Studies of Proteins that Regulate Melanin Synthesis and Distribution

by

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Department of Biochemistry
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Abstract

Melanin is the major component of skin-, hair-, and eye pigmentation in mammals. Synthesis of melanin takes place in specialized organelles in melanocytes, called melanosomes. As melanosomes mature during pigment synthesis, they are transported towards the tips of dendrites in the melanocyte, and eventually transferred to neighbouring keratinocytes to distribute pigment throughout the skin. A large number of proteins regulate melanin synthesis and distribution. Over one hundred genes have been associated with coat colour mutations in mice, and many of these genes have also been identified in human pigmentation disorders. Other proteins involved in pigmentation are part of pathways that are not unique to pigmentation alone, such as the Ras/ERK pathway. In mouse B16 cells, cAMP stimulation leads to the upregulation of melanin synthesis and dendrite extension. However, cAMP also activates the Ras/ERK pathway in these cells, which, upon prolonged stimulation, leads to an inhibition of melanin synthesis and dendrite extension. Here I show that the protein CNrasGEF, which was previously identified in our lab, is responsible for cAMP-dependent Ras activation in B16 cells, and therefore a part of the negative regulatory pathway of melanogenesis. In order to find other proteins involved in pigmentation pathways, I have developed a method to detect melanosomes using Cellomics KineticScan (KSR) high-content image analysis. This system could potentially be used in a high-throughput
RNA interference screen to identify proteins that affect melanosome formation or transport. However, in a pilot study it appeared that knockdown levels achieved upon transient transfection of knockdown constructs from a mouse shRNAmir library against selected targets were in many cases not sufficient to detect an effect on melanocytes, either by confocal microscopy, or by Cellomics KSR analysis. Further reduction of expression levels is necessary before this system can be scaled up to high-content/high-throughput identification of proteins involved in pigmentation.
Acknowledgments

“The Eighth Square at last!’ she cried as she bounded across, and threw herself down to rest on a lawn as soft as moss, with little flower-beds dotted about it here and there.”
– Lewis Carroll (“Through the Looking-Glass”)

This thesis is the final result of six and a half years of research and lab work. As you look at the figures and graphs on its pages, you will learn what I did while I was sitting in the tissue culture room, what I thought about while reading papers, pipetting or peering down microscopes. But the western blots and confocal images will not tell you who where there with me, encouraging me along the way, even at the moments when I thought I’d never get anywhere with my experiments. These first few pages are for them.

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Chapter 1

Introduction
1.1 Thesis Overview

In this thesis I present an analysis of several proteins involved in skin pigmentation.

Chapter 1 begins with a comprehensive summary of known pathways regulating melanin synthesis, dendrite formation, melanosome formation, or melanosome transport in melanocytes. Connections between various steps in the process of pigmentation are discussed, and this section of Chapter 1 ends with a summary of diseases caused by mutations in proteins involved in pigmentation. The next section describes CNrasGEF, a protein previously studied in our lab. CNrasGEF activates Ras in response to cAMP, and Rap1 independently of cAMP. The final section of Chapter 1 gives a brief summary of the use of RNA interference as a tool for molecular biology analysis.

In Chapter 2 I show that CNrasGEF acts as a cAMP-activated Ras guanine nucleotide exchange factor in B16 melanoma cells, where it is part of the Ras/ERK feedback pathway that regulates melanin synthesis and dendrite extension. This work has been published in the Journal of Biological Chemistry (JBC 281 (1), 121-128, 2006)

Chapter 3 describes the development and testing of the detection of melanosomes by a high-content image analysis system, using an antibody against TYRP1 (tyrosinase-related protein 1) as a marker for melanosomes. The use of different cell lines and program settings are discussed in this chapter. I set up this system with the intention of using it as the basis for a high-throughput RNA interference screen for proteins involved in pigmentation.

The next step in testing whether the system described in Chapter 3 can be used in an RNAi screen is testing of selected shRNA library constructs. In Chapter 4 I present a pilot study of twenty-three knockdown constructs against eleven genes involved in pigmentation. In a blinded confocal experiment, two constructs (against TYRP1 and HPS5) showed a significant deviation from control cells in TYRP1 intensity, but Cellomics KSR analysis only convincingly picked up TYRP1, the protein used to label melanosomes. Further analysis showed that many constructs only reduced mRNA levels of target genes by a limited amount, which appears to be insufficient to achieve the phenotypes reported in studies with mutant mouse models or human disease.
Chapter 5 summarizes the material presented in the preceding three chapters and discusses the implications of the work presented in this thesis for the overall understanding of pigmentation processes. Finally, suggestions are given for future experiments that can expand on the knowledge presented here.
1.2 Pigmentation

1.2.1 Melanin and Melanocytes

1.2.1.1 Melanin

Melanin is a brown/black pigment, found in many living organisms, from fungi and plants to mammals. It has different functions in all of these organisms. For example, in invertebrates melanin is part of the immune system, and in the fungus *C. neoformans* it determines virulence (Cerenius, L. et al., 2008, Casadevall, A. et al., 2000). In mammals melanin is the main determinant of skin-, hair-, and eye colour, but it is also found in the substantia nigra area of the brain in the form of neuromelanin (Zecca, L. et al., 2001).

This work focuses on the study of mechanisms of skin pigmentation in mouse and human cells. Skin pigment consists of two forms of melanin: red/yellow pheomelanin and the more abundant brown/black eumelanin. In humans, pigmentation by pheomelanin is noticeable in the (near) absence of eumelanin, e.g. in red-haired, fair-skinned individuals, but levels of eumelanin more closely correlate with phenotypical pigmentation differences (Wakamatsu, K. et al., 2006).

1.2.1.2 Melanocytes

Melanin destined for skin or hair pigmentation is synthesized in melanocytes in the basal layer of the skin, from where it is distributed to neighbouring cells. Melanocytes, like neurons and glial cells, originate from the neural crest during embryo development (Henion, P.D., and Weston, J.A., 1997). Two proteins essential for melanocyte development are the proto-oncogene c-Kit and its ligand “steel factor” (also called stem cell factor (SCF) or mast cell growth factor (MGF)) (Williams, D.E. et al., 1990, Zsebo, K.M. et al., 1990). Studies in mice have shown that loss-of-function mutations in either gene lead to improper migration of melanoblasts outward from the dorsal neural tube, preventing melanocyte formation in the regions most distant from the neural crest. The same is seen in humans, where mutations in c-Kit cause piebaldism, which is characterized by a lack of pigmentation on the forehead, chest, abdomen, and extremities (Fleischman, R.A. et al., 1991). Mutations in the microphthalmia transcription factor (MITF) have a similar effect on melanocyte development, but since MITF also plays an important role in melanin synthesis in mature melanocytes, this transcription factor will be discussed in more detail in a later section.
The pigmentation pattern in piebaldism is the result of a localized absence of melanocytes, but the majority of pigmentation regulation occurs within melanocytes. Studies of mouse models and human pigmentary disorders have elucidated more than a hundred genes involved in the regulation of skin pigmentation, and most of these regulate pigmentation at the subcellular level (Bennett, D.C., and Lamoreux, M.L., 2003, Tomita, Y., and Suzuki, T., 2004). In addition to genetic studies, cell biological and biochemical analyses of human and mouse melanocytes or melanocyte-derived cell lines have provided a more detailed look at pathways of melanin synthesis and distribution. The next section will summarize some of these findings.

1.2.2 Regulation of Pigmentation at the Melanocyte Level

Within melanocytes, melanin is synthesized in specialized organelles called melanosomes, which are a type of lysosome-related organelles. Like lysosomes, they originate from endosomes and multivesicular bodies. Proteins necessary for melanosome formation or melanin synthesis are transported to the melanosome as it matures. As a result, different stages of melanosomes (termed stage I-IV) contain different proteins and amounts of melanin. Maturing melanosomes move outward towards the cell membrane and towards the tips of dendrites. Once they arrive at the cell membrane, melanosomes are transferred to neighbouring keratinocytes in the skin.

This collective pigmentation process can be divided into three sections: melanin synthesis, dendrite extension, and melanosome formation and movement. Regulation of these processes is described below.

1.2.2.1 Regulation of Melanin Synthesis

1.2.2.1.1 Enzymes Involved in Melanin Synthesis

The amount of melanin produced in melanocytes is predominantly determined by the direct or indirect regulation of levels or activity of the enzymes responsible for various steps of the melanin synthesis pathway, shown in Figure 1.1. The first, and rate-limiting, step of melanin synthesis is the conversion of tyrosine to DOPA and dopaquinone by the oxidating enzyme tyrosinase. This step is common to both eumelanin and pheomelanin synthesis (Prota, G., 1980).
Figure 1.1. Melanin synthesis

Simplified pathway representing the synthesis of pheomelanin and eumelanin from tyrosine. Actions of the enzymes Tyrosinase, TYRP1, and TYRP2 are indicated in blue.
From there, synthesis of pheomelanin requires the addition of cysteine to dopaquinone, forming 5-S-cysteinyldopa (5-S-cysteinyl-DOPA) or 2-S-cysteinyldopa. Alternatively, dopaquinone can enter the eumelanin-specific pathway by oxidation to dopachrome, which is further processed to form the two building blocks of eumelanin: DHI (5,6-dihydroxyindole) and DHICA (6-dihydroxyindole-2-carbolic acid) (Ito, S., and Wakamatsu, K., 2008). The formation of eumelanin requires additional enzymes beyond tyrosinase: TYRP1 (tyrosinase-related protein 1) and TYRP2 (tyrosinase-related protein 2, or dopachrome tautomerase).

Mice with mutations in any of the enzymes involved in melanin synthesis show defects in pigmentation, although some more severe than others. Mutations in tyrosinase, the rate limiting enzyme in melanin synthesis, produce entirely white mice (albino), while loss of function mutations in TYRP1 lead to the production of brown rather than black pigment (Jackson, I.J., and Bennett, D.C., 1990, Bennett, D.C. et al., 1990). In humans, mutations in either protein lead to oculocutaneous albinism (OCA) (Tomita, Y., and Suzuki, T., 2004). Many tyrosinase null mutations affect one of the protein's two copper binding regions that are necessary for catalytic activity (Schweikardt, T. et al., 2007).

1.2.2.1.2 MITF

The transcription of tyrosinase, TYRP1, and TYRP2 is regulated by the microphthalmia transcription factor (MITF), which binds a region in the promoters of these genes called M-Box (AGTCATGTGCT) (Bertolotto, C. et al., 1998b, Bertolotto, C. et al., 1998a, Fang, D., and Setaluri, V., 1999, Fang, D. et al., 2002). (TYRP2 may require additional factors for transcription (Yasumoto, K. et al., 1997, Fang, D. et al., 2001).) Variations in MITF levels and activity affect melanin synthesis in cultured mouse or human melanocytes by regulating the transcription of these enzymes. However, MITF is also involved in melanocyte development, and various mutations in MITF in mice lead to a deficiency in melanocytes in skin and ear, and decreased pigmentation and size of the eyes (Hodgkinson, C.A. et al., 1993, Steingrimsson, E. et al., 1994). MITF is mutated in some cases of Waardenburg Syndrome, a disease characterized by pigmentation defects and hearing loss. Other types of Waardenburg Syndrome are caused by mutations in the proteins SOX10 or PAX3, both of which regulate MITF expression (Bondurand, N. et al., 2000).
MITF-mediated regulation of transcription of the enzymes involved in melanin synthesis is a complicated process involving several interacting pathways downstream of the melanocortin-1 receptor.

1.2.2.1.3 MC1R and αMSH

The melanocortin-1 receptor (MC1R) is a seven transmembrane domain G protein-coupled receptor. It is expressed predominantly in melanocytes, but is also found in other skin cells (Schaffer, J.V., and Bologna, J.L., 2001). Other tissues contain melanocortin receptors as well: MC2R in the adrenal cortex, MC3R and MC4R in the brain, and MC5R in peripheral tissues (exocrine glands). The different melanocortin receptors have unique functions (for example, MC4R plays a role in the hypothalamic regulation of food intake (Huszar, D. et al., 1997)) but they are all activated by ligands derived from the precursor protein proopiomelanocortin (POMC) (Schaffer, J.V., and Bologna, J.L., 2001, Cone, R.D., 2006). The predominant POMC-derived ligand produced in skin cells is α-melanocyte stimulating hormone (αMSH), produced by melanocytes, keratinocytes, fibroblasts, endothelial cells and inflammatory cells (Slominski, A. et al., 2000). Stimulation of the MC1R by αMSH leads to intracellular release of the GTP-bound Gsα subunit, which then activates adenylate cyclase to produce cAMP. cAMP interacts with several downstream pathways to regulate melanin synthesis, as well as dendrite extension and melanosome transport. These pathways will be outlined in the next section.

Regulation of melanin synthesis by the MC1R only affects eumelanin, not pheomelanin. Loss-of-function mutations in the MC1R therefore lead to a phenotype in which synthesis of the brown/black pigment eumelanin is impaired, but the red/yellow pheomelanin is still produced in normal levels. This is a relatively common phenotype: most individuals with red hair and pale skin carry MC1R mutations (Valverde, P. et al., 1995). These individuals often show an impaired tanning response: UV radiation upregulates melanin synthesis through several mechanisms, including the increase of αMSH secretion from melanocytes and keratinocytes (Chakraborty, A.K. et al., 1996). Since αMSH needs the MC1R to induce pigmentation, a non-functional MC1R does not respond to the UV-induced increase in αMSH.

The endogenous antagonist of several melanocortin receptors (including MC1R) is the Agouti signaling peptide (ASP), which may even act as an inverse agonist (Schaffer, J.V., and Bologna,
J.L., 2001). Mice with a dominant mutation in Agouti produce excessive amounts of ASP, leading to a yellow coat colour through the MC1R and to obesity through the MC4R (Voisey, J., and van Daal, A., 2002).

The following section will outline several pathways related to melanin synthesis that are affected by MC1R activation of adenylate cyclase and subsequent elevation of intracellular cAMP. While the overall effect of cAMP stimulation is an increase in pigmentation, cAMP is the starting point of several interacting signaling cascades that together fine-tune melanin synthesis.

1.2.2.1.4 cAMP Pathways in Melanin Synthesis

1.2.2.1.4.1 PKA

Protein kinase A (PKA) is a well-studied downstream effector of cAMP. Binding of cAMP to the regulatory subunit of PKA activates the catalytic domain through conformational change, allowing PKA to phosphorylate several intracellular targets, including the transcription factor CREB (cAMP responsive element binding protein) and CREB-binding protein (CBP) (Meinkoth, J.L. et al., 1993). Phosphorylated CREB then activates the expression of genes with CRE consensus sequences in their promoter (Meinkoth, J.L. et al., 1991). One protein that is expressed in response to CREB activation is MITF (Bertolotto, C. et al., 1998a), which in turn activates the transcription of tyrosinase and other melanin synthesizing genes, as described above. To summarize this pathway: cAMP upregulates melanin synthesis through PKA activation, CREB phosphorylation, MITF expression and increased transcription of tyrosinase and related genes. (Figure 1.1)

However, PKA is not the only target of cAMP in melanocytes.

1.2.2.1.4.2 PI3K

Another protein regulated by cAMP in melanocytes is phosphoinositol-3-kinase (PI3K). Class I PI3K consist of a regulatory subunit (p85) and a catalytic subunit (p110). PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP$_2$) to phosphatidylinositol-3,4,5-triphosphate (PIP$_3$), which interacts with downstream targets through lipid-binding domains. The most well-known effector of PI3K is AKT (also known as protein kinase B, or PKB), involved in cell proliferation and metabolism, but PI3K has other effectors as well (reviewed in Hennessy, B.T. et al., 2005).
In B16 mouse melanoma cells, stimulation with cAMP inhibits PI3K, and chemical inhibition of PI3K with LY294002 increases both melanin synthesis and dendrite outgrowth (Busca, R. et al., 1996). Similar to the PKA activation pathway, increase of melanin synthesis through inhibition of PI3K is caused by increased transcription of tyrosinase and TYRP1 through increased expression of MITF (Khaled, M. et al., 2003). The effect of PI3K on melanin synthesis is most likely carried out through PI3K’s downstream target p70S6 kinase (p70S6K), as inhibition of p70S6K increases melanin synthesis but not dendrite outgrowth (Busca, R. et al., 1996). This suggests that other proteins downstream of PI3K are responsible for the cAMP-mediated dendrite outgrowth. The most likely candidates are among the Rho family of GTPases, and their regulation of dendrite extension is discussed in section 1.2.2.2.2.1.

1.2.2.1.4.3 Ras and ERK

A third pathway regulated by cAMP is the Ras/ERK pathway. The group of Robert Ballotti has shown that, in mouse B16 melanoma cells and normal human melanocytes, cAMP-elevating agents (such as forskolin or αMSH) activate MEK-1 and MAPK (ERK) (Englaro, W. et al., 1995, Englaro, W. et al., 1998, Busca, R. et al., 2000). They further showed that this activation is mediated by Ras and B-Raf, but did not identify the guanine nucleotide exchange factor responsible for Ras activation in this pathway (Busca, R. et al., 2000). Guanine nucleotide exchange factors (GEFs) activate Ras by replacing Ras-bound GDP with GTP. This will be discussed in more detail in section 1.3.3.1, and chapter 2 describes work from our lab showing that CNrasGEF fulfills the role of cAMP-responsive Ras GEF in B16 cells.

The effect of the Ras/ERK pathway on melanin synthesis appears to be a negative feedback mechanism for the cAMP and PKA mediated increase in tyrosinase expression as outlined above. When B16 cells are treated with PD98059, a MEK-1 specific inhibitor, tyrosinase levels and activity are increased, indicating that the ERK pathway in these cells has a negative effect on melanin synthesis (Englaro, W. et al., 1998). Indeed, overexpression of dominant negative Ras or MEK-1 also increases tyrosinase expression, while constitutively active mutants of the same proteins inhibit tyrosinase expression (Englaro, W. et al., 1998).

Clues to the molecular mechanism by which the MAPK pathway downregulates tyrosinase expression have come from studies of post-translational modifications of MITF, the transcription factor for the tyrosinase gene. MAPK phosphorylates MITF at Serine 73, which increases the
activity of MITF and hence its transcription of tyrosinase (Hemesath, T.J. et al., 1998, Price, E.R. et al., 1998). This seems to be at odds with the observed downregulatory effect of the MAPK pathway on tyrosinase expression. However this same phosphorylation of MITF on Serine 73 is also essential for ubiquitination and subsequent degradation of MITF (Wu, M. et al., 2000, Xu, W. et al., 2000). Here, ubiquitination is regulated by hUBC9, which is normally an E2 enzyme for sumoylation, but mediates ubiquitination of MITF (Xu, W. et al., 2000). Collectively, these data suggest that MAPK initially induces MITF-mediated tyrosinase expression, but prolonged activation of MAPK eventually leads to downregulation of tyrosinase expression and melanin synthesis (Figure 1.2) (Busca, R., and Ballotti, R., 2000). This also indicates that there might be an initial short-term activation of melanin synthesis upon MAPK activation. However, this stimulatory effect has only been shown at the level of transcriptional activation of tyrosinase by MITF (Hemesath, T.J. et al., 1998).

Figure 1.2 shows the mechanism by which the ERK pathway regulates feedback of PKA-mediated activation of tyrosinase transcription. MAPK not only inhibits melanin synthesis after prolonged activation, but also dendrite extension, as discussed in section 1.2.2.2.2.2.

1.2.2.2 Regulation of Dendrite Extension

1.2.2.2.1 Dendrites

Dendrites in melanocytes are responsible for the delivery of melanosomes to neighbouring keratinocytes in the skin. Without dendrites, the melanocyte wouldn’t be able to interact with as many keratinocytes, and since direct contact is necessary to transfer melanosomes, this suggests that the dendritic phenotype is required for proper skin pigmentation. However, of all known mouse mutants related to pigmentation defects, none has a mutation that affects dendrite extension (Bennett, D.C., and Lamoreux, M.L., 2003). Presumably, genes that regulate dendrite extension in melanocytes are necessary for overall development, and loss-of-function mutations are embryonic lethal.
Figure 1.2. Ras/ERK pathway in melanogenesis

The melanocortin-1 receptor (MC1R) is activated by α-melanocyte-stimulating hormone (αMSH). Agouti Signaling Protein (ASP) acts as an antagonist and inverse agonist, switching the cell to the production of pheomelanin rather than eumelanin. When the MC1R is stimulated, the Gsα subunit of the G-protein coupled to MC1R activates adenylate cyclase (AC), which in turn leads to the production of cyclic AMP (cAMP). Activation of Protein Kinase A (PKA) by cAMP results in binding of CRE-binding protein (CREB) to the cAMP Regulatory Element (CRE) promoter (in the nucleus, shown in yellow), which transcribes the gene for the Microphthalmia Transcription Factor (MITF). MITF, in turn, binds to the M-Box in the promoter of the tyrosinase gene. Increased tyrosinase expression then leads to increased melanin synthesis. Activation of Ras by cAMP (through CNrasGEF, as described in Chapter 2. Indicated here with a blue arrow), leads to activation of ERK1 and ERK2 through B-Raf and MEK. ERK proteins phosphorylate MITF, leading initially to an increase in activation of tyrosinase transcription, but also flagging MITF for ubiquitination by hUbc9 and eventual degradation of the ubiquitinated protein. Removal of MITF attenuates tyrosinase transcription, reducing melanin synthesis. Prolonged inhibition of components of the Ras/ERK pathway has been shown to increase melanin synthesis, most likely by reducing the degradation of MITF.
All current knowledge of dendrite extension in melanocytes comes from cell culture studies, mostly using the B16 mouse melanoma cell line, which responds strongly to cAMP stimulation. In the absence of cAMP, B16 cells have few dendritic extensions, but upon addition of cAMP analogs the number of dendrites per cell increases dramatically (Englaro, W. et al., 1995).

1.2.2.2.2 cAMP-dependent Dendrite Extension

1.2.2.2.2.1 Rac1, RhoA, and CDC42

As mentioned above, inhibition of PI3K by cAMP stimulation has been associated with increased dendrite extension, but the pathway downstream of PI3K is unclear. The most likely candidates are members of the Rho family of small GTPases, RhoA, Rac1, and CDC42, which in many cell types are responsible for the formation of stress fibres, lamellipodia, and filipodia, respectively (Hall, A., 1998). Indeed, these proteins have all been linked to dendrite formation in melanocytes: Busca et al. showed that chemical inhibition of RhoA by Clostridium botulinum C3 exotransferase increases dendrite extension in B16-F10 cells, and overexpression of a constitutively active form of the RhoA target p160ROCK inhibits cAMP-dependent dendrite formation (Busca, R. et al., 1998). Overexpression of a constitutively active form of Rac1, on the other hand, increases dendrite extension in B16-F1 and B16-F10 cells, suggesting that Rac1 and RhoA have opposing effects on dendrite extension (Scott, G.A., and Cassidy, L., 1998, Scott, G., and Leopardi, S., 2003, Scott, G., 2002). Expression of constitutively active CDC42 in human melanocytes increases the formation of small filipodia that connect the melanocytes to keratinocytes (Scott, G. et al., 2002).

It’s not known precisely how PI3K affects RhoA, Rac1 and CDC42 in melanocytes, but PI3K-mediated dendrite extension appears to be cell type specific. For example, in mouse macrophages, activation of the catalytic subunit p110δ negatively regulates RhoA through increased phosphorylation and activation of the GTPase activating protein p190RhoGAP (Papakonstanti, E.A. et al., 2007). In that case, PI3K inhibition activates RhoA, which is the opposite from what is seen in B16 cells, where cAMP levels are linked both to inactivation of PI3K and inactivation of RhoA (Busca, R. et al., 1996, Busca, R. et al., 1998). PC12 rat pheochromocytoma cells are often used as a model for neurite outgrowth. In these cells PI3K activation induces neurite extension through RhoA inhibition and Rac1 activation, while PI3K
inhibition leads to an increase in neurite branching through AKT (Yasui, H. et al., 2001, Nusser, N. et al., 2002, Higuchi, M. et al., 2003). In B16 cells it is PI3K inhibition that leads to RhoA inhibition and Rac1 activation, but the molecular mechanisms remain to be discovered.

### 1.2.2.2.2 Ras and ERK

Prolonged stimulation of Ras and ERK not only decreases tyrosinase levels, as described above, but also decreases dendrite extension (Englaro, W. et al., 1998). How ERK affects dendricity in B16 cells is currently not known, but interactions between ERK and RhoA pathways have been shown in other cell types. For example, in Swiss-3T3 cells transformed with oncogenic (V12) Ras, ERK downregulates p160ROCK, uncoupling RhoA from its downstream effector, and thus affecting cell shape (Sahai, E. et al., 2001). Since in B16 cells prolonged activation of ERK has the same effect on dendrite outgrowth as inhibition of RhoA or p160ROCK, it’s possible that a similar cross-talk between pathways exists in melanocytes.

The overall effects of cAMP on dendrite extension and melanin synthesis are summarized in Figure 1.3

### 1.2.2.3 Other Effects on Dendrites

Melanocyte dendricity is not only regulated by pathways downstream of the melanocortin-1 receptor. For example, exposure of melanocytes to NGF (nerve growth factor) increases dendricity through the NGF receptor (Yaar, M. et al., 1991). Melanocytes from mice with reduced levels of functional Myosin Va protein appear to show a decrease in dendricity as well, but the pigmentation phenotype in these mice is linked to Myosin Va’s role in melanosome transport, as discussed below, and not to dendricity (Edgar, A.J., and Bennett, J.P., 1999, Wei, Q. et al., 1997).

Altogether, formation of dendrites in melanocytes is the least understood level of regulation of skin pigmentation, because there is no physiological phenotype associated with lack of dendrites. Mutations in mouse or human genes that affect pigmentation at the melanocyte level all seem to interfere with either melanin synthesis or melanosome formation or transport.
Figure 1.3. Regulation of dendrite extension and melanin synthesis

cAMP increases melanin synthesis through activation of PKA. Prolonged stimulation of Ras and ERK by cAMP leads to an inhibition of melanin synthesis (see also figure 1.1). Inhibition of PI3K leads to an increase in dendrite extension, most likely through Rac1 activation and RhoA inhibition (see section 1.2.2.2.2.1). Inhibition of PI3K also induces melanin synthesis, and prolonged activation of Ras/ERK increases dendrite extension, but these pathways are currently less well understood.
1.2.2.3 Regulation of Melanosome Formation and Transport

1.2.2.3.1 Melanosome Formation

1.2.2.3.1.1 Origin of Melanosomes

The term “melanosome” was coined by Seiji et al. in 1963, to describe the granular location of tyrosinase and melanin in melanocytes (Seiji, M. et al., 1963). Melanosomes are members of a group of lysosome-related organelles (LROs) – so called because they share several characteristics with lysosomes, including their origin. Like lysosomes, LROs originate from the trans-Golgi network (TGN) and early endosomes, but diverge from lysosomes at the late endosome stage (Raposo, G. et al., 2001).

1.2.2.3.1.2 Stages of Melanosome Development

Melanosomes exist in different stages, defined by shape and melanin content (Seiji, M. et al., 1963). The conventional names for the stages of melanosome development are Stage I to IV, where the first two stages are often referred to as “premelanosomes”. The transition from Stage I to Stage II melanosomes is characterized by the formation of fibrous striations, which form a matrix on which melanin is deposited in later stages. The melanosomal protein pMel17 is necessary and sufficient for this striation formation (Berson, J.F. et al., 2001). Posttranslational processing of pMel17 by furin produces a polypeptide (amino acid 25-467 of pMel17) that forms the striations in Stage II melanosomes (Berson, J.F. et al., 2003).

