Philipps Universität Marburg

Cytoskeletal Components during Myogenesis of *Drosophila melanogaster:* Microtubules vs. Myosins as Actin Motor Proteins

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Content

1 Erklärung des Eigenanteils an den Publikationen

Laut §9, Absatz 1 der Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fachbereiche und des Medizinischen Fachbereichs für seine mathematisch-naturwissenschaftlichen Fächer der Philipps-Universität Marburg (Fassung vom 15.07.2009) können „Publikationen, die aus der Promotionsarbeit hervorgegangen sind und in angesehenen Zeitschriften […] veröffentlicht oder eingereicht wurden, […] als Dissertationsleistung anerkannt werden (kumulative Dissertation). In diesen Fällen ist […] in einer gesonderten Erklärung darzulegen, welchen Anteil die Doktorandin oder der Doktorand an den Publikation hatte." Der Anteil der Doktorandin an den Publikationen wird im Folgenden detailliert erläutert:

Kapitel 5: The syncytial visceral and somatic musculature develops independently of β3-Tubulin during *Drosophila* embryogenesis, while maternally supplied β1-Tubulin is stable until the early steps of myoblast fusion

- Durchführung von 95% der Experimente (Kreuzungsgenetik, Immunhistochemische Proteinnachweise, *In situ*-Hybridisierung, Licht- und Fluoreszenz-Mikroskopie, Generierung von GFP- und Myc-markierten *β3-Tubulin*-Konstrukten und transgenen Fliegen, Zellkulturexperimente, Western Blot),
- Raster-Elektronenmikroskopie in Zusammenarbeit mit Dr. K.-H. Rexer,
- Anfertigung aller Tabellen und Bildtafeln,
- Anfertigung des Manuskripts, in Zusammenarbeit mit Prof. R. Renkawitz-Pohl (Korrektur).
- Dieses Kapitel wurde in der vorliegenden Form im European Journal of Cell Biology am 03.02.2012 online und in Volume 91, Issue 3 im März 2012 veröffentlicht.

Kapitel 6: Myosin heavy chain-like interacts with Rolling pebbles 7 during *Drosophila* myogenesis

- Durchführung von 40% der Experimente (*In situ*-Hybridisierung, Immunhistochemische Proteinnachweise an Embryonen und adulten abdominalen Muskeln, Licht- und Fluoreszenz-Mikroskopie, Generierung von *Mhcl*- und *rols7*-Konstrukten in Zusammenarbeit mit C. Hornbruch-Freitag, Etablierung von transgenen Fliegen, Kreuzungsgenetik),

- Detaillierte Analyse der Transkription von Mhcl im Embryo (Abb. 2) sowie des Expressionsmusters in der adulten abdominalen Muskulatur in Zusammenarbeit mit J. Kuckwa (Abb. 3), Fraß- und Überlebensassay in Zusammenarbeit mit C. Hornbruch-Freitag (Abb. S2), Untersuchung der Lokalisation von ektopisch exprimierten, GFP-markierten Mhcl-Konstrukten im Wildtyp und in Fusionsmutanten (Abb. 5), Supervision der Bachelor-Arbeit von L. Kastl (Tabelle S1),
- Erstellung von 70% aller Bildtafeln,
- Anfertigung des Manuskripts in Zusammenarbeit mit B. Bonn (Anfertigung der Manuskript-Rohfassung, Stand 2010) und Prof. R. Renkawitz-Pohl (Korrektur).
- Dieses Kapitel wurde in der vorliegenden Form am 20.06.2012 in der Zeitschrift Experimental Cell Research eingereicht und befindet sich in Revision.

Kapitel 7: Formation of *Drosophila* longitudinal visceral muscles requires Rolling pebbles 7, Kette and Blow

- Durchführung von 85% der Experimente (Kreuzungsgenetik, Immunhistochemische Färbungen, Licht- und Fluoreszenz-Mikroskopie, Etablierung eines Protokolls zur immunhistochemischen Proteinnachweisen an isolierten Därmen mit umliegender Muskulatur aus Embryonen und Larven),
- Analyse der Entwicklung der Darm-Muskultur im Wildtyp (Abb. 1, 2, 3), Analyse des *rols7*, *blow* und *kette* mutanten Phänotyps in der viszeralen Muskulatur (Abb. 5, 6),
- Anfertigung aller Bildtafeln,
- Anfertigung des Manuskripts in Zusammenarbeit mit Prof. R. Renkawitz-Pohl (Korrektur).

Doktorandin

Betreuerin

2 Summary

The syncytial larval musculature of *Drosophila melanogaster* develops during embryogenesis by fusion of two different cell types, the founder cells (FCs), which determine the identity of an individual muscle, and the fusion competent myoblasts (FCMs). During the fusion events in the somatic mesoderm, which gives rise to the body wall muscles, a Fusion-restricted Myogenic-Adhesive Structure (FuRMAS) is established, consisting of a ring of adhesion molecules and their adaptor proteins as well as of an actin-rich plug on the side of the FCM and an actin sheet on the side of the FC. This FuRMAS is supposed to act as a signaling center, linking cell adhesion to downstream fusion steps.

The actin cytoskeleton is supposed to be important in processes like vesicle transport, fusion pore expansion and force generation at the FuRMAS. This thesis provides analyses of transport processes via microtubules during myogenesis by analyzing the expression of β-Tubulin isoforms, as the structural subunits of microtubules. Although strongly expressed in the mesoderm, the somatic and visceral musculature develops independently of the β3-Tubulin isoform. Furthermore, a low level of maternally supplied β1-Tubulin is sufficient for body wall muscle formation. Thus, it is concluded that newly synthesized microtubules and microtubule-based transport processes are less important for *Drosophila* myogenesis.

Rolling pebbles 7 (Rols7) is an essential adaptor protein at the FuRMAS on the side of the FCs, which interacts *in vitro* with Myosin heavy chain-like (Mhcl). In this thesis, it is shown that Mhcl is also expressed in FCs, and localizes at the contact sites towards the adhering FCM. This unconventional myosin might act as a motor protein for F-actin at the FuRMAS, being involved in vesicle transport or widening of the fusion pore, most likely redundantly to other myosin heavy chains.

The syncytial visceral muscles of the embryo surround the gut as a network of binucleated circular muscles and perpendicularly arranged multinucleated longitudinal muscles. The longitudinal FCs migrate from the caudal visceral mesoderm and are shown in this thesis to fuse with a different FCM type than the circular FCs do. Furthermore, Rols7 is needed during myoblast fusion giving rise to the longitudinal gut muscles, while proteins regulating actin polymerization are involved either already in migration of the longitudinal FCs or also in the fusion processes itself. In conclusion, this muscle type develops distinct to the circular gut muscles and the body wall musculature of *Drosophila*.

3 Zusammenfassung

Die synzytiale larvale Muskulatur von *Drosophila melanogaster* entsteht, indem zwei unterschiedliche Zelltypen miteinander fusionieren, die Identität-vermittelnden Founder Zellen (FCs) mit Fusions-kompetenten Myoblasten (FCMs). Während der Fusion im somatischen Mesoderm wird ein Signalkomplex etabliert, der als Fusion-restricted Myogenic-Adhesive Structure (FuRMAS) bezeichnet wird, bestehend aus einem Ring von Adhäsionsmolekülen sowie einem Aktin-reichen Fokus auf Seiten der FCM und einer Aktin-Schicht auf Seiten der FC. Dieser Signalkomplex verknüpft die Zelladhäsion mit nachfolgenden Fusionsschritten.

Das Aktin-Zytoskelett nimmt während der Myoblastenfusion wahrscheinlich wichtige Funktionen ein, z.B. den Transport von Vesikeln, der Expansion der Fusionspore sowie die Generierung von Kraft, um die FCM in den wachsenden Muskel zu integrieren. In dieser Arbeit wird untersucht, ob zusätzlich zum Aktin-Zytoskelett Transportprozesse über Mikrotubuli involviert sind, indem die Expression von β-Tubulinen, den strukturellen Untereinheiten der Mikrotubuli, analysiert wird. Trotz der starken Expression im Mesoderm entwickelt sich die embryonale Muskulatur unabhängig von der β3-Tubulin Isoform. Desweiteren sind geringe Mengen des maternal bereitgestellten β1-Tubulin ausreichend für die Entwicklung der Körperwandmuskulatur, sodass Mikrotubuli-basierte Transportprozesse während der *Drosophila* Myogenese eine untergeordnete Rolle zu spielen scheinen.

Rolling pebbles 7 (Rols7) stellt ein essentielles Adaptor-Protein an den FuRMAS auf Seiten der FC dar, welches *in vitro* mit Myosin heavy chain-like (Mhcl) interagiert. In dieser Arbeit wird gezeigt, dass Mhcl ebenfalls in FCs exprimiert wird und an der Kontaktstelle hin zur adhärierenden FCM lokalisiert. Dieses unkonventionelle Myosin könnte als Aktin-Motorprotein an den FuRMAS agieren und am Transport von Vesikeln oder dem Weiten der Fusionspore beteiligt sein, wahrscheinlich in Redundanz zu anderen Myosinen.

Die synzytiale viszerale Muskulatur im Embryo umgibt den Darm als Netzwerk aus binukleären zirkulären Muskeln und multinukleären longitudinalen Muskeln. Diese Arbeit zeigt, dass die longitudinalen FCs mit einem anderen FCM-Typ als die zirkulären FCs fusionieren. Das Adapter-Protein Rols7 ist an diesem Fusionsprozess beteiligt, während Proteine, die die Aktin-Polymerisierung während des somatischen Fusionsprozesses regulieren, bereits in der Wanderung der longitudinalen FCs oder ebenfalls in der Entstehung von Synzytien involviert sind.

4 Background

The fruit fly *Drosophila melanogaster* is an intensively studied model organism due to its short generation time and excellent genetic availability. Through the years, it has become clear that many developmental and physiological processes share similar key players on the cellular and molecular level with those in vertebrates. This gave researchers the interesting possibility to study those processes first in *Drosophila* before turning to vertebrate model organisms which are less accessible for genetic manipulation. One of those research fields is the formation of multinucleated myofibers during myogenesis in the *Drosophila* embryo. A lot of research has been carried out regarding the differentiation of mesodermal cells into myoblasts, the fusion of these cells to form a syncytium, the arrangement and the attachment of the myotubes at their precise position within the embryo, and the formation of the sarcomeres as a prerequisite of the larval movement. Establishing multinucleated myotubes requires cell-cell fusion, a mechanism which also applies for other developmental and physiological processes like e.g. sperm-egg fusion during fertilization, somatic cell-cell fusion in the development of the nematode *C. elegans* or fusion of macrophages giving rise to bone-absorbing osteoclasts (reviewed in Oren-Suissa and Podbilewicz, 2010). Studying myoblast fusion in *Drosophila* is well accessible, making use of forward and reverse genetics as well as easily generated transgenic animals. By analyzing the process in detail, researchers hope to reveal involved proteins, interactions and signaling cascades not only common with those in vertebrate myoblast fusion, but also with other cell-cell fusion events.

4.1 Development of the *Drosophila* larval musculature

The musculature of *Drosophila* larvae is established during embryonic development and is subdivided into the somatic body wall musculature, the visceral gut musculature and the heart musculature. While in vertebrates, several myofibers are packed together to form a muscle, one myotube of the *Drosophila* larval body wall represents one single muscle. Every somatic myotube is multinucleated, characterized by a distinct number of nuclei, shape, and position within the embryo. The overall body wall musculature exhibits a highly repetitive pattern of 30 dorsal, ventral and lateral muscles repeating in the abdominal hemisegments (Bate, 1990). The myotubes are attached to the epidermis and allow the movement of the larva after it has hatched out of the eggshell. The visceral muscles, unlike the vertebrate gut musculature, are also syncytial (Klapper et al., 2001), while the heart musculature, located in late stages at the dorsal most part of the embryo, consists of mononuclear cardiomyoblasts arranged in two rows (Lehmacher et al., 2012). All muscle types are of mesodermal origin and become specified from early embryonic stages on.

4.1.1 Determination of the somatic mesoderm

The mesodermal germ layer giving rise to the muscles, the fat body, and the heart, is determined at the ventral most portion of the embryo at the blastoderm stage. The maternally contributed morphogene Dorsal activates the expression of the transcription factor Twist (Twi), which, together with other intrinsic factors as well as with extrinsic factors secreted from the ectoderm, specifies the different derivates by regulating many mesodermal downstream genes (Riechmann et al., 1997; Tixier et al., 2010). During gastrulation, the Twi expressing cells lose their epithelial character, invaginate, and migrate in dorso-lateral direction, forming a monolayer of mesodermal cells along the ectoderm (Leptin and Grunewald, 1990).

The mesodermal cells which are located in the *lethal of scute* (*l'sc*) expression cluster then become divided into two different cell types, into founder cells (FCs) and fusion-competent myoblasts (FCMs) (Bate, 1990; Carmena et al., 1995). By Notch-mediated lateral inhibition, only one cell expressing L'sc becomes specified into a muscle progenitor cell, which then asymmetrically divides into two FCs or in one FC and one adult muscle precursor (Carmena et al., 1995; Ruiz Gómez and Bate, 1997). The remaining cells become FCMs and express the FCM specific transcription factor Lame duck (Lmd) (Duan et al., 2001; Ruiz-Gómez et al., 2002). The FCs are characterized by the differential expression of identity genes which ensure the right number of fusion events as well as the position and orientation within the embryo (reviewed in Tixier et al., 2010). Furthermore, both types of myoblasts are determined by Myocyte enhancing factor 2 (DMef2), which regulates the transcription of several muscle identity genes as well as of genes encoding contractile muscle proteins (Bour et al., 1995; Lilly et al., 1994; Sandmann et al., 2006).

4.1.2 Fusion of somatic myoblasts is characterized by two distinct temporal phases

The mature myofibers arise from cell-cell fusion of the two differently specified cell types, the FCs and the FCMs. The myoblast fusion process itself can be divided into two temporal phases (Bate, 1990; Beckett and Baylies, 2007). In a first fusion phase, one FC fuses with one or two FCMs, forming a precursor cell containing two or three nuclei. The precursor cell then fuses with additional FCMs until a myotube with the determined number of nuclei is formed (Fig. 4.1; Rau et al., 2001). The specific expression of identity genes within the FC determines the number of fusion events; the nuclei of the fused FCMs adopt this expression pattern. A similar two-phase model also applies to the vertebrate myoblast fusion process, although a specialized cell type equivalent to the *Drosophila* FCs has not been identified (reviewed in Pavlath, 2010). It has been proposed that the two temporal phases are also genetically distinct (Berger et al., 2008; Massarwa et al., 2007; Rau et al., 2001) as well as distinct at the ultrastructural level (Doberstein

et al., 1997; Schröter et al., 2004), while another model opposes genetic differences and sets value on the spatial arrangement of FCs and FCMs influencing the frequency of fusion events (Beckett and Baylies, 2007). While the formation of the musculature as a whole takes about 5,5 hours during embryonic development, the individual cell-cell fusion event itself is only a matter of minutes (Beckett and Baylies, 2007).

At the morphological level, the FCMs adopt a teardrop-like shape, migrate towards the FCs/precursor cells and adhere to them. Former studies revealed ultrastructural features after adhesion of the FCM exclusively in the second phase of fusion (Fig. 4.1): at first, electron-dense vesicles, supposed to contain essential molecules of the fusion machinery, pair at both membranes and form a so-called prefusion complex. After that, this prefusion complex gives way to electron-dense plaques as a likely intermediate between the paired vesicles and fusion pore formation. Next, many small fusion pores are established in a way that the membrane vesiculates and breaks down, which eventually leads to a cytoplasmatic continuity of FCM and precursor cell (Doberstein et al., 1997). At last, the several fusion pores unite and expand, so that the content of the FCM is integrated into the growing myotube.

It has been proposed that during fusion, the membranes are brought into close contact, enabling multiple fusions of the outer leaflets to occur via a hemifusion state, which leads to the observed multiple areas of membrane vesiculation (Önel et al., 2011). In an alternative ultrastructural study, one single fusion pore instead of multiple small ones has been observed, with finger-like protrusions from the FCM invading the FC. These structures are being discussed to apply the mechanical forces to bring the membranes of the two cell types in close proximity (Sens et al., 2010).

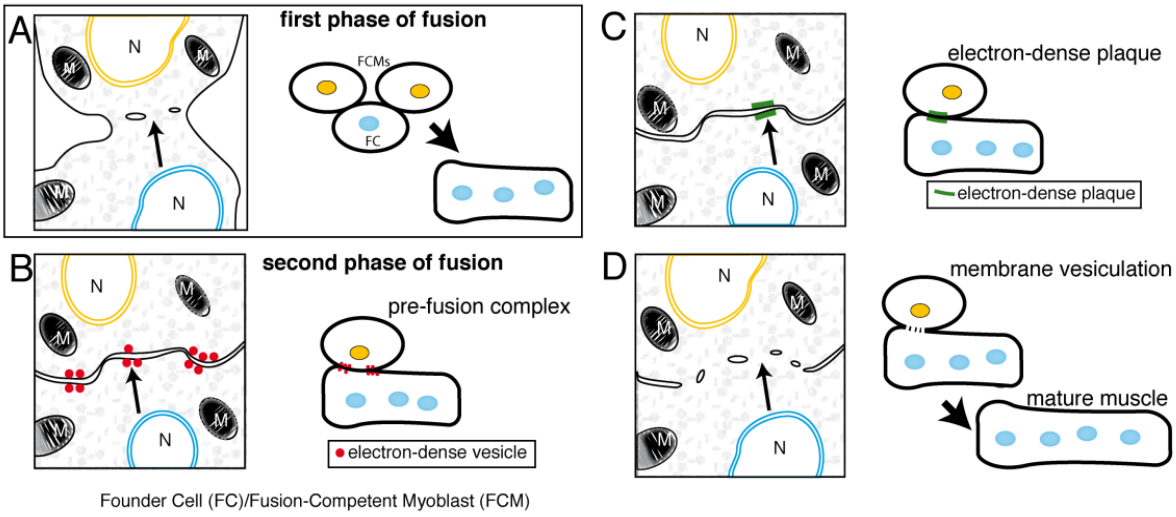

Figure 4.1: Myoblast fusion in the somatic mesoderm of *Drosophila* occurs in two distinct phases. (A) In the 1st phase, one or two FCMs (yellow nuclei) fuse with one FC (blue nuclei). (B-D) In the 2nd phase, this precursor cell fuses with additional FCMs. Ultrastructural features can be detected only at the 2nd phase of fusion, namely electron-dense vesicles (B) and electron-dense plaques (C), before the membrane vesiculates (D). Modified after Önel et al. (2011).

4.1.3 Fusion-restricted Myogenic-Adhesive Structures define the fusion site

Through the past years, several proteins have been shown to participate in the fusion process (Fig. 4.4; reviewed in Abmayr and Pavlath, 2012). The recognition and adhesion between the FCM and the FC/precursor cell is established by molecules of the Immunoglobulin (Ig) super family. Dumbfounded/Kin-of-IrreC (Duf/Kirre) is solely expressed in FCs (Ruiz-Gómez et al., 2000) and acts redundantly to Roughest/Irregular-optic-chiasma-C (Rst/IrreC), which is expressed in FCs as well as in FCMs. A complete block of fusion can only be observed when both genes are deleted (Strünkelnberg et al., 2001). On the side of the FCM, adhesion is mediated by Sticks-and-Stones (Sns) (Bour et al., 2000) by direct binding of its extracellular Ig loops to the Ig loops of Duf; the binding of Sns to Rst has also been proven (Galletta et al., 2004). As ectopic expression of Duf results in FCM migration towards these sites, it has been speculated that the FCMs migrate along a gradient of secreted Duf towards the FCs (Ruiz-Gómez et al., 2000; Strünkelnberg et al., 2001). A fourth Ig-like molecule, Hibris (Hbs), is also expressed in FCMs and interacts with Duf, but not with Rst (Artero et al., 2001; Dworak et al., 2001). Analyses of *sns* and *hbs* double mutants have shown that Hbs can, to a small degree, direct precursor formation in absence of Sns; a partial functional redundancy between both proteins has therefore been proposed (Shelton et al., 2009). In addition to the Ig-like adhesion receptors, the adhesion protein N-cadherin is present in both cell types and presumably needs to be removed from the membrane to allow fusion (Dottermusch-Heidel et al., 2012; see below).

During the second phase of fusion, Duf and Sns are arranged in a ring-like structure surrounding an F-actin focus at the membrane of the FCM (Haralalka et al., 2011; Kesper et al., 2007; Richardson et al., 2007) and a thinner actin sheet at the membrane of the precursor cell, at sites of cell contact (Sens et al., 2010). This structure has been defined as Fusion-restricted Myogenic Adhesive Structure (FuRMAS), which is likely to act as a signaling center. When the FCM adheres to the growing muscle, the FuRMAS ring is first 1 μm in diameter and expands to 5 μm when fusion proceeds. This expansion likely resembles the enlargement of the fusion pore and is driven by F-actin rearrangement (Fig. 4.2; Kesper et al., 2007). Furthermore, F-actin was suggested to be involved in transport of the electron-dense vesicles towards the membrane, in membrane vesiculation and in the integration of the FCM into the growing myotube (Fig. 2). The FuRMAS have been compared to other adhesion structures, namely the immunological synapse, podosomes and invadopodia, with which they share the transient nature as well as the F-actin accumulation and polymerization (reviewed in Önel and Renkawitz-Pohl, 2009).

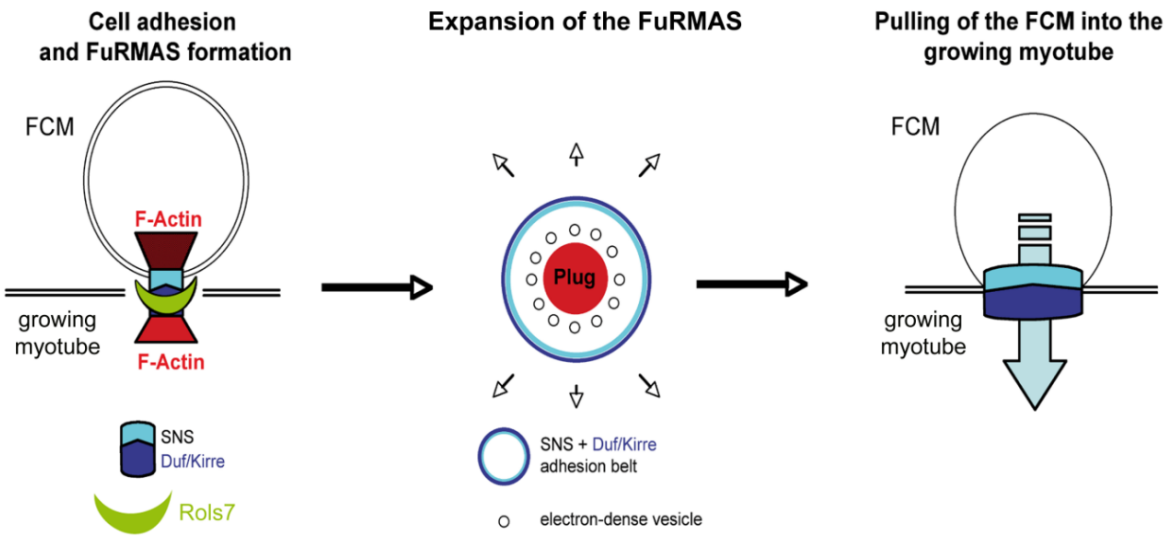

Figure 4.2: F-actin is proposed to be involved in multiple steps during myoblast fusion. An F-actin plug is surrounded by a ring of adhesion molecules during FuRMAS formation. This ring expands and with it the fusion pore. After the cytoplasmatic continuity is achieved, the FCM is pulled into the growing myotube. For all these steps, branched F-actin has been considered important. Modified after Önel and Renkawitz-Pohl (2009).

4.1.4 Rolling pebbles 7 is an essential adaptor protein at the FuRMAS

A further key player during myoblast fusion, the adaptor protein Rolling pebbles 7 (Rols7), is localized at the FuRMAS on the side of the precursor cell; its localization depends on the presence of Duf (Chen and Olson, 2001; Kesper et al., 2007; Menon and Chia, 2001). Rols7 is solely involved in the second phase of fusion, as *rols* deficient embryos exhibit a strong myoblast fusion phenotype, but precursor cells containing two or three nuclei are still present. In *rols* mutants, the FCMs adhere to the growing myotube, but prefusion complexes are not formed (Chen and Olson, 2001; Menon and Chia, 2001; Rau et al., 2001). However, as double mutants for *rols* and *schizo* (*siz*, see below) display only single-nucleated myoblasts, an additional, Duf-independent role for Rols has been suggested (Bulchand et al., 2010).

The *rols* gene encodes for two transcripts which have six of the eight exons in common and use two specifically: Rols6 is expressed in the endoderm, in the Malpighi tubules, and in the apodemes, the muscle attachment sites at the epidermis; it is necessary for Malpighi tubule differentiation (Pütz et al., 2005). Rols7 is exclusively expressed in the mesoderm already in muscle progenitor cells. Both protein isotypes contain several protein-protein interaction domains (Fig. 4.3): (1) A RING finger domain as a form of a zinc finger motif which can mediate protein complex formation (reviewed in Borden, 2000), (2) nine ankyrin repeats organized as a three repeat and a six repeat module, and (3) three tetratricopeptid (TPR) repeats (Chen and Olson, 2001; Menon and Chia, 2001; Rau et al., 2001). TPR repeats are common in multi-protein complexes (reviewed in Blatch and Lässle, 1999); in the case of Rols7, they bind to the

intracellular domain of Duf in a yeast assay (Kreisköther et al., 2006). The TPR and ankyrin repeats are essential for Duf-dependent translocation from the cytoplasm to the membrane and in rescue experiments aiming to restore fusion efficiency in *rols* mutants (Menon et al., 2005). Due to the complex protein structure of Rols7, an interaction with other signaling molecules, actin regulators or motor proteins is plausible. However, a direct interaction partner of Rols7 during fusion, besides Duf, has not been identified *in vivo* yet.

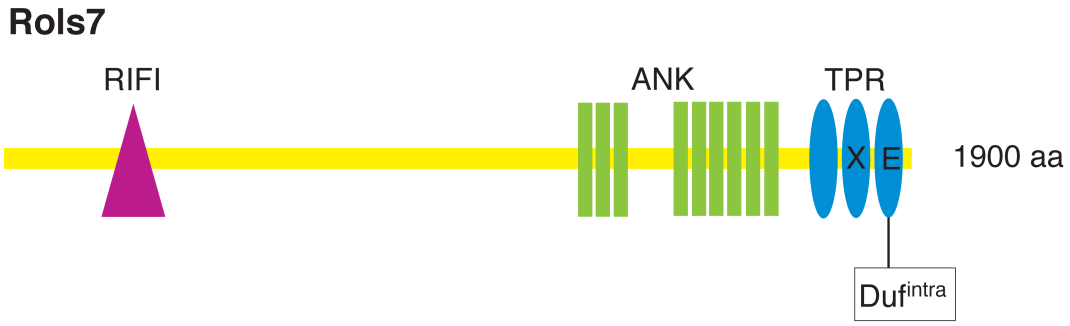

Figure 4.3: Rols7 exhibits many protein-protein interaction domains. RIFI: RING finger, ANK: ankyrin repeats, TPR: tetratricopeptide repeats. TPR-E binds to the intracellular domain of Duf (Duf^{intra}). Modified after Kreisköther et al. (2006).

4.1.5 The link between cell adhesion and F-actin regulation is necessary for the fusion process

As mentioned above, branched F-actin has been proposed to be essential for different events during the fusion process. Actin polymerization is mediated by the Arp2/3-complex in both the FC and the FCM (reviewed in Önel et al., 2011). In the FCM, activation of the Arp2/3 complex depends on (1) the WASp/WIP and (2) the Kette/SCAR complex, while in FCs, only activation by the Kette/SCAR complex has been identified so far (Fig. 4.4).

The Wiskott-Aldrich-Syndrome protein (WASp) functions together with the WASp-interacting protein (WIP)/Verprolin1/Solitary and is required only during the second fusion phase, probably in clearance of membrane remnants (Berger et al., 2008; Kim et al., 2007; Massarwa et al., 2007; Schäfer et al., 2007). The ultrastructural study demonstrating the finger-like protrusions from the FCM invading the precursor cell could show that these structures depend on the presence of the WASp/WIP complex (Sens et al., 2010), and an WASp-independent role of WIP during the first fusion phase has also been hypothesized (Berger et al., 2008). The stability of the WASp/WIP complex is regulated by the PH domain protein Blown fuse (Blow), which is essential in the early precursor formation, as only a few bi- or trinucleated precursor cells are present in the mutant situation (Beckett and Baylies, 2007; Doberstein et al., 1997; Schröter et al., 2004). Blow localizes at the FuRMAS on the side of the FCM (Kesper et al.,

2007) and, by its interaction with the WASp/WIP complex, is indirectly involved in actin polymerization at the foci (Jin et al., 2011; Richardson et al., 2007).

The Kette/SCAR complex regulates both actin foci formation in FCMs redundantly to the WASp/WIP complex as well as formation of the thin actin sheet in FCs/precursor cells. As *kette* mutants arrest fusion after formation of electron-dense plaques, SCAR-dependent signaling might be additionally required to form the small fusion pores (Gildor et al., 2009; Richardson et al., 2007; Schröter et al., 2004; Sens et al., 2010). Kette/SCAR is activated by the redundant function of the small GTPases Rac1 and Rac2, which are in turn activated by the guanine exchange factor (GEF) Myoblast city (Mbc) together with its interaction partner ELMO, on the site of the FCM (Fig. 4.4; Geisbrecht et al., 2008; Haralalka et al., 2011). Mbc is required for the early steps of precursor cell formation, being necessary for the integrity of the F-actin plug in this cell type (Doberstein et al., 1997; Erickson et al., 1997; Haralalka et al., 2011; Rushton et al., 1995). Additionally, a role for Mbc and activated Rac1 in migration of the FCMs towards the fusion sites has been proposed (Gildor et al., 2009). The activation of the Kette/SCAR complex in FCs is, most probably, also achieved by Rac1 and Rac2 (discussed in Haralalka et al., 2011).

The reorganization of the actin cytoskeleton has to be linked to successful cell adhesion by specific adaptor proteins, which are used cell-type specifically (Fig. 4.4). The link between Sns-mediated cell adhesion and actin polymerization regulated by WASp/WIP in FCMs might be achieved by the adaptor protein Crk which can bind to WIP biochemically (Kim et al., 2007). In contrast, Mbc function during myoblast fusion is independent of Crk binding, thus it might interact directly with the intracellular domain of Sns (Balagopalan et al., 2006). Less is known about the link between cell adhesion and actin remodeling in FCs/precursor cells. As mentioned above, Rols has been characterized as the adaptor protein for Duf, although subsequent signaling pathways have not been found so far. The GEF Schizo/Loner (Siz) is a further molecule which is recruited to the membrane in a Duf dependent manner and, like Rols, is likely to sustain Duf localization at the membrane (Fig. 4.4; Bulchand et al., 2010; Chen et al., 2003). The GTPase for Siz, Arf6, has been proposed to activate the SCAR pathway either directly or via Rac1 (Abmayr and Pavlath, 2012; Chen et al., 2003). However, *arf6* mutant embryos display no fusion phenotype (Dyer et al., 2007), so the involvement of Arf6 is not resolved completely. Reanalysis of Siz function could show that Siz is required for the regulation of N-cadherin, most probably for removing this adhesion molecule from the membrane during fusion, and that this interaction is Arf1 dependent (Dottermusch-Heidel et al., 2012). Siz is also present in FCMs outside of the actin foci, but it is not clear whether it has a function in this cell type (Richardson et al., 2007). As another possible adaptor protein, Dock/Nck was shown to bind *in vitro* to the adhesion molecules Duf, Rst, Sns and Hbs as well as to WIP and WASp, suggesting that it might link cell adhesion to actin remodeling in either FCs or FCMs or both (Kaipa et al., 2012).

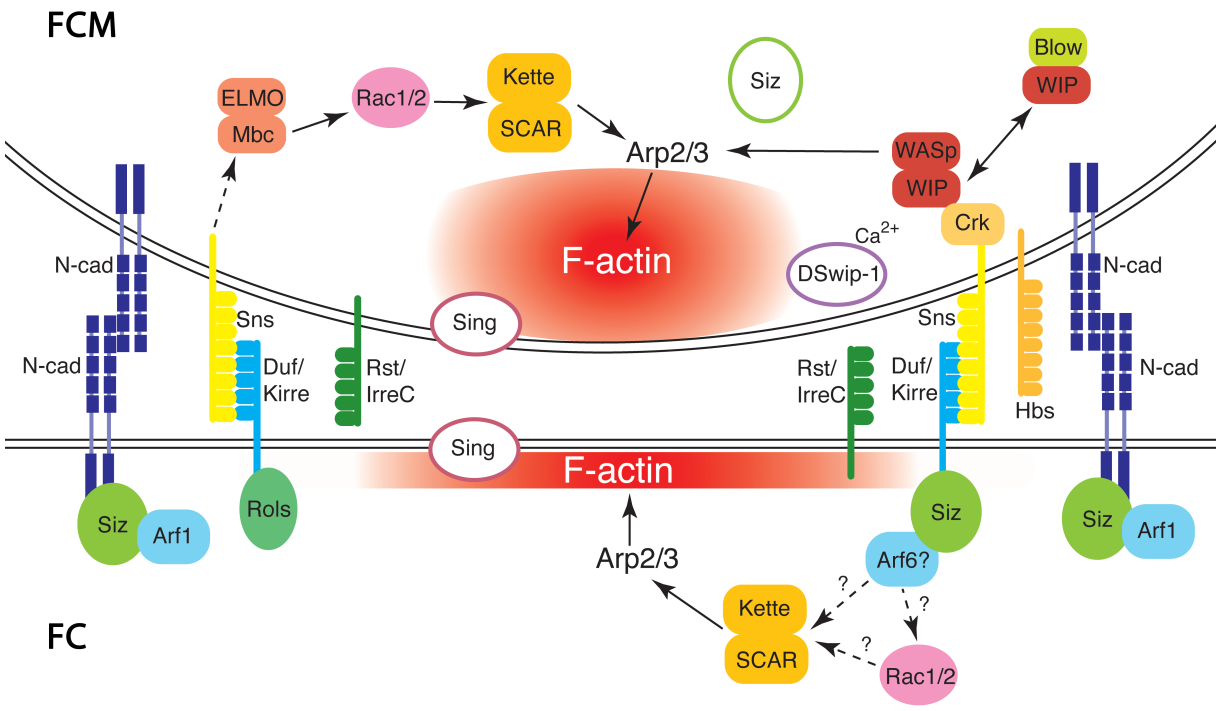

Figure 4.4: Overview of involved proteins during the 2nd fusion phase of *Drosophila* myoblast fusion. Continuous arrows indicate a confirmed activating function, broken arrows indicate a proposed but yet unconfirmed interaction. Proteins indicated with empty circles are yet not integrated into existent pathways. Abbreviations are explained in the text.

There are other proteins involved in myoblast fusion without a direct link to other molecules or pathways: The transmembrane MARVEL domain protein Singles bar (Sing) is expressed in both cell types. Embryos mutant for this protein display an excessive number of prefusion complexes; thus, the protein has been implicated in fusion of the electron-dense vesicles with the membrane (Estrada et al., 2007). As a further FCM-specific protein, DSwiprosin-1 (DSwip-1) accumulates in foci in this cell type, transiently overlapping with F-actin and accumulating in *sing* mutants, indicating that it might take over a function in breakdown of the prefusion complex. As the protein contains two EF-hand-type domains implicated in binding calcium, the presence of DSwip-1 might be a first hint that calcium signaling is involved during myoblast fusion (Hornbruch-Freitag et al., 2011).

4.1.6 Establishing the sarcomere

After the growing myotube has fused to the predetermined number of FCMs, the muscle stretches and attaches to the epidermal tendon cells or apodemes. Extrinsic factors from the ectoderm affect the path finding of the muscles, while the muscle itself secretes factors which initiate the final determination of the tendon cells. The myotendinous junction itself is formed via heterodimerization of α- and β-PS-Integrins expressed in both the myotube and the tendon cell (reviewed in Schweitzer et al., 2010).

To enable movement of the larva, a huge number of proteins have to be arranged into the sarcomeres, which represent the contractible subunits of the muscle. One sarcomere is defined as the distance between two Z-discs and consists of parallel arranged F-actin, called the thin filaments, and myosin thick filaments (Fig. 4.5). The mechanical force needed for contractility is accomplished via the actomyosin ATPase cycle, enabling the sliding of the myosin heads along the passive actin filaments (reviewed in Takagi et al., 2004). In an inactive state, the myosin-binding sites of F-actin are covered with Tropomyosin, which is held in the blocking position by the Troponin complex. In case of Ca^{2+} influx, Tropomyosin changes into an open state, crossbridges between actin and the myosin heads are facilitated and the sliding mechanism and eventually contraction of the sarcomere is achieved (reviewed in Bullard and Pastore, 2011).

The sarcomere is stabilized by a third filament system called connecting filaments, consisting of very large, elastic proteins which link the Z-discs with the thick filaments. The Z-discs themselves are characterized by α-Actinin, which crosslinks the actin filaments (Fig. 4.5; Dubreuil and Wang, 2000; Luther, 2000). In vertebrates, the largest identified protein Titin spans half the length of the sarcomere. It acts like a molecular spring and contributes to the myofibril stiffness and elasticity (reviewed in Clark et al., 2002). In *Drosophila* indirect flight muscles (IFM), the function of Titin is taken over mainly by diagonal Projectin filaments (Fig. 4.5; reviewed in Bullard et al., 2005). The *Drosophila* Titin gene orthologue *sallimus* (*sls*) encodes for several protein isoforms, among others for Kettin, the most abundant isoform (Burkart et al., 2007). Kettin is also part of the connecting filaments in many types of muscles, and is, e.g., responsible for most of the high passive stiffness of IFM. It is bound to α-Actinin and actin, crosslinking the thin filaments to the Z-discs (Kulke et al., 2001; Lakey et al., 1993; van Straaten et al., 1999). As mutants establish only unstable Z-discs and thick and thin filaments, Kettin is further required for correct sarcomere establishment itself (Hakeda et al., 2000).

The FuRMAS adaptor protein Rols7 colocalizes at the Z-discs with α-Actinin and Kettin, and an interaction with α-Actinin and with the smaller Sls isoform Zormin has been observed in yeast. Therefore, a function for Rols7 during sarcomere assembly or as a linker between the connecting filaments, additionally to its role in myoblast fusion, has been suggested (Kreisköther et al., 2006). A full-length Sls isoform, previously called D-Titin, might also be involved in

myoblast fusion, as Sls/D-Titin is enriched on sites of cell contact, and mutants exhibit fusion defects and abnormal muscle morphology (Machado and Andrew, 2000; Zhang et al., 2000).

