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The Role of SPIRE Proteins in Melanosome Dispersion in Skin Melanocytes

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Abstract

A critical physiological process for living cells is to transport their organelles and other material from their site of formation to their site of function. This transport process involves complex cooperation between many different proteins that function as signals, docking factors, motors and tracks. However, the molecular mechanisms that regulate intracellular motility remain mostly obscure. In this study, melanosome of skin melanocytes was used as a model for lysosome-related organelles, and in particular to study the role of SPIRE1/2 (the actin nucleator factors) in intracellular trafficking. More specifically, the role of conserved SPIRE1/2 domains has been investigated in melanosome transport in two ways. Firstly, SPIRE1/2 rescue assay was conducted, in which the translation of endogenous SPIRE1/2-mRNA was disrupted by specific short-interference RNA, and then the cells were rescued by adding siRNA-resistant SPIRE1/2 (full-length, truncations, or mutations). The changes in melanosome distributions were used as a read-out for the effects of SPIRE1/2 on the transport. Secondly, a modified nano-scale pulldown assay was performed in leaden melanocytes (melan-In; melanophilin null melanocytes). In this approach, the active motor and lever arm of myosin-Va was attached to SPIRE1/2 (full-length, truncations, or mutations) and then expressed in melan-In. The changes in melanosome distribution were used as a read-out for the interaction between the SPIRE construct and active Rab27a on the melanosome membrane. The results of siRNA knockdown and rescue assays provided evidence for a crucial role of

SPIRE-nucleated actin in melanosome transport. The interactions with both G-actin and FMN-type formin were found to be essential for this process. The modified nano-scale pulldown assay, along with co-immunoprecipitation, provided consistent evidence of the interaction between SPIRE1 and GTP-Rab27a as well as support considering SPIRE1 as a potential Rab27a effector. The results of the knockdown-rescue assay also revealed the role of SPIRE localisation on melanosome dispersion pattern. SPIRE localising to melanosome membrane via interacting with GTP-Rab27a resulted in a uniformly dispersed distribution, while mislocalised SPIRE that did not interact with Rab27a resulted in an eccentrically dispersed distribution (hyper-dispersed). These results highlight the molecular mechanism of actin-based melanosome transport. However, further investigation is needed to determine the similarities and differences between SPIRE1 and other Rab27a effectors. Additionally, the hyper-dispersed distribution is worth investigating in depth.

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Declaration

This dissertation is the result of my work and includes nothing, which is the outcome of work done in collaboration except where specifically indicated in the text. It has not been previously submitted, in part or whole, to any university or institution for any degree, diploma, or other qualification.

In accordance with the School of Life Science guidelines, this thesis does not exceed 80,000 words, and it contains less than 150 Figures.

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List of abbreviations

Abbreviation	definition
27AUTR1	Rab27a alternative untranslated region 1
3T3-Src+	Mouse embryonic fibroblasts (NIH3T3) cells stably expressing active Src tyrosine kinases(Src+)
ADF	Actin-depolymerising factor
ADP	Adenosine diphosphate
AF	Actin filaments
AFH1	<i>Arabidopsis thaliana</i> Formin Homology protein 1
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli protein
APS	Ammonium Persulfate
ARP	The actin-related proteins
ARP2	Actin Related Protein 2
ARP3	Actin Related Protein 3
ARPC1-5	actin-related protein component 1-5
ATP	Adenosine triphosphate
B16F10	A murine melanoma cell line derived from a C57BL/6J mouse
BF	Barbed end of actin filament associated with formin

BP	Bandpass (microscope filter excitation and emission)
bp	Base pair (in primer oligos)
BS	Barbed end of actin filament associated with SPIRE
BSA	Bovine serum albumin
BSF	Barbed end of actin filament associated with SPIRE and formin
C57BL	"C57 black 6" a common strain of laboratory mouse that is widely used for genetically modification as models of human disease
CA region	The conserved sequence within the nucleation promoting factor facilitates the interaction with ARP2/3
CAAX	A conserved sequence includes cysteine residue (C), two aliphatic residues (AA), and any C-terminal amino acid (X).
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHO	Chinese hamster ovary cell line
CIP	Calf Intestinal Phosphatase
CO ₂	Carbon dioxide
CTCF	The corrected total cell fluorescence

D157/ D425/ D569	Aspartic acid residue in the indicated positions
DAAM	Dishevelled Associated Activators of Morphogenesis
DAD	Dia-autoregulatory domain
DAPI	4',6-diamidino-2-phenylindole
DBP	The vitamin D-binding protein
DCT	Dopachrome tautomerase
DCTN1	Dynactin subunit 1
DENN	Differentially expressed in normal and neoplastic cells
DHI	5,6-dihydroxyl indole
DHICA	5,6-dihydroxylindole-2-carboxylic acid
D-MEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DOPA	Dihydroxyphenylalanine
<i>E. coli</i>	<i>Escherichia coli</i>
E13/ E14/ E32/ E404/ E416/ E51	Glutamic acid residue in the indicated positions
E13Linker / E13L	Mutant SPIRE-E13 with longer linker between KIND and WH2

E416A/ E548A/ E560A	Glutamic acid residue in the indicated position substituted with Alanine
E548K	Glutamic acid residue in the indicated position substituted with Lysine
E9/ E13	Exon no 9 / no 13 within SPIRE1
EB1	End binding protein 1
EDTA	Ethylenediaminetetraacetic acid
EEA1	Early Endosome Antigen protein 1
EGFP	Enhanced Green Fluorescent Protein
EGFR	The epidermal growth factor receptor
EGTA	Ethylene glycol-bis(β -aminoethyl ether)-N, N, N', N'- tetraacetic acid
ER	Endoplasmic reticulum
Fab 1	Yeast orthologue of PIKFYVE (a FYVE finger-containing phosphoinositide kinase)
FBS	Fetal bovine serum
FESEM	Field emission scanning electron microscopy
FH1/ FH2	Formin homology domain 1/2
FHOD	Formin homology domain proteins

FL	Full length
FMN	Formin protein
FMN1/2	Formin1/2
FMNL1	Formin Like 1
FRL	Formin-related proteins in leukocytes
FSI	Formin-SPIRE interaction site
FT	"Farb teiler", German for colour splitter (microscope filter feature)
FYVE/F	Fab1, YOTB, Vac1, and EEA1
G51	Glycine residue in position 51
G51L	Glycine residue in position 51 substituted with Leucine
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green Fluorescent Protein
GS	Griscelli syndrome
GS1/ GS2/ GS3	Griscelli syndrome type 1/ type 2/ type 3
GST	Glutathione S-transferase
GTBM/M	The myosin-V globular tail binding motif

GTD	Globular tail domain of myosin
GTP	Guanosine triphosphate
H/H2	SPIRE C-terminal flanking sequences similar to H2 of SHD-proteins
H73	Histidine in position 73
HCL	Hydrochloric acid
HEK293A	Human embryonic kidney 293A cells
HIV	The human immunodeficiency viruses
Hs	<i>Homo sapiens</i>
HSV	Herpes simplex virus
HVD	Hypervariable domain
I15/ I408	Isoleucine in the indicated position
I408A/ I552A	Isoleucine residue in the indicated position substituted with alanine
IAK	Isoleucine-alanine-lysine motif
IFB	Immunofluorescence buffer
ILVs	Intraluminal vesicles
INF	Inverted-formin
INF2	Inverted-formin 2

IP	Immunoprecipitation
IQ	Isoleucine-glutamine motif
ITRs	Left and right inverted terminal repeats
JNK	Jun N-terminal kinase
K336/ K416/ K419	Lysine residue in the indicated position
K419A	Lysine residue in the indicated position substituted with Alanine
K419W/ K563W	Lysine residue in the indicated position substituted with Tryptophan
KHC	Kinesin heavy chain
KIF	Kinesin superfamily proteins
kif5b	Kinesin-1 heavy chain
KIND	kinase non-catalytic C-lobe domain
KLC	Kinesin light chains
KMSFH	SPIRE construct comprised of KIND, GTBM, SB, FYVE and flanking H2
KW	SPIRE construct comprised of KIND and WH2
KWM	SPIRE construct comprised of KIND, WH2 and GTBM

L400A/ L412A/ L422A/ L423A	Leucine residue in the indicated position substituted with alanine
LALA	Double mutations in which two Leucine residues substituted with alanine
LB	Luria-Bertani medium
LKKT	Leucine-two Lysine-Threonine motif
LP	Long pass (microscope filter Emission)
LROs	Lysosome-related organelles
MADD	MAP Kinase Activating Death Domain
MAP	Microtubule-associated proteins
MAPT	The microtubule-associated protein tau
Mb	<i>Monosiga brevicollis</i>
mDia	Diaphanous-related formin-1
MEF	Mouse embryonic fibroblast cell line
melan-a	Wild-type melanocytes
melan-ash/ ashen melanocytes	Rab27a-null melanocytes
melan-f	Formin deficient melanocytes

melan-ln/	
leaden	Melanophilin-null melanocytes
melanocytes	
MFH	SPIRE construct comprised of GTBM, FYVE and flanking H2
MH	SPIRE construct comprised of GTBM and flanking H2
MHD1/2	Munc13 homology domains 1/2
MLPH	Melanophilin
Mm	<i>Mus musculus</i>
MS	SPIRE construct comprised of GTBM and SB
MSF	SPIRE construct comprised of GTBM, SB and FYVE
MSFH	SPIRE construct comprised of GTBM, SB, FYVE and flanking H2
MTs	Microtubules
MTOC	The microtubule organising centre
MYO	Myosin
MYO5A	Myosin-Va
MYRIP	Myosin VIIA And Rab Interacting Protein
NM	Nanometers
NOC2	No C2 domain
NPF	The nucleation promoting factor

NT	Non-targeting siRNA oligo
NT-SPIRE	NH2-terminus of SPIRE (KIND and WH2)
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEST	Proline, glutamic acid, serine, and threonine
PMA	Phorbol 12- myristate 13-acetate
PMEL/ PMEL17	Pre-melanosome protein
PRD	The proline-rich domain
PTM	Posttranslational modifications
PVDF	Polyvinylidene difluoride membrane
Q137	Glutamine residue in the indicated position
R17/ R196/ R35/ R525/ R537/ R570	Arginine residue in the indicated position
R35W	Arginine residue in the indicated position substituted with Tryptophan
R570A	Arginine residue in the indicated position substituted with alanine
RAB	Ras-related protein in brain

	Mutant Rab27a SF1 (Rab27a <u>YDYLIK</u> <u>F</u> to
Rab27a SF1/F4	Rab3a <u>F</u> DY <u>M</u> F <u>K</u> I) and F4 (Rab27a <u>F</u> FR <u>D</u> AM to Rab3a <u>Y</u> YR <u>G</u> AM)
RabF1-5	Rab superfamily conserved motif 1-5
RAS	Rat sarcomas causing proteins
RBD	Rab27a binding domains
RCF	The Relative centrifugal field
RFU	The relative fluorescence units
RILP	Rab Interacting Lysosomal Protein
RILPL2	Rab Interacting Lysosomal Protein Like 2
RNA	Ribonucleic acid
RNPs	Ribonucleoprotein particles
RPMI	Roswell Park Memorial Institute Medium
RT-PCR	Reverse transcription-polymerase chain reaction
S14/ S145	Serine residue in the indicated position
SB/ S	SPIRE-box domain
SD-1	Subdomain 1 of myosin
SDM	Site-directed Mutagenesis
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide Gel Electrophoresis

SF	SPIRE construct comprised of SB and FYVE
SF1-4	Rab subfamilies conserved sequences
SFH	SPIRE construct comprised of SB, FYVE and the flanking H2
SH	SPIRE construct comprised of SB and the flanking H2
SHD	synaptotagmin-like proteins (Slp) homology domain
SKIP	SifA and kinesin-interacting protein
SLP2	Synaptotagmin-like protein 2
SW	Rab switch regions
T11/ T575	Threonine residue in the indicated position
T401A	Threonine residue in the indicated position substituted with alanine
TARP	Transmembrane AMPA (α -amino-3-hydroxy-5-methyl-4-Isoxazolepropionic acid) receptor Regulatory Proteins
TBE	Tris-boric acid-EDTA buffer
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylene-diamine
TIRF	Total internal reflection fluorescence microscopy
TPR	Tetra-Trico peptide domain
TRP/ TYRP	Tyrosinase-related proteins

TYR	Tyrosinase
UV	Ultraviolet
UVR	Ultraviolet radiation
V18/ V21/ V395/ V411/ V426	Valine residue in the indicated position
V411A/ V555A	Valine residue in the indicated position substituted with alanine
Vac 1	Vesicle transport protein 1
WASP	The Wiskott-Aldrich syndrome proteins
WAVE	WASP-family verprolin-homologous protein
WD	Tryptophan-aspartic acid dipeptide-repeat-containing protein
WH2	Wiskott-Aldrich syndrome protein (WASP) homology two domain
WMSFH	SPIRE construct comprised of WH2, GTBM, SB, FYVE and the flanking H2
Y120K	Tyrosine residue in the indicated position substituted with Lysine
YOTB/ ZK632.12	The nematode orthologue of human PLEKHF2 (pleckstrin homology and FYVE domain containing 2)

Chapter 1: Introduction

1.1 Intracellular Transport

Comprising countless physiological processes, the living cell is an example of complete systems in nature. For such a cell to stay alive, numerous transport events have to be completed into, out of and within the cell. The cargos involved are vary – from small molecules to vesicles and macromolecules. The high density of soluble molecules in the cytosol means that diffusion is insufficient for carrying out transport inside the cell. Instead, active transportation is required for intracellular trafficking. Additionally, since eukaryotic cells are comprised of specialised organelles located in particular regions of the cell, a connection network covering the distance between these organelles is required to facilitate and organise their functions. To meet this requirement, a complex network of cytoskeleton filaments has evolved into a connection and transportation pathways inside the cell. Many molecules function as signalling that regulates the transport while many others function as adaptors or anchors for cargo. Some others serve as vehicles, e.g. molecular motors, which carry the intracellular cargo along the cytoskeleton filament, moving between locations in the cell and creating an elaborate system of intracellular transport (Appert-Rolland et al., 2015, McKay, 2004).

The intracellular trafficking system is considered a crucial element for asymmetric meiotic and mitotic cell division. It drives the movement of the meiotic spindle in the oocyte during polar body formation in mammals

(Pfender et al., 2011, Montaville et al., 2014, Bennabi et al., 2016, McNally, 2013). The intracellular transport system and its elements are utilised to organise the distribution of the organelles between the mitotic cells during cell division (Waterman-Storer et al., 1993). Also, it is a critical system for the autophagy process in starving cell, where some cytoplasmic proteins and organelles become encircled in a double membrane vesicle called an autophagosome. The autophagosomes fuse with the lysosome to be degraded and then the nutrients are released and reused (Xu et al., 2015). The intracellular trafficking system is vital for transporting anti-pathogens (i.e. antimicrobial peptides) as an immune response to an infection. It can also be manipulated by pathogens to facilitate cell invasion (Mostowy and Shenoy, 2015, Abrahams and Hensel, 2006). All this is in addition to the system's significant role in endocytosis (Doherty and McMahon, 2009), exocytosis (Aspengren et al., 2007, Jahn and Fasshauer, 2012), phagocytosis (Araki, 2006), and mRNA localisation (Besse and Ephrussi, 2008).

As it is vital for the living cell, a deficiency in any element of the intracellular trafficking system can lead to failure of the cellular process and subsequent malfunctions and diseases – including cancer and neuronal diseases and various syndromes – in multi-cellular organisms (Olikkonen and Ikonen, 2006). For instance, the hallmark pathology of Alzheimer's disease is the axonal swellings due to the accumulation of microtubule-associated proteins (MAPs), along with molecular motors, vesicles and organelles. These accumulations indicate the failure of intracellular transport, consequently leading to

neuronal death (Perlson et al., 2010, Brady and Morfini, 2017). Similarly, accumulation of the epidermal growth factor receptor (EGFR) affects the level of cell growth and its proliferation, eventually leading to the generation of cancerous cells (Tang and Ng, 2009). That is in addition to many hereditary genetic disorders and syndromes such as Hermansky-Pudlak, Griscelli, and Chediak-Higashi syndromes that have been associated with defects in gene-encoding transport proteins (Olkkonen and Ikonen, 2006).

Therefore, understanding intracellular transport and its elements is not only a necessary precondition for understanding the disease biomarkers and open venues for potential therapeutics but also a significant contributor to our understanding of the fundamental mechanisms of intracellular transport.

Intracellular Transport Elements:

1.2 Cytoskeleton Network

Vertebrate cells have three different types of protein-based filaments that are important for their structure and internal transport. Microtubules (MTs), intermediate filaments, and actin filaments (AFs) have been identified as forming the 'skeleton' of animal cells. The three types integrate to perform these functions. However, only microtubules and actin filaments can generate sufficient forces for cell shape changes and motility. They are more dynamic than intermediate filaments, and these dynamic properties have essential roles in the intracellular trafficking process. The polarised filaments not only spatially organise the organelles but also function as an intracellular 'road

network' for motor proteins (or cellular vehicles) via which cargo transport takes place between different locations in the cell in a directional manner (Appert-Rolland et al., 2015).

1.2.1 Microtubules (MTs)

Microtubules are highly dynamic cytoskeletal structures that are assembled from α and β tubulin (Kamath et al., 2010). The MT's subunits, which comprise α/β heterodimers, polymerise to assemble a protofilament (Figure 1.2.1.A). Twelve to fourteen of those protofilaments wrap into a helical cylinder of 25 nm diameter to form an MT (Figure 1.2.1.B). The asymmetry of the dimers provides two chemically distinguished ends of the filaments, which generate filaments with polarised orientation. In this case, the minus-end (α -tubulin exposed end) is clustered at the microtubule organising centre (MTOC), and the plus-end (β -tubulin exposed end) grows toward the cell periphery (Ikeda et al., 2010, Euteneuer and McIntosh, 1981). This polarity is a substantial feature for intracellular transport in that it determines the walking direction of motor proteins. Besides the polarity, Microtubule arrays are also characterised with a diverse chemical composition of tubulin subunits. This diversity is related to the expression of different α - and β - tubulin isotypes that undergo various posttranslational modifications (PTMs) (Roll-Mecak, 2019). Human cells expressed eight α -tubulin and nine β -tubulin isotypes (Fojo., 2008). Most MTs have combinations of multiple α and β tubulin isotypes (Fojo., 2008). However, some tubulin isotypes are restricted to specific cell types such as β III tubulin (encoded by *TUBB3*) that specifically

express in neurons (Burgoyne et al., 1988). This isotype expresses in non-neuronal cells only during tumorigenesis (Burgoyne et al., 1988, Katsetos et al., 2003). α and β tubulin also undergo a large number of chemically diverse PTMs (Yu et al., 2015). Tubulin may be subjected to abundant PTMs such as methylation, palmitoylation, and phosphorylation (Janke and Magiera, 2020, Yu et al., 2015). Tubulin also undergoes unique PTMs such as the enzymatic, ribosome-independent incorporation of tyrosine (tyrosination), glycine (glycylation and polyglycylation), and glutamate (glutamylolation and polyglutamylolation). Also, detyrosination was observed in α -tubulin in which single tyrosine is enzymatically removed from the C-terminus. Subsequent enzymatic removal of glutamate residues from the detyrosinated α -tubulin C-terminus generates $\Delta 2$ - or $\Delta 3$ -tubulin (Yu et al., 2015, Roll-Mecak, 2019, Janke and Magiera, 2020). The incorporation of the isotypes and PTMs generates tubulin molecular patterns termed 'tubulin code' that regulates intrinsic microtubule properties and their recognition by effectors. For instance, the depolymerizing motors of the kinesin-13 family require tyrosinated MTs as substrates; therefore, complete detyrosination of α -tubulin can stable and protect the MTs from kinesin-13-mediated disassembly (Peris et al., 2009).

Microtubules exhibit special dynamic activity, termed dynamic instability, which is characterised by a stochastic switching between persistent polymerisation and fast depolymerisation. The plus-end growth (polymerisation) occurs by adding a single heterodimer or small oligomers, while fast-shrinking occurs by detaching single protofilaments from the tube.

The alteration from shrinkage to growth is called the rescue, and the one from growth to shrinkage is called catastrophe (Figure 1.2.1.C).

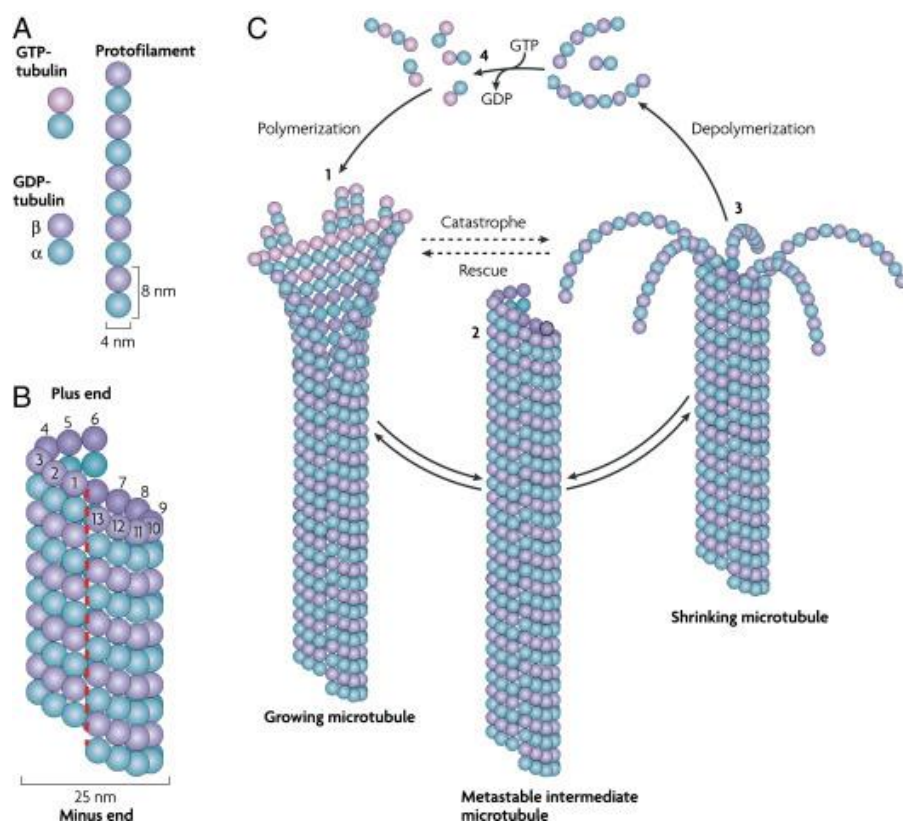


Figure 1.2.1 Microtubule structure and dynamic instability. (A) heterodimers of α/β -tubulin associated with GTP/GDP, assemble into 8 nm protofilaments. (B) About thirteen protofilaments assemble a 25 nm hollow cylinder of MT. (C) the polymerising/depolymerising of MTs (Appert-Rolland et al., 2015)

In vitro study has shown that the speed of MT depolymerisation is not affected by the tubulin concentration in solution, while the speed of polymerisation depends linearly on the tubulin concentration (Walker et al., 1988). However, both processes have relatively low speeds *in vivo* ($\sim 17 \mu\text{m}/\text{minute}$ for polymerisation and $\sim 35 \mu\text{m}/\text{min}$ for depolymerisation) (Brugués et al., 2012, Komarova et al., 2002b). Such that, MTs have persistent polymerisation, and depolymerisation start only when the filaments reach the plasma membrane. Therefore, the contact between MT and the cell

membrane significantly affects the MT dynamics (Komarova et al., 2002b, Ebbinghaus and Santen, 2011).

The plus-end is much more dynamic than the minus-end in MT. This is because the minus end is often attached to the MTOC, which inhibit any dynamic activity of the minus-end. Even if not anchored, the minus-end mostly exhibits depolymerising, while the plus-end exhibits polymerising activity (Appert-Rolland et al., 2015).

A special dynamic activity of MTs, termed treadmilling (Komarova et al., 2002b), was observed in some *in vitro* studies, where the filaments are not attached. In this case, one end of the filament polymerises while the other depolymerises. A treadmilling filament would move in one direction as a whole filament, while the individual subunit is immobile. Treadmilling was also observed in fish melanophore fragments (Rodionov and Borisy, 1997), Chinese hamster ovary (CHO) and normal rat kidney epithelial cells (NRK) (Komarova et al., 2002b), where MTs are not attached *in vivo*.

The GTP (guanosine triphosphate) and GDP (guanosine diphosphate) have a crucial role in the dynamic instability of MTs. During the polymerisation, the α - and β -tubulin bound to free tubulin dimers to assemble a protofilament. After that, the GTP attached to the β -tubulin hydrolyses to GDP (David-Pfeuty et al., 1977) which make most β -tubulins along the filament associate with a GDP with smaller 'islands' of GTP (Dimitrov et al., 2008). The GTP hydrolysis of these 'islands' occurs on different time scales, depending on the position of the associated tubulin subunit – if it is exposed at the plus-end or submerged

inside the MT. Meanwhile, the growing plus-end carries GTP (Mitchison and Kirschner, 1984). The GDP-associated tubulins depolymerise about 100 times faster than GTP subunits (Klemin, 2008). Whenever the GTP cap disappears, rapid depolymerisation events occur along the MT, causing the catastrophe as mentioned above. This catastrophe stops on reaching an island of GTP (a GTP remnant inside the MT), inducing rescue and a new period of growth (Dimitrov et al., 2008).

Apart from the role of the GTP/GDP, the dynamic instability of MTs is also maintained by the interaction of a wide range of proteins with the MT; these are classed in the large family of microtubule-associated proteins (MAPs). MAPs have a broad structure and function and in their interaction with MTs. Some MAPs modify the dynamics of MTs (Schlager and Hoogenraad, 2009). Others depend on MT to carry out their function without any significant effects on the filament. A highly conserved subgroup of MAPs, referred to as +TIPs, are able to bind to the plus-ends of MTs and significantly increase the rescue and reduce the rates of catastrophes (Komarova et al., 2002a).

Consequently, both the GTP-cap and the +TIPs have a qualitatively similar role in the microtubule dynamics and function. Both bind the growing plus-ends and reduce the depolymerisation, thereby positively influencing the average lifetime of MTs (Komarova et al., 2002a). The binding and stabilising effects of both GTP and +TIPs are temporary in that the stabilising function of GTP is abolished by GTP hydrolysis, and the +TIPs unbind from the filament at a given rate. Therefore, the capped MTs are stabilised by and significantly

grows, while unstable MTs grow slowly or pause at the cell membrane (Appert-Rolland et al., 2015).

The dynamical instability of MTs occurs in the existing microtubules. However, nucleation of new microtubules, after complete depolymerisation, occurs on time scales larger than that of the subsequent polymerisation (Kirschner, 1978). Therefore, nucleation is the limiting step in the formation of new microtubules. However, it is a process that helps the cells control the time and position of the MT growth. In many eukaryotic cells, the minus-ends of MTs are attached to the MTOC (Wu and Akhmanova, 2017). Whereas, 13 subunits of the γ -tubulin have been presented to form a γ -tubulin ring complex (γ TuRC), which is highly involved in MT nucleation (Oakley and Oakley, 1989, Oakley, 1992, Zheng et al., 1995). Hundreds of γ TuRC are found on the MTOC *in vivo*, where they cap the minus-end of nucleated MTs to prevent the addition or loss of tubulin dimers at this end. The rings are not completely closed but exhibit helical pitch, which is recovered in the MT structure (Zheng et al., 1995, Moritz et al., 1995).

MTs are vital to the cell cycle, cell motility, the spatial organisation of the cytoplasm, and vesicular transport (Ikeda et al., 2010). For decades, MTs were considered as the only intracellular 'highway' for intracellular bi-directional movements. They are utilised as tracks for mitochondria motility from the cell body to the axonal outgrowth in the neurons. Herpes simplex virus (HSV) and adenoviruses, are transported bi-directionally between the nucleus and cell membrane along the MTs. Phagosomes, secretory vesicles, and

ribonucleoprotein granules have been reported to be transported along MTs as well. The immuno-localisation and biochemical purification of these intracellular cargoes showed binding with MTs' plus- and minus-end motors, which suggests that intracellular cargos employ MT-dependent motors to move bi-directionally (Welte, 2004). However, recent studies indicate the existence of MT-independent long-range transport and suggest actin filaments as an intracellular long-range road (Hume et al., 2001, Evans et al., 2014, Schuh, 2011).

1.2.2 Actin Filaments

The microfilament or actin filament (AF) is structured of two twisted protofilaments with a diameter of only 5–10 nm, which gives AFs more flexibility compared to MTs (Klemin, 2008). However, AFs form highly interconnected networks which as a whole have strong mechanical properties and play critical roles in many cellular processes. These include the maintaining and changing cell shape, cell division, motility and migration. It is also vital for intracellular transport, endo- and exocytosis, and for remodelling the chromatin recovery of damaged DNA (Hume et al., 2001, Hume et al., 2002, Hume et al., 2007, Jahn and Fasshauer, 2012, Belin et al., 2015, Perrin and Ervasti, 2010).

Similar to MTs, actin filaments are polar polymers that have a fast-growing plus- (or barbed-) end and a rapidly disassembled minus- (or pointed-) end, if not stabilised. Some isoforms of AFs have dynamical structures with mechanisms similar to MTs. ATP/ADP nucleotides bind AFs and play a similar

role as the GTP/GDP in the MTs. AFs naturally undergo treadmilling both *in vitro* and *in vivo*. The major difference between MTs and AFs is the time scales of polymerisation-depolymerisation events, which is significantly shorter in case of AFs. This facilitates tracking AF dynamics on microscopic time scales to observe growth and shortening rates (Appert-Rolland et al., 2015).

1.2.2.1 Structure of Actin Monomer

Structurally, an actin filament (AF) is a 5-10 nm a wide polymer assembled from 42-kDa globular-actin (G-actin) subunits (Holmes et al., 1990, Kabsch et al., 1990, Klemin, 2008). G-actin exists in three main isoforms – α , β , and γ – which in turn generate different isoforms of AFs, each of which has a specialised function. Six AF isoforms are conserved in mammalian cells: α -cardiac-actin, α -skeletal-actin, α -smooth-actin and γ -smooth-actin are expressed mainly in cardiac, skeletal, and smooth muscles. They synergise with muscle myosin II to form contractile fibres named actomyosin which resemble small-scale versions of muscle fibres but are also present in non-muscle cells as stress fibres and adhesion belts. They play a major role in cell motility, shape-changing and cytokinesis (Pollard, 1990). While β -cytoplasmic-actin and γ -cytoplasmic-actin are widely expressed function and involved in constructing dynamic actin mesh that vital for a various cellular process (Perrin and Ervasti, 2010). The isoforms share very similar amino acid sequences, with no less than 93% identity between them (Chaponnier and Gabbiani, 2016, Perrin and Ervasti, 2010). The isoforms also undergo posttranslational modifications (PTM). More than 140 PTMs have been

described in actin sequences to date and contribute to the actin folding, localisation, and binding to actin-binding protein (Terman and Kashina, 2013, Varland et al., 2019). For instance, methionine and cysteine residues in the N-terminal of skeletal muscle α -actin are acetylated and cleaved which were found to strengthen the interaction with myosin (actin-based motor) (Abe et al., 2000), the residue Histidine in position 73 (H73) is methylated, and the resulting N-terminal aspartic acid is then reacylated (Dominguez and Holmes, 2011). In mouse embryonic fibroblast (MEF) cells, N-terminally arginylated β -actin is concentrated at the leading edge of lamellipodia. This arginylation is required for normal lamellipodia extension and active migration (Pavlyk et al., 2018). The non-Nt-arginylated actin forms filamentous aggregates *in vitro* and results in impaired lamella formation and cell migration *in vivo* (Karakozova et al., 2006).

Structurally, actin is related to a structural superfamily with sugar kinases, hexokinases, the 70 kilodalton heat shock proteins (Hsp70) (Bork et al., 1992), the actin-related proteins (Arp) (Robinson et al., 2001) and two prokaryotic actin-like proteins MreB (Yeung and Paterson, 2002) and ParM (van den Ent et al., 2002). These proteins share the 375-amino-acid-polypeptide chain of actin that folds into two major α/β -domains – also called the outer and inner domains depending on their location within the actin filament or small and large domains depending on their sizes in electron microscopy images. The major domains are classified into four subdomains. Subdomains 1 and 3 are structurally related, while subdomains 2 and 4 appear as large insertions into

subdomains 1 and 3, respectively (Holmes et al., 2003). Structural studies have revealed contact between the two major domains of actin, where the polypeptide chain passes twice between them. The loop is centred at residue K336 and at the linker helix Q137-S145, which serves as an axis of a hinge between the domains. This structure forms two clefts between the domains (Figure 1.2.2.A). The upper cleft binds the ATP/ADP nucleotides and associated divalent cation (Mg^{2+}), the lower cleft, also called the hydrophobic cleft and the target binding, is located between subdomains 1 and 3 and lined by 11 hydrophobic residues. This cleft mediates important interactions between actin subunits along the filament and constitutes the major binding site for most actin-binding proteins. The contact between the two clefts provides the structural basis for demonstrating the role of changing the nucleotide-dependent conformation on the binding affinities of actin-binding proteins and the strength of inter-subunit contacts in the filament (Dominguez, 2004, Fujii et al., 2010, Oda et al., 2009, Dominguez and Holmes, 2011).

Globular actin is not an effective ATPase, while filamentous actin is. The ATP- and ADP-bound states of G-actin conformation exhibit minor differences involving mainly two loops (Figure 1.2.2.A): the β -hairpin loop that carries residue S14 in subdomain one and is equivalent to the loop containing D157 in subdomain three and the sensor loop carrying the methylated H73.

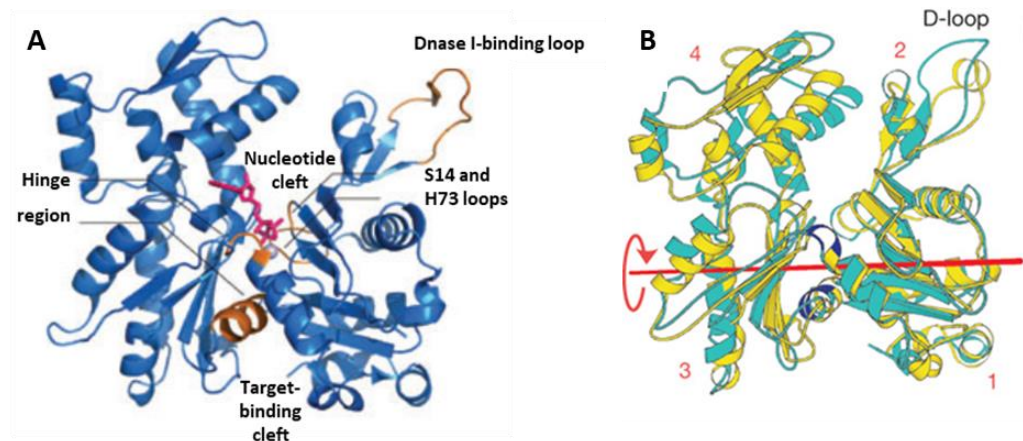


Figure 1.2.2 Monomer actin structure. A) Globular actin structure shows the two clefts and the main loops (Dominguez and Holmes, 2011). B) The vertical propeller rotation of the major domains of globular actin (Oda et al., 2009).

Together, the two loops engulf the phosphates of the nucleotide. Nucleotide-dependent conformational changes begin with S14, which makes a hydrogen-bonding contact with the γ -phosphate in ATP (ATP state). After hydrolysis and the γ -phosphate release, S14 change orientation to form a hydrogen-bonding contact with the β -phosphate of the ADP (ADP state). In the ATP state, S14 also forms a hydrogen-bonding contact with the main chain of the H73 loop. After the rotation of the side chain of S14, in the ADP state, the H73 loop is oriented toward the nucleotide to occupy some of the empty space of the released γ -phosphate. This is because it appears to sense the state of the nucleotide – called sensor loop (Graceffa and Dominguez, 2003, Dominguez and Holmes, 2011).

The sensor loop marks the C-terminal end of subdomain 2. The top of this subdomain contains residues 39–51, which are referred to as the DNase-I-binding loop (D-loop) that mediates the interactions with DNase I (Kabsch et al., 1990). In crystal structures, this loop appears in a variety of

conformations, including, in one case, as β -pleated sheets (Kabsch et al., 1990) and on the other, a short α -helix (Otterbein et al., 2001). Subdomain 2, and the D-loop specifically, plays a critical role in longitudinal contacts of the filament subunit. Therefore, changes in the S14 and sensor loop influence the conformation changing of subdomain two between nucleotide states (Graceffa and Dominguez, 2003). Although the nucleotide-dependent conformational changes are minor, they cause instability of the actin filament in the ADP state. This is because the interaction interface is located between the major domains, releasing γ -phosphate and weakening the linkage between them. Consequently, the domains rotate more freely with respect to one another in two different ways: a scissor-like opening and closing of the nucleotide-binding cleft (Figure 1.2.2.A) (Dominguez and Holmes, 2011) and a vertical propeller rotation of one domain with respect to the other (Figure 1.2.2.B) (Galkin et al., 2002, Oda et al., 2009, Tirion et al., 1995).

The residue Q137, at the beginning of the aforementioned 'hinge helix', plays a critical role in nucleotide hydrolysis (Iwasa et al., 2008), as the domain rotation appears to bring the side chain of Q137 in contact with the γ -phosphate of the nucleotide, thereby stabilising the actin (Oda et al., 2009, Fujii et al., 2010). Similar to the MTs, nucleotide hydrolysis by actin is one of the main factors regulating the transition of G-actin to F-actin (polymerisation/depolymerisation). In this case, the ATP-associated G-actin (ATP state) binds to the growing plus-end of F-actin. In contrast, the monomer in the ADP state disassociates from the minus-end of the filament, resulting in

the treadmilling as mentioned above activity (Wegner and Isenberg, 1983). However, a broad family of various actin-binding proteins are involved in the regulation of actin cytoskeleton dynamics. They serve distinct functions, including actin monomer sequestration, filament severing, and cross-linking and barbed- or pointed-end capping (Pollard and Borisy, 2003). The G-actin-binding proteins belong to different structural families and serve different functions; they also present different nucleotide preferences. However, most of them bind to the actin by inserting an amphiphilic helix into the target-binding cleft of the actin (Pollard and Borisy, 2003). The overall position of the interacting helices is well conserved in these proteins, but the orientation of their polypeptide chains is not. According to the classical view of actin, the directionality of the helices of the formin homology 2 (FH2) domain and the WASP-homology 2 domain (WH2) is from back to front, whereas the helices of the vitamin D-binding protein (DBP) and ADF/cofilin bind in the opposite direction (Dominguez and Holmes, 2011).

1.2.2.2 The *De Novo* Polymerization of actin

Non-muscle cells contain a high concentration of monomer actin ($\leq 100 \mu\text{M}$ in some cells) (Bray and Thomas, 1976, Gordon et al., 1976) mostly bound to ATP and Mg^{2+} (Rosenblatt et al., 1995). This large pool of Mg-ATP-actin monomers has a critical concentration for polymerisation (K_d), of $0.1 \mu\text{M}$ at the barbed end and $0.6 \mu\text{M}$ at the pointed end. At $100 \mu\text{M}$, pure actin, under physiological salt conditions, polymerises in a few seconds, leaving only $0.1 \mu\text{M}$ free monomers. The initiation of a new filament termed nucleation

comprises two steps: first assembly of actin dimers and trimers that are considered seeds (or filament nuclei). Provided Mg-ATP-actin exceeds the critical concentration. This is followed by polymerisation of the barbed end resulting in the filament elongation. The spontaneous nucleation from actin monomers alone is unfavourable because of the instability of actin seed. Kinetic and molecular dynamic simulations have estimated the dissociation equilibrium constant (K_d) for an actin dimer as high as 4–5M (Sept and McCammon, 2001). The lifetimes of dimers and trimers are remarkably short, in the sub-millisecond range, which interfered polymerisation time courses. Quick polymerisation of the barbed ends associated with quick depolymerisation at pointed ends of the actin filaments results in treadmilling dynamic activity (Appert-Rolland et al., 2015). However, spontaneous actin seeds were formed slowly from pure actin, under physiological salt conditions, in a high monomer concentration reaction (Pollard and Cooper, 1986, Pollard et al., 2000).

After nucleation and elongation, actin monomers assemble two chains of actin filament that twist around each other to form a right-handed two-chained helix (Hanson and Lowy, 1963, Dominguez and Holmes, 2011). However, the free single-chain appears as a left-handed helix comprising 13 G-actin subunits that are repeated every six turns at an axial distance of 35.9 nm. The 180° twist per actin subunit in the two chains gives the structure the appearance of a right-handed turn (Figure 1.2.3).

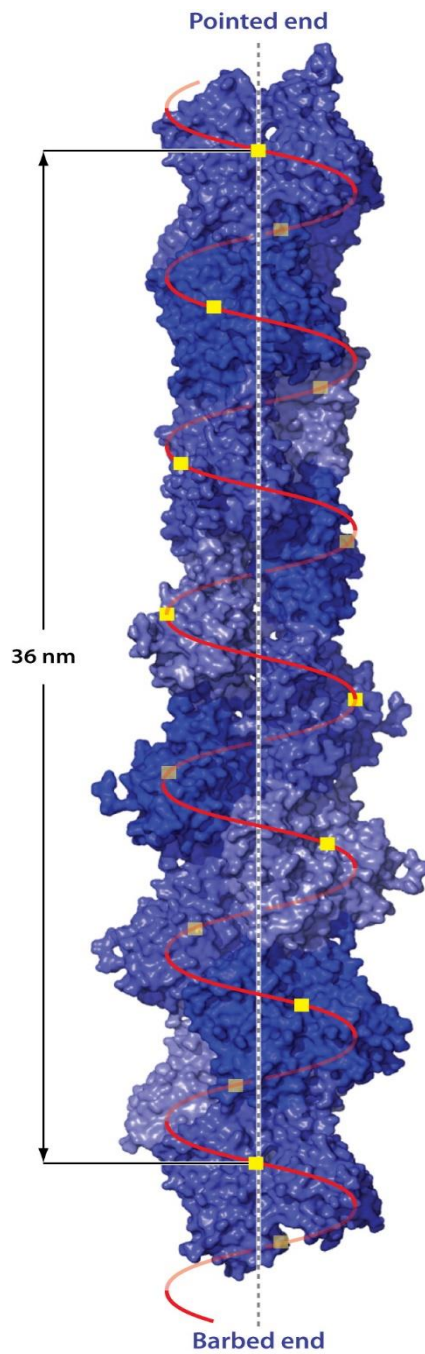


Figure 1.2.3 The helical structure of actin filaments derived from cryo-electron microscopy. The single helix has 13 G-actin subunits repeating in six left-handed turns. The rise is 2.76 nm, and the twist is -166.6 ± 0 per single subunit, that is close to 180° . Therefore, the structure takes on the appearance of a two-strand right-handed helix (Dominguez and Holmes, 2011).

The substantial kinetic barrier to spontaneous nucleation indicates the criticality of the nucleation step in regulating actin polymerisation in cells.

Therefore, the cells evolve various proteins that bind G- and F- actin to

regulate the filament nucleation and polymerisation and to facilitate capping the filament ends to prevent adding or losing monomers (Pollard et al., 2000).

1.2.2.3 Actin Binding Proteins

As one of the most highly abundant and conserved proteins in eukaryotic cells, actin has many interaction partners. They serve different functions. For instance, actin nucleator facilitates filament assembly from monomers. Other groups are involved in the regulation of the actin cytoskeleton. They interact with F-actin, G-actin, or both and serve different functions. Some, for instance, thymosin β 4, inhibits the assembly of the actin filament. Others execute or inhibit nucleotide replacement – for example, profilin can convert the ADP-actin into the ATP-actin, and cofilin acts oppositely. Other groups function as plus- and minus ends cap to stabilise the filaments. Finally, the myosin family – the prototype of a molecular motor – move along actin filaments, mostly towards the barbed end (Winder and Ayscough, 2005).

1.2.2.3.1 Monomer-Binding Proteins

Thymosin- β 4 and profilin are the primary actin monomer-binding proteins in vertebrate cells. Thymosin- β 4 is a small peptide of 43 aa (Safer and Nachmias, 1994), and profilin is a small globular protein of 125–139 aa. Both have overlapping binding sites on actin and compete for binding to it (Safer et al., 1997, Schutt et al., 1993). Thymosin- β 4 binds to ATP-actin (K_d , 1.2–1.6 μ M) and prevents its polymerisation. However, profilin competes with thymosin- β 4 for binding to ATP-actin and promotes its association with filament barbed

end. The concentrations of the binding proteins and the affinities influence division of the ATP-actin pool between profilin and thymosin- β 4 (Pollard and Cooper, 1984, Vinson et al., 1998, Kang et al., 1999, Carlier et al., 1993, Pantaloni and Carlier, 1993). Although profilin-Mg-ATP-actin complexes mediate the barbed end elongation at a similar rate to free actin, the complexes cannot form actin seeds or elongate the pointed end (Pantaloni and Carlier, 1993, Pollard and Cooper, 1984). This similarity between profilin-actin complexes and free actin monomers in the elongation of barbed ends results in a decrease in the concentration of free actin that is required for the barbed end growth (Kang et al., 1999, Pantaloni and Carlier, 1993).

To maintain the concentration of the Mg-ATP-actin pool in the cells, capping proteins bind to the barbed end of the actin filament ($K_d = 0.1$ nM) to prevent the addition of actin monomers. The gelsolin family of actin-capping proteins, includes adseverin, villin, capG, advillin, and supervillin. They are regulated by Ca^{2+} , phosphoinositides, and tyrosine phosphorylation (Silacci et al., 2004). The elongation, in the presence of free barbed ends, rapidly depletes the actin monomers pool in a few seconds. Therefore, capping the barbed end reduces the concentration of the free ends and balances the G-actin level. Gelsolin can be removed from the barbed end (uncapping the end) by interacting with polyphosphoinositides (e.g. Phosphatidylinositol 4,5-bisphosphate $PI_{(4,5)}P_2$). This results in free barbed ends that can be elongated (Janmey, 1994, Schafer et al., 1996, Hartwig et al., 1995).

