Mechanisms of Sorting and Trafficking for Melanosome Biogenesis as Revealed by Studies of the Human Pigment Cell Protein Oca2

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Abstract

Certain cell types harbor specialized lysosome-related organelles (LROs) that derive from the endocytic system like conventional lysosomes but have unique functions. The coexistence of LROs and lysosomes in some cell types implies the existence of sorting mechanisms that divert resident cargo proteins to LROs. Based on the pigment-synthesizing melanosome in melanocytes as a model LRO and two melanosomal resident proteins as model cargoes, current models suggest that cargoes are sorted from early endosomes to melanosomes via one of two independent pathways mediated by the multisubunit complexes AP-3 or BLOC-1, each of which is defective in subtypes of the LRO biogenesis disease Hermansky-Pudlak Syndrome (HPS). An AP-3-related protein complex, AP-1, is thought to function in concert with BLOC-1. In this thesis, I assess the pigment-cell-specific putative transporter protein OCA2, as a third potential cargo protein with which to further dissect the relationships between AP-3, AP-1 and BLOC-1 in melanosomal transport. I first investigate the localization and site of action of OCA2. I use biochemical approaches in combination with site-directed mutagenesis and indirect immunofluorescence microscopic analysis of exogenously-expressed OCA2 in melanocytes to show that OCA2 is indeed a melanosome resident protein and does not function within the endoplasmic reticulum as has been suggested by other models. I show that melanosome localization is essential for OCA2 function and requires an acidic dileucine motif in the N-terminal cytoplasmic domain that can bind to both AP-3 and AP-1. Using site-directed mutagenesis in combination with yeast three hybrid assays and immunofluorescence microscopy analyses in melanocytes derived from mouse models of HPS and controls, I define the features of the OCA2 sorting signal that direct binding to AP-1 or AP-3 and show that OCA2 requires both AP-3 interaction and BLOC-1 for melanosomal localization. My results resolve a controversy regarding OCA2 localization, shed light on the interplay between AP-1 and AP-3 in melanosomal trafficking, and provide the first direct evidence for cooperation between BLOC-1 and AP-3 in trafficking to a LRO.

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MECHANISMS OF SORTING AND TRAFFICKING FOR MELANOSOME BIOGENESIS AS REVEALED BY STUDIES OF THE HUMAN PIGMENT CELL PROTEIN OCA2

Anand Sitaram

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ABSTRACT

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Anand Sitaram

Advisor: Michael S. Marks, Ph.D.

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use biochemical approaches in combination with site-directed mutagenesis and indirect immunofluorescence microscopic analysis of exogenously-expressed OCA2 in melanocytes to show that OCA2 is indeed a melanosome resident protein and does not function within the endoplasmic reticulum as has been suggested by other models. I show that melanosome localization is essential for OCA2 function and requires an acidic dileucine motif in the N-terminal cytoplasmic domain that can bind to both AP-3 and AP-1. Using site-directed mutagenesis in combination with yeast three hybrid assays and immunofluorescence microscopy analyses in melanocytes derived from mouse models of HPS and controls, I define the features of the OCA2 sorting signal that direct binding to AP-1 or AP-3 and show that OCA2 requires both AP-3 interaction and BLOC-1 for melanosomal localization. My results resolve a controversy regarding OCA2 localization, shed light on the interplay between AP-1 and AP-3 in melanosomal trafficking, and provide the first direct evidence for cooperation between BLOC-1 and AP-3 in trafficking to a LRO.
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CHAPTER 1. INTRODUCTION

1.1 Context of the presented work

The human body is composed of many different systems of organs, each specialized to perform particular functions for the benefit of the whole organism. The individual cells of the body too harbor several different types of internal organelles, each specialized for particular functions within the cell. Some of these organelles perform essential cellular functions and are common to the vast majority of the hundreds of different cell types, while other organelles are found only in restricted cell types and allow these cells to carry out their novel activities. The function of an organelle is directly dependent on the cohort of proteins that are localized to it, and thus it is essential for cells to have mechanisms in place to ensure delivery of these resident proteins to the appropriate organelles.

Integral membrane proteins, for example, are synthesized from ribosomes that are docked at the endoplasmic reticulum. Following their synthesis in the endoplasmic reticulum, most proteins must be trafficked either to the cell surface for plasma membrane expression or secretion, or to other intracellular organelles. The existence of several different cellular organelles underscores the need for a sorting system that identifies the correct destination for an integral membrane protein and engages cellular machinery to deliver it there. Numerous studies have identified *cis*-acting signals encoded within cargo proteins as well as *trans*-acting proteins that recognize these determinants and can segregate cargo proteins into directed trafficking routes towards their destinations.
Failure of these processes can lead to organellar, cellular, and ultimately organic dysfunction, and thus ultimately to disease.

In order to better understand how cells direct protein sorting, we have chosen to focus on the sorting of cell type-specific integral membrane proteins to a group of organelles called lysosome-related organelles (LROs), and more specifically to the pigment-producing melanosome – a LRO within pigmented cells such as the melanocytes of the skin, hair bulb, and choroid, and the pigmented epithelial tissues of the eye. Lack of melanosome function causes the hypopigmentation disorder known as albinism, and albinism-associated mutations are found in several genes that encode proteins that direct cargo protein trafficking to the melanosome. Studies of the localization of melanosomal cargo proteins in melanocytic cells that are deficient in some of these trafficking genes have helped us assemble a model of pathways by which cargoes are delivered to melanosomes. However, gaps in our understanding of the relationship between these trafficking proteins and how they function within their respective pathways still remain.

In this thesis I focus on the melanocytic protein OCA2, whose trafficking has not been previously characterized. I first resolve a debate in the literature regarding the steady-state localization and site of function of this protein by showing that it localizes to the melanosome and that mutant forms that remain in the endoplasmic reticulum or mislocalize to the cell surface are non-functional, reaffirming the important link between proper protein localization and function. I also identify critical cis and trans determinants of the melanosomal sorting of OCA2. I then proceed to use OCA2 as a model
melanosomal cargo to probe current models of melanosomal trafficking. I provide new evidence for a previously undescribed pathway that is dependent on two *trans*-acting protein complexes which had been previously thought to act independently. Our work therefore sheds light on both the trafficking of an individual lysosome-related organelle cargo protein as well as on the variety of mechanisms that cells utilize to send different cargo proteins to the same organelle.

In order to put the work into context, this introduction will define lysosome-related organelles and describe melanocytes and their melanosomes as model lysosome-related organelles in greater detail. I then discuss the melanocytic proteins that are critical for melanosome biogenesis, our current understanding of the mechanisms used for their trafficking within cells, and how these mechanisms are disrupted in disease. Finally I introduce the melanocytic protein OCA2 and describe what was known about the protein prior to the work described in this thesis as well as the questions about its trafficking that I wished to address with this body of work.

1.2 Lysosome-related organelles

Lysosome-related organelles (LROs) are a class of intracellular membrane-bound organelles found only in certain cell types (Dell'Angelica *et al.*, 2000; Raposo *et al.*, 2007). They are so named because they share some features in common with conventional lysosomes, the cellular degradative compartment. Like lysosomes, LROs are derived from the endocytic system, they have an acidic lumenal pH, and they can contain a cohort of lysosomal resident proteins such as LAMPs.
Membrane Proteins) or acid hydrolases. However, LROs are distinguished from conventional lysosomes by their ability to carry out additional cell-type-specific functions and by their unique morphologies. LROs are found in diverse tissues and include: melanosomes in skin and choroidal melanocytes and the pigment epithelium of the retina, iris, and ciliary body; dense granules in platelets; lamellar bodies in alveolar epithelial cells; and secretory compartments of hematopoietic cells, including the lytic granules of cytotoxic T cells and natural killer cells, several types of granules in basophils, azurophils, and eosinophils, and the major histocompatibility class II compartments (MIICs) in antigen presenting cells. The diverse morphologies and functions of these LROs are reflections of the distinct cell-type specific resident proteins they harbor. Some LROs, such as the cytotoxic T cell lytic granules, are considered modified lysosomes, carrying out the degradative function in addition to their novel immune function (Burkhardt et al., 1990; Peters et al., 1991). However a subset of LROs, including melanosomes and platelet dense granules, are found to simultaneously coexist in cells with conventional lysosomes, which implies that cells harboring this class of LROs must make use of specialized sorting pathways to segregate LRO resident components away from the general lysosomal delivery pathway and redirect them towards LROs (Raposo et al., 2007). Indeed, melanosomes in melanocytic cells are inaccessible to fluid-phase uptake markers, in contrast to the lysosomes (Fujita et al., 2001; Raposo et al., 2001), and melanosomes of immortalized mouse melanocytes are relatively devoid of the lysosomal marker protein LAMP-2 (Setty et al., 2008; Sitaram et al., 2009). While many of the proteins that are involved in these segregating processes have been identified, determining how they function and interact remains an active area of research.
Melanocytes constitute a model system that is widely used to study mechanisms of LRO trafficking, and thus the following section will discuss melanocytes and melanosomes in more detail.

1.3 Melanocytes and melanosomes - a model of LRO biogenesis

1.3.1 Melanocytes and albinism

Melanocytes are specialized cells that produce melanin pigments found in fish, mammals, and other vertebrates (Seiji et al., 1963; Marks and Seabra, 2001; Raposo and Marks, 2007). In humans, these cells produce the melanin that serves an important light-absorbing function in tissues of the inner eye and gives color and ultraviolet protection to the skin, hair, and eyes. Defects in the biogenesis of melanosomes lead to the hypopigmentation phenotype known as albinism (King, 1998). All types of albinism include phenotypes in the eye (Summers, 2009). Lack of functional melanosomes in the eye tissues causes poor vision and iris transillumination. For reasons that are not completely understood, patients also demonstrate defects in the development of the visual system, including foveal hypoplasia as well as misrouting of optic axons (Guillery et al., 1975), which may underlie the elevated incidence of nystagmus and strabismus seen in these patients. There are four types of oculocutaneous albinism (OCA), in which the eye phenotypes are seen in conjunction with mild to severe hypopigmentation of the skin and hair (King, 1998) (Figure 1.1). OCA patients have an increased risk of skin cancer due to the deficiency of UV-protective melanin. The OCA subtypes are all recessive and linked to different pigment-cell-specific genes important for melanin synthesis or melanosome biogenesis. A distinct disease, X-linked ocular albinism, is caused by mutations in
Figure 1.1

Figure 1.1. Examples of human oculocutaneous albinism.

a) A Brazilian family with two children with normal pigmentation and three children suffering from a form of oculocutaneous albinism.\(^1\) b) A Turkish man suffering from oculocutaneous albinism type 4. Inset: a photo of the man as a youth.\(^2\)

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\(^1\) Reprinted with permission from Reuters.

GPR143 (also known as OA1), which encodes an unusual intracellular G protein coupled receptor of unknown function (Schiaffino et al., 1999). No clinical skin phenotype is observed in X-linked ocular albinism, although ultrastructural examination shows the presence of enlarged melanosomes in pigment cells from skin and eyes (O'Donnell et al., 1976; Cortese et al., 2005).

1.3.2 Melanin synthesis
The range of skin and eye coloration observed in humans can be attributed to differing levels of two types of melanins, eumelanins and pheomelanins. Eumelanins are responsible for black and brown pigmentation, whereas pheomelanins produce red and yellow hues. All melanins are non-protein polymers of modified tyrosine subunits (Figure 1.2a). The rate-limiting initial step in the synthesis of all types of melanin is catalyzed by the pigment-cell-specific protein tyrosinase (Tyr). Mutations in the TYR gene cause OCA type 1, the most common form of OCA in non-Hispanic Caucasians (Hutton and Spritz, 2008). As Tyr is needed for all melanin synthesis, the most severe OCA1 phenotype is a complete lack of pigment, leading to white hair and pale skin. The increased visibility of blood vessels in the eyes causes them to appear pink. The same phenotype is seen in the corresponding albino model mouse, mutated in Tyr, which maps to the c locus (King, 1998). Tyr is a copper-dependent enzyme (Oetting et al., 2003) that catalyzes the hydroxylation of tyrosine to L-DOPA and the further oxidation of L-DOPA to dopaquinone. A series of subsequent oxidation and isomerization steps produces colored indoles and finally a mixture of black and brown eumelanins. Tyr is the only
Figure 1.2

Figure 1.2. Melanin synthesis and melanosome maturation.

a) Eumelanin is a brown-black mixture of polymers of oxidized and cyclized tyrosine subunits. The enzymes tyrosinase (TYR), tyrosinase-related protein (TYRP1), and dopachrome tautomerase (DCT) catalyze the indicated steps of melanin synthesis within the melanosome lumen. Integral membrane proteins including OCA2, SLC45A2, and SLC24A5 are thought to bring about the lumenal conditions needed for melanin synthesis (Braasch et al., 2007). b) Melanosome maturation progresses through four morphologically distinct stages, as shown in an electron micrograph from a human melanoma cell line. Stage II melanosomes are characterized by fibrils containing PMEL. Delivery of Tyr and Tyrp1 to immature melanosomes initiates melanin synthesis and progression to Stages III and IV (Raposo and Marks, 2002).
melanocyte-specific protein needed for melanogenesis, as heterologous expression in
non-melanocytes is sufficient to induce melanin formation (Bouchard et al., 1989a; Ni-
Komatsu and Orlow, 2006).

In melanocytes, two other enzymes can influence the mixture of eumelanins that are
produced. Dopachrome tautomerase (DCT, also known as tyrosinase-related protein 2)
can prevent auto-oxidation of dopachrome (an intermediate downstream of dopaquinone)
to the colored indole intermediate 5,6-dihydroxyindole and instead convert it into the 5,6-
dihydroxyindole-2-carboxylic acid, leading to different eumelanin products (Aroca et al.,
1990; Pawelek, 1991; Orlow et al., 1992). Mutations in Dct underlie the
hypopigmentation phenotype of the slaty mouse (Jackson et al., 1992; Tsukamoto et al.,
1992). Tyrosinase-related protein 1 (Tyrp1) can also influence the ratio of black and
brown eumelanins produced, as demonstrated by the corresponding brown mutant mouse
(Jackson, 1988). Tyrp1 has been proposed to catalyze the oxidation of DHICA, but this
hypothesis is controversial (Sarangarajan and Boissy, 2001). Nevertheless, Tyrp1 is
clearly important in melanosome function, as mutations in TYRP1 cause the more rare
OCA type 3 (Boissy et al., 1996; Manga et al., 1997). OCA type 3 has mostly been
studied in people of African descent and is sometimes called rufous albinism, due to the
yellowish or reddish hair seen in affected patients (Kromberg et al., 1990).

If sufficient cysteine is present, dopaquinone can be modified to cysteinyl L-DOPA, and
the resulting synthesis product is reddish-yellow pheomelanin rather than eumelanin. At
any given time, melanocytes produce primarily one type of melanin or the other; the
alternation between eumelanin production and pheomelanin production within a single hair shaft produces the agouti banding pattern that gives the characteristic brushed appearance to many mammalian coats (Robbins et al., 1993; Cone et al., 1996). The choice of pigment to synthesize is regulated by a signal transduction pathway activated by the binding of alpha melanocyte-stimulating hormone (alpha-MSH) to the melanocortin-1 receptor (MC1R), the target of the murine extension locus e, on the melanocyte surface (Geschwind, 1966; Tamate and Takeuchi, 1984). MC1R signaling via alpha-MSH stimulation leads to the induction of eumelanogenesis. The agouti signaling peptide, encoded by ASIP (Kwon et al., 1994; Wilson et al., 1995), is an inverse agonist of MC1R, and its activity induces pheomelanogenesis (Lu et al., 1994). Mutations in either POMC (Krude et al., 1998)—the gene that encodes the polyprotein that is proteolytically processed to produce alpha-MSH—or MC1R (Valverde et al., 1995) are associated with red-haired phenotypes as a consequence of the inactivation of the eumelanogenesis initiation pathway. Conversely, the widely-used inbred mouse model C57BL/6 is black because of a homozygous inactivation of the a locus, the murine homolog of ASIP (Silvers, 1979; Bultman et al., 1992; Kwon et al., 1994). Initiation of pheomelanogenesis causes the downregulation of several proteins crucial for eumelanogenesis. These include Tyrp1, DCT, PMEL, and the putative transporter OCA2, discussed in more detail below (Barber et al., 1985; Lamoreux et al., 1986; Thody and Burchill, 1992; Kobayashi et al., 1995; Lamoreux et al., 1995; Hirobe et al., 2011). As the vast majority of studies on melanosome biogenesis have focused on eumelanosomes and eumelanogenesis, pheomelanin will not be discussed further. For the
remainder of this thesis, references to melanosomes should be understood to refer to eumelanosomes.

1.3.3 Melanosome biogenesis and maturation

Melanin synthesis occurs within the lumen of the membrane-bound melanosome, the melanocytic LRO. Melanosome biogenesis is considered to occur in four stages, distinguishable on the basis of morphology and visualized by electron microscopy (Seiji et al., 1963) (Figure 1.2b). A stage I melanosome is identified morphologically and functionally as a coated vacuolar domain of an early endosome (Raposo et al., 2001). A portion of the organelle is coated by cytoplasmic clathrin, and a few intralumenal vesicles are visible. In this regard, a stage I melanosome resembles the classical vacuolar early endosome from which multivesicular body formation is initiated (Hopkins and Trowbridge, 1983). However, what distinguishes stage I melanosomes from typical multivesicular early endosomes is the presence of proto-fibrils that radiate from the surface of the intralumenal vesicles (Hurbain et al., 2008). These proto-fibrils are composed primarily of proteolytically-cleaved lumenal fragments of the protein PMEL (Berson et al., 2003). Continued polymerization produces the longitudinally-oriented amyloid-like fibrillar sheets (Birbeck, 1963; Hurbain et al., 2008) that are characteristic of a stage II melanosome (Seiji et al., 1963), a membrane-bound structure spatially and compositionally distinct from the endosome. Melanin-synthesizing enzymes Tyr and Tyrp1 and other proteins such as OCA2 and the copper transporter ATP7A are then delivered to melanosomes (Seiji et al., 1963) from distinct domains of early endosomes (Theos et al., 2005; Setty et al., 2007; Setty et al., 2008; Delevoye et al., 2009; Sitaram et
Melanin synthesis is then initiated, giving rise to stage III melanosomes in which the fibers appear darker and thicker due to the deposition of insoluble melanin upon them (Seiji et al., 1963). As melanin synthesis continues, the internal fibril structure becomes completely obscured, resulting in the black and fully melanized stage IV melanosomes.

In skin melanocytes, which are situated at the basal epidermis, mature melanosomes are trafficked to the cell periphery and transferred to neighboring keratinocytes in the epidermis in a process that remains incompletely understood (Van Den Bossche et al., 2006). The melanosomes in the keratinocytes are distributed in a protective cap that shields the nucleus from UV exposure, and it is these melanosomes found in keratinocytes that give humans their characteristic skin color. Interestingly, the racial differences in skin color do not stem from different numbers of melanocytes (Staricco and Pinkus, 1957); rather, melanocytes in people with darker skin seem to be more active than melanocytes from people with lighter skin (Naeyaert et al., 1991; Iozumi et al., 1993). More importantly, the distribution of melanosomes within host keratinocytes is altered, such that melanosomes in Black keratinocytes are singly dispersed, while melanosomes in Caucasian or Asian keratinocytes are clustered into bundles (Szabo et al., 1969; Toda et al., 1972). Melanocytes in the skin and hair continually synthesize and transfer their melanosomes to the neighboring keratinocytes, but in the pigmented tissues of the eye, the melanosomal maturation process is thought to occur once during early development, and then the melanosomes remain as permanent residents within the pigment cell (Boissy, 1988). However, although temporally distinct, the molecular
processes of melanosome biogenesis are thought to be the same in skin and eye pigment cells.

1.3.4 Transporters that affect melanosome function

In addition to the melanosome contents outlined above, several additional pigment cell-specific proteins are known to be important for proper melanosome biogenesis and function. Many of them are transporters. Mutations in the gene SLC45A2 cause OCA type 4 (Newton et al., 2001) (Figure 1.1b), which is rare worldwide but common in Japan (Inagaki et al., 2004; Inagaki et al., 2005), and the phenotypes in b medaka fish (Fukamachi et al., 2001) and the underwhite mouse (Lehman et al., 2000; Newton et al., 2001). The hypopigmentation in these models might be a consequence of defective tyrosinase processing and trafficking (Costin et al., 2003). Surprisingly, the SLC45A2 protein (also known as Membrane-Associated Transport Protein or MATP, and Antigen in Melanoma 1, or AIM-1) most closely resembles sucrose transporters in plants (Fukamachi et al., 2001; Newton et al., 2001), yet humans do not express any known sucrose transporters. Thus the potential transport substrate of SLC45A2 remains a mystery. Another pigment cell-specific transporter, SLC24A5, is a K⁺-dependent Na⁺/Ca²⁺ exchanger and is the most highly-expressed Na⁺/Ca²⁺ exchanger in melanocytes (Ginger et al., 2008). The SLC24A5 homolog is mutated in the hypopigmented golden zebrafish (Lamason et al., 2005), and the corresponding knockout mouse shows hypopigmentation in the eye and skin (but surprisingly not in the coat) (Vogel et al., 2008). Knockdown of SLC24A5 in a mouse melanoma cell line causes a reduction in cellular melanin levels (Ginger et al., 2008). Single nucleotide polymorphisms in
SLC45A2 and SLC24A5 are associated with normal human variations in skin, hair, and eye color (Lamason et al., 2005; Sturm, 2006; Graf et al., 2007; Norton et al., 2007; Branicki et al., 2008; Mengel-From et al., 2010), suggesting a critical modifying function in melanogenesis.

Other more ubiquitously-expressed transporters are also thought to be critical for regulating the function of melanosomes, in part by regulating lumenal pH (Fuller et al., 2001). Melanosomes have long been considered to be acidic (Devi et al., 1987; Tripathi et al., 1988; Bhatnagar et al., 1993), but the pH is not constant throughout melanosome maturation. Acidification is necessary for proteolytic processing steps that generate fibrillogenic fragments of PMEL (Berson et al., 2001), suggesting that early stage melanosomes are likely acidic. Using quantitative immunoelectron microscopy to quantify the accumulation of DAMP (which accumulates in acidic compartments in proportion to acidity) in melanosomes and endosomal compartments of a human melanoma cell line, Raposo et al. showed that early stage melanosomes are highly acidic, but that melanosomes become less acidic as they mature (Raposo et al., 2001). Such progressive alkalinization is likely necessary for melanization. Both human (Fuller et al., 2001) and mouse tyrosinase (Saeki and Oikawa, 1983; Townsend et al., 1984) show optimal function in vitro at a near-neutral pH, and melanin can form in neutral media into which active Tyr is aberrantly secreted from mutant melanocytes (Potterf et al., 1998; Manga and Orlov, 2001). Melanocytes from Caucasians appear to have lower tyrosinase activity in situ and contain more acidic organelles than the more active melanocytes from Blacks (Ancans et al., 2001b; Fuller et al., 2001), despite similar amounts of tyrosinase.
protein (Iozumi et al., 1993) and identical pH optima (Fuller et al., 2001) in both types of
melanocytes. Treatments that raise organellar pH stimulate tyrosinase activity in
Caucasian melanocytes without concomitant changes in tyrosinase abundance, but they
have little to no effect on Black melanocytes (Ancans et al., 2001b; Fuller et al., 2001).
Similar treatments cause human (Halaban et al., 2002) or murine melanoma cell lines to
become more pigmented, again without changes in tyrosinase protein levels (Saeki and
Oikawa, 1983; Oikawa et al., 1987; Ancans and Thody, 2000), and electron microscopy
confirmed that the induced melanin production takes place in previously immature
melanosomes (Saeki and Oikawa, 1983; Oikawa et al., 1987). These data indicate that
alkalinization is critical during progressive melanosome biogenesis in order to optimize
pigmentation.

How is acidity regulated during melanosome biogenesis? Inhibitors of the vacuolar
proton ATPase inhibit melanosome acidification (Bhatnagar and Ramalah, 1998; Ancans
et al., 2001b; Tabata et al., 2008) suggesting that this ubiquitous endosomal transporter is
necessary for acidification. However, since this complex is responsible for acidification
in the TGN and all endosomal compartments, its activity is likely regulated and/ or
complemented by other transporters. For example, treatments that inhibit Na\(^+\)/H\(^+\)
exchangers (NHEs) – transporters that are used by many cell types to regulate
intracellular and intraorganellar pH levels (Orlowski and Grinstein, 1997) - reduce the
tyrosinase activity of Black but not Caucasian melanocytes (Smith et al., 2004).
Expression analysis demonstrated that several NHE isoforms are expressed in Black and
Caucasian melanocytes and thus may normally act to bring the pH to the optimum level
for tyrosinase activity (Sarangarajan et al., 2001; Smith et al., 2004). Since cytoplasmic pH was not altered by the NHE inhibitor treatments, it was assumed that these NHE proteins localize at the melanosome itself, in agreement with low-resolution indirect immunofluorescence microscopy results (Smith et al., 2004). The regulation of melanosomal pH probably also depends on other unidentified proteins, which likely include some of the melanocyte-specific transporters discussed above and further below.

1.3.5 Transporter localization in melanocytes
Although the transporters described above influence melanosome function, it is not clear whether these effects are direct – i.e. they regulate solute transport into or out of melanosomes – or indirect by affecting precursor compartments, transport intermediates from precursor compartments, or the cytosol. For example, melanocytes derived from Slc45a2-deficient mice suggest that tyrosinase folding in the endoplasmic reticulum might be defective (Costin et al., 2003), implying a potential indirect role for SLC45A2 in regulating the lumenal environment of the endoplasmic reticulum. Similar concerns have been raised for OCA2, the topic of this thesis (see below). Indirect immunofluorescence assays show that SLC24A5 partially localizes to the TGN and partially to punctate structures that might represent melanosomes (Ginger et al., 2008). Proteomic characterization of melanosome-containing fractions has led to proposals that SLC45A2 and SLC24A5 are localized to melanosomes, but this has not been conclusively demonstrated (Chi et al., 2006). Such a localization seems reasonable for SLC24A5, as melanosomes are thought to store high amounts of calcium (Panessa and Zadunaisky, 1981; Salceda and Sanchez-Chavez, 2000). Proteomics analyses of
melanosome fractions have also identified several subunits of the vacuolar ATPase but none of the NHE proteins (Basrur et al., 2003; Chi et al., 2006), although vacuolar ATPase subunits and several NHE proteins have been suggested to be localized to the melanosome by low-resolution indirect immunofluorescence microscopy (Smith et al., 2004; Tabata et al., 2008).

In Chapter 2, I focus studies on the localization of another putative melanocyte transporter, OCA2, for which localization and function in melanosome biogenesis have been controversial. Before entertaining a discussion of what was known about OCA2 before I began my work, I will introduce known mechanisms by which melanosomes acquire their protein contents.

1.4 Protein transport to melanosomes

As mentioned earlier, proper localization of proteins is critical for their ability to carry out their functions. In order to generate functional melanosomes, melanosome contents must be diverted within melanocytic cells from classical endocytic compartments, with which melanosomes coexist. This is the main question addressed in this thesis. Consequently, I will discuss below current models for melanosome protein delivery.

1.4.1 Biogenesis of and protein delivery to early stage melanosomes

The PMEL and GPR143 (OA1) proteins are known to localize to melanosomes but use unusual sorting determinants for their trafficking. PMEL is the major component of the internal fibrillar matrix of the melanosome lumen (Lee et al., 1996; Raposo et al., 2001).
It is synthesized as an integral membrane protein that bears a C-terminal ER exit signal with a critical valine residue and a conventional dileucine motif that serves as a signal directing internalization from the plasma membrane. Disruption of these signals underlies the defect in melanosomal sorting of PMEL in the silver mouse (Theos et al., 2006a). Following endocytosis, PMEL is delivered to the intralumenal vesicles seen in Stage I melanosomes (Berson et al., 2001). This internalization is required for the proteolytic cleavage events that generate the fibrillogenic peptide of PMEL (Berson et al., 2001; Berson et al., 2003). The sorting to the ILVs depends on a luminal determinant (Hoashi et al., 2006; Theos et al., 2006b) that overlaps with the fibrillogenic determinant (Watt et al., 2009). Unlike most other proteins that are sorted to ILVs, PMEL does not require ubiquitination or the activity of the ESCRT machinery for its sorting (Theos et al., 2006b). It is on these ILVs that PMEL initiates fibril formation, ultimately forming the fibrillar sheets that characterize stage II melanosomes (Hurbain et al., 2008). While ILV formation and fibrillogenesis are initiated in stage I melanosomes that are accessible to endocytic tracers, it is yet unknown how these compartments mature into two distinct compartments – late endosomes and stage II melanosomes – with distinct characteristics.

