Universidade de Lisboa

Faculdade de Ciências

Departamento de Biologia Vegetal

Molecular mechanism of melanin transfer from donor melanocytes to recipient keratinocytes

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Sob orientação de:

Prof. Doutor Duarte Barral

Prof. Doutora Rita Zilhão

Dissertação

Mestrado em Biologia Molecular e Genética

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Foreword

All experimental work reported in this dissertation was performed to complete the second year of the Master degree in *Biologia Molecular e Genética* of the Faculty of Sciences, University of Lisbon. The work was developed at the Chronic Diseases Research Centre (CEDOC), NOVA Medical School, NOVA University of Lisbon.

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Abstract

The skin is the largest organ of the human body. From ancient times, the color of skin has been an intriguing feature. Moreover, a large number of diseases are associated with skin disorders such as Griscelli syndrome, Hermansky-Pudlak syndrome and Waardenburg syndrome. Therefore, therapies directed to pigmentation disorders and also cosmetic applications have been intensively studied. Thus it is essential to understand the molecular mechanisms underlying of pigmentation disorders.

The "epidermal-melanin unit" is the functional complex that confers color and photoprotective properties to the skin. This unit is composed by melanocytes and keratinocytes. Epidermal melanocytes are highly specialized cells that synthesize and store the pigment melanin in unique membrane-bound organelles termed melanosomes. Once mature, melanosomes are transferred to neighbor keratinocytes and transported to the apical area of the cell where they form the protective melanin cap. Skin pigmentation results from three sequential processes: (i) the biogenesis of melanin in melanocytes; (ii) the transport from its site of synthesis in the perinuclear area of the cell to the periphery; and finally, (iii) the transfer to receptor keratinocytes. In this work, we focused on the transfer of melanin.

Previous studies from our group support the model of coupled exo-endocytosis of melanin transfer from melanocytes to keratinocytes. As Rab GTPases are master regulators of intracellular trafficking and have already been implicated in several steps of skin pigmentation (melanogenesis and transfer of melanin), the group proposed to continue investigating the molecular mechanisms of the melanin transfer and found that Rab11b mediates the exocytosis and transfer of melanin from melanocytes to keratinocytes.

The aim of this work is to identify Rab11b effectors, which bind to the active form of small GTPases, in melanocytes. Since Rab11-family interacting proteins (Rab11-FIPs) are described to be effectors of Rab11, we studied if they could have a role in the secretion of melanin from melanocytes. Moreover, this analysis was done downregulating each protein and observing if there was a decrease in melanin exocytosis. We found that FIP2 as well as Myosin Va can play a role in this process, since the silencing of each of these proteins impaired melanin secretion by melanocytes. Furthermore, we analyzed the localization of each FIP as well as Myosin Va in melanocytes by overexpressed tagged forms of these proteins. We also analyzed a possible association between these proteins and melanosomes. Relatively to FIP2, we found that this protein co-localize with Rab11b, when both overexpressed, in close proximity with melanosomes along of cytoplasmic membrane. Myosin Va also co-localize with Rab11b but in dendrite tips of melanocytes. At this point, the disposition of FIP3 also

gave us good evidences to play a role in melanin secretion. FIP3 co-localize with Rab11b, when both proteins overexpressed, in microtubule-organizing center, changing the normal localization of Rab11b in the cell. Melanosomes also change its disposition, becoming dispersed in all cytoplasmic region.

In summary, our studies indicate that FIP2, FIP3 and Myosin Va are required for the secretion of melanin by melanocytes.

Keywords: Rab11b, Rab11-family interacting proteins (Rab11-FIPs), melanin, skin pigmentation, vesicular/membrane trafficking.

Resumo Alargado

A pele é o maior órgão do corpo humano e desde sempre a sua pigmentação foi um fator intrigante. Diversas doenças têm sido diagnosticadas e associadas às várias alterações que ocorrem ao nível da pigmentação da pele, maioritariamente hipopigmentação, como a Síndrome de Griscelli, a Síndrome de Hermansky-Pudlak ou mesmo a Síndrome de Waardenburg. Tem havido também um enorme desenvolvimento do mercado das indústrias farmacêuticas e de cosméticos na área da pigmentação da pele, com um contributo na investigação nesta área. Assim, é essencial perceber os mecanismos moleculares que ocorrem nas células envolvidas na pigmentação da pele, de modo a possibilitar um real desenvolvimento de tratamentos que solucionem ou atenuem os sintomas destas doenças.

A "unidade melano-epidérmica" é um complexo que tem a capacidade de conferir cor e propriedades foto-protetoras à pele. Esta unidade é constituída por duas classes de células distintas: os melanócitos e os queratinócitos. Os melanócitos são células epidérmicas altamente especializadas e com a capacidade de sintetizar e armazenar pigmento (melanina) em organelos denominados melanossomas. Uma vez maduros, os melanossomas são transferidos para os queratinócitos adjacentes, nos quais se deslocam para a zona perinuclear apical onde formam um escudo protetor, de modo a conferir a proteção necessária para evitar danos causados no DNA pela radiação ultravioleta. Deste modo, podemos dizer que a pigmentação da pele decorre de três processos essenciais: (i) a biogénese da melanina (melanogénese) e consequente armazenamento nos melanócitos; (ii) transporte dos melanossomas desde o local de síntese, na zona perinuclear da célula, para a periferia; e (iii) transferência dos melanossomas para os queratinócitos adjacentes.

Estudos anteriores realizados pelo nosso grupo, verificaram que a transferência dos melanossomas ocorre maioritariamente através do modelo baseado na exocitose a partir dos melanócitos e posterior endocitose pelos queratinócitos. Além disso, baseando-se no facto das proteínas Rab serem os principais reguladores do tráfego membranar e estando envolvidas em diversas etapas do processo de pigmentação da pele, o grupo propôs-se a continuar o estudo minucioso dos mecanismos moleculares da pigmentação da pele. Assim, foi demonstrado que a proteína Rab11b está envolvida na exocitose e na transferência da melanina dos melanócitos para os queratinócitos. Foi ainda demonstrado que a Rab11b co-localiza com o receptor de transferrina na região perinuclear da célula, indicando que marca maioritariamente os endossomas de reciclagem. Ainda assim, vesículas positivas para Rab11b foram vistas a co-localizar com melanossomas maduros na região periférica da célula.

O principal objetivo deste trabalho parte precisamente deste resultado que envolve a proteína Rab11b. De modo a dar continuidade ao mesmo, o grupo propôs-se a identificar e seguidamente estudar proteínas que pudessem estar envolvidas com a Rab11b na exocitose dos melanossomas. Estas proteínas são designadas efetores e interagem com proteínas G específicas, ajudando-as a desempenharem as suas funções, quando estas se encontram no seu estado ativo, ou seja, ligadas a GTP. Neste sentido, inicialmente procurámos identificar estas proteínas efetoras da Rab11b através da bibliografia mas também através da técnica *"Yeast-two-Hybrid"*. Por conseguinte, foram identificadas *"Rab11-family interacting proteins"*, também designadas Rab11-FIPs e a Miosina Va como as mais promissoras candidatas a interagir com a Rab11b. Seguidamente, o nosso objetivo era observar se aquando do silenciamento de cada um dos genes codificadores de cada proteína existiria ou não uma diminuição na secreção de melanina por parte dos melanócitos. Ou seja, investigar a existência dum fenótipo semelhante ao provocado aquando do silenciamento do gene que codifica a proteína Rab11b. Deste modo, foi demonstrado que a FIP2 poderá estar envolvida neste processo, tendo sido, a par da Miosina Va as proteínas que se apresentaram mais próximas do fenótipo descrito para o silenciamento da Rab11b quando silenciadas nos melanócitos. Contudo, abordamos estes resultados apenas como preliminares devido a algumas dificuldades detetadas na quantificação da melanina segregada. Tendo a FIP2 e a Miosina Va como principais alvos do nosso estudo mas sem poder descartar as restantes proteínas estudadas, iniciámos então a caracterização das mesmas. Para isso, foi essencial sobre-expressar cada uma das FIPs e a Miosina Va sozinhas mas também em simultâneo com a Rab11b, de modo a verificar a existência ou não de co-localização. De modo a complementar o estudo, comparámos também localização de cada proteína estudada com a localização dos melanossomas nos melanócitos, verificando se a proximidade já detetada entre os melanossomas e a Rab11b também se verifica com as FIPs e a Miosina Va. Foi facilmente visualizada a existência de co-localização entre as FIPs e Miosina Va com a Rab11b. Contudo, para cada uma delas foi verificado que esta co-localização se verifica em locais distintos da célula, nuns casos mais próximos do compartimento endocítico de reciclagem (FIP3) e noutros com elevada proximidade à membrana plasmática (FIP2), principalmente nas dendrites do melanócito (Miosina Va). Se do ensaio de exocitose de melanina obtivemos dois alvos preliminares (FIP2 e Miosina Va), observando a localização das FIPs também visualizámos um efeito interessante por parte da sobre-expressão da FIP3 e da sua influência na localização da Rab11b. Isto levou a que fosse também considerada como um potencial alvo para estudos futuros.

Os resultados obtidos no decorrer desta investigação permitem-nos obter informações adicionais sobre os mecanismos moleculares que regulam a transferência de melanina dos melanócitos para os queratinócitos. Em concreto, este trabalho define possíveis alvos, como a FIP2 e

a FIP3, envolvidos nos mecanismos que levam ao transporte e consequente exocitose da melanina dos melanócitos. Os resultados fornecem também boas indicações acerca da influência da Miosina Va no sistema de pigmentação, sendo já conhecida a sua influência nos filamentos de actina, na periferia dos melanócitos. Sugerimos também novos elementos de estudo, principalmente relacionados com o complexo tripartido FIP2-Rab11a-Miosina Vb. Sabendo que a Rab11a quando silenciada não afeta significativamente a exocitose da melanina, contrariamente à Rab11b, podemos colocar a hipótese que a Miosina Vb poderá ter um efeito determinante juntamente com a FIP2 e Rab11b neste processo. Isto principalmente junto da membrana plasmática onde a FIP2 co-localiza com a Rab11b e ambas se encontram adjacentes aos melanossomas. A FIP3, por sua vez, é também interessante pela capacidade que tem em recrutar a Rab11b para o compartimento endocítico de reciclagem, aquando da sobre-expressão de ambas, deixando de se localizar em qualquer outro local da célula. Parece também existir nestas condições um movimento centrípeto dos melanossomas que torna interessante analisar a capacidade de secreção de melanina por parte do melanócito após sobre-expressar esta FIP.

Mais estudos serão necessários para comprovar a possível influência das duas FIPs selecionadas neste modelo de pigmentação. Será também necessário desenvolver um novo método de quantificação da melanina, mais eficaz e preciso, ou melhorar o já utilizado por nós, evitando a minuciosidade, ambiguidade e a demasiada sensibilidade do mesmo. Neste aspeto algumas soluções também são propostas para o efeito sendo que outras foram analisadas, testadas e apresentadas no decorrer da tese.

É importante ter sempre em conta que os resultados e hipóteses para estudos futuros aqui apresentados por nós têm como objetivo primordial melhorar a compreensão dos mecanismos moleculares de pigmentação da pele, permitindo assim servir de base para melhor compreender e interpretar as razões que levam aos diferentes fenótipos das doenças da pigmentação.

Palavras-Chave: Rab11b, Rab11-family interacting proteins (Rab11-FIPs), melanina, pigmentação da pele, tráfego vesicular/membranar

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List of Abbreviations

α-MSH – Alpha-melanocyte-stimulating hormone

AP – Adaptor protein

Arf – ADP-ribosylation factor

ATP – Adenosine triphosphate

BLOC – Biogenesis of lysosome-related organelles complexes

BMP-4 – Bone morphogenic protein 4

BSA – Bovine serum albumin

cDNA – Complementary deoxyribonucleic acid

CT – Cholera toxin

EE – Early endosomes

ER – Endoplasmic reticulum

ERC – Endocytic recycling compartment

ET-1 – Endothelin-1

FBS – Fetal bovine serum

GAP – GTPase-activating protein

GDF – GDI displacement factor

GDI – GDP dissociation inhibitor

GDP – Guanosine diphosphate

GEF – Guanine-nucleotide exchange factor

GGTase – Geranylgeranyl transferase

GS – Griscelli syndrome

GTP – Guanosine triphosphate

GTPase – Guanosine triphosphatase

HPLC – High-performance liquid chromatography

HPS – Hermansky-Pudlak syndrome

ILV – Intraluminal vesicles

KGF – Keratinocyte growth factor

LE – Late endosomes

LRO – Lysosome-related organelle

Lys – Lysosomes

MITF – Microphthalmia-associated transcription factor

Mlph – Melanophilin

MyoVa – Myosin Va

MyoVb – Myosin Vb

OCA – Oculocutaneous albinism

PAR-2 – Protease-activated receptor-2

PBS – Phosphate-buffered saline

PM – Plasma membrane

PMA – Phorbol-12-myristate-13-acetate

PMEL – Premelanosome protein

pp75 – Rab11-interacting protein or 75kDa phospho-protein

qRT-PCR – Quantitative real time polymerase chain reaction

Rab – Ras-like in brain

Rab11-FIP – Rab11-family interacting protein

RabF – Rab family motifs

RabSF – Rab subfamily regions

Ran – Ras-like nuclear

Ras – Rat sarcoma

RBD – Rab11 binding domain

RCP – Rab-coupling protein

RE – Recycling endosomes

REP – Rab escort protein

Rho – Ras homologous

RNA – Ribonucleic acid

RPE – Retinal pigment epithelium

siRNA – small interfering RNA

SNARE – Soluble N-ethylmaleimide-sensitive factor activating protein receptor

TAP – Tandem-affinity purification

TEV – Tobacco etch virus

TGN – Trans-Golgi network

TLC – Thin Layer Chromatography

Tyr – Tyrosinase

TYRP – Tyrosinase-related protein

UVr – Ultraviolet radiation

WS – Waardenburg syndrome

Y2H – Yeast two-hybrid

1. Introduction

1.1 Skin Pigmentation

1.1.1 Human Skin

The skin is the largest organ of the human body and its color plays a very important function in animals. Skin color has been an intriguing feature over the centuries, often causing discrimination among races. Furthermore, various animals have the capacity to change the color of their skin as part of a strategy of camouflage. Presently, business around the skin pigmentation industry are worth billions and this economical exploitation grows with the increase of the concern about physical appearance. Moreover, a large number of diseases are associated with skin color. These are called pigmentation disorders.

