Linking IgSF-mediated cell adhesion with Arp2/3-based actin polymerization during *Drosophila* myoblast fusion

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Erlangung des Doktorgrades

der Naturwissenschaften

(Dr.rer.nat)

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Kaipa Balasankara Reddy

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This analysis was carried out from June 2008 to June 2011 at the Fachbereich Biologie (Entwicklungsbiologie) of Philipps-Universität Marburg under the supervision of Prof.Dr. Susanne Önel.

Vom Fachbereich Biologie

Der Philipps-Universität Marnirg als Dissertation am angenommen.

Erstgutachter: Prof.Dr.Susanne Önel

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Tag der mündlicchen Prüfung:

Dedicated to *My Parents*

Table of Contents

1. Summary

The somatic musculature of *Drosophila* is analogous to vertebrate skeletal muscles and is generated by the fusion of mononucleate myoblasts. Muscle fusion in *Drosophila* involves two distinct cell populations, founder cells (FCs) and fusion-competent myoblasts (FCMs). The recognition and adhesion of both myoblast types is mediated by members of the Immunoglobin superfamily (IgSF) that are expressed myoblast type specifically: Duf and Rst are expressed in FCs. Sns, Hbs and Rst are present in FCMs. The heterophilic interaction of these Ig-domain proteins leads to signal activation and results in the formation of F-actin at the sites of cell-cell contact. Duf and Rst serve in functional redundancy in FCs. The formation of new actin filaments at existing filaments is regulated by the actin-related protein complex (Arp)2/3. The Arp2/3 complex is a multiprotein complex consisting of seven subunits including Arp2 and Arp3. The complex becomes activated by nucleation promoting factors (NPFs), i.e the Wiskott-Aldrich syndrome protein (WASP) and the suppressor of CAMP-receptor (SCAR). Additionally, WASP is activated by the WASP-interacting protein Verprolin1 (WIP). These proteins act together in the WASP-WIP complex. Although WASP and SCAR are activated differently to induce Arp2/3-mediated actin polymerization, they share a common proline-rich region, which is known to bind to SH3 domain containing proteins.

In this study I have addressed two questions. First, I have tried to elucidate the redundant nature of Duf and Rst in FCs. The intracellular domain of Rst and Duf contains three conserved domains, i.e a PADVI, SAIYGNPYLR and NSLLPPLPP domain. Expression of RstΔPADVI in all myoblasts or exclusively in FCs as well as FCMs impaired myoblast fusion. This indicates that the PADVI domain in the intracellular domain of Rst plays an important role during myoblast fusion. To identify Rst specific interaction partners I further performed a yeast two-hybrid screen and identified Actin57B, Papilin and Nidogen as possible Rst interaction partner.

Second, I investigated the role of the SH2-SH3 adopter Dreadlock (Dock) in myoblast fusion. Dock is expressed in FCs and FCMs. In vertebrates, the homologue of Dock - called Nck - links cell adhesion during podocyte formation with actin cytoskeleton rearrangement by binding to the intracellular domain of Nephrin. Nephrin is a homologue of *Drosophila* Sns and Hbs. Biochemical data provided in this study show that Dock is able to bind to the intracellular domain of Sns, Hbs, Duf and Rst and to the proline-rich region of the actin regulators WASP and WIP via its SH3 domains. Interestingly, I found that the SH2 domain of Dock binds to phosphorylated tyrosine at position 1089 in the intracellular domain of Hbs. The SH3 domains of Dock can bind to Sns and Duf and all SH2 and SH3 domains are required to bind to Rst. To demonstrate that these protein interactions are relevant for myoblast fusion, I carried out double mutant experiments. I could show that *dock* interacts genetically with the FC-specific gene *rols*, which encodes for an adaptor protein that binds to the intracellular domain of Duf, and with the FCM-specific cell

adhesion molecule *hbs*. These data indicate that Dock functions in both myoblast populations during myoblast fusion and serves as a linker to transfer the fusion signal from the cell adhesion molecules Duf and Hbs to the actin cytoskeleton, e.g. by interacting with WASP and WIP.

1. Summary

Zusammenfassung

Die somatische Muskulatur von *Drosophila* entsteht analog zur Skelettmuskulatur der Wirbeltiere durch die Fusion von einkernigen Myoblasten. In *Drosophila* unterscheidet man zwischen zwei unterschiedliche Myoblastenpopulationen, die miteinander fusionieren: Gründer-Zellen (FCs) und fusion-kompetent Myoblasten (FCMs). Die Erkennung und Adhäsion der beiden Myoblastentypen wird von Mitgliedern der Immunoglobulin-Superfamilie (IgSF) vermittelt. Diese Zelladhäsionsmoleküle werden FC- und FCM-spezifisch exprimiert: Duf und Rst kommen in FCs vor. Sns, Hbs und Rst werden in FCMs exprimiert. Die heterophile Interaktion der Zelladhäsionsmoleküle führt zur Aktivierung einer Signalkaskade und zur Bildung von F-Aktin am Zell-Zell-Kontakt. Dabei wirken Duf und Rst in FCs in funktioneller Redundanz. Die Bildung neuer Aktinfilamente an bestehende Filamenten wird durch den Aktin-verwandten Protein-Kompalex (Arp)2/3 geregelt. Der Arp2/3-Komplex besteht aus sieben Untereinheiten, einschließlich Arp2 und Arp3. Aktiviert wird der Arp2/3-Komplex durch sogenannte „nucleation promoting"-Faktoren (NPFS), d.h. durch das Wiskott-Aldrich syndrome Protein (WASP) und den Supressor des CAMP-Rezeptors (SCAR). WASP wird durch das WASP-interagierende Protein Verprolin1 (WIP) aktiviert. Obwohl WASP und Scar den Arp2/3-Komplex auf unterschiedliche Weise aktivieren, beinhalten sie eine gemeinsame Prolin-reiche Region an die Proteine mit einer SH3-Domäne binden können.

In dieser Arbeit habe ich mit zwei Fragen befasst. Zuerst habe ich versucht Hinweise auf die redundante Funktion von Duf und Rst in FCs zu erhalten. Die intrazelluläre Domäne von Rst und Duf enthält drei konservierte Domänen, d.h. eine PADVI-, SAIYGNPYLR- und NSLLPPLPP-Domäne. Die Expression von RstΔPADVI in allen Myoblasten bzw. die ausschließliche Expression von RstΔPADVI in FCs oder in FCMs führt zu Fusionsdefekten. Dies lässt darauf schließen, dass diese Domäne eine wichtige Funktion während der Rst-vermittelten Signaltransduktion spielt. Um Interaktionspartner von Rst zu identifizieren habe ich zudem einen Hefe-2-Hybrid Screen durchgeführt und Actin57B, Papilin und Nidogen als mögliche Interaktionspartner identifiziert.

Im zweiten Teil meiner Arbeit habe ich mich mit der Rolle des SH2-SH3 Adaptorproteins Dreadlock (Dock) während der Myoblastenfusion befasst. Das Vertebraten-Homolog von Dock, Nck, verbindet Nephrin-vermittelte Zelladhäsionsprozesse mit der Umstrukturierung des Aktin-Zytoskeeletts bei der Podozyten-Bildung. Nephrin ist verwandt mit *Drosophila* Sns und Hbs. Biochemische Analysen, die in dieser Arbeit durchgeführt wurden zeigen, dass Dock an die intrazelluläre Domäne von Sns, Hbs, Duf und Rst sowie an die Prolin-reiche Region von WASP und WIP binden kann. Interessanterweise konnte ich zeigen, dass die SH2-Domäne von Dock an phosphoryliertes Tyrosin an Position 1089 in der intrazellulären Domäne von Hbs bindet. Die SH3-Domänen von Dock vermitteln dagegen die Bindung mit der intrazellulären Domäne von Sns und Duf. Sowohl die SH2- als auch die SH3-Domänen von Dock vermitteln die

Bindung an die intrazelluläre Domäne von Rst. Um zu zeigen, dass diese Protein-Interaktionen auch während der Myoblastenfusion erfolgen, habe ich eine Reihe von Doppelmutantenanalysen durchgeführt. Dabei konnte ich zeigen, dass *dock* genetisch mit dem FC-spezifischen Gen *rols* interagiert. Rols kodiert für ein Multidomänprotein, das an die intrazelluläre Domäne von Duf bindet. Weiterhin konnte ich eine genetische Interaktion zwischen *dock* und *hbs* aufzeigen. Die genetischen Interaktionsstudien belegen, dass die Funktion von Dock sowohl in FCs als auch in FCMs während der Myoblastenfusion von *Drosophila* benötigt wird. Dies legt die Vermutung nahe, dass Dock das Fusionssignal von den Zelladhäsionsmolekülen Duf und Hbs an Komponenten der Aktin-Regulation, wie WASP und WIP überträgt

2. Introduction:

2.1 *Drosophila melanogaster*

Drosophila melonogaster, the fruit fly, belongs to the class of insectae, Order of dipteria. Its short generation time (10 days if kept at 25^{o} C) and small size which makes breeding and keeping in large numbers possible, are ideal conditions for biological experiment. Furthermore, the development from fertilized egg to a larvae is completed within 24 hours. During this time span, complicated processes such as the development from germ lines to full organs provide an ideal basis for observations and studies on developmental processes. The fruit fly was first introduced in to labs as the beginning of the 20th century by Thomas Hunt Morgan. Because of the advantageous features mentioned above, *Drosophila melanogaster* quickly became established as a model organism. In 2000, the entire genome of *Drosophila melanogaster* was discovered with the help of the "whole genome shot-gun sequencing" method (Adams *et al*.,2000; Celniker *et al*., 2002). The entire genome was distributed over 3 autosomes and 1 gonosome, which makes studies on genomic levels much easier. Now it is important for scientist world-wide to understand the function of the deciphered genes and in which way each of them interact with each other to provide important conclusions for higher organisms, since many functions have been shown to be conserved over most of the animal kingdom.

Drosophila is a model organism for developmental aspects like myogenesis. Studies at the cellular and molecular levels various mutants of *Drosophila* embryos reveal regulatory mechanisms with conserved protein interaction patterns.

2.2 Formation of the Mesoderm in *Drosophila melanogaster*

In *Drosophila* all muscle cells develop from the mesoderm, which is the middle germ layer in theearly embryo. The mesoderm derives from the invagination of the ventral cells of the small epidermis during blastoderm stage. Therefore, the regulatory events controlling dorsal-ventral development in the oocyte and the early embryo are the earliest events in

muscle formation. The first stage in dorsal-ventral development can be traced back to the oocyte, where Gurken-Torpedo signaling establishes dorsal-ventral asymmetry. The ventral half of the oocyte is then allowed to express Pipe, which serves to activate a series of serine proteases. These activation events ultimately lead to the stimulation of the Toll receptor in the ventral side of the early embryo. In the final stage of this Toll maternal cascade there is the formation of the Dorsal protein gradient in the ventral nuclei of the embryo during blastoderm stage. The Dorsal gradient both activates and represses zygotic gene expression to establish mesodermal cell fate and promote mesoderm invagination. The invaginated mesoderm then differentiates into appropriate muscle tissue types according to further positional information. The mesoderm gets subdivided into the fat body and the three myogenic tissue by systematic specific transcription factors: musculature of the body wall equals to the somatic mesoderm, visceral mesoderm and the cardiac mesoderm, depicted In FIG 2.1 Reichmann et al.,(1997).

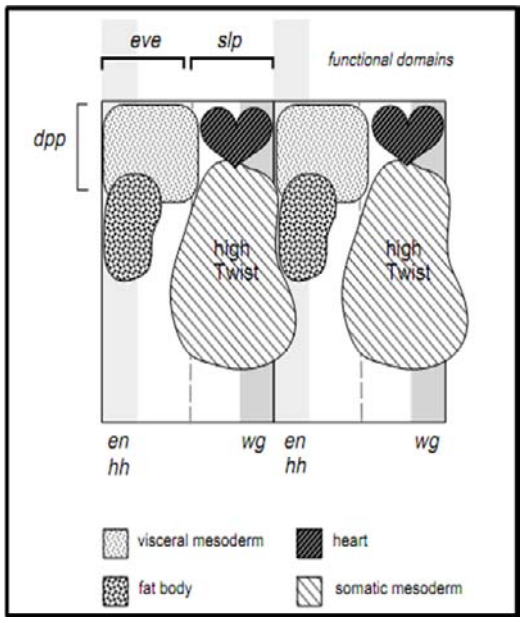


FIGURE 2.1 **Mesoderm subpoupulation tissues.** The anterior (A)- posterior (P) axis is formed by *evenskipped (eve) and sloppy-paired (slp)*. The *slp* domain induces the dorsally located heart precursors and the majority of somatic mesoderm. The somatic muscles development requires high level expression of *twist*. In the *eve* domain dorsally located group cells induces the visceral mesoderm. The fat body originates from mesodermal cells of the anterior segment. The ectoderm induced by *Decapentalegic* (*Dpp*) into visceral mesoderm, heart and dorsal somatic mesoderm. Segment-polarity genes i.e *engrailed (en), hedgehog (hh) and wingless (wg)* regulate the mesoderm subdivision such as heart, fat body, visceral mesoderm and somatic mesoderm. Modified after Reichmann *et al*., (1997).

2.3 Development of Somatic musculature in *Drosophila melanogaster*

The development of the somatic musculature is crucial for this study including the origin and determiation of myoblast, as well as the fusion of myoblasts and mature muscle formation; these steps are controlled by various processes.

The larval bodywall musculature of *Drosophila* is arranged in a highly stereotyped pattern of 30 muscle fibers per hemisegment. Each muscle possesses a distinctive set of properties; size, shape, orientation to the epidermis and specific innervations. The acquisition of these muscle properties during myogenesis depend upon the prior specification of special class of myoblast called founder cells. Each larval muscle is prefigured by a single founder cell, which seeds the muscle formation by fusing with surrounding fusion-competent myoblast. The purpose of these processes is to review the molecules and mechanisms underlying the "birth" off the founder cell and fusion-competent myoblast. These progenitor myoblast are controlled by various transcription and signalling processes to form from mononucleated myoblast to multinucleated matured muscle.

In the next I focus on how these progenitor myoblast are specified. Additionally I present known signalling that triggers the formation of multinucleated muscles.

2.3.1 Determination of Myoblast: Founder cell and Fusion competent cells

The development of the embryonic musculature is intiated by the specification of a so far uniform cell population of the somatic mesoderm that give rise to two classes of myoblasts (FIG. 2.2). The muscle formation is intiated by the specification of a distinctive founder cells in the muscle-forming mesoderm. The founder then fuses with neighbouring fusion-competent myoblasts, recruiting them to its pattern of gene expression and forming a syncytial precursorcell. (Bate, 1990, 1993). These primary cells are formed by strict cell determination mechanisms including the lateral inhibition via the Delta-Notch pathway and asymmetric cell division. Finally Founder cells (FCs) and Fusion-Competent Myoblast (FCMs) are specified by different transcription factors (FIG. 2.2: Richardson *et al*., 2008).

In the beginning, all mesodermal cells which will give rise to somatic musculature contain high amounts of Twist. A cluster of

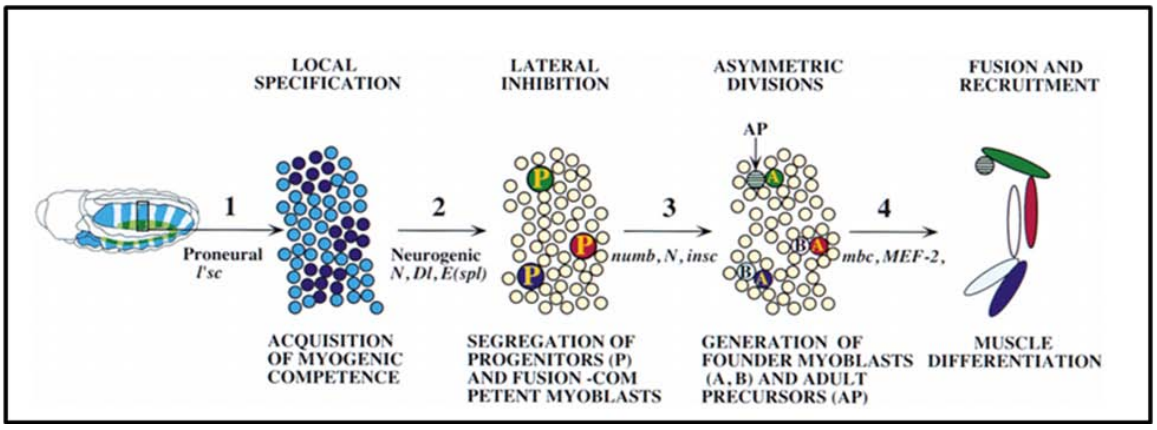


FIG. 2.2 **The development of *Drosophila* larval musculature**. Modulated pattern of twist expression at the embryonic stage 10. **1**. The preneuronal gene *lethal of scute (l'sc)* determines clusters of mesodermal cells as potential muscle progenitors (dark blue). **2.** Single cells becomes into muscle progenitors (P) and the remaining cells in the myogenic cluster behave as fusion-competent myoblasts. **3**. Assymetric division of the progenitors gives rise to a pair of founder or to a founder and adult muscle precursor (AP). **4.** Each founder cell forms a specific muscle by fusing with fusion-competent cells, by specific gene expression to form myotubes (Baylies *et al*., 1998)

small cells starts to express *Lethal-of-Scute (L, Sc)*, a protein known to give rise to muscle progenitors (dark blue cells, FIG 2.2 1&2: Baylies *et al*., 1998; Carmena *et al*., 1995; Nose *et al*., 1998). Lateral inhibition mediated by the proteins Notch and Delta singles out one cell that continous to express *l'Sc* while the surrounding cells are inhibited from continuing expression of *l'Sc* (FIG 2.2 2&3). This single cell is termed progenitor cell (P) that undergoes asymmetric cell division to give rise to either a founder myoblast (FIG 2.2 3 (A, B) and an adult precursor or to two founder cells (FIG 2.2 3; AP). The cells surrounding this progenitor cell become FCMs and begin to expresss a different subset of genes (FIG 2.2 3; cells colored light).

The assymetric cell division is mediated by *inscrutable (insc) and numb (nb)* (Carmena *et al*., 1998; Paululat *et al*., 1999). In *Drosophila*, experiments shows that both proteins localize as

cortical crescents opposing each other. Furthermore, it has been shown that Numb seems to behave antagonistic towards the Notch signalling pathway.

Information stored in FC later determine what type of muscle fibre will be. As stated in a review from Baylies *et al.,* 1998, there are proteins that seem to be responsible for the development of one specific muscle type. These are expressed by the FCs, as well as by precursors and myotubes later on. FCMs fusing with these cells naturally take over the same fate and begin expressing muscle-specific genes as well.

2.4 The processes of myoblast fusion during *Drosophila* embryogenesis

After the the determination of the different myoblast-types the fusion process in the somatic musculature of the embryo occurs in two waves (Rau *et al*., 2001) and is completed after 5.5 hours (Beckett and Baylies, 2007). The first fusion starts at the end of stage 11 in the embryo (Bate, 1990; Beckett and Baylies, 2007). The embryonic muscle formation in the wildtype is completed at stage 16 (Beckett and Baylies, 2007; Dworak and Sink, 2002; Chen and Olsen, 2004).

The intial waves of cell recognition and adhesion between FCMs and FCs can be observed at the light microscopy level during the first fusion wave (FIG 2.3 A; Doberstein *et al*., 1997). Before fusion occurs, FCMs form filopodia towords the FC and the tip of the filopodia attaches to the FC membrane. When both cells are in close contact with each other the membranes fuse and a precursor cell is formed. The second wave of fusion is characterized on electron microscopy level (FIG. 2.3 B; Doberstein *et al*., 1997). So far expression are not observed during the intial waves of fusion process (FIG 2.3 A to B; Schroeter *et al*., 2006). Cell-cell recognition and adhesion between the growing myotube and FCMs is followed by the appearance of a prefusion complex, electron-dense plaque formation and membrane fusion (FIG 2.3 B). Doberstein *et al*.(1997) described that the prefusion complex consists of approximately 15 to 20 electron-dense vesicles at the site of cell contact. These vesicles line up on opposite membranes (Doberstein *et al*., 1997). Doberstein *et al*.(1997) counted the vesicles in serial sections. The great difference between both studies is due to a difference in their counting method and needs to be further analyzed. In a next wave the prefusion

complex resolves into electron-dense plaques. The two myoblast elongate and small fusion pores begins to form. The membrane remnants between the small fusion pores become removed in clear vesicles so that a large fusion pore forms. Finally, the fusion pores expands and the FCM becomes integrated into the growing myotube.

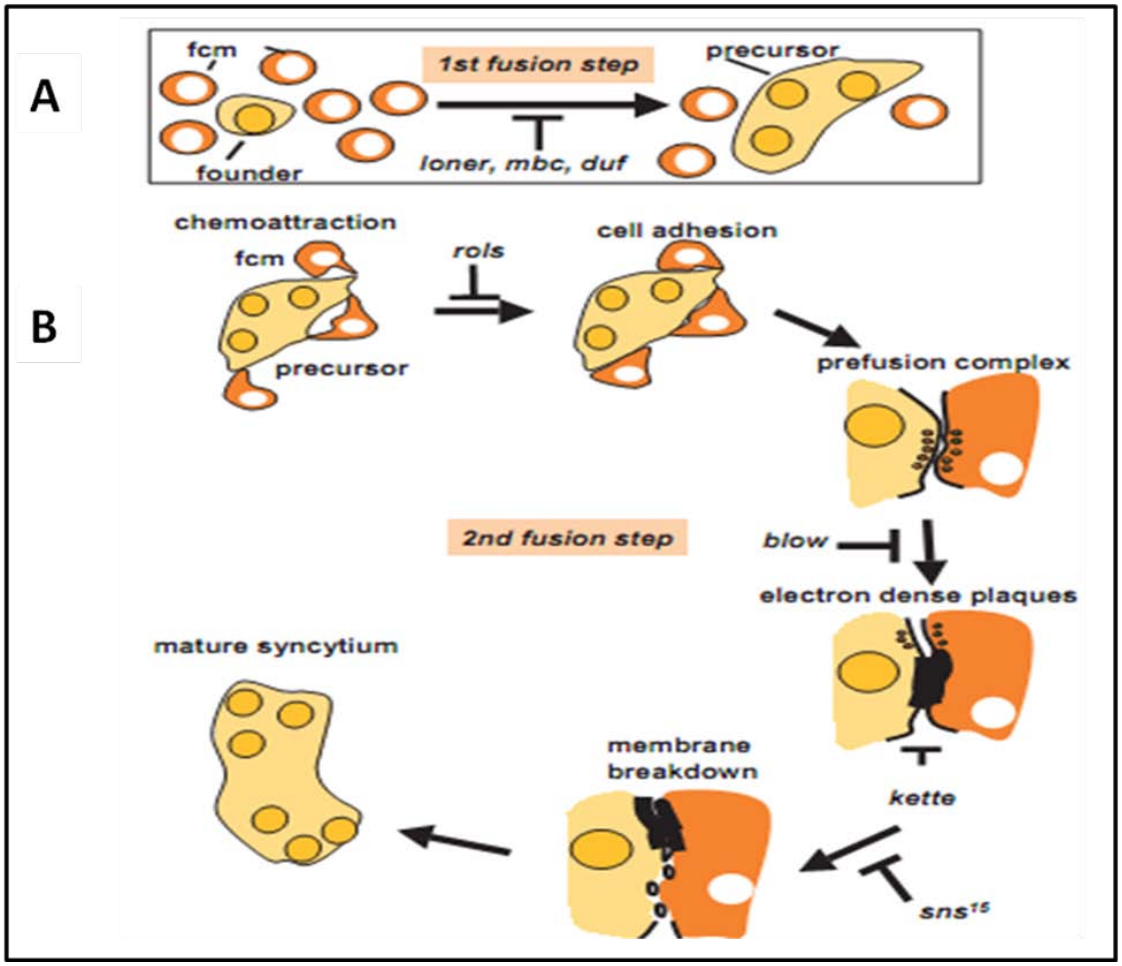


FIGURE 2.3 **Two step myoblast fusion model and important molecular players that trigger the fusion process**. **A.** In the intial fusion wave the founder cell (FC) fuses with two fusion-competent myoblast (FCMs) forming a tri-nucleated precursor cell. This process requires cell-cell recognition and adhesion between both myoblast cell-types. **B.** In the second-wave precursor cell fuses with further FCMs to become a mature muscle fibre. After the recognition and adhesion between precursor cells and FCMs the prefusion complex is formed followed by resolving of this complex into electron-dense plaques. Finally after the second wave the membrane breakdown between both myoblast cells is removed in clear vesicles. Duf, Rst, Hbs and Sns are required for cell-cell recognition

in both fusion waves. Mutations in s*chizo(loner), mbc, sns, rst* and *duf* lead to a halt in fusion. Loss of function of Rols, Blow, Kette, and give rise to precursor cells and unfused FCMs.

The molecular compenents involved in myoblast fusion have been classified by their mutant phenotype to be either required for the first and the second fusion wave. In the first fusion wave, FCs express Dumfounded/Kin of Irre (Duf/Kirre) and Roughest/Irregular-chiasm-C (Rst/IrreC) and serve as chemoattractants for FCMs (Ruiz-Gomez *et al*., 2000; Strunkelnberg *et al*., 2001). In FCMs Sticks and Stones (Sns) is expressed exclusively (Bour *et al*., 2000, Ruiz-Gomez *et al*., 2000). They are the members of the immunoglobin super family (IgSF) proteins. Further experiments on *Drosophila* Schneider cells (SL2) proved the colocalization of Duf/Krre and Sns proteins (Dworak *et al.,* 2001). However certain proteins, such as Roughest/Irregular-chiasm-C (Rst/IrreC), have been found in both cell types as well (Ramos *et al*., 1993). Null mutations in *duf, rst*, or *sns* disrupt the first fusion wave (Bour *et al*., 2000; Ruiz-Gomez *et al*., 2001). Moreover, Hibris (Hbs) is excusively expressed in FCMs and is assumed to be important for myoblast fusion. The role of transmembrane protein Rst is not clear yet, even though its structure highly resembles Duf to have a redundant function in FCs (Ruiz-Gomez *et al*., 2001; Strunkelnberg *et al*., 2001. However, Sns and Hbs act together during fusion is poorly understood. Artero *et al*. (2001) described that Hbs antagonizes Sns function, but in the same year Dworak *et al*. (2001) described that Hbs function is redundant to Sns. Menon *et al.*(2005) described that Hbs and Sns act in functional redundant and also Shelton *et al*.(2009). Recent studies on the extrcellular domains of Duf and Rst can recognize and bind to the extracellular domains of Sns and Hbs (Galletta *et al*., 2004; Dworak *et al*., 2001). The heterophilic interaction between these transmembrane proteins is highly conserved. The vertebrate homolog Nephrin (*Drosophila* Sns and Hbs) and NEPH1 (*Drosophila* Duf and Sns) are essential for podocyte function of the Nephrin family possess putative phosphorylation sites which were shown to mediate a signal to the cytoskeleton after multiple tyrosine phosphorylations (Li *et al*., 2004; Verma *et al*., 2006; Jones *et al*., 2006).The *Drosophila* Nephrin orthologs Sns and Hbs and the Neph1 homologs Duf and Rst also posses phosphorylation sites in their intracellular domain.

During the second wave fusion, the muscle precursor recruits further FCMs. This process is induced by Rolling pebbles7/Antisocial (Rols7/Ants; Rau *et al*., 2001) which serves as an adaptor and links Duf to the components of the cytoskeleton. Rols7 is a cytoplasmic protein that stabilizes the cell adhesion. Recent studies identified that Duf and Sns form a ring –like adhesion structure at cell-cell contact points which contains filamentous (F)-actin in their center and is known as fusion-Restricted Myogenic-Adhesive structure (FuRMAS; Kesper *et al.,* 2007). The actin accumulation is not formed in *duf* and *sns* mutants (Richardson *et al*., 2007), suggesting that beyond the response leading to the reorganization of the actin cytoskeleton (Chen and Olsen, 2004). As a consequence membrane breakdown occurs and fusion takes place.

2.5 Actin Plug/foci form after cell adhesion

In the *Drosophila* embryos, cell-cell adhesion leads to the formation of a ring-like structure, named "FuRMAS" (Fusion-Restricted myogenic-Adhesive structure) (Kesper *et al*., 2007). The FuRMAS contains, F-actin in the center called actin plug or actin foci (FIG 2.4; Kesper *et al*., 2007; Ricchardson *et al*., 2007) suggesting that the FuRMAS is a dynamic structure. In wild-type the size of the ring ranges from 1 to 5 μm. The average area of actin plug is approximately 1.9 um^2 (Ricchardson *et al*., 2007). Based on the size and number of actin plug, Ricchardson *et al*. (2007) divided known muscle mutants into three classes of mutants. The mutants encoding for proteins which are required for cell-cell recognition and adheseion, like Sns/Duf, Rols shows ring intilally in 1 um in diameter in actin plug and no or fewer actin plugs (Ricchardson *et al*., 2007). This indicates that the formation of actin plugs requires successful cell adhesion. In the second class of mutants, absence of intracellular components that regulate the formartion of F-actin such as *schizo/loner* with an increased number of wild type size actin plugs. The third class of mutants is characterized by an increased number and size of actin plugs, such as *blownfuse (blow)*, *kette, rac* and *myoblast city (mbc)*. They all encode for genes which are required for the regulation of actin polymerization. These actin plugs/foci are highly dynamic structures and are established

within 2 min and dissolve completely in less than 1 min, but can be present at the individual fusion take 12 min on average (Ricchardson *et al*., 2007).

The cytoplsmic tails of the cell adhesion molecules are involved in the signalling/ transdusction process (Sink, 2006). The multidomain protein Rolling pebbles 7 (Rols7), also known as Antiscocial (Ants) in *Drosophila* (Chen and Olsen, 2001; Menon and Chia 2001; Kreisköther *et al*., 2006). The Rols and Duf co-localizes in a ring-like manner within the FuRMAS in the FC/growing myotube (FIG 2.4; Kesper *et al*., 2007). Rols links Duf (Rau *et al*., 2001) to the components of the cytoskeleton by additionally binding to *Drosophila Titin (D-Titin)* and *mbc* (Chen and Olsen *et al*., 2001; Menon and Chia 2001; Kreisköther *et al*., 2006).

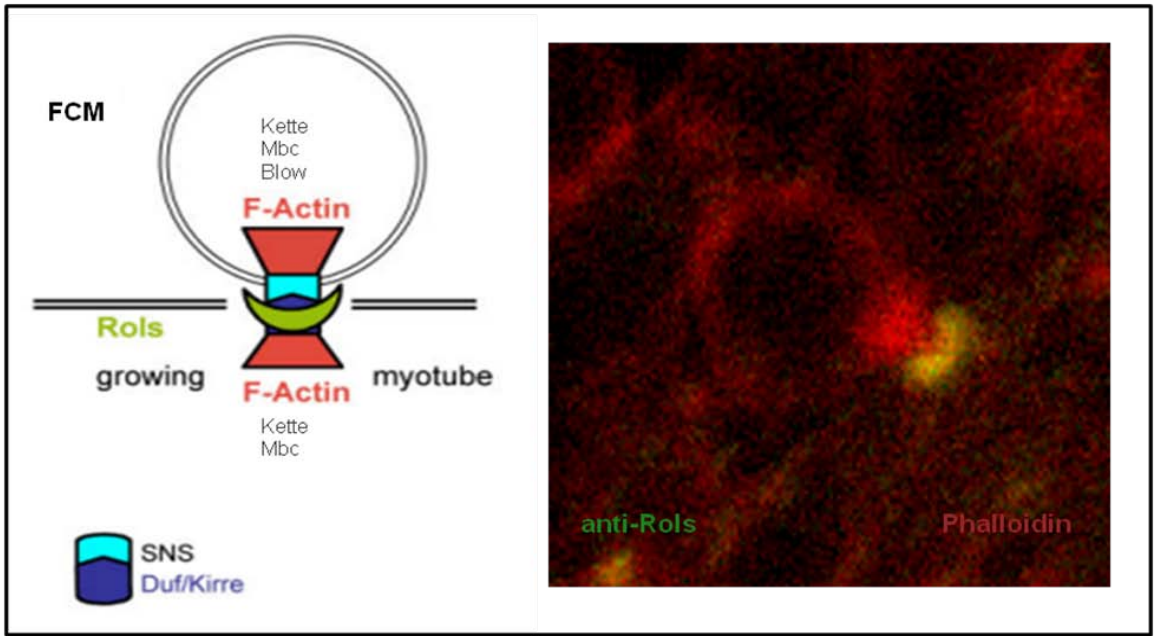


FIGURE 2.4 **Formation of actin foci in fusion process**. During the heterophilic cell adhesion process i.e contact between the FCMs and FCs, Sns (in FCMs) and Duf (in FCs) form a ring-like structure that is stabilized by Rols7. Rols7 co-loalizes with Duf in the growing myotube. The FuRMAS contains F-actin in the center. Other proteins localize with the actin foci e.g. Blow, Kette and Mbc (Kesper *et al*., 2007; Richardson *et al*., 2007

The intacellular domain of Duf was shown to interact in a yeast two-hybrid test with the TPR-repeats of Rols7 (Kreisköther *et al*., 2006). Furthermore, Co-Immunoprecipitation (CoIPs) studies with Duf and Rols7 or Rols7 and Mbc after transfection into S2 cells revealed an interaction of Rols7 with Duf as well as with the guanine nucleotide exchange factor

(GEF) Mbc (Chen and Olsen *et al*., 2001). Mbc, the homolog of human DOCK180 appears to be expressed in both myoblast cell types (FIG 2.4; Rushton *et al*., 1995; Erikson *et al*., 1997; Ricchardson *et al*., 2007). Thus Rols7 serves as a linker between the membrane and the actin cytoskeleton via Mbc and Rac. The loss of Mbc function leads to a complete block of myoblast fusion in *Drosophila* (Rushton *et al*., 1995; Erikson *et al*.,1997) like in *rac1* and *rac2* double mutants. In vertebrates Rac1 is activated by another pathway by the SH2-SH3 adopter protein Crk-II which act in complex with DOCK180 (Kiyokawa *et al*., 1998; Brugnera *et al.,* 2002). The *Drosophila* Crk binds to the Mbc during myoblast fusion (Erikson *et al*.,1997; Nolan *et al*., 1998).

Another signalling molecule that is required for the myoblast fusion is the *Drosophila* GEF *schizo/loner*. Mutation in *schizo/loner* lead to a strong fusion phenotype. Schizo/Loner, a GEF for the Adenosin ribosylation factor (Arf) family GTPases which is exclusively expressed in FCs and was shown to bind arf6 in invitro assays (Chen *et al*., 2003). *arf6* loss-of-function mutants exhibit a wild-type muscle pattern and mutant flies are vital but males are sterile (Dyer *et al*., 2007). This indicates that, Schizo/Loner may activate the Arf GTPase besides Arf6. Recent experiments could show that Schizo/Loner is involved in the activation of the Arf1-GTPase (Dottermusch *et al*., submitted). Richardson *et al.* (2007) describe that Schizo/Loner is expressed in FCs/growing myotubes and FCMs. They also described that Schizo/Loner is expressed outside of the actin plugs/foci and not at the adjacent membranes between FCMs and FCs. Thus, Schizo/Loner seems not to be a prominent component of the FuRMAS. However, Schizo is clearly essential for fusion, but its exact role needs further clarification.

Another actin regulator HEM-2/Nap1-known as Kette in *Drosophila* is also essential for myoblast fusion (Schröter *et al*., 2004). Kette is expressed in both cell types, i.e FCs and FCMs (FIG 2.4; Schröter *et al*., 2004; Ricchardson *et al*., 2007). *kette* was shown to interact genetically with the FCM-specifically expressed gene *blownfuse (blow)* during fusion (Schröter *et al*., 2004), this strongly implies that the actin rearrangement is intiated by FCMs and FCs specific regulatory gene cascades.

Another protein which is involved in myoblast fusion is the WASP-interacting protein (WIP), also known as Verprolin (Vrp1) in *Drosophila* (Kim *et al*., 2007; Massarwa *et al*., 2007; Berger *et al*., 2008). WIP is exclusively expressed in FCMs like Blow (FIG 2.4; Berger *et a*l., 2008; Schäfer *et al*., 2007; Schröter *et al*., 2004; Kim *et al*., 2007; Massarwa *et al*., 2007; Beckett and Baylies, 2006) and homozygous *wip* null mutant display arrested fusion after precursor formation. On the ultra-structural level, Kim *et al*. (2007) observed a mislocalization of the prefusion complex and accumulation of vesicles to the membrane; however the membrane appears to remain intact. Interestingly, *wasp* mutants exhibit a highly similar phenotype to *wip* mutants (Berger *et al*., 2008; Massarwa *et al*., 2007). Until know it is more understood how actin polymerization is regulated in FCMs thanin FCs.

2.6 Filamentous actin polymerization.

Actin is a globular protein, 42-kda moonlighting protein found in all eukaryotes. It is also one of the highly conserved proteins. Actin participates in many important cellular processes including muscle contraction, cell motility, cell division and cytokinesis, vesicle and organelle movement, cell signaling, and the establishment and maintenance of cell junctions, cell shape and cell-cell fusion. Filamentous F-actin consists of monomeric globular (G)-actin. Each asymmetric filament possesses two ends; a fast growing barbed end and a slower growing pointed end that are distinguishable by their structural characteristics and kinetic properties. G-actin can self assemble into F-actin (FIG 2.5), but this process of self nucleation is kinetically unfavourable. ATP hydrolysis in the filament is tightly coupled to polymerization and regulates the kinetics of assembly and disassembly, as well as the association of interacting partners. Spontaneous is a kinetic hurdle in the process of actin polymerization, and, therefore, factors that can accelerate or bypass this step are important for efficient actin assembly in the cell. So, far three classes of protein have been identified that initiates new filament polymerization in various species: the actin-related-protein-2/3 (Arp 2/3) complex, the formins and spire (FIG 2.5; Goley and Welch, 2006).

