

Identifying a Targeting Factor for Rab27a

Antonia Elspeth Grace Booth

Imperial College London

National Heart and Lung Institute

Molecular Medicine

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Declaration

I, Antonia Booth, declare that the following manuscript is in my own works and the data presented is my own work. Any contributions are referenced accordingly.

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Abstract

The Rab family of small GTPases regulate intracellular membrane trafficking pathways and each Rab GTPase localises to a discrete membrane compartment. The mechanism(s) by which this targeting is achieved remains to be elucidated. Previous work identified that the C-terminal hypervariable domain and effector binding were not necessary for Rab27a targeting in melanocytes. However, GEF activity of the non-redundant Rab27a GEF Rab3GEP (R3G) was required but not sufficient for Rab27a targeting. This led to the hypothesis of the involvement of a Rab27a targeting factor.

A Rab27a mutant (Rab27a^{SF1/F4}) had previously been characterised that maintained melanosomal targeting and GTP-loading by R3G but bound to no known Rab27a effectors. Using this mutant, novel Rab27a interacting partners were identified and assessed for their possible role in Rab27a targeting. The current research identified ATP1a1 (the $\alpha 1$ subunit of Na⁺,K⁺-ATPase) as a novel interacting partner for Rab27a in melanocytes, depletion of which resulted in perinuclear clustering of melanosomes, indicative of loss of Rab27a function. Depletion of ATP1a1 did not disrupt Rab27a membrane localisation but did reduce the levels of GTP-bound Rab27a, comparable to what is seen following depletion of R3G. Crucially, depletion of ATP1a1 resulted in displacement of endogenous Rab27a from melanosomal membranes indicating a role in Rab27a targeting. Furthermore, ATP1a1 depletion disrupted the levels of other melanosomal proteins indicative of a disruption in melanogenesis. ATP1a1 is proposed to transiently associate with melanosomes and through its Na⁺,K⁺-ATPase pump function regulate the luminal pH, which indirectly regulates melanosome maturation. How the maturation of the luminal environment is conveyed to Rab27a, and is subsequent recruitment to melanosomes remains to be clarified.

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Abbreviations

AAK1 – AP2-Associated Kinase 1

ACTH – Adrenocorticotrophic Hormone

AP – Adaptor Protein or activator protein

Anxa2 – Annexin A2

APS – Ammonium Persulphate

Arf – ADP-Ribosylation Factor

ARNO – Arf Nucleotide Site Opener

ATP1a1 – Na⁺,K⁺-ATPase α -subunit isoform 1

BCA – Bicinchoninic Acid

BLOC – Biogenesis of Lysosome-related Organelle Complex

CBR – C-terminus Binding Region

CMC – Critical Micellular Concentration

CRMP2 – Collapsing Response Mediator Protein-2

CTL – Cytotoxic T-Lymphocyte

DAMP - *N*-(3-((2,4-dinitrophenyl)amino)propyl)-*N*-(3-aminopropyl)methylamine, dihydrochloride

DD – Death Domain

DENN – Differentially Expressed Normal versus Neoplastic

DKO – Double Knockout

DMSO – Dimethyl Sulphoxide

DNP – Dinitrophenyl

EEA1 – Early Endosome Antigen 1

EGFR – Epidermal Growth Factor Receptor

ER – Estrogen Receptor

EtOH – Ethanol

FBS – Foetal Bovine Serum

FHL – Familial Hemophagocytic Lymphohistiocytosis

FHM – Familial Hemiplegic Migraine

FPH – Familial Progressive Hyper-pigmentation

FRAP – Fluorescence Recovery after Photo-bleaching

GAP – GTPase-Activating Protein

GDF – GDI-Displacement Factor

GDI – Guanine-nucleotide Dissociation Inhibitor

GEF – Guanine-nucleotide Exchange Factor

GEP – Guanine-nucleotide Exchange Protein

GFP – Green Fluorescence Protein

GPMB – Glycoprotein Nonmetastatic Melanoma Protein b

GS – Griscelli Syndrome

GST – Glutathione-s-Transferase

HA – Hemagglutinin

HAP40 – Htt-Associated Protein 40

HPS – Hermansky-Pudlack Syndrome

HRP – Horseradish Peroxidase

HSAN – Hereditary Sensory and Autonomic Neuropathy

HTF – Hemagglutinin- TEV-FLAG

Htt – Huntingtin

IP – Immunoprecipitation

KLD – Kringle-Like Domain

KO – Knockout

LB – Luria-Bertani

MADD – MAPK Activating Death Domain

MAPK – Mitogen-Activated Protein Kinase

MC1R – Melanocortin 1 Receptor

MEL – Mobile Effector Loop

MeOH – Methanol

MITF – Microphthalmia-Associated Transcription Factor

Mlph – Melanophilin

MS – Mass Spectrometry

MTT – (3-4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MyoVa – Myosin-Va

M6PR – Mannose-6-phosphate Receptor

NCKX – K⁺-dependent Na⁺/Ca⁺ Exchanger

NHE – Na⁺/H⁺ Exchanger

NK – Natural Killer

NSF – N-ethylmaleimide Sensitive Factor

NT – Non-Targeting

NTD – N-terminal Domain

OA1 – Ocular Albinism 1

OCA – Oculocutaneous Albinism

OD – Optical Density

PCR – Polymerase Chain Reaction

PBS – Phosphate Buffered Saline

PBST – PBS-Tween

PFA – Paraformaldehyde

PI – Protease Inhibitor

PI(x)P – Phosphatidylinositol Phosphate

PI3K – Phosphatidylinositol-3-Kinase

PKC – Protein Kinase C

PKD – Polycystic Kidney Disease

PNS – Post-Nuclear Supernatant

PRA – Prenylated Rab Acceptor

PS – Phosphatidylserine

PVDF – Polyvinylidene difluoride

P100 – Pellet fraction

Rab – Ras-like in the brain

RabF – Rab Family

RabSF – Rab SubFamily

Rab6IP – Rab6 Interacting Protein 1

Ran – Ras-like in the nucleus

RANKL – Receptor Activator of NF- κ B Ligand

RBD – Rab Binding Domain

RBP – Rab Binding Platform

REP – Rab Escort Protein

RGGT – Rab Geranyl-Geranyl Transferase II

Rho – Ras homologous

ROS – Reactive Oxygen Species

RPE – Retinal Pigment Epithelium

RPMI – Roswell Park Memorial Institute

RPT – repeat

RT – Room Temperature

R3G – Rab3GEP

SCR – Sequence Conserved Region

SCF – Stem Cell Factor

SDS-PAGE – Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SEM – Standard Error of the Mean

SHD – Slp Homology Domain

Slac – Slp homologue lacking C2 domains

Slp – Synaptotagmin-like protein

SNAP – Soluble NSF Attachment Protein

SNARE – SNAP Receptor

S100 – Soluble fraction

TAE – Tris-Acetate EDTA

TAP – Tandem Affinity Purification

TBC – Tre2/Bub2/Cdc16

TCL – Total Cell Lysate

TEMED - N,N,N,N'-Tetramethylethylenediamine

TEV – Tobacco Etch Virus

TGN – Trans Golgi network

TNFR – Tumour Necrosis Factor Receptor

TM – Transmembrane

Tyr – Tyrosinase

TYRP1 – Tyrosinase-Related Protein

UV – Ultraviolet

VARP – Vsp-9 Ankryn-Repeat Protein

VAT1 – Vesicle Amine Transporter 1

VPS – Vacuolar Protein Sorting

VWF – Von Willebrand Factor

WPB – Weibel-Palade Bodies

Yip – Ypt Interacting Protein

1 Introduction

1.1 The Ras Superfamily of Small GTPases

Proteins of the Ras superfamily are small monomeric GTPases that regulate various signalling pathways and impose their regulation through their ability to cycle between the active GTP-bound and inactive GDP-bound states [1].

The Ras GTPase superfamily can be divided into 5 families: Ras, Rho, Rab, Arf and Ran [2]. The Ras GTPase subfamily regulates signalling cascades controlling gene expression, cell proliferation, differentiation and survival. The family has been widely studied due to its involvement in oncogenesis [3]. The Rho GTPase (Ras homologous) proteins are key regulators of actin reorganisation in response to stimuli, therefore regulating cell shape and motility. In addition they regulate cell cycle progression and gene expression [4]. The Rab GTPase (Ras-like in the brain) proteins are the largest family of Ras GTPases and are regulators of intracellular membrane trafficking [5]. The Ran GTPase (Ras-like in the nucleus) proteins are involved in nucleo-cytoplasmic transport of RNA and proteins [6]. The Arf GTPase (ADP-ribosylation factor) proteins regulate vesicular transport, similar to the Rab GTPase proteins [7].

1.1.1 Structure

All Ras GTPases have a GTPase domain consisting of 5 regions (G1-G5) conserved throughout all GTP-binding proteins and comprised of 6 β -sheet strands flanked by five α -helices [1]. The G1 motif (GXXXXGKS/T) is crucial for nucleotide binding, with the conserved Lys residue interacting with the α - and β -phosphates of GTP and, due to its loop like structure, it is also known as the P-loop. The G2 region (also known as the Switch I domain) includes a loop that changes conformation following GTP binding and re-orientates a conserved Thr residue

crucial for coordination of Mg^{2+} . The G3 motif (DXXG) is important for Mg^{2+} binding via a water molecule (Asp) and formation of hydrogen bonds with the γ -phosphate (Gly). The conformation of G3, also known as the Switch II domain, alters dramatically depending on the bound nucleotide. The G4 motif (N/TKXD) forms hydrogen bonds with the GTP guanine ring (Asp) and the Asn and Lys residues are important for stabilising the GTP binding domain. The G5 motif interacts indirectly with GTP by stabilising the Asn and Asp residues in G4. The sequence of G5 varies, however, between the GTPase subfamilies. Hydrolysis of GTP and dissociation of the γ -phosphate releases the Switch domains into a relaxed conformation. The conformational changes in the Switch domains regulate effector binding to the active GTP-bound protein.

1.1.2 Lipid Modification

The majority of small GTPases are post-translationally modified by the addition of lipid moieties to their C-terminus. This modification is essential for their membrane association and subsequent regulatory activity. The Ras and Rho families have a C-terminal-CAAX motif (C= Cysteine, A=aliphatic residue and X= any residue) [8]. The CAAX motif is recognised by farnesyltransferase and geranylgeranyltransferase I, which add farnesyl or geranylgeranyl isoprenoid groups to the cysteine residues, respectively in a process termed prenylation [9]. Rab GTPases have either CC, CXC, CCX, CCXX, CAAX or CCXXXX motifs at the C-terminus, which are modified by Rab geranylgeranyltransferase II (RGGT) with the assistance of Rab escort protein (REP) [10]. All Arf proteins are N-terminally modified with a myristol group [11]. The Rho and Rab GTPases can be removed from the membrane by association with guanine-nucleotide dissociation inhibitors (GDIs), which sequester the prenyl groups and allow solubilisation of the proteins [12].

1.1.3 GDP/GTP Cycle

Small GTPases have high binding affinity for GDP and GTP and also display intrinsic GTPase activity. However, the GDP/GTP cycle is controlled by two sets of regulatory proteins, guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), which exchange GDP for GTP and accelerate the intrinsic GTPase activity of the protein, respectively [13]. Small GTPases perform their regulatory function by acting as molecular switches, cycling between the inactive GDP-bound state, and the active GTP-bound state. When in the GTP-bound state, effectors are recruited to initiate downstream signalling, later the GTPase is inactivated by GAP and some are removed from the membrane by GDI.

1.2 Rab GTPases

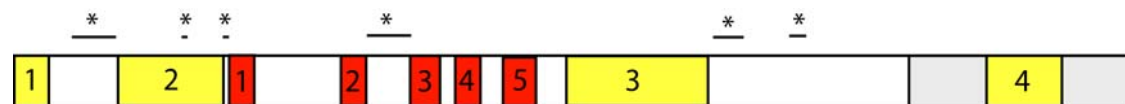
Rab GTPases are the largest family of the Ras GTPase Superfamily, with over 60 encoded in the mammalian genome [14]. Rab GTPases are involved in regulating various stages of membrane trafficking (Figure 1-4) and are exquisitely localised to specific intracellular membranes where they recruit a cohort of effectors to carry out their function.

1.2.1 Rab GTPase Structure

Rab GTPases contain a GTPase domain (section 1.1.1.1), a C-terminal hypervariable domain and a C-terminal prenylation motif that normally contains two Cys residues available for geranylgeranylation; a crucial modification allowing membrane association of Rab GTPases. The C-terminal hypervariable domain is between 35-40 amino acids and displays the greatest divergence between the Rab GTPases. Its involvement in Rab GTPase targeting has been proposed [15], possibly through effector binding [16] but this hypothesis has been questioned [17]. This is discussed in more detail later (Section 1.2.6.1).

Additionally, with the aim of identifying Rab GTPase specific regions, Pereira-Leal and Seabra performed a bioinformatic analysis of sequence information available on protein databases for mammalian Rab GTPases and out-group control GTPases [18]. They identified five conserved motifs, the Rab Family (RabF) motifs 1-5. RabF1 (IGVDF) is in the Switch I domain and RabF2 (KLQIW), RabF3 (RFRSIT), RabF4 (YYRGA) and RabF5 (LVYDIT) motifs are in the Switch II domain (Figure 1-1). Using the RabF motifs as markers of Rab GTPases enabled the identification of a number of new family members. Rab-SubFamily (RabSF1-4) motifs (Figure 1-1), which are conserved within Rab subfamilies, were then identified by comparing previously observed areas of high amino acid conservation [19] or motifs designated for Rab effector binding [20]. Interestingly, the RabSF4 motif is located in the C-terminal hypervariable domain.

The 3D structures of a number of Rab GTPases have been solved whilst in association with nucleotides [21-24], GDI/REP [25, 26] or effectors [22, 27] enabling some general structural features to be elucidated. As mentioned previously, alterations in the Switch domains are the major structural determinant between the inactive and active Rab GTPases. Mapping of the RabSF motifs onto the structure of Rab3 demonstrated two subfamily-specific surfaces (Figure 1-1). One composed of RabSF1, RabSF3 and RabSF4 motifs which forms the interacting surface for its effector, Rabphilin3a [20], whilst the SF2 motif is on the opposite face of the Rab protein, near the Switch I domain. In the structure of Rab27a complexed with the Rab27-binding domain of Slp2-a, it was demonstrated that the RabF1, RabF2, RabF3, RabSF1 and RabSF4 motifs are important for Rab27a binding to the Rab27-binding domain of Slp2-a [27].



- Rab Family Motif
- Rab SubFamily Motif
- C-Terminal Hypervariable Domain

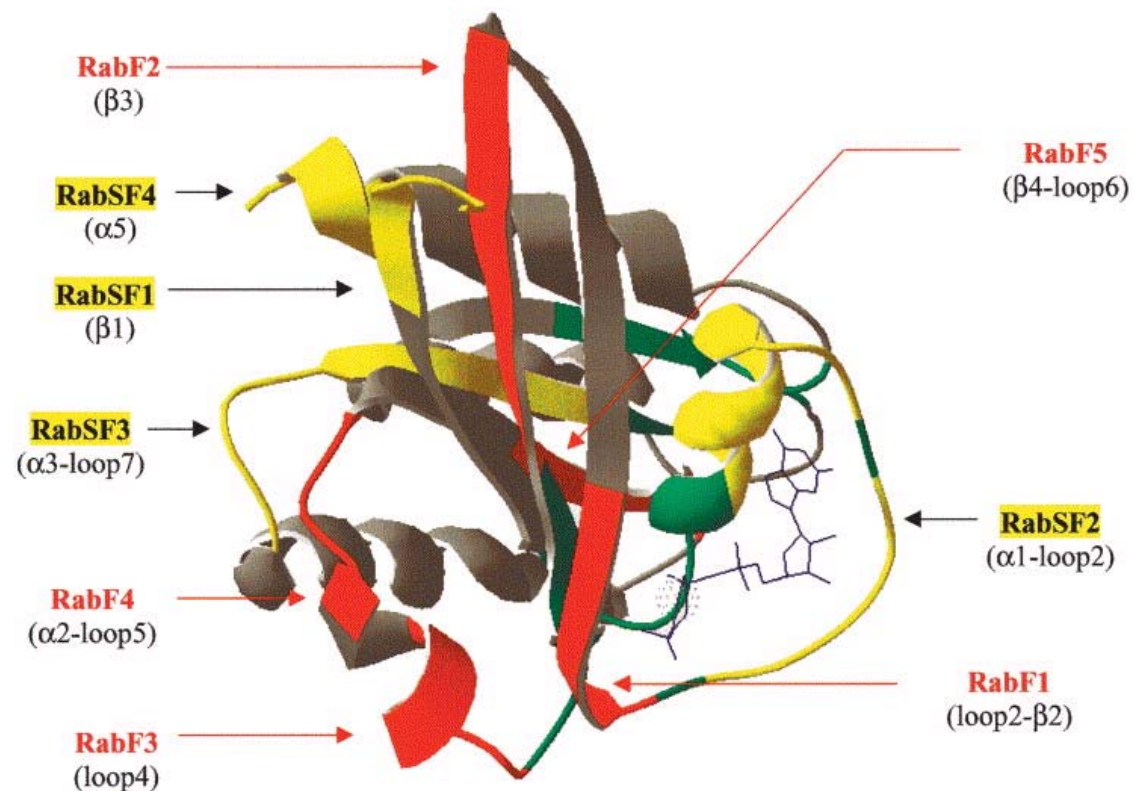


Figure 1-1 Structure of Rab GTPase.

Diagram of the conserved motifs in Rab GTPases and cartoon of Rab3a-GTP 3D structure. RabF motifs are in red, RabSF motifs are in yellow, conserved nucleotide binding residues are indicated by an asterisk (*) or in green. The nucleotide and Mg^{2+} are in blue. Taken, with permission, from Pereira-Leal and Seabra, Journal of Molecular Biology (2000).

1.2.2 Rab GTPase Cycle Model

Rab GTPases achieve stringent regulation of membrane trafficking by cycling between the inactive GDP-bound state, and the active GTP-bound state, normally coinciding with a move from the cytosol to association with membranes respectively (Figure 1-2). Membrane association of Rab GTPases requires C-terminal prenylation by RGGT following binding of the nascent Rab GTPase to REP. REP then transports the prenylated Rab GTPase to its specific membrane (Figure 1-2b). The mechanism by which the precise Rab GTPase targeting to intracellular membranes is achieved is yet to be elucidated, although a number of mechanisms have been proposed, which are described in detail later (Section 1.1.6) but the process could involve a targeting factor (TF). Activation of Rab GTPase requires GTP-loading by RabGEF to enable recruitment of effectors (E1-3) to initiate downstream biological functions (Figure 1-2c). Rab GTPase is inactivated when the bound GTP is hydrolysed to GDP, mediated by RabGAP (Figure 1-2d). The GDP-bound Rab GTPase is removed from the membrane by RabGDI and sequestered in the cytosol (Figure 1-2e). Re-delivery of Rab GTPase to a membrane requires displacement of RabGDI and this is proposed to be mediated by a GDI-displacement factor (GDF) (Figure 1-2b).

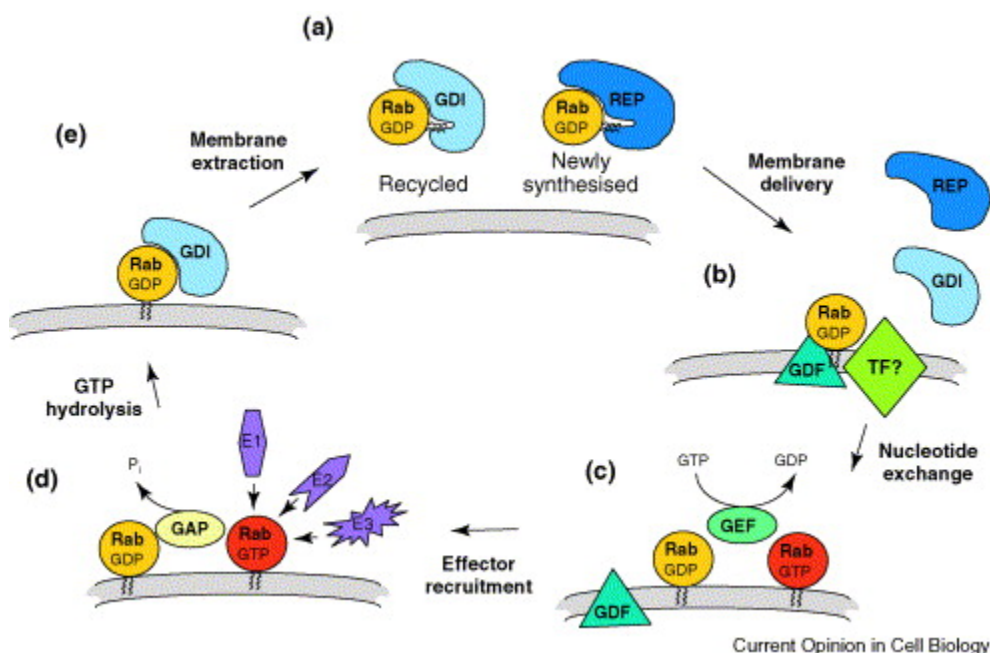


Figure 1-2 Schematic Representation of the Rab Cycle.

(a) GDP-bound Rab proteins form a cytosolic complex with RabGDI or REP. (b) Membrane delivery and RabGDI displacement are mediated by a GDF, probably aided by unidentified targeting factors (TF), followed by (c) Rab activation through GEF-catalysed nucleotide exchange. (d) GTP-bound Rab recruits effector molecules to the membrane. (e) GAP-mediated GTP hydrolysis returns the Rab to its inactive state, resulting in re-extraction from the membrane by GDI. Taken, with permission, from Seabra and Wasmeier *Current Opinion in Cell Biology* (2004).

1.2.3 Rab GTPase Regulation

The Rab GTPase cycle of membrane association and nucleotide binding is regulated by specific Rab GTPase regulators.

1.2.3.1 REP:RGGT and RabGDI

RGGT is a heterodimer belonging to the family of protein prenyltransferases [28]. In the classical pathway, nascent Rab GTPases are bound by REP [29] (in mammals there are two related REP proteins, REP1 (CHM) and REP2 (CHML) [30, 31]) and then recruit RGGT. RGGT then transfers two isoprenoid groups to Rab GTPase whilst still associated with REP [32, 33]. Following dissociation of RGGT, REP then escorts Rab GTPase to the membrane [34]. Alternatively, it was observed that formation of the REP:RGGT complex is stimulated by

prenyl binding to RGGT [35, 36] and that this can associate with Rab GTPases, albeit with a lower affinity. It is therefore proposed that the pathway utilised *in vivo* is dependent on component concentrations [10].

After the initial delivery of Rab GTPase to the membrane, REP is unable to remove it once it is GTP loaded. After GTP hydrolysis, the REP-related protein GDI, which can bind mono-prenylated and bi-prenylated Rab GTPases, is required to extract Rab GTPase from membranes. GDI extracts GDP-bound Rab GTPase from membranes [37-39] and sequesters the prenyl-groups, thereby maintaining a cytosolic pool of inactive, prenylated Rab GTPases.

REP and RabGDI share five sequence conserved regions, SCRs (1A, 1B, 2, 3A and 3B). The crystal structure of bovine GDI α highlighted that the SCRs form two domains [40, 41]. Domain I (SCRs 1A, 1B and 3B) contains the Rab-binding platform (RBP) and Rab C-terminus binding region (CBR) [25, 41, 42] and Domain II (SCR 2 and 3A) contains the mobile effector loop (MEL) and is important for membrane association and Rab GTPase retrieval [40] (Figure 1-3).

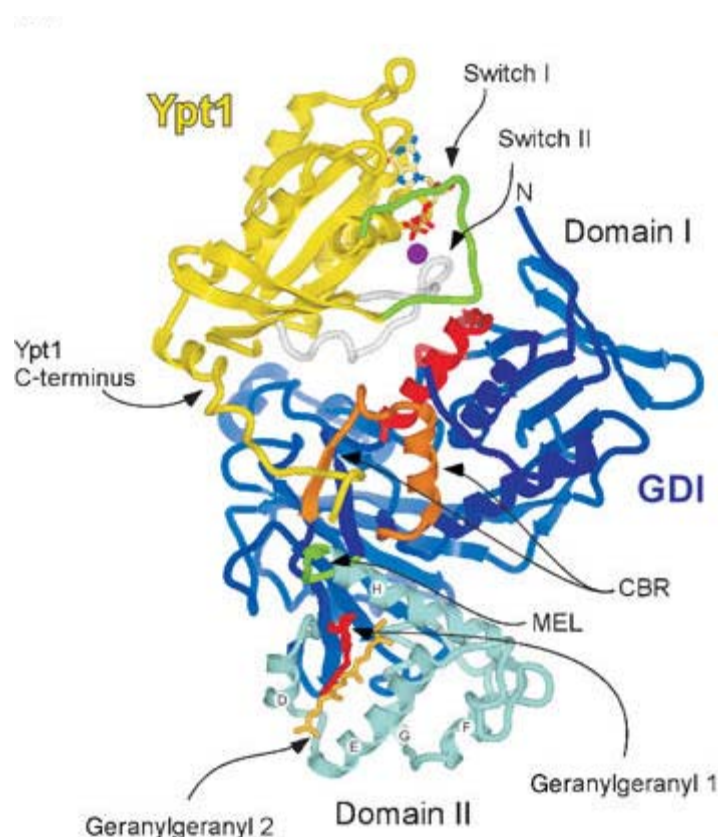


Figure 1-3 Structure of the Doubly Prenylated Ypt1:GDI Complex.

Ribbon representation of GDI (blue) bound to Ypt1 (yellow). Domain I (dark blue), domain II (light blue), the Rab-binding platform (RBP) (red), the C-terminus-binding region (CBR, gold) and the mobile effector loop (MEL, green) of GDI are highlighted. The Switch I and II regions of Ypt1 are highlighted in green and grey, respectively. The helices composing domain II of GDI are marked by letters. The isoprenoid moieties 1 (red) and 2 (dark yellow) are displayed in ball-and-stick representation. The GDP (atomic colours) and Mg^{2+} (magenta) ion are shown in the nucleotide-binding pocket in ball-and-stick and space filling representations, respectively. This was prepared using ICM (Molsoft LLC). Taken, with permission, from Pylypenko O. et al (2006) EMBO Journal.

REP and RabGDI are functionally similar in that they only bind GDP-bound Rab GTPases.

Structural analysis demonstrates that there is very little flexibility in the Switch domains of Rab GTPases when bound to REP/GDI. This ensures REP/GDI maintain Rab GTPases in the GDP-bound state [25, 26].

The interaction of Rab GTPases with REP/GDI involves an initial recognition of the Switch domains and the Rab C-terminus by the RBP and CBR within domain I of REP/GDI [25, 26]. The prenyl-groups then insert into a hydrophobic lipid-binding pocket in Domain II of REP/GDI. The lipid-binding pocket in REP is smaller resulting in REP isoforms preferentially binding to un-prenylated and monoprenylated Rab GTPases, emphasizing their function in facilitating nascent Rab GTPase prenylation by RGGT. Structural analysis proposed that the second prenyl-group displaces the first from the REP lipid-binding pocket. The C-terminus of REP also functions as a lid to the hydrophobic pocket, which is closed when it is in complex with RGGT [43]. Moreover, RabGDI binds poorly to unprenylated Rab GTPases [37, 44] but structural analysis demonstrates its ability to bind bi-prenylated Rab GTPases [37], confirming its role in extraction of fully prenylated Rab GTPases.

The exact mechanism of Rab GTPase extraction from membranes by RabGDI has not been elucidated. Furthermore, the very high affinity of RabGDI for prenylated Rab GTPases indicates that another factor must facilitate RabGDI displacement and return of Rab GTPases to the membrane. This has led to the proposal that GDFs assist the correct membrane targeting of RabGDI complexed Rab GTPases [38, 45], this is discussed later (See Section 1.2.6.3).

1.2.3.2 RabGEF

Once associated with membranes, Rab GTPases require exchange of GDP for GTP resulting in active Rab GTPase that can then interact with a cohort of effectors and initiate downstream biological functions. Although GDP-dissociation can occur intrinsically, this process is relatively slow and in cells is accelerated by RabGEFs (Table 1-1) [46]. The binding of RabGEF to Rab GTPase weakens nucleotide binding [46] as the RabGEF alters the conformation of the P-loop and the Switch domains, ultimately occluding the Mg^{2+} -binding site. With no

identified conserved domains, the precise mechanism by which it achieves this varies between RabGEFs, either binding the Switch I domain [47] or stabilising the P-loop [48].

The Vps9-domain proteins form a large family of RabGEFs thought to be specific for the Rab5 subfamily [49-52]. The Vps-9 domain proteins stabilise the P-loop by contributing an Asp-finger, interacting with a conserved Lys residue [48], similar to the mechanism observed in the Arf Sec7 domain GEF involving a Glu-finger [53]. VARP (Vps-9 ankryn-repeat protein) has been shown to display GEF activity for Rab21 [54] in addition to binding to Rab32/38, but not through the Vps-9 domain [55].

DENN (differentially expressed normal versus neoplastic) domain proteins are another family of proteins with RabGEF activity. The DENN domain is tripartite with the central DENN domain flanked upstream by uDENN and downstream by dDENN domains. These are separated by linker regions of varying lengths [56]. The first evidence that they act as RabGEFs came when it was found that DENN/MADD (MAPK-activating death domain) had GEF activity towards Rab3 and was therefore renamed Rab3GEP (R3G) [57]. However, the DENN domain was not required for GEF activity [58]. R3G has also been identified to have GEF activity towards Rab27a in mammalian melanocytes [59] and the *C. elegans* ortholog AEX-3 has GEF activity towards both Rab3 and Rab27 *C. elegans* orthologs [60]. Corroboration that DENN domain containing proteins (DENNDs) have RabGEF activity came when it was found that the DENND protein, *connecdenn*, preferentially binds to GDP-bound Rab35 [61]. Further investigation then established that *connecdenn*-1, -2 and -3 (entire DENND1 family) have GEF activity towards Rab35 and were more efficient exchange factors for Rab5 than Rabex-5 [62, 63]. More recently, a family wide characterisation of the RabGEF activity of the DENND families has established which Rab GTPases they are functional for [64] (Table 1-1). The importance of the DENN domain for RabGEF activity is still uncertain.

Rab6 interacting protein 1 (Rab6IP) shows DENN domain independent Rab6 binding, but interacts with Rab11-GTP via the DENN domain [65].

There is emerging evidence for the role of a RabGEF cascade in Rab recruitment. The best characterised is the Ypt32/Sec2p/Sec4p cascade in yeast [66]. The yeast RabGTPase Ypt32 recruits Sec2p to secretory vesicles where it then acts as the GEF for Sec4p. Further upstream it has been proposed that a GEF for Ypt32 is recruited by the upstream Rab GTPase Ypt1 [67]. Similar cascades have been proposed involving Rab5/classC VPS:HOPS complex/Rab7 [68] and Rab11/Rabin8/Rab8 [69].

RabGTPase	RabGEF
Rab1	mTrs130 [70]
Rab3	Rab3GEP [57, 58], GRAB [71]
Rab5	Rabex5 [49], Rin 1 [72], Rin 2 [73], Rin 3 [74], Alsln [75], Varp [54], RINL [76], MTMR5 [64]
Rab8	Rabin3 [77], Rabin 8 [77]
Rab9	ST5 [64]
Rab10	IrlB and Dennd4C [64]
Rab12	Dennd3 [64]
Rab13	Connecdenn 3 [64]
Rab18	Dennd3 [64]
Rab21	Varp [54], Rabex5 [48]
Rab22	Rabex5 [48]
Rab27	Rab3GEP [59]
Rab28	MTMR5 and MTMR13 [64]
Rab31	Rin3 [78]
Rab34	ST5 [64]
Rab35	Connecdenn 1/2/3 [64]
Rab39	Rab6IP1 and Rab6IP1-like [64]

Table 1-1 Mammalian RabGEFs.

The table highlights the number of mammalian RabGTPase for which RabGEFs have been identified.

1.2.3.3 RabGAP

Rab GTPases have intrinsic GTPase activity, but this is greatly stimulated by the association of a RabGAP. A number of RabGAPs have been identified, some proposed to have activity on a variety of Rab GTPases *in vitro* [79-84]. Unlike the RabGEFs, RabGAPs contain a

conserved domain, the TBC (Tre2/Bub2/Cdc16) domain, with two conserved sequences, the IxxDxxR and YxQ motifs, which confer the GAP activity. The IxxDxxR motif forms the Arg finger that interacts in the Rab GTP-binding pocket and stimulates GTP hydrolysis, similar to the well documented Ras and Rho GAPs [85, 86]. The Glu residue in the YxQ motif is proposed to coordinate $Mg^{2+}:H_2O$ [87] as would normally be expected of the Glu residue in the DxxGQ motif of RasGTPases [88]. The only non-TBC containing RabGAP identified is Rab3GAP [89].

In addition, it has been proposed that counter-current to the RabGEF cascade, RabGAP cascades would occur and it has been shown in yeast that Ypt32 can recruit Gyp1, a RabGAP for Ypt1, therefore promoting the Rab GTPase conversion [90]. Similarly, in *C. elegans* Rab7 can recruit TBC-2, a RabGAP for Rab5 [91].

1.2.4 Rab GTPases and Membrane Trafficking

Rab GTPases localise to intracellular membranes and regulate membrane trafficking. Different Rab GTPases regulate distinct stages in intracellular trafficking from vesicle formation at the donor membrane, trafficking along the cytoskeleton to vesicle fusion at the acceptor membrane.

1.2.4.1 Cargo Sorting

Formation of a vesicle requires the selective recruitment of cargo and the formation of a coat complex. Membrane curvature, lipid composition and Rab GTPases impart selectivity to coat protein recruitment (Figure 1-4a). Rab9 is involved in cargo-specific coat complex assembly for transport of the mannose-6-phosphate receptors (M6PRs) from late endosomes to the trans-Golgi network (TGN). Rab9 is localised on late endosomes and recruits its effector TIP47. The recruitment of TIP47 by Rab9 not only localises it to late

endosomes but increases the affinity of TIP47 for M6PR, ensuring M6PR sorting into late endosome sorting buds [92]. Similarly, the cargo-selective subcomplex of retromer, which transports M6PR and Wntless protein back to the TGN, has been shown to be recruited to late endosomes by Rab7 [93]. Rab5 has also been implicated in ligand sequestration into clathrin-coated pits [94].

1.2.4.2 Vesicle Un-coating

Following budding of vesicles from donor membranes, the coat is shed to allow subsequent vesicular transport and membrane fusion (Figure 1-4b). As noted above, Rab5 is involved in the formation of the clathrin coat, but it has also been shown to be involved in uncoating. AP2 (adaptor protein-2) association with membranes depends, in part, on phosphorylation of its $\mu 2$ subunit by AP2-associated kinase 1 (AAK1). However, binding of the Rab5 GEF RME-6 to AP2 displaces AAK1 therefore promoting un-coating [95]. In addition, PI(4,5)P₂ (Phosphatidylinositol(4,5)bisphosphate) stabilises AP2 on the membrane and the Rab5 dependant recruitment of PI3K (Phosphatidylinositol-3-Kinase) [96] and Phosphatidylinositol phosphatases [97] most likely disrupts this.

1.2.4.3 Vesicle Motility

Vesicular movement involves both microtubules and actin for long-range and short-range transport respectively and involves the recruitment of motor proteins to facilitate this movement (Figure 1-4c). It is well established that the myosin V family of actin motor proteins can associate with vesicles in a Rab GTPase-dependent manner [98]. The best characterised of these mechanisms is that of Rab27a. In skin melanocytes, Rab27a forms a tripartite complex with Myosin-Va (MyoVa) and the linker protein Melanophilin (Mlph), which is important for the peripheral actin association of melanosomes, lysosome related organelles [99]. Other examples include Rab8, Rab10 and Rab11, which can associate with MyoV isoforms and transport cargo to the plasma membrane [100-102].

Microtubule transport requires kinesins or dyneins for plus-end or minus-end directed transport, respectively. Rab6 and Rab14 have been shown to interact directly with kinesins KIF20A and KIF16B respectively [103, 104], whilst indirect interactions of Rab 3, 6, 9, 11 and 27 occur through adaptor proteins as summarised in [105]. Alternatively, Rab5 alters the lipid composition of early endosomes, as described above (Section 1.2.4.2), resulting in the recruitment of KIF16B through its interaction with PI(3)P [106]. Association of Rab GTPases with dyneins was first shown when Rab4 was found to directly interact with cytoplasmic dynein light intermediate chain 1 [107]. Rab6 isoforms also interact directly with cytoplasmic dynein subunits [108]. Alternatively, indirect associations with dynein motors has been shown for Rab7 and Rab11 via their effectors RILP and FIP3, respectively [109, 110].

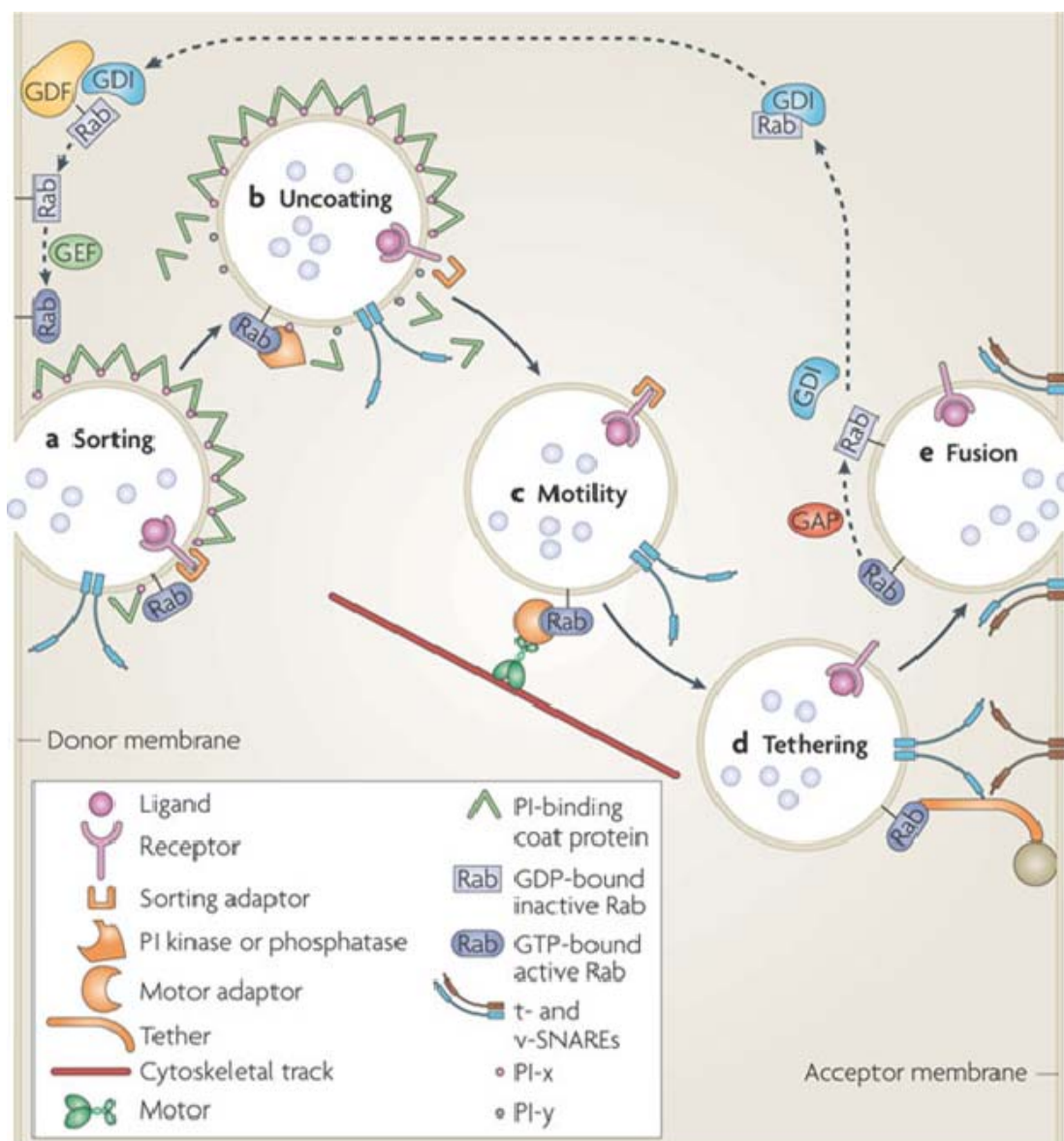


Figure 1-4 Rab GTPase Function in Vesicle Trafficking.

Rab GTPases can regulate distinct membrane trafficking steps. A) Rab GTPase can recruit sorting adaptors to sort receptors into budding vesicles. B) Rab GTPase-recruitment of PI-kinases and phosphatases can change the PI composition (PI-x to PI-y) of a vesicle causing uncoating. C) Rab GTPases can mediate transport along microtubules and actin filaments. D) Rab GTPases can mediate membrane tethering by recruitment of tethering factors which can also interact with and regulate SNARE complex formation and subsequent fusion. E) Following membrane fusion the Rab GTPase is inactivated by RabGAP, removed from the membrane by RabGDI and recycled. Subsequent re-insertion into the membrane is mediated by GDI-displacement factor (GDF) and Rab GTPase is GTP-loaded by RabGEF. Image taken, with permission, from Stenmark H. (2009) Nature Reviews.

1.2.4.4 Vesicle Tethering

In order for membranes to fuse, they need to be brought into proximity to the acceptor membrane to allow formation of a SNARE (SNAP Receptor (Soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein)) complex. Tethering complexes hold vesicles in close proximity to acceptor membranes but also denote selectivity to where the fusion event takes place (Figure 1-4d).

For vesicle fusion at the plasma membrane, vesicles need to be targeted to the correct region of the membrane and this can involve the exocyst tethering complex. In yeast, Sec15p is a component of the exocyst but is also an effector of the Rab GTPase Sec4p on vesicles, therefore active Sec4p is required for correct membrane targeting of secretory vesicles [111]. Rab5 is essential for homotypic fusion of early endosomes with its effectors EEA1 (early endosome antigen 1) and rabenosyn5 acting as two tethering factors [112, 113]. In particular, formation of EEA1 homodimers tethers two Rab5 vesicles together, promoting homo-fusion. Additionally, a number of Rab27a effectors have been implicated in vesicle docking in a variety of cell types. Slp2 (synaptotagmin-like protein 2) tethers vesicles through its interaction with phospholipids [114-116]. Whilst granuphilin tethers Rab27a-positive vesicles at the plasma membrane through its interaction with the SNARE component syntaxin-1 in a Munc18-1 dependent or independent manner [117-119]. Similarly, rabphilin3a, a Rab27a and Rab3a effector, binds SNAP25, another SNARE component [120].

1.2.4.5 Vesicle Fusion

Vesicle fusion occurs following the formation of a SNARE complex (Figure 1-4e). On Rab5 vesicles, Rabenosyn5 is in complex with VPS45 (vacuolar protein sorting-associated protein 45) which is a member of the Sec family of SNARE regulators [113, 121] and EEA1 also interacts with a number of SNARE proteins [122, 123]. Rab27a effectors also bind to SNARE

proteins (syntaxin-1 [119] and SNAP25 [120]) but how these interactions regulate fusion is still unclear.

1.2.5 Rab GTPases and Disease

Considering their important role in membrane trafficking, it is unsurprising that a number of diseases can be associated with Rab GTPases and their regulators or effectors.

There are a number of inherited Rab-related disorders which have contributed to the understanding of the physiological function of Rab GTPases. Mutations of MyoVa, Rab27a, or Mlph result in partial albinism known as Griscelli Syndrome (GS) Types I-III respectively. GSII is associated with immunodeficiency and this is related to the function of Rab27a and the exocytosis of cytotoxic granules from T cells and neutrophils [124]. The X-linked disease Choroideremia occurs due to mutations in the REP1 gene, resulting in under-prenylation of a subset of Rab GTPases in the retinal pigment epithelium (RPE) leading to progressive blindness due to degeneration of the RPE [125]. Hermansky-Pudlak syndrome (HPS) is characterised by albinism and platelet aggregation deficiency arising from defects in lysosome-related organelle biogenesis (melanosomes, platelet dense granules, lamellar bodies of type II alveolar epithelial cells and lytic granules from cytotoxic T-lymphocytes (CTLs)). One of the genes implicated in HPS is Rab38 [126, 127]. A gain of function mutation in Rab7 results in Charcot-Marie-Tooth disease type IIB characterised by neurodegeneration of peripheral neurons [128]. Alternatively, Rab7 defects are associated with HSAN (hereditary sensory and autonomic neuropathy) type I, characterised by loss of pain and temperature sensation [129]. Mutations in Rab3 GAP cause Warburg Micro and Martsolf syndromes, characterised by developmental abnormalities of the eye and central nervous system resulting in severe mental retardation and microgenitalia [130].

Both Rab5 and Rab11 have been implicated in Huntington's disease (HD) [131, 132], which occurs following a mutation in Huntingtin (Htt). Htt-associated protein 40 (HAP40) was

identified as a Rab5 effector, recruiting Htt to early endosomes. HAP40 up-regulation in HD patients is suggested to disrupt endosomal trafficking by displacing early endosomes from microtubules to actin [131]. Rab11 was found to be important for regulating levels of reactive oxygen species in HD transgenic mice models. Rab11 trafficks the glutamate/cysteine transporter EAAC1 to the plasma membrane where uptake of cysteine is crucial for synthesis of the intracellular antioxidant glutathione [132].

The trafficking of GLUT4 is regulated by a number of Rab GTPases [133] and impairment of its translocation to the plasma membrane following insulin stimulation is associated with Type II diabetes. So it is proposed that deregulation of Rab GTPases contributes towards the condition.

Pathogens have been shown to hijack Rab-dependent macropinocytosis pathways [134], halt phagosome maturation [135, 136] or express Rab regulatory proteins and as a result recruit Rabs to replicate intracellular compartments and evade lysosomal degradation [137].

A number of Rab GTPases are associated with cancer including Rab1 [138], Rab23 [139], Rab25 [140], Rab27b [141]. The aberrant regulation of receptor recycling and degradation results in deregulation of cell survival, proliferation and invasiveness, ultimately driving tumourigenesis.

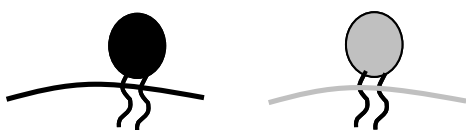
1.2.6 Rab GTPase Targeting

The ability of Rab GTPases to tightly regulate membrane traffic is in part achieved by the specificity with which they associate with distinct intracellular membranes. In most cases this membrane association is tightly linked with GTP loading and activation of Rab GTPase. This ensures strict temporal and spatial regulation of effector recruitment. The mechanism by which precise Rab GTPase targeting is achieved is yet to be elucidated. A number of mechanisms have been proposed (Figure 1-5) [66, 67].

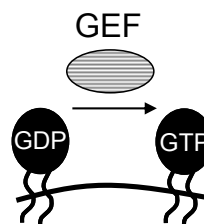
1.2.6.1 C-terminal Hypervariable Domain

The C-terminal hypervariable domain has long been proposed to contain the targeting information required to direct Rab GTPases to their correct membrane (Figure 1-5A). Exchange of the Rab5 C-terminal 35 residues with the corresponding residues of Rab7 resulted in targeting of the Rab5/7 hybrid to Rab7 positive intracellular compartments [15]. Similarly, the C-terminus of Rab5 was sufficient to target a Rab6/5 hybrid to early endosomes and plasma membrane [142]. Comparable results were also seen in yeast [143]. However experiments using chimeric Rab GTPases of different vesicular pathways gave conflicting results [17]. Chimeras of Rab1a, Rab2a, Rab5a, Rab7 and Rab27a were generated, whereby the C-terminal hypervariable domains were exchanged. It was found that the chimeric proteins retained their correct localisation, irrespective of their C-terminal hypervariable domain. Further studies revealed that the RabF and RabSF motifs conveyed the information required for targeting of Rab27a to melanosomes [17]. In agreement with these findings, Aivazian *et al* found that the Rab5 C-terminal domain was dispensable for early endosome localisation [16].

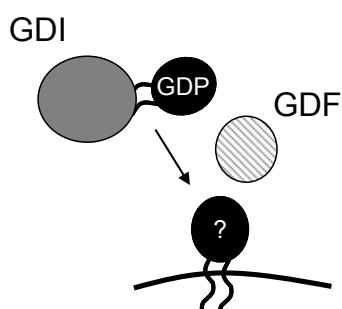
A. C-terminal Hypervariable Domain



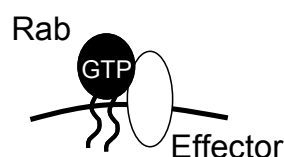
B. RabGEF



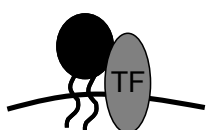
C. RabGDI



D. Rab Effector



E. Targeting Factor



F. Lipid Composition



Figure 1-5 Rab GTPase Targeting.

The mechanisms proposed for Rab GTPase targeting to specific membranes. A) The C-terminal domain encodes a signal sequence for specific membranes. B) RabGEF GTP-loads Rab GTPase and stabilises it on the membrane. C) GDI displaces RabGDI and inserts Rab GTPase into the membrane and may also display RabGEF activity. D) Effectors bind Rab GTPase and stabilise the GTP-loaded protein on membranes E) A putative targeting factor binds and recruits RabGTPases. F) The lipid composition/curvature of the membrane is sensed by RabGTPase and dictates its association.

1.2.6.2 RabGEF

Membrane association and GTP-loading are tightly linked and are thought to occur in quick succession [144]. It has therefore been proposed that RabGEF could aid in membrane targeting by GTP-loading Rab GTPase and stabilising it on the correct membrane as GTP-

bound Rab GTPase can not be retrieved by GDI (Figure 1-5B). In support of this, siRNA depletion of Varp (RabGEF for Rab21) disrupted Rab21 endosomal localisation in HeLa cells [54], similarly depletion of R3G resulted in mis-targeting of Rab27a in melanocytes [145]. However, a Rab27a mutant that undergoes efficient GDP/GTP exchange by R3G *in vitro* failed to localise correctly *in vivo* [145]. This indicates that RabGEF activity is necessary but not sufficient for Rab27a recruitment. Also, the RabGEFs identified to date are all cytosolic and therefore would themselves require targeting to membranes. This is proposed to be achieved by recruitment by upstream Rab GTPases resulting in a RabGEF cascade as detailed previously (see Section 1.1.4.2) [66-69]. In particular, the Rab GTPase Sec4p is recruited to secretory vesicles by the GEF Sec2p which itself is recruited by the upstream Rab GTPase Ypt32 [66]. Similarly, SAND1/Mon1 has been shown to not only displace Rabex5 (Rab5 GEF), but bind the HOPS complex (Rab7 GEF) therefore promoting early-to-late endosome transition by regulating the localisation of sequential RabGEFs [146].

1.2.6.3 GDI-Displacement Factor

It is proposed that RabGDI is responsible for the extraction of some Rab GTPases from membranes and their maintenance in the cytosol. The interaction between RabGDI and Rab GTPase is strong (Rab9 and GDI α Kd=23nM) [147], therefore it is proposed that a GDF is required to allow transfer of Rab GTPase back to membranes (Figure 1-5.C). Dirac-Svejstrup *et al.* first identified a GDF as a membrane component able to dissociate endosomal Rab GTPases from RabGDI [148]. Sivars *et al.* then investigated the involvement of Ypt-interacting proteins (Yips) (Prenylated Rab aceptors (PRAs)) as GDFs due to their ability to interact with prenylated Rab GTPases and RabGDI [149-151]. They showed that Yip3 (PRA-1) acted as a GDF for Rab5 , Rab7 and Rab9, but not for Rab1 or Rab2 [45]. Similarly, Yip1 was shown to regulate the membrane association of Rab6 [152]. Differential subcellular localisation of Yip/PRAs is proposed to contribute to the specific membrane targeting of Rab GTPases [153].

Whether GDFs require any RabGEF activity remains to be clarified. The GDF activity of Yip3 was found to be independent of any RabGEF activity [148]. In conjunction with data proposing that GDP-bound Rab can associate with membranes [144], this implies that GDF activity and membrane association precede GTP-loading. In contrast, in *L. pneumophila*, DrrA was able to displace RabGDI from Rab1 and Rab2, [137] and this was found to be dependent on its RabGEF activity [154]. Whether the combined RabGDF/GEF activity is specific for bacterial proteins remains a possibility. However, the exact mechanism employed by GDF and the involvement of any RabGEF activity remains unclear.

1.2.6.4 Effector Binding

The role of Rab GTPase effectors in membrane association was first proposed following the finding that depletion of TIP47, a Rab9 effector, destabilised Rab9 association with the late endosomal membrane [155]. Using chimeras of Rab1, Rab5 and Rab9, it was established that the Rab9 C-terminal domain was essential and sufficient for TIP47 to bind the GTP-loaded proteins. It was then found that over-expression of TIP47 re-localised Rab5/9 and Rab1/9 chimeras to late endosomes [16]. This led to the proposal that following delivery and GTP-loading of Rab GTPase, competitive interactions with downstream effectors stabilise Rab GTPase on the membrane (Figure 1-5D). However, recent evidence involving Rab27a in melanocytes showed that effectors were not important for Rab27a targeting. A Rab27a mutant, which failed to bind to any known Rab27a effectors was still correctly localised to melanosomes, therefore indicating that Rab27a effector binding is not necessary for Rab27a localisation in melanocytes [145].

1.2.6.5 Targeting Factor

Alternatively, it has been proposed that a putative targeting factor may be responsible for Rab GTPases targeting (Figure 1-5E). Rab GTPases could be recruited to specific membranes by interacting with different proteins, that are already associated with the target membrane.

Alternatively, targeting factors could be stringently targeted to the membrane by an unknown mechanism.

1.2.6.6 Lipid Composition

The lipid composition of intracellular compartments varies considerably and contributes to the overall properties and morphology of the membrane. In particular it plays an important role in regulating membrane deformation as occurs for membrane trafficking, i.e. vesicle budding and membrane fusion [156]. Recent data (M. Kirsten, unpublished data) has found that Rab5a and Rab1a show preferential association with synthetic lipid mixtures of higher interfacial curvature, i.e. they prefer to associate with small vesicles. This is in agreement with their biological role on early endosomes and ER-Golgi trafficking respectively. It is therefore hypothesised that the lipid composition of intracellular compartments may be sensed by RabGTPases and association only occurs with the right lipid composition and membrane curvature (Figure 1-5F).

The discrete localisation of each Rab GTPase conveys their ability to tightly regulate distinct steps of intracellular membrane trafficking. Elucidating the mechanism by which this specificity is achieved would be a significant step in unravelling the complexity of Rab GTPase regulation. The differing reports to date highlight the possibility that a number of factors may contribute towards Rab GTPase targeting. It may involve a combination of any of the afore-mentioned mechanisms, or each Rab GTPase could achieve its membrane localisation through a distinct mechanism.

1.2.7 Secretory Rab GTPases

The Rab27 and Rab3 subfamilies are specialised Rab GTPases for regulated secretion in a variety of cells [157-159]. The function of Rab27a is highlighted by the secretory defects observed in patients with GS-II, where Rab27a is mutated [160]. The GS-II defects (albinism, platelet aggregation defects and immunodeficiency as mentioned in Section 1.2.5) are mirrored in the corresponding *ashen* mouse model (Rab27a null) [161]. Defects in mast cell granule secretion are also observed in *ashen* mice (R. Singh, unpublished data), and Rab27b knockout (KO) and Rab27a/b double knock out (DKO) mice also display defective secretion of dense granules from platelets and mast cells. Similarly, neurons isolated from quadruple Rab3 KO mice display defective Ca^{2+} -triggered synaptic vesicle release [158, 162-164]. Supra-physiological levels of glucose also resulted in a reduced expression of Rab3 and Rab27a in pancreatic β -cells resulting in decreased granule exocytosis, therefore linking the Rab GTPases to Type-II diabetes [165].

Rab27 and Rab3 proteins are proposed to be required for the recruitment/docking of secretory vesicles at the plasma membrane [166, 167]. A decrease in docked vesicles is observed in *ashen* CTLs [168, 169] and pituitary cells from Rab27DKO mice [158] as well as at the neuromuscular junction in Rab3a KO mice [170]. They have also been suggested to have a role in vesicle maturation as it has also been observed that vesicles in both the exocrine pancreas and the parotid gland of Rab3d KO mice are larger [171]. Equally, there is a decrease in dense core granule number in Rab27b KO platelets [162] and a decrease in size but increase in granule density in *ashen* mast cells (R. Singh, unpublished data).

1.2.8 Rab27a

Rab27a is involved in the regulated secretion of lysosome-related organelles and secretory granules from a variety of cell types. As mentioned above (Section 1.2.5), mutations in Rab27a cause immunodeficiency and pigment defects in human GS-II [124] and lightened

coat colour of the *ashen* mouse [161]. Subsequent analysis revealed that Rab27a localised to melanosomes in melanocytes [172, 173] and lytic granules in CTLs [169]. In *ashen* melanocytes, melanosomes cluster in the perinuclear region of the cell and introduction of exogenous wild-type Rab27a restores the peripheral distribution of melanosomes [161, 172, 173]. In *ashen* CTLs, lytic granules are still correctly polarized, but are not secreted, suggesting a role for Rab27a in vesicle docking [168]. In natural killer (NK) cells, Rab27a is proposed to have distinct roles in granule transport at the cell periphery and in the cytosol [174], similar to what has been described for melanosome transport [175]. In endothelial cells, Rab27a is recruited to and regulates the peripheral distribution and secretion of Weibel-Palade Bodies (WPB) [176], ensuring it only occurs once the cargo, Von-Willeband Factor (VWF) is fully multimerised [177]. Rab27a is also involved in secretion of platelet dense granules [178], exosomes [179] and dense-core granules from endocrine cells [180].

The ability of Rab27a to regulate such a variety of secretory pathways in different cells is achieved through its interaction with a host of effector proteins. A total of eleven have been identified to date which interact with GTP-bound Rab27a (Figure 1-6); Rabphilin-3a (Exophilin1) [181, 182], Granuphilin-1 (Exophilin2/Slp4a) [183], Melanophilin (exophilin3/Slp homologue lacking C2 domain (Slac) 2-a) [184, 185], Slp2-a (Exophilin4) [186, 187], Slac2-b (Exophilin5) [185], Slp3 (Exophilin6) [186, 187], Slp1 (Exophilin7/JFC) [186, 188], MyRIP (Exophilin8/Slac2-c) [185], Slp5 (Exophilin9) [189], Noc2 [181, 190] and Munc13-4 [191, 192]. With the exception of Munc13-4 [193], Rab27a effectors have an N-terminal Rab-binding domain (RBD), which in some effectors is divided into subdomains, Slp homology domains (SHDs), separated by zinc-finger motifs. In addition, some have C-terminal C2-domains, consistent with a role in Ca²⁺ regulated membrane association [194]. Biochemical analysis of the Rab27a effectors has revealed they can be divided into low-affinity (Slp3a, Mlph, MyRIP) and high affinity (Slp1, Slp2-1, Granuphilin, Slp5, Slac2-b, Rabphilin and Noc2) effectors, perhaps suggesting a cascade of effectors may be utilised in some Rab27 regulated

pathways [195]. Additionally, the effectors can be subdivided according to their specificities for Rabs. Melanophilin, Slp2-a, Slac2-b and Slp3 interact exclusively with Rab27 whereas the others can interact with other Rabs, including Rab3 isoforms [185]. Finally, in addition to the effectors which bind GTP-bound Rab27, Coronin 3a has been identified to interact with GDP-bound Rab27a in pancreatic β -cells and to regulate endocytosis [196]. Although Rab27a and Rab27b share extensive homology, they have been shown to regulate the same secretory pathways through independent mechanisms by different spatio-temporal interaction with effectors [197].

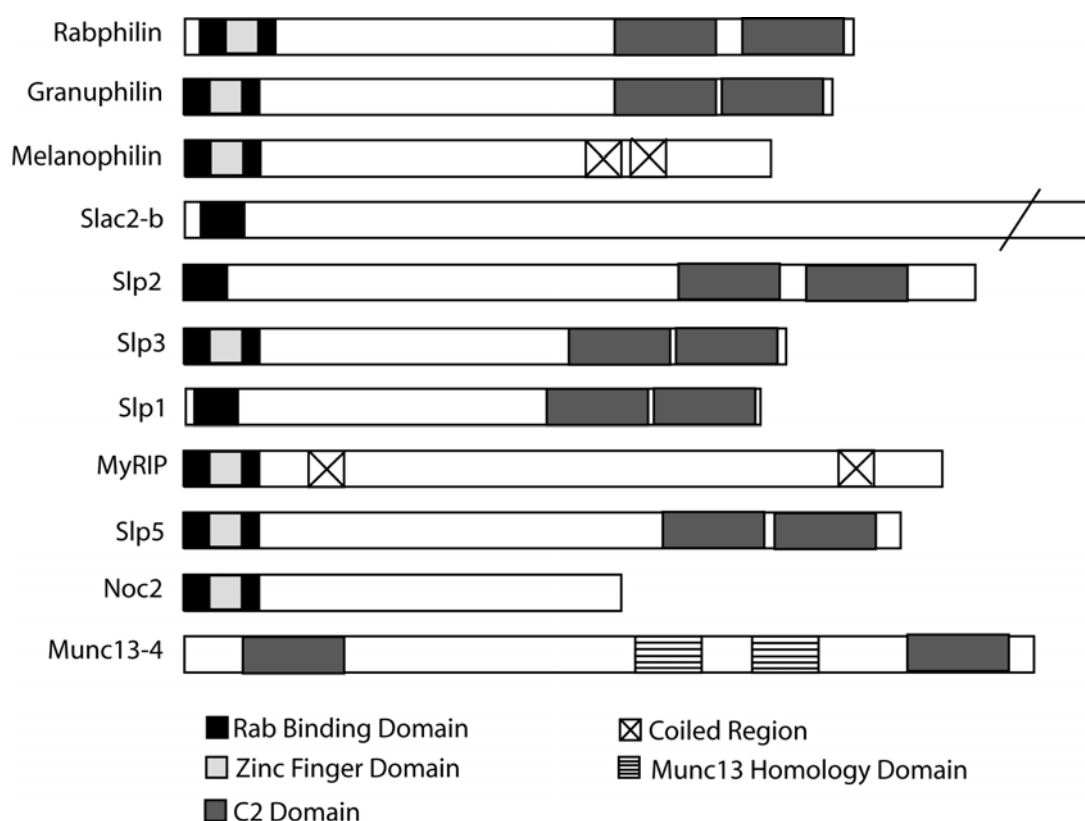


Figure 1-6 Rab27a Effectors.

The schematic depicts the domain structures conserved across the family of Rab27a effectors and an indication of comparative sizes. Adapted from Izumi T. (2007) Endocrine Journal.

1.2.8.1 Effectors

Rabphilin3A (Exophilin1) was initially identified as a Rab3a-interacting partner [182] and enhanced Rab3a regulated secretion from adrenal chromaffin cells [198]. However, analysis of the KO mouse revealed that it is not required for Rab3a function in neurotransmitter release [199]. Conversely, in Rab27a mutant *C. elegans*, Rabphilin3A was mislocalised [60] and subsequently it was found to bind Rab27a in vertebrates [181, 190]. Its interaction with Rab27a recruits it to dense core vesicles in neuroendocrine cells [190]. Rabphilin3A has a C-terminal phospholipid binding domain (C2A domain) which binds phosphatidylserine (PS) [200] and PI(4,5)P₂ [201] in a Ca²⁺ dependent manner. The C2B domain interacts with SNAP-25 and this interaction is thought to regulate the docking of dense-core vesicles [120] or vesicle re-priming [202]. The N-terminal domain interacts with α -actinin and stimulates actin bundle formation [203]. However, this interaction is negatively-regulated by GTP-bound Rab3a suggesting a role of the Rabphilin3A:GTP-Rab3a in actin filament reorganisation [203].

Granuphilin (Exophilin2/Slp4) is expressed in pancreatic β cells where it interacts with Rab27a [180] and the t-SNARE syntaxin-1a and tethers insulin granules at the plasma membrane [119]. However, this interaction with the closed form of syntaxin-1a prevents it forming a SNARE complex therefore preventing granule fusion [118]. Syntaxin-1a KO mice however do not phenocopy the docking defect observed in granuphilin KO mice, suggesting redundancy with syntaxin-2 and -3, all of which have been shown to interact with granuphilin [204]. In PC12 cells, granuphilin was found to bind GDP-bound Rab27a, preventing granule exocytosis [205]. In addition, granuphilin binds to Munc18-1, which also interacts with the closed syntaxin-1a [117]. The C2A domain shows Ca²⁺-independent binding to PS and PIP₂ [183] whilst the C2B domain only binds weakly to PIP₂ [115]. Granuphilin also complexes with Rab27b in parotid acinar cells [206], where it binds syntaxin-2/3 in a Munc18-2-dependent manner [207]. Additionally, granuphilin has also

been suggested to be involved in stimulation-dependent release of RANKL (receptor activator of $\text{NF-}\kappa\text{B}$ ligand) in osteoblasts [208].

Melanophilin (Exophilin3/Slac2-a) binds to Rab27a through the N-terminal RBD and to MyoVa via a medial-domain [99] and C-terminal coiled-coil region [209]. Mlph however lacks C2 domains [184] and does not interact with Rab3 isoforms [181]. Mutations in Mlph are responsible for the melanosome transport defects observed in *leaden* mice [184] and human GS-III [210], supporting its role as a linker protein between Rab27a and MyoVa for melanosome transport. Tracking of melanosomal motility in melanocytes revealed that bi-directional microtubule-dependent movement distributes the melanosomes to the periphery where the Rab27a:Mlph:MyoVa complex tethers it to the cortical actin [211]. Mlph association with the microtubule plus-end tracking protein EB1 is proposed to focus melanosome transfer from microtubules to the actin at the cell periphery [212].

Slp2-a (Exophilin4) was found to bind to Rab27a in B16-F1 melanoma cells and depletion in Melan-a melanocytes using siRNA resulted in dilution of peripheral melanosomes and a more rounded cell morphology [114]. However, no apparent coat colour defect is observed in the mutant mice [213]. Slp2-a was also shown to be expressed in pancreatic α -cells where its interaction with PS and PIP_2 through its C2A domain is important for the targeting of glucagon granules to the plasma membrane [115]. However, unlike Rabphilin3A and Granuphilin, this interaction is inhibited by physiological ranges of intracellular Ca^{2+} induced after secretory stimulus, therefore suggesting a possible swapping of Rab27a effector binding depending on Ca^{2+} concentration [115]. Slp2a has also been proposed to be involved in the docking of secretory lysosomes in CTLs [116] and mucus granules in gastric-surface mucous cells [213].

Slp1 (Exophilin7/JFC1) has the conserved N-terminal RBD and C-terminal C2 domains, which preferentially interact with PIP_3 [188]. Its interaction with Rab27a is important for the

secretion of azurophilic granules from granulocytes [214], docking of secretory lysosomes in CTLs [116], androgen-dependent secretion of prostate-specific markers from prostate epithelia [215] and dense granule secretion in platelets [216]. In neurons, Slp1 has been found to bind the neurotrophin receptor TrkB in a Rab27b dependent manner and CRMP-2 (collapsing response mediator protein-2) connects Slp1 to kinesin-1, therefore it is involved in regulating the anterograde transport of TrkB [217]. It has also been shown to be a substrate for phosphorylation by Akt but this phosphorylation is inhibited when bound to Rab27a [218].

MyRIP (Exophilin8/Slac2-c) bridges Rab27a with MyoVIIa for the transport of melanosomes in RPE cells [219] and Rab27a with MyoVa for the transport of secretory granules in chromaffin cells [220], amylase secretion in parotid acinar cells [206] and WPB in endothelial cells [221]. It has also been shown to regulate insulin secretion from pancreatic β cells but not through association with MyoVa [222]. MyRIP interacts with MyoVa and MyoVII via distinct, non-overlapping domains [223]. Additionally, MyRIP has been found to bind actin, directly linking secretory granules to the actin cortex and controlling their secretion [220]. In contrast to the inhibitory role of granuphilin, MyRIP tethers insulin granules to the cortical actin supplying them for quick release following stimulation [224]. Similarly, in RPE and endothelial cells the tripartite complex is thought to tether melanosomes and WPB onto actin filaments, respectively [177, 225], comparable to the mechanism observed with Mlph:MyoVa in melanocytes. Rab27b has been shown to form complexes with MyRIP in parotid acinar cells [206].

Noc2 shows 40.7% homology to Rabphilin3a but lacks the C2 domains [226]. It binds both Rab27a and Rab3 [181, 190] and it is proposed to regulate exocytosis from endocrine cells when in association with Rab3 [227]. However, its association with Rab27a is involved in secretion of insulin from pancreatic β -cells [228] and dense core vesicles in neuroendocrine

cells [190] therefore suggesting a generic role in exocytotic pathways in exocrine and endocrine cells [229].

