

# The actin nucleator WASp is required for myoblast fusion during adult *Drosophila* myogenesis

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## SUMMARY

Myoblast fusion provides a fundamental, conserved mechanism for muscle fiber growth. We demonstrate here that the functional contribution of *Wsp*, the *Drosophila* homolog of the conserved actin nucleation-promoting factor (NPF) WASp, is essential for myoblast fusion during the formation of muscles of the adult fly. Disruption of *Wsp* function results in complete arrest of myoblast fusion in all muscles examined. *Wsp* activity during adult *Drosophila* myogenesis is specifically required for muscle cell fusion and is crucial both for the formation of new muscle fibers and for the growth of muscles derived from persistent larval templates. Although *Wsp* is expressed both in fibers and individual myoblasts, its activity in either one of these cell types is sufficient. SCAR, a second major Arp2/3 NPF, is also required during adult myoblast fusion. Formation of fusion-associated actin 'foci' is dependent on Arp2/3 complex function, but appears to rely on a distinct, unknown nucleator. The comprehensive nature of these requirements identifies Arp2/3-based branched actin polymerization as a universal mechanism underlying myoblast fusion.

**KEY WORDS:** Actin, *Drosophila*, Myoblast fusion, WASp

## INTRODUCTION

Myoblast fusion constitutes a fundamental aspect of muscle fiber growth, underlying the formation of multinucleated contractile units from pools of individual myoblasts (Rochlin et al., 2009). The musculature of adult *Drosophila* shares many morphological and developmental characteristics with vertebrate somatic muscles, thereby providing an appealing model system for the elucidation of universal molecular and cellular mechanisms governing myogenesis (Dutta et al., 2004; Fernandes and Keshishian, 1999). A prominent example is provided by the establishment of the thoracic indirect flight muscles (IFMs) of the adult fly. These large muscles, which mediate flight by contraction and expansion of the thoracic cuticle, are composed of several sets of bundled fibers, closely resembling vertebrate muscle organization.

Two distinct myogenic programs are employed during IFM formation. The first of these follows a plan similar to that which governs muscle development of the *Drosophila* embryo (Beckett and Baylies, 2006): single 'pioneer' or 'founder' myoblasts seed formation and differentiation of the mature fibers, which grow via repetitive rounds of fusion with neighboring myoblasts (Fernandes et al., 1991; Rivlin et al., 2000). This mode of myogenesis is common to most of the adult musculature, which has to form anew following destruction of nearly all somatic muscles of the *Drosophila* larva during the early stages of pupal development. A separate program is employed during construction of the twelve dorsal-longitudinal muscles (DLMs), which are prominent flight muscles that span the length of the thorax. In this case, a large proliferative population of migratory

myoblasts fuses with a set of persistent larval fibers that survives the general wave of histolysis, and serves as a template for the adult muscle structures (Fernandes et al., 1991; Roy and VijayRaghavan, 1998).

The different developmental modes leading to the formation of the adult *Drosophila* flight musculature thus present opportunities to examine both fusion between individual myoblasts and between myoblasts and maturing fibers. Although classic genetic approaches have proven to be a highly successful tool to study myoblast fusion during embryonic *Drosophila* myogenesis (Abmayr et al., 2008; Chen and Olson, 2004), their application to the study of fusion during adult fly muscle development has been limited. This is due both to functional requirements earlier in development for many of the genes potentially involved in myoblast fusion in the adult and to the syncytial nature of muscles, which restricts the usefulness of clonal analysis.

We have used a combination of genetic approaches to circumvent these difficulties and identify essential contributors to adult myoblast fusion. We report an essential requirement for *Wsp* (*WASp* – FlyBase), a member of the WASp family of actin nucleation-promoting factors (NPFs), which has previously been linked to myoblast fusion in *Drosophila* embryos (Kim et al., 2007; Massarwa et al., 2007; Schafer et al., 2007). *Wsp* is required for all forms of myoblast fusion that lead to the growth of adult somatic muscle fibers. In addition to *Wsp*, the SCAR (also known as WAVE) NPF and associated elements are also implicated in adult myoblast fusion. Furthermore, we describe transient F-actin structures that form in myoblasts near the onset of fusion, under the influence of the Arp2/3 actin polymerization machinery, but independently of the *Wsp* and SCAR nucleating systems. These findings underscore the significant roles played by the actin-based cytoskeleton in the myoblast fusion process (Onel and Renkawitz-Pohl, 2009; Richardson et al., 2008), and, in particular, generalize and accentuate the myogenic role of the WASp pathway.

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## MATERIALS AND METHODS

### *Drosophila* genetics

UAS-dsRNA constructs were commonly expressed together with UAS-*Dicer2* to enhance RNAi activity (Dietzl et al., 2007). Where necessary, the GAL80<sup>ts</sup>/TARGET system (McGuire et al., 2004) was used for temporal control of UAS-based transgene expression. Developing flies were maintained at 18°C, allowing for GAL80-based inhibition of GAL4 activity, and shifted (commonly at 0 hours APF) to 29°C, to inactivate the GAL80<sup>ts</sup> element.

### Mutant alleles

*Wsp*: *Wsp*<sup>1</sup> and Df(3R)3450 (Ben-Yaacov et al., 2001). *D-WIP* (*Vrp1* or *sltr*): *D-WIP*<sup>D30</sup> (Massarwa et al., 2007) and *sltr*<sup>S1946</sup> (Kim et al., 2007).

### GAL4 drivers

*1151-GAL4* (myoblasts) (Roy and VijayRaghavan, 1997); *rp298 (duf)-GAL4* (founder cells) (Menon and Chia, 2001); *Mef2-GAL4* (myogenic mesoderm) (Ranganayakulu et al., 1996).

### UAS-*Wsp* constructs

UAS-*Wsp*, UAS-*Wsp*-GFP, UAS-*Wsp*ΔCA and UAS-*Wsp*<sup>myr</sup> have been described previously (Ben-Yaacov et al., 2001; Bogdan et al., 2005; Massarwa et al., 2007; Tal et al., 2002).

### Other UAS-based transgenes

UAS-RedStinger (nuclear marker) (Barolo et al., 2004); UAS-my-mRFP (membrane marker) (Kandachar et al., 2008); UAS-*Moesin*-GFP (microfilament marker) (Chihara et al., 2003; Dutta et al., 2002); UAS-Rac1<sup>DN</sup> (T17N, a dominant-negative version of Rac1) (Luo et al., 1994); UAS-*Arp3*-GFP and UAS-*Sop2*-GFP (Hudson and Cooley, 2002).

### UAS-dsRNA lines

*Arp2* (JF02785); *kette* (JF03342); *SCAR* (JF01599); *Sop2* (GD42172); *D-WIP* (GD32888); *Wsp* (GD13759). JF lines are from the TRiP (Harvard) collection and GD lines are from the VDRC (Vienna) collection.

### Other lines

MHC-TauGFP (GFP expression in muscles driven by MHC regulatory sequences) (Chen et al., 2003); Actin 88F-lacZ (β-galactosidase expression in adult flight muscles driven by regulatory sequences from the IFM-specific *Actin 88F*) (Hiromi et al., 1986).

### Tissue preparation and histology

Dissected muscle preparations were obtained from staged pupae (collected as white pre-pupae at 0 hours APF). Pupae of the desired age were removed from the pupal case, pinned down on Sylgard plates and dissected in cold PBS (see also Fernandes et al., 1991). Fixation was carried out with 4% paraformaldehyde (PFA) for 30 minutes. Following washes in PBS, tissue was incubated with antibodies diluted in PBS containing 0.1% Triton X-100 and 0.1% bovine serum albumin as blocking reagent. Stained pupal preparations were mounted in 70% glycerol.

Primary antibodies used included: Mab 22C10 (mouse, 1:50, Developmental Studies Hybridoma Bank); anti-Ewg [rabbit, 1:1000 (DeSimone and White, 1993)]; anti-Twist (rabbit, 1:5000, kindly provided by S. Roth, University of Cologne); anti-*Wsp* [rabbit, 1:100 (Ben-Yaacov et al., 2001)]; anti-*D-WIP* [guinea pig, 1:500 (Berger et al., 2008)]; anti-GFP (chicken, 1:500, Aves); anti-DsRed (rabbit, 1:500, Clontech); and anti-β-galactosidase (chicken, 1:200, Abcam). Secondary antibodies conjugated to Alexa Fluor 488 and 568 (Molecular Probes) were used at 1:200. Rhodamine-phalloidin (Molecular Probes) was used at 1:200.

