Zebrafish Melanocytes as a Model System to Investigate Intracellular Transport Regulation

by

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CERTIFICATE OF APPROVAL

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ABSTRACT

Regulated active transport of physiological cargos by molecular motor proteins plays many essential roles in eukaryotic cells, yet the cellular mechanisms that regulate motor proteins to transport cargos to the right place at the right time are still largely unknown. Motor-dependent trafficking is particularly important in asymmetric elongated cells such as neurons. However, investigating motor protein regulation in neurons is difficult because of the number of specialized cargos combined with the lack of knowledge as to what intracellular signaling events are involved in regulating cargo transport. Motor protein regulation can more easily be studied in pigment cells, or melanocytes. Melanocytes contain conspicuous pigment granules, or melanosomes, that are transported using similar cellular mechanisms as cargos in other types of cells, including neurons. Furthermore, many of the signaling pathways involved in regulating melanosome transport have been characterized in *Xenopus* and teleost fish.

Teleost fish melanocytes are a valuable model system for investigating the molecular mechanisms of organelle transport because they aggregate or disperse their melanosomes rapidly and coordinately in response to changes in intracellular levels of cAMP. Dispersion of melanosomes to the cell periphery occurs when intracellular cAMP levels are elevated and protein kinase A (PKA) is activated. Conversely, aggregation of melanosomes to the cell center occurs when cAMP levels are lowered and a phosphatase is activated. The downstream targets of PKA and the phosphatase are still unknown. I am interested in identifying these targets because they are likely regulators of molecular motor proteins.

This dissertation describes using zebrafish (Danio rerio) melanocytes as a model system to investigate the mechanisms of intracellular cargo transport. I designed a forward genetic screening assay, applied it to zebrafish pigmentation mutants, and identified several mutants with melanosome transport defects. One of these mutants, *mlphd*^{j120}, does not completely disperse its melanosomes because of a defect in regulating microtubule-based transport downstream of cAMP signaling. The gene product of *mlphd*^{j120} was identified as melanophilin. Mouse and human orthologs of zebrafish melanophilin have been shown to regulate actin-based melanosome transport, but a role in regulating microtubule-based transport has not been previously described. Therefore, our analysis of *mlphd*^{j120} reveals a new function for melanophilin and sheds light onto how microtubule- and actin- based transport may be coordinated.

Two additional melanosome transport mutants, *smutz* and *b867*, were also identified by the screen. Preliminary analysis of *smutz* mutant melanocytes indicates that regulation of melanosome transfer to and from actin-based transport may be disrupted. The *b867* mutants appear to have a complete loss of actin-based transport – *b867* mutant melanocytes hyper-disperse their melanosomes, which is the same phenotype we observe when actin-based transport is disrupted in wild-type melanocytes. Taken together, these results indicate that the gene products of *b867* and *smutz* may be regulators of actin-based transport.

Thus, the major conclusions of this dissertation are that zebrafish melanocytes are a useful genetic model of organelle transport regulation and that zebrafish melanophilin is a key regulator of microtubule- and actin-based transport.

CHAPTER 1

Introduction

The intracellular transport of cargos along microtubules and actin filaments is fundamental to virtually all aspects of physiology and embryonic development. Examples of such transport include the establishment of cell polarity , intracellular localization of molecules that specify cell fate (Riechmann, 2001 #244), the mechanics of mitosis (Moore and Wordeman 2004), and protein targeting in all cells (Schnapp 2003). While the properties and function of individual motor proteins have been characterized (Mallik and Gross 2004), the cellular mechanisms that regulate motor proteins to transport cargo at the right time and place are still largely unknown. In particular, cargo transport in neurons is enigmatic: the complexity of their cytoskeleton, the diversity of their cargo, and the lack of knowledge of the cellular signaling events that are involved in specific cargo transport have made it difficult to investigate their mechanisms of cargo transport regulation. In contrast, cargo transport regulation can be studied more easily in pigment cells, or melanocytes. Melanocytes contain easily observed pigment granules, called melanosomes, that are transported using similar cellular mechanisms as other cells - including neurons. Furthermore, many of the signaling pathways involved in regulating melanosome transport have been already been characterized in *Xenopus* and teleost fish.

This dissertation describes using zebrafish (Danio rerio) melanocytes as a model system to investigate the regulation of intracellular cargo transport. Following this brief overview, the remainder of Chapter 1 gives background on the scientific problem of molecular motor regulation, describes how studies in the melanocytes of teleost fish,

Xenopus and mammals have shed light on many aspects of organelle transport, and proposes that the question of molecular motor regulation can be well addressed using zebrafish melanocytes as a model system.

Chapter 2 reports the forward genetic screening assay I developed and applied to a subset of previously identified zebrafish pigment mutants to identify mutants with melanosome transport defects. It describes how I characterized melanosome transport in wild-type zebrafish to develop the assay and how I identified several mutants with defective melanosome transport.

Chapter 3 is an in-depth study of one of the mutants identified by the screen. This mutant, *mlpha*^{j120}, slowly and incompletely disperses its pigment because of a defect in regulating microtubule-based transport. The gene product of *mlpha*^{j120}, melanophilin, had been identified as a regulator of melanosome transfer to as well as an activator of actin-based transport in mouse and human melanocytes (Wu, Rao et al. 2002), but a role for microtubule-based transport had not been previously reported. Thus, our analysis of *mlpha*^{j120} sheds light onto how microtubule- and actin-based transport may be coordinated.

Chapter 4 characterizes two additional mutants identified by our screen: *smutz*, and *b867*. *smutz* mutant larvae are unable to completely aggregate their melanosomes, which suggested that they may improperly retain melanosomes in the actin cytoskeleton during aggregation. However, preliminary analysis of *smutz* melanocytes in cell culture showed variable phenotypes during both aggregation and dispersion. Thus, the defective component in *smutz* melanosome transport is still unclear. In contrast, homozygous *b867* mutant larvae and isolated melanocytes share the same conspicuous phenotype: they

hyper-disperse their melanosomes. This is the same phenotype we observe when actin-based transport is disrupted and indicates that the gene product of *b867* is a specific regulator of actin-based transport in zebrafish melanocytes.

Chapter 5 discusses the advantages of zebrafish as a model system to investigate organelle transport, the significance of identifying and cloning zebrafish melanophilin, and future directions. Our major conclusions are that zebrafish melanocytes are a useful genetic model of cytoplasmic motor regulation and that zebrafish melanophilin (Mlpha) is a key regulator in coordinating the actions of both microtubule- and actin-based motors.

The scientific problem of molecular motor regulation

Cargo transport in cells is mediated by three families of molecular motor proteins: kinesin, dynein, and myosin. Motor proteins transport cargos directionally along cytoskeletal tracks: myosin generates short-distance transport along randomly organized cortical actin filaments and the motor proteins kinesin and dynein produce long-distance transport along polarized microtubules with structurally distinct plus and minus ends (Wade and Hyman 1997; Vale 2003). Microtubule-based motors move exclusively in one direction, with most kinesins moving toward the plus-end of microtubules and dynein moving toward the minus-end of microtubules (Goldstein 2001).

Many of the principles regarding how molecular motors organize traffic in the cell have been established. It is understood that processive motors, e.g. Kinesin 1, Kinesin 2 and Myosin V, transduce ATP hydrolysis into a directed walk along their cytoskeletal track, wherein two mechanochemical heads walk arm-over-arm (Mallik and Gross 2004).

Genetic and molecular studies have revealed many of the cellular functions of particular kinesin, myosin, and dynein motors; these functions include intraflagellar transport (Cole 2005), the assembly and function of the mitotic spindle (Mountain and Compton 2000), chromosome dynamics during cell division (Garrett and Kapoor 2003), organelle positioning (Pilling, Horiuchi et al. 2006), and the delivery of specific cargos within cells (Schnapp 2003). Finally, the nature of the linkage between motors and their cargos has been shown to involve specific interactions between motifs or binding domains within the tails of particular motors and various molecules that require localization within the cell (Verhey, Lizotte et al. 1998; Verhey, Meyer et al. 2001; Hoogenraad, Milstein et al. 2005).

Despite all that is currently known about how molecular motors function, remarkably little is known about how microtubule- and actin-based motors are regulated to transport specific cargos to the correct place at the appropriate time. Arguably, the next important question standing before the motor field is the question of how motors are regulated to accomplish this. Two features of motor regulation that are relevant to my dissertation are how bidirectional cargo movement along microtubules is regulated and how cargo is transferred between microtubule- and actin-based motors.

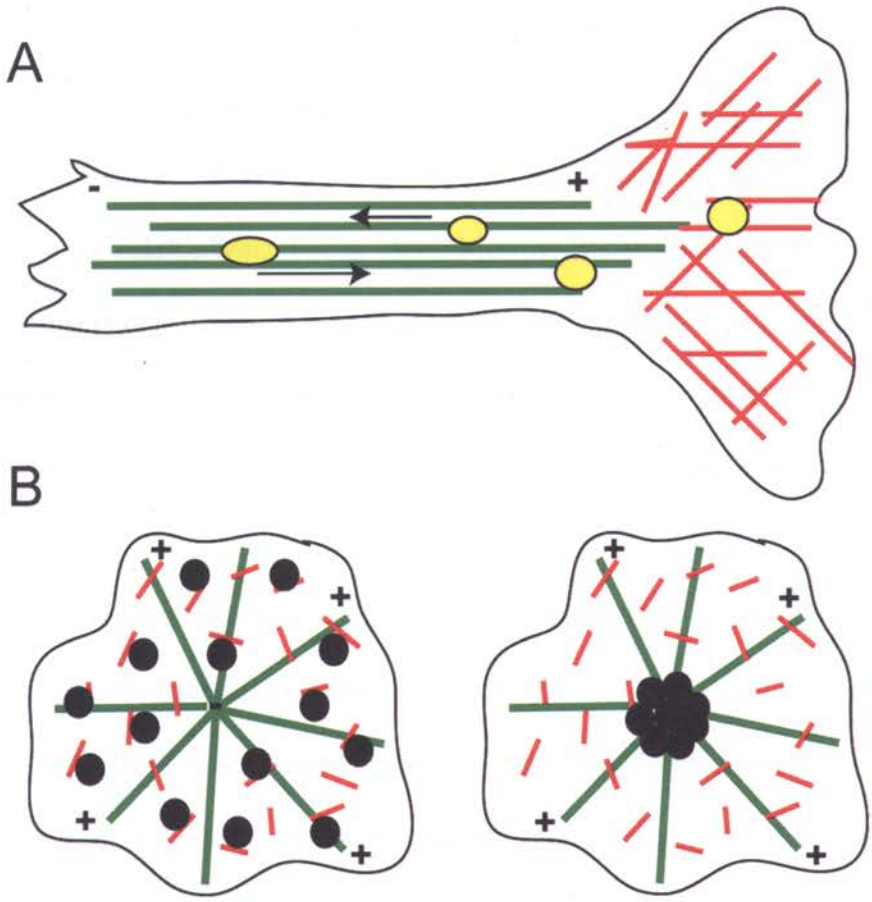

Figure 1.1: Examples of regulated microtubule and actin-based cargo transport in neurons and melanocytes

Green lines represent microtubules (orientation indicated by + and -), and red lines represent actin filaments.

A. Vesicle transport in the axon of a neuron. A variety of different cargos (see text) employ both kinesin and dynein motors to travel long distances on polarized microtubules, then are transferred to myosin motors to travel short distances along randomly oriented actin filaments.

B. Melanosome transport in fish and amphibian melanocytes. In response to external stimuli, melanosomes either evenly distribute through the cell (left side: dispersion) or accumulate at the cell center (right side: aggregation). A balance of forces between plus and minus-end directed microtubule-based motors as well as regulated transfer to and from actin-based transport are required for melanosomes to disperse and aggregate.

Regulated bidirectional transport along microtubules

A large number of cargos, including mitochondria (Morris and Hollenbeck 1993; Pilling, Horiuchi et al. 2006), secretory vesicles (Wacker, Kaether et al. 1997), endosomes (Valetti, Wetzel et al. 1999; Murray, Bananis et al. 2000) and melansomes (Wu, Bowers et al. 1998; Gross, Tuma et al. 2002), employ both kinesin and dynein motors to move bidirectionally along microtubules (Welte 2004). Although bidirectionally moving cargos employ opposite polarity motors, they still achieve polarized distributions in cells. This is accomplished by biasing microtubule-based movement; e.g. more plus-end-directed or less minus-end-directed movement to produce net plus-end displacement (Welte, Gross et al. 1998; Gross, Tuma et al. 2002; Hollenbeck and Saxton 2005). Since these cargos utilize motors of opposite polarities in rapid succession, it is unlikely that simply the attachment and release of opposite polarity motors regulates the direction of their transport (Gross 2004). To bias travel in one direction, bidirectionally moving cargos must either strengthen movement in that one direction or weaken movement in the opposing direction. This could conceivably be accomplished by regulating three parameters: the persistence of travel in one direction (that is, length of time the motors transport cargo unidirectionally before stopping or reversing), the velocity of plus- or minus-end directed motors, or the frequency of travel events in one direction versus the other. Ways in which these parameters could be regulated include changing the number of motors attached to cargo, the number of active motors on the microtubules, or the enzymatic activities of the motors. Thus, by analyzing these parameters of cargo movement, one can elucidate the relative activity of opposing

motors during bidirectional transport and thereby gain insight into bidirectional cargo transport regulation.

An emerging field that has developed in recent years directly examines bidirectional transport regulation by analyzing the movement of individual cargos in vivo. Several seminal studies have addressed how bidirectional microtubule-based transport is regulated by examining the saltatory microtubule-based movement of lipid storage granules in *Drosophila* embryos (Welte, Gross et al. 1998; Gross, Welte et al. 2000; Gross, Welte et al. 2002; Gross, Guo et al. 2003) and the bidirectional movement of melanosomes in fish and *Xenopus* melanocytes (Gross, Tuma et al. 2002; Rodionov, Yi et al. 2003) . These studies directly relate to work reported in Chapter 3 of this dissertation because they established a paradigm of how global transport, meaning bulk changes in cargo distribution, can be understood by analyzing the behavior of individual cargo movement in terms of the three previously described parameters.

The landmark study that demonstrated it is possible to understand global behavior from analyzing the movement of individual cargos examined lipid storage organelle transport in *Drosophila* embryos (Welte, Gross et al. 1998). These lipid droplets are transported along polarized microtubules whose minus-ends are oriented toward the periphery of the embryo. A key advantage of this system is that the lipid droplet population shifts dramatically during different phases of embryogenesis: a period of balanced microtubule-based motion with no net displacement over the population (phase I) is followed by net plus-end directed transport leading to basal droplet accumulation (phase II), then net minus-end directed transport leading to apical droplet accumulation (phase III). To address the question of how the movement of individual

lipid droplets accounts for bulk changes in droplet distribution between phases, high-resolution movies of lipid droplet movement in embryos at all three phases were made and their bidirectional movement tracked over time. The resulting tracks were divided into a series of segments representing continuous movement toward the plus- or minus-ends of microtubules. Three statistics were extracted from each segment: the persistence time (average amount of time spent traveling exclusively in one direction), the average distance traveled during a segment of continuous motion, and the average velocity. These three statistics revealed that net travel toward the minus end of microtubules during phase III is achieved by reducing the persistence of plus-end-directed motors while the other parameters remain unchanged (Welte, Gross et al. 1998). Moreover, the statistics derived from tracking single particles led to an estimate of bulk displacement rates for each phase that accurately predicted the bulk displacement of lipid droplets that were experimentally observed (Welte, Gross et al. 1998). Therefore, this study established the paradigm of analyzing the transport of individual cargos to reveal how motors are regulated to achieve directed bulk displacement of cargos.

In this same study, further insight into the mechanisms of how microtubule motors are regulated came from characterizing the *Drosophilia* mutant *klar* (Welte, Gross et al. 1998). The *klar* mutants appear the same as wild-type during phase II, but during phase III, their lipid droplets do not apically accumulate (Welte, Gross et al. 1998). One would predict that normal basal accumulation during phase II and disrupted apical accumulation during phase III could be due to impaired minus-end-directed or improved plus-end-directed droplet transport. However, analysis of tracks of lipid droplet movement in *klar* revealed that both plus- and minus-end-directed movement are

impaired during phase II and III; the average distance and velocity toward the plus- and minus-end is half of wild-type in phase II and the average distance and velocity toward the minus-end is half of wild-type in phase III. The overall consequence is that *klar* mutants can still achieve net plus-end displacement during phase II because plus- and minus-end directed transport are equally impaired, but cannot accomplish net minus-end movement in phase III because minus-end transport alone is disrupted. Thus, both plus- and minus-end directed transport regulation is disrupted in *klar* mutants. However, these data do not clarify which aspect of motor regulation is disrupted - for example, whether the enzymatic activity of the motors has been disrupted or if there are fewer active motors powering transport.

To distinguish between these possibilities, the forces powering lipid organelle transport were quantified using a laser-based optical trap. An optical trap uses radiation pressure from a focused laser to trap small particles (Block, Goldstein et al. 1990). At a given laser power, a fraction of the small, motor-driven particles (in this case lipid droplets) are stopped by the trap, while the other fraction escape. The fraction of droplets that escape the trap are driven by motors exerting more force than the laser. To measure the forces of lipid droplet transport, this study scored the percentage of droplets escaping the optical trap as a function of the power of the laser, resulting in stall force measurements (Welte, Gross et al. 1998). Stall force measurements provide information about the number of active motors on cargos because, if the load is distributed equally amongst motors, the stall force of two motors should be twice that of one motor (Welte, Gross et al. 1998). In wild-type embryos, the mean stall forces varied markedly at each phase by multiples of approximately 1.1pN (Phase I, 3.3pN, phase II, 5.5pN, phase III,

4.7pN). These data suggest that lipid droplet movement is driven in each phase by 3, 5 and 4 active motors, respectively. In contrast, examination of *klar* mutants revealed that they have a mean stall force (1.2pN) that does not change with developmental phase. Since the unitary force measurement of approximately 1.1pN represents the activity of a single motor, this indicates that *klar* mutant lipid droplets employ only one active motor rather than multiple motors. Thus, the Klar protein appears to coordinate same-direction motors in wild-type embryos during lipid droplet transport. Morevover, plus- and minus-end forces are reduced by the same magnitude in *klar* mutants, which suggests that Klar, in addition to assembling multiple same-polarity motors, also coordinates plus-end with minus-end assemblies of motors. Thus, this study raises the possibility that plus- and minus-end directed motors are coordinated. However, it did not test this conclusively because the interactions between opposite polarity motors were not directly examined, meaning the authors did not disrupt one polarity motor and examine the effect on the other.

To directly address whether opposite polarity motors are coordinated, a subsequent study genetically impaired transport in the minus-end direction and examined whether transport in the plus-end direction was improved or disrupted (Gross, Welte et al. 2002). One would predict that if the activities of motors were not coordinated – for example, if the motors were active simultaneously and engaging in tug-of-war – impairing the function of one motor would put the opposing motor in a better competitive position and improve its function. But if the activities of the motors are coordinated, impairing the function of one motor would either have no effect on the opposing motor or, if the motor coordination itself were affected, have a negative effect on its function.

Since cytoplasmic dynein had been previously shown to power minus-end-directed lipid organelle travel in *Drosophila* (Gross, Welte et al. 2000), this study tested whether opposing motors were coordinated in lipid organelle transport by impairing, but not completely disrupting, minus-end motion by introducing mutations in either the dynein heavy chain (*Dhc*) or a subunit of dynactin (*Gl*), a multimeric cofactor of dynein. Analysis of the movement of individual droplets showed that, while the minus-end mean travel distance was predictably reduced, the mean travel distance toward plus-end was also reduced in *Dhc* and *Gl* mutants compared to wild-type. In addition, optical trap measurements revealed that, even though stall forces of minus-end directed travel were not affected, stall forces of plus-end travel were lower in one *Dhc* mutant allele and the *Gl* mutant. Thus, mutations in dynein heavy chain and dynactin that impair minus-end motion also significantly impair plus-end motion. This supports the model that opposing microtubule-based motors are coordinated and not in a tug-of-war.

Regulated switching between microtubule- and actin-based transport

This brings us to the second aspect of cargo transport regulation, which involves the switching of cargo between microtubule- and actin-based transport systems. That single cargo vesicles can move on both actin and microtubules was first observed in giant squid axoplasm (Kuznetsov, Langford et al. 1992). Later studies indicate that the trafficking of many cargos, including ER-derived vesicles (Tabb, Molyneaux et al. 1998), secretory vesicles (Hirschberg, Miller et al. 1998), mitochondria (Hollenbeck and Saxton 2005), and melanosomes (Rogers and Gelfand 1998; Marks and Seabra 2001) involve transport along both microtubules and actin filaments (Wu, Rao et al. 2002). However, in

these studies, the underlying mechanisms that regulate microtubule- and actin-based motors and how the two transport systems interact to achieve proper cargo transport were not clearly defined.

To investigate how microtubule- and actin-based motors are regulated to cooperatively transport cargos, several other studies also used the strategy of analyzing single cargo movement to uncover mechanisms that regulate bulk cargo displacement, but in the context of a system that employs both microtubule- and actin-based transport. The melanocytes of amphibians and fish, which will be discussed in detail in the next section of this introduction, contain melanosomes that synchronously aggregate to the cell center or disperse throughout the cytoplasm in response to changes in intracellular levels of cAMP. This is in order for the whole animal to rapidly change its color in response to environmental cues (Fujii 2000). During dispersion, melanosomes move bidirectionally along microtubules toward the cell periphery, then are transferred from microtubules to cortical actin, which homogenously distributes the melanosomes (Tuma and Gelfand 1999). Conversely, during aggregation, melanosomes are simultaneously released from cortical actin and transported along microtubules to the cell center. The ability to induce the coordinated actions of both microtubule- and actin- motor proteins provides a powerful tool for investigating intracellular mechanisms that regulate this aspect of cargo transport.

One study determined the parameters of cargo movement that are regulated during microtubule- and actin-based transport as well as examined how the two systems influence one another during dispersion or aggregation of melanosomes. The authors combined tracking the movement of individual melanosomes in *Xenopus* melanocytes

with dominant-negative manipulations of Kinsein 2 and Myosin V (Gross, Tuma et al. 2002); molecular motors previously shown to be involved in melanosome transport (Rogers and Gelfand 1998; Tuma, Zill et al. 1998). Tracks of microtubule-based motion were analyzed in the same way as previous studies examining lipid droplet transport in *Drosophila*, and from these tracks the authors extracted the mean travel distance and mean velocity of travel toward opposing ends of microtubules. In melanocytes where Kinesin 2 activity had been blocked with a dominant-negative construct, the mean travel distance toward the minus-end of microtubules increased during dispersion compared to control melanocytes, but the velocity remained unchanged. Because the velocity of minus-end transport was not affected, dynein motor function was not improved, which supported the model that opposing microtubule-based motors are coordinated rather than engaged in a tug-of-war. However, blocking Myosin V activity, while it had no effect on aggregation, increased the mean distance traveled in the minus-end direction during dispersion. It also dramatically increased the velocity of both plus- and minus-end directed transport. This increase in velocity of microtubule-based transport when Myosin V is blocked during dispersion reveals that microtubule-based motor activity improves in the absence of actin-based motor activity, and it suggests that actin- and microtubule-based transport are not coordinated, but rather are indeed engaged in a tug-of-war.

To further address the differences between the regulation of microtubule- and actin-based transport during aggregation and dispersion, the mean distance traveled and mean velocity of plus- and minus-end transport during aggregation and dispersion were compared in cells expressing a dominant negative Myosin V construct. In control melanocytes, the single parameter that changed between aggregation and dispersion was

the mean travel distance toward the minus-end of microtubules, which decreased during dispersion. However, in melanocytes where Myosin V function was blocked, the mean travel distance toward the plus- and minus-end were equal. Moreover, analysis of the frequency distributions of individual travel events toward the minus-end during dispersion revealed that there were a greater number of relatively long runs when Myosin V was disrupted. Overall, these results indicate that the shift from net aggregation of melanosomes to net dispersion involves decreasing the length of minus-end directed travel events and increasing the activity of Myosin V. It suggests a model where Myosin V selectively ends minus-end directed transport, thus biasing transport along microtubules directed toward the plus end during dispersion. While this study uncovers a role for Myosin V in regulating directed transport along microtubules during dispersion, it does not address whether there may be factors other than molecular motors regulating the relative contributions of actin- and microtubule based transport.

Indeed, a subsequent study in black tetra fish melanocytes suggests that actin-based motor activity is not the only factor biasing net melanosome movement toward the plus-ends of microtubules during dispersion (Rodionov, Yi et al. 2003). In these melanocytes, bulk melanosome movement during dispersion is biased toward the plus-end of microtubules even when actin-based transport is disrupted. Tracks of individual melanosome movement showed that the mean distance traveled toward the plus-end of microtubules was approximately 5-fold greater than toward the minus-end in control melanocytes and 4-fold greater than toward the minus-end in melanocytes treated with latrunculin A, which disrupts actin filaments. This indicates that additional factors are involved in biasing the bidirectional movement of melanosomes toward the plus-ends of

microtubules during dispersion, since movement along microtubules is still strongly biased toward the plus-end even in the absence of actin-based (thereby myosin-driven) transport. This directly relates to Chapter 3 of this dissertation, where I describe how the protein melanophilin is a key regulator of net melanosome displacement during dispersion in zebrafish.

This raises the question: why were the results from fish melanocytes diiferent than *Xenopus*? The reason actin-based transport disruption appears to affect microtubule-based transport in fish and *Xenopus* melanocytes differently is that there is a difference in the relative mean travel distance of minus-end-directed transport during dispersion. In *Xenopus*, there is an approximately 1.3 fold greater mean distance traveled toward the plus-end of microtubules than minus-end during dispersion (Gross, Tuma et al. 2002) whereas, in black tetra, there is an approximately 5-fold greater mean distance toward the plus-end (Rodionov, Yi et al. 2003). Consequently, melanosome transport in fish occurs more rapidly; it takes 30-45 to completely aggregate or disperse melanosomes in *Xenopus* (Rogers, Tint et al. 1997; Reilein, Tint et al. 1998) while it only takes 5-10 minutes in tested fish species (Thaler and Haimo 1990; Sammak, Adams et al. 1992; Rodionov, Yi et al. 2003).

Despite the apparent difference between *Xenopus* and fish, the relative data indicate that Myosin V is playing a similar role during dispersion in both species. In black tetra and *Xenopus*, disrupting actin-based transport increases the mean travel distance of minus-end directed transport and does not affect the mean travel distance of plus-end directed transport (Gross, Tuma et al. 2002; Rodionov, Yi et al. 2003). The result in *Xenopus* is that the mean plus- and minus-end travel distance became equal. Yet

in black tetra, the mean plus-end distance traveled is still approximately 4 fold greater than minus-end, indicating that Myosin V alone did not account for biasing net melanosome movement toward the plus-end during dispersion. Furthermore, a recent biophysical study developed a novel algorithm to compare melanosome transport in black tetra and *Xenopus* and reported that the general mechanisms of regulation are similar between the two species (Zaliapin, Semenova et al. 2005). Thus, the differences observed in between *Xenopus* and fish are likely not due to differences in regulating the interaction of microtubule- and actin-based motors.

While the afore mentioned studies have greatly contributed to our knowledge of cargo transport regulation and a few of the molecular players in certain systems have been identified (Wu, Rao et al. 2002; Gross, Guo et al. 2003), the general questions of how molecular motors are regulated to correctly transport cargo and what proteins are involved in their regulation are still largely unresolved. This dissertation seeks to address how directional microtubule-based transport and the transfer from actin- to microtubule-based transport are regulated in zebrafish melanocytes. I anticipate that, by examining transport regulation in melanocytes and identifying proteins involved in regulating molecular motors, we will enrich what is already known about motor regulation as well as uncover general strategies used by neurons and other types of cells to transport and deliver intracellular cargos at the right place and the right time.

Melanosome Transport in *Xenopus* and Teleost Fish

In the 1960s, Keith Porter introduced the fish melanocyte as a system to investigate intracellular transport mechanisms (Bikle 1966). Forty years later, the

melanocytes of *Xenopus* and various fish species continue to be used successfully as a model system to study cargo trafficking in cells (Levi, Serpinskaya et al. 2006). As described in the previous section, there are several reasons that these melanocytes are an advantageous system for investigating intracellular transport. They contain thousands of melanosomes - dark pigment granules that are easily observed with a light microscope. Melanosomes in fish and amphibian melanocytes synchronously aggregate or disperse in response to neurotransmitters or hormones, and they are transported via the coordinated actions of kinesin, dynein and myosin motors. Molecular studies in *Xenopus* melanocytes have revealed that Kinesin 2 is responsible for plus-end transport of melanosomes along microtubules (Tuma, Zill et al. 1998), cytoplasmic dynein responsible for minus-end transport (Rogers, Tint et al. 1997; Reilein, Serpinskaya et al. 2003), and Myosin V responsible for actin-based transport (Rogers and Gelfand 1998; Rogers, Karcher et al. 1999). Moreover, the Dynein motor that is associated with melanosomes contains a subunit, a variant of the dynein light intermediate chain, that is distinctive from bulk cytoplasmic dynein and may be differentially regulated to permit minus-end, microtubule-based melanosome movement independently of other organelle movement (Reilein, Serpinskaya et al. 2003). Since different isoforms of dynein are thought to provide a means of discrete movement for different types of organelles (Grissom, Vaisberg et al. 2002), investigating transport in fish and *Xenopus* melanocytes can give insight into how differential dynein transport is regulated in cells.

