THE ROLE OF ADP RIBOSYLATION FACTOR GTPASES IN MYOBLAST FUSION IN *DROSOPHILA*

by
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A thesis submitted to Johns Hopkins University in conformity with the requirements for the degree of Master of Science

Baltimore, Maryland
April 2016

Abstract

Myoblast fusion is a crucial step in myogenesis and muscle regeneration and is highly conserved across many species. A guanine nucleotide exchange factor for the Arf GTPase, Loner, is required for myoblast fusion in *Drosophila* presumably by regulating Arf6 GTPase activity. However, other Arfs, such as Arf1, may also be involved in myoblast fusion. In this study, I performed a series of loss-of-function and gain-of-function experiments in embryos, as well as localization experiments in cultured cells and in embryos to determine the roles of Arf1, Arf6, and several other Arfs in myoblast fusion. *arf6* maternal/zygotic mutant embryos did not show fusion defects. Dominant negative and constitutively active Arf1 caused fusion defects, but not dominant negative Arf6 or constitutively active Arf6. All tested Arfs localized to the fusogenic synapse in cultured cells, while Arf6 localized to the fusogenic synapse in embryos, but not Arf1 and Arf4. Therefore, Arf1 and Arf6 appear to have redundant functions in myoblast fusion. This broad study of several Arfs lays the groundwork for future experiments to pinpoint the cellular function of Arfs in myoblast fusion.

Mentor/Principle Investigator: Elizabeth H. Chen, Ph.D.

Advisor: Robert D. Horner, Ph.D.

Acknowledgements

I would like to thank my supervisor, Dr. Elizabeth Chen, for her guidance and support in my years at the lab. She taught me how to work as a scientist, how to think as a scientist, and, most importantly, she taught me to be excited about the world around us as a scientist should. Her passion for scientific discovery has inspired me to never lose my scientific curiosity and to always share it with others.

I would also like to thank current members of the lab – Dr. Ji Hoon Kim, Dr. Khurts Shilagardi, Dr. Rui Duan, Dr. Donghoon Lee, Dr. Jun Shi, Shuo Li, Nathalie Gerassimov, Delgermaa Luvsanjav, Samantha Tienda, and Arnaldo Mercado-Perez – as well as past members of the lab. Dr. Kim, in particular, was my mentor before and throughout this project, and helped me tremendously regarding the design and implementation of experiments. He has been an excellent teacher and role model, and this project would not be possible without him. I would also like to thank Dr. Donghoon Lee, who was a great source of knowledge regarding Arf proteins as well as general advice about my project. I have truly enjoyed working with all the members in the Chen lab and I wish them all the best in their careers.

Thanks to the Johns Hopkins University Department of Biology for their support, especially Dr. Kathryn Tifft for her guidance and advice throughout the course of the program.

Thank you to my family who have always supported me no matter what. I would not be here without them. Thank you to my friends who never fail to provide me with emotional and spiritual support. I am truly grateful to be surrounded by such wonderful people.

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1. Introduction

Membrane fusion occurs in virus-cell fusion, intracellular vesicle fusion and cell-cell fusion (Chen & Olson, 2005). Compared to virus-cell fusion and intracellular vesicle fusion, cell-cell fusion remains poorly understood. Cell-cell fusion is a fundamental biological process in which mononucleated cells fuse to form multinucleated cells in development and homeostasis in multicellular organisms. Cell-cell fusion requires membrane fusion between two cells, where two different lipid bilayers merge into one.

Cell-cell fusion occurs in many organisms and many cell types, including mating in yeast, organ formation in *Caenorhabditis elegans* and myoblast fusion in *Drosophila melanogaster*. In humans and other mammals, cell-cell fusion takes place during fertilization, myogenesis, placenta formation, bone remodeling, immune responses and other processes (Chen & Olson, 2005). The similar cellular processes in different fusion events suggest that cell-cell fusion may share similar mechanisms despite the diversity of cell types. Therefore, it may be possible to understand the general mechanism of all cell-cell fusion events by studying one specific type of cell-cell fusion (Chen & Olson, 2005).

One reason to study cell-cell fusion is its therapeutic potential. The most famous example is fusion of immortal and antibody-secreting B cells to form hybridomas for the production of monoclonal antibodies (Kohler & Milstein, 1975). Cell-cell fusion could also be utilised to repair tissue damage. For example, since myoblast fusion is an essential process in muscle regeneration, enhanced fusion may increase regeneration efficiency in damaged muscles. However, many challenges remain before cell-cell fusion can be therapeutically manipulated. These challenges include the identification of cells to use for reparative fusion, introduction of cells into damaged tissues, activation of cell-cell

fusion, and maintenance of the repaired products (Sullivan & Eggan, 2006).

Myoblast fusion is a crucial step in myogenesis and muscle regeneration, which is necessary for the formation of skeletal muscles. Skeletal muscles are made up of multinucleated muscle fibers that are formed by fusion of hundreds or thousands of mononucleated myoblasts. Due to the complexity of the mammalian musculature, myoblast fusion is a highly regulated process. Muscle cells destined to fuse have to migrate, recognize, and adhere to each other at the appropriate time and place (Abmayr & Pavlath, 2012) by cell adhesion molecules (CAMs). Most of our understanding of myoblast fusion comes from *Drosophila*. The mechanisms of muscle cell fusion in mammals remain not well understood.

1.1 Myoblast fusion in Drosophila

Drosophila is an excellent model organism for genetic and cell biological studies because of its small size, ease of maintenance, short generation time, large number of progeny, and genetic amenability. More importantly, many molecules are conserved between *Drosophila* and humans, as 75% of human disease genes have fly homologs (Reiter et al., 2001). *Drosophila* also has many advantages specific to research on myoblast fusion. Firstly, the ease of visualizing the developing musculature allows easy screening of mutations that affect fusion. Secondly, *Drosophila* myogenesis is conserved in mammals. Thirdly, *Drosophila* musculature develops within 5.5 hours during embryogenesis, compared to the days to weeks in mammals (Bate, 1990). Lastly, cell-cell fusion can be induced in cultured *Drosophila* S2R+ cells by co-expressing *Drosophila* adhesion molecule Sticks and stones (Sns) and *C. elegans* fusogenic protein EFF-1 (Shilagardi et al., 2013).

Myoblast fusion occurs between two types of muscle cells: founder cells and fusion competent myoblasts (FCMs). One founder cell fuses with several FCMs to form a single myofiber. The founder cell determines the size, position, orientation, epidermal attachment, and nerve innervation patterns of the myofiber (Kim et al., 2015). The first step of fusion is cell recognition and adhesion by immunoglobin domain–containing CAMs. The CAMs expressed in the founder cell are Dumbfounded (Duf) and Roughest, which are functionally redundant. The FCM–specific CAMs are Sns and Hibris. Hibris is partially redundant with Sns (Kim et al., 2015). Interaction between the cell type–specific CAMs triggers distinct signaling events in each cell. For example, Antisocial (Ants) is recruited to sites of fusion by Duf to stabilize Duf through a positive feedback loop (Chen & Olson, 2001; Menon et al., 2005).