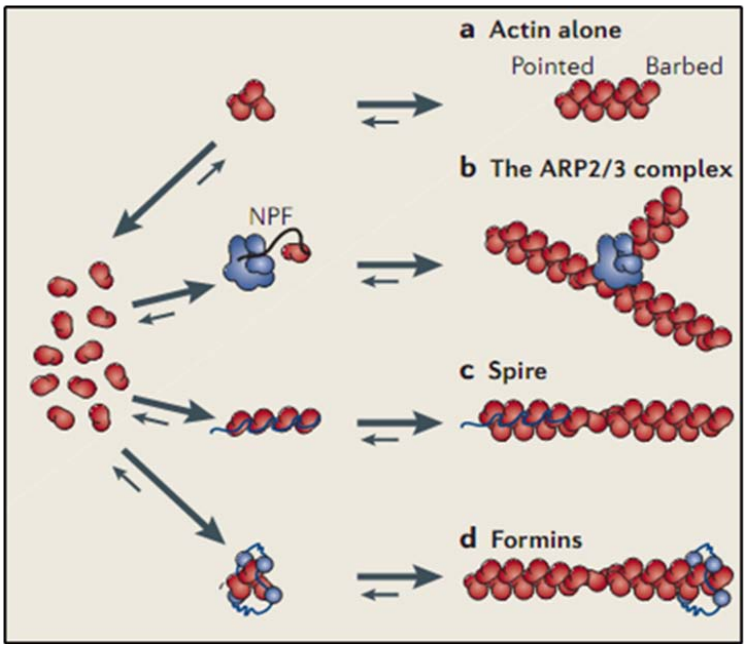


FIGURE 2.5 **Actin nucleation.** Spontaneous initiation of actin-filament assembly requires the formation of a trimeric nucleus is kinetically unfavourable (**a**). Three different classes of proteins or factors that initiates new F-actin polymerization; the actin-related-protein-2/3 (Arp2/3) complex, formins and spire. The actin-related-protein-2/3 (Arp2/3) has a crucial role in the formation of branched-actin-filament networks (**b**). Spire protein mediates longitudinal association of four actin subunits and functions as scaffold for polymerization into an unbranched filament (**c**). The formins also promote the nucleation of unbranched filament, a dimer of formin-homology-2 (FH2) domain stabilizes an actin dimer or trimer to facilitate nucleation event (**d**). Modified after Goley and Welch, 2006. NPF= Nucleation promoting complex.

The actin-related-protein-2/3 (Arp2/3) plays a crucial role in the formation of branched-actin-filament networks. The Formins promote the nucleation of unbranched filaments, a dimer of formin-homology-2 (FH2) domain stabilizes an actin dimer or trimer to facilitate nucleation event. Until now four Formins protein have been identified in a phylogenic analysis of the FH2 domain of *Drosophila*: formin3 (form3), dishevelled associated Activator of Morphogenesis (DAAM), CG32138 (FRL) and diaphanous (dia) (Higgs and Peterson, 2005). The Spire protein contains Wiskott-Aldrich homology domain 2 (WH2) and mediates longitudinal association of four actin subunits and function as scaffold for polymerization

into an unbranched filament. The activation of the Arp2/3 complex, depend on the nucleation promoting factors (NPFs), like Wiskott-Aldrich syndrome protein (WASP) family proteins.

In the next chapters I present short introduction into how linking of immunoglobin super family proteins (IgSF) cell adhesion mediated molecules with Arp2/3-based actin polymerization during *Drosophila* myoblast fusion.

2.7 Linking cell adhesion with Arp2/3-based actin polymerization

The Arp2/3 complex is an important factor for actin polymerization during myoblast fusion. Several questions have been raised, how the cell adhesion molecules mediate the signaling cascades to the actin cytoskeleton.

2.7.1 The cell adhesion molecules in myoblast fusion.

The somatic musculature of *Drosophila* is formed by the cell-cell fusion events. Myoblast requires the recognition of and adhesion depends between the two distinct myoblast cell types, named as founder cells (FCs) and fusion-competent myoblast (FCMs). The process of recognition and adhesion depends on the evolutionary conserved members of the immunoglobulin (Ig) superfamily Dumbfounded/Kin of Irre (Duf/Kirre) and Roughest (Rst) in FCs (Ruiz-Gomez *et al*., 2000; Strunkelnberg *et al*., 2001), and Sticks and stones (Sns) and Hibris (Hbs) in FCMs (Bour *et al*., 2000, Ruiz-Gomez *et al*., 2000). However certain proteins, such as Roughest/Irregular-chiasm-C (Rst/IrreC), have been found in both cell types as well (Ramos *et al*., 1993). Next I introduce the natur, structure, and their known interacting partners and vertebrate homologs of cell adhesion molecules of myoblast fusion.

Sticks and stones (Sns). *Sticks and stones (Sns)* was the first extracellular identified with a role in myoblast fusion (Bour *et al*., 2000). Sns is a member of the IgSF and has eight immunoglobulin (Ig-like) domains, a “degenerate” Ig-like domain, and a Fibronectin type III (FN-III) domain (FIG 2.6; Bour *et al*., 2000). This ectodomain structure is characteristic of the

IgSF subfamily known as Nephrins, which has representatives in *C. elegans*, mouse, rat and human as well as *Drosophila* (Bour *et al*., 2000; Artero et al., 2001; (Dworak *et al*., 2001). The Sns cyotplasmic domain comprises 376 amino acids, with no classical signalling domains; however, it has a potential target site for protein kinase C (PKC) and one for casein kinase II (CK-II). Recent studies identified *Drosophila* Crk is the SH2-SH3 adopter protein as binding partner of Sns (Kim *et al*., 2007). Recent publication described two isoforms of *Drosophila* Crk which differ by 18 amini acids in the SH2 domain (Balagopalan *et al.*, 2006). Because of this difference in the SH2 domain, which could possibly lead to a different interaction between the two isoforms of Crk and Sns. Interrestingly, (Schäfer et al., unpublished data) found that only the long isoform of Crk in the S2 cell lysate and embryo lysate, showing that there is no other isofrom of Crk.

Dumbfounded/Kin of Irre (Duf/Kirre). *Dumbfounded (Duf)* identified in the region spanned by the deletion and found to encode an IgSF protein, Duf (Dworak *et al*., 2001). Duf is new member of the DN-GRASP/BEN/SC1 subfamily (Sotelo *et al*., 1997) and has five Ig-like domains and a cytoplasmic domain comprising 367 amino acids. The cytoplasmic domain has a consensus sequence for the type of autophosphorylation domain found in receptor tyrosine kinase, a candidate PDZ-binding domain, and a putative serine phosphorylation site (Strunkelnberg *et al*., 2001). *duf* is expressed by founder cells and forming myotubes until end of myoblast fusion, deletion of *duf* and its paralogue *rst* displays a myoblast fusion phenotype and the *duf* expression to all of the mesoderm rescues the deletion phenotype (Ruiz-Gomez *et al*., 2000). The Sns and Duf could indeed bind in a heterotypic manner, and also that Duf could bind in a homotypic manner (Dworak *et al*., 2001). The intacellular domain of Duf was shown to interact in a yeast two-hybrid test with the TPR-repeats of Rols (Kreisköther *et al*., 2006). Recent studies showed that Duf intracellular domain between amino acid 687 and 830 of duf plays an important role in translocation of Rols and loner in S2 cells (Bulchand *et al*., 2010). Being its long cytoplasmic domain contains tyrosines, still raises many questions and needs to be further analyzed its role in myoblast fusion.

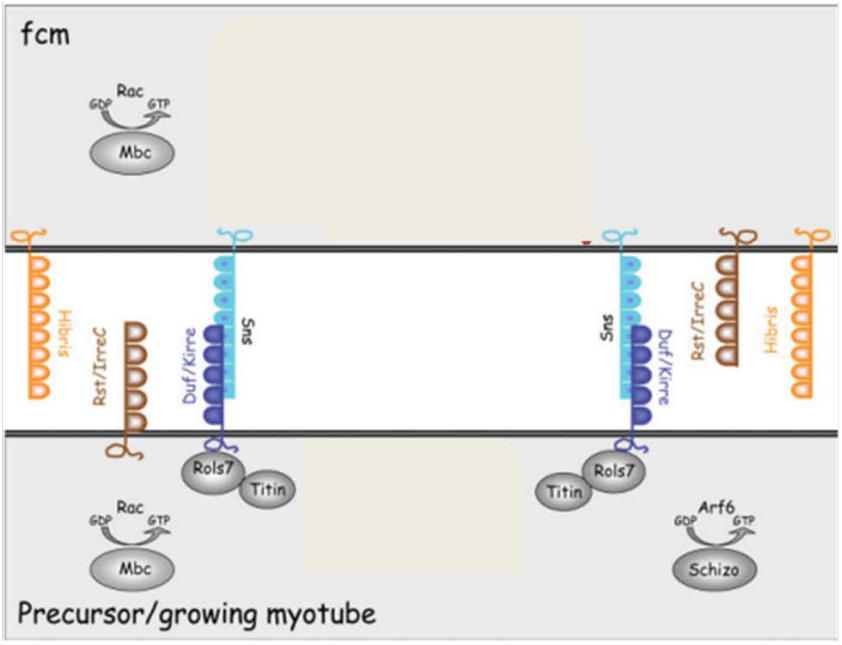


FIGURE 2.6 FIGURE 2.6 **Summary of Cell adhesion molecules**. Duf and Rst expressed in FCs and Sns, Hbs and Rst expressed in FCMs and their known interaction partners of Duf with Rols/Titin.

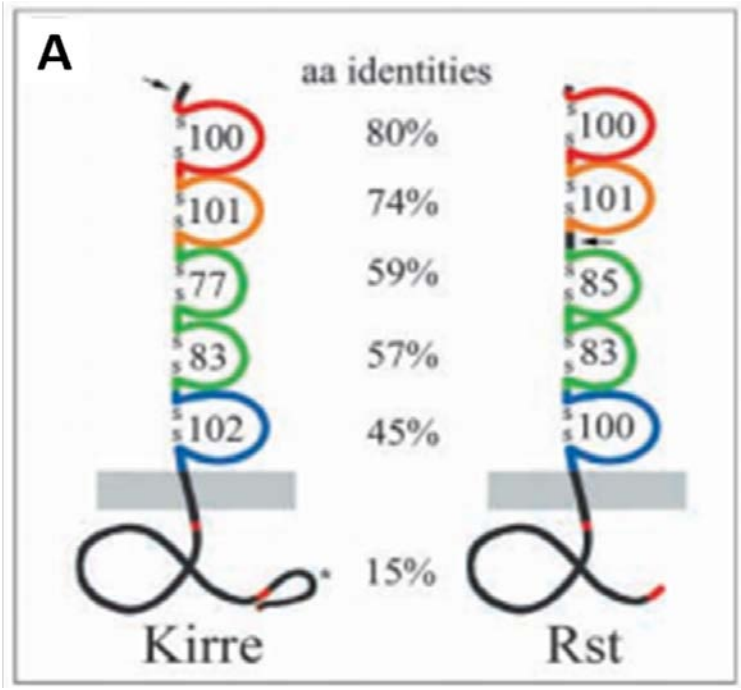
Roughest/Irregular-chiasm-C (Rst/IrreC). Rst was identified (Ruiz-Gomez *et al*., 2000; Strunkelnberg *et al*., 2001), along with its paralogue Duf. Several features of IrreC/Rst overlap with that of Duf/Kirre. *Rst/IrreC* also has five Ig-Like domains and its 166 amino acid cytoplasmic domain has the same motifs found in the Duf/Kirre cytoplasmic domain (Ruiz-Gomez *et al*., 2000; Strunkelnberg *et al*., 2001). The cytoplasmic domain has a consensus sequence for the type of autophosphorylation domain found in receptor tyrosine kinase, a candidate PDZ-binding domain, and a putative serine phosphorylation site (Strunkelnberg *et al*., 2001). *Rst/IrreC* is expressed in founder cells and also expressed in fusion competent myoblasts (Strunkelnberg *et al*., 2001). Deletion of *rst* and its paralogue *duf* displays a myoblast fusion phenotype and the *rst* expression to all of the mesoderm rescues the deletion phenotype (Strunkelnberg *et al*., 2001). Recent S2 cell assays did not uncover an interaction of the IrreC/Rst with either Sns or Duf/Kirre (Dworak *et al*., 2001). So, far Rst interacts with adopter protein X11α/Dmint 1 via its PDZ-binding domain during *Drosophila* eye development (S. Vishnu *et al*., 2006), but these interactions was not shown in myoblast

fusion. In the *Drosophila* database only one new interaction partner fot Rst/IrreC has been discovered: CG10494 was identified in a large two-hybrid screen (Giot *et al*., 2001). Being its cytoplasmic domain contains tyrosine residues , still raises many questions and needs to be further analyzed its role in myoblast fusion.

Hibris (Hbs). The recently reported protein Hibris (Hbs) is the paralogue of Sns (Artero *et a*l., 2001; Dworak *et al*., 2001). Like Sns, the ectodomain of Hbs has eight Ig-like domains, a "degenerate" Ig-like domain, and a FN-III domain. However the Hbs cytoplasmic domain is shorter than that of Sns, with the two Hbs isoforms being 10 and 166 amino acids long. The Hbs cytoplasmic domain has putative target sites for cAMP- and cGMP-dependent protein kinases (PKA and PKG), PKC and Ck-II. The latter two sites are also conserved in Sns (Artero et al., 2001). Both Sns and Hbs are co-expressed in fusion competent myoblasts however, Hbs expression ends slightly sooner than Sns (Artero *et al*., 2001; Dworak *et al*., 2001). In the S2 cell assay hbs does not bind itself or Sns. Surprisingly it also does not bind Rst IrreC/Rst, but to Duf/Kirre (Dworak *et al*., 2001). The binding of Hbs to Duf/Kirre is suggests that Hbs is part of the moleculary machinery in fusion competent myoblast that responds to Duf/Kirre-mediated attraction. In *hbs* and *duf/kirre* mutants one might anticipate a less than-normal ability of fusion competent myoblast to orient towords founder cells, or a qualitative difference in the nature of the contacts made. In the light of the interaction findings, and as the *sns* mutant phenotype is extremely robust compared to Duf/Kirre, Sns must have an additional extracellular binding partners. The cytoplasmic domain of Hbs also contains the tyrosine, still raises many questions and needs to be further analyzed its role in myoblast fusion.

2.7.2 Nature of Redundant functions of Duf/Kirre, Rst/Irre During myoblst fusion

The adhesion mediating transmembrane proteins duf/kirre and Rst/IrreC are functional redundant (Strunkelnberg *et al*., 2001). The *kire* locus maps cytogenetically to region 3C6 and lies 3 kb distal to *Notch*. The *rst* and *kirre* loci are separated by 127 kb and are transcribed from opposite strands with their 5' flanking towords each other.



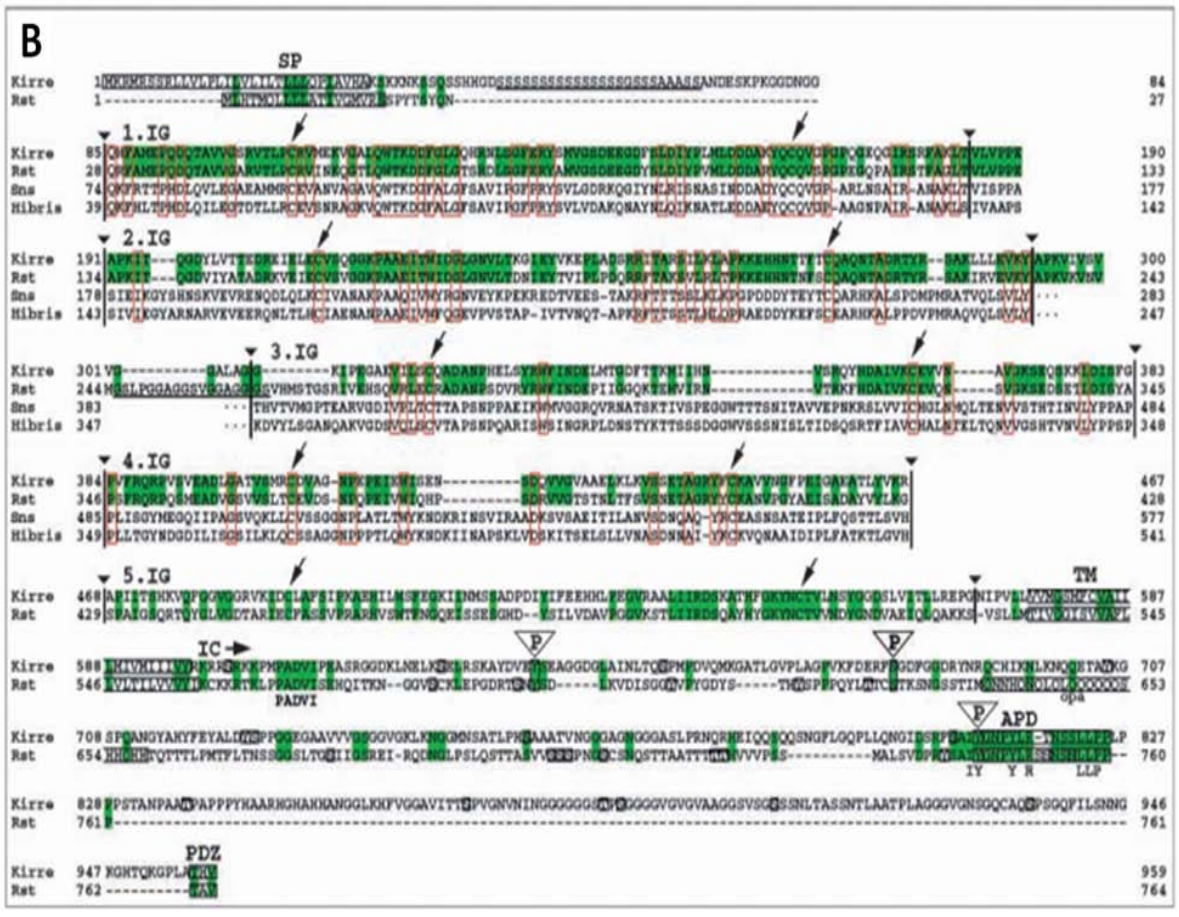


FIGURE 2.7 A **Schematic comparision of Rst and Duf.** Numbers refer to the sizes of immunoglobulin (Ig) domains and to percentages of sequence identities of paralogous Ig domains, respectively.

Arrows indicates the serine-and glycine-rich repeats of Kirre and Rst, respectively. An asterisk marks the sequence stretch separating the autophosphorylation domain and the PDZ-binding motif in Kirre. **(B) Alignment of Rst, Kirre, Sns and Hibris.** Residues identical in Rst and Kirre are with green background, residues identical within all four sequences are marked with red. Borders of IG domains (Ramos *et al*., 1993) are marked by a vertical bar and inverted triangle. Arrows indicate cysteines involved in forming a disulphide bond. Serine-and glycine-rich repeats of Kirre and Rst, respectively are underlined. Putative phosphorylations sites conserved within Kirre and Rst are marked by P in an inverted triangle. Unconserved sites are boxed. APD-autophosphorylation domain with consensus sequence below; IC-intracellular domain; IG-Immunoglobulin domain; PADVI, SAIYGNPYLR and SLLPPLPP are three conserved motifs; opa- *opa*-like repeat; PDZ-PDZ-binding motif; SP-signal peptide; TM-transmembrane domain. Boxed sequence stretches contain the corresponding patterns. (Strunkelnberg *et al*., 2001).

The Kirre sequence shows an overall similarity of 45% to Rst (BLAST algorithm; Altschul *et al*., 1997). Like Rst, the predicted extracellular portion of Kirre protein displays an array of five immunoglobulin (Ig) domains (FIG 2.7 A; Walsh and Doherty, 1997). Stretches of high conservation with Rst reside primarily in the region of the five Ig domains. Within these domains the degree of conservation successively decreases from the N terminus to the transmembrane domain (FIG 2.7 A, boundaries of Ig domains as in Ramos et al., (Ramos *et al*., 1993). Both proteins contain stretches of amino acids with short side chains at differing positions (FIG 2.7 A, arrows; Strunkelnberg *et al*., 2001). Rst contains a stretch of glycines between the second and third immunoglobulin-domain and kirre harbours an array of 18 serine interrupted by a single glycine residue at the N terminus (Strunkelnberg *et al*., 2001).

The intracellular domain of Duf/Kirre and Rst/IrreC is considerably longer than that of Rst and show a low overall homology with the one of Rst. However three highly conserved motifs were detected (FIG 2.7 B; (Strunkelnberg *et al*., 2001). One is located close to the transmembrane consisting of the sequence PADVI, The second and third motifs are close to the C terminus consisting of the sequence SAIYGNPYLR and SLLPPLPP (Strunkelnberg *et al*., 2001). The second and third motifs are corresponds to the consensus sequence of autophosphorylation domains of receptor tyrosine kinases (Yarden and Ulrich, 1998).

Another short motif find at end of the C terminus of both the sequence THV is which corresponds to the consensus sequence of the PDZ-binding motif (Garner *et al*., 2000; Strunkelnberg *et al*., 2001). In addition, one putative tyrosine and one putative serine phosphorylation site are conserved between Rst and Kirre (NetPhos 2.0 algorithm) (Blom *et al*., 1999). A conspicuous difference between the Kirre and Rst proteins is the lack of *opa*-like repeat of Rst in Kirre (Ramos *et al*., 1993).

Similarly searches using the BLAST algorithm showed that the four N-terminal Ig domains of Kirre, Rst, Sns (Bour et al., 2000) and Hibris (GenBank Accession Number, AF210316) are closely related (FIG 2.7 B).

Taken together, these studies help us to dissect down the domains to understand the nature of the different signalling cascades.

2.8 The Role of *Drosophila* Homologe of Nck/Dock during Arp2/3-based actin polymerization

Here I introduce the *Drosophila* homolge of Nck/Dock and finally I introduce the role of actin regulators i.e Wiskott-Aldrich syndrome protein (WASP) family e.g neuronal (N)-WASP and WASP-family Verproline-homolgous protein (SCAR/WAVE).

2.8.1 The SH2-SH3 adopter protein NCK/Dock

After identifying the cell adhesion and actin regulatory proteins, recent studies started to investigate how cell adhesion is linked to the actin cytoskeleton machinery. Different adopter proteins has been identified in last decade: Rols7, Blow and SH2-SH3 adoptar protein to be involved in muscle development. SH2-SH3 adaptor proteins are involved in many signalling pathways. These adaptor proteins are involved in many signal pathways and contain exclusively Src homology 2 (SH2) and Src homology 3 (SH3) domains. SH2-SH3 adaptor molecule Crk interacts with Vrp1 (Kim *et al*., 2007). In vertebrates Crk-II is involved in the activation of the N-WASP-depedent actin polymerization in the smooth musculature (Tang *et al.,* 2005). In vertebrates Nck, another SH2-SH3 adaptor protein, was shown to link phosphorylated Nephrin to the actin cytoskeleton (FIG 2.8. A; Verma *et al*., 2006; Jones *et al*., 2006).

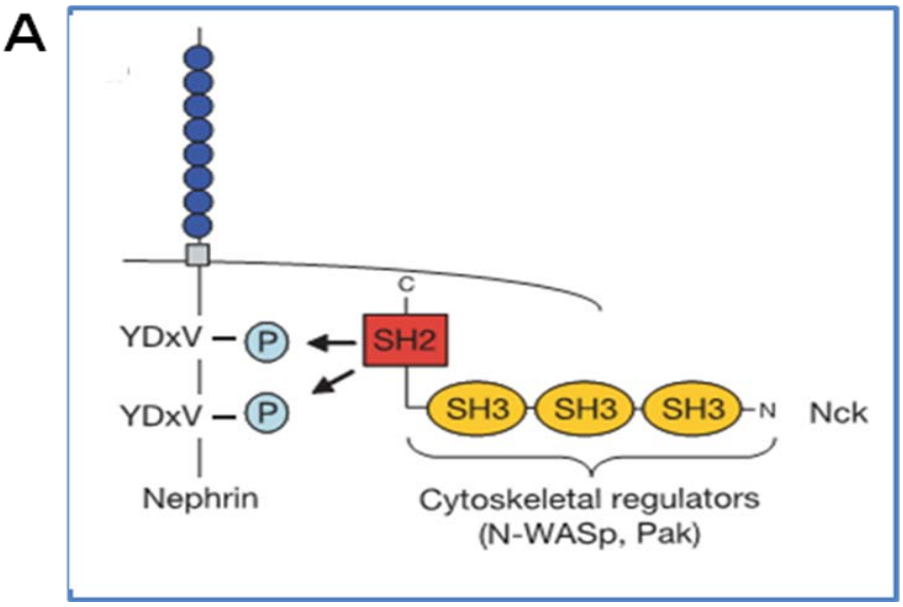


FIGURE 2.8.1 A **The Domain structure of Nck/Dock.** SH2 domain of Nck binds to the conserved phosophotyrosine motif of Nephrin (the Sns homolog). SH3 domain of Nck binds to the proline-rich region of cytoskeletal regulators e.g N-WASP, Pak. Modified after Jones *et al*., 2006.

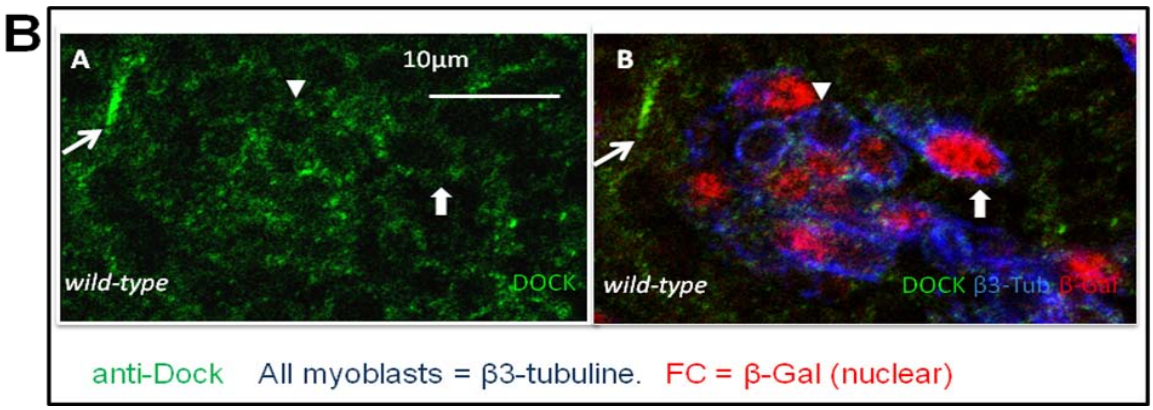


FIGURE 2.8.1 B **Nck/Dock is expressed in FCs and FCMs and localises to the membrane. (A)** anti-Dock staining on embryo expressing a myrristylated Dock construct in wildtype. **(B)** Enhancer trap line rP298 stained for Dock (green), β-gal (red) and β-tubulin (blue). Dock is expressed in both myoblast cell types (FC; arrow, FCM: arrowhead) and is localised at the plasma membrane. Modified after Schäfer et al (in preparation)

Nck (non-catalytic region of tyrosine kinase adaptor protein) is a SH2-SH3 adaptor protein and also known as Dreadlock (Dock) in *Drosophila*. Dock is involved in axonal path finding in *Drosophila*, both in olfactory and in visual senses (Desai *et al*., 1999). Dock binds to WIP directly in a yeast 2-hybrid assay (Giot *et al.,* 2003). However, cell culture experiments on S2 and vertebrate cells could also show that Dock can bind to WASP (Rivero-Lezcano *et al*., 1995; Rohatgi *et al*., 2001; Worby *et al.,* 2001). Nck/Dock is expressed in both myoblast types founder cell and fusion competent cells Schäfer *et al* (in preparation) and is localised to the plasma membrane of the myoblast (FIG 2.8.B).

The binding of these adaptor protein to the proline-rich region depends on their activation at the SH2 domain. The SH2 domain binds with high affinity to proteins with a specific phosphotyrosine-containing motif. Like Nephrin, the adhesion molecules Sns possesses

putative phosphorylation sites which can be bound by the SH2 domain of SH2-SH3 adaptor proteins (Galletta *et al*., 2004; Kim *et al*., 2007). Rescue experiments of *sns* mutants with full length *sns c*onstruct which lacks all phosphotyrosine motifs, were not able to rescue the mutant phenotype completely (Kocherlakota *et al*., 2008). This suggests that the phosphorylation of Sns is essential for the signal transduction during myoblast fusion.

In this study we have examined the role of the Drosophila Nck homolg-Dreadlock(Dock) in myoblast fusion, the SH3 and SH2 domains of Dock links the cell adhesion molecules (IgSF) to the actin regulatory complex such as Wiskott-Aldrich syndrome protein (WASP) family e.g neuronal (N)-WASP and WASP-family Verproline-homolgous protein (SCAR/WAVE). In the next part i introduce about these actin regulators.

2.9 The role of WASP family proteins during Arp2/3-based actin polymerization

The Arp2/3 complex is crucial for actin polymerization and Arp2/3 complex in turn is activated by Wiskott-Aldrich syndrome protein (WASP) family e.g neuronal (N)-WASP and the WASP-family Verprolin-homologous protein (WAVE) (Higgs and Pollard, 1999, 2001; Stradal *et al*., 2004; Tankenawa and Miki, 2001).

The Wiskott-Aldrich syndrome protein (WASP) was first described as a very rarely X-chromosomal immune deficiency (Derry *et al*., 1994). Mammals have two members of WASP subfamily: WASP and N-WASP. The loss of N-WASP function in knock-out mice leads to embryonic lethality and to a disturbed mesoderm development (Lommel *et al*., 20010). While the known *wasp* alleles wsp^{1} and wsp^{3} do not show any defects during myogenesis. A new allele $wsp^{3D3\text{-}035}$ was detected during an EMS collection screen for mutation during myoblast fusion (Hummel *et al*., 1999). This allele revealed severe myoblast fusion defects during the 2nd fusion step (Schäfer *et al*., 2007). WASP possesses an N-terminal WASP homology domain (WH1) to which its interacting partner WIP can bind (Martinez-Quiles *et al*., 2001; FIG 2.9). A regulatory region with binding sites for PIP2 (B-domain) and a GTPase-

binding domain (GBD) to which Cdc42 can bind (Carnon, 2002). In vertebrates, Cdc42 plays an important role in the activation of WASP. However, in *Drosophila*, this does not seem to be the case (Tal *et al*., 2002; Schäfer *et al.,* 2007). *Drosophila* WASP contains the some conserved domains like N-WASP (FIG 2.9; Ben-Yaacov *et al*., 2001; Tal *et al*; 2002).

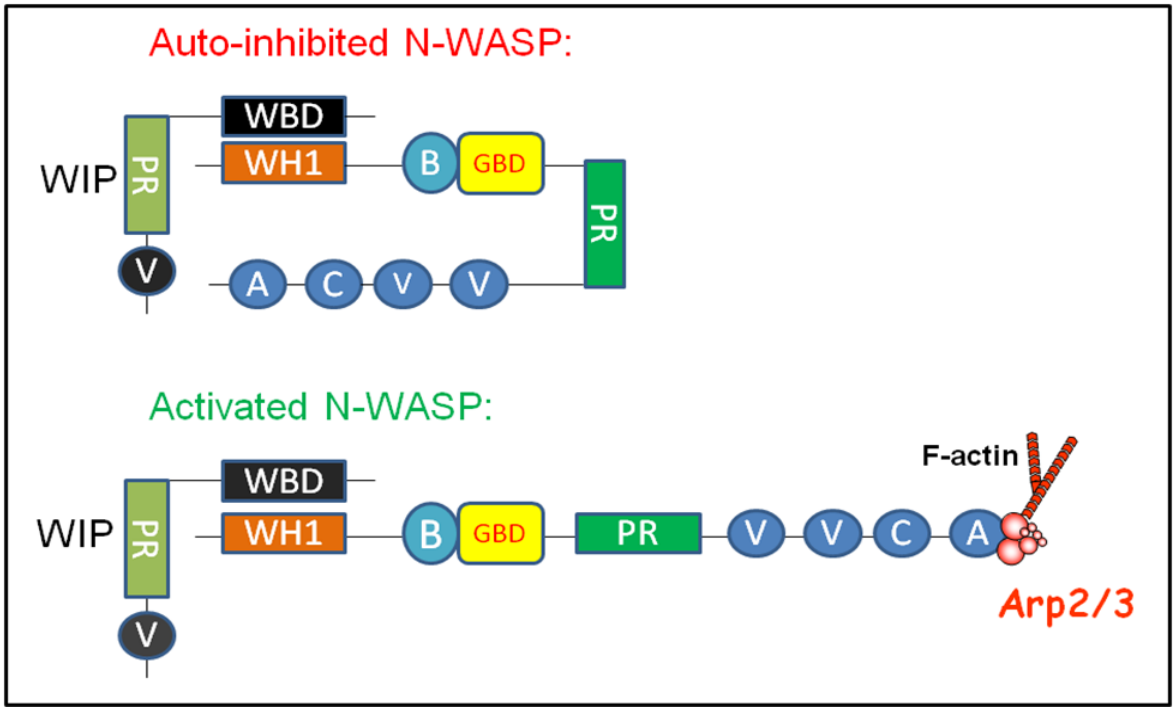


FIGURE 2.9 **N-WASP-WIP complex.** N-WASP contains four regulatory regions at the N-terminus; a WASP-homology region 1 (WH1), a basic domain (B), a GTPase binding domain (GBD), and Proline-rich region (PR) which is activated by SH2-SH3 adaptor protein. VCA module, a actin-cytoskeleton interacting domain consists of two Verprolin homology domains (V), a cofilin homology (C) domain and the most C-terminal acidic tail (A). The activated and auto-inhibited WASP is bound in a complex with Verprolin1 (Vrp 1). Vrp1/WIP possesses a WASP-binding domain (WBD), a Proline-rich region which binds to the SH2-SH3 adaptor protein and a Verprolin homology domain (V). In normal cell auto-inhibited-WASP and VCA-module is masked. The activation of WASP leads to a conformational changes and the VCA module can bind to the actin cytoskeleton machinery.

a proline-rich region (PR), to this SH2-SH3 adaptor protein binds. In vertebrate and in *Drosophila* Schneider cells, Nck/Dock binds to WASP (Rohatgi *et al*., 2001: Worby *et al*., 2001). An actin cytoskeleton interacting domain, the VCA module, the VCA module is responsible for the activation of Arp2/3 complex, resulting in its activation and

polymerization of F-actin (Machesky *et al*., 1999). It contains two Verprolin (V) and Cofilin (C) homologous domain as well as an acidic tail (A). The V domain binds monomeric globular actin (G-actin) and filamentous actin (F-actin) and the CA domain bind to the Arp2/3 complex. The loss of CA domain and the V domain leads to the severe myoblast fusion defects during the 2nd fusion step (Schäfer *et al.,* 2007). N-WASP is usually adopts an auto-inhibited conformation and the VCA module is masked (FIG 2.9). When N-WASP becomes activated the VCA module is able to bind to the Arp2/3 complex and this induces the formation of actin filaments (Machesky *et al*., 1999).

In vertebrates the binding of the WASP-interacting protein (WIP) is able to stabilize auto-inhibited N-WASP (Ho *et al*., 2004) (N-WASP-WIP complex; FIG 2.9). WIP is a member of the Verproline protein family and its protein structure is highly conserved. In human cell experiments, WASP requires the presence of WIP to enable actin polymerization (Tsubio, 2007). This direct interaction was further proved in *Drosophila* in the large screening with the yeast 2-hybrid system (Giot *et al*., 2003). In vertebrates, this interaction occurs between the N-terminal end of WASP and the C-terminal end of WIP (FIG 2.9). WIP possesses a WASP-binding domain (WBD) (FIG 2.9), and a long proline-rich region (PR) and WASP-homology 2 domain (WH2) (FIG 2.9). The proline-rich region is responsible for the binding of SH2-SH3 adaptor protein, such as Nck can bind to WIP.

Another member of the WASP family is WAVE. In Humans three WAVE proteins (WAVE1-3) are described (Machesky and Insall, 1998), but *Drosophila* has only one WAVE protein, which is also known as SCAR (Suppressor of CAMP receptor). The nucleation-Promoting factor WAVE2 contains nearly the same conserved domains like N-WASP and is able to bind the Arp2/3 complex through the conserved VCA module and it has a proline-rich region like N-WASP (FIG 2.10). WAVE2 is bound in complex in the cytoplasm. Known components of the vertebrate WAVE2 complex are specifically Rac-associated protein1 (Sra1), the Abeleson-interacting protein (Abi), the heat shock protein C300 (HSPC300), and the Nck-associated protein 1 (Nap1/hem1/Kette; FIG 2.10; Stradal *et al*., 2004; Ibarra *et al.,* 2005). But the *Drosophila* SCAR//WAVE does not possesss the basic GDB domain and require a different activation pathway. The WAVE complex is normally is in inactive state,

where as the binding of of the small Rac-GTPase seems to activate the WAVE function, which in turn can stimulate the Arp2/3 complex (FIG 2.10; Takenawa and Suetsugu, 2007). In *Drosophila* SCAR/WAVE complex proteins Rac1 and Kette are essential for myoblast fusion. Immunohistochemical studies in *Drosophila* imply that the Hem1 homolog Kette is required for the correct localization of SCAR (Richardson *et al*., 2007).

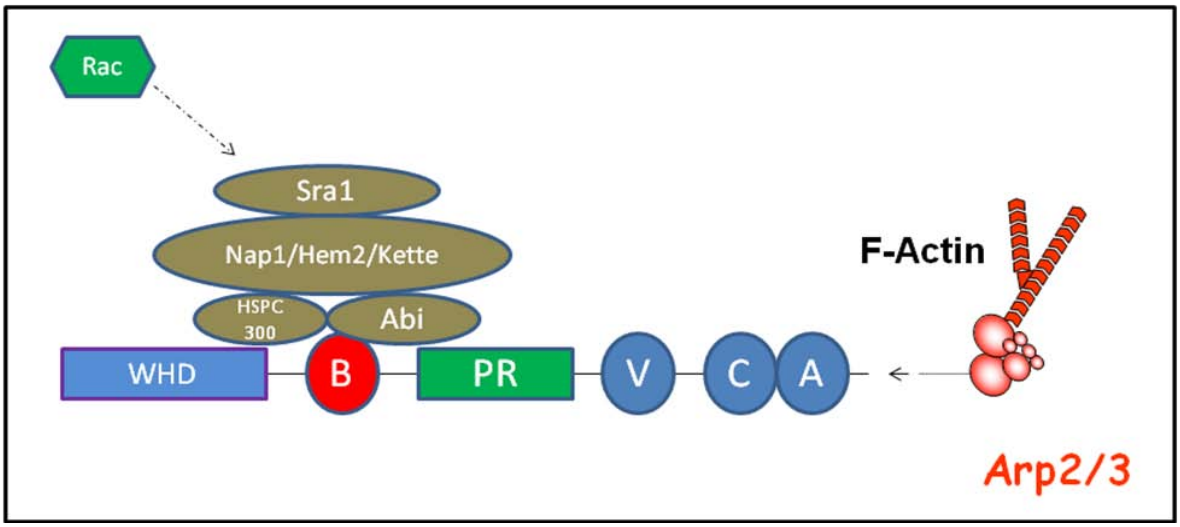


FIGURE 2.10 **SCAR/WAVE complex.** Th regulatory region WAVE contains a WAVE homology domain (WHD), a basic domain (B) and a proline-rich region (PR) which binds to the SH2-SH3 adaptor protein. The VCA domain consists of a Verprolin homology (V), a cofilin homology (C) and an acidic acid (A) sub-domain. Further components of the WAVE complex are shown in grey colour. The WAVE-dependent actin polymerization requires the Rac-GTPase activity.

