer of extremely thin muscles encircling the testes. Proteins that could be involved include Duf and Sns, which belong to the irre cell recognition module (IRM) and Roughest (Rst) as well as Hibris (Hbs) which act in full or partial redundancy to Duf and Sns in embryonic myogenesis, respectively (Shelton et al., 2009; Strünkelnberg et al., 2001). In this study, we focused on Duf and Sns. These proteins function together not only in myoblast fusion but also in cell sorting during eye development and axonal path finding (for a review, see

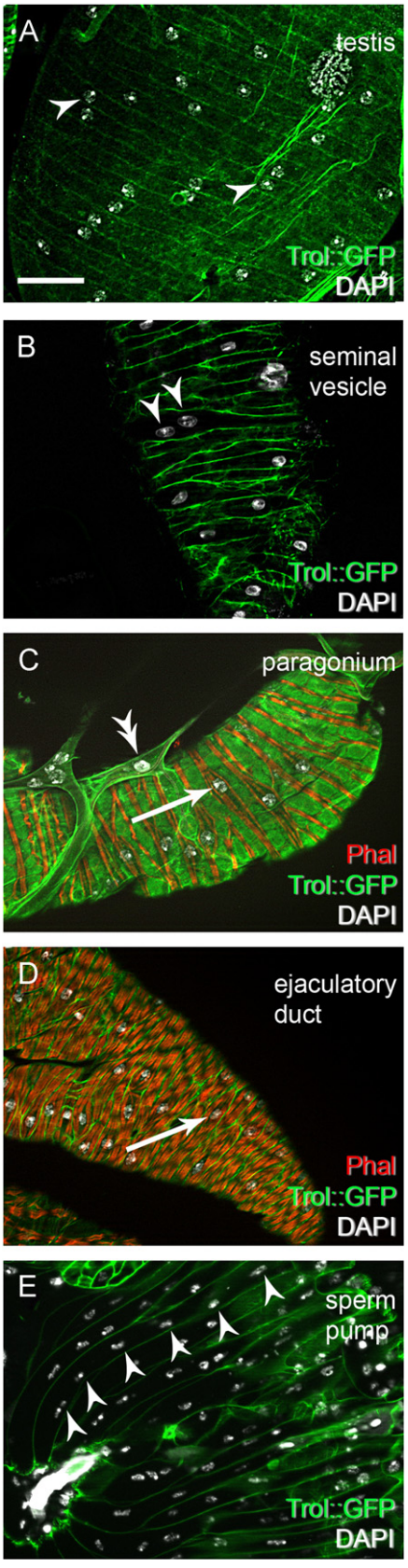

Fig. 5. Muscle tissues of the male reproductive system of adult *D. melanogaster* are mononucleated or multinucleated.A new characterized protein-GFP trap line for Trol visualizes the extracellular matrix (green), additional to DAPI staining (white) in optical sections. (A), (B), (E): In testis (A), seminal vesicle (B) and sperm pump (E) multinucleated myotubes are observed. (C), (D) In paragonia (C) and ejaculatory duct (D) only mononucleated muscles can be found. (C)–(D): The GFP-signal was enhanced by anti-GFP staining and myofibers were visualized with Phalloidin-TRITC, additionally. Arrowheads: nuclei of multinucleated muscle cells. Arrows: nuclei of mononucleated muscles. (C): The double arrowhead points to a trachea. Scale bars: 20 μm.

