Chapter IV

Investigating the role of \textit{Wsp} during myoblast fusion in \textit{Drosophila} adult

\textit{Introduction}

Actin, an evolutionarily conserved protein is a major cellular component of cellular architecture. Cellular processes such as cell migration, cell–cell junction formation, cell polarization, vesicle trafficking and fusion require a well coordinated formation of different dynamic actin networks is required. Actin exists in a globular monomeric form (G-actin) that polymerises to form polar helical filaments (F-actin). Polymerization of G-actin into a thread of polymerised F-actin is a key step for the formation of actin networks responsible for force generation and necessary for cell based movements. Several molecules are known to shape polymerised actin structures among them, the three major players are Arp2/3, Formin and Spire (Discussed in Chapter III).

Arp2/3 (actin related protein), a multi-protein complex of seven protein subunits, is essential for the formation of branched actin filaments. The generation of an actin meshwork pushes the membrane to form pseudopodial structures such as filopodial and lamellipodia that are required for movement (Mogilner and Oster, 1996). Of the seven subunits of Arp2/3, Arp2 and Arp3 mimic actin monomers and initiate nascent actin filament formation at 70° to the pre-existing mother F-actin filament creating branched actin networks. New additions of actin monomers always occur at the barbed (+) end of the actin fiber. Arp2/3
cannot initiate polymerisation by itself but requires activation by a family of nucleation promoting factors (NPF) (Takenawa and Miki, 2001; Thrasher, 2002). These NPFs generate a conformational change in the Arp2/3 complex upon activation which promotes actin nucleation. The Wsp family of proteins which include Wsp/SCAR, plays a major role as nucleation promoting factor of Arp2/3.

*Drosophila* has single *Wsp* and *SCAR* gene in its genome. Genetic analysis of *Wsp* has revealed its role in cell-fate specification of neuronal lineages during mechano-sensory bristle formation (Ben-Yaacov et al., 2001). SCAR is required for a variety of process such as axon development, oogenesis, and adult eye formation in *Drosophila* (Zallen et al., 2002). Both Wsp and SCAR are the major players during myoblast fusion in *Drosophila* embryo (Kim et al., 2007; Massarwa et al., 2007; Gildor et al., 2009; Schäfer et al., 2007).

In the embryonic myoblast fusion studies, the function assigned to Wsp and its interacting protein Wip is quite elusive and varied in different reports (Kim et al., 2007; Massarwa et al., 2007). Wsp/Wip based actin machinery was shown to function at later stages of myoblast fusion, when the fusion pore is already formed and found to be required for expansion of the pore (Massarwa et al., 2007; Scafer 2007). Whereas, in a different study Wip/Wsp was shown to function in a fusion-competent cell-specific manner and required for F-actin accumulation at the sites of fusion (Kim et al., 2007)(discussed in Chapter I).

Using a genetic approach, I decided to investigate the role of Wsp/Wip based actin polymerisation machinery during pupal (imaginal) myogenesis. This study was done to get a general understanding of the molecular function of Wsp/Wsp during fusion. As mentioned before (Chapter-III) two modes of myoblast fusion are utilised in the developing thorax. This gave an opportunity to study *Wsp* function in both the myogenic settings. Analysis of *zygotic* *Wsp* mutants was primarily done on the fusing myogenic cells for DLM formation, a
prominent class of *Drosophila* thoracic flight muscles which are the largest set of muscle fiber and are easily accessible. In this Chapter-IV several aspects of *Wsp* function like its cell-specific requirement, significance of its localisation, and function during fusion pore formation etc have been addressed.

**Results**

4.1 *Wsp* loss-of function inhibit myoblast fusion

The *Drosophila Wsp* gene is 35% identical to mammalian WASPs (Ben-Yaacov et al., 2001). I have examined the role of *Wsp* during myoblast fusion in *Drosophila* pupae. The three *Wsp* mutant alleles used in the study are the result of transgenic deletion and were previously characterised for the study of sensory precursor development. The *Wsp* hemizygotes make a non functional protein which is unable to bind to the cytoskeletal elements and lead to loss of function (Ben-Yaacov et al., 2001). These *Wsp* alleles when used in trans-heterozygote combination (*Wsp1/Df(3450)*) could complete imaginal development without any gross developmental defects, except their failure to eclose and death at late pupal stages. The pharate nature of *Wsp* mutant flies urged look at the thoracic muscles of the fly using a GFP reporter which marks the muscle fiber.