All the nucleation promoting factors are regulated differently. But, WASP, WIP and SCAR have one thing in common; they all possess a proline-rich region, which is further known to bind to the SH2-SH3 adaptor protein.

2.11 Questions to be addressed

The main interest in this thesis focuses on the linking between the immunoglobulin super family (IgSF) mediated cell adhesion molecules with the Arp2/3 based dependant actin polymerization in *Drosophila melanogaster*. The questions addressed are as follows:

1) What is the role of Duf and Rst proteins in the founder cell and redundant nature?
2) Has Rst any interaction partner during myoblast fusion?
3) Does the SH2-SH3 adaptor Dock interact with the cell adhesion molecules Rst, Duf, Sns and Hbs?
4) What is the role of SH2-SH3 adaptor Dock in FCMs during myoblast fusion?
5) Do Hbs and Dock interacts genetically? If interacts what kind of domains are involved ?
6) What kind of domains are involved in the interaction between the Hbs and Dock?
7) What is the role of SH2-SH3 adaptor Dock in FCs during myoblast fusion?
8) Is Dock and Duf interact genetically? If interacts what kind of domains are involved?
9) Which of Dock domain are involved in interaction with Rst?
10) Is Dock acting through the WASP based or Scar/WAVE based Arp2/3 actin polymerization?

With the genetic accessibility in *Drosophila melanogaster* it is feasible to study the various interesting aspects of molecular mechanism involved during myoblast fusion. At the begining of this work I focused on the Duf and Rst chimeras i.e Duf-ET/Rst-IT or Rst-ET/Duf/-IT. The transgenic flies are created and expressed in mesoderm with mesodermal specific Gal4 driver lines to see the effect on myoblast fusion. Later I generated Rst deletion constructs and expressed in mesoderm with mesodermal specific Gal4 driver line to see the effect on myoblast fusion. I identified possible Rst interaction partners i.e Actin 57B, Nidogen and Papilin in a yest two-hybrid assay.

Further I started to analyze the role of the SH2-SH3 adaptor Dock in myoblast fusion. I further analyzed the role of Dock in FCs and FCMs. Later I analyzed the role of Dock

adaptar protein in activating the actin nucleation promoting factors such WASP-WIP and SCAR/WAVE of Arp2/3 complex in actin plomerization.

3 . Materials

3.1 Instruments

Digital camera	Polaroid
Gel Electrophoresis apparatus	University of Marburg
Water Bath 37^{o}C	Julabo U3
Vaccume Centrifuge	Savant SVC 199 H
Vortex Machine	MAGV, Rabenau, Londorf
UV Crosslinker Stratalinker,	UV Stratalinker$^{TM\ 2400,}$ La jolla USA
Thermoblock	Driblock DB.2A, Techne
Stereomicroscope	Stemi SV, Zeiss, Jena
pH-meter	Ultrospec, Pharmacia
Magnetic Stirrer	Variomag, H+P Labortechnik
Confocal Laser Scan Microscope	TCSSP2 Leica, Heidelberg
Videocamera and printer	Botech-Fischer
UV-Transilluminator	Spektroline TS-302
Table top Centrifuge	Biofuge 13, Heraeus
PCR-Machine	Personal Cycler, Biometra
Photometer	Ultrsopec, Pharmacia
Micromanipulator	Leitz
Microinjecter 401,Eppendorf	5242 Hermle ZK
Cool centrifuge	Heraeus megafuge 1.0 R
Fotomicroscope	Zeiss
Capillary extracting equipment	Vertical pipette puller 720
SDS-gel electrophoresis	Biorad, USA

3.2 Chemicals and Growth media

Acrylamide	Serva, Heidelberg
Adenosintriphosphat(ATP)	Roche, Mannheim
Agarose	GibcoBRL, Eggenstein
Acetic acid	Roth, Karlsruhe
Ammoniumpersulfate (APS)	Merck, Darmstadt
Ampicillin (amp)	Roth, Karlsruhe
Agar-Agar	Roth, Karlsruhe
Bovine Serum Albumin (BSA)	Roth, Karlsruhe
5-Bromo-4-chloro-3-indolyl-Phosphate (BCIP, X-Phosphate)	Roche, Mannheim
5-Bromo-4-chloro-3-indoxyl-β-D-thiogalactoside (X-Gal)	Roche, Mannheim

Bromophenol blue	Merck, Darmstadt
Carbon dioxide	Air Liquide, Dusseldorf
Cornmeal	Alnatura, Buckenbach
D-(-) fructose	Roth, Karlsruhe
3, 3' –Diaminobenzidintetrahydrochloride	Sigma, Deisenhofen
Concanovaline A	Sigma, Steinheim
BoricAcid	Roth, Karlsruhe
Di-Natriumhydrogenphosphate	Roth, Karlsruhe
Diethylether	Roth, Karlsruhe
Drop-out medium	Sigma, Steinheim
Dan-Klorix-sodium hypochloride	Fluka, Neu-Ulm
1,4-Dithio-L-threitol (DTT)	
Digoxygenin	Roth, Karlsruhe
Distilled Water	
Ethylene diamino tetraethanoic acid(EDTA)	Roth, Karlsruhe
Epon accelerator DMP 30	Fluka Neu-helm
Epoxy Embediing Media,Hardener DDSA	Fluka Neu-helm
Formaldehyde	Merck, Darmstadt
Flouromount-G	Merk, Darmstadt
Formamide	Merck, Darmstadt
Fetal Bovine Serum	Sigma, Berlin
Glycine	Roth, Karlsruhe
Glucose	Merck, Darmstadt
Glutaraldehyde	Sigma, Deisenhofen
Glycogen	Fluka, Neu-Ulm
Glycerol	Roth, Darmstadt
Heptane	Roth, Karlsruhe
Histidine	Roth, Karlsruhe
Heparin	Sigma, Deisenhofen
Hydrogenperoxide	Roth, Karlsruhe
Harnstoff	Roth, Karlsruhe
Isopropanol	Merck, Darmstadt
Isopropyl-β-D-thiogalactopyranoside (IPTG)	Roth, Karlsruhe
Calciumacetate	Fluka, Neu-Ulm
Calciumhydroxide	Merck, Darmstadt
Leucine	Roth, Karlsruhe
Magnesiumchloride	Merck, Darmstadt
Magnesiumsulfate	Roth, Karlsruhe
Methanol	Roth, Karlsruhe
MOPS (Morpholinopropansulfonsaere)	Roth, Karlsruhe
Nipagin (4-hydroxy benzoic acid methyl ester)	Sigma, Berlin
Non-fat-dry milk	Sigma, Deisenhofen

Sodiumacetate	Sigma, Berlin
Sodiumchloride	Roth, Karlsruhe
Sodiumhydrogenphosphate	Roth, Karlsruhe
Sodiumdodecylsulfate (SDS)	Roth, Karlsruhe
Sodiumhydroxide	Roth, Karlsruhe
4-Nitrotetrazoliumchloride (NBT)	Roth, Karlsruhe
N,N-methylenbisacrylamide	Roth, Karlsruhe
Octylphenolpolyethyleneglycolether	Roche, Mannheim
	Serva, Heidelberg
(TritoX-100)	Serva,Heidelberg
Piperrazin-N,N'-bis(2-ethanolsulfonsaure	
(PIPES)	Sigma, Deisenhofen
Polyoxyethylenesorbitanmonolaurat	
(Tween 20)	Merck, Darmstadt
Paraformaldehyde	Merck, Darmstadt
Pencilline-Streptomycine	Gibco, Karlshruhe
Peptone	Roth, Karlsruhe
PMSF9 (Phenyl methane sulfuronic acid fluoride)	Sigma, Berlin
Potassium Acetate	Roth, Karlsruhe
Propanoic Acid	Roth, Karlsruhe
Raffinose	Roth, Karlsruhe
Salmmon Sperm DNA	Fluka neu-helm
Select agar	Invitrogen, Karlsruhe
Sodium Citrate	Roth, Karlsruhe
SD Base without HULT	Roth, karlsruhe
Sucrose	Roth, karlsruhe
Transfectin Lipid Reagent	Biorad
Tris Base	Roth, karlsruhe
Tryptone	Roth, Karlsruhe
Tryptophane	Roth, Karlsruhe
TEMED (N,N,N,N-tetramethylethylene Glycoether)	Serva, Heidelberg
Uracil	Roth, Karlsruhe
Voltalef oil	Voltalef 10SPCTFE, Atochem, ATO
Yeast Extract	Roth, Karlsruhe
YPD Broth	Roth, Karlsruhe

Media Composition

Cornmeal agar:

0.7% agar Stir in seething water, boil for 20 minutes 60% cornmeal 7.5% fructose 1.2% yeast, dried Dissolved in cold water, add to agar-solution, Boil for 20 minutes cool down at 60^{o}C, 1% sugar beet syrup 0.6% propionic acid0.14% nipagin 10% Fill 40 ml in big, 19 ml in median and 11 ml in small vials 54 g select agar.

Juice agar:

54 g select agar dissolve in 500ml grapevine juice at 60^{o}C add agar solution.

LB-medium:

10g tryptone, 5g Yeast extract, 5g Nacl to 1 L water.

LB-agar: 1.5% select agar in LB-medium.

LB-agar plate(amp)10g peptone: 100mg/100ml

LB-agar plate (kan): 500mg/100ml LB-medium

YPD: 5 g in 100 ml water

YPD-Agar: 1.5% agar in 100 ml YPD-medium

SD Medium: 0.15g ofHULT, 0.15g of each amino acid required, 0.17g Nitrogenbase, 0.5g Ammonium sulphate, to 100ml ddH20

Yeast Induction Agar plates: 10g Agar, 0.75g Nitrogenbase, 2.5g Ammonium sulfate, 0.7g of HULT, 0.7g of aa

YPD Agar plates: 10g YPD broth, 4g Agar, ad 190 ml ddH2O, Autoclave then add 10ml 40% Glucose

3.3 Antibodies and Antiserum:

Anti-Digoxygenin-Fab-fragment - Roche, Manheim

Anti-GFP Polyclonal made in rabbit 1:500 - Abcam, Cambridge

Anti-Myc Monoclonal made in mouse 1:1000 -Marburg-Cappel

Anti-HA Monoclonal made in mouse 1:5000 -Marburg-Cappel

Anti-β3-Tubuline Polyclonal made in rabbit 1:5000 -D.Buttgereit

Anti-β3-Tubuline Polyclonal made in guinea pig 1:5000 -D.Buttgereit

Anti-β-galactosidase(GAL) made in rabbit 1:5000 - Marburg-Cappel

Anti-Nck/Dock Polyclonal made in rabbit 1:1000 - S.Abmayer

Anti-Flag Horseradisch peroxidise (HRP) made in mouse 1:4000 - Sigma

Anti-HA Horseradisch peroxidise (HRP) made in mouse 1:4000 - Sigma

Goat Serum -Vector Laboratories

Horse Serum -Vector Laboratories

Anti-Rabbit Peroxidase 1: 4000 – Vectastatin

Anti-Mouse Peroxidase 1:4000 -Vectastatin

Anti-Rabbit IgG, Cyanine3-conjugated 1:200 -Dianova,Hamburg

Anti-Mouse IgG, Cyanine5-conjugated 1:200 -Dianova,Hamburg

Anti-Guinea pig IgG, Cyanine2-conjugated 1:200 -Dianova,Hamburg

Pholloidine-FITC,TRITC -Sigma

3.4 Molecular biological reagents and Kits.

DIG-DNA-Labeling-Kit	Roche, Mannheim
Elutip-D	Schleicher & Schuell, Dassel
JET star Plasmid Midiprep Kit	Genomed, Bad Oeynahausen
JET sorb Gel Extraction	Genomed, Loehne
pENTR/D-TOPO cloning Kit	Invitrogen, Karlsruhe
ECL™ Plus Western Blotting Detection Reagent	Invitrogen, Karlsruhe
Tyramide Amplification Signal (TSA) System Fluorescein	New England Nuclear Life Science Products
Mass Rular DNA-Ladder,Low Range	MBI Fermentas, St. Leon-Roth
Mass Rular DNA-Ladder, Mix	MBI Fermentas, St. Leon-Roth
Protein Molecar Weight marker (stained)	(PVDF)
Protein Molecular Weight marker	MBI Fermentas, St. Leon-Roth
Site-Directed Mutagenesis Kit	MBI Fermentas, St. Leon-Roth
Hybond P-polyvinyl difluoride membrane	Stratagene

	GE Healthcare, Amersham
Plasmid Kit	Qiagen, Hilden
Vektastain Elite ABC Standard Kit	Vector laboratories, USA

3.5 Enzymes

Deoxyribonuclease I	Roche, Mannheim
Klenow Polymerase	Amersham, Braunschweig
Lysozyme	Serva, Heidelberg
Proteinsae K	Roche, Mannheim
Restriction endonucleases	Fermentas, St, Leon-Roth
RNASe A (1mg/ml)	Roche,Diagnostics,Mannheim
T4 DNA Ligase	Fermentas, St. Leon-Roth
Accu-Prime TM High Fidelity	Invitrogen, Karlsruhe
Taq DNA Polymerase	Roche, Mannheim
Taq DNA Polymerase	Qiagen, Hilden

3.6 Other Materials

Hybond N-Membrane	GE Healthcare, Amersham
Bleach	Colgate Palmolive Gmbh, Hamburg
Whatmanpaper (Blotting-papier GB 2000)	Schleicher & Schuell, Dassel
Parafilm	

3.7 Plasmids

Expression Vector:	Transformation vector containing 5 GAL4 binding sites before the multiple cloning site (Brand and Perrimon, 1993)
pUAST	

TWF dock-flag	3x flag-tagged dock gene in TWF vector (Susan Abmayer, University of Kanas School of Medicine
HA-Duf	HA tagged full Duf with copper activation promotor
pGILDA	Clonetech, Heidelberg
pB42AD	Clonetech, Heidelberg

Cloning Vectors

pBluscript IIKS+	Invitrogen,Karlsruhe
pCRII-TOPO	Invitrogen. karlsruhe
pCR-Blunt	Helperplasmid forP-element transformation encoding P-element-Transposase, (Karess and Rubin 1984)
pπ25.7wc	Stratagene, Heidelberg
pOT2	Stratagene, Heidelberg

3.8 Fly stocks

white	w^1,+ laboratory breed of the working group Prof. Renkawitz-pohl, Marburg
CSTM	*W*;* Cyo/Sp;Sb/Ubx Multiple balancer used for the localization of the P-element
w;If/Cyo^{hglacz}	Balancer line for second chromosome with eye-specific adult marker and embryonic hindgut β-galactosidase Expression (courtesy of A. Michelson)

P {PZ}dock^{04723}/Cyo,ry^{504}	The dock alle was generated by FRT-FLP recombination It is characterized by the insertion of the transposable element P {PZ} in the first intron of the *dock* gene, localized on left arm of the second chromosome. Homozygous mutants of this null allele of *dock* are not viable for the protein expression is totally absent (Bloomington Stock center)
w*; SCAR$^{\Delta 37}$ P{neoFRT}40A/CyO	Berger et al., 2008
Hbs^{459}	Bao and Cagan, 2005; Menon et a*l., 2005*
Df(3L)BK9	Rols deficiency, Kreisköther *et al*., 2006
UAS-Duf-Et/Rst-It	In this study
UAS-Rst-Et/Duf-It	In this study
UAS- *rstΔPADVI*(I),	In this study
UAS- *rstΔSAIYGNPYLR*(II), ΔNSSLLPP(III)	In this study
UAS- UAS-*rstΔPADVI*(I), *ΔSAIYGNPYLR* (II), ΔNSSLLPP (III)	In this study
UAS- *rstΔ* NSSLLPP (III)	In this study
GAL4-Driver lines	twist, Mef2, sns, rP298 from working group Prof. Renkawitz-pohl, Marburg

3.9 Bacterial strains used for transformation and culture (*Escherichia coli*)

DH5α	*Sup E44, ΔlacU160 (ϕ80 lacZΔM15), hsdR17, recA1, endA1,gyrA96,thi-1, relA1*

TOP10	*F^+ (lacq Tn10(Tet^R) mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15, ΔlacX_74, recA1,deoR,araD139, Δ(ara-leu) 7697, galU ,galK, rspL, (str^R), endA1,nupG* Invitrogen, Karlsruhe

3.10 Yeast Strains used for the Yeast two-hybrid assay (*S.cereviciae*)

EGY 48[p80-lacZ]	EGY 48 contains plasmid [p80p-lacZ] *MATα,his3, trp1,ura3*,(estojak et al.,1995 *LexAop(x6, -LEU2 [p80p-lacZ]: LacZ* under control of *lexAop(x8), URA3, amp^R* Clonetech.

3.11 S2 cells used for the transfection of Plasmids(*Drosophila* Schneider 2 (S2) Cells

Drosophila S2 cells are used for heterologous protein expression in the *Drosophila* Expression System (DES). The S2 cell line was derived from a primary culture of late stage (20-24 hours old) *Drosophila melanogaster* embryos (1). This versatile cell line grows rapidly at room temperature without CO2 and is easily adapted to suspension culture. S2 cells are available frozen in both serum-containing (Schneider's *Drosophila* Medium) or serum-free medium (Drosophila SFM).

3.12 Buffers

10X BU Salt: 35g Na2HPO4.2H2O,15g NaH2PO4,ad 500ml ddH2O,pH 7.0

F-PBS: 4g Paraformaldehyde dissolved at 60®C in 1xPBS

Hybridization Solution for In situs: 50% Formamide, 25% 20x SSC (pH 7.O), 0.1%heparin (50 ug/ul), 0.1% Tween 20, 10mg/ml herring or salmon sperm DNA

10X Injection Buffer: 1mM NaHPO4(pH 7.4),50mM KCl

10x Lithium Acetate: 2.0g Lithium acetate dissolved in 20 ml ddH2O,autoclaved

10X Loading Buffer for DNA: 0.1% xylene cyanol,0.1% bromophenol blue, 80% glycerine dissolved in 1x TBE

Lysis Buffer for(protein extracts from embyo): 50mM Tris pH 7.5 with HCl, 150mM KCl, 5mMmgCL2, 250mM Sucrose, 100mM DTT, 2mM PMSF

Lysis Buffer for (protein extracts from S2 cells): 1% Triton X, 1mM PMSF, 1mM DTT,10mM EDTA with protease inhibitor

10x PBS: 1.3 M NaCl, 70mM Na2HPO4, 30mM NaH2PO4

PBT: 1x PBS, 0.1% Tween 20

RF1 Buffer: 100mM RbCl, 30mM KaAc, 10mM CaCl2, 15% Glycine, pH 5.8 with 1M Acetic Acid

RF2 Buffer: 10mM MOPS, 10mM RbCl, 75mM CaCl2, 15% Glycine, pH 6.8 with 1M NaoH, autoclave

E1: 50mM Tris, 10mM EDTA, pH 8.0 with HCL, add 100ug/ul RNase

E2: 200mM NaoH, 1% SDS

E3: 4.2M, Potassium Acetate, pH 5.5 with Acetic Acid

10X SDS-PAGE Running Buffer: 15g Tris, 72g Glycine, 50 ml 10% SDS, ad 500ml H2O

5X SDS-PAGe Sample Buffer: 1.6ml, 10%SDS, 0.4ml β-Mercaptoethanol, bromophenol blue

Transfer buffer: 25mM Tris HCL, 150mM Glycine, 20% MeOH,3M NaCl, 0.3 M NaCl.

SquiB: 10mM Tris HCL (pH 8.2), 1mM EDTA, 25mM NaCl.

20X SSC: 3M NaCl, 3M Sodium Citrate pH 7.0

10X TBE: 500 mM Tris HCl pH 8.2, 500mM Boric Acid, 50mM EDTA

10X TBS: 10mM Tris HCl pH 7.5, 150mM NaCl

Blocking buffer for Western blot: 5% milk powder dissolved in 1l 1xTBS

TBSTT: 0.05% Tween, 2% Triton-X in 1xTBS

10X TE: 0.1mM Tris HCl, pH 7.5, 10mM EDTA

X-GAL: 10%(w/v)X-Gal dissolved in DMF

NBT buffer: 1M Tris HCl pH 9.5, 5M Nacl, 1M MgCl2

NBT buffer for in situs: 1X NBT buffer, 0.1% Tween 20 prior to u

3.13 Synthetic oligonucleotide

Primer name	Sequence
Hbs it EcorI-fwd C-YFP	5’-ATAGAATTCATGCGCCGGCAGAACAAATCTCAG-3’
Hbs.it NotI-rev C-YFP	5’-ATAGCGGCCGCCGTAAGTCACCGGCGGATAGGACATATAT-3’

Hbs-fwd(**Y1088F)** C-YFP	5'-AGCAACGATGATGTGTTCTCCAAGGATGACAGTCAG-3'
Hbs-rev(**Y1088F)** C-YFP	5'- CTGACTGTCATCCTTGGAGAACACATCATCGTTGCT-3'
Duf.it EcorI-fwd C-YFP	5'- ATTAGATCTATGCGACGCAGTCGCAAGAAG-3'
Duf.it EcoRI-rev C-YFP	5'- ATTGCGGCCGCCAACATGAGTGGCCAGAGGTC-3'
Duf.itDprr EcorI-fwd C-YFP Duf.itDprr XhoI-rev C-YFP	5'- ATTAGATCTATGCGACGCAGTCGCAAGAAG-3' 5'- ATTGCGGCCGCCATTACCATAGATGGCGCTAAAGC-3'
Sns.it EcorI-fwd C-YFP	5'- ATTGAATTCATGCATCAGCGCCGCAAGAAAGTG-3'
Sns.it NotI-rev C-YFP	5'- ATAGCGGCCGCCTACGAGGTGTCCGTCCGCATC-3
Sns.it EcorI-fwd **2pxxp** C-YFP	5'- ATTGAATTCATGCATCAGCGCCGCAAGAAAGTG-3'
Sns.it NotI-rev **2pxxp** C-YFP	5'- ATAGCGGCCGCCTACGAGGTGTCCGTCCGCATC-3'
Sns.it EcorI-fwd **2pxxp,y14-f14**	5'- ATTGAATTCATGCATCAGCGCCGCAAGAAAGTG-3'
Sns.it NotI-rev C-YFP **2pxxp,y14-f14**	5'- ATAGCGGCCGCCTACGAGGTGTCCGTCCGCATC-3'
Rst-it BglII-fwd C-YFP	5'- ATAAGATCTATGGTCTACATCAAGTGTAAGAAGC-3'
Rst-it NotI-rev C-YFP	5'- ATAGCGGCCGCCAACGGCAGTGGGTGGCGGCAGCA-3'
Dock-fl-fwd N-YFP	5'- ATTGGATCCGATGTTGGAACACCCCCAGCGGTTTGTG
Dock-fl-rev N-YFP	5'- ATTTCTAGATTACGTGCCATTGGCCTTCGGCAG-3'
Dock-ΔSH2-fwd N-YFP	5'- ATTGGATCCGATGTTGGAACACCCCCAGCGGTTTGTG-3'
Dock-ΔSH2-rev N-YFP	5'- ATTCTCGAGTTACGCCAGATTTGGCCGCTCGATTGGC-3'
Dock-ΔSH3(123)-**I** rev N-YFP	5'- ATTGCGGCCGCCTTCGATCCGGAGCCCTTTTTCACC-3'

Dock-ΔSH3(123)-**I** fwd N-YFP Dock-ΔSH3(123)-I**I** fwd N-YFP	5'- ATTGGATCCGATGTTGGAACACCCCCAGCGGTTTGTG-3' 5'- ATACTCGAGAACGACTACCTGGCCACG-3'
Dock-ΔSH3(123)-I**I** rev N-YFP	5'- ATTTCTAGATTACGTGCCATTGGCCTTCGGCAG-3'
Dock-fl EcorI-fwd YTH	5'- ATAGAATTCATGTTGGAACACCCCCAGCGGTTTGTG-3'
Dock-fl XhoI-rev YTH	5'- ATCCTCGAGTTACGTGCCATTGGCCTT-3'
DockΔSH2-ecorI-fwd YTH	5'- ATAGAATTCATGTTGGAACACCCCCAGCGGTTTGTG-3'
DockΔSH2-xhoI-rev YTH	5'- ATTCTCGAGTTACGCCAGATTTGGCCGCTCGATTGGC
DockΔSH3-1-**I** EcoRI-fwd YTH	5'- ATAGAATTCATGTTGGAACACCCCCAGCGGTTTGTG-3'
Dock-D sh3-1-**I** EcoRI-Rev YTH	5'- ATTGAATTCCTTCGATCCGGAGCCCTTTTTCACC-3'
DockΔSH3-1-**II** XhoI-fwd YTH	5'- ATACTCGAGGGAACGGCTGTGGTCAAGTATAACT-3'
DockΔSH3-1-**II** XhoI-rev YTH	5'- ATCCTCGAGTTACGTGCCATTGGCCTT-3'
DockΔSH3-2-**I** EcorI fwd YTH	5'- ATAGAATTCATGTTGGAACACCCCCAGCGGTTTGTG-3'
DockΔSH3-2-**I** EcorI rev	5'- ATTGAATTCGATGGCCTCTGCGGGATCTGGGG-3'
DockΔSH3-2-**II** XhoI-fwd YTH	5'- ATACTCGAGGATAACGACGGCGAGATCCACACCTAC-3'
DockΔSH3-2-**II** XhoI-rev	5'- ATCCTCGAGTTACGTGCCATTGGCCTT-3'
DockΔSH3-3-**I** EcorI-fwd YTH	5'- ATAGAATTCATGTTGGAACACCCCCAGCGGTTTGTG-3'
DockΔSH3-3-**I** EcorI-rev	5'- ATTGAATTCAAGCACGTTCTCGGCCATTGCGTAG-3'
DockΔSH3-3-**II** XhoI-fwd YTH	5'- ATACTCGAGAACGACTACCTGGCCACG-3'

DockΔSH3-3-**II** rev	5’- ATCCTCGAGTTACGTGCCATTGGCCTT-3’
DockΔSH3(123)-**I** EcorI-fwd YTH	5’- ATAGAATTCATGTTGGAACACCCCCAGCGGTTTGTG-3’
DockΔSH3(123)-**I** EcorI-rev	5’- ATTGAATTCCTTCGATCCGGAGCCCTTTTTCACC-3’
DockΔSH3(123)-**II** XhoI-fwd	5’- ATACTCGAGAACGACTACCTGGCCACG-3’
DockΔSH3(123)-**II** XhoI-rev	5’- ATCCTCGAGTTACGTGCCATTGGCCTT-3’
Wip-fl MfeI-fwd	5’- ATACAATTGATG GCTATTCCGCCACCCCCG-3’
Wip-fl XhoI-rev	5’- ACTCTCGAGCTACATACCATTGGTGGCCTTAAACGTG-3’
Wip-prr EcorI-fwd	5’- ATAGAATTCATGCCACCCTCAACGGCAGATAGCAC-3’
Wip-prr XhoI-rev	5’- ACTCTCGAGCTATGGTGGCGTCGATGGGGCGTT-3’
Wasp-fl EcorI fwd	5’- ATAGAATTCATGAGCAGCGGAATGAGG-3’
Wasp-fl XhoI rev	5’- ATACTCGAGTTAGTCCCACTCCCCTTCGTTGTCC-3’
Wasp-fl prr EcorI-fwd	5’- ATCGAATTCACGGAAACAATGGCGGCTCCCATG-3’
Wasp-fl prr XhoI-rev	5’- ATACTCTTACGGTGCGTGCGTGGTCGTAATGAC-3’
Duf.it EcorI-fwd	5,- ATAGAATTCATGCGACGCAGTCGCAAGAAG-3’
Duf.it XhoI-rev	5’- ATTCTCGAGTTAAACATGAGTGGCCAGAGGTC-3’
Hbs.it EcorI-Fwd	5’- ATAGAATTCATGCGCCGGCAGAACAAATCTCAGAGCGA-3’
Hbs.it XhoI-rev	5’- ATTCTCGAGTAAGTCACCGGCGGATAGGACATATAT-3’
Rst.it EcorI-fwd	5’- ATAGAATTCATGGTCTACATCAAGTGTAAGAAGC-3’
Rst.it XhoI-rev	5’- ATTCTCGAGTAAAACGGCAGTGGGTGGCGGCAGCA-3’
Hbs-fwd(**Y1088F)**	5’- AGCAACGATGATGTGTTCTCCAAGGATGACAGTCAG-3’
Hbs-rev(**Y1088F)**	5’- CTGACTGTCATCCTTGGAGAACACATCATCGTTGCT-3
Hbs.IT-fwd	5’- ATAGAATTCATGCGCCGGCAGAACAAATCTCAG-3’
Hbs.it-rev	5’- ATAGCGGCCGCCGTAAGTCACCGGCGGATAGGACATATAT

Hbs-N_HA_IT-Fwd	5'- ATAGAATTCATGTACCCATACGATGTTCCAGATTACGCTCGCCG GCAGAACAAATCTCAGAGCGA-3'
Hbs-rev-NotI	5'- ATAGCGGCCGCTTAGTAAGTCACCGGCGGATAGGACATATAT-3'
Duf.it fwd-EcorI	5'- ATAGAATTCATGCGACGCAGTCGCAAGAAG-3'
Duf.it rev-NotI	5'- ATTGCGGCCGCCAACATGAGTGGCCAGAGGTC-3'

3.14 Sequencing DNA and Software

Sequencing DNA: Sequencing was done by https://shop.lgcgenomics.com/,Berlin for all the DNA Samples.

Software:

EditSeq- Lasergene DNASTAR®

SeqBuilder Lasergene DNASTAR®

SeqManII Lasergene DNASTAR®

Image processing and assembling:

Adobe Photoshop CS5

Microsoft Power Point 2007

Micrisoft Word 2007

4. Methods

I *Drosophila melanogaster* culture

Drosophila stocks were maintained at 25^0C all the time unless otherwise stated. The stocks were raised and maintained on yeast/glucose/maize medium (15 L ddH20, organic maize (UHURU, Oxford), 380 ml 10% Nipagen (sigma) in ethanol (Hayman) (w/v) and 45 ml propionic acid (sigma) following the standard conditions as described in Roberts, 1998. Flies were anaesthetized with CO2 or Di-ethylether and examined under the microscope.

4.1 P-element mediated germ line transformation in *Drosophila melanogaster*

(*Ruby and Spradling, 1982; Sprading & Ruby 1982)*

4.1.1 Collection of embryos

About 200 to 300 *white* flies of 3-5 days old were collected and transferred to the apple juice agar plates coated with yeast to stimulate egg laying. The embryos for injection were collected every 30 min at 18^0C with 1-2 rounds of precollection on apple juice agar plates. The eggs were transferred with a brush carefully in to a fine-mesh metal filter, washed with 0,7% NaCl solution (Colgate Palmoliv, Hamberg) and water for approximately 1 minute to dechorionate. After through rinsing with 0.7% NaCl, the embryos were transferred with a brush onto a rectangular apple juice agar block and oriented in such a manner which would facilitate the injection. The lined up embryos were attached now onto the glass slide with adhesive (10 ml heptanes desolved adhesive for 10 cm adhesive tape) by giving a slight pressure on the embryos. The embryos were then dried a little bit depending upon air humidity and ambience temperature for 8 to 12 minutes in a dessicator over silicagel, in order to reduce the internal pressure of the embryos. Afterwards the embryos were laminated with miniral oil, in order to prevent further drying.

4.1.2 Microinjection of Embryos

10 ug of DNA, 10X injection buffer (2.5 ul) and 2.5 ul of helper plasmid *pπ25.7wc* (0.5 ug/ul) the
volume was made up to 25 ul with ddH20 and centrifuged (13000 rpm/30 minutes/4^0C) in order to sediment the disturbing floating particles. 1 ul of this DNA mixure was filled from the rear end of the injection needle with the help of Borosilica glass capillary of internal diameter 1.2 mm. The liquid flowed up into the needle through the capillary action. This capillary was connected with the pressure system of the micro injection equipment and was fastened to the micromanipulator. To open the capillary the front part of the capillary was broken off under the microscope (20x objective) by touching to the edge of the glass slide. After introducing the needle to the posterior end of the embryos the DNA solution was injected. Embryos, in which the formation of the pole cells had already taken place and in which the germ cells were inaccessible for transformation, the emergence of non transgenic flies. After the injection few more drops of mineral oil was added over the embryos and the glass slide was transferred onto apple juice agar petriplate. The injected embryos were incubated at 25^0C for approximately 24 hours. The embryos which survived the trauma of microinjection developed into larvae and crawled around in the petriplate. The larvae were collected with the help of a needle and transferred into fly bottles with breading medium. Each larvae which developed into adult flies were collected.

4.1.3 Selection of transformed flies *(klemenz et al. 1987)*

The selection of transformed flies is made possible by the use of the *white*$^+$ gene (red eye colour) as dominant selection marker. Since the insertion of the P-element-construct takes place in the germ line of the injected embryos, the insertion event can be observed only in the G1-generation. The injected animals (G0-generation) which closed immediately crossed with *white* flies. In the G1-generation the transformed individuals can be recognized by the appearance of eye colour ranging from orange to red. Each transgenic animal was crossed now again with *white* flies and the developing heterozygote descendants were further crossed among themselves. The homozygote descendants, which are to be usually recognized by the darker eye colour, were then further establishment of a stable transgenic line. To establish a transgenic line, one of these transgenic flies is crossed against the multiple marker strain *CSTM (Sp/Cyo; TM2/MKRS).* The phenotype of this offspring shows

the localization of the transgene in the chromosome. To further establish the line, the offspring is crossed once more against CSTM.

II Molecular Methods

4.2 Production of chemically competent bacteria (*Escherichia coli*)

(sambrook et al., 1989)

250 ml LB medium was inoculated with 2.5 ml of fresh *E.coli* (1:100) and incubated at 37^0C with continuous shaking till it reached up to OD600 at 0.5-0.6. After 15 minute of incubation on ice the cells were centrifuged at (4000 rpm, 4^0C, 10 min), and the pellet was resuspended in 80 ml cold RF1-buffer and placed again on ice for 15 minutes. The cells were centrifuged to pellet (4000 rpm, 4^0C, 10 minutes) and resuspended in 20 ml RF2-buffer and incubated on ice for 15 minutes. The cells were used immediately for transformation if needed and the remaining aliquots were frozen at -80^0C fo future use.

4.3 Transformation of chemically competent bacteria

200 ul competent cells were thawed on ice for 10-15 minutes, 1-10 ul plasmid DNA solutions or ligation mixture was added and incubated on ice for 30 minutes. The cells were heat shocked in 42^0C waterbath for 60-90 seconds and immediately placed on ice for 5 min. In the mean time LB medium was warmed to room temperature. 900 ul of LB medium was added to the ice cold competent cell mixture and incubated at 37^0C for 1 hour to gain antibiotic resistance. The incubated mixture is spun for 1 minute at 5000 rpm. Pour out majority of the supernatant slowly by vortexing. Plate the entire cell mixture on LB selection plate (ampicillin 1000 mg/ml, Kanamycin 50 mg/ml, IPTG, X-Gal) depending on the type of antibiotic resistant gene used in the construct. Dry the plates and incubated in 37^0C overnight.

4.4 Preparation of plasmid DNA from *E.coli* (mini preparation)

3 ml LB medium (+ antibiotic) is inoculated with a single colony of bacteria and incubated at 37^0C with continuous shaking for 6-7 hours. Centrifuge and pellet

down the cells at 13000 rpm for 30 seconds and discard the supernatant, do the same for the remaining amount of the incubated broth. Add 250 ul of E1-buffer and the resuspend the pellet by vortexing briefly. Add 250 ul of E2-buffer to lyse the cells, incubate at room temperature for 5-10 minutes. Add 250 ul of E3-buffer for neutralization and immediately mix gently, the cell membrane as well as the genomic DNA gets precipitated by giving curd like appearance. Centrifuge at room temp for 10-15 minutes at 13000 rpm. The curd seperates from the supernatant. The supernatant transferred to another eppendorf tube. Add 0.5 V of isopropanol to the supernatant and centrifuge at 13000 rpm for 30 minutes at 4^0C. Decant the supernatant and add 500ul of 70% ethanol and incubate for 15 minutes at room temp for the salt to dissolve. Centrifuge for 15 minutes at room temperature and slowly decant the supernatant. Vaccum dry the pellet and dissolve the DNA in 10-30 ul of ddH2O or TE buffer depending on the concentration required.

4.5 Midi preparation of plasmid DNA (Genomed jet star, according to the manufacturer's instruction given in the manual)

To prepare the large amount of plasmid DNA which is pure and free of all the materials such as SDS and salts midi preparation was done. The incubated 50 ml bacterial culture was transferred into 50 ml Falcon tubes and centrifuged at 4000 rpm for 10 minutes. The pellet was transferred into 50 ml Falcon tubes and centrifuged at 4000 rpm for 10 minutes. The pellet was resuspended in 4 ml of E1-buffer and the cells are finally lysed by addition of 4 ml E2-buffer with gentle mix. This mixure was incubated for 5-10 minutes and 4 ml of E3-buffer is added for neutralization. This mixture was centrifuged at 6000 rpm at room temperature for 20 minutes. The curd seperates from the supernatant containing plasmid DNA. Slowly the supernatant was transferred to the pre-equilibriated anion exchange column with E4-buffer. The solution was let to pass through the column by gravity flow. When all the solution was passed, the column was washed twice with E5-buffer, 10 ml each. The solution was let to pass through the column completely. Finally the plasmid DNA was eluted by adding 5 ml E6-buffer. The eluted DNA was mixed with 0.7 V isopropanol. Centrifuge at 4^0C, 6000 rpm for 15 minutes. The supernatant was decanted and the DNA pellet is vaccum dried. The DNA was dissolved in required amount of ddH2o for the future use.

