UNIVERSITY OF CALIFORNIA,
IRVINE

Elucidating the Role of Phosphoinositides in Melanogenesis: PIKfyve Regulates Melanogenic Processes through the Synthesis of $PI(3,5)P_2$ and PI(5)P

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Sciences

by

Marc Christopher Liggins

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Dissertation Committee:
Associate Professor Anand K. Ganesan, Chair
Professor Bogi Andersen
Professor Xing Dai
Professor Kyoko Yokomori
Assistant Professor Maksim Plikus

2016

Dedication

I dedicate my thesis to my mom, Amalia. She was always my rock. Even in my darkest moments, I could always depend on her. She sacrificed for me, raised me, provided for me, taught me, and gave me the tools I needed to succeed. She drove me to my first day of school and to first day of college and she was there when I earned my Bachelor's Degree. She was the first person I told of my decision to attend the University of California, Irvine, yet she was taken from this world before I could take my first step onto campus as a graduate student. Even from heaven, she's continued to watch over me and has been with me in spirit throughout every step of my graduate journey. Her guidance, her words, eternally resonating within my soul. The greatest lesson that I learned from her is to never give up and to never quit, no matter how long it takes, no matter how many failures along the way, keep pressing forward. It is this lesson that I take deepest to heart and is the core principal that guides me. To my mom up in heaven, thank you for everything, I love you!!!

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List of Abbreviations

α-MSH	α-melanocyte stimulating hormone
AKT	V-Akt Murine Thymoma Viral Oncogene Homolog
AMPKα	Adenosine Monophosphate-Activated Protein Kinase α
AP (-1, -2, -3)	Adaptor Protein (-1, -2, -3)
APLP	Amyloid-like protein
APP	Amyloid precursor protein
ArPIKfyve	Associated Regulator of PIKfyve (Encoded by VAC14 gene)
ATG	Autophagy Related Protein
ATP7A	copper-transporting ATPase 1
BLOC (-1, -2, - 3)	Biogenesis of Lysosome-related Organelles Complex (-1, -2, -3)
ChIP	Chromatin Immunoprecipitation
CHS	Chediak-Higashi Syndrome
CMT4B1/2	Charcot-Marie-Tooth peripheral neuropathy Type 4B1/4B2
CMT4J	Charcot-Marie-Tooth peripheral neuropathy Type 4J
COPI	Coat Protein I
CTI6	Cyc8-Tup1 Interacting protein 6
DAG	Diacylglycerol
DHI	5,6-Dihydroxyindole
DHICA	5,6-Dihydroxyindole-2-carboxylic acid
DIDO	Death Inducer-Obliterator 1
DOPA	L-3,4-dihydroxyphenylalanine

ECM	Extra Cellular Matrix
ER	Endoplasmic Reticulum
FAB1	Forms Aploid and Binucleate cells 1
FCD	Fleck Corneal Dystrophy
FIG4	Factor-Induced Gene 4
FYVE	Fab1 YOTB Vac1 and EEA1
GLUT4	Glucose Transporter 4
HIRA	Histone Cell Cycle Regulator
HPS	Hermansky-Pudlak syndrome
HSV2	Homologous with SVP1
ILV	intraluminal membrane vesicles
ING2	Inhibitor Of Growth Family Member 2
LC3	(Microtubule Associated Protein 1) Light Chain 3
LRO	Lysosome-Related Organelles
MART-1	Melanoma Antigen Recognized by T-cells 1
MCR	Melanocortin Receptor
MC1R	Melanocortin 1 Receptor
MITF (-A, -M)	Microphthalmia-associated Transcription Factor (isoform A / M)
MTM	Myotubularin
MTMR	Myotubularin-Related protein
mTOR	Mammalian Target of Rapamycin
mTORC(1/2)	Mammalian Target of Rapamycin Complex 1/2
MVB	Multivesicular Bodies

OA1	Ocular albinism 1
OCA2	Oculocutaneous Albinism II
PIKfyve	Phosphoinsoitol Kinase FYVE (Encoded by PIKFYVE gene)
PHD	Pleckstrin Homology Domain
PI	phosphoinositide
PI3K	Phosphatidylinositol 3-Kinase
PI3KCII	Phosphatidylinositol 3-Kinase Class I
PI3KCII	Phosphatidylinositol 3-Kinase Class II
PI4K	Phosphatidylinositol 4-Kinase
PI5K	Phosphatidylinositol 5-Kinase
PI5P4K	Phosphatidylinositol-5-Phosphate 4-Kinase
PIP	Phosphatidylinositol
PI(3)P	Phosphatidylinositol-3-Phosphate
PI(3,4)P2	Phosphatidylinositol-3,4-Bisphosphate
PI(3,4,5)P3	Phosphatidylinositol-3,4,5-Trisphosphate
PI(3,5)P2	Phosphatidylinositol-3,5-Bisphosphate
PI(4)P	Phosphatidylinositol-4-Phosphate
PI(4,5)P2	Phosphatidylinositol-4,5-Bisphosphate
PI(5)P	Phosphatidylinositol-5-Phosphate
PI5P4K	PtdIns5P 4-kinase
PMEL	Premelanosome protein
POMC	Proopiomelanocortin
PTEN	Phosphatase and Tensin Homolog

PROPPIN	β-Propellers that bind Polyphosphoinositides
qRT-PCR	Quantitative Real-Time PCR
RAB	RAS-related GTP-Binding protein
RNAi	RNA interference
S6K	(p70) S6 kinase
SAC	SAC domain-Containing protein
SAC3	SAC domain-containing protein 3 (Encoded by FIG4 Gene)
shRNA	short hairpin RNA
siRNA	small interfering RNA
SNARE	Soluble NSF Attachment Protein REceptor
SNX1/2	Sorting Nexin 1/2
SOX10	Sex determining region Y-Box 10
TAF3	TATA-Box Binding Protein Associated Factor 3
TGN	Trans-Golgi Network
TH	Tyrosine Hydroxylase
TOM1	Target Of Myb1
TOM1L1/2	Target Of Myb1 Like 1/2
TMEM	Transmembrane Protein
TPC	Two Pore Channel
TRP	Transient Receptor Channel
TRPML	Transient Receptor Potential cation channel, Mucolipin subfamily
TUP1	dTMP-UPtake
TYR	Tyrosinase

TYRP1	Tyrosinase-Related Protein 1
TYRP2 / DCT	Tyrosinase-Related Protein 2 / Dopachrome Tautomerase
ULK1	Unc-51 Like Autophagy Activating Kinase 1
UVR	UV Radiation
VAC7	Vacuolar segregation protein 7
VAC14	Vacuole morphology and inheritance protein 14
VAC17	Vacuole related protein 17
VPS24	Vacuolar Protein Sorting 24
WIPI	WD-40 repeat-containing protein that Interacts with Phosphotidylinositides

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Acknowledgements

Graduate school is a journey, one that I would not have completed without several remarkable individuals that have each helped me in their own way. It is these individuals that I wish to thank and acknowledge for their contributions. Be it a mentor who has guided me, a colleague who has advised me, a friend who's been there for me, or family who's supported me. It is because of these individuals that I've become the person who I am and it because of them that I have reached end and succeeded in my graduate school journey.

First, I would like to thank **Dr. Anand Ganesan, M.D., PhD.**, for giving me an opportunity and accepting me into his lab. When circumstances would force me to find a new lab during my second year of graduate school, not many would or could offer a hand, yet Anand gave me an opportunity, in turn this drove drive my desire to succeed. Anand has been a valuable scientific mentor during my graduate career, helping me develop the skills needed to become a successful scientist – both at the bench and at the desk. He focused my untamed curiosity and helped me refine and broaden my skillset, ultimately helping define what it means to be a scientist.

I would like to thank my committee members, **Dr. Bogi Andersen, Dr. Xing Dai, Dr. Kyoko Yokomori,** and **Dr. Maksim Plikus** for the scientific advice and mentorship and their part in helping me grow scientist.

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Thank you to my labmates, past and present for their individual contributions. Thank you to **Dr. Elyse Paterson**, for being there for me, not just as an upperclassman, scientific advisor, and labmate, but as a fellow cat lover and friend. Thank you to **Dr. Sohail Jahid** for her advice and help, as well as for being an awesome labmate and an overall great person to chat with. Extra thanks for the awesome Donutery Donuts too ☺. Thanks to **Priya Vasudeva** and **Francisco Espitia** for being great lab managers, labmates, colleagues, and for their contributions in expanding my scientific skillsets. Thank you to my fellow classman, **Rolando Ruiz** for welcoming me into the lab when I

first joined and for guiding me during my adjustment period and for all his help. Thank you to **Chi-Fen Chen** and **Dr. Amy Hopkin** for their scientific advice and for being such pleasant labmates. Thank you to **Jessica Flesher** for the overall pleasant demeanor that she brings to the lab, for helping me finish up with experiments and for all the wonderful homemade desserts!!! Finally, thank you to me awesome (former) undergraduate mentee, **Sam Sandhu** for the extra set of hands and for being a delightful friend.

Research, especially as a graduate student, can particularly stressful. One of the keys to a successful graduate career is having a good outlet. For me, that was taekwondo. When I joined Tom Vo's Taekwondo during my first year, never did I imagine the enormous impact it would have on my life. It gave me an outlet, reignited a passion, built my confidence, taught me to teach, balanced my stress levels, and provided a family. Thereby I wish to thank all of my **Tom Vo's Taekwondo** family. Thank you to **Master Tom Vo Jr.**, who was there for me not just as a taekwondo master, but as a friend, an advisor, and a life mentor. He has tremendously impacted and influenced my life and I am extremely grateful for all that he has done. Thank you to **Ricardo Cruz** for not just being my taekwondo and teaching instructor, but for being one of my closest friends! Thank you to **Billy Phan, Thang Le, Jose Cerda,** and **Juan Amenero** for their help and guidance both on and off the mat. Thank you to **Dr. Amy Huynh** for being a true friend. And thank you to the rest of my TVT family for their friendship and support, **Hai Huynh, Kim Le, Vincent Le, Tony Le, Sung Kim, Colleen Chau, Yuliana Pantaleon, Tina Chu, Richard Zarate,** and the rest of my **Tom Vo's Taekwondo Family.** Finally, a big thank you to **Grandmaster Tom Vo** for founding Tom Vo's Taekwondo and for providing invaluable wisdom.

I would like to thank all the other UCI grad students, past and present that have helped me, guided me, or just been an awesome friend to hang out. Thank you to my upperclassmen, **Dr. Bamboo Dong** and **Dr. Heidi Contreras** for the guidance and friendship. Thank you to my best grad school friend, **Dr. Sonia Flores,** for her wonderful friendship and support, for watching my cats, and all the crazy cat gifts, stories, pictures, and memes we have exchanged! Thank you to **Parker Johnson, Brian Kim, Galina Schmunk, Dr. Irina Ushach, Katie Sanders, Ankita Shukla, Alex Huszagh, Ricardo Ramirez, Elyse Van Spyke,** and **Ohimai Unoje** for being awesome grad friends just to talk and hang out with! After all, who better to understand a graduate student than another graduate student?!

I would also like to thank and acknowledge **Dr. David Fruman** and **Renee Frigo** for their advice and assistance. Especially during my interim period between labs, their assistance was invaluable.

I would like to thank my undergraduate advisor, **Dr. Joseph Nika**. His advice and words prior to entering into my graduate school journey still continue to echo to this day and guide me into the future. I would also like to thank my undergraduate lab mentor, **Dr. Ernesto Abel-Santos**. The opportunities he gave me helped me secure my spot in graduate school. His advice and mentorship helped me grow as a scientist and prepare for my graduate journey ahead. And his friendship and guidance have continued to help me throughout grad school and in preparation for the future.

I would like to thank all my friends for their friendship and support and all the impact that they have made on my life. There are too many to name, but I would like to recognize those that have made the greatest impact, their support and cheers helping me get through graduate school. **Mario Pucci, Elias Benjelloun, Paul Bishop, Loann Larsen, Vanessa De Galacia, Sabrina Rupani, Jasmine Hicks, Natalie Keller, Jianda Li, David Lao, Maria Reynaga, Dr. Jenny Ouyang,** and many others have true friends. I would also like to acknowledge **Deborah Zekany,** a close friend who was like a grandmother to me, taken too soon, early into my undergraduate journey. Yet her iron-will and belief in me gave me inspiration and strength to succeed. I especially would like thank **Dr. Israel Alvarado**, his actions, suggestions, and advice, have both directly and indirectly made a huge impact on my life. Words cannot express how grateful I am to him for his continuing friendship!

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Finally, words cannot express how grateful I am to my Mom up in heaven, **Amalia Liggins.** Her contributions to my life are too lengthy to list; her impact on my life, too great to measure. It is because of her that I am where I am today. She taught me well, provided invaluable lessons, and she has become the greatest motivation to move forward with my life. She taught me to never give up, to stand strong in the face of adversity, and to never back down. It is because of these lessons and values installed within me that I was able to stand up and start graduate school just three months after her passing. It is because of her that I was able to press on and succeed, despite the

difficulties I’ve faced as a graduate student. It is to her that I owe the greatest thanks of them all!

Curriculum Vitae

Marc Christopher Liggins
8304 Palo Verde Road, Irvine, CA 92617
(702) 355-6437
mcliggins@gmail.com
https://www.linkedin.com/pub/marc-liggins/3b/994/4b3

Education

University of California, Irvine
Ph.D. Candidate: Biological Chemistry, School of Medicine — July 2011 – August 2016
Doctor of Philosophy: Biomedical Sciences

University of Nevada, Las Vegas
Post-baccalaureate: Biology — Sept. 2009 – July 2011

University of Nevada, Las Vegas — August 2004 – May 2008
Bachelor of Science: Biological Sciences
Minor: Chemistry

Research Experience

University of California, Irvine
Department of Biological Chemistry — Jan. 2013 – Present
Graduate Research Assistant
Advisor: Dr. Anand K. Ganesan
Thesis Project: *Investigating the role of PIKfyve and phosphoinositides in melanogenesis*

- Characterized the functional role of the PIKfyve kinase in melanocyte function and viability using multiple *in vivo* and *in vitro* approaches
- Prepared manuscript for submission as primary author
- Award NIH Minority supplement for three years of funding
- Presented a poster at local and national conferences and will give an oral presentation at one national conference
- Mentored one undergraduate student in experimental research

University of Nevada, Las Vegas — Sept. 2008 – July 2011
Department of Chemistry
Research Assistant
Advisor: Dr. Ernesto Abel-Santos
Project: *Characterization and inhibition of Clostridium spore germination*

- Conducted extensive microbial and biochemical analysis of spore germination and germination inhibition in three *Clostridium* species
- Published primary and co-authored articles
- Gave oral presentations at one regional and one national conference
- Submitted patent for small molecular inhibitors of *C. perfringens* germination.

- Mentored and supervised multiple undergraduate students in experimental design and procedure

Publications

Liggins, M., Ramirez, N., Magnuson, N**.,** Abel-Santos, E. “Progesterone analogs influence germination of *C. sordellii* and *C*. *difficile* spores *in vitro.”* Journal of Bacteriology. 193: 2776-2783.

Ramirez, N., **Liggins, M.,** Abel-Santos, E. “Kinetic Evidence for the Presence of Putative Germination Receptors in *Clostridium difficile* Spores.” Journal of Bacteriology. 192: 4215-4222.

Manuscripts in Preparation

Liggins, M., Flesher J., Jahid, S., Sandhu, S., Eby, V., Takasuga, S., Sasaki, T. Boissy, R., Sasaki, T., Ganesan, A. “PIKfyve Regulates Melanogenesis Through the Production of PI(5)P and PI(3,5)P_2.” (*In Revision*)

Liggins, M, Pucci, M., Benjelloun, E. Mendoza, J., Ramirez, N, Abel-Santos, E. “Inhibition of Clostridium perfringens spore germination to prevent necrotic enteritis.” (*In Preperation*)

Patents

Liggins, M., Ramirez, R., Abel-Santos, E. “Inhibiting Germination of Clostridium perfringens Spores to Reduce Necrotic Enteritis.” US Patent Pending 62/113,184. Filed February 6, 2015.

Oral Presentations

Liggins, M., Sandhu, S., Flesher, J., Boissy, R., Ganesan, A. “PIKfyve regulates maturation of the melanosome.” Society of Investigative Dermatology Annual Meeting. Scottsdale, AZ. May 14, 2016.

Liggins, M., Mendoza, J., Ramirez, N., Abel-Santos, E. “Germination and Germination and Germination Inhibition of *Clostridium perfringens.”* Wind River Conference on Prokaryotic Biology. Estes Park, Colorado. June 9, 2011.

Liggins, M., Ramirez, N., Abel-Santos, E. “Germination and Germination Inhibition of *Clostridium difficile* Spores by Cholesterol Derivatives.” American Society of Microbiology Regional Meeting, Las Vegas, Nevada. April 16, 2010.

Poster Presentations

Liggins, M., Sandhu, S., Boissy, R., Ganesan, A. “PIKFYVE regulates the maturation of the melanosome.” 2015 Cedars-Sinai Medical Center Graduate Symposium. Los Angeles, CA. October 2, 2015.

Liggins, M., Sandhu, S., Boissy, R., Ganesan, A. "PIKFYVE regulates the maturation of the melanosome." UCI Department of Biological Chemistry Seminar Series, Irvine, CA. PanAmerican Society for Pigment Cell Research Annual Meeting. Orange, CA. September 28-29, 2015.

Teaching Experience

University of California, Irvine, Department of Biological Sciences
Teaching Assistant — Jan. 2013 – March 2013

- Biochemistry Discussion (BIO SCI 098)
- Microbiology Discussion (BIO SCI M122)
- Held discussion sections for both classes
- Assisted in grading and answering questions for main course

University of Nevada, Las Vegas, Department of Life Sciences
Undergraduate Teaching Assistant — August 2010 – May 2011

- General Biology I Laboratory (BIO 196L)
- Assisted in set-up, preparation, and conducting of lab
- Lectured one lab session

Additional Teaching and Mentoring Experience

Vo's Martial Arts
Taekwondo Instructor — December 2012 – Present

- Independently or co-instructed in martial arts instruction of numerous classes
- Instructed wide-variety of students ranging from young children to adults of beginner to advance skill level

University of California, Irvine, Taekwondo Class
Taekwondo Instructor — December 2012 – Present

- Independently or co-instructed in martial arts instruction of numerous classes
- Instructed wide-variety of collegiate students of all skill levels

Awards and Honors

Albert M. Kligman Travel Fellowship — February 2016

UCI School of Medicine Travel Award — February 2016

NIH Minority Supplement Grant — March 2013 – Feb. 2016
3R01AR063116 - 01A1S1 $166,886

NIH Minority Biomedical Research Support Training Grant — July – Sept. 2011
GM-55246 $5,622

Professional Associations

American Society for Microbiology	2011 – Present
American Chemical Society	2011 – 2012

Skills & Techniques

Microbiology
- Bacterial cultivation
  - Aerobic
  - Anaerobic
  - Microaerophilic
  - Endospore
- Bacterial transformation
- Microbial kinetics
- Antimicrobial compound screening

Molecular Biology
- Mammalian cell culture
  - Immortalized
  - Primary
- Cell transfection
- Lentiviral production & infection
- sh/siRNA gene knockdown
- DNA/RNA purification
- Western Blotting
- PCR / RT-PCR / qRT-PCR
- PCR Primer Design
- Tissue fixation, embedding, sectioning
- Immunohistochemistry
- Immunoprecipitation
- Immunofluorescence
- Site-directed mutagenesis

Chemistry and Biochemistry
- Compound screening
- Protein purification
- Germination kinetics
- Differential scanning fluorometry
- Williamson ether synthesis
- Thin-layer chromatography
- Affinity chromatography
- Affinity chromatography
- Michaelis-Menten kinetics

Animal Work
- Mouse husbandry
- Maintenance of mouse colonies
- Mouse handling
- Mouse genotyping

Computer Skills
- Microsoft Word
- Microsoft Excel
- Microsoft Powerpoint
- Adobe Photoshop
- Adobe Illustrator
- ImageJ
- EndNote
- SigmaPlot
- Chemdraw

References

Anand K. Ganesan, M.D., Ph.D.
Associate Professor, Departments of Dermatology and Biological Chemistry
University of California, Irvine
202 Sprague Hall Irvine, CA 92697
(949) 824-2926
aganesan@uci.edu

Ernesto Abel-Santos, Ph.D.
Associate Professor, Department of Chemistry.
University of Nevada, Las Vegas
4505 Maryland Parkway, Chem 218A, Las Vegas NV 89154
(702) 895-2608
ernesto.abelsantos@unlv.edu

Kyoko Yokomori, Ph.D.
Professor, Department of Biological Chemistry
University of California, Irvine
240D Med Sci I, Irvine, CA 92697
(949) 824-8215
kyokomor@uci.edu

Dissertation Abstract

Elucidating the Role of Phosphoinositides in Melanogenesis:
PIKfyve Regulates Melanogenic Processes through the Synthesis
of PI(3,5)P_2 and PI(5)P

By

Marc Christopher Liggins

Doctor of Philosophy in Biomedical Sciences

University of California, Irvine, 2016

Associate Professor Dr. Anand K. Ganesan, Chair

Epidermal melanocytes synthesize melanin within specialized lysosome-related organelles known as melanosomes. These melanosomes are transferred to neighboring keratinocytes where they surround nuclei to protect against ultraviolet-induced UV damage. The synthesis of melanin, a process known as melanogenesis, is subject to complex and intricate regulation at multiple levels. While extensive studies have elucidated many of the transcriptional and regulatory pathways, melanosomal trafficking comparatively far less understood. To further complicate matters, several lysosomal and autophagic components are utilized during these processes, but it is unclear what delineates the specificity of their regulation. To help elucidate these mechanisms, our laboratory conducted a genome-wide siRNA screen, which identified several novel regulators of melanogenesis. Several autophagy components, including the phosphoinositol binding protein WIPI1, were among those implicated in the screen. Through further investigation, it was determined that WIPI1 regulates both melanosomal

transcription and trafficking, but the mechanism by which it coordinated both processes was not thoroughly elucidated. WIPI1 binds to three different phosphoinositides, PI(3)P, $PI(3,5)P_2$, and PI(5)P, the latter two of which are synthesized by the PIKfyve kinase complex. $PI(3,5)P_2$ and PI(5)P have been priorly implicated in regulating membrane trafficking and signaling pathways. Furthermore, mutation in select $PI(3,5)P_2$ effectors or in the PIKfyve complex itself has been shown to affect pigment accumulation, though the mechanisms have not been priorly elucidated. In our studies, we have demonstrated that loss of PIKfyve *in vivo* results in the progressive loss of pigmentation. Disruption of the PIKfyve leads to defects in vesicle trafficking and severe vacuolization. Our *in vitro* and *in vivo* studies have identified similar disruption in melanocytes resulting in the inhibition of melanosome maturation and melanosomal acidification. These mechanisms would suggest an important role of $PI(3,5)P_2$ in melanogenesis. In contrast, partial depletion of PIKfyve depleted WIPI1 and resulted in downregulation of these signaling pathways and expression of MITF-M and TYR, while the addition of PI(5)P had the opposite effect. Taken together these studies demonstrate novel regulatory roles of the PIKfyve complex, elucidating distinct roles of $PI(3,5)P_2$ and PI(5)P in melanogenesis. These studies expand upon the fields of knowledge in both melanocyte and phosphoinositide biology and provide significant insight into the regulatory mechanisms of melanocytes.

Melanogenesis and Its Regulation

The Roles of Phosphoinositides and Phosphoinositide Binding Proteins in Melanosome Biogenesis

Chapter 1

Marc C. Liggins and Anand K. Ganesan

1.1 Abstract

Epidermal melanocytes produce melanin through the process of melanogenesis. Melanin itself is synthesized within lysosome-related organelles known as melanosomes. While extensive studies have elucidated the transcriptional and enzymatic regulatory pathways in melanin synthesis, how the trafficking of melanosomes is regulated is not completely understood. Melanosome biogenesis requires proteins involved in lysosome and autophagosome biogenesis, but it is unclear how these components work together to make the melanosome. Our laboratory conducted a genome-wide siRNA screen, which identified multiple novel regulators of melanogenesis including several autophagy components. WIPI1, a phosphoinositide (PI) binding protein, was among those identified. Further studies determined that WIPI1 coordinated both the transcription of genes that synthesize melanin and vesicle transport; however how WIPI1 does this was not elucidated. WIPI1 is known to bind to two phosphoinositides, PI(3,5)P_2 and PI(5)P, which are synthesized by the PIKfyve kinase complex. Deletion of components of the PIKfyve complex leads to pigment loss *in vivo*. Pigment accumulation was similarly affected by mutation of two PI(3,5)P_2 effectors. Taken together, these studies suggest that WIPI1 may regulate melanogenesis through a mechanism involving phosphoinositides.

1.2 Skin Biology

The skin is the largest organ in the human body; it functions as a physical barrier that serves to maintain homeostasis and protect against environmental insults such as radiation, mechanical, chemical and microbial factors (1, 2). The skin is composed of three layers: the epidermis, dermis, and hypodermis (1, 3). The outermost layer, the epidermis, is devoid of blood vessels and nerves, consisting primarily of keratinocytes and melanocytes (4). The epidermis itself is a stratified epithelium composed of four sublayers, or strata: the stratum corneum, the stratum granulosum, the stratum spinosum, and the stratum basale (1, 4). The stratum corneum is the outermost layer and functions as the skins primary defense (1, 4, 5). It is composed of terminally differentiated keratinocytes called corneocytes that contain abundant keratin and form a barrier to protect from environmental insults and desiccation (1, 4, 5). Directly below is the stratum granulosum which is composed of flattened, nondividing keratinocytes that produce granules of keratinohyalin protein (4). The next layer, the stratum spinosum, contains keratinocytes and antigen-presenting Langerhans' immune cells (4). The basal layer, the stratum basale, is composed of a single layer of cells that are attached to a non-cellular basement membrane that separates the epidermis from the dermis (4). The stratum basale is composed of differentiating and stem cell-like keratinocytes, sensory Merkel cells, and pigment-producing melanocytes (1, 4).

The dermis is situated directly below epidermis (4). It houses the sensory, neural and vascular networks of the skin, immune cells, excretory and secretory glands, as well as hair follicles and nails (4). Even though the dermis is the thickest layer of the

skin, it contains fewer cells (1, 4). The bulk of dermis volume is composed of a fibrous and amorphous extracellular matrix (ECM) which provides the skin with tensile strength and elasticity (1, 4). Fibroblasts are the primary cell type in the dermis and are responsible for the maintenance of the ECM (4). The hypodermis is a fatty tissue that comprises the deepest layer of the skin (3) and is primarily composed of adipocytes which function in thermoregulation, insulation, fat storage, and mechanical protection (3).

1.3 Melanocyte Development and Biology

Melanocytes are neural crest-derived, melanin-producing cells most prominently found the basal layer (stratum basale) of epidermis and the retina, however subpopulations can be found in additional tissues as well (6-10). During mammalian development, neural crest cells develop into melanoblast/glial progenitors and migrate throughout the mesenchyme of the developing embryo until reaching their target sites (4, 8). During development, expression of microphthalmia-associated transcription factor (MITF) and SOX10 commits the progenitors to develop into melanoblasts (4, 8). After migration, expression of tyrosinase-related protein 2 (TYRP2) and kit ligand (KL), in combination with MITF, differentiates melanoblasts into melanocyte stem cells and melanocytes (8). Factors that regulate melanocyte stem cell differentiation and melanocyte renewal are not fully understood. However, there is a gradual increase in melanocyte senescence and loss of the melanocyte population with age, more prominently in the hair bulb than the epidermis (11).

Epidermal melanocytes are specialized melanin-producing cells that are located within the basal layer of the epidermis and within the proximal bulb of the hair follicle (12). Melanocytes comprise 5-10% of the epidermal cell population (11, 13) with greater density observed in the hair follicle (12). Within the epidermis, melanocytes possess an extensive dendritic network in which one cell interacts with approximately 36 keratinocytes as a means of melanin delivery (14, 15). Extensive crosstalk between keratinocytes and melanocytes regulate dendrite formation, proliferation, and melanin production (4, 12). Both endogenous and environmental factors stimulate the release of endocrine, paracrine, autocrine, and intracrine factors to regulate melanogenic processes (16). In response to UV radiation (UVR), keratinocytes upregulate paracrine signaling to melanocytes ultimately resulting in the upregulation of melanin synthesis (4, 17). Melanin itself is produced within lysosome-related organelles called melanosomes in a process known as melanogenesis (4, 17, 18). Melanosomes develop through four distinct stages (4, 17, 18). Mature (stage IV) melanosomes are shuttled through melanocytic dendrites to neighboring keratinocytes to protect cell nuclei from UVR (4). The process of melanogenesis is graphically summarized in Figure 1.1.

1.4 Melanogenic Regulation and Development

Solar ultraviolet (UV) radiation is divided into three wavelengths, UV-A (320 – 400 nm), UV-B (280-320 nm), and UV-C (100 – 280 nm) (17). UV-C is not able to penetrate the atmospheric ozone layer, however UV-A and UV-B are both terrestrially present, accounting for approximately 94% and 6% respectively (17, 19). UV radiation

that penetrates the skin is absorbed by melanin, however the cellular response differs between UV-A and UV-B (17, 19). UV-A has no effect on melanocyte proliferation or total melanin content, instead resulting in oxidative conversion and darkening of melanin (tanning) (4, 17, 19). While UV-A does not induce DNA damage directly, it can result in the production of DNA-damaging free radicals (4, 19). In contrast, UVB is absorbed by DNA leading to the production of thymine dimers and other DNA photoproducts (4, 17). The presence of thymine dimers results in activation of p53 resulting in the transcription of proopiomelanocortin (POMC) in keratinocytes, which in turn triggers the production and release of α-melanocyte stimulating hormone (α-MSH) and other factors (17). UV irradiation also results in the release of other paracrine factors that can independently or synergistically modulate melanocyte function (4, 17).

POMC can be processed into several melanocortin peptides which result in the activation of melanocortin receptors (MCRs) present on the melanocyte cell surface (16, 17, 20). α-MSH and other melanocortins activate the melanocortin 1 receptor (MC1R) resulting in a kinase cascade leading to transcription of the melanocyte-specific isoform of MITF, MITF-M (16, 17, 20, 21). Additional keratinocyte-derived paracrine factors result in the phosphorylative activation of MITF-M (16, 17, 22). Upon activation, MITF-M binds to M-box and E-box consensus sequences resulting in the transcription of factors required for melanosome biogenesis and transport and melanin synthesis (16, 17, 22). MITF-M recognizes consensus sequences associated with several melanogenic factors including MC1R, MART-1, PMEL, tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1), tyrosinase-related protein 2 (TYRP2), RAB27A and other melanogenic factors (23, 24).

While several isoforms of MITF are known, only five are expressed in the skin (22, 25). The MITF-M isoform is specific to melanocytes (16) and is the only isoform that highly expressed within the cell type (22, 25). Loss of the MITF-M isoform only affects dermal pigmentation, resulting in a “black-eyed, white” mouse phenotype *in vivo* (22) and unpublished results). Interestingly, studies have recently identified that both MITF-A and MITF-M are required for melanocytic dendrite formation (25).

1.5 Lysosome-Related Organelles

Melanosomes belong to class of organelles collectively referred to as lysosome-related organelles (LROs) (26-29). LROs are specialized organelles found in select cell types, primarily distinguished by their function and unique cargo (26-29). LROs are found in a number of cell types including several hematopoietic cell types, osteoclasts, sperm cells, endothelial cells, melanocytes, and others (26-29). Lysosomes and LROs share compositional and physiological features, utilize common machinery, share several developmental parallels and often possess low luminal pH (26-29). As such, diseases that affect lysosomal and LRO machinery typically affect multiple tissues, two prime examples include Hermansky-Pudlak syndrome (HPS) and Chediak-Higashi syndrome (CHS) (26-29). HPS most prominently results in oculocutaneous albinism, but patients also exhibit defective blood clotting as other complications can also arise as a result of defective lysosomal function (28, 30). Patients with CHS can exhibit a broader range of symptoms including hypopigmentation, platelet dysfunction, neurological dysfunction, and immunodeficiency (28).

LROs biosynthesis is a multistep process through which the organelles develop by acquiring specialized cargo (26, 27, 29). Vesicles that bud off from the *trans*-Golgi network (TGN) develop into early endosomes or multivesicular bodies (MVB) or through the acquisition of specific factors can develop directly into LROs (26-29). The presence of specific cargo components and other factors determine the developmental fate of early and late endosomes/MVBs – whether they develop into lysosomes or into LROs (26-29).

1.6 Melanosome Development and Melanogenesis

1.6.1 Early Melanosome Development – Stage I and II and their Factors

Melanosomes are LROs that mature through four distinct stages (I-IV) of development (4, 17, 21, 24) (Figure 1.1). Differences in morphology, pigmentation, and the presence or absence of different factors define the maturity, or stage, of the melanosome and distinguish it from other organelles (21). The complex process of melanosome biogenesis is initiated when early endosomes bud off into spherical vacuoles known as stage I melanosomes, marked by the presence of melanoma antigen recognized by T cells 1 (MART-1) and the premelanosome protein (PMEL) (21, 31, 32).