The skin has numerous functions: protects the body against foreign organisms or toxic substances; maintains water and temperature body homeostasis; synthesizes vitamin D; and is essential for sensibility and sensations [1].

Structurally, the skin is composed by three main layers [1,2]:

- *Hypodermis* – the inner layer, mostly composed by adipose tissue and connective tissue.
- *Dermis* – the middle layer, mainly composed by fibroblasts, collagen and elastic fibers with blood vessels that support and nourish the upper layer.
- *Epidermis* – the upper and outermost layer, connected to the dermis by dermal-epidermal junction, is divided in five *strata*: *stratum basale, spinosum, granulosum, lucidum and corneum* (from the lower to the upper stratum, respectively).

Human skin pigmentation is the result of a close interaction between melanocytes and keratinocytes. These cells are present in a ratio of about 1:40, forming the "epidermal-melanin unit" [3]. Melanocytes are highly specialized cells, responsible for the synthesis and storage of the pigment melanin. These cells derive from the neural crest, differentiate from a non-pigmented precursor, the melanoblast, and migrate to the basal layer of the epidermis. The keratinocytes occupy all five layers of the epidermis and are the final recipient cells of melanin [4,5].

1.1.2 Melanin

Melanin is composed of distinct types of monomeric units that are connected by strong carbon-carbon bonds. Melanin is dense and insoluble over a range of pH [6,7] and exists in two forms:

eumelanin and pheumelanin. Both derive from tyrosine, which is oxidized by tyrosinase, leading to dopaquinone. However, pheumelanin needs a cysteine-dependent reduction step whereas eumelanin does not. Moreover, eumelanin is a dark-black insoluble polymer and pheumelanin is a light red-yellow sulphur-containing soluble polymer [6,7]. The primary function of this pigment is to protect the DNA from ultraviolet radiation (UVr), absorbing and converting it into heat, lesser toxic energy. The energy absorbed by melanin is maximal in UV wavelengths [8,9]. The synthesis of melanin occurs within the lumen of specialized organelles called melanosomes in the melanocyte.

1.1.3 Melanosomes

Melanosomes are morphologically and functionally unique organelles within which melanin is synthesized, transported and stored. Melanosomes develop within highly dendritic cells, the melanocytes of the skin, in choroidal melanocytes and retinal pigment epithelial (RPE) cells. Melanosomes are large organelles with a diameter of approximately 500nm. These organelles contain the dark pigment, thus are easily visible by bright-field microscopy [10]. Melanosomes share various features with lysosomes and are considered lysosome-related organelles (LROs) [9,11,12]. Skin pigmentation is a complex process that can be divided in 3 steps: first, the biogenesis of melanin, which occurs within melanosomes in melanocytes; then, the transport of mature melanosome from their site of synthesis in the perinuclear area of the cell to the periphery; and finally, the transfer of melanin to recipient keratinocytes.

The development of melanosomes is divided in four morphological distinct steps. In stage I, non-pigmented vacuolar early endosomes derived from the endosomal system form the pre-melanosomes. Next, the formation of internal fibrous striations begins and, when these are fully formed, the melanosome matures to stage II. At this stage, melanosomes adopt an ellipsoidal shape and these elongated fibrils are visible by electron microscopy. These fibrils serve as a scaffold for the deposit of melanin. Thus, stage III melanosomes are characterized by starts with thickening and darkening of the intraluminal structure that becomes masked by melanin accumulation, leading to stage IV. In this last stage, melanosomes are mature and fully melanised [8–11].

When mature, melanosome are transported to the tip of melanocyte dendrites. Specifically, melanosomes bind to microtubules and initiate a fast, long-distance and bidirectional pathway bound to motor proteins. Once in the periphery of the cell, melanosomes are captured near the plasma membrane. This last step is dependent on actin filaments that allows short-range movements [13–15].

Fully mature melanosomes are then transferred to surrounding keratinocytes where melanin is transported to the apical area, forming a protective supranuclear cap that protects the genetic material from UVr [16]. The specific mechanism of intercellular pigment transfer remains enigmatic and

there are four transfer models accepted in the literature: cytophagocytosis, coupled exocytosis/endocytosis, fusion of plasma membranes and shedding of vesicles [17,18]. Cytophagocytosis consists in the engulfment of an intact part of the melanocyte, usually a dendrite tip containing melanosomes, by a keratinocyte. The tip is pinched off and is then phagocytosed by the keratinocyte. The phagolysosome is transported to the supranuclear region and, finally, disintegrated and the melanin dispersed. Another model is based on the fusion of the melanosomal membranes and the plasma membrane with consequent exocytosis of melanin granules. This leads to the release of naked melanin granules devoid of membrane, also named melanocores, to the intercellular space. These melanocores are then endocytosed by neighbor keratinocytes. The reported ingestion of small and isolated melanin granules is an evidence of this model, as well as the presence of melanin with only one surrounding membrane in keratinocytes [19]. The third model considers the fusion of melanocyte and keratinocyte plasma membranes. The result is a channel that connects the cytoplasm of both cells, allowing the direct passage of melanosomes to keratinocytes. The fourth and last model presented is mediated by vesicles. Melanosomes bud off from melanocyte dendrites and are consequently released to the extracellular space. Then, the keratinocyte internalizes these vesicles, forming a triple membrane compartment, composed of the melanosome with its membrane plus part of the plasma membrane of the melanocyte and the keratinocyte, which is subsequently degraded and the melanin is dispersed in the cytoplasm and transported to the apical perinuclear region.

1.2 Vesicular trafficking

The cytoplasm of an eukaryotic cell is populated with a variety of membrane-bound organelles. The communication between these organelles is a complex and fundamental process that ensures the correct function of intracellular compartments. Vesicular transport ensures this communication and avoids the random movement and fusion of vesicles. Furthermore, this process is also essential for the release of products from the cell (exocytosis) and for internalization of material from the extracellular space (endocytosis) [20]. The first step involved in this process is the budding of a vesicle from a donor compartment. The budding step is mediated by protein coats (such as clathrin) which are recruited from the cytosol to nascent vesicles [21,22]. These proteins play an important function in vesicle budding and in cargo selection, recognizing sorting signals in cytosolic domains of transmembrane cargo proteins [20,23]. Afterwards, the new vesicle is pinched off, uncoated and transported by motor proteins, such as kinesins, dyneins or myosins, via the cytoskeleton (microtubules or actin filaments) to the acceptor compartment [24]. Then, tethering and docking occur, mediated by tethering factors, such as

exocyst complex and SNAREs [25] (Soluble N-ethylmaleimide-sensitive factor activating protein receptors), respectively. These steps ensure the specificity of fusion to the acceptor compartment membrane [20,26,27].

Transport pathways in cells have been divided into endocytic, recycling, transcytic and exocytic pathways [28]. The first one corresponds to the uptake of macromolecules. During this process invaginations develop from the plasma membrane, directing endocytosed components through early endosomes (EE), late endosomes (LE) and lysosomes (Lys). LE and Lys belong to the degradative pathway, where degradation by acid hydrolases occurs [29,30]. Nevertheless, endocytosed cargo can also follow the recycling pathway [19,31,32]. In this case, it returns to the plasma membrane through the endocytic recycling compartment (ERC), also known as recycling endosome (RE). This allows the maintenance of organelle homeostasis, preventing the degradation of components. Some macromolecule cargos need to be transported selectively between different compartments of the cell, which is mediated by the transcytic pathway. This pathway shares some features with the recycling and endocytic pathways. Finally, the exocytic or secretory pathway balances the ratio of cell products, regulating the secretion of new proteins from endoplasmic reticulum (ER) to the plasma membrane (PM), through the Golgi and trans-Golgi network (TGN) [20].

All these steps are regulated by proteins belonging to the Ras superfamily of small guanosine triphosphatases (GTPases), such as Rab proteins, that are specific for different intracellular compartments [14,22,33–36].

1.2.1 Rab GTPase family

The Rab family of proteins is the largest family of the Ras superfamily. This superfamily of GTPases comprises over 150 members in cells. It is divided in five major families: Rat sarcoma (Ras), Ras homologous (Rho), ADP-ribosylation factor (Arf/Sar), Ras-like nuclear (Ran) and Ras-like in brain (Rab) [37,38]. This phylogenetic division was based on sequences obtained from the complete draft of the human genome. Moreover, comparisons between human, fly, yeast and plant species were already performed [39]. The five families of proteins maintain a conserved sequence at the N-terminus, which binds to GTP, named G box GDP/GTP-binding motif elements. Hence, these proteins have the capacity to hydrolyse GTP into GDP [40]. Structurally, this conserved GTPase domain is formed by a six-stranded β-sheet (five parallel strands and one antiparallel one) surrounded by five α-helices which can exchange between two conformations, according to their GDP- or GTP-bound state.

As previously stated, the largest family of the Ras superfamily is the Rab family of proteins (20-25 kDa) with at least 60 *RAB* genes known, found in all eukaryotes studied [41]. However, this number can increase since there is evidence that alternative splicing of these genes exists, resulting in the

production of functionally distinct. Structurally, Rabs share the Ras family characteristics referred above, plus the Rab family (RabF) motifs. These are composed of five conserved distinct amino-acid stretches common to the Rab family members and that distinguish Rabs from the other Ras members, as well as, Rab subfamily (RabSF) regions that can be used to recognize specific Rabs [41,42]. Proteins of the Rab family play an important role as regulators of specific intracellular trafficking pathways, specifically in vesicle budding, interactions with motor proteins and in vesicle tethering and docking. These proteins perform essential roles in controlling anterograde and retrograde trafficking between compartments, to coordinate cargo delivery and membrane recycling. Rab proteins are present in all compartments of the endomembrane system, nucleus, plasma membrane, mitochondria and centrioles, allowing the association of each one with a specialized intracellular compartment, making them good functional markers [43,44].

1.2.2 Rab GTPase cycle model

In order to perform their functions, Rab proteins interconvert cyclically between an active GTP-bound state and an inactive GDP-bound one (Figure 1) [44,45]. These two states correspond to similar conformations but two regions of the protein change conformation namely Switch I (Ras residues: 30-38) and Switch II (59-67) regions [40,46]. Although Rabs are capable of hydrolyzing GTP, they depend on the presence of GTPase-activating proteins (GAP)[47,48] to catalyze the hydrolysis of GTP to GDP, releasing an inorganic phosphate (P_i) and ending the signaling at the acceptor membrane [28,34,36]. This conformation of Rabs is recognized by Rab escort protein (REP)[49,50] that presents Rabs to the geranylgeranyl transferase (GGTase)[49,51]. GDP dissociation inhibitor (GDI)[52,53] recognizes GDP-bound Rabs, thereby stabilizing the inactive form of the proteins. Therefore, REP and GDI function as recycling factors to retrieve Rab proteins from the PM and deliver them to donor membranes. Once targeted to a specific membrane, GDI displacement factors (GDF) are postulated to play a role in the recognition step by disrupting the Rab-GDI complex. Thus, the release of GDI/REP promotes the association between geranylgeranylated Rabs and the donor membrane. Finally, guanine-nucleotide exchange factors (GEFs)[48,54] act in donor compartments, turning ‘on’ the Rabs by exchanging GDP for GTP in the nucleotide-binding pocket. When in the GTP-bound state and associated to membranes, Rabs are considered activated [55]. In this active state can recruit Rab effectors [44].

1.2.3 Rab effectors

Effectors are defined as proteins that have the ability to bind another protein, specifically. Usually, effectors bind to Rab proteins in the GTP-bound state. However, exceptions exist, like protrudin that interacts with the inactive state of Rab11. When Rab proteins return to the GDP-bound state, effectors dissociate, the Rab recycles back and the cycle continues. In many cases, effectors have

been identified by different methods such as yeast two-hybrid (Y2H), genetic screens and affinity purification. The recruitment of effectors by Rabs enables them to control important steps of vesicular transport, like cargo selection, budding, movement, docking and fusion. It is interesting to note that each pathway has its own unique set of effectors. This is the reason why a structural heterogeneity exists and there is a highly specialized and exclusive activity for individual organelles and transport systems. Nevertheless, some Rab effectors share structural features. Consequently, a single Rab protein interacts with multiple effectors [35]. On the other hand, it has been shown possible that a single effector recognizes specifically an isoform of a Rab protein [57]. This shows that effectors also interact with non-conserved regions of Rab proteins, increasing their binding specificity. Given the great structural diversity of Rab effectors and their Rab-interacting domains, further studies are necessary to determine the full range of Rab effector binding interactions [35,58].

