DISSERTATION

DETERMINATION OF THE FUNCTIONS OF RAB32, RAB38, AND THEIR EFFECTOR MYOSIN VC IN THE BIOGENESIS OF MELANOSOMES

Submitted by

Jarred Bultema

Department of Biochemistry and Molecular Biology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2013

Doctoral Committee:

Advisor: Santiago Di Pietro

Eric Ross
Jennifer DeLuca
Chaoping Chen
Anireddy Reddy

ABSTRACT

DETERMINATION OF THE FUNCTIONS OF RAB32, RAB38, AND THEIR EFFECTOR MYOSIN VC IN THE BIOGENESIS OF MELANOSOMES

In mammals, pigment produced within specialized cells is responsible for skin, hair, and eye coloration. Melanocytes are specialized cells that produce pigment within an organelle known as the melanosome. Melanosomes are a member of a specialized class of organelles, known as Lysosome-related organelles (LRO), which are responsible for a number of critical functions in mammals such as pigmentation, blood clotting, lung function, and immune function. LROs are related to the ubiquitous lysosome, and are formed using the same molecular mechanisms as lysosomes that rely upon the Adaptor Protein complexes -1 (AP-1) and -3 (AP-3), and the Biogenesis of Lysosome-related Organelles Complex (BLOC)-2 (BLOC-2). These protein complexes are critical for the trafficking of specialized cargoes to melanosomes required for proper melanin synthesis. But, these complexes are also used for the formation of lysosomes, and no mechanism is known to distinguish between trafficking to lysosomes and melanosomes. The melanosome serves as a model system to study the formation of LROs, and insights from the study of melanosomes help explain the biogenesis of other LROs.

In this dissertation, I present the finding that Rab32 and Rab38 function as melanosome-specific trafficking factors that allow for the use of AP-3, AP-1, and BLOC-2 in melanosome biogenesis. Using biochemical approaches, I show that Rab32 and Rab38 bind directly to AP-3, AP-1, and BLOC-2 on membranes. In microscopy experiments, I demonstrate that Rab32 and Rab38 localize to early endosomal subdomains where AP-3, AP-1, and BLOC-2 function. Using

a combination of biochemical and microscopic approaches, I show that Rab32 and Rab38 serve partially redundant functions in trafficking of specialized cargoes to melanosomes.

I report the discovery that Myosin Vc, a class V myosin motor, interacts with Rab32 and Rab38 and serves novel functions in melanosomes trafficking. I show, using biochemical approaches, that Myosin Vc directly binds to several melanosomal Rab proteins and serves as an effector of these proteins in melanosome biogenesis. Using a combination of approaches, I demonstrate that depletion of Myosin Vc from melanocyte cells causes defects in the trafficking of cargoes to melanosomes, but also causes severe defects in the secretion of mature melanosomes. With biochemical and microscopic approaches, I compare the function and localization of Myosin Vc in melanocytes to related proteins Myosin Va and Myosin Vb, and provide evidence to suggest that all three of these proteins function in distinct steps of melanosome trafficking.

My results answer outstanding questions about the use of ubiquitous trafficking machinery (AP-3, AP-1, and BLOC-2) in trafficking to a specialized organelle. I provide evidence to answer outstanding questions about the mechanism of action of Rab32 and Rab38 in melanosome trafficking through my studies with Myosin Vc. I also establish new areas of research in the comparison of Myosin Va, Myosin Vb, and Myosin Vc in melanosome trafficking. My results address numerous unknown areas in melanosome biogenesis, expand the knowledge of melanosome biogenesis, and provide numerous new avenues of research to explore to understand specialized trafficking to LROs.

ACKNOWLEDGMENTS

Now at the end of my graduate career, I reflect upon the various steps that have brought me to this place. First and foremost, I must thank my advisor Santiago Di Pietro for believing in me and accepting me as one of his first PhD students. Through continuous guidance, advice, and mentorship he has helped shape me into the scientist I am now. My aspiration to be as thoughtful, creative, and dedicated scientist is based on the example set by Santiago. I must also credit Andrea Ambrosio for my development as a scientist. As a core member of the lab and an extremely enjoyable colleague, she was one of my main sources of advice, and in-lab entertainment, and is the reason that the lab has the atmosphere that it does.

Daniel Feliciano and I began our PhD studies with Santiago at the same time, and Daniel has been a constant source of support, encouragement, and inspiration. Knowing how dedicated and committed Daniel is to his work inspired me to work harder and push through the difficult times. The early, Di Pietro lab group felt more like a family, and made the time and frustration associated with graduate school infinitely more bearable. Previous lab member Carolyn Burek kept life in lab entertaining, and contributed to my research efforts with several important experiments. It has been a pleasure to see new members join the Di Pietro lab, and see how things have changed since I began. Thank you to all of the Di Pietro lab members, new and old, for all the help and collaboration. I was lucky to have been a part of this group from the start.

My family and friends have been an enormous support with the sacrifices and stress that are required in graduate school. Thank you for understanding and supporting me.

The frustration of graduate school has brought more to my life than I would have expected. Fellow graduate student, Megan Carter (now Bultema) and I initially bonded by venting about the frustrations of graduate school and the effect that it had on our lives.

Eventually, venting led to dating, an engagement, and a recent wedding. I can say that while earning a PhD is an enormous accomplishment; my proudest moments from graduate school are in meeting and, ultimately, marrying Megan. Thank you Megan for all of your help and support.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Overview

The focus of this dissertation is to investigate the molecular mechanisms underlying the biogenesis of a specialized organelle known as the melanosome. Work presented here focuses on specific steps that occur in the trafficking of proteins to melanosomes that are required for the formation of melanin pigments within the organelle. Pigment production within melanosomes is responsible for skin, hair, and eye pigmentation in mammals. Melanosomes are members of a class of organelles, known as lysosome-related organelles (LROs), which are similar to the ubiquitous lysosome. Lysosomes functions in the degradation of proteins, lipids, and sugars in nearly every cell of the human body. The distinction between a lysosome and a melanosome is through the delivery of melanosome-specific proteins to the organelle, which facilitates the production of melanin pigments. Historically, the melanosome is a well-studied system due to the ease of identification of genetic mutations that result in defects in melanosome biogenesis that manifest as pigmentation disorders.

The formation, or biogenesis, of melanosomes has thus far been shown to depend upon a combination of ubiquitous molecular mechanisms, used in the biogenesis of lysosomes, and a number of cell-type specific proteins found in melanocytes, melanosome producing cells. The endo-lysosomal system is composed of a heterogeneous group of organelles ranging from early endosomes to lysosomes. The early endosome is a hub of intracellular protein trafficking for both ubiquitous and cell-type specific sorting functions. A number of proteins required for the biogenesis of melanosomes are trafficked through the early endosome. It is unclear how proteins

destined for the lysosome or the melanosome are segregated and sorted to the proper organelle as many of these proteins utilize the same molecular machinery for trafficking.

The specific goals of my dissertation are to understand how ubiquitous and cell-type specific proteins functionally interact to distinguish trafficking to these two organelles. I focus on the function of two small GTPases, Rab32 and Rab38, that function as melanosome-specific trafficking factors. The interactions of Rab32 and Rab38 with ubiquitous trafficking machinery are investigated to provide a mechanism for how these melanosome-specific proteins function. In this dissertation, I report novel findings on the functions of Rab32 and Rab38 with adaptor proteins resident on early endosomes and function in the formation of lysosomes and melanosomes (Chapter 2). These findings were published in the Journal of Biological Chemistry in 2012 in which I am the first author. We were also asked to write a commentary on these findings, and how they integrate with the state of knowledge in melanosome biogenesis. This commentary, in which I am the first author, was published in the journal Small GTPases in 2012 (Appendix 1). I also participated, and am a second author, in the publication of a methods paper that established the use of techniques for the biochemical and microscopy study of intracellular protein trafficking, which was published in the Journal of Visualized Experiments (JoVE) in 2011. I also report the novel discovery that Myosin Vc, an actin-based motor, functions with Rab32 and Rab38 in melanosome biogenesis (Chapter 3). Studies on the function of Myosin Vc in melanosome biogenesis have been compiled into a manuscript, which will be submitted to the journal Proceedings of the National Academy of Sciences USA in the coming months.

In order to understand the melanosome-specific trafficking of proteins, it is necessary to first understand the mechanisms used in the cargo trafficking and organelle maturation events of the endo-lysosomal system. In the next section I will discuss certain trafficking proteins,

mechanisms, and events that are critical to the function of the endo-lysosomal system. I will then discuss the formation and different types of lysosome-related organelles, and diseases impacting the biogenesis of lysosome-related organelles. Specific details about the formation of the melanosome and trafficking of melanin synthesizing enzymes will be presented, followed by discussion of the current state of knowledge in the field of melanosome biogenesis (Chapter 1). A list of acronym and abbreviations used may be helpful for a reading of this text (Appendix 2). I will present research findings from experiments I have performed investigating the functions of Rab32 and Rab38 (Chapter 2) and their effector Myosin Vc in melanosome biogenesis (Chapter 3). Finally, I will conclude with a discussion of the impact and significance that my results have on the state of knowledge in the field (Chapter 4).

1.2 Intracellular trafficking of the endo-lysosomal system

Melanosome biogenesis uses aspects of the common endo-lysosomal system for the specialized trafficking of cargoes to the melanosomes. The endo-lysosomal system is a hub of intracellular protein trafficking and specialized trafficking to melanosomes is just one of the many trafficking events that occurs in this system. In order to understand the specialized trafficking that occurs for melanosome biogenesis, it is important to first understand the general mechanisms used in the endo-lysosomal system of organelles. For simplicity, important proteins and protein complexes that are fundamental to understanding of endosome function are explicitly stated by name, but some specific details in the names of proteins – such as the names of specific Rab5 effectors discussed below – are omitted for simplicity. Consultation of the list of acronyms and their definitions may be useful for a reading of Chapter 1.

1.2.1 General structure and function of endosomes

Intracellular trafficking can be simply described as the movement of vesicles; small membrane-bound structures roughly 70-100 nm in diameter, between organelles, larger membrane structures, or the limiting plasma membrane of the cell. Endocytosis, the internalization of material from outside the cell, and secretion, the transport of material to the outside of the cell, account for the majority of intracellular trafficking events that occur in cells and are necessary for numerous cellular functions. Within intracellular trafficking, the endosomal system serves as a major intersection between the intracellular and extracellular protein environments (1). Extracellular material, whether bound to transmembrane receptors or soluble in the extracellular environment, and integral transmembrane proteins embedded in the plasma membrane are internalized via the formation of endocytic vesicles on the cytoplasmic side of the plasma membrane.

Numerous mechanisms exist for the formation and internalization of endocytic vesicles, but a common feature of nearly all endocytic pathways is rapid targeting to distinct organelles called early endosomes. Early endosomes serve as a focal point of intracellular trafficking and sorting to other organelles or recycling back to the plasma membrane (2). The early endosomes, also known as sorting endosomes, are the first endocytic compartment for cargoes internalized from the plasma membrane and are a heterogeneous, dynamic group of organelles that serves as a hub between the extracellular environment and intracellular organelles. They are maintained through constant vesicular and organelle trafficking and remodeling events (3).

Overall, four major routes are used in early endosomal trafficking of non-specialized cells: trafficking to the plasma membrane (via recycling endosomes or direct vesicle transit), trafficking to other endosomal organelles, retrograde trafficking to the trans-golgi network, or

trafficking to the lysosome for degradation (4, 5). The endosomal system can be classified based on lipid identity, morphology, intra-organellar pH, organelle function, and by both lumenal and membrane protein composition (5). The moderately acidic environment of the early endosome, ~ pH 6.3 – 6.8, allows for dissociation of soluble, intraluminal ligands and integral-membrane receptors. Acidification of endosomes, by Vacuolar-type H+ pumps ATPases (V-ATPase) and ion channels, can be utilized by endocytosed cargoes: for instance, the iron-transporting transferrin-receptor (TfR) uses the increased acidification of endosomes to release bound iron ions and allow recycling of the apo-transferrin receptor complex back to the plasma membrane. Upon release from endocytosed receptors, the soluble cargoes can be trafficked out of the endosomes to other organelles or directly into the cytosol.

Early endosomes are composed of thin tubular extensions approximately 60 nm in diameter and larger vacuolar domains roughly 400 nm in diameter (6). The morphologically distinct domains of early endosomes characterize different trafficking pathways and have different protein, lipid, and chemical composition (7, 8). Tubular domains of early endosomes, with a very large surface area to volume ratio, are functionally important in recycling of integral membrane cargoes to the plasma membrane, retrograde transport to the Golgi, and trafficking to other organelles, such as lysosome-related organelles. The vacuolar domain of endosomes contains the majority of soluble content of early endosomes and is associated with degradation via endosomal maturation and fusion with lysosomes (8).

Maturation from early endosomes to late endosomes is characterized by numerous changes in both intra-lumenal and membrane protein content in addition to a shift in membrane lipid composition, morphology, and function. Fundamental steps in endosome maturation: Rab switch from Rab5 to Rab7 on endosome membrane, formation of intraluminal vesicles (ILV),

acidification of lumenal pH, phospho-inositol conversion from PI(3)P to PI(3,5)P2, loss of tubular domains from endosomes, loss of recycling to the plasma membrane, gain of lysosomal proteins, switch in organelle fusion specificity, switch in organelle motility, and intraluminal ion changes are necessary to complete maturation into a late endosome (9).

1.2.2 Rab GTPases are critical members of endo-lysosomal function

Numerous proteins have defined roles in endosomal maturation, but Rab GTPases are important determinants and markers of different organelles and endo-lysosomal maturation events. Rab GTPases compose the largest sub-family of the RAS GTPase superfamily of proteins (10). Rabs are monomeric proteins that localize to specific compartments and membrane subdomains and stably associate with membranes by insertion of a lipid moiety into the lipid bilayer. They serve as pathway-specific "molecular switches" that facilitate the recruitment and function of numerous effector proteins within intracellular membrane trafficking. Rabs function as "molecular switches" via tight regulation of nucleotide state (GTP/GDP), cellular localization, and protein-protein interactions (10). In the GDP state, Rabs are generally bound to a Guanine-dissociation inhibitory protein (GDI) that maintains the Rab in a GDP bound state and inhibits binding of other proteins. Upon specific signaling events, the GDI-protein is displaced by a protein with GTP/GDP-exchange factor (GEF) activity and is generally concomitant with recruitment of a Rab to a membrane (10).

Recruitment of Rabs to membranes is coordinated with insertion of lipid moieties on modified c-terminal Cysteine residues, conserved in all Rabs. Geranylgeranyl-transferases result in the presence of single or double lipid moieties on the C-terminus of Rabs that allows for stable membrane association (10). Rabs are recruited to membranes primarily by GEFs, although it is

also suspected that membrane lipids, SNAREs, and other interacting proteins are also important for recruitment (11).

Upon exchange of GDP for GTP by GEFs, two regions within Rabs, switch I and switch II, undergo conformation changes that facilitate binding to different proteins. In the now "active", GTP-bound membrane-associated state, Rabs can interact with effector-proteins and serve as membrane anchors to functionally couple cytosolic proteins to specific membranes (10). Inactivation of Rabs occurs either via the slow, self-hydrolysis of GTP to GDP or is facilitated by GTPase-activating proteins (GAPs), which also remove GDP-bound Rabs from membranes (10). Now free in the cytoplasm, GDI proteins will bind the Rabs and maintain the inactive state until the cycle is repeated.

1.2.3 Function of Rab5 at early endosomes

Of the more than 60 members of the Rab family, many Rabs have been identified as functioning in endosomal domains. Rab5 is present on endocytic vesicles and early endosomes where it interacts with numerous effector proteins to maintain early endosome function and dynamics (12-16). The initial recruitment of Rab5 to vesicle and early endosome membranes is dependent on a GEF for Rab5 that is capable of binding to ubiquitinated cargoes, either alone or in complex with a Rab5 effector, which interacts with the active form of Rab5 (17, 18). The Rab5 recruitment by its GEF is enhanced by the Rab effector, which creates a positive feedback loop that rapidly recruits additional Rab5 molecules to membranes (19) and facilitates the rapid recruitment of Rab5 effectors.

Through interactions with numerous effector proteins, Rab5 is necessary for the production of phosphatidylinositol-3-phosphate (PI(3)P) lipids on early endosomes (20-22),

homotypic fusion of early endosomes (12), early endosomal signaling pathways (23-25), and early endosome movement on actin and microtubules (26). Many of the Rab5 effectors also bind to the PI(3)P lipids on early endosomes and the lipid composition of these organelles is critical for proper trafficking in early endosomes (27).

Early endosomes undergo numerous remodeling events such as tubulation, dynamic kiss-and-run or stable fusion events, and organelle fission (5). Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are conserved membrane proteins used in the fusion of vesicles and organelles in intracellular trafficking events (28, 29). SNARE proteins are unstructured when monomeric, but in a complex with the correct SNARE binding partners SNARE motifs in proteins form a compacted parallel four-helix bundle helical structure (30, 31, 32). Interaction of the correct SNAREs to form a tetrameric helical complex is restricted to specific pairs of SNAREs, and the presence of correct SNAREs on the vesicle (v-SNAREs) and on target membranes (t-SNAREs) allows a zipper-like function that pulls two membranes together and facilitates membrane fusion (33, 34). Early endosome fusion is mediated by the Rab 5 effector early endosomal antigen-1 (EEA1) in coordination with specific target-SNAREs (35-39). Early endosome fusion events occur through the action of EEA1 proteins bound to Rab5 and PI(3)P on membranes and the interactions of SNAREs. EEA1 recruits this SNARE machinery to early endosomes, and also acts as a tether between two early endosomes by binding to Rab5 on one endosome and PI(3)P on another. The interaction of v-SNAREs and t-SNAREs is facilitated by Rab5 effector the CORVET tethering complex (40, 41). The CORVET complex tethers membranes through interactions with Rab5, and activate and "proof-read" SNARE assembly into a complex (41). These activities cause the fusion of two early endosomes, after which point the SNARE complex dissociates and the SNAREs are recycled (42, 43).

Rab5 effectors are also responsible for the movement of early endosomes through recruiting microtubule and actin based motors (25, 44). The recruitment of Kinesin-3 motor and dynein-dynactin complex is dependent on interactions with the Rab5 effector PI(3)P-kinase (45, 46). Microtubule-based movement is used in the majority of early endosome movement, but a Rab5 effector can drive early endosomes toward actin-based movement using Myosin VI and its interacting partner (26, 44).

1.2.4 Rab5-Rab7 switch in the conversion of endosomes

In addition to functioning in early endosome maintenance, Rab5a also functions in the maturation from early to late endosomes by recruiting Rab7 to organelles (47). The conversion from early to late endosomes is dependent on the formation of a Rab7 domain on an early endosomes, which coexist with Rab5 briefly in a Rab5/Rab7 hybrid organelle (48, 49). It is unclear if early endosomes are directly converted into late endosomes, or if vacuolar regions of early endosomes undergo fission to form a new micro-organelle, which can fuse with other late endosomes (48, 50, 51). Rab5 facilitates the recruitment of Rab7 by interacting with the Rab7-GEF (47, 52), which interacts with both Rab5 and PI(3)P on early endosomes. In addition to functions as a Rab7-GEF function, this protein also disrupts the interaction of the Rab5-GEF with Rab5, and thereby removes the Rab5 activation feedback-loop (47). The accumulation of Rab7 on membranes occurs simultaneously with the deactivation of Rab5 by Rab5-GAP, which catalyzed the conversion of GTP to GDP by Rab5 and results in a loss of Rab5 localization and activity on membranes (53, 54). Recently, it was shown that BLOC-1, an important component for lysosome and melanosome trafficking, regulates the lifetime of Rab5 on membranes through

control of the localization of the Rab5-GAP on membranes, and thus serves to regulate the maturation of early endosomes (55).

1.2.5 Maturation of endosomes

With the recruitment of Rab7 and loss of Rab5, the endosome will undergo a series of changes in membrane and protein composition, morphology, and chemistry resulting in drastically different organelles. Morphologically, the endosome loses the dynamic tubules that are present on early endosomes (48, 56) and increases in the number of intraluminal vesicles (ILV) present in the organelle (9, 57). While small numbers of ILV are present in early endosomes (58) the formation and accumulation of ILV occurs primarily in late endosomes, which because of the increase in ILV are often known as multivesicular bodies/endosomes (MVB/MVE), and is a critical component in late endosome maturation. In respect to cargoes, the formation of ILV effectively reverses the polarity of transmembrane cargoes such that functionally what was the cytosolic tail is now inside the ILV vesicle. ILV formation is critical for the silencing of signaling receptors by hiding the cytosolic tails within ILV and for the degradation of cargoes by lysosomal hydrolases, which are often unable to digest the highly glycosylated luminal domains of transmembrane proteins but are more easily digested on ILV. The formation of ILV occurs using both Endosomal Sorting Complex Required for Transport (ESCRT)-dependent and an ESCRT-independent mechanism that depends on specific lipids (59-70).

The ESCRT machinery is composed of specific types of cytosolic proteins that organize into various sub complexes and enable a unique organelle remodeling events with the formation of ILV. These proteins are individually recruited from the cytosol with ESCRT-0 proteins

arriving first, followed sequentially by ESCRT-I, -II, and –III, which self-assemble and facilitate the formation of inward budding to form ILV. The sorting of proteins into ILV is thought to be regulated, at least in part, by the presence of ubiquitin-modifications on lysine residues and these are recognized by ESCRT proteins to facilitate cargo selection into ILV (71-75). After sorting into the forming ILV, but before vesicle closure, the ubiquitin-modification is removed by ESCRT proteins with deubiquitination activity (76).

ESCRT complex recruitment to endosomal membranes is controlled by binding to ubiquitinated cargo, accessory proteins, clathrin, and specific PI-lipids (9). PIs, found primarily on the cytosolic leaflet of organelles, are important in many membrane fusion and fission events and serve as anchors for the binding of Rabs, and Rab-effectors like EEA1 and the CORVET/HOPS complexes, Vps-C tethering complexes that function in the fusion of early and late endosomes-lysosomes respectively. The maturation of endosomes also encompasses a conversion of the PI-lipids present on membranes from PI(3)P, present on early endosomes, to PI(3,5)P2 on late endosomes (77, 78).

The conversion from PI(3)P to PI(3,5)P2 is regulated by a kinase that binds to enriched PI(3)P regions on membranes, and forms a semi-stable complex with its activator and the PI(3,5)P2 phosphatase that allow for the rapid production and degradation of PI(3,5)P2 (79). In addition to recruitment of the kinase, PI(3)P levels are regulated by the presence of Rab7 on membranes, which functionally decreases the stability of PI(3)P and thus facilitates the conversion to PI(3,5)P2 on Rab7-positive membranes (80).

This change in PI-lipids coincides with a shift in SNARE proteins, tethering complexes CORVET/HOPS that alter the fusion specificity of endosomes. While early endosomes undergo primarily homo-typic fusion, late endosomes will undergo homo-typic fusion with other late

endosomal membranes, and hetero-typic fusion with lysosomes (9). Like early endosomes, late endosomes are a heterogeneous population of organelles composed of different cargo proteins and destined for different fates (57). Fusion events of late endosomes vary, but can be generally categorized as fusion with the Lysosome or autophagosome for cargo and organelle degradation, fusion with the plasma membrane for the secretion of ILV as exosomes for cell-cell communication, or modification of late endosomes into lysosomes or Lysosome-related organelles (9).

1.2.6 Lysosome biogenesis

Lysosomes are cellular organelles that are responsible for the degradation of proteins, organelles, waste material, cellular debris, and extracellular pathogens (9). The various forms of the proteasome are responsible for these degradative functions within the cytoplasm are physically separate from the contents of vesicles and organelles by membranes. Therefore, the lysosome functions as a separate mechanism for the degradation of cargoes that are embedded in the membranes or within the lumen of vesicles and organelles. Lysosomes function through the activity of acid hydrolases, which are only fully functional in the highly acidic environment of the lysosome (~pH 5) (9). Lacking functional lysosomes, cells exhibit severe dysfunction that manifests as lysosomal storage disorders such as Tay-Sachs disease, which results in the progressive nerve degeneration that ultimately results in mental and physical disability (81, 82).

Development of the lysosome occurs through maturation of the early endosomes into late endosomes, invagination of intra-lumenal vesicles to form the multi-vesicular body, and further development into a degradative lysosome via the delivery of vesicles from the Golgi containing the lytic acid hydrolases. Interestingly, the acid hydrolases are present in the late

endosomes and multi-vesicular bodies but are less active than in the lysosome, and are trafficked from the Golgi apparatus to late endosomes/lysosomes primarily using a mechanism dependent on the mannose-6-phosphate receptor that recognizes the mannose-6-phosphate groups that are attached to acid hydrolases within the Golgi network (9).

Trafficking to the lysosome is directed either by the presence of ubiquitin on endocytic cargoes or small peptide motifs, known as sorting signals, on the cytosolic portion of transmembrane proteins (83-85). Two common examples of sorting signals used in lysosomal trafficking are (D/E)xxx(L/I)(L/I) (di-leucine sorting signal) or Yxxϕ (tyrosine sorting signal) where "x" can be any amino acid and ϕ is a bulky hydrophobic amino acid such as valine, isoleucine, leucine, or phenylalanine (86).

Sorting signals themselves do not have active roles in trafficking; signals are recognized by adaptor proteins that will bind the sorting signal and recruit other factors needed for vesicle formation (86). Clathrin-adaptor proteins, such as Adaptor-Proteins 1 and 3 (AP-1 and AP-3) facilitate the formation of a "clathrin coat" by simultaneously binding to tyrosine or di-leucine sorting signals on cytosolic portions of transmembrane proteins, membrane lipids, accessory proteins, and clathrin (87, 88). Clathrin-adaptor proteins function as a nexus that facilitates the concentration of cargo proteins and the protein machinery required for the formation of a vesicle.

Clathrin is a large, hexameric (trimer of dimers) coat protein that is capable of self-oligomerization into a lattice that functions in vesicle formation and invagination in many intracellular trafficking events. The AP (AP-1, AP-2, AP-3, AP-4, AP-5) clathrin adaptor proteins are hetero-tetramers composed of two large subunits (adaptins) that contain a well-folded core domain that links to folded ear domains through flexible, unfolded linker domains, and medium and small subunits that also compose the well-folded core domain (88). The overall

structure of AP clathrin adaptor proteins is said to resemble “mickey mouse” with a well-folded head domain and two ear domains connected via flexible linkers. The core, head-domain is responsible for binding to cargo sorting signals and membrane lipids, while the well-conserved β-subunit is responsible for clathrin binding and the variable subunit is responsible for binding accessories proteins (88). These transient, multimeric protein complexes facilitate the formation of a vesicle that is subsequently released from the endosomal membrane via scission proteins. The clathrin and adaptor-proteins bound to the vesicle quickly disassemble while other accessory proteins already present or subsequently recruited remain bound to facilitate subsequent vesicular trafficking (88). Adaptor proteins function as a critical bridge between transmembrane cargoes, or cargo receptors, and other components of the trafficking machinery required for downstream movement.

1.3 Lysosome-related organelles

The function of the lysosome is a ubiquitous requirement of eukaryotes. However, in some specialized cell-types a similar organelle is formed to serve specific functions. These lysosome-related organelles (LROs) are specialized organelles found in some cell-types and share some characteristics with lysosomes - namely acidic lumen, protein components, and aspects of organelle biogenesis. In addition to lysosomal components, LROs also contain specific proteins that are required for the specialized functions that these organelles serve (89, 90). Based on similarity to lysosomes and studies on specific LROs, it is known that LROs are formed using a combination of ubiquitous lysosome biogenesis machinery and cell-type specific proteins that allow for the formation of an organelle with distinct identity from a lysosome (89, 90).

Lysosome-related organelles are found in specialized cell types such as melanocytes, platelets, lung alveolar type II cells, and some innate and adaptive immune cells (90-94). LROs have critical roles in pigment production, blood clotting, lung surfactant production, lytic activity of the innate immune system and antigen-processing of the adaptive system (90-94). The melanosome has served as the prototypic LRO and has been the most studied due to the easy identifying of protein components involved in melanosome biogenesis and function that manifests as defects in pigmentation (hypopigmentation or hyperpigmentation) in humans, mice, and rats. The availability of the pigmented human melanocyte cell line MNT-1 has served as an important tool in studying melanosome biogenesis (95) and is heavily utilized in studies of melanosome biogenesis.

Produced within melanocyte cells in skin, and retinal pigmented epithelial cells (RPE) in the eye, melanosomes are responsible for the synthesis of photo-protective pigment melanin, responsible for hair and skin pigmentation in mammals. Melanosomes are known to co-exist with lysosomes in melanocytes and RPE cells and, in common with lysosomes, are derived from endosomal domains (91-94). Further, like lysosomes, melanosomes have an acidic lumen that contains acid hydrolases, and membranes that contain the Lysosome-associated membrane proteins (LAMP) 1-3 (89). Melanosomes contain numerous melanosome-specific proteins responsible for organelle biogenesis, pigment production, and organelle secretion (91-94).

Platelet dense granules are another form of LRO (91-94). Found in platelets and in platelet-producing megakaryocytes, dense granules are a component of hemostatic thrombogenesis, better known as blood clotting (91-94). Platelets contain three organelles that serve functions in thrombus (blood clot) formation: α-granules, δ-granules (dense or delta granules), and lysosomes. Dense granules contain high concentrations of serotonin, Calcium,

ATP, ADP, and polyphosphate (89) and are characterized by their electron dense core, as observed in electron micrographs. All three granules (α-granules, δ-granules, and lysosomes) are secreted from platelets during thrombogenesis (91-94). Like the melanosome, dense granules co-exist with lysosomes and have similar mechanisms of biogenesis, presence of LAMPs on the organelle membrane, but do not to contain acid hydrolases or a strongly acid lumen (91-94). Numerous specific ion-channels and pumps are responsible for the concentration of ions and small molecules within dense granules, but the identity and trafficking of most of these components remains a mystery.

Type II alveolar cells of the lung are responsible for production of pulmonary surfactant, which occurs within another type of LRO the lamellar body (91-94). Pulmonary surfactant is a mixture of lipids and proteins that are produced within lamellar bodies, which are ultimately secreted into the alveolar airspaces to assist in proper lung function. While relatively little is known concerning the biogenesis of lamellar bodies, it has been found that many proteins are shared between lamellar bodies and other LROs. Proteomic analysis has found that LROs have a high degree of shared components: a 60% identical proteome was found across all LROs, and lamellar bodies of the lung and the melanosome have a 38% shared proteome (96).

Several LROs are present in cells of the immune system. Lytic granules found in cytotoxic T-cells and natural killer cells are an LRO with shared acidic lumen, acid hydrolases, LAMPs, and endosomal origin as lysosomes (93, 94). Basophil granules are another type of LRO that has functions in allergy response, and shares the same acid hydrolases and LAMPs as lysosomes but may have different biogenesis (93, 94). Azurophil granules in neutrophils are yet another form of LROs of the immune system that also contain lysosomal acid hydrolases, but unlike some other LROs do not have many shared protein components with lysosomes (93, 94).

Within antigen presenting dendritic cells and B-cells, the major histocompatibility-complex type II (MHC-II) compartments where antigens are loaded onto MHC-II complexes are also a type of LRO (93, 94). These compartments also have shared endocytic origin and protein components with lysosomes. Numerous LROs are found in cells of the immune system, but the biogenesis of these organelles has not been as highly studied as in other systems, and therefore less is known about these LROs.

As is evident from the presence of both LROs and lysosomes in several cell types, the production of LROs within cells does not preclude the ability to also form lysosomes. In some cases, such as the lytic granules in cytotoxic T-cells and natural killer cells, it appears that the LRO replaces the lysosome within the cell (93, 94). This raises interesting questions about the mechanisms shared between different types of organelle in the same cells and how the same molecular machinery can simultaneously be used for lysosome and LRO biogenesis within specialized cells where LROs exist.

1.4 Diseases of Lysosome-related organelles

Several genetic disorders result in the loss of functional LROs. Hermansky-Pudlak syndrome (HPS), Chediak-Higashi syndrome (CHS), and Griscelli syndrome (GS) result in deficiency of at least one type of LRO (89, 97) (Figure 1.1). In CHS and HPS both lysosomes and numerous LRO types are affected (89). Research on these disorders has demonstrated that, in addition to shared biogenesis between lysosomes and individual LROs, several types of LROs have common biogenesis mechanisms (94). However, to this point there have not been any genetic disorders discovered that cause defects in formation of all types of LROs. This fact raises several interesting questions. Is there an evolutionary divergence in the biogenesis of

different classes of LROs? What are the proteins and molecular mechanisms responsible for the biogenesis of different LROs? The answers to these questions can be determined, at least in part, by a careful evaluation of the genetic disorders that affect LRO biogenesis.

1.4.1 Hermansky-Pudlak Syndrome

Hermansky-Pudlak syndrome is a group of recessive autosomal disorders that are characterized by hypopigmentation and blood clotting disorders caused by deficiencies in the formation melanosomes and platelet-dense granules (92, 94). Over 15 gene products are identified as causing different forms of HPS in humans or mouse models of the disease (92, 94). All subtypes of HPS have deficiencies of melanosomes and platelet dense-granules, but some HPS forms have additional deficiencies in lung and immune function (92, 94). These additional defects are a result of aberrant LRO biogenesis in lung lamellar bodies, and lytic granules of cytotoxic T-cells, natural killers, and perhaps other immune cells (92). HPS demonstrates that single gene mutations can affect the biogenesis or function of at least two distinct organelles. Genes that cause subtypes of HPS are therefore likely to have general roles in LRO biogenesis, which makes HPS a strong model system to investigate biogenesis and function of LROs (98).

Protein Complex	Mutant gene	Mutant protein	Human disease	Animal model
BLOC-1	DTNBP1	Dysbindin	HPS-7	sandy (m)
	PLDN	Pallidin	HPS-9	pallid (m)
	CNO	Cappuccino	-	cappuccino (m)
	MUTED	Muted	-	muted (m)
	SNAPAP	Snapin	-	-
	BLOC1S1	BLOS1	-	-
	BLOC1S2	BLOS2	-	-
	BLOC1S3/HPS8	BLOS3/HPS8	HPS-8	reduced pigmentation (m)
BLOC-2	HPS3	HPS3	HPS-3	coco (m)
	HPS5	HPS5	HPS-5	ruby-eye 2 (m), pink (f)
	HPS6	HPS6	HPS-6	ruby-eye (m)
BLOC-3	HPS1	HPS1	HPS-1	pale ear (m)
	HPS4	HPS4	HPS-4	light ear (m)
AP-3	AP3B1	AP-3 beta 3A	HPS-2	pearl (m), orange (f)
	AP3D1	AP-3 delta	-	mocha (m), garnet (f)
	AP3M1	AP-3 mu 3A	-	carmine (f)
	AP3S1/AP3S2	AP-3 sigma 3A/3B	-	ruby (f)
HOPS complex	VPS11	VPS11	-	pale gray eyes (m)
	VPS16	VPS16A	-	-
	VPS18	VPS18	-	deep orange (f)
	VPS33A	VPS33A	-	buff (m), carnation (f)
	VPS33B	VPS33B	-	-
	VPS39	VPS39	-	-
	VPS41	VPS41	-	light (f)
-	RABGGTA	RABGGTA	-	gunmetal (m)
-	RAB38	Rab38	-	chocolate (m), fawn-hooded (r)
-	MYO5A	Myosin Va	GS-1	dilute (m)
	RAB27A	Rab27a	GS-2	ashen (m)
	MLPH	Melanophilin	GS-3	leaden (m)
-	CHS1/LYST	CHS1/LYST	CHS	beige (m,r)

HPS = Hermansky-Pudlak Syndrome
GS = Griscelli Syndrome
CHS = Chediak-Higashi Syndrome

m = mouse
f = fruit fly
r = rat

Figure 1. 1
Classification of mutations resulting in pigmentation disorders. Many of the proteins associated with pigmentation disorders organize into stable protein complexes. Adapted from Huizing, et al, 2008 (90).

1.4.2 HPS-associated multi-subunit complexes

Analysis of HPS subtypes led to the discovery that most of the genes associated with HPS are found in four multimeric protein complexes: Biogenesis of Lysosome-related Organelle Complexes (BLOC) -1, -2, and -3; and the adaptor complex-3 (AP-3) (Figure 1.1). The BLOC complexes have very little homology with any proteins of known function, and understanding about the function of the BLOCs has remained unclear (97). All of the BLOC complexes are found within the cytoplasm of cells and associated with specific endosomal membrane domains (91, 94, 97, 99). The localization of BLOC complexes and the general deficiency in melanosomes and dense granules suggest that these complexes serve roles in endosomal dynamics or trafficking.

The BLOC-1 complex is composed of a single copy of eight subunits: Pallidin, Cappuccino, Muted, BLOS3, Dysbindin, Snapin, BLOS1, and BLOS2 (100) (Figure 1.1). Two stable sub-complexes are found within the structure of BLOC-1 that are bridged by a single-chain, unstructured linker giving the complex a flexible elongated structure anticipated to be roughly 300Å long and 30Å in diameter (101). The proteins Pallidin-Cappuccino-BLOS1 form one sub-complex and dysbindin-Snapin-BLOS2 form the second (101). BLOC-1 localizes to early endosomes and functions in vesicle formation through unknown mechanisms (91, 94, 97, 99). The interactions of BLOC-1 with other proteins are consistent with roles in protein trafficking (97) as both subunits of the complex and the entire complex are shown to interact with endosomal and exosomal SNAREs (102-106). Further, BLOC-1 is also responsible for the recruitment of the Rab5-GAP that serves to regulate the lifetime of early endosomes, and this represents a critical regulator in the development of late endosomes, lysosomes, and lysosome-related organelles (107). Of all of the BLOC complexes, BLOC-1 deficient mice display the

most severe hypopigmentation, which demonstrates that BLOC-1 serves a critical function in melanosome biogenesis (90, 92-94).

The function of the BLOC-2 complex - composed of the proteins HPS3, HPS5, and HPS6 – is currently unknown (Figure 1.1). However, some results on BLOC-2 suggests that it may function as a clathrin adaptor protein. BLOC-2 contains a small peptide motif, a clathrin-box, that is capable of binding to clathrin and BLOC-2 partially colocalizes with clathrin in live cells (108). Immunogold labeling in electron micrographs demonstrates that BLOC-2 localizes to specific tubular domains of early endosomes where vesicle formation is known to occur (99). BLOC-2 causes only partial hypopigmentation in mice, far less obvious than BLOC-1 deficient mice, but more severe than BLOC-3 deficient mice (90, 92-94). Unpublished data from our lab also demonstrates that BLOC-2 is able to bind to the sorting signals in the cytosolic tails of melanosomal cargo proteins. The ability to bind clathrin and sorting signals is a hallmark of clathrin-adaptors, but it is currently unclear if BLOC-2 might function as an adaptor protein, or instead serve other functions in intracellular vesicular trafficking.

BLOC-3, composed of HPS1 and HPS4, is a ubiquitously expressed protein complex that exists within the cytoplasm, but also has some degree of membrane localization (89, 109-113) (Figure 1.1). Immunostaining has shown localization in the peripheral regions of cells, but electron micrographs have demonstrated that BLOC-3 is present on tubo-vesicular endosomes, uncoated vesicles, and on early stage melanosomes (112). The structure of BLOC-3 has been determined as anti-parallel coiled-coil α-helices of HPS1 and HPS4 with well-folded domains on both N- and C- terminal regions of the structure and a more disordered central region (114). While BLOC-3 deficient subjects have only mild hypopigmentation, lung fibrosis is prevalent in BLOC-3 deficient patients and animals and this suggests that BLOC-3 also has important roles in

the formation of lamellar bodies in the lung type II epithelial cells (115-117). In fibroblasts, deficiency of BLOC-3 results in altered localization of late endosomes and lysosomes (118).

Unlike the BLOCs, AP-3, the fourth complex affected in HPS, is a well-known protein complex with established functions as a clathrin-adaptor protein (88, 94) (Figure 1.1). As previously described for AP complexes in general, AP-3 is a hetero-tetramer composed of a small subunit, σ3; a medium subunit, μ3; a large, conserved adaptin, β3; and a variable adaptin, δ (88). HPS-2 is caused by a mutation in the β3A adaptin subunit of AP-3 (119-122). Two genes encode for isoforms of σ3 and the same is true for β3 (88). Neuronal AP-3, which contains neuron specific isoforms β3b and σ3a/b, is not affected in HPS2 but is affected in additional HPS subtypes in mice caused by a mutation in the δ subunit of AP-3. These mice have numerous neurological defects in addition to hypo-pigmentation and bleeding disorders underlying the importance of AP-3 in ubiquitous cellular functions (99, 123-127). Conserved from yeast through humans, AP-3 plays a role in cargo selection at early endosomes through interaction with specific sorting signals of cargo proteins, and facilitates sorting of cargoes into vesicles for trafficking to lysosomes or LROs (128, 129).

All of the currently known HPS subtypes in humans are caused by mutations in BLOC-1/2/3 or AP-3 subunits, but additional mouse models of HPS have an additional mutation that affects the HOPS tethering complex (90-94) (Figure 1.1). The *buff* mouse is caused by a mutation in the VPS33A protein, a homologue of a component of the HOPS complex (130). The HOPS complex in yeast is involved in formation of membrane micro domains and membrane fusion events with the yeast vacuole through interactions with the yeast homologue of Rab7, and also functions in late endosome/Lysosome fusion and fission events in mammals through interaction with Rab7 and a late endosome/lysosome SNARE (131-136). The yeast HOPS

complex is both a GEF and effector for the yeast Rab7 homologue, and acts as a tethering complex through simultaneous interactions with the Rab7 homologue, acidic phospholipids, and SNAREs (137-140).

1.4.3 Other HPS-associated genes and proteins

Aside from mouse models of HPS that are caused by defective protein complexes with functions in trafficking, several other rodent models of HPS are caused by defects in monomeric proteins: a Rab geranyl-geranyl transferase and a small GTPase Rab38 (90, 92, 97) (Figure 1.1). The *gunmetal* mouse, caused by a mutation in the protein RABGGTA that catalyzes the transfer of a geranyl-geranyl moiety to the c-terminal cysteines of Rab proteins, which is necessary for the stable membrane association and function of GTP-bound, active Rabs (141). *Gunmetal* mice display hypopigmentation, bleeding disorders, and dysfunctional cytotoxic T-cells, all of which are phenotypes of defects in LRO biogenesis (142, 143). It is likely that RABGGTA is the geranyl transferase used by Rabs involved in LRO biogenesis in these cell types.

The *chocolate* mouse and *fawn-hooded* rat are two animal models caused by defective Rab38 and are characterized by hypopigmentation and bleeding disorders (144, 145) (Figure 1.1). The Rab GTPase Rab38 is a member of the 60+ Rab GTPase family in mammals and its closest homologue in humans is Rab32, with approximately 70% sequence identity (143). Rab32 and Rab38 are suspected to function in the post-Golgi trafficking to maturing LROs in either a redundant or parallel manner (146, 147). These Rabs have been found to colocalize with the cargo proteins required for melanosome biogenesis and with melanin pigment in melanocytes (146, 147). Cells deficient in these Rabs display partial hypopigmentation and also defects in the

biogenesis of other LROS (144, 145, 146, 148). It is clear that Rab38, and likely Rab32, are functional components in the biogenesis of multiple LROs.

1.4.4 Interactions among BLOCs and AP-3

The subcellular localization and knowledge of function for proteins underlying HPS subtypes suggests that these proteins function in early endosomal trafficking. Consistent with this cellular localization, many of these components have been demonstrated to physically interact. BLOC-1 has been demonstrated to physically interact with both BLOC-2 and AP-3 in co-immunoprecipitation experiments (99, 149-153). However, the BLOC-1-BLOC-2 and BLOC-1-AP-3 interacting complexes appear to be distinct as BLOC-2 and AP-3 do not interact and are not all mutually co-precipitated (99). These interactions are likely to occur on early endosomal membranes based on the localization of each of these proteins and the observation that BLOC-1- BLOC-2 and BLOC-1-AP-3 interactions occur in membrane fractions (99, 152) that also contain clathrin (154). However, BLOC-1 and AP-3 have been demonstrated to have somewhat distinct localization on early endosomal domains and function in separate trafficking pathways (87, 99, 153, 155).

1.4.5 Interactions among BLOC-3 and Rabs

It was recently discovered that BLOC-3 functions as a GTP/GDP-exchange factor (GEF) for Rab GTPases (52). BLOC-3 localizes to both endosomes and melanosomes; and, furthermore, BLOC-3 is capable of recruiting Rab32 and Rab38, discussed in detail later, to membranes and acts as a GEF for these Rabs (52). This GEF function was discovered through analysis of regions of small homology between subunits of BLOC-3 and subunits of the yeast

Rab7 GEF Mon1a-Czz1 (52). BLOC-3 has been demonstrated to physically interact with the small GTPase Rab9, which is a known component of endosomal protein trafficking pathways (114). The interaction between BLOC-3 and Rab9 is not using the GEF function of BLOC-3, instead BLOC-3 acts as an effector of Rab9 and this interaction may serve to recruit BLOC-3 to late endosomal membranes where Rab9 functions (114). The discovery of BLOC-3 as a GEF explains a large amount of observations regarding its function in vesicular trafficking, but still leaves many unanswered questions about its interactions with other proteins and role in various trafficking pathways.

1.4.6 HPS complexes and overall roles in melanosome biogenesis

The HPS complexes BLOC-1, -2, -3 and AP-3 appear to serve ubiquitous roles in the trafficking and biogenesis of lysosomes, and also serve functional roles in the biogenesis of several LROs (90-94). The function of BLOC-1 is not clear, but it has an important role in melanosome biogenesis based on the reduction in melanin pigment produced and trafficking of melanosome membrane proteins in BLOC-1 mutant mice (93, 94). A mechanistic role for BLOC-1 is not clear, but it is likely that BLOC-1, BLOC-2, and AP-3 represent different functional steps in vesicular trafficking from early endosomes where BLOC-1 functions upstream of separate BLOC-2- and AP-3-dependent pathways.

The role of AP-3 is best understood as it has been demonstrated to have a vital role in targeting of lysosomal and LRO membrane proteins (93, 94, 99, 156, 157). AP-3 mutant mice exhibit moderate hypopigmentation, as compared to the severely hypo-pigmented BLOC-1 mice (99, 127). AP-3 facilitates vesicle formation from specific early endosomal domains for trafficking of transmembrane proteins to melanosomes and depletion or mutation of AP-3 results

in trafficking defects of melanosome membrane proteins and overall melanosome biogenesis (93, 94, 99).

Phenotypically, BLOC-2 mutants strongly resemble AP-3 mutant mice and BLOC-2/AP-3 double mutant mice are more hypo-pigmented than either mutant alone, or these double-mutant mice resemble BLOC-1 mutant mice (93, 94, 99). While the function of BLOC-2 is not clear, BLOC-2 mutant mice display several defects including melanosome clumping into multi-melanosomal structures and accumulation of melanosome-membrane containing vesicles in the cytoplasm (158-161). A role for BLOC-2 has been established that mirrors that of AP-3 in both its mutant phenotype and interaction with BLOC-1, but BLOC-2 has not been previously established as an adaptor protein (99). Based on phenotypic data and biochemical evidence, discussed in subsequent sections, it appears that BLOC-2 and AP-3 define separate, but partially redundant pathways for biogenesis of melanosomes (99).

BLOC-3 mutant mice display only modest hypo-pigmentation and have severe lung fibrosis (93, 94). The function of BLOC-3 as a GEF and effector of Rabs involved in melanosome biogenesis is supported by the similar hypopigmentation observed in the Rab38-mutant mouse, and it is likely that additional mechanisms are at work in type-II lung alveolar cells that account for the more severe phenotype in those cells (52, 94). BLOC-3 likely functions downstream of BLOC-1, BLOC-2, and AP-3, which appear to function primarily at early endosomes, based both on the localization of BLOC-3 to melanosomes and functional role of BLOC-3 with Rabs that are important in the vesicle-based aspects of transport to melanosomes (52, 147). Consistently, the LRO defect phenotype is more severe in double mutations of BLOC-3 with BLOC-2 or AP-3 (99, 117, 162). BLOC-3 also functions in the movement of

lysosomes in cell types lacking LROs, and it is possible that BLOC-3 also functions, either directly or indirectly through the actions of Rabs and effectors, in the movement of LROs (110).

1.5 Melanocytes and melanosomes - a model of LRO biogenesis

1.5.1 Melanocytes and albinism

In humans, the melanin pigment serves to protect the nucleus of cells from the UV-induced DNA-damage that is known to cause mutations, and is the leading cause of skin cancers (163). Melanin pigments are produced within a specialized Lysosome-related organelle called the melanosome (90-94). In the skin, melanosomes are produced in specialized skin epithelial cells called melanocytes (164). Within the eye, melanosomes are produced within pigmented retinal epithelial cells (93, 94). The biogenesis and function of eye and skin melanosomes is quite similar, but the two systems differ in the storage of melanosomes. Melanosomes produced within retinal epithelial cells reside within the same cells in which there were produced and form a cytosolic, pigmented-cap to protect the nucleus of the cell. Skin melanosomes, on the other hand, are produced in the dermal layer of the skin within melanocytes, but are subsequently transferred to neighboring keratinocytes residing in epidermal skin layers (165). A single skin melanocyte produces and transfers melanosomes to up to 40 neighboring keratinocytes (165). As in the eye, keratinocytes create a cytosolic, pigmented-cap on the nucleus to provide UV protection (165).

The melanin pigments, and the melanosomes themselves, exist for the purpose of UV-protection, but are also responsible for the hair, skin, and eye pigmentation present in all mammals. Melanin functions by efficiently converting the energy of the light into heat through ultrafast internal conversion, rapid conversion of molecules from low-to-high energy states that

subsequently releases energy as heat through molecular vibrations (166). Regulation of melanin synthesis, melanosome biogenesis, or melanosome transfer to keratinocytes results in different degrees of pigmentations locally, which in mammals allows for pigment variations responsible for the wide variety of coat colors observed. Any dysfunction in regulation or the pathways themselves results in coat-color mutants, in most mammals, and albinism, in humans (165). Albinism, characterized as a partial or total loss of pigment, can be caused by various mutations caused by environmental or genetic disorders. Defects in melanin production in the eye cause a variety of problems with vision, and defects in melanin production in skin increase the risk of skin cancer due to UV-exposure (165). The subtypes of albinism are defined based on the proteins affected, which results in various severity and localization of hypopigmentation.

Oculocutaneous albinism affects pigmentation in the hair, skin, and eyes and can be caused by mutations in four genes representing four subtypes: OCA1-4 (167). OCA1 is caused by mutation in the TYR gene that produces the enzyme tyrosinase (167). OCA1 occurs at a frequency of approximately 1:40,000 and is the most severe form of albinism as patients are unable to produce any pigment (167). OCA2 is more prevalent than OCA1, roughly 1:15,000, but less severe as patients with OCA2 are able to produce some pigment (167). OCA2 is caused by the mutation of a protein OCA2 with no known function in melanin synthesis (167, 168, 169). OCA3, caused by mutation in tyrosinase-related protein 1 (Tyrp-1, TYRP1) causes partial albinism less severe than OCA1 and is present primarily in patients of African heritage (167). OCA4, caused by mutation in the protein SLC45A2 – a multi-pass, trans-membrane that is a transporter of unknown cargoes functions in melanosome pH maintenance, is the least common form of albinism and present most often in patients of Japanese ancestry (167, 170, 171).

1.5.2 Melanin synthesis

Two melanin pigments are present in humans, eumelanin, a brown-black polymer composed of dihydroxyindole carboxylic acids and reduced forms, and pheomelanin, a red-brown polymer composed of benzothiazine (165). Both eumelanin and pheomelanin are produced within melanosomes by the conversion of tyrosine to melanin polymers through a series of oxidation and isomerization reactions that occur spontaneously, or are catalyzed by tyrosinase, tyrosinase-related protein-1 (Tyrp-1, 5,6-dihydroxyindole-2-carboxylic acid oxidase), and tyrosinase-related protein-2 (Tyrp-2/TYRP2, also called dopachrome tautomerase) (Figure 1.2).

The synthesis of melanins begins by the oxidation of L-tyrosine to L-DOPA (L-3,4-dihydroxyphenylalanine) and subsequent oxidation of L-DOPA to form DOPAquinone (ortho-quinone of 3,4-dihydroxyphenylalanine), a highly reactive ortho-quinone, by the activity of tyrosinase, a copper-dependent enzyme (172, 173) (Figure 1.2). DOPAquinone can spontaneously react with free cysteines to form cysteinylDOPA isomers, 5-S-cysteinyldopa or 2-S-cysteinyldopa, which are oxidized in a redox reaction with DOPAquinone, and combine to form benzothiazine polymers, which compose the yellow-red pheomelanin pigment (173). As the formation of pheomelanin requires no enzymatic activity other than that of tyrosinase, pheomelanin synthesis is the default synthetic pathway within melanosomes but is highly dependent on the concentration of free cysteines within melanosomes, which is regulated via the activity of specific transporters located within melanosomes (165) (Figure 1.2).

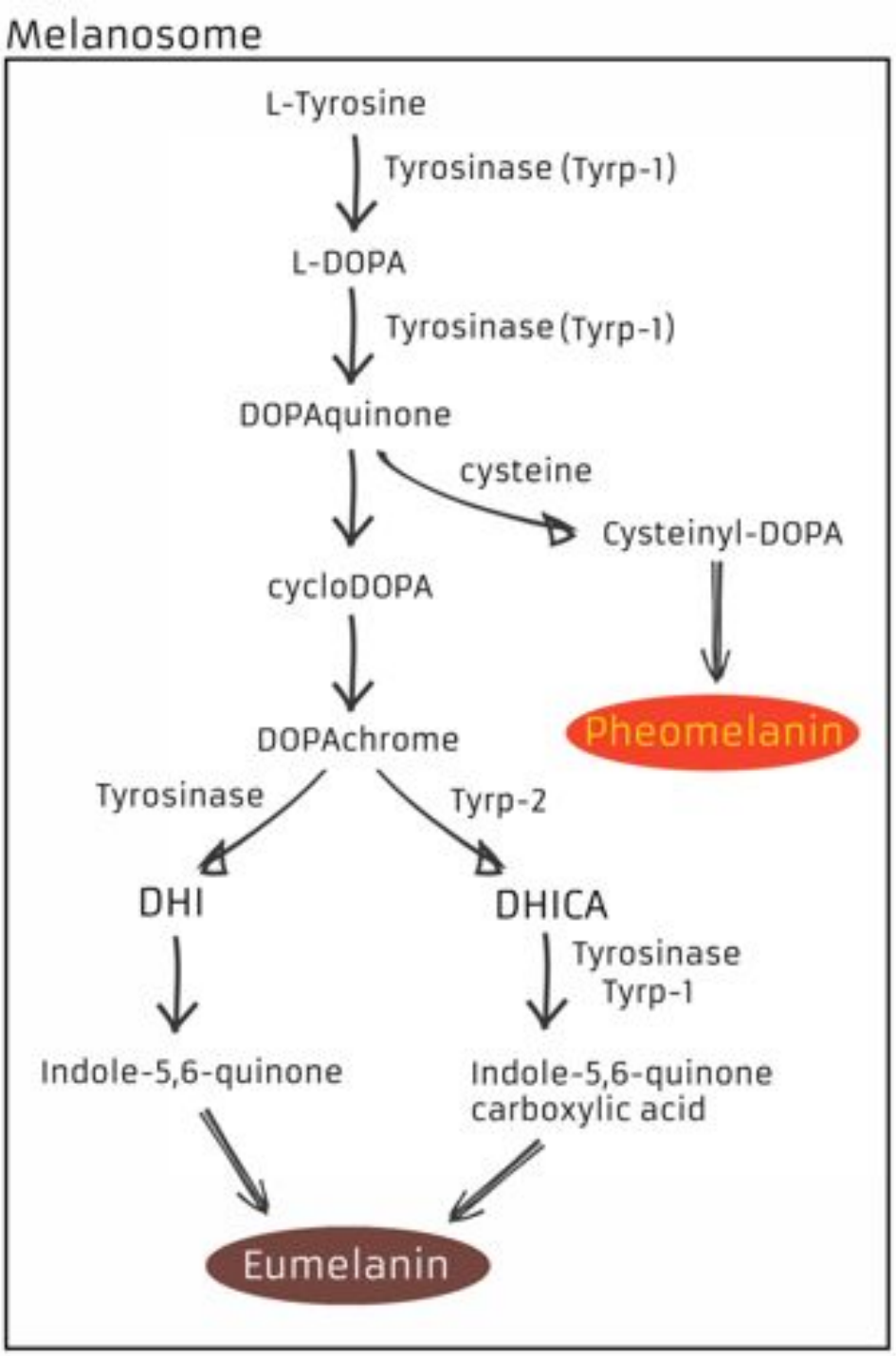

Figure 1. 2
Synthesis of Melanin pigments in melanosomes. Eumelanin and Pheomelanin are produced by the enzymatic activity or tyrosinase, Tyrp-1, and Tyrp-2 using several synthetic routes. Within melanosomes, the synthesis of eumelanin and pheomelanin is dependent on the presence of substrates, such as L-tyrosine and cysteine, catalytic enzymes, and proper intraluminal conditions. Adapted from Simon and Peles, 2010 (192).

In the absence of cysteine, eumelanin are formed following the same initial pathways as pheomelanin synthesis: conversion of L-tyrosine into L-DOPA and finally dopaquinone oxidation by tyrosinase. Dopaquinone spontaneously converts to cycloDOPA by intramolecular cyclization and is rapidly oxidized in a redox reaction with dopaquinone to form dopachrome. The formation of eumelanin from this point can be catalyzed by tyrosinase alone through spontaneous rearrangement to 5,6-dihydroxyindole (DHI), which will be further oxidized and polymerize to form eumelanin pigments (173).