MHC tau-GFP construct was used to detect the GFP which lights up the whole thorax in the wild-type. The huge thoracic muscles comprising DLMs and the DVMs when looked under the fluorescent microscope in the *Wsp* trans-heterozygote mutants seemed to be reduced in size with low GFP signal compared to the wild-type (Fig 4.1-A,B.). When dissected, the thoracic muscles looked smaller and fewer in number than the wild-type (Fig 4.1-D, E). Further examination of thorax confirmed the reduced musculature in *Wsp* mutant much evident from the horizontal section made along the thorax of the fly. The thin sections were
stained with toluidine-blue showed a complete disorganisation of the whole thoracic muscle in Wsp1/Df(3450) (Fig4.1-F, G). The muscle mass was completely lost and the thin fibers seen in the thorax displayed the morphology of a larval template muscle in Wsp1/Df(3450) flies (Fig4.1-G). Similarly, the expression of Wsp without Arp2/3 binding domain (WspΔCA) using the myogenic mef2 promoter phenocopies the Wsp mutant phenotype, thus acting as a dominant negative construct following its inability to activate Arp2/3 complex. These observations specifically indicate a crucial myogenic function of Wsp during muscle development in Drosophila pupae (Fig4.1-C).
Fig.4.1. Defective musculature of Wsp mutant flies.

(A-C) Thoraces of pupae, close to eclosion at 96h APF were imaged, visualized by the expression of MHC-TauGFP in the thoracic muscles. (A) Well-built musculature of a wild-type Drosophila pupa. (B) Wsp<sup>1</sup>/Df(3R)3450 the flight muscles are poorly formed and shows a characteristic loss of muscle mass. (C) Expression of dominant negative form of Wsp using a myogenic promoter constructs mef2>WspCA shows a similar loss of musculature. (D) Dissected out DLMs from the W.T and (E) from Wsp<sup>1</sup>/Df(3R)3450 (F,G) shows the horizontal cross sections of pupal thoraces, counterstained with toluidine blue in (WT) wild-type and Wsp<sup>1</sup>/Df(3R)3450. Muscle are indicated (DLM- dorsal longitudinal flight muscles; DVM- (dorso-ventral flight muscles); JM- Tergal depressor of the trochanter muscle also called jump muscle and the thin groups of remnant larval flight muscle templates are seen (red asterisk) in Wsp<sup>1</sup>/Df(3R)3450 section. Scale bars= 200 µm (A), 50 µm (D), 100 µm (F).

To address the developmental requirement of Wsp during myogenesis, different stages of myogenesis were observed carefully. In order to investigate whether there is an early developmental defect in the embryonic muscle that contribute to the reduced muscle mass
seen in the *Wsp* mutants; early developmental stages like 12h APF were analysed. At this stage the larval muscles which serve as the founder cell marked by *MHC-tau-GFP* (Chen and Olson 2001) and the proliferating myoblast cells at the wing imaginal disc were observed using Rb-Twist antibody (Fig 4.2). The larval template in the *Wsp* mutant looked similar to the larval template in the wild-type (Fig 4.3-A, B).

Normally, reduction of myoblast cell numbers at the wing imaginal disc due to defects in myoblast proliferation is also shown to hinder the fusion process. This is well known in the case of *1151GAL4>DNRac1* which causes disruption of actin cytoskeleton that affects several stages of myoblast fusion starting with myoblast proliferation and migration to fusion (Fernandes et al., 2005). Similar possibility was tested in *Wsp1/Df(3450)*. Anti-Twist as well as *1151GAL4>UAS-nlsRFP* (nuclear localised RFP) was used to detect the number of myoblast nuclei in *Wsp1/Df(3450)* which looked comparative to the wild-type myoblasts covering the notal region of the wing imaginal disc (Fig 4.2-A, B). BrdU incorporation in the myoblast cells at the notal region of the wing imaginal disc further confirmed that the proliferation of the myoblast cells in *Wsp* mutants was normal as the wild-type (Fig 4.2-E, F). These experiments indicate that in *Wsp* mutants have normal embryonic muscles and express correct myoblast identity factors that allow these cells to proliferate and differentiate normally.
Fig. 4.2 Proliferation of myoblast pool is normal in Wsp mutants

(A-D) Dissected third instar larval wing imaginal discs where 1151 promoter was used to drive nlsRFP (nuclear localised RFP) were stained for the Rb–RFP(red) and another marker Rb-Twist to visualised the myoblast nuclei(green). A large population of myoblasts are found throughout the notal region of both WT (C) and Wsp1/Df(3R)3450 (D) discs. (a’b’c’d’) Line drawn along the vertical as well as horizontal axis in both the wild-type and Wsp1/Df(3R)3450 shows a comparable number of myoblast nuclei along the same plane. (E e’, F and f’) BrdU incorporation confirmed the presence of normal proliferating myoblast cells in Wsp1/Df(3R)3450 like in case of wild type. n=6 Scale bar indicates 50µm.