The second step of fusion is enhancing cell membrane proximity, which is achieved by many regulators that regulate Arp2/3 complex–mediated actin polymerization and the formation of F-actin foci in the FCM. The F-actin foci in the FCM were found to cause an inward curvature on the founder cell membrane, and electron microscopy revealed that the foci consist of a cluster of invasive finger–like protrusions (Sens et al., 2010). This protrusive structure shares many similarities with a podosome structure, which is a dynamic cell-matrix adhesion structure (Kim et al., 2015). Thus, this protrusion was named “podosome-like structure” and the interface between the two fusion partners is called a fusogenic synapse (Sens et al., 2010). Proper podosome-like structure invasion by the FCM is necessary for fusion pore formation (Sens et al., 2010; Chen, 2011).

The two major nucleation-promoting factors involved in this process belong to the

Wiskott-Aldrich syndrome protein (WASP) family – Scar (Richardson et al., 2007) and WASP (Kim et al., 2007; Massarwa et al., 2007). Scar functions in the founder cell to create a thin actin sheath at the fusion site, and also functions in the FCM with WASP to create a dense F-actin focus (Sens et al., 2010; Kim et al., 2015). The small GTPase Rac is an upstream activator of the Scar complex (Luo et al., 1994; Hakeda-Suzuki et al., 2002). Rac localization is likely regulated by the small GTPase Arf6 and Loner, the Arf6 guanine nucleotide exchange factor (GEF) (Chen et al., 2003). The signaling pathways are summarized in Figure 1.

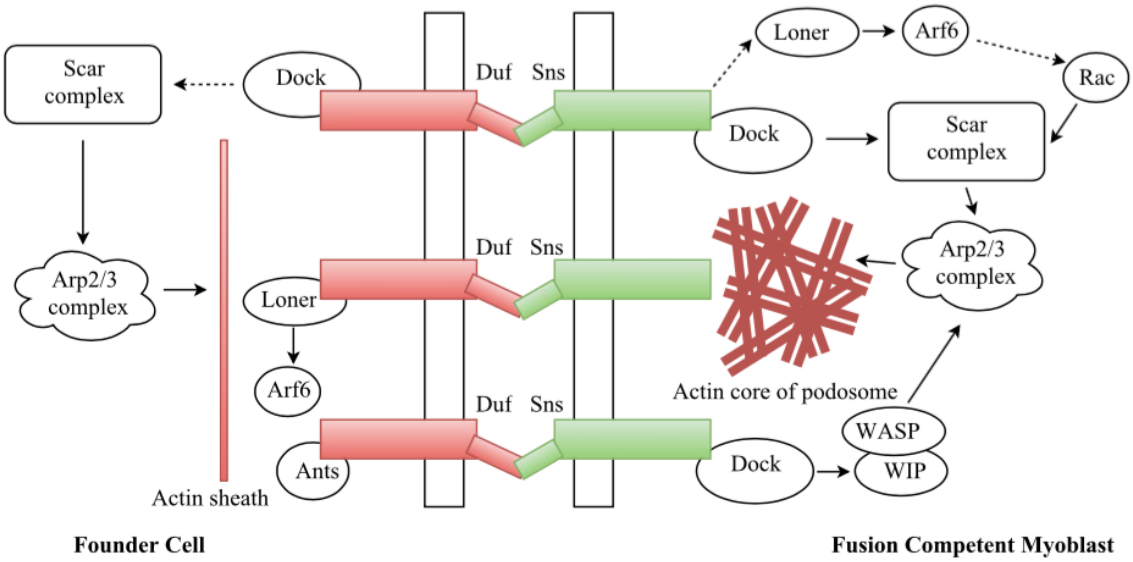

Figure 1. Signaling Pathways in *Drosophila* Myoblast Fusion
Cell-type–specific CAMs Duf and Sns interact and trigger distinct signaling events in each cell. Scar functions in the founder cell to create a thin actin sheath at the fusion site, and also functions in the FCM with WASP to create a dense F-actin focus. Dotted arrows represent signaling events that have yet to be definitively proven.

The third step of fusion is lipid bilayer destabilization. This step is not well understood in *Drosophila*, but studies in cultured mouse C2C12 myoblasts *in vitro* suggest that phosphatidylserine may be flipped to the outer leaflet of the membrane to facilitate fusion (van den Eijnde et al., 2001; Jeong and Conboy, 2011).

1.2 The Arf Family

Arf6 is a member of the ADP-ribosylation factor (Arf) family of small guanine-nucleotide-binding (G) proteins that regulate many different cellular processes, including cytoskeletal dynamics, growth, and membrane trafficking (Bos et al., 2007; Cherfils & Zeghouf, 2013). Like other G proteins, Arf GTPases cycle between GTP-bound and GDP-bound states. GEFs catalyze GDP release and GTP binding, while GTPase–activating proteins (GAPs) catalyze the hydrolysis of GTP-bound Arfs. Based on sequence homology, the six mammalian Arf proteins are divided into three classes: Class I (Arf1, Arf2, Arf3), Class II (Arf4 and Arf5) and Class III (Arf6) (Donaldson & Jackson, 2011). In *Drosophila*, each class has a single Arf ortholog: Class I (Arf1/Arf79F), Class II (Arf4/Arf102F) and Class III (Arf6/Arf51F). The Arf family also includes Arf-like (Arl) proteins, which have a wider range of roles than Arfs, and Sar1, which is functionally similar to Arf1 in coat complex recruitment in vesicle budding (Donaldson & Jackson, 2011).

In mammals, there are 15 Arf GEFs, which are divided into six families, and 31 Arf GAPs, which are divided into nine major subgroups (Donaldson & Jackson, 2011). Two Arl GAPs have been found, but no specific Arl GEFs have been identified (Donaldson & Jackson, 2011). The proper localization of Arf GEFs is crucial because they ensure the correct temporal and spatial activation of Arfs. Besides catalyzing GTP exchange and hydrolysis, Arf GEFs and GAPs also assemble protein complexes at specific sites within cells, thus increasing the versatility of signaling networks (Donaldson & Jackson, 2011). For example, Arf GAPs have multiple functional domains that can interact, independently of their GAP activity, with proteins that regulate

membrane trafficking and the actin cytoskeleton (Inoue & Randazzo, 2007).

Arfs localize to the plasma membrane as well as the membranes of the secretory, endosomal and lysosomal pathways (Donaldson & Jackson, 2011). It appears that Arf1 is released from membranes upon GTP hydrolysis, while Arf4 and Arf6 remain bound to membranes in their GDP-bound state and can bind to membrane-localized partners (Chun et al., 2008; Duijsings et al., 2009). For example, Arf6-GDP binds to the Kalirin family of Rho GEFs, which activates Rac (Koo et al., 2007). Arf6 inactivation is also important for maintaining the podosome in osteoclasts (Heckel et al., 2009). Since both Arf6-GTP and Arf6-GDP interact with proteins that regulate other small G proteins, the regulated cycle of GTP binding and hydrolysis is important for the biological function of Arf6.