A different eumelanin pigment can be formed using a separate pathway with tyrosinase, Tyrp-1, and Tyrp-2. Dopachrome can also rearrange to form to 5,6-dihydroxyindole-2-carboxylic acid (DHICA), instead of DHI, by the activity dopachrome tautomerase (Tyrp-2). As with DHI, DHICA will be further oxidized and will polymerize to form a different eumelanin pigment. While tyrosinase alone is sufficient for the production of eumelanin and pheomelanin, the activities of Tyrp-1 and Tyrp-2 allow for the production of different eumelanin pigments in melanosomes that underlie the range of shades of melanin (174-179). Additionally, the activity and stability of tyrosinase increases in the presence of Tyrp-1 and Tyrp-2; and tyrosinase and Tyrp-1 have been shown to physically interact in *in vitro* and *in vivo* experiments (180).

Multiple melanin pigments can be created via the different actions of enzymes within melanosomes. It has generally been understood that melanocytes produce one type of melanin at a time and alternation between the production of pheomelanin and various eumelanin pigments gives rise to pigmentations patterns present in human hair and mammalian coats (181, 182). However, production of pheomelanin and both types of eumelanin occur simultaneously within melanosomes and it has been proposed that initial melanin production is prevalently the rapid

formation of pheomelanin pigments, which are subsequently coated by slower-forming eumelanin pigments giving a melanosome a pheomelanin core and eumelanin coat (173).

The production of eumelanin or pheomelanin is controlled both by the presence of melanin-specific enzymes (Tyrp-1/Tyrp-2 for eumelanin, cysteine transporter for pheomelanin) within melanosomes; and, at a higher level, by signal transduction pathways of the melanocyte-stimulating hormone (α-MSH) (183). α-MSH binding to the melanocortin-1 receptor leads to the induction of eumelanin synthesis (184). Conversely, stimulation of the melanocortin-1 receptor induces pheomelanin synthesis via the down regulation of proteins necessary for eumelanin synthesis, such as Tyrp-1, Tyrp-2, PMEL (Pmel17), and OCA2 (185-191). Pheomelanin synthesis occurs readily in the presence of cysteine, but red is the least common hair color in humans and pheomelanin accounts for only 25% of melanin within nature (192) . Mutations in α-MSH signaling or melanocortin-1 receptor underlie the production of red hair in humans and other mammals as these mutations inactivate the signaling pathway required for the activation of eumelanin synthesis (193, 194). Red-hair and pale skin has long been correlated with an increased prevalence of skin cancers, and it was believed that this was simply a result of decreased melanin, and a subsequent decrease in UV-protection. However, pheomelanin itself may be a causative factor in skin cancers and ocular diseases through photo activation of oxygen by UV-A light – the lower photo ionization potential of pheomelanin, relative to eumelanin, can result in the generation of mutagenic oxygen radicals -and a much higher incidence of cancer in red haired patients even without direct UV-exposure (192).

1.5.3 Melanosome biogenesis and maturation

Melanin synthesis within melanosomes is dependent on maintenance of the proper pH, enzyme composition, free-amino acids precursor molecules, and structural protein content. Melanosomes are characterized by four, morphologically distinct stages as characterized by electron micrographs (91, 95)(Figure 1.3). Stage I, non-pigmented melanosomes originate from an early/late endosomal organelle and are characterized by enlarged intraluminal vesicles and a rigid, flat clathrin coat on a portion of the limiting membrane of the melanosome (Figure 1.3). These melanosomes are formed by the delivery of the transmembrane, structural protein PMEL, which is cleaved by acid hydrolases into smaller forms that oligomerize into amyloid-like fibrils within the melanosome. Stage II melanosomes are also non-pigmented and characterized by numerous, large PMEL fibrils that extend along the length of the melanosome giving a distinctive striped appearance (Figure 1.3).

The delivery of melanin-synthesizing enzymes tyrosinase, Tyrp-1, and Tyrp-2, among others cargoes, to stage II melanosomes allows for the initial pigment production (Figure 1.3). Melanin is synthesized and deposited along the extended PMEL fibrils (Figure 1.3). Stage III, partially pigmented melanosomes are characterized by the deposition of melanin along the fibrils. Further melanin synthesis within stage III melanosomes eventually fills the entire intraluminal space thus creating the stage IV, fully pigmented melanosome (Figure 1.3). The biogenesis and maturation of the melanosome is dependent on the proper delivery of specific cargoes to different stage melanosomes, and is an intricate case of intracellular trafficking of transmembrane cargoes.

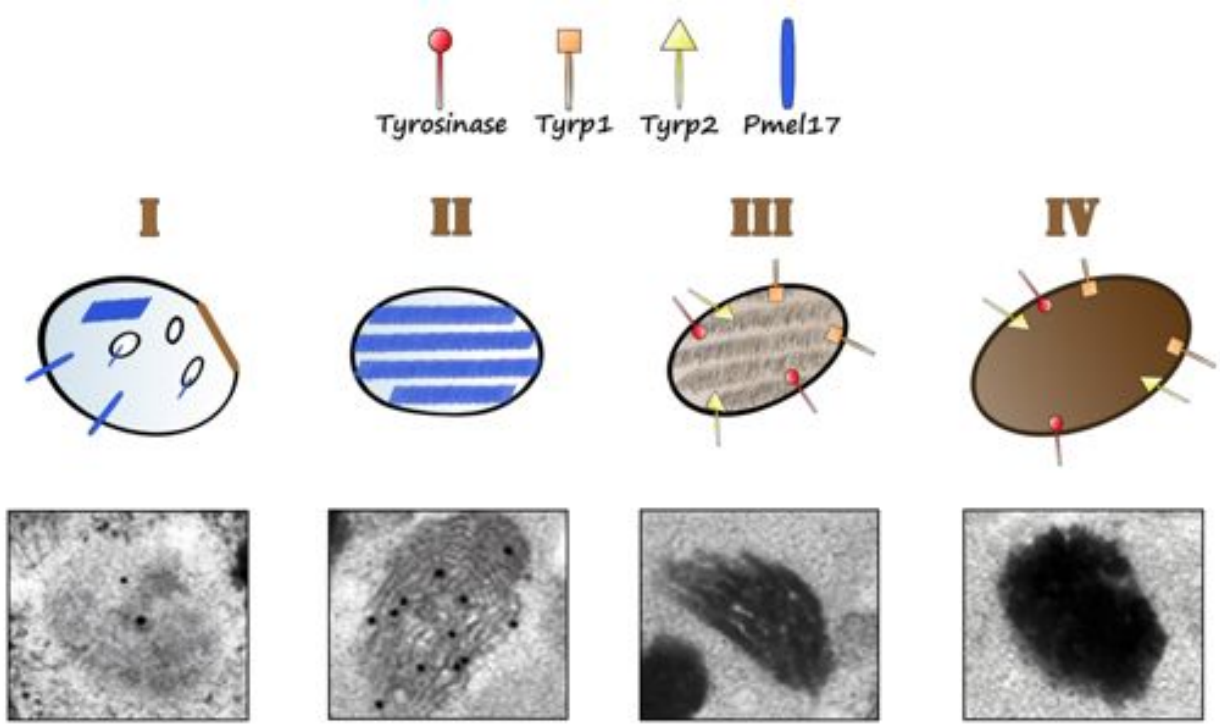

Figure 1. 3
Melanosome maturation progresses through four morphologically distinct phases. Stage I melanosomes contain the protein Pmel17 on the limiting membrane, in intraluminal vesicles, and in small amyloid-like fibrils and a small clathrin lattice. Stage II melanosomes are distinguished by the presence of non-pigmented, Pmel17 fibrils that span the length of the organelle. Delivery of tyrosinase family proteins (tyrosinase, Tyrp-1, Tyrp-2) allows for the synthesis of melanin in stage III melanosomes, which is deposited upon Pmel17 fibrils and gives the appearance of pigmented striations. Stage IV melanosomes are fully pigmented. Electron micrographs demonstrate the morphology of melanosomes in different stages of development. Immuno-gold labeling using a 12nm-gold particle clearly shows the presence of Pmel17 in stage I and stage II melanosomes, but is masked by melanin pigments in stage III and stage IV melanosomes. See chapter 3 for further electron micrographs.

1.6 Transmembrane-protein transport to melanosomes

1.6.1 Biogenesis and trafficking of pre-melanosomes (stage I & II)

Stage I and stage II melanosomes are often referred to as pre-melanosomes as they do not contain the melanin pigment. The formation of non-pigmented, pre-melanosomes has not been definitively characterized, but the pre-melanosome is believed to originate from the vacuolar domain of an early endosomal organelle (91). Studies performed by our lab and other groups that have examined lysosomes using lysosomal-specific markers or using fluorescent-labeled dextran, a complex polysaccharide that is endocytosed and trafficked to lysosomes where it remains un-digested by lysosomal hydrolases, have demonstrated that melanosomes do not originate from lysosomes (195).

A critical component of melanosomes, and a characteristic marker of non-pigmented, pre-melanosomes is the premelanosome protein (PMEL). PMEL is a type I transmembrane glycoprotein with a single transmembrane domain and a lumenal domain that is heavily glycosylated by N- and O-linked oligosaccharides within the golgi (196). Trafficking of PMEL from the golgi to vacuolar early endosomes most likely occurs after rapid transit through the cell surface, followed by endocytosis and trafficking to specific early endosomal domains (196). PMEL is subsequently incorporated into intraluminal vesicles (91, 197, 198) using a mechanism independent of the Endosomal Sorting Complex Required for Transport (ESCRT), the machinery that mediates formation of intraluminal vesicles in Multi Vesicular Bodies (MVBs)/late endosomes and defines the ubiquitous degradative/lysosome pathway (91, 197). In stage I melanosomes, PMEL is cleaved into multiple, small fragments by the activity of furin-family proprotein convertase, a “sheddase” of the disintegrin and metalloproteinase family, and additional melanosome proteases to form the small PMEL fragments that oligomerize to form

striated amyloid fibrils (198). The remaining, non-amyloid PMEL is further cleaved by γ-secretases within stage I and stage II melanosomes (198). The modification and trafficking of PMEL is dependent on interaction with another melanosomal protein, MART-1, that forms a complex with PMEL (199). While MART-1 is also necessary for the proper trafficking of OCA1, an L-DOPA transporter, to melanosomes the function of MART-1 itself is unknown (199).

PMEL is observed both on the limiting membrane of stage I melanosomes, in intraluminal vesicles, and in small amyloid fibrils within pre-melanosomes (91, 197). Stage I melanosomes differ from MVBs by the presence PMEL proteins and by large, flat, clathrin-containing coats on their limiting membrane (95, 165). Initial amyloid fibril formation occurs in stage I melanosomes, but as fibrils increase in size and span the length of the organelle the melanosome is now characterized as a stage II melanosome (91, 95 196, 200).

1.6.2 Trafficking of melanin synthesizing enzymes

Delivery of the type I, single pass transmembrane enzymes tyrosinase and tyrosinase-related proteins-1 and -2 (Tyrp-1 and Tyrp-2) is a requirement of the maturation from stage II to stage III melanosomes (91, 165)(Figure 1.4). Transport of newly synthesized tyrosinase and Tyrp-1 from the ER to the maturing melanosome requires sorting step at the golgi, where enzymes are heavily glycosylated with N-linked oligosaccharides, and to specialized tubular domains of early/recycling endosomes, rather than direct transport from the *trans*-Golgi to the melanosome (91, 127, 153, 201, 202). Packaging of the tyrosinase family proteins into transport vesicles at early/recycling endosome associated tubules is dependent on ubiquitous Adaptor Protein complex (AP)-1 and AP-3 Biogenesis of Lysosome-related Organelles Complex

(BLOC)-1 and BLOC-2 (153, 201); and dependent on the specific sorting signals present in these cargoes.

Tyrosinase contains two sorting signals: a conventional di-leucine sorting motif (EExxxLL) and a tyrosine motif (YxxL) in the short cytoplasmic tail that are bound by AP-1 and AP-3 (87). Similarly, Tyrp-1 also contains two sorting motifs: an unconventional di-leucine motif (DExxxxLL) and tyrosinase motif (YxxL) that are both utilized in the trafficking of the protein. The sorting signals in tyrosinase and Tyrp-1 are recognized by AP-1 and AP-3, which facilitate the packaging of tyrosinase and Tyrp-1 into vesicles at specific early endosome tubular domains (87, 99 , 153). Preliminary data from our lab suggests that BLOC-2 may also be able to bind specific sorting signals in the cytosolic tails of tyrosinase and Tyrp-1 (unpublished data from our lab). BLOC-2 has been shown to bind clathrin through its clathrin-box and also colocalizes with clathrin in cells (107). While BLOC-2 has not been accepted to function as a clathrin-adaptor protein, BLOC-2 has some functional roles of AP-3 and AP-1 in the trafficking of Tyrp-1 (99) and it is possible that BLOC-2 serves an adaptor-like function even if it is not a typical clathrin-adaptor protein.

Several studies have demonstrated that AP-1 and AP-3 define separate, but partially redundant trafficking pathways for tyrosinase and Tyrp-1 (87, 99, 153)(Figure 1.4). Further, BLOC-2 and AP-3 also define separate functional trafficking pathways for tyrosinase and Tyrp-1 (87, 99, 153)(Figure 1.4). Deficiency of both AP-3 and BLOC-2 complexes causes more severe defects in tyrosinase and Tyrp-1 transport and overall pigmentation than either single deficiency (99, 200).

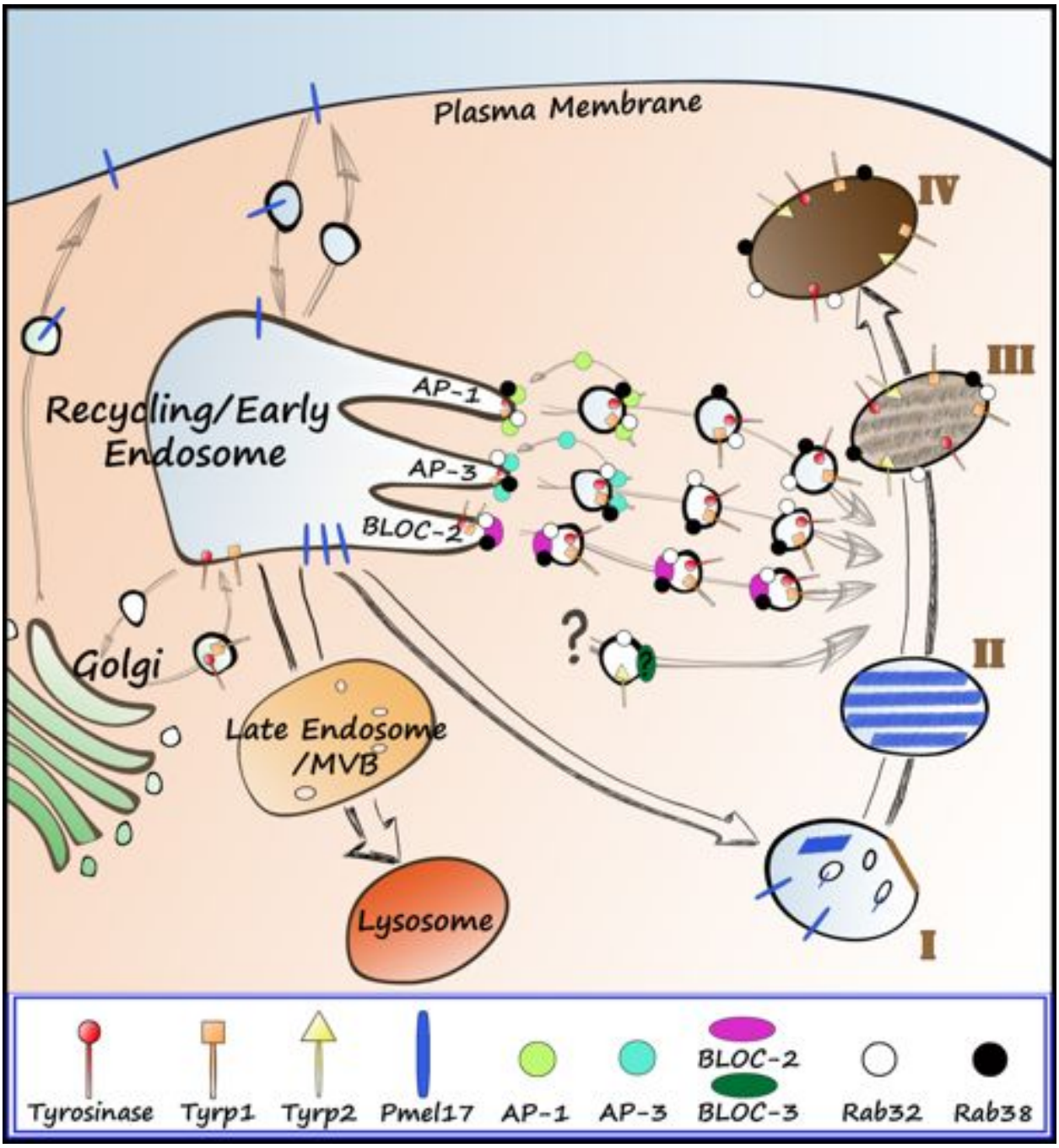

Figure 1. 4
Model for the trafficking of tyrosinase family proteins to maturing melanosomes. The trafficking of tyrosinase and Tyrp-1 to melanosomes uses multiple pathways at specific early endosomal tubules. The presence of AP-3-dependent and AP-3-independent pathways is clear, but it is not clear if AP-1 and BLOC-2 function in the same or in different pathways. The trafficking of Tyrp-2 is not clear, but may use a different route for trafficking to melanosomes. See Appendix 1 for further details.

Analogously, knockdown of AP-1 or AP-3 alter the transport of tyrosinase, and possibly other cargoes, to maturing melanosomes (153). It is unclear if AP-1 and BLOC-2 function in the same pathways, but it is clear that both of these proteins function in a distinct pathway from AP-3. These protein complexes – AP-1, AP-3, and BLOC-2 – define two or three separate trafficking routes for the trafficking of tyrosinase and Tyrp-1 from early endosomes to melanosomes (Figure 1.4). The redundancy of these pathways allows for trafficking of cargoes to the melanosome even upon loss of one of the pathways, although the efficiency of trafficking is diminished upon loss of function of one pathway. These pathways are also used for trafficking of cargoes to lysosomes, and it is striking that separate redundant pathways are redirected for melanosome trafficking (203-206).

Interestingly, BLOC-1 appears to be necessary for the trafficking of tyrosinase and Tyrp-1 whether using an AP-3, AP-1, or BLOC-2 dependent pathway (99, 153). BLOC-1 displays early endosomal localization and interacts directly with AP-3 and BLOC-2 (87, 99, 153). BLOC-1 either functions "upstream" of these pathways, or may function directly in the AP-3 and BLOC-2 dependent pathways for Tyrp-1 trafficking (87, 99, 153).

The necessity of AP-1, AP-3, BLOC-1, and BLOC-2 in the early endosomal step of trafficking of tyrosinase and Tyrp-1 is well accepted, the mechanism by which post-endosomal trafficking occurs is an area of debate (87, 99, 153). Two mechanisms have been suggested for post-endosomal trafficking of tyrosinase and Tyrp-1: vesicle trafficking and direct tubulation between early endosomes and melanosomes. The small GTPases Rab32 and Rab38 serve a functional role in the post-golgi trafficking of tyrosinase and Tyrp-1, and it appears that these Rabs are present on small vesicular structures and melanosomes, which would be consistent with a vesicular trafficking step between early endosomes and melanosomes (146). However, another

study using electron microscopy has suggested that the trafficking of these enzymes from the early endosome occurs via the extension of a tubule that fuses with the melanosome (207). This study suggests that the endosomal tubules, on which AP-3, AP-1, and BLOC-2 are present, are extended from early endosomes and pulled by interaction with Kinesis 13A, a +-end directed microtubule motor. While this model is interesting, it is not an established trafficking mechanism and has not been widely adopted as a likely model in the field. The result of either mechanism is the transport of tyrosinase and Tyrp-1 to melanosomes, where these enzymes are expected to remain to function in melanin synthesis.

The trafficking pathways and molecular mechanisms used for the trafficking of Tyrp-2 remain unclear. Tyrosinase and Tyrp-1 both have di-leucine motifs, which have been shown to be necessary for proper melanosomal trafficking, and tyrosine sorting motifs, which may be used for trafficking to melanosomes (153). Tyrp-2 contains two tyrosine sorting motifs (YRRL and YTPL) that are presumably used for trafficking, but it is not well understood which proteins function in Tyrp-2 trafficking and it remains understudied (208, 209). Several studies have demonstrated that Tyrp-1 and Tyrp-2 utilize distinct routes in trafficking to melanosomes, but that Tyrp-2 is also trafficked through early endosomal compartments (208). Tyrp-2, like Tyrp-1 and tyrosinase, is heavily N-glycosylated, and this glycosylation is necessary for proper targeting to melanosomes: Tyrp-2 lacking glycosylation is degraded by the proteasome (209). In melanoma cells, Tyrp-2 has been localized to the golgi, melanosome, plasma membrane, and other unknown cellular structures, which has led to the model that Tyrp-2 trafficking is more similar to PMEL trafficking: secretion from the golgi to plasma membrane, endocytosis, early endosome sorting, and subsequent trafficking to melanosomes (209).

The trafficking of other melanosome-associated proteins like OCA1, OCA2, Lysosome-associated membrane proteins (LAMPs), and various small molecule transporters like ATP7A and SLC45A2 is less well understood (155, 210-212). OCA2, a melanosomal protein of unknown function, is trafficked to melanosome utilizing a BLOC-1/AP-3 dependent pathway that is similar, or perhaps identical, with tyrosinase and Tyrp-1 trafficking (168, 213). LAMPs are integral membrane proteins that are highly enriched on the lysosome membrane and serve unknown biological functions in lysosome function and movement (214-216). The trafficking of LAMPs is dependent on AP-3, AP-1, and BLOC-2 (99, 217-219). LAMPs contain protein sorting signals in the cytosolic tails of the proteins that are bound by AP-1 and AP-3 in the trafficking of these cargoes to lysosomes (217). The trafficking of LAMPs to melanosomes uses the same trafficking machinery as lysosome traffic: AP-1, AP-3, and BLOC-2, using the same sorting signals as required for lysosomal targeting (99, 217, 220). BLOC-3 is also associated in trafficking of LAMPs and interacts with LAMPs to facilitate lysosomal movement (110, 118). The use of the same ubiquitous early endosomal proteins AP-1, AP-3, and BLOCs by numerous cargoes for the trafficking to both lysosomes and melanosomes demonstrates that melanosome biogenesis relies upon common early endosomal trafficking pathway. However, it is not clear how trafficking to the lysosome and melanosome are differentiated. While LAMPs are simultaneously present in lysosomes and melanosomes, the melanin-synthesizing enzymes (tyrosinase, Tyrp-1, etc) are not normally found within lysosomes, emphasizing the presence of some mechanisms to regulate the specific trafficking the melanosome (221). Further, the redundant nature of AP-1, AP-3, and BLOC-2 in trafficking of both melanosomal and lysosomal cargoes indicates that the melanosome specific trafficking factors must function in multiple trafficking pathways. My work aims at addressing the melanosome specific factors that allow

for the differentiation between the ubiquitous and melanosome trafficking and determining how these factors function.

1.6.3 Maturation of pigmented melanosomes (Stage III & IV)

The distinction between Stage II and Stage III melanosomes is based on a small accumulation of melanin pigments within Stage III melanosomes (87, 91, 196). The delivery of tyrosinase, Tyrp-1, Tyrp-2, and other proteins directly required for melanin synthesis is likely to both stage II and stage III melanosomes. The mechanisms that differentiate different stages of melanosomes and the regulation of melanin synthesis within melanosomes are not clear. Further melanin synthesis produces mature, fully pigmented stage IV melanosomes.

1.6.4 Movement and secretion of mature melanosomes

The movement of maturing melanosomes is dependent on the activity of microtubule based kinesin and dynein motors that act in a tug-of-war model moving the melanosomes toward the perinuclear microtubule organizing center, dynein based movement, or toward the cellular periphery, kinesin based movement (222). As is the case for most organelles and vesicles, melanosomes are coated with microtubule-based kinesin and dynein motors and with actin-based myosin motors that cause the variable speed and directionality of melanosome movement (222). This has led to the use of melanosomes as a system to study organelle motility and cooperativity of multiple classes of motors (98,212, 223).

Stage I and II, non-pigmented melanosomes are primarily localized to perinuclear regions of the cells, while pigmented melanosomes are observed in peripheral regions. The maintenance of non-pigmented melanosomes in the perinuclear region is dependent on dynein and

spectrin/ankyrin activities (224) while the peripheral distribution of pigmented melanosomes is dependent on the activity of kinesins and myosins (225). The speed and directionality of melanosome movement is based both on the presence of motors and upon the differential regulation of those motors through indirect cAMP-signaling and MAP/ERK kinases (115, 226, 227, 228). The retention of stage IV, pigmented melanosomes in the cell periphery is dependent on the transition from microtubules into the polarized actin along the cortical edge of cells and the activity of a class V Myosin, Myosin Va in mammals, on melanosomes (229).

The fate of mature, stage IV melanosomes in skin melanocytes, which transfer melanosomes to keratinocytes, is different from retinal pigmented epithelial cells in the eye, which store melanosomes in cluster that forms a UV-protective perinuclear cap (99, 230). Several mechanisms have been proposed for the transfer of melanosomes from melanocytes to keratinocytes, including: direct melanocyte-keratinocyte membrane fusion; vesicular transfer of individual or multiple melanosomes; secretion of membrane- or non-membrane bound melanosomes followed with phagocytosis by keratinocytes; or phagocytosis by keratinocytes of melanosome-containing filapodia from melanocytes (231-237). However, no consensus has emerged on which mechanisms of melanosome secretion occur, and it is possible that different mechanisms are at play that allow melanosome secretion from perinuclear areas versus the secretion of melanosomes from dendrites (238). Regardless of mechanisms, once in keratinocytes melanosomes are transported to the perinuclear region of the cell, where numerous melanosomes form a distinctive pigmented cap over the nucleus to minimize UV-induced damage.

1.7 Role of Rab GTPases in melanosome biogenesis

The trafficking of structural proteins, transporters, and oxidative enzymes to the maturing melanosome occurs using a combination of common lysosomal protein machinery and cell-type specific proteins. This is especially the case with the Rab GTPases, where melanosome biogenesis uses both common endocytic and exotic features for melanosome formation and subsequent trafficking and secretion (239). There are two major divisions in the function of Rab GTPases with melanosomes: vesicular trafficking of cargoes needed for melanosome biogenesis and mature organelle motility; and each is represented through the recruitment of a combination of ubiquitous and cell-specific Rabs in each pathway.

1.7.1 Function of Endo-lysosomal Rab7 in melanosome biogenesis

Much of the trafficking of cargoes to melanosomes occurs through the early endosomal subdomains, and the vacuolar domains of early endosomes are also important for the formation of stage I melanosomes (91, 196, 197). The early endosomal Rab5s does not appear to function directly in the biogenesis of melanosomes or affect trafficking of cargoes (47, 240). Stage I, early endosome derived melanosomes mature into an organelle that resembles the MVB, and requires the function of MVB components, including the ESCRT complexes and Rab7 (241, 196, 242). The trafficking of cargoes out of early endosomes is dependent on the presence of active Rab7 co-localized with tyrosinase, Tyrp-1, and PMEL at early endosomes (242-244). Rab7 also functions indirectly in the movement of non-pigmented melanosomes through a Rab7-effector protein that recruits dynein-dynactin motors to melanosomes (245, 246). The functions of Rab7 in both early vesicular trafficking and melanosome movement occur simultaneously with the ubiquitous functions of Rab7 using similar or identical effector proteins, which necessitates the

use of cell-type specific Rab GTPases to separate the melanosomes from the late endosome and lysosome pathways.

1.7.2 Post-endosomal trafficking role of Rab32 and Rab38

Two small GTPase Rab38 and Rab32 are tissue specific Rabs that function in melanosome biogenesis and may act as the melanosome specific trafficking factors that function with common early endosomal trafficking components. Rab38 was discovered to have a role in melanosome function through examination of coat-color mutants in mice and rats (144, 247, 248). The closely related homologue of Rab38, Rab32 functions in the formation of melanosome in *Xenopus* and in *Drosophila melanogaster* the homologue of Rab38 and Rab32 functions in pigment formation (249, 250). The Rab38 mutant rodent models are only mildly hypo-pigmented, and no Rab32 mutant model has yet been analyzed (144, 247, 248). However, when melanocytes from Rab38 mutant mice are also subjected to knockdown of Rab32 a more severe hypopigmentation is observed (146). Rab38 and Rab32 are also observed to strongly colocalize with each other, and with melanosome cargoes tyrosinase, Tyrp-1, and pigmented melanosomes (146), suggesting they function in a post-early endosomal sorting step of trafficking. Both Rab32 and Rab38 are found on tubular early endosomes, vesicles, and pigmented melanosomes, in addition to small vesicles containing cargoes transported to melanosomes (146). Such tubules may represent early endosomal sorting domains at which AP-1, AP-3, and BLOC-2 have been observed to function (99, 146). Rab32 and Rab38 also display a highly cell-type specific expression to cells containing LROs and are also important in the formation and function of other LROs (148, 251, 252). The defective trafficking of tyrosinase and Tyrp-1 in Rab38 knockout, or Rab32 knockdown cells demonstrates an important function

for these two proteins in melanosome trafficking. Further, these two proteins appear to be partially redundant in the trafficking of cargoes, a similar function as is observed with the redundant trafficking by early endosome components AP-1, AP-3, and BLOC-2 (99, 146). It is likely that Rab32 and Rab38, therefore, function as LRO-specific biogenesis pathways markers, and may function directly with AP-1, AP-3, and BLOC-2.

It is clear that Rab32 and Rab38 are important for melanosome biogenesis, but the functional role for these Rabs is unclear. The recruitment of Rabs to specific membrane domains is a key component in the pathway specificity and regulation of Rabs. It was recently discovered that BLOC-3, a protein complex that functions in the ubiquitous movement of late endosomes and lysosomes, acts to recruit Rab32 and Rab38 to specific membranes and acts as the GDP/GTP exchange factor (GEF) specifically for Rab32 and Rab38 (52, 118). Lacking BLOC-3, Rab32 and Rab38 do not correctly localize to membranes, and defects in the trafficking of tyrosinase, Tyrp-1, and Tyrp-2 are observed (52). BLOC-3 is responsible for the recruitment of Rabs to specific membranes, as was demonstrated when BLOC-3 was mis-targeted to mitochondria and unmodified Rab32 and Rab38 were found to localize to mitochondria as well (52).

Interestingly, this function of BLOC-3 as a GEF for Rab32 and Rab38 is independent of ubiquitous functions of BLOC-3 in late-endosome and lysosome movement, which is likely facilitated by recruitment of BLOC-3 to membrane by endosomal Rab9 (114, 253). Both Rab32 and Rab38 use the same GEF, but do not necessarily have common GTPase-activating proteins (GAPs). An effector of Rab9, RUTBC1 serves as a GAP for Rab32 but not Rab38 (255). In combination, the connection of Rab9 effectors as regulators of Rab32 and Rab38 function supports a model in which Rab9 and Rab32/38 act in separate, but related pathways in late endosome and LRO biogenesis. However, BLOC-3 mutant mice are less severely hypo-

pigmented than Rab38 mutant mice, and as Rab38 and Rab32 are partially redundant, suggests that BLOC-3 is not solely responsible for the recruitment and function of Rab38 and Rab32 in melanosome biogenesis (111, 144). BLOC-3 serves both ubiquitous and LRO specific functions, and facilitates the activation and recruitment of LRO specific Rab32 and Rab38, but may not be the only mechanism used to active and recruit these Rabs (255).

The function of Rabs in trafficking is not through direct action of the Rabs, but instead through function as an anchor to attach effector proteins to specific membranes to serve trafficking specific functions. Rab32 and Rab38 have one known effector protein, VPS9-ankyrin-repeat protein (Varp), which was discovered in a screen using GTP-locked Rab32 and Rab38 as bait (256, 257). Varp, previously characterized as a GEF for Rab21, was found to interact specifically with the switch II region of Rab38 and Rab32, Valine 78 and 94 respectively, that is a common region of binding for Rab effectors because this regions undergoes conformational change when in the GDP- or GTP-bound state (258). Knockdown of Varp or use of mutants of Varp or Rab32 and Rab38 that disrupt binding result in the mistrafficking of Tyrp-1 to melanosomes and demonstrate that Varp is a functional Rab32 and Rab38 effector in melanosome biogenesis (257).

Further, the Rab32 and Rab38 binding of Varp is required for the formation of dendrites in melanocytes, which are suggested to be necessary for secretion of melanosomes to keratinocytes (200). Varp is known to bind to the VAMP7 (Vesicle-associated membrane protein 7) a vesicle-SNARE protein, and the recruitment of VAMP7 to vesicles is thought to be required for fusion of vesicles carrying Tyrp-1 to melanosomes (200). Interestingly, the regulation of VAMP7 is controlled by binding to Varp with Rab32 or Rab38 (259). When bound by Varp, especially when Varp is also binds to Rab32 or Rab38, VAMP7 is maintained in an

inactive state that does not allow interaction with target SNAREs (t-SNAREs) required for membrane fusion (259). The recruitment of VAMP7 to vesicles is necessary for proper trafficking of Tyrp-1 between early endosomes and melanosomes, and, though it has not been tested, may also be required for proper tyrosinase trafficking (257). This suggests a mechanism in which Varp-Rab32/38 interaction is required for recruitment to vesicles, but that the Varp – Rab32/38 interactions must be disrupted to allow for function of VAMP7 in downstream vesicle fusion. It is unclear how this interaction would be maintained, or would impact interaction of Rab32 and Rab38 with other, unknown, effectors that would be required for vesicle movement, targeting, and fusion with melanosomes.

1.7.3 Trafficking of mature melanosomes via Rab8 and Rab27a

The biogenesis of the early melanosome and trafficking of melanin-synthesizing enzymes to the melanosome is also dependent on Rab GTPases. The fate of pigmented melanosomes is also dependent on the actions of Rabs. Griscelli syndrome is a rare autosomal disorder characterized by hypopigmentation and immune defects (260) (Figure 1.1). Griscelli syndrome is caused by mutation of any of three proteins that function in the trafficking of melanosomes: Myosin Va, Rab27a, or melanophilin (167). Unlike HPS, in which hypopigmentation is a result of defects in melanosome formation, Griscelli syndrome causes hypopigmentation due to mis-localization of mature melanosome in melanocytes that inhibits efficient transfer to keratinocytes in skin and hair (167). Rab27a is also a tissue-specific Rab GTPase and serves functions in organelle trafficking and secretion in multiple cell types, and acts in actin-based movement and secretion of melanosomes (261, 262, 263).

Melanosomes serve as a very nice system to investigate the mechanism that maintains a balance between directed microtubule- and actin-based movement through the regulation of kinesin, dynein/dynactin, and Myosin V family of motors. The attachment of motors to melanosomes is dependent on the presence and function of several Rab GTPases resident on melanosomes at different times. The complete cadre of Rabs on melanosomes has not been determined, and additional Rabs to those already known are likely involved in melanosome movement and transport. The anterograde, kinesin-based movement of melanosomes to the cell periphery has been shown to depend on the localization of Rab1A to melanosomes (265). In contrast, the retrograde movement of melanosomes toward the microtubule organizing center in the perinuclear region of the cell is dependent on the formation of a complex between Rab7 effectors and the p150-Glued subunit of the dynactin complex (266). Both Rab7 and Rab1A are likely to be present simultaneously on melanosomes with a tug-of-war balance between kinesins and dynein occurring. Fast, kinesin-based melanosome movement is also correlated with the presence of Rab32 and Rab38 on pigmented melanosomes (225). Analysis of functions of different Rabs with melanosome movement has recently been an area of focus as more reagents have become available to assess the movement of melanosomes in live cells, and serves as a model system to understand organelle dynamics (212, 222, 267, 268).

Rab32, Rab38, and Rab7 all coexist on stage III melanosomes, but gradually decrease as melanosomes mature to fully pigmented stage IV melanosomes, to be replaced by Rab27a on mature melanosomes (246, 268). Rab27a is the best understood Rab to function with melanosomes: Rab27a mediates the cortical localization of melanosomes via interaction with Myosin Va that is necessary for secretion of melanosomes (246). Rab27a and, the related protein, Rab3A are recruited to mature melanosomes by the previously established GEF for Rab3

(269, 270). In skin melanocytes, Rab27a recruits both melanophilin and Myosin Va to membranes, and these proteins comprise a semi-stable tripartite complex that allows for Myosin Va to pull melanosomes from microtubules and into the polarized cortical actin (264, 271). In retinal pigmented epithelial cells, where melanosomes are trafficked to apical processes within the melanocyte and not secreted, Rab27a recruits Myosin VIIa through a similar tripartite complex with effector protein MYRIP (272, 273). The interactions of Rab27a-Melanophilin-Myosin Va and Rab27a-MYRIP-Myosin VIIa appear to function similarly, but nuances around different functions of Myosins may underlie the differences in the fate of mature melanosomes.

Rab27a facilitates the tethering of melanosomes to cortical regions of melanocytes, but does not function directly in melanosome secretion. Instead, Rab27a is inactivated on mature melanosomes by a specific Rab27a GAP and other Rabs function in melanosome secretion (274, 275). Recycling endosomal Rab17 and Rab11a/b appear to function downstream of Rab27a and have critical roles in melanosome secretion through regulation of melanocyte filapodia and perhaps through a direct trafficking mechanism using Rab11 effectors, such as Myosin Vb (275, 276, 277). The recruitment of recycling endosomal Rabs (Rab17 and Rab11) suggest that melanosome secretion, like early melanosome formation, may utilize ubiquitous trafficking machinery once the specialized organelle is formed by tissue specific factors.

Interestingly, another recycling endosomal Rab, Rab8, is associated with the actin-based movement of melanosomes (278). Rab8 does not appear to function in the trafficking of cargoes to melanosomes, as Tyrp-1 traffics normally in Rab8-siRNA treated cells, but instead Rab8 depletion impacts melanosome trafficking to dendrite tips (279). The role of Rab8 in melanosome movement is also consistent with a function for Myosin Vb in melanosome secretion as both Rab11 and Rab8 are known to recruit Myosin Vb to membranes, and the

function of both Rabs in mature melanosome movement strongly suggests a role for Myosin Vb (280). Rab8 and Rab11 may define different aspects of melanosome movement and transfer as the Rabs are known to function independently in other trafficking pathways (280-282). The precise role for Rab8 in melanosome biogenesis and function is unknown, and it is, therefore, difficult to predict if Rab8 and Rab11 serve similar functions with melanosomes. The association of ubiquitous Rabs from late endosome/lysosome and recycling endosomal pathways to melanosomes demonstrates the use of both general and tissue specific machinery in melanosome trafficking and underlies the importance of Rabs as organelle markers and molecular switches that coordinate and regulate the function of molecular motors (230).

1.8 Role of actin-based Class V Myosin motors in Organelle and vesicle trafficking

1.8.1 General structure and function of class V myosins

The long distance movement of vesicles and organelles is generally understood to occur along microtubules, but once on the ends of microtubule tracks must switch to actin tracks, or be trafficked in a minus-end directed fashion along microtubules. Currently, the only known mechanism for actin-based movement of melanosomes is dependent on the function of Myosin Va. Class V myosins are a non-conventional family of actin-based motors with functions in organelle and vesicle movement that is conserved from yeast to humans (283, 284). There are three members of the class V Myosin family in mammals Myosin Va, Vb, and Vc that have non-redundant functions of vesicle and organelle trafficking (285). The double-headed class V myosins are composed of two heavy chains each of which is characterized by a well-folded actin binding ATPase motor domain, a flexible neck region with six IQ motifs for binding of calmodulin or light chains, extended coiled-coil alpha helical domains that mediate dimerization,

and a well-folded globular tail domain that is necessary for binding to cargoes (283, 286, 287)(Figure 1.5).

The ability of class V myosins to efficiently traffic cargoes is a hallmark of their function in vesicle and organelle trafficking with long 36-nm steps and high processivity giving rise to long run lengths and strong pulling forces that allow single Myosin V motors to travel several hundred nanometers on an actin fiber (288-292). The high processivity of Myosin Vs is thought to be a result both from the dimerization of motors and from association of kinesin, bound to the same structure as a class V myosin, with actin fibers (293) (Figure 1.5). Kinesins, which are not normally associated with actin binding, are thought to weakly bind to actin fibers and act as transient tether, which results in an increased likelihood of Myosin V rebinding to actin (293). Kinesins and myosins often coexist on vesicles and organelles, and the presence of both types of motors on the same cargo may represent a conserved, general mechanism used to increase transport efficiency (293).

1.8.2 Class V Myosins and interacting proteins

The tail-domain of class V myosins, composed of long coiled-coil domains and a c-terminal globular tail, are important in the dimerization and attachment of specific cargoes (Figure 1.5). While the motor activity is regulated primarily by association of Ca^{2+}-dependent calmodulin light-chain binding to IQ motifs, the primary regulation of Myosin V function is dependent on the c-terminal globular tail (284, 294) (Figure 1.5). The structure of the coiled-coil and globular tail domains are highly conserved, and are critical for the recruitment of myosins to membranes and for specific cargo binding (Figure 1.5). Indeed, single amino acid mutations are

able to selectively inhibit binding of secretory cargoes and mutations to another region of the globular tail can inhibit binding of cargoes for organelle movement (295, 296).

Interactions between class V myosins and Rabs occur using both the globular tail and specific regions of the coiled-coil domain that are important for cargo trafficking (230, 297, 298). Tissue specific alternative splicing is a well-established mechanism to generate tissue-specific functions of proteins, and is used by mammalian Myosin Va and Myosin Vb for tissue specific trafficking (230, 297, 299).

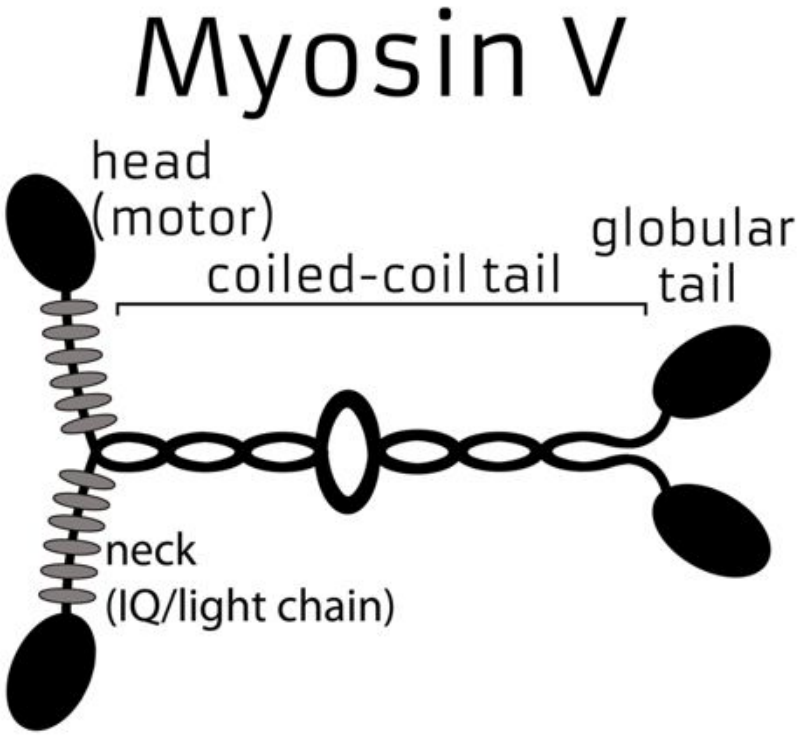

Figure 1. 5
General structure of mammalian class V myosin motors. Functional motors are dimers of monomers that have an ATP-binding motor domain (head), six light-chain binding domains (neck), and a tail domain composed of an extended coiled-coil domain and a folded globular tail.

Tissue specific isoforms functionally impact binding of Rab GTPases, interaction with other trafficking proteins, and subcellular localization of mammalian class V myosins (297, 300-302).

Specificity in interactions of Rab GTPases with Myosin Va, Vb, and Vc varies based on the exon regions used in binding; Rab8 and Rab10 can bind all Myosin Va, Vb, and Vc isoforms using the protein region encoded by exon D, but interactions of other Rabs, which are specific to a single Myosin, occur in other exon regions (297). It is intriguing that some Rabs bind to multiple Myosin family members, suggesting that some pathways utilize multiple Myosin V family members or that a single Rab can operate in more than one pathway.

1.8.3 Yeast Myo2p and Myo4p

Two class V myosins are known to exist in *Saccharomyces cerevisiae*, Myo2p and Myo4p, each with different functions in trafficking. Myo2p is primarily associated with the movement and polarized localization of the vacuole, peroxisomes, and mitochondria (303-308). Myo2p also functions in secretory vesicle trafficking and, as with mammalian class V myosins, binds specific Rabs and other motors through interactions with the globular tail (309, 310). Interestingly, Myo2p interacts with yeast homologues of Rab11 (Ypt32p) and Rab10 (Sec4p), which are both known binding partners for Myosin Va and Vb (Rab11a) or Myosin Va, Vb, and Vc (Rab10) (310, 311). The function of Myo2p as a motor for both organelle and vesicle motility and its interaction with well-conserved Rabs demonstrates the importance of this motor.

The other class V Myosin in yeast, Myo4p functions in the trafficking of specific mRNAs through specific regions in the global tail of Myo4p (312, 313). Myo4p traffics more than 20 different mRNAs associated with polarization to the budding daughter cell through interaction with a series of mRNA-binding adaptor proteins She2p and She3p (314-316). Myo4p is also

responsible for the trafficking of cortical endoplasmic reticulum (ER) into the budding daughter cell and this ER trafficking uses the same mRNA-binding proteins She2p and She3p, but this process occurs completely separate from mRNA trafficking (317).

Unlike mammalian class V myosins, both Myo2p and Myo4p are non-processive motors with relatively low-affinity for actin fibers or bundles (315). Functionally, however, both Myo2p and Myo4p are able to act processively via the presence of multiple motors on the same structures; and though Myo4p does not truly dimerize, it functionally homo-dimerizes through leucine zipper domains (312, 315, 318). It appears that Myo2p is a closer functional homologue to the mammalian class V myosin motors, and conserved interactions with ubiquitous Rab11 and Rab10 suggest that the mammalian Myosin V motors are more closely related evolutionarily to Myo2p.

1.8.4 Mammalian class V myosins

Three class V myosins are present in mammals each with approximately 50% overall sequence identity (319). Interestingly, the tails of the motors are the most divergent regions, and the coiled-coil region of the tail in particular shows low sequence identity that allows for different binding properties and functions of each motor (297, 299, 319). Myosin Va is most abundant in brain and melanocytes, Myosin Vb is expressed in a broad range of epithelial tissues, and Myo5c is expressed broadly but more highly in epithelial and glandular tissues with secretory functions (319, 320). The structure of each of the mammalian class V myosin is very similar, and minor differences in the length and structure of the coiled-coil tail domain are the greatest structural difference. The highly conserved motor and neck domains cause equal step sizes, and classify each myosin as highly processive (321-324). However, some *in vitro* studies

have suggested that Myosin Vc may not function processively as an individual motor, but given physiological conditions and the presence of multiple Myosin Vc motors on the same structure it is predicted that all three myosins are processive *in vivo* (325-327).

1.8.5 Functions of Myosin Va

The function of Myosin Va in both neuronal and non-neuronal cells has been an area of intense research and has resulted to date in the best understanding of function and regulation of any class V myosin (230, 328, 329). Myosin Va is generally understood to function in the capture and dispersal of organelles or vesicles in the cortical actin (329). Interestingly, Myosin Va is capable of specifically localizing to the plus-end of microtubules through indirect binding to the microtubule tip-binding protein EB1, which is facilitated by the Myosin Va binding partner melanophilin and assists to localize Myosin Va to proper cellular location for function (330). This mechanism, in addition to recruitment to membrane via interaction with Rab GTPases, allows for the proper localization of Myosin Va to the polarized cortical actin networks.

In neurons, Myosin Va functions as a point-to-point transporter of IP(3) (inositol 1,4,5-trisphosphate)-sensitive Ca^{2+} stores, mRNA, and endoplasmic reticulum (ER) into dendritic spines, which is reminiscent of the functions of Myo4p in *S. cerevisiae* (329, 331, 332). The trafficking of Ca^{2+} stores, mRNA, and the ER are necessary for dendritic development and functional synaptic plasticity and cerebellar motor learning and mice with mutant versions of Myosin Va displayed defects in cerebellum-dependent motor learning that could be partially rescued by the presence of wild-type Myosin Va (331-334).

In neurons and some non-neuronal secretory tissues, Myosin Va also has a role in the secretion of secretory vesicles and dense core granules (329, 335, 336). A neuron specific splice variant of Myosin Va with the small, 3 amino acid exon B is necessary for interaction with dynein light chains and localization and secretion of neuronal secretory vesicles (335, 337). Myosin Va functions in secretory vesicle and granule trafficking, and in secretion by facilitating the microtubule/actin-transport switch and maintaining localization in the cell periphery by direct binding to the GTP-bound form of Rab3A (338). The interaction between neuronal specific isoform of Myosin Va and Rab3A is directed, but bridging proteins rabphilin-3A and granuphil-a/b are also necessary for the proper trafficking and secretion of neuronal secretory vesicles and secretory granules, respectively (335, 338). Myosin Va also functions in the secretion of other vesicles, such as the high affinity insulin-regulated glucose transporter, GLUT4, in adipocytes where Myosin Va functions with Rab10 to facilitate vesicle-plasma membrane docking and secretion (339). The presence of tripartite complexes of a Rab, class V myosin, and bridging protein may occur in all secretory functions of Myosin Va, including those in which a bridging protein has not been discovered such as the Rab10-Myosin Va dependent GLUT4 vesicle secretion.

The function of Myosin Va is best understood in melanocyte cells in the trafficking of melanosomes, where loss of Myosin Va results in defects in melanosome trafficking and transfer to keratinocytes (340). Myosin Va knockout mice, *dilute*, have a pigmentation defect caused by inability to properly localize melanosomes to the cell periphery, and in humans mutation of Myosin Va underlies Griscelli syndrome type 1 (167). A tissue specific isoform that contains the alternatively spliced exon F of Myosin Va is necessary for melanosome trafficking (341). When exon F is removed melanosome distribution in melanocytes is the same as Myosin Va knockout

cells (341). Myosin Va exon D, another alternatively spliced exon that is present in melanocyte-specific isoform, is not necessary for melanosome trafficking, but is necessary for the Rab8 and Rab10 dependent trafficking of vesicles (280, 339). The localization of Myosin Va to mature melanosomes is dependent on indirect interaction with Rab27a through the bridging protein melanophilin in an exon-F dependent fashion (342, 343). As previously described, the Rab27a/melanophilin/Myosin Va complex is necessary for peripheral melanosome distribution in melanocyte cells, and a variant of this complex using Rab27a/MyRIP/Myosin VIIa functions in a similar fashion in the retinal pigmented epithelial cells (344, 345). The secretion of melanosomes is not directly dependent on the function of Rab27a or Myosin Va/VIIa and may use one of the other class V myosins present in melanocytes (277). Melanocytes from Myosin Va knockout mice have altered distribution of melanosomes, but no significant change in the number of melanosomes consistent with a function in melanosome tethering to the cortical actin, but not a direct role in fusion with the membrane and secretion (275, 346).

1.8.6 Functions of mammalian myosin Vb

Myosin Vb is widely expressed in various epithelial tissues and functions in the ubiquitous plasma membrane recycling systems and in formation and maintenance of membrane polarization in epithelial cells (347-350). Myosin Vb is an effector of multiple Rab GTPases associated with recycling proteins to the plasma membrane (Rab8a, Rab10, Rab11a); and Myosin Vb functions with different Rabs for specific forms of membrane recycling: association with Rab11a is required for transferrin-receptor recycling in non-polarized cells, both Rab8a and Rab11a are required for apical membrane trafficking in epithelial cells, and Rab8a functions with Myosin Vb in the recycling of some cargoes in specialized cells (281, 348).

Myosin Vb is also necessary for the recycling of several neurotransmitter receptors in post-synaptic neurons (351-355). Both the ubiquitous and neurotransmitter receptor recycling are trafficked through recycling endosomes by Rab11a and Rab11-family interacting proteins (Rab11-FIP), which form multi-subunit complexes in a similar fashion as used by Rab27a/melanophilin/Myosin Va (352, 355-358).

Previously, it was unclear if Myosin Vb functions directly in the vesicular trafficking from recycling endosomes to the plasma membrane, or in the earlier perinuclear to peripheral recycling endosome trafficking (359, 360). Results from sub-cellular localization studies and chemical inhibitors suggest that Myosin Vb functions in the long-range movement of vesicles between recycling endosomes and the plasma membrane using a microtubule-independent, actin-dependent mechanism that constitutes a different mode of long-range trafficking in cells (361).

1.8.7 Functions of mammalian myosin Vc

Myosin Vc is the least studied member of the mammalian class V myosin family, but is the most broadly expressed motor that is highly expressed in numerous tissue types (319). Myosin Vc, like Myosin Vb, is expressed in epithelial tissues, but like Myosin Va it is also highly expressed in secretory tissues (319). Myosin Vc is important in the Rab8a-dependent, ubiquitous transferrin-receptor recycling, but does not function or significantly colocalize with Rab11 in transferrin-receptor recycling (319). The recycling of transferrin-receptors is primarily dependent on Rab11 and Myosin Vb, and therefore a Rab8a and Myosin Vc dependent recycling mechanisms may constitute a separate, non-dominant recycling pathway (319, 348). However, little colocalization with Myosin Vb is observed, suggesting that the Myosin Vb and Myosin Vc pathways are not highly overlapping (327).

In some secretory cell types, Myosin Vc facilitates the exocytosis and secretion of secretory vesicles and granules using Rab8a- or Rab27b- dependent mechanisms (327, 362, 363). In these cell types, Myosin Vc shows somewhat polarized localization to specific regions of the cell periphery on one side of the cell, and is characterized in three cellular locations: small and large perinuclear structures, small peripheral structures with polarized cellular distribution, and tubular structures near the basal membrane (327). Myosin Vc punctae and tubular structures in the cell are generally fast moving (327). These tubular structures also appear to colocalize with long actin-bundles near the basal membranes of cells (363), and interestingly nocodazole treatment, a microtubule-depolymerizing agent, disrupts the formation and movement of tubules but not punctate structures (327). In the secretory systems studied, Myosin Vc tubules are found to strongly colocalize with Rab8a, but punctae strongly colocalize with Rab27b or Rab8a (327, 363). Interestingly, Myosin Va and Myosin Vc show little colocalization in these secretory cell types, and Myosin Va does not appear to function in Myosin Vc-dependent secretory pathways in the cell types tested (327). The functions of Myosin Vc in separate functions, cargo recycling and secretion, is also interesting as Rab8 is shown to function with Myosin Vc in both of these functions. Motors themselves are unable to distinguish between separate trafficking pathways, and are regulated by recruitment to and association with Rabs at specific membranes. Regulation of Rabs, therefore, acts as a method to recruit motor activity to different pathways; and interactions with ubiquitous or pathways specific Rabs will underlie the function of Myosin Vc in trafficking. Interactions with these Rabs, therefore, may characterize functions of Myosin Vc in separate trafficking pathways, which can both be utilized for secretion but uses different Rabs.

Myosin Va and Myosin Vb are established as functioning in secretion and cargo recycling, respectively, and functions of Myosin Vc in both of these types of trafficking suggests that Myosin Vc may functionally act as a hybrid of Myosin Va and Myosin Vb. Myosin Vc dependent pathways, however, appear to be distinct from Myosin Va and Myosin Vb dependent pathways. It is notable that Rab8 and Rab10 are capable of interaction with all three mammalian Myosin Vs in a conserved exon-dependent fashion, and that these Rab-Myosin interactions result in similar functions (trafficking of vesicles to the plasma membrane for either exocytosis or recycling) (297). In several cell types all three classes of mammalian myosin V motors are expressed, and often the function of each Myosin in dependent on a Rab that is capable of interacting with Myosin Va, Myosin Vb, and Myosin Vc. It is tempting to suggest that all three myosins function cooperatively in a step-by-step, handoff mechanism for the same trafficking pathway. Low degree of colocalization of Myosin Vc with Myosin Va or Myosin Vb, and the strong phenotypes observed in Myosin Va or Myosin Vb knockdown suggest that Myosin Vc does not serve redundant functions with these motors (167, 348). The functions of Myosin Vc, and the interplay between Myosin Va, Myosin Vb, and Myosin Vc in similar trafficking pathways remain unresolved.