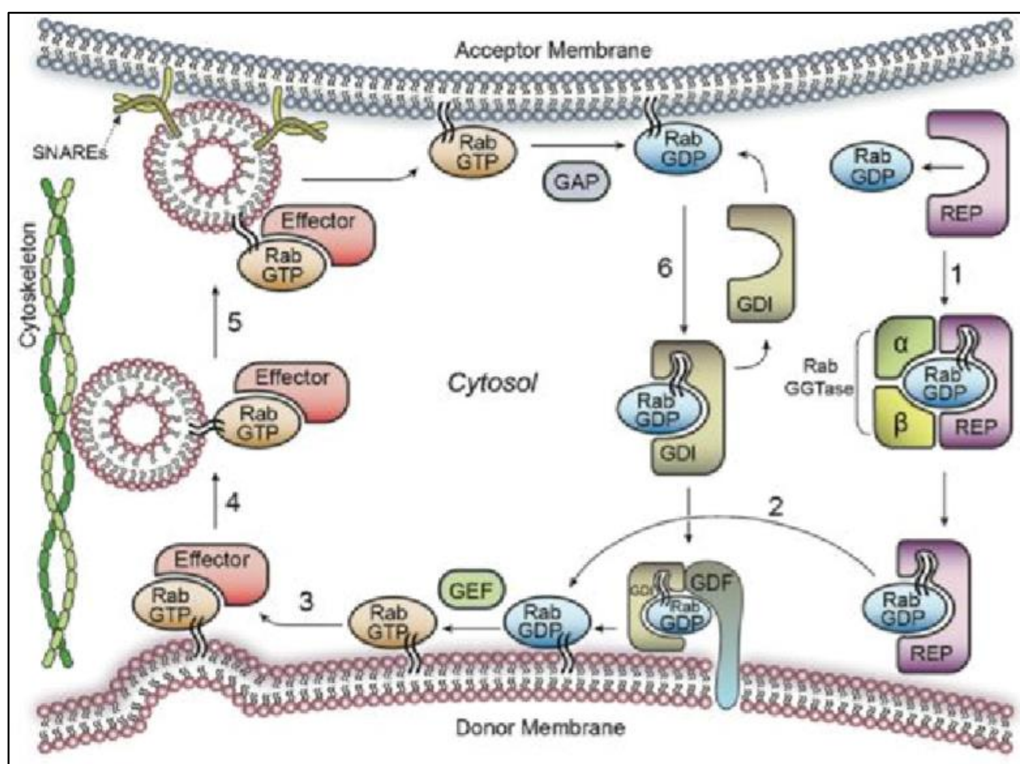

Figure 1 – Model of the cycle of RabGTPases. Rab proteins interconvert cyclically between an active GTP-bound state and an inactive GDP-bound state. For the success of the alternation between these two conformations, several players are needed as shown in this schematic. Taken from Seixas *et al,* 2013 [56].

1.2.4 Rab11 family

The Rab11 family of proteins is composed by Rab11a, Rab11b and Rab25 (or Rab11c). All members of this subfamily are encoded by distinct genes. However, Rab11a and Rab11b share 89% amino acid identity, differing in the C-terminus on 30 amino acids, and each one sharing 61% and 66% identity with Rab25, respectively [59]. Moreover, Rab11 acts in the ERC and studies have shown that these homologs play different roles. Rab11 is involved in the slow endocytic recycling pathway,

transport from TGN to the PM and transport from endosomes to TGN, phagocytosis and apical targeting on epithelial cells [31,32,60]. Rab11 was also implicated in the cleavage of the midbody during cell division. Rab11a and Rab11b functions have been difficult to discriminate and therefore the reference to the isoform is often omitted. Rab11a and Rab11b are expressed ubiquitously; however, Rab25 is expressed almost exclusively in epithelial cells. Several functions played by Rab11 in the cell demonstrate the role of this Rab subfamily in cell traffic. Hence, in the last decade many studies focused on this subfamily of proteins and on identifying Rab11 effectors.

1.2.5 Rab11-family interacting proteins (Rab11-FIPs)

Rab11-family interacting proteins (FIPs) are a set of five proteins that interact with Rab11. These proteins are: Rab-coupling protein (FIP1C or RCP), Rab11-FIP2 (FIP2), Rab11-FIP3 (FIP3 or Eferin or Arfophilin-1), Rab11-FIP4 (FIP4 or Arfophilin-2) and Rab11-interacting protein or 75kDa phospho-protein (FIP5 or Rip11 or pp75 or Gaf1) [59]. Rab11-FIP1 (FIP1) possess at least five transcripts (FIP1A-E), although FIP1C has been the best studied [61,62]. Rab11-FIPs are characterized by the presence of a highly conserved 20 amino acid domain with approximately 90% homology, localized in the C-terminus of the proteins, also designated by Rab11 binding domain (RBD). RBD is predominantly present in α-helical conformation, and contains conserved hydrophobic residues essential for binding to Rab11. However, comparing the sequence of Rab11-FIPs, these proteins only share 14-34% of amino acid identity [59,63]. Despite the low degree of homology, members of this family are grouped into three classes. Class I (FIP1C, FIP2 and FIP5), containing a C2 domain next to the N-terminus of the protein [64]; Class II which includes FIP3 and FIP4 and is characterized by two EF-hands and a proline rich region [65]; and Class III whose single member is FIP1 which does not exhibit homology with any other FIP, besides the RBD domain [63,66]. Furthermore, the number of Rab11-binding proteins is constantly growing, which suggests that interactions between Rab11 and effector proteins account for the diversity of Rab11 functions. However, it remains unclear whether the effector proteins compete with each other for binding to Rab11 or work in a consecutive fashion. (Figure 2)

Nevertheless, the functions of FIPs are poorly understood. It is known that FIPs can be involved in recycling of cargo to the cell surface, delivery of membrane to the cleavage of midbody during cell division or function as linkers between Rab11 and molecular motors. One common speculation is that the interaction between Rab11 and FIPs may serve as targeting complex to interact and recruit to organelles a selective group of proteins that regulates membrane transport [59,63,66].

Briefly, in class I, FIP1C is the only FIP that is known to interact with more than one Rab (Rab11 and Rab4). FIP1C is frequently associated with breast cancer and with the processes of invasive cancer cell migration. Moreover, FIP1C is associated with the recycling pathway but not with degradative

pathway [62,67]. FIP2, is the best characterized FIP and forms a heterotetramer symmetrically arranged with Rab11 (Rab11-$(FIP2)_2$-Rab11) [62,68,69]. FIP2 is involved in early endocytosis and in the endocytic recycling pathway. It is thought to function as a linker in the transport of proteins (aquaporin-2, for instance) by molecular motor proteins like Myosin Vb (MyoVb). Rab11, FIP2 and MyoVb form a ternary complex associated with clathrin or AP-2 at the plasma membrane [68,70–73]. Also, the complex influences the localization of the microtubule motor protein dynein heavy chain and consequently the centripetal movement along microtubules [74]. Finally, FIP5 plays a role in trafficking of the glucose transporter 4 (GLUT4) receptors to the cell surface, in the recycling pathway and in the association with microtubules, through binding to kinesin II-binding protein. FIPs localizes to apical recycling endosomes, regulating the trafficking to the PM [62,75]. Noteworthy, study showed that when the stringency of the Y2H assay increased, only FIP2 and FIP5 showed the interaction with Rab11, rather than all of them [76]. Class II FIPs play different types of functions. FIP3 and FIP4 are associated with trafficking of endosomal cargo during cell division [62,77]. Also, both class II FIPs play a role in cytokinesis. This is evidenced by defects in cell division caused when one of these proteins is downregulated by small interfering RNA (siRNA) and by their localization next to the cleavage furrow/midbody during cell division [78–80]. Another role of this class of proteins is the interaction with ARFs. FIP3 mainly interacts with ARF5 and ARF6, while FIP4 only interacts with ARF5. ARF6 is well known to regulate cell motility, cytokinesis and phagocytosis [65,78,81,82]. FIP3 also binds to microtubule-based motors, namely kinesin I and dynein I [80].

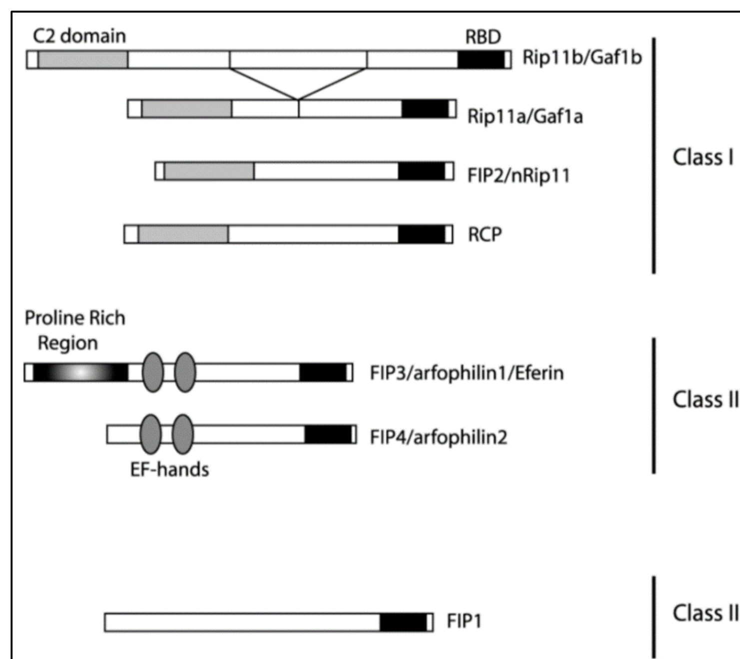

Figure 2 – The structure of Rab11 family interacting proteins (FIPs). This family is grouped into three classes. Class I (FIP1C, FIP2 and FIP5) containing a C2 domain next to the N-terminus of the protein; Class II (FIP3 and FIP4) is characterized by two EF-hands and a proline rich region; and, Class III (FIP1), which does not exhibit homology with any other FIP, besides the RBD domain. Taken from Prekeris, 2003 [66].

1.3 Trafficking in pigmentation

A large number of proteins including Rabs and Rab effectors have been implicated in several processes and pathways related to skin pigmentation, from the biogenesis of melanin and melanosome maturation, to the internalization of melanin by acceptor keratinocytes. These proteins show specificity in their localization, providing a good tool as cell markers [12]. (Figure 3)

In the stage I of melanosome biogenesis, premelanosome protein 17 (PMEL) is recruited to intraluminal vesicles (ILV) of vacuolar early endosomes. PMEL is an important element of the structural intraluminal fibrils and shapes melanosomes [83,84]. A clathrin coat, AP-1 and AP-2 play a role in premelanosome formation and sorting of proteins to the ILVs, although the relation with PMEL sorting is not clear yet [85]. Afterwards, melanin deposits and masks PMEL, allowing the stage I or II of PMEL plus melanosomes identification [86].

The biogenesis of LRO complexes (BLOC) 1 and 2 have similar localization to AP-1 and AP-2 in early endosomes but distinct functions in mediating sorting from there. These proteins are thought to control the exit of cargo from early endosomes (BLOC1) and target it to maturing melanosomes (BLOC2) [87,88].

It was shown that BLOC proteins interact with APs to sort other important regulators of melanogenesis, such as tyrosinase (Tyr) and tyrosinase-related protein 1 (TYRP1) from endosomes to melanosomes [88,89]. These proteins are essential to initiate pigment synthesis and melanosome maturation to stage III and IV. TYRP-1 is a transmembrane protein and a specific marker of mature melanosomes, often used in immunolocalization [90].

Rab proteins play an essential role in biogenesis of melanin. Rab32 and Rab38 were described as crucial in the transport of melanogenic enzymes, like Tyr from the TGN to melanosomes [91]. Moreover, Rab7 has an important role in sorting of TYRP-1 from the endosomal system and in the regulation of PMEL maturation [92,93].

Following maturation, melanosomes are transported from their site of synthesis in the perinuclear area to peripheral areas of the melanocyte and dendrite tips. Cytoskeletal elements play a significant role in intracellular transport, beginning in the cell body with a long-distance process dependent on microtubules. The signals that enable the interaction between melanosomes and microtubules are not known yet. However, it is known that the association of melanosomes with microtubules is dependent on the molecular motors, kinesin and dynein. Fully melanised melanosomes bind to kinesin-I and are then transported to the dendrites of the cell, in a step that is adenosine triphosphate (ATP)-dependent. On the other hand, the retrograde movement depends on

the motor dynein. Therefore the bi-directional movement of melanosomes is important to maintain the equilibrium of melanosomes transport in both ways, according with needs of the cell [94–98]. Rab1a plays a role in microtubule-dependent movement, principally in anterograde movement since its silencing causes the perinuclear aggregation of melanosomes [99].

Furthermore, in the plus end of microtubules, underneath the PM, melanosomes must ultimately be captured and retained to prevent their centripetal re-trafficking. Once this occurs, the transport continues on actin filaments. These short-range movements are dependent on a tripartite complex, composed by Myosin Va (MyoVa), Melanophilin (Mlph) and Rab27a [15,100,101]. MyoVa interacts with the melanosome and with actin filaments, co-localizing with both [13]. This interaction is possible due to the presence of Rab27a on the membrane of melanosome that mediates, through Mlph, a Rab27a effector, the binding between melanosomes and MyoVa [102–105]. Thus, Rab27a is essential in the regulation of the peripheral localization of melanosomes and functions in the tethering of melanosomes to the cortical actin. Other proteins were shown to be involved in this process, such as Rab8. However, more studies are needed to define this mechanism [106].

After being captured at the tips of dendrites, melanosomes are extruded by melanocytes and taken up by keratinocytes. This transfer process is not clearly understood, as mentioned previously. However, some studies showed that Rab11 and Rab17 are involved in melanin transfer since the depletion of each one in melanocytes lead to an accumulation of pigment in these cells [107,108].

The uptake of melanosomes by keratinocytes is far less characterized. In the literature, only one protein has been identified regulating melanin uptake, namely the protease-activated receptor-2 (PAR-2). This protein is present in the keratinocyte PM of keratinocytes and it was shown that the activation of this protein results in the increase of the phagocytic activity in keratinocytes.

1.4 Skin pigmentation modulating factors

Skin pigmentation is primarily determined by the efficiency of melanogenesis and transfer of melanin between recipient and acceptor cells. Hence, it is crucial to understand the mechanisms and factors that regulate these processes. Until now, it is known that melanocyte behavior in skin is largely influenced by environmental signals, as well as autocrine or paracrine signals from keratinocytes [109]. UVr is the most powerful environmental factor known. UVr increases the synthesis and secretion of most keratinocyte-derived factors and decreases the inhibitory ones [110]. Besides, UVr can also up-regulate melanogenesis and melanocyte dendricity. Alpha-melanocyte-stimulating hormone (α-MSH)

is a physiological factor that induces melanogenesis and consequently skin pigmentation, mainly in sun exposed areas. α-MSH, which is released by keratinocytes, increases its action when stimulated by UVr [111]. Others stimulation factors that act directly, or indirectly on TYRP1 and tyrosinase levels, increasing them, are endothelin-1 (ET-1) and keratinocyte growth factor (KGF). Also, the transcription factor Foxn-1 plays an important function in recruiting melanocytes to the epidermal melanin unit or regulate keratinocytes growth and differentiation in skin [112].