Another important advantage to this system is that the microtubules of fish melanocytes are of uniform polarity, with the minus-end at the cell center and the plus-ends at the cell periphery (Euteneuer and McIntosh 1981; Rodionov, Lim et al. 1994).

Uniform polarity of microtubules allows one to easily compare the contribution of plus-end motors and minus-end motors during microtubule-based organelle transport. The microtubules in melanocytes, as in most types of cells, are organized into a radial array by the centrosome in the cell center (Rodionov, Lim et al. 1994). Additionally, in centrosome-free fragments of fish melanocytes, the polarized radial array can rapidly self-organize via a centrosome-independant mechanism in which dynein nucleates the microtubules during melanosome aggregation (Malikov, Kashina et al. 2004; Malikov, Cytrynbaum et al. 2005). This may provide an additional microtubule organizing mechanism in melanocytes by which a uniform orientation of the microtubules is maintained.

Cyclic AMP signaling in fish and amphibian melanocytes

Incontrovertibly, cyclic AMP (cAMP) regulates melanosome transport in fish and amphibia. Melanosomes disperse to the cell periphery when intracellular levels of cAMP increase and melanosomes aggregate to the cell center when levels of cAMP decrease (Rozdzial and Haimo 1986; Potenza and Lerner 1992; Sammak, Adams et al. 1992; Thaler and Haimo 1992; Rodionov, Yi et al. 2003).

Intracellular cAMP levels in melanocytes are modulated via G-protein coupled receptors within the melanocytes. Melanosome dispersion is initiated by the peptide hormone α-melanin stimulating hormone (α-MSH) binding to a G_s-protein coupled receptor (Fujii and Miyashita 1982; Daniolos, Lerner et al. 1990) which activates adenylyl cyclase and increases intracellular cAMP (Rozdzial and Haimo 1986; Sammak, Adams et al. 1992) (Fig. 1.2). Melanosome aggregation is initiated by alpha-2-adrenergic

agonists (e.g. epinephrine) (Fujii and Miyashita 1975) or peptide hormone (Kawauchi, Kawazoe et al. 1983; Sugden 1992; Oshima, Nakamaru et al. 2001) binding to G_i-protein coupled receptors that subsequently inhibit adenylyl cyclase and decrease intracellular levels of cAMP (Fig. 1.2).

The direction of melanosome movement in fish and amphibia is tightly correlated with the dynamics of cAMP levels in the cytoplasm (Sammak, Adams et al. 1992; Rodionov, Yi et al. 2003). This was initially reported by a study that monitored real time changes of cAMP levels in angelfish melanocytes with a fluorescent probe called FlCRhR (Sammak, Adams et al. 1992). FlCRhR consists of a fluorescein-labeled catalytic subunit and a rhodamine-labeled regulatory subunit of Protein Kinase A. In the absence of cAMP, the catalytic and regulatory subunits bind to each other, allowing fluorescence energy transfer (FRET) from fluorescein to rhodamine. In the presence of cAMPthe subunits dissociate, increasing the distance between fluorescein and rhodamine and preventing FRET. Consequently, the levels of cAMP within the cell can be determined by calculating the ratio of fluorescein over rhodamine emission. In melanocytes injected with FlCRhR the fluorescence ratio—that is, the cAMP levels—decreased with melanosome aggregation, and increased when the melanosomes dispersed. Moreover, the degree of melanosome aggregation or dispersion was proportional to the degree of intracellular cAMP reduction or elevation; increasing concentrations of epinephrine caused a progressive lowering of cAMP and increasingly aggregated melanosomes, and increasing concentrations of IBMX (a phosphodiesterse inhibitor that raises intracellular levels of cAMP) caused a progressive increase of cAMP and melanosome dispersion. These results indicate that the directional movement of

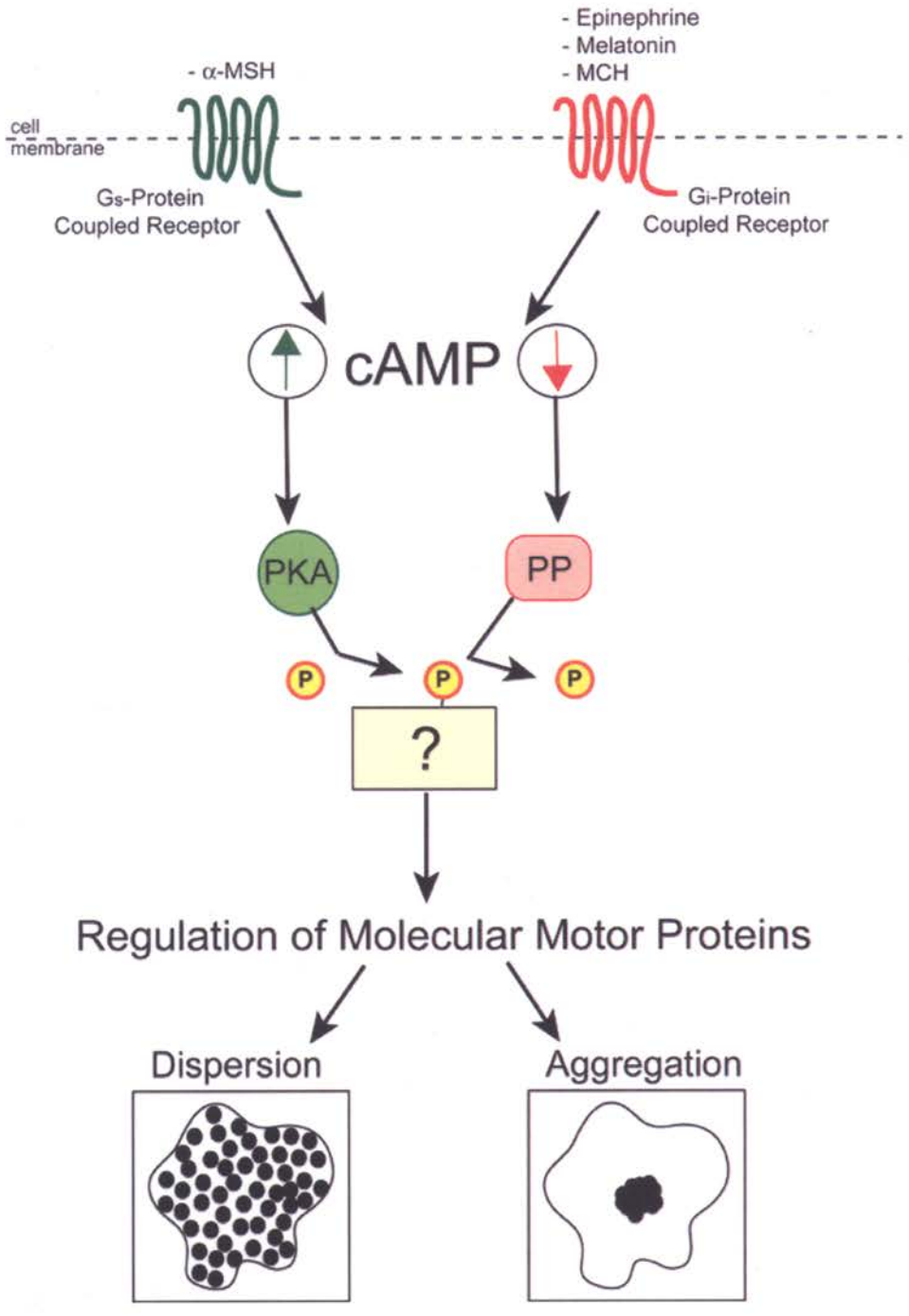

Figure 1.2: cAMP's regulation of melanosome movement in fish and amphibian melanocytes

Stimulation of melanocytes with neurotransmitter or hormones leads to an increase or decrease in intracellular levels cAMP. Elevated cAMP levels activate Protein Kinase A (PKA), which phosphorylates an unknown downstream target and leads to melanosome dispersion (left path). Lower levels of cAMP contribute to the activation of a protein phosphatase (PP), which dephosphorylates the target protein and leads to melanosome aggregation (right path).

melanosomes follows the dynamics of intracellular levels of cAMP. What this study did not address, however, is how the kinetics of cAMP levels correlate with the activity of microtubule- and actin-based motors.

The question of how cAMP signaling relates to the activities of microtubule- and actin- based motors was recently addressed in black tetra melanocytes (Rodionov, Yi et al. 2003). This was accomplished by analyzing, in parallel, the kinetics of single melanosome movement and intracellular cAMP levels. The study tracked the movement of individual melanosomes at increasing time points during dispersion to measure the travel distance of each continuous run toward the plus or minus ends of microtubules. They also measured changes in levels of cAMP with quantitative immunostaining in melanocytes fixed at increasing time intervals after the initiation of dispersion. By these measurements, cAMP levels peaked at approximately 1minute into dispersion, then decreased approximately 2-fold by 4 minutes. When the authors compared cAMP levels with the length of melanosome travel along microtubules, they observed that the mean distance travels toward the plus-end of microtubules (that is, toward the cell periphery) is greatest at the beginning of dispersion, when cAMP levels are at their highest, then decreases as cAMP levels declined (Rodionov, Yi et al. 2003). Moreover, comparing the kinetics of distance traveled toward the microtubule plus-ends with changes in cAMP levels over time reveals a precise correlation. Given that the kinetics of changes in cAMP levels and changes in the length of plus-end runs along microtubules are identical, this strongly suggests that cAMP levels are regulating the actions of microtubule-based motors during dispersion.

In addition, this study examined melanosome displacement along actin filaments by tracking individual melanosome movement in melanocytes treated with the microtubule inhibitor nocodazole (Rodionov, Yi et al. 2003). Melanosome displacement along actin filaments increases as cAMP levels declined, which suggests that reduced levels of cAMP facilitate the switch from microtubule- to actin- based transport during dispersion. To test this possibility, the authors examined melanosome dispersion in cells that had been pharmacologically treated with IBMX and membrane-permeable cAMP to prevent a decline in cAMP levels. When dispersed, the melanosomes in treated cells accumulate at the cell margin, which is similar to the melanosome distribution observed when actin-based transport is disrupted. This result indicates that, when cAMP levels are elevated, the switch from microtubule- to actin- based transport is inhibited and supports the hypothesis that the transfer of melanosomes to actin-based transport occurs when cAMP levels decline.

This decline in cAMP levels facilitates the transfer from microtubule- to actin-based transport by regulating minus-end directed dynein-mediated transport (Rodionov, Yi et al. 2003). Mean kinetic parameters of microtubule-based motion revealed that the distance traveled toward microtubule minus-ends, or the cell center, increase as cAMP levels decrease during dispersion. Moreover, blocking movement toward the minus-ends of microtubules by injecting melanoctyes with a function-blocking dynein antibody shows the same phenotype as when cAMP levels are kept constitutively high. This indicates that minus-end directed movement along microtubules increases as cAMP levels decrease and that switching from microtubule- to actin- based transport occurs

during minus-end moves. Thus, the cumulative results of this study indicate that microtubule- and actin-based transport regulation are coupled through cAMP.

Generally, cAMP-signaling is transduced through protein kinase A (PKA) (Skalhegg and Tasken 2000). This canonical pathway also applies to melanosome transport - increasing cAMP levels in the melanocytes of teleost fish and *Xenopus* leads to dispersion by activating PKA (Rozdzial and Haimo 1986; Sammak, Adams et al. 1992; Reilein, Tint et al. 1998). This was initially suggested by a study investigating the mechanism of cAMP signaling during dispersion (Rozdzial and Haimo 1986). Lysed African cichild melanocytes that underwent dispersion in the presence of radiolabeled phosphate show phosphorylation of a 57kD protein; incorporation of the labeled phosphate is inhibited by protein kinase inhibitor (PKI) – an endogenous peptide that is a potent and specific inhibitor of protein kinase A (Dalton and Dewey 2006). Molecular studies in *Xenopus* and fish further support the role of PKA in melanosome dispersion. Transfection of PKI into *Xenopus* melanocytes blocks MSH induced dispersion (Reilein, Tint et al. 1998) and microinjection of the catalytic subunit of PKA into angelfish melanocytes induces dispersion (Sammak, Adams et al. 1992). Furthermore, blocking PKA activity with PKI or the PKA inhibitor H89 leads to melanosome aggregation (Sammak, Adams et al. 1992; Reilein, Tint et al. 1998; Andersson, Svensson et al. 2003). Thus, PKA activity is required to both bring about and maintain the dispersed state of melanosomes.

Just as melanosome dispersion requires phosphorylation of a target protein by PKA, melanosome aggregation requires dephosphorylation of a target protein by a phosphatase. In *Xenopus* melanophores, protein phosphatase 2A (PP2A) is required

(Reilein, Tint et al. 1998), but the specific phosphatase required for fish melanosome aggregation is unclear (Thaler and Haimo 1990; Sammak, Adams et al. 1992). In the African cichilid melanocyte, inhibition of PP2B phosphatase activity with high concentrations of Okadaic acid (a serine/threonine phosphatase inhibitor), calmodulin antagonists, or a PP2B-specific antibody blocked melanosome aggregation (Thaler and Haimo 1990). However, data presented in this dissertation show that cyclosporine A, a specific inhibitor of PP2B, had no effect on melanosome aggregation in zebrafish (Figure 2.3). Moreover, a study using angelfish melanocytes, as well as data presented in this dissertation, show that Okadaic acid completely inhibits aggregation at 500nM and 1mM, respectively (Sammak, Adams et al. 1992). These are concentrations known to inhibit Protein Phosphatase 1 (PP-1) (IC50 = 315nM) but are far too low to inhibit PP2B (IC50 = 4.5mM). Thus, different fish species may require different phosphatases for aggregation.

Still unclear are the downstream targets of PKA and the phosphatase that regulate melanosome transport. Two studies have reported the phosphorylation of similarly sized proteins during dispersion, suggesting that they had identified the same downstream target: one observed a 57 kD polypeptide that is phosphorylated during dispersion and dephosphorylated during aggregation in lysed fish melanocytes (Rozdzial and Haimo 1986) and another observed phosphorylation of a 53kD protein in *Xenopus* melanocytes treated with MSH (de Graan, Oestreicher et al. 1985). Phosphorylation of a protein of this size directly relates to work reported in Chapter 3 of this dissertation, as I have identified a candidate for PKA phosphorylation that is approximately 60kD. In contrast, a study using *Xenopus* melanocytes that examined purified melansomes rather than whole

cell extract reported different results. Melanosomes purified from melanocytes dispersed in the presence of radiolabled phosphate showed phosphorylation of proteins at 87 kD and 94 kD (Reilein, Tint et al. 1998). Therefore, the microtubule motors themselves might be phosphorylated, since the dynein intermediate chain has a molecular weight of 83 kD and the subunits of Kinesin II have the mol wt of 85 and 95 kD, respectively. But immunoprecipitations of Kinesin II and cytoplasmic dynein from aggregated or dispersed melanocyte cell extract showed no phosphorylation differences, indicating that the motors themselves are not the targets of PKA and PP2A (Reilein, Tint et al. 1998).

One reason for the isolation of PKA-phosphorylated proteins of dissimilar sizes in these studies could be the different approaches used—specifically, examining the phosphorylation state of whole cell extracts (de Graan, Oestreicher et al. 1985; Rozdzial and Haimo 1986) as opposed to examining purified melanosomes (Reilein, Tint et al. 1998). Examining purified melanosomes may have missed the target of PKA phosphorylation because it may be localized elsewhere and not on the melanosome itself. Nevertheless, the targets of PKA and its corresponding phosphatase remain unidentified, and direct evidence that the proteins found to be phosphorylated in these studies are actually involved in melanosome transport regulation is lacking. Identifying and characterizing the function of these targets is of great interest to the field of motor regulation because they are regulating the direction of melanosome transport, thereby the actions of molecular motors.

It is well accepted that PKA signaling is spatially regulated through interacting with A-kinase anchoring proteins (AKAPs). AKAPs function to compartmentalize PKA signaling in cells by localizing it close to a specific phosphorylation target (Smith,

Langeberg et al. 2006). In addition, several studies have shown that signaling molecules can act as scaffolds that link molecular motors to their cargo, which may be a means of linking signaling and trafficking (Verhey, Meyer et al. 2001; Schnapp 2003). Therefore, it is not surprising that recent work in *Xenopus* melanocytes suggests that PKA plays a structural role to localize molecular motors on melanosomes (Kashina, Semenova et al. 2004). This report showed that the regulatory subunit of PKA (RIIα) associates with melanosomes in Xenopus melanocytes via an AKAP and that melanosome aggregation is partially blocked when this interaction is disrupted. Moreover, when endogenous RIIα is displaced from melanosomes with a dominant negative RIIα construct, dynein is also disrupted from the melanosomes, consistent with RIIα disruption from melanosomes inhibiting aggregation. While dispersion is not affected when RIIα was displaced from melanosomes, pull-down assays reveal that all three molecular motors (Kinesin 2, dynein, and Myosin V) interact with RIIα. Hence, there may be two motor regulatory complexes: a dynein-containing aggregation complex that is sensitive to the removal of RIIα and a kinesin- and myosin-containing dispersion complex that, while also interacting with RIIα, is insensitive to its disruption. Thus, the regulatory subunit of PKA may play a structural role to correctly localize molecular motor proteins and signaling molecules within proximity of one another on melanosomes. This arrangement, where motors are directly associated with signaling molecules that regulate their activity, could be a mechanism by which melanocytes efficiently regulate switching between aggregation and dispersion of melanosomes.

Secondary signaling cascades in fish and amphibian melanocytes

This dissertation will focus on the well-established cAMP signaling pathway mediating melanosome transport. However, several other signaling pathways have also been implicated in aggregation and dispersion of fish and *Xenopus* melanosomes. Three additional signaling pathways that have been the focus of research in melanocytes are Protein Kinase C, Mitogen Activated Protein Kinase, and signaling though the second messenger calcium. These signaling pathways likely evolved in fish and amphibia to fine-tune pigment changes, from subtle to dramatic, in order to adapt to a variety of ecological situations (Fujii 2000).

Protein Kinase C (PKC) is an additional kinase involved in melanosome dispersion. PKC is activated in cells when a subunit of an activated G-protein coupled receptor, presumably the $\beta\gamma$ complex of G_s-coupled receptors in melanocytes (Fujii and Miyashita 1982), activates phospholipase-Cβ in the plasma membrane (Newton 1997). The activated phospholipase then hydrolyzes phosphatidylinositol (4.5) biphosphate to form two products: inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG). DAG, either independently or together with calcium released from the endoplasmic reticulum by IP_3, activates PKC (Newton 1997). In *Xenopus* melanocytes, pharmacological activation of PKC with phorbol 12-myristate 13-acetate (PMA) leads to dispersion of melanosomes (Graminski, Jayawickreme et al. 1993; Reilein, Tint et al. 1998). Activation of PKC in the melanocytes appears to depend on DAG but not calcium, because raising intracellular calcium does not lead to melanosome dispersion (Graminski, Jayawickreme et al. 1993).

While PKC is able to induce dispersion, molecular data indicate that it is neither necessary nor sufficient to fully disperse melanosomes (Reilein, Tint et al. 1998). In *Xenopus* melanocytes expressing the PKA inhibitor PKI, pharmacological activation of PKC led to only partial dispersion of melanosomes. Futhermore, expression of a plasmid encoding an epitope-tagged inhibitor of PKC in melanocytes does not block PKA induced dispersion. Thus, PKC signaling can mediate partial dispersion on its own, but is insufficient to fully disperse melanosomes without PKA. Since PKC is able to induce dispersion when cAMP levels, hence PKA activity, is low (Sugden and Rowe 1992) and when PKA activity is blocked (Reilein, Tint et al. 1998), it is possible that PKC may be regulating microtubule-based motors to only partially disperse melanosomes. Consequently, regulation of melanosome transport via PKA- and PKC-mediated pathways may fine-tune pigment changes in melanocytes. Hypothetically, PKC signaling could be a means to partially darken melanocytes while PKA could be a means to fully darken melanocytes in the whole animal.

Another signaling cascade that has been implicated in regulating melanosome transport during both aggregation and dispersion is the Mitogen Activated Protein Kinase (MAPK) pathway. Evidence for MAPK involvement in aggregation was provided by studies in *Xenopus* melanophores that showed that the MAP kinase ERK1 is activated during melatonin-induced aggregation and that blocking its signaling with UO126, a potent inhibitor of MAPK/ERK kinase (MEK), inhibits aggregation of melanosomes (Andersson, Skold et al. 2003; Andersson, Svensson et al. 2003; Deacon, Nascimento et al. 2005). Biochemical data indicate that both MEK (MAPK/ERK kinase) and ERK are present on melanosomes in a functional complex and that MEK and ERK are transiently

phosphorylated during melatonin-induced aggregation (Deacon, Nascimento et al. 2005). ERK signaling appears to be downstream of cAMP signaling; inhibition of PKA with H-89 treatment, which normally causes aggregation of melanosomes (Sammak, Adams et al. 1992), does not cause aggregation in melanocytes where ERK signaling is blocked. This result suggests that MAPK signaling is involved in melanosome aggregation downstream of the cAMP-signaling pathway.

Analysis of individual melanosome movement in *Xenopus* revealed that MAPK signaling plays a role in dispersion as well. Inhibiting ERK signaling greatly reduces the frequency of microtubule-based melanosome motility events without affecting their velocity or travel distance (Deacon, Nascimento et al. 2005). This effect is melanosome specific; tracks of lysosome transport revealed no difference in travel distance or velocity along microtubules when ERK signaling was blocked. Overall, these findings indicate that MAPK is a regulator of the frequency of both plus- and minus-end microtubule-based transport events during aggregation and dispersion in *Xenopus* melanocytes.

In addition to cAMP, calcium has been implicated as another second messenger involved in melanosome transport (Luby-Phelps and Porter 1982; Thaler and Haimo 1992; Kotz and McNiven 1994; Fujii 2000). For years the role of calcium in regulating melanosome transport was controversial because several studies had what appeared to be conflicting reports in fish melanocytes (Luby-Phelps and Porter 1982; Thaler and Haimo 1990; Sammak, Adams et al. 1992; Thaler and Haimo 1992; Kotz and McNiven 1994) . Two reports showed that elevating intracellular calcium in squirrel fish eurythrophores (pigment cells similar to melanocytes containing red pigment) leads to pigment aggregation (Luby-Phelps and Porter 1982; Kotz and McNiven 1994). This finding

suggested that, in addition to cAMP, calcium was an important second messenger in melanosome transport. A later report resolved the role for calcium signaling by revealing that it activates the phosphatase required for melanosome aggregation (Thaler and Haimo 1990). Calcium promotes pigment aggregation in African cichlid (*Tilapia mossambica*) melanocytes by activating protein phosphatase 2B (PP2B) through the calcium signal transducer calmodulin. In whole melanocytes, injection of the calcium chelator BAPTA or calmodulin antagonists blocks melanosome aggregation, and in extensively lysed melanocytes, which are unable to aggregate due to loss of a soluble component required for aggregation, introducing purified PP2B rescues melanosome aggregation (Thaler and Haimo 1990). These authors proposed a model in which pigment aggregation is initiated by increased intracellular calcium, leading to the activation of PP2B and aggregation of melanosomes through dephosphorylation of a target protein.

However, a study performed using angelfish (*Pterphyllum scalare*) melanocytes reported a different result (Sammak, Adams et al. 1992). In melanocytes filled with the calcium indicator Fura-2, Sammak et al. observed a transient rise in intracellular levels of calcium during aggregation. But inducing a rise in intracellular calcium with ionomyacin fails to induce melanosome aggregation and blocking the rise in calcium with BAPTA fails to inhibit aggregation. They concluded that lower intracellular levels of cAMP alone, not calcium, are responsible for inducing aggregation.

The reason these results differ may be because different species of fish require different phosphatases to initiate aggregation. As reviewed earlier in this chapter, angelfish and zebrafish require the actions of a phosphatase for melanosome aggregation, but pharmacological data indicate that this phosphotase is not PP2B for both fish species

(Sammak, Adams et al. 1992). Therefore, different species of fish appear to have evolved different requirements for calcium signaling in melanosome transport.

Genetic studies in mammalian melanocytes

Mammalian melanocytes have also proven to be a robust model system for studying organelle transport. Like fish and amphibia, mammalian melanocytes contain melanosomes that are transported by kinesin, dynein and myosin motors (Marks and Seabra 2001). Mature melanosomes are transported, via the coordinated actions of kinesin and dynein, bi-directionally along microtubules from the center of the melanocyte to the tips of distal processes (Wu, Bowers et al. 1998). Then they are handed off to Myosin-V motors in the actin cytoskeleton and transported to the plasma membrane, where they are transfered to keratinocytes, the epidermal cells that directly pigment mammals (Wu, Bowers et al. 1997). Mammalian melanosomes do not rapidly transport their pigment in response to cAMP; however, cAMP is a key signaling molecule in mammalian melanogenisis. Elevation of intracellular levels of cAMP leads to the expression of melanogenic proteins such as tyrosinase and tyrosinase related protein-1 and -2 (TRP-1 and TRP-2) through a PKA/CREB-dependant pathway (Busca and Ballotti 2000).

A tremendous advantage of studying melanosome transport in mammalian melanocytes is the ability to use a forward genetic approach; that is, uncovering genes by way of their mutant phenotypes to identify interrelated genes that are involved in melanosome transport. The most prominent example of this is the discovery of how mammalian melanosomes connect to the actin motor Myosin Va. This connection was

initially revealed by analyzing three independent, naturally occurring mouse pigment mutations: *dilute, ashen,* and *leaden* (Fukuda, Kuroda et al. 2002; Wu, Rao et al. 2002). These mutations, which encode Myosin Va, Rab27a and Slac2-a/melanophilin (an effector for Rab27a), respectively, produce the same cellular phenotype: a perinuclear accumulation of melanosomes within the melanocytes that leads to hypopigmentation in the whole animal (Fig. 1.2 A). Equivilent mutations in Myosin Va, Rab27a and melanophilin are also observed in humans and are responsible for the three types of Griscelli syndrome, a rare autosomal recessive disorder that is associated with partial albinism (Menasche, Feldmann et al. 2003; Menasche, Ho et al. 2003; Libby, Lillo et al. 2004). Molecular studies in mammalian melanocytes provided evidence that Myosin Va, Rab27a and melanophilin physically interact with one another and suggest a model for how Myosin Va interacts with melanosomes. Rab27a, which is anchored to the melanosome surface, binds to the effector protein melanophilin in a GTP-dependent fashion. Melanophilin then binds the actin cytoskeleton and recruits Myosin Va, thereby forming a link between the motor and the cargo (Fig. 1.2 B). Additional studies have defined mechanistically how melanophilin mediates the interaction between melanosomes and Myosin Va in mammals. Melanophilin belongs to the synaptotagmin-like protein family (Slp) and contains three functional domains that are involved in its interaction with melanosomes and Myosin Va: a Rab27a-binding domain at its N-terminal region, a Myosin Va binding domain, and an actin binding domain at its C-terminus (Kuroda, Ariga et al. 2003). The Rab 27a binding domain contains two Slp homology domains (SHD1 and SHD2) separated by two zinc finger motifs and its Myosin Va binding domain containing two binding sites (MBD1, which interacts with

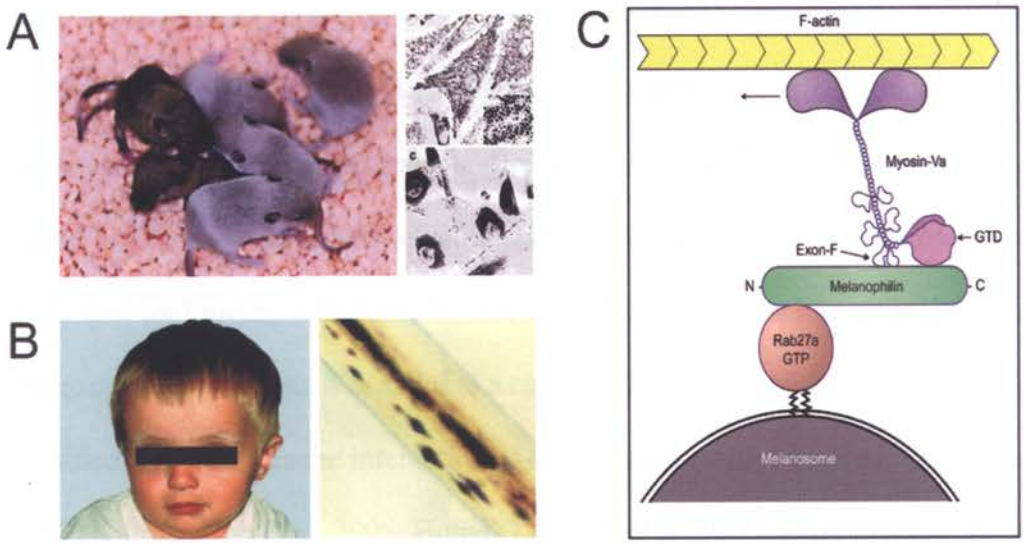

Figure 1.3: Mammalian forward genetics uncovered proteins involved in connecting melanosomes to Myosin Va.