1.9 REFERENCES

1. Wieffer M, Maritzen T, Haucke V. (2009) SnapShot: endocytic trafficking. *Cell* 137(2):382.
2. Hao M, Maxfield FR. (2000) Characterization of rapid membrane internalization and recycling. *J Biol Chem.* 275(20):15279-86.
3. Gruenberg J, Griffiths G, Howell KE. (1989) Characterization of the early endosome and putative endocytic carrier vesicles in vivo and with an assay of vesicle fusion in vitro. *J Cell Biol.* 108(4):1301-16.
4. Clague MJ. (1998) Molecular aspects of the endocytic pathway. *Biochem J.* 336 (Pt 2):271-82.
5. Jovic M, Sharma M, Rahajeng J, Caplan S. (2010) The early endosome: a busy sorting station for proteins at the crossroads. *Histol Histopathol.* 25(1):99-112.
6. Gruenberg J. (2001) The endocytic pathway: a mosaic of domains. *Nat Rev Mol Cell Biol.* 2(10):721-30.
7. Presley JF, Mayor S, McGraw TE, Dunn KW, Maxfield FR. (1997) Bafilomycin A1 treatment retards transferrin receptor recycling more than bulk membrane recycling. *J Biol Chem.* 272(21):13929-36.
8. Mellman I. (1996) Endocytosis and molecular sorting. *Annu Rev Cell Dev Biol.* 12:575-625.
9. Huotari J, Helenius A. (2011) Endosome maturation. *EMBO J.* 30(17):3481-500.
10. Aloisi AL, Bucci C. (2013) Rab GTPases-cargo direct interactions: fine modulators of intracellular trafficking. *Histol Histopathol.* Epub ahead of print.
11. Binder B, Holzhütter HG. (2012) A hypothetical model of cargo-selective rab recruitment during organelle maturation. *Cell Biochem Biophys.* 63(1):59-71.
12. Gorvel JP, Chavrier P, Zerial M, Gruenberg J. (1991) Rab5 controls early endosome fusion in vitro. *Cell.* 64(5):915-25.
13. Bucci C, *et al* (1992) The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. *Cell.* 70(5):715-28.
14. Barbieri MA, Roberts RL, Mukhopadhyay A, Stahl PD. (1996) Rab5 regulates the dynamics of early endosome fusion. *Biocell.* 20(3):331-8.
15. Zerial M, McBride H. (2001) Rab proteins as membrane organizers. *Nat Rev Mol Cell Biol.* 2(2):107-17.
16. Grosshans BL, Ortiz D, Novick P. (2006) Rabs and their effectors: achieving specificity in membrane traffic. *Proc Natl Acad Sci U S A.* 103(32):11821-7.
17. Mattera R, Bonifacino JS. (2008) Ubiquitin binding and conjugation regulate the recruitment of Rabex-5 to early endosomes. *EMBO J.* 27(19):2484-94
18. Horiuchi H, *et al* (1997) A novel Rab5 GDP/GTP exchange factor complexed to Rabaptin-5 links nucleotide exchange to effector recruitment and function. *Cell.* 90(6):1149-59.
19. Lippé R, Miaczynska M, Rybin V, Runge A, Zerial M. (2001) Functional synergy between Rab5 effector Rabaptin-5 and exchange factor Rabex-5 when physically associated in a complex. *Mol Biol Cell.* 12(7):2219-28.
20. Christoforidis S, McBride HM, Burgoyne RD, Zerial M. (1999) The Rab5 effector EEA1 is a core component of endosome docking. *Nature.* 397(6720):621-5.
21. Murray JT, Panaretou C, Stenmark H, Miaczynska M, Backer JM. (2002) Role of Rab5 in the recruitment of hVps34/p150 to the early endosome. Traffic. 3(6):416-27.
22. Hu Y, Chuang JZ, Xu K, McGraw TG, Sung CH. (2002) SARA, a FYVE domain protein, affects Rab5-mediated endocytosis. J Cell Sci. 115(Pt 24):4755-63.
23. Benmerah A. (2004) Endocytosis: signaling from endocytic membranes to the nucleus. *Curr Biol.* 14(8):R314-6.

24. Miaczynska M, *et al* (2004) APPL proteins link Rab5 to nuclear signal transduction via an endosomal compartment. *Cell.* 116(3):445-56.
25. Schenck A, *et al* (2008) The endosomal protein Appl1 mediates Akt substrate specificity and cell survival in vertebrate development. *Cell.* 133(3):486-97.
26. Pal A, Severin F, Lommer B, Shevchenko A, Zerial M. (2006) Huntingtin-HAP40 complex is a novel Rab5 effector that regulates early endosome motility and is up-regulated in Huntington's disease. *J Cell Biol.* 172(4):605-18.
27. van Dam EM, Ten Broeke T, Jansen K, Spijkers P, Stoorvogel W. (2002) Endocytosed transferrin receptors recycle via distinct dynamin and phosphatidylinositol 3-kinase-dependent pathways. *J Biol Chem.* 277(50):48876-83.
28. Bennett MK. (1995) SNAREs and the specificity of transport vesicle targeting. *Curr Opin Cell Biol.* 7(4):581-6
29. Söllner T. (1995) SNAREs and targeted membrane fusion. *FEBS Lett.* 369(1):80-3.
30. Chen YA, Scheller RH. (2001) SNARE-mediated membrane fusion. *Nat Rev Mol Cell Biol.* 2(2):98-106
31. Fasshauer D, Eliason WK, Brünger AT, Jahn R. (1998) Identification of a minimal core of the synaptic SNARE complex sufficient for reversible assembly and disassembly. *Biochemistry.* 37(29):10354-62.
32. Bock JB, Matern HT, Peden AA, Scheller RH. (2001) A genomic perspective on membrane compartment organization. *Nature.* 409(6822):839-41.
33. Hanson, P.I., Roth, R., Morisaki, H., Jahn, R., and Heuser, J.E. (1997). Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch analyzed in microscopy. Cell *90*, 523–535.
34. Lin RC, Scheller RH. (1997) Structural organization of the synaptic exocytosis core complex. *Neuron.* 19(5):1087-94.
35. Mills IG, Jones AT, Clague MJ. (1998) Involvement of the endosomal autoantigen EEA1 in homotypic fusion of early endosomes. *Curr Biol.* 8(15):881-4.
36. McBride HM, *et al* (1999) Oligomeric complexes link Rab5 effectors with NSF and drive membrane fusion via interactions between EEA1 and syntaxin 13. *Cell.* 98(3):377-86.
37. Simonsen A, Gaullier JM, D'Arrigo A, Stenmark H. (1999) The Rab5 effector EEA1 interacts directly with syntaxin-6. *J Biol Chem.* 274(41):28857-60.
38. Mills IG, Urbé S, Clague MJ. (2001) Relationships between EEA1 binding partners and their role in endosome fusion. *J Cell Sci.* 114(Pt 10):1959-65.
39. Jung JJ, Inamdar SM, Tiwari A, Choudhury A. (2012) Regulation of intracellular membrane trafficking and cell dynamics by syntaxin-6. *Biosci Rep.* 32(4):383-91.
40. Epp N, Rethmeier R, Krämer L, Ungermann C. (2011) Membrane dynamics and fusion at late endosomes and vacuoles--Rab regulation, multisubunit tethering complexes and SNAREs. *Eur J Cell Biol.* 90(9):779-85.
41. Solinger JA, Spang A. (2013) Tethering complexes in the endocytic pathway: CORVET and HOPS. *FEBS J.* Epub ahead of print.
42. Söllner TH, Rothman JE. (1996) Molecular machinery mediating vesicle budding, docking and fusion. *Experientia.* 52(12):1021-5.
43. Hong W. (2005) SNAREs and traffic. *Biochim Biophys Acta.* 1744(3):493-517.
44. Chibalina MV, Seaman MN, Miller CC, Kendrick-Jones J, Buss F. (2007) Myosin VI and its interacting protein LMTK2 regulate tubule formation and transport to the endocytic recycling compartment. J Cell Sci. 120(Pt 24):4278-88.
45. Hoepfner S, et al (2005) Modulation of receptor recycling and degradation by the endosomal kinesin KIF16B. *Cell.* 121(3):437-50.

46. Nielsen E, Severin F, Backer JM, Hyman AA, Zerial M. (1999) Rab5 regulates motility of early endosomes on microtubules. *Nat Cell Biol.* 11(6):376-82.
47. Poteryaev D, Datta S, Ackema K, Zerial M, Spang A. (2010) Identification of the switch in early-to-late endosome transition. *Cell.* 141(3):497-508.
48. Rink J, Ghigo E, Kalaidzidis Y, Zerial M. (2005) Rab conversion as a mechanism of progression from early to late endosomes. *Cell.* 122(5):735-49.
49. Vonderheit A, Helenius A. (2005) Rab7 associates with early endosomes to mediate sorting and transport of Semliki forest virus to late endosomes. *PLoS Biol.* 3(7):e233.
50. Driskell OJ, Mironov A, Allan VJ, Woodman PG. (2007) Dynein is required for receptor sorting and the morphogenesis of early endosomes. *Nat Cell Biol.* 9(1):113-20.
51. Mesaki K, Tanabe K, Obayashi M, Oe N, Takei K. (2011) Fission of tubular endosomes triggers endosomal acidification and movement. *PLoS One.* 6(5):e19764
52. Gerondopoulos A, Langemeyer L, Liang JR, Linford A, Barr FA. (2012) BLOC-3 mutated in Hermansky-Pudlak syndrome is a Rab32/38 guanine nucleotide exchange factor. *Curr Biol.* 22(22):2135-9.
53. Nickerson DP, *et al* (2012) Termination of isoform-selective Vps21/Rab5 signaling at endolysosomal organelles by Msb3/Gyp3. *Traffic.* 13(10):1411-28.
54. Lachmann J, Barr FA, Ungermann C. (2012) The Msb3/Gyp3 GAP controls the activity of the Rab GTPases Vps21 and Ypt7 at endosomes and vacuoles. *Mol Biol Cell.* 23(13):2516-26.
55. John Peter AT, *et al* (2013) The BLOC-1 complex promotes endosomal maturation by recruiting the Rab5 GTPase-activating protein Msb3. *J Cell Biol.* 201(1):97-111.
56. van Weering JR, Verkade P, Cullen PJ. (2012) SNX-BAR-mediated endosome tubulation is coordinated with endosome maturation. *Traffic.* 13(1):94-107.
57. Woodman PG, Futter CE. (2008) Multivesicular bodies: coordinated progression to maturity. *Curr Opin Cell Biol.* 20(4):408-14.
58. van Meel E, Klumperman J. (2008) Imaging and imagination: understanding the endo-lysosomal system. *Histochem Cell Biol.* 129(3):253-66.
59. Rieder SE, Banta LM, Köhrer K, McCaffery JM, Emr SD. (1996) Multilamellar endosome-like compartment accumulates in the yeast vps28 vacuolar protein sorting mutant. *Mol Biol Cell.* 7(6):985-99.
60. Doyotte A, Russell MR, Hopkins CR, Woodman PG. (2005) Depletion of TSG101 forms a mammalian "Class E" compartment: a multicisternal early endosome with multiple sorting defects. *J Cell Sci.* 118(Pt 14):3003-17.
61. Razi M, Futter CE. (2006) Distinct roles for Tsg101 and Hrs in multivesicular body formation and inward vesiculation. *Mol Biol Cell.* 17(8):3469-83.
62. Babst M. (2005) A protein's final ESCRT. *Traffic.* 6(1):2-9.
63. Eden ER, White IJ, Futter CE. (2009) Down-regulation of epidermal growth factor receptor signaling within multivesicular bodies. *Biochem Soc Trans.* 37(Pt 1):173-7.
64. Raiborg C, Stenmark H. (2009) The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. *Nature.* 458(7237):445-52
65. Woodman P. (2009) ESCRT proteins, endosome organization and mitogenic receptor down-regulation. *Biochem Soc Trans.* 37(Pt 1):146-50.
66. Hurley JH. (2010) The ESCRT complexes. *Crit Rev Biochem Mol Biol.* 45(6):463-87
67. Hurley JH, Hanson PI. (2010) Membrane budding and scission by the ESCRT machinery: it's all in the neck. *Nat Rev Mol Cell Biol.* 11(8):556-66.
68. Roxrud I, Stenmark H, Malerød L. (2010) ESCRT & Co. *Biol Cell.* 102(5):293-318.

69. Matsuo H, *et al* (2004) Role of LBPA and Alix in multivesicular liposome formation and endosome organization. *Science.* 303(5657):531-4.
70. Trajkovic K, *et al* (2008) Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science.* 319(5867):1244-7.
71. Staub O, *et al* (1997) Regulation of stability and function of the epithelial Na+ channel (ENaC) by ubiquitination. *EMBO J.* 16(21):6325-36.
72. Vaccari T, Bilder D. (2005) The Drosophila tumor suppressor vps25 prevents nonautonomous overproliferation by regulating notch trafficking. *Dev Cell.* 9(5):687-98.
73. Sorkin A, Goh LK. (2008) Endocytosis and intracellular trafficking of ErbBs. *Exp Cell Res.* 314(17):3093-106.
74. Hayer A, *et al* (2010) Caveolin-1 is ubiquitinated and targeted to intralumenal vesicles in endolysosomes for degradation. *J Cell Biol.* 191(3):615-29.
75. Lobert VH, *et al* (2010) Ubiquitination of alpha 5 beta 1 integrin controls fibroblast migration through lysosomal degradation of fibronectin-integrin complexes. *Dev Cell.* 19(1):148-59.
76. Clague MJ, Urbé S, Aniento F, Gruenberg J. (1994) Vacuolar ATPase activity is required for endosomal carrier vesicle formation. *J Biol Chem.* 269(1):21-4.
77. Ikonomov OC, Sbrissa D, Shisheva A. (2006) Localized PtdIns 3,5-P2 synthesis to regulate early endosome dynamics and fusion. *Am J Physiol Cell Physiol.* 291(2):C393-404.
78. Raiborg C, Schink KO, Stenmark H. (2013) Class III phosphatidylinositol 3-kinase and its catalytic product PtdIns3P in regulation of endocytic membrane traffic. FEBS J. 2013 Jan 5. Epub ahead of print
79. Ikonomov OC, *et al* (2009) Kinesin adapter JLP links PIKfyve to microtubule-based endosome-to-trans-Golgi network traffic of furin. *J Biol Chem.* 284(6):3750-61.
80. Sbrissa D, Ikonomov OC, Fenner H, Shisheva A. (2010) ArPIKfyve homomeric and heteromeric interactions scaffold PIKfyve and Sac3 in a complex to promote PIKfyve activity and functionality. *J Mol Biol.* 384(4):766-79.
81. Vellodi A. (2005) Lysosomal storage disorders. *Br J Haematol.* 128(4):413-31
82. Parkinson-Lawrence EJ, *et al* (2010) Lysosomal storage disease: revealing lysosomal function and physiology. *Physiology (Bethesda).* 25(2):102-15.
83. Schaub BE, Nair P, Rohrer J. (2005) Analysis of protein transport to lysosomes. *Curr Protoc Cell Biol.* Chapter 15:Unit 15.8.
84. Callahan JW, Bagshaw RD, Mahuran DJ. (2009) The integral membrane of lysosomes: its proteins and their roles in disease. *J Proteomics.* 72(1):23-33.
85. Saftig P, Klumperman J. (2009) Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function. *Nat Rev Mol Cell Biol.* 10(9):623-35.
86. Mardones GA, *et al* (2013) Structural Basis for the Recognition of Tyrosine-based Sorting Signals by the μ3A Subunit of the AP-3 Adaptor Complex. *J Biol Chem.* 288(13):9563-71.
87. Theos AC, *et al* (2005) Functions of adaptor protein (AP)-3 and AP-1 in tyrosinase sorting from endosomes to melanosomes. *Mol Biol Cell* 16(11):5356-5372.
88. Edeling MA, Smith C, Owen D. (2006) Life of a clathrin coat: insights from clathrin and AP structures. *Nat Rev Mol Cell Biol.* 7(1):32-44.
89. Dell'Angelica EC, Mullins C, Caplan S, Bonifacino JS. (2000) Lysosome-related organelles. *FASEB J.* 14(10):1265-78.
90. Huizing M, Helip-Wooley A, Westbroek W, Gunay-Aygun M, Gahl WA. (2008) Disorders of lysosome-related organelle biogenesis: clinical and molecular genetics. *Annu Rev Genomics Hum Genet.* 9:359-86.

91. Raposo G, Marks MS, Cutler DF. (2007) Lysosome-related organelles: driving post-Golgi compartments into specialization. *Curr Opin Cell Biol.* 19(4):394-401.
92. Wei ML. (2006) Hermansky-Pudlak syndrome: a disease of protein trafficking and organelle function. *Pigment Cell Res.* 19(1):19-42.
93. Raposo G, Fevrier B, Stoorvogel W, Marks MS. (2002) Lysosome-related organelles: a view from immunity and pigmentation. *Cell Struct Funct.* 27(6):443-56.
94. Starcevic M, Nazarian R, Dell'Angelica EC. (2002) The molecular machinery for the biogenesis of lysosome-related organelles: lessons from Hermansky-Pudlak syndrome. *Semin Cell Dev Biol.* 13(4):271-8.
95. Hurbain I, *et al* (2008) Electron tomography of early melanosomes: implications for melanogenesis and the generation of fibrillar amyloid sheets. *Proc Natl Acad Sci U S A.* 105(50):19726-31.
96. Ridsdale R, Na CL, Xu Y, Greis KD, Weaver T. (2011) Comparative proteomic analysis of lung lamellar bodies and lysosome-related organelles. *PLoS One.* 6(1):e16482.
97. Di Pietro SM, Dell'Angelica EC. (2005) The cell biology of Hermansky-Pudlak syndrome: recent advances. *Traffic.* 6(7):525-33.
98. Barral DC, Seabra MC. (2004) The melanosome as a model to study organelle motility in mammals. *Pigment Cell Res.* 17(2):111-8.
99. Di Pietro SM, *et al* (2006) BLOC-1 interacts with BLOC-2 and the AP-3 complex to facilitate protein trafficking on endosomes. *Mol Biol Cell.* 17(9):4027-38.
100. Falcón-Pérez JM, Starcevic M, Gautam R, Dell'Angelica EC. (2002) BLOC-1, a novel complex containing the Pallidin and muted proteins involved in the biogenesis of melanosomes and platelet-dense granules. *J Biol Chem.* 277(31):28191-9.
101. Lee HH, *et al* (2012) Assembly and architecture of biogenesis of lysosome-related organelles complex-1 (BLOC-1). *J Biol Chem.* 287(8):5882-90.
102. Huang L, Kuo YM, Gitschier J. (1999) The pallid gene encodes a novel, syntaxin 13-interacting protein involved in platelet storage pool deficiency. *Nat Genet.* 23(3):329-32.
103. Moriyama K, Bonifacino JS. (2002) Pallidin is a component of a multi-protein complex involved in the biogenesis of lysosome-related organelles. *Traffic.* 3(9):666-77.
104. Ghiani CA, *et al* (2010) The dysbindin-containing complex (BLOC-1) in brain: developmental regulation, interaction with SNARE proteins and role in neurite outgrowth. *Mol Psychiatry.* 15(2):115, 204-15.
105. Ilardi JM, Mochida S, Sheng ZH. (1999) Snapin: a SNARE-associated protein implicated in synaptic transmission. *Nat Neurosci.* 2(2):119-24.
106. Vites O, Rhee JS, Schwarz M, Rosenmund C, Jahn R. (2004) Reinvestigation of the role of snapin in neurotransmitter release. *J Biol Chem.* 279(25):26251-6.
107. John Peter AT, *et al* (2013) The BLOC-1 complex promotes endosomal maturation by recruiting the Rab5 GTPase-activating protein Msb3. *J Cell Biol.* 201(1):97-111.
108. Helip-Wooley A, *et al* (2005) Association of the Hermansky-Pudlak syndrome type-3 protein with clathrin. *BMC Cell Biol.* 6:33.
109. Oh J, *et al* (1996) Positional cloning of a gene for Hermansky-Pudlak syndrome, a disorder of cytoplasmic organelles. *Nat Genet.* 14(3):300-6.
110. Martina JA, Moriyama K, Bonifacino JS. (2003) BLOC-3, a protein complex containing the Hermansky-Pudlak syndrome gene products HPS1 and HPS4. *J Biol Chem.* 278(31):29376-84
111. Nazarian R, Falcón-Pérez JM, Dell'Angelica EC. (2003) Biogenesis of lysosome-related organelles complex 3 (BLOC-3): a complex containing the Hermansky-Pudlak syndrome (HPS) proteins HPS1 and HPS4. *Proc Natl Acad Sci U S A.* 100(15):8770-5

112. Oh J, Liu ZX, Feng GH, Raposo G, Spritz RA. (2000) The Hermansky-Pudlak syndrome (HPS) protein is part of a high molecular weight complex involved in biogenesis of early melanosomes. *Hum Mol Genet.* 9(3):375-85
113. Richmond B, *et al* (2005) Melanocytes derived from patients with Hermansky-Pudlak Syndrome types 1, 2, and 3 have distinct defects in cargo trafficking. *J Invest Dermatol.* 124(2):420-7.
114. Kloer DP, *et al* (2010) Assembly of the biogenesis of lysosome-related organelles complex-3 (BLOC-3) and its interaction with Rab9. *J Biol Chem.* 285(10):7794-804.
115. Andersson TP, Svensson SP, Karlsson AM. (2003) Regulation of melanosome movement by MAP kinase. *Pigment Cell Res.* 16(3):215-21.
116. Guttentag SH, *et al* (2005) Defective surfactant secretion in a mouse model of Hermansky-Pudlak syndrome. *Am J Respir Cell Mol Biol.* 33(1):14-21.
117. Gautam R, *et al* (2006) Interaction of Hermansky-Pudlak Syndrome genes in the regulation of lysosome-related organelles. *Traffic.* 7(7):779-92.
118. Falcón-Pérez JM, Nazarian R, Sabatti C, Dell'Angelica EC. (2005) Distribution and dynamics of Lamp1-containing endocytic organelles in fibroblasts deficient in BLOC-3. *J Cell Sci.* 118(Pt 22):5243-55
119. Feng L, *et al* (1999) The beta3A subunit gene (Ap3b1) of the AP-3 adaptor complex is altered in the mouse hypopigmentation mutant pearl, a model for Hermansky-Pudlak syndrome and night blindness. *Hum Mol Genet.* 8(2):323-30.
120. Zhen L, *et al* (1999) Abnormal expression and subcellular distribution of subunit proteins of the AP-3 adaptor complex lead to platelet storage pool deficiency in the pearl mouse. *Blood.* 94(1):146-55.
121. Yang W, Li C, Ward DM, Kaplan J, Mansour SL. (2000) Defective organellar membrane protein trafficking in Ap3b1-deficient cells. *J Cell Sci.* 113 (Pt 22):4077-86.
122. Peden AA, Rudge RE, Lui WW, Robinson MS. (2002) Assembly and function of AP-3 complexes in cells expressing mutant subunits. *J Cell Biol.* 156(2):327-36.
123. Lane PW, Deol MS. (1974) Mocha, a new coat color and behavior mutation on chromosome 10 of the mouse. *J Hered.* 65(6):362-4.
124. Noebels JL, Sidman RL. (1989) Persistent hypersynchronization of neocortical neurons in the mocha mutant of mouse. *J Neurogenet.* 6(1):53-6.
125. Kantheti P, *et al* (1998) Mutation in AP-3 delta in the mocha mouse links endosomal transport to storage deficiency in platelets, melanosomes, and synaptic vesicles. *Neuron.* 21(1):111-22.
126. Swank RT, Reddington M, Howlett O, Novak EK. (1991) Platelet storage pool deficiency associated with inherited abnormalities of the inner ear in the mouse pigment mutants muted and mocha. *Blood.* 78(8):2036-44.
127. Dell'Angelica EC. (2009) AP-3-dependent trafficking and disease: the first decade. *Curr Opin Cell Biol.* 21(4):552-559.
128. Bonifacino JS, Dell'Angelica EC. (1999) Molecular bases for the recognition of tyrosine-based sorting signals. *J Cell Biol.* 145(5):923-6.
129. Dell'Angelica EC. (2004) The building BLOC(k)s of lysosomes and related organelles. *Curr Opin Cell Biol.* 16(4):458-64
130. Suzuki T, *et al* (2003) The mouse organellar biogenesis mutant buff results from a mutation in Vps33a, a homologue of yeast vps33 and Drosophila carnation. *Proc Natl Acad Sci U S A.* 100(3):1146-50.
131. Raymond CK, Howald-Stevenson I, Vater CA, Stevens TH. (1992) Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E vps mutants. *Mol Biol Cell.* 3(12):1389-402.

132. Fratti RA, Jun Y, Merz AJ, Margolis N, Wickner W. (2004) Interdependent assembly of specific regulatory lipids and membrane fusion proteins into the vertex ring domain of docked vacuoles. *J Cell Biol.* 167(6):1087-98.
133. Zlatic SA, Tornieri K, L'Hernault SW, Faundez V. (2011) Clathrin-dependent mechanisms modulate the subcellular distribution of class C Vps/HOPS tether subunits in polarized and nonpolarized cells. *Mol Biol Cell.* 22(10):1699-715.
134. Caplan S, Hartnell LM, Aguilar RC, Naslavsky N, Bonifacino JS. (2001) Human Vam6p promotes lysosome clustering and fusion in vivo. *J Cell Biol.* 154(1):109-22.
135. Kim BY, *et al* (2001) Molecular characterization of mammalian homologues of class C Vps proteins that interact with syntaxin-7. *J Biol Chem.* 276(31):29393-402.
136. Poupon V, Stewart A, Gray SR, Piper RC, Luzio JP. (2003) The role of mVps18p in clustering, fusion, and intracellular localization of late endocytic organelles. *Mol Biol Cell.* 14(10):4015-27
137. Wurmser AE, Sato TK, Emr SD. (2000) New component of the vacuolar class C-Vps complex couples nucleotide exchange on the Ypt7 GTPase to SNARE-dependent docking and fusion. *J Cell Biol.* 151(3):551-62.
138. Brett CL, *et al* (2008) Efficient termination of vacuolar Rab GTPase signaling requires coordinated action by a GAP and a protein kinase. *J Cell Biol.* 182(6):1141-51.
139. Stroupe C, Collins KM, Fratti RA, Wickner W. (2006) Purification of active HOPS complex reveals its affinities for phosphoinositides and the SNAREVam7p. *EMBO J.* 25(8):1579-89.
140. Hickey CM, Stroupe C, Wickner W. (2009) The major role of the Rab Ypt7p in vacuole fusion is supporting HOPS membrane association. *J Biol Chem.* 284(24):16118-25
141. Zhang Q, *et al* (2002) Cell-specific abnormal prenylation of Rab proteins in platelets and melanocytes of the gunmetal mouse. *Br J Haematol.* 117(2):414-23.
142. Taylor A, *et al* (2011) Impaired prenylation of Rab GTPases in the gunmetal mouse causes defects in bone cell function. *Small GTPases.* 2(3):131-142.
143. Pereira-Leal JB, Hume AN, Seabra MC. (2001) Prenylation of Rab GTPases: molecular mechanisms and involvement in genetic disease. *FEBS Lett.* 498(2-3):197-200.
144. Loftus SK, *et al* (2002) Mutation of melanosome protein RAB38 in chocolate mice. *Proc Natl Acad Sci U S A.* 99(7):4471-6.
145. Oiso N, Riddle SR, Serikawa T, Kuramoto T, Spritz RA (2004) The rat Ruby (R) locus is Rab38: identical mutations in Fawn-hooded and Tester-Moriyama rats derived from an ancestral Long Evans rat sub-strain. *Mamm Genome.* 15(4):307-14.
146. Wasmeier C, *et al* (2006) Rab38 and Rab32 control post-Golgi trafficking of melanogenic enzymes. *J Cell Biol.* 175(2):271-81.
147. Bultema JJ, Ambrosio AL, Burek CL, Di Pietro SM. (2012) BLOC-2, AP-3, and AP-1 proteins function in concert with Rab38 and Rab32 proteins to mediate protein trafficking to lysosome-related organelles. *J Biol Chem.* 287(23):19550-63
148. Ambrosio AL, Boyle JA, Di Pietro SM. (2012) Mechanism of platelet dense granule biogenesis: study of cargo transport and function of Rab32 and Rab38 in a model system. *Blood.* 120(19):4072-81.
149. Newell-Litwa K, *et al* (2010) Hermansky-Pudlak protein complexes, AP-3 and BLOC-1, differentially regulate presynaptic composition in the striatum and hippocampus. *J Neurosci.* 30(3):820-31.
150. Hikita T, *et al* (2009) Proteomic analysis reveals novel binding partners of dysbindin, a schizophrenia-related protein. *J Neurochem.* 110(5):1567-74.

151. Salazar G, *et al* (2009) Hermansky-Pudlak syndrome protein complexes associate with phosphatidylinositol 4-kinase type II alpha in neuronal and non-neuronal cells. *J Biol Chem.* 284(3):1790-802.
152. Salazar G, *et al* (2006) BLOC-1 complex deficiency alters the targeting of adaptor protein complex-3 cargoes. *Mol Biol Cell.* 17(9):4014-26
153. Setty SR, *et al* (2007) BLOC-1 is required for cargo-specific sorting from vacuolar early endosomes toward lysosome-related organelles. *Mol Biol Cell.* 18(3):768-80.
154. Borner GH, Harbour M, Hester S, Lilley KS, Robinson MS. (2006) Comparative proteomics of clathrin-coated vesicles. *J Cell Biol.* 175(4):571-8.
155. Setty SR, *et al* (2008) Cell-specific ATP7A transport sustains copper-dependent tyrosinase activity in melanosomes. *Nature.* 454(7208):1142-6.
156. Baust T, *et al* (2008) Protein networks supporting AP-3 function in targeting lysosomal membrane proteins. *Mol Biol Cell.* 19(5):1942-51.
157. Craige B, Salazar G, Faundez V. (2008) Phosphatidylinositol-4-kinase type II alpha contains an AP-3-sorting motif and a kinase domain that are both required for endosome traffic. *Mol Biol Cell.* 19(4):1415-26
158. Zhang Q, *et al* (2003) Ru2 and Ru encode mouse orthologs of the genes mutated in human Hermansky-Pudlak syndrome types 5 and 6. *Nat Genet.* 33(2):145-53.
159. Gautam R, *et al* (2004) The Hermansky-Pudlak syndrome 3 (cocoa) protein is a component of the biogenesis of lysosome-related organelles complex-2 (BLOC-2). *J Biol Chem.* 279(13):12935-42.
160. Boissy RE, *et al* (2005) Melanocyte-specific proteins are aberrantly trafficked in melanocytes of Hermansky-Pudlak syndrome-type 3. *Am J Pathol.* 166(1):231-40.
161. Helip-Wooley A, *et al* (2007) Improper trafficking of melanocyte-specific proteins in Hermansky-Pudlak syndrome type-5. *J Invest Dermatol.* 127(6):1471-8.
162. Feng L, *et al* (2002) The Hermansky-Pudlak syndrome 1 (HPS1) and HPS2 genes independently contribute to the production and function of platelet dense granules, melanosomes, and lysosomes. *Blood.* 99(5):1651-8.
163. Cao C, Wan Y (2009) Parameters of protection against ultraviolet radiation-induced skin cell damage. *J Cell Physiol.* 220(2):277-84.
164. Sitaram A, Marks MS (2012) Mechanisms of protein delivery to melanosomes in pigment cells. *Physiology (Bethesda).* 27(2):85-99.
165. Kondo T, Hearing VJ (2011) Update on the regulation of mammalian melanocyte function and skin pigmentation. *Expert Rev Dermatol.* 6(1):97-108.
166. Meredith P, Riesz J (2003) "Radiative relaxation quantum yields for synthetic eumelanin". *Photochemistry and photobiology* 79 (2): 211–6
167. Dessinioti C, Stratigos AJ, Rigopoulos D, Katsambas AD (2009) A review of genetic disorders of hypopigmentation: lessons learned from the biology of melanocytes. *Exp Dermatol.* 18(9):741-9.
168. Sitaram A, *et al* (2012) Differential recognition of a dileucine-based sorting signal by AP-1 and AP-3 reveals a requirement for both BLOC-1 and AP-3 in delivery of OCA2 to melanosomes. *Mol Biol Cell.* 23(16):3178-92.
169. Sitaram A, *et al* (2009) Localization to mature melanosomes by virtue of cytoplasmic dileucine motifs is required for human OCA2 function. *Mol Biol Cell.* 20(5):1464-77.
170. Wei AH, *et al* (2013) Exome Sequencing Identifies SLC24A5 as a Candidate Gene for Nonsyndromic Oculocutaneous Albinism. *J Invest Dermatol.* Epub ahead of print.

171. Dooley CM, *et al* (2013) Slc45a2 and V-ATPase are regulators of melanosomal pH homeostasis in zebrafish, providing a mechanism for human pigment evolution and disease. *Pigment Cell Melanoma Res.* 26(2):205-17.
172. King RA, *et al* (2003) Tyrosinase gene mutations in oculocutaneous albinism 1 (OCA1): definition of the phenotype. *Hum Genet.* 113(6):502-13.
173. Ito S, Wakamatsu K (2008) Chemistry of mixed melanogenesis--pivotal roles of dopaquinone. *Photochem Photobiol.* 84(3):582-92.
174. Jackson IJ (1988) A cDNA encoding tyrosinase-related protein maps to the brown locus in mouse. *Proc Natl Acad Sci U S A.* 85(12):4392-6.
175. Bouchard B, Fuller BB, Vijayasaradhi S, Houghton AN (1989) Induction of pigmentation in mouse fibroblasts by expression of human tyrosinase cDNA. *J Exp Med.* 169(6):2029-42.
176. Ni-Komatsu L, Orlow SJ (2006) Heterologous expression of tyrosinase recapitulates the misprocessing and mistrafficking in oculocutaneous albinism type 2: effects of altering intracellular pH and pink-eyed dilution gene expression. *Exp Eye Res.* 82(3):519-28.
177. Aroca P, Garcia-Borron JC, Solano F, Lozano JA (1990) Regulation of mammalian melanogenesis. I: Partial purification and characterization of a dopachrome converting factor: dopachrome tautomerase. *Biochim Biophys Acta.* 1035(3):266-7
178. Chakraborty AK, Orlow SJ, Bolognia JL, Pawelek JM (1991) Structural/functional relationships between internal and external MSH receptors: modulation of expression in Cloudman melanoma cells by UVB radiation. *J Cell Physiol.* 147(1):1-6.
179. Orlow SJ, Chakraborty AK, Pawelek JM (1992) Membrane glycoproteins common to vesicles and melanosomes in mouse melanoma cells. *Pigment Cell Res.* Suppl 2:162-70.
180. Kobayashi T, Hearing VJ (2007) Direct interaction of tyrosinase with Tyrp-1 to form heterodimeric complexes in vivo. *J Cell Sci.* 120(Pt 24):4261-8.
181. Robbins LS, *et al* (1993) Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function. *Cell.* 72(6):827-34.
182. Cone RD, *et al* (1996) The melanocortin receptors: agonists, antagonists, and the hormonal control of pigmentation. *Recent Prog Horm Res.* 51:287-317
183. Geschwind II (1966) Change in hair color in mice induced by injection of alpha-MSH. *Endocrinology.* 79(6):1165-7
184. Tamate HB, Takeuchi T (1984) Action of the e locus of mice in the response of phaeomelanic hair follicles to alpha-melanocyte-stimulating hormone in vitro. *Science.* 224(4654):1241-2.
185. Lu D, *et al* (1994) Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. *Nature.* 371(6500):799-802.
186. Barber JI, Townsend D, Olds DP, King RA (1985) Decreased dopachrome oxidoreductase activity in yellow mice. *J Hered.* 76(1):59-60.
187. Lamoreux ML (1986) Dopachrome conversion and dopa oxidase activities in recessive yellow mice. Catalytic activities of extracts from pheomelanic and eumelanic tissues. *J Hered.* 77(5):337-40.
188. Thody AJ, Burchill SA (1992) Tyrosinase and the regulation of coat color changes in C3H-HeAvy mice. *Pigment Cell Res.* 5(5 Pt 2):335-9.
189. Rios M, *et al* (1999) Catecholamine synthesis is mediated by tyrosinase in the absence of tyrosine hydroxylase. *J Neurosci.* 19(9):3519-26.
190. Lamoreux ML, Zhou BK, Rosemblat S, Orlow SJ (1995) The pinkeyed-dilution protein and the eumelanin/pheomelanin switch: in support of a unifying hypothesis. *Pigment Cell Res.* 8(5):263-70.

191. Hirobe T, *et al* (2011) A novel deletion mutation of mouse ruby-eye 2 named ru2(d)/Hps5(ru2-d) inhibits melanocyte differentiation and its impaired differentiation is rescued by L-tyrosine. *Zoolog Sci.* 28(11):790-801.
192. Simon JD, Peles DN (2010) The red and the black. *Acc Chem Res.* 43(11):1452-60.
193. Krude H, *et al* (1998) Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. *Nat Genet.* 19(2):155-7.
194. Valverde P, Healy E, Jackson I, Rees JL, Thody AJ (1995) Variants of the melanocyte-stimulating hormone receptor gene are associated with red hair and fair skin in humans. *Nat Genet.* 11(3):328-30.
195. Humphries WH 4th, Szymanski CJ, Payne CK (2011) Endo-lysosomal vesicles positive for Rab7 and LAMP1 are terminal vesicles for the transport of dextran. *PLoS One.* 6(10):e26626.
196. Theos AC, *et al* (2006) A lumenal domain-dependent pathway for sorting to intralumenal vesicles of multivesicular endosomes involved in organelle morphogenesis. *Dev Cell* 10(3):343-354.
197. Raposo G, Tenza D, Murphy DM, Berson JF, Marks MS (2001) Distinct protein sorting and localization to premelanosomes, melanosomes, and lysosomes in pigmented melanocytic cells. *J Cell Biol.* 152(4):809-824.
198. Berson JF, *et al* (2003) Proprotein convertase cleavage liberates a fibrillogenic fragment of a resident glycoprotein to initiate melanosome biogenesis. *J Cell Biol.* 161(3):521-533.
199. Hoashi T, *et al* (2005) MART-1 is required for the function of the melanosomal matrix protein PMEL17/GP100 and the maturation of melanosomes. *J Biol Chem.* 280(14):14006-16.
200. Ohbayashi N, Fukuda M (2012) Role of Rab family GTPases and their effectors in melanosomal logistics. *J Biochem.* 151(4):343-351.
201. Peden AA, *et al* (2004) Localization of the AP-3 adaptor complex defines a novel endosomal exit site for lysosomal membrane proteins. *J Cell Biol.* 164(7):1065-1076.
202. Dell'Angelica EC, Shotelersuk V, Aguilar RC, Gahl WA, Bonifacino JS (1999) Altered trafficking of lysosomal proteins in Hermansky-Pudlak syndrome due to mutations in the beta 3A subunit of the AP-3 adaptor. *Mol Cell* 3(1):11-21.
203. Tazeh NN, *et al* (2009) Role of AP-1 in developmentally regulated lysosomal trafficking in Trypanosoma brucei. *Eukaryot Cell.* 8(9):1352-61.
204. Ivan V, *et al* (2012) AP-3 and Rabip4' coordinately regulate spatial distribution of lysosomes. *PLoS One.* 7(10):e48142.
205. Dores MR, *et al* (2012) AP-3 regulates PAR1 ubiquitin-independent MVB/lysosomal sorting via an ALIX-mediated pathway. *Mol Biol Cell.* 23(18):3612-23.
206. Poirier S, *et al* (2013) The cytosolic adaptor AP-1A is essential for the trafficking and function of Niemann-Pick type C proteins. *Traffic.* 14(4):458-69.
207. Delevoye C, *et al* (2009) AP-1 and KIF13A coordinate endosomal sorting and positioning during melanosome biogenesis. *J Cell Biol.* 187(2):247-64.
208. Negroiu G, Dwek RA, Petrescu SM (2005) Tyrosinase-related protein-2 and -1 are trafficked on distinct routes in B16 melanoma cells. *Biochem Biophys Res Commun.* 328(4):914-21.
209. Negroiu G, Dwek RA, Petrescu SM (2003) The inhibition of early N-glycan processing targets TRP-2 to degradation in B16 melanoma cells. *J Biol Chem.* 278(29):27035-42.
210. Jimbow K, *et al* (2000) Intracellular vesicular trafficking of tyrosinase gene family protein in eu- and pheomelanosome biogenesis. *Pigment Cell Res.* 13 Suppl 8:110-7.
211. Dooley CM, *et al* (2013) Slc45a2 and V-ATPase are regulators of melanosomal pH homeostasis in zebrafish, providing a mechanism for human pigment evolution and disease. *Pigment Cell Melanoma Res.* 26(2):205-17.
212. Bruder JM, *et al* (2012) Melanosomal dynamics assessed with a live-cell fluorescent melanosomal marker. *PLoS One.* 7(8):e43465

213. Hoyle DJ, Rodriguez-Fernandez IA, Dell'angelica EC (2011) Functional interactions between OCA2 and the protein complexes BLOC-1, BLOC-2, and AP-3 inferred from epistatic analyses of mouse coat pigmentation. *Pigment Cell Melanoma Res.* 24(2):275-81.
214. Fukuda M (1991). "Lysosomal membrane glycoproteins. Structure, biosynthesis, and intracellular trafficking". *J. Biol. Chem.* 266 (32): 21327–21330
215. Eskelinen EL, *et al* (2002) Role of LAMP-2 in lysosome biogenesis and autophagy. *Mol Biol Cell.* 13(9):3355-68
216. Saftig P, Schröder B, Blanz J (2010) Lysosomal membrane proteins: life between acid and neutral conditions. *Biochem Soc Trans.* 38(6):1420-3
217. Chapuy B, *et al* (2008) AP-1 and AP-3 mediate sorting of melanosomal and lysosomal membrane proteins into distinct post-Golgi trafficking pathways. *Traffic.* 9(7):1157-72.
218. Dell'Angelica EC, Shotelersuk V, Aguilar RC, Gahl WA, Bonifacino JS (1999) Altered trafficking of lysosomal proteins in Hermansky-Pudlak syndrome due to mutations in the beta 3A subunit of the AP-3 adaptor. *Mol Cell.* 3(1):11-21
219. Ihrke G, Kyttälä A, Russell MR, Rous BA, Luzio JP (2004) Differential use of two AP-3-mediated pathways by lysosomal membrane proteins. *Traffic.* 5(12):946-62.
220. Jimbow K, *et al* (2000) Intracellular vesicular trafficking of tyrosinase gene family protein in eu- and pheomelanosome biogenesis. *Pigment Cell Res.* 13 Suppl 8:110-7
221. Fujita H, *et al* (2009) Inulavosin, a melanogenesis inhibitor, leads to mistargeting of tyrosinase to lysosomes and accelerates its degradation. *J Invest Dermatol.* 129(6):1489-99
222. Bouzat S, Levi V, Bruno L (2012) Transport properties of melanosomes along microtubules interpreted by a tug-of-war model with loose mechanical coupling. *PLoS One.* 7(8):e43599
223. Hume AN, Seabra MC (2011) Melanosomes on the move: a model to understand organelle dynamics. *Biochem Soc Trans.* 39(5):1191-6.
224. Kural C, *et al* (2007) Tracking melanosomes inside a cell to study molecular motors and their interaction. *Proc Natl Acad Sci U S A.* 104(13):5378-82.
225. Watabe H, *et al* (2008) Involvement of dynein and spectrin with early melanosome transport and melanosomal protein trafficking. *J Invest Dermatol.* 128(1):162-74.
226. Hume AN, *et al* (2001) Rab27a regulates the peripheral distribution of melanosomes in melanocytes. *J Cell Biol.* 152(4):795-808.
227. Sugden D, Davidson K, Hough KA, Teh MT (2004) Melatonin, melatonin receptors and melanophores: a moving story. *Pigment Cell Res.* 17(5):454-60.
228. Reilein AR, *et al* (2003) Differential regulation of dynein-driven melanosome movement. *Biochem Biophys Res Commun.* 309(3):652-8.
229. Deacon SW, Nascimento A, Serpinskaya AS, Gelfand VI (2005) Regulation of bidirectional melanosome transport by organelle bound MAP kinase. *Curr Biol.* 15(5):459-63.
230. Seabra MC, Coudrier E (2004) Rab GTPases and myosin motors in organelle motility. *Traffic.* 5(6):393-9.
231. Wasmeier C, Hume AN, Bolasco G, Seabra MC (2008) Melanosomes at a glance. *J Cell Sci.* 121(Pt 24):3995-3999.
232. Singh SK, *et al* (2010) Melanin transfer in human skin cells is mediated by filapodia--a model for homotypic and heterotypic lysosome-related organelle transfer. *FASEB J.* 24(10):3756-69.
233. Wu XS, *et al* (2012) Melanoregulin regulates a shedding mechanism that drives melanosome transfer from melanocytes to keratinocytes. *Proc Natl Acad Sci U S A.* 109(31):E2101-9.
234. Scott G (2012) Demonstration of melanosome transfer by a shedding micro vesicle mechanism. *J. Invest Dermatol.* 132(4):1037-4
235. Ando H, *et al* (2012) Melanosomes are transferred from melanocytes to keratinocytes through the processes of packaging, release, uptake, and dispersion. *J Invest Dermatol.* 132(4):1222-9.

236. Ando H, *et al* (2010) Keratinocytes in culture accumulate phagocytosed melanosomes in the perinuclear area. *Pigment Cell Melanoma Res.* 23(1):129-33.
237. Ando H, *et al* (2011) Involvement of pigment globules containing multiple melanosomes in the transfer of melanosomes from melanocytes to keratinocytes. *Cell Logist.* 1(1):12-20.
238. Belleudi F, *et al* (2011) Expression and signaling of the tyrosine kinase FGFR2b/KGFR regulates phagocytosis and melanosome uptake in human keratinocytes. *FASEB J.* 25(1):170-81.
239. Huang Q, *et al* (2013) Ultrastructural observations of human epidermal melanocytes cultured on polyethylene terephthalate film. *Micron.* 48:49-53.
240. Laulagnier K, *et al* (2011) Role of AP1 and Gadkin in the traffic of secretory endo-lysosomes. *Mol Biol Cell.* 22(12):2068-82.
241. Gomes AQ, *et al* (2003) Membrane targeting of Rab GTPases is influenced by the prenylation motif. *Mol Biol Cell.* 14(5):1882-99.
242. van Niel G, *et al* (2011) The tetraspanin CD63 regulates ESCRT-independent and -dependent endosomal sorting during melanogenesis. *Dev Cell.* 21(4):708-21.
243. Kelly JW, Balch WE (2003) Amyloid as a natural product. *J. Cell Biol.* 161, 461–462
244. Hirosaki K, Yamashita T, Wada I, Jin HY, Jimbow K (2002) Tyrosinase and tyrosinase-related protein 1 require Rab7 for their intracellular transport. *J Invest Dermatol.* 119(2):475-80.
245. Hida T, *et al* (2011) Rab7 is a critical mediator in vesicular transport of tyrosinase-related protein 1 in melanocytes. *J Dermatol.* 38(5):432-41.
246. Kawakami A, *et al* (2008) Rab7 regulates maturation of melanosomal matrix protein gp100/Pmel17/Silv. *J Invest Dermatol.* 128(1):143-50.
247. Jordens I, *et al* (2006) Rab7 and Rab27a control two motor protein activities involved in melanosomal transport. *Pigment Cell Res.* 19(5):412-23.
248. Jordens I, *et al* (2001) The Rab7 effector protein RILP controls lysosomal transport by inducing the recruitment of dynein-dynactin motors. *Curr Biol.* 11(21):1680-5.
249. Brooks BP, *et al* (2007) Analysis of ocular hypopigmentation in Rab38cht/cht mice. *Invest Ophthalmol Vis Sci.* 48(9):3905-13.
250. Lopes VS, Wasmeier C, Seabra MC, Futter CE (2007) Melanosome maturation defect in Rab38-deficient retinal pigment epithelium results in instability of immature melanosomes during transient melanogenesis. *Mol Biol Cell.* 18(10):3914-27.
251. Park M, Serpinskaya AS, Papalopulu N, Gelfand VI (2007) Rab32 regulates melanosome transport in Xenopus melanophores by protein kinase a recruitment. *Curr Biol.* 17(23):2030-4
252. Ma J, Plesken H, Treisman JE, Edelman-Novemsky I, Ren M (2004) Lightoid and Claret: a rab GTPase and its putative guanine nucleotide exchange factor in biogenesis of Drosophila eye pigment granules. *Proc Natl Acad Sci U S A.* 101(32):11652-7.
253. Ninkovic I, White JG, Rangel-Filho A, Datta YH (2008) The role of Rab38 in platelet dense granule defects. *J Thromb Haemost.* 6(12):2143-51.
254. Zhang L, *et al* (2011) Rab38 targets to lamellar bodies and normalizes their sizes in lung alveolar type II epithelial cells. *Am J Physiol Lung Cell Mol Physiol.* 301(4):L461-77.
255. Nottingham RM, Ganley IG, Barr FA, Lambright DG, Pfeffer SR (2011) RUTBC1 protein, a Rab9A effector that activates GTP hydrolysis by Rab32 and Rab33B proteins. *J Biol Chem.* 286(38):33213-22.
256. Carmona-Rivera C, *et al* (2011) Clinical, molecular, and cellular features of non-Puerto Rican Hermansky-Pudlak syndrome patients of Hispanic descent. *J Invest Dermatol.* 131(12):2394-400.
257. Wang F, *et al* (2008) Varp interacts with Rab38 and functions as its potential effector. *Biochem Biophys Res Commun.* 372(1):162-7.
258. Tamura K, *et al* (2009) Varp is a novel Rab32/38-binding protein that regulates Tyrp-1 trafficking in melanocytes. *Mol Biol Cell.* 20(12):2900-8.

259. Tamura K, Ohbayashi N, Ishibashi K, Fukuda M (2011) Structure-function analysis of VPS9-ankyrin-repeat protein (Varp) in the trafficking of tyrosinase-related protein 1 in melanocytes. *J Biol Chem.* 286(9):7507-21.
260. Schäfer IB, *et al* (2012) The binding of Varp to VAMP7 traps VAMP7 in a closed, fusogenically inactive conformation. *Nat Struct Mol Biol.* 19(12):1300-9.
261. Griscelli C, Durandy A, Guy-Grand D, Daguillard F, Herzog C, Prunieras M (1978) A syndrome associating partial albinism and immunodeficiency. *Am. J. Med.* 65 (4): 691–702
262. Izumi T, Gomi H, Kasai K, Mizutani S, Torii S (2003) The roles of Rab27 and its effectors in the regulated secretory pathways. *Cell Struct Funct.* 28(5):465-74
263. Fukuda M (2005) Versatile role of Rab27 in membrane trafficking: focus on the Rab27 effector families. *J Biochem.* 137(1):9-16
264. Barral DC, *et al* (2002) Functional redundancy of Rab27 proteins and the pathogenesis of Griscelli syndrome. *J Clin Invest.*110(2):247-57
265. Ishida M, Ohbayashi N, Maruta Y, Ebata Y, Fukuda M (2012) Functional involvement of Rab1A in microtubule-dependent anterograde melanosome transport in melanocytes. *J Cell Sci.* 125(Pt 21):5177-87.
266. Ohbayashi N, Maruta Y, Ishida M, Fukuda M (2012) Melanoregulin regulates retrograde melanosome transport through interaction with the RILP-p150Glued complex in melanocytes. *J Cell Sci.* 125(Pt 6):1508-18.
267. Hume AN, Seabra MC (2011) Melanosomes on the move: a model to understand organelle dynamics. *Biochem Soc Trans.* 39(5):1191-6
268. Hume AN, Wilson MS, Ushakov DS, Ferenczi MA, Seabra MC (2011) Semi-automated analysis of organelle movement and membrane content: understanding rab-motor complex transport function. *Traffic.* 12(12):1686-701
269. Araki K, *et al* (2000) Small Gtpase rab3A is associated with melanosomes in melanoma cells. *Pigment Cell Res.* 13(5):332-6.
270. Figueiredo AC, *et al* (2008) Rab3GEP is the non-redundant guanine nucleotide exchange factor for Rab27a in melanocytes. *J Biol Chem.* 283(34):23209-16.
271. Kukimoto-Niino M, *et al* (2008) Structural basis for the exclusive specificity of Slac2-a/melanophilin for the Rab27 GTPases. *Structure.* 16(10):1478-90.
272. Futter CE, Ramalho JS, Jaissle GB, Seeliger MW, Seabra MC (2004) The role of Rab27a in the regulation of melanosome distribution within retinal pigment epithelial cells. *Mol Biol Cell.* 15(5):2264-75.
273. Klomp AE, Teofilo K, Legacki E, Williams DS (2007) Analysis of the linkage of MYRIP and MYO7A to melanosomes by RAB27A in retinal pigment epithelial cells. *Cell Motil Cytoskeleton.* 64(6):474-87.
274. Imai A, *et al* (2011) EPI64 protein functions as a physiological GTPase-activating protein for Rab27 protein and regulates amylase release in rat parotid acinar cells. *J Biol Chem.* 286(39):33854-62.
275. Beaumont KA, *et al* (2011) The recycling endosome protein Rab17 regulates melanocytic filopodia formation and melanosome trafficking. *Traffic.* 12(5):627-43.
276. Lapierre LA, Goldenring JR (2005) Interactions of myosin Vb with rab11 family members and cargoes traversing the plasma membrane recycling system. *Methods Enzymol.* 403:715-23.
277. Gidon A, *et al* (2012) A Rab11A/myosin Vb/Rab11-FIP2 complex frames two late recycling steps of langerin from the ERC to the plasma membrane. *Traffic.* 13(6):815-33.
278. Chabrillat ML, *et al* (2005) Rab8 regulates the actin-based movement of melanosomes. *Mol Biol Cell.* 16(4):1640-50.

279. Chakraborty AK, Funasaka Y, Araki K, Horikawa T, Ichihashi M (2003) Evidence that the small GTPase Rab8 is involved in melanosome traffic and dendrite extension in B16 melanoma cells. *Cell Tissue Res.* 314(3):381-8.
280. Roland JT, Kenworthy AK, Peranen J, Caplan S, Goldenring JR (2007) Myosin Vb interacts with Rab8a on a tubular network containing EHD1 and EHD3. *Mol Biol Cell.* 18(8):2828-37.
281. Ishikura S, Klip A (2008) Muscle cells engage Rab8A and myosin Vb in insulin-dependent GLUT4 translocation. Am *J Physiol Cell Physiol.* 295(4):C1016-25.
282. Erickson RP, Larson-Thomé K, Valenzuela RK, Whitaker SE, Shub MD (2008) Navajo microvillous inclusion disease is due to a mutation in MYO5B. *Am J Med Genet A.* 146A(24):3117-9.
283. Kalhammer G, Bähler M (2000) Unconventional myosins. *Essays Biochem.* 35:33-42.
284. Hammer JA 3rd, Sellers JR (2011) Walking to work: roles for class V myosins as cargo transporters. *Nat Rev Mol Cell Biol.* 13(1):13-26.
285. Leinwand LA, Saez L, McNally E, Nadal-Ginard B (1983) Isolation and characterization of human myosin heavy chain genes. *Proc Natl Acad Sci U S A.* 80(12):3716-20.
286. Moore JR, Krementsova EB, Trybus KM, Warshaw DM (2004) Does the myosin V neck region act as a lever? *J Muscle Res Cell Motil.* 25(1):29-35.
287. Ganoth A, Nachliel E, Friedman R, Gutman M (2007) Myosin V movement: lessons from molecular dynamics studies of IQ peptides in the lever arm. *Biochemistry.* 46(50):14524-36.
288. Okada T, *et al* (2007) The diffusive search mechanism of processive myosin class-V motor involves directional steps along actin subunits. *Biochem Biophys Res Commun.* 354(2):379-84.
289. Krementsova EB, Hodges AR, Lu H, Trybus KM (2006) Processivity of chimeric class V myosins. *J Biol Chem.* 281(9):6079-86.
290. Veigel C, Schmitz S, Wang F, Sellers JR (2005) Load-dependent kinetics of myosin-V can explain its high processivity. *Nat Cell Biol.* 7(9):861-9.
291. Tanaka H, *et al* (2002) The motor domain determines the large step of myosin-V. *Nature.* 415(6868):192-5.
292. Veigel C, Wang F, Bartoo ML, Sellers JR, Molloy JE (2002) The gated gait of the processive molecular motor, myosin V. *Nat Cell Biol.* 4(1):59-65.
293. Hodges AR, Bookwalter CS, Krementsova EB, Trybus KM (2009) A nonprocessive class V myosin drives cargo processively when a kinesin- related protein is a passenger. *Curr Biol.* 19(24):2121-5.
294. Li JF, Nebenführ A (2008) The tail that wags the dog: the globular tail domain defines the function of myosin V/XI. *Traffic.* 9(3):290-8.
295. Ishikawa K, *et al* (2003) Identification of an organelle-specific myosin V receptor. *J Cell Biol.* 160(6):887-97.
296. Pashkova N, Catlett NL, Novak JL, Weisman LS (2005) A point mutation in the cargo-binding domain of myosin V affects its interaction with multiple cargoes. *Eukaryot Cell.* 4(4):787-98.
297. Roland JT, Lapierre LA, Goldenring JR (2009) Alternative splicing in class V myosins determines association with Rab10. *J Biol Chem.* 284(2):1213-23
298. Casavola EC, *et al* (2008) Ypt32p and Mlc1p bind within the vesicle binding region of the class V myosin Myo2p globular tail domain. *Mol Microbiol.* 67(5):1051-66
299. Lambert J, Naeyaert JM, Callens T, De Paepe A, Messiaen L (1998) Human myosin V gene produces different transcripts in a cell type-specific manner. *Biochem Biophys Res Commun.* 252(2):329-33.
300. Hódi Z, *et al* (2006) Alternatively spliced exon B of myosin Va is essential for binding the tail-associated light chain shared by dynein. *Biochemistry.* 45(41):12582-95.
301. Wagner W, Fodor E, Ginsburg A, Hammer JA 3rd (2006) The binding of DYNLL2 to myosin Va requires alternatively spliced exon B and stabilizes a portion of the myosin's coiled-coil domain. *Biochemistry.* 45(38):11564-77.