Despite its lack of a RBD, Munc13-4 has been shown to bind to Rab27a/b via the region between its C2 domain and Munc 13 Homology Domains [191]. *In vitro* Munc13-4 has been shown to regulate secretion of lysosomes in mast cells [163, 192], an array of secretory organelles in neutrophils [230], basophilic secretory granules in basophils [231] and dense core granules in platelets [191]. Munc13-4 has also been suggested to be involved in stimulation-dependent release of RANKL in osteoblasts [208]. Similar to other Rab27a effectors, Munc13-4 is suggested to tether secretory granules to the plasma membrane [232]. The mutant mouse model *Jinx* [233] and mutations in human Munc13-4 (causing familial hemophagocytic lymphohistiocytosis subtye-3 (FHL3)) are characterised by defective cytotoxicity, attributed to the failed exocytosis of cytotoxic granules despite docking at the plasma membrane [234].

Slp3 (Exophilin3) C2A domain has Ca^{2+} -dependent phospholipid-binding activity [235] and in contrast to granuphilin, Slp3:Rab27a promotes neuroendocrine secretion [236]. Slp5 (Exophilin9) interacts with Rab3a and Rab6a as well as Rab27a [189] and it is expressed in some pancreatic β cell lines [222] as well as in liver [189]. Similar to Munc13-4 and Granuphilin, Slp5 has been suggested to be involved in stimulation-dependent release of RANKL in osteoblasts [208]. Slac2-b (Exophilin 5) has been implicated in Rab27b-regulated exosome secretion [179].

1.2.8.2 Rab27a Regulators

Rab3GEP (R3G) has been characterised as the non-redundant GEF for Rab27a in melanocytes [59]. It was initially identified as a RabGEF for all Rab3 isoforms [58] and the *C. elegans* ortholog AEX-3 showed activity towards Rab3 [237] as well as Rab27a [60]. R3G is able to catalyse nucleotide exchange on Rab27a, Rab27b and Rab3 *in vitro* and depletion in

melanocytes results in a decrease in GTP-loaded Rab27a [59]. As a consequence, R3G depletion results in clustering of melanosomes in the perinuclear region, comparable to the phenotypes seen with depletion of Rab27a, Mlph or MyoVa. Further investigation showed that R3G depletion resulted in mis-targeting of Rab27a [145]. R3G is a large protein (1567 residues) with an N-terminal DENN domain and a C-terminal Death Domain (DD) [238] (Figure 1-7). A conserved fold has been suggested downstream of the DENN domain as database searches identified sequence homology (17% or less) in yeast, bacterial and fungal proteins. Secondary structure predictions have also suggested an α -helical domain surrounding the DD. R3G homologues have high level of sequence conservation across species indicating that the sequence is functionally critical.

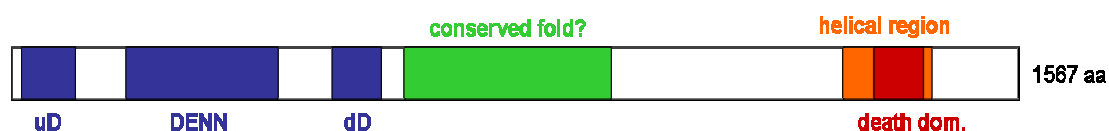


Figure 1-7 Rab3GEP Structure.

The scheme depicts the conserved domains in rat Rab3GEP. The DENN domain, the upstream DENN (uDENN) and the downstream DENN (dDENN) are in blue, the putative conserved fold is in green, the proposed helical region is in orange and the Death Domain is in red.

As described previously (Section 1.1.4.2), the DENND proteins have been implicated in a number of Rab-mediated processes [56]. DDs are protein-protein interacting motifs present in apoptosis-related signalling pathways. The DD links R3G to tumour necrosis factor 1 receptor [238], suggesting a R3G function distinct from Rab GTPase regulation [239], though the DD appears critical for its GEF activity towards Rab3 [58]. The domains crucial for R3G GEF activity on Rab27a still remain to be elucidated.

Rab27a is a poor substrate for RabGDI-mediated extraction (M. Kirsten, unpublished data) suggesting that Rab27a does not readily cycle between the membrane and the cytosol. FRAP (fluorescence recovery after photo-bleaching) analysis of GFP-Rab27a in melanocytes

also suggested that Rab27a does not readily recycle on and off melanosomal membranes (A. Hume, unpublished data).

Over-expression of EPI64, a TBC-domain protein was shown to cause perinuclear clustering of melanosomes in melanocytes and displayed specific GAP activity towards Rab27a *in vitro*, therefore suggesting it is the RabGAP for Rab27a in melanocytes [84]. It has similarly been found to regulate Rab27a GTPase activity in parotid acinar cells [240].

1.2.8.3 Rab27a and Melanosomes

Melanosomes are lysosome-related organelles synthesised in mammalian epidermal melanocytes, choroidal melanocytes and RPE, and in melanophores of lower vertebrates. They are the pigment storage granules whose transfer to surrounding tissues provides colour and photoprotection. Melanosomes are large organelles (0.5µm) allowing easy identification using phase contrast microscopy because of their dense melanin core. Epidermal melanocytes transfer melanosomes to their neighbouring keratinocytes, resulting in skin and hair pigmentation (Figure 1-8). Exposure to ultraviolet (UV) light releases DNA photoproducts that stimulate the release of α -melanocyte-stimulating hormone (α -MSH). α -MSH then activates melanocortin 1 receptor (MC1R) in the melanocyte plasma membrane resulting in cAMP-dependent signalling and stimulation of microphthalmia-associated transcription factor (MITF) [241]. MITF stimulation results in the expression of a multitude of proteins including those involved in melanosome biogenesis (tyrosinase (Tyr), tyrosinase-related protein 1 (TYRP1) and Pmel17) and melanosome transport (Rab27a) [242, 243]. There are four distinct morphological stages of melanosome development [244]. Stage I are non-pigmented vacuoles derived from the endosomal system. Pmel17 is present in Stage I melanosomes on intraluminal vesicles giving rise to the intraluminal fibrils and characteristic striations of the ellipsoidal Stage II melanosomes. Rab7 and MART1 have been suggested to be important for Pmel17 function [245, 246]. The melanogenic enzymes Tyr

and TYRP1 are delivered to Stage II melanosomes, initiating melanin deposition on the fibrils giving rise to Stage III melanosomes. A number of proteins have been implicated in regulating tyrosinase and TYRP1 sorting from the endosomal system including Rab7, Rab32 and Rab38 [247, 248] as well as BLOC1 (biogenesis of lysosome-related organelle complex) in association with BLOC2 or AP3 [246]. Other melanosomal proteins are suggested to be important for melanosome maturation, including MART1 [249-251] and Ocular albinism type I protein (OA1/G-protein coupled receptor 143) in association with G α 13 subunit of heterotrimeric G proteins [252]. In addition OA1 has been suggested to be involved in microtubule-based melanosome transport [253]. Oculocutaneous albinism II (OCA2/P protein) has been suggested to regulate melanosomes pH, absence of the protein results in hypo-pigmentation [254]. In stage IV melanosomes the lumen is full with melanin. Mouse coat-colour mutants have played a key role in identifying proteins involved in melanogenesis [255].

Mature melanosomes are then proposed to be transported to the cell periphery along microtubules. At the cell periphery, Rab27a associated with mature melanosomes interacts with MyoVa via a Mlph linker, which tethers the melanosome to cortical actin [256]. Loss of any member of the tripartite complex results in perinuclear clustering of melanosomes [173, 184, 211]. The mechanism of melanosome transfer to keratinocytes remains to be elucidated (Figure 1-8).

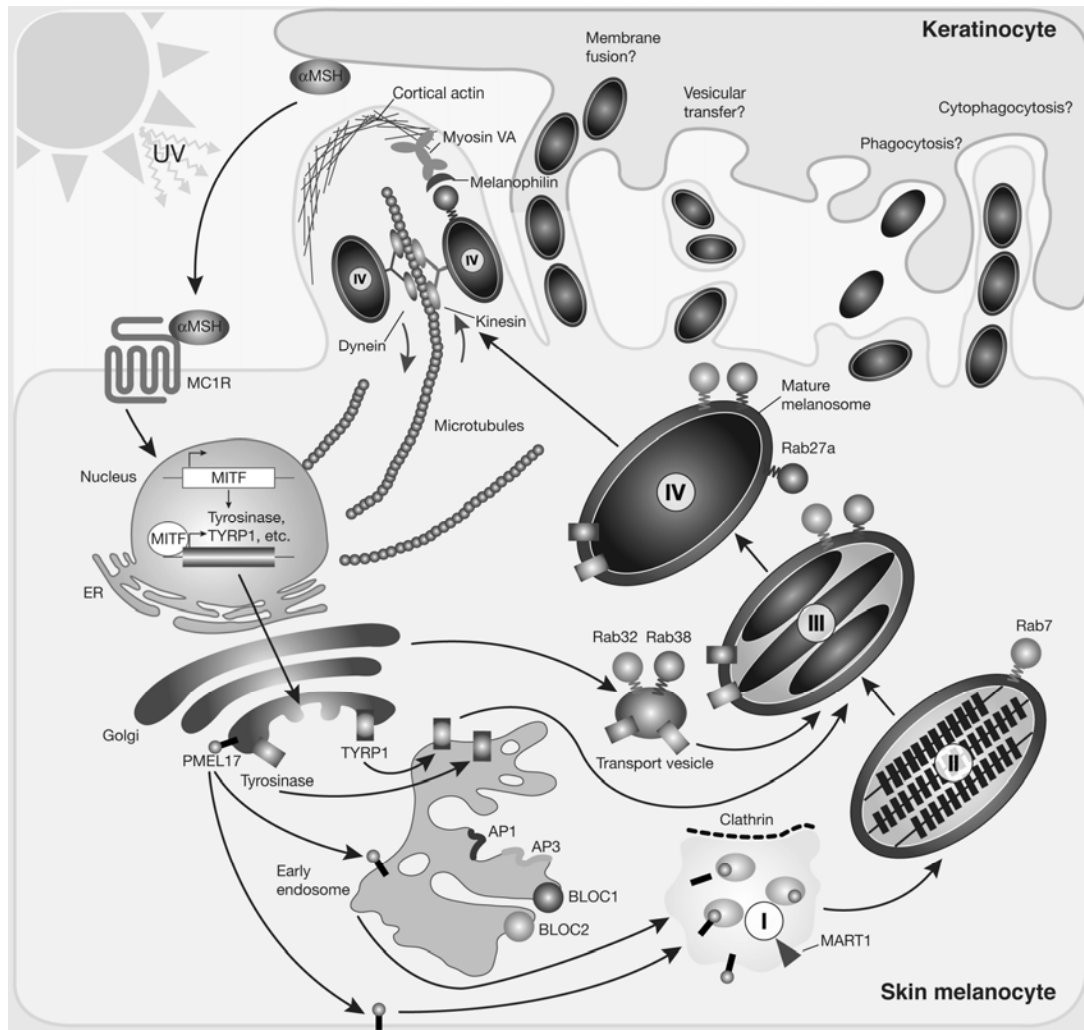


Figure 1-8 Melanosome Biogenesis and Transport in Epidermal Melanocytes.

Stimulation from neighbouring keratinocytes results in MITF-stimulated expression of melanosomal proteins. Melanosome biogenesis occurs through four morphologically distinct stages, with recruitment of melanosomal proteins at distinct stages. Mature melanosomes (Stage IV) are transported along microtubules to the cell periphery and tethered to cortical actin by the tripartite Rab27a:Melph:MyoVa complex. Transfer of melanosomes to keratinocytes then occurs via an uncharacterised mechanism. α-MSH, α-melanocyte-stimulating hormone; AP, adaptor protein; BLOC, biogenesis of lysosome-related organelle complex; MART, melanoma antigen recognised by Tcells; MC1R, melanocortin 1 receptor; MITF, microphthalmia-associated transcription factor; TYRP1, tyrosinase-related protein 1. Image adapted from Wasmeier C. *et al* (2008) *Journal of Cell Science*.

1.3 The P-type ATPase Family

The P-type ATPases are a family of transmembrane pumps, which, following the phosphorylation of a key Asp residue, undergo conformational changes and transport ions (or phospholipids) across cellular membranes. They are evolutionarily conserved with 4 identified in *E. coli* [257], 16 in *S. cerevisiae* [258], 21 in *C. elegans* and 15 in *D. melanogaster* [259]. A genome wide screen has not yet been completed for the murine or human genome.

1.3.1 Structure

All P-type ATPases are multi-transmembrane proteins with cytosolic N- and C-terminal domains. There are four domains which are conserved across the whole family: the phosphorylation domain (P-domain), the nucleotide-binding domain (N-domain), the actuator domain (A-domain) and the membrane domain (M-domain) (Figure 1-9). The P-domain contains the DKTGTLT motif, including the key Asp residue that is phosphorylated following hydrolysis of ATP. The N-domain contains a conserved Lys residue in the nucleotide binding site. The A-domain contains the TGES sequence motif and structural analysis proposes it contacts the P-domain during the ion-transport cycle. The M-domain consists of the structurally conserved transmembrane domains, yet the sequences vary considerably and this is consistent with the wide array of substrates the P-type ATPases transport. Based on sequence homology the P-type ATPases can be divided into five distinct types, and then further into subtypes [257] (Figure 1-10).

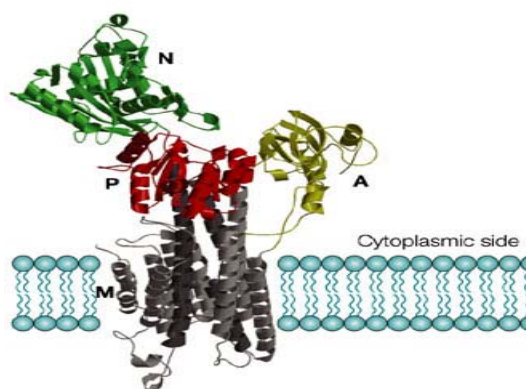


Figure 1-9 P-Type ATPase Structure.

Sarcoplasmic-reticulum (SR) Ca^{2+} -ATPase. The phosphorylation (P)-domain is shown in red, the nucleotide-binding (N)-domain in green, the actuator (A)-domain in yellow, the membrane (M)-domain in grey. The two long helices, M4 and M5, that connect the ion-binding site to the P-domain are shown in a darker shade of grey. Figure adapted from Kuhlbrandt W. (2004) Nature Reviews Molecular Cell Biology with permission.

1.3.1.1 Type I-ATPases

The Type I-ATPases are divided into type-IA and -IB. Type-IA contains the bacterial K^{+} transporter Kdp. Type-IB consists of the heavy metal-ATPases, i.e. Cu^{2+} - and Cd^{2+} -transporters. Most Type-IBs are bacterial, but mutations in the human homologues ATP7a and ATP7b cause hereditary Menkes and Wilsons diseases respectively. These diseases are characterised by copper imbalance resulting in neurological defects, therefore these have been investigated extensively [260, 261].

1.3.1.2 Type II-ATPases

The Type II-ATPases are the most diverse. Type IIAs are the sarcoplasmic reticulum (SR) Ca^{2+} -ATPases and are the most widely investigated as the structure was the first to be elucidated. The plasma membrane Ca^{2+} -ATPases comprise the Type-IIB. Type IIC includes $\text{Na}^{+}, \text{K}^{+}$ -ATPase and the closely related gastric $\text{H}^{+}, \text{K}^{+}$ -ATPase. The Type IIC-ATPases differ from other P-ATPases in that they are hetero-oligomers consisting of a ten transmembrane α -subunit sharing characteristics with the rest of the P-ATPases but also a single-membrane

spanning β -subunit. The Na^+, K^+ -ATPases also have regulatory γ -subunits, also known as FXYD proteins [262]. The Type-II D ATPases are eukaryotic Na^+ -ATPases [257].

1.3.1.3 Type III-ATPases

The Type-IIIA ATPases are H^+ -pumps found exclusively in plant and fungi plasma membranes and the Type-IIIB ATPases are bacterial Mg^{2+} -pumps [263].

1.3.1.4 Type IV-ATPases

Type IV-ATPases are only expressed in eukaryotes and although they share structural homology with the ion transporters, they are involved in the transport of lipids. They are proposed to be important in maintaining lipid-bilayer asymmetry and are otherwise known as flippases. They also associate with the non-catalytic subunit Cdc50 [264].

1.3.1.5 Type V-ATPase

The Type V-ATPases have recently emerged as a separate class. Despite being subdivided according to their subcellular localisations and sequence [265] no known substrate specificities have been assigned.

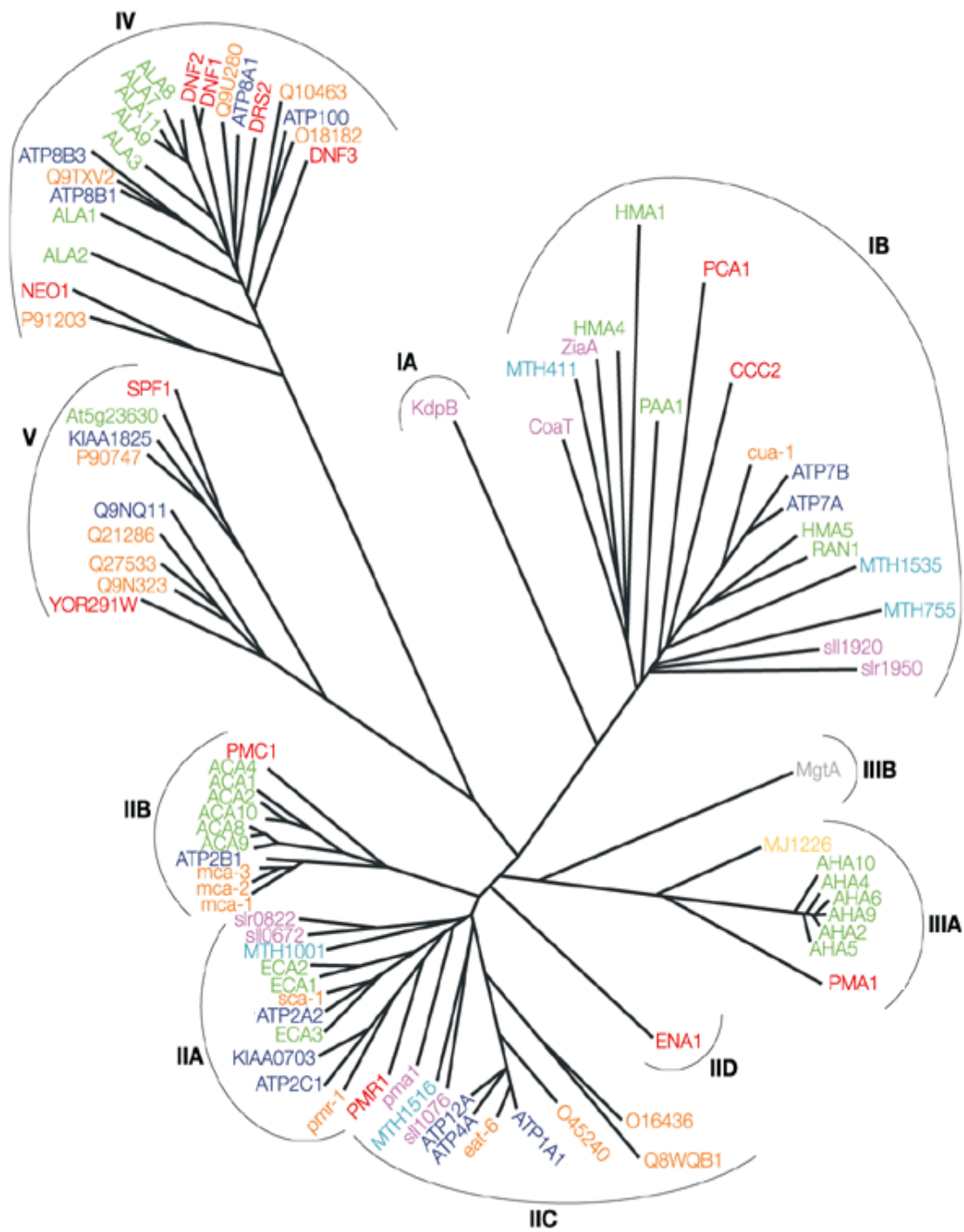


Figure 1-10 Phylogenetic Tree of the P-type-ATPase Family.

Subfamilies cluster according to their ion specificities. Classification according to Axelsen K.B. and Palmgren M.G. (1998) Journal of Molecular Evolution. Representative gene products are colour coded by species: green, genes from *A. thaliana*; orange, *C. elegans*; grey, *E. coli*; dark blue, *H. sapiens*; light blue, *M. thermoautotrophicum*; yellow, *M. jannaschii*; purple, *Synechocystis PCC6803*; and red, *S. cerevisiae*. Figure taken with permission from Kuhlbrandt W. (2004) Nature Reviews Molecular Cell Biology.

1.4 Na⁺,K⁺-ATPase

The Na⁺,K⁺-ATPase was the first P-type ATPase to be discovered [266]. Following the hydrolysis of ATP Na⁺,K⁺-ATPase transports 3 Na⁺ ions across the plasma membrane to the extracellular pool, in exchange for 2 K⁺ ions. This isoelectric function is crucial for ion homeostasis, but also fundamental for many tissue specific functions, including H₂O re-absorption in the kidney proximal tubule, muscle contraction and action potential propagation.

The minimal functional unit is comprised of the α- and β-subunits. There are four isoforms of the α-subunit, and 3 isoforms of the β-subunit. In some tissues, an additional accessory protein has been identified - the γ-subunit, also known as the FXD family proteins. The differential expression of all the subunits results in a wide selection of isozymes, expressed in a tissue- and cell-specific manner, enabling tissue-specific control of ion homeostasis [267].

1.4.1 The α-subunit

The α-subunit family are 110kDa 10-transmembrane domain proteins [268] (Figure 1-11). The extracellular loops are short with the exception of the M7M8 loop, which interacts with the β-subunit [269]. The large intracellular M4M5 loop is approximately 430 residues long and contains the N- and P-domains. The A-domain is within the 120 residue M2M3 loop and the N-terminal 90 residue tail (Figure 1-11). The crystal structure of the phosphate bound protein was solved by Morth *et al.* [270] and, when compared to the previously solved

structure of the Ca^{2+} -ATPase, it shows nearly identical arrangements of the transmembrane helices [271, 272].

Four isoforms have been identified for the α -subunit [272-274], their expression is tissue specific and varies during development [275]. Modelling of the $\alpha 1$ - $\alpha 3$ structures showed very little variation between the isoforms in the M-domains but large variations in the surface-exposed structures of the A- and P-domains [276]. The $\alpha 1$ subunit (ATP1a1) is ubiquitously expressed whilst $\alpha 2$ (ATP1a2) and $\alpha 3$ (ATP1a3) are predominantly expressed in skeletal muscle and brain respectively [277, 278], $\alpha 4$ (ATP1a4) is only expressed in the testis [274]. Interestingly, it has also been shown that intracellular distribution also varies between the isoforms. ATP1a1 is evenly distributed throughout the plasma membrane, whilst both ATP1a2 and ATP1a3 cluster in microdomains in close proximity to the sarcoplasmic reticulum, linking their Na^+ pumping function to the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and hence to Ca^{2+} regulation [279].

Mutations in ATP1a2 and ATP1a3 can result in neurological diseases. ATP1a2 mutations result in familial hemiplegic migraine type 2 (FHM2) [280] and sporadic hemiplegic migraine (SHM) [281] whilst mutations in ATP1a3 are associated with rapid-onset dystonia parkinsonism [282]. Loss of ATP1a4 results in sperm immobility and thus infertility in mice [283]. Single nucleotide polymorphisms (SNPs) in all α -isoforms have been linked to a predisposition to bipolar disorder in humans [284].

ATP1a1 KO mice are non-viable whilst ATP1a2 KO mice survive birth but die a few days later. Heterozygous ATP1a1 and ATP1a2 mice are viable [278]. Heterozygous ATP1a1 mice hearts are hypocontractile whilst heterozygous ATP1a2 mouse hearts are hypercontractile [278]. Tissue specific knockout of ATP1a2 in vascular smooth muscle cells prevents adrenocorticotrophic hormone (ACTH) -induced hypertension [285]

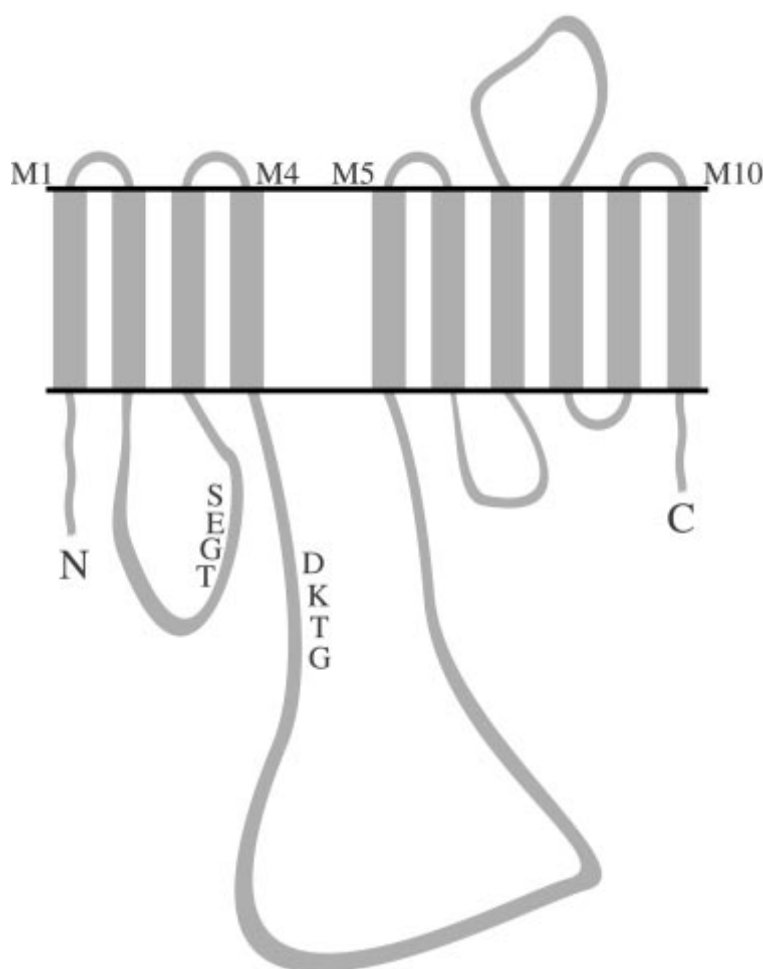


Figure 1-11 The α -subunit.

The diagram depicts the topology of the Na^+, K^+ -ATPase α -subunit in the plasma membrane, demonstrating the small extracellular loops and large M4M5 intracellular loop. Letters depict the conserved A- (TGES) and P- (DKTG) domain motifs. Taken with permission from Kaplan J. H. (2002) Annual Reviews in Biochemistry.

1.4.2 The β -subunit

The β -subunit family are single-membrane-spanning proteins, with the N-terminus in the cytoplasm and a large extracellular C-terminal loop containing three consensus N-glycosylation sequences in addition to three disulphide bridges (Figure 1-12). It is a 370 residue protein with three reported isoforms [286], $\beta 1$ (ATP1b1) is the most abundant, $\beta 2$ (ATP1b2) is expressed in neuronal cells and heart whilst $\beta 3$ (ATP1b3) is expressed primarily in the lung and liver [267, 287]. The extracellular loop can be N-glycosylated, but this is not important for Na^+, K^+ -ATPase function or transport to the plasma membrane [288].

However, N-glycosylation is proposed to be important for intercellular β - β interactions at epithelial junctions [289, 290] and this is proposed to be negatively regulated by FXYD5 as its up-regulation is associated with reduced cell adhesion and increased metastasis [291].

The β -subunit's primary role is that of a chaperone as it is crucial for the transport and correct insertion of the α -subunit at the plasma membrane [292] and the degree of glycosylation has been proposed to be involved in targeting the Na^+, K^+ -ATPase to specific membranes [293]. In addition, the different β -subunit isoforms are proposed to influence the ion affinity of the α -subunit and it was found that reduction of the disulphide bridges resulted in complete loss of Na^+, K^+ -ATPase function through prevention of K^+ occlusion [294]. Mice with cardiac-specific knockout of ATP1 β 1 showed age-related hypertrophy and decreased contractility [295]. ATP1 β 2 KO mice display abnormalities in motor coordination at day 15, worsening to tremors and paralysis and ultimately resulting in death by day 18 [296], but the lethality can be prevented by ATP1 β 1 knock-in [297]. There is no published ATP1 β 3 KO mouse.

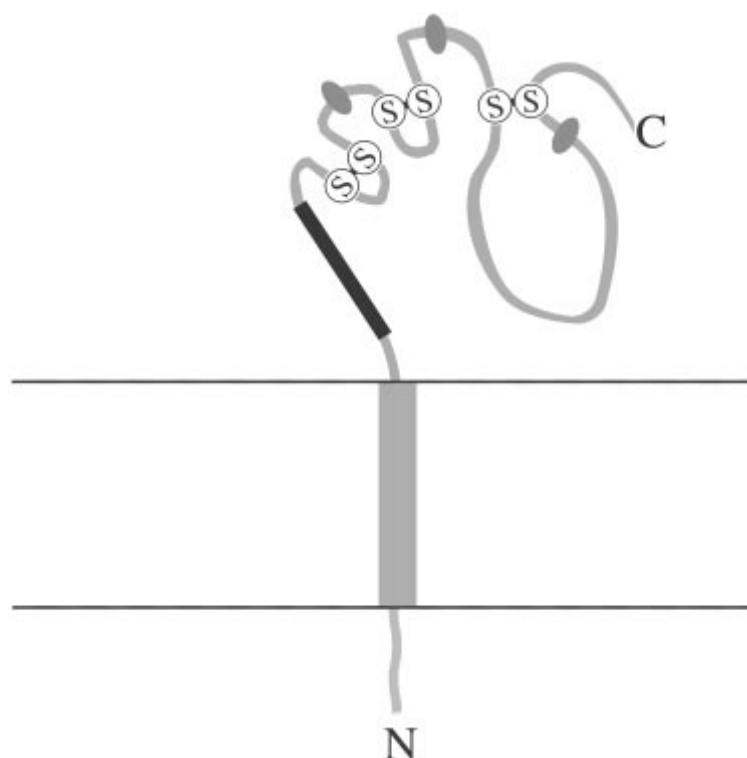


Figure 1-12 The β -subunit.

The diagram depicts the topology of the Na^+, K^+ -ATPase β -subunit in the plasma membrane. The heavy line indicates the domain responsible for interaction with the α -subunit. Ovals represent sites of N-glycosylation. Taken with permission from Kaplan J. H. (2002) Annual Reviews in Biochemistry.

1.4.3 The γ -subunit (FXYP Proteins)

The γ -subunit family are small single-membrane-spanning proteins (7-10kDa). There are 7 members of the mammalian family, characterised by two conserved Gly residues in the transmembrane domain, a conserved Ser residue and an FXYP motif, hence they are alternatively termed the FXYP protein family [298]. They are all known to associate with Na^+, K^+ -ATPase and alter its affinity for ions or ATP [299-301], but they are also proposed to stabilise Na^+, K^+ -ATPases as they have been shown to protect against thermo inactivation or excess detergent *in vitro* [302]. Crosslinking, mutational and structural analysis proposed that the FXYP proteins interact with the α -subunit A-domain [303], M2, M6 and M9 domains [270, 304-306], but reports also propose an interaction with the β -subunit [304].

Phosphorylation of Ser(68) of FXYP1 by protein kinase C (PKC) has been shown to activate the Na^+, K^+ -ATPase in cardiac myocytes [304].

The FXYP proteins have tissue specific expression patterns, suggesting a role in tissue specific regulation of Na^+, K^+ -ATPase. FXYP1 (Phospholemman) is expressed in heart and skeletal muscle [307]; FXYP2 (γ -subunit) in kidney, stomach and heart [308]; FXYP3 (Mat8) in heart, skeletal muscle, liver, breast, brain, lung, colon and stomach; FXYP4 (CHIF) in kidney and colon [300, 309]; FXYP5 (RIC) in heart, brain, lung, skeletal muscle, spleen, kidney and testis [310]; FXYP6 (phosphohippolin) in brain and kidney [311] and FXYP7 exclusively in the brain [312]. FXYP1 KO mice displayed reduced cardiac contractility as a result of hypertrophy and *in vitro* experiments showed increase Na^+, K^+ -ATPase activity [313, 314].

1.4.4 Active Transport and ATP Hydrolysis

It was revealed that P-Type ATPases undergo a large conformational change during the translocation of ions across the membrane [315] and it was proposed that there were two distinct conformations, E_1 and E_2 , both with different affinities for ions and ATP. This has since been elaborated into a complex reaction cycle known as the Post-Albers Scheme [316, 317]. This is shown in Figure 1-13 for Na^+, K^+ -ATPase. In brief, the protein in the E_1 conformation has high affinity for 3 Na^+ and ATP. Hydrolysis of ATP and phosphorylation of the protein in the presence of both Na^+ and Mg^{2+} results in the $E_1\text{P}(3\text{Na})$ conformation following the dissociation of ADP and occlusion of bound Na^+ [318]. Following a conformational change, the 3 Na^+ are released into the extracellular space resulting in the $E_2\text{P}$ conformation. The first Na^+ is readily released, followed more slowly by the remaining two. The $E_2\text{P}$ conformation has a high affinity for K^+ which binds and the subsequent dephosphorylation of the α -subunit results in K^+ occlusion ($E_2(2\text{K})$). Binding of ATP shifts the

protein from the E_2 to the E_1 conformation and catalyses the release of the 2 K^+ into the cytoplasm as the affinity for K^+ in the E_1 conformation is reduced.

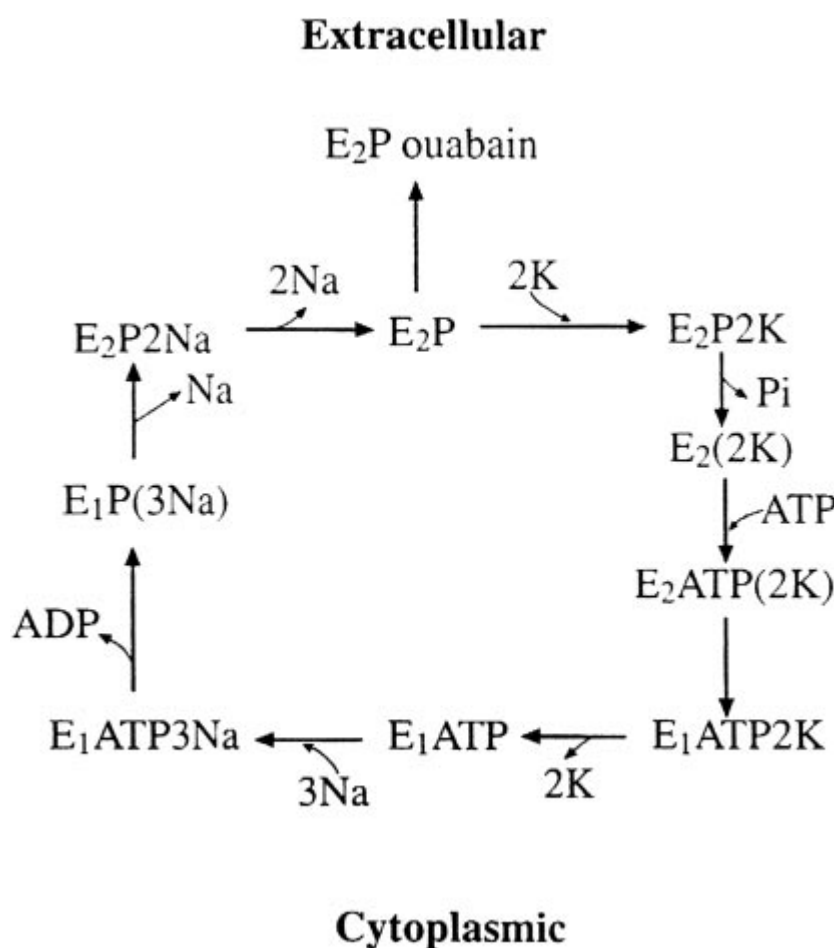


Figure 1-13 The Post-Albers Scheme for the Na^+,K^+ -ATPase.

The sequence of steps involved in the active transport of 3 Na^+ in exchange for 2 K^+ . Taken with permission from Kaplan J.H. (2002) Annual Reviews in Biochemistry.

1.4.4.1 Conformational Changes

Crystal structures of the Ca^{2+} -ATPase revealed details of the large conformational changes that occur during the catalytic cycle of P-type ATPases [271, 319-321]. These structural rearrangements were found to be preserved in the Na^+,K^+ -ATPase [270, 322]. When Na^+ binds, it induces a conformational change in the P-domain through M4 forming a Mg^{2+} binding site. Phosphorylation and bending of the P-domain induces movement of the M1 and M2 by the pull of the A-domain, leading to Na^+ occlusion ($E_1P(3Na)$). Following

phosphorylation, the N-domain undergoes conformational change, allowing the A-domain to rotate and interact with the phosphorylation site (E_2P) [271]. Movement of the A-domain shifts M3 and M4, releasing the 3 Na^+ in exchange for 2 K^+ . This stimulates the conserved Glu residue in the TGES motif on the A-domain to mediate dephosphorylation of the P-domain resulting, in K^+ occlusion ($E_2(2K)$). ATP binds to the N-domain in the E_2 state and promotes transition to the E_1 state (E_1ATP), whereby K^+ is released and the cycle starts again.

1.4.4.2 Cation Binding

The cation-binding pocket in P-Type ATPases has a conserved Glu residue, which interacts with the ions in both the E_1 and E_2 state [323], in addition to a conserved Pro residue [324]. The ion binding sites in the Na^+,K^+ -ATPase have been identified [270], of which some are conserved with the Ca^{2+} binding sites in SERCA [321, 325]. The additional M7-M10 domains of Na^+,K^+ -ATPase contribute to cation binding, allowing additional selectivity for these ions [270, 325].

The first Na^+ binding site involves Thr807 (M6), Asn776, Glu779 (M5) and Gln923 (M9) [326] whilst the second Na^+ binding site involves Glu327 [323, 327], Ala323 and Val325 (M4) and Asp804 and Asp808 (M6) [328]. Two binding sites have been proposed for the third Na^+ , one involving Glu954 (M9) [329-331] and Tyr771 (M5) [331-333], the alternative involving Ser775, Thr774 (M5) [329, 333] Gln854 (M7) and Asp926 (M8) [327, 334, 335]. A channel lined with charged and polar residues has been shown between M5, M7, M8 and M10 [270, 322], which ends at this alternative third Na^+ binding site [334]. This channel is plugged by the C-terminal KETYY sequence, which has been found to be important for Na^+ binding [270, 336].

The Na^+ binding sites are thought to reconfigure and become the sites for K^+ binding. In support of this, structural analysis proposes that Glu327 (M4), Ser775, Asn776 and Glu779 (M5) and Asp804 (M6) are involved in K^+ binding [270].

The importance of the highlighted residues is emphasized by the fact a number of them are mutated in the neurological diseases mentioned in section 1.4.1 [335].

1.4.4.3 ATP Binding

The nucleotide binds in a hydrophobic pocket in the M4M5 loop. In the porcine α -subunit, Arg544 and Lys501 are important for high affinity ATP binding (E_1), whilst Phe475 and Lys480 are more important for the low affinity ATP binding (E_2) [337]. The ⁴⁴²GDASE⁴⁴⁶ sequence in the N-domain has also been implicated in the ATP-binding pocket [338]. This is in agreement with the original research of Ca^{2+} -ATPase activity [339, 340]. Binding of ATP alone induces an open conformation in the M4M5 loop and this is independent of any ion-binding-induced conformational changes in the M-domain (E_2 - E_1 transition). As stated (Section 1.4.4), ATP-binding induces K^+ release. Subsequent Na^+ binding, in conjunction with Mg^{2+} , induces a closed conformation of the M4M5 loop (E_1 - E_2 transition). This then enables ATP hydrolysis and auto-phosphorylation [341].

1.4.5 Assembly and Delivery

The α - and β -subunits are synthesised and assembled into the heterodimer in the ER, they are mutually dependent on each other for the exit from the ER [292]. In particular, the α -subunit C-terminal M-domains fail to insert into the ER membrane without the presence of the β -subunit, thereby exposing degradation signals and resulting in degradation of the whole protein [342]. Association of the γ -subunit is not required for successful $\alpha\beta$ plasma membrane localisation, but the γ -subunit will only associate with the fully formed $\alpha\beta$ complex, and this association is required for stable localisation of the γ -subunit at the plasma membrane [308].

A 26 residue sequence, including an evolutionarily conserved SYGQ motif within the M7M8 extracellular loop of the α -subunit, has been shown to be necessary and sufficient for formation of the $\alpha\beta$ heterodimer and its delivery to the plasma membrane [269, 343].

Deletions in the M-domain [344] or 146-amino acid C-terminal deletion (removing all disulphide bridges and N-glycosylations) of the β -subunit [345] did not disrupt heterodimerisation, but prevented exit from the ER [346]. However, a single point mutation of Pro244 in the extracellular domain of the β -subunit severely disrupts heterodimerisation [347]. Concomitantly, it was found that the ion-transport-deficient α -subunit mutant of Na^+, K^+ -ATPase (αD369A) is correctly delivered to the plasma membrane, therefore signifying that enzyme function is not a pre-requisite for membrane delivery [346].

In polarised epithelia, the Na^+, K^+ -ATPase has polarised distribution and it is only expressed at the basolateral membrane. This specific targeting is proposed to be regulated by the α -subunit N-terminal domain and the glycosylation state of the β -subunit [293, 348].

It has been shown that there is a pool of intracellular Na^+, K^+ -ATPase [349] that is recruited to the plasma membrane following catecholamine stimulation of lung alveolar epithelium [350, 351]. It has been proposed that activation of Akt is necessary and sufficient for Na^+, K^+ -ATPase plasma membrane recruitment. Akt phosphorylates and inactivates AS160 (Rab10 GAP) which promotes Rab10-mediated trafficking of Na^+, K^+ -ATPase to lung alveolar plasma membrane [352, 353]. MyoVa has also been shown to associate with Na^+, K^+ -ATPase containing vesicles in alveolar epithelial cells and negatively regulates their plasma membrane recruitment by restraining Na^+, K^+ -ATPase in its intracellular pool [354].

1.4.6 Cardiotonic Steroids

It has long been known that cardiotonic steroids bind to, and inhibit Na^+, K^+ -ATPase ion transport [355]. Consequently, they are widely used for the regulation of blood pressure and hypertension in humans [356]. Their use has more recently been proposed for bipolar conditions [357] and malignancies [358].

Cardiotonic steroids (digitalis steroids) were first discovered in plants (cardenolides) and toad venom (bufadienolides) and share the same common structure of a steroid nucleus and a lactone ring at C-17 and a hydroxyl group at C-14. Cardenolide-like compounds, ouabain [359-361] and digoxin [362], have been purified from human plasma and the hypothalamus. Bufadienolides have also been identified [363-366]. Studies showed that ouabain is synthesised in the adrenal gland [367, 368], therefore suggesting a function *in vivo*.

Cardiotonic steroids bind to the M1M2 extracellular loop of Na⁺,K⁺-ATPase α -subunit at a highly conserved binding site [369-372]. In particular, the residues at positions 111 and 122 are important as these are altered in ATP1a1 in rodents to positive residues Arg and Asp, resulting in the well characterised, relative ouabain insensitivity of the murine Na⁺,K⁺-ATPase [369, 373]. All human isoforms appear to have the same binding affinity [372, 374].

1.4.7 Signalling Function

The most established role of cardiotonic steroids is the inhibition of Na⁺,K⁺-ATPase transport function [355], but recent work has demonstrated another function in activating signalling cascades independent of its ion-transport inhibitory role. It is proposed that a separate pool of non-pumping Na⁺,K⁺-ATPases mediate the signalling response [349], but that the signalling response varies between cell types depending on the composition of the micro-environment within which the pump is located.

Ouabain inhibition or low extracellular K⁺ both lead to transcriptional up-regulation of Na⁺,K⁺-ATPase [375-377] as well as altering gene expression of a number of other genes (iNOS, VCAM-1, c-fos, c-jun, various cytokines, 14-3-3, PSA) [378-382] and this was first attributed to changes in ion concentrations. However, it was difficult to comprehend how changes in intracellular Na⁺ and K⁺ could be linked to transcriptional regulation, so the question was asked whether the ouabain-induced effects were a result of Na⁺,K⁺-ATPase function inhibition or independent of ion concentration [383, 384].

Extensive research into the effects of ouabain on cardiac myocytes revealed that non-toxic and non-inhibitory levels increased growth and protein synthesis, induced early- and late-response genes and activated the transcription factors AP-1 and NF- κ B, all indicators of cardiac hypertrophy [385-389]. It is now accepted that the initial events that occur following ouabain binding to Na⁺,K⁺-ATPase are independent of Na⁺,K⁺-ATPase activity but involve protein-protein interactions [390].

It was found that ouabain initially induces Src Kinase activation in caveolae [391]. In resting conditions, Na⁺,K⁺-ATPase interacts with, and inactivates Src via the M2M3- and M4M5-loops of the α -subunit. Depletion of ATP1a1 has been shown to increase Src activation [392]. Ouabain treatment causes the Na⁺,K⁺-ATPase to release the kinase domain of Src, enabling the formation of a signalling complex [393], which results in the recruitment of further Src [394]. The active Src then transactivates the epidermal growth factor receptor (EGFR). Ouabain-induced Src Kinase phosphorylation of EGFR is separate from the major Tyr1173 autophosphorylation site [395]. Phospho-activation of EGFR results in recruitment of various downstream signalling molecules (Shc, Grb2, Sos and Ras) and initiates the Ras/Raf/MEK/MAPK signalling pathway [396, 397]. The Na⁺,K⁺-ATPase/Src/EGFR complex also activates PI3K [398].

Some ouabain-induced signalling has been shown to be dependant on extracellular Ca²⁺, calmodulin and PKC [386-388, 397] and involves PLC- γ recruitment to the EGFR-Src complex [397]. The partial inhibition of Na⁺,K⁺-ATPase causes a mild increase in intracellular Na⁺, which activates the sensitive sarcoplasmic reticulum Na⁺/Ca²⁺ exchanger, therefore releasing intracellular Ca²⁺ stores [399-401]. Similarly, ouabain has been shown to induce Ca²⁺ oscillations in embryonic kidney cells through a ouabain-induced Na⁺,K⁺-ATPase effect on the IP3-receptor [402, 403]. Ca²⁺-calmodulin kinase has been shown to phosphorylate Na⁺,K⁺-ATPase resulting in its inhibition [404].

Ras is known to stimulate reactive oxygen species (ROS) production and it is known that ouabain treatment results in increased ROS in mitochondria [390]. The ouabain-induced ROS production has been shown to be independent of the ouabain-induced increase in intracellular Ca^{2+} [390] but it is dependent on Ras [389]. Yet ROS have been shown to inhibit Na^+, K^+ -ATPase [405]. Antioxidants inhibit ouabain-induced increases in skACT and ANF and decreases in $\alpha 3$ -subunit expression through prevention of NF- κ B translocation. Antioxidants also attenuated the MAPK pathway [389].

Despite the majority of this research being undertaken in cardiac myocytes, ouabain-induced protein tyrosine phosphorylation, MAPK activation, ROS production and early response gene expression have been recorded in other cell lines [390, 395, 396].

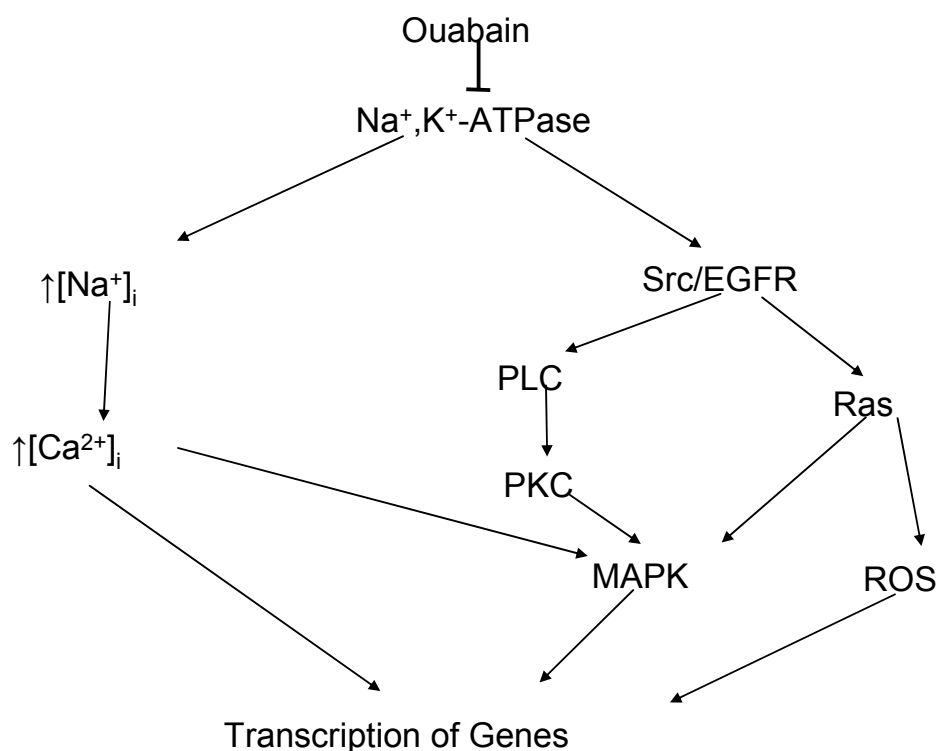


Figure 1-14 Ouabain-induced Signalling Cascade of Na⁺,K⁺-ATPase.

Ouabain binds to the extracellular domain of Na⁺,K⁺-ATPase and transmembrane signal transduction relieves Na⁺,K⁺-ATPase-induced Src inhibition. Higher Ouabain concentrations inhibit Na⁺,K⁺-ATPase pump function resulting in increased intracellular Na⁺. EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; PLC, phospholipase C; PKC, protein kinase C; ROS, reactive oxygen species.

1.5 Project Aims

The aim of this project was to investigate the mechanism of targeting Rab GTPases to their membrane. A number of mechanisms have been proposed for Rab targeting. However, to date, none are conclusive. Rab27a is important for the peripheral distribution of melanosomes in melanocytes and it is a useful model for investigating Rab targeting as disruption of its function results in a distinct phenotype characterised by perinuclear clustering of melanosomes. Previous work has excluded the role of the Rab27a C-terminal hypervariable domain [17] and effector binding [145] in Rab27a targeting. Rab27a has not been found to readily recycle on/off melanosome membranes (A. Hume, unpublished data)

or be extracted by GDI (M. Kirsten, unpublished data), therefore reducing the likely involvement of a GDF. In addition, the GEF activity of the non-redundant Rab27a GEF in melanocytes, R3G, was shown to be important but not sufficient for Rab27a targeting [145]. This led to the hypothesis of a possible targeting factor, a novel interacting partner which is either associated with the target membrane and acts as a dock, or transports the Rab GTPase to the correct membrane. To test this hypothesis, the Rab27a mutant Rab27a^{SF1/F4}, which fails to interact with any known Rab27a effectors yet maintains melanosomal localisation [145], was used to identify novel interacting partners for Rab27a (Chapter 3). Stable melanocyte cell lines were created expressing hemagglutinin-TEV-FLAG tagged Rab27a^{WT} and Rab27a^{SF1/F4} and used for a tandem affinity purification.

Following identification of novel interacting partners, the candidate proteins were investigated for their role in Rab27a targeting (Chapter 4). An siRNA approach was taken to gain insight into the effect that depletion of the candidate proteins had on Rab27a function in melanocytes. Disruption of Rab27a function was assessed by observing the phenotypic changes in melanosome distribution. Following identification that ATP1a1 depletion caused melanosomal clustering, the effect its depletion had on Rab27a expression, activation and localisation were investigated.

Chapter 5 then investigated the localisation of ATP1a1 in melanocytes and the possible mechanisms by which ATP1a1 could regulate Rab27a localisation and its interplay with R3G.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Chemicals were purchased from Sigma-Aldrich unless otherwise specified.

2.1.2 Antibodies

	Raised	Source	Immunoblotting	Immunofluorescence
Rab27a	Mouse	[173]	1:10000	-
	Rabbit	R. Singh (Imperial College, UK)	1:200	1:200
Tubulin	Mouse	Sigma T6557	1:5000	-
ATP1a1	Mouse	Abcam ab7671	1:5000	1:500
	Rabbit	Abcam ab74945	-	1:400
Pme17	Mouse	Dako M0634	1:500	1:50
GPNMB	Goat	R&D Systems AF2330	1:1000	-
AnnexinA2	Rabbit	Gift from J. Ayala-SanMartin (CNRS/UPMN/ENS)	1:5000	-
VAT1	Mouse	Abcam ab89138	1:100	-
MIph	Goat	Everest Biotech EB05444	1:1000	-
	Rabbit	A. Hume (Imperial College, UK)	-	1:50
Calnexin	Rabbit	Stressgen	1:10000	-
TYRP1	Rabbit	C. Wasmeier (Imperial College, UK)	1:10000	1:400
	Mouse	Gift from M. Marks (University of Pennsylvania, USA)	1:500	1:1500
Rab32	Rabbit	E. Mules (Imperial College, UK) [251]	1:50	1:100
Rab38	Rabbit	E. Mules (Imperial	1:200	1:100

		College, UK) [251]		
Rab5	Rabbit	Abcam ab18211	1:1000	1:200
Rab3GEP	Rabbit	Gift from Y. Takai (Osaka Medical Centre for Cancer and Cardiovascular Diseases, Japan)	1:5000	-
Tyr	Rabbit	C. Wasmeier (Imperial College, UK)	1:10000	-
GFP	Goat	Abcam ab6673	1:500	-
V5	Mouse	Invitrogen R96025	1:5000	1:200
ERK1/2	Rabbit	Abcam ab17942	1:1000	-
pERK1/2	Mouse	Abcam ab50011	1:1000	-
ATP1a2	Rabbit	Millipore AB9094	1:250	-
ATP1a3	Mouse	Abcam ab2826	1:250	-
DNP-alexa488	Rabbit	Invitrogen	-	1:50

Table 2-1 Antibodies for Immunoblotting and Immunofluorescence.

For Immunoprecipitation: mouse anti-HA (Roche), mouse anti-FLAG (Sigma F3165), mouse anti-ATP1a1, rabbit anti-ATP1a1.

2.1.3 Tissue Culture Media

Tissue culture media and supplements were from Gibco unless otherwise specified.

2.2 Tissue Culture Methods

2.2.1 Maintenance and Growth of Mammalian Cell Lines

2.2.1.1 Mammalian Cell Lines

Murine wild type melanocytes, Melan-INK4a (Melan-INK), melan-INK4a-ashen (Melan-ash2) and melan-c were a kind gift from Prof. D. C. Bennett (St George's, University of London)

[406]. Murine melanocytes were maintained at 37°C under a humidified atmosphere containing 10% (v/v) CO₂, in Roswell Park Memorial Institute (RPMI) 1640 (+L-Glutamine) supplemented with 10% (v/v) foetal bovine serum (FBS), 100U/ml penicillin, 100U/ml streptomycin, 200nM Phorbol-1,2-myristate 1,3-acetate and 200pM Cholera Toxin.

Human embryonic kidney (HEK) 293A cells (HEK293A) and COS-7 cells were maintained at 37°C under a humidified atmosphere containing 10% (v/v) CO₂, in Dulbecco's modified Eagle's medium (DMEM) (+L-Glutamine) supplemented with 10% (v/v) foetal bovine serum (FBS), 100U/ml penicillin and 100U/ml streptomycin.

2.2.1.2 Sub-culturing of Adherent Mammalian Cell Lines

Cells were sub-cultured by removing the culture medium and washing using sterile Phosphate Buffered Saline (PBS) to remove any remaining serum. 0.25% Trypsin-EDTA was added to the cells and incubated 3-5 min at 37°C to detach the cells from the culture dish. FBS containing media was added to the cells to quench the trypsin activity before centrifuging the cell suspension at 1000xg for 3 min at room temperature (RT). The supernatant was removed and the cells resuspended in the appropriate complete medium and aliquots transferred to a new culture vessel containing the appropriate complete medium to obtain the required dilution.

2.2.2 Freezing and Thawing Cells

Mouse Melanocytes were trypsinised and centrifuged (1000xg for 3min) at RT and resuspended in RPMI 1640 containing 7.5% dimethyl sulphoxide (DMSO). The cell suspension was then aliquoted into cryovials (Nunc) and stored at -80°C for >24h. The cryovials were then transferred to and stored in liquid nitrogen. This gradual freezing process prevented the formation of ice crystals which can damage the cells. HEK293A cells were frozen using the same protocol but resuspended in DMEM containing 10% DMSO.

Cells were thawed by placing the cryovials in a 37°C waterbath until almost all the cells had thawed. The cells were resuspended by gradual addition of complete medium to prevent osmotic shock and cell lysis. The cell suspension was centrifuged as above. The supernatant was removed and the cells resuspended in the appropriate complete medium.

2.2.3 Transfection of Mouse Melanocytes with Plasmid DNA

Cells were plated 24h prior to transfection in the appropriate culture vessel at 30,000cells/ml. Cells were transfected with various constructs using liposomal transfection reagent FuGENE6 (Roche Diagnostics) in a 1:3 ratio of µg of DNA to µl FuGENE6. For each well of a 24-well plate, 0.5µg of DNA was mixed with 1.5µl of FuGENE6 in 25µl of serum-free medium OptiMEM. For each well of a 6-well plate the quantities were multiplied by 5. Transfections were carried out by first diluting the FuGENE6 in OptiMEM and incubating for 5min at room temperature. The plasmid DNA was then added and the mix incubated for >20min at RT to allow formation of the DNA-liposomal complexes. The medium on the cells to be transfected was replaced with OptiMEM and then the transfection mix was added drop-wise. The plates were swirled to ensure even distribution and then incubated for >5h at 37°C. Following the 5h incubation, the media was removed and replaced with RPMI (+L-Glutamine) supplemented with 10% (v/v) FBS and 200nM Phorbol-1,2-myristate 1,3-acetate and 200pM Cholera Toxin.

2.2.4 Generation of a Stable Mouse Melanocyte Cell Lines

Transfection was carried out as described above (2.2.3) but in a 10cm tissue culture dish (quantities multiplied by 10). Two days post transfection, the media was replaced with complete RPMI media supplemented with 100µg/ml Hygromycin B. Cells were grown under selection for three weeks before remaining cells were sub-cultured to one cell per well in a 96-well plate. The isolated clones were expanded and sub-cultured to increasing culture volumes. Frozen stocks were generated as described above (2.2.2).

2.2.5 Transfection of HEK293A Cells with Plasmid DNA

Transfection was carried out as described in Section 2.2.3 for the Mouse Melanocytes but following the 5h incubation, the medium was replaced with DMEM (+L-Glutamine) supplemented with 10% (v/v) FBS only.

Alternatively, Lipofectamine 2000 (Invitrogen) was used as the transfection reagent. Cells were plated 24h prior to transfection in the appropriate culture vessel at 30,000cells/ml. For a 24-well plate, 0.8µg DNA and 0.8µl Lipofectamine 2000 was mixed in 100µl OptiMEM per well and incubated for 20min at RT. This was added to the wells, containing 500µl OptiMEM and incubated for 5h before the medium was replaced with DMEM (+L-Glutamine) supplemented with 10% (v/v) FBS only.

2.2.6 Transfection of Mouse Melanocytes with siRNA

Cells were plated 24h prior to transfection in the appropriate culture vessel at 30,000cells/ml in order to be at 50-70% confluence for the addition of the transfection reagent. Cells were transfected with siRNA (Table 2-2 and Table 2-3) to a final concentration of 100nM using Oligofectamine (Invitrogen). For each well of a 24well plate, 1µl Oligofectamine was diluted in 10µl OptiMEM and 1µl 20µM siRNA was diluted in 50µl OptiMEM and incubated for 5min at RT. The two dilutions were then combined and incubated for >20min at RT to allow complex formation, before adding OptiMEM to a final volume of 200µl. For each well of a 6-well plate the amounts were multiplied by 5 or for a 150cm² flask by 75. The cells to be transfected were washed with OptiMEM and then the siRNA-Oligofectamine complex was added and incubated for >5h at 37°C. Following incubation the media was removed and replaced with complete RPMI medium. For optimal siRNA depletion the siRNA treatment was sometimes repeated after 3 and/or 7day incubations. Cells were visualised and processed at various time points post siRNA transfection.

Gene Target	Dharmacon Cat. No.
ATP1a1	L-052829-00
Annexin A2	L-061993-01
Pmel-17	L-063274-01
Vat1	L-043290-01
GPNMB	L-040019-01
ATP1B3	L-042895-01

Table 2-2 ON-TARGET plus SMARTpool siRNA

Gene Target	Reagent	Sequence	Dharmacon Cat. No.
NT	ON-TARGETplus	-	D-001810-02
Mlph	Custom oligo	GGGCAAAAUACAAAAGGAGU	-
Rab3GEP	siGENOME	GCCCAGACCCACUACUAUA	D-059264-02
ATP1a1 oligo 1	ON-TARGETplus	AGAGGAAGGUCGUCUGAUU	J-052829-05
ATP1a1 oligo 2	ON-TARGETplus	GUAUGAGCCUGCCGCUGUA	J-052829-06
ATP1a1 oligo 3	ON-TARGETplus	GCCAAACGAUGAUCUGUAC	J-052829-07
ATP1a1 oligo 4	ON-TARGETplus	GCUUACACCCUAACCAGUA	J-052829-08

Table 2-3 Individual Oligonucleotides

2.2.7 Compound Treatment of Cells

Melan-INK4a cells were plated 24h prior at 30'000cell/ml. NH_4Cl or NaAc were then added to fresh media up to a final concentration of 80mM, Ouabain was added up to a final concentration of 3mM. Cells were incubated for 4-72h and the phenotype was visualised using a light microscope (Olympus CKX41 using Olympus Soft Imaging System Cell D).

2.2.8 Cytotoxicity Assay

Cells were plated in a 96well plate and treated with increasing concentrations of Hygromycin B. 50µl of 5mg/ml MTT (3-4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in

PBS was added to each well and incubated in the dark with agitation for 5min. Samples were incubated at 37°C for an additional 3h. The media was removed and replaced with 200µl DMSO and shaken for 5min. The optical density at 560nm and 670nm was measured and the latter subtracted from the former (Tecan Sunrise plate reader using Tecan Magellan5 Software). Cell survival was measured relative to the untreated control.

2.2.9 Immunofluorescence and Microscopy

Cells required for immunofluorescence microscopy analysis were plated on coverslips in 24-well plates and experiments carried out as described above (Sections 2.2.3, 2.2.6 or 2.2.7). Prior to immunostaining the coverslips, media was removed and the coverslips washed in PBS before fixation by incubating for 20min at RT in 4% (w/v) paraformaldehyde (PFA) in PBS or ice cold methanol (MeOH) or acetone. Coverslips were then washed twice in PBS.

To immunostain the coverslips they were incubated for 5min at RT in 50mM NH₄Cl in PBS to quench background caused by free aldehyde groups reacting non-specifically with the antibodies. The coverslips were then incubated for 5min at RT in PBS containing 2% FBS and 0.05% Saponin (Buffer A) in order to block non-specific antibody binding and to permeabilise the cells respectively. Coverslips were then incubated for 30min (unless otherwise specified) at RT in primary antibody diluted in Buffer A before washing three times in Buffer A. This was followed by incubating for 30min at RT with the appropriate fluorescently-conjugated secondary antibody (Molecular Probes) diluted 1:400 in Buffer A. The coverslips were then washed three times in Buffer A and then PBS. The coverslips were then rinsed in distilled water and mounted on microscope slides with either ImmunO-fluore mounting medium (MP Biomedicals) or Prolong Gold antifade reagent (Invitrogen). Coverslips were visualised using either widefield microscope (Zeiss Axiovert 200 inverted microscope using C-Imaging Simple-PCI acquisition software) or confocal microscope (Zeiss laser scanning microscope (LSM)-510 inverted confocal microscope using LSM Image Browsers).

2.2.10 Fluorescence pH Measurement

2.2.10.1 DAMP + anti-DNP

Cells were plated on coverslips. When at 70% confluency DAMP (*N*-(3-((2,4-dinitrophenyl)amino)propyl)-*N*-(3-aminopropyl)methylamine, dihydrochloride) was added to the media to a final concentration of 30μM and incubated at 37°C for 20min. Coverslips were fixed in 4% PFA and immunostained as described above (section 2.2.9) using rabbit anti-DNP-alexa488 secondary antibody.

2.2.10.2 LysoTracker

Cells were plated on glass-bottomed dishes (Iwaki Cell Biology). When at 70% confluency, the media was removed and replaced with media lacking FBS but supplemented with 100nM LysoTracker (Invitrogen). Cells were incubated for 30min-2h at 37°C, visualised on a widefield or confocal microscope.

2.3 DNA Techniques

2.3.1 Transformation of *Escherichia coli*

Escherichia coli (*E.coli*) DH5α competent cells (Invitrogen) were thawed on ice. For each transformation, 50μl of cells were aliquoted into pre-cooled tubes and kept on ice. 1-100ng of plasmid DNA was added to the cells and incubated on ice for 30min. Cells were heat-shocked for 20s at 42°C and then returned to ice for an additional 2min incubation. 950μl of pre-warmed Luria-Bertani (LB) medium was added to the tubes and incubated for 1h, 37°C with agitation at 200rpm. 20-200μl of the transformation mixture was plated on selective LB agar containing the appropriate antibiotic (ampicillin 50μg/ml or kanamycin 25μg/ml) and the plates incubated overnight at 37°C.

Single colonies were picked from the LB Agar plates and cultured overnight in 5ml LB medium containing the appropriate antibiotics, at 37°C and with agitation at 200rpm.

LB Medium : 10g/l NaCl, 10g/l Tryptone, 5g/l yeast extract

LB Agar : 10g/l NaCl, 10g/l Tryptone, 5g/l yeast extract, 20g/l agar

2.3.2 Isolation of Plasmid DNA

DNA was isolated using Qiagen kits according to the manufacturer's instructions. For small-scale DNA preparations, the Qiagen mini-prep kit was used. 5ml overnight cultures were pelleted by centrifugation 15min, 4'000xg at 4°C. Pellets were resuspended in 250µl of chilled P1 Buffer and transferred to microcentrifuge tubes. Cells were lysed by the addition of 250µl P2 Lysis Buffer followed by several inversions and neutralisation by adding 350µl of N3 Buffer. The lysate was then centrifuged 10min, 17'000xg, RT and the supernatant loaded onto a DNA binding column. The column was centrifuged for 1min at 17'000xg to bind the DNA onto the column then washed with 0.5ml Buffer PB, then 750µl PE Buffer and the DNA was then eluted by loading 30µl H₂O onto the filter and incubating for 1min at RT before eluting by centrifugation for 1min at 17'000xg. Qiagen midi-prep kit was used for large scale (culture volumes between 50-250ml) DNA isolation following the manufacturer's protocols.

2.3.3 DNA Quantification by Spectrophotometry

DNA concentration was determined by measuring the optical density (OD) at 260nm (OD of 1=50µg/ml) using the Nanodrop Spectrophotometer ND-1000 (Labtech).

2.3.4 Polymerase Chain Reaction (PCR)

Standard PCR was performed using the following PCR Mix: 0.5µM of both forward and reverse primers, 0.2µM dNTPS, 1µl Pfu Turbo (Stratagene), 5µl 10x Buffer, 1µl 1:100 DNA template in a final volume of 50µl with sterile H₂O.

The PCR conditions were as follows (Table 2-4):

Stage	Temperature (°C)	Time (min)	No. Cycles
Initial Denaturation	95	10	1
Denaturation	94	1	30
Annealing	Primer specific	1	30
Extension	72	1/kb	30
Final Extension	72	10	1

Table 2-4 PCR Conditions

2.3.5 Restriction Endonuclease Digestion

DNA digestion was performed in a final volume of 50µl using 1.5µl of each restriction enzyme (1 U/µl, New England Biolabs, Invitrogen) in the appropriate buffer for the single or double digestion according to the manufacturer's instructions, diluted in sterile water. Digestions were incubated at 37°C for 2h to overnight. Digested DNA was subsequently purified using the Qiagen PCR Purification Kit and analysed by agarose gel electrophoresis.

For sequential double digestion of plasmids, the first digestion step was incubated at 37°C for 6h and then heat inactivated according the manufacturer's instructions. 1µl Alkaline Phosphatase and 1.5µl of the second restriction enzyme was added and incubated at 37°C, overnight before heat inactivation according to the manufacturer's instructions. Digested DNA was subsequently purified using the Qiagen PCR Purification Kit and analysed by agarose gel electrophoresis (Section 2.3.9).