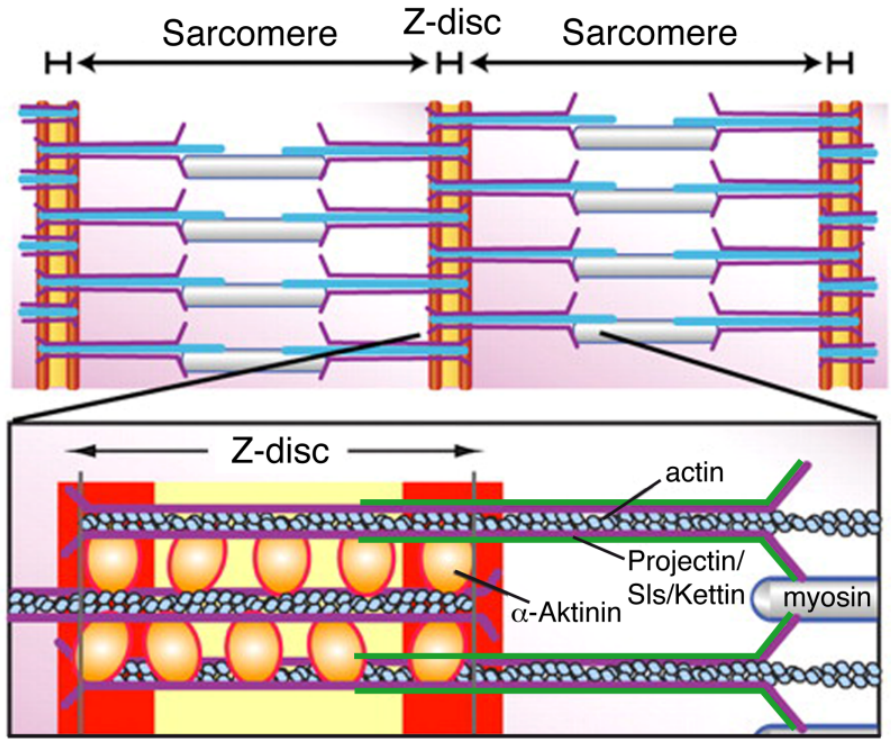

Figure 4.5: Sarcomeric structure of the *Drosophila* IFM. The sarcomere is defined as one subunit of the myotube, flanked by the Z-discs. The Z-discs mainly contain α-Actinin. The actin and myosin filaments are stabilized by connecting filaments consisting of isoforms encoded by the *sls* gene. Modified after Bullard et al. (2005), Clark et al. (2007).

4.2 Microtubules are crucial for vertebrate myogenesis, while their function in *Drosophila* myoblasts is not clear

In vertebrate myogenesis, not only the actin cytoskeleton, but also microtubules have long been considered important. In primary myogenic cell culture systems, microtubules are arranged in longitudinal arrays after myoblast fusion and most likely contribute to the bipolar morphology of the myotube (Bischoff and Holtzer, 1968; Bugnard et al., 2005; Tassin et al., 1985; Toyama et al., 1982; Warren, 1974). The establishment of the sarcomeric structure in cultured skeletal myoblasts also depends on microtubules, namely the arrangement of sarcomeres itself as well as the proper assembly of myosin and Titin molecules within the sarcomere (Pizon et al., 2005; Pizon et al., 2002; Toyama et al., 1982). In addition, the microtubule plus-end binding protein EB1 is required for myoblast differentiation, fusion, and myotube elongation in murine cells (Zhang et al., 2009). Less is known about microtubules in *Drosophila* muscle cells. Ultrastructural studies could show the presence and the position of microtubules within the myofiber: At the end of embryogenesis, a few microtubules are intermingled with thick and thin filaments during sarcomere assembly and are later arranged in parallel as it is the case in mammalian cell culture. Therefore, microtubule-based transport of contractile or connecting proteins might be necessary for establishing the sarcomeres similar to the situation in vertebrates (Dettman et al., 1996). Also later during the development of the IFM in pupae, microtubule sleeves were discussed to serve as a passive support, locating thick and thin filaments at the periphery of the myofibril and thus promote filament assembly (Reedy and Beall, 1993). Microtubule-associated proteins (MAPs) like RacGAP50C and Pavarotti, which regulate the microtubule network via the microtubule nucleator γ-Tubulin during cytokinesis, are needed for correct myotube elongation in embryos (Guerin and Kramer, 2009). Moreover, MAP7 and kinesin-heavy chain are essential in positioning of the nuclei within the myotubes (Metzger et al., 2012); nevertheless, these proteins are dispensable for myoblast differentiation and fusion, and it is not clear whether microtubules themselves are required.

4.2.1 β-Tubulin isoforms are distinctively expressed in *Drosophila*

Microtubules are polarized cytoskeletal elements which are composed of α- and β-Tubulin heterodimer subunits, assembled into linear protofilaments. In *Drosophila,* there are several isoforms of α- and β-Tubulins, which are expressed differently in space and time, and exhibit a high amino acid sequence similarity. β1-Tubulin is already maternally supplied and is part of the microtubules needed for early cell divisions within the embryo. Zygotic expression is restricted to the central nervous system and to muscle attachment sites (Bialojan et al., 1984; Buttgereit et al., 1991; Buttgereit et al., 1996; Raff et al., 1982). The β2-Tubulin isotype is present

in male germ cells, in which it displays the microtubule component during meiotic cell division, and is involved in nuclear shaping of the sperm head; furthermore, β2-Tubulin is part of the sperm axoneme (Fuller et al., 1987; Hoyle and Raff, 1990; Kaltschmidt et al., 1991; Rathke et al., 2010).

β3-Tubulin is strongly expressed in both the somatic and the visceral musculature. Expression starts in early myoblasts, and the protein can be detected in myotubes until the end of embryogenesis as part of the cell's microtubules. The dorsal vessel and the hemocyte-like macrophages are also characterized by the presence of β3-Tubulin, and various regulatory elements regulate the expression of this isotype in all mesodermal derivatives of the embryo (Buttgereit et al., 1996; Damm et al., 1998; Gasch et al., 1989; Hinz et al., 1992; Kimble et al., 1989; Kremser et al., 1999a; Kremser et al., 1999b; Leiss et al., 1988).

4.2.2 β-Tubulins in the *Drosophila* mesoderm - Objective of this study

A lot of research has emphasized the importance of the actin cytoskeleton in vertebrate as well as in *Drosophila* myogenesis (see above). Moreover, transport processes via microtubules appear to be essential for vertebrate myogenesis. It remains unresolved whether these cytoskeletal components are also involved in myogenesis of *Drosophila*, i.e., whether they are essential for myotube formation or for subsequent events like sarcomere assembly. β3-Tubulin is the only isoform zygotically expressed in the mesoderm. This makes it a plausible candidate to elucidate the function of microtubules during myotube formation. Therefore, this study aims at analyzing *β3-Tubulin* mutant embryos regarding the development of the musculature as well as of other organs in which β3-Tubulin is expressed in the wild-type. Furthermore, it is to be elucidated whether the maternally supplied β1-Tubulin isoform as well as a so far undetected expression of β2-Tubulin in the embryo contributes to the microtubule network in muscle cells.

In this study, it is of special interest whether the process of myoblast fusion involves transport processes via microtubules. The FuRMAS at the site of fusion have been compared to podosomes and invadopodia (reviewed in Önel and Renkawitz-Pohl, 2009), and the formation of podosomes as well as podosomal matrix degradation requires kinesin motors along microtubules (Cornfine et al., 2011; Kopp et al., 2006; Linder, 2007; Linder et al., 2000). Therefore, it is of interest to clarify the involvement of microtubules at the FuRMAS, and to elucidate whether they might take over similar functions as in podosomes and invadopodia.

4.3 Myosins act as actin motor proteins in many cellular processes and are conserved across species

Decades ago, myosins have been identified, together with actin, as the contractile elements of striated muscle sarcomeres (see above). Later it became obvious that also non-muscle cells express myosins, which act as motor proteins of the actin cytoskeleton. They are involved in many forms of motility, e.g. cell and organelle movement, cell shape changes, cytokinesis and phagocytosis, and surprisingly also in non-transport mechanisms like transcription or actin organization (reviewed in Hartman et al., 2011).

In case of the classical muscle myosin, the molecules form bipolar filaments and are actually hexamers consisting of two heavy chains, two essential and two regulatory light chains (Fig. 4.6). The heavy chains display a conserved catalytic motor domain (head region), a light chain-binding and regulatory IQ domain (neck region) and a specific tail domain. The motor domain displays Mg^{2+} ATPase activity, converting the ATP stored energy into mechanical force and therefore moving along the actin filaments or translocating other molecules; the regulatory mechanisms and motor properties differ between the several myosins (reviewed in O'Connell et al., 2007). The IQ motifs of the neck region provide binding sites for the light chains as well as for calmodulin or other EF-hand proteins. These motifs ascertain the regulation of the myosin ATPase activity by myosin light chain kinases and phosphatases in dependence of Ca^{2+} (reviewed in Somlyo and Somlyo, 2003). The tail domain contains coiled-coil regions responsible for dimerization and filament formation.

Genomic sequence analyses identified a large number of myosins in animals and plants. Based on the domain structure and the sequence similarity of the head domain, the myosin heavy chains were classified into at least 18 classes, with the conventional muscle myosin grouped into class II. Other members of the superfamily were classified due to other domains present in the molecules, like ankyrin repeats, FERM or PDZ domains (Berg et al., 2001). Another member of class II myosins is the non-muscle myosin heavy chain II. It is especially important as part of the actomyosin network responsible for the cellular contractility in cell migration, cell division and apical constriction (reviewed in Levayer and Lecuit, 2012).

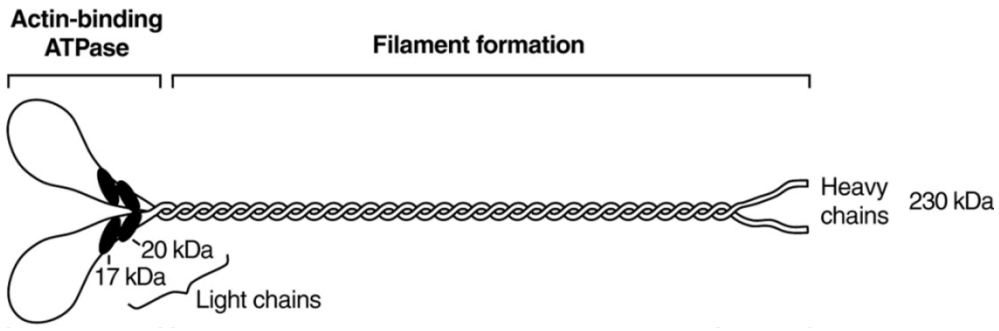

Figure 4.6: Structure of a myosin hexamer. Two myosin heavy chains of a size of 230 kDa each dimerize via the rod domain. The motor domain can bind to actin and can display ATPase activity. The smaller essential and regulatory light chains are bound to the neck region. Modified after Conti and Adelstein (2008).

4.3.1 Conventional and unconventional myosins in *Drosophila*

While the human genome encodes approximately 40 myosins, only 13 myosin heavy chain encoding genes have been identified in *Drosophila* (Foth et al., 2006; Tzolovsky et al., 2002; Yamashita et al., 2000). Only one of them is a classical muscle myosin heavy chain II (Mhc) gene, but it encodes more than 13 protein isoforms with different spatiotemporal expression patterns (George et al., 1989; O'Donnell et al., 1989). For example, alternative Mhc hinge regions are expressed in different types of muscles, like the S2 hinge region used only in the fast flight and jump muscles (Suggs et al., 2007). Mutations of the *mhc* gene have been characterized as dominant flightless and recessive lethal (Swank et al., 2000 and references therein). As already heterozygous mutants display severely disorganized sarcomeres, Mhc might, besides its function as the contractile element, be necessary for sarcomere assembly and integrity as part of the Myosin-Flightin complex (Ayer and Vigoreaux, 2003; O'Donnell and Bernstein, 1988).

The homologue of the vertebrate non-muscle myosin heavy chain IIA is Zipper (Zip), the only other member of myosin class II in flies. Zip, like its counterpart in vertebrates, is involved in processes like cell shape changes, cell polarity and migration. The roles of Zip, its regulatory light chain encoded by the *spaghetti squash (sqh)* gene and a number of regulating kinases and phosphatases are most extensively studied during gastrulation and dorsal closure of the embryo as well as in border cell migration in the egg chamber (reviewed in Conti and Adelstein, 2008; Vicente-Manzanares et al., 2009). Additionally to its function in epithelial cells, Zip is also present at the muscle termini in late embryos and at the Z-discs in larval muscles; analyses of *zip* mutant embryos suggested that Zip is required for proper sarcomeric integrity (Bloor and Kiehart, 2001).

As an unconventional myosin, myosin heavy chain-like (Mhcl) exhibits a PDZ domain at the C-terminal end and has therefore been grouped into class XVIII, displaying approximately 40

% sequence similarity to human and mouse PDZ myosins. Moreover, it exhibits high sequence similarity to class II myosins and, alike the member of this class, its coiled-coil region at the C-Terminus is predicted to mediate homodimerization (Tzolovsky et al., 2002). All six identified transcripts encode for proteins sharing the conserved coiled-coil region, while four isoforms contain a motor domain and only three contain the PDZ domain. The motor domain binds *in vitro* to actin, but does not have ATPase activity, suggesting that Mhcl functions as an actin tether protein (Guzik-Lendrum et al., 2011). The other unconventional *Drosophila* myosin family members have mainly been implicated in the actin-based transport of mRNAs, proteins or vesicles, in a variety of cell types.

4.3.2 The function of Mhcl in the developing muscles - Objective of this study

It is still unknown in which tissue the unconventional myosin heavy chain Mhcl is required and which function it has in *Drosophila*. In first analyses, Mhcl has been shown to interact with Rols7, the adaptor protein at the FuRMAS on the side of the FC (Kreisköther, 2005). Furthermore, Mhcl is expressed in the mesoderm of *Drosophila* embryos (Bonn, 2010). The regulation and polymerization of F-actin plays an important role at the FuRMAS during myoblast fusion (see above), and myosins are plausible candidates to trigger transport processes along F-actin as molecular motors. Furthermore, it is still unknown which pathways are activated by the adaptor protein Rols7. An interaction of Rols7 with Mhcl could thus connect cell adhesion and subsequent fusion steps like vesicle movement or fusion pore expansion. Therefore, this study is aimed on analyzing the localization and function of Mhcl in the developing musculature and on answering the question, whether this unconventional myosin heavy chain could represent the link between cell adhesion and actin-mediated transport processes or force generation at the FuRMAS.

4.4 The visceral musculature develops distinct from the body wall muscles

Unlike in vertebrates, the musculature surrounding the gut of *Drosophila* larvae is also striated and multinuclear. It consists of two types of muscles: the binucleated circular muscles and the longitudinal muscles containing up to six nuclei. The two muscle types are interwoven with each other, establishing a dense network surrounding the endodermal midgut. The outer longitudinal muscles run perpendicularly to the inner lying circular muscles, which are additionally connected by cytoplasmatic bridges (Fig. 4.7). The ectodermal derived hindgut is only surrounded by circular muscles (Bate, 1993; Klapper, 2000; Klapper et al., 2001; Klapper et al., 2002; San Martin et al., 2001).

The circular visceral muscles develop, like the body wall muscles, by fusion of visceral FCs with visceral FCMs. In early embryogenesis, the FCs and FCMs giving rise to the circular muscles are located in metameric clusters within the embryo. These cells change their shape into two continuous layers of columnar cells, forming the trunk visceral mesoderm (TVM) (Azpiazu and Frasch, 1993; San Martin et al., 2001). The visceral FCMs are characterized by expression of the transcription factors Bagpipe (Bap) and Biniou, both essential for TVM formation (Azpiazu and Frasch, 1993; Zaffran et al., 2001). The circular FC fate is induced by signaling of the receptor tyrosine kinase Alk and its ligand Jelly belly, which is secreted from somatic FCs (Englund et al., 2003; Lee et al., 2003; Lorén et al., 2003; Stute et al., 2004; Weiss et al., 2001). The circular FCs fuse one-to-one to the overlaying visceral FCM population to the binucleated syncytia. During closure of the gut, the cells spread dorsally and ventrally, until the whole gut is covered with the circular muscles (Fig. 4.7; Klapper et al., 2002; San Martin et al., 2001; Schröter et al., 2006).

The FCs giving rise to the longitudinal muscles become specified in the caudal visceral mesoderm (CVM) outside of the Twist domain; instead, these cells are determined by the basic Helix-Loop-Helix (bHLH) transcription factor HLH54F (Georgias et al., 1997; Ismat et al., 2010; Kusch and Reuter, 1999). In late stage 10 embryos, the longitudinal FCs start to migrate, under control of FGFR signaling and along the extracellular matrix of the TVM, in anterior direction. During migration, they undergo one mitotic division (Bate, 1993). When they have arrived at the TVM, they have been supposed to fuse with the same FCM population like the circular FCs (Fig. 4.7; Georgias et al., 1997; Kadam et al., 2012; Mandal et al., 2004; Reim et al., 2012; San Martin et al., 2001; Urbano et al., 2011).

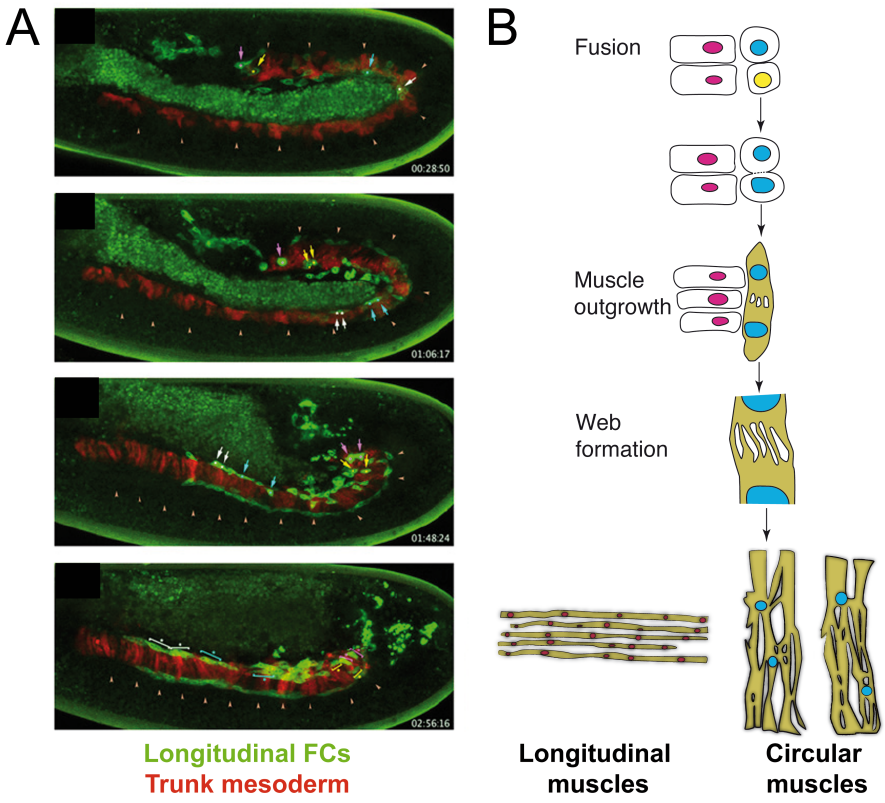

Figure 4.7: The embryonic gut musculature consists of two muscle types. (A) The longitudinal FCs (green) migrate from the posterior tip of the embryo in anterior direction along the trunk mesoderm containing the visceral FCMs (red). Modified after Reim et al. (2012). (B) The circular muscles develop by fusion of one circular FC (blue nucleus) with one visceral FCM (yellow nucleus) via direct membrane breakdown. After that, the cell grows out and cytoplasmatic bridges are formed. Modified after Schröter et al. (2006).

Both FC populations, the circular as well as the longitudinal FCs, express the adhesion molecule Duf, while the visceral FCMs express Sns. In *sns* and *duf* mutants, visceral FCs and FCMs fail to aggregate and fusion is inhibited. Therefore, both adhesion molecules appear to mediate the fusion of the two cell types like in the somatic mesoderm. Mbc is also expressed in visceral FCMs, and *mbc* mutants exhibit aggregated circular FCs and FCMs, but no syncytia are formed (Klapper et al., 2002; San Martin et al., 2001). Interestingly, Blow is present in circular FCs as well as in FCMs of the visceral mesoderm. Nevertheless, formation of the binucleated circular muscles occurs normally, while the stretching around the gut is impaired in both *blow* and *kette* mutants, suggesting that Blow and Kette have different functions in the visceral mesoderm than during the somatic myoblast fusion process. As electron-dense structures cannot be observed during fusion of circular FCs to FCMs, the single fusion event has been compared to the first fusion phase in the somatic mesoderm (Fig. 4.7; Schröter et al., 2006). Mutants for other proteins required for actin polymerization in the somatic myoblast fusion process, such as WIP, WASp and Arp3, display only subtle gut formation phenotypes in the form of a prolonged Duf expression in the visceral mesoderm. The Arp2/3-mediated actin polymerization might therefore be dispensable for visceral myoblast fusion (Eriksson et al., 2010).

After the visceral muscles are established, they subsequently constrict the midgut in four chambers (Campos-Ortega and Hartenstein, 1985; Kusch and Reuter, 1999). The close contact and the interaction between the visceral mesoderm and the underlying endoderm are crucial for correct gut formation (Azpiazu and Frasch, 1993; Reuter et al., 1993; Soplop et al., 2012; Tepass and Hartenstein, 1994). While the outgrowth of the visceral muscles depends on the differentiation of the endoderm as well as on the presence of an extracellular matrix, visceral myoblast fusion is independent of an intact endoderm (Wolfstetter and Holz, 2011; Wolfstetter et al., 2009).

4.4.1 Development of the longitudinal gut muscles - Objective of this study

Many proteins being important during somatic myoblast fusion are also expressed in the visceral mesoderm. The functions of some of them during the visceral myoblast fusion process, giving rise to the binucleated circular muscles, have already been addressed (Eriksson et al., 2010; Klapper et al., 2002; Schröter et al., 2006). However, little is known how the development of the longitudinal muscles takes place, especially whether and to which extent they fuse with visceral myoblasts after they have migrated along the trunk mesoderm. This study is, thus, aimed on analyzing the development of the longitudinal musculature surrounding the *Drosophila* larval midgut. Furthermore, it is still unknown whether proteins with a function during somatic myoblast fusion are also involved in fusion of the longitudinal FCs with the supposed visceral FCMs. Displaying an essential adaptor protein in somatic FCs, the involvement of Rols7 in establishing the longitudinal muscles is to be addressed here. Additionally, the function of the actin regulators Blow and Kette during visceral myoblast fusion is analyzed in this study. These proteins are not necessarily required for the fusion of circular FCs with visceral FCMs (Schröter et al., 2006), and it is intended here to clarify whether these proteins are also dispensable for fusion of longitudinal FCs, which would indicate that indeed the Arp2/3-mediated actin polymerization is less important for the development of the visceral muscles than for the body wall muscles.

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5 The syncytial visceral and somatic musculature develops independently of β3-Tubulin during *Drosophila* embryogenesis, while maternally supplied β1-Tubulin is stable until the early steps of myoblast fusion

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The syncytial visceral and somatic musculature develops independently of β3-Tubulin during *Drosophila* embryogenesis, while maternally supplied β1-Tubulin is stable until the early steps of myoblast fusion

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ABSTRACT

Microtubules are necessary for fusion and elongation of vertebrate muscle cells. In *Drosophila*, several isoforms of β-Tubulin, the functional subunit of microtubules, are expressed in different tissues of the developing embryo, while solely the β3-Tubulin isoform is detected in large amounts during differentiation of the somatic and visceral musculature. Here we show the unexpected result that all mesodermal tissues develop correctly in *β3-Tubulin* loss of function mutants. Furthermore, we show that *β2-Tubulin* transcripts are not detectable in embryos and an exceptional zygotic β1-Tubulin expression in *β3-Tubulin* mutants cannot be observed. Nevertheless, a maternally contributed β1-Tubulin-GFP fusion protein (from protein trap collection, Buszczak et al., 2007, Genetics 175, 1505–1531) acts in a dominant negative way, disturbing embryonic development from early stages on. This effect can be observed to the same extent in a zygotic *β3-Tubulin* mutant situation. Our results indicate that the maternally supplied β1-Tubulin based microtubule network is sufficient for myoblast fusion, myotube elongation and sarcomere formation both during visceral and somatic muscle development in *Drosophila* embryogenesis.

Introduction

Microtubules have long been considered important for vertebrate myogenesis (Bischoff and Holtzer, 1968; Tassin et al., 1985; Toyama et al., 1982; Warren, 1974). From a centrosomal array in myoblasts, microtubules adopt a longitudinal arrangement in myotubes after myoblast fusion; thus, they are possibly involved in maintaining the bipolar morphology of the cell (Bugnard et al., 2005; Tassin et al., 1985; Warren, 1974). In later stages of myotube formation, the lateral alignment of the sarcomeres, the incorporation of sarcomeric myosin, and elongation of Titin are proposed to depend upon a microtubule-dependent transport process, at least in skeletal myoblasts in culture (Pizon et al., 2002, 2005; Toyama et al., 1982). Moreover, the microtubule plus-end binding protein EB1 is required for myoblast differentiation, fusion, and myotube elongation in mouse C2C12 cells (Zhang et al., 2009). Therefore, transport processes via microtubules appear to be essential for the formation of skeletal muscles in vertebrates.

However, less is known about the function of microtubules in the development of *Drosophila* muscle cells, which offer an excellent system to study muscle formation *in vivo* during embryogenesis. As in mammalian myoblasts in culture, microtubules are arranged in arrays parallel to the subsequently formed axis of the myofibers of the larval body wall musculature. The microtubules are intermingled with thick and thin filaments during sarcomere assembly, but only very few microtubules can be detected at the end of embryogenesis (Dettman et al., 1996). Cytokinesis proteins, e.g., RacGAP50C and Pavarotti, which regulate the microtubule network via the microtubule nucleator γ-Tubulin, are necessary for myotube elongation in *Drosophila* embryos (Guerin and Kramer, 2009). Nevertheless, it remains unresolved whether the microtubules themselves are involved in this process. Electron microscopy studies of the developing indirect flight muscles in pupae have revealed that microtubule sleeves might serve as a passive support to concentrate myosin and actin molecules at the periphery of the fibril and facilitate filament assembly (Reedy and Beall, 1993b). It remains to be clarified whether transport mechanisms via microtubules play a role in the formation of the myotubes, i.e., by being necessary for myoblast fusion or elongation, or whether the microtubules are needed solely for the assembly of sarcomeres.

Small gene families encode α- and β-Tubulins in *Drosophila* and are expressed in a highly temporal and spatial pattern. Here we focus on the β-Tubulins, whose individual isoforms exhibit

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90–95% amino acid sequence similarity. β1-Tubulin (βTub56D) is maternally supplied to the embryo and zygotically expressed during neurogenesis and in muscle attachment sites shortly after the insertions of muscles into the epidermis (Bialojan et al., 1984; Buttgereit et al., 1991, 1996; Raff et al., 1982). The β2-Tubulin isotype (βTub85D) is solely expressed during male germ cell development, in which it has multiple functions, including meiotic cell division, nuclear shaping of the sperm head, and axoneme formation (Fuller et al., 1987; Hoyle and Raff, 1990; Kaltschmidt et al., 1991; Rathke et al., 2010). β3-Tubulin (βTub60D) is the only β-Tubulin isotype that is strongly expressed in the differentiating mesoderm until the end of embryogenesis, both in the body wall muscles and in the visceral musculature surrounding the midgut. Additionally, the protein can be detected in other mesodermal derivatives of the embryo, namely the dorsal vessel and macrophages (Buttgereit et al., 1996; Kimble et al., 1989; Leiss et al., 1988). Further transcripts can be found in the chordotonal organs, a subset of mechanosensory organs of the peripheral nervous system (Buttgereit et al., 1996; Dettman et al., 2001). In the individual mesodermal derivatives, β3-Tubulin expression is regulated via various regulatory elements (Damm et al., 1998; Gasch et al., 1989; Hinz et al., 1992; Kremser et al., 1999a,b). During metamorphosis, β3-Tubulin is expressed in the developing adult muscles and optic lobe as well as in imaginal discs; expression in somatic cells of the ovaries and testes persists even in the adult (Currie and Bate, 1991; Hoyle et al., 2000; Kaltschmidt et al., 1991; Kimble et al., 1989).

The genetic analyses of Dettman et al. (1996) provided the first insights into the possible function of β3-Tubulin during embryonic development. Several ethyl methanesulfonate (EMS)-induced mutant alleles of the *β3-Tubulin* gene have been characterized by lethality, male and female sterility, and flightlessness (Kimble et al., 1990). Interestingly, the analysis of the most severe hypomorphic allele ($\beta3t^2$) has revealed defects in gut formation and functioning, yet the body wall musculature is formed in the correct pattern, which indicates that an essential function of β3-Tubulin could lie in the cells of the visceral mesoderm. Dettman et al. (1996) have suggested that the observed lethality of homozygous $\beta3t^2$ larvae is caused by malfunction of the gut because first instar larvae cannot take up nutrients. A possible reason for this malfunction is that the visceral mesodermal cells are required for differentiation of the underlying endodermal cell layer and vice versa (Bienz, 1994; Reuter et al., 1993; Tepass and Hartenstein, 1994; Wolfstetter et al., 2009); therefore, the loss of the microtubule network in the visceral muscles might disturb proper gut formation.

In stage 17 embryos transheterozygous for $\beta3t^2$ and the plexate deletion $Df(2R)Px^2$, few microtubules are present in somatic muscle cells, and sarcomeres are formed correctly. Therefore, Dettman et al. (1996) proposed that sufficient β1-Tubulin allows microtubules to be formed in the somatic mesoderm owing to the long stability of the maternal *β1-Tubulin* mRNA. This mRNA can be detected in all tissues until stage 13 of embryogenesis, in contrast to zygotic expression, which is limited to neurogenesis (Buttgereit et al., 1991). Recently, evidence of *β2-Tubulin* transcripts in the mesoderm has been presented (Jattani et al., 2009). Thus, it is also possible that β2-Tubulin is assembled in the microtubule network of myoblasts when β3-Tubulin is absent and can functionally substitute for β3-Tubulin.

In this study, we addressed whether (1) the visceral and somatic musculature is disturbed in *β3-Tubulin* loss-of-function mutant embryos, which would account for the lethality of homozygous $Df(2R)Px^2$ embryos and the incorrect gut shape and function in $Df(2R)Px^2/\beta3t^2$ larvae observed by Dettman et al. (1996), and (2) whether the absence of β3-Tubulin is compensated by the aberrant expression of β1- or β2-Tubulin isotypes in the visceral and somatic musculature. We show that in *β3-Tubulin* mutant embryos, both the somatic and the visceral musculature are built correctly, as are other tissues in which β3-Tubulin is expressed in the wild-type background. The observed embryonic lethality of embryos carrying the deficiency is likely not to be due to the loss of β3-Tubulin. Our analyses further indicate that an accessory zygotic expression of β1-Tubulin in the mesoderm to replace the β3-Tubulin isoform can be excluded and that an exceptional expression of β2-Tubulin in muscles is very unlikely. Thus, we conclude that zygotically synthesized microtubules are dispensable and that microtubules containing maternally supplied β1-Tubulin are sufficient for the morphogenesis of both the somatic and visceral larval musculature of *Drosophila*.

Material and methods

Drosophila genetics

The following fly stocks were obtained from the Bloomington Drosophila Stock Center: *Df(2R)Exel6082/CyO* (Bl. 7561), $Df(2R)Px^2/CyO$ (Bl. 2604), $\beta Tub60D^2 Kr^{If-1}/CyO$ (Bl. 2), and $P\{GawB\}how^{24B}$ (Bl. 1767). The *β1-Tubulin* protein trap line (CC02069) was obtained from the GFP Protein Trap Database (Buszczak et al., 2007). Double mutants carrying *Df(2R)Exel6082* and the *β1-Tubulin* protein trap locus were generated by meiotic recombination; the obtained fly strains were checked for β3-Tubulin and GFP expression as well as for lethality against $Df(2R)Px^2$.

Flies carrying the reporter construct *crocLacZ* were used for crossings to mark longitudinal founder cells (Häcker et al., 1995). *bapLacZ* is expressed in visceral FCMs (Stute et al., 2004; Zaffran and Frasch, 2002); flies carrying this construct were a gift from M. Frasch, Erlangen. The *rp298LacZ* enhancer trap line (Nose et al., 1998) was used to identify founder cells. Mutant embryos could be distinguished from heterozygous embryos by the *LacZ* insertion marker carried by the balancer chromosome. For scanning electron microscope (SEM) analysis and gut preparations, a balancer chromosome carrying a *GFP* insertion marker (Bl. 6662) was used to identify homozygous embryos and larvae prior to fixation.

Immunohistochemistry and whole-mount in situ hybridization of Drosophila embryos

For immunohistological stainings, the following primary antibodies were used at the indicated dilution: anti-β3-Tubulin 1:3000 (Leiss et al., 1988), anti-β1-Tubulin 1:1000 (Buttgereit et al., 1991), anti-β2-Tubulin 1:50 (Kaltschmidt et al., 1991), anti-Tropomyosin 1:1000 (Abcam plc, Cambridge), anti-eGFP 1:1000 (Abcam plc), anti-β-galactosidase 1:5000 (Biotrend, Köln), anti-Fasciclin3 1:50 (Patel et al., 1987), anti-DMef2 1:500 (Lilly et al., 1994; Nguyen et al., 1994; provided by H. Nguyen, Erlangen), anti-DSwip-1 1:2000 (Hornbruch-Freitag et al., 2011), and anti-myc 1:2000 (Millipore, Schwalbach). Primary antibodies were detected using biotinylated secondary antibodies (1:500, Vector Laboratories, Burlingame) and the reaction was enhanced with the Vectastain ABC Elite Kit (Vector Laboratories). Fluorescent-labeled secondary antibodies were obtained from Dianova (Hamburg) and used at a dilution of 1:200. Embryos were fixed and stained as described previously (Kesper et al., 2007). Embryonic guts were prepared after fixation and further treated the same way as whole-mount embryos.

Whole-mount in situ hybridization was carried out as described in Tautz and Pfeifle (1989). Full-length RNA antisense probes were synthesized by *in vitro* transcription using an RNA-DIG-labeling Kit (Roche, Mannheim) and BDGP clones RE53159 (βTub60D) and GH02051 (βTub85D).

Images were acquired using a Zeiss Axiophot or a Leica TCS SP5 Confocal Laser Scanning Microscope and were processed with Leica

LAS software and Adobe Photoshop CS2. Ovaries from heterozygous *β1-Tubulin*^{CC02069} females were prepared in PBS and the GFP signal was examined using an ApoTom (Zeiss, Jena).

Scanning electron microscopy (SEM)

Guts of first instar larvae were prepared in PBS and fixed for 1 h in 4% glutaraldehyde. Guts were then rinsed twice with water, additionally fixed with 1% osmium tetroxide for 1 h, and dehydrated through a successive ethanol series. After treating three times with absolute acetone dried over copper sulfate, guts were finally dried to the critical point and sputtered with gold. For analysis, a Hitachi S-530 SEM was used with Diss 5 software (Point Electronic).

Constructs and transgenic flies

Full-length cDNA (BDGP clone RE53159) was cloned into pENTR™/D-TOPO vector (Invitrogen, Karlsruhe) and then transferred into the modified Gateway vector (pUAST-attB-rfa-10xMyc kindly provided by S. Bogdan, Universität Münster) by recombination, using LR Clonase® II plus enzyme mix (Invitrogen, Karlsruhe). This 10xMyc-tagged β3-Tubulin full-length construct was injected into X-86Fb flies (Bl. 24749) using a landing site at the third chromosome (Bischof et al., 2007). The construct was additionally used for SL2 cell transfection.

Cell culture and Western Blot analysis

SL2 cells were cultured, transfected and treated with antibodies as described previously (Hornbruch-Freitag et al., 2011). Anti-Myc antibody (Millipore, Schwalbach) was used at a 1:1000 dilution and was detected by the corresponding fluorescent-labeled secondary antibody (1:100, Dianova, Hamburg). Cells were then incubated with Hoechst (1 mg ml^{-1}; Sigma, Steinheim) and phalloidin-TRITC (1:20, Sigma) and finally mounted in Fluoromount G™ (Southern Biotech, Birmingham).

Preparation of protein extracts from embryos as well as Western Blot analysis were performed as described before (Hornbruch-Freitag et al., 2011).

Results

Body wall muscles develop correctly in β3-Tubulin loss-of-function mutants

β3-Tubulin is strongly expressed during the development of the somatic musculature in wild-type *Drosophila* embryos (Fig. 1A; Kimble et al., 1990; Leiss et al., 1988). Previously, the plexate deletion *Df(2R)Px*^{2}, mainly in *trans* to hypomorphic *β3-Tubulin* alleles, has been used to analyze *β3-Tubulin* loss-of-function phenotypes (Dettman et al., 1996; Kimble et al., 1990). Since then, numerous smaller deletions have been created and molecularly characterized. Here we analyzed the deficiency line *Df(2R)Exel6082*, which deletes *β3-Tubulin* and six other genes (*CG3376*, *CG13577*, *nervy*, *CG3394*, *slow border cells*, and part of *blistered*).

Homozygous *Df(2R)Exel6082* embryos and homozygous *Df(2R)Px*^{2} embryos were lethal at late stages of embryogenesis, and β3-Tubulin was clearly absent in all tissues (Fig. 1C and E). This was confirmed by in situ hybridizations with a *β3-Tubulin* probe; transcripts were not detected in either homozygous mutant at all stages of development (not shown). Surprisingly, the body wall muscles visualized with an anti-Tropomyosin antibody in homozygous *Df(2R)Exel6082* embryos (Fig. 1F) as well as in *Df(2R)Px*^{2} embryos (Fig. 1D) developed correctly and attached to the epidermis as in wild-type embryos (Fig. 1B), i.e., myoblast fusion and myotube extension took place normally, as observed previously by Dettman et al. (1996). This was also the case for embryos from egg lays that were incubated at 30 °C (not shown); therefore, we conclude that even in a harsher environment, β3-Tubulin is dispensable for correct somatic muscle formation.

Table 1
Lethality of homozygous or transheterozygous flies used in this and earlier studies.

	Df(2R)Px^2	*Df(2R)Exel6082*	*β3t*^{2}
Df(2R)Px^2	Embryonic lethal (Kimble et al., 1990)		
Df(2R)Exel6082	Embryonic lethal (this study)	Embryonic lethal (this study)	
β3t^2	Larval lethal (Kimble et al., 1990)	Vital (this study)	Larval lethal (Kimble et al., 1990)

Earlier studies have described the EMS-induced *β3t*^{2} allele as the most severe hypomorphic *β3-Tubulin* allele, which leads to larval lethality and feeding defects when homozygous or in *trans* to the large deficiency *Df(2R)Px*^{2} (Dettman et al., 1996; Kimble et al., 1990). Surprisingly, we detected β3-Tubulin in *β3t*^{2} homozygous embryos (Fig. 1G), and the embryos developed a body wall musculature like that of the wild-type (Fig. 1H). As the anti-β3-Tubulin antibody was raised against the last 16 amino acids of β3-Tubulin (Leiss et al., 1988), it is unlikely that the EMS-induced mutation in the *β3t*^{2} allele leads to a truncated and thereby unstable and possibly nonfunctional β3-Tubulin protein. Thus, we addressed whether the larval lethality is caused by the *β3t*^{2} allele or whether this chromosome carries an additional mutation. We verified that *β3t*^{2}*/Df(2R)Px*^{2} transheterozygotes are larval lethal but that *β3t*^{2}*/Df(2R)Exel6082* transheterozygotes are vital (Table 1). Therefore the *β3t*^{2} chromosome likely carries an additional mutation located within the genomic region deleted in *Df(2R)Px*^{2}, but not within the region deleted in *Df(2R)Exel6082*. We conclude that the observed lethality of homozygous *β3t*^{2} larvae is not due to a mutation in the *β3-Tubulin* gene.

Morphogenesis of visceral muscles in β3-Tubulin mutant embryos occurs as in wild-type embryos

With respect to the possible role of microtubules during myogenesis in *Drosophila*, it is of interest that not only are the body wall muscles syncytial, but also the circular visceral muscles (CVMs) and longitudinal visceral muscles (LVMs) surrounding the midgut develop by fusion of myoblasts and are striated (Klapper et al., 2001, 2002; San Martin and Bate, 2001; Schröter et al., 2006; Stute et al., 2004). The CVMs are built first and arise by incomplete fusion of two myoblasts such that the main cell bodies are connected by strings. The CVMs then stretch from ventral to dorsal. The precursors of the LVMs then fuse with visceral myoblasts, giving rise to syncytia containing up to six nuclei. The LVMs are interwoven with the CVMs to create a network in which the LVMs extend from the anterior midgut to the posterior midgut (Schröter et al., 2006).