302. Westbroek W, *et al* (2003) Interactions of human Myosin Va isoforms, endogenously expressed in human melanocytes, are tightly regulated by the tail domain. *J Invest Dermatol.* 120(3):465-75.
303. Tang F, *et al* (2003) Regulated degradation of a class V myosin receptor directs movement of the yeast vacuole. *Nature.* 422(6927):87-92.
304. Fagarasanu A, *et al* (2009) Myosin-driven peroxisome partitioning in S. cerevisiae. *J Cell Biol.* 186(4):541-54.
305. Sheltzer JM, Rose MD (2009) The class V myosin Myo2p is required for Fus2p transport and actin polarization during the yeast mating response. *Mol Biol Cell.* 20(12):2909-19.
306. Fagarasanu A, Fagarasanu M, Eitzen GA, Aitchison JD, Rachubinski RA (2006) The peroxisomal membrane protein Inp2p is the peroxisome-specific receptor for the myosin V motor Myo2p of Saccharomyces cerevisiae. *Dev Cell.* 10(5):587-600.
307. Altmann K, Frank M, Neumann D, Jakobs S, Westermann B (2008) The class V myosin motor protein, Myo2, plays a major role in mitochondrial motility in Saccharomyces cerevisiae. *J Cell Biol.* 181(1):119-30.
308. Matsui Y (2003) Polarized distribution of intracellular components by class V myosins in Saccharomyces cerevisiae. *Int Rev Cytol.* 229:1-42.
309. Schott D, Ho J, Pruyne D, Bretscher A (1999) The COOH-terminal domain of Myo2p, a yeast myosin V, has a direct role in secretory vesicle targeting. *J Cell Biol.* 147(4):791-808.
310. Casavola EC, *et al* (2008) Ypt32p and Mlc1p bind within the vesicle binding region of the class V myosin Myo2p globular tail domain. *Mol Microbiol.* 67(5):1051-66.
311. Babbey CM, Bacallao RL, Dunn KW (2010) Rab10 associates with primary cilia and the exocyst complex in renal epithelial cells. *Am J Physiol Renal Physiol.* 299(3):F495-506.
312. Dunn BD, Sakamoto T, Hong MS, Sellers JR, Takizawa PA (2007) Myo4p is a monomeric myosin with motility uniquely adapted to transport mRNA. *J Cell Biol.* 178(7):1193-206.
313. Heuck A, *et al* (2010) The structure of the Myo4p globular tail and its function in ASH1 mRNA localization. *J Cell Biol.* 189(3):497-510.
314. Takizawa PA, Vale RD (2000) The myosin motor, Myo4p, binds Ash1 mRNA via the adapter protein, She3p. *Proc Natl Acad Sci U S A.* 97(10):5273-8.
315. Bookwalter CS, Lord M, Trybus KM (2009) Essential features of the class V myosin from budding yeast for ASH1 mRNA transport. *Mol Biol Cell.* 20(14):3414-21.
316. Kruse C, *et al* (2002) Ribonucleoprotein-dependent localization of the yeast class V myosin Myo4p. *J Cell Biol.* 159(6):971-82.
317. Estrada P, *et al* (2003) Myo4p and She3p are required for cortical ER inheritance in Saccharomyces cerevisiae. *J Cell Biol.* 163(6):1255-66.
318. Reck-Peterson SL, Tyska MJ, Novick PJ, Mooseker MS (2001) The yeast class V myosins, Myo2p and Myo4p, are nonprocessive actin-based motors. *J Cell Biol.* 153(5):1121-6.
319. Rodriguez OC, Cheney RE (2002) Human myosin-Vc is a novel class V myosin expressed in epithelial cells. *J Cell Sci.* 115(Pt 5):991-1004.
320. McGurk L, Tzolovsky G, Spears N, Bownes M (2006) The temporal and spatial expression pattern of myosin Va, Vb and VI in the mouse ovary. *Gene Expr Patterns.* 6(8):900-7.
321. Zhang C, Ali MY, Warshaw DM, Kad NM (2012) A branched kinetic scheme describes the mechanochemical coupling of Myosin Va processivity in response to substrate. *Biophys J.* 103(4):728-37.
322. Lu H, Kennedy GG, Warshaw DM, Trybus KM (2010) Simultaneous observation of tail and head movements of myosin V during processive motion. *J Biol Chem.* 285(53):42068-74.
323. Sakamoto T, Webb MR, Forgacs E, White HD, Sellers JR (2008) Direct observation of the mechanochemical coupling in myosin Va during processive movement. *Nature.* (7209):128-32.
324. Watanabe S, Mabuchi K, Ikebe R, Ikebe M (2006) Mechanoenzymatic characterization of human myosin Vb. *Biochemistry.* 45(8):2729-38.

325. Takagi Y, *et al* (2008) Human myosin Vc is a low duty ratio, nonprocessive molecular motor. *J Biol Chem.* 283(13):8527-37.
326. Watanabe S, *et al* (2008) Human myosin Vc is a low duty ratio nonprocessive motor. *J Biol Chem.* 283(16):10581-92.
327. Jacobs DT, Weigert R, Grode KD, Donaldson JG, Cheney RE (2009) Myosin Vc is a molecular motor that functions in secretory granule trafficking. *Mol Biol Cell.* 20(21):4471-88.
328. Coudrier E (2007) Myosins in melanocytes: to move or not to move? *Pigment Cell Res.* 20(3):153-60.
329. Desnos C, Huet S, Darchen F (2007) 'Should I stay or should I go?': myosin V function in organelle trafficking. *Biol Cell.* 99(8):411-23.
330. Wu XS, Tsan GL, Hammer JA 3rd (2005) Melanophilin and myosin Va track the microtubule plus end on EB1. *J Cell Biol.* 171(2):201-7.
331. Stiess M, Bradke F (2011) Neuronal transport: myosins pull the ER. *Nat Cell Biol.* 13(1):10-1.
332. Wagner W, Brenowitz SD, Hammer JA 3rd (2011) Myosin-Va transports the endoplasmic reticulum into the dendritic spines of Purkinje neurons. *Nat Cell Biol.* 13(1):40-8.
333. Miyata M, *et al* (2011) A role for myosin Va in cerebellar plasticity and motor learning: a possible mechanism underlying neurological disorder in myosin Va disease. *J Neurosci.* 31(16):6067-78.
334. Rao MV, *et al* (2011) The myosin Va head domain binds to the neurofilament-L rod and modulates endoplasmic reticulum (ER) content and distribution within axons. *PLoS One.* 6(2):e17087.
335. Brozzi F, *et al* (2012) Molecular mechanism of myosin Va recruitment to dense core secretory granules. *Traffic.* 13(1):54-69.
336. Kögel T, Bittins CM, Rudolf R, Gerdes HH (2010) Versatile roles for myosin Va in dense core vesicle biogenesis and function. *Biochem Soc Trans.* 38(Pt 1):199-204.
337. Hódi Z, *et al* (2006) Alternatively spliced exon B of myosin Va is essential for binding the tail-associated light chain shared by dynein. *Biochemistry.* 45(41):12582-95.
338. Wöllert T, *et al* (2011) Myosin5a tail associates directly with Rab3A-containing compartments in neurons. *J Biol Chem.* 286(16):14352-61.
339. Chen Y, *et al* (2012) Rab10 and myosin-Va mediate insulin-stimulated GLUT4 storage vesicle translocation in adipocytes. *J Cell Biol.* 198(4):545-60.
340. Van Gele M, Geusens B, Schmitt AM, Aguilar L, Lambert J (2008) Knockdown of myosin Va isoforms by RNAi as a tool to block melanosome transport in primary human melanocytes. *J Invest Dermatol.* 128(10):2474-84.
341. Au JS, Huang JD (2002) A tissue-specific exon of myosin Va is responsible for selective cargo binding in melanocytes. *Cell Motil Cytoskeleton.* 53(2):89-102.
342. Wu X, Wang F, Rao K, Sellers JR, Hammer JA 3rd (2002) Rab27a is an essential component of melanosome receptor for myosin Va. *Mol Biol Cell.* 13(5):1735-49.
343. Westbroek W, *et al* (2012) Cellular and clinical report of new Griscelli syndrome type III cases. *Pigment Cell Melanoma Res.* 25(1):47-56.
344. Lopes VS, *et al* (2007) The ternary Rab27a-Myrip-Myosin VIIa complex regulates melanosome motility in the retinal pigment epithelium. *Traffic.* 8(5):486-99.
345. Gibbs D, *et al* (2004) Role of myosin VIIa and Rab27a in the motility and localization of RPE melanosomes. *J Cell Sci.* 117(Pt 26):6473-83.
346. Provance DW Jr, Wei M, Ipe V, Mercer JA (1996) Cultured melanocytes from dilute mutant mice exhibit dendritic morphology and altered melanosome distribution. *Proc Natl Acad Sci U S A.* 93(25):14554-8
347. Lapierre LA, *et al* (2001) Myosin vb is associated with plasma membrane recycling systems. *Mol Biol Cell.* 12(6):1843-57.

348. Roland JT, *et al* (2011) Rab GTPase-Myo5B complexes control membrane recycling and epithelial polarization. *Proc Natl Acad Sci U S A.* 108(7):2789-94.
349. Ruemmele FM, *et al* (2010) Loss-of-function of MYO5B is the main cause of microvillus inclusion disease: 15 novel mutations and a CaCo-2 RNAi cell model. *Hum Mutat.* 31(5):544-51.
350. Müller T, *et al* (2008) MYO5B mutations cause microvillus inclusion disease and disrupt epithelial cell polarity. *Nat Genet.* 40(10):1163-5.
351. Gardner LA, Hajjhussein H, Frederick-Dyer KC, Bahouth SW (2011) Rab11a and its binding partners regulate the recycling of the ß1-adrenergic receptor. *Cell Signal.* 23(1):46-57.
352. Wang Z, *et al* (2008) Myosin Vb mobilizes recycling endosomes and AMPA receptors for postsynaptic plasticity. *Cell.* 135(3):535-48.
353. Millman EE, *et al* (2008) Rapid recycling of beta-adrenergic receptors is dependent on the actin cytoskeleton and myosin Vb. *Traffic.* 9(11):1958-71.
354. Volpicelli LA, Lah JJ, Fang G, Goldenring JR, Levey AI (2002) Rab11a and myosin Vb regulate recycling of the M4 muscarinic acetylcholine receptor. *J Neurosci.* 22(22):9776-84.
355. Lisé MF, *et al* (2006) Involvement of myosin Vb in glutamate receptor trafficking. *J Biol Chem. 281*(6):3669-78.
356. Hales CM, Vaerman JP, Goldenring JR (2002) Rab11 family interacting protein 2 associates with Myosin Vb and regulates plasma membrane recycling. *J Biol Chem.* 277(52):50415-21.
357. Brock SC, Goldenring JR, Crowe JE Jr (2003) Apical recycling systems regulate directional budding of respiratory syncytial virus from polarized epithelial cells. *Proc Natl Acad Sci U S A.* 100(25):15143-8.
358. Ducharme NA, Ham AJ, Lapierre LA, Goldenring JR (2011) Rab11-FIP2 influences multiple components of the endosomal system in polarized MDCK cells. *Cell Logist.* 1(2):57-68.
359. Provance DW Jr, *et al* (2004) Chemical-genetic inhibition of a sensitized mutant myosin Vb demonstrates a role in peripheral-pericentriolar membrane traffic. *Proc Natl Acad Sci U S A.* 101(7):1868-73.
360. Provance DW Jr, *et al* (2008) Myosin-Vb functions as a dynamic tether for peripheral endocytic compartments during transferrin trafficking. *BMC Cell Biol.* 9:44.
361. Schuh M (2011) An actin-dependent mechanism for long-range vesicle transport. *Nat Cell Biol.* 13(12):1431-6.
362. Marchelletta RR, Jacobs DT, Schechter JE, Cheney RE, Hamm-Alvarez SF (2008) The class V myosin motor, myosin 5c, localizes to mature secretory vesicles and facilitates exocytosis in lacrimal acini. *Am J Physiol Cell Physiol.* 295(1):C13-28.
363. Xu XF, Chen ZT, Gao N, Zhang JL, An J (2009) Myosin Vc, a member of the actin motor family associated with Rab8, is involved in the release of DV2 from HepG2 cells. *Intervirology.* 52(5):258-65.

CHAPTER 2

BLOC-2, AP-3, AND AP-1 FUNCTION IN CONCERT WITH RAB38 AND RAB32 TO MEDIATE PROTEIN TRAFFICKING TO LYSOSOME-RELATED ORGANELLES[1]

2.1 Summary

Lysosome-related organelles (LROs) are synthesized in specialized cell types where they largely coexist with conventional lysosomes. Most of the known cellular transport machinery involved in biogenesis are ubiquitously expressed and shared between lysosomes and LROs. Examples of common components are the Adaptor Protein complex-3 (AP-3) and Biogenesis of Lysosome-related Organelles Complex (BLOC)-2. These protein complexes control sorting and transport of newly synthesized integral membrane proteins from early endosomes to both lysosomes and LROs such as the melanosome. However, it is unknown what factors cooperate with the ubiquitous transport machinery to mediate transport to LROs in specialized cells. Focusing on the melanosome, we show that the ubiquitous machinery interacts with cell type specific Rab proteins, Rab38 and Rab32, to facilitate transport to the maturing organelle. BLOC-2, AP-3, and AP-1 coimmunoprecipitated with Rab38 and Rab32 from MNT-1 melanocytic cell extracts. BLOC-2, AP-3, AP-1, and clathrin partially colocalized with Rab38 and Rab32 by confocal immunofluorescence microscopy in MNT-1 cells. Rab38- and Rab32-deficient MNT-1 cells displayed abnormal trafficking and steady state levels of known cargoes

[1] Jarred J. Bultema, Andrea L. Ambrosio, Carolyn L. Burek, and Santiago M. Di Pietro
From the Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, Colorado 80523, USA.

of the BLOC-2, AP-3, and AP-1 pathways, the melanin synthesizing enzymes tyrosinase and tyrosinase-related protein-1. These observations support the idea that Rab38 and Rab32 are the specific factors that direct the ubiquitous machinery to mediate transport from early endosomes to maturing LROs. Additionally, analysis of tyrosinase-related protein-2 and total melanin production indicates that Rab32 has unique functions that cannot be carried out by Rab38 in melanosome biogenesis.

2.2 Introduction

Lysosome-related organelles (LROs) are a group of cell type specific membrane-bound compartments with specialized functions (1-4). Examples of LROs include melanosomes, platelet dense granules, lamellar bodies of lung type II epithelial cells, and lytic granules of cytotoxic T lymphocytes and natural killer cells. The physiological functions of these organelles are diverse, from the production and storage of melanin pigments (melanosomes) and the regulation of platelet aggregation (dense granules) to killing virus-infected and tumor cells (lytic granules) (1-4). The LROs share common characteristics with lysosomes such as an acidic luminal pH, the presence of lysosome-associated membrane proteins (LAMPs) in their limiting membrane, and a common biogenesis pathway (1-4). The close relationship between lysosomes and LROs is further demonstrated by certain human genetic disorders, including the Hermansky-Pudlak syndrome (HPS), that cause abnormalities in both organelle types (2,4-6).

HPS is a group of autosomal recessive diseases (OMIM 2033000) that are characterized by oculocutaneous albinism (hypopigmentation of eyes and skin) and prolonged bleeding that result from defects in the biogenesis of melanosomes and platelet dense granules, respectively (2,4-6). Some HPS patients present additional symptoms due to defects in other LROs, for

instance defective lamellar bodies result in fatal pulmonary fibrosis and abnormal lytic granules cause immune deficiency (2,4-6). In humans, different forms of the disease, named HPS-1 through -9, have been associated with mutations in 9 separate genes (2,4-7). Orthologues of those genes and 7 additional genes cause HPS-like disease in 16 mutant mouse strains (4-9). Several of those HPS genes encode proteins that assemble into stable complexes, named Adaptor Protein complex-3 (AP-3), and Biogenesis of Lysosome-related Organelles Complex (BLOC)-1, -2, and -3 (4-10). The function of AP-3 in lysosome biogenesis has been well established: it characterizes a route for trafficking of integral membrane proteins, such as LAMPs, from early endosome-associated tubules to the limiting membrane of late endosomes and lysosomes (11-13). The melanosome has served as a prototype for LRO biogenesis studies in part because of the availability of both mutant HPS-like mice with obvious coat color phenotype and an excellent cell line model system, the MNT-1 pigmented human melanocytic cells (2,4-6,8,9,14-16) Analogous to its function in lysosome biogenesis, AP-3 mediates the transport of cargo integral membrane proteins such as the melanogenic enzyme tyrosinase from early endosome-associated tubules to maturing melanosomes (11,14,15,17). In contrast with AP-3, the function of the BLOCs at the molecular level is not well understood. However, BLOC-1 and -2 also localize to early endosome associated tubules and are part of the machinery that mediates vesicular transport of integral membrane proteins to lysosomes and melanosomes (7,14,17-19). Moreover, BLOC-2 defines a trafficking pathway from early endosomes to maturing melanosomes that is parallel to the AP-3-dependent pathway (3,17). Accordingly, the AP-3 and BLOC-2 single mutant mice are moderately hypopigmented but the AP-3/BLOC-2 double mutant mice display a severe coat color phenotype (17). Consistently, epistatic defects were observed for the trafficking of a melanosomal cargo protein, tyrosinase-related protein 1 (Tyrp-

1), in cultured melanocytes isolated from the corresponding single and double mutant mice (17). Other studies have shown alternative routes for tyrosinase exiting early endosome-associated tubules and presumably leading to maturing melanosomes in MNT-1 cells and mouse melanocytes (15). One such route was defined by AP-3 and the other by another member of the AP family, AP-1, which in melanocytes is also involved in a trafficking pathway from early endosomes to melanosomes (15,20). It has not yet been established if the AP-3-independent pathways originating from early endosome-associated tubules in the different reports (i.e. BLOC-2-dependent and AP-1-dependent) correspond to the same or separate trafficking routes (15,17,20). How are LROs formed in relationship with lysosomes? One possibility is that the LRO replaces the lysosome in the specialized cell and that expression of cell type-specific proteins with lysosomal targeting signals provides the organelle with the LRO function in addition to the degradative role of conventional lysosomes. This may be the case for the lytic granule (1). In contrast, most LROs, including melanosomes and platelet dense granules, coexist with conventional lysosomes as distinct organelles in the same cell (21,22). Therefore, sorting mechanisms must exist to segregate newly synthesized LRO and conventional lysosomal components (11). This problem is particularly puzzling because most of the known elements of the trafficking machinery such as AP-3 and the BLOCs are shared for the biogenesis of both lysosomes and LROs (1,3-7,10,11,17). Moreover, these common components of the trafficking machinery are ubiquitously expressed, as expected for a function in lysosome biogenesis (5,10,11). Thus, an outstanding question is what are the factors that direct the ubiquitous trafficking machinery toward LROs in cells that simultaneously produce conventional lysosomes? (3,11,17)

Rab proteins are GTPases of the Ras superfamily that confer target and timing specificity to vesicle budding, motility, tethering, docking, and fusion within the eukaryotic secretory and endosomal pathways (23,24). Rab38 and its close homolog Rab32 are expressed in a highly tissue specific manner, chiefly in LRO producing cells such as melanocytes, platelets, and lung type II epithelial cells (24-29). Rodent models of HPS with mutations in Rab38 display hypopigmentation, prolonged bleeding, and lung disease due to defective biogenesis of melanosomes, platelet dense granules, and lamellar bodies, respectively (24-32). The pigmentation phenotype elicited by Rab38 deficiency is mild (25). However, when melanocytes isolated from Rab38 mutant mice were subjected to siRNA knock-down of Rab32, the resulting phenotype was much more severe and the trafficking of both tyrosinase and Tyrp-1 was altered (27). This result placed Rab38 and Rab32 as key players in LRO biogenesis and implies that Rab32 is able to functionally compensate for Rab38, at least in part, or that they operate in parallel pathways (27). This apparently epistatic relationship between Rab38 and Rab32 is analogous to the one described between AP-3 and BLOC-2 and raised the exciting possibility that these Rabs could be the specificity factors that work in concert with the ubiquitous trafficking machinery for transport towards LROs (17,27). Despite the initial progress made in the field with the discovery of Rab38 and Rab32 involvement in LRO biogenesis, several questions remain unanswered (27,33). Where do the Rabs function and who are their partners? Can Rab38 functionally compensate for Rab32 deficiency? Do they have non-redundant roles in melanosome biogenesis?

Using gene silencing, biochemical, and imaging approaches, we show that BLOC-2, AP-3, and AP-1 interact physically and functionally with Rab38 and Rab32 to mediate melanosome biogenesis. The results indicate Rab38 and Rab32 operate together with the ubiquitous transport

machinery in pathways from early endosomal domains towards maturing melanosomes. Analysis of the three melanin synthesizing enzymes, tyrosinase, Tyrp-1, and Tyrp-2 and overall melanin production show Rab38 and Rab32 have both redundant and non-redundant roles in melanosome biogenesis.

2.3 Experimental Procedures

2.3.1 Plasmids and Antibodies

The cDNA for Rab32, Rab38 and Rab11 were amplified from total RNA of MNT-1 cells by reverse transcriptase-PCR and subsequently cloned in-frame into pGEX-5X-1 and pET-30a+ bacterial expression vectors (for expression and purification of GST- and poly-histidine-tagged proteins, respectively). Rabbit and rat polyclonal antibodies were generated against purified GST-Rab38 or GST-Rab32. Antibodies were affinity purified from sera using either poly-histidine-Rab38 or GST-Rab32 covalently coupled to Affi-Gel15 beads (Bio-Rad). Anti-Rab32 serum was passed through a GST-Affi-Gel 15 column, to remove anti-GST antibodies.

Other antibodies used: affinity purified rabbit antibody to HPS6 (HP6D, gift from E. C. Dell'Angelica (34)), AP-3 β (Protein Tech); mouse monoclonal antibodies against HPS4 and pallidin (gift from E. C. Dell'Angelica (17,35)) AP-3 δ (SA4, gift from A. A. Peden (13)), AP-3 μ (18/p47A, BD), AP-1 γ (100/3, Sigma), clathrin heavy chain (X22, Abcam), Tyrp-1 (MEL-5/TA99, Santa Cruz), tyrosinase (T311, Santa Cruz), Tyrp-2 (C-9, Santa Cruz), SNX1 (clone 51, BD), EEA1 (clone 14, BD), α-tubulin (DM1A, Sigma). Control rabbit IgG was from Southern Biotechnology (Birmingham, AL). Alexa-488 and Alexa-546 conjugated secondary antibodies were from Invitrogen and horseradish peroxidase (HRP)-conjugated secondary antibodies were from GE Amersham.

2.3.2 Cell culture

Human MNT-1 cell line was cultured as described (36). Transfection for siRNA was performed using the Nucleofector electroporation system (Lonza) and the NHEM-Neo kit with MNT-1 cells sub-cultured 2-3 days before transfection. Two sequential siRNAs treatments were performed on days 1 and 4, cells were analyzed on day 7. Oligonucleotides used for siRNA are as follows: δ subunit of AP-3 (17), HPS6 subunit of BLOC-2 (Sigma, SASI_Hs01_00035287), γ subunit of AP-1 (Sigma, SASI_Hs01_00151148), Rab32 (Sigma, SASI_Hs02_00342400), Rab38 (Sigma, SASI_Hs01_00247037), HPS4 subunit of BLOC-3 and pallidin subunit of BLOC-1 (17).

2.3.3 Biochemical Procedures

Cytosolic and membrane fractions of MNT-1 cells were prepared by homogenization in buffer A (20 mM HEPES, pH 7.4, 50 mM KCl, 1 mM dithiothreitol, 1 mM EGTA, 1 mM $MgCl_2$, 250 mM Sucrose) containing a protease inhibitors mixture (34), followed by centrifugation at 15,000x *g* for 10 min and then ultracentrifugation at 400,000x *g* for 15 min, at 4°C. The final membrane pellet was solubilized in 1 ml of buffer A containing protease inhibitors mixture and 1% (wt/vol) Triton X-100. Triton X-100 was added to the cytosolic fraction to match the detergent concentration. Both fractions were cleared by centrifugation for 10 min at 15,000x *g* before immunoprecipitation, which was performed as described above (34) except for the use of buffer A in all washing steps, three times with 0.1% (w/v) Triton X-100, and then once without detergent. Guanidine nucleotide exchange of GST-Rab fusion proteins and GST pulldown assays was performed as described (37).

For membrane association experiments, cytosol and membrane fractions were obtained from MNT-1 cells by homogenization in buffer B (10 mM HEPES, pH 7.4, 250 mM sucrose, 1 mM dithiothreitol, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.25 mM GTPγS, and protease inhibitors mixture), followed by centrifugation for 5 min at 15,000x *g* and for 15 min at 400,000x *g*, at 4°C. Immunoblotting was performed as described (38).

Quantification of immunoblots was carried out by chemifluorescent detection using a Storm 860 scanner (Molecular Dynamics) with an excitation wavelength of 450 nm and a 520 LP emission filter or by scanning films. Integration of the band intensities was performed using ImageJ (NIH, USA).

2.3.4 Melanin Content

MNT-1 cells were centrifuged at 90x *g* for 10 minutes to pellet cells. Cell pellets were treated and analyzed as described (39) by a spectrophotometric method at 500nm using purified *Sepia officinalis* melanin (Sigma-Aldrich) as a standard.

2.3.5 Immunofluorescence Microscopy and Antibody Internalization assay

Immunofluorescence staining, internalization of antibodies, and leupeptin treatment were performed as described (17,40,41). Rabbit anti-Rab32 and anti-Rab38 were used in all co-staining experiments except for co-staining with BLOC-2 (HPS6 subunit) where the corresponding rat anti-Rab32 and anti-Rab38 were utilized. For labeling lysosomes with dextran, cells were incubated for 15 hours at 37°C in medium containing 50 μg/ml of fixable dextran-Alexa Fluor 647 (Invitrogen) followed by a 4-hour chase period in medium lacking dextran. Immunofluorescence microscopy samples were examined in a temperature controlled chamber at

room temperature on an Olympus IX81 spinning disc confocal microscope with Photometrics Cascade II camera using a 100x/1.40NA objective. Images were acquired and analyzed in Slidebook v5 software (3i, Denver). For determination of the percent of colocalization, each channel was subjected to a Laplacian 2D filter with a 3x3 kernel [-1, 8, -1] and a binary mask was generated using a Ridler-Calvard automated threshold method both on each channel and on the overlap between the two individual channels in Slidebook software. Pixel area overlap from the overlap mask and each individual mask was used to calculate percent colocalization. Negative controls for colocalization were performed with peroxisomal markers (RFP-SKL and PMP34-GFP, gift from P. K. Kim) that give a similar distributed punctate staining as proteins of interest (42). The degree of colocalization between AP-3, BLOC-2, Rab32, or Rab38 and the peroxisomal markers RFP-SKL and PMP-GFP with this method was below 10%. Antibody internalization images were acquired at room temperature using a Nikon Diaphot 300 microscope with Photometrics Cool SNAP camera using Metamorph software under conditions optimized to prevent signal saturation. Images were analyzed for total fluorescence intensity with ImageJ software (NIH, USA) as previously described (17).

2.4 Results

2.4.1 BLOC-2, AP-3, and AP-1 interact physically with Rab38 and Rab32

To study endogenous human Rab38 and Rab32, we raised both rabbit and rat polyclonal antibodies against purified recombinant GST-Rab38 and GST-Rab32 and affinity purified them using the corresponding poly-histidine tagged Rab as a ligand. By immunoblotting analysis, both rabbit and rat antibodies to Rab38 recognized the endogenous protein from MNT-1 cell extracts and did not cross react with Rab32 or any other protein (Figure2.1). Conversely, both rabbit and

rat antibodies to Rab32 recognized the endogenous protein and did not cross react with Rab38 or any other protein (Figure 2.1). Notice Rab32 has a slightly slower mobility than Rab38, consistent with a 14 residue longer amino acid sequence. Rabbit anti-Rab38 and anti-Rab32 antibodies work very well for immunoprecipitation of the corresponding endogenous protein from MNT-1 cell extracts (Figure 2.1). Immunoprecipitated Rab38 was recognized by both rabbit and rat anti-Rab38 antibodies by immunoblotting and immunoprecipitated Rab32 was recognized by both rabbit and rat anti-Rab32 antibodies. Importantly, anti-Rab38 antibodies did not immunoprecipitate Rab32 and, conversely, anti-Rab32 antibodies did not immunoprecipitate Rab38 (Figure 2.1). As expected, knockdown of Rab38 or Rab32 expression by siRNA treatment of MNT-1 cells resulted in a significant decrease of the corresponding polypeptide, further demonstrating the specificity of the antibodies (Figure 2.2, lanes 6-8).

To test for physical association of Rab38 and Rab32 with BLOC-2, AP-3, and AP-1, MNT-1 cell extracts were subjected to immunoprecipitation under non-denaturing conditions using control IgG, anti-Rab38, or anti-Rab32 antibodies. The washed immunoprecipitates were analyzed by immunoblotting for the presence of BLOC-2, AP-3, and AP-1 using antibodies to the HPS6, μ3, and γ subunits, respectively (Figure 2.3). As another specificity control, the immunoprecipitates were also analyzed for the presence of the endocytic adaptor complex AP-2 using antibodies to its α subunit (Figure 2.3). BLOC-2, AP-3, AP-1, AP-2, and Rab proteins exist as both soluble and membrane-associated pools (17,24,27), therefore parallel experiments were carried out using both cytosolic and solubilized membrane extracts.

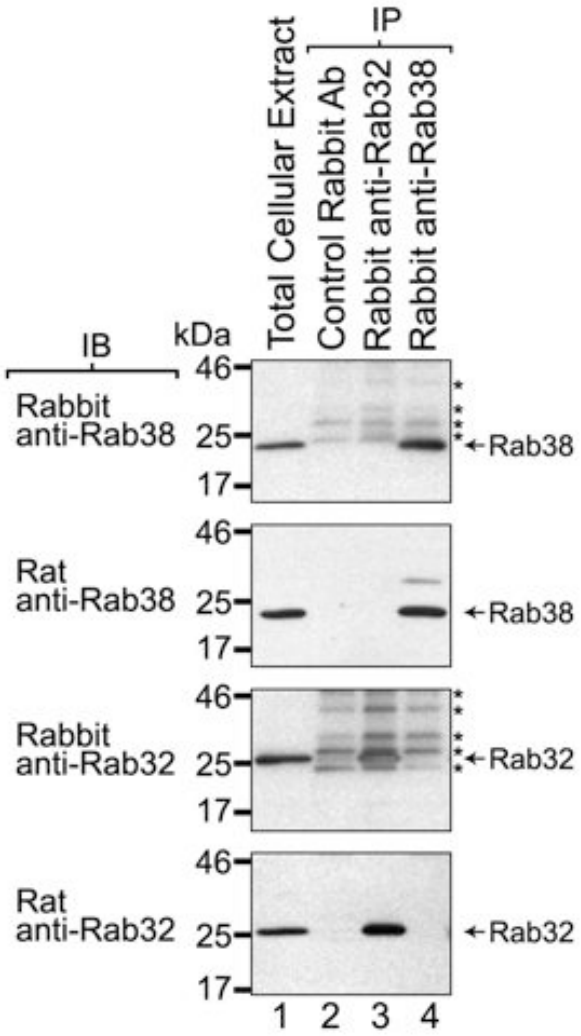

Figure 2. 1

Immunochemical detection of endogenous Rab32 and Rab38. A total extract from MNT-1 cells was divided into aliquots and immunoprecipitated (IP) using irrelevant rabbit IgG (lane 2) or rabbit affinity-purified antibodies against Rab32 (lane 3) or Rab38 (lane 4). Bound proteins, along with a total MNT-1 cellular extract (lane 1), were analyzed by immunoblotting (IB) using rabbit or rat affinity- purified antibodies against Rab38 (top two panels) or Rab32 (bottom two panels). Both rabbit and rat Rab38 and Rab32 antibodies are sufficiently sensitive to detect the corresponding polypeptide in total extracts and recognize only a single band (note that Rab32 mobility is slightly slower than Rab38 due to larger size) (lane 1). Immunoprecipitated Rab32 (lane 3) is detected only in immunoblots for Rab32 and not in Rab38 blots. Similarly, immunoprecipitated Rab38 (lane 4) is detected only in immunoblots for Rab38 and not for Rab32. * indicate bands from the rabbit antibody used in the IP step that is detected by the anti-rabbit secondary antibody in the IB analysis.

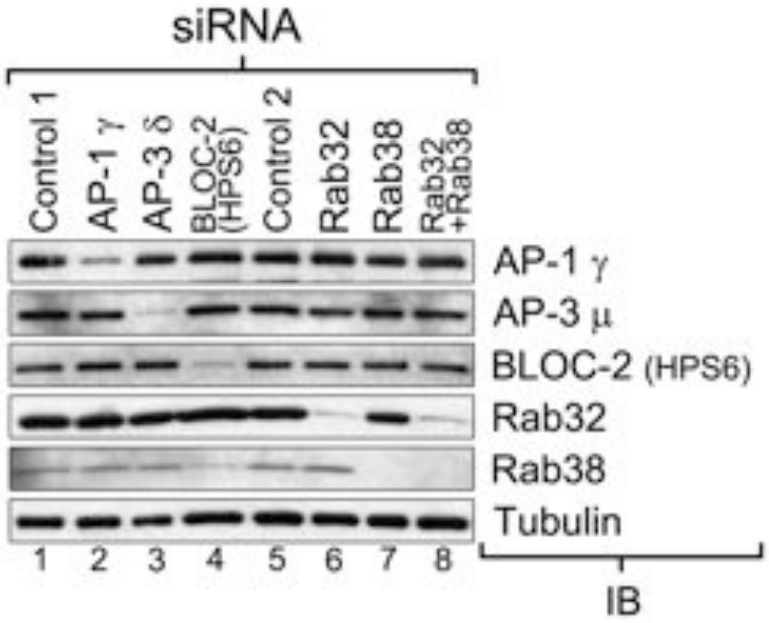

Figure 2. 2
siRNA treatment in MNT-1 cells efficiently and specifically silenced the corresponding targets. The following targets were knocked down in MNT-1 cells by siRNA nucleofection: the γ subunit of AP-1 (lane 2), the δ subunit of AP-3 (lane 3), the HPS6 subunit of BLOC-2 (lane 4), Rab32 (lane 6), Rab38 (lane 7), and both Rab32 and Rab38 (lane 8). Control 1 (lane 1) represents mock nucleofected cells and Control 2 cells nucleofected with an irrelevant siRNA (lane 5). Total extracts were normalized by total protein content and analyzed by immunoblotting (IB) using the indicated antibodies.

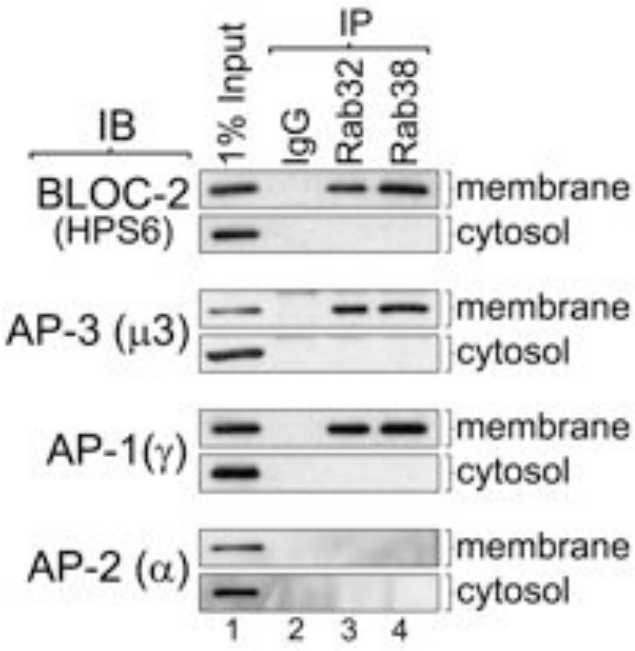

Figure 2. 3
Rab32 and Rab38 coimmunoprecipitate with BLOC-2, AP-3, and AP-1, but not with AP-2. MNT-1 cells were homogenized in the absence of detergents and the homogenate was centrifuged to yield cytosolic and membrane fractions. The membrane fraction was solubilized in buffer containing 1% Triton X-100 and the same concentration of detergent was added to the cytosol to match the buffer compositions (see Materials and Methods). Both cytosolic and solubilized membrane fractions were divided into aliquots and subjected to immunoprecipitation (IP) using irrelevant IgG, anti-Rab32, or anti- Rab38 rabbit antibodies. The immunoprecipitates, together with an aliquot of the input extracts corresponding to 1% of the material available for IP, were analyzed by immunoblotting (IB) using antibodies to the HPS6, μ3, γ, and α subunits of BLOC-2, AP-3, AP-1, and AP-2, respectively.

As shown in Figure 2.3, BLOC-2, AP-3, and AP-1 were detected in the Rab38 and Rab32 immunoprecipitates obtained from solubilized membrane extracts, but not from those obtained from cytosolic extracts. Importantly, AP-2 was not detected in the Rab38 or Rab32 immunoprecipitates obtained from solubilized membranes or cytosolic extracts. These results suggest that endogenous Rab38 and Rab32 interact, directly or indirectly, with BLOC-2, AP-3, and AP-1. The finding that it is the membrane-bound form of the Rabs that participates in the interaction suggests a role of the GTP-bound form of the Rabs. We confirmed the interaction of Rab32 and Rab38 with BLOC-2, AP-3, and AP-1 using pulldown assays. GST-Rab32 and GST-Rab38 were immobilized on glutathione-Sepharose and their bound nucleotide exchanged for GDP or GTPγS, a more stable analog of GTP. Subsequently, each Rab was incubated with MNT-1 cytosolic extract – as a source of free BLOC-2, AP-3, and AP-1 – supplemented with GDP or GTPγS, respectively. As controls, GST and GST-Rab11 were analyzed in parallel following the same procedure. By immunoblotting we observed that BLOC-2, AP-3, and AP-1 bound to GST-Rab32 and GST-Rab38 but not to GST or GST-Rab11 (Figure 2.4). Furthermore, BLOC-2 and AP-3 bound almost exclusively to the GTPγS form of Rab32 and Rab38 compared with the GDP forms (Figure 2.4).

AP-1 also showed a preference for the GTPγS form of Rab32 and Rab38, but less dramatic than BLOC-2 or AP-3 (Figure 2.4). As an additional specificity control we tested for AP-2 which did not bind to either GST-Rab regardless of the nucleotide form (Figure 2.4). These experiments suggest BLOC-2, AP-3 and AP-1 are effectors of Rab32 and Rab38 and that the interactions are likely direct.

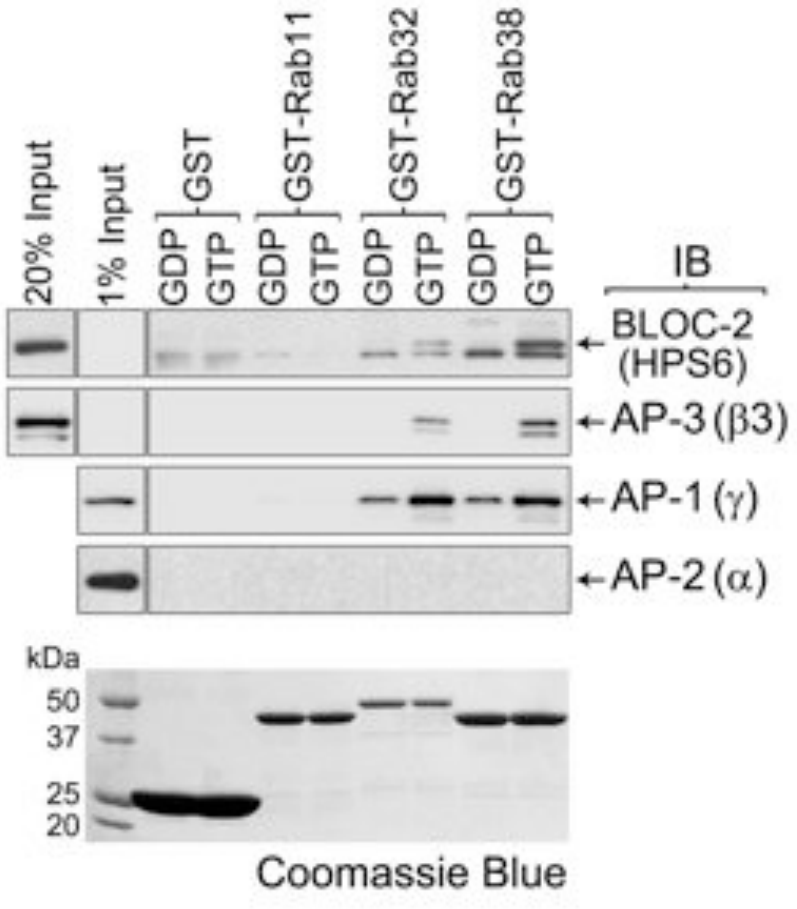

Figure 2. 4
GTP-dependent interaction of Rab32 and Rab38 with BLOC-2, AP-3, and AP-1. GST pulldown using cytosolic extracts from MNT-1 cells and ~ 20 μg each of GST, GST-Rab11, GST-Rab32, or GST-Rab38 immobilized on glutathione-Sepharose and loaded with GDP or the stable GTP analog, GTPγS. The washed glutathione-Sepharose beads, together with an aliquot of the input cytosol corresponding to 1% or 20% of the material available for pulldown, were analyzed by immunoblotting (IB) using antibodies to the HPS6, β3, γ, and α subunits of BLOC-2, AP-3, AP-1, and AP-2, respectively. The bottom panel corresponds to a Coomassie Blue-stained gel and shows the GST fusion proteins.

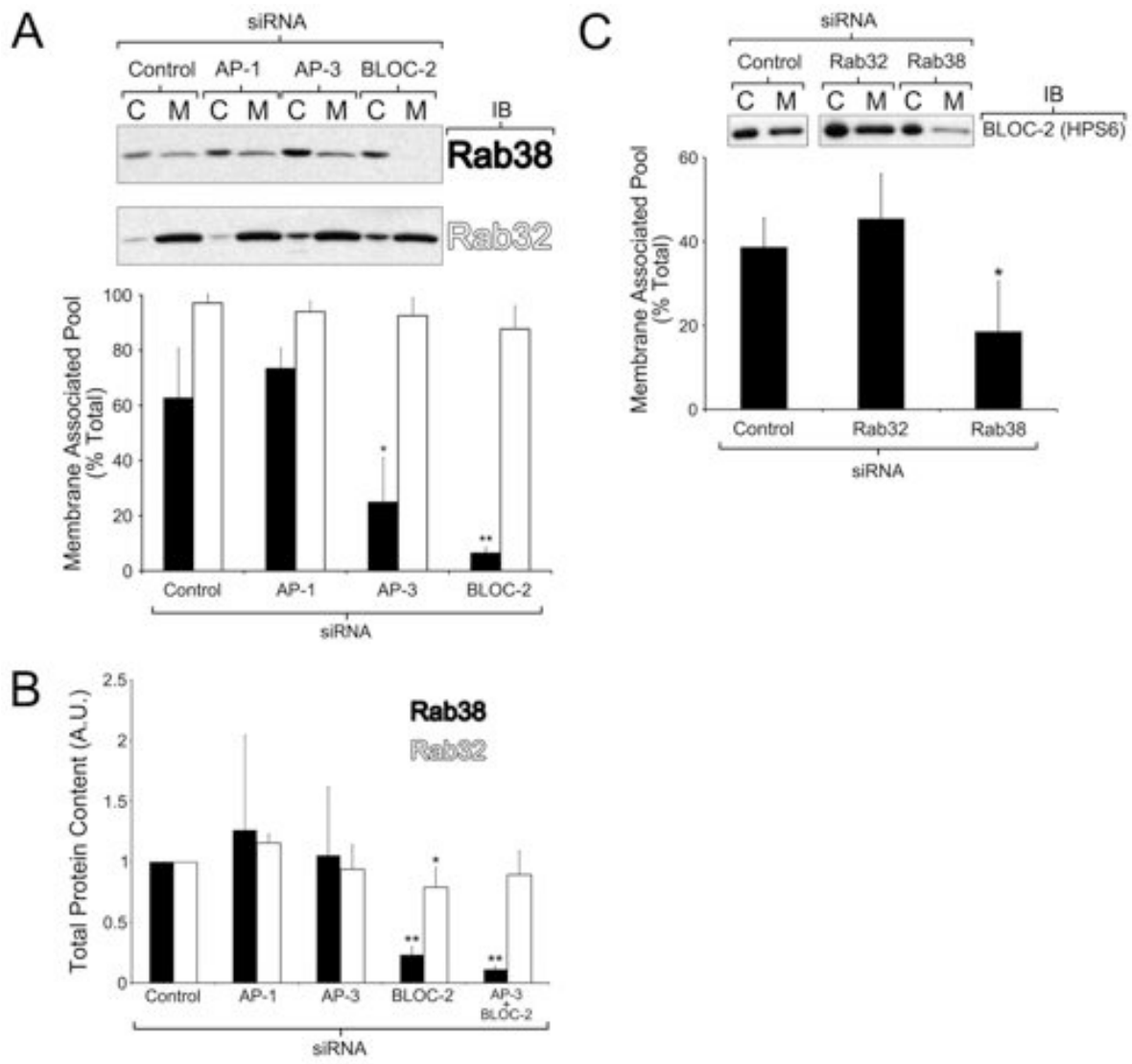

Figure 2. 5

Rab38 association to membranes is altered by AP-3 and BLOC-2 deficiency but only BLOC-2 knock down affects Rab38 stability. A, MNT-1 cells deficient for AP-1, AP-3, or BLOC-2 together with control cells were subjected to a quick homogenization and ultracentrifugation procedure to yield postnuclear membrane (M) and cytosolic (C) fractions (see Materials and Methods). The samples were analyzed by immunoblotting (IB) using antibodies to Rab32 or Rab38. The graph shows the percentage of total Rab38 or Rab32 that was found in the membrane fraction in at least three independent experiments (means ± SD). For each Rab protein, the data corresponding to AP-1, AP-3, or BLOC-2 deficient cells was compared with that of control cells by means of a t test. B, Quantification of the total amount of Rab32 and Rab38 present in extracts of MNT-1 cells treated with the indicated siRNAs relative to control cells. Bars represent means ± SD of at least three independent experiments. One sample t test was used to compare the results obtained from depleted cells with the reference value of 1 set for control cells. C, MNT-1 cells deficient for Rab32 or Rab38 together with control cells were processed as described in (A). The samples were analyzed by immunoblotting using antibodies to BLOC-2. The graph shows the percentage of total BLOC-2 that was found in the membrane fraction in three independent experiments (means ± SD). The data corresponding to Rab32 and Rab38 deficient cells was compared with that of control cells by means of a t test. *$p < 0.05$; **$p < 0.01$.

2.4.2 BLOC-2 and AP-3 regulate the Rab38 association with membranes

In order to perform functional studies we first optimized the conditions to achieve significant knockdown of the targets of interest in MNT-1 cells by siRNA (Figure 2.2). The most efficient gene silencing was obtained using the Nucleofector system for oligonucleotide transfection and subjecting the cells to two sequential siRNA treatments (see *Materials and Methods* for further details) (43). To address the biological significance of the observed physical interactions of BLOC-2, AP-3, and AP-1 with Rab38 and Rab32, we tested whether membrane association of each Rab was affected by deficiencies in their interacting partners. To this end, cytosolic and membrane fractions were obtained from control MNT-1 cells or cells deficient for BLOC-2, AP-3, or AP-1, and the relative amounts of endogenous Rab38 or Rab32 in the membrane fraction were determined by quantitative immunoblotting (17). About 60% of Rab38 and 95% of Rab32 were found in the membrane fractions of control MNT-1 cells, indicating a stronger Rab32-membrane association at steady state (Figure 2.5A). Interestingly, the relative amounts of Rab38 recovered from membranes were reduced to about 25% in AP-3-deficient cells and to less than 10% in BLOC-2- deficient cells (Figure 2.5A). Deficiency of AP-1, however, did not have a statistically significant effect in the fraction of Rab38 recovered from membranes. The relative amount of Rab32 recovered from membranes was somewhat decreased by deficiency in AP-3 or BLOC-2 compared to control cells, but the difference did not reach statistical significance (Figure 2.5A). Deficiency of AP-1 had no effect on the Rab32 membrane-associated pool. In several replicates of these experiments, the overall amount of Rab38 was noticeably reduced in BLOC-2 deficient cells. Hence, quantitative immunoblotting analysis of the total amount of Rab38 was carried out in extracts from control MNT-1 cells or cells deficient for AP-1, AP-3, BLOC-2 or double deficient for AP-3 and BLOC-2.

The total amount of Rab38 was significantly reduced in cells deficient for BLOC-2 or both BLOC-2 and AP-3, but not in AP-1 or AP-3 deficient cells (Figure 2.5B). A similar analysis for Rab32 showed only a marginal decrease in BLOC-2 deficient cells (Figure 2.5B). Together, the data suggest that both AP-3 and BLOC-2 regulate or stabilize the Rab38 association with membranes and that BLOC-2 also regulates overall stability of Rab38. The stronger link between Rab38 and BLOC-2 prompted us to examine the converse relationship, i.e. if the association of BLOC-2 with membranes was affected by deficiency of Rab38. Indeed, the relative amounts of BLOC-2 recovered from membrane fractions decreased from about 40% in control cells to 20% in Rab38 deficient cells (Figure 2.5C). In contrast, deficiency of Rab32 did not have any statistically significant effect on the relative amounts of BLOC-2 recovered from membrane fractions (Figure 2.5C). Overall the data suggests a stronger connection between Rab38 and the ubiquitous components of trafficking machinery (AP-3, BLOC-2), compared to Rab32.

2.4.3 BLOC-2, AP-3, and AP-1 partially colocalize with Rab38 and Rab32

It is well established that the bulk of both AP-3 and AP-1 localize to clathrin coated buds on early endosome-associated tubules in MNT-1 and other melanocytic cells (15,17,20). This has been shown by immunoelectron microscopy and immunofluorescence microscopy with antibodies to the endogenous complexes (15,17,20). Endogenous BLOC-2 was also localized to MNT-1 early endosome-associated tubules by immunoelectron microscopy (17). Given the observed physical association and effect on recruitment to membranes (Figs. 2.3-2.5), we sought to determine whether AP-3, AP-1, and BLOC-2 colocalize with Rab38 and Rab32 in MNT-1 cells. Confocal immunofluorescence microcopy was carried out using the same antibodies to

AP-3, AP-1, or BLOC-2 as in previous reports (15,17,20), and our new antibodies to Rab38 and Rab32 (Figure 2.1 and 2.2). Analysis of untreated cell stainings revealed that Rab38 has more cytosolic distribution compared to Rab32 and that Rab32 stains more structures which is consistent with the biochemical evidence of membrane associated fractions for these Rabs (Figure 2.5A, 2.6, and 2.7). An examination of subcellular distribution of endogenous Rab38 and Rab32 showed that both proteins are mainly found in small punctae in perinuclear and peripheral regions of MNT-1 cells (Figure 2.6 and 2.7). Specificity of the staining with Rab38 and Rab32 antibodies was demonstrated by the drastic signal reduction observed upon siRNA knockdown of the corresponding Rab protein (Figure 2.8). A significant degree of colocalization was observed between the adaptor proteins AP-3 (48 ± 2 %) or AP-1 (57 ± 2 %), and Rab38 in several independent experiments analyzing at least 45 cells (see *Materials and Methods* for further details) (Figure 2.6). There is no discernible difference between AP-3 and AP-1 structures labeled by Rab38 and those without Rab38 and both types of structures are seen throughout the cell. AP-3 and AP-1 also partially colocalized with Rab32 albeit to a lower extent than with Rab38 (35 ± 1 % and 37 ± 1 % respectively) (Figure 2.6 and 2.7), which suggests that the adaptor proteins may function more closely associated with Rab38 than with Rab32. Interestingly, a significant amount of colocalization was observed between clathrin and Rab38 or Rab32 (Figure 2.6 and 2.7). Paralleling the relative levels of colocalization with the adaptors, a higher degree of colocalization was observed between clathrin and Rab38 (49 ± 3 %) than between clathrin and Rab32 (34 ± 1 %).

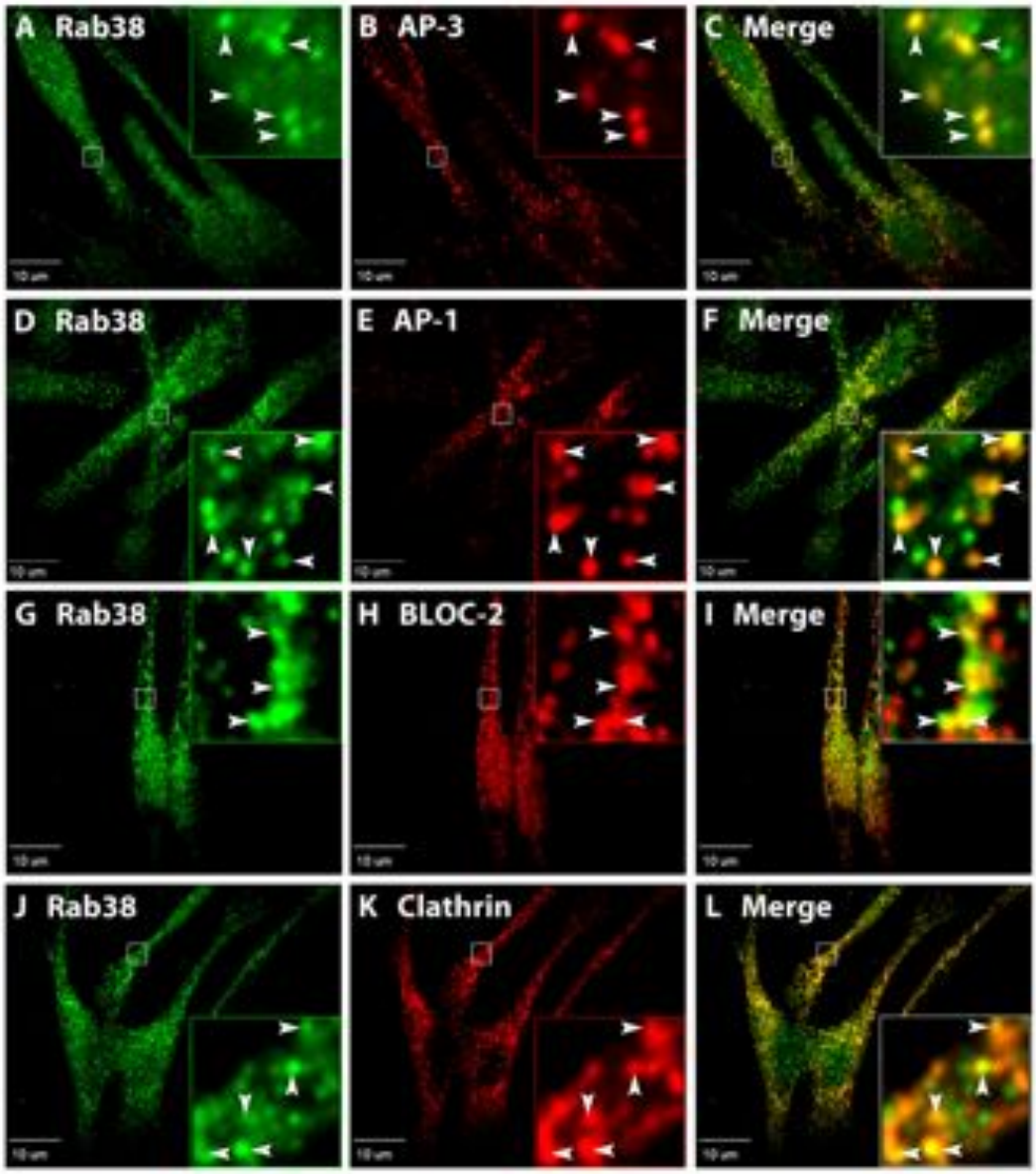

Figure 2. 6
Rab38 partially colocalizes to proteins required in the trafficking from specialized early endosomal domains to melanosomes. MNT-1 cells were fixed/permeabilized and costained with antibodies against Rab38 (A, D, G, J) and the δ subunit of AP-3 (B), the γ subunit of AP-1 (E), the HPS6 subunit of BLOC-2 (H), or the clathrin heavy chain (K). Cells were imaged by confocal fluorescence microscopy and Rab38 was found both on small structures and in diffuse staining distributed throughout the cells. A significant number of AP-3 (B), AP-1 (E), BLOC-2 (H), and Clathrin (K) labeled structures show colocalization with Rab38 in the merged images (C, F, I, and L). Boxed areas are shown in the magnified insets where arrowheads indicate sites of colocalization. Scale bars indicate 10 μm.