Two other components of early stage melanosomes are not limited to these compartments but also present in other compartments within melanocytes. GPR143 localizes to late endosomes, lysosomes, and melanosomes, especially early-stage melanosomes (Piccirillo et al., 2006; Giordano et al., 2009), by virtue of the combined sorting activities of an unusual dileucine motif and a tryptophan-glutamate doublet in the cytoplasmic C-
terminus of the protein (Piccirillo et al., 2006). These two signals are together necessary and sufficient for lysosomal and melanosomal localization of the protein, but the effectors that recognize these signals and effect GPR143 delivery are not known. MART1 (also called melan-a) is an unusual small integral membrane protein with no signal sequence. Like GPR143, MART1 localizes to multiple compartments, including late endosomes and lysosomes, to which it is targeted by ubiquitylation and ESCRT-dependent partitioning to lysosome-bound intralumenal vesicles (Levy et al., 2005; Theos et al., 2006b). MART1 and GPR143 can be coimmunoprecipitated (Giordano et al., 2009), and MART1 has also been suggested to associate with PMEL (Hoashi et al., 2005; Giordano et al., 2009), but the consequences of these interactions are not known.

1.4.2 Trafficking of melanosomal proteins via the AP complexes

Once unpigmented stage II melanosomes are formed, they mature into pigmented stage III and IV melanosomes by the delivery of melanogenic enzymes and transporters. This set of melanosomal proteins is specifically sorted from endosomes towards Stage II premelanosomes via the activity of more conventional sorting signals. These include Tyr and Tyrp1 (Vijayasaradhi et al., 1995; Calvo et al., 1999; Simmen et al., 1999; Huizing et al., 2001; Theos et al., 2005; Setty et al., 2007; Delevoye et al., 2009). Tyr and Tyrp1 are both type I single-pass transmembrane proteins with cytoplasmic acidic dileucine sorting signals. These signals are short, linear peptide motifs in the cytoplasmic domains of transmembrane proteins that have a consensus sequence of [D/E]XXXL[L/I] (Bonifacino and Traub, 2003). Similar signals are found in a wide variety of predominantly endosomal and lysosomal integral membrane proteins and often serve as
independent, transferrable sorting signals for endocytosis and/or for subsequent endosomal sorting. The acidic dileucine motif in the cytoplasmic domain of Tyr is necessary and sufficient for protein localization to late endosomes and lysosomes in non-melanocytes (Calvo et al., 1999; Simmen et al., 1999) and to synaptic vesicles in neuronal cells (Blagoveshchenskaya et al., 1999), and is likely necessary for melanosomal trafficking in melanocytes (Beermann et al., 1995). The Tyrp1 signal is necessary and sufficient for targeting to intracellular compartments in melanocytic and non-melanocytic cells (Winder et al., 1993; Vijayasaradhi et al., 1995).

Acidic dileucine signals effect protein sorting by binding to conserved sites on heterotetrameric Adaptor Protein (AP) complexes. APs are a family of four related complexes that have been extensively studied in their roles as soluble sorting adaptors (Figure 1.3a). They are recruited to membranes, at least in part by binding to phosphoinositides or other prevalent molecules (Gaidarov and Keen, 1999; Zhu et al., 1999; Drake et al., 2000), and cluster integral membrane cargo proteins that contain AP binding sites into coated membrane domains. AP-1, AP-2 and AP-3 also recruit clathrin, and thus facilitate cargo incorporation into clathrin-coated vesicles that bud from these domains at the trans-Golgi network, the cell surface, and endosomes (Boehm and Bonifacino, 2001; Bonifacino and Traub, 2003; Robinson, 2004). Signal-bearing cargo proteins are thus able to engage the vesicle trafficking machinery for directed sorting to target membranes.
Figure 1.3

**a.**

**b.**

**c.**

**d.**

[Diagram images not transcribed]
Figure 1.3. HPS-affected complexes and pigmentation phenotypes.

a) The AP complexes are a family of heterotetrameric soluble adaptors that are recruited from the cytosol to membranes, where they bind signals in the cytoplasmic domains of cargo proteins and cluster those proteins together for subsequent budding into transport carriers (Dell'Angelica et al., 1999). b) The majority of genes affected in Hermansky-Pudlak Syndrome patients and mouse models encode subunits of five multisubunit complexes: AP-3, HOPS, BLOC-1, BLOC-2, and BLOC-3. The color-coding indicates the experimental basis for the interactions depicted between subunits. Subunits in red are mutated in human subtypes of HPS. Subunits in yellow are mutated in additional mouse models of HPS (Di Pietro and Dell'Angelica, 2005). c) The AP-3-deficient pearl mouse—a model for HPS2—(bottom) is hypopigmented relative to a wild-type C57BL/6J mouse (top) (Li et al., 2004). d) Mice deficient in each of the BLOCs show varying degrees of hypopigmentation.³

In melanocytes, AP-1, AP-2 and AP-3 have been implicated in cargo trafficking to melanosomes. The signal in the Tyr cytoplasmic domain interacts with AP-1, AP-2, and AP-3 in vitro (Honing et al., 1998; Theos et al., 2005; Chaudhuri et al., 2009). Tyr can be localized to endosomal buds and tubules that are decorated with AP-1 or AP-3 (Honing et al., 1998; Theos et al., 2005; Chaudhuri et al., 2009), and a significant cohort of Tyr is mislocalized in melanocytes that are genetically deficient in AP-3 (Huizing et al., 2001; Richmond et al., 2005; Theos et al., 2005). By contrast, the Tyrc1 sorting signal interacts with AP-1 but not AP-3 in vitro (Theos et al., 2005) and Tyrc1 can be crosslinked to and co-immunoprecipitated with AP-1 in melanoma cells (Delevoye et al., 2009).

Consistently, Tyrc1 is correctly localized to melanosomes in AP-3-deficient melanocytes (Huizing et al., 2001; Setty et al., 2007). Melanocytes that are genetically deficient in AP-1 are not available since the knockout is embryonic lethal in mice (Zizioli et al., 1999; Meyer et al., 2000), but knockdown of AP-1 in a human melanoma cell line by siRNA causes the majority of Tyrc1 to be mislocalized to vacuolar recycling endosomal domains (Delevoye et al., 2009). AP-1 is also important for the peripheral positioning of endosomes in melanocytes to appose melanosomes, since in the absence of AP-1 recycling endosomes are abnormally shifted to a perinuclear distribution. This is due to the failure of endosomal recruitment of the AP-1 cargo and microtubule motor KIF13A. AP-1 likely regulates the delivery of other cargoes to melanosomes, since its knock down by siRNA leads to a reduction in cellular melanin content (Delevoye et al., 2009).

Finally, AP-2 has been implicated in enhancing the delivery of PMEL to endosomal compartments in melanocytes (Robila et al., 2008), likely by interacting with the previously-mentioned acidic dileucine signal in its cytoplasmic domain (Theos et al.,
From these results it is clear that dileucine motifs and AP complexes play a major role in melanosomal trafficking.

An additional pigment cell-specific protein, OCA2, also bears a putative acidic dileucine motif (Lee et al., 1995), but the trafficking of this protein—before the beginning of this thesis—had not been characterized. OCA2 trafficking will be discussed extensively below.

### 1.5 Diseases of melanosome biogenesis and trafficking

As mentioned previously, melanosomes and some other LROs coexist with conventional lysosomes in their host cells (Raposo et al., 2007), suggesting that specialized sorting pathways are used to segregate LRO cargo from the lysosomal pathway. Some components of this sorting machinery, including AP-3, have been identified by the discovery of human patients in whom LRO function is disrupted (Raposo et al., 2007; Huizing et al., 2008). Genetic diseases in which defective LRO biogenesis is implicated include Chediak-Higashi Syndrome, Griscelli Syndrome, and Hermansky-Pudlak Syndrome (HPS), discussed below. Much of the work that has been performed to characterize these syndromes has been performed in melanocytic models (Raposo and Marks, 2007). As we will see, the genes that are affected in HPS play particularly important roles in trafficking of proteins to melanosomes.

#### 1.5.1 Hermansky-Pudlak Syndrome
Hermansky-Pudlak Syndrome is a set of genetic diseases characterized by phenotypes arising from the loss of LRO function (Di Pietro and Dell'Angelica, 2005; Huizing et al., 2008). The syndrome is divided into several subtypes. All exhibit oculocutaneous albinism and prolonged bleeding, due to the loss of melanosomes and platelet dense granules, respectively. Some HPS subtypes additionally involve lung fibroses that most likely arise due to defective lamellar body function, leading to death in midlife. Some subtypes also exhibit immune deficiencies because of the defect in the biogenesis of LROs in cells of the adaptive and innate immune system, including lytic granules in T lymphocytes and natural killer cells (Clark and Griffiths, 2003; Fontana et al., 2006) and signaling endosomes in plasmacytoid dendritic cells (Blasius et al., 2010; Sasai et al., 2010). It is worth emphasizing that HPS affects several cell types, unlike the primary OCA diseases in which the defects are in genes that are restricted in expression to pigment cells and in which phenotypes are restricted to processes affected only by pigment synthesis. The appearance of multifaceted phenotypes arising from a single defective gene in a given HPS subtype indicates that LRO trafficking has common features conserved across different cell types, but the distinct phenotypes seen in different HPS subtypes also point to some cell-type-specific variations in trafficking.

The eight subtypes are each caused by a defect in a different gene (Di Pietro and Dell'Angelica, 2005; Wei, 2006; Huizing et al., 2008). Although the clinical phenotypes of the disease arise specifically within tissues that harbor LROs, the genes are ubiquitously expressed. There are mouse models of each HPS subtype with defects in the homologous genes, and at least six additional mouse models of the disease for which
patients with defects in the homologous human genes have not been found (Li et al., 2004; Di Pietro and Dell'Angelica, 2005). The majority of the proteins encoded by the affected genes are subunits of five completely distinct multisubunit complexes (Figure 1.3b). These include the Class C Vps complex (a.k.a. homotypic fusion and vacuole sorting complex, or HOPS), AP-3, and the Biogenesis of Lysosome-Related Organelles Complexes-1, -2, and -3 (BLOC-1, -2, and -3).

1.5.1a HPS-associated multisubunit complexes

The yeast HOPS complex comprises the core VPS-C complex (shared with another multisubunit complex) and two distinct subunits Vps39 and Vps41 (Figure 1.3b). These proteins are a guanine nucleotide exchange factor (Wurmser et al., 2000) and an effector (Brett et al., 2008), respectively, for the yeast vacuolar Rab Ypt7, and HOPS is involved in regulating membrane fusion events at the vacuole (Raymond et al., 1992). HOPS acts as a tethering complex that interacts with Ypt7 and acidic phospholipids in the vacuole membrane, and its interaction with SNARE complexes promotes trans-SNARE complex assembly and selectivity (Stroupe et al., 2006; Hickey et al., 2009). The mammalian HOPS complex is less well studied but appears to also be involved in late endosomal and lysosomal fusion events and function in association with the late endosomal SNARE syntaxin 7 (Caplan et al., 2001; Kim et al., 2001; Poupon et al., 2003). None of the HOPS complex subunits are identified as human HPS genes, but a point mutation in the Vps33a subunit underlies the HPS model mouse buff, and mutations in several VPS-C subunit genes in Drosophila melanogaster (including the Vps33a mutant carnation) result in abnormal eye color due to defects in the biogenesis of pigment granules in the
eye (Lloyd et al., 1998; Sevrioukov et al., 1999; Sriram et al., 2003; Richardson et al., 2004; Pulipparacharuvil et al., 2005). Mutations in genes encoding other HOPS subunits in Caenorhabditis elegans cause defects in birefringent gut granules, LROs found in the intestine (Hermann et al., 2005).

The heterotetrameric AP-3 complex (Figure 1.3a, b) has been discussed previously. It consists of one each of beta3, mu3, sigma3, and delta subunits (Robinson, 2004). Beta3A, mu3A, sigma3A or sigma3B, and delta assemble into a ubiquitously-expressed complex, while the neuron-specific beta3B (Darnell et al., 1991; Newman et al., 1995; Gurkan et al., 2005) and mu3B (Pevsner et al., 1994; Gurkan et al., 2005) subunits assemble with sigma3A or sigma3B and delta into a neuron-specific complex. The beta3A subunit is mutated in HPS2 patients and the pearl mouse (Feng et al., 1999; Zhen et al., 1999; Yang et al., 2000; Peden et al., 2002) (Figure 1.3c), and the delta subunit, present in all AP-3 complexes, is mutated in the mocha mouse (Lane and Deol, 1974; Noebels and Sidman, 1989; Kantheti et al., 1998). The mocha mouse has the pigmentation phenotype seen in pearl mice but additionally suffers from neurological disorders such as hyperactivity, seizures, and deafness (Noebels and Sidman, 1989; Swank et al., 1991), presumably because in pearl mice the neuronal beta3B isoform can compensate for the loss of the ubiquitous beta3A subunit, but in mocha mice the loss of the single-isoform delta subunit causes the loss of all AP-3 complexes. C. elegans gut granule defects are linked to mutations in the genes for two subunits (Hermann et al., 2005) and Drosophila eye color mutants have been linked to mutations in all four AP-3 subunits (Ooi et al., 1997;
Simpson et al., 1997; Mullins et al., 1999; Mullins et al., 2000). HOPS and AP-3 are both conserved from yeast to mammals (Dell'Angelica, 2004).

The majority of the HPS-affected genes encode subunits of the BLOC complexes (Figure 1.3b) and were thought to be found only in higher eukaryotes, although more recent low stringency homology searches have revealed potential homologs of BLOC-1 and BLOC-3 in Dictyostelium and S. cerevisiae (Cheli and Dell'Angelica, 2010; Hayes et al., 2011). The subunits of each BLOC complex are not homologous to proteins of known function, and thus the functions of the BLOCs are still not clear. However, a handful of potential interactions with other proteins have been identified and provide some clues regarding function (Di Pietro and Dell'Angelica, 2005). For example, the pallidin subunit of BLOC-1 interacts with the endosomal SNARE syntaxin 13 (Huang et al., 1999; Moriyama and Bonifacino, 2002; Ghiani et al., 2010), and the snapin subunit might interact with the exosomal SNAREs SNAP23 and SNAP25 (Ilardi et al., 1999; Vites et al., 2004); these interactions have been replicated for the complete BLOC-1 (Ghiani et al., 2010), suggesting that the complex might regulate LRO trafficking by affecting endosomal membrane fusion events. As discussed below, data from our laboratory suggest that BLOC-1 and BLOC-2 function during cargo delivery from endosomes to maturing melanosomes, supporting such a role.

BLOC-3 is composed of the HPS1 and HPS4 proteins (Figure 1.3b), mutated in human HPS type 1 and type 4 patients and in the pale ear and light ear mice (Dell'Angelica et al., 2000). Loss of BLOC-3 in the mouse models has a mild effect on coat and eye color
but does cause lightening of the hairless skin of the ears and tail (Figure 1.3d), indicating differences in melanosome biogenesis and development in follicular versus interfollicular melanocytes (Gautam et al., 2006; Nguyen and Wei, 2007). Unique among the BLOCs, loss of BLOC-3 causes melanosomes to become enlarged in the choroid of the eye (Gardner et al., 1997; Feng et al., 2002; Suzuki et al., 2002). Moreover, the lung fibrosis phenotype of HPS is especially prevalent in patients suffering from defects in BLOC-3 (Anderson et al., 2003) and lung function is uniquely affected in mice with mutations in both BLOC-3 and other complexes (Guttentag et al., 2005; Gautam et al., 2006). The heightened severity of LRO phenotypes in double mutants of BLOC-3 in combination with BLOC-2 or AP-3 suggest that BLOC-3 works in a separate pathway from those complexes and perhaps from BLOC-1 as well (Feng et al., 2002; Di Pietro et al., 2006; Gautam et al., 2006). The molecular function of BLOC-3, however, is not clear. Knockdown of BLOC-3 subunit expression in fibroblasts results in altered distribution of late endosomes/lysosomes, suggesting a potential primary role for BLOC-3 in organelle motility (Falcon-Perez et al., 2005), consistent with defects in melanosome transfer observed in hair follicles of pale ear and light ear mice (Nguyen et al., 2002; Nguyen and Wei, 2004).

BLOC-2 comprises HPS3, HPS5, and HPS6 (Figure 1.3b), defective in human HPS types 3, 5 and 6 and the cocoa, ruby-eye 2, and ruby-eye mice, respectively (Di Pietro and Dell'Angelica, 2005). The orthologue to the HPS5 gene is also defective in the Drosophila eye color mutant pink (Falcon-Perez et al., 2007). BLOC-2-deficient mice are more hypopigmented than BLOC-3-deficient mice (Figure 1.3d), and loss of this
complex (but not the other BLOCs) causes choroidal melanosomes to become clumped into multi-melanosomal structures (Zhang et al., 2003; Gautam et al., 2004). Melanocytes from BLOC-2-deficient patients accumulate tyrosinase-containing vesicular structures in the cytoplasm (Boissy et al., 2005; Helip-Wooley et al., 2007), suggesting that BLOC-2 might play some role in vesicular fusion during melanosome biogenesis. We will discuss this potential role further below.

BLOC-1 comprises at least eight subunits (Di Pietro and Dell'Angelica, 2005) (Figure 1.3b). The dysbindin subunit is mutated in HPS7 and the sandy mouse and the BLOS3 subunit is mutated in HPS8 and the reduced pigmentation mouse. Another three HPS mouse models—pallid, muted, and cappuccino—are defective in the pallidin, muted, and cappuccino subunits. Of the three BLOC complexes, BLOC-1 mutant mice are the most severely hypopigmented (Dell'Angelica, 2004) (Figure 1.3d) and BLOC-1 is epistatic to BLOC-2 and BLOC-3 in pigmentation (Di Pietro et al., 2006; Gautam et al., 2006).

1.5.1b Other HPS-associated genes and proteins
The remaining murine HPS-associated genes are presented briefly here for the sake of completeness. Slc7a11, mutated in the subtle gray (sut) mouse, encodes a cystine transporter that is critical for cystine import and maintenance of cellular glutathione levels in cultured melanocytes and other cell types (Chintala et al., 2005). Accordingly, pheomelanin but not eumelanin production is drastically reduced in sut mice. Dense granule numbers are also reduced in platelets from these mice (Swank et al., 1996). Rab38 is mutated in the chocolate mouse (Loftus et al., 2002). In melanocytes, Rab38 is
associated with melanosomes and is needed for proper Tyrp1 trafficking (Loftus et al., 2002; Wasmeier et al., 2006), consistent with the similarity of the coat color phenotypes in chocolate and Tyrp1-deficient brown mice (Loftus et al., 2002). Rabggta, mutated in the gunmetal mouse, encodes Rab geranylgeranyltransferase alpha, required for the prenylation and membrane-anchoring of several Rabs (Detter et al., 2000). Rabggta deficiency leads to phenotypes in multiple tissues (Seabra et al., 2002). One target of Rabggta prenylation is Rab27a. Together with its effector protein melanophilin, melanosome-associated Rab27a recruits myosin VA, enabling transfer of melanosomes from microtubules onto the peripheral actin network for subsequent transfer to keratinocytes (Wu et al., 2001; Hume et al., 2002). Mutations in the genes encoding the subunits of the Rab27a/melanophilin/myosin VA complex lead to perinuclear clustering of melanosomes and give rise respectively to the hypopigmentation phenotype of ashen, leaden, and dilute mice, models of the three subtypes of Griscelli Syndrome, another LRO biogenesis disorder (Pastural et al., 1997; Menasche et al., 2000; Menasche et al., 2003).

1.5.2 Interactions among BLOCs and AP-3

How do the HPS complexes interact? A portion of BLOC-1 and BLOC-2 can be co-immunoprecipitated with each other from detergent extracts of membranes from fibroblasts and neuronal cell lines (Di Pietro et al., 2006; Salazar et al., 2006), suggesting that they functionally interact. A separate cohort of BLOC-1 can also be coimmunoprecipitated from membrane extracts with AP-3 (Di Pietro et al., 2006), and BLOC-1 can be isolated from immunoaffinity purified neuronal subcellular fractions
enriched in AP-3 complexes (Salazar et al., 2006) as well as from clathrin-coated vesicle fractions of HeLa cells (Borner et al., 2006). This suggests that a cohort of BLOC-1 might function together with AP-3, consistent with data from AP-3- and BLOC-1-deficient neurons that show similar patterns of cargo trafficking defects (Newell-Litwa et al., 2009; Salazar et al., 2009). However, this has to be reconciled with data in melanocytes that AP-3 and BLOC-1 localize to distinct domains of early endosomes (Theos et al., 2005; Di Pietro et al., 2006; Setty et al., 2007), and with functional data discussed below that implicate BLOC-1 and AP-3 in distinct cargo trafficking pathways to melanosomes (Theos et al., 2005; Setty et al., 2007; Setty et al., 2008). Data presented in this thesis will attempt to clarify this controversy.

1.5.3. HPS complexes and melanosome biogenesis

Melanocytes have proven to be an excellent model in which to dissect the function of the BLOCs and AP-3 in cargo trafficking to LROs. This has been supported by the availability of primary melanocytes from both HPS patients and HPS mouse models, as well as immortalized melanocytes from HPS mouse models. Studies in primary melanocytes derived from human HPS patients suggest that Tyr is mislocalized in AP-3-deficient cells (Richmond et al., 2005). As discussed earlier, studies in mouse melanocytes showed that Tyr was largely mislocalized to late endosomes and vacuolar early endosomes in AP-3-deficient melanocytes (Theos et al., 2005), but a cohort of Tyr was present in melanosomes. By contrast, in BLOC-1-deficient cells 60% of the wild-type cohort of Tyr is correctly localized to melanosomes. In contrast, whereas Tyrp1 appears to be localized normally to melanosomes in AP-3-deficient cells (Huizing et al.,
2001; Setty et al., 2007), nearly all Tyrp1 in BLOC-1-deficient melanocytes is depleted from melanosomes and instead redistributed largely to vacuolar domains of early endosomes, demonstrating a degree of cargo selectivity by BLOC-1 (Setty et al., 2007). Analysis of the dynamics of Tyrp1 in these cells indicates that Tyrp1 exit from these vacuoles in melanosome-bound transport carriers is defective, such that instead Tyrp1 is “stuck” in an endless recycling loop to the plasma membrane. Interestingly, the compartments in which Tyrp1 becomes entrapped in BLOC-1-deficient cells are positive for the early endosomal SNARE syntaxin 13 (Setty et al., 2007). Like Tyrp1, the copper transporter ATP7A, defective in the hypopigmented mottled mouse (Levinson et al., 1994) and in patients suffering from Menkes disease (Tumer and Moller, 2010), is also largely mislocalized to vacuolar early endosomes in BLOC-1-deficient cells but correctly localized in AP-3-deficient cells (Setty et al., 2008). These data suggest that both Tyrp1 and ATP7A are cargoes of an AP-3-independent, BLOC-1-dependent transport pathway to melanosomes.

Interestingly, the effects of BLOC-1 deficiency on Tyrp1 trafficking and on pigmentation are similar to those observed in a human pigmented melanoma cell line upon knockdown of the AP-3-related adaptor, AP-1 (Delevoye et al., 2009). As discussed earlier, AP-1 – like AP-3 -- also binds to many melanosomal targeting signals, and in other cell types has been implicated in cargo delivery to or from endosomes. Like BLOC-1, AP-1 has been localized to tubulovesicular structures in proximity to melanosomes (Theos et al., 2005; Delevoye et al., 2009). Because of these similarities, it has been thought that AP-1 and BLOC-1 might work together in Tyrp1 trafficking to melanosomes. How such
cooperation might occur is unclear, as a biochemical interaction between these two complexes has not been seen (Salazar et al., 2009).

Several studies in primary melanocytes derived from human HPS patients have also suggested that Tyr and Tyrp1 are mislocalized in the absence of BLOC-2 (Boissy et al., 2005; Richmond et al., 2005; Helip-Wooley et al., 2007; Huizing et al., 2009). BLOC-2 also seems to regulate Tyrp1 trafficking in mouse melanocytes, and it may act from an endosomal intermediate downstream of BLOC-1 (Setty et al., 2007). Immunoelectron microscopy in these cells has demonstrated that both BLOC-1 and BLOC-2 localize to tubulovesicular elements that are closely associated with melanosomes (Di Pietro et al., 2006; Setty et al., 2007). The presence of transferrin in these tubules after long periods of uptake identifies them as deriving from recycling endosomal domains. Importantly, such domains have been shown to be involved in the formation of direct tubular contacts between vacuolar endosomes and maturing melanosomes (Delevoye et al., 2009), suggesting that BLOC-1 and BLOC-2 might play critical roles in either the formation of these domains or the integration of cargoes into them. Like in BLOC-1-deficient cells, Tyrp1 is largely redistributed to STX13-containing early endosomes and to the plasma membrane in BLOC-2-deficient cells (Setty et al., 2007). However, Tyrp1 in BLOC-2-deficient melanocytes is also aberrantly delivered to lysosomes (Setty et al., 2007) and transported in a retrograde manner to the Golgi (A. Mantegazza, M. Dennis, D. Tenza and G. Raposo, unpublished data), suggesting that BLOC-2 may help direct where the Tyrp1-containing tubules deliver their cargo. BLOC-1/BLOC-2 double mutant mice show the more severe hypopigmented phenotype of BLOC-1-deficient mice, also
consistent with BLOC-2 working downstream of BLOC-1 (Di Pietro et al., 2006; Gautam et al., 2006).

1.6 A model for melanosomal trafficking

The differential effect of loss of AP-3 or BLOC-1 on trafficking of Tyr versus Tyrp1 and ATP7A suggests a model by which two distinct pathways, one controlled by each complex, exist for anterograde trafficking of integral membrane proteins to melanosomes (Raposo and Marks, 2007; Setty et al., 2007). Specifically, the model describes an AP-3-mediated pathway taken by a large cohort of Tyr, and a BLOC-1-dependent pathway taken by nearly all of the Tyrp1 and ATP7A proteins and by a portion of Tyr (Figure 1.4). The Tyrp1 trafficking studies suggest that the BLOC-1-dependent pathway also utilizes AP-1 and BLOC-2. This model of distinct BLOC-1 and AP-3 pathways is consistent with the observation that a mouse that is doubly homozygous for BLOC-1 and AP-3 mutations is more hypopigmented in the coat, choroid, and retinal pigment epithelium and has more pronounced lamellar body abnormalities than either the BLOC-1 or AP-3 single mutant mouse (Di Pietro et al., 2006; Gautam et al., 2006).

The model of distinct BLOC-1-mediated and AP-3-mediated trafficking pathways is at odds with data showing a robust biochemical interaction between BLOC-1 and AP-3 in primary mouse neurons, a neuronal cell line, and mouse melanocytes, and with the ability of one to affect the membrane recruitment of the other (Di Pietro et al., 2006; Salazar et al., 2006; Newell-Litwa et al., 2009; Salazar et al., 2009), unpublished results from our lab). AP-3 helps generate synaptic vesicles from endosomes in neurons
Figure 1.4. Current model of melanosomal trafficking from endosomes.

Fluid phase cargoes (green arrows) follow the classical endocytic pathway through the early endosome (EE)/stage I coated endosome (St I/CE) to late endosomes (LE) and finally to lysosomes (Lys), but do not access maturing melanosomes. The model of directed endosome-to-melanosome trafficking is derived from studies of the trafficking of tyrosinase (orange arrows) and Tyrp1 (pink arrows). Tyrp1 utilizes a BLOC-1-dependent and AP-3-independent pathway for trafficking from endosomes to melanosomes. A minor cohort of tyrosinase may also use this pathway, while the majority of tyrosinase uses a BLOC-1-independent, AP-3-dependent pathway for trafficking to Stage II and III melanosomes (St II, St III). Melanin synthesis in Stage II melanosomes leads to melanin accumulation and melanosome maturation through Stages III and IV (St III, St IV).
(Faundez et al., 1998), and a subset of AP-3-dependent cargo is selectively enriched in synaptic vesicles from brain tissues from BLOC-1-deficient mice (Newell-Litwa et al., 2009), though trafficking in these cells is more complex due to the presence of the additional neuronal AP-3 complex. AP-3-dependent CD63 and VAMP7-T1 trafficking appear to be altered in fibroblasts lacking BLOC-1, but there are also conflicting data as to whether the trafficking of AP-3-dependent LAMP1 is altered in BLOC-1-deficient cells (Di Pietro et al., 2006; Salazar et al., 2006). It is also possible that increases in the surface levels of AP-3-dependent cargo in BLOC-1 deficient cells reflect indirect effects of the saturation of the internalization machinery due to the mislocalization of BLOC-1 cargo (Setty et al., 2007) rather than a direct requirement for BLOC-1 in the trafficking of the AP-3 cargo proteins. Thus although the possibility of a common trafficking pathway mediated by both BLOC-1 and AP-3 has been proposed in other cell types, the studies of Tyr, Tyrp1, and ATP7A have not provided direct evidence of such a pathway in melanocytic trafficking. Moreover, no evidence exists for such a pathway in trafficking to other LROs.