2.3.6 Ligation of DNA

DNA ligation was performed using the Ready-To-Go T4 DNA Ligase Kit (Amersham Bioscience) according to the manufacturer's instructions. Digested insert DNA and digested plasmid DNA were added in a 3:1 molar ratio to the tube, made up to 20µl using sterile

water and incubated for 5min at RT. Negative control ligation reactions were performed in parallel without the addition of any digested insert DNA. The ligation reactions were then mixed and incubated 40min at 16°C. 2µl of the ligation mix were used to transform bacteria (Section 2.3.1) and the newly ligated plasmid isolated as described previously (Section 2.3.2).

2.3.7 Colony PCR

Prior to isolation of newly ligated plasmids, colony PCR was performed to ensure the isolated colony contained the expected insert. Half the colony was picked using a sterile pipette tip and added to a PCR Mix: 0.1µM of both forward and reverse primers, 0.2µM dNTPS, 3mM MgCl₂, 0.5µl Taq Polymerase (Invitrogen), 5µl 10x Taq Buffer, made up to 50µl with H₂O. The PCR conditions were as described in Table 2-4.

The PCR product was then purified using Qiagen PCR purification Kit and analysed by agarose gel electrophoresis (2.3.9).

2.3.8 Site-directed Mutagenesis by PCR

Complimentary oligonucleotides containing the desired mutation flanked by unaltered nucleotide sequence were designed and sent for synthesis (Thermo). PCR Mix: 0.5µM of both forward and reverse primers, 0.2µM dNTPS, 1µl Pfu Turbo, 5µl 10x Buffer, 50ng template DNA made up to 50µl with H₂O.

The PCR conditions were as follows (Table 2-5):

Stage	Temperature (°C)	Time (min)	No. Cycles
Initial Denaturation	95	5	1
Denaturation	94	1	25
Annealing	Primer specific	1	25
Extension	68	1/kb	25
Final Extension	68	10	1

Table 2-5 Site-directed Mutagenesis Conditions

Following the PCR, the sample was incubated with 1µl Dpn1 (New England Biolabs) for 1h at 37°C to digest the methylated template DNA. The remaining DNA was then used to transform competent cells for amplification (Section 2.3.1).

2.3.9 Agarose Gel Electrophoresis

DNA separation was performed using 1% (w/v) Tris-Acetate EDTA (TAE) agarose gels. Agarose was dissolved by heating in a microwave and then cooled. Ethidium Bromide (1:50000) was then added and the mixture poured into a gel castor. DNA samples, mixed with the appropriate volume of 5x DNA loading buffer were loaded along with a 1kB DNA Ladder (Invitrogen) and gels were electrophoresed at 100V in TAE Buffer. DNA stained with ethidium bromide was visualised using UV illumination on a BioRad Gel Doc System.

TAE Buffer : 40mM Tris Acetate, 1mM EDTA

5x DNA Loading Buffer : 40% Sucrose, 0.25% Bromophenol Blue

2.3.10 Plasmid Constructs

HTF-ER (Hemagglutinin-TEV-FLAG-tagged Estrogen Receptor) plasmid was a gift from S. Baron (Stanford University) and it had been derived from the cPSG5PL vector background [407]. pEGFP-C2-Rab27a^{WT} (M. Strom, Imperial College) -Rab27a^{SF1/F4} and Rab27a^{SF2} (C.

Wasmeier, Imperial College) were prepared as described previously [145]. pCMV-AC-GFP-ATP1a1 and pCMV-AC-ATP1b3 were purchased from OriGene.

2.3.11 DNA Sequencing

Primer Extension sequencing was carried out by Cogenics (Beckman Coulter Genomics). 10µl of 100ng/µl plasmid were sent along with 10µl of 10µM primers unless one of their custom primers was suitable.

2.3.12 HTF-Rab27a Cloning

Rat Rab27a^{WT}, Rab27a^{SF1/F4} and Rab27a^{SF2} were PCR amplified (PCR Mix: 2µM primers, 0.25mM dNTPs, 1.5mM MgCl₂, 5µl 10xBuffer, 1µl Taq Polymerase, made up to 50µl with H₂O) from pEGFP-C2 vectors using the primers described below to incorporate NheI and SacI restriction sites (underlined) at the N- and C-termini respectively.

5'-CTAGCTAGCTCGGATGGAG-3'

5'-CGAGCTCTCAACAGCCGC-3'

PCR Conditions were as follows (Table 2-6):

Stage	Temperature (°C)	Time (min)	No. Cycles
Initial Denaturation	95	0.5	1
Denaturation	94	0.5	35
Annealing	55	1	35
Extension	72	1.5	35
Final Extension	72	10	1

Table 2-6 HTF-Rab27a Cloning Conditions

PCR products were purified using Qiagen Gel Extraction Kit and then cloned into pCR2.1-TOPO vector according to the manufacturer's instructions. The newly generated plasmids

(TA-Rab27a^{WT}, TA-Rab27a^{SF1/F4}, and TA-Rab27a^{SF2}) were then transformed into DH5α *E.coli* cells, plated on LB Agar + Ampicillin and the plasmid isolated using Qiagen Miniprep Kit (Section 2.3.2).

The HTF-ER plasmid underwent site-directed mutagenesis to remove a NheI restriction site outside of the multiple cloning site. The primers below were designed (underlined indicates altered base), synthesised (Thermo) and used as described above (Section 2.3.8) to produce the HTF-ER^{-NheI} plasmid.

5'-GACTAGTGCCAGCCCCGAACG-3'

5'-GTTCTGGGGGCTGGCACTAGTC-3'

The HTF-ER^{-NheI} plasmid then underwent restriction digestion using NheI and SacI to remove the ER insert. The plasmid DNA (HTF-plasmid) was isolated using the Qiagen Gel Purification Kit.

The TA-Rab27a plasmids then underwent restriction digestion using NheI and SacI and the digested DNA purified using Qiagen Gel Extraction Kit. The Rab27a inserts were then ligated into the digested HTF-plasmid, transformed into XL10 Gold *E.coli* (Stratagene) and the new HTF-Rab27a plasmids isolated using the Qiagen Miniprep Kit (Section 2.3.2).

2.4 Protein Techniques

2.4.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were prepared by boiling 5-10min in SDS Loading Buffer (5xBuffer:- 0.3M Tris-HCl pH 6.8, 10% (w/v) SDS, 40% (v/v) Glycerol, 0.02% (w/v) Bromophenol Blue, 10% (v/v) β-mercaptoethanol). Proteins were separated on either 10 or 12.5% (v/v) SDS-PAGE gels. Resolving gels (10 or 12.5% acrylamide) were made using 30% acrylamide polymerised using

Ammonium Persulfate (APS) and N,N,N',N'-Tetramethylethylenediamine (TEMED) in 375mM Tris pH8.8 with 1% SDS. Stacking gels (6% acrylamide) were made as above but with 125mM Tris pH6.8. Gels were cast using the Hoeffer Mighty Small gel casting system and electrophoresed in the Hoeffer Mighty Small apparatus using SDS-PAGE Running Buffer (24mM Tris, 192mM Glycine, 0.1% (w/v) SDS) at 40mA per gel. A molecular weight standard (BioRad) was run alongside samples for molecular weight calibration.

2.4.2 Silver Staining of SDS Gels

In the preparation of SDS gels for Silver Staining, particular care was taken to avoid keratin contamination. This involved washing all the gel casting equipment and glass troughs with Nitric Acid. SDS-PAGE protein gels were fixed in Fixer (50% MeOH, 12% Acetic Acid, and 0.05% Formalin) for 2h at RT. Gels were then washed 3x20min in 35% ethanol (EtOH) before sensitising in 0.02% $\text{Na}_2\text{S}_2\text{O}_3$ for 2min then washing 3x5min in H_2O . The gels were stained in Silver Nitrate (0.2% AgNO_3 , 0.076% Formalin) and washed 2x1min in H_2O before adding to Developer (6% Na_2CO_3 , 0.05% Formalin, and 0.0004% $\text{Na}_2\text{S}_2\text{O}_3$). Developing was stopped when the edges of the gel started to turn brown by transferring to Stop Solution (50% MeOH, 12% Acetic Acid) for 5min.

2.4.3 Western Blotting

Immobilon-FL Polyvinylidene difluoride (PVDF) membrane (Millipore) was pre-soaked in 100% Methanol before equilibrating in Transfer Buffer (25mM Tris, 192mM Glycine, 20% Methanol). Proteins were transferred to the PVDF membrane using the Hoefer TE22 mini tank transfer unit at 500mA for 1.5-3h. Following transfer, the membrane was washed in PBS+0.1% Tween (PBST), then non-specific binding was blocked by incubating the membrane in 10% (w/v) Semi-Skimmed Milk in PBST for 1h, RT. The membrane was then incubated with the primary antibody diluted in 10%Milk-PBST for 1h at RT or overnight at 4°C. The membrane was then washed 3x5min in PBST. Appropriate HRP-conjugated secondary

antibodies (Dako) were then diluted 1:10'000 in 10%Milk-PBST and incubated with the membrane for 1h, RT. The membrane was then washed 3x in PBST. The membrane was incubated with ECLplus Reagent (GE Healthcare) and chemiluminescence was detected by subsequent exposure of Amersham Hyperfilm ECL (GE Healthcare) and development of the films in a Xograph Compact X4 Developer. Quantitative analysis was performed using Image J Software.

2.4.4 Coomassie Blue Staining of PVDF Membrane

PVDF membranes were stained in Coomassie Staining Solution (0.25% (w/v) Coomassie, 45% (v/v) MeOH, and 10% (v/v) Acetic Acid) by agitation for 5min at RT. The membrane was then destained in Destain Solution (30% (v/v) MeOH, 10% (v/v) Acetic Acid) until the background was white.

2.4.5 Determination of Protein Concentration

Protein concentrations of cell lysates and recombinant proteins were measured using either the colourimetric bicinchoninic acid (BCA) Assay (Thermo Scientific) or using a Nanodrop Spectrophotometer ND-1000 (Labtech).

2.4.5.1 BCA Assay

BCA assay was carried out according to manufacturer's protocol. Briefly, a protein standard curve was created using bovine serum albumin (BSA) 2-30µg/ml. 5µl of sample was added to 2ml of the A+B Buffer Mix (50:1 v/v) in cuvettes and incubated at 30°C for 30min, the absorbance was then measured at 562nm. This method was used when detergent was present in the sample.

2.4.5.2 Nanodrop

Protein concentration was calculated by measuring the OD at 280nm (OD of 1 = 1mg/ml). The extinction coefficient and molecular weight were taken into account when measuring

recombinant protein. This method is not compatible with high levels of detergent as bubbles interfere with the readings.

2.4.6 Preparation of Cell Lysates

2.4.6.1 Cell Harvesting

Cells grown in flasks were detached with trypsin as detailed before (2.2.1.2). The cells were resuspended in the appropriate volume of Buffer (depending on the experiment).

Alternatively, cells grown in plates or dishes were washed using PBS, then the appropriate buffer was added and the cells were scraped from the culture dish.

2.4.6.2 Cell Lysis

Cells were then lysed by passage through a 25G needle 20 times. Alternatively, for gentle cell lysis and maintenance of the intracellular structures, cells were passed through a cell cracker with a 7 μ m gap, 30 times. The total cell lysate (TCL) could then be clarified by centrifugation at 700 $\times$ g, 10min, 4°C, resulting in the post-nuclear supernatant (PNS).

2.4.7 Subcellular Fractionation

For basic subcellular fraction, whereby the membrane and soluble fractions are separated, the TCL or PNS were centrifuged at 100'000 $\times$ g, 1h, 4°C in a Beckman TLA45 rotor in a Beckman Optima TL Benchtop Ultracentrifuge to separate the soluble supernatant (S100) fraction, and the membrane insoluble pellet (P100) fraction.

In experiments where more extensive subcellular fractionation was required a sucrose density gradient was used. Melan-INK4a cells were grown in 2x150cm² flasks until 90% confluent. Cells were harvested by trypsinisation and pelleted. The cells were resuspended in 5ml Buffer (10mM HEPES pH 7.2, 1mM EDTA, 0.25M Sucrose and 1x Protease Inhibitor Cocktail (PI; Roche)) and lysed using a cell cracker. A sucrose gradient was created with 2ml

layers of increasing concentrations of sucrose from 2.0-0.25M. The PNS was layered on top of the gradient and the sample centrifuged at 100'000 g , 1h, 4°C (Beckman L7-65 Ultracentrifuge) in a swinging bucket rotor (Sorvall Surespin 630). Fractions were collected by removing 2ml fractions from the top. These were made up to 25ml in the above buffer and centrifuged at 17'000 g , 30min, 4°C. Following removal of the supernatant the pellets were resuspended in a small volume of buffer and analysed by separation by SDS-PAGE and immunoblotting.

For immunofluorescence analysis, each fraction was diluted 1:10 and 500 μ l were aliquoted onto coverslips in a 24-well plate. The plate was centrifuged 1'500 g , 5min, 4°C. Coverslips were fixed in 4% PFA and immunostained as described above (2.2.9).

Immunofluorescence images were taken on a fluorescence confocal microscope.

2.4.8 Melanosome Purification

Melanosome purification was adapted from [408]. Cells were grown until confluent in a 150cm² flask and then harvested by trypsinisation. Washed cells were then resuspended in 5ml of chilled Homogenisation Buffer (HB) (250mM Sucrose, 50mM Imidazole, 1mM EDTA, 10mM MgCl₂, 0.15mg/ml Casein, 1mM DTT and 1xPIs) and, lysed on ice using a cell cracker. PNS was then collected by centrifugation at 600 g for 5min at 4°C. The PNS was then centrifuged 3x10min at 2500 g at 4°C and the pellets collected and pooled (P2500). The P2500 was resuspended in 2ml of chilled HB and 500 μ l aliquots layered on top of 500 μ l 50% Percoll in microcentrifuge tubes. The sample was centrifuged 20min, 8'000 g 4°C. The pellets were collected, pooled and diluted up to 1.5ml HB and centrifuged 10min, 5'000 g , 4°C. The final pellet was resuspended in 200 μ l HB.

For immunofluorescence analysis, the purified melanosomes were diluted 1:500 in HB then 500 μ l aliquoted onto coverslips in a 24-well plate. The plate was then centrifuged 1500 g ,

5min, 4°C. Coverslips were then fixed in 4% PFA and immunostained as described above (2.2.9).

Immunofluorescence images were taken on a fluorescence confocal microscope. The images were then analysed using Simple PCI Software (Hamamatsu) to measure the average fluorescence intensity of each melanosome.

2.4.9 Effector-Pulldown Assay

Cells from a single well in a 6-well plate were washed in PBS, then 150µl Buffer (50mM Tris pH7.5, 150mM NaCl, 1mM DTT, 1x PI) was added. Cells were then harvested by scraping and lysed by passage through a 25G needle. PNS was then collected by centrifugation at 800xg, 10min 4°C. Following protein quantification, 25µg total protein PNS, 20µl pre-washed Glutathione-Sepharose beads (Amersham Biosciences) and 20µM of either purified GST or GST-Slp1 (produced previously by A. C. Figueiredo and A. K. Tarafder [59]) were combined. Samples were incubated for 45min at RT. Sepharose beads were pelleted, the supernatant removed and then the sepharose beads were washed three times with Buffer (as above). Samples were then prepared for SDS-PAGE and immunoblotting for Rab27a.

2.4.10 Immunoprecipitation

The appropriate cells were harvested and lysed. The PNS was then solubilised using 1% CHAPS and pre-cleared with equilibrated Protein A/G Ultralink Resin (Protein A/G Beads; Thermo Scientific). Antibodies against the protein of interest or its tag were added to the sample and incubated >2h, 4°C, rotating. This was then added to equilibrated Protein A/G beads and incubated a further 2h. The un-bound supernatant was removed and the beads washed three times prior to resuspending in SDS-Loading Buffer and boiling for 5min. Immunoprecipitation was assessed by separation by SDS-PAGE and immunoblotting.

2.4.11 Tandem-Affinity-Purification (TAP)

For each TAP, 3x150cm² flasks of stable HTF-Rab27a cells were used (4x10⁷ cells). These were harvested by trypsinisation and pellets collected and flash frozen in liquid nitrogen and stored at -80°C.

2.4.11.1 Protein Solubilisation

Cell pellets were resuspended in 300µl Buffer A (50mM Tris pH7.5, 150mM NaCl, 5mM MgCl₂, 1mM DTT, 1xPI) and lysed by passage through a 25G needle. 1% CHAPS was then added to solubilise Rab27a and incubated 1h, 4°C, rotating. The solubilised fraction (S100) was then collected by subcellular fractionation (2.4.7).

2.4.11.2 HA-Immunoprecipitation

S100 was pre-cleared by incubation with 100µl pre-equilibrated Protein A/G Beads, 1h, 4°C, rotating. 50µg mouse anti-HA antibody was then added to the pre-cleared S100 and incubated 2h, 4°C, rotating. This was then added to 250µl pre-equilibrated Protein A/G Ultralink Resin and incubated for 2h, 4°C, rotating. The supernatant (HA-IP S) was then removed and the beads washed 3 times in Buffer A (HA-IP-B).

2.4.11.3 TEV Cleavage

The washed beads were then resuspended in 200µl Buffer A including 10µl (10U) AcTEV Protease (Invitrogen) and incubated overnight, 4°C, rotating. The supernatant (TEV S) was then isolated and the beads washed 3 times in Buffer A (TEV-B).

2.4.11.4 FLAG-Immunoprecipitation and Elution

13.6µg mouse anti-FLAG antibody was added to TEV S and incubated 2h, 4°C, rotating. This was then added to 50µl pre-equilibrated Protein A/G Beads and incubated 2h, 4°C, rotating. The supernatant (FLAG-IP S) was then isolated and the beads washed 3 times in Buffer A (FLAG-IP-B). The beads were then resuspended in 100µl Buffer A including 45µg FLAG

peptide (Sigma) and incubated 2h, 4°C, rotating. The supernatant (pep S) was then isolated and the beads washed 3 times in Buffer A (pep-B).

2.4.12 Mass Spectrometry Analysis

Preliminary analyses were performed by Dr. P. Hitchin (Biochemistry Department, Imperial College) but final analyses were performed by Dr. P. Ajuh (Dundee Cell Products).

2.4.12.1 In-Solution Digestion Sample Preparation

FLAG-eluted samples were dialysed (Snakeskin Pleated Dialysis Tubing, 10,000 MWCO, Thermo Scientific 68100) in x1000 volume of sample Buffer (50mM Tris pH7.5, 150mM NaCl, 5mM MgCl₂, 1mM DTT) over night, 4°C. Buffer was then replaced and samples dialysed for a further 4h.

2.4.12.2 In-Gel Digestion Sample Preparation

FLAG-eluted samples were separated by SDS-PAGE and Silver Stained (as detailed above). Bands of interest were excised, transferred to microcentrifuge tubes and shipped at RT.

2.4.12.3 In-solution Digestion by Paul Hitchen

For in-solution digestion, the samples were dried then dissolved in 25 µL of 8M urea containing 50mM ammonium bicarbonate (pH 8.4), reduced with DTT, alkylated with iodoacetic acid, and digested in solution with sequencing grade modified trypsin (Promega sequencing grade modified trypsin, prepared according to manufacturer's instructions). Tryptic peptides were then dried on a vacuum centrifuge and resuspended in 80 µL 0.1% trifluoroacetic acid, prior to mass spectrometry.

2.4.12.4 Gel Electrophoresis and In-gel Digestion by Paul Hitchen

Samples were run on a 10% pre-cast gel (Invitrogen) and stained with colloidal Coomassie staining solution (Novex, Invitrogen). Bands were excised, destained in 400µl of 50% (v/v) acetonitrile in 0.1M ammonium bicarbonate, pH 8.4 and dried using a SpeedVac. Gel slices

were reduced with 10mM DTT at 56°C and alkylated with 55mM iodoacetic acid for 30min at RT. The peptides were then digested with 20µl of trypsin working solution and incubated overnight at 37°C. The supernatant was removed and the tryptic digest halted by addition of 50 µl of 0.1% trifluoroacetic acid (v/v) for 15min at 37°C, followed by the addition of 100 µl acetonitrile for a further 15min at 37°C. The supernatant was removed and pooled. The extraction step was repeated with the pooled supernatants. The volume of the combined supernatants was subsequently reduced using a SpeedVac drying apparatus.

2.4.12.5 NanoLC-MS/MS Analysis by Paul Hitchen

For offline liquid chromatography MALDI TOF/TOF-MS analysis, tryptic peptides were separated using the Ultimate 3000 LC system (Dionex, Sunnyvale CA), fitted with a Pepmap analytical C-18 nanocapillary (75 µm internal diameter x15 cm length; Dionex). The digest was loaded onto the column and eluted using solvent A (0.1% (v/v) formic acid in 2% (v/v) acetonitrile and solvent B (0.1% (v/v) formic acid in 90% (v/v) acetonitrile), in the following gradient: 0-60% solvent B (0- 60 min), 60-90% solvent B (60-61 min), 90% solvent B (61-66 min) and 100% solvent A (66-67 min). Eluting fractions were mixed with α-cyano-hydroxy cinnamic acid matrix and spotted onto a metal MALDI target plate using a Probot (LC Packings, Dionex, Sunnyvale, CA). MALDI TOF/TOF MS was performed using an Applied Biosystems 4800 mass spectrometer in the positive reflectron mode and set for delayed extraction. MS precursor acquisition was followed by interpretation and data-dependent MS/MS acquisition with the CID on. Data interpretation was configured to select a maximum of 10 precursor ions per fraction with a minimum signal-to-noise ratio of 50. Sequazyme peptide mass standards were used as external calibrants.

2.4.12.6 Database Searches by Paul Hitchen

LC-MS/MS data were used to search the SwissProt database (Release 57.8) with the Mascot database search algorithm (version 2.1.0, www.matrixscience.com) for peptide sequences

consistent with the ion fragments observed. For each search, the parameters used were: peptide masses fixed as monoisotopic, partial oxidation of methionine residues considered, partial carboxymethylation of cysteine residues considered, mass tolerance 100 ppm, fragment ion tolerance 0.3 Da, tryptic digests assumed to have no more than one missed cleavage, and taxonomy group searched *Mus musculus*. Probability-based Mowse scores were generated for each peptide sequence identified and only values of greater than 95% significance were considered. Positive identification of a protein required identification of two or more unique peptide sequences identified with ions scores of greater than 95% significance.

2.4.12.7 Gel Electrophoresis and In-gel Digestion by Dundee Cell Products

Samples were reduced in SDS-PAGE loading buffer containing 10mM DTT and alkylated in 50mM iodoacetamide before boiling and separation by 1-D SDS PAGE on a 4-12% Bis-Tris Novas mini-gel (Invitrogen). Proteins were visualised by colloidal Coomassie staining (Novex, Invitrogen). The entire lane was excised and divided into 10 gel slices. Each slice was subjected to in-gel digestion with trypsin. The tryptic peptides were extracted by 1% formic acid, acetonitrile and lyophilized in a speedvac (Helena Biosciences) and resuspended in 1% formic acid.

2.4.12.8 LC-MS/MS by Dundee Cell Products

Trypsin digested peptides were separated using an Ultimate U3000 (Dionex Corporation) nanoflow LC-system consisting of a solvent degasser, micro and nanoflow pumps, flow control module, UV detector and a thermostated autosampler. 10 µl of sample (a total of 2µg) was loaded with a constant flow of 20µl/min onto a PepMap C18 trap column (0.3 mm id x 5 mm, Dionex Corporation). After trap enrichment peptides were eluted onto a PepMap C18 nano column (75 µm x 15 cm, Dionex Corporation) with a linear gradient of 5-35% solvent B (90% acetonitrile with 0.1% formic acid) over 65 minutes with a constant flow of

300 nl/min. The HPLC system was coupled to a LTQ Orbitrap XL (Thermo Fisher Scientific Inc) via a nano ES ion source (Proxeon Biosystems). The spray voltage was set to 1.2 kV and the temperature of the heated capillary was set to 200°C. Full scan MS survey spectra (m/z 335-1800) in profile mode were acquired in the Orbitrap with a resolution of 60,000 after accumulation of 500,000 ions. The five most intense peptide ions from the preview scan in the Orbitrap were fragmented by collision induced dissociation (normalised collision energy 35%, activation Q 0.250 and activation time 30 ms) in the LTQ after the accumulation of 10,000 ions. Maximal filling times were 1,000 ms for the full scans and 150 ms for the MS/MS scans. Precursor ion charge state screening was enabled and all unassigned charge states as well as singly charged species were rejected. The dynamic exclusion list was restricted to a maximum of 500 entries with a maximum retention period of 90 seconds and a relative mass window of 10 ppm. The lock mass option was enabled for survey scans to improve mass accuracy [409]. Data were acquired using the Xcalibur software.

2.4.12.9 Database Searches by Dundee Cell Products

The derived peak list was searched with the Mascot search engine (version 2.1.04; Matrix Science, London, UK) against a concatenated database combining 80,412 proteins from International Protein Index (IPI) protein database version 3.6 (forward database), and the reversed sequences of all proteins (reverse database). Alternatively, database searches were done using Mascot (Matrix Science) as the database search engine and the results saved as a peptide summary. During the search, proteins are successively included starting with the best-identified ones until a false discovery rate of 1% is reached; an estimation based on the fraction of reverse protein hits. Enzyme specificity was set to trypsin allowing for cleavage N-terminal to proline and between aspartic acid and proline. Carbamidomethylation of cysteine was searched as a fixed modification, whereas *N*-acetyl protein, and oxidation of methionine were searched as variable modifications.

3 Identification of Candidate Targeting Factors for Rab27a

3.1 Introduction

Rab proteins are exquisitely localised to their specific intracellular membrane and this specificity is crucial to enable the tight regulation of intracellular trafficking. The exact mechanism by which this precise targeting is achieved remains to be elucidated. A number of mechanisms have been proposed including the involvement of the Rab C-terminal hypervariable domain [15], GEF cascades [66], GDF activity [148] and effector binding [16] as detailed previously (Section 1.2.6). Previous research in the Seabra laboratory has focused on investigating the putative role of the C-terminal hypervariable domain [17], effector binding and GEF activity [145] in the targeting of Rab27a to melanosomal membranes in melanocytes. Rab27a targeting to melanosomes is a good model for investigating Rab targeting as its non-redundant role in melanosome transport is well documented and loss of Rab27a targeting/function gives rise to an easily distinguishable phenotype of perinuclear melanosomal clustering [173]. In addition, the Rab27a GEF in melanocytes has been identified (R3G) [59], as have a number of Rab27a effector proteins. Finally Rab27a is not readily extracted from the membrane by GDI (M. Kirsten unpublished data) so any loss of melanosomal association is unlikely to be a result of membrane extraction.

The C-terminal hypervariable domain has long been proposed to contain the targeting information required to direct Rabs to their correct membrane. Exchange of the Rab5 C-terminal 35 residues with the corresponding residues of Rab7 resulted in targeting of the Rab5/7 hybrid to Rab7 intracellular compartments [15] in a number of systems [142, 143].

Following on from these findings, the role of the C-terminal hypervariable domain in targeting of Rab27a to melanosomes was investigated. Exchanging the C-terminal domain of Rab27a with those of Rab5 or Rab1 did not re-target the Rab27a hybrid to early endosomes or the Golgi respectively, and both Rab27a hybrids were able to rescue the *ashen* phenotype (i.e. peripheral distribution of melanosomes was restored). Similarly, the C-terminal hypervariable domain of Rab27a was not sufficient to re-target Rab5 or Rab1 [17]. These results suggest that the Rab27a C-terminal hypervariable domain is not required for subcellular targeting of Rab27a to melanosomes in melanocytes.

As the hypervariable domain does not appear to contain targeting information for Rab27a, other regions of Rab27a were examined, specifically the RabF (Family) and RabSF (Sub-Family) motifs [18]. Rab27a/Rab5 chimeras were created exchanging RabF or RabSF motifs and their intracellular localisation was assessed. Rab27a^{SF2} (Rab27a VLYQYTDGKFNNSKFIT to Rab5 LVLRVFKGQFHEFQES) and Rab27a^{SF3} (Rab27a LNVRNWISQLQMHAYCENPDIVLC to Rab5 ARAKNWVKELQRQASPNIIVALS) mis-targeted Rab27a to the ER/Golgi [17]. The same mis-targeting defect was observed with the Rab5^{SF3} (Rab5 ARAKNWVKELQRQASPNIIVALS to Rab27a LNVRNWISQLQMHAYCENPDIVLC) and Rab5^{F4} (Rab5 APMYYRGA to Rab27a TTAFFRDA). It was proposed that this ER/Golgi localisation represented the default location for mis-targeted Rabs. Of note, though the YYRGA sequence of the RabF4 motif is generally conserved between Rabs, the Rab27a sequence is significantly different (FFRDA), yet the substitution of the RabF4 motif of Rab27a had no effect on its localisation [17].

The RabSF2 and RabF4 motifs are within the Switch I and Switch II domains respectively, suggesting their possible involvement in effector binding. It had previously been shown that binding to the effector TIP47 was crucial for stabilising Rab9 on its specific membrane [155] and that over-expression of TIP47 re-targeted hybrid Rabs with the Rab9 C-terminus to late endosomes [16]. To investigate the role of effector binding in Rab27a localisation,

Rab27a chimeras were generated where various RabF or RabSF motifs of Rab27a were substituted with the corresponding motif of either Rab3a or Rab5a. These chimeras were assayed for their effector binding capacity and ability to localise to melanosomes [145]. Rab27a^{F4} (Rab27a TTAFFRDA to Rab3a TTAYYRGA) lost interaction with Mlph, MyRIP, Rabphilin3a, Slp3 and Munc13-4 but retained binding to Slp1, 2, 4 and 5. Rab27a^{SF1} (Rab27a YDYLIKF to Rab3a FDYMEKI) lost interaction with Mlph. Strikingly, the combination mutant Rab27a^{SF1/F4} was unable to interact with any known Rab27a effectors. Localisation experiments showed that Rab27a^{SF1/F4} retained the ability to target to melanosomes [145]. Conversely, Rab27a^{SF2} (Rab27a VLYQYTDGKFNSKFIT to Rab5a LVLRFVKGQFHEFQES) retained binding to representatives of all Rab27a effector subgroups, but as mentioned previously, localisation experiments showed that Rab27a^{SF2} failed to target to melanosomes [17]. All the mutants were correctly prenylated and were substrates for R3G, therefore these results suggest that effector binding may not be necessary for Rab27a localisation in melanocytes [145].

Finally, a role for RabGEFs in Rab recruitment has been proposed, either through the prevention of GDI-extraction or the involvement of a Rab cascade, as it has been observed for Rab Ypt32/Sec2p/Sec4p, Rab5/classC VPS:HOPS complex/Rab7 and Rab11/Rabin8/Rab8 [66-69] (see section 1.2.6.2). Following the identification of R3G as the non-redundant GEF for Rab27a in melanocytes [59] and the known presence of Rab32/Rab38 as upstream Rabs to Rab27a, a similar mechanism could be utilised for Rab27a targeting to melanosomes. Therefore the role of R3G activity in Rab27a targeting was investigated. R3G depletion caused melanosomal clustering due to an inability to activate Rab27a [59]. In addition, in R3G depleted melanocytes, both Rab27a^{WT} and Rab27a^{SF1/F4} failed to localise to melanosomal membranes (Figure 3-1) [145]. This demonstrated that GEF activity was necessary for Rab27a targeting, but it cannot be sufficient as Rab27a^{SF2} remained a substrate for R3G, yet still failed to localise to the melanosomes.

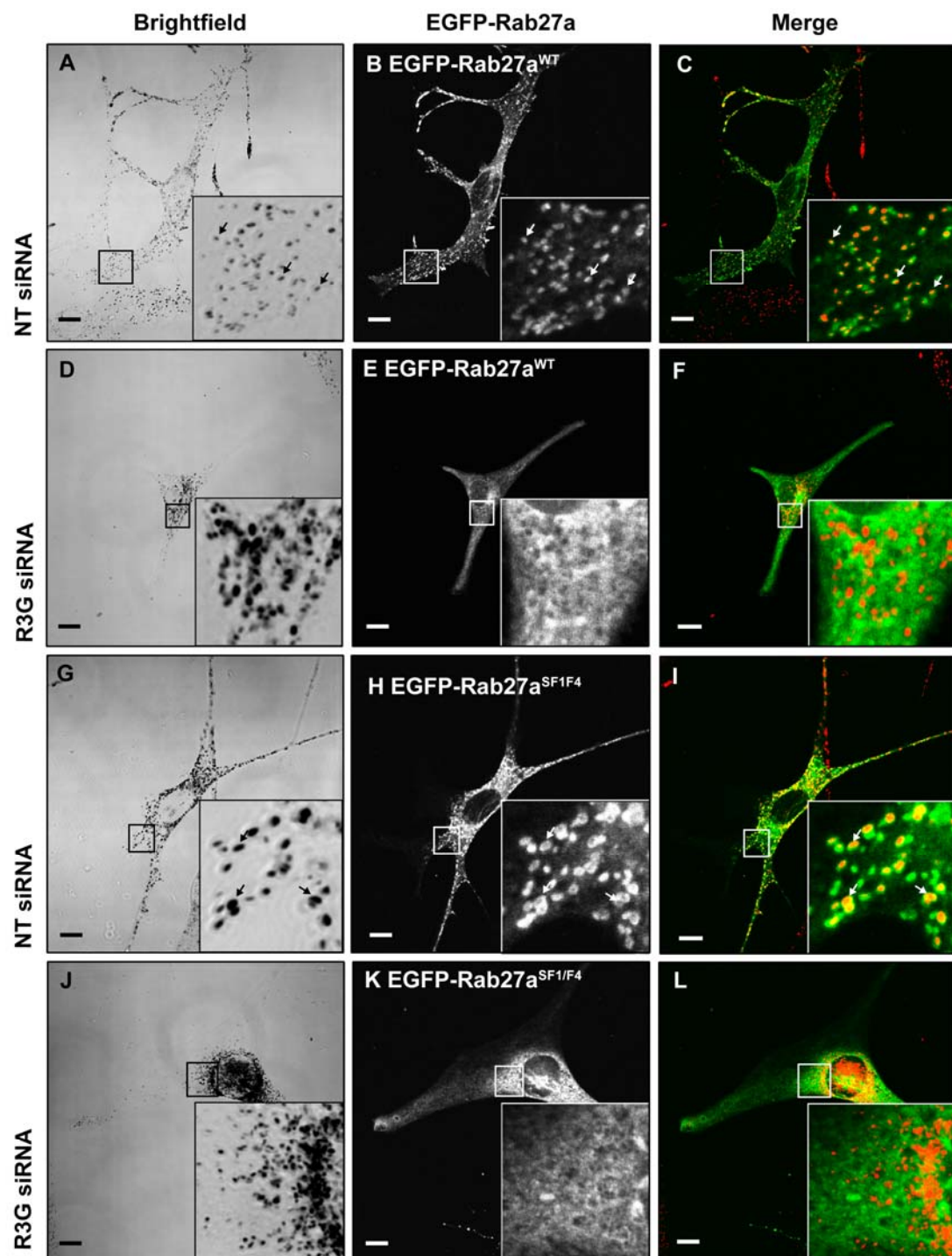


Figure 3-1 Localisation of EGFP-Rab27a^{WT} and EGFP-Rab27a^{SF1/F4} Mutants in R3G-depleted Melan-INK4a.

Melan-INK4a cells were plated on coverslips and siRNA treated with NT or R3G oligonucleotides for 72h and then transiently transfected with EGFP-Rab27a^{WT} or EGFP-Rab27a^{SF1/F4}, as indicated. Cells were fixed with 4% PFA. Images were taken on a confocal microscope. Brightfield images (A, D, G, and J) show melanosome distribution. Pigment is inverted and pseudo-coloured red to aid colocalisation with the GFP signal (B, E, H, and K) in the merge panels (C, F, I, L). Insets are a higher magnification of the boxed area. Arrows indicate colocalisation. Scale bar represents 10µm.

These findings, taken together, led to the hypothesis that Rab targeting could occur through Rab binding to a specific targeting factor. Using the previously created Rab27a mutant, Rab27a^{SF1/F4}, which is unable to bind to any known Rab27a effectors yet maintains melanosomal localisation, the aim of this project was to identify novel Rab27a interacting partners, which may then be associated to a targeting function in melanocytes, using Tandem Affinity Purification (TAP).

3.1.1 Tandem Affinity Purification

The Tandem Affinity Purification (TAP) method was first introduced by Rigaut *et al* [410] and is described as a generic protein purification method for protein complex isolation, characterization and proteome exploration (Figure 3-2). It has two major advantages over other purification methods: 1) the protein complex is formed in native conditions and 2) purification involves two rounds of immunoprecipitation resulting in less contamination of the sample from non-specific binding proteins. A standard TAP tag includes two small tags separated by a TEV (Tobacco Etch Virus) protease cleavage site. In the first instance, DNA encoding the protein of interest is cloned into the plasmid containing the tag (Figure 3-2 Step 1), which is then transfected into mammalian cells (Figure 3-2 Step 2). A stable cell line is generated, expressing the tagged-protein, through antibiotic selection and expansion of the transfected cells (Figure 3-2 Step 3). The stable cells are lysed (Figure 3-2 Step 4) and the tagged-protein (in association with its interacting partners) is solubilised and purified from

the stable cell lysates by immunoprecipitation of the first tag (Figure 3-2 Step 5). The immunoprecipitated proteins are eluted by cleavage at the TEV cleavage site with TEV Protease (Figure 3-2 Step 6). The tagged-proteins are then purified again by another round of immunoprecipitation with the second tag (Figure 3-2 Step 7). The tagged-proteins are eluted by competition for the antibody by excess of the tag peptide (Figure 3-2 Step 8). The final eluted sample is then analysed using Mass Spectrometry (MS) to identify proteins within the eluate (Figure 3-2 Step 9).

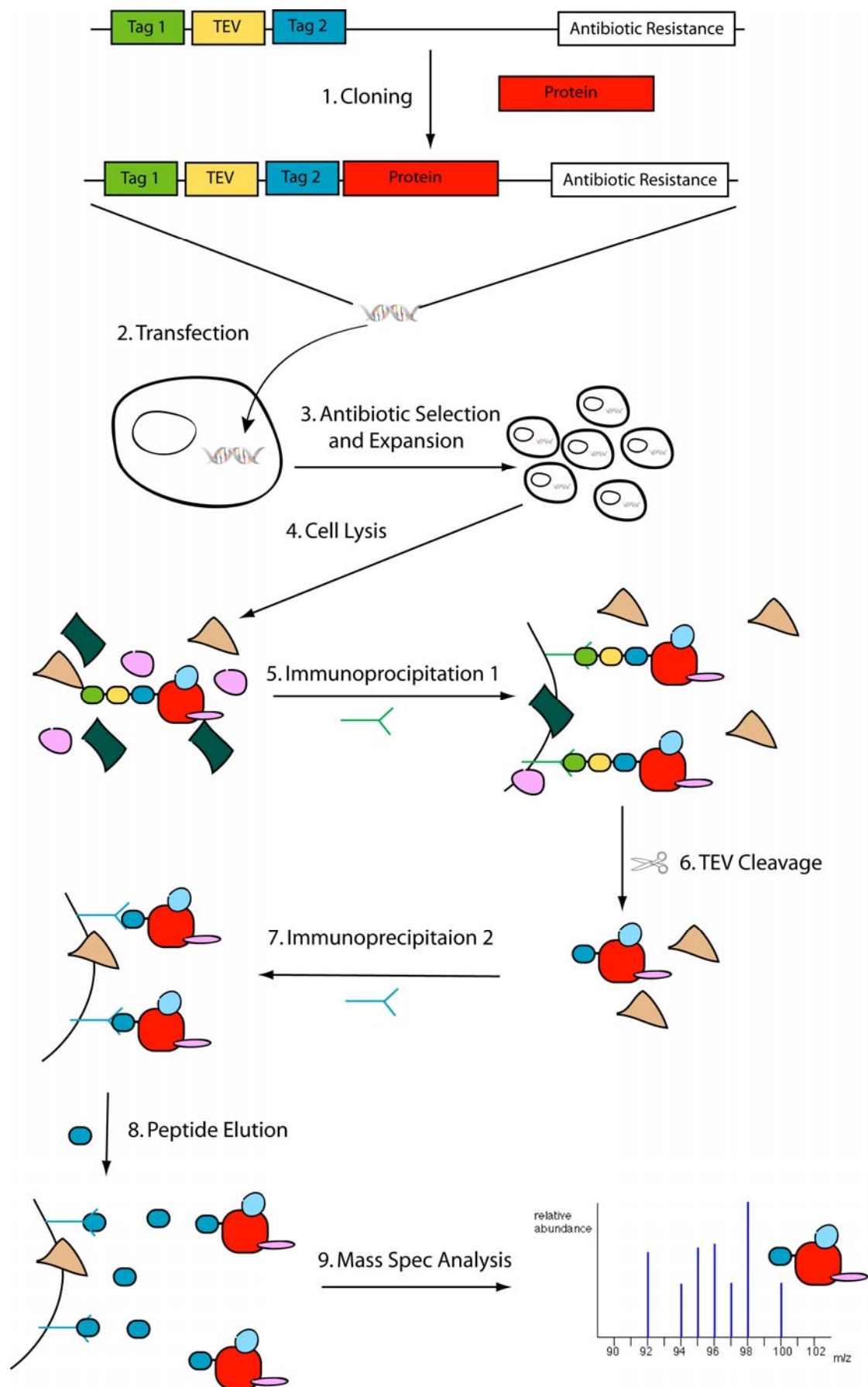


Figure 3-2 Schematic of Tandem Affinity Purification

A multistep process for the purification of protein complexes formed *in vivo*. The protein of interest is cloned downstream of a TEV Cleavage site flanked by two different tags (Step 1.). Steps 2-4; A Stable cell line is created expressing the tagged construct and the lysate of these cells is used for the TAP. Steps 5-8; TAP involves two round of immunoprecipitation separated by a TEV Cleavage Step (Step 6). Following final elution, the sample is analysed using MS (Step 9).

3.2 Site-directed Mutagenesis of HTF-plasmid

A plasmid encoding a Hemagglutinin – TEV –FLAG (HTF) tag was kindly donated by S. Baron (Stanford University).

```
AATTATGAGCTACCCATACGATGTTCTGACTATGCGGGCGGCGCCTA
TCCCTATGACGTCCCGGATTACGCAAGGAGCGGATTATGATATTCCAAC
TACTGCTAGGAATTACGAGAATTTGTATTTTCAG↓GGTGAGCTGCAGC
ACTACAAGGACGACGATGACAAAGGTACCATTACAAAGATGATGAC
GACAAGGCTAGC.....GAGCTC
```

Figure 3-3 HTF-tag sequence.

ATG translation start site underlined. HA-tag highlighted in green. TEV Protease sequence highlighted in yellow, arrow indicates TEV Protease cleavage site. FLAG-tag highlighted in blue. NheI and SacI restriction enzyme sites in bold italics.

The hemagglutinin (HA) tag is a 9-amino acid sequence (YPYDVPDYA) derived from the influenza hemagglutinin protein. The TEV cleavage site is a 7-amino acid sequence (ENLYFQG) which is specifically cleaved between the Q and G residues by TEV Protease. This enables immunoprecipitated proteins to be eluted from immobilised beads by incubation with TEV Protease (Figure 3-2 Step 6), without the requirement of changing the assay conditions which could affect the protein complex interactions. The FLAG tag is an 8-amino acid sequence (DYKDDDDK) which unlike other epitopes, was specifically designed for detection and purification of proteins. It is highly hydrophilic and therefore unlikely to affect the structure of the protein to which it is fused [411]. The plasmid also encodes Hygromycin

B resistance enabling selection of transfected cells and the creation of a stable cell line (Figure 3-2 Step 3). The plasmid also encodes NheI and SacI restriction sites for the cloning of the DNA sequence encoding the protein of interest (Figure 3-2 Step 1). However, before use, a second NheI restriction site downstream of the cloning site was deleted using site-directed mutagenesis. This was achieved via a PCR reaction using primers designed to introduce a cytosine in place of a thymine, therefore removing the NheI restriction site sequence (see Materials and Methods). The success of the mutagenesis was assessed by digestion with NheI. Successful mutation of the second NheI site resulted in a linearised plasmid following NheI digestion (see Figure 3-4 lane 1). Digestion of the original plasmid (HTF-ER plasmid) confirmed activity of the NheI enzyme as a 1965bp fragment (HTF-ER) was excised (Figure 3-4 lane 2). The mutated plasmid was used for subsequent cloning and referred to as the HTF-ER^{NheI}-plasmid.

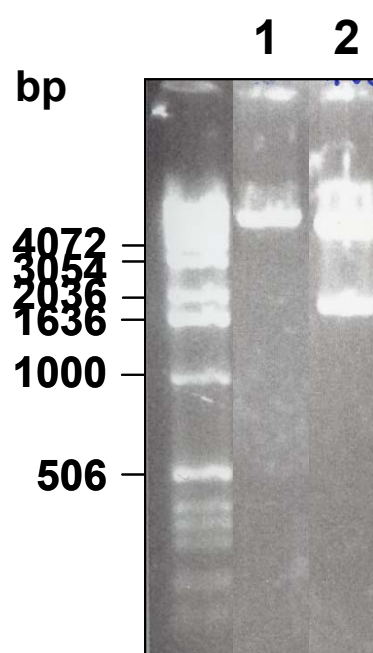


Figure 3-4 Site-directed Mutagenesis of HTF-ER Plasmid.

The original HTF-ER plasmid was mutagenised to remove a second NheI restriction site. The mutagenesis was assessed by digestion with NheI (1). The original HTF-ER plasmid was used as a control for the restriction enzyme function (2).

3.3 Generation of HTF-Rab27a Constructs

Rab27a^{WT}, Rab27a^{SF1/F4} and Rab27a^{SF2} were PCR amplified from mammalian (pEGFP-C2) expression vectors (described previously [145]) using primers to incorporate NheI and SacI restriction sites. The PCR products were sub-cloned into the pCR2.1-TOPO vector and subsequently transformed into bacteria for amplification. Digestion of the purified plasmids with NheI and SacI restriction enzymes isolated DNA fragments of the size expected for Rab27a (669bp) (Figure 3-5 A lanes 2-4). The HTF-ER^{NheI} plasmid was also digested with NheI and SacI to excise the ER insert, from which the linearised empty plasmid (HTF^{NheI}-plasmid) was isolated (Figure 3-5 A lane 1). The HTF^{NheI}-plasmid and Rab27a inserts were gel purified and the inserts ligated into the HTF^{NheI}-plasmid. NheI and SacI digestion of the newly ligated purified plasmids, following bacterial amplification, demonstrated that the Rab27a inserts had been sub-cloned into the HTF^{NheI}-plasmid as 669bp fragments were excised from the plasmids (Figure 3-5 B arrow). Digestion of the HTF^{NheI}-ER plasmid was used as a control for the restriction enzymes, and successfully excised the original ER insert (1790bp). One clone of Rab27a^{WT} (colony 3) and Rab27a^{SF1/F4} (colony 2) and Rab27a^{SF2} (colony 6) were amplified, sequenced and used for subsequent experiments.

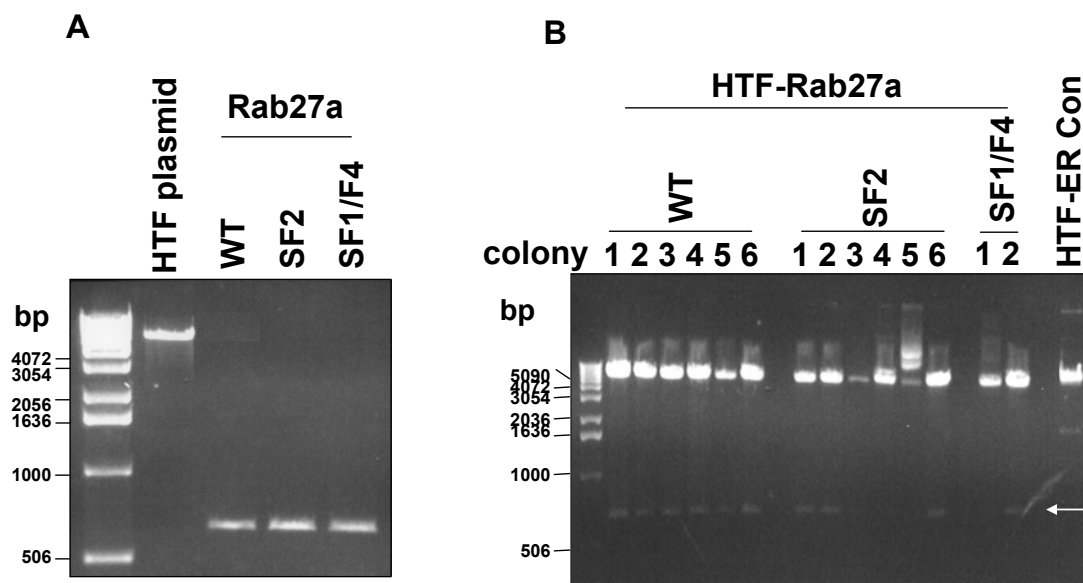


Figure 3-5 Sub-Cloning of Rab27a into HTF^{Nhe}-plasmid

Rab27a^{WT}, Rab27a^{SF1/F4} and Rab27a^{SF2} were PCR amplified from pEGFP-C2 vectors using primers to introduce 5'-NheI and 3'-SacI restriction sites. A) HTF^{Nhe}-plasmid following digestion with NheI and SacI and removal of the ER insert (lane 1) and Rab27a PCR products following digestion with NheI and SacI (lane 2-4). B) Purification of the digestion products and ligation of the inserts into the HTF^{Nhe}-plasmid resulted in HTF-Rab27a plasmids as confirmed by NheI and SacI digestion. The arrow indicates the excised Rab27a insert. The HTF^{Nhe}-ER plasmid was used for a control for the restriction enzymes.

3.4 Generation of a Stable Melanocyte Cell Line

Expressing HTF-Rab27a^{WT} and HTF-Rab27a^{SF1/F4}

The TAP method requires the creation of stable cell lines expressing the tagged protein of interest for optimal yield. The HTF-plasmid contains the DNA sequence for antibiotic resistance to Hygromycin B so the cytotoxicity of Hygromycin B treatment on melanocytes needed to be assessed to enable antibiotic selection for transfected cells. Melan-ash (Rab27a^{-/-} melanocytes), a murine melanocyte cell line, (Kind gift from D. Bennett, St George's) was used to create the stable cell line to eliminate competition from endogenous Rab27a. Melan-ash cells were treated with increasing concentrations of Hygromycin B and cytotoxicity was assessed at two time points (Figure 3-6). At days 5 and 7, 100µg/ml

Hygromycin B was sufficient to cause over 90% cell death. These results indicated that a Hygromycin B concentration of 100µg/ml was a suitable dose for antibiotic selection.

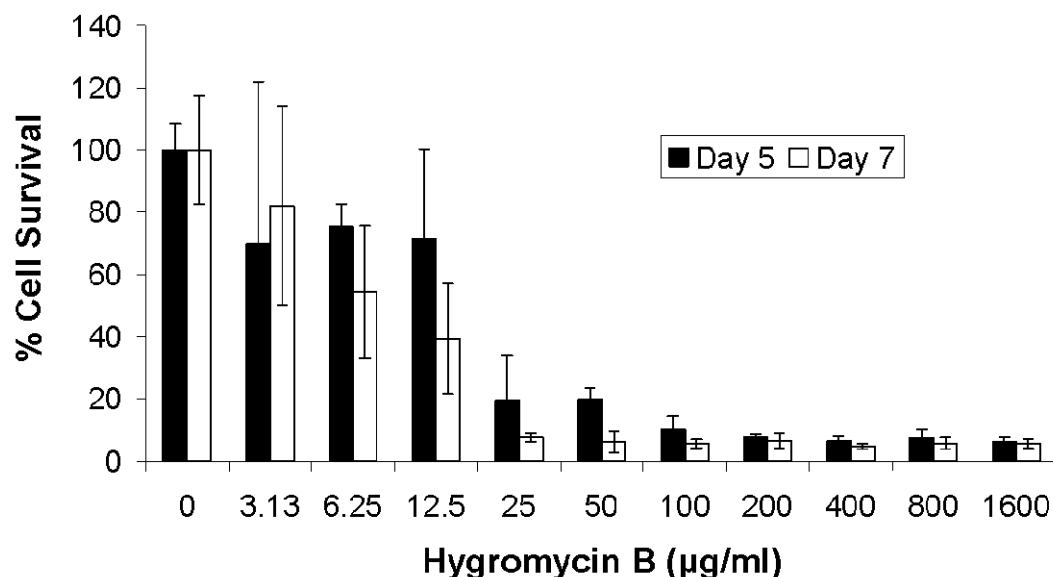


Figure 3-6 Hygromycin B Cytotoxicity.

Melan-ash cells were treated with increasing concentrations of Hygromycin B for up to 7 days. Cytotoxicity was measured as described in Material and Methods 2.2.8. Error bars indicate Standard deviation, n=3.

Melan-ash cells were transfected with HTF-Rab27a^{WT}, HTF-Rab27a^{SF1/F4} or HTF-Rab27a^{SF2} (Figure 3-2 Step 2). Rab27a^{SF2} was to be used as a negative control as it would not be expected to interact with a targeting factor as it fails to correctly localise. After transfection, the melanocytes were grown for 72h before the addition of 100µg/ml Hygromycin B to select for melanocytes containing the plasmid, resulting in stable cell lines (Figure 3-2 Step 3). After a week in selection the cells were harvested and a single cell suspension produced to result in one cell per well in a 96-well plate. The cells were then left to expand to give colonies derived from single cells before further expansion.

Before TAP purification, the stably transfected cell lines were characterised to ensure that the HTF-Rab27a constructs mimicked the properties of their GFP-Rab27a counterparts, which have been described previously [145].

3.5 Membrane-Association of HTF-Rab27a

First, membrane-association of HTF-Rab27a constructs in stably transfected melanocytes was analysed. Endogenous Rab27a is prenylated, which allows association with membranes and partitioning into the membrane fraction after sub-cellular fractionation by centrifugation at 100,000xg. In order to verify that the HTF-Rab27a constructs were correctly membrane-associated, stably transfected melanocytes were harvested and the TCL partitioned into the soluble (S) and membrane pellet fractions (P) and immunoblotted with anti-Rab27a antibody. With all the HTF-Rab27a constructs, the majority of the fusion protein partitioned into the membrane fraction indicating that the protein was associated with membranes and had undergone correct post-translational prenylation (Figure 3-7). Immunoblotting for calnexin was used as a control for protein loading and also as an indicator of efficient partitioning between membrane and soluble fractions as calnexin is a membrane associated protein (Figure 3-7). Two different HTF-Rab27a^{SF1/F4} clones were tested, highlighting that the level of expression varied between clones. It was decided that the F4 clone was preferable as it had a higher expression level despite this leading to a larger pool of soluble Rab27a, which was also seen in the HTF-Rab27a^{WT} and HTF-Rab27a^{SF2} samples. Addition of the HTF tag to Rab27a possibly interfered with 100% efficient post-translational processing, as is often the case with tagged proteins. Alternatively, over-expression of HTF-Rab27a overwhelmed the prenylation machinery, both of which would result in a small fraction of soluble protein. Although endogenous Rab27a is primarily membrane-associated, it is unknown whether the putative targeting factor binds Rab27a in the soluble fraction and takes it to the membrane or if it is itself a membrane

bound protein. Hence, it was decided that exclusion of the soluble pool of HTF-Rab27a from the analysis would be detrimental as it could possibly eliminate a fraction of HTF-Rab27a bound to the targeting factor but not yet at the membrane.

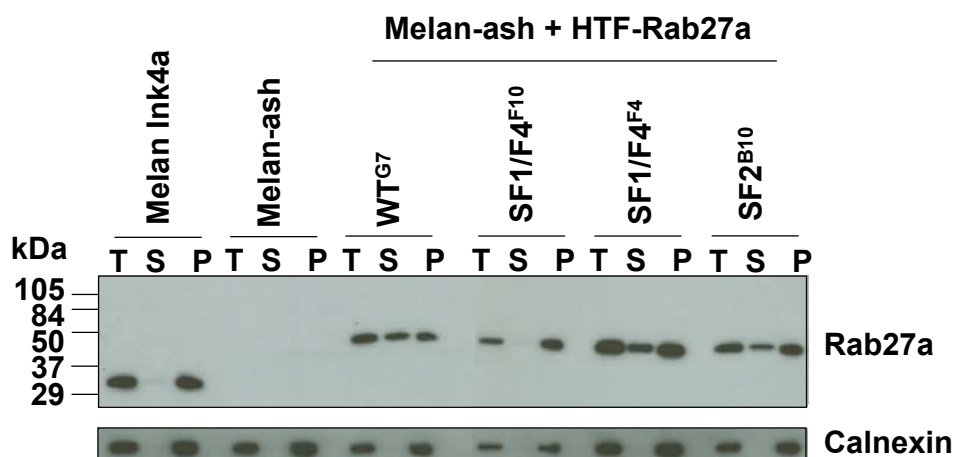


Figure 3-7 HTF-Rab27a Membrane Association in Stably Transfected Melan-ash Cells.

Melan-ash cells were stably transfected with HTF-Rab27a. The TCL (T) was partitioned into the membrane containing pellet fraction (P), and the soluble fraction (S) by centrifugation at 100,000xg. Rab27a was analysed by immunoblotting using mouse anti-Rab27a antibody. Calnexin was used as a loading control. Melan-INK4a cells express endogenous Rab27a (30kDa). Melan-ash cells express no endogenous Rab27a. HTF-Rab27a transfected Melan-ash melanocytes express higher molecular weight Rab27a (45kDa). Superscript lettering indicates clone number.

3.6 Localisation and Functionality of HTF-Rab27a

In melan-ash melanocytes, with no endogenous Rab27a, melanosomes are clustered in the perinuclear region of the cell (Figure 3-8 D), but addition of HTF-Rab27a^{WT} enabled the peripheral distribution of melanosomes by formation of the tripartite complex with Mlph and MyoVa (Figure 3-8 G). The restoration of melanosome distribution is a clear indicator of correct Rab27a function. Immunofluorescence staining with anti-HA antibody indicated that HTF-Rab27a^{WT} extensively co-localised with melanosomes (Figure 3-8 I). HTF-Rab27a^{SF1/F4} localised to melanosomes (indicated by arrows on zoom), yet failed to restore the peripheral distribution of melanosomes (Figure 3-8 J-L). HTF-Rab27a^{SF2} failed to localise to

melanosomes, therefore it did not restore the peripheral distribution of melanosomes (Figure 3-8 M-O). These results are comparable to those seen previously with the EGFP-tagged Rab27a constructs [145], therefore indicating that the HTF-tagged Rab27a^{WT} is fully functional and the HTF-Rab27a mutants behave as expected.

As membrane-association, localisation and functionality of these clones were as expected, the cells were expanded for their use in the TAP. However, analysis of the HTF-Rab27a^{SF2} cells after extensive expansion showed that in a proportion of cells, the fusion protein began to localise onto the melanosomes, which was highly unexpected. The cell line was therefore abandoned as it would no longer serve its function as a negative control. Instead, untransfected melan-ash melanocytes were used as a negative control for nonspecific binding in the TAP method.

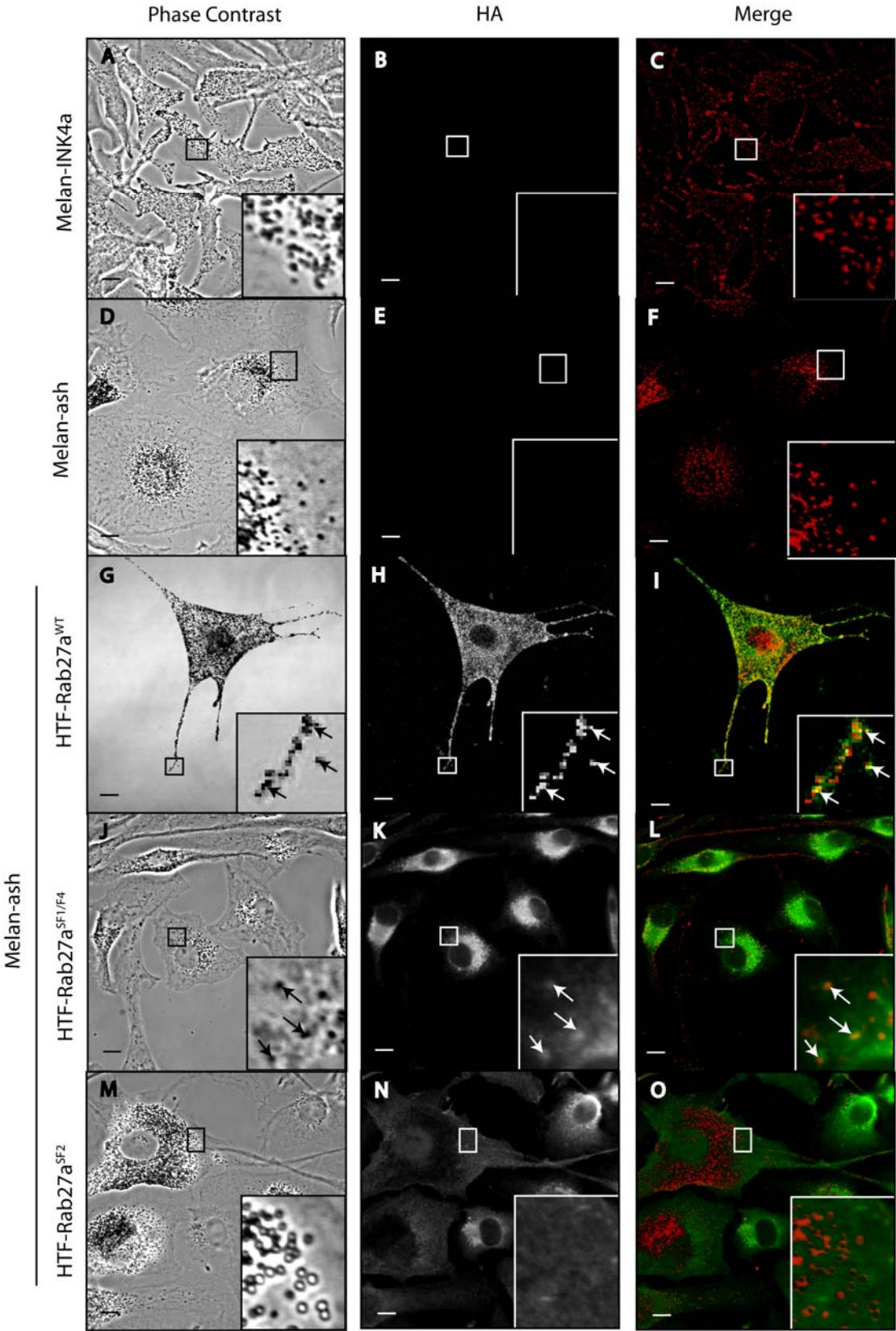


Figure 3-8 Localisation and Functionality of HTF-Rab27a Constructs in Melan-ash Melanocytes.

Melan-ash melanocytes were stably transfected with HTF-Rab27a constructs. Cells were grown on coverslips and fixed with 4% PFA. Cells were permeabilised and blocked with FBS before immunostaining with mouse anti-HA antibody and Alexa-488 conjugated anti-mouse secondary antibody. Phase contrast panels (A, D, G, J, and M) show melanosome distribution. Panels B, E, H, K and N show HA immunostaining. Pigment is inverted and pseudo-coloured red to aid colocalisation with HA (green) in the merge panels (C, F, I, L and O). Insets are a higher magnification of the boxed area. Arrows indicate colocalisation. Scale bar represents 10µm.

3.7 Tandem Affinity Purification of HTF-Rab27a^{WT} and HTF-Rab27a^{SF1/F4}

TAP is a multistep purification process requiring optimisation of each stage of the purification process.

3.7.1 Solubilising Rab27a

Rab27a is associated with the membrane and therefore requires solubilising prior to TAP. In addition, a proportion of its interacting partners may be membrane proteins that require solubilisation too. All conditions therefore had to favour extraction of the proteins from membranes without destabilising protein-protein interactions. Complete solubilisation of HTF-Rab27a was used as an indicator that interacting partners would most likely also be solubilised. Three different detergents were tested, TritonX-100, CHAPS and Cholic Acid, each an example of a non-ionic, a zwitterionic and an ionic detergent, respectively. In general, non-ionic and zwitterionic detergents are milder and less denaturing than ionic detergents and suitable for solubilising membrane proteins whilst maintaining protein:protein interactions. Ionic detergents are strong solubilising agents but can often denature proteins. Increasing concentrations of each detergent, with the lowest below the critical micellar concentration (CMC), were assessed for their ability to displace HTF-Rab27a from the membrane into the soluble supernatant (S). Immunoblot analysis of HTF-

Rab27a following detergent treatment and 100,000 $\times$ g centrifugation showed that >1% CHAPS, >0.1% TritonX-100 and >10mM Cholic Acid solubilised Rab27a (Figure 3-9).

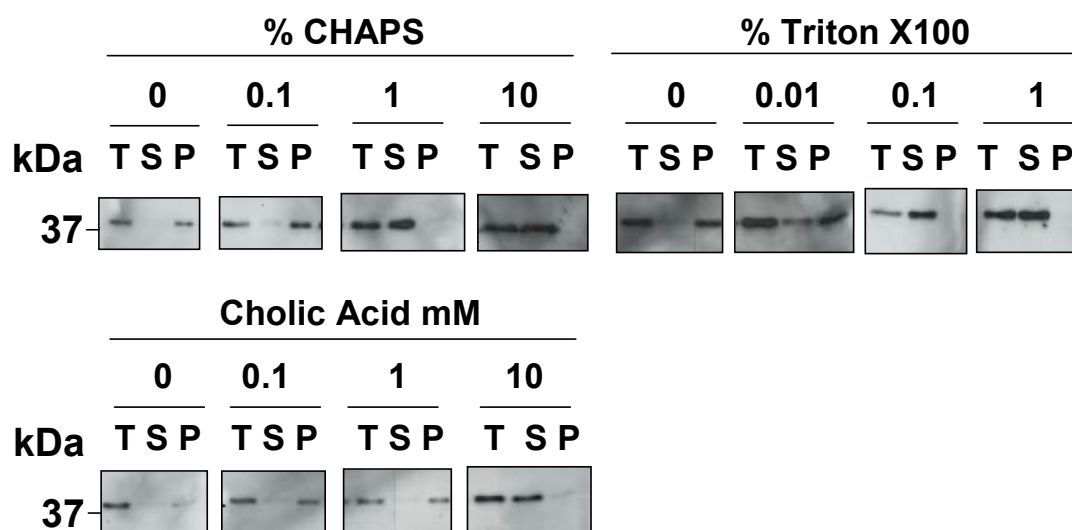


Figure 3-9 Solubilisation of HTF-Rab27a Using Different Detergents.

Melan-ash cells transiently transfected with HTF-Rab27a^{SF1/F4} were lysed and detergents added to the TCL (T) as indicated. Samples were incubated for 1h before centrifugation at 100,000 $\times$ g to separate the soluble (S) and membrane (P) fractions. HTF-Rab27a solubilisation was assessed by immunoblotting with mouse anti-Rab27a antibody. Equal volumes of T, S and P fractions were loaded for each condition.

3.7.2 HA Immunoprecipitation

Following solubilisation of HTF-Rab27a, the first round of purification involves immunoprecipitation (IP) using the HA-tag. Mouse anti-HA antibody was added to the S100, which was solubilised using various detergent conditions (1% CHAPS, 0.1% TritonX100, 10mM Cholic Acid). The efficiency of HTF-Rab27a IP was analysed by immunoblotting for Rab27a in the un-bound supernatant (S) and bound Bead (B) fractions. It was found that all three buffer conditions efficiently allowed purification of the majority of the HTF-Rab27a using the mouse anti-HA antibody (Figure 3-10).



Figure 3-10 HA-Immunoprecipitation of HTF-Rab27a^{WT} in Different Detergent Conditions.

Melan-ash melanocytes stably transfected with HTF-Rab27a^{WT} were harvested and the TCL solubilised with the indicated detergent. The TCL was separated into membrane and soluble (S100) fractions by centrifugation at 100,000xg. The S100 was pre-cleared with protein G Sepharose Beads and then incubated for 2h at 4°C with 1µl mouse anti-HA antibody per 50µl S100. Protein G Sepharose beads were added for an additional 2h. The un-bound supernatant fraction (S) was removed and the beads (B) washed, resuspended in the same volume of SDS-Loading buffer and boiled. Rab27a was analysed by immunoblotting with mouse anti-Rab27a antibody. Equivalent volumes were loaded per sample.