1.3 Loner and the Arf Family in Myoblast Fusion

Rac localization to the fusogenic synapse is likely regulated by the small GTPase Arf6 and its GEF Loner/Schizo (Chen et al., 2003; Onel et al., 2004). Loner was discovered in a genetic screen for muscle development regulators in *Drosophila*. Loner is required for myoblast fusion and is recruited by Duf in the founder cell, although it may be present in the FCM as well (Richardson et al., 2007; Bulchand et al., 2010). The Sec7 domain of Loner possesses GEF activity towards Arf6. Arf6 likely recruits Rac to the membrane (Chen et al., 2003), activates the Scar complex (Koronakis et al., 2011), and/or regulates actin remodeling through lipid metabolism (D'Souza-Schorey & Chavrier, 2006). Myoblast fusion is impaired when dominant negative Arf6 protein ($Arf6^{T27N}$) is expressed (Chen et al., 2003). Arf6 has also been shown to regulate mammalian myoblast fusion through PLD activation and $PI(4,5)P_2$ production (Bach et al., 2010).

Although Loner and Arf6 have been shown to play a role in *Drosophila* myoblast

fusion, studies have suggested that other Arfs may interact with Loner. One study showed that myoblast fusion is not affected in *arf6* maternal/zygotic null mutants, which suggests that Arf6 may be functionally redundant with another Arf protein (Dyer et al., 2007). Another study showed that dominant negative (DN) Arf1, but not DN-Arf6, causes myoblast fusion defects that resemble *loner* mutants (Dottermusch-Heidel et al., 2012). In the same study, constitutively active (CA) Arf1, but not CA-Arf6, partially rescued *loner* myoblast fusion defect. In a yeast two-hybrid assay, DN-Arf1 ($Arf1^{T31N}$), but not DN-Arf6, CA-Arf1, or CA-Arf6, interacts with Loner (Dottermusch-Heidel et al., 2012). These studies suggest that Loner may target another GTPase in addition to Arf6. It is also possible that Arf6 and Arf1 are functionally redundant. Furthermore, it is unknown whether Arf1 and Arf6 function in the founder cell, in the FCM, or both. In addition, the role of other Arfs, such as Arf4, Arl1 and Arl2, in myoblast fusion is unknown. The fusion phenotypes of the aforementioned Arf genotypes are listed in Table 1.

The goal of this study was to investigate the function of several Arf GTPases in myoblast fusion in *Drosophila*, especially Arf1 and Arf6. My hypothesis was that Arf1 and Arf6 are functionally redundant in *Drosophila* myoblast fusion, since both may be downstream of Loner and necessary for myoblast fusion. To pinpoint which Arfs are involved in myoblast fusion, both loss-of-function and gain-of-function experiments were performed in embryos, and localization experiments were performed in cultured cells and in embryos.

Arf Genotype	Fusion Phenotype	Reference
Maternal/zygotic *arf6* mutant	wt	Dyer et al., 2007
DN-Arf6 ($Arf6^{T27N}$)	Fusion defect	Chen et al., 2003
DN-Arf6 ($Arf6^{T27N}$)	wt	Dotterbusch-Heidel et al., 2012
DN-Arf1 ($Arf1^{T31N}$)	Fusion defect	Dotterbusch-Heidel et al., 2012
CA-Arf1	Rescued *loner* mutant	Dotterbusch-Heidel et al., 2012
CA-Arf6	Did not rescue *loner* mutant	Dotterbusch-Heidel et al., 2012

Table 1. Summary of known fusion phenotypes of different Arf genotypes
Wildtype phenotype is indicated by "wt". DN-Arf6 has been shown to impair myoblast fusion (Chen et al., 2003), but a later study showed that it does not impair myoblast fusion (Dotterbusch-Heidel et al., 2012).

2. Results

2.1 Maternal/zygotic arf6 Mutant Embryos Did Not Show Fusion Defects

To test my hypothesis that Arf1 and Arf6 are functionally redundant, I observed the fusion phenotype of *arf6*;*arf1* zygotic double mutant embryos. No fusion defects were observed in *arf6*;*arf1* zygotic mutants (data not shown). Since both Arf1 and Arf6 have strong maternal contribution (Fisher et al., 2012), there may still be Arf1 and Arf6 activity in the *arf6*;*arf1* zygotic mutants. To test whether a more complete knockdown of Arf6 would produce a fusion defect, I generated *arf6/arf6;arf1*/+ embryos without maternal contribution of *arf6*. These embryos did not show fusion defects (Fig. 2).

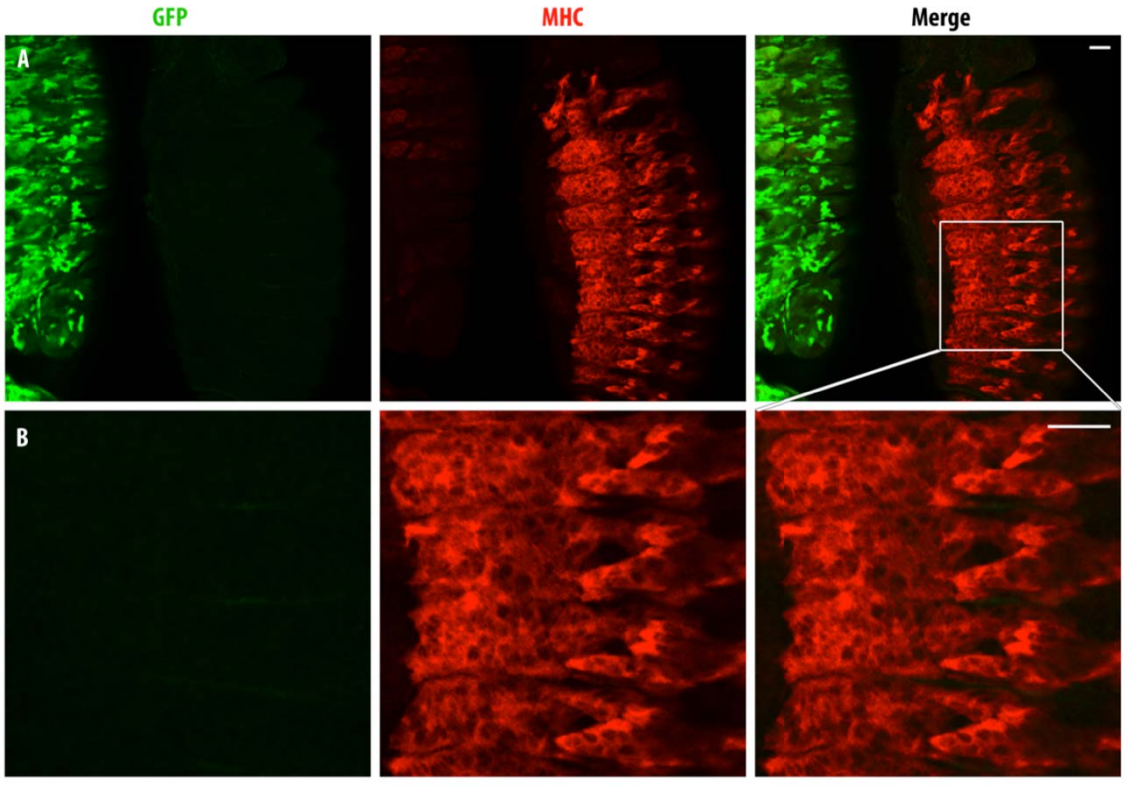

Figure 2. *arf6* maternal/zygotic mutant embryos did not show fusion defects
arf6/arf6;arf1/+ embryos without maternal contribution of *arf6* were labeled with α-GFP (green) and α-MHC (red). *arf6* maternal/zygotic mutants were identified by their lack of GFP expression (see methods for details). Embryos were magnified (B), as indicated by the white box (A). Scale bars, 20μm.