A. Grey *ashen* mutants (right side) next to black WT mice (left side). Mutant *dilute* and *leaden* mice also have the same silver hair phenotype. Inset – WT (top) and *ashen* (bottom) melanocytes in cell culture. WT melanosome are distributed to the tip of the dendrites, while *dilute, ashen,* and *leaden* mutant melanosomes accumulate at the cell center. (Adapted from Wilson et al (2000), PNAS, **97**:7933-7938)

B. A child with Griscelli syndrome (Type 2). A conspicuous feature of all three types of Griscelli syndrome is hypopigmentation of skin and hair. The right panal shows abnormal accumulations of pigment in the hair shaft due to disrupted melanosome transfer to keratinocytes. (Adapted from Patirodlu et al (2000), Turk J Haematol, **17(2)**: 85-87)

C. Model of how Myosin Va interacts with melanosomes in mammals. Activated Rab27a attached to the melanosome membrane interacts the effector protein melanophilin. Melanophilin then binds to Myosin Va, thereby forming a link between the cargo and the motor. (Adapted from Wu et al (2002), Nat.Cell Biol., **4**:271-278)

the globular tail of Myosin, and MBD2, which interacts with an alternatively spliced exon in the Myosin Va tail called exon F (Wu, Rao et al. 2002; Hume, Tarafder et al. 2006)) (Figure 2C, (Kuroda, 2005 #99; Hume, 2006 #143). Biochemical and imaging studies examining truncated and site-directed mutagenized melanophilin cDNA expressed in mouse melanocyte cell lines have reported that SHD1 is necessary and sufficient for binding with Rab27a, that MBD2 binding exon F is critical for binding melanophilin to Myosin Va, and that a coiled-coil region in the actin binding domain significantly enhances the interaction of melanophilin with Myosin Va (Wu, Rao et al. 2002; Kuroda, Itoh et al. 2005; Hume, Tarafder et al. 2006). Moreover, a study examining purified Myosin Va and truncated melanophilin proteins in vitro reported that melanophilin, through its MBD1 binding site, stimulates Myosin Va ATPase activity, thereby its motor activity (Li, Ikebe et al. 2005). Thus, melanophilin appears to regulate Myosin Va activation in melanocytes as well as link it to melanosomes.

Zebrafish as a genetic model system

The primary aim of this thesis was to apply the forward genetic approach that had been successfully used in mouse melanocytes in the context of a fish melanocyte system. To accomplish this, we turned to the genetically tractable organism zebrafish (*Danio rerio*). Zebrafish have many practical advantages for genetic analysis: a short generation time, transparent embryos that develop outside of the parent, and robust breeding all year round (Westerfield 2000; Beis and Stainier 2006). They also have theoretical advantages and have been used effectively for more than twenty years to address important questions

in developmental biology and, more recently, cell biology and human disease (Kimmel 1989; Roest Crollius and Weissenbach 2005). In addition, much of the zebrafish genome has been sequenced, which provides a powerful tool for identifying mutant gene products of interesting mutants.

A recent study reporting the identification of the zebrafish *golden* gene provides an exciting example of how studies in zebrafish can identify functional roles for proteins in mammals (Lamason, Mohideen et al. 2005). Zebrafish *golden* is a recessive pigment mutation that is characterized by delayed melanocyte development and reduced melanosomes, which results in fish with pale melanocytes and retinal pigment epithelium (RPE). Positional cloning revealed the *golden* gene to be SLC24A5, a putative cation exchanger that is thought to be involved with organellar calcium uptake. Subsequent analysis revealed that SLC24A5 is functionally conserved between fish and humans. Moreover, genetic polymorphisims within SLC24A5 contribute to skin color variation within different human populations. These results indicate that SLC24A5 plays an important role in the pigmentation of fish and humans, and they demonstrate that zebrafish can be used as a model organism to give important insight into gene functions in mammals.

Conclusions

It has been suggested that in neurons, vesicles are transported along microtubules to nerve termini, where they are then transferred to Myosin-V motors that subsequently transport them in the actin-rich nerve terminus (Brown, Simonetta et al. 2001; Langford 2002; Libby, Lillo et al. 2004). This kind of directed movement along microtubules

followed by transfer from microtubule-based motors to actin-based Myosin-V motors is also responsible for proper melanosome trafficking in melanocytes. Therefore, information gained from analyzing melanosome transport may give insight into how cargo transport is regulated in other types of cells, such as neurons.

The goal of this dissertation was to identify molecules required for regulating molecular motors by identifying genes required for melanosome transport in zebrafish melanocytes. Using zebrafish as a model system allows us to combine the advantage of synchronous melanosome transport in response to well-defined intracellular signaling pathways found in fish and amphibian melanocytes with the advantage of forward genetics previously used in mammalian melanocyte studies. Work presented here will show that we can identify in zebrafish pigmentation mutants with melanosome transport defects and, subsequently, genes involved in melanosome transport.

The function of the gene I identified, zebrafish melanophilin, is to bias movement of melanosomes toward the plus-ends of microtubules as well as to regulate the transfer of melanosomes from microtubule- to actin-based transport. Therefore, I have shown that a single protein is responsible for regulating the actions of both microtubule- and actin-based motors during melanosome dispersion in zebrafish and that this protein biases bidirectional microtubule-based transport and coordinates the transfer of melanosomes from microtubules to actin. Conceivably, this strategy may be used in other cell types to regulate both biased movement of cargos along microtubules and transfer between microtubules and actin, thereby regulating the special distribution of cargo in the cell.

CHAPTER 2

Characterization of Zebrafish Melanocytes for Genetic and Functional Studies of Melanosome Motility

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Introduction

Pigment organelle transport within pigment cells, or melanocytes, has long been recognized as a valuable experimental model system for investigating intracellular cargo transport (Byers and Porter 1977). Melanocytes have a large population of membrane-bound organelles, called melanosomes, whose dynamics are easily observed in a light microscope because they contain the dark biological pigment melanin. Proper trafficking of melanosomes involves the cooperation of actin and microtubule motors (Rodionov, Hope et al. 1998; Rogers and Gelfand 1998; Tuma, Zill et al. 1998; Wu, Bowers et al. 1998). The microtubule motors dynein and kinesin carry melanosomes along radially organized microtubule tracks toward or away from the cell center, respectively, while the actin motor myosin interacts with melanosomes to transport them within the actin cortex (Rodionov, Lim et al. 1994; Rodionov, Hope et al. 1998; Rogers and Gelfand 1998). A similar functional coordination of microtubule-and actin-based transport underlies the delivery cargo vesicles to the plasma membrane in neurons and secretory vesicles in neuroendrocrine cells (Kamal and Goldstein 2000; Bridgman 2004).

As discussed in Chapter 1, studies in mouse and human melanocytes demonstrate how the genetic analysis of pigmentation mutants can identify interrelated molecular components of organelle transport. The molecular linkage of mammalian melanosomes to Myosin Va was discovered by analyzing three mammalian pigment mutations (Hammer and Wu 2002) that encode Myosin Va, Rab27a and melanophilin (an effector for Rab27a) (Wu, Rao et al. 2001; Wu, Wang et al. 2002; Wu, Rao et al. 2002). Thus,

previous genetic studies in mammalian melanocytes reveal how the analysis of pigment mutants can give insight into the mechanisms of motor regulation.

The melanocytes of both mammals and teleost fish transport melanosomes using similar cellular machinery. However, they differ in terms of the function, regulation, and dynamics of melanosome transport. Mammalian melanocytes distribute melanosomes to the tips of their processes, where they are transferred to keratinocytes, the cells that directly pigment mammals (Wu and Hammer 2000). The movement of mammalian melanosomes is not synchronous, and the signaling cascades regulating melanosome transport have not been characterized. Fish melanocytes, in contrast, cause the organism to lighten or darken directly by rapidly and synchronously aggregating or dispersing their melanosomes in response to neurotransmitters or hormones that modulate intracellular cAMP (Sammak, Adams et al. 1992; Reilein, Tint et al. 1998; Rodionov, Yi et al. 2003). This close correlation of intracellular levels of cAMP with the coordinated activities of microtubule- and actin-motor proteins gives melanocytes in fish an important advantage for investigating transport regulation.

Fish melanosomes aggregate to the cell center when cAMP levels are lowered and a phosphatase is activated, whereas they evenly disperse throughout the melanocyte when intracellular cAMP levels are elevated and protein kinase A (PKA) is activated (Rozdzial and Haimo 1986; Sammak, Adams et al. 1992; Rodionov, Yi et al. 2003). The activities of PKA lead to rapid, directed movement of melanosomes along microtubules toward the periphery of the cell, followed by the transfer of the melanosomes to Myosin motors in the actin cytoskeleton, which evenly distributes the pigment within melanocytes. Thus, factors downstream of PKA appear to regulate the actions of microtubule- and actin-

based motors. However, the downstream targets of PKA in melanocytes are still unknown, and attempts to biochemically isolate them have been inconclusive (Rozdzial and Haimo 1986; Reilein, Tint et al. 1998). Identifying these downstream proteins is of great interest because it would provide critical insight into how microtubule- and actin-based motors are coordinated to successfully traffic intracellular cargo.

To identify proteins downstream of cAMP signaling and PKA, we took advantage of the genetic approach pursued previously in mice but in the context of fish melanocytes. Here, we report that this is possible using the vertebrate genetic organism zebrafish (*Danio rerio*). We have characterized the signaling pathways that regulate the synchronous aggregation and dispersion of melanosomes in zebrafish melanocytes and developed a screening assay that specifically identifies mutants with defective melanosome transport. Using this assay, we have identified several mutations that disrupt the process of melanosome transport downstream of known signaling molecules. Thus, zebrafish is a useful organism for uncovering novel genes responsible for the regulated intracellular transport of cargoes along microtubules or actin filaments.

Materials and Methods

Fish Stocks: WIK, AB and TU lines were obtained from Zebrafish International Resource Center (ZIRC). They were maintained as described (Johnson, Midson et al. 1994).

Larvae melanosome transport screening assay. We sedated 5 day old larvae with a mixture of 200 ppm quinaldine and 0.1 mg ml^{-1} lidocaine in E3 solution (5mMNaCl, 0.17

mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$). We avoided the sedative Tricaine because it elicits melanosome dispersion in larvae. After exposing the larvae for 10 min to 5 μM MCH (Bachem, King of Prussia, PA) diluted in sedative solution containing 0.1% DMSO, we examined them for aggregation defects. Following this we incubated the same larvae in 10-30 μM MSH (Sigma-Aldrich, St. Louis, MO) for 10 – 20 min and then screened them for dispersion defects. We observed larvae under brightfield using a Leica MZFLII stereomicroscope equipped with a 1.0x PlanApo lens (Leica Microsystems, Wetzlar, Germany). Digital photos were taken with a Magnifire-SP digital camera (Optronics, Goleta, California).

Primary melanocyte cultures. Prior to culturing, prim 5 stage (24 hour) embryos were bleached and dechorionated (Westerfield 2000). When these larvae were five to seven days old they were anesthetized and then minced in ice-cold calcium-free Ringers solution (116 mM NaCl, 2.9 mM KCl, 5 mM HEPES (pH 7.2)). The minced tissue was rinsed three times in 50 ml sterile calcium-free Ringers, and incubated in 0.05% Trypsin solution with EDTA (Invitrogen, Carlsbad, California) for twenty min at room temperature. While in Trypsin solution, the tissue was triturated every five minutes with a fine tipped glass pipette. The dissociated cells were then rinsed and resuspended to a density of 125 -500 x 10^3 ml^{-1} in L-15 media (Cellgro, Mediatech Inc., Herndon VA) supplemented with 10% FBS, 0.2 % gentamycin, 0.5% penicillin, 0.5% streptomycin (Invitrogen, Carlsbad, California) and 0.8 mM CaCl2. Cells were plated onto clean 25 mm diameter No. 1 glass coverslips (Fisher Scientific, Hampton, NH) that had been coated with a mixture of 20 μg ml^{-1} laminin and 10 μg ml^{-1} fibronectin (Roche, Basel,

Switzerland) or 0.2 mg ml^{-1} E-C-L (Upstate, Charlottesville, VA). Approximately 0.25 ml of the cell suspension was incubated with each coverslip for 4-8 hours. After plating, each coverslip was cultured at room temperature for up to three weeks in 2.5 ml of L15 media with the supplements listed above.

Imaging cultured melanocytes. Coverslips with attached cells were sealed into a closed recording chamber (Model RC-21BR, Warner Instruments, Hamden, CT) in Ringers solution (116 mM NaCl, 2.9 mM KCl 5 mM, HEPES, pH 7.2, 1.8mM $CaCl_2$). Cells were imaged by differential interference contrast or by brightfield using a Zeiss Axioplan (Zeiss, Oberkochen, Germany) microscope equipped with a custom fiber optic illuminator coupled to a Hg arc lamp. We used the following Plan-Neofuor oil immersion objectives: 40x (N.A. = 1.3); 100x (N.A. = 1.3). Digital images were acquired with a Photometrics CoolSNAP CCD camera (Roper Scientific, Trenton, NJ) and stored on a personal computer, all under the control of MetaMorph imaging software (Molecular Devices Corporation, Sunnyvale, CA).

Pharmacological studies of cultured zebrafish melanocytes. The following reagents (from Sigma-Aldrich, St. Louis, MO, unless otherwise noted) were diluted in Ringers and perfused into the microscope chamber: 0.1 -10 mM MCH (Bachem, King of Prussia, PA), 0.2 – 20 mM MSH, 0.01mM epinephrine, 5 mM caffeine, 200 mM forskolin, 0.1 - 1mM SQ 22536, 1 mM IBMX, 40 mM H-89 and 0.5 -10 mM okadaic acid. Melanocytes in culture media were preincubated with okadaic acid overnight prior to exposure to MCH or epinephrine (Namboodiripad and Jennings 1996; Reilein, Tint et al. 1998).

Results

Screening Assay

Many different types of zebrafish pigment mutations have been identified in previous screens (Kelsh, Brand et al. 1996; Golling, Amsterdam et al. 2002); however, zebrafish with melanosome transport defects have not been described. This is because previous screens were designed for the purpose of identifying defects in the developmental biology of pigment cells, e.g. their differentiation, patterning and survival. The genes involved in these processes are likely different from those involved in melanosome transport. We therefore developed a screening assay to specifically identify zebrafish mutants with defective melanosome transport.

In wild-type five-day-old swimming larvae, it is simple to discern the dispersion and aggregation of melanosomes in response to background light or various chemicals. We observed aggregation of pigment when larvae were exposed to direct light or MCH, while complete dispersion of melanosomes occurred when they were sedated with 0.02% tricaine or when they were exposed to MSH (Fig. 2.1 A). DMSO alone, which was used as a drug carrier in our assay, did not cause aggregation or dispersion of melanosomes (data not shown).

In order to characterize melanosome transport in further detail, we developed a means to isolate melanocytes from zebrafish larvae in primary cell culture. We dissociated whole zebrafish larvae at 6-8 days old and plate the cells at a low density onto glass coverslips coated with extracellular matrix proteins. A subset of the cells that adhered to the coverslips were melanocytes. The melanocytes are distinctive and easily

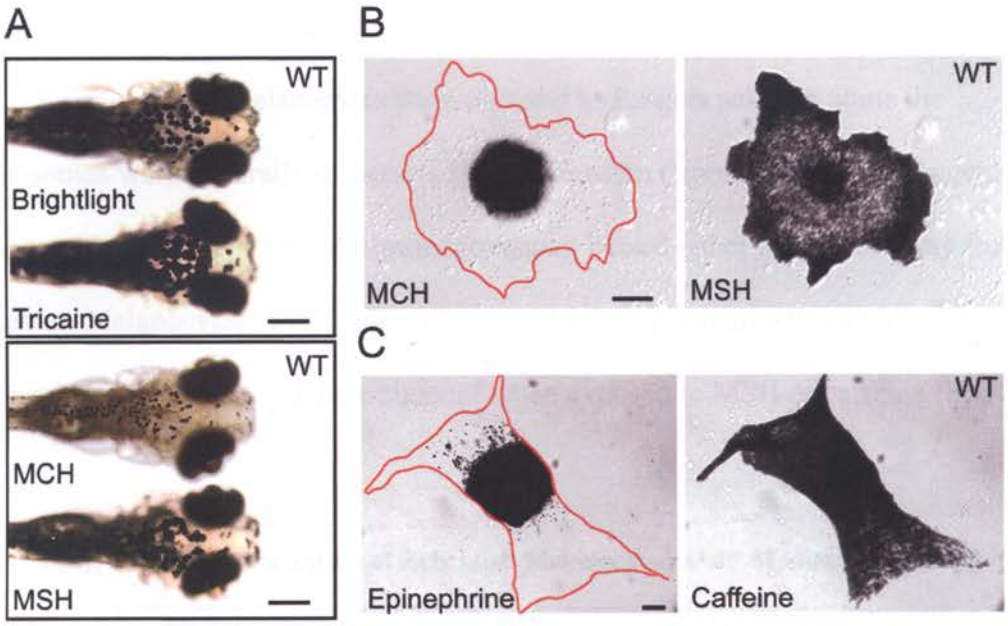

Figure 2.1: Melanosome transport screening assay

A. Melanosome transport screening assay in whole zebrafish larvae. Wild-type, 5 day old swimming larvae aggregated their melanosomes in response to bright light or 5 μM Melanin Concentrating Hormone (MCH) and dispersed their in response to 0.02% tricaine or 10 μM α-Melanin Stimulating Hormone (MSH). Larvae exposed to MCH and MSH were sedated with quinaldine/lidocane and exposed to each ligand for 10 minutes. (Scale Bars = 200 μm)

B. Images of cultured melanocytes exposed to MCH and MSH. Cultured melanocytes aggregated their melanosomes when exposed to 1 μM MCH and dispersed their melanosomes when exposed to 0.5 μM MSH. Bright field illuminated images were taken 5 minutes after the application of each hormone. The melanocytes were exposed to each hormone for a total of 10 minutes.

C. Images of cultured melanocytes exposed to epinephrine and caffeine. Cultured melanocytes aggregated their melanosomes when exposed to 0.1 mM epinephrine and dispersed their melanosomes when exposed to 5 mM caffeine (10 minutes each). Brightfield illuminated images were taken 5 minutes after the application of each reagent. (Scale Bars = 10 μm)

identified amongst other cells. They can be used for live imaging experiments within 48 hours of plating, but we observed they were ideal at 6-14 DIV (days in vitro).

When cultured melanocytes were exposed to Ringers solution alone the melanosomes were generally dispersed. However, when exposed to various reagents, they responded with rapid melanosome movement biased either toward or away from the cell center. Melanocytes in cell culture aggregated their pigment when exposed to MCH or epinephrine and dispersed their pigment when exposed to MSH or caffeine (Fig. 2.1 B, C).

Table 2.1: Characterization of Zebrafish Melanocyte cAMP Mediated Signaling

Reagent	Mechanism	Effect on Wild-type
α-MSH (0.2-20 μM)	Activates adenylyl cyclase via Gs-protein coupled receptor	**Dispersion**
MCH (0.1-1.0 μM)	Inhibits adenylyl cyclase via Gi-protein coupled receptor	**Aggregation**
Caffeine (5 mM)	A1 adenosine receptor antagonist; elevates intracellular cAMP	**Dispersion**
Epinephrine (0.1 mM)	A2-adrenergic receptor agonist; inhibits adenylyl cyclase via Gi-protein coupled receptor	**Aggregation**
Forskolin (200 μM)	Adenylyl cyclase activator	**Dispersion**
sp-cAMP (30 μM)	Membrane permeable cAMP analogue	**Dispersion**
SQ 22,536 (100μM-1mM)	Adenylyl cyclase inhibitor	**Inhibits Dispersion**
Okadaic Acid (1μM)	Serine/Threonine Phosphatase inhibitor	**Inhibits Aggregation**
Cyclosporin A (20μM)	Protein Phosphatase 2B inhibitor	**No Effect**
H-89 (40μM)	Protein Kinase A inhibitor	**Aggregation**

The effect of modulating cAMP levels and inducing melanosome movement has been examined in many species of fish as well as *Xenopus* (Sammak, Adams et al. 1992;

Thaler and Haimo 1992; Kotz and McNiven 1994; Rodionov, Yi et al. 2003). To verify that the cAMP signaling pathways that regulate melanosome transport in other fish species also do so in zebrafish, we exposed isolated melanocytes to reagents that activate or inhibit known players in the signaling pathways (Table 2.1). Dispersion of melanosomes occurred when the melanocytes were exposed to forskolin, which elevates cAMP levels by activating adenylyl cyclase (Fig. 2.2 A), and when exposed to a membrane permeable analogue of cAMP. In contrast to previous reports, aggregation of melanosomes did not occur when the melanocytes were exposed to SQ 22,536 (100 mM-1 mM), which lowers cAMP levels by inactivating adenylyl cyclase. Instead, it appeared to preferentially block the action of drugs that cause dispersion (Fig. 2.2 B).

The effects of inhibiting the actions of PKA or phosphatases in fish melanocytes have also been studied (Rozdzial and Haimo 1986; Reilein, Tint et al. 1998; Rodionov, Yi et al. 2003). We observed aggregation of melanosomes when zebrafish melanocytes were exposed to the PKA inhibitor H-89 (Fig. 2.3 A). We also observed that the action of aggregating drugs was blocked when dispersed zebrafish melanocytes were exposed to 1 μM Okadaic Acid, a general phosphatase inhibitor (Fig. 2.3 C). These results confirm the importance of cAMP modulation and the activity of PKA and a phosphatase in the regulation of zebrafish melanosome movement and suggest that the overall pathways that regulate dispersion and aggregation in zebrafish are similar to what has been established in other species of fish. Thus, zebrafish can be used as a model system to identify proteins that regulate melanosome transport downstream of the phosphatase and PKA.

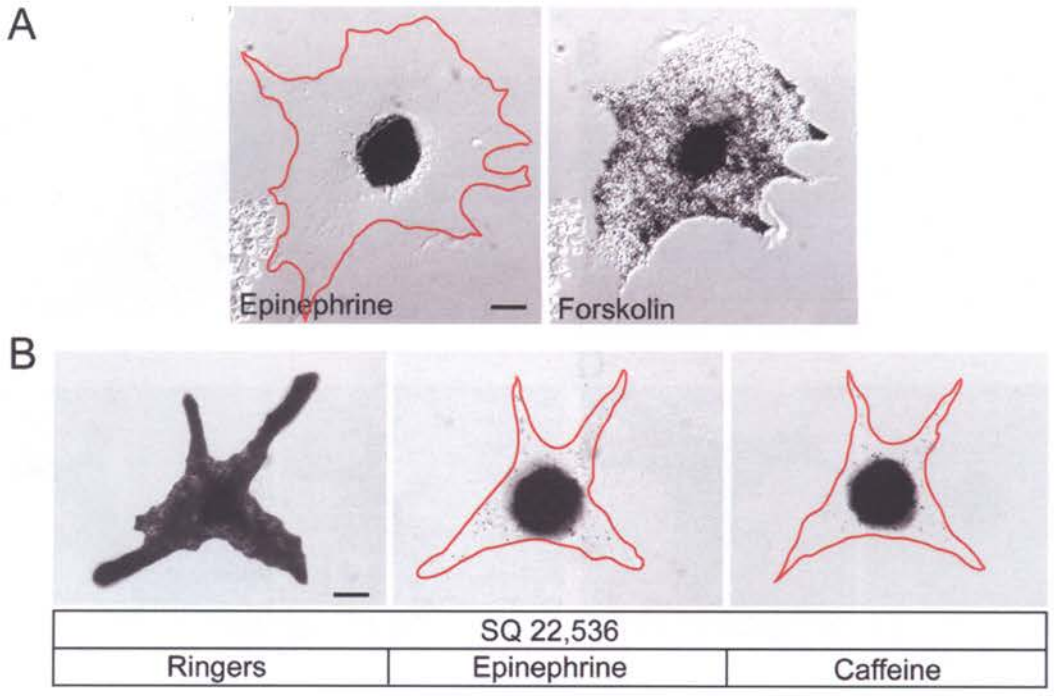

Figure 2.2: Zebrafish melanocytes aggregate and disperse their melanosomes in response to cAMP signaling.
A. Exposure of melanocytes to compounds that raise intracellular levels of cAMP. Cultured melanocytes in cell culture were exposed to epinephrine for 10 minutes to fully aggregate their pigment, then exposed to 200 μM forskolin, which raised intracellular levels of cAMP and induced dispersion of pigment. Differential interference contrast (Mehta, Rock et al.) images were taken 5 minutes after the application of each reagent.
B. Inhibition of intracellular cAMP elevation in cultured melanocytes. Melanocytes were treated with 1 mM SQ 22536 (a reagent that inhibits adenylyl cyclase), then exposed to 0.1mM epinephrine for 10 minutes followed by 5mM caffeine for 30 minutes. Exposure to SQ 22536 inhibited caffeine induced dispersion. Brightfield images were taken 5 minutes after exposure to and 30 minutes after exposure to caffeine.
(Scale Bars = 10 μm)

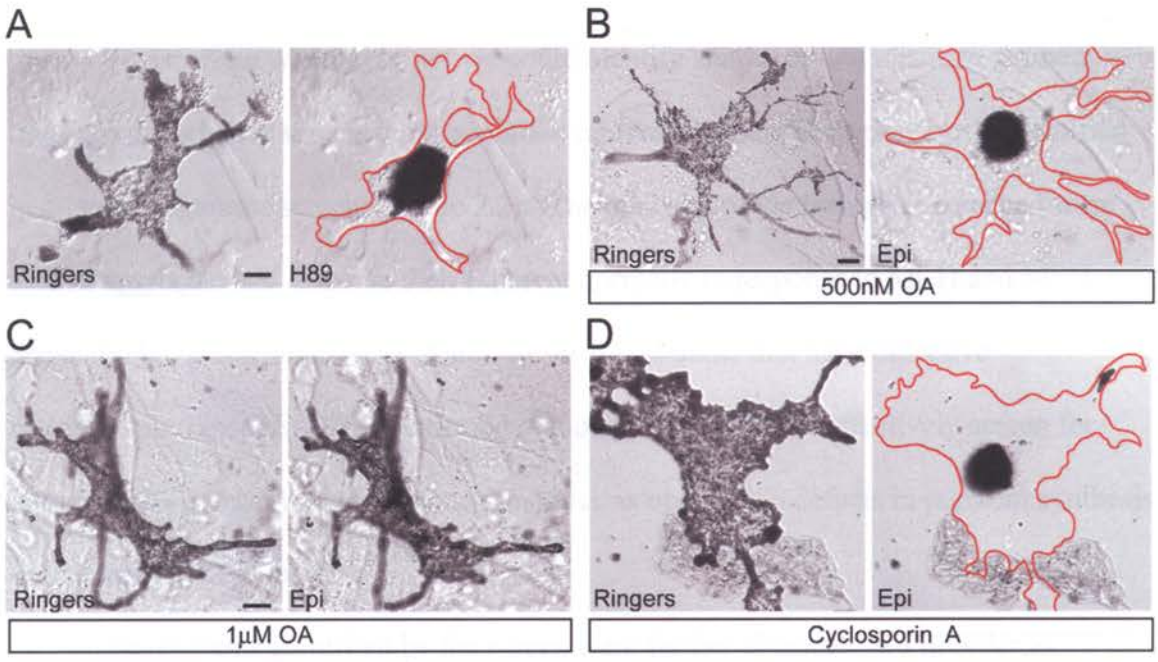

Figure 2.3: PKA is involved in dispersion and a phosphatase is involved in aggregation in zebrafish melanocytes.

A. Inhibition of PKA in cultured zebrafish melanocytes. Exposure to H89 (a reagent that inhibits PKA activity) for 5 minutes generated melanosome aggregation in isolated melanocytes. This indicates that PKA signaling maintains the dispersed state of melanosomes in zebrafish melanocytes.

B. and C. Inhibition of the phosphatase responsible for dispersion with Okadaic Acid (OA) treatment. OA blocks melanosome aggregation at intermediate concentrations. Cultured melanocytes were treated with 500 nM or 1 μM OA, then exposed to 0.1 mM epinephrine. 1μM OA blocked epinephrine-induced melanosome aggregation.

D. Cultured melanocytes treated with 20 μM cyclosporine A (an inhibitor of (PP2B), then exposed to 0.1 mM epinephrine. Exposure to cyclosporine A did not block melanosome aggregation.

(Scale Bars = 10 μm)

Small scale screen for melanosome transport mutants

To ascertain whether or not we could identify zebrafish mutants with pigment transport defects, we screened pigmentation mutants that had been previously identified in other mutagenesis screens (Table 2.2). The majority of the larvae we screened were able to aggregate and disperse their pigment normally in response to MCH and MSH, respectively. Nevertheless, we identified several mutants that have defective melanosome transport. This confirmed that our assay is able to effectively screen for mutants with defects in melanosome dynamics as opposed to defects in pigment synthesis or melanocyte degeneration.

The mutants identified by the screen were further characterized in order to determine whether or not the defects observed are due to mutations in proteins that act downstream of cAMP signaling. The first mutant we examined, *fata morgana*, was identified because it did not aggregate its melanosomes in response to light (Fig. 2.4 A) or MCH (Fig. 2.4 B). Isolated *fata morgana* melanocytes in cell culture were completely unresponsive to MCH, but aggregated normally in response to epinephrine (Fig. 2.4 C). This indicates that the mutation found in *fata morgana* is involved in MCH receptor signaling. We did not further characterize or clone this mutant because it is upstream of cAMP signaling and likely not involved in molecular motor regulation.