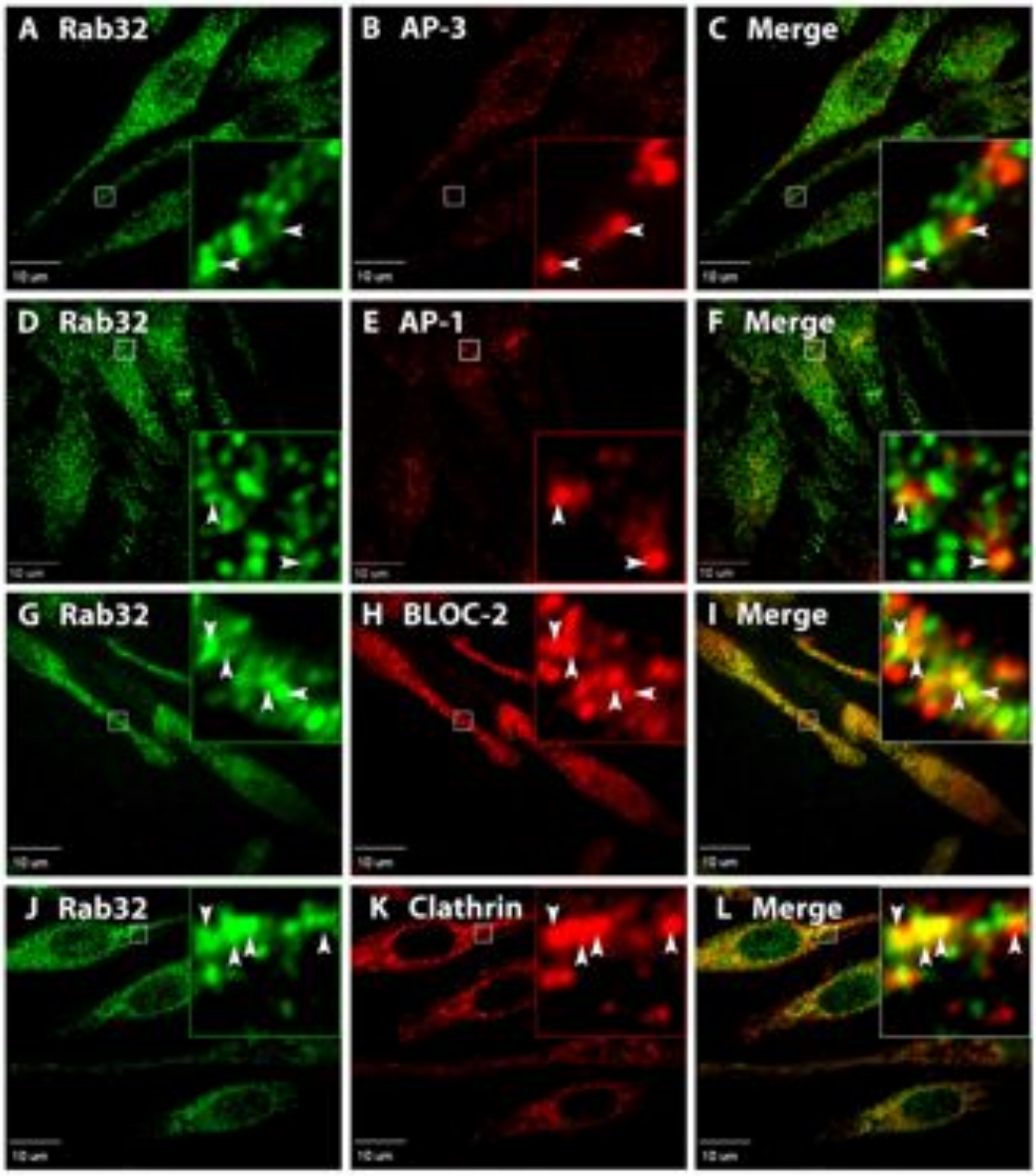

Figure 2. 7
Rab32 partially colocalizes to proteins required in the trafficking from early endosomes to melanosomes. MNT-1 cells were fixed/permeabilized and costained with antibodies against Rab32 (A, D, G, J) and the δ subunit of AP-3 (B), the γ subunit of AP-1 (E), the HPS6 subunit of BLOC-2 (H), or the clathrin heavy chain (K). Cells were imaged by confocal fluorescence microscopy and Rab32 was mainly found on small structures distributed throughout the cells. Numerous AP-3 (B), AP-1 (E), BLOC-2 (H), and Clathrin (K) labeled structures show colocalization with Rab32 in the merged images (C, F, I, and L), although to a lesser extent than with Rab38. Boxed areas are shown in the magnified insets where arrowheads indicate sites of colocalization. Scale bars indicate 10 μm.

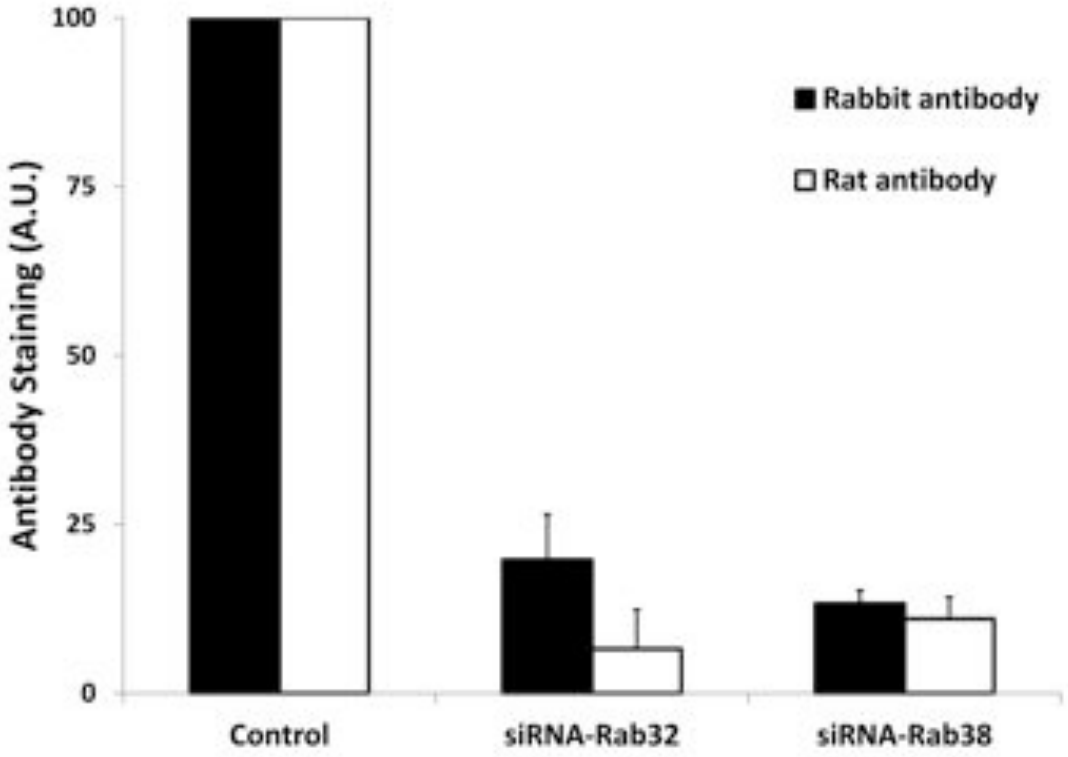

Figure 2. 8
Validation of antibodies against Rab32 and Rab38 for immunofluorescence microscopy. MNT-1 cells were subjected to Rab32 or Rab38 siRNA treatment, fixed/permeabilized, and stained with affinity-purified antibodies against Rab32 or Rab38 generated in both rat and rabbit. Cells were imaged by confocal fluorescence microscopy and the Slidebook software was used to generate automated masks covering numerous individual cells and total fluorescence intensity was quantified and represented relative to control stainings.

Together the data indicates Rab38 and Rab32 are likely recruited to early endosomes during the vesicle budding process before the clathrin coat has disassembled. The relative levels of colocalization of AP-3, AP-1, and clathrin with each Rab showed a consistent difference, perhaps indicating Rab38 is recruited earlier than Rab32 during coat formation. In addition, BLOC-2 labeled structures also showed partial colocalization with Rab38 or Rab32 but to a similar extent (Figure 2.6 and 2.7). Although the molecular function of BLOC-2 is not clear, it has been suggested to operate both at early endosome associated tubules and also in downstream transport intermediates (14,17,18). It is possible that the Rabs are recruited to nascent vesicles, where they interact with AP-3, AP-1, or BLOC-2, and then remain associated with the vesicles along with BLOC-2.

The above data is compatible with a role of Rab38 and Rab32 in a pathway from specialized early endosome associated tubules – defined by AP-3, AP-1, and BLOC-2 – to maturing melanosomes (Fig. 2.9). As a control for colocalization between the Rabs and other early endosomal domains, we tested for colocalization with EEA1 and the retromer complex subunit SNX1 which label the vacuolar domain of early endosomes and a retrieval pathway to the *trans*-Golgi network, respectively (Figure 2.10). Rab38 showed a very low level of colocalization with EEA1 (4 ± 1 %) and SNX1 (4 ± 1 %) (Figure 2.10) that was comparable with the colocalization of Rab38 and peroxisomal markers used as negative controls and quantified with the same image analysis procedure (see *Materials and Methods* for further details). Likewise, Rab32 displayed very low level of colocalization with EEA1 (4 ± 1 %) and SNX1 (5 ± 1 %) (Figure 2.10).

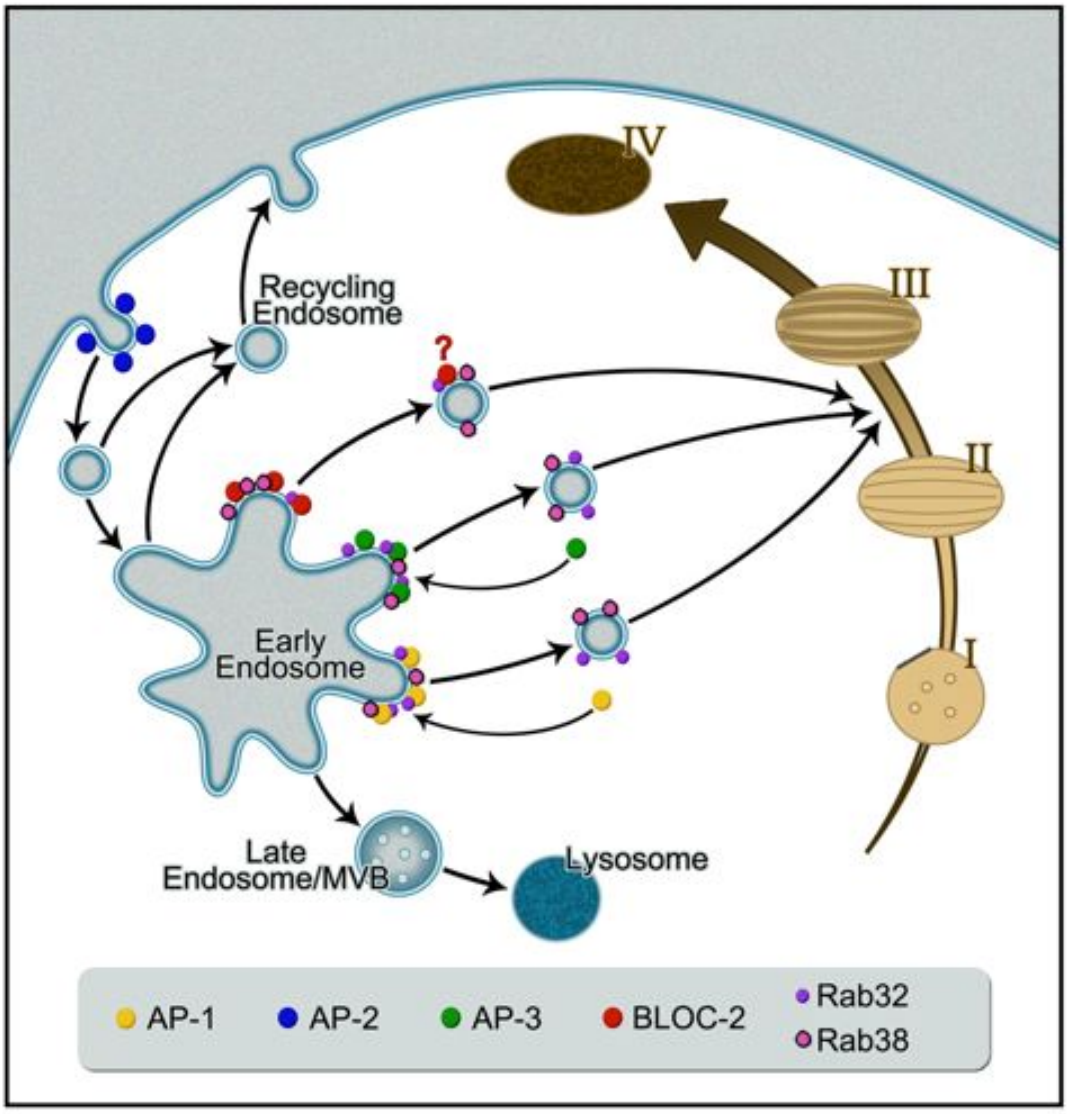

Figure 2. 9
Model for AP-3, AP-1, and BLOC-2 cooperation with Rab38 and Rab32 to mediate transport to maturing melanosomes. Rab38 and Rab32 interact with AP-3, AP-1, and BLOC-2 at early endosome membrane domains where cargo such as the tyrosinase family proteins are concentrated and packaged into transport intermediates. Upon budding, some components of the coat (AP-3, AP-1, clathrin) dissociate from the vesicle but others remain bound (Rab32, Rab38, and possibly BLOC-2) to mediate further transport, tethering, and fusion with maturing melanosomes. During melanosome biogenesis, transition between stage II and stage III occurs upon incorporation of the melanogenic enzymes with the concomitant beginning of melanin synthesis, thus vesicles defined by Rab32 and Rab38 likely target this melanosome maturation stage. Deficiency in different components of these pathways elicits cargo accumulation in the early endosomes that eventually leaks into other pathways such as the recycling pathway towards the plasma membrane or the late endosome/multi vesicular body (MVB) degradative pathway.

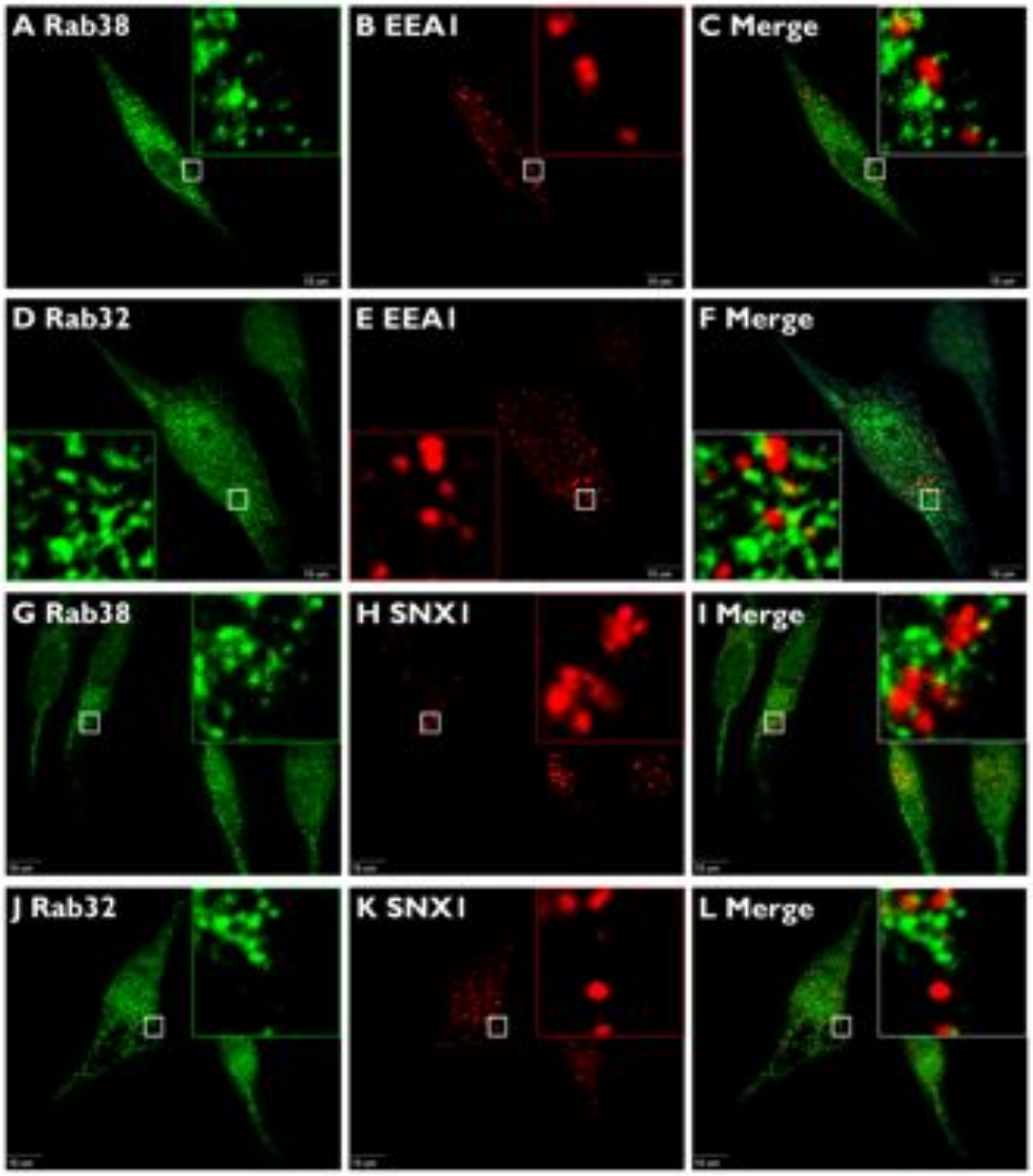

Figure 2. 10
Rab38 and Rab32 do not colocalize to proteins that label early endosome vacuolar domains or the retrieval pathway to the trans-Golgi network. MNT-1 cells were fixed/permeabilized and costained with antibodies against Rab38 (A, G) or Rab32 (D, J) and Early Endosome Antigen 1, EEA1 (B, E), or the retromer subunit Sorting Nexin 1, SNX1 (H, K). Cells were imaged by confocal fluorescence microscopy and only background levels of colocalization were detected in the merged images (C, F, I, and L). Boxed areas are shown in the magnified insets. Scale bars indicate 10 μm.

We also tested for colocalization between each of Rab38 and Rab32 with melanosome and lysosome markers. We used Tyrp-1 as a well-established melanosomal marker that at steady state primarily labels stage III and IV melanosomes and internalized dextran chased for 4 hours to label mature lysosomes (Figure 2.11). Both Rab38 and Rab32 showed partial colocalization with Tyrp-1 (36 ± 7 % and 36 ± 6 %, respectively) and negligible colocalization with internalized dextran (1 ± 2 % and 2 ± 2 %) (Figure 2.11).

2.4.4 Melanocytes deficient for Rab38 and Rab32 display abnormal cargo trafficking and steady state levels

In cells deficient for the ubiquitous components of the trafficking machinery, such as AP-3, AP-1 and BLOC-2, newly synthesized integral membrane protein cargo are not properly sorted and accumulate in early endosomes. This has been shown both for lysosomal cargo such as LAMP-1 in non-specialized cells and for melanosomal cargo such as tyrosinase and Tyrp-1 in melanocytes (3,11,13-15,17-20,40,44). Cargo accumulated in early endosomes can subsequently leak into the recycling pathway to the plasma membrane or enter into the ESCRT-dependent multivesicular body (MVB) pathway for degradation (Figure 2.9) (3,11,13-15,17-20,40,44). Therefore, as a consequence of disrupting the normal early endosome to melanosome transport, cargo proteins exhibit increased traffic via the plasma membrane and/or are subjected to degradation (3,11,13-15,17-20,40,44). Melanocytes isolated from AP-3 or BLOC-2 deficient mice showed both phenotypes (recycling through the plasma membrane and degradation) when endogenous Tyrp-1 was analyzed (17). Melanocytes from AP-3/BLOC-2 double mutant mice showed a more severe defect than each single mutant (17).

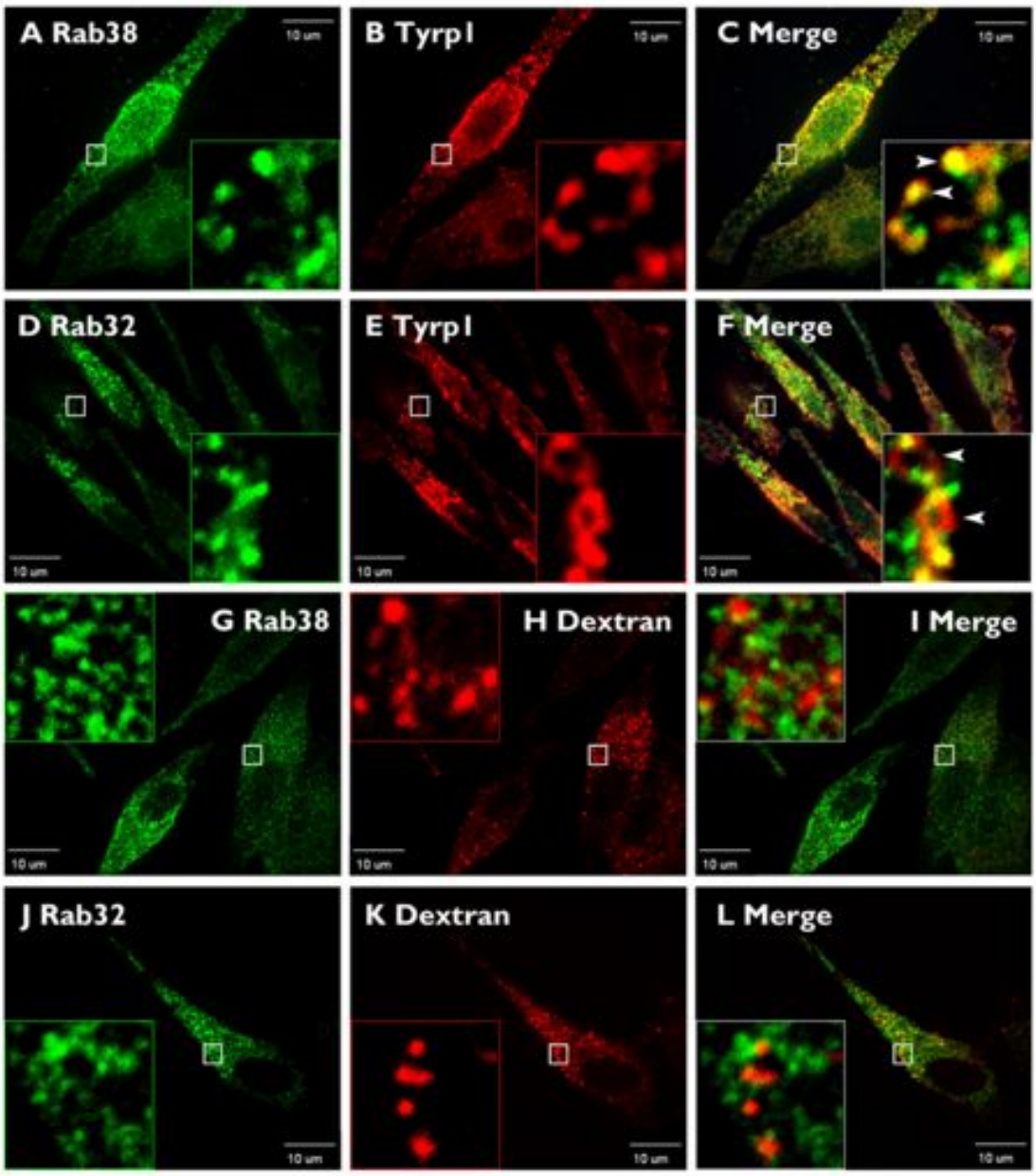

Figure 2. 11

Rab38 and Rab32 partially colocalize to melanosomes but not to lysosomes. MNT-1 cells were fixed/permeabilized and costained with antibodies against Rab38 (A) or Rab32 (D) and the melanosomal protein Tyrp-1 (B, E). Alternatively, cells were allowed to internalize dextran-Alexa-647, followed by a chase period of 4 hours to ensure specific labeling of mature lysosomes (H, K), fixed/permeabilized and stained with antibodies against Rab38 (G) or Rab32 (J). Cells were imaged by confocal fluorescence microscopy and both Rab38 and Rab32 were found to partially colocalize with the melanosome marker Tyrp-1 (C, F) but not with lysosomes (I, L) in the merged images. Boxed areas are shown in the magnified insets. Scale bars indicate 10 μm.

Here, we sought to determine if Rab38 and Rab32 deficiency also elicits such phenotypes. Live MNT-1 cells subjected to siRNA treatment targeting each Rab alone or both simultaneously were incubated with antibodies to the luminal domain of Tyrp-1 for 20 minutes and then fixed/permeabilized and processed for fluorescence microscopy. The total fluorescence intensity signal per cell (internalized antibody) was determined for numerous cells per treatment (48 to 231 cells per treatment), averaged, and normalized to that of control cells (Figure 2.12). Deficiency of either Rab38 or Rab32 produces a statistically significant but modest recycling phenotype.

Simultaneous deficiency of both Rabs elicits a more pronounced recycling phenotype than either Rab alone. Thus, these results suggest Rab38 and Rab32 are involved in Tyrp-1 transport from early endosomes to maturing melanosomes. In order to compare the relative severity of the Tyrp-1 recycling phenotype, similar experiments were carried out in parallel with cells deficient for AP-3 or AP-1 (Figure 2.12). The level of Tyrp-1 recycling in Rab deficient cells was comparable to that observed in AP-1 deficient cells but less severe than in AP-3 deficient cells assayed under the same conditions.

A possible caveat with the antibody internalization assay is that the observed surface expression of Tyrp-1 could be secondary to increases in total Tyrp-1 expression. This possibility was addressed by quantitative Tyrp-1 immunoblotting analysis of crude extracts from MNT-1 cells subjected to the corresponding siRNA treatment (Figure 2.13A). Single Rab38 or Rab32 deficiency caused a marginal decrease in the total levels of Tyrp-1, compared to control cells. Deficiency in both Rabs caused a more marked decrease in overall Tyrp-1 levels (Figure 2.13A). Therefore, the observed increase in Tyrp-1 recycling in Rab deficient MNT-1 cells is not due to

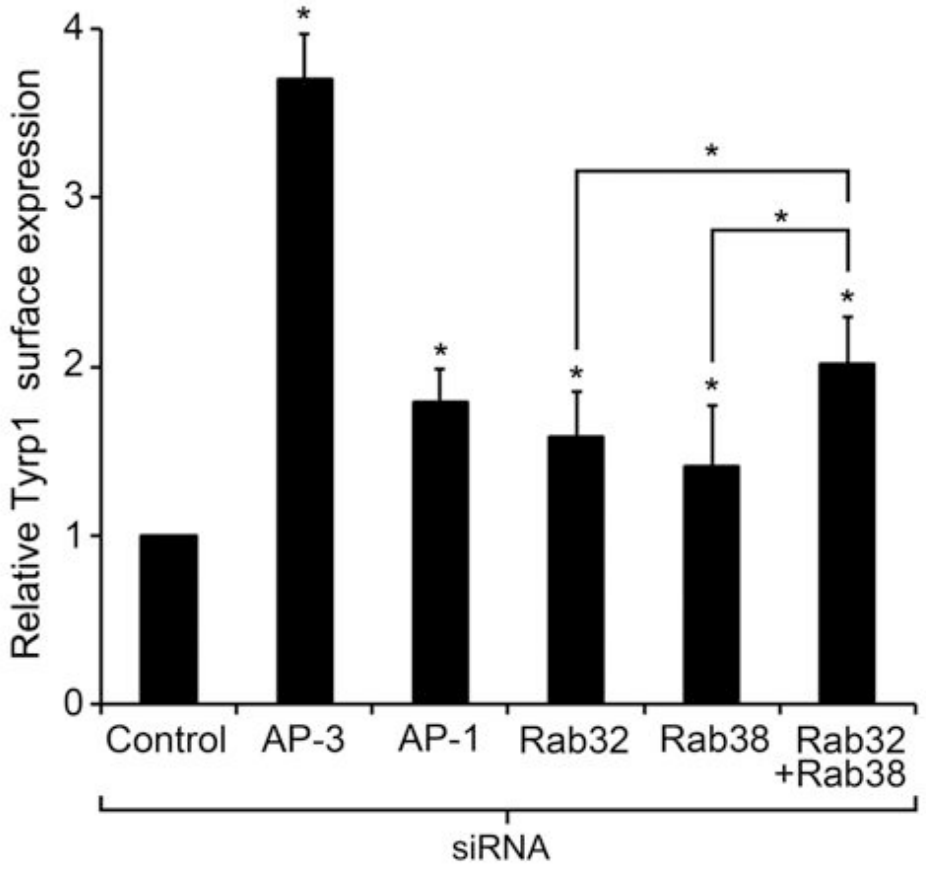

Figure 2. 12
Knockdown of Rab32 or Rab38 cause mistrafficking of Tyrp-1. Live MNT-1 control cells or cells deficient for AP-3, AP-1, Rab32, Rab38, or both Rab32 and Rab38 were incubated in media containing a mouse anti-Tyrp-1 antibody for 20 minutes at 37°C and subsequently fixed, permeabilized, and immunostained with an Alexa-488 conjugated anti-mouse IgG. Cells were imaged using an epifluorescent microscope and the relative amounts of internalized anti-Tyrp-1 antibody was estimated as the average fluorescence intensity per cell determined with ImageJ and normalized to control cells (means ± SD). Result from at least three independent experiments, $N > 48$ for each treatment, were compared with control cells (or between the Rab32/Rab38 double knockdown and the corresponding single knockdowns) by means of a t test, * $p<0.05$.

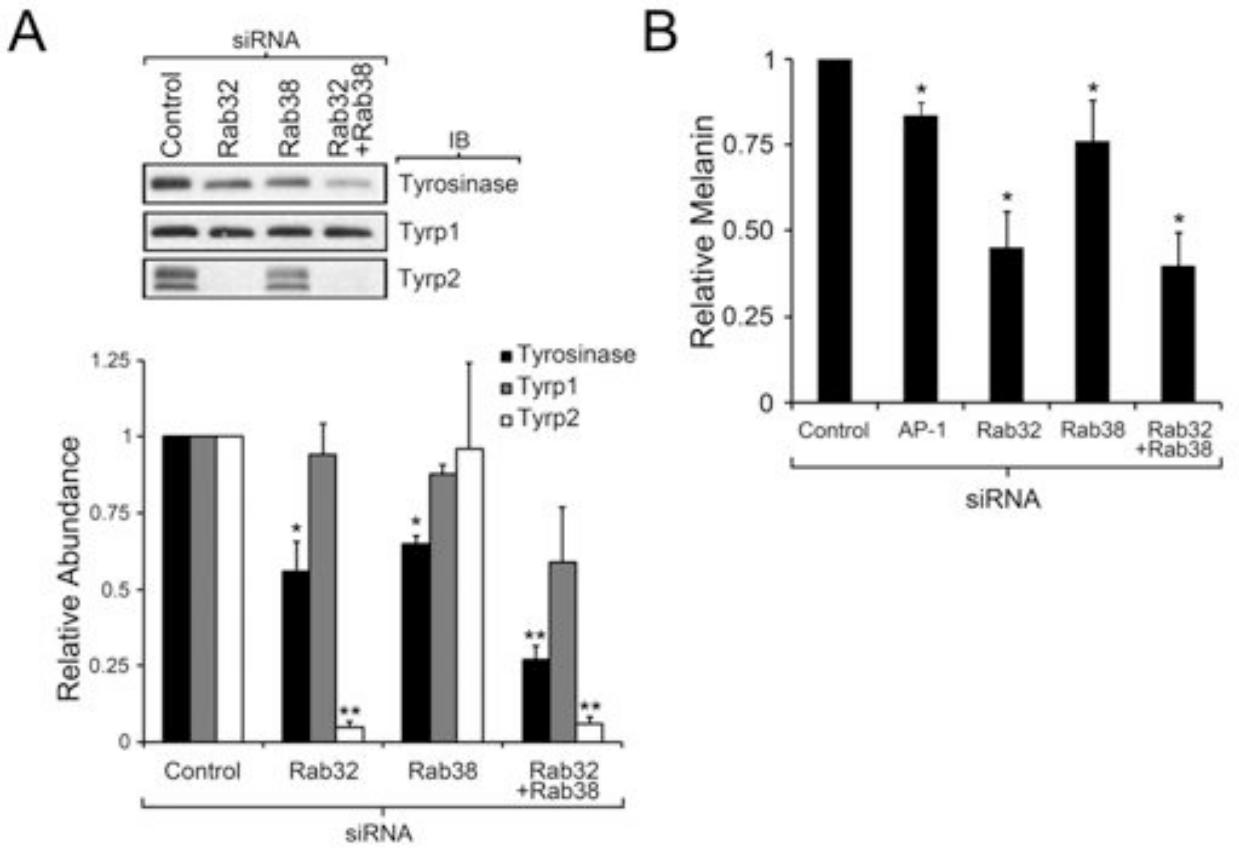

Figure 2. 13

Rab32 and Rab38 are required for normal steady state levels of tyrosinase, Tyrp-1, and Tyrp-2 and are not fully redundant in overall melanosome biogenesis. A, Immunoblotting analysis of total cell extracts from control MNT-1 cells and cells deficient for Rab32, Rab38, or both Rab32 and Rab38 was performed and quantified to determine the total abundance of tyrosinase (black), Tyrp-1 (grey), and Tyrp-2 (white) relative to control cells. Results correspond to three independent experiments normalized to number of cells, and compared using the t test, * $p<0.05$, ** $p<0.01$. B, Melanin was extracted from MNT-1 control cells or cells deficient for AP-1, Rab32, Rab38, or both Rab32 and Rab38 and quantified by a spectrophotometric method. Results correspond to at least three independent experiments normalized to number of cells and abundance of melanin in control cells, * $p<0.05$.

an overall increase in Tyrp-1 expression. Moreover, a lower steady state level of Tyrp-1 in Rab deficient cells would be consistent with a scenario in which the melanogenic enzyme cannot properly traffic from early endosomes to maturing melanosomes, thus accumulating in early endosomes and leaking into the degradative MVB pathway (Figure 2.9). Following the same approach, extracts from MNT-1 cells deficient for each Rab or both simultaneously were analyzed for the total amounts of tyrosinase and Tyrp-2, the other two enzymes responsible for melanin synthesis in melanosomes. Tyrosinase was somewhat reduced in Rab38 or Rab32 deficient cells and more so in the double deficient cells, compared to control cells (Figure 2.13A). Tyrosinase and Tyrp-1 followed a similar trend although the defect was more marked for tyrosinase.

Notably, Tyrp-2 showed a very strong reduction in Rab32 deficient cells but normal levels in Rab38 deficient cells (Figure 2.13A). The steady state levels of tyrosinase, Tyrp-1, and Tyrp-2 in single and double Rab-deficient cells were partially rescued by incubation with the lysosomal protease inhibitor leupeptin in agreement with mistrafficking to the degradative pathway in the absence of Rab32/Rab38 (Fig 2.14). Taken together, these results are consistent with a model in which the Rabs function in the traffic of all three tyrosinase family members from early endosomes to maturing melanosomes. In addition, these results underscore similarities and differences between the Rabs in melanosome biogenesis. On the one hand, both Rabs appear to be involved in the transport of tyrosinase and Tyrp-1 in an epistatic fashion such that deficiency in one Rab is not severe because the other Rab can still carry out transport, but deficiency in both Rabs results in a more important phenotype. On the other hand, Rab32 is strictly necessary to maintain normal Tyrp-2 steady state levels, suggesting Rab32 has unique functions in Tyrp-2 transport to the maturing melanosome.

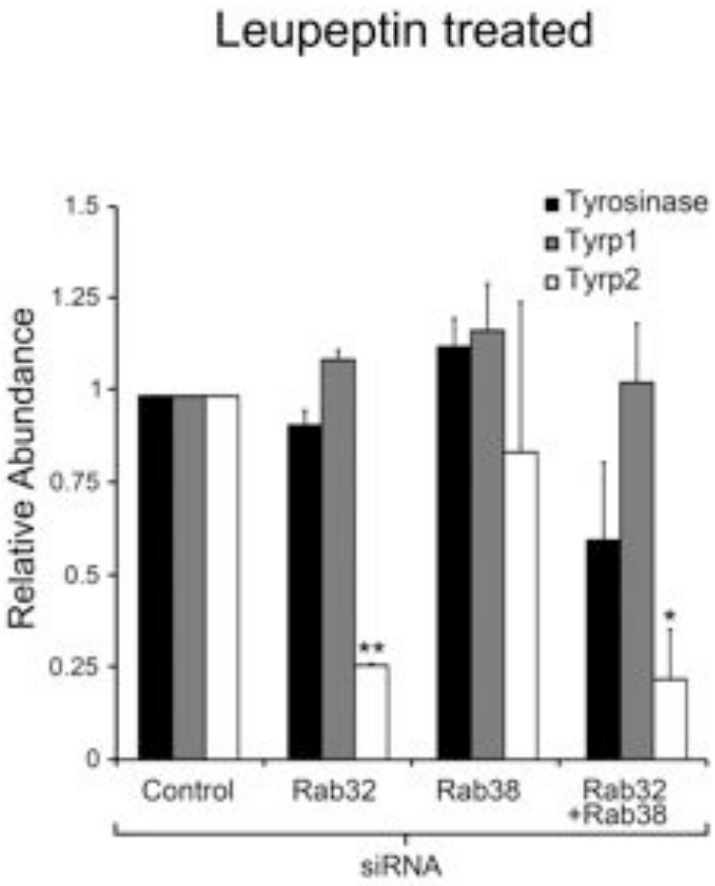

Figure 2. 14
Partial rescue of tyrosinase, Tyrp-1, and Tyrp-2 steady state levels by leupeptin treatment on Rab32- and Rab38-deficient cells. Control MNT-1 cells and cells deficient for Rab32, Rab38, or both Rab32 and Rab38 were incubated with leupeptin for 6 hours, total cell extracts were prepared, and the total abundance of tyrosinase (black), Tyrp-1 (grey), and Tyrp-2 (white) analyzed by immunoblotting. Results correspond to three independent experiments normalized to number of cells, represented relative to control cells, and compared using the t test, *$p<0.05$, ** $p<0.01$. Notice the higher abundance of tyrosinase, Tyrp-1, and Tyrp-2 (rescue) compared to knockdown cells not treated with leupeptin (Figure 2.13).

2.4.5 Rab32 and Rab38 functions in melanosome biogenesis are partially redundant

Another unresolved matter concerns the relative overall relevance of each Rab for melanosome biogenesis (27,33). Are they able to functionally compensate for each other? The fact that the Rab38 mutant mice, *chocolate*, is hypopigmented indicates that Rab32 cannot fully compensate for Rab38 deficiency (25). Is the converse also true? Analysis of the cargo enzymes suggests partial functional compensation between the Rabs for tyrosinase and Tyrp-1 traffic to maturing melanosomes but Rab32 appears to be strictly necessary for normal Tyrp-2 traffic (Figs. 2.12 and 2.13).

Analysis of particular cargo proteins gives important clues but also has its limitations and we do not yet know the identity or roles of all the cargo proteins involved in melanosome formation (45). Therefore, overall melanin production is probably the most faithful and quantitative measure of proper melanosome biogenesis and an adequate way to determine to what extent a particular component is needed (9,17,20,46). By visual inspection, we consistently noticed that Rab32 deficient MNT-1 cells were significantly hypopigmented, even more so than cells deficient for Rab38. This was particularly apparent after collecting the cells by centrifugation. To test whether the observed differences were significant, total melanin was extracted and quantified from MNT-1 control cells or cells deficient for Rab38, Rab32, or simultaneously both Rab38 and Rab32. Several independent replicates were carried out and AP-1 deficient cells were also analyzed for comparison. Consistent with the mild hypopigmentation of *chocolate* mice, Rab38 deficiency caused a moderate loss of melanin in MNT-1 cells (Figure 2.13B). Importantly, Rab32 deficient MNT-1 cells contained less melanin than control cells (and Rab38 or AP-1 deficient cells) (Figure 2.13B). This result suggests Rab32 is a critical component for mature melanosome biogenesis and that Rab38 cannot fully compensate for

Rab32 deficiency. Consistent with a significant role for Ra32 in overall melanosome biogenesis, Rab32/Rab38 double deficiency did not significantly worsen the phenotype elicited by Rab32 single deficiency (Figure 2.13B). One trivial explanation for these results is that MNT-1 cells may express substantially more Rab32 than Rab38 but we found comparable amounts of both Rabs by quantitative immunoblotting of total cell extracts. In fact, Rab38 is expressed at slightly higher rather than lower levels compared to Rab32 (130 versus 89 pg of Rab per μg of total proteins) (Figure 2.15). Additionally, no compensatory up-regulation of Rab32 was observed upon siRNA knockdown of Rab38 or vice versa (Figure 2.2). Taken together, these results imply that the Rabs cannot fully functionally compensate for each other during biogenesis of mature melanosomes.

2.4.6 Melanocytes deficient for BLOC-3 but not BLOC-1 display abnormal Tyrp-2 steady state levels

To test the possibility that Tyrp-2 transport depends on BLOC-1 or BLOC-3, we carried out quantitative Tyrp-2 immunoblotting analysis of crude extracts from MNT-1 cells subjected to the corresponding siRNA treatment (Figure 2.16). MNT-1 cells deficient for BLOC-3 displayed a statistically significant decrease in Tyrp-2 steady state levels, while BLOC-1 deficiency had no effect (Figure 2.16).

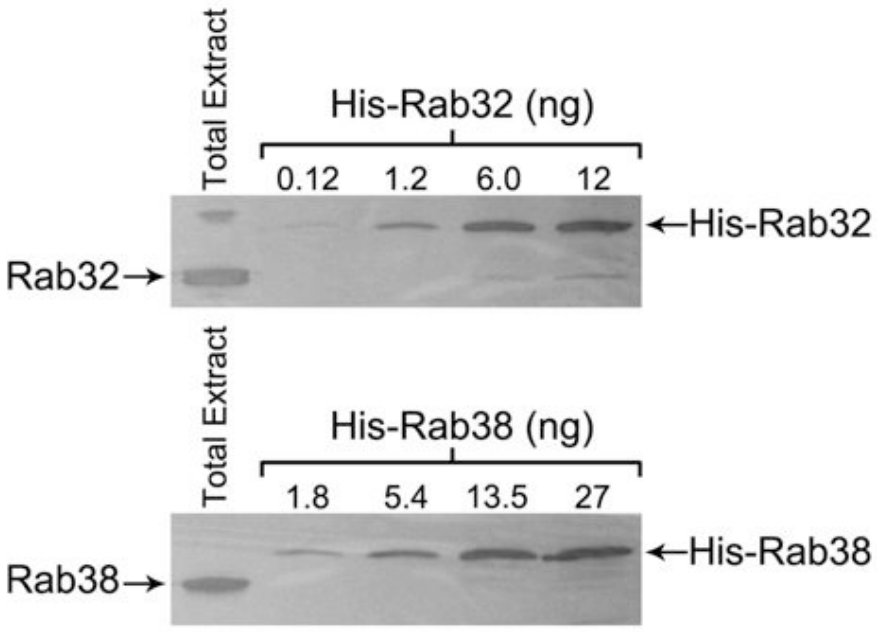

Figure 2. 15
MNT-1 cells express similar amounts of Rab32 and Rab38. MNT-1 total extracts (77 µg) were analyzed by immunoblotting using the corresponding rabbit anti-Rab32 or anti-Rab38 antibodies together with His-Rab32 or His-Rab38 samples containing the indicated mass of protein. The intensity of the bands was determined by chemifluorescent detection and the amount of Rab32 and Rab38 in MNT-1 total extract was calculated by comparison with the His-Rab32 and His-Rab38 calibration curves, respectively.

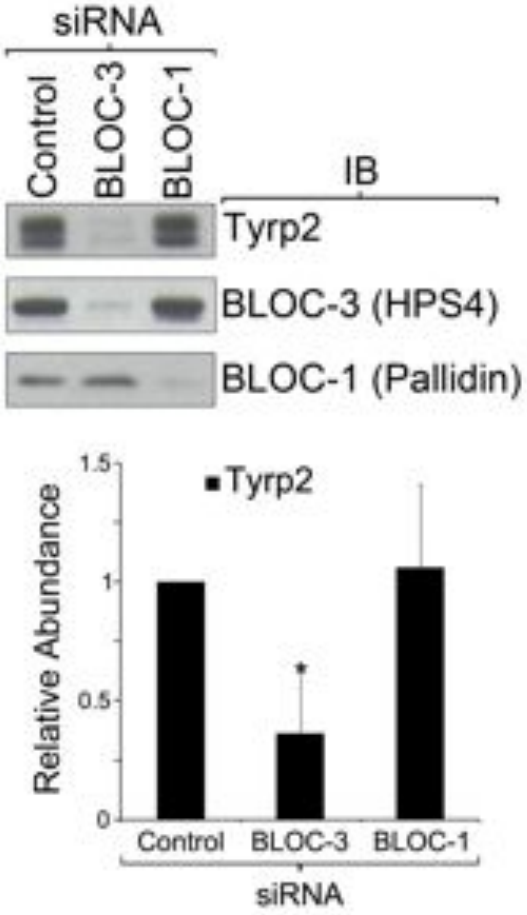

Figure 2. 16
Cells deficient for BLOC-3 display abnormal Tyrp-2 steady state levels. Immunoblotting analysis of total cell extracts from control MNT-1 cells and cells deficient for BLOC-3, or BLOC-1 was performed and quantified to determine the total abundance of Tyrp-2 relative to control cells. Results correspond to three independent experiments normalized to number of cells, and compared using the t test, * $p<0.05$.

2.5 Discussion

The finding that cell type specific Rab38 and Rab32 are involved in the biogenesis of LROs raised the possibility that these proteins could operate together with the ubiquitous trafficking machinery for transport towards LROs in specialized cell types (24-31). However, no evidence has been reported for a link between the BLOC or AP complexes involved in LRO biogenesis and Rab38 or Rab32. In this study, we demonstrate that AP-3, AP-1, and BLOC-2 interact physically and functionally with Rab38 and Rab32 to mediate transport of integral membrane proteins from specialized early endosomal domains to maturing melanosomes.

We show that endogenous Rab38 and Rab32 can associate with AP-3, AP-1, and BLOC-2 into macromolecular assemblies that are stable enough to allow detection by coimmunoprecipitation (Figure 2.3). Our results show the interactions between the Rabs and AP-3, AP-1 and BLOC-2 occur on membranes but not in the cytosol, suggesting a role of the GTP-bound form of the Rabs. Indeed, in GST-Rab pulldown experiments the interactions showed a strong preference for the GTP form of the Rabs. Since cytosolic AP-3, AP-1, and BLOC-2 presumably represent the "free form" of these complexes, their interaction with purified GST-Rab38 and GST-Rab32 are likely direct, although the possibility of a bridging protein cannot be ruled out. Consistent with these physical interactions a significant degree of colocalization was observed between AP-3, AP-1, and BLOC-2 and the Rabs (Fig 2.6 and 2.7). The bulk of AP-3 and AP-1 has been shown to localize to coated structures budding off from distinct tubular early endosomes or vesicles in MNT-1 and other melanocytic cells (Figure 2.9) (15,17,20). In line with these observations clathrin partially colocalized with the Rabs but markers of other endosomal domains, EEA1 and the retromer complex, did not. Moreover,

clathrin partially colocalized with Rab38 and Rab32 to a similar degree as the clathrin adaptors AP-3 and AP-1, suggesting that the Rabs are recruited in the budding process.

In support of the idea that the interactions of AP-3, AP-1, and BLOC-2 with the Rabs are biologically relevant, we show that knockdown of either Rab38 or Rab32 or both simultaneously elicits the same phenotypes as deficiency in AP-3, AP-1, or BLOC-2. On the one hand, deficiency in the Rabs causes enhanced Tyrp-1 recycling through the plasma membrane, a defect previously observed in primary melanocytes from AP-3 and BLOC-2 mutant mice and also detected here in MNT-1 cells deficient for AP-3 or AP-1 (Figure 2.12) (17). On the other hand, Rab deficiency elicits reduced overall levels of the tyrosinase family members paralleling the results obtained with melanocytes from AP-3 and BLOC-2 mutant mice, or from HPS patients with mutations in AP-3 and BLOC-2 (Figure 2.13A) (17,47,48). These cargo trafficking data, together with the physical interactions and colocalization results, are most consistent with a model in which the Rabs function in pathways from early endosome associated tubules to maturing melanosomes (Figure 2.9). Such a location fits well with the established model for AP-3-, AP-1-, and BLOC-2-dependent transport of tyrosinase and Tyrp-1 from early endosomes to maturing melanosomes and places the Rabs as acting downstream of the early endosome with potential roles in vesicle motility or fusion events (3,14,15,17,18,20,44). This model also provides a satisfactory explanation to the observed cargo phenotypes. Disruption of transport from early endosomes towards melanosomes causes accumulation of the cargo in early endosomes and leakage into the recycling pathway to the plasma membrane and/or into the degradative MVB/lysosomal pathway. This model is also compatible with a previous electron microscopy study that found overexpressed GFP-Rab38 in both MNT-1 cells and mouse melanocytes localized to tubules, vesicles and melanosomes (27). These three structures

(tubules, vesicles, and melanosomes) contained tyrosinase and Tyrp-1 and would correspond to the model donor compartment, transport intermediate, and target organelle, respectively (Figure 2.9). In fact we also found that both endogenous Rab38 and Rab32 partially colocalize with melanosomes, thus supporting the proposed model (Figs. 2.9 and 2.11). Finally, this model is consistent with the recently discovered interaction between the Rab38/32 effector protein Varp and VAMP7 (46,49). Varp plays a role in Tyrp-1 transport to the melanosome and VAMP7 is a well-known vesicle SNARE protein of the AP-3- and BLOC-1-dependent pathway involved in vesicle fusion with late endosomes/lysosomes (11,19). Alternatively or in parallel, Rab32 and Rab38 might participate in a post-Golgi route that goes from the *trans*-Golgi Network (TGN) directly to the maturing melanosomes – without an early endosome step in between the TGN and melanosomes (27). For all the reasons discussed above we favor the early endosome route as the main Rab32/Rab38-dependent pathway to maturing melanosomes.

The Tyrp-1 recycling phenotype found in AP-3 depleted MNT-1 cells (Figure 2.12) recapitulates a similar observation made with melanocytes from AP-3 mutant mice using the same approach (17). This result seems to be at variance with that of a previous article reporting a relatively normal steady state localization of Tyrp-1 in fixed melanocytes from patients with AP-3 deficiency (48). However, we note that the Tyrp-1 immunofluorescence staining shown in that report in AP-3–deficient melanocytes appears to be significantly less intense than in normal melanocytes (48), thus in agreement with our previous results with melanocytes from AP-3 mutant mice (17) and results presented here with MNT-1 cells. It is likely that in AP-3 deficient cells a proportion of Tyrp-1 molecules eventually reach the melanosomes using the AP-1/BLOC-2 route(s) thus explaining the fixed cell analysis in the earlier report (48). The recycling assay with live cells is designed to detect molecules transiently exposed at the cell surface with high

sensitivity that would be difficult to appreciate at steady state by confocal fluorescence or thin section electron microscopy. A complete understanding of adaptor requirement for transport of each tyrosinase family member will require further elucidation.

Results presented here shed light on aspects of Rab38 and Rab32 that were previously unexplored (33). Our findings suggest Rab38 and Rab32 have functional similarities and differences in melanosome biogenesis. For instance, both Rabs interact with AP-3, AP-1, and BLOC-2 however the connection with Rab38 is stronger than with Rab32 and our data suggests it is possible that Rab38 operates earlier than Rab32 in the pathways from early endosomes to maturing melanosomes. First, AP-3, AP-1, and clathrin display a higher degree of colocalization with Rab38 than with Rab32 (Figure 2.6 and 2.7). Second, Rab38 association with membranes is decreased after knockdown of AP-3 or BLOC-2, but Rab32 is only marginally affected (Figure 2.5A). The functional relationship between Rab38 and BLOC-2 is particularly strong. BLOC-2 deficiency affected most severely the Rab38 association with membranes and also compromised the overall Rab38 stability (Figure 2.5A and 2.5B). Conversely, Rab38 (but not Rab32) deficiency affected BLOC-2 association with membranes (Figure 2.5C). This level of interdependence between BLOC-2 and Rab38 resembles the one observed between subunits of the same BLOC or AP complex (10,40). For instance absence of a BLOC-2 subunit such as HPS3 causes a destabilization (although not complete absence) of the other 2 subunits (HPS5 and HPS6) (50). Further confirming the strong Rab38-BLOC-2 connection, the coat color of the chocolate mouse (with a Rab38 mutation) is very similar to the BLOC-2 mutant strains, cocoa (HPS3), ruby-eye (HPS6) and ruby-eye-2 (HPS5) (25,29,50). Nevertheless, the Rab38 association with BLOC-2 is likely transient rather than as components of a stable complex. Unlike AP-3 and AP-1, the molecular function of BLOC-2 is unknown. The tight association

with Rab38 discovered here will help future elucidation of BLOC-2 function at the molecular level.

Our results show additional similarities and differences between Rab38 and Rab32 in terms of their requirement for trafficking of specific cargoes and overall relevance for melanosome biogenesis. Single deficiency in each Rab causes similar and relatively modest Tyrp-1 recycling phenotype or decrease in the steady state levels of tyrosinase and Tyrp-1 (Figure 2.12, 2.13A). In contrast, we observed drastically different effects for Tyrp-2 steady state levels, which were severely reduced in Rab32-deficient cells but not affected in Rab38-deficient cells. In addition, tyrosinase and Tyrp-1 trafficking phenotypes are more severe in cells simultaneously deficient for both Rabs, compared to single deficient cells (Figure 2.12, 2.13A). Thus, Rab32 appears to more clearly cooperate with Rab38 in pathways utilized by cargo such as tyrosinase and Tyrp-1, which are also known to depend on AP-3, AP-1, and BLOC-2 (4,11,14,15,17,18,20,47,48,51). Interestingly, the trafficking of Tyrp-2 has been far less characterized compared to that of tyrosinase and Tyrp-1. Our data suggests Tyrp-2 utilizes a pathway in which Rab32 is strictly necessary and that it is at least partially different from those of tyrosinase and Tyrp-1 (Figure 2.13A). This result is consistent with the finding that Tyrp-1 but not Tyrp-2 associates with tyrosinase in murine melanocytes *in vivo* (52). This apparently Rab38-independent pathway towards melanosomes utilized by Tyrp-2, and perhaps other melanosome components would provide an explanation for the incomplete compensation by Rab38 in Rab32 deficient cells as assessed by melanin content (Figure 2.13B). Conversely, incomplete compensation by Rab32 in Rab38 deficient MNT-1 cells (Figure 2.13B) is consistent with the pigmentation defect of the *chocolate* mouse (25). Therefore the emerging picture is one in which the Rabs are only partially redundant for melanosome biogenesis.

An interesting question is how does Rab32 carry out its unique, non-Rab38 redundant functions in melanosome biogenesis such as Tyrp-2 traffic? As stated above, this trafficking route appears to be different from the routes used by tyrosinase and Tyrp-1, which are also known to depend on AP-3, AP-1, and BLOC-2 (4,11,14,15,17,18,20,47,48,51). It is possible that such Tyrp-2/Rab32 route is dependent on other components of the ubiquitous machinery involved in melanosome biogenesis, such as BLOC-1 or BLOC-3. Analysis of MNT-1 cells deficient for BLOC-3 showed significantly decreased steady state levels of Tyrp-2, while BLOC-1 deficiency had no effect (Figure 2.16). The Tyrp-2 phenotype caused by BLOC-3 deficiency resembles that elicited by Rab32 deficiency (Figure 2.13A). This result raises the possibility that Rab32 participates in a separate route to melanosomes that is dependent on BLOC-3 and used by cargo such as Tyrp-2. The molecular function of BLOC-3 is unknown. Our results suggest a framework for the Rab32 unique functions (non-redundant with Rab38) and will be important for future studies aimed at elucidating the function of BLOC-3. Rab32 may have additional unique functions unrelated to LRO biogenesis as it was also reported to modulate the mitochondrial-associated membrane properties (53).

In conclusion, our results point to a novel mechanism for directing the ubiquitous trafficking machinery from early endosomes towards maturing melanosomes and likely other LROs. Tissue specific Rab proteins, Rab38 and Rab32, interact with AP-3, AP-1, and BLOC-2 and likely identify specialized early endosomal domains for budding of transport intermediates destined to maturing melanosomes. It is possible that an ubiquitous Rab could define analogous early endosomal domains for transport of membrane proteins to conventional lysosomes. This could be the function of Rab7, which is involved in transport to lysosomes and has recently been shown to be co-recruited with AP-3 to artificial membranes containing the endosomal

phospholipid phosphatidylinositol-3 phosphate (54). We also obtained evidence that the Rabs have both overlapping and exclusive functions for overall melanosome biogenesis and traffic of specific cargoes. Further study is needed to dissect the mechanisms by which Rab38 and Rab32 execute redundant and unique functions in trafficking to LROs.

2.6 Acknowledgments

We thank Andrew A. Peden, Esteban C. Dell'Angelica, and Peter K. Kim for their generous gifts of reagents, and Vincent J. Hearing for guidance with the Nucleofector system for MNT-1 transfection. This work was supported by American Heart Association award 09SDG2280525 and NIH grant 1R01HL106186-01A1 to S.D. Microscopes used in this work are supported in part by the Microscope Imaging Network core infrastructure grant from Colorado State University.