2.2 Dominant Negative Arf1 Impairs Myoblast Fusion in Drosophila Embryos, but Not Dominant Negative Arf6

To study whether Arf1 and Arf6 play a role in myoblast fusion, I expressed dominant negative (DN) forms of Arf1 and Arf6 in all muscles in embryos using a muscle–specific *GAL4* driver. Many unfused myoblasts were observed when DN-Arf1 was expressed (Fig. 3A). No unfused cells were observed when DN-Arf6 was expressed in the muscles (Fig. 3B). Therefore, DN-Arf1 impairs myoblast fusion, while DN-Arf6 does not.

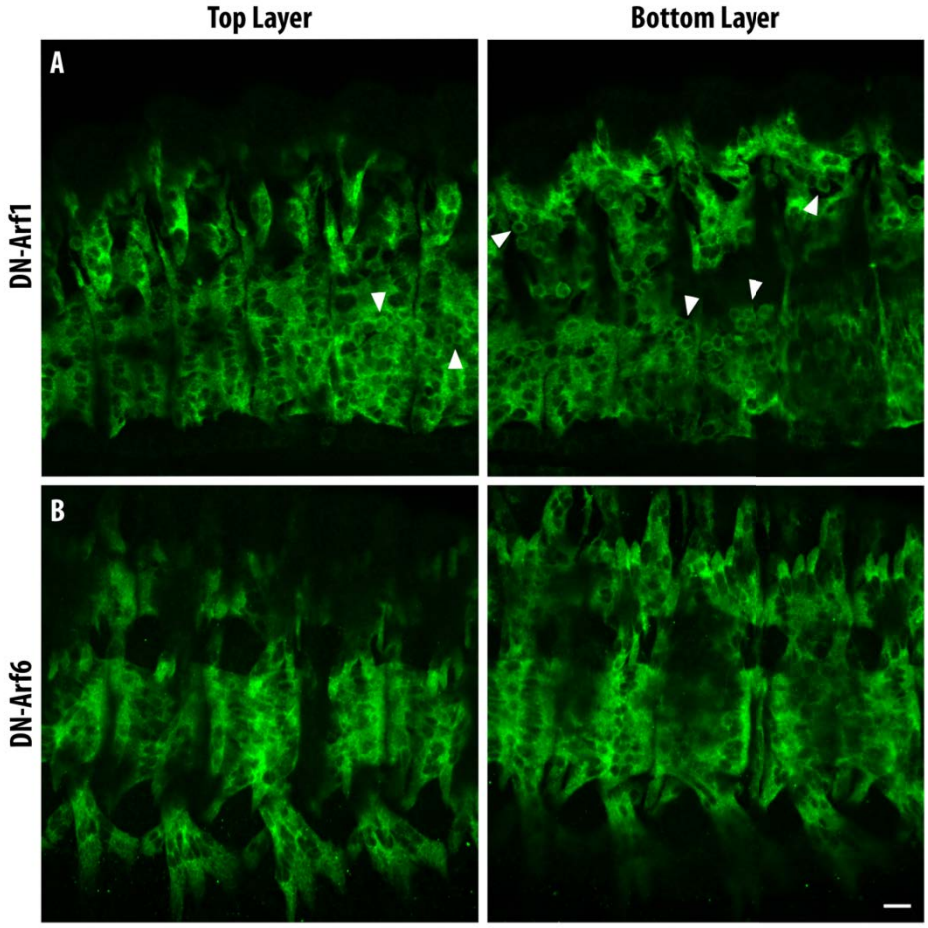

Figure 3. Expression of DN-Arf1 and DN-Arf6 in embryos
DN-Arf1 and DN-Arf6 were expressed in the muscles using a *twist (twi)-GAL4* driver. Muscles were labeled with α-myosin heavy chain (MHC) (green). The top layer and bottom layer of the musculature are shown. Arrows indicate unfused cells. Scale bar, 10μm.

2.3 Constitutively Active Arf1 Impairs Myoblast Fusion in Drosophila Embryos More Severely than Constitutively Active Arf6

Gain-of-function experiments can provide further insight to the role of Arf1 and Arf6 in myoblast fusion. I thus expressed constitutively active (CA) Arf1 and CA-Arf6 in all muscles in *Drosophila* embryos. No developed musculature can be observed in embryos expressing CA-Arf1 (Fig. 4A).

Defects in myoblast fusion cause an increase in the number of mononucleate myoblasts present in muscles because the myoblasts are unable to fuse. However, many "holes" can be observed in the embryos when CA-Arf1 is expressed (Fig. 4A), which reflect a loss of muscle cells. Since Arf1 is required in secretory pathways (Donaldson &

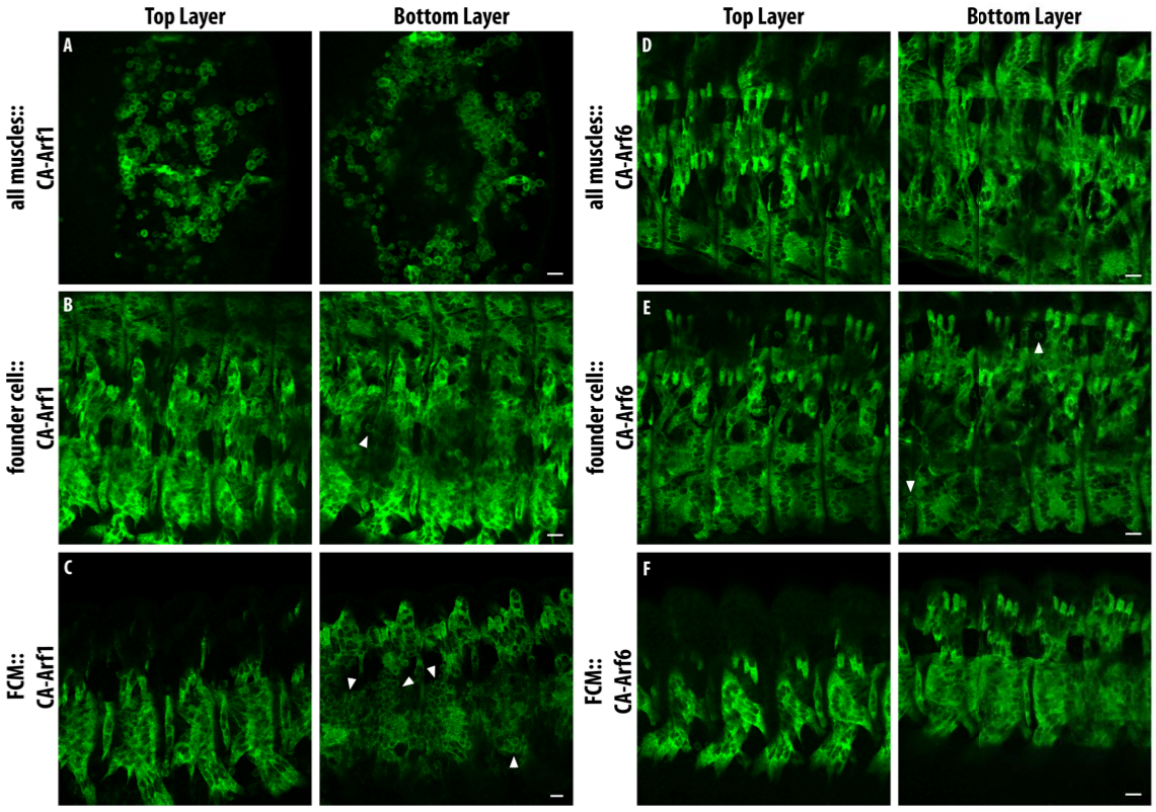

Figure 4. Expression of CA-Arf1 and CA-Arf6 in embryos
CA-Arf1 and CA-Arf6 were expressed in muscles using muscle–specific (*twi-GAL4*) (A, D), founder cell–specific (*rp298-GAL4*) (B, E), and FCM–specific (*sns-Gal4*) (C, F) *GAL4* drivers. Muscles were labeled with α-MHC (green). Arrows indicate unfused cells. The top layer and bottom layer of the musculature are shown. Scale bars, 10μm.