For histological sections, thoraces of staged 96 hour APF pupae were separated from the head and abdomen, fixed in 4% PFA overnight, dehydrated in an alcohol series and embedded in JB-4 (Electron Microscopy Sciences). Sections (10 μm) were cut using a microtome, stained with Toluidine Blue and mounted in DPX (Fluka).

### Immunofluorescence and microscopy

Fluorescent images of the (GFP-labeled) thoracic musculature of live pupae were collected using a Leica MZ16 F stereomicroscope equipped with a Nikon Digital Sight camera. Images of immunofluorescent samples were acquired using Zeiss LSM 510 and Olympus FV1000 confocal scanning

systems and processed using Adobe Photoshop CS3. Area determination of actin foci was performed as previously described (Gildor et al., 2009; Richardson et al., 2007).

## RESULTS

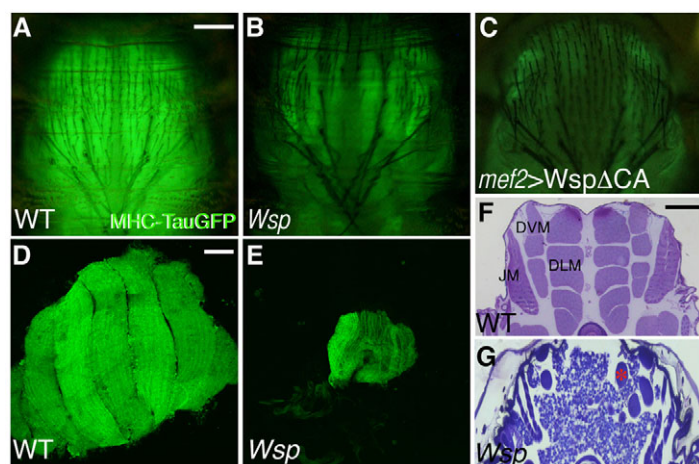
### Adult *Wsp* mutant flies possess poorly developed flight muscles

Although *Wsp* performs essential roles throughout *Drosophila* development, flies lacking zygotic *Wsp* function survive until late pupal/early adult stages because maternally contributed *Wsp* gene products are sufficient for proper embryonic and larval development. The external morphology of *Wsp* mutant flies is generally normal at the time of eclosion, except for the absence of many mechanosensory bristles (Ben-Yaacov et al., 2001). To assess whether myogenesis proceeds properly in these flies, we incorporated the muscle-specific reporter construct MHC-TauGFP (Chen and Olson, 2001) into a *Wsp* mutant background. In contrast to the very bright GFP signal normally provided by the substantial flight musculature housed within the adult fly thorax, *Wsp* mutant flies displayed a faint signal indicative of under developed or missing muscles (Fig. 1A,B). To verify that the defects in adult muscle organization arose during pupal development, we made use of *Wsp*ΔCA, a dominant-negative construct that lacks the crucial C-terminal Arp2/3-binding domain of *Wsp* and has been shown to strongly disrupt *Wsp* function during embryonic myoblast fusion (Massarwa et al., 2007; Tal et al., 2002). Limiting expression of UAS-*Wsp*ΔCA to muscle tissue only, following the onset of pupariation, was achieved by using the myogenic driver *Mef2-GAL4* (Ranganayakulu et al., 1996) in combination with the GAL80<sup>ts</sup>/TARGET system for temporally restricted GAL4 activity (McGuire et al., 2004). Such flies were able to eclose but were incapable of flight and displayed a general reduction of thoracic muscle mass, similar to that seen in the *Wsp* zygotic mutants (Fig. 1C). These observations are consistent with a specific requirement for *Wsp* during adult muscle development.

We initially chose to concentrate on the DLMs, the most prominent class of *Drosophila* thoracic flight muscles. In the adult fly, the DLMs comprise two sets of six massive groups of fibers. Sets of DLMs dissected from *Wsp* pupae were unusually small and lacked coherent organization (Fig. 1D,E). These abnormalities were accentuated in matched histological sections of wild-type and *Wsp* pupal thoraces (Fig. 1F,G). Striking differences in both the size and organization of the DLM patterns between late wild-type and *Wsp* pupae are thus readily apparent, consistent with the general reduction in adult muscle mass observed in *Wsp* mutant flies.

### The *Wsp* flight muscle defects result from failure of myoblast fusion

To determine the basis for the abnormalities in *Wsp* mutant flight muscle structure, we followed their formation during the early stages of pupal development. The adult DLMs develop from sets of three mesothoracic larval myofibers, which survive the wave of histolysis that initiates in the early pupa and destroys almost all of the musculature generated during the embryonic and larval stages (Fernandes et al., 1991). Sets of DLM progenitor fibers of normal shape and size were found in *Wsp* pupae 12 hours after puparium formation (APF), demonstrating that the templates upon which the adult DLMs are constructed form appropriately in the mutants (Fig. 2A,B). The extensive growth of the DLM fibers initiates at ~12 hours APF and is accomplished by fusion of the persistent DLM progenitors with numerous individual myoblasts, which migrate to the thorax from their original position near the wing imaginal disc



**Fig. 1. Defective musculature of *Wsp* mutant flies.**

(A–C) Thoracic flight muscles of intact, live pupae close to eclosion, visualized with MHC-TauGFP. (A) Robust musculature of a wild-type (WT) *Drosophila* pupa. (B,C) Poorly developed flight muscles are characteristic of *Wsp*<sup>1</sup>/Df(3R)3450 pupae (B) and of pupae in which the dominant-negative construct UAS-*Wsp*ΔCA is expressed in myogenic tissue only (via the *Mef2*-GAL4 driver) following the onset of pupariation (C). (D,E) Dissected sets of MHC-TauGFP-expressing DLMs from hemithoraces of wild-type (D) and *Wsp*<sup>1</sup>/Df(3R)3450 (E) pupae at 72 hours APF. (F,G) Transverse sections through pupal thoraces counterstained with Toluidine Blue. Large groups of bundled flight muscle fibers are present in wild-type pupae (F). Major muscle groups are indicated: DLM, dorsal longitudinal flight muscles; DVM, dorsoventral flight muscles; JM, leg jump muscle. Thoracic muscle bundles are markedly absent from *Wsp*<sup>1</sup>/Df(3R)3450 pupae (G), in which only a few thin groups of larval flight muscle templates remain (asterisk). Scale bars: 200 μm in A; 50 μm in D; 100 μm in F.

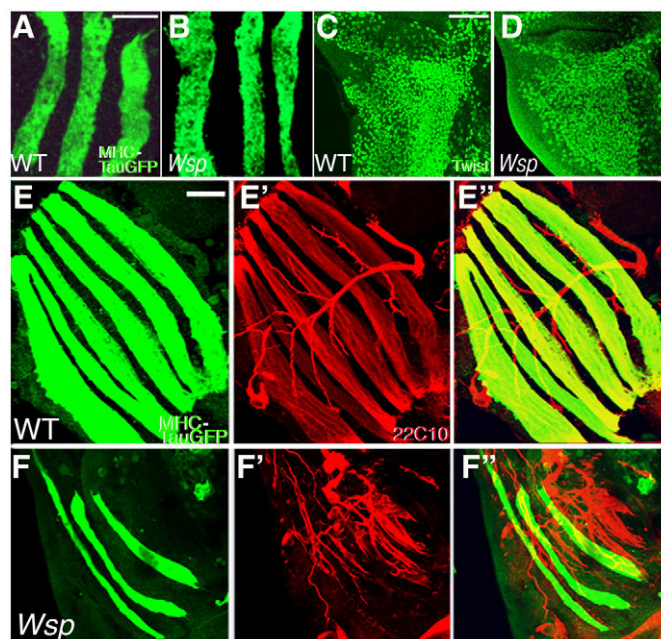
(Bate et al., 1991; Fernandes et al., 1991). Visualization using the myoblast-specific marker Twist demonstrated that *Wsp* mutants harbor a normally sized array of proliferating wing disc-associated myoblasts (Fig. 2C,D). Taken together, these observations imply that the two cell populations from which DLM fibers are constructed – larval template fibers and myoblasts – undergo normal specification and morphogenesis in *Wsp* zygotic mutants.