For the final MS analysis it was crucial that the sample be highly concentrated to maximise the identification rate. Therefore experiments were scaled up from using 1x10cm dish of cells to 3x15cm dishes (approx 4x10⁷ cells). The previous optimisation experiments were performed with an antibody which was later found to no longer be commercially available. Therefore a new antibody was purchased and following scaling up of cell numbers utilised, increasing amounts of antibody were tested to maximise the efficiency of the IP. Increasing the antibody used per reaction to 30µg saturated the beads as antibody bands could now be seen in the supernatant (Figure 3-11). However 20µg of mouse anti-HA antibody was sufficient to immobilise almost 100% of HTF-Rab27a onto 100µl protein A/G Beads (Figure 3-11).

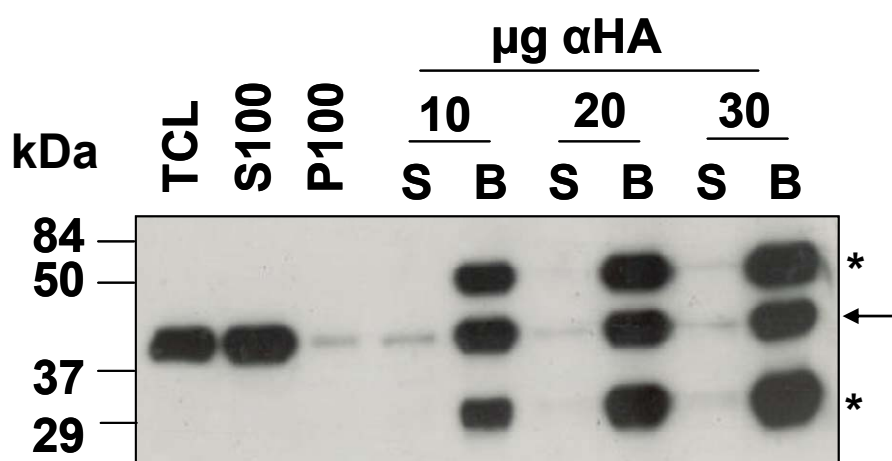


Figure 3-11 Optimisation of Mouse anti-HA Antibody Concentration and Protein A/G Bead Volume for Efficient HA-IP.

3x15cm dishes of stably transfected HTF-Rab27a^{WT} were lysed and the TCL solubilised in 0.1% Triton X-100. S100 fraction was isolated, pre-cleared and incubated with increasing amounts of mouse anti-HA antibody (10-30µg) for 2h at 4°C. 100µl of Protein A/G beads were added and incubated for 2h at 4°C. Supernatant was collected (S) and the beads (B) washed, resuspended in the same volume of SDS-Loading buffer and boiled. Equivalent volumes were loaded per sample. HA-IP was analysed by immunoblotting for Rab27a using mouse anti-Rab27a antibody. The arrow indicates HTF-Rab27a. Asterisk (*) indicates cross reactivity of the anti-mouse secondary antibody with the mouse anti-HA immunoglobulin heavy and light chains.

On consultation with the facility performing the downstream MS analysis (Paul Hitchen, Imperial College), it was noted that if the samples were to be analysed using in-solution digestion then the analysis would be hampered by the presence of detergent, which interferes with the liquid chromatography step. Previous experiments had used Triton X-100 which is non-dialysable, however CHAPS is readily dialysable and therefore could be removed from the sample prior to analysis. As shown previously (Figure 3-9), 1% and 10% CHAPS solubilised endogenous Rab27a, so it was investigated whether these conditions were compatible with large scale solubilisation and HA-IP of stably transfected HTF-Rab27a^{WT}. 1% CHAPS solubilised the majority of HTF-Rab27a, yet 10% CHAPS was more efficient (Figure 3-12 A). However, HA-IP of the S100 was greatly reduced in 10% compared to 1% CHAPS (Figure 3-12 B). It is possible that the high detergent levels interfered with the

epitope:antibody interaction. Therefore, despite the slightly lower solubilising efficiency, 1% CHAPS was chosen as the optimal condition for the TAP.

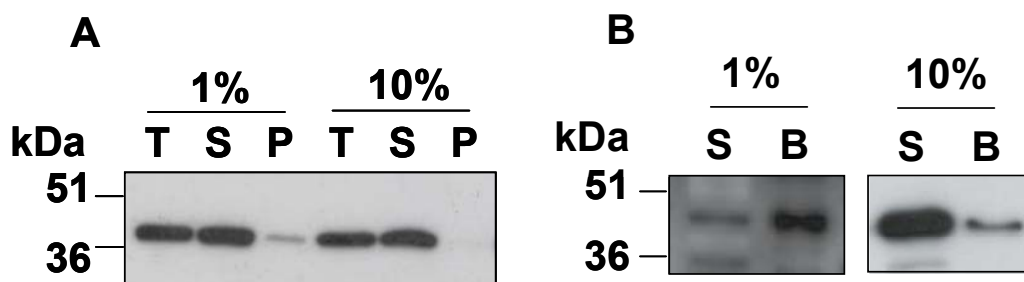


Figure 3-12 Analysis of CHAPS Conditions for Solubilising HTF-Rab27a and Efficient HA-IP.

A) 3x15cm dishes of stably transfected HTF-Rab27a^{WT} were lysed and the TCL solubilised in 1 or 10% CHAPS. S100 fraction was isolated, pre-cleared and incubated with 40µg mouse anti-HA antibody for 2h at 4°C. 200µl Protein A/G beads were added and incubated 2hr at 4°C. Supernatant was collected (S) and the beads (B) washed, resuspended in the same volume of SDS-Loading buffer and boiled. Equivalent volumes were loaded per sample. Experiments were analysed by immunoblotting using mouse anti-Rab27a antibody.

3.7.3 Optimisation of TEV Cleavage

The next stage of TAP makes use of the specificity of the TEV Protease, enabling elution of the purified HA-tagged protein by cleavage downstream of the HA-tag therefore releasing it from the immobilised antibody. As at this stage the ultimate buffer conditions for MS were not confirmed, the activity of the TEV Protease was investigated using different detergent conditions (1%CHAPS, 0.1%Triton X-100, 10mM Cholic Acid). The efficiency of the TEV cleavage was assessed after 2h and 5h incubations. After 2h the sample was divided in two and one half continued incubating up to 5h whilst the other was stopped by removing the supernatant and washing the beads. The TEV cleavage was assessed by immunoblotting the supernatant and beads for Rab27a after 2h and 5h incubations. After 2h incubation with the TEV protease (10U) (Figure 3-13 A), in the presence of 0.1% Triton X-100, the vast majority of HTF-Rab27a was released into the supernatant (band at 37kDa) and therefore cleaved from the beads. This was unsurprising as the commercial protease buffer contains 0.1% Triton X-100. With 1% CHAPS, over 50% of the HTF-Rab27a had been eluted, this increased further

with longer incubation (Figure 3-13 B, relative intensity of Rab27a in B and S). However with 10mM Cholic Acid, the TEV Protease failed to cleave the HTF-Rab27a from the beads, even with extended incubation (HTF-Rab27a is still in the beads fraction).

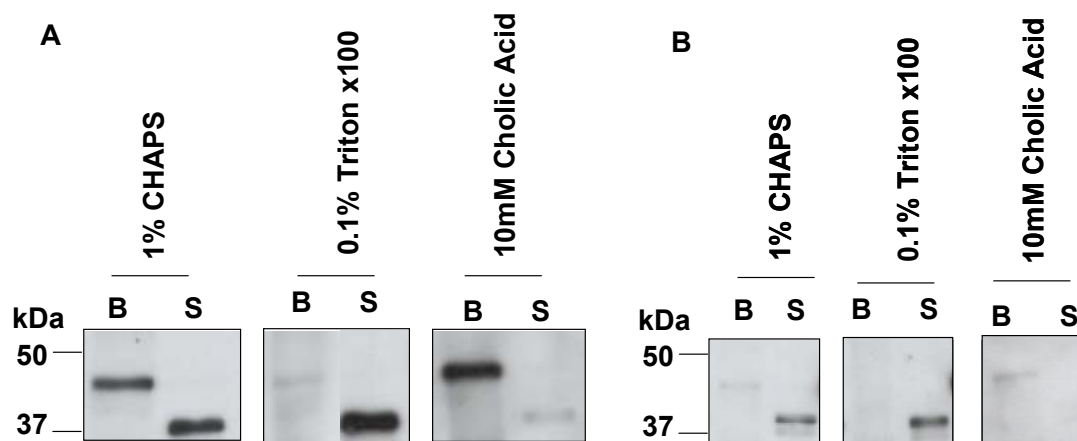


Figure 3-13 TEV Protease Activity Under Various Detergent Conditions.

Following HA-IP from 10cm dish of HTF-Rab27a^{WT}, the beads were washed, and resuspended in 100µl of buffer including 10U AcTEV Protease and incubated at 4°C for A) 2h or B) 5h. The supernatant was then removed (S) and the beads (B) washed, resuspended in the same volume of SDS-Loading buffer and boiled. Equivalent volumes were loaded per sample. TEV cleavage was analysed by immunoblotting using mouse anti-Rab27a antibody.

3.7.4 Optimisation of FLAG Immunoprecipitation

The second stage of purification (Figure 3-2) involved incubating the TEV cleaved samples (TEV S) with anti-FLAG antibodies for the second immunoprecipitation. Initial optimisation was in 0.1% Triton X-100 and all the FLAG-tagged Rab27a was immunoprecipitated (Figure 3-14 A). Furthermore the efficiency was unaffected by scaling up the number of cells used or altering the detergent to 1% CHAPS (Figure 3-14 B).

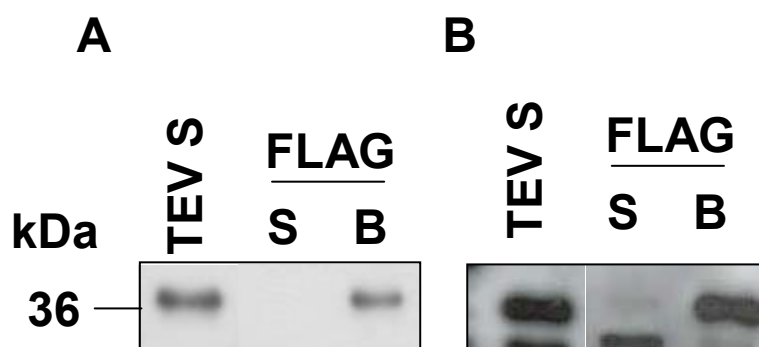


Figure 3-14 FLAG-IP Optimisation.

TEV-cleaved HTF-Rab27a (TEV S) from small (A ; 10cm dish) or large scale (B; 3x15cm dishes) TAP in A) 0.1% Triton or B) 1% CHAPS was incubated with mouse anti-FLAG antibodies (A; 3 μ g, B;13.8 μ g) for 2h at 4°C then added to A) 12.5 μ l or B) 50 μ l Protein A/G beads for a further 2h. Supernatant (S) was then removed and the beads (B) washed, resuspended in the same volume of SDS-Loading buffer and boiled. Equivalent volumes were loaded per sample. FLAG-IP was analysed by immunoblotting using mouse anti-Rab27a antibody.

3.7.5 Optimisation of FLAG-peptide Elution

The final stage of TAP involves elution of the immunoprecipitated tagged protein from the Protein A/G beads (FLAG B) (Figure 3-2). For this, FLAG peptide was added in excess to compete with the FLAG-tag for antibody binding. As with the FLAG IP, cell number and detergent did not affect the efficiency of the elution of the tagged-Rab27a as it was successfully eluted into the supernatant (Figure 3-15 pep S).

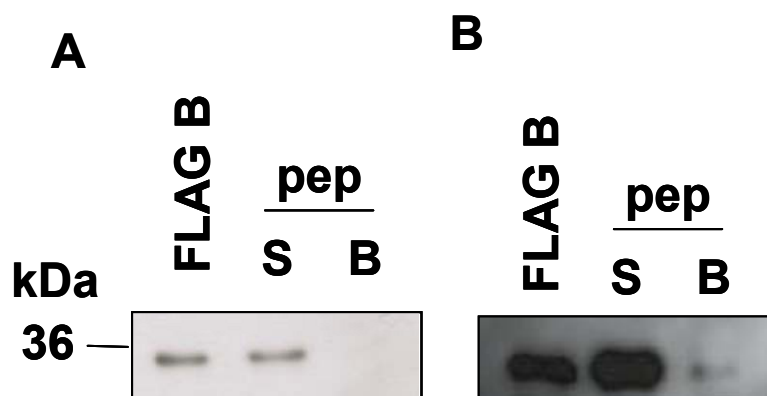


Figure 3-15 FLAG-peptide Elution.

FLAG-immunoprecipitated HTF-Rab27a (FLAG B) from small (A; 10cm dish) or large scale (B; 3x15cm dishes) TAP in A) 0.1% Triton or B) 1% CHAPS was incubated with FLAG peptide (A; 20 μ g, B; 60 μ g) for 2h at 4°C. Supernatant (S) was then removed and the beads (B) washed, resuspended in the same volume of SDS-Loading buffer and boiled. Equivalent volume was loaded per sample. FLAG peptide elution was analysed by immunoblotting using mouse anti-Rab27a antibody.

As mentioned previously, detergent would be detrimental for MS analysis and in addition, the presence of protease inhibitors (PI) could interfere with the crucial trypsin digestion step. Therefore, following FLAG-peptide elution in buffer containing 1% CHAPS and PI, the sample was dialysed. By immunoblotting, it is clear that the dialysis step resulted in a loss of protein, as it is also apparent when the samples were analysed by silver staining (Figure 3-16). To compound this, the sample was sent for MS analysis and there was insufficient protein for reliable MS analysis. It was uncertain whether the loss of protein was a result of protease degradation or the dialysis process.

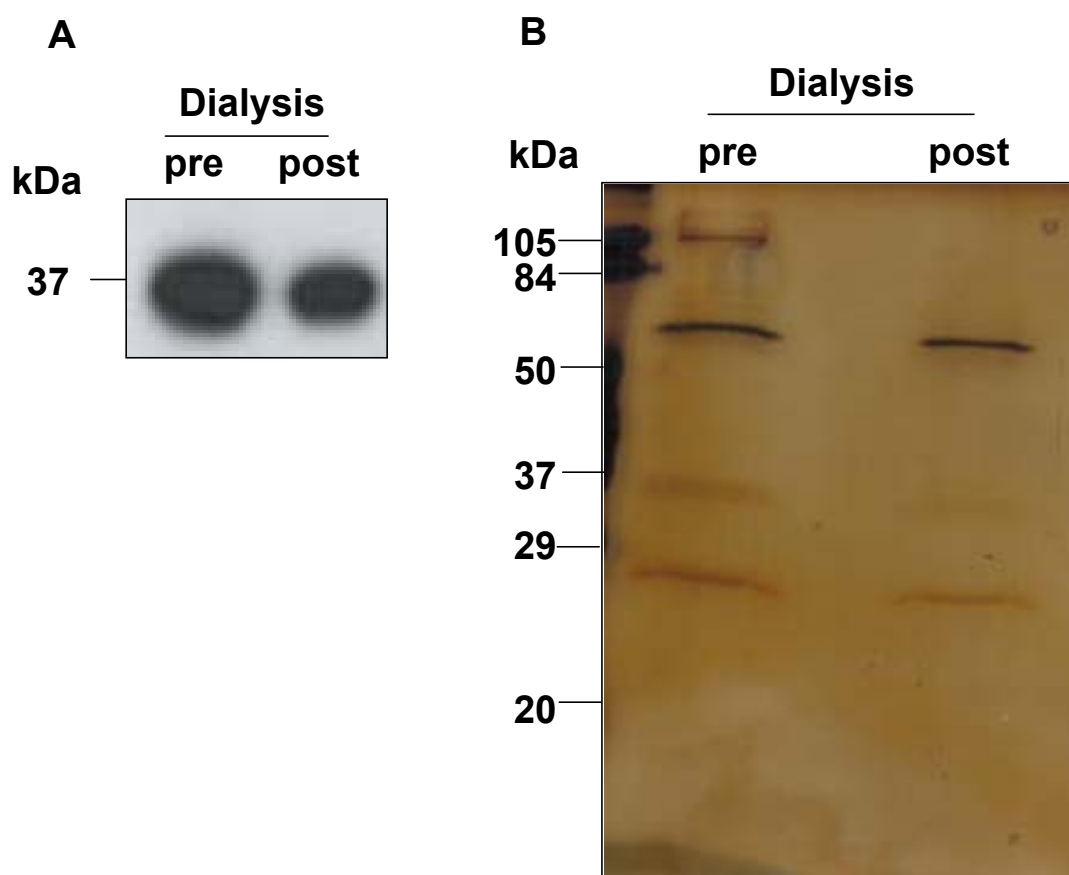


Figure 3-16 Dialysis of the FLAG-peptide Eluted Sample.

TAP was performed from 3x10cm dishes of HTF-Rab27a^{WT} cells in 1% CHAPS. After FLAG-peptide elution, an aliquot was taken (pre) and the remaining 150µl dialysed in buffer without CHAPS or PI. Samples were analysed by A) immunoblotting using mouse anti-Rab27a antibody and B) silver staining. Equal volumes were loaded per sample.

As an alternative approach, rather than removing the CHAPS and PI after elution, the final FLAG-peptide elution step was tested in the absence of detergent and/or PI. Removal of detergent prevented the efficient elution with FLAG as the majority of Rab27a was still associated with the beads (Figure 3-17 lane 2), probably a result of non-specific binding to the beads due to the absence of detergent. Removal of PI however did not affect the elution of the protein (+/+ compared to -/+).

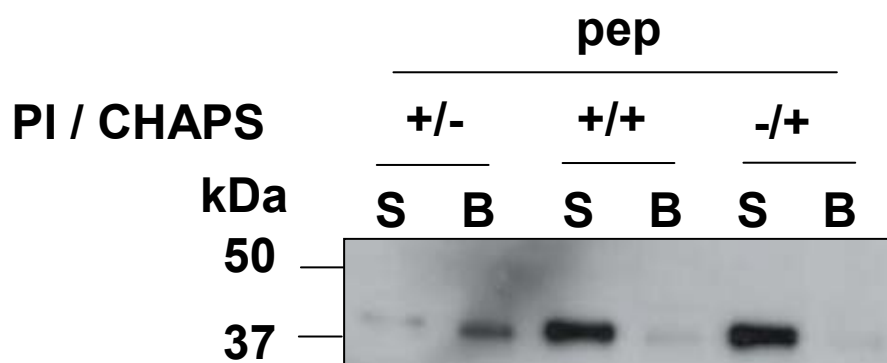


Figure 3-17 FLAG-peptide Elution Without Detergent and/or PI.

FLAG-immunoprecipitated HTF-Rab27a was washed and resuspended in 100µl buffer with or without 1%CHAPS or PI. 45µg FLAG peptide was added to each and incubated for 2h at 4°C. Supernatant (S) was removed and the beads (B) washed, resuspended in the same volume of SDS-Loading buffer and boiled. FLAG peptide elution was analysed by immunoblotting using mouse anti-Rab27a antibody. Equivalent volumes were loaded per sample.

This therefore suggested that the detergent could not be removed and so if in-solution digestion was to be used for MS analysis then it would need to be removed by dialysis, therefore requiring very high protein concentration beforehand to ensure sufficient protein after dialysis. In order to overcome the issue of concentration, multiple TAPs were performed and the samples pooled. Ultimately, TAPs were performed with HTF-Rab27a^{SF1/F4} (total of 9.2×10^8 cells), HTF-Rab27a^{WT} (total of 3.6×10^8 cells) and untransfected (total of 2.0×10^8 cells) melan-ash melanocytes.

The optimal conditions used for each TAP are detailed in Table 3-1 and an example experiment is depicted in Figure 3-18.

Step	Conditions	Incubation
Cell Number	3x15cm dishes Stably Transfected Melanocytes	-
Cell Lysis and Solubilisation	1ml Buffer A (50mM Tris pH7.5, 150mM NaCl, 5mM MgCl ₂ , 1mM DTT, Protease Inhibitor Cocktail, 1% CHAPS)	30min, 4°C
HA-IP	1ml S100, 50µg mouse anti-HA antibody + 250µl Protein A/G Beads	2h, 4°C
TEV Cleavage	100µl Buffer A, 100U TEV Protease	Overnight, 4°C
FLAG-IP	TEV S, 13.8µg mouse anti-FLAG antibody + 50µl Protein A/G Beads	2h, 4°C
FLAG-peptide Elution	100µl Buffer A without PI, 45µg FLAG peptide	2h, 4°C

Table 3-1 Optimal Conditions for TAP.

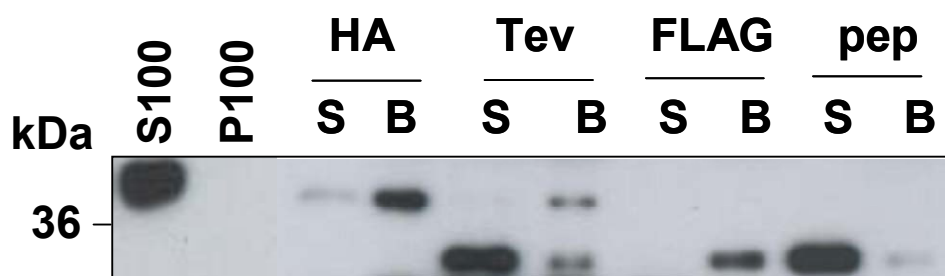


Figure 3-18 Optimised TAP of HTF-Rab27a^{WT}.

3x15cm dishes of stably HTF-Rab27a^{WT} transfected melan-ash cells were lysed and TCL solubilised in 1% CHAPS. The S100 fraction was used for TAP, using HA-IP (HA), TEV Cleavage (TEV) and FLAG-IP (FLAG) and FLAG-peptide elution (pep) steps as detailed in Table 3-1. TAP was analysed by immunoblotting using mouse anti-Rab27a antibody. Equivalent volumes were loaded per sample.

The samples were first analysed by silver staining to identify any protein bands of interest (Figure 3-19). The bands present in the untransfected melan-ash melanocyte sample indicate contamination, most likely keratin or immunoglobulins. A few bands are present in the HTF-Rab27a^{WT} (lane 2) and HTF-Rab27a^{SF1/F4} (lane 1) samples (asterisk) which are absent from the untransfected cells, therefore indicating that these could be interacting partners for Rab27a.

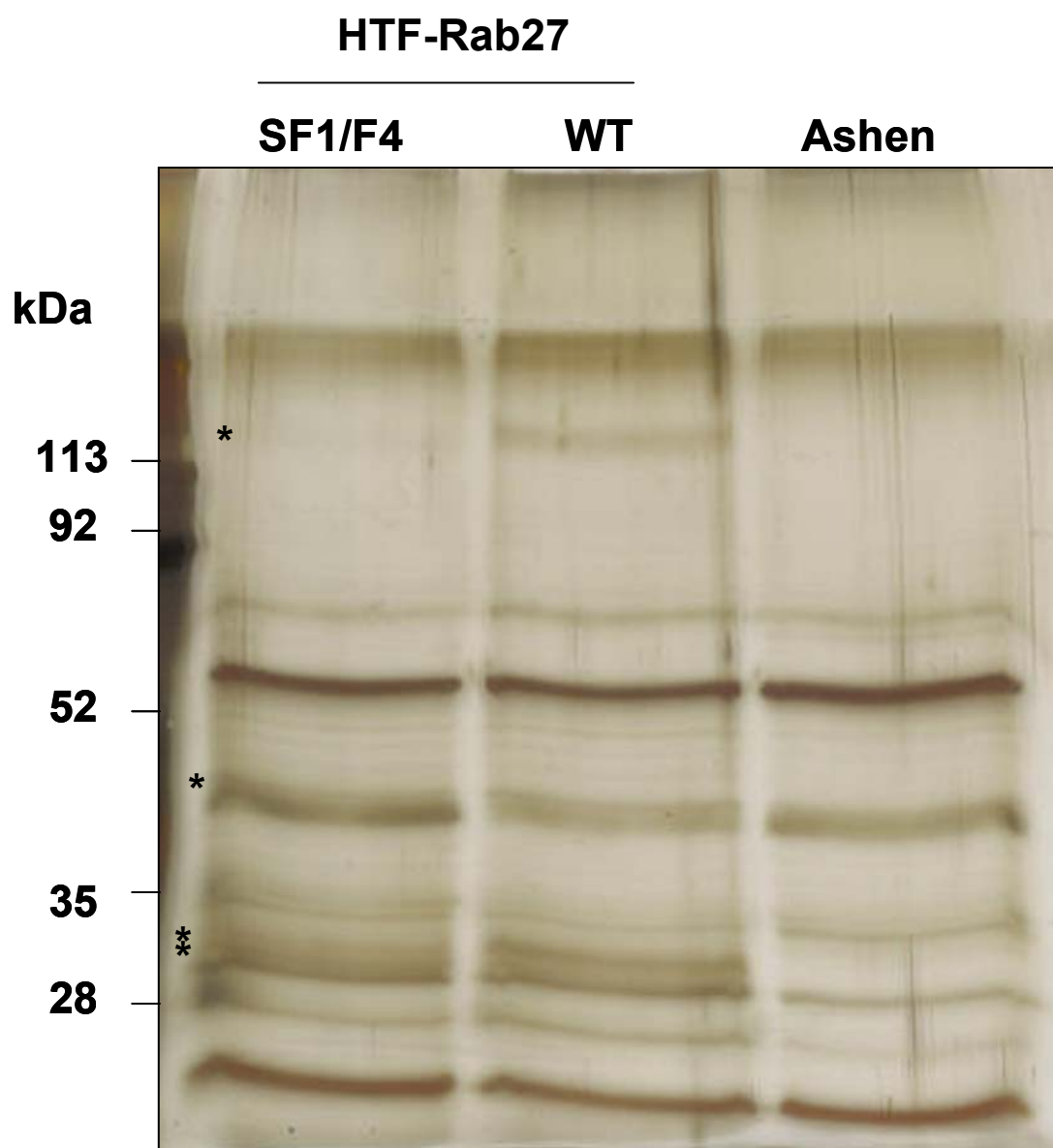


Figure 3-19 Silver Stain of the Final TAP Samples of HTF-Rab27a^{WT}, HFT-Rab27a^{SF1/F4} and Untransfected Melan-ash Melanocytes.

The final FLAG-peptide eluted samples were pooled and 50µg of each was run on a 9% gel and then proteins detected using silver staining. Asterisk (*) indicates possible Rab27a interacting proteins.

3.8 Mass Spectrometry Analysis

MS is the method of choice for analysing the components of the final eluted samples from TAPs. Routinely, samples are separated by SDS-PAGE, silver stained and specific bands excised for analysis. It was decided, however, that this method had limitations as it was not

certain that all proteins of interest would be at sufficient abundance for the sensitivity of the staining. In addition, the staining itself is known to be detrimental to subsequent MS analysis, therefore lowering the sensitivity again. There was also fear of contamination from keratin during the process which commonly compromises MS analysis by overshadowing the true proteins of interest. To confound this fear, when a band corresponding to Rab27a was excised from a silver stained gel and sent for analysis, the analysis did identify Rab27a but there was significant keratin contamination in the sample.

Following this test and discussion with those performing the analysis, it was decided that a preferable method would be to try in-solution digestion. This required the removal of the detergent as described above, however on analysis it was found that the sample was still not sufficiently concentrated.

Finally, the method settled upon was for 1ml of each of the combined FLAG-peptide eluted samples to be sent to Dundee Cell Products. There the samples were concentrated, protein separated using SDS-PAGE and the whole lane excised and analysed in 10-piece segments. On examination of the Coomassie stained gels (Figure 3-20) it was apparent that a number of protein bands were present in both HTF-Rab27a^{WT} and HT-Rab27a^{SF1/F4}, some of which could corresponded to the bands seen in the silver stained gel (Figure 3-19), whilst only very faint bands were present in the untransfected sample.

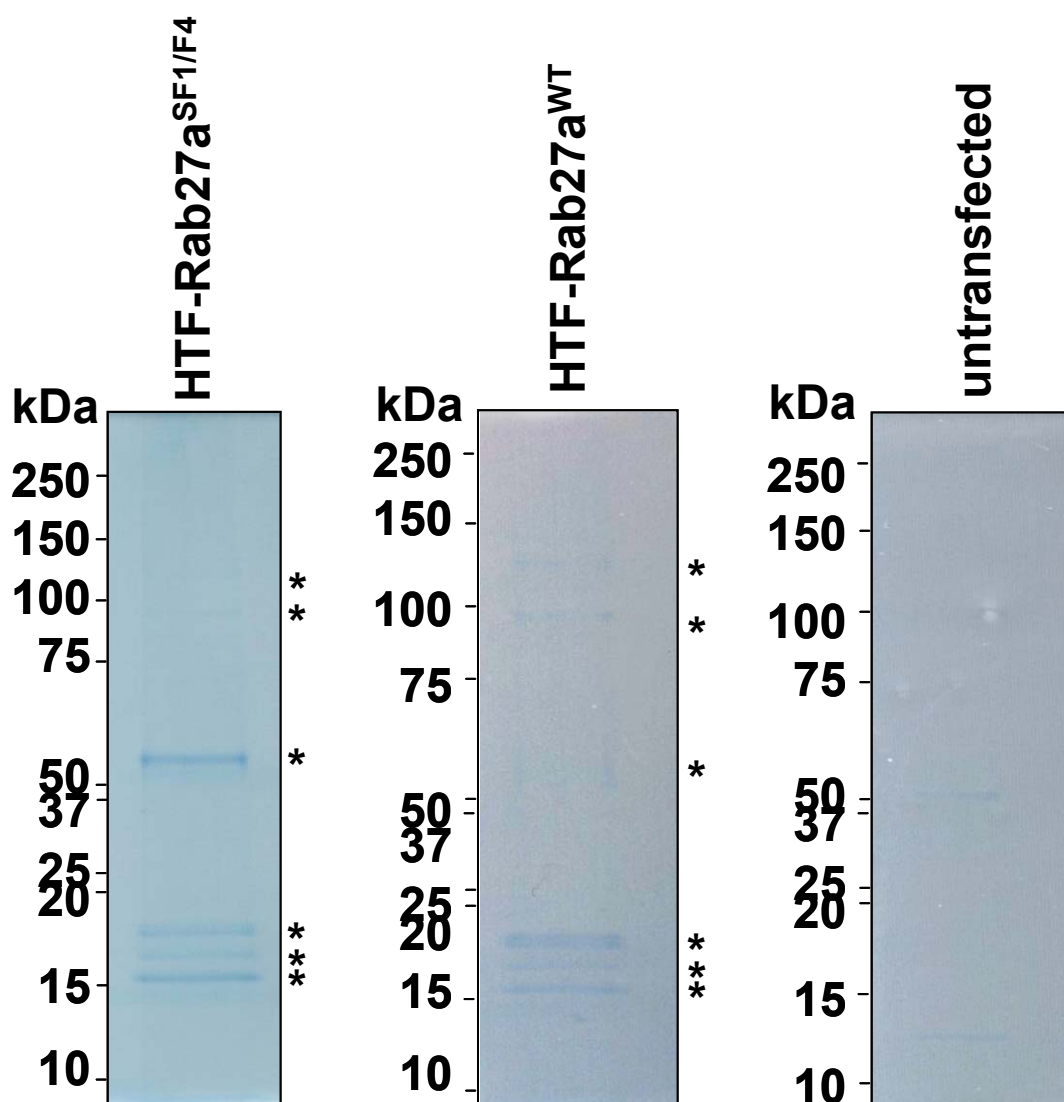


Figure 3-20 Coomassie Staining of Concentrated FLAG-peptide Eluted Samples.

Samples were separated by SDS PAGE on a 4-12% Bis-Tris Novas mini-gel. Proteins were visualised by colloidal Coomassie staining. Courtesy of Dundee Cell Products.

Gel slices were digested in trypsin and then analysed using LC-MS/MS as detailed in the methods section (2.4.12.8).

3.9 Candidate Protein Selection

The MS analysis returned a large number of hits. The untransfected melan-ash negative control returned hits for keratin, cytoskeletal proteins and trypsin. This was expected as the

Coomassie staining of this sample revealed there was very little protein, as expected for the negative control, an indication that the TAP was clean. Removal of the negative control hits from the results for HTF-Rab27a^{SF1/F4} sample resulted in 273 proteins, of which 93 also featured in the HTF-Rab27a^{WT} sample (See Appendix for complete list of HTF-Rab27a^{SF1/F4} hits).

In order to narrow the investigation of candidate proteins, a list of criteria was established. The protein had to be i) identified in both the HTF-Rab27a^{WT} and HTF-Rab27a^{SF1/F4} TAP and ii) identified in at least two of the three previous melanosomal proteomics studies [412-414]. In addition, proteins were highlighted if they iii) had a predicted molecular weight that matched the bands identified in the silver stain, iv) are peripheral/integral membrane proteins and v) known to be involved in vesicular transport.

After applying the criteria to the protein hits, a final list of candidate proteins was compiled and it is detailed in Table 3-2. Annexin AII (Anxa2) was identified in only one of the proteomics studies but was included as melanosome transport defects have been observed in the RPE of the knock-out mouse (S. Moss, UCL Institute of Ophthalmology; personal correspondence).

Official Symbol	Alternative Names	Function	Gene ID
Pmel17	Pmel, Silver, GP100, ME20M	Melanosome pigmentation [244]	Mouse: 20431 Human: 6490
GPNMB	lpd, Dchil, Osteoactivin	Uncertain. Melanogenesis?[415]	Mouse: 93695 Human: 10457
VAT1	FLJ20230	Unknown	Mouse: 26949 Human: 10493
ATP1a1	-	α -subunit of Na ⁺ ,K ⁺ ATPase. [416]	Mouse: 11928 Human: 476
TYRP1	TRP, TRP1, RP11-3L8.1, CAS2, CATB, GP75, b-Protein	Melanogenesis [417]	Mouse: 22178 Human: 115501
Anxa2	P36, ANX2, LIP2, LPC2, CAL1H, LPC2D, ANX2L4, PAP-IV	Ca ²⁺ and phospholipid binding protein [418]	Mouse: 12306 Human: 302

Table 3-2 Candidate Proteins.

Gene symbols, alternative names, functions and ID numbers as published on NCBI Gene Database.

All the above proteins represent putative Rab27a interacting partners that may be involved in Rab27a function, through regulation of its targeting to melanosomes. In order to assess their putative role in Rab27a targeting, the first step involved assessing the effect their depletion had on melanosome distribution, as an indicator of Rab27a function. TYRP1 however was not investigated further as immortalised melanocytes (Melan-b) from the TYRP1 mutant mice (*brown*) show normal melanosome distribution, indicative of normal Rab27a localisation and function [419].

3.10 Conclusions

Melan-ash melanocytes stably expressing HTF-Rab27a^{WT} or HTF-Rab27a^{SF1/F4} were created.

Using these cell lines, a TAP was optimised to precipitate HTF-Rab27a in association with putative novel interacting partners. These candidates will be verified for their involvement in Rab27a function and localisation in the next chapter.

4 Characterisation of Candidate Proteins

4.1 Introduction

The Tandem Affinity Purification of HTF-Rab27a^{SF1/F4} identified a selection of putative novel Rab27a interacting partners: Pmel17, Glycoprotein nonmetastatic melanoma protein b (GPNMB), TYRP1, Annexin A2 (Anxa2), the α 1-subunit of Na⁺,K⁺-ATPase (ATP1a1) and vesicle amine transporter 1 (Vat1). All these candidates had previously been identified in proteomic studies as melanosomal proteins [412-414]. In addition, MITF, the critical transcription factor for melanocyte development and survival [420] which transcriptionally regulates the genes involved in pigmentation, including Rab27a [242], TYRP1 [421] and Pmel17 [422] also regulates those candidate genes not previously associated with a pigimentary function: GPNMB [423], Vat1 and ATP1a1 [424].

Pmel17

Pmel17 (gp100/gp95/gp85/ME20/RPE1/SILV/MMP115) is the product of the *silver* gene, mutation of which causes the mouse coat colour mutant *silver* [425]. Pmel17 consists of a 24-residue N-terminal signal sequence [426], a 578 residue luminal domain, a 24-residue transmembrane domain and a cytosolic C-terminal targeting EXXPL motif, similar to the targeting motif seen in TYRP1 [427] and tyrosinase (Tyr) [428]. The luminal domain is composed of the N-terminal domain (NTD) which contains glycosylation sites [426], a polycystic kidney disease (PKD) domain, the Repeat (RPT) domain (a region rich in acidic residues, Pro, Ser and Thr forming repeat sequences) [429] and the Kringe-like domain

(KLD). Following the RPT domain is a proteolytic cleavage site, critical for Pmel17 function [430] (Figure 4-1).

In stages I-II of melanogenesis, Pmel17 forms intraluminal proteinaceous-fibrils providing a matrix for the subsequent deposition of melanin (stages III-IV) [244]. In stage I melanosomes, Pmel17 is cleaved into M α (luminal) and M β (membrane) fragments by a pro-protein convertase. M α then forms the proteinaceous fibrils in Stage II melanosomes. The fate of the small M β fragment after cleavage is unclear, but it is proposed to be present only on Stage I and II melanosomes [431, 432]. Given that Rab27a associates with Stage III and IV melanosomes and that only the C-terminal cytosolic domain would be accessible for Rab27a interaction, it is unclear how the two proteins would interact

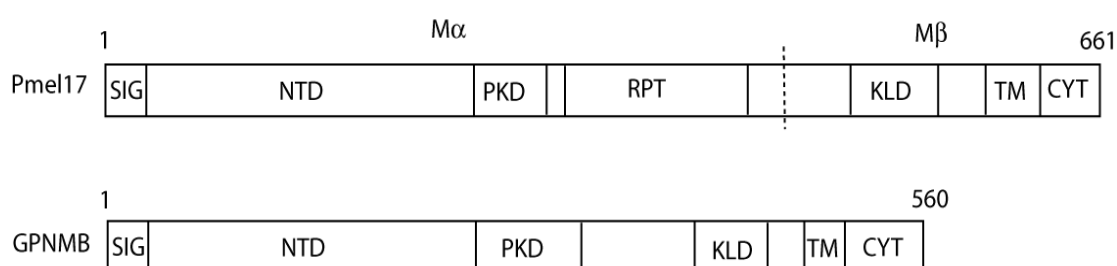


Figure 4-1 Domain Structure of Pmel17 and GPNMB.

There is high homology between Pmel17 and GPNMB. N-terminal signal domain (SIG), N-terminal domain (NTD), polycystic kidney domain (PKD), repeat domain (RPT) kringle like domain (KLD), transmembrane domain (TM) and cytosolic domain (CYT). The numbers indicate amino acid numbers. The dotted line represents the cleavage site.

GPNMB

GPNMB was first cloned from human metastatic melanoma cells and its over-expression decreased tumour growth [433]. It is a 560-residue protein with high homology to Pmel17 but lacks the RPT domain crucial for Pmel17 function (Figure 4-1). Mutations in the GPNMB gene cause iris pigment dispersion in mouse pigmentary glaucoma [415]. It is a melanosomal protein [412, 413] enriched on Stage III and IV melanosomes [434], but it has also been observed to localise at the plasma membrane [435]. The localisation of GPNMB in

the plasma membrane has been proposed to be important for melanocyte adhesion to neighbouring keratinocytes [435], but GPNMB is also thought to be released following proteolytic cleavage by metalloproteases [434]. The function of GPNMB on melanosomes remains unclear, but its enrichment on mature melanosomes could act as a maturation signal for Rab27a recruitment.

Annexin A2

Annexins bind to negatively charged phospholipids in a Ca^{2+} -dependent manner and cycle between the cytosol and distinct cellular membranes [418], showing some similarity to Rab proteins. Anxa2 interacts with $\text{PI}(4,5)\text{P}_2$ [436] via its annexin core whilst its N-terminal domain interacts with a protein named S100A10 [437]. The formation of a heterotetrameric complex with S100A10 acts as a membrane scaffold and can result in vesicle aggregation [438].

Anxa2 has been implicated in vesicular trafficking. In Anxa2 depleted cells a striking perinuclear clustering of early endosomes is observed [439]. More interestingly, Anxa2 in complex with S100A10 regulates Ca^{2+} -dependent release of von Willebrand Factor (vWF) [440] and chromaffin granule secretion [441]. Moreover, Anxa2^{-/-} mice display disrupted melanosome transport in RPE cells although they display no obvious coat colour defect (S. Moss, UCL, personal communication). All these transport processes are regulated by Rab27a and so it is possible that Rab27a and Anxa2 participate in the same transport pathways.

Na^+, K^+ -ATPase $\alpha 1$ subunit (ATP1a1)

The Na^+, K^+ -ATPase is a heterodimeric transmembrane complex, found primarily at the plasma membrane (Figure 4-2). It consists of a 10-transmembrane α -subunit and a regulatory, single transmembrane spanning β -subunit [416]. A γ -subunit has also been implicated in regulation of the protein. At the plasma membrane it has two functional roles. It is best characterised by its ion pump function which results in the export of 3 Na^+ in

exchange for 2 K⁺ following hydrolysis of ATP [416]. However, in recent years, a novel signalling function has also been characterised whereby binding of the cardiotonic steroid ouabain results in activation of Src kinase [442]. Four isoforms of the α -subunit have been identified [273, 274] with the $\alpha 1$ isoform (ATP1a1) being ubiquitously expressed [277]. ATP1a1 KO mice are non-viable and die at the blastocyst stage [278]. To date the role of ATP1a1 in membrane trafficking has not been investigated.

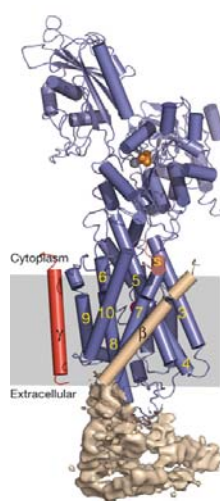


Figure 4-2 Crystal Structure of Na⁺,K⁺-ATPase.

The α -, β - and γ -subunits are coloured blue, cream and red, respectively. Helices are represented by cylinders and β -strands by arrows. The β -ectodomain is shown by surface representation of the experimental electron density. The transmembrane segments of the α -subunit are numbered (yellow) starting with the most N-terminal. The small C-terminal helix (S, for switch) is light red. Mg²⁺, MgF₄²⁻ and Rb⁺ ions are grey, orange and pink, respectively. Taken with permission from Morth *et al.* Nature 2007.

VAT1

VAT1 was identified as a major synaptic vesicle component in the Pacific electric ray *Torpedo californica* [443]. It is a 41kDa integral membrane protein, it was isolated from a murine breast cancer cell line and showed ATPase activity [444]. However, functional information for VAT1 is limited. A C-terminal Ca²⁺-binding domain displays some homology to the Ca²⁺-binding domains of the annexins [445]. It has been shown that siRNA depletion of VAT1 in glioma cells significantly reduced their migration [446], a phenotype also seen on Anxa2

depletion in glioma cells [447]. More recently it was found in neutrophils that VAT1 localises onto secondary and tertiary granules and the plasma membrane in resting cells. Upon stimulation, it translocates to the plasma membrane [448]. To date, no VAT1 KO mouse has been generated.

4.2 siRNA Depletion of the Candidate Proteins

Following the identification of candidate proteins, the first step was to assess the effect their depletion had on Rab27a function which, in melanocytes, is easily assessed as it is crucial for the peripheral distribution of melanosomes. Loss of either Rab27a [173], Mlph [184], MyoVa [449] or R3G [59] results in perinuclear clustering of melanosomes, which is easily observed using light microscopy. Candidate proteins whose depletion affects Rab27a function will then be assessed for their effect on Rab27a localisation to melanosomes.

For all candidate proteins (Table 3-2), siRNA SMART-pools were purchased from Dharmacon (Table 2-2). These were a mixture of four different oligonucleotides and the company guaranteed depletion of the target protein. The negative control was non-targeting (NT) siRNA and Mlph depletion was used as a positive control for disruption of Rab27a function [184].

In the first instance, melan-INK4a cells were treated with siRNA, cultured for 3 days, sub-cultured and a second round of siRNA treatment applied. Following the two rounds (a total of 7 days treatment), the cells were lysed and depletion of the candidate proteins assessed by immunoblotting the TCL with commercially available antibodies against each candidate protein. This initial experiment showed that Pmel17 (Figure 4-3 A), GPNMB (Figure 4-3 B), and ATP1a1 (Figure 4-3 C) were successfully depleted under these experimental conditions as there was a decrease in protein levels in the siRNA treated samples compared to the non-

treated samples, relative to the Rab27a loading control. The same conditions depleted 97% of Mlph (Figure 4-3 E). However, Anxa2 depletion was minimal (Figure 4-3 D). The commercial antibody available for VAT1 failed to identify any bands (expected molecular weight of 42kDa) on a Western blot of NT siRNA treated melan-INK4a (data not shown). According to the manufacturer's datasheet the antibody was raised against human VAT1 and MCF7 cells were cited as the positive control. Using MCF7 cell TCL, immunoblotting revealed a very faint band of the approximate predicted molecular weight (42kDa, indicated by the arrow Figure 4-3 Fi) in addition to an unspecific band as expected according to the manufacturer's datasheet (indicated by *). However, neither band could be identified in TCLs of HEK293A or melan-INK4a cells despite attempts to optimise the conditions (Figure 4-3 Fii). Therefore immunoblotting using the available antibody was not suitable for assessing whether the siRNA SMARTpool had successfully depleted VAT1. A possible alternative method would be to perform quantitative real time PCR (qRT-PCR) to assess mRNA levels.

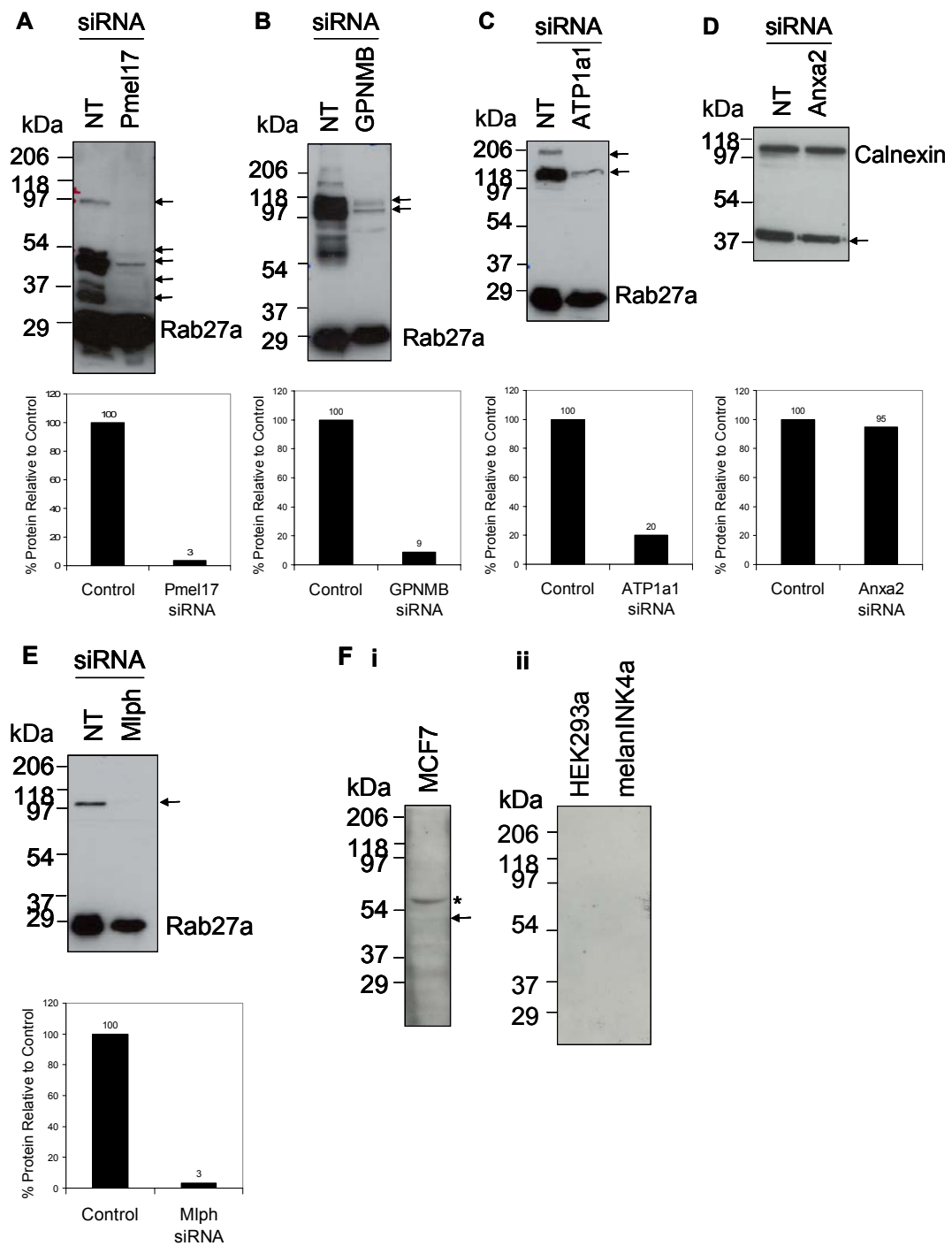


Figure 4-3 Depletion of the Candidate Proteins Using siRNA SMARTpools.

Melan-INK4a cells were treated with two rounds of siRNA (7 days incubation) prior to cell lysis. TCLs were analysed for protein depletion by immunoblotting for the candidate proteins and comparing to the non-treated control. A) Pmel17, B) GPNMB, C) ATP1a1, D) Anxa2, E) Mlph. Rab27a or calnexin were used as loading controls. Band intensities were measured using Image J, normalised to the loading control and % protein quantified relative to non-treated control. F) Assessment of the VAT1 antibody using (i) MCF7 cells TCL as a positive control (ii) HEK293A and melan-INK4a TCLs. The arrows indicate predicted sizes of candidate proteins. * indicates known nonspecific band.

Candidate protein depletion was measured by quantifying the density of the bands following immunoblotting using Image J, normalising to the loading control and comparing to the control treatment. Altering the experiment to only one siRNA treatment or up to three repeat treatments (without sub-culturing) was assessed to optimise the candidate protein depletion. For Pmel17, the optimal depletion was achieved with a single siRNA treatment (99%) (Figure 4-4 A), this decreased with increasing siRNA treatments (x2 – 53%, x3 – 3%). GPNMB was optimally depleted using three siRNA treatments (92%) (Figure 4-4 B) (x1 -12%, x2 – 49%). ATP1a1 was unaffected by altering the number of rounds of siRNA depletions, 93% was achieved with just one treatment (Figure 4-4 C). Anxa2 depletion was very inconsistent varying from 5% depletion with 1x siRNA treatment, 80-97% depletion with 2x siRNA (Figure 4-4 D) and 5-68% depletion with 3x siRNA.

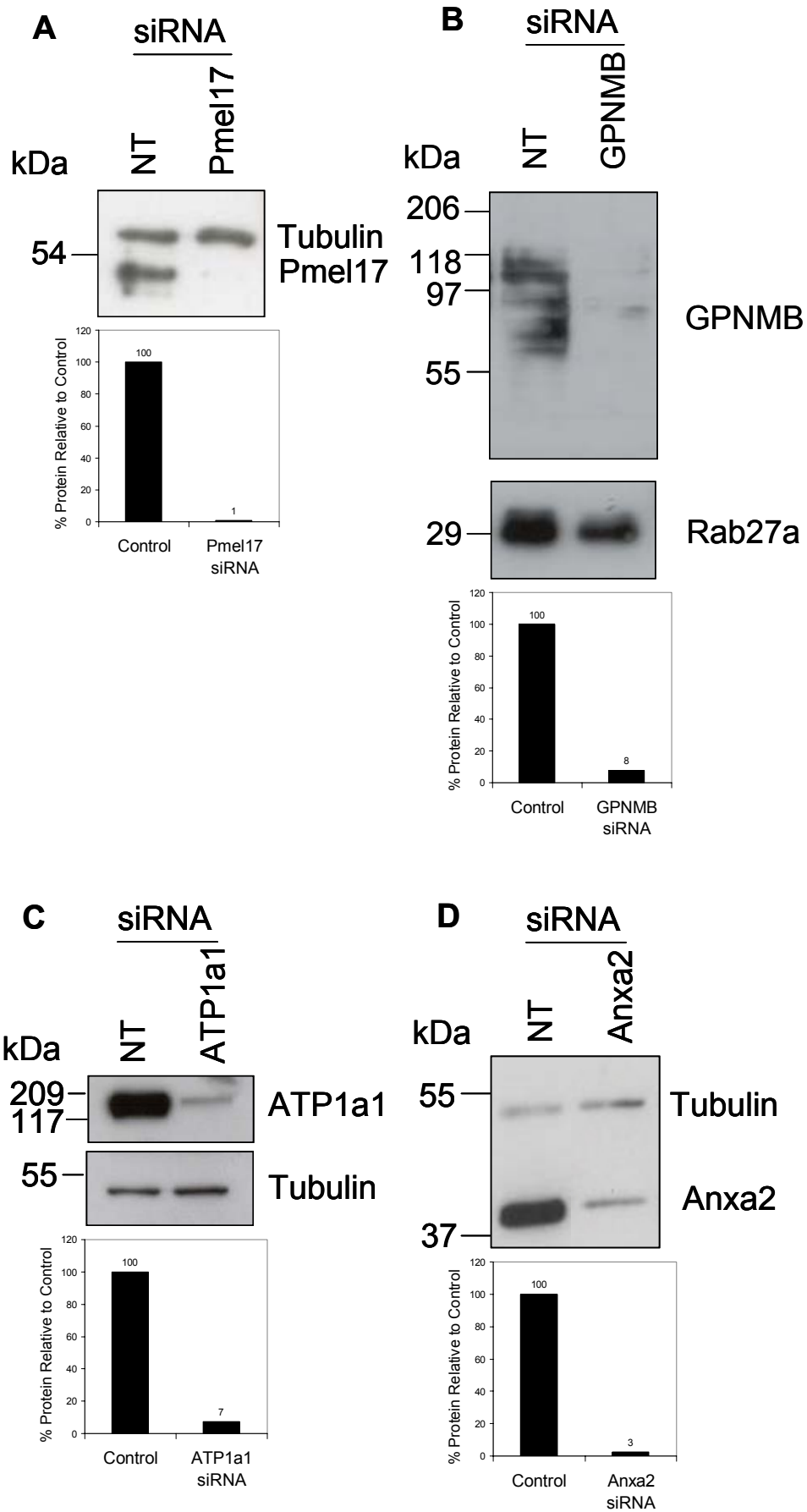


Figure 4-4 Optimal Depletion of the Candidate Proteins Using siRNA SMARTpools.

Experimental conditions were varied to achieve optimal siRNA depletion. Representative blots of depletion of A) Pmel17, B) GPNMB, C) ATP1a1 and D) Anxa2 are shown. Equal amounts of protein were loaded, as demonstrated by the Tubulin or Rab27a loading controls. Band intensities were measured using Image J, normalised to the loading control and the percentage of protein was quantified relative to non-targeting (NT) siRNA control.

4.3 The Effect of Candidate Protein Depletion on Rab27a Function

Once optimal conditions for depletion of the candidate proteins were achieved the effect this had on Rab27a function was assessed by examining the distribution of melanosomes. Mlph siRNA depletion was used as a positive control and resulted in distinct perinuclear clustering of melanosomes as indicated by the arrows (Figure 4-5). Optimal depletion of either Pmel17 or GPNMB had no apparent effect on Rab27a function as melanosome distribution was unaffected. In the majority of cases, Anxa2 depletion did not cause melanosome clustering but a small percentage of clustered cells was observed, which may indicate that Anxa2 could play a role in Rab27a function. However, as 100% depletion of Anxa2 was never achieved, the phenotype could not be reliably reproduced across the cell population. Depletion of ATP1a1 on the other hand reliably caused melanosomal clustering in the perinuclear region of the majority of the cells (arrowed cells), indicative of loss of Rab27a function and comparable to Mlph depletion-induced clustering.

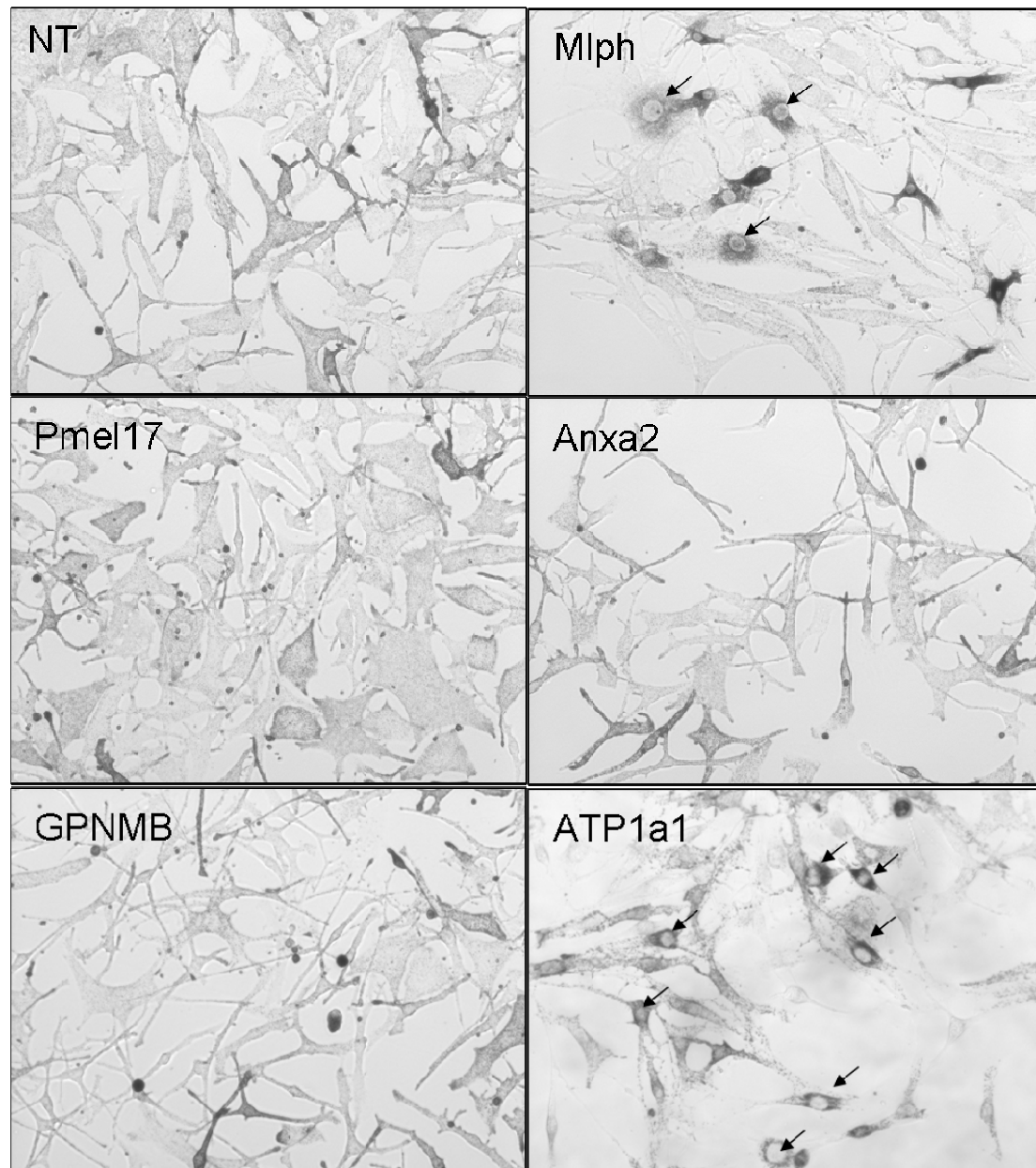


Figure 4-5 Melanosome Distribution Following Candidate Protein Depletion.

Melan-INK4a cells were treated with siRNA for Pmel-17, GPNMB, Anxa2 and ATP1a1 under optimal conditions. Mlph siRNA was used as a positive control. The cells were then visualised using light microscopy. Arrows indicate clustered cells.

4.4 Depletion of ATP1a1 with Individual siRNA Oligonucleotides Causes Melanosome Clustering

Following the finding that the ATP1a1 SMARTpool of oligonucleotides was able to cause a melanosome clustering phenotype in melan-INK4a cells, the individual oligonucleotides were purchased to assess if the same phenotype could be replicated and to minimise any possible off-targeting effects. After normalisation to the tubulin loading control, oligonucleotides 1, 2 and 4 were able to deplete ATP1a1 relative to NT control after only one round of siRNA treatment (Figure 4-6).

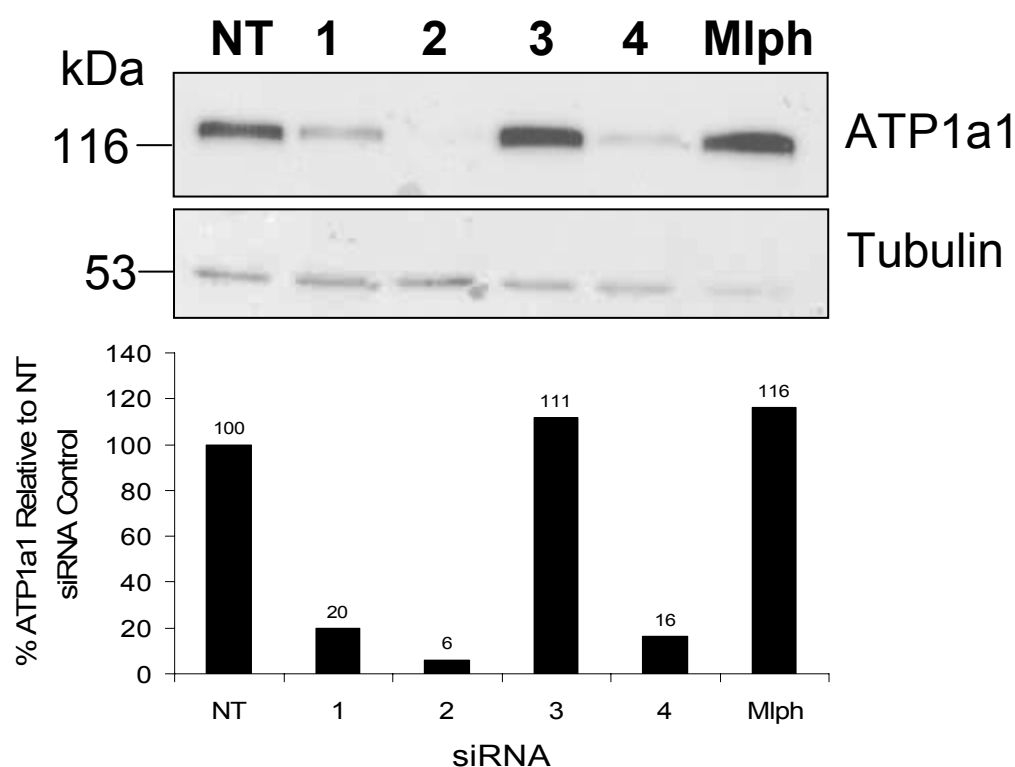


Figure 4-6 Depletion of ATP1a1 Protein Levels with Individual siRNA Oligonucleotides.

Individual oligonucleotides were purchased from Dharmacon. Melan-INK4a cells were treated with the siRNA and kept in culture for 3 days, lysed and the protein levels in the TCL assessed by immunoblotting using mouse anti-ATP1a1 and mouse anti-tubulin antibodies. Tubulin was used as a loading control. Band intensities were measured using Image J, normalised to the loading control and the percentage of protein was quantified relative to NT siRNA control.

Consistent with efficacy of ATP1a1 depletion, oligonucleotides 1, 2 and 4 also caused melanosomal clustering phenotypes (Figure 4-7). In particular, oligonucleotide 2 depleted 94% of ATP1a1 and resulted in the strongest clustering phenotype. From this point onwards all ATP1a1 depletion was achieved using oligonucleotide 2.

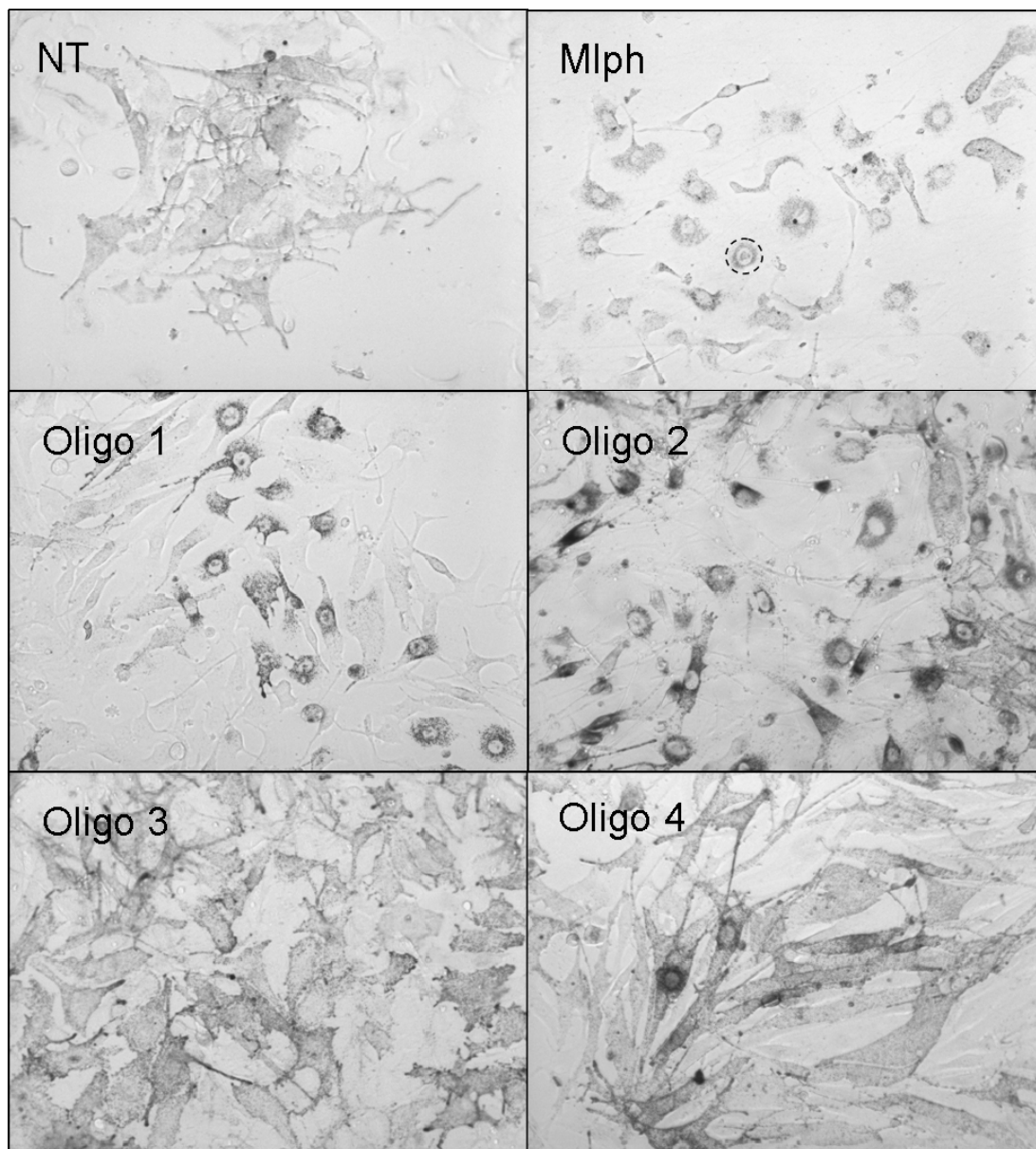


Figure 4-7 Melanosome Distribution Following ATP1a1 Depletion with Individual Oligonucleotides.

Melan-INK4a cells were treated with the siRNA and kept in culture for 3 days before melanosome distribution relative to NT and Mlph controls was assessed by light microscopy. Dashed circle indicates perinuclear region.

Clustering induced by ATP1a1 depletion was then compared to that induced by Mlph depletion. A perinuclear region was arbitrarily set using the Mlph control and included the nucleus and core surrounding melanosomes (as indicated by the dashed circle in Figure 4-7). This region was then applied to ATP1a1 depleted cells with apparent clustering. The percentage of melanosomes within the perinuclear region compared to the whole cell was then calculated. Using this method, NT siRNA treated cells had 30% of their melanosomes in the perinuclear region compared with 66% and 83% in the ATP1a1 and Mlph siRNA treated cells respectively (Figure 4-8). These results show a significant increase in melanosomes in the perinuclear region after ATP1a1 ($p=1.56 \times 10^{-36}$) and Mlph ($p=4.77 \times 10^{-55}$) depletion compared with NT siRNA treated cells. However, there was still a significant difference ($p=7.95 \times 10^{-10}$) between the ATP1a1 and Mlph depleted cells.