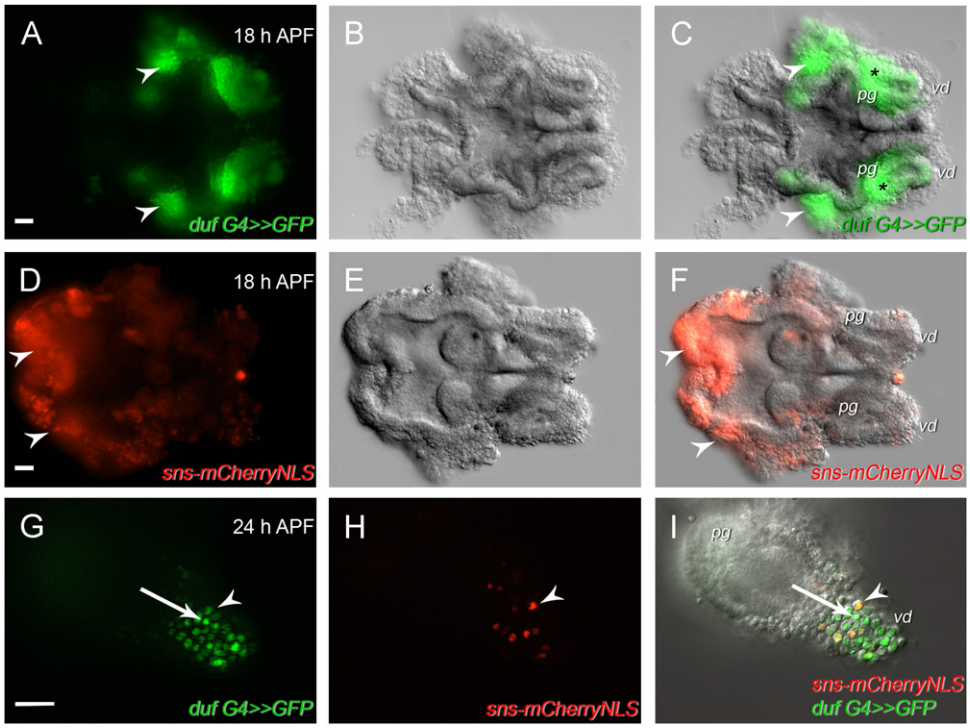

Fig. 6. *duf* and *sns* are active in myoblasts of the genital imaginal disc (A)–(C): *duf-Gal4* drives GFP expression in the myoblasts occupying the genital imaginal disc in a ventral view at 18 h APF. Arrowheads in (A) and (C): The GFP signal is visible in myoblasts occupying the genital disc. The myoblasts migrating over the paragonia (pg) and the vas deferens (vd) are GFP-positive as well. (B): Light microscopy of the same genital disc depicted in (A). (C): Merge of (A) and (B). The epithelia at the tip of the paragonia, giving rise to the secondary cells show *duf*-activity (asterisks). (D)–(F): Sns-mCherryNLS expression in the nuclei of myoblasts of the genital disc in a ventral view at 18 h APF. Arrowheads in (D) and (F): Sns-mCherryNLS is expressed in myoblasts on the genital disc. (E): Light microscopy of the same genital disc depicted in (D). (F): Merge of (D) and (E). (G)–(I): Depicted are the myoblasts occupying the vas deferens (vd) of pupae carrying duf-Gal4 ≫ UAS-GFP and *sns-mCherryNLS* 24 h APF in an optical section. The markers for *duf* and *sns* activity do not colocalize in all, but in some of the depicted myoblasts (arrowheads). Arrows show a cell just positive for duf-Gal4 ≫ UAS-GFP. Scale bars: 20 μm.

Fischbach et al., 2009). We again used the UAS-Gal4 system (Brand and Perrimon, 1993), this time driving GFP expression under the control of *duf-Gal4* or *sns-Gal4*. We dissected the testes of pupae at different stages to follow the activity of the promoter regions of *duf* and *sns*.

The regulatory elements of both genes are activated in the primary myotubes of the pupal testes. We observed *sns*- (Fig. 7A and B, arrows) and *duf*-driven GFP expression (Fig. 7D and E, arrows) when the primary myotubes just reached the testis. At about 40 h APF, loosely spaced GFP-positive muscles fibers enclosed the testis (Fig. 7C and F). In agreement with observations mentioned above, we detected several nuclei in these primary myotubes (Fig. 7C, E and F, arrowheads). These myotubes migrated over the testis from the distal to the proximal end during the development of its muscle layer (Fig. 7E and F).