MART-1 and PMEL expression is regulated by MITF-M and serve as stage markers for Stage I and II melanosomes, respectively (23, 32-34). While indispensable, the function of MART-1 is not well defined (33, 34). MART-1 is a small membrane-

bound protein that is localized to the Golgi, ER, and stage I melanosomes (18, 33, 34). Even though MART-1 has no known enzymatic activity, it is required for expression, stability, processing, and trafficking of PMEL along with ocular albinism 1 (OA1) (31, 33). Within post-Golgi compartments, OA1 and MART-1 stabilize PMEL (31, 33, 34). OA1 also regulates melanosome size and composition, however its interactions with L-DOPA could suggest that it also functions in melanin synthesis as well (31).

PMEL is a large pre-protein that is glycosylated in the ER and Golgi (32). From the trans-Golgi network, PMEL associates with the adaptor protein 2 (AP-2) complex via di-leucine-based consensus and is transported to early endosomes along with MART-1 (32, 34). Within early endosome vacuolar domains, PMEL is sorted into intraluminal membrane vesicles (ILVs) (32). Acidification induces the proteolytic cleavage and maturation of PMEL into fragments which can then be polymerized (32). The transition between early endosomes and stage I melanosomes is poorly understood as it is debatable whether the two are distinguished from each other.

Stage I melanosomes are multivesicular early endosomes, or premelanosomes, that possess a poorly organized internal structure, marked by the presence of MART-1 and PMEL (17, 32-34). PMEL begins to generate fibrils from ILVs through association with the endosomal protein CD63 (26, 32). Membrane fusion induces the loss of ILVs and allows PMEL fibrils to be organized into amyloid sheets marking the transition to stage II (32). These sheets organize into a fibrillary matrix that is characteristic of stage II melanosomes and ultimately serve as a scaffold for melanin polymerization and deposition (17). Prior to the initiation of melanin synthesis, deacidification of the

melanosome is required for proper enzymatic function (16), however it is unknown when this occurs.

While tyrosinase (TYR), and to the lesser extent TYRP1 and TYRP2, are essential for melanin production, these factors are initially absent from melanosomes (16). Once glycosylated, these proteins are packaged into adaptor protein-3 (AP-3) or -1 (AP-1) clatharin-coated transport vesicles, which are transported to and fuse with stage II melanosomes (35). Tyrosinase allows for the initiation of melanin synthesis (16, 17). The deposition of melanin onto PMEL fibrils marks the transition to stage III melanosomes (16, 17).

1.6.2 Mechanisms Underlying Melanosomal Trafficking

The initiation of melanin synthesis requires the delivery of key melanogenic transporters and enzymes such as TYR, TYRP1, TYRP2, and OCA2 (oculocutaneous albinism II) (18). Within the ER and Golgi, TYR, TYRP1, and TYRP2 are folded and heavily glycosylated before being transported to the TGN (18). Within the *trans*-Golgi network, TYR, TYRP1, and OCA2 associate are recognized by adaptor protein (AP) complexes via acidic dileucine consensus sequences (18, 36). In non-melanocytic cells these sequences confer trafficking to late endosomes and lysosomes (36, 37), thus it is not fully understood what confers melanocytic specificity in the presence of both melanosomes and lysosomes. TYR and OCA2 are packaged within AP-1 or AP-3 clatharin-coated vesicles, however TYRP1 only interacts with AP-1 vesicles (18, 35, 36, 38). Association via dileucine consensus sequences packages TYR, TYRP1, and OCA2

in clathrin-coated AP-1/AP-3 vesicles for delivery to stage II melanosomes (18, 35). Interestingly, a dileucine consensus sequence is absent from TYRP2 (38), which instead utilizes a tyrosine based motif consensus sequence that normally directs proteins to the lysosome (36, 39). It is not fully understood what signals mediate the delivery of TYRP2 to the melanosome.

To facilitate vesicular transport, AP-complexes work in conjunction with biogenesis of lysosome-related organelles complex (BLOC) -1, -2, and -3 complexes (18, 40). The BLOC complexes are further assisted by RAB32 and RAB38, functioning as guanine-exchange factors to activate these factors and facilitate localization (40-43). Other RAB proteins are also believed to be involved in melanosome biogenesis, but their roles are less defined (18, 44, 45). BLOC -1, -2, and -3, in conjunction with RAB32 and RAB38 facilitate trafficking of TYR and TYRP1, while TYRP2 trafficking appears to be solely dependent on RAB32 (18, 42, 43). Studies have shown that AP clatharin-coated vesicles, in conjunction with RAB32/38 and BLOC complexes, function to transport cargo to melanosomes (18, 40, 42, 43). However the mechanisms underlying the specificity of vesicular targeting and fusion are poorly understood. It has been suggested that phosphotidylinositides may be involved in melanosome biogenesis and could thus elucidate this pathway (18, 42).

1.6.3 Tyrosinase is required for the initiation of melanogenesis

To facilitate melanin production, TYR, TYRP1, TYRP2, OCA2, and ATP7A are transported to stage II melanosomes (18). The initiation of melanin synthesis marks the

transition to stage II melanosomes (4). OCA2 and ATP7A both function as transport proteins for tyrosine and copper respectively (46, 47) while TYR and related proteins 1/2 are key enzymes involved in melanin synthesis (48).

BLOC-1 also facilitates transport of ATP7A to stage II melanosomes (18, 47). Copper is a necessary co-factor for tyrosinase function, and while TYR is believed to acquire copper in the TGN, supplementation by ATP7A is necessary for pigment production (18, 47). The function of OCA2 is less defined, but is necessary for melanin synthesis (18, 48, 49). While studies initially suggest that OCA2 functions as a tyrosine transporter (46, 49), recent studies would suggest that it functions as a Cl^- transporter and contributes tyrosinase stability by increasing melanosomal pH (18, 48, 50). Tyrosinase initiates the melanogenic process by converting tyrosine and L-DOPA to DOPAquinone, thus serving as the rate limiting step in melanogenesis (4, 16, 51).

Tyrosinase (TYR) and tyrosine hydroxylase (TH) are two unrelated mammalian proteins capable of hydroxylation of L-tyrosine to L-DOPA (52). TH is only expressed in neural tissues, specifically within the adrenal medulla and dopaminergic neurons (53) while TYR is melanocyte specific (52). Activation of tyrosinase requires the reduction of two copper ions (Cu^{2+} to Cu^{1+}) and is only optimally functional at neutral to basic pH (16). At lower pH, tyrosinase activity is reduced and is totally lost at acidic pH (16). Tyrosinase mediates the rate-limiting step in the production of both eumelanin and pheomelanin, utilizing L-tyrosine or L-DOPA as substrates to synthesize DOPAquinone, thus initiating (eu-/pheo-)melanogenesis (16, 51, 54). Normal melanocytes produce mixtures of pheomelanin and eumelanin partly determined by the availability of cysteine (51). The addition of cysteine to DOPAquinone initiates pheomelanogenesis permitting

sufficient cysteine concentrations are available (51). While TYR is required for melanin synthesis, recent studies would suggest that low levels of TYR are sufficient for pigment production (54).

1.6.4 Types of Melanin

Three types of melanin are produced in mammals as the end result of L-tyrosine transformation and polymerization – eumelanin, pheomelanin, and neuromelanin (4, 16, 44). While both eumelanin and pheomelanin are produced within melanocytes, neuromelanin is exclusively produced by dopaminergic neurons resulting in pigmentation of the substantia nigra (4, 16, 55, 56). Neuromelanin is synthesized via tyrosine hydroxylase and is believed to function in the chelation of potentially damaging metals to prevent neurodegeneration (4, 9, 16, 55, 56). Nevertheless, the synthesis, function, and role of neuromelanin is not well defined.

Eumelanin (brown/black pigments) and pheomelanin (red/yellow pigments) are exclusively produced by melanocytes as part of the melanogenic process, however only eumelanin possesses UV protective properties (4, 16, 44, 57, 58). While pheomelanin is of evolutionary significance, no biological role has been identified in humans (59). Furthermore, oxidation of pheomelanin by UV radiation results in increased oxidative damage and has been correlated with increased cancer risk (16, 59, 60). While most studies have been focused the production of eumelanin, eumelanogenesis, regulatory pathways involving pheomelanin production are not well understood. It is also unclear what factors determine whether melanosomes develop into eumelanosomes or

pheomelanosomes, though studies have identified proteins and mutations exclusive to pheomelanin synthesis (57, 60-64). The morphology of eumelanosomes and pheomelanosomes differs, where eumelanosomes are fibrillar during maturation while pheomelanosomes maintain a rounded appearance (4).

1.6.5 Eumelanogenesis and Melanosome Transport

The initiation of melanin synthesis marks the transition to stage II melanosomes (4). Tyrosinase initiates the eumelanogenic process by converting tyrosine and L-DOPA to DOPAquinone, thus serving as the rate limiting step in melanogenesis (4, 16, 51). In a two-step reaction, DOPAquinone is converted to DOPAchrome, which then spontaneously decomposes to produce to 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) in a 70:1 ratio (4, 16, 51). However, TYRP2 shifts this ratio by acting as a dopachome tautomerase (DCT), catalyzing the conversion of DOPAchrome to DHICA (4, 16, 51). TYRP1 and/or tyrosinase then catalyze the oxidation DHICA (4, 16, 51). DHI and DHICA, both in reduced and oxidized states, are synthesized into eumelanin polymers, which are then concentrated on PMEL fibrils (4, 16, 51). The completion of melanin synthesis, characterized by complete darkening of the melanosome and minimal TYR activity, signify the completion of melanogenesis and are thus termed stage IV melanosomes (4).

TYRP1 and TYRP2 exhibit significant functional and structural homology to tyrosinase but are only functional in the eumelanogenic pathway (16, 52). Specifically, TYRP1 and TYRP2 share ~40% amino acid homology with TYR, but utilize different

metal ions, iron and zinc respectively, as catalysts (16). TYRP1 and TYRP2 function to catalyze the conversion of downstream DOPA reaction products during the eumelanogenic process (4, 16, 51). While not necessary for melanin synthesis per se, their presence further catalyzes eumelanogenesis increasing both the quality and quantity of eumelanin produced (16, 51). Furthermore, TYRP2 also plays a key role in melanocyte development and survival (16).

As melanosomes mature, microtubules, actin filaments, and myosin, in conjunction with RAB27A, move melanosomes towards the extremities of the cell and into the dendrites (17, 21, 65, 66). Upon completion of melanin synthesis, mature (stage IV) melanosomes are transferred to keratinocytes through a process that is not well understood (17, 21, 65). Melanosome transfer may involve exocytosis, cytophagocytosis, or fusion of the keratinocyte and melanocyte membranes (17). Coordination between melanocytes and keratinocytes mediate these processes are upregulated in a UV-dependent manner (17, 21, 65) (Figure 1.1).

1.7 Identification of Novel Melanogenic Regulators utilizing genome-wide siRNA screen

Extensive studies have greatly increased our knowledge of melanogenesis, yet the process is far from fully understood. Most prominently, the mechanisms underlying melanosome development, delivery and transport have been poorly elucidated. In particular, there is significant overlap in the machinery utilized in the development of

LROs and it is less clear what mediates the delivery of proteins specifically to melanosomes.

To gain further insight into these mechanisms, a genome-wide siRNA screen against 21,127 unique human genes was conducted to identify novel regulators of melanogenesis (67). The screen was conducted in MNT-1 melanoma cells utilizing a Dharmacon library consisting of 84,508 siRNAs corresponding to four unique siRNA duplexes per gene (67). This study utilized a 5 day post-transfection incubation period was established using tyrosinase depletion as a positive control for quantitative determination of melanin impairment (67). Melanin was spectrophotometrically quantified using an ATP-dependent luminescence cell viability assay (CellTiter-Glo) to normalize these results on a per cell basis by measuring absorbance at 405 nm for melanin quantitation (67). MNT-1 melanoma cells were utilized in the screen as the cell line is most similar to normal human epidermal melanocytes and produce substantial melanin quantities, having been previously established in prior studies (35, 67-69).

Using a cutoff of 2 standard deviations below the mean, the screen identified 98 genes as regulators of melanogenesis (67). Individually synthesized siRNA pools were directed against 35 randomly selected genes from the initial 98 identified in the primary screen were selected for further screening to validate their impact on cellular pigmentation and four false positives were eliminated (67). Tyrosinase was utilized as a positive control and keratin 7 as a negative control that did not affect pigmentation (67).

The remaining candidate genes were examined for their impact on MITF and TYR accumulation (67). These genes were screened in MNT-1 cells as well as darkly- and moderately-pigmented human epidermal melanocytes (67). Approximately half

were found to affect tyrosinase accumulation in MNT-1 and darkly-pigmented melanocytes, and moderately-pigmented melanocytes (67). The autophagy protein, WIPI1 (WD-40 repeat-containing protein that interacts with phosphotidylinositides), was found to significantly impact tyrosinase accumulation in all three cell types (67). Several other autophagy components were also implicated in melanogenesis (67).

WIPI1 is an autophagy protein that is believed to function in autophagosome formation through interactions with phosphatidylinositol-3-phosphate [PI(3)P] (70, 71). WIPI1 can also bind to phosphatidylinositol-3,5-bisphosphate [$PI(3,5)P_2$] and phosphatidylinositol-5-phosphate [PI(5)P] (70, 71) however the function of these interactions has not been elucidated. WIPI1 is one of four mammalian PROPPINs (β-propellers that bind polyphosphoinositides) along with WIPI2, WIPI3, and WIPI4 (67, 68). WIPI1 and WIPI2 share the greatest homology with 80% protein identity (68, 69). Despite WIPI1 homology, WIPI2 only moderately affected pigmentation while WIPI3 and WIPI4 did not (67).

1.8 The Role of Autophagic Regulators in Melanogenesis

Autophagic machinery can work in conjunction with LROs (67, 72). Recent observations highlighted that autophagosomes accumulate in cells defective for melanosome maturation (67, 72). Autophagy proteins LC3 and ATG5 were found to colocalize with melanosomes (67) (Figure 1.2). Not surprisingly, in addition to WIPI1, additional autophagy proteins identified in the screen were also found to significantly inhibit pigment accumulation *in vitro* (67, 73). Prominently, beclin1 inhibited pigment

accumulation *in vitro* and *in vivo,* specifically haploinsufficent mice were hypopigmented compared to wild-type controls (67, 73).

Other studies have identified several autophagy proteins which impact melanogenesis via a pathway that is distinct from canonical autophagy further expanding upon the autophagy-melanogenesis relationship (24, 73-76). Induction or suppression of mTORC1 were found to reciprocally modulate autophagy and melanogenesis, suggesting an antagonistic relationship between the two processes (24, 76). Depletion of the autophagic regulator ULK1 resulted in increased melanogenic transcription and pigment production, further highlighting the antagonistic relationship between autophagy and melanogenesis (74). In contrast, loss of several autophagy components has been found to negatively affect melanogenesis (24, 67, 73, 75). Intriguingly, starvation induced autophagy has not been found to impact to melanogenesis (24, 74). In some cases autophagy may function antagonistically to melanogenesis; however these studies would suggest that several autophagic components are necessary for melanogenic processes (24, 73). Taken together, it is clear that autophagy and melanogenesis are closely related processes; however dissecting the nature of the relationship is a significant conundrum. The complex relationship between autophagy and melanogenesis is further emphasized in follow-up studies that sought to define the functional role of WIPI1 in melanogenesis.

1.9 WIPI1 Coordinates Melanogenic Transcription and Trafficking

Initial studies identified the autophagy protein, WIPI1, as a novel regulator of melanogenesis (67). siRNA knockdown resulted in significant pigment loss, and decreased tyrosinase and MITF mRNA (67). Further investigation reaffirmed these findings and confirmed decreased mRNA and protein accumulation of genes under MITF-M regulation as a result of RNAi knockdown of WIPI1 (24).

To validate the transcriptional regulatory role of WIPI1, MNT-1 cells were transfected with WIPI1 overexpression or siRNA constructs (24). 24 hours post-transfection, cells were co-transfected with a *Renilla* luciferase reporter and firefly luciferase promoters driven by tyrosinase or MITF-M promoters and after 48 hours were evaluated for luciferase activity (24). Core promoter activity of both MITF-M and tyrosinase was decreased as a result of WIPI siRNA knockdown (24). Complimentary to these findings, WIPI1 overexpression increased core promoter activity of both (24).

Given that WIPI1 depletion results in downregulation of many MITF-M target genes essential for melanosome development and melanogenesis (24), this raised the question to whether or not melanosome maturation is affected a result. To answer this question, WIPI1 shRNA-expressing primary melanocytes were evaluated by transmission electron microscopy (24). WIPI1 depleted melanocytes exhibited significantly lower numbers of advanced stage (III and IV) melanosomes, instead accumulating stage I and II melanosomes and abnormal atypical vacuole-like vesicles (24). While other studies have shown that tyrosinase depletion resulted in decreased numbers of advanced stage melanosomes (54), the accumulation of immature melanosomes as a result of WIPI1 knockdown would suggest that tyrosinase is not

being trafficked to melanosomes (24). Taken together these defects would suggest that WIPI1 depletion results in abnormal melanosomal trafficking (24).

Studies have shown that suppression of mTORC1 upregulates melanogenesis (24, 77, 78) and autophagy (79) providing further evidence for the linkage between melanogenesis and autophagy. Similarly, WIPI1 depletion upregulated mTORC1 signaling while resulting in the downregulation of the mTORC2 pathway (24). mTORC2 promotes the transcription of MITF-M, thus as a result of downregulation, melanogenic transcription was reduced (24). Treatment with rapamycin rescued the phenotypes that resulted from WIPI1 knockdown (24). Given these findings, it was concluded that WIPI1 regulates melanogenesis via suppression of mTORC1 and activations of mTORC2 (24) (Figure 1.2).

It was thoroughly established that WIPI1 impacts melanogenesis *in vitro* (24, 67), likely through modulating mTOR activity, however the exact regulatory mechanism remains unclear. Yet intriguingly, mTORC1 has been shown to suppress autophagy through the suppression of ULK1 (79), which itself has been shown to repress negatively impact melanogenesis (74). As both mTORC1 and WIPI1 interact with PI(3)P and PI(3,5)P_2 (70, 71, 80), it remains plausible that phosphoinositides are the missing link in the regulation of these two pathways.

1.10 PROPPIN Family

WIPI1, along with WIPI2, WIPI3, and WIPI4 are the four mammalian members of the PROPPIN family (81, 82). PROPPINs adopt a seven-bladed propeller fold that

mediates protein interactions (81, 82). They contain phosphoinositide (PI) binding sites which mediate interactions with PI(3)P, PI(3,5)P_2, and PI(5)P (70, 71) through several evolutionary conserved residues found in blades 5 and 6 (71, 81-83). Interestingly, these residues have been found to mediate binding to both PI(3)P and to a lesser degree PI(3,5)P_2 as mutation attenuated all PI binding (71, 81-83). Mutational analysis has not been conducted for PI(5)P binding.

Saccharomyces cervisiae (yeast) possess three members of the PROPPIN family: ATG18, ATG21, and HSV2 (81-84). ATG18 possess two mammalian homologs, WIPI1 and WIPI2, which share 80% protein identity with each other (82-84). ATG21 does not possess a mammalian homolog, but possesses functional homology to WIPI2 (82-85). HSV2 also possess two mammalian homologs, WIPI3 and WIPI4, however there is greater divergence from HSV2 and there is reduced functional homology (82-84).

In yeast, ATG18 is able to form several diverse autophagy complexes which are mediated or regulate PI binding (86-88). ATG18 binds to the yeast-specific type V myosin adaptor, VAC17, to mediate cytoskeletal rearrangement and vacuole transport (88-90). A mammalian VAC17 homolog has not been identified, nor has a WIPI1-myosin V relationship been identified. Interestingly however, the melanosomal protein, melanophillin (which is part of the RAB27a complex) functions similarly as a myosin V adaptor (91). Furthermore, keratinocyte derived endothelin-1, regulates the expression of both WIPI1 and RAB27A in melanocytes (92). However, whether WIPI1 plays a similar roll in myosin V melanosome transport remains to be elucidated.

ATG18 also forms a complex with ATG2 which is recruited during phagophore development through interaction PI(3)P (87, 93). While it has been suggested that WIPI1/2 mediate this function in mammals, only WIPI4 was found to bind to ATG2 and is believed to regulate this function to date(83, 93). Of interest, ATG18 is also able to modulate activity of the phosphoinositol kinase, FAB1, through complex formation. Binding of ATG18 to the FAB1 complex results in the downregulation of kinase activity, thus resulting in decreased levels of $PI(3,5)P_2$ (94). Given that ATG18 binds to both PI(3)P and $PI(3,5)P_2$, this could thus be indicative of an autoregulatory relationship, however the role of ATG18-$PI(3,5)P_2$ relationship has not been elucidated. While the components of the FAB1 (PIKfyve in mammals) core complex are evolutionary conserved, a mammalian relationship between WIPI1/2 and PIKfyve has not been established.

WIPI1 and WIPI2 are both expressed ubiquitously, however expression is highly variable by cell type (83, 95, 96). Interestingly, WIPI1 is highly expressed in melanocytes and melanoma cells (70, 96). In response to UVR, keratinocytes upregulate synthesis and secretion enthothelin-1, which in turn stimulates and activates MCR1 in melanocytes, as well as upregulating WIPI1 expression thus resulting upregulation of melanogenic gene expression and melanogenesis (16, 17, 92). WIPI2 possesses five different isoforms which play different roles in autophagy regulation (85). During autophagy, WIPI1 and WIPI2 have both been shown to localize with autophagosomes, however studies have yielded mixed results to whether WIPI1 and WIPI2 have agnostic or antagonistic rolls in autophagy (71, 83-85, 97, 98). Recent studies have downplayed the role of WIPI1 in autophagy suggesting that WIPI2b

(isoform b) is required for ATG16L1 recruitment and LC3 conjugation (84, 85, 97, 99). Furthermore, pulldown analysis would suggest that WIPI1 only weakly binds ATG16L1 but binds COPI vesicle transport machinery with high affinity, while WIPI2b co-immunoprecipitated ATG16L1 but not COPI factors (85). Phosphoinositol binding by WIPI2 is required for induction of autophagy mediated induction of autophagy (83, 85, 97, 99). Interestingly, while both PI(3)P and PI(5)P can induce autophagy via WIPI2b as a result of amino acid starvation, only PI(5)P induces autophagy under glucose starvation (83, 85, 97, 99). While WIPI2b and WIPI2d may have redundant functions (84, 85, 97), the functions of isoforms a, c, and e have not been elucidated. Interestingly expression of WIPI1 is upregulated in several cancer lines (83, 100), however the exact function of WIPI1 remains to be determined as no significant phenotype has been demonstrated thus far due to loss of WIPI1 *in vivo*. Furthermore, it is unclear if and how $PI(3,5)P_2$ acts a WIPI1/2 effector.

HSV2 and its mammalian homologs, WIPI3 and WIPI4, are poorly understood in terms of function (71, 81-83). WIPI3 is ubiquitously expressed however its function has yet to be defined (83). WIPI4 is an autophagy protein, functioning in autophagosome formation and possibly downstream of WIPI2 in LC3 lipidation, however these functions have not been well characterized (83). *In vivo*, loss of WIPI4 results in prominent neuropathological symptoms including axonal swelling, impaired memory and learning, behavioral abnormalities, and motor impairment (101). Cellularly, neural cells exhibited impaired autophagic flux and vacuolization (101). Given that the autophagy and neurological defects are milder compared to the loss of other autophagy proteins, this could be indicative of functional redundancy with other WIPI proteins (101). WIPI4 has

reduced affinity for PIs as a result of a hydrophobic residue in the binding region (81) however its functional relationship with the different PIs has yet to elucidated.

Overall, with the exception of WIPI2b, the mammalian functions of the WIPI family have not been well elucidated. Studies have suggested that some WIPI functions may be functionally redundant between members, however many questions remain unanswered. Furthermore, the polygamous binding to different phosphoinositides may regulate different pathways as demonstrated by WIPI2 (71, 81-83, 97, 99). How WIPI1 functions in autophagy, melanogenic transcription, and melanogenic regulation could be mediated through the recruitment of different phosphoinositides.

1.11 Overview of Phosphoinositides

Phosphoinositides are an important class of phospholipids that mediate several cellular functions including membrane trafficking, signal transduction and cellular regulation, and nuclear processes (102-108). Within the ER, phosphatidylinositol synthase mediates the condensation of cytidine diphosphodiacylglycerol and *myo*-inositol generating phosphatidylinositol (PtdIns) (102, 109). Phosphatidylinositol (PIP) is composed of hydrophilic inositol head group linked to glycerol and coupled to two fatty acyl chains and serves as the primary building block of all phosphoinositides (PIs) (102, 109). The inositol ring can be reversibly phosphorylated at the 3, 4, and 5 positions generating mono-, bis-, and tri-phosphoinositides (102-108).

As summarized in Figure 1.3, multiple kinases and phosphatases result in the interconversion of different phosphoinositides, or the inositol head by phospholipase C

can be released to act as a soluble signaling molecule (102-108). The subcellular localization of the individual PIs is highly variable in their distribution (102-108). PIs have been found to be localized to plasma membrane, various organelles, vesicles, and within the nucleus (102-108). Similarly, individual PIs mediate several functions which vary highly depending upon localization and associated effectors (102-108).

Phosphoinositides play several important roles in membrane dynamics including regulation of membrane traffic, cytoskeletal rearrangement, endocytosis and exocytosis events, and vesicular fusion (104, 105, 110). Segregation of different phosphoinositides ensures proper trafficking of vesicles to different organelles and membranes (104, 105, 110). Several clatharin adaptor protein complexes, including AP-1 and AP-2, associate with phosphoinositides to deliver cargo (104, 105, 110-113). To initiate membrane fusion, additional factors including SNARE proteins are recruited by PIs which function in the tethering and fusion of vesicles, however the actual fusion process is not well understood (104, 105, 110-114).

Classically, one major PI regulatory pathway occurs through the hydrolysis by PKC to generate diacylglycerol (DAG) and inositol(1,4,5)triphosphate to trigger signaling cascades in various pathways (102, 103, 105). Phosphoinositides are also involved in the direct regulation of several cellular processes through the recruitment of different factors (102-105). PIs mediate and regulate several processes including autophagy and lysosome biogenesis and function (80, 97, 102-104, 113, 115-121). The of role PI(3)P in autophagy regulation has been well established, however recent studies have implicated roles of PI(3,5)P_2 and PI(5)P as well (97, 103, 113, 121, 122). Studies would suggest that phosphoinositides regulate autophagy at multiple levels from activation of

autophagy pathways, to induction, to lysosomal transport and function (97, 103, 113, 121-126).

The role of nuclear phosphoinositides remains the most enigmatic. It is unclear whether phosphoinositides are transported to the nucleus or synthesized within it. Most phosphoinositides are present within the nucleus, specifically within both membranes, the nuclear interior, and in association with chromatin, however their nuclear roles are poorly defined (102, 127-129). However, several cytoplasmic and nuclear transcription factors do contain phosphoinositide binding domains (102, 127-129). Furthermore, it is unclear how the fatty acid tails are constrained, however studies would suggest that different mechanisms may be utilized to sequester that tails (102). Within the nucleus, studies would suggest that PIs function in chromatin remodeling and transcriptional regulation through interactions with various factors (102, 127-129).

1.12 Do WIPI1 and Phosphoinositides coordinate to regulate melanogenesis?

The precise mechanism through which WIPI1 regulates melanogenic trafficking and transcription has not been determined (24). The importance of its melanogenic role is further highlighted as WIPI1 is highly expressed in melanocytes and melanoma cells (70, 96). Furthermore, given that WIPI1 is a phosphoinositide binding protein that binds to PI(3)P, PI(3,5)P_2, and PI(5)P (70, 71), it is reasonable to postulate that WIPI1 coordinates melanogenic functions through phosphoinositide binding. Interestingly, two of these phosphoinositides, PI(3,5)P_2 and PI(5)P, are synthesized by one enzyme, PIKfyve (130, 131). Furthermore, the yeast homologs of WIPI1 and PIKfyve, ATG18

and FIG4 respectively, form a regulatory complex (94). It is unclear if a functional relationship exists in mammals as well.

1.13 Biochemistry of The FAB1 and PIKfyve Complexes

1.13.1 Functional biochemistry of the yeast PI5-kinase: FAB1

FAB1 is a lipid kinase in *Saccharomyces cerevisiae* (yeast) is the only class III PI5 lipid kinase and is responsible for the generation of $PI(3,5)P_2$ by phosphorylating PI(3)P at the 5-position (88, 132, 133). For efficient generation of $PI(3,5)P_2$ two additional components are required, FIG4 and VAC14 (88, 132-134). FIG4 is a PI5-phosphatase that mediates the conversion of $PI(3,5)P_2$ to PI(3)P, yet paradoxically is a necessary activating component of the FAB1 complex (94, 132). VAC14 forms a scaffolding dimer that mediates interactions with FAB1, FIG4, as well as two regulatory components, VAC7 and ATG18 (88, 94, 132, 134). FAB1, FIG4, and dimerized VAC14 form the core $PI(3,5)P_2$ kinase complex, which is evolutionarily conserved in mammals (94, 135). In *S. cerevisiae*, VAC7 and ATG18 respectively function as inhibitors or activators of kinase activity through binding to VAC14 (94), however, these interactions are not conserved in mammals. VAC7 does not possess a known mammalian homologue. Even though ATG18 possess two mammalian homologues, WIPI1 and WIPI2, they have not been shown to interact or regulate kinase activity.

1.13.2 Functional biochemistry of the mammalian PI5-kinase: PIKfyve

The mammalian PI5-kinase core complex is composed of the kinase PIKfyve, the phosphatase SAC3 (encoded by the *FIG4* gene*)*, and a scaffolding VAC14/ArPIKfyve dimer (135-137). Like yeast, PIKfyve is the only type III PI5 kinase (130). While FAB1 has only been shown to utilize PI(3)P as a substrate, mammalian studies have suggested that PIKfyve can utilize either PI(3)P or phosphatidylinositol (PIP) to produce $PI(3,5)P_2$ and PI(5)P respectively (130, 131). PIKfyve is responsible for the generation of ~85% of PI(5)P both directly through phosphorylation of PIP and indirectly via $PI(3,5)P_2$ hydrolysis (131, 138). PI(5)P can also be produced through dephosphorylation of $PI(4,5)P_2$ (130, 138). Other mammalian proteins have been shown to interact with the PIKfyve complex (139, 140), however only APP and APLP2 have been suggested to modulate kinase activity though direct interaction (141, 142).

1.13.3 Molecular biochemistry of PIKfyve

PIKfyve is a large cytosolic protein consisting of 2,052 – 2098 amino acid residues (143, 144). PIKfyve possesses several domains, specifically a phosphoinositol-binding FYVE domain, a DEP domain, a chaperonin-like domain, and a C-terminal kinase domain (143, 145, 146). The FYVE domain specifically binds to PI(3)P with high affinity and is essential for PIKfyve localization (145). The DEP domain is only present in PIKfyve from higher eukaryotes (147). While it normally functions in contributing to protein stability and targeting, its PIKfyve function has not been elucidated (147).

Similarly, the PIKfyve Cpn60_TCP1 chaperonin domain function has not well been defined (147). Deletion of the chaperonin domain did not affect kinase activity (147).

Three critical amino acid residues have been identified in the catalytic C-terminal domain (147). Lysine 1831 is the predicted to mediate ATP-binding given that kinase activity is detected upon mutation (147). Adjacent lysines (1999 and 2000) in the kinase domain also possess phosphoinositide binding affinity, but to a significantly lesser degree than the FYVE domain (145). Only PIP and PI(3)P have been demonstrated to function as substrates in kinase driven phosphorylation at the 5-position (115, 131, 145). PI(3)P is the preferred kinase substrate, however mutagenesis studies have demonstrated that mutation of K2000 can shift substrate preference towards PI5P while K1999 mutagenesis had the reverse effect (115, 131, 145). Despite greater affinity for PI(3)P, PIKfyve inhibition or knockdown resulted in a relatively greater decrease in PI(5)P levels in comparison to $PI(3,5)P_2$ (131). As a possible explanation for this discrepancy, it has been suggested that different PIKfyve pools are responsible for generating PI(5)P verses $PI(3,5)P_2$ (115, 131).

PIKfyve possesses multiple predicted phosphorylation sites (148), however among the four sites characterized (149-151), only phosphorylation of Ser^{318} was found to directly modulate kinase activity (151). *In vitro,* AMPKα phosphorylates PIKfyve at Ser^{307} to facilitate PIKfyve translocation (150). It has been suggested that P-Ser^{307} may promote GLUT4 translocation, as PIKfyve knockdown reduced insulin dependent glucose uptake (150). Insulin and glucose have been shown to increase $PI(3,5)P_2$ (80, 150) and PI(5)P levels both directly and indirectly (152). Specifically insulin and glucose

inhibit the SAC3 phosphatase (153) and stimulate AKT to phosphorylate PIKfyve at Ser^{318} and promote kinase activity (151).

1.13.4 Molecular biochemistry of VAC14

VAC14, also known as ArPIKfyve, does not possess enzymatic activity, but is an essential part of the PIKfyve complex (136). VAC14 possesses multiple HEAT repeats that mediate interactions with PIKfyve and SAC3 (94, 135, 136). Interaction with VAC14 is essential for SAC3 and PIKfyve to elicit enzymatic activity (94, 135, 154). The VAC14 C-terminal domain mediates dimerization, as monomeric VAC14 is unable to bind PIKfyve and SAC3 resulting in significant loss of enzymatic activity (134, 135). VAC14 is essential for SAC3 protein stability (155). In *VAC14* null mice, VAC14 and SAC3 are lost, but transcriptional levels of SAC3 are not affected (155). In contrast, loss of PIKfyve does not affect protein levels or complex formation between SAC3 and VAC14 (138). While a few proteins have been reported to bind directly to PIKfyve (139-142), VAC14 has been suggested to have a very large interactome (156). However, very few of these interactions have been verified (156) and it is unknown if and how these mediate kinase activity or what complexes form with VAC14.