4.6 Preparation of genomic DNA from *Drosophila* *(pirotta, 1986; Steller & pirotta 1986)*

10 anesthetised flies were homogenized in 100 ml extraction buffer with a pestle in a glass homogeniser. Whole extract was transferred into a 1.5 ml micro reaction container and incubated for 30 minutes at 65^0C water bath. After addition of 14 ul of 3M KAc the mixture was incubated on ice for 30 minutes and centrifuged at 13000 rpm at 4^0C for 15 minutes. The supernatant was transferred to a new reaction container which contained genomic DNA and 0.5 V of isopropanol was added. The mixture was centrifuged for 30 minutes at 4^0C /13000 rpm. The pellet was washed twice with 70% ethanol, centrifuge and pellet was air dried. Finally the genomic DNA was dissolved in 20-100 ul ddH2O and stored at -20^0C for future use.

4.7 Agarose gel electrophoresis *(Sambrook et al., 1989)*

DNA molecules were separated on the basis of size by agarose gel electrophoresis. The horizontal gels of the size 6 cm x 9 or 8 cm x 12 were used. For proper separation based on the size of the DNA to be analyzed the agarose concentration 0.8-2% 9 (w/v) in 1x TAE buffer was used to which Ethidium bromide (0.5 ug/ul) was added. The DNA samples to be loaded on to the gels were mixed with 1/10 V of loading buffer. The gel was run at 60-100 V. Subsequently the gel was documented with UV light and the picture was printed out.

4 .8 Isolation of DNA from agarose gels (*JET sorb Gel Extraction*)

The desired fragment was cut with a sharp scalpel under the UV light from the agarose gel and transferred to 1.5 ml micro reaction tube. To every 100 mg of piece of gel 10 ul of captured buffer and 300 ul of solution A was added and incubated at 60^0C for 15 minutes with brief vortexing for every 2-3 min in order to dissolve the agarose. After the agarose was dissolved completely, the solution was transferred to solution A mix well and centrifuged for 30 seconds for 13000 rpm. Remove the supernatant and wash with 300 ul of solution A and centrifuge at 13000 rpm/30 seconds. Take off the supernatant and wash pellet second time with 3000 ul of solution B at 13000 rpm/30 seconds. Repeat the same step, dry the pellet, add 10 ul of ddH2O or 1X TE buffer to pellet and dissolve at 55^0C for 5

minutes. Centrifuge at 13000/30 seconds. Transfer supernatant to a new micro tube and discard pellet.

4.9 Estimation of DNA concentration using Spectrophotometer

Nucleic acids can be quantified due to their maximum absorption at the wavelength of λ=260 nm using spectrophotometer. From the absorption (optical density = OD) the nucleic acid concentration can be computed in ug/ml using a quartz cuvette with the thickness of 1 cm considering the following parameters.

Double standard DNA OD_{260} X 50 X Dilution factor

Single standard DNA OD_{260} X 50 X Dilution factor

RNA OD_{260} X 37 X Dilution factor

4.10 Enzymatic manipulation of nucleotides

4.10.1 Digestion of DNA with the help of Restriction endonucleases

The following formula is generally used to determine the units of restriction enzyme required to cleave the DNA with a particular concentration.

$$\text{Required Units/ug DNA} = \frac{\text{48.5 Kb (=bp λ) X Nuber of restriction sites in targetDNA}}{\text{Get-DNA(bp) X Number of restriction sites in λ-DNA}}$$

Restriction endonuclease digestion were performed using the enzymes and buffer from (Roche, Mannheim) or Amersham Pharmacia Biotec (Freiburg) according to the given instruction in the manual. Reactions were generally performed in the total volume of 30 ul. For some enzymes (*Ecor I*) which show the star activity the total volume of 50 ul was taken to prevent star activity. Double digestion were done with suitable buffers which are comparable with both the chosen enzymes. All digestions were done at 37^0C for 2 hours.

4.10.2 Ligation of DNA fragments

The ligation of DNA fragments requires the presence of compatible ends. 100-150 ng of vector DNA were used together with a three to fivefold molar excess of insert depending

upon the insert size with 1/10 V of ligation buffer and 2 units of T4-DNA ligase in a final reaction volume of 15-20 ul. The ligation mixture was incubated at 16^0C overnight.

4.10.3 Polymerase chain reaction (PCR) *(Saiki et al, 1988; Qiagen, Stratagene according the manufacturers manual)*

The amplification of specific DNA fragments is facilitated by PCR. The synthetically manufactured oligonucleotides serves as specific primers to amplify the DNA of interest. The thermostable polymerase plays a major role in synthesis of complementary DNA from the template. By the cyclic denaturation and renaturation the DNA region of interest amplifies exponentially. The reaction was done in a total volume of 50 ul by adding the following reaction components.

For pfu DNA polymerase

X ul of Template-DNA (5-100 ng)
1.5 ul Sense-Primer (100 pmol/ul)
1.5 ul Anti-sense-Primer (100 pmol/ul)
1.5 ul dNTP-Mix (10 mM dNTP)
5.0 ul 10X pfu polymerase buffer
1.0 ul of 50 mM $MgSO_4$
1.0 ul pfu-DNA-Polymerase (5 U/ul)
Make up the volume to 50 ul with ddH2O

For Vent Polymerase

X ul Template-DNA (5-100 ng)
1.0 ul Sense-Primer (25 pmol/ul)
1.0 ul Antisense-Primer (25 pmol/ul)
1.0 ul dNTP-Mix (10mM dNTP)
5.0 ul 10 X Thermo Polymerase buffer
3.0 ul of 50 mM $MgSO_4$
1.0 ul Vent –DNA –Polymerase (5U/ul)
Make up the volume to 50 ul with ddH2O

For Taq polymerase

X ul Template-DNA (5-50 ng)
1 ul Sense-Primer (25 pmol/ul)
1 ul Antisense primer (25 pmol/ul)

1 ul dNTP-Mix (10mM dNTP)
5 ul 10 X PCR-Puffer
1 ul Taq- DNA – Polymerase (5U/ul)
Make up the volume to 50 ul with ddH2O

The following programme was used for the PCR in the thermocycler.

Step 1. Denaturation	95 ^{0}C	300s
Step 2.Denaturation	94 ^{0}C	10 s
Step 3. Primer anneling	40-60 ^{0}C	60s
Step 4. Polymerization	72 ^{0}C	30-180 s
Step 5. Polymerization	72 ^{0}C	300s

After completion of the DNA synthesis in step 4 the reaction was taken up again to step 2. The cycles were repeated 30-35 times. After the completion of reaction it was stored at 4 ^{0}C in the final step.

4.10.4 Cloning with the TOPO vector

Cloning with the TOPO vector is an easy procedure. By this method PCR product can be cloned directly by following procedure i.e

0.5 ul TOPO or 0.5 ul TOPO blunt vector

0.5-4ul PCR product

X ul ddH2O, Make total volume to 6 ul.

Incubate at room temperature for about 10 minutes, then stop reaction by putting on ice. Transform the 3ul of the reaction mixture on to TOP 10 or *E.Coli DH5α* chemically competent cells and proceed with standard transformation techniques.

4.10.5 Cloning with the other vector

Choose appropriate restriction enzymes and digest both fragment and vector of interest. Because the fragments usually are cloned into other vector, it is necessary to extract the fragment alone by means of DNA elution. The vector, if cut with only one enzyme will need to be treated with alkaline phosphatise to avoid re-ligating with itself. The entire digested

vector sample is digested with 2.5 ul alkaline phosphatise and 4 ul 10 X phosphatise buffer. Add enough water to a total volume of 40 ul and incubate at 37^0C for 40 minutes . To deactivate enzyme, put sample for 20 minutes at 68 ^{0}C.

The actual ligation step needs to be calculated, depending on the sizes of the fragment and vector.

$$\frac{\text{(50 ng vector X insert Kb)}}{\text{(vector kb)}}$$

The result is multiplied by three. This is how much fragment you have to use for 50ng Vector. Add 1 U T4 ligase and 2ul 10X ligation buffer. Mix with enough ddH2O to reach a volume of 20 ul. Depending on size of insertion fragment, incubate sample over night at a temperature between 14^0C – 16 ^{0}C. The smaller a fragment is, the higher the temperature can be set.

4.11 Precipitation of DNA

Precipitation of DNA with Ethanol

Add 1.5 X volume of 100% EtOH and 1/10 5M NaAc to solution with DNA**.** Mix carefully and centrifuge at 13000 rpm for about 20 minutes. Take off supernatant and wash DNA pellet with 70% EtOH by centrifuging for another 20 minutes at 13000 rpm. Take of supernatant and dry pellet completely to remove any traces of alcohol before resuspending the pellet in suitable amounts of 1X TE buffer or sterile ddH2O.

Precipitation of DNA with Isopropanol

Add 1.5-2 X volume of Isopropanol. Mix carefully and precipitate DNA by centrifuging at full speed for at least 30 minutes. Take off supernatant and wash pellet in 70% EtoH, same procedure as above. Resuspend pellet in either 1x TE buffer or sterile ddH2O.

4.12 Site-Directed mutagenesis (*QuikChange™ Site-Directed Mutagenesis Kit according to manufacturer Stratagene, USA)*

PRIMER DESIGN

Mutagenic primers introduce specific experimental mutations. The mutagenic oligonucleotide primers for use in this protocol must be designed individually according to the desired mutation. The following considerations should be made for designing mutagenic site.

Setting Up the Reactions

1. Prepare the control reaction

5 µl of 10× reaction buffer
2 µl (10 ng) of pWhitescript™ 4.5-kb control plasmid (5 ng/µl)
1.25 µl (125 ng) of oligonucleotide control primer #1 [34-mer (100 ng/µl)]
1.25 µl (125 ng) of oligonucleotide control primer #2 [34-mer (100 ng/µl)]
1 µl of dNTP mix
Double-distilled water (ddH2O) to a final volume of 50 µl
Then add
1 µl of *PfuTurbo* DNA polymerase (2.5 U/µl)

2. Prepare the sample reaction

5 µl of 10× reaction buffer
2 µl (10 ng) of dsDNA Template (5 ng/µl)
1.25 µl (125 ng) of oligonucleotide [34-mer (100 ng/µl)]
1.25 µl (125 ng) of oligonucleotide [34-mer (100 ng/µl)]
1 µl of dNTP mix
Double-distilled water (ddH2O) to a final volume of 50 µl
Then add
1 µl of *PfuTurbo* DNA polymerase (2.5 U/µl)

Cycling Parameters for the QuikChange Site-Directed Mutagenesis Method			
Segment	**Cycles**	**Temperature**	**Time**
1	1	95°C	30 seconds
2	12–18	95°C	30 seconds
		55°C	1 minute
		68°C	2 minutes/kb plasmid

Digesting the Products:

Add 1 µl of the *Dpn* I restriction enzyme (10 U/µl) directly to each amplification reaction below the mineral oil overlay using a small, pointed pipet tip. Gently and thoroughly mix each reaction mixture by pipetting the solution up and down several times. Spin down the reaction mixtures in a microcentrifuge for 1 minute and immediately incubate each reaction at 37°C for 1 hour to digest the parental (i.e., the nonmutated) supercoiled dsDNA.

Transforming into *Epicurian Coli XL1-Blue* Supercompetent Cells:

Transfer 1 of the *Dpn* I-treated DNA from each control and sample reaction to separate aliquots of the supercompetent cells. Preoceed the standard Transformation protocol. Finally the mutation was confirmed by sequencing the plasmid.

4.13 Yeast two-hybrid assay *(clonetch, Matchmaker Lex A Two-Hybird System based on Gyuris et al., 1993).*

By yeast two-hybrid system, protein interaction can be tested by means of growth of yeast clones. The proteins of interest are cloned into two different vectors. The pB42AD vector is the so-called 'bait' vector. The first protein of inerest is cloned into the pB42AD. Coresponding to that, there is pGilda, the library protein. This is where the second protein of interest is cloned into. In pB42AD, Lex A is also coded but cannot work alone. In pGilda however, there is a peptide sequence that can activate LexA. This means that if bait and library proteins physically cololate, i.e interact with each other, the peptide sequence can activate LexA. LexA in turn, activates a LacZ machinery that produces β-galactosidase. This β-galactosidase can be easily detected by looking for colonies that are colored blue.

Cultivation of Yeast

The basic yeast strain used for experiments is EGY48 b[*p80p-lacZ*]. It already contains the reporter plasmid which can be activated by LexA. Furthermore, It can synthetize uracil by itself, thus enabling selection on yeast media without uracil. The

cultures are gown at 30°C overnight on either agar plates or liquid media. Transformed yeast clones need longer incubation times of at least 3 days.

Transformation of Yeast

Depending on the size of the colonies of the stock agar plate, inoculate 50ml of SD-Ura media with one to several colonies of yeast. Incubate in a shaker at 30°C until the OD_{600nm} 0.5 -1.5. Centrifuge the culture down at 3000 rpm for 3 minutes and discard media. Resuspend the pellet in a suitable amount of 1X LiAc. The suitable amount to dependent on the amount of transformations. For 12 transformations, add 1300ul LiAc, for 5 transformations, add 600ul LiAc, etc. Mix well to solve pellet properly. Denature 17.8 ul salmons sperm DNA (15 min, at 95°C, cool on ice) and mix with 1-2 ug DNA. Add 100ul re-dissolved cells and 500ul 50% PEG/1:1 1XTE/1XLiAc and vortex to mix well. Incubate at room temperature for 35 minutes. Add 50 ul DMSO and heat shock for 15 minutes at 42°C. Cool samples down on ice. Inoculate 20 ml YPD medium with samples and incubate flasks at 30 °C in shaker for one hour to let cells recover. Pellet the culture by centrifuging at 1000 rpm for 5 minutes. Re-dissolve pellet in 10 ul 1X TE buffer and plate samples on respective selective SD agar plates. Incubate upside-down at 30°C for at least 3 days untile colonies form.

Transformatiom Efficiency

Before continuing the experiments, it is necessary to determine the transformation efficiency of the yeast cells. This is calculated.

$$\frac{\text{cfy}}{\text{ug DNA}} = \frac{\text{CFU X total transformation volume (ul)}}{\text{(plated Volume ul) x amount of transformed DNA (ug)}}$$

The result provides information of how many colony forming units (cfu) per ug DNA used arise. A co-transformation should usually reach about 1X cfu/ug DNA.

Induction Test

This is the actual test to see interaction between two proteins. Co-transformed colonies are plated on selective media plates (-HULT/+Raf/+Gal/+X-Gal). The yeast culture is now forced to express both the bait and library protein and thus the proteins of interest along with it, in order to survive. Colonies arising from this either turn blue (β-Galactosidase is expressed with LacZ and with X-Gal, produces a blue color) or simple growth. The latter is controlled by Leucine, which is encoded by the yeast strain. Only if interaction occurs, Leucine can be expressed and thus the cell can survive on media without Leucine. Results were scanned by Hp laser jet.

III Histological methods.

4.14 Fixation of the *Drosophila* embryos

Carefully collect embryos from apple juice or grape juice plates with PBT or water and a small brush and transfer to a small net. Wash several times with PBT or water to rid off food rests. Dechorionize in a 1:1 Chlorine/Water solution for about 2 minutes. Wash off solution twice with plain water and dry well.

Heat FiXation

Collect embryos with PBT and transfers into a cup or falcon tube, depending on amount of embryos. Remove PBT and add hot PBT to embryos. Cook in hot water bath for 10 to 40 seconds before cooling down on ice and replacing with fresh PBT. Remove PBT and add equal volumes of heptane and methanol. Shake vigorously for at least one minute to de-vittelinize embryos. You can see the embryos have been successfully de-vittelinized if white embryos sink to the bottom of the cup/tube and the rest swim to the interphase between heptane and methanol. Wash twice with methanol and store at 4°C for furthure use.

Formaldehyde Fixation

Collect embryos with heptane and transfer into a cup or falcon tube, depending on amount of embryos. Change heptane and add equal amount of 4% F-PBS. Fix embryos on shaker at room temperature for about 15 minutes. Take off the water phase at the bottom of the cup first, before taking of the rest of the organic phase, swimming at the top. The embryos should swim directly in between. Add equal parts of methanol and heptane and shake vigourously for one minute to de-vittelinize embryos. Take off entire solution and embryos swimming in the interphase, leaving the de-vittelinized embryos at the bottom of the cup/tube. Wash twice with methanol and store at 4°C fo further use.

4.15 In situ hybridisation on *Drosophila* embryos

4.15.1 Preparation of DIG labelled Riboprobes

As RNA is very sensitive, it is important to work at all times in a clean environment to ensure no RNase gets transferred into cups containing your riboprobes. To create DIG-labelled riboprobes, digest DNA of interest with appropriate enzymes to linearize DNA. Use proper RNA polymerase to generate an anti-sense and sense string

1ug linearized DNA
2ul appropriate RNA polymerase
2 ul 10x transcription buffer
X ul dNTP's marked with DIG
X ul ddH2o, sterile
Make up total volume to 20 ul

Incubate mixture at 37°C for a minimum of 3 hours. Precipitate riboprobe with method of choice and disolve dried pellet in 20 ul 1:1 sterile ddH2O/hybridization solution. Sample can now be stored at -20 °C for further use.

4.15.2 Test of the DIG labelled DNA probe (Dot Blot)

To test whether transcription has actually occurred, prepare a dot blot. Cut a small piece of Hybond N-membrane and blot 1 ul riboprobe in the following dilutions: 1:25, 1:50, 1:1000, 1:1000. Let dots dry completely, then fix the samples by cross-linking them to the membrane by UV light. Wash the membrane once for 5 minutes in PBT, then incubate at room temperature with the α-DIG antibody at a 1:5000 dilution for about 1 hour. Take off antibody (without Tween). Add 9ul NBT and & 7 ul BCIP per 2 ml NBT buffer and incubate in the dark. If transcription of riboprobes was successful, small dark spots should visible on the membrane. Determine dosage for in situ hybridization by picking the highest dilution in which a positive reaction is still visible.

4.15.3 In situ Hybridisation and staining on Drosophila Embryos

Rehydrate the embryos 3X10 minutes with PBT, Cook samples in boiling water for 6 minutes, then cool down on ice. Change PBT twice and wait until all embryos have sunk to the bottom of the tube. Re-fix embryos for 15 minutes in 4% F-PBS. Wash off fixation solution 5 X 5 minutes with PBT. Incubate 15 minutes in 1:1 solution HS:PBT at room temperature, then incubate for 20 minutes in pure HS at 55°C. Change HS and incubate the samples for 1.5 hours at the same temperature. Cook riboprobes diluted appropriately in HS for about 15 minutes. Remove HS from samples and add the hot riboprobes. Incubate overnight at 55°C. Remove riboprobes from samples and wash them 3 X 20 minutes in warm HS. It is crucial to keep samples and HS at 55°C during this process. Incubate samples in 1:1 solution of HS and PBT for 15 minutes, then wash samples 3 X 10 minutes in PBT at room temperature. Incubate samples with α-DIG antibody with a concentration of 1:2000 in PBT for about 2 hours. Wash off the antibody 5 X 10 minutes with PBT then change buffer to NBT. Wash samples 2 X 20 minutes in NBT buffer containing tween.

To start staining reaction, add 4.5 ul NBT and 3.5 ul BCIP. To stop reaction, take of solution and wash thoroughly with PBT. Embed samples in epon and let epon dry overnight at 60°C in incubator.

4.15.4 Embedding *Drosophila* Embryos

With Glycerine

To embed with glycine, remove as much PBT as possible from the stained embryos. Add a small amount (e.g. 300-500 ul) 70% glycerine and allow embryos to sink to the bottom of the cup. This usually takes several hours, so it is best to do this overnight at 4°C. Pipette as little as possible of the glycerine but with as much embryos as possible on to a slide and cover.

With Epon

Epon is usually stored at 20°C and should be treated with care as it is hazardous. Stained embryos need to be slowly dehydrated with alcohol. Start first with a 10 minute wash in 25% EtOH and keep increasing concentration until the embryos are washed in 96% EtOH. Heat epon to 37°C in water bath so that it becomes liquid enough for usage. Pipette a small drop of epon on slide. Take a small droplet of EtOH containing the stained embryos and carefully pipette drop on epon. Carefully cover side (no bubbles) dry epon at 60°C for at least in an incubator.

IV. Immunihistochemistry

4.16 Whole mount fluorescent antibody staining on *Drosophila* Embryos

Slide mounting of Immunofluorescent specimen enables microscope study and storage, Formaldehyde fixated embryos were washed three times for 10 minutes with 1X PBT. The primary antibodies in 1X PBT are added to the embryos for overnight shaking at 4°C. 3X wash with 1X PBT followed by blocking of unspecific binding sites via 500 ul 2% NGS/PBT for 30 minutes. After the blocking solution is replaced with fluorophore-coupled secondary antibodies (1:200). The embryos are incubated on shaker for 2 hours in a dark box. Samples are finally washed three times for 10 minutes with 1X PBT. Then the supernatant is totally substituted by mounting by mounting medium. Embryos are transferred and arranged on

microscope slide. Sealed with cover slip, the conserved samples are ready for microscope examination and permanent storage.

4.17 Transfection and immunostainig of SL2 cells

$3x10^5$ S2 cells were seeded onto a 24 well dish and incubated at 24 degree for 24 hours, followed by 0.5 ug of each constructs and 0.5 ug of actin GAL4 were transfected using biorad transfectine reagent and incubated for 24 hours. After 24 hours of post transfection cells were washed, transferred in to a concanovaline A coated coverslips (incubation time is 40 minutes at RT) and the cells were incubated on coverslips for 2.3 hours at room RT and after that cells were washed with 1xPBS. Cells were fixed in 4% F-PBS about 15 minutes. Removed the excess cells, wash with two times with 1xPBS and cells were permeabilised by 0.5% Triton x-100 in 1xPBS for 2.5 minutes at RT. Cells were washed with 1xPBS two times for 5 minutes. Cells were blocked in 3% BSA in 1xPBS for 30 minutes at RT and one time wash with 1xPBS.

For split YFP assay incubate cells with phalloidine staining 1:40 and nuclei were labelled with Hoechest (1:200 in 1xPBS). Cells were washed with 1xPBS and cells were mounted using Southern Biotech flouromount-G, images were obtained under a confocal mikroskope (LEICA TCS sp2).

For primary antibody staining, cells with anti-Ha mouse(1:1000), anti-Myc mouse(1:1000) and incubated at RT for about 2 hours, wash with 1xPBS two times. Cells were incubated with secondary antibody anti-mouse cy2 and cy3 (1:250) for 1hour at RT. Cells were washed with 1xPBS two times ,nuclei were labelled with Hoechest. Cells were washed with 1xPBS and cells were mounted using Southern Biotech flouromount-G, images were obtained under confocal mikroskope (LEICA TCS sp2).

4.18 Western blotting

Western blot provides a possibility to analize proteins extracted from either cells or Drosophila embryos, e.g Whether a certain protein is expressed at all. In this work, western blots were done using proteins extracted from *Drosophila* embryos.

Protein extraction from *Drosophila* embryos.

Collect embryos of desired genotype and transfer to net. Dechorionize embryos by putting net in a 1:1 H2O/chlorine solution for about 2 minutes. Wash off chlorine by immersing net in water twice. Dry net well and add 1X PBT. Transfers embryos into a cup and remove as much PBT as possible. Determine the total volume of embryos and add just enough lysis buffer to easily mush the embryos. Once embryos have been homogenized as possible, add more lysis buffer to a total of 2X original volume of embryos. Add 1/7x original volume of 7X complete protease inhibitor cocktail. From here on, always put lysate on ice to avoid proteins being destroyed. Centrifuge at 4°C for about 15 minutes. You will notice a thin layer on top of the supernatant into a new cup. Centrifuge again for another 15 minutes and transfer supernatant without the lipid layer. Store proteins at 4°C.

Protein extraction from *Drosophila* SL2 cells

The drosophila S2 cells were collected from the cultured plates using cell scraper in to falcon tube. The cell were spun down at 4°C/2000 rpm for 2-5 minutes. The supernatant was removed and the pellet was dissolved in appropriate volume of cell lysis buffer and mix well with the pipett. Keep down the cells on ice for 5 minute and lysate were transferred into eppendorf, centrifuged at 13000 rpm/10 minutes/4°C. Collect the supernatant which contains the proteins and stored the proteins at 4°C for further use.

Protein estimation (Bradford assay)

To determine the concentration of protein in the embryo extract, you will first need a BSA test series. The test series provides a graph from which the concentration of the sample can be determined. To make test series, pipette 0 ug/ul, 1ug/ml, 5ug/ml, 10ug/ml and 20ug/ml into separate cups. Add enough ddH2O to a total volume of 800ul. Add 200ul Bradford solution and mix well. Incubate at room temperature for 5 minutes, then measure absorption at OD595nm. For the sample, pipette 5 ul and add 800 ul ddH20 and 200 ul

Bradford. Mix well and incubate at room temperature for 5 minutes to measure absorption. Draw graph with the BSA dilution series. Read concentration of sample from graph with the help of the absorption measured.

SDS-PAGE Protein Gel *(Sambrook et al)*

The SDS PAGE gel separates proteins based on their mass in KDa. Depending on the concentration of the gel, these proteins will travel faster (less concentrated) or slower (more concentrated).

To force proteins in a more homogenous matter, SDS, which denatures proteins, is used. Usually, travelling of proteins through an electric field is dependent on several factors: the nett charge of the protein, friction coefficient and the electric strength applied. With SDS, the mass of the protein plays the only the role anymore when travelling through the acryl amide gel. This is because SDS is so negatively charged that when it binds to the protein, the actual charge of the protein does not matter anymore.

The gel itself is prepared in two steps. The first step requires making the separation gel which separates the protein extract into bands according to their mass in KDa. Above this gel comes a "stacking gel" which collects the protein mix and makes sure that the proteins run evenly at the same speed to reduce falsification of results. The results are prepared like this:

5% SDS-PAGE (stacking gel)	**8% SDS-PAGE**
4.4 ml ddH2O	2.3 ml ddH2O
830 ul acryl amide	1.3 ml acryl amide
630 ul Tris pH 6.8	1.3 ml Tris pH 8.8
50 ul 10%SDS	50 ul 10% SDS
50 ul 10%APS	100 ul 10%APS
5 ul TEMED	10 ul TEMED
Make the total volume to 5.0 ml	Make the total volume to 5.0 ml

10% SDS-PAGE

1.9 ml ddH2O
1.7 ml acryl amide
1.3 ml Tris pH 6.8
50 ul 10% SDS
100 ul 10 SDS
10 ul TEMED
Make the total volume to 5.0 ml

Prepare the SDS-PAGE gel. As soon as APS and TEMED have been added to the mixture, the gel will begin to polymerize, so from here on you will need to work quickly. Pour the SDS-PAGE gel in between two galss plates that have been separated with spacers. To make the top of the gel as even as possible, carefully pour some isopropanol on top of the gel. The gel will poymeize pretty quickly (about half an hour). Remove all the isopropanol and prepare stacking gel. Carefully pour stacking gel over the other gel and insert the comb. Be sure to avoid air bubbles when putting the comb into the liquid stacking gel. Let gel polymerize (will take at least half an hour) before further use.

Assemble the machinery for running the gel according to manufacturer's instructions. Generously pour 1X SDS Running Buffer to cover the gel completely, as well as fill the bottom of the gel chamber. Remove comb and wash pockets with the Running buffer. Make sure there are no bubbles at the bottom of gel chamber and the gel itself.

Dilute protein sample to a concentration of 20 ug within a volume of maximum 8 ul. Mix with 2 ul 5X SDS loading buffer and cook samples for 5 minutes at 94°C. Cool down on ice before loading samples into pockets. Load appropriate marker in a separate pocket. Run gel at 100-150 V until the sample have reached the border of the stacking gel, then change current to 200V and let samples run through until the blue marker of the sample has completely runout of the gel.The gel can be stained with staining techniques such as comassie blue or the gel can be used for western blotting.

Western Blotting (BioRad, USA)

The western Blotting technique transfers the proteins from the gel onto a special membrane by means of electricity. The membrane can then be used for immunochemistry, i.e antibody staining.

The gel is carefully removed from the glass plates and transferred into 1X Transfer Buffer for about 10 minutes. In the meantime, 12-16 Whatman-Filters cut to the size of the gel are prepared and drenched in Transfer Buffer as well. Cut out a piece of Hybond P-membrane to the exact size of the gel and soak in Me0H. On the blotting machine assemble stack as the following.

- 6-8 whatman filters
- Hybond P-membrane
- Gel
- 6-8 Whatman filters

For about 1 hour, run current through gel which is calculated by the following:

$$0.8\ mA/cm^{2\ filter}\ area$$

After blotting, you should see the marker bands clearly on the membrane. Carefully remove stack to take membrane and wash with protein side up in 1X TBS for about 10 minutes at room temperature. Block membrane for 1 hour in 5% milk powder/TBS. Wash off milk powder with TBSTT for 10 minutes, then add primary antibody diluted to the right concentration with TBS. Incubate overnight at 4°C. The next day, wash antibody off twice with TBSTT for each 10 minutes at room temperature. Incubate membrane with secondary antibody diluted 1:4000 in 5% milk powder/TBS for 1 hour at room temperature. Remove the secondary antibody and wash 4X 10 minutes in TBSTT. After this, detection of protein can be started by immersing the membrane in 1 ml Solution A mixed with 1 ml Solution B from the ECL Kit provided by the company Amersham. Incubate for less than a minute.

Embed the membrane between the plastic membrane foil and proceed to make autoradiograph films with the first exposure time being 1 minute.

4.19 Co-immunoprecipitation:

$3x10^5$ number of cells were seeded onto a 2x 8ml cell culture petri dishes at 25 degrees at 24 hours prior to transfection. 0.4 ug of each constructs (ex.HA-Hbs.it and Dock-Flag) and co transfection of these constructs into these cells using Biorad tansfectin reagent. Cells were harvested (>48 hours post transfection) by centrifugation 1500 rpm and washed with 1X PBS. Cells were resuspended in 700ul of ice cold IP buffer (10mM EDTA, 1mM protease inhibitor, 1% Triton-x 100, EDTA free protease inhibitor tablets (Roche), in 1xPBS. Cells were centrifuged at 12000 rpm for 10 minutes and pellet was collected. 50 ul of 2xLaemmli buffer was added to the 50 ul of the supernatant and boiled for 10 minutes, this was used as input. For Ha- Hbs.it and Dock-flag co-ip 50 ul of anti-Ha (Sigma) agarose beads were taken and washed with the two times with 750 ul of IP buffer and the rest of the supernatent was added to the anti-HA agarose beads these were left on a roller in 4 degree for 2-3 hours.The mixture was spun down at 4^o C for 30 sec at 2000 rpm and washed in cold IP buffer. This was repeated for 2 times with interval of 5 minutes with occasionally mixing. After the final centrifugation equal volume of 2X laemmli buffer was added and the sample boiled for 5 minutes.

The co-ip of HA-Hbs.it and Dock flag with anti-Flag (Sigma) beads would be the same.

The co ip of HA-Duf-Fl and Dock-flag with anti-Flag (Sigma) beads would be the same.

The co ip of HA-Duf-fl and Dock-flag with anti-HA (Sigma) beads would be the same.

The co ip of Duf.it Myc and Dock-flag with anti-Myc (Sigma) beads would be the same expect the incubation period is overnight/8hours.

The co ip of Duf.it Myc and Dock-flag with anti-Flag (Sigma) beads would be the same expect the incubation period is overnight hours.

After completing the Co-IP protocol samples were analyzed by SDS-PAGE, followed by Western blot. The final results were analyzed.

5. Results:

I. Cell adhesion molecules that mediate myoblast fusion in FCs

In *Drosophila melanogaster* Ig-SF molecules are involved in myoblast fusion. The Ig-SF are Duf/Kirre, Rst/Irre, Sns, Hbs. In founder cells Duf, Rst are the myoblast attractants, they are redundant to each other. But, in contrast to Duf, Rst is also expressed in FCMs. Duf and Rst has five Ig-like domains at the extracellular domain and high conservation reside primarily in the region of five Ig domains. The intracellular domain of Duf and Rst is considerably longer than that of Rst and show a low overall homology with the one of Rst. However three highly conserved motifs were detected (FIG 2.7 B; Strunkelnberg *et al*., 2001). I have created chimeric constructs of both protein, i.e Duf extra-Rst intra celluar domains and Rst extra-Duf intra cellular domains to see the nature of redundant functions of both proteins during myoblast fusion. Furthermore, I created the Rst/Irre deletion constructs to determine which of the conserved domain is essential for fusion.

To anlayze the interaction partner of cell adhesion molecule i.e Rst in founder cells I have performed a global yeast-two hybrid screen by using embryonic *Drosophila* cDNA library *(clonetch, Matchmaker Lex A Two-Hybird System based on Gyuris et al., 1993)*. Rst serves redundant functions with Duf in FCs, but is also expressed in FCMs. So in the intracellular domain of Rst there must exist different regions that might mediate the redundant function in FCs and mediate the signalling in FCMs. To elucidate these possible differences I have used different approaches. First, I have tried to identify Rst interaction partner. Maybe there exist different interaction partners in FCs and in FCMs that transfer the fusion signal from the site of cell-cell recognition into the cell. Second, I created chimeric constructs. The processes of cell adhesion is result of the Ig-domains of Duf interact heterophilically with the Ig-domains of Sns. Rst however interacts homophilically. So, by creating chimeric constructs that carry the Ig-domain of Duf and the intracellular domain of Rst, I expect that expression of that construct in FCs does not disturb myoblast fusion. However, the expression of this construct in FCMs might distrub myoblast fusion in a wild-type background. The chimeric construct carrying the extracellular domain of Rst and the

intracellular domain of Duf might disturb myoblast fusion when expressed in a wild-type background in FCMs.

5.1 Creating Duf/Kirre and Rst/Irre chimera and Rst deletion constructs to analyze the nature of the redundant function of both proteins in myoblast fusion

Rst and Duf act redundantly during the embryonic muscle development in *Drosophila* (Strunkelnberg *et al*., 2001). To analyze the redundant nature of Duf and Rst in myoblast fusion in *Drosophila,* I have created chimeras and expressed them in wild-type myoblasts using the *twist*-GAL4 and *Mef2*-GAL4 driver line and myoblast-type specifically, i.e in founder cells (FCs) with rP298-GAL4 and in fusion competent cells (FCMs) with sns-GAL4. The muscle pattern of embryos expressing the constructs was assessed for muscle impairment by using the *β3-Tubulin* antibody that marks all mononucleated, growing and mature muscle.

5.1.1 Expression of Duf-ET/Rst-IT or Rst-ET/Duf/-IT does not impair myoblast fusion

To create chimeric construct i.e the Rst-Et/Duf-IT, I first generated fragment containing *rst-et* domain (1.695 kb) by PCR from *rst* full length cDNA (*rst* cDNA RE01586: 2.388 kb), which contains *Mfe I* and *Bgl II* restriction sites and were cloned into pUAST plasmid at *EcoR I* and *Bgl II* sites respectively. The second fragment containing duf-it (1.15 kb) was generated by PCR from *duf* full length cDNA (*duf* cDNA: 2.988 kb), which contains *Bgl II* and *Xba I* restriction sites and was cloned into pUAST plasmid which contains *rst-et* domain (FIG. 5.1 A). For the second chimeric constructs Duf ET/Rst-IT, I first generated fragment containing *duf-et* (1.7 Kb) by PCR from *duf* full length cDNA (*duf* cDNA: 2.988 kb), which contains *Bgl II* and *Kpn I* restriction sites and was cloned into pUAST plasmid at *Bgl II* and *Kpn I* restriction sites. The second fragment containing *rst-it* (0.699 kb) was generated by PCR from *rst* full length cDNA (*rst* cDNA RE01586; 2.388 kb), which contains *Kpn I* and *Xba I* restriction sites, were cloned into pUAST plasmid which contains *duf-et* domain (FIG. 5.1 A).

The generated chimeric constructs were injected into the germ line of w^{118} embryos by microinjection at the syncytial blastoderm stage. (Refer to materials and methods; 4.1)

A

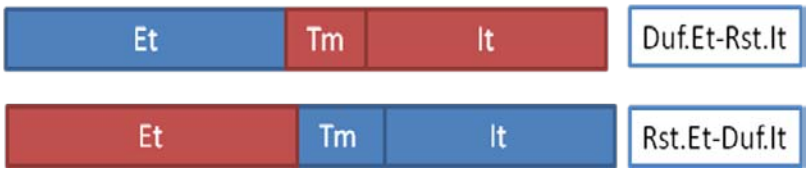


FIGURE 5.1 A **Generating chimeric constructs UAS-*rst-et/duf-it* and UAS-*duf-et/rst-it***. *rst-et/duf-it* were cloned into pUAST plasmid at *Mfe I, Bg lII and Xba I* restriction sites, respectively and *duf-et/rst-it* were cloned into pUAST plasmid at Bgl II, Kpn I and XbaI restriction sites, respectively.

The expression of these chimeric constructs i.e UAS-*rst-it/duf-it* and UAS-*duf-et/rst-it* was driven by using the mesodermal driver lines such as t*wist-GAL4*, *Mef2*-GAL4 which drives in both myoblast (FCs, FCMs), and specifically *rP298*-GAL4 which drives in FCs and *sns*-GAL4 driver lines which drives only in FCMs.