Proteins are sorted to the melanosome at different stages of its development, depending on their function. For example, as described above, pMel17 is necessary for the formation of striations and the transformation into Stage II melanosomes, so it is sorted to Stage I melanosomes (Raposo, G. et al., 2001, Theos, A.C. et al., 2006). TYRP1 is necessary for the later stages of eumelanin synthesis, which doesn’t occur until the melanosome has matured. Reflecting the requirement of TYRP1 in later stages of melanosomal development, it bypasses Stage I and is sorted from the trans-Golgi network to Stage II melanosomes (Raposo, G. et al., 2001).
1.2.2.3.1.3 Melanosomal Sorting Complexes

The sorting of melanosomal proteins to various stages of melanosomes is mediated by sorting complexes such as AP-3 (adaptor protein 3) and biogenesis of lysosome-related organelles complex (BLOC)-1, -2, and -3.

There are four known classes of AP complexes, which are involved in receptor-mediated endocytosis (AP-2) as well as in sorting from the trans-Golgi network to early endosomes (AP-1A), and sorting to lysosomes and lysosome-related organelles (AP-3, AP-4) (Nakatsu, F., and Ohno, H., 2003). AP-1 and AP-2 were originally identified as heterotetrameric protein complexes interacting with clathrin to form clathrin-coated vesicles (Keen, J.H., 1987). AP-3 was discovered based on cDNA homology with AP-1 and AP-2 subunits, and is involved in the sorting of proteins from the TGN or early endosomes to lysosomes and lysosome-related organelles such as melanosomes (Dell'Angelica, E.C. et al., 1997). Like other APs, AP-3 consists of four subunits: δ, β3A (which binds clathrin), μ3A and σ3. The β3A subunit is mutated in Hermansky-Pudlak Syndrome Type 2 (HPS2), which leads to hypopigmentation, prolonged bleeding, and immune defects (Huizing, M. et al., 2002, Fontana, S. et al., 2006). The pigmentation defect is caused by a missorting of melanosomal cargo, as AP-3 mediates the transport of tyrosinase from endosomes to melanosomes (Theos, A.C. et al., 2005, Richmond, B. et al., 2005). The other hallmarks of HPS2 are caused by sorting defects in platelet dense granules and T-cell lytic granules, which are also AP-3-dependent. AP-3 does not regulate the transport of all proteins destined for melanosomes. For example, TYRP1 sorting can occur independently of AP-3, but requires two other protein complexes: BLOC-1 and BLOC-2 (Setty, S.R. et al., 2007, Di Pietro, S.M. et al., 2006).

BLOCs are composed of several subunits and while Hermansky-Pudlak Syndrome 2 (HPS2) is caused by a mutation in a subunit of AP-3, all other known types of HPS (HPS1 and HPS3-8) are caused by mutations in subunits of BLOC-1, -2, and -3 (Wei, M.L., 2006). Other subunits of BLOCs found in mice have not been linked to human disease phenotypes (Li, W. et al., 2004). Table 1.1 gives an overview of the proteins that compose the four sorting complexes known to be involved in melanosome development, together with mouse strains and subtypes of Hermansky-Pudlak Syndrome related to mutations in these subunits.
<table>
<thead>
<tr>
<th>Complex</th>
<th>Subunit</th>
<th>Mouse strain</th>
<th>Hermansky-Pudlak Syndrome Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-3</td>
<td>β3A</td>
<td>Pearl</td>
<td>HPS2</td>
</tr>
<tr>
<td></td>
<td>δ</td>
<td>Mocha</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>μ3</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>σ3</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>BLOC-1</td>
<td>Pallidin</td>
<td>Pallid</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>Cappuccino</td>
<td>Cappuccino</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>Muted</td>
<td>Muted</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>BLOS3</td>
<td>Reduced pigmentation</td>
<td>HPS8</td>
</tr>
<tr>
<td></td>
<td>Dysbindin</td>
<td>Sandy</td>
<td>HPS7</td>
</tr>
<tr>
<td></td>
<td>Snapin</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>BLOS1</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>BLOS2</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>BLOC-2</td>
<td>HPS3</td>
<td>Cocoa</td>
<td>HPS3</td>
</tr>
<tr>
<td></td>
<td>HPS5</td>
<td>Ruby eye-2</td>
<td>HPS5</td>
</tr>
<tr>
<td></td>
<td>HPS6</td>
<td>Ruby eye</td>
<td>HPS6</td>
</tr>
<tr>
<td>BLOC-3</td>
<td>HPS1</td>
<td>Pale ear</td>
<td>HPS1</td>
</tr>
<tr>
<td></td>
<td>HPS4</td>
<td>Light ear</td>
<td>HPS4</td>
</tr>
</tbody>
</table>

**Table 1.1**  Components of sorting complexes

AP-3 and BLOCs consist of multiple subunits, which are mutated in the eight known types of Hermansky Pudlak Syndrome and in several mouse coat colour variations. However, so far not all subunits have been linked to mouse coat colour mutations or Hermansky-Pudlak Syndrome, indicated by question marks in the table.
AP-3 and the BLOCs interact with each other both physically and genetically. Co-immunoprecipitation studies have shown that BLOC-1 interacts with both BLOC-2 and AP-3, but AP-3 and BLOC-2 do not interact with each other, while BLOC-3 does not interact with any of the other complexes (Di Pietro, S.M. et al., 2006). The study of double and triple mutant mice, in which multiple BLOCs and/or AP-3 were deficient, does show an additional lightening of coat colour in mice with defects in AP-3 or BLOC-2 in combination with BLOC-3 (Gautam, R. et al., 2006). Double mutants of AP-3 and BLOC-2 show a phenotype reminiscent of BLOC-1 single mutants, both at the melanocyte level as well as in mouse coat colour (Di Pietro, S.M. et al., 2006, Gautam, R. et al., 2006). This suggests that AP-3 and BLOC-2 function in separate pathways downstream of BLOC-1. However, AP-3 can also act independently of BLOC-1, for example in the sorting of tyrosinase (Huizing, M. et al., 2001). As mentioned above, TYRP1 sorting is independent of AP-3 but requires BLOC-1 and BLOC-2. The sorting pathway of pMel17, which is required in Stage I melanosomes, is independent of BLOC-2 component HPS5 (Helip-Wooley, A. et al., 2007). Other than targeting proteins involved in melanin synthesis, AP-3 and BLOC-1 are also responsible for the correct targeting of fusion proteins necessary for melanosome formation, such as the R-(v)-SNARE VAMP-TI (Salazar, G. et al., 2006).

Figure 1.4 summarizes the current understanding of some of the sorting pathways discussed here. The precise mechanisms by which distinct proteins are sorted to different stages of melanosomes by AP-3 or BLOCs is not yet understood.
Figure 1.4. Melanosome development

A. Proteins are sorted to different stages of melanosomes by a number of protein complexes. BLOC-1 and BLOC-2, but not AP-3, are required for the sorting of TYRP1 to melanosomes. BLOC-1 and AP-3 sort tyrosinase to melanosomes, but tyrosinase is also properly sorted in the absence of BLOC-1, by AP-3 alone. pMel17 is sorted to premelanosomes, and requires BLOC-1.

B. Schematic representation of stages of melanosomes. Stage I melanosomes (premelanosomes or coated endosomes) convert to Stage II melanosomes with the formation of fibrous striations. Stage III and IV melanosomes are defined by increased melanin levels.
1.2.2.3.2 Melanosome Transport

The function of melanosomes in skin pigmentation requires not only their proper formation, but also their transport within the cell. Melanosomes are transported along microtubules towards actin near the plasma membrane and in the tips of dendrites, from where they are transferred to keratinocytes (Scott, G. et al., 2002). In melanocytes, the transport of melanosomes is carried out by a complex of three proteins: Rab27A, Myosin Va and melanophilin (Fukuda, M. et al., 2002). The names of the mouse strains that have mutations in these genes reflect their grey coat colour: ashen (Rab27A), leaden (melanophilin), and dilute (MyoVa) (Matesic, L.E. et al., 2001, Wilson, S.M. et al., 2000, Mercer, J.A. et al., 1991). These mice produce normal amounts of melanin, and melanosomes are properly formed, but melanosomes are clustered near the nucleus of the melanocyte, and do not reach the cell membrane or keratinocytes.

1.2.2.3.2.1 Mechanism of Melanosome Transport

Rab27A is a GTPase associated with the melanosome membrane (Gomes, A.Q. et al., 2003). A S1p homology domain (SHD) in melanophilin interacts with the Switch I region in Rab27A, recruiting melanophilin to the melanosomes (Westbroek, W. et al., 2008). Another domain in melanophilin, the Myosin Va binding domain (MBD) in turn binds Myosin Va (Kuroda, T.S. et al., 2005, Provance, D.W. et al., 2002). Myosin Va is a two-headed processive motor, capable of ATP-dependent stepwise movement along F-actin tracks (Vale, R.D., 2003, Desnos, C. et al., 2007a). Together, Rab27A, melanophilin and Myosin Va form a complex which is sufficient for in vitro transport along F-actin (Figure 1.5) (Wu, X. et al., 2006). Melanophilin not only serves as a linker between Rab27A and Myosin Va, but its interaction with Myosin Va also increases the ATPase activity of Myosin Va required for movement (Li, X.D. et al., 2005). In addition, melanophilin has an actin-binding domain (ABD) with which it can directly bind to actin. In B16 cells, this interaction is stimulated by cAMP, acting through RhoA (Passeron, T. et al., 2004). A melanophilin mutant without the actin binding domain is incapable of normal melanosome transport (Kuroda, T.S. et al., 2003). However, a coiled coil region within the ABD enhances binding of Myosin Va to the MBD of melanophilin, so it is not clear to what extent melanophilin’s interaction with actin is required for transport (Hume, A.N. et al., 2006).
Figure 1.5. Melanosome on F-actin

Schematic diagram showing the complex of Rab27A, melanophilin, and Myosin Va that transports melanosomes along actin. Rab27A binds to the melanosome membrane by prenylation motifs. Melanophilin acts as a linker between Ras27A and Myosin Va, and activates the ATPase activity of Myosin Va that drives movement along actin.
Myosin Va is responsible for the movement of melanosomes along actin, but does not interact directly with microtubules. Nevertheless, melanocytes in *dilute* mice, lacking Myosin Va, show reduced movement of melanosomes along microtubules. It appears that even though Myosin Va does not directly regulate transport along microtubules, it influences the direction in which melanosomes travel (da Silva Bizario, J.C. et al., 2002). In this model, melanosomes travel along microtubules in both directions – outward and inward – but the recruiting of Myosin Va by melanophilin pulls the melanosomes from the microtubule towards actin, favouring outward movement. The outward movement of melanosomes on microtubules is regulated by the interaction of melanophilin with EB1, a microtubule plus end-tracking protein (+TIP) (Wu, X.S. et al., 2005). Through this interaction, melanosomes travel on the tip of growing microtubules. Furthermore, in *Xenopus laevis* melanophores, melanosomes are transported outward along microtubules by kinesin II (Tuma, M.C. et al., 1998). Zebrafish studies have shown that movement along microtubules towards the nucleus is mediated by dynein, and that melanophilin inhibits this retrograde transport, thus shifting the overall transport of melanosomes outward towards the plasma membrane and dendrites (Sheets, L. et al., 2007).

### 1.2.2.3.2.2 Griscelli Syndrome

Griscelli Syndrome is a disease characterized by mutations in Rab27A, Myosin Va or melanophilin. While all mutations lead to hypopigmentation, mutations in any of the three proteins cause different secondary symptoms. Griscelli Syndrome Type 1 is caused by a mutation in Myosin Va (Pastural, E. et al., 1997). In addition to a pigmentation defect, patients of Griscelli Syndrome Type 1 also suffer from neurological impairment (Ménasché, G. et al., 2002, Pastural, E. et al., 2000). Griscelli Type 2, caused by a mutation in Rab27A, does not include neurological symptoms, but patients are immunodeficient (Pastural, E. et al., 2000, Ménasché, G. et al., 2000, Stinchcombe, J. et al., 2004). Finally, Griscelli Syndrome Type 3 is characterized by hypopigmentation alone, without neurological or immunological symptoms. This last type of Griscelli Syndrome is caused by mutations in melanophilin, or by a mutation in the F-exon of Myosin Va (Ménasché, G. et al., 2003).

Intracellular movement of other lysosome-related organelles is regulated in a similar manner as melanosome transport. The differences in symptoms between the three types of Griscelli Syndrome can be explained in terms of tissue-specific protein functions: Myosin Va is not only
involved in the transport of melanosomes in melanocytes, but also in the trafficking of secretory granules in neuroendocrine cells (Desnos, C. et al., 2007b, Eichler, T.W. et al., 2006). Here Myosin Va is shown to be involved in Ca^{2+}-regulated exocytosis by interaction with the T-SNARE syntaxin-1A (Watanabe, M. et al., 2005). A mutation in this protein therefore causes both a pigmentation defect and neurological impairment. However, Myosin Va is not involved in lytic granule secretion in cytotoxic T-lymphocytes, explaining the absence of immunological symptoms in Griscelli Syndrome Type 1 (Bossi, G. et al., 2005). Rab27A, however, is required for secretion of lytic granules, and its mutation leads to the immunological defects in Griscelli Syndrome Type 2 (Stinchcombe, J.C. et al., 2001). Although Rab27A is also required for the transport of secretory granules in neuroendocrine cells, its function can be taken over by another protein, Rab27B, and mutation in Rab27A therefore does not lead to the neurological symptoms seen in Griscelli Syndrome Type 1 (Chen, Y. et al., 2002). Rab27B is not expressed in cytotoxic T-lymphocytes, thus explaining the immunodeficient phenotype of Type 2 patients (Barral, D.C. et al., 2002). Griscelli Syndrome Type 3 is limited to decreased pigmentation, without other symptoms, suggesting that expression of melanophilin and the F-exon of Myosin Va is limited to melanocytes, or that their function is taken over by other proteins in other cell types (Ménasché, G. et al., 2003). Indeed, in retinal pigment epithelial (RPE) cells, melanosome transport is mediated by a different complex, consisting of Rab27A, MyRIP, and Myosin VIIa (Kuroda, T.S., and Fukuda, M., 2005, Lopes, V.S. et al., 2007). MyRIP is also involved in the exocytosis of insulin from pancreatic β-cells (Waselle, L. et al., 2003). Melanophilin has, however, been found in mouse cortical collecting ducts, where it is involved in aldosterone-induced trafficking of the epithelial sodium channel ENaC, but this has not been linked to a phenotype of Griscelli Syndrome Type 3 (Martel, J.A. et al., 2007).

1.2.2.3.2.3 Interaction of Melanosome Transport with Other Pigmentation Pathways

Regulation of melanosome transport is not entirely independent of the regulation of other aspects of pigmentation, such as melanin synthesis or dendrite extension. Initial analysis of dilute mouse melanocytes suggested that the Myosin Va mutation caused decreased dendricity as well as reduced melanosome transport, but this was later disproved (Wei, Q. et al., 1997). Nevertheless, a reduction in dendricity has also been reported in an experiment where Myosin Va was knocked down (Edgar, A.J., and Bennett, J.P., 1999). A potential molecular mechanism for this reported
and disputed dendricity defect in Myosin Va deficit cells is currently unknown. In B16 cells, the interaction of melanophilin with actin is stimulated by inhibition of RhoA, which also increases dendrite extension through actin remodeling (Busca, R. et al., 1998, Passeron, T. et al., 2004). Finally, there are links between melanin synthesis and melanosome transport. Rab27A is transcribed by MITF, connecting melanosome transport and melanin synthesis (Chiaverini, C. et al., 2008). In addition, melanosome transport along microtubules in *Xenopus* melanophores is inhibited in both directions upon inhibition of ERK (Deacon, S.W. et al., 2005). It is evident that even though pigmentation can be considered as separate steps (melanin synthesis, dendrite extension, melanosome formation and melanosome transport), mutations in proteins controlling one aspect of pigmentation may also affect another.

1.2.3 Pigmentation-Related Diseases

The previous sections have mentioned several pigmentation-related disease phenotypes. Lack of pigmentation can be the result of a defect in melanocyte development, as is the case in Piebaldism or Waardenburg Syndrome (Fleischman, R.A. et al., 1991, Bondurand, N. et al., 2000). Mutations in genes required for melanin synthesis, such as tyrosinase or TYRP1, cause oculocutaneous albinism (OCA) (Tomita, Y., and Suzuki, T., 2004, King, R.A. et al., 2003). While OCA has no other symptoms than albinism, mutations affecting melanosome formation or transport also affect other organs that depend on lysosome-related organelles (Tomita, Y., and Suzuki, T., 2004, Wei, M.L., 2006, Ménasché, G. et al., 2002, Ménasché, G. et al., 2003). There are no known pigmentation disorders related to the formation of dendrites.

Table 1.2 provides a summary of several diseases and corresponding mutated proteins that have been discussed in this chapter.
<table>
<thead>
<tr>
<th>Disease</th>
<th>Mutated protein</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Melanocyte development</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Piebaldism</td>
<td>c-Kit</td>
<td>lack of pigmentation on the forehead, chest, abdomen, and extremities</td>
</tr>
<tr>
<td>Waardenburg Syndrome</td>
<td>MITF*</td>
<td>Piebaldism with hearing loss</td>
</tr>
<tr>
<td><strong>Melanin Synthesis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oculocutaneous albinism (OCA) 1</td>
<td>Tyrosinase</td>
<td>Hypopigmentation</td>
</tr>
<tr>
<td>Oculocutaneous albinism (OCA) 3</td>
<td>TYRP1</td>
<td>Hypopigmentation</td>
</tr>
<tr>
<td><strong>Melanosome Formation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hermansky-Pudlak Syndrome Type 1</td>
<td>HPS1</td>
<td>Hypopigmentation, prolonged bleeding</td>
</tr>
<tr>
<td>Hermansky-Pudlak Syndrome Type 2</td>
<td>AP-3</td>
<td>Hypopigmentation, prolonged bleeding, immune defects</td>
</tr>
<tr>
<td>Hermansky-Pudlak Syndrome Type 3</td>
<td>HPS3</td>
<td>Mild hypopigmentation and decreased vision</td>
</tr>
<tr>
<td>Hermansky-Pudlak Syndrome Type 4</td>
<td>HPS4</td>
<td>Similar to HPS1</td>
</tr>
<tr>
<td>Hermansky-Pudlak Syndrome Type 5</td>
<td>HPS5</td>
<td>Mild hypopigmentation and bleeding disorder</td>
</tr>
<tr>
<td>Hermansky-Pudlak Syndrome Type 6</td>
<td>HPS6</td>
<td>**</td>
</tr>
<tr>
<td>Hermansky-Pudlak Syndrome Type 7</td>
<td>Dysbindin</td>
<td>**</td>
</tr>
<tr>
<td>Hermansky-Pudlak Syndrome Type 8</td>
<td>BLOS3</td>
<td>**</td>
</tr>
<tr>
<td>Chediak-Higashi Syndrome</td>
<td>LYST</td>
<td>Hypopigmentation, immune defects, bleeding disorder, neurological symptoms</td>
</tr>
<tr>
<td><strong>Melanosome Transport</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Griscelli Syndrome Type 1</td>
<td>Myosin Va</td>
<td>Hypopigmentation and neurological impairment</td>
</tr>
<tr>
<td>Griscelli Syndrome Type 2</td>
<td>Rab27A</td>
<td>Hypopigmentation and immune defect</td>
</tr>
<tr>
<td>Griscelli Syndrome Type 3</td>
<td>Melanophilin***</td>
<td>Hypopigmentation</td>
</tr>
</tbody>
</table>

* Waardenburg Syndrome is also caused by mutations in the transcription factors SOX10 or PAX3
** Not all clinical phenotypes of variations of Hermansky Pudlak syndrome are well-described.
*** Griscelli Syndrome Type 3 is also caused by mutations in the tissue-specific F-exon of Myosin Va.

Table 1.2 A summary of several diseases caused by mutations in proteins discussed in this chapter.
1.3 CNrasGEF

1.3.1 Discovery of CNrasGEF

CNrasGEF was identified in our lab in a screen of a 16 day mouse embryo expression library for proteins interaction with the second WW domain of rat Nedd4. Nedd4 is an E3 ubiquitin ligase containing three or four WW domains, which bind substrates that contain PY motifs (Ingham, R.J. et al., 2004, Kanelis, V. et al., 2006, Kanelis, V. et al., 2001). Our lab has previously carried out a screen of a 16-day mouse embryo expression library for proteins that bind the WW2 domain of human Nedd4. Among 17 positive hits of this screen was a mouse homolog of an at the time unknown human gene (KIAA0313), encoding a putative Ras guanine exchange factor (Pham, N. et al., 2000). This protein has been renamed CNrasGEF (cyclic nucleotide Ras guanine exchange factor) (Pham, N. et al., 2000).

Three other groups identified CNrasGEF simultaneously through other screens: Ohtsuka et al. identified the protein from a fetal mouse brain expression library screen as a binding partner for synaptic scaffolding molecule (S-SCAM). They named the protein nRapGEP (neural Rap guanine exchange protein) (Ohtsuka, T. et al., 1999). A yeast two hybrid screen for Ras-binding proteins in Caenorhabditis elegans, carried out by Liao et al., identified the C.elegans protein Ce-RA-GEF as a homolog of human KIAA0313 (Liao, Y. et al., 1999). Finally, De Rooij et al. identified KIAA0313 in a database search of the human genome as a potential Rap1 guanine exchange factor, and named the protein PDZ-GEF1 (de Rooij, J. et al., 1999).

For consistency, the protein will be referred to as CNrasGEF for the remainder of this thesis.

1.3.2 Domain Architecture and Tissue Expression of CNrasGEF

1.3.2.1 Domain Architecture

Human CNrasGEF is a ~180 kDa protein, containing several domains and motifs as shown in Figure 1.6. Starting from the N-terminus, these domains are: a cyclic nucleotide binding domain (cNMP-BD), Ras exchange motif (REM), PDZ domain, Ras-association (RA) domain, CDC25-related GEF domain, two PY motifs (PPGY and PPDY) and a carboxy-terminal SxV (PDZ-binding) motif (Pham, N. et al., 2000, Ohtsuka, T. et al., 1999, Liao, Y. et al., 1999, de Rooij, J. et al., 1999).
Figure 1.6. Domain architecture of CNrasGEF

From N-terminus to C-terminus, CNrasGEF contains a cyclic nucleotide binding domain (cNMP-BD), Ras Exchange Motif (REM), Ras association (RA) domain, CDC25 domain, two PY motifs, and an SxV (PDZ-binding) motif.
1.3.2.2 Tissue Expression and Conservation between species

CNrasGEF is predominantly expressed in the brain and in other tissues of neural origin (Pham, N. et al., 2000, Ohtsuka, T. et al., 1999). However, it is also found in other tissues, including heart, placenta, lung, liver, skeletal muscle, kidney, and pancreas (de Rooij, J. et al., 1999).


The high level of conservation and widespread tissue distribution suggest that CNrasGEF may play an important role in one or more biological processes.

1.3.3 Regulation of CNrasGEF by Nedd4

1.3.3.1 Ubiquitination

Ubiquitination is the covalent attachment of ubiquitin (a 76 aa polypeptide) to proteins. Ubiquitin is conjugated by its C-terminal carboxy group to the ε-amino group of a lysine residue in the substrate (Pickart, C.M., 2001). Ubiquitin can itself be ubiquitinated, allowing a chain of ubiquitin molecules to be attached to the same lysine residue on the substrate protein (polyubiquitination). Polyubiquitinated proteins are recognized and degraded by the 26S proteasome (Weissman, A.M., 2001). Through this pathway, ubiquitination is a means of regulating the total cellular levels of a protein. This happens, for instance, in the regulation of the total levels of MITF in cAMP-regulated melanin synthesis in melanocytes (see section 1.2.2.1.4.3) (Xu, W. et al., 2000). However, ubiquitination regulates other processes as well. While addition of a chain of four or more ubiquitin proteins to a lysine residue targets a protein to the proteasome, the addition of one ubiquitin molecule (mono-ubiquitination) has been shown
to be involved in processes such as internalization of membrane receptors, endosomal and lysosomal sorting, and histone regulation (Staub, O., and Rotin, D., 2006, Hicke, L., 2001b).

The addition of a ubiquitin group to a lysine residue requires the sequential action of three enzymes: activating enzyme (E1), conjugating enzyme (E2) and ubiquitin ligase (E3). E1 activates the C terminus of ubiquitin for nucleophilic attack, E2 carries the activated ubiquitin molecule as a conjugated thiol ester, and E3 transfers the activated ubiquitin from E2 to the substrate (Pickart, C.M., 2001). There is only one E1 gene, but there are multiple E2 and E3 enzymes. The most variation is found in E3 enzymes, which are required to recognize a range of substrates. E3 ubiquitin ligases can be divided into two groups: RING finger E3 ligases and HECT domain E3 ligases. RING (Really Interesting New Gene) finger E3 ligases exist as a monomeric polypeptide or a multimeric protein complex. Both types of RING finger E3 ligases mediate the transfer of ubiquitin from E2 to the substrate (Weissman, A.M., 2001). HECT domain E3 ligases consist of one polypeptide chain, and covalently bind ubiquitin before transferring it to the substrate. One of the best described classes of HECT domain E3 ligases is the group of C2-WW-HECT E3 ligases, of which Nedd4 is a member (Kee, Y., and Huibregtse, J.M., 2007).

1.3.3.2 Nedd4

Nedd4 is conserved among several species, and has a lipid-binding C2 domain, three or four WW domains, and a catalytic HECT domain. The WW domains recognize substrates by their PY motifs (PPxY). Not all Nedd4 proteins contain the same WW domains. For example, human Nedd4-1 contains four WW domains, but its rat homolog lacks the third WW domain (Henry, P.C. et al., 2003).

Substrates of human Nedd4 studied in our lab include the epithelial sodium channel ENaC, LAPTM5, and CNrasGEF (described below) (Henry, P.C. et al., 2003, Staub, O. et al., 2000, Lu, C. et al., 2007, Pak, Y. et al., 2006, Pham, N., and Rotin, D., 2001). Other known substrates of Nedd4 include, for example, melanosomal protein MART-1, which is redistributed from melanosomes to lysosomes upon ubiquitination (Levy, F. et al., 2005).
1.3.3.3 CNrasGEF is a Nedd4 Substrate

CNrasGEF was identified by its interaction with the second WW domain of human Nedd4 (Pham, N. et al., 2000). Further analysis has shown that CNrasGEF is indeed a substrate of ubiquitination by Nedd4: Ubiquitination of CNrasGEF is increased upon overexpression of wild-type Nedd4, but inhibited in cells overexpressing a catalytically inactive mutant of Nedd4. Furthermore, the half-life of CNrasGEF that lacks PY motifs (and is therefore unable to bind Nedd4) is prolonged from 10 to 14 hours (Pham, N., and Rotin, D., 2001). The physiological function of regulation of CNrasGEF levels is currently not understood, although it is known that binding of Ras or Rap1 to the catalytic domain (but not necessarily their activation) is required for ubiquitination of CNrasGEF by Nedd4 (Pham, N., and Rotin, D., 2001).

1.3.4 Function of CNrasGEF

1.3.4.1 Guanine Nucleotide Exchange Factors

The Ras superfamily of small GTPases includes the Ras, Rho, Arf, Rab, and Ran families. These proteins carry out a number of different cellular functions. The Ras family (including Ras, Rap and Ral proteins) is involved in cell growth and differentiation. The Rho family (RhoA, Rac1, and CDC42) regulates cytoskeletal organization. The family of Arf (ADP ribosylation factor) GTPases regulates the formation of coated vesicles. Ran regulates nuclear import and export, and the Rab family mediates vesicular transport (Wennerberg, K. et al., 2005). In the previous sections on pigmentation, many of these small GTPases have been discussed in terms of their role in melanocytes: Ras is part of a pathway leading to the phosphorylation of the transcription factor MITF, RhoA inhibition in melanocytes leads to dendrite extension, and Rab27a is part of a molecular complex that transports melanosomes to the tips of dendrites. In addition, Arf1 plays a role in melanosome sorting by mediating the targeting of AP-3 to membranes (Ooi, C.E. et al., 1998). In total, the Ras GTPase superfamily in humans comprises approximately 150 proteins. Table 1.3 gives some examples of GTPases in each family.