2.7. REFERENCES

1. Dell'Angelica EC, Mullins C, Caplan S, Bonifacino JS. (2000) Lysosome-related organelles. FASEB J. 14(10):1265-78.
2. Spritz RA, Chiang, P. W., Oiso, N., and Alkhateeb, A. (2003) Human and mouse disorders of pigmentation. *Curr Opin Genet Dev* 13, 284-289
3. Raposo G, Marks MS, Cutler DF. (2007) Lysosome-related organelles: driving post-Golgi compartments into specialisation. Curr Opin Cell Biol. 19(4):394-401.
4. Huizing M, Helip-Wooley A, Westbroek W, Gunay-Aygun M, Gahl WA. (2008) Disorders of lysosome-related organelle biogenesis: clinical and molecular genetics. Annu Rev Genomics Hum Genet. 9:359-86.
5. Di Pietro SM, Dell'Angelica EC. (2005) The cell biology of Hermansky-Pudlak syndrome: recent advances. Traffic. 6(7):525-33.6. Wei, M. L. (2006) *Pigment Cell Res* 19, 19-42
6. Wei, M. L. (2006) Hermansky-Pudlak syndrome: a disease of protein trafficking and organelle function. *Pigment Cell Res* 19, 19-42
7. Cullinane, *et al* (2011) A BLOC-1 mutation screen reveals that PLDN is mutated in Hermansky-Pudlak Syndrome type 9. *Am J Hum Genet* 88, 778-787
8. Li W, *et al* (2004) Murine Hermansky-Pudlak syndrome genes: regulators of lysosome-related organelles. *Bioessays* 26, 616-628
9. Gautam R, *et al* (2006) Interaction of Hermansky-Pudlak Syndrome genes in the regulation of lysosome-related organelles. Traffic. 7(7):779-92.
10. Dell'Angelica EC. (2004) The building BLOC(k)s of lysosomes and related organelles. Curr Opin Cell Biol. 16(4):458-64.
11. Dell'Angelica EC. (2009) AP-3-dependent trafficking and disease: the first decade. Curr Opin Cell Biol. 21(4):552-559.12.
12. Bonifacino JS, and Traub, L. M. (2003) Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu Rev Biochem* 72, 395-447
13. Peden AA, *et al* (2004) Localization of the AP-3 adaptor complex defines a novel endosomal exit site for lysosomal membrane proteins. *J Cell Biol.* 164(7):1065-1076.
14. Raposo G and Marks MS (2007) Melanosomes--dark organelles enlighten endosomal membrane transport. *Nat Rev Mol Cell Biol* 8, 786-797
15. Theos AC, *et al* (2005) Functions of adaptor protein (AP)-3 and AP-1 in tyrosinase sorting from endosomes to melanosomes. *Mol Biol Cell* 16(11):5356-5372.
16. Hurbain I, *et al* (2008) Electron tomography of early melanosomes: implications for melanogenesis and the generation of fibrillar amyloid sheets. *Proc Natl Acad Sci U S A.* 105(50):19726-31.
17. Di Pietro SM, *et al* (2006) BLOC-1 interacts with BLOC-2 and the AP-3 complex to facilitate protein trafficking on endosomes. *Mol Biol Cell.* 17(9):4027-38.
18. Setty SR, *et al* (2007) BLOC-1 is required for cargo-specific sorting from vacuolar early endosomes toward lysosome-related organelles. *Mol Biol Cell.* 18(3):768-80.
19. Salazar G, *et al* (2006) BLOC-1 complex deficiency alters the targeting of adaptor protein complex-3 cargoes. *Mol Biol Cell.* 17(9):4014-26
20. Delevoye C, *et al* (2009) AP-1 and KIF13A coordinate endosomal sorting and positioning during melanosome biogenesis. *J Cell Biol.* 187(2):247-64.
21. Marks MS and Seabra, M C (2001) The melanosome: membrane dynamics in black and white. *Nat Rev Mol Cell Biol* 2, 738-748
22. Rendu F and Brohard-Bohn B (2001) The platelet release reaction: granules' constituents, secretion and functions. *Platelets* 12, 261-273
23. Zerial M and McBride H (2001) Rab proteins as membrane organizers. *Nat Rev Mol Cell Biol* 2, 107-117

24. Hutagalung AH, and Novick PJ (2011) Role of Rab GTPases in membrane traffic and cell physiology. *Physiol Rev* 91, 119-149
25. Loftus SK, *et al* (2002) Mutation of melanosome protein RAB38 in chocolate mice. *Proc Natl Acad Sci U S A* 99, 4471-4476
26. Cohen-Solal KA, *et al*(2003) Identification and characterization of mouse Rab32 by mRNA and protein expression analysis. *Biochim Biophys Acta* 1651, 68-75
27. Wasmeier C, *et al* (2006) Rab38 and Rab32 control post-Golgi trafficking of melanogenic enzymes. *J Cell Biol* 175, 271-281
28. Brooks BP, *et al* (2007) Analysis of ocular hypopigmentation in Rab38cht/cht mice. *Invest Ophthalmol Vis Sci* 48, 3905-3913
29. Osanai K, *et al* (2008) A mutation in Rab38 small GTPase causes abnormal lung surfactant homeostasis and aberrant alveolar structure in mice. *Am J Pathol* 173, 1265-1274
30. Oiso N, Riddle SR, Serikawa T, Kuramoto T, and Spritz RA (2004) The rat Ruby (R) locus is Rab38: identical mutations in Fawn-hooded and Tester-Moriyama rats derived from an ancestral Long Evans rat sub-strain. *Mamm Genome* 15, 307-314
31. Ninkovic I, White JG, Rangel-Filho A, and Datta YH (2008) The role of Rab38 in platelet dense granule defects. *J Thromb Haemost* 6, 2143-2151
32. Lopes VS, Wasmeier C, Seabra MC, and Futter CE (2007) Melanosome maturation defect in Rab38-deficient retinal pigment epithelium results in instability of immature melanosomes during transient melanogenesis. *Mol Biol Cell* 18, 3914-3927
33. Marks MS (2006) Darkness descends with two Rabs. *J Cell Biol* 175, 199-200
34. Di Pietro SM, Falcon-Perez JM, and Dell'Angelica EC (2004) Characterization of BLOC-2, a complex containing the Hermansky-Pudlak syndrome proteins HPS3, HPS5 and HPS6. *Traffic* 5, 276-283
35. Nazarian R, *et al* (2008) An immunoblotting assay to facilitate the molecular diagnosis of Hermansky-Pudlak syndrome. *Mol Genet Metab* 93, 134-144
36. Dell'Angelica EC, *et al* (2000) Molecular characterization of the protein encoded by the Hermansky-Pudlak syndrome type 1 gene. *J Biol Chem* 275, 1300-1306
37. Kloer DP, *et al* (2010) Assembly of the biogenesis of lysosome-related organelles complex-3 (BLOC-3) and its interaction with Rab9. *J Biol Chem* 285, 7794-7804
38. Nazarian R, Starcevic M, Spencer MJ, and Dell'Angelica EC (2006) Reinvestigation of the dysbindin subunit of BLOC-1 (biogenesis of lysosome-related organelles complex-1) as a dystrobrevin-binding protein. *Biochem J* 395, 587-598
39. Ozeki H, Ito S, Wakamatsu K, and Hirobe T (1995) Chemical characterization of hair melanins in various coat-color mutants of mice. *J Invest Dermatol* 105, 361-366
40. Dell'Angelica EC, Shotelersuk V, Aguilar RC, Gahl WA, and Bonifacino JS (1999) Altered trafficking of lysosomal proteins in Hermansky-Pudlak syndrome due to mutations in the beta 3A subunit of the AP-3 adaptor. *Mol Cell* 3, 11-21
41. Dell'Angelica EC, Ooi CE, and Bonifacino JS (1997) Beta3A-adaptin, a subunit of the adaptor-like complex AP-3. *J Biol Chem* 272, 15078-15084
42. Kim PK, Mullen RT, Schumann U, and Lippincott-Schwartz J (2006) The origin and maintenance of mammalian peroxisomes involves a de novo PEX16-dependent pathway from the ER. *J Cell Biol* 173, 521-532
43. Valencia JC, *et al* (2006) Sorting of Pmel17 to melanosomes through the plasma membrane by AP1 and AP2: evidence for the polarized nature of melanocytes. *J Cell Sci* 119, 1080-1091
44. Truschel ST, *et al* (2009) ESCRT-I function is required for Tyrp-1 transport from early endosomes to the melanosome limiting membrane. *Traffic* 10, 1318-1336
45. Setty SR, *et al* (2008) Cell-specific ATP7A transport sustains copper-dependent tyrosinase activity in melanosomes. *Nature* 454, 1142-1146

46. Tamura K, *et al* (2009) Varp is a novel Rab32/38-binding protein that regulates Tyrp-1 trafficking in melanocytes. *Mol Biol Cell* 20, 2900-2908
47. Helip-Wooley A, *et al* (2007) Improper trafficking of melanocyte-specific proteins in Hermansky-Pudlak syndrome type-5. *J Invest Dermatol* 127, 1471-1478
48. Huizing M, *et al* (2001) AP-3 mediates tyrosinase but not TRP-1 trafficking in human melanocytes. *Mol Biol Cell* 12, 2075-2085
49. Tamura K, Ohbayashi N, Ishibashi K, and Fukuda M (2011) Structure-function analysis of VPS9-ankyrin-repeat protein (Varp) in the trafficking of tyrosinase-related protein 1 in melanocytes. *J Biol Chem.* 286(9):7507-7521
50. Gautam R, *et al* (2004) The Hermansky-Pudlak syndrome 3 (cocoa) protein is a component of the biogenesis of lysosome-related organelles complex-2 (BLOC-2). *J Biol Chem* 279 (13):12935-12942
51. Huizing M, *et al* (2009) Clinical and cellular characterisation of Hermansky-Pudlak syndrome type 6. *J Med Genet* 46 (12):803-810
52. Kobayashi T and Hearing VJ (2007) Direct interaction of tyrosinase with Tyrp-1 to form heterodimeric complexes in vivo. *J Cell Sci* 120 (Pt24):4261-4268
53. Bui M, *et al* (2010) Rab32 modulates apoptosis onset and mitochondria-associated membrane (MAM) properties. *J Biol Chem* 285 (41):31590-31602
54. Baust T, *et al* (2008) Protein networks supporting AP-3 function in targeting lysosomal membrane proteins. *Mol Biol Cell* 19, 1942-1951

CHAPTER 3

NOVEL FUNCTION OF MYOSIN VC IN MELANOSOME BIOGENESIS THROUGH INTERACTIONS WITH MELANOSOMAL RAB GTPASES [2]

3.1 Summary

Class V myosins are actin-based motors with conserved functions in vesicle and organelle trafficking. Myosin Vc, the third member of the mammalian Myosin V family, functions in secretory trafficking and ubiquitous recycling functions in specific cell types. Herein, we report the novel discovery of a function for Myosin Vc in melanosome biogenesis as an effector of melanosome-associated Rab GTPases. We demonstrate direct physical interaction between GTP-bound Rab7a, Rab8a, Rab32, and Rab38 and the tail of Myosin Vc in yeast 2-hybrid assays and GST-pulldowns. The binding of Rab32 and Rab38 to Myosin Vc depends on residues in the switch II region of the Rabs, and Rab binding is mapped to specific, partially overlapping regions of the coiled-coil tail domain of Myosin Vc. Knockdown of Myosin Vc in MNT-1 melanocytes causes defects in the trafficking of Tyrp-1 and Tyrp-2 to melanosomes, but also significantly increases the abundance of pigmented melanosomes. Fractionation of MNT-1 cells demonstrates that Myosin Vc is not abundant on mature melanosomes and likely functions in vesicle trafficking, as opposed to Myosin Va and Myosin Vb that are both present on

[2] Jarred J. Bultema, Judith A. Boyle, Aparna Jorapur, Parker Malenke, Richard E. Cheney, and Santiago M. Di Pietro
Department of Biochemistry and Molecular Biology, Colorado State University,
Fort Collins, Colorado 80523, USA.

melanosomes. A comparison of the localization of Myosin Va, Myosin Vb, and Myosin Vc suggests that these motors function in different aspects of melanosome biogenesis and secretion.

3.2 Introduction

The pigmentation of tissues is responsible for the formation of hair and skin coloration in mammals, and serves the underlying function of protecting from UV-induced DNA damage. In skin melanocytes, specialized cells responsible for pigment production, melanins are formed in a specialized lysosome-related organelle known as the melanosome. Melanosomes, like all lysosome-related organelles, are similar to the ubiquitous lysosome; and the biogenesis of melanosomes uses similar mechanisms as the formation of lysosomes (1, 2, 3). The melanosome is derived from an endosomal organelle through a combination of organelle remodeling and vesicular trafficking events that transport melanosome-specific proteins toward maturing melanosomes (4).

The development, or maturation, of melanosomes is known to depend on discrete trafficking events and can be characterized into four distinct stages. The least mature melanosomes, stage I, are formed by an unknown organelle remodeling event from an endosomal membrane. Stage I melanosomes are characterized by the presence of the structural protein Pmel17 on the organelle limiting membrane and in intraluminal vesicles within the organelle, and by a distinctive clathrin lattice on the limiting membrane (4). Within the environment of stage I melanosomes, Pmel17 proteins are cleaved by acid hydrolases (5, 6, 7). In stage II melanosomes, these Pmel17 fragments oligomerize and form amyloid-like fibrils that span the length of the melanosome, and are clearly evident in electron micrographs (8, 9). The delivery of enzymes tyrosinase, Tyrp-1, and Tyrp-2 is needed for the synthesis of melanin and

facilitates the production of melanin pigments that coat the Pmel-fibrils to form partially pigmented stage III melanosomes (10). Additional melanin synthesis generates fully pigmented stage IV melanosomes (10). In skin, the stage IV melanosomes are transferred to keratinocytes, using unclear mechanisms, where they remain and form a pigmented cap around the keratinocyte nucleus (11, 12).

In endosomal trafficking, Rab GTPases serve numerous functions in organelle remodeling and in vesicle trafficking; and serve as specific markers to distinguish endosomal trafficking pathways (13). Melanosome biogenesis is known to utilize a combination of ubiquitous endosomal Rabs and cell-type specific Rabs for different stages of melanosome maturation (4). Cell-type specific Rab27a functions in the regulation of the sub-cellular distribution of melanosomes through interaction with the actin-based motor Myosin Va, in a tripartite complex with melanophilin (14, 15). Other cell-type specific proteins, Rab38 and Rab32 are important in the biogenesis of melanosomes and function in the vesicle trafficking of tyrosinase, Tyrp-1, and Tyrp-2 from early endosomes to melanosomes. Rab32 and Rab38 act as tissue-specific proteins that allow for the trafficking of cargoes to the melanosome using ubiquitous early endosomal trafficking components AP-3, AP-1, and BLOC-2 (16, 17). Less is known about the mechanisms used by ubiquitous Rabs, such as Rab7a, Rab8a, Rab17, and Rab11a/b that are known to function in cargo and organelle trafficking, that have been implicated with functions on melanosomes (18, 19, 20, 21, 22).

The most clearly understood mechanism used by Rabs in melanosome biogenesis is the movement of stage IV melanosomes by the semi-stable complex composed of Rab27a, melanophilin, and Myosin Va. Class V myosins are a well-conserved family of processive actin motors that function in vesicle and organelle trafficking events (23). In melanocytes, the long-

range movement of melanosomes is dependent on the functions of microtubule-based kinesin and dynein motors, but the proper transport of melanosomes to keratinocytes also depends on the actin-based transport of melanosomes using Myosin Va. A cooperative capture model has been used to describe the movement of melanosomes as they are pulled off of microtubule tracks and into the cortical actin meshwork at the cell periphery by Myosin Va (24, 4). In wild-type melanocytes, pigmented melanosomes accumulate along the plasma membrane of cells, and are especially enriched within dendritic projections that have been shown to be sites of melanosome transfer to keratinocytes (25). Lacking functional Myosin Va, Rab27a, or melanophilin pigmented melanosomes accumulate in perinuclear regions of cells and transfer to keratinocytes is diminished (14, 25, 26). The other members of the mammalian class V Myosin family, Myosin Vb and Myosin Vc, function in the ubiquitous recycling of endocytic cargoes, such as the transferrin-receptor, and in some polarized trafficking and secretion functions in specialized cell types (27, 28, 29, 30). Additional recycling endosomal Rabs, Rab17 and Rab11a/b have been implicated in melanosome biogenesis and secretion, but to date no direct evidence has demonstrated that other recycling endosomal machinery is required for normal melanosome trafficking (22).

Here we report the novel discovery that Myosin Vc functions as an effector of Rab7a, Rab8a, Rab32, and Rab38 in the biogenesis and secretion of melanosomes. Myosin Vc serves multiple functions in both the trafficking of cargoes to melanosomes and in the secretion of mature melanosomes, but interestingly is not abundant on mature melanosomes. Further, we demonstrate that Myosin Vb is enriched on melanosomes, and that all three mammalian class V myosins are implicated in non-redundant functions in melanosome trafficking.

3.3 Results and Discussion

3.3.1 Interaction of Myosin Vc with melanosome-associated Rab GTPases

Previous work has identified Rab32 and Rab38 as key components in the biogenesis of lysosome-related organelles (16, 17, 31), but the mechanisms of action of these Rabs remains unclear. To better understand the functions of Rab38, a yeast 2-hybrid screen was performed using the constitutively-active GTP-locked Rab38-Q69L mutant against a whole bone marrow library to identify Rab38 effector proteins. Among the candidates, Myosin Vc appeared as a strongly interacting binding partner and was further tested for Rab38 binding. Myosin Vc is found to interact preferentially with the constitutively-active, GTP-locked mutants of Rab38 and with GTP-locked Rab32, but not with wild-type proteins (Figure 3.1A).

Interestingly, Myosin Vc also interacts with constitutively-active Rab7a, and both the wild-type and constitutively-active Rab8a (Figure 3.1A). A function for Myosin Vc as an effector of multiple Rabs in melanosome biogenesis is an interesting possibility. However, controls for specificity of the interaction of Rabs with Myosin Vc are necessary to validate functional interactions. Two obvious controls for binding are Rab11a and Rab27a, known binding partners of Myosin Vb and Myosin Va (27, 14). Neither Rab11a, nor Rab27a interact with Myosin Vc (Figure 3.1A), suggesting that interactions between melanosome-associated Rabs and Myosin Vc are specific.

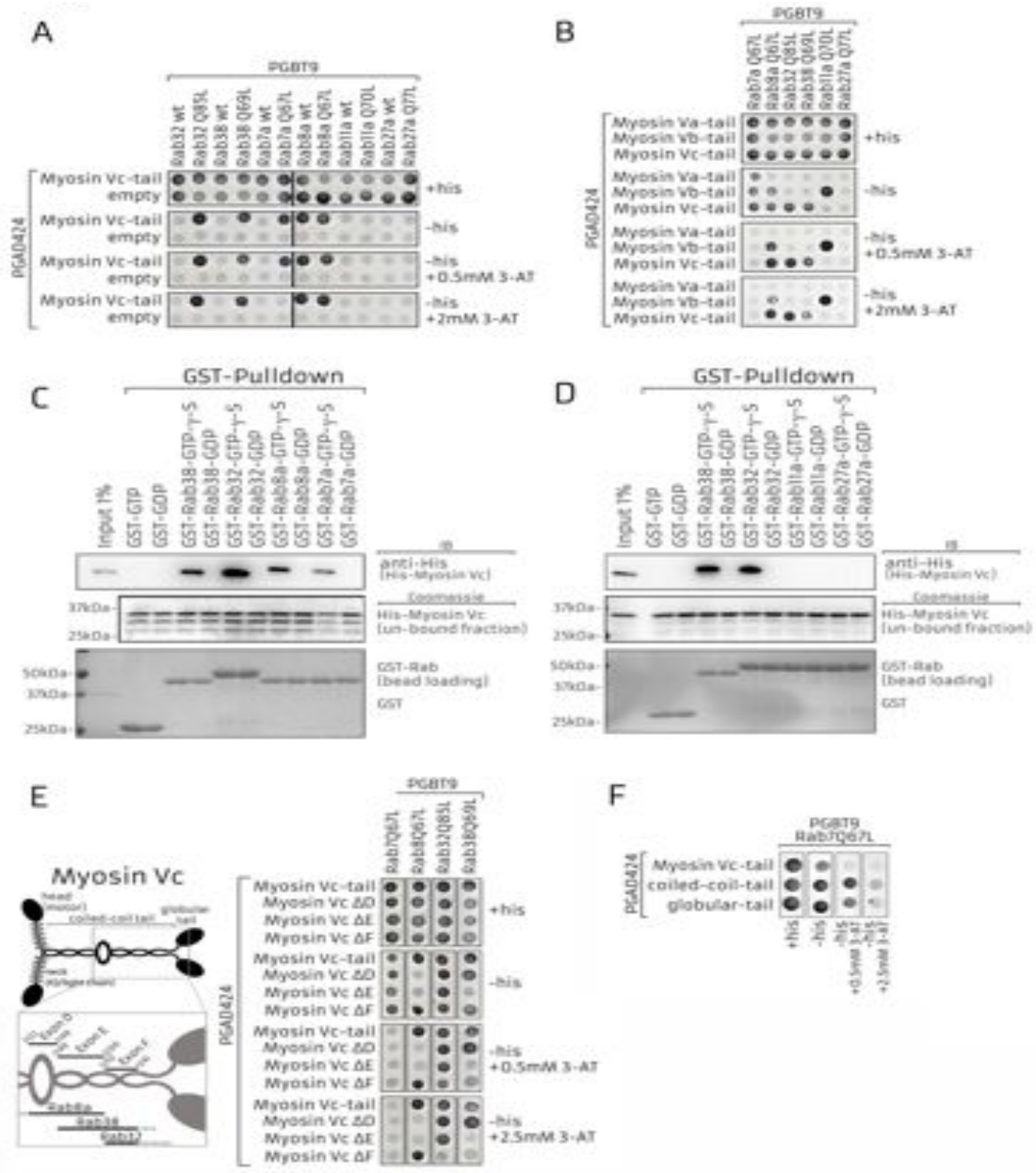

Figure 3. 1

Interaction of early melanosomal Rabs with Myosin Vc. Yeast 2-hybrid assays with the full-tail of human Myosin Va (aa.908 –1855), Myosin Vb (aa.905-1848), and Myosin Vc (aa.898-1742) binding to human Rab GTPases associated with melanosome biogenesis in the GTP-locked, constitutively active state (Q-L mutations). Cell growth in panels lacking histidine indicates physical interaction between Myosin V and Rabs. Growth in higher concentrations of 3-amino-1,2,4-triazole (3-AT) demonstrates higher affinity binding. (A) Myosin Vc binds to constitutively active Rab7a, Rab8a, Rab32, and Rab38, but not with Rab11a or Rab27a. (B) Specificity of Rab interactions with class V Myosins. Rab32 and Rab38 bind only to Myosin Vc, but Rab7a and Rab8a bind other class V myosins. (C,D) In vitro GST-pulldowns demonstrate the direct binding of specific Rabs to His-Myosin Vc(aa.1121-1350). (E) Mapping binding of Rab8, Rab32, and Rab38 to Myosin Vc tail. (F) Rab7a independently binds to the coiled-coil (aa.898-1350) and globular tail domains (aa.1350-1742) of Myosin Vc.

In addition to the specificity of Myosin Vc binding, the specificity of Rab binding to other human class V myosins was tested (Figure 3.1B). Rab7a has the least specificity in binding and is observed to weakly bind to Myosin Va, Myosin Vb, and Myosin Vc (Figure 3.1B). Rab8a, previously demonstrated to function in separate trafficking pathways with either Myosin Vb or Myosin Vc, is observed to interact with Myosin Vb and Myosin Vc, but not Myosin Va (Figure 3.1B). Interestingly, Rab38 and Rab32 have the most specific binding to Myosin Vc, and neither Rab38 nor Rab32 bind Myosin Va or Myosin Vb (Figure 3.1B). Rab11a binds only Myosin Vb, as expected; and Rab27a shows no direct binding to any of the Myosins tested, which is expected based on the known necessity of melanophilin for Rab27a-Myosin Va interaction (32). The specificity of constitutively active Rab32 and Rab38 binding to only Myosin Vc strongly suggests that Myosin Vc functions as an effector protein for these Rabs. Does Myosin Vc interact with multiple Rabs present in the same trafficking pathway? Rabs are known to bind the globular tail and coiled-coil domains of class V myosins (33). The globular tail of Myosin Vc alone does not interact with Rab38 and Rab32 and this region of the tail is not the binding site for these two Rabs. To test the binding of these Rabs to Myosin Vc, *in vitro* GST-pulldowns were performed using purified GST-Rab fusion proteins and His-tagged Myosin Vc coiled-coil tail domain (aa.1121 – 1350) (Figure 3.1C, 3.1D). GST-Rab38, GST-Rab32, GST-Rab8a, and GST-Rab7a are found to bind directly to the coiled-coil region of Myosin Vc only when the Rabs are pre-loaded with GTP-γ-S, a non-hydrolysable analogue of GTP (Figure 3.1C). Rab32 appears to bind most strongly to Myosin Vc, while Rab7a shows the weakest binding (Figure 3.1C). Consistent with yeast 2-hybrid assays (Figure 3.1A), GST-Rab11a and GST-Rab27a do not bind to the portion of Myosin Vc coiled-coil that binds Rab7a, Rab8a, Rab32, and Rab38 (Figure 3.1D).

Tissue specific alternative splicing of Myosin V-tail exons in the coiled-coil domain are prevalent and important for regulating specific Rab interactions (33). In melanocyte cells, Myosin Va exists as an alternatively splice isoform containing exon F that is required for localization to melanosomes (34). Rab8a binding to Myosin Vc has been shown to dependent on the presence of exon D in the coiled-coil tail (33), but is this region also important for binding of Rab38, Rab32, and Rab7a? Candidate exons in the tail of Myosin Vc were removed from the full-length tail and tested in yeast 2-hybrid assays with Rab7a, Rab8a, Rab32, and Rab38 (Figure 3.1E). Rab8a and Rab38 have partially overlapping binding sites that both utilize exon E (Figure 3.1E). Similarly, Rab38 and Rab32 have partially overlapping binding sites with exon F (Figure 3.1E). The binding of Rab7a is not mapped to these exons, but Rab7a is capable of binding both the coiled-coil and globular tail domains in the tail of Myosin Vc (Figure 3.1C, 3.1E, 3.1F). In MNT-1 melanocytes, the only isoform of Myosin Vc detected contained all of the exons D, E, and F - naming based on alignment with Myosin Va and Myosin Vb (29). The possibility exists that Myosin Vc could simultaneously bind to specific Rabs with non-overlapping binding sites (Figure 3.1E), but this cannot be determined by these experiments and will be an area of focus of future research.

3.3.2 Myosin Vc binding depends on the switch II region of Rab38 and Rab32

Previous studies investigating Rab-effector binding have found that exposed hydrophobic residues in the switch I, switch II, and hydrophobic triad of Rabs are important for interactions of Rabs with effectors (35, 36, 37). Further, the only previously known effector of Rab38 and Rab32, Varp, interacts with specific residues in the switch II region of these Rabs (38). Alanine-based mutagenesis was performed for amino acids in the switch I, switch II, and hydrophobic

triad regions and a sub-family specific loop, shared by Rab9a, Rab38, and Rab32, was deleted (Figure 3.2A), and these mutants were tested for ability to interact with Myosin Vc (Figure 3.2B). Only mutation of the tyrosine residues present in the switch II regions of Rab38 (Y79A) and Rab32 (Y95A) disrupt the interaction with Myosin Vc (Figure 3.2B). As both Varp and Myosin Vc bind to the switch II region, we mutated residues important for Rab interactions with Varp to determine if the same site is used to interact with Myosin Vc (Figure 3.2C, 3.2D). Yeast 2-hybrid experiments were performed with a longer growth period to detect subtle defects in binding of Rab mutants. Mutation of adjacent valine residues individually in the switch II region does not appear to significantly decrease binding of Rab38 or Rab32 with Myosin Vc, but combined mutation of valine and tyrosine results in a loss of interaction with Myosin Vc (Figure 3.2A, 3.2C). *In vitro* GST-pulldowns show that mutation of adjacent valine and tyrosine residues in the switch II region significantly decreases the binding of Rab32 and Rab38 to Myosin Vc (Figure 3.2D). Therefore, Varp and Myosin Vc both act as Rab38 and Rab32 effectors that utilize the same binding site, but the functional implications of both effectors binding to the same domain are not clear.

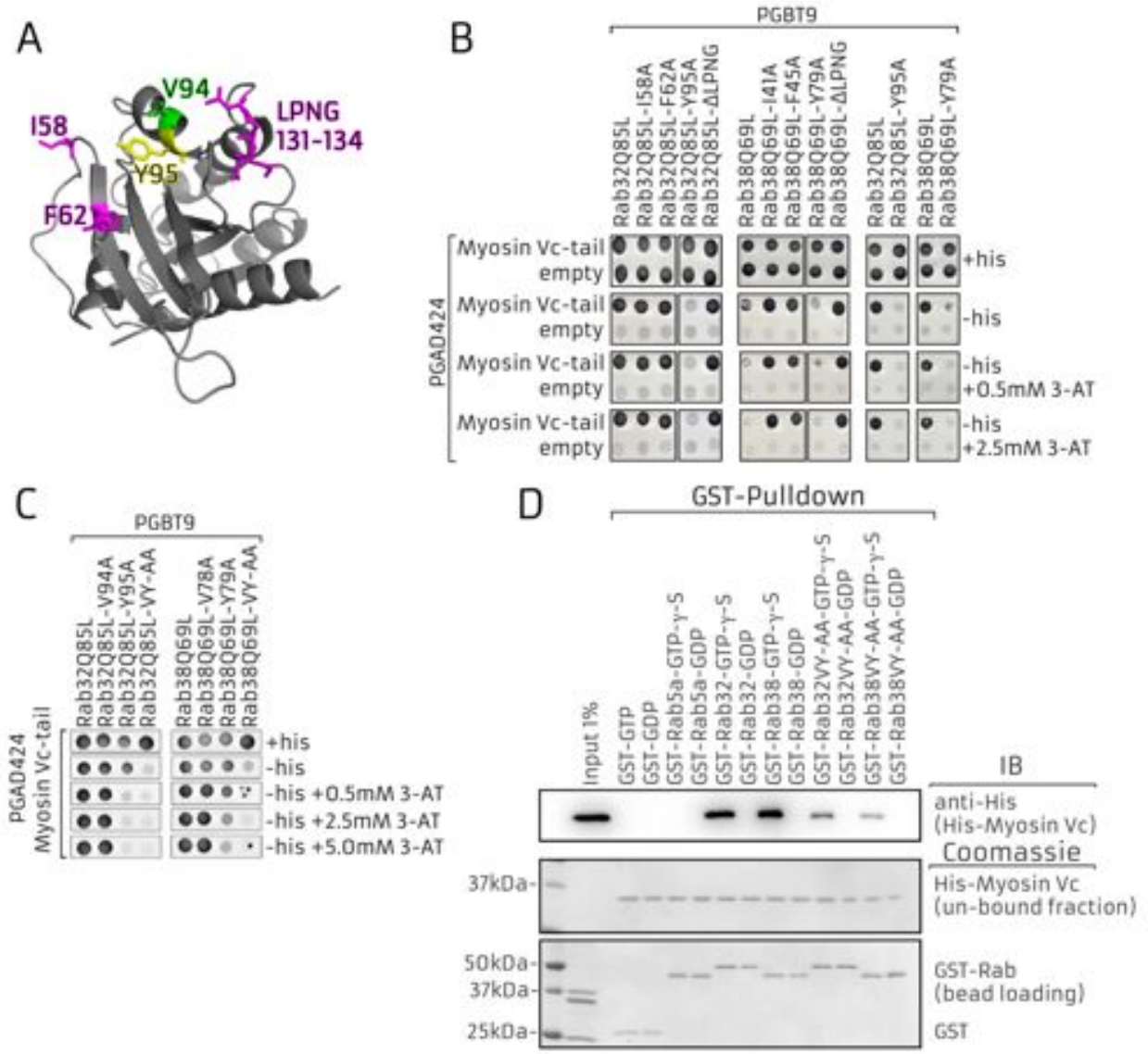

Figure 3. 2

Mapping Myosin Vc binding sites on Rab32 and Rab38. (A) Homology modeling of Rab32 onto the crystal structure of Rab4a (PDB 2bme). Residues in pink are not required for the binding to Myosin Vc. Green residue, Valine94, is required for the binding of Rab32 effector Varp. Yellow residue, Tyrosine95, is critical for the binding of Myosin Vc. (B) Yeast 2-hybrid assay with alanine mutants of constitutively active Rab38 and Rab32. Mutation of Rab38Q69L-Y79 and Rab32Q85L-Y95 specifically disrupt interaction with Myosin Vc tail. (C) Yeast 2-hybrid assays with mutations that disrupt interactions with Rab38 and Rab32 effectors Varp and Myosin Vc. (D) In vitro GST-pulldowns with Rab double mutants disrupt binding to Myosin Vc.

3.3.3 Myosin Vc knockdown alters melanosome secretion

The interaction between Rab38 and Rab32 with Myosin Vc is intriguing, but it is unclear if this interaction has any functional importance in melanosomes, or if Myosin Vc serves any functions in melanosome biogenesis. To determine if Myosin Vc functions in melanocytes, siRNA-mediated knockdown against Myosin Vc was performed and cells analyzed after two siRNA treatments. Importantly, the siRNA oligonucleotide found to cause significant knockdown of Myosin Vc does not knockdown related proteins Myosin Va or Myosin Vb (Figure 3.3A). Rather, knockdown of Myosin Vc, but not scrambled siRNA oligonucleotide, results in a significant increase in the abundance of Myosin Va and Myosin Vb (Figure 3.3A). Further, knockdown of Myosin Vc causes a significant increase in the amount of melanin in MNT-1 melanocytes (Figure 3.3B). Transmission-electron micrographs of control and Myosin Vc knockdown cells demonstrate that the increase in melanin is due to an increase in the abundance of pigmented stage III and IV melanosomes suggesting a role of Myosin Vc in secretion (Figure 3.3C). As Myosin Va is found on mature melanosomes, the increase in Myosin Va and Myosin Vb may be caused by the increased melanosome content. Alternatively, the increase in Myosin Va and Myosin Vb might reflect compensation for the loss of Myosin Vc functions by up regulation of Myosin Va and Myosin Vb. The latter possibility would suggest that some degree of redundancy exists in the functions of Myosin Vc with Myosin Va or Myosin Vb. Myosin Va knockdown results in perinuclear clustering of melanosomes without significantly affecting melanosome number or cell morphology (24, 39). Myosin Vc knockdown shows no obvious defects in melanosome localization or cellular morphology of MNT-1 cells, but significantly increases the number of pigmented melanosomes (Figure 3.3C). Pigmented melanosomes are frequently observed in close proximity to the plasma membrane (Figure 3.3C).

The distinct phenotypes of Myosin Va and Myosin Vc knockdown suggest that Myosin Vc functions independently from Myosin Va in melanocytes.

Consistent with increased abundance of pigmented melanosomes, Myosin Vc knockdown also causes an accumulation of some melanosome proteins (Figure 3.4A). The melanin synthesizing enzymes tyrosinase and Tyrp-1 are necessary for the production of melanin pigment within melanosomes, and these proteins are highly enriched in Myosin Vc knockdown samples (Figure 3.4A). The SNARE VAMP7, thought to be a component of melanosome biogenesis or trafficking (38) is also enriched in knockdown samples (Figure 3.4A). Does this enrichment of melanosome proteins reflect an increase in the number of pigmented of melanosomes (Figure 3.3C), or is it an indirect result of up regulation of melanosomal production resulting from Myosin Vc knockdown? Immunogold labeling of Pmel17 in electron micrographs of MNT-1 cells demonstrates that knockdown of Myosin Vc results in an increase in the number of pigmented melanosomes, but has no effect on the abundance of less mature, striated melanosomes, which should have been increased if Myosin Vc knockdown was causing an increase in melanosome production (Figure 3.4C). The increase in melanosomal proteins, therefore, reflects an increase in the abundance of pigmented melanosomes and is not a result of up regulation of melanosome production, and suggests that the accumulation of pigmented melanosomes in Myosin Vc knockdown cells is a result of defective melanosome secretion.

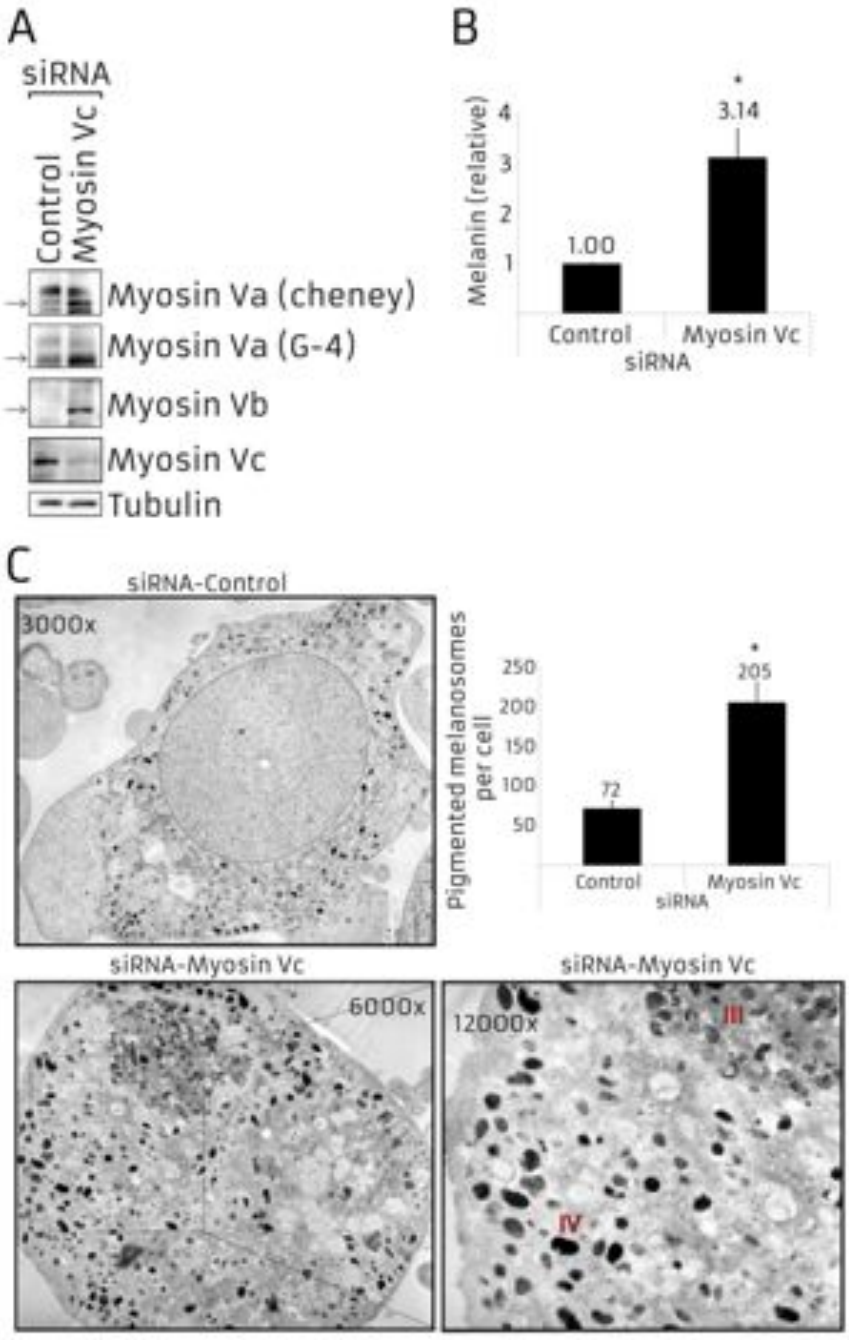

Figure 3. 3

Myosin Vc knockdown causes accumulation of pigmented melanosomes. (A) Immunoblotting of MNT-1 extracts subjected to control or Myosin Vc siRNA knockdown. Myosin Vc is highly depleted upon siRNA treatment, but Myosin Va and Myosin Vb are enriched in Myosin Vc depleted extracts. (B) Myosin Vc knockdown increases melanin content in MNT-1 detected in a spectroscopic method based on absorbance at 500 nm. (C) Myosin Vc knockdown results in accumulation of pigmented, stage III and IV, melanosomes in MNT-1 cells by ultra-thin Transmission Electron Microscopy. Error bars represent standard error, * = $p < 0.05$, $N \geq 4$ experiments.

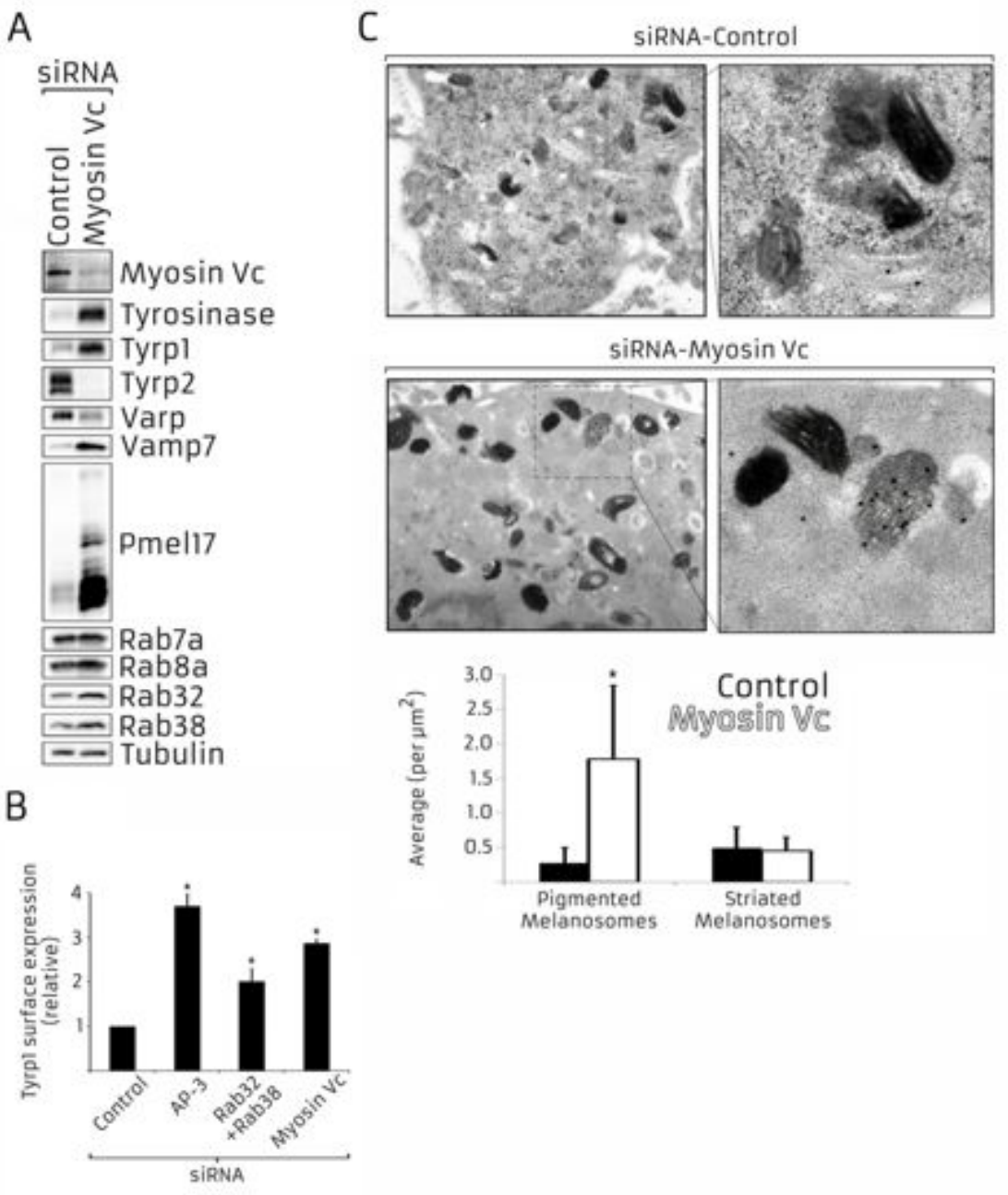

Figure 3. 4

Myosin Vc knockdown disrupts trafficking of specific melanosomal cargoes. (A) Immunoblotting of extracts from siRNA control and Myosin Vc treated MNT-1 cells. Depletion of Myosin Vc results in an accumulation of melanin synthesizing enzymes tyrosinase and Tyrp-1, but a severe reduction in Tyrp-2. Pmel17 is highly enriched in Myosin Vc depleted samples. (B) Myosin Vc depletion results in mistrafficking of Tyrp-1 at early endosome exit sites in a Tyrp-1 recycling assay. (C) Electron micrographs (20,000x magnification) and analysis of Pmel17-immunogold (12mm) labeled melanosomes. "Pigmented melanosomes" reflects any pigmented organelle and "Striated melanosomes reflects any organelle structure labeled with Pmel17 that has visible striations. Error bars represent standard error, * = $p < 0.0001$, $N > 10$ cells.

3.3.4 Myosin Vc knockdown causes defects in cargo trafficking to melanosomes

In addition to defects in melanosome secretion, Myosin Vc depletion also causes defects in the trafficking of cargoes to melanosomes. The trafficking of Tyrp-1 is disrupted, as detected in a recycling assay that demonstrates trafficking deficiency from early endosomes to melanosomes, by Myosin Vc knockdown (Figure 3.4B) (3, 17). Further, Tyrp-2 abundance is severely decreased upon Myosin Vc knockdown (Figure 3.4A). Previously it was shown that knockdown of Rab38 or Rab32 cause mistrafficking of Tyrp-1 (Figure 3.4B), and that Rab32 knockdown reduces Tyrp-2 levels (17). Interestingly, knockdown of Myosin Vc results in increased levels of multiple Rabs, including Rab32 (Figure 3.4A). Therefore, the cargo mistrafficking phenotypes of Myosin Vc knockdown suggest functions of Myosin Vc in Tyrp-1 and Tyrp-2 trafficking pathways that might depend on interaction with Rab32.

The trafficking of Tyrp-2 is believed to utilize a different route than the trafficking of tyrosinase and Tyrp-1, which relies on transport through recycling endosomes (40, 41). Defects in the trafficking pathways used by Tyrp-1 and Tyrp-2 suggests that Myosin Vc may function at a recycling or early endosomal trafficking step, in addition to defects in melanosome secretion observed in Myosin Vc knockdown (Figure 3.3). Nevertheless, sufficient trafficking to melanosomes occurs to allow the production of pigmented melanosomes (Figure 3.3B, 3.3C). The bimodal phenotypes in Myosin Vc knockdown may reflect dual functions in melanosome biogenesis and trafficking, or instead may be due to defects in the trafficking of components necessary for melanosome secretion.

Based on the severe melanosome secretion defect and cargo mistrafficking observed by Myosin Vc knockdown it is clear that Myosin Vc serve an important function in these trafficking pathways. Is there a functional connection between Myosin Vc and the melanosome-associated

Rabs (Rab7a, Rab8a, Rab32, Rab38) to which it binds? Knockdown of Rab38 and Rab32 can partially recapitulate defects in cargo trafficking observed in Myosin Vc knockdown (Figure 3.4B) (17), but do interactions of Myosin Vc with Rab7a or Rab8a account for the melanosome secretion defects? Melanosome-associated Rabs were knocked down and cells were assayed for the abundance of melanosome proteins observed to be altered by Myosin Vc knockdown (Figure 3.5). Knockdown of Rab7a, Rab8a, or Rab32 demonstrate a mistrafficking of Tyrp-2 (Figure 3.5). Accumulation of Pmel17 is also observed in Rab8a knockdown, but not significantly from knockdown of any other Rabs tested (Figure 3.5). Knockdown of Rab27a, while only partially depleted, does not demonstrate the same phenotypes as observed with Myosin Vc knockdown, and reinforces a distinct phenotype of Myosin Vc knockdown than observed for Myosin Va. Knockdowns of multiple Rabs are able to partially recapitulate the phenotypes observed with Myosin Vc knockdown (Figure 3.5). Notably, knockdown of some Rabs causes both the up regulation and down regulation of other Rabs: Rab7a knockdown appears to results in increased abundance of Rab8a and decreased abundance of Rab32; Rab8a knockdown results in increased Rab7a abundance; and Rab27a knockdown increases Rab7a and Rab8a abundance (Figure 3.5). This connection between Rabs may reflect redundancy and interdependence in the function and recruitment to melanosomes. Myosin Vc may function in multiple steps in the biogenesis and secretion of melanosomes through interactions with different Rabs. Myosin Vc functions as a Rab effector with functions in melanosome biogenesis (Ra7a, Rab8a, Rab32, and Rab38), and also serves a critical function in melanosome secretion.

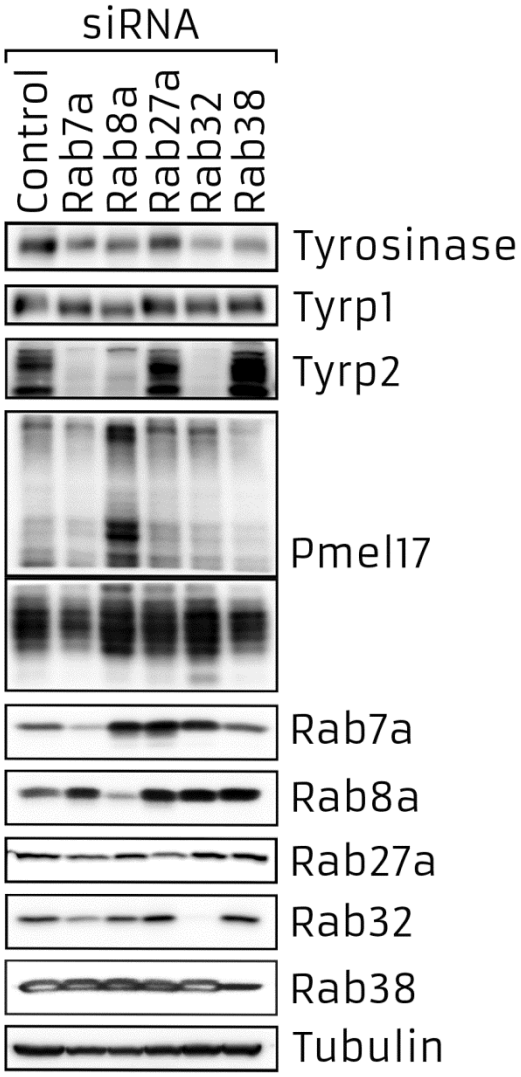

Figure 3. 5
Knockdown of melanosome-associated Rabs demonstrates divergent effects in melanosome biogenesis and secretion. Immunoblots of extracts from siRNA-treated MNT-1 cells demonstrate that depletion of melanosome-associated Rabs have different effects in the trafficking and abundance of melanosome cargoes tyrosinase, Tyrp-1, Tyrp-2, and Pmel17. Depletion of several Rabs partially recapitulates the effects observed upon depletion of Myosin Vc. Only knockdown of Rab8a results in Pmel17 accumulation; but Rab7a, Rab8a, and Rab32 knockdown all result in drastic reduction of Tyrp-2.

Tyrp-1 and Tyrp-2 cargo trafficking defects suggest that Myosin Vc functions at early endosomes, where the other Rab38/32 effector Varp functions. Therefore, both effectors may function in the same step of trafficking between early endosomes and melanosomes. Surprisingly, knockdown of Myosin Vc results in decreased Varp abundance (Figure 3.4A). Such an effect is observed for proteins that functionally interact on membranes and form transient complexes, such as BLOC-2 and Rab38 (17). It is unclear if Myosin Vc and Varp function in the same trafficking steps to melanosomes or if they interact with Rab38 and Rab32 during discreet steps, but these two effectors appear to be functionally connected.

3.3.5 Myosin Va and Myosin Vb are present on melanosomes but Myosin Vc is associated with vesicles

To determine on which structures Myosin Vc functions in melanosome biogenesis, the subcellular distribution of Myosin Vc was assayed using sucrose gradient fractionation of untreated MNT-1 cells (Figure 3.6). Melanosomes are concentrated in fractions 6-10 based on the fractionation patterns of melanosome proteins tyrosinase, Tyrp-1, Tyrp-2, and Pmel17 (Figure 3.6). SNARE VAMP7 and melanosome Rabs are also enriched in these fractions (Figure 3.6). Rabs also are highly enriched in fractions 1-2, which represent cytosolic and small vesicle fractions. Interestingly, Myosin Vc is not abundant in melanosome fractions 6-10, but rather is enriched in fractions 1-2, and to some degree in fractions 3-5, which suggests that Myosin Vc functions primarily in a vesicle or endosomal trafficking step in melanosomes biogenesis instead of residing on melanosomes (Figure 3.6). Myosin Va is primarily abundant in melanosome fractions 6-10, but also exists in some less dense fractions 4-5 that may correspond to other endosomal organelles (Figure 3.6). Surprisingly, Myosin Vb is also present in dense

melanosome fractions 7-9, but is found most in vesicle fraction (fraction 2) and fractions that may correspond to recycling endosomes (fractions 5-6). The resolution of this technique is not sufficient to fully differentiate between different endosomal organelles, but it does clearly demonstrate that Myosin Va and Myosin Vb are present on melanosomes while Myosin Vc is primarily present in vesicle and cytosolic fractions (Figure 3.6).

It is also interesting to note that Varp and Myosin Vc have partially overlapping fractionation patterns. Both Myosin Vc and Varp are present in cytosolic and vesicles fractions (fractions 1-2), and have partially overlapping distribution in less dense endosomal fractions (fractions 3-6). However, it does not appear as Myosin Vc and Varp have identical distribution in endosomal fractions (fractions 3-6) and the identity of organelles in these fractions is not clear (Figure 3.6). Based on the fractionation pattern of recycling endosomal Rab11a (fractions 1-5) and Myosin Vb (fractions 5-6), which are similar to the distribution of Myosin Vc, and suggests that Myosin Vc may also be present on recycling endosomes (Figure 3.6), as has previously been shown for other cell-types (29, 30). Myosin Vc is not abundant on mature melanosomes, but rather appears to function in vesicles and at a less-dense endosomal membrane (Figure 3.6). The defect in melanosome secretion observed from Myosin Vc knockdown is likely to occur through an indirect mechanism and be the result of mistrafficking of proteins to melanosomes that are required for melanosome secretion.

3.3.6 Non-redundant functions of Myosin Va, Myosin Vb, and Myosin Vc in melanosomes?

Previous studies have shown that Myosin Va is necessary for the peripheral localization of mature melanosomes, but that Myosin Va is not required for melanosome secretion (25). The enrichment of both Myosin Va and Myosin Vb in melanosome fractions suggests that Myosin

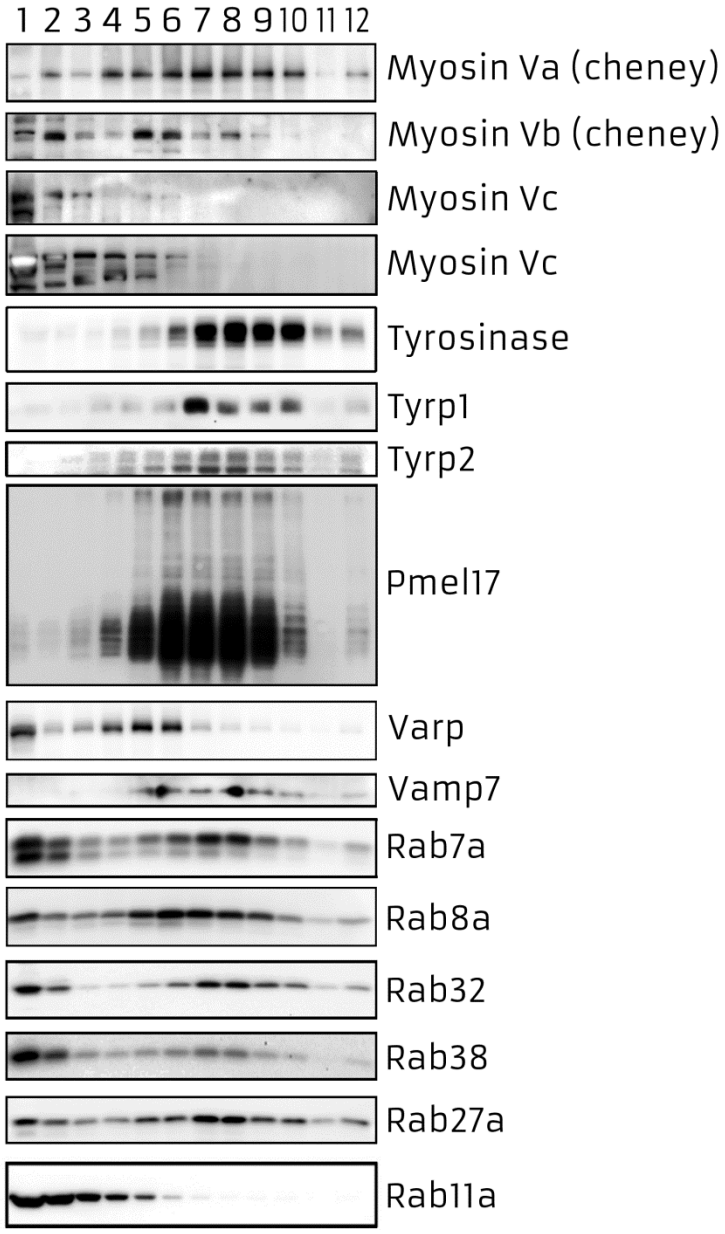

Figure 3. 6

Myosin Vc is not abundant in melanosome-enriched cellular fractions. 20-55% sucrose gradients with ultracentrifugation at 55,000xg (?) were used to separate non-detergent treated MNT-1 extracts. Melanosome markers tyrosinase, Tyrp-1, Tyrp-2, and Pme17 should be primarily present in melanosomes at steady state. Gradient fractions 6-10 are enriched in melanosome markers, and melanosome-associated Rabs Rab7a, Rab8a, Rab32, Rab38, and Rab27a. Myosin Va is highly enriched in fractions 6-10, consistent with localization to mature melanosomes. Myosin Vb is present in several fractions, including fractions enriched for melanosome markers. Alternatively, Myosin Vc is present primarily in fractions 1-4, and most enriched in fractions 1 and 2, corresponding to cytosol and vesicles.

Vb might also be involved in the melanosome movement and secretion (Figure 3.6). Myosin Vc knockdown causes an accumulation of many proteins resident on melanosomes (Figure 3.3, 3.4, 3.6). Both Myosin Va and Myosin Vb are enriched in Myosin Vc knockdown extracts suggesting that Myosin Vb may also be present on melanosomes.

An examination of the cellular distribution of Myosin Va, Myosin Vb, and Myosin Vc demonstrates that these three proteins exhibit partial colocalization, but have distinct distribution in MNT-1 cells (Figure 3.7A, 3.7B, 3.7C, 3.7D). Colocalization of Myosin Va, Myosin Vb, and Myosin Vc is observed in some perinuclear clustered structures (Figure 3.7F). Myosin Vb also strongly colocalizes with Myosin Va in dendrite projections of cells, which are known sites of melanosome secretion (Figure 3.7B, 3.7C, 3.7D, 3.7E) (25). Myosin Vb partially colocalizes with Rab27a in dendrite projections and in some large Myosin Vb structures near the plasma membrane that are not in dendrites (Figure 3.8H), and may correspond to non-dendritic structures at which melanosome secretion has been shown to occur (25). A role of recycling endosomes in melanosome secretion has previously been suspected due to roles of recycling endosomal Rab17, and potentially Rab11a/b, in secretion of melanosomes (22), and results presented here suggest that these functions also involve Myosin Vb (Figure 3.3A, 3.7E, 3.8).