Jackson, 2011), expressing CA-Arf1 in the mesoderm may have caused defects in other cellular processes such as cell differentiation. Therefore, CA-Arf1 and CA-Arf6 were expressed in the founder cell or FCM exclusively to reduce the effects of CA-Arf1 on processes other than myoblast fusion. CA-Arf1 caused fusion defects when expressed in the FCM (Fig. 4C) and marginal defects when expressed in the founder cell (Fig. 4B). CA-Arf6 did not cause fusion defects when expressed in all muscles (Fig. 4D), caused marginal defects when expressed in the founder cell (Fig. 4E) and did not cause fusion defects when expressed in the FCM (Fig. 4F). The fusion phenotypes of embryos expressing DN-Arfs and CA-Arfs are summarized in Table 2.

Arf expressed		All muscles	Founder Cell	FCM
Arf1	DN (T48N)	–	ND	ND
	CA (Q71L)	– –*	wt	–
Arf6	DN (T44N)	wt	ND	ND
	CA (Q67L)	wt	wt	wt

Table 2. Summary of fusion phenotypes of embryos expressing DN-Arfs and CA-Arfs

DN-Arfs and CA-Arfs were expressed in all muscles, the founder cell, and FCM specifically using *twi-GAL4*, *rp298-GAL4*, and *sns-GAL4* respectively. Minus signs (-) indicate the severity of the fusion defects, wt indicates wildtype phenotype or marginal fusion defects, and ND (not determined) indicate experiments that were not performed. The asterisk (*) indicates that expression of CA-Arf1 likely affected processes other than myoblast fusion (Fig. 4A).

2.4 Several Arfs are Enriched at the Fusogenic Synapse in Drosophila S2R+ Cells

Proper localization of fusion regulators is important because the correct timing and positioning of the fusogenic synapse is needed for proper musculature formation.

Thus, I investigated the subcellular localization of Arf proteins in *Drosophila* S2R+ cells that have been induced to fuse. Although S2R+ cells are not muscle cells, the induced fusion between S2R+ cells is mediated by a similar podosome-like stucture as in *Drosophila* embryos. I expressed GFP-Arf1 (Fig. 5A), GFP-Arf4 (Fig. 5B), GFP-Arf6 (Fig. 5C), GFP-Arl1 (Fig. 5D) and GFP-Arl2 (Fig. 5E) constructs in S2R+ cells, then used an α-GFP antibody to detect the localization of the Arfs. To visualize the fusogenic synapse, F-actin was labeled by phalloidin and V5-tagged Sns was labeled with an α-V5 antibody. All tested GFP-Arfs co-localized with the F-actin focus and Sns. Therefore, GFP-Arf1, GFP-Arf4, GFP-Arf6, GFP-Arl1 and GFP-Arl2 are enriched at the fusogenic synapse in cultured cells.

2.5 Arf6 is Enriched at the Fusogenic Synapse in Drosophila Embryos

Since *in vitro* experimental results are not always reflective of the *in vivo* system, I then investigated the localization of Arf1-GFP and Arf6-GFP, as well as Arf4-GFP in embryos. UAS-Arf-GFP transgenes were expressed in all muscles using muscle–specific *GAL4* drivers. F-actin was labeled with phalloidin, and Duf or Ants were labeled with antibodies to visualize the fusogenic synapse. Arf6-GFP was enriched at the fusogenic synapse (Figures 6E-F), while Arf1-GFP (Fig. 6A-B) and Arf4-GFP (Fig. 6C-D) were not. There was some localization of Arf1 and Arf4 around the fusogenic synapse, as indicated by the “fuzzy” area around the fusogenic synapse (Fig. 6B, 6D).