All small GTPases bind GTP or GDP, and are only active in the GTP-bound state (Figure 1.7) (Vetter, I.R., and Wittinghofer, A., 2001). Two regions of the GTPase, called Switch I and Switch II, interact with the γ-phosphate group of GTP, which pulls both Switch regions into the
The Ras superfamily of small GTPases is divided into five families: Ras, Rho, Ran, Rab, and Arf. Several family members are shown in this summarized overview. In total there are more than 150 human small GTPases.

<table>
<thead>
<tr>
<th>Family</th>
<th>Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ras</td>
<td>Classic Ras: H-Ras, N-Ras, K-Ras, R-Ras, RalA, Rap1, Rap2</td>
</tr>
<tr>
<td></td>
<td>Rho</td>
</tr>
<tr>
<td></td>
<td>Ran</td>
</tr>
<tr>
<td></td>
<td>Rab</td>
</tr>
<tr>
<td></td>
<td>Arf</td>
</tr>
</tbody>
</table>
Figure 1.7. Activation and deactivation of Ras GTPases

Ras is active in the GTP bound state. Hydrolysis of GTP to GDP deactivates the protein. This reaction is spontaneous, but is accelerated by a GTPase activating protein (GAP). Replacement of GDP by GTP is catalyzed by guanine exchange factors (GEF), which release bound GDP. Both GDP and GTP can bind the Ras protein, but a high local concentration of GTP leads to an overall net displacement of GDP by GTP upon GEF catalysis.
active conformation. GTP hydrolysis to GDP releases the γ-phosphate group, and the conformation switches to the inactive state (Hall, B.E. et al., 2002). GTP hydrolysis occurs spontaneously, but is accelerated by GTPase activating protein (GAP). Activation of Ras GTPases requires the replacement of GDP by GTP. This reaction is mediated by guanine nucleotide exchange factors (GEFs) (Cherfils, J., and Chardin, P., 1999). GDP and GTP both bind the small Ras GTPase by interacting with a P loop (phosphate binding loop) in the protein. A Mg$^{2+}$ ion further increases binding affinity. The GEF catalyzes removal of the guanine nucleotide by interacting with the Switch regions and causing a conformational change. That leads to removal of the Mg$^{2+}$ ion and disturbance of the P loop, eventually releasing GDP. After removal of GDP and the GEF, GTP spontaneously re-enters the binding site, bringing the Ras GTPase back to the active state (Guo, Z. et al., 2005).

There is great variation in types of GEFs, but within subfamilies there is some conservation of particular protein domains: GEFs for Ras proteins have a CDC25 domain, while all Rho-GEFs have a DH (Dbl homology) domain (Vetter, I.R., and Wittinghofer, A., 2001).

1.3.4.2 CNrasGEF is a Ras- and Rap-GEF

1.3.4.2.1 Ras

Within the Ras family of small GTPases, the Ras subfamily consists of the products of three genes, H-Ras, N-Ras, and K-Ras, which are mutated in over 30% of human cancers (Boettner, B., and Van Aelst, L., 2002, Crespo, P., and Leon, J., 2000). Ras is involved in many intracellular signaling pathways, mainly regulating proliferation and differentiation. Among the known Ras effectors are Raf, PI3K, and Ral-GDS (Crespo, P., and Leon, J., 2000, Rodriguez-Viciana, P. et al., 1994). Activation of downstream targets of Ras varies with cell type, depending in part on variation in expression of H-Ras, N-Ras, or K-Ras (Voice, J.K. et al., 1999, Hamilton, M., and Wolfman, A., 1998). In addition, species specific variations control which effectors are activated: the same activating mutation in oncogenic H-Ras acts through the Raf pathway in mice and the RalGDS pathway in humans (Boettner, B., and Van Aelst, L., 2002, Hamad, N.M. et al., 2002). This makes the study of Ras signaling pathways complex, as cell type specificity should always be considered (Shields, J.M. et al., 2000).
1.3.4.2.1.1 Ras Guanine Nucleotide Exchange Factors

All GEFs of the superfamily of Ras GTPases function according to the mechanism described in section 1.3.4.1. There are currently four known guanine nucleotide exchange factors for the subfamily of Ras proteins: Sos, RasGRF, RasGRP, and CNrasGEF (Pham, N. et al., 2000, Chardin, P. et al., 1993, Farnsworth, C.L. et al., 1995, Ebinu, J.O. et al., 1998). The most well-studied Ras GEF is Sos (mammalian homolog of son-of-sevenless), which signals downstream of receptor tyrosine kinases. Autophosphorylation of these receptors forms a binding site for the protein Grb2, which then recruits Sos to the plasma membrane, where it activates Ras (Buday, L., and Downward, J., 1993). RasGRF proteins (RasGRF1 (or CDC25Mm) and RasGRF2) are involved in neuronal differentiation, and activate Ras in response to Ca\(^{2+}\) stimulation (Farnsworth, C.L. et al., 1995, Fam, N.P. et al., 1997). RasGRFs can also activate the Rho family of GTPases though a DH domain (Yang, H., and Mattingly, R.R., 2006). Four members of the family of RasGRP (Ras guanine release protein) are expressed predominantly in haematopoietic cells, where RasGRP1 is involved in processes such as thymocyte differentiation (Ebinu, J.O. et al., 1998, Ebinu, J.O. et al., 2000, Dower, N.A. et al., 2000). The fourth known type of Ras GEF is CNrasGEF, which is described in more detail in the following section.

1.3.4.2.1.2 CNrasGEF Activates Ras in Response to cAMP

Work from our lab has shown that CNrasGEF is able to activate the small GTPase Ras, and that this activation is dependent on binding of cyclic nucleotide cAMP to the cNMP-BD of CNrasGEF (Pham, N. et al., 2000, Pak, Y. et al., 2002). Overexpressed wild-type CNrasGEF in HEK-293T cells activates Ras in the presence of 8-Br-cAMP, a cAMP analog. This activation does not occur upon deletion of either the cNMP-BD or CDC25 domain (Pham, N. et al., 2000). The CNrasGEF PDZ domain is involved in binding of CNrasGEF to the SxV motif of the \(\beta1\) adrenergic receptor (\(\beta1\)-AR). This G protein-coupled receptor activates adenylate cyclase through Gs\(\alpha\) upon agonist activation. In cells overexpressing both the \(\beta1\)-AR and CNrasGEF, stimulation of the receptor with its agonist isoproterenol leads to Ras activation. This activation is blocked by either absence of isoproterenol, deletion of the CDC25 domain, deletion of the cNMP-BD, or mutation of the SxV motif (VA) of the \(\beta1\)-AR, indicating that there is indeed a direct interaction between the receptor and CNrasGEF that mediates Ras activation (Pak, Y. et al., 2002).
Until recently, information about the role of CNrasGEF as a Ras GEF has come from overexpression studies. In Chapter 2 I will present experiments showing Ras activation by endogenous CNrasGEF in melanocytes.

1.3.4.2.2 Rap1

Rap1 was originally identified as a gene that reverts the phenotype of cells oncogenically transformed by K-Ras. This observation, combined with a high sequence similarity between Ras and Rap1, has led to the long-held assumption that Rap1 is merely a Ras antagonist, competing with Ras for the same downstream effectors. However, in recent years Ras-independent Rap1 signaling pathways have been elucidated. Most notably, Rap1 is involved in the regulation of integrin-mediated cell adhesion and the formation of cell-cell junctions (Caron, E., 2003, Bos, J.L. et al., 2001, Tsukamoto, N. et al., 1999, Kooistra, M.R. et al., 2007, Rangarajan, S. et al., 2003).

1.3.4.2.2.1 Rap1 activation by CNrasGEF

CNrasGEF not only activates Ras, but it is also a guanine nucleotide exchange factor for Rap1 (Pham, N. et al., 2000, Ohtsuka, T. et al., 1999, Liao, Y. et al., 1999, de Rooij, J. et al., 1999). However, unlike Ras activation, Rap1 activation by CNrasGEF is independent of cAMP binding (Pham, N. et al., 2000).

The only known downstream effector pathway of CNrasGEF-activated Rap1 is the ERK pathway. The *Drosophila* homolog of CNrasGEF (dPDZ-GEF) activates the MAPK pathway through Rap1, independently of Ras (Lee, J.H. et al., 2002). In rat hippocampal neurons, CNrasGEF has also been reported to activate ERK through Rap1 in response to activation of the TrkA receptor, leading to neurite extension in these cells (Hisata, S. et al., 2007). This response to ERK is the opposite of that observed in melanocytes, where prolonged ERK inhibition (not activation) leads to dendrite extension (see section 1.2.2.2.2.2).

1.3.4.2.2.2 Epac

Although Rap1 activation by CNrasGEF is independent of cAMP, a cAMP-dependent Rap1 GEF does exist: Epac (exchange protein directly activated by cAMP) has a structure that resembles that of CNrasGEF, with both a cNMP-BD and CDC25 domain, and activates Rap1 in
response to direct cAMP binding (de Rooij, J. et al., 1998). One interesting difference between
the cAMP-dependent regulation of the GEF activity of CNrasGEF and Epac is the effect seen
upon deletion of the cNMP-binding domain: When the cNMP-BD is deleted, CNrasGEF no
longer activates Ras, even in the presence of cAMP, while Epac becomes a constitutive activator
of Rap1 when the cNMP-BD is removed (Pham, N. et al., 2000, de Rooij, J. et al., 1998).

1.3.4.3 Function of CNrasGEF in Melanocytes

In melanocyte pigmentation, activation of Ras/ERK leads to an inhibition of melanin synthesis
and dendrite extension, as described in section 1.2.2.1.4.3. In Chapter 2 I show that CNrasGEF is
the Ras GEF responsible for the cAMP-dependent activation of Ras in melanocytes. To study the
effect of endogenous CNrasGEF in melanocytes, I used RNA interference. This technique is
described in detail in the next section.
1.4 RNA Interference

1.4.1 Tools for Mammalian RNAi

Gene silencing by long double-stranded RNA (dsRNA) has long been used to specifically knock down target genes in *D. melanogaster* and *C. elegans*. In mammals, long dsRNA leads to the interferon response, in which the dsRNA activates protein kinase PKR, which leads to non-specific mRNA degradation and inhibition of translation (Manche, L. et al., 1992). The discovery that the mediator of RNA interference in flies and worms is a short (21-22 nucleotides) RNA fragment rather than the full-length dsRNA, has led to the development of a working, target-specific, mammalian RNAi tool in the form of small interfering RNA (siRNA) (Elbashir, S.M. et al., 2001).

To use siRNA as a knockdown tool, the siRNA is chemically synthesized to be complementary to a ~21 bp region of the mRNA of the gene to be silenced. After transfection, siRNA is processed by the RNAi machinery of the cell, and one strand of the duplex is incorporated into RISC (RNA-induced silencing complex). The loaded RISC recognizes the region in the mRNA with a sequence complementary to the siRNA, and cleaves the target mRNA (Filipowicz, W., 2005).

A drawback of siRNA is that it is short-lived, and cannot be used to study long-term effects. To this end, small hairpin RNA (shRNA) vectors have been developed. Such vectors contain a stretch of DNA that, when transcribed in the cell, forms an RNA hairpin. The hairpin is cleaved by the RNase III enzyme Dicer to generate siRNA, which is then incorporated into RISC as described above. The advantages of shRNA over siRNA include the ability to stably incorporate the hairpin DNA, allowing for long-term knockdown, as well as the possibility of tracking transfected cells by expressing a fluorescent marker protein from the same construct (Cullen, B.R., 2006). In Chapter 2 I describe the use of the shRNA vector pSuper-EGFP to study the function of CNrasGEF in melanocytes.

1.4.2 Off-target Effects

Both the use of siRNA and shRNA can lead to off-target effects (Cullen, B.R., 2006). Two types of off-target effects are common to RNA-interference:
1. Non-specific immune responses, such as the interferon response (described above). Even though the interferon response is not as prevalent in siRNA and shRNA as it is with dsRNA, it may still be activated, especially when too much siRNA or shRNA is present in the cell.

2. Sequence-specific off-target effects. One or two mismatches in siRNA or shRNA may still interact with mRNA and knock down the corresponding gene. This means that siRNA/shRNA designed against one gene can cross-react with a sequence in another gene that differs by only a few nucleotides.

1.4.3 shRNA Libraries

The development of large scale mammalian siRNA and shRNA libraries has made it possible to study knockdown phenotypes in high-throughput systems (Bernards, R. et al., 2006, Buchholz, F. et al., 2006, Root, D.E. et al., 2006, Paddison, P.J. et al., 2004, Westbrook, T.F. et al., 2005, Silva, J.M. et al., 2005, Schlabach, M.R. et al., 2008, Moffat, J. et al., 2006, Kittler, R. et al., 2004, Echeverri, C.J., and Perrimon, N., 2006). Several of these libraries are currently available. In Chapter 4 I use several knockdown constructs from the second generation shRNAmir library, developed by the labs of Hannon and Elledge (Silva, J.M. et al., 2005). This library is based on micro RNA (miRNA), the endogenous mammalian RNA interference system. miRNAs are transcribed by RNA polymerase II to form pri-miRNAs (primary microRNAs). This pri-miRNA is then cleaved by the RNase III enzyme Drosha and produces the precursor pre-miRNA, which is transported by Exportin 5 from the nucleus to the cytoplasm where it is processed by Dicer into miRNA (Bohnsack, M.T. et al., 2004, Cullen, B.R., 2004). This pathway is shown in Figure 1.8.

Regular shRNA products, such as those transcribed from pSuper-EGFP, resemble pre-miRNA, but the second generation Hannon/Elledge shRNAmir library resembles pri-miRNA. The transcript produced by these vectors is therefore processed at an earlier step in the RNAi pathway, leading to more effective silencing (Silva, J.M. et al., 2005, Chang, K. et al., 2006).

To reduce the risk of false positives in RNAi screening, the shRNAmir library contains multiple shRNA constructs against each gene (Paddison, P.J. et al., 2004, Silva, J.M. et al., 2005). The use of multiple hairpins against the same gene also validates smaller scale experiments: Two
constructs that both knock down the same gene should lead to the same downstream effects. Deviations from this pattern may indicate off-target effects (Cullen, B.R., 2006).

**Figure 1.8.** Processing of microRNA

pri-miRNA is processed in the nucleus by Drosha. The product, pre-miRNA, is then exported to the cytoplasm, where it is cleaved by Dicer. One strand of the remaining double stranded RNA product is loaded onto the RISC complex, which then silences (through degradation or inhibition of transcription) mRNA containing a complementary nucleotide sequence.
1.5 Goals and Rationale

The synthesis and distribution of melanin in mammalian melanocytes is regulated by a large number of proteins and signaling pathways. To date, over a hundred mouse genes have been linked to coat colour changes, and many of these genes have human homologs that are also involved in pigmentation (Bennett, D.C., and Lamoreux, M.L., 2003). Within melanocytes, pigmentation requires the synthesis of melanin, the formation of dendrites, and the formation and transport of melanosomes. A defect in any of these steps affects skin pigmentation.

The study of skin pigmentation can offer valuable insights into several diseases: Several proteins involved in the synthesis of melanin (B-Raf, Ras) are mutated in human cancers. In addition, pigmentation is linked to skin cancer because the absence of melanin poses an increased risk of UV-induced DNA damage. Other diseases that are intrinsically linked to mechanisms of pigmentation are those caused by defects in the formation and transport of lysosome-related organelles, such as Hermansky-Pudlak Syndrome, Griscelli Syndrome, and Chediak-Higashi Syndrome (Tomita, Y., and Suzuki, T., 2004). These diseases are characterized by defects in pigmentation as the result of impaired formation or intracellular transport of melanosomes as well as lysosome-related organelles in other cell types. Therefore, the study of melanosome formation and transport in melanocytes is a model for organelle formation and transport in general (Barral, D.C., and Seabra, M.C., 2004, Marks, M.S., and Seabra, M.C., 2001).

The goal of the work presented in this thesis is to study various aspects of pigmentation:

In B16 mouse melanoma cells, activation of Ras by cAMP triggers a pathway that negatively regulates melanin synthesis. I hypothesize that the cAMP-activated Ras guanine exchange factor CNrasGEF is the Ras GEF involved in this pathway (Figure 1.9). This is investigated in Chapter 2.

I further hypothesize that a high-content/high-throughput RNA interference screen can serve as a tool for identifying proteins that regulate melanogenesis. Two immediate goals towards this investigation are to develop a method to detect melanosomes using high-content image analysis (Chapter 3) and to test this system using a small set of shRNA constructs against proteins known to be involved in melanosome formation, transport, or other aspects of pigmentation.
Figure 1.9. Goal: identification of cAMP-dependent Ras GEF in B16 cells

One of the goals of the work presented in this thesis is to investigate whether CNrasGEF, a cAMP-dependent Ras GEF identified in our lab as a substrate for Nedd4, is the GEF responsible for the activation of Ras in response to cAMP in B16 cells.
Chapter 2
The Guanine Nucleotide Exchange Factor CNrasGEF Regulates Melanogenesis and Cell Survival in Melanoma Cells

The work presented in this chapter was published in the Journal of Biological Chemistry:


I carried out most of the experiments in this chapter, with the following exceptions: Nam Pham investigated the levels of endogenous CNrasGEF (Figure 2.1) and performed the Ras activation assays for overexpression (Figure 2.2A). I carried out the Ras activation assays for knockdown constructs together with Youngshil Pak (Figure 2.2 B and C).
2.1 Summary

cAMP-dependent Ras activation has been demonstrated in numerous cell types, particularly of neuronal and endocrine origin, but the Ras activator involved in these cell types has not been identified. In B16 melanoma cells, cAMP activates the Ras/ERK pathway, leading initially to stimulation of melanogenesis (dendrite extension and melanin production), but subsequently to long-term (>24hrs) inhibition of these processes. Here we identify CNrasGEF as the Ras GEF involved. We demonstrate that CNrasGEF is expressed endogenously in B16 melanoma cells, and that cAMP-mediated activation of Ras and ERK1/2 in these cells can be augmented by CNrasGEF overexpression and reduced by its knockdown by RNAi. Moreover, we show that CNrasGEF participates in the regulation of melanogenesis. Knockdown of CNrasGEF leads to increased dendrite extension and melanin production, observed ~50hrs after forskolin/IBMX treatment. This suggests that CNrasGEF inhibits melanogenesis in the long term, providing further evidence for its role in the Ras/ERK pathway. In addition, we find that overexpression of CNrasGEF leads to apoptosis, whereas its knockdown by RNAi enhances cell proliferation, independent of cAMP. Collectively, these results suggest that CNrasGEF regulates melanogenesis, but that it also has a distinct role in regulating cell proliferation/apoptosis.
2.2 Introduction

Cyclic AMP (cAMP) exhibits differential mitogenic effects in different cell types (Stork, P.J., and Schmitt, J.M., 2002). In several endocrine, neuronal or Schwann cells and in some 3T3 cells, cAMP promotes cell proliferation, often by activating Ras. For example, in thyroid cells, cAMP-stimulated mitogenesis following thyroid stimulating hormone binding to its receptor is mediated via Ras activation, independent of PKA (Tsygankova, O.M. et al., 2000). Moreover, cAMP-dependent activation of ERK in NIH-3T3 cells was recently demonstrated to be carried out independently of Rap1-Epac, and instead proposed to be mediated via Ras activation (Enserink, J.M. et al., 2002). The cAMP-dependent Ras activator involved in these cases has not been identified. Recently, a pathway for cAMP-dependent Ras/ERK activation (independent of PKA or Rap1) has been proposed in melanoma cells (Busca, R. et al., 2000).

Melanocytes are specialized epidermal cells originating from the neural crest. They synthesize melanin and are responsible for skin pigmentation and protection from UV radiation (Busca, R., and Ballotti, R., 2000, Busca, R., 2002). Transformed melanocytes can give rise to a very aggressive skin cancer, melanoma. B16 mouse melanoma cells, particularly B16-F10, have been characterized extensively with regard to their tumorigenic and metastatic potential (e.g., Hill, R.P. et al., 1984, Cillo, C. et al., 1987, Hill, S.E. et al., 1990), and the signaling pathways responsible for melanin production (Busca, R., and Ballotti, R., 2000). Melanin synthesis takes place in intracellular organelles called melanosomes. Upon stimulation by ultraviolet radiation several factors are released from melanocytes and neighbouring skin cells (keratinocytes), including α-melanocyte stimulating hormone (α-MSH), a strong melanogenic factor (Hunt, G. et al., 1994). α-MSH acts by binding to the melanocortin receptor MC1R, a G protein-coupled receptor (GPCR) coupled to Gsα. Stimulation of MC1R by α-MSH causes elevation of intracellular cAMP, which is a critical regulator of melanin synthesis, formation and extension of dendrites, and the movement of melanosomes towards the tips of dendrites. These melanogenic effects of α-MSH can be mimicked by elevation of intracellular cAMP (e.g. with forskolin and IBMX) (Busca, R., and Ballotti, R., 2000).

Earlier work has investigated the role of cAMP in the signaling pathways regulating melanogenesis: Activation of PKA and CREB by elevated cAMP levels leads to production of
the Microphthalmia transcription factor (MITF) (Hodgkinson, C.A. et al., 1993, Steingrimsson, E. et al., 1994), in turn causing activation of transcription of the tyrosinase gene promoter (Bertolotto, C. et al., 1998b, Bertolotto, C. et al., 1998a). Tyrosinase is the rate limiting enzyme in melanin synthesis (Hearing, V.J., 1999), and its increased transcription thus promotes melanogenesis. However, cAMP-dependent and PKA-independent pathways have also been linked to the regulation of melanogenesis, including the PI3K and the Ras/ERK pathways (Busca, R. et al., 2000). Ballotti and colleagues have demonstrated that cAMP activates Ras (but not Rap1) in B16 melanoma cells, leading to activation of B-Raf, MEK and ERK (Englaro, W. et al., 1995, Englaro, W. et al., 1998, Busca, R. et al., 2000, Busca, R., and Ballotti, R., 2000). Ras/ERK activation in these cells has two effects: initially, phosphorylation of MITF by ERK (on Ser73) leads to increased transcriptional activity of the tyrosinase promoter, increasing melanogenesis (Bertolotto, C. et al., 1998a, Englaro, W. et al., 1995, Hemesath, T.J. et al., 1998). However, after prolonged stimulation of the Ras/ERK pathway (>24 hrs) melanogenesis is inhibited. The latter effect was unraveled by demonstrating sustained inhibition (observed after 48 hrs) of melanogenesis upon overexpression of constitutively active Ras or MEK, or activation of melanogenesis with a specific MEK inhibitor (PD98059) (Englaro, W. et al., 1998). This long-term effect is believed to be caused by Ser73 phosphorylation of MITF as well, resulting in its ubiquitination and subsequent proteasomal degradation, leading to attenuation of melanogenesis (Xu, W. et al., 2000, Busca, R., and Ballotti, R., 2000).

Based on the critical role played by cAMP-mediated Ras activation in melanogenesis, Busca et al. have proposed the existence of a cAMP-dependent Ras activator (GEF) in melanoma cells (Busca, R. et al., 2000). We and others have previously identified a guanine nucleotide exchange factor, called CNrasGEF (aka PDZ-GEF1, nRapGEF, RA-GEF, RAPGEF2) (Pham, N. et al., 2000, Ohtsuka, T. et al., 1999, Liao, Y. et al., 1999, de Rooij, J. et al., 1999). Our previous work showed that, when expressed heterologously in HEK293T cells, CNrasGEF can activate Ras in response to elevation of cAMP levels (independent of PKA), achieved by treatment with 8-Br-cAMP, or forskolin plus IBMX (Pham, N. et al., 2000), or by agonist (isoproterenol) stimulation of co-expressed β1 adrenergic receptor, a GPCR that activates Gsα and leads to elevation of intracellular cAMP levels (Pak, Y. et al., 2002). CNrasGEF can also activate Rap1, but this activation is independent of cAMP (Pham, N. et al., 2000, Ohtsuka, T. et al., 1999, Liao, Y. et al., 1999, de Rooij, J. et al., 1999). We therefore investigated whether CNrasGEF could
participate in the cAMP-dependent Ras activation in melanoma cells. Our work here shows that CNrasGEF is highly expressed in B16 melanoma cells, it can activate Ras and ERK1/2 in these cells upon cAMP elevation, and it plays an important role in regulating melanogenesis. We also show that CNrasGEF regulates cell survival of B16 cells, an effect not dependent on cAMP.
2.3 Experimental Procedures

2.3.1 Constructs and Vectors

FLAG-tagged WT-CNrasGEF and FLAG-tagged ΔCDC25-CNrasGEF (lacking the catalytic Ras/Rap1 activating domain) were expressed from a pCMV5 vector as described previously (Pham, N. et al., 2000). Constructs expressing GFP-tagged WT- and ΔCDC25-CNrasGEF were generated by cloning the respective cDNA sequences from the pCMV5 constructs into pEGFP-c3 (WT), or pEGFP-c2 (ΔCDC25-CNrasGEF). To produce the knockdown construct pSuper-EGFP-CNrasGEF-RNAi a 64 bp oligo was inserted into the multiple cloning sites (MCS) of pSuper-EGFP, a modified pEGFP-c1 vector (Clontech) that incorporates the promoter from pSuper H1 RNA flanked by MCS (kindly provided by Dr. C.C. Hui, The Hospital for Sick Children, Toronto). The 64 bp oligo was designed to form a small hairpin RNA targeting the following sequence unique to the N-terminal region of mouse CNrasGEF (mKIAA0313): AAACAGCCTGACTGACAGC. As a control, a scrambled version of the above oligo was designed, containing the following sequence (not recognizing any coding regions in the mouse genome): AAGAACCTGTCAGCCACCTAC. The oligo strands were purchased from Sigma Genosys, and were annealed and phosphorylated before cloning into pSuper-EGFP.

pCGT vectors containing T7-tagged V12-H-Ras or N17-H-Ras were kindly provided by Dr. Dafna Bar-Sagi (State University of New York, Stony Brook, NY)

2.3.2 Cell Culture and Transfections

B16-F10 murine melanoma cells (hereafter called B16 cells), purchased from ATCC, were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained at 37°C and 5% CO2. Cells were transfected using Lipofectamine (Invitrogen) according to the manufacturer’s instructions.

2.3.3 Analysis of Cell Proliferation and Morphology

Cells were transfected with pEGFPc1, pSuper-EGFP-CNrasGEF-RNAi (hereafter called GFP-CNrasGEF-RNAi or CNrasGEF-RNAi for short), pEGFPc3-WT-CNrasGEF (called WT-CNrasGEF), or pEGFPc2-ΔCDC25-CNrasGEF (called ΔCDC25-CNrasGEF) and sorted by fluorescence-activated cell sorting (FACS) the next day to obtain a homogenously transfected
population, since transfection efficiencies ranged from ~30%-80%. The GFP-positive cells were reseeded at low density onto 6-well plates in medium containing 2% FBS and penicillin/streptomycin, since complete serum depletion did not allow the cells to survive for more than 24 hours. After attaching overnight, cells were stimulated (or not) with 20 μM forskolin and 20 μM IBMX (Sigma) for periods of up to 72 hours.

Cells were photographed using a Leica DM IRE2 microscope with Openlab software at different sections of the wells, at 0, 8, 24, 48 and 72 hours after stimulation, returning to the same cells at every time point. Cells with and without dendrites were counted, where “dendrites” were cell extensions that appeared over time. Only cells with more than two dendrites were scored, and the length of the dendrites was equal to or more than the width of the cell body. The percentage of cells with dendrites was calculated and the 95% confidence interval for proportions of cells with dendrites ($\pm 1.96\sqrt{p(1-p)/N}$, $p=$ proportion, $N=$ total number of cells (Motulsky, H., 1995)) was determined and shown as error bars where appropriate.