1.13.5 Molecular biochemistry of SAC3

SAC3 is encoded by the *FIG4* gene (136). SAC3 is stabilized by binding to VAC14 (155) through which it functions as an activator of PIKfyve (136). SAC3 belong

to the SAC phosphatase family along with SAC1 and SAC2 (125, 137). While there is conservation in the phosphatase active site between all three members, the VAC14 binding site is not conserved (125, 137). Furthermore, SAC1 and SAC2 utilize different phosphoinositol substrates than SAC3 (125, 157-161). SAC3 is the only known 5-phosphotase to utilize $PI(3,5)P_2$ as a substrate in the generation of PI(3)P (125, 136). Interestingly, SAC3 only exhibits phosphatase activity in complex with both VAC14 and PIKfyve (136). Impaired binding of SAC3 to VAC14 impairs PIKfyve enzymatic function (155, 158). Expression of wild-type or phosphatase-deficient SAC3 or in a *FIG4* null background resulted in significant rescue of vacuolization *in vitro,* suggesting that phosphatase activity is not necessary for PIKfyve to exhibit kinase activity (158). Depletion of SAC3, or abolishment of phosphatase activity, stimulates glucose uptake thus promoting the activation of PIKfyve (151, 153). Similarly, insulin decreases SAC3 phosphatase activity (153). Interestingly, expression of phosphatase-deficient SAC3 in a *FIG4*$^{-/-}$ background only resulted in partial-rescue *in vivo,* thus suggesting that SAC3 possesses both catalytic and non-catalytic functions, the latter of which are poorly understood (158). While SAC3 is necessary for PIKfyve function (155), it is not fully understood how the PIKfyve complex functions both as a phosphatase and kinase as studies would suggest multiple factors modulate the balance between the two activities.

1.14 PIKfyve Function and Disorder

1.14.1 PIKfyve function and pathology

PIKfyve is a ubiquitously expressed kinase found to varying degrees in all tissues (137, 146, 162). Most of the protein is cytosolic or associated with internal structures, while a small fraction is membrane associated (146). Intracellular localization is determined by the FYVE domain, primarily resulting in localization to early endosomes and MVBs (143-145). PIKfyve, through the production of phosphoinositides, has been implicated to function in autophagy (97, 122, 123), endosome maturation (163), lysosome function and biogenesis (124, 164-168), cytokine production (169), and vesicular trafficking (167, 170).

At the cellular level, PIKfyve inhibition or disruption of any complex components results in abnormal vesicular trafficking and severe vacuolization (123, 136, 138, 167). As a result, vacuolization, disruption of autophagy, and lysosomal inhibition ultimately lead to cell death (123, 124, 136, 163). Organismal disruption of PIKfyve or complex members results in dysfunction of multiple organ systems (138, 171), most prominently affecting the nervous system (94, 122, 123, 138, 155, 158, 171-173). Mutation or loss of any complex member results in severe neurodegeneration and juvenile lethality (94, 138, 155). Loss of PIKfyve itself is non-viable and results in pre-implantation lethality (138). PIKfyve has also been implicated in playing an essential role in several other tissues to different degrees (124, 163, 171, 174). PIKfyve is essential for cellular function and viability as homozygous loss of any complex member is lethal, however the presence of one functional allele does not impair development (175). Only four human diseases have been linked to mutations in the PIKfyve complex (176). Among the complex members, only VAC14 has not been linked to any human disease.

1.14.2 Human diseases and the PIKfyve complex

Several single nucleotide polymorphisms in the *PIKFYVE* gene have been identified as the causative agent of fleck corneal dystrophy (FCD), resulting in primarily frameshift mutations that result in premature truncation (176-178). FCD is relatively mild disease characterized by the accumulation of lipid and polysaccharide containing vesicles around eye keratocyte nuclei resulting in the appearance of “white flecks” which eventually clear up (176-178). The molecular pathogenesis of the disease has not been well characterized. Despite being autosomal dominant it is unclear why pathology is limited to the cornea (176-178).

Mutations in the *FIG4* gene are associated with Charcot-Marie-Tooth peripheral neuropathy type 4J (CMT4J) (155, 171), Yunis-Varón syndrome (179, 180) and seizures and psychiatric features (181). Additionally, polymorphisms in *FIG4* have also been found in 2% of patients with amyotrophic and primary lateral sclerosis and may serve as a risk factor, however a clear linkage has not been established (172).

CMT4J is a rare autosomal recessive disorder neurological disorder that with childhood or adult onset (155, 171), however the triggers for age of onset are unclear. As a result of extensive demyelination and neurodegeneration, there is a progressive loss of motility and eventually death ensues (155, 171). The disease itself is caused by compound heterozygosity at the *FIG4* locus, specifically a missense mutation I41T, combined with null mutation in the other allele resulting in partial loss of function (155, 171). As a result, SAC3 exhibits defective interaction with VAC14, leading to destabilization of the protein, thus resulting in extensive vacuolization and eventually

cell death (155). Biallelic null mutations, both heterozygous and non-heterozygous, in *FIG4* are associated with Yunis-Varón syndrome (179, 180). A more severe phenotype that is reminiscent of SAC3 null mice is observed and early juvenile lethality results (155, 179, 180). A novel, autosomal recessive missense mutation in SAC3, D783V, causes polymicrogyria and cortical malformation, resulting in epileptic seizures and behavioral abnormalities (181). Only minimal characterization of the D783V has been conducted but studies would suggest only partial loss of function (181).

1.15 PtdIns(3,5)P_2

1.15.1 Molecular Biochemistry of PtdIns(3,5)P_2

PI(3,5)P_2 is among the least abundant phosphoinositides, accounting for about 0.025%~0.1% of total phosphoinositides (131, 138, 182). PIKfyve is solely responsible generating all cellular PI(3,5)P_2 through the phosphorylation of PI(3)P (138). Complete *ex vivo* depletion of PIKfyve from mouse fibroblasts resulted in undetectable levels of PI(3,5)P_2. PI(3,5)P_2 is required for survival both *in vitro* and *in vivo* (131, 138, 182). Loss of PI(3,5)P_2 results in severe vacuolization *in vitro* and *in vivo* and prominently leads to severe neurodegeneration *in vivo* (131, 138, 182). Determining the exact functional role of PI(3,5)P_2 has presented two major caveats. First, as PIKfyve is responsible for the synthesis of both PI(3,5)P_2 and PI(5)P, it can be difficult to distinguish their individual roles and effectors. Secondly, many PI binding proteins have affinity for more than one phosphoinositide. Nevertheless, studies have shown that PI(3,5)P_2 can function in

several cellular processes acting as both an effector molecule and in regulation of membrane trafficking and protein sorting (182). Alternatively, $PI(3,5)P_2$ can be hydrolyzed to PI(3)P or PI(5)P via MTMR phosphatases (131, 138).

The transcriptional role of $PI(3,5)P_2$ is poorly understood as PIKfyve is not known to localize to localize to the nucleus, as a result the nucleus is devoid of the phospholipid (102). Nuclear PI(5) is produced in a PIKfyve-independent manner (102). $PI(3,5)P_2$ is not known to have a transcriptional role in mammals, however it has been shown to regulate transcription in yeast through interactions with TUP1 and CTI6, thus resulting in their activation and nuclear localization (183). TUP1 does possess a mammalian homologue, HIRA, however its function in relation to $PI(3,5)P_2$ has not been determined.

1.15.2 Cytoplasmic PtdIns(3,5)P_2 Functions and Effectors

Cellularly, $PI(3,5)P_2$ is primarily localized to endosomal and lysosomal compartments as well as MVBs, functioning in both trafficking and functional regulation of the organelles (102, 123, 184). Studies would suggest that trafficking and regulation of endolysosomal pathways is mediated by a number of known and potential $PI(3,5)P_2$ effectors (156, 164-166, 182, 184-188). The role of $PI(3,5)P_2$ in membrane trafficking has been best characterized in yeast where it plays an essential role in protein sorting in MVBs and retrograde trafficking from endosomes to the TGN (170, 182, 189). In mammals, $PI(3,5)P_2$ functions to regulate MVB trafficking through binding to the ESCRT-III component, VPS24 (182, 188). Loss of $PI(3,5)P_2$ or the VPS24 lipid-binding

domain results in the accumulation of large intracellular vacuoles (131, 188). While a definite effector of retrograde trafficking has not been identified, SNX1 and SNX2 are both PI(3)P and $PI(3,5)P_2$ binding proteins that function in retrograde trafficking (170, 185). The exact role of $PI(3,5)P_2$ in their regulation of these proteins is unclear. $PI(3,5)P_2$ has been shown to regulate vacuole acidification in yeast and plants (182, 190) suggesting that it may play a similar role in mammalian cells, however studies have yielded mixed results (124, 163). While studies would suggest that $PI(3,5)P_2$ is essential for lysosomal trafficking, its role in lysosomal acidification remains unclear as studies have shown contradictory results (124, 163, 191). Furthermore, It has however been suggested that the $PI(3,5)P_2$ regulated ion channels could function in regulating lysosomal pH (192, 193).

While no PIKfyve regulated proton channels have yet been identified, $PI(3,5)P_2$ has been found to regulate two groups of endolysosomal cation channels – specifically two-pore channels (TPC1 and TPC2) (194) and transient receptor potential cation channel, mucolipin subfamily channels (TRPML1, TRPML2, and TRPML3) (165). Both TPC and TRPML channels were found to be specifically activated by $PI(3,5)P_2$ with EC_{50}'s in the nanomolar range (165, 194).

TPC1 and TPC2 are Na^+-selective ion channels that upon stimulation by $PI(3,5)P_2$ trigger membrane depolarization of endolysosomes by releasing sodium ions into the cytosol (194, 195). However, recent studies have suggested that TPC2 may function as a Ca^{2+} channel (196). Furthermore, studies would suggest that TPC channels regulate vacuolar size as overexpression resulted in enlarged endolysosomes, possible due to dysregulation in endolysosomal fusion/fission events (126, 164, 194).

Furthermore, TPCs have been found to co-immunoprecipitate several RAB-GTPases (164). Similarly, inhibition of RAB nucleotide binding resulted in decreased TPC channel activity (164). TPC2 function was directly implicated in pigment production as overexpression of TPC2 or inhibition of RAB-GTPase activity resulted in decreased pigmentation (164, 195, 196). Interestingly, recent studies have found suggested that TPC2 functions as a $PI(3,5)P_2$ regulated melanosomal cation channel (195, 196). TPC2 was found to regulate both melanosomal pH and size (195, 196). Overexpression of TPC2 or the addition of exogenous $PI(3,5)P_2$ decreased melanosomal pH thus resulting in decreased pigmentation (164, 195, 196). Recent studies have also implicated TPCs in membrane and endolysosomal trafficking (196, 197), however it is unclear whether TPCs also function in melanosome trafficking.

TRPMLs form a subfamily within the transient receptor channel (TRP) superfamily of cation channels (198) distinguished by several N-terminal conserved basic amino acid residues that mediate $PI(3,5)P_2$ binding (165). The TRPMLs possess 75% amino acid similarity and function as endolysosomal Ca^{2+}-channels (192, 193). TRPMLs primarily function in endolysosomal trafficking, however studies have suggested additional roles including signal transduction, autophagy, and in lysosomal function and pH homeostasis (192, 193). Interestingly, overexpression or loss TRPMLs results in impaired autophagy (192). TRPML1 is the best characterized among the TRPML members and is the only member that is ubiquitously expressed (192, 193). Mutations in TRPML1 are associated with the neurodegenerative, mucolipidosis type IV (165, 166, 192, 193). *In vitro*, loss or mutation of TRPML1 is phenotypically similar to loss of $PI(3,5)P_2$ resulting in enlarged endolysosomes, trafficking defects, and impaired

lysosomal function (165, 166, 192, 193). TPRML2 and TRPML3 are expressed in tissue specific manners and are not associated with any human disease (192, 193). TRPML2 and TRPML3 are less characterized and while all three members retain the same core functions, there are some differences in subcellular localization and function (192, 193).

Studies have implicated mTORC1 as a regulator of melanogenesis and autophagy, however recent studies could suggest that $PI(3,5)P_2$ is required for its activation (24, 77, 78, 80). Raptor, a component specific to the mTORC1 complex, binds to $PI(3,5)P_2$ to mediate both mTORC1 activation and localization suggesting it could be a key effector (80). It has been suggested that distinct pools of $PI(3,5)P_2$ localize mTORC1 to late endosomal and lysosomal structures to mediate the release of amino acid under starvation conditions (80, 186) and may function in the regulation of lysosomal pH as well (199). Furthermore, $PI(3,5)P_2$ activation of mTORC1 results in downregulation of autophagy (80, 186). It has been proposed that pathway may mediate function conjunction with PIKfyve suppressors to mediate autophagy (186). Homeostatic balance between $PI(3,5)P_2$ and PI(5)P may function in autophagy regulation as studies have shown opposing effects on autophagy function (80, 84, 97, 186).

1.15.3 Myotubularin-related proteins convert $P(3,5)P_2$ to PI(5)P *in vitro*

Two sets of phosphatases hydrolyze $PI(3,5)P_2$, SAC3 which generates PI(3)P and the myotubularin-related protein (MTMR) phosphatase family which generates PI(5)P (125, 130, 200-202). SAC3 is the only phosphatase known to use $PI(3,5)P_2$ as a

substrate in the generation of PI(3)P (125, 136). In contrast, several members of the MTMR family have been proposed to possess 3-phosphatase activity, utilizing PI(3)P and/or PI(3,5)P_2 as substrates (125, 130, 200-202). MTM1, MTMR1-4, MTMR6-8, and MTMR14 possess sequence homology containing an active CX_5R 3-phosphatase motif and are predicted to dephosphorylate of PI(3,5)P_2 to PI(5)P (125, 203, 204). Among these, most have been shown to modulate mammalian PI(3,5)P_2 levels i*n vitro* (97, 200, 203-207). Mammalian PIKfyve studies and reviews by the Shisheva and Weisman groups have opposing viewpoints on the contributions of MTMRs to the PI(5)P pool (125, 131, 182, 208), thus the exact *in vivo* contributions of mammalian MTMRs in the production of PI(5)P remains unclear.

MTMRs have been shown to regulate a wide variety of cellular processes and some are expressed in a tissue specific manner (207, 209). Mutations and polymorphisms in various MTMRs are associated with or severe as risk factors for several diseases (209, 210) however the molecular pathogenesis is poorly understood. Interestingly, mutation or loss of MTMR2 or MTMR13 are associated with CMT neuropathies type 4B1 and 4B2 respectively (CMT4B1/2) (211-213). Even though MTMR13 is catalytically inactive, it serves as a regulatory binding partner to MTMR2 (212, 214). Loss of MTMR2 or MTMR13 alone does not result in vacuolization, however combined loss results neural demyelination which is further exacerbated in combination with SAC3 loss (211, 213). MTMR2 dephosphorylates PI(3)P and PI(3,5)P_2 (214) and is involved in pathways regulating myelin production (215), AKT signaling (214), and late endosomal trafficking (216). Taken together with the seemingly redundant phosphatase activity shared with other MTMRs it is difficult to understand the molecular

pathology of CMT4B and to ascertain the involvement of the individual phosphoinositides.

1.16 PtdIns5P

1.16.1 Molecular Biochemistry of PtdIns(5)P

PI(5)P is about ten-times more abundant than $PI(3,5)P_2$ in mammalian cells comprising about 0.25% of total phosphoinositides (131). PIKfyve is responsible for the production of most cellular PI(5)P either through direct phosphorylation of PIP or indirectly by dephosphorylation of $PI(3,5)P_2$ by MTMRs (115, 131, 138). Studies by Shisheva's group have demonstrated that PIKfyve utilizes both PIP and PI(3)P as substrates in the production of PI(5)P and $PI(3,5)P_2$ respectively (115, 131, 217). While studies have shown that MTM and MTMRs do possess $PI(3,5)P_2$ phosphatase activity (9, 31, 79-81) there is limited evidence to support that this is the major PI(5)P synthetic pathway in mammalian cells (115, 131). Nevertheless, the dephosphorylation pathway may be more prevalent in a subcellular or tissue specific manner. Regardless, PIKfyve is responsible for the generation of majority of cellular PI(5)P (115, 131, 138). Loss, knockdown, or inhibition of PIKfyve results in decreased levels of both $PI(3,5)P_2$ and PI(5)P (138). Similarly, mutation of PIKfyve's ATP-binding or catalytic residues results in decreased levels of both (217).

Alternatively, PI(5)P is produced by dephosphorylation of $PI(4,5)P_2$ by type I and type II $PI(4,5)P_2$ 4-phosphatases (218). The extent to which these phosphatases

contribute to PI(5)P production is unknown and varies by cell type (115). Type I phosphatases are the sole contributors in generation of the nuclear PI(5)P pool (102, 115). A minor proportion of PI(5)P is produced in a PIKfyve-independent manner by type I and type II PI(4,5)P_2 4-phosphatases (218). Both phosphatases are ubiquitously expressed but expression levels do vary by tissue (218). Both localize to late endosomes and lysosomes (115, 218) and it has been suggested that this may be of more significance in cell types expressing low levels of PIKfyve (115). Furthermore, type I phosphatases can translocate to the nucleus in response to DNA damage is believed to be solely responsible for generation of nuclear PI(5)P, resulting in an increased PI(5)P levels (102, 115). PI(5)P is suggested to function in the regulation of multiple cellular functions, both nuclearly and cytosolically (115). While it has been suggested that phospholipase C-*δ*1 may function in hydrolysis of PI(5)P to PIP, this mechanism has not been well elucidated (115). The majority of PI(5)P is removed by phosphorylation to PI(4,5)P_2 by PIP4 kinases.

1.16.2 Nuclear PtdIns(5)P and Factors

Nuclear PI(5)P is found throughout the nucleus and within chromatin-enriched fractions (102). Nuclear PI(5)P is able to bind to factors involved in stress response, cell proliferation, cell differentiation through interactions with PHD-finger domain containing factors, affecting chromatin association as a result (102, 115, 127-129). Within the nucleus, PI(5)P levels are relatively low and controlled by type I phosphatases and the beta isoform of the PIP4 kinase (PIP4Kβ) (102, 128). UV radiation and oxidative stress

increase nuclear PI(5)P levels by translocating type I phosphatases to the nucleus and decreasing PIP4Kβ activity (102, 115, 128). ING2, and several other PHD-finger containing nuclear factors were found to interact with PI(5)P and mediate association with chromatin, however only three of these factors (ING3, TAF3, and DIDO1) interacted with modified histone tails (129). PI(5)P binds to the nuclear transcription factor, ING2, to mediate its translocation and drive expression of ING2-regulated DNA damage response genes (127, 128). Even though PHD-containing proteins have the highest affinity for PI(5)P, other phosphoinositides [primarily PI(3)P and PI(4,5)P_2] are also able to bind and activate these factors (129). The consequences of these interactions are not fully understood and only ING3 and TAF3 interactions have been characterized in detail thus far (127, 129).

1.16.3 Cytoplasmic PtdIns(5)P and factors

Studies suggest that PI(5)P is involved both nuclear and cytoplasmic cellular processes. PI(5)P is able to coordinate several cellular stress responses both directly through nuclear synthesis and indirectly through modulation of signaling pathways mediated by AKT activation (115). Cytoplasmically, PI(5)P is localized the ER and Golgi, and early endosomes (115, 118) where it has been implicated to function in glucose uptake, actin remodeling, membrane trafficking, autophagy, and AKT signaling pathways (97, 115). How PI(5)P modulates many of these processes remains unclear as very few cytoplasmic PI(5)P effectors have been identified (97, 119).

Salmonella and *Shigella* both produce virulence factors that function as $PI(4,5)P_2$ phosphatases to increase PI(5)P levels resulting in the recruitment of TOM1 to early endosomes. Even though TOM1, as well as TOM1L1 and TOM1L2, all preferentially bind PI(5)P (119), the functions of the latter two with respect to lipid binding have not be elucidated. Recruitment of TOM1 by PI(5)P ultimately results in lysosomal inhibition thus leading to AKT activation (115, 117-119). This would suggest that PI(5)P may act in an opposing manner to $PI(3,5)P_2$ in the regulation of lysosomal function. Similarly, PI(5)P and $PI(3,5)P_2$ may play opposing roles in autophagy.

Insulin and glucose have been shown to increase PI(5)P levels, promoting GLUT4 translocation and breaking down actin-stress fibers ultimately resulting increased glucose uptake (115, 152, 219). Complimentary to these findings, glucose-starvation was also found to upregulate autophagy and promote autophagosome biogenesis through WIPI2-PI(5)P interactions in a PI(3)P-independent manner (97). This is further exemplified as WIPI1 knockdown or deletion resulted in upregulation of autophagy (97, 220). Upon further investigation, the authors concluded the PI(5)P is essential for inducing autophagy under glucose withdrawal (97). Mechanistically, it is likely that PI(5)P dependent phosphorylation of AKT functions in a feedback loop to promote PIKfyve kinase activity (117, 151). As, $PI(3,5)P_2$ activation of mTORC1 results in downregulation of autophagy (80, 186), there are likely additional factors that can tip the balance towards PI(5)P production.

Given that PIKfyve synthesizes both $PI(3,5)P_2$ and PI(5)P, further complicated the promiscuity with many PI-binding proteins, it is often difficult to discern the roles of the individual phosphoinositides in pathogenesis. Among the identified PI(5)P effectors,

none thus far have been clearly implicated in disease pathology. Thus PI(5)P remains the most enigmatic phosphoinositide.

1.16.3.1 PtdIns5P 4-kinases phosphorylate PI(5)P to PI(4,5)P_2

PtdIns5P 4-kinases (PI5P4Ks) are responsible for the removal of PI(5)P by phosphorylating it at the 5-position to PI(4,5)P_2 (97, 220-224). Three PI5P4K isoforms (α, β, and γ) have been identified, differing in both enzymatic activity and localization (97, 220-224). PI5P4Kα is the most active isoform and is primarily localized cytoplasmically (220, 222). PI5P4Kα can dimerize with PI5P4Kβ to translocate to the nucleus (220, 222). Interestingly, PI5P4Kβ exhibits great nuclear activity (220, 222). PI5P4Kα and PI5P4Kβ have been found to function in insulin signaling, autophagy, stress responses, gene regulations, and cancer (220, 223).

PI5P4Kγ is the least understood among the PI5P4K isoforms, exhibiting the lowest kinase activity among the three (222, 225). PI5P4Kγ phosphorylation by mTORC1 or heterodimerization with PI5P4Kα alters its cellular localization (222, 224), prominently associating with autophagosomes (97). Interestingly, PI5P4Kγ reciprocally regulates mTORC1 activity (220, 221). PI5P4Kγ deletion results in hyperactivation of mTORC1, several downstream pathways including autophagy regulation are not affected (220, 221). This stands in complement to the findings that that PI5P4Ks were found to negatively affect autophagy as a result of PI(5)P depletion (97). *In vivo*, germline loss of PI5P4Kα or PI5P4Kβ did not result in decreased viability or disease phenotypes and only the double knockout resulted in perinatal fatality (221), suggesting

functional compensation between the isoforms. However, loss of PI5P4Kγ resulted in immune hyperactivity and autoimmunity, though it is unclear if and how autophagy is affected (221).

1.17 The role of PIKfyve and Phosphoinositides in Melanogenesis

While multiple studies have focused on the function and pathogenesis of PIKfyve and its effectors, the role of PIKfyve in melanogenesis has not been well elucidated. Studies have noted pigment phenotypes associated with VAC14 and SAC3 mutation, however these have not been characterized in detail (94, 155, 171). Specifically, *VAC14 ingls a*nd *FIG4* pale tremor mice both exhibited significantly depigmented coats in addition to the severe neurodegenerative phenotype (94, 171). Furthermore, a partial loss of function SAC3 mouse mutant exhibited both increased longevity and intermediate restoration of coat color (155, 158)

Similarly, mutations in two $PI(3,5)P_2$ effectors have been associated with pigment abnormalities *in vivo*, however $PI(3,5)P_2$ has not been directly implicated in one case (164, 195, 196, 226, 227). TPC2 was found to be activated by $PI(3,5)P_2$ and negatively regulate pigmentation *in vitro* in an expression dependent context (164, 195, 196). Furthermore, single-nucleotide polymorphisms in TPC2 have also been identified in humans that are correlated with skin, eye, and hair pigment variation (227). Even though TPC2 has also been implicated in endolysosomal trafficking (196, 197), its melanosomal contributions are unclear. Finally, mice mutant for TRPML3 exhibit

hypopigmentary phenotypes (226). TRPML1 and TRPML2 have not been characterized for their effect on melanogenesis.

Published studies revealed that mutations to VAC14 and SAC3 exhibit aberrant pigmentation (94, 155, 171), however these phenotypes have been poorly characterized and no studies have directly implicated the PIKfyve kinase itself in pigment regulation. Furthermore, studies have also identified abnormal pigmentation associated with the $PI(3,5)P_2$ effectors TPC2 and TRPML3 (164, 195, 196, 226, 227), further implicating a role of PIKfyve in melanogenesis. While many studies have focused on the regulatory functions of $PI(3,5)P_2$ and PI(5)P, these studies have failed to elucidate how these phosphoinositides function in cellular trafficking. Similarly, WIPI1 was implicated to function in both melanosome regulation and trafficking (24) and binds to both PIKfyve synthesized phosphoinositides. The implications of such have not yet been elucidated. Taken together, these studies strongly suggest that PIKfyve and its phosphoinositide products play a significant role in melanogenesis. Thus investigating and elucidating the role of PIKfyve and its phosphoinositol products is the focus of my thesis project.

1.18 References

1. Tobin DJ. Biochemistry of human skin--our brain on the outside. Chem Soc Rev. 2006;35(1):52-67.

2. Proksch E, Brandner JM, Jensen JM. The skin: an indispensable barrier. Exp Dermatol. 2008;17(12):1063-72.

3. Kanitakis J. Anatomy, histology and immunohistochemistry of normal human skin. Eur J Dermatol. 2002;12(4):390-9; quiz 400-1.

4. Costin GE, Hearing VJ. Human skin pigmentation: melanocytes modulate skin color in response to stress. FASEB J. 2007;21(4):976-94.

5. Elias PM. The skin barrier as an innate immune element. Semin Immunopathol. 2007;29(1):3-14.

6. Theriault LL, Hurley LS. Ultrastructure of developing melanosomes in C57 black and pallid mice. Dev Biol. 1970;23(2):261-75.

7. Nichols SE, Jr., Reams WM, Jr. The occurrence and morphogenesis of melanocytes in the connective tissues of the PET/MCV mouse strain. J Embryol Exp Morphol. 1960;8:24-32.

8. Mort RL, Jackson IJ, Patton EE. The melanocyte lineage in development and disease. Development. 2015;142(7):1387.

9. Barden H, Levine S. Histochemical observations on rodent brain melanin. Brain Res Bull. 1983;10(6):847-51.

10. Markert CL, Silvers WK. The Effects of Genotype and Cell Environment on Melanoblast Differentiation in the House Mouse. Genetics. 1956;41(3):429-50.

11. Tobin DJ. Aging of the hair follicle pigmentation system. Int J Trichology. 2009;1(2):83-93.

12. Cichorek M, Wachulska M, Stasiewicz A, Tyminska A. Skin melanocytes: biology and development. Postepy Dermatol Alergol. 2013;30(1):30-41.

13. Thingnes J, Lavelle TJ, Hovig E, Omholt SW. Understanding the melanocyte distribution in human epidermis: an agent-based computational model approach. PLoS One. 2012;7(7):e40377.

14. Kippenberger S, Bernd A, Bereiter-Hahn J, Ramirez-Bosca A, Kaufmann R. The mechanism of melanocyte dendrite formation: the impact of differentiating keratinocytes. Pigment Cell Res. 1998;11(1):34-7.

15. Scott G. Rac and rho: the story behind melanocyte dendrite formation. Pigment Cell Res. 2002;15(5):322-30.

16. Slominski A, Tobin DJ, Shibahara S, Wortsman J. Melanin pigmentation in mammalian skin and its hormonal regulation. Physiol Rev. 2004;84(4):1155-228.

17. Park HY, Kosmadaki M, Yaar M, Gilchrest BA. Cellular mechanisms regulating human melanogenesis. Cell Mol Life Sci. 2009;66(9):1493-506.

18. Sitaram A, Marks MS. Mechanisms of protein delivery to melanosomes in pigment cells. Physiology (Bethesda). 2012;27(2):85-99.

19. Miyamura Y, Coelho SG, Schlenz K, Batzer J, Smuda C, Choi W, et al. The deceptive nature of UVA tanning versus the modest protective effects of UVB tanning on human skin. Pigment Cell Melanoma Res. 2011;24(1):136-47.

20. Tsatmali M, Ancans J, Thody AJ. Melanocyte function and its control by melanocortin peptides. J Histochem Cytochem. 2002;50(2):125-33.

21. Yamaguchi Y, Hearing VJ. Physiological factors that regulate skin pigmentation. Biofactors. 2009;35(2):193-9.

22. Yajima I, Sato S, Kimura T, Yasumoto K, Shibahara S, Goding CR, et al. An L1 element intronic insertion in the black-eyed white (Mitf[mi-bw]) gene: the loss of a single Mitf isoform responsible for the pigmentary defect and inner ear deafness. Hum Mol Genet. 1999;8(8):1431-41.

23. Hoek KS, Schlegel NC, Eichhoff OM, Widmer DS, Praetorius C, Einarsson SO, et al. Novel MITF targets identified using a two-step DNA microarray strategy. Pigment Cell Melanoma Res. 2008;21(6):665-76.

24. Ho H, Kapadia R, Al-Tahan S, Ahmad S, Ganesan AK. WIPI1 coordinates melanogenic gene transcription and melanosome formation via TORC1 inhibition. J Biol Chem. 2011;286(14):12509-23.

25. Jo IS, Sohn K-C, Kim CD, Lee YH. Effect of MITF-M and MITF-A Overexpression on the Dendrtic Formation in Melanocytes. Korean J Phys Anthropol. 2016;29(1):27-34.

26. Marks MS, Heijnen HF, Raposo G. Lysosome-related organelles: unusual compartments become mainstream. Curr Opin Cell Biol. 2013;25(4):495-505.

27. Raposo G, Marks MS, Cutler DF. Lysosome-related organelles: driving post-Golgi compartments into specialisation. Curr Opin Cell Biol. 2007;19(4):394-401.

28. Dell'Angelica EC, Mullins C, Caplan S, Bonifacino JS. Lysosome-related organelles. FASEB J. 2000;14(10):1265-78.

29. Huizing M, Helip-Wooley A, Westbroek W, Gunay-Aygun M, Gahl WA. Disorders of lysosome-related organelle biogenesis: clinical and molecular genetics. Annu Rev Genomics Hum Genet. 2008;9:359-86.

30. Richmond B, Huizing M, Knapp J, Koshoffer A, Zhao Y, Gahl WA, et al. Melanocytes derived from patients with Hermansky-Pudlak Syndrome types 1, 2, and 3 have distinct defects in cargo trafficking. J Invest Dermatol. 2005;124(2):420-7.

31. Giordano F, Bonetti C, Surace EM, Marigo V, Raposo G. The ocular albinism type 1 (OA1) G-protein-coupled receptor functions with MART-1 at early stages of melanogenesis to control melanosome identity and composition. Hum Mol Genet. 2009;18(23):4530-45.

32. Watt B, van Niel G, Raposo G, Marks MS. PMEL: a pigment cell-specific model for functional amyloid formation. Pigment Cell Melanoma Res. 2013;26(3):300-15.

33. De Maziere AM, Muehlethaler K, van Donselaar E, Salvi S, Davoust J, Cerottini JC, et al. The melanocytic protein Melan-A/MART-1 has a subcellular localization distinct from typical melanosomal proteins. Traffic. 2002;3(9):678-93.

34. Hoashi T, Watabe H, Muller J, Yamaguchi Y, Vieira WD, Hearing VJ. MART-1 is required for the function of the melanosomal matrix protein PMEL17/GP100 and the maturation of melanosomes. J Biol Chem. 2005;280(14):14006-16.

35. Theos AC, Tenza D, Martina JA, Hurbain I, Peden AA, Sviderskaya EV, et al. Functions of adaptor protein (AP)-3 and AP-1 in tyrosinase sorting from endosomes to melanosomes. Mol Biol Cell. 2005;16(11):5356-72.

36. Calvo PA, Frank DW, Bieler BM, Berson JF, Marks MS. A cytoplasmic sequence in human tyrosinase defines a second class of di-leucine-based sorting signals for late endosomal and lysosomal delivery. J Biol Chem. 1999;274(18):12780-9.

37. Dell'Angelica EC. AP-3-dependent trafficking and disease: the first decade. Curr Opin Cell Biol. 2009;21(4):552-9.

38. Vijayasaradhi S, Xu Y, Bouchard B, Houghton AN. Intracellular sorting and targeting of melanosomal membrane proteins: identification of signals for sorting of the human brown locus protein, gp75. J Cell Biol. 1995;130(4):807-20.

39. Ohno H, Aguilar RC, Yeh D, Taura D, Saito T, Bonifacino JS. The medium subunits of adaptor complexes recognize distinct but overlapping sets of tyrosine-based sorting signals. J Biol Chem. 1998;273(40):25915-21.