Colocalization of Myosin Va, Myosin Vb, and Myosin Vc in the perinuclear area of cells occurs at recycling endosomal structures where the transferrin-receptor is also present (Figure 3.7D, 3.7F, 3.7J, 3.7K, 3.7L). A single perinuclear cluster with Myosin Vb, Myosin Vc, and transferrin-receptor is observed in each cell, and in this area the highest colocalization of Myosin Vb and Myosin Vc occur (Figure 3.7J, 3.7K, 3.7L). The function of this perinuclear cluster is unclear as little colocalization with melanosomal proteins is observed in this area (Figure 3.8). Only Tyrp-2 is found to strongly colocalize with the perinuclear clusters in which Myosin Vb

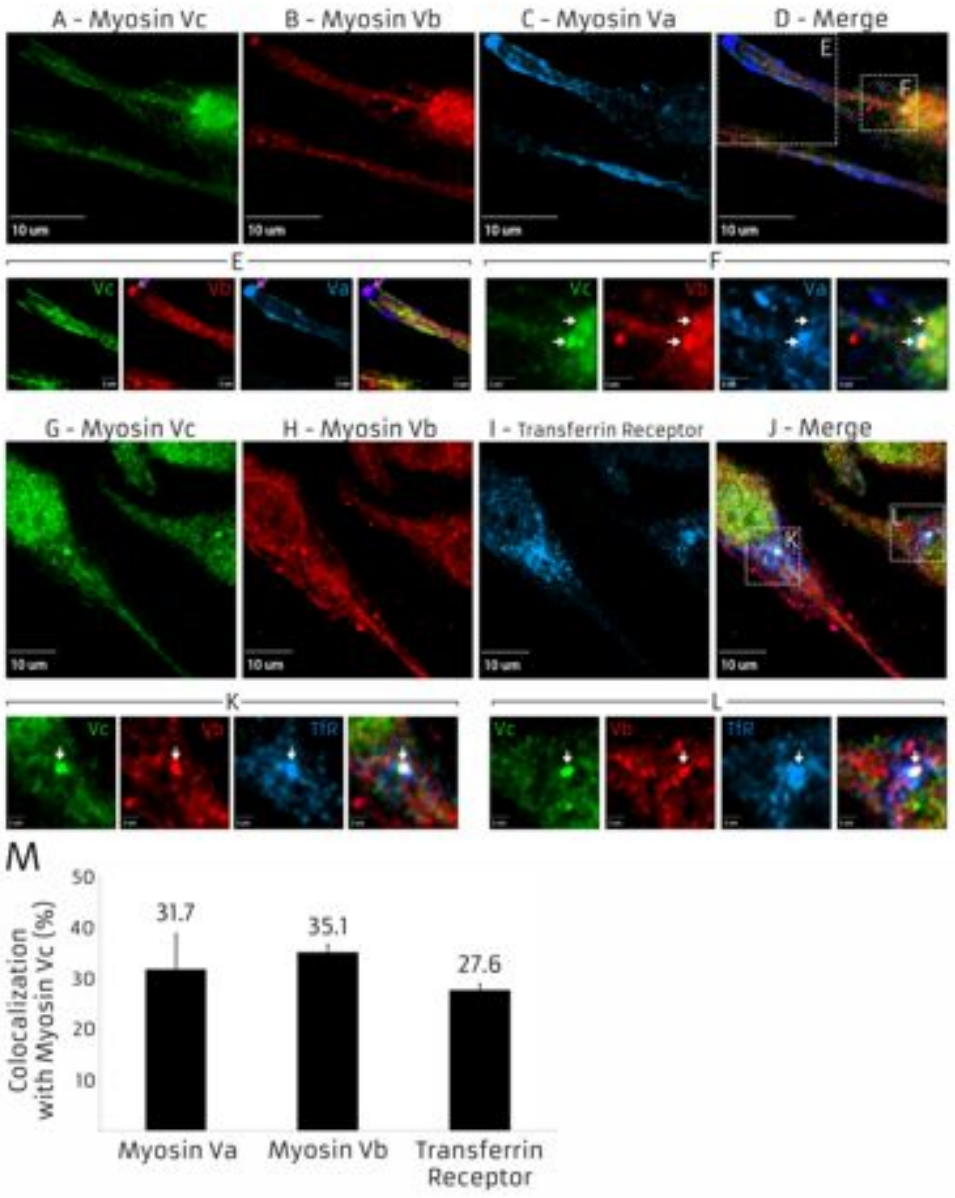

Figure 3. 7

Sub-cellular distribution of human class V Myosins. Confocal immunofluorescence microscopy images of untreated MNT-1 cells. (A) Myosin Vc is present primarily in perinuclear regions of cells in small and large punctuate structures. (B) Myosin Vb is found in small and large structures in perinuclear areas, and also in large structures at the ends of dendrite tips. (C) Myosin Va is found enriched along dendrites, it dendrite tips, and in some perinuclear clusters. (D) Merge of Myosin Vc, Vb, and Va. Colocalization occurs between Myosin Vb and Va at dendrite tips (E), which do not contain Myosin Vc (purple arrows). In perinuclear areas (D and F), Myosin Vc, Vb, and Va are observed to colocalize in punctuate clusters (white arrows). Some perinuclear structures with Myosin Vc (G) and Myosin Vb (H) also colocalize with transferrin-receptor (I, J, K, and L), but do not colocalize in more peripheral localizations (J, K, and L). A single, large cluster of Myosin Vc, Vb, and transferrin-receptor is present in each cell (J). (M) Analysis of colocalization with Myosin Vc. Error bars represent standard error, $N \geq 10$ cells.

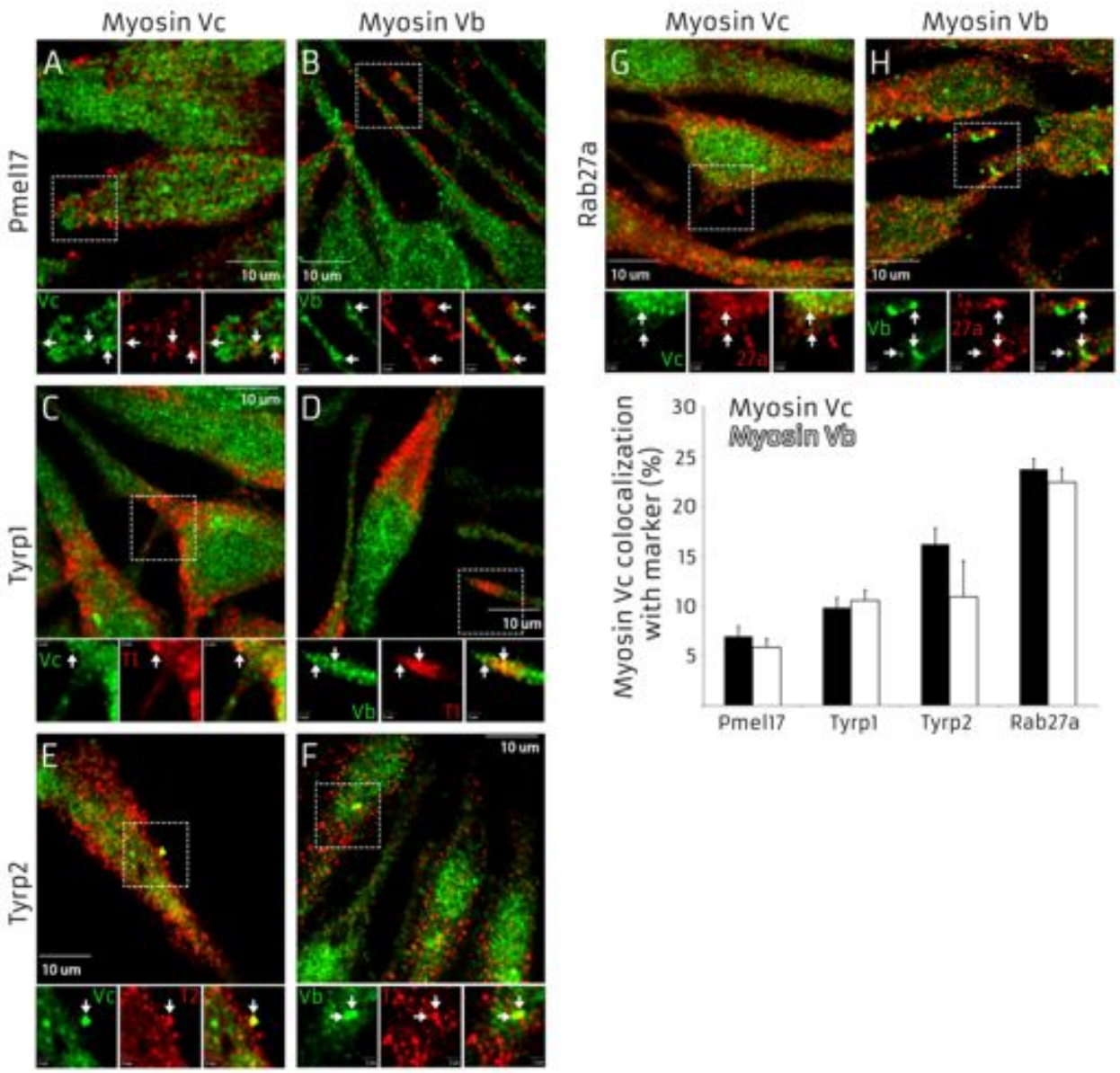

Figure 3. 8

Myosin Vc and Myosin Vb display similar localization to melanosome markers. Confocal microscopy images of immunolabeled Myosin Vc (Alexa 488)(A,C,E,G), Myosin Vb (Alexa 488) (B,D,F,H) and melanosome markers Pmel17, Tyrp-1, Tyrp-2, and Rab27a (Alexa 546). Both Myosin Vc (A,C,E,G) and Myosin Vb (B,D,F,H) show partial colocalization with early, Pmel17-labeled, melanosomes (A,B) and with more mature, pigmented melanosomes with Tyrp-1, Tyrp-2, and Rab27a labeling (C-H). Differences in the subcellular staining patterns and colocalization of Tyrp-2 with Myosin Vc and Myosin Vb suggest that it may utilize a different trafficking pathway, which is dependent on Myosin Vc and Myosin Vb (E,F,I), to melanosomes than is used by Tyrp-1. Error bars represent standard error, $N \geq 10$ cells.

and Vc are present (Figure 3.8E, 3.8F), underlying previous reports that the trafficking of Tyrp-2 may utilize a recycling endosomal pathway (40). Overall, the cellular distribution of Myosin Vc strongly resembles Myosin Vb (Figure 3.7), and both motors colocalize with melanosome markers to a similar degree (Figure 3.8). Myosin Vc exhibits only partial colocalization with melanosome markers Tyrp-1, Tyrp-2, Pmel17, and Rab27a (Figure 3.8), but sucrose gradient fractionation suggests that Myosin Vc is present primarily in vesicles at steady-state (Figure 3.6). Based on interaction with multiple melanosome-associated Rabs (Figure 3.1A) and the phenotypic connection of Myosin Vc with multiple Rabs (Figure 3.4A, 3.5), it is unclear if these Rabs function with Myosin Vc in a redundant or independent fashion. The degree of colocalization of Myosin Vc with Rab7a, Rab8a, Rab32, and Rab38 suggests that Myosin Vc functions with each of these Rabs to some degree (Figure 3.9J). Interestingly, Myosin Vc colocalizes with Rabs in two distinct cellular areas: perinuclear clusters of Myosin Vc at which Rab7a and Rab8a are present (Figure 3.9A, 3.9C, 3.9D, 3.9E) and the transition from cell body to dendrite extension where colocalization with Rab7a, Rab32, and Rab38 is observed (Figure 3.9A, 3.9C, 3.9F, 3.G, 3.9H, 3.9I). Despite strong binding and a functional connection with Rab8a (Figure 3.1A, 3.1D, 3.5), Myosin Vc colocalizes least with Rab8a and is found to colocalize most with Rab32 and Rab38 (Figure 3.9E, 3.9G, 3.9I, 3.9L). The degree of colocalization observed between Myosin Vc with Rab32 and Rab38 is similar to the degree of colocalization observed between Rab32 and Rab38 with early endosomal vesicle budding sites (labeled by AP-3, AP-1, BLOC-2, and clathrin) or melanosomal cargoes Tyrp-1 and Tyrp-2 (17). The colocalization with Rab7a, Rab8a, Rab32, and Rab38 is consistent with Myosin Vc function with each of these Rab GTPases in melanosome biogenesis.

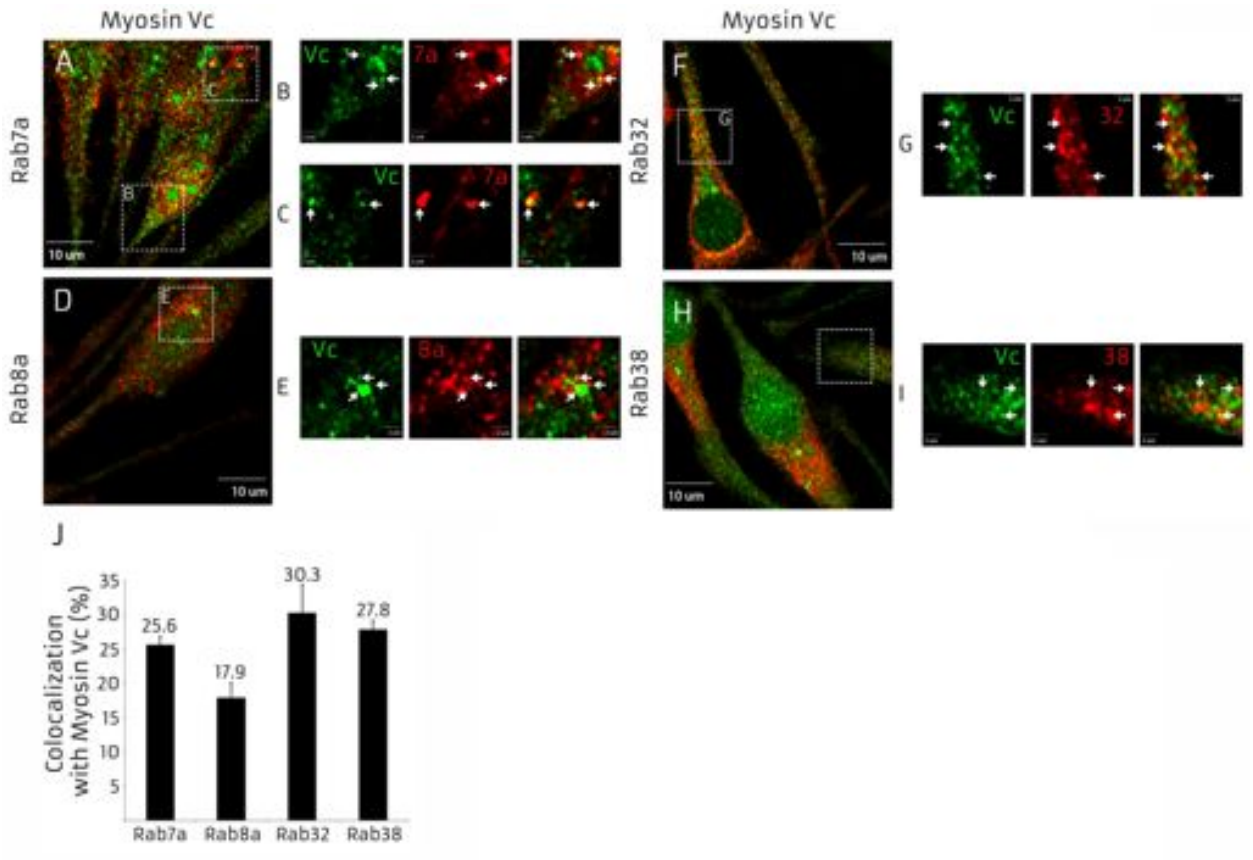

Figure 3. 9

Localization of melanosome-associated Rabs with Myosin Vc. Confocal immunofluorescence images of Myosin Vc (Alexa 488) and Rab7a, Rab8a, Rab32, Rab38, and melanosome SNARE VAMP7 (Alexa 546) (A-J). Myosin Vc is observed to colocalize more strongly with Rab38, Rab32, and Rab7a than Rab8a (A-I). Colocalization with Rab7a and Rab8a occur often in perinuclear areas (A-E). Rab7a is also found in beginnings of dendrite extensions, as are Rab38 and Rab32 (A-C, F-I). (J) Analysis of colocalization. Error bars represent standard error, $N \geq 10$ cells.

3.3.7 Discussion

We demonstrate that Myosin Vc directly binds the active form of Rab7a, Rab8a, Rab32, and Rab38 and colocalizes with these Rabs in MNT-1 melanocyte cells (Figure 3.1, 3.2, 3.9). Myosin Vc likely functions as an effector of several of these Rabs in melanosome biogenesis as knockdown of each of these Rabs does not mimic the phenotypes observed in Myosin Vc knockdown (Figure 3.5). Myosin Vc knockdown causes a dramatic increase in the number of pigmented melanosomes in MNT-1 cells, and these melanosomes do not shown any obviously altered localization compared to control (Figure 3.3). Myosin Vc serves an important role in the trafficking of melanin-synthesizing enzymes to melanosomes, but mature melanosomes are still formed in cells with Myosin Vc knockdown (Figure 3.3, 3.4). Myosin Va and Myosin Vc have different subcellular localization in MNT-1 cells, and Myosin Vc is found primarily in vesicles and does not strongly colocalize with sites of melanosome secretion (Figure 3.7, 3.6, 3.8).

Notably, Myosin Vb also localizes to sites of melanosome secretion, is present in melanosome fractions in sucrose gradients, and shows a similar enrichment upon Myosin Vc knockdown as with several other melanosomal proteins (Figure 3.3, 3.6, 3.7). However, Myosin Vb does not strongly colocalize with melanosome markers, and may function downstream of Myosin Va in the secretion of melanosomes (Figure 3.8). A role for recycling endosomes has been suggested in the secretion of melanosomes, but to date no role for Myosin Vb in melanosome secretion has been suggested (22). Localization of Myosin Vb at dendrite tips, where Myosin Va is enriched, and in some sites with Rab27a suggests that Myosin Vb and recycling endosomes serve a role in mature melanosome secretion (Figure 3.7, 3.8).

The function and location of Myosin Vc on vesicles or organelles in melanosome biogenesis is not yet resolved. Interactions of Myosin Vc with Rab7a, Rab32, and Rab38 - all

demonstrated to be present on non-melanosomal structures and on less mature melanosomes – are consistent with a role of Myosin Vc in melanosome biogenesis, rather than in melanosome secretion (19, 42). Rab32 and Rab38 are established components in the vesicle, or perhaps tubular, trafficking between early endosomes and melanosomes (16, 17, 43). Rab8a has been implicated in the non-Myosin Va dependent, actin-dependent movement of melanosomes (20, 21), but it is unclear if these are functions of Myosin Vc, Myosin Vb, or both. The localization of Rab8a on specific stages of melanosomes has not been determined, and may act in melanosome secretion.

Complicating the understanding of Myosin Vc functions in this pathway are the interactions and colocalization with multiple Rabs in different cellular locations (Figure 3.1, 3.9). Does Myosin Vc serve different functions in different cellular locations with different Rabs? Or do Rabs function redundantly to recruit Myosin Vc to membranes? Unanswered questions also remain about the regulation of Myosin Va, Myosin Vc, and potentially Myosin Vb in this pathway. How are the interactions of multiple Myosins with a single Rab regulated, and do different motors use the same Rab in this pathway? Knockdown of Myosin Va and Myosin Vc have distinct phenotypes, suggesting that little functional overlap between these motors exist. What are the functions of Myosin Vb in this pathway? Outstanding questions about the function and redundancy of Rabs and class V myosins in melanosome biogenesis and secretion will certainly constitute a significant area of research in the coming years.

3.4 Methods

Yeast 2-hybrid experiments were performed using PGBT9 and PGAD424 vectors (Clontech) using AH109 s. cerevisiae cells grown on Synthetic Dropout media lacking leucine and tryptophan as selection markers, as previously described (44). 3-amino-1,2,4-triazole (Alfa Aesar) was used to test for higher binding stringency. Expression and purification of GST-Rabs and His-Myosin Vc were performed as previously described (17,44). In vitro GST-pulldowns were performed as previously described (17,44) using 50µg GST-Rab and 250µg His-Myosin Vc. Transfection of MNT-1 cells was performed as previously described (17) using Nucleofector kit NHEM-Neo (Lonza). Briefly, $1.5x10^6$ cells were transfected with universal negative control siRNA (Sigma-Aldrich, SIC001), Myosin Vc siRNA (Sigma-Aldrich, SASI_Hs01_00184026), Rab7a (Sigma-Aldrich, SASI_Hs01_00104357), Rab8a (Sigma-Aldrich, SASI_Hs02_00339466), Rab27a (Sigma-Aldrich, SASI_Hs01_00172591), Rab32 (Sigma-Aldrich, SASI_Hs02_00342400), Rab38 (Sigma-Aldrich, SASI_Hs01_00247037) on days 1 and 4 as previously described (17). Cells here harvested on day 7 for melanin assay as previously described (17), electron microscopy, or immunoblotting. Total cell extracts were prepared using as previously described (17). Antibodies used: Rabbit a-Myosin Va and Chicken a-Myosin Vb were the generous gifts of Richard Cheney (29), Mouse a-Myosin Va (Sigma-Aldrich, G-4), Rabbit a-Myosin Vc (SDIX), Mouse a-tyrosinase (Santa Cruz, T311), Mouse a-Tyrp-1 (Santa Cruz, clone MEL5), Mouse a-Tyrp-2 (Santa Cruz, C-9), Mouse a-Pmel17 (Dako, HMB45), Rabbit a-Varp (Abcam), Mouse a-VAMP7 (Abcam, SYBL1), Mouse a-Rab7a (Sigma-Aldrich, Rab7-117), Mouse a-Rab8a (BD, 610845), Mouse a-Rab27a (Santa Cruz, E12A-1), Rabbit a-Rab11a (Invitrogen, 71-5300), Mouse a-Rab32 (Sigma-Aldrich, 1C7) and Mouse a-Rab38 (Santa Cruz, 11B-7) used for immunofluorescence, Rabbit a-Rab32 and Rabbit a-Rab38

used for immunoblotting (17), Mouse a-Transferrin-receptor (Invitrogen, 136800), and Mouse a-αTubulin (Sigma-Aldrich, T9026). For immunofluorescence, cells were grown on matrigel coated coverslips and fixed in a 50/50 mixture of cold (-20C) methanol/acetone at room temperature for 10 minutes, allowed to dry, and stored in PBS + 0.1% azide at 4C. Cells were permeabilized and blocked (17), incubated with primary antibody for 1 hour, and incubated with species specific secondary antibodies conjugated to Alexa-488, Alexa-546, or Alexa-647 (Invitrogen). Images were acquired on a spinning disc confocal microscope (3i, Denver) and analyzed using Slidebook software (3i). Degree of colocalization was performed as previously described (17). Tyrp-1 recycling assay performed as previously described (17). Fixation for electron microscopy, immunogold labeling, and electron micrographs were performed as previously described (31).

3.5 REFERENCES

1. Bonifacino JS (2004) Insights into the biogenesis of lysosome-related organelles from the study of the Hermansky-Pudlak syndrome. Ann N Y Acad Sci. 1038:103-14.
2. Theos, A. C., et al (2005). Functions of adaptor protein (AP)-3 and AP-1 in tyrosinase sorting from endosomes to melanosomes. Mol. Biol. Cell 16, 5356-5372.
3. Di Pietro, et al (2006). BLOC-1 interacts with BLOC-2 and the AP-3 complex to facilitate protein trafficking on endosomes. Mol. Biol. Cell 17, 4027-4038.
4. Wasmeier C, Hume AN, Bolasco G, Seabra MC (2008) Melanosomes at a glance. J Cell Sci. 121(Pt 24):3995-9.
5. Harper DC, et al (2008) Premelanosome amyloid-like fibrils are composed of only golgi-processed forms of Pmel17 that have been proteolytically processed in endosomes. J Biol Chem. 283(4):2307-22.
6. Kummer MP, et al (2009) Formation of Pmel17 amyloid is regulated by juxtamembrane metalloproteinase cleavage, and the resulting C-terminal fragment is a substrate for gamma-secretase. J Biol Chem. 284(4):2296-306.
7. Leonhardt RM, Vigneron N, Rahner C, Cresswell P (2011) Proprotein convertases process Pmel17 during secretion. J Biol Chem. 286(11):9321-37.
8. Berson JF, et al (2003) Proprotein convertase cleavage liberates a fibrillogenic fragment of a resident glycoprotein to initiate melanosome biogenesis. J Cell Biol. 161(3):521-33.
9. Hoashi T, et al (2006) The repeat domain of the melanosomal matrix protein PMEL17/GP100 is required for the formation of organellar fibers. J Biol Chem. 281(30):21198-208.
10. Raposo, G. and Marks, M. S. (2007). Melanosomes-dark organelles enlighten endosomal membrane transport. Nat. Rev. Mol. Cell. Biol. 8, 786-797.
11. Boissy, R. E. (2003). Melanosome transfer to and translocation in the keratinocyte. Exp. Dermatol. 12, 5-12.
12. Van Den Bossche, K., Naeyaert, J. M. and Lambert, J. (2006). The quest for the mechanism of melanin transfer. Traffic 7, 769-778.
13. Seabra, M. C. and Coudrier, E. (2004). Rab GTPases and myosin motors in organelle motility. Traffic 5, 393- 399.
14. Wu X, Rao K, Bowers MB, Copeland NG, Jenkins NA, Hammer JA 3rd (2001) Rab27a enables myosin Va-dependent melanosome capture by recruiting the myosin to the organelle. J Cell Sci. 114(Pt 6):1091-100.
15. Kuroda TS, Itoh T, Fukuda M (2005) Functional analysis of slac2-a/melanophilin as a linker protein between Rab27A and myosin Va in melanosome transport. Methods Enzymol 403:419-31.
16. Wasmeier C, et al (2006) Rab38 and Rab32 control post-Golgi trafficking of melanogenic enzymes. J Cell Biol. 175(2):271-81.
17. Bultema JJ, Ambrosio AL, Burek CL, Di Pietro SM (2012) BLOC-2, AP-3, and AP-1 proteins function in concert with Rab38 and Rab32 proteins to mediate protein trafficking to lysosome-related organelles. J Biol Chem. 287(23):19550-63.
18. Gomez PF, et al (2001) Identification of rab7 as a melanosome-associated protein involved in the intracellular transport of tyrosinase-related protein 1. J Invest Dermatol. 117(1):81-90.
19. Jordens I, et al (2006) Rab7 and Rab27a control two motor protein activities involved in melanosomal transport. Pigment Cell Res. 19(5):412-23.
20. Chabrillat ML, et al (2005) Rab8 regulates the actin-based movement of melanosomes. Mol Biol Cell. 16(4):1640-50.
21. Chakraborty AK, Funasaka Y, Araki K, Horikawa T, Ichihashi M (2003) Evidence that the small GTPase Rab8 is involved in melanosome traffic and dendrite extension in B16 melanoma cells. Cell Tissue Res. 314(3):381-8.

22. Beaumont KA, et al (2011) The recycling endosome protein Rab17 regulates melanocytic filopodia formation and melanosome trafficking. Traffic 12(5):627-43.
23. Hammer JA 3rd, Sellers JR (2011) Walking to work: roles for class V myosins as cargo transporters. Nat Rev Mol Cell Biol. 13(1):13-26.
24. Wu X, Bowers B, Rao K, Wei Q, Hammer JA 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.
25. Wu XS, et al (2012) Melanoregulin regulates a shedding mechanism that drives melanosome transfer from melanocytes to keratinocytes. Proc Natl Acad Sci USA. 109(31):E2101-9.
26. Kuroda TS, Itoh T, Fukuda M (2005) Functional analysis of slac2-a/melanophilin as a linker protein between Rab27A and myosin Va in melanosome transport. Methods Enzymol. 403:419-31.
27. Roland JT, Kenworthy AK, Peranen J, Caplan S, Goldenring JR (2007) Myosin Vb interacts with Rab8a on a tubular network containing EHD1 and EHD3. Mol Biol Cell. 18(8):2828-37.
28. Roland JT, et al (2011) Rab GTPase-Myo5B complexes control membrane recycling and epithelial polarization. Proc Natl Acad Sci USA. 108(7):2789-94.
29. Rodriguez OC, Cheney RE (2002) Human myosin-Vc is a novel class V myosin expressed in epithelial cells. J Cell Sci. 115(Pt 5):991-1004.
30. Jacobs DT, Weigert R, Grode KD, Donaldson JG, Cheney RE (2009) Myosin Vc is a molecular motor that functions in secretory granule trafficking. Mol Biol Cell. 20(21):4471-88.
31. Ambrosio AL, Boyle JA, Di Pietro SM (2012) Mechanism of platelet dense granule biogenesis: study of cargo transport and function of Rab32 and Rab38 in a model system. Blood. 120(19):4072-81.
32. Hume AN, Ushakov DS, Tarafder AK, Ferenczi MA, Seabra MC (2007) Rab27a and MyoVa are the primary Mlph interactors regulating melanosome transport in melanocytes. J Cell Sci. 120(Pt 17):3111-22.
33. Roland JT, Lapierre LA, Goldenring JR (2009) Alternative splicing in class V myosins determines association with Rab10. J Biol Chem. 284(2):1213-23.
34. Wu X, Wang F, Rao K, Sellers JR, Hammer JA 3rd (2002) Rab27a is an essential component of melanosome receptor for myosin Va. Mol Biol Cell. 13(5):1735-49.
35. Tamura K, et al (2009) Varp is a novel Rab32/38-binding protein that regulates Tyrp-1 trafficking in melanocytes. Mol Biol Cell. 20(12):2900-8.
36. Kloer DP, et al (2010) Assembly of the biogenesis of lysosome-related organelles complex-3 (BLOC-3) and its interaction with Rab9. J Biol Chem. 285(10):7794-804.
37. Stein M, et al (2012) The interaction properties of the human Rab GTPase family--comparative analysis reveals determinants of molecular binding selectivity. PLoS One. 7(4):e34870
38. Tamura K, Ohbayashi N, Ishibashi K, Fukuda M (2011) Structure-function analysis of VPS9-ankyrin-repeat protein (Varp) in the trafficking of tyrosinase-related protein 1 in melanocytes. J Biol Chem. 286(9):7507-21.
39. Edgar AJ, Bennett JP (1999) Inhibition of dendrite formation in mouse melanocytes transiently transfected with antisense DNA to myosin Va. J Anat. 195(Pt 2):173-84.
40. Negroiu G, Dwek RA, Petrescu SM (2003) The inhibition of early N-glycan processing targets TRP-2 to degradation in B16 melanoma cells. J Biol Chem. 278(29):27035-42.
41. Negroiu G, Dwek RA, Petrescu SM (2005) Tyrosinase-related protein-2 and -1 are trafficked on distinct routes in B16 melanoma cells. Biochem Biophys Res Commun. 328(4):914-21.
42. Hume AN, Wilson MS, Ushakov DS, Ferenczi MA, Seabra MC (2011) Semi-automated analysis of organelle movement and membrane content: understanding rab-motor complex transport function. Traffic. 12(12):1686-701.

43. Delevoye C, et al (2009) AP-1 and KIF13A coordinate endosomal sorting and positioning during melanosome biogenesis. J Cell Biol. 187(2):247-64.
44. Feliciano D, Di Pietro SM (2012) SLAC, a complex between Sla1 and Las17, regulates actin polymerization during clathrin-mediated endocytosis. Mol Biol Cell. 23(21):4256-72.

CHAPTER 4

CONCLUSIONS AND IMPLICATIONS FROM STUDIES ON RAB32, RAB38, AND MYOSIN VC IN MELANOSOME BIOGENESIS

4.1 Summary

My studies on Rab32, Rab38 and Myosin Vc have helped to address the molecular mechanisms of trafficking from the early endosome to the melanosome and expand the knowledge of melanosome biogenesis. My results also establish roles for multiple motor proteins in melanosome biogenesis, which acts as an interesting model system to study the coordination of distinct trafficking steps. Below I discuss the implications of my findings in detail.

4.2 Rab32 and Rab38 are a tissue-specific bridge underlying trafficking to melanosomes

Prior to the start of this dissertation, one of the major questions in melanosome biogenesis was how ubiquitous lysosomal biogenesis machinery is used in melanosome biogenesis. Some of the ubiquitous machinery had been established, and parallel pathways formed by AP-3, A-1 and BLOC-2 were known to serve critical functions in the trafficking of melanosomal cargoes at early endosomes (1, 2). These complexes are critical for the formation of vesicles at early endosome exist sites, but, as AP-3, AP-1, and BLOC-2 also direct trafficking to the lysosomes, it was not clear how separate melanosome trafficking could co-exist (1, 2). Rab32 and Rab38 had been shown to function in melanosome trafficking downstream of the early endosome and were implicated in the formation of other lysosome-related organelles (3).

Therefore, Rab32 and Rab38 were candidates that might serve as the functional links between the ubiquitous lysosomal trafficking machinery and specialized melanosome traffic (3-7).

My studies on interactions of Rab32 and Rab38 with ubiquitous trafficking machinery have served to functionally connect these Rabs to AP-3, AP-1, and BLOC-2 in melanosome trafficking. Rab32 and Rab38 physically interact with AP-3, AP-1, and BLOC-2 in membrane fractions that can be stably co-immunoprecipitated (Figure 2.1). These interactions occur with Rab32 and Rab38 when they are in the active, GTP-bound form only on membranes; and the presence of Rab38 and Rab32 on membranes is co-dependent with the presence of AP-3 and BLOC-2 (Figure 2.2, 2.3). The membrane stability and cellular abundance of Rab38 is highly dependent on interactions with AP-3 and BLOC-2, but Rab32 is only mildly impacted by depletion of BLOC-2 or AP-3 (Figure 2.3). The interactions of active Rab32 and Rab38 with early endosomal trafficking components is consistent with a function for these Rabs as factors that direct trafficking to melanosomes, instead of lysosomes.

These findings highlight differences between Rab32 and Rab38 in both the degree of membrane localization of each Rab and in the functional dependence on AP-1, AP-3, and BLOC-2. Rab32 is found to be associated with membranes to a much higher degree than Rab38, and does not seem to be impacted significantly by depletion of these early endosomal trafficking components (Figure 2.3). In contrast, Rab38 has a lower degree of membrane localization at steady state and the membrane stability of Rab38 is much more sensitive to depletion of AP-3 or BLOC-2 (Figure 2.3). The functional connection between Rab38 and BLOC-2 is quite strong, as depletion of Rab38 results in decreased BLOC-2 membrane localization (Figure 2.3). This interdependence of Rab38 and BLOC-2 on membranes is indicative of a transient complex, and suggests that BLOC-2 functions somewhat differently than AP-1 or AP-3 (1). These differences

between Rab38 and Rab32 could reflect differences in sites of action, where Rab32 functions more downstream of early endosomes, or that Rab32 interacts with different early endosomal components that are responsible for recruitment and maintenance of Rab32 at specific early endosomal exit sites.

In the MNT-1 melanocyte cell line, a well-established system of melanosome biogenesis, studies on the localization of Rab38 and Rab32 have also shown a function connection to the early endosome (Figure 2.3, 2.6, 2.7) (3). Rab38 and Rab32 strongly colocalize with AP-3, AP-1, and BLOC-2 on membranes (Figure 2.6, 2.7). Colocalization of Rab38 and Rab32 with the coat protein clathrin suggests that these structures are vesicle budding sites (Figure 2.6, 2.7). As AP-3, AP-1, and BLOC-2 have all been shown to function at early endosomal budding sites, this implicates Rab38 and Rab32 with early endosomal trafficking (1, 2). The localization of Rab38 and Rab32 to specific early endosomal subdomains labeled by AP-3, AP-1, and BLOC-2, but not to other endosomal domains that function in distinct trafficking pathways, suggests that these Rabs function in melanosome specific trafficking (Figure 2.6, 2.7, 2.10).

My experiments show that Rab38 and Rab32 also localize to melanosomes and to other smaller structures containing tyrosinase and Tyrp-1, two enzymes necessary for melanosome synthesis. Tyrosinase and Tyrp-1 are known to traffic through AP-3, AP-1, and BLOC-2 dependent pathways in early endosomes, but are primarily enriched in melanosomes at steady state (Figure 2.10, 2.11, 3.6). Notably, Rab38 and Rab32 do not localize to ubiquitous lysosomes, but are present on intermediate maturity stage II-IV melanosomes, demonstrating that these Rabs localize to a melanosome specific trafficking pathway (Figure 2.11)(3). A previous study observed localization of over-expressed Rab38 to melanosomes and non-pigmented organelles by electron microscopy, and demonstrated that Rab38 partially colocalizes with

pigmented melanosomes (3). My findings refine the localization of endogenous Rab38 and Rab32 to both early endosomes trafficking sites and to melanosomes, and show that Rab32 and Rab38 are not present at other early endosomal subdomains or lysosomes. Based on these results, Rab32 and Rab38 appear to function in a trafficking step between early endosomes and melanosomes.

Do Rab32 and Rab38 serve functions in vesicle trafficking or melanosome trafficking events? A study published in recent years has compared the presence of Rab32, Rab38, and Rab27a on pigmented melanosomes, and observed that Rab32 and Rab38 are abundant on less mature, faster moving melanosomes that are associated with microtubule dependent transport (8). However more mature, slower moving melanosomes, associated with Myosin Va dependent tethering in cortical actin, were found to contain Rab27a but very little Rab32 or Rab38 (8). This result suggests that Rab32 and Rab38 are removed from melanosomes prior to Rab27a-dependent functions, and that they define trafficking between early endosomes and stage II melanosomes (3, 8). Therefore, the main function of Rab32 and Rab38 may be in the vesicle trafficking to melanosomes, but these Rabs may also serve some undefined functions on melanosomes themselves.

4.3 Function for Rab32 and Rab38 in early endosome to melanosome cargo trafficking

Consistent with Rab38 and Rab32 dependent vesicle trafficking pathways, I found that Rab38 and Rab32 are functionally important for the trafficking of tyrosinase family proteins (Figure 2.12, 2.13 2.14). These Rab proteins serve partially redundant roles in the trafficking of tyrosinase and Tyrp-1, but not in the trafficking of Tyrp-2 (Figure 2.13). Earlier studies had shown that depletion of Rab38 or Rab32 altered the distribution of tyrosinase and Tyrp-1, but

had not assessed where the trafficking defect occurred (3). I show that the mistrafficking of these tyrosinase family proteins resulting from Rab38 or Rab32 depletion is caused by diminished trafficking from the early endosome (Figure 2.12, 2.13 2.14). The partial redundancy of Rab38 and Rab32 in the trafficking of these enzymes matches extremely well with previous data on the redundancy of AP-3, AP-1, and BLOC-2 in trafficking from the early endosome (1, 2). My studies on the redundancy of Rab32 and Rab38 also provide a better explanation for the partial hypopigmentation observed in Rab38 mutant models, implying that Rab32 is able to partially compensate for the loss of functional Rab38 in these animals (5, 6).

Rab32 and Rab38, however, are not fully redundant in melanosome biogenesis as demonstrated by the trafficking of Tyrp-2 (Figure 2.13, 2.14). The trafficking of tyrosinase and Tyrp-1 are defined by early endosomal pathways dependent on AP-1, AP-3, and BLOC-2 (1, 2). The trafficking of Tyrp-2 has been suggested to utilize an alternate route from tyrosinase and Tyrp-1, one that involves sorting through the plasma membrane. The necessity for AP-1, AP-3, or BLOC-2 in Tyrp-2 trafficking has not been tested (9, 10). Interestingly, Tyrp-2 is not necessary for regulation of normal pigmentation, and functions in only one pathway of eumelanin synthesis (Figure 1.2), so it is difficult to predict if any of the hypo-pigmented animal models have Tyrp-2 trafficking defects. The necessity of Rab32 in an alternative pathway for the trafficking of Tyrp-2, which does not utilize Rab38, suggests that Tyrp-2 may not trafficking through AP-1-, AP-3-, or BLOC-2-dependent pathways. Consistent with additional Rab38-independent functions for Rab32, knockdown of Rab32 causes more severe hypopigmentation than Rab83 knockdown (Figure 2.13). Our results demonstrate that Rab32 and Rab38 function in parallel or redundant pathways for the trafficking of some cargoes, but that Rab32 may also function in separate trafficking pathways to melanosomes.

4.4 Interacting partners of Rab32 and Rab38

When I performed these studies, there was only a poor understanding of the functions of Rab38 and Rab32 in melanosome trafficking (3). The one known interacting partner of Rab38 and Rab32, Varp, was also discovered very close to the time I began work in this project (11, 12). Two studies have demonstrated that Varp functions in the trafficking of Tyrp-1 and VAMP7, a SNARE, to melanosomes and that Varp is dependent on interactions with Rab38 and Rab32 (12). VAMP7 is likely to be recruited to specific early endosomal subdomains through interactions with AP-3 and Varp, which facilitate loading into vesicles bound for melanosomes (13). VAMP7 is important for the fusion of vesicles trafficked to lysosomes, and it is likely that it is also important for the fusion of vesicles with melanosomes, which explains why a defect in Tyrp-1 trafficking to melanosomes would also occur in cells in which VAMP7 was not present (13). A mechanism for the trafficking of VAMP7 by Rab38 and Rab32 is still not fully understood, despite interesting work on the interactions between Rab38 and Rab32 with Varp that has occurred since I began working on this project (14, 15). My studies on the function of Rab38 and Rab32 at endosomes have served to expand knowledge of the trafficking functions of Varp, which is thought to utilize the vesicle trafficking pathways dependent on Rab32 and Rab38.

The mechanism used to recruit Rab32 and Rab38 to endosomal sites to function with AP-3, AP-1, and BLOC-2 was not known until recently (16). Initially, we suspected that Rab38 and Rab32 might be recruited to membranes directly by interactions with AP-3 and BLOC-2 (Figure 2.3). The finding that Rab38 and Rab32 bound to AP-3, AP-1, and BLOC-2 only when in the active, GTP-bound form suggested that other proteins were responsible for the recruitment and activation of Rab38 and Rab32 (Figure 2.4). In our experiments, we observed that knockdown

of BLOC-3 resulted in a similar trafficking defect of Tyrp-2 as observed by Rab32 knockdown, which we suggested was a specific result of disruption of a Rab32-BLOC-3 dependent pathway (Figure 2.16, Appendix 2) (Chapter 2). Recently, it was discovered that BLOC-3 functions as the GEF that is responsible for the recruitment of Rab38 and Rab32 to membranes, and for the GTP-loading of these Rabs (16). Our previous findings regarding defects in the trafficking of Tyrp-1 were repeated in this study, and it was found that knockdown of BLOC-3 reproduced these trafficking defects (16). BLOC-3, however, is not likely to be the only GEF that functions with Rab38 and Rab32 as BLOC-3 knockdown samples do not have as severe of trafficking defects as observed for Rab32 or Rab38 alone (16). BLOC-3 mutant mice are less severely hypo-pigmented than Rab38 mutant mice (17, 18), suggesting that other mechanisms exist for Rab38 and Rab32 recruitment and activation in this pathway. Simultaneous knockdown of Rab32 and Rab38 results in more severe defects in the trafficking of tyrosinase and Tyrp-1 than either knockdown alone (Figure 2.12, 2.13), and if BLOC-3 acted as the lone GEF for Rab32 and Rab38 in this pathway we expect a severe hypopigmentation resulting from BLOC-3 knockdown.

4.5 Interaction of Myosin Vc with melanosomal Rab proteins

My work on the functions of Rab32 and Rab38, and other work published in the field since I began this project, has expanded understanding of how Rab32 and Rab38 function at early endosomes in the trafficking to melanosomes. It is still unclear, however, how Rab32 and Rab38 function in the trafficking of cargoes after vesicle budding from early endosomes. Therefore, the discovery that Myosin Vc functions as an effector of Rab32 and Rab38 in melanosome biogenesis is an important expansion of the field. Myosin Vc was discovered as an

interacting partner for Rab38 in a screen against the active, GTP-bound mutant of the protein, and the interaction with only the constitutively-active, GTP-locked mutant suggest that Myosin Vc is a Rab effector protein. I discovered that Myosin Vc also acts an effector of Rab32, and furthermore that Myosin Vc is also an effector protein of other Rabs associated in melanosome biogenesis, Rab7a and Rab8a (Figure 3.1) (19-25). Direct interaction between Myosin Vc and Rabs with different functions in melanosome biogenesis implicates Myosin Vc in melanosome biogenesis and is a novel function for Myosin Vc.

The finding that Myosin Vc interacts with multiple melanosomal Rabs raises interesting questions about the specificity of these interactions: are these Rabs non-specifically binding to any class V myosin? I found that while Rab7a and Rab8a bound to Myosin Va or Myosin Vb, Rab32 and Rab38 bind only to Myosin Vc (Figure 3.1B). Additionally, Rab32 and Rab38 bind more strongly to Myosin Vc than either Rab8a or Rab7a, which may have functional implications on the ability of Rab-Myosin Vc interactions to occur *in vivo* (Figure 3.1C). Even if Rab-Myosin Vc interactions are low affinity, binding to multiple Rabs would increase the avidity of Myosin Vc interactions, and the ability to bind multiple Rab proteins may be crucial to the function of Myosin Vc in these cells.

Experiments to map the binding sites of Rab7a, Rab8a, Rab32, and Rab38 show that some of these Rabs have partially overlapping binding sites within the coiled-coil tail of Myosin Vc. Additionally, Rab7a binding to Myosin Vc is dependent on both the coiled-coil and globular tail domains, suggesting that Rab7a either has multiple binding sites in the two tail domains or that it binds the coiled-coil and globular tail simultaneously (Figure 3.1C, 3.1E, 3.1F). To date, numerous studies have been performed about the necessity for specific coiled-coil regions for the binding to Rab proteins, but no studies have investigated simultaneous binding of multiple Rabs

(26-28). Notably, interaction between Rabs and effectors can also occur using Rab dimers that interact with effectors in a 2:2 ratio, and raises possibility of hetero- or homo-dimers of Rabs interacting with Myosin Vc, which functions as a dimer (29). Based on these studies, it is unclear if these binding sites would be compatible with simultaneous binding to multiple Rabs, or perhaps certain pairs of Rabs that do not have overlapping binding sites (Figure 3.1E). The binding of Rab7a, Rab8a, Rab32, and Rab38 to Myosin Vc serves as an excellent case to investigate the mechanisms used by class V myosins that are able to bind multiple Rabs.

In addition to mapping the binding of Rabs to Myosin Vc, I also investigated the sites on Rab32 and Rab38 used for binding to Myosin Vc (Figure 3.2). I found that mutation of specific tyrosine residues in the switch II region of Rab32 or Rab38 disrupted interaction with Myosin Vc (Figure 3.2B). The finding that the switch II region is required for effector binding is consistent with previous findings that have shown effector binding in regions that undergo conformational change between in-active, GDP-bound Rabs and active, GTP-bound Rabs (29-35). The interaction of Varp with Rab32 and Rab38 was also recently mapped to the switch II region of the Rabs, and is dependent specifically on a valine residue that is directly adjacent to the tyrosine that is required for Myosin Vc binding (14). Interestingly, Varp binding to Rab32 and Rab38 is very specific, as other residues in the switch II region were not required for the binding of Varp, but the necessity of the tyrosine residue for binding to Varp was not tested in this study (14). Mutation of valine residue in the switch II region of Rab32 and Rab38, important for binding to Varp, does not significantly diminish binding to Myosin Vc unless the valine and tyrosine residues are both mutated (Figure 3.2C, 3.2D). Mutation of both valine and tyrosine residues reduces the degree of binding to Myosin Vc, and the degree of growth observed in yeast 2-hybrid assays (Figure 3.2C, 3.2D). This finding that the only known effectors of Rab32 and Rab38,

Varp and Myosin Vc, bind to the same site on Rab32 and Rab38 raises questions about the mechanisms that control Rab-effector binding. Do these two effectors compete for binding to Rab32 and Rab38? Or do they function in distinct trafficking steps in melanosome biogenesis?

4.7 Functions of Myosin Vc in melanosome trafficking

Experiments investigating the effects of Myosin Vc knockdown in MNT-1 cells suggest that Myosin Vc and Varp may function in similar trafficking steps to melanosomes. I show that Myosin Vc knockdown results in defects in both the trafficking of Tyrp-1 and Tyrp-2 to melanosomes, but also causes a major defect in melanosome secretion (Figure 3.3, 3.4). The efficiency of Tyrp-1 trafficking from early endosomes to melanosomes is reduced by Myosin Vc knockdown, such that Tyrp-1 accumulates in early endosomes and is present on the plasma membrane of cells (Figure 3.4B). This effect has been shown to occur when the early endosomal exit sites are disabled by knockdown of AP-3, AP-1, BLOC-2, and Rab32 or Rab38 (Figure 2.12) (1). This result is consistent for a trafficking function of Myosin Vc that depends on Rab32 and Rab38. The defect in Tyrp-1 resulting from Myosin Vc is more severe than Rab32 and Rab38 combined knockdown, and suggests that Myosin Vc serves functions in the trafficking of cargoes beyond those facilitated by interactions with Rab32 and Rab38 (Figure 2.13, 3.4). Varp also functions in the trafficking of Tyrp-1, and Myosin Vc and Varp may function in the same steps of trafficking to melanosomes.

The defect in melanosome secretion that occurs from Myosin Vc knockdown suggests that Myosin Vc also serves Rab32- and Rab38-independent trafficking functions. The accumulation of mature, pigmented melanosomes is quite distinct from the effect observed upon knockdown of Rab32 or Rab38 (Figure 2.12, 2.13, 3.3, 3.4). Rab32 and Rab38 knockdown

causes a reduction in pigment production, and likely represent an increase in the ratio of non-pigmented : pigmented melanosomes (Figure 2.13). Myosin Vc knockdown does not cause an increase in the number of immature or striated melanosomes, and only causes an increase in the number of pigmented melanosomes (Figure 3.3). The fact that melanosome are still formed by Myosin Vc knockdown, which shows defects in the trafficking of cargoes to melanosomes, reflects that some trafficking of tyrosinase and Tyrp-1 occurs. This suggests that Myosin Vc is not absolutely required for the trafficking of tyrosinase and Tyrp-1, but that Myosin Vc is critical for other functions in melanosome biogenesis.

The function of Myosin Vc in multiple pathway of melanosome biogenesis is also suggested by a comparison of Myosin Vc knockdown to knockdown of different melanosomal Rabs (Figure 3.5). Knockdown of Myosin Vc shows some of the cargo trafficking defects observed by Rab32 or Rab38 knockdown, but also shows an accumulation of melanosomal proteins that does not occur in Rab32 or Rab38 knockdown (Figure 3.5). Knockdown of Rab7a and Rab8a show some of the same changes in abundance of melanosomal proteins, but only Rab8a shows the increase in Pmel17 abundance that suggests a defect in secretion (Figure 3.5). A melanosome secretion defect by Rab8a knockdown may be consistent with previous studies that have shown functions for Rab8a in melanosome movement, independent of Rab27a (24, 25). The colocalization of Myosin Vc with Rab7a, Rab8a, Rab32, and Rab38 suggests that Myosin Vc serves multiple functions in melanosome biogenesis (Figure 3.9). Colocalization of Myosin Vc with Rab7a or Rab8a is observed in large, perinuclear clusters (Figure 3.9). Conversely, Myosin Vc is observed to colocalize with Rab7a, Rab32, or Rab38 in smaller structures in more distal regions of cells, in the transition from cell body to dendrite projection (Figure 3.9). The

distinct sites of colocalization with melanosome associated Rab proteins are consistent with the conclusion that Myosin Vc serves multiple functions in melanosome biogenesis and trafficking.

In MNT-1 cells, Myosin Vc is not shown to co-fractionate with Rab7a, Rab8a, Rab32, or Rab38 in melanosomal or other endosomal fractions (Figure 3.6). The lack of co-fractionation with melanosomal markers suggests that Myosin Vc is not abundant on melanosomes, and may function instead in vesicle trafficking events (Figure 3.6). The low degree of colocalization of Myosin Vc with melanosomal markers in immunofluorescence microscopy experiments also reflects that Myosin Vc is not abundant on mature melanosomes (Figure 3.8). Notably, Myosin Vc does interact with some structures labeled by Tyrp-2, which may reflect a Tyrp-2 trafficking pathway (Figure 3.8). The fact that knockdown of only some Rabs results in defects in Tyrp-2 trafficking, further indicates that Myosin Vc functions in distinct trafficking steps (Figure 3.5). Different colocalization patterns with different Rabs may be indicative of different sites of function, and different functions of Myosin Vc in melanosome trafficking.

Defects in trafficking of Tyrp-1 and Tyrp-2 suggest a function for Myosin Vc in vesicle trafficking to melanosomes. How does Myosin Vc function in melanosome secretion? As Myosin Vc is not abundant on melanosomes it is unlikely that Myosin Vc is directly associated with melanosome secretion (Figure 3.4, 3.6). At least two models could explain these divergent functions of Myosin Vc in melanosome trafficking and secretion. The first model would suggest that Myosin Vc functions primarily in the trafficking of cargoes to melanosomes, and that a defect in melanosome trafficking is an indirect effect of defects in cargo trafficking. Perhaps other components of melanosomes, necessary for secretion, may also be trafficked along the same pathway as Tyrp-2; and that lacking Myosin Vc these melanosome components do not reach melanosomes resulting in decreased melanosome secretion. Rab32 may serve non-

redundant trafficking functions for this pathway, which would explain the similarity of lack of Tyrp-2 by knockdown of Myosin Vc or Rab32 (Figure 2.12, 2.13, 3.4, 3.5). However, Rab32 knockdown does not result in any obvious defect in melanosome secretion, suggesting that Myosin Vc functions in Rab32-independent functions upstream or downstream of Rab32-dependent trafficking steps (Figure 2.13).

The trafficking of Tyrp-2 is not clearly established, but appears to be different from tyrosinase and Tyrp-1 (9,10). Tyrp-2 is likely to be directly transported from the golgi to plasma membrane, and subsequently endocytosed and trafficked through recycling endosomes where it may intersect with the tyrosinase and Tyrp-1 trafficking pathways (9, 10). Specific trafficking defects in Tyrp-2 could occur in the golgi to plasma membrane, endocytic, or recycling trafficking of the protein, but previously established function of Myosin Vc at recycling endosomes suggests that knockdown of Myosin Vc would cause a recycling endosomal defect (38). Recycling endosomal functions of Myosin Vc are consistent with the colocalization of Myosin Vc with Myosin Vb and Transferrin-receptor (Figure 3.7). Sites of colocalization with Myosin Vb or Transferrin-receptor appear similar to perinuclear sites where colocalization of Myosin Vc with Rab7a and Rab8a occurs (Figure 3.7). Further, Rab8a is implicated in recycling endosomal trafficking functions with Myosin Vb in other cell types, suggesting that it might serve a cargo trafficking function at recycling endosomes for melanosome trafficking (39). Therefore, the first model would suggest that Myosin Vc functions in both recycling endosomal and early endosomal steps in cargo trafficking to melanosomes using different Rab proteins for distinct trafficking steps.

Some of my experiments provide evidence to support the first model to explain functions of Myosin Vc in melanosome secretion. As previously discussed, knockdown of Myosin Vc

results in an increase in most melanosomal proteins, but also causes defects in the trafficking of Tyrp-2 (Figure 3.4). Notably, Myosin Vc also changes the abundance of Varp and VAMP7, but in quite distinct ways (Figure 3.4). Myosin Vc knockdown causes a significant decrease in the abundance of Varp, which we would expect would cause defects in the trafficking of VAMP7 to melanosomes (Figure 3.4) (12, 14, 15). The mechanisms by which Myosin Vc knockdown would cause loss of Varp are not clear, but may reflect a functional collaboration of Varp and Myosin Vc in melanosome trafficking (Figure 2.5).

In contrast, we observe a large accumulation of VAMP7 as a result of Myosin Vc knockdown (Figure 3.4). It is unclear if the accumulation of VAMP7 represents proteins present on mature melanosomes, and thus is reflective of the defect in melanosome secretion, or if VAMP7 accumulation reflects a defect in trafficking to melanosomes (Figure 3.4). A role for VAMP7 in melanosome secretion has not been established, but loss of a critical SNARE for melanosome secretion could explain the melanosome secretion defect observed in Myosin Vc knockdown. It is possible that the functions of Varp and Myosin Vc are functionally connected in some way, and establishing a connection between the two Rab32 and Rab38 effectors represents an interesting new direction for research projects. The effects that Myosin Vc knockdown have on Varp and VAMP7 are striking, and this connection is potential mechanism to explain the function of Myosin Vc in melanosomes.

A second model to explain the trafficking defects observed in Myosin Vc knockdown would also explain defects in melanosome secretion. In this second model, Myosin Vc functions at early endosomes for the trafficking of some cargoes to melanosomes through interactions with Rab32 and Rab38, but the melanosome secretion is a direct function of Myosin Vc, rather than an indirect effect resulting from cargo mistrafficking suggested by the first model. Myosin Vc

may still function in recycling endosomes, but for the purposes of melanosome secretion. A previous study investigating melanosome secretion has shown that recycling endosomal Rab17 and Rab11a/b are important for melanosome secretion (38). A direct role for recycling endosomes in melanosome secretion that depends on Myosin Vc could occur, but this model would likely not involve a direct interaction of Myosin Vc and Rab11a, as these proteins are not seen to physically interact in conditions we tested (Figure 3.1). Myosin Vc, therefore, could function with Rab17 or Rab8a in recycling endosome dependent melanosome secretion. It is also possible both of these models occur, and that Myosin Vc functions with recycling endosomes in cargo trafficking and melanosome secretion. However, it is not possible to draw conclusions about these models based on the currently known information about melanosome secretion.