B

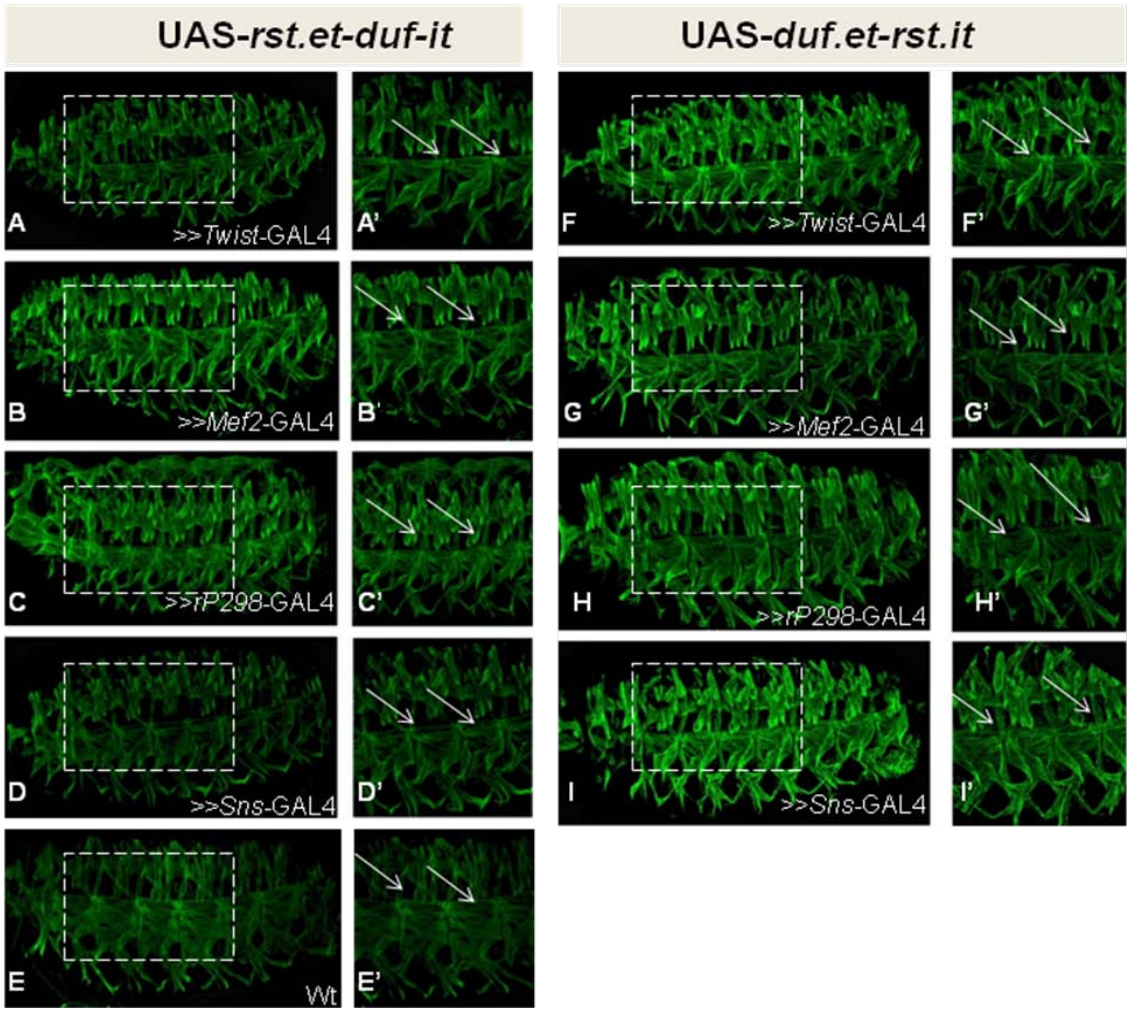


FIGURE 5.1 B **Expression of Duf-ET/Rst-IT or Rst-ET/Duf/-IT does not impair myoblast fusion**. Lateral view of stage 16 embryos stained with anti-β3-Tubulin. Expression of UAS-*rst-it/duf-it* in the mesoderm does not impair fusion, but shows 20-30% attachment defects. This was observed by using t*wist, Mef2, rP298, sns*-GAL4 driver lines respectively (A-D) and in high magnifications, arrow point to attachments sites (A'-D') in comparision with wild- type (E-E'). Expresssion of UAS-*duf-et/rst-it shows* (G-I) in mesoderm does not impairs fusion, but shows 20-30% attachment defects was observed with all *twist, Mef2, rP298 sns*-GAL4 driver lines respectively (F-I) and in high magnifications, arrows point to attachment sites (F'-I') in comparision with wild-type (E-E'). However, on the whole the muscle pattern is reminiscent of the wild-type muscle pattern.

None of these chimeric constructs showed severe impairement of myoblast fusion. The UAS-*rst-et/duf-it* construct shows only 20-30% attachment defects were observed in all GAL4 driver lines (FIGURE 5.1 B). The UAS-*duf-et/rst-it* constructs shows minor attachment defects were observed in all GAL4 driver lines (FIGURE 5.1 A). These experiments were done in a wild type background.

Taken together, the above data shows that the expression of the UAS-*rst-et/duf-It* and UAS-*duf-et/rst-it* chimeric constructs in wild-type embryos has no effect on myoblast fusion. Duf also plays a role in muscle guidance and is expressed at attachment sites (Kreisköther *et al*., 2006). Our data indicate that driving expression of the chimeric constructs in wild-type embryos has effect a minor on attachement.

5.1.2 The deletion of the conserved domain I in Rst induces severe defects during myoblast fusion when expressed in FCs and FCMs

The Rst and Duf have three conserved domains at the intracellular region (Introduction 27.2). To examine their role in *Drosophila* myoblast fusion, I have deleted the conserved domains in Rst I domain, II and III domain, I II and III domain, III domain .

I have generated the UAS-*rstΔPADVI (*I*)* (I; 2.370 kb) construct by PCR from *rst* full length cDNA (*rst* cDNA RE01586; 2.388 kb), the first part was cloned at *Bgl II-Not I* restriction sites and second part was cloned at *Not I* and *Xba I* sites into pUAST plasmid (FIG 5.1.2 A). The second UAS-*rstΔSAIYGNPYLR*(II), ΔNSSLLPP(III) (2.337 kb) construct was generated by PCR from *rst* full length cDNA (*rst* cDNA RE01586; 2.388 kb), and were cloned at *Bgl II* and *Xba I*

restriction sites into pUAST plasmid (FIG 5.1.2 A). The third UAS-*rstΔPADVI*(I), *ΔSAIYGNPYLR* (II), ΔNSSLLPP (III) (2.319 kb) construct was generated by PCR from *rst* full length cDNA (*rst* cDNA RE01586; 2.388 kb), the first part was cloned at at *Bgl II-Not I* restriction sites and second part was cloned at *Not I* and *Xba I* sites into pUAST plasmid (FIG 5.1.2 A). The fourth UAS-*rst*ΔNSSLLPP (III) 2.367 kb) construct was generated by PCR and were cloned at *Bgl II* and *Xba I* restriction sites into pUAST plasmid (FIG 5.1.2 A).

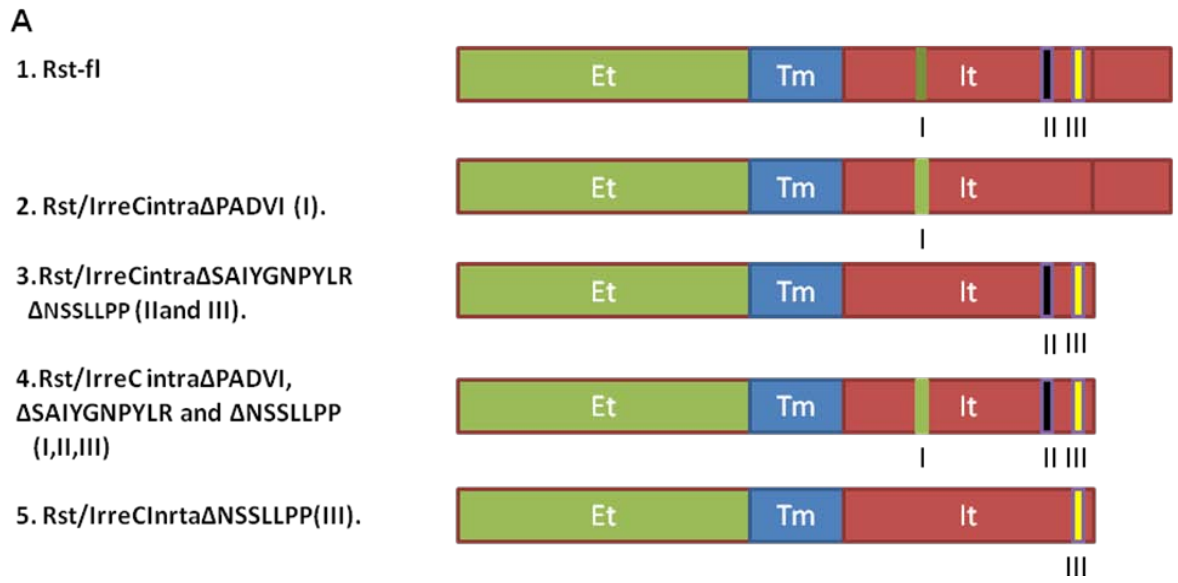
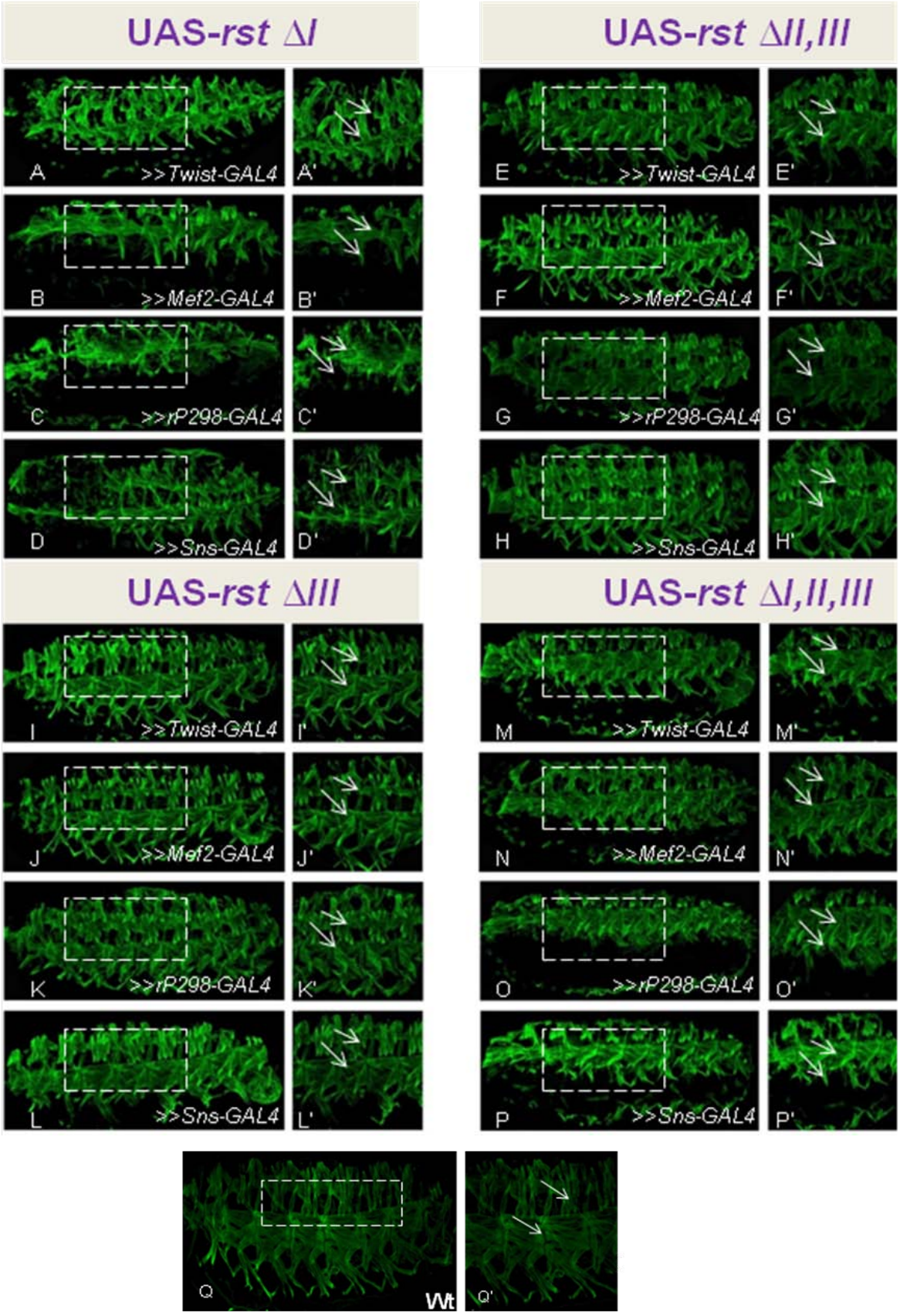


FIGURE 5.1.2 A. **Generation of UAS-*rst* deletion constructs**. The Rst-fl has three conserved domains i.e PADVI, SAIYGNPYLR, NSSLLPP (1). In first combination I have generated the UAS-*rstΔPADVI*(I), were cloned into pUAST plasmid at *Bgl II, Not I* and *Xba I* restriction sites respectively (2). In the second combination I have generated the UAS-*rstΔSAIYGNPYLR* (II), Δ*NSSLLPP* (III), were cloned into pUAST plasmid at *Bgl II* and *Xba I* restriction sites respectively (3). In the third combination I have generated the UAS-rstΔ*PADVI* (I), Δ*SAIYGNPYLR* (II), Δ*NSSLLPP* (III), were cloned into pUAST plasmid at *Bgl II, Not I* and *Xba I* restriction sites respectively (4). In the fourth combination I have generated the UAS-rstΔ*NSSLLPP* (III), were cloned into pUAST plasmid at *Bgl II, a*nd *Xba I* restriction sites respectively (5). The color codes green indicates deletion of domain I, blue indicates the deletion of domain II and yellow indicates the deletion of III domain from the respective constructs.

The generated chimeric constructs were injected into the germ line of w^{118} embryos by microinjection at the syncytial blastoderm stage, (Refer to materials and methods; 4.1). To drive the expression of Rst deletion constructs i.e UAS-*rstΔPADVI*(I), UAS-*rstΔSAIYGNPYLR*(II), *ΔNSSLLPP*(III),

B


UAS-rst ΔI
UAS-rst ΔII,III
UAS-rst ΔIII
UAS-rst ΔI,II,III
A >>Twist-GAL4
A'
B >>Mef2-GAL4
B'
C >>rP298-GAL4
C'
D >>Sns-GAL4
D'
E >>Twist-GAL4
E'
F >>Mef2-GAL4
F'
G >>rP298-GAL4
G'
H >>Sns-GAL4
H'
I >>Twist-GAL4
I'
J >>Mef2-GAL4
J'
K >>rP298-GAL4
K'
L >>Sns-GAL4
L'
M >>Twist-GAL4
M'
N >>Mef2-GAL4
N'
O >>rP298-GAL4
O'
P >>Sns-GAL4
P'
Q Wt
Q'

5.1.2 B. **The deletion of the conserved domain I in Rst induces severe defects during myoblast fusion when expressed in FCs and FCMs.** Lateral view of stage 16 embryos stained with anti-β3-Tubulin. In all cases each UAS constructs was expressed in mesoderm and driven by *twis*t-GAL4, *Mef2*-GAL4, *rP298*-GAL4, and *sns*-GAL4 (expect in panel-Q). UAS-*rstΔPADVI(*I*)* expression in mesoderm in both myoblast types and specific set of myoblast, impaired myoblast fusion (FIG 5.1.2 B, A-D) and higher magnification with arrows point at unfused myoblasts (FIG 5.1.2 B, A'-D'). UAS-*rstΔSAIYGNPYLR(II), ΔNSSLLPP(III)* expression in mesoderm in both myoblast and in specific set of myoblast does not impair myoblast fusion (FIG 5.1.2 B, E-H) and higher magnification with arrows point at unimpaired myoblasts (FIG 5.1.2 B, E'-H'). UAS-*rst ΔNSSLLPP* (III) expression in mesoderm in both myoblasts as well as specific set of myoblasts does not impairs myoblast fusion (I-L) and higher magnification with arrows point at unimpaired myoblast FIG 5.1.2 B, (I'-L'). UAS-*rstΔPADVI (*I*), ΔSAIYGNPYLR* (II), and *ΔNSSLLPP* (III) expression in mesoderm in both myoblast and in specific set of myoblast Impairs myoblast fusion (FIG 5.1.2 B, M-P) and higher magnification with arrows point at unfused myoblast (FIG 5.1.2 B, M'-P').

UAS-*rstΔPADVI*(I), *ΔSAIYGNPYLR*(II), *ΔNSSLLPP*(III) and UAS-*rst*ΔNSSLLPP(III) in the mesoderm I used *twist*-GAL4, *Mef2*-GAL4 which drives in bothmyoblasts (FCs, FCMs) and and *rP298*-GAL4 which drives in FCs and *sns*-GAL4 driver lines which expresses only in FCMs (FIG 5.1.2 B).

Only the expression of UAS-*rstΔPADVI*(I) in all myoblast, FCs and FCMs severly impaired myoblst fusion was observed. It indicating the essentiality of this domain in both myoblast during myoblast fusion in *Drosophila.* I also further drive this UAS-*rstΔPADVI*(I) construct in mesoderm by using a FCs specific *rP298*-GAL4 and FCMs specific *sns*-GAL4 severely impaired myoblast fusion indicating the essentiality of this domain in specific myoblast during myoblast fusion in *Drosophila (*FIG 5.1.2 B).

I have expressed the UAS-*rstΔSAIYGNPYLR* (II), ΔNSSLLPP (III) construct in the mesoderm by using *twist*-GAL4 and *Mef2*-GAL4, this does not impairs myoblast fusion which indicates that these domain are not required to mediate myoblast fusion in *Drosophila* FCs and FCMs. Also the myoblast specific expression of UAS-*rstΔSAIYGNPYLR*(II), ΔNSSLLPP(III) in FCs with *rP298*-

GAL4 and FCMs with *sns*-GAL4 does not impair myoblast fusion, which indicates that this domain are not required in specific myoblasts during myoblast fusion in *Drosophila (*FIG 5.1.2 B).

I have expressed the UAS-*rstΔPADVI(*I*), ΔSAIYGNPYLR* (II), and*Δ NSSLLPP*(III) construct in mesoderm by using the *twist*-GAL4 and *Mef2*-GAL4 drivers. Myoblast fusion was severly impaired when all three domains were deleted. Since expression of (II) and (III) domain did not disturb myobalst fusion, the fusion phenotype seems to be due to loss of domain I. I also further driven this UAS-*rstΔPADVI(*I*), ΔSAIYGNPYLR* (II), and Δ *NSSLLPP*(III) constructs in FCs with *rP298*-GAL4 and FCMs *sns*-GAL4 driver line impairs myoblast fusion, which further indicates essentiality of this domain in specific myoblast during myoblast fusion in *Drosophila.*

I have expressed the UAS-*rst* Δ*NSSLLPP*(III) construct in mesoderm by using the *twist*-GAL4 and *Mef2*-GAL4 does not impairs myoblast fusion which indicates that this domain is not required in both myoblast during myoblast fusion in *Drosophila.* I also further drive this UAS-*rst* ΔNSSLLPP(III) constructs in FCs with *rP298*-GAL4 and in FCMs with *sns*-GAL4 does not disturb myoblast fusion, which indicates that this domain is not required in specific myoblast during myoblast fusion in *Drosophila (*FIG 5.1.2 B).

However on the whole the expression of UAS-*rstΔSAIYGNPYLR (II), ΔNSSLLPP (III) and* UAS-*rst* Δ*NSSLLPP* (III) constructs in mesoderm shows muscle pattern is reminiscent of the wild-type muscle pattern (Q-Q'). The expression of UAS-*rstΔPADVI (*I*)* and UAS-*rstΔPADVI (*I*), ΔSAIYGNPYLR* (II), and*Δ NSSLLPP*(III) constructs in mesoderm shows the deletion of the conserved domain I in Rst induces severe defects during myoblast fusion when expressed in FCs and FCMs.

Taken together, the deletion of RstΔPADVI(I) impairs the severe myoblast fusion and effects both myoblasts FCs and FCMs. Rst serves redundant functions with Duf in FCs, but is also expressed in FCMs. This domain acts as dominant- negative effect when deleted during myoblast fusion. So in the intracellular domain I (PADVI) of Rst that might mediate the redundant function in FCs Duf and might act in similar signalling pathway.

5.1.3 A yeast two-hybrid screen to identify Rst interaction partners

May be there exist common interaction partner in FCs and in FCMs that transfer the fusion signal from the site of cell-cell recognition into the cell. To further study the role of the cell adhesion molecule Rst during myoblast fusion in *Drsosophila.* I have performed a yeast two-hybrid screen to identify Rst interaction partners. I have cloned the *rst-it* (0.630 kb) into pGBKT7 plasmid at *EcoR* I restriction site. The *Drosophila* genomic libraray was provided by *clonetch, Matchmaker Lex A Two-Hybird System based on Gyuris et al (1993).* Screening was performed according to the manufacturer's protocol. Finally the positive clones was reselected 3-times on high stringency medium. The potential interacting partners were selected and plasmids were isolated and sequenced. The sequenced clones were blasted against the *Drosophila* genomic sequence. Finally three potential interacting partners were selected which are described below in the table.

TABLE 1 **The interesting partners from the yeast-two hybrid screen**

Clone no	**Gene/Annotaion symble**	**Function/Biological process.**
15 or 27	***Actin57B***	Its **molecular function** is described as: structural constituent of cytoskeleton; glucuronosyltransferase activity; ATP binding; protein binding. It is involved in the **biological processes**: cytoskeleton organization; cytokinesis; heart development; chondroitin sulfate biosynthetic process; heparan sulfate proteoglycan biosynthetic process; positive regulation of NFAT protein import into nucleus.
18	***Nidogen/entactin***	Its **molecular function** is described as calcium ion binding. It is involved in the **biological processes**: bioluminescence; cell-matrix adhesion; protein-chromophore linkage.

45.	***Papilin***	Its **molecular function** is described as: extracellular matrix structural constituent; metalloendopeptidase activity; zinc ion binding; serine-type endopeptidase inhibitor activity. It is involved in the **biological process** extracellular matrix organization. 5 alleles are reported. The **phenotypes** of these alleles are annotated with: embryonic/larval somatic muscle; Malpighian tubule; embryonic/larval tracheal system.

In situ hybridisation were performed to determine the mRNA expression pattern during *Drosophila* embryo. Expression pattern was observed whether these potential interaction partners are expressed in myoblasts when fusion occurs.

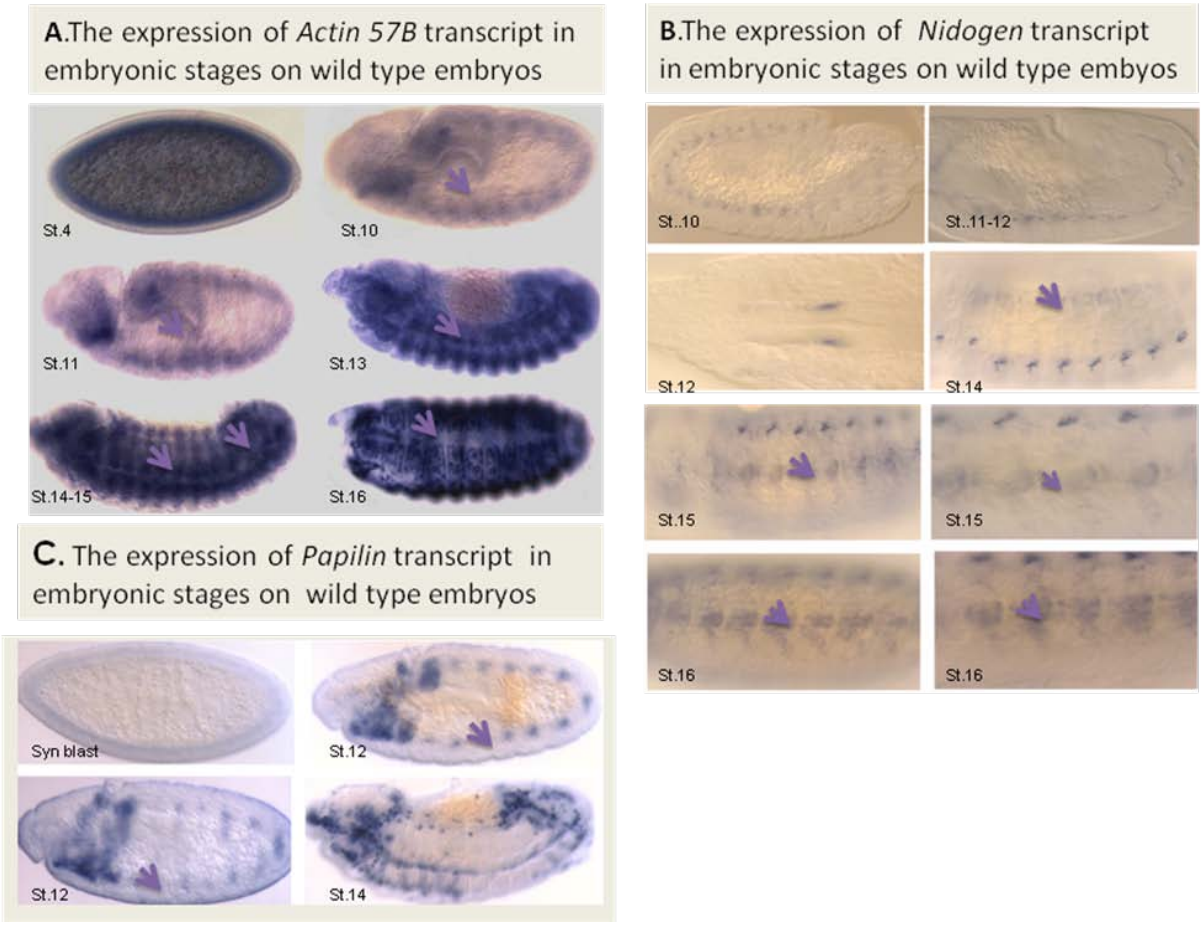


FIGURE 5.1.3 **The expression pattern of *Actin 57B, Nidogen* and *Papilin* during embryogenesis**. A) *Actin 57B* expression pattern from stage 4 to 16 using *Actin 57B m*RNA probe, from stage 10-12 starts expressing in mesoderm and stage 13-16 expression in somatic musculature and it may suggests that it has a role in myoblast fusion. **B)** *Nidogen* expression pattern from stage 10-16 using

*Nidogen m*RNA *probe,* at stage.14. *Nidogen* expressed in somatic musculature shown with arrows and also expressed at later stage 15 and 16, Arrows point at muscle attachment sites and suggests that it may play a role after the myoblast fusion. **C)** *Papilin* expression pattern from stage 4-14. At stage 12-14 it is expressed in the mesoderm shown with arrows and it may suggests that it may play a role in myoblast fusion.

These clones initially were cloned into pBLUSCRIPT plasmid and DIG-labelled RNA probes were prepared according to the protocol (Methods 4.12).

The expression of the transcript of these genes is shown FIGURE 5.1.3. *Actin57B* is expressed in the somatic mesoderm and also at later stages of embryos (FIGURE 5.1.3 A), which indicates it has a role in myoblast fusion and at later stages it may be involving in the muscle attachment sites in *Drosophila.*

Nidogen is expressed in some muscle groups of embryo at later stages 14-16 (FIGURE 5.1.3 B), which indicates that it has no role in myoblast fusion, but it may be involving in muscle maturation in *Drosophila* embryo.

Papilin is expressed in embryo at stage 12-14 (FIGURE 5.1.3 C), which indicates that it has a role in myoblast fusion in *Drosophila* embryo.

However the further investigation was studied about these potential interacting patners of Rst. Mutant analysis of these Rst intearaction partner suggests that muscle phenotype was not observed during myoblast fusion in *Drosophila* embryo.

II. Linking cell adhesion with Arp2/3-based actin polymerization

5.2 A conserved function of the adaptor protein Nck/Dock in signalling cell adhesion

In vertebrates, Nck bind to Nephrin an orthologous of Sns (Jones *et al*., 2006). In vertebrates Nephrin and Nep1 mediate cell-cell adhesion in kidney podocytes. The loss of Nephrin or Neph1 functions results in an abnormal podocyte formation during kidney development (Lie *et al*., 2004; Verma *et al*., 2006., Jones *et al*., 2006). *Drosophila* Sns and Hbs are orthologs of vertebrate Nephrin (Dworak *et al*., 2001); and Duf and Rst are homologs of Neph1 (Dworak *et al*., 2001). In *Drosophila* homolog of SH2-SH3 adaptor Nck is Dock. It has an important role in development of *Drosophila* such as axonal path finding, both in olfactory and in visual senses (Desai *et al*., 1999). Dock is expressed in FCs and FCMs (from Schafer *et al*, in preparation). Due to this evidence I have studied the role of Dock in myoblast fusion. To examine whether Dock directly binds to the cell adhesion molecules, I performed a yeast two-hybrid assay, Biomolecular fluorescence (BiFC).

5.2.1 The SH2-SH3 adaptor Dock interacts with Rst, Duf, Sns and Hbs in a yeast two-hybrid assay.

To study the interaction of cell adhesion molecules (Rst, Duf, Sns and Hbs) with Dock and to determine which domain of Dock is involved in binding to the cell adhesion molecules. I have cloned the *dock-fl (*1.644 Kb), *dock-ΔSH2* (1.393 Kb), into *EcoR I* and *Xho I* sites respectively and the *dock-ΔSH3-1(1.563 Kb), dock-ΔSH3-2 (1.479 Kb), dock-ΔSH3-3 (1.470 Kb) and dock-ΔSH3 (123) (1.398 Kb)* into the yeast expression vector pB42AD. The first fragment *dock-ΔSH3 (123)* was cloned in to *EcoR I* and second fragment were cloned into *Xho I* site respectively into pB42Ad prey plasmid. The intracellular domain of the cell adhesion molecules (rst 0.63 Kb, duf ~0.9 Kb, sns ~1.1 Kb and hbs ~0.5Kb) were cloned into bait plasmid pGILDA. The remaining protocol was performed according to the manufacturer's.

First, I analyzed if Duf.it interacts with Dock-fl in yeast. I have observed the interaction between these two proteins within two days after induction (FIG 5.2.1 A). I further studied the interaction between Duf.it and Dock deletion constructs (FIG 5.2.1 B), I have observed the interaction between the Duf.it and Dock-ΔSH2 (FIG 5.2.1 B), This indicates no requirement of Dock-ΔSH2 for binding to Duf.it.

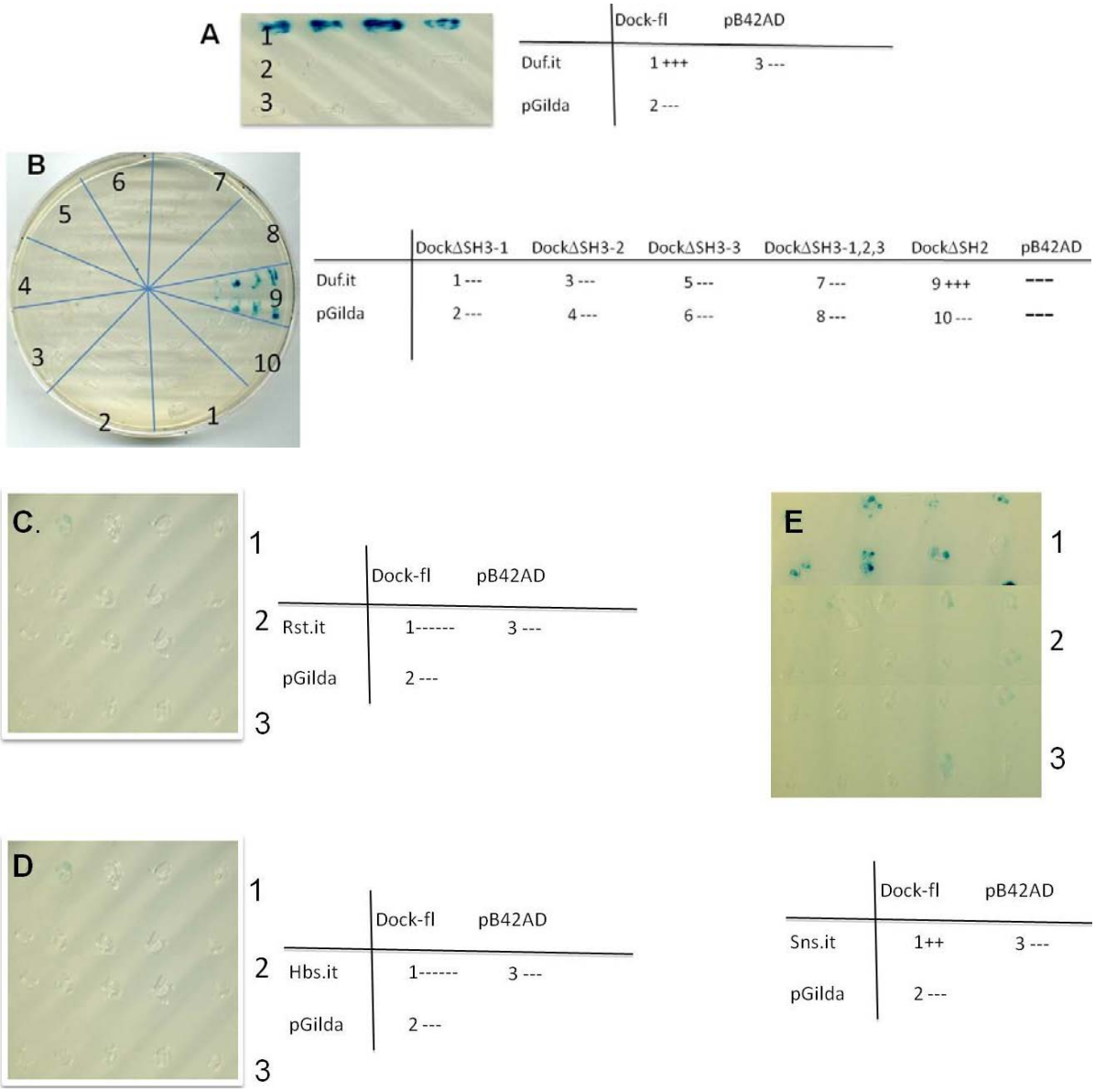


FIGURE 5.2.1 **Dock interacts with Duf and Sns in a yeast two-hybrid assay**. In all these experiments, yeasts were grown on synthetic dropout plates (SD) lacking histidine, tryptophan, uracil and leucine with Galactose /raffinose as carbon source with X-Gal (80 mg/L), in all panels (A-E). Interaction of Dock-fl with Duf.it (A) was observed within two days. The deletion of Dock-ΔSH2 (B,9) still shows an interaction with Duf.it and no interaction was observed between the Duf.it and SH3 deletions of

Dock i.e Dock-ΔSH3-1, Dock-ΔSH3-2, Dock-ΔSH3-3 and Dock-ΔSH3 (123) (B,1, 3, 5 and 7). Rst.it does not interact with Dock-fl (C). Hbs.it does not interacts with Dock-fl (D). Sns.it interacts with Dock-fl but it seems to be very week. All the above interactions were observed within two days after induction.

Similarly I studied the interaction between Duf.it and all deletions of Dock-ΔSH3 i.e Dock-ΔSH3-1, Dock-ΔSH3-2, Dock-ΔSH3-3 and Dock-ΔSH3 (123) individually (FIG 5.2.1 B). None of these Dock deletion shows interaction with the Duf.it and this indicates that all SH3 domains of Dock are required for binding to Duf.

Next, I studied the Rst.it and Dock-fl, Rst did not interacts with Dock (FIG 5.2.1 C). It indicates that it may be due to no interaction or interaction, but secondary modifications required. In the yeast secondary modifications such as phosphorylation of tyrosine residues does not occur.

I have further investigated if Hbs.it interacts with Dock-fl. No interaction was observed between Hbs and Dock (FIG 5.2.1 D). It indicates that it may be due to no interaction or interaction, but secondary modifications required. In the yeast secondary modifications such as phosphorylation of tyrosine residues does not occur.

I have further investigated if Sns.it interacts with Dock-fl, shows week interaction even after three days of induction (FIG 5.2.1 E). It indicates that it may be due to interaction, but secondary modifications required. In the yeast secondary modifications such as phosphorylation of tyrosine residues does not occur.

Taken together the above experiment suggest that Dock interacts with the cell adhesion molecules, with Duf via SH3 domain, Rst and Hbs shows no interaction with Dock and Sns shows interaction with Dock, but it is very week. Based on Rst and Hbs results, I have further confirmed these interactions by Biomolecular Fluorescence Complementation (BiFC) in *Drosophila* SL2 cells.

5.2.2 The SH2-SH3 adaptor Dock interacts with Rst, Dock, Sns and Hbs in the Biomolecular Fluorescence Complementation (BiFC) in *Drosophila* SL2 cells

Based on the above results, I have further confirmed the interaction of Dock with cell adhesion molecules such as Duf, Rst, Hbs and Sns by Biomolecular Fluorescence Complementation (BiFC) in *Drosophila* SL2 cells. Direct protein interactions can be studied by the BiFC assay. The BiFC assay is based on the finding that the flourescence of splitted GFP or YFP fragments can be restored after protein interaction. The protein expressed as either N-terminal or C-terminal fusions with the split YFP fragments, often referred to as *YN* and *YC*, respectively (Hu *et al*., 2002; Bracha-Drori et al., 2004; Citovsky *et al.*, 2006; Kerppola, 2006). Using different combinations of *YN* and *YC* fusion pairs is advisable since the orientation of the fusion can greatly affect YFP complex formation (Bracha-Drori *et al*., 2004). The flexible spacer i.e *HA* tag introduced at the C-terminal part of *YN* of split *YFP* (FIGURE 5.2.2 A) and *Myc* tag was introduced at the N-terminal part of *CN* of split *YFP,* these vectors obtained from Verena.G (FIGURE 5.2.2 A). *YN* of Split *YFP* was cloned into *pUAST* plasmid at *EcoR I* restriction site and *CN* of split *YFP* was cloned into pUAST plasmid at *Xho I* restriction site respectively.

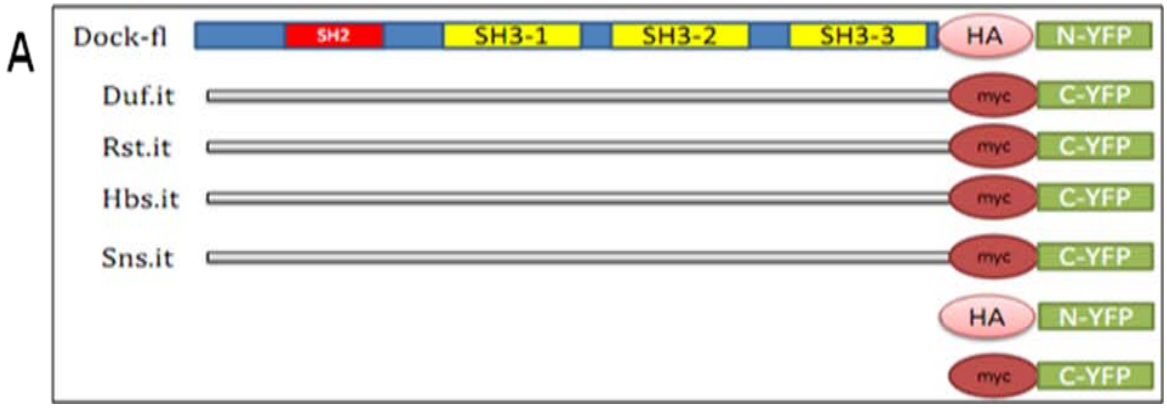


FIGURE 5.2.2 **A Generation of Split *YFP* constructs. *YN* with *HA* of Split *YFP* and *CN* with *Myc* of split YFP.** Dock-fl was fused at *YN* of Split *YFP* and the intracellular domain of all cell adhesion molecules i.e *rst (*0.63 kb*), duf* (~0.9 Kb), *sns* (~1.1 kb) and *hbs (* ~0.5kb) was fused at *CN* of Split *YFP*.