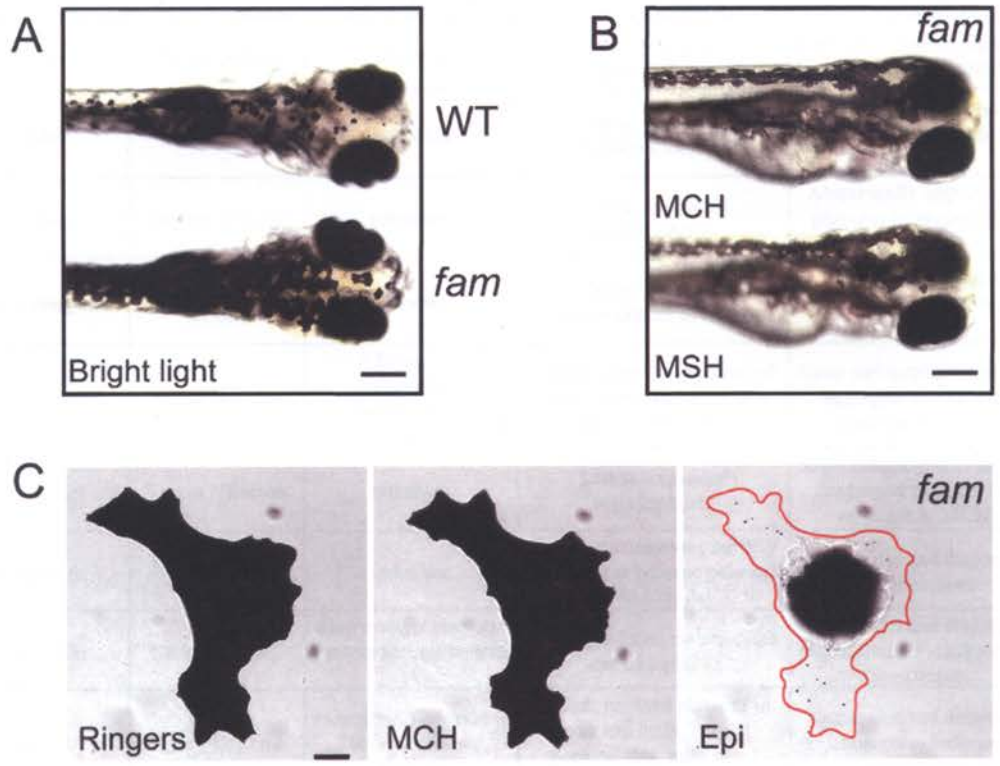

Figure 2.4: Characterization of *fata morgana*; a melanosome transport mutant that effects MCH signaling

A. Wild-type and fata morgana mutant larvae response to light. Five day old, awake larvae were exposed to bright light, which normally leads to aggregation of pigment. In contrast to wild-type, *fata morgana* mutants did not aggregate their melanosomes in response to bright light. (Scale Bar = 200 μm)

B. Melanosome transport screening assay applied to *fata morgana* mutants and wild-type siblings. Five day old *fata morgana* mutant larvae did not aggregate their melanosomes when exposed to 5 μM MCH for 20 minutes. Full dispersion of melanosomes was confirmed by exposing the mutants to 10 μM MSH. (Scale Bar = 200 μm)

C. Images of cultured *fata morgana* mutant melanocytes exposed to MCH and epinephrine. Exposure of the melanocyte to 1 μM MCH for 10 minutes did not lead to melanosome aggregation. However, exposure to 0.1 mM epinephrine lead to robust and complete aggregation.

Table 2.2: Pigment Mutants Screened

Name	Source	Affected Gene	Unique Features	Screen
j120	Stephen Johnson	unknown	Viable, constricted melanophores	**Does not disperse pigment in response to MSH**
b867	Judith Eisen	unknown	Lethal, individual melanophores look blotchy	**Unevenly disperses pigment in response to MSH**
smutz	Stephen Johnson	unknown	Viable, expanded melanophores	**Abnormally aggregates pigment in response to MCH**
fata morgana	Tübingen Stock Center	unknown	Viable, expanded melanophores in larvae	**Does not aggregate pigment in response to MCH**
hi112	Nancy Hopkins	ATPase, H+ transporting, Lysosomal Interacting Protein 1	Lethal; mixed population of dark puctate and dispersed melanophores at d5	Some melanophores do not aggregate pigment in response to MCH
stardust	Teresa Nicolson	unknown	Lethal; expanded melanophores	Some melanophores do not aggregate pigment in response to MCH
delayed fade	Tübingen Stock Center	unknown	Lethal; melanocytes initially normal but become pale and spot-like on day 3	Aggregates and disperses melanosomes
hi2499a	Nancy Hopkins	deep orange/ vacuolar protein sorting protein 18	Lethal; reduced melanocytes and iridophores	Aggregates and disperses melanosomes- melanocyte degeneration
hi1207	Nancy Hopkins	vacuolar ATP synthase 16kD proteolipid subunit	Lethal; reduced pigment in eyes and body, ruffled pectoral fins, mild brain necrosis	Aggregates and disperses melanosomes- melanocyte degeneration
hi923	Nancy Hopkins	ATPase, H+ transporting, lysosomal, V1 subunit H	Lethal; reduced pigmentation, d3 brain necrosis	Aggregates and disperses melanosomes- melanocyte degeneration
hi577a	Nancy Hopkins	vacuolar ATP synthase subunit E	Lethal; reduced pigment, small otoliths, arches/jaws misshapen, touch insensitive	Aggregates and disperses melanosomes
hi1463	Nancy Hopkins	lysosome membrane protein II	Lethal: reduced pigmentation, variable mispaterning of hindbrain, notochord defects	Aggregates and disperses melanosomes
black and tan	James Lister	unknown	Viable, subpopulation of melanocytes small and pale	Aggregates and disperses melanosomes- melanocyte degeneration
256G	James Lister	unkown	Lethal; small, pale melanocytes, reduced iridophores	Aggregates and disperses melanosomes- melanocyte degeneration
280E	James Lister	unknown	Lethal; small, pale melanocytes and small eyes	Aggregates and disperses melanosomes- melanocyte degeneration

On the other hand, three other identified mutants (*j120*, *b867* and *smutz)* may be due to defects downstream of cAMP signalling, which makes them strong candidates for regulators of organelle transport. The results of their characterization and, in the case of *j120* subsequent identification, are reported in Chapter 3 and 4.

Discussion

Zebrafish research has centered primarily on human disease modeling, embryogenesis, or physiology. Few studies have exploited zebrafish for cell biology research. Yet the short generation time, transparency, and tractable genetics of zebrafish provide many advantages for investigating cell biological questions. Here we explored whether zebrafish melanocytes are an appropriate model system for uncovering novel genes responsible for intracellular transport of cargoes along microtubules and actin filaments. Using a two-step approach, we identified mutations that disrupt the process of melanosome transport. First we applied a screening assay to zebrafish, which identifies mutants with melanosome transport defects. We then isolated zebrafish melanocytes in cell culture to address whether the mutations affect genes downstream of known signaling molecules and to characterize more completely the nature of the defect.

Our screening assay has two distinct advantages. One advantage is that zebrafish melanocytes respond to hormone quickly and robustly. This means that this assay could be used to easily screen a large number of zebrafish mutants. A second advantage is that, because we are screening larvae, we are also able to easily screen for lethal mutations. This is in contrast to other vertebrate organisms, such as mice. Since many mutations that disrupt organelle trafficking are critical to the organism's survival (Homma, Takei et al. 2003; Sasaki, Mori et al. 2005), we predict that this will allow us to uncover novel genes involved in transport regulation that would be difficult to identify in mammals.

To ascertain whether we could successfully identify zebrafish mutants with melanosome transport defects, we applied our screening assay to zebrafish that had been previously classified as pigment mutants. We discovered that the melanocytes of most of

the mutants were either able to aggregate and disperse their pigment normally or had degraded too severely to assay for melanosome movement (Table 2.2). However, we did identify four mutants with melanosome transport defects. Since we were able to find them in our small-scale screen, we predict that many more could be identified in a large-scale genetic screen. This is because previous screens identified mutants with blatant abnormalities the appearance of their melanocytes. Subtle defects in melanosome transport that would not have been uncovered in these previous screens could be identified with our assay in a large-scale screen of zebrafish mutants.

The reason that the melanocytes of teleost fish are a powerful system for investigating organelle transport regulation is that intracellular levels of cAMP tightly regulate synchronous melanosome movement. While cAMP modulation has been shown to regulate melanosome transport in several fish species (Sammak, Adams et al. 1992; Thaler and Haimo 1992; Rodionov, Yi et al. 2003), it had not been examined in zebrafish melanocytes. Thus, we needed to confirm that cAMP modulation is involved in zebrafish melanosome transport. But it is not possible to assay this in whole larvae because cAMP modulation affects many other cellular processes in the fish. In order to examine the effects of chemicals that modulate the signaling pathways involved in regulating melanosome transport, we developed a method to isolate zebrafish melanocytes in cell culture. When reagents were applied that activated or inhibited known signaling molecules, isolated zebrafish melanocytes responded in the same way as isolated melanocytes from other species of teleost fish (Fig 2.2, Fig. 2.3,Table 2.1). These results confirm that zebrafish are a useful system to identify molecules downstream of known

players in the signaling pathways. This is important because downstream targets of known signaling molecules are likely novel regulators of melanosome transport.

The question of transport regulation at this particular time exemplifies the type of research problem that would benefit from a forward genetic approach. Very little is understood about how transport is regulated and, most importantly, we lack molecular candidates. We anticipate that cloning genes responsible for defective melanosome transport in zebrafish will identify factors that regulate motor activity. This will give us valuable insight into how motors may be regulated in mammalian cells.

CHAPTER 3

Zebrafish melanophilin (mlpha) brings about melanosome dispersion by suppressing dynein motility

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Introduction

Fish and amphibians constantly adjust their pigmentation in order to blend with backgrounds that vary from light to dark. Background adaptation depends on melanocytes that rapidly aggregate and disperse melanin-containing membrane-bound organelles called melanosomes.

Melanosome aggregation and dispersion involves transport on both microtubules and actin. Because the melanocyte's microtubules are arrayed with their minus-ends at the cell center, Kinesin-2, a plus-end motor, carries melanosomes outward, leading to dispersion (Tuma, Zill et al. 1998), while the minus-end motor dynein carries melanosomes inward, which leads to aggregation (Tuma, Zill et al. 1998; Reilein, Serpinskaya et al. 2003). Melanosomes also interact with myosin V (Rogers and Gelfand 1998), which transfers outwardly moving melanosomes from microtubules to short actin filaments that intermingle with the microtubules (Rodionov, Hope et al. 1998; Rogers and Gelfand 1998). This transfer is crucial for darkening the organism. If it is prevented, e.g. by disassembling actin, melanosomes move to the minus ends of the microtubules and accumulate at the cell cortex, which leaves most of the cell pigment free (Rodionov, Hope et al. 1998).

As a population melanosomes aggregate and disperse, but the motion of individual melansomes is saltatory and bidirectional, i.e. episodes of travel in each direction are interspersed with each other and with periods of pause or small scale undirected motion that reflects either a stopped state on the microtubule or a switch to actin-based motility (Gross, Tuma et al. 2002). Net aggregation or dispersion is achieved

through biasing the frequency and/or persistence of dynein vs kinesin motility (Gross, Tuma et al. 2002; Rodionov, Yi et al. 2003).

The mechanisms underlying melanosome transport regulation presumably operate in other cells, as saltatory, bidirectional movement characterizes microtubule-based transport generally, from chromosome movement on the mitotic spindle (Mountain and Compton 2000) to organelle transport in neurons (Gunawardena and Goldstein 2004). Switching from microtubule- to actin-based transport also occurs in other cells: e.g. post-Golgi transport vesicles destined for the plasma membrane must leave microtubules and penetrate the cortical actin network in order to reach their final destinations (Allan and Schroer 1999).

The advantage of fish melanocytes for investigating the mechanisms underlying motor regulation is that melanosome transport is tightly regulated by cAMP levels. A rise in cAMP activates Protein Kinase A (PKA) and leads to melanosome dispersion; a decline in cAMP enhances the phosphatase activity that reverses PKA dependent phosphorylation (Rozdzial and Haimo 1986; Sammak, Adams et al. 1992; Rodionov, Yi et al. 2003). During dispersion, cAMP levels rise and fall with precise kinetics that correlate with both a rise and fall in the run lengths of plus-end motility events (Rodionov, Yi et al. 2003). As plus-end travel declines, minus-end travel and actin-based movements increase. These and other findings have led to a model wherein the phosphorylation state of an unknown target protein(s) changes during dispersion, and this is what is responsible for varying the activities of the kinesin, dynein, and myosin motors during the course of dispersion. A number of experiments suggest that the hand-off from

microtubules to actin occurs during dynein-driven motility events (Gross, Tuma et al. 2002; Rodionov, Yi et al. 2003).

To this point, our understanding of how phosphorylation controls the three melanosome motors is phenomenological: the molecules that operate downstream of PKA to regulate the motors are not known. Here we identify one of these molecules through the analysis of the zebrafish pigment mutant, *j120*.

Materials and Methods

Fish Stocks. WIK, AB and TU lines were obtained from the Zebrafish International Resource Center (ZIRC).

Larvae Screening Assay: The screening assay was applied as previously described in Chapter 2.

Primary Melanocyte Culture and Isolated Melanocyte Imaging: Performed as previously described in Chapter 2.

Analysis of global melanosome dispersion and melanocyte cytoskeletal organization. Immediately following drug application, digital images of individual melanocytes were collected for a period of 5 – 10 min using a camera exposure time of 150 ms. To graph the time course of global melanosome dispersion, Metamorph software was used to trace the perimeter of the cell and to compute the enclosed area. A threshold applied to the frame identified the pixels occupied by melanosomes, and the summed area of the

melanosomes was divided by the total cell area to compute the percent of the cell area filled by melanosomes. This parameter was measured in successive frames to graph the time course of dispersion.

To evaluate the organization of the microtubule and actin cytoskeletons, melanocytes were fixed with 4% paraformaldehyde in 1X PBS (137mM NaCl, 2.7 mM, 4.3mM Na_2HPO_4 * $7H_2O$, 1.4mM KH_2PO_4), immunostained with a primary antibody against a-tubulin (Sigma-Aldrich), and then labeled with rhodamine phalloidin (Molecular Probes, Invitrogen, Carlsbad, California). Fluorescent cells were observed with epi-illumination using the Plan-Neofuor 40x (N.A. = 1.3) oil emersion lens (Zeiss, Oberkochen, Germany) and FITC and rhodamine filter sets (Chroma, Rockingham, VT). Digital images were processed using MetaMorph and Adobe Photoshop software.

Single melanosome tracking during dispersion. Melanocytes were first exposed to 1μM MCH or 0.1 mM epinephrine to bring about the aggregation of their melanosomes. They were then stimulated with 5 mM caffeine or 5 μM MSH to initiate dispersion. 1.5 and 3 min after initiating dispersion. High resolution image sequences were generated using the Plan-Neofuor 100x (N.A. = 1.3) oil emersion lens (Zeiss, Oberkochen, Germany). Images were captured at three frames per second at 150 or 300 ms exposure, and majority of image sequences lasted 30s. The movements of individual melansomes were analyzed with the centroid-tracking function of MetaMorph software. Using this function, the distribution of a melanosome's position within a fixed cell was Gaussian with a mean of 2.7 nm and a SD of 3.8 nm.

Melanosome tracks were manually decomposed into three motion regimes: linear outward (plus-end) movement; linear inward (minus-end) movement; and episodes of non-directed movement or pause. Movement along the microtubule axis was defined as linear melanosome displacement toward or away from the cell center that was greater than 30 nm per 300ms. The endpoint of a linear run was determined by a pause or reversal of movement along the microtubule axis. Pauses were defined as episodes wherein melanosome displacement was less than 30 nm over 300 ms. Random movement was defined as apparent non-directed melanosome displacement that was greater than 30 nm per 300ms and persisted at least 900ms, though it generally persisted much longer. To measure run lengths associated with linear travel along microtubules, we used a simple least squares method to compute the best fit line to the trajectory and then summed the components of the motion along this line.

Positional cloning and molecular analysis of *mlpha*^{j120} *j120/j120* fish in a C32 background were outcrossed with wild-type WIK fish to make heterozygous *j120* carriers in a C32/WIK background. These map cross fish were mated and their progeny screened at 4 to 5 days old for the *j120* phenotype at 4 to 5 days post fertalization. Screened larvae were fixed in methanol, and their genomic DNA isolated. Linkage analysis was performed by PCR using known markers as primers. Linkage to chromosome 6 was established using SSLP markers. Fine mapping was performed using SSR markers found in the following databases: The Zebrafish Information Network, zfin.org; Tübingen Map of Zebrafish Genome, wwwmap.tuebingen.mpg.de/; and Zebrafish SSR Search, danio.mgh.harvard.edu/markers/ssr.html.

To sequence the wild-type and mutant *mlpha* genes we PCR amplified and sequenced overlapping fragments of the gene, using information from the Zebrafish Genome-Sequencing Project (www.ensembl.org/Danio_rerio/index.html) to design the PCR and sequencing primers. To make *mlpha* cDNA, we isolated mRNA from wild-type and *j120* larvae using the (Poly(A)Purist™ Kit (Ambion Inc, Austin, TX) and then performed RT-PCR using the Advantage RT for PCR kit (BD Biosciences, San Jose, CA). The primers for amplifying the entire Mlpha protein coding sequence from cDNA obtained from wild-type larvae were TCCTGAAAGACTTGGCAACTG and CGTCTTTATGCCAGTCTGTCAAA. The primers for amplifying a 251 bp fragment that contains the premature stop codon in the mutant were TCACTCTCGCAGACAGTCTGT and TCTACCTGGACACTTCAGAGGAAGAGGATA. To sequence identify the point mutation in *j120*, genomic DNA was amplified with the primers GAAGAAAATATATTAGGAAATACGGT and CATCACTGATTAAGGCGCTTTA, and then sequenced.

In situ Hybridization: Whole mount in situ hybridization was performed per Thisse et al. [Westerfield, 2000 #94] in 0.003% PTU treated larvae at 24 hours, 48 hours, 3 days and 4 days post fertilization. Digoxigenin labeled RNA probes were synthesized using linearized plasmid DNA containing zebrafish melanophilin as the template (Open Biosystems, clone ID 8008178, Accession DT063943.1).

Morpholino Injection: Morpholinos (Gene Tools, Philomath, OR) were dissolved in distilled water, diluted to 1mM in 1X Danieau solution (58mM NaCl, 0.7mM KCl, 0.4mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES pH 7.6), and injected into 1-2 cell wild-type embryos with 0.05% phenol red as a marker. The sequence of the melanophilin morpholino was 5'-GACAGGTCCAACTTCTTGTCCATGT-3'. A standard control morpholino with fluorescein (5'-CCTCTTACCTCAGTTACAATTTATA-3') was also injected to confirm that the phenotype observed was not due to toxicity.

Results and Discussion

The mutation *j120* interferes with melanosome dispersion downstream of cAMP.

j120 is a recessive allele generated by ENU mutagenesis . *j120* / *j120* fish are fully viable and appear normal apart from an inability to adapt their pigmentation to a dark background. Like other fish, zebrafish aggregate and disperse their melanosomes in response to melanin concentrating hormone (MCH) and melanin stimulating hormone (MSH), respectively (Fig. 3.1 A). Mutant fish appear normal when the fish are in MCH, but upon exposure to MSH, the spots of pigment in the mutant remain small whereas those in wild-type fish expand dramatically (Fig. 3.1A).

To analyze melanosome transport in more detail we employed a digital light microscope to make movies of melanocytes in primary cell cultures of dissociated five-day old larvae (Fig. 3.1B). *j120* mutant melanosomes aggregate normally in response to MCH or epinephrine (Fig. 3.1B), but in MSH or caffeine, they disperse very slowly and

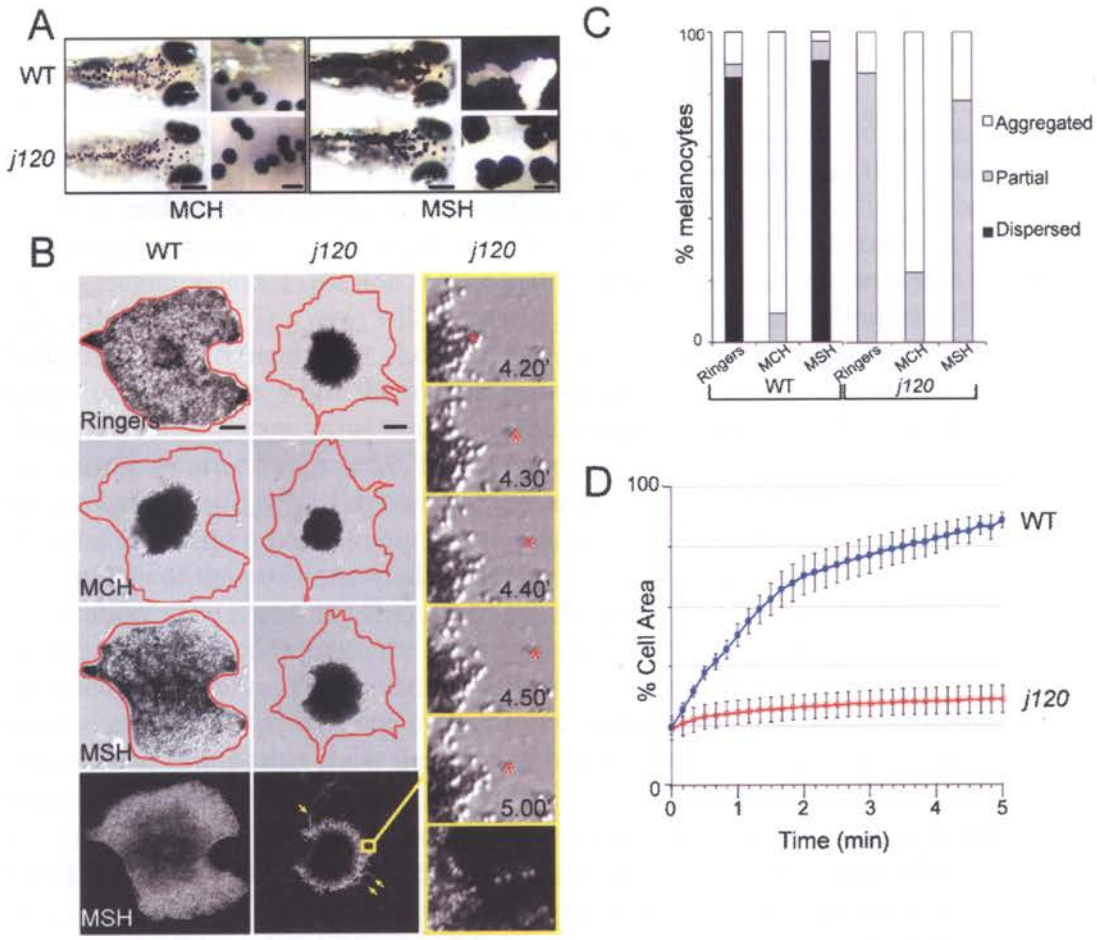

Figure 3.1: Characterization of melanosome transport in wild type and *j120* melanocytes – figure legend on next page

Figure 3.1: Characterization of melanosome transport in wild type and *j120* melanocytes

A. Aggregation and dispersion in wild type and mutant five-day old larvae. In response to MCH, pigment appears similarly aggregated in mutant and wild type fish. In response to MSH, melanocytes within wild type fish disperse their pigment fully, while melanocytes in mutant fish do not. (Scale Bars = 200 μm; 50 μm in inset).

B. Aggregation and dispersion in cultured wild type and *j120* melanocytes visualized by DIC microscopy. Top row: wild type and *j120* melanocytes in Ringers solution. In Ringers or in L15 media (not shown), the wild type cell maintains its melanosomes in a fully dispersed state, while the mutant cell maintains its melanosomes in a partially dispersed state. Second row: wild type and mutant melanocytes fully aggregate their melanosomes after 5 min in MCH. Aggregation appears normal in the mutant melanocyte. Third row: Exposure of the cells to MSH for 5 min elicits complete dispersion in wild type, but only partial dispersion in mutant melanocytes. Bottom row: Summation of the positions of all melanosomes during the 5 min period following MSH application. The preceding frame was subtracted from each new frame and the differences summed through the recording period. This reveals that dispersion in *j120* melanocytes involves the movement of individual melansomes along linear paths (arrowheads). The path of one melanosome is shown in the inset. Above the inset is the sequence of frames that show the moving melanosome that contributes to the difference image (Scale bars = 10 μm).

C. Fraction of cultured wild type and mutant melanocytes that respond to MCH and MSH fully or partially. Melanocyte cultures were treated for 10 min with 0.5 μM MSH or 1 μM MCH. Partial dispersion or aggregation of melanosomes was evident in a minor fraction of wild type melanocytes. Mutant melanocytes never dispersed their melanosomes fully (n = 96 and 75 melanocytes, for wild type and mutant, respectively).

D. Kinetics of global melanosome movement in cultured wild type and *j120* mutant melanocytes. Wild type and mutant melanocytes with melanosomes aggregated were stimulated to disperse, and the percent of the projected cell area filled with melanosomes was measured at 10 s intervals. Datapoints are averages from five mutant and five wild-type melanocytes (Error Bars = SEM).

spread only part way to the melanocyte margin (Fig. 3.1B,C,D). Spreading plateaus 5-8 minutes after applying MSH.

We performed pharmacological experiments to address whether the *j120* phenotype is caused by a defect in the signal transduction pathway that leads to cAMP elevation. Drugs that elevate intracellular cAMP downstream of the MSH receptor, including forskolin, an activator of adenylyl cyclase, and sp-cAMP, a membrane permeable analogue of cAMP, elicit full dispersion in wild-type melanocytes (Fig. 3.2A) (Sammak, Adams et al. 1992) but do not affect *j120* mutant melanosome dispersion, which is again slow and incomplete (Fig 3.2A). Therefore, the *j120* gene product does not function between MSH binding to its receptor and cAMP elevation. To address whether the partial dispersion phenotype is caused by elevated activity of the opposing phosphatase, we exposed *j120* mutant melanocytes to 1 μM of the phosophatase inhibitor okadaic acid (Fig. 3.2B). This blocks melanosome aggregation in wild-type and *j120* mutant melanocytes (Fig. 3.2B), indicating inhibition of the phosphatase opposing PKA phosphorylation, but dispersion in the mutant melanocytes remains incomplete. Thus, the *j120* phenotype does not result from defects in the signaling pathway controlling PKA dependent phosphorylation.

The slow and incomplete melanosome dispersion in *j120* melanocytes is unaffected by actin disruption and occurs through the transport of individual melanosomes along microtubules.

The microtubule and actin cytoskeletons of *j20* mutant melanocytes, visualized by anti-tubulin immunofluorescence and rhodamine-phalloidin labeling (Fig. 3.3A), appear

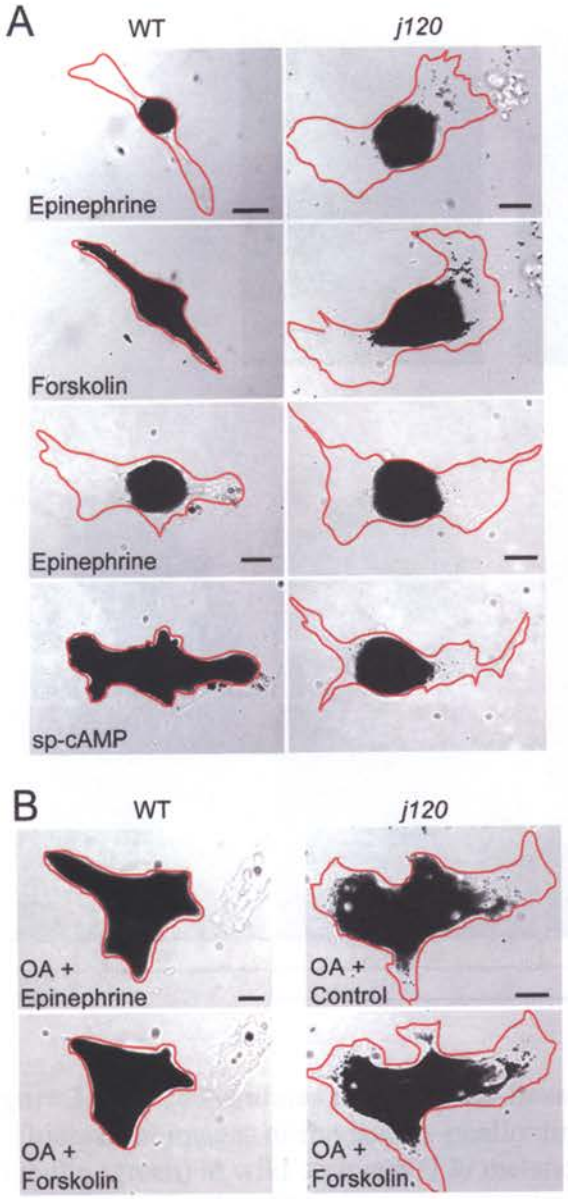

Figure 3.2 The *j120* gene product functions downstream of cAMP signaling

A. Images of cultured wild type and *j120* mutant melanocytes five minutes after applying compounds that lower or elevate intracellular cAMP levels. First and third rows: melanocytes were exposed to 0.1 mM epinephrine, which induces aggregation. Second and fourth row: forskolin (200 μM) or sp-cAMP (30 μM) elicits full melanosome dispersion in wild type but only partial dispersion in mutant melanocytes.