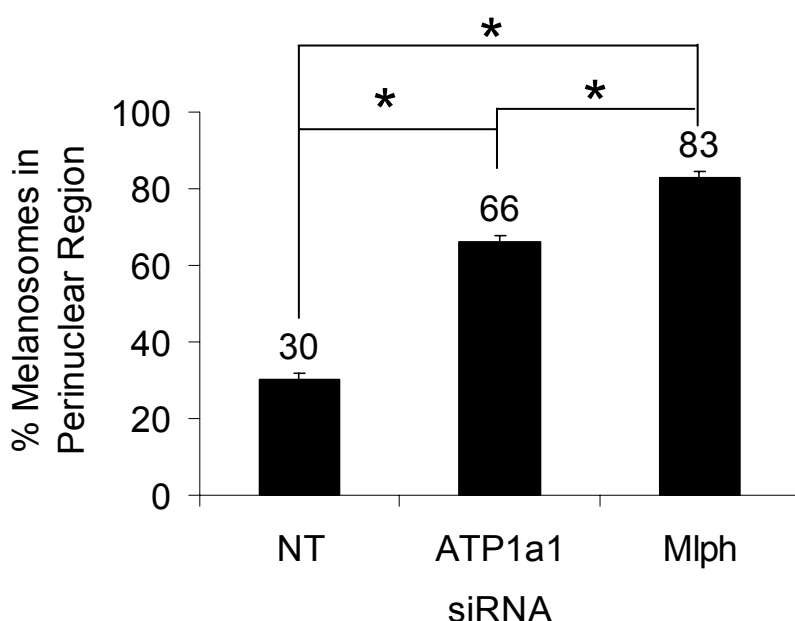


Figure 4-8 Quantification of ATP1a1 siRNA Induced Melanosomal Clustering.

Melan-INK4a cells were treated with the indicated siRNA and kept in culture for 3 days, before cells were imaged by light microscopy to assess melanosome distribution relative to NT and Mlph controls. The perinuclear region was set using the Mlph siRNA positive control. The percentage of melanosomes in the perinuclear region as a percentage of the whole cell was calculated. Data was collected from 4 independent experiments. Statistical analysis using two sample t-test assuming unequal variance.

These results indicated that depletion of ATP1a1 had a negative effect on Rab27a function. This could be as a result of a number of possibilities: i) Prenylation was affected resulting in loss of membrane association of Rab27a, ii) Rab27a expression was decreased, iii) Rab27a was no longer GTP-loaded and so failed to recruit its effector Mlph to melanosomes or iv) Rab27a was incorrectly targeted/removed from the melanosomal membrane.

4.5 Depletion of ATP1a1 Has No Effect on Rab27a Expression or Membrane-Association

In order to assess membrane-association of Rab27a following ATP1a1 depletion (an indirect measure of Rab27a prenylation) melan-INK4a cells were treated with ATP1a1 siRNA, the cells harvested and the TCL separated into cytosolic and membrane fractions by centrifugation at 100,000xg. The levels of Rab27a were then assessed by immunoblotting. Mlph depletion was used as a control for loss of Rab27a function without affecting Rab27a prenylation. R3G depletion was also included as it is known to cause melanosomal clustering as a result of reduced activation of Rab27a and loss of melanosomal localisation of Rab27a [145]. Tubulin was used as a control for effective separation of soluble and insoluble fractions. Comparison to the tubulin control showed that all endogenous Rab27a separated into the membrane fraction after siRNA depletion of Mlph, R3G and ATP1a1 (Figure 4-9). Depletion of ATP1a1 therefore had no effect on Rab27a membrane-association, comparable to that seen for Mlph or R3G depleted cells. Also, quantification of the levels of Rab27a normalised to the tubulin loading control showed that ATP1a1 depletion did not cause a decrease in Rab27a expression.

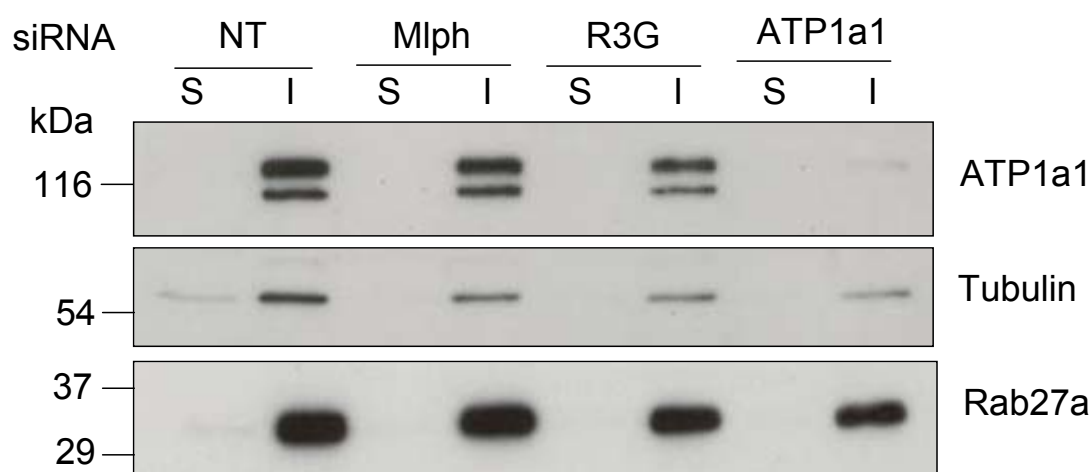


Figure 4-9 Solubility and Expression of Rab27a Following ATP1a1 Depletion.

Melan-INK4a cells were treated with NT, Mlph, R3G or ATP1a1 siRNA and cultured for 3 days. The TCL was then separated into the insoluble (I) and soluble (S) fraction by centrifugation at 100,000xg. Rab27a, ATP1a1 and Tubulin levels were assessed by immunoblotting. Using mouse anti-Rab27a, mouse anti-ATP1a1 and mouse anti-Tubulin antibodies

4.6 Depletion of ATP1a1 Decreases GTP-bound Rab27a

A key requirement for Rab27a function and localisation is R3G [145]. As mentioned, in the absence of R3G, Rab27a is inactivated, resulting in perinuclear clustering of melanosomes [145]. This raises the question whether the clustered phenotype observed following ATP1a1 depletion, which is comparable to that seen following R3G depletion, could be as a result of decreased Rab27a activation. In order to assess the levels of GTP-bound Rab27a in a cell, a simple technique is used, taking advantage of the knowledge that Rab27a predominantly binds to its effector Slp1 when in the GTP-bound form. Purified GST and GST-tagged Slp1 were utilised to assess the effects of ATP1a1 depletion on Rab27a activation using the same protocol as previously published [59]. R3G was used as a positive control. It was observed that depletion of ATP1a1 caused a 50% decrease in GTP-bound Rab27a ($p=0.01$), comparable to that observed with R3G depletion (53% $p=0.03$) (Figure 4-10). Interestingly, the combined

depletion of ATP1a1 and R3G (A+R) resulted in a further decrease in active Rab27a to 22% ($p=0.002$), indicating a combinatorial effect although not significant from the single depletion. This could suggest that ATP1a1 and R3G work in distinct pathways to regulate the activity of Rab27a or that R3G requires ATP1a1 to function to its full capacity. R3G is known to be the non-redundant GEF for Rab27a but the function of ATP1a1 in Rab27a function remains unclear.

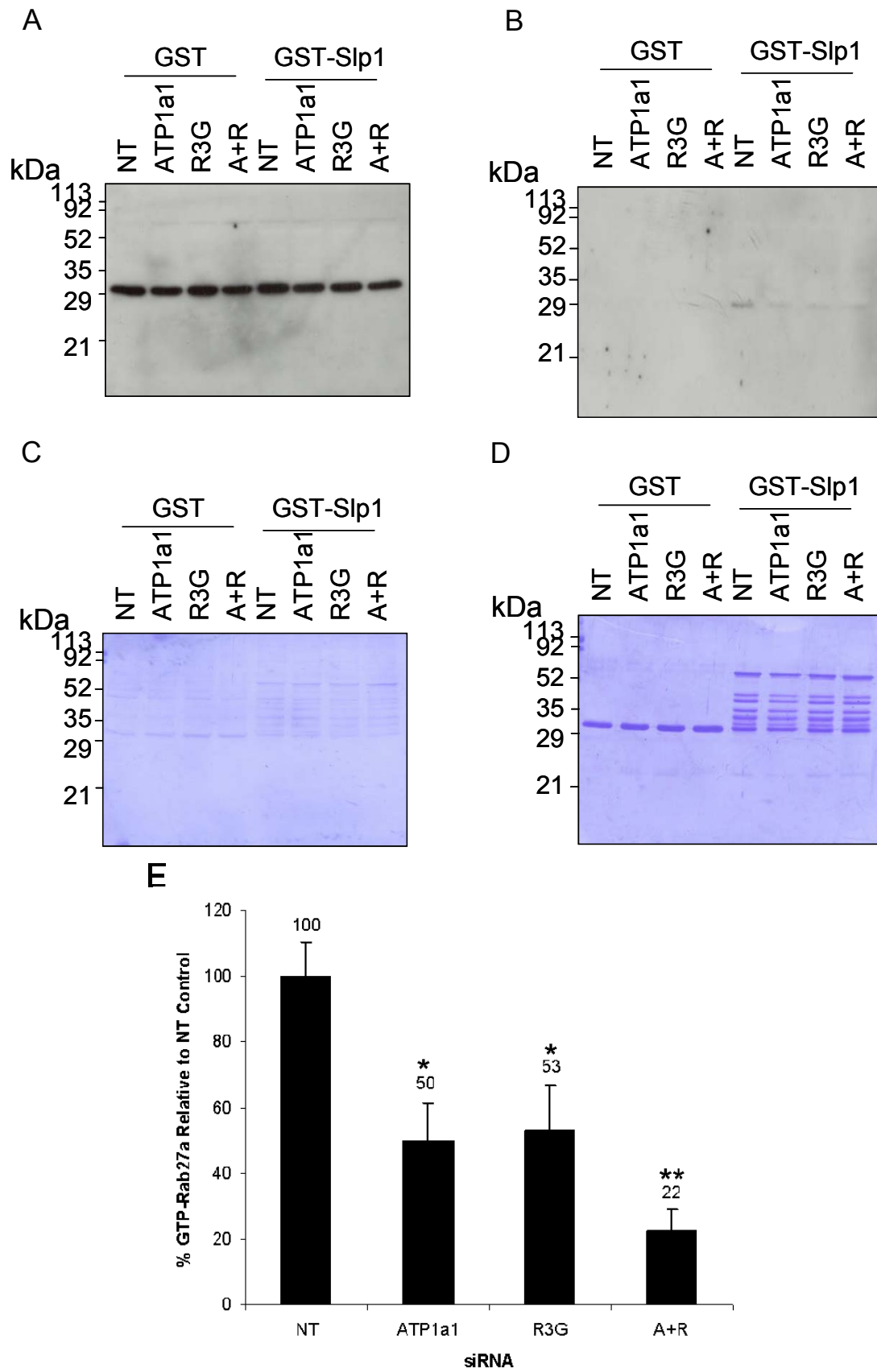


Figure 4-10 GTP-bound Rab27a Following ATP1a1 and/or R3G Depletion.

Melan-INK4a cells were treated with two rounds of NT, ATP1a1, R3G or A+R (ATP1a1 and R3G combined) siRNA. Cell lysates were incubated with immobilised GST-Slp1 or GST. Rab27a was quantified by immunoblotting the A) supernatant and B) bound to the beads using mouse anti-Rab27a antibody. Coomassie staining of the membranes demonstrated C) equal protein loading and D) immobilization of GST and GST-Slp1. E) Rab27a immobilized by GST-Slp1 was normalized to the coomassie and quantified relative to the NT control using Image J. The experiment was repeated 5 times. Immunoblots and coomassie blots are representative of one experiment. Quantification is of all experiments. Error bars are SEM. Significance calculated using two-sample t-test assuming equal variance. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$

4.7 Depletion of ATP1a1 Disrupts Rab27a Targeting to Melanosomes

Following the finding that ATP1a1 depletion affects Rab27a function and GTP-loading, the main aim was to assess whether ATP1a1 depletion had any effect on Rab27a targeting to melanosomes, as has been seen previously in the case of R3G depletion. This was assessed in fixed melanocytes as well as purified melanosomes.

4.7.1 Depletion of ATP1a1 Disrupts GFP-Rab27a Targeting to Melanosomes

In order to assess the effect ATP1a1 depletion has on targeting of newly translated Rab27a, melan-INK4a cells were treated with ATP1a1 siRNA, cultured for 24h, before transfection with GFP-Rab27a. Transfected cells were then visualised using fluorescence microscopy. It was observed that GFP-Rab27a localised onto melanosomes in NT and Mlph siRNA treated cells (Figure 4-11 A-F), as indicated by arrows in the inset. Yet in the ATP1a1 siRNA depleted cells, GFP-Rab27a did not localise to melanosomes and appeared throughout the cytosol with some stronger staining in the perinuclear region (Figure 4-11 G-I). This is comparable to Rab27a localisation following R3G depletion [145].

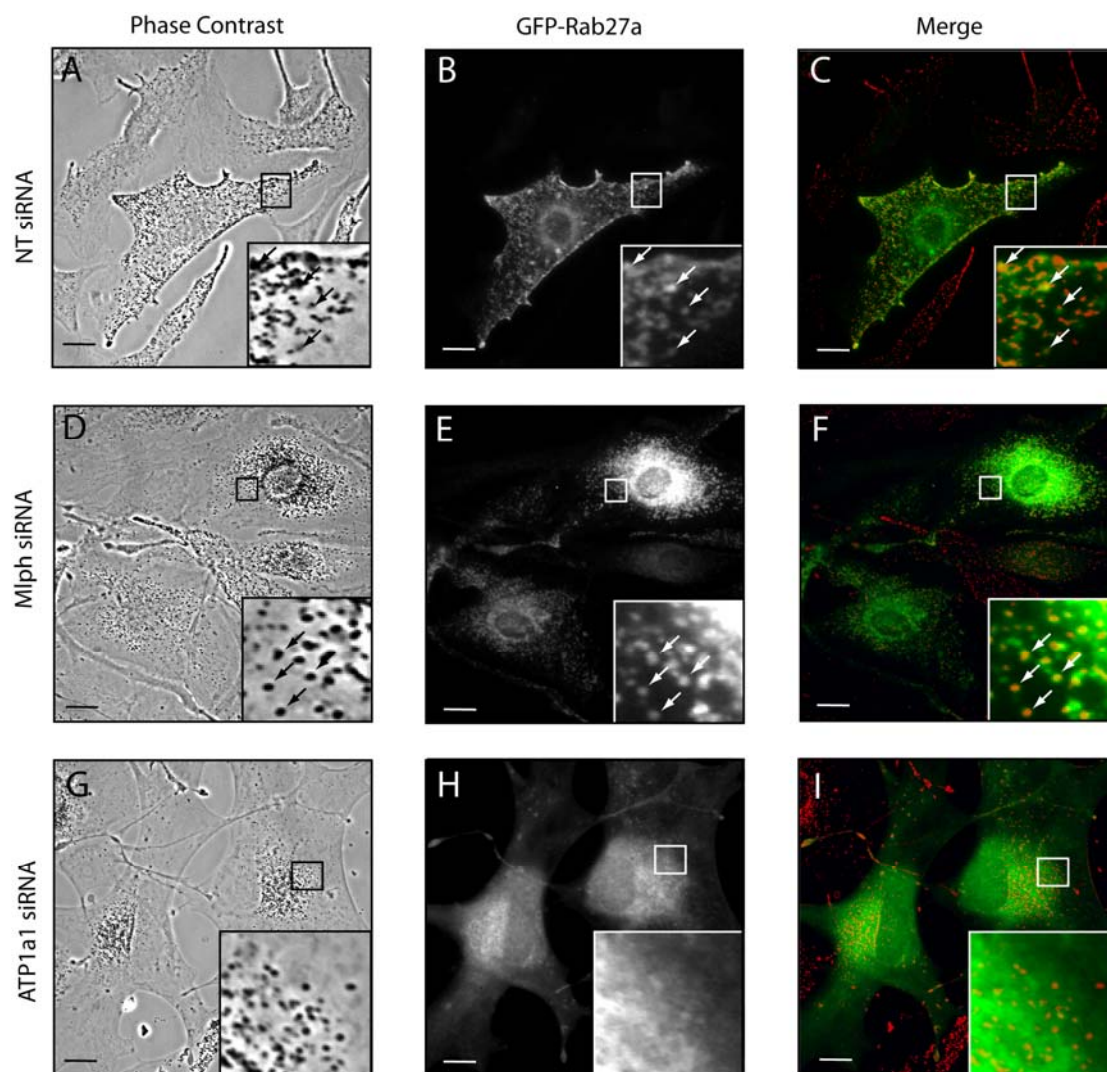


Figure 4-11 GFP-Rab27a Localisation Following ATP1a1 Depletion in Melan-INK4a.

Melan-INK4a cells were grown on coverslips and treated with either NT (A-C), Mlph (D-F) or ATP1a1 (G-I) siRNA and cultured for 24h before transfection with GFP-Rab27a. After 48h, cells were fixed with 4% PFA. Images were taken using a confocal microscope. Phase contrast panels demonstrate melanosome distribution (A, D and G). Panels B, E and H show GFP-Rab27a localisation. Pigment is inverted and pseudo-coloured red to aid colocalisation with the green GFP signal in the merge panels (C, F, I). Insets are a higher magnification of the boxed area. Arrows indicate colocalisation. Scale bar represents 10µm. Images representative of two independent experiments.

In a similar experiment melan-ash melanocytes were treated with either NT or ATP1a1 siRNA before GFP-Rab27a transfection. This again was to check the targeting of newly expressed Rab27a, but eliminating any possible competition from endogenous Rab27a. In the NT siRNA treated cells (Figure 4-12 A-C) GFP-Rab27a localised to melanosomes and was able to restore the peripheral distribution of the melanosomes. However, in ATP1a1

depleted melan-ash melanocytes, despite high expression, GFP-Rab27a failed to localise to melanosomes and therefore failed to restore the peripheral melanosomal distribution (Figure 4-12 D-F). This again highlighted that ATP1a1 depletion prevented targeting of newly synthesised Rab27a to melanosomal membranes.

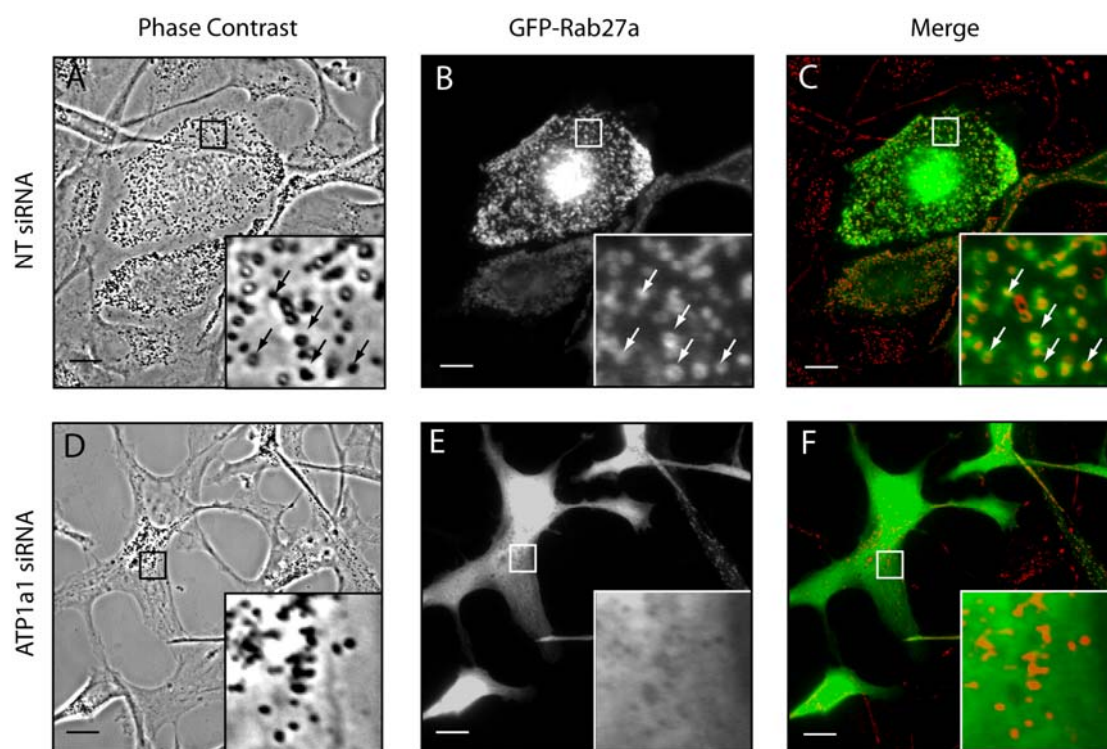


Figure 4-12 GFP-Rab27a Localisation Following ATP1a1 Depletion in Melan-ash.

Melan-ash cells were grown on coverslips and treated with NT (A-C) or ATP1a1 (D-F) siRNA and cultured for 24h before transfection with GFP-Rab27a. After an additional 48h, cells were fixed with 4% PFA. Images were taken on a confocal microscope. Phase contrast images demonstrate the melanosome distribution (A and D). Panels B and E show GFP-Rab27a localisation. Pigment is inverted and pseudo-coloured red to aid colocalisation with the green GFP signal in the merge panels (C and F). Insets are a higher magnification of the boxed area. Arrows indicate colocalisation. Scale bar represents 10 μ m. Images representative of one experiment

4.7.2 Depletion of ATP1a1 Disrupts Endogenous Rab27a Targeting to Melanosomes

Depletion of ATP1a1 prevented correct targeting of newly synthesised GFP-Rab27a to the melanosomes, but the question remained whether the loss of ATP1a1 was able to affect

endogenous Rab27a localisation to melanosomes. Rab27a is not proposed to be recycled from melanosome membranes (A. Hume, unpublished data), similarly it is not readily extracted from membranes by GDI (M. Kirsten, unpublished data). It was therefore thought that once Rab27a was targeted to melanosomal membranes it would not readily be removed. However, to assay this, melan-INK4a cells were treated with siRNA against NT, Mlph or ATP1a1 and cultured for 3 days before immunostaining and visualising using fluorescence confocal microscopy. Endogenous Rab27a localised to melanosomes in NT siRNA treated cells and they remained distributed throughout the cytosol (Figure 4-13 A-C). In the Mlph siRNA treated cells, the melanosomes clustered in the perinuclear region as the loss of Mlph prevented the peripheral tethering of the melanosomes, but endogenous Rab27a was still localised to the clustered melanosomes (Figure 4-13 D-F). However, in ATP1a1 depleted cells, the clustered phenotype was evident but endogenous Rab27a appeared to lose its localisation to melanosomes (Figure 4-13 G-I) suggesting that loss of ATP1a1 caused displacement of endogenous Rab27a. This has also been seen in R3G depleted cells [145]. On closer inspection of the perinuclear region in ATP1a1 depleted cells (Figure 4-13 J-L), there appeared to be punctate Rab27a staining distinct from the pigment, therefore suggesting that endogenous Rab27a was on a vesicular structure (in agreement with the membrane-association data see Figure 4-9) distinct from the melanosome, perhaps a 'Rab27a delivery vesicle'. Of note, it has been published that expression of EGFP-Rab27a in *ashen* melanocytes identified a subpopulation of Rab27a-positive vesicles that lacked a melanin core (14% of total) [175].

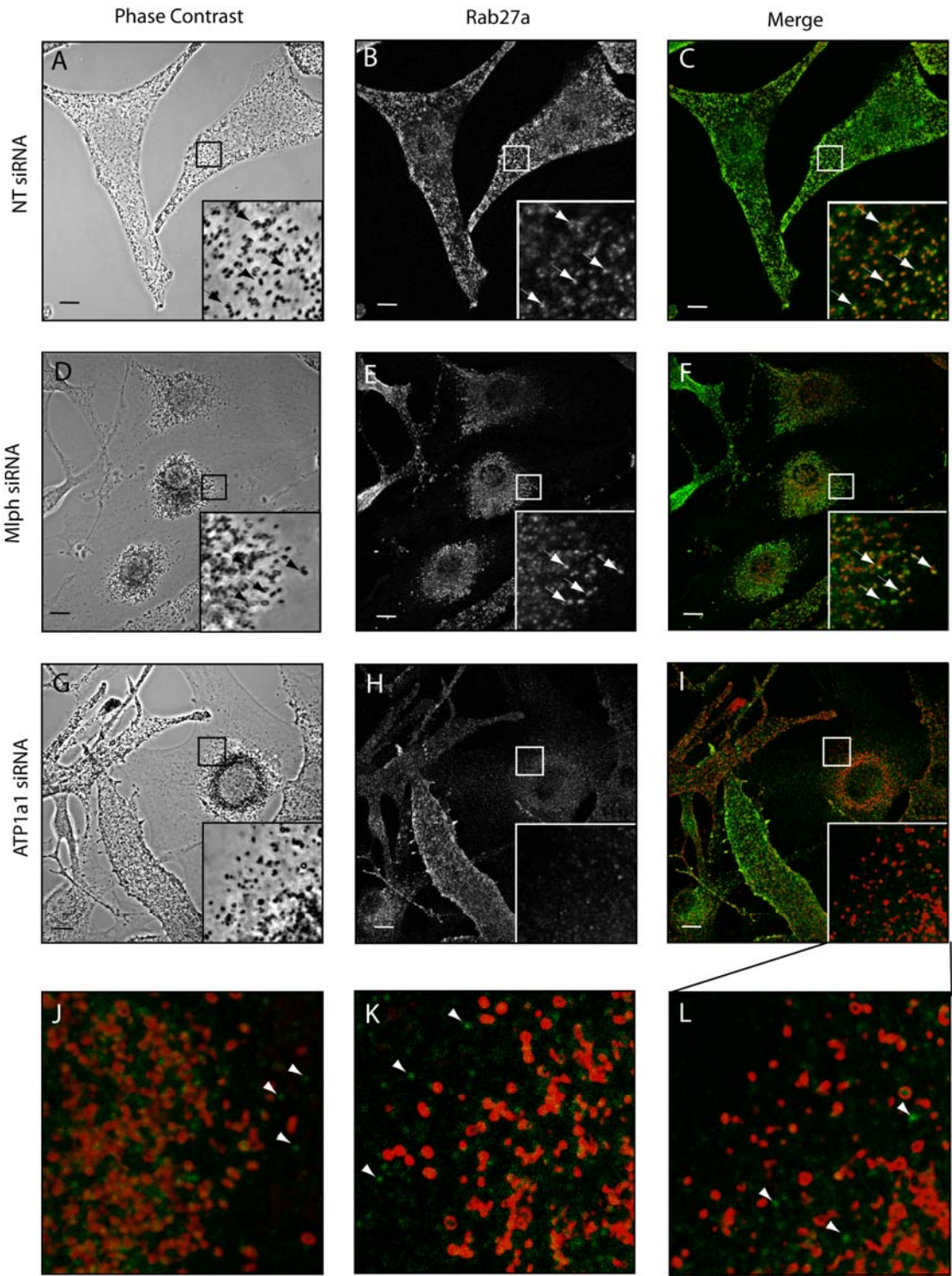


Figure 4-13 Endogenous Rab27a Localisation Following ATP1a1 Depletion.

Melan-INK4a cells were grown on coverslips and treated with NT, Mlph or ATP1a1 siRNA and cultured for 3 days. Cells were fixed with 4% PFA, permeabilised, blocked with FBS and immunostained with rabbit-anti Rab27a antibody and Alexa-488 conjugated-anti rabbit secondary antibody. Images were taken with a fluorescence confocal microscope. Phase contrast panels demonstrate melanosome distribution (A, D and G). Panels B, E and H show endogenous Rab27a localisation. Pigment is inverted and pseudo-coloured red to aid colocalisation with Rab27a (green) in the merge panels (C, F and I). Insets are a higher magnification of the boxed area. Arrows indicate colocalisation. Zoom of perinuclear area of ATP1a1 depleted cells demonstrating Rab27a positive vesicles distinct from mature melanosomes indicated by arrowheads (J, K and L). Scale bar represents 10µm. Images are representative of four independent experiments.

To confirm the effect ATP1a1 depletion has on Rab27a localisation to the melanosomes, it was investigated in more depth by purifying melanosomes from siRNA treated cells. Melanosomes were purified using a method adapted from that of Chabrilat *et al.* [408]. Protein concentrations were measured and equal amounts of protein separated on a SDS-PAGE gel and analysed by immunoblotting (Figure 4-14). Rab27a levels in the ATP1a1 depleted PNS appeared reduced but when normalised to the tubulin loading control showed no difference from NT siRNA treated cells. However, immunoblotting of the ATP1a1 depleted melanosomes suggested that Rab27a was reduced, Rab32 increased and TYRP1 levels slightly reduced. However there was no loading control to assure equal loading as tubulin was not present in the melanosome preparation. Also, there appeared to be no ATP1a1 on the purified NT siRNA melanosomes which was of concern.

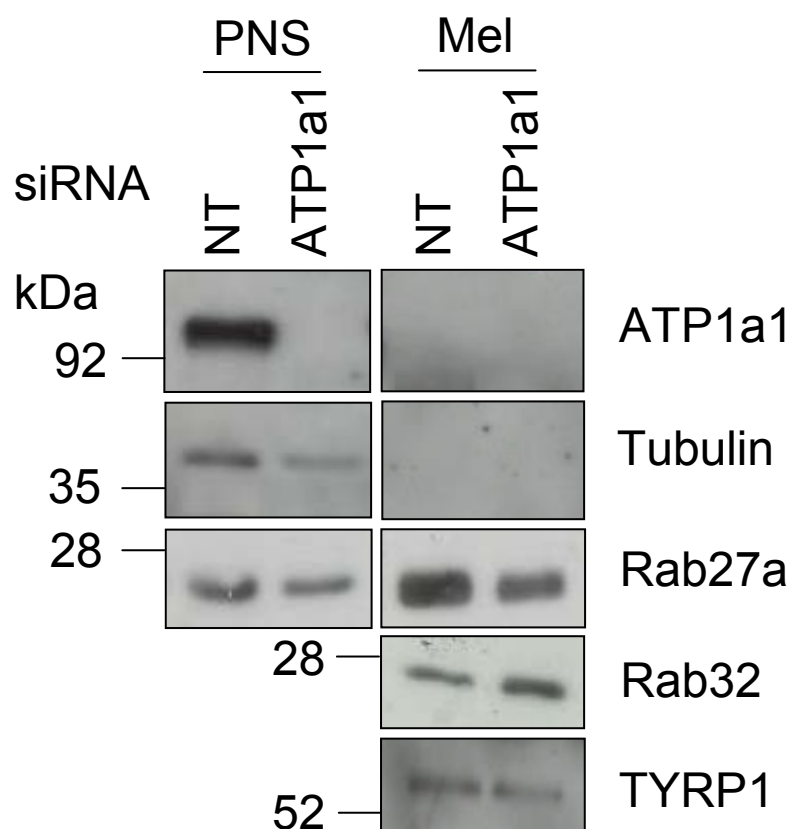


Figure 4-14 Protein Association to Purified Melanosomes Following ATP1a1 Depletion.

Melan-INK4a cells were treated with NT or ATP1a1 siRNA and cultured for 3 days. Cells were harvested and melanosomes purified. Equal protein concentrations were separated by SDS-PAGE and protein levels were analysed by immunoblotting using mouse anti-ATP1a1, mouse anti-Tubulin, mouse anti-Rab27a, rabbit anti-Rab32 and rabbit anti-TYRP1. n=1

This highlighted the difficulty in assessing the effect of ATP1a1 depletion on Rab27a association using immunoblotting and so a novel method was developed. Melanosomes were purified from NT or ATP1a1 siRNA treated melan-INK4a and untreated melan-ash cells. The purified melanosomes were centrifuged onto coverslips, fixed, immunostained and analysed by immunofluorescence microscopy (Figure 4-15). The same microscope settings were used for acquisition of all images. Simple PCI Image Software (Hamamatsu) was used to quantify the fluorescence intensity of endogenous Rab27a associated with the purified melanosomes. Melanosomes purified from melan-ash melanocytes were used as a

background control and its average measurement was subtracted from all measurements for NT or ATP1a1 siRNA treated cells. Quantification of the endogenous Rab27a associated with the purified melanosomes found that there was a 59% decrease with the ATP1a1 siRNA treated cells ($p=1.7 \times 10^{-6}$). This was in agreement with the preliminary immunoblotting result and with the immunofluorescence images of the whole cells.

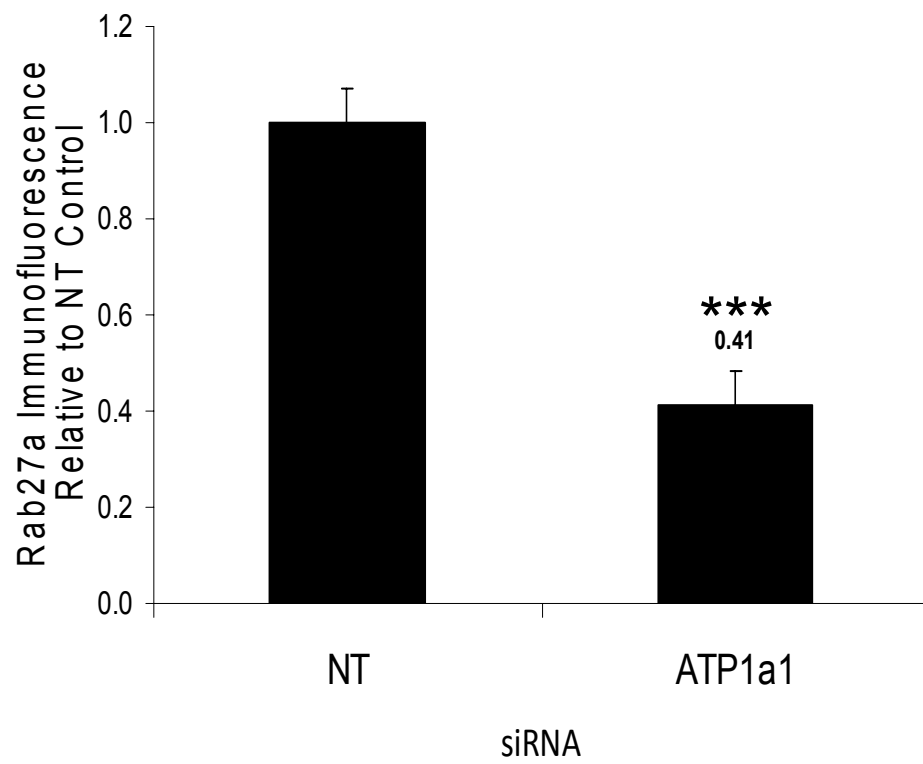
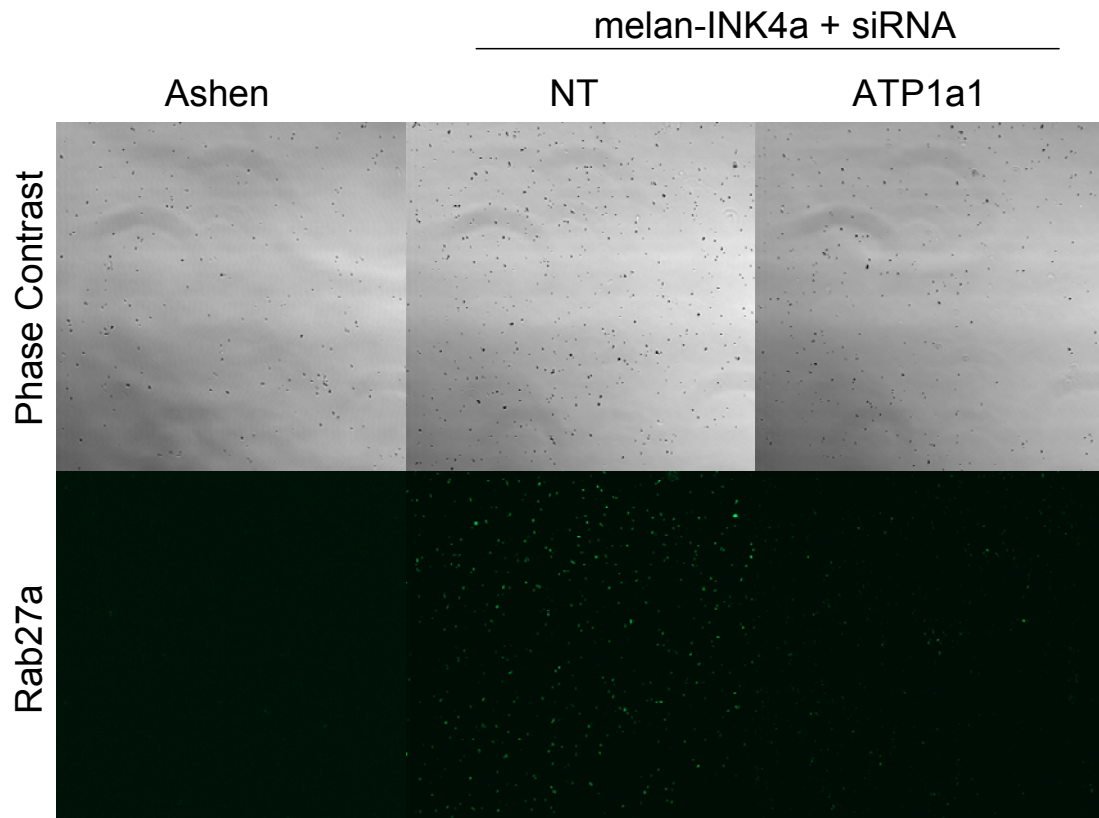


Figure 4-15 Rab27a Association to Purified Melanosomes Following Short-term ATP1a1 Depletion.

Melan-INK4a cells were treated with NT or ATP1a1 siRNA and cultured for 3days. Cells were harvested and melanosomes purified. Non-treated melan-ash cells were used as a negative control. Purified melanosomes were centrifuged onto coverslips, fixed with 4% PFA, permeabilised, blocked in FBS and immunostained using rabbit anti-Rab27a antibody and Alexa-488 conjugated anti-rabbit secondary antibody. Images were acquired on a confocal microscope and then analysed using SimplePCI analysis software, subtracting the measurements for the melan-ash melanosomes. The experiment was repeated twice and statistical analysis performed on the NT control normalised data sets. Error bars are SEM. Significance calculated using t-test. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ (Two-sample assuming unequal variances)

4.8 Depletion of ATP1a1 and R3G Further Disrupts

Endogenous Rab27a Targeting to Melanosomes

To continue, the effect R3G siRNA treatment individually and in combination with ATP1a1 depletion (A+R) had on endogenous Rab27a was investigated. Similarly to that observed following ATP1a1 depletion, depletion of R3G and ATP1a1 and R3G in combination resulted in disruption of localisation of endogenous Rab27a to melanosomes (Figure 4-16).

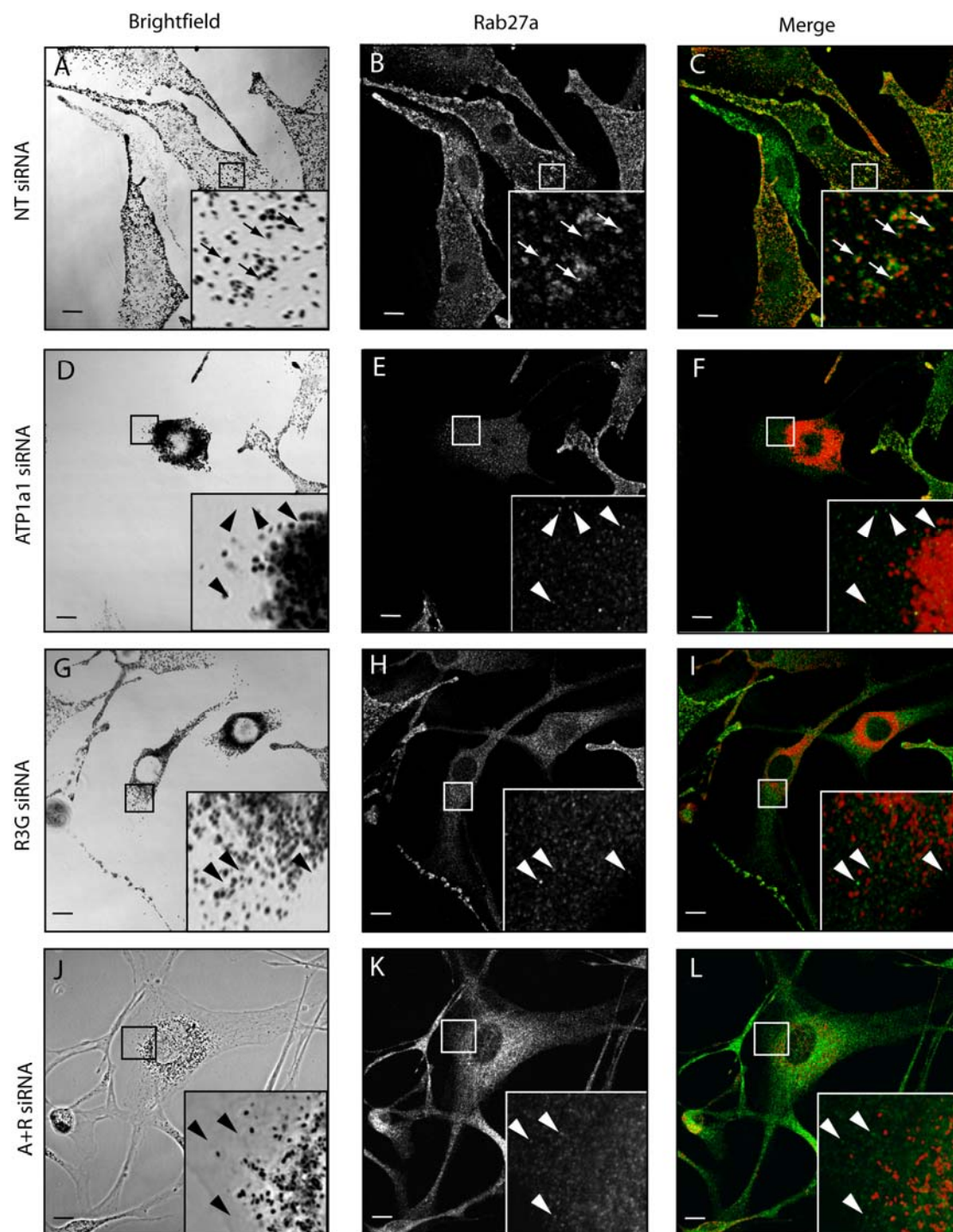


Figure 4-16 Endogenous Rab27a Localisation Following ATP1a1, R3G and A+R siRNA Depletion.

Melan-INK4a cells were grown on coverslips and treated with NT, ATP1a1, R3G or A+R siRNA twice and cultured for 7 days. Cells were fixed with 4% PFA, permeabilised, blocked with FBS and immunostained with rabbit-anti Rab27a antibody and Alexa-488 conjugated-anti rabbit secondary antibody. Images were taken with a fluorescence confocal microscope. Brightfield panels demonstrate melanosome distribution (A, D, G and J). Panels B, E, H and K show endogenous Rab27a localisation. Pigment is inverted and pseudo-coloured red to aid colocalisation with Rab27a (green) in the merge panels (C, F, I and L). Insets are a higher magnification of the boxed area. Arrows indicate colocalisation, arrowheads show Rab27a devoid of melanin. Scale bar represents 10µm. Images are representative of one experiment.

Further analysis of purified melanosomes observed a significant decrease in Rab27a immunofluorescence on melanosomes following depletion of ATP1a1 ($p=0.02$), R3G ($p=0.004$) or both ($p=1.2 \times 10^{-8}$) (Figure 4-17). Interestingly, the combined depletion of ATP1a1 and R3G showed significantly reduced Rab27a levels on melanosomes compared to that of the single depletion of ATP1a1 ($p=0.02$), but not R3G.

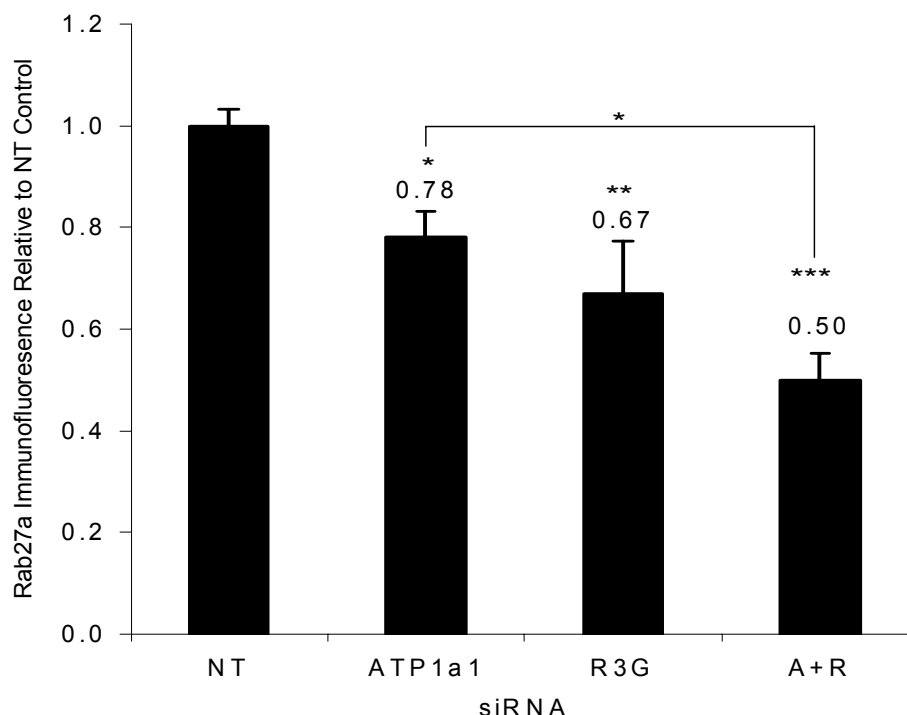


Figure 4-17 Rab27a Association to Purified Melanosomes Following ATP1a1 and/or R3G Depletion.

Melan-INK4a cells were treated with NT, ATP1a1, R3G or ATP1a1+R3G (A+R) siRNA and cultured for 3 days before repeating the siRNA treatment. Cells were harvested and melanosomes purified. Melan-ash cells were used as a negative control. Purified melanosomes were centrifuged onto coverslips, fixed with 4% PFA, permeabilised and blocked with FBS. Coverslips were then immunostained using rabbit anti-Rab27a antibody and Alexa-488 conjugated anti-rabbit secondary antibody. Images were taken on fluorescence confocal microscope and then analysed using SimplePCI analysis software. Experiment was repeated 5 times and statistical analysis performed on the NT control normalised data sets. Error bars are SEM. Significance calculated using t-test. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ (Two-sample assuming unequal variances)

In a complimentary experiment, the levels of Mlph associated with purified melanosomes were assessed as an indirect indicator of Rab27a associated with melanosomes and its nucleotide-bound state. It was observed that depletion of ATP1a1 resulted in a decrease in Mlph associated with melanosomes compared to NT siRNA control (Figure 4-18) ($p = 0.00019$). R3G depletion resulted in a further loss of Mlph association ($p = 0.00001$) however the combined depletion (A+R) did not enhance the decrease ($p = 0.00003$). These results were comparable with the Rab27a data (Figure 4-17). The difference between

ATP1a1 and R3G depletion was also significant ($p=0.048$), similarly ATP1a1 or the dual depletion and ashen ($p=0.009$ and 0.002 respectively).

The finding that A+R depletion did not further reduce Rab27a or Mlph association with melanosomes from the single R3G depletion, yet caused a further decrease in GTP-bound Rab27a could suggest that R3G depletion is sufficient to displace Rab27a from the melanosomes and hence reduce Mlph association but ATP1a1 depletion is necessary to fully deplete GTP-bound Rab27a.

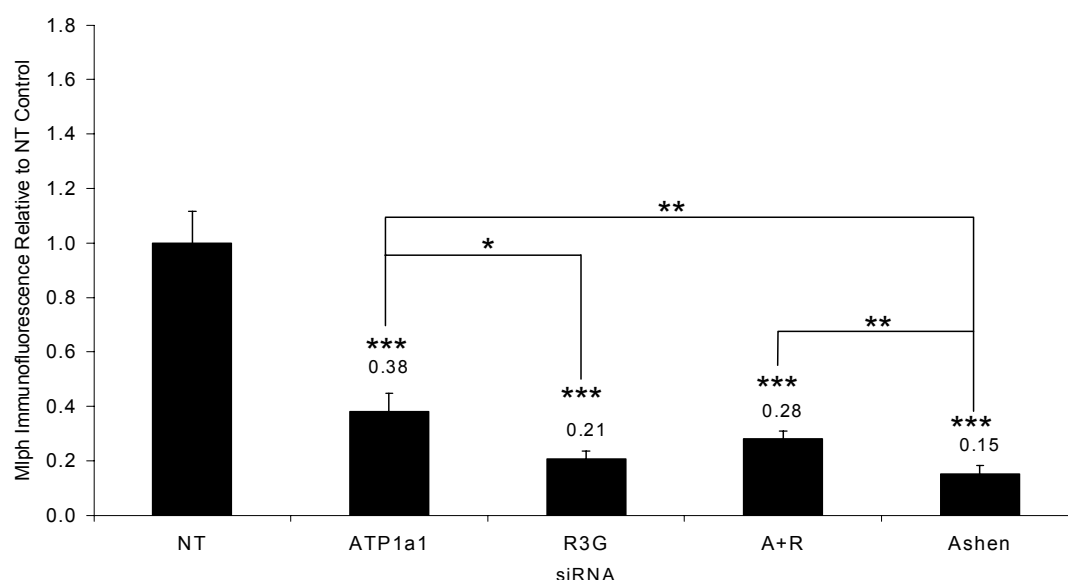


Figure 4-18 Mlph Association with Purified Melanosomes Following ATP1a1 and/or R3G Depletion.

Melan-INK4a cells were treated with two rounds of NT, ATP1a1, R3G or A+R siRNA. Non-treated melan-ash cells were used as a negative control. Purified melanosomes were centrifuged onto coverslips, fixed with 4% PFA, permeabilised and blocked with FBS. Coverslips were then immunostained using rabbit anti-Mlph antibody and alexa-488 conjugated anti-rabbit secondary antibody. Images were taken on a fluorescence confocal microscope and then analysed using SimplePCI analysis software. Experiment was repeated in triplicate and statistical analysis performed on the NT control normalised data sets. Error bars are SEM. Significance calculated using t-test . * $p<0.05$, ** $p<0.005$, *** $p<0.001$ (Two-sample assuming unequal variances)

4.9 Depletion of ATP1a1 Affects Other Melanosomal Proteins

Demonstrating that ATP1a1 depletion affects Rab27a function and localisation raised the question whether this was specific to Rab27a or if it affected any other melanosomal proteins. To investigate this further, ATP1a1 was depleted using one round of siRNA, the melanosomes purified and the association of TYRP1 and Rab38 investigated by immunofluorescence microscopy (Figure 4-19). Fluorescence data was analysed by Simple PCI Software. siRNA depletion of ATP1a1 had no significant effect on TYRP1 association with melanosomes ($p=0.73$). Despite the suggestion of an increase in Rab38, the observed increase was not significant ($p=0.19$).

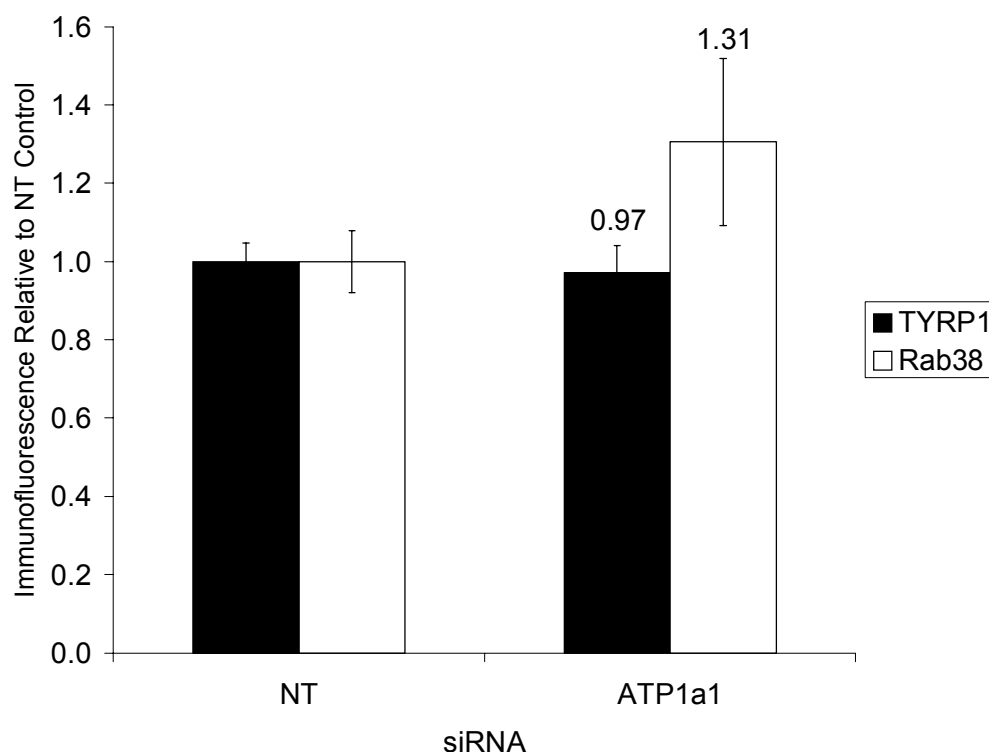


Figure 4-19 Melanosomal Protein Association with Purified Melanosomes Following Short-term ATP1a1 Depletion.

Melan-INK4a cells were treated with NT or ATP1a1 siRNA and cultured for 3 days. Cells were harvested and melanosomes purified. Purified melanosomes were then centrifuged onto coverslips, fixed with 4% PFA, permeabilised and blocked with FBS. Coverslips were then immunostained with rabbit anti-TYRP1 or rabbit anti-Rab38 antibodies and Alexa-488 conjugated anti-rabbit secondary antibody. Images were taken on fluorescence confocal microscope and then analysed using SimplePCI analysis software. The experiment was repeated twice. Statistical analysis performed on the NT control normalised data sets. Error bars are SEM. Significance calculated using t-test. (Two-sample assuming unequal variances)

Using two rounds of siRNA treatment ATP1a1, R3G and A+R siRNA treated melanosomes were investigated (Figure 4-20). After the longer depletion time, ATP1a1 ($p=1.28 \times 10^{-5}$) and A+R ($p=0.0001$) siRNA treated melanocytes showed a significant decrease in associated TYRP1, whilst the effect of R3G depletion was not significant ($p=0.07$). Although showing a trend towards an increase, neither ATP1a1 ($p=0.25$) nor R3G ($p=0.25$) depletion caused a significant increase in Rab38 association with the melanosomes, however the increase was significant following A+R depletion ($p=0.05$).

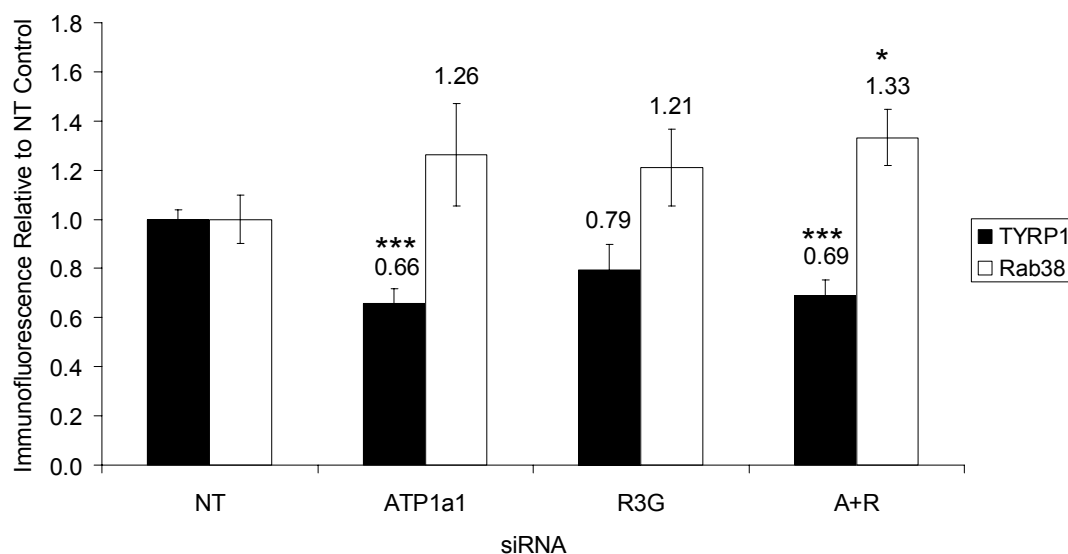


Figure 4-20 Melanosomal Protein Association with Purified Melanosomes Following Long-term ATP1a1 and/or R3G Depletion.

Melan-INK4a cells were treated with NT, ATP1a1, R3G or A+R siRNA and cultured for 3days before repeating the siRNA treatment (7days total). Cells were harvested and melanosomes purified. Purified melanosomes were then centrifuged onto coverslips, fixed with 4% PFA, permeabilised and blocked in FBS. Coverslips were then immunostained with rabbit anti-TYRP1 or rabbit anti-Rab38 antibodies and Alexa-488 conjugated anti-rabbit secondary antibody. Images were taken on fluorescence confocal microscope and then analysed using SimplePCI analysis software. The experiment was repeated in triplicate. Statistical analysis performed on the NT control normalised data sets. Error bars are SEM. Significance calculated using t-test. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ (Two-sample assuming unequal variances)

This data suggests that depletion of ATP1a1 and R3G had a time-dependent effect on melanosomes, initially disrupting Rab27a association with melanosomes and possibly consequently affecting upstream melanosomal proteins TYRP1 and Rab38.

4.10 Depletion of ATP1a1 Increases the Melanin Content of Melanocytes.

Following on from the apparent disruption ATP1a1 depletion has on melanosomal protein balance, it was regularly observed that depletion of ATP1a1 resulted in darker TCLs which was visible by eye (Figure 4-21).

Quantification of the absorbance of the TCL at 492nm wavelength (Nanodrop) relative to the protein concentration and normalised to the NT siRNA treated control, demonstrated that there was a two-fold increase in melanin. However this difference did not occur following R3G depletion and was therefore the first instance where depletion of ATP1a1 caused a phenotype not phenocopied by R3G depletion.

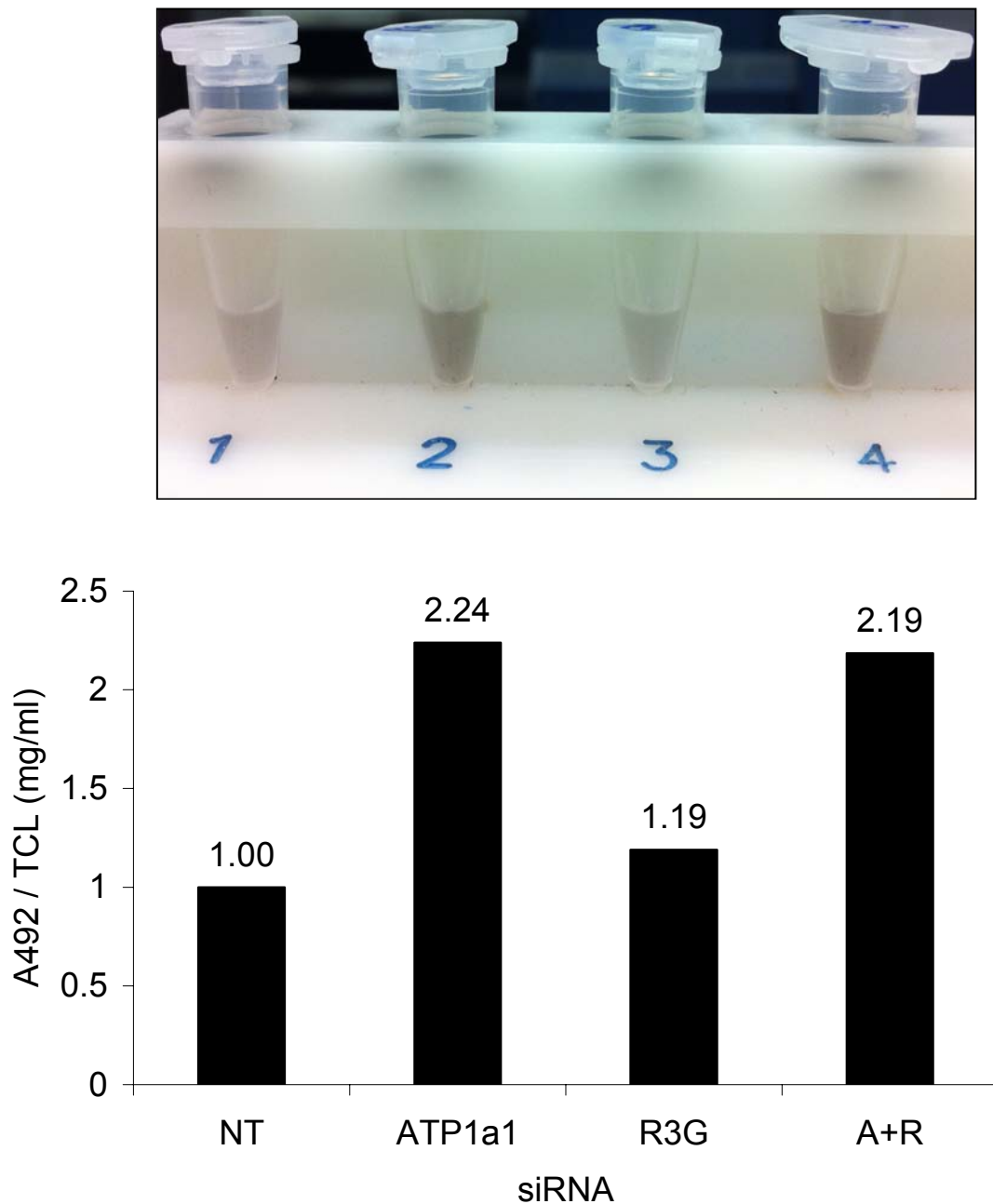


Figure 4-21 Pigmentation Following ATP1a1 and/or R3G Depletion.

Melan-INK4a cells were treated twice with NT, ATP1a1, R3G or A+R siRNA for up to 7 days. Cells were harvested and protein concentration and absorbance at 492nm measured using the nanodrop. Quantification of the A492 per mg/ml of protein was then calculated and normalised to NT siRNA. Figure represents one experiment.

4.11 Depletion of ATP1a1 Does Not Affect EGFP-Rab5 or EGFP-Rab7 Vesicle Distribution.

Demonstrating that ATP1a1 depletion affects the peripheral distribution of melanosomes raised the question whether this was a specific effect or if ATP1a1 depletion would cause clustering of other vesicles/organelles in melanocytes. NT or ATP1a1 siRNA treated melan-INK4a cells were transfected with EGFP-Rab5 (a marker for early endosomes) or EGFP-Rab7 (a marker for late endosomes/lysosomes). The distribution of the EGFP-Rabs was then assessed by fluorescence microscopy. Both EGFP-Rab7 (Figure 4-22 B) and EGFP-Rab5 vesicles (Figure 4-22 H) were evenly distributed throughout the cell and ATP1a1 depletion did not affect this (Figure 4-22 E and K).

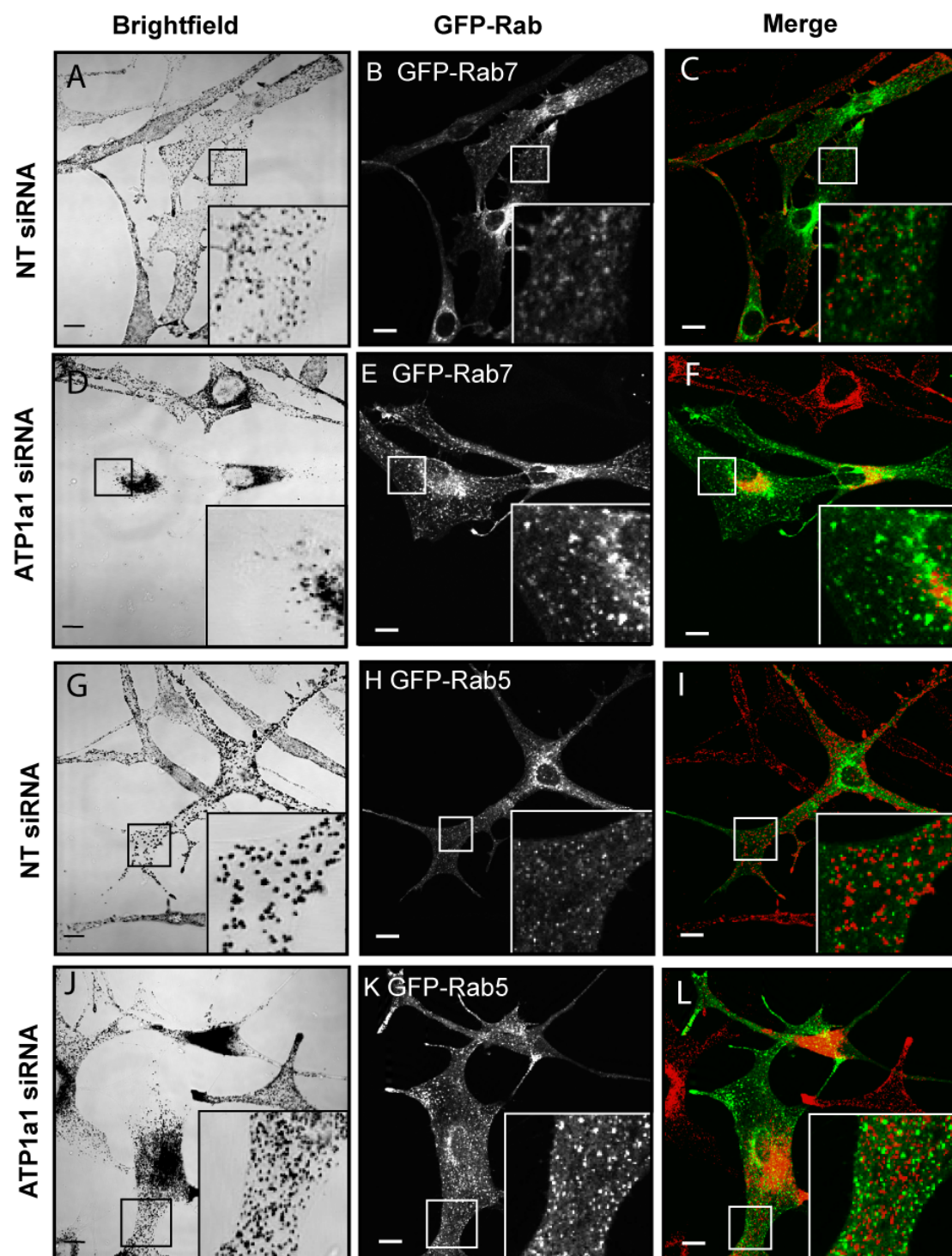


Figure 4-22 EGFP-Rab5 and EGFP-Rab7-positive Vesicle Distribution Following ATP1a1 Depletion.

Melan-INK4a cells were grown on coverslips and treated with either NT or ATP1a1 siRNA and cultured for 24h before transfection with EGFP-Rabs. After an additional 48h, cells were fixed with 4% PFA and then analysed using fluorescence microscopy. Brightfield image demonstrates the melanosome distribution (A, D, G and J). Panels B and E show EGFP-7 localisation and panels H and K show EGFP-Rab5 localisation. Pigment is inverted and pseudo-coloured red to aid colocalisation with the EGFP signal in the merge panels (C, F, I and L). Insets are a higher magnification of the boxed area. Scale bar represents 10µm. Images representative of two experiments.

Further analysis looked at the effect of ATP1a1 depletion on endogenous Rab5.

Immunostaining for endogenous Rab5 showed punctuate staining throughout the cell (Figure 4-23 B and C), similar to the EGFP-Rab5 (Figure 4-22) which was unaffected by ATP1a1 depletion (Figure 4-23 E and F).