My study suggest that the muscle defects observed in Wsp mutants arise at later stages of the myogenesis. A marker to label the muscle fiber and the neuron Mab22C10 was used to detect any deformity at 24h APF. The disorganisation of muscles due to fusion arrest in Wsp1/Df(3450) leads to defective neuronal morphology. This defective neuronal pattern could be attributed to non availability of axon targets in a highly reduced muscle fiber. Dramatic differences were observed in the muscle as well as the neuronal pattern of Wsp1/Df(3450) demonstrates the requirement of Wsp during the later steps which is during myoblast fusion in pupae (Fig 4.3 C, D).
Fig 4.3. Wsp mutants contains normal larval muscle but defective muscles later

(A, B) The larval muscle expressing MHC-TauGFP at 12 h APF in the wild-type and in Wsp^{1}/Df(3R)3450 pupae.(C, D) Immuno-labeling of 22C10 in wild-type and Wsp^{1}/Df(3R)3450 DLMs. While the wild-type fiber set splits in two and grows in size to yield six mature fibers in each hemithorax, the underdeveloped DLM templates in Wsp^{1}/Df(3R)3450 pupae remain thin and fail to split shown by the white asterisk. n=5 Scale bars indicates 50 µm.
4.2 Fusion is compromised during thoracic myogenesis in \textit{Wsp} mutants.

Myoblast fusion can be divided into several different steps (discussed in Chapter 1). One of the primary steps is migration of myoblast cells from the wing imaginal disc towards the DLM template in order to fuse. The ability of the cellular migration is mainly attributed to the actin cytoskeletal rearrangement within the cell (Pollard and Borisy, 2003) (discussed in chapter 1). When the myoblast nuclei covering the whole thorax were examined, a uniform migratory pattern was observed in \textit{Wsp1/Df(3450)} visually similar to the wild-type migratory patterns forbidding any role of \textit{Wsp} in myoblast migration during \textit{Drosophila} imaginal myogenesis. However, live imaging of the migrating myoblast cells is required to ascertain this observation.

Thoracic myoblast fusion commences at 10-12 h and continues till 24h to 30h APF. Myoblast cells begin to make attachments to the myofibers as early as 10h APF and the fusion between the myoblast cells and the larval template for the construction of DLMs starts at 12 h APF and peaks around 20h APF. At around 24 h almost all the fusion events are nearly complete. Dual colour labeling of muscle fiber with \textit{MHC-GFP} and myoblast cell nuclei in \textit{1151GAL4>UAS-nlsRFP} (myoblast specific promoter driving the expression of UAS-nlsRFP) was employed to detect any abnormality during myoblast fusion in the fixed preparations.
4.4 Flight muscle defects in *Wsp* mutants arise due to fusion defect in developing pupal muscle.

(A, B) Myoblast nuclei (red, visualized by *1151-GAL4>*UAS-RFP-nls/RedStinger) and (A’, B’) DLM fibers (green, visualized with MHC-GFP and (A’’ and B’’) Mab-22C10. In wild-type (A-A’’) and *Wsp*¹/Df(3R)3450 (B-B’’) pupae at 18 h APF. Wild-type DLMs contain many myoblast nuclei, while the immature fibers in *Wsp*¹/Df(3R)3450 mutant pupae have not incorporated any myoblast nuclei. *Scale bar indicated 50µm*
Fig 4.5 Myoblast fusion does not occur in Wsp mutants

(A-D) Shown here are the number of nuclei incorporated in the wild-type and the Wsp$^1$/Df(3R)3450 using two nuclear marking strategies. (A) Myoblast nuclei are visualised by using 1151GAL4>nls-RFP and the marking the muscle fiber with standard Mab22C10 in wild-type and panel (B) Wsp$^1$/Df(3R)3450. (C) Anti-Erectwing staining also indicates a lack of nuclei inclusion in the Wsp$^1$/Df(3R)3450 muscle fiber due to fusion defect when compared to wild-type(D). Black circular figures within these fibers correspond to the enlarged, polyploid nuclei of larval muscles. Scale bar indicates 50µm

Upon observation of different stages of wild-type and Wsp$^1$/Df(3450) mutant animals, not a single myoblast nuclei was found to be incorporated into the larval muscle fiber of the Wsp mutant when compared to large number of nuclei fused into the wild-type DLM fiber (Fig 4.5-A, B). The Erect-wing staining clearly shows the presence of large founder nuclei within the undeveloped larval myofibers of the Wsp mutants however, not a single inclusion of the small size nuclei, representing the myoblast nuclei was seen. While, the wild-type muscle
fiber contained hundreds of nuclei in parallel rows within the muscle fiber (Fig 4.5-C,D). When a membrane tethered form of RFP was used to mark the myoblast membrane in the genotype 1151GAL4>UAS-myRFP the RFP expressing membrane clearly mixes with the MHC-GFP expressing membrane. This suggests that of membranes intermingle between the two fusing cells. Such membrane merger makes a case of formation of well characterised hemi-fusion complex when the lipid mixing occurs during cell fusion (Chernomordik and Kozlov, 2003). In a stark contrast to the wild type, the myoblast cells in Wsp1/Df(3450) which fail to fuse were left unfused around the thin DLM muscle fiber. Strikingly, the two coloured labeling of opposing membranes in Wsp1/Df(3450) show a visually apparent difference compared to wild-type situation. The membrane lipid mixing does not appear to take place between the two mono layer of the cell plasma membrane (Fig 4.6 A, B, C and D, E, F respectively). Strikingly, the two coloured labeling of opposing myoblast cells in Wsp1/Df(3450) shows a visually apparent difference where membrane lipid mixing never occur between the two mono layer of the cell plasma membrane (Fig 4.6 A,B,C and D,E,F respectively). Also when looked at 36h APF the fully differentiated DLM of genotype 1151GAL4/UAS-myRFP; MHC-GFP no unfused myoblast was seen and the two membranes completely merge. In contrast, several unfused myoblast can be seen near Wsp1/Df(3450) muscle with no evidence of merge of membranes (Fig4.7 A’’,B’’).
Fig: 4.6. Membrane fusion fails to occur between the template fibers and surrounding myoblasts in Wsp mutants.