40. Marks MS. Organelle biogenesis: en BLOC exchange for RAB32 and RAB38. Curr Biol. 2012;22(22):R963-5.

41. Gerondopoulos A, Langemeyer L, Liang JR, Linford A, Barr FA. BLOC-3 mutated in Hermansky-Pudlak syndrome is a Rab32/38 guanine nucleotide exchange factor. Curr Biol. 2012;22(22):2135-9.

42. Bultema JJ, Ambrosio AL, Burek CL, Di Pietro SM. BLOC-2, AP-3, and AP-1 proteins function in concert with Rab38 and Rab32 proteins to mediate protein trafficking to lysosome-related organelles. J Biol Chem. 2012;287(23):19550-63.

43. Bultema JJ, Di Pietro SM. Cell type-specific Rab32 and Rab38 cooperate with the ubiquitous lysosome biogenesis machinery to synthesize specialized lysosome-related organelles. Small GTPases. 2013;4(1):16-21.

44. Hida T, Sohma H, Kokai Y, Kawakami A, Hirosaki K, Okura M, et al. Rab7 is a critical mediator in vesicular transport of tyrosinase-related protein 1 in melanocytes. J Dermatol. 2011;38(5):432-41.

45. Kawakami A, Sakane F, Imai S, Yasuda S, Kai M, Kanoh H, et al. Rab7 regulates maturation of melanosomal matrix protein gp100/Pmel17/Silv. J Invest Dermatol. 2008;128(1):143-50.

46. Lee ST, Nicholls RD, Jong MT, Fukai K, Spritz RA. Organization and sequence of the human P gene and identification of a new family of transport proteins. Genomics. 1995;26(2):354-63.

47. Setty SR, Tenza D, Sviderskaya EV, Bennett DC, Raposo G, Marks MS. Cell-specific ATP7A transport sustains copper-dependent tyrosinase activity in melanosomes. Nature. 2008;454(7208):1142-6.

48. Kamaraj B, Purohit R. Mutational analysis of oculocutaneous albinism: a compact review. Biomed Res Int. 2014;2014:905472.

49. Morya VK, Dung NH, Singh BK, Lee HB, Kim EK. Homology modelling and virtual screening of P-protein in a quest for novel antimelanogenic agent and in vitro assessments. Exp Dermatol. 2014;23(11):838-42.

50. Bellono NW, Escobar IE, Lefkovith AJ, Marks MS, Oancea E. An intracellular anion channel critical for pigmentation. Elife. 2014;3:e04543.

51. Kondo T, Hearing VJ. Update on the regulation of mammalian melanocyte function and skin pigmentation. Expert Rev Dermatol. 2011;6(1):97-108.

52. Bentley NJ, Eisen T, Goding CR. Melanocyte-specific expression of the human tyrosinase promoter: activation by the microphthalmia gene product and role of the initiator. Mol Cell Biol. 1994;14(12):7996-8006.

53. Okimoto DK, Blaus A, Schmidt MV, Gordon MK, Dent GW, Levine S. Differential expression of c-fos and tyrosine hydroxylase mRNA in the adrenal gland of the infant rat: evidence for an adrenal hyporesponsive period. Endocrinology. 2002;143(5):1717-25.

54. Paterson EK, Fielder TJ, MacGregor GR, Ito S, Wakamatsu K, Gillen DL, et al. Tyrosinase Depletion Prevents the Maturation of Melanosomes in the Mouse Hair Follicle. PLoS One. 2015;10(11):e0143702.

55. Zecca L, Zucca FA, Costi P, Tampellini D, Gatti A, Gerlach M, et al. The neuromelanin of human substantia nigra: structure, synthesis and molecular behaviour. J Neural Transm Suppl. 2003(65):145-55.

56. Zecca L, Tampellini D, Gerlach M, Riederer P, Fariello RG, Sulzer D. Substantia nigra neuromelanin: structure, synthesis, and molecular behaviour. Mol Pathol. 2001;54(6):414-8.

57. Thody AJ, Higgins EM, Wakamatsu K, Ito S, Burchill SA, Marks JM. Pheomelanin as well as eumelanin is present in human epidermis. J Invest Dermatol. 1991;97(2):340-4.

58. Ozeki H, Ito S, Wakamatsu K, Thody AJ. Spectrophotometric characterization of eumelanin and pheomelanin in hair. Pigment Cell Res. 1996;9(5):265-70.

59. Galvan I, Ghanem G, Moller AP. Has removal of excess cysteine led to the evolution of pheomelanin? Pheomelanogenesis as an excretory mechanism for cysteine. Bioessays. 2012;34(7):565-8.

60. Hennessy A, Oh C, Diffey B, Wakamatsu K, Ito S, Rees J. Eumelanin and pheomelanin concentrations in human epidermis before and after UVB irradiation. Pigment Cell Res. 2005;18(3):220-3.

61. Ozeki H, Ito S, Wakamatsu K, Hirobe T. Chemical characterization of hair melanins in various coat-color mutants of mice. J Invest Dermatol. 1995;105(3):361-6.

62. Lamoreux ML, Wakamatsu K, Ito S. Interaction of major coat color gene functions in mice as studied by chemical analysis of eumelanin and pheomelanin. Pigment Cell Res. 2001;14(1):23-31.

63. Le Pape E, Wakamatsu K, Ito S, Wolber R, Hearing VJ. Regulation of eumelanin/pheomelanin synthesis and visible pigmentation in melanocytes by ligands of the melanocortin 1 receptor. Pigment Cell Melanoma Res. 2008;21(4):477-86.

64. Chintala S, Li W, Lamoreux ML, Ito S, Wakamatsu K, Sviderskaya EV, et al. Slc7a11 gene controls production of pheomelanin pigment and proliferation of cultured cells. Proc Natl Acad Sci U S A. 2005;102(31):10964-9.

65. Boissy RE. Melanosome transfer to and translocation in the keratinocyte. Exp Dermatol. 2003;12 Suppl 2:5-12.

66. Hume AN, Collinson LM, Rapak A, Gomes AQ, Hopkins CR, Seabra MC. Rab27a regulates the peripheral distribution of melanosomes in melanocytes. Journal of Cell Biology. 2001;152(4):795-808.

67. Ganesan AK, Ho H, Bodemann B, Petersen S, Aruri J, Koshy S, et al. Genome-wide siRNA-based functional genomics of pigmentation identifies novel genes and pathways that impact melanogenesis in human cells. PLoS Genet. 2008;4(12):e1000298.

68. Hoek K, Rimm DL, Williams KR, Zhao H, Ariyan S, Lin A, et al. Expression profiling reveals novel pathways in the transformation of melanocytes to melanomas. Cancer Res. 2004;64(15):5270-82.

69. Kushimoto T, Basrur V, Valencia J, Matsunaga J, Vieira WD, Ferrans VJ, et al. A model for melanosome biogenesis based on the purification and analysis of early melanosomes. Proc Natl Acad Sci U S A. 2001;98(19):10698-703.

70. Proikas-Cezanne T, Waddell S, Gaugel A, Frickey T, Lupas A, Nordheim A. WIPI-1alpha (WIPI49), a member of the novel 7-bladed WIPI protein family, is aberrantly expressed in human cancer and is linked to starvation-induced autophagy. Oncogene. 2004;23(58):9314-25.

71. Gaugel A, Bakula D, Hoffmann A, Proikas-Cezanne T. Defining regulatory and phosphoinositide-binding sites in the human WIPI-1 beta-propeller responsible for autophagosomal membrane localization downstream of mTORC1 inhibition. J Mol Signal. 2012;7(1):16.

72. Smith JW, Koshoffer A, Morris RE, Boissy RE. Membranous complexes characteristic of melanocytes derived from patients with Hermansky-Pudlak syndrome type 1 are macroautophagosomal entities of the lysosomal compartment. Pigment Cell Res. 2005;18(6):417-26.

73. Ho H, Ganesan AK. The pleiotropic roles of autophagy regulators in melanogenesis. Pigment Cell Melanoma Res. 2011;24(4):595-604.

74. Kalie E, Razi M, Tooze SA. ULK1 regulates melanin levels in MNT-1 cells independently of mTORC1. PLoS One. 2013;8(9):e75313.

75. Zhang CF, Gruber F, Ni C, Mildner M, Koenig U, Karner S, et al. Suppression of autophagy dysregulates the antioxidant response and causes premature senescence of melanocytes. J Invest Dermatol. 2015;135(5):1348-57.

76. Kim ES, Chang H, Choi H, Shin JH, Park SJ, Jo YK, et al. Autophagy induced by resveratrol suppresses alpha-MSH-induced melanogenesis. Exp Dermatol. 2014;23(3):204-6.

77. Ohguchi K, Banno Y, Nakagawa Y, Akao Y, Nozawa Y. Negative regulation of melanogenesis by phospholipase D1 through mTOR/p70 S6 kinase 1 signaling in mouse B16 melanoma cells. J Cell Physiol. 2005;205(3):444-51.

78. Busca R, Bertolotto C, Ortonne JP, Ballotti R. Inhibition of the phosphatidylinositol 3-kinase/p70(S6)-kinase pathway induces B16 melanoma cell differentiation. J Biol Chem. 1996;271(50):31824-30.

79. Jewell JL, Russell RC, Guan KL. Amino acid signalling upstream of mTOR. Nat Rev Mol Cell Biol. 2013;14(3):133-9.

80. Bridges D, Ma JT, Park S, Inoki K, Weisman LS, Saltiel AR. Phosphatidylinositol 3,5-bisphosphate plays a role in the activation and subcellular localization of mechanistic target of rapamycin 1. Mol Biol Cell. 2012;23(15):2955-62.

81. Baskaran S, Ragusa MJ, Boura E, Hurley JH. Two-site recognition of phosphatidylinositol 3-phosphate by PROPPINs in autophagy. Mol Cell. 2012;47(3):339-48.

82. Krick R, Busse RA, Scacioc A, Stephan M, Janshoff A, Thumm M, et al. Structural and functional characterization of the two phosphoinositide binding sites of PROPPINs, a beta-propeller protein family. Proc Natl Acad Sci U S A. 2012;109(30):E2042-9.

83. Proikas-Cezanne T, Takacs Z, Donnes P, Kohlbacher O. WIPI proteins: essential PtdIns3P effectors at the nascent autophagosome. J Cell Sci. 2015;128(2):207-17.

84. Polson HE, de Lartigue J, Rigden DJ, Reedijk M, Urbe S, Clague MJ, et al. Mammalian Atg18 (WIPI2) localizes to omegasome-anchored phagophores and positively regulates LC3 lipidation. Autophagy. 2010;6(4):506-22.

85. Dooley HC, Razi M, Polson HE, Girardin SE, Wilson MI, Tooze SA. WIPI2 links LC3 conjugation with PI3P, autophagosome formation, and pathogen clearance by recruiting Atg12-5-16L1. Mol Cell. 2014;55(2):238-52.

86. Watanabe Y, Kobayashi T, Yamamoto H, Hoshida H, Akada R, Inagaki F, et al. Structure-based analyses reveal distinct binding sites for Atg2 and phosphoinositides in Atg18. J Biol Chem. 2012;287(38):31681-90.

87. Obara K, Sekito T, Niimi K, Ohsumi Y. The Atg18-Atg2 complex is recruited to autophagic membranes via phosphatidylinositol 3-phosphate and exerts an essential function. J Biol Chem. 2008;283(35):23972-80.

88. Efe JA, Botelho RJ, Emr SD. Atg18 regulates organelle morphology and Fab1 kinase activity independent of its membrane recruitment by phosphatidylinositol 3,5-bisphosphate. Mol Biol Cell. 2007;18(11):4232-44.

89. Peng Y, Weisman LS. The cyclin-dependent kinase Cdk1 directly regulates vacuole inheritance. Dev Cell. 2008;15(3):478-85.

90. Weisman LS. Organelles on the move: insights from yeast vacuole inheritance. Nat Rev Mol Cell Biol. 2006;7(4):243-52.

91. Lipatova Z, Tokarev AA, Jin Y, Mulholland J, Weisman LS, Segev N. Direct interaction between a myosin V motor and the Rab GTPases Ypt31/32 is required for polarized secretion. Mol Biol Cell. 2008;19(10):4177-87.

92. Murase D, Hachiya A, Kikuchi-Onoe M, Fullenkamp R, Ohuchi A, Kitahara T, et al. Cooperation of endothelin-1 signaling with melanosomes plays a role in developing and/or maintaining human skin hyperpigmentation. Biol Open. 2015;4(10):1213-21.

93. Fullgrabe J, Klionsky DJ, Joseph B. The return of the nucleus: transcriptional and epigenetic control of autophagy. Nat Rev Mol Cell Biol. 2014;15(1):65-74.

94. Jin N, Chow CY, Liu L, Zolov SN, Bronson R, Davisson M, et al. VAC14 nucleates a protein complex essential for the acute interconversion of PI3P and PI(3,5)P(2) in yeast and mouse. EMBO J. 2008;27(24):3221-34.

95. Stelzer G, Rosen N, Plaschkes I, Zimmerman S, Twik M, Fishilevich S, et al. The GeneCards Suite: From Gene Data Mining to Disease Genome Sequence Analyses. Curr Protoc Bioinformatics. 2016;54:1 30 1-1 3.

96. Uhlen M, Fagerberg L, Hallstrom BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Proteomics. Tissue-based map of the human proteome. Science. 2015;347(6220):1260419.

97. Vicinanza M, Korolchuk VI, Ashkenazi A, Puri C, Menzies FM, Clarke JH, et al. PI(5)P regulates autophagosome biogenesis. Mol Cell. 2015;57(2):219-34.

98. Mauthe M, Jacob A, Freiberger S, Hentschel K, Stierhof YD, Codogno P, et al. Resveratrol-mediated autophagy requires WIPI-1-regulated LC3 lipidation in the absence of induced phagophore formation. Autophagy. 2011;7(12):1448-61.

99. Vicinanza M, Rubinsztein DC. Mirror image phosphoinositides regulate autophagy. Mol Cell Oncol. 2016;3(2):e1019974.

100. Thost AK, Donnes P, Kohlbacher O, Proikas-Cezanne T. Fluorescence-based imaging of autophagy progression by human WIPI protein detection. Methods. 2015;75:69-78.

101. Zhao YG, Sun L, Miao GY, Ji CC, Zhao HY, Sun HY, et al. The autophagy gene Wdr45/Wipi4 regulates learning and memory function and axonal homeostasis. Autophagy. 2015;11(6):881-90.

102. Shah ZH, Jones DR, Sommer L, Foulger R, Bultsma Y, D'Santos C, et al. Nuclear phosphoinositides and their impact on nuclear functions. FEBS J. 2013;280(24):6295-310.

103. Dall'Armi C, Devereaux KA, Di Paolo G. The role of lipids in the control of autophagy. Curr Biol. 2013;23(1):R33-45.

104. Simonsen A, Wurmser AE, Emr SD, Stenmark H. The role of phosphoinositides in membrane transport. Current Opinion in Cell Biology. 2001;13(4):485-92.

105. Di Paolo G, De Camilli P. Phosphoinositides in cell regulation and membrane dynamics. Nature. 2006;443(7112):651-7.

106. Sasaki T, Takasuga S, Sasaki J, Kofuji S, Eguchi S, Yamazaki M, et al. Mammalian phosphoinositide kinases and phosphatases. Progress in Lipid Research. 2009;48(6):307-43.

107. Majerus PW, York JD. Phosphoinositide phosphatases and disease. J Lipid Res. 2009;50:S249-S54.

108. Liu Y, Bankaitis VA. Phosphoinositide phosphatases in cell biology and disease. Progress in Lipid Research. 2010;49(3):201-17.

109. Antonsson B. Phosphatidylinositol synthase from mammalian tissues. Bba-Lipid Lipid Met. 1997;1348(1-2):179-86.

110. Poccia D, Larijani B. Phosphatidylinositol metabolism and membrane fusion. Biochem J. 2009;418(2):233-46.

111. McMahon HT, Boucrot E. Molecular mechanism and physiological functions of clathrin-mediated endocytosis. Nat Rev Mol Cell Bio. 2011;12(8):517-33.

112. Haucke V. Phosphoinositide regulation of clathrin-mediated endocytosis. Biochem Soc T. 2005;33:1285-9.

113. He SS, Ni DJ, Ma BY, Lee JH, Zhang T, Ghozalli I, et al. PtdIns(3)P-bound UVRAG coordinates Golgi-ER retrograde and Atg9 transport by differential interactions with the ER tether and the beclin 1 complex. Nature Cell Biology. 2013;15(10):1206-U192.

114. James DJ, Khodthong C, Kowalchyk JA, Martin TFJ. Phosphatidylinositol 4,5-bisphosphate regulates SNARE-dependent membrane fusion. Journal of Cell Biology. 2008;182(2):355-66.

115. Shisheva A. PtdIns5P: news and views of its appearance, disappearance and deeds. Arch Biochem Biophys. 2013;538(2):171-80.

116. Jha A, Ahuja M, Patel S, Brailoiu E, Muallem S. Convergent regulation of the lysosomal two-pore channel-2 by Mg(2)(+), NAADP, PI(3,5)P(2) and multiple protein kinases. EMBO J. 2014;33(5):501-11.

117. Pendaries C, Tronchere H, Arbibe L, Mounier J, Gozani O, Cantley L, et al. PtdIns5P activates the host cell PI3-kinase/Akt pathway during Shigella flexneri infection. EMBO J. 2006;25(5):1024-34.

118. Ramel D, Lagarrigue F, Pons V, Mounier J, Dupuis-Coronas S, Chicanne G, et al. Shigella flexneri infection generates the lipid PI5P to alter endocytosis and prevent termination of EGFR signaling. Sci Signal. 2011;4(191):ra61.

119. Boal F, Mansour R, Gayral M, Saland E, Chicanne G, Xuereb JM, et al. TOM1 is a PI5P effector involved in the regulation of endosomal maturation. J Cell Sci. 2015;128(4):815-27.

120. Vergne I, Delgado-Vargas M, Ponpuak M, Deretic V. Regulation of autophagy by PI3P phosphatases. Autophagy. 2009;5(6):906-.

121. Vergne I, Deretic V. The role of PI3P phosphatases in the regulation of autophagy. Febs Letters. 2010;584(7):1313-8.

122. Ferguson CJ, Lenk GM, Meisler MH. Defective autophagy in neurons and astrocytes from mice deficient in PI(3,5)P2. Hum Mol Genet. 2009;18(24):4868-78.

123. Martin S, Harper CB, May LM, Coulson EJ, Meunier FA, Osborne SL. Inhibition of PIKfyve by YM-201636 dysregulates autophagy and leads to apoptosis-independent neuronal cell death. PLoS One. 2013;8(3):e60152.

124. Min SH, Suzuki A, Stalker TJ, Zhao L, Wang Y, McKennan C, et al. Loss of PIKfyve in platelets causes a lysosomal disease leading to inflammation and thrombosis in mice. Nat Commun. 2014;5:4691.

125. Takasuga S, Sasaki T. Phosphatidylinositol-3,5-bisphosphate: metabolism and physiological functions. J Biochem. 2013;154(3):211-8.

126. Boccaccio A, Scholz-Starke J, Hamamoto S, Larisch N, Festa M, Gutla PV, et al. The phosphoinositide PI(3,5)P(2) mediates activation of mammalian but not plant TPC

proteins: functional expression of endolysosomal channels in yeast and plant cells. Cell Mol Life Sci. 2014;71(21):4275-83.

127. Bua DJ, Martin GM, Binda O, Gozani O. Nuclear phosphatidylinositol-5-phosphate regulates ING2 stability at discrete chromatin targets in response to DNA damage. Sci Rep. 2013;3:2137.

128. Jones DR, Bultsma Y, Keune WJ, Halstead JR, Elouarrat D, Mohammed S, et al. Nuclear PtdIns5P as a transducer of stress signaling: an in vivo role for PIP4Kbeta. Mol Cell. 2006;23(5):685-95.

129. Stijf-Bultsma Y, Sommer L, Tauber M, Baalbaki M, Giardoglou P, Jones DR, et al. The basal transcription complex component TAF3 transduces changes in nuclear phosphoinositides into transcriptional output. Mol Cell. 2015;58(3):453-67.

130. Sasaki T, Takasuga S, Sasaki J, Kofuji S, Eguchi S, Yamazaki M, et al. Mammalian phosphoinositide kinases and phosphatases. Prog Lipid Res. 2009;48(6):307-43.

131. Shisheva A, Sbrissa D, Ikonomov O. Plentiful PtdIns5P from scanty PtdIns(3,5)P2 or from ample PtdIns? PIKfyve-dependent models: Evidence and speculation (response to: DOI 10.1002/bies.201300012). Bioessays. 2015;37(3):267-77.

132. Botelho RJ, Efe JA, Teis D, Emr SD. Assembly of a Fab1 phosphoinositide kinase signaling complex requires the Fig4 phosphoinositide phosphatase. Mol Biol Cell. 2008;19(10):4273-86.

133. Dove SK, McEwen RK, Mayes A, Hughes DC, Beggs JD, Michell RH. Vac14 controls PtdIns(3,5)P(2) synthesis and Fab1-dependent protein trafficking to the multivesicular body. Curr Biol. 2002;12(11):885-93.

134. Alghamdi TA, Ho CY, Mrakovic A, Taylor D, Mao D, Botelho RJ. Vac14 protein multimerization is a prerequisite step for Fab1 protein complex assembly and function. J Biol Chem. 2013;288(13):9363-72.

135. Sbrissa D, Ikonomov OC, Fenner H, Shisheva A. ArPIKfyve homomeric and heteromeric interactions scaffold PIKfyve and Sac3 in a complex to promote PIKfyve activity and functionality. J Mol Biol. 2008;384(4):766-79.

136. Ikonomov OC, Sbrissa D, Fenner H, Shisheva A. PIKfyve-ArPIKfyve-Sac3 core complex: contact sites and their consequence for Sac3 phosphatase activity and endocytic membrane homeostasis. J Biol Chem. 2009;284(51):35794-806.

137. Sbrissa D, Ikonomov OC, Fu Z, Ijuin T, Gruenberg J, Takenawa T, et al. Core protein machinery for mammalian phosphatidylinositol 3,5-bisphosphate synthesis and turnover that regulates the progression of endosomal transport. Novel Sac phosphatase joins the ArPIKfyve-PIKfyve complex. J Biol Chem. 2007;282(33):23878-91.

138. Zolov SN, Bridges D, Zhang Y, Lee WW, Riehle E, Verma R, et al. In vivo, Pikfyve generates PI(3,5)P2, which serves as both a signaling lipid and the major precursor for PI5P. Proc Natl Acad Sci U S A. 2012;109(43):17472-7.

139. Ikonomov OC, Fligger J, Sbrissa D, Dondapati R, Mlak K, Deeb R, et al. Kinesin adapter JLP links PIKfyve to microtubule-based endosome-to-trans-Golgi network traffic of furin. J Biol Chem. 2009;284(6):3750-61.

140. Ikonomov OC, Sbrissa D, Mlak K, Deeb R, Fligger J, Soans A, et al. Active PIKfyve associates with and promotes the membrane attachment of the late endosome-to-trans-Golgi network transport factor Rab9 effector p40. J Biol Chem. 2003;278(51):50863-71.

141. Currinn H, Guscott B, Balklava Z, Rothnie A, Wassmer T. APP controls the formation of PI(3,5)P(2) vesicles through its binding of the PIKfyve complex. Cell Mol Life Sci. 2016;73(2):393-408.

142. Currinn H, Wassmer T. The amyloid precursor protein (APP) binds the PIKfyve complex and modulates its function. Biochem Soc Trans. 2016;44(1):185-90.

143. Shisheva A, Sbrissa D, Ikonomov O. Cloning, characterization, and expression of a novel Zn2+-binding FYVE finger-containing phosphoinositide kinase in insulin-sensitive cells. Mol Cell Biol. 1999;19(1):623-34.

144. Cabezas A, Pattni K, Stenmark H. Cloning and subcellular localization of a human phosphatidylinositol 3-phosphate 5-kinase, PIKfyve/Fab1. Gene. 2006;371(1):34-41.

145. Sbrissa D, Ikonomov OC, Shisheva A. Phosphatidylinositol 3-phosphate-interacting domains in PIKfyve. Binding specificity and role in PIKfyve. Endomenbrane localization. J Biol Chem. 2002;277(8):6073-9.

146. Sbrissa D, Ikonomov OC, Shisheva A. PIKfyve, a mammalian ortholog of yeast Fab1p lipid kinase, synthesizes 5-phosphoinositides. Effect of insulin. J Biol Chem. 1999;274(31):21589-97.

147. Shisheva A. PIKfyve: Partners, significance, debates and paradoxes. Cell Biol Int. 2008;32(6):591-604.

148. Hornbeck PV, Zhang B, Murray B, Kornhauser JM, Latham V, Skrzypek E. PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. Nucleic Acids Res. 2015;43(Database issue):D512-20.

149. Hill EV, Hudson CA, Vertommen D, Rider MH, Tavare JM. Regulation of PIKfyve phosphorylation by insulin and osmotic stress. Biochem Biophys Res Commun. 2010;397(4):650-5.

150. Liu Y, Lai YC, Hill EV, Tyteca D, Carpentier S, Ingvaldsen A, et al. Phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) is an AMPK target participating in contraction-stimulated glucose uptake in skeletal muscle. Biochem J. 2013;455(2):195-206.

151. Er EE, Mendoza MC, Mackey AM, Rameh LE, Blenis J. AKT facilitates EGFR trafficking and degradation by phosphorylating and activating PIKfyve. Sci Signal. 2013;6(279):ra45.

152. Sbrissa D, Ikonomov OC, Strakova J, Shisheva A. Role for a novel signaling intermediate, phosphatidylinositol 5-phosphate, in insulin-regulated F-actin stress fiber breakdown and GLUT4 translocation. Endocrinology. 2004;145(11):4853-65.

153. Ikonomov OC, Sbrissa D, Ijuin T, Takenawa T, Shisheva A. Sac3 is an insulin-regulated phosphatidylinositol 3,5-bisphosphate phosphatase: gain in insulin responsiveness through Sac3 down-regulation in adipocytes. J Biol Chem. 2009;284(36):23961-71.

154. Zhang Y, Zolov SN, Chow CY, Slutsky SG, Richardson SC, Piper RC, et al. Loss of Vac14, a regulator of the signaling lipid phosphatidylinositol 3,5-bisphosphate, results in neurodegeneration in mice. Proc Natl Acad Sci U S A. 2007;104(44):17518-23.

155. Lenk GM, Ferguson CJ, Chow CY, Jin N, Jones JM, Grant AE, et al. Pathogenic mechanism of the FIG4 mutation responsible for Charcot-Marie-Tooth disease CMT4J. PLoS Genet. 2011;7(6):e1002104.

156. Schulze U, Vollenbroker B, Braun DA, Van Le T, Granado D, Kremerskothen J, et al. The Vac14-interaction network is linked to regulators of the endolysosomal and autophagic pathway. Mol Cell Proteomics. 2014;13(6):1397-411.

157. UniProt: a hub for protein information. Nucleic Acids Res. 2015;43(Database issue):D204-12.

158. Lenk GM, Frei CM, Miller AC, Wallen RC, Mironova YA, Giger RJ, et al. Rescue of neurodegeneration in the Fig4 null mouse by a catalytically inactive FIG4 transgene. Hum Mol Genet. 2016;25(2):340-7.

159. Bajaj Pahuja K, Wang J, Blagoveshchenskaya A, Lim L, Madhusudhan MS, Mayinger P, et al. Phosphoregulatory protein 14-3-3 facilitates SAC1 transport from the endoplasmic reticulum. Proc Natl Acad Sci U S A. 2015;112(25):E3199-206.

160. Mesmin B, Bigay J, Moser von Filseck J, Lacas-Gervais S, Drin G, Antonny B. A four-step cycle driven by PI(4)P hydrolysis directs sterol/PI(4)P exchange by the ER-Golgi tether OSBP. Cell. 2013;155(4):830-43.

161. Hsu F, Hu F, Mao Y. Spatiotemporal control of phosphatidylinositol 4-phosphate by Sac2 regulates endocytic recycling. J Cell Biol. 2015;209(1):97-110.

162. Lindskog C. The potential clinical impact of the tissue-based map of the human proteome. Expert Rev Proteomics. 2015;12(3):213-5.

163. Kim GH, Dayam RM, Prashar A, Terebiznik M, Botelho RJ. PIKfyve inhibition interferes with phagosome and endosome maturation in macrophages. Traffic. 2014;15(10):1143-63.

164. Lin-Moshier Y, Keebler MV, Hooper R, Boulware MJ, Liu X, Churamani D, et al. The Two-pore channel (TPC) interactome unmasks isoform-specific roles for TPCs in endolysosomal morphology and cell pigmentation. Proc Natl Acad Sci U S A. 2014;111(36):13087-92.

165. Dong XP, Shen D, Wang X, Dawson T, Li X, Zhang Q, et al. PI(3,5)P(2) controls membrane trafficking by direct activation of mucolipin Ca(2+) release channels in the endolysosome. Nat Commun. 2010;1:38.

166. Li X, Saitoh S, Shibata T, Tanimura N, Fukui R, Miyake K. Mucolipin 1 positively regulates TLR7 responses in dendritic cells by facilitating RNA transportation to lysosomes. Int Immunol. 2015;27(2):83-94.

167. de Lartigue J, Polson H, Feldman M, Shokat K, Tooze SA, Urbe S, et al. PIKfyve regulation of endosome-linked pathways. Traffic. 2009;10(7):883-93.

168. Nicot AS, Fares H, Payrastre B, Chisholm AD, Labouesse M, Laporte J. The phosphoinositide kinase PIKfyve/Fab1p regulates terminal lysosome maturation in Caenorhabditis elegans. Mol Biol Cell. 2006;17(7):3062-74.

169. Cai X, Xu Y, Cheung AK, Tomlinson RC, Alcazar-Roman A, Murphy L, et al. PIKfyve, a class III PI kinase, is the target of the small molecular IL-12/IL-23 inhibitor apilimod and a player in Toll-like receptor signaling. Chem Biol. 2013;20(7):912-21.

170. Rutherford AC, Traer C, Wassmer T, Pattni K, Bujny MV, Carlton JG, et al. The mammalian phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) regulates endosome-to-TGN retrograde transport. J Cell Sci. 2006;119(Pt 19):3944-57.

171. Chow CY, Zhang Y, Dowling JJ, Jin N, Adamska M, Shiga K, et al. Mutation of FIG4 causes neurodegeneration in the pale tremor mouse and patients with CMT4J. Nature. 2007;448(7149):68-72.

172. Chow CY, Landers JE, Bergren SK, Sapp PC, Grant AE, Jones JM, et al. Deleterious variants of FIG4, a phosphoinositide phosphatase, in patients with ALS. Am J Hum Genet. 2009;84(1):85-8.

173. Zhang X, Chow CY, Sahenk Z, Shy ME, Meisler MH, Li J. Mutation of FIG4 causes a rapidly progressive, asymmetric neuronal degeneration. Brain. 2008;131(Pt 8):1990-2001.

174. Takasuga S, Horie Y, Sasaki J, Sun-Wada GH, Kawamura N, Iizuka R, et al. Critical roles of type III phosphatidylinositol phosphate kinase in murine embryonic visceral endoderm and adult intestine. Proc Natl Acad Sci U S A. 2013;110(5):1726-31.

175. Ikonomov OC, Sbrissa D, Delvecchio K, Xie Y, Jin JP, Rappolee D, et al. The phosphoinositide kinase PIKfyve is vital in early embryonic development: preimplantation lethality of PIKfyve-/- embryos but normality of PIKfyve+/- mice. J Biol Chem. 2011;286(15):13404-13.

176. Gee JA, Frausto RF, Chung DW, Tangmonkongvoragul C, Le DJ, Wang C, et al. Identification of novel PIKFYVE gene mutations associated with Fleck corneal dystrophy. Mol Vis. 2015;21:1093-100.

177. Li S, Tiab L, Jiao X, Munier FL, Zografos L, Frueh BE, et al. Mutations in PIP5K3 are associated with Francois-Neetens mouchetee fleck corneal dystrophy. Am J Hum Genet. 2005;77(1):54-63.

178. Kotoulas A, Kokotas H, Kopsidas K, Droutsas K, Grigoriadou M, Bajrami H, et al. A novel PIKFYVE mutation in fleck corneal dystrophy. Mol Vis. 2011;17:2776-81.

179. Nakajima J, Okamoto N, Shiraishi J, Nishimura G, Nakashima M, Tsurusaki Y, et al. Novel FIG4 mutations in Yunis-Varon syndrome. J Hum Genet. 2013;58(12):822-4.

180. Campeau PM, Lenk GM, Lu JT, Bae Y, Burrage L, Turnpenny P, et al. Yunis-Varon syndrome is caused by mutations in FIG4, encoding a phosphoinositide phosphatase. Am J Hum Genet. 2013;92(5):781-91.

181. Baulac S, Lenk GM, Dufresnois B, Ouled Amar Bencheikh B, Couarch P, Renard J, et al. Role of the phosphoinositide phosphatase FIG4 gene in familial epilepsy with polymicrogyria. Neurology. 2014;82(12):1068-75.

182. Jin N, Lang MJ, Weisman LS. Phosphatidylinositol 3,5-bisphosphate: regulation of cellular events in space and time. Biochem Soc Trans. 2016;44(1):177-84.

183. Han BK, Emr SD. Phosphoinositide [PI(3,5)P2] lipid-dependent regulation of the general transcriptional regulator Tup1. Genes Dev. 2011;25(9):984-95.