In several cases, we also observed that the muscle fiber formation begins at the distal end. During the next hours, these muscles became more abundant and more tightly packed. *duf*-driven GFP expression was observed during migration and development of primary myotubes (Fig. 7D–F). Just when the GFP-positive cells reached the testes, they were already multinucleated, displaying mostly two to three nuclei (Fig. 7G, arrowheads). This indicates a further function of Duf and Sns besides myoblast fusion while primary myotubes migrate over the seminal vesicles and the testes.

At the tip of the paragonia, the so-called secondary cells are located; these cells contain large vacuoles (Bairati, 1968; Bertram et al., 1992). Interestingly, during pupal stages as well as in adult flies, we found these binucleated secondary cells to be active for *duf* and *sns* (Fig. 7H, I and data not shown). In addition we analyzed the epithelial sheath of the paragonia, consisting of the main cells, and found it being binucleated. We also investigated the epithelial sheaths of the other organs and found them to be mononucleated (for details see supplement and Fig. S1).

During the testis muscle sheath formation, Duf and Sns might be involved in muscle filament arrangement

To gain more insights into Sns and Duf function during development of the testes musculature, we knocked down the level of these proteins with an UAS-based RNAi experiment (Dietzl et al., 2007). For this approach, we used the testes muscle-sheath-specific driver line *C855a*, as the adult muscle precursor-specific driver-line *1151-Gal4* (Roy and VijayRaghavan, 1997) is not active in this tissue (data not shown) and *DMef2-Gal4* disrupts the development of the larval muscles.

Driving a UAS-*duf*-RNAi- or a UAS-*sns*-RNAi-construct during the development of the testes musculature, adult males do not show defects in movement and are entirely fertile. Thus, we analyzed the adult testes musculature of these flies with an anti-Tropomyosin antibody and the F-actin marker Phalloidin to visualize sarcomeres. The analysis of pupal testes 40 h APF revealed that nascent myotubes are indistinguishable from the wild-type situation and these nascent myotubes migrate towards the testis and are multinucleated (Table 1).

In adult testis additionally to the smooth muscle fibers encircling the testis, some scattered filaments, arranged like the little rods in the Mikado game (Mikado phenotype), can be observed, overlying the regular muscle sheath (arrowheads in Fig. 8A and B and higher magnifications). The pattern of other muscles of the male reproductive system is not altered. These Mikado phenotype filaments have never been observed in testes of *C855a* flies (Fig. 8C). This indicates that the alteration in myofilament arrangement is not due to the