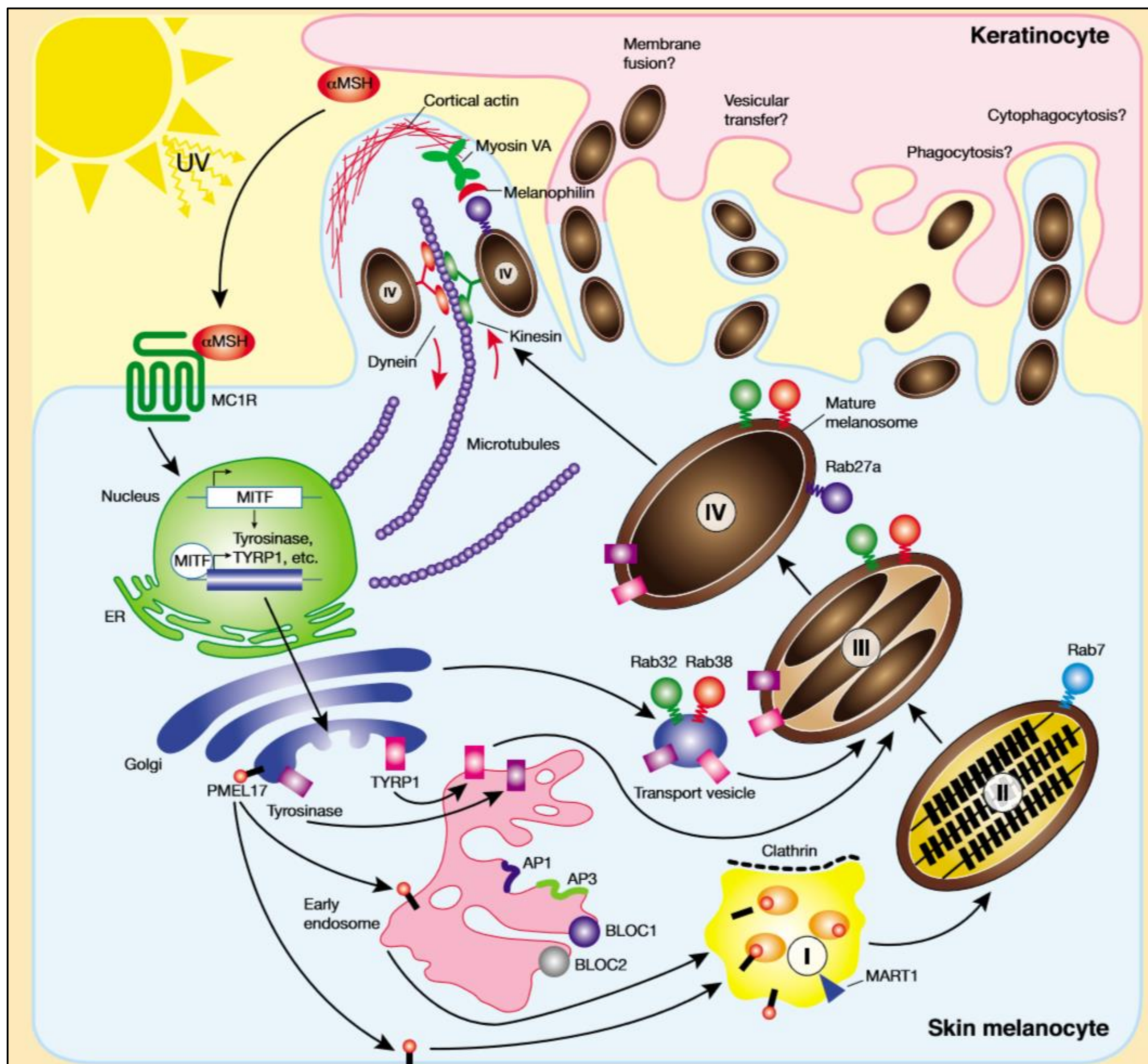

Figure 3 – The role of membrane trafficking in skin pigmentation. Schematic representative of several steps in formation and development of melanosomes in melanocytes. The four models proposed for melanosome transfer from donor melanocytes to recipient keratinocytes are also represented. Taken from Wasmeier *et al,* 2008 [10].

1.5 Skin pigmentation disorders

It is crucial to recognize the molecules and mechanisms governing skin pigmentation to unravel the basis of diseases that involve the pigmentary system [36,56,113]. Defects in any step of this system can lead to pigmentary disorders, commonly characterized by a reduction or even absence of pigmentation in skin, hair and eyes. However, these disorders are also associated with immunodeficiency or bleeding diathesis, among others, which are associated with LRO defects.

Defects on melanoblast migration in embryos from the neural crest to the skin can lead to diseases like piebaldism or Waardenburg syndrome (WS). The cause of this dysregulation results from mutations in genes like *Microphthalmia-associated transcription factor* (MITF). In these cases, white patches in skin and hair result from loss of melanocytes [114].

Pigmentary diseases also occur when the melanin is not produced, leading to an absence or deficiency of pigment in viable melanocytes. Mutations in *TYR* and *TYRP1* genes can be cause of this effect and are present in the autosomal recessive disease called Oculocutaneous albinism (OCA) [11,115].

A disease that is in the boundary between anomalies in melanin formation and defects in melanosomes constitution is the Hermansky-Pudlak syndrome (HPS). In this case, there is impaired trafficking of Tyr and TYRP1 leading to partial albinism and bleeding diathesis. The genes involved in HPS are *HPS1-9* and encode subunits of several proteins involved in melanosome biogenesis and function, such as AP-3, BLOC-1, BLOC-2 and BLOC-3 [12,56].

Griscelli syndrome (GS) is perhaps the most studied pigmentary disorder. It is an autosomal recessive disease characterized by hypopigmentation of the hair and skin. GS occurs when there is a defect in at least one protein of the tripartite complex involved in binding of melanosomes to actin. Thus, this syndrome is divided in three types: GS1[116,117], GS2 (or Elejalde syndrome)[118] and GS3 [116]. These three subtypes of the disease are characterized by deficiency in MyoVa, Rab27a or Mlph, respectively. Interestingly, there are mutant mice for each protein of the tripartite complex protein: *dilute*[119], *ashen*[104] and *leaden*[120] [15,121,122] .

Finally, investigation of the molecular machinery involved in melanosome motility is essential to elucidate the processes that regulate organelle motility and membrane trafficking in general. It is important to apply these studies to specific models, such as the pigmentary system, in order to increase the knowledge about pigmentary disorders, which can lead to the development of new therapies or cosmetics products.

2 Previous work

Previous work by our group focused on the regulation of melanogenesis and the molecular mechanism of melanin secretion from melanocytes, leading to the accumulation of evidence pointing to coupled exo-endocytosis as the predominant mechanism of melanin transfer in human skin.

The first line of evidence is that melanocores can be found in the extracellular space between melanocytes and keratinocytes. The presence of melanin granules lacking a surrounding membrane suggests exocytosis of the melanosome from melanocyte to the extracellular space, following the fusion between the melanosomal membrane and the melanocyte plasma membrane. Furthermore, melanin endocytosed by keratinocytes is only surrounded by a single limiting membrane. Finally, this membrane lacks the melanosomal membrane marker TYRP1. Hence, the melanosomal membrane is not present in keratinocytes, supporting the model of melanin exocytosis followed by endocytosis by keratinocytes as the major mechanism of melanin transfer in the skin [19].

It was also shown that keratinocytes are capable of inducing melanin exocytosis by melanocytes in co-culture assays. This is specific for keratinocytes as the same does not occur in co-cultures between melanocytes and fibroblasts, where the secretion levels are similar to those achieved when melanocytes are cultured alone. These results are consistent with other studies indicating that keratinocyte-derived factors are essential for promoting melanogenesis and melanosome transport in melanocytes, as well as pigment transfer [19].

Our group also identified that Rab11b regulates the secretion of melanin from melanocytes. When Rab11b is depleted from melanocytes, there is a decrease of keratinocyte-induced melanin exocytosis and also transfer to keratinocytes. Nevertheless, melanogenesis is not disrupted and melanosomes remain accumulated in melanocytes. On the other hand, a 2-fold increase in melanin exocytosis was observed when Rab11b was overexpressed in primary melanocytes in the presence of keratinocytes. These evidences was identified specifically to Rab11b assays, not occurring when performed to Rab11a. Furthermore, Rab11b presents a punctate distribution throughout the cytoplasm with accumulation at the periphery of the cell, where it co-localizes with the transferrin receptor, suggesting a localization to recycling endosomes. These observations are in agreement with previous studies that found Rab11-positive structures in close proximity to mature melanosomes in the cell periphery [19].

3 Hypothesis and Objectives

Despite all studies performed, the detailed mechanism of melanin exocytosis is still not fully understood. Therefore, the objective of this study was to characterize the molecular mechanisms of Rab11b-mediated melanin exocytosis and transfer. The question raised was whether Rab11b effectors could be involved in this mechanism mediating the role of Rab11b. Therefore, the specific aims of this work are:

Aim 1 – Identification of Rab11b effectors implicated in the exocytosis of melanin from melanocytes.

Aim 2 – Characterization of the localization of the Rab11b-effectors in melanocytes.

Aim 3 – Investigation of the interaction between Rab11b and the effectors found.

4 Materials and Methods

4.1 Cell Culture

Melan-ink4a melanocytes were cultured in RPMI 1640 (Gibco/Invitrogen), supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mM L-Glutamine, 200 nM Phorbol-12-myristate-13-acetate (PMA, Calbiochem), 200 µM Cholera toxin (CT, Gentaur), 100 U/ml penicillin G, and 100 U/ml streptomycin. XB2 keratinocytes were cultured in DMEM (Gibco/Invitrogen), supplemented with 10% FBS, 2 mM L-Glutamine, and 100 U/mL penicillin, 100 U/mL streptomycin. Both types of cells were maintained in a humidified incubator at 37ºC and 10% CO2.

4.2 Silencing

Silencing of *RAB11B*, *FIP1-5* and *MYOVA* was achieved using siGENOME SMARTpool (Thermo Scientific) specific for *Mus musculus*. Control siRNA was a non-targeting siRNA pool (Thermo Scientific).

For 12-well plates, 50 nM of gene specific siRNA were added to 50 µL of Opti-MEM (Gibco/Invitrogen). In parallel, 1.5 µL of Dharmafect 4 transfection reagent (Thermo Scientific) were added to 50 µL of Opti-MEM. After 5 minutes of incubation at room temperature, these two mixtures were combined, mixed gently, and incubated for 20 minutes at room temperature. During this time, the growth medium was removed from cells seeded the day before transfection and 400 µL of RPMI 1640 was added to each well. After 20 minutes at room temperature, 102 µL of silencing mix (siRNA + transfection reagent + Opti-MEM) were added to the wells. Cells were incubated for 4 hours at 37ºC / 10% CO_2 and then the medium was changed to keratinocyte conditioned medium, obtained by culturing with XB2 keratinocytes for 2 days. The medium was then removed, filtered (pore diameter = 0.45 µm) and CT and PMT were added at the same concentrations described above.

4.3 Melanin exocytosis

Melan-ink4a melanocytes (75 x 10^3 cells) were seeded onto 12-well plates. After 24 hours, cells were transfected with siRNA. The medium containing siRNA oligos was removed after 4 hours, and XB2 conditioned medium with CT and PMA was added. Cells were incubated 4 or 5 days, depending on the growth of the cells and the secretion of melanin to the medium. After that time, medium containing exocytosed melanin was centrifuged at 800 *g* for 5 minutes to pellet cell debris. The supernatant was then centrifuged at 20,000 *g*, 45 minutes at 4°C to pellet melanin. Melanin pellets were washed with absolute ethanol : diethyl ether (1:1 v/v) and dissolved in NaOH/20% DMSO (Sigma) at 60°C for 1 hour. Melanin content was measured as optical density at 340 nm and normalized to the number of

melanocytes. Total cell number for each well/condition was counted using a Neubauer chamber (Celeromics) at the end of the assay.

4.4 Immunofluorescence

Cells were grown on coverslips and fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in 1X Phosphate-buffered saline (PBS) for 20 minutes at room temperature. Excess fixative was removed by extensive washing in PBS. Cells were blocked and permeabilized with 1% Bovine Serum Albumin (BSA, Sigma), 0.05% saponin (Sigma) in PBS for 30 minutes. Fixed cells were then incubated with primary antibodies for 1 hour, washed extensively 4 times with PBS and incubated for 1 hour with appropriate secondary antibodies conjugated with a fluorophore (Molecular Probes). All antibody incubations were made in PBS, 0.5% BSA, 0.05% saponin. To visualize the nuclei, cells were incubated with DAPI (Invitrogen) for 5 minutes. Coverslips containing fixed cells were mounted in MOWIOL mounting medium (Calbiochem).

4.5 Antibodies

Antibodies used are summarized in the Supplementary Table 1. Secondary antibodies (Alexa) were from Invitrogen and used at 1:1000.

4.6 RNA extraction, cDNA production and real-time quantitative polymerase chain reaction

Total Ribonucleic acid (RNA) was isolated from cells using an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. RNA was reverse transcribed into complementary deoxyribonucleic acid (cDNA) by incubating RNA with, 10 mM dNTPs mix (Thermo Scientific) and random primers (Sigma) at 65ºC for 5 minutes. Samples were then incubated with 5x buffer (Invitrogen), DTT (Invitrogen) and RNAseOUT (Invitrogen) at 25ºC for 5 minutes. Lastly, Superscript II (Invitrogen) was added and the samples incubated at 25ºC for 10 minutes, at 42ºC for 50 minutes and finally at 70ºC for 15 minutes. For the real-time quantitative polymerase chain reaction (qRT-PCR), Brilliant SYBR® Green QPCR Master Mix (Roche) was used according to manufacturer's instructions and the analysis was done in a qPCR Roche LyghtCycler 96 system (Roche). For each protein, gene expression was calculated relative to control wells and standardized using α-tubulin as a housekeeping gene. The primers used are summarized in Supplementary Table 2.