Once initiated, the process of DLM growth involves multiple rounds of template-myoblast cell fusion and is accompanied by splitting of the fibers, generating a mature fiber set by ~24 hours APF (Fernandes et al., 1991; Roy and VijayRaghavan, 1998). Examination of the DLMs at this stage revealed dramatic morphological differences between the wild-type thoracic muscles and those that form in *Wsp* mutants (Fig. 2E–F’). As readily observed by monitoring the expression of MHC-TauGFP, *Wsp* DLMs did not increase in size and failed to split (Fig. 2E,F). Thus, each *Wsp* hemithorax contained three immature fibers at 24 hours APF, in contrast to the enlarged six-fiber set of corresponding wild-type DLMs. Staining for 22C10 (Futsch, a microtubule-associated protein expressed in both mature DLM fibers and peripheral nerves (Dutta et al., 2004; Hummel et al., 2000a), underscored the poor differentiation of DLMs in *Wsp* mutants, which were nonetheless innervated.

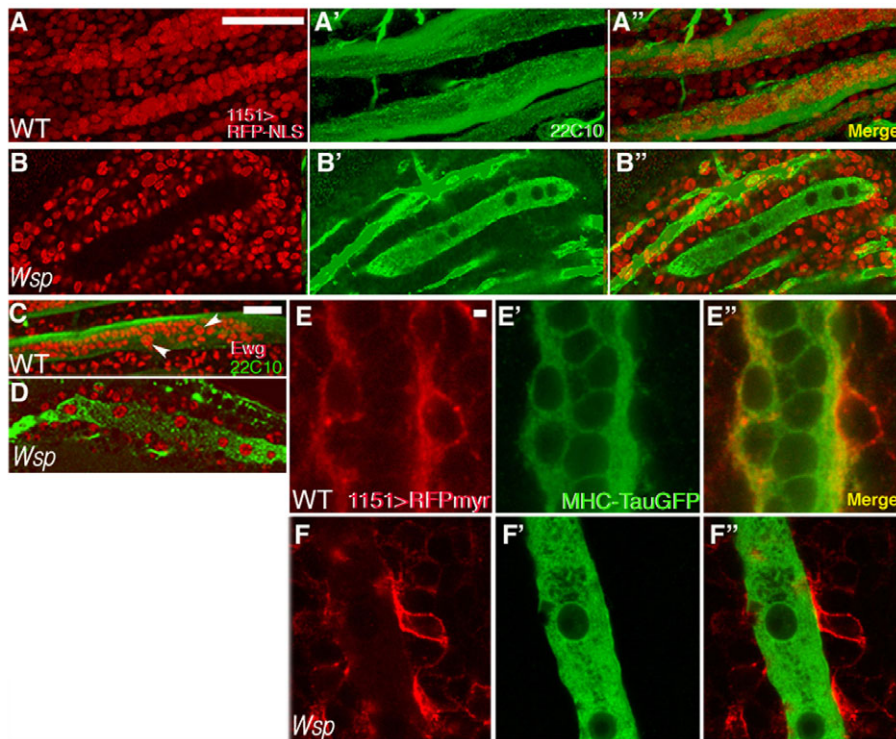
The observations described above define the period of 12–24 hours APF as a time window during which the absence of zygotic *Wsp* function leads to abnormal flight muscle formation. We therefore observed, simultaneously, the developing DLM fibers and the population of myoblasts that contributes to DLM growth during this period (Fig. 3). Fibers were viewed using template-specific markers and myoblasts were visualized with the aid of *1151*-GAL4, a myoblast-specific GAL4 driver (Roy and VijayRaghavan, 1997). An initial experiment utilized *1151*-GAL4-based expression of UAS-RedStinger (Barolo et al., 2004), an RFP variant that localizes to the nucleus (Fig. 3A–B’). Wild-type DLMs at 18 hours APF contained many RFP-expressing nuclei (Fig. 3A–A’), reflecting the massive incorporation of myoblasts into the larval templates via cell fusion. In stark contrast, the DLMs in age-matched *Wsp* pupae, although surrounded by myoblasts, were completely devoid of RFP-expressing nuclei and retained their larval stage morphology and size (Fig. 3B–B’).

To provide an alternative fusion assay, we made use of the transcription factor Erect wing (Ewg), which localizes to the nuclei of both larval muscle templates and fusing myoblasts (DeSimone et al., 1996; Fernandes and Keshishian, 1996). At 18 hours APF,

wild-type DLMs are filled with many Ewg-positive nuclei (Fig. 3C). Most of these are myoblast-derived, whereas a handful of larger, polyploid nuclei represent the original larval set (Roy and VijayRaghavan, 1998). DLMs from 18 hour APF *Wsp* mutant pupae contained only  $9 \pm 2.2$  ( $n=8$ ) large nuclei (Fig. 3D), which is similar to the established number (8–13) of template nuclei prior to the onset of fusion (Dutta et al., 2004) and indicative of a complete arrest of myoblast fusion in *Wsp* mutants. Failure of fusion between



**Fig. 2. Flight muscle defects in *Wsp* mutants arise during early pupal development.** (A,B) DLM templates expressing MHC-TauGFP at 12 hours APF. Templates in *Wsp*<sup>1</sup>/Df(3R)3450 *Drosophila* pupae (B) resemble those found in wild-type pupae (A). (C,D) Dissected third instar larval wing imaginal discs stained for the nuclear myoblast marker Twist. A large population of myoblasts is found throughout the notum of both wild-type (C) and *Wsp*<sup>1</sup>/Df(3R)3450 (D) discs. (E–F’’) Visualization of DLMs at 24 hours APF in wild-type (E–E’’) and *Wsp*<sup>1</sup>/Df(3R)3450 (F–F’’) pupae via MHC-TauGFP expression (green) and staining with Mab 22C10 (red). Whereas the wild-type fiber set splits in two and grows in size to yield six mature fibers in each hemithorax, the underdeveloped DLM templates of *Wsp*<sup>1</sup>/Df(3R)3450 pupae remain thin and fail to split. Scale bars: 50 μm.



**Fig. 3. Failure of fusion between template fibers and surrounding myoblasts in *Wsp* mutant pupae. (A–B'')** Myoblast nuclei (red, visualized by 1151-GAL4>UAS-RFP-NLS/RedStinger) and DLM fibers (green, visualized with Mab 22C10) in wild-type (A–A'') and *Wsp*<sup>1/Df(3R)3450</sup> (B–B'') *Drosophila* pupae at 18 hours APF. Wild-type DLMs contain many myoblast nuclei, whereas the immature fibers in *Wsp* mutant pupae have not incorporated any myoblast nuclei. The black circular figures within these fibers correspond to the enlarged polyploid nuclei of larval muscles (Dutta et al., 2004). (C,D) DLM preparations from wild-type (C) and *Wsp*<sup>1/Df(3R)3450</sup> (D) pupae at 18 hours APF, stained with anti-Ewg (red) and Mab 22C10 (green). Arrowheads (C) point to the large, persisting larval nuclei. Such nuclei are the only ones present in *Wsp* mutant fibers (D). (E–F'') Myoblasts (red, visualized by 1151-GAL4>UAS-myr-mRFP) and DLM fibers (green, visualized with MHC-TauGFP) in wild-type (E–E'') and *Wsp*<sup>1/Df(3R)3450</sup> (F–F'') pupae at 18 hours APF. Incorporation of the membrane marker into DLM membranes, as is observed in wild type, does not appear to take place in *Wsp* mutants. Scale bars: 50 μm in A,C; 2 μm in E.

myoblasts and the DLM templates was further illustrated by myoblast-specific expression of UAS-myr-mRFP (Kandachar et al., 2008), a membrane-tethered RFP construct (Fig. 3E–F''). Incorporation of the myoblast-derived myr-mRFP into wild-type DLM membranes was readily apparent at 18 hours APF (Fig. 3E–E''), whereas no such incorporation was observed in *Wsp* mutant pupae, despite the close apposition of myoblasts to the template fibers (Fig. 3F–F'').