*Dock-fl (*1.644 kb) was cloned into the restriction sites of *Bgl II and Not I* of YN of split YFP *pUAST* expression vector. The intracellular cell adhesion molecules i.e *rst* 0.63 kb, *duf* ~0.9

kb, *sns* ~1.1 kb and *hbs* ~0.5 kb was cloned at *CN* of split YFP pUAST. *rst.it* 0.63 kb was cloned into *CN* of *split YFP pUAST* plasmid at *Bgl II and Not I* restriction sites (FIG 5.2.2 A). *duf*.it~0.9 kb was cloned into *CN* of *split YFP pUAST* plasmid at *Bgl II and Not I* restriction sites (FIG 5.2.2 A). *Sns.it(*~1.1 kb) was cloned into *CN* of *split YFP pUAST* plasmid at *Bgl II and Not I* restriction sites (FIG 5.2.2 A). *hbs.it(*~0.5kb) was cloned into *CN* of *split YFP-pUAST* plasmid at *Bgl II and Not I* restriction sites (FIG 5.2.2 A). All these above clones were sequenced.

Constructs expression was induced through co-transfection with Actin-GAL4 (pWA-GAL4). After transfection the cells were incubated under normal growth condition for 48 hours (refer to methods; 4)

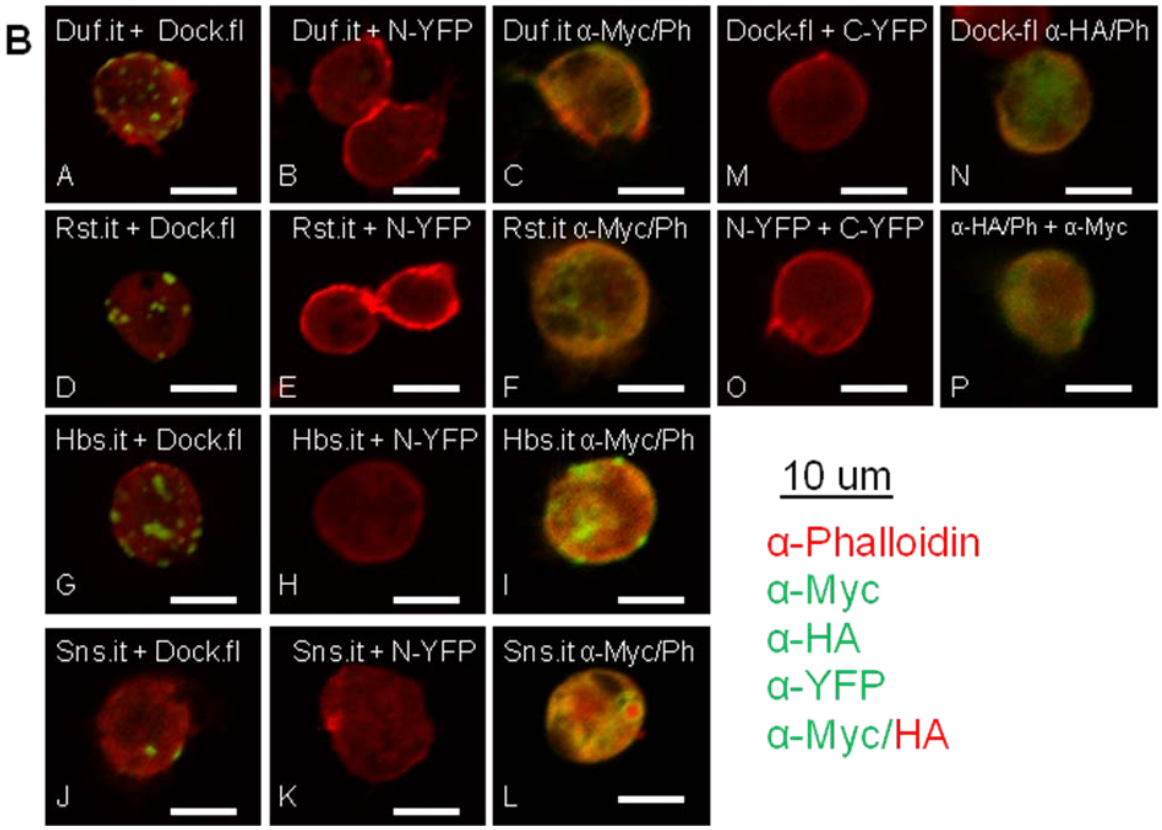


FIGURE 5.2.2 B **Dock interacts with Rst, Dock, Sns and Hbs in the Biomolecular Fluorescence Complementation (BiFC) in *Drosophila* SL2 cells.** In all the panels cells were stained with phalloidin (Red) except panel (P). The restoration YFP signal were observed in the panel (A, D, G, J). Duf.it

interacts with Dock-fl signal was observed in green (A), and the respective controls were with Duf.it and N-YFP, no signal was observed (B). Duf.it expression was confirmed by anti-*Myc* in green (C). Rst.it interacts with Dock-fl signal was observed (D), and the respective controls were with Rst.it and N-YFP, no signal was observed (E). Rst.it expression was confirmed by anti-*Myc* in green (F). Hbs.it interacts with Dock-fl signal was observed (G), and the respective controls were with Hbs.it and N-YFP, no signal was observed (H). Hbs.it expression was confirmed by anti-*Myc* in green (I). Sns.it interacts with Dock-fl signal was observed (J), and the respective controls were with Sns.it and N-YFP, no signal was observed (K). Sns.it expression was confirmed by anti-*Myc* in green (L). Dock-fl with C-YFP, no signal were observed (M). Dock-fl expression was confirmed by anti-HA in green (N). The controls i.e N-YFP with C-YFP, no restoration of YFP signal was observed (O) and their expression was confirmed with anti-Myc (green) and anti-HA red (P).

All constructs were detectable in transfected cells, The cytoskeleton staining was visible (FIG 5.2.2 B) except (P) with *Myc and HA* antibody stainin . Panel (O) no restoration YFP signal with splited YFP fragments i.e C-YFP and N-YFP. All the cell adhesion molecules interact with Dock-fl in (FIG 5.2.2 B and A, D, G, J). The controls shown no interaction (FIG 5.2.2 B).

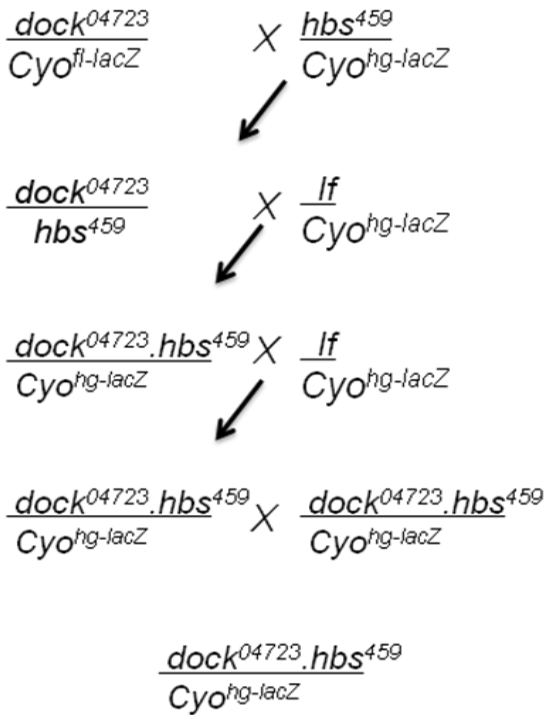
Taken together all the interactions, I have observed in yeasts and additionally observed an interaction of Dock with Rst and Hbs in BiFC assay, which I did not see in yeasts. Thus, it is possible that this interaction is based on secondary modification which do not occur in yeasts. Furthermore the observed biochemical interaction was verified during myoblast fusion in *Drosophila*.

5.3 Analyzing the role of Dock in FCMs during myoblast fusion

Dock is expressed in FCMs and also in FCs (from Schafer et al, in preparation). An interaction of Dock with cell adhesion molecules i.e Rst, Duf, Sns and Hbs was confirmed in vitro by Biomolecular Fluorescence Complementation (BiFC) in *Drosophila* SL2 cells. Based on these results, I first started to investigate the role of Dock in FCMs, if the observed interactions occur during myoblast fusion.

5.3.1 The SH2-SH3 adaptor *dock* interacts genetically with *hbs*

To gain further insights into the signal transductional pathway and to clarify the role of Dock during myoblast fusion in fusion competent cells (FCMs), I have generated and analyzed *dock hbs* double mutants. For the genetic interaction studies, I used the $dock^{04723}$ null allele and hbs^{459} null allele. The recombination were performed in the following manner.



Mutant phenotype was examined by *β3-Tubulin* antibody staining which marks all myoblasts and mature myoblasts. *hbs*^{459} mutant embryos display no strong fusion defects, but few unfused myoblast were observed (FIG 5.3.1 B-B') unlike in wild-type (FIG 5.3.1 A-A'). *dock*^{04723} mutant embryos display no strong fusion defect , but few unfused myoblast were observed (FIG 5.3.1 C-C') unlike in wild-type (FIG 5.3.1 A-A'). Homozygous *dock*^{04723} *hbs*^{459} double mutant embryos shows strong fusion defect in comparision to the single mutants (FIG 5.3.1 D-D').

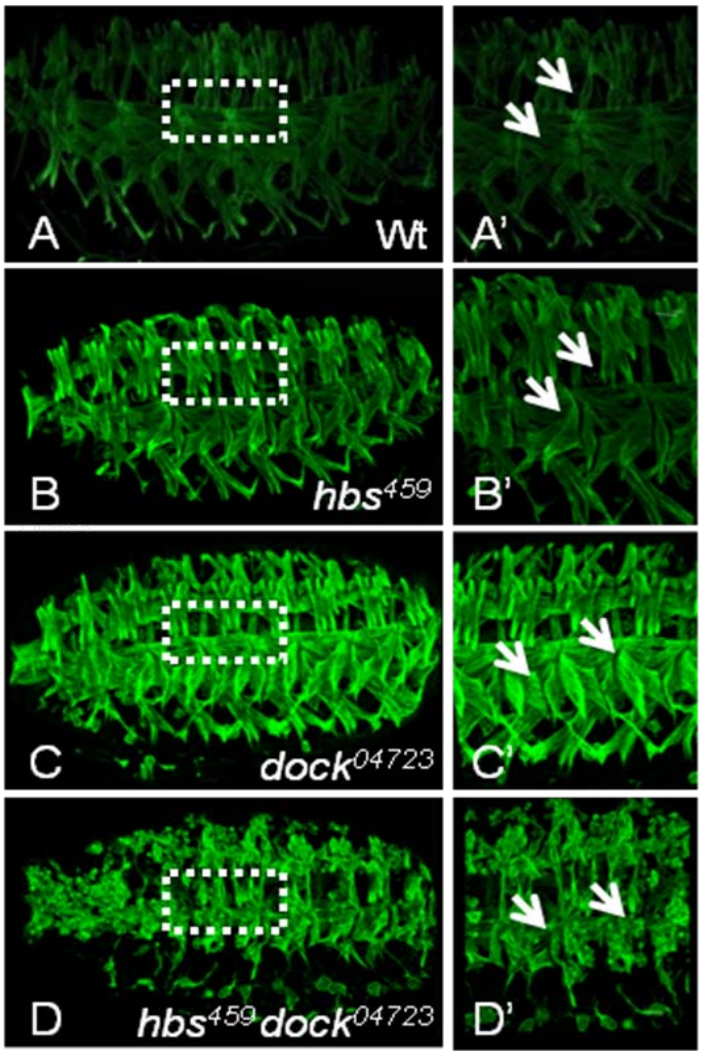


FIGURE 5.3.1 **The SH2-SH3 adaptor *dock* interacts genetically with *hbs*.** Whole mount embryos stained with *β3-Tubulin* (A-D), ventral view of stage 16 embryos. A'-D' are magnified view of the respective images. Wild-type muscle pattern (FG 5.3.1 A-A'). A few unfused myoblasts are visible in

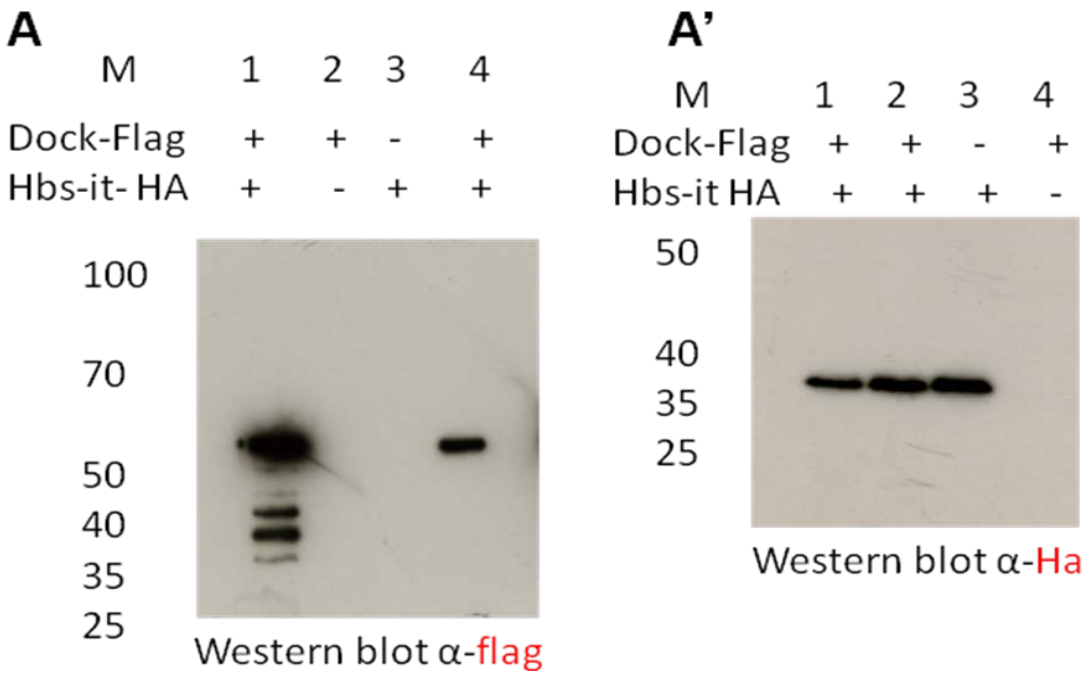
dock^{0472} mutant embryos (FG 5.3.1 B-B'). *hbs*^{459} mutant embryos shows few unfused myoblasts attached to growing myotubes (FG 5.3.1 C-C'). Homozygous *dock*^{047} *hbs*^{459} double mutant display stronger muscle phenotype than single mutant (FG 5.3.1 D-D').

Taken together, these experiments show that *dock* and *hbs* genetically interact during *Drosophila* muscle development.

5.3.2 The physical interaction between Dock and Hibris depends on the SH2 domain and on the phospohrylation of Tyrosin 1089

To examine Dock and Hbs interaction studies, I further performed CO-IP with Flag-tagged Dock and HA-Hbs.it in *Drosophila* Schneider (SL2) cells. The Dock-Flag tag construct was obtained from the group of Susan Abmayer and HA-Hbs.it (~0.5 kb) was cloned into *pUAST* plasmid at *Bgl II* and *Not I* restriction sites respectively.

To investigate a possible interaction between Dock and Hbs.it on protein level, I expressed Dock-Flag and HA-Hbs.it in *Drosophila* Schneider (SL2) cells.



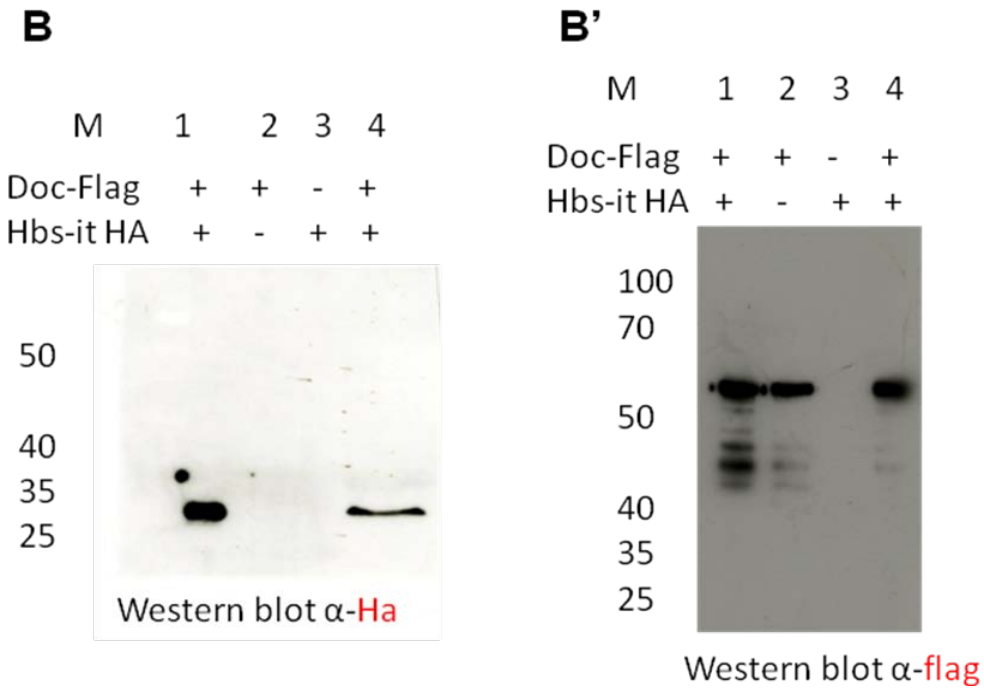


FIGURE 5.3.2 **CO-IP of Dock-Flag with HA-Hbs.it in SL2 cells.** Co-IP with HA-tagged agarose beads; Dock interacts with HA-Hbs.it, confirmed by western blot with α-Flag (A) and the control blot with α-HA (A'). CO-IP with Flag-tagged agrose beads; Dock interacts with HA-Hbs.it beads, confirmed by western blot with α-HA (B), the control blot with α-Flag (B').

Co-immunoprecipitation assays were performed to test the interactions. As shown in the western blot analysis in FIG 5.3.2 A and A', Hbs is able to pull down Dock. Here Co-IP was performed by using the HA-tagged agarose beads and western blot was performed by α-Flag (FIG 5.3.2 A), the control blot with α-HA to make sure that HA-Hbs.it binds to the beads (FIG 5.3.2 A').

In the vice versa the CO-IP was performed by Flag tagged beads, western blot was developed with α-HA FIG 5.3.2 B), Dock interacts with Hbs.it and the control blot with α-Flag to make sure that Dock-Flag binds to the beads (FIG 5.3.2 B'). Suggesting that the above results indicates that Dock interacts with the Hbs on protein leve in Sl2 cells of *Drosophila*.

Recently, In vertebrates, Nck was shown to bind to Nephrin via putative phosphorylation sites (Jones *et al*., 2006). The SH2 domain of Nck binds to Nephrin by YDXV motif. Based on this evidence, Nephrin ortholog Hbs in *Drosophila*, I found closest motif i.e YDEQ (1089) with

YDXV motif of Nephrin. Hbs contains 12 tyrosines in the cytodomain, I used *hbs.it* construct in which *Y1089* tyorsine in the cytodomain have been mutated to F1089. By using the *Hbs.it* and *Hbs.it Y1089F* constructs (FIG 5.3.2 C), I have performed Biomolecular Fluorescence Complementation (BiFC) in *Drosophila* SL2 cells with SH2 and SH3 Dock deletions to determine which of domain of Dock are required for binding to Hbs (FIG 5.3.2 C).

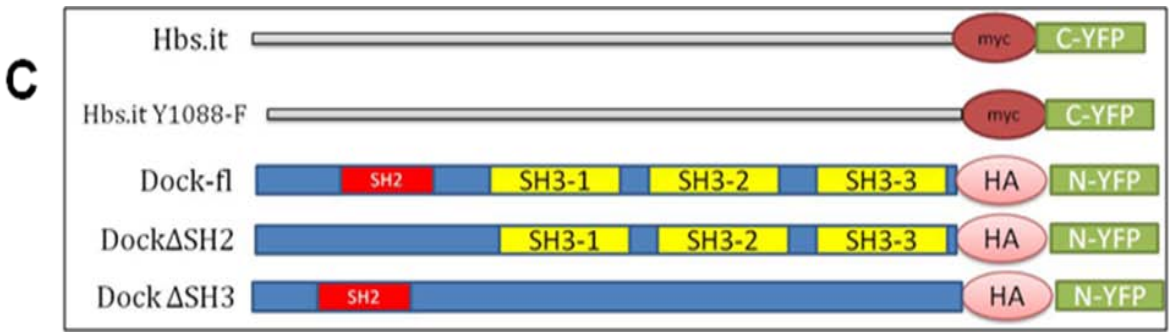


FIGURE 5.3.2 C **Generation of Split *YFP* constructs.** *Hbs.it* and Hbs.it *Y1089F* were cloned into *CN* of pUAST plasmid. *Dock-fl*, *Dock-ΔSH2* and *Dock-ΔSH3* were cloned at *YN* of pUAST plasmid.

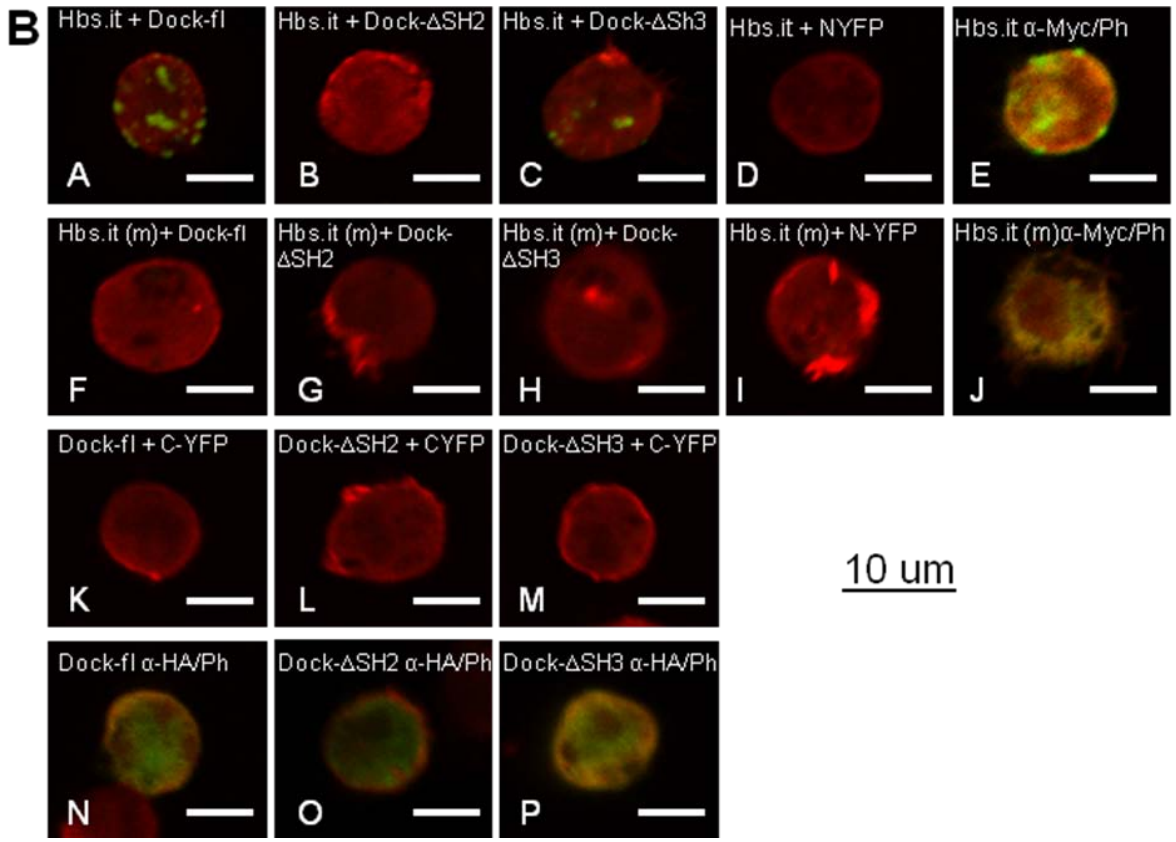


FIGURE 5.3.2 D Dock interacts with Hbs.itY1089F via its SH2 domain by using Biomolecular Fluorescence Complementation (BiFC) in *Drosophila* SL2 cells. In all the panels cells were stained

with phalloidin (Red). The restoration of Split YFP signal in panel (A). Hbs.it interacts with Dock-fl restoration of YFP signal was observed (A). No interaction with Dock-ΔSH2 (B) and interacts with Dock-ΔSH3 restoration of YFP signal (C) and the respective control were with Hbs.it and N-YFP, no signal was observed (D). Hbs.it expression was confirmed by anti-*Myc* shown in green (E). Hbs.itY1089F does not interacts with Dock-fl signal was observed (F), no interaction with Dock-ΔSH2 (G) and no interaction with Dock-ΔSH3 (H) and the respective control were done by transfecting Hbs.it with empty NYFP vector, no signal was observed (I). Hbs.it Y1089F expression was confirmed by anti-*Myc* in green (J). Dock-fl, Dock-ΔSH2 and Dock-ΔSH3 controls with C-YFP, no restoration of YFP signal was observed (K, L, M). Dock-fl, Dock-ΔSH2 and Dock-ΔSH3 expression was confirmed by anti-HA in green (N, O, P).

*dock-fl (*1.644 kb), *dock-ΔSH2* (1.393 kb), were cloned into *Bgl II* and *Xba I* restriction sites respectively and *dock-ΔSH3 (123) (1.398 kb)* were cloned into *Bgl II*, *Not I* and *Xba I* restriction sites into *YN* of pUAST plasmid respectively. Hbs.it (~0, 5 kb) and Hbs.it *Y1089F* (~0, 5 kb) into *Bgl II* and *Not I* restriction sites into *CN of pUAST* plasmid respectively.

Hbs.it interacts with Dock-fl (FIG 5.3.2 D A), and does not interacts with Dock-ΔSH2 (FIG 5.3.2 D B), and interacts Dock-ΔSH3 (FIG 5.3.2 D C). This indicates that the interaction between Dock and Hbs.it is mediated by the SH2 domain of Dock. For Nck it was reported in Jones *et al*. (2006) that the interaction between Nck and Nephrin is mediated by the SH2 domain of Nck. The above data indicated that the interaction is mediated by the SH2 domain on the site of Dock. The SH2 domain of Nck binds to Nephrin by YDXV motif. I have next searched for possible phosporylated tyrosines that might be responsible for the interaction with Dock on the site of Hbs. None of the Dock deletions does not interacts with Hbs.it Y1089F (FIG 5.3.2 D: F, G, H). The above data further indicated that the SH2 domain of Dock binds to the Hbs (Y1089F) via YDEQ motif.

5.3.3 The physical interaction between Dock and Sns depends on SH3 domain of Dock

In vertebrates, Nck bind to Nephrin an orthologous of Sns (Jones *et al*., 2006). In *Drosophila* whether this interaction is really required during myoblast fusion, garland cell development or eye development was not assessed. Yeast and BiFC assays suggested that Dock binds to Sns. To determine which of the Dock domains bind to the Sns and also which motifs of Sns

bind to Dock, I have cloned the *Sns.it* (1.1 kb) into *CN* of *pUAST* plasmid at *Bgl II* and *Not I* restriction sites respectively. I have further cloned Sns.itΔ2pxxp (1.1 kb) and Sns.itΔ2pxxp, ΔY14-F14 (1.1 kb) obtained from the group of Susan Abmayer were cloned into *CN* of *pUAST* plasmid at *Bgl II* and *Not I* restriction sites respectively.

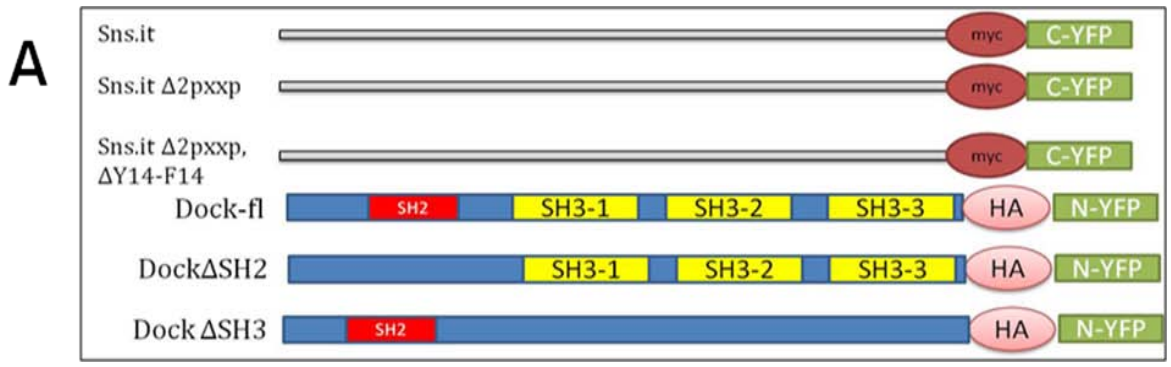


FIGURE 5.3.3 **A Generation of Split *YFP* constructs**. Sns.it, Sns.it Δ2pxxp (1.1 kb) and Sns.itΔ2pxxp, ΔY14-F14 (1.1 kb) were cloned into *CN* of pUAST plasmid. *Dock-fl*, *Dock-ΔSH2* and *Dock-ΔSH3* were cloned at *YN* of pUAST plasmid.

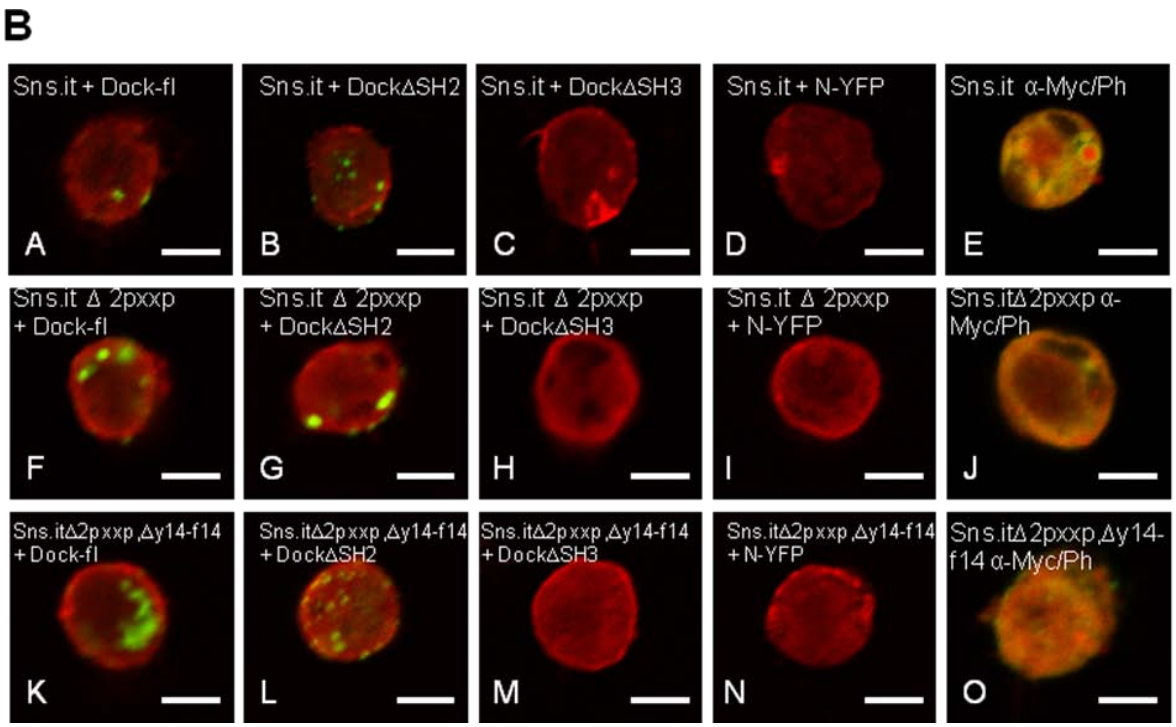


FIGURE 5.3.3 B **Dock interacts with Sns.it via its SH3 domain by Biomolecular Fluorecence Complementation (BiFC) in *Drosophila* SL2 cells.** In all the panels cells were stained with phalloidin (Red).The restoration of split YFP signal was observed in (A, B, F, G, K, L). Sns.it interacts with Dock-fl

and Dock-ΔSH2, restoration of YFP signal was observed (A) and (B) and no interaction with Dock-ΔSH3 (C) and the respective controls were with Sns.it and N-YFP, restoration of YFP signal was observed (D). Sns.it expression was confirmed by anti-*Myc* in green (E). Sns.it Δ2pxxp interacts with Dock-fl and Dock-ΔSH2, restoration of YFP signal was observed (F) and (G) and no interaction with Dock-ΔSH3 (H) in green and the respective controls were with Sns.it Δ2pxxp and N-YFP, no signal was observed (I). Sns.itΔ2pxxp expression was confirmed by anti-*Myc* in green (J). Sns.it Δ2pxxp,ΔY14-F14 interacts with Dock-fl and Dock-ΔSH2, restoration of YFP signal was observed (K) and (L) and no interaction with Dock-ΔSH3 (M) and the respective controls were with Sns.itΔ2pxx p,ΔY14-F14 and N-YFP, no restoration of YFP signal was observed (N). Sns.itΔ2pxxp, ΔY14-F14 expression was confirmed by anti-*Myc* in green (O). By the above evidence shows that Dock-ΔSH3 domain is required to bind to Sns.it and is not depending on the proline motif and tyrosine residues.

*dock-fl (*1.644 kb), *dock-ΔSH2* (1.393 kb), were cloned into *Bgl II* and *Xba I* restriction sites respectively and *dock-ΔSH3 (123) (1.398 kb)* were cloned into *Bgl II*, *Not I* and *Xba I* restriction sites into YN of pUAST plasmid respectively.

I studied the interaction between Sns.it and Dock-fl (FIG 5.3.3 B, A), and Dock-ΔSH2 restoration of YFP signal was observed (FIG 5.3.3 B, B), and no interaction was observed with Dock-ΔSH3 (FIG 5.3.3 B, C). It indicates that Dock-ΔSH3 domain binds to the Sns. Further interaction studied with Sns.itΔ2pxxp, ΔY14-F14 (1.1 kb) and Dock deletion constructs by split YFP assay (FIG 5.3.3 K, L, M). Sns.it Δ2pxxp, ΔY14-F14 (1.1 kb) interacts with Dock-fl (FIG 5.3.3 B, K), and Dock-ΔSH2 (FIG 5.3.3 B, L), restoration of YFP signal was observed. No interaction was observed with Dock-ΔSH3 (FIG 5.3.3 B, M). It is suggested that the SH3 domain is required for the bindingto Sns, but it is not depending on the proline motif and phosphorylated tyrosine residues of Sns.

Taken together from the above data, SH2 domain of Dock binds to the intracellular domain of phosphorylated tyrosine residue of Hbs (Y1089) and SH3 domain of Dock binds to the intracellular domain of Sns and is not dependent on PxxP and tyrosine motif. We had a collabaration with the group of Susan Abmayr. They did a *dock sns* recombinant and unless like my *dock hbs* recombinant, they found no evidence for a genetic interaction between

dock and *sns.* It therefore remains unclear whether the observed interaction in my study is really required for myoblast fusion.

5.4 Analyzing the role of Dock in FCs during myoblast fusion

Since Dock is not only expressed in FCMs, but also in FCs (Schafer *et al,* in preparation). An interaction of Dock with cell adhesion molecules i.e Rst, Duf, Sns and Hbs was confirmed in vitro by Biomolecular Fluorescence Complementation (BiFC) (FIG 5.2.2 B) in *Drosophila* SL2 cells. Based on these results, I further started to investigate the role of Dock in FCs, if the observed interactions occur in FCs during myoblast fusion.

5.4.1 The SH2-SH3 adaptor *dock* interacts genetically with the FC-specific gene *rols*

Rols directly binds to the intracellular domain of Duf (Kreisköther *et al*., 2006; Bulchand et al., 2010).To examine the role of Dock in FCs, I have generated and analyzed *dock rols* double mutants. For the recombination I used the $dock^{04723}$ null allele and *rols* deficiency i.e *Df(3L)BK9*).

Mutant phenotype was examined by staining with the *β3-Tubulin* antibody that marks all myoblast and mature myoblasts. *Df(3L)BK9* mutant embryos display strong fusion defect, but few muscles was observed (FIG 5.4.1 B-B') unlike in wild-type (FIG 5.4.1 A-A'). $dock^{04723}$ mutant embryos display no strong fusion defect , but few unfused myoblast were observed (FIG 5.4.1 C-C') unlike in wild-type (FIG 5.4.1 A-A'). Homozygous $dock^{04723}$ *Df(3L)BK9* double mutant embryos show strong fusion defect with complete block of fusion in comparision to the single mutant *Df(3L)BK9* (FIG 5.4.1 D-D').