184. Li X, Wang X, Zhang X, Zhao M, Tsang WL, Zhang Y, et al. Genetically encoded fluorescent probe to visualize intracellular phosphatidylinositol 3,5-bisphosphate localization and dynamics. Proc Natl Acad Sci U S A. 2013;110(52):21165-70.

185. Carlton J, Bujny M, Peter BJ, Oorschot VM, Rutherford A, Mellor H, et al. Sorting nexin-1 mediates tubular endosome-to-TGN transport through coincidence sensing of high- curvature membranes and 3-phosphoinositides. Curr Biol. 2004;14(20):1791-800.

186. Jin N, Mao K, Jin Y, Tevzadze G, Kauffman EJ, Park S, et al. Roles for PI(3,5)P2 in nutrient sensing through TORC1. Mol Biol Cell. 2014;25(7):1171-85.

187. Feng X, Huang Y, Lu Y, Xiong J, Wong CO, Yang P, et al. Drosophila TRPML forms PI(3,5)P2-activated cation channels in both endolysosomes and plasma membrane. J Biol Chem. 2014;289(7):4262-72.

188. Whitley P, Reaves BJ, Hashimoto M, Riley AM, Potter BV, Holman GD. Identification of mammalian Vps24p as an effector of phosphatidylinositol 3,5-bisphosphate-dependent endosome compartmentalization. J Biol Chem. 2003;278(40):38786-95.

189. Odorizzi G, Babst M, Emr SD. Fab1p PtdIns(3)P 5-kinase function essential for protein sorting in the multivesicular body. Cell. 1998;95(6):847-58.

190. McCartney AJ, Zhang Y, Weisman LS. Phosphatidylinositol 3,5-bisphosphate: low abundance, high significance. Bioessays. 2014;36(1):52-64.

191. Kerr MC, Wang JT, Castro NA, Hamilton NA, Town L, Brown DL, et al. Inhibition of the PtdIns(5) kinase PIKfyve disrupts intracellular replication of Salmonella. EMBO J. 2010;29(8):1331-47.

192. Cheng X, Shen D, Samie M, Xu H. Mucolipins: Intracellular TRPML1-3 channels. FEBS Lett. 2010;584(10):2013-21.

193. Puertollano R, Kiselyov K. TRPMLs: in sickness and in health. Am J Physiol Renal Physiol. 2009;296(6):F1245-54.

194. Wang X, Zhang X, Dong XP, Samie M, Li X, Cheng X, et al. TPC proteins are phosphoinositide- activated sodium-selective ion channels in endosomes and lysosomes. Cell. 2012;151(2):372-83.

195. Bellono NW, Escobar IE, Oancea E. A melanosomal two-pore sodium channel regulates pigmentation. Sci Rep. 2016;6:26570.

196. Ambrosio AL, Boyle JA, Aradi AE, Christian KA, Di Pietro SM. TPC2 controls pigmentation by regulating melanosome pH and size. Proc Natl Acad Sci U S A. 2016;113(20):5622-7.

197. Sakurai Y, Kolokoltsov AA, Chen CC, Tidwell MW, Bauta WE, Klugbauer N, et al. Ebola virus. Two-pore channels control Ebola virus host cell entry and are drug targets for disease treatment. Science. 2015;347(6225):995-8.

198. Montell C. The TRP superfamily of cation channels. Sci STKE. 2005;2005(272):re3.

199. Hu Y, Carraro-Lacroix LR, Wang A, Owen C, Bajenova E, Corey PN, et al. Lysosomal pH Plays a Key Role in Regulation of mTOR Activity in Osteoclasts. J Cell Biochem. 2016;117(2):413-25.

200. Schaletzky J, Dove SK, Short B, Lorenzo O, Clague MJ, Barr FA. Phosphatidylinositol-5-phosphate activation and conserved substrate specificity of the myotubularin phosphatidylinositol 3-phosphatases. Curr Biol. 2003;13(6):504-9.

201. Kim SA, Taylor GS, Torgersen KM, Dixon JE. Myotubularin and MTMR2, phosphatidylinositol 3-phosphatases mutated in myotubular myopathy and type 4B Charcot-Marie-Tooth disease. J Biol Chem. 2002;277(6):4526-31.

202. Blondeau F, Laporte J, Bodin S, Superti-Furga G, Payrastre B, Mandel JL. Myotubularin, a phosphatase deficient in myotubular myopathy, acts on phosphatidylinositol 3-kinase and phosphatidylinositol 3-phosphate pathway. Hum Mol Genet. 2000;9(15):2223-9.

203. Yoo KY, Son JY, Lee JU, Shin W, Im DW, Kim SJ, et al. Structure of the catalytic phosphatase domain of MTMR8: implications for dimerization, membrane association and reversible oxidation. Acta Crystallogr D Biol Crystallogr. 2015;71(Pt 7):1528-39.

204. Bong SM, Son KB, Yang SW, Park JW, Cho JW, Kim KT, et al. Crystal Structure of Human Myotubularin-Related Protein 1 Provides Insight into the Structural Basis of Substrate Specificity. PLoS One. 2016;11(3):e0152611.

205. Walker DM, Urbe S, Dove SK, Tenza D, Raposo G, Clague MJ. Characterization of MTMR3. an inositol lipid 3-phosphatase with novel substrate specificity. Curr Biol. 2001;11(20):1600-5.

206. Berger P, Schaffitzel C, Berger I, Ban N, Suter U. Membrane association of myotubularin-related protein 2 is mediated by a pleckstrin homology-GRAM domain and a coiled-coil dimerization module. Proc Natl Acad Sci U S A. 2003;100(21):12177-82.

207. Shen J, Yu WM, Brotto M, Scherman JA, Guo C, Stoddard C, et al. Deficiency of MIP/MTMR14 phosphatase induces a muscle disorder by disrupting Ca(2+) homeostasis. Nat Cell Biol. 2009;11(6):769-76.

208. Ikonomov OC, Sbrissa D, Venkatareddy M, Tisdale E, Garg P, Shisheva A. Class III PI 3-kinase is the main source of PtdIns3P substrate and membrane recruitment signal for PIKfyve constitutive function in podocyte endomembrane homeostasis. Biochim Biophys Acta. 2015;1853(5):1240-50.

209. Hnia K, Vaccari I, Bolino A, Laporte J. Myotubularin phosphoinositide phosphatases: cellular functions and disease pathophysiology. Trends Mol Med. 2012;18(6):317-27.

210. Kioschis P, Wiemann S, Heiss NS, Francis F, Gotz C, Poustka A, et al. Genomic organization of a 225-kb region in Xq28 containing the gene for X-linked myotubular myopathy (MTM1) and a related gene (MTMR1). Genomics. 1998;54(2):256-66.

211. Vaccari I, Dina G, Tronchere H, Kaufman E, Chicanne G, Cerri F, et al. Genetic interaction between MTMR2 and FIG4 phospholipid phosphatases involved in Charcot-Marie-Tooth neuropathies. PLoS Genet. 2011;7(10):e1002319.

212. Robinson FL, Dixon JE. The phosphoinositide-3-phosphatase MTMR2 associates with MTMR13, a membrane-associated pseudophosphatase also mutated in type 4B Charcot-Marie-Tooth disease. J Biol Chem. 2005;280(36):31699-707.

213. Bolino A, Bolis A, Previtali SC, Dina G, Bussini S, Dati G, et al. Disruption of Mtmr2 produces CMT4B1-like neuropathy with myelin outfolding and impaired spermatogenesis. J Cell Biol. 2004;167(4):711-21.

214. Berger P, Tersar K, Ballmer-Hofer K, Suter U. The CMT4B disease-causing proteins MTMR2 and MTMR13/SBF2 regulate AKT signalling. J Cell Mol Med. 2011;15(2):307-15.

215. Bolis A, Coviello S, Visigalli I, Taveggia C, Bachi A, Chishti AH, et al. Dlg1, Sec8, and Mtmr2 regulate membrane homeostasis in Schwann cell myelination. J Neurosci. 2009;29(27):8858-70.

216. Tsujita K, Itoh T, Ijuin T, Yamamoto A, Shisheva A, Laporte J, et al. Myotubularin regulates the function of the late endosome through the gram domain-phosphatidylinositol 3,5-bisphosphate interaction. J Biol Chem. 2004;279(14):13817-24.

217. Ikonomov OC, Sbrissa D, Mlak K, Kanzaki M, Pessin J, Shisheva A. Functional dissection of lipid and protein kinase signals of PIKfyve reveals the role of PtdIns 3,5-P2 production for endomembrane integrity. J Biol Chem. 2002;277(11):9206-11.

218. Ungewickell A, Hugge C, Kisseleva M, Chang SC, Zou J, Feng Y, et al. The identification and characterization of two phosphatidylinositol-4,5-bisphosphate 4-phosphatases. Proc Natl Acad Sci U S A. 2005;102(52):18854-9.

219. Grainger DL, Tavelis C, Ryan AJ, Hinchliffe KA. Involvement of phosphatidylinositol 5-phosphate in insulin-stimulated glucose uptake in the L6 myotube model of skeletal muscle. Pflugers Arch. 2011;462(5):723-32.

220. Droubi A, Bulley SJ, Clarke JH, Irvine RF. Nuclear localizations of phosphatidylinositol 5-phosphate 4-kinases alpha and beta are dynamic and independently regulated during starvation-induced stress. Biochem J. 2016;473(14):2155-63.

221. Shim H, Wu C, Ramsamooj S, Bosch KN, Chen Z, Emerling BM, et al. Deletion of the gene Pip4k2c, a novel phosphatidylinositol kinase, results in hyperactivation of the immune system. Proc Natl Acad Sci U S A. 2016;113(27):7596-601.

222. Clarke JH, Irvine RF. Evolutionarily conserved structural changes in phosphatidylinositol 5-phosphate 4-kinase (PI5P4K) isoforms are responsible for differences in enzyme activity and localization. Biochemical Journal. 2013;454:49-57.

223. Clarke JH, Giudici ML, Burke JE, Williams RL, Maloney DJ, Marugan J, et al. The function of phosphatidylinositol 5-phosphate 4-kinase gamma (PI5P4K gamma) explored using a specific inhibitor that targets the PI5P-binding site. Biochemical Journal. 2015;466:359-67.

224. Giudici ML, Clarke JH, Irvine RF. Phosphatidylinositol 5-phosphate 4-kinase gamma (PI5P4Kgamma), a lipid signalling enigma. Adv Biol Regul. 2016;61:47-50.

225. Clarke JH, Giudici ML, Burke JE, Williams RL, Maloney DJ, Marugan J, et al. The function of phosphatidylinositol 5-phosphate 4-kinase gamma (PI5P4Kgamma) explored using a specific inhibitor that targets the PI5P-binding site. Biochem J. 2015;466(2):359-67.

226. Xu H, Delling M, Li L, Dong X, Clapham DE. Activating mutation in a mucolipin transient receptor potential channel leads to melanocyte loss in varitint-waddler mice. Proc Natl Acad Sci U S A. 2007;104(46):18321-6.

227. Sulem P, Gudbjartsson DF, Stacey SN, Helgason A, Rafnar T, Jakobsdottir M, et al. Two newly identified genetic determinants of pigmentation in Europeans. Nat Genet. 2008;40(7):835-7.

Figure 1.1: Graphical Summary of Melanogenesis. Melanogenesis is an intricate process that is subject to complex regulation. UV radiation stimulates keratinocytes to produce αMSH and other factors. αMSH activates a signaling kinase cascade leading to the transcription of MITF-M. Stem Cell Factor activates MITF-M to drive transcription of several melanosomal factors. These factors are processed and several are packaged into AP-clatharin coated vesicles and trafficked to the developing melanosome. MART-1, PMEL, and OA1 are trafficked to developing endosomes / stage I melanosomes. Acidification of stage I melanosomes induces proteolytic cleavage of PMEL. The PMEL fragments are polymerized into extend striations resulting in an elongated morphology associated with stage II melanosomes. TYR and other factors are delivered to stage II melanosomes. Deacidification and the initiation of melanin synthesis defines the third stage in melanosome development. Upon complete melanization, the mature, stage IV, melanosomes are transferred to keratinocytes where they surround the nucleus to protect from UV-induced DNA damage.

Figure 1.1

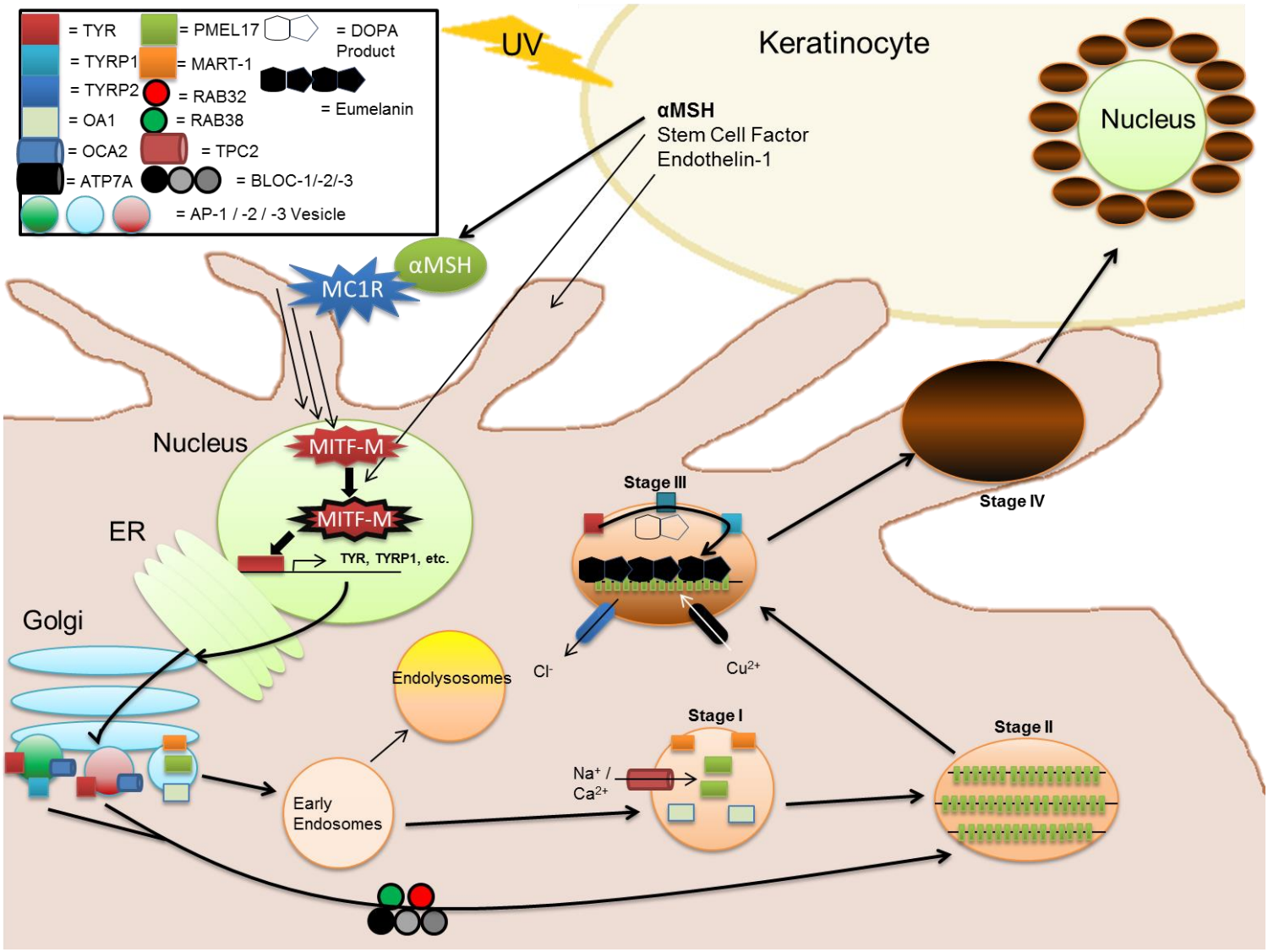
= TYR
= TYRP1
= TYRP2
= OA1
= OCA2
= ATP7A
= PMEL17
= MART-1
= RAB32
= RAB38
= TPC2
= BLOC-1/-2/-3
= AP-1 / -2 / -3 Vesicle
= DOPA Product
= Eumelanin
UV
Keratinocyte
αMSH
Stem Cell Factor
Endothelin-1
Nucleus
αMSH
MC1R
Nucleus
MITF-M
MITF-M
TYR, TYRP1, etc.
ER
Golgi
Stage III
Stage IV
Cl-
Cu2+
Endolysosomes
Stage I
Stage II
Na+ / Ca2+
Early Endosomes

Figure 1.2: Autophagy factors are involved in melanosome biogenesis. A) Autophagy factors catalyze LC3 lipidation. LC3-II then coats the outer membranes of both autophagosomes and melanosomes. B) The autophagy factor, WIPI1, modulates mTORC1 activity leading to stabilization of β-Catenin and increased MITF-M transcription. MITF-M can then bind to consensus sequences associated with melanosomal factors and drive their transcription.

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Figure 1.2

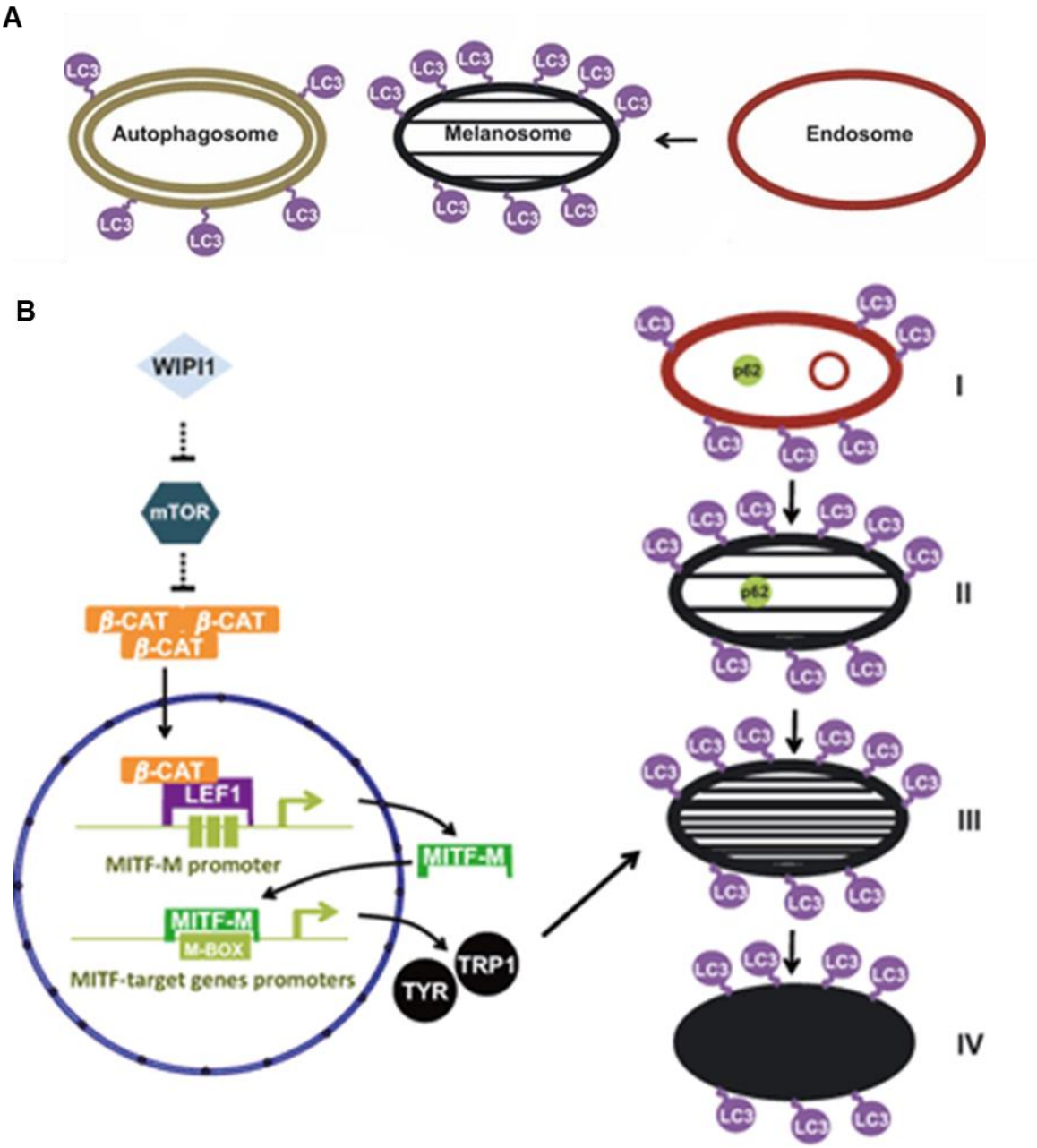
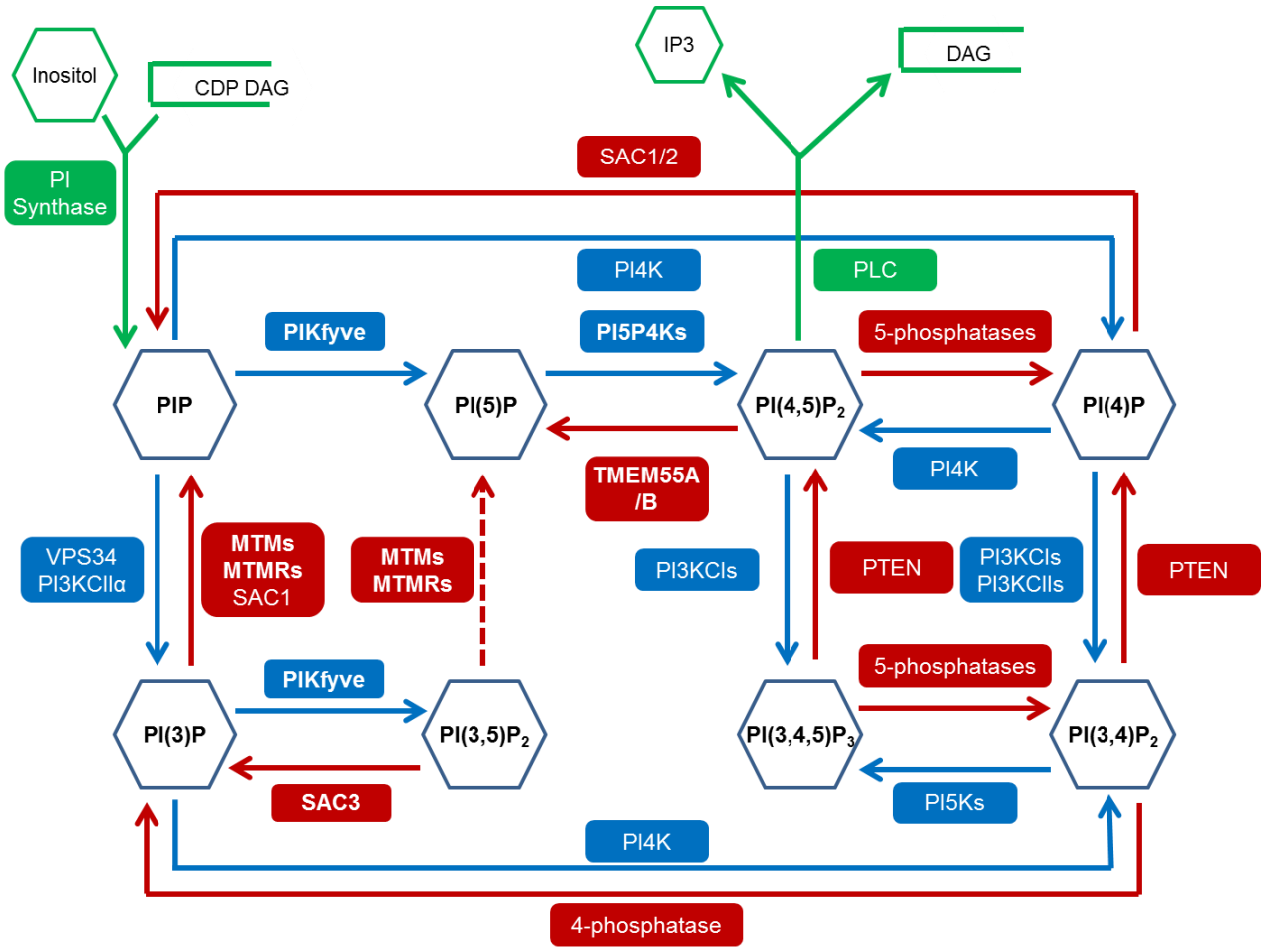

A
LC3
Autophagosome
Melanosome
Endosome
B
WIPI1
mTOR
β-CAT
β-CAT
β-CAT
β-CAT
LEF1
MITF-M promoter
MITF-M
MITF-M
M-BOX
MITF-target genes promoters
TYR
TRP1
p62
I
II
III
IV

Figure 1.3: Phosphoinositide Biosynthetic Pathway.

Bold texted enzymes are actively discussed. Highlighted in red are phosphatases and associated pathways. Kinases and associated pathways are highlighted in green. Pathways that mediate the synthesis or breakdown of phosphoinositides are highlighted in green. PIKfyve mediates the conversion of PIP to PI(5)P as well as the conversion of PI(3)P to $PI(3,5)P_2$. $PI(3,5)P_2$ can be dephosphorylated by the MTM/MTMR phosphatase family to produce PI(5)P. Dephosphorylation of $PI(4,5)P_2$ can contribute to the PI(5)P pool as well. PI(5)P can be phosphorylated to $PI(4,5)P_2$ which then feeds into second PI-biosynthetic pathway.

Figure 1.3


Inositol
CDP DAG
PI Synthase
IP3
DAG
SAC1/2
PI4K
PLC
PIKfyve
PI5P4Ks
5-phosphatases
PIP
PI(5)P
PI(4,5)P2
PI(4)P
TMEM55A /B
PI4K
VPS34 PI3KCIIα
MTMs MTMRs SAC1
MTMs MTMRs
PI3KCIs
PTEN
PI3KCIs PI3KCIIs
PTEN
PIKfyve
5-phosphatases
PI(3)P
PI(3,5)P2
PI(3,4,5)P3
PI(3,4)P2
SAC3
PI5Ks
PI4K
4-phosphatase

PIKfyve Regulates Melanogenesis Through the Production of PI(5)P and PI(3,5)P_2

Chapter 2

Marc C. Liggins, Jessica L. Flesher, Sohail Jahid, Smanpreet Sandhu, Victoria Eby, Shunsuke Takasuga, Junko Sasaki, Takehiko Sasaki, Raymond E. Boissy, and Anand K. Ganesan

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2.1 Abstract

PIKfyve, VAC14, and SAC3 form a complex that catalyzes the production of PI(3,5)P_2 which can be converted to PI(5)P. While disruption of this complex leads to defects in vesicle trafficking and organelle biogenesis, the precise roles of PI(3,5)P_2 and PI(5)P in these processes is unknown. In this study, we used melanogenesis as a model system to dissect the contributions of individual phosphoinositides to organelle biogenesis. Melanocyte-specific PIKfyve knockout mice exhibit greying of the mouse coat, indicating that PIKfyve deletion modulates melanogenesis *in vivo*. PIKfyve inhibition blocked melanosome and lysosome maturation inducing cell death *in vitro*, phenotypes which could be rescued by the addition of PI(3,5)P_2. Partial depletion of PIKfyve in melanocytes downregulated the expression of tyrosinase and MITF, while addition of PI(5)P induced tyrosinase expression. Taken together, these studies define distinct roles for PI(5)P and PI(3,5)P_2 in regulating transcription and vesicle trafficking, respectively.

2.2 Author Summary

Transcription and vesicle trafficking must be coordinated in both time and space to precisely deliver proteins to the correct cellular organelle. In this study, we used melanosome biogenesis as a model system to identify signaling pathways that coordinate vesicle trafficking with transcriptional events. We identify PI(5)P and $PI(3,5)P_2$ as critical regulators of transcription and vesicle trafficking, defining a PIKfyve complex that can coordinately regulate organelle biogenesis.

2.3 Introduction

Melanin, a pigment produced within uveal and epidermal melanocytes (1), absorbs UV radiation, protecting the eyes and skin from UV-induced DNA damage (2). As melanin synthesis generates a significant amount of reactive oxygen species (ROS) (3, 4), eukaryotic cells have evolved precise mechanisms to sequester melanin precursors and ROS within the melanosome. The melanosome is a lysosome-related organelle that develops through four distinct stages that are readily distinguishable by electron microscopy (5-7). Several human monoallelic disorders that present with hypopigmentation also have deficits in the biogenesis of lysosomes and lysosome-related organelles (8), highlighting the utility of melanosome biogenesis as a model system to define pathways that regulate organelle biogenesis (9, 10).

The complex process of melanosome biogenesis initiates when specialized early endosomes bud off into spherical vacuoles known as stage I melanosomes, marked by the presence of melanoma antigen recognized by T-cells 1 (MART-1) and the premelanosome protein (PMEL) (5, 11, 12). Cleavage and polymerization of PMEL marks the transition to stage II melanosomes (12) where PMEL fibrils serve as scaffold for melanin polymerization and deposition (7). Tyrosinase (TYR), and tyrosinase-related proteins 1 and 2 (TYRP1 & TYRP2) are three key enzymes involved in producing melanin (7). These proteins are glycosylated within the Golgi and packaged into adaptor protein-3 (AP-3) or -1 (AP-1) clatharin coated transport vesicles, which are transported to and fuse with stage II melanosomes (13). The initiation of tyrosinase enzymatic activity allows for the production of melanin, which is then deposited onto PMEL fibrils in

stage III melanosomes (7, 14). Mature, stage IV melanosomes are highly pigmented vesicles, which are opaque structures on electron microscopy filled with electron dense melanin (7, 15). Finally, mature melanosomes are transferred to neighboring keratinocytes (14, 16) through a process that is poorly understood.

A number of studies have focused on determining how proteins are precisely delivered to the melanosome. Consensus sequences on TYR and TYRP1 are recognized by conserved sites on AP complexes (6, 13, 17), which then assist in sorting and packaging TYR and TYRP1 into AP-1 and AP-3 clathrin coated vesicles for delivery to stage II melanosomes (6, 13). While these motifs are required for trafficking they do not delineate specificity- when expressed in non-melanocytes these motifs target TYR and TYRP1 to late endosomes or lysosomes (18, 19). AP complexes work in conjunction with Rab32, Rab38, and biogenesis of lysosome-related organelles complex (BLOC) -1, -2, and -3 complexes to mediate vesicle trafficking of TYR, TYRP1, and TYRP2 from early endosomes to maturing stage II melanosomes (6, 20-23). These trafficking pathways are not specific to the melanosome as they are involved in both lysosome and lysosome-related organelle trafficking (20). While both the BLOC trafficking machinery and AP complexes are critical to deliver proteins to the melanosome, it is clear that other factors must also be involved to direct AP-coated vesicles to the right location.

In an effort to identify proteins that convey specificity for melanosome delivery, our lab conducted a genome wide RNAi screen, which identified 94 novel positive regulators of melanogenesis including several autophagy genes (24). Other studies have identified several autophagy proteins, which impact melanogenesis via a pathway

that is distinct from canonical autophagy (25-28). WIPI1 and WIPI2, human homologues to the yeast protein ATG18 (29, 30), were among the autophagy proteins identified in the screen as novel regulators of melanogenesis (24). WIPI1 and WIPI2 possess a phosphoinositide (PI) binding domain that allow them to bind to PI(3)P, PI(3,5)P_2, and PI(5)P (31, 32). Phosphoinositides have been implicated in controlling the fusion of transport vesicles with its desired target membrane (33, 34). Knockdown of WIPI1 was found to effect the transcription of melanosomal proteins and disrupt vesicular trafficking, leading to accumulation of “vacuole-like” aberrant vesicles in melanocytes (24, 25). Interestingly, knockout of the WIPI1/2 yeast homologue ATG18 also results in vacuolization (35) similar to that observed with WIPI1 (24, 25). ATG18 (the yeast WIPI homologue) can form a regulatory complex with a yeast PI5K kinase that generates PI(3,5)P_2 (36). A similar complex between PIKfyve (the only mammalian PI5K kinase) and WIPI1/2 has not been identified. In mammalian cells, PIKfyve forms a complex with SAC3 and VAC14 (36, 37) and converts PI(3)P to PI(3,5)P_2 (38) and PIP to PI(5)P (39, 40). Intriguingly, SAC3 (41, 42) and VAC14 (36) mutant mice exhibit vesicle trafficking defects in both the central nervous system (CNS) and have noted coat color defects, implicating a role for the PIKfyve protein complex in both vesicle trafficking and organelle biogenesis.

While published studies have revealed that PIKfyve complex mutants have macroscopic coat color defects (36, 41, 42), it is currently unclear how the PIKfyve complex regulates melanogenesis at the molecular level or which phosphoinositides are involved in this process. In this study, we examined the role of PIKfyve and the PIKfyve complex in regulating melanogenesis. We determined that complete inhibition of

PIKfyve blocks melanosome formation while partial suppression of PIKfyve activity blocks the accumulation of melanosome proteins via a mechanism involving the phosphoinositide PI(5)P. Taken together, these studies define a novel role for phosphoinositides in coordinating transcription and vesicle trafficking events required for organelle biogenesis.