To construct growth curves, the total cell number per mm$^2$ was plotted against growth time. The 95% confidence interval for counted cells ($\pm 1.96\sqrt{N}$, $N=$ total number of cells) was determined, corrected for cells per mm$^2$ and shown as error bars where applicable (Motulsky, H., 1995). The growth rate was determined by normalizing the number of cells per mm$^2$ to the number obtained at the first measured time point, calculating the $2\log$ value, and plotting against time.

Short-term dendrite formation was analyzed as follows: B16 cells were transfected as described above. Transfected cells were recovered for 15 hrs in DMEM with 10% FBS, penicillin/streptomycin, and with or without 20 μM forskolin/IBMX. Cells were analyzed using a Leica DM IRE2 microscope with Openlab software, by counting GFP-fluorescent cells with and without dendrites, as described above.

2.3.4 Apoptosis Analysis

Cells were transfected and sorted as described above, and reseeded onto 4-well polystyrene vessel glass slides (BD Falcon). After 20-24 hours, cells were fixed in 1% para-formaldehyde (PFA) in PBS (pH 7.4) and permeabilized using ethanol:acetic acid (2:1). Fixed cells were stained for DNA damage using the ApopTag Red kit (Chemicon) according to the manufacturer’s protocol, and mounted using Vectashield with DAPI (Vector Laboratories). Cells
were visualized and photographed using a Leica DM IRE2 microscope with Openlab software. Cells stained for apoptosis were counted and presented as percentage of total cells. The 95% confidence interval for proportions (±1.96√(p(1-p)/N), p=proportion, N=total number of cells (Motulsky, H., 1995)) was determined.

2.3.5 Determination of Melanin Production

B16 cells were grown to 50% confluence and transfected (or not) with CNrasGEF-RNAi or the scrambled control as described above. The next day, cells were re-seeded 1:2 into new dishes and the medium was replaced with DMEM containing 2% FBS, penicillin/streptomycin, and with or without 20 μM forskolin/100 μM IBMX. After 54 hours of stimulation, cells were collected by trypsinization and divided into two equal batches. Half of the collected cells were lysed for protein analysis as described below. The other half was used to isolate melanin, according to a previously described protocol with slight modifications (Ando, H. et al., 1999). Briefly, cells were washed twice in PBS, re-suspended in 200 μl ddH2O and 1 ml ethanol:ether (1:1), and incubated at room temperature for up to one hour to remove impurities. Subsequently, the pellet was re-suspended in 0.5 ml 1N NaOH/10% DMSO and melanin was dissolved for 15-30 minutes at 70°C. Melanin absorption was measured with a Beckman Coulter DU640B spectrophotometer at 470 nm.

To determine the effect of Ras on melanin synthesis, cells were transfected with V12-Ras or N17-Ras, and collected 48 hours after stimulation with forskolin/IBMX. Melanin was isolated as described above.

2.3.6 Western Blot Analysis

For protein analysis in growth-, morphology-, and melanin production experiments, cells were washed once with PBS and lysed in lysis buffer (150 mM NaCl, 50 mM Hepes, 1% Triton-X, 10% glycerol, 1.5 mM MgCl2, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM PMSF). Total protein levels were determined using the BIORAD protein assay. An antibody generated against the carboxyl terminus of human CNrasGEF (Pham, N. et al., 2000) was used to detect mouse CNrasGEF in all experiments. β-Actin (antibody from Sigma Aldrich) was used as a loading control and to ensure that knockdown (where applicable) was not affecting all proteins in the cell non-specifically. Relative CNrasGEF levels were quantified as
protein band intensities using FluorChem densitometry software and normalized to β-actin levels.

### 2.3.7 Ras Activation Assay

B16 cells were transfected, serum starved overnight and then subjected to treatments with 500 μM 8-Br-cAMP (Sigma) for 15 min. Cells were lysed with lysis buffer (25 mM Hepes, pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% Na deoxycholate, 10% glycerol, 25 mM NaF, 10 mM MgCl₂, 1 mM EDTA, 1 mM NaVO₄, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 250 μM PMSF) and the level of Ras-GTP in the lysates was determined using an activation specific probe, as described (de Rooij, J., and Bos, J.L., 1997). Briefly, to determine the levels of active Ras (Ras-GTP) in cells, agarose-bound GST fusion protein of the Ras-binding domain (RBD) of Raf-1 (GST-Raf1-RBD; Upstate Biotechnology Inc.) was used to precipitate Ras-GTP from cell lysates, and the amount of Ras-GTP was determined by immunoblotting with anti H/N-Ras antibodies (Quality Biotech), since our unpublished work demonstrated endogenous expression of N- and H-Ras (but not K-Ras) in B16 cells. Relative Ras and CNrasGEF levels were quantified as protein band intensities using FluorChem densitometry software and normalized to total Ras levels.

### 2.3.8 Analysis of ERK1/2 Activation

B16 cells were transfected with GFP-CNrasGEF-RNAi, GFP-scrambled RNAi (control), GFP-WT-CNrasGEF or GFP-ΔCDC25-CNrasGEF, and FACS sorted the next day. Sorted cells were reseeded in 6-well dishes and grown in DMEM containing 20% FBS for 24 hours before serum starvation overnight. To study the effect of cAMP elevation, cells were stimulated (or not) with 20μM forskolin/100μM IBMX for 15 minutes and lysed on ice in a buffer with phosphatase inhibitors (100 mM NaCl, 50 mM Hepes pH 7.4, 1% Triton-X-100, 1mM NaVO₄, 5mM EDTA, 50 mM NaF, 1 mM NaPPi, 10 mM PNPP, 10 μg/ml Aprotinin, 10 μg /ml Leupeptin, 1mM PMSF). Western blot was carried out on these lysates using antibodies against CNrasGEF and phosphorylated ERK1/2 (Promega). Total ERK1/2 levels were determined after stripping the blot and re-probing with an antibody against ERK1/2 (Promega).
2.3.9 cAMP Production Assay

B16 cells were cultured as described above, and grown overnight in medium with different serum concentrations (0, 2 or 10% FBS). Cells were stimulated (or not) with 20μM forskolin/IBMX for 1 hour, or 500 μM 8Br-cAMP for 15 minutes. Intracellular cAMP levels were measured using the cAMP Biotrak Enzymeimmunoassay (EIA) system (Amersham Biosciences) according to the manufacturer’s protocol.
2.4 Results

2.4.1 CNrasGEF is Expressed Endogenously in B16 Melanoma Cells and is Able to Activate Ras and ERK1/2 in These Cells.

To study the role of CNrasGEF in B16 cells, we first tested the level of its endogenous expression in these cells. As seen in Figure 2.1, CNrasGEF is highly expressed in B16 (F10) melanoma cells relative to other cell lines analyzed. We have previously shown that overexpressed CNrasGEF in HEK293T cells activates Ras upon cAMP elevation (Pham, N. et al., 2000, Pak, Y. et al., 2002). Here we tested whether it can also activate Ras in B16 cells in response to cAMP. Figure 2.2A and C demonstrate that treatment of B16 cells with the membrane permeant analog of cAMP, 8-Br-cAMP, leads to Ras activation, in agreement with previous studies of Busca et al. (Busca, R. et al., 2000). Overexpression of CNrasGEF in these cells causes an increase in Ras activation, which is further augmented by treatment with 8-Br-cAMP (Figure 2.2A). Thus, CNrasGEF can enhance Ras activation in B16 melanoma cells.

To further analyze the requirement of CNrasGEF for Ras activation in B16 cells, we knocked down CNrasGEF in these cells by RNAi. To achieve knockdown, we utilized a modified pEGFPc1 vector with an incorporated pSuper H1 RNA promoter and multiple cloning sites, where we introduced the CNrasGEF-RNAi hairpin oligo (see Experimental Procedures). This vector (called GFP-CNrasGEF-RNAi, or CNrasGEF-RNAi for short) expresses EGFP from a CMV promoter, separate from the promoter transcribing the shRNA. As seen in Figure 2.2, overexpression of the CNrasGEF-RNAi construct in B16 cells resulted in a substantial reduction in the amount of CNrasGEF (but not of total Ras), suggesting that our knockdown strategy is effective. Some (variable) non-specific knockdown due to overexpression of GFP alone or the scrambled control was also seen (Figure 2.2B and C, bottom), but it was not as pronounced as the knockdown by the specific CNrasGEF-RNAi. On average, specific knockdown by RNAi accounted for a ~35% difference in CNrasGEF levels compared to the scrambled control. Transfection efficiency of both knockdown construct and scrambled control was ~40-50%. We then tested the effect of knockdown of CNrasGEF on cAMP-stimulated Ras activation in these cells. FACS sorting to isolate only the GFP-expressing cells could not be performed for these experiments because the yield of sorted cells is too low for biochemical assays measuring Ras activation. Nevertheless, Figure 2.2A and C demonstrate that reduction of CNrasGEF levels with
Figure 2.1  Endogenous expression of CNrasGEF in B16 melanoma cells.

Cell lysate (50 μg protein/lane) from HEK293T (293T), NG108-15 (NG108) or B16-F10 (B16) cells, as well as HEK293T cell overexpressing CNrasGEF, were immunoblotted with anti CNrasGEF antibodies (right) or pre-immune serum (left). The rightmost lane (HEK293T-transfected) represents lower exposure time than the rest of the autoradiogram.
**Figure 2.2** CNrasGEF leads to cAMP-dependent Ras and Erk1/2 activation in B16 melanoma cells.

**A-C.** Activation of Ras by CNrasGEF: B16 cells were transfected (or not) with wild type Flag-tagged CNrasGEF (Flag-CNrasGEF), CNrasGEF-RNAi, or a scrambled sequence used as a control for the RNAi (panel B). Cells were then treated (or not) with the cAMP analogue, 8-Br-cAMP, and activated Ras (Ras-GTP) precipitated from lysates with GST-Raf1-RBD. The lower panels in A and C (bottom) represent controls for total Ras, amount of transfected CNrasGEF (immunoblotted with anti Flag antibodies), and amount of endogenous CNrasGEF, depicting knockdown of CNrasGEF with RNAi, and some none-specific knockdown with the scrambled control. There was some variability in the extent of knockdown by the scrambled control (compare panel B to C, top), but it was consistently smaller than the CNrasGEF-RNAi. The knockdown of CNrasGEF depicted in panels B and C is from cells treated with 8-Br-cAMP, but a similar effect was seen with untreated cells. Figure 2A-C are representatives of 7 experiments. Cells were not FACS-sorted for analysis of Ras activation.

**D.** Activation of Erk1/2 by CNrasGEF: Cells were transfected (or not) with GFP-WT-CNrasGEF, GFP-ACDC25-CNrasGEF, or GFP-tagged CNrasGEF-RNAi or a scrambled control. The population of transfected cells was enriched by FACS sorting. Sorted cells were treated (or not) with forskolin/IBMX. Active ERKs (middle panels) were detected using Western blot with an antibody against phosphorylated ERK1/2, and levels were normalized to levels for total ERK1/2 (lower panels), detected after stripping and reprobing of the blot. The levels of endogenous or ectopic CNrasGEF were determined using an antibody against CNrasGEF (top panels). Left and right panels of Fig 2D are representative of 3 experiments.
RNAi resulted in a corresponding reduction in cAMP-dependent Ras activation. When normalized to levels of total Ras and actin, activated Ras levels after RNAi and stimulation with 8Br-cAMP were ~50% lower than levels in untransfected cells (n=7), and ~25% lower than in cells transfected with the scrambled control (n=2).

To examine the effect of CNrasGEF on the activation of ERK1/2, we transfected cells with GFP-tagged constructs expressing wildtype CNrasGEF, the inactive ΔCDC25 mutant, CNrasGEF-RNAi, or the scrambled control. Cells were FACS-sorted and reseeded the day after transfection in order to measure the levels of activated ERK1/2 in transfected cells only. Figure 2.2D (left panel) shows that, consistent with its activation of Ras, CNrasGEF mediates the cAMP-dependent activation of ERK1/2. In response to forskolin/IBMX, the levels of phosphorylated ERK1/2 increase in untransfected cells. Overexpression of WT-CNrasGEF (but not the catalytically inactive ΔCDC25 mutant) further augments the cAMP-dependent increase of phosphorylated ERK1/2. Knockdown of CNrasGEF with RNAi (with ~30-80% efficiency relative to scrambled controls) reveals a reduction of ~10-40% in levels of phosphorylated ERKs (normalized to total ERK, Figure 2D, right panel), with higher levels of knockdown resulting in greater reduction of phospho-ERK levels. The reduction was stronger with ERK1 (top band) than ERK2.

Taken together, these results suggest that CNrasGEF is able to activate Ras and ERK1/2 in B16 cells in response to cAMP stimulation, and that its knockdown by RNAi reduces this activation.

2.4.2 CNrasGEF Inhibits the Sustained (Long Term) Dendrite Extension in B16 Melanoma Cells

Melanoma cells, including B16 cells, are derived from the neural crest and extend dendrites, which are needed for pigment distribution. Dendrite extension and melanin production are the hallmarks of melanogenesis, and are regulated by cAMP (Busca, R., and Ballotti, R., 2000). The net stimulatory effect of cAMP on dendrite extension is the combined result of several cAMP-dependent pathways: those stimulating dendrite extension and those inhibiting it. Upon prolonged cAMP stimulation, the Ras/ERK pathway has been shown to inhibit dendrite extension. To analyze the role of CNrasGEF in dendrite formation/extension, we transfected B16 cells with GFP alone or GFP-tagged CNrasGEF-RNAi, WT-CNrasGEF, or the catalytically
inactive ΔCDC25-CNrasGEF, in which the CDC25 domain has been deleted and which can not bind Ras. For these experiments, transfected cells were FACS-sorted to isolate only the cells that took up the corresponding (GFP-expressing) plasmids. Figure 2.3A demonstrates the effectiveness of knockdown of CNrasGEF by overexpression of GFP-CNrasGEF-RNAi and FACS-sorting. Following FACS-sorting, cells were seeded and grown for 15 hrs in medium with low (2%) serum prior to the addition of forskolin + IBMX, to stimulate cAMP production (Table 1). Dendrite extension was analyzed at 0 and 48 hours after stimulation with forskolin/IBMX, or in low-serum medium alone at corresponding time points.

Data for dendrite extension of control cells and cells with knocked down CNrasGEF were collected only from experiments in which knockdown exceeded 50%. Figure 2.3B depicts the morphology of B16 melanoma cells after 48 hour treatment with forskolin/IBMX (bottom panels) or in low-serum medium at the same time point. Extensive dendrite formation is observed in the stimulated cells expressing GFP, CNrasGEF-RNAi, or ΔCDC25-CNrasGEF, but not in cells overexpressing WT-CNrasGEF. The number of cells with and without dendrites under every condition were counted, and represented as percentage of cells with dendrites in Figure 2.3C and 2.3D. As seen in Figure 2.3D, the percentage of cells that extended dendrites after 48 hours of stimulation with forskolin/IBMX was increased in the cells in which CNrasGEF was knocked down, relative to cells expressing GFP alone or cells overexpressing the catalytically inactive ΔCDC25-CNrasGEF mutant. In contrast, there was a strong reduction in the proportion of cells extending dendrites in cells overexpressing WT-CNrasGEF. Similar results (although more modest) were seen at 24 hours, but not at 8 hours (not shown) after forskolin/IBMX stimulation, suggesting that the effect of CNrasGEF on inhibition of dendrite extension is a late effect. Although the effect of knocking down CNrasGEF on dendricity appears modest, it is both statistically significant (Chi² test P values <0.0001) and highly reproducible, and may reflect the fact that the RNAi approach did not produce a complete knockdown. Moreover, in experiments where RNAi did not produce a visible knockdown, no increase in dendricity was observed. Dendrite extension in all transfected cells grown in the presence of 2% serum but in the absence of forskolin/IBMX treatment was quite low (Figure 2.3C) and correlates with lack of elevation of intracellular cAMP levels (Table 2.1).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>fmol (mean±SEM)</th>
<th>cAMP/μg (mean±SEM)</th>
<th>protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% FBS</td>
<td>1.65±0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2% FBS</td>
<td>1.89±0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0% FBS</td>
<td>3.81±0.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2% FBS + 20μM forskolin+20μM IBMX (1h)</td>
<td>&gt;1600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0% FBS + 500μM 8-Br-cAMP (15 min)</td>
<td>&gt;1600</td>
<td></td>
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</tbody>
</table>

**Table 2.1**  cAMP (or its analog) levels in B16 melanoma cells.

Cells were grown in DMEM with the indicated amount of serum (FBS) and treatments. cAMP levels were measured as described in Experimental Procedures.
Figure 2.3  CNrasGEF inhibits long-term dendrite formation in B16 melanoma cells.

A. Upper panel: knockdown of endogenous CNrasGEF following transfection with GFP-CNrasGEF-RNAi vector and FACS sorting. The GFP-vector alone was used as a control. Knockdown in this example was ~70% after quantification. Lower panel depicts the same blot re-probed with β-actin, to verify equal protein loading and that the RNAi targeted CNrasGEF and did not knock down the expression of all cellular proteins non-specifically.

B. Morphology of B16 melanoma cells after 48 hr treatment with forskolin/IBMX (e-h) or in the absence of forskolin/IBMX at the same time point (a-d). Cells were transfected with the GFP-vector alone (GFP)(a, e), CNrasGEF-RNAi (b, f), wild type CNrasGEF (WT-CNrasGEF) (c, g), or CNrasGEF lacking its catalytic CDC25 domain (ΔCDC25-CNrasGEF)(d, h), all GFP-tagged. Cells were FACS-sorted and reseeded as described above. Arrows depict examples of dendrites.

C, D. Quantification of cells with dendrites after two days in the absence (C) or presence (D) of forskolin/IBMX: The percentage of all unstimulated cells that show dendrite extension over time was analyzed as described in the Experimental Procedures. Only experiments in which knockdown exceeded 50% were included. Error bars represent 95% confidence intervals. Data are a summary of 2-5 independent experiments. Numbers in the bars indicate the total number of cells counted per condition. Asterisks indicate P values from Chi² tests (*=P<0.05, ***=P<0.0001)
Collectively, these results suggest that CNrasGEF is involved in the cAMP-dependent, sustained (long-term) inhibition of melanogenesis. This inhibitory effect was not related to the pro-apoptotic effect of CNrasGEF (see below) because dendrite formation was measured only in living, non-apoptotic cells.

In addition to inhibiting melanogenesis in the long run, Ras has also been proposed to stimulate melanogenesis early (<15 hrs) after its activation by cAMP (Englaro, W. et al., 1995). Our results suggest that the early stimulatory effect is also regulated by CNrasGEF, since overexpression of CNrasGEF increased early dendrite formation, an effect requiring the presence of its CDC25 (catalytic) domain. However, cAMP-stimulation of CNrasGEF did not further augment this short-term dendrite formation (Figure 2.4).

### 2.4.3 CNrasGEF Inhibits Melanin Production

To directly test melanin production, B16 melanoma cells transfected as above were treated with forskolin + IBMX for 54 hrs and harvested (FACS sorting was not possible due to insufficient yield of sorted cells for spectrophotometrical detection of melanin). Melanin levels were analyzed at 470 nm, as described (Ando, H. et al., 1999), and normalized for total protein concentration. As shown in Figure 2.5A, melanin production in untransfected cells increased upon prolonged stimulation with forskolin/IBMX. Melanin levels were increased even further in cells in which CNrasGEF was knocked down with RNAi (Figure 2.5A, inset). Use of a scrambled control revealed partial decrease in CNrasGEF levels (inset) and accordingly, resulted in intermediate amounts of melanin produced. (The average % knockdown by CNrasGEF-RNAi was 57% relative to untransfected cells and 33% relative to the scrambled control). These data suggest that CNrasGEF inhibits long-term melanin production in B16 melanoma cells. This effect was only seen in the presence of forskolin/IBMX, since melanin levels were similar in all unstimulated cells regardless of CNrasGEF levels. Similar results were obtained at 72 hrs post stimulation with forskolin/IBMX (not shown). These results are in agreement with the above data (Figure 2.3) describing dendrite extension, and again demonstrate the sustained inhibition of melanogenesis by CNrasGEF. This inhibition can also be mimicked by overexpression of a constitutively active V12-Ras, but not by a dominant negative N17-Ras (Figure 2.5B).
Figure 2.4  Short term stimulation of dendrite formation by overexpression of CNrasGEF.

A. EGFP fluorescence images showing elaborate dendrite extension (exemplified by arrows) in B16 melanoma cells shortly (~15 hrs) after they were transfected with GFP-tagged WT-CNrasGEF (panels c,d), but less dendrites in cells transfected with GFP-vector alone (panels a,b) or with the catalytically inactive ΔCDC25-CNrasGEF (panels e,f). Panels a,c,e: untreated cells. Panels b,d,f: cells treated with forskolin/IBMX.

B. Quantification of the percentage of cells with dendrites in the absence or presence of forskolin/IBMX stimulation. Data are percentage of cells with dendrites ±95% confidence interval. They are a summary of 2 independent experiments, counting 1995-3768 cells per treatment. (Chi² test: P<0.05 (*) for GFP or ΔCDC25-CNrasGEF expressing cells treated with forskolin/IBMX relative to untreated cells, and P>0.05 for WT-CNrasGEF expressing cells treated with forskolin/IBMX relative to untreated cells).
A. 

<table>
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<th>GFP</th>
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B. 

- Horizontal axis: % dendritic cells
- Vertical axis: % dendritic cells

- Bars represent forskolin/IBMX
- Grey bars represent unstimulated

Bar chart comparisons:
- GFP
- WT-CNrasGEF
- ΔCDC25-CNrasGEF

* indicates statistical significance.
Figure 2.5  Sustained (long term) melanin production in B16 melanoma cells is stimulated by knockdown of CNrasGEF.

A. B16 cells were transfected (or not) with CNrasGEF-RNAi or a scrambled control, and stimulated (or not) with forskolin/IBMX 24 hrs after transfection (= time 0). At 54 hrs post stimulation, cells were harvested and the amount of melanin produced per mg protein analyzed, as described in the Experimental Procedures. The average % knockdown by CNrasGEF-RNAi was 57% vs untransfected cells and 33% vs the scrambled control. Error bars represent average ±SEM of 2 separate experiments, where protein levels were measured in duplicates and melanin absorption was determined at two different concentrations. Asterisks indicate P values from Chi² tests (*=P<0.05, **=P<0.01. For CNrasGEF-RNAi P<0.01 relative to both untransfected cells and scrambled control.). B. B16 cells were transfected (or not) with V12-Ras (constitutively active) or N17-Ras (dominant negative) and stimulated (or not) with forskolin/IBMX. Melanin levels were analyzed at 48 hrs post stimulation. Error bars represent average ±SEM of 3 independent experiments. Asterisks indicate P values relative to untransfected cell, as described in panel A above.
The effect of overexpression of wildtype CNrasGEF on melanin synthesis could not be analyzed, since the apoptotic effect of CNrasGEF overexpression began at an earlier stage than the time point at which melanin production could be measured (see below).

2.4.4 CNrasGEF Inhibits Proliferation of B16 Cells and Promotes Their Apoptosis

In the course of our experiments, we noticed extensive cell death in cells overexpressing WT-CNrasGEF. To quantify the effect, we transfected B16 cells with GFP alone, WT-CNrasGEF, CNrasGEF-RNAi, or the catalytically-inactive ΔCDC25-CNrasGEF (all GFP tagged), FACS-sorted them and analyzed the effect of CNrasGEF on cell proliferation. As shown in Figure 2.6A, the rate of B16 cell proliferation was slowest in cells overexpressing WT-CNrasGEF relative to those overexpressing GFP alone, ΔCDC25-CNrasGEF, or those in which CNrasGEF was knocked down by RNAi; In fact, the latter exhibited the fastest proliferation rate, suggesting that CNrasGEF provides inhibitory signals for cell proliferation of B16 melanoma cells.

We subsequently analyzed whether the retarded proliferation of the B16 melanoma cells overexpressing WT-CNrasGEF was due to apoptosis, using a TUNEL assay. Figure 2.6B demonstrates that cells overexpressing WT-CNrasGEF showed an approximate 4-5-fold increase in the number of apoptotic cells relative to control cells (transfected with the GFP vector alone), and more than 2 fold increase in apoptotic cells relative to those overexpressing the catalytically-inactive ΔCDC25-CNrasGEF construct. Both proliferation rate and apoptosis were not affected by cAMP (data not shown). These results therefore, show that B16 cells are exquisitely sensitive to the amounts of CNrasGEF they express, and that overexpression of this GEF leads to enhanced apoptosis.

Overall, our data suggest that in B16 cells, CNrasGEF plays critical roles in regulating cell proliferation and cAMP-dependent differentiation.
Figure 2.6  CNrasGEF attenuates proliferation and promotes apoptosis in B16 melanoma cells.

A. Growth curves of B16 melanoma cells transfected with GFP-control, WT-CNrasGEF, CNrasGEF-RNAi or ΔCDC25-CNrasGEF. Data represent cells/mm² ±95% confidence interval (N=2500-5000 cells per transfection at 87 hours after seeding) from 2 (knockdown) to 5 (overexpression) experiments. Only experiments in which knockdown exceeded 50% were included. Inset: logarithmic growth curve (see Experimental Procedures). Although data shown are of cells stimulated with forskolin/IBMX, identical results were obtained with unstimulated cells (not shown). B. TUNEL assays performed on B16 melanoma cells overexpressing GFP-control, WT-CNrasGEF, CNrasGEF-RNAi or ΔCDC25-CNrasGEF (all GFP-tagged), revealing extensive apoptosis in cells overexpressing the wild type CNrasGEF at 24 hours after sorting and re-seeding (48 hours after transfection). Data are percentage of apoptotic cells ±95% confidence interval (N=450-850 cells counted per transfection), taken from 3 independent experiments.
2.5 Discussion

The process of melanogenesis, manifested as dendrite extension and melanin production, heavily relies on the elevation of cAMP, and is tightly regulated by numerous factors. An important one is cAMP-dependent Ras activation that leads to ERK activation, a process that is not mediated by PKA or Rap1 (Busca, R. et al., 2000). Our work presented here suggests that CNrasGEF could be the (or an) exchange factor that mediates this cAMP-dependent Ras activation. In support of this notion, we demonstrate here that cAMP-dependent Ras and ERK1/2 activation in B16 melanoma cells (first documented in (Busca, R. et al., 2000)) can be augmented by overexpression of CNrasGEF, or decreased by knockdown of this GEF (Figure 2.2).

Prolonged activation of Ras and ERK in B16 melanoma cells inhibits the cAMP-dependent formation of dendrites and synthesis of melanin (Englaro, W. et al., 1998). This raises the possibility that Ras activation via CNrasGEF in these cells could be involved in this process. Our data clearly demonstrate that alterations in the levels of expression of CNrasGEF (by overexpression or knockdown) affect B16 dendricity and melanin production in response to cAMP (Figure 2.3 and 2.5), whereby CNrasGEF inhibits long-term dendrite extension and melanin synthesis. We propose that this negative regulatory effect is the result of Ras activation by CNrasGEF. We confirmed that Ras indeed inhibits melanin synthesis by using constitutively active and dominant negative Ras mutants (Figure 2.5B). These data support previous findings (Englaro, W. et al., 1998), and offer additional evidence that CNrasGEF indeed has a role in the Ras-pathway in B16 cells.

As well as inhibiting long-term melanogenesis, CNrasGEF is also capable of stimulating early (<15 hrs) dendrite formation in B16 cells, an effect previously proposed to be Ras-dependent (Englaro, W. et al., 1995). Our work shows, however, that this effect was not cAMP-dependent, although it required catalytically active CNrasGEF. The pathway(s) involved in this function of CNrasGEF is currently unknown.