5 Results

5.1 Identification of putative Rab11b effectors

As referred above, Rab11 is a protein involved in several trafficking processes such as: control the slow endosomal recycling pathway, the transport of material from peripheral sorting endosomes to the ERC, or from TGN to ERC. Rab11b has been suggested to mediate calcium-induced exocytosis in neuronal cells, also to mediate exocytosis of insulin granules in pancreatic β-cells, as well to mediate the exocytosis of melanosomes in melanocytes cells [59]. To perform these functions, Rab11 interacts with different effector proteins when in the GTP-bound (active) state.

The first aim of this work was to identify the proteins that interact with Rab11b. For this, literature searches were very important to define candidates. Moreover, we performed a yeast-two-Hybrid (Y2H) screen. This technique is very useful to discover protein-protein interactions and protein-DNA interactions, testing the physical interactions of the proteins involved. In our case, the screen was performed by Hybrigenics Services using as prey a library of human melanocyte proteins. Furthermore, the constitutively active form of *Mus musculus* Rab11b (Q70L), fused with the promotor of a reporter gene (*HIS3*) that was used as bait. If Rab11b-Q70L interacted with a protein from the library, the reporter gene was expressed, allowing yeast cells to grow in a medium lacking histidine. The DNA of the positive clones was then sequenced and analyzed to identify Rab11b-Q70L binding partners.

The Y2H screen showed that Rab11b-Q70L interacts with several proteins with Class I FIPs (FIP1, FIP2 and FIP5), MyoVa and Rab11-binding protein (Rab11BP, also named WDR44) with the highest likelihood, as well as Class II FIPs (FIP3 and FIP4) with a lower likelihood.

Therefore, we decided to analyze in detail the role of FIPs and MyoVa in melanin exocytosis. Importantly, there are no studies that investigate the role of FIPs in pigmentation. The reason to MyoVa was that it has already been described in the literature as playing a role in tripartite complex that accept melanosomes from microtubules and help to attach them with PM in melanocytes, being interesting to confirm its relationship with Rab11b.

5.2 Role of Rab11b effectors in melanin exocytosis from melanocytes.

After identifying putative Rab11 effectors, we proposed to verify whether any of these has plays an important role in pigmentation, particularly in melanin exocytosis. We began by performing an assay where each one of the FIPs or MyoVa were silenced. For this, we transfected Melan-Ink4a

melanocytes with siRNA pools, targeting each one of these proteins and we added XB2-conditioned medium to stimulate the secretion of melanin from melanocytes. The cells were incubated for 4-6 days, depending on cell viability, confluence, capacity to secrete melanin and the melanin present in the culture medium quantified. The silencing levels were measured by qRT-PCR in all cases.

First, we analyzed the darkness of the culture medium after removing the cell debris. This led us to conclude that the negative controls (mock and siControl) presented the darkest medium, followed by FIP2 and MyoVa which showed lighter medium. As expected, the silencing of Rab11b always corresponded to the lightest medium color, confirming the previous results from our group [19] (Figure 4 A).

Then, we isolated melanin from the medium and quantified the amount by spectrophotometry. (Figure 4 B) At this point, we noticed that the quantification did not show the same trued that we observed when we analyzed the darkness of the medium. For instance, the amount of melanin in the case of Rab11b silencing was higher than the siControl. Nevertheless, we pursued with a normalization of the amount of melanin *per* cell since there are different numbers of cells in each well due to some mortality that occurs during the transfection (Figure 4 C). In this case, FIP2 silencing showed a reduction in the amount of melanin in the medium. However, we did not see a decrease in the case of Rab11b silencing, which showed the most striking difference on the analysis of the medium color. For both graphs, melanin was quantified at 340 nm.

After some attempts we obtained a more interesting result, reflecting more closely the observation of medium darkness (Figure 4 D). However, the reduction in melanin present in medium from Rab11b depleted medium decrease when compared to siControl, was not as striking as suggest by the medium color darkness. Interestingly, we confirmed the decrease in melanin secreted by cells silenced for FIP2. In the case of MyoVa, we also observed a decrease of melanin in the medium *per* cell but with a smaller difference to siControl than FIP2. In this independent experiment, the melanin was also measured at 340 nm. The quantification of mRNA expression in all cases is shown on Supplementary Figure 1 and Supplementary Figure 2 .

To improve the accuracy and reliability of the melanin quantification assay, we analyzed the spectrum of absorbance of the melanin isolated from the medium and compared it with synthetic melanin. We found that the peak of melanin absorbance is around 250nm (Supplementary Figure 3). We then measured the purified melanin absorbance at 250 nm, 340 nm and 492 nm and compare to the synthetic melanin. We observed that at 250 nm the differences between samples are more prominent. However the curve is more irregular in this area. On the other hand, at 492 nm the differences are minimal between samples, measuring in the limit of capacity of the our nanodrop.

Thus, we continued using 340 nm since at this wavelength the curve is regular and smooth and has enough resolution to distinct between samples.

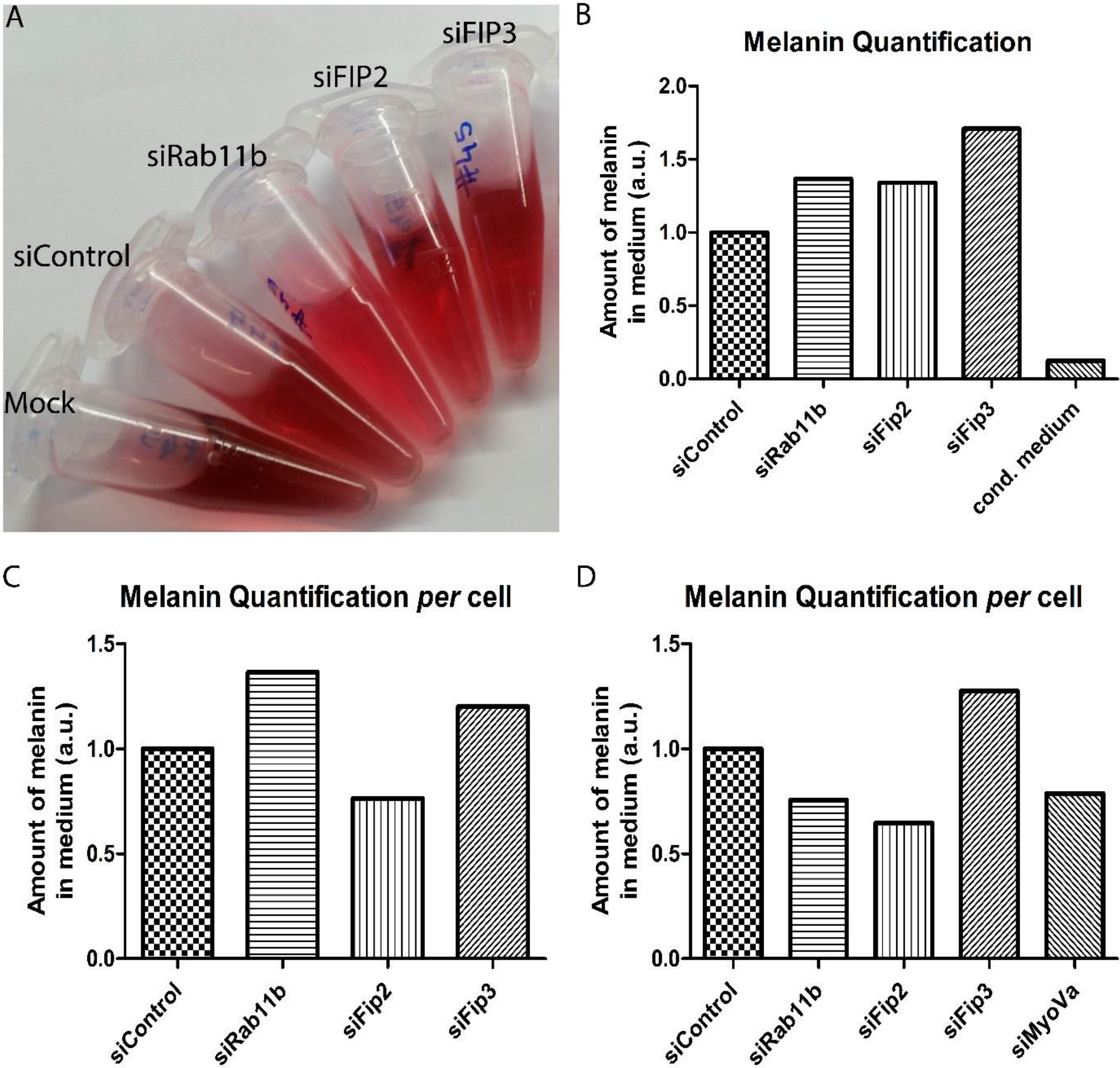

Figure 4 - Melanin exocytosis is reduced by silencing of Rab11b, FIP2 and MyoVa. **(A)** Culture medium after 5 days. The medium is lighter in the case of Rab11b and FIP2 silencing when compared with controls, indicating a decrease of amount of melanin secreted from Melan-ink4a melanocytes. **(B)** Results of melanin quantification after isolation from the medium shown in (A). **(C)** Same results as in (B) but normalized to the number of cells at the end of experiment. **(D)** Results of melanin quantification *per* cell, from a different experiment than in (A). Values of amount of melanin were measures at 340 nm and normalized to siControl.

5.3 Localization of Rab11b effectors in Melan-Ink4a and proximity to melanosomes

We obtained evidence that FIP2 and MyoVa could be involved in melanin exocytosis. However, due to the reasons explained above we could not exclude the other effectors. Therefore, we attempted to localize FIPs and MyoVa in Melan-ink4a cells. For this, we overexpressed each protein individually and with Rab11b. To compare the localization of the FIPs, Rab11b and MyoVa with melanosomes, we used TYRP1 as a specific marker for the melanosomal membrane. We also used the brightfield. Rab11b was described by our group to localize to the ERC and near the plasma membrane in melanocytes dendrites (Figure 5 A, D). Although it does not co-localize with melanosomes, Rab11b was found in close proximity to these organelles. Indeed we confirmed that (Figure 5 C, F).

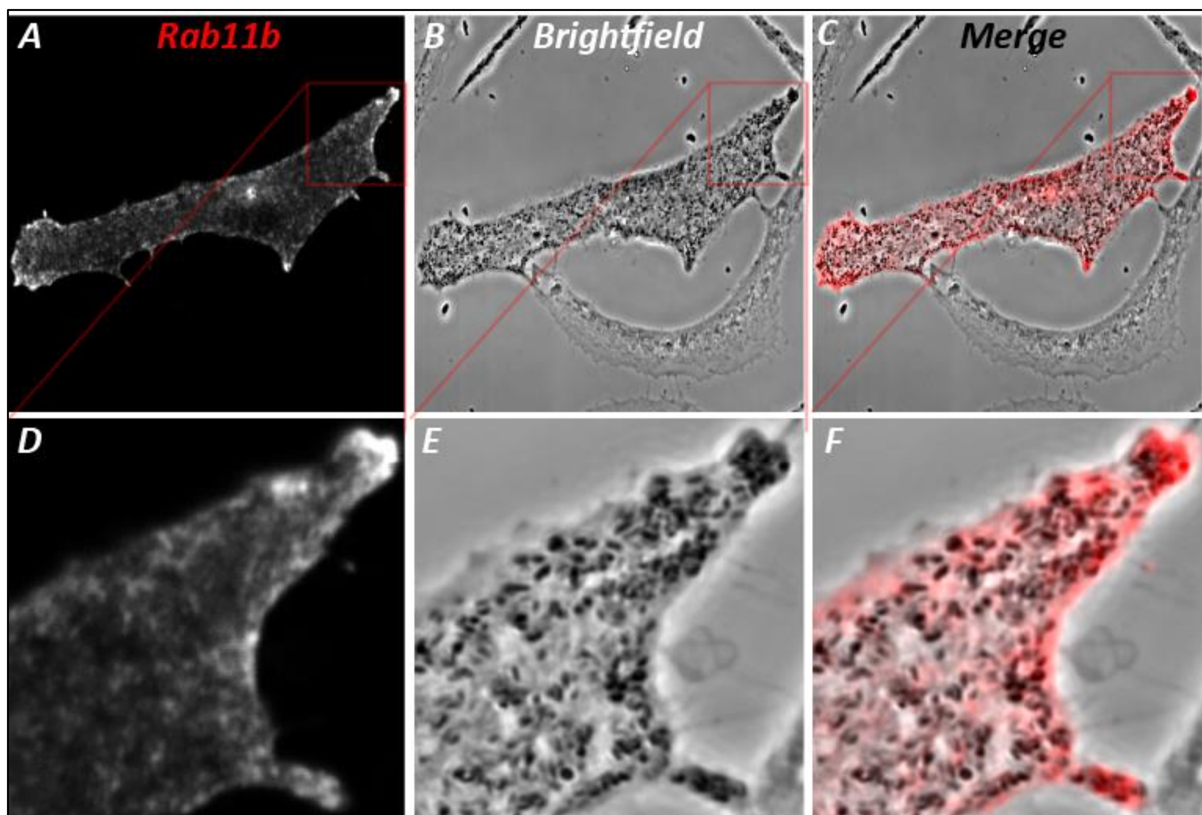

Figure 5 – Localization of Rab11b in Melan-ink4a cells. Rab11b was overexpressed alone in Melan-INK4a cells. **(A)** Rab11b localizes mainly to the ERC and dendrite tips (see zoom **(D)**). **(B)** Brightfield, used to visualize melanosomes (dark spots) on respective zoom **(E)**. Melanosomes accumulate in dendrite tips. **(C)** Merge of the two channels where the proximity between Rab11b and melanosomes can be appreciated.