B. Response of cultured wild type and *j120* mutant melanocytes to the inhibition of the phosphatase that directs aggregation. Pre-treatment of cultures with 1 μM okadaic acid inhibited aggregation in wild type melanocytes, indicating that it is blocking phosphatase responsible for aggregation. *j120* melanocytes did not fully disperse their melanosomes when exposed to forskolin after treatment with 1μM okadaic acid overnight. (Scale Bar = 10 μm)

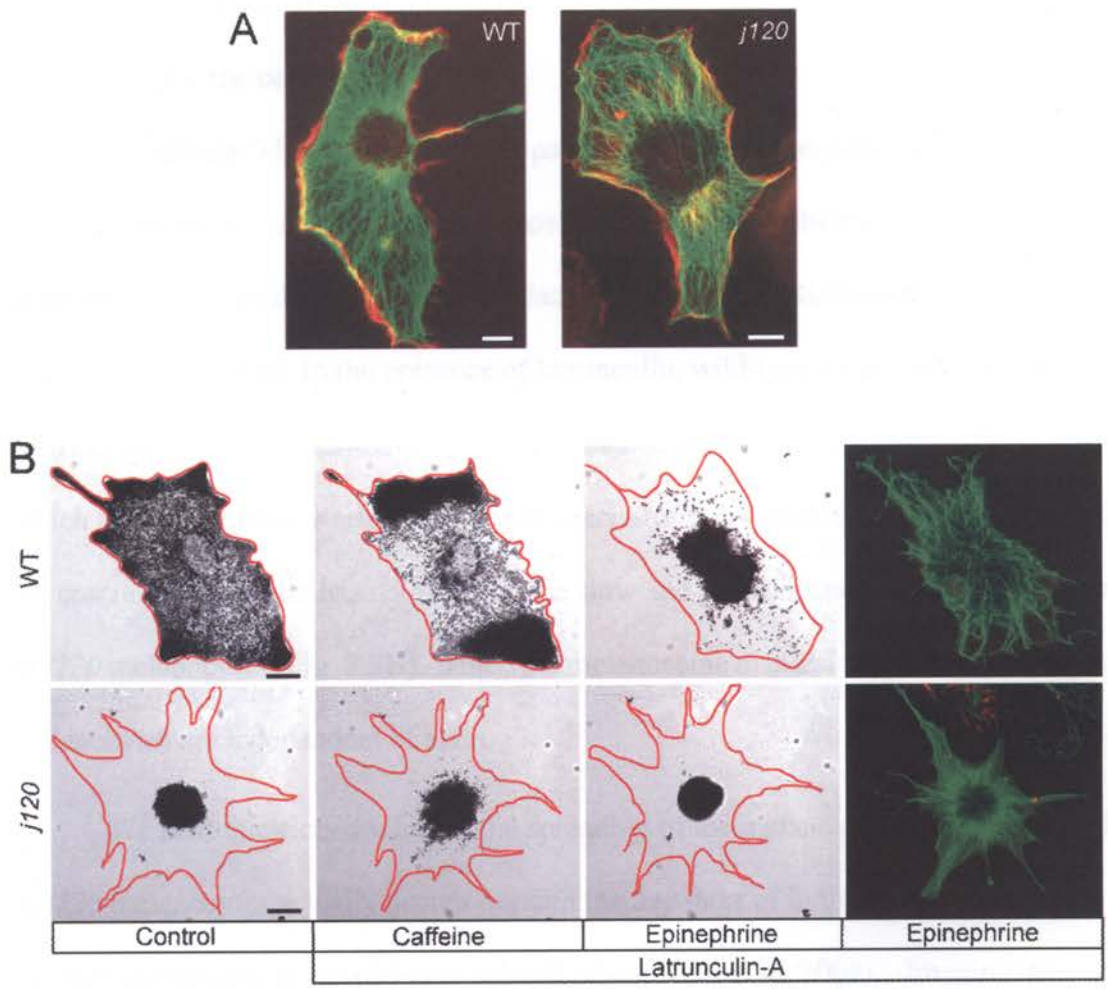

Figure 3.3: *j120* regulates microtubule-based transport
A. Fluorescent images of rhodamine-phalloidin labeled actin (red) and immuno-labeled α-tubulin (green) in wild-type and *j120* melanocytes. Overall cytoskeletal morphology appears normal in the *j120* melanocytes, and its microtubules extend to the periphery of the cell.
B. Aggregation and dispersion of melanosomes in the absence of cortical actin. Melanocytes were treated with 5 μM latrunculin A for 15 minutes to disrupt actin polymerization, then with caffeine to induce dispersion. WT melanocytes hyperdisperse their melanosomes when actin is disrupted. In contrast, *j120* was unaffected by actin disruption and still only partially dispersed its melanosomes. Rhodamine phalloidin staining confirmed the loss of actin in the treated melanocytes and microtubules were labeled green by immuno-staining α-tubulin.
(Scale Bar = 10 μm)

normal. Thus, the partial dispersion of *j120* melanosomes cannot be attributed to microtubules that fail to reach the periphery, or to actin filaments that are abnormally concentrated at the cell center.

To address whether the slow and partial melanosome transport in the *j120* mutant is caused by premature transfer of melanosomes from microtubules to actin early in dispersion, we treated melanocytes with latrunculin A, which disassembles actin filaments (Fig. 3.3B). In the presence of latrunculin, wild-type melanocytes respond to MSH or caffeine by hyper-dispersing their melanosomes to the cell margin (Fig. 3.3B), which confirms earlier work in a different species of fish (Rodionov, Hope et al. 1998). By contrast, latrunculin has no effect on the slow and partial dispersion of melanosomes in *j120* melanocytes (Fig. 3.3B). Thus, the melanosome dispersion defects in *j120* melanocytes are independent of actin.

We next questioned whether the spreading of the melanosomes during dispersion in *j120* melanocytes actually occurs through the transport of individual melanosomes along microtubules, as in wild-type cells (Rodionov, Yi et al. 2003). Imaging the movement of single melanosomes during dispersion in melanocytes from *j120* mutants is difficult because the melanosomes overall do not spread far and their images overlap. Therefore, we examined sequences of difference images created by subtracting from each incoming frame the previous frame (Schnapp, Crise et al. 1990). The summed stack of difference images reveals only what has moved during the sequence (Fig. 3.1B). This analysis indicates that the dispersing mass of melanosomes appears as amalgamated outwardly directed tracks, where each of these tracks corresponds to the movement of an individual melanosome along a linear path, consistent with transport along microtubules

(Fig. 3.1B, inset). On this basis we conclude that *j120* mutant melanosome dispersion is not driven by diffusion and that *j120* melanosomes are not "stuck" together in an aggregated mass or permanently stalled on microtubules. They do load onto and move along microtubules. The finding that a few melanosomes escape the pack through microtubule-based transport (Fig. 3.1B inset sequence) suggests that we can analyze the kinetics of their movements to understand further how the mutation impacts the regulation of microtubule-based transport.

_j120_ inhibits the frequency and persistence of minus-end travel episodes during dispersion.

The motion of individual melanosomes is normally bidirectional, with a bias toward minus- or plus-end travel depending on whether the population as a whole is aggregating or dispersing. An attenuated rate of dispersion like that seen in *j120* mutant melanocytes could result from either abnormally high plus-end travel or abnormally low minus-end travel. To address which of these two possibilities underlies the *j120* phenotype, we undertook a kinetic analysis of melanosome dispersion by tracking the movement of individual melanosomes.

We tracked single melanosomes (Fig. 3.4 and Table 3.1) 1.5 and 3 minutes after applying MSH. Melanosome tracks (Fig. 3.4A) consist of episodes of continuous outward (plus-end) or inward (minus-end) microtubule-based movement interspersed with episodes of undirected motion or pause (Gross, Tuma et al. 2002; Rodionov, Yi et al. 2003). The latter category, which we henceforth term "undirected motion" presumably corresponds to either a stopped state on the microtubule or to actin-based

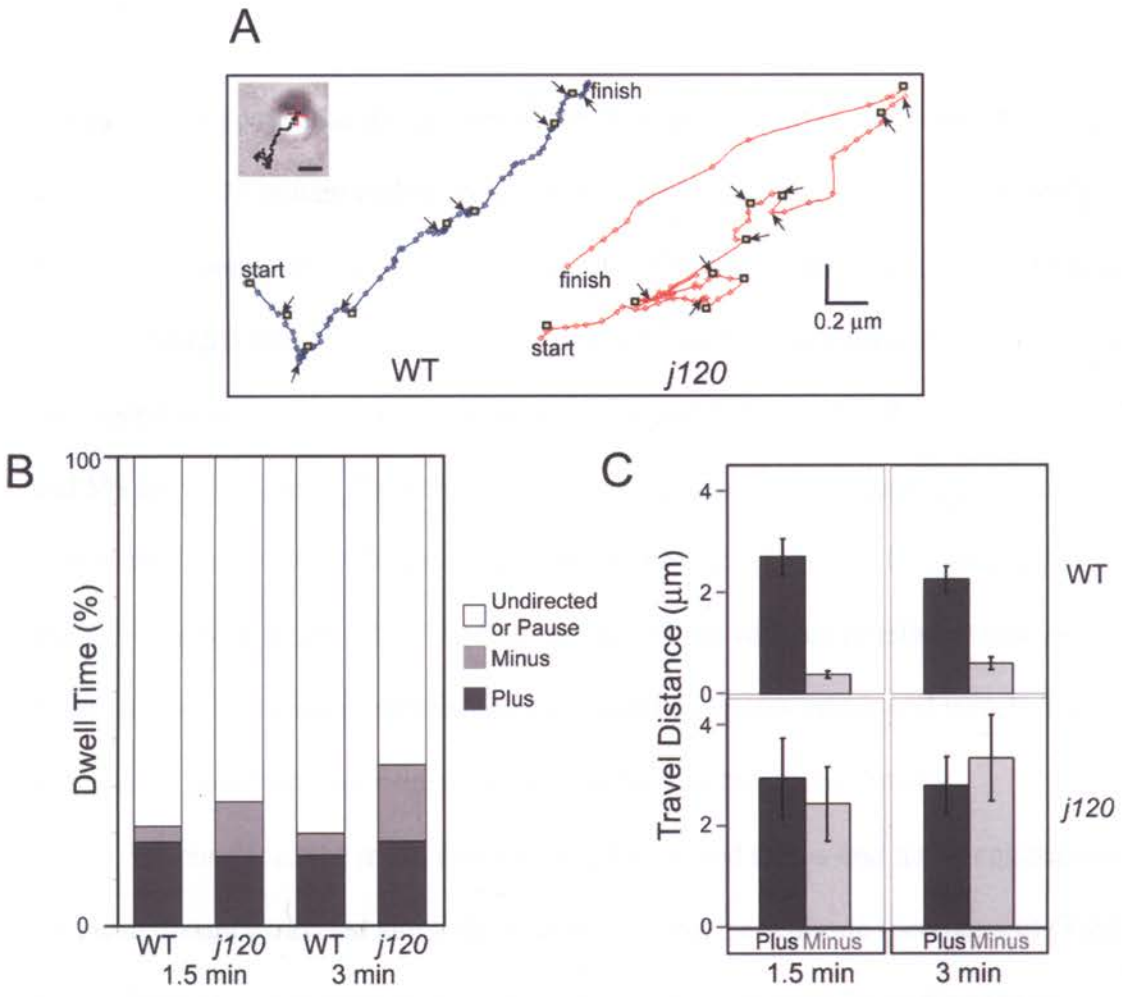

Figure 3.4 Kinetic parameters of individual melanosome movement during dispersion

A. Representative tracks from a wild-type and *j120* melanocytes. Each yellow box indicates the beginning of a directed move along the microtubule axis, and black arrows indicate pauses or periods of random movement. The tracks illustrate net movement toward the cell periphery (top-right side) of the wild-type melanosome and very little net-movement of the *j120* melanosome. Inset - DIC image and track of an individual melanosomes in a wild type melanocyte over 30 seconds; 1.5 minutes after the initiation of dispersion. (Scale Bar = 1 µm)

B. Fraction of time spent by melanosomes in each of the three different states. Graphs represent the cumulative fraction of time spent in each state for all melanosomes tracked. *j120* melanosomes spend more time moving toward the minus-end of microtubules during dispersion than wild-type.

C. Average travel distance of wild type and *j120* melanosomes along microtubules. Graphs show the product of the average run length and frequency of events found in Table 2. The distance traveled per minute toward the minus-end of microtubules is significantly greater in *j120* at both time points. (Error Bars = SEM)

transport. We pooled the dwell times in the three motion states across all tracks from wild-type or *j120* mutant melanosomes (Fig. 3.4B). Zebrafish melanosomes spend the majority of time in undirected motion (66% and 73% for *j120*; 79% and 80% for wild-type at 1.5 and 3 min). *j120* melanosomes spend less time in undirected motion because they spend more time in minus-end travel (12 % and 16% at 1.5 and 3 min for *j120*; 3% and 5% for wild-type). The percentage of time melanosomes engage in plus-end travel is very similar between *j120* and wild-type melanocytes (15% and 18% for *j120*; 18% and 15% for wild-type). These findings are consistent with the possibility that slow dispersion of *j120* mutant melanosomes is caused by excess minus-end travel. To address this possibility we measured travel in the two directions directly.

We computed the mean frequencies of plus- and minus-end travel episodes and the mean distance traveled along the microtubule axis per episode (persistence) (Table 3.1). These kinetic parameters together determine the average travel distance (d) that a melanosome moves toward the plus- versus the minus-end of a microtubule (Fig. 3.4C). Whereas mutant and wild-type melanosomes on average travel similar distances toward microtubule plus-ends at the two time points (2.9 ± 0.8 μm and 2.8 ± 0.6 μm for *j120*; 2.7 ± 0.4 μm and 2.3 ± 0.3 μm for wild-type), *j120* mutant melanosomes travel much further toward microtubule minus-ends (2.4 ± 0.7 μm and 3.3 ± 0.9 μm for *j120*; 0.4 ± 0.1 μm and 0.6 ± 0.1 μm for wild-type). Increases in both the frequency and persistence of minus-end motility episodes underlie the abnormally high minus-end travel of *j120* melanosomes (Table 3.1). Persistence frequency distributions (Figure 3.5), which are described by the sum of two decaying exponentials, reflecting short and long classes of travel episodes (Gross, Welte et al. 2000; Gross, Tuma et al. 2002), indicate that an

excess of long episodes is largely responsible for elevating the mean persistence of *j120* minus-end motility. In summary, the ratios of mean plus- to minus-end travel at the two time points are ~ 6:1 and 4:1 for wild-type, consistent with rapid global dispersion, while the *j120* ratios are ~ 1:1.

Table 3.1: Mean Kinetic Parameters of Microtubule Based Movement

	WT	WT	*j120*	*j120*	WT	WT	*j120*	*j120*
Travel direction	Plus-end	Plus-end	Plus-end	Plus-end	Minus-end	Minus-end	Minus-end	Minus-end
Time (minutes)	1.5	3	1.5	3	1.5	3	1.5	3
Run length (μm) (SEM)	0.32 (± 0.04)	0.27 (± 0.03)	0.43 (± 0.10)	0.23 (± 0.05)	0.13 (± 0.01)	0.16 (± 0.02)	0.40 ** (± 0.10)	0.32 * (± 0.10)
Events min^{-1} (SEM)	8.8 (± 0.9)	8.3 (± 0.9)	6.9 (± 1.3)	12.0* (± 1.7)	3.0 (± 0.5)	3.7 (± 0.6)	6.1 * (± 1.2)	11.3 ** ± 1.4)
# of cells	5	5	4	4	5	5	4	4
# of melanosomes tracked	86	76	36	39	86	76	36	39

The mean distance of travel events (run length) and the mean frequency of events per minute (events min^{-1}) in the minus-end direction are significantly different between WT and *j120* mutants at both 1.5 and 3 min post initiation of dispersion. In addition, the mean frequency of events per minute (events min^{-1}) in the plus-end direction are significantly different between WT and *j120* mutants at 3 min. (Student's T-test: **, $p < 0.0001$; *, $p < 0.05$)

We conclude that in wild-type cells, the function of the normal gene product is to suppress minus-end travel in response to MSH, and that this is essential for driving the rapid outward spreading of the melanosome population during dispersion. This fits with previous studies of black tetra fish and *Xenopus* melanocytes, which also show that the shift from aggregation to dispersion entails a decrease in minus-end travel (Gross, Tuma et al. 2002; Rodionov, Yi et al. 2003).

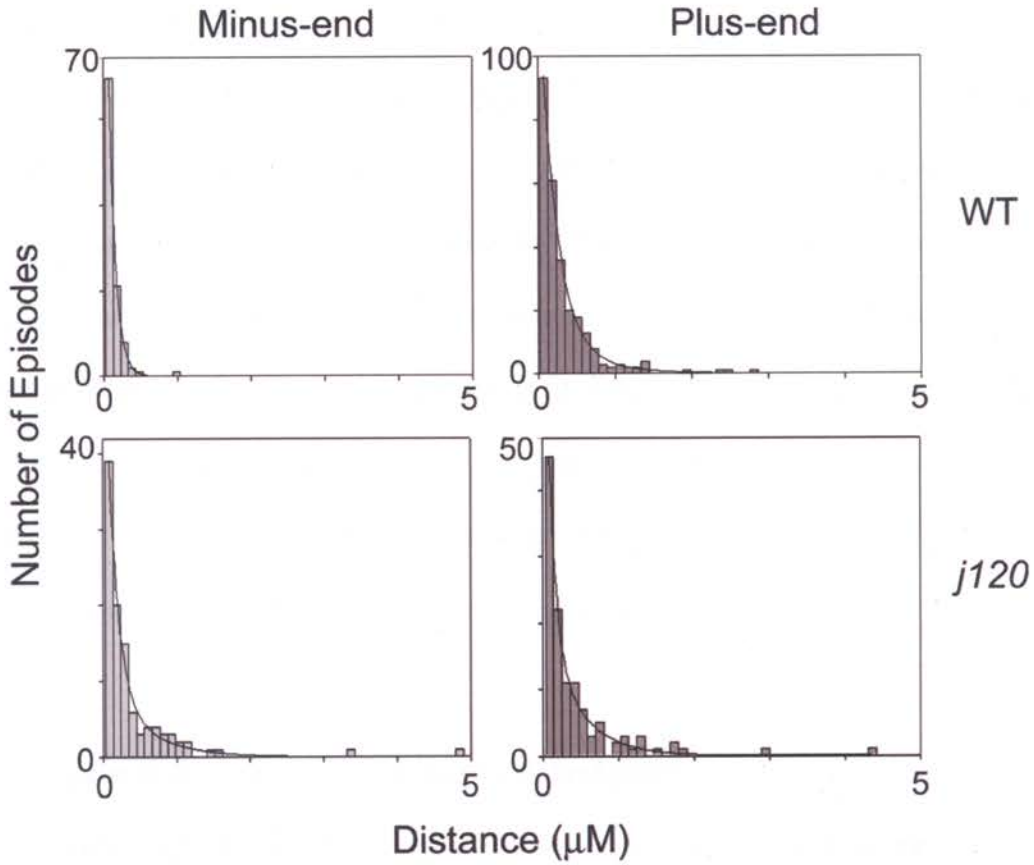

Figure 3.5: *j120* mutant melanosomes have a greater number of long travel episodes toward the minus-ends of microtubules

Distribution of lengths of WT and *j120* mutant melanosome travel toward the plus- or minus-ends of microtubules (0.1 μm bins). Frequency histograms shown are the run distances of melanosomes at 1.5 min post dispersion initiation. In WT and *j120* mutant melanocytes, both plus- and minus end directed histograms are well fit by the sum of two decaying exponentials (solid lines), indicating that there are two states of droplet travel - short runs and long runs (Gross, Guo et al. 2003). **Top row:** In WT there are far fewer long runs (greater than 0.7 μm) toward the minus-end of microtubules (left panel) than toward the plus-end (right panel). **Bottom Row:** For *j120* mutant melanosome travel events, the general shape of both frequency histograms is the same. Note the increase in long runs toward the minus-end of microtubules (bottom left panel) relative to WT (top left panel).

The mean frequency and persistence of travel episodes are constant from 1.5 to 3 min in wild-type melanocytes, while in mutant melanocytes the travel episode frequencies increase ~ two-fold for both directions (Table 3.1). This result indicates that in addition to its effects on the minus-end motor, the *j120* gene product also affects plus-end motor function. Furthermore, mutant melanosome travel episodes not only increase in frequency, they undergo a decrease in persistence such that, for each direction, there is no change in mean travel at the two time points (Fig. 3.4C). Thus, the mutation unmasks a novel property of the motor coordination machinery, which is to balance travel in the two directions through compensatory changes in travel episode persistence and/or frequency. In wild-type cells, this balance is off-set to achieve rapid global dispersion.

The *j120* gene is the zebrafish ortholog of melanophilin

To shed light on how *j120* regulates melanosome motor activities, we characterized the molecular basis of the mutation. We mapped the gene to Chromosome 6, to within 0.16 cM (0 crossovers in 1208 meioses) of an SSLP marker developed from BAC sequence BX649631 (Fig 3.6A). Because the ratio of physical to genetic distance in zebrafish is approximately 1000 Kb/cM, this indicates that our tightly linked marker may be within 160 Kb of the *j120* mutation. Accordingly, we examined a region 160 Kb on either side of our marker for candidate genes. Among 6 genes evident within this interval, the only one with a known relationship to melanosome transport is an ortholog (see below) of melanophilin (MLPH). In mouse and human, MLPH is genetically required for the accumulation of melanosomes at the dendritic tips of melanocytes (Fukuda, Kuroda et al. 2002; Wu, Rao et al. 2002; Menasche, Ho et al. 2003).

Furthermore, the mouse MLPH mutant phenotype, called *leaden* (Wu, Rao et al. 2002), is similar to that of *j120* in zebrafish: melanosomes are clustered in the cell center and not dispersed throughout the cell.

The *mlpha*^{j120} cDNA sequence (designated *mlpha* because of a predicted gene duplication, Figure 3.8 A,B) reveals a premature stop codon that would truncate the protein after residue 238 (Fig. 3.6B). This is consistent with the *mlpha*^{j120} genomic DNA sequence, wherein a point mutation at a canonical acceptor splice site is predicted to introduce this stop codon into Exon 7 through mis-splicing (Fig. 3.6D). As predicted, RT-PCR reveals that the mis-spliced mRNA is evident in fish homozygous and heterozygous for the mutation, but not in wild-type fish (Fig. 3.6C). Premature stop codons usually lead to mRNA degradation through nonsense mediated decay (Maquat 2002) which would create a null. To address this possibility, we used in situ hybridization to evaluate *mlpha* mRNA expression in 2-day-old embryos. Whereas *mlpha* mRNA staining is conspicuous in the melanocytes of wild-type embryos, it is weak in *j120* embryos (Fig 3.6E), consistent with a null mutation.

To confirm whether a mutation in *mlpha* can account for the *j120* mutant phenotype, we reduced expression of *mlpha* mRNA in wild-type embryos with a morpholino anti-sense oligonucleotide against the translation initiation site of the endogenous gene. This reproduces the *j120* phenotype (Fig 3.7). Melanosome dispersion recovered within 7 days (not shown), which is likely due to loss of morpholino oligonucleotide effectiveness by dilution (Heasman, 2002 #243). Taken together, the molecular analysis of *j120* and the morpholino phenocopy experiments indicate that the gene product of *j120* is zebrafish Mlpha.

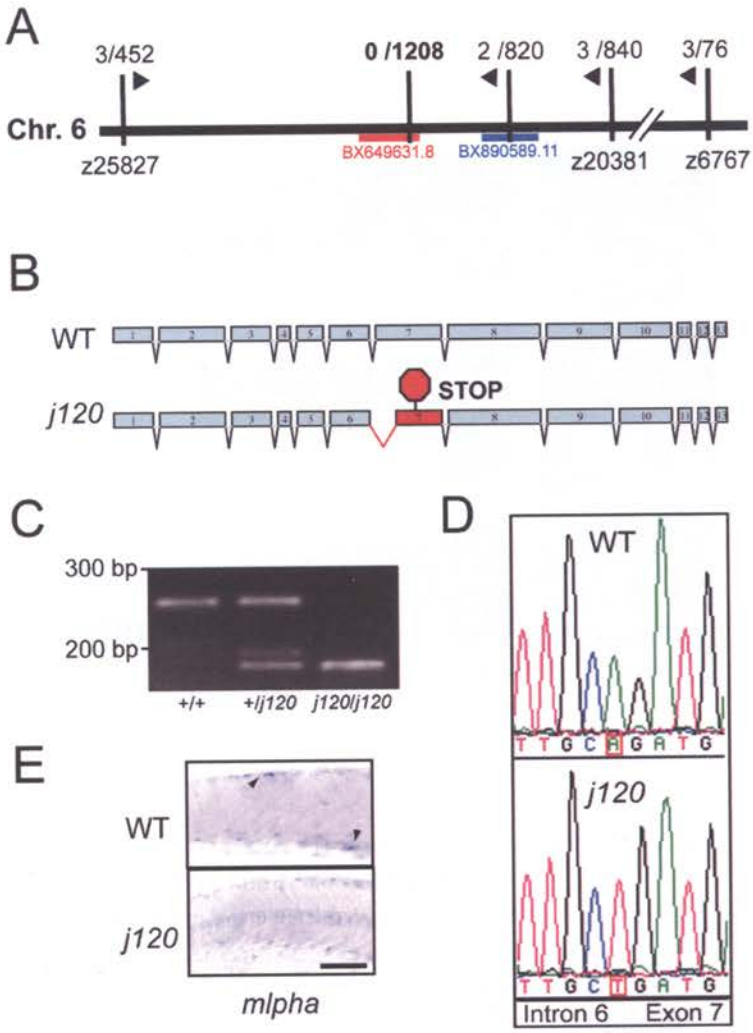

Figure 3.6: Identification of *j120* as zebrafish melanophilin
A. Linkage analysis of *j120*. The *j120* gene is located on chromosome 6 and linked to a SSR marker in the BAC clone BX649631.8, which contains zebrafish melanophilin.
B. Intron-exon schematic of zebrafish *mlpha*. A mutation in the *j120* gene leads to abnormal splicing of exon 7 and the introduction of a stop codon.
C. RT-PCR of *mlpha* exon 7. Gel electrophoresis reveled a 80 bp smaller product in the *j120* mutant than in wild-type. This smaller product is the size we would predict from the abnormal splicing of exon 7.
D. Genomic DNA sequence of Mlpha intron/exon 7. Alignment of wild type and mutant sequence revealed a mutation (A to T) in the splice acceptor site of intron 7 in *j120/j120*.
E. In situ hybridization of 2 day old embryos with anti-sense probe for *mlpha*. Arrows indicate expression in cells that correspond with melanocytes. (Scale Bar = 100μm)

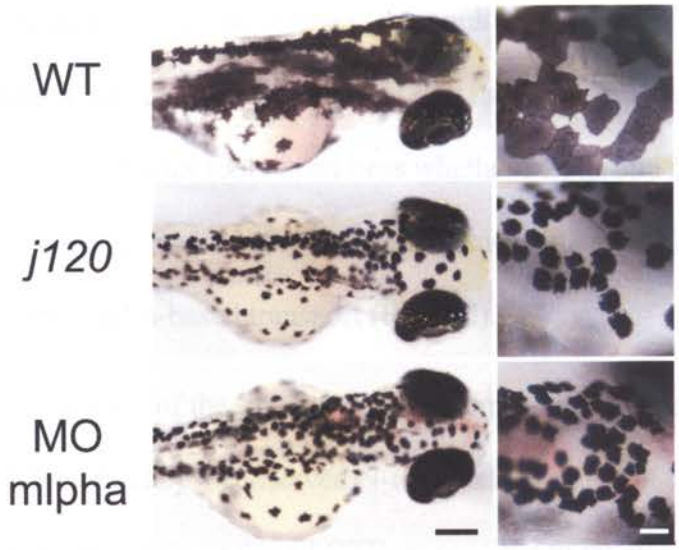

Figure 3.7: Morpholino knockdown of Mlpha phencopies the *j120* mutant
Morpholino knock-down of Mlpha in 3 day old embryos. Translation of *mlpha* mRNA was blocked with a morpholino antisense oligo targeted to the 5'UTR. Knockdown of Mlpha in wild type embryos exactly phenocopies *j120* mutants. (Scale Bar = 200 μm, 50 μm in inset)

The identification of *j120* as Mlpha creates a paradox, as on the one hand the mutant phenotype affects microtubule-based transport and is independent of actin (Figs. 3.3 and 3.4), while on the other mammalian melanophilin is an organelle associated receptor for myosin V and integral for actin-based transport. To investigate this disparity further, we used bioinformatics tools to address whether Mlpha and mammalian MLPH are indeed direct orthologs, and whether the amino acid sequence of Mlpha is consistent with a potential role in actin-based transport (Fig. 3.8).

MLPH is a member of the synaptotagmin like protein (Slp) family (Fukuda, Kuroda et al. 2002) marked by a conserved N-terminal domain that interacts with GTP activated Rab27a, a membrane anchored GTPase required in many cell types for the transport of particular vesicular organelles, e.g. secretory vesicles (Tolmachova, Anders et al. 2004). This family's most thoroughly characterized member is mammalian melanophilin (MLPH). MLPH is an adaptor through two structurally distinct interaction motifs (Fig. 3.8B), one for Rab27a, the other for myosin V, it links myosin V to the melanosome membrane. This enables melanosomes to switch from microtubules to cortical actin, and to accumulate within the melanocyte dendritic tip (Li, Ikebe et al. 2005; Hume, Tarafder et al. 2006) where they are then taken up by the keratinocyte (Wu and Hammer 2000). Homologous proteins in other cells also serve as adaptors that couple a myosin to Rab27a, though in these cases Rab27a is anchored in the membranes of other organelles (Kuroda, 2005 #100; Desnos, 2003 #242).