In addition to revealing a regulatory role for CNrasGEF in melanogenesis, we also observed very striking effects of CNrasGEF on cell survival of B16 cells (Figure 2.6), where this GEF appeared to promote apoptosis, independent of cAMP. Interestingly, we also found extensive cell death in another cell line of neuronal origin, the rat glial/mouse neuroblastoma hybrid cell line NG108-
15, when transfected with WT-CNrasGEF (data not shown). This apoptotic effect may not be universal, however, since HEK293T cells do not seem to be adversely affected by overexpression of CNrasGEF (Pham, N. et al., 2000). In both B16 and NG108-15 cells, the pro-apoptotic effects of CNrasGEF were not dependent on cAMP, but were dependent on its intact catalytic activity, as little apoptosis was seen upon overexpression of ΔCDC25-CNrasGEF. Although the mechanisms involved are currently not known, it is possible that Rap1 activation via CNrasGEF plays a role in regulating cell survival/apoptosis. Rap1 is expressed in B16 melanoma cells (Busca, R. et al., 2000), and has been shown to be involved in regulating cell survival/apoptosis in some cells, such as thyroid cells (eg. (Saavedra, A.P. et al., 2002)), where TSH-mediated Akt phosphorylation is augmented by Rap1 activation (Tsygankova, O.M. et al., 2001), and hepatocytes (Cullen, K.A. et al., 2004). However, in the latter cases, cAMP appears to be involved, whereas in our studies survival rate was similar in the presence or absence of cAMP stimulation, and previous work from several groups, including ours, demonstrated that CNrasGEF-mediated activation of Rap1 was not dependent on cAMP (Pham, N. et al., 2000, Liao, Y. et al., 1999, de Rooij, J. et al., 1999). This suggests that other pathways, not yet known, may play a role in the CNrasGEF-dependent pro-apoptotic effects. Similar to other cell types, numerous pro and anti apoptotic factors participate in the regulation of melanocyte survival (eg. (Hussein, M.R. et al., 2003, McGill, G.G. et al., 2002, Larribere, L. et al., 2004)), and it remains to be shown which of these participate in the pro-apoptotic effect(s) of CNrasGEF.

The findings described here have some broad implications. First, cAMP-dependent Ras and ERK activation (independent of PKA and Epac/Rap1) has been proposed in several cellular systems in addition to melanoma cells, particularly in neuronal and endocrine cells (Enserink, J.M. et al., 2002, Saavedra, A.P. et al., 2002, Cheng, G. et al., 2003), where a cAMP-dependent Ras GEF has been sought after for quite some time. We propose that CNrasGEF could fulfill this role at least in some of these cells. Second, melanogenesis itself, which includes melanosome exocytosis, provides an example of a specialized case of lysosomal movement/exocytosis, where defects in such a process lead to genetic disorders often associated with albinism (Stinchcombe, J. et al., 2004). The involvement of CNrasGEF in regulating melanogenesis may suggest that CNrasGEF could be involved in some disorders in which aberrant signaling would result in defective melanosome exocytosis. Future work is required to test these hypotheses.
Chapter 3
Developing a Method for High-Content Analysis of Melanosomes

I designed and performed all experiments in this chapter.
3.1 Summary

Melanosomes are lysosome-related organelles in which melanin is synthesized and transported within melanocytes. Many proteins that regulate formation and movement of melanosomes are also involved in the formation or movement of other organelles. Several of these proteins have been identified through the study of mutant mouse models or human pigmentation disorders. Our aim was to develop a high-content cellular imaging system in combination with RNA interference (RNAi) to screen for proteins involved in melanosome formation or transport. Here I describe the development of a system to detect melanosomal properties by high-content image analysis. Using a Cellomics KineticScan HCS Reader combined with Spot Detector BioApplication we were able to measure dispersion of melanosomes in B16 cells in response to cAMP stimulation, as well as an increase in TYRPI (tyrosinase-related protein 1) levels within melanosomes upon longer (24 hours) stimulation. However, we noticed that the proliferation pattern of B16, causing them to grow in clumps, was a drawback in using these cells for automated cellular image analysis. Melan-a was thus selected as a cell line better suited for the study of melanosomes in this system. We attempted to use the Cellomics setup to detect a decrease in melanosome movement in melan-a cells upon knockdown of Myosin Va, a protein known to be involved in melanosome transport. Despite a significant decrease in protein levels we were not able to see the expected pattern of melanosomes clustering around the nucleus by direct microscopy, and Cellomics analysis was unconvincing. This indicates that before using Cellomics analysis in combination with RNA interference, a closer analysis of the effect of knockdown of proteins involved in melanosome formation and transport is required.
3.2 Introduction

Melanosomes are specialized, lysosome-related organelles in melanocytes, in which melanin is synthesized and transported along dendrites in the cell, to finally be distributed to neighbouring keratinocytes (Seiji, M. et al., 1963). The mechanisms involved in formation of melanosomes and their movement towards the plasma membrane and the tips of dendrites within melanocytes are similar to those of lysosome-related organelles in other cell types, including platelet dense granules and lytic granules in cytotoxic T lymphocytes (Stinchcombe, J. et al., 2004, Dell'Angelica, E.C. et al., 2000, Orlow, S.J., 1995, Dell'Angelica, E.C., 2003). Diseases in which formation or movement of melanosomes is impaired, such as Hermansky-Pudlak Syndrome, Chediak-Higashi Syndrome or Griscelli Syndrome, are therefore characterized not only by a pigmentation defect, but also by defects in related organelles. Hermansky-Pudlak Syndrome (HPS) patients have bleeding disorders and pigmentation defects related to mutations in any of several genes necessary for the proper sorting of cargo proteins to platelet dense granules and melanosomes (Setty, S.R. et al., 2007, Di Pietro, S.M. et al., 2006, Helip-Wooley, A. et al., 2007, Boissy, R.E. et al., 2005, Di Pietro, S.M. et al., 2004). Chediak-Higashi Syndrome manifests itself as hypopigmentation with immunodeficiency, and is caused by mutations in the protein LYST, leading to the formation of giant melanosomes and lysosomes (Page, A.R. et al., 1962, Burkhardt, J.K. et al., 1993, Fukai, K. et al., 1996). The pigmentation defect in Griscelli Syndrome is caused by mutations in either Rab27a, Myosin Va, or melanophilin (Pastural, E. et al., 1997, Ménasché, G. et al., 2000, Ménasché, G. et al., 2003). Together, these proteins form a complex that transports melanosomes towards the cell membrane and the tips of dendrites (Matesic, L.E. et al., 2001, Wu, X. et al., 2006, Provance, D.W.,Jr et al., 1996, Bahadoran, P. et al., 2001). In melanocytes of Griscelli Syndrome patients, this complex is dysfunctional and melanosomes remain clustered around the nucleus. Myosin Va and Rab27a are also required for neuron function and the release of lymphocyte cytotoxic granules respectively (Stinchcombe, J.C. et al., 2001, Bridgman, P.C., 1999, Haddad, E.K. et al., 2001). As a result, individuals with Griscelli Syndrome Type 1, who have a mutation in Myosin Va, do not just have a pigmentation defect but also exhibit a developmental delay (Pastural, E. et al., 1997). Griscelli Syndrom Type 2 patients, with a mutation in Rab27a, have an immune defect in addition to hypopigmentation (Ménasché, G. et al., 2000).
The common mechanisms of formation and transport of melanosomes and other lysosome
related organelles make melanosomes a good model for the study of organelle transport in

Current knowledge of many of the proteins involved in melanosome development or transport
comes from human disease phenotypes and mouse models. However, it is very likely that not all
proteins involved in these processes have been identified this way. Many proteins required for
organelle formation and sorting are necessary for proper development, and in that case a loss-of-
function mutant might not result in a viable phenotype. This is the case with the protein UBPY
(USP8), which regulates deubiquitination of cargo required for endosomal sorting: A UBPY
knockout mouse is embryonic lethal and can not be studied as a loss-of-function mouse model
(Niendorf, S. et al., 2007)

All proteins involved in melanosome function, including those for which there is no disease or
viable knockout mouse model, can also be studied at the cellular level using RNA interference
(RNAi). Therefore, high-throughput cellular image analysis combined with RNA interference
could be a valuable tool for high-throughput discovery of proteins involved in melanosome (or
lysosome) formation or transport. Several libraries of shRNA vectors against the entire human or
mouse genomes are currently available (e.g. (Silva, J.M. et al., 2005, Moffat, J. et al., 2006) ) ,
but there is not yet a high-throughput method to detect melanosomes in individual melanocytes.

Here we describe the development of a detection system that would allow for high-content and
high-throughput analysis of melanosome properties, such as number of melanosomes or amount
of a fluorescently labeled melanosomal protein. We used the Cellomics KineticScan HCS Reader
(Cellomics KSR) to visualize melanosomes labeled with an antibody against tyrosinase-related
protein 1 (TYRP1), and analyzed quantified data with the Spot Detector BioApplication
(Cellomics). Using this method we detected distribution of melanosomes or level of the
melanosomal protein TYRP1 in mouse melanoma B16 cells, but were not able to see an effect of
knockdown of Myosin Va in either B16 cells or in the mouse melanocyte cell line melan-a.
3.3 Experimental Procedures

3.3.1 Constructs and Vectors

pSuper-EGFP shRNA vectors were produced as described previously (Chapter 2, Amsen, E.M. et al., 2006). The unique hairpin sequences were as follows: pSuper-EGFP-Myo5a-RNAi targets GAGATACCTGTGTATGCAG in mouse myosin Va (as was used as siRNA target in (Varadi, A. et al., 2005)) pSuper-EGFP-TYRP1-RNAi targets GCGCACAACTCACCCTTTA in mouse TYRP1. pSuper-EGFP-scrambled (AAGAACCT GTCAGCCACCTAC) does not recognize any regions in the mouse genome.

3.3.2 Cell Culture and Transfection Methods

B16-F10 cells (hereafter, B16 cells) were cultured as described previously (Chapter 2, (Amsen, E.M. et al., 2006)) Melan-a cells were obtained from the Wellcome Trust Functional Genomics Cell Bank and cultured at 37°C and 10% CO₂ in RPMI-1640 with L-glutamine (Invitrogen, cat. 11875) supplemented with 10% FBS, 100U/ml penicillin, 100 μg/ml streptomycin, 7.5 μg/ml phenol red, 0.1 mM 2-mercaptoethanol, 2.7 mM HCl, and 200 nM PMA (added prior to use). MNT-1 cells were kindly provided by Dr. Vincent Hearing (National Institutes of Health, Bethesda, MD) and were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) with 20% FBS, 100U/ml penicillin, and 100μg/ml streptomycin. WM35 cells were a gift from Dr. Chi-Hung Siu (University of Toronto) and were cultured in RPMI1640 (Invitrogen) with 10% FBS, 100U/ml penicillin, and 100μg/ml streptomycin. HEK293T cells were cultured in DMEM with 10% FBS, 100U/ml penicillin, and 100μg/ml streptomycin. B16, MNT-1, WM35, and HEK293T cells were all grown at 37°C and 5% CO₂.

We investigated several lipid-based transfection methods, as outlined in the Results section. The most successful methods selected for the transfection of B16 and melan-a cells were the following: B16 cells were transfected using ESCORT V Transfection Reagent (Sigma), by preparing 0.12 μg DNA in 10 μl buffer and 0.36 μl ESCORT V in 10 μl buffer per sample and adding 12.5 μl of this mix to 100 μl in a 96–well plate. Melan-a cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol, using 0.25 μg DNA per well and 0.6 μl lipofectamine 2000 per well in a 96-well plate.
3.3.3 Microscopy

To visualize TYRP1 staining in various cell types, and to investigate whether TYRP1 is a suitable label for melanosomes, cells were grown in multiwell plates, and transfected (or not) as described. Cells were fixed in 1% paraformaldehyde (PFA) (or 4% for melan-a) in PBS for 20 minutes and permeabilized with a buffer of 0.1% Triton-X 100 and 1% BSA in PBS. Cells were blocked for 1 hour in 5% donkey serum (Jackson ImmunoResearch Laboratories). Samples were incubated for 1 hour with a mouse monoclonal antibody against TYRP1 (AbCam) at 1:200 dilution (or 1:1000 for cells fixed in 4% PFA) in 1%BSA in PBS, washed three times with 1%BSA in PBS, and incubated 45 minutes with Alexa 488-labeled goat-anti-mouse antibody (Molecular Probes) (or with Cy3-labeled donkey-anti-mouse F(ab')$_2$ (Jackson ImmunoResearch Laboratories) for melan-a cells) at 1:1000 in 1% BSA in PBS. Imaging was carried out with a Leica DM IRE2 microscope with Openlab software.

Confocal analysis was used to visualize the location of TYRP1 vs. LAMP1. Melan-a cells were grown in 4-well polystyrene vessel glass slides (BD Falcon). Samples were first stained for 5 minutes with Alexa-488 labeled Concanavalin A (Molecular Probes). The cells were then washed in PBS and fixed in 4% paraformaldehyde (PFA) in PBS for 20 minutes and permeabilized with a buffer of 0.1% Triton-X 100 and 1% BSA in PBS. Cells were blocked for 1 hour in 5% donkey serum (Jackson ImmunoResearch Laboratories). Samples were incubated for 1 hour with a mouse monoclonal antibody against TYRP1 (AbCam) at 1:1000 dilution and a rat monoclonal antibody against LAMP1 (BD Pharmingen) at 1:1000 in 1%BSA in PBS, washed three times with 1%BSA in PBS, and incubated 45 minutes with Cy3-labeled donkey-anti-mouse F(ab')$_2$ (Jackson ImmunoResearch Laboratories) at 1:1000 and Alexa-647 labeled donkey-anti-rat antibody (Molecular Probes) at 1:1000 dilution in 1% BSA in PBS. After washing with PBS, slides were mounted using DAKO® Fluorescent mounting medium. All steps were carried out at room temperature and without shaking to avoid cells detaching from the slide, and in the dark to avoid photobleaching. Confocal analysis was carried out on a Zeiss Axiovert 200 inverted fluorescence microscope with 63X 1.2 water immersion C-Apochromat objective and analyzed with LSM510 software. TYRP1 intensities were quantified with Volocity software.
3.3.4 Cellomics Analysis

Cells were grown in clear 96-well plates (Corning), transfected as described, and fixed at the indicated times in 4% PFA in PBS. After fixing, permeabilization and immunostaining were carried out as described above until the final PBS wash. Then cells were incubated for 5 minutes with DAPI nucleic acid stain (Molecular Probes), washed again, and kept in PBS (100 μl per well).

Plates were scanned on a Cellomics KineticScan® HCS Reader with 10x Plan-Neofluar objective. Images were acquired of 3-20 fields per well (as indicated per experiment) and in the following channels: DAPI was detected in XF93 Hoechst (0.040 second exposure), Cy3 in XF93 TRITC (0.500 second exposure) and when cells were transfected with a GFP-expressing construct this was measured in the XF93 FITC channel (0.500-1.000 second exposure). Each well was measured once (kinetic scan set to “single time point”).

To analyze melanosomal properties (as visualized by TYRP1-labeling) the Spot Detector BioApplication was used and various conditions were tried out as described under Results.

3.3.5 Western Blot Analysis

To investigate knockdown of Myosin Va by pSuper-EGFP constructs, B16 cells were sorted by fluorescence-activated cell sorting (FACS) the day after transfection and the GFP-expressing cells were reseeded in 6-well plates to enrich the population of transfected cells. Cells were lysed the following day (48 hours after transfection) in lysis buffer (150 mM NaCl, 50 mM Hepes, 1% Triton-X, 10% glycerol, 1.5 mM MgCl2, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM PMSF). Total protein levels were determined using the BIORAD protein assay and samples were run on a 7% SDS-PAGE gel. Western blotting was carried out using a rabbit polyclonal antibody against Myosin Va (DIL2, kindly provided by Dr. Vladimir Gelfand (Northwestern University, Chicago, IL)). Primary antibody dilution was 1:1000 in 5% BSA in PBS+0.1% Triton X-100 and secondary HRP-tagged goat-anti-rabbit (Calbiochem) antibody was used at 1:10,000 dilution in 3% skimmed milk in PBS+0.1% Triton X-100. β-Actin was used as a loading control.

To investigate TYRP1 levels in response to cAMP, untransfected B16 cells were treated with 20μM forskolin and 100 μM IBMX at 5 hours or 24 hours prior to cell lysis (or not at all).
Because the antibody against TYRP1 (AbCam) does not recognize the denatured form of the protein, non-reducing sample buffer (without DTT or SDS) was added to cell lysates (not boiled) and samples were run on a 10% non-denaturing non-reducing gel at pH 7 (Gallagher, S.R., 2001). After blotting, the membrane was incubated with primary antibody against TYRP1 at 1:1000 in 5% BSA in PBS+0.1% Triton X-100, and secondary anti mouse antibody (Jackson labs) at 1:10,000 in 3% skimmed milk in PBS+0.1% Triton X-100.
3.4 Results

3.4.1 Melanosome Labeling with Antibody Against TYRP1

We set out to develop a method to monitor melanosomes using high-content analysis on the Cellomics KineticScan HCS Reader (Cellomics KSR). Even though melanosomes in highly pigmented cells are visible in bright field microscopy, the Cellomics KSR only detects fluorescent signals. To visualize melanosomes by immunofluorescence, fixed cells were labeled with an antibody against TYRP1 (tyrosinase-related protein 1), a protein involved in melanin synthesis. TYRP1 is exclusively expressed in melanocytes, where it is localized predominantly to late stage melanosomes. Figure 3.1 shows images of several cell lines stained with DAPI and an antibody against mouse and human TYRP1. Melanocyte-derived cell lines B16, MNT-1 (a human melanoma cell line), and melan-a all show expression of TYRP1, whereas no TYRP1 expression is observed in HEK293T, a human embryonic kidney cell line. In addition, stimulation of B16 cells with 20 μM forskolin and 100 μM IBMX for 24 hours increases TYRP1 expression (Figure 3.1 B).

Labeling with secondary antibody alone does not show any fluorescent staining (Figure 3.1 F), indicating that the signal is specific to the primary antibody. To further confirm that the TYRP1 antibody does not cross-react with non-specific targets, we knocked down TYRP1 using pSuper-EGFP shRNA against TYRP1. Even though there is a large variation in knockdown between cells (see also Chapter 4), we expect to see cells with almost no residual TYRP1 protein upon knockdown. Indeed, Figure 3.2 shows reduction of TYRP1 levels in cells transfected with pSuper-EGFP against TYRP1, but not in cells transfected with a non-silencing control. Although not all cells showed an equally high TYRP1 reduction (making the overall knockdown effect not as high as that seen in the pictured cells), the fact that it is possible to remove most of the TYRP1 signal upon knockdown in at least several cells indicates that the antibody is specific to TYRP1, and does not cross-react with other proteins that are not being knocked down. In this figure, MNT-1 cells were used, but the same phenomenon was also observed repeatedly in melan-a cells using another knockdown construct against TYRP1 (Chapter 4).
**Figure 3.1.** TYRP1 labels only melanocytes

TYRP1 antibody labels melanocyte-derived cell lines of mouse and human origin. Mouse melanoma cell line B16, human melanoma cell line MNT-1, and human embryonic kidney cell line HEK293T were labeled with an antibody against TYRP1 and an Alexa-488 tagged secondary antibody. Mouse melanocyte cell line melan-a was labeled with an antibody against TYRP1 and a Cy3-tagged secondary antibody. All TYRP1 staining is shown in red for consistency. All nuclei were stained with DAPI (blue). Melanocyte-derived cell lines B16 (A,B), MNT-1 (C) and melan-a cells (E) all show TYRP1 staining, while human kidney cells HEK293T do not (D). In the absence of secondary antibody, no signal is seen, shown here for MNT-1 cells (F). Stimulation of B16 cells with forskolin/IBMX for 24 hours shows an increase in TYRP1 signal intensity and more TYRP1 reaches the cell membrane (B) compared to unstimulated cells (A). Field G shows a phase-contrast image of the melan-a cells shown in field E.
Figure 3.2. Low background of TYRP1 antibody

In MNT-1 cells, TYRP1 staining with a secondary Cy3-tagged antibody (shown in red) is reduced upon knockdown of TYRP1 with a pSuper-EGFP construct against TYRP1 (top two panels), indicating that the antibody does not cross-react with other proteins. Transfection with a scrambled control does not show reduction of TYRP1 (bottom two panels).
**Figure 3.3.** TYRP1 labels melanosomes, but not lysosomes

Melan-a cells labeled with an antibody against TYRP1 (secondary antibody Cy3-tagged donkey-anti-mouse F(ab')2, shown here in red) and an antibody against LAMP-1 (secondary antibody Alexa647-tagged donkey-anti-rat antibody, shown here in green) show no colocalization of the two fluorophores in the cytoplasm (only in the TGN), indicating that TYRP1 and LAMP-1 localize to different organelles. This figure shows that TYRP1 is able to selectively label melanosomes, and does not cross-react with lysosomes.
Finally, to make sure that TYRP1 antibody specifically labeled melanosomes and not lysosomes, we costained B16 and melan-a cells with both a TYRP1 and a LAMP1 antibody. Figure 3.3 shows that in melan-a cells, TYRP1 and LAMP1 are colocalized in the perinuclear region (likely the trans-Golgi network), but label separate organelles in the rest of the cytoplasm. This was also observed in B16 cells (not shown). The perinuclear costaining is not surprising, as both melanosomes and lysosomes are initially formed from the same membranes, originating from the trans-Golgi network and early endosomes (Raposo, G. et al., 2001, Orlow, S.J., 1995). The cytoplasmic separation of TYRP1 and LAMP1 signal indicates, however, that the proteins are sorted to different later stage organelles (melanosomes and lysosomes, respectively). This observation corresponds to those of Setty et al. who also showed TYRP1 and LAMP1 immunofluorescent staining in separate organelles (Setty, S.R. et al., 2007).

These combined results suggested that using TYRP1 as a melanosomal marker would allow us to specifically monitor melanosomes using the Cellomics KSR.

3.4.2 Cellomics KSR with Spot Detector BioApplication Detects cAMP-induced Melanosome Dispersion and Increased TYRP1 Expression in B16 Cells

Our initial experiments were carried out with B16 cells. These cells have low basal pigmentation, but in response to cAMP stimulation they increase melanin synthesis, dendrite extension and melanosome movement. We took advantage of the cAMP-dependent effect on melanosome movement and melanin synthesis in B16 cells to optimize detection and analysis parameters on the Cellomics KSR with the Spot Detector BioApplication to allow us to measure these effects.

The Spot Detector BioApplication is one of several protocols available for analysis of data recorded on the Cellomics KSR. It uses an object-recognition algorithm to detect spots by local increases in fluorescence intensity. In this case, spots are defined as localized areas of increased TYRP1 signal, which we see in the melanosomes, as well as in a larger region closer to the nucleus (most likely the TGN). The area of the cell in which measurements take place can be defined as either a ring or a circle around the nucleus, which in turn is recognized by the software as an area of increased DAPI fluorescence intensity. Finally, we used a third channel to separate transfected and untransfected cells by their level of GFP expression.
Figure 3.4. Different protocol settings for the same experiment showing cAMP-dependent melanosome transport in B16 cells

A, B. Spot Detector BioApplication measurements of TYRP1-labeled melanosomes in B16 cells, stimulated with 20 μM forskolin and 100μM IBMX for 5 hours or 24 hours. The measured number of spots per cell increases after 5 hour stimulation using both the Ring Protocol (A) and the Circle Protocol (B). Intensity of spots increases after 24 hour stimulation using both protocols, and this change is also reflected as an increase in spot number when the Ring Protocol is used. Error bars indicate standard error of the mean.

C. Western blot of total lysate from B16 cells is used to confirm that there is indeed an increase in TYRP1 levels after 24 hours of stimulation with forskolin/IBMX, but not after 5 hours. Samples were run on a non-denaturing, non-reducing gel. The ponceau stain of the blot (before treatment with antibody against TYRP1) shows that total protein levels between lanes did not change, and the increase is specific to TYRP1. (Protein size marker not shown: preboiled protein marker ran faster than unboiled samples, and marker bands did not reflect correct size.)
### Table 3.1 Differences in Spot Detector BioApplication parameters between Ring protocol and Circle protocol.

All parameters for the Circle protocol are listed in Appendix A.1. See figure 3.5 for a schematic representation of the areas where spots are detected in the Ring and Circle protocol.
Figure 3.5. Schematic representation of ring and circle areas used in Spot Detector BioApplication

A. In the Ring Protocol, spots are detected in an area defined by a ring around the nucleus. The width of the ring and the distance from the nucleus are variable. B. In the Circle Protocol, spots are detected in a circle that includes the area of the nucleus. This allows for the detection of spots that are close to the nucleus, where the location of the nucleus is calculated by the intensity of the DAPI signal.
In this experiment, we looked at the effect on melanosome dispersion upon stimulation of untransfected B16 cells with forskolin/IBMX for 5 or 24 hours. Figure 3.4A and 3.4B show the result of these measurements using two different versions of the Spot Detector protocol with different parameters (Table 3.1). The main difference between the two protocols is the area of the cell selected for measurements: “Ring protocol” measures spots in an area defined by a ring around the nucleus, 4 pixels wide. “Circle protocol” measures spots in a circle in the middle of the cell of a radius 5 pixels larger than the radius of the nucleus. See Figure 3.5 for a schematic representation of the area in which spots are detected. Using both protocols, we saw an increase in the number of spots in the selected region after 5 hours of stimulation with forskolin/IBMX. The Ring protocol showed a further increase in spot number at 24 hours after stimulation, but this was not observed when using the Circle protocol. In the latter case, melanosome dispersion did not increase further between 5 and 24 hours of forskolin/IBMX stimulation. Furthermore, both protocols showed an increase in spot intensity after 24 hours of stimulation with forskolin/IBMX. Since spots are defined by labeling with a TYRP1 antibody, and TYRP1 expression is known to be upregulated in response to cAMP activation (Bertolotto, C. et al., 1998b), this was most likely due to an increase in TYRP1 levels. Indeed, western blotting confirmed that TYRP1 levels in B16 cells increased after 24 hours of stimulation with forskolin/IBMX, but not yet after 5 hours (Figure 3.4C). This suggests that the inconsistent increase in spot number at 24 hours as measured with the ring protocol was an artifact of increased TYRP1 signal overall. Further experiments were carried out with the Circle protocol (See Appendix A.1).

3.4.3 Choice of Cell Line and Transfection Method

Even though B16 cells appeared as a good test system due to their cAMP-responsiveness, they were not suitable for our intended purpose of setting up a system to use RNA interference to find proteins involved in melanosomal transport or formation. During the initial test screens with B16 it became apparent that the cells grow as clumps, even at lower seeding density, as shown by screen-captures from the Cellomics KSR (Figure 3.6, top four panels). This creates a problem when using the Cellomics KSR applications to select individual cells. Cells growing on top of each other could be eliminated by setting the Spot Detector BioApplication to reject cells with nuclei that were within a certain range of each other, but this greatly reduced the number of valid
Figure 3.6. Proliferation patterns of B16 and melan-a cells

Screen captures of cells imaged in several runs of the Cellomics KSR in both DAPI and Cy3 fields show that B16 cells do not spread as a monolayer, but rather grow on top of each other, making it difficult to identify individual cells using Cellomics’ image analysis software. Melan-a cells grow slower but spread out more evenly over the wells.
objects (cells) in each field. We therefore switched to another mouse melanocyte-derived cell line, melan-a. These cells grow in a monolayer at both confluent and subconfluent conditions (Bennett, D.C. et al., 1987) (Figure 3.6, bottom four panels). This facilitates the selection of individual cells with image analysis software. Table 3.2 summarizes the differences in settings for the Spot Detector BioApplication for B16 cells and melan-a cells. The background signal, especially in the DAPI channel, was higher in melan-a cells than in B16 cells, requiring a different setting for the Background Correction parameter. Effectively this means that the background in all channels was calculated over a larger area for melan-a cells than for B16 cells. To reject cells that are too close together in the experiments using B16 cells, the nuclei were located by peak intensity and considered separate when the centers of the nuclei were 14 pixels apart or more. Cells that were too close together were grouped as a whole and rejected by size.