To analyze the development of the visceral musculature in *β3-Tubulin* loss-of-function embryos in detail, we used specific markers to follow longitudinal founder cells (FCs) and visceral fusion-competent myoblasts (FCMs). After differentiation, wild-type longitudinal FCs migrate from a primordium at the posterior tip of the embryo toward the anterior to the site of fusion with the visceral FCMs of the trunk mesoderm and adopt a characteristic outstretched, spindle shape (Fig. S1A; Georgias et al., 1997; Klapper et al., 2002; Kusch and Reuter, 1999; San Martin and Bate, 2001; Schröter et al., 2006; Tepass and Hartenstein, 1994). The longitudinal FCs express the fork head domain transcription factor Crocodile (Häcker et al., 1995) and thus can be followed by expression of β-galactosidase under the control of a *croc* promoter (*crocLacZ*).

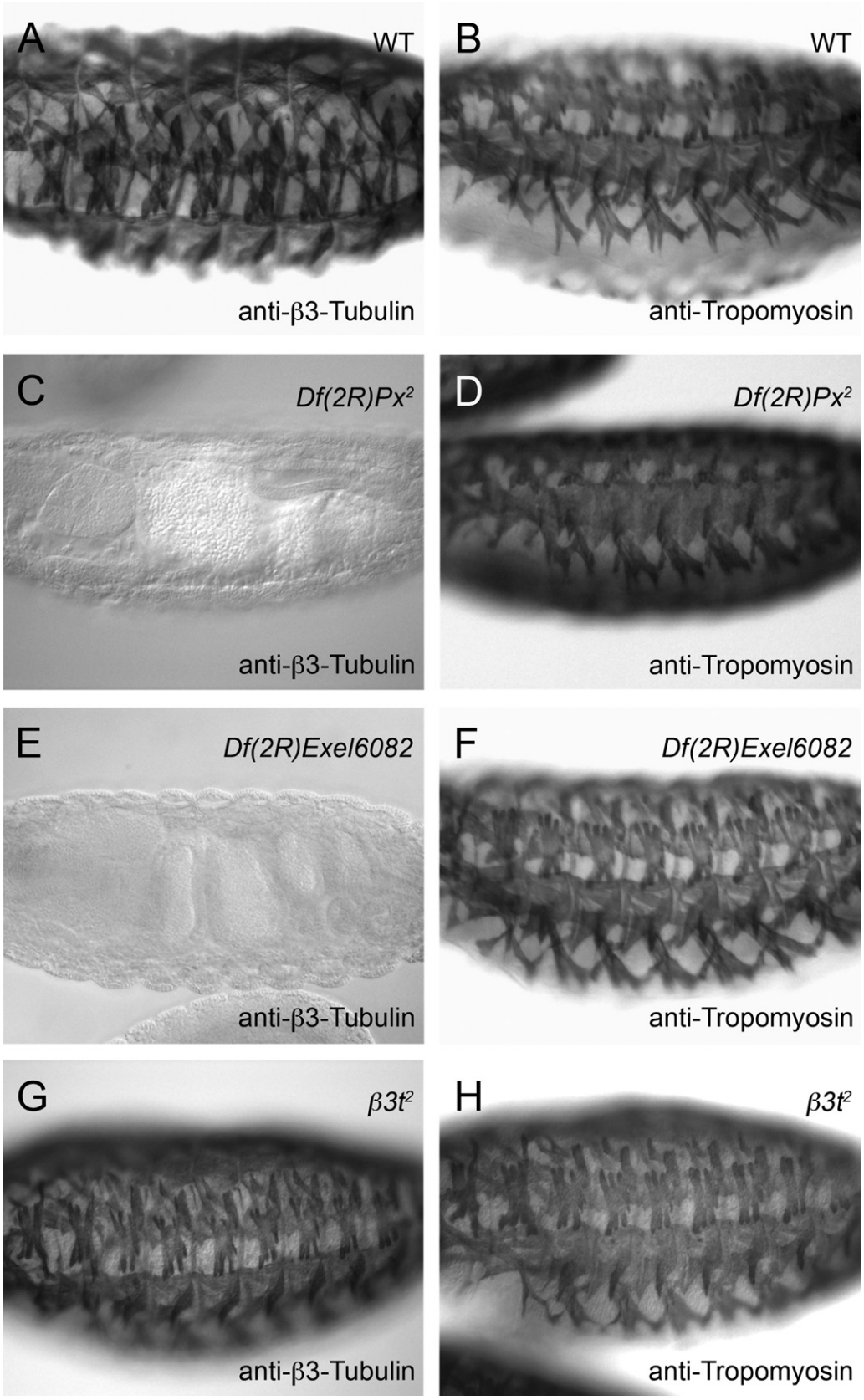

Fig. 1. *β3-Tubulin* loss-of-function mutants develop a normal somatic musculature. In this and the following figures, embryos are oriented anterior to the left and dorsal to the top. (A) Late-stage wild-type embryos stained with anti-β3-Tubulin and (B) anti-Tropomyosin. (C, D) Homozygous embryo of the deficiency line *Df(2R)Px²* stained with (C) anti-β3-Tubulin and (D) anti-Tropomyosin. (E, F) Homozygous embryo of the deficiency line *Df(2R)Exel6082* stained with (E) anti-β3-Tubulin and (F) anti-Tropomyosin. In both *Df(2R)Px²* and *Df(2R)Exel6082*, the *β3-Tubulin* gene is deleted. (G, H) Homozygous embryos carrying the hypomorphic allele *β3t²* stained with (G) anti-β3-Tubulin and (H) anti-Tropomyosin.

In *β3-Tubulin* mutants, the migration and shaping of longitudinal FCs occurred as in wild-type embryos (Fig. S1B). To analyze the fusion efficiency in the visceral mesoderm, we used the reporter construct *bagpipeLacZ* (*bapLacZ*) to follow the fate of the visceral FCMs (Bodmer et al., 1990; Zaffran et al., 2001). In the wild-type, most visceral FCMs fuse with longitudinal and circular FCs, while 30–40% of the visceral FCMs migrate into the somatic mesoderm and fuse with somatic FCs (Azpiazu and Frasch, 1993). *β3-Tubulin* mutant embryos did not have a higher number of unfused or abnormally migrating visceral myoblasts after fusion was completed (Fig. S1D).

To form a fully constricted gut, the visceral muscles must develop correctly (Bienz, 1994; Reuter and Scott, 1990). Analysis of late-stage embryos revealed that the gut elongated and gut constrictions formed in *β3-Tubulin* loss-of function mutants (Fig. 2B) and in hypomorphic $\beta3t^2$ mutants (not shown) as in the wild-type (Fig. 2A). Therefore, even though microtubule bundles are concentrated at sites where gut constrictions form (Reuter and Scott, 1990) and β3-Tubulin is expressed there at higher levels compared to the rest of the visceral mesoderm because of the increased gene transcription (Dettman et al., 1996; Hinz et al., 1992; Kremser et al., 1999b), our results indicated that the presence of the β3-Tubulin isoform was dispensable for this process.

Since previous SEM analysis revealed that the visceral musculature consists of a network of CVMs and LVMs (Schröter et al., 2006), we questioned whether the formation of this network occurs normally in *β3-Tubulin* loss-of-function embryos. Since such mutants carrying the deficiency *Df(2R)Exel6082* were embryonic lethal and SEM analysis of embryonic guts was not possible, we prepared guts from homozygous mutant embryos and examined them immunohistochemically using an anti-Tropomyosin antibody, which marks both somatic and visceral muscles. In these preparations, the network of longitudinal and circular visceral muscles was visible in the wild-type (Fig. 2C) as well as in mutant embryos (Fig. 2D), i.e., in *β3-Tubulin* loss-of-function embryos, visceral muscles were built correctly, the longitudinal and circular FCs fused with visceral FCMs, and the muscles stretched and formed the characteristic network.

Dettman et al. (1996) have shown that larvae carrying the hypomorphic EMS-induced allele $\beta3t^2$ have a smaller gut and die as first instar larvae by starvation, even though the visceral muscles function normally, which indicates that the larvae have problems absorbing nutrients. Since an inductive role of the visceral mesoderm for the underlying endoderm has long been observed (Immerglück et al., 1990; Reuter, 1994; Reuter et al., 1993) and a failure in the development of the larval visceral muscle pattern could explain the starvation phenotype of $\beta3t^2$ larvae, we examined the pattern and shape of the larval gut musculature of these hypomorphic mutants using SEM. In wild-type (Fig. 2E) as well as in homozygous $\beta3t^2$ larvae (Fig. 2F), the longitudinal and circular visceral muscles formed the characteristic net-like structure around the gut. We therefore conclude that the observed malfunction of the gut in $\beta3t^2$ homozygous larvae is not due to a failure in the formation of the visceral musculature.

Lethality of Df(2R)Exel6082 embryos is not caused by the loss of β3-Tubulin

Since *β3-Tubulin* loss-of-function mutant embryos did not exhibit a defective somatic or visceral muscle phenotype, we questioned whether defects in other β3-Tubulin-expressing tissues or the loss of one of the other deleted genes causes the observed lethality of homozygous *Df(2R)Exel6082* embryos.

β3-Tubulin is expressed not only in somatic and visceral muscles, but also in four cardioblasts per hemisegment (Damm et al., 1998; Leiss et al., 1988). We analyzed the formation of the dorsal vessel using an anti-DMef2 antibody that marks the nuclei of the musculature and all cardioblasts (Lilly et al., 1994; Nguyen et al., 1994). In late-stage wild-type embryos, when dorsal closure takes place, the cardioblasts were arranged in one row on the dorsal side of the embryo (Fig. 3A); this characteristic alignment of the cardioblasts was also observed in *β3-Tubulin* loss-of-function mutant embryos (Fig. 3B). Also the nuclei in the somatic musculature of the mutant embryos (Fig. 3D) were arranged throughout the myotubes as in wild-type embryos (Fig. 3C).

Another cell type in which β3-Tubulin is expressed in wild-type embryos is the macrophage-like hemocytes which are dispersed throughout the embryo and are important for the development of the central nervous system (CNS) on the ventral side (Evans and Wood, 2011, and references therein). These cells are also characterized by expression of the EF-hand protein DSwip-1, which does not overlap with β3-Tubulin (Hornbruch-Freitag et al., 2011); we therefore used an antibody against DSwip-1 as a marker for correct macrophage shaping and migration. The macrophages in the *β3-Tubulin* loss-of-function mutants were present beneath the CNS, and had a shape and established arm-like structures (Fig. 3F) like the wild-type (Fig. 3E). Even though these macrophage arm-like structures contain the microtubule-binding protein CLIP170 and therefore were thought to be composed of microtubules (Stramer et al., 2010), our results indicated that at least the β3-Tubulin isoform is not necessary for their formation.

We also analyzed the peripheral nervous system (PNS) and the chordotonal organs, in which *β3-Tubulin* transcripts have also been detected (Buttgereit et al., 1996). Also these mechanosensory organs in *β3-Tubulin* mutants were formed correctly (not shown). However, with our methods, we could not detect axon outgrowth defects in the PNS, as described for *nervy* loss-of-function mutants; the *nervy* gene is located in close to the *β3-Tubulin* gene and is also deleted in *Df(2R)Exel6082* embryos. Thus, we cannot exclude the possibility of a muscle innervation defect in these mutants.

We observed that at the end of embryogenesis, *Df(2R)Exel6082* embryos were able to move in the eggshell and could contract their muscles, although not as frequently and vigorously as wild-type embryos (not shown). Also Kimble et al. (1990) observed an infrequent and uncoordinated muscle contraction of homozygous $Df(2R)Px^2$ embryos at this stage of development. This indicates that sarcomeres are formed in these mutant embryos and that the muscles themselves are functional; nevertheless, the animals failed to hatch. Thus, we questioned whether the failure to hatch is due to the loss of β3-Tubulin or the deletion of one of the other six genes. We established transgenic *Drosophila* lines carrying *UAS-β3-Tubulin-10xMyc* using a modified Gateway-phiC31 system and a landing site at the third chromosome (Bischof et al., 2007). We analyzed the functionality of this construct in SL2 cell culture, which are hemocyte like cells (Armknecht et al., 2005) and express β3-Tubulin (not shown) and we detected the overexpressed Myc-tagged β3-Tubulin protein in the microtubule cytoskeleton of these cells (Fig. 3G). In rescue experiments, we expressed *UAS-β3-Tubulin-10xMyc* in the mesoderm of homozygous *Df(2R)Exel6082* embryos. The ectopic protein was visualized via its Myc-tag in the mesoderm and in established somatic muscles in late-stage embryos (Fig. 3H). The anti-Myc staining of the ectopic Myc-tagged β3-Tubulin protein resembled the staining with the anti-β3-Tubulin antibody, which also detected microtubules in the cytoplasm around the nuclei and accumulated at the muscle ends. We therefore conclude that the transgenic β3-Tubulin was integrated correctly into the microtubule network. Surprisingly, homozygous *Df(2R)Exel6082* embryos overexpressing UAS-β3-Tubulin-10xMyc were also lethal and did not hatch. Hence we conclude that the deletion of another gene in the deficiency mutant *Df(2R)Exel6082* leads to the embryonic lethality.

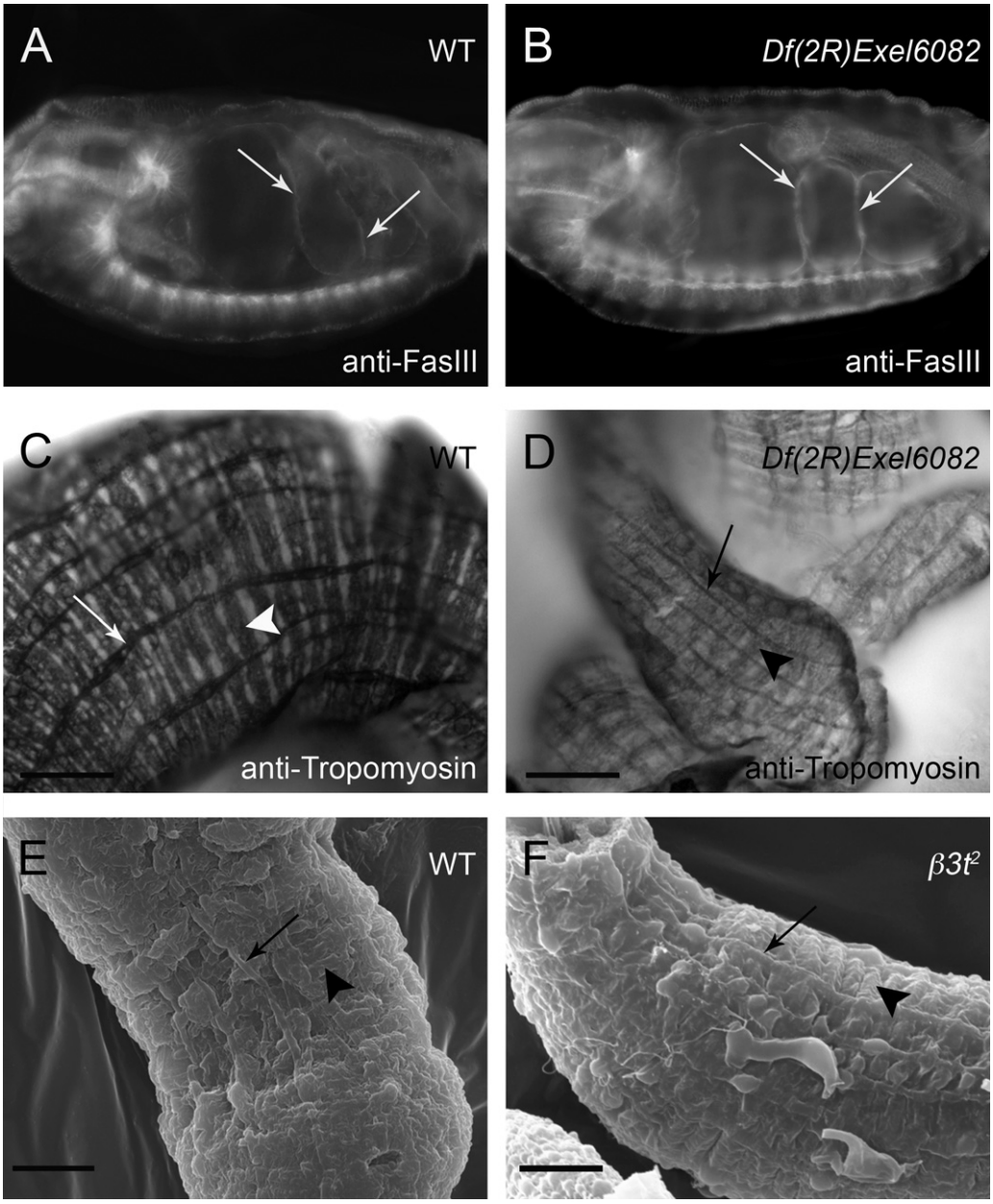

Fig. 2. The network of visceral muscles surrounding the midgut is established correctly in *β3-Tubulin* mutants. (A) Wild-type and (B) *β3-Tubulin* loss-of-function mutant *Df(2R)Exel6082* late-stage embryos; arrows point to gut constrictions. (C, D) Embryonic guts of (C) wild-type and (D) *β3-Tubulin* loss-of-function mutant *Df(2R)Exel6082*, incubated with anti-Tropomyosin antibody; arrowheads point to circular visceral muscles; arrows point to longitudinal visceral muscles; scale bars: 20 μm. (E, F) Guts of first instar (E) wild-type larvae and (F) larvae homozygous for $\beta 3t^2$ analyzed by SEM; arrows point to longitudinal visceral muscles; arrowheads point to circular visceral muscles; scale bars: 10 μm.

The β2-Tubulin isoform is not zygotically expressed in embryos

Previous studies suggested a role for other β-Tubulin isoforms in mesodermal tissues in the absence of β3-Tubulin (Dettman et al., 1996; Jattani et al., 2009); we therefore examined the expression of these isoforms in the *β3-Tubulin* mutant. In Northern Blots, *β2-Tubulin* transcripts have been detected only in testes (Bialojan et al., 1984), in agreement with protein expression and genetic data (Raff et al., 1982). However, Jattani et al. (2009) recently detected *β2-Tubulin* transcripts in the wild-type somatic and visceral trunk mesoderm using a full-length RNA antisense probe and in situ hybridization. Since *β2-Tubulin* might be expressed at a low level in embryos, Northern Blots might not detect the transcripts. Therefore, we used in situ hybridization of wild-type embryos with a full-length *β2-Tubulin* RNA antisense probe and indeed observed a signal in the somatic and visceral mesoderm (Fig. 4A). However, since the *β3-Tubulin* gene shows 87–88% sequence similarity to the *β1-* and *β2-Tubulin* genes (Michiels et al., 1987; Rudolph et al., 1987), the full-length *β2-Tubulin* probe might have hybridized with *β3-Tubulin* transcripts. We tested this possibility using homozygous *β3-Tubulin* loss-of-function mutant embryos in the in situ hybridization; the signal in the mesoderm was absent (Fig. 4B), which indicates that the probe indeed hybridized to *β3-Tubulin* and not to *β2-Tubulin* transcripts in wild-type embryos. We then used an anti-β2-Tubulin antibody against the 14 C-terminal isotype-specific amino acids (Buttgereit et al., 1991; Kaltschmidt et al., 1991; Leiss et al., 1988) and detected a signal only in the sperm tail (Fig. 4C), which persists in the embryo after fertilization and is enclosed in the gut when gut formation is completed (Karr, 1991). Given our mRNA and protein expression data, it is unlikely that

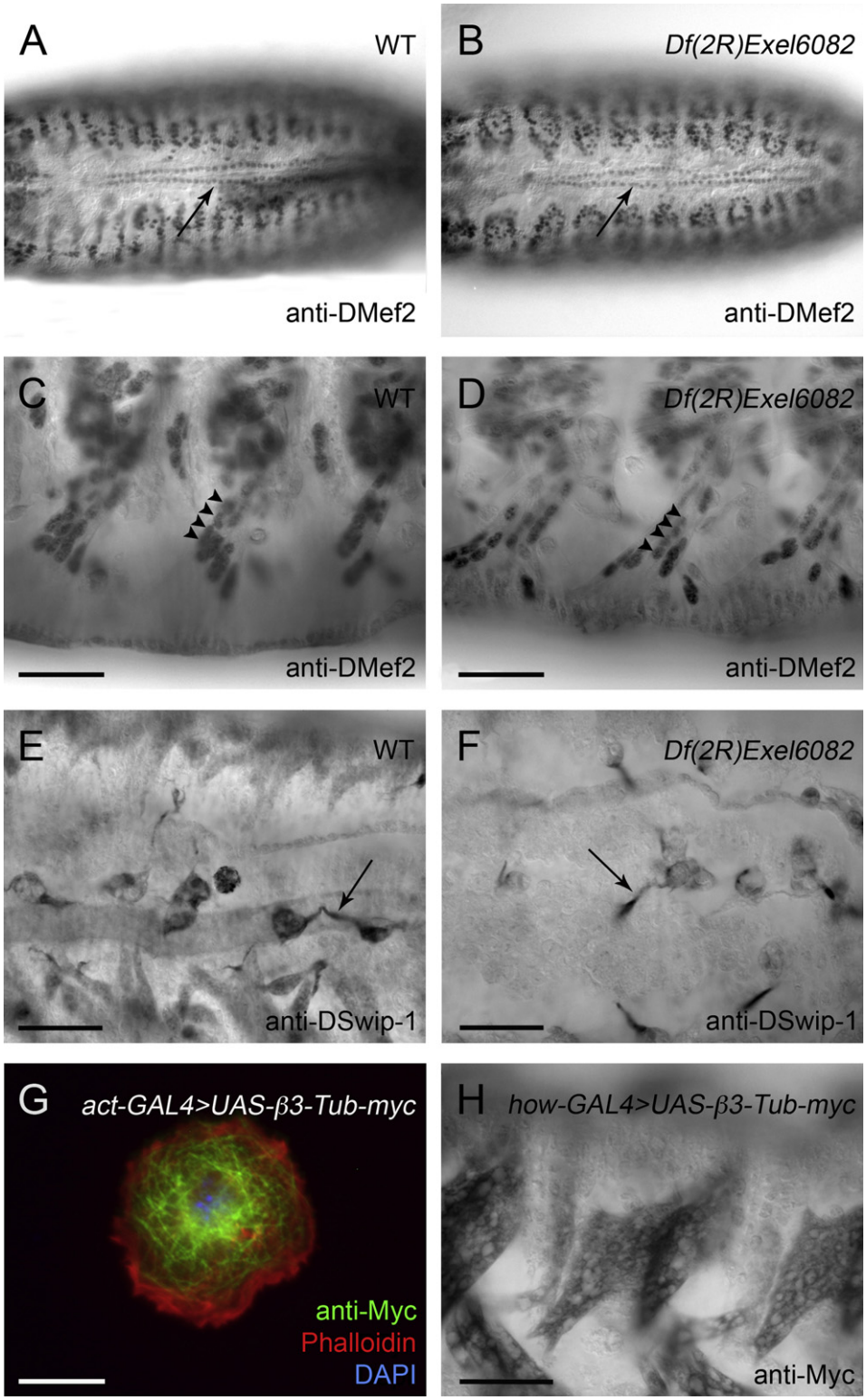

Fig. 3. Cardioblasts of the dorsal vessel and macrophages are shaped normally in homozygous *Df(2R)Exel6082* embryos, and nuclei are properly arranged within muscles. (A, B) Dorsal vessels of a late-stage (A) wild-type embryo and (B) an embryo of the *β3-Tubulin* loss-of-function mutant *Df(2R)Exel6082* visualized with anti-DMef2 antibody; arrows mark nuclei of cardioblasts. (C, D) Nuclei of VA1 and VA2 muscles in (C) wild-type and (D) *Df(2R)Exel6082* mutant embryo; arrowheads point toward nuclei; scale bars: 20 μm. (E) Wild-type and (F) *β3-Tubulin* loss-of-function mutant *Df(2R)Exel6082* macrophages on the ventral side of embryos expressing DSwip-1, visualized with anti-DSwip-1 antibody; arrows point to DSwip-1 positive arm-like structure of macrophages; scale bars: 20 μm. (G) Expression of a Myc-tagged *β3-Tubulin* transgene overexpressed in S2 cells, detected with anti-Myc (green); costained with Phalloidin (red) and DAPI (blue); scale bar: 5 μm. (H) Rescue experiments in which the Myc-tagged *β3-Tubulin* transgene was expressed in the mesoderm of homozygous *Df(2R)Exel6082* embryos and detected with an anti-Myc antibody; scale bar: 20 μm.

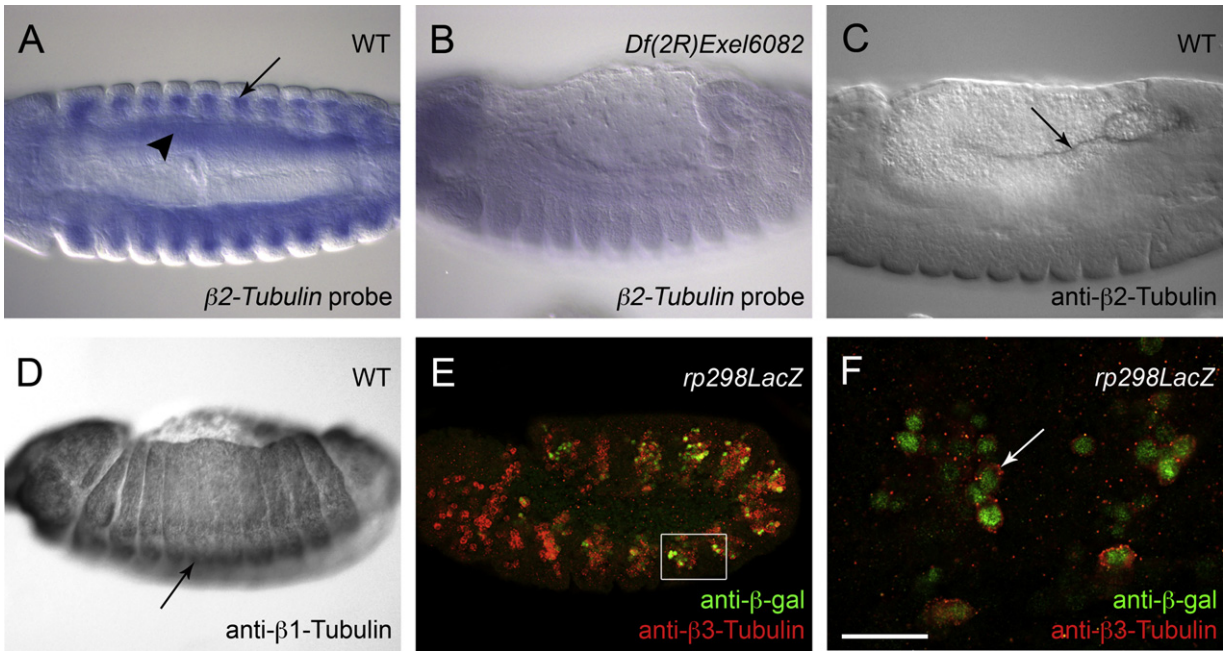

Fig. 4. β2-Tubulin is not zygotically expressed in the embryo, while β1-Tubulin is strongly maternally contributed. (A) In situ hybridization of a wild-type embryo with a full-length *β2-Tubulin* RNA antisense probe; arrow points to somatic mesoderm; arrowhead points to visceral mesoderm. (B) In situ hybridization of a *β3-Tubulin* loss-of-function mutant embryo with a full-length *β2-Tubulin* RNA antisense probe. (C) Wild-type embryo stained with an anti-β2-Tubulin antibody against the 14 C-terminal isotype-specific amino acids; arrow points to sperm tail, which persists after fertilization. (D) Wild-type embryo in mid-embryogenesis stained with anti-β1-Tubulin antibody; arrow points to slightly higher signal in the CNS. (E) β3-Tubulin expression (red) in embryos coexpressing the Duf reporter construct *rp298LacZ* (green). (F) Higher magnification of boxed area in (E); arrow points to β3-Tubulin signal (red) surrounding the *rp298LacZ* positive nuclei (green); scale bars: 20 μm.

β2-Tubulin is expressed in the embryo at all, even when the *β3-Tubulin* gene is deleted.

A maternally supplied β1-Tubulin-GFP fusion protein disturbs embryonic development

The β1-Tubulin isoform can be detected throughout wild-type embryos during mid-embryogenesis using an anti-β1-Tubulin antibody (Buttgereit et al., 1991; Kaltschmidt et al., 1991; Leiss et al., 1988) because it is strongly contributed maternally (Buttgereit et al., 1991). Zygotic expression started in stage 11 in the developing central nervous system (Fig. 4D) and later in the apodemes, the epidermal muscle attachment cells, while muscles remain β1-Tubulin negative (Buttgereit et al., 1991).

Recently, Dobi et al. (2011) have shown that the microtubule-depolymerizing chemical nocodazole has an inhibitory effect on fusing myoblasts in culture when the myoblasts are isolated before stage 9 of embryonic development. Since we did not observe a myoblast fusion defect when β3-Tubulin was absent, we determined when β3-Tubulin expression begins and whether the β1-Tubulin isoform is still present at this time. Using the Duf reporter construct *rp298LacZ*, we detected β3-Tubulin in the mesoderm from stage 11 onwards (Fig. 4E and F), at the same time point when the nuclei of somatic and visceral founder cells become *rp298LacZ* positive, just prior to the first fusion events (Nose et al., 1998). At this stage of development, the maternally contributed β1-Tubulin is still detectable (Buttgereit et al., 1991). Therefore, myoblasts in culture before stage 10 are likely to contain the β1-Tubulin isoform.

We tested whether the *β1-Tubulin*^{CC02069} allele, originating from a protein trap collection, is suitable for investigating myogenesis in the absence of zygotically expressed functional wild-type β1-Tubulin. In this allele, a GFP-encoding sequence is inserted within the single intron of the *β1-Tubulin* gene (Buszczak et al., 2007), which results in a fusion protein with GFP presumably integrated in the very N-terminal part which is supposed to be involved in nucleotide binding of the Tubulin dimer (Nogales et al., 1998). Despite of 50% maternally contributed wild-type β1-Tubulin pool, a homozygous *β1-Tubulin*^{CC02069} situation leads to lethality, which indicates that this fusion protein is not functional. We detected GFP in the epidermis above the growing myotubes in stage 14 embryos (Fig. 5A) and in the developing CNS and the apodemes in late-stage (Fig. 5B) homozygous *β1-Tubulin*^{CC02069} embryos; muscles expressing β3-Tubulin were not stained. Furthermore, a GFP signal could be detected in the oocyte, but was absent from nurse and follicle cells in heterozygous *β1-Tubulin*^{CC02069} female flies (Fig. S2A–C). This demonstrates that β1-Tubulin-eGFP is already expressed prior to fertilization and is contributed maternally, as was observed before for the wild-type protein (Buttgereit et al., 1991).

Next we examined embryos carrying the *β1-Tubulin*^{CC02069} chromosome and found severe germ band retraction and organ formation defects in 10% of the embryos even in the heterozygous situation; another 30% of the animals displayed minor myoblast fusion and muscle guidance defects (Fig. 5D and F, heterozygous mothers and heterozygous embryos). Therefore, the *β1-Tubulin*^{CC02069} allele caused a dominant negative effect in about 40% of all animals, indicating that already one copy of the maternally contributed fusion protein disturbed the microtubule network in all tissues from the beginning of embryogenesis. Nevertheless, up to 60% of *β1-Tubulin*^{CC02069} heterozygous and homozygous embryos developed a muscle pattern like that of the wild-type (Fig. 5C and E; for statistics see Table S1). Thus, the fusion protein does not cause additional organ formation defects when zygotically homozygous. The very inhomogeneous developmental defects in 40% of either homozygous or heterozygous *β1-Tubulin*^{CC02069} embryos can possibly be explained by the low stability of the fusion protein, as the integrated GFP presumably disturbs the GTP-binding domain of β1-Tubulin and

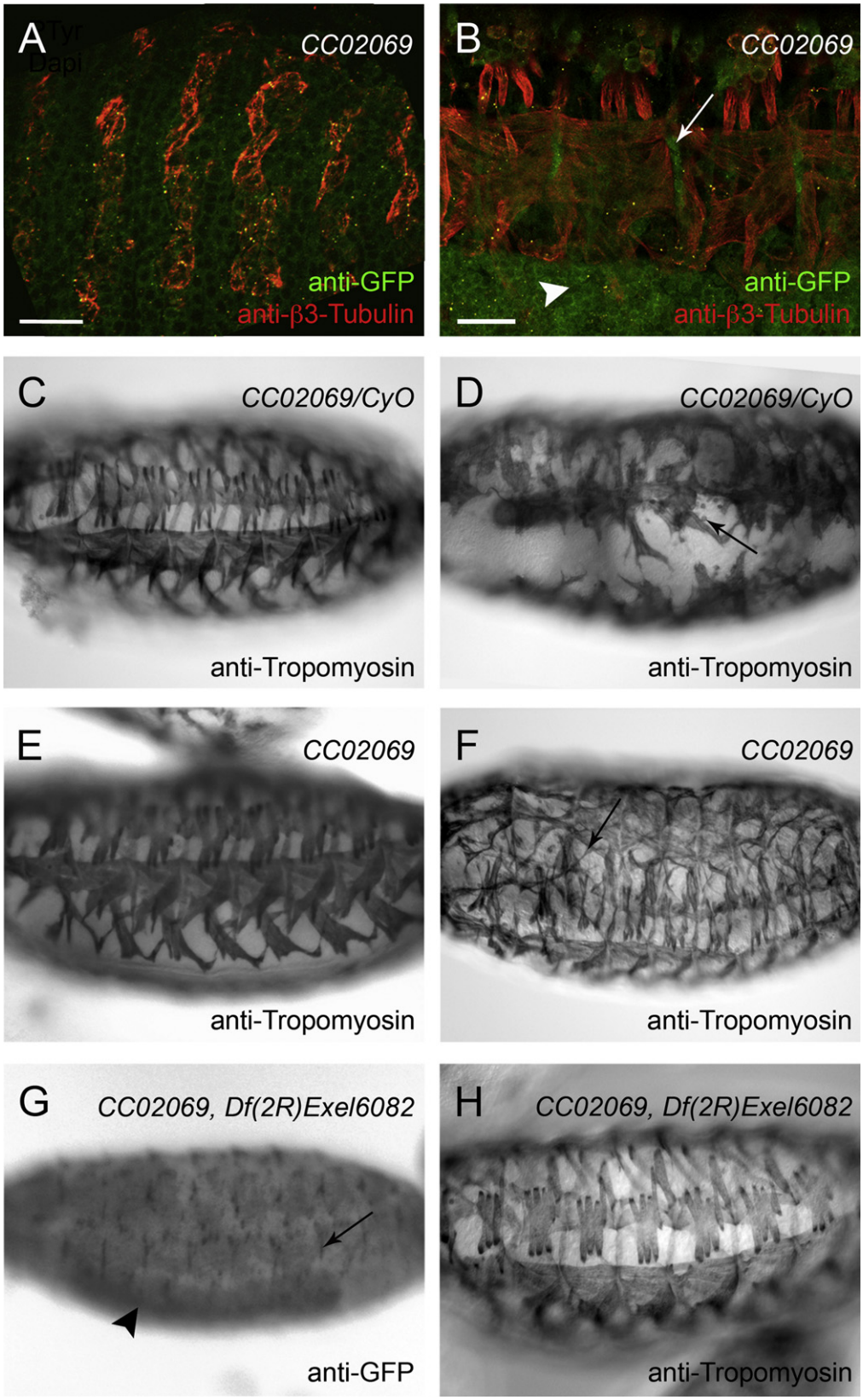

Fig. 5. The protein trap allele *β1-Tubulin*^{CC02069} causes developmental defects already when heterozygous, but GFP expression could not be detected in the mesoderm. (A, B) Stage 13 (A) and late-stage (B) homozygous *β1-Tubulin*^{CC02096} embryos; GFP expression was detected with anti-GFP (green); arrow points to epidermal muscle attachment cells; arrowhead points to CNS; muscles detected with anti-β3-Tubulin (red); scale bars: 20 μm. (C–F) Somatic musculature developed by (C, D) heterozygous and (E, F) homozygous *β1-Tubulin*^{CC02069} embryos; exemplary for (C, E) wild-type muscle pattern and myoblast fusion defects (D) and muscle outgrowth and guidance defects (F); muscles detected with anti-Tropomyosin, arrows point to unfused myoblast (D) and misguided muscle (F). (G, H) *β1-Tubulin*^{CC02069}, *β3-Tubulin* mutant embryos; (G) GFP expression was detected with anti-GFP; arrow points to apodemes; arrowhead points to the CNS; (H) muscles detected with anti-Tropomyosin.

Tubulin monomers are known to be very instable. This was further confirmed by Western Blot analysis: β1-Tubulin-eGFP could be detected in very low amounts in comparison to the wild-type protein contributed by the balancer chromosome (Fig. S2D). Unfortunately, further RNAi analyses would not be reasonable in this case: β1-Tubulin RNAi knock-down already in oogenesis would most likely result in a failure of early cell divisions in the syncytial blastoderm stage of the embryo and would probably lead to a completely block of embryogenesis, as β1-Tubulin is the only isoform expressed in ovaries (Leiss, 1989). We performed RNAi using a mesoderm-specific driver line, but did not detect any muscle defects (not shown). This was not surprising, as the maternal contributed wild-type β1-Tubulin could not be knocked down using this method.

Our data so far might be interpreted in a way that the maternally derived β1-Tubulin persists into stages when myogenesis occurs. To clarify whether maternally supplied β1-Tubulin is sufficient for mesoderm differentiation, we analyzed muscle development in homozygous double mutant embryos. We constructed a mutant with both the *Df(2R)Exel6082* deletion and the *β1-Tubulin*^{CC02069} allele on the same chromosome by meiotic recombination. In *β3-Tubulin* mutant situation, the GFP signal was not altered, i.e., the anti-GFP antibody stained mainly the apodemes and the CNS (Fig. 5G), which indicates that β1-Tubulin-eGFP expression did not extend significantly to mesodermal tissues. Thus, zygotic β1-Tubulin expression was not activated in the mesoderm in the absence of β3-Tubulin. Furthermore, up to 35% of these mutant embryos, which lacked β3-Tubulin and zygotically expressed a presumably nonfunctional β1-Tubulin isoform, displayed the same defects in the somatic musculature and all other tissues as embryos carrying the *β1-Tubulin*^{CC02069} allele alone, while more than 60% showed a wild-type muscle pattern (Fig. 5H, Table S2), indicating that the zygotically expressed β1-Tubulin-GFP fusion protein and the lack of β3-Tubulin do not contribute to stronger defects in these animals.

In summary, our results demonstrate that one copy of maternally supplied wild-type β1-Tubulin was sufficient for wild-type muscle development in more than half of the embryos, even in absence of β3-Tubulin, while in 40% the dominant negative effect of the maternally expressed β1-Tubulin-GFP fusion protein disturbed embryonic muscle development.

Discussion

β3-Tubulin is the only β-Tubulin isoform that is strongly expressed in mesodermal tissues, namely in the somatic and visceral musculature, the heart, and hemocytes. Nevertheless, we showed that these organs develop normally in the absence of β3-Tubulin. Although it has already been demonstrated that the somatic musculature is not disturbed in hypomorphic *β3-Tubulin* mutants (Dettman et al., 1996), we could now show that the larval lethality and the variable gut phenotype of the previously analyzed allele was not due to a mutation in the *β3-Tubulin* gene. This explains why Kimble et al. (1990) were not able to rescue the lethality of homozygous *β3t*^{2} larvae by expressing a *β3-Tubulin* transgene.

In *β3-Tubulin* loss-of-function mutants, the formation of the visceral muscles was not disturbed, and the heart developed normally. Moreover, we did not detect any defects in the structure of the PNS and chordotonal organs with our methods. However, these loss-of-function mutants homozygous for the deficient chromosome *Df(2R)Exel6082* died at late stages of embryogenesis and did not hatch, even though they showed normal movements in the eggshell, as has been observed before (Dettman et al., 1996; Kimble et al., 1990). In rescue experiments, we showed that the lethality was not due to the loss of β3-Tubulin. The analyzed deficiency line deletes, among others, the gene *nervy* (*nvy*), which has been shown to be important for axon guidance; *nvy* loss-of-function mutants show axon repulsion phenotypes in the PNS and defective muscle innervations in late-stage embryos (Parrish et al., 2006; Terman and Kolodkin, 2004), which might explain the lethality of homozygous *Df(2R)Exel6082* embryos.