In contrast to profilin, the cofilin family binds Mg-ADP-actin with a higher affinity than ATP-actin. ADF/cofilin is involved in actin turnover mainly by filament depolymerisation. Members of this family include actin-depolymerising factor (ADF), cofilin-1 (in non-muscle cells), cofilin-2 (in muscle cells), and twinfilin, which contains two ADF domains. The rapid depolymerisation of the pointed end by ADF/cofilin proteins is related to the severing of actin filaments, creation of more ends, and the increasing of the rate of subunit dissociation from the pointed ends (Maciver, 1998). The hydrolysis of ATP bound to actin filament subunits and the release of the γ -phosphate marks filaments for depolymerisation by ADF/cofilin proteins (Maciver et al., 1991). The ATP rapidly exchanges with ADP on the terminal subunits at the barbed ends of filaments, which contributes to the stability of barbed ends where no nucleotide exchange is observed on pointed ends (Teubner and Wegner, 1998).

1.2.2.3.2 Actin-Binding Proteins Involved in *De Novo* Actin Polymerisation

A group of actin-binding proteins includes actin filament nucleation and elongation factors that regulate the *de novo* formation of actin filaments. Thus, they are considered one of the most vital groups on cytoskeleton structure (Campellone and Welch, 2010, Dominguez, 2010, Pollard, 2007). This group includes three different types of polymerisation machinery: Arp2/3 complex, formins, and a family of nucleation-promoting factors (NPFs) including SPIRE, WASP, WAVE, Cobl, VopL/VopF, TARP and Lmod that regulate polymerisation time and location and influence the structures of formed actin

networks. NPFs such as SPIRE use the Wiskott-Aldrich syndrome protein (WASP) homology two domain (WH2) for interaction with G-actin. Formins are exceptional among actin filament nucleators, as they interact with actin via the FH2 domain and promote not only nucleation but also processive plus-end elongation (Amann and Pollard, 2001, Goode and Eck, 2007).

1.2.2.3.2.1 THE ACTIN RELATED PROTEIN (ARP2/3) COMPLEX

ARPs were the first identified actin nucleation factors. The 220kDa ARP2/3 complex is composed of seven conserved stably-associated polypeptides; two of the subunits are related to ARP2 and ARP3 subfamilies (the complex named after them). The other five subunits are termed actin-related protein complex 1-5 (ARPC1-5) (Figure 1.2.4 A, B, and C). Only ARPC1 is a tryptophan-aspartic acid (WD) dipeptide-repeat-containing protein, while the other four ARPCs have no common sequence motifs (Lees-Miller et al., 1992, Winter et al., 1997, Goley and Welch, 2006, Winder, 2003).

The complex drives filament branching and branch-pointed-end capping (Rotty et al., 2013). ARP2/3 complex binds to pre-existing actin filaments and nucleates daughter filaments on 70° to the mother filament resulting in a Y-shaped branch structure (Figure 1.2.4 D) (Campellone and Welch, 2010). ARP2/3 complex is considered essential for the formation of the branching actin filament, yet it is insufficient to nucleate actin seed. Its function needs to be stimulated by the nucleation promoting factor (NPF) proteins that facilitate binding the complex to the actin seed to nucleate the branch (Campellone and Welch, 2010).

Multiple NPFs can activate ARP2/3 complex to nucleate actin polymerisation and to organise actin networks. These include, for example, the evolutionarily conserved Wiskott-Aldrich syndrome protein (WASP) (Yarar et al., 1999, Winter et al., 1999) and N-WASP (Rohatgi et al., 2001). NPFs are diverse in their structure and functions, but they share some common characteristics. All NPFs have conserved an Arp2/3-binding sequence called a CA region comprise of connector region (or C) and acidic amino acids region (or A). This sequence binds four subunits within the Arp2/3 complex (Arp2, Arp3, ARPC1, and ARPC3) (Zalevsky et al., 2001b, Zalevsky et al., 2001a). To activate the Arp2/3 complex, NPF forms a ternary complex with Arp2/3 complex and G-actin (or F-actin for some NPFs). Within this structure, the CA region of the NPF causes an activating conformational change in ARP2/3 complex (Zalevsky et al., 2001a) and G-actin that bind to WH2 domain, facilitating the formation of an actin seed. Further interaction between the ternary complex and a pre-existing “mother filament” results in full activation of the Arp2/3 complex (Goley and Welch, 2006, Machesky et al., 1999). The ternary complex interact with the side of “mother filament” and pointed ends of “daughter filaments” (Mullins et al., 1998), via the F-actin-binding activity of the Arp2/3 complex, then the complex starts to assemble a daughter filament (Figure 1.2.4) (Gournier et al., 2001, Mullins et al., 1998, Volkman et al., 2001).

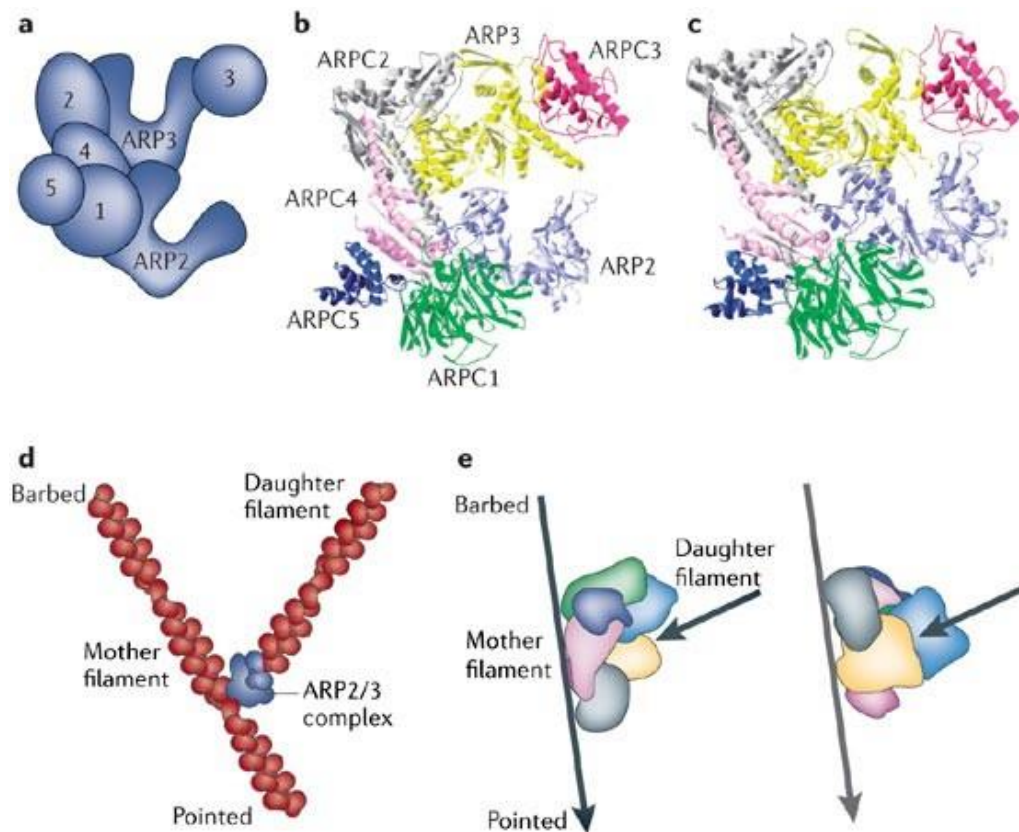


Figure 1.2.4. Actin nucleation by actin-related protein-2/3 (ARP2/3) complex. a) schematic representation of the subunit structure of the inactive ARP2/3 complex. b and c) Ribbon diagram of the crystal structure of the inactive and active conformation, respectively, of ARP2/3 complex with subunits labelled and presented in different colours. d) schematic representation of actin nucleation by the ARP2/3 complex. The complex interacts with the mother filament and the daughter filament in a γ -branch at a $\sim 70^\circ$ angle. e) A representation of the two models of the complex orientation at a γ -branch junction. Subunits are coloured as in part b. Both models propose that ARP2 and ARP3 associate with the pointed end of the daughter filament, and other subunits mediate contacts with the mother filament, from (Goley and Welch, 2006).

Some actin-binding proteins such as tropomyosin inhibit filament branching by binding the sides of actin filaments and blocking Arp2/3 complex binding sites (Blanchoin et al., 2001). Other binding proteins, such as ADF/cofilin, facilitate debranching by ATP hydrolysis and γ -phosphate release in the daughter filament, which triggers branch disassembly, followed by the complex disassociation from the pointed end (Blanchoin et al., 2000).

The branched actin mesh that is nucleated by the ARP2/3 complex has a critical role in cellular and developmental functions. Losing the ARP2/3 complex affects cortical actin cytoskeletal organisation, endocytosis, and phagocytosis (May et al., 2000, Bailly et al., 2001, Campellone and Welch, 2010, Welch and Mullins, 2002). It also affects *Drosophila* embryonic nervous system and eye development (Zallen et al., 2002).

1.2.2.3.2.2 FORMIN

cappuccino (*Drosophila* formin) are first identified for maternal effects on both the dorsal-ventral and anterior-posterior axes of the egg and embryo of *Drosophila* (Manseau and Schupbach, 1989). The mammalian Formin was first described as a protein product of the limb deformity gene in mice, which suggested its role in the formation of several organ systems (Mass et al., 1990, Woychik et al., 1990). After this discovery, a diverse family of formin proteins were discovered and considered as actin nucleation and elongation factors. The members of this family are relatively large multi-domain proteins (120–220 kDa). They are defined by the presence of a catalytic formin homology domains. However, the diversity in the flanking regions between different formins provides them with the ability to interact with different partner and perform various cellular functions and regulation mechanisms (Schönichen and Geyer, 2010, Heimsath and Higgs, 2012, Harris et al., 2004, Favaro et al., 2003, Goode and Eck, 2007).

Mammals have 15 formin genes classified based on FH2 sequencing into seven different subclasses: formin (FMN), inverted-formin (INF), formin-

homology domain proteins (FHOD), formin-related proteins in leukocytes (FRL), Diaphanous (Dia), Dishevelled Associated Activators of Morphogenesis (DAAM), and Delphinin (Higgs and Peterson, 2005, Chesarone et al., 2010). The role of formins in regulating MTs and AFs dynamics results in the assembly of the elaborate cytoskeletal structures that are essential for a range of cellular functions, including cell adhesion, division and motility—also causing different diseases including cancer (Zhu et al., 2008, Favaro et al., 2003, Lizárraga et al., 2009, Haga and Ridley, 2016) and immune diseases (Higgs and Peterson, 2005, Eisenmann et al., 2007, Colucci-Guyon et al., 2005).

Formins assemble linear (unbranched) actin filaments that are present in the filopodia, stress fibres, and the cytokinetic ring (Goode and Eck, 2007). The FH1 domain located toward the middle of the proteins is predicted to be rope-like, based on a lack of secondary structure. It has a profilin-binding pro-rich region that binds profilin–actin complexes. Profilin is a ubiquitous G-actin binding protein. Profilin–actin complexes are the primary supplier for actin assembly *in vivo* (Kaiser et al., 1999, Kursula et al., 2008). Therefore, the interactions between profilin and the FH1 domain are crucial for the recruitment of G-actin to the nucleated filament and accelerating the assembly (Kovar et al., 2006, Romero et al., 2004). At the C-terminus to FH1 is the actin-binding FH2 domain. It is essential for the dimerisation of formin and forming a head-to-tail doughnut-shaped dimer that encircles the barbed end of the filament (Figure 1.2.5 A, B) (Chesarone et al., 2010, Higgs and Peterson, 2005). FH2 can bind both G- and F-actin (Pruyne et al., 2002, Pring et al.,

2003a, Zigmond, 2004). Therefore, it mediates the nucleation and elongation of the two actin strands. FH2 also mediates assembly of linear F-actin by remaining bound to the plus-end of the filament and preventing branch formation (Romero et al., 2004, Zigmond et al., 2003, Higashida et al., 2004). In some formins (Bnr1p, AFH1, FMNL1, and mDia2), FH2 has F-actin bundling activity (Harris et al., 2006).

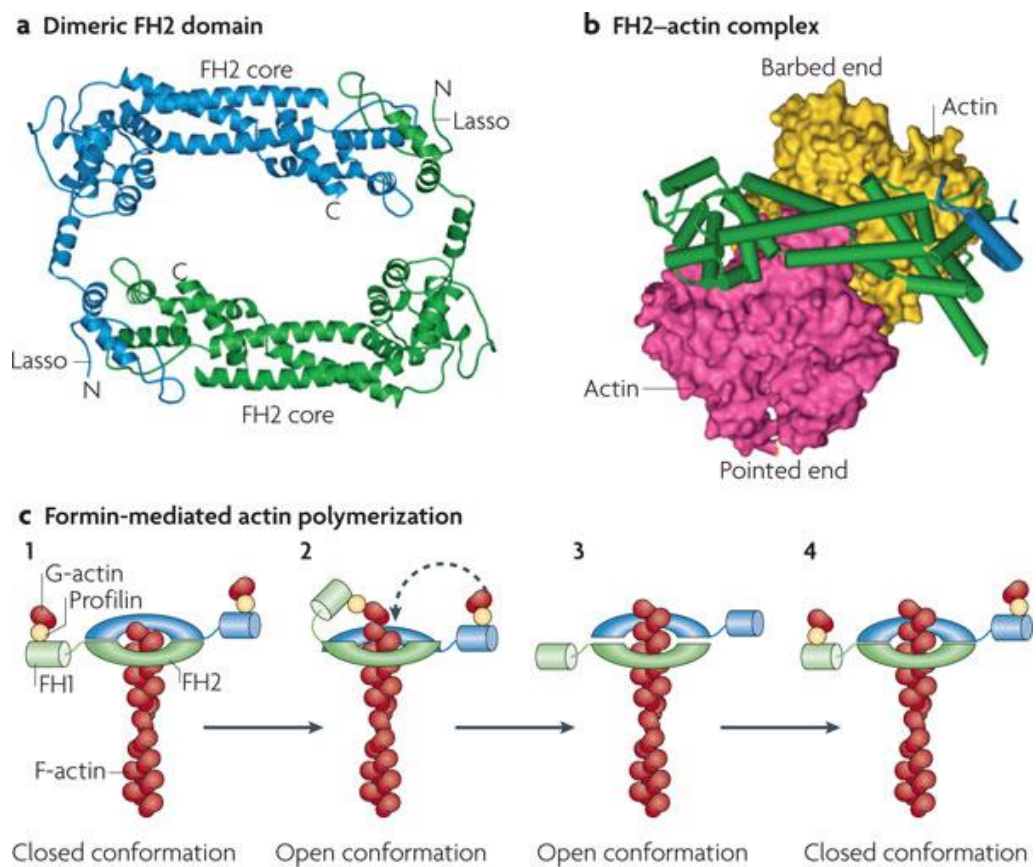


Figure 1.2.5. Actin nucleation by formin proteins. a) a ribbon diagram of the dimeric formin homology 2 (FH2) domain. b) | The FH2 domain encircles actin filament. c) A schematic representation actin polymerisation by formin-mediated, from (Campellone and Welch, 2010).

FH2 cannot recruit multiple monomers to form a stable trimer or tetramer G-actin seed for polymerisation (Chesarone et al., 2010). Therefore, it has been hypothesised that the FH2 recruit and stabilises the spontaneously formed

actin dimers and trimers to accelerate filament polymerisation (Chesarone and Goode, 2009, Pring et al., 2003b). This could explain the sufficient polymerisation function of FH2 when using purified actin monomers *in vitro* (Sagot et al., 2002). However, actin monomers *in vivo* bind profilin and thymosins, which inhibits spontaneous dimer and trimer formation. Only specific events in living cells, such as rapid filament depolymerisation, may increase the free G-actin concentration to stimulate formin-mediated nucleation (Higashida et al., 2008). Nevertheless, some formins, including INF2, FMNL3, mDia1, DAAM1, Bni1, and Bnr1, contain WH2 or WH2-like domains near their C-terminal which facilitate the formation of actin monomer seeds for the polymerisation (Chhabra and Higgs, 2006, Heimsath and Higgs, 2012, Gould et al., 2011, Dominguez, 2016). Other formins have nucleation cofactors, termed Nucleation-Promoting Factors (NPFs). Well-characterised examples are the synergistic interaction between the mDia1 with adenomatous polyposis coli (APC) and between FMN-type formin and SPIRE1/2 (Breitsprecher et al., 2012, Quinlan et al., 2007, Pechlivanis et al., 2009).

The C-terminal flanking region of FMN-type formin features a conserved sequence of 29 amino acids unique for the FMN subgroup. This sequence (named the formin-SPIRE interaction (FSI) site) provides the main binding site for the SPIRE-KIND domain (kinase non-catalytic C-lobe domain)(Quinlan et al., 2007). Together, FMN1/2 and SPIRE1/2 nucleate dynamic actin that is essential for different cellular processes.

1.2.2.3.2.3 SPIREs

SPIRE proteins are highly conserved in metazoans (Kollmar et al., 2019).

Vertebrate genomes encode two SPIRE genes, SPIRE1 and SPIRE2, that have high sequence similarity between them, especially evolutionarily conserved domains (37% identity, 50% similarity for the mouse proteins at the amino acids level) (Kollmar et al., 2019, Ciccarelli et al., 2003, Schumacher et al., 2004). Three alternatively spliced isoforms of SPIRE1 share the core functional domains but distinct regarding highly variable encoding of exons toward the middle of the protein. SPIRE1, SPIRE1 with exon 9 (E9), and SPIRE1 with exon13 (E13) (also termed SPIRE1C (Manor et al., 2015)), along with single SPIRE2 results in four SPIRE proteins in mammals (Figure 1.2.6) (Kollmar et al., 2019). The function of the SPIRE1 exon 9 is unknown, yet it is identically co-localised with SPIRE1 on transported vesicles, while SPIRE exon 13 is co-localised in the mitochondrial outer membrane (Kollmar et al., 2019, Manor et al., 2015), where it nucleates actin filaments that provide the force for mitochondrion constriction and fission (Manor et al., 2015). SPIRE1-E9 and E13 are only expressed at relatively low amounts in the brain and small intestine tissues, never exceeding 9% for E9 and 5% for E13 of the total SPIRE1 expression (Kollmar et al., 2019).

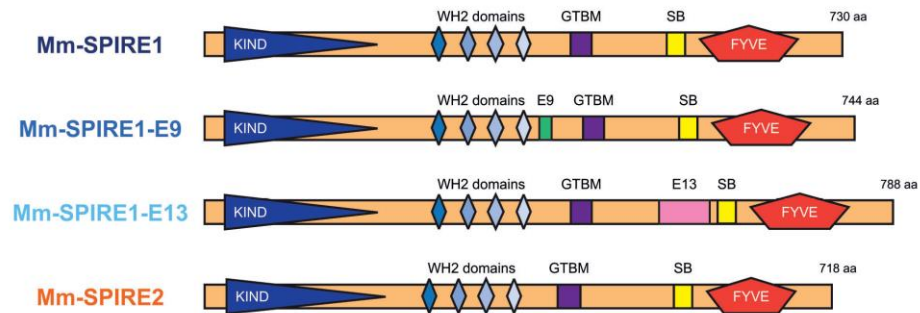


Figure 1.2.6. A schematic representation of the domain structure of mouse SPIRE proteins (Kollmar et al., 2019)

SPIRE1/2 contain an N-terminal KIND domain (kinase non-catalytic C-lobe domain) which interacts directly with the C-terminal formin-SPIRE interaction motif (FSI) of FMN-subgroup formin (Pechlivanis et al., 2009, Quinlan et al., 2007). KIND of SPIRE1 also interacts with mDia1 *in vitro* (Lagal et al., 2014), and KIND of mitochondrial SPIRE1 (SPIRE1C) interacts with inverted formin 2 (INF2) (Manor et al., 2015). C-terminus of the KIND, SPIREs contain a cluster of four WH2 domains (Wiskott-Aldrich homology region 2) that are a defining feature of these proteins. The four WH2s bind to four actin monomers to nucleate actin (Quinlan et al., 2005). The WH2 domain is a short 17–27 stretch of amino acids. Its N-terminal region forms a helix that binds in the target-binding cleft of actin. Following this helix, the WH2 domain contains an extended region that contains a conserved four residue motif LKKT (Dominguez and Holmes, 2011). The four WH2 domains of SPIREs (named A, B, C, and D) have different affinities for actin monomers: removing or mutating the essential hydrophobic residues of WH2-A resulted in the smallest effect on actin-binding and nucleation, while progressively greater effects result in the case of WH2-B and WH2-C. In contrast, mutating or

removing WH2-D markedly reduced nucleation activity. The short linker (~15 aa) between WH2-C and -D also exhibit nucleation activity (Quinlan et al., 2005). At the middle of SPIRE protein sequences is the myosin-V globular tail binding motif (GTBM) that binds to the subdomain SD-1 of the GTD of the three isoforms of myosin-V (a,b, and c) (Kd ~ 728 nM, 377 nM, and 2.88 μ M for MyoVa, MyoVb, and MyoVc, respectively) (Pylypenko et al., 2016). In the C-terminus region of SPIREs, the SPIRE-Box domain (SB) is a highly conserved region and shares sequence similarity with alpha-helical N-terminal extension of the rabphilin-3A FYVE finger-related structure. This extension in rabphilin-3A mediates its interaction with active Rab3A and Rab27a GTPase (Kerkhoff et al., 2001). SPIRE-Box of human SPIRE1/2 shares conserved residues with SHD1 of well-established Rab27a effectors (e.g. MLPH, MYRIP, Rabphilin3A, and NOC2) (Alzahofi et al., 2020). SPIRE1 localise with Rab3A in invadosomes of 3T3-Src⁺ cells, and the protein interaction was verified by co-immunoprecipitated from the same cells (Lagal et al., 2014). This is followed by the modified FYVE (named after the cysteine-rich proteins Fab1, YOTB, Vac1, and EEA1)-type zinc finger. FYVE comprises of eight cysteine residues capable of binding two zinc ions and forms a hydrophobic “turret loop”. The structure facilitates SPIREs’ sub-cellular localisation by interacting with negatively charged lipid (phosphatidylinositol) in the vesicular membrane and by intruding into the lipid bilayer via the hydrophobic contributions of the turret loop. M-FYVE also regulates the SPIRE function by forming back-folded inhibited conformation with N-terminal KIND domain (Tittel et al., 2015). Finally, the C-terminus flanking alpha-helical domain named due to the

sequence similarity with H2 of SHD in Rab27a effectors (Alzahofi et al., 2020). It is phosphorylated by Jun N-terminal kinase (JNK)-interacting protein (an effector of Rac and Cdc42 GTPases involved in actin reorganisation) (Otto et al., 2000). It also shares some similarity with SHD2 of Rab27a effectors (Alzahofi et al., 2020).

1.2.2.3.2.4 SPIRE-FORMIN INTERACTION TO NUCLEATE ACTIN FILAMENTS

SPIRE (also named spir) and formin cappuccino (Capu) were first identified in screens for genes affecting embryonic pattern formation in *Drosophila melanogaster* (Manseau and Schüpbach, 1989). Mutation of either SPIRE or formin genes results in the premature onset of microtubule-dependent fast cytoplasmic streaming during oogenesis, loss of oocyte polarity, gross defects in embryonic morphology, and female sterility (Theurkauf, 1994, Emmons et al., 1995). Mutation of *Drosophila* profilin (Chickadee) or addition of the actin-depolymerising toxin cytochalasin-D also results in the same effects (Emmons et al., 1995, Manseau et al., 1996b). These studies linked SPIRES with actin filaments and suggested that actin structures assembled by SPIRE, cappuccino (Capu), and chickadee are involved in regulating oocyte development (Serbus et al., 2005). The same interaction and functional cooperation happens between the mammalian orthologs, FMN-type formin (formin 1/2) and SPIRE1/2, during oocyte maturation, melanosomes and vesicular transportation, and damaged DNA repair (Schuh, 2011, Pfender et al., 2011, Kerkhoff et al., 2001, Alzahofi et al., 2020). The direct interaction between SPIRE and formin is required for their function and has been intensively studied biochemically and structurally (Quinlan, 2013, Quinlan et al., 2007, Zeth et al., 2011, Vizcarra et

al., 2011, Pechlivanis et al., 2009). Formin dimers bind to two molecules of SPIRE with ~100 nM affinity via interaction of their FSI and KIND domains. The electrostatic interactions of a positively charged basic cluster of the FMN-FSI sequence and a negatively charged acidic groove within the KIND domain enhance the actin nucleation activity of SPIRE. In contrast, KIND competes with barbed ends for formin binding and inhibiting actin nucleation by formin (Ciccarelli et al., 2003, Pechlivanis et al., 2009, Quinlan et al., 2007, Rosales-Nieves et al., 2006, Zeth et al., 2011).

The mechanism in which this interaction regulates actin cytoskeleton and intracellular trafficking has still not been fully revealed. Firstly, in mouse oocytes, SPIRE1/2, Fmn2, and Myosin-Vb are localised on Rab11-positive vesicles. The vesicles are located at nodes of the actin mesh that fills the oocyte (Schuh, 2011). The dynamic mesh is involved in spindle positioning and long-distance transport of the Rab11-positive vesicles towards the cortex. A proposed model for the long-distance transport includes actin filaments nucleated by SPIRE and formin growing with their barbed ends remaining near the vesicles. These filaments serve as tracks for myosin-V to transport the adjacent vesicles (Figure 1.2.7) (Schuh, 2011).

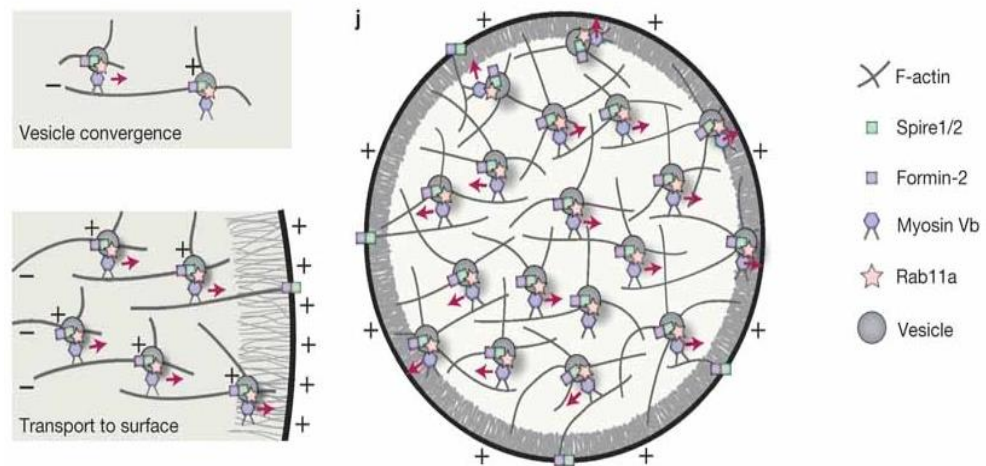


Figure 1.2.7. a schematic representation of actin-dependent vesicle transport as proposed in (Schuh, 2011)

Then, combining quantitative analysis of fertility with an examination of the actin mesh and temporal control of fast streaming in *Drosophila* oocytes suggested the so-called “Hand-off” model for SPIRE/FMN function. In this model, SPIREs serve as actin nucleators, while formin (Capu in the published model, Figure 1.2.8) acts as an elongation factor, such that the dimerised SPIRE with eight WH2 domain provides (handing off) Capu with a filament seed of eight actin monomers. The seed serves as a two-start actin filament helix which is processively polymerised at the barbed end by Capu. In the final step, SPIRE and Capu separate so that they can nucleate and elongates a new filament (Figure 1.2.8) (Quinlan, 2013).

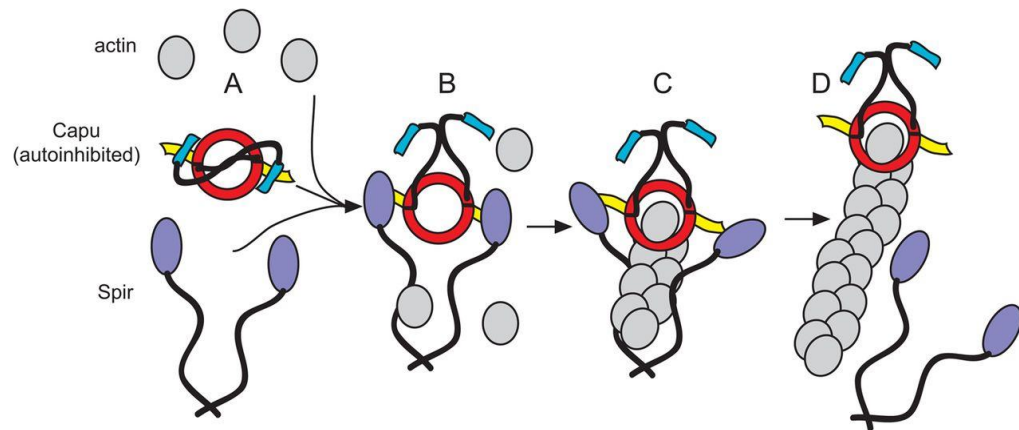


Figure 1.2.8. A schematic representation of the synergy between SPIRE/spir and Capu to nucleate actin filament (Hand-off model) (Quinlan, 2013)

Another proposed model for SPIRE/FMN function is 'Ping-pong' model.

Investigating actin assembly in bulk solution, and measuring the individual filament kinetics led to the proposal of this model. In this model, SPIRE and Fmn2 alternatively bind to the barbed ends of the actin filament. SPIRE acts as a cap on the growing end and facilitates Fmn2 association with barbed ends. Fmn2 binding to the barbed ends is followed by rapid processive elongation activity and results in releasing SPIRE from the barbed ends. This processive assembly continues until SPIRE again caps the barbed end and removes Fmn2 (Figure1.2.9) (Montaville et al., 2014).

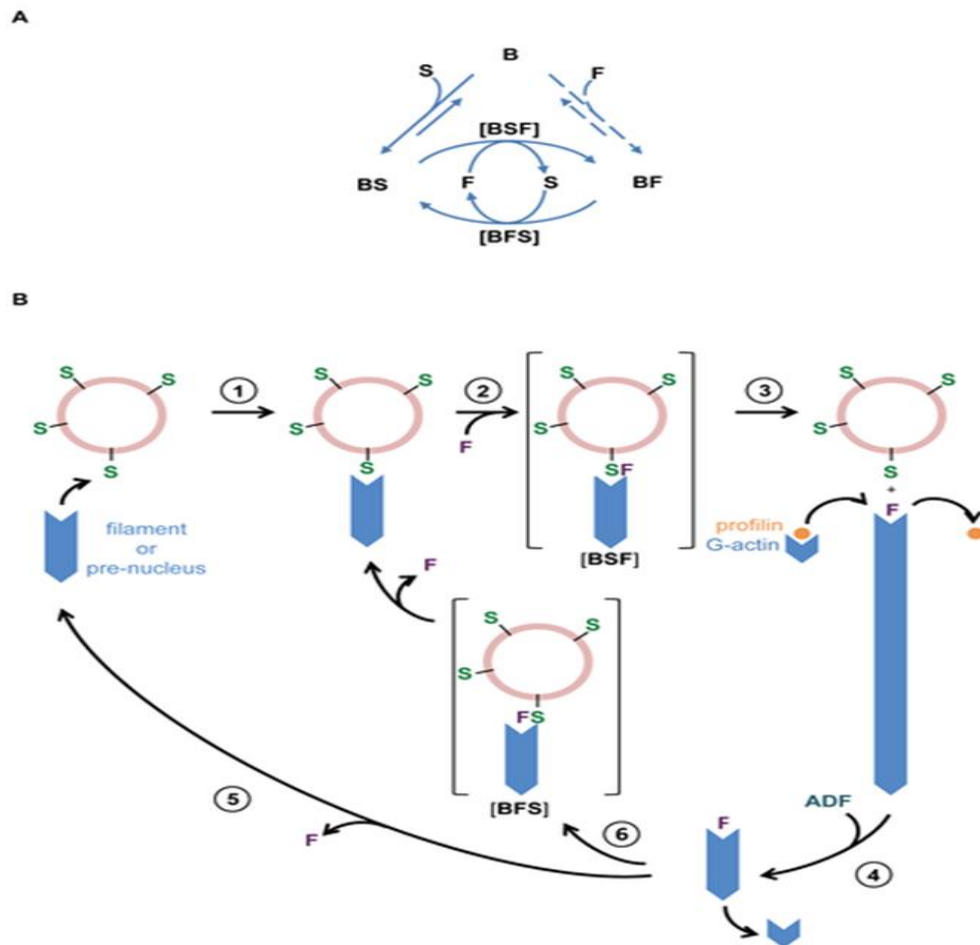


Figure 1.2.9. A schematic representation of synergy between SPIRE and Fmn2 in actin polymerisation in the ping-pong model. Abbreviations: the barbed end actin filament (B), Nt-SPIRE (S), Fmn2 (F), barbed-SPIRE (BS), barbed-Fmn2 (BF), barbed-SPIRE-Fmn2 (BSF), From (Montaville et al., 2014).

Recent biochemistry and fly genetics study revealed the ability of SPIRE to interact with both barbed and pointed ends of actin filaments (Bradley et al., 2020). In the study, the N-terminus construct of *Drosophila* SPIRE (KIND and WH2 domains) immobilised on streptavidin-coated microspheres and mixed with the free C-terminus construct of Capu (FH1/2 and FSI domains). Adding G-actin and profilin and visualising the nucleated filaments with total internal reflection fluorescence (TIRF) microscopy revealed extensive and rapid polymerisation of filaments. The filaments had pointed ends retained at the

beads and barbed ends oriented away from beads while the filaments near the beads were released over time. The same results were obtained when G-actin was added to the attached NT-SPIRE without Capu and profilin. It also suggested that the SPIRE bound the pointed ends of filaments for ~100 seconds. Furthermore, the researchers observed the ability of NT-SPIRE, via WH2-A, to “capture” the barbed end of a filament nucleated on the neighbouring bead for more than 10 minutes (Figure 1.2.10) (Bradley et al., 2020).

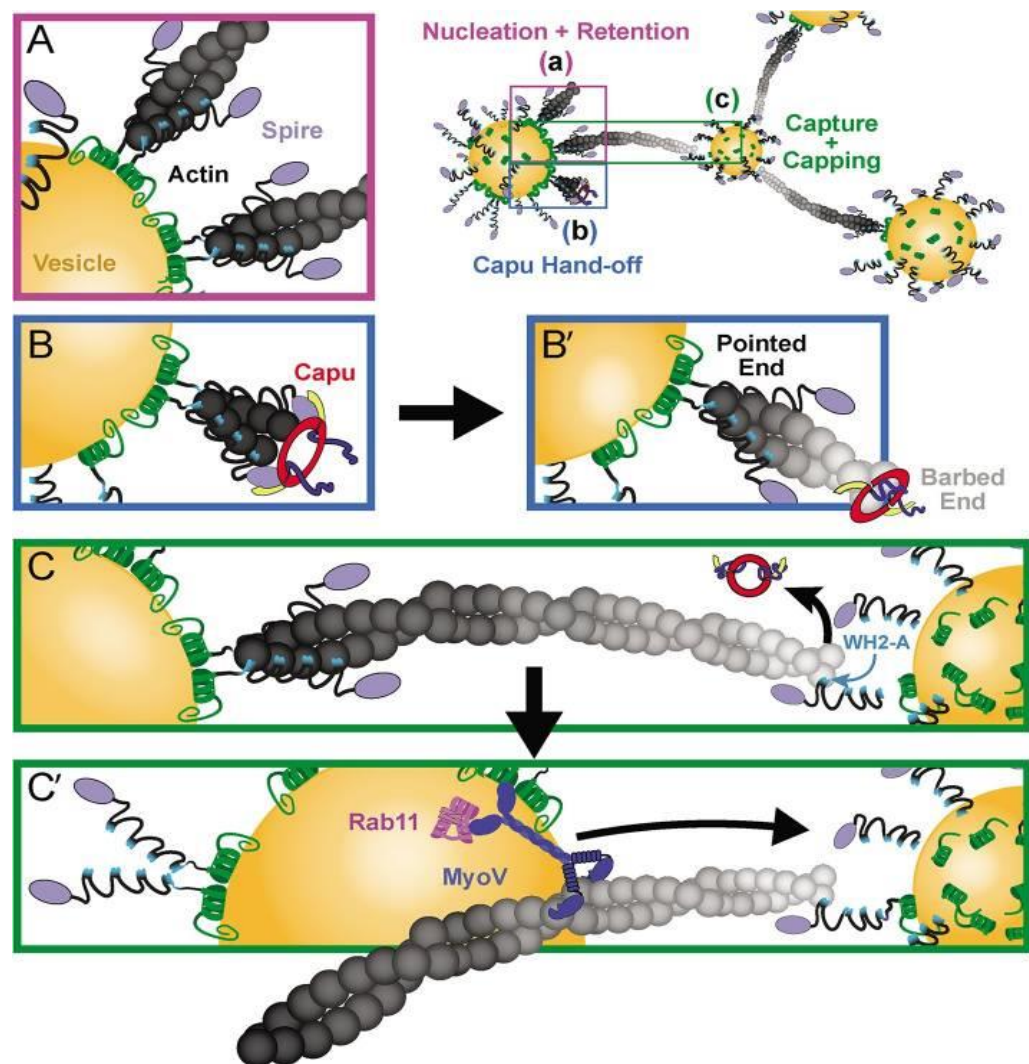


Figure 1.2.10. SPIRE-Capu synergy to nucleate actin filament as proposed by (Bradley et al., 2020).

There are some similarities and differences between the findings of Bradley et al. (2020) and the previously proposed model of synergising between SPIRE and Capu (formin). Consistent with the ping-pong model, the researchers observed the transfer of Capu-bound barbed ends to SPIRE but not in the other way (from SPIRE to Capu). In which, After the filament elongation by Capu, SPIRE in the adjacent bead can capture the barbed end, but did not give it back to Capu. However, contrary to the ping pong model, they found that the assembly of profilin–actin *in vitro* was improved in the absence of SPIRE binding to barbed ends. The nucleation rate in SPIRE and Capu was significantly higher compared to the nucleation by SPIRE alone (with or without binding to barbed ends). They also observed that ceasing the nucleation by SPIRE by removing WH2 resulted in the termination of the synergy with Capu. This suggested a significant contribution of the SPIRE's nucleation to synergy, contrary to SPIRE's assumed to function as an NPF or passive actin-binding protein (Bradley et al., 2020).

Considering the geometry of SPIRE/FMN assembly in their *in vitro* assays similar the *in vivo* geometry, Bradley et al. (2020) suggested that the barbed ends of actin filaments were oriented opposite to the previously proposed models in Schuh (2011) and Montaville et al. (2014). Their findings suggested that SPIRE could conduct long-distance transport by capturing a growing filament and myosin V could transport the vesicle from which the filament was nucleated (Figure 1.2.10 C). The long duration of barbed-end capture

compared to pointed-end binding supported this conclusion (Bradley et al., 2020).

1.2.2.4 Actin Filaments and the Binding Proteins in Long-Range Intracellular Trafficking

A part of actomyosin, dynamic actin plays a critical role in intracellular trafficking. Because of the short length of actin filaments, it has been thought that they serve as the tracks for short-range transport, akin to 'local roads' in human transport systems (Wu et al., 1998). However, many studies have revealed the capability of actin to drive long-range transport in cells. For example, HIV can invade cells and transport to the nucleus in the absence of MTs (Fackler and Kräusslich, 2006). Also, the transport of the nucleocapsid of the Marburg virus toward the cell membrane is not affected by the absence of MTs while disassembled actin filaments block the transport of Marburg nucleocapsids over long distances from the viral replication centres to the budding sites entirely (Schudt et al., 2013). Also, the recycling of transferrin from the perinuclear region to the plasma membrane is an actin-dependent transport in the hepatoma cell line (Durrbach et al., 1996). Disassembled actin filaments or knockdown actin nucleation proteins block long-range vesicle transport (Schuh, 2011, Kerkhoff et al., 2001) and melanosome centrifugal transport in skin melanocytes (Evans et al., 2014, Alzahofi et al., 2020). Actin also contributes to the transport of endoplasmic reticulum (ER) vesicles in neurons, Golgi-derived vesicles in epithelial cells, and cytoplasmic vesicles in living *Drosophila* embryos (DePina and Langford, 1999). Together, these

observations indicate that actin filaments can be used as a track for long-range transport independently from the MTs.

1.3 Cytoskeletal Motors

Motor proteins are molecules that have the ability to move along MTs or polar actin filaments using the energy derived from ATP hydrolysis. The conformational changes induced by ATP hydrolysis results in a step along the filament. The motors divide into two groups depending on the cytoskeleton filaments (the track) they are attached to (and move along): Microtubules-based motors including kinesins and dyneins (Vale, 2003) and actin-based motors are myosins (Goode et al., 2000, Hartman et al., 2011, Ross et al., 2008). The motors are also categorised into two groups depending on the number of steps (or conformation changes) the motor can perform during a single attachment to the filament. A processive motor can perform many steps before detaching, while a non-processive motor mostly disassociates the filament after a single step. Muscle myosins II is an example of non-processive motors. In contrast, other myosins and MT-based motors are processive motors. They can individually interact with cytoskeleton filaments for an extended period of time and move over long distances (Schliwa and Woehlke, 2003, Appert-Rolland et al., 2015, Sweeney and Holzbaaur, 2018).

1.3.1 Kinesins

Conventional kinesin (also named kinesin-1) was the first molecular motor discovered to transport cargo along microtubules filaments (Vale et al., 1985).

A broad superfamily of kinesin-related proteins was found to have a highly conserved, ~340-amino acid residue motor domain at their N-terminus.

Kinesin superfamily is divided into 14 families based on sequence comparisons (Lawrence et al., 2004). However, all kinesins featured with the kinesin heavy chain (KHC) that contains conserved motor domain (the head) binds to the MT and ATP and unique cargo-binding domains that bind specifically to different intracellular cargos (Appert-Rolland et al., 2015, Sweeney and Holzbaur, 2018). The head is linked to the cargo-binding domain by a neck-linker region that is subject to conformational change during ATP hydrolysis followed by an extended series of coiled coils. Additional domains were characterised to serve specific regulatory control, such as IAK motif (isoleucine-alanine-lysine) that is essential for autoinhibition (Sweeney and Holzbaur, 2018, Hackney et al., 2009). The heterotetrameric Kinesin-1 contains two Kinesin heavy chains (KHC) and two Kinesin light chains (KLC) (Figure 1.3.1). The KLC contains N-terminal coiled-coil domain that binds to the C-terminal region of the KHC-coiled coils. The C-terminal region of KLC contains six repeats of tetra-tripeptide (TPR) domains, which interact with cargo adaptors and also regulate kinesin activity by self-interaction to form inhibitory conformation (Gindhart and Goldstein, 1996, Bowman et al., 2000, Gauger and Goldstein, 1993, Yip et al., 2016)

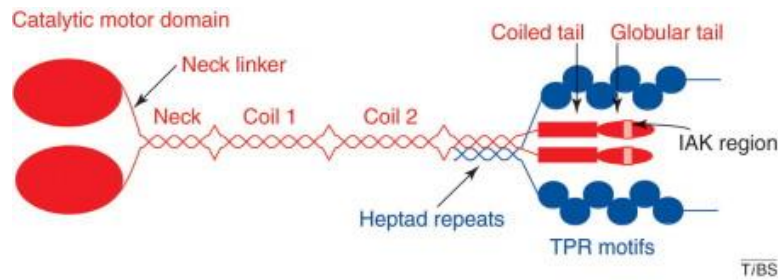


Figure 1.3.1. A schematic representation of the kinesin-1 domain structure. Kinesin heavy chain (shown in red) contains the motor domain, the neck linker, neck domains, the coil 1/2 regions of the stalk domain, and the globular and coiled regions of the tail domain. The regulatory IAK motif in the globular tail. Kinesin light chain (shown in blue) contains an α -helical heptad repeat region and six TPR (tetratricopeptide repeat) motifs (Verhey and Rapoport, 2001).

The superfamily members perform diverse functions in the cell. However, most of them function as motors and contribute to intracellular trafficking, including vesicles, organelles, chromosomes, protein complexes, and RNPs transport. Two other kinesin groups regulate microtubule dynamics. One can move processively along microtubules and selectively modulate microtubule dynamics, like kinesin-8. The other acts solely to destabilize or depolymerize microtubule with no motor activity, like kinesin-13 (Friel and Howard, 2011, Hirokawa, 1998, Sweeney and Holzbaur, 2018).

The movement of the Kinesin motors along the MT depends on ATP hydrolysis; however, the kinetic cycles are distinct among the different family members. In dimeric kinesin-1, ATP hydrolysis affects the head affinity to bind MT filament. Hydrolysis of ATP and release of γ -phosphate weaken the head's ability to bind MT, resulting in detaching the head. At the same time, the ATP-associated-head binds MT strongly. Exchanging ADP with ATP in the detached head induces a conformational change of the neck-linker, resulting in re-attaching the head to the front (Figure1.3.2). The ATP then hydrolyses, and

the cycle repeats until the motor entirely disassociates the filament. This mechanism, called hand-over-hand allows kinesin-1 to move approximately 100 steps processively before disassociating the filament (Block et al., 1990). Hydrolysis of a single ATP molecule results in an 8-nm step which equals the length of one tubulin subunit (Schnitzer and Block, 1997, Coy et al., 1999, Sweeney and Holzbaur, 2018, Appert-Rolland et al., 2015, Yildiz et al., 2004b). Kinesin-1 is a fast motor, moving at 0.5–1 $\mu\text{m}/\text{sec}$ unidirectionally toward the microtubule plus end, *in vitro* as well as *in vivo*. However, some kinesins move in the opposite direction towards the minus end (Appert-Rolland et al., 2015, Sweeney and Holzbaur, 2018).

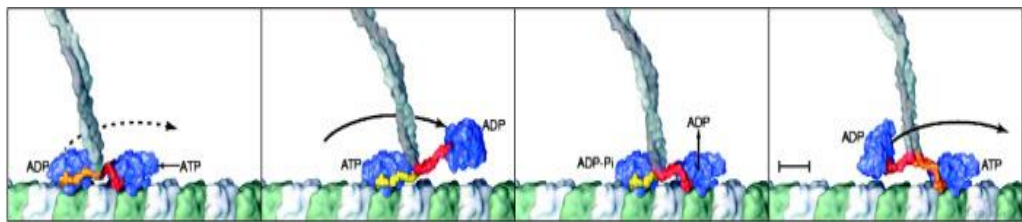


Figure 1.3.2. A schematic representation of the hand-over-hand movement mechanism of kinesin-1. Hydrolysis of ATP and release of γ -phosphate weaken the head's ability to bind MT, resulting in detaching the head. At the same time, the ATP-bounding head binds MT strongly. Exchanging ADP with ATP in the detached head induces a conformational change of the neck-linker, resulting in re-attaching the head to the front (Appert-Rolland et al., 2015).