Initially, we also investigated the possibility of using a human melanocyte-derived cell line. However, none of the human cell lines we studied were suitable for our purposes. We first acquired WM35, a non-metastatic cell-line derived from the primary radial growth phase of human melanoma (Herlyn, M., 1990), but this cell line is not pigmented enough. No pigmentation was visible by eye, and tyrosinase, the rate limiting enzyme in pigmentation, showed minimal expression and enzymatic activity in these cells (not shown). We then studied MNT-1, a highly pigmented human melanoma cell line. In these cells we could easily visualize melanosomes using a TYRP1 antibody, which recognizes both mouse and human TYRP1 protein (figure 3.1 C), but we found that MNT-1 cells were very difficult to transfect, and could not reach transfection efficiencies higher than about 7% (see Table 3.3).

Ease of transfection was an important criterion in the selection of a cell line, as we intended this system to be used for medium- to high-throughput RNAi screening without using viral infection. We investigated lipid-based transfection methods, as they generally give high transfection efficiencies and are easily adapted for different culture volumes or number of simultaneous transfections. Table 3.3 lists the maximum transfection efficiencies achieved with various cell lines and methods after optimization. It’s clear that B16 is the most easily transfectable cell line of the ones we tried, but melan-a transfection levels are high enough to ensure that there will be transfected cells in all fields imaged on Cellomics KSR. Additionally, increasing the number of measured fields also increases the number of imaged cells and with it the accuracy of the measurements and the statistical relevance of the data.
<table>
<thead>
<tr>
<th></th>
<th>B16 cells</th>
<th>Melan-a cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background Correction</td>
<td>Background correction calculated over an area with</td>
<td>Background correction calculated over an area with</td>
</tr>
<tr>
<td></td>
<td>radius 1 pixel</td>
<td>radius 50 pixels</td>
</tr>
<tr>
<td></td>
<td>(BackgroundCorrection = 1)</td>
<td>(BackgroundCorrection = 50)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Identifying individual cells</td>
<td>Nuclei are identified by peak intensity and considered</td>
<td>Nuclei are identified based on shape with a typical radius of 5</td>
</tr>
<tr>
<td></td>
<td>separate when they are at least 14 pixels apart</td>
<td>pixels per nucleus</td>
</tr>
<tr>
<td></td>
<td>(ObjectSegmentationCh1 = -7)</td>
<td>(ObjectSegmentationCh1 = 5)</td>
</tr>
</tbody>
</table>

**Table 3.2** Differences in Spot Detector BioApplication parameters between protocols run on B16 cells or melan-a cells.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Escort V</th>
<th>Lipofectamine</th>
<th>Lipofectamine 2000</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16</td>
<td>70-95%</td>
<td>20-50%</td>
<td>not used</td>
</tr>
<tr>
<td>MNT-1</td>
<td>&lt;5%</td>
<td>5%</td>
<td>not used</td>
</tr>
<tr>
<td>Melan-a</td>
<td>0%</td>
<td>not used</td>
<td>40-50%</td>
</tr>
</tbody>
</table>

**Table 3.3**  Approximate maximal transfection efficiencies obtained with various lipid-based transfection reagents.

Transfection efficiencies were calculated by transfecting cells with a GFP-expressing construct (pSuper-EGFP) and using Cellomics or OpenLab software to compare the number of GFP-expressing cells to the total number of cells.
We increased the number of fields per well from 3-10 for B16 cells to 10-20 for melan-a cells in order to keep the number of measured transfected cells constant at approximately 300 cells measured per well in both cell lines.

We transfected melan-a cells with Lipofectamine 2000 rather than Lipofectamine, because successful transfection of melan-a with Lipofectamine 2000 had already been reported previously by various groups (Loftus, S.K. et al., 2002, Suzuki, T. et al., 2001). The main practical difference between the two reagents is the transfection medium. Lipofectamine calls for serum-free media, while Lipofectamine 2000 works in medium with serum, but in the absence of antibiotics.

For further experiments, we used the melan-a cell line in combination with knockdown constructs against mouse mRNA sequences. Another advantage of this cell line is that it has previously been used in several studies of melanosomes, suggesting that it would be suitable for our purposes as well (Kuroda, T.S. et al., 2005, Kuroda, T.S. et al., 2003, Suzuki, T. et al., 2001).

3.4.4 No Effect of Knockdown of Myosin Va on Melanosome Transport

Myosin Va is necessary for transport of melanosomes, and loss-of-function mutations in Myosin Va lead to pigmentation defects in mice and humans (Wei, Q. et al., 1997, Pastural, E. et al., 1997, Provance, D.W., Jr et al., 1996). At the cellular level, this is visible in bright field microscopy as a clustering of melanosomes around the nucleus, rather than a distribution throughout the cytoplasm as seen in wild-type melanocytes. This clustering has also been visualized by fluorescently labeling melanosomes with an antibody against TYRP1 (Wei, Q. et al., 1997).

Because we detected variations in melanosome distribution in response to cAMP in B16 cells using the Cellomics KSR with the Spot Detector BioApplication (Figure 3.4A and B), we expected to be able to detect a decrease in melanosome distribution upon Myosin Va knockdown. I created a pSuper-EGFP knockdown construct against mouse Myosin Va, using a target sequence that was previously used by Varadi and colleagues to successfully knock down this protein (Varadi, A. et al., 2005). To confirm that this construct was able to knock down Myosin Va, B16 cells were transfected with the generated pSuper-EGFP construct against Myosin Va, or with pSuper-EGFP constructs without insert or with a hairpin with a non-
silencing sequence (pSuper-EGFP-scrambled). The next day, cells were FACS-sorted by GFP fluorescence to enrich for transfected cells, and lysed for western blot analysis at 48 hours after transfection. Figure 3.7 shows that pSuper-MyoVa successfully knocks down Myosin Va in B16 cells. (Two separate transfections with the knockdown construct are shown, both knocking down equally well, with 80-90% of protein reduced compared to non-silencing control and normalized against actin levels). Unfortunately, we were not able to repeat this experiment using melan-a cells, as these cells died shortly after FACS-sorting: They could not be reseeded after sorting, and they did not yield enough cells directly after sorting to carry out western blot analysis.

However, even though Myosin Va was knocked down, melanosome location did not seem to be affected. None of the melan-a or B16 cells transfected with the knockdown construct showed the typical clustering of melanosomes around the nucleus that is seen in melanocytes of dilute mice, which have a defect in Myosin Va. Initially, Cellomics KSR analysis of melan-a cells transfected with pSuper-EGFP constructs against Myosin Va or TYRP1 did show a reduction in spot number compared to cells transfected with non-silencing controls (Figure 3.8, top panel). However, when the intensity of the TYRP1 signal is analyzed in this same experiment, it is clear that there is no reduction in TYRP1 level upon transfection with pSuper-TYRP1 compared to cells transfected with negative control constructs (Figure 3.8, bottom panel). This indicates that there is no knockdown of TYRP1 in this experiment, and that therefore the observed change in spot number upon transfection with pSuper-TYRP1 was a false positive. This makes the reduction in spot number for pSuper-Myosin Va transfection suspect as well. Indeed, these results were not reproducible.
pSuper-EGFP shRNA against Myosin Va knocks down Myosin Va, as shown here for FACS-sorted B16 cells. Two separate transfections with the construct both reduce Myosin Va levels significantly compared to non-silencing controls (top panel). β-Actin was used as a loading control (bottom panel). Normalized reduction in protein levels is 80-90% of control in cells transfected with knockdown constructs.
Figure 3.8. Cellomics KSR analysis of Myosin Va and TYRPI knockdown

Melan-a cells transfected with knockdown constructs against Myosin Va or TYRPI seem to show a decrease in spot number per cell when normalized over untransfected cells in the same wells (top), but knockdown of TYRPI is not significant enough to reduce TYRPI levels (bottom). The decrease in spot number was not reproducible. Error bars indicate standard error of the mean.
3.5 Discussion

Automated high-content image analysis of cells has been successfully used in several studies where the desired output was not one single phenotype, but rather a range of possible phenotypes. An example is the use of high-content screening in combination with RNAi to identify genes whose suppression led to any of several phenotypes associated with mitotic spindle defects (Rines, D.R. et al., 2008).

We proposed the use of high-content cellular image analysis combined with RNAi to study melanosomes, because it would have allowed for the detection of changes in melanosome formation, proper sorting of cargo, or melanosome transport – all of which may lead not only to pigmentation defects but may also be involved in organelle formation and transport in general. In this chapter we investigated the use of the Cellomics HCS KineticScan Reader combined with Spot Detector BioApplication to carry out high-content analysis of melanosome properties, with the goal of ultimately scaling up this system to a medium- or high-throughput RNAi screen.

Even though melanosomes in highly pigmented melanocytes are visible by bright field microscopy, the Cellomics KSR only detects fluorescently labeled targets. We showed that a TYRP1 antibody with fluorescently tagged secondary antibody successfully labeled melanosomes in several cell lines of melanocytic origin (Figure 3.1 – 3.3). It is interesting to note that there is a clear distinction between TYRP1 and LAMP1 labeled organelles. A similar observation was made by others using both EM and immunofluorescent imaging (Raposo, G. et al., 2001, Setty, S.R. et al., 2007). Raposo et al. also reported a colocalization of TYRP1 and LAMP1 in vesicles near the Golgi area, corresponding with a colocalization we observe in that region (Figure 3.3)(Raposo, G. et al., 2001). Some literature suggests that there is LAMP1 present in melanosomes, but these studies focus on early stage organelles common to both lysosomes and melanosomes or are based on biochemical techniques in which contamination from other organelles is possible (Zhou, B.K. et al., 1993, Chi, A. et al., 2006). For our purposes, however, our data clearly indicate that TYRP1 can be used to label (late stage) melanosomes as distinct organelles in the cytoplasm.

In our initial experiments we successfully used the Cellomics KSR and Spot Detector BioApplication software to detect cAMP-induced changes in melanosomal properties in B16
cells. These cells are known to increase melanosome movement after stimulation with cAMP (Passeron, T. et al., 2004, Chiaverini, C. et al., 2008). Indeed, after 5 hours of stimulation, a cAMP-induced dispersion of melanosomes was registered as an increase in spot number (Figure 3.4). This increase in spot number was still seen after changing several parameters of the Spot Detector protocol (Table 3.1), suggesting that this detection method was quite robust. It should be kept in mind, however, that the number of spots in the selected target area is not only a measure of melanosome dispersion, but also of overall melanosome number. In a screen for proteins involved in either formation or transport of melanosomes, both phenotypes would likely register as a change in spot number and further analysis would be required to determine the cause of the change in spot number. Furthermore, the number of spots is not a direct measurement of the number of melanosomes but merely a representation. Changes in cutoff values, background correction, and digital smoothing of spots all contribute to a change in the spot number output value (Table 3.1). In addition, TYRP1 is also present in other cell bodies before it is sorted to the melanosome, and a fraction of TYRP1 is always seen in the trans-Golgi network, which is also considered a “spot” by the software.

We see the effect of changes in parameters on spot detection quite clearly when comparing the measured change in spot number for B16 cells stimulated with forskolin/IBMX for 24 hours. At this time point, an increase in TYRP1 levels is visualized by both Cellomics analysis and western blotting. One of the Spot Detector settings used, the Ring protocol, also registers this change as a change in spot number, while the Circle protocol does not see a further increase in spot number between 5 hours and 24 hours of stimulation. This indicates that changing the detection parameters may influence output, but it also shows that the biologically significant information - in this case an increase in TYRP1 intensity upon 24 hour stimulation with forskolin IBMX and an increase in melanosome dispersion as soon as 5 hours after stimulation - is detected quite robustly by both protocols, as spot intensity and spot number respectively.

B16 cells not only show increased melanosome transport upon cAMP stimulation, but also increased melanin synthesis and dendrite extension. There are two BioApplications available for Cellomics that detect neurite extension from a cell, but neither of them was able to detect the overall morphological changes that occur in B16 cells when dendrites are formed. This was caused in part by the proliferation pattern of B16 cells, which also proved problematic for spot detection (see below). Other melanocyte-derived cell lines, such as melan-a, do not form as
many dendritic extensions upon cAMP stimulation as B16 does, so dendrite extension was not further investigated as a parameter for Cellomics KSR analysis. In addition, knockdown of RhoA does not show a consistent effect on dendricity of B16 cells, even though chemical inhibition of RhoA is known to increase dendrite extension (Busca, R. et al., 1998) (Appendix A.3).

Even though B16 was successfully used to set up screening parameters for spot detection, it is not a suitable cell line to use for our intended experiments, in which measurements would only be taken in transfected cells (identified by GFP expression): B16 cells do not grow in a monolayer but in clumps, and when cells are on top of each other the software cannot properly distinguish transfected cells from untransfected cells. We settled on the pigmented mouse melanocyte cell line melan-a as a suitable alternative, but not until after investigating various transfection methods to transfect the cells with pSuper-EGFP knockdown constructs. While B16 cells were most successfully transfected with Escort V, this method did not work well for melan-a cells. Transfection of melan-a cells with lipofectamine 2000 yielded enough transfected cells to carry out Cellomics analysis. We also noticed that there is a lot of variation in TYRP1 staining between cells and between wells. Much of this variation is inherent to the cells themselves, but to rule out any changes in TYRP1 staining due to local variations in antibody concentrations or other artifacts, we normalized all data collected from transfected cells over the values calculated from untransfected cells in the same well.

We tested whether knockdown of Myosin Va would affect spot number. Since Myosin Va is essential for melanosome transport, we expected to see a reduction in melanosome movement upon its knockdown. However, even though a change in spot number was detected on Cellomics, no change in melanosome location was visible by eye in transfected cells, and a detected change in spot number for TYRP1 knockdown did not correlate with the TYRP1 intensity for this sample (Figure 3.7). Myosin Va knockdown can’t be checked in the same sample as used for spot detection. An independent analysis of Myosin Va knockdown in B16 cells shows that, at least in that cell line, the knockdown construct is very efficient (reducing protein levels by 80-90%) but it’s unknown whether it is equally efficient in melan-a cells or how much reduction of Myosin Va is required to see an effect on melanosome location. This issue is addressed in the next chapter, which describes the systematic search for proteins whose knockdown can serve as a positive control for our intended screen.
Chapter 4
Investigation of the Potential Use of RNA-interference as a Tool to Find Proteins Involved in Melanosome Formation or Transport

I designed and performed all experiments in this chapter.
4.1 Summary

Melanosomes are lysosome-related organelles that produce and transport the pigment melanin within melanocytes. Mutations in proteins required for melanosome transport and formation lead to a range of changes at the cellular level, including perinuclear clustering of melanosomes, or reduced sorting of melanosomal cargo such as tyrosinase-related protein 1 (TYRP1). Our intention was to investigate the possibility of using RNA interference screening and high-content cellular imaging to detect new proteins involved in melanosome development or transport.

We previously set up a system to detect and analyze TYRP1-labeled melanosomes using Cellomics KSR high-content analysis (Chapter 3). Here we investigate whether this system is suitable for RNAi screening. We analyzed mouse melan-a cells transfected with shRNAmir constructs against the following eleven genes: TYRP1, pallidin, cappuccino, dysbindin, HPS5, LYST, Myosin Va, melanophilin, RhoA, UBPY and mahogunin (Table 4.1). In a blinded confocal experiment, the only reproducible change observed in melan-a cells was a decrease in TYRP1 levels upon transfection with knockdown constructs against TYRP1 itself, or one of three constructs targeting HPS5 – the gene mutated in Hermansky-Pudlak Syndrome 5. However, upon analysis with Cellomics KSR, only the construct with the most severe effect on TYRP1 levels was detected: the knockdown construct against TYRP1 itself.

RT-PCR analysis showed that many of the shRNAmir constructs reduced mRNA levels, but often by less than 60%, suggesting that this level of reduction of mRNA and proteins is not enough to detect effects at the melanosome level. This is further examined for melanophilin, a protein necessary for melanosome transport. Perinuclear clustering is observed only in cells where melanophilin levels are extremely low, but in most cells melanophilin levels are not significantly reduced upon transfection with the knockdown construct.

Altogether, the inability to detect the effect of knockdown of known regulators of melanosome formation, transport, or other aspect of pigmentation makes this system not sensitive enough for use in an RNA interference screen for unknown proteins affecting melanosome properties.
4.2 Introduction

Melanosomes are specialized organelles in melanocytes, responsible for the synthesis and distribution of the pigment melanin (Seiji, M. et al., 1963). The mechanisms and proteins involved in formation and movement of melanosomes are similar to those of lysosome-related organelles in other cell types, such as synaptic vesicles in neurons, platelet dense granules, and lytic vesicles in T-cells (Stinchcombe, J. et al., 2004, Barral, D.C., and Seabra, M.C., 2004, Orlow, S.J., 1995). In diseases such as Hermansky-Pudlak Syndrome, Chediak-Higashi Syndrome, or Griscelli Syndrome, hypopigmentation caused by a defect in either formation of movement of melanosomes is accompanied by defects in other lysosome-related organelles, causing, for example, bleeding disorders, immunological abnormalities, or neurological symptoms (Wei, M.L., 2006, Pastural, E. et al., 1997, Ménasché, G. et al., 2000, Ménasché, G. et al., 2003, Page, A.R. et al., 1962, Burkhardt, J.K. et al., 1993, Hermansky, F., and Pudlak, P., 1959). This makes melanosomes an interesting model to study organelle formation and transport in general.

We have set up a high-content analysis system on the Cellomics HCS KineticScan Reader (Cellomics KSR) to investigate changes in melanosome formation or movement using TYRP1 as a melanosomal label (Chapter 3). To test whether the Cellomics KSR can be used in an RNA interference screen for proteins affecting melanosome formation or movement, we have previously transfected cells with a knockdown construct against Myosin Va (Chapter 3). This protein is involved in melanosome transport, and mice with a mutant, inactive, form of Myosin Va show a clustering of melanosomes around the nucleus of melanocytes (Wei, Q. et al., 1997, Provance, D.W.,Jr et al., 1996). However, when Myosin Va was knocked down in melan-a cells, we did not observe such a clustering (Chapter 3).

Before considering scaling up Cellomics KSR analysis of melanosomes to a medium- or high-throughput RNA interference screen, it is necessary to find control shRNA constructs for which we detect a clear change in phenotype upon target knockdown, and knockdown of Myosin Va did not seem to fit this purpose.

In this chapter I describe the search for other genes whose knockdown may serve as a positive control for a high-content RNAi analysis of melanosomes using Cellomics KSR. To this end I
selected eleven proteins to be knocked down: cappuccino, pallidin, dysbindin, HPS5, Myosin Va, melanophilin, LYST, RhoA, UBPY, mahogunin, and TYRP1.

Cappuccino, pallidin, and dysbindin were originally identified as proteins whose mutations caused a severe pigmentation defect in mice (Li, W. et al., 2004, Nguyen, T. et al., 2002). These three proteins are components of the BLOC-1 complex, required for the proper sorting of cargo (such as TYRP1) from early endosomes to lysosome-related organelles (Setty, S.R. et al., 2007). In humans, mutations in dysbindin are associated with Hermansky-Pudlak Syndrome type 7 and with an increased susceptibility to schizophrenia (Chen, X.W. et al., 2008, Li, W. et al., 2003). The latter is related to the formation of synaptic vesicles, highlighting the conserved pathways between melanosomes and other lysosome-related organelles. HPS5, a protein which, when mutated, leads to Hermansky-Pudlak Syndrome type 5, is a component of the protein complex BLOC-2, which acts downstream of BLOC-1 and is also involved in the sorting of proteins to lysosome-related organelles (Di Pietro, S.M. et al., 2006, Helip-Wooley, A. et al., 2007). Myosin Va and melanophilin play a role in melanosome transport and are mutated in Griscelli Syndrome (Wei, Q. et al., 1997, Fukuda, M. et al., 2002, Matesic, L.E. et al., 2001, Pastural, E. et al., 1997, Ménasché, G. et al., 2003, Kuroda, T.S., and Fukuda, M., 2004). LYST is mutated in Chediak-Higashi syndrome, where it produces giant lysosomes and melanosomes (Burkhardt, J.K. et al., 1993, Fukai, K. et al., 1996, Tchernev, V.T. et al., 2002). Chemical inhibition of RhoA in mouse melanoma B16 cells increases dendrite extension, suggesting that its knockdown might have the same effect (Busca, R. et al., 1998). UBPY is a deubiquitinating enzyme required for endosomal sorting (Mizuno, E. et al., 2006). Mahogunin is an E3 ubiquitin ligase involved in regulating the melanocortin-1 receptor-mediated balance between the brown/black pigment eumelanin and the yellow/red pigment pheomelanin (Bagher, P. et al., 2006, Kim, B.Y. et al., 2007, Phan, L.K. et al., 2006). Finally, TYRP1 is the label used in these experiments to detect melanosomes. It is also involved in melanin synthesis, and its sorting to melanosomes is regulated in part by pallidin, cappuccino, dysbindin, and HPS5 (Ito, S., and Wakamatsu, K., 2008, Setty, S.R. et al., 2007, Helip-Wooley, A. et al., 2007). The known functions and predicted effects of knockdown of all proteins mentioned above are listed in Table 4.1.

Here we studied the effect of knockdown of all target proteins in a blinded experiment using confocal microscopy, and found that knockdown of TYRP1 and HPS5 both showed a reproducible decrease in TYRP1 levels, but that only knockdown of TYRP1 itself is detected
using the Cellomics KSR. Limited knockdown and residual protein expression appear to be the major cause for the lack of effect of knockdown of any of the other selected targets.

The lack of detection of any of the proteins known to dramatically affect melanosome location or TYRPI sorting or levels unfortunately makes the system unsuitable at this point for use in a medium- or high-throughput screen for unknown proteins affecting melanosome properties.
Table 4.1  Targets for pilot study

Proteins selected for knockdown in experiments searching for a positive control for Cellomics analysis. These targets have been linked to melanin synthesis (TYRP1, Mahogunin), development of melanosomes or other lysosome-related organelles (cappuccino, pallidin, dysbindin, HPS5, LYST, UBPY), transport of melanosomes (Myosin Va, melanophilin) or dendrite extension (RhoA).
<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cappuccino</td>
<td>Component of BLOC-1. (Li, W. et al., 2004, Nguyen, T. et al., 2002).</td>
</tr>
<tr>
<td>Pallidin</td>
<td>Component of BLOC-1. Mice with a mutation in pallidin have almost no mature melanosomes, and TYRP1 sorting is impaired (Setty, S.R. et al., 2007, Li, W. et al., 2004, Nguyen, T. et al., 2002).</td>
</tr>
<tr>
<td>Dysbindin</td>
<td>Component of BLOC-1. Mice with a mutation in dysbindin have almost no mature melanosomes. In humans, dysbindin mutations are associated with Hermansky-Pudlak Syndrome type 7 and with schizophrenia (Chen, X.W. et al., 2008, Li, W. et al., 2003).</td>
</tr>
<tr>
<td>HPS5</td>
<td>Component of BLOC-2. Mutated in Hermansky-Pudlak Syndrome type 5 patients, and reduces number of mature melanosomes and TYRP1 levels in these patients (Helip-Wooley, A. et al., 2007).</td>
</tr>
<tr>
<td>LYST</td>
<td>Mutated in Chediak-Higashi Syndrome. Mutation leads to formation of giant lysosomes and melanosomes. (Burkhardt, J.K. et al., 1993, Tchernev, V.T. et al., 2002).</td>
</tr>
<tr>
<td>Myosin Va</td>
<td>Mutated in Griscelli Syndrome Type 1. Necessary for transport of melanosomes. (Wei, Q. et al., 1997, Pastural, E. et al., 1997).</td>
</tr>
<tr>
<td>UBPY (USP8)</td>
<td>Deubiquitinating enzyme, necessary for proper sorting to endosomes. Knockdown in HeLa cells affects shape of endosomes and MVBs. Mouse mutant is embryonic lethal. (Niendorf, S. et al., 2007, Mizuno, E. et al., 2006).</td>
</tr>
<tr>
<td>Mahogunin</td>
<td>Required for pigment type switching between eumelanin and pheomelanin. Mahogunin is an E3 ubiquitin ligase required for endosome to lysosome trafficking. (Bagher, P. et al., 2006, Kim, B.Y. et al., 2007, Phan, L.K. et al., 2002).</td>
</tr>
<tr>
<td>RhoA</td>
<td>Chemical inhibition in B16 cells induces dendrite outgrowth (Busca, R. et al., 1998).</td>
</tr>
</tbody>
</table>
4.3 Experimental Procedures

4.3.1 Cell Culture and Constructs

Melan-a cells were obtained from the Wellcome Trust Functional Genomics Cell Bank and cultured at 37°C and 10% CO₂ in RPMI-1640 with L-glutamine (Invitrogen, cat. 11875) supplemented with 10% FBS, 100U/ml penicillin, 100 µg/ml streptomycin, 7.5 µg/ml phenol red, 0.1 mM 2-mercaptoethanol, 2.7 mM HCl, and 200 nM PMA (added prior to use).

Mouse genomic pGIPZ-shRNAmir vectors from the Hannon-Elledge library were purchased from Open Biosystems. These vectors express TurboGFP and transcribe an shRNAmir hairpin, both using a CMV promotor (Silva, J.M. et al., 2005). pGIPZ constructs used are listed in Table 4.2.

Cells for confocal analysis were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol, using 1µg DNA and 3 µl lipofectamine 2000 per well in a 4-well chamber slide, seeded with 100,000 cells the previous day. Cells for Cellomics analysis were transfected as described in Chapter 3.

4.3.2 Immunostaining:

For confocal analysis, cells were grown in 4-well polystyrene vessel glass slides (BD Falcon). At 48 or 72 hours after transfection with pGIPZ vectors, cells were fixed and stained for immunofluorescence. All steps were carried out at room temperature and without shaking to avoid cells detaching from the slide, and in the dark to avoid photobleaching. Samples were first stained for 5 minutes with Alexa-647 labeled Concanavalin A (Molecular Probes) to label the plasma membrane. The cells were then washed in PBS and fixed in 4% paraformaldehyde (PFA) in PBS for 20 minutes and permeabilized with a buffer of 0.1% Triton-X 100 and 1% BSA in PBS. Cells were blocked for 1 hour in 5% donkey serum (Jackson ImmunoResearch Laboratories). Samples were incubated for 1 hour with a mouse monoclonal antibody against TYRP1 (AbCam) at 1:1000 dilution and a rabbit polyclonal antibody against TurboGFP (Evrogen) at 1:10,000 dilution in 1%BSA in PBS. After this, cells were washed three times with 1% BSA in PBS, and incubated 45 minutes with Cy3-labeled donkey-anti-mouse F(ab')₂ (Jackson ImmunoResearch Laboratories) at 1:1000 and FITC-labeled donkey-anti-rabbit
Table 4.2  pGIPZ constructs

pGIPZ constructs transcribing hairpins from the Hannon-Ellegde shRNAmir library were obtained from OpenBiosystems. Every available shRNAmir hairpin against each gene is listed. GenBank IDs refer to the mouse genome.
<table>
<thead>
<tr>
<th>Gene name</th>
<th>GenBank ID (mouse)</th>
<th>Open Biosystems Construct ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>TYRP1</td>
<td>NM_031202</td>
<td>v2LMM_12467</td>
</tr>
<tr>
<td>Cappuccino</td>
<td>NM_133724</td>
<td>v2LMM_21767</td>
</tr>
<tr>
<td></td>
<td></td>
<td>v2LMM_250967</td>
</tr>
<tr>
<td>Pallidin</td>
<td>NM_019788</td>
<td>v2LMM_24372</td>
</tr>
<tr>
<td></td>
<td></td>
<td>v2LMM_28461</td>
</tr>
<tr>
<td></td>
<td></td>
<td>v2LMM_36292</td>
</tr>
<tr>
<td>Dysbindin</td>
<td>NM_025772</td>
<td>v2LMM_76067</td>
</tr>
<tr>
<td>HPS5</td>
<td>NM_001005248</td>
<td>v2LMM_196168</td>
</tr>
<tr>
<td></td>
<td></td>
<td>v2LMM_92618</td>
</tr>
<tr>
<td></td>
<td></td>
<td>v2LMM_224489</td>
</tr>
<tr>
<td>LYST</td>
<td>NM_010748</td>
<td>v2LMM_4222</td>
</tr>
<tr>
<td></td>
<td></td>
<td>v2LMM_11529</td>
</tr>
<tr>
<td></td>
<td></td>
<td>v2LMM_10733</td>
</tr>
<tr>
<td>Myosin Va</td>
<td>NM_010864</td>
<td>v2LMM_4868</td>
</tr>
<tr>
<td></td>
<td></td>
<td>v2LMM_7485</td>
</tr>
<tr>
<td></td>
<td></td>
<td>v2LMM_17673</td>
</tr>
<tr>
<td>Melanophilin</td>
<td>AF384098</td>
<td>v2LMM_73840</td>
</tr>
<tr>
<td></td>
<td></td>
<td>v2LMM_70729</td>
</tr>
<tr>
<td></td>
<td></td>
<td>v2LMM_62443</td>
</tr>
<tr>
<td>UBPY</td>
<td>NM_019729</td>
<td>v2LMM_37945</td>
</tr>
<tr>
<td>Mahogunin</td>
<td>NM_029657</td>
<td>v2LMM_66590</td>
</tr>
<tr>
<td></td>
<td></td>
<td>v2LMM_75408</td>
</tr>
<tr>
<td>RhoA</td>
<td>NM_016802</td>
<td>v2LMM_50451</td>
</tr>
<tr>
<td>Non-silencing control</td>
<td></td>
<td>RHS4346*</td>
</tr>
</tbody>
</table>

* catalog number
(Jackson ImmunoResearch Laboratories) secondary antibody in 1% BSA in PBS. After washing with PBS, slides were mounted using DAKO® Fluorescent mounting medium.