Previous studies have suggested that the expression of β3-Tubulin contributes to the low levels of β1-Tubulin, which is maternally derived and can be detected throughout the embryo. Therefore, β3-Tubulin would only be needed to increase the Tubulin pool in mesodermal tissues as an evolutionary advantage for the fly (Dettman et al., 1996). We showed here that β1-Tubulin expression was not enhanced in *β3-Tubulin* loss-of-function mutants. In addition, the developmental defects which could be seen even in 40% of *β1-Tubulin*^{CC02069} heterozygous embryos were not enhanced in *β1-*, *β3-Tubulin* double mutants. It was also shown previously on the ultrastructural level that only a very low number of microtubules is present in the musculature when *β3-Tubulin* is deleted (Dettman et al., 1996). Thus, in summary we hypothesize that a large pool of microtubules is not required in the formation of the larval musculature and that, although not detectable with antibodies, maternal-derived wild-type β1-Tubulin is sufficient to build functional microtubules in a *β3-Tubulin* mutant. Such a case of sufficient maternal supply has been shown for the Wiskott-Aldrich syndrome protein (WASP) in myoblast fusion (Mukherjee et al., 2011; Schäfer et al., 2007).

In *β3-Tubulin* mutant embryos, longitudinal and cross sections on the ultrastructural level reveal no abnormalities in sarcomeric structure (Dettman et al., 1996) and the embryos are able to move in the eggshell; therefore, microtubules do not seem to be essential for sarcomere assembly. In vertebrate skeletal myocytes, in contrast, an intact microtubule array is required for this process (Pizon et al., 2002, 2005; Toyama et al., 1982), but not for sarcomeric organization of cardiac myocytes (Ng et al., 2008). The very low number of microtubules in late-stage wild-type embryos and first instar larvae (Dettman et al., 1996) might guide the filaments to their destination, but this process might also occur when microtubules are not present, considering that the sarcomeres in larval muscles are much less organized than in other types of muscles, e.g., adult flight muscles (Reedy and Beall, 1993a,b).

The formation of multi-nucleated myotubes in the *Drosophila* embryo by myoblast fusion has been shown to be characterized by local F-actin accumulation and to depend on the actin cytoskeleton, with F-actin arranged in a Fusion-Restricted Myogenic Adhesive Structure (FuRMAS) (Haralalka et al., 2011; Kesper et al., 2007; Kim et al., 2007; Massarwa et al., 2007; Önel and Renkawitz-Pohl, 2009; Richardson et al., 2007; Schäfer et al., 2007). In a recent study, a decreased number of myotubes per field have been observed during fusion assays with isolated *Drosophila* myoblasts in culture when treated with nocodazole (Dobi et al., 2011), suggesting the importance of microtubules in the fusion process. Nevertheless, when microtubules are completely disassembled in embryonic myotubes through overexpression of the AAA ATPase Spastin, the muscles are partially detached from the epidermis, but myoblast fusion occurs to a normal extent (Sherwood et al., 2004). Moreover, some actin–microtubule linkage molecules known to be required in cell division have been implicated in myotube extension, but myoblast fusion is not disturbed in the mutants (Guerin and Kramer, 2009). Given our results that the strongly mesodermally expressed β3-Tubulin isoform was not required for myoblast fusion, we propose that transport mechanisms in this process do not involve newly synthesized microtubules and that a maternally derived microtubule network is sufficient for muscle formation. The electron-dense vesicles at the fusion site that can be observed at the ultrastructural level (Doberstein et al., 1997) could probably

be transported via actin, in agreement with Kim et al. (2007), who showed using immuno-electron microscopy that vesicles located within the actin foci and at the plasma membrane of adherent myoblasts are actin-coated, while only vesicles at a greater distance are associated with microtubules.

Besides actin foci, FuRMAS share many further features with podosomes/invadopodia and the immunological synapse (reviewed in Önel and Renkawitz-Pohl, 2009). Podosome formation and podosomal matrix degradation require microtubules, kinesin motors, and microtubule acetylation proteins (Cornfine et al., 2011; Destaing et al., 2005; Kopp et al., 2006; Linder et al., 2000; Linder, 2007). Considering the analyses presented here, the necessity of high level microtubules in podosome/invadopodia formation and myoblast fusion seems to differ, even though these structures share similarities in the regulation of the actin cytoskeleton and the formation of rings of cell-adhesion or cell matrix connecting molecules.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejcb.2011.11.002.

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Supplementary material

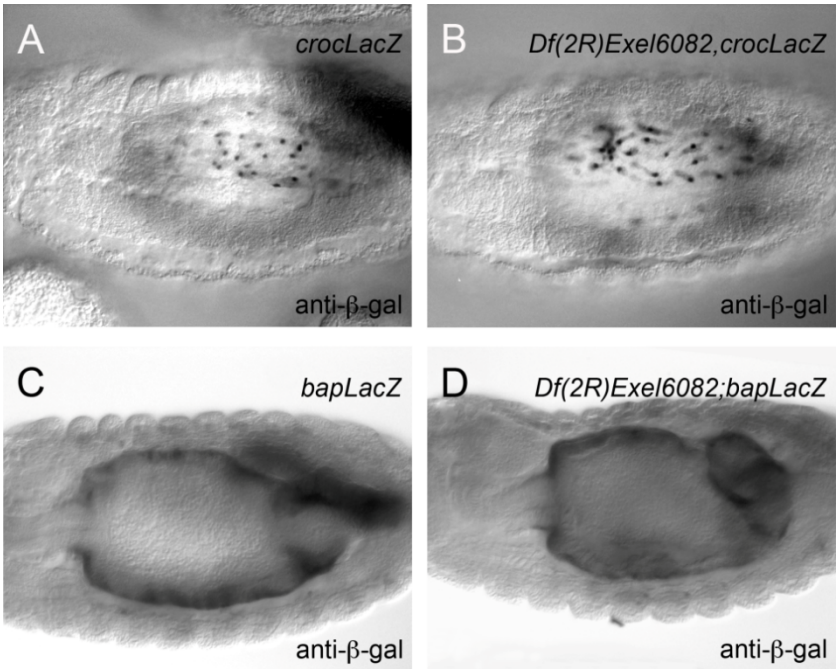

Fig. S1. Longitudinal founder cells migrate and visceral myoblast fusion takes place in *β3-Tubulin* mutants. (A) Wild-type embryo and (B) *β3-Tubulin* loss-of-function mutant *Df(2R)Exel6082* embryo; longitudinal founder cells (FCs) expressing *crocodileLacZ* (*crocLacZ)*, detected with anti-β-galactosidase antibody. (C) Wild-type and (D) *β3-Tubulin* loss-of-function mutant *Df(2R)Exel6082* after completion of visceral myoblast fusion; unfused visceral myoblasts that expressed *bagpipeLacZ (bapLacZ*) were detected with anti-β-galactosidase antibody.

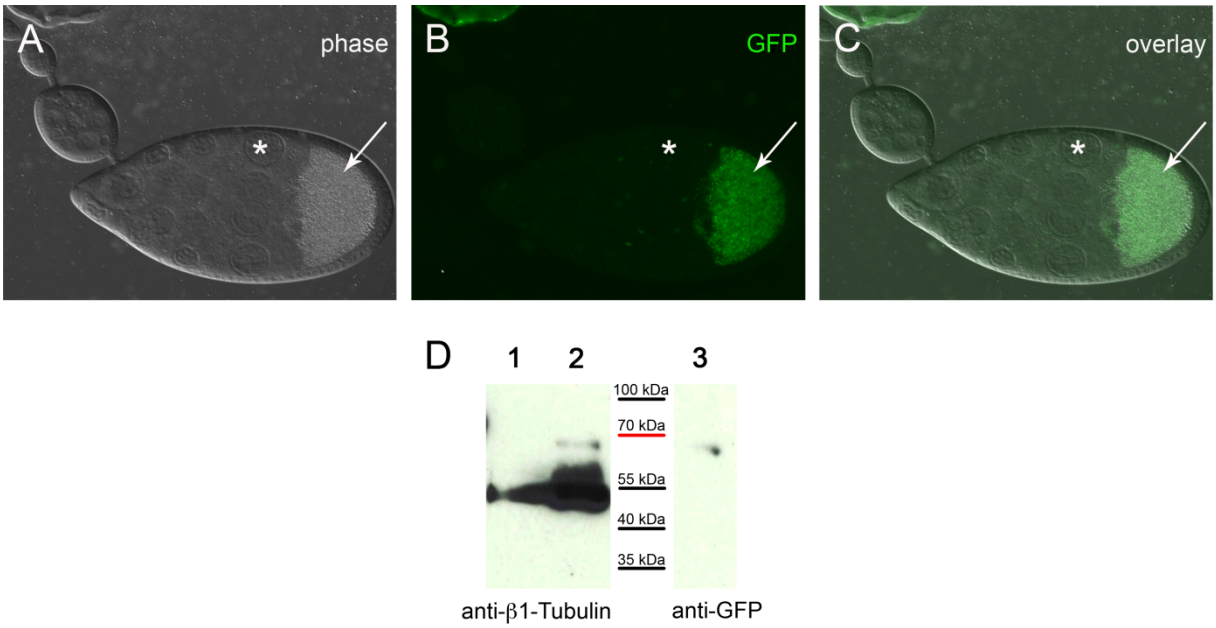

Fig. S2. β1-Tubulin-eGFP is already expressed in oocytes and can only be detected in low amounts in embryos. (A-C) Ovaries from heterozygous *β1-Tubulin*^{CC02069} females. (A) Phase contrast, (B) Detection of GFP signal, (C) overlay of both. Arrow points to oocyte, asterisks mark nurse cell next to the oocyte. (D) Western Blot analysis of protein extracts from wild-type (1) and heterozygous *β1-Tubulin*^{CC02069} (2, 3) embryos; wild-type β1-Tubulin could be detected with anti-β1-Tubulin (1, 2); β1-Tubulin-eGFP was detected with anti-β1-Tubulin (2) and anti-GFP (3).

Table S1

Number of heterozygous and homozygous *β1-Tubulin^{CC02069}* embryos displaying a wild-type and disturbed muscle pattern, respectively.

Sample	**1**	**2**	**3**
N	267	57	158
***β1-Tubulin^{CC02069} /CyO^{HgLacZ}* and *CyO^{HgLacZ}/CyO^{HgLacZ}* embryos** (Zygotically expressed β1-Tubulin-eGFP fusion protein: 50% and 0%)	163	42	113
Wild-type muscle pattern	99 (61%)	24 (57%)	75 (66%)
Strong developmental defects	17 (10%)	4 (9%)	10 (9%)
Mild myoblast fusion and outgrowth defects	47 (29%)	14 (33%)	28 (25%)
***β1-Tubulin^{CC02069}/β1-Tubulin^{CC02069}* embryos** (Zygotically expressed β1-Tubulin-eGFP fusion protein: 100%)	50	15	32
Wild-type muscle pattern	25 (50%)	7 (47%)	19 (59%)
Strong developmental defects	6 (12%)	3 (20%)	2 (6%)
Mild myoblast fusion and outgrowth defects	19 (38%)	5 (33%)	11 (34%)
Strong developmental defects, not possible to determine genotype	54 (20%)	0 (0%)	13 (8%)

All analyzed embryos contain maternally contributed β1-Tubulin: 50% wild-type β1-Tubulin protein and 50% β1-Tubulin-eGFP fusion protein.

Table S2

Number of heterozygous and homozygous *Df(2R)Exel6082, β1-Tubulin^{CC02069}* embryos displaying a wild-typic and disturbed muscle pattern, respectively.

Sample	**1**	**2**	**3**
N	82	128	246
***Df(2R)Exel6082, β1-Tubulin^{CC02069}/CyO^{HgLacZ}* and *CyO^{HgLacZ}/CyO^{HgLacZ}* embryos** (Zygotically expressed β1-Tubulin-eGFP fusion protein: 50% and 0%; Zygotically expressed β3-Tubulin: 50% and 100%)	45	63	132
Wild-type muscle pattern	35 (78%)	49 (78%)	95 (72%)
Strong developmental defects	2 (4%)	4 (6%)	9 (7%)
Mild myoblast fusion and outgrowth defects	8 (18%)	10 (16%)	28 (21%)
Df(2R)Exel6082, β1-Tubulin^{CC02069} / Df(2R)Exel6082, β1-Tubulin^{CC02069} embryos (Zygotically expressed β1-Tubulin-eGFP fusion protein: 100%; Zygotically expressed β3-Tubulin: 0%)	32	50	106
Wild-type muscle pattern	21 (66%)	35 (70%)	75 (71%)
Strong developmental defects	3 (9%)	1 (2%)	6 (6%)
Mild myoblast fusion and outgrowth defects	8 (25%)	14 (28%)	25 (24%)
Strong developmental defects, not possible to determine genotype	5 (6%)	15 (12%)	8 (3%)

All analyzed embryos contain maternally contributed β1-Tubulin: 50% wild-type β1-Tubulin protein and 50% β1-Tubulin-eGFP fusion protein.

6 Myosin heavy chain-like interacts with Rolling pebbles 7 during *Drosophila* myogenesis

Myosin heavy chain-like interacts with Rolling pebbles 7 during *Drosophila* myogenesis

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Abstract

The body wall muscles of *Drosophila* larvae develop through the highly regulated fusion of two different cell types: founder cells (FCs) and fusion competent myoblasts (FCMs). A protein complex establishes at the site of cell–cell contact (Fusion-restricted Myogenic-Adhesive Structure or FuRMAS). This complex acts as a signaling center and consists of a ring of adhesion molecules and an actin-rich core. Branched F-actin is likely needed to widen the fusion pore and to integrate the myoblast into the growing muscle. We present evidence that an unconventional myosin, Myosin heavy chain-like (Mhcl), interacts *in vitro* with Rolling pebbles 7 (Rols7), a protein necessary for myoblast fusion. *Mhcl* was transcribed during fusion-relevant stages most prominently in FCs. *Mhcl* deficient embryos developed a wild-type somatic and visceral musculature, but died as first or second instar larvae. The endogenous protein was detected adjacent to the sarcomeric Z-discs in mature muscles, while an ectopically expressed GFP-tagged Mhcl localized at the FC membrane at sites of cell–cell contact towards the attached FCM. We postulate that Mhcl interacts with Rols7 and acts at the FuRMAS as motor protein for F-actin in redundancy to other myosin heavy chains.

Keywords: Myoblast fusion, Mhcl, Rols7, myosin, FuRMAS, sarcomeres

Introduction

Intensive research has provided deep insight into the process of myoblast fusion in the model organism *Drosophila melanogaster* (Abmayr and Pavlath, 2012; Massarwa et al., 2007; Rochlin et al., 2010; Sens et al., 2010; Önel and Renkawitz-Pohl, 2009). To build up the approximately 30 mature muscle fibers per hemisegment of *Drosophila* larvae, two different mesodermal cell types — the founder cells (FCs) and the fusion competent myoblasts (FCMs) — must recognize and adhere to each other, and fuse. The FCs express various transcription factors and thus provide information about the final muscle size, shape, and orientation within the embryo as well as the attachment to the epidermis (Baylies et al., 1998; Richardson et al., 2008). All FCMs are characterized by their expression of the transcription factor Lame duck (Lmd) (Duan et al., 2001; Furlong et al., 2001; Ruiz-Gómez et al., 2002).

Analysis of mutant embryos has revealed that muscle development proceeds in two consecutive temporal phases. In the first temporal phase, one FC fuses with a few FCMs, giving rise to a precursor cell; in the second fusion phase, this precursor cell recruits further FCMs until the mature myofiber size is reached (Bate, 1990). In one model of this process, these phases are distinguishable by genetic means (Bate, 1990). In another model, the phases are not distinguished genetically because the involved proteins are instead needed for efficient progress of the fusion event (Beckett and Baylies, 2007). Various structures exclusive to the second fusion step have been described (Doberstein et al., 1997; Önel and Renkawitz-Pohl, 2009). After establishment of the cell–cell contact between an FCM and the growing muscle, electron-dense vesicles originating from the Golgi apparatus accumulate on opposing membranes to form the so-called prefusion complex. These vesicles are thought to deliver molecules crucial for proper fusion. From these prefusion complexes, electron-dense plaques resembling desmosomes are assumed to arise and can be detected at the opposing membranes. Near these plaques, cytoplasmic continuity is achieved by vesiculating and removing membranes (Doberstein et al., 1997). Fusion is completed when the myoblast is integrated into the growing muscle.

Various classes of proteins are involved in myoblast fusion. The extracellular parts of immunoglobulin proteins manage the recognition of FCs and FCMs and maintain their cell–cell contact. Dumbfounded/Kin of Irre C (Duf/Kirre) is expressed specifically in FCs and interacts with the FCM-specific protein Sticks and Stones (Sns) (Bour et al., 2000; Galletta et al., 2004; Ruiz-Gómez et al., 2000). The Sns paralog Hibris (Hbs) is detectable only in FCMs, and Roughest/Irregular Chiasm C (Rst/Irre C) acts in redundancy to Duf/Kirre but is expressed in both cell types (Dworak et al., 2001; Strünkelnberg et al., 2001). Signaling via the intracellular parts of these proteins to adaptor molecules eventually leads to reorganization of the actin cytoskeleton, membrane degradation, and successful myoblast fusion. The intracellular domain of Duf/Kirre might interact directly with the TPR-E repeat of the FC-specific protein Rolling

pebbles 7 (Rols7), as suggested by results of *in vitro* experiments (Chen and Olson, 2001; Kreisköther et al., 2006). *rols* mutant embryos display small syncytia and many unfused myoblasts, which indicates an essential role of Rols7 in the second phase of fusion. Ultrastructural analyses of *rols* mutant embryos have shown initial cell adhesion of FCMs with the precursor cell, but no prefusion complexes; therefore, Rols7 most likely stabilizes the cell–cell contacts of FCMs and growing muscles (Rau et al., 2001). Rols7 contains multiple protein–protein interaction domains: one RING finger, nine ankyrin repeats, and three tetratricopeptide repeats (TPR) (Chen and Olson, 2001; Menon and Chia, 2001; Rau et al., 2001). Deletion of the ankyrin repeats results in failure to rescue the *rols* mutant phenotype, which suggests an essential role of this domain in the myoblast fusion process (Menon et al., 2005). After myogenesis is completed, Rols7 colocalizes and putatively interacts with α-Actinin and Zormin (a Sls isoform) at the Z-discs of the sarcomeres, as well as with Duf/Kirre at the terminal Z-discs. There Rols7 is presumably needed for the attachment of the mature muscle to the epidermis (Kreisköther et al., 2006).

Rols7 and multiple other cytoplasmic proteins are assumed to be involved in transducing the fusion signal to the actin cytoskeleton and its regulators. At the cell–cell contact, an F-actin plug on the side of the FCMs and a thinner actin sheet on the side of the FC/precursor cell are surrounded by a ring of the adhesion molecules and their interaction partners; this represents a structure defined as FuRMAS (Fusion-Restricted Myogenic-Adhesive Structure), which is thought to act as a signaling center (Kesper et al., 2007; Richardson et al., 2007; Önel and Renkawitz-Pohl, 2009). FuRMAS restricts the area of membrane breakdown and is assumed to represent the site where the prefusion complex is established. Analyses of different F-actin regulators essential for myoblast fusion have suggested that the actin cytoskeleton is involved in transporting electron-dense vesicles to the site of fusion, widening the fusion pore within the FuRMAS, and integrating the myoblast into the growing muscle (Berger et al., 2008; Kesper et al., 2007; Kim et al., 2007; Massarwa et al., 2007).

For all these tasks in the process of myoblast fusion, one or more molecular motors are likely to be involved in the transport along F-actin or in the movement of these filaments against each other. Plausible candidates are myosins, which can occur as hexamers consisting of two heavy chains, two essential light chains, and two regulatory light chains. The myosin heavy chains contain a motor domain responsible for ATP-dependent movement, a regulatory neck region with one or more light chain binding IQ domains, and a specific N-terminal tail often with coiled-coil motifs. These motifs mediate the homodimerization of the heavy chains, which are classified according to the sequence similarity of their motor domain (Berg et al., 2001; Tzolovsky et al., 2002). The conventional muscle-specific myosin heavy chain (Mhc) belongs to class II and is, in a complex with the essential and regulatory light chains, responsible for contraction of muscles (reviewed in Takagi et al., 2004). Mhc is present already in myoblasts

prior to fusion and in the late somatic and visceral musculature of the embryo; the protein persists in the larval musculature as well as in adult flight and jump muscles as the major component of sarcomeric thick filaments. *mhc* mutations have been characterized as dominant flightless and recessive lethal. In an *mhc* null mutant, establishment of sarcomeric thick filaments fails, and embryos die at late stages; analyses of *mhc* point mutations have revealed the absence of thick filaments in the adult musculature and have shed light on the isoform diversity of Mhc in flies (Swank et al., 2000 and references therein). Further unconventional myosins are known to be involved in many forms of motility, such as cell shape changes, cytokinesis, and cell migration, as well as in transporting organelles and proteins, organizing actin filaments, and even in transcription (reviewed in Hartman et al., 2011). Zipper (Zip), the non-muscle myosin heavy chain II of *Drosophila*, has been implicated in morphogenetic processes, such as gastrulation and dorsal closure of the embryo as well as in border cell migration in the egg chamber (reviewed in Conti and Adelstein, 2008; Vicente-Manzanares et al., 2009). Zip also functions as an essential component of the larval Z-discs and seems to be responsible for the integrity of sarcomeres; furthermore, subtle defects in the establishment of muscles VA 1–3 have been observed (Bloor and Kiehart, 2001).

Here we present evidence that the unconventional myosin heavy chain-like (Mhcl) interacts via its C-terminal tail in a yeast two-hybrid (Y2H) assay with the ankyrin repeats of Rols7. We show that *Mhcl* is transcribed during the fusion relevant stages in FCs of the somatic and the visceral mesoderm, and that a GFP-tagged version of Mhcl localized at the contact sites of myoblasts on the side of the growing muscle. Our results indicate a function for Mhcl as an interaction partner of Rols7 during myogenesis, and we propose that Mhcl might be involved in actin-dependent processes during myoblast fusion. The localization of the Mhcl protein indicates a further function in the Z-discs of the sarcomeres in the larval and adult musculature.

Material and methods

Drosophila melanogaster stocks and genetics

The following fly strains were obtained from the Bloomington *Drosophila* Stock Center, unless otherwise indicated: *white*^{1118} (wild-type, Bl. 6326), *rP298-LacZ* (enhancer trap line in the locus of *duf/kirre;* Nose et al., 1998), *sls-GFP* (ZCL2144 from Flytrap collection; Morin et al., 2001), *P(EPgy2)EY00454* (Bl. 15025, P-element located in the putative 5´-UTR of *Mhcl*), *Df(3R)BSC728* (Bl. 26580, amongst others, N-terminal part of Mhcl deleted), *lmd*^{1}*/Tm3,Sb* (Hummel et al., 2000), *Notch*$^{264\text{-}39}$*/FM4,B*$^{+}$ (Bl. 730), *blow*^{2}*/CyO* (Doberstein et al., 1997), *mbc*$^{D11.2}$*/TM3* (Bl. 4952; Bour et al., 2000), *Df(3L)BK9/TM3,Sb* (Bl. 2991, *rols* deficient fly strain), *zip*^{2}*/CyO* (Bl. 8739, *zip* LOF allele), and UAS-GFP-DN-*zip* (Franke et al., 2005; kindly provided by

D. Kiehart, Duke University, NC). Mutant embryos could be distinguished from heterozygous embryos by a *LacZ* insertion marker carried by the balancer chromosome. *Mhcl* deficient embryos for feeding and lethality assays were identified using a balancer chromosome carrying a GFP insertion marker (Bl. 6663).

As mesoderm-specific driver lines, we used *DMef2-Gal4* (Ranganayakulu et al., 1996), *twist-Gal4* (SG24, kindly provided by A. Michelson, National Heart, Lung and Blood Institute, Bethesda, MD), *sns-pro3-Gal4* (kindly provided by S. Abmayr, Stowers Institute for Medical Research, Kansas City, MO), and *rp298-Gal4* (Menon and Chia, 2001). The adult muscle precursor (AMP)-specific driver line *1151-Gal4* (Roy and VijayRaghavan, 1997) was obtained from L. S. Shashidhara, IISER Pune, India.

P{XP}msps^{d03376} (d03376) and *PBac{WH}f03086* (f03086, Exelixis Collection at Harvard) were used for FLP-FRT-mediated recombination of the *Mhcl* locus as described in Parks et al. (2004). The established white-eyed flies (Δ*Mhcl*) were checked for an occurring recombinant hybrid element via PCR (primers XP5' plus and WH5' minus).

We used meiotic recombination in females to generate flies that carry either *Df(3L)BK9* and Δ*Mhcl*, or *Df(3L)BK9* and *UAS-GFP-Mhcl*, on the third chromosome. Recombinant flies were checked for Mhcl expression and lethality against the *rols*^{XX117} allele. To remove one copy of *rols* (or *Mhcl*) in homozygous *Mhcl* (or *rols*) mutants, virgins of recombinant flies were collected and crossed against males containing only one deficiency.

In situ hybridization

Paraformaldehyde-fixed embryos in whole mounts were hybridized *in situ* essentially as described in Tautz and Pfeifle (1989). DIG-labeled RNA probes were synthesized from linearized cDNA using appropriate RNA polymerases (according to DIG RNA Labeling Kit, Roche Diagnostics GmbH, Mannheim). We used the *Mhcl* cDNA GH15471 (obtained from *Drosophila* Genomics Resource Center, USA) as a template for transcribing the RNA probe using SP6 polymerase (Roche Diagnostics).

To identify embryos carrying balancer chromosomes tagged with a *LacZ* marker or to stain embryos carrying additional *LacZ* reporter constructs, the embryos after hybridization were immunostained with anti-β-galactosidase (Biotrend).

Immunohistochemistry

Staged embryos were collected from grape juice agar plates, rinsed with TNX (0.7% NaCl and 0.01% Triton-X-100), dechorionized with 50% bleach, and fixed for 15 min in 4% paraformaldehyde/heptane. They were subsequently devitellinized by intensive agitation in a 1:2 vol/vol heptane/methanol solution. After rehydration in PBT, embryos were incubated overnight at 4 °C with primary antibodies in PBT at the following concentrations: anti-β-

galactosidase (rabbit, Biotrend, Köln) 1:3000, anti-β3-Tubulin (rabbit; Leiss et al., 1988) 1:3000, anti-Fasciclin III (mouse; Patel et al., 1987) 1:50, anti-Kettin (rabbit, kindly provided by B. Bullard, University of York, UK) 1:1000, anti-GFP (rabbit, Abcam plc, Cambridge) 1:1000, and anti-Myc (mouse, Millipore, Schwalbach) 1:2000. After blocking with 2% normal goat serum, the primary antibodies were detected with biotinylated anti-rabbit or anti-mouse secondary antibodies (Vector Laboratories, Burlingame) at a 1:500 dilution for 2 h at room temperature. After amplifying the reaction using the Vectastain ABC Elite Kit (Vector Laboratories), embryos were stained with diaminobenzidine, H_2O_2, and NiCl. Fluorescent-labeled secondary antibodies (Dianova, Hamburg) were used at a 1:200 dilution.

1st and 3rd instar larvae were fixed with 4% paraformaldehyde, carefully dissected to allow access of the antibody, and fixed again for 20 min. Adult abdominal muscles were prepared and fixed as described in Currie and Bate (1991). Immunohistochemistry was carried out as described above, using additionally Cy5-coupled phalloidin (Dyomics, Jena) at a 1:100 dilution. Specimens were mounted in Epon or Fluoromount G™ (Southern Biotech, Birmingham) and examined using a Zeiss Axiophot or Zeiss Apotome microscope.

Yeast two-hybrid assays

A cDNA library, obtained from 0–24 h *Drosophila* embryos, in pB42AD vectors was screened using the Matchmaker™ LexA Two-Hybrid System (Clontech) and a fragment carrying the ankyrin repeats of Rols7 (RolsANK) in pGilda (Kreisköther et al., 2006). The obtained *Mhcl* fragment in pB42AD corresponds to the C-terminal 2.3 kb of the gene, starting shortly behind the IQ domains and ending at the stop codon. This fragment was recloned into pGilda. Additional Y2H assays were carried out with *rols* fragments R1 (encoding aa 367–633), R2 (encoding aa 634–1035), and R3 (encoding aa 1038–1369) in pGBKT7 using the Matchmaker™ 3 System (Clontech).

The yeast strain EGY48 [p8op-LacZ] was co-transformed with the appropriate constructs in the pGilda and pB42AD vectors. Co-transformants were grown on suitable plates, and the colonies were transferred on induction plates containing X-gal, either with or without leucine, to test for activation of an additional reporter gene. Blue staining of the colonies after one or two days indicated an interaction of the tested constructs.

Constructs for cell culture experiments and generation of transgenic flies

Full-length versions of *Mhcl* and *Rols7* and the coiled-coil region of Mhcl (Mhcl-fl, Rols-fl, Mhcl-CC) were amplified via PCR using the following respective primer pairs: Mhcl-fl-fwd (3'-CACCATGACGCACATCGAGAGCC-5') and Mhcl-fl-rev (3'-CTTGGCGTTATTTCTCGGGCT-5'), Rols-fl-fwd (3'-CACCATGCCCTCGCTGCAG-5') and Rols-fl-rev (3'-CAAATCGGTGATCTCATGATGAG-5'),

and Mhcl-CC-fwd (3'-CACCATGCCCCTGCTCAACGTTCATCG-5') and Mhcl-CC-rev (3'-GAACACCACGGTGCGGG-5').

Fragments were cloned into the pENTR™/D-TOPO® vector (Invitrogen, Karlsruhe). Recombination was catalyzed by the LR Clonase® II plus enzyme mix (Invitrogen), and the constructs were cloned into modified Gateway vectors (kindly provided by S. Bogdan, Münster University). The constructs with N- or C-terminal tagged GFP and 10×Myc tags were injected into embryos of fly strains Bl. 24484 and Bl. 24749 (the construct integrates into the second and third chromosome, respectively; Bischof et al., 2007).

SL2 cell culture, transfection, and immunostainings

SL2 cells were transfected and stained with antibodies to analyze the localization of Mhcl and Rols7 as described in Hornbruch-Freitag et al. (2011). The antibodies used were anti-GFP (Abcam plc) 1:1,000, anti-Myc (Millipore) 1:1,000, and fluorescent-labeled secondary antibodies (Dianova) 1:100.

Generating an anti-Mhcl antibody

An 822 bp C-terminal fragment of the gene (red line in Fig. 1A) was cloned into the expression vector pET44a (Novagen, Merck, Darmstadt), and the corresponding 274 aa were ectopically expressed in *Escherichia coli* cells. After purification of the recombinant protein via a His tag, the tags provided by the vector were removed by recombinant enterokinase (Novagen). This purified protein fragment was used to immunize rabbits (performed by Pineda Antikörper-Service, Berlin). The obtained sera were purified using protein A Sepharose and NHS-activated Sepharose (GE-Healthcare, Munich). The antibody was used at a dilution of 1:1,000 in immunofluorescent stainings of larval and adult muscles. To test for specificity, the antibody was incubated with the purified peptide for 1 h at room temperature prior to immunohistochemical analysis of adult abdominal muscles.

Lethality and feeding assay

Wild-type and *Mhcl* deficient embryos were staged to the same age, dechorionized, and bedded in oil on corn meal agar plates with yeast paste. After five days, the number of dead and alive 1st, 2nd, and 3rd instar larvae were counted.

To examine the size and shape of the gut, wild-type and *Mhcl* deficient 1st instar larvae were bedded on corn meal agar plates containing approximately 0.005% bromophenol blue and were allowed to feed for 2 days before embedding in Epon; the fixation step was omitted.

Results

The myosin heavy chain Mhcl interacts with Rols7

Because of the numerous genetic redundancies in *Drosophila*, it is difficult to identify new genes relevant for myoblast fusion via forward genetic strategies. Therefore, we screened a cDNA library with the ankyrin repeats of Rols7 (RolsANK; Kreisköther et al., 2006) in a Y2H assay to identify unknown interaction partners of this protein. In this yeast system, we identified Myosin heavy chain-like (Mhcl) as an interaction partner of RolsANK (Supplementary Fig. S1). According to its mesodermal transcription during myoblast fusion (see next paragraph) and with respect to many actin-based processes during myogenesis, we considered Mhcl as a particular interesting candidate to focus on.

Screens with other Rols fragments did not reveal likely candidates for interaction with Rols7 during myoblast fusion (Supplementary Table S1), as none were expressed in the mesoderm and the corresponding mutant embryos were not defective in musculature development. As the TPR repeats of Rols7 are known to interact with the adhesion molecule Dumbfounded (Kreisköther et al., 2006) and the RING finger domain interacted with itself in the Y2H assay (not shown), we concluded that RolsANK used to screen the library was the only fragment that revealed a new potential interaction partner of Rols7 during myogenesis.

The unconventional myosin Mhcl belongs to myosin class XVIII (Tzolovsky et al., 2002). Like other myosins, Mhcl contains an actin-binding motor domain (Guzik-Lendrum et al., 2011), which probably is responsible for the movement of Mhcl along F-actin. Mhcl also contains two IQ domains that probably bind light chains or related calmodulins and a C-terminal tail consisting of two sections of coiled-coil domains, known as the rod domain. As a characteristic criterion of myosins of class XVIII, Mhcl contains a PDZ domain, known as a protein–protein interaction module (Fig. 1A). The fragment interacting with RolsANK in the Y2H assay is located at the C-terminus of Mhcl (Fig. 1A; Rols-interacting region). To analyze whether Mhcl is able to homodimerize via its coiled-coil region, we co-transformed yeast cells with the corresponding fragment of the gene (Mhcl-CC) in pGilda and pB42AD. The induced growth of blue yeast colonies occurred more rapidly than in other cases, which indicated a very strong homodimerization of this protein via its rod domain (Supplementary Fig. S1).

We verified the interaction of Mhcl and Rols via transfection of the full-length proteins alone and together in SL2 cells. GFP-Mhcl transfected alone localized in vesicles (Fig. 1B, B', B''), whereas Myc-Rols transfected alone localized in small spots throughout the cytoplasm (Fig. 1C, C', C''). When we cotransfected both GFP-Mhcl and Myc-Rols, most of the Myc-Rols protein colocalized with GFP-Mhcl in vesicles instead of throughout the cytoplasm (Fig. 1D, D', D''),

which thus demonstrated the interaction of Rols7 and Mhcl in SL2 cells. Furthermore, we verified the interaction of Rols7 and Mhcl by co-immunoprecipitation (not shown).

Mhcl is expressed predominantly in somatic and visceral FCs during myoblast fusion

To investigate the relevance of Mhcl during myogenesis, we first analyzed its transcript distribution via *in situ* hybridization. Hardly any transcripts were detectable in embryonic stages 0–10 (not shown), which strongly implies that the mRNA is not provided maternally. Zygotic *Mhcl* transcription started in stage 11 in both the somatic and the visceral mesoderm (Fig. 2A) and continued through stage 14 (Fig. 2B). At the end of embryogenesis, the transcript was present in segmental epidermal stripes and in the esophagus; we observed no signals of *Mhcl* transcripts in mature myotubes (not shown).

To identify the cell type transcribing *Mhcl*, we used *rp298-LacZ* embryos, which express β-galactosidase in the nuclei of all mesodermal FCs because of an enhancer trap insertion in the FC specific transcribed *duf/kirre* gene (Nose et al., 1998). In embryos hybridized with an *Mhcl* probe, the *Mhcl* mRNA was detected in FCs of the somatic mesoderm, but not in the surrounding FCMs (Fig. 2C). Furthermore, in stages in which the visceral *rp298-LacZ*-positive FCs are arranged in one row with the FCMs lying above them (Klapper et al., 2002), *Mhcl* transcripts were only detected in the row of visceral FCs (Fig. 2D). To confirm the prominent transcription of *Mhcl* in FCs, we analyzed *Notch* and *lmd* null mutant embryos. Homozygous *Notch* mutants differentiate more FCs at the cost of FCMs (Corbin et al., 1991); in these mutants, the *Mhcl* transcription pattern was much broader than in wild-type embryos (Fig. 2E). Embryos carrying a loss-of-function allele of the FCM-specific transcription factor Lmd exhibit more FCMs than the wild-type (Duan et al., 2001). Here, the *Mhcl* transcription pattern was not extended (Fig. 2F). From these *in situ* hybridization data, we concluded that *Mhcl* is most likely transcribed solely in the FCs of the mesoderm and that transcription is shut down after successful myoblast fusion.

An anti-Mhcl antibody detects the protein in ectopic conditions and endogenous protein adjacent to the Z-lines of sarcomeres

To analyze the distribution of the Mhcl protein, we raised an antibody against a 274 aa fragment of the rod domain (Fig. 1A, dark red bar). We tested the specificity of the antibody in Western blots of protein extracts of SL2 cells transfected with *Mhcl-fl* or the N-terminally shortened version *Mhcl-CC*. The antibody bound to the corresponding proteins of the expected size in transfected cells and to endogenous Mhcl in untransfected cells (Fig. 3A). Although we also detected ectopically expressed Mhcl in the epidermis of embryos using a *wingless-Gal4* driver line, we did not detect a specific signal of endogenous Mhcl in the mesoderm (not shown). Therefore, we postulate that Mhcl might be a part of a protein complex in the mesoderm and is thus not accessible for the antibody *in vivo*.

We then analyzed the mature larval and adult musculature using the anti-Mhcl antibody. The sarcomeres are characterized by the Z-line component Kettin (Hakeda et al., 2000), which is derived from the *sls* gene. We visualized the Z-discs by anti-GFP staining of flies carrying the protein trap allele *sls-GFP* (Morin, 2003). In muscles of 3rd instar larvae (Fig. 3B) and in adult abdominal muscles (Fig. 3C), the anti-Mhcl antibody bound to two distinct bands adjacent to the GFP signal (Fig. 3B', B'', C', C''). The signal did not resemble the pattern one would expect for the sarcomeric muscle myosin, i.e., the thick filaments between the Z-discs (Fig. 3 C''). We tested the signal specificity by incubating the antibody with the purified peptide used for raising the antibody prior to incubation with adult abdominal muscles. In the musculature, the signal obtained with the preincubated anti-Mhcl antibody was strongly reduced (Fig. 3D). We then checked whether Mhcl ectopically expressed and N-terminally tagged with GFP localizes with a similar pattern when driven in the adult musculature using the *1151-Gal4* driver line (Roy and VijayRaghavan, 1997). The ectopic protein also localized to the Z-lines, although the pattern detected was less distinct than that obtained with the antibody (Fig. 3E). From this we concluded that the anti-Mhcl antibody specifically detected the endogenous protein adjacent to the sarcomeric Z-discs of mature myotubes. As Rols7 can also be found in Z-lines of the sarcomeres (Kreisköther et al., 2006), it is possible that the two proteins interact in myotubes, in addition to their potential interaction at the FuRMAS during myoblast fusion.

Homozygous ΔMhcl embryos develop a wild-type somatic and visceral musculature

The mRNA distribution in the mesoderm, the likely interaction of Mhcl with Rols7, and the lack of any other myosin known to be essential for myoblast fusion led us to postulate that Mhcl could be involved in myoblast fusion. Therefore, we deleted *Mhcl* via FLP-FRT mutagenesis. In the fly line used, Δ*Mhcl* and five additional genes have been removed, among them *CG32855* and the sex-specific phospholipase *sxe2*, both of which are located within introns of *Mhcl*, and also *Akt1*, a member of the mTOR pathway controlling growth, survival, and proliferation of cells (reviewed in Hietakangas and Cohen, 2009). We verified the deletion of *Mhcl* in these embryos via PCR (not shown) and *in situ* hybridization (Fig. 4A). Surprisingly, Δ*Mhcl* embryos displayed a somatic muscle pattern like that of the wild-type (Fig. 4B), and attachment to the epidermis was unaffected (not shown). The morphology of the gut was also like that of the wild-type (Fig. 4C), even though Mhcl is strongly expressed in the gut (Fig. 2A, B, D). Furthermore, when we examined other tissues of homozygous Δ*Mhcl* embryos, we did not detect any irregularities in dorsal closure or in the formation of the CNS and the heart (not shown).