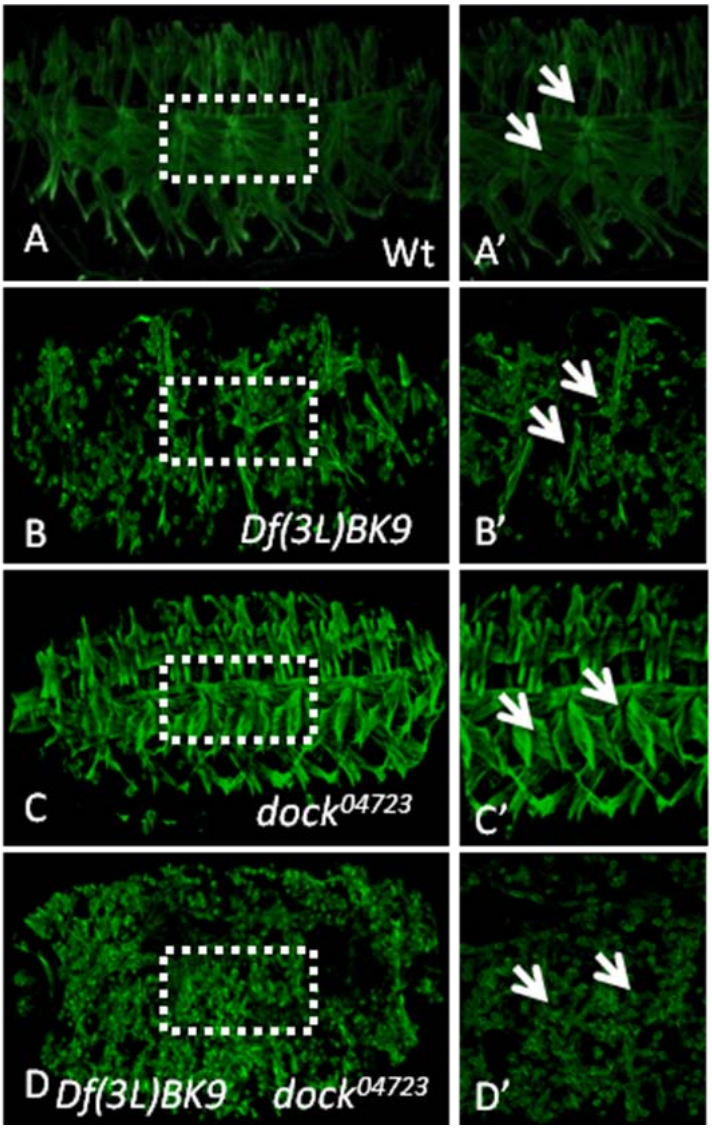
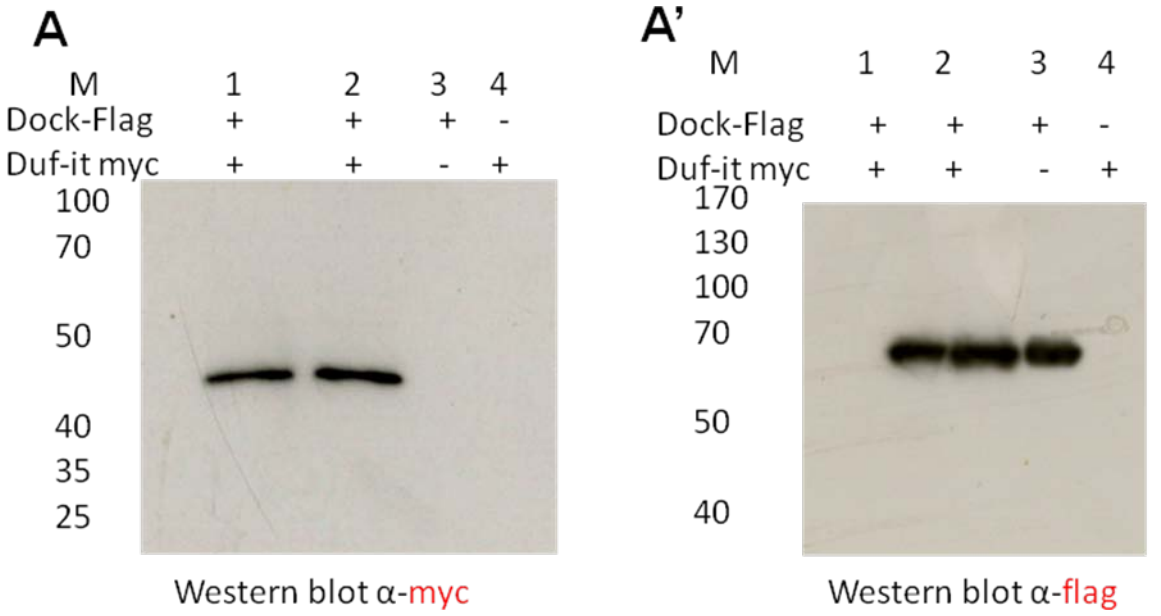


FIGURE 5.4.1 **The SH2-SH3 adaptor *dock* interacts genetically with *rols*.** Whole mount embryos stained with *β3-Tubulin*, (A-D) ventral view of stage 16 embryos. A'-D' are magnified view of the respective images. Wild-type muscle phenotype (FG 5.4.1 A-A'). *Df(3L)BK9* mutant embryos display strong fusion defect, but few muscles was observed (FG 5.3.1 B-B'). A few unfused myoblast was visible in $dock^{04723}$ mutant embryos (FG 5.4.1 C-C'). Homozygous $dock^{04723}$ *Df(3L)BK9* double mutant display stronger muscle phenotype than single mutant *Df(3L)BK9* (FG 5.4.1 D-D').

Taken together, these experiments shows that *dock* and *rols* genetically interact during *Drosophila* muscle development. Since, Duf interact with Rols (Kreisköther *et al*., 2006; Bulchand *et al*., (2010). This suggests that the interaction between the intracellular domain of Duf and Dock-fl is essential to mediate signalling in fusion myoblasts.

5.4.2 The physical interaction between Dock and Duf depends on proline rich region of Duf and SH3 domain of Dock

To examine further interaction between the Dock and Duf, I have performed CO-IP with Flag-tagged Dock and *Myc*-Duf.it in *Drosophila* Schneider (SL2) cells. Myc-Duf.it (~0.9 kb) was cloned into pUAST plasmid at *Bgl II* and *Not I* restriction sites respectively.



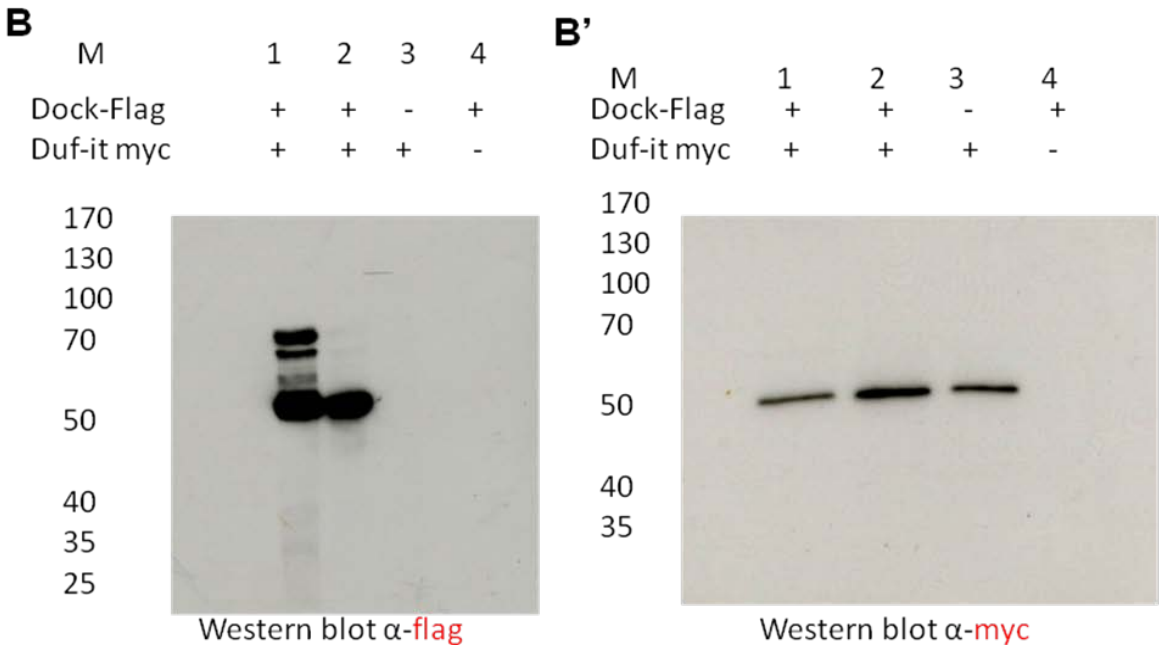


FIGURE 5.4.2 **CO-IP of Dock-Flag with Myc-Duf.it In SL2 cells.** Co-IP with Myc-tagged agarose beads, Dock interacts with Myc-Duf.it, confirmed by western blot with α-Myc (A), the control blot with α-Flag (A'). CO-IP with Flag-tagged agrose beads, Dock interacts with Myc-Duf.it beads, confirmed by western blot with α-Flag (B), the control blot with α-Myc (B').

To study a possible interaction between Dock and Duf.it, I expressed Dock-Flag and Myc-Duf.it in *Drosophila* Schneider (SL2) cells. As shown in the western blot analysis in FIG 5.4.2 A and A', Duf is able to pull down Dock. Co-IP was performed by using the Flag-tagged agarose beads and western blot was performed by α-Myc (FIG 5.4.2 A), the control blot with α-Flag to make sure that Flag-Dock binds to the beads (FIG 5.4.2 A').

In the viceversa the CO-IP was performed by Myc tagged beads and western blot was developed with α-Flag (FIG 5.4.2 B), Dock interacts with Duf.it and the control blot with α-Myc to make sure that Myc-Duf.it binds to the beads (FIG 5.4.2 B'). The above data suggesting that the intracellular domain of Duf physically interacts with Dock.

Based on the above evidence, I started to determine which of the Dock domain required to bind to the intracellular domain of Duf and also which region of intracellular domain of Duf required to bind to Dock by BiFC.

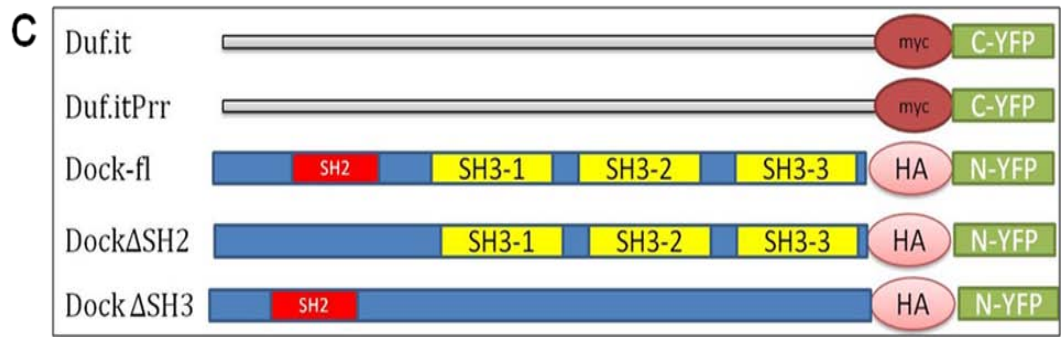


FIGURE 5.3.2 C **Generation of Split *YFP* constructs**. Duf.it (~0.9 kb) and Duf.it ΔPPP (~0.7 kb) were cloned into *CN* of pUAST plasmid. *Dock-fl*, *Dock-ΔSH2* and *Dock-ΔSH3* were cloned at *YN* of pUAST plasmid.

I have cloned the *Duf.it* (0.9 kb) into *CN* of *pUAST* plasmid at *Bgl II* and *Not I* restriction sites respectively. I have further cloned Duf.it ΔPPP (0.7 kb) into *CN* of *pUAST* plasmid at *Bgl II* and *Not I* restriction sites respectively. *dock-fl (*1.644 kb), *dock-ΔSH2* (1.393

kb), were cloned into *Bgl II* and *Xba I* restriction sites respectively and *dock-ΔSH3 (123) (1.398 kb)* were cloned into *Bgl II*, *Not I* and *Xba I* restriction sites into YN of pUAST plasmid respectively.

Duf.it interacts with Dock-fl (FIG 5.4.2 D, A), and also interacts with Dock-ΔSH2 (FIG 5.4.2 D, B), and no interaction with Dock-ΔSH3 (FIG 5.4.2 B D, C). It indicates that Dock-ΔSH3 domain binds to the Duf.it. Further interaction with Duf.itΔPPP and Dock deletion constructs (FIG 5.4.2 D F, G, H) by split YFP assay. Duf.it ΔPPP interaction with Dock-fl (FIG 5.4.2 D, F), Dock-ΔSH2 (FIG 5.4.2 D, G) and Dock-ΔSH3 (FIG 5.4.2 D, H) no interaction was observed.

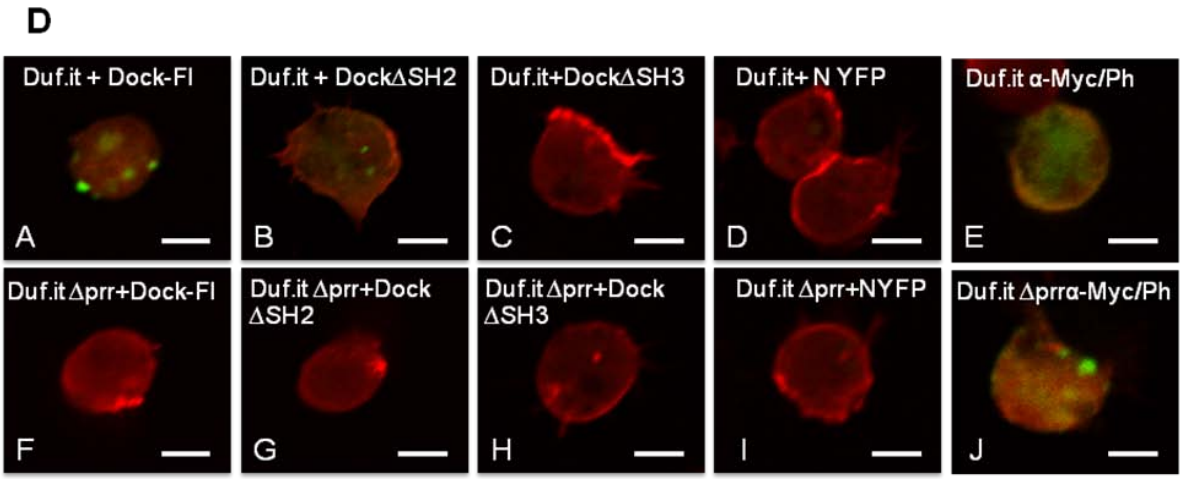


FIGURE 5.4.2 D **Dock interacts with Duf.it via proline rich region of Duf and SH3 domain of Dock by Biomolecular Fluoresence Complementation (BiFC) in Drosophila SL2 cells**. In all the panels cells were stained with phalloidin (Red). The restoration of split YFP signal was observed in (A). Duf.it interacts with Dock-fl and Dock-ΔSH2 signal was observed (A) and (B) and no interaction with Dock-ΔSH3 (C) and the respective controls were with Duf.it and N-YFP, no signal was observed (D). Duf.it expression was confirmed by anti-*Myc* in green (E). Duf.it ΔPPP interacts with Dock-fl and Dock-ΔSH2 restoration of split YFP signal were observed (F) and (G) and no interaction with Dock-ΔSH3 (H) and the respective controls were with Duf.itΔ PPP and N-YFP, no restoration of YFP signal was observed (I). Duf.it ΔPPP expression was confirmed by anti-*Myc* in green (J).

Taken together, CoIP and data from the BiFC assay suggest that Dock and Duf physically interact. Moreover, by using Dock deletion constructs I was able to show that the interaction between Dock and Duf is mediated by the SH3 domains on the site of Dock. Since SH3 domains are known to bind to proline-rich regions I searched for proline-rich

regions in the intracellular domain of Duf and identfied a short proline-rich sequence of PxxP. When I deleted the part of intracellular domain contains a short proline-rich sequence I lost interaction.

5.4.3 The physical interaction between Dock and Rst depends on the SH2 domain of Dock.

Based on the above data in FIGURE 5.2.2 B, D, I have further studied the role of Dock in FCs. To determine which of Dock domain required to bind with intracellular domain of Rst, by BiFC.

I have cloned the *rst.it (*0.6 kb) into *CN* of *pUAST* plasmid at *Bgl II* and *Not I* restriction sites respectively. *dock-fl (*1.644 kb), *dock-ΔSH2* (1.393 kb), were cloned into *Bgl II* and *Xba I* restriction sites respectively and *dock-ΔSH3 (123) (1.398 kb)* were cloned into *Bgl II*, *Not I* and *Xba I* restriction sites into *YN* of pUAST plasmid respectively.

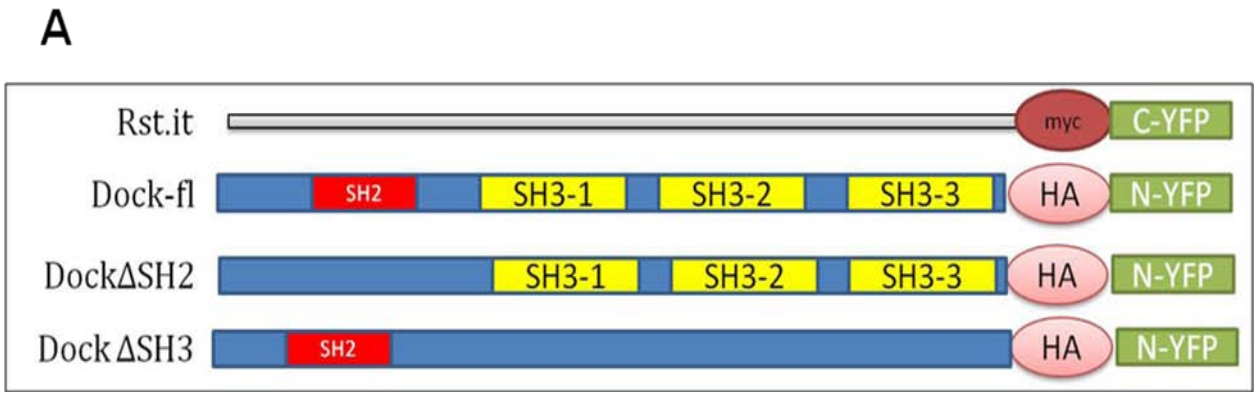


FIGURE 5.4.3 A **Generation of Split *YFP* constructs**. *rst.it* (~0.6 kb) were cloned into *CN* of pUAST plasmid. *Dock-fl*, *Dock-ΔSH2* and *Dock-ΔSH3* were cloned at *YN* of pUAST plasmid.

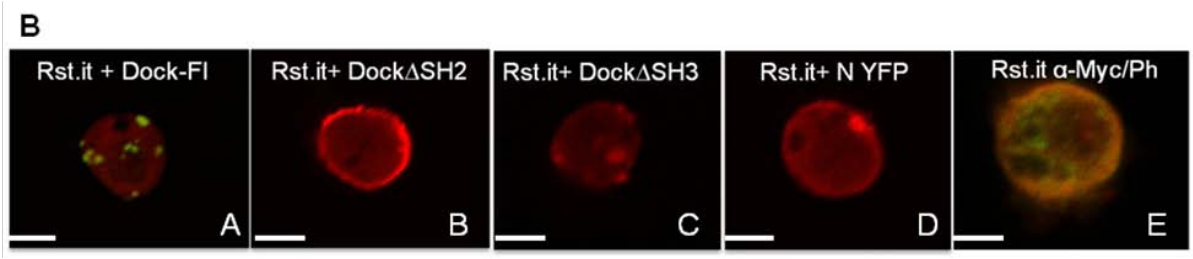


FIGURE 5.4.3 B **Dock interacts with Rst.it via SH3 domain of Dock by Biomolecular Fluorescence BiFC assay in *Drosophila* SL2 cells.** In all the panels cells were stained with phalloidin (Red). The

restoration of split YFP signal was observed in (A). Rst.it interacts with Dock-fl and (A) and no interaction with Dock-ΔSH2 and Dock-ΔSH3 signal were observed in green (B) and (C) and the respective contorls were with Rst.it and N-YFP, no restoration of YFP signal was observed (D). Rst.it expression was confirmed by anti-*Myc* in green (E).

Rst.it interacts with Dock-fl (FIG 5.4.3 B, A), and no interaction with Dock-ΔSH2 (FIG 5.4.3 B, B), and no interaction with Dock-ΔSH3 (FIG 5.4.3 B, C). It indicates that Dock-ΔSH2 and Dock-ΔSH3 domain binds to the Rst. By the above evidence it indicates that Rst binds to the Dock viaΔSH2 and ΔSH3. However I have further studied the role of Dock and Rst interaction genetically, but no genetic interaction was observed. Therefore it still remains unclear whether the Rst- Dock interaction required for myoblast fusion.

5.5 Dock links cell adhesion with Arp2/3-based actin polymerization

The Arp2/3-based actin complex is regulated by WASP-WIP and SCAR/WAVE pathway. The above data suggests that SH2 and SH3 domain of Dock binds to the cell adhesion molecules. In vertebrate and in *Drosophila* Schneider cells, Nck/Dock bind to WASP (Rohatgi *et al*., 2001: Worby *et al*., 2001). Since, all these WASP, WIP and SCAR/WAVE actin nucleation promoting factors contain the proline-rich region. Whether proline-rich region of all actin regulators can bind to th SH3 domain of Dock. To answer this question, I performed interactions between the Dock and WASP-WIP and SCAR/WAVE by yeast two hybrid assay and genetic interactions respectively.

5.5.1 The SH2-SH3 adaptor Dock binds to the Arp2/3-regulator Wasp and the Wasp interaction partner Wip via its proline-rich region

To study the interaction between the Dock and WASP-WIP complex, *dock-fl (*1.644 kb), was cloned into *EcoR I* and *Xho I* site respectively into pB42AD prey plasmid. The *WASP-fl* (1.569 kb), *WASP-PPP* (0.319 kb), *WASP-ΔPPP* (~1.2 kb) was cloned into bait plasmid *pGILDA* at *EcoR I* and *Xho I* restriction sites respectively. The *WIP-fl* (2.124 kb), *WIP-PPP* (0.864 kb), *WIP-ΔPPP* (~1.2 kb) was cloned into bait plasmid *pGILDA* at *EcoR I* and *Xho I* restriction sites respectively. The remaining protocol was performed according to the manufacturer's protocol.

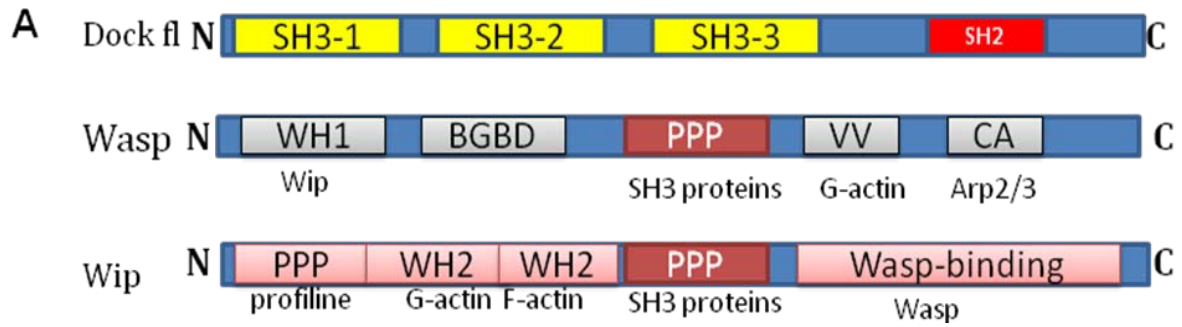
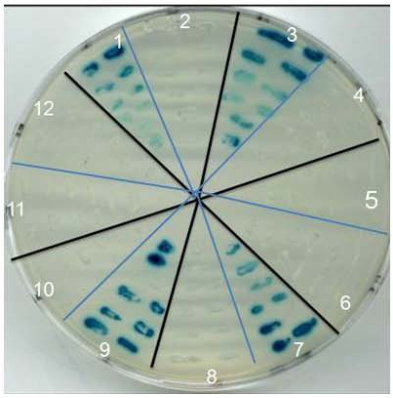


FIGURE 5.5.1 A **Generation of Dock-fl and WASP and WIP constructs.**

First, I have performed the interaction between Dock-fl and WIP-fl, WIP-PPP and WIP-ΔPPP by yeast-two hybrid assay, I have observed the interaction between these proteins within two days after induction (FIG 5.5.1 B). Dock-fl interacts with WIP-fl (FIG 5.5.1 B, 1), WIP-PPP (FIG 5.5.1 B, 3) and no interaction were observed with WIP-ΔPPP (FIG 5.5.1 B, 5). It indicates that Dock binds to the proline rich region of WIP (FIG 5.5.1 B). Similarly I have performed the interaction between the Dock and all deletions of WASP proteins i.e WASP-fl, WASP-PPP, WASP-ΔPPP. Dock-fl interacts with WASP-fl (FIG 5.5.1 B, 7), WASP-

B



	Wip-Fl	Wip-Prr	WipΔPrr
Dock-fl	1 +++	3 +++	5 ---
pB42AD	2 ---	4 ---	6 ---

	Wasp-fl	Wasp-prr	WaspΔPrr	pGilda
Dock-fl	7 +++	9 +++	11 ---	---
pB42AD	8 ---	10 ---	12 ---	---

FIGURE 5.5.1 B **The SH2-SH3 adaptor Dock binds to the Arp2/3-regulator WASP and the WASP interaction partner WIP via its proline-rich region.** For all these interaction studies, yeast were grown on synthetic dropout plates (SD) lacking histidine, tryptophan, uracil and leucine with Galactose /raffinose as carbon source with X-Gal (80 mg/L). In all panels (1-12) interaction) was observed within two days. Dock-fl interacts with WIP-fl (1) , WIP-PPP (3) and no interaction was observed with WIP-ΔPPP (5). The respective controls with WIP-fl and pB42AD (2), WIP-PPP and pB42AD (4) and WIP-ΔPPP (6), no interaction was observed. Dock-fl interacts with WASP-fl (7) , WASP-PPP (9) and no interaction was observed with WASP-ΔPPP (11). The respective controls with WASP-fl and pB42AD (8), WASP-PPP and pB42AD (10) and WASP-ΔPPP (12), no interaction was observed. Dock-fl with pGILDA, no interaction was observed (FIG 5.5.1 B).

PPP (FIG 5.5.1 B, 9) and no interaction were observed with WASP-ΔPPP (FIG 5.5.1 B, 11). It indicates that Dock binds to the Proline rich region of WASP (FIG 5.5.1 B)

I have further studied the interaction between the WASP-PPP and WIP-PPP with Dock deletions.

5.5.2 SH3-1 and SH3-3 are essential for binding WIP and all SH3 domains are required to bind to WASP

Based on the above studies, I have further tested the interactions between the WIP-PPP and WASP PPP with Dock deletions i.e Dock-ΔSH2, Dock-ΔSH3-1, Dock-ΔSH3-2, Dock-ΔSH3-3 and Dock-ΔSH3 (123). *dock-ΔSH2* (1.393 kb), was cloned into *EcoR I* and *Xho I* sites respectively and the *dock-ΔSH3-1 (1.563 kb), dock-ΔSH3-2 (1.479 kb), dock-ΔSH3-3 (1.470 kb) and dock-ΔSH3 (123) (1.398 kb)* of these constructs, the first part was cloned in to *EcoR I* and second part was cloned into *Xho I* site respectively into pB42AD prey plasmid. The *WASP-PPP* (0.319 kb) was cloned into bait plasmid *pGILDA* at *EcoR I* and *Xho I* restriction sites respectively. The *WIP-PPP* (0.864 Kb) was cloned into bait plasmid *pGILDA* at *EcoR I* and *Xho I* restriction sites respectively. The remaining protocol was performed according to the manufacturer's.

First, I have performed the interactions between WIP-PPP and Dock-ΔSH2, Dock-ΔSH3-1, Dock-ΔSH3-2, Dock-ΔSH3-3 and Dock-ΔSH3 (123). By yeast-two hybrid assay, i have observed the interaction between these proteins within two days after induction (FIG 5.5.2 B). WIP-PPP interacts with Dock-ΔSH2 (FIG 5.5.2 B, 9), Dock-ΔSH3-2 (FIG 5.5.2 B, 9), indicates no requirement these domains and no interaction were observed with Dock-ΔSH3-1 (FIG 5.5.2 B, 1), Dock-ΔSH3-3 (FIG 5.5.2 B, 5) and Dock-ΔSH3 (FIG 5.5.2 B, 7). It indicates that WIP-PPP binds to Dock by Dock-ΔSH3-1 and Dock-ΔSH3-3 (FIG 5.5.2 B).

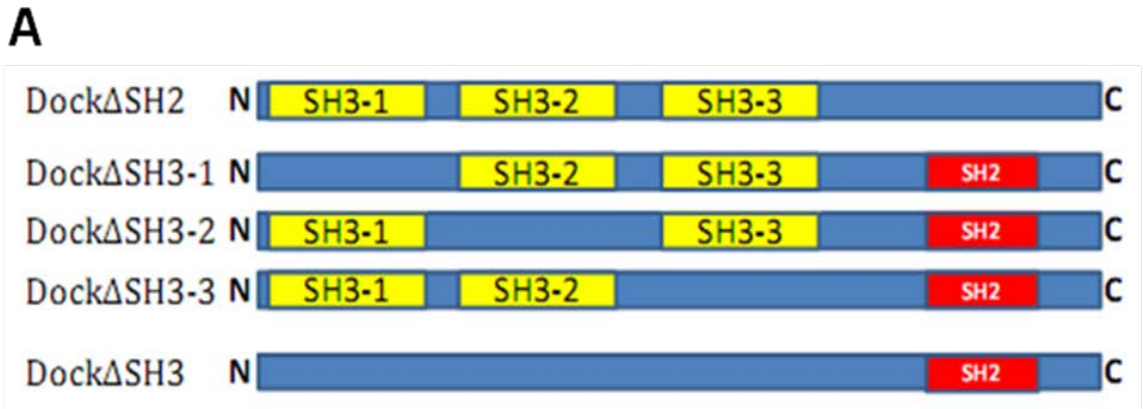
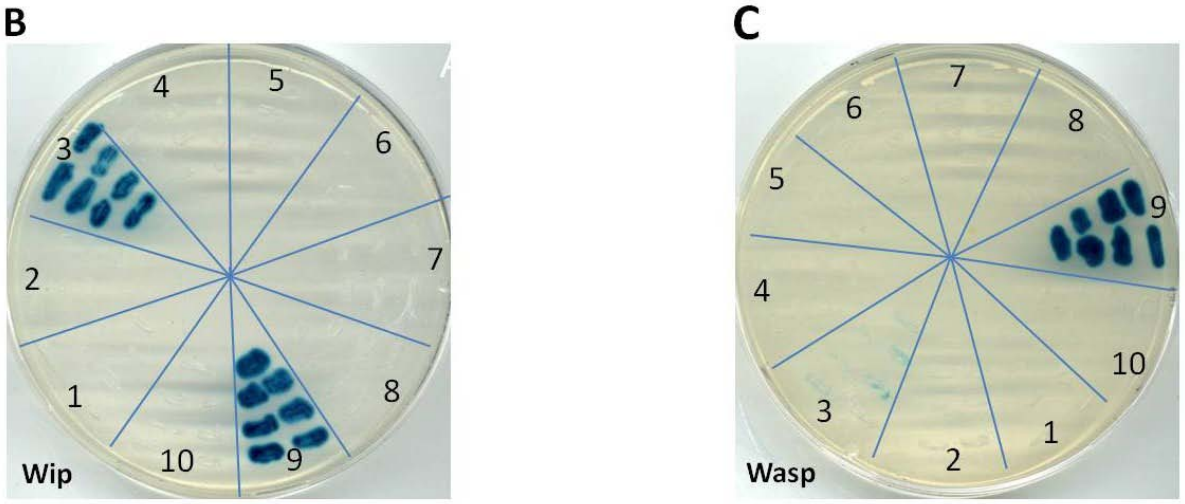


FIGURE 5.5.2 A **Generation of Dock deletion constructs** i.e. Dock-ΔSH2, Dock-ΔSH3-1, Dock-ΔSH3-2, Dock-ΔSH3-3 and Dock-ΔSH3 (123).



	DockΔSH3-1	DockΔSH3-2	DockΔSH3-3	DockΔSH3-1,2,3	DockΔSH2	pB42AD
Wip-PPP	1 ---	3 +++	5 ---	7 ---	9 +++	
pGilda	2 ---	4 ---	6 ---	8 ---	10 ---	
Wasp-PPP	1 ---	3 ---	5 ---	7 ---	9 +++	

FIGURE 5.5.2 B, C **SH3-1 and SH3-3 are essential for binding WIP and all SH3 domains are required to bind to WASP.** For all these interaction studies yeast were grown on synthetic dropout plates (SD) lacking histidine, tryptophan, uracil and leucine with Galactose /raffinose as carbon source with X-Gal (80 mg/L). In all panels (1-12) interaction was observed within two days. WIP-PPP interacts with Dock-ΔSH2 (9), Dock-ΔSH3-2 (2). No interaction was observed with Dock-ΔSH3-1 (1), Dock-ΔSH3-3 (5), Dock-ΔSH3 (7). Respective controls shows no interaction with Dock-ΔSH3-1 and pGILDA (2), Dock-ΔSH3-2 and pGILDA (4), Dock-ΔSH3-3 and pGILDA (6) and Dock-ΔSH3 and pGILDA (6). Similarly WASP-PPP interacts with Dock-ΔSH2 (9) and no interaction was observed with Dock-ΔSH3-1 (1), Dock-ΔSH3-2 (3), Dock-ΔSH3-3 (5) and Dock-ΔSH3 (7). Respective controls show no interaction with Dock-ΔSH3-1 and pGILDA (2), Dock-ΔSH3-2 and pGILDA (4), Dock-ΔSH3-3 and pGILDA (6) and Dock-ΔSH3 and pGILDA (6) and also shows no interaction between WIP-PPP and PB42AD, WASP-PPP and pB42AD.

Similarly, I have performed the interactions between WASP-PPP and Dock-ΔSH2, Dock-ΔSH3-1, Dock-ΔSH3-2, Dock-ΔSH3-3 and Dock-ΔSH3 (123). By yeast-two hybrid assay, I have observed the interaction between these proteins within two days after

induction (FIG 5.5.2 B). WASP-PPP interacts with Dock-ΔSH2 (FIG 5.5.2 C, 9), Indicates no requirement of these domain. WASP does not interacts with Dock-ΔSH3-1 (FIG 5.5.2 C, 1), Dock-ΔSH3-2 (FIG 5.5.2 C, 3), Dock-ΔSH3-3 (FIG 5.5.2 C, 5) and Dock-ΔSH3 (FIG 5.5.2 C, 7). It indicates that WASP-PPP binds to Dock by Dock-ΔSH3-1, Dock-ΔSH3-2, Dock-ΔSH3-3 and Dock-ΔSH3 (FIG 5.5.2 C).

Taken together from the above data suggests that SH3-1 and SH3-3 domain of Dock binds to the WIP-PPP and All SH3 domains are required to bind to WASP-PPP.

5.5.3 The SH2-SH3 adaptor Dock does not act through the Arp2/3-regulator Scar during myoblast fusion

Previous genetic interaction studies from Gritt Schäfer showed that dock and *wip* interact genetically with *dock* and that no myoblast fusion occurs in the double mutant whereas some muscles are formed in *wip* mutants and *dock* mutants show no muscle defects.

Arp2/3-based actin polymerization is essential for myoblast fusion and I want to link cell adhesion with F-actin formation. An interesting candidate is Dock in transferring the signal from the cell adhesion molecule to Arp2/3-based actin polymerization. But the Arp2/3 complex can be activated by different ways such WASP-WIP and SCAR/WAVE pathway. So, in the above chapter I have looked at WASP-WIP based path way. Now I started to study whether the other pathway plays a role i.e SCAR/WAVE based path way.

I generated and analyzed *dock scar* double mutants. For the genetic interaction studies, I have used the *dock*^{04723} null allele and *scar*$^{\Delta 37}$ null allele. The recombination was performed in the following manner.

$$\frac{dock^{04723}}{Cyo^{fl\text{-}lacZ}} \times \frac{Scar^{\Delta 37}}{Cyo^{hg\text{-}lacZ}}$$

$$\frac{dock^{04723}}{Scar^{\Delta 37}} \times \frac{lf}{Cyo^{hg\text{-}lacZ}}$$

$$\frac{dock^{04723}, Scar^{\Delta 37}}{Cyo^{hg\text{-}lacZ}} \times \frac{lf}{Cyo^{hg\text{-}lacZ}}$$

$$\frac{dock^{04723}, Scar^{\Delta 37}}{Cyo^{hg\text{-}lacZ}} \times \frac{dock^{04723}, Scar^{\Delta 37}}{Cyo^{hg\text{-}lacZ}}$$

$$\frac{dock^{04723}, Scar^{\Delta 37}}{Cyo^{hg\text{-}lacZ}}$$

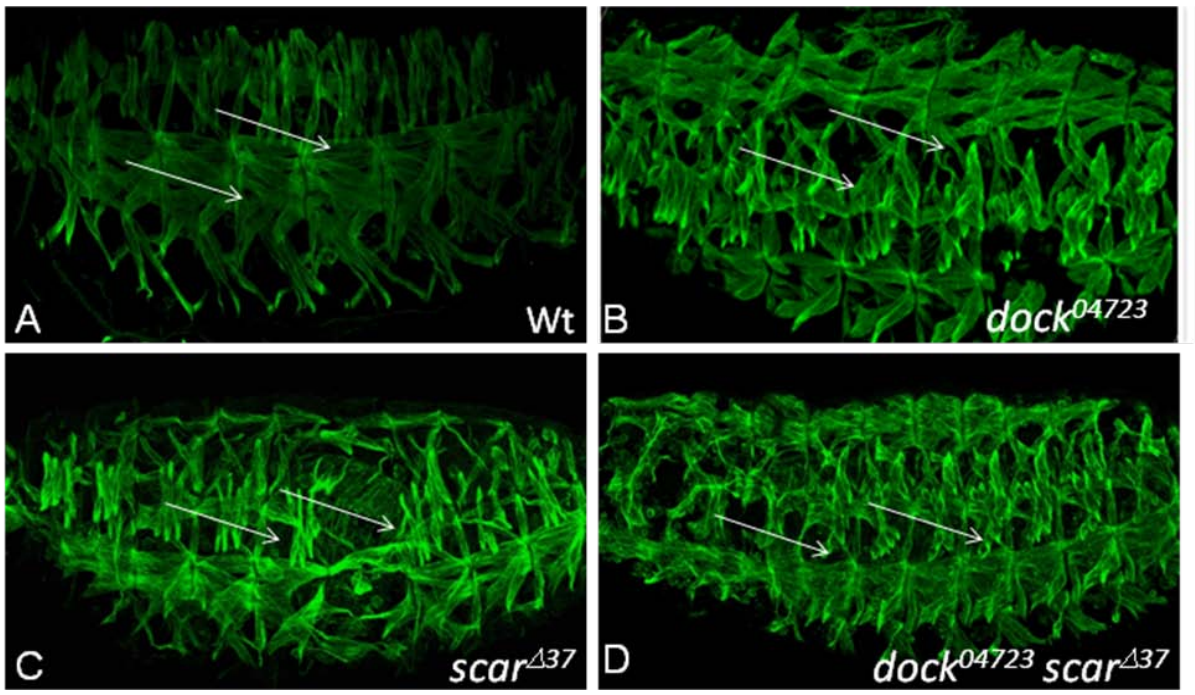


FIGURE 5.5.3 **The SH2-SH3 adaptor *dock* does not interact genetically with *scar*.** Whole mount embryos stained with *β3-Tubulin* (A-D) at stage 16 embryo of ventral view. Wild-typemuscle phenotype (FG 5.5.3 A'). A few unfused myoblast are visible in $dock^{04723}$ mutant embryos (FG 5.5.3 B'). $scar^{\Delta 37}$ mutant embryos show few unfused myoblasts (FG 5.5.3 C). Homozygous $dock^{047}$ $scar^{\Delta 37}$ double mutant does not display stronger muscle phenotype than single mutant ((FG 5.5.3 D).