2.4 Results

Loss of PIKfyve leads to pigment loss *in vivo*

PIKfyve forms a complex with VAC14 and SAC3 (36, 37) which then phosphorylates PI(3)P to PI(3,5)P_2 (38) and PIP to PI(5)P (39, 40). An intact ternary complex consisting of PIKfyve, VAC14 and SAC3 is required for PIKfyve enzymatic activity (37). Published studies have shown that the PIKfyve complex is necessary for cell function and survival in different cell types (38, 43, 44), however its role in melanocytes has not been well characterized. PIKfyve knockout mice die during embryonic development (38), making it difficult to assess the effects of PIKfyve on melanogenesis. However, VAC14 and SAC3 mutants not only are characterized by early lethality and accumulation of vacuoles in the CNS but also by coat color defects (36, 41, 42). To better elucidate the role of the PIKfyve complex in melanogenesis, we generated melanocyte-specific inducible PIKfyve knockout mice by crossing an established *PIKFYVE*$^{Flox/Flox}$ strain (43) with an established melanocyte-specific, inducible Cre strain under a tyrosinase promoter (45), both generated on a pure C57/B6 background. The resulting *TyrCreER*^{T2} *PIKFYVE*$^{Flox/Flox}$ mice were administered tamoxifen-containing chow for 29 days beginning at P21 (Figure 1A) to induce Cre-mediated excision of the PIKfyve allele. Mice were photographed (Figure S1A), shaved and depilated at approximately P35 and hairs were allowed to regrow. *TyrCreER*^{T2} *PIKFYVE*$^{Flox/Flox}$ mice that were fed tamoxifen for one month resulted in the accumulation of numerous white hairs that were visually apparent at P85 (Figure 1B,

Figure S1B). The same phenotype was not observed in Cre negative *PIKFYVE*$^{Flox/Flox}$ mice fed tamoxifen for one month or *TyrCreER*^{T2} *PIKFYVE*$^{Flox/Flox}$ mice that were administered a normal diet (Figure 1B, Figure S1B). Hair from the backs of experimental mice was solubilized and the relative accumulation of melanin in the hair was quantified using standard spectrophotometric methods (46, 47). Hairs from the *TyrCreER*^{T2} *PIKFYVE*$^{Flox/Flox}$ mice that were fed tamoxifen accumulated 50% less melanin as compared to mice that were not fed tamoxifen or Cre negative controls (Figure 1C). To better assess whether this phenotype was exclusively related to an effect on melanogenesis, we allowed the hairs to regrow after shave depilation and observed whether there was an increased accumulation of white hair post removal of tamoxifen feed. More white hairs were visually apparent after the mice were fed a normal diet for an additional 20 days (Figure 1D, Figure S1B & S1C). In addition, even less melanin accumulated in the hair of *PIKFYVE*$^{Flox/Flox}$ mice after they were taken off tamoxifen chow (Figure 1E), indicating that the phenotype was either a result of a profound effect of PIKfyve on melanogenesis or a result of stem cell loss. Skin biopsies were taken at p105 and stained with TRP2, a marker that can recognize melanocyte stem cells (48). PIKfyve deletion resulted in only a slight decrease of TRP2 staining in the hair follicle (Figure S1D), indicating that the observed phenotype was mostly due to a direct effect on melanogenesis, although there might be a more subtle secondary effect of the deletion on melanocyte stem cell survival. To determine whether PIKfyve deletion could inhibit the expression of melanogenesis genes, skin was harvested from mice at p50 before grey hairs were observed. Gene expression analysis indicated that mice that were fed tamoxifen for one month had decreased expression of TYR, TYRP1,

and TYRP2 (Figure 1F). Taken together, these studies demonstrate that like VAC14 and SAC3 knockout mice, knocking out PIKfyve specifically within melanocytes influences melanin accumulation within the hair *in vivo,* likely by directly modulating the expression of enzymes that synthesize melanin. These findings spurred us to pursue *in vitro* approaches to better dissect the effects of the PIKfyve complex on melanogenesis.

Pharmacologic inhibition of PIKfyve blocks melanosome maturation.

PIKfyve is responsible for generating all cellular $PI(3,5)P_2$ and most intracellular PI(5)P (38-40). Previous studies have determined that only a small amount of intracellular PIKfyve is required for normal cellular function, as near complete knockdown of PIKfyve was required to observe depletion of intracellular $PI(3,5)P_2$ and PI(5)P pools (38). In contrast, pharmacological inhibition of PIKfyve has been shown to potently and acutely block enzymatic activity (38, 49) and significantly reduce phosphoinositide levels (38). Our mouse studies revealed that complete loss of PIKfyve had effects on melanin accumulation (Figure 1). In order to better understand how PIKfyve directly modulates melanogenesis, we sought to examine the effects of acute inhibition of PIKfyve activity and partial depletion of PIKfyve on melanogenesis *in vitro*. MNT-1 melanoma cells that produce stage I-IV melanosomes *in vitro* were treated with two PIKfyve inhibitors, YM-201636 and apilimod, the latter of which has been noted for increased potency and specificity (50). MNT-1 cells treated with YM-201636 or apilimod accumulated less melanin as compared to vehicle treated melanocytes (Figure 2A, 2B), similar to what was observed in experimental mice (Figure 1). Once we determined that

pharmacologic inhibition of PIKfyve resulted in decreased melanin production, we sought to determine how acute inhibition of PIKfyve blocks melanogenesis. PIKfyve inhibition did not inhibit the accumulation of tyrosinase, but it did induce the accumulation of the stage II melanosome marker PMEL in inhibitor treated cells (Figure 2C). PMEL maturation requires acidic proteolytic cleavage prior to synthesis of polymeric fibrils that are required for melanin deposition (12). Intriguingly, the PIKfyve inhibitor prevented the normal processing of PMEL, as a greater ratio of full size PMEL was present in inhibitor treated cells (Figure 2C). PIKfyve inhibition also blocked the pH dependent cleavage of the lysosomal protein cathepsin D, leading to an increased accumulation of unprocessed species (Figure 2C). Interestingly, a similar increased accumulation of unprocessed cathepsin D was observed in PIKfyve knockout platelets (51). While it remains unclear if PIKfyve is necessary for the acidification of lysosomes, there is general consensus that PIKfyve controls lysosomal trafficking (49, 51). Taken together, our studies indicate that acute inhibition of PIKfyve blocks the maturation of proteins in both the melanosome (PMEL) and lysosome (cathepsin D).

To determine how PIKfyve regulates the formation of melanosomes, the effect of PIKfyve inhibition on melanosome biogenesis was assessed at the ultrastructural level. Established cultures of normal human melanocytes were treated with various dosages of YM-201636 (YM) and processed for routine and DOPA histochemistry electron microscopy. Low magnification images of YM-201636 (1000 nM) versus vehicle treated melanocytes demonstrated that YM treated cells were dramatically hypopigmented (Figure 3A). Upon DOPA incubation, melanosomes in the treated melanocytes contained melanin reaction product almost to the extent of control melanocytes (Figure

3A). However, the melanin deposition within melanosomes in treated melanocytes appeared irregular and less homogeneous. Higher magnification of vehicle versus YM treated cells demonstrated that within the Golgi zone, melanosomes of all stages existed in the control, whereas primarily Stage I and a few Stage II melanosomes existed in the YM treated melanocytes (Figure 3B). In regions lateral to the Golgi zone and within dendrites, all stages of melanosomes existed in the YM treated melanocytes as oppose to predominantly Stage IV in the control treated melanocytes (Figure 3B). Melanosome stages were quantified in all YM-201636 treated melanocytes. As the concentration of YM increased the melanosome profile increased in percent Stage I and concomitantly decreased in Stage 4 in a dose dependent manner (Figure 3C, Figure S2A).

Once we realized that the percentage of “Stage 1” melanosomes increased with YM treatment, these primitive organelles were subjected to further scrutiny. It has been demonstrated that tyrosinase exits the Golgi in 50 nm trafficking vesicles and en route to the Stage II melanosome enters the multivesicular body (MVB) and then rapidly is re-recruited by a complex containing AP-3 into vesicles that ultimately transports this cargo to Stage II melanosomes (22). Without DOPA incubation it is difficult to ultrastructurally discern these multivesicular bodies from Stage I melanosomes particularly when melanofibrils are not apparent. After DOPA incubation, the multivesicular bodies appear with DOPA reaction peripherally around their limiting membranes due to the fact that tyrosinase is a transmembrane enzyme with its catalytic carboxy end protruding into the lumen (Figure S2B). These MVBs can occasionally appear in the Golgi zone of control melanocytes however subjectively many more appear in the YM treated

melanocytes and possibly in the dendrites (Figure S2C). Melanosome density within both the cell body and the dendrites of control and YM treated melanocytes with and without DOPA treatment was quantified. There was a statistically significant increase in melanosome density in the cell body of YM treated melanocytes versus control in both the non-DOPA and DOPA treated group (Table S1 and S2). In contrast, no difference in melanosome density was observed in the dendrites. The density of 50 nm vesicles containing tyrosinase cargo was quantitated in DOPA processed melanocytes. In the Golgi area the density of DOPA positive 50 nm vesicles was significantly increased in the YM versus the vehicle treated melanocytes (Figure S2B), indicating the accumulation of stage I melanosomes.

Previous studies have indicated that stage I melanosomes containing MART-1 are primarily localized to the perinuclear region (52). Immunofluorescence microscopy revealed that MART-1 positive vesicles accumulate in PIKfyve inhibitor treated cells, suggesting that PIKfyve inhibition blocks the maturation of Stage I vesicles (Figure 3D). If PIKfyve inhibitors block the generation of stage II melanosomes, then PIKfyve inhibition should also result in the mislocalization of proteins normally delivered to the stage II, III and IV melanosomes. PIKfyve inhibitor treatment blocked the normal trafficking of TYRP1, resulting in the accumulation of TYRP1 in a perinuclear region (Figure 3D). Taken together, these studies further support the notion that PIKfyve regulates the maturation of the stage II melanosome.

The PIKfyve complex regulates the expression of genes required for melanin synthesis.

While transgenic mouse studies have shown that the PIKfyve complex impacts vesicle trafficking in melanocytes, the exact contribution of each member of this complex to melanogenesis remains unclear. While the inhibitor experiments indicate that PIKfyve regulates lysosome and melanosome maturation (Figure 2), prolonged blockade of PIKfyve eventually results in cell death (data not shown), making it difficult to elucidate the role of individual phosphoinositides in vesicle trafficking via lipid complementation. To gain a better appreciation of the role of the PIKfyve complex and phosphoinositides in melanogenesis, we utilized an shRNA-based loss of function strategy to more subtly modulate PIKfyve levels. Previous studies have demonstrated that PIKfyve knockdown can significantly inhibit the accumulation of $PI(3,5)P_2$ without completely eliminating the production of this lipid (38). To further elucidate the role of the PIKfyve complex in melanogenesis, melanoma and melanocyte lines or strains expressing shRNAs directed towards PIKfyve complex components were generated. shRNA mediated suppression of PIKfyve expression resulted in decreased accumulation of MITF-M and MITF-M regulated proteins (TYR, TYRP1) in all cell lines (Figure 4A). Quantitative RT-PCR studies revealed that PIKfyve knockdown inhibited the accumulation of MITF-M, TYR, and TYRP1 mRNAs in the majority of cells examined (Figure 4B). Similarly, VAC14 or SAC3 depletion also inhibited the accumulation of tyrosinase protein and mRNA transcripts (Figure 4C and 4D). While acute inhibition of PIKfyve resulted in an abrupt block in vesicle maturation, it did not affect the transcription of genes that regulate melanogenesis (Figure 2). In contrast, depletion of PIKfyve results in a downregulation of the expression of genes that regulate

melanogenesis, suggesting that melanogenesis could be modulated by the accumulation of specific phosphoinositide intermediates.

PIKfyve modulates the stability of WIPI1, a protein known to regulate the accumulation of proteins that regulate melanogenesis

WIPI1 and WIPI2 are both homologous to yeast ATG18 (29, 30) which itself can regulate kinase activity of the yeast PIKfyve homologue (FAB1) (35, 36). Yeast-two hybrid studies and mutant analysis have further suggested that the yeast WIPI1/2 homologue (ATG18) can bind to the yeast PIKfyve homologue (FAB1), acting as a negative regulator of kinase activity (36). Previous studies from our laboratory demonstrated that WIPI1 knockdown not only blocked the formation of stage III melanosomes in melanocytes but also inhibited the expression of genes that regulate melanogenesis (25), suggesting that WIPI1 and PIKfyve could interact. To determine whether there is a functional interaction between the PIKfyve complex and WIPI1 in mammalian cells, the consequences of PIKfyve knockdown on WIPI1 levels were examined. PIKfyve knockdown inhibited the accumulation of WIPI1 protein (Figure 5A), while having no effect on the accumulation of WIPI1 or WIPI2 mRNA (Figure 5B). To investigate whether PIKfyve directly binds to and stabilizes WIPI1/2, we immunoprecipitated WIPI1/2 or PIKfyve and probed for WIPI1, WIPI2, PIKfyve, SAC3, or VAC14 (Figure 5D). Additional experiments revealed that treating cells with a PIKfyve inhibitor did not inhibit the accumulation of WIPI1 (Figure 5C), indicating that the phenotypes observed in our shRNA experiments were likely the result of depletion of

PIKfyve itself or more likely the result of an effect of PIKfyve depletion on phosphoinositide levels. PIKfyve or VAC14 antibodies were able to immunoprecipitate PIKfyve, VAC14, and SAC3, but were not able to precipitate WIPI1 or WIPI2. Similarly, the members of the PIKfyve complex were not immunoprecipitated with the WIPI1 or WIPI2 antibodies. Taken together these results indicate that the PIKfyve complex regulates melanogenesis by influencing both the accumulation of enzymes that synthesize melanin and by influencing melanosome maturation, similar to what was observed with WIPI1 (25).

A Novel Role for PI(5)P in Stimulating Melanogenesis

WIPI1 contains a phosphoinositide binding motif that can mediate binding to PI(3)P, PI(3,5)P_2 and PI(5)P (32). WIPI1's ability to interact with PI(3)P is believed to function primarily in autophagy (53), but its functional relationship with the other phospholipids has yet to elucidated. PIKfyve is responsible for generating PI(3,5)P_2 and most cellular PI(5)P (38-40). Moreover recent studies suggest that PI(3,5)P_2 plays a more substantial role in cellular trafficking (39) while the functions PI(5)P are relatively less defined. Our results show that PIKfyve does not directly interact with WIPI1 although PIKfyve knockdown does result in decreased WIPI1 accumulation (Figure 5). It is conceivable that PIKfyve could stabilize WIPI1 by catalyzing the production of phosphoinositides, which then bind WIPI1 and promote protein stability. To better define which phosphoinositide may be involved in WIPI1 stabilization, we overexpressed WIPI1 in HEK293T cells and examined its ability to bind to different phosphoinositides.

The overexpressed WIPI1 construct was able to bind to all three phosphoinositides with preference for monophosphates (Figure 6A). Recent studies have implicated WIPI2-PI(5)P binding in noncannonical autophagy (31) however the nature of WIPI1's interaction has yet to be elucidated. To elaborate on the roles of the phospholipids in melanogenesis we treated parental MNT-1 cells (Figure 6B, 6C) with either PI(3)P, $PI(3,5)P_2$ and PI(5)P and appropriately charged matched carriers. Addition of PI(3)P and PI(5)P increased the accumulation of TYR protein (Figure 6B) and mRNA (Figure 6C), indicating that these phosphoinositides modulate melanogenesis. Interestingly, only PI(3)P increased WIPI1 accumulation, consistent with the hypothesis that this phosphoinositide stabilizes WIPI1 and modulates melanogenesis via WIPI1. In contrast, PI(5)P induced tyrosinase accumulation but did not modulate WIPI1 levels (Figure 6B). Recent studies have indicated that loss of $PI(3,5)P_2$, as opposed PI(5)P, results in cellular vacuolization leading to cell death (39). Additional studies revealed that $PI(3,5)P_2$ complemented the effect of 1000 nM YM-201636 on cell death (Figure 6D). Taken together our studies suggest that PI(3)P and PI(5)P modulate melanogenesis, while $PI(3,5)P_2$ influences vesicle trafficking and cell survival.

2.5 Discussion

The studies presented in this manuscript demonstrate that the PIKfyve complex regulates melanogenesis directly (Figure 1) as lineage-dependent PIKfyve knockout mice retain melanocyte stem cells as identified by TRP2 staining (Figure S1A) yet accumulate progressively less pigment in the hair over time. These observations are slightly more dramatic than what was observed in both *VAC14 ingls* (36) and *FIG4* pale tremor mice (42), both of which had lightening of the coat. *Vac14* and *Fig4* constitutive knockout mice as well as the *PIKfyve* gene-trap mouse also had severe neurologic disease, which ultimately resulted in early lethality (36, 38, 42) preventing a full examination of the effects of these genes on melanogenesis. Interestingly, partial loss of function mutations in *FIG4* resulted in a beige coat color and increased longevity (41), implicating a specific role for the PIKfyve complex in melanogenesis. The lineage specific knockout studies presented here further confirm that the PIKfyve complex plays a specific role in melanogenesis.

Previous studies have implicated an important role for PIKfyve and $PI(3,5)P_2$ in lysosomal function and trafficking (51, 54-56). Dysregulation of autophagy (44) and impaired lysosomal function are known to contribute to vacuolization and cell death associated with PIKfyve inhibition or loss (51, 55). In this study, we demonstrate that PIKfyve inhibition results in the accumulation of proteins that would normally be processed by the lysosome (Cathepsin D) (Figure 2C), consistent with prior *in vivo* studies that demonstrated a critical role for *PIKfyve* in lysosome biogenesis in platelets (51). We also show that PIKfyve inhibition blocks melanin accumulation (Figure 2), and

prevents the maturation of stage I to stage II melanosomes by blocking PMEL cleavage. This ultimately results in the accumulation of vesicles marked by MART-1 (Figure 3) and an overall reduction in the accumulation of melanin. Taken together these results explain the results observed in SAC3 (42) and VAC14 mice (36), which accumulate less melanosomes within their hair.

The PIKfyve complex functions as a critical phosphoinositol kinase in mammalian cells as it is responsible for generating the majority of PI(5)P and all $PI(3,5)P_2$ (38-40). In this manuscript, we sought to examine the contributions of PI(5)P and $PI(3,5)P_2$ to melanogenesis and melanosome biogenesis. Recent studies have indicated that loss of $PI(3,5)P_2$, as opposed to PI(5)P, result in trafficking defects that lead to cellular vacuolization (39). Our studies support these findings, as the addition of $PI(3,5)P_2$ was able to attenuate cell death as a result of PIKfyve inhibition (Figure 6D). Intriguingly, other published studies suggest that $PI(3,5)P_2$ may regulate melanosome biogenesis. Two families of cation channels, the TRPMLs and the TPCs, act as $PI(3,5)P_2$ effectors and function in vesicular fusion (55, 57, 58). Interestingly, TPC2 mutations in *Xenopus* oocytes (54) result in decreased pigment accumulation while single-nucleotide polymorphisms in TPC2 in humans is associated with skin, eye, and hair color variation (59). Similarly, mice mutant for TRPML3 (60) exhibit hypopigmentary phenotypes, further implicating a role for $PI(3,5)P_2$ in melanogenesis. Future studies will focus on determining whether the spatial production of $PI(3,5)P_2$ and the cellular location of these channels is critical for controlling vesicle fusion and organelle biogenesis.

Published studies indicate that PI(5)P also functions in autophagy and endolysosomal formation, but often in a functionally distinct manner from $PI(3,5)P_2$ (40,

61). In this study, we sought to better understand the contributions of PI(5)P to melanogenesis. PIKfyve knockdown resulted in the downregulation of genes that catalyze the synthesis of melanin (Figure 4). Additional experiments revealed that addition of PI(5)P induced the expression of genes that catalyzed melanogenesis (Figure 6). These results suggest that PIKfyve modulates the expression of melanogenesis genes via a mechanism involving PI(5)P. The observations presented here are strikingly similar to previous studies indicating that WIPI1 regulates both melanogenesis and the maturation of the melanosome (25). While WIPI1 does not form a complex with PIKfyve (Figure 5D) in mammalian cells as it does in yeast (36), PIKfyve knockdown does regulate the accumulation of WIPI1 suggesting a functional interaction (Figure 5A). WIPI1 can bind to both PI(3)P, PI(3,5)P_2, and PI(5)P (Figure 6A) and is highly expressed in melanocytes and melanoma cells as compared to other cell types (62, 63). Future studies will investigate whether WIPI1 is the phosphoinositide effector protein that regulates melanogenesis.

PI(5)P is a relatively rare phosphoinositide and remains poorly characterized (40). We observed that the addition of PI(5)P can induce the accumulation of tyrosinase protein (Figure 6B). PI(5)P is present in the nucleus where it has been shown to interact with several PHD-domain containing transcription factors (64). PI(5)P can upregulate the expression of specific genes through an epigenetic mechanism involving the epigenetic regulator ING2 (65). In addition, PI(5)P can also activate the differentiation of myoblasts through its interaction with TAF3 (64). Other studies have indicated that WIPI1, a putative phosphoinositide effector in melanocytes, can localize to the nucleus and bind to transcription factors (62). Taken together, these studies

suggest that WIPI1 and the phosphoinositide PI(5)P may regulate melanogenesis by controlling gene expression (Figure 7). As phosphoinositides can rapidly be interconverted within cells (66), it is often difficult to definitively determine which phosphoinositide regulates a particular step in the melanogenesis pathway. Future studies will focus on better defining potential nuclear and cytoplasmic PI(5)P effectors to determine more precisely how PI(5)P regulates transcription.

Melanosome biogenesis is a precisely orchestrated process involving the coordinated transcription of multiple genes and the timed delivery of enzymes to the melanosome as it matures (10). While the transcription of genes that regulate melanosome transport and melanin synthesis are coordinately regulated by MITF, it is currently unclear whether the transcription of melanin synthesizing enzymes is coordinated with the biogenesis of the melanosome itself. Previous work from our group implicated a role for the phosphoinositide binding protein WIPI1 in coordinating melanosome biogenesis with the transcription of enzymes that synthesize melanin (25). In this study, we implicate a role for phosphoinositides themselves in this coordinate regulation. While this coordinate regulation is novel, it is not entirely unexpected as $PI(3,5)P_2$ can act as a transcriptional regulator through interactions with Tup1 and Cti6 in yeast (68) while also regulating vesicle traffic (38). Future studies will focus on defining the precise role for specific phosphoinositides and their effectors in vesicle trafficking and transcription *in vivo*.

2.6 Materials and Methods

Antibodies and Primers

All antibodies used in experimental assays are listed in Table S3. *PIKfyve* genotyping primers are described in (43) and other genotyping primers were taken from the mouse mutant resource website, (Jackson Laboratory Bar Harbor, ME). RT-qPCR primers were designed using Primer3web (http://www.primer3plus.com) and are listed in Table S4.

Cell Culture

Human MNT-1 cells were cultured in DMEM (Genesee Scientific) supplemented with 15% fetal bovine serum (Corning), AIM-V medium (Life Technologies), MEM vitamin solution (Invitrogen), and antibiotic-antimycotic (Life Technologies). For melanin quantification experiments MNT-1 cells were switched to DMEM minus phenol RED (Fisher Scientific) supplemented with 10% fetal bovine serum, L-glutamine (Invitrogen), and antibiotic-antimycotic. Human deeply pigmented neonatal epidermal melanocytes (Life Technologies) were cultured in Medium 254 (Life Technologies) supplemented with Human Melanocyte Growth Supplement 2 (Life Technologies) and antibiotic-antimycotic. B16 cells were cultured DMEM supplemented with 10% fetal bovine serum (Life Technologies), L-Glutamine, MEM non-essential amino acids (Life Technologies), antibiotic-antimycotic, and 0.075% sodium bicarbonate (Sigma-Aldrich). Epidermal

melanocytes isolated from C57BL/6J mice were obtained from Antonella Bacchiocchi (Yale University). These cells were cultured in Ham's F-12 Nutrient Mixture (Life Technologies) supplemented with 8% fetal bovine serum, bovine pituitary extract (Life Technologies), antibiotic-antimycotic, 5 ng phorbol 12-myristate 13-acetate (Abcam), and 0.00022% 3-Isobutyl-1-methylxanthine (Sigma-Aldrich). HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotic-antimycotic.

RNA Interference

We utilized the pLKO.1 lentiviral based shRNA expression system (Open Biosystems) for our shRNA experiments. MNT-1 cells, B16 cells, human melanocyte, and mouse melanocyte shRNA-expressing cell lines were established as described (24, 25). The following constructs were purchased from Open Biosystems: pLKO.1-PIKFYVE #1, RHS3979-98831506; pLKO.1-PIKFYVE #2, RHS3979-98831520; pLKO.1-VAC14 #1, RHS3979-99218405; pLKO.1-VAC14 #2, RHS3979-99218425; pLKO.1-SAC3 #1, RHS3979-98058306; pLKO.1-SAC3 #2, RHS3979-98058322; and pLKO.1 non-targeting control. In B16 cells and mouse melanocytes a mouse specific construct purchased from Sigma-Aldrich was also used (pLKO.1-PIKFYVE #3 (TRCN0000025096, Clone ID:NM_011086.1-949s1c1) along with pLKO.1-PIKFYVE #2 which recognizes both the human and mouse sequence. Gene knockdown was quantified by real-time quantitative RT-PCR and immunoblotting. A Student's two-tailed

t-test was used to calculate the statistical significance in comparison to non-targeting controls.

Real-time quantitative PCR for mRNA quantitation

E.Z.N.A. Total RNA Kit I (Omega Bio-Tek) was used to lyse cells and purify RNA. A high-capacity RNA-to-cDNA kit (Applied Biosystems) was then utilized to generate cDNA for RT-qPCR. Quantitative RT PCR was performed with cDNA using Power SYBR Green PCR Master Mix (Applied Biosystems). A 7900HT Fast-Real TIME PCR System (Applied Biosystems) and SDS 2.4 (Applied Biosystems) were utilized to determine Ct values. Each sample was normalized to at least one housekeeping gene (GAPDH, β-Actin, and/or HPRT) and analyzed using the relative quantification model (Pfaffl). A Student's two-tailed t-test was used to calculate the statistical significance in comparison to vehicle-treated control.

Drug treatment

MNT-1 cells were plated in 6-well plates at a concentration of 2×10^5 cells per well and allowed to adhere overnight. Cells were then incubated with varying concentrations of YM-201636 (Cayman Chemical) or Apilimod (US Biological) dissolved in normal MNT-1 media. For the control, an equal amount of DMSO (0.1%) was added to the media. Media was refreshed after 48 hours and cells were lysed with RIPA buffer after 72 hours of treatment.

Pigment Measurement

MNT-1 cells were plated in 96-well plates at a concentration of 1.5 x 10^4 cells per well and allowed to re-attach overnight. Media was refreshed for drug treated cells every 48 hours. After five days of treatment cells were lysed with Cell-Titer-Glo reagent (Promega). Relative melanin accumulation was quantified by measuring absorbance at 405 nm and normalizing this value to luminescence to determine cell number as determined by the Cell-Titer-Glo assay as previously described (24). Pigment percent was quantified relative to vehicle control. A Student's two-tailed t-test was used to calculate the statistical significance in comparison to vehicle-treated control.

Treatment of cells by addition of exogenous lipids

All carriers and phospholipids were obtained from Echelon Biosciences. Unlabeled PI(3)P, PI(5)P, and PI(3,5)P_2 were constituted in DMSO:H_2O (10:1); carrier 2 and carrier 3 were reconstituted in H_2O. Carriers and lipids were combined at 1:1 molar ratio and incubated for 15 minutes at room temperature. Charge matched carriers were used to optimize lipid delivery – carrier 3 was used to reconstitute PI(3)P or PI(5)P; carrier 2 was used to reconstitute PI(3,5)P_2. The mixture was then diluted in MNT-1 media and incubated with cells for 48 hours. Media was refreshed and cells were incubated for another 24 hours. Cells were lysed with RIPA lysis buffer at the completion of treatment

and subjected to western blot analysis. Media combined with carrier 2 or 3 alone served as a negative control and lipids were tested at 2 – 10 μM final concentrations.

Cell Survival Assay

MNT-1 cells were plated in 96-well plates at a concentration of 1.5 x 10^4 cells per well and allowed to re-attach overnight. Phospholipids were prepared as previously described and combined with media containing drug or vehicle control. Media containing drug and lipid was refreshed on cells every 48 hours. After five days of treatment cells were lysed with Cell-Titer-Glo reagent (Promega). The luminescence value was used to determine cell survival as determined by the Cell-Titer-Glo assay as previously described (69). A Student's two-tailed t-test was used to calculate the statistical significance in comparison to vehicle-treated control.

Immunoprecipitation

Cells were lysed from 10 cm plates with 1 mL of lysis/wash buffer (20 mM Tris HCl, 150 mM NaCl, 5% Glycerol, 1% NP40, 1 mM EDTA, protease inhibitors). Lysates were centrifuged at 14000 RPM and the supernatant was homogenized by passing through a syringe with a 22-gauge needle. Lysates were loaded on to Protein A/G PLUS agarose (Santa Cruz Biotechnologies), incubated with PIKfyve, SAC3, VAC14, WIPI1, WIPI2 antibodies or control IgG overnight, washed 3x with wash buffer, and eluted with

Laemmli sample buffer. Eluted lysates were subjected to SDS-PAGE followed by immunoblotting with the indicated antibodies.

Lipid Beads Pulldown Assay

Lysates from HEK293T cells overexpressing WIPI1 were generated in lipid-binding buffer (20mM Tris-HCl, 150mM NaCl, and 1mM EDTA [pH 7.5]). Cells were passed ten times through a G25 needle and sonicated on ice. Insoluble debris was removed by centrifugation at 13,000xg for 1 hr at 4°C. 100 µL of PI(3)P, PI(3,5)P_2, PI(5)P, or control bead slurry (Echelon Bioscience) was added to 40 µg of cell lysate and incubated on a rotator for 4 hours at 4°C. The beads were washed five times with lipid-wash buffer (10mM HEPES [pH 7.4], 150mM NaCl, 0.25% NP40). Bound proteins were eluted with 2X SDS. Protocol modified from previously described (31).

Electron Microscopy

Darkly pigmented melanocytes were treated with various dosages of YM-201636 (0 – 1000 nM) for 72 hours. Cells were then fixed for 4 hours in Karnovsky's fixative, pH 7.2, before being washed with sodium cacododylate buffer (0.2 M). Samples were then processed for routine DOPA histochemistry electron microscopy. Melanosome stages (I – IV) were quantified visually in the electron micrographs and melanosome stage percentage was assessed versus vehicle treated controls. Electron microscopy on whole mouse skin was obtained and processed as previously described by (47).

Immunofluorescence Microscopy

MNT-1 cells were plated in 12-well plates with coverslips at a concentration of 1 x 10^4 cells per well and allowed to adhere overnight. Cells were then treated with 100 nM Apilimod, 1000 nM YM-201636, or DMSO control overnight. Alternatively, control and shRNA expressing cells were plated in 12-well plates at a concentration of 1 x 10^4 cells per well and allowed to re-attach overnight. Cells were fixed with 4% paraformaldehyde for 1 hour. Coverslips were rinsed with PBS and permeablized with 0.1% Triton X-100 (Fisher Scientific) and subsequently blocked in 2% BSA in PBS containing 0.1% Tween 20 for 1 hour. Cells were then incubated with primary antibodies (Supplemental Table 1) followed by secondary antibodies conjugated to Alexa Fluor 594 or 488 (Invitrogen) and were mounted in a solution containing DAPI. Confocal images were acquired using a LSM 780 confocal multiphoton microscope and images were processed in Zen lite (Zeiss).

Mouse Strains and Genotyping

All experiments involving mice conform to the NIH guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Irvine, approval number 2011–3020. C57BL/6 *PIKFYVE*$^{Flox/Flox}$ mice on a pure C57BL/6 background were obtained from Dr. Takehiko Sasaki (Akita University, Akita, Japan). *PIKFYVE*$^{Flox/Flox}$ were crossed to *Tyrosinase::CreER*^{T2} (JAX stock no: 012328) on a pure

C57BL/6 background. The resulting *Tyrosinase::CreER*^{T2,} *PIKFYVE*$^{Flox/+}$ progeny were backcrossed to *PIKFYVE*$^{Flox/Flox}$ to generate *CreER*^{T2,} *PIKFYVE*$^{Flox/Flox}$ mice. Upon weaning, mice were placed on tamoxifen feed (Harlan Laboraties, 250 mg/kg) for 30 days. Genomic DNA was isolated from mouse tail biopsies using the Quick Genotyping DNA Preparation Kit (Bioland Scientific, LLC) according to the manufacturer's instructions.

Mouse hair

Dorsal hairs of mice at P50 or P100 were shaved and 1 mg was dissolved overnight in 1 mL of hot (65°C) of 9:1 Soluene-350 (PerkinElmer) and water. Quadruplicate 150 µL aliquots for each mouse hair sample were then analyzed for absorbance values at 405 nm as previously described (47).

RNA isolation on whole mouse skin and Nanostring Analysis

4 mm punch biopsies of mouse skin was harvested from anesthetized mice and stabilized overnight in RNAlater (Life Technologies) at 4°C. RNA Purification and Nanostring gene expression analysis were carried out as previously described (47).

Tissue Immunofluorescence

Whole mouse skin was formalin fixed and paraffin embedded. Immunofluorescence staining was carried out using a TRP2 primary antibody and a FITC-conjugated Alexa Fluor 488 secondary antibody. Specific antibodies used are listed in Table S3.

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2.8 References

1. Markert CL, Silvers WK. The Effects of Genotype and Cell Environment on Melanoblast Differentiation in the House Mouse. Genetics. 1956 May;41(3):429-50.

2. Costin GE, Hearing VJ. Human skin pigmentation: melanocytes modulate skin color in response to stress. FASEB J. 2007 Apr;21(4):976-94.