Multiple lines of evidence indicate that Mlpha and mammalian MLPH are orthologs. Among all mammalian Rab 27a effectors, the *mlpha* nucleotide sequence is most similar to melanophilin (Fig. 3.8A). Mlpha shares 33% and 37% amino acid

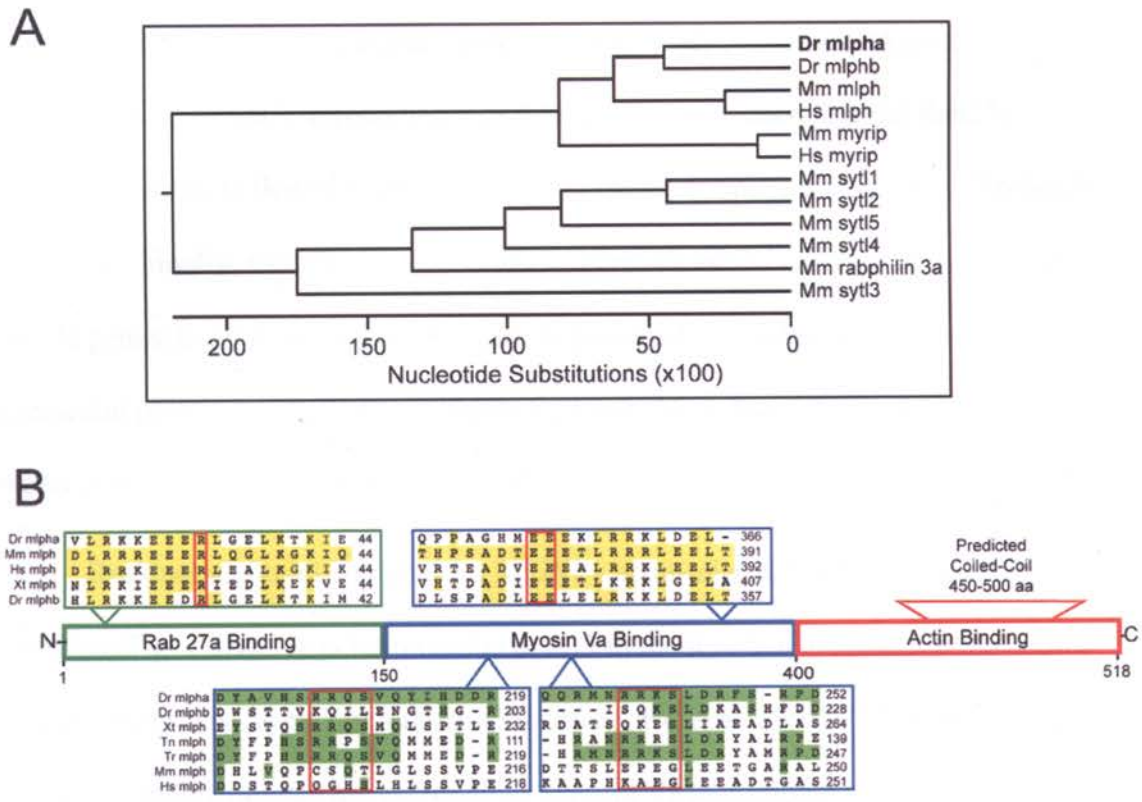

Figure 3.8: Zebrafish Mlpha is an ortholog of mammalian melanophilin
A. A phylogenetic tree derived from an alignment of zebrafish Mlpha with mammalian members of the synapotagmin-like protein family. Mlpha is most closely related to mouse and human melanophilin (MLPH).
B. Sequence alignments of zebrafish Mlpha with mammalian MLPH. These alignments uncovered sequence conservation in regions shown to be critical for linking melanosomes to actin in mammals (Menasche, Ho et al. 2003; Kuroda, Itoh et al. 2005; Hume, Tarafder et al. 2006). Yellow highlights residues matching mouse MLPH sequence, and reveals conservation of a critical arginine residue in the Rab27a binding domain and two critical acidic residues in the Myosin Va binding domain. In addition, residues at the C-terminal region of Mlpha are predicted to be coiled-coils (Paircoil program). Since a coiled-coil in the actin binding region is essential for Myosin Va recruitment to melanosomes in mammals (Hume, Tarafder et al. 2006), this further supports a role for Mlpha in actin-based melanosome transport in zebrafish. Sequences of two predicted PKA phosphorylation motifs (RxxpS) in zebrafish were also compared with other species. Green highlights residues matching zebrafish Mlpha sequence, and revealed conservation of predicted PKA phosphorylation sites between Mlpha and predicted Mlph sequences from *Xenopus* and two other fish species (*Takifugu rubripes* and *Tetraodon nigroviridis*), but not human and mouse.

identity with the mouse and human proteins, respectively, which themselves are only 65% identical. *mlpha*'s intron/exon structure in the region encoding the Rab27a binding domain is similar to that of mammalian melanophilin, but differs from other Slp family members. Finally, there is syntany between zebrafish *mlpha* and mouse and human MLPH genes: in all three species this gene is proximal to leucine rich repeat (in FLII) interacting protein 1 (LRRFIP1). Taken together, these data indicate that zebrafish *mlpha* is the ortholog of mammalian MLPH.

Residues and motifs critical for MLPH's actin-related function are conserved in zebrafish (Fig. 3.8B). A point mutation at R35 in the Rab 27a binding domain causes Griscelli Syndrome Type 3 in humans (Menasche, Ho et al. 2003); E380 and E381 in the myosin V binding domain are essential for myosin Va recruitment to melanosomes in mammals (Kuroda, Itoh et al. 2005; Hume, Tarafder et al. 2006); and Mlpha's C-terminus contains the predicted coiled-coil region that enhances the binding of MLPH to myosin Va in mouse melanocytes (Hume, Tarafder et al. 2006). These observations suggest that the zebrafish protein likewise links myosin V to melanosomes for the purpose of switching transport from microtubules to actin during dispersion.

If Mlpha's sole function were to attach myosin V to melanosomes, the mutant would hyper-disperse its melanosomes to the cell margin (Fig. 3.3B). Instead the mutation slows dispersion due to a failure to suppress minus-end travel on microtubules. The observation that this phenotype is unaffected by actin disassembly is very significant, as this is what excludes the possibility that the mutation *mlpha*^{j120} alters minus-end travel on microtubules indirectly by disrupting the melanosome-myosin V linkage. Were it not for this observation, one might have suspected that the failure to suppress minus-end

motility in the mutant results from disruption of the myosin V – actin interaction, as in *Xenopus* melanocytes, there is evidence that the tension placed on the melanosome through myosin V walking on actin shortens minus-end travel episodes, thereby enabling plus-end travel to dominate during dispersion (Gross, Tuma et al. 2002). Our findings indicate a second basis for interplay between Myosin V and dynein motor activities, through Mlpha's dual functions (Fig. 3.9).

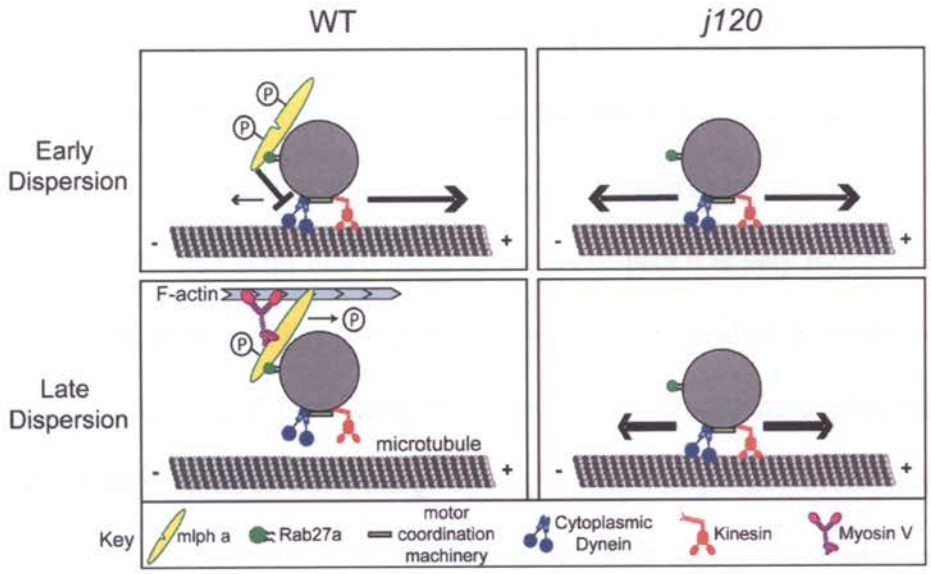

Figure 3.9: Model of Mlpha's function in zebrafish melanocytes
Left panels: during early dispersion, when cAMP levels are high, Mlpha suppresses dynein-mediated movement in WT melanocytes. The result is melanosome movement along microtubules predominantly toward the periphery of the cell. Later in dispersion, as cAMP levels decline, melanophilin releases its inhibition on dynein-mediated movement and implements the transfer of melanosomes from microtubule- to actin-based transport. The actions of melanophilin may be modulated by differing levels of PKA phosphorylation during dispersion i.e. maximal phosphorylation early in dispersion (top left), followed by decreased phosphorylation late in dispersion (bottom left). Right panels: in the absence of melanophilin, as observed in *j120* mutants dynein-mediated moves are not suppressed and the melanosomes are not transferred to the cortical actin meshwork. This results in the overall population of melanosomes in *j120* mutant melanocytes never reaching the cell periphery.

What coordinates Mlpha's dual functions during dispersion? To achieve a uniform distribution of melansomes, an initial period of outwardly directed microtubule-based transport is followed by a period of ever increasing actin-based transport (Rodionov, Yi et al. 2003). The time course of the transition from microtubule to actin-based transport is crucial for achieving an even distribution of melanosomes in the dispersed state. Because this time course correlates with the rise and fall of cAMP levels during dispersion (Rodionov, Yi et al. 2003), this implies that the phosphorylation state of a protein with one or more PKA phosphorylation sites likewise changes, and that the phosphorylation state of this protein leads to changes in the relative activities of microtubule- and actin-based motors. In this respect it is very interesting that highly conserved, putative PKA phosphorylation motifs are evident in mlpha and its homologues in other lower vertebrates, but are not present in mammalian melanophilin (Fig. 3.8B), which do not undergo aggregation and dispersion in response to cAMP. One obvious possibility is that the phosphorylation state of these sites in Mlpha are modulated by the changing cAMP levels during dispersion, and that depending on the phosphorylation state, either the myosin-V or dynein suppression functions of Mlpha are brought into play (Fig. 3.9). Although there is no direct evidence for the dynamic phosphorylation of melanophilin or any other molecularly identified protein during dispersion, a study many years ago in African cichilid reported that a 57 kD polypeptide, similar in size to zebrafish Mlpha, is phosphorylated during dispersion (Rozdzial and Haimo 1986). Addressing whether Mlpha phosphorylation has a role in the regulation of melanosome transport is an obvious subject for further research.

CHAPTER 4

Characterization of *smutz* and *b867* - melanosome transport mutants with apparent defects in actin-based transport

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Introduction

Short-range transport along actin filaments plays an important role in the delivery of intracellular cargo such as dense-core secretory vesicles (Varadi, Tsuboi et al. 2005), melanosomes (Wu, Bowers et al. 1997), and synaptic vesicles (Libby, Lillo et al. 2004; Watanabe, Nomura et al. 2005). As discussed in Chapters 1 and 3, much of what is known about the mechanisms of actin-based transport regulation has been uncovered by studies in *Xenopus* and fish melanocytes (Rodionov, Yi et al. 2003; Snider, Lin et al. 2004). Moreover, many of the proteins involved in actin-based transport in mammalian melanocytes have been identified through forward genetics (Wu, Bowers et al. 1997; Wu and Hammer 2000; Matesic, Yip et al. 2001). Yet even though great progress has been made through these studies, there are still deficiencies in our understanding of how actin-based motors are regulated to efficiently transport cargos on actin-filaments.

The molecular motor that has been implicated in short-range actin-based transport in many types of cells, including neurons and melanocytes, is Myosin V (Brown 1999; Langford 2002). Class V myosins are processive actin-based motors that function in mRNA transport, membrane trafficking, and cell polarity (Provance and Mercer 1999). Members of the Mysoin V family function as dimers to transport cargo in a "hand-over-hand" manner along actin filaments (Mehta, Rock et al. 1999; Vale 2003). Each Myosin V monomer is composed of a globular cargo binding domain, a tail domain containing several segments of coiled-coil, a neck domain containing six IQ motifs that bind calmodulin, and a head region containing the actin and nucleotide binding sites (Wang, Thirumurugan et al. 2004). Of the three isoforms of Myosin V that exist in vertebrates, Myosin Va is the most abundant and is highly enriched in neurons (Watanabe, Nomura et

al. 2005). Mutations in Myosin Va in humans cause Griscelli Syndrome Type 1 which, in addition to partial albinism, is associated with severe neurological abnormalities (Pastural, Barrat et al. 1997).

There are several potential mechanisms by which Myosin Va may be regulated to engage and release cargo. One mechanism that has been discussed in detail in this dissertation is binding to melanophilin, which both engages and activates Myosin Va activity in melanocytes. Another is phosphorylation of the cargo-binding domain by Calcium-Calmodulin-Dependant Protein Kinase II (CamKII). During mitosis, CamKII phosphorylates the globular tail domain of Myosin V, which leads to the dissociation of Myosin V from vesicular cargo (Karcher, Roland et al. 2001; Wollert, Weiss et al. 2002) and the inhibition of Myosin V-driven transport. A third potential mechanism of regulation is calcium signaling through calmodulin. Binding of calmodulin to the IQ motifs converts Myosin V from a folded-inactive conformation to an open-active conformation in vitro (Wang, Thirumurugan et al. 2004). Finally, dephosphorylation of an unknown target protein by PP2A is required for the transfer of melanosomes from actin- to microtubule-based transport in melanocytes (Reilein, Tint et al. 1998). While these studies shed light into how Myosin Va may be regulated, the mechanisms by which these signals temporally and spatially regulate Myosin Va transport are still unclear.

In our small-scale screen of zebrafish pigment mutants, I identified several additional mutations that disrupt the process of melanosome transport. Here I characterize two mutants that appear to have defects in actin-based transport: *smutz* and *b867*. *smutz* fish melanocytes were unable to completely aggregate their pigment, which suggested that the *smutz* gene might be involved in regulating the release of melanosomes

from actin, hence inactivating Myosin Va, during dispersion. However, preliminary analysis of *smutz* melanocytes in cell culture showed variable responses to peptide hormones that initiate aggregation and dispersion. These results indicate that the defect in *smutz* is not as simple as the whole-larvae phenotype suggests.

In contrast, *b867* mutant melanocytes exhibited an unambiguous phenotype both in larvae and in cell culture during dispersion – an accumulation of melanosomes at the melanocyte periphery, or hyper-dispersion. This is the same phenotype observed when actin-based transport is disrupted in fish melanocytes, and this finding suggests that the *b867* gene is critical for actin-based, but not microtubule-based, transport. Preliminary mapping of the *b867* gene revealed its position to be on chromosome 18 in a genetic interval containing Myosin Va, which is a strong candidate gene. However, analysis of *b867* cDNA revealed that it contains no apparent mutation in the coding region of Myosin Va. This raises the interesting possibility that the *b867* gene may be a novel regulator of actin-based transport.

Materials and Methods

Larvae Screening Assay: The screening assay was applied as previously described in Chapter 2.

Primary Melanocyte Culture and Isolated Melanocyte Imaging: Performed as previously described in Chapter 2.

Analysis of Whole Cell Melanosome Distribution: Performed as previously described in Chapter 3.

Positional Cloning: Homozygous *b867* in an AB background strain were outcrossed with wild-type WIK strain fish to make heterozygous WIK/AB *b867* carriers. These fish were mated and their progeny screened for the *b867* phenotype, then fixed in methanol. Mapping was performed as described in reference (Dahm 2002). Linkage to chromosome 6 was established using SNP markers and was graciously performed by Robert Geisler (Max Planck Institute for Developmental Biology). Additional genetic mapping was performed using SSR markers found in the following databases: The Zebrafish Information Network, zfin.org; Tübingen Map of Zebrafish Genome, wwwmap.tuebingen.mpg.de/; and Zebrafish SSR Search, danio.mgh.harvard.edu/markers/ssr.html. Zebrafish cDNA was synthesized via RT-PCR (BD Biosciences, San Jose, CA; Advantage RT for PCR kit) using mRNA isolated from wild-type and homozygous *b867* larvae per manufacturers instructions (Ambion Inc, Austin, TX; Poly(A)Purist™ Kit). Zebrafish Myosin Va cDNA was amplified with the primers GTGATTTGCAGGAACACCG and GCTGATTATCATAGAAATCAATGAGAG; CAAACTACAGGCTATAAACGCCC and GGCTGGTCTCTAGAGTGTTCAAA; GGCATGGAGAACAAGATCATG and TTTCTTCAGCTTACGAACGGTC; ATGCTGACTACATCAACGATGAC and GTGGGGTGATTGACAGGTCTA.

Primers were designed with information obtained from the Zebrafish Genome-Sequencing Project, ensembl.org/Danio_rerio/index.html.

Results

smutz – a mutation with varying defects in actin-based transport

The zebrafish pigment mutant *smutz* is a recessive allele generated in an ENU mutagenesis screen. It was identified it as a melanosome transport mutant because *smutz* larvae are unable to completely aggregate their melanosomes when exposed to MCH (Fig. 4.1A). The mutant's name is based on "schmutz," the German-derived Yiddish term for "dirt," because accumulations of melanosome remain stuck in the periphery of the melanocyte during aggregation, giving the melanocyte a dirty appearance. This phenotype suggested that *smutz* mutants are unable to completely release their melanosomes from the actin cytoskeleton during aggregation. The defect is likely melanocyte-specific because homozygous *smutz* mutants otherwise appear normal and are viable.

To further analyze melanosome transport in *smutz* mutants, I isolated their melanoctyes in cell culture and characterized their responses to MCH, MSH, and epinephrine. Surprisingly, most *smutz* melanocytes completely aggregated their pigment in response to epinephrine or MCH (Fig. 4.1B,C), which is unlike the phenotype observed in screened larvae. This result indicated that the defect in *smutz* melanocytes was not melanosome release from the actin cytoskeleton during aggregation. Moreover, nearly half the melanocytes abnormally dispersed their melanosomes in response to MSH, which is something we also did not observe in *smutz* larvae; approximately 13% of the melanocytes examined only partially dispersed their melanosomes, while 33% unevenly dispersed their melanosomes as seen by accumulations at the periphery of the cell (Fig 4.1C). While these results are preliminary, they indicate that a cellular

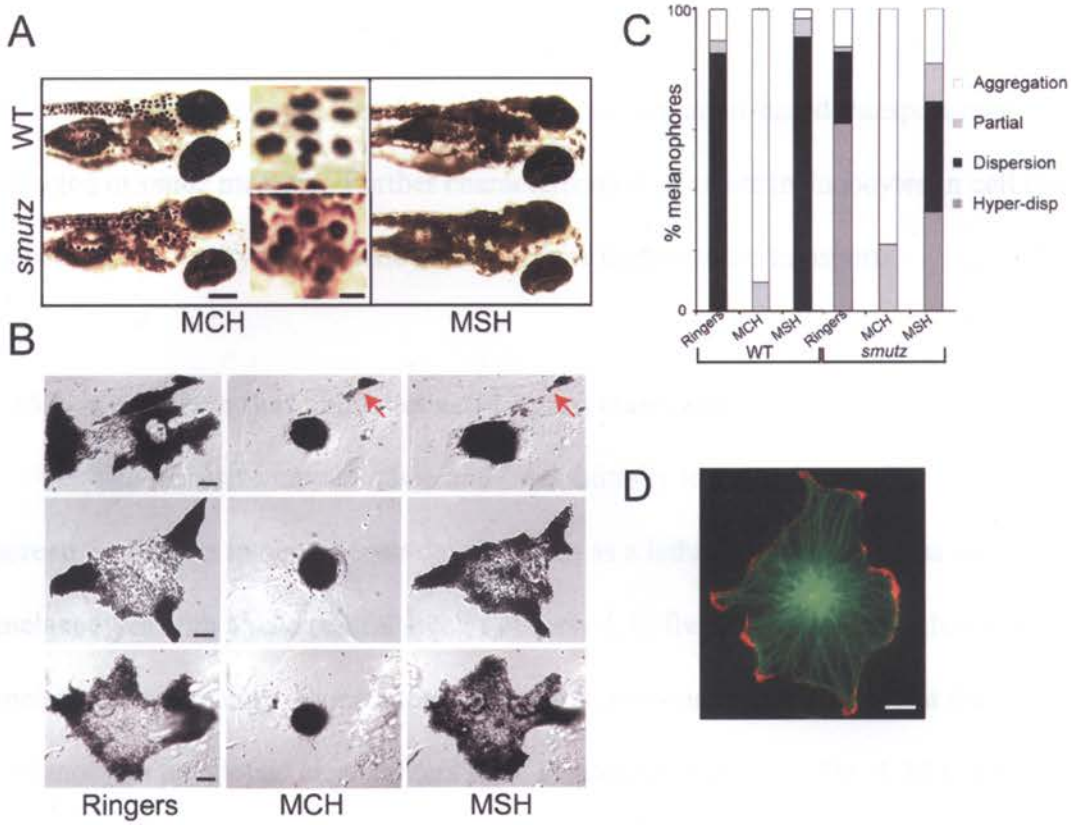

Figure 4.1: Characterization of melanosome movement in *smutz* mutants

A. Melanosome transport screening assay applied to *smutz* mutants. Five day old WT and *smutz* mutant larvae were exposed to 5 μM MCH followed by 10 μM MSH for 10 min each. In contrast to WT, *smutz* mutants did not completely aggregate their melanosomes when exposed to MCH. When exposed to MSH, pigment dispersion in *smutz* mutants appeared normal. (Scale Bars = 200 μm; 50 μm in inset)

B. Representative images of *smutz* mutant cultured melanocytes after exposures to 1 μM MCH and 0.5 μM MSH for 10 min each. All three melanocytes appeared hyperdispered in Ringers prior to application of hormone. Top row: following exposure to MCH for 10 min, a subset of melanosomes failed to aggregate and were retained in one of the processes (red arrow). Exposure to MSH for 10 minutes led to only partial dispersion. Middle row: exposure to MCH led to normal aggregation, but exposure to MSH led to hyperdispersion of melanosomes. Bottom Row: aggregation in response to MCH and dispersion in response to MSH appeared normal.

C. Fraction of cultured wild-type and mutant smutz melanocytes that respond to MCH and MSH with aggregation, partial displacement, full dispersion, or hyperdispersion. Melanocyte cultures were treated for 10 min with 0.5 μM MSH or 1 μM MCH. The majority of both WT and smutz melanocytes aggregated their melanosomes in response to MCH. However, in response to MSH, over half of the smutz mutant melanocytes examined responded abnormally. (n = 96 and 55 melanocytes, for wild type and mutant, respectively)

D. Fluorescent images of rhodamine-phalloidin labeled actin (red) and immuno-labeled α-tubulin (green) in wild-type and *smutz* melanocytes. Overall cytoskeletal morphology appears normal in the *smutz* melanocytes. (Scale Bar = 10 μm)

mechanism regulating the transfer of melanosomes to actin-based transport may be affected in *smutz* mutants. Further characterization of *smutz* melanocytes in cell culture is needed to identify the specific defect in their melanosome transport.

b867 - a mutation that abolishes actin-based transport

The recessive mutant allele *b867* was initially identified in an ENU mutagenesis screen for defects in neural-crest development as a lethal mutation that has stellate melanocytes with a pale central area. I observed, in five-day-old larvae, that *b867* melanocytes unevenly disperse their pigment in response to MSH and that the melanocytes developed clear centers after 10 minutes exposure (Fig. 4.2A). Exposure of isolated *b867* melanocytes in culture to caffeine or MSH elicited a striking defect in melanosome dispersion. Instead of evenly dispersing throughout melanocytes, *b867* melanosomes accumulated at the periphery of the cell (Fig. 4.2B,D). This hyper-dispersion phenotype was identical to that of wild-type zebrafish melanocytes that have been treated with latrunculin A to disrupt the actin cytoskeleton (Fig. 4.2C, Fig. 3.3B). However, the actin network within *b867* melanocytes appears normal when visualized with rhodamine-phalloidin staining (Fig. 4.2B). Thus, *b867* appears to disrupt actin-based transport in zebrafish melanocytes, but not through a direct effect on the actin filaments themselves.

Subsequent genetic mapping indicated that the *b867* gene was located on chromosome 18 between z7142 and z8343, and additional linkage analysis revealed that *b867* was closely linked to marker z58894 (0 recombinations/546 meiosis) (Fig. 4.2E).

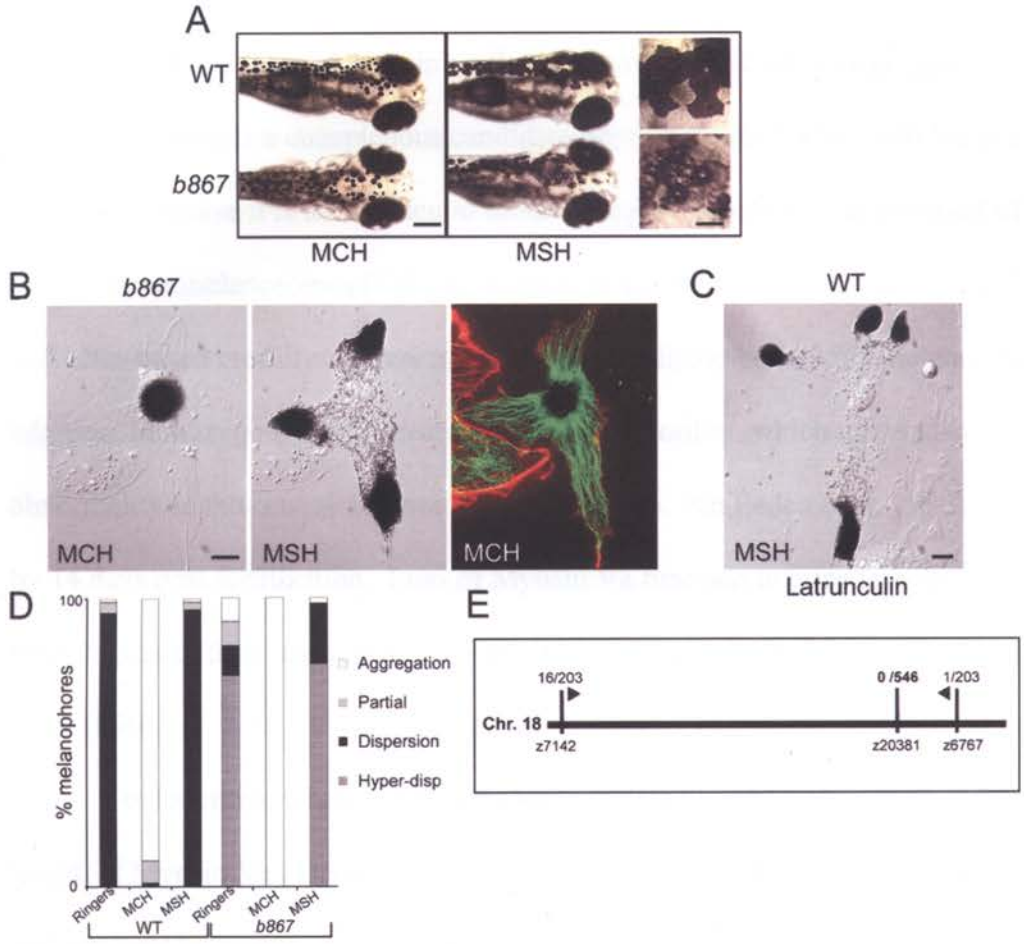

Figure 4.2: Characterization of melanosome movement in *b867* mutants

A. Melanosome transport screening assay applied to *b867* mutants. Five day old WT and *b867* mutant larvae were exposed to 5 μM MCH followed by 10 μM MSH for 10 min each. Following exposure to MCH, aggregation of pigment in *b867* mutants appeared normal. However, following exposure to MSH, *b867* mutant melanocytes did not evenly disperse their pigment. (Scale Bars = 200 μm; 50 μm in inset)

B. Images of *b867* mutant cultured melanocytes after exposures to 1 μM MCH and 0.5 μM MSH for 10 min each. The mutant melanocyte aggregated its melanosomes normally in response to MCH, but hyperdispersed it's melanosomes in response to MSH. Fluorescent image of rhodamine-phalloidin labeled actin (red) and immuno-labeled α-tubulin (green) shows that cortical actin is present *b867* mutant melanocytes. (Scale Bar = 10 μm)

C. Image of WT melanocyte dispersion in the absence of cortical actin. WT melanocytes were treated with 5 μM latrunculin A for 15 minutes to disrupt actin polymerization, then with 0.5 μM MSH to induce dispersion. WT melanocytes hyperdisperse their melanosomes when actin is disrupted, which is the same phenotype observed in *b867* mutant melanocytes with intact cortical actin. (Scale Bar = 10 μm)

D. Fraction of cultured wild-type and mutant b867 melanocytes that respond to MCH and MSH with aggregation, partial displacement, full dispersion, or hyperdispersion. Melanocyte cultures were treated for 10 min with 0.5 μM MSH or 1 μM MCH. The majority of both WT and b867 melanocytes aggregated their melanosomes in response to MCH. However, in response to MSH, 77% of b867 mutant melanocytes hyperdispersed. (n = 77 and 48 melanocytes, for wild type and mutant, respectively)

E. Linkage analysis of *b867*. The *b867* gene is located on chromosome 18 and linked to SSR marker z20381.

While the genetic interval containing the *b867* mutation is fairly large (approximately 366 kb), it contains a conspicuous candidate gene - Myosin Va. Myosin Va is a strong candidate because it is the molecular motor involved in actin-based transport of mammals and *Xenopus* melanosomes (Fukuda, Kuroda et al. 2002; Kashina, Semenova et al. 2004) and actin-based motility appears to be completely disrupted in *b867* melanocytes. In addition, homozygous *b867* larvae have reduced motility, which may indicate an abnormality in the central nervous system (Granato, van Eeden et al. 1996), and they die by 14 days post fertilization. Loss of Myosin Va function in mice is associated with neurological defects and results in death at two to three weeks of age (Strobel, Seperack et al. 1990).