Taken together, these phenotypes imply that *Wsp* myoblasts migrate from the imaginal wing disc and attach to the DLM fibers, but that the fibers and adhered myoblasts fail to fuse. The adult flight muscle abnormalities characteristic of zygotic *Wsp* mutants therefore result from a specific and apparently complete disruption of myoblast fusion capabilities in these flies.

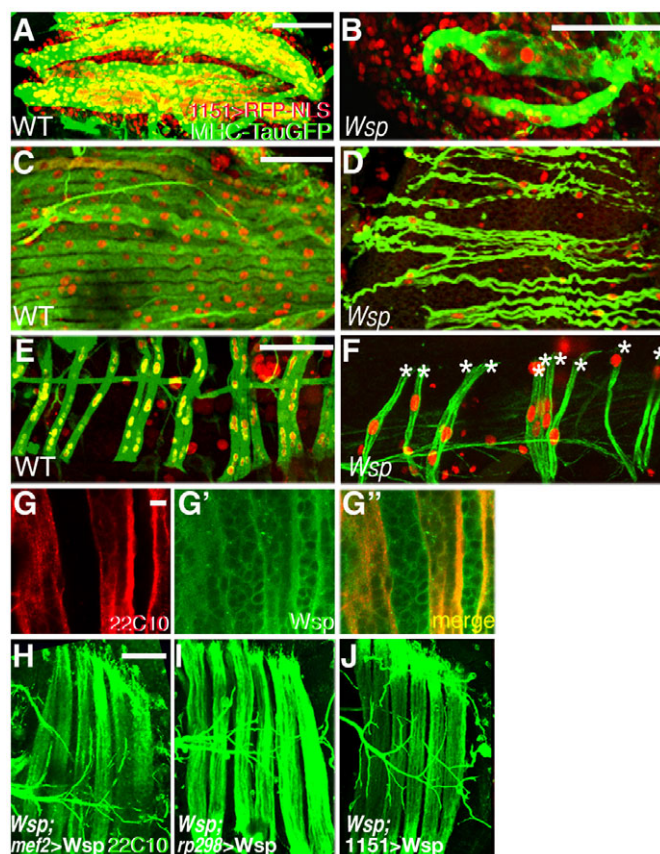
### ***Wsp* is a general mediator of adult myoblast fusion, acting in both muscle fibers and myoblasts**

Whereas DLM fiber growth is based on fusion between wing disc-associated myoblasts and persistent larval templates, most adult *Drosophila* muscles are formed by an alternative ('de novo') myogenic program, in which individual founder or pioneer myoblasts seed formation of fibers via fusion with neighboring myoblasts (Dutta et al., 2004; Fernandes and Keshishian, 1999). Prominent examples include the thoracic dorsoventral muscles (DVMs), which constitute a second major set of IFMs, and the various groups of abdominal muscles, which are derived from nerve-associated myoblasts (Currie and Bate, 1991; Fernandes and Keshishian, 1999; Rivlin et al., 2000; Atreya and Fernandes, 2008). We monitored the development of these different muscle types in *Wsp* mutant pupae and observed severe defects in the construction of all muscles examined (Fig. 4A–F). *Wsp* DVMs were considerably smaller than their wild-type counterparts and contained a minimal number of nuclei, as opposed to the dozens of nuclei present within the wild-type muscles (Fig. 4A,B).

Significantly, these muscles displayed established morphological characteristics of DVM founder myoblasts, including an elongated shape and an enlarged nucleus (Fernandes et al., 2005; Rivlin et al., 2000), consistent with the proper initiation of their specialized myogenic differentiation program, followed by arrest at the onset of fiber growth via fusion.

Since DVMs in *Wsp* mutants were often difficult to identify, we examined the abdominal muscles as a more reliable preparation for quantification of fusion events during de novo muscle formation. Both lateral and dorsal abdominal muscles were similarly underdeveloped in *Wsp* mutant pupae (Fig. 4C–F). Both abdominal muscle types were properly arranged in parallel arrays, but the fibers that formed were very thin and commonly contained only a single nucleus ( $1.1 \pm 0.3$ ,  $n=10$  dorsal muscle nuclei), in contrast to the thicker, multinucleated ( $9.7 \pm 1.8$ ,  $n=13$ ) wild-type fibers. These observations suggest that, as for the thoracic flight muscles, the specification and differentiation aspects of the adult abdominal myogenic program are properly initiated in *Wsp* zygotic mutants, but that fiber growth and maturation are arrested owing to an inability to incorporate myoblasts through cell fusion.

The fusion events that underlie all programs of muscle growth in *Drosophila* occur between distinct cell types, namely growing syncytial fibers and individual myoblasts. The employment of separate genetic programs by fibers and myoblasts is a general, established feature of myogenesis (Buckingham et al., 2003; Estrada et al., 2006), raising the issue of a differential mechanistic contribution to the fusion process by the pairs of fusing cells (Chen and Olson, 2004). We therefore sought to determine whether any muscle cell type bias exists in the utilization of *Wsp* during pupal muscle cell fusion. We chose to study this issue during growth of the DLM fibers, as fibers and myoblasts are clearly defined in this muscle system from the outset. We first used anti-*Wsp* antibodies to assess the expression pattern of *Wsp* protein in developing DLM flight muscles, and observed a general



**Fig. 4. Wsp function is required in all fusing muscles and is sufficient when provided from either myofibers or myoblasts.** (A–F) DVM thoracic flight muscles at 24 hours APF (A,B), and lateral (C,D) or dorsal (E,F) abdominal muscles at 48 hours APF of wild-type (A,C,E) and *Wsp*<sup>1/Df(3R)3450</sup> (B,D,F) *Drosophila* pupae. Fibers are visualized with MHC-TauGFP and myoblast nuclei are visualized using 1151-GAL4>UAS-NLS-RFP/RedStinger. DVMs and abdominal muscles of *Wsp* mutant pupae assume characteristic morphologies but fail to fuse with surrounding myoblasts. Asterisks (F) mark individual muscle fibers. (G–G'') Maturing DLMs at 18 hours APF stained with Mab 22C10 (red) to mark the larval templates and with anti-Wsp (green). Wsp expression is observed in both growing fibers and adjacent myoblasts. (H–J) Rescue of the *Wsp* mutant DLM defects, as visualized by Mab 22C10, can be achieved by driving UAS-*Wsp* in all muscle cells using *Mef2*-GAL4 (H) as well as by myofiber-specific expression using *rp298*-GAL4 (a GAL4 insertion in the *duf* locus) from 0 hours APF (I), or by myoblast-specific expression obtained with the myoblast driver 1151-GAL4 (J). Scale bars: 50 μm in A–C, E, H; 5 μm in G.

distribution in both the growing fibers and the adjacent myoblasts (Fig. 4G–G''), suggesting that both muscle cell types could serve as a source of Wsp.

To address the possibility of a functional bias, we used different muscle cell type GAL4 drivers to express UAS-*Wsp* in a *Wsp* mutant background. Phenotypic rescue of the *Wsp* DLM mutant phenotype was readily achieved using the pan-myogenic *Mef2*-GAL4 driver (Fig. 4H). Rescue of the mutant phenotype was similarly obtained (Fig. 4I) when *Wsp* was specifically expressed in the persistent larval templates, under *rp298* (*duf*)-GAL4 (also known as *kirre* – FlyBase) (Menon and Chia, 2001). Although a previous detailed study concluded that this driver is not active in myoblasts (Atreya and Fernandes, 2008), we again used the GAL80<sup>ts</sup>/TARGET system (McGuire et al., 2004) to ensure

restriction of GAL4 activity to pupal stages (see Fig. S1 in the supplementary material). Robust rescue of DLM growth in *Wsp* mutant pupae was similarly observed following expression of *Wsp* via the myoblast-specific driver 1151-GAL4 (Fig. 4J). These experiments therefore suggest that Wsp activity in either fibers or myoblasts is sufficient to ensure fusion-based growth of DLM muscles.