1.3.2 Dynein

The dynein family was first discovered and purified from the axonemes of cilia and flagella of eukaryotic cells (Gibbons and Rowe, 1965, Sweeney and Holzbaur, 2018). The family includes two groups: axonemal dyneins, which contribute to the motion of cilia and flagella, and cytoplasmic dyneins, which contribute to intracellular trafficking. The two groups share both structural and sequence similarity. Cytoplasmic dynein motor functions as the primary

minus-end microtubule motor in the cell and facilitates the transport of different intracellular cargoes. This ability is facilitated by different multifunctional adaptors such as dynactin, which regulates dynein motor activity (Kardon and Vale, 2009).

Dyneins are the largest cytoskeletal motors (0.5–3 MDa) and the most complicated ones. Each dynein is a complex of between 1 and 3 heavy chains (DHC), along with a number of intermediate and light chains (King, 2000). The heavy chain forms a globular head with the stalk and tail structures emerging from it (Goodenough and Heuser, 1984, Goodenough et al., 1987). The head includes six tandem repeats of “ATPases associated with diverse cellular activities” (AAA) domain. These six domains are present in a polypeptide that folds to form a hexameric ring (Carter et al., 2016, Kon et al., 2004). A linker domain stretches out from the ring, connecting AAA1 to the tail domain (Figure 1.3.3) (Kon et al., 2012, Roberts et al., 2012). The stalk domain has the ATP-sensitive microtubule-binding domain (Gee et al., 1997, Vallee and Gee, 1998, Koonce and Tikhonenko, 2000). The tail domain at the N-terminus of DHC can homodimerize and can also interact with different dynein subunits such as dynein intermediate-, light intermediate-, and associated light-chains in a cytoplasmic dynein. The distinct isoforms of these subunits provide specialization regarding intracellular targeting and regulation. For example, the dynein-intermediate chain is expressed in mammals in both ubiquitous and neuron-specific isoforms. The latter preferentially binds tyrosine receptor kinase (Trk) to facilitate the signalling complexes motility required for

neuronal development and function (Mitchell et al., 2012, Sweeney and Holzbaur, 2018).

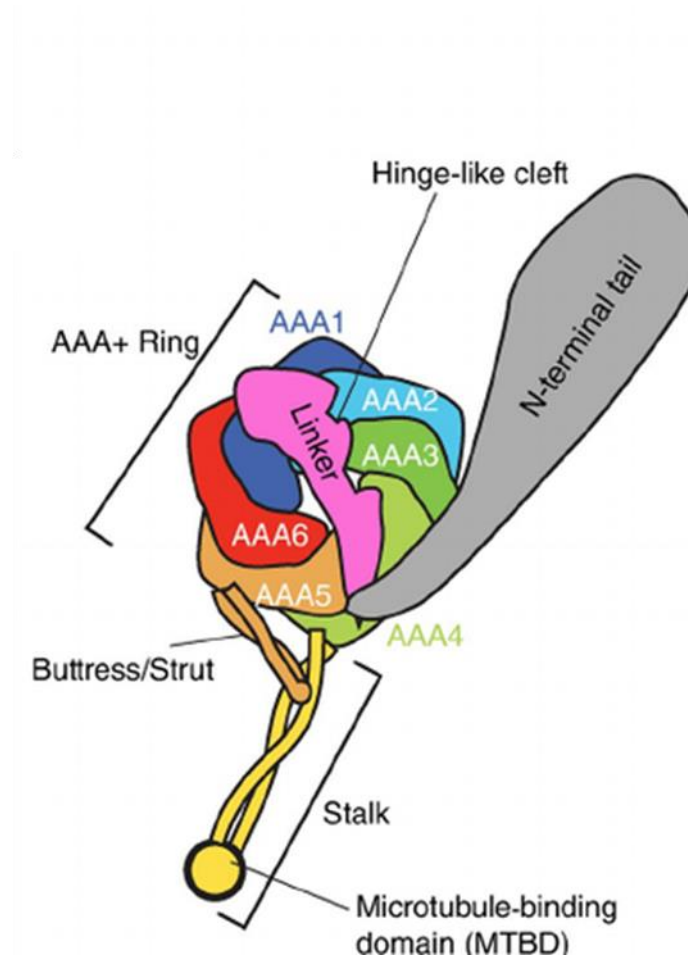


Figure 1.3.3. A schematic representation of the structure of cytoplasmic dynein heavy chain (Kikkawa, 2013).

Like kinesin, cytoplasmic dynein movement requires a cycle of nucleotide-dependent conformational changes in the motor domain. ATP binding weakens the motor association with MT and results in detachment. Then the ATP hydrolysis on the detached head results in rebinding to the MT. The structural change drives a processive side-steps movement along MT filaments (Figure 1.3.4). The binding sites of dynein and kinesin on the MT surface are overlapped, and thus, the motors may compete for binding sites

and blocks each other on encounters in bidirectional transport on MTs (Holzbaur and Johnson, 1989, Wang et al., 1995, Reck-Peterson et al., 2006, Sweeney and Holzbaur, 2018, Appert-Rolland et al., 2015, Carter, 2013).

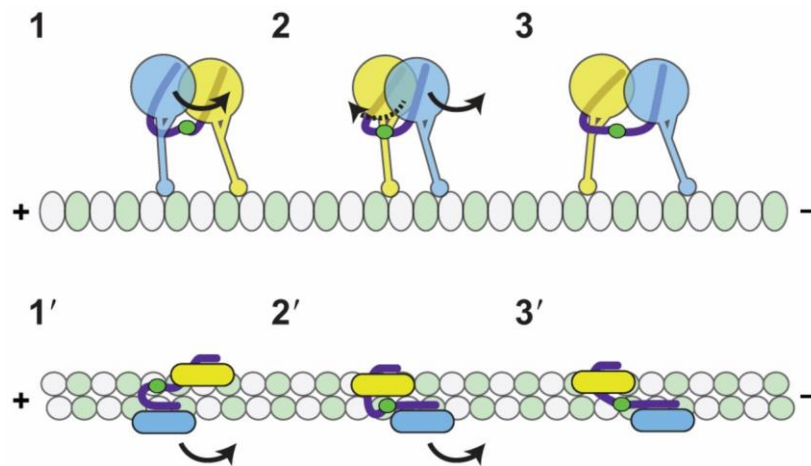


Figure 1.3.4. A schematic representation of the dimeric dynein motor walking ‘side-steps’ toward the minus-end of the MT viewed from the side (the upper panel) and from above (the lower panel). The linker domains are shown in purple, and the site of dimerization is represented by a green oval. (1) The yellow motor domain in front (leading motor). (2) the blue motor domain has passed the yellow one to take the lead. If the leading motor domain moves again, it is as likely to move forward as it is to move backwards (broken arrow). (3) the blue motor domain (leading) has undergone a second forwards step; it detached from the microtubule, reattached in a forward position. The increase of the distance between the motor domains stimulates yellow motor domain stepping forwards (Carter, 2013).

1.3.3 Myosin (Actin-Based Motor)

The myosin superfamily is the only known actin-based motor proteins (Goode et al., 2000, Hartman et al., 2011, Ross et al., 2008). The various myosins family members are grouped into more than 40 classes and are involved in several cellular pathways (Woolner and Bement, 2009, Krendel and Mooseker, 2005, Foth et al., 2006, Odronitz and Kollmar, 2007).

Conventional myosin (called myosin II) is present in muscles and non-muscle cells. It interacts with actin to form actomyosin, which is responsible for muscle contraction and cell motility. All myosin motors move toward the

barbed end of actin filaments, except myosin VI, which moves towards the pointed end (Wells et al., 1999, Bresnick, 1999, Sweeney and Holzbaur, 2018).

All known myosin feature three distinct domains: the N-terminal motor or head domain, that contains actin- and ATPase binding sites. The head also includes a converter subdomain, which is attached to an extended light chain helix (neck region). The neck region contains a variable number of IQ motifs that bind light chains of calmodulin or calmodulin-like to form a “lever arm.” In two-headed myosins, the lever arm is followed by a coiled-coil region that facilitates protein dimerisation. Finally, the C-terminal globular tail domain (GTD or targeting domain) undergoes cargo binding and dimerisation (Figure. 1.3.5) (Krendel and Mooseker, 2005, Sweeney and Holzbaur, 2018).

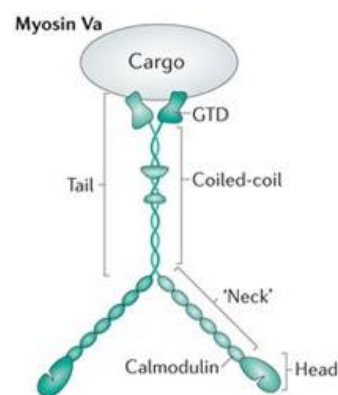


Figure 1.3.5. A schematic representation of dimeric myosin-Va motor. This shows the main structural features of myosin family proteins (Hammer and Sellers, 2012).

Myosin motors alternatively bind to actin filament and hydrolyse ATP. Binding actin stimulates myosin to hydrolyse ATP and release γ -phosphate followed by the release of Mg^{2+} and ADP, while rebinding ATP detaches myosin from the filament, allowing the hydrolysis and repeating the cycle. The reversible conformational changes during ATP hydrolysis result in a significant

movement of the lever arm. The lever arm movement is known as a power stroke, and it generates the force required for myosin movement. In contrast, the lever arm movement associated with ATP hydrolysis is known as the recovery stroke and involves a rapid isomerisation with a low free-energy barrier (Sweeney and Holzbaur, 2018). All myosin classes share the same basic kinetic cycle. However, the transition rates between the states are highly variable. Multiple interactions with actin without detaching from the filament allow some two-headed myosins to walk processively on an actin filament (De La Cruz et al., 1999, Mehta et al., 1999, Rock et al., 2001, De La Cruz et al., 2001). In this walk, the binding of ATP detaches one head from actin while the other head remains attached to the filament long enough for the detached head to hydrolyse the ATP and reattach to the distant actin-binding site in the front (Figure 1.3.6). This hand-over-hand mechanism allows myosin V, for example, to transport organelles and vesicles in large steps 36nm in length, which is equal to the actin helix pitch (Ökten et al., 2004, Yildiz et al., 2003, Yildiz et al., 2004a, GM, 2000).

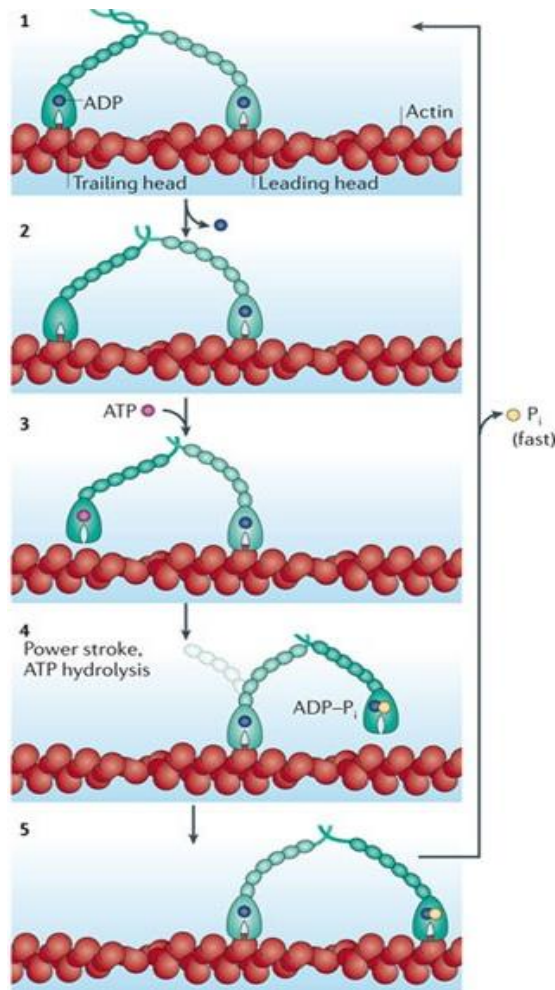


Figure 1.3.6. A schematic representation of the hand-over-hand mechanism of myosin movement along the actin filament. The binding of ATP detaches one head from actin while the other head remains attached to the filament long enough for the detached head to hydrolyse the ATP and reattach to the distant actin-binding site in the front (Hammer and Sellers, 2012).

Dimeric motors such as myosin-V can fold into an autoinhibited confirmation form. In this form, the globular tail domains (GTD) of the dimer interacts with the heads trapping it in the pre-power stroke state with bound Mg-ADP and Pi (inorganic phosphate). They are unable to release Pi and are thus frozen in the inactive state (Wendt et al. 2001). The inhibited conformation is supported by different intramolecular interactions, including head- GTD interactions, GTD-GTD interactions, and interactions between the GTD and the C-terminus of the first coiled-coil segment (Figure 1.3.7) (Zhang et al., 2018).

The phosphorylation of the regulatory light chains, Ca^{2+} or cargo binding via adaptor proteins, frees the head and forms the active conformation (Zhang et al., 2018, Cao et al., 2019).

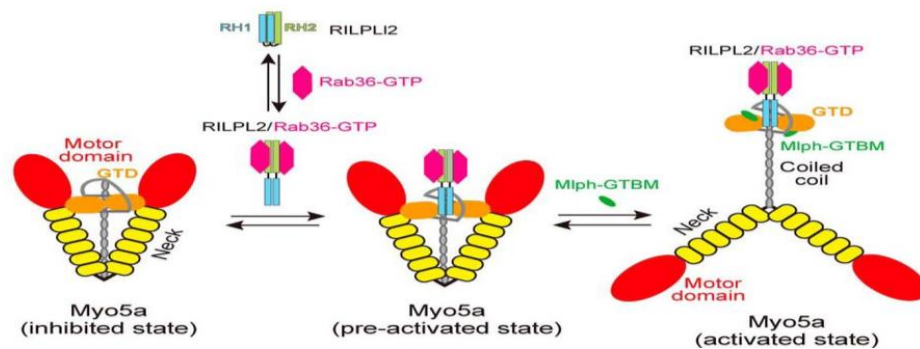


Figure 1.3.7. A schematic representation of the reversible conformational transformation between the active and inhibited state of myosin-Va by the cargo adaptor proteins melanophilin and RILPL2 (Cao et al., 2019).

1.4 Adaptor Proteins and Anchorage of Motor Proteins to Cargoes

Cytoskeletal motors organise organelle spatial localisation and support vesicular transport. In order to transport the specific cargo to the appropriate site, motors need to accurately and selectively recognise the vesicle/organelle to be transported. The organelle-associated ‘cargo-adaptor’ proteins in cellular transport have sequences and structural features that regulate motor activation, motility and motor-cargo association. Those adaptors play a crucial regulatory role in enabling the cell to finely control membrane trafficking and organelle dynamics (Cross and Dodding, 2019).

1.4.1 Rab GTPases

Rab GTPases family and their effectors (binding partners) constitute one of the most significant adaptors that regulate intracellular trafficking. Rab-

GTPases are the largest family of small GTPases within the Ras superfamily. It is also evolutionarily conserved in metazoan. Their specific localisation within the cell allows them to specifically recruit the appropriate motor protein to a given cargo. Therefore, they control the specificity and the directionality of membrane trafficking (Seabra et al., 2002, Zhen and Stenmark, 2015, Pfeffer, 2013).

Rab family features cysteine prenylation motif conjuncts with five distinct motifs named RabF1 to RabF5 (Pereira-Leal and Seabra, 2001). The subfamilies have distinct conserved sequences named SF1–4, which define the sites of interactions for their respective effectors (Figure 1.4.1) (Pereira-Leal and Seabra, 2001). Like other regulatory GTPases, Rab family is also characterised by two ‘switch regions’ that bind γ -phosphate of GTP and exhibit considerable conformational alternation between the active and inactive states (Dumas et al., 1999, Stroupe and Brunger, 2000).

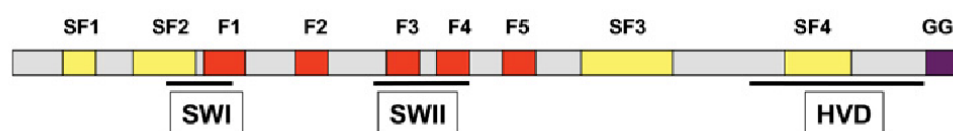


Figure 1.4.1. A schematic representation of the domain structure of a generic Rab-GTPase. RabF motifs are shown in red and RabSF motifs are shown in yellow. The prenylation motif (GG) is located at the C-terminal end of the protein (purple). The location of the switch regions (SW I and SW II) and the hypervariable domain (HVD) are indicated by black bars (Ali and Seabra, 2005).

Rab activity, like all other small GTPases, is regulated by cycling between an active GTP-bound and inactive GDP-bound form (Figure 1.4.2). The switch between these two forms is controlled by regulatory proteins such as guanine nucleotide exchange factors (GEFs), which mediate the exchange of GDP to

GTP, and GTPase-activating proteins (GAPs), which stimulate Rab GTPase activity. The cycling allows Rab to switch from being membrane-associated (active state) to being cytosolic and binding with the effectors (inactive state) (Seabra et al., 2002, Guadagno and Progida, 2019, Pylypenko et al., 2018).

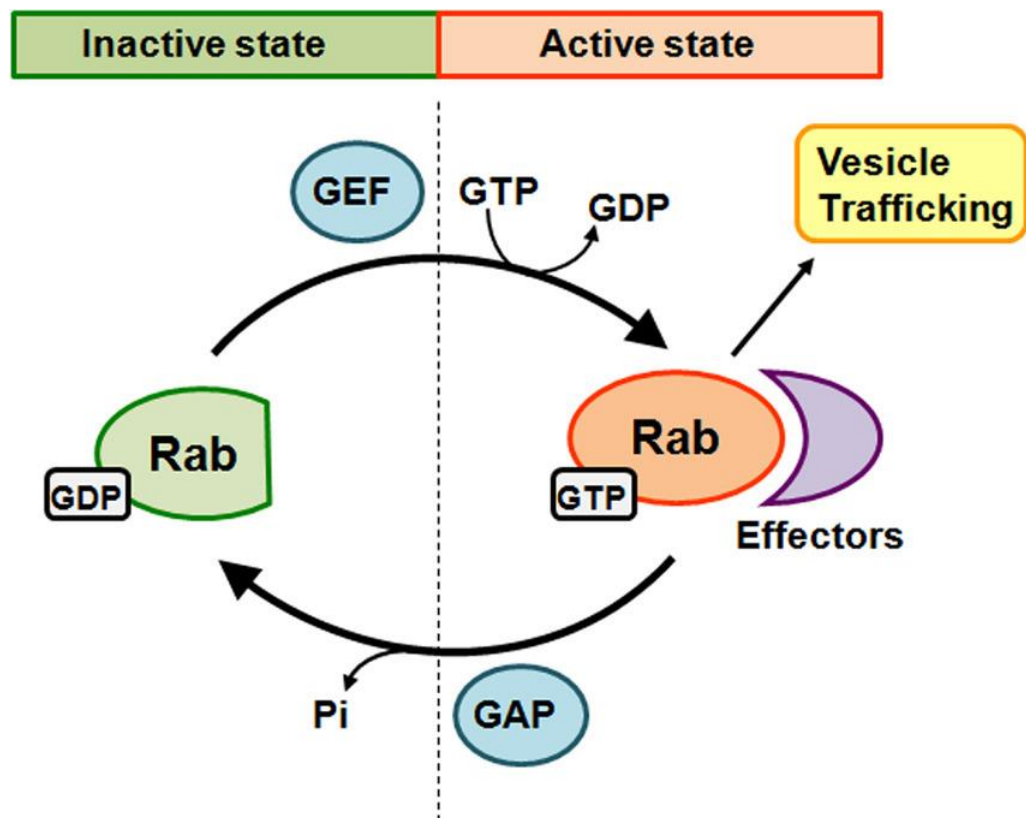


Figure 1.4.2. A schematic representation of Rab-GTPase nucleotide cycling. A guanine nucleotide exchange factor (GEF) catalyzes transition from GDP-bound (inactive form) to GTP-bound (active form). The active-form of Rab can bind specific effectors to facilitate vesicle transport. The intrinsic GTP hydrolysis of Rabs along with the effect of GTPase-activating protein (GAP) leads to Rab inactivation and effector dissociation (Wang et al., 2017).

Effector molecules are a broad group of Rab-isoform specific interacting proteins, such as motor proteins, coat proteins, phosphatases and tethering factors. They are essential to exert Rab transport functions (Strom et al., 2002, Lürick et al., 2017, Angers and Merz, 2011, Jean and Kiger, 2012). Rab GTPases are crucial in maintaining cell homeostasis and specialised cellular functions. Therefore, a lack of the same contributes to many diseases that result in

alterations in intracellular trafficking and signalling. A specific group of cancer, neurodegenerative and immunity disorders are associated with a defect in specific Rab27 members or their effectors (Guadagno and Progida, 2019, Seabra et al., 2002).

1.4.1.1 Rab27

Metazoans' widely conserved Rab27 is involved in the regulation of secretory granules in exocytic pathways. Two isoforms, Rab27a and Rab27b, are present in vertebrates. They share similar, but not identical, functions regarding their interactions with effectors (Izumi, 2007, Pereira-Leal and Seabra, 2001). While Rab27b expression in rescue melanocytes deficient for Rab27a, Rab27a and Rab27b play non-redundant roles in the distribution and biogenesis of secretory granules of mast cells, platelets and the exosomal pathway (Mizuno et al., 2007, Tolmachova et al., 2007, Barral et al., 2002, Ostrowski et al., 2009). However, they share the same effector proteins, and Rab27b compensates for any loss of function caused by Rab27a deficiency (Fukuda, 2013, Pereira-Leal and Seabra, 2001).

Eleven distinct Rab27 effectors that interact with the active GTP form of Rab27 have been identified in humans and mice thus far (Fukuda, 2013, Fukuda, 2005). They are categorised into three groups based on their domain organisation. The first group is characterised by an N-terminal Rab27-binding synaptotagmin-like proteins (Slp) homology domain (SHD) as well as C-terminal tandem C2 domains that bind phospholipids. This group includes synaptotagmin-like proteins (Slps) and rabphilin. The second group contains

the N-terminal SHD but lacks C2 domains and includes Slac2 (Slp homologue lacking C2 domains) and Noc2 (no C2 domain) (Kuroda et al., 2002, Strom et al., 2002, Wu et al., 2002, Fukuda et al., 2002, Fukuda, 2003, Fukuda et al., 2001). The Slp-type Rab27 binding domains may also be divided into two groups based on whether they contain a Zn⁺² finger domain (Figure 1.4.3). The final group includes only Munc13-4, which contains two separate C2 domains, and two Munc13 homology domains (named MHD1 and MHD2), which are distinct types of Rab27-binding sites that differ from the SHD (Neeft et al., 2005, Yamamoto et al., 2004).

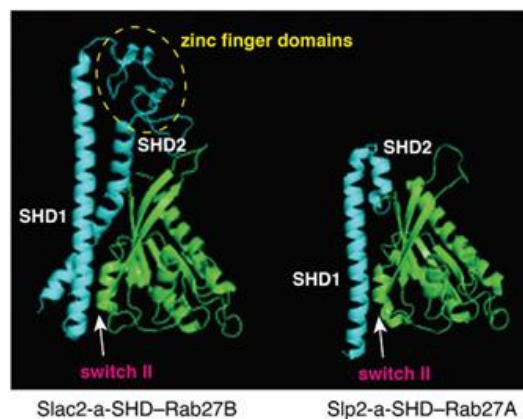


Figure 1.4.3. The structure of Rab27 in complex with effectors. The complex of Rab27 (green) with two types of effectors (blue); Slac2a-SHD with zinc finger motif between the SHD1 and SHD2 (broken yellow circle) and Slp2a without zinc finger motifs (Fukuda, 2013).

Rab3GEP (also known as MAP kinase activating death domain MADD, or DENN) is one of the Rab27a-GEFs in melanocytes (Tarafder et al., 2011, Sanzà et al., 2019). That Rab3GEP is a targeting factor that contributes to the association of Rab27a with melanosome membranes. Rab3GEP-Knockout melanocytes exhibit a mixture of clustered and dispersed melanosome distributions. That suggested Rab3GEP-independent-pathway(s) for Rab27a activation and targeting. This pathway is thought to be related to the high

intrinsic nucleotide exchange activity of Rab27a or involve other Rab-GEFs factors (Sanzà et al., 2019).

Rab27a was the first Rab protein to be associated with human hereditary diseases. A defect in Rab27a or its binding partner causes several human diseases. For example, Type 2 Griscelli syndrome (GS), which is characterised by pigment dilution of the hair (silvery hair), results from defects in melanosome transport in melanocytes, and immunodeficiency results from defects in granule exocytosis by cytotoxic T lymphocytes. Moreover, the defect in Rab27a-effector Melanophilin (MLPH) (also named Exophilin3/ Slac2-a,) causes type 3 GS, which is also characterised by deficiencies in melanosome transport in melanocytes (Seabra et al., 2002). Type 1 GS is associated with a defect in myosin-Va that associates with melanophilin as a cargo adaptor. It characterises with albinism and a severe primary neurological impairment. (Seabra et al., 2002).

1.4.2 Melanophilin (Exophilin3/ Slac2-a)

The genetic study of Griscelli syndrome and the corresponding mice mutations revealed a shared function pathway between Rab27a, myosin-Va and Melanophilin (MLPH). MLPH is a Rab27 effector that functions as a linker protein between Rab27a on the cytoplasmic face of melanosome and myosin-Va. MLPH directly and simultaneously interacts with the GTP-bound form of Rab27A via the N-terminal Rab27-binding domain, which contains two SHD domains separated by a putative Zn²⁺ finger motif but lacks tandem C2 domains. The central region of MLPH has two binding sites for myosin-Va: the

globular tail domain and the alternatively spliced exon F motif (Figure 1.4.4).

The tripartite protein complex that is composed of Rab27A, MLPH and myosin-Va is essential for transporting melanosomes toward the cell cortex where they can be transferred to keratinocytes to exert their photoprotective function (Wu et al., 2002, Strom et al., 2002, Hume et al., 2002, Hume et al., 2006, Fukuda et al., 2002, Nagashima et al., 2002). MLPH also interacts with actin filaments, which are critical for melanosome transport (Kuroda et al., 2003). The myosin-Va- and actin-binding domains at the C terminus of MLPH contain multiple PEST-like sequences (potential signals for rapid protein degradation) that play a role in protein turnover and tripartite protein complex dissociation. MLPH also interacts with Microtubule plus-end-tracking protein EB1 (end binding 1). The interaction with EB1 is said to facilitate tracking of MLPH to the melanosome dendrite tips on the plus-end tips of growing microtubules (Wu et al., 2005). However, knockdown EB1 or disturbing MLPH-EB1 interaction did not affect MLPH localisation to melanosome or melanosome transport to peripheral dendrites via myosin-Va (Hume et al., 2007).

MLPH functions not only as a cargo adaptor for myosin-V but also as a regulator. By acting as a tether between the motor and the actin filament,

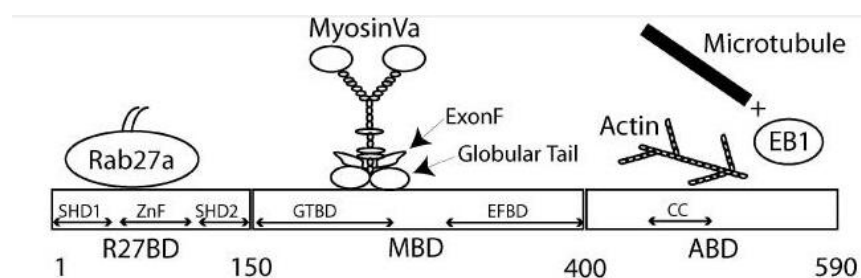


Figure 1.4.4 A schematic representation of domains structure of MLPH. The N-terminus Rab27-binding domains contain SHD1 and 2 with zinc finger motif in between. They were followed by two Myosin-Va binding domains in the middle of the protein. Finally, the actin and EB1- binding domains in the C-terminus region (Hume et al., 2006).

MLPH significantly increases the number of motors recruited to actin filaments. These changes increase myosin-Va run-length along actin filaments (Sckolnick et al., 2013). MLPH and Rab-interacting lysosomal protein-like 2 (RILPL2) contribute to myosin-V activation (Li et al., 2005, Cao et al., 2019, Sckolnick et al., 2013).

1.5 Melanosomes and Melanocytes as A Model For Understanding Intracellular Cargo Trafficking

Notwithstanding the intensive research that has been conducted in the field of intracellular transport, the molecular mechanisms that govern and regulate this process remain largely obscure. Many organelles lack intrinsic contrast *in vivo* and require a specific indicator, often a fluorescent label, which on excitation during the study could harm the cell and interfere with the physiological processes, including trafficking, or could be bleached and become invisible. Thus, to better study intracellular transport *in vivo*, utilising a high contrast cargo/organelle that can be observed under phase contrast or a bright field microscope facilitates the process by simplifying the analysis of cargo movement. From this perspective, the melanosome of brown/black eumelanin is a suitable model of intracellular cargo for studying protein/protein interactions and their contributions to the transport process.

1.5.1 The Melanosome as A Model Cargo for Intracellular Trafficking

Melanosomes of skin melanocytes are specialised organelles in which melanin (polyanionic pigment) is synthesised and stored (Wasmeier et al., 2008). The biological features of melanin provide a defence mechanism against environmental radiation and toxicity. That is due to the ability of melanin to absorb and isolate UV radiation, heavy metals and some drugs such as antimalarials (chloroquine) (Karlsson and Lindquist, 2016, Boulton and Dayhaw-Barker, 2001, Miyamura et al., 2007, Hong and Simon, 2007). Animal melanocytes produce two major types of melanin: brown-black eumelanin and red-yellow pheomelanin. Melanin synthesis in melanosomes is a pathway for melanogenesis and is comprised of many enzymatic reactions.

Melanogenesis starts with tyrosinase (TYR) hydrolysing L-tyrosine to L-DOPA and then oxidising L-DOPA into dopaquinone. Next, the availability of L-cysteine determines the types of melanin that are produced. Eumelanin is synthesised in the absence of L-cysteine; dopachrome tautomerase (DCT) converts dopaquinone into two intermediate molecules, 5,6-dihydroxyl indole (DHI), and 5,6-dihydroxylindole-2-carboxylic acid (DHICA), which undergo an enzymatic reaction with TYR and tyrosinase-related protein 1/2 (TYRP1/2 and TRP1/2) to form eumelanin eventually (Figure 1.5.1). If L-cysteine is present, it facilitates the conversion of dopaquinone into cysteinyl-DOPA, by subjecting it to many enzymatic reactions that involve oxidation and polymerisation to form pheomelanin (Figure 1.5.1) (Sturm, 2009, Solano, 2018, Lai et al., 2018).

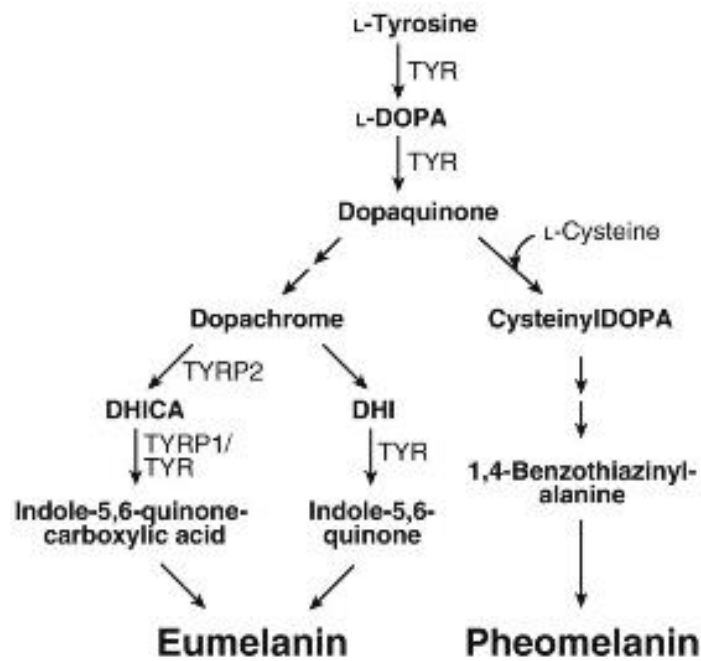


Figure 1.5.1. Melanogenesis Pathway to form Eumelanin and Pheomelanin, from (Wiriyasermkul et al., 2020)

The development and maturation of melanosome from early endosomes occurs in four different stages in a process named melanosome biogenesis. Melanosomes at stage I is endosome-like organelles that contain intraluminal vesicles (ILVs). They lack melanin but contain pre-melanosome protein (PMEL) (also referred to as PMEL17 and gp100). PMEL starts forming amyloid fibrils in the organelle's lumen. The PMEL fibril formation is completed in stage II. Melanosome then adopts an ellipsoidal shape, and the fibrils are organised and elongated as arrays of parallel sheets to serve as a matrix for melanin deposition. At stage III, electron-dense melanin is synthesised to completely cover the PMEL fibrils, which then thickens and darkens at stage IV (Figure 1.5.2) (Raposo and Marks, 2007, Raposo et al., 2001, Watt et al., 2013, Wasmeier et al., 2008, Wiriyasermkul et al., 2020).

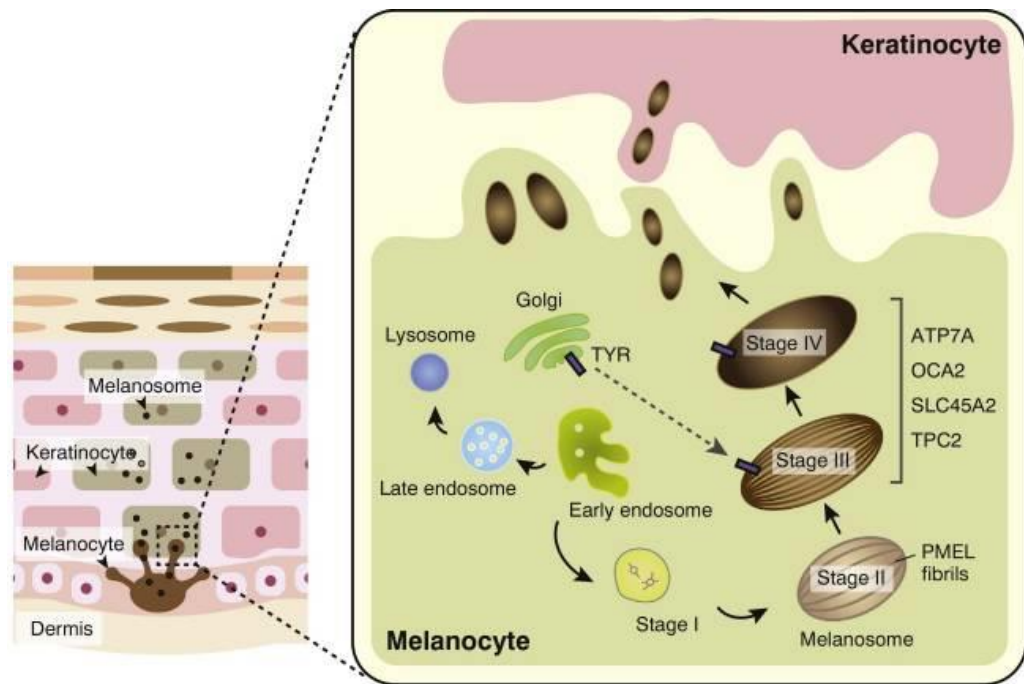


Figure 1.5.2. A schematic representation of epidermis layers and melanosome biogenesis in melanocytes (Wiriyasermkul et al., 2020).

Melanosomes are continuously produced in skin melanocytes. The mature melanin-packed melanosomes are transferred to a large number (~40) of neighbouring keratinocytes where melanin forms a supranuclear cap called ‘microparasol’ that protects the genetic material by absorbing and scattering UVR (Randolph Byers et al., 2003, Boissy, 2003).

Melanosome is a member of lysosome-related secretory organelles (LROs), which are a group of specialised organelles that share the morphological features of secretory lysosomes and have distinct functions. Genetic studies of human syndromes, such as Hermansky–Pudlak, Chediak–Higashi and Griscelli syndromes, and corresponding mouse mutations revealed similarities between melanosomes and other LROs in biogenesis, maturation, trafficking, recycling and migration (Raposo et al., 2007, Stinchcombe et al., 2004, Marks and Seabra, 2001). These syndromic diseases each feature a defect in

melanosome biology associated with dysfunction in other organelles. For example, Griscelli syndrome type II, as mentioned before, is characterised by defect in melanosome cytosolic granules (the LRO in T-cells) transport. This relationship makes the melanosome a good model for understanding the molecular basis of genetic disorders and the molecular mechanisms governing intracellular trafficking and organelle biogenesis among this class of organelle. Besides, mammalian melanocytes are amenable to genetic modification by siRNA (short interfering RNA)/shRNA (short hairpin RNA) knockdown and transgene expression. Further, the genetic association with different coat colours in mutant mice and the differentials of the shape, size and dispersion patterns of melanosome in mutant mice make them a good model for studying transgene expression effects on protein functions in organelle transport. Besides, the high contrast of brown/black eumelanin can be observed under phase contrast, or a bright field microscope can assist in studying the process by simplifying the analysis of cargo movement *in vivo* without interfering the process by fluorescent excitation or any artificial labelling (Hume and Seabra, 2011).

1.5.2 Melanosome Transport in Skin Melanocytes

The mechanism of melanosome transport in mammalian melanocytes has been a controversial topic. Melanosome dispersion was believed to be entirely actin-dependent (Koyama and Takeuchi, 1980). In which, depleted actin filaments by cytochalasin-B results in melanosome aggregation/ clustering in the perinuclear area. Then, a cooperative model was proposed

(Wu et al., 1998). In this model, MTs are considered a highway for melanosome transport through which MT-dependent motors of KIF and dynein transport melanosomes bi-directionally between the cell centre and the dendritic periphery. Melanosomes are then 'captured' and tethered at the cell periphery by the actin filaments and myosin motor (Figure 1.5.3) (Wu et al., 1998). Supporting this model, it has been found that knockdown Rab1a, its effector SKIP (SifA and kinesin-interacting protein), or the heavy chain of the motor kinesin-1 (Kif5b) resulted in perinuclear clustering of melanosomes, which suggested that Rab1a–SKIP–kinesin-1 complex regulates melanosome transport to the melanocytes periphery (Ishida et al., 2015). It also determined that Melanoregulin (Mreg) regulates microtubule-dependent retrograde melanosome transport (Ohbayashi et al., 2012). In which, a complex of Mreg- RILP (Rab interacting lysosomal protein) -p150^{Glued} (dynactin subunit 1, DCTN1)-and dynein-dynactin motor transport melanosome toward perinuclear region along the MT. Overexpressing Mreg or RILP resulted in perinuclear clustering of melanosome in wild-type melanocytes. Also, knockdown Mreg or disrupt the function of the complex resulted in peripheral melanosome distribution in ashen melanocytes (Rab27a- null melanocytes) (Ohbayashi et al., 2012).

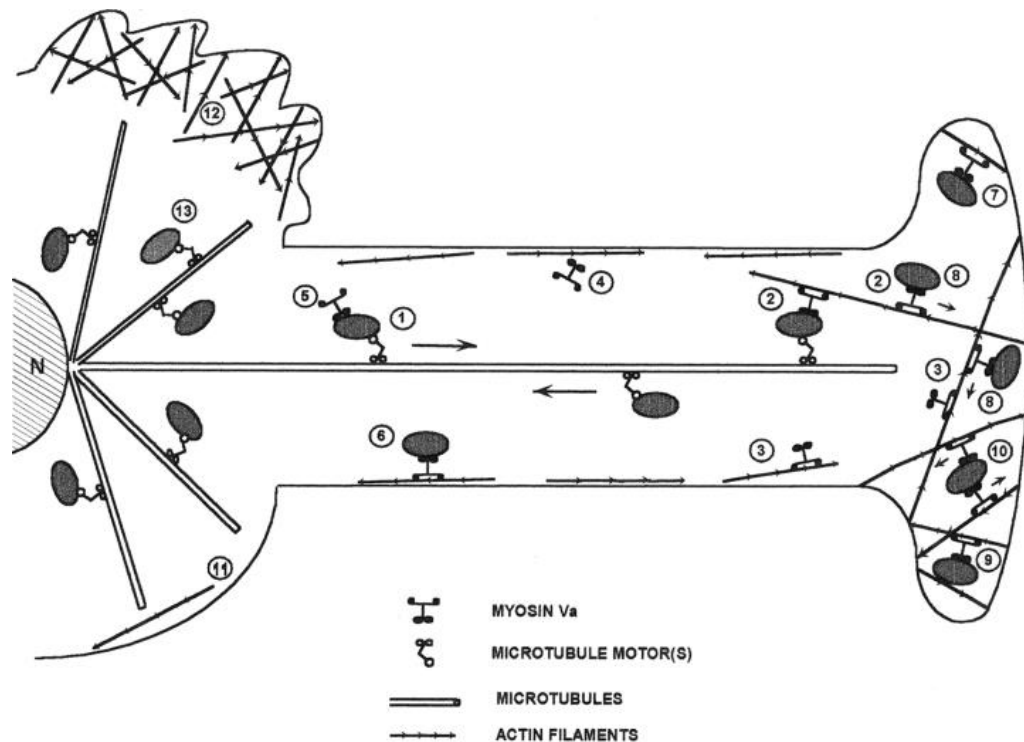


Figure 1.5.3 The cooperative model of melanosome transport. Melanosomes transport for long-distance bidirectionally along the MTs and only captured and tethered for a short distance along actin filament (Wu et al., 1998).

Genetic studies of Griscelli syndrome and the corresponding mutant mice reveal the vital role of the tripartite protein complex of GTP-Rab27a, MLPH and myosin-Va (actin-based motor) in melanosome anterograde/centrifugal transport. The dysfunction of any of these proteins results in melanosomes clustering in the perinuclear cytoplasm of melanocytes, which suggests that actin filament can be used as a track for long-range transport (i.e. from the perinuclear area to the cortex) and not only for tethering. In contrast, no mammalian pigmentary disorder has been reported as a result of dysregulation of microtubule-dependent anterograde melanosome transport. Additionally, recent studies used siRNA-mediated knockdown and disrupting the polymerisation of MTs to show that the retrograde/centripetal transport of melanosomes but anterograde transport was MT-dependent (Evans et al.,

2014). In further support of this idea, knocking down heavy chains of kinesin-1 (Kif5b) in melanocytes did not affect anterograde transport of melanosomes (Robinson et al., 2017) which contradicts Ishida et al. (2015) findings. This different observation could be related to the low percentage of cells with clustering melanosome (~40%) in the two Kif5b-specific siRNA-treated cells in Ishida et al. (2015) while two of seven Kif5b-specific siRNA that used in Robinson et al. (2017) resulted in ~10% when the other five results in no melanosome clustering. Also, Disturbing the actin-f polymerisation or knocking down actin nucleation proteins blocked the anterograde transport and did not affect the retrograde ones (Evans et al., 2014, Alzahofi et al., 2020). These findings suggested that microtubules and actin filaments play opposing roles in melanosome transport, whereby melanosome retrograde transports move toward the cell centre along microtubules and use actin filaments for anterograde, long-range transport toward the cell periphery.

Gaining a better understanding of melanosome trafficking and proteins involved could lead to a better understanding of other LROs traffic and the related diseases

1.6 Hypothesis and Aims of the project

In preliminary work previous PhD student Christopher Robinson used siRNA knockdown to test the role of more than 130 different actin regulatory proteins in melanosome dispersion. This revealed that expression of the actin nucleation proteins of SPIRE1/2 and actin-elongation protein of formin1 is required for anterograde transport of melanosomes (Alzahofi et al., 2020).

These results suggested that SPIRE1/2 as actin nucleators have a significant role in melanosome dispersion in skin melanocytes. However, the contribution of SPIRE1/2 and their domains in melanosome transport is poorly understood. Therefore, the main aims of this study were as follows:

- Investigate the contribution of SPIRE1/2 and their well-known domains in melanosome transport using short-interference RNA and rescue assay.
- Investigate the interaction between SPIRE and Rab27a in mutant melanocytes (melan-leaden) using chimeric proteins in the modified nanoscale pull-down assay.
- Investigate the role of SPIRE localisation on melanosome dispersion pattern.

Together, these aims were expected to enhance the comprehension of the molecular mechanisms involved in actin-based transport and help further the understanding of dysfunction and resultant diseases, ultimately in the design of appropriate therapeutic improvement

Chapter 2: Materials and Methods

2.1 Molecular Biology

2.1.1 Polymerase Chain Reaction (PCR)

PCR primers (Sigma) were designed as complementary to a target gene sequence to be amplified during the routine PCR. Otherwise, to construct a restriction site during site-directed mutagenesis, of which, primers were designed to mutate a maximum of 8 base-pairs. At least 20bp of the complementary sequence were included either side of the target mutation to ensure the primers would still anneal tightly to the template DNA.

Sequence	Purpose of primer
TACAATGCGGCCGCGAAGAAATTAGACGGAGCAGA	to construct NotI restriction site (GC/GGCCGC) before the start of GTBM domain
TACAATGCGGCCGCTCCAGGCAGAGCTCACGGTCT	to construct NotI restriction site (GC/GGCCGC) before the start of Spire-Box domain
GCTAAGCGGCCGCGCCCTGAAAAAAGGAAAGCTCT	to construct NotI restriction site (GC/GGCCGC) before the start of FYVE domain

TCGTCCCGGGTTCACCTCGGAGATGAACTTTTT	to construct XmaI restriction site (CCCGGG) at the end of FYVE domain
TCGTCCCGGGtTCAGATCTCGTTGATAGTCCGTTC	to construct XmaI restriction site (CCCGGG) at the end of H2 domain

Table 2.1 PCR Primers that were used in routine PCR to construct restriction sites within Mm-SPIRE1 as a step to construct truncations that tested in this study.