Further investigation of anterograde melanosomal trafficking pathways will require the characterization of additional melanosomal cargo proteins. As mentioned earlier, the pigment-cell-specific protein OCA2 has a putative acidic dileucine sorting motif in its cytoplasmic domain and thus appears poised to use AP-1 and/or AP-3 for trafficking to post-Golgi organelles. As such, it was an ideal candidate to be a melanosomal cargo protein that could be utilized to characterize trafficking mediated by HPS-associated complexes. In the next section we describe OCA2 in greater detail.
1.7 OCA2

1.7.1 The OCA2 gene and mutant phenotypes

The OCA2 protein is the product of the OCA2 gene (Brilliant, 2001). Recessive mutations in the gene cause oculocutaneous albinism type 2, the most common form of albinism among people of African heritage and worldwide (King, 1998). It has a reported incidence of 1:17,000 in Blacks or 1:30,000 in Caucasians in the United States (Witkop, 1985) to as high as 1:2000 among the Navajo (Yi et al., 2003) and 1:669 in Tuvalu (Johanson et al., 2010). Whereas OCA1 stems from a deficiency in tyrosinase and therefore displays a complete lack of pigment, OCA2 is often referred to as tyrosinase-positive albinism, since hypopigmented hairbulbs plucked from these patients, but not ones with OCA1, will become pigmented when incubated with the tyrosinase substrates L-tyrosine or L-DOPA (Kugelman and Van Scott, 1961; Witkop et al., 1973). Indeed, OCA2 patients can exhibit light to moderate pigmentation. They are born with light skin and lightly pigmented blond, reddish, or brown hair (King, 1998; King et al., 2003) (Figure 1.5a), consistent with the finding that OCA2 deficiency affects production of black-brown eumelanin but not yellow-red pheomelanin (Rinchik et al., 1993; Lamoreux et al., 1995; Hirobe et al., 2011). The hair may darken throughout life and some tanning is possible (Brilliant, 2001). They may also develop pigmented lentigines or nevi (Bothwell, 1997). Like all other OCA subtypes, OCA2 patients present phenotypes that are restricted to tissues involving dysfunction due to loss of pigment. For example, platelet dense granules are normal in patients with tyrosinase-positive albinism (OCA2).
Figure 1.5

a. Fraternal twin girls born to a couple of Congolese origin. One girl has normal pigmentation while the other has oculocutaneous albinism type 2 and shows the typical hypopigmentation phenotype. b) Defects in the murine Oca2 gene give rise to the pink-eyed dilution mouse. On a non-agouti background, these mice have light gray coats and pink eyes (Silvers, 1979). c) The proposed structure of OCA2 is that of a 12-transmembrane domain glycoprotein with a long N-terminal cytoplasmic domain and short C-terminal cytoplasmic domain. The sequence shows homology with transporters in a superfamily with members expressed from bacteria to humans (Sitaram et al., 2009).

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(Witkop et al., 1973). This is consistent with the pigment cell-restricted expression of OCA2 and the other OCA-associated genes (Gardner et al., 1992).

The mutations identified within the OCA2 gene in OCA2 patients and in controls are informative regarding the function of the OCA2 protein. While a high percentage of people of African heritage who suffer from OCA2 have a homozygous 2.7-kb deletion allele (Durham-Pierre et al., 1994), many cases of OCA2 in Africans and other ethnic groups are attributed to point mutations that result in small amino acid substitutions in the protein. These substitutions are mostly found in the C-terminal half of the protein in amino acid residues that are conserved in murine OCA2 (Lee et al., 1995). Non-pathological polymorphisms in human OCA2 and in the adjacent gene HERC2 are thought to modify OCA2 gene expression, and are strongly linked to ethnic differences in skin color and to the emergence of blue eye color in humans (Lao et al., 2007; Norton et al., 2007; Sulem et al., 2007; Eiberg et al., 2008; Sturm et al., 2008; Branicki et al., 2009; Mengel-From et al., 2010).

The OCA2 model mouse is named pink-eyed dilution, and the affected murine gene had originally been named p but is now referred to as Oca2. Over 100 mutant alleles of the murine gene have been isolated (Brilliant, 2001), and some are associated with phenotypes besides hypopigmentation, such as cleft palate, male sterility, and neurological disorders. However these alleles were induced by radiation treatment and the additional phenotypes derive from effects on physically-linked genes. The mutant Oca2 allele found in the original pink-eyed dilution mice arose spontaneously
(Gruneberg, 1952) and produces no transcript (Brilliant, 2001). These mice, when bred on a black, non-agouti background, have pink eyes and light gray coats, consistent with the OCA phenotype observed in humans (Figure 1.5b). Immortalized melan-p1 skin melanocytes used for cell culture experiments are derived from the \( p^{sp}/p^{25H} \) mouse; \( p^{sp} \) homozygotes have cleft palates and die soon after birth (Silvers, 1979), while \( p^{25H} \) homozygotes have abnormal reproduction (Hunt and Johnson, 1971), but the mixed heterozygotes are not documented as having these phenotypes (Lyon et al., 1992).

Neither OCA2 allele produces a transcript, and melan-p1 cells cultured from this mouse have very few mature, pigmented melanosomes, but instead display an abundance of small, hypopigmented compartments morphologically resembling Stage II melanosomes (Sidman et al., 1965; Rosemblat et al., 1998). The hypopigmentation phenotype indicates that Tyr activity in intact \( Oca2^{-/-} \) cells is reduced compared to controls. Surprisingly, \textit{in vitro} Tyr activity levels in these cells are similar to or even higher than in wild-type cells (Manga et al., 2001; Chen et al., 2002; Toyofuku et al., 2002). Localization of Tyr activity \textit{in situ} by DOPA histochemical assays and electron microscopy shows that a significant amount of Tyr correctly localizes to the non-pigmented melanosomes but is apparently inactive \textit{in vivo}. In this regard OCA2 deficiency resembles the hypopigmentation phenotype seen in BLOC-1 and BLOC-2 mutant cells, in which a significant amount of Tyr is correctly localized to melanosomes yet inactive (Boissy et al., 2005; Helip-Wooley et al., 2007; Setty et al., 2007; Setty et al., 2008), suggesting that the defect in OCA2-deficient cells might be due to failure to raise the lumenal pH or facilitate copper transport. Tyr activity in OCA2-deficient melanocytes is also abnormally localized to 50-nm cytoplasmic vesicles distributed throughout the cell, and
particularly near the plasma membrane (Manga et al., 2001). Again, this phenotype is reminiscent of the increased number of Tyr-containing cytoplasmic vesicles found in BLOC-2-deficient melanocytes cultured from HPS-3 and HPS-5 patients (Boissy et al., 2005; Helip-Wooley et al., 2007). Consistently, Tyr in OCA2-deficient cells appears to be aberrantly cleaved and secreted from cells, as is shown by a shift in Tyr protein and activity from the detergent phase to the aqueous phase and by immunoprecipitation of cleaved Tyr from the culture media, and the media itself contains an unusually high amount of melanin (Potterf et al., 1998; Manga et al., 2001; Manga and Orlow, 2001; Chen et al., 2002; Toyofuku et al., 2002; Chen et al., 2004). These data suggest that the loss of OCA2 function causes defects in the lumenal melanin-synthesizing environment as well as in delivery or retention of Tyr at the immature melanosomes.

1.7.2 The OCA2 protein – controversial function and localization

The OCA2 gene encodes a 12-transmembrane domain protein (Rinchik et al., 1993) that is highly conserved throughout vertebrate evolution (Gardner et al., 1992; Rinchik et al., 1993; Fukamachi et al., 2004; Protas et al., 2006) (Figure 1.5c), such that the C-terminal two-thirds of the human and mouse homologues share >90% amino acid sequence identity (Lee et al., 1995). The sequence shows homology to the ArsB/NhaD superfamily of permeases, with members expressed from bacteria to humans that transport a variety of substrates including arsenate and Na\(^+/\)H\(^+\) exchange. No transport substrate has been identified for OCA2. It had been hypothesized that the protein might serve as a melanosomal tyrosine transporter supplying the tyrosine substrates for melanin production, and that loss of this activity contributed to the hypopigmentation of OCA2-
deficient melanocytes. Consistent with this interpretation, supplementation of the culture media of these cells with extra tyrosine stimulated melanin synthesis (Sidman et al., 1965; Rinchik et al., 1993; Potterf et al., 1998). However, tyrosine uptake was found to be comparable across the plasma membrane of OCA2-deficient and wild-type melanocytes, as well as into melanosome-containing granular fractions from these cells (Gahl et al., 1995; Potterf et al., 1998), suggesting that the effect of tyrosine supplementation was indirect. Saccharomyces cerevisiae expressing exogenous OCA2 become more susceptible to chemicals typically detoxified by glutathione, owing to localization of the transgene to the vacuolar membrane and an increase in vacuolar delivery and degradation of glutathione (Staleva et al., 2002). Direct involvement of OCA2 in glutathione transport was not assayed in the yeast, and although mouse melanocytes expressing endogenous OCA2 are also more sensitive to these toxic chemicals than OCA2-deficient cells, cellular glutathione levels are not different between the two types of melanocytes (Staleva et al., 2002). The protein has also been proposed to control melanin synthesis by directly regulating the lumenal pH of melanosomes (Puri et al., 2000). Consistent with this hypothesis are several studies in which treatment of OCA2-deficient pigmented cells with lysosomotropic agents or vATPase inhibitors causes an increase in melanin content, leading to the suggestion that OCA2 functions to raise melanosomal pH for optimal tyrosinase activity (Ancans et al., 2001b; Manga and Orlow, 2001; Chen et al., 2004; Ni-Komatsu and Orlow, 2006). Others have claimed that OCA2 functions to acidify melanosomes, based on the observation that Tyrp1-containing compartments in Oca2-deficient melanocytes accumulate less DAMP, a marker of acidification, than those in wild-type melanocytes (Puri et al., 2000). However, this
hypothesis is complicated by the fact that Tyrp1 is apparently mislocalized in OCA2-deficient cells (Manga et al., 2001).

Not only is the function of the OCA2 protein unclear, but its subcellular site of action has also been debated. There are several pieces of evidence that OCA2 is a bona fide melanosomal protein. A putative acidic dileucine sorting signal present within the long, cytoplasmically exposed N-terminal domain of OCA2 was predicted to serve a melanosomal sorting function (Lee et al., 1995), analogous to the signals in the cytoplasmic C-terminal domains of Tyr and Tyrp1 (Peng et al., 1994; Vijayasaradhi et al., 1995; Calvo et al., 1999; Simmen et al., 1999). In addition, OCA2 co-fractionated with Tyrp1 and Tyr in wild-type cultured melanocytes (Rosemblat et al., 1994), and low-resolution immunofluorescence microscopy analysis was interpreted to show some colocalization with the early stage melanosomal protein PMEL (Toyofuku et al., 2002). Moreover, like PMEL, OCA2 was difficult to extract from heavily melanized cells using non-ionic detergents, suggesting a close association with melanin (Rosemblat et al., 1994; Donatien and Orlow, 1995). However, other data were interpreted to suggest that OCA2 localizes to and functions within the endoplasmic reticulum. OCA2 co-fractionated with ER markers as well as melanosomal markers, and exogenously expressed OCA2 displayed some colocalization with ER markers by indirect immunofluorescence microscopy in melanocytes and non-melanocytes (Chen et al., 2002). The shift of steady-state Tyr to more immature, endoglycosidase H-sensitive glycoforms in OCA2-deficient cells has been interpreted to indicate that OCA2 modulates Tyr folding within and consequent exit from the endoplasmic reticulum (Chen
et al., 2002; Toyofuku et al., 2002; Chen et al., 2004; Ni-Komatsu and Orlow, 2006); this would be consistent with a role for OCA2 in modifying cellular glutathione levels. However, none of these data were definitive in identifying the functional cellular localization for OCA2 in wild-type melanocytes, or in testing whether OCA2, like other melanosomal proteins, was mislocalized in melanocytes derived from models of trafficking disorders such as Hermansky-Pudlak syndrome. Thus the question of where precisely OCA2 localizes and functions within melanocytes required further, definitive investigation.

1.8 Goals of this thesis

As discussed, previous work in the literature had not resolved the question of the subcellular localization and site of function of OCA2 in promoting melanin synthesis, suggesting either the ER or the melanosome as the putative site. In Chapter 2, I describe how biochemical analysis indicates that OCA2 must function in a post-ER compartment, consistent with the acquisition of resistance to endoglycosidase H treatment. The OCA2 protein localizes to late endosomes/lysosomes when exogenously expressed in non-melanocytes or to the mature melanosome in melanocytes, as expected for a melanosomal protein. Furthermore, similar to findings in Tyr and Tyrp1, I find that melanosomal localization depends on an acidic dileucine motif in the cytoplasmic domain, and that this motif is a binding site for the adaptor protein complexes AP-1 and AP-3.
We have previously described how studies with Tyrp1 and Tyr have led to a model of endosome-to-melanosome trafficking mediated by two largely distinct pathways. One is mediated by AP-3 and is utilized by a large cohort of Tyr, while another pathway, used by Tyrp1, is mediated by BLOC-1, with contributions from AP-1 and BLOC-2. But questions still remain regarding how distinct these two pathways are. Following our discovery that OCA2 binds both AP-1 and AP-3, I exploited OCA2 as a useful tool to dissect the relative roles of each complex in melanosomal trafficking and to interrogate our trafficking model. In Chapter 3, I describe how I characterized the dependence of the localization and AP-interacting activity of the OCA2 acidic dileucine motif on primary sequence rather than position within the protein. I also describe the isolation of two point mutations in the acidic dileucine motif that differentially affect binding of AP-1 or AP-3. I determined the localization of these mutants in wild-type, AP-3-deficient, and BLOC-1-deficient melanocytes. My results suggest that OCA2 exploits an anterograde trafficking pathway that requires both AP-3 and BLOC-1, suggesting the existence of a third as yet unappreciated pathway for melanosome cargo delivery and providing a functional correlate for the AP-3-BLOC-1 interaction previously observed. The results also show that chimeric expression of the acidic dileucine motif from a BLOC-1-independent protein does not confer BLOC-1 independence on OCA2, suggesting that the determinants of BLOC-1 dependence are distinct from those of AP complexes.
CHAPTER 2: LOCALIZATION TO MATURE MELANOSOMES BY VIRTUE OF CYTOPLASMIC DILEUCINE MOTIFS IS REQUIRED FOR HUMAN OCA2 FUNCTION

2.1 Abstract

Oculocutaneous albinism type 2 is caused by defects in the gene OCA2, encoding a pigment cell-specific, 12-transmembrane domain protein with homology to ion permeases. The function of the OCA2 protein remains unknown, and its subcellular localization is under debate. Here, we show that endogenous OCA2 in melanocytic cells rapidly exits the endoplasmic reticulum (ER) and thus does not behave as a resident ER protein. Consistently, exogenously expressed OCA2 localizes within melanocytes to melanosomes, and, like other melanosomal proteins, localizes to lysosomes when expressed in nonpigment cells. Mutagenized OCA2 transgenes stimulate melanin synthesis in OCA2-deficient cells when localized to melanosomes but not when specifically retained in the ER, contradicting a proposed primary function for OCA2 in the ER. Steady-state melanosomal localization requires a conserved consensus acidic dileucine-based sorting motif within the cytoplasmic N-terminal region of OCA2. A second dileucine signal within this region confers steady-state lysosomal localization in melanocytes, suggesting that OCA2 might traverse multiple sequential or parallel trafficking routes. The two dileucine signals physically interact in a differential manner with cytoplasmic adaptors known to function in trafficking other proteins to melanosomes. We conclude that OCA2 is targeted to and functions within melanosomes but that residence within melanosomes may be regulated by secondary or alternative targeting to lysosomes.
2.2 Introduction

Melanin pigments are synthesized by specialized cell types, including dermal and epidermal melanocytes and retinal pigment epithelial cells, within unique organelles known as melanosomes (Raposo and Marks, 2007). Melanosomes are members of a class of tissue-specific “lysosome-related organelles” characterized by an acidic lumenal pH and the presence of some lysosomal proteins (Dell'Angelica et al., 2000; Griffiths, 2002). Among lysosome-related organelles, melanosomes represent a subclass that coexists with bona fide lysosomes in their host cells (Raposo and Marks, 2007). Melanosomes are distinguished from lysosomes by the presence of cell-type-specific cargo proteins that confer unique functional and morphological properties. How these cargo proteins are diverted from traditional lysosomes and delivered to and maintained within melanosomes is incompletely understood, and the degree of cargo cross-talk between melanosomes and lysosomes is not known.

Melanosomes undergo a program of maturation within melanocytes by the ordered delivery of cargoes to nascent melanosomes via specialized trafficking pathways (Raposo and Marks, 2007). Some components of the melanosomal trafficking machinery are known, largely from analyses of the sorting of well-characterized cargo proteins, such as the melanogenic enzyme tyrosinase (Tyr) and tyrosinase-related protein 1 (Tyrp1), in both wild-type melanocytes and melanocytes derived from patients or mouse models of genetic hypopigmentary diseases such as Hermansky–Pudlak Syndrome (HPS) (Di Pietro and Dell'Angelica, 2005; Wei, 2006). Tyr and Tyrp1 contain cytoplasmic acidic dileucine
motifs that are required for melanosomal sorting (Vijayasaradhi et al., 1995; Calvo et al., 1999; Simmen et al., 1999) and that are bound within endosomal intermediates by heterotetrameric adaptor proteins (APs) AP-1 or AP-3. AP-1 and AP-3 each bind the Tyr sorting signal (Honing et al., 1998; Theos et al., 2005), but they participate in distinct delivery pathways toward melanosomes (Theos et al., 2005). Consistently, Tyr is largely (but not completely) missorted in melanocytes derived from human HPS type 2 patients and HPS model pearl mice that bear mutations in the gene encoding the β3A subunit of AP-3 (Huizing et al., 2001; Theos et al., 2005). By contrast, the acidic dileucine-based sorting signal in Tyrp1 has been shown to bind AP-1 but not AP-3 (Huizing et al., 2001; Theos et al., 2005), and accordingly Tyrp1 accumulates normally on melanosomes in AP-3-deficient melanocytes (Huizing et al., 2001; Setty et al., 2007) (although an unusually large cohort cycles through the plasma membrane; (Di Pietro et al., 2006)). These results corroborate the dependence on AP-1 and AP-3, respectively, for in vitro budding of Tyrp1 and tyrosinase from Golgi/endosomal membrane fractions (Chapuy et al., 2008). Interestingly, both lysosomal and melanosomal proteins depend on AP-1 and AP-3 for their trafficking, and it is not known how trafficking to these distinct organelles in melanocytes is distinguished. Additional components that influence Tyr and Tyrp1 trafficking to melanosomes include the biogenesis of lysosome-related organelles complex (BLOC)-1 and BLOC-2, subunits of which are defective in other forms of HPS, and the tissue-specific Rab proteins Rab32 and Rab38 (Richmond et al., 2005; Di Pietro et al., 2006; Wasmeier et al., 2006; Helip-Wooley et al., 2007; Setty et al., 2007). Tyrp1 and Tyr trafficking toward melanosomes or lysosomes is also regulated by luminal
interactions with glycosphingolipid-dependent membrane microdomains, presumably on endosomes (Sprong et al., 2001; Groux-Degroote et al., 2008).

The mechanisms regulating the delivery of other melanosomal proteins to melanosomes are less understood. Pmel17, a component of the fibrillar melanosome matrix on which melanins deposit, becomes incorporated into early stage melanosomes in a manner that seems to be unaffected in most, if not all, forms of HPS. Pmel17 is first delivered to endosomes by a dileucine-based internalization motif (Theos et al., 2006a) and is subsequently sorted to the internal membranes of multivesicular endosomes in a step that precedes and is required for fibril formation (Hoashi et al., 2006; Theos et al., 2006b). This latter step requires a lumenal determinant within Pmel17 but does not require components of the classical multivesicular body sorting machinery such as Hrs and endosomal sorting complex required for transport subunits (Theos et al., 2006b). The G protein-coupled receptor OA1 is targeted to melanosomes by a distinct class of sorting signal (Piccirillo et al., 2006), but the effectors that regulate its sorting are not known.

OCA2, also called pink-eyed dilution or P protein, is an enigmatic protein with a critical function in pigmentation. The OCA2 gene is mutated in oculocutaneous albinism (OCA) type 2, one of the most common forms of human albinism (Brilliant, 2001). In addition, nonpathological polymorphisms in both coding and noncoding regions of the OCA2 gene have been implicated as major determinants of skin color (Lao et al., 2007; Norton et al., 2007; Sulem et al., 2007), and variation in OCA2 expression through a polymorphism in an adjacent gene, HERC2, is thought to underlie blue eye color in humans (Eiberg et al.,
OCA type 2 patients exhibit severe hypopigmentation of the skin, hair, and eyes. Defects in the corresponding murine gene (p) give rise to the pink-eyed dilution mouse (Rinchik et al., 1993), which has similar eye and coat hypopigmentation. Melanocytes from this model mouse contain melanosomes that are small, immature, and hypopigmented relative to normal melanosomes (Sidman et al., 1965; Rosemblat et al., 1998). Although these features clearly implicate OCA2 as being critical for pigmentation, the molecular function of OCA2 is still unclear. Its sequence predicts that OCA2 is a 12-transmembrane domain protein with homology to a superfamily of permeases (Rinchik et al., 1993; Lee et al., 1995). However, no transport substrate has yet been identified.

Not only is OCA2 function not known, but its subcellular site of action is also debated. OCA2 was originally thought to localize predominantly to mature melanized melanosomes based on subcellular fractionation of melanocytes (Rosemblat et al., 1994), poor extraction by detergent from melanized melanocytes relative to nonmelanized melanocytes (Donatien and Orlow, 1995), and interpretation of results from confocal immunofluorescence microscopy (IFM) analyses (Toyofuku et al., 2002). Tyrp1-containing compartments in melanocytes from OCA2-deficient mice are less acidic than in wild-type melanocytes, suggesting that OCA2 not only localizes to melanosomes but also modulates their pH (Puri et al., 2000), although this interpretation is disputed because Tyrp1 does not localize to melanosomes properly in OCA2-deficient melanocytes (Manga et al., 2001). The possibility that melanosomal pH might be affected by OCA2 deficiency is supported by an observed increase in melanin synthesis upon
neutralization of organellar pH in otherwise hypopigmented OCA2-deficient melanocytes (Ancans et al., 2001b). Other evidence, however, suggests that OCA2 localizes to and functions from the endoplasmic reticulum (ER). OCA2-immunoreactive subcellular fractions from melanocytes contain ER markers as well as melanin, and OCA2 colocalized extensively with ER markers by indirect IFM (Chen et al., 2002). Moreover, cells that lack OCA2 show a reduction in terminal glycosylation of Tyr (Chen et al., 2002; Toyofuku et al., 2002), suggesting that OCA2 activity might provide an optimal environment within the ER to facilitate Tyr folding. Thus far, this controversy has not been resolved.

To resolve this debate and extend our understanding of melanosomal protein trafficking, we attempted to clarify the subcellular localization and site of action of OCA2 and to define the determinants required for its localization. Our results demonstrate that human OCA2 is rapidly processed by Golgi enzymes, suggesting that it is not a resident ER protein. Consistently, ectopically expressed OCA2 localizes predominantly to melanosomes in a manner that depends on a cytoplasmic dileucine-based sorting signal, similar to those found in Tyr and Tyrp1. A second dileucine-based signal in human OCA2 facilitates steady-state lysosomal but not melanosomal localization. We further show that melanosomal localization of OCA2 correlates with its function in supporting melanin synthesis. These results strongly suggest that OCA2 is active in melanosomes and raise the possibility that OCA2 activity may be limited by additional sorting to lysosomes.
2.3 Results

OCA2 Transits to a Post-ER Compartment and Becomes Terminally Glycosylated.

Analysis of the primary sequence of human OCA2 reveals three evolutionarily conserved consensus $N$-glycosylation sites within the first predicted lumenal loop (at Asn residues 214, 218, and 273) and additional sites elsewhere, but mouse OCA2 was suggested to be nonglycosylated based on lack of a mobility shift in tunicamycin-treated cells (Rosemblat et al., 1994). We thus tested whether human OCA2 was in fact $N$-glycosylated using endoglycosidase treatment in a metabolic pulse/chase and immunoprecipitation experiment. MNT-1 human melanoma cells that endogenously express OCA2 or HeLa cells transiently transfected with OCA2 were pulse-labeled with $^{35}$S-cysteine/methionine, chased for various times, and then lysed and subjected to immunoprecipitation with the NOCA2 anti-OCA2 antibody. Immunoprecipitates were mock treated or digested with endoH or PNGase F. Endogenous OCA2 from pulse-labeled MNT-1 cells is entirely sensitive to digestion by both endoH and PNGase F, indicating that it is modified by the addition of core $N$-linked oligosaccharides. By 1 h of chase, a cohort of OCA2 becomes resistant to cleavage by Endo H (Figure 2.1a, compare M and D, arrows), indicating modification by $N$-acetylglucosamine transferase in the medial Golgi. By 4 h of chase, all detectable OCA2 is resistant to endoH. As a control, PNGase F digestion increases the mobility of OCA2 at all time points. Similar results were obtained in transiently transfected HeLa cells expressing OCA2, although processing in the Golgi seemed to be faster (Figure 2.1b). Note that processed OCA2 disappeared rapidly from detergent lysates in melanocytes, consistent with earlier results, suggesting close association with melanin (Donatien and Orlow, 1995), but much less rapidly in transfected HeLa cells.
**Figure 2.1.** Human OCA2 is N-glycosylated and is not an ER resident protein.

(a and b) Endogenous OCA2 in MNT-1 human melanoma cells (a) or transfected OCA2 in HeLa cells (b) was immunoprecipitated after metabolic labeling and indicated chase. Immunoprecipitates were digested with endoglycosidases, fractionated by SDS-PAGE, and analyzed by phosphorimaging. M, fully mature protein; P, precursor protein with incomplete glycosylation; D, deglycosylated protein; -, mock treatment; H, endoH treatment; F, PNGase F treatment; *, nonspecific band. All markers are indicated in kilodaltons.
The reduction in $M_r$ resulting from PNGase F or endoH treatment of OCA2 was ~7 kDa, consistent with loss of two $N$-linked oligosaccharide chains. Consistently, the $M_r$ of OCA2 isolated from normal human melanocytes or OCA2-transfected COS-7 cells was reduced if cells were first treated with the $N$-glycosylation inhibitor tunicamycin or the mannosidase inhibitor deoxymannojirimycin (data not shown). Together, these results indicate that OCA2 is a glycoprotein and is not a resident of the ER but rather traffics to at least the medial Golgi.

OCA2 Localizes to Melanosomes in Melanocytes and Lysosomes in Nonmelanocytes.

Because OCA2 is largely exported from the ER and thus does not behave like an ER resident protein, we next sought to determine the steady-state localization of OCA2 in melanocytes and nonmelanocytic HeLa cells. Endogenous OCA2 in human melanocytic cells could not be detected by IFM (data not shown), but a full-length human OCA2 transgene product, with or without a triple HA epitope tag inserted in-frame within the first lumenal loop of the protein (OCA2-HA; see Figure 2.5a) could be detected in several melanocytic cell lines after transient transfection. The HA tag did not affect OCA2 function (see Figure 2.3) and permitted detection of the OCA2-HA transgene with an anti-HA antibody. When expressed in pigmented melan-Ink4a mouse melanocytes, both OCA2 and OCA2-HA were distributed in either a reticular or vesicular pattern as revealed by labeling with NOCA2 or anti-HA antibodies and analysis by IFM and image deconvolution (Figure 2.2a; data not shown). The reticular pattern was similar to that observed with antibodies to ER markers (data not shown). Qualitative observations of transfected cells did not reveal an obvious correlation between transgene expression level
Figure 2.2. OCA2 localizes to melanosomes in melanocytes and to lysosomes in nonmelanocytes.

(a–c) Melan-Ink4a melanocytes were transfected with human OCA2-HA and stained with anti-HA antibodies (a; magenta in the merge, c). The bright field image of pigmented melanosomes (b) is inverted and colored green in the merge (c). (d–f) HeLa cells transfected with human OCA2-HA were treated with 50 µg/ml cycloheximide for 3 h before fixation. Cells are stained with antibodies to HA (d; magenta in the merge, f) and the lysosomal marker LAMP-1 (e; green in the merge, f). Insets, 3X magnification of boxed regions. Arrows point to regions of overlap between OCA2-HA and melanosomes (a–c) or lysosomes (d–f). Asterisk indicates an overexpressing cell. Bar, 10 µm.
and the subcellular distribution of the protein. In cells with vesicular labeling, the anti-OCA2 or anti-HA label overlaps almost entirely with that of the melanosomal marker Tyrp1 (data not shown), as well as with pigment granules visualized by bright field microscopy (Figure 2.2, b and c). These data suggest that although a cohort of exogenously expressed OCA2 accumulates in the ER, OCA2 that exits the ER is targeted to melanosomes.

Most melanosomal proteins localize to late endosomes or lysosomes when expressed in nonmelanocytic cells (Bouchard et al., 1989b; Vijayasaradhi et al., 1995; Calvo et al., 1999; Simmen et al., 1999; Berson et al., 2001). We therefore tested the localization of OCA2-HA expressed in nonmelanocytic HeLa cells. As with melanocytes, OCA2 or OCA2-HA was distributed either in a predominantly reticular pattern, characteristic of the ER, or a predominantly vesicular pattern (30.4 and 69.5% of transfected cells, respectively; Figure 2.2d; data not shown). The vesicles overlapped nearly completely with labeling for LAMP-1, a membrane protein that accumulates in late endosomes and lysosomes (Figure 2.2, e and f). Treatment of HeLa cells for 3 h with the protein synthesis inhibitor cycloheximide increased the percentage of cells displaying predominantly vesicular HA staining to 81.7%. Additionally, the fraction of cells exhibiting vesicular localization increased with increasing time after transfection (data not shown). A similar cycloheximide treatment in transfected melan-Ink4a melanocytes did not alter the ratio of cells with predominantly ER or vesicular staining, although it did significantly reduce the fraction of cells with any detectable labeling (data not shown). This is likely a consequence of the rapid degradation of OCA2 observed in melanocytic
cells (Figure 2.1a), such that OCA2 fails to accumulate to detectable levels without continuous biosynthetic input. We therefore interpret these results to suggest that the ER-localized pool of OCA2 is transient and that OCA2 traffics to melanosomes in melanocytes and to lysosomes in nonmelanocytes. We cannot exclude the possibility that microheterogeneity in our cell culture system contributes to differential rates of ER exit or stability within melanosomes.