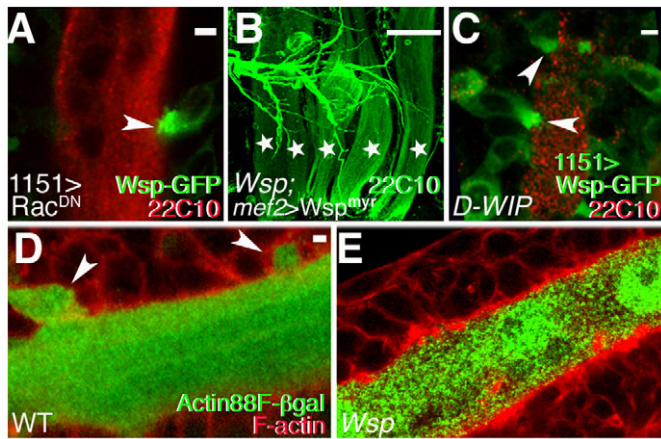
Taken together, the *Wsp* fusion-arrest mutant phenotype observed in all pupal muscles, the general expression pattern of endogenous Wsp protein, and the full functionality of *Wsp* regardless of cell type source, imply that *Wsp* provides a general fusion-related function that is common among myogenic cells. Furthermore, the ability to obtain full rescue when supplying Wsp from either of the fusion partners is likely to have important mechanistic implications.

### Wsp acts at adult myoblast cell membranes prior to fusion pore formation

Arp2/3-based actin polymerization in cells commonly takes place in the vicinity of the plasma membrane, and the forces generated by this activity mediate a variety of membrane-associated processes (Takenawa and Suetsugu, 2007). To monitor the subcellular localization pattern of Wsp in fusing myoblasts, we expressed a functional Wsp-GFP fusion protein (Massarwa et al., 2007) together with a dominant-negative form of the small GTPase Rac1 (Rac<sup>DN</sup>), the expression of which in myoblasts leads to the arrest of muscle cell fusion (Dutta et al., 2004; Fernandes et al., 2005). Such an approach is commonly employed in studies of embryonic myoblast fusion to allow for the accumulation of fusion-related elements at their sites of activity (Galletta et al., 2004; Massarwa et al., 2007; Menon et al., 2005). A prominent concentration of Wsp-GFP was observed at the myoblast attachment sites in 1151-GAL4>UAS-Rac<sup>DN</sup> pupae (Fig. 5A), suggesting that Wsp localizes normally to the membrane regions that are actively involved in fusion. Furthermore, expression in myogenic cells of Wsp<sup>myr</sup>, a plasma membrane-tethered form of Wsp (Bogdan et al., 2005), substantially rescued the *Wsp* mutant phenotype, underscoring the functional significance of targeting Wsp to muscle cell membranes (Fig. 5B).

D-WIP (Vrp1 or Sltr), the sole *Drosophila* member of the WIP protein family (Moreau et al., 2000; Sasahara et al., 2002), acts in a membrane recruitment capacity to mediate the involvement of Wsp in embryonic myoblast fusion (Kim et al., 2007; Massarwa et al., 2007). Furthermore, disruption of D-WIP function during pupal development generates a *Wsp*-like fusion-arrest phenotype (see Fig. S2 in the supplementary material). To assess whether D-WIP is required for fusion site localization of Wsp, we used the 1151-GAL4 driver to express Wsp-GFP in the *D-WIP* mutant background. Surprisingly, Wsp-GFP remained prominently localized to the membrane attachment sites between myoblasts and immature DLM fibers (Fig. 5C), suggesting that Wsp accumulates at these sites independently of D-WIP function. Taken together, these observations imply an essential role for D-WIP function during adult myoblast fusion, which is somewhat distinct, however, from its embryonic role as a Wsp-recruiting element.

The demonstration of a conserved requirement for *Wsp* function during adult myoblast fusion raises the issue of the specific cellular tasks that this element performs. Observations made on *Wsp* mutant embryos have suggested a role in fusion pore expansion, as fusion arrests at a relatively late stage, following the formation of nascent fusion pores (Berger et al., 2008; Gildor et al., 2009; Massarwa et al., 2007). To address the possibility that this role is



**Fig. 5. Wsp acts at fusing myoblast cell membranes prior to fusion pore initiation.** (A) In 1151-GAL4>Rac<sup>DN</sup> *Drosophila* pupae, Wsp-GFP accumulates at the interface (arrowhead) between fusion-arrested myoblasts and larval templates (red, Mab 22C10). (B) Expression of Wsp<sup>myr</sup>, a membrane-tethered form of Wsp, almost fully restores the normal DLM pattern (green, Mab 22C10) in Wsp<sup>1</sup>/Df(3R)3450 pupae. Individual DLMs are marked with stars. (C) Wsp-GFP (green) accumulates at attachment sites (arrowheads) between fusion-arrested myoblasts and larval templates (red, Mab 22C10) in a fusion-arrested *D-WIP*<sup>D30/sltr</sup><sup>51946</sup> pupa. (D,E) Lack of cytoplasmic transfer in *Wsp* mutant pupae. (D)  $\beta$ -galactosidase (green), specifically expressed in DLM templates by the *Actin 88F* enhancer trap, transfers and accumulates in attached myoblasts (arrowheads) upon the onset of fusion. Myoblast contours (cortical F-actin) are visualized with phalloidin (red). (E) No such transfer is observed in the many fusion-arrested myoblasts attached to the DLM templates of Wsp<sup>1</sup>/Df(3R)3450 pupae. Scale bars: 2  $\mu$ m in A,C,D; 50  $\mu$ m in B.

conserved during adult myoblast fusion, we monitored cytoplasmic transfer between myoblasts as a criterion for the stage at which the fusion process is impaired. We made use of the *Actin 88F-lacZ* enhancer trap transgene, in which  $\beta$ -galactosidase ( $\beta$ -gal) expression is limited to growing fibers, but is absent from individual myoblasts (Fernandes et al., 1991; Hiromi et al., 1986). Close examination of wild-type *Actin 88F-lacZ* pupae during DLM maturation revealed incorporation of  $\beta$ -gal into a small population of attached myoblasts (Fig. 5D), demonstrating that cytoplasmic transfer between partially fused cells could be detected in this setting. Transfer of this type was never observed, however, in *Wsp* mutant pupae bearing *Actin 88F-lacZ*, as none of the numerous unfused myoblasts attached to the persistent templates appeared to contain  $\beta$ -gal (Fig. 5E). These observations imply that the fusion process in *Wsp* mutant pupae arrests before the initiation of cytoplasmic continuity.

### The Arp2/3 nucleation-promoting factor SCAR is required for adult myoblast fusion

WASP family proteins act as NPFs, stimulating the capacity of the conserved Arp2/3 protein complex to nucleate actin polymerization and generate branched microfilament arrays (Goley and Welch, 2006). Metazoan cells commonly employ WASP family proteins and the related SCAR elements as the major Arp2/3 NPFs (Pollitt and Insall, 2009). Although the two classes of NPFs share a common mode of interaction with the Arp2/3 complex, they recognize and are activated by distinct molecular machineries (Derivery and Gautreau, 2010). *Drosophila* possesses single