To detect melanophilin knockdown by immunofluorescence, the same protocol was used, but with a goat polyclonal antibody against melanophilin (AbCam, 1:1000 dilution) instead of TYRP1 antibody, and secondary antibody 1:1000 donkey-anti-goat Cy3 (Jackson ImmunoResearch Laboratories) instead of donkey-anti-mouse F(ab’)2

For Cellomics KSR analysis, cells were grown in 96-well plates (Corning), transfected as described, and fixed at the indicated times in 4% PFA in PBS. After fixation, permeabilization and immunostaining were carried out as described above until the final PBS wash step. Then cells were incubated for 5 minutes with DAPI nucleic acid stain (Molecular Probes), washed again, and kept in PBS (100 μl per well).

4.3.3 Confocal Analysis

Confocal analysis was carried out on a Zeiss Axiovert 200 inverted fluorescence microscope with 63X 1.2 water immersion C-Apochromat objective and analyzed with LSM510 software. To quantify levels of TYRP1 per cell, Volocity software was used to measure the total intensity of the Cy3 signal in all GFP-expressing cells, as well as the total intensity of the Cy3 signal in a similar number of cells that did not express GFP in the same fields, to account for variations in signal intensity between samples. Results were analyzed and presented using GraphPad, where error bars show the Standard Error of the Mean (S.E.M.) and unpaired T-test analysis was used to determine statistical significance between transfected and untransfected cells. The same protocol was followed to quantify melanophilin knockdown by immunofluorescence.

4.3.4 Cellomics KSR Analysis

Plates were scanned on a Cellomics KineticScan® HCS Reader with 10x Plan-Neofluar objective. Images were acquired in 20 fields per well and in the following channels: DAPI was detected in XF93 Hoechst (0.040 second exposure), Cy3 in XF93 TRITC (0.500 second exposure) and FITC-labeled TurboGFP in the XF93 FITC channel (0.500 second exposure). Each well was measured once (kinetic scan set to “single time point”).
To determine TYRP1 levels, the SpotDetector BioApplication was used as described in Chapter 3. Briefly, two protocols were used to measure spots: one measured transfected cells (SpotAvgIntenCh3 min = 25) and the other measured untransfected cells (SpotAvgIntenCh3 max = 24.999).

4.3.5 RNA Isolation and First Strand Synthesis

To analyse mRNA levels after knockdown, cells were transfected with pGIPZ constructs and GFP-expressing cells were collected on a BD Aria-RITT sorter by fluorescence-activated cell sorting (FACS) after 72 hours. Collected cells were immediately lysed in 800 µl TRIZOL Reagent (Invitrogen) according to the manufacturer’s protocol. After phase separation with chloroform, 0.5 µl glycogen was added as a carrier to the aqueous phase. Final RNA pellet was taken up in 12 µl DEPC-treated water. After determining RNA concentration in the sample, 8 µl was converted to cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR (real-time PCR) (Invitrogen) according to the manufacturer’s protocol.

4.3.6 RT-PCR

After first-strand synthesis, cDNA levels of genes of interest were determined using quantitative PCR with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), using 12.5 µl SYBR Green, 1 ml each of forward and reverse primer (10 µM), 8.5 µl H2O and 2 µl cDNA template. Primers are listed in table 4.3. All primers were designed to cross intron-exon boundaries, except primers for Cappuccino, which only has one exon. (For Cappuccino, RNA samples were treated with DNase to ensure removal of genomic DNA.) Samples were run for forty cycles (30 seconds 95ºC, 30 seconds 54 or 55ºC (depending on primers), 30 seconds 72ºC) on a Bio-Rad Chromo 4 Real-Time PCR detector and analyzed with Opticon Monitor and Genex (Bio-Rad) software. Expression levels were normalized to levels in cells transfected with the non-silencing control.
Table 4.3  Primers used for RT-PCR.

To avoid amplifying residual genomic DNA all primers were designed (with PerlPrimer) to cross intron-exon boundaries with the exception of those against the single-exon gene Cappuccino.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TYRP1</td>
<td>ACACCTTGTAAACAGCAGCAG</td>
<td>ATCATTTGGAGACAAATGGGT</td>
<td>290</td>
</tr>
<tr>
<td>Dysbindin</td>
<td>GAAGCCTTCAAAGCTGAACCTC</td>
<td>ACATCACAATGTTGTCTCTCTC</td>
<td>300</td>
</tr>
<tr>
<td>Pallidin</td>
<td>CTGCTGTCCCCACTACTTACC</td>
<td>CATCTCCTTCCCTATATGCACCAAGGACT</td>
<td>200</td>
</tr>
<tr>
<td>Cappuccino</td>
<td>CAGGATGGAGGAGCAGGTC</td>
<td>CTCGGTCCGAACAGGACT</td>
<td>163</td>
</tr>
<tr>
<td>HPS5</td>
<td>ACACCAAGTCAAAGCAGCTCC</td>
<td>GCTACATCTGAAATATCCTGTGAC</td>
<td>230</td>
</tr>
<tr>
<td>Melanophilin</td>
<td>ATGTAAGCCCTTTACCTCTG</td>
<td>CTCCAAGATCTGAGTCTCAGG</td>
<td>243</td>
</tr>
<tr>
<td>Myosin Va</td>
<td>GAGAGGAAGTTAGTAAAGAGAG</td>
<td>ATAGGGCACTTCAGCATCAG</td>
<td>152</td>
</tr>
<tr>
<td>LYST</td>
<td>CCATCATAGGCTGACATTCTC</td>
<td>GACACTCTTCTTCTTCTCC</td>
<td>162</td>
</tr>
<tr>
<td>RhoA</td>
<td>CTTCAATCCAGAAAGAATCTGTG</td>
<td>GCGGTCAAATCTGTTCTTGC</td>
<td>204</td>
</tr>
<tr>
<td>UBPY</td>
<td>TATTTCAACGAAAACACTGCTACC</td>
<td>CCGATTGTCAGCCTATTTCAG</td>
<td>260</td>
</tr>
<tr>
<td>Mahogunin</td>
<td>ATGATGAGCTGAAACTTTGACC</td>
<td>TTGGTTCTGTTCTCGATGCC</td>
<td>228</td>
</tr>
<tr>
<td>Actin</td>
<td>GATGACCCAGATCATTTTGGAG</td>
<td>CTTCTCTTTGATGTACGCAC</td>
<td>291</td>
</tr>
</tbody>
</table>
4.4 Results

4.4.1 Blinded Confocal Experiment

In order to find a suitable control for Cellomics KSR analysis, a blinded experiment was carried out to find proteins that, when knocked down, change the properties (e.g. number, size) of TYRP1-labeled melanosomes in such a way that they can be clearly detected by confocal imaging. If confocal analysis shows an obvious change in TYRP1 staining levels or location upon knockdown of one of the proteins, this could then be used as a control on Cellomics analysis, with the ultimate goal of setting up a system to find new regulators of melanosome properties.

Twenty-three pGIPZ constructs, against eleven target genes, were used to transf ect melan-a cells in a blinded experiment, for which samples were relabeled and reordered. Cells were grown in four-well chamber slides. On every slide one well was transfected with non-silencing control and three wells were transfected with blinded knockdown vectors. After fixing and staining, twenty confocal images were taken of every well. During this process two things were immediately obvious: First, many transfected cells did not look any different than untransfected cells, and second, there was a lot of variation in phenotype even among untransfected cells or non-silencing controls. The only obvious phenotype seen in some samples was a reduction in TYRP1 levels as visualized by a Cy3-tagged antibody. Figure 4.1 shows this reduction at 72 hour knockdown for two samples, which, after unblinding, were identified as samples in which either TYRP1 (top) or HPS5 (bottom) was knocked down.

To quantify this observation, TYRP1 levels in transfected cells (identified by TurboGFP expression) were compared to TYRP1 levels in untransfected cells in the same well. TYRP1 levels per cell were measured using Volocity software and analyzed in GraphPad, where T-test analysis was used to determine whether the difference in TYRP1 levels between transfected and untransfected cells was statistically significant. The P-values obtained from this analysis are shown in Table 4.4, and graphs are shown in Figure 4.2 (48 hour knockdown) and Figure 4.3 (72 hour knockdown).
Figure 4.1. Reduction of TYRP1 levels

After 72 hour transfection, some cells transfected with the pGIPZ constructs v2LMM_12467 (against TYRP1, top) or v2LMM_224489 (against HPS5, bottom) showed a decrease in TYRP1 levels (arrows in second column). Deviations in TYRP1 levels were observed in a blinded experiment, and samples were later identified as those transfected with v2LMM_12467 or v2LMM_224489 respectively.
Figure 4.2. Quantified TYRP1 levels per cell after 48 hour transfection with pGIPZ constructs.

For each sample of melan-a cells grown in 4-well chamber slides, TYRP1 levels per cell were determined using Volocity software for both transfected and untransfected cells, to account for variations in TYRP1 intensity between wells or slides. The differences between values for transfected and untransfected cells per sample were evaluated using an unpaired T-test, and asterisks indicate statistical significance: *=P<0.05, **=P<0.01, ***=P<0.001. P-values for these samples are ranked in Table 4.4. Data were collected in a blinded experiment and later annotated with the correct pGIPZ ID. Error bars indicate standard error of the mean.
**Figure 4.3.** Quantified TYRP1 levels per cell after 72 hour transfection with pGIPZ constructs.

For each sample of melan-a cells grown in 4-well chamber slides, TYRP1 levels per cell were determined using Volocity software for both transfected and untransfected cells, to account for variations in TYRP1 intensity between wells or slides. The differences between values for transfected and untransfected cells per sample were evaluated using an unpaired T-test, and asterisks indicate statistical significance: *=P<0.05, **=P<0.01, ***=P<0.001. P-values for these samples are ranked in Table 4.4. Data were collected in a blinded experiment and later annotated with the correct pGIPZ ID. Error bars indicate standard error of the mean.
To quantify results from the confocal experiments (see Figures 4.2 and 4.3), TYRP1 levels were measured using Volocity software for both transfected and untransfected cells in each sample, and a ranking order was based on P-values for unpaired T-test between measurements from transfected and untransfected cells. Samples that showed a statistically significant (P<0.05) difference between transfected and untransfected cells at both 48 hour and 72 hour knockdown were ranked highest, followed by those which only showed a significant difference at 72 hour knockdown, followed by samples only significant at 48 hour knockdown or not at all.

At both time points (48 and 72 hour knockdown), two out of eight negative controls samples (transfected with non-silencing pGIPZ plasmid) showed a difference in TYRP1 levels between transfected and untransfected cells with P<0.05 (see Figures 4.2 and 4.3). Therefore, any samples that only showed up once with P<0.05 were considered no more significant than negative controls. This is indicated by a horizontal line in table 4.4.

The top samples (above the horizontal line) were retested at 72 hour knockdown, and only v2LMM_12467 and v2LMM_224489 showed a reproducible effect on TYRP1 levels.

Data were collected in blinded experiments and later annotated with the corresponding pGIPZ ID numbers.
<table>
<thead>
<tr>
<th>pGIPZ ID</th>
<th>gene</th>
<th>Knockdown 48 hr</th>
<th>Knockdown 72 hr</th>
<th>Retested 72hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>v2LMM_12467</td>
<td>TYRP1</td>
<td>**</td>
<td>**</td>
<td>***</td>
</tr>
<tr>
<td>v2LMM_76067</td>
<td>Dysbindin</td>
<td>*</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>v2LMM_196168</td>
<td>HPS5</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>v2LMM_224489</td>
<td>HPS5</td>
<td>***</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>v2LMM_7485</td>
<td>Myosin Va</td>
<td>***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>v2LMM_37945</td>
<td>UBPY</td>
<td>**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>v2LMM_75408</td>
<td>Mahogunin</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>v2LMM_36292</td>
<td>Pallidin</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>v2LMM_50451</td>
<td>RhoA</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>v2LMM_73840</td>
<td>Melanophilin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>v2LMM_4222</td>
<td>LYST</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>v2LMM_4868</td>
<td>Myosin Va</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>v2LMM_21767</td>
<td>Cappuccino</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>v2LMM_17673</td>
<td>Myosin Va</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>v2LMM_24372</td>
<td>Pallidin</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>v2LMM_28461</td>
<td>Pallidin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>v2LMM_92618</td>
<td>HPS5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>v2LMM_70729</td>
<td>Melanophilin</td>
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<td></td>
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</tr>
<tr>
<td>v2LMM_11529</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>v2LMM_250967</td>
<td>Cappuccino</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
At 72 hour knockdown, more samples show a higher statistically significant difference in TYRP1 levels between transfected and untransfected cells than at 48 hour knockdown. This is not surprising, as longer knockdown will have reduced protein levels further, and any observed effects of this knockdown are stronger. However, a statistically significant difference between transfected and untransfected cells does not necessarily correlate with a biologically significant difference. This is clear from the data collected from non-silencing controls. After both 48 hour and 72 hour transfections, two out of eight non-silencing controls show a statistically significant (P<0.05) reduction in TYRP1 levels in transfected cells compared to untransfected cells in the same wells. This means that there is a strong possibility that about a quarter of all “hits” for which P<0.05 are a false positive. To indicate this, the samples are ranked by reproducibility and P value in Table 4.4. Samples where a significant change in TYRP1 levels was observed at both 48 and 72 hour knockdown are at the top, followed by samples that were only significant at 72 hour knockdown with P<0.01 or P<0.001. Under the horizontal line are all samples that only showed up as statistically significant for one time point with P<0.05 – in other words, all the samples that were no more significant than 25% of the negative controls.

To investigate reproducibility, six of the top hits were analyzed again using confocal microscopy on cells fixed 72 hours after transfection. In this experiment, only two samples showed the same effect as before: knockdown of TYRP1 with construct v2LMM_12467 and knockdown of HPS5 with construct v2LMM_224489 (Table 4.4).

### 4.4.2 Cellomics Analysis

Next, we investigated whether Cellomics KSR analysis would be able to pick up the same samples as the ones that show a reproducible reduction in TYRP1 on confocal analysis. We have previously optimized detection settings for melanosomes using this system (Chapter 3). The Cellomics KSR is a high-content imaging platform, which calculates several output values using specific image analysis algorithms called BioApplications, for which parameters can be adjusted by the user. The parameters used in Spot Detector BioApplication analysis to optimally detect melanosomes are listed in Appendix A.1.

The Spot Detector BioApplication defines spots as localized TYRP1 intensity in a predefined area around the nucleus of every cell. Because TYRP1 is specifically sorted to melanosomes,
spots are roughly equivalent to melanosomes in this model, although the trans-Golgi network is also labeled and detected as one big spot (Chapter 3). The algorithm returns several output values after image analysis, such as the number or size of spots per cell, or the signal intensity per spot or per cell.

To reproduce the confocal analysis on Cellomics, melan-a cells grown in a 96-well plate were transfected with the constructs giving the strongest effects in the confocal experiment (v2LMM_12467 against TYRP1, v2LMM_76067 against dysbindin, v2LMM196168 and v2LMM_224489 against HPS5, v2LMM_7485 against Myosin Va, and v2LMM_37945 against UBPY) or with a non-silencing control. The plate was fixed and stained after 72 hours. Because the phenotype observed in confocal analysis was a reduction in TYRP1 levels, we expected the Spot Detector BioApplication output value SpotTotalIntenPerObjectCh2 (reporting the total intensity of the Cy3-labeled TYRP1 per cell) to most closely resemble results obtained on confocal analysis.

We ran the Spot Detector BioApplication twice: once reporting output values for TYRP1-labeled spots in transfected cells and the second time carrying out the same measurements on untransfected cells. Transfected cells were detected on the Cellomics KSR in the FITC channel. The pGIPZ vector expressed TurboGFP, which is itself fluorescent, but bleaches upon fixing cells with PFA. The TurboGFP signal was enhanced by labeling cells with an antibody against TurboGFP and a secondary FITC-labeled antibody. To emulate the comparison of transfected and untransfected cells per well, which was expressed using P-values in confocal analysis, the SpotTotalIntenPerObjectCh2 output value for transfected cells was normalized over the value obtained from untransfected cells in the same well. For wells where the TYRP1 intensity is not very different between transfected and untransfected cells, this value approaches 1. For wells where the difference between transfected and untransfected cells is large (corresponding to a low P-value in the confocal experiment) the normalized TYRP1 intensity on Cellomics analysis is low. Other output values, such as the number or size of the spots, were calculated by Cellomics KSR but showed no difference between any of the samples and the negative controls, so only the TYRP1 intensity is reported here.

Figure 4.4 shows the normalized result of a Cellomics scan for the top hits of the confocal analysis, reported in grid fashion corresponding to the order of the samples on the plate.
Figure 4.4. Cellomics analysis

The knockdown constructs that led to the highest reduction in TYRP1 levels in melan-a cells on confocal analysis were tested using Cellomics KSR high content analysis. Here TYRP1 levels were measured as the output value SpotTotalIntenPerObjectCh2 of the Spot Detector BioApplication. To correct for variations between wells, the output for transfected cells in every well was normalized to that for untransfected cells in the same well. In this figure, this normalized value is indicated per well by a colour as shown in the legend. Samples are shown in their respective location in a 96-well plate. Only v2LMM_12467 shows a detectable reduction in TYRP1 levels compared to non-silencing controls in Cellomics analysis. See Appendix A.2 for data.
To better visualize the data, colours were assigned to normalized levels on the red-green scale, where red represents the lowest normalized values and green the values closest to 1. It is immediately obvious from this figure that only knockdown of TYRP1 itself reduced TYRP1 enough to be detected by Cellomics KSR with Spot Detector BioApplication. In this figure, all six transfections with pGIPZ against TYRP1 correspond to the six wells with the lowest TYRP1 levels in transfected cells. In a repeat experiment, five out of six TYRP1 knockdown transfections had the lowest reported TYRP1 values of the plate (not shown). None of the other transfections are detected as hits in this analysis. V2LMM_224489, the knockdown construct against HPS5 that was a reproducible hit in the confocal microscopy experiment does not appear to show any further TYRP1 reduction than non-silencing controls.

4.4.3 Validation of Knockdown

The above results indicate that, other than TYRP1 itself, none of the knockdown constructs reduce TYRP1 levels enough to be detectable in Cellomics KSR analysis. However, we selected these target genes based on predicted effects on melanosome properties or other aspects of pigmentation. Based on the literature we expected either reduction of TYRP1 levels for knockdown of BLOC-1 or BLOC-2 components (pallidin, dysbindin, cappuccino, HPS5) or altered distribution of melanosomes upon knockdown of Myosin Va or melanophilin. The fact that only one of three knockdown constructs against HPS5 showed a reproducible reduction in TYRP1 in confocal analysis suggests that perhaps knockdown of these target proteins was incomplete.

To determine knockdown levels, we used quantitative real time PCR (RT-PCR) on cDNA prepared from FACS sorted cells transfected with pGIPZ constructs. The residual mRNA levels in every RT-PCR experiment were normalized to mRNA levels for the protein of interest in cells transfected with a non-silencing control plasmid. Figure 4.5 shows the results of these experiments. Every sample was measured at least in triplicate (most samples in quadruplicate) and some experiments were repeated in their entirety to confirm that results were reproducible.

The results indicate that of the investigated proteins, TYRP1 is most effectively knocked down by its corresponding shRNA construct, reducing mRNA levels to approximately 5-15% of the levels in non-silencing control (results from two separate experiments each measured in
Figure 4.5. RT-PCR results

RT-PCR results showing residual mRNA levels after knockdown with pGIPZ shRNA mir constructs vs. non-silencing control. All samples were FACS-sorted before RNA isolation, so only cells containing the TurboGFP-expressing pGIPZ vector were analyzed. Error bars indicate standard deviation. A. Knockdown efficiency of constructs that showed a reproducible effect on TYRP1 levels in confocal analysis. B. Knockdown efficiency of constructs that showed a non-reproducible effect on TYRP1 levels in confocal analysis. C. Knockdown efficiency of constructs that showed no effect on TYRP1 levels in confocal analysis.
A. 
TYRP1

% expression vs non-silencing
Y2LMM_22461
Y2LMM_72661

HPS5

% expression vs non-silencing
Y2LMM_19469
Y2LMM_17540

B. 
UBPY

% expression vs non-silencing
Y2LMM_57045
Y2LMM_19618

Dysbindin

% expression vs non-silencing
Y2LMM_7465
Y2LMM_78607

C. 
Pallidin

% expression vs non-silencing
Y2LMM_44372
Y2LMM_49681
Y2LMM_90352

Cappuccino

% expression vs non-silencing
Y2LMM_51787
Y2LMM_25967

RhoA

% expression vs non-silencing
Y2LMM_50451

HPS5

% expression vs non-silencing
Y2LMM_98618

Mahogunin

% expression vs non-silencing
Y2LMM_88999
Y2LMM_17540

Myosin Va

% expression vs non-silencing
Y2LMM_4568
Y2LMM_71673

LYST

% expression vs non-silencing
Y2LMM_42722
Y2LMM_75823
Y2LMM_10729

Melanophilin

% expression vs non-silencing
Y2LMM_89443
Y2LMM_10729
Y2LMM_75840
quadruplicate). Since TYRP1 was used as melanosomal label in all confocal and microscopy experiments we also know the approximate reduction in protein level for this knockdown construct. Cellomics analysis of spot intensity shows that TYRP1 protein levels were reduced to ~50-60% compared to both TYRP1 levels in untransfected cells in the same well, and to TYRP1 levels in cells transfected with a non-silencing control in the same plate.

While TYRP1 mRNA levels were reduced by about 90%, knockdown of other proteins, such as mahogunin or LYST, was minimal, even at the mRNA level. This may explain why cells transfected with these knockdown constructs did not differ from negative controls. Other constructs, such as those targeting cappuccino, show a reduction of about 50% at the mRNA level, but no effect on TYRP1 levels in confocal analysis.

4.4.4 Melanophilin Knockdown

Many of the proteins targeted in these experiments have only previously been studied using cells from mutant mice or from human patients with pigmentation disorders, but not using knockdown in cultured melanocytes. Only knockdown of melanophilin in melan-a cells has been previously reported (Kuroda, T.S., and Fukuda, M., 2004). Kuroda and Fukuda showed an expected perinuclear distribution in cells in which melanophilin levels were reduced, but we did not observe a similar distribution pattern in cells transfected with any of the three pGIPZ constructs against melanophilin. RT-PCR results show that none of these constructs strongly reduce melanophilin levels. Only v2LMM_73840 reduces mRNA levels by about 50%.

In these studies we determined knockdown efficiency by RT-PCR rather than a direct measurement of protein levels for several reasons: melan-a cells did not yield enough cells after FACS sorting to carry out western blot analysis, FACS-sorted cells did not survive reseeding to increase cell number, and for many of the proteins investigated no antibodies were available. However, there is a commercially available antibody against melanophilin, and using this antibody we were able to measure residual protein levels upon melanophilin knockdown using confocal analysis and Volocity software, as was done for TYRP1 levels (see section 4.4.1). After Volocity analysis of melanophilin levels, it became clear that even though there is a slight reduction of melanophilin at the mRNA level, the protein levels are not markedly reduced (Figure 4.6). However, there is a large variation in melanophilin levels between individual cells,
as shown by the distribution of intensities in Figure 4.6. Figure 4.7 shows the confocal images of a cell with very little melanophilin levels. The bright field image of this particular cell does appear to show a perinuclear localization of melanosomes, and in a repeat experiment, a few other cells with very low melanophilin levels also showed perinuclear localization. This suggests that the protein levels need to be very low to detect any effect of melanophilin reduction.

4.4.5 Non-melanosomal Phenotypes

From mouse studies it is known that the most severe pigmentation phenotypes occur in mice with mutations in proteins affecting melanosome formation, such as the components of BLOC-1 and BLOC-2 (Li, W. et al., 2004, Nguyen, T. et al., 2002). Cellular studies have also shown that TYRP1 sorting is affected by these proteins, as reflected by overall TYRP1 levels (Setty, S.R. et al., 2007, Helip-Wooley, A. et al., 2007). Indeed, when carrying out confocal analysis, a change in TYRP1 levels was the only discernible phenotype. However, among the proteins targeted in this study we included those affecting stages of pigmentation other than melanosome formation and transport, such as RhoA, which is required for dendrite extension. Experiments investigating the effect of RhoA knockdown on dendrite extension as visualized by confocal imaging were carried out simultaneously with experiments briefly described in Chapter 3, that showed that Cellomics KSR cannot easily distinguish dendrites. Regardless of the difficulty of detecting cAMP-induced dendrite formation in B16 cells on Cellomics, it appears that RhoA knockdown does not significantly affect the phenotype of transfected cells. Figure 4.5C shows that RhoA mRNA levels are reduced by approximately 50% in melan-a cells, yet none of the cells transfected with the v2LMM_50451 knockdown construct against RhoA show an increase in dendricity, even though chemical inhibition of RhoA in B16 cells increases dendrite extension (Busca, R. et al., 1998). To rule out a cell-specific effect, we also transfected B16 cells with the same construct, where it effectively reduced RhoA protein levels, but did not see a change in dendricity in B16 cells either (Appendix A.3).
Figure 4.6. Melanophilin protein levels

Melanophilin levels per cell, quantified using Volocity software from confocal images of cells labeled with an antibody against Melanophilin and secondary Cy3-labeled F(ab')2 antibody fragment. Cells were transfected with pGIPZ constructs transcribing shRNAmir hairpins against melanophilin, or with a non-silencing control. Untransfected cells in each well were measured to account for variation in signal intensities between wells.
Figure 4.7. Melanosome transport is inhibited at very low melanophilin levels

In a cell where melanophilin levels are extremely low (last panel, arrow) upon 72 hour knockdown with v2LMM_73840, melanosomes are localized near the nucleus instead of spreading throughout the cell (middle panel).
The change in TYRP1 levels observed upon TYRP1 or HPS5 knockdown was the only phenotype detected on confocal microscopy, and therefore the only phenotype investigated on Cellomics analysis. The Spot Detector BioApplication, and other BioApplications are capable of reporting a wide range of cellular measurements, but since no change in cell morphology was observed on confocal analysis upon knockdown of any of the target proteins, these Cellomics applications were not used.
4.5 Discussion

In Chapter 3 we showed that melanosomes can be identified on the high-content analysis system Cellomics KSR by using an antibody against TYRP1 in combination with a secondary fluorescently labeled antibody (Chapter 3). While we were able to detect a cAMP-induced change in melanosome dispersion in B16 cells, the effect of knockdown of Myosin Va on melanosome distribution in melan-a cells was not apparent, even though mice lacking this protein show a strong perinuclear localization of melanosomes (Wei, Q. et al., 1997). Since knockdown of Myosin Va did not show the predicted effect on melanosome location, this could not be used as a control in a RNAi screen. Before commencing a screen for unknown proteins involved in melanosome properties, it was necessary to first find suitable positive controls.