*ER-localized OCA2 Cannot Rescue Pigmentation of OCA2-deficient Melanocytes.*

Because a significant cohort of exogenously expressed OCA2 resides in the ER at steady state, we tested whether OCA2 in the ER is functional. We took advantage of the fact that expression of human OCA2 in nonpigmented, OCA2-deficient mouse melan-p1 melanocytes can restore melanin synthesis and consequent pigmentation (Sviderskaya *et al.*, 1997), and we asked whether purposeful retention of OCA2 in the ER is compatible with this function.

We first constructed two OCA2 mutants that are constitutively localized to the ER. OCA2-HMGCR was created by fusing the C-terminal cytoplasmic domain of human HMG CoA reductase (HMGCR), containing a canonical dilysine ER retrieval signal (Jackson *et al.*, 1990), to the cytoplasmic C terminus of OCA2-HA (Figure 2.3a). OCA2-HMGCR was effectively retained in the ER when expressed in MNT-1 melanoma cells, as judged by colocalization with calnexin by IFM (Figure 2.4, a–c). As controls, we constructed two mutants in which the lysine residues were mutagenized to alanine (OCA2-HMGCR-AATA) or in which the targeting signal was deleted (OCA2-HMGCR-
Figure 2.3

<table>
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<tr>
<th>OCA2-HA (WT)</th>
<th>OCA2 ΔN91</th>
<th>MHRRR-OCA2</th>
<th>MHAAAI-OCA2</th>
<th>OCA2-HMGCR</th>
<th>OCA2-HMGCR-AATA</th>
<th>OCA2-HMGCRΔKKTA</th>
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<td>GACTAATA</td>
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OCA2 (anti-HA) | Bright field | OCA2/ Bright field

b. OCA2-HA (WT)  

e. MHRRR-OCA2  

h. MHAAAI-OCA2  

k. OCA2-AA123  

n. % melanized tdl. cells

WT | AA123 | ΔN91 | RRR | AAA | HMGCR | AATA | ΔKKTA
---|------|-----|-----|-----|-------|------|------
| 100 | 60   | 40  | 80  | 80  | 60    | 40   | 80   |
Figure 2.3. OCA2 function correlates with localization to melanosomes.

(a) Scheme of HA-tagged, chimeric, and truncated OCA2 proteins as described in the text. Added sequences in fusion proteins are indicated at the N or C termini. All constructs bear a triple HA epitope tag (3xHA, indicated by a black bar). Gray boxes indicate predicted transmembrane domains, and N-glycosylation sites are indicated by branching. (b–m) OCA2-deficient melan-p1 mouse melanocytes were transfected with human OCA2-HA (b–d), MHRRROCA2-ΔN91 (e–g), MHAAM-OCA2-ΔN91 (h–j), or OCA2-AA123 (k–m; see Figure 3.4a). Transfected cells were identified by anti-HA staining (b, e, h, and k) and visually inspected for the presence of pigmented melanosomes by bright field microscopy (c, f, i, and l). In the merged images (d, g, j, and m), the bright field image is inverted and colored green and anti-HA is colored magenta. Insets, 5X magnification of boxed regions. Arrows point to regions of overlap between OCA2-HA and melanosomes (b–d, h–j). Bar, 10 µm. (n) Bar graph of pigmentation rescue by each OCA2 construct. Shown is the percentage of transfected cells expressing each indicated construct that contained pigmented melanosomes (% pigmented cells). The degree of rescue induced by any construct bearing an ER targeting signal or lacking endogenous dileucine sorting signals was significantly different from rescue induced by wild-type OCA2-HA. *, p < 0.05; ***, p < 0.001.
**Figure 2.4.** ER targeting signals alter localization of OCA2 chimeras.

MNT-1 human melanoma cells were transfected with OCA2-HMGCR (a-c), OCA2-ΔN91 (d-f) or MHRRR-OCA2-ΔN91 (g-i), and analyzed for colocalization between each OCA2 construct, visualized by anti-HA staining (a, d, g), and either the ER marker calnexin (b, h) or pigmented melanosomes (bright field, e). c, f, and i are merged images in which anti-HA is colored green and anti-calnexin (c, i) or the inverted bright field image (f) is colored magenta. Arrows indicate colocalization between OCA2-ΔN91 and pigmented melanosomes (g-i). Bar = 10 µm.
ΔKKTA) (Figure 2.3a); these mutants were weakly and strongly localized to melanosomes, respectively (data not shown). Another OCA2 chimeric construct bore an N-terminal cytoplasmic ER-targeting arginine motif, MHRRR, derived from the p35 form of human Invariant chain (Schutze et al., 1994). This signal did not confer ER retention when fused to full-length OCA2 (data not shown). However, when appended to a deletion mutant that lacked the first 91 amino acids of OCA2 (OCA2-ΔN91) and that localized by IFM in pigmented MNT-1 melanoma cells to melanosomes like fulllength OCA2-HA (Figure 2.4, d–f), the resultant MHRRR-OCA2-ΔN91 fusion protein was effectively retained in the ER (Figure 2.4, g–i); the Δ91 deletion presumably brought the N-terminal ER retention signal near enough to the membrane anchor to allow for interactions with membrane-proximal effectors. By contrast, when the triple arginine signal was mutagenized to a triple alanine (MHAAA-OCA2-ΔN91), melanosomal localization in melan-Ink4a was restored (data not shown, but see Figure 2.3).

To test whether ER-retained OCA2 is functional, each of these mutants was expressed in hypopigmented, OCA2-deficient mouse melan-p1 cells and restoration of pigmentation was scored by bright field microscopy. As expected, expression of human OCA2-HA was sufficient to rescue pigmentation in 80% of transfected melan-p1 cells (Figure 2.3, b–d, n), indicating that the addition of the luminal triple HA tag does not negatively affect OCA2 function. Likewise, the level of rescue induced by OCA2-ΔN91, 69%, was not significantly different from wild-type, indicating that the first 91 amino acids of OCA2 are not essential for protein function (Figure 2.3n). Strikingly, expression of OCA2-HMGCR or MHRRR-OCA2-ΔN91, both of which localized in melan-p1 cells to a
reticular pattern characteristic of ER as in MNT-1 cells (Figure 2.3, e–g; data not shown), rescued pigmentation in only 16 and 17% of transfected cells, respectively (Figure 2.3n). The corresponding fusion proteins with mutated ER targeting signals—MHAAA-OCA2-ΔN91 and OCA2-HMGCR-ΔKKTA—showed restored vesicular/melanosomal localization (Figure 2.3, h–j; data not shown) and induced pigmentation in a fraction of melan-p1 cells that was not significantly different from that induced by wild-type OCA2-HA (Figure 2.3n); OCA2-HMGCR-AATA rescued more efficiently than OCA2-HMGCR but not as efficiently as OCA2-HMGCR-ΔKKTA, consistent with its partial restoration of melanosome localization. This might reflect misfolding induced by the elongated C-terminal domain, as similar observations were made with other C-terminally extended OCA2 variants (data not shown). Overall, our results demonstrate that OCA2 is unable to promote melanin synthesis from the ER of melanocytes and therefore must function in a post-ER compartment.

**OCA2 Localization Requires Cytoplasmic Dileucine Motifs.**

To begin to understand the mechanism by which OCA2 is sorted to melanosomes, we next investigated the cis-acting signals required for OCA2 localization. The cytoplasmic C-terminal domains of the melanosomal proteins Tyr and Tyrp1 contain sequences conforming to the [D/E]XXL[L/I] consensus for acidic dileucine motifs that are necessary for sorting of these proteins to melanosomes (Vijayasaradhi et al., 1995; Calvo et al., 1999; Simmen et al., 1999). Mutagenesis of the two leucine residues within these motifs results in mislocalization to the cell surface. Three sequences conforming to the acidic dileucine consensus are present in the human OCA2 cytoplasmic N-terminal
domain (Figure 2.5a). We tested whether these motifs are required for steady-state localization of OCA2-HA to melanosomes by simultaneously mutagenizing the leucine residues in all three motifs to alanine and examining the localization of the resultant mutant, expressed transiently in wild-type melan-Ink4a melanocytes, by IFM. Whereas wild-type human OCA2-HA localizes to melanosomes in these cells (Figure 2.5, b–d), the triple dileucine mutant (OCA2-AA123) localizes to the cell periphery (Figure 2.5, e–g). HA-positive structures within the cell perimeter did not colocalize with melanosomes (Figure 2.5g), but rather they seemed to correspond to surface projections. As with wild-type OCA2, the labeling pattern of OCA2-AA123 in transiently transfected melanocytes was heterogeneous, in that some cells showed a reticular pattern rather than peripheral protein localization. Similarly, whereas wild-type OCA2 localized to lysosomes in HeLa cells, OCA2-AA123 localized to the cell periphery in these cells as well (Figure 2.6). To confirm localization to the cell surface, wild-type OCA2-HA or OCA2-AA123 was transiently expressed in pigmented melan-Ink4a melanocytes and incubated at 4°C with anti-HA antibodies to detect the lumenally exposed (extracellular) triple HA tag on the cell surface. Cells were then washed, fixed, permeabilized, and stained with NOCA2 to identify all transfected cells (NOCA2 binding is not affected by mutagenesis of the N-terminal dileucine motifs). Cells expressing OCA2-AA123 were labeled with the anti-HA antibody, whereas cells expressing wild-type OCA2-HA were not (Figure 2.7, a–f).

Together, these data show that the dileucine motifs in the N-terminal cytoplasmic domain of human OCA2 are necessary for melanosome/lysosome localization and that in their absence OCA2 localizes by default to the plasma membrane.
Figure 2.5. OCA2 melanosomal sorting requires intact dileucine motifs.

(a) Topology of human OCA2 and sequence of the cytoplasmic N terminus with putative acidic dileucine motifs indicated in magenta. (b–g) Wild-type OCA2-HA (b–d) or the triple dileucine mutant OCA2-AA123 (e–g) were expressed in pigmented melan-Ink4a melanocytes. Transfected cells are identified by anti-HA staining (b and e; colored magenta in merged images, d and g). Bright field images (c and f) were inverted and
colored green in merged images (d and g). Insets, 4X magnification of boxed regions. Arrows point to regions of overlap between OCA2-HA and melanosomes (b–d). Bar, 10 µm.
Figure 2.6. Overexpression of OCA2 intact dileucine motifs induces lysosomal swelling and clustering in HeLa cells.

a-f) HeLa cells were transfected with either human OCA2-HA (a-c) or the OCA2-AA123 mutant (d-f) and transgenic proteins were visualized by staining with anti-HA antibodies (a, d). Lysosomes were identified by staining of the marker LAMP-1 (b, e). c and f are merged images in which anti-HA labeling is colored green and anti-LAMP-1 is colored magenta. Note the swelling and perinuclear clustering of lysosomes only in cells expressing OCA2-HA (insets 2) relative to untransfected cells (insets 1). g-i) HeLa cells
were transfected with OCA2-TfR and stained with NOCA2 to identify transgenic proteins (g) and with anti-LAMP-1 (h). Merged image (i) shows anti-OCA2 in green and anti-LAMP-1 in magenta. Note lysosomal clustering in transfected cells (inset 2) but not in untransfected cells (inset 1). Bar = 10 µm.
Figure 2.7

**Figure 2.7.** Surface localization of OCA2-AA123.

Pigmented melan-Ink4a melanocytes expressing human OCA2-AA123 (a-c) or OCA2-HA (d-f) were incubated with anti-HA antibodies at 4°C to label surface-localized OCA2 proteins (a, d) and then washed, fixed, permeabilized, and stained with NOCA2 (b, e) to mark all transfected cells. Bright field images show melanosomes in c, f. Bar = 10 μm.
To determine whether sorting to melanosomes was required for OCA2 function, we tested whether the cell surface localized OCA2-AA123 could restore pigmentation upon expression in melan-p1 cells. As with cells expressing the ER-localized OCA2 fusion proteins, only 3% of melan-p1 cells expressing OCA2-AA123 were pigmented (Figure 2.3, k–n). This indicates that OCA2 cannot function on the cell surface and must function from an intracellular, post-ER compartment. Interestingly, lysosomes in HeLa cells that overexpressed OCA2-HA tended to be unusually clustered around the nucleus and swollen compared with untransfected cells (Figure 2.6, compare insets 1 and 2); a similar phenomenon has been seen in HeLa cells expressing tyrosinase (Calvo et al., 1999). By contrast, lysosomes in HeLa cells expressing the surface-localized OCA2-AA123 seemed similar to those in untransfected cells (Figure 2.6). These results suggest that overexpression of the intact OCA2 cytoplasmic sorting signals can alter late endosome/lysosome function or morphology, further supporting a functional role for these motifs in sorting to lysosome-like organelles.

**OCA2 Dileucine Motifs Are Sufficient for Lysosomal Localization in Nonmelanocytes and Intracellular Targeting in Melanocytes.**

To determine whether the OCA2 dileucine motifs were sufficient to cause trafficking to lysosomes or melanosomes, we assessed their ability to direct the localization of a reporter molecule. We generated chimeric proteins in which the N-terminal cytoplasmic domain of either wild-type human OCA2 or the dileucine mutant, OCA2-AA123, was fused to the transmembrane and lumenal domains of human transferrin receptor (hTfR) to generate OCA2-TfR and AA123-TfR, respectively (Figure 2.8a). hTfR, which normally
Figure 2.8

a. hTfR, OCA2-TfR, and AA123-TfR with their respective domains labeled: cytoplasmic, transmembrane (TM), and extracellular.

b. Immunofluorescence images of hTfR with anti-TfR and LAMP-1 antibodies.

c. Zoomed-in images of the boxed areas in (b).

d. Anti-TfR/LAMP-1 co-staining.

e. OCA2-TfR images with anti-HA and anti-TfR antibodies.

f. Zoomed-in images of the boxed areas in (e).

g. Anti-TfR/LAMP-1 co-staining.

h. AA123-TfR images with anti-HA and anti-TfR antibodies.

i. Zoomed-in images of the boxed areas in (h).

j. Anti-TfR/LAMP-1 co-staining.

k. Untreated cells showing anti-HA, anti-TfR, and LAMP-1 staining.

l. Zoomed-in images of the boxed areas in (k).

m. Anti-TfR/LAMP-1 co-staining.

n. Cells treated with NH₄Cl showing anti-HA, anti-TfR, and LAMP-1 staining.

o. Zoomed-in images of the boxed areas in (n).

p. Anti-TfR/LAMP-1 co-staining.
Figure 2.8. OCA2 dileucine motifs are sufficient for lysosomal localization in nonmelanocytes.

(a) Schematic of OCA2-transferrin receptor chimeras. Green, hTfR sequences; blue, cytoplasmic N terminus of human OCA2; TM, hTfR transmembrane domain; green circles, intact OCA2 dileucine motifs; white X’s, disrupted dileucine motifs. Binding regions of relevant antibodies are indicated. The NOCA2 epitope does not overlap the dileucine motifs. (b–j) Chinese hamster ovary cells were transfected with hTfR (b–d), OCA2-TfR (e–g), or AA123-TfR (h–j), and the transgenic proteins were localized at steady-state by anti-hTfR staining (b, e, and h). The lysosomal marker LAMP-1 is stained in c, f, and i, and images are merged in d, g, and j with anti-hTfR in green and anti-LAMP-1 in magenta. Insets, 4X magnification of boxed regions. (k–p) Chinese hamster ovary cells were cotransfected with wild-type OCA2-HA (k and n) and OCA2-TfR (l and o) and incubated with (n–p) or without (k–m) 50 mM NH₄Cl before fixation in order to inhibit lysosomal degradation. Lysosomes are marked by LAMP-1 staining (m and p). Insets show 3X magnifications of two-color merged images of OCA2-HA (green), OCA2-TfR (red), and LAMP-1 (blue). Arrows point to regions of overlap among the three proteins (n–p). Bar, 10 μm.
cycles between the plasma membrane and early endosomes, was chosen as a fusion partner because it is a type II integral membrane protein and thus allowed us to maintain the proper orientation of the OCA2 N-terminal domain. hTfR or the chimeric proteins were transiently expressed in CHO cells, which do not endogenously express human OCA2 or hTfR, and localization was determined by IFM. As expected, heterologously expressed hTfR localized to the cell surface and to peripheral intracellular puncta that did not overlap with the late endosome/lysosome marker LAMP-1 and that likely represent early endosomes (Figure 2.8, b–d). By contrast, OCA2-TfR localized exclusively to intracellular compartments that were predominantly accumulated in the perinuclear area (Figure 2.8, e–g). Mutagenesis of the three dileucine motifs in AA123-TfR restored cell surface localization (Figure 2.8, h–j). Surprisingly, the puncta to which OCA2-TfR localized did not appreciably overlap with LAMP-1. We reasoned that the hTfR lumenal domain, to which the antibody was directed, might be degraded within LAMP-1-containing late endosomes and lysosomes. To test this possibility, CHO cells that were cotransfected with both the OCA2-TfR chimera (Figure 2.8, l and o) and full-length OCA2-HA (Figure 2.8, k and n) were analyzed by IFM after treatment with 50 mM NH₄Cl to neutralize lysosomes and interfere with proteolysis. Whereas neither protein colocalized with LAMP-1 in untreated cells (Figure 2.8, k–m), both OCA2-TfR and OCA2-HA redistributed to vesicular structures that colocalized with LAMP-1 in NH₄Cl-treated cells (Figure 2.8, n–p). Consistently, OCA2-TfR localized to LAMP-1-positive compartments in HeLa cells even without lysosomal inhibition, and induced enlargement and perinuclear clustering like full-length OCA2-HA (Figure 2.6). These results confirm that the OCA2 dileucine motifs are sufficient to direct trafficking to lysosomes in
nonmelanocytes. Moreover, the enlargement and clustering of LAMP-1-positive compartments in HeLa cells induced by expression of OCA2-TfR indicates that these effects are a consequence of overexpression of the cytoplasmic sorting signals and not of OCA2 ion transport activity. Similar observations were made upon overexpression of a chimeric protein with the tyrosinase cytoplasmic domain (Calvo et al., 1999). It is unclear why this effect on lysosome morphology is not seen in transfected CHO cells, but it may reflect the apparently heightened sensitivity of lysosomally-localized OCA2 constructs to degradation in this cell type.

When expressed in melanocytic cells, OCA2-TfR was detected intracellularly on perinuclear vesicular structures that overlapped minimally with pigment granules and the melanosome marker Tyrp1 (Figure 2.9). We were unable to alter its distribution by treatment with inhibitors of lysosomal proteases or deacidification reagents; however, similar results were obtained with other chimeras that target to lysosomes in other melanocytic cells (data not shown), suggesting that lysosomal proteolysis might be more difficult to disrupt in melanocytes than in other cell types. We thus could not directly determine whether the dileucine motifs were sufficient for localization to melanosomes. However, by labeling transfected melan-Ink4a cells with anti-hTfR antibodies without permeabilization at 4°C, we tested whether the dileucine motifs confer steady-state intracellular localization. Whereas intact cells expressing full-length hTfR (Figure 2.10, a–c) or AA123-TfR (Figure 2.10, g–i) were strongly labeled at the cell surface by anti-hTfR antibodies, cells expressing OCA2-TfR were not (Figure 2.10, d–f). These data indicate that the N-terminal OCA2 dileucine motifs are sufficient to confer intracellular
**Figure 2.9**

Wild-type melan-Ink4a melanocytes were transfected with OCA2-TfR and stained with antibodies against hTfR (a) and the melanosomal marker Tyrp1 (b). Images are merged in c (anti-hTfR, green; anti-Tyrp1, magenta). Bar = 10 µm.
Figure 2.10. OCA2 dileucine motifs confer internal localization in melanocytes.

Wild-type melan-Ink4a melanocytes were transfected with hTfR (a–c), OCA2-TfR (d–f), or AA123-TfR (g–i) and incubated at 4°C with antibodies to the extracellular region of hTfR (b, e, and h). After subsequent washing, fixation, and permeabilization, cells were labeled with NOCA2 antibody (c, f, and i) to identify transfected cells expressing chimeric proteins. Bright field images are shown in a, d, and g. Bar, 10 µm.
localization in melanocytes, and they are consistent with the notion that they may confer melanosome localization.

*Individual OCA2 Dileucine Motifs Are Not Functionally Equivalent.*

To determine whether individual OCA2 dileucine motifs had distinct or redundant functions, we mutagenized each of the three motifs individually or in all possible combinations within the context of OCA2-HA (Figure 2.11a). The three motifs are designated LL1, LL2, and LL3, with LL1 being the most N-terminal and LL3 the most C-terminal. The corresponding OCA2 mutant proteins are designated OCA2-AA1, -AA2, and -AA3. Mutants were expressed in wild-type melan-Ink4a melanocytes and localization was determined by IFM. Disruption of LL3 (OCA2-AA3) did not affect steady-state localization of OCA2-HA to melanosomes (data not shown), whereas disruption of LL1 and LL2 together—leaving only LL3 intact (OCA2-AA12)—ablated localization to melanosomes and resulted in surface expression as seen with the triple mutant (Figure 2.11, c–e). Therefore, LL3 is dispensable for OCA2 trafficking.

Strikingly, mutants in which LL2 alone (OCA2-AA2) or both LL2 and LL3 (OCA2-AA23) were mutagenized, leaving LL1 intact, were as efficiently localized at steady state to melanosomes as the wildtype protein (Figure 2.11, f–h; data not shown). This indicates that, within the context of full-length OCA2, LL1 is sufficient to confer melanosomal localization. Moreover, LL1 is necessary for steady-state melanosomal localization, because mutants in which LL2 was present but LL1 was disrupted (OCA2-AA1 and OCA2-AA13) did not colocalize with pigment granules (Figure 2.11, i–k; data not
Figure 2.11

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**b. melan-p1 rescue**

% pigmented cells

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**c. OCA2 (anti-HA)**

**d. BF**

**e. OCA2/BF**

**f. OCA2 (anti-HA)**

**g. BF**

**h. OCA2/BF**

**i. OCA2 (anti-HA)**

**j. BF**

**k. OCA2/BF**

**l. OCA2 (anti-HA)**

**m. BF**

**n. LAMP-2**
**Figure 2.11.** Localization and function of OCA2 dileucine mutants.

(a) Schematic of the panel of human OCA2 dileucine mutants with the relevant N-terminal region of OCA2 highlighted. A green circle indicates the presence of LL1, LL2, or LL3, and a white X indicates its absence. (b) Dileucine mutants were expressed in melan-p1 cells, and transfected cells were visually inspected for the presence of pigmented melanosomes. Shown is the percentage of transfected cells expressing each indicated construct that contained pigmented melanosomes (% pigmented cells). Columns with asterisks were significantly different from rescue by OCA2-HA. **, p < 0.01. (c–n) IFM analysis of melan-Ink4a cells expressing selected mutant OCA2 variants. Melan-Ink4a melanocytes were transfected with OCA2-AA12 (c–e), OCA2-AA2 (f–h), or OCA2-AA1 (i–n). Transgenes were visualized with anti-HA antibodies (c, f, i, and l), melanosomes were visualized by bright field (BF) microscopy (d, g, j, and m), and late endosomes/lysosomes were visualized by labeling with anti-LAMP-2 antibody (n). e, h, and k are merged OCA2-HA (magenta) and inverted bright field (green) images. All insets show 3.5X magnified images of the boxed region, and insets in l–n show paired merged images of anti-HA (green), anti-LAMP-2 (red), and inverted bright field (blue). Arrows point to regions of overlap of OCA2 constructs with melanosomes (f–h) or with LAMP-2 (l–n). Bars, 10 µm.
shown). Rather, these mutants localized at steady state to nonpigmented, cytoplasmic vesicles that were also labeled by antibodies to LAMP-2 (Figure 2.11, l–n; data not shown), a lysosomal marker (Granger et al., 1990) that is largely excluded from pigment granules in melan-Ink4a cells. Together, these data indicate that LL1 is necessary and sufficient for melanosomal trafficking in the context of full-length OCA2, whereas LL2 confers steady-state localization to lysosomes, but not to melanosomes, in a manner that is masked by LL1 function. In HeLa cells, which do not have a separate melanosomal trafficking pathway, the presence of either LL1 or LL2 was sufficient to confer lysosomal localization of full-length OCA2 mutants, whereas the simultaneous loss of both motifs caused accumulation on the cell surface similar to that seen with the triple mutant (data not shown).

To determine whether melanosomal localization correlated with OCA2 function, we next tested how mutagenesis of distinct dileucine motifs affected the ability of OCA2 to rescue pigmentation when expressed in melan-p1 cells (Figure 2.11b). Transfected cells expressing a mutant in which LL1 was disrupted (AA1, AA12, and AA123) showed a significant reduction in the percentage of pigmentation, suggesting that melanosomal localization is needed for maximal OCA2 function in pigmentation. By contrast, disruption of either LL2 (AA2) or LL3 (AA3) had no significant effect, and a mutant in which only LL1 was intact (AA23) rescued pigmentation as efficiently as the wild-type protein. The surface-localized OCA2-AA12 mutant, lacking both LL1 and LL2, was as inefficient as the triple mutant in restoring pigmentation. These results corroborate the finding that of the three dileucine motifs in the N terminus of OCA2, LL1 is the most
important for both localization to melanosomes and function. Interestingly, mutants lacking LL1 but retaining LL2 (OCA2-AA1 and -AA13) were able to partially restore pigmentation to melan-p1 cells. Because these mutants localize at steady state to lysosomes, one possible interpretation of this result is that a cohort of melanosome-localized OCA2 that is below the limit of detection is sufficient to rescue pigmentation in these cells. Alternatively, OCA2 might either transiently traverse melanosomes en route to late endosomes/lysosomes or function from late endosomal compartments.

**OCA2 Binds Adaptors AP-1 and AP-3 in a Dileucine-dependent Manner.**

Previous studies have shown that acidic dileucine signals bind to members of the clathrin-associated heterotetrameric adaptor protein family (Bonifacino and Traub, 2003; Janvier et al., 2003; Chaudhuri et al., 2007; Doray et al., 2007). The acidic dileucine signal in Tyr mediates an interaction with both AP-3 and AP-1 (Honing et al., 1998; Theos et al., 2005), whereas the corresponding signal in Tyrp1 has been shown to interact with AP-1 only (Theos et al., 2005). We therefore used an affinity “pull-down” assay to test whether the dileucine motifs in the OCA2 cytoplasmic domain can be recognized by heterotetrameric adaptors. Recombinant fusion proteins consisting of GST fused to the cytoplasmic N terminus of wild-type (GST-OCA2_{NT}) or mutagenized human OCA2 were purified, immobilized onto glutathione-Sepharose beads, and then incubated with MNT-1 or HeLa cell lysates. Bound proteins were analyzed by immunoblotting. As shown in Figure 2.12a, GST-OCA2_{NT} bound the adaptor proteins AP-1, AP-2, and AP-3 but not the unrelated proteins β-actin, α-tubulin, Rab5, or the BLOC-1 subunit pallidin. We did
Figure 2.12

(a) GST alone (GST) or fused to the N-terminal cytoplasmic domain of human OCA2 (GST-OCA2<sub>NT</sub>) was bound to glutathione-Sepharose and then incubated without (No Lys) or with MNT-1 cell lysates. Bound proteins were fractionated by SDS-PAGE and immunoblotted with antibodies to the proteins indicated at left. The first lane includes lysate before incubation (1% of the total used for the pulldowns). (b) GST fused to wild-type (OCA2<sub>NT</sub>) or the indicated mutants of the N-terminal cytoplasmic region of human OCA2, or to the C-terminal cytoplasmic domain of OA1 as a negative control (OA1<sub>CTS</sub>), were bound to glutathione-Sepharose, incubated without (No Lys) or with HeLa cell lysate, and then analyzed by immunoblotting. Arrows point to the relevant bands in the immunoblot. Arrowheads point to cross-reactivity of the anti-AP-3 µ3A antibody with GST-fusion proteins. Shown at bottom is a Coomassie-stained gel of identical reactions showing the GST-fusion proteins.

Figure 2.12. OCA2 cytoplasmic dileucine-based motifs differentially bind AP proteins.
not detect binding of AP-1 or AP-3 to GST fused to the C-terminal cytoplasmic domain of the melanosomal/lysosomal protein OA1 (GST-OA1_{CT5}). This domain contains an unrelated sorting signal that does not conform to any canonical AP-binding consensus sequences (Figure 2.12b) (Piccirillo et al., 2006). These data suggest that the cytoplasmic domain of OCA2 interacts specifically with AP-1, AP-2, and AP-3.