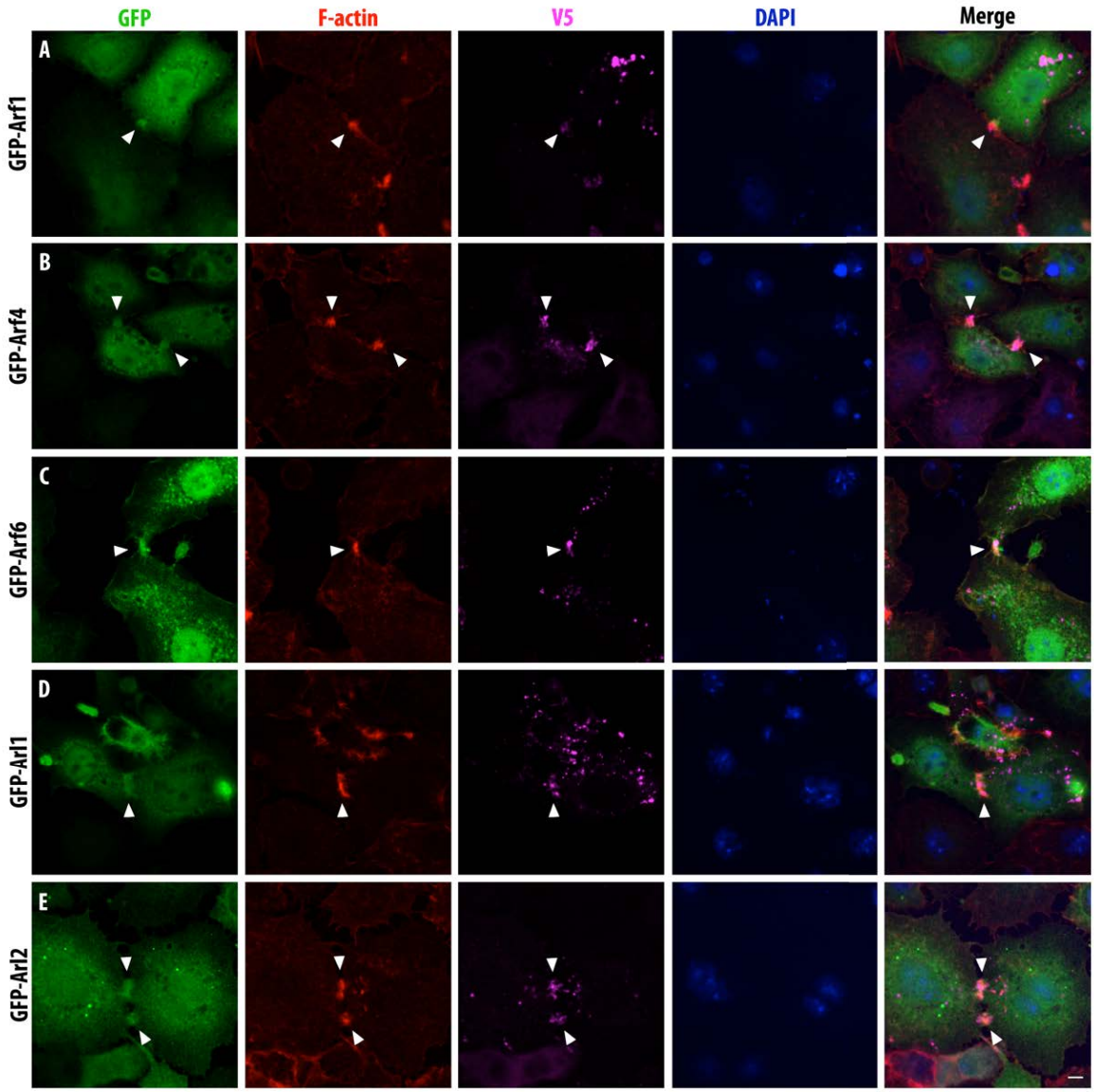

Figure 5. Enrichment of GFP-Arfs at the fusogenic synapse in *Drosophila* S2R+ cells

S2R+ cells were transfected with Sns-V5, Eff1-HA to induce fusion, as well as appropriate GFP-Arf constructs. Cells were labeled with DAPI (blue), α-GFP (green), phalloidin (red) and α-V5 (pink). Arrows indicate the fusogenic synapse. Scale bar, 5μm.

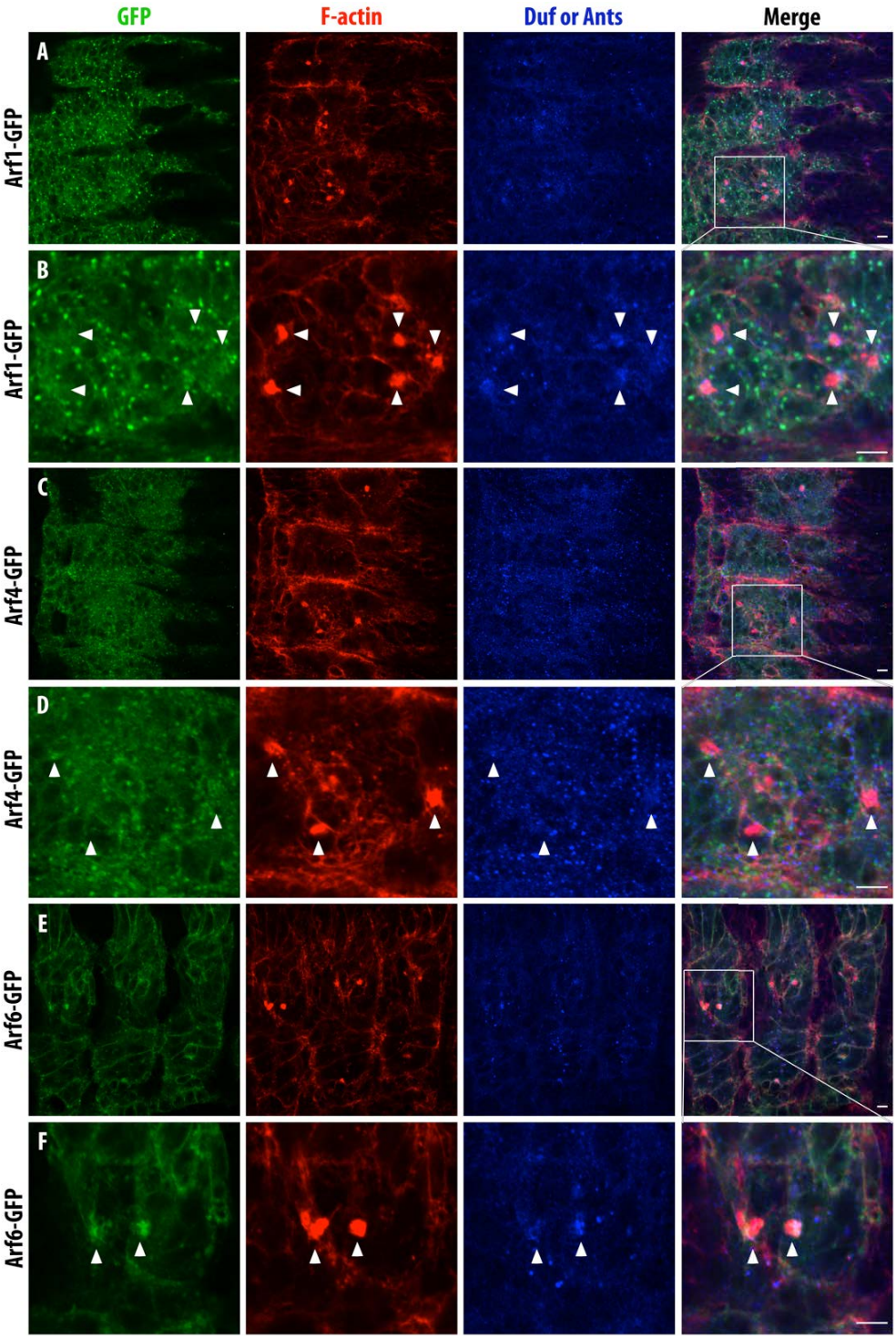

Figure 6. Enrichment of Arf-GFP at fusogenic synapses in embryos
Arf1-GFP and Arf6-GFP were expressed in the muscles using a *dmef2-GAL4* driver (A, B, E, F), while Arf4-GFP was expressed using a *twi-GAL4* driver (C, D). Embryos were labeled with α-GFP (green), phalloidin (red) and α-Duf (A, B, E, F) or α-Ants (blue) (C, D) to visualize the fusogenic synapses. Fusogenic synapses were magnified (B, D, F), as indicated by the white boxes (A, C, E). Arrows indicate the fusogenic synapse. Scale bars, 5μm.

3. Discussion

I showed that Arf1 and Arf6 are the most likely candidates to have a function in myoblast fusion. First, *arf6* maternal/zygotic mutant embryos did not show fusion defects. These embryos were derived from $arf6^{KO}/arf6^{1}$ mothers and were also heterozygous *arf1* mutants (Fig. 2). My results are consistent with the study by Dyer et al., who showed that embryos derived from $arf6^{1}/arf6^{1}$ mothers did not show fusion defects (Dyer et al., 2007). Since Arf6 is strongly enriched at the fusogenic synapse in the embryo (Fig. 6E-F) and likely has a function in myoblast fusion, the results from the *arf6* mutant experiment suggest that another protein compensated for Arf6 function. Given results from the dominant negative experiments (Fig. 3) and data from other labs (Dottermusch-Heidel et al., 2012), Arf1 likely compensated for Arf6 function.