3. Urabe K, Aroca P, Tsukamoto K, Mascagna D, Palumbo A, Prota G, et al. The inherent cytotoxicity of melanin precursors: a revision. Biochim Biophys Acta. 1994 Apr 28;1221(3):272-8.

4. Jenkins NC, Grossman D. Role of melanin in melanocyte dysregulation of reactive oxygen species. Biomed Res Int. 2013;2013:908797.

5. Yamaguchi Y, Hearing VJ. Physiological factors that regulate skin pigmentation. Biofactors. 2009 Mar-Apr;35(2):193-9.

6. Sitaram A, Marks MS. Mechanisms of protein delivery to melanosomes in pigment cells. Physiology (Bethesda). 2012 Apr;27(2):85-99.

7. Slominski A, Tobin DJ, Shibahara S, Wortsman J. Melanin pigmentation in mammalian skin and its hormonal regulation. Physiol Rev. 2004 Oct;84(4):1155-228.

8. Wei AH, Li W. Hermansky-Pudlak syndrome: pigmentary and non-pigmentary defects and their pathogenesis. Pigment Cell Melanoma Res. 2013 Mar;26(2):176-92.

9. Raposo G, Marks MS. Melanosomes--dark organelles enlighten endosomal membrane transport. Nat Rev Mol Cell Biol. 2007 Oct;8(10):786-97.

10. Wasmeier C, Hume AN, Bolasco G, Seabra MC. Melanosomes at a glance. J Cell Sci. 2008 Dec 15;121(Pt 24):3995-9.

11. Giordano F, Bonetti C, Surace EM, Marigo V, Raposo G. The ocular albinism type 1 (OA1) G-protein-coupled receptor functions with MART-1 at early stages of melanogenesis to control melanosome identity and composition. Hum Mol Genet. 2009 Dec 1;18(23):4530-45.

12. Watt B, van Niel G, Raposo G, Marks MS. PMEL: a pigment cell-specific model for functional amyloid formation. Pigment Cell Melanoma Res. 2013 May;26(3):300-15.

13. Theos AC, Tenza D, Martina JA, Hurbain I, Peden AA, Sviderskaya EV, et al. Functions of adaptor protein (AP)-3 and AP-1 in tyrosinase sorting from endosomes to melanosomes. Mol Biol Cell. 2005 Nov;16(11):5356-72.

14. Park HY, Kosmadaki M, Yaar M, Gilchrest BA. Cellular mechanisms regulating human melanogenesis. Cell Mol Life Sci. 2009 May;66(9):1493-506.

15. Hirobe T. Origin of melanosome structures and cytochemical localizations of tyrosinase activity in differentiating epidermal melanocytes of newborn mouse skin. J Exp Zool. 1982 Dec 30;224(3):355-63.

16. Boissy RE. Melanosome transfer to and translocation in the keratinocyte. Exp Dermatol. 2003;12 Suppl 2:5-12.

17. Vijayasaradhi S, Xu Y, Bouchard B, Houghton AN. Intracellular sorting and targeting of melanosomal membrane proteins: identification of signals for sorting of the human brown locus protein, gp75. J Cell Biol. 1995 Aug;130(4):807-20.

18. Dell'Angelica EC. AP-3-dependent trafficking and disease: the first decade. Curr Opin Cell Biol. 2009 Aug;21(4):552-9.

19. Calvo PA, Frank DW, Bieler BM, Berson JF, Marks MS. A cytoplasmic sequence in human tyrosinase defines a second class of di-leucine-based sorting signals for late endosomal and lysosomal delivery. J Biol Chem. 1999 Apr 30;274(18):12780-9.

20. Bultema JJ, Ambrosio AL, Burek CL, Di Pietro SM. BLOC-2, AP-3, and AP-1 proteins function in concert with Rab38 and Rab32 proteins to mediate protein trafficking to lysosome-related organelles. J Biol Chem. 2012 Jun 1;287(23):19550-63.

21. Bultema JJ, Di Pietro SM. Cell type-specific Rab32 and Rab38 cooperate with the ubiquitous lysosome biogenesis machinery to synthesize specialized lysosome-related organelles. Small GTPases. 2013 Jan-Mar;4(1):16-21.

22. Marks MS. Organelle biogenesis: en BLOC exchange for RAB32 and RAB38. Curr Biol. 2012 Nov 20;22(22):R963-5.

23. Gerondopoulos A, Langemeyer L, Liang JR, Linford A, Barr FA. BLOC-3 mutated in Hermansky-Pudlak syndrome is a Rab32/38 guanine nucleotide exchange factor. Curr Biol. 2012 Nov 20;22(22):2135-9.

24. Ganesan AK, Ho H, Bodemann B, Petersen S, Aruri J, Koshy S, et al. Genome-wide siRNA-based functional genomics of pigmentation identifies novel genes and pathways that impact melanogenesis in human cells. PLoS Genet. 2008 Dec;4(12):e1000298.

25. Ho H, Kapadia R, Al-Tahan S, Ahmad S, Ganesan AK. WIPI1 coordinates melanogenic gene transcription and melanosome formation via TORC1 inhibition. J Biol Chem. 2011 Apr 8;286(14):12509-23.

26. Ho H, Ganesan AK. The pleiotropic roles of autophagy regulators in melanogenesis. Pigment Cell Melanoma Res. 2011 Aug;24(4):595-604.

27. Kalie E, Razi M, Tooze SA. ULK1 regulates melanin levels in MNT-1 cells independently of mTORC1. PLoS One. 2013;8(9):e75313.

28. Zhang CF, Gruber F, Ni C, Mildner M, Koenig U, Karner S, et al. Suppression of autophagy dysregulates the antioxidant response and causes premature senescence of melanocytes. J Invest Dermatol. 2015 May;135(5):1348-57.

29. Polson HE, de Lartigue J, Rigden DJ, Reedijk M, Urbe S, Clague MJ, et al. Mammalian Atg18 (WIPI2) localizes to omegasome-anchored phagophores and positively regulates LC3 lipidation. Autophagy. 2010 May;6(4):506-22.

30. Krick R, Busse RA, Scacioc A, Stephan M, Janshoff A, Thumm M, et al. Structural and functional characterization of the two phosphoinositide binding sites of PROPPINs, a beta-propeller protein family. Proc Natl Acad Sci U S A. 2012 Jul 24;109(30):E2042-9.

31. Vicinanza M, Korolchuk VI, Ashkenazi A, Puri C, Menzies FM, Clarke JH, et al. PI(5)P regulates autophagosome biogenesis. Mol Cell. 2015 Jan 22;57(2):219-34.

32. Baskaran S, Ragusa MJ, Boura E, Hurley JH. Two-site recognition of phosphatidylinositol 3-phosphate by PROPPINs in autophagy. Mol Cell. 2012 Aug 10;47(3):339-48.

33. Di Paolo G, De Camilli P. Phosphoinositides in cell regulation and membrane dynamics. Nature. 2006 Oct 12;443(7112):651-7.

34. Poccia D, Larijani B. Phosphatidylinositol metabolism and membrane fusion. Biochem J. 2009 Mar 1;418(2):233-46.

35. Efe JA, Botelho RJ, Emr SD. Atg18 regulates organelle morphology and Fab1 kinase activity independent of its membrane recruitment by phosphatidylinositol 3,5-bisphosphate. Mol Biol Cell. 2007 Nov;18(11):4232-44.

36. Jin N, Chow CY, Liu L, Zolov SN, Bronson R, Davisson M, et al. VAC14 nucleates a protein complex essential for the acute interconversion of PI3P and PI(3,5)P(2) in yeast and mouse. EMBO J. 2008 Dec 17;27(24):3221-34.

37. Sbrissa D, Ikonomov OC, Fenner H, Shisheva A. ArPIKfyve homomeric and heteromeric interactions scaffold PIKfyve and Sac3 in a complex to promote PIKfyve activity and functionality. J Mol Biol. 2008 Dec 26;384(4):766-79.

38. Zolov SN, Bridges D, Zhang Y, Lee WW, Riehle E, Verma R, et al. In vivo, Pikfyve generates PI(3,5)P2, which serves as both a signaling lipid and the major precursor for PI5P. Proc Natl Acad Sci U S A. 2012 Oct 23;109(43):17472-7.

39. Shisheva A, Sbrissa D, Ikonomov O. Plentiful PtdIns5P from scanty PtdIns(3,5)P2 or from ample PtdIns? PIKfyve-dependent models: Evidence and speculation (response to: DOI 10.1002/bies.201300012). Bioessays. 2015 Mar;37(3):267-77.

40. Shisheva A. PtdIns5P: news and views of its appearance, disappearance and deeds. Arch Biochem Biophys. 2013 Oct 15;538(2):171-80.

41. Lenk GM, Ferguson CJ, Chow CY, Jin N, Jones JM, Grant AE, et al. Pathogenic mechanism of the FIG4 mutation responsible for Charcot-Marie-Tooth disease CMT4J. PLoS Genet. 2011 Jun;7(6):e1002104.

42. Chow CY, Zhang Y, Dowling JJ, Jin N, Adamska M, Shiga K, et al. Mutation of FIG4 causes neurodegeneration in the pale tremor mouse and patients with CMT4J. Nature. 2007 Jul 5;448(7149):68-72.

43. Takasuga S, Horie Y, Sasaki J, Sun-Wada GH, Kawamura N, Iizuka R, et al. Critical roles of type III phosphatidylinositol phosphate kinase in murine embryonic visceral endoderm and adult intestine. Proc Natl Acad Sci U S A. 2013 Jan 29;110(5):1726-31.

44. Martin S, Harper CB, May LM, Coulson EJ, Meunier FA, Osborne SL. Inhibition of PIKfyve by YM-201636 dysregulates autophagy and leads to apoptosis-independent neuronal cell death. PLoS One. 2013;8(3):e60152.

45. Bosenberg M, Muthusamy V, Curley DP, Wang Z, Hobbs C, Nelson B, et al. Characterization of melanocyte-specific inducible Cre recombinase transgenic mice. Genesis. 2006 May;44(5):262-7.

46. Ozeki H, Ito S, Wakamatsu K, Thody AJ. Spectrophotometric characterization of eumelanin and pheomelanin in hair. Pigment Cell Res. 1996 Oct;9(5):265-70.

47. Paterson EK, Fielder TJ, MacGregor GR, Ito S, Wakamatsu K, Gillen DL, et al. Tyrosinase Depletion Prevents the Maturation of Melanosomes in the Mouse Hair Follicle. PLoS One. 2015;10(11):e0143702.

48. Nishimura EK, Jordan SA, Oshima H, Yoshida H, Osawa M, Moriyama M, et al. Dominant role of the niche in melanocyte stem-cell fate determination. Nature. 2002 Apr 25;416(6883):854-60.

49. Kim GH, Dayam RM, Prashar A, Terebiznik M, Botelho RJ. PIKfyve inhibition interferes with phagosome and endosome maturation in macrophages. Traffic. 2014 Oct;15(10):1143-63.

50. Cai X, Xu Y, Cheung AK, Tomlinson RC, Alcazar-Roman A, Murphy L, et al. PIKfyve, a class III PI kinase, is the target of the small molecular IL-12/IL-23 inhibitor apilimod and a player in Toll-like receptor signaling. Chem Biol. 2013 Jul 25;20(7):912-21.

51. Min SH, Suzuki A, Stalker TJ, Zhao L, Wang Y, McKennan C, et al. Loss of PIKfyve in platelets causes a lysosomal disease leading to inflammation and thrombosis in mice. Nat Commun. 2014;5:4691.

52. De Maziere AM, Muehlethaler K, van Donselaar E, Salvi S, Davoust J, Cerottini JC, et al. The melanocytic protein Melan-A/MART-1 has a subcellular localization distinct from typical melanosomal proteins. Traffic. 2002 Sep;3(9):678-93.

53. Proikas-Cezanne T, Takacs Z, Donnes P, Kohlbacher O. WIPI proteins: essential PtdIns3P effectors at the nascent autophagosome. J Cell Sci. 2015 Jan 15;128(2):207-17.

54. Lin-Moshier Y, Keebler MV, Hooper R, Boulware MJ, Liu X, Churamani D, et al. The Two-pore channel (TPC) interactome unmasks isoform-specific roles for TPCs in endolysosomal morphology and cell pigmentation. Proc Natl Acad Sci U S A. 2014 Sep 9;111(36):13087-92.

55. Dong XP, Shen D, Wang X, Dawson T, Li X, Zhang Q, et al. PI(3,5)P(2) controls membrane trafficking by direct activation of mucolipin Ca(2+) release channels in the endolysosome. Nat Commun. 2010;1:38.

56. Li X, Saitoh S, Shibata T, Tanimura N, Fukui R, Miyake K. Mucolipin 1 positively regulates TLR7 responses in dendritic cells by facilitating RNA transportation to lysosomes. Int Immunol. 2015 Feb;27(2):83-94.

57. Bellono NW, Oancea EV. Ion transport in pigmentation. Arch Biochem Biophys. 2014 Dec 1;563:35-41.

58. Feng X, Huang Y, Lu Y, Xiong J, Wong CO, Yang P, et al. Drosophila TRPML forms PI(3,5)P2-activated cation channels in both endolysosomes and plasma membrane. J Biol Chem. 2014 Feb 14;289(7):4262-72.

59. Sulem P, Gudbjartsson DF, Stacey SN, Helgason A, Rafnar T, Jakobsdottir M, et al. Two newly identified genetic determinants of pigmentation in Europeans. Nat Genet. 2008 Jul;40(7):835-7.

60. Xu H, Delling M, Li L, Dong X, Clapham DE. Activating mutation in a mucolipin transient receptor potential channel leads to melanocyte loss in varitint-waddler mice. Proc Natl Acad Sci U S A. 2007 Nov 13;104(46):18321-6.

61. Boal F, Mansour R, Gayral M, Saland E, Chicanne G, Xuereb JM, et al. TOM1 is a PI5P effector involved in the regulation of endosomal maturation. J Cell Sci. 2015 Feb 15;128(4):815-27.

62. Proikas-Cezanne T, Waddell S, Gaugel A, Frickey T, Lupas A, Nordheim A. WIPI-1alpha (WIPI49), a member of the novel 7-bladed WIPI protein family, is aberrantly expressed in human cancer and is linked to starvation-induced autophagy. Oncogene. 2004 Dec 16;23(58):9314-25.

63. Uhlen M, Fagerberg L, Hallstrom BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Proteomics. Tissue-based map of the human proteome. Science. 2015 Jan 23;347(6220):1260419.

64. Stijf-Bultsma Y, Sommer L, Tauber M, Baalbaki M, Giardoglou P, Jones DR, et al. The basal transcription complex component TAF3 transduces changes in nuclear phosphoinositides into transcriptional output. Mol Cell. 2015 May 7;58(3):453-67.

65. Bua DJ, Martin GM, Binda O, Gozani O. Nuclear phosphatidylinositol-5-phosphate regulates ING2 stability at discrete chromatin targets in response to DNA damage. Sci Rep. 2013;3:2137.

66. Duex JE, Nau JJ, Kauffman EJ, Weisman LS. Phosphoinositide 5-phosphatase Fig 4p is required for both acute rise and subsequent fall in stress-induced phosphatidylinositol 3,5-bisphosphate levels. Eukaryot Cell. 2006 Apr;5(4):723-31.

67. Howe AG, McMaster CR. Regulation of vesicle trafficking, transcription, and meiosis: lessons learned from yeast regarding the disparate biologies of phosphatidylcholine. Biochim Biophys Acta. 2001 Dec 30;1534(2-3):65-77.

68. Han BK, Emr SD. Phosphoinositide [PI(3,5)P2] lipid-dependent regulation of the general transcriptional regulator Tup1. Genes Dev. 2011 May 1;25(9):984-95.

69. Ho H, Aruri J, Kapadia R, Mehr H, White MA, Ganesan AK. RhoJ regulates melanoma chemoresistance by suppressing pathways that sense DNA damage. Cancer Res. 2012 Nov 1;72(21):5516-28.

Figure 2.1. Melanocyte specific PIKfyve knockout mice exhibit hair greying. A) Timeline of *in vivo* experiments. Five *Tyrosinase::Cre*^{ERT2}; *PIKFYVE*$^{Flox/Flox}$ and 5 *PIKFYVE*$^{Flox/Flox}$ mice were administered tamoxifen chow for 29 days. A control group of 5 *Tyrosinase::Cre*^{ERT2}; *PIKFYVE*$^{Flox/Flox}$ mice were fed a control diet throughout the course of the experiment. All mice were shave depilated at p35 and subsequently fed normal chow beginning at p50 for the subsequent 50 days. Mice were photographed at both p85 and p105 and melanin quantitation of mouse hair was performed. Gray bar denotes the duration that mice, with the exception of the control group, were on tamoxifen feed. Littermates were photographed at P85 (B) and P105 (D). Mice hair was dissolved in solune-350 and melanin quantitation was performed as described. The relative amount of melanin in the hair was calculated relative to *Cre*- controls at P85 (C) and P105 (E) Data shown are mean ± S.D. (n = 5 as indicated by error bars). F) 4mm skin biopsies were obtained from experimental mice at P50. Extracted RNA was subjected to Nanostring analysis using a codeset monitoring the expression of the genes listed below the graph. Normalized counts of each mRNA (n = 3 mice per group) were averaged for each gene. Data shown are mean ± S.D. (as indicated by error bars). *, $p < 0.05$; **, $p < 0.01$; or ***, $p < 0.001$ using a two-tailed Student's paired T test.

Figure 1

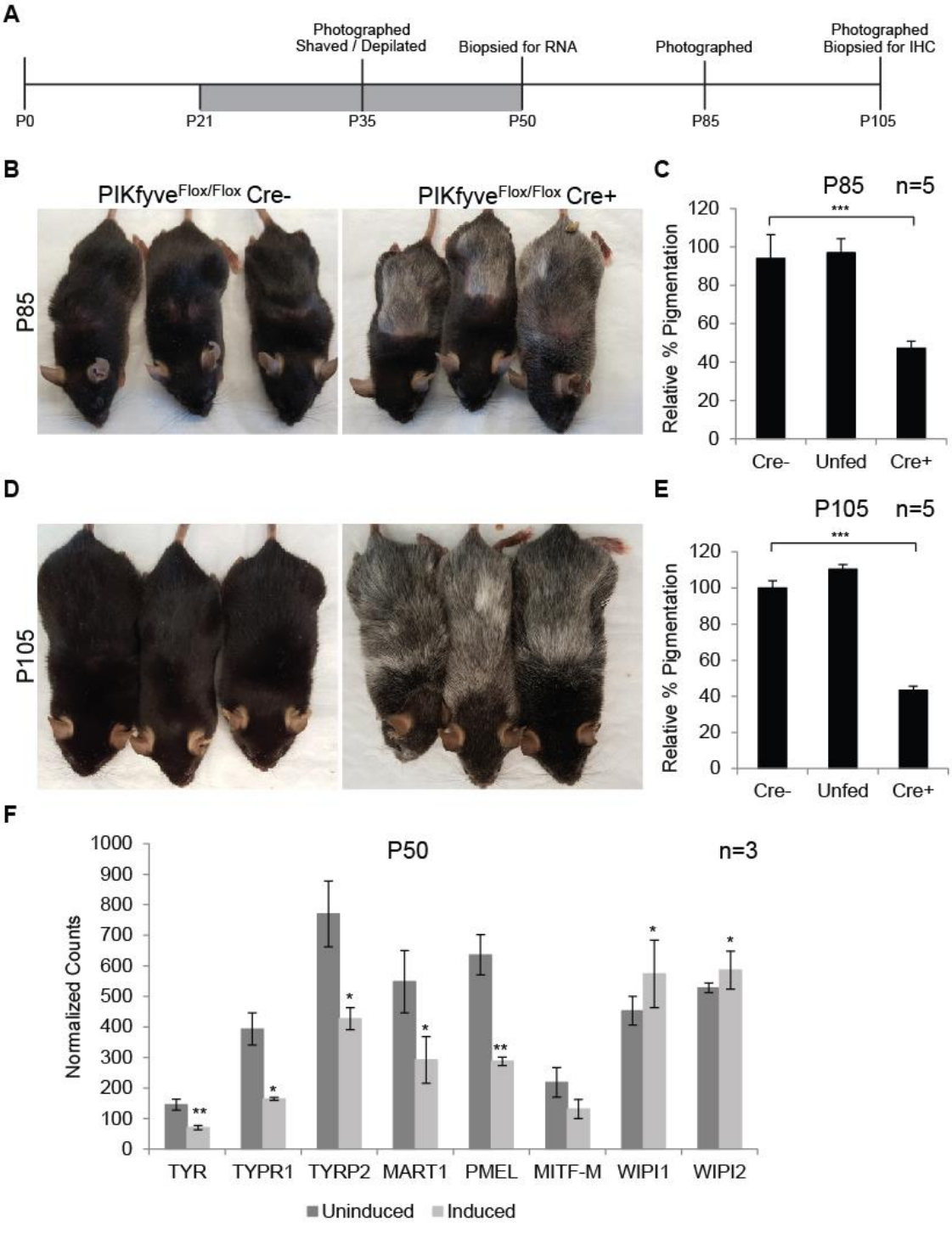

A
Photographed
Shaved / Depilated
Biopsied for RNA
Photographed
Photographed
Biopsied for IHC
P0
P21
P35
P50
P85
P105
B
PIKfyve Flox/Flox Cre-
PIKfyve Flox/Flox Cre+
P85
C
P85 n=5
***
Relative % Pigmentation
120
100
80
60
40
20
0
Cre-
Unfed
Cre+
D
P105
E
P105 n=5
***
Relative % Pigmentation
120
100
80
60
40
20
0
Cre-
Unfed
Cre+
F
P50
n=3
Normalized Counts
1000
900
800
700
600
500
400
300
200
100
0
TYR
TYPR1
TYRP2
MART1
PMEL
MITF-M
WIPI1
WIPI2
Uninduced
Induced

Figure 2.2. PIKfyve inhibitors block lysosomal trafficking pathways and inhibit melanin accumulation. A) MNT-1 cells were treated with PIKfyve inhibitors YM-201636 (YM), apilimod or vehicle control for five days. A spectrophotometric melanin quantitation assay was used to measure the amount of accumulated melanin. (n = 6 as indicated by error bars). B) Equal numbers of MNT-1 cells treated with the indicated doses of YM or Apilimod were pelleted. Photographs of the cell pellets were obtained to document that YM and Apilimod treatment inhibited the accumulation of melanin. C) MNT-1 cells treated with PIKfyve inhibitors for 72 hours. The relative accumulation of TYR, and processed and unprocessed forms of PMEL and Cathepsin D was measured by immunoblotting. Average size of glycosylated TYR is indicated. Sizes indicate full-sized protein unless otherwise indicated. Preprocathepsin D is 43 KD and procathepsin D is 46 kD. Each experiment was performed three times in triplicate. For all experiments, data shown are mean ± S.D *, $p < 0.05$; **, $p < 0.01$; or ***, $p < 0.001$ using a Student's paired T test versus vehicle treated control.

Figure 2

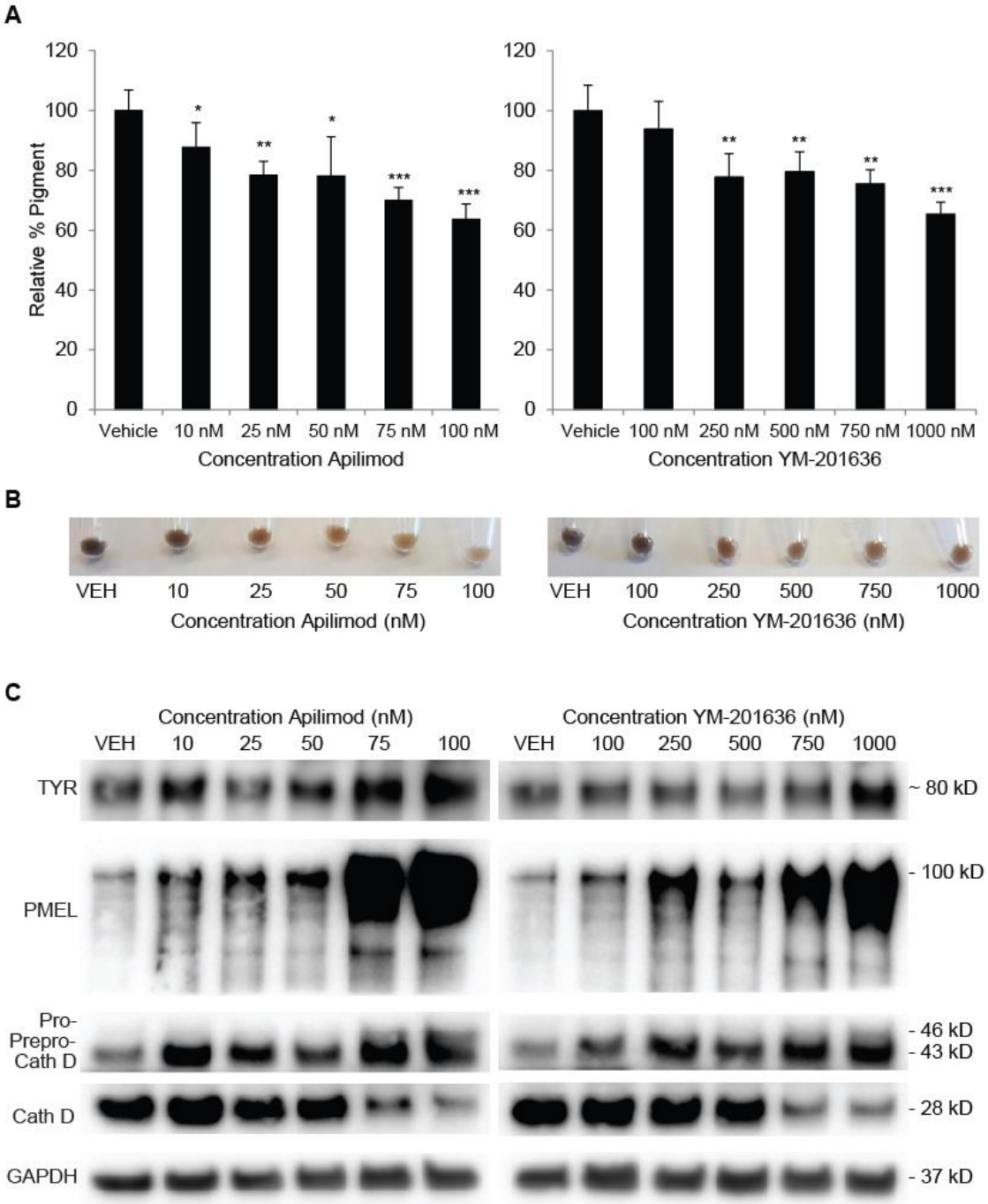

A
Relative % Pigment
120
100
80
60
40
20
0
Vehicle
10 nM
25 nM
50 nM
75 nM
100 nM
Concentration Apilimod
Vehicle
100 nM
250 nM
500 nM
750 nM
1000 nM
Concentration YM-201636
B
VEH
10
25
50
75
100
Concentration Apilimod (nM)
VEH
100
250
500
750
1000
Concentration YM-201636 (nM)
C
Concentration Apilimod (nM)
VEH
10
25
50
75
100
Concentration YM-201636 (nM)
VEH
100
250
500
750
1000
TYR
~ 80 kD
PMEL
- 100 kD
Pro-
Prepro-
Cath D
- 46 kD
- 43 kD
Cath D
- 28 kD
GAPDH
- 37 kD

Figure 2.3. PIKfyve inhibition results in decreased number of advanced stage melanosomes. Normal human melanocytes (NHM) were treated with 1000 nM YM-201636 or vehicle without (Control) or with DOPA histochemistry (DOPA Tx) and (A) observed by electron microscopy *Scale bar*, A = 5 µm and B = 2 µm. (B) Pie graphs representing quantification of melanosome stages as percentage in NHM treated with 100, 500, or 1000 nM YM-021636 or vehicle. C) MNT-1 cells treated with 1000 nM YM-201636 (YM), 100 nM apilimod (AP), or vehicle (VEH) control were stained with anti-MART-1 or anti-TYRP1 antibodies and imaged by confocal microscopy. Percent quantification of cytosolic MART-1 or TYRP1 per signal area in MNT-1 cells treated with PIKfyve inhibitors relative to vehicle controls. Data shown are normalized to vehicle control. *Scale bar*, 10 µm. For all experiments, all data are mean ± S.D. (n = 3 as indicated by error bars). A Student's paired t-test with a two-tailed normal distribution versus non-targeting scramble control. *, $p < 0.05$; or **, $p < 0.01$. Each experiment was performed with three biological replicates and three technical replicates.

Figure 3

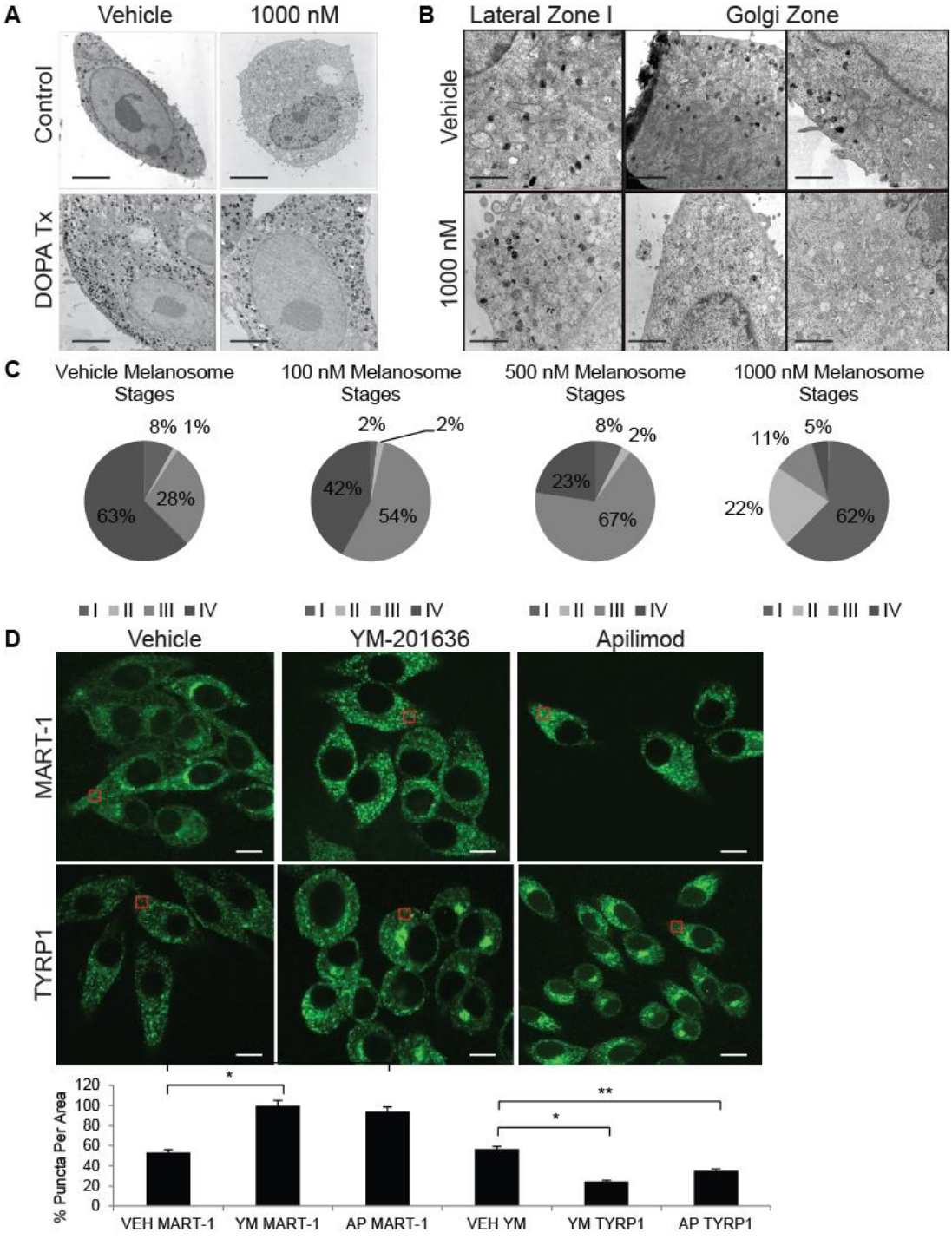

A
Vehicle
1000 nM
Control
DOPA Tx
B
Lateral Zone I
Golgi Zone
Vehicle
1000 nM
C
Vehicle Melanosome Stages
100 nM Melanosome Stages
500 nM Melanosome Stages
1000 nM Melanosome Stages
8% 1%
28%
63%
2%
2%
42%
54%
8%
2%
23%
67%
11%
5%
22%
62%
I II III IV
D
Vehicle
YM-201636
Apilimod
MART-1
TYRP1
% Puncta Per Area
120
100
80
60
40
20
0
*
**
*
VEH MART-1
YM MART-1
AP MART-1
VEH YM
YM TYRP1
AP TYRP1

Figure 2.4. PIKfyve knockdown inhibits the accumulation of proteins that synthesize melanin. MNT-1 human melanoma, darkly pigmented (DP) human epidermal melanocytes, B16 mouse melanoma cells, and C57/B6 mouse epidermal melanocytes were transduced with lentiviruses expressing independent shRNA's against PIKfyve (A,B), or SAC3 or VAC14 (C,D) or control, and selected with puromycin. Protein levels for MITF-M, TYR, and TYRP1 were accessed via immunoblotting. MITF-M was detected on a separate blot. (A,C) and quantified via densitometry analysis relative to GAPDH. mRNA levels of shRNA expressing cells were quantified via quantitative RT-PCR as described (B,D). For all experiments, all data are mean ± S.D. (n = 3 as indicated by error bars). A Student's paired t-test with a two-tailed normal distribution versus non-targeting scramble control. *, $p < 0.05$; **, $p < 0.01$; or ***, $p < 0.001$. Each experiment was performed with three biological replicates and three technical replicates.