(A-L) Myoblasts (red, visualized by 1151-GAL4>UAS-myr-mRFP) and DLM fibers (green, visualized with MHC-TauGFP) in wild-type (A, B, C) and Wsp\textsuperscript{1}/Df(3R)3450 (D,E,F) pupae at 18 h APF. (G-L) Is the high magnification of the same. Incorporation of the membrane marker into DLM membranes is indicated by white arrow, observed in wild-
type (I), which does not appear to take place in Wsp mutants (L) when observed at high magnification. \( n=5 \) Scale bars = 50 µm (A-F), 2 µm (G-L)

Fig 4.7 Underdeveloped muscles fibers in Wsp1/Df(3450) at 36h APF

(A) In 1151GAL4>UAS-myrRFP;MHC-GFP at 36hAPF the muscle fiber(green) and myoblast cell(red).\((A'')\) Membrane merge is seen in the fiber in the upper panel. \((B-B'')\)In Wsp1/Df(3450) unfused myoblast (red) is seen around the immature myotube(green). Scale barr indicate 50 µm

The myoblasts in the wild-type acquires a characteristic rounded shape that spread over the surface of muscle fiber giving more area of contact between the two layers of the membrane however, in Wsp1/Df(3450) fusion compromised situation the cells were able to adhere normally to the muscle fiber but retain a characteristic spindle shape organisation with a minimum surface of contact between the two membranes (Fig 4.6 G, H, I and J, K, L). Similar bipolar conformation of unfused myoblast cells attached to the myotube surface is
seen in 1151 GAL4 > DN Rac1 (Chapter-III). All the experiments done indicated an essential requirement of Wsp function during myoblast fusion and upon disruption of Wsp function the muscles fail to develop to an adult fly.

4.3 Wsp is required in both the fusing cell types

Considering the differential genetic expression in FC/FCM and their distinct contribution during myoblast fusion (Chen and Olson, 2004; Estrada et al., 2006; Buckingham et al., 2003), I decided to test whether similar molecular biasness regarding the Wsp function exists in the fusing cells. Wsp protein was uniformly distributed in both the myogenic cell types depicted from the anti-Wsp staining. When anti-Wsp staining was done in Wsp1/Df(3450) the expression was found to be completely lost from the membranes (Fig4.8-B, D). In order to determine whether Wsp distribution mirrors its functional contribution, a genetic situation was designed where the Wsp function was restricted only to one cell type in Wsp1/Df(3450). Using both cell type specific drivers in Wsp compromised situation functional Wsp was introduced back using UAS-Wsp full length construct and any rescue of the fusion defect were analysed. Firstly, D-mef2 GAL4 (Gogineni Ranganayakulu et al., 1996) driven expression of UAS-Wsp was done which showed a complete rescue of the defective muscle phenotype of Wsp1/Df(3450). This confirmed that the phenotypes seen are truly because of the myogenic defects and not because of any neuronal component (Fig-4.8 E).

In order to provide a restricted Wsp expression in the muscle fiber, duf-GAL4 (insertion in the Duf locus) was used (Ruiz-Gómez et al., 2000). The limited expression of duf-GAL4, only in the muscle fiber during pupal myogenesis was obtained by using GAL80ts. The reason behind using GAL80ts was a previous report, where a mild expression of Duf was seen in the proliferating myoblast cells (Dutta et al., 2004). Even though, a recent report
ruled out any activity of the *duf-GAL4* in the wing imaginal disc I still wanted to be sure that the muscle specific expression is able to rescue the *Wsp* phenotype (Atreya and Fernandes, 2008). Thus, the temperature sensitive GAL80ts strain was used with muscle fiber (*duf GAL4*; GAL80ts) specific driver with Wsp (UAS-Wsp-full length) where functional Wsp was supplied back into the *Wsp1/Df(3450* Selective supply of Wsp only in the muscle fiber gave a complete rescue of the severely defective fusion phenotype at 24hAPF (Fig 4.8-F).

Similar rescue was obtained when UAS-*Wsp* transgenic construct was expressed under *1151GAL4*, which is exclusive to the myoblast populations (Roy and VijayRaghavan, 1997) (Fig 4.8-G). In all the rescue experiments, pharate adults were obtained due to the developmental defects caused by the loss of Wsp function. The rescue experiments strongly suggest that Wsp protein is necessary as well as sufficient when provided from either myogenic cell type. Thus, it can be said that *Wsp* has a general myoblast fusion related function as it can promote fusion from either the fiber or the myoblast cell.
Fig. 4.8. Wsp function is sufficient when provided from either myofibers or myoblasts.