Sequence	Purpose of primer
CAGTTTCTGCCACCAGCATGCTCCAGGCAGAGCTCA	to construct SphI restriction site (GCATG/C) at the end of GTBM domain
TGAGCTCTGCCTGGAGCATGCTGGTGGCAGAACTG	
TTTCTGCCACCATAGCCCGGGTCCAGGCAGAGCTCA	to construct XmaI restriction site (CCCGGG) at the end of GTBM domain
TGAGCTCTGCCTGGACCCGGGCTATGGTGGCAGAAA	
AAAAAAGGAAAGCTCTGACCCGGGTGCTTCTGTTGCCGA	to construct XmaI restriction site (CCCGGG) at the end of Spire-Box domain
TCGGCAACAGAAGCACCCGGGTCAGAGCTTTCCTTTTTT	
AAAAAAGGAAAGCTCGCATGCTGCTTCTGTTGCCGA	

TCGGCAACAGAAGCAGCATGCGAGCTTTCCTTTTTT	to construct SphI restriction site (GCATG/C) before the start of FYVE domain
ACGATGGAGGTGTGTGCATGCGTGGACTGCAAAAAG	to construct SphI restriction site (GCATG/C) at the end of FYVE domain
CTTTTTGCAGTCCACGCATGCACACACCTCCATCGT	
ATCTCCGAGATCTAGCCCGGGATCAGCTCAAGCCGG	to construct XmaI restriction site (CCCGGG) at the end of FYVE domain
CCGGCTTGAGCTGATCCCGGGCTAGATCTCGGAGAT	
ACGATGGAGGTGTGTGCGGCCGCGTGGACTGCAAAAAG	to construct NotI restriction site (GC/GGCCGC) before the start of H2 domain
CTTTTTGCAGTCCACGCGGCCGCACACACCTCCATCGT	

Table 2.2 PCR Primers that were used in site-directed mutagenesis to construct restriction sites within *Mm-SPIRE1* as a step to construct truncations that tested in this study.

The PCR reaction was mixed on ice by adding 38µl Milli-Q water, 5µl 10X Pfu Polymerase Buffer (Promega), 0.5µl 20mM dNTPs (Promega), 1.5 units Pfu Polymerase (Promega), 2.5µl 10µM 5' primer, 2.5µl 3' primer, and 1µl 50 ng/µl cDNA template in a 200µl thin-walled PCR tube. Then the reaction was mixed gently, centrifuged briefly, and then subjected to the PCR cycles using a Sensoquest thermocycler as indicated in table 2-2.

Step	Temperature °C	Time (min)		Cycles	
		R-PCR	SDM	R-PCR	SDM
Initial Denaturation	95	1		1	1
Denature	95	1		35	18
Anneal primers	55	1		35	18
Extend DNA	72	10	12	35	18
Final Extension	72	10		1	1

Table 2.3 Thermocycler setting used for routine PCR (R-PCR) and Site-directed Mutagenesis (SDM)

In Site-directed Mutagenesis, the parental DNA plasmid that would not contain the mutation(s) were digested using methylation-dependent endonuclease (DpnI) (Figure 2.1.1).

16 µl PCR reaction was mixed with 1µl DpnI restriction enzyme (NEB) and 2µl cut-smart buffer (NEB). The mixture was incubated for 1 hour at 37°C and the DpnI then heat-inactivated for 20 minutes at 80°C.

The linear PCR product was then transferred into competent *Escherichia coli* (*E. coli*) DH5α cells to be amplified and the purified and collected as explained below.

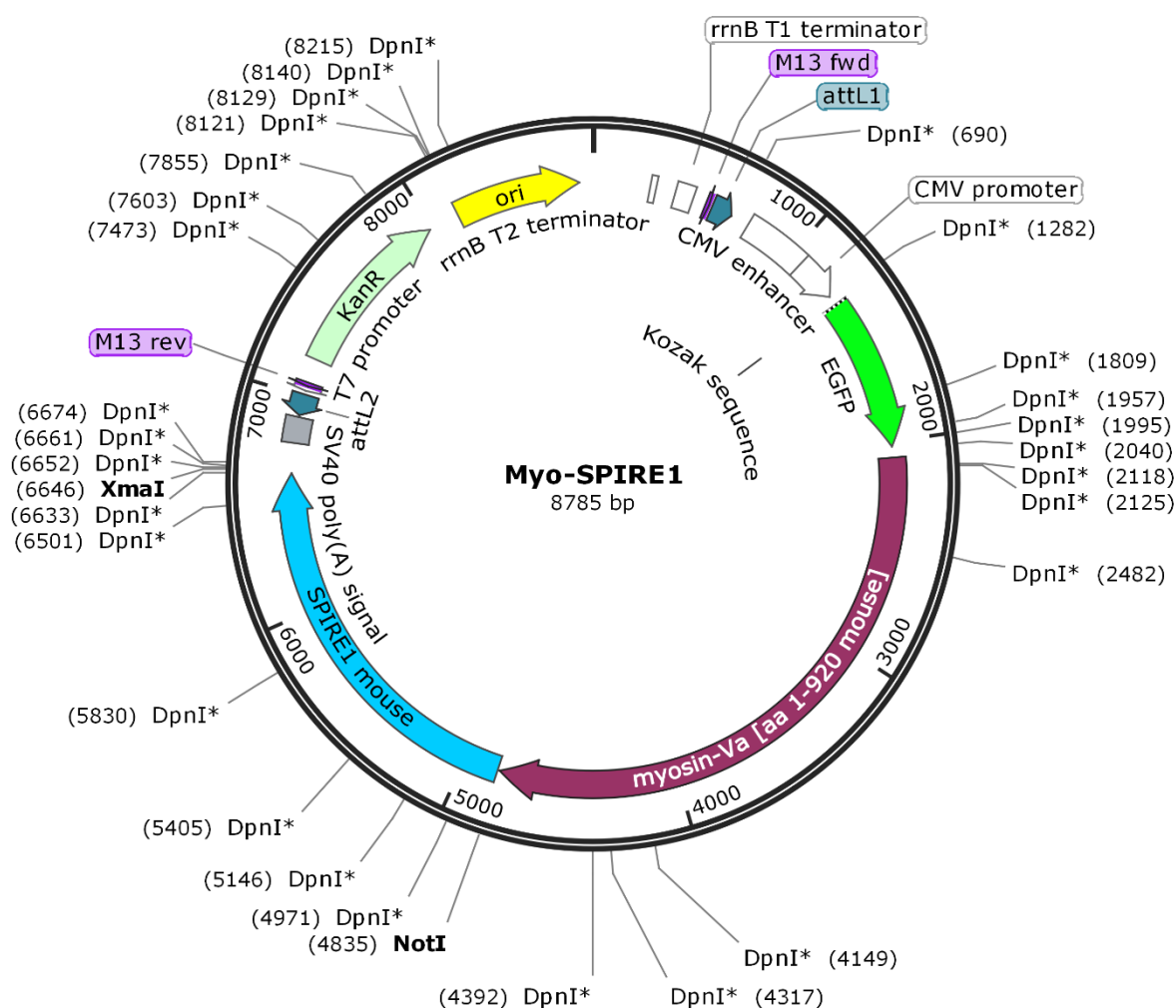


Figure 2.1.1. A map of the pENTR GFPC- Myo-SPIRE1 plasmid encoding Myo-SPIRE1 full-length shows the plasmid feature (e.g. kanamycin resistance (KanR), CMV promoter and the interested restriction sites).

2.1.2 Restriction Digest

Restriction enzyme digestion reactions were prepared by mixing 1 µg of DNA of interest with 10 units of restriction endonucleases (NEB® or Promega) with 2 µl of an appropriate buffer (according to manufacturer's cloning technical guide) and MilliQ water to a final volume of 20 µl. The mixture was then incubated at 37°C for at least 3 hours.

The reaction then was ceased by heat to the appropriate temperature according to the manufacture's guide or by DNA clean-up kit. Then, gel electrophoresis was used to separate the digested DNA fragments. That was by running 5 µl of the reaction on 1% agarose gel

using undigested DNA as control. The digested products were separated depends on their size then visualised under a UV lamp.

2.1.3 Ligation

To insert a gene of interest into a plasmid backbone, both DNA were digested using the same restriction enzyme to generate compatible sticky ends. The DNA would be extracted from the agarose gel and purified using a NucleoSpin® gel and PCR clean-up (Machery Nagel Ref. 740609.250). The manufacturer's instructions have been followed to heat-solubilising the gel and excise DNA. The required amount of DNA in a ratio of 3 insert: 1 vector, was calculated using the Ligation Calculator website http://www.insilico.uni-duesseldorf.de/Lig_Input.html. The insertion and vector were mixed with 1 µl (400 units) of T4 DNA ligase enzyme (Promega), 2 µl 10X T4 DNA buffer and MilliQ water were added to a final volume of 20 µl. The reaction then was incubated at 16°C for 18 hours. A control experiment was carried out by DNA-ligase the DNA vector alone without adding a DNA insert. The process was assessed by diagnostic restriction digest and agarose gel images as described in the previous section.

In some cases, the vector was dephosphorylated before ligation to reduce the probability of self-ligation. After the restriction digest, the vector was incubated for 1 hour at 37°C with 1 µl of Calf Intestinal Phosphatase (CIP) (NEB®). Dephosphorylation reactions were ceased by PCR clean-up kit (Machery Nagel®), and then the ligation was performed as described above. The successful ligation then transformed into competent (*E. coli*) DH5α cells to propagate the ligated clones.

2.1.4 Heat-Shock Transformation of Chemically Competent Bacteria

The plasmid DNA was transferred into competent *E. coli* DH5 α cells to facilitate their storage and amplification. For this, a 50 μ l aliquot of competent *E. coli* DH5 α cells (provided kindly from Dr Hume lab) were thawed on ice for 30 minutes and followed by gently mixed with 5 μ l of plasmid DNA in an Eppendorf tube. The cell-DNA mixture was then incubated for an additional 30 minutes on ice. After which time, the mixture was heat-shocked by heating in a water bath at 42°C for up to 90 seconds and returning immediately to the ice to cool for further 2 minutes. Then, the transformation mixture was incubated with 250 μ l of sterile LB broth medium (4g NaCl, 4g tryptone, 2g yeast extract in 400 ml distilled water) in a 37°C shaking incubator (200 rpm) for one hour. No antibiotics were added to the mixture to allow the bacteria to grow and generate antibiotic-resistant proteins as that encoded in the plasmid of interest. Then the cells were spread on an agar plate containing the appropriate antibiotic (50 μ g/ml Kanamycin or 100 μ g/ml ampicillin). The bacteria with target plasmid then would grow and form colonies after 18 hours incubation on 37°C.

2.1.5 Inoculating Overnight Cultures, Production Glycerol Stock, and Plasmid Mini-Preparation

A single bacterial colony from an agar plate was transferred under aseptic conditions into 5 ml LB broth with an appropriate antibiotic (50 μ g/ml Kanamycin or 100 μ g/ml ampicillin). The cultures were incubated in a 37°C-shaking incubator (200 rpm) for 18 hours to allow the bacteria to grow to a high density. After that, transformed cells were stored for the long term in a glycerol stock by mixing 500 μ l of the overnight culture with 500 μ l of 30% sterile glycerol in a cryovial and stored at -80°C.

To extract the plasmid of interest from the *E. coli* cells, NucleoSpin® Plasmid kit (Machery Nagel Ref. 740499.250) was used, and the manufacturer's instructions have been followed. In this process, SDS/alkaline lysis was used to denature plasmid- and the bacterial genomic-DNA. The genomic DNA and cell debris then removed by 10 minutes centrifugation. The plasmid DNA then neutralised and bound to a silica membrane, washed by ethanolic buffer and eluted in 5 mM Tris/HCl, pH 8.5 that was provided with the kit. The purified DNA is then suitable for other molecular biology applications.

2.1.6 DNA Quantification

The concentration and purity of DNA were assessed by NanoDrop 2000 (Thermo Scientific), with elution buffer used as a blank. The 260nm/280nm ratio was used to determine the DNA purity from protein and RNA, considering a range of 1.8- 2.00 considered as acceptable purity for DNA.

2.1.7 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to separate DNA depending on their size, which could help in the DNA fragment purification and analysis.

1% Agarose gel was prepared by mixing agarose powder with 1X TBE (1.08% w/v Tris base, 0.55% w/v boric acid, 0.0585% EDTA w/v in dH₂O, adjusted to pH8.0). Then, the gel mixture was heated by microwave and allowed to cool down to about 45 °C before 0.5 µl ethidium bromide was added to label the running DNA.

An appropriately sized casting tray was prepared with a compatible comb. The agarose solution was then poured into the tray to a depth of about 5 mm and allowed to solidify at room temperature for 15-20 mins.

For tracking of DNA migration during DNA-electrophoresis, 5-10ul of the DNA-sample was added to 6x DNA loading buffer (40% sucrose, 0.25% bromophenol blue in Milli-Q water) to make a final concentration of 1X.

After gel solidification, the casting tray was placed in an electrophoresis tank to run the samples from the negative electrode (black) to the positive one (red). The tank was then filled with 1X TBE buffer, and the comb removed. Three μ l of 1 kb DNA marker (NEB®) (500 μ g/ml) was used as a size indicator, and 5-10 μ l of the sample was loaded in each well separately. Then the gel was run at 98 V till the dye reach the end of the gel. The gel then imaged by UV in a GelDoc system using GenoSnap 7.12 software.

2.1.8 Sequencing

To assess the efficacy of the cloning process and to ensure no functional mutation has occurred in the gene of interest, sequencing DNA was performed via SourceBioscience. A 6 μ l containing 600 ng plasmid DNA was sent along with 10 μ l of 3.2 μ M primer unless company stock primers were used.

2.1.9 Adenovirus Production

Recombinant adenovirus was generated using the ViraPower™ Adenoviral Gateway® Expression Kit, catalogue no. K4940-00 of pAd/PL-DEST™ Gateway® Vector, (Invitrogen) in accord with the manufacturer's instructions with some modification. In this process, the

gene of interest was transferred from pENTR plasmid vector into pAd/PL-DEST (hereafter referred to pAd vector) using Gateway® LR clonase II enzyme mix. In this process, 100 ng of the gene of interest in pEnter- vector was mixed with 150 ng of pAd-DEST vector in Tris-EDTA (TE) buffer solution at pH 8.0 to a final volume of 8 µl. Then, 2µl of enzyme mix was added to the reaction mixture, and this was incubated at 25°C overnight. The reaction was ceased by incubation with 2 µg of proteinase-k and for 10 minutes at 37°C. The reaction mixture was then transformed into *E-coli* DH5α cells, and recombinant clones of pAd and the gene of interest were selected using ampicillin (as described in section 2.1.3). DNA from ampicillin-resistant clones was then purified using the mini-prep kit (as described in section 2.1.4). Bacterial DNA was then removed, and the encoding gene for the viral production (named left and right inverted terminal repeats ITRs) were exposed by digest 5 ug of pAd vector with PacI restriction endonucleases overnight at 37°C. The digestion was then terminated by heating at 65 °C for 20 minutes. Subsequently, the digested pAd vector was used in HEK293A transfection to generate viral particles as described in section 2.3.1.

2.2 Cell Cultures Techniques

2.2.1 Maintaining Immortal Cells

Immortal murine melanocytes cell lines of wild-type (melan-a), leaden (melan-ln), ashen (melan-ash) and FMN1-deficient melanocytes (melan-f). The cell lines were derived from non-agouti/black C57BL/6 mice as described in (Lavado et al., 2005, Alzahofi et al., 2020, Bennett et al., 1989). All cell lines were maintained in a complete medium of RPMI-1640 medium (Invitrogen) that was supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco), 10% (v/v) fetal bovine serum (FBS) (Gibco), and 40 nM PMA (phorbol 12- myristate 13-acetate) (Sigma), and incubated at 37°C with 10% CO₂ (Hume et al., 2007,

Hume et al., 2006). The melanocyte cell lines described here are available from the Wellcome Trust Functional Genomics Cell Bank <http://www.sgul.ac.uk/depts/anatomy/pages/WTFGCB.htm>.

Murine melanoma cell line B16F10 were maintained in RPMI-1640 medium that was supplemented with 100 U/ml penicillin, 100 µg /ml streptomycin (Gibco), 5% (v/v) FBS and incubated at 37°C with 10% CO₂.

Human embryonic kidney 293A cells (HEK 293A) were maintained in a complete medium of D-MEM (L-Glutamine-high glucose medium) (Invitrogen) supplemented with 100 U/ml penicillin, 100 µg /ml streptomycin, 10% (v/v) FBS, and incubated at 37° C with 10% CO₂.

All the aforementioned cell lines were typically passaged when the confluency (the amount of surface area covered by cells) is in 80-90%. For that, the cells were trypsinised using 1X Trypsin-EDTA (Gibco) and transferred into filter cap flasks either T25 (Nunc) or T75 (Corning).

2.2.2 HEK293A Transfection

Cells were trypsinised and seeded in 6-well plates (Corning) in a density of 5×10^5 cells/well and allowed to attach and spread overnight. For each well transfection, 10 µl of the transfection reagent Fugen6 (Invitrogen) was diluted in 1.5 ml of opti-MEM (serum-free medium) (Gibco) and incubated at room temperature for 5 minutes. After that, 1 µg (10 µl) of Pac I digested pAd of the gene of interest (prepared in section 2.1.8) were added and incubated for further 15 minutes at room temperature to allow the DNA-Fugen6 complex to form. After which time, the cell's growth medium was replaced with transfection mixture and incubated at 37°C with 10% CO₂ overnight. Then the transfection mixture was replaced

with the complete growth medium. Cells were then typically incubated for 5-7 days in order to allow the virus to amplify. When the cell reached 80-90% confluency, they were trypsinised and transferred into a sterile 10 cm tissue culture plate (Corning) with 10 ml complete D-MEM medium. Virus production was visualised using a 10x/0.25 Ph1 lens of Zeiss Axiovert S100 inverted microscope with an appropriate fluorescent channel (as described in section 2.4.2.), and images were captured using an AxioCam MR3 CCD camera. The success of virus production was confirmed by observation of virus plaque formation and expression of EGFP. The medium and virus-infected cell then collected in falcon tube and lysate by three cycles of freezing at -80 °C for 30 mins then thaw at 37 °C for 15 mins. The cell debris was pelleted after that by centrifugation at 3000 rpm for 15 mins, and the viral particles were harvested under sterile conditions and stored in cryovials at -80 °C. A second amplification process was carried out to generate higher viral titres by infecting 90% confluency HEK 293A cells (3×10^6 cells) in 10 cm plate (in complete growth medium) with 100 µl of the crude viruses. The viral particles were collected typically after 3 days using a similar method to that described above.

2.2.3 The siRNA-Mediated Knockdown

SPIRE1/2-double knockdown was performed in melan-a cells, using non-targeting (NT), Rab27a, and FMN1- mediated siRNA knockdown as controls to assess the efficiency of the process as described in (Hume et al., 2007, Robinson et al., 2017). In brief, cells were trypsinised and seeded onto a 13 mm glass coverslip in a 24-well plate (Nunc) at a density of 5×10^4 cells/well. The cells then were allowed to attach and spread overnight. After that time, a total of 1 µl of (20 µM) oligo-siRNA (Sigma) and 1 µl of the of transfection reagent oligofectamine (Invitrogen) were diluted separately, for each well, with 40 and 7.5 µl of opti-

MEM, respectively, and incubated at room temperature for 5 minutes. The two dilutions were mixed together and incubated for further 15 mins at room temperature to form DNA-oligo complex. Then an additional 200 µl of Opti-MEM/transfection-well was added to the mixture. After that, the growth medium was replaced by the transfection mixture at 37°C with 10% CO₂ overnight. After which time, the transfection mixture was replaced with complete growth medium. Cells were then typically incubated for 72-96 hours in order to allow the siRNA-specific mRNA to be knocked down.

Name of oligo	Sense oligonucleotide sequence
Non-targeting (NT)	UAGCGACUAAACACAUCAA
Rab27a (27AUTR1*)	UUGAGAAUCCAGAUUUCAA
FMN1	GGACAAACCUGAACAAUUU
SPIRE1#3	GGACGACAUUCGGUGCAAA
SPIRE2#4	CAAAGAACACUGCACGAGA

Table 2.4 The Sense oligonucleotide sequence of siRNA used in this study.

2.2.4 Transduction of Melanocytes with Adenovirus

After siRNA knockdown of SPIRE1/2 mRNA in melan-a cells, adenovirus encoding siRNA-resistant EGFP-SPIRE was added to the growth media. Cells then were incubated with the viruses overnight at 37°C with 10% CO₂ to allow SPIRE encoding gene to express in the cells.

The cells were then fixed and stained for imaging using a fluorescence microscope, as described below to assess the level of protein expression.

2.3 Microscopy

2.3.1 Fixing of Mammalian Cells

Growth medium was removed, and cells were washed gently with 1X phosphate-buffered saline (PBS) (Invitrogen). 200 µl of 4% paraformaldehyde (pH 7.0) (Sigma) was then added to each of well for 15 minutes at room temperature. Then the cells washed three times with 1X PBS and then immersed in 50mM ammonium chloride (Sigma) to reduce auto-fluorescence for 10 mins. A further two 1X PBS washes were performed. Cells may then be stored in PBS at 4°C or stained directly.

2.3.2 Indirect Immunofluorescence Staining

The PBS was then discarded, and fixed cells were incubated in immunofluorescence buffer (IFB) (0.5% bovine serum albumin (BSA) (Fisher Scientific), 0.05% saponin (Sigma) in 1X PBS for 15 minutes to permeabilise cell membrane and allow antibodies to access intracellular antigens and block unspecific binding of primary antibody at the same time. Cells were then incubated in primary antibody (mouse anti-EGFP (Roche) diluted in a ratio of 1:300 in IFB) for 30 minutes. Then, cells were washed five times by PBS to remove unbound primary antibody. Subsequently, cells were incubated in secondary antibody (goat-anti-mouse (Alexa) in a ratio of 1:500) for 30 minutes. Cells were then washed three times and nucleus staining by incubating the cells in 600nM DAPI (4',6-diamidino-2-phenylindole) for 5 minutes and washed three times as previously. All incubations were performed in darkness to prevent any bleaching of the fluorochrome. Cells were then mounted by inverting the

coverslip on a glass slide onto a ~6 µl droplet of immunofluor mounting medium (MP Biomedicals) and stored at 4°C as soon the medium hardened.

2.3.3 Image Capture

Stained cells were visualised and imaged in fluorescence and phase contrast mode using a Zeiss Axiovert S100 microscope equipped with CP-Achromat 10x/0.25 Ph1 (Zeiss 440931-0000-000) and 40x/1.30 Ph3 oil immersion Neofluar lenses and fluorescence filters as shown in table 3.2.

Filter set no.	Excitation	Beam Splitter	Emission
02	G 365	FT 395	LP 420
10	BP 450-490	FT 510	BP 515-565
15	BP 546/12	FT 580	LP 590

Table 2.5 Features of fluorescence filters used in Zeiss Axiovert S100 microscope.

2.4 Data Collection and Analysis

2.4.1 Quantifying Melanosome Distribution in Melanocytes

To quantify the distribution of melanosomes in melanocytes, the captured images were analysed manually via ImageJ® software in two different ways. First, cells in a low magnification (10x) fields of view were counted using the "multi-point" tool and manually scored as containing a dispersed, clustered, or hyper-dispersed distribution of melanosomes. Then the percentage of each phenotype was calculated by dividing the

number of cells with each distribution on the total of the cells in the field of view x 100. The mean of at least three of this biological repeats was considered as one technical replicate.

Second, the extent of melanosome dispersion distance (pigment area) was calculated for cells in higher magnification (40x) fields of view by drawing around the cell membrane using the "freehand selection" tool and determine the total area via the software. Then the same process applied to determine the area of the melanosomes mass within the cell.

Subsequently, melanosome area as a proportion of cell area was determined by the following calculation, melanosome area/cell area x 100, where 100% indicates full melanosomes dispersion, i.e. wild type phenotype and less than 50% indicates a melanosome clustered phenotype. Each cell represents a biological replicate sample, and a total of 10 cells considered in one technical replicate.

2.4.2 Quantifying Actin Fluorescence Intensity

To compare the fluorescence intensity of actin filaments in experimental cells, the treated cells were fixed and stained with texas-red-phalloidin (1:300) (as described previously). Then, the fluorescence intensity of cells in higher magnification (40x) fields was measured by using ImageJ®. By drawing around the cell membrane using the "freehand selection" tool and determine the total area and the Integrated Density via the software. The same process was applied for three areas outside the cell, and the average intensity of these areas was taken as the background. The corrected total cell fluorescence (CTCF) was calculated using the formula: Integrated Density – (Area of selected cell X Mean fluorescence of background readings). Each cell represents a biological replicate sample, and a total of 10 cells considered in one technical replicate.

2.4.3 Statistical Analyses

The data statistically analysed and compared by One- and two- way and ANOVA with Tukey's multiple comparisons test via Prism software.

2.5 Biochemistry

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

2.5.1.1 Gel Preparation

Gels were prepared by mixing components outlined in Table 2.5 in the order they appear.

(ammonium persulfate (APS) (Fisher Scientific) was made fresh on the day the gels were prepared). The resolving gel then was poured firstly between glass plates and 1.5mm spacers in the BioRad Mini- PROTEAN Tetra Cell casting frames leaving ~2 cm from the top for stacking gel. A 500 µl water-saturated butanol was overlaid the resolving gel to smooth, and level surface on top of the gel.

After complete polymerisation, the butanol overlayer was removed and stacking gel was poured over the resolving gel and suitable comb was insert.

Reagents	10% resolving gel	stacking gel
ddH ₂ O	4 ml	3.4 ml
30% acrylamide	3.3 ml	0.83 ml

1.5M Tris-HCl pH 8.8	2.5 ml	-
1M Tris-HCl pH 6.8	-	0.63 ml
10% SDS	0.1 ml	0.05 ml
Ammonium persulfate (APS)	0.1 ml	0.05 ml
N,N,N',N'- tetramethylethylenediamine (TEMED)	0.004 ml	0.005 ml

Table 2.6 Components of SDS-PAGE gels.

The gel cassettes then placed in the Mini-PROTEAN Tetra Cell tank (BioRad) and covered by SDS-PAGE- running buffer (25mM Tris-HCl, 192mM glycine, 0.1% (w/v) SDS).

2.5.1.2 Samples Preparation

The protein samples were prepared by boiling 5 volume of the sample with 1 volume of 6X SDS-sample buffer (375mM Tris-HCl pH 6.8, 6% SDS, 48% glycerol, 0.03% bromophenol blue, 9% β -mercaptoethanol) for 10 minutes at 95°C. The samples then run using 40mA current/per gel cassette until the dye had reached the bottom of the gel (typically ~120 minutes).

2.5.1.3 Transferring to Membrane

Separated proteins were transferred to Polyvinylidene difluoride (PVDF) membrane using the Geneflow wet transfer system. PVDF membrane was activated by pre-soaking in 100%

methanol for 30-60 seconds and then incubated in transfer buffer (25mM Tris, 192mM glycine, 10% methanol) for 2-5 minutes. The gel was then layered on top of the PVDF membrane and placed in the transfer sandwich in the Geneflow cassette using the following order: (three pieces of 10x 10 cm 3mm-filter paper, PVDF transfer membrane, the gel and three pieces of filter paper). The filter paper must be pre-soaked in transfer buffer beforehand. The cassette was then placed in transfer tank filled with the ice-cold transfer buffer with the PVDF membrane closest to the cathode. Transfer took place at 4°C, using either 200V for 3 hours or 30V for 20 hours.

2.5.1.4 Blocking and Antibody Staining

After transfer completion, the PVDF membrane was then blocked with 5% skimmed milk (MARVEL) in 1x PBS, 0.1% Tween-20 and for 1 hour at room temperature to reduce the background and prevent the non-specific binding to the primary antibody. The membrane was then incubated with the primary antibody diluted in the blocking buffer, as indicated in table 2.6. for 1-3 hours at room temperature or 20 hours at 4°C. Subsequently, the membrane was washed 3x30 minutes using 1x PBS, 0.1% Tween-20. Then the membrane was incubated with the diluted-secondary antibody and washed as for the primary antibody.

Primary antibody	Source	Organism raised in	Dilution
Anti-GFP	Abcam ab290	Rabbit	1:10000
Anti-Rab27a	AB2345-200	Goat	1:10000
Anti-Rab27	sicgen ab7223-200	Goat	1:10000
Anti-spire1	H00056907-M01	Mousse	1:5000
Anti-spire1	Santa Cruz sc-85162	Rabbit Polyclonal	1:1000
Anti-mouse-spire-1 sa2133	Kindly was provided from Eugen Kerkhoff (14-12-03)	Rabbit Polyclonal	1:40

Table 2.7 The primary antibodies used in this study

Secondary Antibody	Source	Dilution
Donkey anti-Goat IR800	Licor	1:15000
Donkey anti-Mouse IR680	Licor	1:15000
Donkey anti-Rabbit IR680	Licor	1:15000

Table 2.8 The secondary antibodies used in this study

2.5.1.5 Imaging the PVDF Membrane

The stained PVDF membranes were scanned using the Licor Odyssey instrument. The image was acquired by Image Studio (Version 5.2) software applying the following settings: resolution: 84 μ m, quality: high, offset: 0.5mm, intensity: 7.5.

2.5.2 Immunoblotting

Melan-a melanocytes were trypsinised when reached 80-90% confluency by using 1X Trypsin-EDTA (Gibco) (500 μ l for T25) and seeded at 6-well-plates (Nunc) in the amount of 3×10^5 /well and allowed to attach and spread overnight. After that, a different amount of adenovirus vectors encoding the target proteins were added to the growth media of melanocytes. Cells then were incubated with the viruses overnight at 37°C with 10% CO₂ to allow encoding gene to express in the cells. The cells were then trypsinized and collected in Eppendorf tube, centrifuged and washed by complete medium to inhibit trypsin by the serum then washed by PBS. The proteins then denatured by SDS-sample buffer and separated depends on their mass using SDS-PAGE and detected by immunoblotting as described above.

2.5.3 Immunoprecipitation

The cells were prepared and lysed as described previously in (Hume et al., 2001) with some modification. In details, Melan-a cells were collected from 30X T75 flask (Corning) to 80-90% confluency. The cells were Trypsinized and re-suspended in complete growth media, for which the serum inhibit trypsin, then centrifuged and washed twice by PBS. The cells then were then lysed in IP-buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EGTA,

1% CHAPS, and 0.5 protease inhibitor cocktail tablet (Cat. No. 05-892-791-001). Then, cells debris was removed by centrifugation the lysate on 10000 g for 15 min at 4°C.

The Protein A/G UltraLink Resin (beads) were washed 3 times by ice-cold IP buffer (without protease inhibitor) and centrifuged on 800 rpm for 1 min at 4°C. Then, divided into 3 groups:

- 1) for pre-clearing the lysate to reduce the non-specific binding and reduce the background.
- 2) for negative control.
- 3) incubated with 1 ug goat polyclonal Rab27a antibody (sicgen-ab7223-200) for 1 hour at 4°C then centrifuge at 3000 x g for 2 min at 4°C. then the supernatants were discarded, and the pellets were washed twice with IP buffer.

The lysate pre-cleaned by incubating with washed beads for 60 minutes at 4°C with gentle rotation. The beads then discarded after centrifugation on 10000 g for 10 mins, and the lysate was divided into two equal amount; 1) incubated with washed beads without antibody as a negative control. 2) incubated with beads with antibody-immobilised beads. Both incubated under rotary agitation overnight at 4°C.

After which time the mixtures were centrifuged on 1000 rcf (1000 xg) for 5 min, and the supernatant was discarded. The pellets were washed by IP buffer 3 times to remove any non-specific binding. Then, the IP-products on the pellets either sent for mass spectrometry analysis or the protein was eluted using SDS sample buffer and analysed by immunoblotting technique.

Chapter 3: SPIRE Proteins Maintain Melanosome Dispersion by Nucleating Actin Filament in Cooperation with FMN-Type Formin

3.1 Introduction

A critical physiological process for living cells is to transport their organelles and other material from their formation site to their functional destination inside or outside the cell. This process involves complex cooperation between different proteins which function as signalling, docking, motors and tracks for the transport. Understanding the molecular mechanisms and the role of proteins involved would help understand the dysfunction and resultant diseases and ultimately in the design of appropriate therapeutic improvement. Melanosomes of skin melanocytes are considered a model for lysosome-related organelles. After being synthesised in the perinuclear cytoplasm of melanocytes, melanosomes are transported to the tip of melanocyte dendrites as a crucial step to be transferred to the surrounded keratinocytes and then serve its main function as a photo protector. Despite the intensive studies conducted on melanosome transport in mammalian melanocytes, the molecular mechanisms that involve and regulate this process remain largely obscure. The genetics and *in vitro* studies on Griscelli syndrome and the proteins involved revealed a crucial role of a tripartite complex for melanosome centrifugal/ anterograde transport. This complex comprises of the small-GTPase Rab27a which is associated with the cytoplasmic face of the melanosome membrane. GTP-Rab27a then recruits myosin-Va to the

melanosome membrane through its effector Melanophilin (Hume et al., 2001, Lydia et al., 2001, Hume et al., 2002, Fukuda et al., 2002, Nagashima et al., 2002).

However, the involvement of microtubules (MTs)- and actin (AFs)- based transport and their related proteins has been controversial. Recent studies have questioned the previously formulated- 'co-operative capture' model of melanosome transport (Evans et al., 2014, Alzahofi et al., 2020, Robinson et al., 2017). These studies used siRNA-mediated-knockdown assays and disrupting the MTs and AFs polymerisation. They showed that the retrograde/centripetal transport of melanosomes is MT-dependent while the anterograde/centrifugal transport is AF-dependent (Evans et al., 2014, Alzahofi et al., 2020, Robinson et al., 2017).

The contribution of actin nucleation factors and regulatory proteins in melanosome dispersion of wild-type melanocytes was investigated using siRNA mini-library comprised of 130 pools (4 target-specific oligonucleotide duplexes/pool) (Alzahofi et al. (2020) and Hume and Robinson unpublished study). The study showed that depleting both the actin nucleation proteins of SPIRE1/2 or actin-elongation protein formin1 (FMN1) induced perinuclear melanosome clustering similar to that characterises ashen, leaden and dilute mice melanocytes (Wilson et al., 2000, Hume et al., 2001, Wu et al., 2001, Ménasché et al., 2003, Menasche et al., 2000, Mercer et al., 1991, Wu et al., 1998, Pastural et al., 1997). The mean percentage of cell area that occupied with melanosomes (mean pigment area % total) was $52.56 \pm 6.868\%$ and $45.07 \pm 8.061\%$ in the cells treated with SPIRE1/2- and FMN1-specific siRNA respectively compared to $86 \pm 10.28\%$ and $31.28 \pm 8.242\%$ in the cells treated with non-targeting- and Rab27a-specific siRNA (Figure 3.1.1.). The specificity of the siRNA effects was verified by adding back siRNA-resistant of SPIRE1/2, and FMN1 in SPIRE1/2

depleted melan-a and immortal FMN1-deficient melanocytes (melan-f). That resulted in restoring melanosome dispersion to the level of wild-type melanocytes. This indicates that both SPIRE1/2 and FMN1 are the primary actin-related proteins that regulate the anterograde transport of melanosomes (Alzahofi et al., 2020).

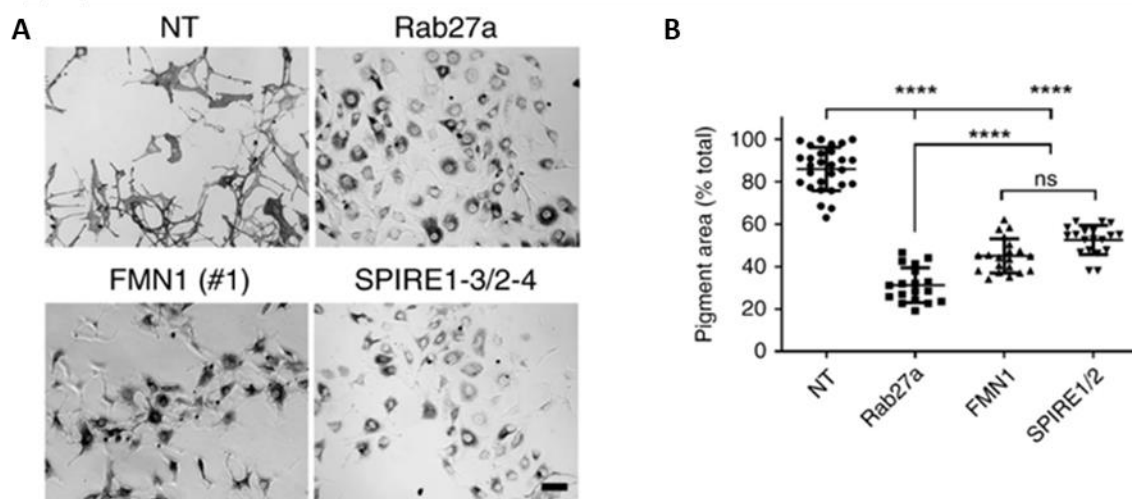


Figure 3.1.1. Depleting SPIRE1/2 and FMN1 resulted in clustering melanosome at the perinuclear cytoplasm of melanocytes. A) Bright-field- microscopic images of melan-a after 72 hours of siRNA-transfection. B) A scatter plot shows the percentage of the pigments area in individual cells transfected with the indicated siRNA (n (cells)= 28 (NT), 18 (Rab27a), 20 (FMN1) and 22 (SPIRE1/2)). ****= $P < 0.0001$ ns= no significant differences between the compared populations as determined by one-way ANOVA (Alzahofi et al., 2020).

SPIRE/spir and cappuccino (*Drosophila* formin) are first identified for maternal effects on both the dorsal-ventral and anterior-posterior axes of the egg and embryo of *Drosophila* (Manseau and Schupbach, 1989). The same effect was observed after inhibiting actin polymerisation using cytochalasin-D. This linked SPIRE function with the actin cytoskeleton (Manseau et al., 1996a). Mammalian formin1 and formin2 (FMN1/2) and *Drosophila* cappuccino (Cappu) belong to the FMN-subgroup of the formin family of actin-nucleation factors. The C-terminal regions of FMN proteins feature with a common formin homology 1 and 2 domains (FH1/2) that facilitate actin polymerisation (Otomo et al., 2005). Followed by

formin-SPIRE interaction motif (FSI) that mediates direct interaction with an acidic groove at the surface of the KIND domain of SPIRE (Pechlivanis et al., 2009) (Figure 3.1.2).

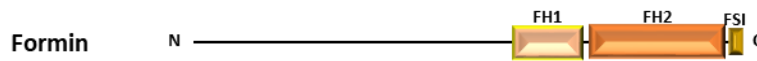


Figure 3.1.2. A schematic representation of the domain structure of actin nucleation region of FMN-type Formins. Actin nucleation region mainly exists within the C-terminal domains of these formins. It comprised of the FH1 domain that binds profilin–actin while FH2 binds barbed-end of the actin filament. FSI binds to SPIRE which starts two-strands actin filament that is elongated by formin.

SPIRE expresses in mammalian tissues as three isoforms of SPIRE1 (SPIRE1, SPIRE1-exon 9, and SPIRE1-exon 13) and a single SPIRE2 (Kollmar et al., 2019). All share the core functional domains that are: an N-terminal KIND domain (kinase non-catalytic C-lobe domain) which interacts directly with the C-terminal formin-SPIRE interaction motif (FSI) of FMN-subgroup formin. The KIND is followed by a cluster of four of WH2 domains (Wiskott-Aldrich homology region 2) which are a defining feature of SPIRE proteins. The four WH2 bind to four actin monomers to nucleate an actin filament (Quinlan et al., 2005). At the middle of SPIRE protein sequences are the myosin-V globular tail binding motif (GTBM) that binds to the globular tail domain (GTD) of the three isoforms of myosin-V (a,b, and c) (Pylypenko et al., 2016). In the C-terminal region of SPIRE is the SPIRE-Box domain, which is a highly conserved region and shares sequence similarity with SHD1 of Rab27a effectors (Alzahofi et al., 2020). The SPIRE-Box is followed by the modified FYVE (m-FYVE named after the cysteine-rich proteins Fab1, YOTB, Vac1 and EEA1)-type zinc finger comprising eight cysteine residues capable of binding two zinc ions and forms a hydrophobic “turret loop”. The structure facilitates SPIREs sub-cellular localisation by interacting with negatively charged lipid (phosphatidylinositol) in the vesicular membrane and by intruding the lipid bilayer via the hydrophobic contributions of the turret loop. M-FYVE also regulates the SPIRE function by interacting with N-terminal KIND domain to form back-folded inhibited conformation

(Kerkhoff, 2011). Finally, the C-terminus flanking alpha-helical domain that is phosphorylated by Jun N-terminal kinase (JNK)-interacting protein (Otto et al., 2000). It also shares some sequence similarity with SHD2 of Rab27a effectors (Alzahofi et al., 2020) (Figure 3.1.3). SPIRE1-Exon-9 identically localised with SPIRE1 on transported vesicles while SPIRE1-Exon-13 localised in mitochondrial outer-membrane and mediate mitochondrion constriction and fission (Kollmar et al., 2019, Manor et al., 2015).

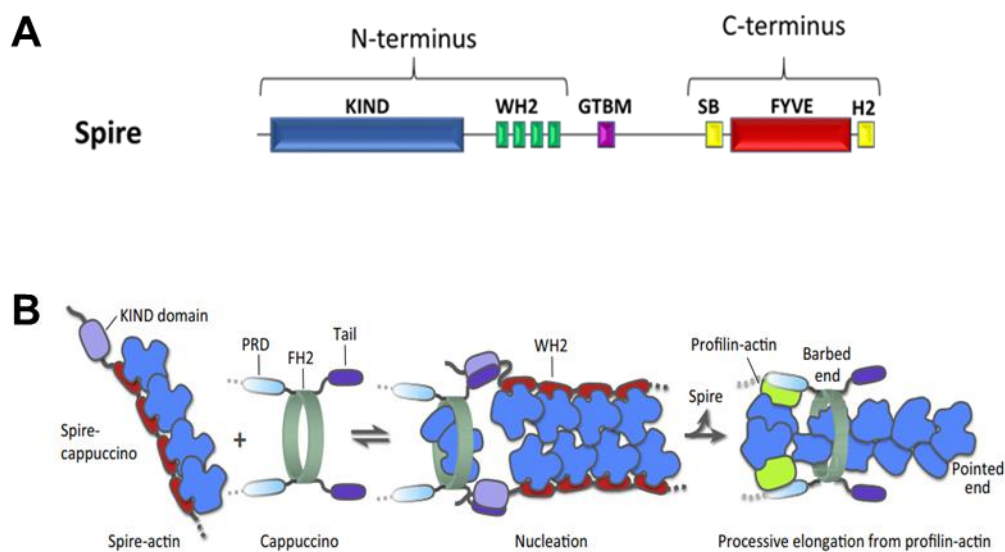


Figure 3.1.3 A) A schematic representation of the domain structure of SPIRE proteins. B) The proposed actin nucleation mechanism of spire: formins (bottom) (from (Dominguez, 2016): in this mechanism, FSI motifs of a dimer of formin interact with KIND domain of two spire monomers. Then the eight actin monomers that bind to spire-WH2 domain serve as a seed for actin filament helix. Subsequently, formin elongates the seed by adding profilin-actin via PRD (FH1).

Based on the results of several studies, the direct interaction between SPIRE and FMN to nucleate actin filaments is considered crucial for different cellular and physiological functions in the fruit fly and mammalian. Including fertility (Pfender et al., 2011, Wen et al., 2018), cell metastasis (Lagal et al., 2014), pathogens-host interaction (Kuhbacher et al., 2015, Daniel et al., 2016). DNA repair (Belin et al., 2015), behavioural learning (Pleiser et al., 2014), and long-range intercellular transport (Kerkhoff et al., 2001, Alzahofi et al., 2020, Schuh, 2011).

In this chapter, the role and involvement of SPIRE domains in melanosome anterograde transport were investigated. To this end, a siRNA-mediated knockdown and rescue assay was carried out in cultured melanocyte (Figure 3.1.4 A). In this assay, the endogenous SPIRE1/2 were depleted using short-interference RNA (siRNA). Subsequently, siRNA-resistant truncations or mutations of human-derived SPIRE1 or 2 (Figure 3.1.4 B-F) were transiently expressed in SPIRE1/2-depleted melan-a. The cells then fixed and stained and the melanosomes distributions visualised using a fluorescence microscope. The cells then scored manually as they have clustered, dispersed or hyper-dispersed melanosomes, and the percentage of each phenotype was used as a read-out for the domain contribution in this process.