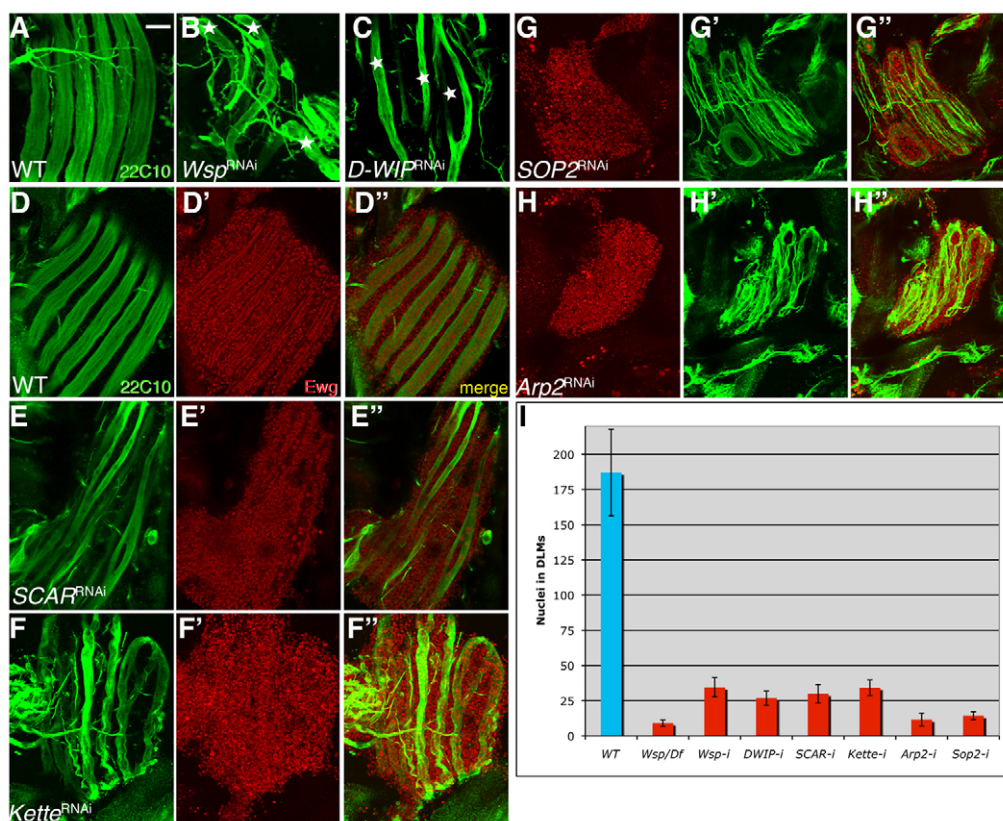
homologs for WASP (Wsp) and SCAR/WAVE (SCAR), which generally operate in separate developmental and cellular settings (Zallen et al., 2002). Embryonic myoblast fusion is exceptional in this regard, as both NPFs have been ascribed essential roles in the fusion process (Kim et al., 2007; Massarwa et al., 2007; Richardson et al., 2007; Schafer et al., 2007; Schroter et al., 2004). Direct comparisons, however, reveal separate and temporally distinct roles for the two Arp2/3 NPFs in which SCAR acts prior to Wsp, raising questions regarding the mechanistic basis by which such functional distinctions are achieved (Berger et al., 2008; Gildor et al., 2009). To determine the functional interplay of these factors during the establishment of adult *Drosophila* muscles, we first sought to complement the above analysis of *Wsp* mutants by disrupting the activity of *SCAR* and its associated molecular machinery in developing pupae.

Unlike *Wsp*, the maternal contribution of *SCAR* and related elements is not sufficient to overcome zygotic requirements during embryonic and larval stages. We therefore chose to pursue restricted tissue and developmental stage expression of UAS-based RNA-interference (RNAi) transgenes (Dietzl et al., 2007; Schnorrer et al., 2010), as an alternative method of disrupting gene activity during establishment of the adult fly musculature. To verify the usefulness of this approach in the context of adult myoblast fusion, we first assessed the effects of expressing UAS-*Wsp*-RNAi and UAS-*D-WIP*-RNAi constructs using the muscle-specific *Mef2*-GAL4 driver. Severe myogenic phenotypes resembling those obtained with loss-of-function alleles were observed in both cases (Fig. 6A-C). Incorporation of UAS-*Dicer2*, an established tool for enhancing RNAi activity (Dietzl et al., 2007), proved useful in this context as it led to more severe and consistent defects in flight muscle formation. We therefore applied this protocol to examine the involvement of SCAR and associated elements in adult myogenesis.

Although a number of transgenic RNAi constructs directed against *SCAR* and functionally associated elements failed to elicit an effect, *Mef2*-GAL4-based expression of a *SCAR*-RNAi construct from the potent VALIUM series (Ni et al., 2008; Ni et al., 2009) strongly disrupted flight muscle development, generating a characteristic myoblast fusion-arrest phenotype of unfused myoblasts congregated around thin, underdeveloped DLM templates (Fig. 6D-E'). Such myogenic defects (Fig. 6F-F') were also observed following similar utilization of a VALIUM series RNAi construct targeting *kette*, which encodes the conserved Nap1 (Hem - FlyBase) component of the SCAR regulatory complex (Hummel et al., 2000b). Zygotic loss-of-function mutations in this element result in a strong arrest of embryonic myoblast fusion and have been used as a major tool in the study of *SCAR* function in this context (Richardson et al., 2007; Schroter et al., 2004). The specificity of the *kette*-RNAi construct was verified by restoration of normal myogenesis following co-expression of UAS-*kette* (see Fig. 6). As expected, strong fusion-arrest phenotypes were observed following RNAi-based targeting of *Sop2* and *Arp2* (Hudson and Cooley, 2002), which encode two subunits of the Arp2/3 complex (Fig. 6G-H'), the downstream target of both the Wsp and SCAR NPFs. Quantification of DLM nuclear content (Fig. 6J) underscored the strong inhibition of myoblast fusion by RNAi targeting the Arp2/3 complex or either of its major NPF systems.

### Arp2/3, but neither of its major NPFs, mediates formation of fusion-associated actin foci

The demonstrated involvement of actin polymerizing factors during adult myoblast fusion implies the formation of microfilament structures that contribute to the fusion process. To