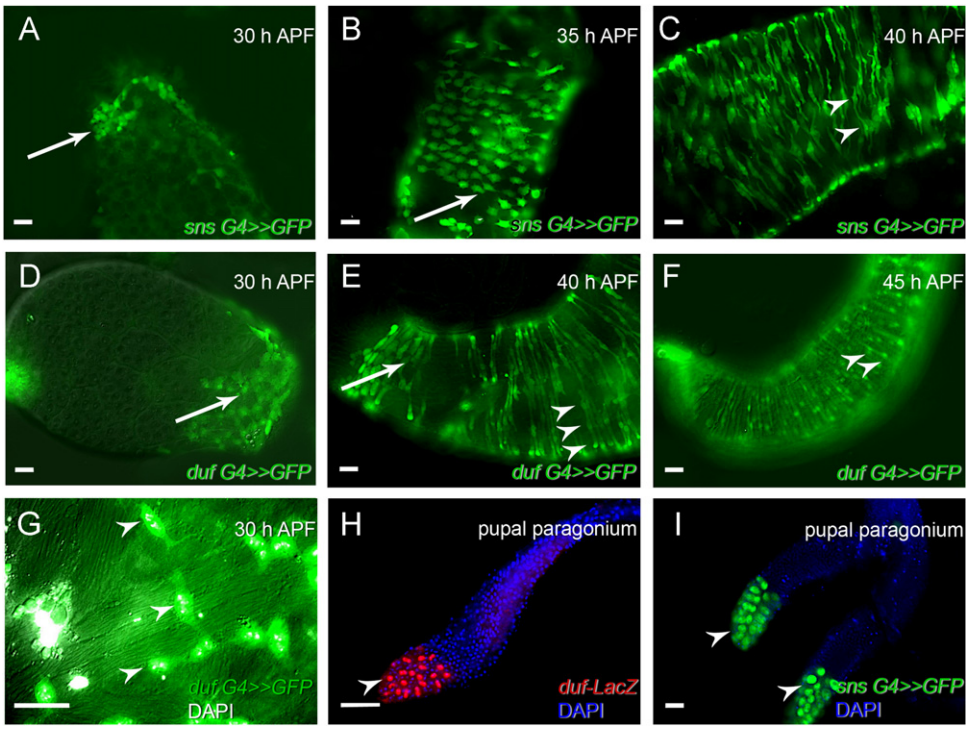

Fig. 7. The promoter regions of the cell-adhesion molecules *duf* and *sns* are active in myotubes forming the syncytial muscle layer of the testes. (A)–(C): GFP expression in pupal testes under the control of *sns-Gal4*. (A): Pupal testis at 30 h APF. Arrow: Primary myotubes at the distal end. (B): pupal testis at 35 h APF. Arrow: Primary myotubes migrating from the distal end to the proximal end in the developing testes. (C): Pupal testis at about 40 h APF. Arrowheads: Myofibers forming at the proximal end of the testes. (D)–(F): GFP expression in pupal testes under the control of *duf-Gal4*. (D): Pupal testis at 30 h APF. Arrow: GFP expression at the distal end of the testis. (E): Pupal testis at 40 h APF. Arrow: a single primary myotube at the distal end of the testis; arrowheads: Muscles that contain more than one nucleus surrounding the proximal part of the testis. (F): Pupal testis at around 45 h APF. Arrowheads: Multinucleated muscle fibers. (G): Pupal testis round about 30 h APF. The primary myotubes that migrate over the testis show GFP expression under control of *duf* promoter regions. These myotubes have two to three nuclei (arrowheads). DAPI staining is depicted in white. Scale bar: 42 μm. (H): Pupal paragonium shortly before eclosion, expressing the reporter construct Duf-LacZ. Arrowhead: *duf* expression domain in secondary secretory cells located at the distal tip of the paragonium. (I): Pupal paragonium expressing *sns-Gal4*-driven GFP in the secondary secretory cells (arrowheads). Scale bars: 20 μm (except for G).

Table 1
RNAi experiments revealed a Mikado phenotype in the testes musculature of adult males by driving expression of *duf*, *sns* and *hbs* RNAi constructs.

	Fly-line	40 h APF testes nascent myotubes	Adult testes muscles
duf	v3111	Multinucleated	Mikado phenotype
rst	v951	Multinucleated	Wild-type
	BL28672	Multinucleated	Wild-type
sns	v109442	Multinucleated	Mikado phenotype
hbs	v40898	Multinucleated	Mikado phenotype
Driver line	C855a	Multinucleated	Wild-type

driver line itself. In addition we tested the potential redundancy partners of Sns and Duf. Driving a UAS-*hbs*-RNAi constructs the same way, the testes muscles observe the displayed Mikado phenotype as well (Table 1). In contrast, testes of flies driving a UAS-*rst*-RNAi -construct during the development of the testes musculature look like wild-type testes (Table 1).

In summary, we propose from these findings that these adhesion molecules might be involved in arranging muscles around the testes in a regular pattern.

Discussion

Our analysis of *Drosophila's* muscle sheaths of the inner male reproductive system revealed that the organs are surrounded by a distinct circular muscle layer. The only exception is the sperm pump, whose muscle sheath consists of linear muscle fibers. The muscles of the male genitalia share some similarities with the muscles of the female reproductive system and with the adult musculature of the abdomen and the leg, but also have major differences. Our gained knowledge about the muscles of the *D. melanogaster* male reproductive system is summarized in Fig. 9.