Homozygous Δ*Mhcl* animals died during the transition from the 1st to the 2nd instar larvae (Supplementary Fig. S2). In a feeding assay, we showed that these mutant larvae could take up yeast paste containing bromophenol blue and that the size and shape of the gut is regular (compare the wild-type in Fig. 4D to the mutant in Fig. 4E), as was already observed in

embryos. Thus, the development as well as the function of the gut did not seem to be affected in *Mhcl* mutant larvae.

We detected an anti-Mhcl antibody signal adjacent to Kettin-positive Z-discs, and we speculated that Mhcl is necessary for sarcomere formation. Therefore, we analyzed the establishment of larval sarcomeres using an anti-Kettin antibody. In homozygous Δ*Mhcl* larvae, the Z-discs of the sarcomeres were clearly visible all over the myotube and at the terminal end of the myotube (Fig. 4G) and did not differ from the wild-type (Fig. 4F). Thus, we concluded that sarcomeres are formed correctly in Δ*Mhcl* larvae. Their proper formation was confirmed by the normal motility of 1st instar larvae (not shown).

As the imprecise excision of a P-element located in the putative 5'-UTR of *Mhcl* (*EY00454*) produced lethal fly lines mutated in *Akt1* (data not shown), we speculated that the deletion of *Akt1* was responsible for the lethality of Δ*Mhcl* 1st or 2nd instar larvae. Moreover, earlier studies have pointed out that hypomorphic mutations of Akt1 as well as P-element insertions upstream of *Akt1* are semi-lethal (Gao et al., 2000; Stocker et al., 2002), but that expression of a mutated, catalytically inactive Akt1 protein leads to embryo death (Staveley et al., 1998). Therefore, we analyzed embryos transheterozygous for Δ*Mhcl* and the deficiency line *Df(3R)BSC728.* In these embryos, Akt1 is expressed and only the coding regions of two other proteins, CG10185-P and Msps, as well as the C-terminal part of Mhcl, are deleted. Nevertheless, also these flies were not viable, even though their body wall muscles developed normally (not shown). Therefore, we propose that the complete deletion of *Akt1* is not the only reason for the lethality of the generated Δ*Mhcl*.

In epistasis experiments, we tested whether the deletion of *Mhcl* enhances the phenotype of the *rols* deficiency line *Df(3L)BK9*. Homozygous $rols^{Df(3L)BK9}$ embryos exhibited the characteristic pattern of unfused FCMs and so-called mini muscles that stopped myogenesis after the first fusion phase (Fig. 4H; Chen and Olson, 2001; Menon and Chia, 2001; Rau et al., 2001). We observed the same muscle phenotype in the $rols^{Df(3L)BK9}$ and Δ*Mhcl* double mutant embryos (Fig. 4I). In contrast, removing one copy of *rols* in an *Mhcl*-deficient background did not disturb somatic muscle development (Fig. 4J).

Although Mhcl was strongly expressed in the developing visceral and somatic mesoderm, these tissues in Δ*Mhcl* mutant embryos and larvae were not obviously defective. We thus proposed that Mhcl can be functionally replaced by other myosin heavy chains when Mhcl is absent. To test for functional redundancy during myoblast fusion, we chose the non-muscle myosin heavy chain II/Zipper (Zip). Zip is strongly expressed already in oogenesis and, in the developing embryo, in the leading edge cells of the lateral epidermis and in muscle attachment sites (Bloor and Kiehart, 2001; Franke et al., 2005); furthermore, Zip shows 30% sequence similarity at the protein level to Mhcl (http://blast.ncbi.nlm.nih.gov/Blast.cgi), which made it an interesting candidate for functional redundancy. We created $\Delta Mhcl;zip^{2}$ double mutants and

analyzed the somatic and visceral muscle pattern as well as fusion efficiency. Unfortunately, Δ*Mhcl;zip²* double mutants were strongly defective in dorsal closure and germ band retraction, as described before for *zip²* mutants alone (Young et al., 1993). These defects made it impossible to determine whether myoblast fusion in the double mutants was more disrupted than in single mutants (not shown). Therefore, we expressed a dominant negative version of Zip (*UAS-GFP-DN-zip;* Franke et al., 2005) in the mesoderm of Δ*Mhcl* mutant embryos; this DN-Zip causes dorsal closure defects when expressed in the ectoderm (Franke et al., 2005). The dominant negative protein accumulated strongly in the developing musculature; however, we did not detect any defects in muscle formation in Δ*Mhcl* embryos additionally expressing this fusion protein (Supplementary Fig. S3). From these results, we concluded that either the construct did not have any dominant negative effect in the mesoderm or Zip is not involved in myoblast fusion, neither alone nor in redundancy to Mhcl.

An ectopically expressed GFP-Mhcl localizes in FCs at the membrane towards the adhering FCM

Since the generated anti-Mhcl antibody did not produce a specific signal in embryos, we created transgenic flies for full-length Mhcl N-terminal tagged with GFP and induced expression in the mesoderm or in specific cell types. This did not result in an abnormal muscle phenotype (not shown); thus, excess protein does not disturb myoblast fusion. When expressed using *twi-Gal4*, GFP-Mhcl was detected from stage 11 onwards; in fusion-relevant stages, a transient signal was obtained at the contact sites of FCMs and the growing muscle (Fig. 5A, A'). When we expressed GFP-Mhcl only in FCs, we observed the same localization as a thin sheet at the membrane (Fig. 5B, B'). This localization was lost when the protein was ectopically expressed only in FCMs; the signal was randomly distributed at the membrane of unfused myoblasts (Fig. 5C). In rare cases, we detected a signal at the contact sites of FCMs towards the growing muscle (Fig. 5C'). Nevertheless, as we only observed a specific localization there in very late myotubes, we proposed that this represents ectopically expressed GFP-Mhcl that had been contributed by earlier fused myoblasts.

We then examined the localization of GFP-Mhcl in characterized fusion mutants in which myoblast fusion is completely arrested. The guanine exchange factor (GEF) Mbc has been shown to act solely in FCMs during myoblast fusion; *mbc$^{D11.2}$* mutant embryos lack F-actin foci at the site of the FCM (Haralalka et al., 2011). In these mutants, GFP-Mhcl still localized as a thin sheet at the site of the FC with adhering FCMs when expressed by *rp298-Gal4* (Fig. 5D, D'). We observed this localization only in very rare cases, as only a few unfused myoblasts still adhere to an FC in *mbc* mutant embryos (Doberstein et al., 1997). In *blow²* mutant embryos, myoblast fusion does not proceed beyond the prefusion complex, so that myoblasts adhere to the FCs, but no cytoplasmic continuity is established (Doberstein et al., 1997; Jin et al., 2011). When driven in

FCs of *blow²* mutants, GFP-Mhcl localized at distinct spots at the membrane of these cells and also accumulated within the FCs (Fig. 5E, E'). Although the *blow²* mutant phenotype is characterized by many myoblasts adhering at the growing myotube, known as the "bunches of grapes" phenotype (Doberstein et al., 1997), GFP-Mhcl localized only in rare cases at the contact sites towards FCMs (Fig. 5E). Then we analyzed the localization of GFP-Mhcl in FCs of *rols* LOF mutants, using the *rols* deficiency *Df(3L)BK9*. In these embryos, GFP-Mhcl was detected in the cytoplasm of the FCs (Fig. 5F, F'), while contact sites of these cells with adhering FCMs did not exhibit the strong signal we obtained at contact sites of myoblasts in wild-type embryos.

At last checked whether the coiled-coil region of Mhcl (Mhcl-CC, Fig. 1A) was sufficient for the localization at contact sites of the growing muscle during myoblast fusion. To this aim, we expressed Mhcl-CC-10×Myc in the whole mesoderm using *DMef2-Gal4* (Fig. 5G, G'). With the shorter protein, we detected the Myc tag in the mesoderm in all stages of development. In fusion-relevant stages, Mhcl-CC localized randomly in speckles and close to the membrane of unfused FCMs (Fig. 5G) and the growing muscle (Fig. 5G'); we did not observe a specific signal at contact sites of the FCMs and myotubes. This localization pattern resembles that in *blow²* mutants, which also accumulate GFP-Mhcl at sites where no FCMs adhere at the FC (compare to Fig. 5E, E').

Our results showed that GFP-Mhcl localizes at contact sites in the wild-type after recognition and attachment has occurred between the growing muscle and the FCM. Furthermore, GFP-Mhcl did not localize at contact sites of unfused FCMs in the wild-type and localized randomly at the myotube membrane in *blow²* mutant embryos. GFP-Mhcl did also not localize at myoblast contact sites in *rols* null mutant embryos, indicating that the correct localization of Mhcl depends on the presence of Rols7. Finally, Mhcl-CC-Myc did not localize at contact sites in FCs. From this, we concluded that the region of Mhcl that is deleted in Mhcl-CC is needed for correct localization of the protein.

Discussion

We present the unconventional myosin Mhcl as a new potential player in myogenesis of *Drosophila* embryos. In this study, we showed that the protein *in vitro* and in SL2 cells interacts via its C-terminal coiled-coil domain with the ankyrin repeats of Rols7. This part of the fusion-relevant molecule is already described as being essential for muscle development (Menon et al., 2005). *Mhcl* is expressed during the fusion-relevant stages in both the somatic and the visceral mesoderm, and most prominently in the FCs. Owing to its mesoderm-specific expression pattern, we postulated an essential function for Mhcl in myoblast fusion. When ectopically expressed in the mesoderm, GFP-Mhcl localized at the contact sites of already adhered myoblasts, predominantly on the side of the growing myotube and even in mutants that stop

myoblast fusion after recognition and adhesion. For this localization, the motor domain of Mhcl is necessary. Nevertheless, deletion of *Mhcl* did not result in a myoblast-fusion defect. Therefore, other myosin motor proteins are likely involved in the same cellular processes during myoblast fusion.

Rols7, the interaction partner of Mhcl, is part of the FuRMAS (Kesper et al., 2007), a transient signaling center needed for successful myoblast fusion. We postulate a model in which Mhcl affects widening of FuRMASs in redundancy to a so far unidentified motor protein (Fig. 6). In our model, the Ig domain protein Duf/Kirre is inserted into the membrane of the FC/growing muscle and its intracellular part interacts with the TPR-E of Rols7 (Kreisköther et al., 2006). Via the essential ankyrin repeats (Menon et al., 2005), Rols7 is necessary to recruit the C-terminal part of Mhcl. GFP-Mhcl localizes at the contact sites in the growing myotube, which are also characterized by a thin F-actin sheet; Mhcl-CC lacking its motor domain does not localize correctly. As Guzik-Lendrum et al. (2011) demonstrated that actin is bound by the motor domain of Mhcl, we therefore postulate that the presence of Rols7 alone is not sufficient to localize Mhcl at the FuRMAS, but that actin is also needed for correct localization. We thus hypothesize that Mhcl is recruited to the actin filaments in the precursor cell and, after the FuRMAS is established and fusion is initiated, widens the fusion pore by moving along these filaments in the actin-containing center of the FuRMAS. An actin-free zone before membrane breakdown has been described at the ultrastructural level in fusing myoblasts (Sens et al., 2010), and therefore Mhcl and redundantly operating molecules are possibly required for the establishment of this actin-free area, similar to the non-muscle myosin heavy chain Zip, "pushing" determinants into daughter cells during asymmetric cell division of neuroblasts (Barros et al., 2003).

Successful fusion initiation seems to be necessary for Mhcl localization, as GFP-Mhcl only localized at the contact sites in *mbc* mutants in the rare cases when an FCM adheres towards a growing muscle. However, the ectopically expressed fusion protein localized only insufficiently at the contact sites in FCs in *blow* mutant embryos. It has been shown that Blow functions at the site of the FCM in regulating the WASp/WIP complex necessary for Arp2/3-mediated actin polymerization, and that the FuRMAS and with them the fusion pores do not expand in *blow* mutants (Jin et al., 2011; Kesper et al., 2007). Therefore, we suggest that actin branching is also necessary at the site of the FCM for widening the fusion pore and that Mhcl might only be localized when fusion pore widening is initiated correctly.

The FuRMASs share many characteristics with other transient cell-adhesion structures, as immunological synapses, podosomes, or invadopodia (reviewed in Önel and Renkawitz-Pohl, 2009; Önel et al., 2011). Immunological synapses usually connect an antigen-presenting cell with a T cell, and regulate the spatial and temporal communication between the two cells. The formation of such an immunological synapse involves the orchestrated movement of T cell

antigen receptor microclusters, which is dependent on Myosin IIA, the vertebrate non-muscle myosin II (Ilani et al., 2009). Podosomes and invadopodia are detectable in cells derived from the monocytic lineage as well as in epithelial cells. With the help of these actin-containing structures, cells adhere to and degrade the extracellular matrix, which can ultimately lead to invasion. There is evidence that myosin II forms circular structures within the podosomes to regulate their dynamics and to change their size or shape (Collin et al., 2008). Therefore, an involvement of myosin motor proteins in regulation of the FuRMAS size during myoblast fusion seems plausible.

Also the exocytosis of cytolytic granules at the actin-rich immunological synapse between a human natural killer cell and its target cell requires Myosin IIA (Andzelm et al., 2007). Particularly Myosin IIA is constitutively associated with these lytic granules to enable their interaction with F-actin and their penetration at the immunological synapse to the synaptic membrane (Sanborn et al., 2009). In addition, Zip has been shown to act at the *Drosophila* larval neuromuscular junction in synaptic vesicle trafficking (Seabrooke et al., 2010; Seabrooke and Stewart, 2011). Mhcl could therefore alternatively or additionally be involved in the transport of electron-dense vesicles to the FuRMAS; the vesicles are thought to contain essential molecules of the fusion machinery (Doberstein et al., 1997). These vesicles have been described as being coated with actin (Kim et al., 2007). Moreover, a transport process via microtubules seems unlikely, as the microtubule network plays only a minor role in myogenesis of the *Drosophila* embryo (Rudolf et al., 2012). However, vesicle transport has been so far only described for members of other classes of myosins, e.g., for MyoV, which is required for apical transport of secretory vesicles along actin filaments in epithelial tubes (Massarwa et al., 2009). Mhcl itself is likely to lack ATPase activity (Guzik-Lendrum et al., 2011), which supports the suggestion that a redundant motor protein must be involved in the proposed fusion pore widening or vesicle transport mechanism. Unfortunately, analysis of mutants in both *Mhcl* and the non-muscle heavy chain gene *zip,* which display severe defects in many tissues other than muscles, did not contribute to the identification of a redundant function. As also the classical muscle-myosin is present in myoblasts already prior to fusion, we cannot exclude the possibility that Mhc can replace Mhcl when deleted.

Using an anti-Mhcl antibody, we showed that Mhcl is additionally present in the sarcomeres of larval and adult muscles adjacent to the Z-discs. This pattern resembles the localization of the vertebrate homologue MYO18B in skeletal and cardiac muscles of mice (Ajima et al., 2008) and the distribution of muscle LIM protein, which was therefore thought to stabilize the sarcomeres together with D-Titin at the Z-disc boundary by capping the thin filaments (Clark et al., 2007). Moreover, vertebrate non-muscle heavy chains IIA and IIB, the *Drosophila* non-muscle heavy chain Zip, as well as the fusion-relevant protein Mind bomb 2 localize at the Z-discs (Bloor and Kiehart, 2001; Carrasco-Rando and Ruiz-Gómez, 2008; Takeda et al., 2000), and

the presence of a functional actin cytoskeleton in proximity to the Z-discs involved in structural integrity has been suggested before (reviewed in Kee et al., 2009). Because of the distribution of Mhcl in mature muscles and because *Mhcl* mutant larvae move and feed normally, Mhcl is unlikely to take over the muscle contraction and function of Mhc in the sarcomeric thick filaments. Mhcl might rather form protein complexes to establish and/or to maintain the sarcomere as part of the connecting filaments, possibly in a complex with its interaction partner Rols7, which also localizes at the Z-discs and therefore might take over similar functions (Kreisköther et al., 2006).

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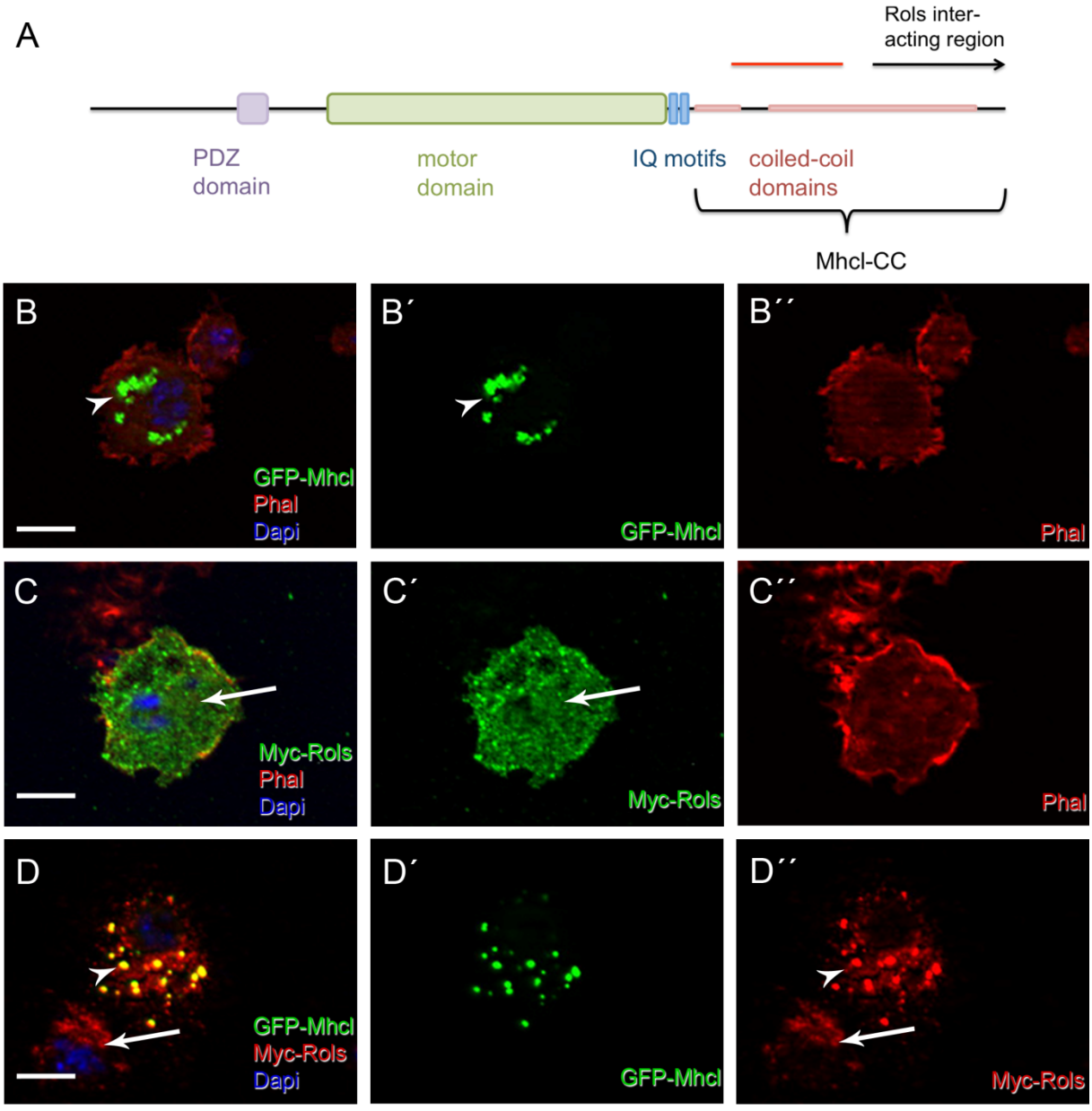
Figures


A
Rols inter-
acting region
PDZ
domain
motor
domain
IQ motifs
coiled-coil
domains
Mhcl-CC
B
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B´
GFP-Mhcl
B´´
Phal
C
Myc-Rols
Phal
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C´
Myc-Rols
C´´
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D
GFP-Mhcl
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Dapi
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GFP-Mhcl
D´´
Myc-Rols

Fig. 1. Mhcl and Rols colocalize in SL2 cells. (A) The domain structure classifies Mhcl as a group XVIII myosin. The 2148 aa Mhcl isoform PA contains the N-terminal PDZ domain, the motor domain, two IQ motifs, and the C-terminal tail, which consists of two coiled-coil domains (modified from flybase and http://smart.embl-heidelberg.de/). An anti-Mhcl antibody was raised against the indicated 274 aa (dark red bar). A C-terminal fragment of the protein that interacted with the ankyrin repeats of Rols (black arrow) was identified in a yeast two-hybrid assay. The C-terminal fragment Mhcl-CC was used for further studies. (B, B', B'') SL2 cells transfected with Mhcl-GFP and counterstained with phalloidin, showing localization of Mhcl-GFP in small vesicles (arrowhead). (C, C', C'') SL2 cells transfected with Rols-Myc, showing localization of Rols-Myc throughout the cytoplasm (arrows). (D, D', D'') SL2 cells cotransfected with Mhcl-GFP and Rols-Myc, showing colocalization of Rols-Myc with Mhcl-GFP in small vesicles (arrow head), and SL2 cells negative for Mhcl-GFP transfected with Rols-Myc, showing localization of Rols-Myc in the cytoplasm (arrow). Scale bars: 6 μm.

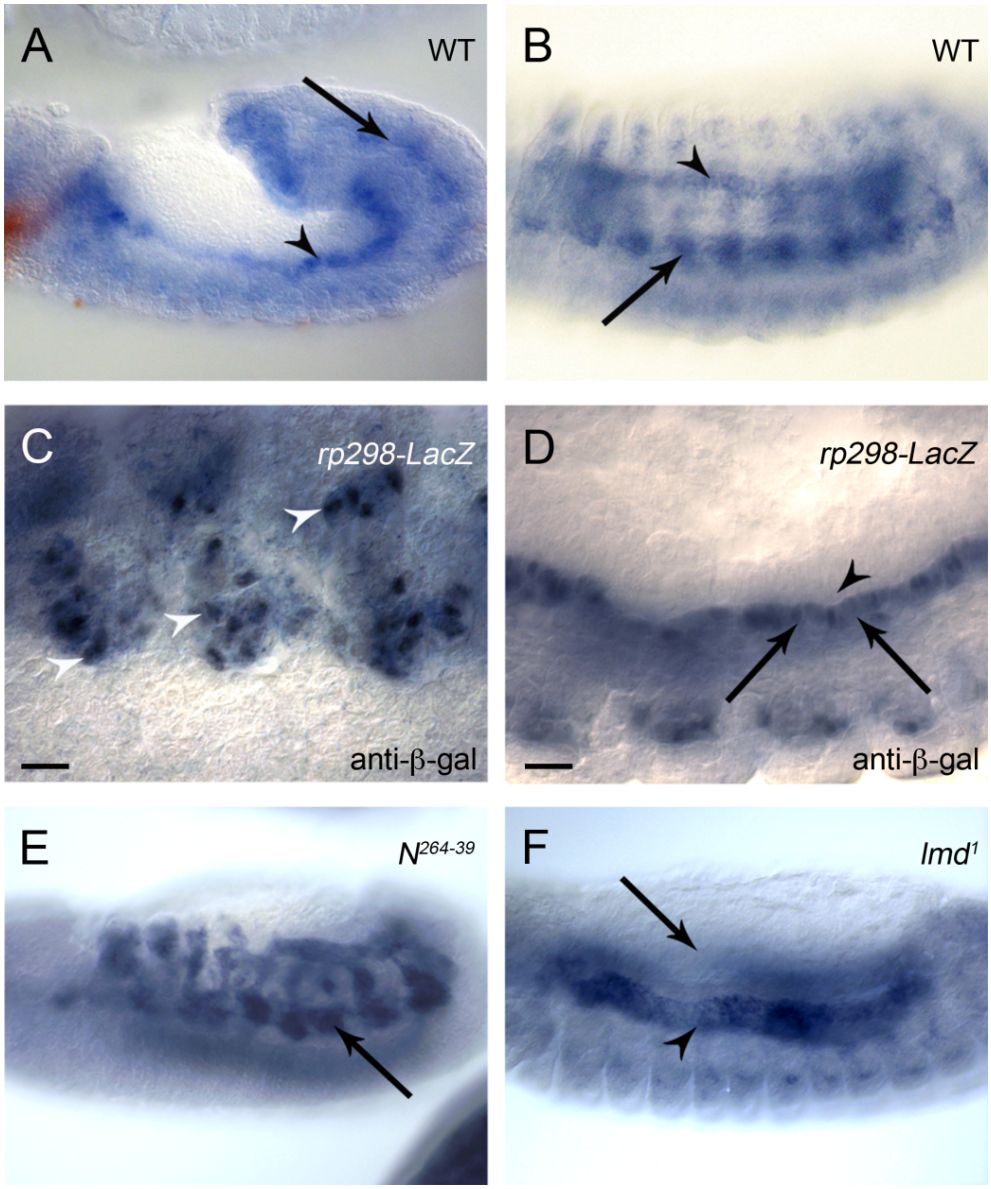


Fig. 2. *Mhcl* is expressed in the mesoderm predominantly in FCs during fusion-relevant stages. (A–F) *In situ* hybridization of embryos with a full-length *Mhcl* probe. (A, B) *Mhcl* mRNA in the somatic mesoderm (arrow) and in the visceral mesoderm (arrowhead) in (A) stage 11 embryos and (B) fusion-relevant stage 14. (C, D) *In situ* hybridization of *rp298-LacZ* embryos. Black areas, FC nuclei. Scale bars: 10 μm. (C) *Mhcl* mRNA in *rp298-LacZ*-positive cells of the somatic mesoderm (arrowheads). (D) *Mhcl* mRNA in one row of cells representing *rp298-LacZ*-positive FCs of the visceral mesoderm (arrows); visceral FCMs lying above this row contained no *Mhcl* mRNA (arrowhead). (E) *In situ* hybridization of *Notch* null mutant embryo showing broader *Mhcl* mRNA signal in the somatic mesoderm (arrow). (F) *In situ* hybridization of *lmd*^{1} mutant showing no broader *Mhcl* signal in the somatic mesoderm (arrow) and *Mhcl* transcripts in the visceral mesoderm (arrowhead).

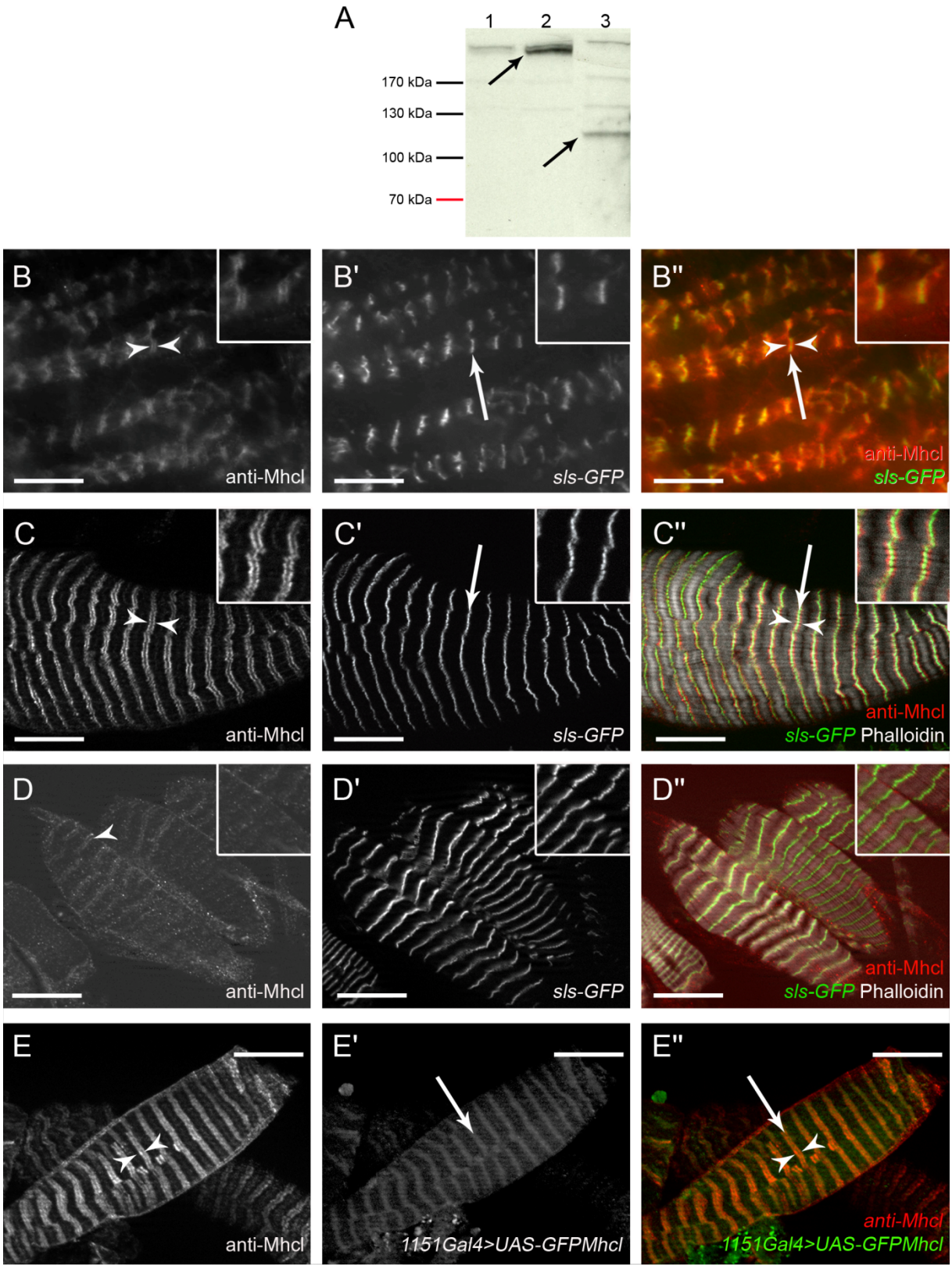

A
1
2
3
170 kDa
130 kDa
100 kDa
70 kDa
B
anti-Mhcl
B'
sls-GFP
B"
anti-Mhcl
sls-GFP
C
anti-Mhcl
C'
sls-GFP
C"
anti-Mhcl
sls-GFP Phalloidin
D
anti-Mhcl
D'
sls-GFP
D"
anti-Mhcl
sls-GFP Phalloidin
E
anti-Mhcl
E'
1151Gal4>UAS-GFPMhcl
E"
anti-Mhcl
1151Gal4>UAS-GFPMhcl

Fig. 3. An anti-Mhcl antibody detects the protein in sarcomeres adjacent to the Z-lines. (A) Western blot of protein extracts of (lane 1) untransfected SL2 cells, (lane 2) cells transfected with *Mhcl-fl*, and (lane 3) cells transfected with *Mhcl-CC*. The anti-Mhcl antibody bound to proteins of the expected size of Mhcl-fl (230 kDa) and Mhcl-CC (90 kDa) (arrows). (B–D) Immunohistochemical analysis with the anti-Mhcl antibody of (B) 3rd instar larvae and (C, D) adult abdominal muscles of flies carrying the protein trap allele *sls-GFP*. (B, C) Anti-Mhcl signal (arrowheads, B, B'', C, C'') close to the GFP signal of the Z-line marker Kettin (arrows, B', B'', C', C''); actin filaments were visualized with phalloidin (C''). (D, D', D'') The anti-Mhcl antibody was preincubated with the purified peptide used for raising the antibody before incubation with the adult abdominal muscles; strongly diminished signal adjacent to the Z-lines was observed (arrowhead, D). (E, E', E'') Mhcl N-terminally tagged with GFP and ectopically expressed in the adult musculature using the *1151-Gal4* driver line. Ectopic protein at the Z-discs was detected by anti-GFP (arrow, E', E''), endogenous Mhcl was detected by anti-Mhcl (arroheads, E, E''). Scale bars: 10 μm.

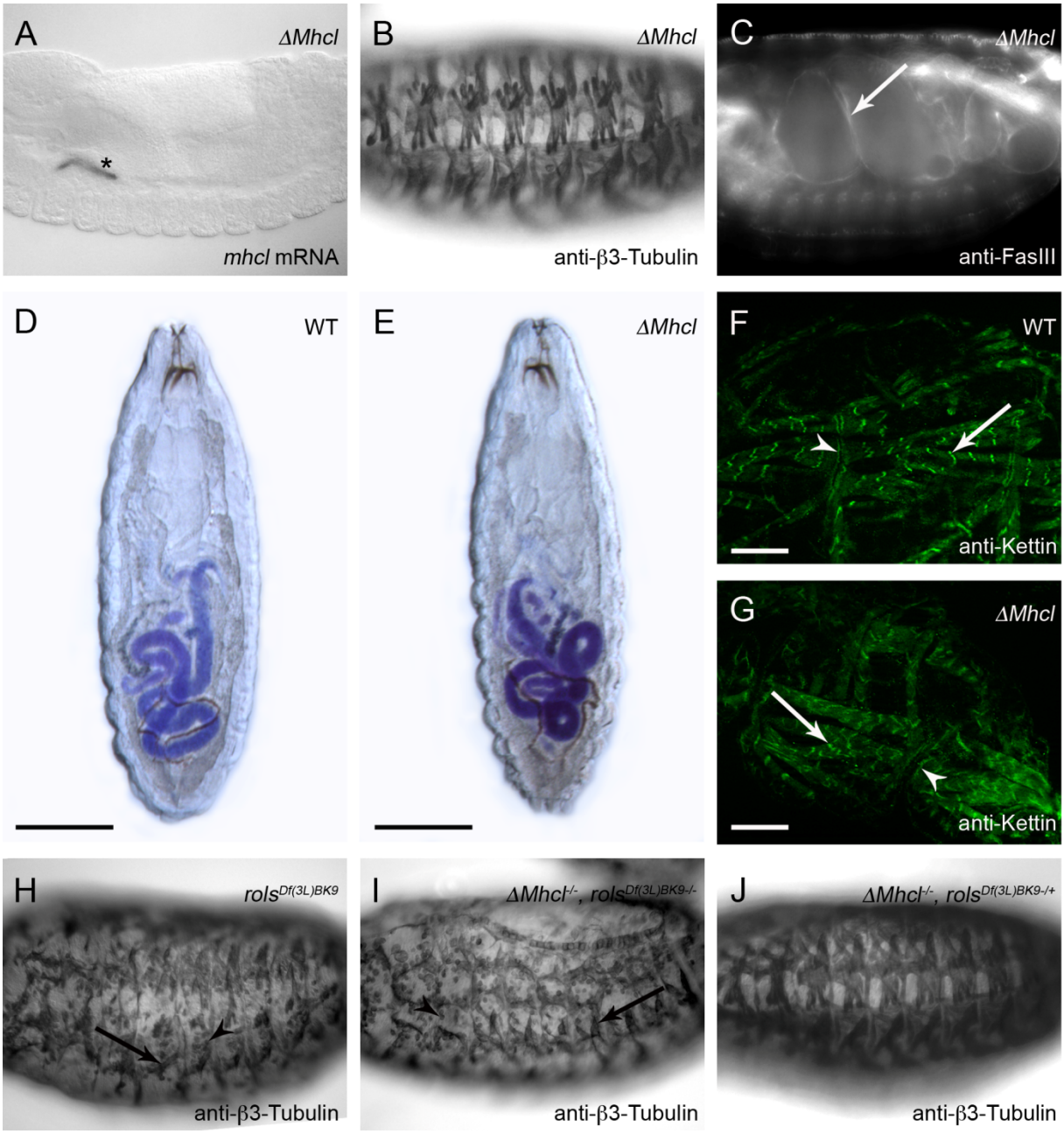

A
ΔMhcl
*
mhcl mRNA
B
ΔMhcl
anti-β3-Tubulin
C
ΔMhcl
anti-FasIII
D
WT
E
ΔMhcl
F
WT
anti-Kettin
G
ΔMhcl
anti-Kettin
H
rols^Df(3L)BK9
anti-β3-Tubulin
I
ΔMhcl-/-, rols^Df(3L)BK9-/-
anti-β3-Tubulin
J
ΔMhcl-/-, rols^Df(3L)BK9-/+
anti-β3-Tubulin

Fig. 4. The somatic and visceral musculature develops normally in *Mhcl* loss-of-function mutants. (A) *In situ* hybridization with a full-length *Mhcl* probe of a Δ*Mhcl* loss-of-function embryo generated via FRT/FLP mutagenesis; asterisk indicates an unspecific signal in the salivary gland. (B) The somatic musculature of a homozygous Δ*Mhcl* embryo visualized with an anti-β3-Tubulin antibody revealsed no defects. (C) Immunohistological analysis of the gut of a Δ*Mhcl* embryo using an anti-Fasciclin III antibody; properly formed gut constrictions were observed (arrow). (D-E) Wild-type (D) and (E) Δ*Mhcl* mutant 1st instar larvae with guts stained with bromophenol blue; scale bars: 200 μm (F–G) Larval somatic musculature of (F) wild-type and (G) Δ*Mhcl* 1st instar larvae stained with an anti-Kettin antibody; Z-discs within one myotube (arrows), terminal Z-discs of myotubes (arrowheads). Scale bars: 20 μm. (H–J) Body wall musculature visualized with an anti-β3-Tubulin antibody developed by (H) a *rols*-deficient embryo, (I) a homozygous Δ*rols,*Δ*Mhcl* double mutant embryo, and (J) a heterozygous Δ*rols*, homozygous Δ*Mhcl* embryo; mini muscles (arrows); unfused myoblasts (arrowheads).