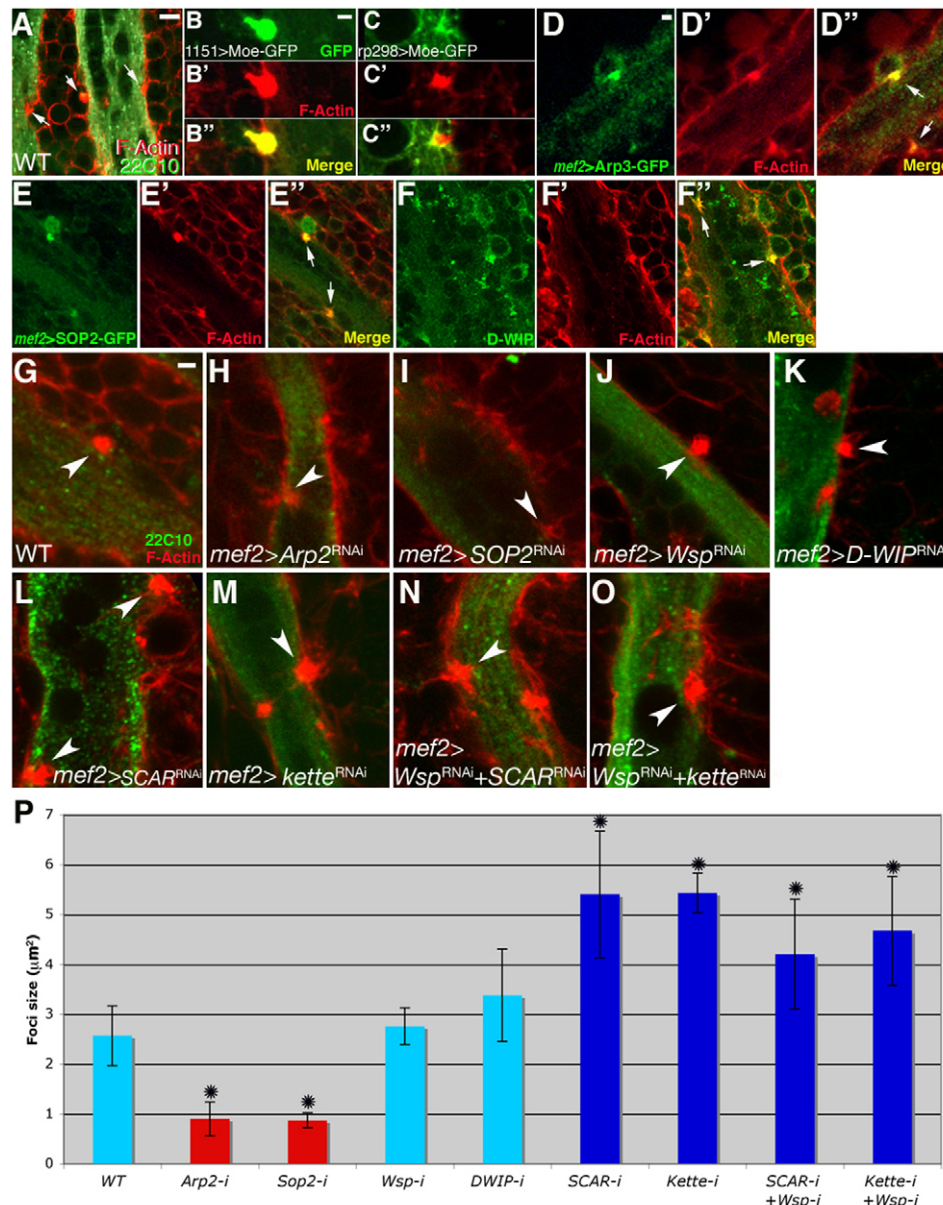


**Fig. 6. Muscle-specific RNAi expression reveals a requirement for the SCAR NPF in adult myoblast fusion.** (A–C) Dissected *Drosophila* DLMs at 24 hours APF visualized with Mab 22C10. DLM templates (stars) fail to grow and split following expression of UAS-RNAi targeted against *Wsp* (B) and *D-WIP* (C) via *Mef2*-GAL4. (D–H'') DLMs at 24 hours APF following *Mef2*-GAL4-based expression of transgenic UAS-RNAi constructs. Fibers are visualized with Mab 22C10 (green) and nuclei with anti-Ewg (red). Ewg is expressed in all nuclei within the fibers as well as in nuclei of surrounding myoblasts. The wild-type images (D–D'') represent the driver on its own. RNAi constructs used were targeted against *SCAR* (E–E''), *kette* (F–F''), *Sop2* (G–G'') and *Arp2* (H–H''). (I) The extent of DLM fusion was quantified by counting Ewg-positive nuclei within single DLM fibers at 24 hours APF. Wild-type fibers contain  $187 \pm 30.7$  nuclei ( $n=4$ ). Quantification of Ewg-positive nuclei within DLMs of *Wsp*<sup>1/Df(3R)3450</sup> mutant pupae serves as the measure of fiber nuclear content in the complete absence of fusion (see also Fig. 3D). Fusion is strongly inhibited following expression in muscles of *D-WIP*-RNAi ( $26.8 \pm 5$  nuclei,  $n=10$ ), *Wsp*-RNAi ( $34.5 \pm 6.8$  nuclei,  $n=8$ ), *SCAR*-RNAi ( $29.9 \pm 6.6$ ,  $n=10$ ), *kette*-RNAi ( $34.2 \pm 5.6$ ,  $n=10$ ), *Arp2*-RNAi ( $11.6 \pm 4.7$ ,  $n=9$ ) and *Sop2*-RNAi ( $14.3 \pm 2.8$ ,  $n=6$ ). Co-expression of UAS-*kette* (Hummel et al., 2000b) together with the *kette*-RNAi construct significantly rescues the fusion defect ( $129.1 \pm 13.5$  nuclei,  $n=9$ ), verifying the specificity of the RNAi construct. The minimal degree of fusion observed for all RNAi transgenes, including the *Wsp*-targeting construct, attests to the difficulty of obtaining complete disruption of gene activity by this approach. Error bars indicate s.d. Scale bar: 50  $\mu$ m.

try to identify such structures, we monitored the subcellular distribution of phalloidin-stained microfilaments during DLM formation. This analysis revealed that single, prominent, spherical F-actin structures form at the interface between DLM fibers and attached myoblasts, on the verge of fusion (Fig. 7A). We refer to these structures as actin ‘foci’, a term used to describe F-actin structures of similar appearance and size ( $2\text{--}3 \mu\text{m}^2$  in area) that are associated with myoblast fusion during *Drosophila* embryogenesis (Gildor et al., 2009; Kesper et al., 2007; Kim et al., 2007; Richardson et al., 2007; Sens et al., 2010). Such foci were also observed during construction of the adult de novo forming DVMs (see Fig. S3 in the supplementary material), suggesting that they are a general feature of myoblast fusion in *Drosophila*. To assess the manner in which the foci are distributed between DLM fibers and fusing myoblasts, we visualized them following cell type-specific expression of the GFP-tagged F-actin-binding domain of Moesin (Moe) (Chihara et al., 2003; Dutta et al., 2002). Full overlap (Fig. 7B–B'') was commonly observed between *1151*-GAL4-expressed Moe-GFP

and phalloidin (19/20 foci examined), whereas overlap of *rp298*-GAL4-expressed Moe-GFP and phalloidin was rarely seen (2/18 foci), and even then it was restricted to a minimal portion of the structure (Fig. 7C–C''). The strong bias obtained using this approach suggests, therefore, that the actin foci reside primarily, if not exclusively, within myoblasts.

We next sought to determine whether the Arp2/3 complex and its associated NPFs contribute to formation of the fusion-associated actin foci. We first monitored the localization pattern of two subunits of the Arp2/3 complex, Arp3 and Sop2 (Arpc1), by expression of GFP-tagged forms (Hudson and Cooley, 2002) in fusing adult myoblasts. Both constructs were found to strongly co-localize with the actin foci, consistent with a role for Arp2/3 in their establishment (Fig. 7D–E''). Indeed, following expression of RNAi constructs targeted against the Arp2/3 subunits Arp2 and Sop2 via *Mef2*-GAL4, the bright, spherical foci were replaced by small and diffuse accumulations of actin (Fig. 7G–I), implying that Arp2/3 function is essential for the proper construction of the fusion-associated foci.



**Fig. 7. Arp2/3-dependent F-actin foci form during adult myoblast fusion.** (A) Spherical foci containing F-actin (arrows) are present at the interface between DLM templates (visualized with Mab 22C10, green) and attached myoblasts in an 18 hour APF *Drosophila* pupa. Foci and myoblast outlines are visualized with phalloidin (red). (B-B'') An 18 hour APF pupa expressing the F-actin-binding protein Moe-GFP under the control of the myoblast-specific driver *1151*-GAL4. The anti-GFP (B, green) and phalloidin (B', red) staining patterns are entirely coincident (B'', merge). (C-C'') By contrast, when expressing Moe-GFP via the fiber-specific driver *rp298* (*duf*)-GAL4, the anti-GFP (C, green) and phalloidin (C', red) staining patterns are mostly separate (C'', merge) in the rare instances in which any overlap is detected at all. The actin focus therefore resides primarily, if not exclusively, within myoblasts. (D-E'') Pupae at 18 hours APF expressing GFP-tagged versions of the Arp2/3 subunits Arp3 (D-D'') and Sop2 (E-E''). Both subunits, visualized using anti-GFP (green), strongly co-localize with F-actin (phalloidin, red) at focus structures (arrows in merges). (F-F'') Endogenous D-WIP in an 18 hour APF pupa, as visualized with anti-D-WIP antibodies (green), is also found to co-localize with the F-actin (phalloidin, red) focal structures (arrows in merge). (G-O) Morphology of 18 hour APF actin foci (visualized with phalloidin, red) following RNAi-based targeting of Arp2/3 and related NPFs via the *Mef2*-GAL4 driver. DLM template muscles are visualized with Mab 22C10 (green) in all panels. (G) Wild-type-sized focus in the absence of RNAi construct expression. Weak, diffuse foci are observed following targeting of the Arp2/3 subunits *Arp2* (H) and *Sop2* (I). Targeting of *Wsp* (J) and *D-WIP* (K) does not affect focus size or morphology, whereas enlarged somewhat irregular foci are found in pupae expressing RNAi targeting *SCAR* (L), the SCAR complex element *kette* (M) and both *Wsp* and *SCAR* (N) or *Wsp* and *kette* (O). (P) The areas of actin foci in pupae expressing different RNAi constructs. Values represent the average area of ~25 foci for each genotype. Asterisks indicate foci size values that differ significantly ( $P < 0.005$ ) from wild-type values. Error bars indicate s.d. Scale bars: 5 μm in A; 2 μm in B,D,G.

We next used RNAi-based disruption of gene activity to determine the identity of the NPF system that stimulates Arp2/3 activity in this context (Fig. 7J-P). Although D-WIP co-localized with the actin foci

(Fig. 7F), expression of RNAi constructs targeting *Wsp* (Fig. 7J) and *D-WIP* (Fig. 7K) did not interfere with the capacity to form foci of normal size. Turning to the SCAR-based NPF system, we observed

that expression of RNAi constructs targeting either *SCAR* or the *SCAR* complex element *kette* led to the formation of enlarged, irregularly shaped actin foci (Fig. 7L,M). Enlarged foci were similarly generated following simultaneous RNAi-based targeting of both the *Wsp*- and *SCAR*-based NPF systems (Fig. 7N,O). Thus, neither separate nor combined *Wsp* or *SCAR* complex NPF function appears to directly mediate the contribution of the Arp2/3 complex to actin focus construction, implying the involvement of a distinct, as yet unknown system for stimulation of Arp2/3 actin nucleation activity in this context.