The musculature of the male reproductive system is characterized by striated mono- and multinucleated as well as smooth multinucleated muscles

The male genitalia of *D. melanogaster* are surrounded by distinct muscle sheaths. These sheaths are comprised of mono-nucleated or multinucleated muscles (Fig. 9A). These muscle fibers of the male reproductive tract, except the sperm pump, are thin. In agreement with the former work of Kozopas and colleagues, we found that the myoblasts giving rise to these muscles are derived from the genital imaginal disc.

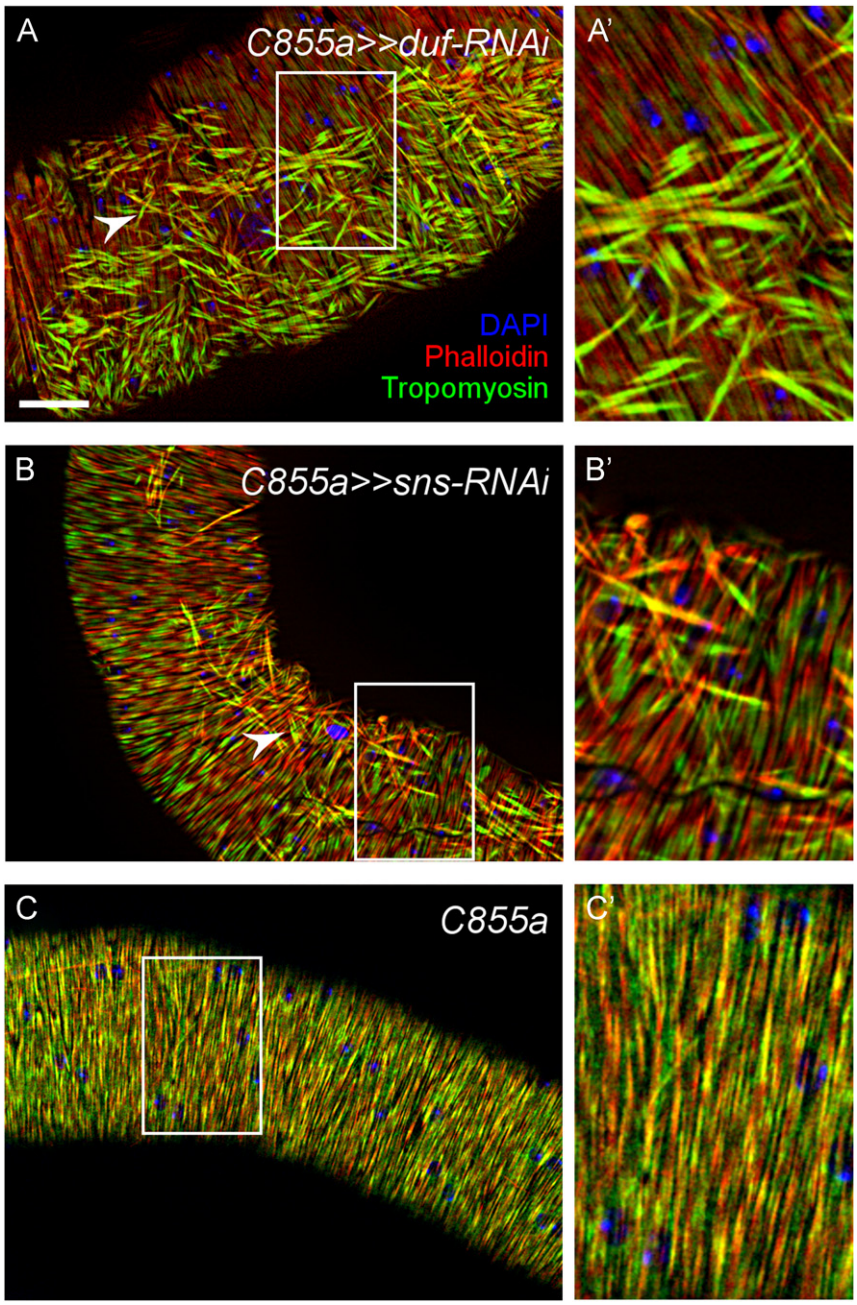

Fig. 8. *duf* and *sns* knockdown during development of the testes musculature leads to scattered myofilaments in adult testes RNAi-knockdown for *duf* and *sns*, using the testis muscle sheath-specific driver line *C855a*. Muscle filaments are visualized with Phalloidin-TRITC (red) and anti-Tropomyosin staining (green) in optical sections to observe the testes muscle pattern. Nuclei are labeled with DAPI (blue). (A)–(B): Knocking down *duf* or *sns* leads to a scattered distribution of single filaments (arrowheads) at many areas. These filaments seem to lie above the regular muscle sheath of the testes. (C): The driver line *C855a* shows a filament pattern resembling wild-type testes. Boxed areas depict the higher magnifications shown on the right-hand side. Scale bar: 20 μm.

The musculature of the female genitalia of *D. melanogaster* consists of three different circular layers. An epithelial sheath covers each of the sixteen ovarioles that comprise one ovary. A peritoneal sheath surrounds each ovary. The third muscle sheath consists of large muscles that surround the oviduct. The epithelial and peritoneal sheaths are mononucleated, whereas the oviduct muscles are multinucleated and resemble the indirect flight musculature (Hudson et al., 2008; Middleton et al., 2006).