To determine whether adaptor binding was mediated by the dileucine-based motifs, the experiment was repeated using GST fused to OCA2 cytoplasmic domain mutants in which one or more dileucine motifs were disrupted by dileucine-to-dialanine mutations (Figure 2.12b). Whereas AP-2 binding to different mutants was detected inconsistently over multiple trials (data not shown), AP-3 was consistently bound to GST-OCA2_{NT} fusions containing an intact LL1 (OCA2-AA2, -AA3, and -AA23) but not with fusions containing a disrupted LL1 (OCA2-AA1, -AA12, -AA13, and -AA123). Although low levels of bound AP-1 were observed even with a GST-OCA2_{NT} fusion protein bearing mutations in all three dileucine motifs (OCA2-AA123), much higher levels of bound AP-1, as for AP-3, were observed with fusion proteins containing an intact LL1. Interestingly, intermediate binding to AP-1 was observed with a mutant bearing only an intact LL2 (OCA2-AA13). Detection of all AP complexes was dependent on incubation with cell lysates (Figure 2.12b, “No Lys”), and identical results were observed using lysates derived from MNT-1 cells (data not shown). These data suggest that LL1 mediates strong binding to AP-1 and AP-3, whereas LL2 mediates moderate binding to AP-1 and LL3 has no appreciable affinity for the adaptors we tested. Thus, strong binding to AP-1 and AP-3 correlates with steady-state localization to melanosomes and with
OCA2 function, and modest binding to AP-1 correlates with steady-state lysosomal sorting. These data further corroborate the different roles of the dileucine motifs and implicate AP-1 and/or AP-3 in regulating the trafficking of OCA2.

2.4 Discussion

OCA2-deficient melanocytes have clear defects in melanosome morphology and function, but previous reports have ascribed functions for OCA2 within different subcellular locations. Recent reports suggest that melanosome malformation in OCA2-deficient melanocytes results from misfolding of melanosomal proteins in the ER, and that OCA2 localizes to the ER (Chen et al., 2002). Our data indicate that endogenous OCA2 in melanocytic cells and OCA2 transgene products in nonmelanocytic cells reside only transiently within the ER and that spatial restriction of OCA2 to the ER or to the cell surface impairs the function of OCA2 in melanogenesis. IFM and deconvolution analyses show that exogenously expressed OCA2 localizes to pigmented structures in mouse melanocytes that bear markers of mature melanosomes. Moreover, like other melanosomal proteins, the targeting of OCA2 to melanosomes is dependent on cytoplasmic dileucine-based motifs that bind to AP-3 and AP-1 adaptors and mediate lysosomal sorting in nonmelanocytic cells. These data indicate that OCA2 traverses intracellular sorting pathways common to other melanosomal proteins. We conclude that OCA2 localizes to and functions primarily within melanosomes.

The molecular function of the OCA2 protein is not yet known, but it is predicted to be a transmembrane ion transporter based on sequence homology to the ArsB/NhaD family of
permeases. Our results suggest that this proposed activity would be required to regulate the intraluminal ion concentration within melanosomes to promote melanin synthesis. The lack of melanin synthesis in OCA2-deficient cells is thus likely a direct consequence of the dysregulation of ion content. This model is consistent with in vitro data showing that tyrosinase activity is sensitive to pH and redox state (Townsend et al., 1984). Ion dysregulation could in turn have indirect consequences on other processes in OCA2-deficient cells. For example, alterations in intraluminal ion balance or pH can affect cellular membrane fusion events (Peters and Mayer, 1998; Ungermann et al., 1999; Pryor et al., 2000), perhaps explaining the accumulation of melanosomal cargo in vesicular structures in OCA2-deficient melanocytes (Manga et al., 2001). Moreover, disruption of OCA2 transport activity across the melanosomal membrane may indirectly alter ion concentrations or pH in the cytosol; for example, exogenous expression of OCA2 in Saccharomyces cerevisiae led to a depletion of cytoplasmic glutathione due to glutathione transport into the vacuole (Staleva et al., 2002). Alterations in substrate levels could in turn have downstream effects on ion transport between the cytosol and other compartments, such as the ER. Such effects might alter folding or disulfide bond formation of proteins such as tyrosinase in the ER, perhaps explaining the reduced rate of processing and ER exit of tyrosinase in OCA2-deficient melanocytes (Toyofuku et al., 2002). Tyrosinase contains 15 lumenal Cys residues and many intramolecular disulfide bonds (Wang and Hebert, 2006), so changes in the disulfide bonding capacity of the ER could have a significant effect on tyrosinase maturation. A similar indirect effect on cellular ion levels and consequent tyrosinase missorting has been hypothesized to underlie the pigmentation defects in cells bearing a mutated form of SLC24A5, a pigment
cell-specific potassium-dependent sodium-calcium exchanger that was suggested to localize to the trans-Golgi network (Ginger et al., 2008).

OCA2 is unique among melanosomal proteins by virtue of having multiple functional dileucine-based trafficking signals. The three motifs we identified in the N terminus of OCA2 are the only cytoplasmic sequences in the protein that conform to the acidic dileucine consensus. Of these, only the first motif (LL1) has been specifically noted in the past (Lee et al., 1995). The cytoplasmic N terminus of OCA2 is poorly conserved across species, and LL1 is the only one of the three consensus dileucine signals in the human homologue that is conserved in the mouse. This raises the question of how human and murine OCA2 trafficking might differ. No posttranslational modifications of OCA2 (other than N-glycosylation) have been reported, but the N terminus contains many serine residues that could be potential sites of regulatory phosphorylation. It is also possible that the combined activities of both human dileucine signals are incorporated into the single mouse dileucine signal, which differs in specific sequence from human LL1.

Consistently, the single dileucine signal of tyrosinase seems to be sufficient to target tyrosinase to either of two distinct pathways to the melanosome (Theos et al., 2005; Setty et al., 2007). Although dileucine-based signals clearly play crucial roles in melanosomal trafficking, it is worth noting that none of the documented pathological point mutations in human Tyr, Tyrp1, or OCA2 occur in the acidic dileucine motifs of the proteins. The ability of the dileucine motifs to direct lysosomal localization of the OCA2-TfR chimera in nonmelanocytes suggests that these motifs are likely sufficient for OCA2 trafficking, but the existence of additional and/or potentially interacting melanosomal trafficking
determinants cannot be ruled out because we could not visualize OCA2-TfR on melanosomes in melanocytes.

The change in steady-state localization that arises from mutation of individual dileucine motifs raises the possibility that different motifs mediate different steps of the trafficking itinerary of human OCA2. IFM data suggest that LL2 confers steady-state localization to lysosomes but is dominated by LL1, which confers steady-state localization to melanosomes. The existence of two signals suggests either of two possible models for OCA2 trafficking within melanocytes. One model posits that distinct cohorts of OCA2 use LL1 and LL2 for melanosomal and lysosomal trafficking, respectively. Mutagenesis of either motif would thus render the mutant protein subject to the direction of the remaining signal. Because LL1 mutants localize at steady state to lysosomes and yet stimulate substantial melanin synthesis in melan-p1 cells, this model would require that OCA2 be able to support optimal melanin synthesis in the melanosome either from late endosomes/lysosomes but not from the ER or the cell surface, or via a cohort of melanosome-localized OCA2 that is below the level of detection by IFM. A second model posits that OCA2 traffics through both melanosomes and lysosomes sequentially by virtue of sequential utilization of LL1 and LL2. In this case, LL2 would function as a timer to limit the residence of OCA2 within melanosomes by targeting it to lysosomes for subsequent degradation. OCA2 mutants that lack the strong melanosomal targeting of LL1 but still stimulate significant melanin synthesis might be targeted inefficiently to melanosomes via the weak AP-1-binding activity of LL2 and subsequently travel more efficiently to lysosomes, which would constitute the predominant accumulation at steady
state. This model would explain the short half-life of OCA2 in melanocytes and can potentially be vigorously tested by live cell imaging experiments. In either case, the differential localization of OCA2 mutants with only LL1 or LL2 intact indicates that cytoplasmic targeting signals, irrespective of potential integral membrane or lumenal targeting determinants, can be differentially decoded to confer steady-state localization to lysosomes or melanosomes. This contrasts with the targeting of Tyrp1, in which lumenal determinants seem to be required to distinguish these two fates (Groux-Degroote et al., 2008).

LL1 and LL2 have differing abilities to bind adaptor proteins, thus suggesting a potential mechanism behind the complex trafficking pathway of OCA2. Like that of tyrosinase, the OCA2 cytoplasmic domain binds both AP-1 and AP-3. This binding depends critically on the presence of LL1. LL2 binds AP-1 more weakly and does not bind significantly in our assay to AP-3. Thus, steady-state localization of OCA2 to melanosomes, seen with all constructs containing an intact LL1, correlates well with either AP-3 binding or high-affinity binding to either AP protein, similar to the situation for tyrosinase. By contrast, a weak affinity for AP-1 alone may be sufficient to drive targeting of constructs containing LL2 but lacking LL1 toward lysosomes, either directly from endosomes or secondarily from melanosomes. Interestingly, lysosomal enlargement is seen in nonmelanocytes that overexpress fusion proteins containing the wildtype dileucine motifs from OCA2 or Tyr, which possess both AP-1 and AP-3 binding ability; enlargement is not seen when the fusion protein contains the dileucine motif of Tyrp1, which binds only AP-1 (data not shown). Thus, in this case a functional alteration of the target organelle seems to correlate
with the cargo protein’s capacity for interaction with multiple AP proteins. How differences in affinity might distinguish melanosomal from lysosomal targeting in melanocytes remains to be elucidated. Moreover, whether the two adaptor proteins distinguish the dileucine motifs by virtue of differences in their sequence or in their position within the protein is not clear. Future experiments will more closely probe the sequence of events in the trafficking of OCA2 to define the relationship between melanosomal and lysosomal targeting and the role each plays in OCA2 function.

2.5 Acknowledgments

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CHAPTER 3: DIFFERENTIAL SEQUENCE PREFERENCES FOR AP-1 AND AP-3 IN AN ACIDIC DILEUCINE-BASED SORTING SIGNAL REVEAL A REQUIREMENT FOR BOTH BLOC-1 AND AP-3 IN DELIVERY OF OCA2 TO MELANOSOMES

3.1 Abstract

Cell types that harbor unique lysosome-related organelles (LROs) require specialized intracellular trafficking pathways to divert cargoes from conventional endocytic organelles toward nascent LROs. In melanocytes, cargo delivery to melanosomes – the LRO in which melanin pigments are synthesized and stored – correlates with cargo binding via cytoplasmically exposed targeting signals to either of two clathrin-associated adaptors, AP-1 or AP-3. Some cargoes, such as tyrosinase, have acidic dileucine-based sorting signals that can bind either adaptor, but how binding to each adaptor functions during melanosome delivery of such cargoes is not clear. Moreover, analyses of melanocytes from mouse models of Hermansky-Pudlak syndrome suggest that AP-3 and BLOC-1 function in mutually exclusive cargo transport pathways, whereas in other cell types they appear to function together. Here, we use the pigment cell-specific putative transporter, OCA2, to dissect the relative roles of AP-1 and AP-3 and their cooperativity with BLOC-1 in melanosome transport. We show that interaction of the OCA2 acidic dileucine-based melanosome targeting motif with AP-1 or AP-3 depends on the primary sequence and not the position of the motif within the cytoplasmic domain. Directed mutagenesis of the motif led to the isolation of mutants that show preferred binding to either AP-1 or AP-3 by yeast two-hybrid analysis. Each mutant was able to traffic toward melanosomes when expressed in wild-type melanocytes and rescue melanin synthesis in OCA2-deficient melanocytes, but robust AP-3 binding was necessary for
steady-state melanosome localization. Importantly, despite its dependence on AP-3, OCA2 trafficking to melanosomes required BLOC-1. This dependence on BLOC-1 was observed even when the endogenous OCA2 sorting motif was replaced with that of tyrosinase, a single membrane-spanning domain enzyme that traffics to melanosomes in an AP-3-dependent/ BLOC-1-independent fashion. The dependence of OCA2 on both AP-3 and BLOC-1 for melanosomal localization indicates that BLOC-1 can cooperate with either of several adaptors during cargo transport, thus reconciling melanosome cargo transport with previously reported physical and functional interactions between AP-3 and BLOC-1.

3.2 Introduction

Lysosome-related organelles (LROs) are cell type-specific organelles that share some characteristics with lysosomes, such as an acidic lumenal pH, the presence of some lysosomal proteins, and derivation from the endocytic system (Dell'Angelica et al., 2000; Raposo et al., 2007), but are distinguished from true lysosomes in that they also contain unique cargo proteins that confer distinct morphological and functional characteristics. Some cell types simultaneously harbor LROs and lysosomes and consequently must utilize unique sorting and trafficking pathways to divert specific cargo proteins from the general endocytic pathway and deliver them to LROs (Dell'Angelica et al., 2000; Raposo and Marks, 2007; Raposo et al., 2007). For example, melanocytes contain LROs called melanosomes, organelles that serve as the site of synthesis of the melanin pigments that give color to the skin, hair, and eyes (Marks and Seabra, 2001; Hearing, 2005). Melanosomal cargoes are biosynthetically transported from endosomes to melanosomes
(Raposo and Marks, 2007), but fluid-phase cargoes and the endosomally recycling transferrin receptor are both excluded from melanosomes, consistent with the idea that the melanosomal cargoes must be specifically sorted from endosomes into trafficking pathways that are targeted to these organelles (Raposo et al., 2001; Delevoye et al., 2009). Several studies have implicated both the AP-1 and AP-3 heterotetrameric adaptors as being important for the trafficking of melanosomal cargo proteins from endosomes (Huizing et al., 2001; Theos et al., 2005; Setty et al., 2007; Setty et al., 2008; Delevoye et al., 2009). These complexes seem to regulate distinct LRO cargoes, but what determines this selectivity is still unclear. Moreover, other components that facilitate cargo delivery during or after AP-mediated sorting have not been clarified.

The AP complexes are a family of cytosolic trafficking adaptors that couple clathrin recruitment to clustering of integral membrane cargo via interactions with short sorting signals in the cytoplasmic domains of the target proteins (Boehm and Bonifacino, 2001; Bonifacino and Traub, 2003; Robinson, 2004). One class of signals recognized by the AP complexes is the acidic dileucine motifs, with the consensus [D/E]XXXL[L/I] (Bonifacino and Traub, 2003). The melanosomal sorting of tyrosinase (Tyr) and tyrosinase-related protein 1 (Tyrp1), two enzymes that carry out the synthesis of melanin, depends on critical acidic dileucine sorting motifs in their cytoplasmic domains (Vijayasaradhi et al., 1995; Calvo et al., 1999; Simmen et al., 1999; Huizing et al., 2001; Theos et al., 2005; Setty et al., 2007; Delevoye et al., 2009). As expected, these two motifs interact with adaptors, but they show differing levels of dependence on AP-1 and AP-3. AP-1 binds the signals in the cytoplasmic domains of both Tyr and Tyrp1 in vitro
while AP-3 binds only the signal in Tyr (Honing et al., 1998; Theos et al., 2005). Those features of acidic dileucine-based sorting signals that distinguish between AP-1 and AP-3 binding have not been identified. While a crystal structure has identified the interaction site between the AP-2 core complex and an acidic dileucine-like signal from CD4 (Kelly et al., 2008), comparable structures of AP-1 or AP-3 with signals have not been described and thus the molecular basis for distinctive interaction patterns is not known.

Consistent with the in vitro binding, both Tyr and Tyrp1 are localized to AP-1-coated endosomal buds and tubules that accumulate near melanosomes in melanocytic cells, whereas only Tyr is found in the AP-3-coated structures in similar subcellular regions (Raposo et al., 2001; Theos et al., 2005; Delevoye et al., 2009). In hypopigmented AP-3-deficient pearl mouse melanocytes or melanocytes derived from AP-3-deficient human patients, Tyr is largely mislocalized to early endosomes and multivesicular late endosomes, suggesting that AP-3 is required to efficiently sort Tyr out of the classical endosomal degradation pathway and toward melanosomes (Huizing et al., 2001; Richmond et al., 2005; Theos et al., 2005). However, the amount of Tyr that localizes to AP-1-coated buds also increases in these cells, suggesting that AP-1 might be able to partially substitute for AP-3 under certain circumstances (Theos et al., 2005). By contrast to Tyr, Tyrp1 (and another melanosome resident, ATP7A; (Setty et al., 2008)) is not mislocalized like Tyr in AP-3-deficient melanocytes but is correctly delivered to melanosomes (Huizing et al., 2001; Setty et al., 2007). By contrast, depletion of AP-1 from a pigmented human melanoma cell line causes substantial mislocalization of Tyrp1 to vacuolar domains of recycling endosomes and a reduction in cellular melanin content.
At least part of AP-1 function is mediated by its interaction with a kinesin motor, Kif13A, which is required for the generation of tubules that emerge from recycling endosomes, make direct contacts with melanosomes, and correlate with cargo transfer (Delevoye et al., 2009). Thus AP-1 and AP-3 each function in the delivery of distinct cargoes from endosomes toward melanosomes, but how they discriminate among cargo proteins, cooperate in the delivery of cargoes that are recognized by both complexes, and interface with other components of the trafficking machinery are not well understood.

The importance of AP-3 in melanosomal trafficking is further underscored by mutations in the beta3A subunit that underlie the LRO biogenesis defect of Hermansky-Pudlak Syndrome (HPS) type 2 and the pearl mouse (Feng et al., 1999). HPS is a group of genetic diseases that result from impaired LRO biogenesis in multiple cell types (Di Pietro and Dell'Angelica, 2005; Huizing et al., 2008). Phenotypes common to all HPS subtypes include oculocutaneous albinism and prolonged bleeding, due respectively to defects in the biogenesis of melanosomes and of platelet dense granules, and LRO defects in other tissues lead to additional phenotypes in some HPS subtypes. Mutations in any of at least 15 genes give rise to murine models of HPS, eight of which correspond to those affected in human HPS subtypes (Di Pietro and Dell'Angelica, 2005; Wei, 2006). Most of these genes encode subunits of five distinct multisubunit protein complexes, at least four of which are thought to regulate endosomal membrane dynamics required for LRO biogenesis. Among the murine HPS gene products are two subunits of AP-3 as well as five of at least eight subunits of the Biogenesis of Lysosome-Related Organelles Complex 1 (BLOC-1). AP-3 has been well-characterized as a cargo adaptor complex,
but BLOC-1 has not been successfully demonstrated to physically interact with any melanosomal cargo proteins. In fact, the function of BLOC-1 is still unknown, although its demonstrated interactions with several SNARE proteins suggest a potential role in regulating membrane fusion events (Huang et al., 1999; Ilardi et al., 1999; Moriyama and Bonifacino, 2002; Vites et al., 2004; Ghiani et al., 2010). While AP-3 is detected primarily on buds of sorting endosomal tubules, BLOC-1 has been localized primarily on tubular domains of early endosomes (Di Pietro et al., 2006; Setty et al., 2007). Consistently, BLOC-1 deficiency leads to mislocalization of distinct melanosomal cargoes from those affected by AP-3 deficiency. For example, in BLOC-1-deficient melanocytes from *pallid* or *muted* HPS model mice, Tyr was only partially mislocalized, with a large cohort capable of accessing melanosomes, but Tyrp1 and ATP7A failed to access melanosomes at all, and Tyrp1 was largely trapped in an endocytic recycling loop (Setty et al., 2007; Setty et al., 2008). Based on this differential sensitivity of melanosomal proteins to perturbations in AP-3 and BLOC-1 expression, melanosomal transmembrane cargoes have been proposed to follow one of two pathways from early endosomes to melanosomes, one controlled by AP-3 and another by BLOC-1 (Raposo and Marks, 2007; Setty et al., 2007). Moreover, the similarity of the Tyrp1 trafficking phenotypes seen with AP-1 knockdown or genetic deficiency of BLOC-1 has led to the additional proposal that AP-1 and BLOC-1 act together to promote Tyrp1 trafficking to melanosomes via recycling endosomal tubules (Delevoye et al., 2009).

A model in which AP-1 and BLOC-1 cooperate in one dedicated cargo delivery pathway to melanosomes and AP-3 functions in a separate dedicated pathway fails to account for
several published observations. First, a robust physical interaction has been detected between AP-3 and BLOC-1 in detergent extracts of membranes from fibroblasts, brain, and liver (Di Pietro et al., 2006; Salazar et al., 2006); our lab has also observed this interaction in lysates from melanocytes (S. Gangi Setty, unpublished observation). By contrast, despite several efforts, AP-1 has not been demonstrated to biochemically interact with BLOC-1 (Salazar et al., 2009). Moreover, in neurons, AP-3 and BLOC-1 appear to cooperate in the delivery of a set of cargoes (Newell-Litwa et al., 2009; Salazar et al., 2009). Indeed, although AP-3 and BLOC-1 localize to distinct domains of early endosomes in melanocytes, BLOC-1 has been consistently detected in immunoaffinity purified AP-3-coated vesicles from neurons (Newell-Litwa et al., 2009; Salazar et al., 2009), as well as in clathrin-coated vesicles from HeLa cells (Borner et al., 2006). As mentioned, it is also unclear how signals such as those in tyrosinase that are capable of binding either AP-3 or AP-1 «choose» between the two distinct pathways.

In order to further dissect models for melanosomal protein sorting, we turned to an additional melanosomal protein, OCA2, also known as pink-eyed dilution or P protein (Rinchik et al., 1993). OCA2 is a 12-transmembrane domain protein that is expressed only in pigment cells and that bears homology to a superfamily of transporters (Rinchik et al., 1993; Lee et al., 1995). Mutations in the OCA2 gene underlie oculocutaneous albinism (OCA) type 2, the most common form of OCA worldwide (King, 1998). We have previously shown that OCA2 localizes to melanosomes by virtue of one of three acidic dileucine sorting signals in the cytoplasmic N-terminal domain (Sitaram et al., 2009). As for the Tyr sorting signal, the OCA2 sorting signal binds to both AP-1 and AP-
3, as shown using a GST pulldown assay. Here, we sought to define those features of the OCA2 acidic dileucine-based sorting signal that confer the ability to bind AP-1 or AP-3, and then to exploit mutants that only bind to one of these complexes to dissect the respective role of each adaptor in OCA2 melanosome transport. Our results define sequence features of acidic dileucine-based sorting signals that can distinguish between AP-1 and AP-3 binding, show that OCA2 absolutely requires AP-3 interaction for stable localization to melanosomes, and provide evidence for two distinct AP-3-dependent melanosomal transport pathways in melanocytes – one that is independent of BLOC-1 and one that requires BLOC-1. Together, these data suggest that BLOC-1 can cooperate with multiple adaptors to effect cargo delivery to cell type-specific LROs.

3.3 Results

The OCA2 Acidic Dileucine-based Melanosome Targeting Signal Binds to AP-1 and AP-3 Hemicomplexes in a Yeast Three-Hybrid Assay

The N-terminal cytoplasmic domain of human OCA2 contains three consensus acidic dileucine-based sorting motifs termed LL1, LL2 and LL3 (Figure 3.1a). We have previously shown that substitution of the two critical leucines of LL1 for alanine (AA1N) resulted in a loss of steady state localization to melanosomes, implicating LL1 as the critical sorting signal for melanosome delivery. Moreover, a fusion protein consisting of the OCA2 N-terminal cytoplasmic domain fused to glutathione S-transferase (GST) was capable of binding to both AP-1 and AP-3 from human melanoma or HeLa cell cytosol, and this interaction required intact LL1 (Sitaram et al., 2009). We first sought to determine those features of LL1 that permitted its interaction with AP-1 and/or AP-3 and
Figure 3.1

a. Human OCA2

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</tr>
<tr>
<td>hTyr</td>
<td>H\textsubscript{N}---GAL4 BD</td>
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b. OCA2N

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c. OCA2- AA23N

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**Figure 3.1.** The cytoplasmic domain of OCA2 shows dileucine-dependent interaction with adaptor proteins in a yeast three-hybrid system.

(a) Sequences of the three acidic dileucine sorting motifs in the cytoplasmic N-terminus of human OCA2 and the single acidic dileucine motif in the cytoplasmic C-terminus of human tyrosinase. (b) Schematic of OCA2 N-terminal domain Gal4 fusion constructs used in the assay. GAL4 BD, Gal4 binding domain; green square, intact dileucine motif; white X, disrupted dileucine motif. Black numbers within the green square indicate whether the primary sequence of the LL1, LL2, or LL3 motif is inserted at the indicated position within the domain. Below, a yeast three-hybrid assay of various OCA2 constructs co-expressed with hemicomplexes of the AP-1 (gamma/sigma1A), AP-2 (alpha/sigma2), and AP-3 (delta/sigma3A) complexes. A protein-protein interaction leads to expression of HIS3, allowing growth on His-deficient media. All transformed yeast grow on His-containing control media. (c) Schematic of OCA2 constructs used in the assay. Below, yeast three-hybrid assay of the OCA2 constructs in which dileucine motif sequences are re-positioned within the OCA2 cytoplasmic domain.
thus distinguished it from the nearly inactive LL2 and completely inactive LL3. In order to more effectively assay in parallel the interaction between various OCA2 mutants and adaptor protein complexes, we adapted a yeast three-hybrid (Y3H) assay that was previously used to study acidic dileucine motifs in the melanogenic enzyme tyrosinase and the HIV protein Nef (Janvier et al., 2003; Theos et al., 2005; Chaudhuri et al., 2007; Chaudhuri et al., 2009). The assay involves coexpression of Gal4 fusion proteins of the test cytoplasmic domain with two of the four subunits of each AP complex, namely the small subunit (sigma1A, sigma2, or sigma3A) and the corresponding large subunit (gamma, alpha, or delta) for AP-1, -2, and -3, respectively, which comprise the surface that interacts with acidic dileucine sorting motifs (Janvier et al., 2003; Doray et al., 2007; Kelly et al., 2008) (note that the sigma4/epsilon hemicomplex of AP-4 has not been demonstrated to bind an acidic dileucine motif). The full cytoplasmic N-terminal region of human OCA2 (amino acids 1-173) was fused to the C-terminus of the yeast Gal4 binding domain. One of the three sigma subunits was expressed from the MET5 promoter within a second transcription unit in the same plasmid, and the gamma, alpha, or delta subunits were fused to the C-terminus of the Gal4 activation domain in a separate plasmid. The *Saccharomyces cerevisiae* strain HF7c was co-transformed with both plasmids and grown on Met-deficient selective media to express all three proteins. An interaction between the OCA2 cytoplasmic domain and an AP hemicomplex activates Gal4-dependent expression of *HIS3* and allows for growth on His-deficient media. The cytoplasmic domain of human tyrosinase (from amino acid 499 through the C-terminus), previously shown to interact with all three AP hemicomplexes in this assay (Janvier et al., 2003; Chaudhuri et al., 2009), served as a positive control.
As expected, the tyrosinase cytoplasmic domain interacted with hemicomplexes from AP-1, AP-2 and AP-3, as indicated by growth of all three sets of transformants on His-deficient media (Figure 3.1b). The full-length human OCA2 cytoplasmic N-terminal domain also interacted with all three AP hemicomplexes, confirming previous results obtained with GST-cytoplasmic domain fusion proteins (Sitaram et al., 2009); consistent with previous results, the strength of interaction with the AP-2 alpha/sigma2 hemicomplex varied among experiments (not shown). Also consistently, the interaction was ablated when all three acidic dileucine motifs were mutagenized to substitute the three dileucine pairs to dialanines (OCA2-AA123N), and the restoration of the critical LL1 motif (OCA2-AA23N) was sufficient to restore the interaction with the AP hemicomplexes. These data confirm that the Y3H assay robustly recapitulates the ability of LL1 to interact with AP complexes.

*Acidic dileucine motif position does not affect AP binding or sorting activity.*

Our previous analyses of immortalized melanocytes that transiently express full-length OCA2 mutant constructs revealed that neither the second nor third acidic dileucine motif (LL2 or LL3) alone could support steady-state localization of OCA2 to melanosomes in wild-type melanocytes or fully restore melanin synthesis when expressed in OCA2-deficient melanocytes (Sitaram et al., 2009). Moreover, GST fusion proteins with cytoplasmic domains in which LL1 was ablated by mutagenesis showed impaired binding to AP complexes. To determine why these motifs were inactive in melanosome sorting
and AP binding, we considered two parameters: position within the cytoplasmic domain and sequence of the intervening “X” residues within the EXXXLL consensus.