(A-A’’) Wsp expression is observed in both growing fibers and adjacent myoblasts in wild-type at 20h APF (B-B’’) whereas the Wsp expression is almost lost from the
Wsp¹/Df(3R)3450 muscles as well as myoblast cells. Rescue of the Wsp mutant DLM defects, visualized by Mab 22C10, can be achieved by driving UAS-Wsp in all muscle cells using mef2-GAL4 (C) as well as by myofiber-specific expression, using rp298-GAL4 (a GAL4 insertion in the duflocus) (D), or by myoblast-specific expression, obtained with the myoblast driver 1151-GAL4 (E). n=5 Scale bars= 50 µm (A-G)

4.4 Membrane localisation of Wsp

The Arp2/3 based actin polymerisation machinery is known to function at the vicinity of plasma membrane and are responsible for the cellular movements (Takenawa and Suetsugu, 2007). As expected, Wsp localizes to the membrane along the cortex of the myoblast cell which was determined by using anti-Wsp staining (Fig-4.9 A). The expression of Wsp at the membrane shows a spatial variation of expression in wild-type. At early stages like 14h APF, Wsp staining appears to be faint but once the fusion process begins to rapid the Wsp levels also increases at the membranes at around 20h APF. Again it goes down at the later stages which suggest that Wsp is highly expressed when most of the fusion events happen and it is rather low at stages of less action. This certainly means that the Wsp protein levels increases at the time of its function.

Immuno-labeling of Wsp antibody in wild-type is never seen at the site of fusion but was rather distributed all over the membrane. As seen previously, the proteins with high turnover can be visualised in a set up where fusion is arrested and protein is not recycled (Galletta et al., 2004; Massarwa et al., 2007; Menon et al., 2005). Expression of DNRAc gave such an opportunity to catch specific localisation of Wsp. UAS-Wsp-GFP construct was used in a background of 1151GAL4>DNRAc where the fusion deprived myoblast cells are stuck near the myofiber (Chapter III). Immuno-staining of anti-Wsp showed an exclusive and
concentrated localisation at the fusion sites, implying that it functions at the site of fusion (Fig 4.9 B).

**Fig 4.9:** Wsp is functionally active near the membrane

(A) Membrane associated function of Wsp which is one of the characteristic of its function was observed using Rb anti-Wsp which uniformly localise near the membrane of the myoblast cell and the muscle fiber. In order to visualise Wsp near the fusion site a fusion 1151GAL4>DnRac, Wsp-GFP (B) combination was used where Wsp could be seen at the point of attachment between the myofibers and the myoblast. Membrane attached function of Wsp was establish by using mef2GAL4>Wsp-myr; GAL80ts, Wsp1/Df(3R)3450. Where the Wsp-myr was able to rescue the Wsp mutant fusion defects(C). Scale bars= 50 µm (A, C), 2 µm (B)

From the antibody staining and GFP expression, Wsp was observed to be associated with the membrane which encouraged us to ask whether the membrane attachment of Wsp is sufficient to carry a fusion related function during muscle development. A transgenic construct UAS-Wsp-myr, a membrane-tethered form of Wsp was expressed under mef2GAL4 promoter in all the myogenic cells (Bogdan et al., 2005) This over-expression construct UAS-Wsp-myr gave a fusion phenotype itself. Therefore, a temperature regulated
expression of UAS-\textit{Wsp-myr} was employed using the GAL80ts (temperature sensitive) in a 
\textit{Wsp} mutant background, to direct rescue of the mutant phenotype. A partial but substantial 
amount of rescue of the mutant phenotype was observed upon expression of membrane 
bound Wsp in all the myogenic cells. The muscles detected in the rescue animals were near 
to normal when compared to the \textit{Wsp} deficient animals (Fig-4.9 C). The \textit{Wsp-myr} construct 
used here may be interfering with the normal Wsp function at the membrane, as a membrane 
attached form may hinder normal Wsp function. The defective phenotype as well as the 
partial rescue seen may be due to such hindrance caused by the construct. Although, the 
partial rescue of \textit{Wsp} mutant shown by the expression of \textit{Wsp-myr} construct reveal that Wsp 
indeed functions at the vicinity of plasma membrane, a typical domicile for its action in 
order to promote myoblast fusion.

\textbf{4.5 Formation of fusion pores}

To seek a definitive role of the microfilament nucleation promoting factor Wsp I examined 
the different cellular processes that are known to occur during fusion process. Different steps 
of the fusion process were analysed starting from cell proliferation, migration, recognition 
and attachment. All of the mentioned steps were found to be normal and do not seem to 
affect the fusion process in \textit{Wsp1/Df(3450)}. Embryonic studies suggest that the expansion of 
fusion pore is a prime function of \textit{Wsp} dependent Arp2/3 polymerisation machinery during 
fusion (Berger et al., 2008; Gildor et al., 2009, Massarwa et al., 2007).