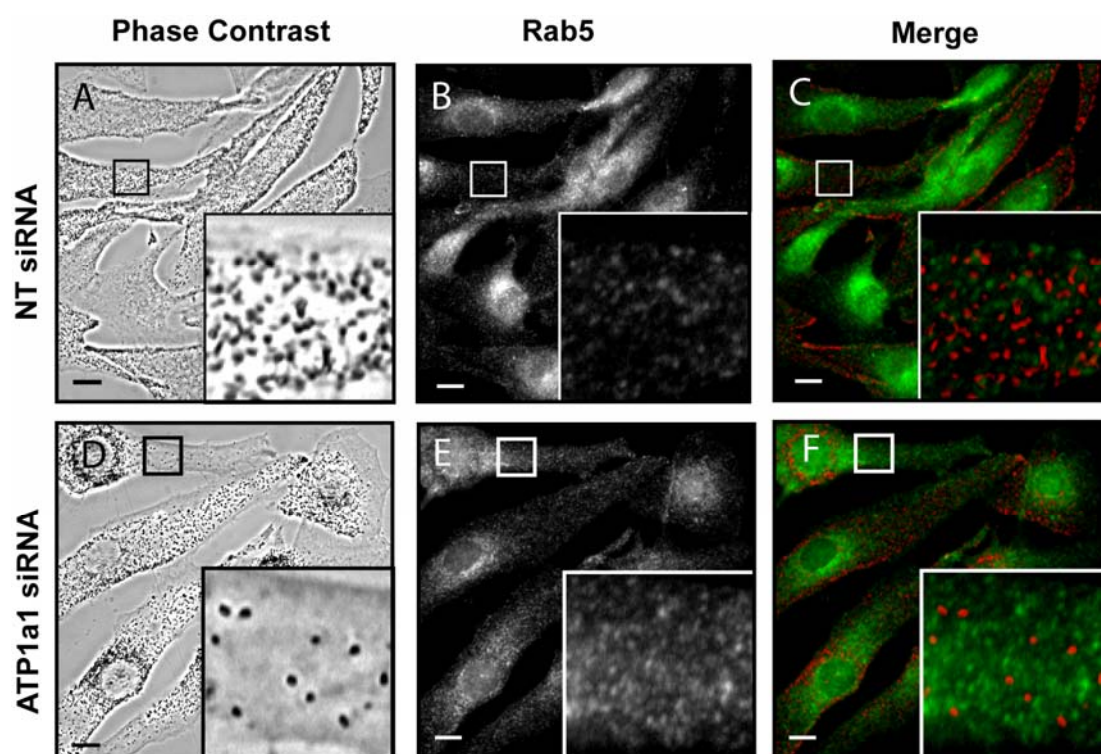


Figure 4-23 Endogenous Rab5-vesicle Distribution Following ATP1a1 Depletion.

Melan-INK4a cells were grown on coverslips and treated with either NT or ATP1a1 siRNA and cultured for 72h then fixed with 4% PFA, permeabilised and blocked with FBS. Coverslips were immunostained with rabbit anti-Rab5 antibody and Alexa-488 conjugated anti-rabbit secondary antibody then analysed using fluorescence microscopy. Phase contrast images demonstrate the melanosome distribution (A and D). Panels B and E show endogenous Rab5 localisation. Pigment is inverted and pseudo-coloured red to aid colocalisation with Rab5 (green) in the merge panels (C and F). Insets are a higher magnification of the boxed area. Scale bar represents 10µm. Images represent one experiment.

4.12 Confirmation of Rab27a and ATP1a1 Interaction

ATP1a1 was identified as a candidate protein following its identification from a tandem affinity purification using Rab27a as bait. To further confirm this association, the interaction was further investigated by performing the reverse immunoprecipitation.

Melan-INK4a cells were lysed and incubated with mouse anti-ATP1a1 antibody. The IP was assessed by immunoblotting for Rab27a and ATP1a1. Probing for both proteins simultaneously revealed a band at the expected molecular weight of endogenous Rab27a (30kDa, indicated by lower arrow) in the ATP1a1 IP B sample (Figure 4-24 A lane 3) that was

not present in the negative control (no antibody IP) (Figure 4-24 A lane 5). However there appeared to be no immunoprecipitated ATP1a1 in the sample (indicated by upper double arrows). Repeat analysis of an increased amount of IP sample and immunoblotting for ATP1a1 showed that indeed it had been immunoprecipitated (Figure 4-24 B, indicated by upper double arrows). However it did highlight unspecific reactivity of the immunoblotting antibody (asterisk *) with a band of approx. 30kDa, comparable with endogenous Rab27a, therefore suggesting that the band previously thought to be endogenous Rab27a could be cross-reactivity of the anti-mouse secondary antibody with the mouse anti-ATP1a1 antibody used for the IP. Similarly, the double bands between 50-60kDa (asterisks *) are likely to be due to cross-reactivity with the immunoglobulin heavy chain. Repeat analysis of the samples by immunoblotting with a rabbit antibody for Rab27a also showed a 30kDa band in the ATP1a1 IP-B sample (Figure 4-24 C), but this was also present in the IP-B negative control (asterisk *) suggesting the rabbit secondary cross-reacted with the boiled beads.

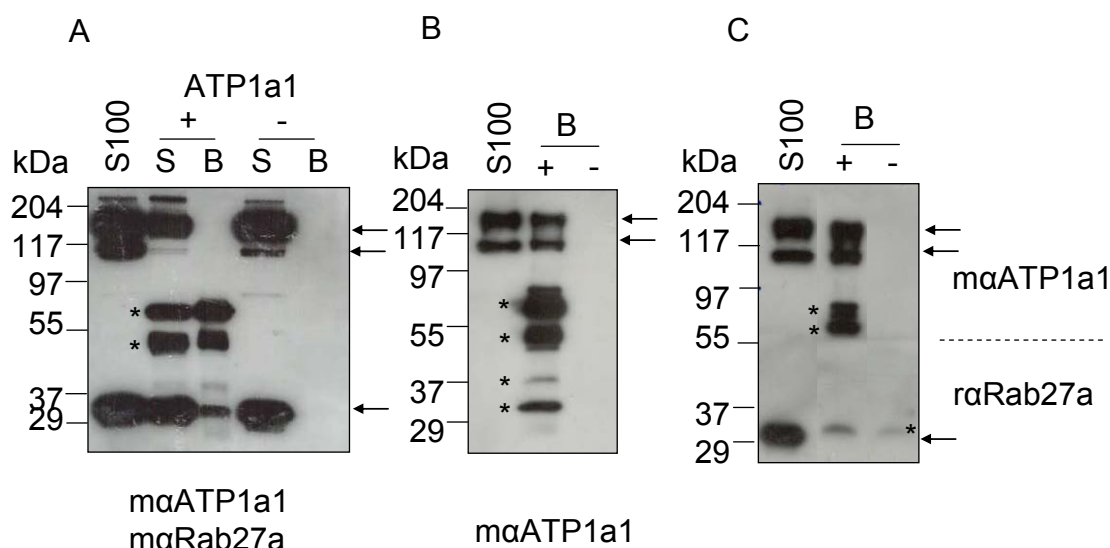


Figure 4-24 Co-Immunoprecipitation of Endogenous Rab27a with ATP1a1.

Melan-INK4a cells were lysed and proteins solubilised in 1% CHAPS. Samples were then incubated with or without 5µg mouse anti-ATP1a1 and 50µl Sheep anti-mouse Dynabeads overnight. A) 30µl of each sample was analysed by immunoblotting with mouse anti-Rab27a and mouse anti-ATP1a1 antibodies. B) 5µl S100 and 60µl B and immunoblotted with mouse anti-ATP1a1 antibody. C) 5µl S100 and 60µl B, blot divided and top half immunoblotted with mouse anti-ATP1a1 antibody and the bottom rabbit anti-Rab27a antibody. Arrows indicate Rab27a and ATP1a1 bands. Asterisk * indicate unspecific bands.

One possible solution to these issues was to use a rabbit antibody for the IP and immunoblot with a mouse antibody. Melan-INK4a and melan-ash cells were lysed, and solubilised in 1% CHAPS and a rabbit anti-ATP1a1 was used for the IP. However, the rabbit anti-ATP1a1 failed to IP any ATP1a1 as no protein band is apparent in the ATP1a1 IP-B sample (Figure 4-25 upper arrow indicates ATP1a1) Therefore, it was decided that probing for endogenous Rab27a using the available beads and antibodies was not possible due to unspecific cross-reactivity with either the beads or the antibodies. Crosslinking the antibody to the beads prior to IP may resolve this problem or using low pH to elute from the beads as opposed to boiling. Similarly, using a tagged Rab27a would aid the analysis as it has a higher molecular weight than endogenous Rab27a.

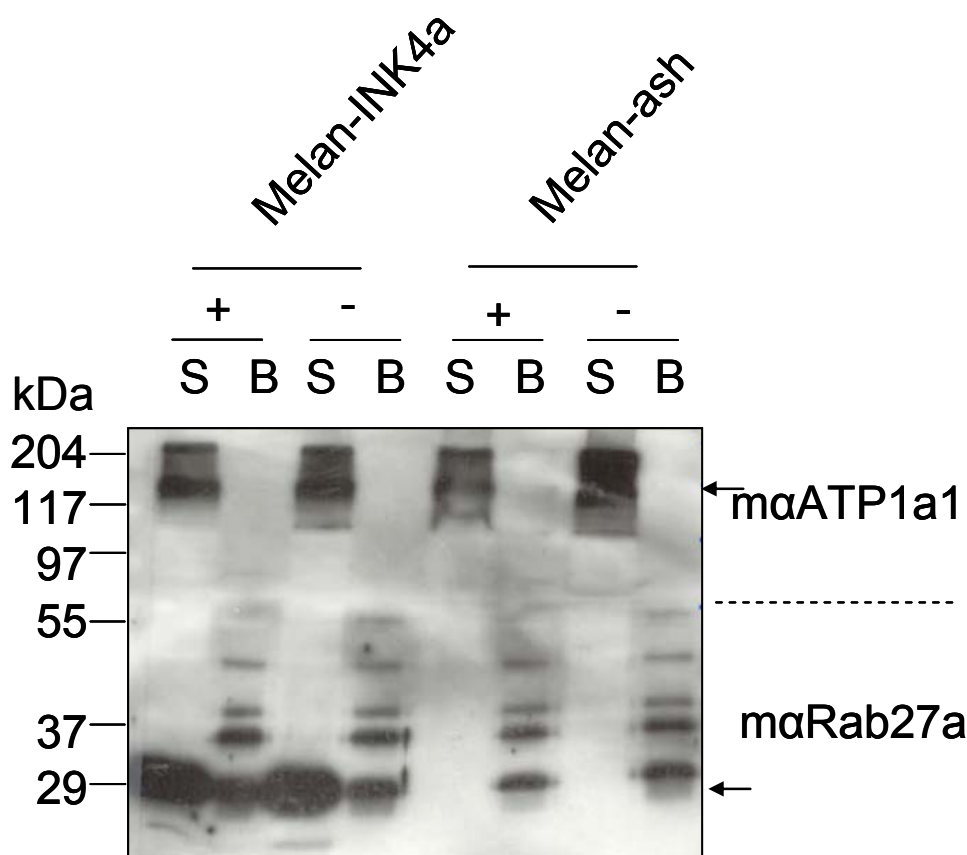


Figure 4-25 Co-Immunoprecipitation of Endogenous Rab27a with ATP1a1.

Melan-INK4a and Melan-ash cells were lysed and proteins solubilised in 1% CHAPS. Samples were then incubated with or without 4µl rabbit anti-ATP1a1 antibody and 30µl A/G Beads overnight. 30µl of each sample was analysed by immunoblotting with mouse anti-Rab27a and mouse anti-ATPa1 antibodies.

In this vein, the HTF-tagged Rab27a stable cells were used which have the added advantage as there is no endogenous Rab27a to compete for ATP1a1 binding. Cell pellets of stable HTF-Rab27a^{WT} (Figure 4-26 A), HTF-Rab27a^{SF1/F4} (Figure 4-26 B) and HTF-Rab27a^{SF2} (Figure 4-26 C) cells were solubilised in 1% CHAPS and HTF-Rab27a or ATP1a1 were immunoprecipitated using mouse anti-HA or anti-ATP1a1 antibodies respectively or no antibody as a negative control. Initial analysis of the HA IP-B lanes suggested that ATP1a1 did not co-IP with HTF-Rab27a (Figure 4-26 A), however re-analysis of the HTF-Rab27a^{WT} samples using a higher concentration of antibody for the immunoblotting highlights that a small amount of ATP1a1 did co-IP with HTF-Rab27a^{WT} (Figure 4-26 E). Endogenous ATP1a1 was successfully immunoprecipitated with the mouse anti-ATP1a1 antibody (Figure 4-26 A-C lane 7). In all

samples, the levels of ATP1a1 were very low in the S100 yet very strong bands were visible in the ATP1a1 IP-B samples, indicative of efficient IP of the protein. Analysis of the ATP1a1 IP revealed a band with the expected molecular weight for HTF-Rab27a (45kDa) in the precipitated samples (Figure 4-26 A-C lane 7, indicated by arrows). The smaller sized band at about 37kDa was believed to be a degradation product of HTF-Rab27a (Figure 4-26 A-C arrowhead). The bands at about 60kDa and 29kDa in both the HA and ATP1a1 IPs were cross-reactivity of the anti-mouse secondary immunoblotting antibody with the heavy and light chains of the IP-antibody respectively (Figure 4-26 A-C asterisk *). Similarly, the band at about 50kDa in the ATP1a1 IP was also cross-reactivity of the anti-mouse secondary immunoblotting antibody with the IP-antibody (Figure 4-26 A-C asterisk *). This was confirmed by re-analysis of the HTF-Rab27a^{WT} samples and only immunoblotting with the anti-mouse secondary antibody (Figure 4-26 D). This suggests that ATP1a1 is able to co-IP HTF-Rab27a. Further from this, comparison of the amount of Rab27a relative to ATP1a1 in the mαATP1a1-B lane suggests an 80% decrease in Rab27a co-immunoprecipitated by ATP1a1 from the HTF-Rab27a^{SF2} samples relative to the HTF-Rab27a^{SF1/F4} (Figure 4-26 B and C). This indicated that the HTF-Rab27a^{SF2} mutant had a weakened association with ATP1a1 compared to the HTF-Rab27a^{WT} and HTF-Rab27a^{SF1/F4} mutant, therefore suggesting a reason for its lack of melanosomal targeting if association with ATP1a1 is important. Of particular note however was that the HTF-Rab27a^{SF2} cell line was not used for the TAP as HTF-Rab27a^{SF2} was showing signs of localising to melanosomes, therefore not suitable for a negative control. If an interaction between Rab27a and ATP1a1 is required for Rab27a localisation to melanosomes, this low level of interaction could explain why a small population of cells showed localisation.

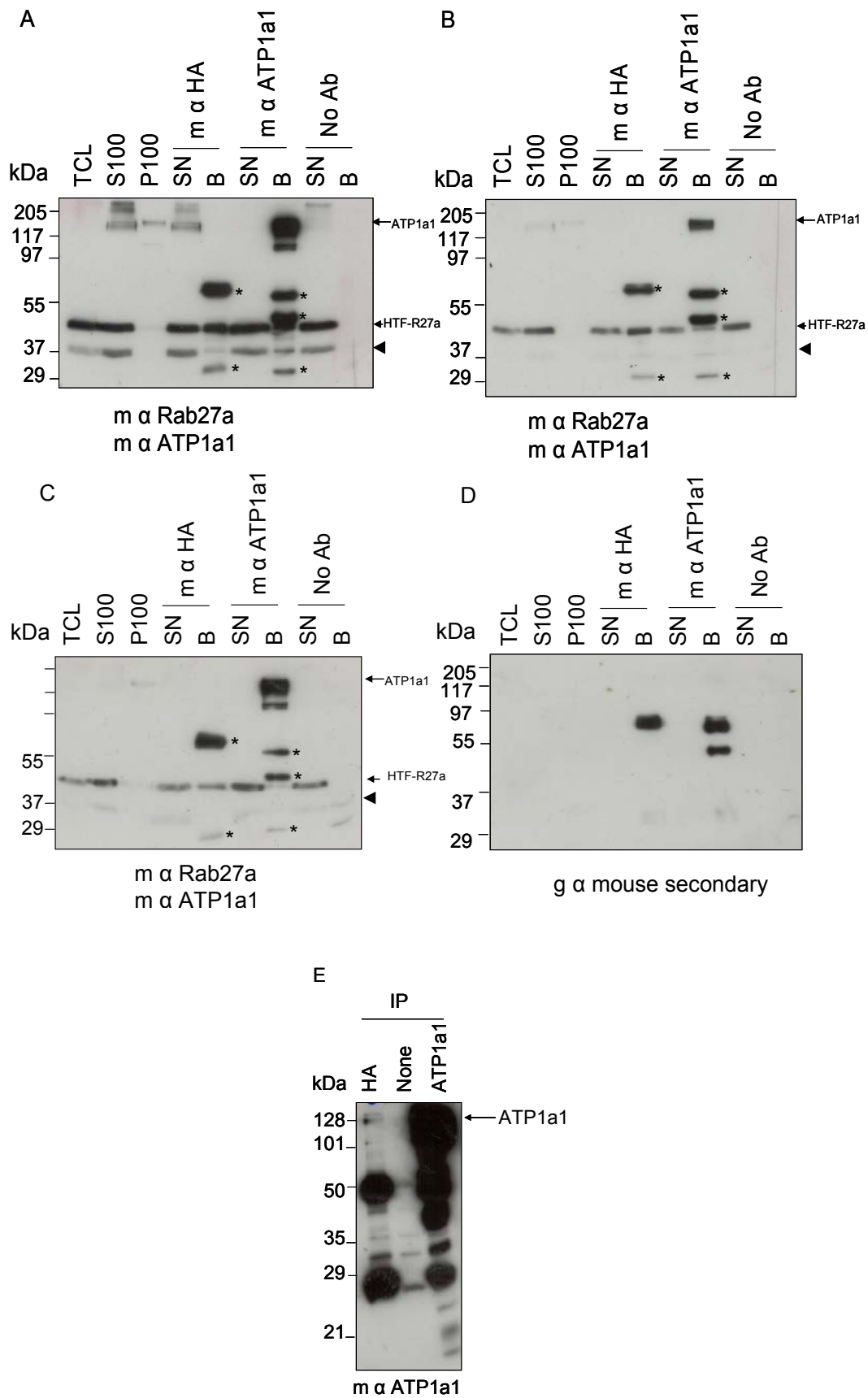


Figure 4-26 Co-Immunoprecipitation of HTF-Rab27a with ATP1a1.

HTF-Rab27a^{WT} (A), HTF-Rab27a^{SF1F4} (B) and HTF-Rab27a^{SF2} (C) cell pellets were lysed and proteins solubilised in 1% CHAPS. S100 was then incubated with 10µg mouse anti-ATP1a1 antibody, 10µg mouse anti-HA antibody or no antibody. This was added to 100µl A/G Beads and incubated overnight. 40µl of each sample was analysed by immunoblotting with mouse anti-Rab27a and mouse anti-ATP1a1 antibodies. D) HTF-Rab27a^{WT} samples were analysed by immunoblotting with goat anti-mouse-HRP secondary antibody only. E) Overexposure of HTF-Rab27a^{WT} immunoprecipitated samples following immunoblotting with mouse anti-ATP1a1 antibody. Arrows indicate HTF-Rab27a and ATP1a1. Arrowheads indicate HTF-Rab27a degradation. Asterisk * indicate unspecific bands.

4.13 Conclusions

These data provide compelling evidence that the depletion of ATP1a1 has a detrimental effect on Rab27a function and its association with melanosomes. This phenotype is exacerbated by simultaneous depletion of R3G. ATP1a1 and R3G depletion also affects the balance of other melanosomal proteins. However the disrupted vesicle distribution is specific to Rab27a-regulated traffic. The interaction of Rab27a with ATP1a1 (directly or indirectly) may be required for the correct membrane targeting of Rab27a.

5 Characterisation of ATP1a1

Involvement in Rab27a Recruitment

5.1 Introduction

ATP1a1 ($\alpha 1$ subunit of Na^+, K^+ -ATPase) was identified as a novel interacting partner for Rab27a (Chapter 3). Subsequent analysis found that depletion of ATP1a1 resulted in decreased GTP-bound Rab27a and displacement of Rab27a from melanosomal membranes. In addition, ATP1a1 and R3G depletion altered the association of Rab38 and TYRP1, suggesting a possible disruption in melanosome biogenesis (Chapter 4).

The Na^+, K^+ -ATPase has two functional roles: 1) ion homeostasis as it exports 3 Na^+ in exchange for 2 K^+ and 2) signalling function through regulation of Src Kinase. A number of questions still remain as to the mechanism by which ATP1a1, a subunit of Na^+, K^+ -ATPase, could be regulating Rab27a: i) is ATP1a1 a component of melanosomal membranes and does it interact directly with Rab27a or ii) is melanosome associated-Rab27a interacting with plasma membrane ATP1a1? iii) Is Na^+, K^+ -ATPase at the plasma membrane regulating cellular pH, which in turn affects Rab27a? iv) Does Na^+, K^+ -ATPase function as an ion pump on melanosomes, altering the lumenal environment and regulating melanosome maturation? v) Is the ATP1a1 signalling function involved in Rab27a targeting?

The following investigations aimed to elucidate the localisation and function of ATP1a1 in melanocytes and establish a mechanism for its role in regulating Rab27a targeting to melanosomal membranes.

5.2 ATP1a1 Intracellular Localisation

The TAP and immunoprecipitation experiments described previously (Chapters 2 and 3) indicated that Rab27a interacts with ATP1a1, however Na^+, K^+ -ATPase is a plasma membrane protein and little is known regarding its function/presence on intracellular membranes. It has been shown that there is a pool of intracellular Na^+, K^+ -ATPase [349] that in lung alveolar epithelium it is recruited to the plasma membrane following catecholamine stimulation [350, 351]. It has been proposed that activation of Akt is necessary and sufficient for Na^+, K^+ -ATPase plasma membrane recruitment. Akt phosphorylates and inactivates AS160 (Rab10 GAP) which promotes Rab10-mediated trafficking of Na^+, K^+ -ATPase to lung alveolar plasma membranes [352, 353]. MyoVa has also been shown to associate with Na^+, K^+ -ATPase containing vesicles in alveolar epithelial cells and negatively regulates their plasma membrane recruitment by restraining Na^+, K^+ -ATPase in its intracellular pool [354]. In melanocytes, ATP1a1 has been proposed to be a melanosomal protein by proteomic studies [412, 414]. However, following purification of melanosomes, immunoblotting failed to identify ATP1a1 in the purified fraction (Figure 4-14).

5.2.1 ATP1a1 Localises to Intracellular Vesicles in Melanocytes

In order to assess the localisation of ATP1a1 in melanocytes, melan-INK4a cells were fixed and immunostained for ATP1a1. Cell fixation with 4% PFA followed by immunostaining with mouse anti-ATP1a1 antibody resulted in extensive staining of the cytosol (Figure 5-1) but the expected plasma membrane staining was not apparent. Altering the antibody concentration did not alter that staining pattern. A degree of punctate staining was seen with high concentrations of antibody (1:250 dilution), perhaps indicating vesicular association (Figure 5-1 E, F). However, no definite co-localisation with melanin could be ascertained.

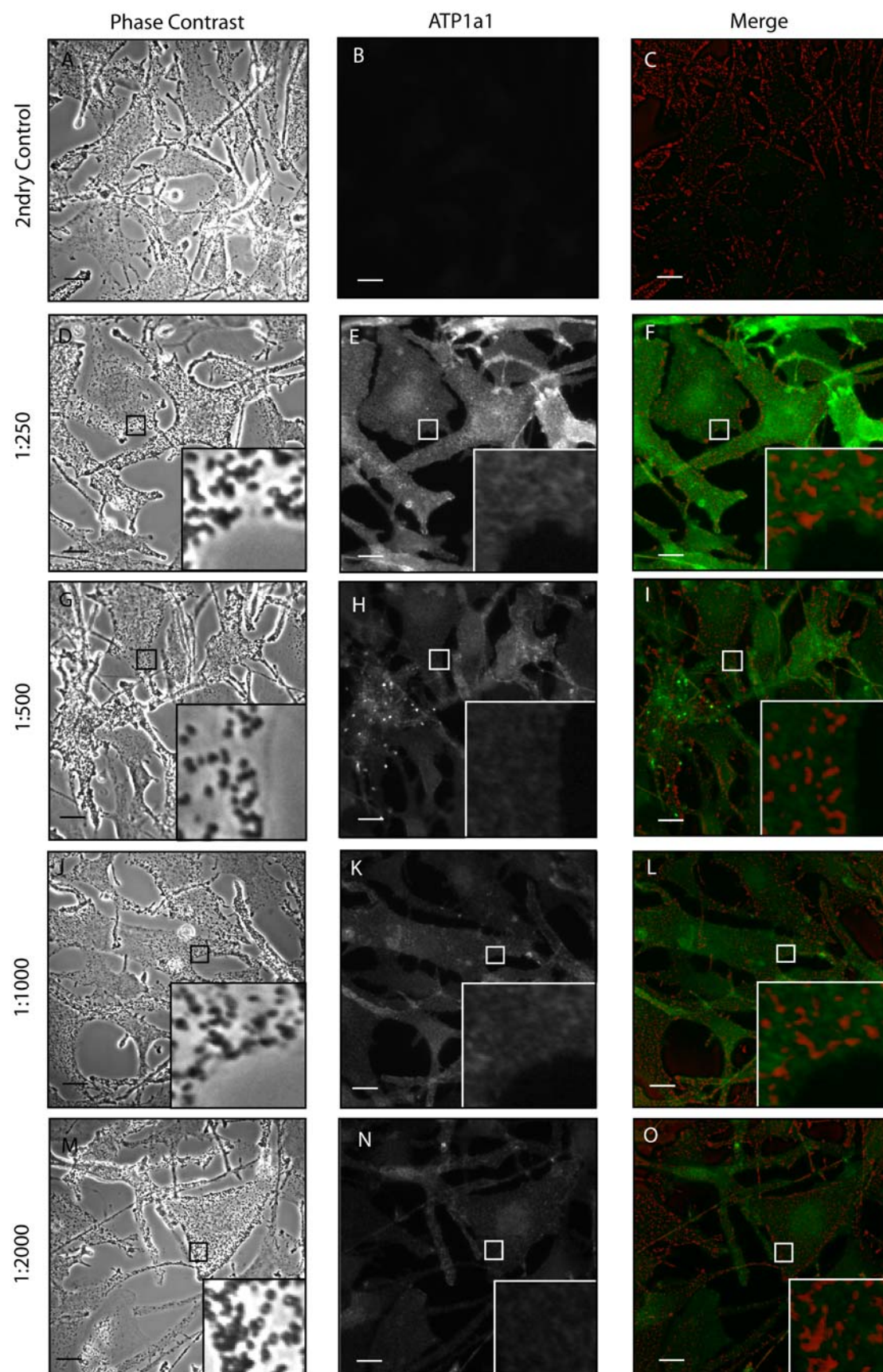


Figure 5-1 ATP1a1 Immunostaining of Melan-INK4a After PFA Fixation

Melan-INK4a cells were grown on coverslips and fixed with 4% PFA. Cells were permeabilised and blocked with FBS prior to immunostaining with mouse anti-ATP1a1 antibody in varying concentrations and Alexa-488 conjugated anti-mouse secondary antibody. A secondary only sample was used as a negative control (A-C). Immunofluorescence images were taken on a fluorescence widefield microscope. Phase contrast panels show melanosome distribution (A, D, G, J and M). Panels B, E, H, K and N show ATP1a1 immunostaining. Pigment is inverted and pseudo-coloured red to aid colocalisation with ATP1a1 (green) in the merged panels (C, F, I, L and O). Insets are a higher magnification of the boxed area. Scale bar represents 10µm. Images representative of three experiments.

Using MeOH as a fixative resulted in similar irregular cytosolic staining as seen with PFA (Figure 5-2 A-C). Acetone fixation resulted in more distinct punctate cytosolic staining in addition to some plasma membrane staining (Figure 5-2 D-F) but there was no apparent colocalisation with melanosomes. The epitope for the mouse antibody has not been mapped and, if present on the extracellular/luminal domains, then detection by immunofluorescence may have been hampered due to quenching from the melanin. A rabbit polyclonal antibody raised against an N-terminal synthetic peptide of ATP1a1 was therefore purchased. Immunofluorescence using the rabbit antibody showed similar punctate staining with optimal staining apparent with acetone fixation (Figure 5-2 J-L) rather than 4% PFA (Figure 5-2 G-I) as a fixing reagent. However there was no colocalisation with the melanin in any condition and there was no plasma membrane staining (Figure 5-2 C, F, I, L).

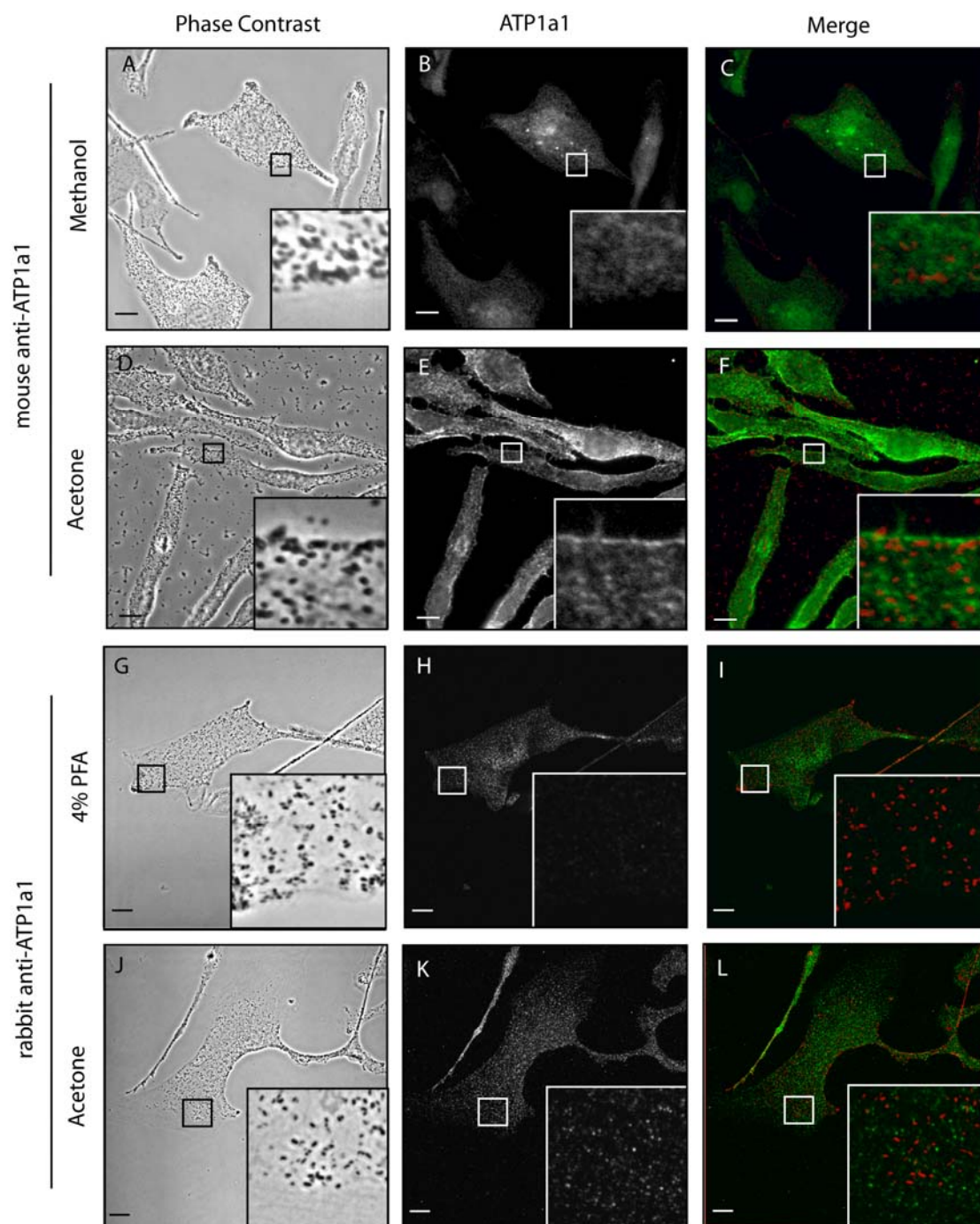


Figure 5-2 ATP1a1 Immunostaining of Melan-INK4a After Methanol and Acetone Fixation.

Melan-INK4a cells were grown on coverslips and fixed with methanol (A-C), acetone (D-F, J-L) or 4% PFA (G-I). Cells were permeabilised and blocked with FBS prior to immunostaining with mouse anti-ATP1a1 (A-F) or rabbit anti-ATP1a1 (G-L) antibodies and Alexa-488 conjugated anti-mouse or anti-rabbit secondary antibodies. A secondary only sample was used as a negative control (data not shown). Images were taken on a fluorescence widefield (A-F) or confocal (G-L) microscope. Phase contrast panels show melanosome distribution (A, D, G and J). Panels B, E, H and K show ATP1a1 immunostaining. Pigment is inverted and pseudo-coloured red to aid colocalisation with ATP1a1 (green) in the merge panels (C, F, I and L). Insets are a higher magnification of the boxed area. Scale bar represents 10µm. Images representative of one experiment.

Immunostaining using the mouse antibody following cell fixation with acetone was deduced to be the most effective. However, to check whether the observed staining was accurate, immunostaining of melan-INK4a cells after ATP1a1 depletion was performed. Despite the sample preparation resulting in extensive bubbles, depletion of ATP1a1 resulted in a weakened immunofluorescence signal indicating that the staining observed was specific for ATP1a1 in melanocytes (Figure 5-4 B and D). These results therefore suggest that in addition to the plasma membrane, ATP1a1 is localised on intracellular vesicles in melan-INK4a.

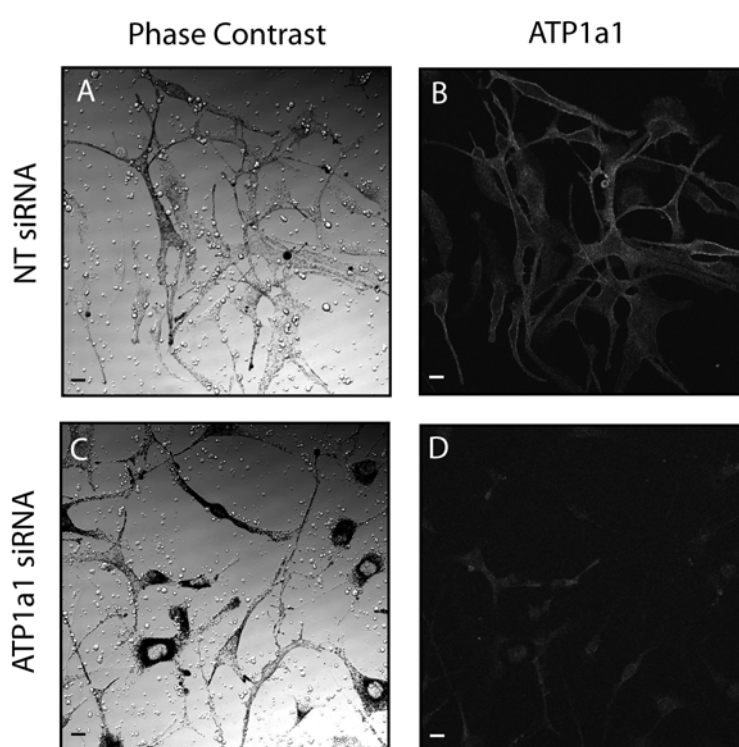


Figure 5-3 ATP1a1 Staining of ATP1a1 Depleted Melan-INK4a.

Melan-INK4a cells were grown on coverslips and treated with NT or ATP1a1 siRNA for 3 days. Cells were fixed with acetone, permeabilised and blocked with FBS prior to immunostaining with mouse anti-ATP1a1 antibody and Alexa-488 conjugated anti-mouse secondary antibody. Images were taken on a fluorescence confocal microscope. Phase contrast panels show melanosome distribution (A and C). Panels B and D show ATP1a1 immunostaining. Scale bar represents 10 μ m. Images representative of one experiment.

Acetone fixation of cells and immunostaining with the mouse anti-ATP1a1 antibody appeared to give optimal staining so this was assessed with co-staining for endogenous Rab27a in NT and ATP1a1 siRNA treated cells (Figure 5-4). However, acetone fixation

disrupted the efficiency of Rab27a immunostaining and the signal was very weak relative to the background. The poor staining was emphasized by the observation that co-localisation of Rab27a with melanin was not as clear as normally observed with PFA fixation (Figure 5-4 H arrows). The ATP1a1 intracellular immunostaining was punctate but extensive (Figure 5-4 B and D) therefore preventing convincing co-localisation analysis with Rab27a (Figure 5-4 F). Despite the difficulties, there appeared to be a few melanosomes which were positive for both Rab27a and ATP1a1 (Figure 5-4 I arrowheads) suggesting a transient association.

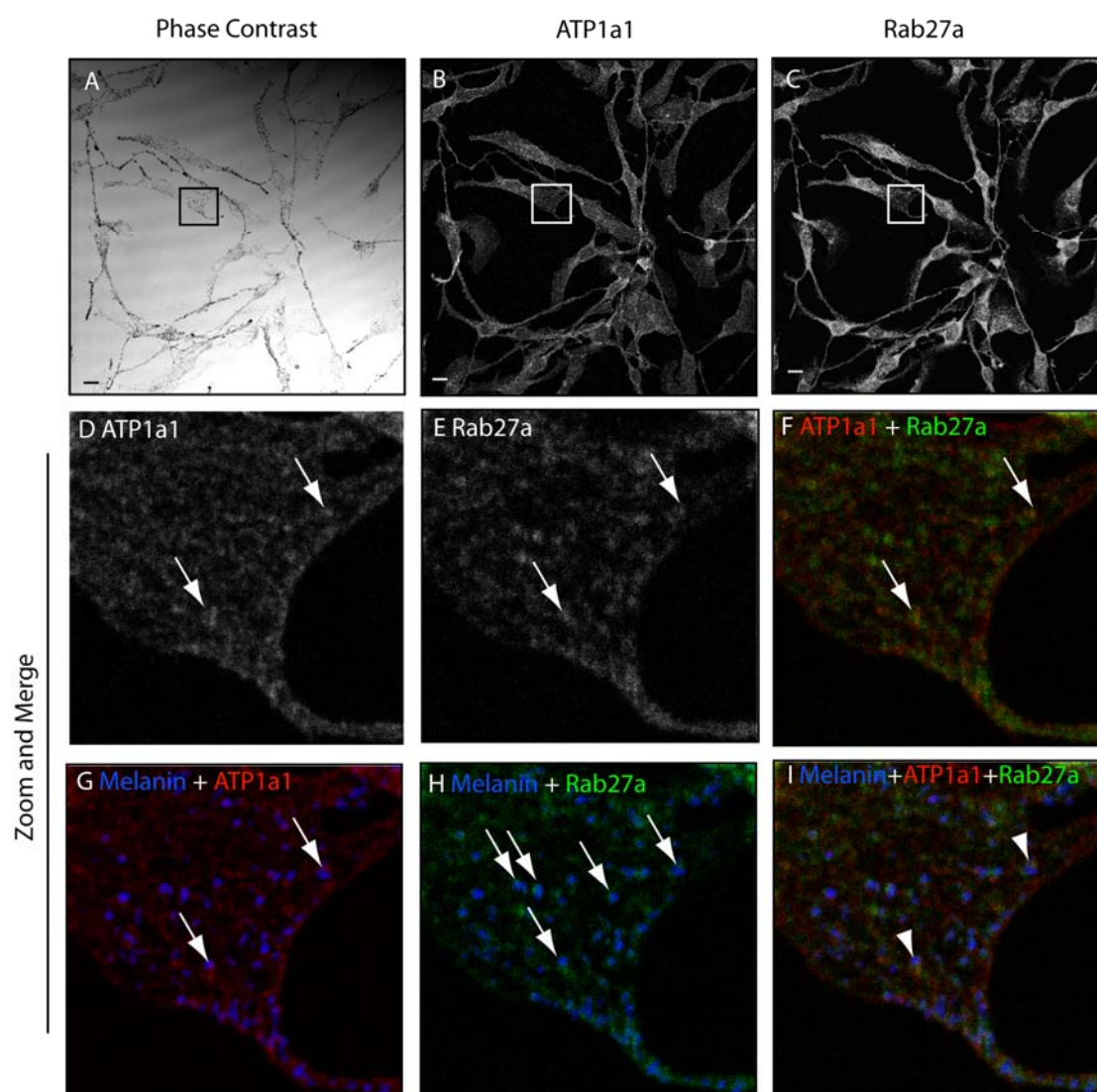


Figure 5-4 ATP1a1 and Rab27a Co-staining of Melan-INK4a.

Melan-INK4a cells were grown on coverslips before fixing with acetone. Cells were permeabilised and blocked with FBS prior to immunostaining with mouse anti-ATP1a1 and rabbit anti-Rab27a antibodies and Alexa-568 conjugated anti-mouse and Alexa-488 conjugated anti-rabbit secondary antibodies. A secondary antibody only sample was used as a negative control (data not shown). Images were taken on a fluorescence confocal microscope. Phase contrast panel shows melanosome distribution (A). Panels B and D show ATP1a1 immunostaining and panels C and E show Rab27a immunostaining. Panel F shows the merge of ATP1a1 (red) and Rab27a (green). Pigment is inverted and pseudo-coloured blue (G-I) to aid co-localisation with ATP1a1 (G, red), Rab27a (H, green) or both (I ATP1a1:red, Rab27a:green). Zoom panels are a higher magnification of the boxed area. The arrows indicate co-localisation, the arrowheads show triple-colocalisation. Scale bar represents 10µm. Images representative of one experiment.

5.2.2 GFP-ATP1a1 Transfection of Melanocytes

The antibody detection was not good enough to distinguish the possible small amounts of ATP1a1 on melanosomes and so a plasmid encoding GFP-tagged human ATP1a1 was purchased. It was thought that over expression of exogenous ATP1a1 may aid identification of its intracellular localisation. Melan-INK4a cells were transfected with the construct under various transfection conditions and an EGFP construct was used as a control for transfection. The EGFP construct was expressed, resulting in green fluorescence cells. However, none of the transfection conditions assayed resulted in any GFP-ATP1a1 expression as assessed by GFP fluorescence or anti-GFP immunofluorescence staining. The experiment was repeated in HEK293A cells as melan-INK4a cells are known to be difficult to transfect, but still no GFP-ATP1a1 expressing cells were identified.

The β -subunit of Na^+, K^+ -ATPase is required for the exit of the α -subunit from the ER [292] so it is possible that exogenous expression of ATP1a1 required the exogenous expression of the β -subunit. ATP1b3 (β subunit, isoform 3 of Na^+, K^+ -ATPase) was identified as a melanosomal protein in proteomics studies [412] and as a putative interacting partner for Rab27a by HTF-Rab27a^{SF1/F4} TAP (Chapter 3 and Appendix) and so a plasmid encoding the DNA for ATP1b3 was purchased (pCMV-AC-ATP1b3). Melan-INK4a cells were transfected with the plasmids (pCMV-GFP-ATP1a1 and pCMV-AC-ATP1b3) individually and in combination. However, harvesting the cell lysates and immunoblotting for ATP1b3 or GFP yielded no signal, and immunofluorescence microscopy identified no GFP-positive cells. The ATP1b3 antibody also failed to identify any bands in untransfected melan-INK4a TCLs therefore questioning its reactivity. It was therefore uncertain whether dual-transfection was achieved. Tagging the ATP1b3 construct would aid in analysing its expression, but dual transfection still requires optimisation. The plasmids were not sequenced prior to transfection as the company stated they were 'transfection ready', however maybe there was a sequence error.

Additionally, transfer of the gene into an alternative plasmid, known to express well in melan-INK4a, may promote the expression.

5.2.3 ATP1a1 Localises to a Fraction of Purified Melanosomes

An alternative method was to purify melanosomes from melanocytes and immunostain for endogenous ATP1a1. Using the method described previously (Chapter 4), purified melanosomes were centrifuged onto coverslips and fixed with 4% PFA before immunostaining for ATP1a1. Although the majority of melanosomes were not positive for ATP1a1, a small population were (Figure 5-5 A-F arrows), and this was decreased in the ATP1a1 siRNA treated cells (Figure 5-5 D-F).

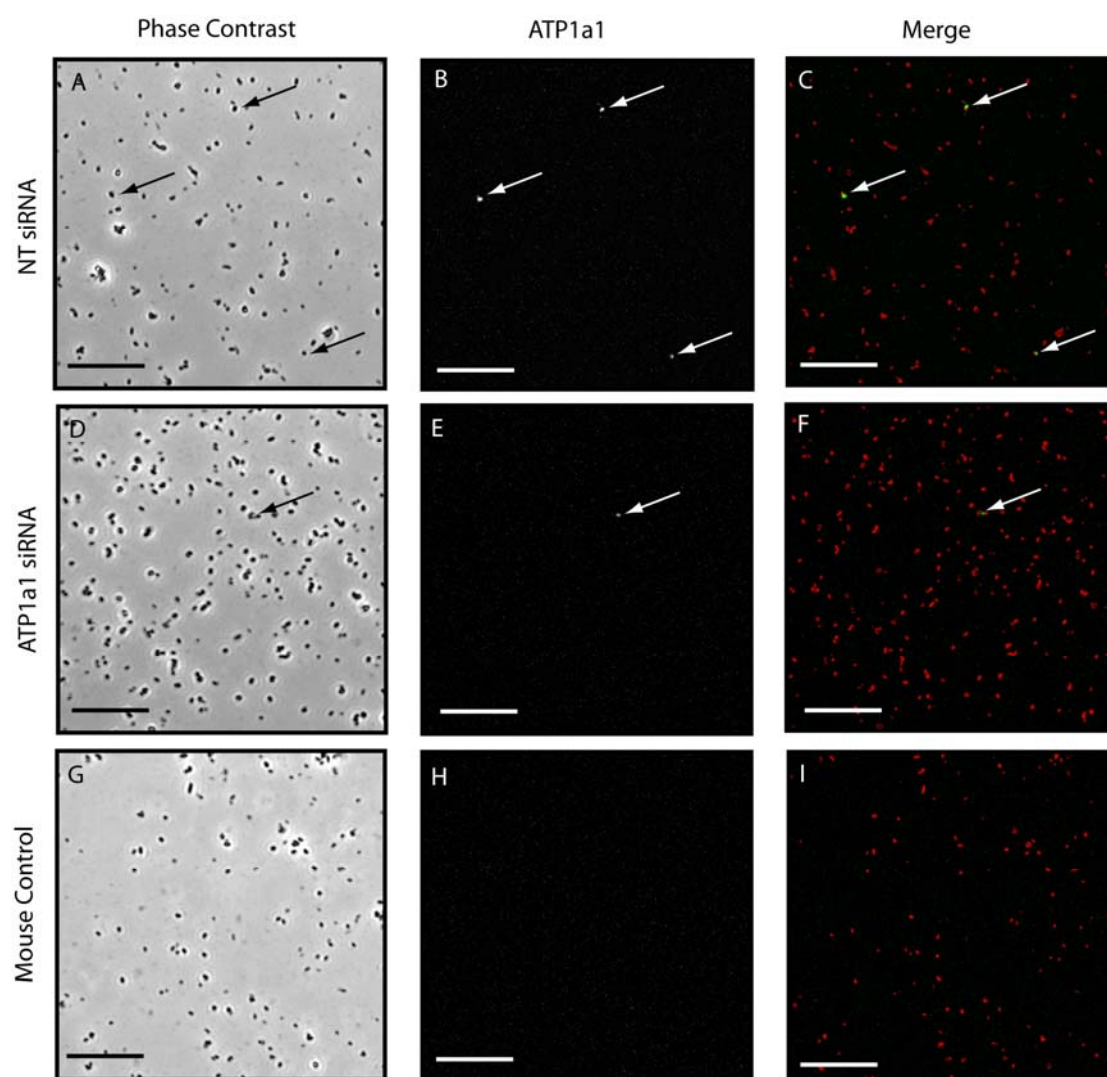


Figure 5-5 ATP1a1 Staining of Purified Melanosomes After PFA Fixation.

NT or ATP1a1 siRNA treated melan-INK4a cells were lysed and the melanosomes purified and centrifuged onto coverslips. The coverslips were fixed with 4% PFA, permeabilised and blocked with FBS prior to immunostaining with mouse anti-ATP1a1 antibody and Alexa-488 conjugated anti-mouse secondary antibody. A secondary only sample was used as a negative control (G-I). Images were taken on a fluorescence confocal microscope. Phase contrast panels show melanosomes (A, D and G) and panels B, E and H show ATP1a1 immunostaining. Pigment is inverted and pseudo-coloured red to aid colocalisation with ATP1a1 (green) in the merge panels (C, F and I). The arrows indicate co-localisation. Scale bar represents 10 μ m. Images representative of two experiments.

Similar results were seen when melanosomes were fixed with acetone (Figure 5-6). In both NT and ATP1a1 siRNA treated cells, 75% of the ATP1a1 positive structures co-localised with melanin (Figure 5-6 A-F). Following ATP1a1 depletion the number of ATP1a1 positive

structures was reduced by 70% (Figure 5-6 D-F). These results suggested that ATP1a1 may transiently associate with pigmented melanosomes.

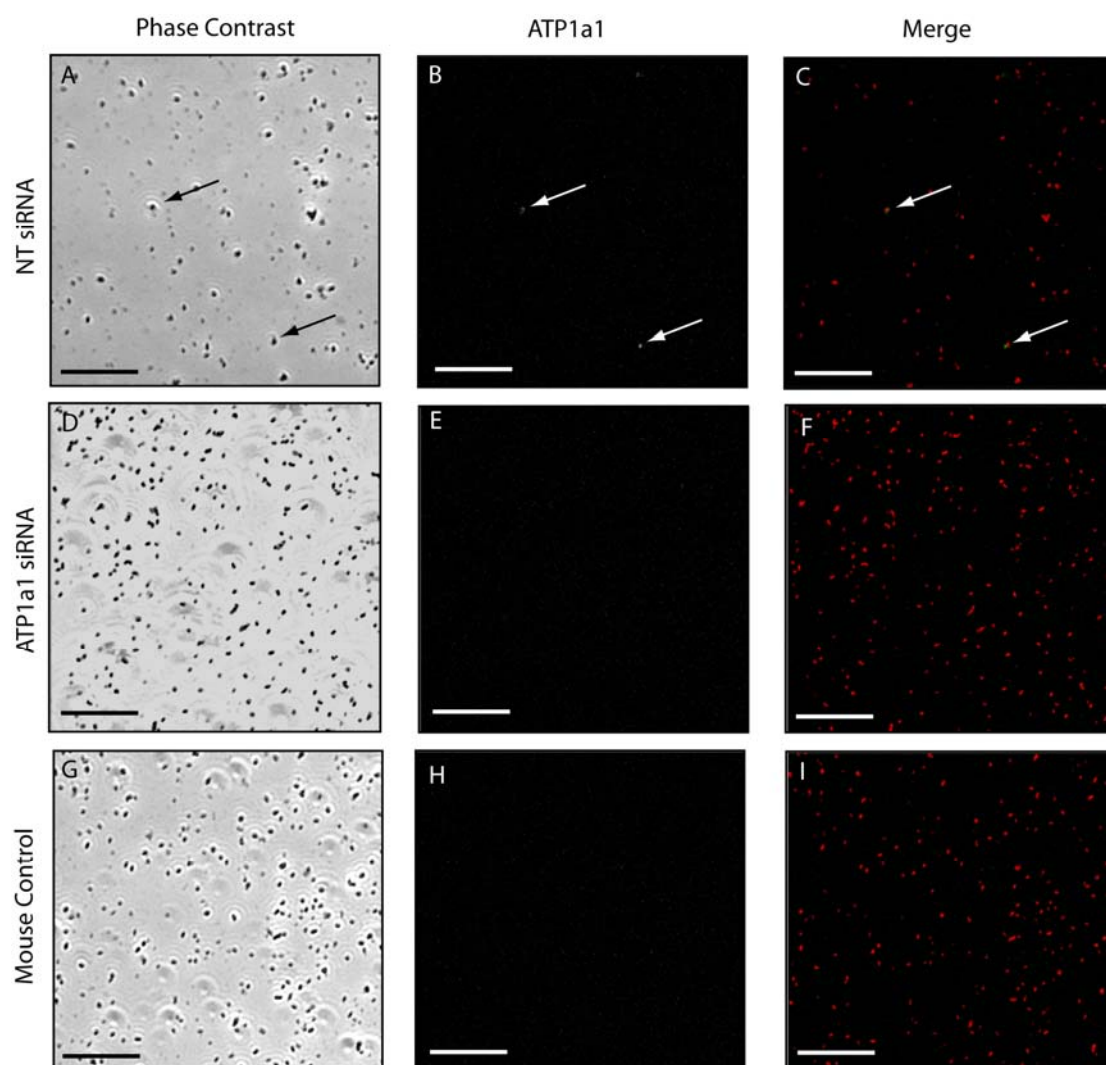


Figure 5-6 ATP1a1 Staining of Purified Melanosomes After Acetone Fixation.

NT or ATP1a1 siRNA treated melan-INK4a cells were lysed and the melanosomes purified and centrifuged onto coverslips. The coverslips were fixed with acetone, permeabilised and blocked with FBS prior to immunostaining with mouse anti-ATP1a1 antibody and Alexa-488 conjugated anti-mouse secondary antibody. A secondary only sample was used as a negative control (G-I). Images were taken on a fluorescence confocal microscope. Phase contrast panels show melanosomes (A, D and G) and panels B, E and H show ATP1a1 immunostaining. Pigment is inverted and pseudo-coloured red to aid colocalisation with ATP1a1 (green) in the merge panels (C, F and I). The arrows indicate co-localisation. Scale bar represents 10µm. Images representative of one experiment.

5.2.4 ATP1a1 Partially Co-Fractionates with Rab27a

The method of melanosome purification described above purifies dense mature melanosomes [450], therefore excluding any analysis of early stage melanosomes. In addition, as Rab27a has been observed on non-melanised vesicles [175], a possible Rab27a delivery vesicle, this could be the site for Rab27a and ATP1a1 co-localisation. Cell fractionation has been used to demonstrate that ATP1a1 associated with MyoVa [354] and Rab10 [352] containing vesicles. Also, sucrose density-gradient ultracentrifugation has previously been used to separate early and late stage melanosomes [431]. Therefore a similar approach was taken to fractionate melan-INK4a cells and probe for Rab27a and ATP1a1 and assess whether they co-fractionated. PNS of melan-INK4a cells was layered on top of a discontinuous sucrose gradient ranging between 0.25-2.0M with 14 x 2ml steps. Previous reports have proposed that stage I and II melanosomes fractionate at 1.0-1.2M sucrose, stage III melanosomes fractionate in 1.4-1.6M sucrose and the dense sucrose fractions contain stages III and IV. Immunoblot analysis of the fractions showed that Rab27a was present in those fractions approx. >1.0M, corresponding to all stages of melanogenesis (Figure 5-7). ATP1a1 fractionated in the fractions equivalent to approx. 1.0-1.4M sucrose only (Figure 5-7) similar to what has been published previously [352, 354] and corresponding to stages I-III melanosomes. Although ATP1a1 was not fractionating with late stage melanosomes, these results highlighted that Rab27a and ATP1a1 co-fractionated with earlier stage melanosomes.

The co-fractionation of the two proteins could suggest they co-localise, whether this be on melanosomes or a separate Rab27a-positive vesicle. However, this colocalisation appears transient, as ATP1a1 is lost with the maturation of melanosomes and their fractionation into the denser sucrose fractions, whilst Rab27a is maintained. Therefore suggesting that ATP1a1 may fulfil its function in recruiting Rab27a but is then recycled from the late stage

melanosomes. Alternatively, ATP1a1 present in the fractions could be a result of contamination with plasma membrane, therefore these results required further investigation. Analysis of the fractions with a plasma membrane marker would aid assessment of the plasma membrane contamination. Similarly, immunoblotting for an early stage melanosome marker (i.e. Pmel17) would aid identification of the early stage melanosome fractions.

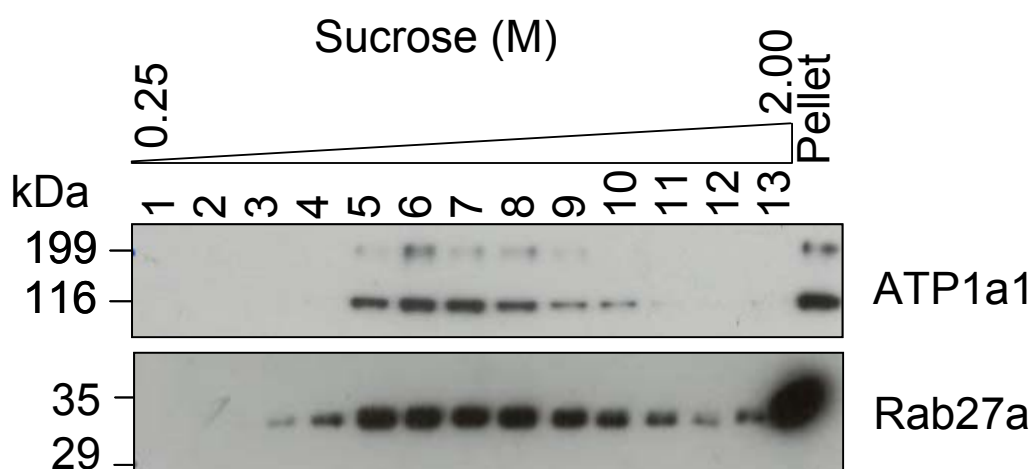


Figure 5-7 ATP1a1 Partially Co-fractionates with Rab27a.

Melan-INK4a cells were lysed and the PNS layered on top of a 0.25-2.0M discontinuous sucrose gradient with 2ml steps. This was centrifuged 100'000 *xg* in a swinging bucket rotor for 1h at 4°C. 2ml layers were removed from the top down, diluted in buffer and centrifuged 17'000 *xg* 30min, 4°C. The pellet was resuspended in 500µl buffer. Pellet fraction represents the pellet after 100'000 *xg* spin. Equal volumes of each fraction were separated by SDS-PAGE and analysed by immunoblotting for Rab27a or ATP1a1. Immunoblot representative of three experiments.

To investigate the fractions further, aliquots were centrifuged onto coverslips and co-immunostained for Rab27a and ATP1a1. Fractions 5-8 and the pellet fraction were analysed for Rab27a and ATP1a1 co-localisation. It was found that Rab27a and ATP1a1 co-localised on some structures, primarily in Fraction 7 (Figure 5-8). In addition, Rab27a and melanin co-localised in the pellet fraction which appeared comparable to the purified melanosomes investigated previously (Figure 5-5 and Figure 5-6). The structures where Rab27a and

ATP1a1 co-localise are most likely stage III-IV melanosomes as they appeared pigmented and as a result were visible in the phase contrast images (Figure 5-8 E, I, M, Q).

Despite distinct bands following immunoblotting, there was very little ATP1a1 immunostaining in the fractions (Figure 5-8). The unidentified ATP1a1-positive vesicles could be small and light, therefore failed to pellet onto the coverslips using the current method. Alternatively, it could be a consequence of the sample preparation as it was previously shown that ATP1a1 immunostaining is optimal with acetone fixation (5.2.1), however Rab27a co-staining requires fixation with 4% PFA. This again highlighted the difficulty in co-staining for Rab27a and ATP1a1 using the available antibodies. In addition, it raised the possibility that additional ATP1a1-positive structures could be visualised if acetone fixation was used but this would limit co-localisation with Rab27a. However, other melanosomal markers (i.e. Pmel17, Tyr or TYRP1) could be tested for their compatibility with acetone fixation to enable analysis of ATP1a1 co-localisation with melanosomes. In particular, stage specific melanosomal markers would aid characterisation of the melanosome when ATP1a1 co-localises, i.e. Pmel17 for early stage melanosomes.

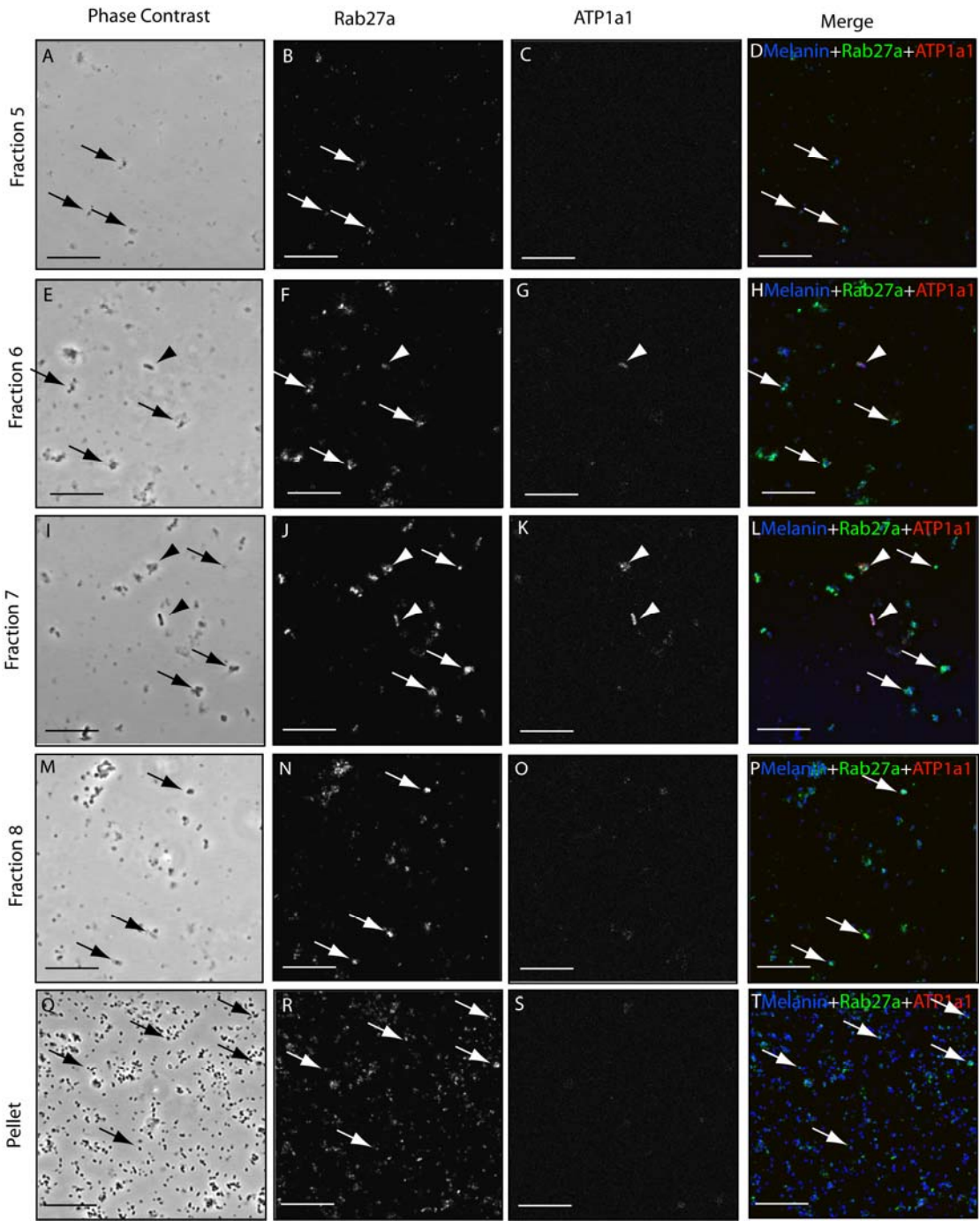


Figure 5-8 Rab27a and ATP1a1 Staining of Sucrose Fractions.

Melan-INK4a cells were fractionated as described previously. Aliquots of each fraction were centrifuged onto coverslips and fixed with 4% PFA. Coverslips were then permeabilised and blocked with FBS prior to immunostaining with rabbit anti-Rab27a, mouse anti-ATP1a1, Alexa-488 conjugated anti-rabbit and Alexa-568 conjugated anti-mouse antibodies. Images were taken on a fluorescence confocal microscope. Phase contrast images show melanosomes (A, E, I, M and Q). Panels B, F, J, N, and R show Rab27a immunostaining and C, G, K, O and S show ATP1a1 immunostaining. Pigment was inverted and pseudo-coloured blue to aid co-localisation with Rab27a (green) and ATP1a1 (red) in the merge panels (D, H, L, P and T). The arrows indicate colocalisation of Rab27a with pigment. The arrow heads indicate colocalisation of Rab27a, ATP1a1 and pigment. Scale bar represents 10µm. Images representative of one experiment.

The sucrose density fractionation experiment again identified instances where Rab27a and ATP1a1 co-localised, comparable to the previous melanosome purification data (Figure 5-5 and Figure 5-6). However, the low frequency of the co-localisation further suggests a transient association and the nature of the structures remained elusive.

5.3 Na⁺,K⁺-ATPase and pH Regulation

The Na⁺,K⁺-ATPase is primarily an ion transporter and its function at the plasma membrane has been widely studied [416, 451, 452]. Disruption of Na⁺ and K⁺ ion transport is likely to have a profound effect on the intracellular environment. Although other Na⁺ and K⁺ ion transporters may be able to compensate the Na⁺ and K⁺ ion imbalance, depletion of ATP1a1 is likely to alter the electropotential and pH of the intracellular environment. In addition, when on intracellular membranes, as proposed in melanocytes, the Na⁺,K⁺-ATPase would maintain its Na⁺ and K⁺ ion pumping properties, thereby regulating the luminal environment of the intracellular compartment. It has been observed that the ATP-driven acidification of endosomes is electrogenic [453] and it is proposed that Na⁺,K⁺-ATPase (also electrogenic) generates an interior-positive membrane potential which opposes the ATP-driven H⁺ uptake by V-type H⁺-ATPase (V-ATPase) [454]. It has also been shown that ouabain, a potent Na⁺,K⁺-ATPase inhibitor, induces endosomal acidification [454-456], therefore suggesting a role for Na⁺,K⁺-ATPase in regulating the pH of intracellular vesicles.

5.3.1 ATP1a1 and R3G Depletion Alters Melanosomal pH

As melanosomes are lysosome-related organelles, it was proposed that they would be acidic. Initial analysis supported this hypothesis [457] and revealed that acidic conditions were optimal for melanogenesis [458]. DAMP (an acidotropic reagent) staining further highlighted the acidic nature of melanosomes in C57BL/6 melanocytes [459]. However, using MNT-1 cells it was found that stage III and IV melanosomes accumulated less DAMP than Stage II, suggesting that melanosomes alkalinise as they mature [460]. Similarly, recent data proposed that neutralising melanosomal pH or treating melanocytes with Bafilomycin A (V-ATPase inhibitor) to inhibit acidification, promoted melanogenesis [461, 462]. Also, the lysosomotropic agent NH_4Cl was shown to stimulate tyrosinase activity [463] as well as melanosome maturation [464]. Finally, it has been shown that activation of the cAMP pathway by forskolin or by the melanocyte stimulating hormone, αMSH , resulted in melanosome alkalinisation through regulating transcription of V-ATPase and ion transporters [465]. Taken together, the literature suggests that melanosomes are initially acidic but then alkalinise during maturation, and therefore the luminal pH is likely to be tightly regulated.

This led to the hypothesis that Na^+, K^+ -ATPase, in opposition to the V-ATPase, could regulate melanosomal pH in a similar mechanism to that proposed for endosomes [454]. Through regulation of the luminal pH, it could contribute to the regulation of melanosome maturation. Depletion of ATP1a1 could lead to luminal acidification, which could indicate melanosomal immaturity and result in the absence of Rab27a recruitment. Concomitantly, ATP1a1 is a transmembrane protein and could be proposed to convey the pH status of the lumen through structural changes resulting in binding and recruitment of Rab27a.

In order to investigate this proposed hypothesis, melan-INK4a cells were treated with NT, Mlph or ATP1a1 siRNA and cultured for 3 days before incubation with $30\mu\text{M}$ DAMP for 20

min. The cells were then fixed and immunostained with anti-DNP antibody conjugated to Alexa-488. Anti-DNP immunostaining resulted in punctate staining throughout the cytosol in the NT siRNA treated cells (Figure 5-9 B), indicating that DAMP accumulated in vesicular structures. However, these structures did not co-localise with TYRP1 stained melanosomes (Figure 5-9 D). In clustered cells (Figure 5-9 E, I) the anti-DNP staining appeared less punctate, in particular the staining in the perinuclear region appeared reduced/absent. This could be a consequence of the melanin within the clustered melanosomes quenching the luminal fluorescence signal. To overcome this issue, the experiment was repeated in melan-c melanocytes, which lack tyrosinase and therefore produce no pigment. In these cells the anti-DNP staining was also punctate throughout the cytosol (Figure 5-9 N), but there was no definitive colocalisation with the TYRP1 staining (Figure 5-9 N-P), comparable to what was observed in melan-INK4a melanocytes. Mlph and ATP1a1 depletion caused perinuclear clustering of melanosomes in melan-c as indicated by the TYRP1 staining (Figure 5-9 S, W). The DAMP containing vesicles also appeared to cluster in the perinuclear region (Figure 5-9 R, V), however the DAMP staining did not co-localise with the TYRP1 vesicles (Figure 5-9 T, X). These results therefore suggested that mature melanosomes are not acidic and that neither Mlph nor ATP1a1 depletion caused a decrease in melanosomal pH as detectable by DAMP accumulation.