For all muscles of the male reproductive tract except those of the testes, we found sarcomere structures of striated musculature (Fig. 9B), which has also been described for the muscle layers of the female reproductive system (Hudson et al., 2008; Middleton et al., 2006). Muscles encircle the testes, seminal vesicles, paragonia, and ejaculatory duct perpendicular to the *A*/*P* axis, like the muscles of the peritoneal and epithelial sheaths of the ovary (Hudson et al., 2008).

The main difference between the muscle layers of the male and female genitalia is the presence of multinucleated smooth muscles in the male genitalia, which encircle the testes. The Sls/Kettin and F-actin distribution of this muscle layer did not resemble the well-ordered disc-like pattern of striated muscles. Instead, Sls/Kettin and the actin fibers in the testes were distributed in a chevron pattern and were missing a typical Z-disc, thereby resembling the smooth muscles of vertebrates (Herrera et al., 2005). Our findings are the first description of multinucleated smooth muscles in *D. melanogaster*.

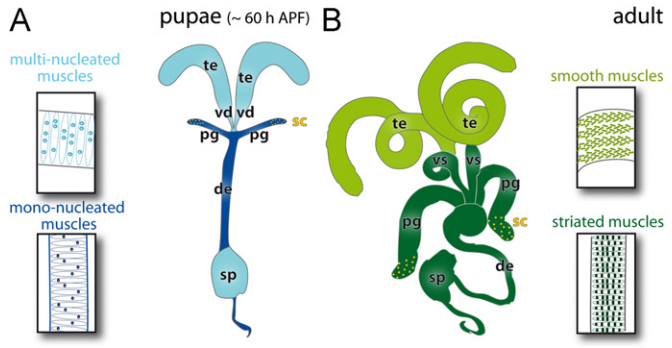

Fig. 9. Schematic overview of the muscle sheaths of the male reproductive system of *D. melanogaster*. (A): Pupal reproductive system at about 60 h APF. Light-blue shading: multinucleated muscles; dark-blue shading: mononucleated muscles. (B): Muscle types in adult males. Light-green shading: muscle sheath with a chevron distribution of sarcomere components, reminiscent of the smooth muscles of vertebrates; dark-green shading: striated sarcomere composition similar to that in all other characterized muscles of *D. melanogaster*; yellow dots: secondary cells (sc). te: testis; pg: paragonium; vs: seminal vesicle; vd: vas deferens; de: ejaculatory duct and sp: sperm pump.