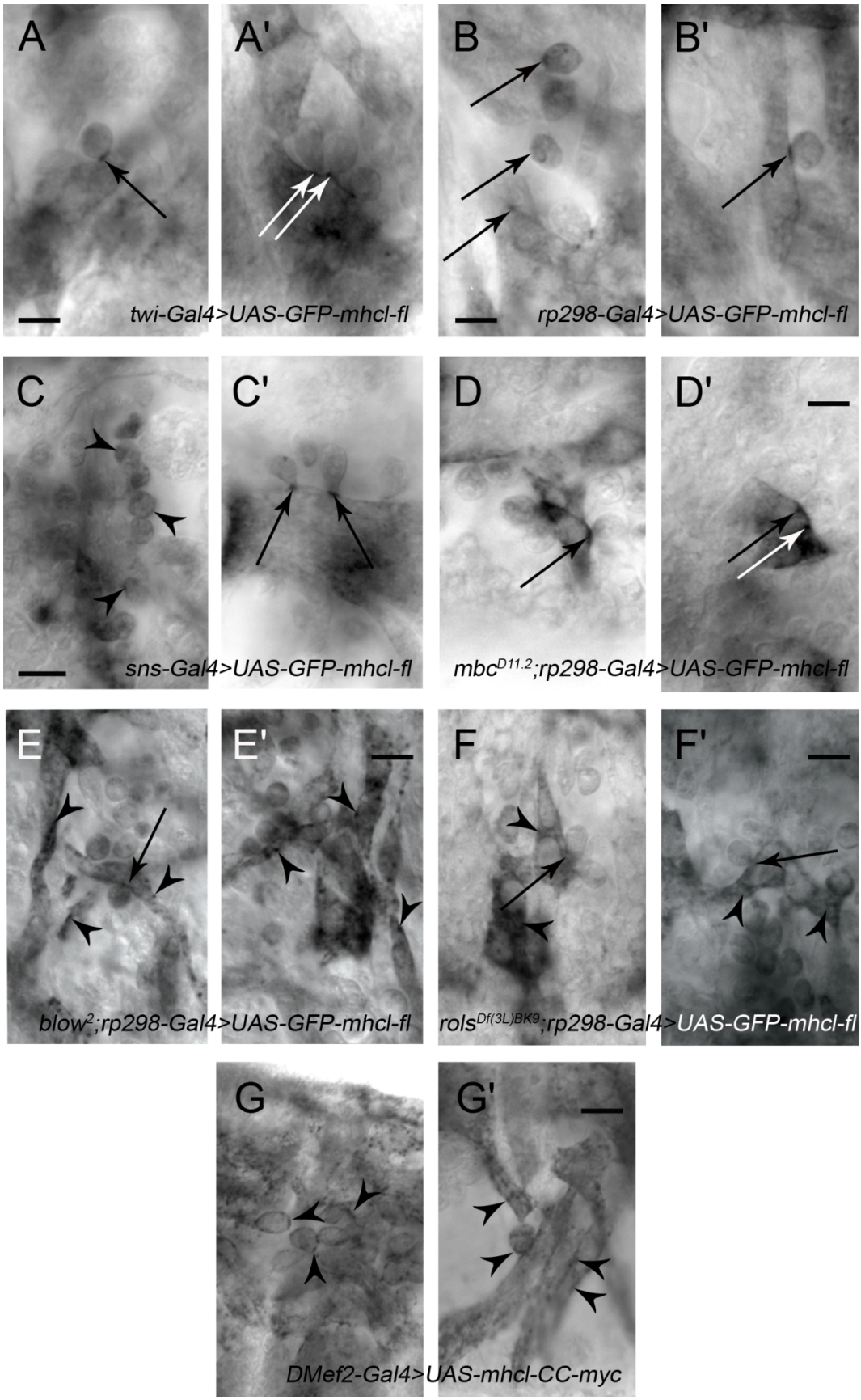

A
A'
B
B'
twi-Gal4>UAS-GFP-mhcl-fl
rp298-Gal4>UAS-GFP-mhcl-fl
C
C'
D
D'
sns-Gal4>UAS-GFP-mhcl-fl
mbc^D11.2;rp298-Gal4>UAS-GFP-mhcl-fl
E
E'
F
F'
blow^2;rp298-Gal4>UAS-GFP-mhcl-fl
rols^Df(3L)BK9;rp298-Gal4>UAS-GFP-mhcl-fl
G
G'
DMef2-Gal4>UAS-mhcl-CC-myc

Fig. 5. GFP-Mhcl localizes at the contact sites of the growing muscle towards FCMs during myoblast fusion. (A–C) Embryos with GFP-Mhcl-fl expression driven by (A) *twi-Gal4,* (B) *rp298-Gal4,* and (C) *sns-Gal4*. (A, A', B, B') The anti-GFP antibody produced a signal at the site of the growing muscle, where an FCM adheres when expression is induced in FCs; contact sites of myoblasts with growing muscles (arrows). GFP-Mhcl was detected at random sites when driven in FCMs (arrowheads in C), and only in rare cases at contact sites within a growing muscle (arrows in C'). (D, D') $mbc^{D11.2}$ mutant embryos in which GFP-Mhcl expression is driven by *rp298-Gal4*. GFP-Mhcl localized in FCs at sites where FCMs are adhering (arrows). (E, E') $blow^{2}$ mutant embryo in which GFP-Mhcl expression is driven by *rp298-Gal4*. GFP-Mhcl was detected at random sites at the membrane within FCs (arrowheads) and a localization at contact sites was only faint (arrow). (F, F') $rols^{Df(3L)BK9}$ mutant in which GFP-Mhcl expression is driven by *rp298-Gal4*. The anti-GFP antibody detected the protein in the cytoplasm (arrowheads), and contact sites with adhering myoblasts did not exhibit a stronger signal (arrows). (G, G') Embryos in which Mhcl-CC-Myc expression was driven by *DMef2-Gal4*. The anti-Myc antibody detected a signal randomly distributed at the membrane of FCMs (arrowheads in G) and the growing muscle (arrowheads in G'). Scale bars: 5 μm.

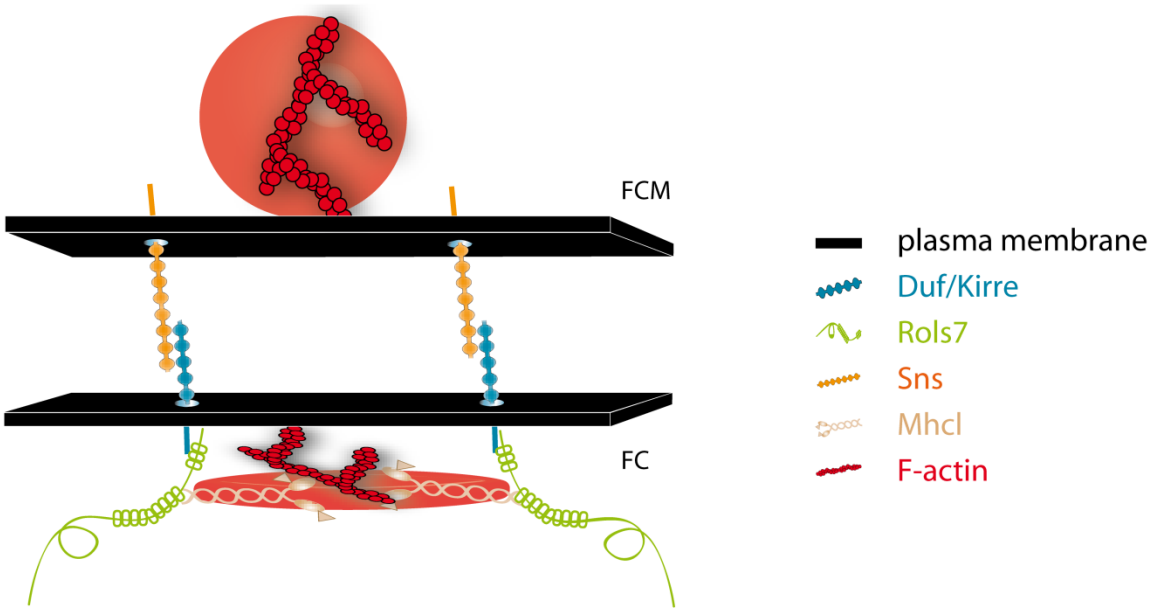
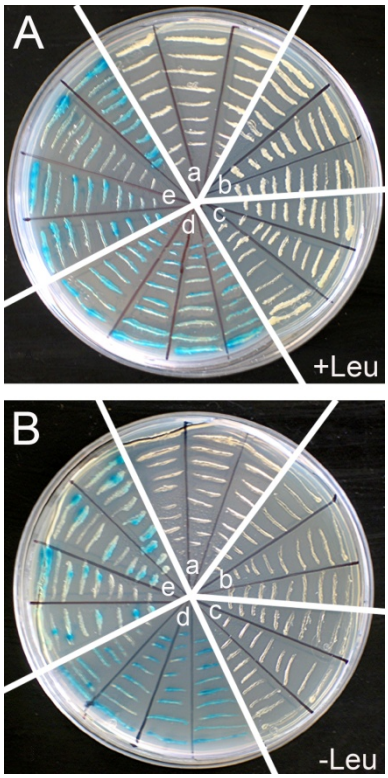
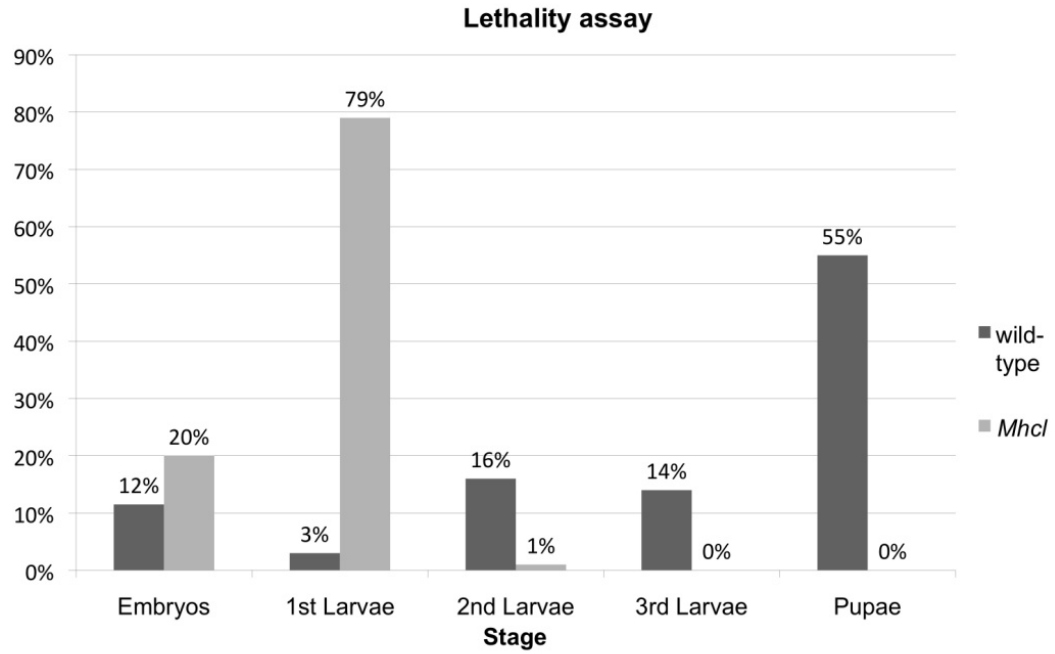


Fig. 6. Model of the function of Mhcl in widening of the fusion pore during myoblast fusion. Duf/Kirre is inserted into the plasma membrane of FCs/growing myotubes, where it interacts with the FCM-specific adhesion molecule Sns. Via its intracellular part, Duf/Kirre interacts with the TPR-E repeat of Rols7. The ankyrin repeats of Rols7 interact with the C-terminal part of Mhcl and recruit it to the site of fusion, where its motor domain moves along the actin sheet (only the actin sheet within the FuRMAS is shown), at the site of the growing myotube. This leads to widening of the fusion pore.

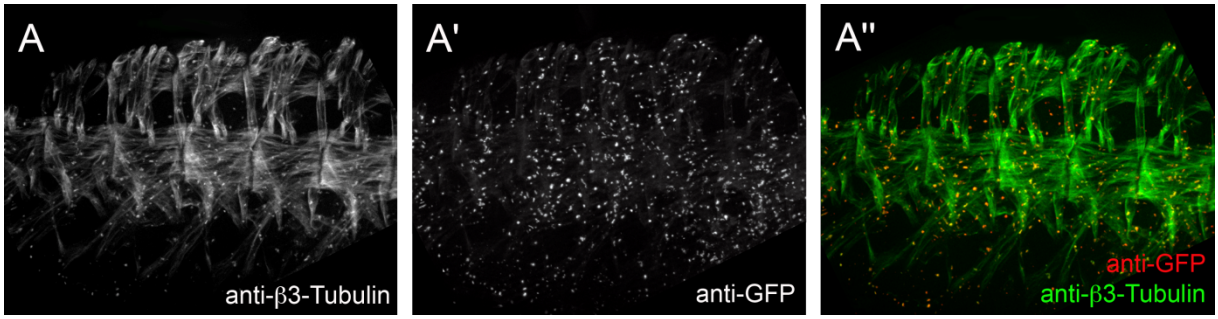
Supplementary material



Supplementary Fig. S1. Mhcl and Rols7 interact in yeast two-hybrid assays. To test for activation of the reporter genes *LEU2* and *β-galactosidase,* yeast co-transformants were plated on plates (A) containing leucine or (B) lacking leucine. (a) Negative controls: Yeast cells co-transformed with empty vectors pGilda and pB42AD (B) cannot produce Leu for growth and (A) do not transcribe *ß-galactosidase*. (b, c) Tests for auto-activation: (b) Mhcl-CC in pGilda and (c) Mhcl-CC in pB42AD co-transformed with the empty counterpart vector; (A) growth is only induced on plates with Leu, but colonies stay white. (d) The coiled-coil region of Mhcl (Mhcl-CC) mediates homodimerization of the protein, as yeast co-transformants with this fragment in either vector pGilda or vector pB42AD are able to activate the transcription of both reporter genes *ß-galactosidase* and *LEU2.* (e) Co-transformation with constructs encoding the Rols-interacting region of Mhcl and RolsANK induce transcription of both reporter genes *ß-galactosidase* and *LEU2.*



Supplementary Fig. S2. *Mhcl*-deficient larvae die at the 1st or 2nd instar larval stage. Wild-type and Δ*Mhcl* mutant embryos were collected and allowed to develop to larval stages. After 6 days, the number of dead embryos, dead 1st, 2nd and 3rd instar larvae, and living pupae were counted.



Supplementary Fig. S3. **Δ*Mhcl* mutant embryos expressing a GFP-tagged DN-Zipper develop a normal musculature.** (A, A', A'') Homozygous Δ*Mhcl* embryos in which *UAS-GFP-DN-*Zip is expressed by *DMef2-Gal4*, stained with anti-β3-Tubulin and anti-GFP. Note that ectopic GFP-DN-Zip accumulates strongly in the musculature (A', A'').

Supplemental Table S1 – Proteins found to interact with RI, RII, and RIII fragments of Rols7 in a yeast two-hybrid screen

Fragment	Interacting protein	Expression pattern	Protein function and involved processes	Analyzed alleles
RI	CG7109 (Mts)*	Maternal, CNS (Mayer-Jaekel et al., 1992), visceral mesoderm (this study)	Stabilization of microtubules during cell division and at the neuromuscular synapse (Snaith et al., 1996; Viquez et al., 2006)	*P(lacW)mts^{s5286}; P(PZ)mts^{02469}; mts$^{XE-2258}$; mts$^{XE-2258}$/Df(2L)ED12527*
	CG11949 (Cora)*	Foregut, hindgut, epidermis, chordotonal organs, salivary glands, trachea (Fehon et al., 1994)	Adult somatic muscle development (Schnorrer et al., 2010); assembly and component of septate junctions in epithelial cells (Fehon et al., 1994; Laprise et al., 2009)	*P(LacW)cora^{k08713};Df(2R)Exel6069*
	CG4532 (Pod1)*	CNS, PNS, epidermis (Rothenberg et al., 2003)	Crosslinking of actin and microtubules(Rothenberg et al., 2003); cellularization, axon guidance	n.d.
	CG8110 (Syd)*	CNS, weak mesodermal	Kinesin-dependent axonal transport (Bowman et al., 2000)	*P(EPgy2)syd^{EY02303}; P(EPgy2)syd^{EY02303}/Df(3L)Exel6279*
	CG11901 (Ef1y)		Component of translationelongation factor complex (Lasko, 2000)	n.d.
	CG12788		Predicted chromatin associated (McQuilton et al., 2012)	n.d.
RII	CG2677 (EIF2b)		Initiation of translation (Lasko, 2000)	n.d.
	CG12079		Predicted component of the respiratory chain in mitochondria (McQuilton et al., 2012)	n.d.
	CG5468 (TweedleM)		Cuticula protein (Guan et al., 2006)	n.d.
RIII	CG6506		Bromodomain transcription factor, component of SAGA acetyltransferase complex (Weake et al., 2009)	n.d.
	CG5407 (Sur-8)*	CNS, hindgut, midgut (this study)	Unknown; predicted RasGTPase binding (McQuilton et al., 2012)	*PBac(PB)Sur-8^{e02803}; PBac(PB)Sur-8^{e02803}/Df(3R)BSC564*
	CG2150		Egg shell protein (McQuilton et al., 2012)	n.d.

Using the yeast two hybrid system, we screened an embryonic library with fragments of Rols7 containing no specific protein–protein interaction domains (fragments R1, R2, and R3). From these screens, we identified several other potential interaction partners. Plausible candidates are marked with *; their expression pattern and the muscle phenotype of embryos carrying the indicated alleles were analyzed further. n.d.: not determined.

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7 Formation of *Drosophila* longitudinal visceral muscles requires Rolling pebbles 7, Blown fuse and Kette

Formation of *Drosophila* longitudinal visceral muscles requires Rolling pebbles 7, Blown fuse and Kette

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Abstract

The visceral musculature of *Drosophila* larvae is composed of circular muscles tightly interwoven with longitudinal muscles. While the circular muscles arise by fusion of a circular founder cell (FC) to a visceral fusion competent myoblast (FCM) from the trunk visceral mesoderm, the FCs of the longitudinal muscles become specified in a different primordium, the caudal visceral mesoderm, and migrate anteriorly during embryogenesis. Here we show that the longitudinal FCs do not fuse to the same type of FCMs to which the circular FCs fuse, but most probably to other cells expressing the FCM specific adhesion protein Sns. We further show that the adaptor protein Rolling pebbles 7 (Rols7) is expressed in both FC types of the visceral mesoderm. The longitudinal FCs of *rols7* mutants form syncytia at later stages of gut development than in the wild-type, and mononucleated longitudinal gut muscles are detectable at the end of embryogenesis, at least in the anterior part of the gut. We thus suggest that Rols7 is responsible for fusion efficiency of the longitudinal FCs. Moreover, we show here that most longitudinal FCs stay mononucleated in mutants of the fusion gene *blow*, while multinucleated longitudinal gut muscles are present in *kette* mutants, although the latter exhibit longitudinal FC migration defects at earlier stages. This study thus provides evidence that fusion relevant proteins known from the somatic myoblast fusion process are also needed in the formation of syncytial longitudinal gut muscles in *Drosophila*.

Keywords: Drosophila myogenesis, myoblast fusion, longitudinal gut muscles, Rols7, Blow, Kette, Sns, Duf, HLH54F, Trol

Introduction

The body wall musculature of *Drosophila melanogaster* arises during embryogenesis and metamorphosis by fusion of two cell types, the founder cells (FCs) and the fusion competent myoblasts (FCMs), giving rise to syncytial myotubes which allow the movement of larvae and adults (reviewed in Abmayr and Pavlath, 2012; Maqbool and Jagla, 2007). It has long been noticed that also the visceral muscles surrounding the mid- and hindgut are syncytial, enabling the contraction of the gut and therefore movement of the food through the digestion tract. The visceral musculature forms a web-shaped syncytium around the gut, consisting of binucleated circular muscle fibers, which are interwoven with multinucleated longitudinal muscles. Both muscle types persist during metamorphosis (Goldstein and Burdette, 1971; Klapper, 2000; Klapper et al., 2001; San Martin et al., 2001; Sandborn et al., 1967; Schröter et al., 2006).

At least the circular visceral muscle type in *Drosophila* was also shown to arise by fusion of a FC with a FCM. While a proposed common pool of visceral FCMs exists, the circular and longitudinal FCs are distinct and originate from different mesodermal primordia. The circular FCs as well as the visceral FCMs become differentiated in the trunk visceral mesoderm (TVM), which is characterized by expression of the homeodomain transcription factor Bagpipe (Bap) and the FoxF gene Biniou (Azpiazu and Frasch, 1993; Tremml and Bienz, 1989; Zaffran et al., 2001). The circular FC fate is determined, on the one hand, by Delta/Notch signaling, and on the other hand, by Ras/MAPK signaling via the receptor tyrosine kinase Alk and its ligand Jelly Belly (Englund et al., 2003; Lee et al., 2003; Lorén et al., 2003; Stute et al., 2004). These FCs fuse one-to-one with a visceral FCM. After that, the binucleate cells stretch until they enclose the whole gut, still connected by cytoplasmatic bridges (Klapper et al., 2002; San Martin et al., 2001; Schröter et al., 2006). The correct establishment of the circular muscles is a prerequisite for subsequent gut development, as they constrict the gut into four chambers at the end of embryogenesis (Campos-Ortega and Hartenstein, 1985; Kusch and Reuter, 1999).

Just as in the somatic mesoderm, fusion of visceral myoblasts to form the binucleate circular muscles is dependent on the presence of the Immunoglobulin super family receptors Dumbfounded/Kin of Irre (Duf/Kirre) and/or Roughest/Irregular chiasm C (Rst/IrreC) in the circular FCs and Sticks and Stones (Sns) in the visceral FCMs. In *duf,rst* double mutants as well as in *sns* mutants, the FCMs fail to adhere to the circular FCs and fusion is completely blocked. Therefore, the adhesion molecules appear to mediate the heterotypic adhesion between these two cell types. Furthermore, mutants of the GEF encoding gene *myoblast city* (*mbc*) display aggregated, but mononuclear visceral myoblasts and lacking gut constrictions; thus, Mbc appears to be also involved in myoblast fusion in the visceral mesoderm (Erickson et al., 1997; Klapper et al., 2002; San Martin et al., 2001).

Other studies could show that some molecules with an essential function during the somatic fusion process, although expressed in the visceral mesoderm, play a different role in morphogenesis of the gut muscles. Mutants of the actin regulators Wiskott-Aldrich-syndrome protein (WASp) and D-WIP/Verprolin1/Solitary, which bind and activate the Arp2/3 complex and enable actin polymerization during somatic myoblast fusion (Berger et al., 2008; Gildor et al., 2009; Kim et al., 2007; Schäfer et al., 2007; Sens et al., 2010), exhibit no obvious defects in gut muscle development (Eriksson et al., 2010). In *blown fuse* (*blow*) and *kette* mutants, stretching and outgrowth of the circular visceral muscles is disturbed, while fusion itself is not affected (Schröter et al., 2006). Blow overlaps with the actin plugs on the side of the FCM in the somatic mesoderm and was recently found to stabilize the WASp/D-WIP complex there (Jin et al., 2011; Kesper et al., 2007). The actin regulating factor Kette is needed during the second fusion phase as part of the Kette/SCAR complex, which regulates actin foci formation on the side of the FCM as well as formation of the thinner actin sheet on the side of the FC, also by activating the Arp2/3 complex, which is essential for somatic myoblast fusion in both cells (Gildor et al., 2009; Richardson et al., 2007; Schröter et al., 2004). Because mutants of these two actin polymerization promoting factors, as well as mutants for the Arp2/3 complex itself, can form binucleate circular muscles, it has been supposed that Arp2/3 mediated actin polymerization is not essential in the process of visceral myoblast fusion (Eriksson et al., 2010).

Fusion of circular FCs with the visceral FCMs has been shown to be incomplete and lack the ultrastructural features observed in the somatic myoblast fusion process, namely electron-dense vesicles and electron-dense plaques (Schröter et al., 2006). Less is known about myoblast fusion leading to the formation of the visceral longitudinal muscles, running perpendicularly to the circular muscles. They contain up to six nuclei and are therefore supposed to develop through several fusion events. The longitudinal FCs originate from the caudal visceral mesoderm (CVM) at the posterior tip of the embryo. They are determined by the basic Helix-Loop-Helix transcription factor HLH54F and migrate under control of FGFR signaling along the TVM in anterior direction (Georgias et al., 1997; Ismat et al., 2010; Kadam et al., 2012; Kusch and Reuter, 1999; Reim et al., 2012). After one mitotic division, the cells are arranged along the band of circular FCs and visceral FCMs (Bate, 1993). It has long been supposed that these longitudinal FCs, once having reached the TVM, fuse with the remaining visceral FCMs after circular fusion is finished.

It is still not known which proteins are involved and whether known fusion genes are essential for the fusion events after the longitudinal FCs have arrived at the TVM. First analyses of the syncytial visceral muscles demonstrated that mutants of *mbc* display not only a block of fusion of circular FCs with visceral FCMs, but also mononucleated longitudinal muscles (San Martin et al., 2001). Here we present evidence that the correct development of multinucleated longitudinal myotubes depends on the adaptor protein Rolling pebbles 7 (Rols7). Rols7 is

expressed and binds to Duf at the site of FCs in the somatic mesoderm as part of the Fusion-restricted Myogenic-Adhesive Structure (FuRMAS), which were postulated as an essential signaling center at sites of cell contacts. Rols7 is required for completion of the second fusion phase as an adaptor protein linking cell recognition and adhesion via Duf supposedly to vesicle transport, actin remodeling or widening of the adhesion ring within the FuRMAS (Chen and Olson, 2001; Kesper et al., 2007; Menon and Chia, 2001; Rau et al., 2001). We further show that Blow is necessary for the formation of syncytial longitudinal muscles, additionally to its role in somatic and circular muscle development. In contrast, Kette appears to be not absolutely required for fusion, but for early cell migration of the longitudinal FCs. Finally, this study presents evidence that the longitudinal muscles arise most probably by fusion of the longitudinal FCs with Sns positive FCMs of a different primordium than the FCMs which fuse with the circular FCs, sheding light on a separated pool of visceral FCMs for the two different multinucleated gut muscle types in *Drosophila*.

Material and methods

Drosophila stocks

The following fly stocks were used in this study: *Df(3L)BK9/TM3,Sb,DfdLacZ* (fly stock deficient for *rols7,* Bl. 2991, Bloomington *Drosophila* Stock Center, Indiana), *rols^{XX117}/TM3,Sb,DfdLacZ* (Rau et al., 2001), *blow^{2}/CyO* (Doberstein et al., 1997), *kette$^{J4\text{-}48}$/TM6,Tb* (Hummel et al., 2000; Bl. 8753), *rp298-LacZ* (Nose et al., 1998), *sns-mCherry-NLS* (Haralalka et al., 2011; provided by S. Abmayr, Stowers Institue for Medical Research, Kansas City), *bagpipe(bap)-LacZ* (Azpiazu and Frasch, 1993), *HLH54F-LacZ* (Ismat et al., 2010), *bap-Gal4* (Zaffran et al., 2001; this and the former two stocks were kindly provided by M. Frasch, University of Erlangen), *P{UASp-GFP.Act57B}$^{10\text{-}2}$* (Bl. 9255), and the protein trap lines *sallimus(sls)::GFP* (Morin et al., 2001; ZCL2144, obtained from Flytrap) and *terribly reduced optic lobes(trol)::GFP* (Susic-Jung et al., 2012; provided by C. Klämbt, University of Münster). In order to distinguish homozygous mutants from heterozygous ones, balancer chromosomes carrying *LacZ* or *GFP* insertion markers (Bl. 6662 and Bl. 6663) were used. For wild-type reference, we used *w^{1118}* or balanced sibling embryos.

Immunohistochemistry on Drosophila embryos

Fixation of embryos and immunohistochemistry were performed as described before (Kesper et al., 2007). Embryonic and larval guts were prepared after fixation with 4% paraformaldehyde and stained the same way as embryos, skipping the methanol step. Antibodies were used at the following dilution: anti-Rols300 (rabbit, directed against the first

300 amino acids of Rols7) 1:500, anti-Blow (Schröter et al., 2006) 1:200, anti-Fasciclin3 (Patel et al., 1987, obtained from the Developmental Studies Hybridoma Bank, Iowa) 1:50, anti-Tropomyosin (abcam plc, Cambridge) 1:1000, anti-β3-Tubulin (Leiss et al., 1988) 1:3000, anti-DMef2 (provided by H. Nguyen, University of Erlangen) 1:500, anti-GFP (abcam plc) 1:1000, anti-RFP (provided by S. Abmayr) 1:1000, and anti-β-galactosidase (Biotrend, Cologne) 1:5000. Primary antibodies were detected either using biotinylated secondary antibodies in a dilution of 1:250 and Vectastain ABC Elite Kit (Vector Laboratories, Burlingame) or using fluorescent-labeled antibodies in a dilution of 1:200 (Dianova, Hamburg). Hoechst reagent (Sigma-Aldrich, Steinheim) was used at 50 μg ml^{-1} and TRIC-coupled Phalloidin (Sigma-Aldrich) was used at 10 μg ml^{-1}. Anti-Rols7 and anti-Blow stainings were performed on heat-fixed embryos and the reaction was enhanced using an Individual Indirect Tyramide Reagent Pack (Perkin-Elmer, Waltham). Specimens were embedded in Epon or Fluoromount-G™ (Southern Biotech, Birmingham) and analyzed using a Zeiss Axiophot or a Zeiss AxioObserver Z.1 inverse microscope (Zeiss, Stuttgart).

Fluorescent in situ hybridization (FISH)

A DIG-labeled *rols7* probe was synthesized by *in vitro* transcription on *rols7* cDNA LD1 (Rau et al., 2001) according to DIG-RNA labeling Kit (Roche Diagnostics, Mannheim). Fluorescent *in situ* hybridization on paraformaldehyde fixed embryos was carried out essentially as described in Lécuyer et al. (2008). The biotinylated anti-DIG antibody (Roche Diagnostics) was used in a dilution of 1:2000 and was detected using the TSA™ Fluorescein System (Perkin-Elmer).

Generation of transgenic flies carrying promoter constructs

The sequence of the first intron of the *rols* gene was cloned into pChabHsp43-LacZ vector (Thummel et al., 1988). The construct was injected into w^{1118} flies according to standard procedures.

Results

Longitudinal visceral muscles are interwoven with the circular muscles and its Trol/Perlecan based ECM

The gut musculature of *Drosophila* larvae is multinuclear and striated (Klapper, 2000; Klapper et al., 2001; San Martin et al., 2001), consisting of a dense network of circular and longitudinal muscles (Schröter et al., 2006). One subunit of a striated muscle fiber is defined as the sarcomere, mainly consisting of contractile actin and myosin filaments, flanked by the Z-discs. At least in sarcomeres of the flight muscles, the connecting filament protein Kettin, encoded by the *sls* gene, links α-Aktinin to the actin filaments (Kulke et al., 2001; Lakey et al., 1993; van Straaten et al., 1999). We used flies carrying the protein trap allele *sls::GFP* (Morin et al., 2001), which encodes a fusion protein localized to the Z-discs in the muscle sheaths surrounding the *Drosophila* male reproductive system (Susic-Jung et al., 2012). Also in sarcomeres of the larval gut musculature, the Z-discs were characterized by Sls-GFP localization, flanking the sarcomeric actin filaments (Fig. 1A). In these experiments, we observed that the sarcomeres of the visceral muscles were of a large size: While the body wall muscles were shown to contain sarcomeres of 1-2 μm length (Dettman et al., 1996), we measured the sarcomeres of the circular muscles to be approximately 10 μm (Fig. 1A). This is even longer as sarcomeres within the skeletal muscles of vertebrates, which are about 2 μm long (reviewed in (Burkholder and Lieber, 2001) and demonstrates the enormous length invertebrate sarcomeres can reach (reviewed in Paniagua et al., 1996).

An intact extracellular matrix (ECM) of the endodermal layer has been shown to be required for the outgrowth and the formation of the network of circular and longitudinal muscles (Wolfstetter and Holz, 2011). Furthermore, the ECM of the trunk mesoderm is required for the migration of the longitudinal FCs along the TVM (Urbano et al., 2011). We analyzed embryos carrying the protein trap allele *trol::GFP*, which encodes for the extracellular matrix protein Trol, or Perlecan, tagged internally with GFP (Friedrich et al., 2000; Susic-Jung et al., 2012) and could observe that the network of gut muscles was embedded into a thick layer of ECM when development is completed (Fig. 1B). The circular muscles are binuclear and have been shown to arise by one-to-one fusion of a circular FC from the TVM with a visceral FCM (Klapper et al., 2002; San Martin et al., 2001). In embryos carrying *rp298-LacZ,* which is a reporter construct in the locus of the FC specific gene *duf/kirre*, the nuclei of somatic and visceral FCs are characterized by β-gal expression (Nose et al., 1998). In *rp298-LacZ* 1st instar larvae, the FC-derived nucleus in the small syncytia stayed β-gal positive, while the nucleus derived from the visceral FCM was β-gal negative (Fig. 1C), as known from embryos at stage 12

(Klapper et al., 2002). This is in contrast to the somatic mesoderm, as in mature body wall myotubes, all nuclei of the FCMs become β-gal positive after fusion (Nose et al., 1998).

The longitudinal visceral FCs migrate and form multinucleated syncytia during embryogenesis

As far less is known about the development of the syncytial longitudinal muscles, which are interwoven with the circular muscles, we concentrated on that issue and made use of flies carrying the reporter construct *HLH54F-LacZ,* in which longitudinal FCs are marked by β-gal expression (Ismat et al., 2010). These cells migrate from the CVM anteriorly along the TVM (Georgias et al., 1997; Ismat et al., 2010). We observed that they were mononucleated when they arrived at the TVM in early stage 12 of development. They formed protrusion at that time, and in some cases, we observed adhering cells (Fig. 2A). The longitudinal FCs were arranged dorsally and ventrally of the TVM when they migrated (Fig. 2B). At late stage 12, we detected bi- and trinucleated nascent myotubes, as well as cells connected by thin cytoplasmatic bridges (Fig. 2C). At a time when the circular muscles had stretched dorsally, the longitudinal FCs were arranged perpendicularly to the circular muscles, and contained already three or four nuclei (Fig. 2D, E). At the end of embryogenesis, when the gut was already constricted, the longitudinal gut muscles covered the whole gut evenly. The cells themselves appeared very thin, not much wider than the nuclei, with even thinner protrusions between them (Fig. 2F).

Longitudinal FCs do not fuse with Bap expressing visceral FCMs

It has long been suggested that the longitudinal FCs fuse with the remaining visceral FCMs of the TVM, which express the transcription factor Bagpipe (Bap) and which lie in close proximity to the longitudinal FCs after they have migrated. The longitudinal FCs themselves do not express Bap (Zaffran et al., 2001). Therefore, we used the driver line *bap-Gal4* to express actin-GFP only in the visceral FCMs. At the end of embryogenesis, the longitudinal muscles, but not the circular muscles, were characterized by FasIII expression, and the characteristic cytoplasmatic projections of the former (Klapper, 2000) were clearly visible (Fig. 2G'). We found that the actin-GFP positive circular muscles, which had developed by fusion of one circular FC with a *bap* expressing FCM, were interwoven with the FasIII positive, but actin-GFP negative longitudinal muscles (Fig. 2G-G'').

This finding, that the longitudinal FCs appeared not to fuse with Bap expressing FCMs out of the TVM, was very surprising, and we analyzed this further by using reporter constructs for Duf and Sns expression in the visceral FCs and FCMs. The adhesion molecule Duf is expressed in the circular FCs, while Sns is expressed in all somatic and visceral FCMs. It has already been shown that Sns expression decreases at a time when the circular muscles have formed and the longitudinal FCs have arrived at the TVM (Klapper et al., 2002). We observed that in embryos

carrying *rp298-LacZ,* the nuclei of the longitudinal FCs were β-gal positive in stages in which fusion presumably occurred (Fig. 3B, B'), concordant with earlier data which showed *rp298-LacZ* positive nuclei in migrating longitudinal FCs as well as in the mature longitudinal visceral muscles (Klapper et al., 2002; San Martin et al., 2001). Therefore, the adhesion molecules Duf and/or Rst appear to be expressed. Interestingly, we observed that the cells were also positive for expression of mCherry under control of the *sns* promoter in these stages (Fig. 3B''), when we analyzed embryos simultaneously expressing *rp298-LacZ* and *sns-mCherry* (Haralalka et al., 2011). In order to analyze the *sns* transcription in the longitudinal FCs further, we established flies carrying *sns-mCherry* and *HLH54F-LacZ*. In stages in which the longitudinal FCs migrated and were still mononucleated, *sns-mCherry* positive FCMs from the somatic mesoderm and the TVM laid in close proximity to the *HLH54F-LacZ* positive FCs. We observed that the FCs were negative for *sns* transcription in these stages (Fig. 3C). However, when we looked at bi- and tri-nucleated longitudinal FCs in later stages, we observed a weak mCherry signal in these cells (Fig. 3D). At the end of embryogenesis, we also detected a signal in some nuclei of the multinucleated syncytia (not shown), as the mCherry protein under control of the *sns* promoter additionally contained a nuclear localization signal (NLS).

We could show here that the longitudinal FCs do not fuse with Bap positive visceral FCMs. Furthermore, the mCherry protein is present in longitudinal FCs of *sns-mCherry* embryos. These data allow two different conclusions: At first, it may be that the longitudinal FCs themselves expressed mCherry under control of the *sns* promoter, simultaneously to expression of *rp298-LacZ*, which would indicate that Sns as well as Duf and/or Rst are present in this cell type. In this case, Sns expression appears to start when the longitudinal FCs are already syncytial. Second, it may be that the longitudinal FCs fuse with *sns-mCherry* positive cells, and that the mCherry protein is contributed to the longitudinal FCs by Sns expressing FCMs. These fusing FCMs would then originate from a different source than from the visceral FCMs which express Bap.

Rols7 is expressed in FCs of the visceral mesoderm, and rols7 *transcription is guided by enhancer elements in the first intron of the rols gene*

Several genes relevant for the somatic fusion process have been shown to be also transcribed in the visceral mesoderm (Eriksson et al., 2010; Klapper et al., 2002; Schröter et al., 2006). The adaptor protein Rols7 is specifically expressed in FCs of the somatic mesoderm, where it links Duf-dependent cell adhesion to downstream events (Chen and Olson, 2001; Menon and Chia, 2001; Menon et al., 2005; Rau et al., 2001). We analyzed whether Rols7 is also present in the visceral FCs. At first, we performed fluorescent *in situ* hybridisation on embryos carrying *rp298-LacZ*, and observed that indeed, *rols7* was transcribed in the circular FCs (Fig. 4A-A''). Next we used an antibody directed against the first 300 amino acids of Rols7 and

detected the protein in the visceral mesoderm in the extended germ band stage, prior to visceral myoblast fusion (Fig. 4B). Due to their position, we identified them to be circular FCs. In the FCMs lying above, we did not detect Rols7 (Fig. 4B). Both *rols7* mRNA and Rols7 protein were distributed in a punctated pattern in these cells (compare Fig. 4A' and Fig. 4B). After the binucleated circular muscles had formed and started to stretch along the endoderm, Rols7 expression declined and was only weakly detectable at or above the TVM (Fig. 4B'). Finally, we also detected the protein in the CVM, giving rise to the longitudinal FCs, in stages prior to cell migration (Fig. 4B'').

The *rols* gene is regulated by two promoters leading to *rols7* and *rols6* transcripts with selective 5´ exons (Rau et al., 2001). We analyzed the promoter regions responsible for transcription of *rols7* and *rols6* in the different tissues by establishing and analyzing LacZ reporter gene strains (not shown). In the case of *rols7*, we observed that the region 2 kb upstream of the transcription start site was required for the strong expression in the somatic mesoderm. A further regulatory element for *rols7* transcription in these cells laid in the intron between exons 1 and 2, which was mainly required for transcription in the visceral mesoderm. Rols6 is expressed in the endoderm and the Malpighian tubules (Pütz et al., 2005). The transcription of *rols6* in both tissues was dependent on 1,2 kb upstream of the transcription start site. A schematic drawing of the different promoter regions is given (Fig. 4C). For all regulatory modules, no already known transcriptional regulators could be identified. Moreover, a DMef2-binding site in the *rols7* promoter region was dispensable for *rols7* transcription (not shown), in agreement with genome wide analysis of DMef2 target genes (Sandmann et al., 2006).

In later stages of development, the Rols7 antibody gave a very weak signal in the developing gut muscles, making it impossible to address whether Rols7 expression persists in the longitudinal FCs after they have migrated from the CVM (not shown). Therefore, we made use of the *LacZ* reporter construct containing the intron between exons 1 and 2 (*roIn1-LacZ*), in which we identified the regulatory element of *rols7* transcription in the visceral mesoderm (Fig. 4C). We could observe that in the embryos transcribing *roIn1-LacZ*, the longitudinal FCs were marked by β-gal staining when they migrated along the TVM in mid-embryogenesis (Fig. 4D). The signal was detectable from stage 10 onwards. Also later, when the circular muscles stretched dorsally, we detected β-gal in the longitudinal FCs which were already binucleated (Fig. 4D'). We obtained a weaker signal in the circular muscles behind, which again showed that *rols7* is transcribed in the circular FCs as well.

In conclusion, we could show that Rols7 is expressed in the CVM and FC specifically in the TVM, and that visceral FCMs do most probably not express Rols7. The observed expression pattern in the circular FCs, using the anti-Rols7 antibody, was very transient, which is concordant with earlier data (Rau et al., 2001). The reporter construct experiments revealed that *rols7* is additionally transcribed in the longitudinal FCs, although the long persistence of the

β-gal protein did not allow us to determine whether Rols7 is present until the fusion of these cells. However, given the fact that the transcription of *rols7* in the somatic and visceral mesoderm is tightly regulated by different promoter regions, an additional function of Rols7 during the development of the visceral muscles is plausible.