Because proximity to the membrane has previously been reported to affect the ability of a consensus sorting motif to function and to bind adaptors (Rohrer et al., 1996; Geisler et al., 1998; White et al., 1998), we used the Y3H assay to determine whether the inability of the LL2 or LL3 motif to bind APs was due to its placement within the cytoplasmic domain. We made variants of the OCA2-AA23N construct in which the sequence of the active LL1 signal was replaced in situ by that of LL2 (OCA2-AA23N LL1-2) or LL3 (OCA2-AA23N LL1-3) (Figure 3.1c). Whereas all three AP hemicomplexes interact with OCA2-AA23N bearing intact LL1, no AP interacted with either the LL1-2 or LL1-3 mutant, indicating that the sequences of the LL2 and LL3 motifs were inherently inactive. We then performed the complementary experiment in which the LL1 sequence was placed in the position of the second or third motif (in the context of an inactivated LL1 motif at position 1 in construct OCA2-AA1N). Whereas the disruption of the LL1 motif in OCA2-AA1N ablates the interaction with all three AP hemicomplexes, reintroduction of the LL1 sequence into the second position (OCA2-AA1N LL2-1) or the third position (OCA2-AA12N LL3-1) within the cytoplasmic domain restores this interaction. These data indicate that the LL1 motif is active in AP binding regardless of its position within the cytoplasmic domain, implicating specific sequence elements as critical to its AP binding activity.
To determine whether motif position affected the activity of the sorting signal, the mutations described above were engineered into the full-length, HA-tagged human OCA2 and transiently expressed in wild-type pigmented melan-Ink4a mouse melanocytes. By indirect immunofluorescence microscopy (IFM) analysis in these cells, OCA2-AA1 LL2-1 – in which LL1 is placed in position 2 - distributes largely in HA-positive rings that surround pigmented structures (Figure 3.2, a-c), similar to the staining pattern seen with expression of wild-type HA-tagged or native OCA2 (Sitaram et al., 2009). To determine whether this construct retained function, we expressed it in non-pigmented OCA-2-deficient melan-p1 mouse melanocytes; we have previously shown that OCA2 function correlates with melanosomal localization, since expression of melanosomally-localized but not surface-localized human OCA2 constructs in melan-p1 melanocytes restores pigment synthesis (Sviderskaya et al., 1997; Sitaram et al., 2009). Consistently, transiently-expressed OCA2-AA1N LL2-1 localized to vesicular structures in melan-p1 cells as in wild-type cells (Figure 3.2, d-f), and rescued pigmentation in 54% of transfected cells, compared to 69% of cells expressing wild-type OCA2 (Figure 3.2j).

By contrast, full-length OCA2 constructs bearing the LL1-2 and LL1-3 mutations were mislocalized to the cell surface when expressed in wild-type melanocytes (see Figure 3.3j and data not shown) or melan-p1 cells (Figure 3.2, g-i) and were incompetent to rescue melanin synthesis in melan-p1 cells (Figure 3.2, g-j and data not shown). Together, these data confirm that the LL2 and LL3 sequences are intrinsically poor at interacting with AP complexes and at effecting melanosome localization, even when placed in a "favorable" position within the protein. Conversely, the LL1 sequence is intrinsically competent to bind AP complexes in a relatively position-independent manner.
Figure 3.2. Localization and function of OCA2 dileucine motif position mutants.
a-i) IFM analysis of melan-Ink4a cells (a-c) or melan-p1 cells (d-i) expressing selected mutant OCA2 variants. Melan-Ink4a melanocytes were transfected with OCA2-AA1
LL2-1 (a-c) and melan-p1 cells were transfected with OCA2-AA1 LL2-1 (d-f) or OCA2-AA23 LL1-2 (g-i). Transgenes were visualized with anti-HA antibodies (a, d, g) and melanosomes were visualized by bright field microscopy (b, e, h). c, f, and i are merged OCA2-HA (magenta) and inverted bright field (green) images. All insets show 5X magnified images of the boxed region. Arrows point to regions of overlap of OCA2 constructs with melanosomes. Bar = 10 µm. (j) Dileucine mutants were expressed in OCA2-deficient melan-p1 cells, and transfected cells were visually inspected for the presence of pigmented melanosomes. Shown is the percentage of transfected cells expressing each indicated construct that contained pigmented melanosomes (% pigmented cells). All columns were significantly different from rescue by OCA2-HA. *** p < 0.001
Acidic dileucine motif sequence requirements for AP binding and melanosome targeting activity.

To begin to dissect those primary sequence features of the OCA2 dileucine motifs that conferred AP binding and sorting activity, we compared the sequence of the inactive LL3 motif in OCA2 to that of active acidic dileucine sorting signals. In particular, the sole acidic dileucine sorting motif in human Tyr, EKQPLL, differs at only two positions from L3 motif in human OCA2, EKGDLL (Figure 3.3a). Whereas AP hemicomplexes did not interact with the OCA2-AA23N LL1-3 mutant, in which the LL3 sequence in position 1 is the only intact motif (Figures 3.1c, 3.3b), all three AP hemicomplexes interacted with an OCA2-AA23N construct bearing the EKQPLL motif of human Tyr at position 1 (OCA2-AA23N hTyr; Figure 3.3b). However, constructs bearing either of the two single amino acid substitutions that change the middle residues of the Tyr signal to those in the inactive LL3 motif (OCA2-AA23N TyrPD or OCA2-AA23N TyrQG) do not interact with any AP hemicomplexes (Figure 3.3b). The AP binding ability of the motifs correlated completely with sorting activity, since full-length OCA2-AA23 bearing endogenous LL1 (Figure 3.3, d-f) and OCA2-AA23N hTyr bearing the intact tyrosinase signal localize to melanosomes at steady-state when expressed in melan-Ink4a melanocytes (Figure 3.3, d-i) and stimulate melanin synthesis in a high proportion of transfected melan-p1 cells (Figure 3.3c), but neither of the two “LL3-like” single point mutants chimeric constructs did. Rather, these mutants localized to the plasma membrane (Figure 3.3, k and l). Taken together, our results indicate that the "X" amino acids of the [D/E]XXX[L/I] consensus can greatly affect AP binding and sorting activity in the context of OCA2, and that neither glycine at position -2 nor an aspartate at
Figure 3.3. Internal sorting signal residues affect AP complex interaction.
(a) Comparison of the LL3 acidic dileucine motif from human OCA2 and the sole acidic
dileucine motif in human tyrosinase. OCA2-AA23N hTyr constructs were made in
which the first acidic dileucine motif of the OCA2 cytoplasmic domain was replaced with
the sequence from tyrosinase, with or without the indicated amino acid substitutions. (b)
Yeast three-hybrid analyses of AP hemicomplex interaction with the OCA2-AA23N hTyr
constructs. (c) Melan-p1 rescue assay performed as in Figure 3.2j. n.s., not significant. (d-
l) Melan-Ink4a mouse melanocytes were transiently transfected with full-length HA-
tagged OCA2-AA23 (d-f), OCA2-AA23 hTyr (g-i), OCA2-AA23 LL1-3 (j), OCA2-
AA23 hTyrPD (k), or OCA2-AA23 hTyrQG (l) constructs bearing the identical
mutations used in the yeast three-hybrid assay. Cells were stained with anti-HA
antibodies (d, g; colored magenta in merged pictures f, i-l) and subjected to indirect
immunofluorescence microscopy. Bright field images (e, h) were inverted and colored
green in merged pictures. Arrows point to regions of overlap between OCA2 constructs
and melanosomes. Bar = 10 µm.
position -1 (relative to the first leucine) can support either activity.

**OCA2 Interactions With AP-1 and AP-3 are Separable**

To further define the sequence requirements for AP binding and for melanosome targeting, we compared LL1 to acidic dileucine-based sorting signals from other melanocytic proteins. The LL1 motif in human OCA2 is the only one of the three human motifs that has a counterpart in OCA2 homologs in the mouse, Japanese medaka fish, or Mexican cavefish. Although the general consensus for acidic dileucine motifs is [D/E]XXXL[L/I], the three nonhuman OCA2 homologs and the LL1 (but not LL2 or LL3) motif of human OCA2 share a more restricted consensus of EXXPLL (Figure 3.4a). Moreover, the sequences of the cytoplasmic sorting motifs found in Tyr and Tyrp1 also correspond to the more restricted motif, suggesting the specific importance of a -4 glutamate and -1 proline for sorting to melanosomes. We tested the effect of a conservative Glu-to-Asp mutation of the OCA2 LL1 motif (OCA2-AA23N E96D) and of a non-conservative Pro-to-Ala mutation (OCA2-AA23N P99A; Figure 3.4b). The E96D mutant ablated the interaction of OCA2-AA23N with AP-3 hemicomplexes by Y3H assay (Figure 3.4c). Its interactions with AP-1 and AP-2 hemicomplexes were reduced but not eliminated; this manifested as variability in yeast growth over several trials (compare Figure 3.6b). Conversely, the P99A mutation abolished interaction of OCA2-AA23N with AP-1 hemicomplexes, but, although reduced, the interaction with AP-3 hemicomplexes was consistently retained. The P99A mutation consistently impaired the interaction with AP-2 hemicomplexes (data not shown). These data suggest that AP-1 but not AP-3 is tolerant of an aspartate residue at position -4, whereas AP-3 but not AP-1
Figure 3.4

a.

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Figure 3.4. AP-1 and AP-3 interaction with the OCA2 LL1 motif are separable.

a) Comparison of sequences of acidic dileucine motifs in OCA2 isoforms from human, mouse, Japanese medaka fish, and Mexican cavefish. All motifs are underlined, and gray boxes highlight conserved elements in the OCA2 motifs as well as in motifs from human and mouse tyrosinase and Tyrp1. b) Single amino acid substitutions were made in the
OCA2-AA23N construct in order to test for changes in interaction with AP complexes. c) Yeast three-hybrid analyses of AP interaction with OCA2 sorting motif mutants. Transformants were spotted in 5-fold serial dilutions. E96D and P99A show reciprocal interaction with AP-1 and AP-3.
is tolerant of an alanine at position -1.

To determine what features at position -4 were necessary for binding to AP-1 hemicomplexes, we further mutagenized the -1 Pro residue to test the suitability of a range of side groups for the residue in that position (Figure 3.5a). Of Ala, Asp, Asn, Lys, Phe, and Ser, only the serine substitution interacted with AP-3 hemicomplexes at a level that was comparable to that of the wild-type OCA2 LL1 motif; importantly, like the alanine substitution, the serine substitution did not interact with AP-1 hemicomplexes (Figure 3.5b). The phenylalanine substitution also interacted variably with both AP-1 and AP-3 hemicomplexes. Together, these data suggest that interaction of an acidic dileucine motif with AP-1 requires a proline or perhaps a large hydrophobic amino acid residue at position -1, whereas AP-3 binding can tolerate small, uncharged residues such as serine or alanine at this position. As predicted by the individual mutations, incorporation of both the E96D and P99S mutations together within the LL1 motif of OCA2-AA23N ablated the interaction with either AP-1 or AP-3 hemicomplexes (Figure 3.6a, b). Although each individual mutant retained the ability to bind to the AP-2 hemicomplex, the combined mutant also ablated this interaction (Figure 3.6b).

OCA2 Interaction with AP-3 is Required for Melanosomal Localization But Not Function
As we had isolated OCA2 constructs that showed contrasting preferences for AP-1 or AP-3 binding—E96D for AP-1 interaction and P99S or P99A for AP-3 interaction—we next tested the role of each complex in melanosomal trafficking of OCA2 by determining whether the changes in interactions with AP complexes affected localization of OCA2.
Figure 3.5. AP-3 is more tolerant than AP-1 of substitutions at the -1 position in the OCA2 LL1 sorting motif.
a) Schematic of OCA2-AA23N constructs containing a range of amino acid substitutions at the conserved proline of the LL1 sorting motif of human OCA2. b) Yeast three-hybrid analyses of the interaction of OCA2-AA23N proline substitution constructs with AP-3 or AP-1.
**Figure 3.6**