When we overexpressed FIP1C, we observed that it localizes throughout the cell with an accumulation in the perinuclear region (Supplementary Figure 4 A, C). On the other hand, almost complete co-localization between FIP1C and Rab11b is observed when both are overexpressed

simultaneously. Moreover, the location of FIP1C changes when Rab11b is also overexpressed, becoming more punctate. This punctate signal is mainly perinuclear, and almost does not exist in other cell location. Other interesting observation show us the presence of FIP1C non-co-localizing (green color in merge channel) when overexpressed with Rab11b. However, the same does not occur with Rab11b, always being co-localized with FIP1C. (Supplementary Figure 4 D-K). With melanosomes we cannot obtain several conclusions. There are a presence of some numbers of melanosomes that have not FIP1C associated. However, we can observe that where we see FIP1C (co-localized or not with Rab11b) also is closely associated at least one melanosome. (Supplementary Figure 4 K)

In the case of FIP2, when we overexpressed this protein, we detected a punctate distribution in Melan-Ink4a in several places of the cell. Moreover, there is some accumulation in perinuclear region, probably in the ERC. (Figure 6 A, C) When we overexpressed FIP2 and Rab11b simultaneously (Figure 6 D-K), we observed significant co-localization although not as extensive as with FIP1C. FIP2 is found mainly closed to plasma membrane. However, the proximity to melanosomes can occur but is not totally obvious, observing the results from overexpression. (Figure 6 I, K) Since FIP2 is one of the most prominent candidate effector to mediate the role of Rab11b in melanin secretion (see previous section), we obtained an anti-FIP2 antibody to label the endogenous protein. With it, we confirm that FIP2 is always found close to melanosomes, further suggesting a role for this protein in melanosome secretion (Figure 6 L-Q).

Regarding FIP3 shows a striking to a perinuclear dot when FIP3 is overexpressed. Using gamma-tubulin staining to mark the microtubule-organizing center (MTOC) we showed that FIP3 localizes in this area of the cell, where the ERC also localizes (Supplementary Figure 5 A-D). Importantly, whereas the localization of FIP3 does not change when it is overexpressed with Rab11b, the localization of Rab11b is dramatically affected by the presence of overexpressed FIP3, since it becomes clustered in the central dot where FIP3 is (Supplementary Figure 5 E-H).

FIP4 overexpressed shows a dispersion in the cell, with concentration in the perinuclear region, probably in the ERC (Supplementary Figure 6 A-F). When FIP4 and Rab11b were both overexpressed we observed a striking co-localization near the nucleus. Moreover, we can observe more alone dots of FIP4 than with other FIPs in same conditions. Close proximity between FIP4 and melanosomes was observed in the cell body but not in dendrites (Supplementary Figure 6 G-N).

Finally, FIP5 was found dispersed throughout the cytoplasm when overexpressed alone (Supplementary Figure 7 A-C). However, when FIP5 was overexpressed with Rab11b they show co-localization. However, Rab11b do not stay restricted to that place (as in some of others FIPs) and continues localized in its habitual places in cytoplasm. When overexpressed with Rab11b, FIP5 shows

a more punctate distribution than when overexpressed alone. The localization is also more proximal to plasma membrane especially in dendrites tips. There we can observe the excellent proximity to melanosomes (Supplementary Figure 7 D-K).

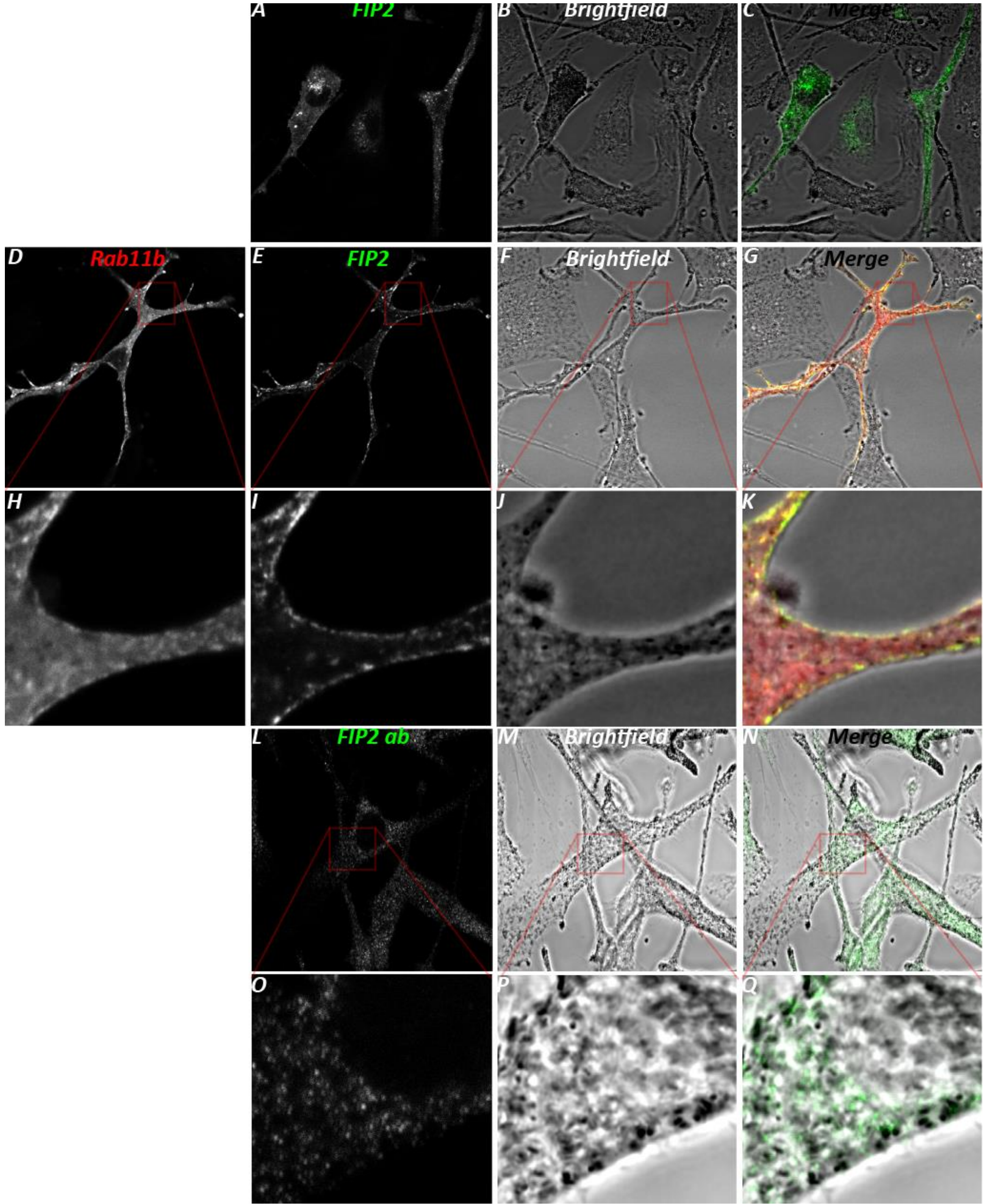

Figure 6 – Localization of FIP2. **(A-C)** Overexpression of FIP2 alone in Melan-INK4a cells. **(D-G)** Overexpression of Rab11b and FIP2 simultaneously, with corresponding zoom **(H-K)**. **(L-N)** Immunostaining and corresponding zoom **(O-Q)**. **(D,H)** Rab11b staining (red). **(A,E,I,L,O)** FIP2 staining (green). **(B,F,J,M,P)** Brightfield. **(C,G,K,N,Q)** Merged images.

MyoVa shows the same localization when overexpressed alone or together with Rab11b. MyoVa has a very strong signal in dendrite tips where it is known to have an important role in melanosomes docking to actin filaments. In dendrite tips there is co-localization between MyoVa and the Rab11b. However, Rab11b is not restricted to that area and is also present in other places like ERC, although with lower intensity (Figure 7).

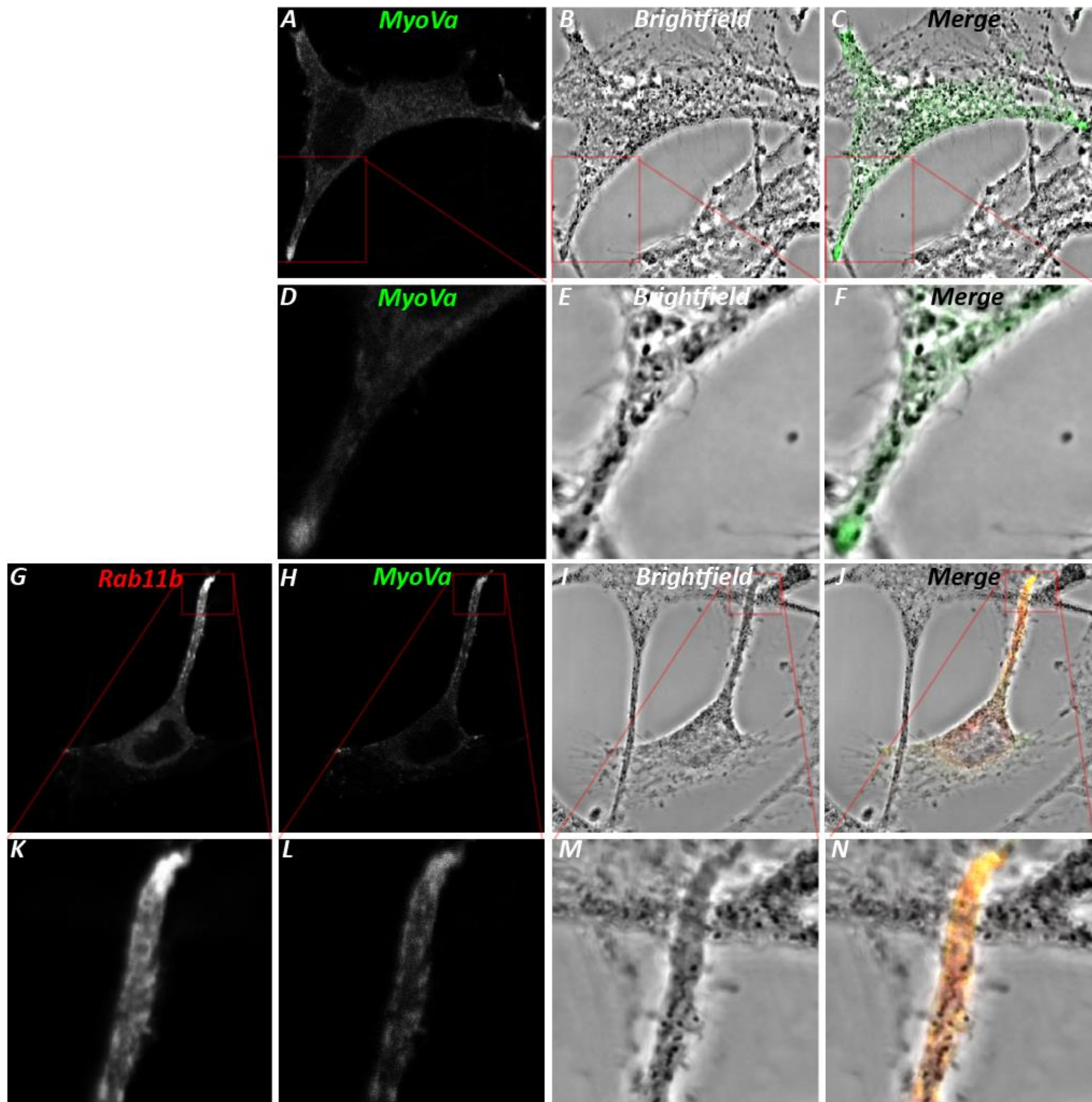

***Figure 7 – Localization of Myosin Va. (*A-C)** Myosin Va overexpressed in Melan-ink4a localizes to dendrite tips as zoom ins are shown **(D-F)**. **(G-J)** Overexpression of Rab11b and MyosinVa simultaneously, with corresponding zoom **(K-N)**. **(G,K)** Rab11b staining (red). **(A,D,H,L)** MyosinVa staining (green). **(B,E,I,M)** Brightfield. **(C,F,J,N)** Merged images.

6 Discussion and Future Perspectives

When we started this work we proposed to identify Rab11b-effectors that could play a role in pigmentation and specifically in melanin exocytosis from donor melanocytes. Moreover, we performed a Y2H screen to identify candidate effector proteins that could interact with Rab11b. As a result, MyoVa and Class I FIPs (FIP1, FIP2 and FIP5) were identified with a very high confidence, while FIP3 and FIP4 were also identified but with a lower confidence score. Thus, we investigated if FIPs and/or MyoVa could mediate the function of Rab11b in the regulation of melanin exocytosis.

By qrt-PCR, we found that in Melan-ink4a melanocytes, FIPs have lower expression levels than Rab11b (10-20% of Rab11b expression, data not shown). This suggests that FIPs may act only in restricted places and only perform very specific functions.

After obtaining a good silencing for the FIPs and MyoVa, we analyzed the melanin present in the cell culture medium that is the result of melanin exocytosis by Melan-ink4a melanocytes. For this we counted the number of viable cells at the end of the experiment, analyze the darkness of the medium and isolated melanin for quantification by spectrophotometry, normalizing for the number of cells. Upon a careful analysis of the results, we realized that the analysis of the medium did not match the quantification. This was obvious in the case of the silencing of Rab11b, our positive control. When Rab11b was downregulated, melanin secretion to the medium was impaired since medium was lighter than the controls. However, when we quantified the melanin in the medium we could not observe this trend.

Nevertheless, we confirmed that when Rab11b is silenced, there is less melanin secreted to the medium. We analyzed the effects of silencing to FIPs and MyoVa, too. Then, in the case of FIP1C, FIP3, FIP4 and FIP5 the medium was always as dark as the controls. However, silencing of FIP2 always resulted in lighter medium than the negative controls, suggesting that melanin exocytosis is impaired. A similar result was obtained with MyoVa.