Rols7 is required for fusion of longitudinal FCs at the right time frame

Next, we analyzed *rols7* deficient embryos, which express different reporter constructs that mark visceral FCs or FCMs. The transcription factor Bagpipe (Bap) is expressed in all visceral FCMs. After these cells have fused one-to-one with the circular FCs, the remaining FCMs were suggested to fuse with longitudinal FCs, but about 40% of them migrate into the somatic mesoderm where they can fuse with somatic precursor cells (Azpiazu and Frasch, 1993). Shortly before the gut became constricted, the interstitium between the gut and the somatic muscles was free of Bap expressing cells in the wild-type (Fig. 5A), and some nuclei of somatic myotubes were β-gal positive in *bap-LacZ* embryos (Fig. 5A'). The visceral FCMs apparently cannot fuse with somatic precursor cells in *rols7* mutant embryos; instead, we found that the cells were located in the interstitium between somatic and visceral mesoderm more closely towards the gut muscles than towards the somatic mesoderm (Fig. 5B-B'). This enlarged number of unfused visceral FMCs in *rols7* mutants might be due to the myoblast fusion defect in the somatic mesoderm of these embryos. However, we wanted to know whether this could also be caused by fusion defects in the visceral mesoderm, because an enlarged number of Bap expressing cells has also been observed for *mbc* mutants, which are known to exhibit a complete block of fusion not only in the somatic, but also in the visceral mesoderm (Klapper et al., 2002; Wolfstetter et al., 2009).

At first, we analyzed whether the gut is constricted correctly in *rols7* mutants. The gut constrictions develop by local contractions of the circular muscles and their presence is hence an indication of normal visceral muscle development (Kusch and Reuter, 1999). Using an anti-FasIII antibody, we could show that the gut became constricted rather normally in *rols7* mutants in late embryogenesis (Fig. 5C). Earlier in development, we found that after the circular FCs had fused with the visceral FCMs, the circular muscles stretched normally in dorsal direction (Fig. 5D). We thus conclude that the fusion of circular FCs with the visceral FCMs is unaffected in *rols7* mutants. We further suppose that the enhanced number of unfused Bap positive visceral FCMs is due to the strong somatic phenotype of *rols7* mutants, as we have shown that the longitudinal FCs do most probably not fuse with the this FCM type.

Next we concentrated our analysis on the development of the longitudinal gut muscles in *rols7* LOF mutants. To this aim, we crossed flies deficient for *rols7* to those carrying the reporter construct *bHLH45F-LacZ* and analyzed the arrangement of longitudinal FCs in these embryos. We found that the longitudinal FCs migrated correctly along the circular muscles during mid-

embryogenesis and were arranged dorsally and ventrally of them (compare Fig. 5D with the wild-type situation in Fig. 2B). Nevertheless, shortly before the formation of constrictions, the cells did not run entirely perpendicular to the circular muscles; instead, they formed protrusions in other directions and were mainly mononucleated (Fig. 5E), at a stage when bi- and trinucleated syncytia were detectable in the wild-type (Fig. 2C, D). In later stages, above the completed constricted gut, we observed gaps in the normally evenly distributed cells (compare Fig. 5F with the wild-type in Fig. 2F). We sometimes detected mononucleated cells which stretched out extensions in every direction, and we rarely detected stretched, multinuclear cells in the anterior part of the gut. Interestingly, the posterior part of the gut was still surrounded by the dense stripes of longitudinal muscles in late embryogenesis (Fig. 5F). When we looked at the cells in a higher magnification, we saw that these cells were mainly binucleated, stretching protrusions in the correct orientation (Fig. 5G). In summary, we could show that although the longitudinal FCs migrate correctly at the first place, fusion of these cells was disturbed in *rols7* mutant embryos, with the consequence that only binucleated syncytia were detectable at the end of embryogenesis. In the anterior part of the gut, the phenotype was even more severe. Furthermore, we could show that fusion appeared to be time-delayed, as mononucleated cells were present at stages when gut constrictions already formed.

For most of our analyses, we used the *rols* deficiency allele *Df(3L)BK9*, which means that also the *rols6* variant is not expressed in the deficient embryos. Therefore, Rols6 could also be responsible for the mutant phenotype we observe. However, analysis of the *rols6-LacZ* reporter constructs did not reveal a *rols6* transcription in the somatic or visceral mesoderm, but only in the endoderm and in the cells of the malpighian tubules (not shown). Furthermore, *rols6* mRNA was not found in previous studies to be present in the visceral mesoderm (Pütz et al., 2005; Rau et al., 2001). Therefore we conclude that Rols7 is the variant involved in visceral muscle morphogenesis, and not Rols6.

Longitudinal muscle development requires Blow and Kette

Some proteins relevant for actin polymerization during somatic myoblast fusion are dispensable for fusion of visceral myoblasts giving rise to the circular gut muscles, although they are also present in the visceral mesoderm (Eriksson et al., 2010; Schröter et al., 2006). For instance, Blow has been shown to be expressed in both the circular FCs and visceral FCMs, although Blow as well as the actin regulator Kette are not needed for the formation of these binucleated syncytia (Schröter et al., 2006). We wanted to know whether both fusion relevant genes are also dispensable for formation of the longitudinal muscles. Using an anti-Blow antibody, we detected Blow also in the CVM, i.e. in the longitudinal FCs prior to migration (Fig. 6A). Kette is broadly transcribed in the embryo until stage 14 and additionally maternally contributed (Hummel et al., 2000). When we analyzed whether the gut constrictions were

correctly formed in *kette* and *blow* mutants, we could observe that although constricted, the gut was often smaller and abnormally shaped in very late stages of embryogenesis (not shown). We suppose that this is due to the former observed fact that the circular muscles do not stretch properly around the gut (Schröter et al., 2006), although we cannot exclude the possibility that this might be due to more general defects of the whole embryo, as many organs appeared to be disturbed because of the absent body wall musculature in these mutants (not shown).

We now wanted to know whether the longitudinal muscles develop correctly in *blow* and *kette* mutants, which display even stronger fusion defects in the somatic mesoderm than *rols7* mutants do (Beckett and Baylies, 2007; Doberstein et al., 1997; Hummel et al., 2000; Schröter et al., 2004). In *blow* mutants, the longitudinal FCs migrated correctly along the TVM; however, we observed that the cells were slightly more rounded up than in the wild-type situation, and that they did not entirely run dorsally and ventrally of the stretching circular muscles (Fig. 6B). In later stages, the longitudinal FCs stretched protrusions not only in anterior-posterior direction, but also in dorsal-ventral direction (Fig. 6C). When we additionally stained embryos with anti-DMef2 to visualize the nuclei of these cells, we observed that they were mononucleated before the gut became constricted (Fig. 6D, D'). In stages after constriction formation, we saw that the cell protrusions were much thinner than in the wild-type, often without reaching the neighbor cell, and that the cells were mostly still mononucleated at the end of gut development (Fig. 6E).

In *kette* mutants, the longitudinal FCs also migrated along the TVM, but we detected some cells outside, migrating away or never having reached the dorsal/ventral side of the stretching circular muscles (Fig. 6F). In later stages, the cells mainly formed protrusions in the correct direction (not shown). However, at late embryogenesis, we observed that although some longitudinal FCs had formed thin cell protrusions and had connected to each other, the overall number of longitudinal muscles present appeared to be reduced, and some areas along the circular muscles were not covered with longitudinal muscles (Fig. 6G). In double staining with anti-DMef2, we detected bi- and multinucleated syncytia at that time (Fig. 6H).

In conclusion, we could show here that fusion of longitudinal FCs was severely disturbed in *blow* mutant embryos. Here, the increase in cell protrusions formation without forming syncytia was most prominent. In contrast, the phenotype of *kette* mutants was less severe, as binucleated cells were detectable, although the muscle number was reduced, which might be due to the observed cell migration defect in early stages.

Discussion

In this study, we have analyzed the development of the syncytial longitudinal visceral muscles of *Drosophila*, which are interwoven with the circular muscles and are embedded into a thick ECM layer. Determined by the transcription factor HLH54F, the longitudinal FCs migrate from the CVM, under control of FGFR signaling, anteriorly along the trunk mesoderm (Georgias et al., 1997; Ismat et al., 2010; Kadam et al., 2012; Reim et al., 2012). Here, we provide evidence that the longitudinal FCs fuse with *sns* transcribing cells to multinucleated syncytia. Moreover, we showed that these cells most probably do not derive from the 11 mesodermal clusters of Bap expressing visceral FCMs, which fuse with the circular FCs (Azpiazu and Frasch, 1993; Klapper et al., 2002; San Martin et al., 2001). It has been suggested before that cells of the TVM exist which do not derive from the "Bap patches", instead lying between these patches and align to the continuous band of the TVM together with the visceral Bap positive FCMs as well as with the circular FCs (Azpiazu and Frasch, 1993). These cells could be the source of the FCM pool capable of fusing with the longitudinal FCs, supported by analyses of San Martin et al. (2001), who showed that in the absence of a TVM, the longitudinal FCs stay mononucleated. Another possibility is that these FCs fuse with the Sns positive, Bap negative somatic FCMs, which lie in close proximity. This would indicate that a mixed pool of somatic and visceral FCMs exists. The visceral FCMs are known to be capable of fusing with somatic FCs in mutants of visceral FC determination (Englund et al., 2003; Eriksson et al., 2010; Lee et al., 2003; Stute et al., 2004), and are here shown to be "left over" when somatic myoblast fusion is inhibited in *rols7* mutants. In contrast to that, visceral and somatic FCMs have been shown to be genetically distinct, as ectopic expression of the visceral specification factor Biniou in the somatic mesoderm results in myoblast fusion defects (Zaffran et al., 2001). Further analyses of the origin of the FCM type which fuses with the longitudinal FCs are needed to determine whether somatic FCMs can also contribute to a visceral muscle type. However, as the observed mCherry signal reflecting *sns* transcription was weak in the longitudinal FCs in comparison to the signal in the somatic myotubes, we suggest that the Sns positive FCMs, presumably from the somatic mesoderm, contribute only to a minor amount to the mass of the longitudinal muscles.

Another possibility is that the longitudinal FCs can fuse with themselves to give rise to the longitudinal muscles. Thus, a situation in which the FCs seed the information and the FCMs contribute to the mass of the muscle would only account for the body wall and the circular visceral muscles. It would be the first time that homotypic cell fusion is described in *Drosophila*, comparable to vertebrate myoblast fusion, occurring without different subsets of myoblasts, neither during embryogenesis nor during muscle regeneration (reviewed in Abmayr and Pavlath, 2012). In this case, the longitudinal FCs appear to express Sns as well as Duf and/or Rst at the same time. Cell culture studies showed that Sns can mediate the association of cells by

binding to Duf as well as by binding to Rst, but association between cells which only express Duf has also been observed (Dworak et al., 2001; Galletta et al., 2004). If homotypic cell fusion occurs between the longitudinal FCs, further analyses are needed to determine which molecules allow the homotypic cell adhesion at the surface of the FCs. Nevertheless, as we have shown that the mCherry signal in these cells of *sns-mCherry* embryos is detectable after syncytia have already formed, we support the view that the mCherry protein has been contributed by Sns expressing FCMs. This is in concordance with Mandal et al. (2004), who showed that in longitudinal syncytia, one nucleus is positive for the longitudinal FC specific transcription factor Couch potato (Cpo), while the others are Cpo negative and therefore have to derive from another cell type than from the longitudinal FCs.

We have further demonstrated that proteins, which are essential in the somatic myoblast fusion process, are also essential in longitudinal gut muscle development. The adaptor protein Rols7 is expressed in the longitudinal FCs, and might bind to Duf to allow fusion efficiency via a positive feedback loop, as it is the case in the somatic mesoderm (Menon et al., 2005). We showed that in mutants for *rols7*, the development of syncytial longitudinal muscles is disturbed, most prominent in the anterior part of the gut. Additionally, we showed that syncytia formation was strongly time-delayed. As the posterior part of the gut was still covered with multinucleated longitudinal muscles, we propose that only in areas where the cells lie in very close proximity, fusion of the longitudinal FCs might function without Rols7, analogous to the first phase of fusion in the somatic mesoderm, which is independently of Rols7 function (Chen and Olson, 2001; Menon and Chia, 2001; Rau et al., 2001).

We further showed that fusion of longitudinal FCs is not necessarily disturbed in *kette* mutants, but that these cells exhibit a migration defect prior to fusion, with the consequence that less longitudinal muscles are present at the end of embryogenesis. We thus suppose that Kette is thus rather required for the oriented migration of the longitudinal FCs along the stretching circular muscles. Strikingly, the *kette* mutant phenotype in the longitudinal musculature resembled the mutants for the fibroblast growth factors (FGF) encoding genes *pyramus* (*pyr*) and *thisbe* (*ths*). Here, the longitudinal FCs do not migrate properly anymore due to the failed signaling via the FGF-receptor Htl, with the consequence that less longitudinal muscles are present at the end of gut development (Reim et al., 2012). Kette might be required for the cytoskeletal organization in the longitudinal FCs, analogously to its role in migration of glial cells and axonal pathfinding (Hummel et al., 2000). However, we cannot exclude the possibility that the observed gaps in the longitudinal visceral musculature above the fully constricted gut is due to the previously observed stretching failure of the circular muscles (Schröter et al., 2006), as longitudinal FC migration depends on an intact TVM (Reim et al., 2012; Zaffran et al., 2001). However, we detected gut constrictions in both *kette* and *blow* mutants, indicating only subtle defects of the circular musculature. Overall, mutants for *kette* develop wild-typic gut

constrictions (Eriksson et al., 2010) and exhibit regular fusion of circular FCs with visceral FCMs (Schröter et al., 2006) as well as binucleated longitudinal muscles (this study). Thus, the overall visceral phenotype of *kette* is only subtle in comparison to the somatic muscle phenotype. The examination mutant embryos of SCAR and other actin nucleation promoting factors would shed more light on the requirement of the Arp2/3 mediated actin polymerization during fusion of longitudinal FCs.

In contrast to that, cell migration was unaffected in *blow* mutants, but we detected many mononucleated longitudinal FCs, stretching cell protrusions randomly before and very thin protrusions after gut constriction formation. We thus suppose a function for Blow in fusion of the longitudinal FCs. Interestingly, Blow is expressed in both FC types as well as in the FCMs of the visceral mesoderm (Schröter et al., 2006 and this study), so the place of Blow function in the visceral myoblast fusion process has to be addressed. It will be interesting for future studies to elucidate the role of WASp in longitudinal muscle development, as Blow is needed in stabilization of the WASp/WIP complex during somatic myoblast fusion (Jin et al., 2011). A first study concerning this question could show that mutants of *d-wip/vrp1/slt* display a normal longitudinal muscle morphology (Eriksson et al., 2010); therefore, it might be that Blow acts independently of the WASp/WIP complex in the formation of the longitudinal muscles. Finally, the findings that *blow*, but not *kette* and *d-wip/vrp1/slt* mutants, display mononucleated longitudinal muscles, although all three take part in regulating actin polymerization in the somatic FCMs and exhibit severe somatic myoblast fusion defects, contradict again the assumption that the longitudinal FCs fuse with this cell type.

Nevertheless, in the analyses of *rols7*, *blow* and *kette* mutant embryos, we cannot exclude the possibility that the lacking structural support of an intact body wall musculature affects fusion of the longitudinal FCs forming syncytia. Furthermore, not only the correct formation of the endoderm, but also an intact ECM is required for visceral muscle development (Wolfstetter and Holz, 2011; Wolfstetter et al., 2009), and the structural support of the longitudinal muscles was shown to be essential for circular muscle arrangement (Ismat et al., 2010). Thus, we propose that the development of the gut might be easily disturbed by fusion defects in the somatic mesoderm, and that mutants for essential, fusion relevant genes have to be examined carefully.

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Figures

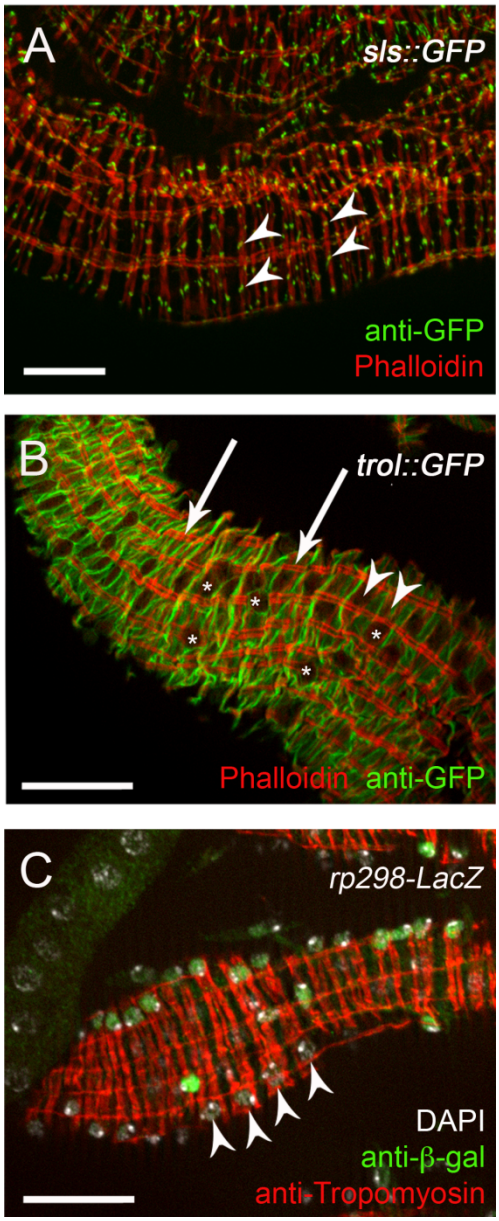

Fig. 1. A dense network of striated muscles surrounds the larval gut. (A) Guts isolated out of 1st instar larvae carrying the protein trap allele *sls::GFP*, counterstained with Phalloidin to visualize sarcomeric actin filaments. Arrowheads mark GFP positive Z-discs. (B) The GFP fusion protein Trol-GFP localizes to the ECM of the 1st instar larval gut musculature. Arrows mark longitudinal muscles, arrowheads mark circular muscles (mainly out of focus), asterisks mark positions of nuclei. (C) 1st instar gut muscles of *rp298-LacZ* larvae. Nuclei were counterstained with DAPI, muscles were visualized using an anti-Tropomyosin antibody. Arrowheads mark nuclei of the circular muscles which were β-gal negative, arrows mark β-gal positive nuclei of the longitudinal muscles. Scale bars: 20 μm.

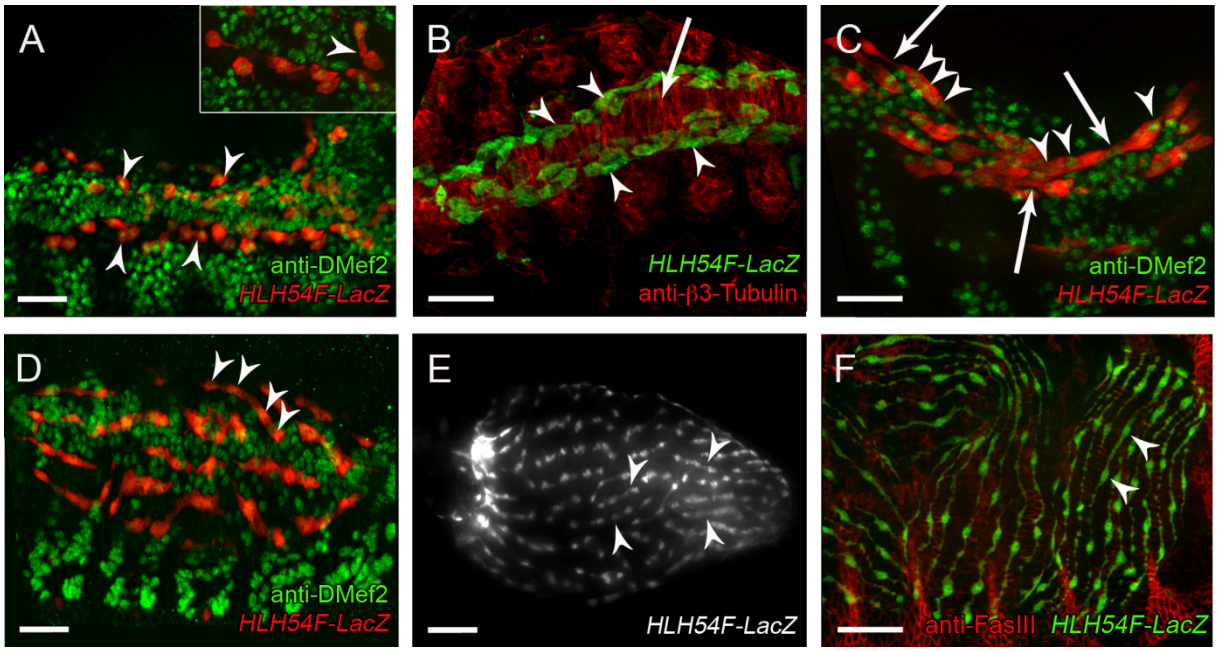

Fig. 2. Longitudinal FC migration and fusion leads to the formation of the longitudinal visceral muscles. (A-F) Embryos in which longitudinal FCs express *HLH54F-LacZ.* Nuclei of mesodermal cells in (A, B, D) were marked by anti-DMef2 staining. (A) Mononucleated, migrating longitudinal FCs (arrowheads) in early stage 12 embryos. Magnification in the right hand corner exhibited cells contacting each other (arrowhead). (B) Longitudinal FCs (arrowheads) were arranged along the stretching, β3-Tubulin expressing circular muscles (arrow) in stage 12 embryos. (C) Late stage 12 embryo with multinucleated longitudinal FCs. Arrowheads point to nuclei of bi- and trinucleated cells, arrows point to cell contacts. (D, E) At the time when the circular muscles stretched, the multinucleated longitudinal FCs stretched perpendicularly. Arrowheads in D point to nuclei of one multinucleated cell, arrowheads in E point to cells stretching in anterior-posterior direction. (F) Embryo at the end of development. Longitudinal muscles covered the gut evenly. Arrowheads mark nuclei of multinucleated muscle. Scale bars: 20 μm.

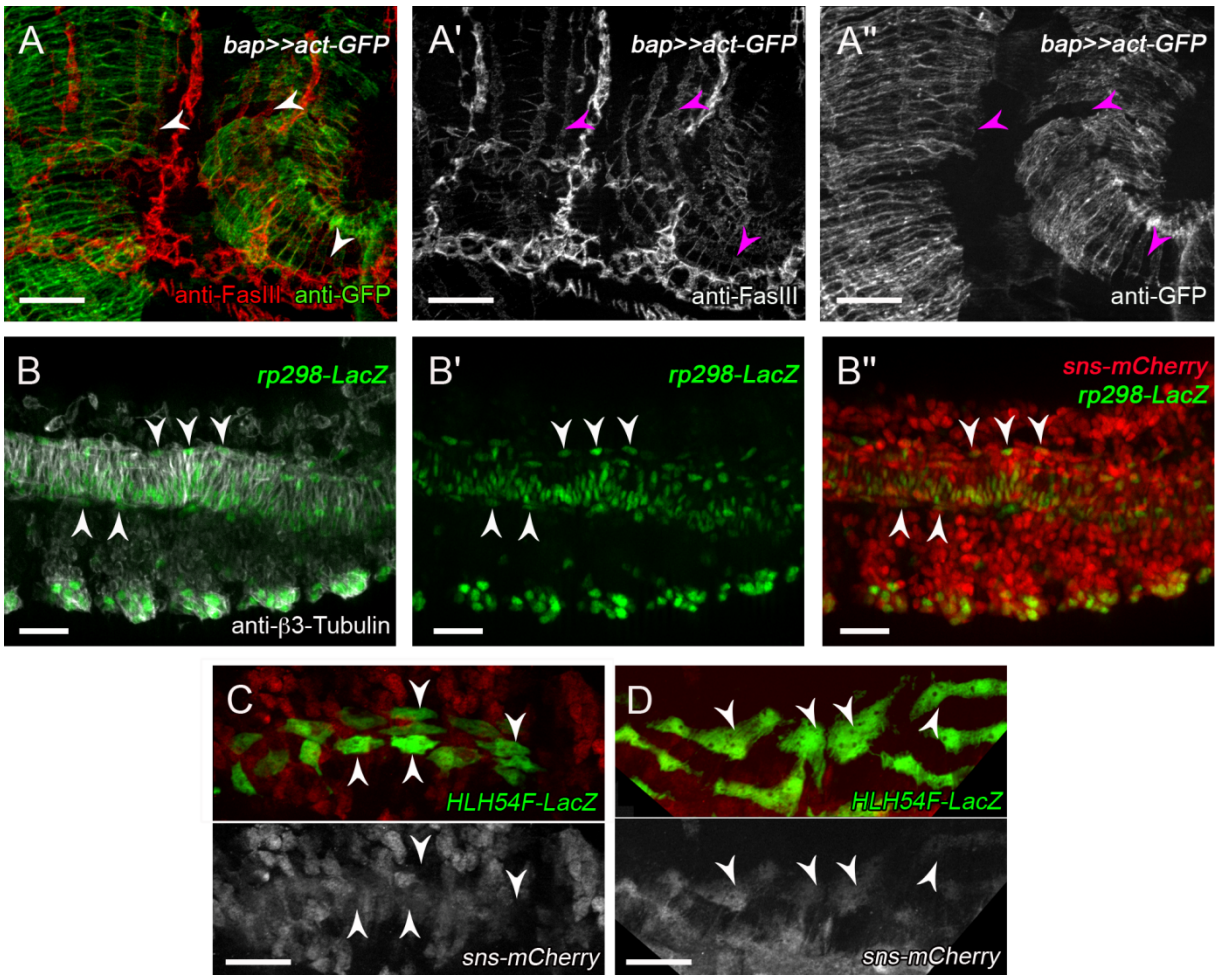

Fig. 3. Longitudinal FCs are *rp298-LacZ* and *sns-mCherry* positive, but do not fuse with *bap* transcribing visceral FCMs. (A-A'') Late staged embryo in which act-GFP was driven with *bap-Gal4*. Longitudinal muscles (arrowheads) were visualized with anti-FasIII staining (A, A') and were largely negative for GFP (A''). (B-B'') Embryo expressing *rp298-LacZ* in somatic and visceral FCs, and *sns-mCherry* in somatic and visceral FCMs. Mesodermal cells in (B) were visualized with anti-β3-Tubulin. Arrowheads point to β-gal positive longitudinal FCs. (C, D) Embryos expressing *sns-mCherry* and *HLH54F-LacZ*. Upper region of the pictures displays anti-β-gal signal, lower region displays anti-mCherry signal. (C) Early stage embryo, in which the longitudinal FCs (arrowheads) were still mononucleated. (D) Embryo in later stage with multinucleated, mCherry positive syncytia (arrowheads). Scale bars: 20 μm

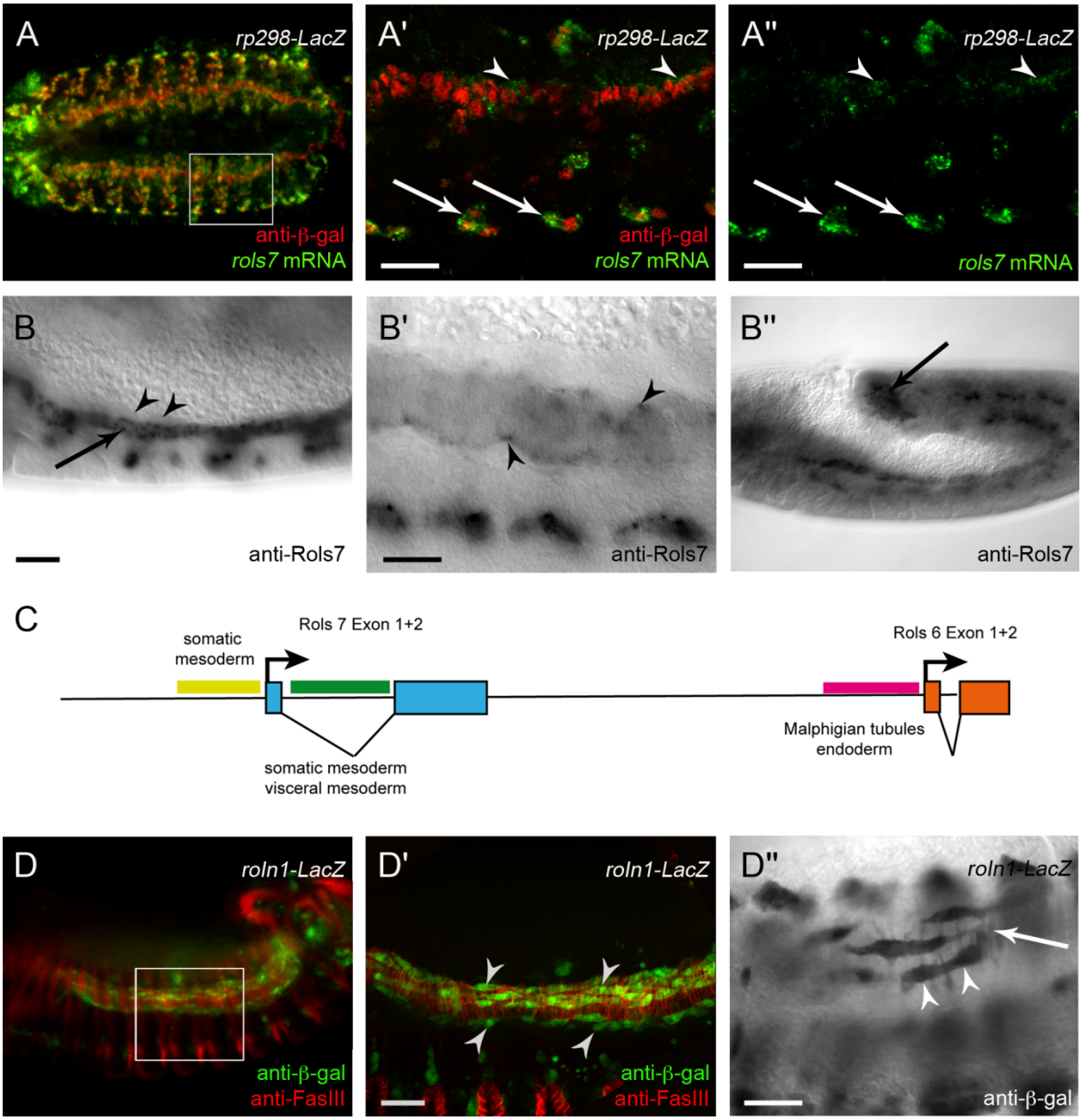
A
rp298-LacZ
anti-β-gal
rols7 mRNA
A'
rp298-LacZ
anti-β-gal
rols7 mRNA
A''
rp298-LacZ
rols7 mRNA
B
anti-Rols7
B'
anti-Rols7
B''
anti-Rols7
C
somatic mesoderm
Rols 7 Exon 1+2
somatic mesoderm
visceral mesoderm
Rols 6 Exon 1+2
Malphigian tubules
endoderm
D
roln1-LacZ
anti-β-gal
anti-FasIII
D'
roln1-LacZ
anti-β-gal
anti-FasIII
D''
roln1-LacZ
anti-β-gal

Fig. 4. Rols7 is expressed in the TVM and CVM. (A-A'') *In situ* hybridization using a *rols7* probe on *rp298-LacZ* embryos, in which nuclei of FCs were marked by anti-β-gal staining. The boxed area in (A) is magnified in (A'-A''). Arrowheads mark *rols7* mRNA in β-gal positive visceral FCs, arrows mark *rols7* mRNA in β-gal positive somatic FCs. (B-B'') Wild-type embryos stained with anti-Rols7. (B) Stage 11 embryo, arrow marks circular FCs. Note that visceral FCMs above (arrowheads) were Rols7 negative. (B') Embryo with stretching circular muscles. Rols7 was only weakly detectable (arrowheads). (B'') Rols7 detected in the CVM (arrow), in stages prior to migration of longitudinal FCs. (C) Schematic drawing of the *rolling pebbles* promoter region. For *rols7*, 2kb of upstream sequence (yellow box) is required for maximum expression in the somatic mesoderm. Control elements for transcription in the visceral mesoderm as well as for somatic muscles (green box) reside in the intron between exons 1 and 2 of *rols7*. For *rols6*, approx. 1, 2 kb of sequences upstream of the transcription start site (red box) are essential for expression in the endoderm and the Malpighian tubules. (D-D'') Expression of *roIn1-LacZ* reporter construct, in which the regulatory region between exon 1 and 2 of the *rols* gene drives expression of β-gal. The boxed area in (D) is magnified in (D'). β-gal was detected in longitudinal FCs (arrowheads in D') along the TVM, marked by anti-FasIII staining. Besides the longitudinal FCs (arrowheads), the stretching circular muscles were also β-gal positive (arrow in D''). Scale bars: 20 μm.

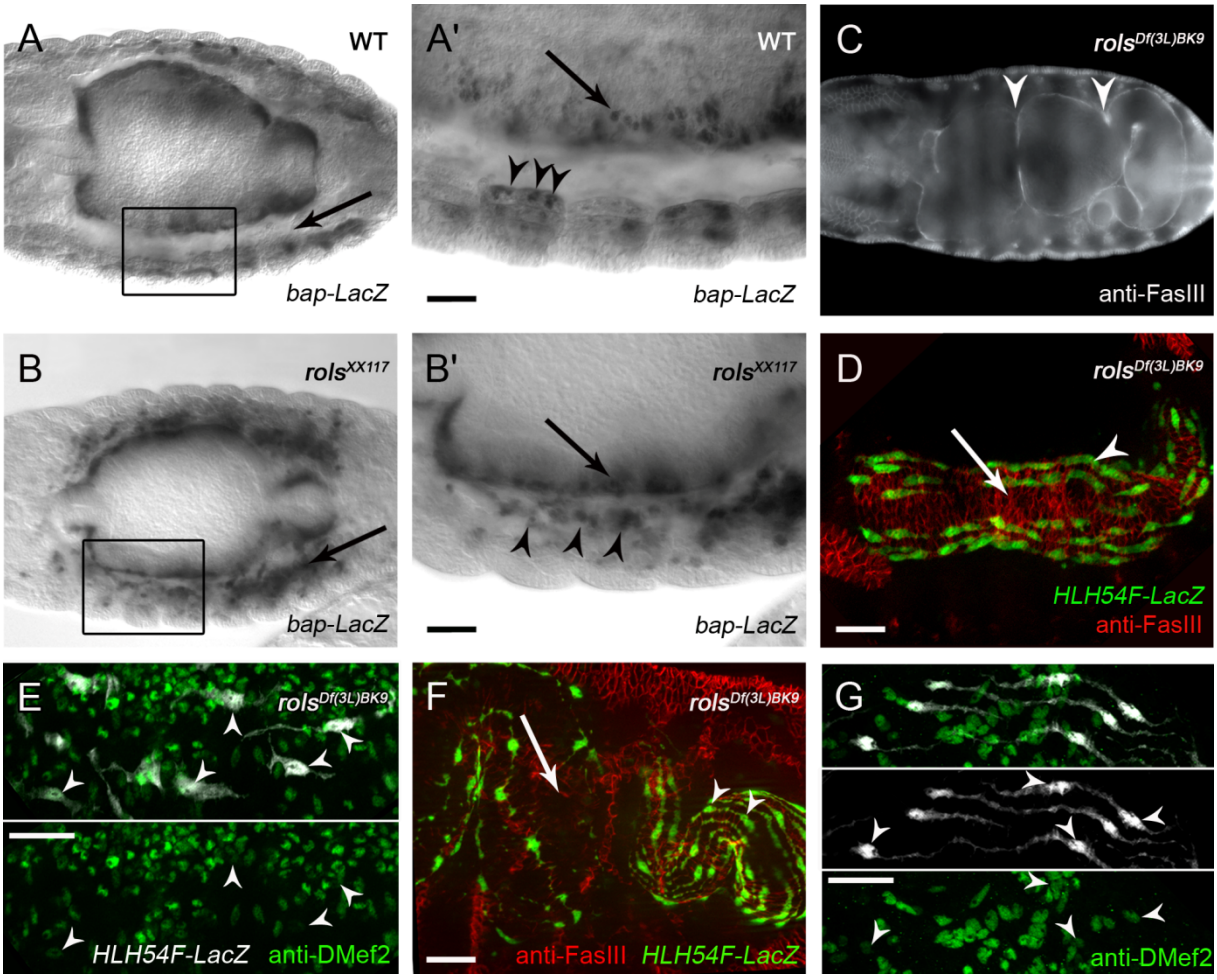
A
WT
bap-LacZ
A'
WT
bap-LacZ
C
rols^Df(3L)BK9
anti-FasIII
B
rols^XX117
bap-LacZ
B'
rols^XX117
bap-LacZ
D
rols^Df(3L)BK9
HLH54F-LacZ
anti-FasIII
E
rols^Df(3L)BK9
HLH54F-LacZ
anti-DMef2
F
rols^Df(3L)BK9
anti-FasIII
HLH54F-LacZ
G
anti-DMef2

Fig. 5. Longitudinal muscle development requires Rols7. (A, A') Wild-type embryo transcribing the reporter construct *bap-LacZ*. (A) Arrow marks interstitium between developing gut and body wall muscles, which is free of visceral FCMs. (A') Magnification of boxed area in (A). Arrowheads mark β-gal positive nuclei in body wall myotubes, arrow marks β-gal positive nucleus of circular muscles. (B, B') *rols7* mutant embryo transcribing *bap-LacZ*. (B) Arrow marks interstitium between gut and somatic mesoderm, containing β-gal positive visceral FCMs. (B') Magnification of boxed area in (B). Arrowheads mark unfused visceral FCMs, arrow marks β-gal positive nuclei in circular muscles. (C) Anti-FasIII staining of *rols7* LOF mutants. Gut constrictions are marked by arrowheads. (D-G) Longitudinal FCs in *rols7* mutant embryos marked by *HLH54F-LacZ* expression. Nuclei of mesodermal cells in (E) and (G) are marked by anti-DMef2 staining. (D) In mid-embryogenesis, the longitudinal FCs (arrowhead) are located dorsally and ventrally of the stretching, FasIII positive circular muscles (arrow). (E) Later stage of gut development, shortly before gut constrictions were formed. Lower region of the picture displays anti-DMef2 signal, upper region displays overlay of anti-DMef2 and anti-β-gal. Arrowheads mark nuclei of mononucleated cells stretching protrusions. (F) Stage after the gut became constricted. Arrow marks area not covered with longitudinal muscles, arrowheads mark parallel longitudinal muscles in the posterior region of the gut. (G) Longitudinal gut muscles of *rols* mutants at the end of embryogenesis. Central region of the picture displays anti-β-gal signal, lower region displays anti-DMef2 signal, and upper region displays overlay of both. Arrowheads point to nuclei of binucleated longitudinal muscles. Scale bars: 20 μm.