We obtained pGIPZ knockdown constructs from the Hannon-Elledge shRNAAmir library against eleven genes that are either known to be directly involved in melanosome development or transport, or likely to affect pigmentation or organelle formation. This set did not include constructs targeting CNrasGEF, a protein previously studied in our lab, as knockdown of this protein increases pigmentation (Chapter 2) and this pilot study focused on decrease of pigmentation upon knockdown. This is further discussed in Chapter 5.

Of the eleven proteins knocked down in these experiments, only TYRP1 knockdown (using v2LMM_12467) was high enough to be repeatedly detected by both confocal and Cellomics analysis (Figures 4.2 and 4.4). Not only was this the protein that showed the largest decrease in mRNA levels upon knockdown, but its knockdown is also the direct output value measured in these systems. Knockdown of all other proteins is measured indirectly in terms of their effect on TYRP1 levels. The Cellomics KSR was not able to accurately detect the indirect effect of HPS5 knockdown by v2LMM_224489 on TYRP1 levels, even though this is detected in confocal analysis (Figures 4.2 and 4.4). Strangely, only one knockdown construct against HPS5 is picked up in this analysis, even though v2LMM_196168 and v2LMM_92618 reduce HPS mRNA levels by approximately the same amount (Figures 4.5). However, while measurement of residual mRNA levels were reproducible in independent experiments, it should be noted that the samples in which knockdown was determined were prepared independently from the confocal and Cellomics measurements. In our previous work with CNrasGEF (Chapter 2) we noticed that there can be a large variation in knockdown efficiency between transfections, and it is possible
that such a variation accounts for the fact that not all HPS5 knockdown constructs are picked up. The observed reduction of TYRP1 levels in response to HPS5 knockdown by v2LMM_224489 is, however, in line with observations by others: In melanocytes of HPS5 patients, TYRP1 levels are significantly reduced (Helip-Wooley, A. et al., 2007). HPS5 is a component of the BLOC-2 complex, which is involved in the sorting of TYRP1 to melanosomes (Di Pietro, S.M. et al., 2006). Based on this, we also expected to see an effect of knockdown of dysbindin, pallidin, and cappuccino. These proteins are all components of the BLOC-1 complex, which is also required for TYRP1 sorting and acts upstream of BLOC-2. Mice lacking any of the BLOC-1 proteins are extremely light - almost white (Li, W. et al., 2004). However, we did not pick up pallidin and cappuccino at all, and an initially observed effect of transfection with the dysbindin knockdown construct on TYRP1 levels was not reproducible (Figures 4.2 - 4.4 and table 4.4). RT-PCR showed negligible knockdown of dysbindin (Figure 4.5). Cappuccino and pallidin knockdown constructs reduce mRNA levels to about 50-70% of control levels, but this appears to have no effect on TYRP1 levels, even though a relation between pallidin and TYRP1 levels has been shown by others (Setty, S.R. et al., 2007). However, in that example mice with mutant inactive pallidin were studied, and in our experiments at least 50% of residual pallidin is still present (possibly more at the protein level). Perhaps this is enough for the BLOC-1 complex to function normally, but this remains to be determined.

It appears that many of the genes we investigated are not sufficiently knocked down by the pGIPZ constructs from the Hannon-Elledge library, which accounts for the lack of downstream effects. This library reportedly achieved 60% reduction of RNA levels or more for about 80% of the constructs in a test set (Silva, J.M. et al., 2005). We observe a similar reduction in only 6 of 23 tested constructs (Figure 4.5). It is possible that knockdown is cell-line specific, or that this particular set of constructs shows below average knockdown efficiency. On top of this, a reduction in mRNA levels may have little effect on protein levels. This is clear when we look at knockdown of melanophilin. Reduction of melanophilin levels should be visible as a perinuclear localization of melanosomes (Kuroda, T.S., and Fukuda, M., 2004). However, we did not observe perinuclear localization of melanosomes in most cells transfected with melanophilin knockdown constructs, This can easily be explained in terms of residual protein level: even though v2LMM_73840 appeared to reduce melanophilin levels to approximately 50% of control, no overall reduction was detected at the protein level (Figures 4.5C and 4.6). Only in cells with
extremely low melanophilin protein levels (intensity ~20% of the mean melanophilin intensity) was perinuclear localization visible (Figure 4.7)

Temporary knockdown will always leave some residual protein. Often this is not a problem, and a partial reduction in protein levels will still translate to a detectable effect. But if minimal amounts of protein still show a wildtype phenotype, it is not possible to use such a system for high-throughput RNA interference screening. We show this here for melanophilin, but a recent study also showed that Myosin Va, which is part of the same transport complex, requires prolonged knockdown in order to see an effect on melanosome location (Van Gele, M. et al., 2008).

In conclusion, investigation of knockdown of a small set of proteins suggests that it is not feasible to use Cellomics KSR in combination with transient transfection of pGIPZ RNAi constructs to identify proteins involved in melanosome formation or transport: Many constructs show only limited knockdown at the mRNA level, residual protein (even after mRNA removal) may still elicit an effect, and the melanosomal system appears to be particularly robust to changes in protein levels, requiring near depletion of proteins to elicit an effect. However, these problems can potentially be overcome by reducing the protein levels further than we are able to do in this system: The pGIPZ construct can be used for lentiviral infection, which allows the hairpin to integrate into the genome and reduce protein levels over a longer period of time. This might reduce protein levels enough to see effects similar to those observed in mice lacking the functional protein altogether.
Chapter 5
Discussion
5.1 General Discussion

The skin pigment melanin is synthesized in specialized organelles in melanocytes, called melanosomes, which are transported to the cell membrane and the tips of newly formed dendrites, from where they are transferred to neighbouring keratinocytes. A network of intracellular pathways regulates the synthesis of melanin, formation of dendrites, and formation or transport of melanosomes. In this thesis I described work investigating proteins that are involved in several of these processes.

5.1.1 Study of the Role of CNrasGEF in Melanin Synthesis

Previous work by others has shown that in mouse B16 melanoma cells prolonged cAMP-dependent activation of the Ras/ERK pathway leads to a downregulation of expression of tyrosinase – the rate-limiting enzyme in melanin synthesis (Englaro, W. et al., 1998, Busca, R. et al., 2000). These studies have further shown that the guanine nucleotide exchange factor involved in this pathway is not Sos, and that PKA activation by cAMP is not responsible for Ras activation (Busca, R. et al., 2000). It was suggested that an unknown cAMP-dependent Ras activator might be involved in this pathway. In Chapter 2 of this thesis I present work that provides evidence that the cAMP-dependent Ras activator CNrasGEF, previously identified in our lab, fulfills the role of the guanine nucleotide exchange factor in cAMP stimulated Ras/ERK activation in B16 cells. I showed this by first demonstrating that both overexpressed and endogenous CNrasGEF in B16 cells are capable of activating Ras and ERKs in response to cAMP, and that knockdown of CNrasGEF reduces this activation. I further showed that knockdown of CNrasGEF leads to an increase in melanin production compared to controls, indicating that CNrasGEF is indeed part of a negative feedback mechanism for melanin synthesis. The Ras/ERK pathway is also known to affect dendricity in a similar manner as melanin synthesis, and I confirmed that in the presence of prolonged (48 hour) cAMP activation the number of B16 cells with a dendritic phenotype increases when CNrasGEF is knocked down and decreases upon overexpression of WT-CNrasGEF. Overexpression of a mutant lacking the catalytic domain (ΔCDC25-CNrasGEF) does not have any effect on dendricity compared to control cells, suggesting that this mutant does not compete with endogenous CNrasGEF.
One question that remains to be answered directly with regards to this pathway is whether CNrasGEF is the only guanine nucleotide exchange factor that activates Ras in B16 cells. Knockdown of endogenous CNrasGEF levels by about 50% (in unsorted cells) leads to a reduction in Ras activation by approximately 40%. This indicates that CNrasGEF is responsible for the majority, if not all Ras activation in this system. However, we cannot say with certainty whether complete removal of CNrasGEF would lead to complete inactivation of Ras. Even in FACS sorted cells, CNrasGEF is not entirely removed, but reduced to approximately 50-90%. Unfortunately, Ras activation could not be studied directly after FACS sorting, because it requires a large amount of cell lysate. Nevertheless, the data presented in Chapter 2 clearly show that CNrasGEF fulfills the requirements of a previously predicted cAMP-dependent Ras activator in the known Ras/ERK pathway regulating melanin synthesis.

Interestingly, in carrying out these experiments it became clear that overexpression of wildtype CNrasGEF, but not the mutant lacking the catalytic CDC25 domain, leads to decreased cell proliferation and to apoptosis. This effect is not dependent on cAMP, and it is therefore tempting to speculate that it might act through Rap1, since activation of Rap1 by CNrasGEF is not dependent on cAMP (Pham, N. et al., 2000). However, this remains to be studied. (See also Future Directions).

5.1.2 Cellomics Analysis of Melanosomes

In Chapter 3 I describe the development of a high-content image analysis system to measure melanosome properties. To our knowledge, this is the first time the Cellomics KSR system with Spot Detector BioApplication has been used to monitor lysosome-related organelles. A search of the literature suggests that until now the Spot Detector BioApplication program has only been used to monitor endocytosis or phagocytosis (Steinberg, B.E. et al., 2007, Ghosh, R.N. et al., 2005, Zhang, L. et al., 2007).

While melanosomes of highly pigmented cells such as melan-a are visible by bright-field microscopy as black dots, Cellomics KSR only measures fluorescence. Therefore, I labeled melanosomes using an antibody against TYRP1 and a Cy3-tagged secondary antibody. The subcellular location of TYRP1 has been extensively studied by several groups (Setty, S.R. et al., 2007, Di Pietro, S.M. et al., 2006, Helip-Wooley, A. et al., 2007, Boissy, R.E. et al., 2005, Xu, Y. et al., 1997). TYRP1 is found in late stage melanosomes (stage III and stage IV) but there is
also a significant fraction in the trans-Golgi network (TGN). The Spot Detector BioApplication of the Cellomics KSR cannot distinguish melanosomes from the TGN, and counts every localized region of TYRP1 fluorescence as a spot. Since the TGN spot is larger than the melanosomes, this may affect measurements: if the TYRP1 signal coming from the TGN is half of the total intensity, a complete lack of TYRP1 post-Golgi transport would only show as an approximately 50% decrease. It is possible to adapt the detection system by labeling another melanosomal protein, but since all melanosomal proteins originate in the TGN, this might not make a difference. The system introduced in Chapter 3 can be easily adapted to study other organelles and other cells as well, by using a different label. For example, it would be possible to study lysosomes in any cell type by labeling with LAMP1.

5.1.3 Knockdown of Proteins Involved in Melanosome Formation and Transport

In Chapter 4 I describe the use of confocal microscopy to study the effect of knockdown of several proteins in cells where melanosomes were labeled with TYRP1, the same label used in Cellomics analysis. Several of these proteins are known to affect TYRP1 sorting: Cappuccino, pallidin, and dysbindin are components of BLOC-1, which is required for TYRP1 sorting to melanosomes. HPS5 is a component of BLOC-2, which is also involved in the same sorting mechanism. Myosin Va and melanophilin are both required for transport of melanosomes, and knockdown of melanophilin in melan-a cells, the cell system used here, is known to lead to a phenotype of melanosomes clustered around the nucleus. In a blinded confocal experiment in which all these proteins (and others) were knocked down using all available shRNAmir constructs against these targets, I only picked up a slight overall reduction in TYRP1 levels upon HPS5 knockdown. Although the overall effect was small, in some cells TYRP1 was completely reduced. I was able to investigate melanophilin knockdown more closely by using an antibody against melanophilin suitable for immunofluorescent staining. In doing so, I found that there was a lot of variation in knockdown efficiency between cells, and that only cells with minimal amount of residual melanophilin protein showed the expected phenotype of melanosome clustering. Clearly this variation in knockdown between cells is a problem when the average effect does not reach detectable levels. In the Future Directions below I give some suggestions to improve this system.
Even though Chapter 2 indicates that CNrasGEF is involved in pigmentation, I did not include this protein in the set of knockdown targets to be tested. Knockdown of CNrasGEF slightly increases melanin synthesis and dendrite extension, but since I was not able to detect an effect of knockdown of RhoA (see Appendix A.3), which has a more direct effect on dendricity, I did not further pursue the detection of dendrites on Cellomics analysis. The detection of melanosomes by the Cellomics Spot Detector BioApplication worked well in cAMP-stimulated B16 cells. It is possible that CNrasGEF is involved in melanosome formation or transport, and it would have been a worthwhile protein to study in this system, but since its exact role in these processes is not known it would not make a good control for a pilot study. Furthermore, CNrasGEF is cAMP-dependent, and melan-a cells, which proved most suitable for Cellomics analysis (see Chapter 3) do not respond strongly to cAMP. Their basal pigmentation is so high that adding cAMP does not further increase it, and they do not have the heavily dendritic phenotype of cAMP-stimulated B16 cells. Since the effect of CNrasGEF was most obvious with regards to the specific cAMP-dependent effects on B16, it would be more difficult to study it in melan-a cells.
5.2 Future Directions

While the work presented in this thesis has contributed to an increased understanding of regulation of various processes in pigmentation at the melanocyte level, it also leads to new questions. In this final section I will propose several research directions for which the work presented in this thesis forms an initial starting point.

5.2.1 Investigate the Potential Role of Rap1 as a CNrasGEF Effector in B16 Cells

In FACS-sorted cells where CNrasGEF levels are reduced to approximately 50-90%, downstream ERK activation is only reduced by at most 40%, suggesting that there may be other pathways interacting with the Ras/ERK pathway that also lead to ERK activation. In some systems Rap1 is known to upregulate ERK. Busca et al. ruled out a role for Rap1 in the cAMP-dependent ERK activation in B16 cells, but others have shown that Rap1 appears to be responsible for at least partial ERK activation in melanoma cells, and that this is involved in metastasis (Busca, R. et al., 2000, Gao, L. et al., 2006). It would be interesting to see whether CNrasGEF has any effect on metastasis, potentially through Rap1. Such studies can be done using the same cell system, B16 melanoma cells, which are well established as a system to study metastasis in mice.

Rap1 may also be involved in the mediation of the apoptotic effect of overexpression of wildtype CNrasGEF in B16 cells, since this was independent of cAMP, but not seen upon overexpression of the ΔCDC25 mutant, which lacks the catalytic domain. There is a known correlation between Rap1 upregulation and apoptosis in human melanoma (Kobayashi, Y. et al., 2006). It will be interesting to investigate whether Rap1 also induces apoptosis in B16 melanoma cells, and whether this is CNrasGEF dependent. Functional studies of CNrasGEF in various systems have thus far focused on either Ras or Rap1, but not on both effectors in the same cell system.

5.2.2 Investigate How the Ras/ERK pathway Regulates Dendrite Extension

Although it has been shown, by me and others, that activation of the Ras/ERK pathway in B16 cells leads to an inhibition of dendrite extension, the mechanism by which this occurs is currently unknown (Englaro, W. et al., 1998, Amsen, E.M. et al., 2006). It seems likely that there is an
interaction between this pathway and the Rac1/RhoA system, but the molecular mechanism remains to be discovered. Inhibition of MEK, directly upstream of ERK, leads to an increase in dendrite extension, indication that the cross-interaction with the Rac1/RhoA pathway is either at the level of MEK or ERK, or a result of phosphorylation of transcription factors by ERK, but not further upstream at the level of B-Raf, or Ras.

5.2.3 Analyze the Intracellular Targeting of CNrasGEF in B16 Cells

Previous work in our lab has shown that in HEK293T cells overexpressing both CNrasGEF and the β1-adrenergic receptor, CNrasGEF is targeted to the membrane through interaction of its PDZ domain with the SxV C-terminal motif of the β1-AR (Pak, Y. et al., 2002). In that system, CNrasGEF activates Ras when the receptor activates adenylate cyclase through Gsα, increasing local cAMP levels. In B16 cells, CNrasGEF activates Ras in response to cAMP produced by adenylate cyclase downstream of the melanocortin-1 receptor (MC1R). Initially, this suggests a similar targeting mechanism might be in place, but the MC1R does not have a PDZ-binding motif. CNrasGEF needs a high local cAMP concentration to activate Ras, requiring controlled intracellular targeting. I suggest investigating whether CNrasGEF is able to interact with the MC1R, either directly or indirectly. CNrasGEF has both a PDZ domain and a PDZ-binding motif, offering multiple potential interactions, perhaps through intermediate proteins.

5.2.4 Investigate the Role of CNrasGEF in Melanosome Transport

The Ras/ERK pathway, with CNrasGEF as cAMP-dependent Ras activator, is known to affect melanin synthesis and dendrite extension. Specifically, the Ras/ERK pathway inhibits these aspects of pigmentation upon prolonged cAMP stimulation. Nevertheless, in another system, Xenopus melanophores, melanosome transport is inhibited upon inhibition of ERK, suggesting a role for ERK in the stimulation of melanosome transport (Deacon, S.W. et al., 2005). It is not clear whether ERK has a similar effect on melanosome transport in mouse melanocytes. The relation between ERK and melanosome transport needs to be investigated further in mouse melanocytes before investigating the effect of upstream activators of ERK such as CNrasGEF.

5.2.5 Increase Knockdown of shRNAmir targets by Lentiviral Infection

Investigation of knockdown levels of the target proteins used in the blinded confocal study of melan-a cells described in Chapter 4 shows that reduction of mRNA levels of many of these
proteins is only around 50% after 72 hour transfection with the knockdown construct. For several of the investigated proteins it is known that a complete lack of functional protein affects melanosome formation or transport, and indeed I saw that cells that contain minimal levels of melanophilin do show a lack of melanosome movement similar to that seen in mice lacking functional melanophilin or in Griscelli Syndrome Type 3 patients. I suspect that incomplete knockdown of target proteins is a major reason for the lack of predicted effects on TYRP1 levels or melanosome location. In support of this, it should be noted that Griscelli Syndrome is a recessive disorder. Carriers of the disease, with only one mutated allele of either Rab27A, Myosin Va or melanophilin, show no phenotype (Pastural, E. et al., 1997, Ménasché, G. et al., 2000, Ménasché, G. et al., 2003).

The shRNAmir constructs I used to knock down proteins of interests are originally designed for lentiviral infection. I did not use lentiviral infection in these experiments, because the goal was to find a suitable control that could eventually be used in high-throughput, high-content screening. Our lab is not equipped to safely carry out high-throughput lentiviral infections, so I focused on transient transfection. However, since it now appears that transient transfection does not adequately reduce protein levels, it may be worthwhile to investigate whether lentiviral infection improves the knockdown efficiency for the selected target set. If lentiviral infection greatly improves knockdown, and the control set shows the expected phenotypes, then a high-throughput screen might be feasible, provided there are appropriate safety conditions for lentiviral infection.

5.2.6 Double Knockdown Studies of Proteins Involved in Melanosome Formation

Double knockout mice lacking components of more than one BLOC complex show a more severe pigmentation defect than mice lacking only one protein from one BLOC (Gautam, R. et al., 2006). It seems that BLOC complexes can partially take over each other’s function. Perhaps the phenotype seen upon partial knockdown of a protein component of one BLOC can also be exacerbated when another BLOC is not functioning properly either. I proposed the use of double knockdown studies to investigate this. Specifically, the double knockdown of HPS5 and pallidin is worthy of study. Both are proteins that are known to affect TYRP1 sorting to melanosomes. Pallidin is a part of BLOC-1 and HPS5 is part of BLOC-2. Furthermore, the effect of HPS5 knockdown was already seen in my experiments, although not very strongly. Such an experiment
does require the fluorescent label on one of the knockdown constructs to be switched to another fluorophore, and this may not be amenable to high-throughput analysis.

In addition, it is worthwhile testing the effect of single protein knockdown in cells derived from mice already lacking one of the BLOC proteins. The effect of HPS5 knockdown should be more severe in cells from mice with a dysfunctional BLOC-1 complex.

5.2.7 Investigate the Role of Ubiquitination in Melanosome Sorting

Our initial aim was to develop a high-throughput screen for proteins involved in melanosome formation or transport. Unfortunately, the system we tested is not yet suitable to be scaled up to a screen. If the work presented in this thesis eventually leads to a functioning screen, for example by using lentiviral infection (see 5.2.5), then our lab would be especially interested in the role of ubiquitination in melanosome formation.

As discussed in Chapter 1, melanosomes and lysosomes share a common origin. Since ubiquitin is involved in the sorting of proteins to lysosomes, it might also play a role in melanosome development. The sorting of internalized plasma membrane receptors to lysosomes by way of early endosomes is regulated by mono-ubiquitination of cargo proteins as well as by mono-ubiquitination of the sorting machinery itself (Hicke, L., 2001b, Shih, S.C. et al., 2002). Mono-ubiquitinated cargo proteins are recognized by ubiquitin-binding domains (UBDs), such as ubiquitin-interacting motifs (UIMs) in components of endosomal sorting complexes required for transport (ESCRTs) (Williams, R.L., and Urbe, S., 2007). The complexes ESCRT-0 (Hrs-STAM complex), ESCRT-I and ESCRT-II recruit ubiquitinated proteins, while ESCRT-III recruits deubiquitinating enzymes, which remove ubiquitin from the cargo before it enters the intralumenal vesicles (ILVs) of the MVBs.

Much of the current knowledge of lysosomal sorting pathways comes from the study of yeast vacuolar sorting. Forty vacuolar protein sorting mutants (Vps mutants) identified in *Saccharomyces cerevisiae* have been classified into six groups (A-F) (Raymond, C.K. et al., 1992). The mammalian ESCRT orthologs consist of homologs of the Class E Vps proteins. Yeast was also the model system in which ubiquitination was first shown to be involved in sorting from the TGN to endosomes or lysosomes (Hicke, L., 2001a).
As of yet there is little evidence to suggest that the ubiquitination-dependent sorting system found in the lysosomal pathway is also required for the sorting of cargo to melanosomes. In fact, sorting of pMel17 to multi-vesicular bodies has been shown to be independent of the ESCRT system (Theos, A.C. et al., 2006). Another melanosomal protein, MART-1 (required for pMel17 function (Hoashi, T. et al., 2005)), is missorted to lysosomes instead of melanosomes when it is ubiquitinated by the ubiquitin ligase Nedd4 (Levy, F. et al., 2005). Nevertheless, other lines of evidence do support a role for ubiquitination in melanosome formation. For example, zebrafish with a mutation in the ubiquitin ligase Vps18 (a Class C Vps) show reduced skin pigmentation and decreased number of melanosomes in retinal pigment epithelium (Maldonado, E. et al., 2006). Human Vps18 (hVps18) has not been directly linked to pigmentation, but it ubiquitinates endosomal sorting protein GGA3 (Golgi-localized, γ-ear-containing, ADP ribosylation factor (ARF)-binding protein 3) (Yogosawa, S. et al., 2006). GGA3 is involved in AP-dependent sorting pathways (including AP-3) from the TGN to endosomes and lysosomes, and interacts with ubiquitinated cargo through a ubiquitin-binding domain (Dell'Angelica, E.C. et al., 2000, Puertollano, R., and Bonifacino, J.S., 2004, Scott, P.M. et al., 2004). Work from our own lab has shown that Nedd4 is able to ubiquitinate GGA3 in the presence of Nedd4 binding partner LAPTM5. The association of LAPTM5 with Nedd4 is required for GGA3-mediated sorting of LAPTM5 to lysosomes (Pak, Y. et al., 2006). It is currently not known whether GGA3 is involved directly in pigmentation, but its involvement in sorting to lysosomes and its ubiquitination by Vps18, which is linked to pigmentation in zebrafish, do suggest that it may be involved in sorting to melanosomes as well. Another confirmed link between ubiquitination and pigmentation is through the protein mahogunin. Mahogunin is an E3 ubiquitin ligase (Phan, L.K. et al., 2002). In agouti mice, a null mutation in mahogunin switches melanin production from pheomelanin to eumelanin, suggesting that it inhibits the effect of the agouti signaling protein on the melanocortin-1 receptor (Phan, L.K. et al., 2002). Another, more severe, mahogunin mutation is not only associated with pigmentation, but also with spongiform neurodegeneration (He, L. et al., 2003). This latter effect is thought to be linked to TSG101, a substrate for monoubiquitination by mahogunin (Kim, B.Y. et al., 2007). TSG101 is a component of ESCRT-1, and its ubiquitination plays an important role in endosomal trafficking, thus linking a coat colour defect to a ubiquitin-dependent sorting pathway. This is the most compelling evidence to date for a role of ubiquitination in melanosome sorting, and the connection between ubiquitination and the melanosome system is worthwhile of further investigation.
5.3 Concluding remarks

Work presented in this thesis has shown that the protein CNrasGEF is responsible for cAMP-dependent activation of Ras in B16 mouse melanoma cells (Chapter 2). Through this pathway, it exerts a negative effect on melanin synthesis and dendrite extension. While my findings indicate that CNrasGEF fulfills the role of a previously postulated camp-dependent Ras activator in these cells, it is not yet known if it is the only guanine nucleotide exchange factor to act in this pathway. In addition, CNrasGEF is also a Rap1 GEF, although not dependent on cAMP. It remains to be investigated whether Rap1 activation by CNrasGEF plays a role in B16 cells.

I have also optimized a method to analyze melanosome properties by high-content analysis (Chapter 3). The aim of this project was to develop a high-content/high-throughput RNA interference screen for proteins that affect pigmentation. However, a pilot study of a subset of shRNAmir constructs against target proteins involved in pigmentation indicates that upon transient transfection (72 hours) knockdown is not effective enough to achieve phenotypes that have been reported in the literature for inactive mutations of these proteins (Chapter 4). Further work in this area should first focus on increasing knockdown levels, for example through viral infection. If this is successful, and high-content/high-throughput analysis of melanosome properties can be achieved, this can then be used to find new proteins involved in pigmentation pathways.
References


Appendix A.1

Selection parameters and assay setting for Spot Detector BioApplication

These settings of the Spot Detector BioApplication (V2; version 4.1.0.2029) are referred to as “Circle protocol” in Chapters 3 and 4, and used to identify melanosomes on Cellomics KSR.

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Appendix A.2

Data from Cellomics analysis used to create Figure 4.4

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Appendix A.3

Knockdown of RhoA in B16 cells has no net discernable effect on dendrite extension.

A. Transfection with v2LMM_50451 shRNAmir against RhoA reduces RhoA protein levels compared to non-silencing controls.

B. In unstimulated B16 cells, transfection with RhoA knockdown construct has no effect on cell morphology. In stimulated cells, the majority of cells transfected with RhoA show no dendrites (middle panels) while some appear to increase the formation of long dendritic extensions (bottom panels) but the overall effect of RhoA knockdown is negligible, and not as strong as the overall effect of stimulating B16 cells with cAMP.

In melan-a cells, no effect on dendricity was observed at all (not shown).