To further study which Arfs are involved in myoblast fusion, I tested loss-of-function and gain-of-function Arfs for fusion defects. I first sought to replicate previous experiments by expressing DN-Arf6 and DN-Arf1 in *Drosophila* embryos. Expressing DN-Arf6 in embryos did not cause fusion defects, while expressing DN-Arf1 impaired myoblast fusion (Fig. 3). A study by Dottermusch-Heidel et al. also showed that DN-Arf1, but not DN-Arf6, causes myoblast fusion defects that resemble *loner* mutants (Dottermusch-Heidel et al., 2012). However, a previous study in my lab showed that DN-Arf6 expressed in the founder cell does cause fusion defects (Chen et al., 2003), which was not the case in this study. One thing to note is that the DN-forms of Arf1 and Arf6 used by Chen et al. and Dottermusch-Heidel et al. were "nucleotide-free" Arf1 and Arf6 ($Arf1^{T31N}$ and $Arf6^{T27N}$), whereas "GDP-locked" Arf1 and Arf6 were used in this study ($Arf1^{T48N}$ and $Arf6^{T44N}$), which may explain the variation in fusion phenotypes observed.

Another explanation for the difference in fusion phenotypes is the variation in the expression level of DN-Arf6, since it is unclear how many copies of the GAL4 driver and the UAS transgene were used in previous studies. If insufficient DN-Arf6 was expressed, $Arf6^{T27N}$ and $Arf6^{T44N}$ may both be unable to completely inhibit endogenous Arf6 and perhaps other highly homologous Arfs, such as Arf1. Increasing the copies of the *GAL4* driver and the *UAS* transgene, as well as increasing the experimental temperature may lead to more consistent fusion defects. Furthermore, the founder-cell specific *rp298-GAL4* driver (which is on the X chromosome) used by Chen et al. causes fusion defects in embryos hemizygous for the *rp298-GAL4* driver (Sens et al., 2010). Although male flies carrying the *rp298-GAL4* driver were used to drive the expression of $Arf6^{T27N}$ to reduce fusion defects from the *rp298-GAL4* driver, genetic interactions cannot be ruled out. The *twi-GAL4* driver used by Dottermusch-Heidel et al. and in this study does not have such genetic interactions. To conclude, my study shows that one copy of UAS-DN-Arf1, but not UAS-DN-Arf6, causes fusion defects.

CA-Arf6 did not cause fusion defects (Fig. 4D-F), while expressing CA-Arf1 in the FCM caused fusion defects (Fig. 4C). Expressing constitutively active Arf1 in all muscles likely caused defects in many cellular processes other than fusion (Fig. 4A), because Arf1 is an essential protein that acts as a regulator in secretory pathways (D'Souza-Schorey & Chavrier, 2006), while Arf6 is not an essential protein (Dyer et al., 2007). GTP hydrolysis of Arf1 is required for the dissociation of COPI from transport vesicles (D'Souza-Schorey & Chavrier, 2006). Defects in myoblast fusion result in many unfused cells. However, a decrease in the number of myoblasts was observed in embryos expressing CA-Arf1. Expressing CA-Arf1 in the FCM (Fig. 4C), but not in the founder

cell (Fig. 4B), caused fusion defects. It is therefore possible that Arf1 localizes to the fusogenic synapse and affects myoblast fusion exclusively in the FCM.

However, conclusions cannot be made solely based on gain-of-function experiments. Firstly, the effects of CA-Arfs, or DN-Arfs, may not be specific to myoblast fusion. Secondly, constitutively active Arfs may not accurately reflect the function of the wildtype Arfs. While Dottermusch-Heidel et al. showed that CA-Arf1, but not CA-Arf6, rescued *loner* mutants (Dottermusch-Heidel et al. 2012), this per se cannot prove that wildtype Arf1, not Arf6, is downstream of Loner. Although CA-Arf1 can compensate for the lack of *loner* signaling, this experiment does not show the role of wildtype Arf1 in myoblast fusion. While gain-of-function experiments can provide hints as to which Arf is involved in myoblast fusion, conclusions should not be made solely based on these experiments.

Furthermore, I expressed GFP-tagged Arfs in *Drosophila* cells due to the experimental efficiency of the S2R+ cell line. Arf1, Arf4, Arf6, Arl1 and Arl2 all localized at the fusogenic synapse (Fig. 5), which implies that all tested Arfs may have a function in myoblast fusion. I also showed that Arf6 was highly enriched at the fusogenic synapse in embryos, while Arf1 and Arf4 showed limited enrichment around the fusogenic synapse (Fig. 6). This suggests strongly that Arf6 has a role in fusion but does not rule out roles for Arf1 and Arf4. However, *in vitro* experimental results are not always reflective of the *in vivo* system. One explanation for the localization of all tested Arfs *in vitro* is that GFP tagging may have affected the localization of Arfs. Using other tags or generating antibodies for Arfs may circumvent this issue. Another possible explanation is that upstream regulators in cultured cells, such as Loner, are not as active

compared to upstream regulators in the embryo, causing the localization of multiple Arfs to compensate for the Arf primarily involved in myoblast fusion. Furthermore, while it is possible that Loner recruited all tested Arfs in S2R+ cells, other recruiters of Arfs may be present at the fusogenic synapse in S2R+ cells.

Although all tested Arfs localized at the fusogenic synapse in S2R+ cells, it is unlikely that all of them are functionally redundant with each other. It is possible that some Arfs, especially Arl1 and Arl2, perform a supporting role in myoblast fusion. Studying the localization of Arfs in different *arf* mutants should provide more hints as to which Arfs are more important in fusion.

It is still unclear why only Arf6 is highly enriched at the fusogenic synapse in the embryo, but only DN-Arf1 and CA-Arf1 show fusion defects. One key experiment to test my hypothesis that Arf1 and Arf6 are functionally redundant is to express both DN-Arf1 and DN-Arf6 in the embryo and observe whether there is a synthetic phenotype. An alternative and more conclusive experiment would be to generate *arf1 arf6* double mutants without maternal contribution of either Arf. One caveat of this experiment is that Arf1 is an essential protein in the *Drosophila* embryo. Thus, *in vitro* experiments using S2R+ cells should be informative, since RNAi can be used to inhibit expression of different Arfs. By using different combinations of dsRNA and observing the fusion phenotypes, we can elucidate the relationship between Arf1 and Arf6, as well as between other Arfs.

Furthermore, my findings suggest that Arf4, which is not well characterized, may also have a redundant role with other Arfs in myoblast fusion. Generating *arf4* knockout mutants and embryos expressing dominant negative Arf4 should help elucidate this role.

Using CRISPR to generate *arf1 arf4 arf6* triple mutant flies would reveal whether these three Arfs are functionally redundant. Another interesting experiment would be to investigate the interactions between Loner and Arfs, as it is currently unknown whether Loner interacts with Arfs other than Arf6.

This broad study of multiple Arfs lays the foundation for future experiments to determine the cellular function of Arfs in myoblast fusion in *Drosophila*. Understanding the complex signaling pathways in myoblast fusion is a crucial step towards tapping into the therapeutic potential of cell-cell fusion.

4. Materials and Methods

Molecular Biology

Full-length cDNAs of Arf1, Arf4, Arf6, Arl1 and Arl2 were amplified by PCR using primers listed in Table 3 from *Drosophila* embryonic cDNA and tagged with GFP at the N-terminus using the Gateway cloning system (Invitrogen). All constructs were verified by sequencing analysis.