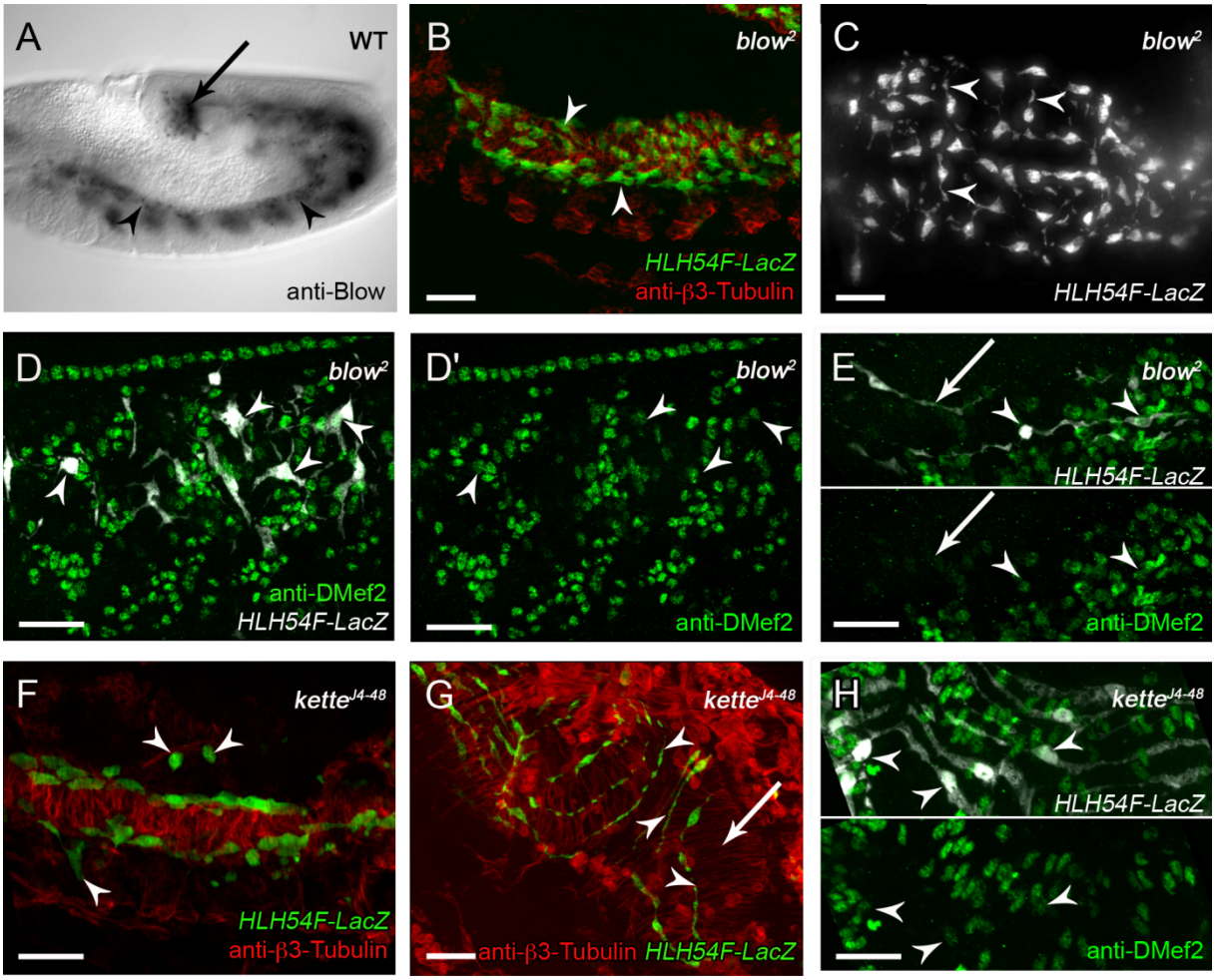
A
WT
anti-Blow
B
blow2
HLH54F-LacZ
anti-β3-Tubulin
C
blow2
HLH54F-LacZ
D
blow2
anti-DMef2
HLH54F-LacZ
D'
blow2
anti-DMef2
E
blow2
HLH54F-LacZ
anti-DMef2
F
kette J4-48
HLH54F-LacZ
anti-β3-Tubulin
G
kette J4-48
anti-β3-Tubulin HLH54F-LacZ
H
kette J4-48
HLH54F-LacZ
anti-DMef2

Fig. 6. Longitudinal muscle devlopment is disturbed in *blow* and *kette* mutants. (A) Wild-type embryo stained with anti-Blow antibody. A signal was detected in the TVM (arrowheads) and the CVM (arrow). (B-H) *blow*^{2} (B-E) and *kette*$^{J4\text{-}48}$ (F-H) mutant embryos carrying the reporter construct *bHLH54F-LacZ*. Nuclei of all mesodermal cells in (D, D', E, H) are marked with anti-DMef2. (B) *blow* mutant in mid-embryogenesis, in which longitudinal FCs (arrowheads) were detected along the circular muscles, marked with anti-β3-Tubulin staining. (C) Mononucleated longitudinal FCs forming protrusions (arrowheads) in ventral/dorsal direction. (D, D') Mononucleated longitudinal FCs in a stage in which gut constrictions formed. Arrowheads point to single nuclei of longitudinal FCs. (E) *blow* mutant embryo at the end of embryogenesis. Longitudinal muscles with two nuclei (arrowheads) at the most were present. Upper region displays β-gal and DMef2 signal, lower region displays the DMef2 signal alone. (F) *kette* mutant in which longitudinal FCs were detected along the β3-Tubulin expressing circular muscles. Arrowheads mark cells migrating away from the developing circular muscles. (G) Embryo at the end of embryogenesis, in which the longitudinal FCs formed thin cell protrusions (arrowheads). Note that in some regions of the gut surface, β3-Tubulin positive circular muscles are present, while longitudinal muscles are missing (arrow). (H) Longitudinal muscles at the end of gut development. Arrowheads point to binucleated myotubes. Upper region displays the β-gal and DMef2 signal, lower region displays the DMef2 signal alone. Scale bars: 20 μm.

8 Conclusions and Perspectives

Myoblast fusion in the *Drosophila* embryo is a highly regulated matter. A lot of research has been conducted to unravel the exact mechanisms of this heterotypic cell fusion of a founder cell (FC) with a fusion competent myoblast (FCM) in the somatic mesoderm. In particular, the regulation of F-actin polymerization is important, and transport or force generating processes have been supposed to be taken over by the actin cytoskeleton. In this thesis, it was shown that newly synthesized microtubules play only a minor role in the development of the larval musculature, and the implications of this study are reviewed here. Furthermore, evidence was presented that the unconventional myosin heavy chain Mhcl is expressed in the mesoderm of *Drosophila* embryos. A possible role of Mhcl at the Fusion-restricted Myogenic-Adhesive Structures (FuRMAS) between the FC and the FCM is discussed in this chapter. At last, the development of the longitudinal gut musculature has been analyzed in this thesis, providing evidence that genes essential for the somatic myoblast fusion process are also involved in fusion of the longitudinal visceral FCs. This chapter discusses a model how fusion leads to the formation of the syncytial longitudinal visceral muscles, and which proteins are most likely involved.

8.1 Newly synthesized microtubules are dispensable for myogenesis in the *Drosophila* embryo

In vertebrate primary cell culture systems, in parallel arranged microtubules are essential for sarcomere assembly in the myotube (Pizon et al., 2005; Pizon et al., 2002; Toyama et al., 1982). This thesis addressed the requirement for microtubules in the *Drosophila* embryonic musculature by analyzing mutants for β-Tubulins, the subunits of the heterodimers which, together with α-Tubulins, constitute the microtubule protofilaments. Without one type of these subunits, there are no functional microtubules assembled (reviewed in Hammond et al., 2008). It was shown that the zygotic expression of the β1- and β3-Tubulin isoforms in the embryo is not necessarily needed for correct completion of myogenesis. Furthermore, it became evident that the β2-Tubulin isoform is most likely not expressed in the embryo. The fact that a maternally expressed β1-Tubulin-GFP fusion protein disturbs embryonic development confirmed the hypothesis that low levels of maternally contributed β1-Tubulin are sufficient to form a functional microtubule network in embryos in general and in the developing musculature in particular (Rudolf et al., 2012). Heterologous β-Tubulin can be incorporated into functional microtubules, as shown by injection experiments into cultured mammalian cells (Saxton et al., 1984), sea urchin embryos (Salmon et al., 1984) and *Drosophila* embryos (Kellogg et al., 1988).

Although the ectopic expression of the β3-Tubulin isoform cannot substitute the function of β2-Tubulin in meiosis in the male germline and in formation of the sperm axoneme (Hoyle and Raff, 1990), the present study provides evidence that a β-Tubulin isoform, here β1-Tubulin, is likely able to substitute for the strongly expressed β3-Tubulin isoform during the development of the *Drosophila* embryo. Therefore, the function of β3-Tubulin appears not to be very specialized, and it might contribute to the ubiquitously present β1-Tubulin pool as an evolutionally advantage for the fly, as it has been suggested before (Dettman et al., 1996).

There are only few microtubules present in myoblasts and myotubes of the embryo (Dettman et al., 1996). As low levels of maternal derived β1-Tubulin allows the development of the musculature (Rudolf et al., 2012), microtubules appear to play only a minor role in myogenesis of the embryo in general and in myoblast fusion in particular. This sheds light on the importance of the actin cytoskeleton during this process. In many years of research, several EMS-induced mutageneses resulted in mutant fly strains, which revealed defects in myoblast fusion and were identified as mutants for genes encoding actin regulators (Berger et al., 2008; Rushton et al., 1995; Schröter et al., 2004; Schäfer et al., 2007). However, mutants for genes encoding structural elements of microtubules or encoding microtubule-associated proteins have not been found in such screens so far. The FuRMAS contain an F-actin plug in the center, and the loss of several actin regulators results in strong fusion defects (reviewed in Önel et al., 2011). For example, in mutants for the *mbc* gene, the F-actin plug is absent in adhering myoblasts, with the consequence that myoblast fusion is completely blocked (Haralalka et al., 2011; Rushton et al., 1995). Therefore, F-actin must be polymerized at the FuRMAS for correct fusion, especially on the side of the FCM. Moreover, the electron-dense vesicles, which derive from the Golgi and can be detected at the ultrastructural level, are coated with actin (Doberstein et al., 1997; Kim et al., 2007). It is thus likely that these vesicles are transported via the actin cytoskeleton, although long-range transports of vesicles through the cell are often managed by microtubules, e.g. via the interaction of microtubule motors with vesicle-associated Rab GTPases (reviewed in Horgan and McCaffrey, 2011).

Recently, *Drosophila* myoblasts were successfully taken in culture. The fusion ability and myotube formation was reduced when these cells treated with the microtubule depolymerisator nocodazole (Dobi et al., 2011). However, it is imaginable that secondary effects of the drug, like inhibition of cell migration, caused the observed fusion defects of myoblasts in culture rather than an inhibition of fusion itself. Sherwood et al. (2004) could show *in vivo* that microtubule destabilization by overexpression of the AAA ATPase Spastin in the mesoderm results in myotube detachment from the epidermis, but not in myoblast fusion defects, which supports the model that myoblast fusion can occur *in vivo* with low numbers of microtubules. This can be confirmed by further experiments: Nocodazole injected into embryos of the right age, in stages

of beginning myogenesis, could demonstrate the muscle development in absence of microtubules. A protocol for injections of drugs or dye in living embryos has already been established (Rand et al., 2010). However, these analyses have to be performed very carefully. The injection of the drug could possibly result in secondary defects in the musculature, due to the fact that the microtubules in all tissues would be destabilized, including the epidermis, at which the muscles attach at the end of embryogenesis. Depending on the exact time frame in which the drug should be injected, which is already hard to determine, it could also be possible to inhibit myoblast migration towards the growing myotube as an unwanted side effect.

Podosomes and invadopodia are detectable in cells derived from the monocytic lineage as well as in epithelial cells. With the help of these structures, cells adhere to and degrade the extracellular matrix (ECM), which, in case of the invadopodia of cancer cells, can ultimately lead to invasion (reviewed in Linder, 2007). The FuRMAS share many features with podosomes and invadopodia such as the formation of ring-like structures containing adhesion molecules, F-actin plugs and regulation of the actin cytoskeleton (reviewed in Önel and Renkawitz-Pohl, 2009). For example, the podosomes in macrophages and osteoclasts are F-actin rich, and the actin polymerization in these structures depends on WASp (reviewed in Murphy and Courtneidge, 2011), analogous to the situation at the FuRMAS (Schäfer et al., 2007). The protrusive activity of podosomes and invadopodia is most probably achieved by coupling actin polymerization with membrane deformation (reviewed in Gimona, 2008; Murphy and Courtneidge, 2011). In addition, microtubules are essential for podosome dynamics (Kopp et al., 2006; Linder et al., 2000). In vertebrate monocytes, the strong ECM degradation within the podosomes requires the interaction of the microtubule motor kinesin with vesicle-associated flotilin proteins, and the vesicles containing proteases and signaling molecules are likely to be transported via microtubules towards the sites of ECM degradation in podosomes and invadopodia (Cornfine et al., 2011; Linder, 2007; Poincloux et al., 2009). As newly synthesized microtubules are not that needed in the myoblast fusion process of *Drosophila* embryos, the necessity of high-level microtubules displays a difference between the formation of the FuRMAS on the one hand and the formation of podosomes and invadopodia on the other hand. As a possible reason, strong ECM degradation has not been observed during myoblast fusion so far.

A recent study presented evidence that a microtubule-associated protein (MAP) and kinesin-dependent transport along microtubules in *Drosophila* myotubes is required for the correct arrangement of nuclei. In mutants for *MAP7/ensconsin* as well as in mutants for *kinesin heavy chain*, the myonuclei are clumped together, with the consequence that these mutants exhibit a reduced larval locomotion (Metzger et al., 2012). Nevertheless, myonuclei in *Drosophila* myotubes are correctly arranged in mutants for *β3-Tubulin* at the end of embryogenesis (Rudolf et al., 2012). Again it may be that a low number of β1-Tubulin containing microtubules are

sufficient for correct nuclei arrangement in these mutants. Positioning of myonuclei in *C. elegans*, *Drosophila* and vertebrates commonly depend on nesprins, which connect the nuclear envelope mainly with the actin cytoskeleton, but which can also associate with microtubule motors (reviewed in Starr and Fridolfsson, 2010). Therefore, both cytoskeletal elements are supposed to keep the nucleus at the correct position within the cell. Moreover, the uniform array of microtubules, their linkage with the nucleus and with it myotube elongation in *Drosophila* depends on cytokinesis-relevant proteins, which were speculated to provide a link of microtubules with the actin cytoskeleton within the myotube (reviewed in Guerin and Kramer, 2009). This research field is of great interest, as the human muscle diseases, called centronuclear myopathies, display abnormal positioned nuclei within the myofibers, along with muscle weakness and disorganized sarcomeres (reviewed in Jungbluth et al., 2008). Thus, further research concerning the connection between microtubule- and actin-dependent nuclei positioning and muscle function in *Drosophila* and in vertebrates could lead to new therapies for genetically caused muscular dystrophies.

8.2 Mhcl is an interaction partner of Rols7 and might be involved in actin-based processes during myoblast fusion

Multiple functions of F-actin during myoblast fusion in *Drosophila* are hypothesized (see above). For all these tasks, motor proteins are likely to exist, which might allow actin-based transport processes or force generation. Rolling pebbles 7 is an essential adaptor protein at the FuRMAS, linking Duf-dependent cell adhesion to so far unknown downstream pathways (Kesper et al., 2007). In this thesis, it was shown that Rols7 interacts with the unconventional myosin heavy chain Mhcl (Myosin heavy chain-like) *in vitro* via its essential ankyrin repeats (Chapter 6).

Although *Mhcl* is strongly transcribed in the mesoderm, on the side of the FCs already prior to fusion, *Mhcl* deficient embryos exhibit a wild-typic musculature. As myoblast fusion is highly regulated and presumably secured by redundant proteins, other myosin heavy chains might participate in muscle development and can possibly substitute for Mhcl as the heavy chain subunit of the myosin molecule. The non-muscle myosin heavy chain Zipper (Zip) is also expressed in FCs prior to fusion (Estrada et al., 2006) as well as in mature myotubes at the end of embryogenesis (Bloor and Kiehart, 2001). Unfortunately, muscle development of *ΔMhcl,zip* double mutants were not analyzable due to the strong germ band retraction and dorsal closure defects (Franke et al., 2005, Chapter 6); thus, it remains elusive whether Zip and Mhcl act redundantly. Although a function of the conventional muscle Myosin heavy chain (Mhc) before sarcomere assembly has not been described so far, Mhc is already expressed in myoblasts (Swank et al., 2000 and references therein), and future studies should thus address *ΔMhcl,Mhc* double mutants as well. Furthermore, the Myosin regulatory light chain Spaghetti squash (Sqh)

is the only regulatory light chain which is ubiquitously expressed in the embryo (Zhang and Ward, 2011). Mhcl contains an IQ motif which is expected to bind to calmodulin or related proteins like myosin light chains (Berg et al., 2001; Tzolovsky et al., 2002), so Sqh might be part of the myosin hexamer interacting with the heavy chains encoded by *Mhcl*. First experiments to knock down *sqh* expression in the mesoderm via RNA interference (RNAi) did not result in a myoblast fusion defect (unpublished observations); however, this might be due to the fact that RNAi in the *Drosophila* embryo often does not knock down the expression of proteins involved in myoblast fusion, as the expression levels of the RNAi constructs are low (D. Buttgereit, personal communication) and the fusion process is only a matter of minutes (Beckett and Baylies, 2007). Thus, other genetic strategies to impair the function of a hypothesized myosin molecule during muscle development have to be established in the future.

The class XVIII myosin heavy chain Mhcl is most similar to members of the non-muscle myosin II subfamily, which are crucial in, e.g., cell migration, adhesion and cytokinesis (reviewed in Conti and Adelstein, 2008). Phylogenetic analyses provided evidence that the members of class II and class XVIII share a common origin (Foth et al., 2006). The motor domain of Mhcl binds transiently to actin, but it exhibits no ATPase activity, indicating that Mhcl rather functions as an actin tether protein like α-Actinin (Guzik-Lendrum et al., 2011). Mhcl is most closely related to mammalian Myosin-18A (Berg et al., 2001; Tzolovsky et al., 2002). Both, Mhcl and Myo-18A, contain PDZ domains, which are commonly found in proteins establishing molecular complexes (Doyle et al., 1996). Furthermore, PDZ-domain containing proteins are often involved in vesicle transport or display adaptors of the actin cytoskeleton and the membrane (reviewed in Sierralta and Mendoza, 2004). Myo-18A is part of a complex which is responsible for assembling actomyosin bundles in lamellipodia of mammalian cells in culture (Tan et al., 2008). Mhcl might also form complexes with other proteins, and due to its ability to bind actin, it is possibly involved in actin polymerization, actin localization or bundling of actin filaments. One other myosin heavy chain of class 18, Myo-18B, has been identified in vertebrates, which lacks the PDZ domain and is expressed in human myogenic cells (Salamon et al., 2003). Due to its localization within the nucleus, a function during gene transcription has been proposed (Redowicz, 2007), which sheds light on the multiple functions unconventional myosins can take over within the cell. However, localization at the nucleus has not been observed for Mhcl, thus a function during gene regulation is unlikely.

Although the *ΔMhcl* embryos do not exhibit any muscle defects, an ectopically expressed GFP-Mhcl fusion protein localizes clearly at the contact sites of a FCs towards the FCM during myoblast fusion, and this localization depends on the presence of Rols7. In the previously described model, Mhcl interacts with Rols7 after successful cell adhesion during the second phase of fusion, enabling actin-based processes at this site (Chapter 6). In rat myogenic cells in

culture, a cortical actin wall is present in aligned myoblasts, which is reorganized prior to vesicle pairing at both membranes. This actin reorganization as well as subsequent fusion of the cells depends on non-muscle Myosin IIA (Duan and Gallagher, 2009). These first observations that myosins are crucial in vertebrate myoblast fusion lead to the assumption that a myosin is also involved in *Drosophila* myoblast fusion. In the following, possible functions for Mhcl at the FuRMAS are discussed:

At first, Mhcl could be involved in the transport of the electron-dense vesicles towards the fusion site. These vesicles are actin-coated and were discussed to deliver fusion-relevant molecules towards the membrane (Kim et al., 2007). The FuRMAS have been compared, additionally, to podosomes and invadopodia, to the immunological synapse (IS) between an antigen-presenting cell and a T cell (Önel and Renkawitz-Pohl, 2009). In Natural Killer cells, the transport and exocytosis of lytic vesicles towards the IS depend on Myosin IIA, which enables the interaction of these vesicles with actin and their transit through the actin-rich core of the IS towards the membrane (Andzelm et al., 2007; Sanborn et al., 2009). Analogous to the situation at the IS, the transport of vesicles towards the fusion site at the FuRMAS could require a myosin, mediating translocation along the actin cytoskeleton. In *Drosophila*, vesicle transport has already been shown to depend on myosins: At the larval neuromuscular junction, Zip is required for transport of synaptic vesicles within the nerve terminal bouton (Seabrooke et al., 2010; Seabrooke and Stewart, 2011). In other model systems and in other cell types, vesicle transport mechanisms have so far been described for unconventional myosins of the groups I, II, V and VI (reviewed in Bond et al., 2011). However, the mammalian Mhcl orthologue Myo-18A forms complexes with a Golgi-associated protein and links the actin cytoskeleton to the Golgi complex as an actin tether (Dippold et al., 2009). Therefore, Mhcl could alternatively function as a linker of actin and the Golgi-derived electron-dense vesicles at the FuRMAS, rather being involved in vesicle exocytosis than in transport itself.

A second possible function for Mhcl might be the widening of the fusion pore. At the FuRMAS, the ring-like structure of the adhesion molecules becomes widened from 1 μm to 5 μm, and with it the fusion pore. This was proposed to depend on branched F-actin (Kesper et al., 2007). There is evidence that myosin II forms circular structures within podosomes to regulate their dynamics and to change their size or shape (Collin et al., 2008). Several myosin hexamers can assemble into “minifilaments”, which then form networks with actin. These actomyosin networks can provide contractile activity (reviewed in Levayer and Lecuit, 2012). It is possible that Mhcl could, together with F-actin, provide the tension for regulating the size of the FuRMAS during myoblast fusion, analogous to the function of myosin II in podosomes.

Third, establishing a protein-free zone is a prerequisite of cytoplasmatic continuity during myoblast fusion. Ultrastructural studies showed that membrane breakdown is preceded

by the creation of an actin-free zone (Sens et al., 2010). Moreover, it has been proposed that a prerequisite for correct fusion is the clearance of the adhesion molecule N-cadherin from the membrane, which appears to depend on the GEF Schizo (Siz) (Dottermusch-Heidel et al., 2012). Analogous, Zip is important for the endocytosis of E-cadherin during epithelial morphogenesis in the early embryo, (Levayer et al., 2011). Furthermore, a "pushing" function for Zip has been described during the asymmetric cell division of neuroblasts; in these cells, Zip is needed for translocating determinants along the membrane into the daughter cell (Barros et al., 2003). Removing the adhesion molecules from the fusion site could be achieved by Mhcl, either by transport along the F-actin filaments, analogous to Zip function in neuroblasts, or by enabling endocytosis of the membrane-bound receptors. This clearance is likely to accompany and to be closely associated with the widening of the fusion pore discussed earlier.

Lastly, some unconventional myosins are directly involved in actin polymerization by interacting with WASp and WIP, and are further needed for tension sensing at the membrane as well as for cell migration (reviewed in Hartman et al., 2011). Myosin IIA is required for the integrity of the IS itself, and actomyosin dynamics and contraction are required for T cell receptor signaling and clustering (Babich et al., 2012; Yi et al., 2012). In conclusion, multiple functions for myosins interacting with the actin cytoskeleton, the membrane or vesicles are possible at the FuRMAS, yet the exact role for Mhcl there has to be addressed.

Additionally to its early expression in FCs of the embryo and its localization at the FuRMAS, Mhcl localizes in mature myotubes at the Z-discs of the sarcomere (Chapter 6). The vertebrate homologue Myo18B as well as other non-muscle myosin heavy chains of vertebrates and *Drosophila* localize in the same way and have been suggested to be responsible for sarcomeric integrity (Ajima et al., 2008; Bloor and Kiehart, 2001; Takeda et al., 2000). In addition to the thin filaments required for contraction of the sarcomere, a functional actin cytoskeleton is present in mature murine myotubes. Those filaments are supposed to be important in vesicle transport at the sarcoplasmatic reticulum and are a component of the costameres, the attachments of the sarcomere to the sarcolemma; furthermore, an actin cytoskeleton can be detected at the Z-discs with a proposed, but yet unproved function in stabilization of the Z-discs (reviewed in Kee et al., 2009). Mhcl might be associated with the latter actin cytoskeleton, providing structural integrity of the sarcomere. It is of notion that in *Drosophila*, some fusion-relevant proteins are additionally present at the Z-disc of mature myotubes, e.g. Mind bomb 2 (Carrasco-Rando and Ruiz-Gómez, 2008), Duf, and Rols7 (Kreisköther et al., 2006), which interacts with Mhcl. This unconventional myosin thus could take over two different functions, one at the FuRMAS and one at the Z-disc of the sarcomere.

8.3 The longitudinal visceral musculature develops distinctly from the circular muscles, and fusion-relevant proteins are also needed for fusion efficiency of longitudinal FCs

This thesis addressed the development of the *Drosophila* larval longitudinal visceral muscles, which are interwoven with the circular muscles to form a network embedded into a thick layer of ECM. The longitudinal FCs migrate along the trunk visceral mesoderm (TVM), where the circular FCs as well as the visceral FCMs are located, but they surprisingly do not fuse with the same type of FCMs as the circular FCs do. Nevertheless, this type of FCs appears to fuse to cells expressing the FCM specific adhesion molecule Sns. It has already been discussed which cells might contribute to the syncytial longitudinal muscles (Chapter 7): (1) FCMs out of the TVM, which do not express the transcription factor Bagpipe (Bap) and are therefore distinct from the circular FCMs, (2) FCMs out of the somatic mesoderm, which lie in close proximity, (3) only longitudinal FCs, which eventually fuse homotypically to each other.

In stage 10 of embryogenesis, i.e., before the longitudinal FCs start to migrate, the transcription factor Bap is expressed in 11 segmental patches consisting of 17 cells. After one mitotic division, these patches of cells rearrange and eventually merge with each other, giving rise to the continuous band of the TVM. Because of a lacking marker, cells initially lying between the Bap patches could not have been followed so far, but they might also contribute to the TVM (Azpiazu and Frasch, 1993). These cells might be the source of FCMs to which the longitudinal FCs fuse. The longitudinal FCs do not form syncytia in *eve* mutants, which do not determine the TVM (San Martin et al., 2001); thus, hypothesis (1) is very likely.

The second hypothesis would indicate that the FCMs, which contribute to the somatic and both types of visceral muscles, are interchangeable to a greater extent than suggested before (Klapper et al., 2002; San Martin et al., 2001; Schröter et al., 2006). In mutants, which do not specify the circular FCs, the visceral FCMs originating from the TVM can fuse with somatic FCs (Englund et al., 2003; Eriksson et al., 2010; Lee et al., 2003; Stute et al., 2004). This is, although to a lower extent, even the case in the wild-type situation (Azpiazu and Frasch, 1993); furthermore, the number of unfused visceral FCMs is strongly enhanced if fusion in the somatic mesoderm is disturbed (Chapter 7). These data provide evidence that visceral FCMs are in principle capable of fusing with somatic FCs. In contrast to that, Zaffran et al. (2001) showed that the ectopic expression of the visceral FCM specification factor Biniou in the somatic mesoderm results in myoblast fusion defects, indicating that both types of FCMs are well distinguishable regarding their genetic program. Moreover, several mutants of fusion-relevant genes with an essential function in somatic FCMs during myoblast fusion exhibit only subtle defects in the visceral mesoderm (Eriksson et al., 2010; Schröter et al., 2006), which contradicts the view that somatic

FCMs contribute to the visceral muscles. Further analyses should address the different expression profiles of visceral and somatic FCMs as well as their ability to fuse to all three types of FCs, in order to clarify whether all FCMs of the *Drosophila* embryo can be regarded as one FCM pool.

The hypothesis that the longitudinal FCs fuse homotypically, is the most unlikely one, as this has not been observed before in *Drosophila*. Homotypic fusion events occur, e.g., in the development of *C. elegans*, in macrophage fusion forming osteoclasts, and also in the development of vertebrate muscles (reviewed in Oren-Suissa and Podbilewicz, 2010). During vertebrate myogenesis, myoblasts which fuse to each other appear genetically identical (reviewed in Abmayr and Pavlath, 2012). In contrast to that, the differential expression of identity genes within the FCs control the size, shape, and orientation of the muscle within the *Drosophila* embryo, while the FCMs contribute only to the mass of the muscle (reviewed in Tixier et al., 2010). Looking at the shape and the number of the longitudinal FCs migrating along the TVM, one might indeed have the impression that these cells fuse with themselves, as the fully developed longitudinal muscles contain only very few nuclei in relevance to their enormous length. However, a strong argument against the possibility of homotypic cell fusion is the finding of (Mandal et al. (2004), who showed that in syncytial longitudinal FCs, one nucleus is positive for the FC specific transcription factor Cpo, while the others are not. Therefore, a different cell population is likely to fuse with the longitudinal FCs.

This thesis further showed that the fusion relevant proteins Rols7, Blown fuse (Blow) and Kette are involved in longitudinal muscle formation. *rols7* as well as *kette* mutant embryos establish binucleated longitudinal muscles, but these syncytia have only been observed at the posterior part of the gut in *rols7* mutants. In contrast, most of the longitudinal FCs stay mononucleated in embryos mutant for *blow*. However, further experiments need to be done in order to confirm that the observed phenotypes are indeed due to the lack of the proteins in the visceral mesoderm, and that the absence of an intact body wall musculature does not interfere with the development of the longitudinal muscles. To this aim, rescue experiments could be performed, expressing Rols7, Blow or Kette in the somatic mesoderm of the corresponding mutants, and analyzing fusion efficiency of the longitudinal FCs. Furthermore, all three mutant alleles used in the study might delete other genes which could be additionally involved in gut muscle formation: The analyzed *rols7* deficiency *Df(3L)BK9* deletes several other genes (Rau et al., 2001), while the EMS induced $kette^{J4\text{-}48}$ chromosome is known to carry more hits than *kette* (S. Önel, personal communication). Moreover, the chromosome carrying the $blow^{2}$ allele contains a mutated *fasciclin III* gene (Sickmann, 2010). FasIII is present in the membrane of both types of visceral FCs as well as in the membrane of the visceral FCMs (Kusch and Reuter, 1999; San Martin et al., 2001; Stute et al., 2004), fulfilling a yet unknown function. It is therefore important

to analyze gut muscle formation in transheterozygous embryos, carrying different mutant alleles.

Blow is expressed in both, the longitudinal FCs and the circular FCs, as well as in the visceral FCMs. Interestingly, fusion of circular FCs is unaffected in the mutant situation (Schröter et al., 2006). In the somatic mesoderm, the Blow protein is indirectly involved in actin polymerization at the actin plugs of the somatic FCMs by competing with D-WIP/Vrp1/Slt for WASp binding (Jin et al., 2011). However, mutants for *d-wip/vrp1/slt* develop a longitudinal musculature like that of the wild-type (Eriksson et al., 2010). These observations lead to two different hypotheses: First, given the previously discussed possibility that the longitudinal FCs might fuse with somatic FCMs, and given that Blow acts on the side of the somatic FCM, the strong phenotype of longitudinal muscle formation in *blow* mutants could be explained. However, Blow function would in this case be independent of competing with D-WIP. Second, given that the longitudinal FCs fuse with Bap negative FCMs from the TVM, a function for Blow in this process is possible. In this case, it has to be determined whether or not it acts on the side of the longitudinal FCs and/or on the side of the longitudinal FCMs. To this aim, cell type-specific rescue experiments could be done. Analyses of *WASp* mutant embryos addressing the number of nuclei within the longitudinal muscles would further demonstrate whether WASp-dependent actin polymerization is involved like in the somatic FCMs (Jin et al., 2011) and whether Blow might also regulate WASp/D-WIP dynamics in this type of visceral FCMs.

The *kette* mutant phenotype in the somatic mesoderm is comparable to the *blow* phenotype: precursor cells are established, but the second fusion step is completely inhibited in both *kette* and *blow* mutants. Furthermore, the two proteins interact with each other genetically (Schröter et al., 2004). In contrast, while longitudinal myoblast fusion clearly requires Blow, embryos lacking the actin polymerization-regulating factor Kette still exhibit binucleated longitudinal syncytia, although the overall number of longitudinal muscles is reduced at the end of embryogenesis (Chapter 7). The broad expression pattern of Kette as well as the maternal contributed *kette* mRNA (Hummel et al., 2000) does not allow determining whether or not the protein is present in the longitudinal FCs at all. Double labeling of embryos with an anti-Kette antibody and cell type-specific markers could answer this question. The observed gaps in the gut musculature of *kette* mutants might be due to the partially misguided longitudinal FCs along the TVM, which then would not be at the right place to fuse to another or the same cell type. Indeed, the longitudinal muscles of *kette* mutant embryos resemble the ones which have been described for mutants of the fibroblast growth factors (FGF) encoding genes *pyramus* (*pyr*) and *thisbe* (*ths*). Here, the FGF receptor Htl expressed in the longitudinal FCs does not receive signals of the TVM in form of its ligands Pyr and Ths, with the consequence that the FCs do not migrate properly and less longitudinal muscles are present at the end of gut development (Kadam et al., 2012; Reim et al., 2012). Kette might be involved in actin polymerization at the leading edge of the

longitudinal FCs, analogous to its proposed role in glial cell migration (Hummel et al., 2000), and might provide a link from the actin cytoskeleton to FGFR signaling there. However, it cannot be excluded that Kette acts additionally during longitudinal myoblast fusion, as the longitudinal muscles still exhibit less nuclei as in wild-type embryos.

Additionally to the somatic FCMs, F-actin-rich plugs are also present in the visceral FCMs, as shown in experiments in which actin-GFP expression was driven by the circular FCM-specific driver line *bap-Gal4* (Berger, 2010). Thus, actin polymerization appears to be required during fusion of the circular FCs with the Bap positive visceral FCMs. Interestingly, this process is not disturbed in *kette* mutant embryos (Schröter et al., 2006); together with the results presented in Chapter 7, this indicates that the Kette/SCAR complex might not be required for actin polymerization in the visceral mesoderm at all. Future studies need (1) to elucidate whether or not F-actin is enriched at points of cell contacts in the longitudinal FCs and/or FCMs as it is the case in the somatic and circular visceral mesoderm, and (2) whether the Arp2/3 complex is activated, leading to the formation of branched F-actin there. Mutants of the Arp2/3 complex itself develop at least gut constrictions, an indication of normal circular muscle development (Kusch and Reuter, 1999), but exhibit a prolonged Duf expression in the visceral mesoderm at later stages, which cannot be observed in the wild-type. This was discussed to indicate a fusion arrest to a smaller degree in the longitudinal muscles (Eriksson et al., 2010). It would further be interesting whether other actin nucleation-promoting factors like WASp and WAVE are involved, also regulating the Arp2/3 complex and thus enabling the assembly of branched actin filaments (reviewed in Padrick and Rosen, 2010).

The adaptor protein Rols7 is also present in the visceral mesoderm in general and in the longitudinal FCs in particular. In *rols7* deficient embryos, the longitudinal FCs migrate correctly, but fuse time-delayed and less efficient at places where the cells lie farther away from each other (Chapter 7). Rols7 might connect cell adhesion via Duf and/or Rst to so far unknown downstream events in the longitudinal FCs and might enable the progress of fusion, perhaps via a positive feedback loop between Duf and Rols7, which was described in somatic FCs (Menon et al., 2005). As binucleated syncytia are detectable in both the somatic and visceral mesoderm of *rols7* mutants, the role of Rols7 in sustaining fusion beyond the bi- and trinucleated precursor stage appears to be the same in both mesodermal types of FCs.

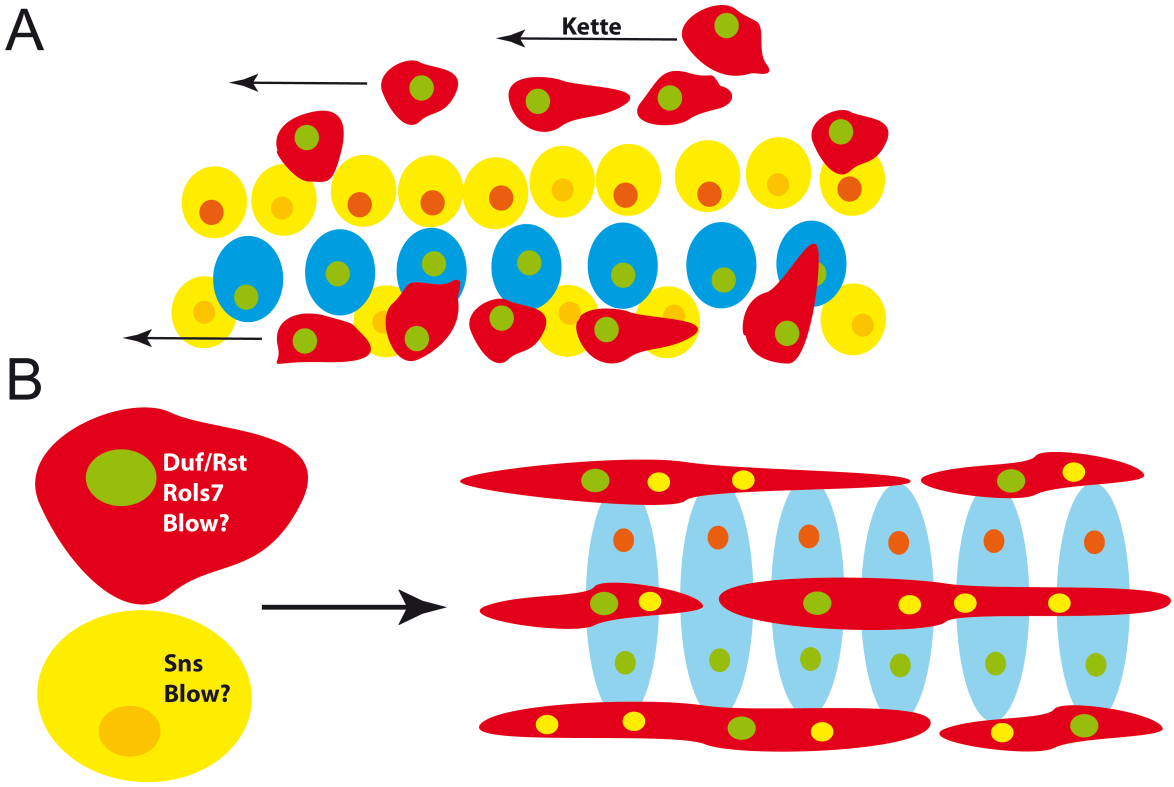

Figure 8.1: Schematic presentation of longitudinal muscle morphogenesis in *Drosophila* embryos. (A) Longitudinal FCs (red) with *rp298-LacZ* positive nuclei (green) migrate along the TVM in anterior direction (i.e., to the left). This process requires Kette. The TVM consists of *rp298-LacZ* positive circular FCs (blue), Bap positive (yellow with orange nuclei) and Bap negative (yellow with light orange nuclei) visceral FCMs. (B) The longitudinal FCs fuse with Bap negative FCMs, dependent on Duf/Rst and Rols7 in the FCs and Sns in the FCMs. Blow might be involved in one or in both cell types. After fusion, the stretching multinucleated longitudinal muscles are arranged perpendicular to the stretching binucleated circular muscles (light blue).

In the most supported working model, the longitudinal FCs migrate from the caudal visceral mesoderm (CVM) along the TVM, a process in which Kette is involved, presumably by regulating actin polymerization during cell migration. The FCs then fuse with Bap negative FCMs from the TVM, after binucleated circular muscles have been established (Fig. 8.1 A). Duf and/or Rst are present in this type of FCs, and might bind to Sns on the surface of the Bap negative FCM. On the side of the FC, Rols7 might be the adaptor protein for Duf and/or Rst. In FCs lying more closely to the FCMs, i.e. in the posterior part of the gut, fusion occurs without Rols7, comparable to the first phase of fusion in the somatic mesoderm. Furthermore, Blow is required for efficient fusion, in the longitudinal FCs, the visceral FCMs, or both, independently of D-WIP (Fig 8.1 B). The multinucleated longitudinal muscles then stretch perpendicularly to the circular muscles, until the network of both muscle types envelop the whole gut evenly at the end of embryogenesis.

8.4 References

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Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation

Cytoskeletal Components during Myogenesis of *Drosophila melanogaster:* Microtubules vs. Myosins as Actin Motor Proteins

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