The formation of the multinucleated testes muscles shows parallels to embryonic myogenesis and adult muscle formation

The *duf* and *sns* genes encode cell-adhesion proteins of the immunoglobulin superfamily. We found both genes to be active in myoblasts/nascent myotubes migrating over the developing imaginal disc. We know that GFP-positive cells under control of the *duf* promoter and Sns-positive cells at this stage are already multinucleated. This might be caused by reprogramming the Sns-positive nuclei to a FC cell fate after fusion occurred as it is observed in somatic embryonic myoblast fusion (for review see Abmayr and Pavlath, 2012). The few Sns-mCherryNLS expressing cells on the vas deferens might indicate ongoing fusion events. A more detailed analysis of fusion events requires the identification of suitable markers for the myoblast population leading to multinucleated muscles; e.g., for the testes and the sperm pump as well as markers to distinguish between the Duf and Sns expressing binucleated secondary cells of the paragonia. Additionally, we found that the promoters of *duf* and *sns* are active in primary myotubes of the testes musculature during migration along the testes, which indicates that Sns and Duf are possibly involved in the arrangement of the testes musculature.

Our findings complement previous investigations of the developing leg and flight musculature from adepithelial cells of leg or wing imaginal discs during adult myogenesis. These muscles are multinucleated and probably arise from fusing FCs and FCMs. In both cases, Duf-positive cells were observed that fused with the myoblasts that were forming aggregates around them and did not express Duf (Atreya and Fernandes, 2008; Dutta et al., 2004; Kozopas and Nusse, 2002; Soler et al., 2004). We observed that multinucleated primary myotubes arrive at the testes and express Duf and/or Sns during their further migration and differentiation between the epithelial layer and basal membrane of the testes. Therefore, we hypothesize that myogenesis of the musculature of the testes takes place in several waves: (i) Sns- and Duf-dependent fusion during migration of the myoblasts/primary myotubes over the genital imaginal disc, (ii) myoblast fusion during myoblast migration before reaching the testes and (iii) myofiber arrangement during migration along the testes.

Duf and Sns might have an additional function in testis muscle cell arrangement

As the decreased amount of Sns or Duf in the emerging myotubes leads to scattered myofilaments (Mikado phenotype) above the regular muscle sheath of the testis, we assume that Duf and Sns are required for proper localization of the myofilaments encircling the testes. The RNAi experiments indicate a possible second role of these adhesion molecules in the arrangement of the myofibers in late stages of developing testis muscle sheath. Several functions of *sns* and *duf* have been reported so far in *D. melanogaster* (for a review, see Fischbach et al., 2009). Besides their role in recognition and attachment of myoblasts during embryonic myoblast fusion (Bour et al., 2000; Ruiz-Gómez et al., 2000), they mediate the ommatidia spacing in eye development (Bao et al., 2010) and are essential for the formation of the slit diaphragm-like structure in nephrocytes (Weavers et al., 2009; Zhuang et al., 2009). Altogether, the adhesion molecules Duf and Sns function frequently in cell sorting and arrangement. That might also be the case in establishing the testes musculature, as Sns and Duf are apparently expressed until late stages of pupal testes muscle development.

There are proteins acting redundantly to Duf and Sns: in embryonic myoblast fusion, cell adhesion is only disrupted in *duf/rst* double mutants (Strünkelnberg et al., 2001) and *hbs* acts partially redundant to *sns* (Shelton et al., 2009). Furthermore *duf* is upregulated in *rst*-knockdown situation during pupal retina development (Machado et al., 2011). Knock down of *hbs* during testes muscle development results in the phenotype observed with *duf*- or *sns*-RNAi, whereas expression of *rst*-RNAi constructs do not lead to severe defects. As we were not able to check the efficiency of the *rst* knockdown, we cannot exclude a relevance of Rst in this context. During pupal testes muscle development, Duf as well as Sns and likely also the closely related molecule Hbs seem to be involved at least in proper muscle- or muscle filament arrangement after multinuclear myotube formation.