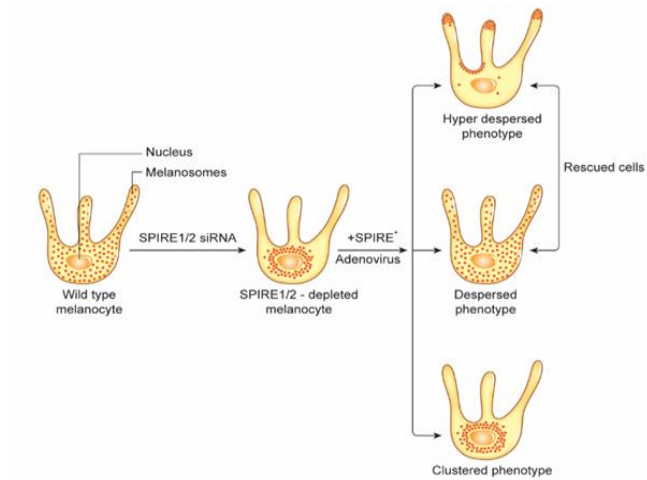
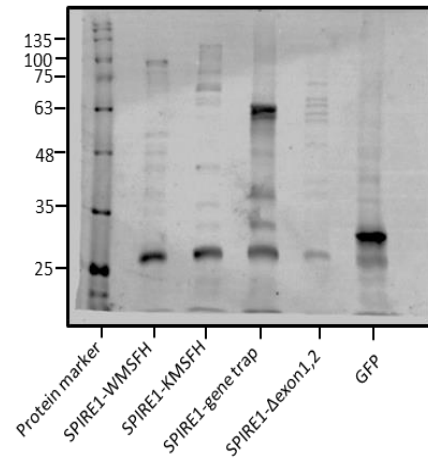
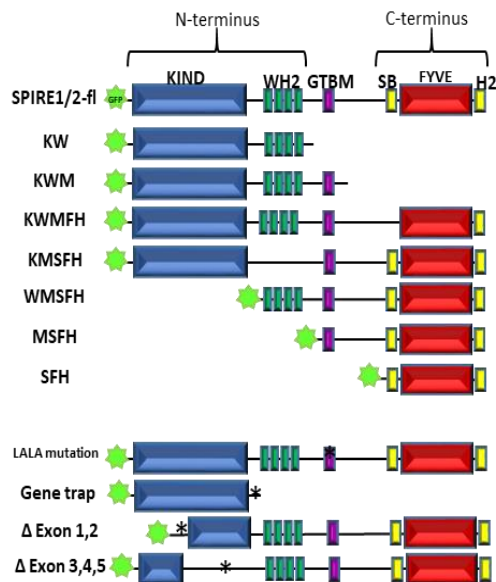
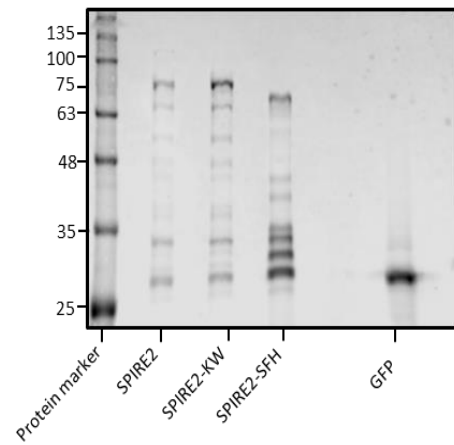
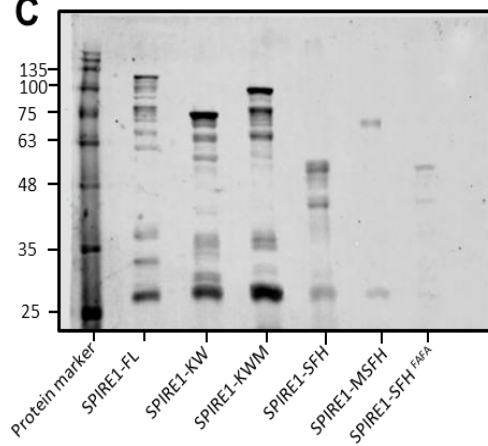
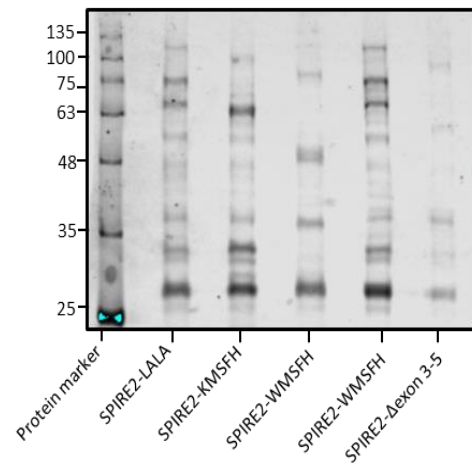
A**D****B****E****C****F**

Figure 3.1.4 SPIRE1/2 rescue assay. A) A schematic representation of the functional assay/ SPIRE rescue assay conducted in this chapter; B) A schematic representation of the domain structure of human SPIRE1/2 and the correspondence with truncations and mutations used in this chapter fused to EGFP at their N-termini. C, D, E, F) The integrity and the size of proteins used in this chapter: adenoviruses encoding the indicated proteins were expressed in melan-a melanocytes, the protein expression was monitored by fluorescence optics. After confirming protein expression, the cells were harvested by Trypsin-EDTA. The cells then lysate in SDS-sample buffer and the proteins separated by 10% SDS-PAGE and immunoblotted using mouse anti-GFP. Abbreviation: K, KIND; W, WH2; M, GTBM, globular tail domain-binding motif; S, SB, SPIRE-Box; F, FYVE (named after the cysteine-rich proteins Fab1, YOTB, Vac1 and EEA1); H, H2, C-terminal flanking sequences similar to H2 of SHD-proteins.

3.2 Functional Disruption of SPIRE1/2 Affects Melanosome Dispersion

To confirm the role of SPIRE proteins in long-range actin/myosin-Va driven organelle transport, melanosomes of skin melanocytes have been used as a model cargo, and the effects of SPIREs depletion on melanosome distribution were assessed. For this, immortal wild-type mouse melanocytes (melan-a) were transfected with SPIRE1 and SPIRE2 (SPIRE1/2) specific siRNA oligonucleotides (SPIRE1#3 and SPIRE2#4 that were previously found to be effective in depletion of SPIRE1/2 (Alzahofi et al., 2020). A non-targeted (NT) siRNA oligonucleotide was used as a negative control as it is predicted not to target any mRNA in melanocytes thus would not affect melanosomes distribution in treated cells. Rab27a and formn1 specific siRNA oligonucleotides were used as positive controls as Rab27a is considered a critical element in melanosomes centrifugal transport and the dispersed distribution of melanosomes (Hume et al., 2001) and formin1 knockout melanocytes (melan-f) exhibit same perinuclear melanosome clustering similar to mutant melanocytes of ashen, dilute and leaden (Wilson et al., 2000, Hume et al., 2001, Wu et al., 2001, Ménasché et al., 2003, Menasche et al., 2000). The cells were incubated for 18-20 hours in the transfection mixture then in complete growth medium for a total of 96 hours to knock down the target mRNA. Subsequently, the cells were fixed and immunostained as described in the methods and visualised using an immunofluorescence microscope.

Ninety-six hours after siRNA-transfection, all the six treated populations (NT, SPIRE1, SPIRE2, SPIRE1/2, formn1, and Rab-27a depleted cells) exhibited a mixture of dispersed and clustered melanosome distribution phenotypes in different ratio among them. Another phenotype observed clearly in SPIRE1 depleted cells was where melanosome accumulates in the cortical cytoplasm leaving the centre of cells with few or no melanosome hereafter termed “hyper-dispersed” (Figure 3.2.1 A).

To quantify the differences between treated cell populations, the cells were manually scored as containing a dispersed, clustered, or hyper-dispersed distribution of melanosomes, disregarding dividing cells that cluster their melanosome in the perinuclear cytoplasm in order to divide them equally into the daughter cells; these cells can be recognized easily as they contain two nuclei (Figure 3.2.1 A).

As shown in Figure 3.2.1 B, C knocking down individual SPIRE2 alone has no significant effect on the dispersed melanosome distribution compared with the negative control NT (the mean of cells with clustered melanosome is $15.46\% \pm 8.532$ for SPIRE2 treated cells compared with $7.749\% \pm$ for NT-treated cells ($P > 0.1$)). The ratio significantly increased in SPIRE1-siRNA transfected cells to $28.62\% \pm 14.97$ ($P < 0.01$) yet was significantly lower in both positive controls ($P < 0.0001$). Double knockdown SPIRE1 and 2 in melan-a results in a significantly higher percentage of cells with clustered melanosomes compared to NT in a similar level to the positive control Rab27a and formn1 ($80.15\% \pm$ for SPIRE1/2 compared with $7.749\% \pm$ ($P < 0.0001$) for NT and with $84.2\% \pm 6.877$, $77.22\% \pm 2.86$ ($P > 0.1$) for Rab27a and FMN1, respectively). These results indicated a similarity between Rab27a, FMN and SPIREs in the role of establishing the dispersed distribution of melanosomes. Accordingly, the depletion of SPIRE1/2 FMN or Rab27a blocked melanosomes’ anterograde transport and

resulted in melanosome clustering in perinuclear cytoplasm. This was confirmed by measurement of the percentage of the cell area that was occupied with melanosomes (pigments area) to provide a more detailed read-out of the melanosome dispersion within the cell. As shown in Figure 3.2.1 D, no significant differences were found between the extent of melanosome dispersion as reported by the % of cytoplasm containing melanosomes in the SPIRE2-depleted cells and NT-treated cells (a mean of $78.69\% \pm 20.24$ in SPIRE2 compared with $95.48\% \pm 7.6$ in NT ($P > 0.1$)). The pigment area significantly decreased in SPIRE1 ($65.51\% \pm 25.16$ ($P < 0.001$)) yet remained significantly higher than the positive controls. The mean percentage of pigment area of SPIRE1/2-depleted melan-a ($58.52\% \pm 28.15$) showed no significant differences with both FMN1- ($56.75\% \pm 22.49$) and Rab27a-depleted cells ($56.88\% \pm 24.82$). These results indicate that depletion of SPIRE1/2 leads to melanosome transport defects similar to those induced by the single depleting of FMN1 and Rab27a. This supports the argument that melanosome anterograde transport is an actin-based transport and that actin is SPIRE-1/2 nucleated.

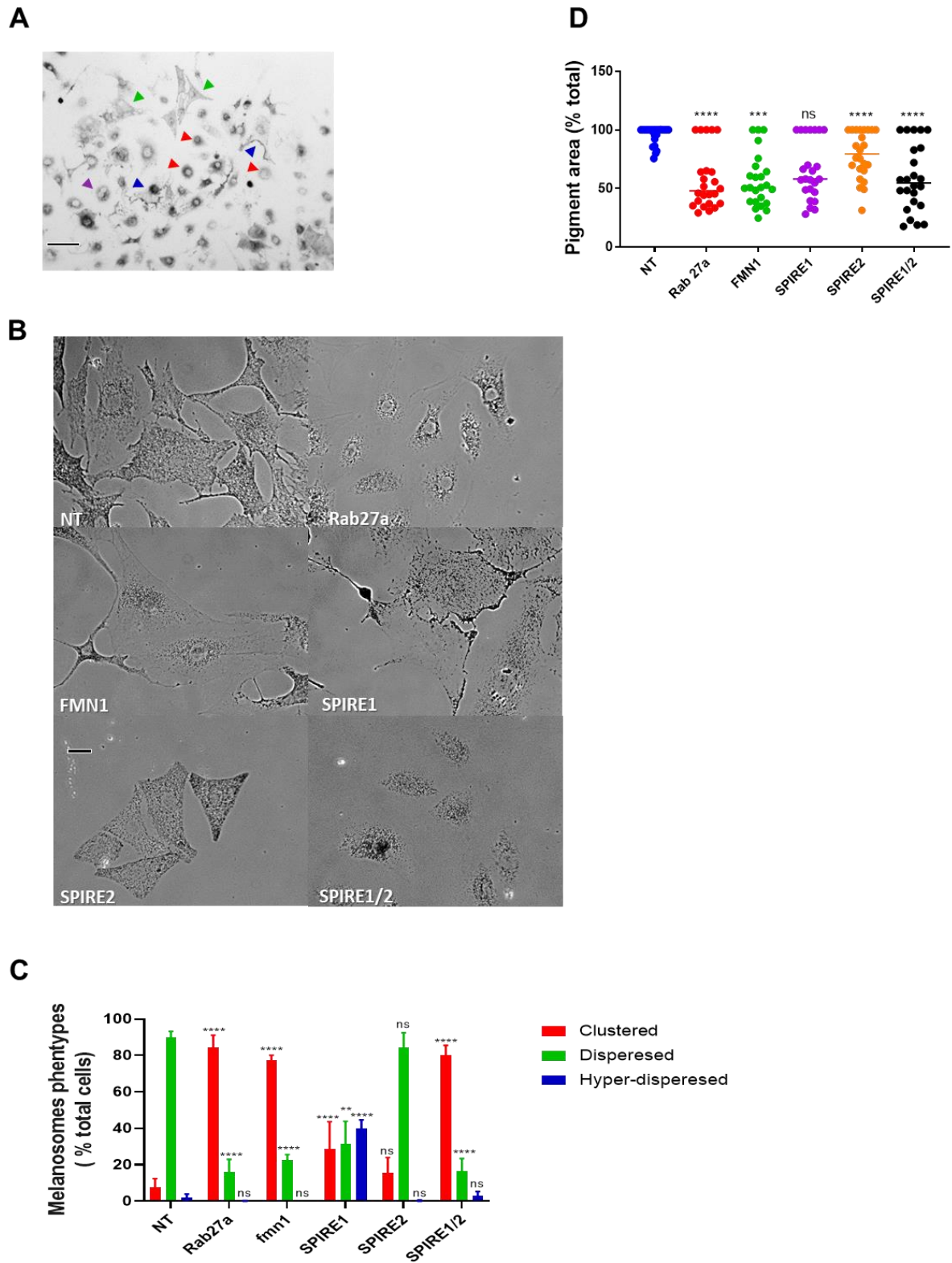


Figure 3.2.1. Functional disruption of SPIRE1/2 affects anterograde transport of melanosomes: melan-a cells were transfected by siRNA in order to deplete SPIRE1 and /or 2, Rab27a, FMN1. Cells were fixed 72 hours after the transfection and imaged using phase-contrast optics to observe melanosome distribution. A) a low magnification (10x) fields of view represent the three different melanosome distribution phenotypes observed after depleting the mentioned protein: red arrows indicate the clustered phenotype, blue arrows: hyper dispersed phenotype, green arrows: dispersed phenotype, purple arrow: dividing cell.; scale bar 100 μ m. B)

higher magnification (40x) fields of view represent melanosome distribution in melan-a melanocytes after depleting the indicated protein, scale bar 20 μ m. C) The percentage of melan-a exhibiting clustered, dispersed, or hyper-dispersed phenotype (coloured as in A) after depleting the indicated protein (n=3 low magnification fields of view in three independent experiments for each tested siRNA). D) A scatter plot showing the extent of melanosome dispersion in individual melan-a after depleting the indicated proteins (n=24 cells for each treatment in 3 repeats) Asterisks indicate the significance of the difference of the dataset compared with NT treated cells: ****=P<0.0001 ns= no significant differences as determined by one-way ANOVA.

3.3 The Effect of SPIRE1/2 Knockdown on Actin Filament in Melan-A

To confirm the defect in melanosome transport that was shown in the previous experiment is a result of actin filament depletion, the actin mesh abundance in melanocytes was assessed after depletion of SPIRE1/2. Therefore, the siRNA-mediated knockdown was performed for all the proteins that were used in the previous experiment. The cells were then fixed and stained with phalloidin to facilitate analysis of the relative amount of actin filaments. After visualising the treated cell populations, the fluorescent integrated intensity of groups of individual whole cells in each population was measured using ImageJ as described in the methods. The results show that although there was a visually observed reduction in the fluorescent signal in SPIRE1/2- and Rab27a-depleted cells compared to negative control and other treatments, no statistically significant differences were reported among the six treated cell populations (Figure 3.3.1 A, B).

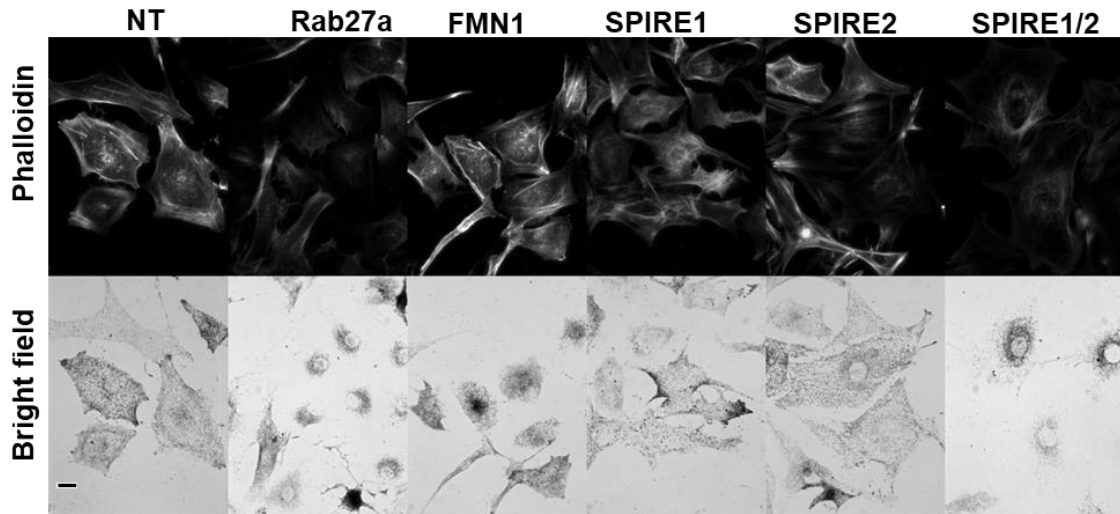
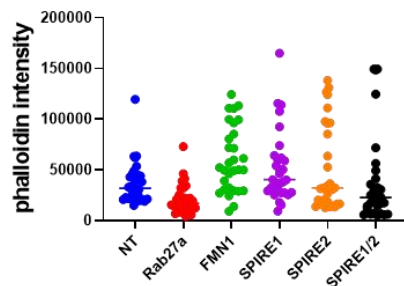
A**B**

Figure 3.3.1 The effect of SPIRE1/2 knockdown on actin filament in melan-a. A) melan-a cells were depleted of the indicated proteins by siRNA transfection and fixed 72 hours later, stained with texas-red-phalloidin to fluorescently labelled actin filaments. Cells were then imaged using bright-field and fluorescence optics to observe melanosome and actin intensity. B) the actin intensity indicated by the phalloidin fluorescent was measured by ImageJ, and the corrected total cell fluorescence (CTCF) was calculated using the formula: Integrated Density – (Area of selected cell X Mean fluorescence of background readings) represented in a scatter plot. Each dot represents CTCF of an individual cell (n=30 cells in three independent experiments for each tested protein). No significant differences were indicated between the target treatments and NT as determined by one-way ANOVA.

3.4 SPIRE1 and SPIRE2: Partial Redundant Function.

SPIRE1/2 have been characterised as having redundant functions, for example, re-introduction of either SPIRE1 or 2 into SPIRE1/2-depleted oocytes was able to restore spindle positioning in oocytes (Pfender et al., 2011). Similarly, as shown in the previous experiment, knockdown of individual SPIRE1 or 2 in melanocytes did not block melanosome transport, although melanosome distributions were slightly different. These observations

indicate that SPIRE1 and 2 share similar functions, and in the absence of one of them, the other would perform sufficiently.

In order to further test the possible functional redundancy between SPIRE1 and SPIRE2 and to verify the specificity of siRNA double knockdown, a rescue assay (hereafter knockdown-rescue experiment) performed in which EGFP-labelled *Homo sapiens* (Human) SPIRE1 (NM-001128626.1) or SPIRE2 (CAD-19439.1) was transiently expressed in SPIRE1/2-depleted melan-a melanocytes. Hs-SPIRE1/2 are resistant to the siRNA used here that specifically targets mouse SPIRE-mRNA. In this experiment, serial volumes of adenoviruses lysate that allowing the expression of EGFP-SPIRE1, EGFP-SPIRE2 and EGFP-alone, as a negative control, were added to cell growth medium after 72 hours of knockdown of endogenous SPIRE1/2. The cells were fixed 24 hours after infection with adenovirus and stained for immunofluorescence using an anti-EGFP antibody. Thereafter, melanosome and protein distribution were detected using a microscope as described in the method.

As shown in Figure 3.4.1, the results show that re-introduction of either SPIRE1 or SPIRE2 in SPIRE1/2-depleted melan-a sufficiently rescued the clustered phenotype and restored melanosome anterograde transport in the majority of the cell. Only $6.37\% \pm 6.4$ and $1.74\% \pm 2.1$ of the cells expressing EGFP-SPIRE1 and EGFP-SPIRE2, respectively, remained with their melanosomes clustered in the perinuclear cytoplasm compared to $80.6\% \pm 11.83$ in EGFP-alone expressing cells ($P < 0.0001$). This confirms the specificity of siRNA-mediated knockdown and highlights the crucial role of SPIRE proteins in establishing melanosome dispersion. Although individual SPIREs were able to restore the dispersed melanosome distribution, the majority of the cells expressing EGFP-SPIRE2 exhibited a hyper-dispersed phenotype with a mean of $53\% \pm 10.3$ at the lowest expression level compared to

0.66%±0.75 and 2.82%±2.02 in EGFP-SPIRE1, and EGFP-alone treated cells ($P<0.0001$). In the cells with the higher expression level of EGFP-SPIRE2 (mean GFP intensity 82.58±45.19), the percentage of cells exhibiting the hyper-dispersed phenotype decreased to 4.76%±4.19. That indicates differences between SPIRE1 and SPIRE2 in their ability to restore dispersed melanosome distribution.

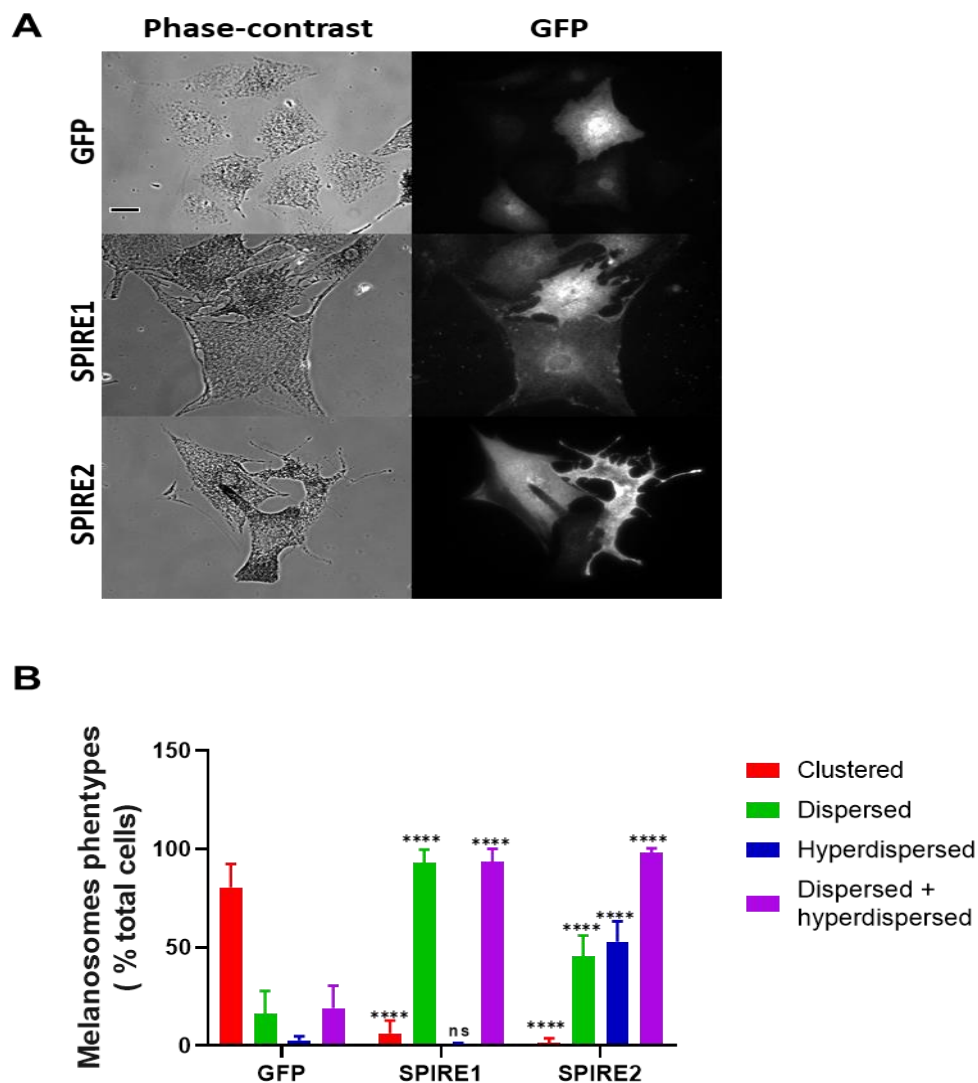


Figure 3.4.1 SPIRE1 and SPIRE2: partial redundant function: A) melan-a cells were transfected with SPIRE1/2 specific siRNA 72 hours later infected with adenoviruses expressing the indicated proteins. Cells were fixed 16-24 hours later, immunofluorescently stained with mouse anti-GFP and imaged using phase-contrast and fluorescence optics to observe melanosome and protein distribution; scale bar 20 μ m. B) The percentage of melan-a exhibiting clustered, dispersed, and/or hyper-dispersed phenotype after expressing the indicated proteins (n=3 low magnification fields of view in three independent experiments for each tested protein). Asterisks indicate the significance of the difference of the dataset compared with EGFP expressing cells unless otherwise indicated; ****= $P<0.0001$ ns= no significant differences as determined by two-way ANOVA.

3.5 Actin Nucleation: the Essential SPIRE Function in Melanosome Dispersion

After confirming the role of SPIRE protein in melanosome dispersion, the involvement of SPIRE domains and the interaction with other proteins in this process was investigated. To that end, adenovirus encoding EGFP- N-terminal-actin nucleating domains KIND and WH2 (KW) (amino acids (aa) 2-402 of SPIRE1 and aa 1-384 of SPIRE2) and another containing EGFP - C-terminal membrane-binding region, i.e. SB, FYVE and α -helical domains (SFH) (aa 525-742 of SPIRE1 and 375-728 of SPIRE2) were expressed in SPIRE1/2-depleted melan-a with EGFP-alone expressed as a negative control and full-length human SPIRE1/2 as positive controls. The cells were then fixed and immuno-stained, and the distribution of melanosomes was scored and statistically analysed.

As shown in Figure 3.5.1 and 3.5.2, the expression of the C-terminus region of both SPIRE1/2 was unable to restore dispersed melanosomes distribution. Instead, The majority of the cells remained with perinuclear melanosomes clustering (a mean of $87.59\% \pm 2.1$ and $89.22\% \pm 9.65$ of the cells expressing SFH truncation of SPIRE1 and 2, respectively compared to $88.01\% \pm 5.63$ in EGFP-alone expressed cells ($P > 0.9999$)).

On the other hand, the expression of the N-terminus region of both SPIREs resulted in the restoration of dispersed melanosome distribution. Only a mean of $4.458\% \pm 4.704$ and $14.20\% \pm 3.539$ of the cells expressing the N-terminus of SPIRE1 and 2 respectively, remained their melanosome clustered compared to $3.671\% \pm 3.384$ and $8.657\% \pm 0.9$ of cells expressing full-length SPIRE1 and 2, respectively ($P > 0.1$). These results indicate that SPIREs establish melanosome centrifugal transport by nucleating actin filaments via the interaction of SPIRE-WH2 domain with globular-actin and SPIRE-KIND with formin protein. These interactions are sufficient to restore dispersed melanosomes distribution even in the absence of membrane

association. However, a high ratio of hyper-dispersed phenotype recorded in cells expressing N-terminal truncation of SPIRE1 (a mean of $36.69\% \pm 7.75$ compared to 0% of cells expressing the full-length of SPIRE1 (P -value < 0.0001)) indicates a role for C-terminus function as a membrane association or as activity regulator -by form inhibited conformation with KIND- to achieve uniform melanosome dispersion. This ratio has no significant difference with the full-length SPIRE2 ($P > 0.1$) which suggests that SPIRE1/2 has redundancy function of N-terminal actin nucleating region and differs in the C-terminal membrane region. However, more investigations are required to test this hypothesis.

To investigate in depth the role of C-terminal domains in the melanosome dispersion, adenovirus of a SPIRE2 construct that comprised of KIND, WH2, GTBM, FYVE, and H2 (i.e. lacks SPIRE-Box domain (Δ SB)) was expressed in SPIRE1/2-depleted melan-a. Interestingly, removing the SPIRE-box motif reduced the percentage of the cells that exhibit hyper-dispersed phenotype compared with the full-length SPIRE2 ($P < 0.1$) with no significant differences in the total rescued cells (dispersed + hyper-dispersed) (Figure 3.5.1 and 3.5.2). This reduction highlights a possible role of SB of SPIRE2 in the hyper-dispersed phenotype and supports the notion of the discrepancy between the C-terminal region of SPIRE1/2.

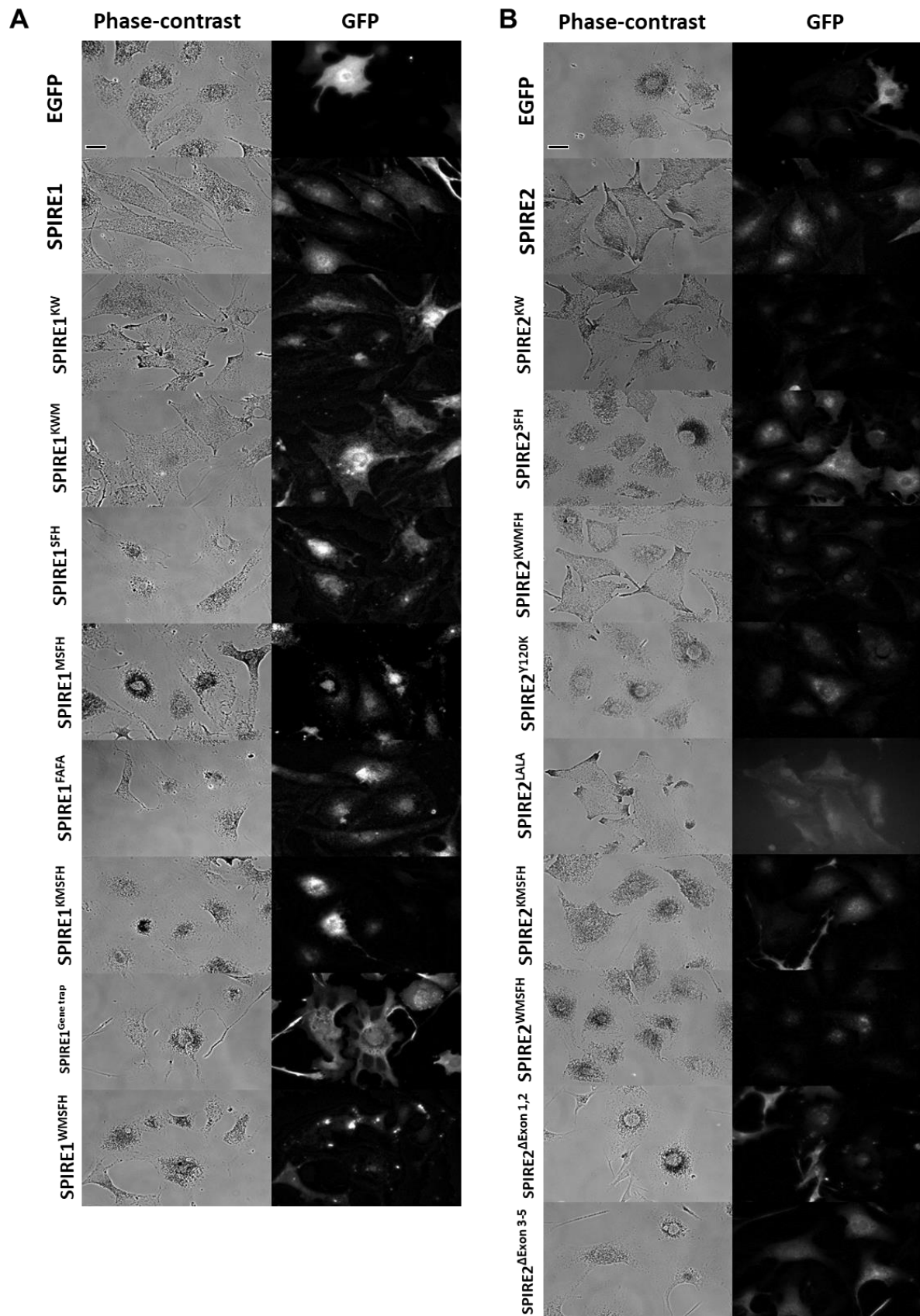
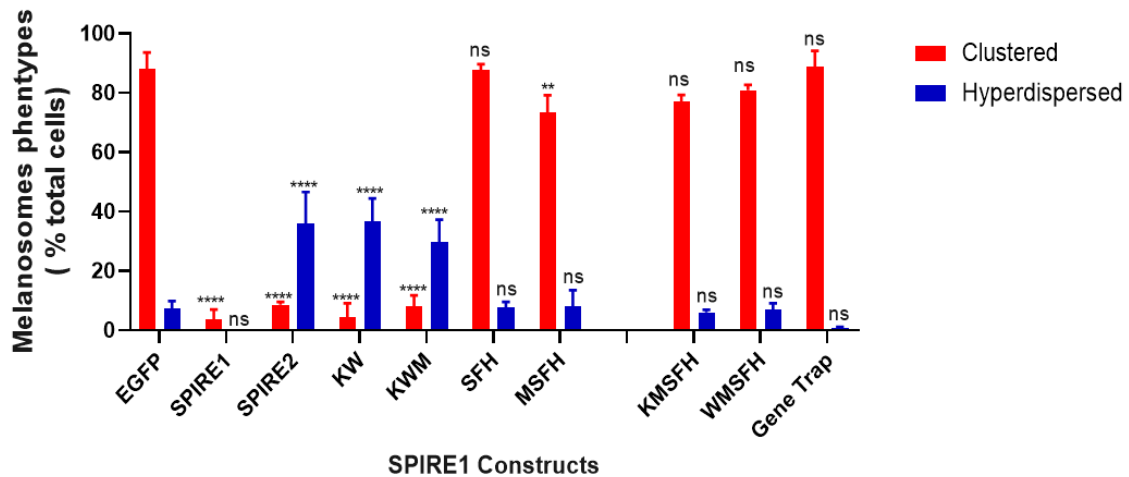


Figure 3.5.1 SPIRE1/2 restore melanosome dispersed distribution by interacting with both formin and G-actin monomer. melan-a cells were transfected with SPIRE1/2 specific siRNA 72 hours later infected with

adenoviruses expressing the indicated proteins. A) SPIRE1 truncations and mutations, and B) SPIRE2 truncations and mutations. Cells were fixed 16-24 hours later, immunofluorescently stained with mouse anti-GFP and imaged using phase-contrast and fluorescence optics to observe melanosome and protein distribution; scale bar 20 μ m. Abbreviation: K, KIND; W, WH2; M, globular tail domain-binding motif; S, SPIRE-Box; F, FYVE modified FYVE type zinc finger domain; H, C-terminal flanking sequences similar to H2 of SHD-proteins.

A



B

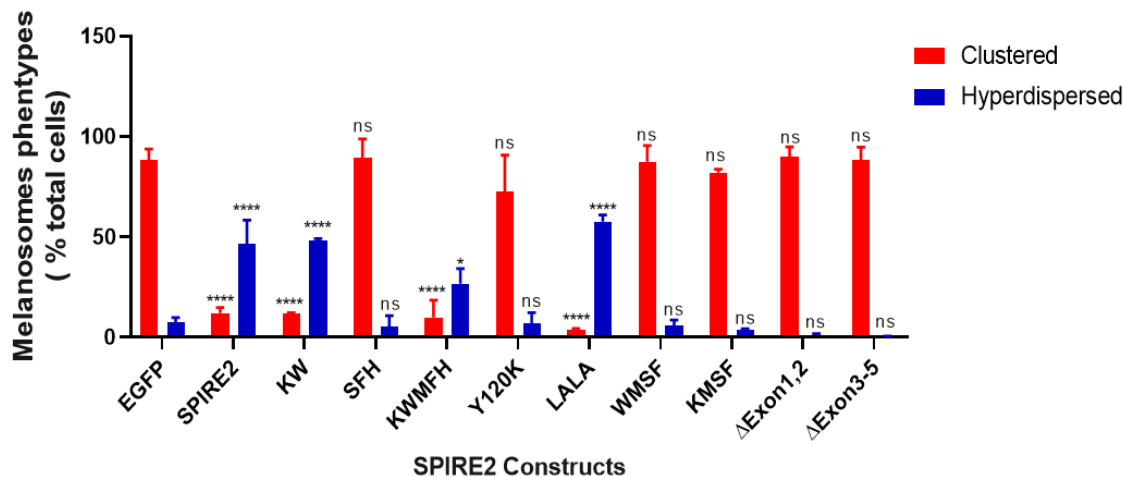


Figure 3.5.2 The percentage of melan-a exhibiting clustered and hyper-dispersed phenotype after expressing the indicated constructs of SPIRE1 (A) or SPIRE2 (B) in SPIRE1/2-depleted melan-a (n=3 low magnification fields of view in three independent repeats); Asterisks indicate the significance of the difference of the dataset compared with EGFP expressing cells unless otherwise indicated; **** indicates significant difference $p < 0.0001$, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.1$, ns = no significant differences as determined by two-way ANOVA

3.6 SPIRE1/2 Disperse Melanosomes by Interacting with both Formin And G-actin Monomer

To test the role of KIND and WH2 individually in melanosome dispersion, adenoviruses encoding SPIRE1/2 constructs that contain KMSFH and WMSFH were expressed in SPIRE1/2 depleted melan-a with EGFP-alone expressed as a negative control and full-length SPIRE1/2 as positive controls. The cells then prepared and visualised, and the data was collected and statistically analysed as previously described. As shown in Figure 3.5.1 and 3.5.2, neither truncation could restore the dispersed melanosome distribution, and the majority of the cells remained with perinuclear clustered melanosomes ($91\% \pm 6$ and $92.89\% \pm 3$ in SPIRE2-WMSFH and KMSFH, respectively compared to $92.77\% \pm 3.4$ and $5.3\% \pm 11.3$ in EGFP and SPIRE2 treated cells, respectively. As well as a mean of $80.99\% \pm 1.69$ and $97.40\% \pm 7.793$ cells with clustered melanosomes in SPIRE1-WMSFH and -KMSFH expressed cells compared to ($85.05\% \pm 3.82$ ($P > 0.1$)) in EGFP-alone expressed cells.

Similar results were observed in experiment expressing several other KIND mutants and truncations. The mean percentage of SPIRE1/2-depleted melan-a that remained with clustered melanosomes after expressing SPIRE1-gene trap was ($88.75\% \pm 5.49$). Gene trap is described to disrupt the SPIRE1 mRNA translation at sequences encoding a region after KIND domain- i.e. this construct expresses the SPIRE1-KIND domain only. The mean was $89.79\% \pm 5.03$ in the case of SPIRE1- Δ exon 1,2 (i.e. Δ first half of KIND) and $88.39\% \pm 6.24$ in SPIRE2- Δ Exon-3,4,5 (i.e. Δ second half of KIND) expressed cells. No significant differences were recorded between in all the mentioned proteins compared to EGFP-alone expressed cells ($85.05\% \pm 3.82$ ($P > 0.1$)) (Figure 3.5.1 and 3.5.2). Overall, these data This indicate that

interaction with both formin and G-actin is crucial for melanosome anterograde transport and supports the claim that melanosomes use SPIRE/formin-nucleated actin for this process.

3.7 The Role of Interaction with Myosin-Va in Melanosome Dispersion

SPIRE interact with the three isoforms of myosin-V (a, b, and c) via a motif termed The myosin-Va Globular Tail Binding Motif (GTBM) in the middle of SPIRE. The contribution of GTBM to the efficiency of melanosome transport has not identified yet. To determine the role of SPIRE: myosin-V interaction in melanosome anterograde transport, adenoviruses encoding EGFP-fused- N and C-terminus of SPIRE1 including GTBM were expressed in SPIRE1/2-depleted melanocytes. The truncation consistent of KIND, WH2, and GTBM (KWM 2-524 amino acids) and the other is GTBM, SB, FYVE, and H2 (MSFH 388-742 aa) with the previous truncations of KW and SFH were used for comparison. The cells then prepared and visualised, and the distribution of melanosomes was scored and statistically analysed as previously described. As shown in Figure 3.5.1 and 3.5.2, no significant differences were recorded between KWM and KW truncation. However, significant differences were recorded between the percentage of cells with clustered melanosomes expressing SPIRE1-MSFH and -SFH truncations ($73.30425\% \pm 5.9$ and $87.59\% \pm 2.09$, respectively ($P < 0.01$)) indicating that the interaction with myosin-Va may have effects on melanosome transport. However, further investigation is needed.

To support the previous results, a parallel experiment was conducted using adenovirus encoding EGFP-SPIRE2 with L422A L423A mutation (double mutations within GTBM that were described to impair the interaction between SPIRE and myosin-Va) was expressed in SPIRE1/2-depleted melan-a. The cells then prepared and visualised, and the distribution of melanosomes was scored and statistically analysed as previously described. As shown in

Figure 3.5.1 and 3.5.2, no significant differences were recorded between mutant SPIRE2-LALA and full-length SPIRE2. That indicates the interaction between SPIRE and myosin-V is not essential to melanosome dispersion.

3.8 Discussion and Conclusion

This study was designed to characterise SPIRE1/2 function and the role of specific domains within these proteins in melanosome dispersion. The interaction between SPIRE1/2 domains and their partner proteins and the interaction functions have been intensively studied (summarized in figure 3.). In details, SPIRE1/2 contain an N-terminal KIND domain that interacts directly with the C-terminal formin-SPIRE interaction motif (FSI) of FMN-subgroup formin (Pechlivanis et al., 2009, Quinlan et al., 2007, Zeth et al., 2011). Then, four WH2 domains bind to four actin monomers (Quinlan et al., 2005). SPIREs interaction with formin and G-actin is essential for nucleating actin filaments (Manseau and Schupbach, 1989, Quinlan et al., 2005, Quinlan et al., 2007, Pfender et al., 2011). At the middle of SPIRE protein sequences, the myosin-V globular tail binding motif (GTBM) that binds to the subdomain SD-1 of the GTD of the three isoforms of myosin-V (a,b, and c) (Pylypenko et al., 2016). Then, the C-terminus region of SPIREs that contains the SPIRE-Box domain (SB) which interacts with Rab3A (Lagal et al., 2014), the modified FYVE that interacts with phosphatidylinositol (Tittel et al., 2015), and the C-terminus flanking alpha-helical domain. Together, C-terminal domains facilitate associating SPIRE to the vesicular membrane (Lagal et al., 2014, Tittel et al., 2015, Jo et al., 2019).

Therefore, in this study, using constructs of SPIRE1/2 that missing domain(s) would highlight the role of these domains in melanosome dispersion, consequently, the role of their function in this process.

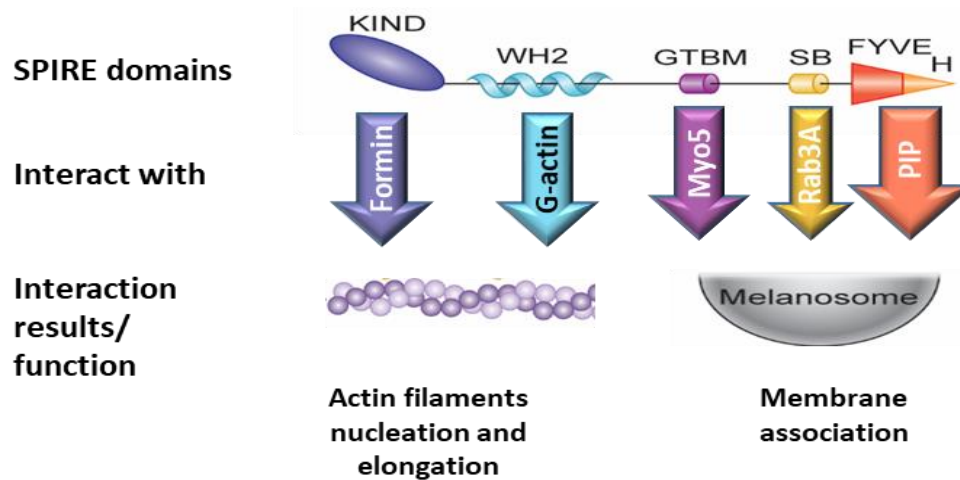


Figure 3.8.1 A schematic representation of SPIRE domains, their interaction partners and functions as described in the literature.

Collectively, the rescue of SPIRE1/2-depleted melan-a cells with different truncated and mutated versions of SPIRE indicates the critical role of the actin nucleation function of these proteins in establishing full dispersion of melanosomes distribution. Re-introduction a construct comprised of the N-terminal actin nucleation domains of KIND and the tandem WH2 in SPIRE1/2-depleted melan-a rescued the melanosome dispersion. Both the dispersed and hyper-dispersed phenotypes that observed after expressing SPIRE construct include the long-range transport of melanosome from the centre of the cells toward the cell cortex. This supports the actin-dependence of centrifugal melanosome transport (Evans et al., 2014, Alzahofi et al., 2020). Moreover, removing the gene sequence or disturb the mRNA translation of the tandem WH2 domains of SPIRE (i.e. disrupting the integrity of binding G-actin) as such as removing the whole KIND sequence or part of it (as in the case of SPIRE1 Δ exon 1,2 and SPIRE2 Δ exon 3-5) (i.e. disrupting the interaction between SPIRE and FMN) had similar results of suppression melanosome dispersion. The essential nature of the synergy between KIND and the tandem WH2 domains is indicated in many physiological processes that are regulated by actin filaments such as mammalian neural functions (Pleiser et al., 2014), vesicle transport (Kerkhoff et al., 2001, Pylypenko et al., 2016, Schuh, 2011),

meiotic division (Montaville et al., 2014, Pfender et al., 2011, Quinlan, 2013), DNA repair (Belin et al., 2015) and now melanosome anterograde transport ((Alzahofi et al., 2020, Evans et al., 2014) and as shown in this study).

On the other hand, re-introduction of C-terminal membrane-binding domains of SPIRE-box, FYVE, and H2 in siRNA-mediated double knockdown SPIRE1/2 in melan-a melanocyte failed to restore melanosome dispersed distribution. Interestingly, including the myosin-Va binding motif to the C-terminal membrane-binding domains in the rescue assay significantly reduced the cell with clustered melanosome in unclear reasons. Together, these suggested that SPIRE association with melanosome membrane is not essential for melanosome anterograde transport. However, the role of the interaction with myosin-Va in this process needs more investigations.

The effects of depleting SPIRE1/2 on actin filaments could not be recorded in this study. Although a reduction in phalloidin intensity can be visually observed, no statistically significant differences were recorded. That could be related to the weak signal specificity in the images obtained by a wide-field microscope used in this study. The reduction of the actin filaments after SPIRE1/2 knockdown can be observed clearly with high-resolution field emission scanning electron microscopy (FESEM) ((Alzahofi et al., 2020) and Hume and Bailly unpublished data). In their study, a meshwork of actin filaments can be seen surrounded melanosomes of melan-a. That filaments appear to originate from the surface of melanosomes and link them to adjacent melanosomes forming a complex melanosome/actin network. Knockdown of SPIRE1/2 and Rab27a results in a dramatic reduction in the intensity and the length of actin network surrounding melanosomes compared to normal or NT-treated melan-a ((Alzahofi et al., 2020) and Hume and Bailly

unpublished data) which support the role of actin filaments on melanosome transport.