In the future, the group should on improving the melanin quantification method or replacing it by an alternative method. Although we have tried to improve the current method, we ended up concluding that the wavelength of 340 nm is the best for melanin quantification. The first reason is because melanin has the highest absorption in UV and not in visible spectrum. However, at wavelengths lower than 340 nm the absorption curves is more irregular and has some small peaks. Therefore, at 340 nm the absorption is still high and the curve is smooth without clear interferences. Nevertheless, the method is not very robust and shows high variability. Thus, there are some

alternatives methods that can be tried. The first one is to perform the experiment in 6-well plates to obtain a higher amount of melanin secreted to the medium. This would allow increase in the amount of melanin that can be quantified, decreasing the variability of the measurements. Another is to measure the absorbance directly from the medium without isolating the melanin. This would decrease the number of steps involved and the variability introduced by the melanin isolation protocol. On the other hand, it might difficult to distinguish the melanin from the other components of the medium based only on the wavelength. We could also change the way of measuring the melanin by using thin-layer chromatography (TLC). This technique relies on the separation of the samples on a silica gel or cellulose matrix. Finally, the last method that we propose to try is the high-performance liquid chromatography (HPLC) that is a technique normally very useful to separate, identify and quantify the different components present in any mixture sample. This technique would be more complex and expensive, however would give us more precise and clear results.

Although we cannot take definitive conclusions, FIP2 and MyoVa seem to be the most interesting candidates. Moreover, we investigated the intracellular location of each FIP and MyoVa in Melan-ink4a cells by overexpression of these proteins alone or with Rab11b. Interestingly, we found that the distribution of the FIPs is clearly different when they are overexpressed alone or coupled with Rab11b. When overexpressed together with Rab11b the FIPs (except FIP3) present a more punctate distribution. FIP1C showed a good co-localization with Rab11b, specially near the nucleus and is clear that one alter the localization of the other when both are overexpressed simultaneously. FIP2 overexpressed alone also localizes predominantly to the perinuclear region. Importantly, when overexpressed, FIP2 shows a similar location to that obtained by immunostaining of the endogenous protein. When FIP2 and Rab11b are overexpressed, Rab11b does not alter its localization. However, FIP2 is much more prominent in the dendrites, mainly near the plasma membrane. Moreover, only near plasma membrane we could detect co-localization of both proteins. This suggest that this protein can be involved in processes of transport that allow the anchoring of melanosomes to the plasma membrane. The localization of FIP3 is completely different from the other FIPs, showing a very intense dot in the MTOC region, as demonstrated by gamma-tubulin. Moreover, the overexpression of FIP3 completely changes the localization of Rab11b, co-localizing with it. This has also been reported to occur in HeLa cells and could demonstrate that the overexpression of FIP3 enhances the centripetal movement of the vesicles that it binds to [123]. The localization of FIP4 is similar but less intense. FIP4 is mainly found in ERC when overexpressed with Rab11b. On its own, overexpressed FIP4 is dispersed in the cell with a small amount in the ERC region. In this FIP4 only exist co-localization with Rab11b in ERC and areas involved. Finally, FIP5 is found throughout the cell when overexpressed alone. However, when overexpressed with Rab11b both localize very close to the plasma membrane, in dendrite tips.

When we analyzed the localization of the FIPs relative to the melanosomes, FIP1C was always found next to these organelles. However, there also many melanosomes without FIP1C next to them. Interestingly, the FIP1C plus positive vesicles that are next to melanosomes also possess Rab11b. Regarding FIP2, when it is overexpressed with Rab11b is not clear that they always localize next to melanosomes. Noteworthy, the number of melanosomes seems to be decreased in cells overexpressing FIP2 and Rab11b. This could indicate that the overexpression can enhance melanosome exocytosis, but more studies are needed to conclude this. Since FIP2 was the most promising candidate effector to regulate melanosome exocytosis, we analyzed its localization using a specific antibody. We observed a close association between FIP2 and melanosomes, which localized at the periphery, near the plasma membrane. Upon overexpression of FIP3 alone or with Rab11b, we did not detect any co-localization or close association with melanosomes. However, FIP3 shows a striking accumulation near the MTOC and mislocalizes Rab11b to the same location. In the case of FIP4, the proximity to melanosomes resembles that of FIP3 but with much less intensity. When overexpressed with Rab11b, FIP5 localizes next to melanosomes and around some of them. Interestingly, Rab11b and FIP5 co-localize on the dendrite tips, very close to the plasma membrane.

MyoVa has been shown to associate with melanosomes via a tripartite complex of proteins, that includes Mlph and Rab27a, and is important for the capture of melanosomes at the cell periphery and interaction with the actin cytoskeleton. We observed that overexpressed Rab11b and MyoVa through with co-localize in the dendrite tips and that both associate with melanosomes.

After establishing the FIP(s) that is(are) involved in melanin secretion, it would be necessary to confirm the interaction with Rab11b in Melan-ink4a melanocytes. This could be done by normal immunoprecipitation or using tandem affinity purification (TAP) method. Briefly, this method consists in fusing a TAP tag with the target protein (Rab11b, in this case). This TAP tag has a cleavage site for the tobacco etch virus (TEV) protease inserted between two tags: one is the FLAG-tag (a polypeptide with the sequence DYKDDDDK) and the other is the HA-tag (human influenza hemagglutinin, a surface glycoprotein). Essentially, this method devolves an immunoprecipitation with anti-HA antibody to allowed by cleavage with TEV protease and a second immunoprecipitation with an anti-FLAG antibody. An advantage of this multi-step method is the identification of protein complexes, eliminating the background caused by proteins binding non-specifically.

Since there are five FIPs expressed in Melan-Ink4a melanocytes, they could have redundant roles. Therefore, the lack of phenotype upon silencing of one FIP could be explained by compensation from another FIPs. To investigate this, it would be interesting to silence two, three, four or even all FIPs, simultaneously. This test is already interesting to do with FIP2 and MyoVb because is proved that

they form a tripartite complex with Rab11a, playing roles in actin filaments that are essential in translocation of melanosomes from microtubules to plasma membrane. One possibility would be Rab11b also forms a complex with MyoVb and FIP2, allowing the interaction of melanosomes with actin filaments and consequently with plasma membrane. Indeed, we found that FIP2 are associated with Rab11b and melanosomes at the cell periphery [70]. A different possible hypothesis to justify the importance of FIP2 in the model is that it interact with clathrin and AP-1 that are also involved in Stage I of melanosomes maturation (premelanosomes). This proteins are important to recruit anothers proteins to ILV (probably PMEL, although the relation was not confirmed yet) essential to maturation of melanosomes, formation of fibrils and correct shape.

In the case of FIP3 it is interesting to note that it is associated with kinesin-I during cytokinesis [63,80]. Kinesin-I also is a microtubule-dependent motor protein that moves anterogradely, transporting melanosomes from the perinuclear region to the periphery. We found that overexpressed FIP3 shows a striking localization at the MTOC. Moreover, when overexpressed, FIP3 induces mislocalization of Rab11b to the MTOC. Furthermore, when FIP3 is overexpressed, the melanosomes seem dispersed throughout the cytoplasm, rather than in the dendrites. Therefore, it is possible that FIP3 causes a dominant-negative effect by binding to kinesin-I and not allowing it to be available to transport melanosomes to the cell periphery. Hence, it would be interesting to investigate the localization of kinesin in FIP3-overexpressing cells. Following our hypothesis, kinesin should be found clustered near the MTOC when FIP3 is overexpressed.

However, is still poorly understood the function of this family of proteins and how they are related with Rab11b. Moreover, it is not known if FIPs have any role in melanosomes biology. Nevertheless, would be interesting continue to improve the knowledge in relation between these two areas: skin pigmentation and the vesicular traffic of Rab11-family interacting proteins.

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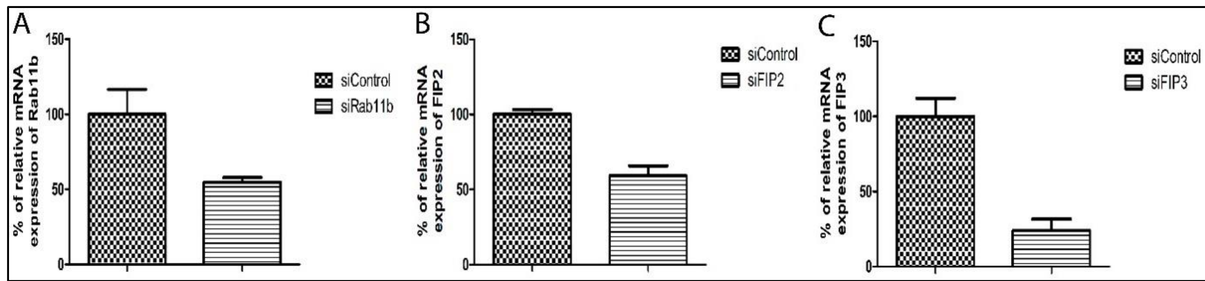
Supplementary Data

Supplementary Table 1 – Antibodies used in this study.

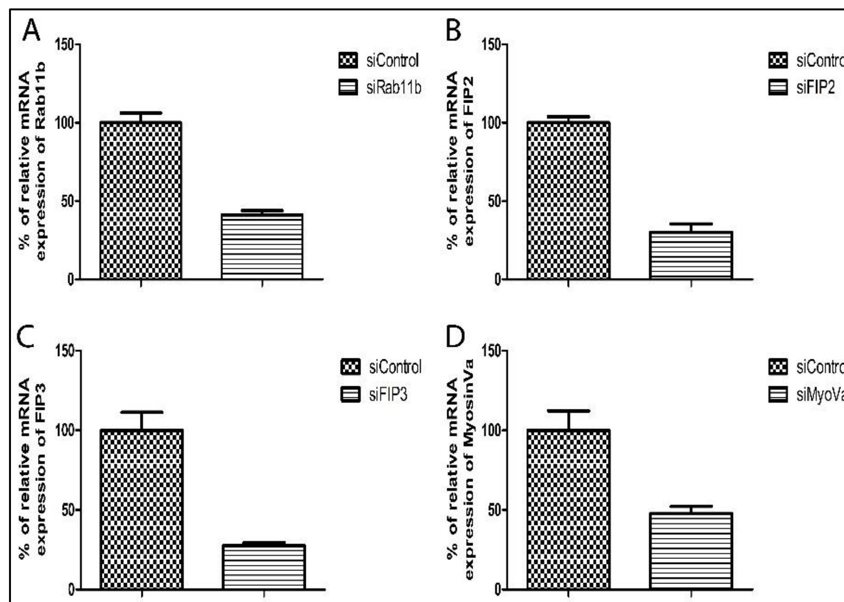
Antibody	Source	Catalogue number	Raised in	Fixed with	Dilution used	Concentration (mg/ml)
α – FIP2	Abcam	Ab76892	Rabbit	PFA	1:100	1
α – TYRP1	Abcam	Ab3312	Mouse	PFA	1:1000	0.2
α – γ-Tubulin	SIGMA	T5326	Mouse	PFA	1:200	1

Supplementary Table 2 – Sequences of primers used in real-time quantitative polymerase chain reaction (qRT-PCR).

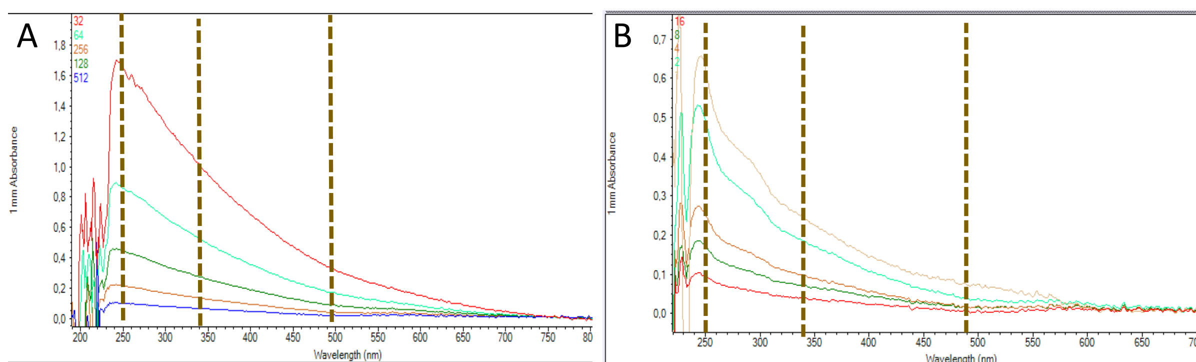
Gene	Sequence (5'-3')	
α-TUBULIN	Forward	GGTGGATCTAGAACCTGGG
	Reverse	CCCAGTGAGTGGGTCAGC
RAB11B	Forward	AAGGAGCTGCGGGATCATGC
	Reverse	ACAGGCTCTGGCAGCACTGC
FIP1C	Forward	AGGCTTGATCACACACTCCA
	Reverse	TCGCCGTCTGACTTAAACCT
FIP2	Forward	ACAATCCTTTCGATGCCACG
	Reverse	AGGATGCTGGGTGTCTCTTC
FIP3	Forward	GGAGCGTGAGAAGAGCATTG
	Reverse	CGATGTTGGCCTTCAGACAG
FIP4	Forward	GACATGCCTACAACAGCGAG
	Reverse	GCTGTGCTTGAGATCTTCCG
FIP5	Forward	TCCAGGTGACCATCCAGTTC
	Reverse	CTTGGAAGAATCGCTGAGG
Myosin Va	Forward	CCGAGTCCTCATGGAACAGC
	Reverse	CATCCTTGGGTTGGATGGCT