In order to address whether the role of \textit{Wsp} is conserved in this context I assayed the fusion 
pore formation between the two cell types. During fusion once a proper attachment between 
the two cells is made a “fusion pore” is generated that allow membrane merger as well as 
cytoplasmic mixing between the two cellular contents. To monitor the cytoplasmic 
continuity between the muscle fiber and the fusing myoblast, a cytoplasmic protein was
expressed in one cell type and leakage between the opposing cells was observed. Towards this end, \textit{Actin88F-lacZ}, a transgenic construct in which cytoplasmic β-galactosidase (β-gal) put under the control of a muscle-specific \textit{Act88f} promoter was used (Hiromi et al., 1986) so that the expression is limited to the growing fibers, but is absent from the individual myoblasts (Fernandes et al., 1991). Close examination of wild-type \textit{Actin88F-lacZ} pupae during DLM maturation, reveals incorporation of β-gal into a small population of attached myoblasts at an advanced stage of fusion with the fibers (Fig-4.10 A, B) demonstrating that cytoplasmic transfer between partially fused cells can be detected in this setting as well.

Next, I examined the distribution of \textit{Actin88F-lacZ} derived β-gal in fusion-arrested pupae in \textit{Wsp1/Df(3450}. None of the numerous, unfused myoblasts attached to the persistent templates appear to contain any β-gal staining (Fig 4.10 B). Additionally, to determine the cytoplasmic continuity between the myoblast cells and the muscle fiber the GFP labeled myoblast cells were monitoring which show cytoplasmic GFP transfer into the muscle fiber. Similar to the previous experiment the transfer from \textit{GFP} expressing myoblast in \textit{1151>eGFP; Wsp1/Df(3450)} was never observed, suggestive of a possible role of \textit{Wsp} in the construction of fusion pore between the two opposing cells (Fig 4.10 G, H). Similar experiments were conducted in the background of \textit{1151>DNRac} animals. Fusion pore do not form in this case implying on function of Rac dependent actin polymerization for fusion pore formation (Fig 4.11 A,B,C and D,E,F). These observations imply that in \textit{Wsp} mutants the fusion process arrests before the initiation of fusion pore. Thus, Wsp activity appears to be essential for the generation of nascent fusion pores, which is interestingly different to its proposed function during embryonic myogenesis (Massarawa et. al, 2007). Recently EM analysis of the \textit{Wsp} mutant done in the lab also has confirmed that fusion pores are not formed in \textit{Wsp} mutants.
Fig 4.10: Formation of fusion pore is abolished in Wsp mutants

To assess the pore formation in wild-type and Wsp^{1}/Df(3R)3450, Act88F-lacZ transgenic construct was used in the back ground of Wsp^{1}/Df(3R)3450(B) and compared to wild-type(A). The cytoplasmic lacZ expressed only in the myofibers was visualised by βeta gal staining in wild-type (A) and Wsp^{1}/Df(3R)3450 (B). Also, using GFP expression in the myoblast cells (1151>eGFP in Green and phalloidin in Red) only indicated similarly the lack of cytoplasmic continuity between the myoblast and the muscle fiber (E) compared to wild-type (D) Scale Bar indicates 2µm
Fig 4.11 Fusion pores are not formed in 1151>DNRac muscles.

Fusion pore between the myoblast cells and the myofibers are assayed using Act88f-GFP in 1151>UAS-DNRac. The Anti-GFP staining is not observed in the attached myoblast cells stained with phalloidin. Also, the contribution of GFP from the myoblast is not seen in 1151>UAS-DNRac;eGFP myofibers shown by asterisks. Scale bar indicates 5µm.

4.6 Disruption of Wsp function leads to defects in DVM’s and abdominal muscle development

Unlike DLM formation, most of the other adult somatic muscles in Drosophila are formed by an alternative myogenic program, in which individual founder or pioneer myoblasts seed formation of fibers via fusion with the neighbouring myoblasts (Fernandes et al., 1991; Fernandes and Keshishian, 1999). Major examples include, the thoracic dorsal-ventral muscles (DVMs), a second major set of indirect flight muscles (Rivlin et al., 2000; Fernandes et al., 1991) and the various groups of abdominal muscles, which are derived from nerve-associated myoblasts (Currie and Bate, 1991) (discussed in Chapter-3). When
the development of these different muscle types were monitored in \( Wsp \) mutant pupae, severe defects in the construction of all muscles forms were seen (Fig 4.10). \( Wsp \) DVMs, which often are difficult to identify altogether, found to be considerably smaller than their wild-type counterparts, and contain no more than fewer nuclei, as opposed to the numerous nuclei present within the wild-type muscles (Fig.4.12-A, B). Both lateral (Fig.4.12-C, D) and dorsal (Fig.4.12-E, H) abdominal muscles are similarly under-developed in \( Wsp \) mutant pupae. Although, the abdominal muscles are properly arranged in parallel arrays, but the muscle fibers are very thin, and commonly contain only single nuclei incorporated within the thin muscle fiber, in contrast to the thicker, multi-nucleated, wild-type muscle fibers.