Therefore, using sufficient microscope (i.e. confocal microscope) to test the knockdown affects the actin filaments intensity is worth conducting.

However, using phase-contrast microscopy, the effect of SPIRE1/2 knockdown and transient expression human SPIRE1/2 proteins on melanosome distribution of melan-a melanocytes was clearly observed. Regarding this, this study presented differences in melanosome distribution between cells expressing SPIRE1 and 2 that expressing SPIRE1 resulted in uniform dispersed distribution similar to that of wild-type while expressing SPIRE2 (or knockdown SPIRE1) results in hyper-dispersed distribution. This result challenges the notion of redundancy between the two proteins. The non-redundant function between SPIRE1/2 was previously observed only in pathogen-host interaction studies. In this work, the invasion of mammalian cells by *Listeria monocytogene* was reduced by SPIRE2 but not SPIRE1 (Kuhbacher et al., 2015). Meanwhile, in other studies, SPIRE1 depletion reduced the binding of *Salmonella typhimurium* to the mammalian cell, while SPIRE2 depletion affected only the pathogen replication in host cells (Daniel et al., 2016). The unknown differences in SPIRE1/2 role in cellular processes can be indicated by the differences in the expression pattern between SPIRE1 and SPIRE2. Although both SPIREs start with similar high expression level in mouse oocytes (Pfender et al., 2011), SPIRE1/2 exhibited different expression pattern in adult mice that SPIRE1 is expressed strongly in neurones of the nervous system and to a lesser extent in the testes and spleen. Meanwhile SPIRE2 exhibited a broad expression pattern, which includes the epithelial cells of the digestive tract and spermatocytes in the testes, in addition to neurones (Pleiser et al., 2010a, Pleiser et al., 2010b). While both SPIREs are expressed in neuronal cells of the adult brain, *in situ* hybridizations of oblique cross-sections show only a minor overlap in their expression pattern. This is confirmed by the

behavioural study on mutant SPIRE1 mouse in which the insertion of a gene-trap cassette into the mouse SPIRE1 gene locus impaired the expression and function of endogenous SPIRE1 which results in enhancing fear expression in male mutant mice (Pleiser et al., 2014) (i.e. the endogenous SPIRE2 could not substitute SPIRE1 functions in this case).

The C-terminus region seems to have a role in the differences between SPIRE1 and 2 and in achieving a uniform dispersed distribution in SPIRE1. The cells expressing N-terminal truncation of SPIRE1 exhibit the hyper-dispersed distribution, which is observed full-length SPIRE2-, but not SPIRE1-, expressed cells. Also, removing SPIRE-box of SPIRE2 reduced the percentage of cells exhibit hyper-dispersed phenotype in SPIRE1/2-depleted melan-a which suggested an association between SPIRE-box motif of SPIRE2 and hyper-dispersed phenotype. This could also be related to the interaction with Rab-GTPase. *in vitro* pulldown assay showed that Rab27a interacts with SPIRE1 stronger than with SPIRE2, while SPIRE2 interact with Rab8 stronger than SPIRE1 (Kollmar et al., 2019).

In conclusion, N-terminal domains of SPIRE1/2 play a crucial role in melanosome dispersion while membrane-targeting domain has yet an unidentified role in achieving an even melanosome dispersed distribution. This role would be investigated later in Chapter 7. The capability of the human SPIRE to rescue mouse melanocytes in this study has aroused a question about the conservation of N-terminal function of nucleating actin filament in synergy with FMN-type formin among SPIREs of different species, i.e. their capability to substitute endogenous mouse SPIRE, which will be investigated in the next chapter.

Chapter 4: The Role of Other SPIREs on Melanosome Dispersion

4.1 Introduction

SPIRE is identified in Ichthyosporea, Choanoflagellida, and Metazoa (Kollmar et al., 2019).

Choanoflagellates are the closest single-celled relatives of metazoans and provide the basis for studying the developmental processes in animals. *Monosiga brevicollis* (Mb), as a model organism for Choanoflagellates, share signalling and adhesion molecular mechanisms with metazoan thus provide a good model to understand the evolution of metazoan multicellularity (Hoffmeyer and Burkhardt, 2016). SPIRE of Mb is considered the origin of the holozoan-SPIRE proteins as it contains the SPIRE domain arrays and functions have been conserved throughout the animal evolution. Consistent with this *in vitro* pulldown assay showed that Mb-SPIRE could interact with FMN-subgroup formin, Myosin-V, and Rab8 of Mb (Kollmar et al., 2019). During the evolution, the SPIRE gene is lost in many invertebrate phyla apart from the Salmon louse *Lepeophtheirus* and the sea squirt *Ciona intestinalis*. On the other hand, genome duplications in vertebrates have generated three SPIRE genes (SPIRE1, 2, 3) two of which are conserved in mammals (SPIRE1 and SPIRE2) (Kollmar et al., 2019). The exon-intron structures of the three vertebrate SPIRE genes are identical except a highly variable exons encoding region in the protein sequence between the GTBM and SB domain. For instance, SPIRE1 contains differentially included exons before the GTBM (exon 9) and before the SB domain (exon13) results in four SPIRE proteins in mammals, three SPIRE1 variants and a single SPIRE2 protein. The function of the SPIRE1 exon 9 (E9) is unknown, yet it identically co-localised with SPIRE1 on transported vesicles. While SPIRE

exon 13 (E13) (also termed SPIRE1C(Manor et al., 2015)) co-localised to mitochondrial outer-membrane (Kollmar et al., 2019, Manor et al., 2015) where it cooperated with inverted formin 2 (INF2) to nucleate actin filaments that provide the force for mitochondrion division (Manor et al., 2015). SPIRE1-E9 and E13 were only expressed in minor amounts in the brain and small intestine tissues never exceeding 9% for E9 and 5% for E13 of the total SPIRE1 expression (Kollmar et al., 2019).

In this chapter, the capability of the transiently expressed *Monosiga brevicollis* (Mb)-SPIRE and mitochondrial-anchored SPIRE1-E13 isoform (both ends of SPIRE evolution spectrum) to restore dispersed melanosome distribution in SPIRE1/2-depleted mouse-melan-a was tested as an indicator of the conserved function of SPIRE: formin interaction throughout the evolution.

4.2 Mb-SPIRE Rescue Melanosome Dispersion in Mouse Melanocytes

To investigate the evolutionary conservation of SPIRE: formin interaction, the capability of Mb-SPIRE of to restore melanosomes dispersed distribution in SPIRE1/2-depleted mouse melanocytes (melan-a) was investigated. For this purpose, a serial amount of adenovirus encoding EGFP-Mb-SPIRE was added to the cells growth medium with EGFP-fused human SPIRE1/2 and EGFP-alone as controls. The cells were fixed 16–24 hrs after gene transduction and stained. Thereafter, melanosome and protein distributions were detected using a fluorescence microscope and the data statically analysed.

As presented in Figure (4.2.1 A, B), Mb-SPIRE successfully restores melanosome dispersed distribution of SPIRE1/2-depleted melan-a. The total rescued cells (i.e. dispersed + hyper-dispersed) in Mb-SPIRE expressed cells has no significant differences with the positive controls (Hs-SPIRE1/2). A mean of $93.07\% \pm 1.78$ of the cells expressing Mb-SPIRE showed

rescued melanosome distributions compared to $24.35\% \pm 9.31$ ($P < 0.0001$) in EGFP-alone expressed cells. However, a mean of $34.50\% \pm 8.14$ of the cells expressing Mb-SPIRE exhibit hyper-dispersed melanosome distribution which is significantly different ($P < 0.0001$) from all controls used in this experiment ($2.93\% \pm 1.50$, $0.87\% \pm 0.65$, and $63.53\% \pm 17.30$ in EGFP-, SPIRE1-, and SPIRE2-expressed cells, respectively).

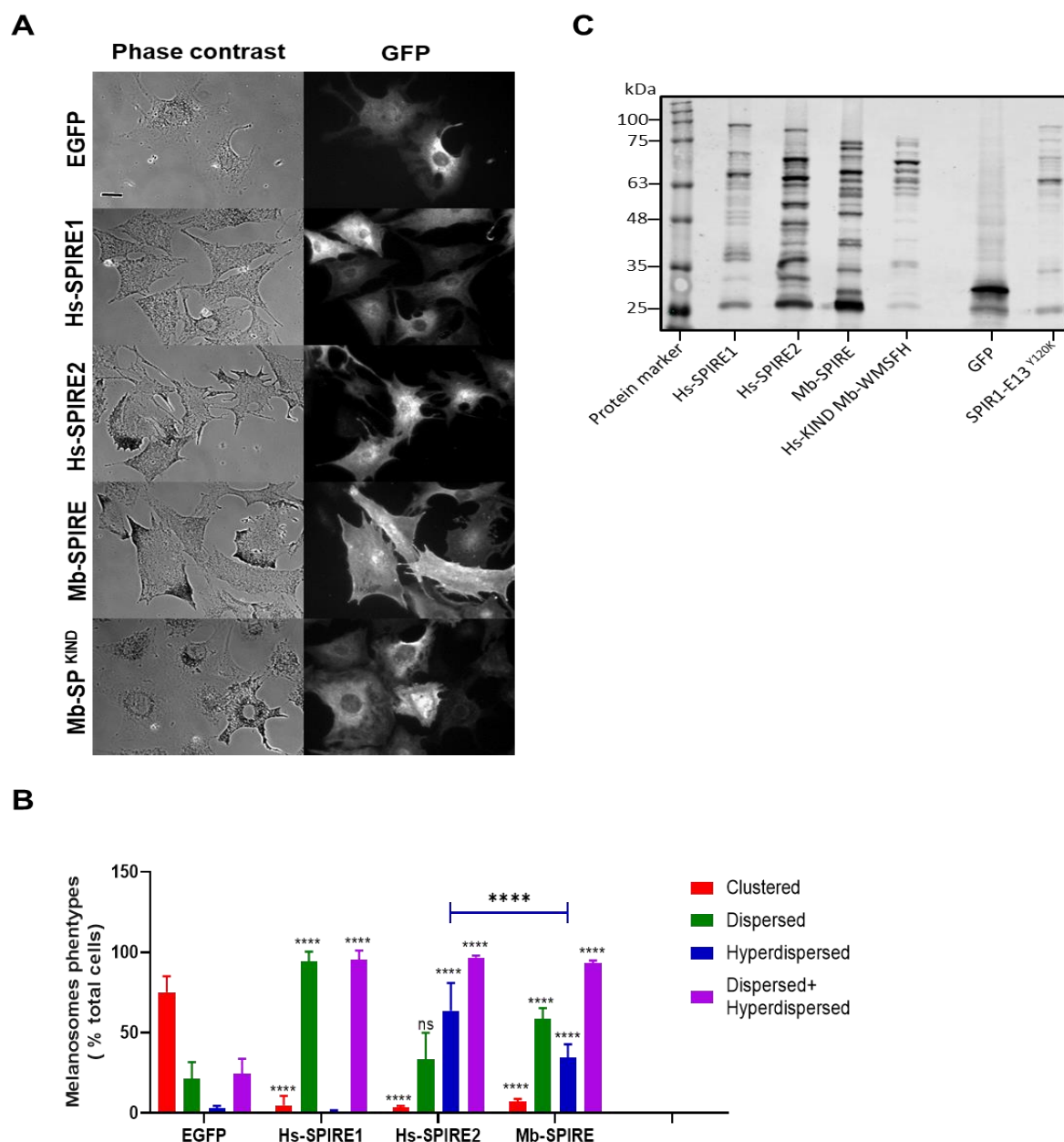


Figure 4.2.1 *Monosiga brevicollis* (Mb) SPIRE is able to restore melanosome dispersed distribution in mouse melanocytes A) melan-a cells were plated onto glass coverslips and depleted of SPIRE1/2 proteins by siRNA transfection. After 72 hours, cells were infected with adenoviruses expressing the indicated proteins. Cells

were fixed after 16-20 hours, processed for immunofluorescence and imaged using phase contrast and fluorescence optics to observe melanosome and protein distribution/expression. Scale bar represents 20 μm . B) The percentage of melan-a exhibiting clustered, dispersed, and/or hyper-dispersed phenotype after expressing the indicated protein (n=5 low magnification fields of view in four independent experiments). Asterisks indicate the significance of the difference of the dataset compared with EGFP expressing control unless otherwise indicated; **** and n.s indicate significant difference $p < 0.0001$, and no significant $P > 0.1$ as determined by two-way ANOVA. C) The integrity and the size of proteins used in this chapter: adenoviruses encoding the indicated proteins were expressed in melan-a melanocytes, the protein expression was monitored by fluorescence optics. After confirming protein expression, the cells were harvested using Trypsin-EDTA. The cells were then lysed in SDS-sample buffer and the proteins separated by 10% SDS-PAGE.

4.3 Mitochondrial SPIRE1 (SPIRE1-E13/ SPIRE1C)

4.3.1 SPIRE1-E13 Restore the Melanosome Dispersed Distribution of SPIRE1/2-Depleted

Melanocytes.

To test the ability of mouse-E13 to establish dispersed melanosome distribution, adenovirus encoding full-length-E13 expressed in SPIRE1/2-depleted melan-a melanocytes using EGFP-alone as a negative control and individual Hs-SPIRE1/2 as positive controls. The E13 with longer linker (E13Linker) were used for comparison. The cells were then fixed, stained and visualised as described previously.

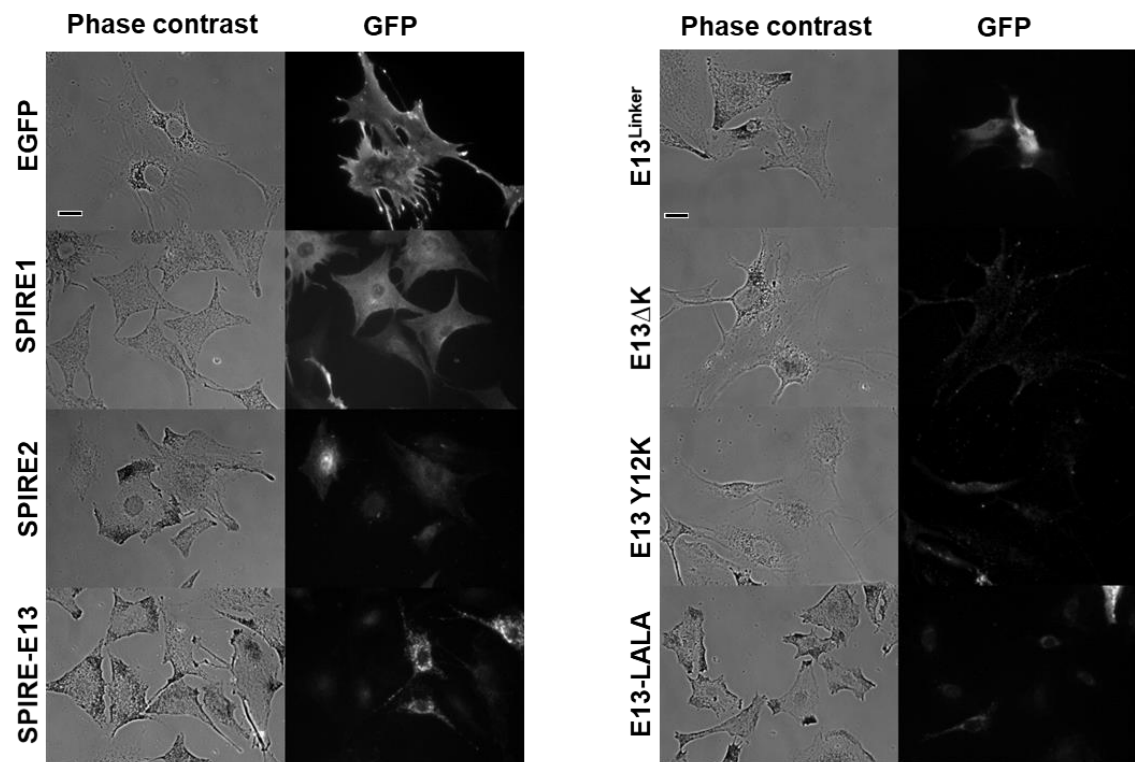
As shown in Figure (4.3.1 A, B), both E13 and E13Linker successfully restored the melanosome dispersed distribution. Only $10.21\% \pm 6.96$ and $18.64\% \pm 1.13$ of the cells expressing E13 and E13Linker, respectively remained with perinuclear melanosomes clustering compared to $73.03\% \pm 10.68$ in EGFP-alone expressed cells ($P < 0.0001$). However, a majority of the cells exhibited the hyper-dispersed phenotype seen in SPIRE2-expressing cells. A mean of $69.50\% \pm 17.93$ and $66.75\% \pm 3.044$ in E13 and E13Linker, respectively, compared with $55.98\% \pm 10.35$ in SPIRE2-treated cells ($P > 0.1$). That indicates that the sub-cellular localisation has no effect on E-13 function in melanosome dispersion; however, it could have a role in the pattern of melanosome distributions (i.e. the hyper-dispersed distribution).

4.3.1 Spire1-E13 Restore Melanosome Dispersion by Nucleating Actin Filaments in Cooperation With Formin

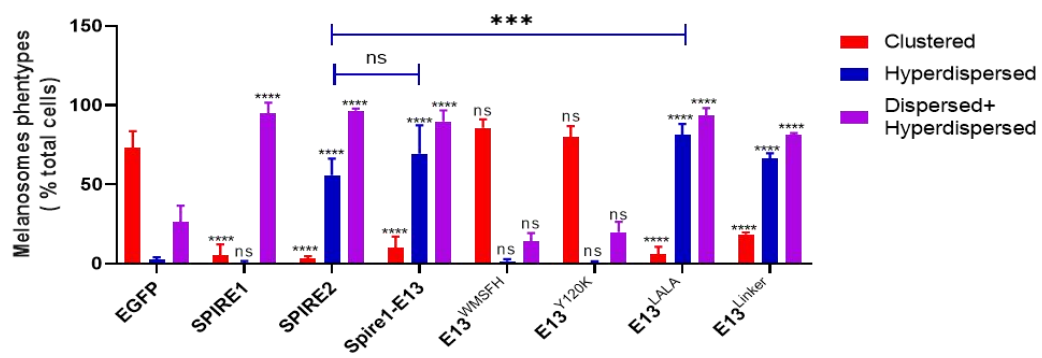
To identify the essential interaction that E13 required to restore the melanosome dispersion, adenovirus encoding truncated and mutant E13 were expressed in SPIRE1/2-depleted melan-a. Specifically, truncated E13 with WH2, GTBM, SB, FYVE, and H2 (Δ KIND) and E-13 with Y120K mutation (a point mutation that has been described to disturb the interaction between SPIRE-KIND and FSI of formin (Zeth et al., 2011) were used to identify the interaction with formin. LALA mutant E-13 was used to identify the contribution of the interaction with myosin-Va globular tail. The full-length Hs-SPIRE1/2 and EGFP-alone were used as controls.

As shown in Figure (4.3.1 A, B), similar to the Hs-SPIRE1/2 results that were described in the previous chapter, disrupting the interaction between E-13 and formin ceased the restoration of melanosome dispersed distribution in SPIRE1/2-depleted melan-a. A mean of $85.81\% \pm 5.21$ and $80.12\% \pm 6.70$ of the cells remained with their melanosomes clustered in E-13 Δ KIND and Y120K respectively compared to $10.21\% \pm 6.96$ ($P < 0.0001$) and $73.03\% \pm 10.68$ ($P > 0.1$) in FL-E13 and EGFP-alone expressed cells, respectively. While disturbing the myosin interaction using the LALA mutant had no effect on the SPIRE function and successfully restored the melanosome dispersed distribution. Only a mean of $6.26\% \pm 4.44$ of the cells remained with clustered phenotype. However, the percentage of cells exhibited the hyper-dispersed phenotype significantly increased in LALA expressed cells ($81.47\% \pm 6.67$) compared with fl-E13 ($69.50\% \pm 17.92$) and SPIRE2 ($55.98\% \pm 10.34$) ($P < 0.01$), which indicate a role of the interaction between SPIRE and myosin-Va in melanosome dispersion that worth further investigation.

A



B



C

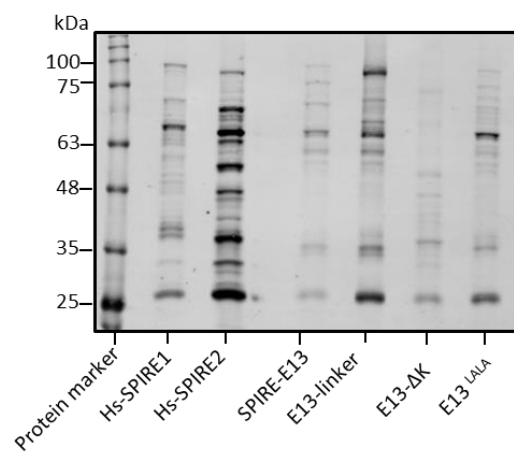


Figure 4.3.1. SPIRE1-E13 restore melanosome dispersed distribution by nucleating actin filaments in cooperation with formin: A) melan-a cells were plated onto glass coverslips and depleted of SPIRE1/2 proteins by siRNA transfection. After 72 hours, cells were infected with adenoviruses expressing the indicated proteins. Cells were fixed after 24 hours, processed for immunofluorescence and imaged using phase contrast and fluorescence optics to observe melanosome and protein distribution/expression. Scale bar represents 20 μ m. B) The percentage of melan-a exhibiting clustered, and hyper-dispersed, and dispersed phenotype after expressing the indicated protein; n = 5 microscopic fields in three independent experiments for each tested protein. Asterisks indicate the significance of the difference of the dataset compared with EGFP expressing cells unless otherwise indicated; **** indicates significant difference $P < 0.0001$, *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.1$, ns = no significant differences as determined by two-way ANOVA. C) the integrity and the size of proteins used in this chapter: adenoviruses encoding the indicated proteins were expressed in melan-a melanocytes, the protein expression was monitored by fluorescence optics. The cells harvested by Trypsin-EDTA when the protein expression was confirmed. The cells then lysate in SDS-sample buffer and the proteins separated by 10% SDS-PAGE.

4.3.2 E-13 Restore Melanosome Dispersed Distribution by Interacting with FMN-Type Formin.

SPIRE1-E13 (SPIRE1C) was described as interacting with INF to nucleate actin filaments around the mitochondrial outer membrane to facilitate mitochondrion division (Manor et al., 2015). To confirm whether the melanosome dispersion observed after expressing E13 in SPIRE1/2-depleted melan-a is a result of interaction with FMN-type formin or INF, the adenovirus encoding FL-E13 was expressed in FMN1 knockout cell melanocytes (melan-f). Adenovirus encoding EGFP-alone and Formin1 were used as controls and Hs-SPIRE1/2 were expressed for comparison.

As shown in Figure (4.3.2 A, B), neither E13 nor SPIRE1/2 were able to rescue the clustered melanosome phenotype in melan-f. All the cells remained with their melanosome clustered at the centre of the cells compared to the full rescue of the cells that results from expressing the missing Formin1 protein. This was confirmed by measuring the melanosome dispersed distance (the area of the cells occupied by the pigments). The data shows the cells treated with E13 had their melanosome cover the mean of $61.50\% \pm 12.83$ compared to $95.72\% \pm 6.356$ ($P < 0.0001$) in the cells treated with formin1 and $57.82\% \pm 13.61$, $58.23\% \pm 12.35$, and $59.31\% \pm 12.53$ ($P > 0.1$) in the cells expressing EGFP, SPIRE1 and SPIRE2,

respectively which indicates that E13 similar to SPIRE1/2 can restore melanosome dispersed distribution by nucleating actin filaments in cooperation with FMN-type formin.

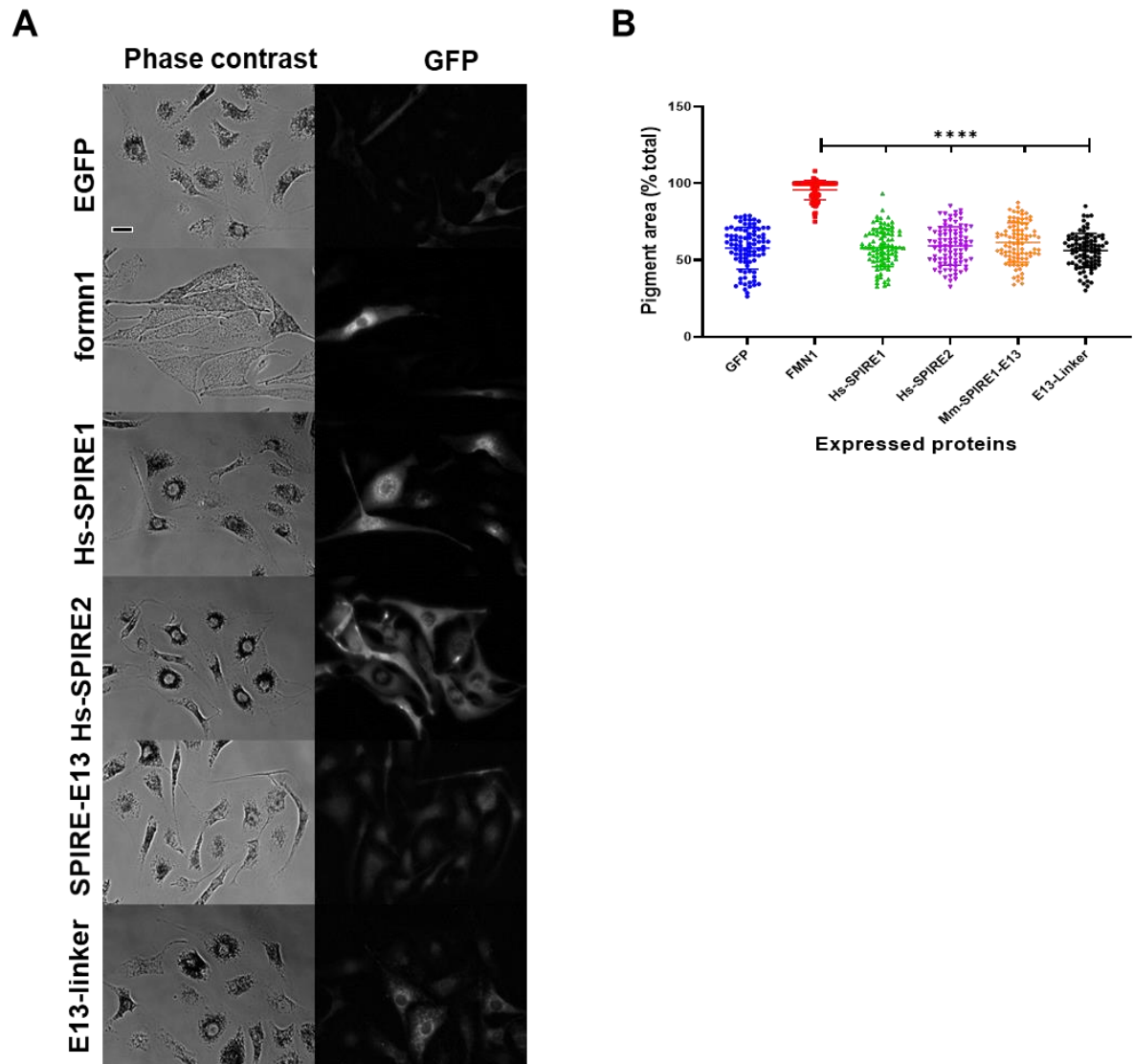


Figure 4.3.2 E-13 restore melanosome distribution by interacting with FMN-type formin. A) Melan-f seeded in coverslips and transfected with adenoviruses encoded the indicated proteins. The cells were fixed after 16 hours, processed for immunofluorescence and imaged using phase contrast and fluorescence optics to observe melanosome and protein distribution/expression. Scale bar represents 20 μ m B. A scatter plot showing the extent of pigment dispersion in cells shown in A. n = 30 cells in three independent experiments for each tested protein. Asterisks indicate the significance of the difference of the dataset compared with EGFP expressing cells unless otherwise indicated; **** indicates significant difference $p < 0.0001$, *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.1$, ns = no significant differences as determined by two-way ANOVA.

4.4 Discussion and Conclusion

The previous chapter presented evidence that the interaction between SPIRE1/2 and FMN-type formin plays an essential role in restoring dispersed melanosome distribution. The well-established SPIRE: formin interaction to nucleate actin filaments is conserved in *Drosophila* and mammalian cells (Quinlan et al., 2007, Kerkhoff, 2011, Montaville et al., 2014, Pechlivanis et al., 2009, Quinlan, 2013, Schumacher et al., 2004, Zeth et al., 2011, Pfender et al., 2011). In this chapter, the functional conserved of SPIRE: formin interaction was investigated using *choanoflagellates*- and mitochondrial anchored-SPIRE (both end of SPIRE evolution spectrum) to rescue mouse melanocytes after depleting the endogenous SPIREs. Also, Conducting SPIRE1/2-knockdown/rescue experiments on SPIRE1-E13 provides an insight into the effect of non-melanosomal localisation on SPIRE function. Despite the hyper-dispersed distribution observed in the expressed cells, both Mb-SPIRE and SPIRE1-E13 were successfully able to restore melanosome dispersion at the same level as Hs-SPIRE1/2. This indicates that the functional interaction between SPIRE and formin was conserved through animal evolution.

SPIRE localisation seems to have a role in melanosome distribution pattern. Although mitochondrial localisation did not affect SPIRE1-E13 function to nucleate actin filament essential for melanosome dispersion, the hyper-dispersed distribution indicates differences with SPIRE1. The hyper-dispersed phenotypes increased when the interaction with myosin-Va was disrupted by LALA mutation, which supports the hypothesis that linked this phenotype with membrane-associated domains and their function of localising SPIRE to melanosome membrane.

The GST-pulldown assay showed the ability of KIND domain of E13 to interact with inverted formin INF to nucleate actin on the mitochondrial outer membrane (Manor et al., 2015). To test whether this interaction is the cause of the hyper-dispersed distribution in melan-a, the rescue experiments were conducted in the FMN1 knockout cells. E13, as well as Hs-SPIRE1/2, failed to rescue melanosome distribution of the FMN1 knockout cells indicates that the melanosome dispersion and the hyper-distribution observed in E13-expressed melan-a are results of the known interaction between SPIRE and FMN-type formin.

In conclusion, The functions of SPIRE/FMN interaction on melanosome dispersion is conserved among animal evolution. However, the localisation on melanosome membrane seems to have a role in this process that worth more investigation in the following chapters.

Chapter 5: SPIRE1/2 as a Potential Rab27a Effectors

5.1 Introduction

A small GTPase and a member of the RAS oncogene family, Rab27a governs different types of intracellular trafficking, including melanosome anterograde transport, by interaction with distinct effectors. Melanosome dispersion in mammalian melanocytes is an actin-based transport. In this pathway, a crucial tripartite complex formed of GTP-Rab27a on the melanosome membrane which recruits its effector, melanophilin (mlph), which in turn recruits myosin-Va (actin-based-motor) to transport melanosome along actin filaments (Figure 5.1.1 A) (Hume et al., 2001, Hume et al., 2002, Hume et al., 2006, Matesic et al., 2001, Kazuaki et al., 2002, Fukuda et al., 2002). Any dysfunction in one of those three proteins impaired melanosome dispersion which is a pathology hallmark for the three types of human Griscelli Syndrome (GS) and mouse-coat colour. That is, GS-I and the corresponded dilute mutant in mice (d) encodes myosin-Va (MyoVa) (Mercer et al., 1991, Ménasché et al., 2003, Pastural et al., 1997, Wu et al., 1998). GS-II and the corresponded ashen mutant mice (ash) encodes Rab27a (Wilson et al., 2000, Hume et al., 2001, Wu et al., 2001, Ménasché et al., 2003, Menasche et al., 2000), GS-III and the corresponded leaden mutant mice (ln) encodes MLPH (Hume et al., 2002, Matesic et al., 2001, Ménasché et al., 2003, Fukuda et al., 2002). Each of these is characterised by melanosomes aggregated/clustered in the perinuclear area of melanocytes, in contrast to the wild-type melanocytes where melanosomes are dispersed throughout the cytoplasm (Figure 5.1.1 B)

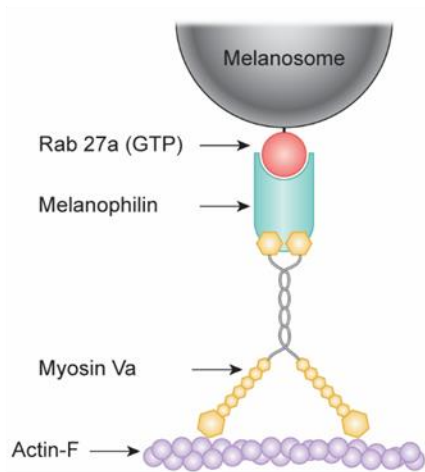
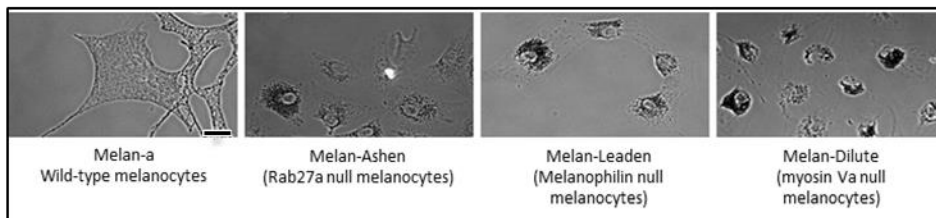
A**B**

Figure 5.1.1 A) schematic representation of the tripartite complex shows melanophilin (MLPH) interacting with GTP-Rab27a on the melanosome membrane and recruits the motor myosin Va (myoVa) which transport melanosome along actin filament toward plus end. B) high magnification (40x) fields of view represent melanosome distribution in the three types of melanocytes mutations compared with wild type. Scale bar represents 20 μm .

MLPH/Slac2a is related to a Rab27a-effector family characterized by a Rab27-binding Slp homology Domain (SHD) at their N-terminus (Fukuda, 2013, Fukuda et al., 2002). The crystal structure of Rab27 complex with Rab27a-binding domains, which contains or lacks zinc finger motifs, has been determined and serves in defining the specific molecular contributions in SHD: Rab27a interaction (Chavas et al., 2008, Fukuda, 2013, Fukuda et al., 2002).

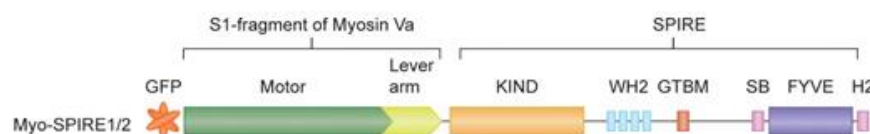
The notion of the necessity of protein-protein interaction for SPIRE in order to accomplish specific sub-cellular localisation arose in the study that showed the high affinity and non-

specific interaction between modified FYVE type motifs of SPIRE with a different type of negatively charged lipids (phosphoinositides) which are located on a wide variety of membrane that there needs to be some factor, e.g. protein-protein interactions involving SPIRE, to ensure that there is a specificity of interaction with certain membranes (Tittel et al., 2015). Since then, SPIRES have been associated with different Rab-GTPase that regulate vesicular trafficking. In this case, sequence alignment reveals a shared sequence between Spire-Box domain with alpha-helical N-terminal extension of the rabphilin-3A FYVE finger-related structure. This extension mediates the interaction between rabphilin-3A and the GTP-form of Rab3A and Rab27a GTPases. It has been shown that both the full-length and the C-terminus region of SPIRE1 interact with Rab3A *in vitro* (Lagal et al., 2014). SPIRE also localised with Rab11 vesicles, and this localisation was abolished after removing the spire-box domain (Kerkhoff et al., 2001). Recently, more evidence has been uncovered that SPIRE can interact with Rab27a on the cytoplasmic face of the melanosome membrane. An alignment of the C-terminal domains (Spire-Box (SB), FYVE (F), and α -helical (H) domains) of human SPIRE1/2 of with SHD1/2 of well-established Rab27a effectors (e.g. MLPH, MYRIP, Rabphilin3A and NOC2) reveals that SPIRE shares conserved residues in SB and H along with the conserved cysteine in FYVE domain (Alzahofi et al., 2020). Consistent with this similarity, the full-length and C-terminus region of Hs-SPIRE1/2 interact with GTP-form of Rab27a in GST pull-down assay. The proteins interact in a bimolecular fluorescence complementation assay (BiFC) as well (Alzahofi et al., 2020). Moreover, SPIRE1 co-localises with melanosomes in MLPH-null melanocytes (melan-l_n) but not in Rab27a-null melanocytes (melan-ash). These findings tie together to support the interaction between SPIRE and Rab27a (Alzahofi et al., 2020).

GST pull-down assay is considered a standard method to indicate protein-protein interaction *in vitro*; however, the artificial buffers that are used in this approach could interfere with the interaction leading to false results (Bird et al., 2017). On the other hand, the *in vivo* fluorescent measurement such as BiFC demands sophisticated equipment, fluorescent probe optimisation and/or intricate post-processing of data. Localisation can also identify a false interaction, especially if the interacting proteins diffuse throughout the cytoplasm (Bird et al., 2017). However, targeting the interacting proteins artificially to a subcellular area (such as an organelle) enhances the specificity of detecting the interaction (Bird et al., 2017, Bear et al., 2000, Henry et al., 2013, Gallego et al., 2013, Zolghadr et al., 2008). For instance, targeting the vesicle-associated proteins to microtubule-based motors redirects the interacting proteins to a distinct cellular area and provides a highly specific read-out of the interaction (Bird et al., 2017, Bentley and Banker, 2015, Bentley et al., 2015, Jenkins et al., 2012). Therefore, in this chapter, a novel interaction assay has been used to support the hypothesis that SPIRE can interact with Rab27a on the melanosome membrane and to characterize the interaction sites within SPIRE *in situ*. Melan-In (mlph-null melanocytes) provides one such reagent with which to study SPIRE: Rab27a interaction. In (Evans et al., 2014) study, Melan-In and melan-d (myoVa-null melanocytes) were rescued (i.e. their melanosomes were significantly dispersed to a higher level compared with the negative control) as a result of transiently expressed chimeric protein that contains the Rab27a-binding domain (RBD) of synaptotagmin-like protein 2-a (Sytl2a) fused to the S1-fragment of myosin-Va (the active motor domain and lever arm) (Evans et al., 2014). In this chimaera (hereafter termed mini-Va), the RBD targets the motor to melanosome (the cargo) by interacting with Rab27a. The function of this interaction can be observed in the ability of the motor to transport melanosomes towards the cell membrane (melanosome

redistribution). In this chapter, this approach (hereafter termed modified nanoscale pulldown assay) is adapted, such that the SPIRE (full length, truncations or mutants) was fused in the C-terminus of the S1-fragment of myosin-Va (Figure 5.1.2 A). Then the fused SPIRE shall target the active motor to the melanosome membrane in melan-In by interaction with Rab27a. There, the associated motor can transport melanosome toward the plus end of actin filaments, while melanosome dispersed distance (as measured by the pigment area) serves as a read-out of Rab27a interaction (Figure 5.1.2 B). Co-immunoprecipitation/mass spectrometry was also used to confirm the ability of endogenous Rab27a to pulldown SPIRE1 from the melan-a and melanoma lysate.

A



B

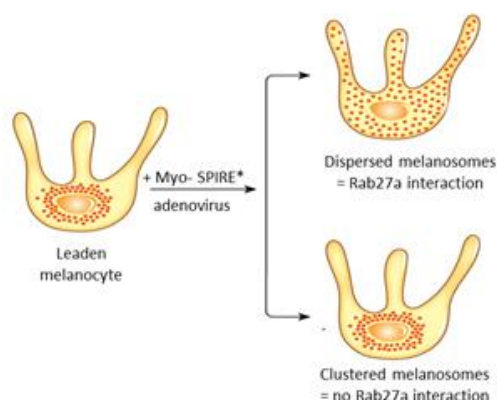


Figure 5.1.2 modified nanoscale pulldown assay (myo-SPIRE assay). A) schematic representation of the domain structure of the chimeric protein of active motor of myosin Va and the full length of SPIRE protein fluorescently labelled by EGFP was fused to N-terminus. B) A schematic representation of the adapted approach (modified nanoscale pulldown assay) that was used in this chapter. SPIRE construct within myo-SPIRE chimaera either has Rab27a-interaction site(s) thus, the interaction would target the motor to the melanosome membrane and facilitate melanosome dispersion. If the SPIRE construct within the chimaera has no Rab27a-interaction site, the membrane cannot associate to melanosome membrane, and no dispersion would occur.

5.2 Myosin-SPIRE1/2 Chimera Rescue the Defect of Melanosome Anterograde Transport of Leaden Melanocytes in Different Efficiency

To test the ability of SPIRE1/2 to interact with Rab27a on the cytoplasmic face of the melanosome membrane, adenoviruses encoding EGFP-fused chimeric proteins of S1-fragment of myosin-Va and full-length of SPIRE1 or SPIRE2 (hereafter referred to as myo-SPIRE*) were expressed in leaden-melanocytes (melan-In). EGFP-alone was expressed as a negative control, melanophilin (MLPH) (melan-In missing protein) mini-va chimaera (S1 fragment of myosin-Va fused to Rab27a-binding domain of synaptotagmin-like protein 2-a (Sytl2a)) were used as positive controls. The extent of melanosome dispersion (pigment area) was measured by calculating the percentage of the cell area occupied by melanosomes to provide a more detailed read-out of the function of the expressed protein (e.g. myo-SPIRE1). As shown in Figure (5.2.1 A, C), expressing myo-SPIRE1 and –SPIRE2 significantly restored melanosome dispersion in melan-In compared with the negative control (EGFP-alone). The mean pigment area was $78.44\% \pm 11.28$ and $68.81\% \pm 12.03$ in cells expressing myo-SPIRE1 and myo-SPIRE2, respectively, compared with $46.16\% \pm 12.47$ in cells expressing EGFP-alone ($P < 0.0001$). No significant difference was recorded between cells expressing myo-SPIRE1 and MLPH ($77.14\% \pm 16.60$) or mini-Va ($77.01\% \pm 12.13$). However, the pigment area in cells expressing myo-SPIRE2 is significantly lower ($P < 0.001$) than MLPH and mini-Va. This indicates the capability of SPIRE1 to target the motor to melanosome membrane by interacting with Rab27a is on the same level as the well-established effectors. It also highlights the difference between SPIRE1 and 2 in this capability.

To determine whether the melanosome redistribution seen in melan-In was a result of a specific interaction between SPIRE1/2 and Rab27a, the adenoviruses encoding myo-SPIRE1,

myo-SPIRE2 and mini-Va were expressed in melan-ashen (Rab27a null melanocytes) using Rab27a and EGFP-alone as controls. As shown in Figure 5.2.1 B, C, there was no significant change in melanosome distribution in all three chimaeras compared with the negative control (mean % of pigment area were 47.8 ± 8 , 41 ± 8.6 , and 43.5 ± 8.4 in cells expressing mini-Va, myo-SPIRE1, and myo-SPIRE2 respectively, compared with 44 ± 10.23 in EGFP-alone and 83.7 ± 8 in Rab27a expressing cells). These results indicate that the melanosome redistributions that was observed in melan-In, expressing the three mentioned proteins are related to Rab27a interaction.

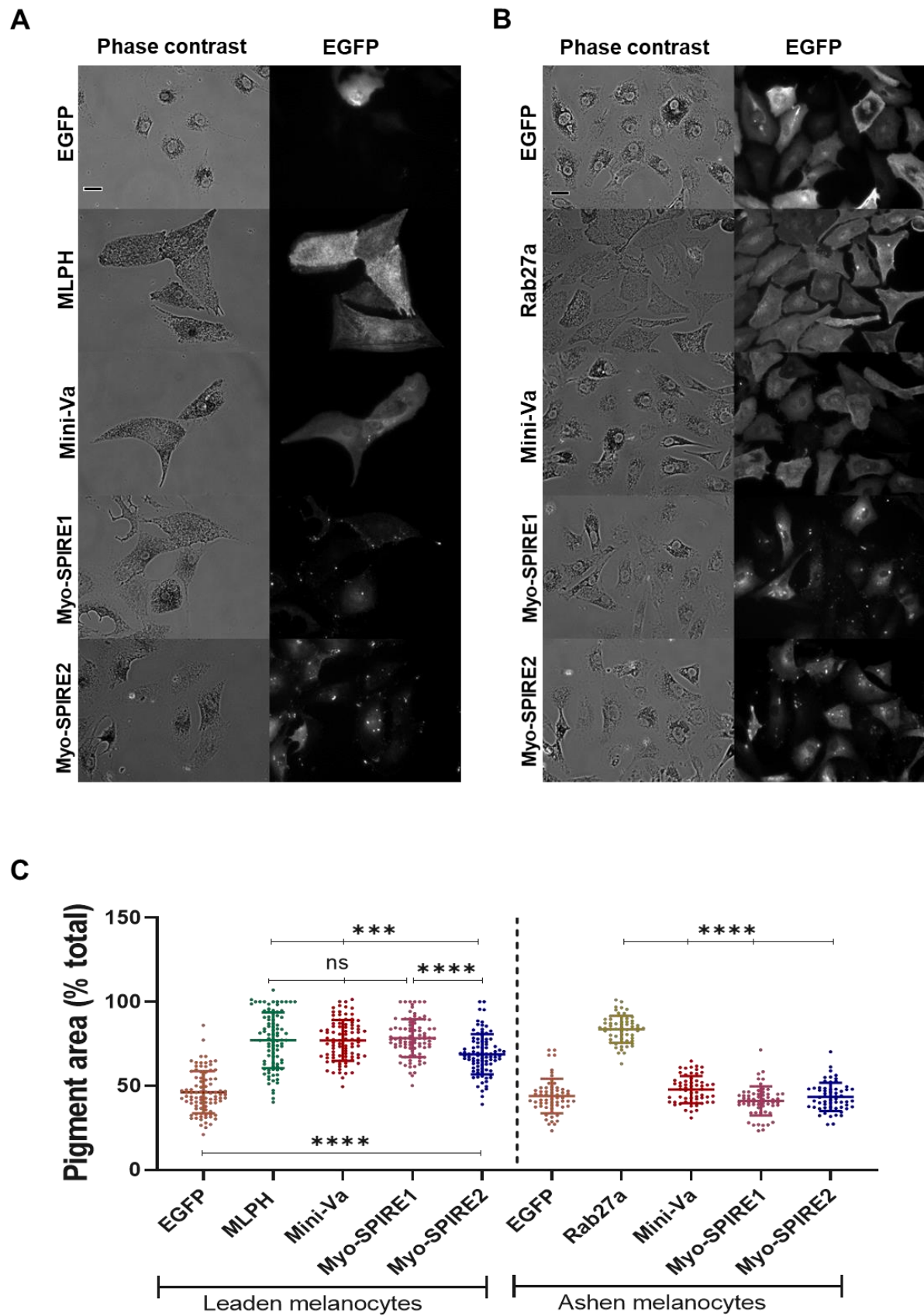


Figure 5.2.1 Myo-SPIRE1/2 expression results in redistribution of melanosome in melan-In but not melan-ash as a result of SPIRE interact with Rab27a on the melanosome membrane in melan-In. A-B) melan-In and

-ash cells were seeded on coverslips for 24 hours then infected with adenoviruses expressing the indicated proteins. Cells were fixed 16-24 hours later, immunofluorescently stained with mouse anti-GFP and imaged using phase-contrast and fluorescence optics to observe melanosome and protein distribution; scale bar represents 20 μ m. C) A Scatter plot shows a comparison of the measurement of the extent of melanosome dispersed distance (pigment area) among the treated cell populations in melan-In and melan-ash; each spot represents an individual cell data. The horizontal bar represents SD and the mean at the middle; Asterisks indicate the significance of the difference of the dataset compared with the positive control (above the data) and compared with negative control (below) **** = $p < 0.0001$, *** = $P < 0.001$; ** = $P < 0.01$, * = $P < 0.1$, ns = no significant differences as determined by One-Way ANOVA. Data shown are from at least three independent experiments $n=90$ cells for each tested protein.