As discussed below, the nature of the involvement of the WASp and *SCAR* pathways in actin focus formation in adult myoblasts mirrors observations made during myoblast fusion in *Drosophila* embryos. The ability to directly monitor Arp2/3 complex function is, however, unique to the adult system and leads us to suggest that the fusion-associated foci are Arp2/3-dependent branched microfilament structures and that a novel NPF mediates the involvement of Arp2/3 in this process.

## DISCUSSION

The universal nature of muscle fiber formation and growth via myoblast fusion suggests that common molecular mechanisms underlie the fusion process. The myogenic processes leading to the formation of adult *Drosophila* muscles provide a promising, yet generally unexplored, setting in which conserved elements of this type can be identified and characterized. This is particularly true for establishment of the prominent thoracic IFMs, which exhibit similarities in their arrangement and program of differentiation to both *Drosophila* embryonic myogenesis and muscle formation in vertebrates, including mammals (Fernandes and Keshishian, 1999; Dutta and VijayRaghavan, 2006). Whereas a detailed genetic analysis of myoblast fusion has been undertaken for the *Drosophila* embryo (Beckett and Baylies, 2006; Maqbool and Jagla, 2007; Abmayr et al., 2008), few, if any, mutants affecting the corresponding adult process have been reported. In the current study, we have utilized a combination of genetic approaches to initiate progress along these lines via the study of the adult myogenic requirements for *Wsp*, the sole *Drosophila* WASp family member, and additional elements of the cellular actin polymerization machinery.

### *Wsp* function is essential during adult myoblast fusion

A major finding of our study is that *Wsp* performs an essential role during adult fly myogenesis, which is crucial for the growth of muscle fibers via myoblast fusion. This requirement is highly specific to the fusion process, as other features and characteristics of adult myoblast development (e.g. the definition of a myoblast pool, myoblast proliferation levels and the different patterns of myoblast migration) appear unaffected in the absence of *Wsp* activity. The functional requirement for *Wsp* is comprehensive and universal in character. A complete arrest of myoblast fusion is observed in *Wsp* mutants for all classes of somatic muscle groups examined. Furthermore, reliance on *Wsp* activity during fusion is common to both of the major adult myogenic programs, namely the de novo construction of muscle fibers from individual myoblasts and fiber growth by fusion of myoblasts with persistent larval templates, as exemplified by the prominent DLM flight muscles (Roy and VijayRaghavan, 1999).

Whereas de novo muscle fiber formation in the adult resembles the *Drosophila* embryonic program of myogenesis, in which *Wsp* was previously shown to mediate myoblast fusion (Kim et al.,

2007; Massarwa et al., 2007; Schafer et al., 2007), DLM growth presents a distinct paradigm. The incorporation of myoblasts into pre-existing fibers is characteristic of several aspects of skeletal muscle growth in vertebrates, including the repair of injured muscle by satellite cell-derived myoblasts (Charge and Rudnicki, 2004) and the initiation of the second wave of mammalian embryonic myogenesis (Jansen and Pavlath, 2008). The essential and functionally conserved involvement of *Wsp* in diverse programs of *Drosophila* somatic muscle formation and growth leads us to propose that the actin nucleation-promoting activity of WASp family proteins constitutes a fundamental aspect of the cell fusion events that accompany myogenesis.

### Involvement of the Arp2/3 nucleation system in the formation of fusion-associated actin foci

Spherical F-actin-rich structures closely associated with myoblast fusion pore formation during *Drosophila* embryogenesis have been recently described and extensively characterized (Onel and Renkawitz-Pohl, 2009; Richardson et al., 2008; Richardson et al., 2007; Sens et al., 2010). Our observation of very similar structures during the period of adult muscle growth via fusion now suggests that these foci are a universal feature of the myoblast fusion process. Although a variety of alterations in embryonic actin focus morphology have been described for various mutants, the molecular mechanism governing their formation is still unknown. A key lingering question is the identity of the actin nucleation machinery that is responsible for establishment of the foci. The Arp2/3 nucleation system is an obvious candidate for such a role, given the well-documented involvement of its different components in embryonic myoblast fusion. However, direct assessment of Arp2/3 complex function during embryogenesis is difficult because the maternal contribution of complex subunits, which is essential for oogenesis (Hudson and Cooley, 2002; Zallen et al., 2002), is sufficient to overcome zygotic gene disruption. The capacity of RNAi to efficiently disrupt gene function during adult myogenesis thus provides a unique opportunity to study the contribution of the Arp2/3 complex to actin focus formation. Our data suggest that Arp2/3 indeed plays a crucial role in this process, as focus formation is significantly impaired upon expression of RNAi targeting two of the complex subunits.

Such a requirement for Arp2/3 is somewhat surprising because, as shown above, actin foci persist or grow following separate or simultaneous disruption of the two major Arp2/3 NPF systems centered on *Wsp* and *SCAR*. Furthermore, these behaviors closely match those reported following disruption of *Wsp* and *SCAR* system activity by a variety of means during embryogenesis (Gildor et al., 2009; Richardson et al., 2008; Richardson et al., 2007). Although the WASp and *SCAR* systems continue to be regarded as primary NPFs for Arp2/3, a growing list of alternative nucleators has recently emerged (Campellone and Welch, 2010), suggesting that a novel element might well activate Arp2/3 in this context. Identification of this novel NPF should provide genetic tools for determining the functional significance of the actin foci, which has so far remained elusive owing to the lack of information regarding the mechanism by which they are constructed.

### Functional aspects of the cellular role performed by *Wsp* in fusing muscles

A central and ongoing issue is the mechanistic role played by *Wsp* during myoblast fusion. We find several of the observations reported in our study to be instructive in this context.

The capacity to fully rescue the adult *Wsp* mutant phenotype by providing functional Wsp in either fibers or individual myoblasts suggests that interactions with cell type-specific factors are not a crucial mechanistic feature. It is therefore likely that the fusion-associated microfilament dynamics nucleated by Wsp are performed and utilized by mechanisms and molecular elements common to all muscle cells. This conclusion is particularly noteworthy considering the significance assigned to cell type-specific pathways in some models of embryonic myoblast fusion.

As suggested in the embryo, localization to sites of fusion between muscle cell membranes appears to be an important aspect of Wsp activity, although the means by which Wsp is targeted to these sites during adult myogenesis is not clear. D-WIP appears to provide a key membrane-targeting function during embryonic myogenesis (Massarwa et al., 2007), but our observations suggest that Wsp reaches adult fusion sites independently of D-WIP. Establishment of the mechanism by which Wsp localizes to adult myoblast fusion sites thus requires further study. These findings also leave open the role performed by D-WIP. A direct effect of D-WIP on cytoskeletal organization, as suggested during embryonic myoblast fusion (Kim et al., 2007), might be relevant in this context.

The transfer of cytoplasmic material between attached, fusion-arrested muscle cells, which is a hallmark of the embryonic myoblast fusion phenotype in *Wsp* mutant embryos (Gildor et al., 2009; Massarwa et al., 2007), is not observed following disruption of *Wsp* function during adult myogenesis. The basis for this difference is unknown and will require better elucidation of the particular aspects of the fusion process that rely on Wsp activity. One explanation is an expanded role for Wsp during adult myoblast fusion. An additional functional requirement prior to fusion pore formation might mask any subsequent participation in fusion pore expansion. Finally, it is of interest to note that, in the adult, disruption of either the Wsp or SCAR NPF systems leads to the strong arrest of myoblast fusion, similar to the embryonic scenario. Our observations on adult myogenesis thus serve as a reaffirmation that the fusion process requires non-overlapping functional contributions from the two major Arp2/3 NPFs, even though they operate in close spatial and temporal proximity.

In summary, the observations reported in our study generalize the involvement of the Arp2/3 complex and its associated cellular machinery during the construction of muscle fibers via myoblast fusion. In particular, a prominent and potentially universal role is assigned to the WASP NPF as a mediator of these events.

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#### Competing interests statement

The authors declare no competing financial interests.

#### Supplementary material

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