These observations suggest that, similar to thoracic flight muscles, the specification and differentiation aspects of the adult abdominal myogenic cells are properly initiated in \( Wsp \) zygotic mutants, but fiber growth and maturation is arrested due to a nearly complete failure to add myoblasts into the muscle fiber through cell-fusion.
Fig 4.12 Wsp is a general mediator of myoblast fusion.

(A-F) DVM thoracic flight muscles at 24h APF and Abdominal muscles at 50hrs APF (A,B) lateral (C,D) and dorsal (E,F) at 48h APF of wild-type (A,C,E) and Wsp¹/Dif(3R)3450 (B,D,F) pupae. Fibers in all panels visualized with MHC-TauGFP, while myoblast nuclei are visualized using 1151-GAL4>UAS-nls-RFP/Red-Stinger. DVMs and abdominal muscles of Wsp mutant pupae undergo characteristic fibrous morphologies, but fail to fuse with the surrounding myoblasts. (Scale bar indicates 50µm)

Discussion:

Arp2/3 activator Wsp is essential during imaginal myogenesis

The current knowledge regarding the molecular players of myoblast fusion comes from the extensive studies done during embryonic myogenesis. The signal transduction from the cell surface through the trans-membrane proteins to the cytoskeletal elements is well illustrated in various investigations done in past two decades. Arp2/3 complex which generates branched actin networks near the plasma membrane has emerged as a central regulator of multiple signaling cascade recently (Higgs and Pollard, 2001) and shown to be a major participant of signaling mechanisms operating during myoblast fusion in Drosophila (Massarwa et al., 2007, Sens et al., 2010a). Examination of Arp2/3 activator Wsp, in this context has shed light upon a different aspect of actin other than the lamellipodia and filopodial formation (Yarar et al., 1999). Fusion of myoblast nuclei and the cellular contents for the formation of multinucleated muscle fiber is severely affected in Wsp zygotic mutants, in spite of normal migration patterns and formation of proper cellular attachments indicate towards a unique molecular perspective of Wsp function during imaginal myoblast fusion.
The myoblast cells in \( Wsp \) mutants retain the spindle shaped conformation, normally utilised for the migration of myoblast cell contrary to the rounded shape of the wild-type myoblast cell upon attachment that leads to an increase in the surface of contact between the two plasma membranes. Recent study indicates that the increase of surface of contact is directly related to the number of fusion pore formed which could be the reason for \( Wsp \) deficient myoblast retaining a bipolar shape rather than a rounded conformation (Chen et al., 2008).

Conventionally, Wsp is located near places of actin dynamics in the vicinity of the plasma membrane of the cell providing the protruding forces for cellular movements (Takenawa and Suetsugu, 2007, Kurisu and Takenawa, 2010). The membrane localisation of Wsp and membrane associated function goes well with the known fact that actin functions near the membrane in a much localised manner that powers different cellular process. I find that the membrane associated function of Wsp is important for imaginal myoblast fusion. Based on the observation, it can be assumed that \( Wsp \) deficiency could lead to reduction in the efficiency of the Arp2/3 complex which in turn affects the branching of actin near the membrane that affects fusion by some unknown mechanism which needs further investigation. Also, the connection between the Wsp based actin with the membrane raise a possible role for membrane binding proteins during myoblast fusion (B Qualmann et al., 2000).

\textbf{Wsp functions at the site of fusion to allow membrane merge and fusion pore formation.}

Fusion between two cells requires union of the two bi-lipid membranes. Plasma membrane of each cell contains two mono-layers of lipids and proteins embedded in between them. The
present knowledge of monolayer merger comes from studies done in the context of viral fusion (Shmulevitz and Duncan, 2000, Kielian and Rey, 2006). There are two different scenarios for fusion between the two membranes that are taken into consideration. First, the direct fusion event where the generation of fusion pores is required in both the opposing membranes. Here the edges of fusion pore from both the cellular partners join leading to the generation of an aqueous connection and cytoplasmic mixing. The alternative mechanism of fusion between the membranes would be the merger of the outer leaflet and formation of a hemi fusion complex. This further causes lipid mixing between the inner as well as the outer membrane of both the fusing cells. The later scenario seems to be more evident during myoblast fusion in *Drosophila* as the membrane mixing between the myoblast cells and the myofibers is observed using a two coloured labeling of the opposing membranes. Although, lipid mixing is not the decisive factor for cytoplasmic mixing between two cells but, it can be considered as a primary step for initiating the fusion process (Dennison et al., 2006; Chan et al., 2009). During fusion of myoblast cells the presence of trans-membrane fusion proteins like Duf, Rst and Sns, Hibris initiate recognition as well as the attachment of the myoblast cell membrane to the myofibers but what triggers the membrane merger is not known.