5.3 SPIRE1 Interacts with Rab27a at the Endogenous Level

To identify the physiologically relevant protein-protein interactions between endogenous SPIRE1 and Rab27a in melan-a melanocytes, co-immunoprecipitation was performed to precipitate Rab27a/SPIRE1 complex from melan-a lysate. To this end, Rab27a- specific antibody (Ab) immobilized to Protein A/G UltraLink Resin was used to precipitate Rab27a/SPIRE1 complex from melan-a lysate containing 3.204 mg/ml of proteins. Negative control was set by incubating the same amount of the cell lysate with the same amount of the beads without Rab27a Ab. The beads then washed, and the co-IP products were then eluted as described in the methods. The precipitated proteins were separated depending on in SDS-PAGE, and SPIRE1-specific antibody was used to detect SPIRE1 in the co-IP products.

As shown in Figure 5.3.1 A. SPIRE1 Ab detected a single band around 60kDa in the samples immunoprecipitated with Rab27a Ab. The same band was detected in melan-a lysate (Figure 5.3.1 B).

To confirm the interaction between SPIRE1 and Rab27a, co-IP combined with mass spectrometry (MS) was performed. To this end, EGFP-SPIRE1 was transiently expressed in the B16F10 cell line with EGFP-MLPH and EGFP-alone expressed as controls. The relative fluorescence units (RFU) of the cell lysates are represented in table 5.1. The cell lysates were then incubated with Rab27a Ab immobilized to Protein A/G UltraLink Resin. Subsequently,

the co-IP products were eluted and separated depending on their mass in SDS-PAGE, while EGFP-specific antibody was used to detect EGFP- fused proteins in the co-IP products. A parallel experiment was sent for mass spectrometry (MS) analysis (conducted by Dr Amanda K Miles).

Expressed protein	EGFP-alone	EGFP-MLPH	EGFP-SPIRE1
Protein concentration (mg/ml)	105.08	88.97	92.24
RFU/ μ l	1166.7	253.88	78.78

Table 5.1 The protein concentration was measured by Bradford protein assay using the absorbance of 595nm, and the Relative Fluorescence Units (RFU) as measured by CLARIOstar using excitation of 470-15 and gain of 1000.

As shown in Figure (5.3.1 C), the EGFP-Ab detects all the three transiently expressed proteins in co-IP products. However, the MS detected only two peptides of EGFP in the samples of EGFP-alone, 31 peptides of MLPH in EGFP-MLPH and 18 peptides specific for SPIRE1 in EGFP-SPIRE1. That support the immunoblotting results and confirm the interaction between SPIRE1 and endogenous Rab27a.

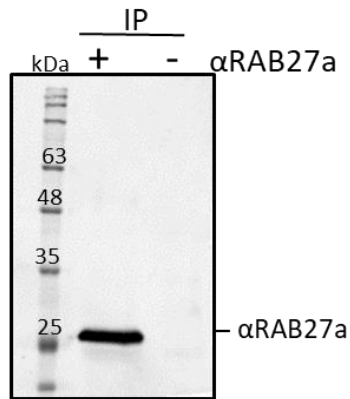
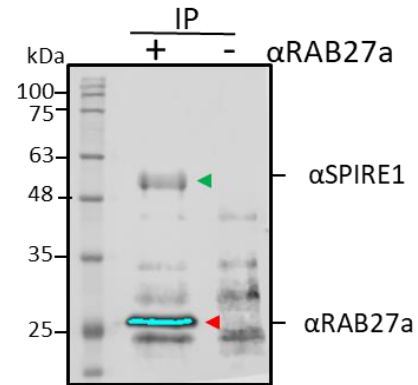
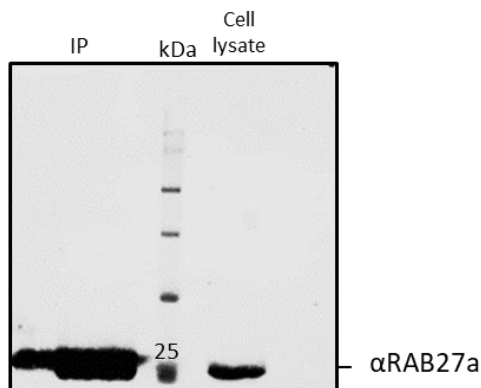
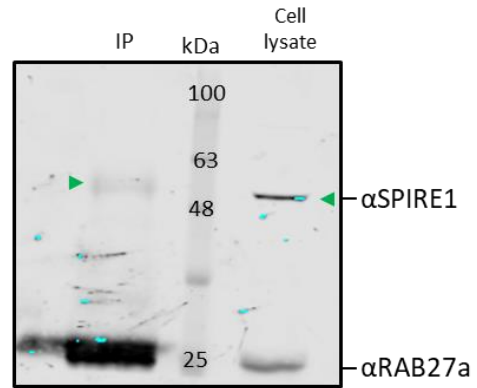
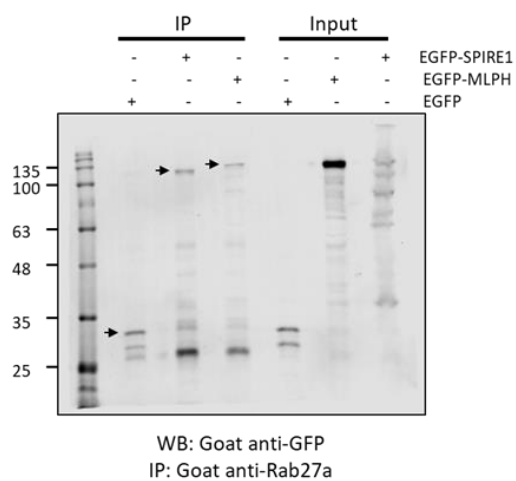
A**B****C****D****E**

Figure 5.3.1 Endogenous SPIRE1 interacts with Rab27a .A, B, C, D) melan-a lysate was incubated with beads with and without Rab27a- specific antibody (goat) for 20 hours then the beads were washed, and the IP products were eluted from the beads and separated depends on their mass in 10% SDS-Page. After wet transfer of the proteins to PVDF membrane, the membrane was immunoblotted using mouse anti-Rab27a

then **donkey anti-mouse IR680** (A, C) then Rabbit anti-mouse-SPIRE1 then **donkey anti-rabbit IR800** (B, D). A, B) comparing the eluate of the beads with Rab27a Ab and the beads without Rab27a-Ab (the negative control). C,D) compare the elute of the beads with Rab27a Ab (same as A) and the cell lysate (the input). E) B16F10 cell line expressed EGFP-SPIRE1, EGFP-MLPH and EGFP-alone were lysate and incubated with beads with Goat-anti-Rab27a. The IP products were eluted, separated on SDS-page and detected using goat-anti-GFP then donkey anti-goat IR800.

5.4 Discussion and Conclusion

In this chapter, the first section focused on using myo-SPIRE fusion proteins and melanosome dispersion as a cell-based assay system to detect SPIRE interaction with endogenous Rab27a at the melanosome membrane. The results presented showed a noticeable difference between the capability of SPIRE1 and 2 to target the active motor to the melanosome membrane and establish a strong interaction that was sufficient for melanosome anterograde transport (restore dispersed phenotype). SPIRE1 was capable of establishing the dispersed distribution in a level similar to MLPH and mini-Va, containing Rab27a-binding domain of the well-established Rab27a-effector (Sytl2a). SPIRE2, in the meantime, was capable of establishing the dispersed distributions but to a significantly shorter distance compared to SPIRE1 and the positive controls. These results are consistent with the *in vitro* pull-down assay that showed differences in Rab27a interaction affinity between SPIRE1 and 2 such that SPIRE2 has lower interaction affinity than SPIRE1 (Kollmar et al., 2019, Alzahofi et al., 2020). That determines SPIRE1 as a potential Rab27a-effector. These differences also supported the results presented in chapter 3, which indicated a difference between SPIRE1 and 2 according to the melanosome distributions in the melan-a rescued cells. There, SPIRE1-rescued cells exhibit wild-type dispersed distributions, while SPIRE2- and the N-terminus construct of SPIRE1- rescued cells showed hyper-dispersed distribution. Same distribution was also observed in mitochondrial anchored SPIRE1-E13

which linked the hyper-dispersed to SPIRE localisation at melanosome membrane that can be achieved by interacting with Rab27a.

In the second section, the attempt to provide more evidence of the interaction between SPIRE1 and Rab27a by using co-immunoprecipitation faced many complications. The specific antibody against SPIRE1 recognized a single band in co-IP products and the melan-a lysate that was shorter than the well-established mouse-SPIRE1 sequence (68.46kDa). The specificity of the SPIRE1 antibody used in the immunoblotting support that the detected bands are SPIRE1. Also, a record of short sequence variant of SPIRE1 of 59,390 kDa was identified as A0A286YDN9-1 in UniProt contains the C-terminus domains (MSFH) essential for Rab27a-interaction. However, no sequencing was processed for the detected band or the lysate to confirm if it is the same variant. Therefore, co-IP for transiently expressed EGFP-human-SPIRE1 was performed with EGFP-alone and EGFP-MLPH as controls. Reducing the wash steps results in high background proteins in EGFP-alone, which is one of the co-IP drawbacks. Therefore, to confirm the presence of EGFP-SPIRE1 in IP-products, mass spectrometry analysis was performed to identify Rab27a potential partners in co-IP. The immunoblotting here overestimated the EGFP-alone and underestimated the SPIRE1 in co-IP products. The same samples prepared under the same conditions for immunoblotting and MS resulted in two peptides of EGFP-alone in MS, compared to 18 specific peptides of SPIRE1, which were in contrast with immunoblotting results.

In conclusion, protein: protein interactions are crucial regulators of physiological processes in living cells. Understanding their mechanism and regulation would enhance our understanding of various cellular physiological processes and their deficiencies. Despite the different methodologies that have been used to identify protein: protein interaction, no

single one can eliminate false positives and/or false negatives, due to their disparities in specificity and sensitivity (Bird et al., 2017, Yu-Chi et al., 2010, Pascal et al., 2008). Using the modified nanoscale pulldown assay (myo-SPIRE assay) where the interaction between the expressed SPIRE fusion protein interacts with endogenous Rab27a was detected directly with no complications or drawbacks, and its results are consistent with the *in vitro* GST-pulldown assay and co-IP/MS results. Therefore, the modified nanoscale pulldown assay will be used to characterise and map the interaction site(s) within SPIRE1 in the next chapter.

Chapter 6: Characterizing Rab27a Interaction-Sites within SPIRE1

6.1 Introduction

SPIRE1/2 have been associated with Rab27a in various experiments. *In vitro* GST-pulldown, bimolecular fluorescence complementation (BiFC), and co-localisation assays (Kollmar et al., 2019, Alzahofi et al., 2020). An alignment of the C-terminal domains (Spire-Box (SB), FYVE (F), and α -helical (H) domains) of human SPIRE1/2 of with SHD1/2 of well-established Rab27a effectors (e.g. MLPH, MYRIP, Rabphilin3A and NOC2) reveals that SPIREs shares conserved residues in SB and H along with the conserved cysteine in FYVE domain (Alzahofi et al., 2020).

The previous chapter concluded with evidence that SPIRE1 can be considered as a Rab27a-effector and verified the modified nanoscale pulldown approach (i.e. rescue leaden melanocytes using a chimaera of myosin-Va active motor and SPIRE1) in the determination of the interaction with GTP-form of Rab27a.

In this chapter, the same approach will be used to map the interaction site(s) within SPIRE1. For that, adenoviruses encoding different truncations and mutations of SPIRE1 (Figure 6.1.1) were expressed in leaden melanocytes, and the pigment area was used as a read-out for the ability of the SPIRE1 domains to interact sufficiently with Rab27a.

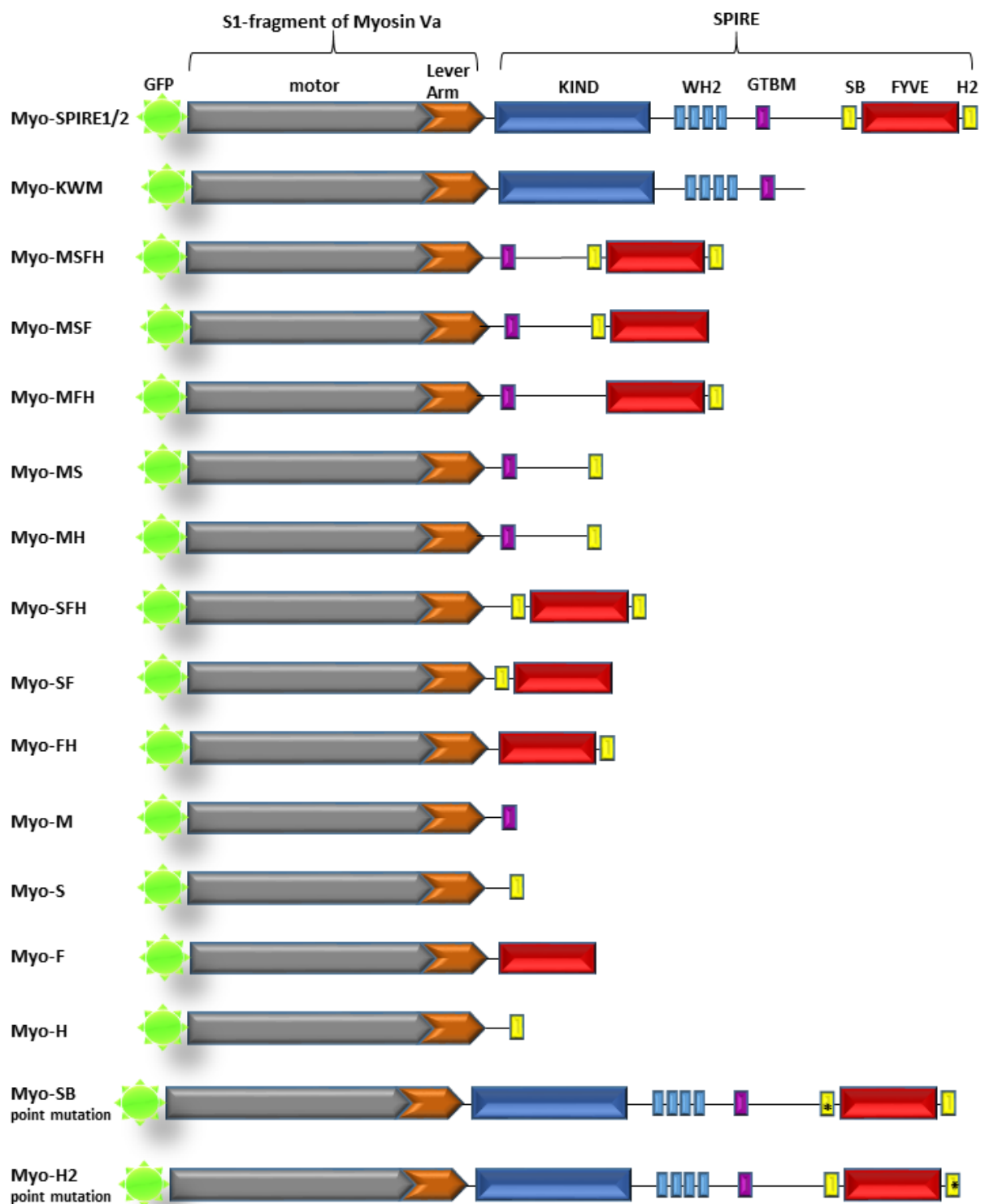


Figure 6.1.1 Schematic representations of the domain structure of a chimeric protein of active motor of myosin Va and the full-length of SPIRE protein fluorescently labelled by GFP was fused to N-terminus and the correspondence with SPIRE truncations and mutations used in this chapter.

6.2 C-Terminal Region of SPIRE1 has Rab27a Interaction Site(S)

In order to confirm that the C-terminal membrane-binding region (MSFH) of SPIRE1 contains the Rab27a-interaction sites, adenovirus encoding the N- and C-terminus regions of SPIRE1 (KWM aa 1-380) and (MSFH aa 231-598) respectively, were fused to S1-fragment of myosin-Va (as all chimaeras used in this chapter). myo-KWM and myo-MSFH were expressed in melan-In using myo-SPIRE1 (full-length) as a positive control and EGFP-alone as a negative control. The cells were fixed 16-20 hours after the transfection, immuno-stained, and melanosome dispersed distance (pigment area) was measured as previously described.

As shown in Figure 6.2.1, significant restoration of dispersed melanosome distribution was recorded in myo-MSFH but not in myo-KWM expressing cells, in comparison with EGFP-alone. The mean of pigment area percentage was $32.44\% \pm 9.08$ and $76.6\% \pm 11.82$ in the cells expressing myo-KWM and myo-MSFH, respectively, compared to $34.32\% \pm 10.51$ and $78.44\% \pm 11.3$ in the cells expressing EGFP-alone and myo-SPIRE1, respectively. These results indicate that N-terminal domains have no Rab27a interaction sites and the interaction sites are within the C-terminus membrane binding region of SPIRE1.

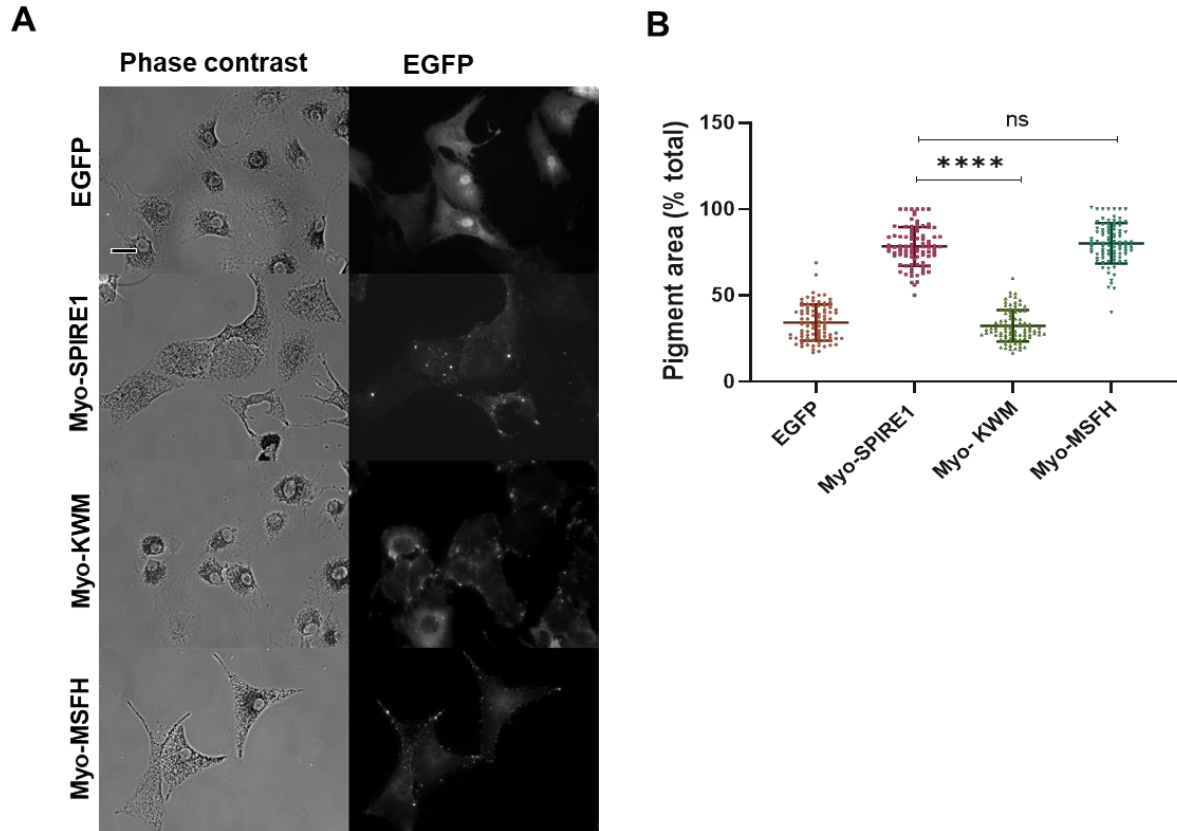


Figure 6.2.1 C-terminal region of SPIRE1 has Rab27a interaction site(s). A) melan-In were seeded on coverslips for 24 hours then infected with adenoviruses expressing the indicated proteins. Cells were fixed 16-24 hours later, immunofluorescently stained with mouse anti-EGFP and imaged using phase-contrast and fluorescence optics to observe melanosome and protein distribution; scale bar represents 20 μ m. B) A Scatter plot shows a comparison of pigment area among the treated cell populations; each spot represents an individual cell data. The horizontal bar represents SD and the mean at the middle; Asterisks indicate the significance of the difference of the dataset compared with positive control **** = $p < 0.0001$, ns= no significant differences as determined by One-Way ANOVA. Data shown are from at least three independent experiments $n=90$ cells for each tested protein.

6.3 Investigating the Role of Individual Domains in Rab27a Interaction

To identify the ability of the individual domains within the C-terminus of SPIRE1 (Globular tail binding motif aa 230-380 (M), SPIRE-Box aa 380-388 (S), FYVE aa 388-598 (F), and α -Helical aa 544-598 (H) domains) to interact with Rab27a, adenoviruses encoding chimaera of myo-M, -S, -F, and -H were expressed in melan-In with myo-SPIRE1 and EGFP-alone as controls. The cells were then fixed and prepared as previously described and the melanosome dispersed distance was measured and analysed.

As shown in Figure 6.3.1, none of the individual domains was able to restore the melanosome dispersion of melan-In. The mean pigment area percentage was $44.91\% \pm 12.57$, $44.82\% \pm 12.38$, $44.04\% \pm 11.62$, and $46.57\% \pm 11.93$ in the cells expressing myo-M, myo-S, myo-F and myo-H, respectively, compared to $48.9371\% \pm 10.98$ ($P > 0.1$) and $82.84\% \pm 9.246$ ($P < 0.0001$) in the cells expressing EGFP-alone and myo-SPIRE1, respectively. This indicates that none of the individual domains is able to establish an interaction with Rab27a sufficient for the motor to disperse melanosomes of melan-In.

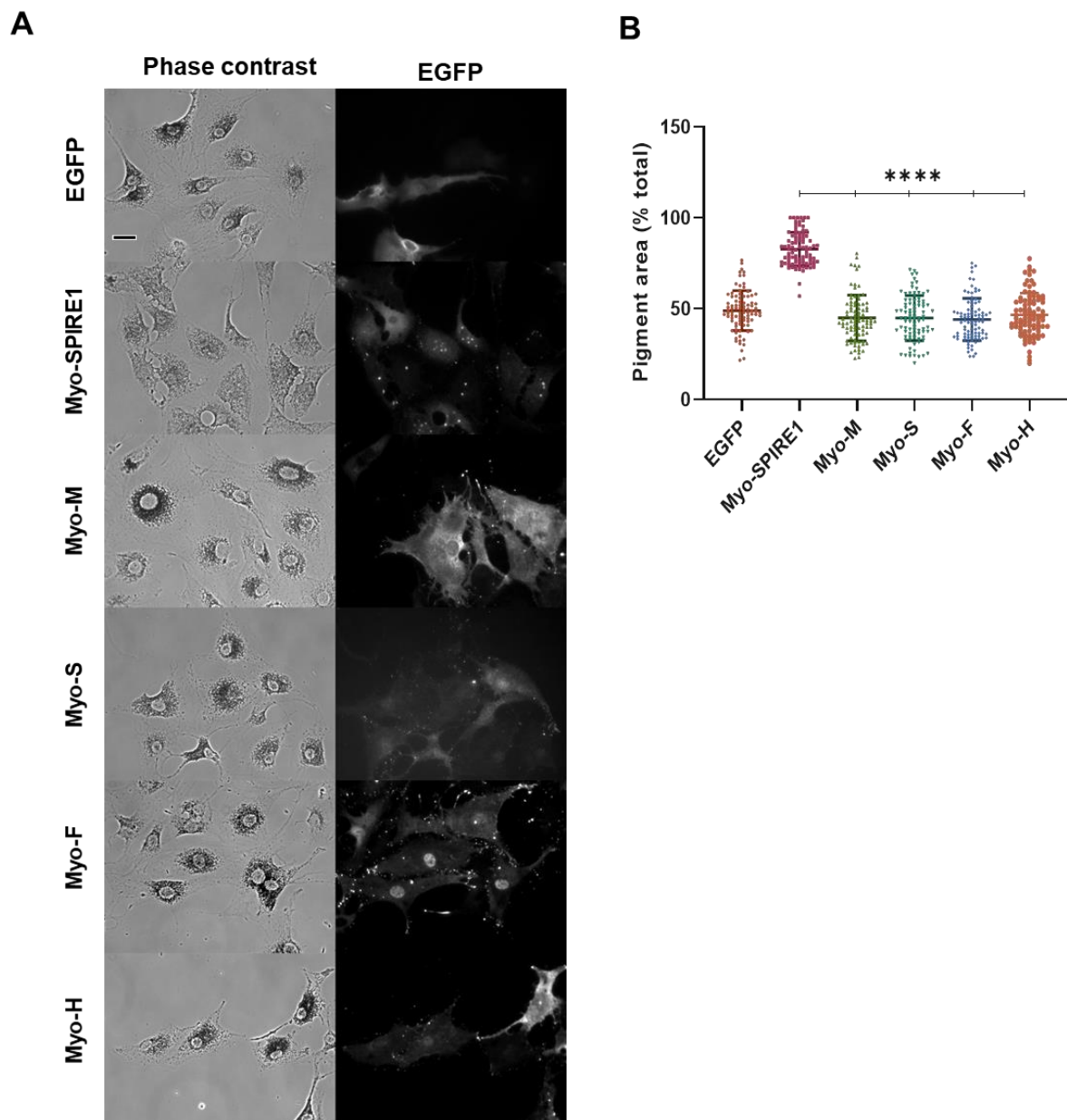


Figure 6.3.1 Investigating the role of individual domains in Rab27a interaction. A) melan-In were seeded on coverslips for 24 hours then infected with adenoviruses expressing the indicated proteins. Cells were fixed 16-24 hours later, immunofluorescently stained with mouse anti-EGFP and imaged using phase-contrast and fluorescence optics to observe melanosome and protein distribution; scale bar represents 20 μ m. B) A Scatter plot shows a comparison of pigment area among the treated cell populations; each spot represents an individual cell data. The horizontal bar represents SD and the mean at the middle; Asterisks indicate the significance of the difference of the dataset compared with positive control **** = $p < 0.0001$ as determined by One-Way ANOVA. Data shown are from at least three independent experiments $n=90$ cells for each tested protein.

6.4 Characterising the Contribution of C-Terminus Domains in the Interaction with Rab27a

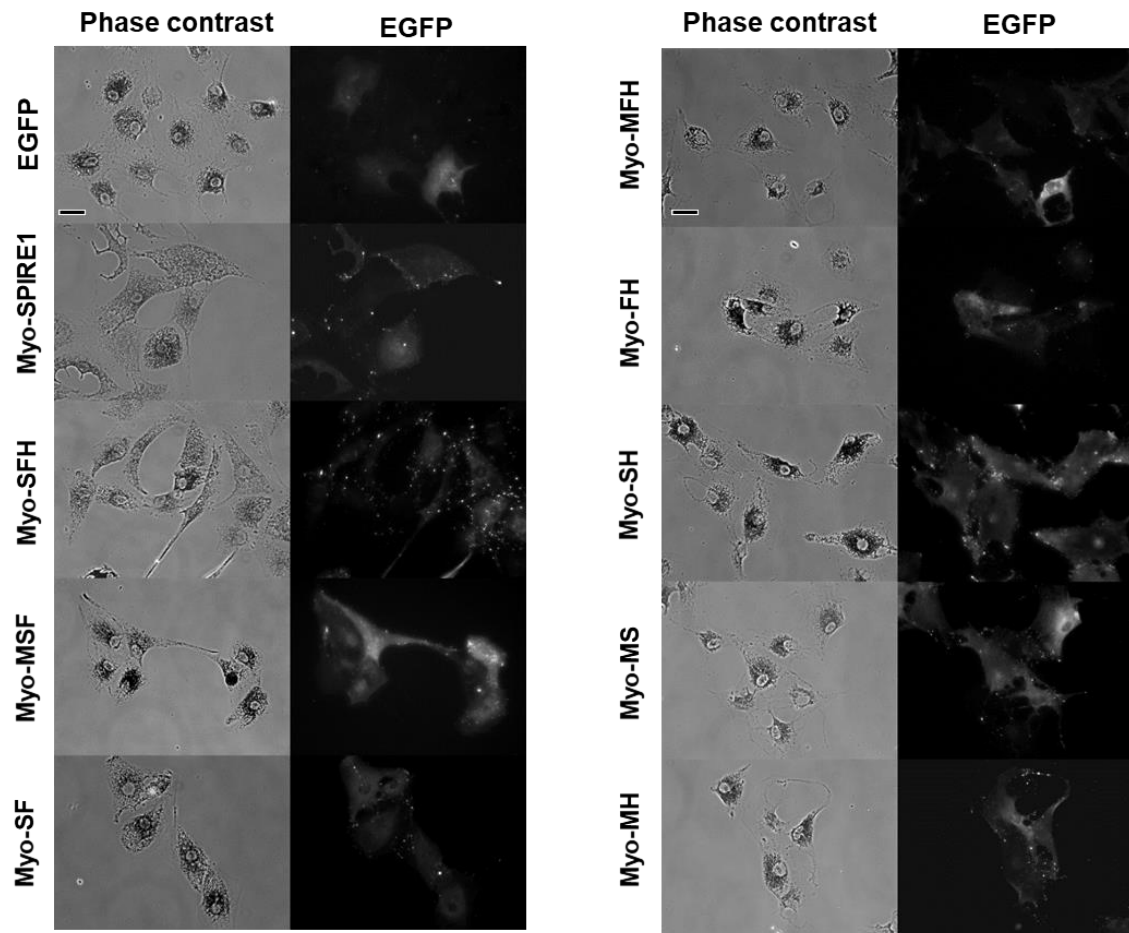
To identify the smallest truncated SPIRE1 able to establish sufficient interaction with Rab27a for melanosome dispersion, a combination of C-terminus domains of SPIRE1 were used in myosin chimera to rescue melan-In. To that end, chimera of myo-SFH (aa 381-598) , -MSF (aa 230-554) , -SF (aa 381-553) , -MFH (aa 230-380, 428-598), -FH (428-598 aa), SH (aa 380-388, 544-598), MS (aa 230-388), and MH (aa 230-380, 544-598) were expressed in melan-In through adenovirus expressing system, with myo-SPIRE1 and EGFP-alone used as controls. The cells were then fixed and prepared as described, and the data was collected and analysed.

As shown in Figure 6.4.1, SFH was able to target the motor to melanosome membrane and restore the melanosome dispersed distribution to a similar level of full-length of SPIRE1 (a mean of $76.60\% \pm 11.82$ of pigment area in the cells expressing myo-SFH compared to $81.78\% \pm 13.03$ ($P > 0.1$) and $50.16\% \pm 11.83$ ($P < 0.0001$) in the cells expressing myo-SPIRE1 full-length and EGFP-alone, respectively). While deleting α -helical domain from the C-terminus (i.e. myo-MSF and SF) partially restored the dispersion with pigment area significantly smaller than the positive control and higher than the negative control (a mean of $67.65\% \pm 15.54$ and $60.00\% \pm 14.29$ of pigment area in the cells expressing myo-MSF and -SF, respectively, compared to both myo-SPIRE1 and EGFP-alone ($P < 0.0001$)). Interestingly,

deleting FYVE or SPIRE-box domain seems to cease the interaction with Rab27a, such that all myo-MFH, -FH, SH, and -MH failed to restore melanosome dispersion. The mean percentage of pigment area was $47.12\% \pm 12.47$, $44.31\% \pm 12.14$, $45.03\% \pm 13.26$ and $49.19\% \pm 14.82$ for the cells expressing chimaera of MFH, FH, SH and MH respectively, recording no significant differences compared to EGFP-alone ($P > 0.1$). Similarly, deleting both FIVE and α -helical domains abolishes the ability of the C-terminal region to restore the melanosome dispersion in melan-In. Thereby, the mean percentage of pigment area in cells expressing myo-MS was $44.42\% \pm 11.71$ with no significant differences with EGFP-alone.

These results indicated a significant role of the SPIRE-Box and FYVE domains in the interaction with Rab27a and highlighted a role of H domain in this interaction. However, the presence of the three domains (SPIRE-Box, FYVE, and α -Helical domains) is essential to establish an interaction with Rab27a that is sufficient to allow the motor to disperse melanosome to the level of the full-length SPIRE1.

A



B

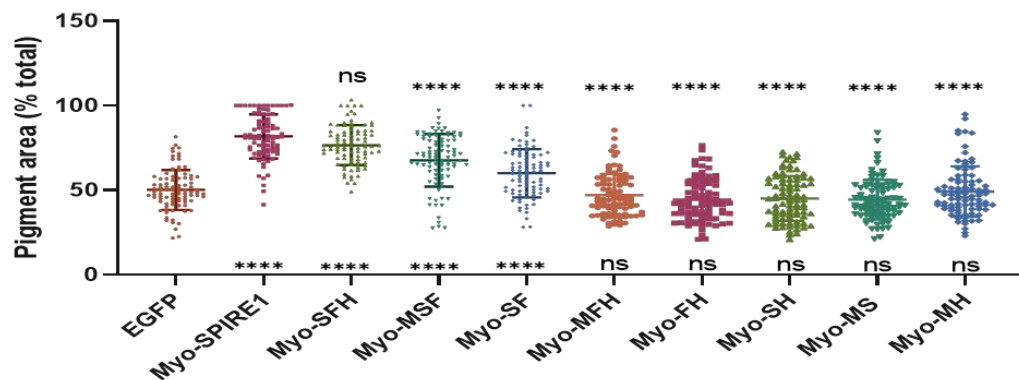


Figure 6.4.1 Characterising the contribution of C-terminus domains in the interaction with Rab27a. A) melan-In were seeded on coverslips for 24 hours then infected with adenoviruses expressing the indicated proteins. Cells were fixed 16-24 hours later, immunofluorescently stained with mouse anti-EGFP and imaged using phase-contrast and fluorescence optics to observe melanosome and protein distribution; scale bar represents 20 μ m. B) A Scatter plot shows a comparison of pigment area among the treated cell populations; each spot represents an individual cell data. The horizontal bar represents SD and the mean at the middle; Asterisks indicate the significance of the difference of the dataset compared with positive control **** = $p < 0.0001$, ns= no significant differences as determined by One-Way ANOVA. Data shown are from at least three independent experiments $n=90$ cells for each tested protein.

6.5 Identifying the Crucial Residue(S) for The SPIRE: Rab27a Interaction within C-terminus of SPIRE1

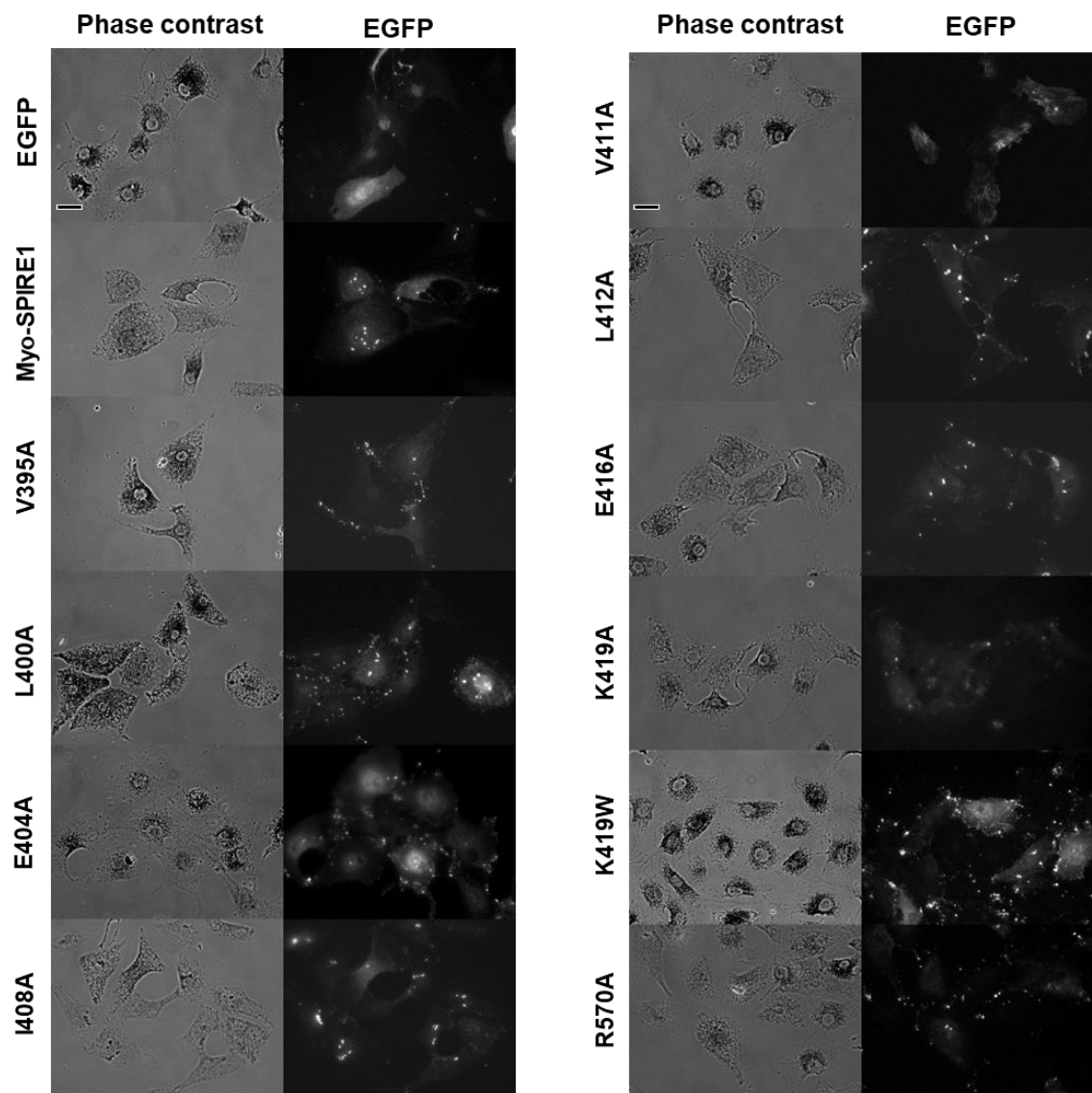
To identify the crucial residue(s) for the SPIRE1: Rab27a interaction, the conserved residues shared between SPIRE1 and Rab27a-binding domain of the well-known Rab27a-effectors (e.g. mlph and myrip) were substituted with alanine by site-directed mutagenesis. The tested residues V395, L400A, E404, I408, V411, L412, E416, and K419, are within the SPIRE-Box domain and R570 is within the α -helical domain of SPIRE1. The SPIRE1 mutants were then expressed as myosin chimaera in melan-In with myo-SPIRE1 (full-length/ wild-type) as a positive control and EGFP-alone as a negative control. The cells were then fixed and prepared for immunofluorescence, and the data was collected and analysed as previously described.

As shown in Figure 6.5.1 (A, B, C), the results with the mutant constructs indicate that the naturally occurring residues contribute differently to the interaction. Most of them (V395A, I408A, L412A, E416A, K419A and R570A) maintained the interaction, but the melanosome distribution distance was significantly less than the positive control (partially rescued melan-In). The mean of the pigment area percentage was $66.96\% \pm 13.56$, $70.49\% \pm 15.81$, $70.06\% \pm 16.15$, $66.04\% \pm 14.47$, $64.91\% \pm 13$, and $64.1\% \pm 16.67$ in the cells expressing V395A, I408A, L412A, E416A, K419A, and R570A, respectively, compared to $80.44\% \pm 10.16$ and $34.32\% \pm 10.5$ in cells expressing myo-SPIRE1 and EGFP-alone. Only the mutant L400A had no significant differences with myo-SPIRE1, where the mean of the pigment area percentage was $85.52\% \pm 11.29$ compared to $80.44\% \pm 10.16$ in cells expressing myo-SPIRE1. On the other hand, only two tested mutations (E404A and V411A) entirely ceased the interaction and the pigment area in the cell expressing those proteins showed no significant differences,

compared to the negative control. The mean pigment area percentage was $31.97\% \pm 9.981$ and $35.45\% \pm 8.551$ in cells expressing the chimaera of E404A and V411A, respectively, compared to $34.32\% \pm 10.5$ in cells expressing EGFP-alone. These results indicate that the residues E404 and V411 within S domain are crucial for the SPIRE1: Rab27a interaction.

Interestingly, substituting lysine 419 (K419) with tryptophan instead of alanine had a strong effect on the interaction with Rab27a. As shown in Figure 6.5.1 (A, C), expressing the K419W chimaera in melan-In failed to restore melanosome dispersion. The mean percentage of the pigment area was $39.66\% \pm 10.94$ in cells expressing K419W chimaera with high significant differences compared to and in the cells expressing myo-SPIRE1 ($P < 0.0001$) and no significant differences compared to EGFP-alone ($P > 0.1$). That indicated the capability of the point mutation K419W to cease the interaction with Rab27a and highlighted the strong effect of tryptophan to abolish the said interaction, compared to alanine.

A



B

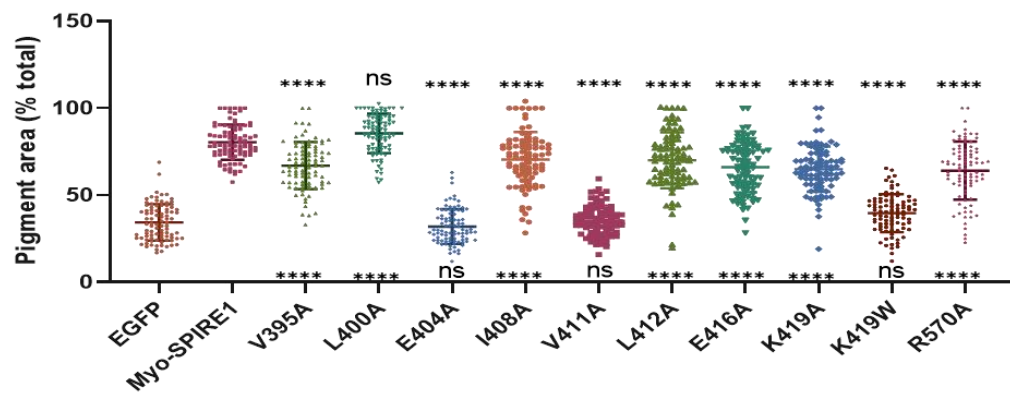


Figure 6.5.1 Identifying the crucial residue(s) for the SPIRE: Rab27a interaction within C-terminus of SPIRE1.

A) melan-In were seeded on coverslips for 24 hours then infected with adenoviruses expressing the indicated proteins. Cells were fixed 16-24 hours later, immunofluorescently stained with mouse anti-EGFP and imaged using phase-contrast and fluorescence optics to observe melanosome and protein distribution; scale bar represents 20 μm . B) A Scatter plot shows a comparison of pigment area among the treated cell populations; each spot represents an individual cell data. The horizontal bar represents SD and the mean at the middle; Asterisks indicate the significance of the difference of the dataset compared with positive control **** = $p < 0.0001$, ns= no significant differences as determined by One-Way ANOVA. Data shown are from at least three independent experiments $n=90$ cells for each tested protein.

6.6 Chapter Discussion and Conclusion

In this chapter, the ability of the MYO-SPIRE chimeric protein to disperse melanosomes was used to map the Rab27a interaction sites within the C-terminal region of SPIRE1. To this end, truncation and mutations of SPIRE1 were generated in myo-SPIRE1 chimera and then expressed in melan-In., melanosome dispersion distance, as indicated by the area the melanosome covered in the cell (pigment area), was then used as a read-out for the ability of the truncated/mutated SPIRE1 to target the active motor of myosin-Va to melanosome by interacting with Rab27a. The results were consistent with the *in vitro* study (Alzahofi et al., 2020), with SPIRE1 interacting with Rab27a through C-terminal membrane-binding domains and not the N-terminal. However, the results showed that none of the individual domains of the C-terminus was able to establish the interaction needed for the motor to disperse melanosome and that the presence of the three domains (SPIRE-Box, FYVE, and α -helical domains) is essential to provide the motor with sufficient interaction for melanosome dispersion. The presence of myosin-globular tail binding domain (M) in the C-terminal construct has no significant effect on its ability to interact with Rab27a. However, the results highlighted a significant, yet unknown role of α -Helical domain and a crucial role of FYVE and SPIRE-Box domains in this interaction.