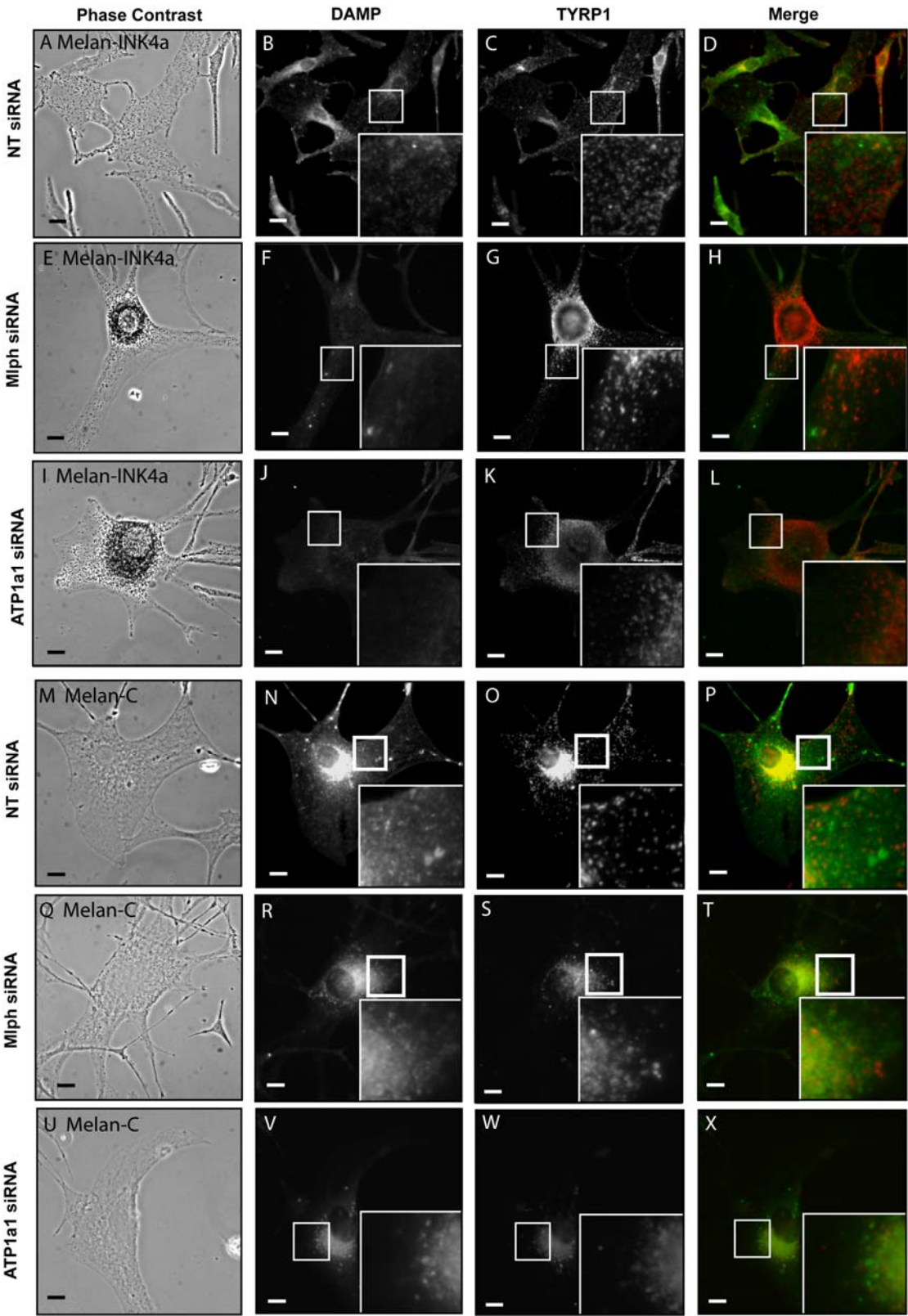


Figure 5-9 Melanocyte Staining with DAMP Following ATP1a1 Depletion.

Melan-INK4a (A-L) and Melan-c (M-X) cells were grown on coverslips and treated with NT, Mlph or ATP1a1 siRNA and incubated for 3 days. 30 μ M DAMP was added to the cells and incubated for 20min. Cells were then fixed with 4% PFA, permeabilised, blocked with FBS and immunostained with anti-DNP-Alexa-488 conjugate, mouse anti-TYRP1 antibody and Alexa-568 conjugated anti-mouse secondary antibody. Images were taken on a fluorescence widefield microscope. Phase contrast panels show melanosome distribution (A, E, I, M, Q and U). Panels B, F, J, N, R and V show DAMP accumulation and C, G, K, O, S and W show TYRP1 immunostaining. The merge panels (D, H, L, P, T and X) show DAMP (green) and TYRP1 (red) co-staining. Insets are a higher magnification of the boxed area. Scale bar represents 10 μ m. Images representative of one experiment.

Another fluorescence probe frequently used for analysis of organelle pH is LysoTracker (Invitrogen). Melan-INK4a cells were incubated with media supplemented with 100nM LysoTracker for varying time points and visualised to determine whether there was co-localisation with melanin. LysoTracker required live cell imaging as fixation quenched the signal. After 1.5h incubation, LysoTracker localised to a proportion of mature melanosomes, indicating an acidic lumen, as assessed by LysoTracker accumulation (Figure 5-10).

Although it had been reported previously that DAMP staining was reduced in stage III and IV melanosomes [460] it was unexpected to see contrasting results between DAMP and LysoTracker. A possible explanation for the unexpected difference was that cells were only incubated with DAMP for 20 min (as detailed in the literature [465]) but LysoTracker co-localisation with melanin only occurred after 1.5h incubation. Therefore, perhaps similar co-localisation would be seen with DAMP if the cells were incubated for longer.

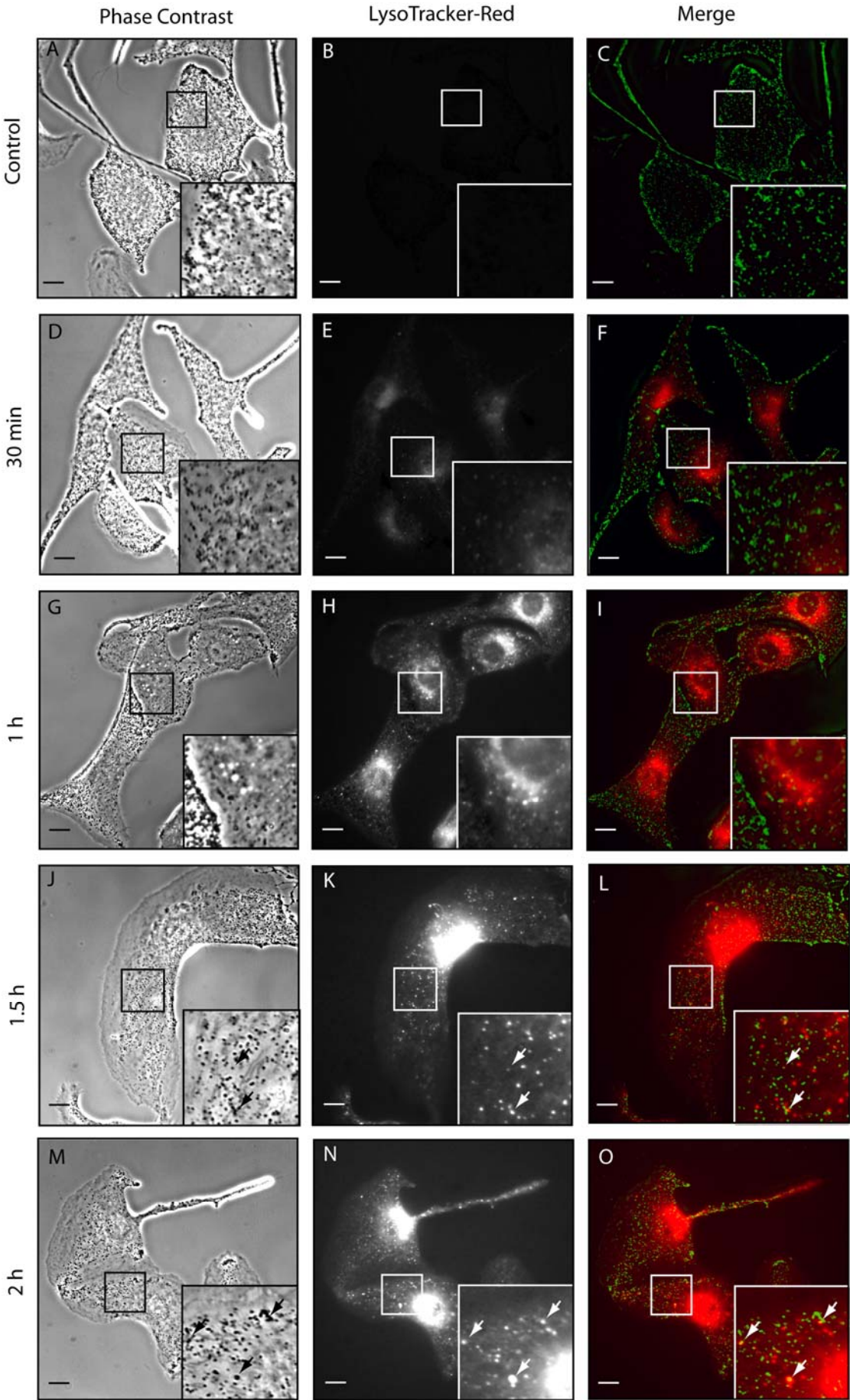


Figure 5-10 Melanocyte Staining with LysoTracker-Red.

Melan-INK4a cells were grown in glass-bottomed dishes and incubated in media supplemented with 100nM LysoTracker-Red for 30min-2h. Images were taken on a fluorescence widefield microscope. Phase contrast panels show melanosome distribution (A, D, G, J and M). Panels B, E, H, K and N show LysoTracker accumulation. Pigment is inverted and pseudo-coloured green to aid colocalisation with the LysoTracker-Red signal in the merge panels (C, F, I, L, O). The arrows indicate colocalisation. Insets are a higher magnification of the boxed area. Scale bar represents 10µm. Images representative of one experiment.

The experiment was repeated using NT, ATP1a1 or R3G siRNA treated cells. The cells were incubated with media supplemented with 100nM LysoTracker-Red for 2h prior to visualisation on a fluorescence confocal microscope. Initial analysis of the NT siRNA treated cells highlighted the variability between experiments as there was distinctly less perinuclear LysoTracker staining compared to the previous experiment (Figure 5-10). However there still remained a proportion of pigmented melanosomes which were positive for LysoTracker accumulation in the NT siRNA treated cells (Figure 5-11 A-C arrows). However, in the ATP1a1 and R3G siRNA treated cells (Figure 5-11 D-I) there was a distinct increase in LysoTracker positive vesicles in the perinuclear region which co-localised with melanosomes, indicative of acidification of melanosomes.

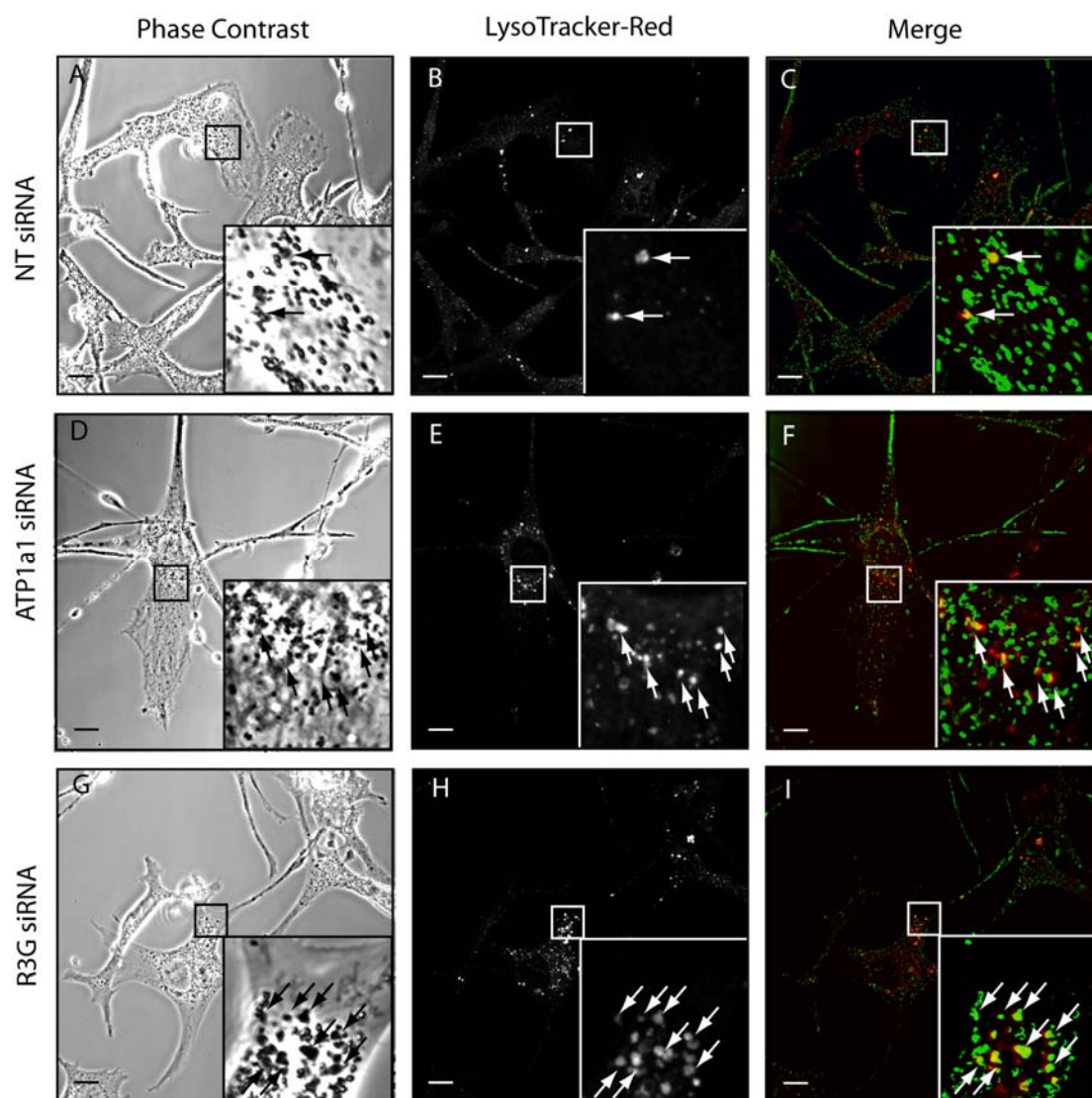


Figure 5-11 Melanocyte Staining with LysoTracker-Red Following ATP1a1 and R3G Depletion.

Melan-INK4a cells were grown in glass-bottomed dishes and treated with NT, ATP1a1 or R3G siRNA for 3 days then stained with LysoTracker by incubation in media supplemented with 100nM LysoTracker-Red for 2h. Images were taken on a fluorescence confocal microscope. Phase contrast panels show melanosome distribution (A, D and G). Panels B, E and H show LysoTracker accumulation. Pigment is inverted and pseudo-coloured green to aid colocalisation with the red LysoTracker signal in the merge panels (C, F and I). The arrows indicate colocalisation. Insets are a higher magnification of the boxed area. Scale bar represents 10µm. Images representative of one experiment.

The observation that ATP1a1 depletion caused an accumulation of LysoTracker in melanosomes suggested that Na^+/K^+ -ATPase played a role in regulating melanosome luminal pH. It was interesting that R3G depletion resulted in similar LysoTracker accumulation as this suggested that R3G depletion also affected melanosomal pH. Alternatively, the

accumulation of melanosomes in the perinuclear region could be triggering a degradation pathway and melanosomes could be fusing with lysosomes for subsequent degradation. However, as a hyper-pigmentation phenotype was observed in the ATP1a1 depleted cells and no hypo-pigmentation phenotype was observed in the R3G depleted cells it seemed unlikely that melanosomes were being degraded. Currently, with the data so far it is not possible to distinguish whether melanosome acidification is dependent on the loss of ATP1a1/R3G or a consequence of the cellular location of melanosomes. It would be interesting to assess the LysoTracker staining of Mlph siRNA treated or *ashen* melanocytes. However, a possible hypothesis is that, following ATP1a1 or R3G depletion, melanosomes were failing to mature and retained an acidic lumenal environment.

5.3.2 Increasing Cytoplasmic pH Increases Melanosomal Protein Association

As the previous data suggested that melanosomal pH was altered following ATP1a1 depletion, the question remained whether chemical manipulation of the cytoplasmic pH could also affect melanosome distribution and Rab27a localisation. Treatment of melanocytes with NH₄Cl would disrupt proton gradients therefore promoting alkalinisation of the cytosol and intracellular compartments. Conversely, acetate treatment would promote acidification. It could then be assessed whether alkalinisation or acidification of the whole cell could phenocopy the effect of ATP1a1 and R3G depletion on melanosome distribution and Rab27a localisation.

5.3.2.1 NH₄Cl Treatment Causes Melanosomal Clustering

Melan-INK4a cells were treated with increasing concentrations of NH₄Cl (10-80mM) to assess the effect on Rab27a function. It was found that <40mM NH₄Cl treatment resulted in perinuclear clustering of melanosomes after just 4h incubation (Figure 5-12). However

treatment was limited as high dose or long incubations were toxic to the cells. The clustering of melanosomes suggested that NH_4Cl could be disrupting Rab27a function.

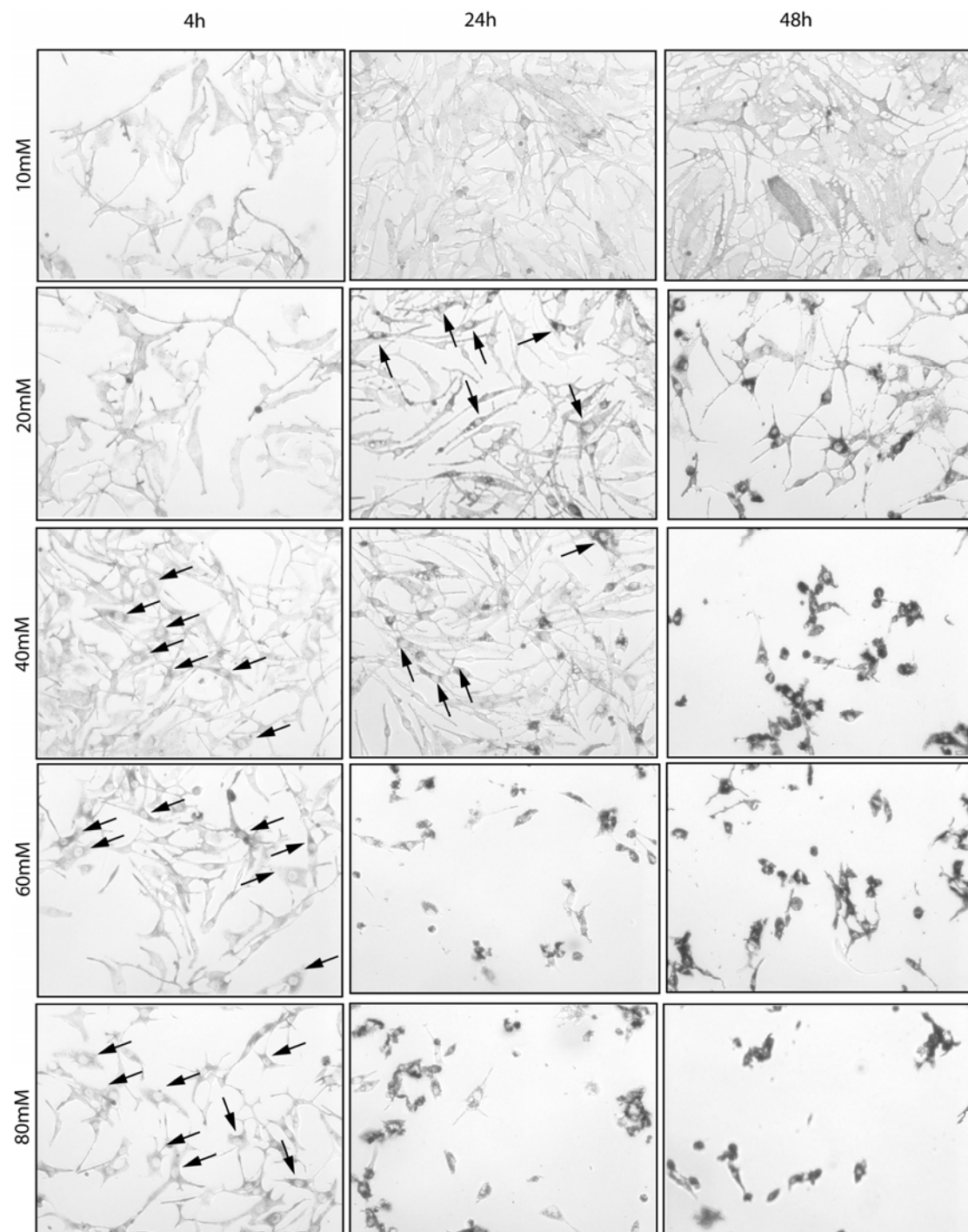


Figure 5-12 Melanosome Distribution Following NH_4Cl Treatment.

Melan-INK4a cells were treated with 10-80mM NH_4Cl for 4-48h and then visualised using a light microscope. Arrows indicate clustered cells. Images representative of two experiments.

5.3.2.2 NH₄Cl Treatment Does Not Affect Rab27a Expression or Solubility

To examine the effect NH₄Cl treatment was having on Rab27a, the expression and solubility of endogenous Rab27a was assessed. Separation of the soluble and membrane fractions following NH₄Cl treatment showed that Rab27a partitioned into the membrane fraction (Figure 5-13) therefore suggesting that its membrane association was unaffected. Also, relative to the tubulin loading control the expression levels of Rab27a were unaffected.

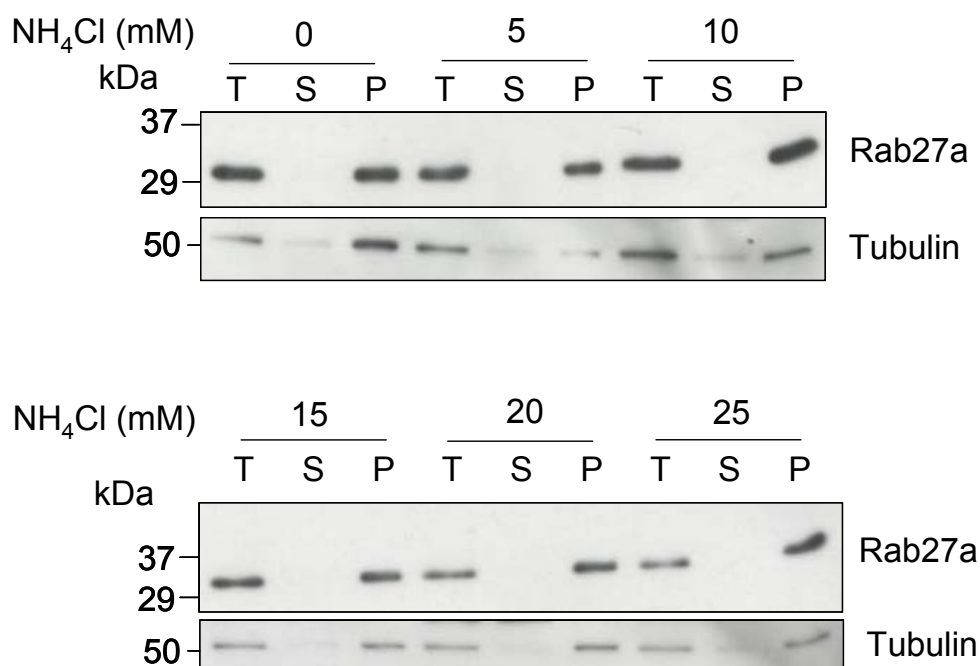


Figure 5-13 Rab27a Solubility Following NH₄Cl Treatment.

Melan-INK4a cells were treated with 5-25mM NH₄Cl for 24h. Cells were harvested and lysed and TCL was separated into S100 (S) and P100 (P) by centrifugation at 100,000xg for 1h. Equal volumes were loaded per sample. Immunoblotted for Rab27a and Tubulin. N=1

5.3.2.3 NH₄Cl Treatment Causes a Decrease in the Levels of GTP-bound Rab27a

As expression and solubility were unaffected, the activation of Rab27a was investigated. Using the effector pulldown assay, it was found that NH₄Cl treatment resulted in a 48% decrease in GTP-loaded Rab27a (Figure 5-14) (p=0.008), comparable to that observed following ATP1a1 or R3G depletion (Figure 4-10).

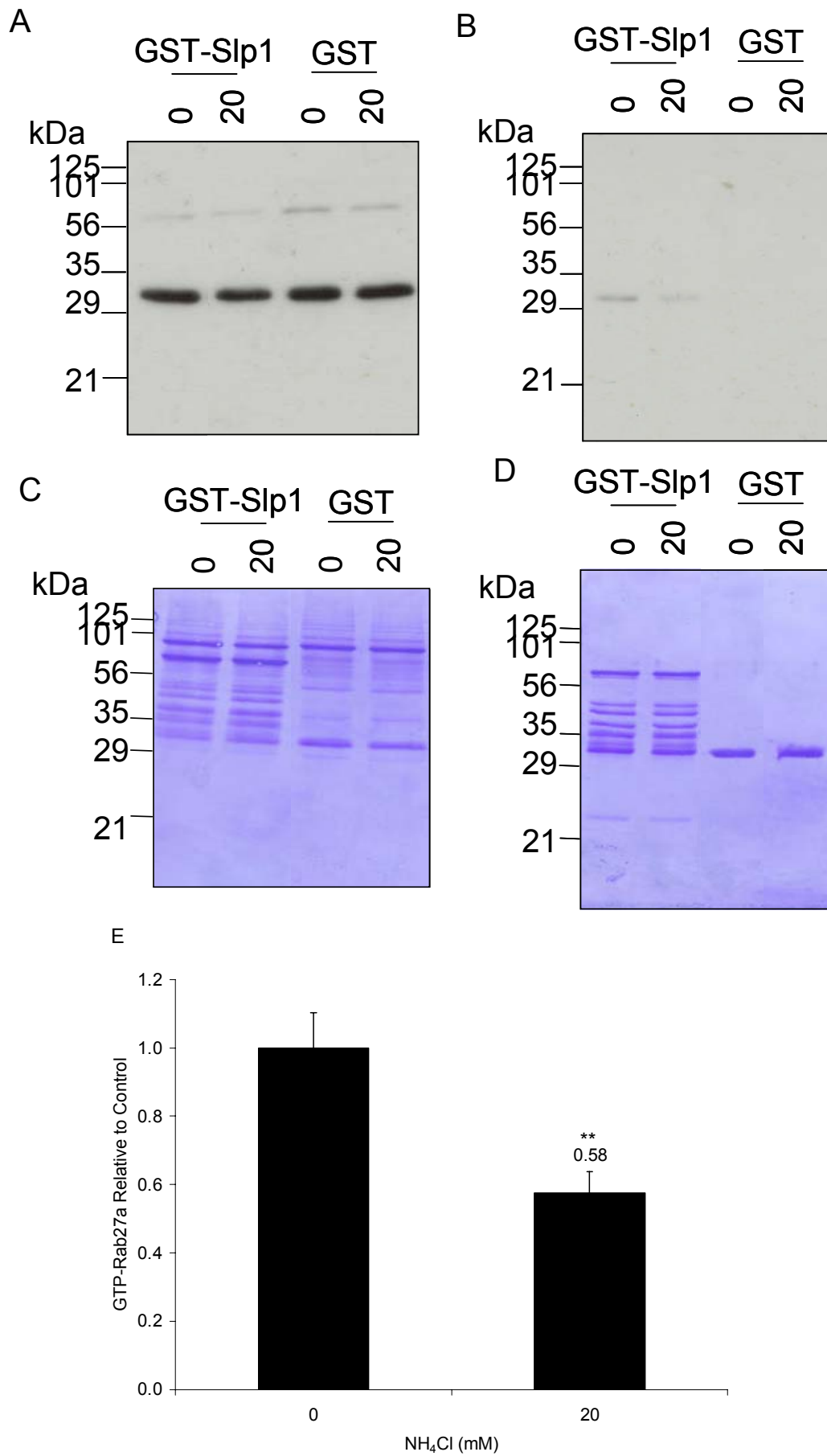


Figure 5-14 GTP-Bound Rab27a Following NH₄Cl Treatment.

Melan-INK4a cells were treated with 0mM or 20mM NH₄Cl for 24h. Cell lysates were incubated with immobilised GST-Slp1 or GST. Rab27a was quantified by immunoblotting the A) supernatant and B) bound to the beads with mouse anti-Rab27a antibody. Coomassie staining of the blots demonstrated C) equal protein loading and D) immobilization of GST and GST-Slp1. E) Rab27a immobilized by GST-Slp1 relative to the NT control was quantified across 3 experiments. Immunoblot and coomassie blots representative of one experiment independently repeated three times. Error bars are SEM. Significance calculated using t-test . * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ (Two-sample assuming equal variances).

5.3.2.4 NH₄Cl Treatment Alters Endogenous Rab27a Localisation to Melanosomes

NH₄Cl treatment resulted in decreased GTP-Rab27a without affecting the membrane association of the protein and so it was important to assess its intracellular localisation following NH₄Cl treatment. Melan-INK4a cells were treated with 20mM NH₄Cl and then immunostained for endogenous Rab27a. In cells with clustered melanosomes (Figure 5-15 D and G) the immunostaining for endogenous Rab27a appeared perinuclear with distinct Rab27a positive structures devoid of pigment in some cells (Figure 5-15 E and H arrowheads) whilst still co-localising with melanin in others (Figure 5-15 H, I, arrows). The distinct Rab27a positive vesicles devoid of pigment are comparable to those observed following ATP1a1 and/or R3G depletion (Figure 4-13 and Figure 4-16). However, as co-localisation was still apparent in some cells (Figure 4-13 G-I), the effect NH₄Cl treatment had on Rab27a localisation was ambiguous.

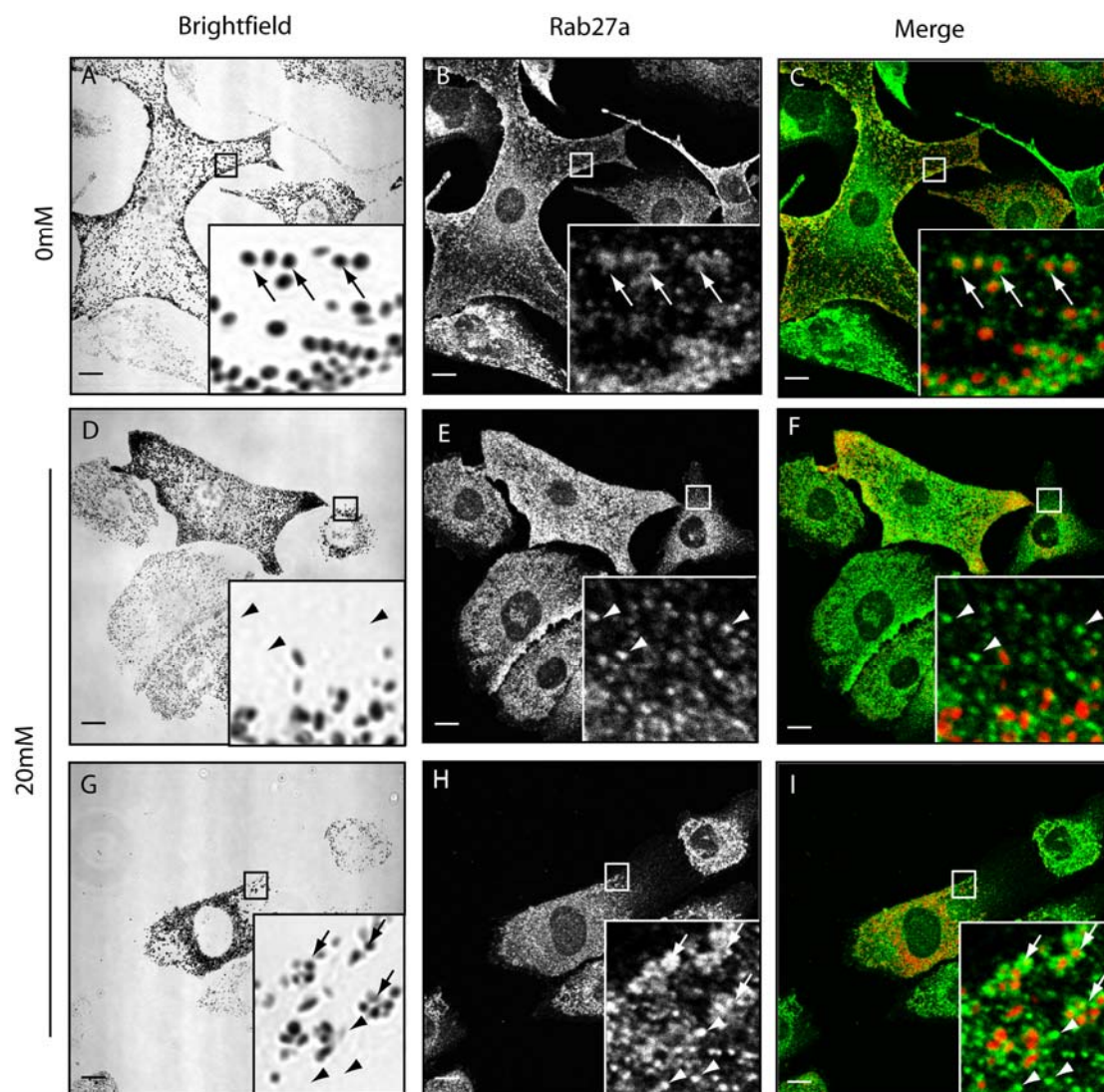


Figure 5-15 Endogenous Rab27a Localisation in Whole Cells Following NH_4Cl Treatment.

Melan-INK4a cells were grown on coverslips and treated with 0 or 20mM NH_4Cl for 24h. Cells were fixed with 4% PFA, permeabilised and blocked with FBS. Cells were then immunostained for endogenous Rab27a using rabbit-anti Rab27a antibody and Alexa488-conjugated anti-rabbit secondary antibody. Images were taken using a confocal microscope. Brightfield panels show the melanosome distribution (A, D and G). Panels B, E and H show endogenous Rab27a localisation. Pigment is inverted and pseudo-coloured red to aid colocalisation with Rab27a (green) in the merge panels (C, F and I). Insets are a higher magnification of the boxed area. The arrows indicate co-localisation, the arrowheads indicate Rab27a vesicles without pigment. Scale bar represents 10 μm . Images representative of one experiment.

As analysis of Rab27a association with melanosomes was inconclusive from immunostained whole cells, melanosomes were purified and the levels of associated Rab27a assessed. Interestingly, it was observed that NH_4Cl treatment resulted in an increase in Rab27a

associated with purified melanosomes (Figure 5-16) ($p=4.65 \times 10^{-7}$). This is in contrast to the results following ATP1a1 and R3G (Figure 4-15 and Figure 4-17).

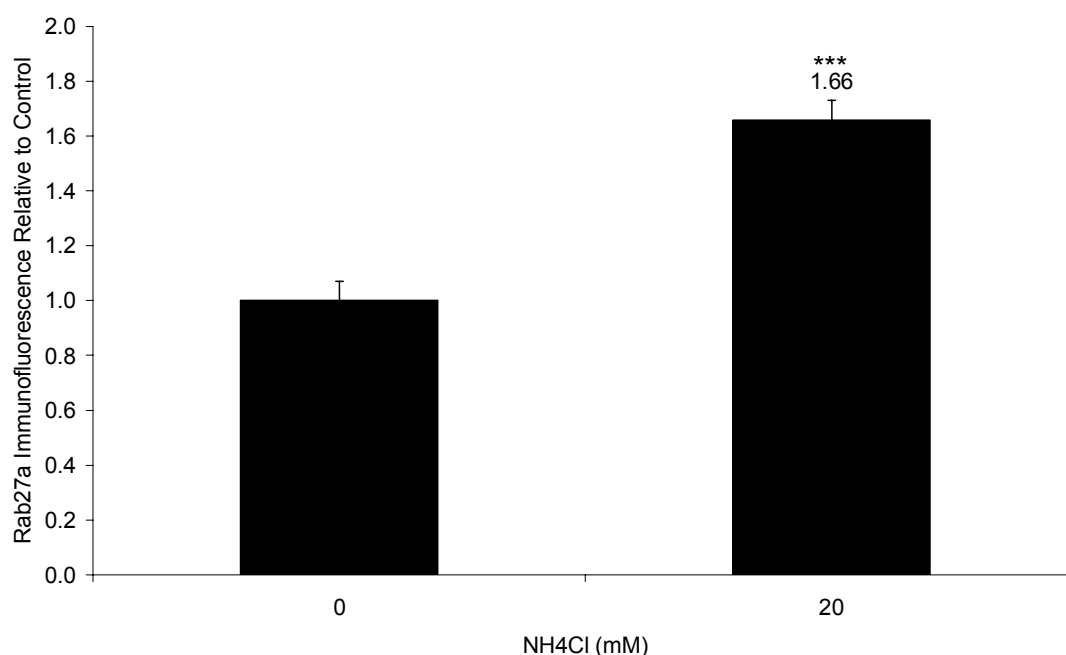


Figure 5-16 Rab27a on Purified Melanosomes Following NH₄Cl Treatment.

Melan-INK4a cells were treated with 20mM NH₄Cl for 24h. Cells were harvested and melanosomes purified. Non-treated melan-ash cells were used as a negative control. Purified melanosomes were centrifuged onto coverslips. They were then fixed with 4% PFA, permeabilised, blocked with FBS and immunostained using rabbit anti-Rab27a antibody and Alexa-488 conjugated anti-rabbit secondary antibody. Images were taken on a fluorescence confocal microscope and then analysed using SimplePCI analysis software. Experiment was independently repeated three times and statistical analysis performed on the untreated control normalised data sets. Error bars are SEM. Significance calculated using t-test. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ (Two-sample assuming unequal variances).

It has been proposed previously that NH₄Cl treatment promotes melanogenesis [464]. If the NH₄Cl treatment alkalinised the melanosomal lumen and if this were an indication of melanosome maturation, this would favour Rab27a recruitment. However, as GTP-loading of Rab27a was inhibited, Rab27a would ultimately fail to retain melanosomes at the periphery as its association with Mlph was lost resulting in clustering of melanosomes. It would be beneficial to investigate the melanosome pH following NH₄Cl treatment to ascertain if it is alkalinised.

5.3.2.5 NH₄Cl Treatment Increases Rab38 and TYRP1 Association with Melanosomes

Following the results for Rab27a association with melanosomes and the possible role of NH₄Cl in promoting melanogenesis, the effect NH₄Cl treatment had on Rab38 and TYRP1 melanosome association was assessed. Analysis of Rab38 associated with melanosomes after NH₄Cl treatment showed there to be an increase (Figure 5-17) ($p=0.02$), similar to that seen with ATP1a1 and/or R3G depletion (Figure 4-20). However, analysis of TYRP1 associated with melanosomes saw a significant increase (Figure 5-17) ($p=0.0002$), contrary to that observed following ATP1a1 and/or R3G depletion (Figure 4-20).

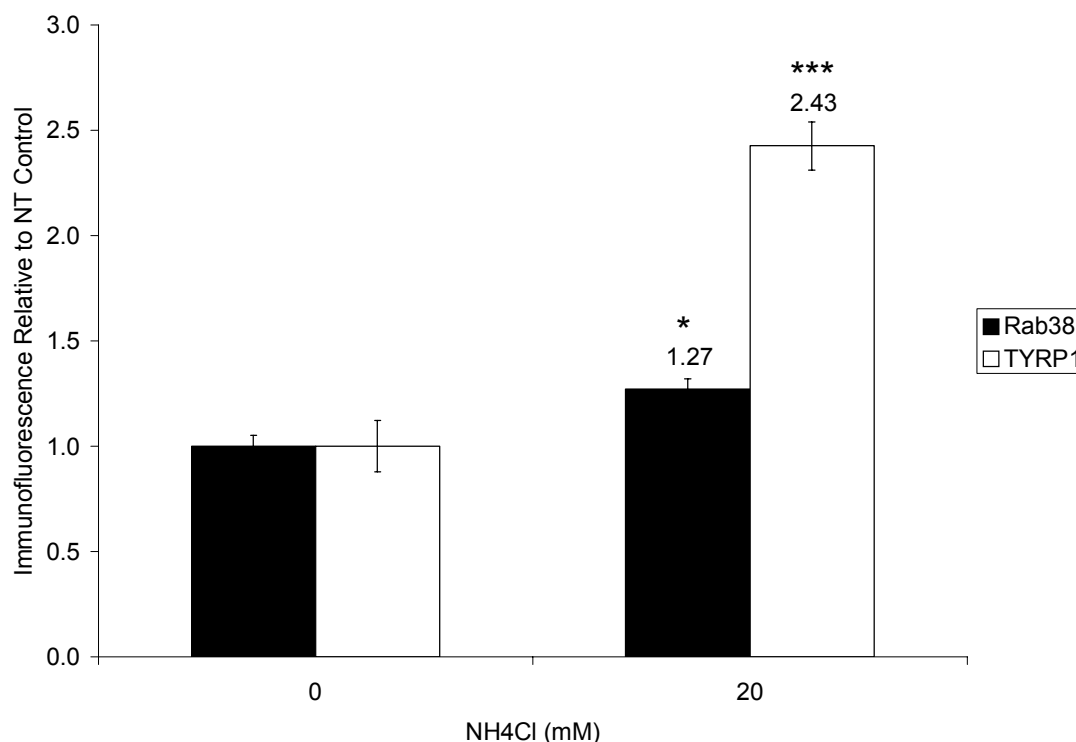


Figure 5-17 Melanosomal Proteins on Purified Melanosomes Following NH₄Cl Treatment.

Melan-INK4a cells were treated with NH₄Cl for 24h. Cells were harvested and melanosomes purified. Secondary antibody only was used as a negative control. Purified melanosomes were centrifuged onto coverslips, fixed with 4% PFA, permeabilised, and blocked with FBS. Coverslips were then immunostained with rabbit anti-Rab38 or rabbit anti-TYRP1 antibodies and Alexa-488 conjugated anti-rabbit secondary antibody. Images were taken on a fluorescence confocal microscope and then analysed using SimplePCI analysis software. Experiment was repeated independently three times and statistical analysis performed on the untreated control normalised data sets. Error bars are SEM. Significance calculated using t-test. * p<0.05, ** p<0.005, *** p<0.001 (Two-sample assuming unequal variances).

Previous research had shown that NH₄Cl treatment stimulated Tyr activity in Caucasian melanocytes [463, 466] and although this has been suggested to occur without an increase in Tyr expression [467], others have observed an increase in Tyr and TYRP1 in Negroid melanocytes [468, 469]. Delivery of Tyr and TYRP1 to melanosomes is dependent on Rab32/38 transport from early endosomes. Therefore if NH₄Cl treatment stimulated Tyr activity by increasing the expression and Rab32/38-dependent delivery of Tyr and TYRP1 to melanosomes, this would support the observed increase in both these proteins.

Overall, the NH_4Cl treatment resulted in melanosome clustering in a subset of cells. The mechanism of action in this subset of cells is unclear but it is most likely a result of the decreased GTP-loading of Rab27a. This therefore suggests that NH_4Cl treatment disrupted R3G activity therefore affecting melanosome distribution similar to ATP1a1 and R3G depletion. However, NH_4Cl treatment had an opposing effect on Rab27a and TYRP1 association with melanosomal membranes compared to ATP1a1 and/or R3G depletion. This could be a consequence of NH_4Cl -induced alkalinisation of the melanosomal lumen, supporting the hypothesis that ATP1a1 and R3G depletion acidifies the lumen.

5.3.2.6 Acetate Treatment Causes Melanosomal Clustering

Following on from the NH_4Cl experiments where the cells were alkalinised, initial experiments were carried out to assess the effect of acidifying cell cytoplasm. Melan-INK4a cells were grown in media containing increasing concentrations of NaAc for 24-72h. 80mM NaAc treatment resulted in melanosomal clustering from 24h, and >40mM caused melanosomal clustering after 48h treatment suggesting Rab27a function was disrupted (Figure 5-18). Long-term treatment did not result in cell death, therefore suggesting cells can withstand acidification conditions better than alkalinisation.

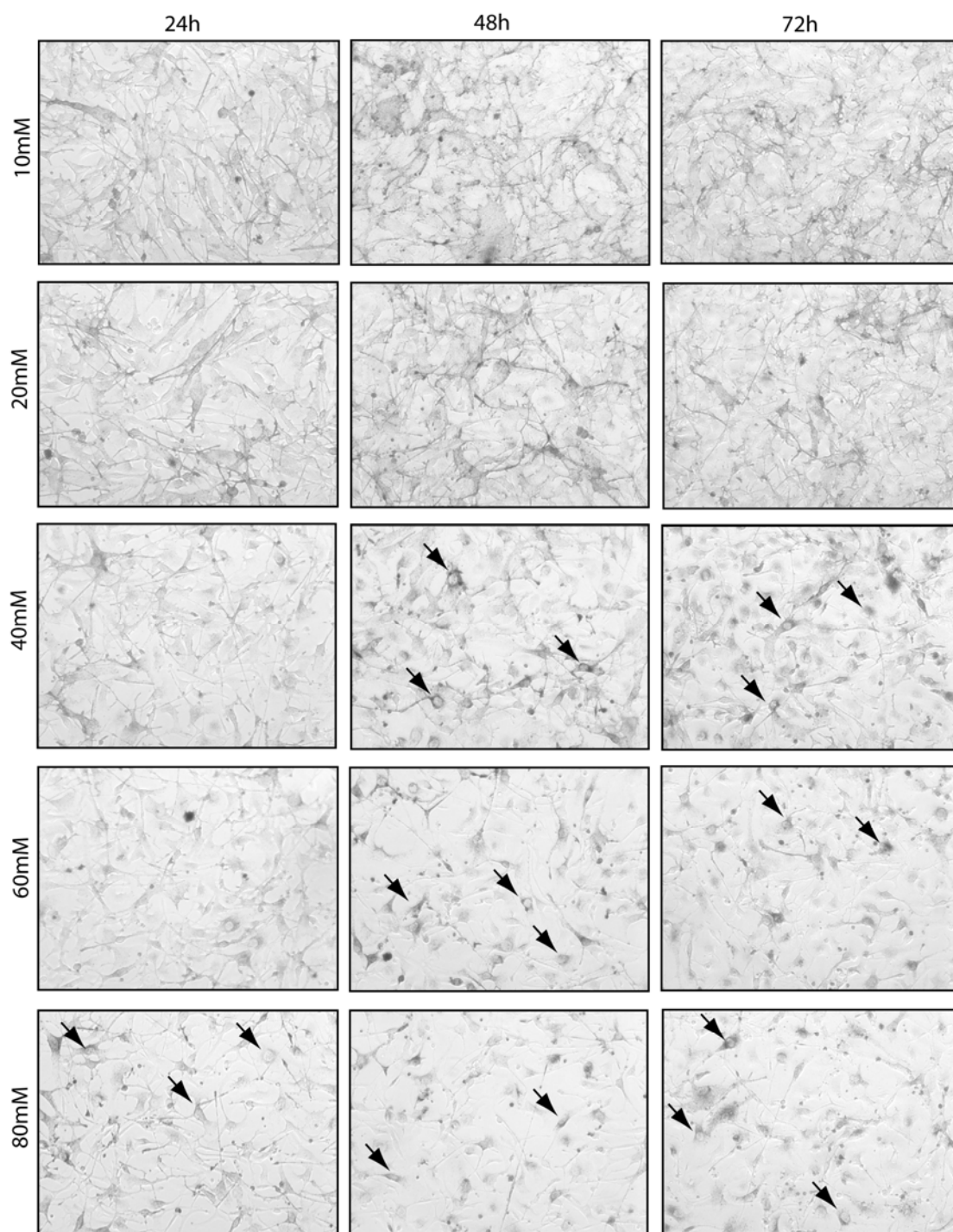


Figure 5-18 Melanosome Distribution Following Acetate Treatment.

Melan-INK4a cells were treated with 10-80mM NaAc for 24-72h and then visualised using a light microscope. The arrows indicate clustered cells. Images representative of three experiments.

5.3.2.7 Acetate Treatment Decreases GTP-bound Rab27a

As acetate treatment caused clustering of melanosomes, the effect acetate treatment had on GTP-loading of Rab27a was assessed. Melan-INK4a cells were treated with 80mM NaAc

for 48h and then the levels of GTP-Rab27a assessed using the effector pulldown assay. Initial results observed a 48% decrease in GTP-bound Rab27a following NaAc treatment (Figure 5-19).

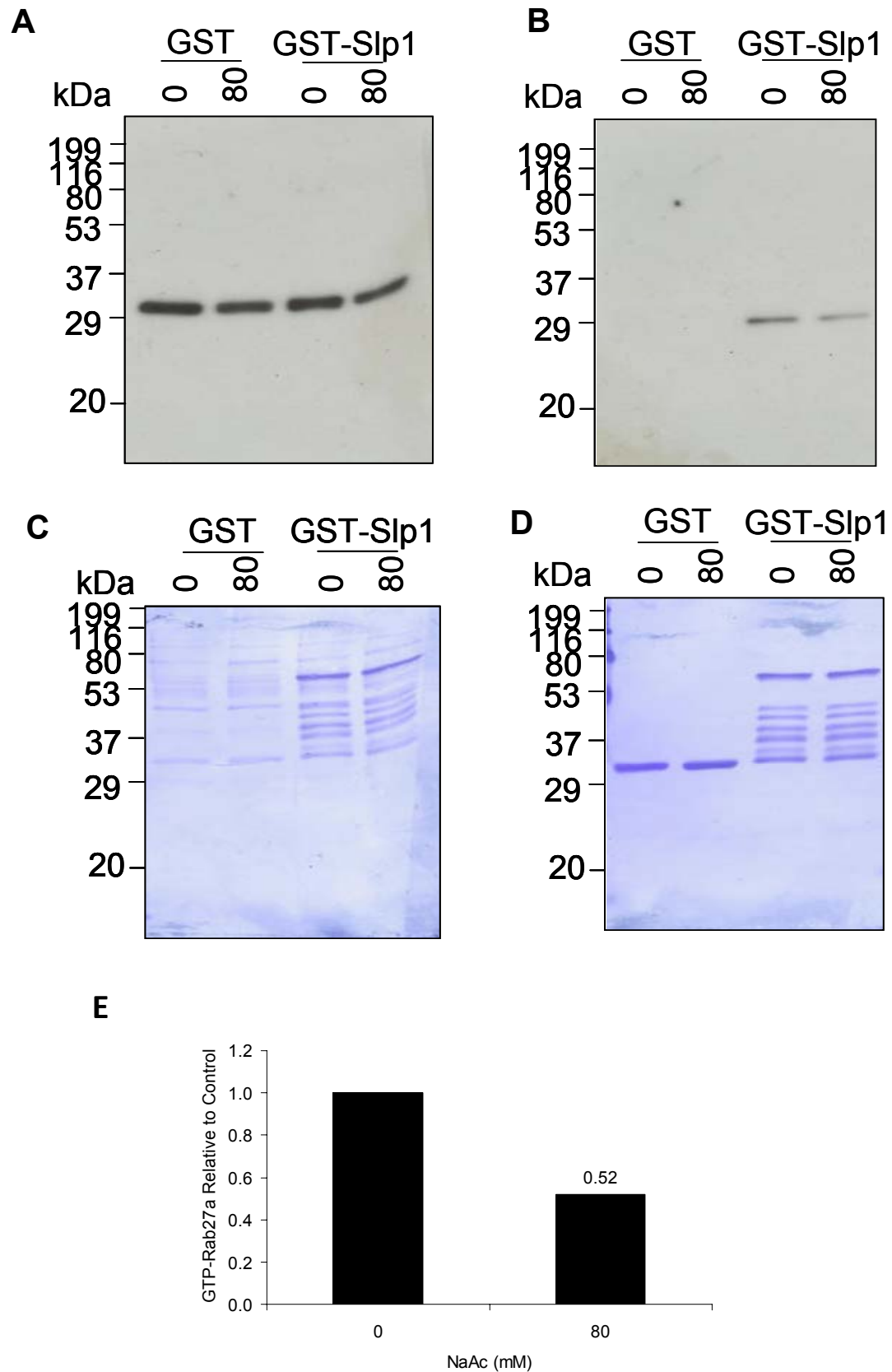


Figure 5-19 GTP-Bound Rab27a Following Acetate Treatment.

Melan-INK4a cells were treated with 80mM NaAc for 48h. Cell lysates were incubated with immobilised GST-Slp1 or GST. Rab27a was quantified by immunoblotting the A) supernatant and B) bound to the beads with mouse anti-Rab27a antibody. Coomassie staining of the blots demonstrated C) equal protein loading and D) immobilization of GST and GST-Slp1. E) Rab27a immobilized by GST-Slp1 relative to the NT control was quantified. n=1.

5.3.2.8 Acetate Treatment Does Not Affect Rab27a, Rab38 or TYRP1 Association with Melanosomes

As GTP-loaded Rab27a was decreased, the effect NaAc had on Rab27a association with purified melanosomes was assessed to see if it was comparable to ATP1a1/R3G depletion or NH₄Cl treatment. Melan-INK4a cells were grown in media supplemented with 80mM NaAc, lysed and melanosomes purified. Immunofluorescence analysis of melanosomes showed there to be no change in the levels of endogenous Rab27a associated with melanosomes (Figure 5-20).

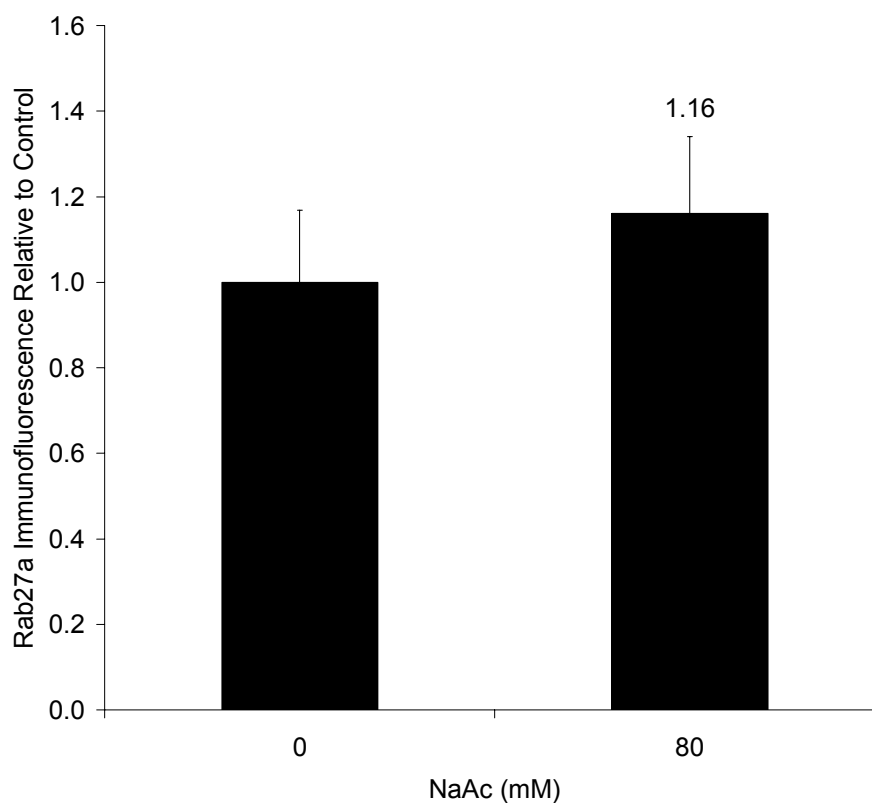


Figure 5-20 Rab27a Association with Purified Melanosomes Following Acetate Treatment.

Melan-INK4a cells were treated with 80mM NaAc for 48h. Cells were harvested and melanosomes purified. Non-treated melan-ash cells were used as a negative control. Purified melanosomes were centrifuged onto coverslips. They were then fixed with 4% PFA, permeabilised and blocked in FBS. Coverslips were then immunostained using rabbit anti-Rab27a antibody and Alexa-488 conjugated anti-rabbit secondary antibody. Images were taken on a fluorescence confocal microscope and then analysed using SimplePCI analysis software. Error bars are SEM. n=1.

Similarly, analysis of Rab38 and TYRP1 levels on purified melanosomes showed the NaAc treatment had no effect on their association with melanosomes (Figure 5-21).

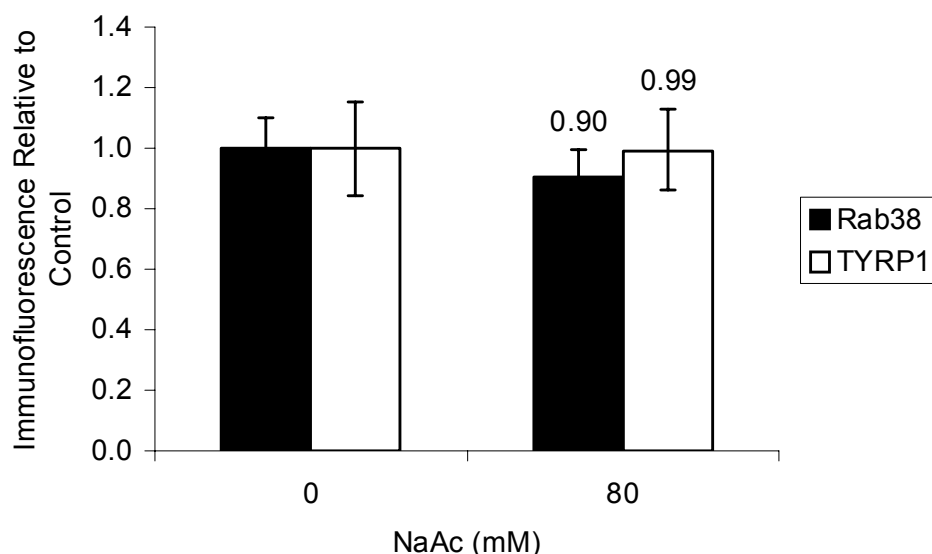


Figure 5-21 Melanosomal Proteins Association with Purified Melanosomes Following Acetate Treatment.

Melan-INK4a cells were treated with 80mM NaAc for 48h. Cells were harvested and melanosomes purified. Purified melanosomes were centrifuged onto coverslips, fixed with 4% PFA, permeabilised and blocked with FBS. Coverslips were then immunostained with rabbit anti-Rab38 or rabbit-anti-TYRP1 antibodies and Alexa-488 conjugated anti-rabbit secondary antibody. Images were taken on a fluorescence confocal microscope and then analysed using SimplePCI analysis software. Error bars are SEM. n=1.

Despite NaAc treatment resulting in clustered melanosomes and reduced GTP-bound Rab27a, NaAc had no effect on association of the melanosomal proteins, including Rab27a. This highlights again that reduced GTP-loading does not automatically result in displacement of Rab27a, however it would no longer be able to recruit Mlph and so would result in a clustered phenotype. As long-term NaAc treatment was not toxic, it would be interesting to repeat the above experiments following a longer incubation. It is possible that displacement of melanosomal proteins required longer treatment. Rab27a displacement in ATP1a1 or R3G siRNA treated cells only occurred after the clustered phenotype had been apparent for 24-48h. It would be hypothesised that NaAc treatment could acidify the melanosomal lumen comparable to that proposed following ATP1a1 and R3G depletion. Then if an acidic melanosomal lumen functioned as an indicator for melanosome maturation, a similar decrease in Rab27a and TYRP1 association would be observed following NaAc treatment.

Interestingly, it could be hypothesised that the brief NH_4Cl treatment is sufficient to alkalinise the melanosome lumen before overall toxic effects occur. Alkalinisation of the melanosome lumen could signify melanosome maturation and favour Rab27a recruitment. Conversely disruption of Na^+, K^+ -ATPase function on melanosome membranes following ATP1a1 depletion acidifies the melanosome lumen and results in Rab27a displacement after sufficient time.

It was observed that both alkalinisation and acidifying media reduced the GTP-loading of Rab27a in melanocytes. This therefore suggested that altering intracellular pH disrupted normal R3G function resulting in clustering of melanosomes. The question remains whether this is a R3G specific effect or if other RabGEFs are also affected by cellular pH.

5.4 Ouabain Treatment

In parallel, the role ATP1a1 has in cell signalling was investigated. It has long been known that cardiotonic steroids (e.g. Ouabain) bind the α -subunit of Na^+, K^+ -ATPase and inhibit ion transport [355]. Ouabain is proposed to be impermeable to the cell membrane therefore only inhibits plasma membrane Na^+, K^+ -ATPase. Ouabain is extensively used to regulate blood pressure and hypertension in humans [356] and also in cell culture as a Na^+, K^+ -ATPase inhibitor. In addition, it is known that low ouabain concentrations stimulate Src signalling pathways by relieving α -subunit binding and inhibition of Src kinase. The ouabain-induced signalling cascade occurs at concentrations that do not disrupt Na^+, K^+ -ATPase ion transport function [385, 389]. It was shown in rat ventricular myocytes that concentrations of Ouabain higher than 1mM were required for complete inhibition of Na^+, K^+ -ATPase ion pump function but that up to 50% inhibition was tolerated before any cell toxicity [385]. In addition, depletion of ATP1a1 has been shown to increase Src kinase activation [392],

therefore the Src signalling cascade could be involved in regulating Rab27a melanosomal targeting.

5.4.1.1 Ouabain Treatment Stimulates the MAPK Cascade

Ouabain treatment is known to stimulate the MAPK pathway by releasing ATP1a1 inhibition of Src Kinase. Src then activates Ras, which can stimulate the MAPK cascade (Raf-MEK-ERK1/2). To assess if ouabain treatment resulted in phosphorylation of ERK1/2 in melanocytes, melan-INK4a cells were treated with 0.5 and 1mM Ouabain for 72h and then the PNS assessed for ERK1/2 and pERK1/2 levels. Initial experiments suggest that the ouabain treatment resulted in increased phosphorylation of ERK1/2 compared to the untreated cells (Figure 5-22)

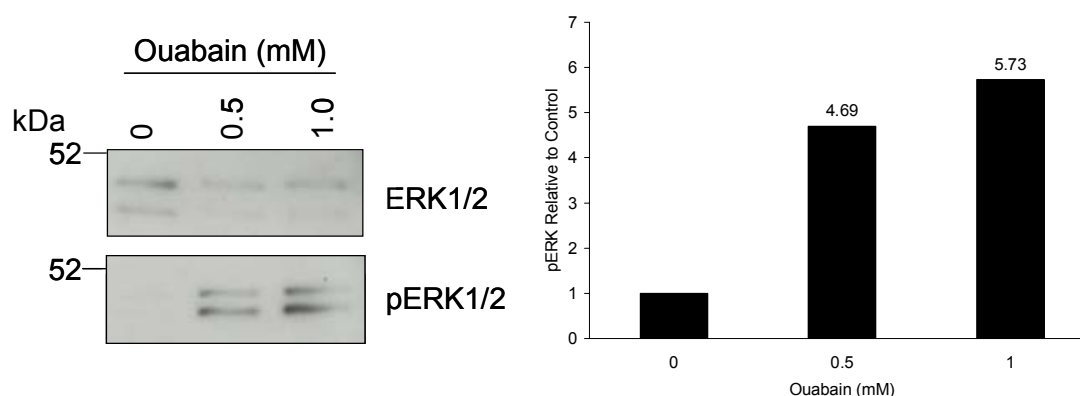


Figure 5-22 MAPK Cascade Activation in Melanocytes Following Ouabain Treatment.

Melan-INK4a cells were treated with 0, 0.5 or 1.0mM Ouabain for 72h. PNS were then collected and immunoblotted to analyse ERK1/2 and pERK1/2 levels. Equal protein was loaded per sample. ERK1/2 and pERK1/2 levels were normalised to coomassie staining and the relative pERK1/2 to ERK1/2 calculated, then normalised to control. n=1

5.4.1.2 Ouabain Treatment Causes Melanosomal Clustering

In order to assess whether Rab27a function was affected by ouabain-induced signalling, melan-INK4a cells were treated with varying doses of ouabain. The cells were then visualised on a light microscope to assess whether the treatment resulted in melanosome clustering. Ouabain doses >0.2mM induced melanosomal clustering in a subset of cells

(Figure 5-23). Doses >1mM were toxic (Figure 5-23) in support of the argument that complete loss of Na^+, K^+ -ATPase function at the plasma membrane is toxic. Additionally, this leads to the hypothesis that depletion of ATP1a1 could be compensated for by other α -subunit isoforms. Proteomic studies have found ATP1a3 to be present in melanocytes [412], however immunoblot analysis of melan-INK4a TCLs for ATP1a2 and ATP1a3 has so far failed to identify any bands.

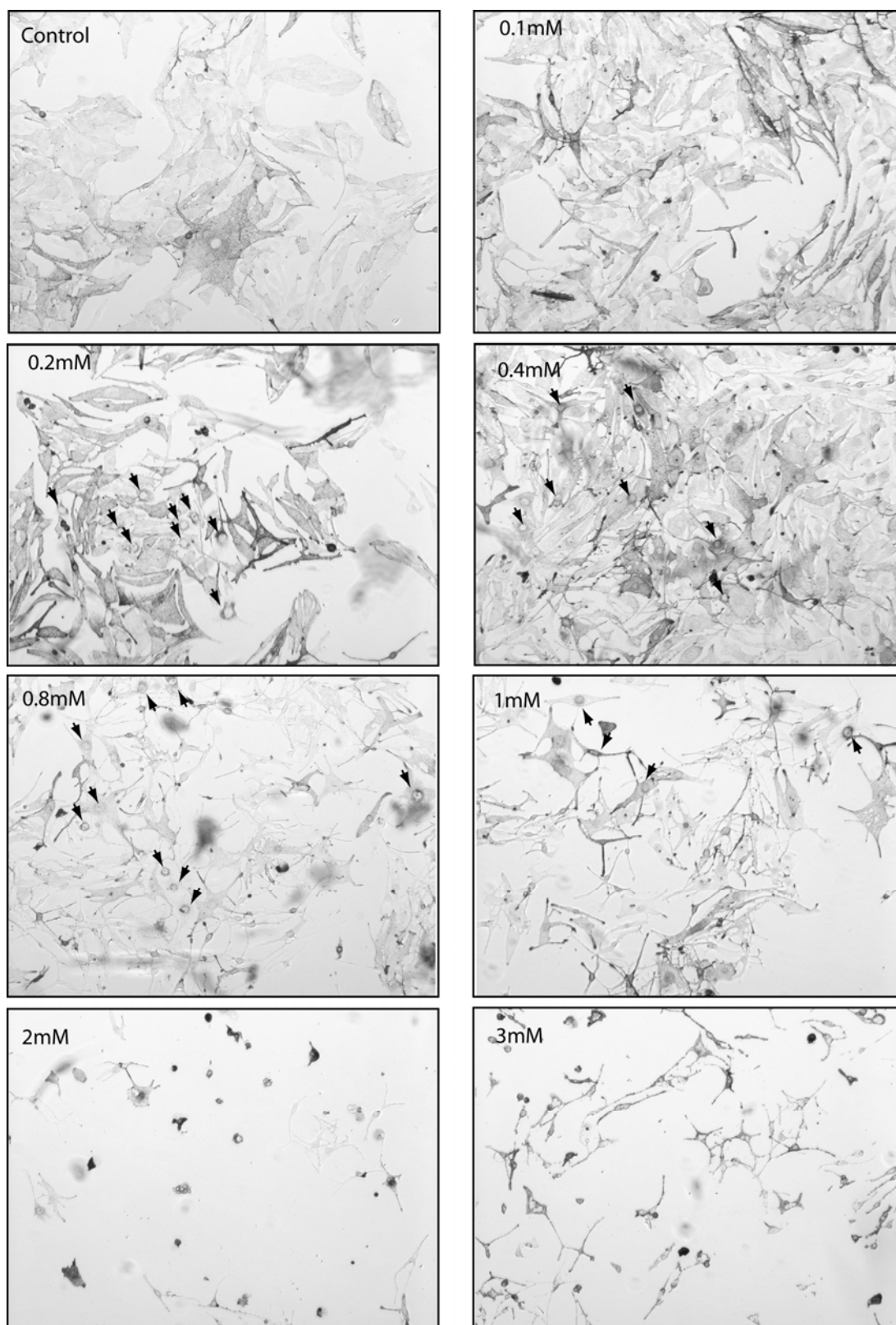


Figure 5-23 Melanosome Distribution Following Ouabain Treatment.

Melan-INK4a cells were treated with Ouabain (0.1-3mM) for 72h. The cells were visualized on a light microscope. Arrows indicate cells with clustered melanosomes. Images representative of three experiments.

5.4.1.3 Ouabain Treatment Disrupts Endogenous Rab27a Localisation to Melanosomes

As ouabain treatment resulted in melanosomal clustering in a subset of cells, the effect the treatment had on Rab27a localisation in these cells was assessed. Melan-INK4a cells were treated with ouabain and then immunostained for endogenous Rab27a. The treatment resulted in melanosomal clustering (Figure 5-24 D, G, J). In addition, the endogenous Rab27a immunostaining appears more diffuse, suggesting displacement from melanosomes. Similarly, discrete Rab27a positive vesicles were apparent (Figure 5-24 D-L).