To determine whether Mysosin Va is mutated in *b867*, we sequenced the full-length of Myosin Va cDNA obtained from homozygous *b867* mutants, heterozygous siblings, and homozygous wild-type fish of C32 and WIK background. Alignments of the sequences showed no notable differences between wildtype and *b867* Myosin Va cDNA. While this doesn't rule out Myosin Va as the affected gene, it does indicate that the *b867* mutant phenotype it is not due a mutation in the coding sequence of Myosin Va.

Discussion

Here I report two zebrafish mutants with phenotypes that indicate defective actin-based melanosome transport. One of the mutants, *smutz*, retained many of its melanosomes in the periphery of melanocytes during aggregation in the whole larvae, which initially suggested a defect in transferring a subset of the melanosomes from actin- to microtubule- based transport. However, in *smutz* melanocytes isolated in culture, most

melanocytes aggregated their melanosomes normally. Instead, two types of defective transport during dispersion were observed: melanoctyes that did not completely disperse their melanosomes and, more frequently, melanocytes that did not evenly disperse their melanosomes. Both phenotypes suggest a problem transferring the melanosomes in *smutz* to or from actin-based transport. The variability in melanocyte phenotypes indicates that further work needs to be done to pinpoint the defective mechanism in *smutz* mutant melanosome transport.

There are several possible causes for the *smutz* phenotype that will need to be examined in future studies. One is that cAMP levels may not be decreasing during dispersion, leading to abnormally high PKA activity. Inappropriately high cAMP levels could explain both of the phenotypes we observed in the isolated cells; high levels of cAMP have been shown to inhibit aggregation and lead to hyper-dispersion of melanosomes in fish melanocytes (Rodionov, Yi et al. 2003). Another possible cause is that actin dynamics may be abnormal in *smutz* melanoctyes, which may affect the special regulation of Myosin Va (Jay 2000). Finally, there may be a hypomorphic mutation in *smutz* that reduces the function of a gene involved in actin-based transport regulation. Reduced function in the *smutz* gene could conceivably lead to variable responses to the modulation of intracellular cAMP in melanocytes. Very speculatively, this may explain the difference between the *smutz* larval phenotype and melanocytes isolated in culture - MCH and MSH applied directly to melanocytes may more effectively modulate intracellular cAMP levels than hormones applied to whole embryos.

A second mutant we identified, *b867*, hyper-disperses its melanosomes in response to caffeine or MSH. This response is exactly what is observed when cortical

actin is disrupted in wild-type fish melanocytes (Rodionov, Hope et al. 1998), which suggests that actin-based transport is specifically disrupted. Alternatively, the phenotype we see in *b867* could also be caused by constitutively high intracellular levels of cAMP disrupting the transfer of melanosomes from microtubules to actin (Rodionov, Yi et al. 2003). However, we think this is unlikely because *b867* melanocytes aggregate their melanosomes normally. One would predict that if intracellular cAMP levels remained elevated (e.g. cAMP phosphodiesterase was not functioning properly) then aggregation would be severely impaired.

Preliminary mapping results revealed that the *b867* mutation is linked to a marker on Chromosome 18 that is proximal to Myosin Va. However, sequence alignment of *b867* Myosin Va cDNA with wild-type revealed no apparent mutations, which indicates that the *b867* phenotype is not due to a mutation within the coding sequence of Myosin Va. Myosin Va could nevertheless be the affected gene; mutations in regulatory or cis-acting elements in non-coding regions of DNA could result in reduced transcription of Myosin Va (Shin, Priest et al. 2005). Finer mapping of the genetic interval containing *b867* will determine whether it is indeed linked to Myosin Va. In addition, anti-sense morpholino knock-down of Myosin Va would clarify whether the phenotype we observe in *b867* can be attributed to an effect on the Myosin Va gene. If it turns out that Myosin Va is not the affected gene, then it raises the exciting possibility that the *b867* gene may be a novel regulator of actin-based transport. The two genes other than melanophilin that have been previously identified as critical for actin-based transport in mouse and human melanocytes, Rab 27a and Rab geranylgeranyl transferase, are not currently mapped to the interval on Chromosome 18 containing the *b867* gene.

The identification and characterization of the pigment transport mutants *b867*and *smutz* further support that zebrafish melanocytes are a useful model system for identifying multiple regulators of organelle transport. In Chapter 3, I reported the characterization and cloning of Mlpha; a protein that regulates the transfer from microtubule- to actin- based transport. Since the phenotypes of *smutz* and b867 mutants are much different from *mlphaj*^{120} , further characterization and cloning the affected genes of these two mutants should provide additional information into how actin-based transport is regulated in melanocytes. Uncovering the molecular regulation of actin-based transport in melanocytes may give us insight into the general strategies used by other types of cells, such as neurons and secretory cells, to regulate Myosin V-mediated transport and intracellular cargo delivery.

CHAPTER 5

Conclusions

Zebrafish are a useful genetic model organism for elucidating the mechanisms of melanosome transport regulation

I began this dissertation by considering the scientific problem of intracellular cargo transport regulation - that is, how cargo is transported to the right place at the right time. Two aspects of cargo transport regulation that my work addressed are how bidirectional movement of cargo on microtubules is regulated and how cargos are transferred from microtubules, where transport occurs over long distances, to actin, where transport is more localized (Vale 2003; Welte 2004).

In the introduction, I reviewed how fish and *Xenopus* melanocytes have been used to elucidate the mechanisms underlying these two aspects of transport regulation. In these melanocytes, tracking the movement of individual melanosomes has revealed how the actions of the kinesin, dynein, and myosin motors that direct cargo transport change during aggregation and dispersion; such studies have also clarified the relative contributions of microtubule- and actin-based transport (Gross, Tuma et al. 2002; Rodionov, Yi et al. 2003). These studies demonstrate how the specific activities of molecular motors are regulated to properly distribute melanosomes spatially. Moreover, the activity of microtubule- and actin-based motors change in direct correlation to intracellular levels of cAMP (Rodionov, Yi et al. 2003). This provides a means of temporal melanosome transport regulation in melanocytes that delivers cargo to the right place at the right time.

While these findings give phenomenological insight into how microtubule and actin-based motors are regulated to transport cargos to the right place at the right time, they do not identify the proteins responsible for molecular motor regulation. Identifying these proteins is critical to gaining a deeper understanding of how cargo transport is successfully regulated. Since molecular motor regulation is tightly correlated with cAMP levels in fish and amphibian melanocytes (Lonart and Sudhof 1998; Rodionov, Yi et al. 2003), proteins that are downstream of cAMP-mediated signaling are strong candidates for factors that regulate molecular motors.

Previous studies in mammalian melanocytes have demonstrated that factors regulating molecular motors can be uncovered through forward genetics (Wu, Bowers et al. 1997; Fukuda, Kuroda et al. 2002; Wu, Rao et al. 2002; Menasche, Ho et al. 2003). However, mammalian melanocytes do not have the advantage, found fish and amphibia, of synchronous melanosome transport in response to a well-defined signaling cascade. In order to identify genes involved in melanosome transport regulation—as well as to uncover their mechanisms of transport regulation through biophysical analysis of individual melanosome movement—we sought to combine the advantage of synchronous melanosome movement in response to cAMP found in fish with the advantage of forward genetics found in mice.

Consequently, I established zebrafish melanocytes as a genetic model to study intracellular organelle transport regulation. I developed assays to identify zebrafish mutants with melanosome transport defects, then performed a small-scale forward genetic screen on zebrafish pigmentation mutants. From this screen, I identified three mutants with distinct transport phenotypes. This work demonstrates that zebrafish are

well suited for identifying factors involved in transport regulation through a forward genetic screen. Furthermore, the screening assay applied to mutant larvae (exposing them to hormones that initiate aggregation and dispersion) is rapid and simple, and it could be easily applied in a large-scale screen of zebrafish mutations. By these means additional mutants could be identified and characterized, thereby uncovering additional factors involved in transport regulation.

Our characterization and subsequent identification of zebrafish melanophilin (Mlpha) reveals that not only can we identify genes involved in melanosome transport in zebrafish pigmentation mutants, but we can also uncover new functions for orthologs previously identified in mammals. Through biophysical analysis of individual melanosome movement, we observed that Mlpha regulates bidirectional microtubule-based movement during melanosome dispersion and that it does so by inhibiting movement toward the minus-ends of microtubules. Our data, combined with the functional analysis of melanophilin previously described in mammals (Kuroda, Itoh et al. 2005; Hume, Tarafder et al. 2006), suggest that melanophilin coordinates the transfer from microtubule- to actin-based transport in zebrafish melanocytes by regulating the actions both microtubule- and actin-based motors. To our knowledge, this is the first time a single protein regulating both microtubule- and actin-based motors has been described, and this single-protein regulation may be a strategy used to coordinate transport of other types of cargos.

Potential mechanisms of Mlpha function

It seems likely, though future experiments would need to confirm it, that the cellular mechanism by which Mlpha mediates the transfer to actin-based transport is similar to that which has already been reported in mice and humans (Menasche, Ho et al. 2003; Kuroda, Itoh et al. 2005; Hume, Tarafder et al. 2006). What remains unclear is how Mlpha may be regulating microtubule-based motors to inhibit travel toward the minus-ends of microtubules. We observed in *mlpha*^{j120} mutants that the mean frequency of events and the distance traveled toward the plus- and minus- end of microtubules were nearly equal to one another at both of the time points we examined (Table 3.1). This indicates that Mlpha may be working through a cellular mechanism responsible for coordinating the actions of microtubule-based motors.

Numerous studies have reported that plus-end and minus-end microtubule motors are coordinated to prevent simultaneous activity (Waterman-Storer, Karki et al. 1997; Martin, Iyadurai et al. 1999; Gross, Welte et al. 2000; Gross, Welte et al. 2002; Kural, Kim et al. 2005). Several of these studies have indicated that dynactin, a large, multimeric complex that binds dynein to organelles, may be responsible for this coordination: microtubule-based transport in both directions was inhibited when extruded squid axoplasm was treated with an antibody against a component of the dynactin complex (Waterman-Storer, Karki et al. 1997); genetic interactions among dynein, kinesin, and dynactin were observed in *Drosophila* fast axonal transport (Martin, Iyadurai et al. 1999); and dynactin was shown to play a crucial role in coordinating the actions of plus- and minus-end directed microtubule motors involved in lipid-droplet transport in *Drosophila* embryos (Gross, Welte et al. 2002). Moreover, studies in *Xenopus*

melanocytes observed that the actions of dynein and kinesin II are coordinated and that both motors physically interact with dynactin (Tuma, Zill et al. 1998; Gross, Tuma et al. 2002). Taken together with our results, this finding suggests that Mlpha may work through dynactin during dispersion to inhibit dynein-mediated movement.

Speculatively, Mlpha may interact with dynactin via its C-terminal actin-binding domain. Dynactin contains two morphologically distinct domains: a projecting arm, which binds to dynein and other motors, and a larger, rod-like domain that contains actin-related protein 1 (Arp1) and whose function is poorly defined (Schroer 2004). Arp1 is structurally similar to conventional actin but short and very stable. Thus, Mlpha may regulate microtubule-based transport through an interaction with the Arp-1 rod of dynactin. Examining whether dynactin and Mlpha interact with *in vitro* protein-protein interaction experiments–for example, co-immunoprecipitation analysis and glutathione s-transferase (GST) pull-down assays (Smith and Johnson 1988; Masters 2004)–could provide another useful piece of information for clarifying the mechanism of Mlpha-mediated transport regulation.

Analysis of Mlpha may give insight into the mechanisms of trafficking regulation by related Rab effector proteins

I hypothesize that by identifying and characterizing genes involved in zebrafish melanosome transport regulation we will uncover strategies used by other types of cells, such as neurons, to regulate intracellular organelle. Yet it remains to be seen how discoveries made in zebrafish melanocytes will contribute to our understanding of organelle trafficking in other cell types. For example, our discovery that Mlpha regulates

both microtubule- and actin-based transport during dispersion uncovers a previously unidentified strategy of cargo transport regulation. However, the function of Mlpha is melanocyte-specific; both mammals and fish that have lost melanophilin function are relatively normal in every aspect other than melanosome transport (Matesic, Yip et al. 2001; Menasche, Ho et al. 2003). Therefore, a question that must be addressed is how zebrafish melanophilin may contribute to our understanding of cargo transport in other cell types.

There is mounting evidence that many types of cells, including neurons and secretory cells, use the same cellular machinery as melanocytes (e.g. Rab 27a, dynein, Myosin V) to transport cargos (Libby, Lillo et al. 2004; Hollenbeck and Saxton 2005; Tsuboi and Fukuda 2005; Mahoney, Liu et al. 2006). This demonstrates that different cells types may use specific homologues of melanophilin to regulate cargo transport. Recent work examining rabphilin (rabphilin 3a), a Rab effector protein related to melanophilin (Figure 3.7 A,B), gives an alluring indication as to how further characterization of Mlpha may give insight into how related proteins regulate trafficking in other cell types. Rabphilin is a cytosolic neuronal protein that is involved in trafficking vesicles to synapses (Gonzalez and Scheller 1999). It was originally identified as an effector of Rab3 (Shirataki, Kaibuchi et al. 1993); however, several reports have shown that Rabphilin preferentially interacts with Rab27 in both vertebrates and invertebrates (Fukuda, Kanno et al. 2004; Fukuda and Yamamoto 2005; Mahoney, Liu et al. 2006). Recent studies have just begun to characterize rabphilin's role in vesicle trafficking through its interaction with Rab27. In PC12 cells, it was revealed that rabphilin regulates dense-core vesicle docking at the synapse through a specific

interaction with Rab27a (Fukuda, Kanno et al. 2004; Tsuboi and Fukuda 2005), and a genetic study in *Caenorhabditis elegans* provided evidence that Rab27 regulates synaptic transmission through rabphilin, further supporting the model that rabphilin is required to localize vesicles to release sites (Mahoney, Liu et al. 2006). Moreover, rabphilin is phosphorylated by PKA *in vitro* (Fykse, Li et al. 1995; Lonart and Sudhof 2001), in cultured cerebellar granule cells (Fykse 1998), and in nerve terminals (Lonart and Sudhof 1998), but the function of PKA phosphorylation remains unknown. While indirect, the similarities in function and potential regulation suggest that rabphilin and melanophilin may use similar strategies to regulate intracellular trafficking in different types of cells. Consequently, investigating the mechanisms of Mlpha transport regulation may give additional insight into the functions of homologous proteins in cargo trafficking.

Future Directions

Since future experiments characterizing *b867* and *smutz* mutants are discussed in Chapter 4, this section will focus primarily on follow-up experiments further characterizing Mlpha. Our identification of Mlpha in zebrafish and its role in regulating microtubule-based transport raises a series of tractable questions. As our data, combined with previous work (Rodionov, Yi et al. 2003), suggest that Mlpha regulates molecular motors in response to cAMP, one key question that needs to be addressed is whether Mlpha is the protein that tranduces PKA signaling in melanocytes. Another question for future studies is whether there is a critical domain of the Mlpha protein that is necessary and sufficient to regulate microtubule-based transport. Identification of this domain would allow us to investigate microtubule-based transport more deeply by identifying other proteins that contain this domain as well as identifying factors that interact with this

domain. Finally, because we identified a predicted gene-duplicate of Mlpha, follow-up work is needed to examine whether it also has a role in melanosome transport in zebrafish.

Is Mlpha the target of PKA?

Our data, combined with previous work (Rodionov, Yi et al. 2003), suggest that Mlpha regulates microtubule- and actin-based motors in response to cAMP-signaling and the actions of PKA. Comparative analysis of Mlpha protein sequence with that of two other fish species (Figure 3.7 C) reveals two predicted PKA phosphorylation sites that are not conserved in human or mouse melanophilin. Moreover, a seminal study in fish melanocytes reported that a 57 kD protein is phosphorylated during dispersion (Rozdzial and Haimo 1986), which is approximately the same molecular weight as Mlpha (60 kD). Cumulatively, this evidence makes Mlpha a strong candidate protein for PKA phosphorylation.

The first step in examining whether Mlpha is a substrate of PKA is to determine whether PKA phosphorylates Mlpha efficiently *in vitro*. A routine method for determining whether a kinase phosphorylates a protein efficiently is to incubate the purified candidate protein substrate (in this case, Mlpha) with the kinase (in this case, the catalytic subunit of PKA) in the presence of radiolabeled phosphate ([γ^{32}P] ATP) (Berwick and Tavare 2004; Peck 2006). By comparing the incorporation of the radiolabeled phosphate in Mlpha with equivalent amounts of a non-substrate and a known PKA substrate, one can determine whether Mlpha is efficiently phosphorylated by PKA. If phophorylation of Mlpha is observed *in vitro*, the next step is to identify the specific

residues that are phosphorylated by PKA (Peck 2006). Because we have identified two putative phosphorylation sites for PKA in Mlpha (Figure 3.6), we could test whether these sites are specifically phosphorylated by mutating one or both of their serine residues into an unphosphorylatable residue (e.g. alanine), then repeating the *in vitro* assays to see if the mutations abolish PKA phosphorylation. In addition, the identity of these sites can also be confirmed with mass spectrometry sequencing (Mann, Ong et al. 2002).

While *in vitro* experiments can indicate whether Mlpha is phosphorylated by PKA, they do not reveal the phosphorylation status of the protein *in vivo*; nor do they determine if PKA phosphorylation is physiologically relevant to Mlpha's function. In theory, increased phosphorylation of Mlpha early in dispersion contributes to its suppressing minus-end microtubule-based movement, and decreased phosphorylation of Mlpha later in dispersion allows it to interact with Myosin Va and release the inhibition of minus-end moves, thus facilitating the transfer from microtubule- to actin-based transport. Therefore, Mlpha may be transiently phosphorylated by PKA, with the highest amount of phosphorylation occurring early in dispersion.

To determine whether endogenous Mlpha is transiently phosphorylated during dispersion, one strategy would be to examine Mlpha's phosphorylated state in melanocyte extracts collected at increasing time points after a-MSH exposure. Since phosphorylated proteins often migrate at a higher apparent molecular mass during sodium dodecyl sulfate (SDS) gel electrophoresis, a simple method for monitoring the phosphorylation status of Mlpha would be to look for an apparent band shift on a western blot probed with a Mlpha specific antibody (Wegener and Jones 1984). If Mlpha is

transiently more phosphorylated early in dispersion, then one would observe a stronger comparatively high molecular weight band in melanocyte extracts isolated during early verses late dispersion. There are, however, two caveats to this approach: first, a band-shift does not occur for every phosphoprotein and, second, posttranslational modifications other than phosphorylation can also alter protein migration on a gel (Peck 2006). Therefore, one could alternatively probe western blots with, in addition to a pan Mlpha antibody, antibodies that are specific to the PKA phosphorylated residues of Mlpha (Berwick and Tavare 2004). Additionally, extracts from melanocytes treated with α-MSH and a PKA inhibitor, such as H-89, should also be examined to verify that observed phosphorylation is PKA-specific.

To examine whether PKA phosphorylation is physiologically relevant, one could compare transient expression of full-length epitope-labeled Mlpha cDNA in the mutant background with expression of Mlpha cDNA containing point mutations at the sites of PKA phosphorylation. Two types of mutations could be introduced: either unphosphorylatable residues (e.g. serine to alanine) or constitutively phosphorylated residues (e.g. serine to glutamate). If unphosphorylatable Mlpha fails to rescue the phenotype at the same concentrations that led to successful rescue with wild-type cDNA, or if constitutively phosphorylated Mlpha leads to a hyperdispersion phenotype similar to what is observed when intracellular cAMP levels are maintained at a high level (Rodionov, Yi et al. 2003), then phosphorylation of Mlpha may be physiologically relevant to its function.

Transient expression experiments could initially, and most easily, be performed by injecting plasmid DNA into 1-cell stage embryos, then examining the resulting

phenotype in whole larvae. However, this would only reveal how the expression of different recombinant Mlpha constructs affect overall melanosome displacement. To examine changes in the kinetics of melanosome transport in detail, it would be ideal to transiently express cDNA in melanocytes isolated in cell culture. Yet, in spite of trying several techniques, among them electroporation (Hamm, 2002 #245), lipofection (Dalby, Cates et al. 2004), and nonliposomal-multicomponent transfection (Jacobsen, Calvin et al. 2004), I have had limited success in transfecting plasmid DNA into primary zebrafish melanocyte cell culture. Therefore, additional work is needed to develop an effective method of expressing cDNA in cultured melanocytes.

Does Mlpha contain a specific domain that regulates microtubule-based transport?

Another question that needs to be addressed in future studies is whether there is a specific cellular domain in Mlpha that regulates microtubule-based transport. Most of what has been uncovered about the cellular domains in melanophilin that regulate its interaction with Rab27a and Myosin Va has been through molecular studies examining the expression of truncated or mutated melanophilin in wild-type and *leaden* melanophilin mouse melanocytes in cell culture (Wu, Rao et al. 2002; Kuroda, Itoh et al. 2005; Hume, Tarafder et al. 2006). Thus, one strategy that could be used in zebrafish would be to transiently express mutated or truncated Mlpha in *mlpha*^{j120} mutant background, then examine the resulting phenotype.

Before we can define a domain of Mlpha that is responsible for microtubule-based transport regulation, we must first examine whether its regulation of microtubule-based movement and its interaction with the actin-based motor are functionally separable. This

could be directly examined by transiently expressing in *mlpha*^{j120} mutants Mlpha cDNA with point mutations in the conserved acidic residues shown in mammals to be critical for binding Myosin V (Kuroda, Itoh et al. 2005; Hume, Tarafder et al. 2006). Hypothetically, if Mlpha's regulation of microtubule-based movement is functionally separable from its regulation of actin-based movement, then specifically disrupting its interaction with Myosin V should lead to a hyper-dispersion phenotype.

If it is established that microtubule- and actin-based transport regulation are functionally separable, then the domain responsible for microtubule-based transport regulation could be identified by transiently expressing various truncations of Mlpha cDNA in the mutant background. I predict that if a truncation containing a domain of Mlpha that is necessary and sufficient for regulating microtubule-based transport were expressed in *mlpha*^{j120} mutant melanocytes, it would lead to hyper-dispersion of melanosomes. Subsequently, by identifying the minimal fragment of Mlpha that leads to hyper-dispersion, the specific domain of the Mlpha protein that is involved in suppressing dynein-mediated transport could be isolated. This microtubule-motor regulation domain could then be used as bait in co-immunoprecipitation and GST pull-down assays (Smith and Johnson 1988; Masters 2004) to identify factors that it interacts with, thereby providing another useful piece of information for clarifying the mechanism of Mlpha mediated transport regulation. Additionally, the nucleic acid and protein sequences of the identified domain could be used in database searches (i.e. BLAST) to identify other proteins that may be involved in microtubule motor regulation.

Does the predicted gene duplicate (Mlphb) play a role in zebrafish melanosome transport?

Finally, in addition to uncovering the mechanisms of Mlpha transport regulation, future work is needed to characterize its predicted gene duplicate – Mlphb. Zebrafish often have two copies of a gene that is present as a single copy in mammals. This is due to a genome wide duplication event that is thought to have occurred after zebrafish (the ray fin fish lineage) diverged from birds and mammals (the lobe fin lineage). We uncovered Mlphb on Chromosome 9 with synteny between it and mouse and human melanophilin, indicating that this is likely a bona fide duplication of chromosome segment (Postlethwait, Woods et al. 2000). There are three potential fates for duplicate gene pairs (Force, Lynch et al. 1999). One is that the duplicate gene may incur a mutation in coding or regulatory sequence that leads to non-function, or a pseudogene. Another is that each duplicate gene may have a sub function, and that expression of both genes is required to fulfill the functions of the ancestral locus. Finally, the mutation in the duplicate may have led to a new function in the gene that diverges from the ancestral locus. Therefore, cloning the Mlphb and examining its expression in zebrafish will be necessary in order to define the mechanism of Mlpha function. Theoretically, if Mlphb is also expressed in cells that correspond with melanocytes in the same developmental time frame as Mlpha, then both Mlpha and Mlphb may be required for melanosome transport regulation. In addition, blocking Mlphb translation with an antisense morpholino could give insight into whether Mlphb is also necessary for proper melanosome transport.

Further characterization of identified mutants and a large-scale screen

The ultimate goal of this work is to use zebrafish melanocytes to identify multiple regulators of cargo transport. Therefore, future studies should also focus on further characterizing and cloning the two other melanosome transport mutants we identified: *smutz* and *b867*. In addition, the simplicity of screening larvae for melanosome transport mutations makes our assay well suited for a large-scale screen of zebrafish mutants. Ideally, detailed characterization of multiple mutants identified in a large-scale screen would deepen our knowledge about the spatial and temporal regulation of intracellular cargos in cells.

REFERENCES

Allan, V. J. and T. A. Schroer (1999). "Membrane motors." Curr Opin Cell Biol **11**(4): 476-82.

Andersson, T. P., H. N. Skold and S. P. Svensson (2003). "Phosphoinositide 3-kinase is involved in Xenopus and Labrus melanophore aggregation." Cell Signal **15**(12): 1119-27.

Andersson, T. P., S. P. Svensson and A. M. Karlsson (2003). "Regulation of melanosome movement by MAP kinase." Pigment Cell Res **16**(3): 215-21.

Beis, D. and D. Y. Stainier (2006). "In vivo cell biology: following the zebrafish trend." Trends Cell Biol **16**(2): 105-12.

Berwick, D. C. and J. M. Tavare (2004). "Identifying protein kinase substrates: hunting for the organ-grinder's monkeys." Trends Biochem Sci **29**(5): 227-32.

Bikle, D., Tilney, L.G., Porter, K.R. (1966). "Microtubules and pigment migration in the melanophores of Fundulus heteroclitus." Protoplasma **61**(Numbers 3-4): 322-345.

Block, S. M., L. S. Goldstein and B. J. Schnapp (1990). "Bead movement by single kinesin molecules studied with optical tweezers." Nature **348**(6299): 348-52.

Bridgman, P. C. (2004). "Myosin-dependent transport in neurons." J Neurobiol **58**(2): 164-74.

Brown, J. R., K. R. Simonetta, L. A. Sandberg, P. Stafford and G. M. Langford (2001). "Recombinant globular tail fragment of myosin-V blocks vesicle transport in squid nerve cell extracts." Biol Bull **201**(2): 240-1.

Brown, S. S. (1999). "Cooperation between microtubule- and actin-based motor proteins." Annu Rev Cell Dev Biol **15**: 63-80.

Busca, R. and R. Ballotti (2000). "Cyclic AMP a key messenger in the regulation of skin pigmentation." Pigment Cell Res **13**(2): 60-9.

Byers, H. R. and K. R. Porter (1977). "Transformations in the structure of the cytoplasmic ground substance in erythrophores during pigment aggregation and dispersion. I. A study using whole-cell preparations in stereo high voltage electron microscopy." J Cell Biol **75**(2 Pt 1): 541-58.

Cole, D. G. (2005). "Intraflagellar transport: keeping the motors coordinated." Curr Biol **15**(19): R798-801.

Dahm, C. N.-V. a. R. (2002). Zebrafish, Oxford University Press.

Dalby, B., S. Cates, A. Harris, E. C. Ohki, M. L. Tilkins, P. J. Price and V. C. Ciccarone (2004). "Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications." Methods **33**(2): 95-103.

Dalton, G. D. and W. L. Dewey (2006). "Protein kinase inhibitor peptide (PKI): a family of endogenous neuropeptides that modulate neuronal cAMP-dependent protein kinase function." Neuropeptides **40**(1): 23-34.

Daniolos, A., A. B. Lerner and M. R. Lerner (1990). "Action of light on frog pigment cells in culture." Pigment Cell Res **3**(1): 38-43.

de Graan, P. N., A. B. Oestreicher, H. Zwiers, W. H. Gispen and F. C. van de Veerdonk (1985). "Characterization of alpha-MSH-induced changes in the phosphorylation of a 53 kDa protein in Xenopus melanophores." Mol Cell Endocrinol **42**(2): 127-33.

Deacon, S. W., A. Nascimento, A. S. Serpinskaya and V. I. Gelfand (2005). "Regulation of bidirectional melanosome transport by organelle bound MAP kinase." Curr Biol **15**(5): 459-63.

Euteneuer, U. and J. R. McIntosh (1981). "Polarity of some motility-related microtubules." Proc Natl Acad Sci U S A **78**(1): 372-6.