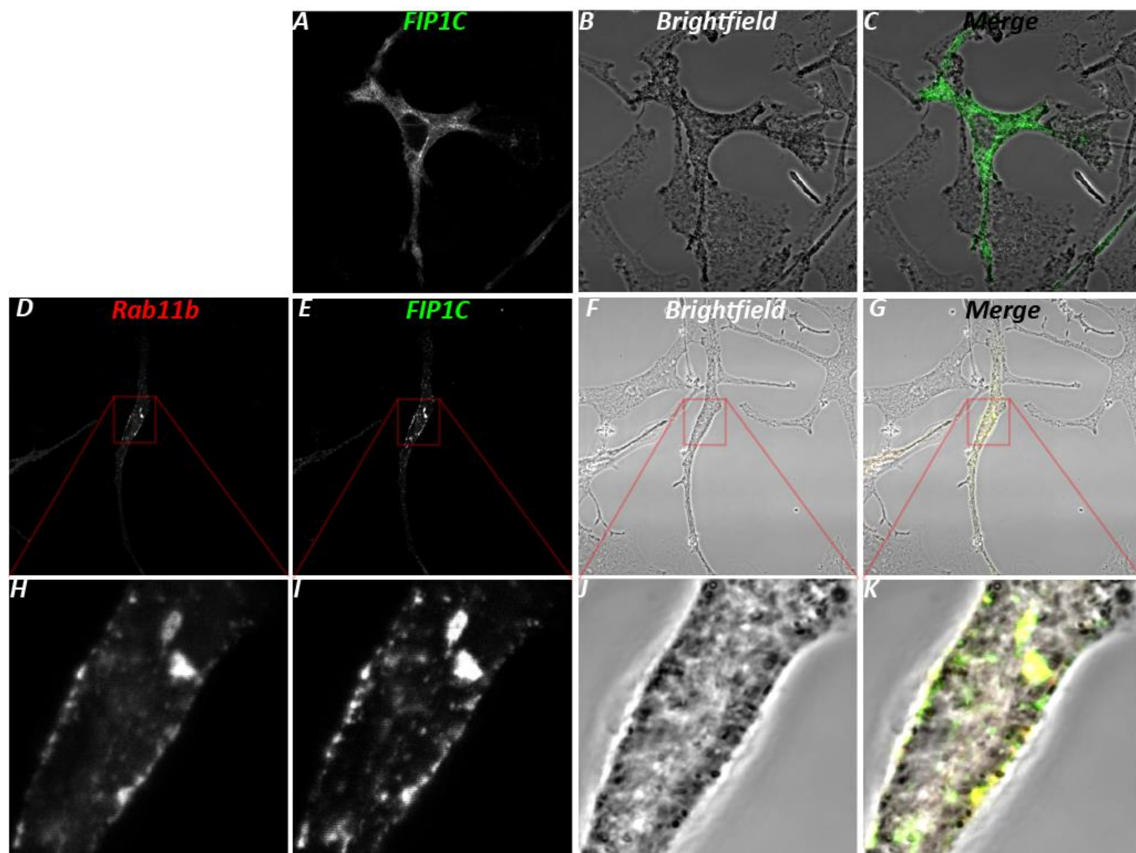
Supplementary Figure 1 – Quantification of Rab11b, FIP2 and FIP3 silencing (corresponding to the results shown in Figure 4 A-C). Percentage of relative mRNA expression for **(A)** Rab11b, **(B)** FIP2 and **(C)** FIP3, quantified by qrt-PCR.



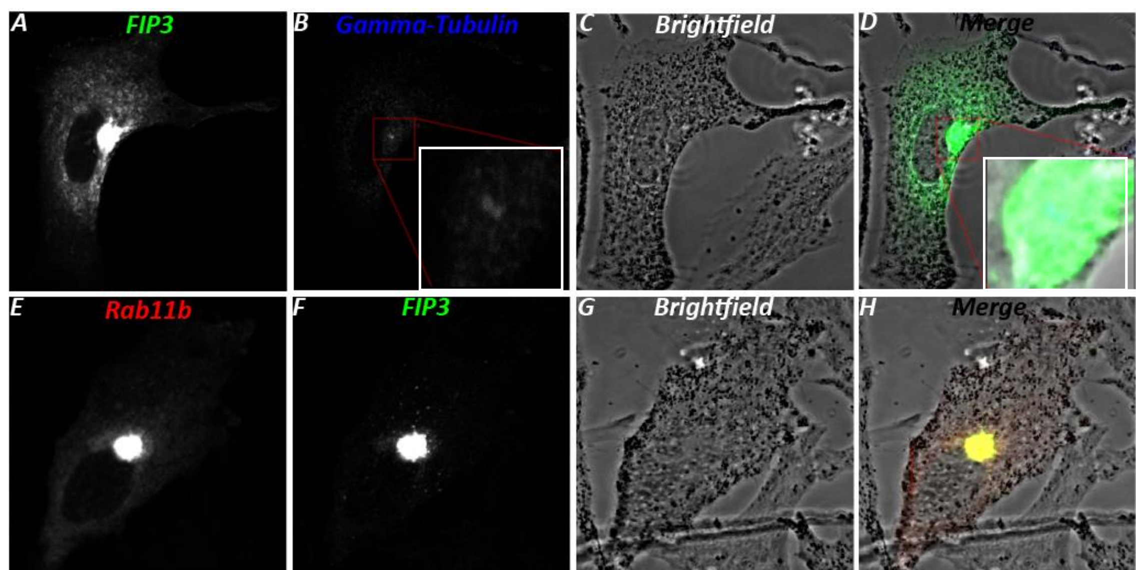
Supplementary Figure 2 – Quantification of Rab11b, FIP2 and FIP3 silencing (corresponding to the results shown in Figure 4 D). Percentage of relative mRNA expression for **(A)** Rab11b, **(B)** FIP2, **(C)** FIP3 and **(D)** MyosinVa, quantified by qrt.PCR.



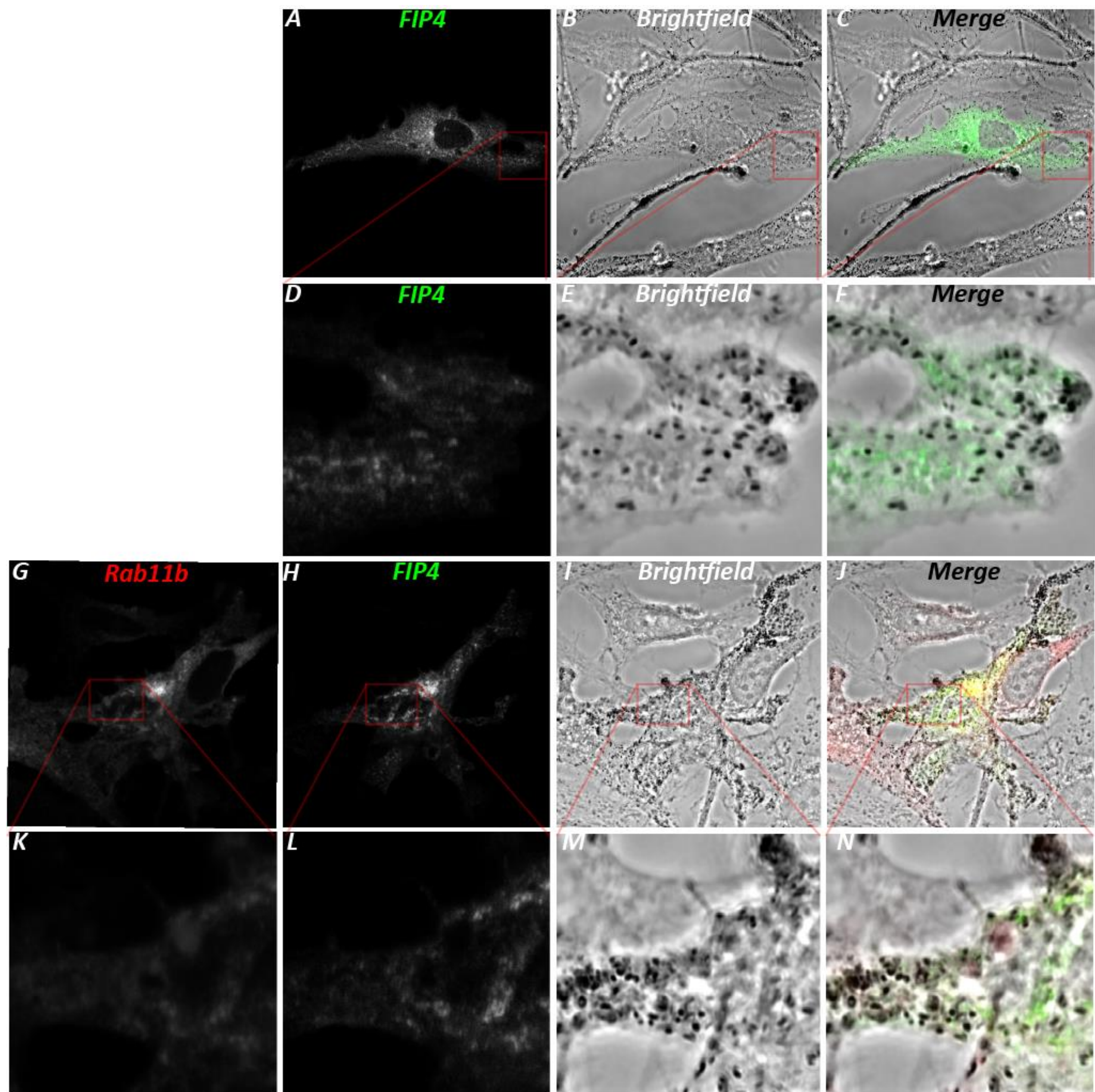
Supplementary Figure 3 – Melanin absorption spectrum. **(A)** Spetrum of synthetic melanin curves correspond to different dilutions (1:32, 1:64, 1:128, 1:256 and 1:512). Wavelengths 250, 340 and 492 nm are marked. **(B)** Spectrum of isolated melanin from the siControl shown in Figure 4 D..



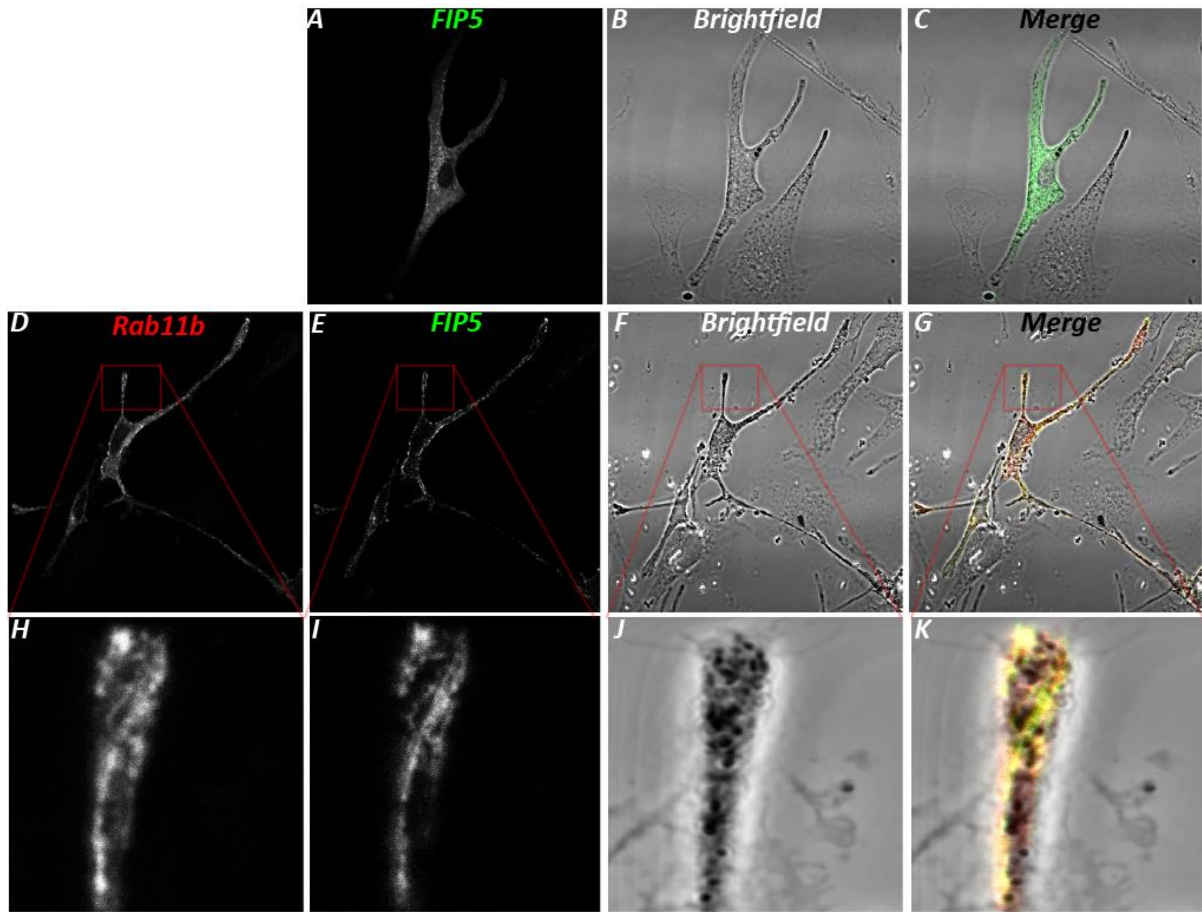
Supplementary Figure 4 – Localization of FIP1C. (**A-C)** Overexpression of FIPC alone in Melan-ink4a melanocytes. **(D-G)** Overexpression of Rab11b and FIP1C simultaneously, and corresponding zoom **(H-K)**. **(D,H)** Rab11b staining (red). **(A, E, I)** FIP1C staining (green). **(B, F, J)** Brightfield. **(C, G, K)** Merged images.



Supplementary Figure 5 – Localization of FIP3. (**A-D)** Overexpression of FIP3 alone in Melan-ink4a melanocytes. MTOC gamma-tubulin **(B)**. **(E-H)** Overexpression of Rab11b and FIP3 simultaneously. **(E)** Rab11b staining (red). **(A, F)** FIP3 staining (green). **(B)** Gamma-tubulin staining (blue). **(C,G)** Brightfield. **(D,H)** Merged images.



***Supplementary Figure 6 – Localization of FIP4. (*A-C)** Overexpression of FIP4 alone in Melan-ink4a melanocytes and corresponding zoom **(D-F)**. **(G-J)** Overexpression of Rab11b and FIP4 simultaneously and corresponding zoom **(K-N)**. **(G,K)** Rab11b staining (red). **(A,D,H,L)** FIP4 staining (green). **(B,E,I,M)** Brightfield. **(C,F,J,N)** Merged images.



Supplementary Figure 7 – Localization of FIP5. (**A-C)** Overexpression of FIP5 alone in Melan-ink4a melanocytes. **(D-G)** Overexpression of Rab11b and FIP5 simultaneously and corresponding zoom **(H-K)**. **(D,H)** Rab11b staining (red). **(A,E,I)** FIP5 staining (green). **(B,F, J)** Brightfield. **(C, G, K)** Merged images.