I find that in *Wsp* compromised situation, membrane mixing is not clearly observed. However, the ability to resolve the two bi-lipid membranes is not enough to determine the membrane mixing. But the membrane merge in the wild-type context is strikingly distinct from what is seen in *Wsp* mutant tissue preparation. The formation of fusion pores between the myoblast cells and the muscle fiber is completely abolished in *Wsp* mutants during pupal myogenesis. This result is in contrast to the known embryonic function of *Wsp* gene during fusion, where in *Wsp* mutant fusion pore is seen that could allow transfer of cytoplasmic GFP from one cell to another but the incorporation of the whole cytoplasm was ceased
(Massarwa et al., 2007). Thus, indicating the role of Wsp during fusion is to expand the fusion pores, as it is assumed that the pushing forces are required for the membrane to move apart (Kaksonen et al., 2006). But contrary to the idea of pushing force exerted sidewise to open the pore wide, a recent study indicates that upon de polymerisation of actin, the fusion pore size does not change, implicating on the role of membrane bending proteins for expansion of the nascent pore formed (Chen et al., 2008). The embryonic Wsp function needs more critical investigation. My study shows that the formation of fusion pore itself is compromised during imaginal myoblast fusion which hints towards a distinct feature of Wsp function during this context.

How Wsp is regulating the fusion pore formation remains unanswered in this context. One possibility could be the transfer of actin dependent vesicle mediated fusogen at the site of fusion which cause the membrane merger as well as the fusion pore formation. Several electron-microscopy studies on different developmental cell fusion reactions reported the presence of vesicles in the lumen of the growing pores, suggesting that pore growth proceeds by membrane vesiculation (Doberstein et al., 1997; Mohler et al., 1998). As observed ultra structurally, the presence of vesicle near the fusion site during embryonic myogenesis as well as in mammalian cell culture may raise a hope for a possible actin driven mechanism by which the actin coated vesicle transport an unknown fusogen to allow integration of the cytoplasmic material (Duan and Gallagher, 2009; Kim et al., 2007). It can be well assumed that the transport of fusogen required for fusion pore formation is inhibited in Wsp mutants that lead to lack of fusion pore formation and cytoplasmic continuity between the fusing cells. My study has identified a novel aspect of Wsp function which is to initiate fusion pore formation during fusion. However, the mechanistic understanding of this process still poses a challenge to our understanding of Wsp function during imaginal myoblast fusion.
“Wsp” a general mediator of fusion in myogenic cells

Specialized somatic muscles formed during the second phase of myogenesis in adult flies include the indirect flight muscles (IFMs), the tergal depressor of the trochanter (TDT, or jump muscle), direct flight muscles at the base of the wing, as well as head and leg muscles (Bernstein et al., 1993b). The adult *Drosophila* body wall comprise of diverse groups of muscle fibers attached to the epidermis utilizing tendons cells at each junction (Ghazi et al., 2003). Several groups of muscles display distinct shape, size as well as the mode of formation. Differential myogenic programs are utilised for formation of muscles to architect the body wall of *Drosophila* adult. The muscle fibers which are formed *de novo* resemble the myogenic program of embryogenesis where the single founder cell is selected among the pool of several myoblast cells which seeds the formation of muscle fiber upon fusing to its neighbouring myoblast cells. This single cell behaves as founder cell and expresses founder cell specific markers. The DVMs and the abdominal muscle are formed similar to embryonic muscle fiber generation. The abdominal muscles are thinner and surround the abdomen like a sheet attached to the epidermis. Lack of fusion in the abdominal as well as the DVM suggest a complete arrest of *de novo* mode of fusion in *Wsp* mutants. This clearly illustrates the requirement of *Wsp* mediated Arp2/3 dependent actin polymerisation machinery required for myoblast fusion is used for construction of all types of skeletal muscles in *Drosophila*.

Thus, *Wsp* demonstrates a wider developmental role in the myogenic cells. Recent study in the embryo show that the dynamicity of the actin structure in these PLS require modulation of Wsp-Wip mediated actin polymerisation which induces invasion into the founder membrane leading to myoblast fusion (Jin et al., 2011). These possibilities need to be tested in this context.
Summary

My study illustrates a novel role of $Wsp$ during pupal myoblast fusion. Mutation in $Wsp$ gene leads to the disruption of normal fusion process is all the muscle types indicate a comprehensive role of Wsp protein in different myogenic cell types during development. $Wsp$ dependent Arp2/3 actin polymerisation machinery acts near the membrane and plays a significant role in either cell type (Muscle fiber /FCM). Contrast to its known embryonic function, which is to expand the newly formed pore (Massarwa et al., 2007), in pupa $Wsp$ function is required for initiation of fusion pore formation itself as no cytoplasmic continuity was observed between myofibers and the attached myoblast cells. Also the ultrastructural study supports this finding. Thus, my investigation emphasis that the $Wsp$ mediated actin polymerisation machinery is distinctly required during the formation of adult musculature.

Intriguing question which may arise from this study are well be that how the loss of $Wsp$ function leads to lack of fusion pore formation? What are the molecular mechanisms by which the branched actin networks are commanding the formation of fusion pore? Understanding a definitive function of $Wsp$ remains a challenge in the arena of myoblast fusion.