Deleting α -helical domain from C-terminus results in partial rescue of melan-In, which indicates that the SPIRE-box and FYVE were able to establish an interaction with Rab27a,

but the interaction was not sufficient to allow the motor to transport melanosome for long-distance at the level of the full-length SPIRE1. Deleting FYVE abolishes the interaction completely and results in no significant differences in the pigment area compared with the negative control, which is consistent with the essentials of the zinc finger and the hydrophobic turret loop of FYVE-type zinc finger domain in localising SPIRE to the oocyte cortex and cytoplasmic face of the vesicular membrane (Tittel et al., 2015, Jo et al., 2019). In both the studies, removing the zinc or disrupting the loop by point mutations results in a homogeneous distribution of SPIRE in the cytoplasm (Tittel et al., 2015, Jo et al., 2019). Similar to FYVE, deleting the SPIRE-box also blocked the interaction with Rab27a and all the truncated SPIRE1 failed to target the motor to melanosome membrane. This supports the similarity between SPIRE-box of SPIRE1 with SHD1 (synaptotagmin-like protein homology domain) of Rab27a-effectors (Kerkhoff et al., 2001, Alzahofi et al., 2020). *In vitro* pull-down assay showed that SHD1 of Slp1–3 and Slac2-a/b interact specifically and directly with GTP-form of Rab27a (Kuroda et al., 2002).

However, the similarity between the interaction interface of SPIRE1 and Rab27a effectors needs to be interpreted with caution. Amino acid sequence alignment of C-terminal regions of human SPIRE1 and SPIRE2 with N-terminal regions of human MLPH, MYRIP, Rabphilin3A and NOC2 through Clustal Omega Multiple Sequence Alignment tool showed that SPIRE1/2 shared conserved residues with the aforementioned effectors (Alzahofi et al., 2020). In order to investigate the crucial residues among them for SPIRE1: Rab27a interaction, myosin chimaera of mutant SPIRE1 comprised of a single point mutation in one of the conserved residue (mainly within SPIRE-box domain) was used to rescue melan-In. Based on the results of the myo-SPIRE assay that it seems that the residues make different contributions to the

Rab27a interaction. Mutation L400A had no effect on the interaction. While V395A, I408A, L412A, E416A, K419A, and R570A slightly reduced the interaction, the motor was not able to restore melanosome dispersion to the level of non-mutate SPIRE1 chimaera. However, mutations of E404A and V411A (and T401A from Alzahofi and McNutt unpublished data) completely blocked the interaction such that the chimaera of mutant SPIRE1 failed to rescue melan-In, which indicates the requirement of these residues for the SPIRE1: Rab27a interaction. These results were consistent with an *in vitro* study that indicated the E404 and V411 corresponding residues in MLPH (E14 and V21, respectively) are essential for MLPH: Rab27a interaction (Fukuda, 2002). Also, the crystal structure of SLP2-Rab27a complex reported the indispensability of the residue V18 in SLP2 (corresponding to V411 of SPIRE1) in the interaction with Rab27a. Nevertheless, in some of the effectors' residues corresponding to ones identified here as essential for SPIRE/Rab interaction are not essential there that a single mutation of the residue E51 in Noc2 (corresponding to E404 of SPIRE1) only reduces the binding affinities *in vitro* and *in vivo* assays (Fukuda et al., 2004). Also, the mutation I408A in SPIRE1 slightly affects the interaction, but the corresponding residue I15 in SLP2 was reported as an essential residue for the interaction (Chavas et al., 2008). Interestingly, substituting the residue K419 of SPIRE1 with alanine only reduced the efficiency of the interaction, while substituting it with tryptophan completely blocked the interaction with Rab27a. Mutant K419W was designed to mimic the corresponding residue R35W in MLPH (according to Hume and Kerkhoff unpublished alignment) while aligns with D569 in Hs-SPIRE1 according to (Alzahofi et al., 2020) that corresponding to D425 in Mm-SPIRE1. R35W is the main cause of Griscelli syndrome III in human and lavender phenotype in chickens (Vaez et al., 2008, Ménasché et al., 2003, Fukuda, 2002). Although K419 does not align with R35W, the high hydrophobic features of the indole ring, in-R group of tryptophan

(W) has a strong effect on the interaction with Rab27a. Moreover, substituting either E416 or K419 in SPIRE1 with alanine reduced the efficiency of the interaction with Rab27a, while MLPH E32 corresponding to K416 is in the region that is responsible for the leaden coat colour in mice (REEERLQ) and the deletion of the corresponding region in SLP2 impairs the effector function (Matesic et al., 2001).

SPIRE1		Rab27a effectors		
		corresponding residues		
Residue	Effect on Rab27a interaction	Effector	residue	Effect on Rab27a interaction
V395	±	x		
L400A	-	x		
T401A	+	MLPH	T11	+
E404	+	MLPH	E14	+
		NOC2	E51	±
I408	±	MLPH	V18	+

V411	+	MLPH	V21	+
		SLP2	V18	+
L412	±	x		
E416	±	MLPH	E32	+
K419	±	x		
R570	±	x		

Table 6.1. A comparison of the contribution of the SPIRE residues in Rab27a interaction and the corresponding residues of established Rab27a-effector. (+) critical for the interaction, (-) has no effect on the interaction, (±) has a partial effect on the interaction, (X)= there is no tested residue in the literature (thus far).

Together, the crucial residues shed light on the similarity between the interaction interface of SPIRE1 and MLPH with Rab27. More investigations are needed, though. These findings highlight the mechanism by which Rab27a regulates actin-based transport of melanosome as a model of lysosome-related organelles.

Chapter 7: The Effects of SPIRE Spatial Localisation on Melanosome Distribution

7.1 Introduction

The diversity of physiological processes in living cells has been achieved by the distinct organelles and their associated proteins. Most of the translated proteins must be translocated to at least one cellular membrane to exert their functions, thus achieving physiological diversity (Hung and Link, 2011, Chacinska et al., 2009). The translocation of a protein into distinct organelles involves particular mechanisms. However, a standard feature for the protein trafficking mechanism is the presence of signal sequences encoded within the target protein that can be recognised by specific receptors (Hung and Link, 2011). Appropriate protein localisation provides access to interacting partners and the post-translational modification machinery. On the other hand, abnormalities in the subcellular localisation of a protein are mainly caused by mutations that result in the dysregulation of the protein trafficking machinery or change in the transport receptors' expressions. Subsequently, the mislocalisation of the proteins can lead to their inactivation or exert the toxic gain of function that has been associated with different human diseases (Hung and Link, 2011). For example, the mislocalisation of nuclear tumour-suppressor proteins to the cytoplasm leads to inactivation effects, thus increasing uncontrolled cell proliferation and the onset of disease (Hung and Link, 2011, Fabbro and Henderson, 2003, Salmena and Pandolfi, 2007, Turner et al., 2012, Yashiroda and Yoshida, 2003). Further, the mislocalisation of the microtubule-associated protein tau (MAPT) to dendritic spines has

neurotoxicity effects that lead to synaptic dysfunction and consequently, neurodegeneration (Hung and Link, 2011, Hoover et al., 2010).

Correspondingly, manipulating the protein localisation is a promising strategy for developing anticancer, antiviral, and anti-inflammatory therapies. Therapeutic strategies aimed either to restore normal protein folding and localisation or deliberately cause protein mislocalisation, therefore, inhibit the function of disease-causing proteins. For this reason, gaining an understanding of subcellular localisation and mislocalisation of proteins is essential to the comprehension of the mechanisms involved in the pathogenesis of human diseases and therapeutic interventions.

In this chapter, the effects of SPIRE localisation on melanosome distributions is investigated. The wild-type melanocyte has a uniform dispersed distribution, while mutant melanocytes have perinuclear clustering melanosomes (Chapter 5, Figure 5.1.1 B). However, eccentric melanosome distribution is observed in the SPIRE1/2-rescue assay (Chapters 3 and 4). The hyper-dispersed distribution is featured with an accumulation of melanosomes at dendritic and cell cortex (Figure 7.1.1). The hyper-dispersed distribution of melanosomes is observed in cells expressing Hs-SPIRE2, N-terminus domains of Hs-SPIRE1 (KIND and WH2), Mm-SPIRE1-exon13, and Mb-SPIRE, which are all described with weak or no interactions with Rab27a (i.e., they do not localise to melanosome membrane). This observation suggests an association between the SPIRE spatial localisation and the pattern of melanosome distribution.

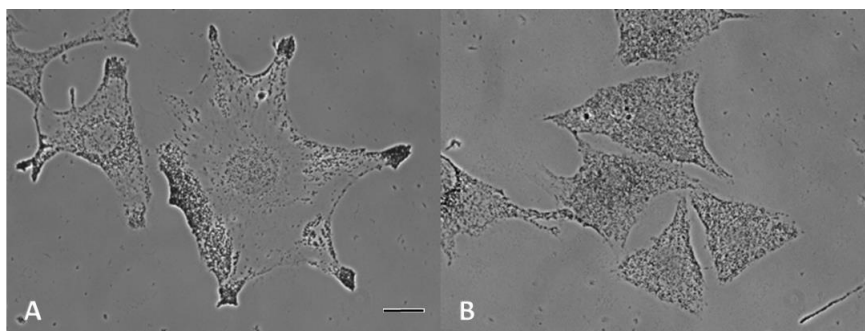


Figure 7.1.1 High magnification (40x) fields of view represent the differences in melanosome distribution after expression of SPIRE2 and SPIRE1 in SPIRE1/2-depleted melan-a. A) expressing SPIRE2 resulted in hyper-dispersed distribution in which melanosomes accumulate at the dendritic. B) expressing SPIRE1 resulted in uniform dispersed distribution similar to that of wild-type melan-a; Scale bar represents 20 μ m.

To test this hypothesis, the effects of mislocalised SPIRES on melanosome distributions was investigated in a rescue assay of SPIRE1/2-depleted melan-a. To this end, SPIRE was mislocalised in two ways, namely, a) using mutant Hs-SPIRE1 genes that equivalent to mutant Mm-SPIRE1 that described in the previous chapter (in myo-SPIRE1 chimaera). That has point mutation that impaired Rab27a interaction and b) manipulating SPIRE2 localisation by fusing N-terminus domains (KIND and WH2) with well-known membrane-associated proteins to target the actin nucleation function of the SPIRE to other organelles (e.g., Golgi apparatus or plasma membrane). Mislocalised SPIRE1/2 were transiently expressed in SPIRE1/2-depleted melan-a, and the percentage of cells with dispersed and hyper-dispersed melanosomes was used as a read-out for the effects of mislocalised SPIRE on melanosome distributions.

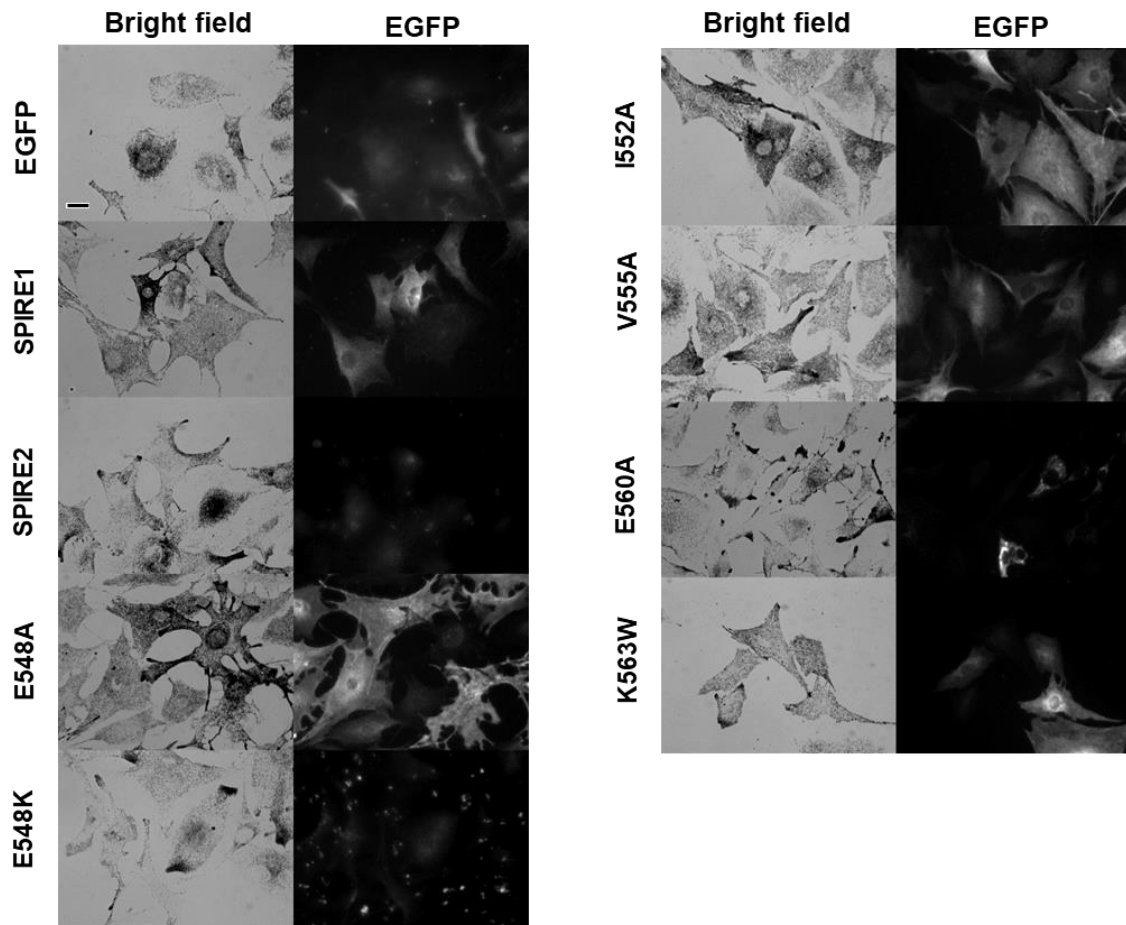
7.2 The Effects of Disrupting the Interaction Between SPIRE1 and Rab27a on Melanosome Distribution

To test the SPIRE-localisation to melanosome membrane, by Rab27a interaction, on melanosome distributions, mutant Hs-SPIRE1 that have point mutations equivalent to those described in the previous chapter to impair Rab27a interactions entirely or partially were

used in the SPIRE1/2 rescue assay. In detail, mutant Hs-SPIRE1-E548A, E548K, -I552A, -V555A, E560A, and -K563W that were equivalent to Mm-SPIRE1-E404A, I408A, V411A, E416A, and K419W, respectively (chapter 6), were expressed in SPIRE1/2-depleted melan-a via the adenoviruses expressing system. FL-SPIRE1/2 and EGFP-alone were used as controls. The cells were then fixed, stained using an anti-GFP antibody, and their melanosomes distribution data were collected and analysed.

As shown in Figure 7.2.1, all mutant SPIRE1 caused hyper-dispersed distribution similar to SPIRE2. A part of mutants I552A and K563W, all the other mutants caused a significantly high ratio of hyper-dispersed distribution in their expressing cells ($P < 0.0001$ and < 0.001) compared to non-mutant SPIRE1. The mean of cells with hyper-dispersed distribution was $25.88\% \pm 15.49$ (E548A), $56.85\% \pm 1.98$ (E548K), $11.26\% \pm 9.72$ (I552A), $43.97\% \pm 10.84$ (V555A), $59.16\% \pm 8.76$ (E560A), and $13.40\% \pm 9.59$ (K563W) compared to $0.69\% \pm 0.37$, $55.98\% \pm 10.35$, and $5.63\% \pm 1.55$ of the cells expressing SPIRE1, SPIRE2, and EGFP-alone, respectively. These findings associate melanosome distributions with SPIRE-Rab27a interaction (SPIRE localisation at melanosome membrane) and linked hyper-dispersed distribution with mislocalised SPIRE.

A



B

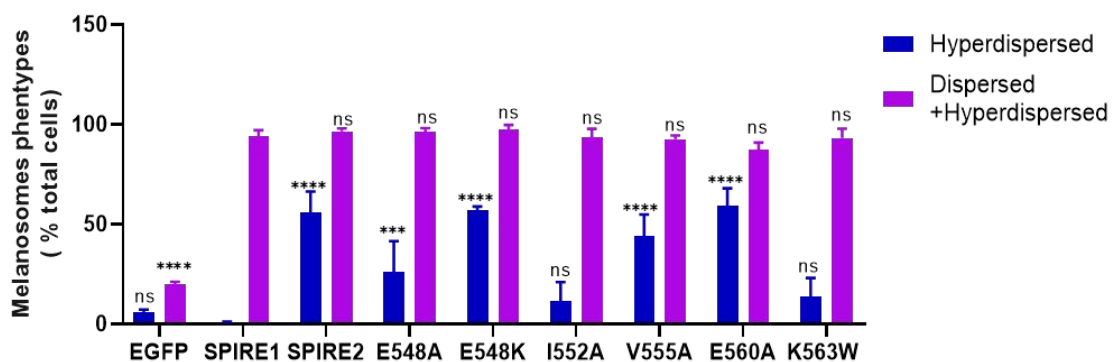


Figure 7.2.1 The effects of disrupting the interaction between *SPIRE1* and *Rab27a* on melanosome distribution. A) melan-a cells were plated onto glass coverslips and depleted of *SPIRE1/2* proteins by siRNA transfection. After 72 hours, cells were infected with adenoviruses expressing the indicated proteins. Cells were fixed after 24 hours, processed for immunofluorescence and imaged using bright field and fluorescence optics to observe melanosome and protein distribution/expression. Scale bar represents 20 μ m. B) The percentage of melan-a with melanosome hyper-dispersed and the total dispersed distribution after expressing the indicated proteins (n=3 low magnification fields of view in three independent experiments for each tested

protein). Asterisks indicate the significance of the difference of the dataset compared with SPIRE1 expressing cells; ****= $P < 0.0001$ ns= no significant differences as determined by two-way ANOVA.

7.3 The Effect of the Spatial Distribution of SPIREs on Melanosome Distribution

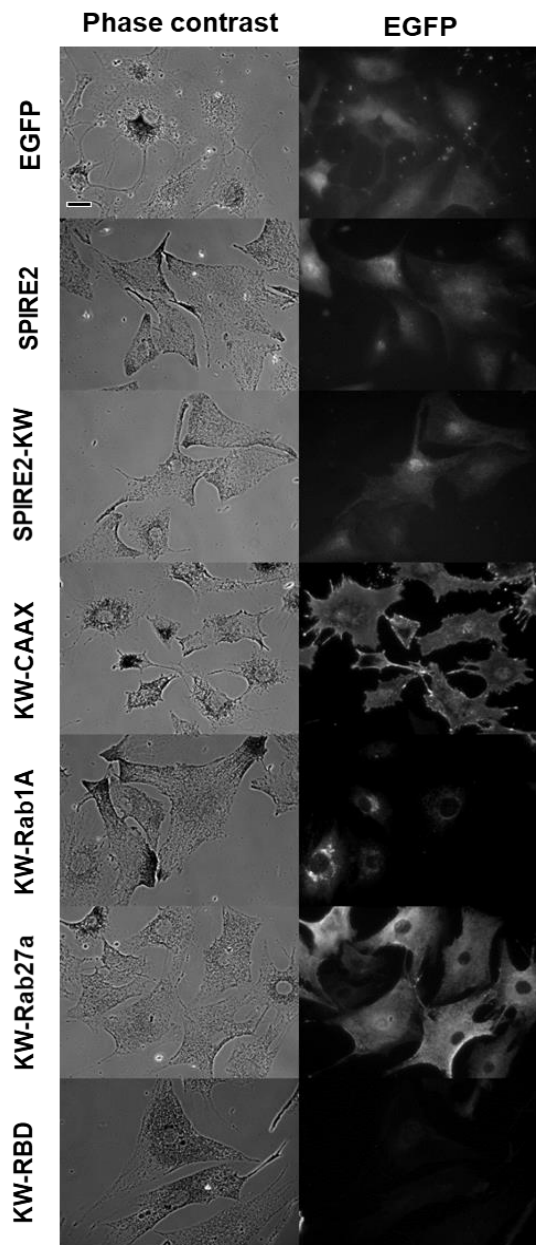
To confirm the effects of the SPIRE localisation on melanosome distributions, the impact of SPIRE2 localisation on melanosome distributions was investigated. To this end, the actin nucleation function of SPIRE2 was harnessed for different organelles. To elaborate, chimeric proteins comprised of N-terminus (KIND and WH2) of the Hs-SPIRE2 fused to a) the CAAX motif of Ras proteins (named after a specific amino acid sequence: C is cysteine, AA are two aliphatic residues, and X represents any C-terminal amino acid on different proteins. CAAX motif targets the cytoplasmic face of the cell membrane, b) Rab1a that targets Golgi apparatus, c) inactive mutant Rab27a SF1/F4 that is described to be targeting melanosome membrane, and d) the Rab27a-binding domain (RBD) of synaptotagmin-like proteins 2-a (Sytl2a) that also target melanosome membrane, were expressed in SPIRE1/2-depleted melan-a melanocytes. EGFP-alone FL- and N-terminus regions of SPIRE2 were used as controls. The cells were then fixed and immuno-stained, and further, melanosome distribution was visualised, and data collected and analysed as previously described.

As shown in Figure 7.3.1, targeting the actin nucleation function of SPIRE2 to the cytoplasmic face of the plasma membrane via the CAAX domain resulted in a clustered melanosome. A mean percentage of cells with clustered melanosomes was $72.14\% \pm 8.35$ of the cells expressing KW-CAAX, compared to $82.33\% \pm 5.27$ ($P > 0.1$), $4.78\% \pm 4.23$ ($P < 0.0001$), and $10.79\% \pm 1.41$ ($P < 0.0001$) in EGFP-alone, FL-, and KW-SPIRE2 expressed cells, respectively. Only a mean of $9.81\% \pm 3.22$ of the cells exhibited hyper-dispersed distribution in KW-CAAX, compared to $2.48\% \pm 1.44$ ($P > 0.1$), $67.93\% \pm 17.47$ ($P < 0.0001$), and $54.25\% \pm 2.93$ ($P < 0.0001$) in the EGFP-alone, FL-, and KW-SPIRE2 expressed cells, respectively.

In contrast, targeting the actin nucleation function of SPIRE2 to the Golgi apparatus via Rab1a rescued the clustered distribution with a significantly higher percentage/proportion of the cell population exhibiting hyper-dispersed distribution. A mean of $3.18\% \pm 2.66$ of the cells expressing KW-Rab1A their melanosomes remains clustered in the perinuclear cytoplasm ($P < 0.0001$, > 0.1 , and > 0.1 in comparison with EGFP-alone, FL-, and KW-SPIRE2 expressed cells, respectively), while a mean of $44.73\% \pm 10.80$ of the cells exhibited hyper-dispersed distribution ($P < 0.0001$, < 0.1 , and > 0.1 , in comparison with EGFP-alone, FL-, and KW-SPIRE2 expressed cells, respectively).

Finally, supporting the hypothesis, targeting the actin nucleation function of SPIRE2 to the melanosome membrane via fusion of the KW active fragment of SPIRE2 to the mutant Rab27a^{SF1F4} or RBD of SLP2 rescued both the clustered and hyper-dispersed distribution of the melanosome. A mean of $92.84\% \pm 2.22$ and $81.04\% \pm 3.33$ of the cells expressing KW-Rab27a and KW-RBD respectively, exhibit uniform dispersed distribution similar to that of wild-type melanocytes. Only, a mean of $1.74\% \pm 2.79$ and $3.46\% \pm 2$ of the cells expressing KW-Rab27a and KW-RBD, respectively, exhibit hyper-dispersed distribution compared to FL- and KW-SPIRE2 expressing cells, respectively ($P < 0.0001$). That support the hypotheses that melanosome distributions are associated with the subcellular localisation of SPIRE1/2. The SPIREs melanosomal localisation via Rab27a-interaction is essential for achieving the uniform dispersed distributions that characterises wild-type melanocytes. Mislocalised SPIRE to other organelles or disrupting Rab27a interaction results in the hyper-dispersed distribution.

A



B

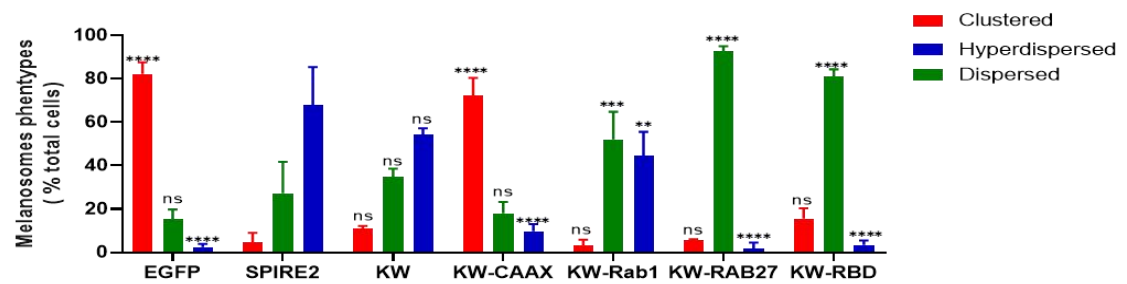


Figure 7.3.1 The Effect of the Spatial Distribution of SPIREs on Melanosome Distribution. A) melan-a cells were plated onto glass coverslips and depleted of SPIRE1/2 proteins by siRNA transfection. After 72 hours, cells were infected with adenoviruses expressing the indicated proteins. Cells were fixed after 24 hours,

processed for immunofluorescence and imaged using bright field and fluorescence optics to observe melanosome and protein distribution/expression. Scale bar represents 20 μm . B) The percentage of melan-a with melanosome clustered, dispersed and hyper-dispersed phenotypes after expressing the indicated proteins (n=3 low magnification fields of view in three independent experiments for each tested protein). Asterisks indicate the significance of the difference of the dataset compared with SPIRE2 expressing cells; ***=P<0.0001 ns= no significant differences as determined by two-way ANOVA.

7.4 Chapter Discussion and Conclusion

In this chapter, the effects of SPIRE localisation on melanosome distributions have been investigated. SPIREs localisations appeared to have a critical role in their function. In mouse oocytes, SPIRE1/2 localises to the cortex and Zn^{+2} -rich vesicles; removing FYVE domain or introducing point mutations on the crucial cysteine residues resulted in mislocalised SPIREs which impair the asymmetric division and cytokinesis processes (Jo et al., 2019). The previous chapter results (chapter 6) associated SPIRE localisation to the melanosome membrane with Rab27a. Such that disrupting the interaction with Rab27a by truncation or point mutation results in mistargeting the motor in the chimeric protein, subsequently failed to disperse melanosomes. In this chapter, SPIRE1/2 knockdown/rescue assay was conducted using mutants SPIRE1 that cannot interact with Rab27a (thus assuming it non-melanosomal localised). These Hs-SPIRE1 mutants are equivalents to the Mm-SPIRE1 mutants that were tested in myo-SPIRE1 chimaera and resulted in no melanosome dispersion. The expression of mutant Hs-SPIRE1-E548A, E548K, -I552A, -V555A, E560A, and -K563W) in SPIRE1/2-depleted melan-a showed that the mutants retain the actin nucleation function, thus facilitating the rescue of melanosome transport. However, losing the interaction with Rab27a causes the accumulation of melanosomes at the edge of the cells in the hyper-dispersed distribution that is different from the wild-type and the well-studied mutant melanocytes (e.g. leaden, ashen, and dilute). This finding was consistent with the previous observations (Chapters 3 and 4). There, hyper-dispersed distribution was observed after

rescue melan-a with Hs-SPIRE2 which interacts weakly with Rab27a ((Kollmar et al., 2019), and as seen in chapter 5), N-terminus of the Hs-SPIRE1 (which lacks membrane-associated region), Mb-SPIRE which interacts with Rab8 (Kollmar et al., 2019), and Mm-SPIRE-E13 which localises to mitochondria (Kollmar et al., 2019, Alzahofi et al., 2020).

To confirm the effect of SPIRE subcellular localisation on melanosome distributions, The actin nucleation function of SPIRE2 was fused to well-known membrane-associated protein to target other organelles. Supporting to the hypotheses, targeting the KW region of SPIRE2 to the Golgi apparatus via fusion with Rab1A results in a high percentage of hyper-dispersed distribution, which consistent with the previous observation—in contrast, targeting the cytoplasmic faces of cell membranes via CAAX results in no rescue that indicates the effect of SPIRE localisation on melanosome distributions. Consistent with these findings, targeting the actin nucleation function of SPIRE2 via fusion with Rab27a and Rab27a-binding domain to melanosome membrane results in uniform melanosome distribution similar to that of Wild-type melanocytes.

In conclusion, the spatial distribution of SPIRES plays a significant role in melanosome distributions. Mis-localised SPIRES facilitate melanosome dispersion but cause an abnormal distribution. The hyper-dispersed distribution can be rescued by restoring the appropriate localisation at the melanosome membrane via Rab27a interaction. However, further investigation is needed to determine the differences in melanosome transport that cause uniform dispersion or the hyper-dispersed phenotype. Also, the effects of the hyper-dispersed distribution on melanosome transfer to keratinocytes are worth to be investigated.

Chapter 8: Thesis Discussion and Conclusion

This project aimed to determine the role of the actin nucleation proteins named SPIRE1/2 in melanosome transport for better understanding of intracellular trafficking. Melanosomes of skin melanocytes provide such a reagent to study intracellular trafficking. The similarity in biological features of melanosomes and lysosome-related organelles (LROs) makes them a good studying model for such organelles (e.g. cytotoxic granules and dense platelet granules) (Marks and Seabra, 2001). Therefore, a better understanding of melanosome trafficking could lead to a better understanding of other LROs traffic and related diseases. Besides, the contrast provided by melanin in melanosome facilitates tracking their movement without any requisite for sophisticated equipment.

In this project, SPIRE1/2 role, and their well-studied domains in melanosome transport have been investigated in two ways. First, the SPIRE1/2 rescue assay is conducted by which the translation of endogenous SPIRE1/2-mRNA is disrupted by specific short-interference RNA, then rescuing the cells by adding siRNA-resistant SPIRE1/2 (full-length, truncations, or mutations). The change in melanosome distributions is used as a read-out for the effects of SPIRE1/2 on the transport.

This approach results in different findings. First, it provides evidence to support that melanosomes dispersion in skin melanocytes is actin-dependent, and this actin is nucleated by the cooperation between SPIRE and FMN-type formin. The results showed that depleting SPIRE1/2 resulting in perinuclear clustering of melanosome. It also shows that without the domains requisite for the interaction with FMN and globular actin (i.e. KIND and WH2), SPIRE1/2 was not able to restore melanosome dispersion in SPIRE1/2-depleted melan-a

(chapter 3). This is consistent with previous studies, which showed that depleting dynamic actin using latrunculin-A or inducing actin stabilization using jasplakinolide resulted in the perinuclear clustering of melanosomes (Evans et al., 2014). Also, the reduction in actin mesh after depleting SPIRE1/2 in mice oocytes was similar to that induced by Latrunculin-B (Pfender et al., 2011).

SPIRE was associated with formin since the discoveries of their maternal effects in the genetic screen of *Drosophila melanogaster* (Manseau and Schupbach, 1989). Mutation in either SPIRE or cappuccino (*Drosophila* formin) has similar effects of losing oocyte polarity thus female sterility (Theurkauf, 1994, Emmons et al., 1995, Rosales-Nieves et al., 2006, Quinlan, 2013). The SPIRE: formin interaction has been intensively studied biochemically and by structural biology (Quinlan et al., 2007, Pechlivanis et al., 2009, Vizcarra et al., 2011, Zeth et al., 2011). These studies showed a direct interaction between KIND domain of *Drosophila* SPIRE and FSI motif in cappuccino and same domain interaction between mammalian SPIRE1/2 and the homologues FMN1/2. Tie with this interaction, *in vivo* studies showed that depleting SPIRE1/2 shared the same effects of depleting formin in mice oocyte (Pfender et al., 2011, Schuh, 2011). In this project, depleting SPIRE1/2 has a similar effect of clustering melanosomes as the depletion FMN1. Also, expressing construct of spire1/2 that have a mutant KIND domain or lack it (i.e. disturbing the interact with FMN) in SPIRE1/2-depleted melan-a results in a similar effect as depleting SPIRE1/2 or FMN1 (i.e. perinuclear clustering of melanosome) (Chapter 3). Even with association with other organelles SPIRE1-E13 (mitochondrial anchored isoform SPIRE) also was capable of restoring melanosome dispersion in Mm-SPIRE1/2-depleted melan-a (chapter 4). Disturbing the interaction between KIND and FMN in E13 via point mutation causes perinuclear clustering of

melanosomes. That suggested that E13 also interact with FMN to nucleate actin filament that essential for melanosome dispersion. E13 was unable to rescue melan-f (FMN1 knockout melanocytes), suggesting the indispensability of the interaction between SPIRE1-E13 with FMN1 to establish melanosome dispersion (chapter 4). However, an *in vitro* GST-pulldown assay and fluorescence anisotropy showed the ability of KIND domain of E13 to interact with the endoplasmic reticulum-anchored isoform of the formin protein inverted formin2 (INF2) to nucleate actin filaments that facilitate mitochondrial division (Manor et al., 2015). This could indicate the flexibility of KIND domain of E13 to interact with different types of formin depending on the actin function. Further investigation is needed to determine the interaction between E13-KIND and FMN via GST-pulldown assay or immunoprecipitation as well as determine whether INF2 is expressed in melanocytes via RT-PCR. In addition to this, the results indicate no effect of SPIRE localisation on actin nucleation function of the protein. This will be explained later in this section.

The results also determine that the ability of KIND to interact with FSI was conserved among SPIRE and FMN through animal evolution. The results showed that the Choanoflagellate SPIRE of *Monosiga brevicollis* (Mb) was able to rescue SPIRE1/2-depleted Mm- melan-a. That suggested the ability of Mb-SPIRE to interact with Mm-FMN to nucleate actin that needed as a track for melanosome dispersion (chapter 4). This is consistent with an *in vitro* GST-pulldown assay that showed KIND domain of Mb-spire could interact strongly with the C-terminal region of Mm-FMN1 and to a lower extent with Mm-FMN2 (Kollmar et al., 2019). Collectively, the results determine that the actin filament nucleated by the cooperation between SPIRE1/2 and FMN-type formin is the melanosome dispersion track.

The second method used in this project is modified Nano-Scale Pulldown Assay (Bird et al., 2017) in leaden melanocytes (melan-In). In this, the mini-Va chimaera described in the study by (Evans et al., 2014) was modified by replacing the Rab27a-binding domain of SLP2 with SPIRE1/2 (full-length, truncations, or mutations) and then expressed in melan-In. This approach aims to investigate the ability of SPIRE to target the active motor of myosin-Va to the melanosome membrane by interacting with Rab27a. The change in melanosome distribution was used as a read-out for the said interaction. In which the interaction with Rab27a will result in dispersed melanosome, otherwise, melanosome will remain clustered at perinuclear area of melan-In .

The main finding obtained using this approach confirmed the ability of SPIRE1 to interact with Rab27a on melanosome membranes. That was supported by immunoprecipitation and mass spectrometry analysis. In this, SPIRE1 in myo-SPIRE1 chimaera was able to target the motor to melanosome membrane, which results in melanosome dispersion to the same level as expressing the missing protein (melanophilin). Also, GFP-fused Hs-SPIRE1 precipitated in complex with endogenous Mm-Rab27a, which was detected in both immunoblotting and mass spectrometry analysis. However, further investigation is needed for the smaller isoform (or truncated) endogenous Mm-SPIRE1 that precipitated in complex with endogenous Mm-Rab27a, via sequencing and RT-PCR. Nevertheless, the results indicate that SPIRE1 is a potential Rab27a effector (chapter 5).

The second finding is that SPIRE1 interacts with Rab27a via its membrane-associated domains. Using truncated SPIRE1 in myo-SPIRE1 chimaera showed that spire-box motif and FYVE are crucial domains for the said interaction. Deleting spire-box or FYVE domain blocked the interaction with Rab27a, and the truncated SPIRE1 failed to target the motor to

melanosome membrane, thus resulting in no dispersion in melan-In. Deleting α -helical domain, however, results in partial dispersion that is significantly lower than that results of full-length SPIRE1 suggesting a significant role for α -helical in this interaction (chapter 6). These results are tied with the similarity between spire-box of SPIRE1 and SHD1 (synaptotagmin-like protein homology domain) of Rab27a-effectors (Alzahofi et al., 2020, Kerkhoff et al., 2001). That as an amino acid sequence alignment showed that spire-box shares most of the conservative residues with the SHD1 of well-established Rab27a effectors (Alzahofi et al., 2020). *In vitro* pull-down assay showed that SHD1 of Slp1–3 and Slac2-a/b interact specifically and directly with GTP-form of Rab27a (Kuroda et al., 2002). The results also concurred with the essential requirement for the zinc finger and the hydrophobic turret loop of FYVE-type zinc finger domain in localising SPIRE to the oocyte cortex and cytoplasmic face of the vesicular membrane. In which, removing the zinc finger or disrupting the loop by point mutations results in a homogeneous distribution of SPIRE in the cytoplasm (Tittel et al., 2015, Jo et al., 2019). On the other hand, the α -helical domain has the fewest shared conserved residues with SHD1I compared to SB and FYVE ((Alzahofi et al., 2020) and Hume and webb unpublished data) Together, the results show that the presence of the three domains (spire-box, FYVE, and α -helices) is essential for sufficient binding with Rab27a to restore melanosome dispersion to the same level of full-length-SPIRE1 considering SPIRE1 as Rab27a effector (chapter 6).

Using mutant SPIRE1 in the same approach revealed crucial residues within the spire-box for binding Rab27a. The single mutation of T401A, E404A, and V411A entirely blocked the ability of SPIRE1 to target the melanosome membrane, thus resulting in no melanosome dispersion in melan-In, while V395A, I408A, L412A, E416A, K419A, and R570A partially

blocked the interaction resulting in lower dispersion level compared to non-mutant SPIRE1. Interestingly, substituting the residue K419 of SPIRE1 with alanine (A) partially affects the interaction with Rab27a, while replacing it with tryptophan (W) completely blocked it. Alanine is widely used in site-directed mutagenesis due to its chemical features. The small methyl side chain (-CH₃) prevents side-chain interactions without affecting the protein conformation. Also, the non-polar, non-charged features prevent the formation of hydrogen bonds or electrostatic interactions with the partner proteins. On the other hand, tryptophan featured with high hydrophobic indole ring in the side chain may affect the protein conformation, thus the binding with the partners (chapter 6).

The tested residues within SPIRE1 have corresponding residues in other Rab27a-effectors that are considered crucial for binding Rab27a (table 6.1). However, more investigation is needed to determine the similarity (or differences) between the interaction interface of SPIRE1 with Rab27a. One way to do this is by testing more residues within SPIRE1, for example, the residue V426 which is equivalent to R35 in MLPH and MYRIP and T575 in Mm-SPIRE1 is equivalent to G51 in SLP2. The mutant G51L was constructed to generate a Rab27a super-effector and was aimed to stabilize the interaction between SLP2 and Rab27a, thus inhibiting the interaction between MLPH and Rab27a. However, G51L disrupted the interaction of the super-effector with Rab27a (Hume and Al-Saad unpublished data). Glycine in position 51 is within SHD1I of SLP2 and is highly conservative in Rab27a effector except in slac2 a/c (leucine) and SPIRE1/2 (threonine). Testing T575 within the α -helical domain of SPIRE1 could shed light on the role of this domain in the interaction with Rab27a. Another way to investigate the similarity between SPIRE1 and Rab27a-effectors is by crystal structure

modelling the protein interaction and a computer-based analysis like molecular dynamics simulation.

The results of both SPIRE1/2 rescue assay and modified nanoscale pulldown assays show no significant contribution to the interaction between spire and myosin-Va in Spire functions. In which the results of expressing the KIND and WH2 in SPIRE1/2-depleted melan-a has no significant differences with KIND, WH2 and GTBM. Also using LALA mutant of SPIRE2 and E13 has no significant differences compared to the non-mutant proteins. Also, expressing the myo-SPIRE chimaera that includes GTBM, SB, FYVE, and α -helices have no significant differences with the chimaera that include only SB, FYVE and α -helices. That suggested no effects of the interaction between SPIRE and the globular tail domain of myosin-Va in the ability of SPIRE to nucleate actin filaments and to interact with Rab27a (chapter 3,4, and 6). However, the interaction could be significant for SPIRE functions in other cells worth investigated.

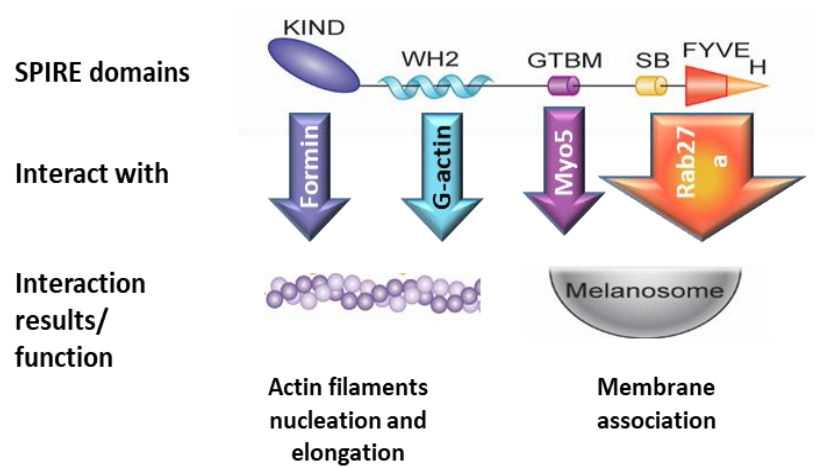
Both approaches showed differences between SPIRE1 and 2 in melanosome dispersion. Expressing SPIRE2 in SPIRE1/2-depleting melan-a results in abnormal melanosome distribution termed hyper-dispersed distribution. Expressing SPIRE1 under the same conditions results in a uniform distribution similar to that of wild-type melan-a. Observing an equal hyper-dispersed distribution in the cells expressing SPIRE1 construct that lack the membrane-binding domains suggested that the differences between SPIRE1/2 are within the C-terminal region. That was supported by modified nano-scale pulldown assay, where the results showed that the chimeric SPIRE1 is capable of restoring melanosome dispersion to the same level of the positive controls. In contrast, chimeric SPIRE2 results in partial dispersion that is significantly lower than the positive controls (chapter 3,5). This is

consistent with the *in vitro* GST-pulldown assays, which showed that the interaction between Rab27a and SPIRE2 is weaker than the interaction with SPIRE1. However, Both SPIRE1/2 interact at the same level as Rab8A (strong resulted band) and Rab3A (weaker band) (Kollmar et al., 2019, Alzahofi et al., 2020). That would suggest an association between the interaction with Rab27a and the pattern of melanosome distribution. This is supported by the observation of hyper-dispersed distribution in cells rescued by Mb-SPIRE that interact with Rab8 and the cells rescued by E-13 that localised to the mitochondrial outer membrane (chapter 4). Also, this hypothesis is supported by results showing that disrupting the interaction between SPIRE1 and Rab27a by a single point mutation (E548A, E548K, -I552A, -V555A, E560A, and -K563W) resulting in hyper-dispersed distribution while targeting the actin nucleation function of SPIRE2 to the melanosome membrane results in uniform distribution (chapter 7). However, the mechanism by which the interaction with Rab27a would impact melanosome distribution needs further investigation that includes the effects on the actin filaments length or stability, or the motor attached and detached to the filaments or the cargo during this process. The hyper-dispersed distribution is also worth further investigation regarding whether it impacts the melanosome transfer as well or if it is linked to dermal diseases (such as hyperpigmentation and post-inflammatory pigmentation). The effects of the mislocalised SPIRE on other LROs trafficking is also worth investigating.

In conclusion, the purpose of this project is to determine the role of SPIRE1/2 in melanosome transport as a model for intracellular cargo. The project support that melanosome anterograde transport (melanosome dispersion) is SPIRE-actin dependent. It also shows that SPIRE localises to melanosome membrane by interacting with GTP-Rab27a.

This suggested that after the association with the melanosome membrane, SPIRE then nucleate actin filaments by interacts with globular actin and cooperates with FMN-type formin which use as track for myosin-Va to transport melanosome to the edge of the cells (Figure 7.4.1) – a crucial step for melanosome to be transferred to the adjacent keratinocytes and serve its main function of photoprotection. This project suggests differences between SPIRE1 and SPIRE2 and associated melanosome distribution with the interaction between spire and Rab27a on melanosome membrane. The results of this project revealed some questions worth further investigation.

A



B

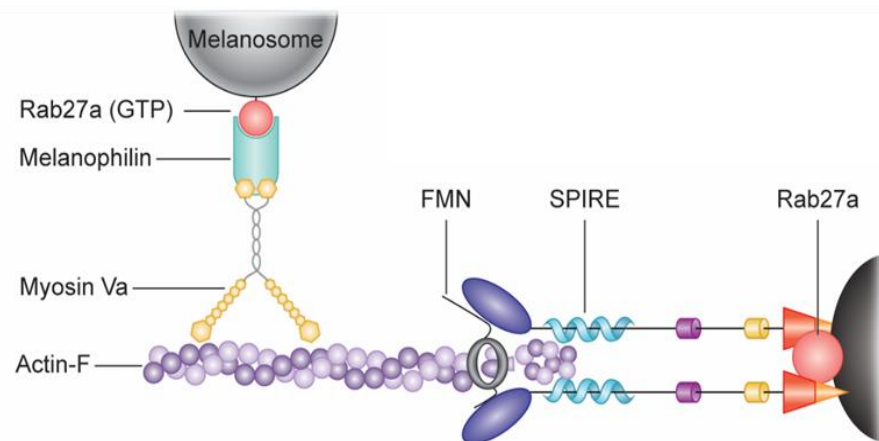


Figure 7.4.1 The role of SPIRE in melanosome dispersion in skin melanocytes. A) A schematic representation of SPIRE domains and their interaction partners as described in the literature and as found in this project (i.e. the interaction between C-terminus domains of SPIRE and Rab27a) along with the interaction functions as described in the literature. B) A schematic representation of The mechanism in which SPIRE nucleate actin filaments that used as a track for melanosome dispersion.

Chapter 9: References

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