*a.*

```
OCA2-AA23   94  T E N P L L R N...
OCA2-AA23 E96DP99S  F D N T S L L R N...
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OCA2-AA23N  H2N-GAL4 BD  L-X-COOH  E73
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**b.**

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**c.**

**d.**

Figure 3.6. The E96DP99S combined dileucine mutant is nonfunctional.
(a) The OCA2 LL1 motif was subjected to two amino acid substitutions in the OCA2-AA23N yeast three-hybrid construct to create the OCA2-AA23N E96DP99S mutant. (b) Yeast three-hybrid results show that the E96DP99S construct does not interact with the three AP complexes. (c) Full-length OCA2-AA23 E96DP99S was transiently transfected into melan-Ink4a melanocytes. The transgene is visualized by anti-HA antibodies (magenta) and pigmented melanosomes are visualized by Bright field microscopy (inverted and colored green). Bar = 10 µm. (d) OCA2-AA23 E96DP99S was transiently transfected into melan-p1 melanocytes and assayed for rescue of melanin synthesis as in Figure 3.2j. The graph shows the results from three independent transfections.
Each sorting motif mutation was placed into full-length HA-tagged human OCA2 and transiently expressed in melan-Ink4a melanocytes. In these cells, most of the visualized OCA2-AA23 localizes to rings, and approximately 49% of these structures surround pigmented melanosomes (Figure 3.3, d-f; 3.7g). When transiently expressed in these cells, the AP-3-biased OCA2-AA23 P99S or P99A constructs consistently showed a significant amount of steady-state staining at the surface of the cell, reminiscent of the staining seen with OCA2-AA123, which contains no functional dileucine motifs (Figure 3.7, a-c). However, this construct also showed a variable ability to localize properly to rings, about 40% of which enclose pigmented structures (Figure 3.7g). The ability of the P99A and P99S constructs to form the HA-positive rings suggests that OCA2 interaction with AP-1 might not be absolutely required for trafficking. By contrast, the AP-1-biased OCA2-AA23 E96D construct localized to structures that formed partial coats around pigmented melanosomes (Figure 3.7, d-f). These structures showed a reduced amount of overlap with melanin, approximately 25%, suggesting that an ability to bind AP-3 rather than AP-1 might be necessary for a late step of melanosomal trafficking of OCA2. As expected for a mutant that was unable to bind to any of the adaptors, full length OCA2-AA23N bearing the double E96DP99S mutation localized to the cell surface when expressed in wild-type melan-Ink4a melanocytes (Figure 3.6c).

To confirm the role of AP-3 in melanosomal trafficking of OCA2, we also expressed the constructs in AP-3-deficient melan-pearl mouse melanocytes. These cells are significantly pigmented, but they exhibit mislocalization of a large cohort of tyrosinase (Theos et al., 2005). Melanosomal localization of Tyrp1 is largely intact, consistent with
**Figure 3.7.** Localization of dileucine motif point mutants in wild-type melanocytes.

a-i) IFM analysis of melan-Ink4a cells expressing selected mutant OCA2 variants. Melan-Ink4a melanocytes were transfected with OCA2-AA23 (a-c), OCA2-AA23 P99A (d-f), or OCA2-AA23 E96D (g-i). Transgenes were visualized with anti-HA antibodies (a, d, g) and melanosomes were visualized by bright field microscopy (b, e, h). c, f, and i are merged OCA2-HA (magenta) and inverted bright field (green) images. All insets show 5X magnified images of the boxed region. Arrows point to regions of overlap of
OCA2 constructs with melanosomes. Bar = 10 µm. (j) Deconvolved IFM images of each mutant were converted to binary images and analyzed for marker overlap. Cells transfected with OCA2-AA23 P99A or OCA2-AA23 P99S were combined in the analysis. Each data point represents one cell. Shown is the percentage of punctate/vesicular HA staining that overlapped with pigment in each cell (% of OCA2 with pigment). Each point mutant was compared to OCA2-AA23. n.s., not significant. ****, p < 0.0001
an interaction of Tyrp1 with AP-1 but not AP-3 in the yeast three-hybrid assay (Theos et al., 2005; Setty et al., 2007). In melan-pearl cells, transiently expressed OCA2-AA23 localized to punctate structures that were near or adjacent to pigmented melanosomes (Figure 3.8, a-c). About 25% of these structures overlapped with pigment (Figure 3.8j), a level similar to the overlap seen with the AP-1-biased E96D construct in wild-type cells. This result is consistent with a model in which AP-3 is necessary for melanosomal delivery of OCA2 from adjacent endosomal structures. Both the E96D and P99S constructs are also found on structures near pigmented melanosomes (Figure 3.8, d-i), and the amount of co-localization of either mutant with pigment was not statistically different from that of OCA2-AA23 (Figure 3.8j). Thus in all cases, the lack of AP-3 appeared to prevent efficient melanosomal delivery of OCA2.

We next tested whether changes in steady-state localization of the mutants affected function by utilizing the melan-p1 pigmentation rescue assay. Expression of OCA2-AA23 leads to rescue in 86% of transfected cells, while the expression of the surface-localized OCA2-AA123 and OCA2-AA23 E96DP99S mutants led to a minimal increase in pigmentation (Figure 3.8k and Figure 3.6d). The expression of the AP-3-biased P99S construct stimulated pigment synthesis as well as OCA2-AA23, despite the reduced efficiency of melanosomal delivery compared to OCA2-AA23. Since the E96D mutant is defective in melanosomal localization in wild-type cells, we expected it would be less able to rescue melanin synthesis in melan-p1 cells. Surprisingly, the AP-1-biased E96D was only slightly less efficient than OCA2-AA23. The high amount of rescue effected by the E96D construct suggests that OCA2 may in fact be able to function from within the
**Figure 3.8.** Localization of dileucine motif point mutants in AP-3-deficient melanocytes.

(a-i) IFM analysis of AP-3-deficient cells expressing selected mutant OCA2 variants.

Melan-pearl melanocytes were transfected with OCA2-AA23 (a-c), OCA2-AA23 P99S
(d-f), or OCA2-AA23 E96D (g-i). Transgenes were visualized with anti-HA antibodies (a, d, g) and melanosomes were visualized by bright field microscopy (b, e, h). c, f, and i are merged OCA2-HA (magenta) and inverted bright field (green) images. All insets show 5X magnified images of the boxed region. Bar = 10 µm. (j) Deconvolved IFM images of each mutant were converted to binary images and analyzed for marker overlap. Each data point represents one cell. Shown is the percentage of punctate-vesicular HA staining that overlapped with pigment in each cell (% of OCA2 with pigment). Neither point mutant was significantly different (n.s.) from OCA2-AA23. (k) OCA2-deficient melan-p1 melanocytes were rescued as in Figure 3.2j. All columns were compared to OCA2-AA23. n.s., not significant. *, p < 0.05, ***, p < 0.001
endosomal structures adjacent to melanosomes to which the construct localizes.

**OCA2 Trafficking to Melanosomes is Dependent on BLOC-1**

Finally, melanosomal trafficking of Tyr and Tyrp1 show very different levels of dependence on BLOC-1. In order to investigate the dependence of the melanosomal trafficking of OCA2 constructs on the BLOC-1 complex, we examined their localization following transient expression in BLOC-1-deficient melan-pallid mouse melanocytes. We previously showed that Tyrp1 does not localize to melanosomes in these cells but is instead mislocalized to vacuolar endosomes (Setty et al., 2007). In addition, there is increased steady-state staining of Tyrp1 at the cell surface thought to be due to a shift into a recycling loop as a result of the failure of BLOC-1-dependent siphoning towards melanosomes. About 50% of OCA2-AA23 staining in the melan-pallid cells colocalizes with internal Tyrp1 in melan-pallid cells (Figure 3.9, a-c; j), suggesting that melanosomal trafficking of OCA2 is similarly BLOC-1-dependent. The AP-1-biased E96D construct shows an equivalent amount of overlap with Tyrp1 (Figure 3.9, d-f; j). The AP-3-biased P99S construct, however, appears to be mislocalized to the cell surface to a much greater extent than Tyrp1 and consequently shows little overlap with the marker (Figure 3.9, g-i). A possible explanation may be a combination of increased delivery of OCA2 from endosomes to the surface in the same manner as Tyrp1 as well as potentially decreased endocytosis of the P99S construct that could be a consequence of its decreased interaction with AP-2 relative to the other OCA2 constructs. Interestingly, OCA2-AA23 hTyr, bearing the dileucine motif from human tyrosinase, is unable to traffic to melanosomes in melan-pallid melanocytes, as shown by a lack of colocalization with
Figure 3.9. Localization of dileucine motif point mutants in BLOC-1-deficient melanocytes.
(a-i) IFM analysis of BLOC-1-deficient cells expressing selected mutant OCA2 variants. Melan-pallid melanocytes were transfected with OCA2-AA23 (a-c), OCA2-AA23 E96D (d-f), or OCA2-AA23 P99S (g-i). Transgenes were visualized with anti-HA antibodies (a, d, g) and endogenous Tyrp1 was visualized in b, e, and h. c, f, and i are merged OCA2-HA (magenta) and Tyrp1 (green) images. All insets show 5X magnified images of the boxed region. Bar = 10 µm. (j) Deconvolved IFM images of OCA2-AA23 and OCA2-AA23 E96D were converted to binary images and analyzed for marker overlap. The P99S mutant was not analyzed. Each data point represents one cell. Shown is the percentage of punctate/vesicular HA staining that overlapped with Tyrp1 in each cell. (% of OCA2 with Tyrp1). E96D was not significantly different (n.s.) from OCA2-AA23.
PMEL, a marker of the unpigmented melanosomes in these cells (Figure 3.10). This result suggests that the transplantable dileucine motif from tyrosinase can confer AP-interacting ability but not BLOC-1-independence on OCA2. Together, our data suggest a model for melanosomal trafficking of OCA2 that is dependent on both AP-3 and BLOC-1, complexes that were previously hypothesized to mediate distinct melanosomal trafficking pathways.

3.4 Discussion

Based on the behaviour of model cargo proteins studied to date, two routes have been proposed to regulate cargo delivery from endosomes towards maturing melanosomes in melanocytic cells: one mediated by AP-3 and utilized largely by Tyr (Huizing et al., 2001; Theos et al., 2005), and the other mediated by AP-1 and BLOC-1 and utilized by Tyrp1 (Setty et al., 2007; Delevoye et al., 2009), the copper transporter ATP7A (Setty et al., 2008), and perhaps a cohort of Tyr (Theos et al., 2005). By analyzing the behavior of OCA2 and targeting signal mutants that bind selectively to AP-1 or AP-3, we now extend this model by providing evidence for a pathway that requires both BLOC-1 and AP-3. Our data confirm previous findings that OCA2 can bind to both AP-1 and AP-3 via a critical acidic dileucine sorting motif, but show for the first time that the identity of particular residues within the core motif--but not the placement of the motif within the cytoplasmic domain--underlie binding specificity for each adaptor. The distinct intracellular localization of mutants that interact by Y3H assay preferentially with either AP-1 or AP-3 provide evidence that AP-3 mediates a final step in the delivery of OCA2 to melanosomes, highlighting an expanded role for this complex within the scope of LRO
Figure 3.10

Figure 3.10. The dileucine motif does not determine BLOC-1 dependence.

The OCA2-AA23 hTyr construct was transiently transfected into BLOC-1-deficient melan-pallid melanocytes. The transgene was visualized with anti-HA antibodies (a) and hypopigmented melanosomes were visualized by antibodies to PMEL (b). OCA2 (magenta) and PMEL (green) images were merged in (c). All insets show 5X magnified images of the boxed region. Bar = 10 µm.
biogenesis.

The AP complexes are well known for their role in coupling clathrin coat recruitment to cargo selection via recognition of cytoplasmic sorting motifs and consequent cargo clustering into buds for directed vesicular trafficking within the endosomal system (Bonifacino and Traub, 2003; Robinson, 2004). The cytoplasmic domains of a number of melanosomal proteins, including Tyr and Tyrp1, contain acidic dileucine-based sorting signals that have been shown to be necessary for melanosomal transport (Vijayasaradhi et al., 1995; Calvo et al., 1999; Simmen et al., 1999). Human OCA2 has three consensus acidic dileucine-based motifs, but only the most distal motif, LL1, confers melanosomal localization and full function (Sitaram et al., 2009). Unlike acidic dileucine signals in other melanosomal and lysosomal proteins, which tend to be positioned within 6-11 residues of the transmembrane domain, the LL1 motif of human OCA2 is located 70 amino acid residues from the transmembrane domain. Motifs at such a distance tend to be limited for use in internalization rather than targeting to lysosomes or LROs (reviewed in (Bonifacino and Traub, 2003)), as seems to be the case for an acidic dileucine signal in the distal cytoplasmic domain of PMEL (Lepage and Lapointe, 2006; Theos et al., 2006a; Robila et al., 2008), but in this case it is likely that protein:protein or protein:lipid interactions bring the motif close enough to the membrane to engage AP-1 and AP-3. Nevertheless, the data presented here demonstrate that the LL1 signal functions in a position-independent manner – at least within the membrane proximal 100 amino acid residues of OCA2 - and is capable of interacting with AP complexes and effecting melanosome targeting when moved to sites harboring inactive LL2 and LL3 motifs. We
conclude that the primary determinant of acidic dileucine-based targeting activity is ultimately a property of its sequence rather than its context. In this regard, the LL1 sequence is different from other acidic dileucine motifs such as a CD4/CD3-gamma chimera (Geisler et al., 1998), the distal motif of lip31 (Pond et al., 1995), and the motif of Vam3p (Darsow et al., 1998), all of which were affected by the residue immediately upstream of the acidic residue. It may be that OCA2 folds in such a way that only the core motif is presented in the correct orientation for AP binding, without the engagement of nearby residues. Interestingly the LL1 sequence is exactly conserved in nearly all of the predicted mammalian homologs of OCA2, with the exception of mouse and rat, suggesting that the sequence may have evolved to optimize function.

While the adjacent residues do not seem to be important for LL1 interaction with AP complexes, the so-called X residues within the core [D/E]XXXL[L/I] motif are. In our mutagenic study with the human tyrosinase motif and the similar but nonfunctional OCA2 LL3 motif, the mutation of the -2 Gln in the Tyr motif to the Gly of the LL3 motif (OCA2-AA23 hTyrQG) completely abolished the interaction with all three AP complexes and caused mislocalization of the full-length protein to the cell surface, despite the fact that this motif still conforms to the EXXPLL consensus that seems to be a hallmark of melanosomal sorting signals. Since the motifs were all placed in the same position within the OCA2 domain, the difference in AP interaction between the Q-motif and the G-motif must reflect differences in the direct interaction between the core motif and the AP complexes. Our experiments with the Tyr motif and LL3 show that the binding of the OCA2 acidic dileucine motifs to AP complexes can be extraordinarily
sensitive to the identity of the X residues of the motif. There is not yet a crystal structure
of an acidic dileucine motif in complex with AP-1 or AP-3, but there is one for AP-2 with
the motif from CD4 (Kelly et al., 2008). The side chains for residues at the -2 and -3
positions of the motif point into the solvent in vitro and thus do not contribute
significantly to binding affinity, in stark contrast to the large effect on AP binding that we
observed upon mutation in the -2 position. AP-2 is used for endocytosis of a wide variety
of proteins from the cell surface and a less rigid binding orientation is thought to
contribute to its broad specificity as a cargo adaptor (Kelly et al., 2008). It may be that
AP-1 and AP-3 have more rigid binding surfaces that are more sensitive to the identities
of the X residues than AP-2.

The E to D mutation in OCA2 greatly reduced but did not eliminate AP-1 interaction and
had less of an effect on AP-2 interaction, consistent with mutagenesis results with the
distal acidic dileucine motif of LRP9 (Doray et al., 2007). Our data additionally show
that the conservative E to D mutation eliminates AP-3 interaction. Acidic residues
upstream of the dileucine pair are known to be necessary for AP-3 binding to LIMP-II
(Honing et al., 1998) and Vam3p (Darsow et al., 1998), but to our knowledge this study
represents the first report of specificity for the identity of the acidic residue. In contrast
to the E to D mutant, the P to A/S mutants of OCA2 eliminated AP-1 interaction and
greatly reduced AP-2 interaction, but maintained a significant level of AP-3 interaction.
Proline has been specifically noted as a common -1 residue in several acidic dileucine
motifs (Pond et al., 1995; Darsow et al., 1998; Honing et al., 1998; Doray et al., 2007).
Consistent with the importance of the -1 Pro for AP-1 and AP-3 interaction, mutagenesis
of a -1 Pro in the distal motif of LRP9 eliminates binding to AP-1 and severely reduces its interaction with AP-2, and CI-MPR can acquire the ability to interact with AP-1 and AP-2 from a heterologous motif that includes a -1 Pro (Doray et al., 2008). The -1 Pro does not seem to be required for AP-3 interaction since the same position could accommodate alanine (with a small hydrophobic side chain), serine (polar uncharged side chain), and phenylalanine (bulky hydrophobic side chain) in our yeast three-hybrid assay. However, neither OCA2 LL1 (Figure 3.5) nor the OCA2-AA23 hTyrPD chimera (Figure 3.3) was able to tolerate an acidic residue (asparatate) in this position. In the AP-2 crystal structure the -4 acidic residue binds in a pocket that has some flexibility, the two leucine residues bind in a separate hydrophobic pocket, and the -1 position protrudes into the solvent. It is again tempting to speculate that the differential requirements for the identity of the acidic residue or the -1 Pro will be reflected in structural differences between AP-1 and AP-3 dileucine motif-interacting surfaces.

Although the E96D and P99S mutants show opposite preferences for AP interaction, full-length OCA2 bearing either mutation was found to localize to post-endosomal structures and to confer nearly complete function to OCA2-deficient melanocytes. This indicates that either AP-1 or AP-3 alone is sufficient to direct proteins into the endosomal system and into endosomal domains bound for melanosomes. However, steady-state melanosomal localization of OCA2 seemed to critically depend on an OCA2/AP-3 interaction, because (a) the AP-1-biased E96D mutant localized to endosomal structures closely apposed to melanosomes rather than properly to melanosomal “rings” in wild-type melanocytes, and (b) no construct expressed in AP-3-deficient melanocytes
localized properly to melanosomes. The localization of the E96D mutant to endosomal tubules that are physically continuous with melanosomes in wild-type cells and the high amount of melanin synthesis induced by this construct in OCA2-deficient cells raise the intriguing possibility that melanosomal localization of OCA2 may not be absolutely required for function. OCA2 is thought to function as a transporter, and thus mislocalization of OCA2 itself might be overcome by diffusion of its putative substrate into melanosomes via passage through the tubules. Such a model would still be consistent with studies that suggested the protein localized to the melanosome membrane on the basis of subcellular fractionation, low-resolution immunofluorescence microscopy, and effects on melanosomal luminal pH (Rosemblat et al., 1994; Donatien and Orlow, 1995; Puri et al., 2000; Sitaram et al., 2009) and still disfavor an alternate proposal that the protein functions from the endoplasmic reticulum (Chen et al., 2002; Toyofuku et al., 2002).

The trafficking of OCA2 differs from that of Tyrp1 in that it is AP-3 dependent. However, like Tyrp1 and unlike Tyr, all OCA2 constructs were mislocalized in BLOC-1-deficient melanocytes; indeed, OCA2 in these cells largely overlapped with Tyrp1, which becomes entrapped primarily in early endosomal vacuoles (Setty et al., 2007). This dependence on BLOC-1 differentiates OCA2 trafficking from that of Tyr, for which a substantial cohort can be found in melanosomes in BLOC-1-deficient cells (Setty et al., 2007; Setty et al., 2008). Thus we have identified a new mode of endosome-to-melanosome trafficking that is both BLOC-1-dependent and AP-3-dependent. This
pathway provides a critical biological context in a LRO-generating cell type for a previously described physical interaction between AP-3 and BLOC-1 (Di Pietro et al., 2006; Salazar et al., 2006). Whether AP-3 functions independently of BLOC-1 – as for tyrosinase – or dependent on BLOC-1 – as for OCA2 – likely reflects some feature of the cargo. The failure of OCA2-AA23 hTyr, bearing the sorting motif of the largely BLOC-1-independent tyrosinase, to localize to immature melanosomes in BLOC-1-deficient melanocytes suggests that the dileucine motif is not the determinant of the BLOC-1-dependence of a cargo. The identity of that feature will have to await further experimentation. We speculate that the 12 membrane spanning domains might preclude entry of OCA2 into early sorting endosomal domains from which tyrosinase accesses AP-3 for the BLOC-1-independent pathway (Peden et al., 2004; Theos et al., 2005).

The unusual structures to which OCA2 localized in the absence of AP-3 or a strong AP-3 binding signal were adjacent to and partially overlapping with melanin. This pattern of localization resembles that of AP-1 and recycling endosomal markers in a human pigmented melanoma cell line, and correlates with tubular transport intermediates that physically connect recycling endosomal domains with melanosomes (Delevoye et al., 2009). This similarity thus suggests that OCA2 accumulates in such tubular extensions when it is capable of binding to AP-1 but not to AP-3. By contrast, deficiency in either AP-1 or BLOC-1 prevents tubular localization of Tyrp1 and instead causes its mislocalization to vacuolar domains of recycling endosomes (Setty et al., 2007; Delevoye et al., 2009). Based on these results, we proposed a model in which AP-1 and BLOC-1 work together to specify Tyrp1 entry into these tubules. Our results here suggest that
OCA2 might also enter these tubules in a manner that is similarly dependent on BLOC-1, but the successful delivery of the AP-3-biased P99S mutant to melanosomes in wild-type cells suggests that entry of OCA2 into the tubules might not require direct interaction with AP-1 and could instead be regulated by cooperation between BLOC-1 and AP-3. This interpretation would align our previous model of distinct BLOC-1-mediated and AP-3-mediated melanosomal trafficking pathways with observations in neurons that suggest that BLOC-1 and AP-3 work together in cargo distribution (Newell-Litwa et al., 2009; Salazar et al., 2009).

3.5 Acknowledgments

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CHAPTER 4. DISCUSSION

Prior to the start of this thesis, the localization of OCA2 was unclear, and the trafficking of the protein had not been explored at all. My results have resolved to a large extent the question of the site of action of OCA2 and defined important sorting determinants that control trafficking of the protein. The protein also has proved useful as a model melanosomal cargo and revealed new mechanisms for the trafficking of proteins from endosomes to melanosomes, expanding our understanding of trafficking to these unique organelles. Below I discuss the implications of some of these findings in more detail.

4.1 Sorting signals in OCA2

4.1.1 The LL1 motif

Although many of the initial reports characterizing OCA2 function suggested that the protein localizes to melanosomes (Rosemblat et al., 1994; Donatien and Orlow, 1995; Puri et al., 2000; Toyofuku et al., 2002), a competing hypothesis that OCA2 might function from within the ER introduced a legitimate controversy within the field (Chen et al., 2002). Previously published immunofluorescence microscopy and subcellular fractionation analyses were inconclusive and could have been used to support either interpretation (Rosemblat et al., 1994; Chen et al., 2002; Toyofuku et al., 2002). My results presented in Chapter 2 demonstrate that although OCA2 can be detected primarily in the ER of a substantial fraction of melanocytes, the steady-state localization of OCA2 to the ER is not compatible with its function in promoting melanin synthesis. Instead, melanin synthesis induction correlated with localization of the protein to mature
melanosomes. As expected for a melanosomal protein, this localization in turn depended on intact acidic dileucine motifs that serve as binding sites for the AP complexes. Of the three dileucine motifs found within OCA2, I found that the first motif alone is both necessary and sufficient for steady-state localization of OCA2 to melanosomes. This finding correlates well with the observation that the primary sequence of this motif—ENTPLL—is nearly invariant among the predicted homologs of OCA2 in placental mammals, the mouse and rat being notable exceptions. A less-restricted EXXPLL consensus expands the conservation to nearly all the vertebrates, confirming the importance of the LL1 motif. However, the role of acidic dileucine motifs in the trafficking of OCA2 is more complex. For example, an LL3 EXXXLL motif, which is neither necessary nor sufficient for melanosomal trafficking or function of human OCA2, is conserved among the majority of predicted OCA2 homologs, so simple conservation is not enough to determine which motifs are most critical.

We noted during the domain swap assays that the LL1 motif of human OCA2 is located 70 amino acid residues from the transmembrane domain, much longer in primary sequence from the 6-11 residue distance that is more typical for lysosomal or LRO targeting motifs (Bonifacino and Traub, 2003). We hypothesized that protein:protein or protein:lipid interactions might bring the motif close enough to the membrane to engage AP-1 and AP-3. This is consistent with our earlier study, in which an ER retention motif added to the N-terminus of OCA2 functioned more efficiently when the first 91 amino acids of OCA2 were first removed, bringing the signal very close to the LL1 motif (Sitaram et al., 2009). I hypothesized that the OCA2 cytoplasmic domain might adopt a
secondary structure that facilitates an interaction of that region with membrane-proximal
retention machinery. However, the domain swapping experiments demonstrated that the
LL1 signal functions in a position-independent manner – at least within the context of the
first 100 residues of OCA2 - and is capable of interacting with AP complexes and
effecting melanosome targeting when moved to sites previously harboring inactive LL2
and LL3 motifs.

Since acidic dileucine motif position within the cytoplasmic domain was not critical for
AP interaction, it follows that AP complex binding to the OCA2 LL1 sequence may be
relatively independent of contributions from residues outside the core motif, a property
that differs from some other acidic dileucine motifs. The addition of a basic arginine
immediately upstream of the acidic residue in a CD4/CD3-gamma chimera reduced the
internalization and degradation of the protein (Geisler et al., 1998). An asparagine
upstream of the distal motif of mouse invariant chain Iip31 was important for proper
internalization kinetics (Pond et al., 1995), and an asparagine upstream of the Vam3p
sorting motif was identified in a mutant screen as a residue that was important for
interaction with AP-3 (Darsow et al., 1998). The interaction was disrupted when the
asparagine was mutated to the hydrophobic isoleucine. The residues upstream of the
acidic residue in the OCA2 LL1 and LL3 positions--threonine and serine, respectively--
are also polar uncharged residues like asparagine. However, the residue upstream of the
LL2 position is hydrophobic tryptophan, and an OCA2 construct with the LL1 sequence
in that position (OCA2-AA1 LL2-1) can still interact with AP-3 and localize properly to
the melanosome membrane, showing that an upstream polar residue is not necessary for
LL1 interaction with AP complexes (though the tryptophan itself may be special, as discussed below). In another instance, AP-1 and AP-2 could not bind to fusion proteins consisting of GST fused to the cytoplasmic domains of LRP3 and LRP12 in which their nonfunctional acidic dileucine motifs were mutagenized to the sequence found in the functional AP-interacting motif from LRP9 (Doray et al., 2008). The authors speculated that amino acids specific to the parent proteins and outside of the core motif might have interfered with AP binding. In our mutagenic study with the human tyrosinase motif and the nonfunctional OCA2 LL3 motif, we saw that a single point mutation in an X residue was able to inactivate the tyrosinase motif embedded in OCA2, despite still conforming to the EXXPLL melanosomal sub-consensus. Since the surrounding residues were the same between the two different motifs, we can conclude that the difference in binding stems from differences in the ability of the AP complexes to engage the core motif itself. The experiment also demonstrates the importance of the so-called X residues. The only crystal structure available of an AP complex bound to an acidic dileucine motif is that of AP-2 with an acidic dileucine-containing peptide from CD4 (Kelly et al., 2008). There was no interaction between the side chains of the -2 and -3 amino acids and the AP-2 complex, but the authors of that study hypothesized that their contribution to AP interaction might be apparent in vivo (Kelly et al., 2008). The -3 position is occupied by polar Asn in the LL1 motif of human OCA2, acidic Asp in the motif from mouse OCA2, and basic Lys or Arg in the motif from predicted OCA2 homologs of birds, amphibians, reptiles, and fish, suggesting that this position may indeed be relatively free. The -2 position is occupied by Thr in the majority of OCA2 homologs, and by Ser in the rest, while in Tyr and Tyrp1 from human and mouse the position holds Gln, suggesting a need
for a hydrophilic amino acid at this position. Interactions of the AP complex with other peptide residues surrounding the motif in the crystal were not described.

The effect of the E to D mutation in LL1 on AP-1 and AP-2 interaction was consistent with different mutagenesis assays of other proteins such as the acidic dileucine motif of LRP9 (Doray et al., 2007). A similar E to D mutation in Iip31 (Pond et al., 1995) and CD4/CD3-gamma chimeras (Geisler et al., 1998) had no effect on internalization, consistent with a more minimal effect on AP-2 interaction. Whether this position requires an acidic residue at all seems to depend on the individual motif; substitution by alanine at this position within the endogenous Iip31 motif or a transferred CI-M6PR motif within an Iip31 chimeric protein disrupted internalization (Pond et al., 1995), and a similar E to A mutation within the CD3-gamma motif impairs internalization of a CD4/CD3-gamma chimera (Geisler et al., 1998), but internalization of LIMP-II, Nef, or native CD3-gamma was not impaired by a similar mutation (Letourneur and Klausner, 1992; Pond et al., 1995; Bresnahan et al., 1998; Sandoval et al., 2000). Although the E to A mutation in the CD3 gamma motif in an Iip31/CD3-gamma chimera (Pond et al., 1995) or in LIMP-II (Sandoval et al., 2000) did not disrupt internalization, these mutants failed to reach their proper compartments within the endosomal system. This suggested that loss of the acidic residue affected another sorting pathway such as AP-1-mediated sorting at the TGN (Sandoval et al., 2000). Our data show that the conservative E to D mutation also eliminates interaction with AP-3. Though the importance of a -4 acidic residue for AP-3 interaction has been previously described (Darsow et al., 1998; Honing et al., 1998), to our knowledge ours is the first report of specificity for the acidic residue.
Unlike the E→D mutant, the P→A/S mutants of the OCA2 LL1 motif eliminated the interaction with AP-1 interaction and greatly reduced the interaction with AP-2, but only modestly diminished or maintained interaction with AP-3. Proline has been specifically noted as a common -1 residue in acidic dileucine motifs, including in Vam3p (Darsow et al., 1998), LIMP-II (Honing et al., 1998), Iip31 (Pond et al., 1995), and LRP9 (Doray et al., 2007). Consistent with our Y3H results, mutation of the -1 Pro to an alanine eliminated binding of a GST fusion of LRP9 to recombinant AP-1 and severely reduced interaction with recombinant AP-2; binding to AP-3 was not tested (Doray et al., 2008). Although a cohort of the OCA2 LL1 proline mutants localize effectively to melanosomes, they were also consistently enriched at the cell surface. The enriched surface accumulation of these mutants is likely a consequence of the weakened interaction with AP-2 for a cohort of OCA2 that accesses the plasma membrane either through normal biosynthetic traffic like PMEL (Theos et al., 2006a) or post-melanosomal fusion with the plasma membrane like Tyrp1 (Truschel et al., 2009). This hypothesis is supported by observations that Ala substitution for the -1 Pro halves the internalization rate of Iip31 bearing either its native motif or the LIMP-II motif (Pond et al., 1995). An alternative explanation for the increased surface localization of the Pro mutants may be that the loss of AP-1 interaction with OCA2 mimics the effect of the genetic loss of BLOC-1 on Tyrp1 trafficking (Setty et al., 2007) or of AP-3 on LAMP1 trafficking (Peden et al., 2004) in enhancing biosynthetic and endosomal recycling to the plasma membrane as a consequence of the failure to divert the cargo towards melanosomes/lysosomes. This hypothesis is consistent with the idea that AP-1 and
BLOC-1 work together at an early step of melanosomal diversion of cargo proteins. The importance of the -1 Pro for binding to AP-1 and AP-2 is supported by surface plasmon resonance assays of GST-fusions of Ii showing a 70-80% reduction in binding to AP-1 or AP-2-enriched cell fractions when the Pro was mutated (Kongsvik et al., 2002). Furthermore, the mutagenized GGA-binding SDEDLL motif of CI-MPR acquired AP-1 and AP-2 binding when altered to EDEPLL (found naturally in LRP9), but not when altered to EDEDLL lacking the P\(^{-1}\) (Doray et al., 2008). Unlike AP-1 and AP-2, the -1 Pro does not seem to be required for AP-3 interaction since the same position could accommodate alanine, serine, or phenylalanine (but not aspartate) in our Y3H assay. That this position in the OCA2 motif could be more flexible for AP-3 interaction is supported by the fact that a mutagenesis screen in the Vam3p cytoplasmic domain identified every residue in the acidic dileucine sorting motif as being critical for AP-3 interaction except for the -1 Pro (Darsow et al., 1998).

4.1.2 The LL2 motif

The LL1 motif is not the only motif in OCA2 that confers sorting information to OCA2. The OCA2-AA1 mutant, bearing an intact LL2 motif in its endogenous position in combination with an inactivated LL1 motif, localizes at steady state to lysosomes in melanocytes yet is still competent to restore pigment synthesis in a substantial and statistically significant fraction of OCA2-deficient melan-p1 cells (Sitaram et al., 2009). We hypothesized that OCA2-AA1 passes transiently through melanosomes and continues trafficking towards lysosomes by virtue of a weak interaction between the intact LL2 motif and AP-1, as demonstrated in the GST pulldown assays. However, we could not
confirm this interaction by Y3H assay. We also noted that the OCA2 constructs that localize at steady state to the melanosome had in common strong binding to AP-3, in contrast to the mislocalized OCA2-AA1 mutant with the weak AP-1-binding ability. This differing ability of AP-1 and AP-3 to determine the steady-state localization foreshadowed the results of the subsequent experiments described in Chapter 3.

In Chapter 3, we extended this investigation into acidic dileucine-mediated trafficking of OCA2, ultimately analyzing the \textit{in vivo} behavior of the AP-1-biased E96D construct and the AP-3-biased P99A and P99S constructs. Analysis of the trafficking of these OCA2 mutants in wild-type melanocytes indicated that the E96D construct could localize to structures that abut melanosomes and that likely represent the recycling endosome-derived tubules that tether endosomes to melanosomes. As with the OCA2-AA1 mutant, the E96D variant rescued pigmentation in a substantial fraction of melan-p1 cells without localizing at steady state to melanosomes.

Why do the OCA2-AA1 and E96D mutants have differing steady-state localizations if both exhibit weak binding to AP-1 and negligible binding to AP-3? We cannot exclude the possibility that OCA2 interacts with other \textit{trans}-acting trafficking determinants besides the AP complexes. In particular, we noted that the steady-state lysosomal sorting activity of LL2 is masked when the stronger LL1 melanosomal sorting signal is intact. The glutamate of the LL2 motif is preceded by a tryptophan residue and thus may act as an additional sorting signal analogous to the WE motif in the cytoplasmic domain of the melanocytic G protein-coupled receptor GPR143; this signal has been shown to be
sufficient to sort chimeric proteins to vesicular structures in a human melanoma cell line, although the factors that recognize this signal are not known (Piccirillo et al., 2006). As before, this sorting activity would be masked in the E96D mutant, in which the stronger LL1 signal is altered but still functional and directs steady-state localization to the endosomal tubules. The lack of the upstream tryptophan may also explain the apparent position-dependence of the LL2 sequence, which can facilitate lysosomal localization when in its endogenous position in OCA2-AA1, but is inactive when placed in the LL1 position in the OCA2-AA1 LL1-2 mutant. This position-dependence stands in contrast to the position-independence of the LL1 sequence, which functions in both the LL2 position and the LL3 position. Further support for the importance of the WE sequence stems from the fact that a WEXXXLL motif is conserved in the LL2 position among nearly all sequenced or predicted mammalian OCA2 homologs. (In mouse and rat the sequence is WEXXXML, but a Met could substitute for Leu in this position, as seen in human Lip31). Characterization of tryptophan substitution mutants should help resolve whether OCA2 utilizes this additional sorting mechanism.

4.2. OCA2 melanization-promoting function within the endocytic system

Despite the differences observed in the steady-state localization of the OCA2-AA1 and E96D mutants, the differences in their ability to stimulate melanin synthesis in OCA2-deficient melan-p1 cells were not statistically significant (data not shown), whereas OCA2 lacking any endosomal sorting signals – such as in the AA123 or E96DP99S mutants – were completely non-functional in “rescuing” OCA2 deficiency. Taken together, the results suggest that OCA2 function may be generally required within the
endocytic system if not at the melanosome itself. One possible explanation for this result might be connectivity between the recycling endosomal domains and melanosomes, which have been observed by electron tomography and by live cell imaging (Delevoye et al., 2009); unpublished results, A. Mantegazza and M. E. Dennis). Because endosomal tubules physically connect the lumenal environment of endosomes and melanosomes, the passage of active OCA2 through the endocytic system may allow for its putative substrate to access endosomes and diffuse into melanosomes despite its absence from the melanosomal membrane. Alternatively, the steady state distribution might reflect a transient presence within melanosomes, which might provide ample time to concentrate the putative substrate within melanosomes directly.

A final alternative might be that the transport activity of OCA2 might be required within the transport intermediates themselves, or within endosomes to generate the transport intermediates. Could the endosomal tubules be the normal site of action for OCA2? The indirect immunofluorescence assays (and unpublished immunoelectron microscopy) that we have performed showing steady-state melanosomal localization of OCA2 were all conducted in cells that were transiently overexpressing an epitope-tagged form of human OCA2. Moreover, two proteomics studies of melanosome-enriched fractions from a pigmented human melanoma cell line have not identified OCA2 (Basrur et al., 2003; Chi et al., 2006). It is not clear whether this reflects a lack of melanosomal localization or if the endogenous levels of OCA2 are too low to detect. Consistent with the latter possibility, our antibody directed against a human OCA2 peptide can immunoprecipitate the endogenous protein and can be used to detect the transiently overexpressed OCA2 by