The duf and sns genes are active in the specialized epithelial cells of the Drosophila melanogaster paragonia

The paragonia are essential for the reproduction of *D. melanogaster* (Rylett et al., 2007), and their structure and function have been well investigated also in other insect species (Freitas et al., 2007; Radhakrishnan et al., 2009). The epithelium of the paragonia secretes a variety of peptides and protein hormones into the seminal fluid. This leads to behavioral changes in post-mated females, such as an increase in ovulation, loss of receptivity to males, and increased sperm storage (reviewed in Chen, 1984; Wolfner, 1997). In *D. melanogaster*, two morphologically distinct epithelial cells can be discriminated: the so-called secretory 'main' cells and the secretory 'secondary' cells. The secondary cells are located in the distal third of the paragonia, are larger than the main cells and contain large vacuoles (Bairati, 1968; Bertram et al., 1992).

We showed that cells of the epithelial sheath surrounding the paragonia are binucleated, as previously reported (Bairati, 1968; Bertram et al., 1992), and we observed in pupae *sns*- and *duf*-driven GFP expression in the binucleated secondary cells. Therefore, one could speculate that these binucleated cells arise from fusion, and that Duf and Sns are needed for this process. But since also the main cells are binucleated and we observed neither Duf nor Sns in these cells, we regard it rather unlikely that cell fusion is the function or sole function of Duf and Sns in secondary cells. Moreover, we also observed *sns*- and *duf*-driven GFP expression in secondary cells of paragonia of adult flies. This is in agreement with Duf and Sns involvement in the formation of a slit-diaphragm-like structure in *D. melanogaster* nephrocytes (Weavers et al., 2009; Zhuang et al., 2009). The two proteins are located at the entry point of the nephrocyte diaphragm into the labyrinthine channels and are co-expressed in the same cell, as we observed for the secondary cells. These two proteins are vital for the formation of the filtration slit diaphragm, which is needed for the uptake of molecules into

labyrinthine channels for processing by endocytosis (Weavers et al., 2009; Zhuang et al., 2009). Hence, we hypothesize that Sns and Duf serve an adherence function in the secretory secondary epithelial cells of the paragonia.

Conclusions

Interestingly we found three different muscle types constituting the musculature of the male reproductive system: the multinucleated striated muscles in the sperm pump and the seminal vesicles, the mononucleated muscles surrounding the ejaculatory duct and the paragonia, and the smooth multinucleated muscles enclosing the testes. With emphasis on myoblasts we followed the development of the male genitalia during metamorphosis. In agreement with the former work of Kozopas et al. (1998), we observed that the myoblasts forming these muscles originate from the male genital imaginal disc. These mesodermal cells migrate over the paragonia and vas deferens during metamorphosis, and after the attachment, finally, onto the testes. During migration over the vas deferens we could observe multinucleated cells that form the muscles of the testes.

The two genes *duf* and *sns* encode cell adhesion molecules, which are essential for myoblast fusion. Both genes were found active in the secondary cells of the paragonia and more importantly in the myoblasts/primary myotubes forming the musculature of the male reproductive system. We therefore hypothesize that the multinucleated smooth muscles of the testes and the multinucleated striated musculature are formed by myoblast fusion. We assume that, like in somatic larval muscle fusion, the two cell adhesion molecules Sns and Duf are involved in this process. At what time point fusion occurs still needs further investigation. Besides that, we assume a function for Sns and Duf in the proper arrangement of the testis muscles itself or their filaments after they reached their final position on the testis.

Acknowledgements

We thank Christina Rathke for introducing us to the dissection and analysis of the male reproductive system of D. melanogaster. We thank Anja Rudolf and Helga Kisselbach-Heckmann for help with the specimens for SEM. We thank Katja Gessner for excellent secretarial assistance, Detlev Buttgereit and Christine Dottermusch-Heidel for critical reading, and Karen A. Brune for excellent editing. Gratefully we acknowledge the receipt of fly strain sns-mCherryNLS from Susan M. Abmayr, Stowers Institute for Medical Research, Kansas City, MO, USA. Trol::GFP is a protein trap line kindly provided by Christian Klämbt, Universität Münster, Germany. We received gratefully fly strains from Vienna Drosophila RNAi Center, Austria. This work was supported by the Deutsche Forschungsgemeinschaft Re 628/14-3, Re 628/15-2, and GK 1216.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.07.022.

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