Arf cDNA	Primer Pairs	
Arf1	Forward	TACCGAGCATCCGAAA
	Reverse	GTTAGTTGTATTCATATTGTTGCTTT
Arf4	Forward	GCTTTTTCCTATACGAAATAAAATAG
	Reverse	AGAACAGCAATTCCTATCTT
Arf6	Forward	GGCTAAGGTTTAATGGATG
	Reverse	TACATGCCATATACATACATATAAG
Arl1	Forward	GGGAGCGCCAGCACTAGA
	Reverse	CACAGGGATCTGGGGGTCTGA
Arl2	Forward	CATAGCGTTACTAAAATACA
	Reverse	GGAAGTAAAATAAATTGTATTTAA

Table 3. Primers used to amplify full-length Arfs cDNAs
Full-length cDNAs of Arf1, Arf4, Arf6, Arl1 and Arl2 were amplified by PCR from *Drosophila* embryonic cDNA using the listed primers.

Cell culture and Transfection

Drosophila S2R+ cells were cultured in Schneider's medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and penicillin/streptomycin (Sigma). Cells were transfected using Effectene (Qiagen) according to the manufacturer's

protocol.

Reconstitution of Cell-Cell Fusion in Cultured Cells

S2R+ cell fusion was induced as previously described (Shilagardi et al., 2013). To summarize, S2R+ cells were transfected with Sns-V5, Eff1-HA to induce fusion, as well as appropriate GFP-Arf constructs. After 48 hours, cells were fixed and labeled as follows.

Immunocytochemistry

Two days after transfection in PBS, cells were fixed with 4% formaldehyde and washed in PBST (PBS with 0.1% Triton X-100) and PBSBT (PBST with 0.2% BSA) consecutively, and stained with the following primary antibodies in PBSBT: mouse α-V5 (1:2000; Invitrogen) and chicken α-GFP (1:2000). Secondary FITC-, Cy5-, or Cy3-conjugated antibodies (Jackson Immunoresearch) were used at 1:500 in PBST. DAPI stain (1:1000) and Alexa 568-conjugated phalloidin (1:500; Invitrogen) were added with the secondary antibodies in PBST.

Fly Genetics

Fly stocks were obtained from the Bloomington Stock Center (Bloomington, IN), except for the following: *UAS-Arf1::GFP*; *UAS-Arf4::GFP*; *UAS-Arf6::GFP; UAS-Arf6 [T44N]*; *UAS-Arf6 [Q67L]*; *UAS-Arf1 [Q71L]*; *UAS-Arf1 [T48N]*; $arf1^{182\text{-}1}$*/TM3*; $arf6^{KO}$*/CyO,twi-Gal4,UAS-GFP* , $arf1^{182\text{-}1}$*/TM3,twi-GAL4,UAS-GFP* (unpublished, courtesy of Dr. Donghoon Lee, generated at Dr. Tony Harris' lab at the University of Toronto); $arf6^{1}$*/CyO* (Dyer et al., 2007); $arf6^{KO}$*/CyO* (Huang et al., 2009).

To express genes in all muscle cells, founder cells, and FCMs, females carrying transgenes downstream of an *UAS* promoter were crossed with *twi-GAL4* or *dmef2-*

GAL4, *rP298-GAL4* (Menon & Chia, 2001) and *sns-GAL4* (Kocherlakota et al., 2008) males respectively.

arf6 zygotic mutant embryos without maternal contribution were produced by first crossing $arf6^{KO}$ females with $arf6^{1}$ males. $arf6^{KO}/arf6^{1}$ females were then crossed with $arf6^{KO}$/*CyO,twi-Gal4,UAS-GFP;*$arf1^{182\text{-}1}$/*TM3,twi-GAL4,UAS-GFP* males to produce $arf6^{KO}/arf6^{KO}$;$arf1^{182\text{-}1}$/+ embryos. Mutant embryos were identified by the lack of GFP expression.

All crosses were done on standard fly food at 25°C.

Immunohistochemistry

Stage 15-17 embryos were fixed and stained as previously described (Kim et al., 2007; Sens et al., 2010). The following antibodies were added in PBSBT: α-muscle myosin heavy chain (1:1000), α-GFP (1:500; Invitrogen), α-Ants (1:1000) (Chen and Olson, 2001) and α-Duf (1:500) (Sens et al., 2010). The following secondary antibodies were added at 1:200: Alexa488- (Invitrogen), Cy3-, and Cy5-conjugated (Jackson). Vectashield Mounting Medium (Vector Laboratories) was used to mount the embryos.

To stain F-actin, embryos were fixed for 50-60 min at room temperature in formaldehyde-saturated heptane (1:1 mix of 37% formaldehyde/heptane and shaken well), then devitellinized by hand in PBST. Alexa568-conjugated phalloidin (Invitrogen) was then added at 1:200.

Fluorescent images were taken on an LSM 700 Meta confocal microscope (Zeiss) and acquired with LSM software (Zeiss) and processed using Adobe Photoshop CS5.1 (Adobe).

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6. Curriculum Vitae

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Master of Science in Molecular & Cellular Biology

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Coursework
Emerging Strategies in Biomedical Research; Advanced Cell Biology; Molecular Biology; Developmental Biology; Evolution; Reproductive Physiology; Cell Biology; Cell Biology Lab; Biochemistry; Biochemistry Lab; Genetics; Genetics Lab; Organic Chemistry I, II; Introduction to Organic Chemistry Lab; General Physics I, II; General Physics Lab I, II; Calculus I, II

Work Experience
Research Assistant at The Johns Hopkins University School of Medicine
September 2013-Present
- Conduct research under Dr. Elizabeth Chen at the Department of Molecular Biology & Genetics
- Conduct independent research on the role of Arf6 GTPase in myoblast fusion in *Drosophila melanogaster*

Teaching Assistant at The Johns Hopkins University
August 2015-Present
- Independently lead a General Biology Lab section of more than 20 students
- Explain biological concepts and demonstrate laboratory skills
- Evaluate homework and tests to ensure students understand course concepts
- Assist professors with General Biology lectures

Program Assistant at The Johns Hopkins University Center for Talented Youth
July-August 2014, 2015

- Program assistant of The Physics of Engineering course at The University of Hong Kong and The Independent Schools Foundation Academy sites
- Taught 5th-6th grade gifted youth basic physics and engineering concepts
- Led engineering activities and projects

Volunteer Teacher at Vibrant Inspirations Children's Education Fund Limited
June-August 2013, August 2014

- In 2014, designed and taught an English class tailored for 5 underprivileged Hong Kong children in grades 2-3
- In 2013, designed and taught a leadership training class and a science class 25 hours per week tailored for underprivileged Hong Kong teenagers, mostly immigrants from Mainland China
- Raised students' self-esteem and exposed students to science through interesting experiments and activities

Teaching Assistant at Chinese International School in Hong Kong
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- Substituted for a Year 1 English teaching assistant
- Assisted teachers in teaching Year 1 students English, mathematics, and science
- Explained basic mathematical and scientific concepts, and helped teachers prepare for lessons

Research Assistant at The University of Hong Kong
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- Conducted research under Dr. Irene Ng at the Department of Pathology
- Conducted research on miR-210 and the relationship between cancer and hypoxia
- Performed experiments independently, including molecular cloning, immunoblotting, quantitative-PCR and luciferase reporter assay

Research Assistant at The Chinese University of Hong Kong
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- Conducted research under Dr. Eric Wong at the Department of Clinical Oncology
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- Learnt basic laboratory techniques, including Western blotting and cell culture techniques