4.8 The role of multiple class V myosins in melanosome trafficking

Myosin Va has been well-classified with specific functions in melanosome movement, and my experiments demonstrating that Myosin Vc functions in melanosome biogenesis provide the first direct evidence of multiple class V myosin motors functioning in the same pathway (40). A comparison of Myosin Va knockdown or knockout cells and Myosin Vc knockdown cells demonstrates that the two motor proteins serve distinct functions in melanosome trafficking (Figure 3.3, 3.4) (40-42). Myosin Va knockdown cells demonstrate a perinuclear clustering of melanosomes, with no obvious defects in melanosome formation or a dramatic increase in the number of melanosomes (41). In contrast, Myosin Vc knockdown cells have normal distribution of melanosomes, but defects in melanosome cargo trafficking and secretion, with a significant increase in the amount of pigmented melanosomes (Figure 3.3, 3.4). The subcellular localization

of Myosin Va and Myosin Vc also appears to be quite distinct, and the two proteins are only observed to strongly colocalize in some large perinuclear structures (Figure 3.7). The absence of Myosin Vc from the tips of dendrite extensions, where Myosin Va is abundant, further suggests that Myosin Va and Myosin Vc have different functions with melanosomes (Figure 3.7). Myosin Vc is primarily found in sucrose gradient fractions corresponding to the cytosol and vesicles while Myosin Va is found to be primarily associated with melanosomal markers in dense fractions (Figure 3.6). Myosin Va and Myosin Vc appear to serve distinct functions in melanosome biogenesis and trafficking.

In my experiments, however, I also find evidence for a role of Myosin Vb in melanosome trafficking (Figure 3.3, 3.6, 3.7, 3.8). First, Myosin Vb strongly colocalizes with Myosin Va in dendrite projections, a known site of melanosome secretion (42). Previous work about melanosome secretion implies that these sites are specific to melanosome secretion, and localization of Myosin Vb to these sites suggests a direct function with melanosomes (42). Second, in Myosin Vc knockdown experiments an enrichment of melanosomal proteins is observed, including Myosin Va, and Myosin Vb is also enriched by Myosin Vc knockdown (Figure 3.3). This enrichment could represent a compensation for the loss of Myosin Vc by up regulation of Myosin Va and Myosin Vb, but that would not explain other data suggesting Myosin Vb functions with melanosomes. Third, Myosin Vb co-fractionates with melanosomal markers in sucrose gradients (Figure 3.6). The presence of Myosin Vb on these fractions with mature melanosome markers is not likely to represent the ubiquitous, recycling endosomal functions of Myosin Vb, but rather implies a role for Myosin Vb directly with melanosomes. Finally, Myosin Vb partially colocalizes with Rab27a, a marker of mature melanosomes (Figure 3.8). As Rab27a is not present on melanosomes directly before secretion, this result would be

consistent with a localization of Myosin Vb to a subset of mature melanosomes (38). What functions would be predicted for Myosin Vb in melanosome trafficking?

A function for Myosin Vb in melanosome trafficking would be consistent with the function of recycling endosomal Rab17 and Rab11a/b in melanosome secretion (38), and with some of the observed localization of Myosin Vc in cells. Notably, Myosin Vb and Myosin Vc are observed to strongly colocalize with specific structures that contain the transferrin-receptor and are likely to be recycling endosomes (Figure 3.7). Also, Myosin Vb and Myosin Vc have similar colocalization with melanosome markers, and both Myosin Vb and Myosin Vc are present in some structures that also contain accumulated Tyrp-2 (Figure 3.8). Based on the previous discussion of Tyrp-2 trafficking, it is likely that these structures are recycling endosomes through which Tyrp-2 is trafficked (9, 10). Alternatively, the presence of Myosin Vb at the tips of dendrite tips, where Myosin Va is also present, could represent the functions of Myosin Vb at recycling endosomes in melanosome secretion (Figure 3.7).

Investigations of the function of Rab17 and Rab11a/b in melanosome secretion has demonstrated that Rab27a is not critical for melanosome secretion, and suggested that melanosome secretion occurs using mechanisms downstream of Rab27a and Myosin Va that are dependent on Rab17 and Rab11a/b (38). Melanosome secretion functions downstream of Rab27a could explain the modest colocalization of Myosin Vb with Rab27a labeled melanosomes (Figure 3.8). The low-degree of colocalization of Myosin Vb with melanosome markers could also be explained by ubiquitous functions for Myosin Vb in melanocytes, or that the Myosin Vb associates with mature melanosomes only briefly before melanosome secretion (Figure 3.8). However, Rab8a and Myosin Vc are not found in dendrite tips, suggesting that such a role for the recycling endosome in melanosome secretion would be independent of

Myosin Vc (Figure 3.7, 3.9). The sub-cellular localization of Myosin Vb has similarities with both Myosin Va and Myosin Vc, and notably there are some perinuclear structures in MNT-1 cells where all three motors are present (Figure 3.7). The identity and function of these perinuclear clusters is unclear, but may represent clusters of melanosomes at the perinuclear microtubule-organizing center or clustered recycling endosomes. No direct role for Myosin Vb in melanosome trafficking has been established, but evidence from my experiments and recent publications strongly suggest a role for recycling endosomes and Myosin Vb in melanosome secretion.

What are the functional implications of Rabs that are capable of interacting to different class V myosins? Some melanosomal Rabs show interaction with only specific class V myosins: Rab27a interacts with Myosin Va, Rab11a interacts only with Myosin Vb, and Rab32 and Rab38 bind only to Myosin Vc (Figure 3.1). The use of these Rabs to recruit specific Myosins for functions in melanosome trafficking is easy to interpret. Are Rab proteins that interact with multiple Myosins, such as Rab7a with Myosin Va, Myosin Vb, and Myosin Vc, able to functionally bind to each of these motors in melanosome trafficking (Figure 3.1)? The localization of Rab7a and Rab27a to distinct melanosomes suggests that Rab7a is not enriched on melanosomes that contain Myosin Va (22). Rab7a is associated more with functions in melanosome biogenesis, but is also implicated with fast, microtubule-based melanosome movement (19-23). It is, therefore, unclear if Rab7a is able to function with Myosin Vb and Myosin Vb in melanosome trafficking, but it is unlikely that Rab7a functions with Myosin Va.

Colocalization of Rab7a and Myosin Vc is observed at both perinuclear clusters and more distal structures, so it is possible that Myosin Vc functions in some trafficking pathways with Rab7a (Figure 3.9). Based on the fractionation of Myosin Vc in sucrose gradients, these

functions would likely involve vesicle trafficking steps (Figure 3.6). The trafficking defects in Rab7a knockdown are similar to defects caused by Rab32 or Rab38 knockdown (Figure 3.5). And these three Rab proteins have similarities in their subcellular distribution (Figure 3.9). It is possible that Myosin Vc functions with Rab7a, Rab32, and Rab38 in some cargo trafficking steps. However, not enough is known about the functions of these Rabs in melanosome biogenesis and a careful comparison of the functions of Rab7a, Rab32, and Rab38 in melanosome biogenesis is needed.

Rab8a is able to bind to both Myosin Vb and Myosin Vc, but does it function with both motors in melanosome trafficking? Rab8a functions with Myosin Vb in polarized trafficking from the recycling endosome in polarized cell types, and in the recycling of glucose-receptors in muscle cells (37, 43, 44). Rab8a also functions with Myosin Vc in secretion of secretory vesicles in certain cell types (45, 46). Rab8a has been shown to function in Rab27a-independent, actin-based movement of melanosomes (24). In melanosome biogenesis, some of the functions of Myosin Vc may be dependent on interactions with Rab8a, as suggested by comparison of the phenotypes for Rab8a and Myosin Vc knockdown (Figure 3.3, 3.4, 3.5). In MNT-1, colocalization of Rab8a and Myosin Vc occurs on structures that would be consistent with recycling endosomes, and not melanosomes (Figure 3.9). This would suggest that that Myosin Vc is not responsible for the Myosin Va-independent, Rab8a-dependent actin-based movement of melanosomes (24). Perhaps these functions are dependent on Myosin Vb. Myosin Vb is found to localize to dendrite tips, where actin-based movement of melanosomes is known to occur, and this could be a site where Rab8a and Myosin Vb could functionally interact in melanosome trafficking. The ability, however, for Rabs to bind to multiple class V myosin

motors, and the ability for motors to interact with multiple Rabs in melanosome biogenesis, makes it difficult to dissect the functions of these Rabs and Myosins in melanosome trafficking.

4.9 Concluding remarks

Experiments that I have performed investigating the functions of Rab32 and Rab38 in melanosome biogenesis have addressed outstanding questions about the mechanism used to recruit ubiquitous lysosome trafficking machinery for melanosome specific trafficking. Results on the redundant and non-redundant functions of Rab32 and Rab38 also match the known parallel pathways established by AP-3, AP-1, and BLOC-2 (1, 2). The presence of Rab38-independent functions for Rab32 for the trafficking of Tyrp-2 suggests that another trafficking pathway is also important for melanosome biogenesis. Analysis of the redundant and non-redundant functions of Rab32 and Rab38 will be certain to yield interesting information about the molecular mechanisms that regulate these two similar Rabs in melanosome biogenesis.

The discovery that Myosin Vc is an effector of multiple Rabs in melanosome biogenesis is an important finding, but one that raises numerous questions about the function of this motor in melanosome trafficking. Outstanding questions about the ability for Rab32 and Rab38 to interact with two effectors in the same trafficking pathway will certainly yield better understand of Rab – effector interactions in general. The combination of ubiquitous and cell-type specific Rabs in melanosome biogenesis serves as a model to investigate the collaboration of multiple Rabs in specialized trafficking pathways. How are the membrane localization and function of these Rabs regulated, and how are interactions with a shared effector, such as Myosin Vc, controlled? The function of Myosin Vc in melanosome biogenesis is undefined. Does Myosin Vc function in multiple trafficking steps in melanosome biogenesis, or do we observe a

combination of ubiquitous and melanosome specific trafficking functions in MNT-1 cells? Additional experiments will be required to define the functions of Myosin Vc, and define the molecular mechanisms that control Myosin Vc in melanosome trafficking.

The presence of multiple class V myosin motors in melanosome trafficking raises questions about the functions of these motors. Do all of these motors serve specific trafficking functions in melanosome trafficking or does an overlap of ubiquitous and cell-type specific trafficking occur? Do these motors function in distinct or redundant steps in melanosome trafficking? And ultimately, what are the mechanisms that are able to regulate the functions of so many Rab proteins and Myosin V motors in melanosome trafficking? Melanosome biogenesis serves as an excellent model to study the complicated cell biology and biochemical events that underlie cell-type specific organelle formation.

4.10 REFERENCES

1. Di Pietro SM, *et al* (2006) BLOC-1 interacts with BLOC-2 and the AP-3 complex to facilitate protein trafficking on endosomes. *Mol Biol Cell.* 17(9):4027-38.
2. Theos AC, *et al* (2005) Functions of adaptor protein (AP)-3 and AP-1 in tyrosinase sorting from endosomes to melanosomes. *Mol Biol Cell* 16(11):5356-5372.
3. Wasmeier C, *et al* (2006) Rab38 and Rab32 control post-Golgi trafficking of melanogenic enzymes. *J Cell Biol.* 175(2):271-81.
4. Ninkovic I, White JG, Rangel-Filho A, Datta YH (2008) The role of Rab38 in platelet dense granule defects. *J Thromb Haemost.* 6(12):2143-51.
5. Brooks BP, *et al* (2007) Analysis of ocular hypopigmentation in Rab38cht/cht mice. *Invest Ophthalmol Vis Sci.* 48(9):3905-13.
6. Lopes VS, Wasmeier C, Seabra MC, Futter CE (2007) Melanosome maturation defect in Rab38-deficient retinal pigment epithelium results in instability of immature melanosomes during transient melanogenesis. *Mol Biol Cell.* 18(10):3914-27.
7. Park M, Serpinskaya AS, Papalopulu N, Gelfand VI (2007) Rab32 regulates melanosome transport in Xenopus melanophores by protein kinase A recruitment. *Curr Biol.* 17(23):2030-4
8. Hume AN, Wilson MS, Ushakov DS, Ferenczi MA, Seabra MC (2011) Semi-automated analysis of organelle movement and membrane content: understanding rab-motor complex transport function. *Traffic.* 12(12):1686-701
9. Negroiu G, Dwek RA, Petrescu SM (2005) Tyrosinase-related protein-2 and -1 are trafficked on distinct routes in B16 melanoma cells. *Biochem Biophys Res Commun.* 328(4):914-21.
10. Negroiu G, Dwek RA, Petrescu SM (2003) The inhibition of early N-glycan processing targets TRP-2 to degradation in B16 melanoma cells. *J Biol Chem.* 278(29):27035-42.
11. Wang F, *et al* (2008) Varp interacts with Rab38 and functions as its potential effector. *Biochem Biophys Res Commun.* 372(1):162-7.
12. Tamura K, *et al* (2009) Varp is a novel Rab32/38-binding protein that regulates Tyrp-1 trafficking in melanocytes. *Mol Biol Cell.* 20(12):2900-8.
13. Salazar G, *et al* (2006) BLOC-1 complex deficiency alters the targeting of adaptor protein complex-3 cargoes. *Mol Biol Cell.* 17(9):4014-26.
14. Tamura K, Ohbayashi N, Ishibashi K, Fukuda M (2011) Structure-function analysis of VPS9-ankyrin-repeat protein (Varp) in the trafficking of tyrosinase-related protein 1 in melanocytes. *J Biol Chem.* 286(9):7507-21.
15. Schäfer IB, *et al* (2012) The binding of Varp to VAMP7 traps VAMP7 in a closed, fusogenically inactive conformation. *Nat Struct Mol Biol.* 19(12):1300-9.
16. Gerondopoulos A, Langemeyer L, Liang JR, Linford A, Barr FA. (2012) BLOC-3 mutated in Hermansky-Pudlak syndrome is a Rab32/38 guanine nucleotide exchange factor. *Curr Biol.* 22(22):2135-9.
17. Nazarian R, Falcón-Pérez JM, Dell'Angelica EC. (2003) Biogenesis of lysosome-related organelles complex 3 (BLOC-3): a complex containing the Hermansky-Pudlak syndrome (HPS) proteins HPS1 and HPS4. *Proc Natl Acad Sci U S A.* 100(15):8770-5
18. Loftus SK, *et al* (2002) Mutation of melanosome protein RAB38 in chocolate mice. *Proc Natl Acad Sci U S A.* 99(7):4471-6.
19. Hirosaki K, Yamashita T, Wada I, Jin HY, Jimbow K (2002) Tyrosinase and tyrosinase-related protein 1 require Rab7 for their intracellular transport. *J Invest Dermatol.* 119(2):475-80.
20. Hida T, *et al* (2011) Rab7 is a critical mediator in vesicular transport of tyrosinase-related protein 1 in melanocytes. *J Dermatol.* 38(5):432-41.
21. Kawakami A, *et al* (2008) Rab7 regulates maturation of melanosomal matrix protein gp100/Pmel17/Silv. *J Invest Dermatol.* 128(1):143-50.

22. Jordens I, *et al* (2006) Rab7 and Rab27a control two motor protein activities involved in melanosomal transport. *Pigment Cell Res.* 19(5):412-23.
23. Jordens I, *et al* (2001) The Rab7 effector protein RILP controls lysosomal transport by inducing the recruitment of dynein-dynactin motors. *Curr Biol.* 11(21):1680-5.
24. Chabrillat ML, *et al* (2005) Rab8 regulates the actin-based movement of melanosomes. *Mol Biol Cell.* 16(4):1640-50.
25. Chakraborty AK, Funasaka Y, Araki K, Horikawa T, Ichihashi M (2003) Evidence that the small GTPase Rab8 is involved in melanosome traffic and dendrite extension in B16 melanoma cells. *Cell Tissue Res.* 314(3):381-8.
26. Roland JT, Lapierre LA, Goldenring JR (2009) Alternative splicing in class V myosins determines association with Rab10. *J Biol Chem.* 284(2):1213-23
27. Lambert J, Naeyaert JM, Callens T, De Paepe A, Messiaen L (1998) Human myosin V gene produces different transcripts in a cell type-specific manner. *Biochem Biophys Res Commun.* 252(2):329-33.
28. Westbroek W, *et al* (2003) Interactions of human Myosin Va isoforms, endogenously expressed in human melanocytes, are tightly regulated by the tail domain. *J Invest Dermatol.* 120(3):465-75.
29. Kawasaki, M., Nakayama, K. and Wakatsuki, S. (2005) Membrane recruitment of effector proteins by Arf and Rab GTPases. *Curr. Opin. Struct. Biol.* 15, 681–689
30. Overmeyer JH, Wilson AL, Erdman RA, Maltese WA (1998) The putative "switch 2" domain of the Ras-related GTPase, Rab1B, plays an essential role in the interaction with Rab escort protein. *Mol Biol Cell.* 9(1):223-35.
31. Grosshans BL, Ortiz D, Novick P. (2006) Rabs and their effectors: achieving specificity in membrane traffic. *Proc Natl Acad Sci U S A.* 103(32):11821-7.
32. Kloer DP, *et al* (2010) Assembly of the biogenesis of lysosome-related organelles complex-3 (BLOC-3) and its interaction with Rab9. *J Biol Chem.* 285(10):7794-804.
33. Sultana A, *et al* (2011) The activation cycle of Rab GTPase Ypt32 reveals structural determinants of effector recruitment and GDI binding. *FEBS Lett.* 585(22):3520-7.
34. Fernandes H, *et al* (2009) Structural aspects of Rab6-effector complexes. *Biochem Soc Trans.* 37(Pt 5):1037-41.
35. Pereira-Leal JB, Seabra MC (2000) The mammalian Rab family of small GTPases: definition of family and subfamily sequence motifs suggests a mechanism for functional specificity in the Ras superfamily. *J Mol Biol.* 301(4):1077-87.
36. Rodriguez OC, Cheney RE (2002) Human myosin-Vc is a novel class V myosin expressed in epithelial cells. *J Cell Sci.* 115(Pt 5):991-1004.
37. Roland JT, *et al* (2011) Rab GTPase-Myo5B complexes control membrane recycling and epithelial polarization. *Proc Natl Acad Sci U S A.* 108(7):2789-94.
38. Beaumont KA, *et al* (2011) The recycling endosome protein Rab17 regulates melanocytic filopodia formation and melanosome trafficking. *Traffic.* 12(5):627-43.
39. Van Gele M, Dynoodt P, Lambert J (2009) Griscelli syndrome: a model system to study vesicular trafficking. *Pigment Cell Melanoma Res.* 22(3):268-82.
40. Van Gele M, Geusens B, Schmitt AM, Aguilar L, Lambert J (2008) Knockdown of myosin Va isoforms by RNAi as a tool to block melanosome transport in primary human melanocytes. *J Invest Dermatol.* 128(10):2474-84.
41. Dessinioti C, Stratigos AJ, Rigopoulos D, Katsambas AD (2009) A review of genetic disorders of hypopigmentation: lessons learned from the biology of melanocytes. *Exp Dermatol.* 18(9):741-9.
42. Wu XS, *et al* (2012) Melanoregulin regulates a shedding mechanism that drives melanosome transfer from melanocytes to keratinocytes. *Proc Natl Acad Sci U S A.* 109(31):E2101-9.

43. Roland JT, Kenworthy AK, Peranen J, Caplan S, Goldenring JR (2007) Myosin Vb interacts with Rab8a on a tubular network containing EHD1 and EHD3. *Mol Biol Cell.* 18(8):2828-37.
44. Ishikura S, Klip A (2008) Muscle cells engage Rab8A and myosin Vb in insulin-dependent GLUT4 translocation. Am *J Physiol Cell Physiol.* 295(4):C1016-25.
45. Jacobs DT, Weigert R, Grode KD, Donaldson JG, Cheney RE (2009) Myosin Vc is a molecular motor that functions in secretory granule trafficking. *Mol Biol Cell.* 20(21):4471-88.
46. Xu XF, Chen ZT, Gao N, Zhang JL, An J (2009) Myosin Vc, a member of the actin motor family associated with Rab8, is involved in the release of DV2 from HepG2 cells. *Intervirology.* 52(5):258-65.

APPENDIX 1

CELL-TYPE SPECIFIC RAB32 AND RAB38 COOPERATE WITH THE UBIQUITOUS LYSOSOME BIOGENESIS MACHINERY TO SYNTHESIZE SPECIALIZED LYSOSOME-RELATED ORGANELLES [3]

A.1.1 Summary

Lysosome-related organelles (LROs) exist in specialized cells to serve specific functions and typically co-exist with conventional lysosomes. The biogenesis of LROs is known to utilize much of the common protein machinery used in the transport of integral membrane proteins to lysosomes. Consequently, an outstanding question in the field has been how specific cargoes are trafficked to LROs instead of lysosomes, particularly in cells that simultaneously produce both organelles. One LRO, the melanosome, is responsible for the production of the pigment melanin and has long been used as a model system to study the formation of specialized LROs. Importantly, melanocytes, where melanosomes are synthesized, are a cell type that also produces lysosomes and must therefore segregate traffic to each organelle. Two small GTPases, Rab32 and Rab38 are key proteins in the biogenesis of melanosomes and were recently shown to redirect the ubiquitous machinery – BLOC-2, AP-1, and AP-3 – to traffic specialized cargoes to melanosomes in melanocytes. In addition, the study revealed Rab32 and Rab38 have both redundant and unique roles in the trafficking of melanin-producing enzymes and overall

[3] Jarred J. Bultema and Santiago M. Di Pietro
Department of Biochemistry and Molecular Biology, Colorado State University,
Fort Collins, Colorado 80523, USA.

melanosome biogenesis. Here we review these findings, integrate them with previous knowledge on melanosome biogenesis, and discuss their implications for biogenesis of other LROs.

A.1.2 Introduction

In humans, the pigment melanin is responsible for pigmentation of hair, skin, and eyes and serves to minimize the damage caused by exposure to the UV radiation from sunlight. Melanin is produced in a specialized organelle, the melanosome, which is found in melanocyte cells, in skin and hair follicles, and retinal and iris pigmented epithelial cells in the eyes (1-4). The formation of melanosomes has been heavily studied both because of disease implications caused by defects in melanosome formation and because the melanosome is a prototype of the specialized class of organelles called Lysosome-related organelles (LROs) (1, 3-6). Lysosome-related organelles are found in specialized cell types such as melanocytes, platelets, lung alveolar type II cells, and some innate and adaptive immune cells and have critical roles in pigment production, blood clotting, lung surfactant production, lytic activity of the innate immune system and antigen-processing of the adaptive immune system, respectively (4-8). LROs are so called because of shared acidic lumen, protein components and because LROs utilize similar biogenesis pathways as lysosomes (4-6). Melanosome maturation is characterized by four morphologically distinct phases as observed in electron micrographs (1, 9). Stage I melanosomes are formed by the delivery of the transmembrane, structural protein Pmel17 to vacuolar early endosomes, most likely after rapid transit through the cell surface, and subsequent sorting to intraluminal vesicles (Figure A1.1) (1, 10).

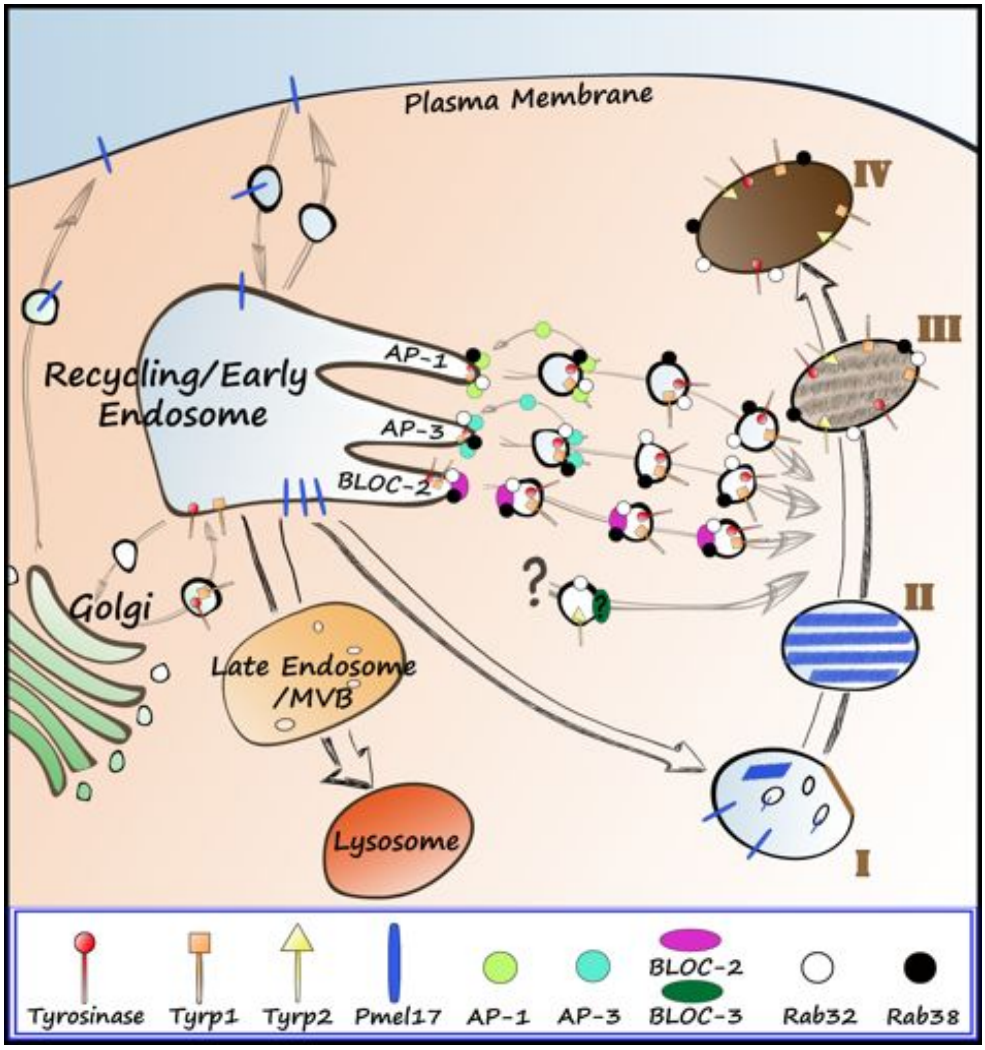

Figure A. 1

Model of melanosome biogenesis. Schematic diagram of the four stages of melanosome maturation (I-IV), endosomal organelles, and biosynthetic transport pathways followed by the cargo integral membrane proteins Pmel17, tyrosinase, tyrosinase-related protein-1 (Tyrp-1) and Tyrp-2. Melanosomal cargoes derive from the Golgi complex and traverse early/recycling endosomal domains either directly or through the cell surface. Sorting of Pmel17 to intraluminal vesicles from the limiting membrane of vacuolar early endosomal domains mark Stage I melanosomes. This process initiates the segregation of pre-melanosomes from the degradative late endosome/Multi Vesicular Body (MVB) pathway to lysosomes. Formation of Pmel17 fibrils across the length of the organelle characterizes stage II melanosomes. Tyrosinase and Tyrp-1 reach the maturing melanosome from specialized tubular domains of early/recycling endosomes and catalyze the synthesis of the melanin pigment observed in stage III and IV melanosomes. Rab32 and Rab38 interact with AP-1, AP-3, and BLOC-2 on early/recycling endosome tubules where cargo such as tyrosinase and Tyrp-1 are loaded into vesicles or transport intermediates. This trafficking machinery is organized into at least two parallel or alternate routes for transport of cargo to the maturing melanosome such that deficiency of one component typically causes a partial defect rather than complete failure of melanosome biogenesis. Rab32, Rab38, and possibly BLOC-2 remain associated with the vesicles or transport intermediates to promote their motility, tethering and fusion with the maturing melanosome. The pathway taken by Tyrp-2 is not known, but it is at least partially different from that of tyrosinase or Tyrp-1 and it depends strictly on Rab32 – not on Rab38 – and BLOC-3.

This Pmel17 sorting is independent of the Endosomal Sorting Complex Required for Transport (ESCRT), the machinery that mediates formation of intraluminal vesicles in Multi Vesicular Bodies (MVBs)/late endosomes and defines the ubiquitous degradative/lysosome pathway (Figure A1.1) (1, 11). Stage I melanosomes are also differentiated from MVBs by the presence of large, flat, clathrin-containing coats on their limiting membrane (9, 12). Pmel17 is then cleaved in the luminal region of the protein by a proprotein convertase and the luminal Pmel17 fragments form amyloid fibrils across the length of the organelle, thus characterized as stage II melanosomes (Figure A1.1) (1, 9, 11, 13). Stage I and II melanosomes are occasionally referred to as pre-melanosomes and they do not yet contain the melanin pigment. Delivery of the transmembrane enzymes tyrosinase and tyrosinase-related proteins-1 and -2 (Tyrp-1 and Tyrp-2), the main proteins responsible for melanin synthesis, is required to drive maturation from stage II to stage III melanosomes (Figure A1.1) (1, 12). Tyrosinase, Tyrp-1, and Tyrp-2 form large melanin polymers that are deposited upon Pmel17 fibrils to form partially pigmented stage III melanosomes (1, 9, 14). Further melanin synthesis produces mature stage IV melanosomes which are fully pigmented and are transported to the cell periphery for transfer to keratinocytes, in the case of skin melanocytes, or long-term storage, in the case of retinal pigmented epithelial cells in the eye (2, 15).

Transport of newly synthesized tyrosinase and Tyrp-1 to the maturing melanosome requires a sorting step at specialized tubular domains of early/recycling endosomes, rather than direct transport from the *trans*-Golgi network (Figure A1.1) (1, 16-18). Packaging of the tyrosinase family proteins into transport vesicles at early/recycling endosome associated tubules is dependent on ubiquitous Adaptor Protein complex (AP)-1 and AP-3, and Biogenesis of Lysosome-related Organelles Complex (BLOC)-1 and BLOC-2 (16, 17). Furthermore, AP-3 and

BLOC-2 define parallel pathways for melanosome biogenesis, thus deficiency of both complexes causes a more severe defect in Tyrp-1 transport and overall pigmentation than either single deficiency (Figure A1.1) (17). Analogously, AP-1 and AP-3 provide alternate routes for transport of tyrosinase and possibly other cargoes to maturing melanosomes (Figure A1.1) (16). While it has not been established if the AP-3 independent pathways – i.e. BLOC-2- and AP-1-dependent, respectively – are separate or the same trafficking route, it is clear that the early/recycling endosomal system is a key sorting station for delivery of cargo to maturing melanosomes (1). Remarkably, lysosome integral membrane proteins such as LAMPs reach the lysosome limiting membrane from analogous early/recycling endosome tubules in vesicles formed by the same AP and BLOC complexes (17, 19-21). It is therefore puzzling how specialized cells such as the melanocyte simultaneously produce and maintain lysosomes and specialized LROs such as the melanosome. How does the cell define separate early/recycling endosomal tubular domains and utilize at least partially overlapping machinery to mediate distinct integral membrane protein transport to different organelles (1, 6, 21)? What other factors are involved in the biogenesis to allow for the separate, but concurrent existence of the two pathways and organelle types?

One possible mechanism to facilitate the production and existence of both lysosomes and LROs is the expression of cell-type specific proteins that function in LRO biogenesis and not in lysosome biogenesis (22). Two closely related small GTPases of the Rab family, Rab32 and Rab38 are expressed in selected cell types such as melanocytes and other cells where LROs are present (23-25). Rab32 and Rab38 have been shown to work on melanosome biogenesis and to mediate transport of tyrosinase and Tyrp-1 by an unknown mechanism (23). Rab38 deficient mice and rats are hypopigmented and Rab32 silencing in melanocytes isolated from Rab38

mutant mice further enhances the melanosome biogenesis defect (23, 26, 27). This cooperation between Rab32 and Rab38 for melanosome biogenesis resembles that of BLOC-2, AP-3, and AP-1 described above. These Rabs could therefore be the cell-type specific factors that interact with the ubiquitous trafficking machinery to mediate transport to maturing melanosomes (22). Such a scenario would be in agreement with the known functions of the Rab family proteins, which belong to the Ras superfamily and operate as elegant "switches" that regulate vesicular trafficking through interactions with effector proteins (28-30). However the specific function and partners of Rab32 and Rab38 in the biogenesis of melanosomes or other LROs had remained poorly characterized (23, 31).

A.1.3 Rab32 and Rab38 interact physically and colocalize with BLOC-2, AP-1, and AP-3

To study endogenous Rab32 and Rab38 using biochemical and immunofluorescence microscopy approaches, antibodies against the Rabs were produced and validated (31). In immuno-precipitation experiments, endogenous Rab32 and Rab38 were found to interact with BLOC-2, AP-1, and AP-3 in membrane, but not cytosolic fractions of MNT-1 melanocyte cells (31). These results suggest a specific interaction with BLOC-2, AP-1, and AP-3 on membranes, where the adaptors are known to function. In GST-Rab pulldown assays, Rab32 and Rab38 showed preferential binding to BLOC-2, AP-1, and AP-3 when bound to GTP instead of GDP (31). This GTP-bound specificity is consistent with the idea that Rab32 and Rab38 have an active, functional role in the trafficking pathway mediated by BLOC-2, AP-1, and AP3. The exciting possibility emerges that Rab32 and Rab38 may function with the ubiquitous machinery in trafficking cargoes to maturing melanosomes and could be the cell-type specific factors that differentiate trafficking pathways to melanosomes instead of lysosomes.

Adaptor proteins orchestrate the formation of membrane coats that mediate cargo selection and vesicle budding (32-34). Adaptor proteins are recruited to membranes through interactions with membrane lipids, the cytoplasmic tails of transmembrane cargoes and, at least in some cases, ARF proteins – another family of GTP-binding proteins of the Ras superfamily (32-34). While it was initially suspected that AP-3 may be clathrin-independent, subsequent research has shown that AP-3 and AP-1 act as clathrin-binding adaptor proteins, and it is also possible that BLOC-2 functions as a clathrin-binding adaptor (19, 35-41). Clathrin is a structural component that defines one of the major classes of transport vesicle coats (34). Adaptor proteins also help recruit a host of proteins involved in downstream vesicle functions such as motility through motor protein-cytoskeleton interactions, and vesicle tethering and fusion with the target organelle (32). An RNAi approach was used to determine if the recruitment or stabilization of Rab32 and Rab38 on membranes is dependent on the presence of AP-1, AP-3, or BLOC-2 (31). Depletion of AP-3 or BLOC-2 causes a significant decrease in the percentage of Rab38 that is associated with membranes at steady state, but has a modest effect on Rab32 membrane association. Depletion of AP-1 has no effect on either Rab32 or Rab38 membrane association. Furthermore, depletion of BLOC-2, but not AP-1 or AP-3, causes a large reduction in the total amount of Rab38 in cells, but modest reduction of total amounts of Rab32, which is likely due to Rab protein destabilization. Given the strong effect that depletion of BLOC-2 has on Rab38 membrane association and stability, RNAi depletion of Rab32 and Rab38 was performed to determine if there is any effect on BLOC-2 membrane association. Depletion of Rab38 significantly decreases the membrane association of BLOC-2, but Rab32 depletion has no effect on BLOC-2 levels. These results indicate that while both Rab32 and Rab38 interact physically

with BLOC-2, AP-1, and AP-3 on membranes, the association may be functionally stronger between Rab38 and AP-3 and even stronger between Rab38 and BLOC-2 (31).

Confocal immunofluorescence microscopy experiments show that a significant percentage of structures labeled with endogenous AP-1, AP-3, and BLOC-2 co-localize with endogenous Rab32 and Rab38 in many locations throughout MNT-1 melanocytes (31). A larger percentage of structures labeled by AP-1, AP-3, or BLOC-2 co-localize with Rab38 than with Rab32. This is consistent with the stronger membrane association defects observed with Rab38 compared to Rab32 upon depletion of AP-3 and BLOC-2. The localization of Rab32 and Rab38 is likely to specific tubular domains of early/recycling endosomes that contain AP-1, AP-3, or BLOC-2 (16, 17). In support of that idea, Rab32 and Rab38 do not colocalize with the early endosome vacuolar domain marker EEA1 or tubular domains involved in the retrieval pathway to the *trans*-Golgi network labeled by the retromer complex (31). Interestingly, both Rab38 and Rab32 partially co-localize with the coat protein clathrin (31). Upon vesicle budding, the clathrin coat and adaptors disassemble and return to the cytosolic pool so they can be reutilized in further rounds of traffic (Figure A1.1). Therefore, Rab32 and Rab38 are probably loaded onto the transport vesicles on endosomal tubular domains during the budding process or soon after vesicle release, but before vesicle uncoating. Rab32 and Rab38 likely remain bound to vesicles upon disassembly of AP-1, AP-3, and clathrin from vesicles (Figure A1.1). It has been suggested that BLOC-2 may also be present in downstream vesicles or transport intermediates and may not undergo quick dissociation as is expected for AP-1, AP-3, and clathrin (1). This possibility would be consistent with the stronger Rab38-BLOC-2 membrane association described above. Rabs are ideal candidates to serve as mediators of trafficking between endosomal tubules and downstream organelles through interactions with specific effector proteins (28-30). The

specificity of Rab32 and Rab38 function in endosome to melanosome trafficking is demonstrated by their localization to stage III and IV melanosomes and not to lysosomes (Figure A1.1) (23, 31). These results indicate that Rab32 and Rab38 operate in the same pathways previously defined for AP-1, AP-3, and BLOC-2 and suggest they are the specific proteins that divert AP-1, AP-3, and BLOC-2 dependent cargoes to maturing melanosomes and away from lysosomes.

A.1.4 Rab32 and Rab38 serve critical functions in the trafficking of melanin-producing enzymes

If Rab32 and Rab38 are redirecting protein trafficking from early/recycling endosomal domains to melanosomes instead of lysosomes, then depletion of these Rab proteins should cause mis-trafficking of the cargo proteins. RNAi depletion of Rab32 or Rab38 causes incorrect trafficking of the melanin-producing enzyme Tyrp-1 to the plasma membrane in MNT-1 melanocytes (31). The same phenotype is produced by deficiency of either of AP-1, AP-3, or BLOC-2 (17, 31), consistent with the idea that Rab32 and Rab38 participate in a transport step mediated by AP-1, AP-3, and BLOC-2. This mistrafficking likely represents a blockage in transport to compartments downstream the early/recycling endosomes, which results in accumulation of cargo in early endosomes and leakage into the recycling pathway to the plasma membrane (Figure A1.1). Simultaneous depletion of Rab32 and Rab38 elicits a more severe trafficking defect for Tyrp-1, thus suggesting partially redundant roles for Rab32 and Rab38 (31).

Examination of the total abundance of tyrosinase and Tyrp-1 further demonstrates the importance of Rab32 and Rab38 for correct, functional trafficking. Independent depletion of either Rab32 or Rab38 causes a significant decrease in overall tyrosinase abundance, but only

modest decrease in Tyrp-1 abundance (31). However, simultaneous depletion of both Rab32 and Rab38 further reduces the abundance of tyrosinase and Tyrp-1 (31). Inhibition of lysosomal hydrolases partially restores tyrosinase and Tyrp-1 to steady-state levels even with simultaneous depletion of Rab32 and Rab38. These results demonstrate that Rab32 and Rab38 are critical to direct trafficking of melanosome-specific cargoes to maturing melanosomes, instead of the default trafficking to lysosomes (Figure A1.1). These results also resemble those obtained with melanocytes deficient for AP1-, AP3, or BLOC-2, reinforcing the notion of functional cooperation with Rab32 and Rab38 in the traffic of cargo to melanosomes (17, 42, 43).

It appears that Rab32 and Rab38 function in parallel roles for the trafficking of tyrosinase and Tyrp-1 and are able to partially compensate for the loss of the other Rab. Such a result is also observed upon depletion of AP-1, AP-3, or BLOC-2, where each protein can partially compensate for the loss of the other (16, 17). As mentioned above, this observation has been interpreted as evidence of parallel AP-1, AP-3, or BLOC-2 dependent pathways in the trafficking of tyrosinase and Tyrp-1. Given the physical interaction and colocalization of Rab32 and Rab38 with AP-1, AP-3, and BLOC-2, these Rabs likely serve all of the AP-3, AP-1, and BLOC-2 dependent pathways in the trafficking of tyrosinase, Tyrp-1, and possibly additional cargoes required for melanosome biogenesis (Figure A1.1) (31).

A.1.5 Evidence for Rab32 unique roles in melanosome biogenesis

In contrast with the cooperation displayed between Rab32 and Rab38 in the transport of tyrosinase and Tyrp-1, the Rabs do not appear to have redundant or compensatory roles in the trafficking of Tyrp-2 (31). Depletion of Rab32, but not Rab38, causes a dramatic loss of Tyrp-2 within MNT-1 melanocytes, which is likely due to mistrafficking to lysosomes (31). What is

more, depletion of Rab32 or Rab38 causes a reduction in the total amount of melanin within MNT-1 melanocytes, an indication of deficient melanosome biogenesis (31). However, Rab32 depletion has a much more severe effect than depletion of Rab38, and simultaneous depletion of both Rabs elicits similar melanin levels as depletion of Rab32 alone. These results imply there are key Rab32 roles in melanosome biogenesis, such as Tyrp-2 transport, that Rab38 cannot carry out. Rab32 and Rab38 appear to have partial functional redundancy, but differ in the membrane association dependency with BLOC-2 and AP-3, the extent of co-localization with AP-3 and AP-1, trafficking of Tyrp-2, and overall function in melanin production. Both Rab32 and Rab38 are present at similar levels within MNT-1 cells, demonstrating that non-redundant functions of Rab32 are not secondary to overall protein abundance (31). Ultimately, Rab32 and Rab38 have some redundant functions, but some unique function of Rab32 is required to maintain normal pigmentation within melanocytes.

In stark contrast with the transport of tyrosinase and Tyrp-1, the trafficking pathway followed by Tyrp-2 to reach maturing melanosomes is unknown. The above results suggest it may use a distinct pathway that requires Rab32 but not Rab38. Provocatively, depletion of BLOC-3, but not BLOC-1, both components of the machinery involved in melanosome biogenesis, also causes substantial loss of Tyrp-2 within MNT-1 melanocytes, suggesting a possible independent role for Rab32 in the trafficking of Tyrp-2 through a BLOC-3 dependent pathway (31). The function of BLOC-3 and its localization in specialized LRO-producing cells are unknown, although it has been shown to impact the localization of late endosomes/lysosomes in fibroblasts (44) and to interact physically with Rab9 (45).

A.1.6 Implications for other Lysosome-related organelles and future directions

Several genetic disorders exist with simultaneous defects in the production of melanosomes and other LROs, consistent with the idea that LROs share a common biogenesis mechanism (1, 3, 4, 6, 46, 47). For example, Hermansky-Pudlak Syndrome (HPS) patients and the corresponding animal models have abnormal melanosomes, platelet dense granules, and lamellar bodies of lung type II epithelial cells. These defects produce partial oculocutaneous albinism, bleeding diathesis, and lung disease, respectively (1, 3, 4, 6, 46-48). Mutations in subunits of AP-3, BLOC-1, BLOC-2, and BLOC-3 underlie many forms of HPS (4, 20, 49). Importantly, Rab38 deficiency in rodent disease models causes biogenesis defects in melanosomes, platelet dense granules, and lamellar bodies (25-27, 50-52). Therefore it is likely that the cooperation between Rab38 and the ubiquitous transport machinery uncovered in melanocytes also functions in the biogenesis of other LROs such as platelet dense granules and lamellar bodies. In future studies it will be important to assess the potential contribution of Rab32 to the biogenesis of other LROs in addition to melanosomes.

Recruitment of the Rabs occurs at specific early/recycling endosome tubular domains but they are also present on melanosomes, suggesting that they remain bound to vesicles until fusion with the downstream melanosome (23, 31). These results constitute a step forward in our understanding of these pathways but also open other questions. What are the functions of Rab32 and Rab38? What are the effectors of Rab32 and Rab38 that facilitate trafficking to and fusion with melanosomes? What proteins are important for the regulation of Rab32 and Rab38? Little is known about the protein effectors of Rab32 and Rab38 beyond interaction with the protein Varp, which is implicated in the recruitment of the v-SNARE VAMP-7/TI-VAMP and Tyrp-1 traffic (15, 53, 54). This Rab-Varp interaction is likely important for the vesicle-melanosome fusion

event. Potential interactions of Rab32 and Rab38 with tethering proteins such as the HOmotypic fusion and Protein Sorting (HOPS) complex could also facilitate trafficking or fusion of vesicles with melanosomes (55-57). Supporting this possibility, mutation of the HOPS complex subunit Vps33a results in deficient melanosome biogenesis in the *buff* mouse (55). However, the precise mechanisms that control the movement and targeting of vesicles remain unclear. The mechanisms used by Rab32 and Rab38 in the trafficking of transmembrane cargoes to melanosomes, and perhaps other LROs, will doubtless be investigated in future studies.

A.1.7 REFERENCES

1. Raposo G and Marks MS (2007) Melanosomes--dark organelles enlighten endosomal membrane transport. *Nat Rev Mol Cell Biol* 8(10):786-797.
2. Wasmeier C, Hume AN, Bolasco G, Seabra MC (2008) Melanosomes at a glance. *J Cell Sci* 121(Pt 24):3995-3999.
3. Spritz RA, Chiang PW, Oiso N, Alkhateeb A (2003) Human and mouse disorders of pigmentation. *Curr Opin Genet Dev* 13(3):284-289.
4. Huizing M, Helip-Wooley A, Westbroek W, Gunay-Aygun M, Gahl WA (2008) Disorders of lysosome-related organelle biogenesis: clinical and molecular genetics. *Annu Rev Genomics Hum Genet* 9:359-386.
5. Dell'Angelica EC, Mullins C, Caplan S, Bonifacino JS (2000) Lysosome-related organelles. *FASEB J* 14(10):1265-1278.
6. Raposo G, Marks MS, Cutler DF (2007) Lysosome-related organelles: driving post-Golgi compartments into specialisation. *Curr Opin Cell Biol* 19(4):394-401.
7. Fontana S, *et al* (2006) Innate immunity defects in Hermansky-Pudlak type 2 syndrome. *Blood* 107(12):4857-4864.
8. Sugita M, *et al* (2002) Failure of trafficking and antigen presentation by CD1 in AP-3-deficient cells. *Immunity* 16(5):697-706.
9. Hurbain I, *et al* (2008) Electron tomography of early melanosomes: implications for melanogenesis and the generation of fibrillar amyloid sheets. *Proc Natl Acad Sci U S A* 105(50):19726-19731.
10. Theos AC, *et al* (2006) Dual loss of ER export and endocytic signals with altered melanosome morphology in the silver mutation of Pmel17. *Mol Biol Cell* 17(8):3598-3612.
11. Theos AC, *et al* (2006) A lumenal domain-dependent pathway for sorting to intralumenal vesicles of multivesicular endosomes involved in organelle morphogenesis. *Dev Cell* 10(3):343-354.
12. Raposo G, Tenza D, Murphy DM, Berson JF, Marks MS (2001) Distinct protein sorting and localization to premelanosomes, melanosomes, and lysosomes in pigmented melanocytic cells. *J Cell Biol* 152(4):809-824.
13. Berson JF, *et al* (2003) Proprotein convertase cleavage liberates a fibrillogenic fragment of a resident glycoprotein to initiate melanosome biogenesis. *J Cell Biol* 161(3):521-533.
14. Kondo T, Hearing VJ (2011) Update on the regulation of mammalian melanocyte function and skin pigmentation. *Expert review of dermatology* 6(1):97-108.
15. Ohbayashi N, Fukuda M (2012) Role of Rab family GTPases and their effectors in melanosomal logistics. *Journal of biochemistry* 151(4):343-351.
16. Theos AC, *et al* (2005) Functions of adaptor protein (AP)-3 and AP-1 in tyrosinase sorting from endosomes to melanosomes. *Mol Biol Cell* 16(11):5356-5372.
17. Di Pietro SM, *et al* (2006) BLOC-1 interacts with BLOC-2 and the AP-3 complex to facilitate protein trafficking on endosomes. *Mol Biol Cell* 17(9):4027-4038.
18. Setty SR, *et al* (2007) BLOC-1 is required for cargo-specific sorting from vacuolar early endosomes toward lysosome-related organelles. *Mol Biol Cell* 18(3):768-780.
19. Peden AA, *et al* (2004) Localization of the AP-3 adaptor complex defines a novel endosomal exit site for lysosomal membrane proteins. *J Cell Biol* 164(7):1065-1076.
20. Dell'Angelica EC, Shotelersuk V, Aguilar RC, Gahl WA, Bonifacino JS (1999) Altered trafficking of lysosomal proteins in Hermansky-Pudlak syndrome due to mutations in the beta 3A subunit of the AP-3 adaptor. *Mol Cell* 3(1):11-21.
21. Dell'Angelica EC (2009) AP-3-dependent trafficking and disease: the first decade. *Curr Opin Cell Biol* 21(4):552-559.
22. Marks MS (2006) Darkness descends with two Rabs. *J Cell Biol* 175(2):199-200.

23. Wasmeier C, *et al* (2006) Rab38 and Rab32 control post-Golgi trafficking of melanogenic enzymes. *J Cell Biol* 175(2):271-281.
24. Cohen-Solal KA, *et al* (2003) Identification and characterization of mouse Rab32 by mRNA and protein expression analysis. *Biochim Biophys Acta* 1651(1-2):68-75.
25. Zhang L, *et al* (2011) Rab38 targets to lamellar bodies and normalizes their sizes in lung alveolar type II epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 301(4):L461-477.
26. Loftus SK, *et al* (2002) Mutation of melanosome protein RAB38 in chocolate mice. *Proc Natl Acad Sci U S A* 99(7):4471-4476.
27. Oiso N, Riddle SR, Serikawa T, Kuramoto T, Spritz RA (2004) The rat Ruby (R) locus is Rab38: identical mutations in Fawn-hooded and Tester-Moriyama rats derived from an ancestral Long Evans rat sub-strain. *Mamm Genome* 15(4):307-314.
28. Hutagalung AH, Novick PJ (2011) Role of rab GTPases in membrane traffic and cell physiology. *Physiol Rev* 91(1):119-149.
29. Zerial M, McBride H (2001) Rab proteins as membrane organizers. *Nat Rev Mol Cell Biol* 2(2):107-117.
30. Stenmark H (2009) Rab GTPases as coordinators of vesicle traffic. *Nat Rev Mol Cell Biol* 10(8):513-525.
31. Bultema JJ, Ambrosio AL, Burek CL, Di Pietro SM (2012) BLOC-2, AP-3, and AP-1 Proteins Function in Concert with Rab38 and Rab32 Proteins to Mediate Protein Trafficking to Lysosome-related Organelles. *J Biol Chem* 287(23):19550-19563.
32. Owen DJ, Collins BM, Evans PR (2004) Adaptors for clathrin coats: structure and function. *Annu Rev Cell Dev Biol* 20:153-191.
33. Bonifacino JS, Traub LM (2003) Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu Rev Biochem* 72:395-447.
34. Brodsky FM, Chen CY, Knuehl C, Towler MC, Wakeham DE (2001) Biological basket weaving: formation and function of clathrin-coated vesicles. *Annu Rev Cell Dev Biol* 17:517-568.
35. Dell'Angelica EC, Klumperman J, Stoorvogel W, Bonifacino JS (1998) Association of the AP-3 adaptor complex with clathrin. *Science* 280(5362):431-434.
36. Dell'Angelica EC, *et al* (1997) AP-3: an adaptor-like protein complex with ubiquitous expression. *EMBO J* 16(5):917-928.
37. Faundez V, Horng JT, Kelly RB (1998) A function for the AP3 coat complex in synaptic vesicle formation from endosomes. *Cell* 93(3):423-432.
38. Simpson F, *et al* (1996) A novel adaptor-related protein complex. *J Cell Biol* 133(4):749-760.
39. Simpson F, Peden AA, Christopoulou L, Robinson MS (1997) Characterization of the adaptor-related protein complex, AP-3. *J Cell Biol* 137(4):835-845.
40. Borner GH, Harbour M, Hester S, Lilley KS, Robinson MS (2006) Comparative proteomics of clathrin-coated vesicles. *J Cell Biol* 175(4):571-578.
41. Helip-Wooley A, *et al* (2005) Association of the Hermansky-Pudlak syndrome type-3 protein with clathrin. *BMC cell biology* 6:33.
42. Delevoye C, *et al* (2009) AP-1 and KIF13A coordinate endosomal sorting and positioning during melanosome biogenesis. *J Cell Biol* 187(2):247-264.
43. Helip-Wooley A, *et al* (2007) Improper trafficking of melanocyte-specific proteins in Hermansky-Pudlak syndrome type-5. *J Invest Dermatol* 127(6):1471-1478.
44. Nazarian R, Falcon-Perez JM, Dell'Angelica EC (2003) Biogenesis of lysosome-related organelles complex 3 (BLOC-3): a complex containing the Hermansky-Pudlak syndrome (HPS) proteins HPS1 and HPS4. *Proc Natl Acad Sci U S A* 100(15):8770-8775.
45. Kloer DP, *et al* (2010) Assembly of the biogenesis of lysosome-related organelles complex-3 (BLOC-3) and its interaction with Rab9. *J Biol Chem* 285(10):7794-7804.
46. Di Pietro SM, Dell'Angelica EC (2005) The cell biology of Hermansky-Pudlak syndrome: recent advances. *Traffic* 6(7):525-533.

47. Wei ML (2006) Hermansky-Pudlak syndrome: a disease of protein trafficking and organelle function. *Pigment Cell Res* 19(1):19-42.
48. Li W, *et al* (2004) Murine Hermansky-Pudlak syndrome genes: regulators of lysosome-related organelles. *Bioessays* 26(6):616-628.
49. Dell'Angelica EC (2004) The building BLOC(k)s of lysosomes and related organelles. *Curr Opin Cell Biol* 16(4):458-464.
50. Ninkovic I, White JG, Rangel-Filho A, Datta YH (2008) The role of Rab38 in platelet dense granule defects. *J Thromb Haemost* 6(12):2143-2151.
51. Osanai K, *et al* (2008) A mutation in Rab38 small GTPase causes abnormal lung surfactant homeostasis and aberrant alveolar structure in mice. *Am J Pathol* 173(5):1265-1274.
52. Lopes VS, Wasmeier C, Seabra MC, Futter CE (2007) Melanosome maturation defect in Rab38-deficient retinal pigment epithelium results in instability of immature melanosomes during transient melanogenesis. *Mol Biol Cell* 18(10):3914-3927.
53. Tamura K, *et al* (2009) Varp is a novel Rab32/38-binding protein that regulates Tyrp-1 trafficking in melanocytes. *Mol Biol Cell* 20(12):2900-2908.
54. Tamura K, Ohbayashi N, Ishibashi K, Fukuda M (2011) Structure-function analysis of VPS9-ankyrin-repeat protein (Varp) in the trafficking of tyrosinase-related protein 1 in melanocytes. *J Biol Chem* 7507-7521.
55. Suzuki T, *et al* (2003) The mouse organellar biogenesis mutant buff results from a mutation in Vps33a, a homologue of yeast vps33 and Drosophila carnation. *Proc Natl Acad Sci U S A* 100(3):1146-1150.
56. Brocker C, Engelbrecht-Vandre S, Ungermann C (2010) Multisubunit tethering complexes and their role in membrane fusion. *Curr Biol* 20(21):R943-952.
57. Zlatic SA, Tornieri K, L'Hernault S W, Faundez V (2011) Metazoan cell biology of the HOPS tethering complex. *Cellular logistics* 1(3):111-117.

LIST OF ABBREVIATIONS

ADP: Adenosine diphosphate

AP-1: Adaptor Protein -1

AP-3: Adaptor Protein -3

ATP: Adenosine triphosphate

BLOC-1: Biogenesis of Lysosome-related organelle complex -1

BLOC-2: Biogenesis of Lysosome-related organelle complex -2

BLOC-3: Biogenesis of Lysosome-related organelle complex -3

CHS: Chediak-Higashi syndrome

CORVET: class C core vacuole/endosome tethering

DHI: 5,6-dihydroxyindole

DHICA: 5,6-dihydroxyindole-2-carboxylic acid

DOPAquinone: ortho-quinone of 3,4-dihydroxyphenylalanine

EEA1: Early endosome antigen 1

ER: Endoplasmic reticulum

ERK kinase: extracellular-signal-regulated kinases

ESCRT: Endosomal Sorting Complex Required for Transport

GAP: GTPase-activating proteins

GDI: Guanine-dissociation inhibitory proteins

GDP: Guanosine diphosphate

GEF: GTP/GDP-exchange factor

GLUT4: high affinity insulin-regulated glucose transporter

GS: Griscelli syndrome

GST: glutathione *S*-transferase

GTP: Guanosine-5'-triphosphate

HOPS: homotypic fusion and vacuole protein sorting

HPS: Hermansky-Pudlak syndrome

ILV: intralumenal vesicles

IP(3): inositol 1,4,5-trisphosphate

LAMP: Lysosome-associated membrane protein

L-DOPA: L-3,4-dihydroxyphenylalanine

LRO : Lysosome-related organelle

MAP kinase: Mitogen-activated protein kinase

MHC-II: major histocompatibility-complex type II

MNT-1: highly pigmented human melanoma cells

mRNA: messenger RNA

MVB/MVE: multivesicular bodies/endosomes

OCA: Oculocutaneous albinism

PI(3)P: phosphotidylinositol-3-phosphate

PI(3,5)P2: phosphotidylinositol-3,5-bisphosphate

PMEL: premelanosome protein

Rab: small GTPases of the Ras superfamily

RNAi: RNA interference

RPE: retinal pigmented epithelial cells

siRNA: Small interfering RNA / short interfering RNA /silencing RNA

SNARE: Soluble N-ethylmaleimide-sensitive factor attachment protein receptor

SNX1: Sorting nexin-1

TfR: transferrin-receptor

t-SNARE: target SNARE

Tyrp-1: Tyrosinase-related protein -1; 5,6-dihydroxyindole-2-carboxylic acid oxidase

Tyrp-2: Tyrosinase-related protein -2; dopachrome tautomerase

VAMP7: Vesicle-associated membrane protein 7

Varp: VPS9-ankyrin-repeat protein

V-ATPase: Vacuolar-type H+ pumps ATPases

v-SNARE: vesicle SNARE

α-MSH: melanocyte-stimulating hormone