Mutant phenotype was examined by staining with the *β33-Tubulin* antibody that marks all myoblast and mature myoblasts. $dock^{04723}$ mutant embryos display no strong fusion defect , but few unfused myoblast were observed (FIG 5.5.3. B) unlike in wild-type (FIG 5.5.3 A). $scar^{\Delta 37}$ mutant embryos display no strong fusion defect, but few unfused myoblast were observed (FIG 5.5.3 C) unlike in wild- type (FIG 5.5.3.1 A). In homozygous $dock^{04723}$ $scar^{\Delta 37}$ double mutant embryos does not shows strong fusion defect in comparision to the single mutant (FIG 5.5.3 D). Taken together, these experiments show that *dock* and *scar* does not interact genetically during *Drosophila* muscle development.

Taken together above experiments suggests that Dock does not acting through the Arp2/3-regulator SCAR/WAVE, but through the Arp2/3-regulator WASP and the WASP interacting partner WIP during myoblast fusion.

6. Disccussion

Actin participates in many important cellular processes including muscle contraction, cell motility, cell division and cytokinesis, vesicle and organelle movement, cell signaling, and the establishment and maintenance of cell junctions, cell shape and cell-cell fusion. The somatic musculature of *Drosophila* is formed by the cell-cell fusion events between two myoblast cell types. Myoblast requires the recognition and adhesion of the two distinct myoblast cell types, named founder cells (FCs) and fusion-competent myoblast (FCMs). The process of recognition and adhesion depends on the evolutionary conserved members of the immunoglobulin (Ig) superfamily Dumbfounded/Kin of Irre (Duf/Kirre) and Roughest (Rst) in FCs (Ruiz-Gomez *et al*., 2000; Strunkelnberg *et al*., 2001), and Sticks and stones (Sns) and Hibris (Hbs) in FCMs (Bour *et al*., 2000, Ruiz-Gomez *et al*., 2000). However certain proteins, such as Roughest/Irregular-chiasm-C (Rst/IrreC), have been found in both cell types as well (Ramos *et al*., 1993; Strunkelnberg *et al*., 2001). Kesper et al. (2007) and Richardson et al. (2007) show that F-actin is present at the contact between the myoblast (Chapter 2.5, FIG 2.4). The actin-related-protein-2/3 (Arp2/3) plays a crucial role in the formation of branched-actin-filament networks in *Drsosophila* myoblast fusion. Arp2/3 complex is activated by nucleation promoting factors WASP and SCAR (Chapter 2.8.2). Linking between the immunoglobulin super family (IgSF) mediated cell adhesion molecules with the Arp2/3 based dependant actin polymerization is poorly understood in *Drosophila melanogaster.* My studies on Duf and Rst chimera and Rst deletions could try to show the redundant function of both proteins in myoblast fusion and also I identified the relavent interaction partners of Rst. Further studies on Dock in cell culture could show that Dock interacts with Sns and Hbs via SH3 domain and SH2 domain respectively and this might link to the WASP-WIP-based actin polymerization. hbs and dock interacts genetically in FCMs during myoblast fusion. The SH3 domain of Dock interacts with Duf via its proline-rich region and also interacts with Rst via the SH2 and SH3 domain of Dock. Dock and Duf interacts in FCs during myoblast fusion.

6.1 Duf and Rst proteins in the founder cell are redundant nature

To analyze the redundant functions of both proteins in myoblast fusion. Duf and Rst has five Ig-like domains at the extracellular domain and high conservation reside primarily in the region of five Ig domains. The intracellular domain of Duf, Rst shows a low overall homology. Yet, there seems to be three consereved motifs: PADVI, SAIYGNPYLR and SLLPPLPP (Strunkelnberg *et al*., 2001). Furthermore, the intracellular domain of Rst is much shorter than the intacellular domain of Duf. The less conserved regions of Rst intra might play a role during the signal transduction in FCMs. Following expression of Duf and Rst in *duf, rst* deficient embryos completely restore the wild-type muscle pattern (Ruiz-Gomez *et al*., 2000; Strunkelnberg *et al*., 2001). This also suggests that Duf can substitute for Rst and vice versa.

To test these possibilities, I created the chimeric constructs that carry either the extracellular domain of Duf and intracellular domain of Rst (Duf-Et/Rst-It) and extracellular domain of Rst and intracellular domain of Duf (Rst-Et/Duf-It). I expressed the transgenes carrying the chimeric constructs using the UAS-GAL4 system (Brand and Perrimon, 1993) in the entire mesoderm of wild-type embryos. Expression of UAS-*duf-et/rst-it* chimera in all myoblasts using *twist*-GAL4*, Mef2*-GAL4 does not effect myoblast fusion. But it shows minimal muscle attachment defects. The chimera was also expressed in specific sets of myoblasts i.e FCs and FCMs using FCs specific *rP298*-GAL4 and FCMs specific *sns*-GAL4. This did not effect myoblast fusion, but shows again minimal muscle attachment defects. The expression of the second chimeric constructs UAS-*rst-et/duf-it* in all the myoblast and specific sets of myoblasts with *twist*-GAL4, *Mef2*-GAL4, *rp298*-GAL4, *sns*-GAL4 also does not impair myoblast fusion. Muscle attachment defects like in embryos expressing UAS-*duf-et/rst-it* were observed. Thus, the chimeras do not effects the myoblast fusion and this suggests that chimeras can not acts as a dominant negative effect. Since, wild-type embryo contains the endogenous Duf and Rst .

I further analyzed the intracellular domain of Duf and Rst. Since both Rst and Duf proteins contain three conserved motifs. I have deleted three motif from Rst: PADVI, SAIYGNPYLR and SLLPPLPP (Chapter 2.7.2, FIG 2.7). I first deleted the

PADVI motif from Rst and expressed an UAS-*rstΔPADVI* construct in the entire mesoderm using UAS-GAL4 system in wild-type embryos. I expressed the Rst deletions in both myoblasts i.e FCs and FCMs by using *twist*-GAL4 and *Mef2*-GAL4 and found that this disrupts myoblast fusion. Also expression in specific set of myoblasts by using FCs specific *rp298*-GAL4 and FCMs specific *sns*-GAL4 impairs myoblast fusion. This indicates that RstΔPADVI motif is required for myoblast fusion in FCs as well as FCMs. Next I deleted the second and third conserved motif SAIYGNPYLR and SLLPPLPP from Rst. I expressed an UAS-*rstΔSAIYGNPYLR, ΔSLLPPLPP* construct in both myoblast i.e FCs and FCMs by using *twist*-GAL4 and *Mef2*-GAL4 driver line and found that this does not disrupt myoblast fusion. Also expressed in specific set of myoblasts by using FCs specific *rP298*-GAL4 and FCMs specific *sns*-GAL4 driver line does not impair myoblast fusion. This indicates that Rst*ΔSAIYGNPYLR, ΔSLLPPLPP* motif is not required for the myoblast fusion.

I further deleted the third conserved motif SLLPPLPP in the intracellular domain of Rst. Expression of UAS-*rstΔSLLPPLPP* in FCs and FCMs by using *twist*-GAL4 and *Mef2*-GAL4 and also expression in a specific set of myoblasts by using FCs specific *rP298*-GAL4 and FCMs specific *sns*-GAL4 does not impair myoblast fusion. This indicates that the Rst*ΔSLLPPLPP* motif is not required for myoblast fusion. Finally I deleted all conserved motives of Rst i.e Rst*ΔPADVI*, *SAIYGNPYLR* and *ΔSLLPPLPP.* I expressed an UAS-*rstΔPADVI, SAIYGNPYLR, ΔSLLPPLPP* construct in the both myoblasts i.e FCs and FCMs by using *twist*-GAL4 and *Mef2*-GAL4 driver line and found that this disrupt myoblast fusion like RstΔPADVI. I also expressed UAS-*rstΔPADVI, SAIYGNPYLR, ΔSLLPPLPP* in a specific set of myoblasts by using FCs specific rP298-GAL4 driver line and FCMs specific sns-GAL4 driver line. this impaired myoblast fusion. This indicates that the conserved motif PADVI, which is a common motif in Rst and Duf is required for myoblast fusion. It further indicates that the Duf and Rst are redundant in FCs . Furthermore, these results also supports the Bulchand *et al*. (2010). The complete deletion of Duf intracellular region, also PADVI motif were unable to rescue the *duf, rst deficient embryos*. This also further indicates that PADVI conserved motif of Rst and Duf are required during myoblast fusion and Rst, Duf may be involved in the common signal transduction pathway by interacting with common interaction partner.

Another open question is which of the common protein interacts with Rst and Duf and I will further discuss some interaction partners of Rst. So, far only one protein have been found i.e CG10494 was identified in a large two-hybrid screen (Giot *et al*., 2001). During development CG10494 is expressed ubiquitously in *Drosophila* embryo. Rst interacts with adopter protein X11α/Dmint 1 via its PDZ-binding domain during *Drosophila* eye development (S. Vishnu *et al*., 2006), but these interactions was not shown in myoblast fusion. Further I identified three potential interaction partners of Rst in a yeast two-hybrid screening. I used the intracellar domain of Rst against the *Drosophila* embryonic cDNA library. I found that Actin57B, Nidogen/Entactin and Papilin are potential interaction partners of Rst. Actin57B is expressed in the somatic mesoderm and also in later stages of embryonic development, which indicates it has a role in myoblast fusion and at later stages it may be involving in the muscle attachment sites in *Drosophila*. Nidogen is expressed in embryo at later stages 14-16 which indicates it has no role in myoblast fusion, but it may be involving in muscle attachment sites in *Drosophila* embryo. Papilin is expressed in embryo at stage 12-14 which indicates it may be required for myoblast fusion in *Drosophila* embryo. Actin57B is a basic structural constituent of *Drosophila* cytoskeleton (Kelly et al., 2002), Nidogen is a cell matrix protein (Murshed et al., 2000) and Papilin is *Drosophila* extracellular matrix. Protein (Kramerova *et. al*. 2003) However, none of the identified Rst interaction partners has no clear role in myoblast in myoblast fusion.

The common interaction partner of cell adhesion molecules i.e Dock/Nck wiil be further discussed in the following chapter.

6.2 Dock/Nck links cell adhesion with Arp2/3-based actin polymerization

The genetic and biochemical data presented in this study provide new insights into the signal transduction from the transmembrane molecule Duf, Rst, Sns and Hbs to the actin nucleation factor WASP-WIP during the *Drosophila* myoblast fusion. Recently different adaptor proteins e.g Rols7 and the

SH2-SH3 adaptor could be identified to be involved in the regulation of actin reorganization. SH2-SH3 adaptor proteins are involved in many signalling pathways, e.g activation of actin polymerization. In vertebrates Crl-II is involved in the activation of N-WASP-dependent actin polymerization in the smooth musculature (Tang *et al*., 2005). In *Drosophila* Kim *et al*. (2007) suggest that Crk binds to the proline-rich region of WIP during myoblast fusion. Therefore, *Drsophila* Crk could possiblly bind to the proline rich region of WASP or WIP. In vertebrates, The SH2 domain of Nck can bind with high affinity to proteins with specific phosphotyrosine-containing peptide motif (pYDXV, Jones *et al*., 2006; pYEEL, Anderson *et al.,* 1990; mayer *et al*., 1991). Through this interaction the SH2-SH3 adaptor protein is activated and the SH3 domains can bind to SH3-binding motif in the proline-rich region (PxxP) of other proteins, e.g WASP, WIP and SCAR. In this study we show that the SH2-SH3 adaptor protein *Drosophila* Dreadlocks (Dock) is required for myoblast fusion.

6.2.1 Dock/Nck required in signalling cell adhesion

In vertebrates SH2 domain of Nck (Homolog of Dock in *Drosophila*), is able to bind to phosphorylated Nephrin, an ortholog of Sns and thus induces actin polymerization (Jones *et al*., 2006; Verma *et al*., 2006; Garg et al., 2007). Beside Sns, *Drosophila* has another Nephrin homolog – Hibris (Hbs; Dworak *et al*., 2001), which also possesses tyrosine phosphorylation sites. Hbs has the same conserved domains as Nephrin or Sns and is required for myoblast fusion. Hbs can acts in functional redundancy to Sns (Menon *et al*., 2005). Therefore, it is possible that Dock could bind to the intracellular domain of Hbs during myoblast fusion. In vertebrates Nck is not only able to interact with Nephrin, but also with phosphorylated Neph1 (Garg *et al*., 2007). *Drosophila* FCs express two Neph1 orthologs, Duf and Rst. Sequence analysis of Duf and Rst intracellular domain possesses tyorosine residues (Strunkelnberg *et al*., 2001) which can be phosphorylated and could be bound by Dock. Using immunohistochemical studies it was shown that Dock is expressed in both myoblast types i.e founder cell (FCs) and fusion competent cells (FCMs) Schäfer *et al* (in preparation) and is localised at the plasma membrane of myoblasts (FIG 2.8.B, Chapter 2.8.1). Here I discussed the role of Dock in

signalling myoblast fusion by transferring the fusion signal cell adhesion molecules to the actin cytoskeleton regulators WIP and WASP.

I analyzed the role of Dock in FCs Duf, Rst and FCMs Sns, Hbs cell adhesion molecules by yeast two-hybrid assay and split YFP assay. I first did yeast two-hybrid assay with Dock full length and the intracellular domain of Duf (FIG 5.2.1). This result were further confirmed by SH3 and SH2 deleted domains of Dock with Duf. Interestingly Duf.intracellular domain binds to the SH3 domain of Dock and not by SH2 domain unlike in vertebrate Neph1 (Garg *et al.,* 2007). Since, Nck binds to phosphorylated Neph1 (Garg *et al*., 2007), is ortholog to *Drosophila* Duf. This suggest that SH3 domain of Dock can bind to proline-rich region of Duf.

I have further investigated the interaction between Rst.intracellular domain with Dock-fl and found no interaction using yeast two-hybrid system (FIG 5.2.1 C). It indicates that it may be due to secondary modifications or no interaction. In yeast secondary modifications does not occur. This results further confirmed by split YFP assay in *Drosophila* SL2 cells. Interestingly Dock and Rst.intracellular domain interact in SL2 cells (FIG 5.2.2 B, D). This indicates that the interaction between the Dock and Rst may be due to secondary modification such as phosphorylation, which do not occur in yeast. This results supports that in anology to vertebrates *Drosophila* homologue of Nck is able to bind to the Neph1 orthologue, e.g Rst. (and Duf; Garg *et al*., 2007).

Taken together, these results support that the *Drosophila* Dock/Nck could be the potential interaction partners for Duf and Rst, in FCs which are redundant each other. Hence, further analysis are required to clear the role of Dock in FCs and will discussed in following chapter (6.2.3).

I further investigated the role of Dock interaction with the nephrin homolog e.g. Sns and Hbs by using yeast two-hybrid assay and split YFP assay. Dock does not interacts with Hbs by yeast two-hybrid assay (FIG 5.2.1 G). It indicates that it may be due to secondary modifications or no interaction. In yeast secondary modifications does not occur. This result further confirmed by split YFP assay in *Drosophila* SL2 cells, interestingly Dock and Hbs.intracellular domain interacts in SL2 cells (FIG 5.2.2 B, G). This indicates that the

interaction between the Dock and Hbs may be due to secondary modifications such as phosphorylation, which do not occur in yeast. This result supports the interaction of Nck to the Nephrin in vertebrates (Jones *et al*., 2006; Verma et al., 2006) is homolog to *Drosophila* Hbs. I also tested the interaction of Dock with Sns by using yeast two-hybrid assay and split YFP assay. Interestingly Dock interacts with Sns in both assays, but in yeast two-hybrid assay interaction is very weak. This may be due secondary modification. This results supports that in anology to vertebrates *Drosophila* homologue of Nck is able to bind to the nephrin homolog e.g Sns and Hbs(Jones *et al*., 2006; Verma et al., 2006).

Taken together, these result supports the *Drosophila* Dock/Nck binds to the intracellular domain of Hbs and Sns. Hbs can acts in functional redundancy to Sns (Shelton *et al.,* 2009). Further analysis are required to clear the role of Dock in FCMs and will discussed in the following chapter.

6.2.2 Dock required in FCMs during myoblast fusion

In this study I found that the SH2-SH3 adaptor protein Dreadlocks (Dock) is required in FCMs during myoblast fusion. Dock is expressed in both myoblast types fusion competent cells (FCMs) and also in founder cell (FCs) Schäfer *et al* (in preparation) and is localised to the plasma membrane of the myoblast (FIG 2.8.B, Chapter 2.8.1). Dock interacts genetically with the transmembrane protein Hbs, the *Drosophila* homolog of Human Nephrin. This results was confirmed by Co-immunoprecipitations (FIG 5.3.1; FIG 5.3.2). And also the SH2 domain of Dock interacts with transmembrane protein Hbs by split assay (FIG 5.3.2 D). Interestingly, it was shown in vertebrates that the SH2 domain of Nck is able to bind to phosphorylated Nephrin and thus induces actin polymerization (Jones *et al*., 2006; Verma *et al*., 2006; Garg et al., 2007; Blastig *et al*., 2008). Until now four members of the nephrin protein family are identified on mammals i.e Nephrin, Neph1, Neph1, Neph2 and Neph3. Nephrin expressing cells can form Nephrin form clusters via interacting with its extracellular domain (Koshnoodi *et al*., 2003). Neph1 and Nephrin were shown to mediate a signal to the cytoskeleton after multiple

tyrosine phosphorylation (Lie *et al*., 2004; Verma *et al.,* 2003). The loss of Nephrin or Neph1 functions results in an abnormal podocyte formation. Members of the Src kinase protein family can phosphorylate multiple tyrosine (Y) residues in the intracellular domain of Nephrin and thus is required for the binding of Nck. The Y^{1191}, Y^{1208} and Y^{1232} in the pYDXV motif of Nephrin are essential for the interaction with the SH2 domain of SH2-SH3 adaptor protein Nck (Jones *et al*., 2006; Verma *et al*., 2006). Sequence analyses were not able to identify a pYDXV motif in *Drosophila hbs*, but a $YDEQ^{1089}$ motif. The split YFP assay with a mutated *hbs* constructs $YDEQ^{1089}$ to $FDQV^{1089}$ reveled that $YDEQ^{1089}$ site of Hbs are involved in the binding of Dock.

Beside Hbs, *Drosophila* has another Nephrin homolog Sns can bind the Dock (Jones *et al*., 2006). Sns also possesses tyrosine phosphorylation sites. Sns has the same conserved domains as Nephrin and is required for myoblast fusion. Sns can acts in functional redundancy to Hbs (Menon *et al*., 2005). Interestngly, I found that SH3 domain of Dock binds to the Sns not by SH2 domain by split YFP assay. Further Split YFP assay with mutated *sns* constructs which lacks all pY sites and deletion of PXXP motif revealed that the 14 Tyr and the PxxP sites of Sns are not involved in the binding of Dock. But, it depends on SH3 domain of Dock (FIG 5.3.3 B). This is unique to the *Drosophila*, but in *Drosophila* whether this interaction is really required during myoblast fusion, garland cell development or eye development was not assessed.

Taken together, this results supports that *Drosophila* Dock/Nck binds to the intracellular domain of FCMs Hbs and Sns. Hbs can acts in functional redundancy to Sns (Menon *et al.,* 2005). In vertebtates the adaptor protein Nck can bind to the Nck-binding site in WIP (Anton *et al*., 1998). Furthermore, in a yeast two-hybrid assay Dock/Nck interacts with WIP (Giot *et al.,* 2003). Cell culture experiments on *Drosophila* S2 cells and vertebrate cells could also show that Dock/Nck can bind to WASP (Rivero-Lezcano *et al*., 1995; Rohatgi et al., 2001; Worby *et al*., 2002). Therefore, Dock could be the potential linker between the Nephrin homolog Sns and/or Hbs and the WASP dependent actin polymerization and will discuused in the following chapter (chapter 6.3).

6.2.3 Dock required in FCs During myoblast fusion

In this study I found that the SH2-SH3 adaptor protein Dreadlocks (Dock) is required in FCs during myoblast fusion. Dock is expressed in both myoblast types founder cell (FCs) and also in fusion competent cells (FCMs) Schäfer *et al* (in preparation) and is localised to the plasma membrane of the myoblast (FIG 2.8.B, Chapter 2.8.1). *Drosophila* FCs express two Neph1 orthologs, Duf and Rst (Garg *et al*., 2007). Sequence analysis of Duf and Rst contains tyrosines (Strunkelnberg *et al*., 2001), which can become phosphorylated and could be bound by Dock. To test these possibilities I conducted genetic experiments with Dock and Duf. In *Drosophila* intracellular domain of Duf was shown to interact by a yeast two-hybrid test with the TPR-repeats of Rols (Kreisköther *et al*., 2006). Recent studies showed that Duf intracellular domain between amino acid 687 and 830 of duf plays an important role in translocation of Rols and loner in S2 cells (Bulchand *et al*., 2010). Multidomain protein Rols Stabilizes Duf during cell adhesion. I did genetic studies on FCs *rols* with *dock*. Since, *duf* single mutants is not available. *dock* genetically interacts with FCs specific *rols* (FIG 5.4.1). This mean that Dock interacts genetically with Duf during myoblast fusion. Further the interaction of Dock with Duf was confirmed by Co-immunoprecipitation (FIG 5.4.2). I further analyzed the domain analysis of Dock and Duf by split YFP assay. Interestingly, SH3 domain of Dock binds to the Duf intracellular domain. Since, SH3 domain of Dock binds to the proline-rich region and sequence analyses were able to identify a short proline-rich motif i.e Pxxp in the intracellular domain of Duf. Split YFP assay with deleted *duf* constructs which lacks proline rich motif (PxxP) revealed that the PxxP motif of Duf are involved in the binding of Dock SH3 domain (FIG 5.4.2 D). Taken together Dock binds to Duf in FCs during myoblast fusion. *Drosophila* FCs express another Neph1 ortholog, Rst (Garg et al., 2007). Rst possesses tyrosine phosophorylation sites. Rst and Duf are redundant (Strunkelnberg *et al*., 2001). I found that the SH3 and SH2 domain of Dock interacts with Rst intracellular domain by split YFP assay. Unlike other cell adhesion molecules Rst is expressed in FCs and also in FCMs (Strunkelnberg *et al*., 2001). But, genetic interaction studies realed that *dock* does not interacts with *rst*. Hence, further analysis are required to clear the role of Dock interaction with Rst during myoblast fusion.

Taken together, this results support the *Drosophila* Dock/Nck could be the potential interaction partners of Duf and Rst in FCs, which are redundant to each other. However, cell culture experiments on S2 and vertebrate cells could also show that Dock/Nck can bind to WASP (Rivero-Lezcano *et al*., 1995; Rohatgi et al., 2001; Worby *et al*., 2002). This indicates that Dock could link the WASP based actin polymerization in growing myotube. Hence, further analysis are required to clear the role of Dock in FCs and will discussed in following chapter.

6.3 Dock activates the WASP-WIP complex during Arp2/3-based actin polymerization

After the signalling of Dock with cell adhesion molecules in FCs Duf and Rst and in FCMs Hbs and Sns. In this study I started to analyze how the WASP complex becomes activated during myoblast fusion (Schäfer G. *et al.,* 2007). The Arp2/3 complex is crucial for actin polymerization and Arp2/3 complex in turn is activated by Wiskott-Aldrich syndrome protein (WASP) family e.g neuronal (WASP and the WASP-family Verprolin-homologous protein (WAVE) (Higgs and Pollard, 1999, 2001; Stradal *et al*., 2004; Tankenawa and miki, 2001; Schäfer *et al*., 2007; Berger et al., 2008). In vertebtates the adaptor protein Nck can bind to the Nck-binding site in WIP (Anton *et al*., 1998). Furthermore, in a yeast two-hybrid assay Dock/Nck interacts with WIP (Giot *et al.,* 2003). Cell culture experiments on *Drosophila* S2 cells and vertebrate cells could also show that Dock/Nck can bind to WASP (Rivero-Lezcano *et al*., 1995; Rohatgi et al., 2001; Worby *et al*., 2002). WIP is expressed only in FCMs (Kim et al., 2007; Massarwa *et al*., 2007; Berger *et al*., 2008). However, WASP-WIP is not present in the FCs before fusion; this indicates that another actin regulator must be required. Recently SCAR could be identified to be essential for myoblast fusion (Richardson *et al.,* 2007; Berger et al., 2008). WASP-WIP and the SCAR/WAVE complex have different roles during myoblast fusion (Berger *et al*., 2008).

I first studied the role of Dock in FCMs for the activation of the WASP-WIP complex. Double mutant experiments with

enhanced muscle phenotype in *wip dock* double mutants Schäfer *et al* (in preparation). To approve theses studies I performed the biochemical experiments with Dock and WASP-WIP by a yeast two-hybrid assay. Since, all members of WASP family and WIP posses a proline-rich region which can be bound by the SH3 domain of SH2-SH3 adaptor protein (Chapter 2.8.2, FIG 2.9 and 2.10). I found that all SH3 sub-domains (Dock-ΔSH3-1, Dock-ΔSH3-2 and Dock-ΔSH3-3) of Dock are required to bind the proline-rich region of WASP (FIG 5.5.2 C). Interestingly Dock-ΔSH3-1 -DockΔSH3-3 of Dock are required to bind proline-rich region of WIP (FIG 5.5.2 B). Taken together my biochemical interactions approved that Dock activates the WASP-WIP complex with Arp2/3-based actin polymerization during *Drosophila* myoblast fusion.

Another open question to which of the pathway Dock is acting e.g WAP-WIP and SCAR/WAVE. WAS-WIP and SCAR/WAVE complex have different roles during myoblast fusion (Berger *et al*., 2008). To prove this assumption, I performed the gene doses experiments with *scar* and *dock*. *scar dock* double mutant does not enhance the muscle phenotype (FIG 5.53). This proved that Dock activates the WASP-WIP complex with Arp2/3-based actin polymerization in FCMs during *Drosophila* mypblast fusion.

Besides the Dock-WASP-WIP pathway in FCMs, the role of Dock in FCs and WIP express only in FCMs not in FCs. However, WASP-WIP complex is not present in the FCs before fusion; this indicates that another actin regulator must be required and needs to further analyzed.

6.4 Fusion model: Dock Links igSF-mediated cell adhesion with Arp2/3-based actin polymerization during *Drosophila* myoblast fusion

The data presented in this study are summarized in a model in Figure 1. The function and localization of WASP, WIP, Dock, SCAR, Arp3, Rols and cell adhesion molecules Duf, Rst, Hbs and Sns are described together with other proteins which are required for Drosophila myoblast fusion in Table 1.

Cell-cell fusion of *Drosophila* myoblasts require Duf, Rst in FCMs and Sns, Hbs and also in FCMs (FIG. 1). The formation of new branched actin filaments requires the activity of Arp2/3 complex, which becomes activated by WASP and SCAR. The Arp 2/3 complex needs to bind an existing actin filament to induce branched actin formation. It remains unknown how the existing unbranched actin filament is build during *Drosophila* myoblast fusion.

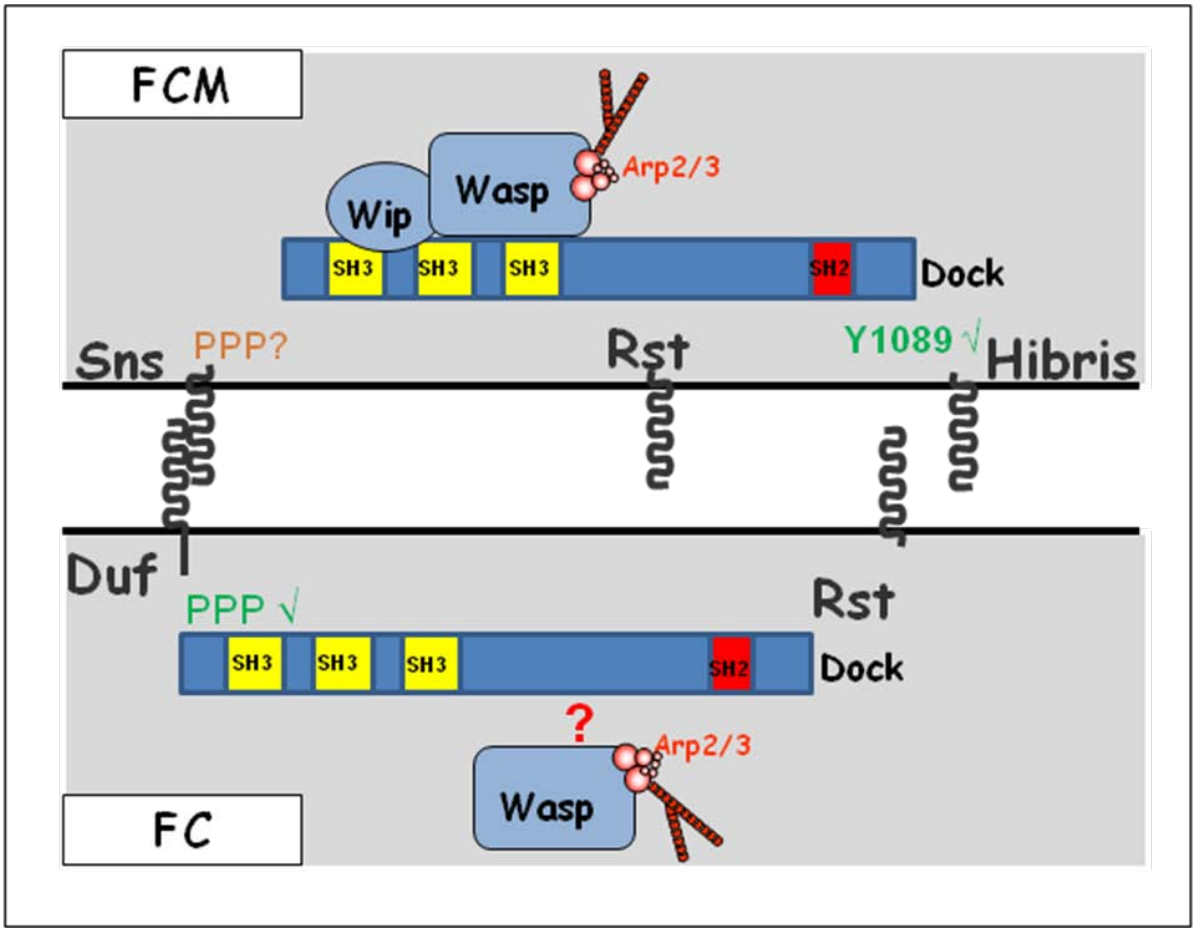


FIGURE 1. Regulation of actin polymerization during myoblast fusion. Cell adhesionis mediated by Duf and Rst in FCs and Sns and Hbs in FCMs. Dock expression FCs and FCMs (Schäfer *et al* (in preparation). **In FCMs**, SH2 domain binds to Hbs Hbs Y^{1089} and to Sns via SH3 domain. Arp2/3 nucleation promoting factors WASP and WIP binds to SH3 domain of Dock. Thus it suggest that Dock regulates the actin by WASP-WIP complex in FCMs. **In FCs**, SH3 domain of Dock binds to the Duf PXXP motif and Dock also binds to the Rst via SH2 and SH3 domain. But, in FCs WIP does not exist only WASP is present. Dock colud be activate the Arp2/3 complex through the WASP with other actin regulator. Further information of proteins can be found in Table 1.

I started to analyze the role of Dock in signaling with cell adhesion molecules of FCs and FCMs. Since Dock is expressed in FCs as well as FCMs. My genetic and biochemical studies on FCMs Hbs and Dock implies that Dock interacts with Hbs Y^{1089} via its SH2 domain (FIG .1). Dock also interacts with Sns via SH3 domain (FIG. 1). Furthermore, my biochemical studies imply that Dock interacts with WASP via all SH3 domains (FIG. 1). Dock also binds to WASP interacting protein WIP via SH3-3-1 and SH-3-3 (FIG. 1). My further genetic studies on *docks.scar* revealed that no genetic interaction between Dock and SCAR is visible. Taken together, Docks links Hbs and/or Sns with WASP-WIP based Arp2/3 actin polymerization in FCMs during *Drosophila* myoblast fusion. Furthermore, my biochemical and genetic studies in FCs Duf, Rst with Dock revealed that SH3 domain of Dock binds to Duf PxxP motif (FIG. 1). Dock also interacts with Rst via SH2 and SH3 domain by biochemically (FIG. 1). WIP is not expressed in FCs, It could suggests that Dock activates the Arp 2/3 complex along with WASP by other actin regulator.

Table 1; Proteins involved during Drosophila myoblast fusion

Protein	**Homololog**	**Function**	**Localization**	**Fusion steps**	**Reference**
Duf/Kirre	Neph1	Cell-adhesion	FCs	First and Second	Ruiz-Gomez *et al*., 2000
Rst/IrreC	Neph1	Cell-adhesion	FCs, FCMs	First and Second	Strunkelberg *et al*., 2001
Sns	Nephrin	Cell-adhesion	FCMS	First and	Bour *et al*.,

				Second	2000
Hbs	Nephrin	Cell-adhesion	FCMs	First and Second	Artero *et al*., 2001; Dowark *et al*., 2001
Dock	Nck	SH2-SH3 adoptor	FCs, FCMs	First and second	Schäfer *et al*., in preparation
WASP	N-WASP	F-actin regulation, Activates Arp2/3 complex	FCs, FCMs	Second	Massarwa *et al* 2007; Schäfer *et al*., 2007
Vrp1	WIP	F-actin regulation, WASP interacting partner	FCMs	First and Second	Kim et al., 2007; Massarwa *et al*., 2007; Berger *et al*., 2008
SCAR	WAVE	F-actin regulation, Activates Arp2/3 complex	FCs, FCMs	First and Second	Richardson *et al* 2007; Berger *et al.*, 2008
Rols	-	Multidomain protein, Stabilizes Duf	FCs	Second	Chen *et al*., 2001; Menon *et al*., 2001; Rau *et al*., 2001
Kette	Hem1	F-actin regulation	FCs, FCMs	Second	Schröter *et al*., 2004
Blow		F-actin regulation	FCM	First and Second	Doberstein *et al*.,1997; Schröter *et al*., 2006
D-Titin	Titin	Structural constituent of the actin cytoskeleton	FCs, FCMs	-	Machado *et al*., 200; Zhang *et al*., 2000
Crk	Crk-II	SH2-SH3 adaptor	FCs, FCMs	-	Erickson *et al*., 1997; Balagopalan *et*

					al., 2006
Rac1, Rac2	Rac	Rac-Gtpase, F-actin regulation	-	First and Second	Hakeda-Suzuki *et al*., 2002
Schizo	Arf	GEF	FCs, FCMs	First and Second	Chen *et al*., 2003; Richardson *et al*., 2007
Arp3	Arp3	Subunit of the Arp2/3 complex, F-actin regulation	-	First and Seond	Richardson *et al*., 2007; Berger *et al*., 2008
Mbc	Dock180	Gef	FCs, FCMs	First and Second	Rushton *et al*., 1995; Erickson *et al*., 1997

(**Modified after berger *et al*., 2008**)

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Abbrevations

aa	Amino acid
A.dest	Aqua bidestilled
APS	Ammoniumpersulfate
bp	Basepairs
dNTPs	Deoxyribonucleotide phosphates
ddNTPs	Dideoxyribonucleotide
cDNA	Complementary DNA
CIP	Alkalinephosphatase from calf intestine
DNA	Deoxyribonuclic acid
DNase	Deoxyribonuclease
DMSO	Dimethylsulfoxide
kb	Kilobase
KDa	Kilodalton
M	Molar
mRNA	Messenger RNA
ORF	OPen Reading Frame
PCR	Polymerase Chain Reaction
pI	Iso electric point
SDS	Sodium dodecyl sulfate
Rpm	Rotations per minute
w/v	Weight per volume
RNase	Ribonuclease
YFP	Yellow fluorescent protein
TritonX- 100	Octylphenolpolyethylenglycolether
Tween 20	Polyoxyethylensorbitanmonolaurate
UV	Ultraviolet ligh

FC	Founder cells
FCMs	Fusion competent cells
Et	Extracellular domain
It	Intracellular domain

Acknowledgements

My thanks go to all the people who helped me during the time of my PhD thesis. These people are.

I am deeply indebted to reverend Prof. Dr. Susanne Önel for giving me an opppurtunity to pursue this research in her laboratory, for helpful discussion and her encouragement.

Prof. Dr. Renate Renkawitz-Pohl for being second reviewer with all the efforts this implies and for her continues support.

A lot of thanks to Dr. Detlev Buttgereit and Dr. Christina Rathke for their suggestions and support.

Sabina Huhn for her technical assistance with everything that could have gone wrong in the lab.

I thanks to Ruth Hyland, Inge Simon for their aminable attitude towords me and microinjection of my transformation constructs.

I thanks Dr. Franz Grolig for helping me with the confocal pictures.

I thanks to Susan Abmayr, PhD for providing reagents.

My thanks to Verena Growth, Gritt Schäfer and Christine Dottermusch for being very friendly co-workers during this project.

I thank all my collegues in this lab for the cheerful and friendly ambience created among ourselves.

DFG and GK1216 for financing my PhD and several travels.

I thanks the GK1216 “Intra- and Intercellular Transport and Communication” for their support in this research.

Thank you also to all my friends near and especially far- for sharing your insights with me.

My thanks go out to my family on both sides who provided me with heaps of moral support.

Declaration

I declare that I myself wrote this study and carried out the experimental work described in it, without using any other sources and aids than those that are stated. Title of my thesis is

Linking IgSF-mediated cell adhesion with Arp2/3-based actin polymerization during *Drosophila* myoblast fusion.

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Schäfer, G*., **Sankara Reddy, K. B***., Önel, S-F

Dock links igSF-mediated cell adhesion with Arp2/3-based actin polymerization during *Drosophila* myoblast fusion.

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To be Submited in December 2011.