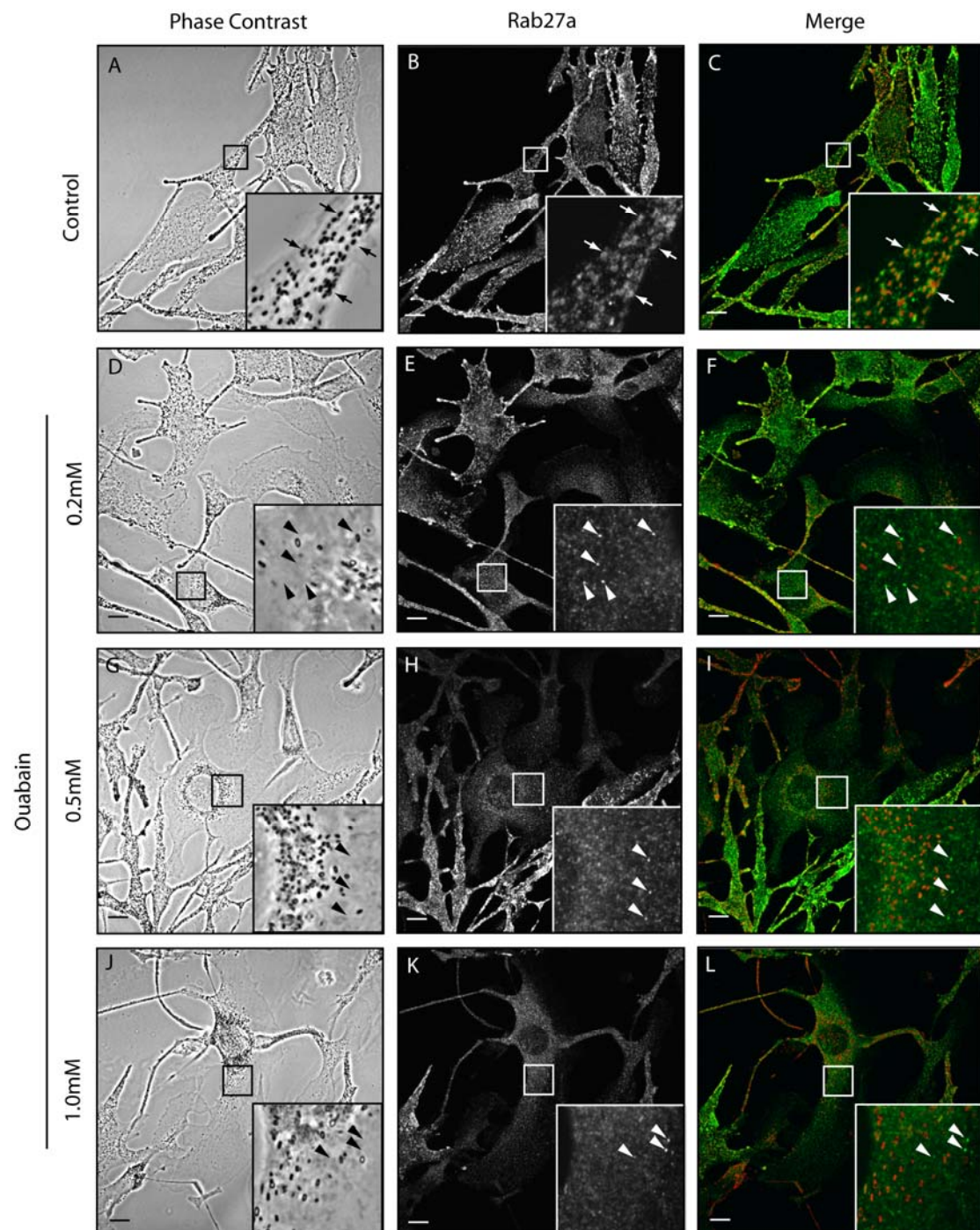


Figure 5-24 Endogenous Rab27a Localisation Following Ouabain Treatment.

Melan-INK4a cells were grown on coverslips and treated with 0.2-1.0mM Ouabain for 72h. Cells were fixed with 4% PFA, permeabilised and blocked with FBS. Cells were then immunostained for endogenous Rab27a using rabbit anti-Rab27a antibody and Alex-488 conjugated anti-rabbit secondary antibody. Images were taken using a confocal microscope. Phase contrast image demonstrates the melanosome distribution (A, D, G and J). Panels B, E, H and K show endogenous Rab27a localisation. Pigment is inverted and pseudo-coloured red to aid colocalisation with Rab27a (green) in the merge panels (C, F, I and L). Insets are a higher magnification of the boxed area. Arrows indicate colocalisation. Arrowheads indicated Rab27a vesicles without pigment. Scale bar represents 10µm. Images representative of one experiment.

These data suggest that ouabain treatment can stimulate the MAPK signalling cascade in melanocytes, however melanosomal clustering only occurred in a subset of cells. This therefore questions whether MAPK activation has any role in inducing melanosome clustering. It could be suggested that ATP1a1 expression levels vary in a cell population. As a result, in the subset of cells with reduced ATP1a1, inhibition of the plasma membrane Na^+/K^+ -ATPase results in redistribution of the intracellular ATP1a1 to the plasma membrane to ensure maintenance of ion homeostasis. This would therefore deplete intracellular ATP1a1 that could be necessary for Rab27a targeting therefore resulting in the subset of clustered cells. Immunofluorescence analysis of endogenous ATP1a1 localisation after ouabain treatment in cells with clustered melanosomes would clarify this hypothesis.

5.4.1.4 Depletion of ATP1a1 and R3G has Differing Effects on MAPK Signalling

In order to assess the role of MAPK signalling in ATP1a1 and/or R3G-depletion-induced clustering, the levels of phosphorylated ERK1/2 were assessed following ATP1a1 and/or R3G depletion. R3G was first identified as MADD (MAPK activating death domain) due to its association with TNFR1 (tumour necrosis factor receptor 1) via its DD and regulation of MAPK pathways [238]. It was found that over-expression of R3G in COS cells resulted in increased pERK1/2, therefore depletion of R3G would be expected to result in a decrease in

pERK1/2, contrary to that expected for ATP1a1 depletion. In order to test what occurs in melanocytes, ATP1a1, R3G and A+R depleted melan-INK4a cells were immunoblotted for ERK1/2 and pERK1/2 (Figure 5-25). As predicted, pERK1/2 was increased and reduced following ATP1a1 and R3G depletion, respectively. It was observed that two rounds of siRNA treatment for ATP1a1 depletion resulted in an increase in pERK1/2 comparable to ouabain treatment (Figure 5-22). As ouabain treatment never resulted in extensive clustering, despite similar increases in pERK1/2 following ATP1a1 depletion, this suggested that ERK1/2 activation is not wholly responsible for the clustered phenotype.

Interestingly, MAPK signalling activation and hyper-pigmentation are the two phenotypes observed following ATP1a1 depletion but not R3G depletion therefore suggesting that the two could be linked. Favouring this hypothesis, activation of MAPK signalling has been linked to MITF up-regulation and this is discussed further in the next chapter. This could therefore suggest that activation of MAPK results in the hyper-pigmentation phenotype. Investigating the effect ouabain treatment has on melanocyte pigmentation would contribute to clarifying this hypothesis. Similarly, Src kinase specific inhibitors could be used to assess whether they can prevent ATP1a1-depletion-induced hyper-pigmentation.

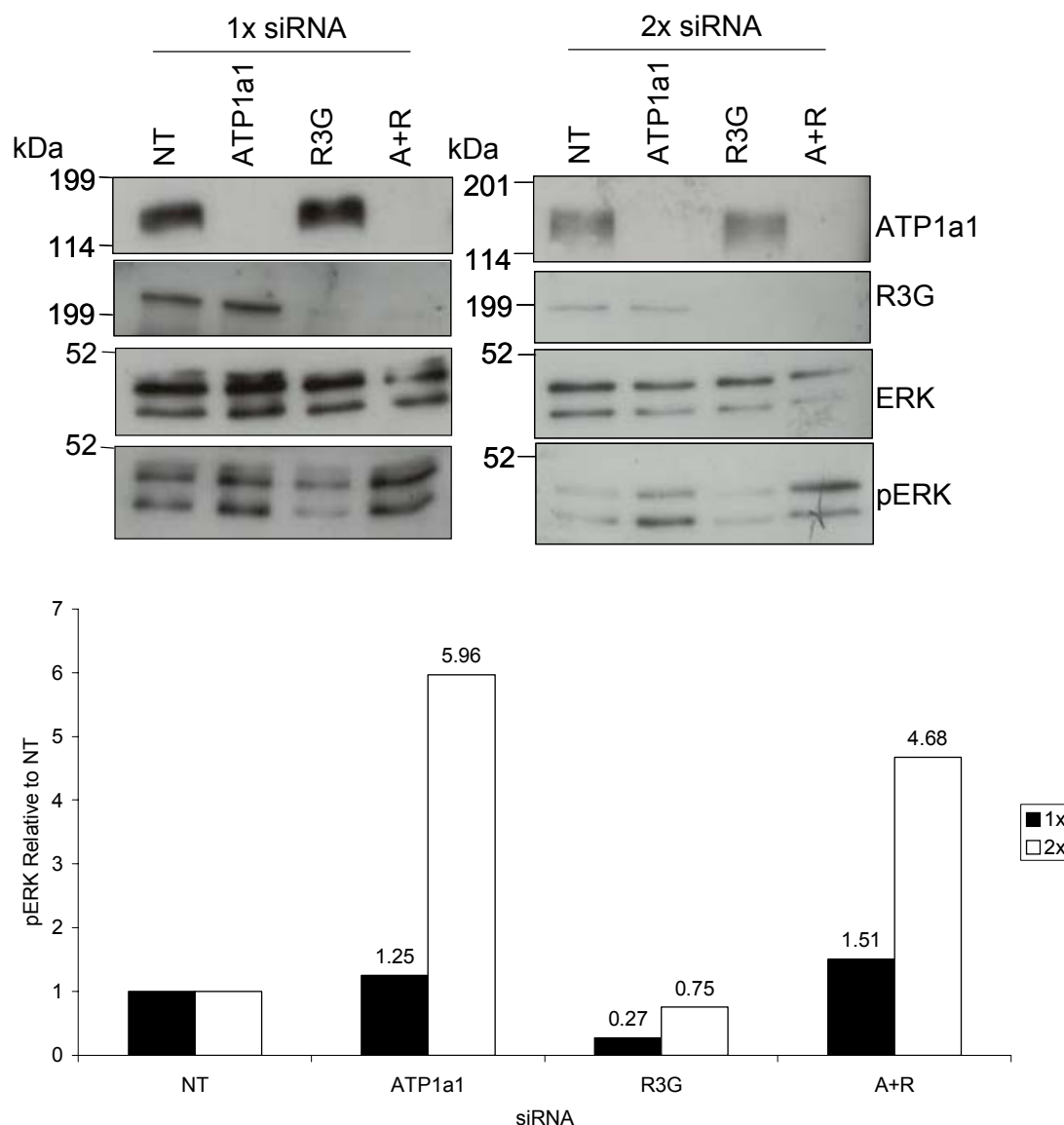


Figure 5-25 MAPK Cascade Following ATP1a1 and/or R3G Depletion Melanocytes.

Melan-INK4a cells were treated with NT, ATP1a1, R3G or A+R siRNA. PNS were then collected and immunoblotted to analyse ATP1a1 and R3G depletion and ERK1/2 and pERK1/2 levels. Equal protein was loaded per sample. ERK1/2 and pERK1/2 levels were normalised to coomassie staining and the relative pERK1/2 to ERK1/2 calculated then normalised to NT siRNA control. Immunoblots each representative of two independent experiments.

5.5 Conclusions

Investigation of the localisation of ATP1a1 in melanocytes identified its association with intracellular structures, with transient association with melanosomes. This suggested that Na^+, K^+ -ATPase may function on intracellular vesicles. Following ATP1a1 and R3G depletion,

LysoTracker accumulation suggested that the luminal pH of melanosomes reduced. Interestingly, NH_4Cl treatment, which could be proposed to alkalinise luminal environments, had an opposing effect on melanosomal protein association compared to ATP1a1 and R3G depletion. Taken together these results suggest that melanosome luminal pH could be a defining factor for melanosome maturation and protein association, including Rab27a recruitment. The ion pumping function of Na^+, K^+ -ATPase across melanosomal membranes could contribute to regulation of melanosome luminal pH regulation. The mechanism by which the luminal environment is conveyed to the cytosol and related to Rab27a recruitment remains to be clarified. Similarly, the mechanism by which R3G could be involved in luminal pH regulation remains elusive. Additionally, altering cytosolic pH by NH_4Cl and NaAc treatment resulted in decreased GTP-bound Rab27a, suggesting that altering the pH balance could disrupt R3G activity.

In parallel to the pH investigations, the role of the MAPK cascade in the observed phenotypes was investigated. It is proposed that the increased activation of ERK1/2 following depletion of ATP1a1 could be linked to the hyper-pigmentation phenotype.

6 Discussion

The Rab family of small GTPases tightly regulates numerous intracellular membrane trafficking pathways and they achieve this by their discrete targeting to specific membranes. The mechanism(s) by which this targeting is achieved remains to be elucidated. In the Seabra group, the role of the C-terminal hypervariable domain [17], effector binding and GEF activity [145] in the targeting of Rab27a to melanosomes in melanocytes have been investigated. It is proposed that the GEF activity of the non-redundant Rab27a GEF R3G was required, but not sufficient, for Rab27a targeting and led to the hypothesis that a targeting factor may also be involved. The Rab27a mutant Rab27a^{SF1/F4} maintains melanosomal targeting and R3G mediated GTP-loading, but it interacts with no known Rab27a effectors [145]. This mutant was used to discern novel Rab27a interacting partners with the aim of identifying a putative targeting factor.

The current research identified the $\alpha 1$ subunit of Na^+, K^+ -ATPase, ATP1a1, as a novel interacting partner for Rab27a in melanocytes following tandem affinity purification (TAP) (Chapter 3). Depletion of ATP1a1 resulted in perinuclear clustering of melanosomes, suggestive of loss of Rab27a function. Further analysis established that Rab27a still partitioned into the membrane fraction, indicative of correct prenylation, but that GTP-loading was diminished, comparable to that seen following depletion of R3G. Importantly, depletion of ATP1a1 resulted in displacement of endogenous Rab27a from melanosomal membranes, indicating a role in Rab27a targeting. In addition, ATP1a1 depletion disrupted the association of other melanosomal proteins and altered the melanin content, indicative of disruption in melanogenesis (Chapter 4). Endogenous ATP1a1 was found to localise to intracellular structures, and associate transiently with melanosomes (Chapter 5). It has been proposed that melanosomal Na^+, K^+ -ATPase may function to regulate luminal pH and

therefore indirectly regulate melanosome maturation. Depletion of ATP1a1 was also found to stimulate the MAPK cascade and this was proposed to result in the ATP1a1-depletion-specific hyper-pigmentation phenotype. The mechanism by which ATP1a1 regulates Rab27a recruitment to melanosomes still remains to be elucidated.

6.1 Rab27a and ATP1a1 Interaction

The identification of ATP1a1 following TAP of Rab27a inferred that the two proteins interact and subsequent immunoprecipitation experiments supported this. However, a direct interaction remains to be established. The large cytosolic loop between transmembrane domains 4 and 5 (ATP1a1-M4M5) is the most likely interacting domain. This loop has been shown to dimerise [470] and bind a number of intracellular components including cofilin [471], AP2 [472], ankyrin [473], Src [393], PLC- γ 1 [474], AS160 [353] arrestin and spinophilin [475]. Purification and direct interaction assays utilising ATP1a1-M4M5 and Rab27a would be beneficial. Of note, an interaction between R3G and Rab27a has so far proved elusive and R3G does not co-localise with Rab27a in melanocytes. Nevertheless, it is firmly established that Rab27a function is dependent on R3G in melanocytes [59], therefore would entail a transient interaction. A transient association between Rab27a and ATP1a1 could be sufficient to target Rab27a, therefore explaining the minimal co-localisation observed for Rab27a and ATP1a1 (Figure 5-5). In addition, the absence of ATP1a1 in the dense sucrose fractions corresponding to late stage melanosomes (Figure 5-7) suggests ATP1a1 is recycled from melanosomal membranes. However, proteomics studies suggest it is a common protein present in all stages of melanogenesis [412]. Considering its ubiquitous expression and its localisation at the plasma membrane, a direct interaction can not be the sole determinant of Rab27a localisation. As R3G activity has previously been shown to be

required for Rab27a targeting [145] and the finding that the combined depletion of ATP1a1 and R3G exacerbates the phenotypes, this suggests that the two may function in tandem.

6.2 GEF Activity

GTP-loading was proposed to aid Rab GTPase membrane association by destabilising the association with GDI and promoting membrane association of the Rab GTPase. Supporting this, depletion of the RabGEFs Varp or R3G resulted in mis-targeting of Rab21 and Rab27a, respectively [54, 145]. Interestingly, our data showed that ATP1a1 depletion reduced GTP-bound Rab27a (Figure 4-10), comparable to that observed after R3G depletion, therefore suggesting that ATP1a1 depletion disrupts GTP loading. Additionally, depletion of both ATP1a1 and R3G resulted in a further reduction. The question remains, how does ATP1a1 regulate R3G function? Does ATP1a1 recruit R3G to melanosomes/Rab27a delivery vesicles? Or is R3G recruited by the upstream Rabs, Rab32/38, proposing a Rab cascade? Do Rab27a, ATP1a1 and R3G form a complex? Does R3G require Rab27a to be on melanosomal membranes before activation, therefore ATP1a1 depletion restricts Rab27a loading? Alternatively, both NH₄Cl and acetate treatment similarly reduced GTP-bound Rab27a, suggesting that disruption to pH may perturb GEF activity, perhaps inactivating R3G or disrupting its association with Rab27a. Could the depletion of ATP1a1 affect the cytosolic pH sufficiently to disrupt R3G function? It would be interesting to assess the GEF activity of other RabGEFs, in particular DENN domain containing Rab GEFs, after ATP1a1 depletion to ascertain whether the disruption is specific for R3G. Similarly, assessing the pH-sensitivity of GEF activity could be achieved by varying the pH of the buffer in the *in vitro* GDP/GTP exchange assay.

6.3 Rab Cascade

A Rab GAP cascade has been proposed whereby downstream Rab GTPases recruit the Rab GAPs for upstream Rab GTPases and promote their removal from the membrane. In yeast, Ypt32 can recruit Gyp1, a RabGAP for Ypt1, therefore promoting the Rab GTPase conversion [90]. Similarly, in *C. elegans* Rab7 can recruit TBC-2, a RabGAP for Rab5 [91]. This model has not been investigated for Rab32/38 and Rab27a. However, it is a reasonable mechanism as Rab32/38 regulated melanosome biogenesis needs to be completed before Rab27a-mediated melanosome transport. The current results show an inverse correlation between the association of Rab27a and Rab38 with melanosomal membranes (Figure 4-20), suggesting a Rab GTPase conversion during melanosome maturation. Interestingly, it has recently been published that Rab27a and Rab32/38 are present on slow-moving/static and fast-moving melanosomes, respectively [175]. It was observed that the speed of melanosome movement was inversely correlated to the level of associated Rab27a and vice versa for Rab32/38. The author suggests that a co-ordinated Rab conversion promotes melanosome transfer from microtubule transport to actin tethering, in time with melanosome maturation. Failure to recruit Rab27a to melanosome membranes could prevent recruitment of a RabGAP for Rab32/38 and result in the accumulation of Rab32/38 on melanosomes. No RabGAP has been identified for Rab38, but RUTBC1 has recently been identified to have GAP activity towards Rab32 and over-expression of RUTBC1 reduced Rab32 association with its effector VARP [476]. Investigating the melanocyte expression and interaction between Rab27a and RUTBC1 would contribute to elucidating a Rab GTPase conversion hypothesis. Additionally, investigating the association of Rab32 on melanosomes in ATP1a1 depleted cells would be of interest as Rab32 and Rab38 are proposed to be redundant in melanocytes [251].

Altogether, a model could be proposed involving all the above mechanisms for Rab27a delivery and melanosome maturation (Figure 6-1). Na^+, K^+ -ATPase in the melanosomal membrane binds Rab27a on Rab27a-delivery vesicles. Fusion of the delivery vesicle with melanosomes inserts Rab27a into the melanosomal membrane. Rab27a is stabilised in the melanosomal membrane by R3G which is itself recruited by ATP1a1 or Rab32/38. Active Rab27a then recruits a GAP for Rab32/38 which results in removal of Rab32/38 from melanosomal membrane. The Rab27a-enriched melanosome is then tethered at the cell periphery through a Mlph-MyoVa link to cortical actin.

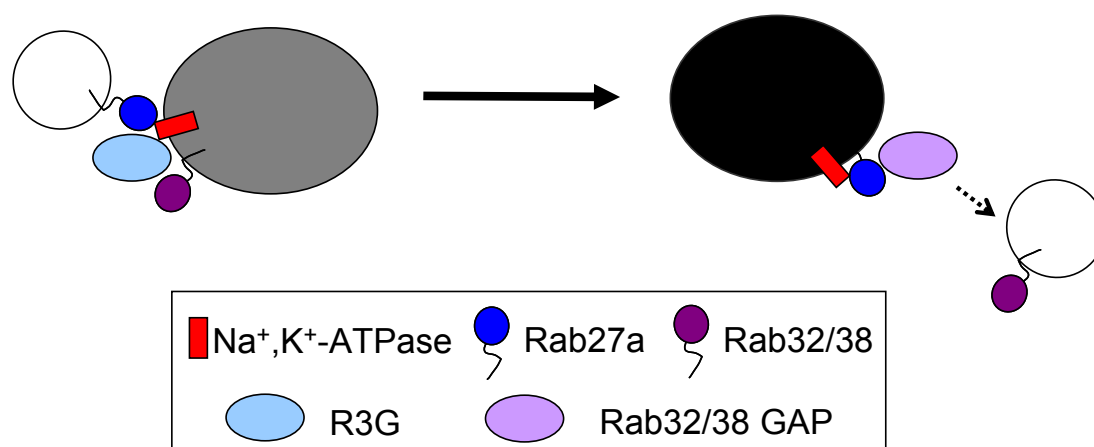


Figure 6-1 Rab27a Delivery to Melanosomes.

The figure depicts a mechanism for Rab27a delivery and melanosome maturation. Rab27a binds to ATP1a1 and is activated by R3G. This stabilises Rab27a on the membrane and enables recruitment of Rab27a effectors. Rab27a recruits a GAP for Rab32/38 resulting in their removal from the melanosome membrane.

6.4 Na^+, K^+ -ATPase and Luminal Acidification

The first indication that vesicular pH may regulate intracellular trafficking came when it was discovered that Arf recruitment to pancreatic microsomal vesicles was dependant on the luminal pH [477]. Subsequent analysis found that Arf-1 mediated cytosolic coatamer protein recruitment to early endosomes was dependent on endosomal acidification [478,

479]. Similarly, endosome acidification promotes recruitment of ARNO (Arf nucleotide site opener) (Arf6 GEF) and Arf6 to endosomal membranes through interaction with the N-terminus of the α 2-subunit and c-subunit of V-ATPase, respectively [480, 481]. These findings led to the proposal of a transmembrane pH sensing protein [482] which in this instance, was assigned to the α 2-subunit of V-ATPase as ARNO association was dependent on luminal acidification [483, 484]. A number of mammalian pH-sensing transmembrane proteins have since been identified, including subunits of V-ATPase and other ion transporters [483, 485-490]. In all of these, histidine residues have been identified as crucial for pH-sensing capabilities.

A principal hypothesis for the role of ATP1a1 in melanocytes was through the possible function of Na^+, K^+ -ATPase in regulating melanosomal pH and, indirectly, melanosome maturation. It was then hypothesised that the luminal pH could be relayed to the cytosol, indicating the maturation of the melanosome and subsequent Rab27a recruitment. It could similarly be hypothesised that similar to ARNO, R3G recruitment is luminal pH-dependent.

Na^+, K^+ -ATPase has been shown to regulate the luminal pH of endosomes by opposing the electrogenic V-ATPase H^+ uptake [453, 454], highlighting the importance of ion balance in regulating luminal pH. A model has previously been proposed for the role of ion transporters in regulating pH in melanosomes [491] and/or TGN [492] which involved the active transport of protons into the lumen by V-ATPase coupled to a Na^+/H^+ exchanger (NHE) and subsequently a potassium-dependent $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCKX) (Figure 6-2). The model proposed that in the presence of NHE and NCKX, the luminal accumulation of protons mediated by the pumping V-ATPase is negated by the coupled proton export by NHE, therefore preventing proton accumulation and luminal acidification (Figure 6-2).

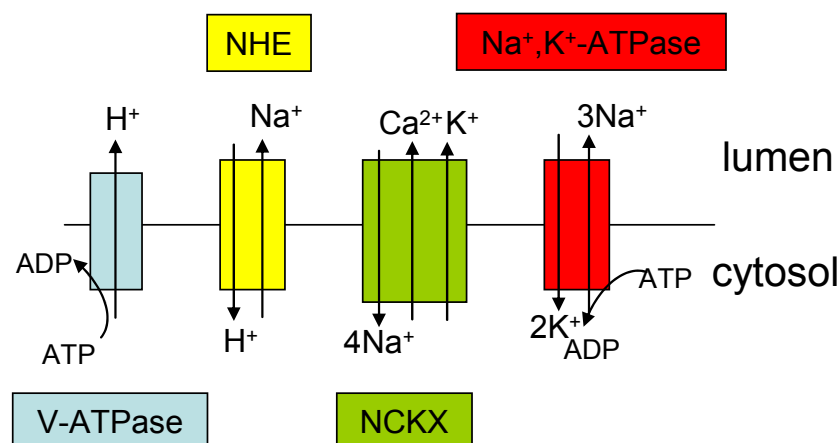


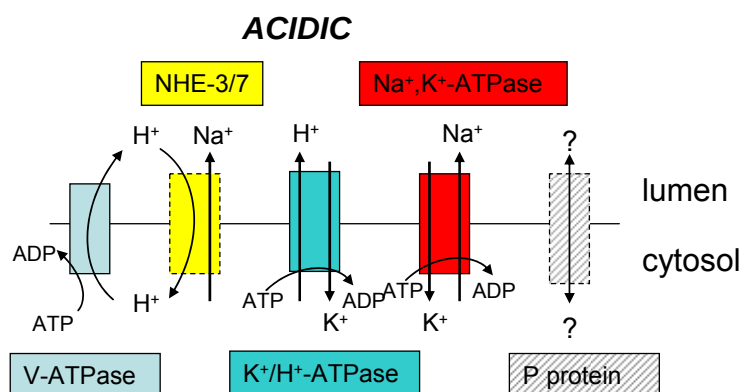
Figure 6-2 Ion Transporters.

The figure shows the ion transporters (V-ATPase, NHE and NCKX) previously suggested to regulate ion balance across melanosomal membranes. The number and direction of their ion transport is shown and highlights how Na^+,K^+ -ATPase could contribute by counter-transport of Na^+ and K^+ ions.

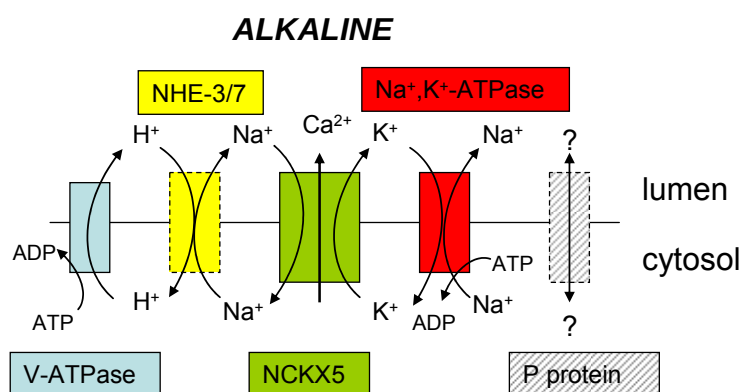
However this model did not account for the counter-transport of K^+ ions, therefore allowing the incorporation of the Na^+,K^+ -ATPase that would extrude 2 K^+ ions from the lumen in exchange for 3 Na^+ forming a 'pH regulating ion transport chain' (Figure 6-2).

Subunits of the V-ATPase have been localised to melanosomes [412, 413, 493, 494], NHE-3 and NHE-7 co-localise with TYRP1 [493] and NCKX5 is a melanosomal NCKX [412]. Based on proteomics [412] a K^+/H^+ -ATPase is present in early melanosomal membranes but absent in late stage melanosomes (Figure 6-3 A), supporting the alkalinisation hypothesis. Similarly, NCKX5 is only associated with late stage melanosomes [412] suggesting the coupled ion transport chain only occurs in late stage melanosomes (Figure 6-3 B). However, no NHE isoforms were identified in melanosome proteomics but this could be a result of failure to solubilise the protein. Overall, a model linking ion transporters and melanosome biogenesis could be proposed (Figure 6-3 C). The ion transporters present on early stage melanosomes maintain an acidic luminal environment, but remodelling of the melanosomal membrane and delivery of NCKX5 and perhaps NHE3/7 to stage III melanosomes alters the ion balance, resulting in an alkaline luminal environment in mature melanosomes.

A. Stage I + II



B. Stage III + IV



C. Melanosome Maturation

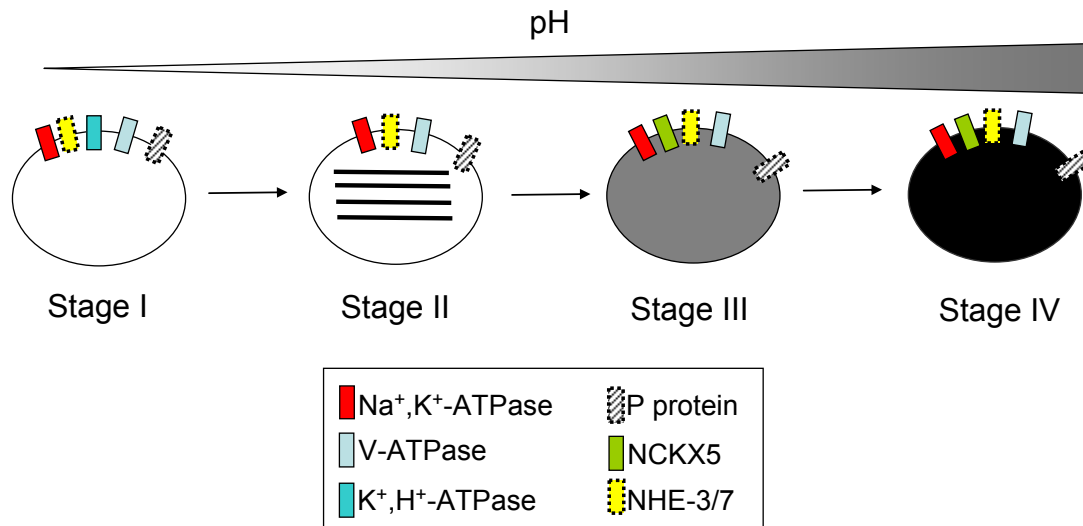


Figure 6-3 The Role of Ion Transporters in Melanosomal Luminal pH Regulation.

The figure shows ion transporters proposed to be on A) stage I and II and B) stage III and IV melanosomes and a scheme depicting melanosome maturation (C). Dotted outlines indicate the protein was not identified by proteomics studies.

The accumulation of Ca²⁺ in the melanosome lumen from the NCKX5 was suggested to activate the processing of Pmel17 which is mediated by a furin-like protein [430], whose

activity is Ca^{2+} dependent [495]. Both NCKX and Na^+, K^+ -ATPase are electrogenic (NCKXs are proposed to exchange 4 Na^+ for 1 K^+ and 1 Ca^{2+} [496]), but in opposing directions, therefore the 4 Na^+ extruded by NCKX is counterbalanced by the Na^+ ions imported by NHE (1 Na^+) and Na^+, K^+ -ATPase (3 Na^+). Together, NHE, NCKX and Na^+, K^+ -ATPase maintain ion charge balance across the membrane therefore proton uptake by V-ATPase would form a self-limiting electrochemical gradient, therefore limiting lumenal acidification. Alternatively, the positive charge could be eliminated by the putative anion transporting function of p protein [255]. The p protein is an uncharacterised protein whose mutation results in Oculocutaneous Albinism Type 2 (OCA2) in humans, pink-eyes and light-grey fur in mice and decreased pigmentation in p-null melanocytes (Melan-p1) resulting from defects in melanosome biogenesis [497] and trafficking of Tyr and TYRP1 [498, 499]. Concomitantly, melan-p1 cells contain predominantly stage I-II melanosomes and a few stage III indicating maturation defects [499].

Overall, it could be predicted that loss of any of the ion transporters would break the chain and NHE would no longer export protons and the ion charge balance would be disrupted, resulting in acidification of the melanosome lumen.

The relationship between the ion transporters and melanogenesis is suggested to be linked to Tyr activity. Depletion of NCKX5 reduced melanin production and expression of melanocyte markers [492] and NHE inhibition caused a dose-dependent decrease in Tyr activity [493]. Both have been proposed to result in disrupted Tyr and TYRP1 processing and trafficking [492, 493], similar to that observed in OCA2 [498, 499]. Amelanotic melanocytes display increased V-ATPase activity and disrupted Tyr trafficking [500] and BafA1 (V-ATPase inhibitor) treatment was able to restore normal Tyr trafficking [501]. Similarly, BafA1 or NH_4Cl treatments result in alkalinisation, increased Tyr activity [461, 466] and melanosome maturation [461] in Caucasian melanocytes and induce melanin synthesis and Tyr processing

in melan-p1 cells [502, 503]. Indirectly, this supports the current finding that NH_4Cl treatment resulted in increased TYRP1 association with melanosomes (Figure 5-17). Tyr and TYRP1 are proposed to be delivered to melanosomes by Rab32/38-positive vesicles from the TGN via endosomal intermediates [251, 504]. As NHE-1 and NHE-7 have been shown to co-localise with TYRP1 [493] it could be proposed that these ion transporters are delivered to stage III melanosomes on the same vesicle (Figure 6-4). Both NCKX5 and Na^+, K^+ -ATPase also localise to endosomes and possibly in conjunction with NHE regulate the luminal microenvironment in budding vesicles, promoting the sorting of Tyr and TYRP1 into buds for trafficking to melanosomes.

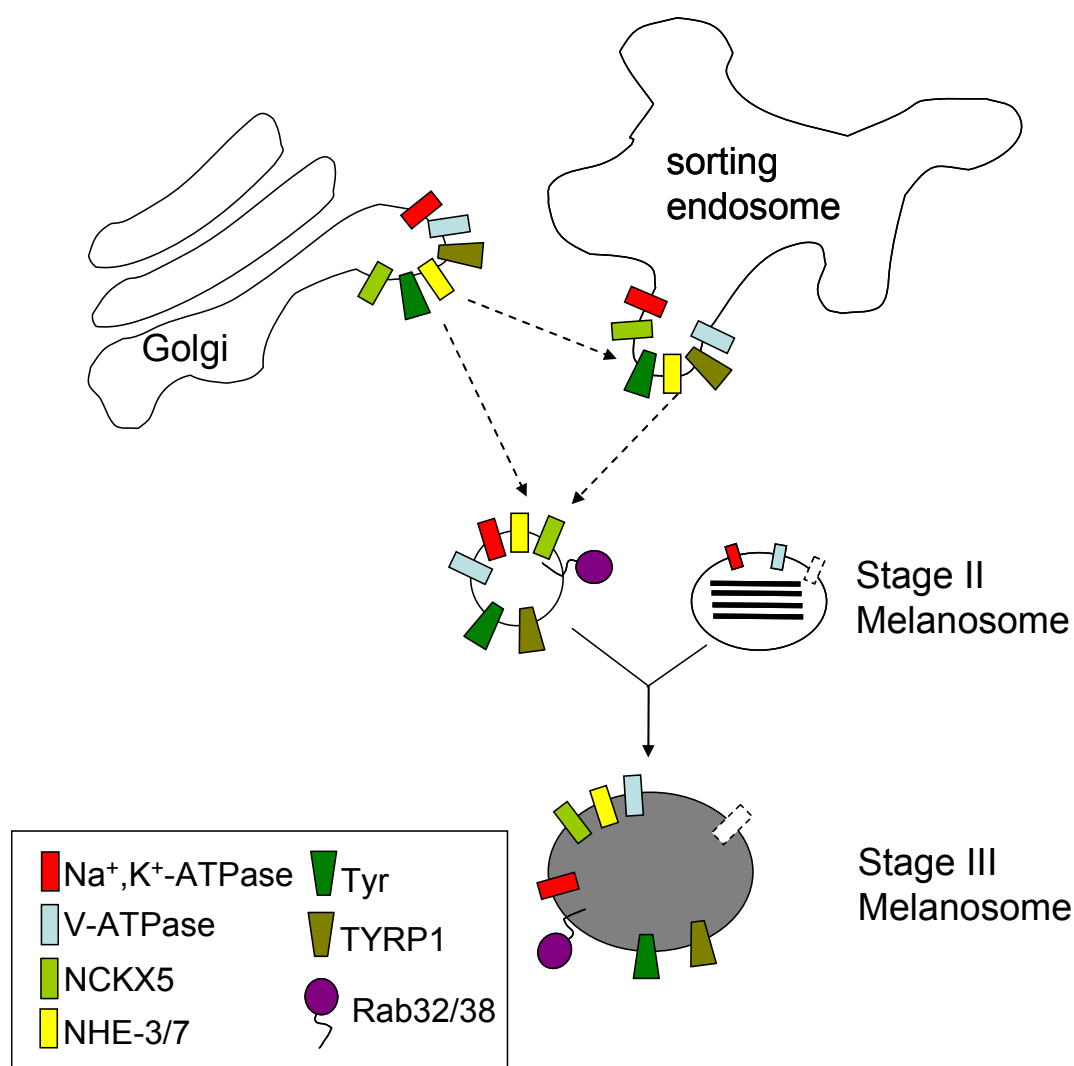


Figure 6-4 Tyr and TYRP1 Delivery to Melanosomes.

Figure depicts the proposed mechanism of Tyr and TYRP1 delivery to melanosomes from TGN/sorting endosome. The ion pumping of a host of ion transporters is suggested to regulate the sorting of Tyr and TYRP1 into Rab32/38 delivery vesicles, therefore regulating melanosome maturation.

Taken together, these findings argue that regulation of luminal pH (melanosome, endosomal and/or TGN) is required for correct trafficking of Tyr and TYRP1 to melanosomes for melanogenesis. A similar pH-dependent endosomal sorting of TGN38, Furin and GPP130 has been seen for transport to the TGN [505, 506]. The observed reduction in TYRP1 associated with melanosomes following ATP1a1 depletion (Figure 4-20) could therefore be a comparable redistribution due to acidification. It would be interesting to assess the Tyr activity of ATP1a1 depleted cells. More importantly, the effect BafA1 treatment has on

Rab27a function and the ATP1a1 depletion-induced clustering, would clarify the role the V-ATPase plays in the phenotype.

Rab27a sorting and budding of the delivery vesicles could similarly be pH mediated. Alternatively, it could be proposed that the presence of the pH regulating ion transport chain signals that the melanosome is alkalinised and therefore mature. Concomitantly, ATP1a1 may sense the pH of the lumen and convey this across the membrane through conformational changes similar to that proposed for V-ATPase/ARNO/Arf6 [480, 481]. However ATP1a1 has no His residues in its intra-lumenal domains therefore suggesting no similarity to other pH-sensing proteins. Alternatively, other transmembrane proteins may be responsible, including the V-ATPase. R3G and/or Rab27a melanosomal recruitment could be suggested to be dependant on a pH-sensing induced conformational change of V-ATPase. Interestingly, subunits of the V-ATPase were identified as interacting partners for Rab27a in the HTF-Rab27a^{SF1/F4} TAP (Appendix), however they were not present in the HTF-Rab27a^{WT} TAP.

Taken together it could be proposed that in the absence or inhibition of any of the ion transporters, Tyr and TYRP1 endosomal sorting is perturbed. Tyr and TYRP1 remain in endosomes but Rab32/38 are still delivered to melanosomes. However the melanosome lumen does not alkalinise as the ion transporters are absent. Subsequently, Rab27a endosome sorting and/or pH-dependent association with the melanosome membrane would fail. A number of questions need to be addressed to investigate this model. Does depletion of the other ion transporters affect Rab27a localisation? Where are Rab27a delivery vesicles derived from? Is the ATP1a1 intracellular staining endosomal? Does Rab27a or R3G interact directly with ATP1a1?

6.5 Proposed Model for Rab27a Targeting

Taking all the above information into account, a model for pH-dependant melanosome maturation and Rab27a targeting can be proposed. The maturation of a melanosome would be tightly regulated to ensure the correct processing and deposition of melanin, prior to its transfer to neighbouring keratinocytes. In particular, the timely delivery of melanogenic proteins to melanosomes. Tyr and TYRP1 are pH-dependently sorted and delivered to Stage III melanosomes via Rab32/38 vesicles (Figure 6-4), in conjunction with NHEs and other ion transporters. After delivery to melanosomes, the proton transporting function of NHE counteracts the previously functioning V-ATPase, commencing luminal alkalinisation. Concomitantly, K^+, H^+ -ATPase is removed from melanosomal membranes, also promoting alkalinisation (Figure 6-3). Once an alkaline luminal environment is created, this is conveyed to the cytosol via a conformational change in a pH-sensing protein. Rab27a is then recruited by interacting with the pH-sensing protein, ATP1a1 (Figure 6-1). In parallel, Rab32/38 or the pH-sensing protein, recruit R3G which activates Rab27a and promotes its stable association with the melanosomal membrane. Active Rab27a then recruits a Rab32/38 RabGAP, promoting their removal from melanosomal membranes (Figure 6-1).

6.6 ATP1a1 and MAPK Induced Hyper-pigmentation

ATP1a1 depletion was observed to cause hyper-pigmentation (Figure 4-21) despite previous reports proposing acidification of the melanosome lumen results in hypo-pigmentation [492, 501]. ATP1a1 and/or R3G depletion resulted in melanosomal clustering, reduced GTP-Rab27a, displacement of Rab27a and TYRP1 from and increased Rab38 association with melanosomes in addition to acidification of the melanosomal lumen. However hyper-pigmentation and increased pERK1/2 (Figure 5-25) were the only changes observed following ATP1a1 depletion but not R3G depletion suggesting they could be linked. Interestingly, MAPK signalling has been linked to regulation of MITF, the transcription factor

critical for melanocyte development and survival [420]. The cAMP-independent trans-activation of the tyrosine kinase receptor c-Kit following α -MSH binding to MC1R results in stimulation of the Src/Raf/MEK/ERK signalling cascade [507] (Figure 6-5). ERK phosphorylation of MITF on Ser73 is suggested to activate transcription of Tyr [508] and can occur following UVA radiation resulting in melanogenesis [509]. ERK2 activation of the serine/threonine kinase p90 Rsk-1 also results in phosphorylation of MITF at Ser409. Stem cell factor (SCF) is the ligand for c-Kit and a gain-of-function SCF mutant has been identified to cause Familial Progressive Hyper-pigmentation (FPH) resulting in increased Tyr activity [510], again highlighting the role of the c-Kit/MAPK pathway in melanin synthesis. Contrary to this, MAPK inhibition has been shown to promote melanocyte differentiation and sustained ERK1/2 activity was shown to inhibit melanogenesis [511]. It has since been shown that phosphorylation of MITF couples its activation and degradation as it enhances association of the transcriptional co-activator p300 [512] but also reduces the half life by targeting MITF for proteosome degradation [513] (Figure 6-5). This is highlighted by the finding that double-phosphorylation deficient MITF is transcriptionally inert but stable [514], however single MITF Ser73 or Ser409 mutants can rescue the white coat colour phenotype of the MITF mutant mouse [515].

The above data suggests that the observed increase in pERK1/2 levels following ATP1a1 depletion (Figure 5-25) could result in an initial increase in Tyr and TYRP1 expression as these are regulated by MITF [421, 516]. In this study, hyper-pigmentation was observed in ATP1a1 depleted cells following a single round of siRNA treatment (Figure 4-21) whilst significant decreases in TYRP1 melanosomal association only occurred after two rounds of siRNA treatment (Figure 4-19 and Figure 4-20). This could suggest that the initial depletion of ATP1a1 activates ERK1/2, resulting in increased TYRP1 and Tyr production and activity, leading to hyper-pigmentation. However, with long-term ATP1a1 depletion the prolonged ERK1/2 activation would target phosphorylated MITF for degradation and the pH-dependent

defective Tyr and TYRP1 trafficking prevents any further hyper-pigmentation. R3G depletion resulted in a decrease in pERK1/2 levels (Figure 5-25), which supports the finding that R3G depletion had no effect on pigmentation. A key experiment would be a time-course to investigate the phosphorylation and expression levels of MITF following ATP1a1 depletion.

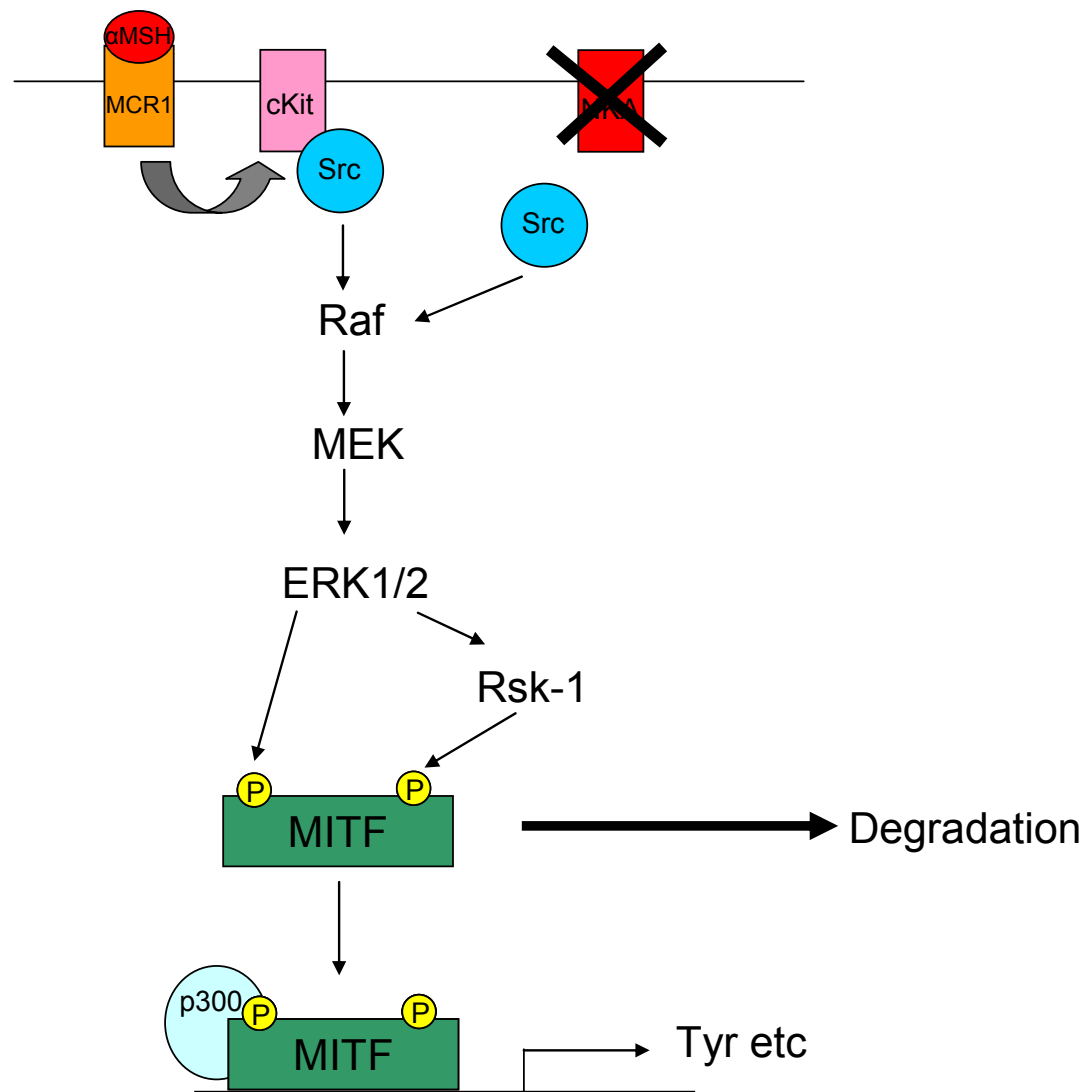


Figure 6-5 ERK1/2 and MITF Activation in Melanocytes.

The figure shows the signalling cascade from activation of MCR1 by α MSH and trans-activation of cKIT. Activation of the MAPK pathways through Src/Raf/MEK/ERK1/2 results in phosphorylation and activation of MITF leading to transcription of melanocyte specific proteins. Prolonged ERK1/2 activation results in MITF degradation. Depletion of ATP1a1 relieved its inhibition of Src Kinase and activated the MAPK pathway.

6.7 Future work

Investigating a direct interaction between Rab27a and ATP1a1 would be the most informative experiments towards an understanding of the mechanism of action. As ATP1a1 is a ten transmembrane protein it could prove difficult to purify and so initial experiments are likely to use purified cytosolic domains. In particular, the large loop between transmembrane domains 4 and 5 (ATP1a1-M4M5) (See Figure 1-11) as it has been implicated in interactions with other proteins in the cytosol [353, 393, 471-475]. GST tagged ATP1a1-M4M5 could be used to assess a direct interaction with Rab27a. If a direct interaction is identified, subsequent experiments would involve assessing the affinity of the different Rab27a mutants, as the Rab27a^{SF2} mutant would not be expected to bind if an interaction is necessary for Rab27a targeting. Also, it would be interesting to assess whether ATP1a1 interacts with R3G, possibly forming a Rab27a targeting/activating complex or perhaps acting as a linker between Rab27a and R3G.

Characterisation of the ATP1a1 positive vesicles in melanocytes is paramount and this could initially be assessed by co-staining with markers for various compartments i.e. TGN and EE. Key to investigate this would be cyro-immuno electron microscopy (EM) analysis of melanocytes with immunogold labelling for ATP1a1. EM analysis would be particularly useful as different intracellular compartments have distinctive characteristics, so aiding characterisation of the ATP1a1-positive structures. Additionally, it is important to characterise the Rab27a vesicles observed after ATP1a1 and R3G depletion. Similarly, EM analysis of ATP1a1 depleted melanocytes would enable analysis of the effect on melanogenesis as the different stages are distinguishable. In addition, assessment of the localisation of TYRP1, Tyr and Rab32/38 in melanocytes following ATP1a1 depletion using both immunofluorescence microscopy and EM would aid in clarifying the proposed model.

Of great interest would be investigating the effect depletion of other ion transporters has on Rab27a localisation. This would clarify the importance of ion balance and the role of the putative pH regulating ion transport chain in melanogenesis. In particular, it would be interesting to assess whether inhibition of V-ATPase using BafA1 is able to prevent/rescue the ATP1a1-induced phenotype. Conversely, if acidification of the cell/melanosomes can phenocopy ATP1a1 depletion, this would support the importance of pH in melanogenesis.

A time-course experiment assessing the phosphorylation state and expression levels of MITF to pERK1/2 following ATP1a1 depletion would clarify its involvement in the hyper-pigmentation phenotype. If the proposed mechanism is true, following ATP1a1 depletion an initial increase in phosphorylated MITF would be observed but rapidly followed by its degradation. Similarly, Src kinase specific inhibitors could be used to assess if they can prevent ATP1a1-depletion-induced hyper-pigmentation

6.8 Conclusions

The identification of the α -subunit of Na^+, K^+ -ATPase as being important for targeting of Rab27a to melanosomal membranes was initially controversial due to its primary function at the plasma membrane. However, depletion of ATP1a1 resulted in perinuclear clustering of melanosomes, reduced GTP-Rab27a and displacement of Rab27a and TYRP1 from melanosomes. Interesting, R3G depletion resulted in comparable phenotype.

It is unlikely that a direct interaction between Rab27a and ATP1a1 is the sole determinant of Rab27a localisation. It is more likely that an assortment of interactions and/or processes need to occur which characterises the microenvironment of the membrane. This would include RabGEF recruitment and activation, resulting in recruitment and stabilisation of a RabGTPase on a distinct membrane.

Although still only preliminary, results suggest the involvement of intracellular Na^+, K^+ -ATPase in regulating ion balance and pH of luminal environments. It is then proposed that subsequent RabGTPase recruitment is mediated by pH-induced RabGTPase and/or RabGEF interactions with a pH-sensing protein. Each intracellular compartment may have distinct assortments or concentrations of ion transporters and as a result have discrete luminal pH environments and cytosolic binding surfaces. This proposes a model whereby organelle luminal pH changes are linked to organelle maturity and subsequent Rab GTPase recruitment. The mechanism could be applied to the maturation of endosomes, Golgi cisternae, autophagosomes or any other maturing organelle where Rab recruitment/conversion requires temporal regulation.

7 Appendix

The list below contains all hits from the HTF-Rab27a^{SF1/F4} TAP sample after exclusion of the negative control (untransfected ashen TAP). Highlighted in grey are those also featured in the HTF-Rab27a^{WT} TAP sample. References highlight those hits previously identified from melanosomal proteomics.

Syt12 - Isoform 1 of Synaptotagmin-like protein 2

Syt12 - Isoform 5 of Synaptotagmin-like protein 2

Rab27a Ras-related protein Rab-27A [412]

Actg1 Putative uncharacterized protein

Actb Actin, cytoplasmic 1

Iqgap1 Ras GTPase-activating-like protein IQGAP1 [412]

Krt5 Keratin, type II cytoskeletal 5

Tubb5 Tubulin beta-5 chain [412]

Syt12 synaptotagmin-like 2 isoform 1

Tuba1b Tubulin alpha-1B chain

Hspa5 78 kDa glucose-regulated protein [412]

Atp5a1 ATP synthase subunit alpha, mitochondrial [412]

Atp5b ATP synthase subunit beta, mitochondrial [412]

Tuba1a Tubulin alpha-1A chain

Krt6a Keratin, type II cytoskeletal 6A

Tubb2c Tubulin beta-2C chain [412]

Syt14 Isoform 1 of Synaptotagmin-like protein 4

Krt14 Keratin, type I cytoskeletal 14

Tuba4a Tubulin alpha-4A chain

LOC100042025;Gapdh;Gm10293;Gm10359;Gm12033;Gm2574;Gm5138;Gm3272
Glyceraldehyde-3-phosphate dehydrogenase [412]

Gstp1 Glutathione S-transferase P 1 [412]

Ppia Peptidyl-prolyl cis-trans isomerise [412]

Gm5506;Eno1;LOC100044223 Alpha-enolase

Krt42 Keratin, type I cytoskeletal 42

Pkm2 Isoform M2 of Pyruvate kinase isozymes M1/M2 [412]

Anxa5 Annexin A5 [412]

Mdh2 Malate dehydrogenase, mitochondrial

Rab27b Ras-related protein Rab-27B [414]

Vim Vimentin [412]

Unknown - Putative uncharacterized protein (Fragment)

Gpnmb Transmembrane glycoprotein NMB [412-414]

Pgk1 Phosphoglycerate kinase 1 [412]

Hadha Trifunctional enzyme subunit alpha, mitochondrial

Aldoa Fructose-bisphosphate aldolase [412]

Anxa1 Putative uncharacterized protein [412]

Krt16 Keratin intermediate filament 16a

Hspd1 Isoform 1 of 60 kDa heat shock protein, mitochondrial

Lgals1 Galectin-1 [412]

Ywhaz 14-3-3 protein zeta/delta [412]

Sec23b Protein transport protein Sec23B

Eef1a1 Elongation factor 1-alpha 1 [412]

Slc25a5 ADP/ATP translocase 2 [412]

Krt76 Keratin, type II cytoskeletal 2 oral

Hspa9 Stress-70 protein, mitochondrial

Pfn1 Profilin-1 [412]

Cltc Clathrin heavy chain 1 [412]

Cfl1 Cofilin-1 [412]

Slc25a4 ADP/ATP translocase 1

Anxa2 Annexin A2 [412]

Gm9145 Pterin-mimicking anti-idiotope heavy chain variable region (Fragment)

ldh2 Isocitrate dehydrogenase [NADP], mitochondrial

Eef2 Elongation factor 2 [412]

Igkv6-25 Ig kappa chain V-V region MPC11

Prdx1 Peroxiredoxin-1 [412]

Ppib Peptidyl-prolyl cis-trans isomerase B [412]

Eif2s1 Eukaryotic translation initiation factor 2 subunit 1 [412]

Tpt1;Gm14456 Translationally-controlled tumor protein [412]

Krt79 Keratin, type II cytoskeletal 79

Oxct1 Succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial

Mif Macrophage migration inhibitory factor [412]

Serpinh1 Serpin H1 [412]

Typr1 5,6-dihydroxyindole-2-carboxylic acid oxidase [412-414]

Hsp90aa1 Heat shock protein HSP 90-alpha [412]

Ywhae 14-3-3 protein epsilon [412]

Gpi1 Glucose-6-phosphate isomerase

Eif5a Eukaryotic translation initiation factor 5A-1 [412]

Pdia6 Putative uncharacterized protein [412]

Uba52;2810422J05Rik;Gm11808 ubiquitin A-52 residue ribosomal protein fusion product 1

Hsp90b1 Endoplasmin [412]

Tpi1 triosephosphate isomerase 1 [412]

Alb Serum albumin

Dbi Acyl-CoA-binding protein

Krt14 Type I epidermal keratin (Fragment)

Vcp Transitional endoplasmic reticulum ATPase [412]

Vat1 Synaptic vesicle membrane protein VAT-1 homolog [412, 413]

Rab7 Ras-related protein Rab-7a [412, 413]

Ighg;lgh-1b;lgh-1a Ighg protein

Atp1a1 Sodium/potassium-transporting ATPase subunit alpha-1 [412, 414]

Sec23a Protein transport protein Sec23A [412]

Etfa Electron transfer flavoprotein subunit alpha, mitochondrial [412]

Rnh1 Ribonuclease inhibitor [412]

Akr1a4 Alcohol dehydrogenase [NADP+]

Gm5409 Try10-like trypsinogen

Pebp1 Phosphatidylethanolamine-binding protein 1 [412]

Prdx2 Peroxiredoxin-2 [412]

Msn Moesin [412]

Eef1g Elongation factor 1-gamma [412]

Hspe1-rs1 CPN10-like protein [412]

Fdps Farnesyl pyrophosphate synthetase

Csrp1 Cysteine and glycine-rich protein 1

Ywhaq Isoform 1 of 14-3-3 protein theta [412]

Pgd 6-phosphogluconate dehydrogenase, decarboxylating [412]

Golt1b Vesicle transport protein GOT1B [412]

Sec24b Sec24 related gene family, member B

Myh9 Myosin-9 [412]

Ywhab Isoform Long of 14-3-3 protein beta/alpha [412, 414]

Eps8 Putative uncharacterized protein [412]

Atp5f1 ATP synthase subunit b, mitochondrial [412]

Eif1 Eukaryotic translation initiation factor 1 [412]

Eif4a1 Eukaryotic initiation factor 4A-I [412]

Etf1 Eukaryotic peptide chain release factor subunit 1

Got2 Aspartate aminotransferase, mitochondrial [412]

Atp5d ATP synthase subunit delta, mitochondrial

Krt8 Keratin, type II cytoskeletal 8

Nme2 Nucleoside diphosphate kinase B [412]

Arhgdia Rho GDP-dissociation inhibitor 1 [412]

mt-Co2 Cytochrome c oxidase subunit 2 [412]

Ddx6 Probable ATP-dependent RNA helicase DDX6 [412]

Igh-6 Ig mu chain C region membrane-bound form

Rab10 Ras-related protein Rab-10 [412]

Ahnak2 similar to KIAA2019 protein

Lgals3 Galectin-3 [412]

Unknown- 11 kDa protein

Rhoc Rho-related GTP-binding protein RhoC [412]

Mdh1 Malate dehydrogenase, cytoplasmic [412]

Copg Putative uncharacterized protein

Hspa4 Heat shock 70 kDa protein 4 [370]

Gsta4 Glutathione S-transferase A4

Tmed10 Isoform 1 of Transmembrane emp24 domain-containing protein 10 [412]

Ywhag 14-3-3 protein gamma [412]

Rab1 Ras-related protein Rab-1A [412]

Phb2 Prohibitin-2 [412]

Sod1 Superoxide dismutase [Cu-Zn] [412]

Tpm1 tropomyosin 1 alpha chain isoform 10

Phb Prohibitin [412]

Atp5o;LOC100047429 ATP synthase subunit O, mitochondrial [412]

Pcna Proliferating cell nuclear antigen

P4hb Putative uncharacterized protein [412]

Tkt Transketolase [412]

LOC100045999;Ran GTP-binding nuclear protein Ran

Pycr2 Pyrroline-5-carboxylate reductase 2

Ephx1 Epoxide hydrolase 1, microsomal, isoform CRA_c [412]

Csnk1a1 Isoform 1 of Casein kinase I isoform alpha

Banf1 Barrier-to-autointegration factor

Atp6v1a Isoform 1 of V-type proton ATPase catalytic subunit A [412]

Sept2 Septin-2 [412]

- Ig kappa chain V-III region PC 7175

Mtpn Myotrophin [412]

Copa Coatomer subunit alpha [412]

Ptma Prothymosin alpha

Ifitm3 MCG22590

Si Silver, isoform CRA_a [412-414]

Akr1b3 Aldose reductase

Tln1 Talin-1 [412]

Ugp2 Isoform 1 of UTP--glucose-1-phosphate uridylyltransferase [412]

Dpysl2 Dihydropyrimidinase-related protein 2 [412]

Rcc1 Regulator of chromosome condensation 1

Calml3 Calmodulin-like protein 3

Dsp desmoplakin

Mgll Putative uncharacterized protein

Ola1 Isoform 1 of Obg-like ATPase 1

Clic1 Chloride intracellular channel protein 1 [412]

Gyg Glycogenin-1

Aldh2 Aldehyde dehydrogenase, mitochondrial

Bcap31 B-cell receptor-associated protein 31 [412]

Atp5c1 ATP synthase subunit gamma, mitochondrial [412]

Pdia3 Protein disulfide-isomerase A3 [412]

Cct6a T-complex protein 1 subunit zeta [412]

Surf4 Surfeit locus protein 4 [412]

Capzb Isoform 2 of F-actin-capping protein subunit beta [412]

Rtn4 Isoform 3 of Reticulon-4 [413]

Pcmt1 Putative uncharacterized protein

Hnrnpf Isoform 1 of Heterogeneous nuclear ribonucleoprotein F

Eif2s3x Eukaryotic translation initiation factor 2 subunit 3, X-linked

Uqcrc2 Cytochrome b-c1 complex subunit 2, mitochondrial [412]

Ctsd Cathepsin D [412]

Cotl1 Coactosin-like protein

Coro1c Coronin-1C [412]

Vdac2 Voltage-dependent anion-selective channel protein 2 [414]

Calr Calreticulin [412]

Gdi2 Isoform 1 of Rab GDP dissociation inhibitor beta

Rpn2 Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2

Canx Calnexin [412, 414]

Atp5k ATP synthase subunit e, mitochondrial

Slc16a1 Monocarboxylate transporter 1 [412]

Eef1d Isoform 1 of Elongation factor 1-delta

Prdx6 Peroxiredoxin-6 [412]

Hsd17b11 Isoform 1 of Estradiol 17-beta-dehydrogenase 11

Ndrg1 Protein NDRG1

Unknown - similar to SMT3B protein isoform 3

Rtn3 Isoform 3 of Reticulon-3

Ldhb L-lactate dehydrogenase B chain [412]

Ndufa4 NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4

Slc25a3 Phosphate carrier protein, mitochondrial [412]

Idh1 Putative uncharacterized protein

Eef1b2 Elongation factor 1-beta

Ctsb Cathepsin B [412]

Hint1 Histidine triad nucleotide-binding protein 1 [412]

Cops3 COP9 signalosome complex subunit 3

Cycs;Gm10053 Cytochrome c, somatic [412]

Ddx39 Isoform 1 of ATP-dependent RNA helicase DDX39

Myl6b Myosin light chain 6B [412]

Cs Citrate synthase, mitochondrial

Gm10145 similar to LOC446231 protein

Gm5436 hypothetical protein

Nedd8 NEDD8

Ldha L-lactate dehydrogenase A chain [412]

Rasa3 Ras GTPase-activating protein 3

Ak2 Isoform 2 of Adenylate kinase 2, mitochondrial

Unknown 13 kDa protein

Tagln2 Transgelin-2 [412]

Gtf2b Transcription initiation factor IIB

Kpnb1 Importin subunit beta-1 [412]

Hnrnpk Isoform 1 of Heterogeneous nuclear ribonucleoprotein K [412]

Rps6ka1 Ribosomal protein S6 kinase alpha-1

Pgam1 Phosphoglycerate mutase 1 [412]

Ube2n Ubiquitin-conjugating enzyme E2 N [412]

Tmod3 Tropomodulin-3 [412]

Atp6v1e1 V-type proton ATPase subunit E 1 [412]

Reep5 receptor accessory protein 5

Aldh1a1 Retinal dehydrogenase 1

Chid1 chitinase domain containing 1 isoform 2

Prpf19 Isoform 2 of Pre-mRNA-processing factor 19

Hadhb Trifunctional enzyme subunit beta, mitochondrial

Pabpn1 Isoform 1 of Polyadenylate-binding protein 2

Vapa Vesicle-associated membrane protein-associated protein A [412]

Akr1b10 aldo-keto reductase family 1, member B10

Cdc42 Isoform 2 of Cell division control protein 42 homolog [412]

Psmc1 26S protease regulatory subunit 4 [412]

Phgdh D-3-phosphoglycerate dehydrogenase [412]

Mtap S-methyl-5'-thioadenosine phosphorylase

Psmb5 Proteasome subunit beta type-5

Fabp5 Fatty acid-binding protein, epidermal

Cct4 T-complex protein 1 subunit delta [412]

Hnrnpa2b1 Isoform 3 of Heterogeneous nuclear ribonucleoproteins A2/B1

Atp1b3 Sodium/potassium-transporting ATPase subunit beta-3 [412]

Rap2b Ras-related protein Rap-2b

Ppp1cc Isoform Gamma-1 of Serine/threonine-protein phosphatase PP1-gamma catalytic subunit [412]

Epb4.1l3 Isoform 1 of Band 4.1-like protein 3

Flna Isoform 1 of Filamin-A [412]

Cox6c Cytochrome c oxidase subunit 6C

Actr3 Actin-related protein 3 [412]

Epb4.1l2 4.1G protein

Prmt1 Isoform 1 of Protein arginine N-methyltransferase 1

Suclg1 Succinyl-CoA ligase [GDP-forming] subunit alpha, mitochondrial

Lta4h Leukotriene A-4 hydrolase

Hmgb1;Gm15387 High mobility group protein B1 [412]

Pcbp1 Poly(rC)-binding protein 1 [412]

EG436523;Tcrb-V20;Gm5771;Prss1;Prss3 protease, serine, 1

Atp5j2 ATP synthase subunit f, mitochondrial [412]

Tmed7 transmembrane emp24 protein transport domain containing 7

Sub1 Activated RNA polymerase II transcriptional coactivator p15

St13 Hsc70-interacting protein [412]

Grn granulin [412]

Rab44 Ras-related protein Rab-44

Chchd2 Coiled-coil-helix-coiled-coil-helix domain-containing protein 2, mitochondrial [412]

Idh3a Isoform 1 of Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial [412]

Gstm1 Glutathione S-transferase Mu 1

Oat Ornithine aminotransferase, mitochondrial [412]

1110007L15Rik Isoform 1 of UPF0363 protein C7orf20 homolog

Cdk3 Isoform 1 of Cell division protein kinase 3 [412]

Zyx Zyxin

Hmgcl Hydroxymethylglutaryl-CoA lyase, mitochondrial

Ptges3;Gm9769;LOC100048119 Prostaglandin E synthase 3 [412]

Lmna Isoform C of Lamin-A/C

Maoa Amine oxidase [flavin-containing] A

Sars Seryl-aminoacyl-tRNA synthetase, isoform CRA_b [412]

Tceb2 Transcription elongation factor B polypeptide 2

Fah Fumarylacetoacetase [412]

Hnrnpab Heterogeneous nuclear ribonucleoprotein A/B

Apex1 DNA-(apurinic or apyrimidinic site) lyase

Sec22b Vesicle-trafficking protein SEC22b [412]

Ipo5 Isoform 2 of Importin-5

Nceh1 Neutral cholesterol ester hydrolase 1

Npm1 Nucleophosmin [412]

Esd S-formylglutathione hydrolase [412]

Dstn Destrin [412]

Rpn1 Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1 [412]

Cct7 T-complex protein 1 subunit eta [412]

Hmcn2 Hemicentin 2

Hnrnpd Isoform 2 of Heterogeneous nuclear ribonucleoprotein D0

mt-Atp6;mt-Atp8 ATP synthase protein 8

Pcbp2 Isoform 1 of Poly(rC)-binding protein 2 [412]

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