immunofluorescence microscopy in these cells but cannot detect the endogenous protein by immunofluorescence microscopy (Sitaram et al., 2009). It may be that the melanosomal localization in overexpressing wild-type cells represents spillover of extra protein from the endosomal localization into the melanosomes, a possibility that is supported by the behavior of a putative platelet dense granule protein when transiently expressed in melanocytes (A. Sitaram, R. Meng and K. Skinner, unpublished data). Since OCA2 is not detected on melanosomes in AP-3-deficient melanocytes or in wild-type cells that express the E96D mutant, such spillover would necessarily be facilitated by interaction with AP-3. Ultimately, this question will be difficult to address without a means of detecting endogenous OCA2.

4.3 Expansion of the endosome-to-melanosome trafficking model

4.3.1 The addition of the BLOC-1- and AP-3-dependent pathway

What do the studies of OCA2 localization tell us about cargo sorting from endosomes to melanosomes? Our model to date was based on studies of the trafficking of Tyr, Tyrp1, and ATP7A in cells lacking the HPS-associated trafficking complexes AP-3 or BLOC-1. The model posited one pathway of melanosomal trafficking from endosomes that relies on AP-3 and is used by a large cohort of Tyr, while a second pathway used by a small cohort of Tyr and the majority of Tyrp1 (and ATP7A) depends on BLOC-1. Since AP-1 deficiency phenocopies the BLOC-1-deficiency in terms of Tyrp1 localization, it had also been proposed that BLOC-1 and AP-1 work together to promote Tyrp1 recruitment to the recycling endosomal tubules that physically connect to melanosomes. Since Tyrp1 and ATP7A are physically associated and could potentially thus traffic in concert (Setty et al.,
2008), this model could be considered to be based on only two independent cargoes and thus had the potential to substantially benefit from data contributed by the investigation of the trafficking of an additional cargo. Our studies with OCA2 demonstrated its value as an additional model cargo and surprisingly revealed a trafficking “phenotype” that incorporates features of both Tyr and Tyrp1/ATP7A (Figure 4.1). OCA2 binds both AP-1 and AP-3 but appears to depend heavily on AP-3 for steady-state melanosomal localization like Tyr, but OCA2 is largely mislocalized in BLOC-1-deficient melanocytes like Tyrp1/ATP7A. Thus, OCA2 is trafficked in a manner distinct from that of Tyr, Tyrp1, and ATP7A.

4.3.2 Cargo selection and entry into endosomal tubules

As discussed in the Introduction, the molecular function of BLOC-1 is not known. What is clear is that BLOC-1-deficiency causes a buildup of Tyrp1 in vacuolar endosomal domains and a dramatic increase in the flux of Tyrp1 through the endocytic recycling pathway to the cell surface (Setty et al., 2007). Transferrin-positive tubules that are depleted of Tyrp1 are detectable emanating from endosomes in wild-type melanocytes as well as in BLOC-1-deficient melanocytes, suggesting that BLOC-1 activity primarily directs recruitment of cargo and/or effectors into the tubules, although since tubules also mediate recycling to the plasma membrane and the Golgi, the destination of the tubules observed in BLOC-1-deficient cells is not clear and thus a role for BLOC-1 in the formation or stabilization of melanosome-bound tubules cannot be ruled out.
**Figure 4.1.** New model of endosome to melanosome trafficking.

Previous research suggested a model of endosome to melanosome trafficking with two independent pathways, one mediated by AP-3 and used by the majority of tyrosinase, and one mediated by BLOC-1 in conjunction with AP-1 and used by Tyrp1. The research presented here suggest that OCA2 utilizes a distinct pathway that is both BLOC-1- and AP-3-dependent.
The similarity of the AP-1 knockdown and BLOC-1-deficiency phenotypes on Tyrp1 trafficking suggest that BLOC-1 and AP-1 functionally cooperate in cargo recruitment to the endosomal tubules (Setty et al., 2007; Delevoye et al., 2009). Localization of OCA2 has not been investigated in melanocytes in which AP-1 has been knocked down, but in wild-type cells, the OCA2 proline mutants, deficient in AP-1 binding but competent to bind AP-3, are still able to traffic to melanosomes, albeit less efficiently. This suggests that BLOC-1 can additionally work with AP-3 to facilitate cargo entry into the endosomal tubules (Figure 4.2). The role of the AP complex in BLOC-1 cargo selection might be in the regulation of BLOC-1 membrane recruitment. The percentage of BLOC-1 that cofractionates with membranes relative to cytosol from mouse skin fibroblasts is reduced when AP-3 is genetically deficient (Di Pietro et al., 2006). Furthermore, AP-3 and BLOC-1 interactions appear to be regulated by cargo. For example, AP-3 and BLOC-1 can be co-immunoprecipitated with epitope-tagged PI4KIIalpha, an AP-3 cargo protein, from HEK293T cells in which the cargo has been transiently expressed (Salazar et al., 2009), but coprecipitation of both is reduced in cells that express a PI4KIIalpha with a mutation in its dileucine motif; this suggests that BLOC-1 recruitment requires an AP-3/PI4KIIalpha interaction. An alternative explanation for this result is that both BLOC-1 and AP-3 interact simultaneously with cargo proteins via the dileucine motif, but it is difficult to imagine how two complexes could simultaneously engage such a small peptide, especially considering the tight fit of the acidic and leucine residues into pockets within the AP complex. Moreover, BLOC-1 has never been shown to bind to the cytoplasmic domains of any of its cargoes. Finally, the OCA2 LL1 signal, like the acidic
Figure 4.2 Model of endosome-to-melanosome trafficking of OCA2.

Transferrin-positive tubules emanating from recycling endosomal domains making physical connections with melanosomes have been visualized in a human melanoma cell line. Tyrp1 has also been visualized on AP-1 coated endosomal tubules, and deficiency in BLOC-1 or AP-1 causes mislocalization of Tyrp1 to vacuolar endosomal domains, suggesting that BLOC-1 and AP-1 together mediate Tyrp1 entry into tubules. Our results from studies of OCA2 trafficking in mouse melanocytes are consistent with a model in which entry of OCA2 (blue) into tubules is also BLOC-1-dependent. Final delivery of OCA2 to melanosomes requires interaction with AP-3, predicted to take place in AP-3-coated buds analogous to the AP-1-coated buds that are proposed to traffic Tyrp1. (Modified from (Delevoye et al., 2009))
dileucine-based signal of tyrosinase (Theos et al., 2005), interacts with both AP-1 and AP-3, but unlike tyrosinase, OCA2 is highly dependent on BLOC-1 for its trafficking. This reliance on BLOC-1 is maintained even when the LL1 signal on OCA2 is replaced by that of tyrosinase, as indicated by the lack of overlap of OCA2-AA23 hTyr with PMEL-containing melanosomes in BLOC-1-deficient cells. This indicates that the sorting signal itself is not sufficient to determine whether or not the cargo uses a BLOC-1-dependent or -independent pathway.

Other determinants must distinguish the choice of whether to utilize the BLOC-1 pathway (Figure 4.1). I can only speculate on the identity of such determinants, but one possibility is suggested by the exclusion of transferrin from melanosomes during the formation of transient, tubular connections between recycling endosomes and melanosomes. Transferrin remains associated with transferrin receptor—a dimeric, single transmembrane domain containing type II integral membrane protein—throughout the endocytic system (Dautry-Varsat et al., 1983). One might speculate that a “gating” mechanism that prevents transferrin/transferrin receptor accumulation in the melanosome is more generally used to prevent similarly composed integral membrane proteins from indiscriminately diffusing into the melanosome. Such a gate might function by excluding proteins of a certain size, membrane spanning domain content, or lipid microdomain. OCA2 is a 12-transmembrane domain protein and is therefore much larger than tyrosinase, which is a single-pass protein like transferrin receptor. Although Tyrp1
traverses the tubules and is approximately the same size as tyrosinase, previously published (Setty et al., 2008) and unpublished evidence suggests that Tyrp1 traffics to melanosomes together with ATP7A; indeed, ATP7A depletion results in the mistargeting of Tyrp1 to lysosomes and in its degradation (unpublished results). ATP7A is a P-type ATPase with 8 membrane-spanning domains (Lutsenko et al., 2007), and thus might have properties more in common with OCA2. Finally, when the OCA2 N-terminal cytoplasmic domain was fused to the transmembrane and luminal domains of human TfR and expressed in non-melanocytic Chinese hamster ovary cells, the chimera was able to localize to lysosomes in a dileucine motif-dependent manner (Sitaram et al., 2009), as expected for a melanosomal protein expressed in a non-melanocytic cell. However, in melanocytes the chimera localized to an unidentified intracellular compartment that did not colocalize with pigment, perhaps falling prey to the same mechanism that prevents melanosomal accumulation of native transferrin receptor. These examples suggest the existence of a gating mechanism through the BLOC-1-dependent pathway that might only permit entry to proteins or protein complexes with multiple membrane spanning domains.

The gating mechanism might not function at the level of the proteins themselves, but rather through lipid interactions. Tyrp1 and tyrosinase both require glycosphingolipids for proper intracellular trafficking to melanosomes (Sprong et al., 2001; Groux-Degroote et al., 2008). Furthermore, experiments with chimeric proteins consisting of luminal and
cytoplasmic domain swaps between Tyrp1 and the lysosomal protein, LAMP1, or between Tyr and LAMP1 showed that the determinants for the lipid-dependent behavior reside in the lumenal domains of the proteins. The failure of the OCA2 cytoplasmic domain chimera to traffic to melanosomes could thus be explained if similar interactions between lipids and OCA2 lumenal determinants regulated trafficking and passage through the endosome/melanosome “gate”. It will be interesting to see whether OCA2 trafficking similarly depends on glycosphingolipids.

4.3.3 BLOC-2 and AP-3
What happens once OCA2 has entered the endosomal tubular transport intermediates? As discussed above, this localization might be sufficient to allow for OCA2 transport activity to rescue melanin synthesis in OCA2-deficient cells. However, BLOC-2 is likely to facilitate the ultimate delivery of OCA2 via these tubular intermediates to melanosomes. Although we currently do not yet understand BLOC-2 function at the molecular level, analyses of Tyrp1 trafficking in our laboratory implicate BLOC-2 as functioning downstream of BLOC-1 in directing Tyrp1 towards melanosomes via the tubular intermediates (Setty et al., 2007). As in BLOC-1-deficient cells, a cohort of Tyrp1 in BLOC-2-deficient cells is aberrantly localized to early endosomes and the plasma membrane, but substantial cohorts are also delivered to the TGN and to multivesicular endosomes/lysosomes. This steady state redistribution of Tyrp1, together with live cell imaging analyses, suggests that BLOC-2 facilitates the directed fusion of the tubular intermediates with melanosomes. Is OCA2 also dependent on BLOC-2 for
trafficking? Preliminary results suggest that OCA2 is indeed mislocalized in BLOC-2-deficient melanocytes derived from the *cocoa* mouse model of HPS type 3, but further experiments are needed. A genetic interaction between OCA2 and BLOC-2 has also been suggested by results from Hoyle et al, in which BLOC-2-deficient mice were observed to progressively lose pigmentation with the loss of one or both alleles of OCA2 (Hoyle et al., 2011). Because OCA2 mutations alone are typically recessive and phenotypes are not typically observed in heterozygotes, Hoyle et al interpreted their results to suggest that OCA2 requires BLOC-2 to traffic to melanosomes. Thus, depletion of BLOC-2 compromises the melanosomal content of OCA2 such that gene dosage effects become apparent. In further support of a role for BLOC-2 in regulating OCA2 delivery to melanosomes, Tyr activity in both OCA2-deficient mouse melanocytes and BLOC-2-deficient melanocytes cultured from human patients is normal or increased relative to wild-type melanocytes *in vitro*, but Tyr activity is diminished *in vivo* (Manga et al., 2001; Chen et al., 2002; Toyofuku et al., 2002; Boissy et al., 2005; Helip-Wooley et al., 2007) – likely a reflection of altered pH or copper content in melanosomes. Moreover, Tyr in both models is mislocalized to cytoplasmic vesicles that are not themselves pigmented but that stain positively in DOPA histochemical assays, suggesting that in intact cells the lumenal environment in these vesicles does not support melanin formation (Manga et al., 2001; Boissy et al., 2005; Helip-Wooley et al., 2007). The similarity of the phenotypes in the two cell lines suggest that OCA2 relies in part on BLOC-2 for its trafficking. BLOC-1-deficient melanocytes are more severely hypopigmented than BLOC-2-deficient melanocytes, at least partially due to a failure in BLOC-1-deficient cells to deliver ATP7A, the copper transporter, to melanosomes to supply Tyr with its cofactor, copper.
(Setty et al., 2008). The higher pigmentation level of BLOC-2-deficient melanocytes suggests that like OCA2, ATP7A is also likely to be weakly dependent on BLOC-2 for its localization to melanosomes. The partial mislocalization of Tyrp1 and reduced but not ablated activities of OCA2, and ATP7A in BLOC-2-deficient melanocytes is consistent with a role for BLOC-2 as a tether, such that melanosomal targeting of tubular transport structures is impaired but not entirely eliminated in its absence.

Interestingly, the BLOC-2 and AP-3 double mutant mouse is reported to be as severely hypopigmented as the BLOC-1-deficient mouse in both coat and eye color (Di Pietro et al., 2006; Gautam et al., 2006). Dependence of OCA2 on both BLOC-2 and AP-3 suggests one possible contributing factor to this severe hypopigmentation. If OCA2 melanosomal localization and function is hampered but not eliminated by either BLOC-2 or AP-3 deficiency, the combined loss of the two might result in a severe loss of OCA2 localization and function, phenocopying the loss of BLOC-1, which most likely prevents entry of OCA2 into the tubular transport intermediates. Although the hypopigmentation phenotype of BLOC-1-deficient cells has been attributed largely to loss of copper-loading of tyrosinase due to ATP7A mislocalization (Setty et al., 2008), OCA2 functional loss could also contribute. The in vitro assays showing that DOPA activity is restored in BLOC-1-deficient cells upon addition of copper do not reveal whether in vivo pH dysregulation attributed to OCA2 deficiency was also a factor contributing to low in vivo Tyr activity.
Current data suggest that BLOC-1 functions upstream of BLOC-2 (Setty et al., 2007), but where does AP-3 act? As described above, I propose that AP-3 can function together with BLOC-1 to divert cargo into melanosome-bound endosomal tubular transport intermediates (Figure 4.2), but whether AP-3 is additionally needed downstream for delivery from within the tubules to melanosomes is unclear. At the light microscopy level it may not be possible to tell whether the structures to which E96D is localized are the tubules themselves or the vacuolar endosomes, reflecting a simple block in initial entry to the tubules. Both AP-1 and AP-3 coated buds have been observed on the endosomal tubules near melanosomes by immunoelectron microscopy (Theos et al., 2005; Delevoye et al., 2009), and the AP-1 coated buds are proposed to help deliver Tyrp1 to the adjacent melanosomes (Delevoye et al., 2009). Moreover, by electron tomography, unidentified buds are observed to extend from the tubules that connect endosomes to melanosomes (Delevoye et al., 2009). AP-3 has previously been localized to buds on endosomal tubules that are decorated with BLOC-1 in spatially distinct areas (Di Pietro et al., 2006); whether these tubules are the same as those that are continuous with melanosomes is not clear (Di Pietro et al., 2006). We speculate that AP-3-coated buds will be found on these tubular transport intermediates continuous with melanosomes, and that the buds function in anterograde transport from the tubules to melanosomes (Figure 4.2). If true, this would provide evidence that AP-3 plays a sorting role downstream of BLOC-1/AP cargo selection. One might postulate that OCA2 is “handed off” from BLOC-1/AP-3 to BLOC-2 and perhaps finally to AP-3 again for melanosomal delivery. This would be consistent with evidence for physical interactions between BLOC-1 and BLOC-2, between BLOC-1 and AP-3 (Di Pietro et al., 2006), and more recently between BLOC-2 and AP-3 (Salazar
et al., 2009) in skin fibroblasts. Careful examination at the light and electron microscopy levels of OCA2 trafficking in cells deficient in BLOC-2 or AP-3 will be required to sort out the spatiotemporal order of these complexes in melanosomal trafficking.

4.4 OCA2 function and Tyr trafficking

What do my results imply about OCA2 function? In OCA2-deficient cells, an unusually high proportion of Tyr is sensitive to endoglycosidase H at steady state (Chen et al., 2002; Toyofuku et al., 2002; Chen et al., 2004). This has been interpreted as a defect in Tyr maturation and ER exit. Moreover, Tyr is abnormally cleaved and secreted from these cells, and is also found in small vesicles distributed throughout the cytoplasm (Manga et al., 2001; Manga and Orlow, 2001; Chen et al., 2002; Toyofuku et al., 2002; Chen et al., 2004; Ni-Komatsu and Orlow, 2006). How does one explain these findings in the context of our model?

4.4.1 OCA2 as a pH regulator

Sequence homology places OCA2 in the ArsB/NhaD family of transporters. The NhaD founder is a Na⁺/H⁺ exchanger, and since OCA2 is thought to regulate pH, some researchers have proposed that OCA2 might also be a proton transporter (Lee et al., 1995; Ancans et al., 2001a), though this hypothesis is disputed (Brilliant and Gardner, 2001; Brilliant, 2001). The regulation of lumenal pH appears to be important for melanin synthesis. Fluorescent assays show a higher steady-state pH in Black melanocytes than in Caucasian melanocytes (Fuller et al., 2001). Treatment of Caucasian melanocytes with chemicals that raise organellar pH stimulates melanin synthesis in these cells; a similar
induction in melanin synthesis is seen in OCA2-deficient melanocytes receiving the same treatments (Ancans et al., 2001a; Ancans et al., 2001b; Fuller et al., 2001; Manga and Olow, 2001; Chen et al., 2002; Chen et al., 2004; Ni-Komatsu and Olow, 2006). NHE activity has been demonstrated to be a determinant of the difference in melanin synthesis activity between Caucasian and Black melanocytes (Smith et al., 2004), and population genetics analyses consistently demonstrate strong linkage between OCA2 polymorphisms and racial differences in skin color (Shriver et al., 2003; Lao et al., 2007; Norton et al., 2007). However, the NHE activity that was seen to be a factor in the activity in the different melanocytes was sensitive to derivatives of the fairly promiscuous NHE inhibitor amiloride (Sarangarajan et al., 2001; Smith et al., 2004), and researchers noted that the amino acid sequence that confers EIPA sensitivity on an NHE is not present within OCA2 (Smith et al., 2004), making it unlikely to be the critical NHE. My unpublished results also suggest that OCA2 does not directly act as an NHE.

Heterologous expression of a plasma membrane-localized mutant of OCA2 (lacking active LL1 and LL2) in NHE-deficient fibroblasts was not sufficient to rescue these cells from death following intracellular acid loading, while cells expressing a bona fide NHE protein survived. pH-sensitive live-cell fluorescence assays gave similar results, showing that the same OCA2-expressing fibroblasts could not recover their pH after short-term acid loading in a flow chamber apparatus. These experiments do not rule out pH regulation by OCA2 via some other mechanism within melanocytes.

The proposed role of OCA2 as a pH regulator may also explain the appearance of the aberrant cytoplasmic Tyr vesicles seen in OCA2-deficient melanocytes. The origin of
these vesicles is not completely clear. Tyrosinase has been visualized in AP-3 and AP-1 coated endosomal buds near melanosomes in wild-type cells (Theos et al., 2005). If OCA2 acting within the endosomes/melanosomes is required to promote correct targeting of endosomally-derived Tyr-containing vesicles, then OCA2 deficiency could lead to the accumulation of vesicles that bud appropriately but are unable to fuse with their target compartments. The fact that the vesicles detected in OCA2-deficient cells are not pigmented but stain positively for Tyr activity by classical DOPA histochemistry (Manga et al., 2001) indicates that the Tyr in these vesicles is inactive in vivo but likely already loaded with copper (if it were not copper loaded, excess copper would be needed during DOPA histochemistry; (Setty et al., 2008)). This might reflect a failure to strip copper in endosomes (Setty et al., 2008) in OCA2-deficient cells, such that copper remains bound in endosome-derived transport vesicles. Failure to strip copper in endosomes due to loss of OCA2-mediated pH regulation is consistent with the appearance of some induced melanin deposition in endosome-like structures in OCA2-deficient melanocytes that have been treated with the vATPase inhibitor bafilomycin A1 (Chen et al., 2004), though the majority of the induced activity was correctly localized to melanosomes.

Alternatively, the accumulated Tyr vesicles could originate from the TGN instead of endosomes. In this case, OCA2 might be predicted to function at the endosome to facilitate targeting and/or fusion of the incoming Tyr vesicles. In support of the idea that OCA2-mediated pH regulation could affect TGN-derived Tyr-containing vesicle fusion at the endosome, modulation of lumenal pH has previously been proposed to play a role in membrane fusion at the yeast vacuole (Ungermann et al., 1999), and mutations in the
endosomal Ca^{2+} transporter MCOLN3 affect endosomal acidification as well as the rate of homotypic endosomal fusion in vitro (Lelouvier and Puertollano, 2011). Interestingly, a gain-of-function mutation in MCOLN3 gives rise to the varitint-waddler mouse, which exhibits hypopigmentation among other phenotypes (Di Palma et al., 2002).

4.4.2 Could OCA2 act elsewhere?

Other researchers have suggested that OCA2 might facilitate transmembrane glutathione transport, since heterologous expression in yeast leads to localization of the transgene to the vacuolar membrane and a correlated reduction in cellular glutathione levels due to degradation following import into the vacuolar lumen (Staleva et al., 2002). Because the Tyr glycosylation and cleavage/secretion defects seen in OCA2-deficient melanocytes reflect problems at relatively early steps in Tyr biogenesis, Chen, Manga and Orlow have proposed that OCA2 acts within the endoplasmic reticulum to promote Tyr maturation (Chen et al., 2002). Tyr contains several disulfide bonds, and its maturation might thus be particularly sensitive to changes in the redox state of the ER (Wang and Hebert, 2006). If OCA2 were an ER-localized glutathione transporter for example, it could greatly affect Tyr biosynthesis. My results from rescue assays performed with ER-localized OCA2 mutants show that OCA2 must traffic to a post-Golgi compartment for melanin synthesis to take place. I speculate that substrate gradients generated by OCA2 in distal endosomal compartments might be transduced back to the ER and correct the early defects in Tyr maturation. However, another untested possibility is that OCA2 actually functions in both the ER and the endosome/melanosome. In the ER it could act to promote Tyr folding and glycosylation while in the endosome/melanosome it could facilitate fusion of Tyr-
containing vesicles or promote the lumenal conditions needed for melanin synthesis in melanosomes. Our rescue assay only examined the most distal phenotype, melanin synthesis. It would be informative to see whether ER-restricted OCA2 mutants could correct the observed differences in Tyr glycosylation, cleavage and secretion, or mislocalization to scattered cytoplasmic vesicles.

4.5 Concluding remarks

The completion of the work in this thesis has led to the resolution of a debate surrounding the localization of the OCA2 protein, the confirmation that a putative sorting signal does in fact play a critical role in OCA2 trafficking to melanosomes, and the reconciliation of our model of melanosomal trafficking with data from studies in several cell types. But beyond that, my initial work with this protein has also paved the way for its use in further investigation of the determinants of AP complex interaction with dileucine motifs and characterization of the melanosomal trafficking pathway in additional mutant cells. The differences in structure and sorting between OCA2 and Tyr/Tyrp1 should additionally provide new testable hypotheses regarding the mechanism for determining the BLOC-1-dependence of LRO cargo proteins, perhaps ultimately leading to the elucidation of BLOC-1 function.

The question of the function of OCA2 itself, however, also remains unanswered. Though use of OCA2 as a trafficking model does not strictly require knowledge of the protein’s function, we have seen for example that the localization of the protein appears to be somewhat flexible within the endocytic system, and I believe that future elucidation of
the putative transport function of OCA2, likely to be achieved via *in vitro* studies or expression in heterologous systems, will undoubtedly contribute to the determination of its “true” site of action. Solving the riddle of OCA2 function will also help us understand its biological role in normal pigmentation and how this function is disrupted by the numerous disease-causing mutations that have been cataloged.
CHAPTER 5: MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Cell Culture and Transgene Expression

All culture reagents were purchased from Invitrogen (Carlsbad, CA) unless stated otherwise. All cells were grown in media that included 1% penicillin/streptomycin and incubated at 37°C and 10% CO₂ unless stated otherwise. HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT). Immortalized mouse melanocyte cell lines melan-Ink4a and melan-pallid (Sviderskaya et al., 2002), melan-p1 (Sviderskaya et al., 1997), and melan-pearl (Theos et al., 2005) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 200 nM phorbol 12-myristate-13-acetate. Melan-pearl growth media was additionally supplemented with 200 nM cholera toxin. MNT-1 human melanoma cells (Cuomo et al., 1991) were grown in DMEM supplemented with 15% fetal bovine serum, 10% AIM-V, 1% nonessential amino acids, 1% sodium pyruvate (Mediatech, Herndon, VA), and antibiotics. Chinese hamster ovary (CHO) cells subclone K1 were a gift from Dr. Monty Krieger (Massachusetts Institute of Technology, Cambridge, MA). Chinese hamster ovary (CHO) cells were grown in minimal essential medium with 5% fetal bovine serum (HyClone Laboratories) in 5% CO₂. Transfections in HeLa, CHO, and mouse melanocytes were performed using FuGENE-6 (Roche...
Diagnostics, Indianapolis, IN) according to manufacturer’s instructions with 1–2 µg of DNA. Transfections in MNT-1 cells were performed using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions, with 1.6 µg of DNA and Opti-MEM (Invitrogen).

Construction of OCA2 Expression Plasmids
To clone the full-length human OCA2, a human melanoma library in the Uni-Zap vector (Stratagene, La Jolla, CA) was screened using a 953-base pair probe derived from the central portion of OCA2 cDNA by reverse transcription-polymerase chain reaction (RT-PCR) (with Pfu DNA polymerase; Promega, Madison, WI) from total melanocyte cDNA synthesized with the SuperScript preamplification system kit (Invitrogen). Among several independent positive clones isolated, two contained full-length OCA2 cDNA with an internal deletion of 72 base pairs encompassing transmembrane domain 3 and corresponding to exon 10 of the OCA2 gene; the deleted region was reinserted by subcloning an encompassing unique AccI–HindIII fragment obtained by RT-PCR into AccI–HindIII-digested OCA2 cDNA. The entire cDNA was subcloned into the BamHI and XhoI sites of pCR3 (Invitrogen), and its sequence was confirmed (Bio Molecular Research, DNA sequencing service at the University of Padua, Italy; http://bmr.cribi.unipd.it/). Compared with the originally published sequence (Rinchik et al., 1993), the pCR3/OCA2 contains four silent mismatches and the previously reported nonpathologic polymorphism D257A (Oetting et al., 1998). In most experiments (except those in Figure 1), the entire 5´-untranslated region was replaced by a synthetic Kozak consensus sequence immediately upstream of the OCA2 translational start site. To
generate a lumenally exposed epitope-tagged form of OCA2, three consecutive copies of an influenza hemagglutinin epitope tag (3xHA) were introduced into the first luminal loop as follows. The codons for the unconserved R\textsuperscript{243}P\textsuperscript{244} residues in this loop were subjected to site-directed mutagenesis by using two-step amplification (Higuchi et al., 1988) with AmpliTaq (Applied Biosystems, Foster City, CA) to generate a BsiWI restriction site (converting P\textsuperscript{244} to T). The 3xHA tag with a 5´ BsiWI site and a 3´ Acc65I site was amplified by thermal cycling with AmpliTaq from pCI-pallidin-HA (Moriyama and Bonifacino, 2002) and subcloned into the new BsiWI site on OCA2, creating OCA2-HA. To construct OCA2-HMGCR, we amplified a fragment beginning 5´ of the unique BstXI site, encoding the C terminus of human OCA2 fused to the last eight amino acids of human HMG CoA reductase (GACTKKTA), and followed by an XhoI site, and the product was subcloned in place of the BstXI–XhoI fragment of pCR3/OCA2-HA. Mutated forms of OCA2-HMGCR were constructed in a similar manner. To construct OCA2-ΔN91, an oligonucleotide encoding amino acids 92-102 of OCA2 and flanked by BamHI and EcoRI sites was subcloned to replace the BamHI–EcoRI fragment of pCR3/OCA2-HA, excising amino acids 2-91 of OCA2. MHRRROCA2-ΔN91 and MHAAA-OCA2-ΔN91 were constructed in the same way using oligonucleotides that encoded the sequences MHRRR (the first five amino acids of the p35 form of human Invariant chain; (Schutze et al., 1994) or MHAAA (in which the tribasic ER retention signal is mutated) fused to amino acid 92 of OCA2. To alter the leucine residues within the N-terminal cytoplasmic domain of OCA2, the codons for the indicated pairs of leucine residues were replaced with those for two alanines by site-directed mutagenesis using two-step amplification. Expression vectors for chimeric
OCA2-human transferrin receptor (hTfR) proteins were constructed from pCR3-OCA2 and pCDM8-hTfR (Marks et al., 1996) by site-directed mutagenesis using two-step amplification with primers that bridged the junction between the OCA2 N-terminal cytoplasmic domain and the transmembrane domain of human transferrin receptor. The final chimera contained amino acids 1-173 of human OCA2 and amino acids 62-761 of hTfR. To generate the GST fusion proteins, the 5’ end of the OCA2 cDNA, corresponding to the first 162 amino acids, was amplified from pCR3-OCA2 and cloned into the BamHI and SalI sites of the prokaryotic expression vector pGEX5X-1 (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Constructs containing the various combinations of mutations at the dileucine motifs were generated by amplification from the corresponding pCR3 vectors. The construction of the yeast three-hybrid pBridge plasmids containing the sigma constructs and the cytoplasmic domain of tyrosinase (Theos et al., 2005) and the construction of the pGADT7 plasmids containing fusions of murine gamma1, rat alphaC, and human delta to yeast Gal4-AD (Janvier et al., 2003) have been previously described. The yeast three-hybrid OCA2-containing pBridge constructs were made by PCR amplification of the N-terminal portion of the appropriate pCR3/OCA2-containing plasmid with primers that added a 5’ EcoRI site and 3’ SalI site. The products were subcloned into the same restriction sites downstream of yeast Gal4-BD in pBridge plasmids containing human sigma1A, rat sigma2, or human sigma3A in the second transcription unit. Unless stated otherwise, all recombinant plasmids were verified by automated sequencing by the University of Pennsylvania Cell Center or the Nucleic Acid/Protein Research Core Facility at the Children's Hospital of Pennsylvania. Details of PCR primers, sequences, and conditions can be provided upon request.
Antibodies

Antibodies used were as follows: rat anti-HA 3F10 and mouse B3/25 antitransferrin receptor were from Roche Diagnostics, TA99 (Mel-5) anti-Tyrp1 was from American Type Culture Collection (Manassas, VA), mouse H4A3 anti-human lysosome-associated membrane protein (LAMP)-1 and rat GL2A7 anti-mouse LAMP-2 were from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA), rabbit anti-LAMP-1 was from Affinity Bioreagents (Golden, CO), mouse anti-HA 16B12 was from Covance Research Products (Princeton, NJ), mouse MAB3126 anti-calnexin was from Millipore (Billerica, MA), HMB45 anti-PMEL was from LabVision (Fremont, CA), mouse anti-AP-3 μ3A (anti-p47A) was from BD Biosciences Transduction Laboratories (Lexington, KY), mouse anti-Rab5 was from Synaptic Systems (Göttingen, Germany), and the mouse monoclonal antibodies 100/3 anti-AP-1γ, 100/2 anti-AP-2α, anti-α-tubulin, and anti-β-actin were from Sigma-Aldrich. The mouse anti-pallidin monoclonal antibody (mAb) 2G5 was described previously (Nazarian et al., 2008). Goat anti-immunoglobulin G (IgG) secondary antibodies conjugated to Alexa-488 and Alexa-594 were from Invitrogen. Donkey anti-IgG secondary antibodies conjugated to 7-amino-4-methylcoumarin-3-acetic acid or conjugated to DyLight 488 and unconjugated donkey anti-mouse IgG antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Conjugation of Alexa-488 to monoclonal antibodies was performed using a protein labeling kit from Invitrogen. The NOCA2 anti-OCA2 antibody was generated by conjugating the first thirteen amino acids of human OCA2 (MHLEGDRGRRYPG) with a C-terminal cysteine to keyhole limpet hemocyanin and immunizing rabbits from
Genemed Biosynthesis (San Francisco, CA) with the conjugate. NOCA2 was further affinity purified against the antigenic peptide conjugated to SulfoLink coupling gel (Pierce Chemical, Rockford, IL) according to the manufacturer’s instructions.

**Glutathione Transferase (GST) Pull-Down Assays**

The GST-OCA2 fusion constructs were expressed in *Escherichia coli* and purified as described previously (Starcevic and Dell'Angelica, 2004). Detergent extracts of MNT-1 or HeLa cells were prepared in MNT-1 buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 1 M MgCl$_2$, 1 mM NaF, and 0.5% NP-40) or HeLa buffer (25 mM HEPES, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1 mM NaF, 0.5 mM MgCl$_2$, and 0.5% Triton X-100), respectively, containing protease inhibitor mixture [1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 µg/ml leupeptin, 5 µg/ml aprotinin, and 1 µg/l pepstatin A]. The MNT-1 extract was subsequently diluted with 1 volume of MNT-1 buffer lacking detergent and precleared by incubation with glutathione-Sepharose 4 Fast Flow beads (GE Healthcare) and centrifugation. Aliquots of the cleared detergent extracts were incubated for 1 h at 4°C with GST-fusion proteins (20 µg) that had been immobilized onto 15 µl of glutathione-Sepharose beads. After the incubation period, beads were collected by brief centrifugation and washed three times with MNT-1 buffer containing 0.1% NP-40 or HeLa buffer containing 0.1% Triton X-100, respectively, and one time with buffer lacking detergent. Proteins bound to the beads were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and subjected to immunoblotting as described previously (d'Addio *et al*., 2000).
Metabolic Labeling and Endoglycosidase Assays

Metabolic labeling of MNT-1 cells or transiently transfected HeLa cells and immunoprecipitation from Triton X-100 cell lysates were done essentially as described previously (Berson et al., 2000) by using EXPRE\(^{35}\text{S}\text{S}\text{S}\text{S}\)-cysteine/methionine mixture (PerkinElmer Life and Analytical Sciences, Boston, MA). Cells were labeled for 30 min at 37°C and chased for the indicated times. For endoglycosidase treatments, immunoprecipitates were denatured, split into equal aliquots, and mock treated or incubated with endoglycosidase H (endoH) or peptide N-glycosidase F (PNGase F) (both from New England Biolabs, Ipswich, MA), according to manufacturer’s instructions, for 4 h with the addition of phenylmethlysulfonyl fluoride, leupeptin (Roche Diagnostics), and 1% NP-40 (Roche Diagnostics). Samples were fractionated by SDS-PAGE using 8% acrylamide gels, dried, and analyzed using a Storm 860 PhosphorImager and ImageQuest software (GE Healthcare) as described.

Transfections and Immunofluorescence Microscopy

For HeLa and CHO cells, cells were seeded onto glass coverslips in six-well dishes at 1.5x10\(^5\) per well. Mouse melanocytes were seeded at 1x10\(^5\) per well in six-well dishes or at 0.5-0.75x10\(^5\) per well in 12-well dishes on coverslips coated with Matrigel (BD Biosciences, San Jose, CA) according to manufacturer’s instructions. The next day, cells were transfected with 1–2 \(\mu\)g of the relevant OCA2 construct by using FuGENE-6 reagent. Two days later, cells were fixed for 30 min in 2% formaldehyde (Thermo Fisher Scientific, Waltham, MA), stained with primary and fluorochrome-conjugated secondary antibodies, and mounted onto glass slides as described previously (Calvo et al., 1999).
some experiments, transfected HeLa cells were incubated in fresh medium with or without 50 µg/ml cycloheximide for 3 h before fixation. For lysosomal inhibition, transfected CHO cells were incubated in fresh medium with or without 50 mM NH₄Cl (Thermo Fisher Scientific) for 4 h before fixation. For surface staining, chilled cells were labeled with 1 µg/ml anti-HA antibody for 10–30 min on ice, and then unbound antibody was removed by rinsing in ice cold phosphate-buffered saline before fixation as described. Slides were analyzed on a DM IRBE microscope (Leica, Wetzlar, Germany) equipped with an Orca digital camera (Hamamatsu, Bridgewater, NJ) or a Retiga Exi Fast 1394 digital camera (Qimaging, Surrey, BC, Canada). Images were captured and manipulated using OpenLab software (Improvision, Lexington, MA), with the volume deconvolution package. Final images were prepared using Adobe Photoshop (Adobe Systems, Mountain View, CA).

**Yeast Culture, Transformation, and Three-hybrid Assays**

The *Saccharomyces cerevisiae* strain HF7c (Clontech), was maintained on complete YPD plates. Co-transformation with pBridge and pGADT7 plasmids was performed by a modification of the lithium acetate procedure as described in the Yeast Protocols Handbook from Clontech. HF7c transformants were selected by spreading on plates lacking leucine, tryptophan, and methionine. For colony growth assays, HF7c transformants were pooled and spotted once or in 5-fold serial dilutions on plates lacking leucine, tryptophan, methionine, and histidine, and allowed to grow at 30° C for 3–5 days.
Statistical Analysis

Quantification of melanin synthesis rescue was performed as follows. Transfected cells were identified by positive labeling with anti-HA antibody by immunofluorescence microscopy and then assessed for the presence of pigmented melanosomes by bright field microscopy. Within each experiment, the percentage of transfected cells (>100 cells/experiment) that were positive for pigment rescue was calculated. Graphs represent rescue by each construct averaged over three independent experiments. In Figures 2.3 and 2.11, an initial repeated measures analysis of variance was performed on the matched sets, followed by Dunnett’s multiple comparison test to compare rescue by each mutant to rescue by wild-type OCA2. In Figure 2.3, a two-tailed t test was used to compare rescue by OCA2-HMGCR and OCA2-HMGCR-AATA. To quantify overlap of OCA2 dileucine mutants with pigment or with Tyrp1, deconvolved paired images were rendered binary in ImageJ by density slicing and the total area of overlap between them was calculated for objects containing more than 5 pixels, excluding the densely labeled perinuclear area. Seven to 10 cell profiles were quantified for each pairwise comparison. To compare colocalization with pigment, a one-way analysis of variance was performed on the unmatched sets, followed by Bonferroni's multiple comparison test. To compare the colocalization of OCA2-AA23 or OCA2-AA23 E96D with Tyrp1, an unpaired two-tailed t test was performed. All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA).
REFERENCES


(cocoa) protein is a component of the biogenesis of lysosome-related organelles complex-2 (BLOC-2). J Biol Chem 279, 12935-12942.


Ni-Komatsu, L., and Orlow, S.J. (2006). Heterologous expression of tyrosinase recapitulates the misprocessing and mis trafficking in oculocutaneous albinism


evidence for a prevacuolar compartment in class E vps mutants. Mol Biol Cell 3, 1389-1402.


Genetic determinants of hair, eye and skin pigmentation in Europeans. Nat Genet 39, 1443-1452.