Force, A., M. Lynch, F. B. Pickett, A. Amores, Y. L. Yan and J. Postlethwait (1999). "Preservation of duplicate genes by complementary, degenerative mutations." Genetics **151**(4): 1531-45.

Fujii, R. (2000). "The regulation of motile activity in fish chromatophores." Pigment Cell Res **13**(5): 300-19.

Fujii, R. and Y. Miyashita (1975). "Receptor mechanisms in fish chromatophores-I. Alpha nature of adrenoceptors mediating melanosome aggregation in guppy melanophores." Comp Biochem Physiol C **51**(2): 171-8.

Fujii, R. and Y. Miyashita (1982). "Receptor mechanisms in fish chromatophores--V. MSH disperses melanosomes in both dermal and epidermal melanophores of a catfish (Parasilurus asotus)." Comp Biochem Physiol C **71C**(1): 1-6.

Fukuda, M., E. Kanno and A. Yamamoto (2004). "Rabphilin and Noc2 are recruited to dense-core vesicles through specific interaction with Rab27A in PC12 cells." J Biol Chem **279**(13): 13065-75.

Fukuda, M., T. S. Kuroda and K. Mikoshiba (2002). "Slac2-a/melanophilin, the missing link between Rab27 and myosin Va: implications of a tripartite protein complex for melanosome transport." J Biol Chem **277**(14): 12432-6.

Fukuda, M. and A. Yamamoto (2005). "Assay of the Rab-binding specificity of rabphilin and Noc2: target molecules for Rab27." Methods Enzymol **403**: 469-81.

Fykse, E. M. (1998). "Depolarization of cerebellar granule cells increases phosphorylation of rabphilin-3A." J Neurochem **71**(4): 1661-9.

Fykse, E. M., C. Li and T. C. Sudhof (1995). "Phosphorylation of rabphilin-3A by Ca2+/calmodulin- and cAMP-dependent protein kinases in vitro." J Neurosci **15**(3 Pt 2): 2385-95.

Garrett, S. and T. M. Kapoor (2003). "Microtubule assembly: catastrophe factors to the rescue." Curr Biol **13**(20): R810-2.

Goldstein, L. S. (2001). "Molecular motors: from one motor many tails to one motor many tales." Trends Cell Biol **11**(12): 477-82.

Golling, G., A. Amsterdam, Z. Sun, M. Antonelli, E. Maldonado, W. Chen, S. Burgess, M. Haldi, K. Artzt, S. Farrington, S. Y. Lin, R. M. Nissen and N. Hopkins (2002). "Insertional mutagenesis in zebrafish rapidly identifies genes essential for early vertebrate development." Nat Genet **31**(2): 135-40.

Gonzalez, L., Jr. and R. H. Scheller (1999). "Regulation of membrane trafficking: structural insights from a Rab/effector complex." Cell **96**(6): 755-8.

Graminski, G. F., C. K. Jayawickreme, M. N. Potenza and M. R. Lerner (1993). "Pigment dispersion in frog melanophores can be induced by a phorbol ester or stimulation

of a recombinant receptor that activates phospholipase C." J Biol Chem **268**(8): 5957-64.

Granato, M., F. J. van Eeden, U. Schach, T. Trowe, M. Brand, M. Furutani-Seiki, P. Haffter, M. Hammerschmidt, C. P. Heisenberg, Y. J. Jiang, D. A. Kane, R. N. Kelsh, M. C. Mullins, J. Odenthal and C. Nusslein-Volhard (1996). "Genes controlling and mediating locomotion behavior of the zebrafish embryo and larva." Development **123**: 399-413.

Grissom, P. M., E. A. Vaisberg and J. R. McIntosh (2002). "Identification of a novel light intermediate chain (D2LIC) for mammalian cytoplasmic dynein 2." Mol Biol Cell **13**(3): 817-29.

Gross, S. P. (2004). "Hither and yon: a review of bi-directional microtubule-based transport." Phys Biol **1**(1-2): R1-11.

Gross, S. P., Y. Guo, J. E. Martinez and M. A. Welte (2003). "A determinant for directionality of organelle transport in Drosophila embryos." Curr Biol **13**(19): 1660-8.

Gross, S. P., M. C. Tuma, S. W. Deacon, A. S. Serpinskaya, A. R. Reilein and V. I. Gelfand (2002). "Interactions and regulation of molecular motors in Xenopus melanophores." J Cell Biol **156**(5): 855-65.

Gross, S. P., M. A. Welte, S. M. Block and E. F. Wieschaus (2000). "Dynein-mediated cargo transport in vivo. A switch controls travel distance." J Cell Biol **148**(5): 945-56.

Gross, S. P., M. A. Welte, S. M. Block and E. F. Wieschaus (2002). "Coordination of opposite-polarity microtubule motors." J Cell Biol **156**(4): 715-24.

Gunawardena, S. and L. S. Goldstein (2004). "Cargo-carrying motor vehicles on the neuronal highway: transport pathways and neurodegenerative disease." J Neurobiol **58**(2): 258-71.

Hammer, J. A., 3rd and X. S. Wu (2002). "Rabs grab motors: defining the connections between Rab GTPases and motor proteins." Curr Opin Cell Biol **14**(1): 69-75.

Hirschberg, K., C. M. Miller, J. Ellenberg, J. F. Presley, E. D. Siggia, R. D. Phair and J. Lippincott-Schwartz (1998). "Kinetic analysis of secretory protein traffic and characterization of golgi to plasma membrane transport intermediates in living cells." J Cell Biol **143**(6): 1485-503.

Hollenbeck, P. J. and W. M. Saxton (2005). "The axonal transport of mitochondria." J Cell Sci **118**(Pt 23): 5411-9.

Homma, N., Y. Takei, Y. Tanaka, T. Nakata, S. Terada, M. Kikkawa, Y. Noda and N. Hirokawa (2003). "Kinesin superfamily protein 2A (KIF2A) functions in suppression of collateral branch extension." Cell **114**(2): 229-39.

Hoogenraad, C. C., A. D. Milstein, I. M. Ethell, M. Henkemeyer and M. Sheng (2005). "GRIP1 controls dendrite morphogenesis by regulating EphB receptor trafficking." Nat Neurosci **8**(7): 906-15.

Hume, A. N., A. K. Tarafder, J. S. Ramalho, E. V. Sviderskaya and M. C. Seabra (2006). "A Coiled-Coil Domain of Melanophilin Is Essential for Myosin Va Recruitment and Melanosome Transport in Melanocytes." Mol Biol Cell.

Jacobsen, L. B., S. A. Calvin, K. E. Colvin and M. Wright (2004). "FuGENE 6 Transfection Reagent: the gentle power." Methods **33**(2): 104-12.

Jay, D. G. (2000). "The clutch hypothesis revisited: ascribing the roles of actin-associated proteins in filopodial protrusion in the nerve growth cone." J Neurobiol **44**(2): 114-25.

Johnson, S. L., C. N. Midson, E. W. Ballinger and J. H. Postlethwait (1994). "Identification of RAPD primers that reveal extensive polymorphisms between laboratory strains of zebrafish." Genomics **19**(1): 152-6.

Kamal, A. and L. S. Goldstein (2000). "Connecting vesicle transport to the cytoskeleton." Curr Opin Cell Biol **12**(4): 503-8.

Karcher, R. L., J. T. Roland, F. Zappacosta, M. J. Huddleston, R. S. Annan, S. A. Carr and V. I. Gelfand (2001). "Cell cycle regulation of myosin-V by calcium/calmodulin-dependent protein kinase II." Science **293**(5533): 1317-20.

Kashina, A. S., I. V. Semenova, P. A. Ivanov, E. S. Potekhina, I. Zaliapin and V. I. Rodionov (2004). "Protein kinase A, which regulates intracellular transport, forms complexes with molecular motors on organelles." Curr Biol **14**(20): 1877-81.

Kawauchi, H., I. Kawazoe, M. Tsubokawa, M. Kishida and B. I. Baker (1983). "Characterization of melanin-concentrating hormone in chum salmon pituitaries." Nature **305**(5932): 321-3.

Kelsh, R. N., M. Brand, Y. J. Jiang, C. P. Heisenberg, S. Lin, P. Haffter, J. Odenthal, M. C. Mullins, F. J. van Eeden, M. Furutani-Seiki, M. Granato, M. Hammerschmidt, D. A. Kane, R. M. Warga, D. Beuchle, L. Vogelsang and C. Nusslein-Volhard (1996). "Zebrafish pigmentation mutations and the processes of neural crest development." Development **123**: 369-89.

Kimmel, C. B. (1989). "Genetics and early development of zebrafish." Trends Genet **5**(8): 283-8.

Kotz, K. J. and M. A. McNiven (1994). "Intracellular calcium and cAMP regulate directional pigment movements in teleost erythrophores." J Cell Biol **124**(4): 463-74.

Kural, C., H. Kim, S. Syed, G. Goshima, V. I. Gelfand and P. R. Selvin (2005). "Kinesin and dynein move a peroxisome in vivo: a tug-of-war or coordinated movement?" Science **308**(5727): 1469-72.

Kuroda, T. S., H. Ariga and M. Fukuda (2003). "The actin-binding domain of Slac2-a/melanophilin is required for melanosome distribution in melanocytes." Mol Cell Biol **23**(15): 5245-55.

Kuroda, T. S., T. Itoh and M. Fukuda (2005). "Functional analysis of slac2-a/melanophilin as a linker protein between Rab27A and myosin Va in melanosome transport." Methods Enzymol **403**: 419-31.

Kuznetsov, S. A., G. M. Langford and D. G. Weiss (1992). "Actin-dependent organelle movement in squid axoplasm." Nature **356**(6371): 722-5.

Lamason, R. L., M. A. Mohideen, J. R. Mest, A. C. Wong, H. L. Norton, M. C. Aros, M. J. Jurynec, X. Mao, V. R. Humphreville, J. E. Humbert, S. Sinha, J. L. Moore, P. Jagadeeswaran, W. Zhao, G. Ning, I. Makalowska, P. M. McKeigue, D. O'Donnell, R. Kittles, E. J. Parra, N. J. Mangini, D. J. Grunwald, M. D. Shriver, V. A. Canfield and K. C. Cheng (2005). "SLC24A5, a putative cation exchanger, affects pigmentation in zebrafish and humans." Science **310**(5755): 1782-6.

Langford, G. M. (2002). "Myosin-V, a versatile motor for short-range vesicle transport." Traffic **3**(12): 859-65.

Levi, V., A. S. Serpinskaya, E. Gratton and V. Gelfand (2006). "Organelle transport along microtubules in Xenopus melanophores: evidence for cooperation between multiple motors." Biophys J **90**(1): 318-27.

Li, X. D., R. Ikebe and M. Ikebe (2005). "Activation of myosin Va function by melanophilin, a specific docking partner of myosin Va." J Biol Chem **280**(18): 17815-22.

Libby, R. T., C. Lillo, J. Kitamoto, D. S. Williams and K. P. Steel (2004). "Myosin Va is required for normal photoreceptor synaptic activity." J Cell Sci **117**(Pt 19): 4509-15.

Lonart, G. and T. C. Sudhof (1998). "Region-specific phosphorylation of rabphilin in mossy fiber nerve terminals of the hippocampus." J Neurosci **18**(2): 634-40.

Lonart, G. and T. C. Sudhof (2001). "Characterization of rabphilin phosphorylation using phospho-specific antibodies." Neuropharmacology **41**(6): 643-9.

Luby-Phelps, K. and K. R. Porter (1982). "The control of pigment migration in isolated erythrophores of Holocentrus ascensionis (Osbeck). II. The role of calcium." Cell **29**(2): 441-50.

Mahoney, T. R., Q. Liu, T. Itoh, S. Luo, G. Hadwiger, R. Vincent, Z. W. Wang, M. Fukuda and M. L. Nonet (2006). "Regulation of synaptic transmission by RAB-3 and RAB-27 in Caenorhabditis elegans." Mol Biol Cell **17**(6): 2617-25.

Malikov, V., E. N. Cytrynbaum, A. Kashina, A. Mogilner and V. Rodionov (2005). "Centering of a radial microtubule array by translocation along microtubules spontaneously nucleated in the cytoplasm." Nat Cell Biol **7**(12): 1213-8.

Malikov, V., A. Kashina and V. Rodionov (2004). "Cytoplasmic dynein nucleates microtubules to organize them into radial arrays in vivo." Mol Biol Cell **15**(6): 2742-9.

Mallik, R. and S. P. Gross (2004). "Molecular motors: strategies to get along." Curr Biol **14**(22): R971-82.

Mann, M., S. E. Ong, M. Gronborg, H. Steen, O. N. Jensen and A. Pandey (2002). "Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome." Trends Biotechnol **20**(6): 261-8.

Maquat, L. E. (2002). "Nonsense-mediated mRNA decay." Curr Biol **12**(6): R196-7.

Marks, M. S. and M. C. Seabra (2001). "The melanosome: membrane dynamics in black and white." Nat Rev Mol Cell Biol **2**(10): 738-48.

Martin, M., S. J. Iyadurai, A. Gassman, J. G. Gindhart, Jr., T. S. Hays and W. M. Saxton (1999). "Cytoplasmic dynein, the dynactin complex, and kinesin are interdependent and essential for fast axonal transport." Mol Biol Cell **10**(11): 3717-28.

Masters, S. C. (2004). "Co-immunoprecipitation from transfected cells." Methods Mol Biol **261**: 337-50.

Matesic, L. E., R. Yip, A. E. Reuss, D. A. Swing, T. N. O'Sullivan, C. F. Fletcher, N. G. Copeland and N. A. Jenkins (2001). "Mutations in Mlph, encoding a member of

the Rab effector family, cause the melanosome transport defects observed in leaden mice." Proc Natl Acad Sci U S A **98**(18): 10238-43.

Mehta, A. D., R. S. Rock, M. Rief, J. A. Spudich, M. S. Mooseker and R. E. Cheney (1999). "Myosin-V is a processive actin-based motor." Nature **400**(6744): 590-3.

Menasche, G., J. Feldmann, A. Houdusse, C. Desaymard, A. Fischer, B. Goud and G. de Saint Basile (2003). "Biochemical and functional characterization of Rab27a mutations occurring in Griscelli syndrome patients." Blood **101**(7): 2736-42.

Menasche, G., C. H. Ho, O. Sanal, J. Feldmann, I. Tezcan, F. Ersoy, A. Houdusse, A. Fischer and G. de Saint Basile (2003). "Griscelli syndrome restricted to hypopigmentation results from a melanophilin defect (GS3) or a MYO5A F-exon deletion (GS1)." J Clin Invest **112**(3): 450-6.

Moore, A. and L. Wordeman (2004). "The mechanism, function and regulation of depolymerizing kinesins during mitosis." Trends Cell Biol **14**(10): 537-46.

Morris, R. L. and P. J. Hollenbeck (1993). "The regulation of bidirectional mitochondrial transport is coordinated with axonal outgrowth." J Cell Sci **104 (Pt 3)**: 917-27.

Mountain, V. and D. A. Compton (2000). "Dissecting the role of molecular motors in the mitotic spindle." Anat Rec **261**(1): 14-24.

Murray, J. W., E. Bananis and A. W. Wolkoff (2000). "Reconstitution of ATP-dependent movement of endocytic vesicles along microtubules in vitro: an oscillatory bidirectional process." Mol Biol Cell **11**(2): 419-33.

Namboodiripad, A. N. and M. L. Jennings (1996). "Permeability characteristics of erythrocyte membrane to okadaic acid and calyculin A." Am J Physiol **270**(2 Pt 1): C449-56.

Newton, A. C. (1997). "Regulation of protein kinase C." Curr Opin Cell Biol **9**(2): 161-7.

Oshima, N., N. Nakamaru, S. Araki and M. Sugimoto (2001). "Comparative analyses of the pigment-aggregating and -dispersing actions of MCH on fish chromatophores." Comp Biochem Physiol C Toxicol Pharmacol **129**(2): 75-84.

Pastural, E., F. J. Barrat, R. Dufourcq-Lagelouse, S. Certain, O. Sanal, N. Jabado, R. Seger, C. Griscelli, A. Fischer and G. de Saint Basile (1997). "Griscelli disease maps to chromosome 15q21 and is associated with mutations in the myosin-Va gene." Nat Genet **16**(3): 289-92.

Peck, S. C. (2006). "Analysis of protein phosphorylation: methods and strategies for studying kinases and substrates." Plant J **45**(4): 512-22.

Pilling, A. D., D. Horiuchi, C. M. Lively and W. M. Saxton (2006). "Kinesin-1 and Dynein are the primary motors for fast transport of mitochondria in Drosophila motor axons." Mol Biol Cell **17**(4): 2057-68.

Postlethwait, J. H., I. G. Woods, P. Ngo-Hazelett, Y. L. Yan, P. D. Kelly, F. Chu, H. Huang, A. Hill-Force and W. S. Talbot (2000). "Zebrafish comparative genomics and the origins of vertebrate chromosomes." Genome Res **10**(12): 1890-902.

Potenza, M. N. and M. R. Lerner (1992). "A rapid quantitative bioassay for evaluating the effects of ligands upon receptors that modulate cAMP levels in a melanophore cell line." Pigment Cell Res **5**(6): 372-8.

Provance, D. W. and J. A. Mercer (1999). "Myosin-V: head to tail." Cell Mol Life Sci **56**(3-4): 233-42.

Reilein, A. R., A. S. Serpinskaya, R. L. Karcher, D. L. Dujardin, R. B. Vallee and V. I. Gelfand (2003). "Differential regulation of dynein-driven melanosome movement." Biochem Biophys Res Commun **309**(3): 652-8.

Reilein, A. R., I. S. Tint, N. I. Peunova, G. N. Enikolopov and V. I. Gelfand (1998). "Regulation of organelle movement in melanophores by protein kinase A (PKA), protein kinase C (PKC), and protein phosphatase 2A (PP2A)." J Cell Biol **142**(3): 803-13.

Rodionov, V., J. Yi, A. Kashina, A. Oladipo and S. P. Gross (2003). "Switching between microtubule- and actin-based transport systems in melanophores is controlled by cAMP levels." Curr Biol **13**(21): 1837-47.

Rodionov, V. I., A. J. Hope, T. M. Svitkina and G. G. Borisy (1998). "Functional coordination of microtubule-based and actin-based motility in melanophores." Curr Biol **8**(3): 165-8.

Rodionov, V. I., S. S. Lim, V. I. Gelfand and G. G. Borisy (1994). "Microtubule dynamics in fish melanophores." J Cell Biol **126**(6): 1455-64.

Roest Crollius, H. and J. Weissenbach (2005). "Fish genomics and biology." Genome Res **15**(12): 1675-82.

Rogers, S. L. and V. I. Gelfand (1998). "Myosin cooperates with microtubule motors during organelle transport in melanophores." Curr Biol **8**(3): 161-4.

Rogers, S. L., R. L. Karcher, J. T. Roland, A. A. Minin, W. Steffen and V. I. Gelfand (1999). "Regulation of melanosome movement in the cell cycle by reversible association with myosin V." J Cell Biol **146**(6): 1265-76.

Rogers, S. L., I. S. Tint, P. C. Fanapour and V. I. Gelfand (1997). "Regulated bidirectional motility of melanophore pigment granules along microtubules in vitro." Proc Natl Acad Sci U S A **94**(8): 3720-5.

Rozdzial, M. M. and L. T. Haimo (1986). "Bidirectional pigment granule movements of melanophores are regulated by protein phosphorylation and dephosphorylation." Cell **47**(6): 1061-70.

Rozdzial, M. M. and L. T. Haimo (1986). "Reactivated melanophore motility: differential regulation and nucleotide requirements of bidirectional pigment granule transport." J Cell Biol **103**(6 Pt 2): 2755-64.

Sammak, P. J., S. R. Adams, A. T. Harootunian, M. Schliwa and R. Y. Tsien (1992). "Intracellular cyclic AMP not calcium, determines the direction of vesicle movement in melanophores: direct measurement by fluorescence ratio imaging." J Cell Biol **117**(1): 57-72.

Sasaki, S., D. Mori, K. Toyo-oka, A. Chen, L. Garrett-Beal, M. Muramatsu, S. Miyagawa, N. Hiraiwa, A. Yoshiki, A. Wynshaw-Boris and S. Hirotsune (2005). "Complete loss of Ndel1 results in neuronal migration defects and early embryonic lethality." Mol Cell Biol **25**(17): 7812-27.

Schnapp, B. J. (2003). "Trafficking of signaling modules by kinesin motors." J Cell Sci **116**(Pt 11): 2125-35.

Schnapp, B. J., B. Crise, M. P. Sheetz, T. S. Reese and S. Khan (1990). "Delayed start-up of kinesin-driven microtubule gliding following inhibition by adenosine 5'-[beta,gamma-imido]triphosphate." Proc Natl Acad Sci U S A **87**(24): 10053-7.

Schroer, T. A. (2004). "Dynactin." Annu Rev Cell Dev Biol **20**: 759-79.

Shin, J. T., J. R. Priest, I. Ovcharenko, A. Ronco, R. K. Moore, C. G. Burns and C. A. MacRae (2005). "Human-zebrafish non-coding conserved elements act in vivo to regulate transcription." Nucleic Acids Res **33**(17): 5437-45.

Shirataki, H., K. Kaibuchi, T. Sakoda, S. Kishida, T. Yamaguchi, K. Wada, M. Miyazaki and Y. Takai (1993). "Rabphilin-3A, a putative target protein for smg p25A/rab3A p25 small GTP-binding protein related to synaptotagmin." Mol Cell Biol **13**(4): 2061-8.

Skalhegg, B. S. and K. Tasken (2000). "Specificity in the cAMP/PKA signaling pathway. Differential expression,regulation, and subcellular localization of subunits of PKA." Front Biosci **5**: D678-93.

Smith, D. B. and K. S. Johnson (1988). "Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase." Gene **67**(1): 31-40.

Smith, F. D., L. K. Langeberg and J. D. Scott (2006). "The where's and when's of kinase anchoring." Trends Biochem Sci **31**(6): 316-23.

Snider, J., F. Lin, N. Zahedi, V. Rodionov, C. C. Yu and S. P. Gross (2004). "Intracellular actin-based transport: how far you go depends on how often you switch." Proc Natl Acad Sci U S A **101**(36): 13204-9.

Strobel, M. C., P. K. Seperack, N. G. Copeland and N. A. Jenkins (1990). "Molecular analysis of two mouse dilute locus deletion mutations: spontaneous dilute lethal20J and radiation-induced dilute prenatal lethal Aa2 alleles." Mol Cell Biol **10**(2): 501-9.

Sugden, D. (1992). "Effect of putative melatonin receptor antagonists on melatonin-induced pigment aggregation in isolated Xenopus laevis melanophores." Eur J Pharmacol **213**(3): 405-8.

Sugden, D. and S. J. Rowe (1992). "Protein kinase C activation antagonizes melatonin-induced pigment aggregation in Xenopus laevis melanophores." J Cell Biol **119**(6): 1515-21.

Tabb, J. S., B. J. Molyneaux, D. L. Cohen, S. A. Kuznetsov and G. M. Langford (1998). "Transport of ER vesicles on actin filaments in neurons by myosin V." J Cell Sci **111 (Pt 21)**: 3221-34.

Thaler, C. D. and L. T. Haimo (1990). "Regulation of organelle transport in melanophores by calcineurin." J Cell Biol **111**(5 Pt 1): 1939-48.

Thaler, C. D. and L. T. Haimo (1992). "Control of organelle transport in melanophores: regulation of Ca2+ and cAMP levels." Cell Motil Cytoskeleton **22**(3): 175-84.

Tolmachova, T., R. Anders, J. Stinchcombe, G. Bossi, G. M. Griffiths, C. Huxley and M. C. Seabra (2004). "A general role for Rab27a in secretory cells." Mol Biol Cell **15**(1): 332-44.

Tsuboi, T. and M. Fukuda (2005). "The C2B domain of rabphilin directly interacts with SNAP-25 and regulates the docking step of dense core vesicle exocytosis in PC12 cells." J Biol Chem **280**(47): 39253-9.

Tuma, M. C. and V. I. Gelfand (1999). "Molecular mechanisms of pigment transport in melanophores." Pigment Cell Res **12**(5): 283-94.

Tuma, M. C., A. Zill, N. Le Bot, I. Vernos and V. Gelfand (1998). "Heterotrimeric kinesin II is the microtubule motor protein responsible for pigment dispersion in Xenopus melanophores." J Cell Biol **143**(6): 1547-58.

Vale, R. D. (2003). "Myosin V motor proteins: marching stepwise towards a mechanism." J Cell Biol **163**(3): 445-50.

Vale, R. D. (2003). "The molecular motor toolbox for intracellular transport." Cell **112**(4): 467-80.

Valetti, C., D. M. Wetzel, M. Schrader, M. J. Hasbani, S. R. Gill, T. E. Kreis and T. A. Schroer (1999). "Role of dynactin in endocytic traffic: effects of dynamitin overexpression and colocalization with CLIP-170." Mol Biol Cell **10**(12): 4107-20.

Varadi, A., T. Tsuboi and G. A. Rutter (2005). "Myosin Va transports dense core secretory vesicles in pancreatic MIN6 beta-cells." Mol Biol Cell **16**(6): 2670-80.

Verhey, K. J., D. L. Lizotte, T. Abramson, L. Barenboim, B. J. Schnapp and T. A. Rapoport (1998). "Light chain-dependent regulation of Kinesin's interaction with microtubules." J Cell Biol **143**(4): 1053-66.

Verhey, K. J., D. Meyer, R. Deehan, J. Blenis, B. J. Schnapp, T. A. Rapoport and B. Margolis (2001). "Cargo of kinesin identified as JIP scaffolding proteins and associated signaling molecules." J Cell Biol **152**(5): 959-70.

Wacker, I., C. Kaether, A. Kromer, A. Migala, W. Almers and H. H. Gerdes (1997). "Microtubule-dependent transport of secretory vesicles visualized in real time with a GFP-tagged secretory protein." J Cell Sci **110 (Pt 13)**: 1453-63.

Wade, R. H. and A. A. Hyman (1997). "Microtubule structure and dynamics." Curr Opin Cell Biol **9**(1): 12-7.

Wang, F., K. Thirumurugan, W. F. Stafford, J. A. Hammer, 3rd, P. J. Knight and J. R. Sellers (2004). "Regulated conformation of myosin V." J Biol Chem **279**(4): 2333-6.

Watanabe, M., K. Nomura, A. Ohyama, R. Ishikawa, Y. Komiya, K. Hosaka, E. Yamauchi, H. Taniguchi, N. Sasakawa, K. Kumakura, T. Ushiki, O. Sato, M. Ikebe and M. Igarashi (2005). "Myosin-Va regulates exocytosis through the submicromolar Ca2+-dependent binding of syntaxin-1A." Mol Biol Cell **16**(10): 4519-30.

Waterman-Storer, C. M., S. B. Karki, S. A. Kuznetsov, J. S. Tabb, D. G. Weiss, G. M. Langford and E. L. Holzbaur (1997). "The interaction between cytoplasmic dynein and dynactin is required for fast axonal transport." Proc Natl Acad Sci U S A **94**(22): 12180-5.

Wegener, A. D. and L. R. Jones (1984). "Phosphorylation-induced mobility shift in phospholamban in sodium dodecyl sulfate-polyacrylamide gels. Evidence for a protein structure consisting of multiple identical phosphorylatable subunits." J Biol Chem **259**(3): 1834-41.

Welte, M. A. (2004). "Bidirectional transport along microtubules." Curr Biol **14**(13): R525-37.

Welte, M. A., S. P. Gross, M. Postner, S. M. Block and E. F. Wieschaus (1998). "Developmental regulation of vesicle transport in Drosophila embryos: forces and kinetics." Cell **92**(4): 547-57.

Westerfield, M. (2000). The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio). 4th ed. Univ. of Oregon Press, Eugene.

Wollert, T., D. G. Weiss, H. H. Gerdes and S. A. Kuznetsov (2002). "Activation of myosin V-based motility and F-actin-dependent network formation of endoplasmic reticulum during mitosis." J Cell Biol **159**(4): 571-7.

Wu, X., B. Bowers, K. Rao, Q. Wei and J. A. Hammer, 3rd (1998). "Visualization of melanosome dynamics within wild-type and dilute melanocytes suggests a paradigm for myosin V function In vivo." J Cell Biol **143**(7): 1899-918.

Wu, X., B. Bowers, Q. Wei, B. Kocher and J. A. Hammer, 3rd (1997). "Myosin V associates with melanosomes in mouse melanocytes: evidence that myosin V is an organelle motor." J Cell Sci **110 (Pt 7)**: 847-59.

Wu, X. and J. A. Hammer, 3rd (2000). "Making sense of melanosome dynamics in mouse melanocytes." Pigment Cell Res **13**(4): 241-7.

Wu, X., K. Rao, M. B. Bowers, N. G. Copeland, N. A. Jenkins and J. A. Hammer, 3rd (2001). "Rab27a enables myosin Va-dependent melanosome capture by recruiting the myosin to the organelle." J Cell Sci **114**(Pt 6): 1091-100.

Wu, X., F. Wang, K. Rao, J. R. Sellers and J. A. Hammer, 3rd (2002). "Rab27a is an essential component of melanosome receptor for myosin Va." Mol Biol Cell **13**(5): 1735-49.

Wu, X. S., K. Rao, H. Zhang, F. Wang, J. R. Sellers, L. E. Matesic, N. G. Copeland, N. A. Jenkins and J. A. Hammer, 3rd (2002). "Identification of an organelle receptor for myosin-Va." Nat Cell Biol **4**(4): 271-8.