Figure 4

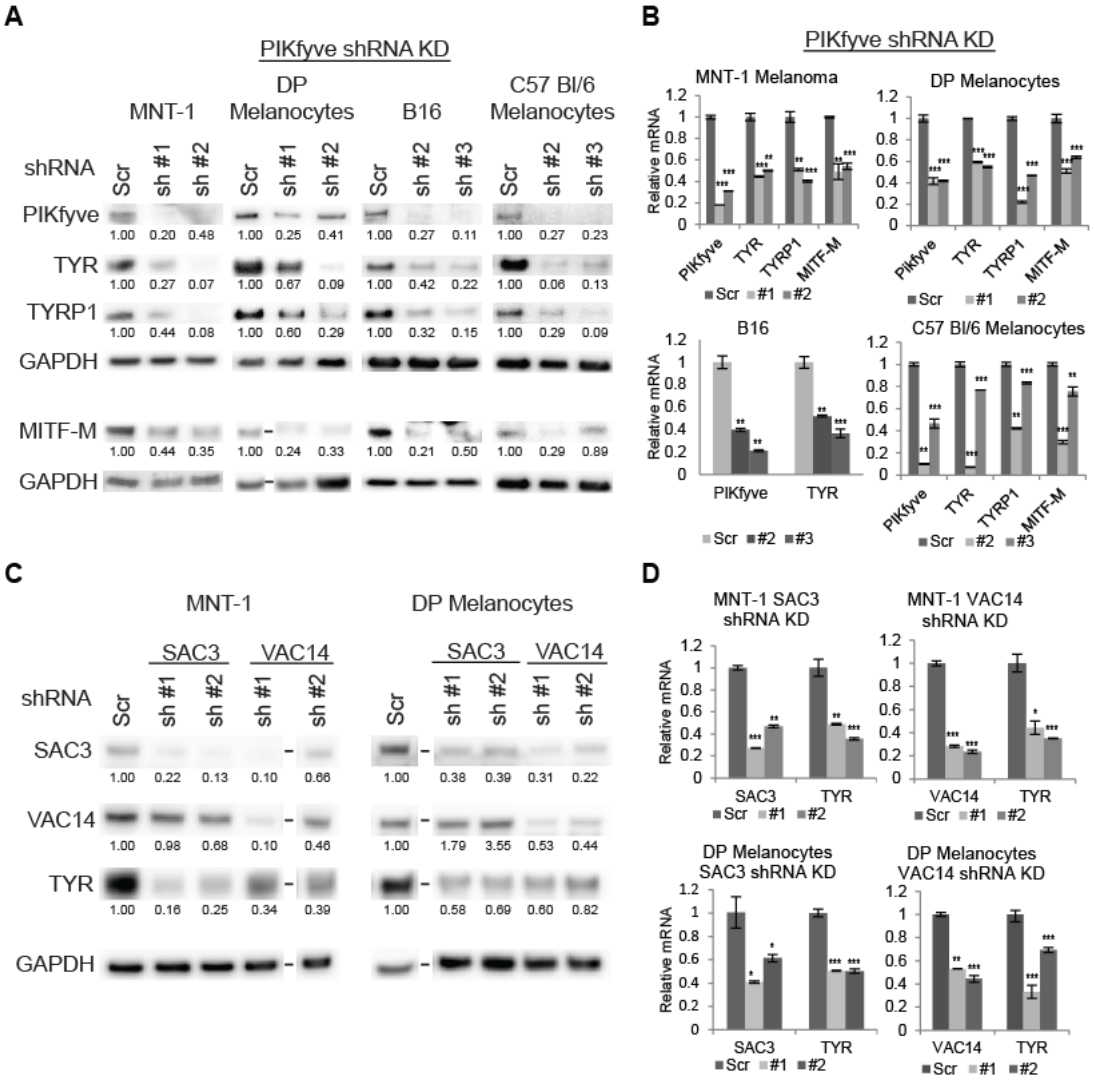

A
PIKfyve shRNA KD
MNT-1
DP Melanocytes
B16
C57 Bl/6 Melanocytes
shRNA
Scr
sh #1
sh #2
sh #3
PIKfyve
TYR
TYRP1
GAPDH
MITF-M
B
PIKfyve shRNA KD
MNT-1 Melanoma
DP Melanocytes
B16
C57 Bl/6 Melanocytes
Relative mRNA
PIKfyve
TYR
TYRP1
MITF-M
Scr
#1
#2
#3
C
MNT-1
DP Melanocytes
SAC3
VAC14
D
MNT-1 SAC3 shRNA KD
MNT-1 VAC14 shRNA KD
DP Melanocytes SAC3 shRNA KD
DP Melanocytes VAC14 shRNA KD

Figure 2.5. PIKfyve regulates melanogenic transcription by stabilizing WIPI1. A) MNT-1 cells, darkly pigmented (DP) melanocytes, B16 mouse melanoma cells, and C57/B6 mouse epidermal melanocytes expressing shRNA's against PIKfyve or non-targeting scramble control were analyzed. The amount of accumulated WIPI1 and WIPI2 proteins was assessed relative to GAPDH using immunoblotting. Densitometry values for each lane are reported. B) mRNA levels of shRNA expressing cells described in (A) were quantified via quantitative RT-PCR. C) MNT-1 cells treated with PIKfyve inhibitors for 72 hours were lysed and the relative accumulation of WIPI1 and WIPI2 proteins was measured via immunoblotting. D) MNT-1 cell lysates were immunoprecipitated with the indicated antibodies (PIKfyve, VAC14, WIPI1, WIPI2, or mouse IgG control) and probed with the antibodies indicated. All data are mean ± S.D. (n = 3 as indicated by error bars). A Student's paired t-test with a two-tailed normal distribution versus controls. *, $p < 0.05$; **, $p < 0.01$; or ***, $p < 0.001$. Each experiment was performed with three biological replicates and three technical replicates.

Figure 5

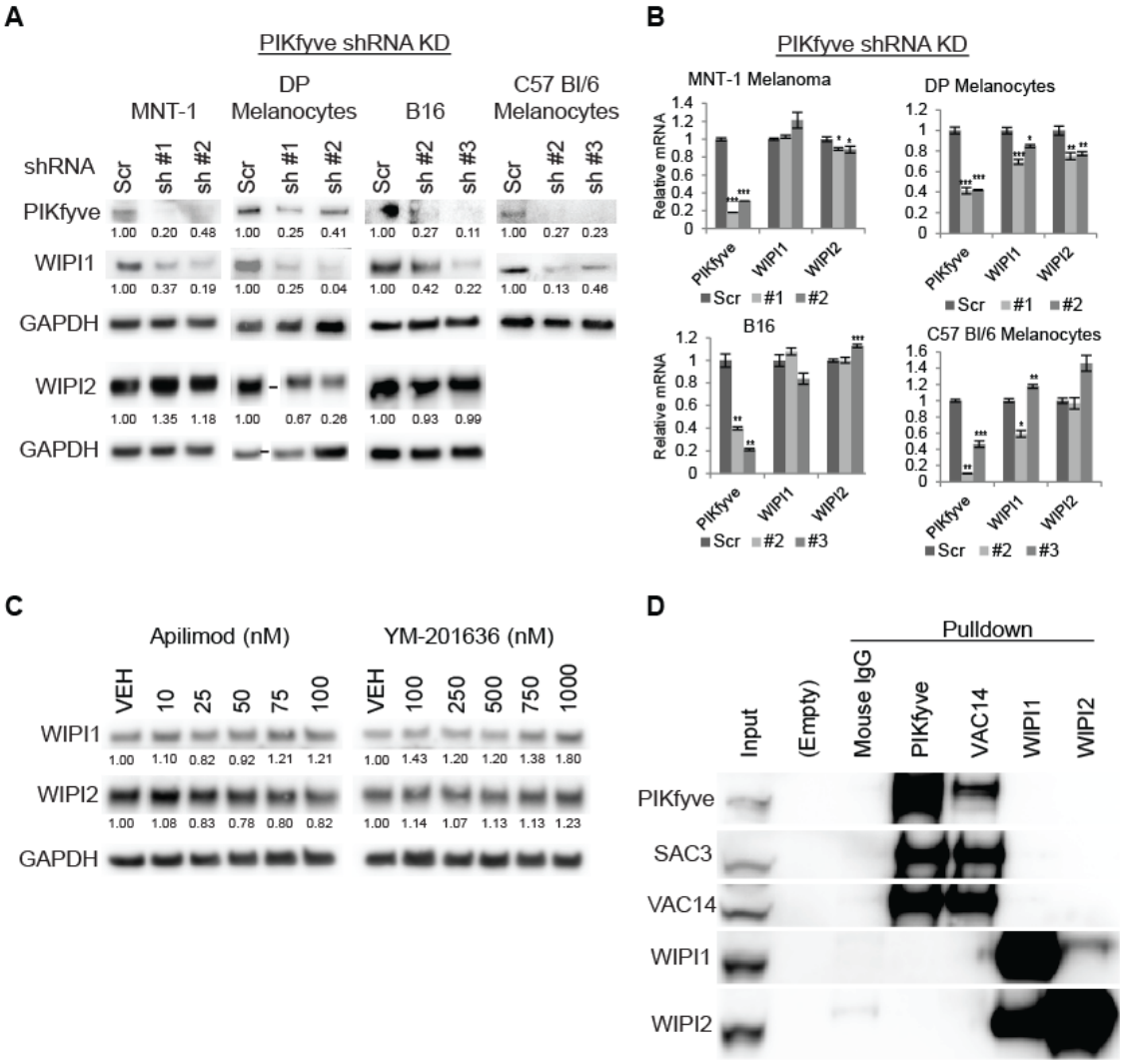

A
PIKfyve shRNA KD
MNT-1
DP Melanocytes
B16
C57 Bl/6 Melanocytes
shRNA
Scr
sh #1
sh #2
sh #3
PIKfyve
WIPI1
GAPDH
WIPI2
GAPDH
B
PIKfyve shRNA KD
MNT-1 Melanoma
DP Melanocytes
B16
C57 Bl/6 Melanocytes
Relative mRNA
PIKfyve
WIPI1
WIPI2
Scr
#1
#2
#3
C
Apilimod (nM)
YM-201636 (nM)
VEH
10
25
50
75
100
250
500
750
1000
WIPI1
WIPI2
GAPDH
D
Pulldown
Input
(Empty)
Mouse IgG
PIKfyve
VAC14
WIPI1
WIPI2
PIKfyve
SAC3
VAC14
WIPI1
WIPI2

Figure 2.6. PI5P stimulates melanogenesis. A) Lysates from HEK293T cells overexpressing WIPI1 were incubated with agarose beads coated with PI(3)P, $PI(3,5)P_2$, or PI(5)P. Bound proteins were eluted and assessed by immunoblotting. Uncoated beads were used as a control. MNT-1 cells were treated with 10 µM of phospholipids [PI(3)P, $PI(3,5)P_2$, PI(5)P] or equimolar amounts of appropriate carriers for 72 hours before lysis. B) The amount of TYR and WIPI1 protein that accumulated in lipid treated cells was measured by immunoblotting and quantified by protein densitometry relative to a GAPDH loading control. C) Relative mRNA levels of the genes in phospholipid treated cells were quantified via quantitative RT-PCR as described. PI(3)P and PI(5)P were quantified relative to carrier 3. $PI(3,5)P_2$ was quantified relative to carrier 2. D) MNT-1 cells were treated with 1000 nM YM-201636 or vehicle control for five days and 0.5 µM of phospholipids [PI(3)P, $PI(3,5)P_2$, PI(5)P] or carrier alone. Relative cell survival was quantified using a Cell-Titer-Glo assay described previously (69). All data are mean ± S.D. (n = 3 as indicated by error bars). A Student's paired t-test with a two-tailed normal distribution versus controls. *, $p < 0.05$; **, $p < 0.01$; or ***, $p < 0.001$. Each experiment was performed with three biological replicates and three technical replicates.

Figure 6

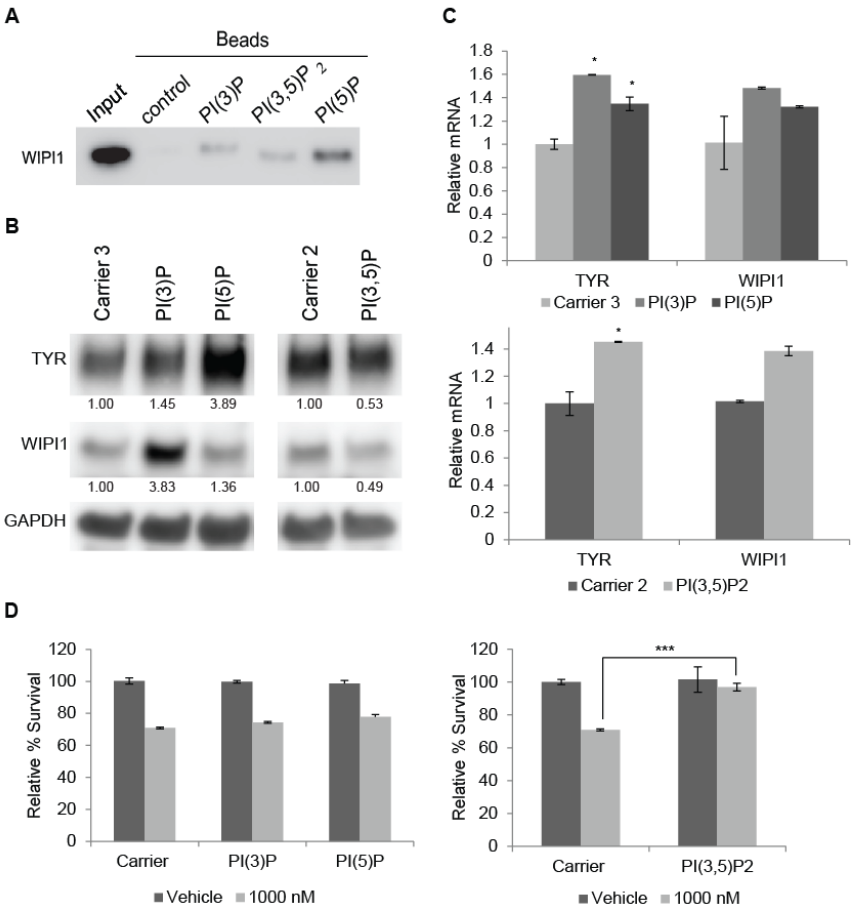

A
Beads
Input
control
PI(3)P
PI(3,5)P 2
PI(5)P
WIPI1
B
Carrier 3
PI(3)P
PI(5)P
Carrier 2
PI(3,5)P
TYR
1.00 1.45 3.89 1.00 0.53
WIPI1
1.00 3.83 1.36 1.00 0.49
GAPDH
C
Relative mRNA
TYR
WIPI1
Carrier 3 PI(3)P PI(5)P
Carrier 2 PI(3,5)P2
D
Relative % Survival
Carrier
PI(3)P
PI(5)P
PI(3,5)P2
Vehicle 1000 nM
***

Figure 2.7. PIKfyve regulates melanogenesis and melanocyte survival. Synthesis of PI(3,5)P_2 by PIKfyve regulates both melanosome biogenesis and the survival of melanocytes. PIKfyve also stimulates melanogenesis through the production of PI(5)P by either a direct or indirect mechanism.

Figure 7

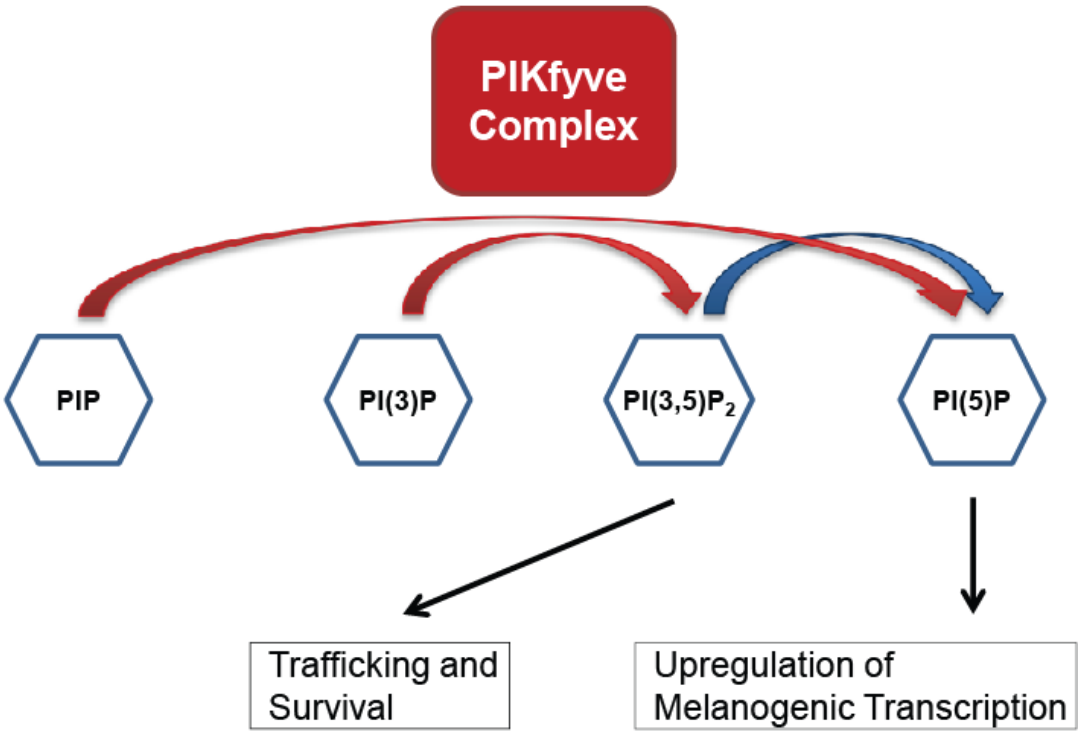

PIKfyve Complex
PIP
PI(3)P
PI(3,5)P2
PI(5)P
Trafficking and Survival
Upregulation of Melanogenic Transcription

Figure 2.S1. Melanocyte specific PIKfyve knockout mice exhibit hair greying. A) Representative photographs of littermates from each group. Unfed = *Tyrosinase::Cre*^{ERT2}; *PIKFYVE*$^{Flox/Flox}$ that were only administered normal feed. Cre- = *PIKFYVE*$^{Flox/Flox}$. Cre+ = *Tyrosinase::Cre*^{ERT2}; *PIKFYVE*$^{Flox/Flox}$. Side-by-side comparison of female littermates photographed at p35 prior to shave depilation and induction of a new hair cycle. *Tyrosinase::Cre*^{ERT2}; *PIKFYVE*$^{Flox/Flox}$ (left two) and *PIKFYVE*$^{Flox/Flox}$ (middle two) were administered tamoxifen feed from day 21 to 35. The two mice on the far right (*Tyrosinase::Cre*^{ERT2}; *PIKFYVE*$^{Flox/Flox}$) were administered normal feed for days 21-35. B) Representative photographs of individual littermates from each group taken at p85 (left three) or p105 (right three). C) Side-by-side comparison of representative female littermates photographed at p105. D) Four-mm skin biopsies taken at P105 were formalin fixed, dehydrated, and paraffin embedded. Skin was sectioned and immunostained with a TRP2 antibody. TRP2 expressing cells were then identified using a FITC-conjugated secondary antibody. Representative 10x images are shown.

Supplemental Figure 1

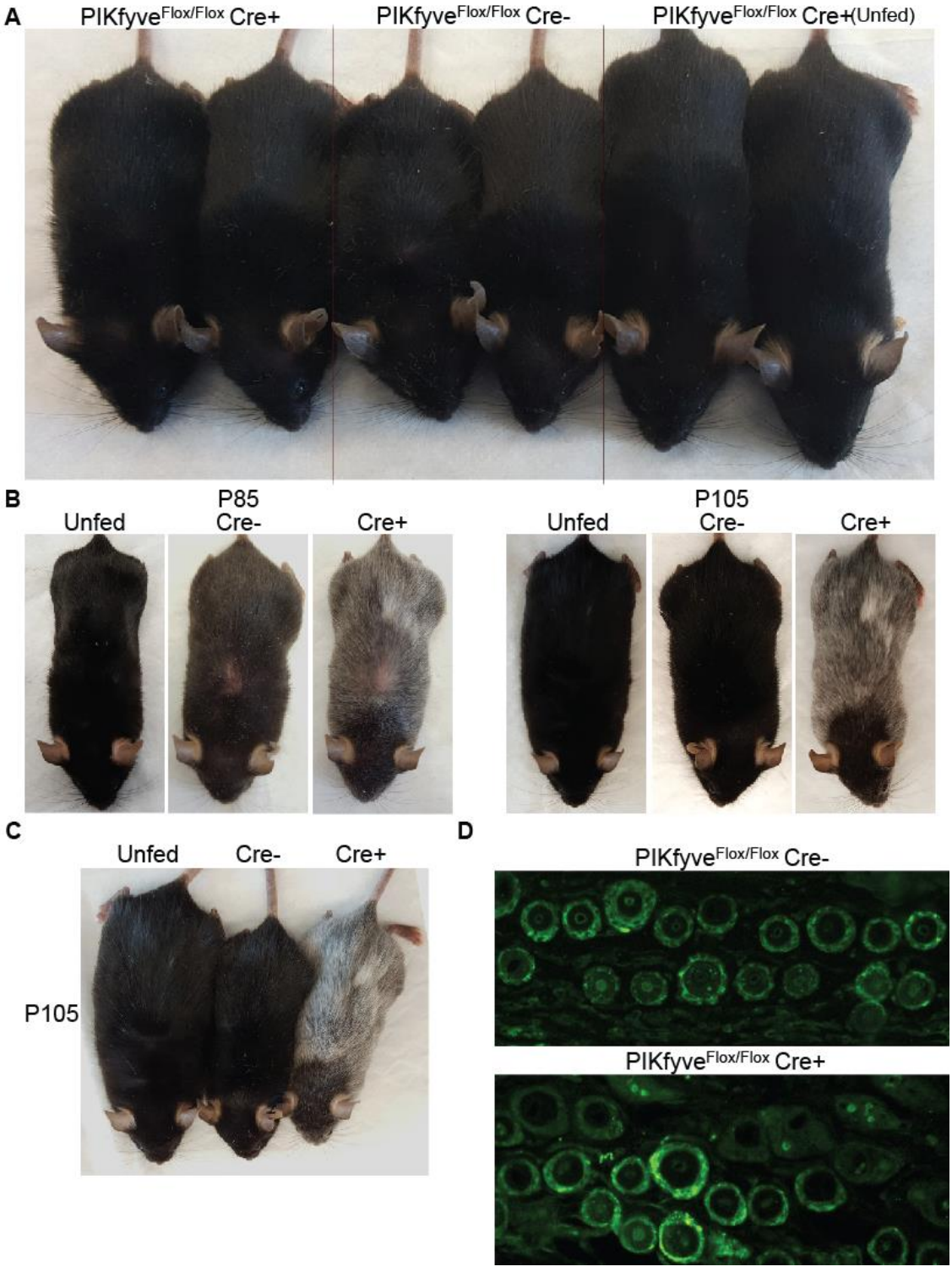


Figure 2.S2. PIKfyve inhibition results in decreased number of advanced stage melanosomes. A) Quantification of melanosome stages as percentage in NHM treated with 100, 500, or 1000 nM YM-021636 or vehicle. B) Normal human melanocytes (NHM) were treated with 1000 nM YM-201636 or vehicle and observed by electron microscopy. Arrows indicate multivesicular bodies that after DOPA histochemistry appear to have reaction product peripherally around their limiting membranes. *Scale bar*, 2 µm. C) Normal human melanocytes (NHM) were treated with 1000 nM YM-201636 or vehicle, processed for DOPA histochemistry and observed by electron microscopy. The density of DOPA positive 50nm vesicles in the Golgi area was quantitated. *Scale bar*, 2 µm. D) Numeration of melanosome density if the cell body and dendrite of NHM treated with 1000 nM YM-201636 or vehicle without or with DOPA histochemistry and P values determined by Student t-Test of the density data.

Supplemental Figure 2

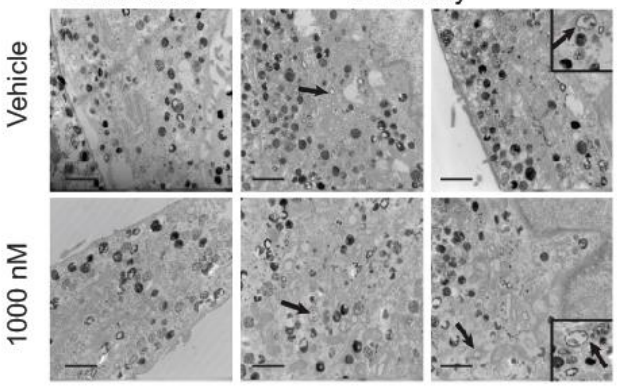
A

YM-201636 Treatment

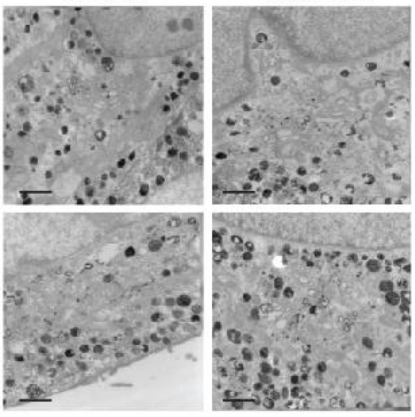
Melanosome Stages	Vehicle	100 nM	500 nM	1000 nM
Stage I	8.12 ± 7.4	1.71 ± 4.7 **	7.48 ± 10.9 *	62.42 ± 18.4 **
Stage II	1.31 ± 4.3	1.89 ± 3.6 *	2.42 ± 2.8 *	21.86 ± 12.0 **
Stage III	28.78 ± 11.9	54.33 ± 12.7 **	67.72 9.3 **	11.27 ± 10.2 **
Stage IV	63.78 ± 15.9	42.06 ± 12.9 **	22.78 ± 10.0 **	4.45 ± 8.0 **

* = P ≤ 0.05 ** = P ≤ 0.005 (Relative to Vehicle Control)

B



C



Vesicles / 100 μm²

Vehicle	38.89
1000 nm	77.52

P=0.016

Table 2.S1. Numeration of melanosome density of the cell body and dendrite of NHM treated with 1000 nM YM-201636 or vehicle without or with DOPA histochemistry.

Treatment	Histochemistry	Cell Area	Average # melanosomes/100 μm^2
Vehicle	No DOPA	Cell Body	129
Vehicle	DOPA	Cell Body	139
1000 nM YM-201636	No DOPA	Cell Body	171.3
1000 nM YM-201636	DOPA	Cell Body	179.4
Vehicle	NO DOPA	Dendrite	142.5
Vehicle	DOPA	Dendrite	138.5
1000 nM YM-201636	No DOPA	Dendrite	139.5
1000 nM YM-201636	DOPA	Dendrite	140.6

Table 2.S2. P values determined by Student t-Test of the density data presented in Table 2.S1.

t-Test	Site	P values
Vehicle-no DOPA vs Vehicle-DOPA	Cell Body	0.323
YM 1000 nM-no DOPA vs YM 1000 nM-DOPA	Cell Body	0.352
Vehicle-no DOPA vs YM 1000 nM-no DOPA	Cell Body	0.067
Vehicle-DOPA vs YM 1000 nM-DOPA	Cell Body	0.022
Vehicle-no DOPA vs Vehicle-DOPA	Dendrite	0.137
YM 1000 nM-no DOPA vs YM 1000 nM-DOPA	Dendrite	0.484
Vehicle-no DOPA vs YM 1000 nM-no DOPA	Dendrite	0.154
Vehicle-DOPA vs YM 1000 nM-DOPA	Dendrite	0.462

Table 2.S3. List of antibodies used in experiments.
WB = Western Blot. IP = immunoprecipitation. IF = Immunofluorescence. If two applications are listed, concentrations used depict respective antibody concentrations used.

Antibody	Host	Application	Dilution	Company	Catalog No.
Anti-Cathepsin D	Rabbit	WB	1:1000	Cell Signaling	2284
Anti-SAC3	Mouse	WB	1:1000	Antibodies Inc.	75-201
Anti-GAPDH	Rabbit	WB	1:5000	Cell Signaling	5174
Anti-HMB45 (PMEL)	Mouse	WB	1:1000	Thermo Scientific	MA5-13232
Anti-Melan-A (MART1)	Mouse	IF	1:2500	Santa Cruz	Sc-20032
Anti-MITF	Mouse	WB	1:500	Invitrogen	18-0369
Anti-PIP5K3 (PIKfyve)	Mouse	WB / IP	1:1000 / 1:500	Abnova	H00200576-M01
Anti-Tyrosinase (TYR)	Mouse	WB	1:3000	Abcam	ab738
Anti-Tyrosinase (TYR)	Goat	WB	1:1000	Santa Cruz	Sc-7833
Anti-TRP1	Mouse	WB / IF	1:500 / 1:5000	Abcam	ab3312
Anti-TRP2	Goat	IF	1:50	Santa Cruz	Sc-10451
Anti-VAC14	Mouse	WB / IP	1:1000 / 1:200	Santa Cruz	Sc-271831
Anti-WIPI49 (WIPI1)	Mouse	WB / IP	1:1000 / 1:1250	Abnova	H00055062-M02
Anti-WIPI2	Mouse	WB / IP	1:1000 / 1:1000	AbD Serotec	MCA5780GA
Anti-Goat IgG, HRP-Linked	Rabbit	WB	1:5000	Santa Cruz	Sc-2768
anti-rabbit IgG, HRP-linked	Goat	WB	1:5000	Cell Signaling	7074
anti-mouse IgG, HRP-linked	Horse	WB	1:5000	Cell Signaling	7076
EasyBlot anti-mouse IgG, HRP-linked		WB	1:5000	GeneTex	GTX221667-01
Normal Mouse IgG	Mouse	IP	1:200	Santa Cruz	Sc-2025

Table S4. List of primers used in experiments and their sequences, 5’ to 3’.

Primer	Sequence 5' to 3'	Animal	Application
Cre	GCGGTCTGGCAGTAAAAACTATC	Mouse	Genotyping
Cre	GTGAAACAGCATTGCTGTCACTT	Mouse	Genotyping
PIKfyve^{Flox}	GAGAAAGGGGACAGTGTTTGGC	Mouse	Genotyping
PIKfyve^{Flox}	CCAGATCTTGCACTGTAACCACAAACCAC	Mouse	Genotyping
Pikfyve-Forward	TGGCCACAGATGATAAGACGT	Human	Q RT-PCR
Pikfyve-Reverse	GGGGCTCATCTTGATCAGGAG	Human	Q RT-PCR
Pikfyve-Forward	TCATGAAATGGCCACAGATG	Mouse	Q RT-PCR
Pikfyve-Reverse	TGGTCAGGAGTCAAGGGTTT	Mouse	Q RT-PCR
Tyr-Forward	AGGCAGAGGTTCCTGTCAGA	Human	Q RT-PCR
Tyr-Reverse	ATCCCATGAAGTTGCCAGAG	Human	Q RT-PCR
Tyr-Forward	GGGCCCAAATTGTACAGAGA	Mouse	Q RT-PCR
Tyr-Reverse	ATGGGTGTTGACCCATTGTT	Mouse	Q RT-PCR
Tyrp1-Forward	CGCACAACTCACCCTTTATTT	Human	Q RT-PCR
Tyrp1-Reverse	CTTTCCTGTCCTACCCCAAG	Human	Q RT-PCR
Tyrp1-Forward	ACCCATTTGTCTCCCAATGA	Mouse	Q RT-PCR
Tyrp1-Reverse	CCTCCTTAGCCATTCGTCAA	Mouse	Q RT-PCR
Vac14-Forward	GCTAAGGAGTCGCGAGGTTC	Human	Q RT-PCR
Vac14-Reverse	TTCCGCTTTTCGTACAGCTT	Human	Q RT-PCR
Vac14-Forward	CAACCTCGGGCTAAGGTGT	Mouse	Q RT-PCR
Vac14-Reverse	CAACCTTCCGCTTTTCGTAG	Mouse	Q RT-PCR
Fig4-Forward	GTGTTGTTCCTGGCTGGACT	Human	Q RT-PCR
Fig4-Reverse	TTCTGGACCGAGCTGATGAT	Human	Q RT-PCR
Fig4-Forward	GTCTGGTGTGCTGGAGGTCT	Mouse	Q RT-PCR
Fig4-Reverse	TTCTGGACCGAGCTGATGAT	Mouse	Q RT-PCR
MITF-M-Forward	ACCT TCTCTTTGCCAGTCCA	Human	Q RT-PCR
MITF-M-Reverse	CCAATCCAGTGAGAGACGGT	Human	Q RT-PCR
MITF-M-Forward	TATGGTGCCTTCTTTATGCC	Mouse	Q RT-PCR
MITF-M-Reverse	AGCATAGCAAGGTTTCAGG	Mouse	Q RT-PCR
Wipi1-Forward	TGAAATCCCGGACGTCTACA	Human	Q RT-PCR
Wipi1-Reverse	GCCGTGGTTTTGTGTGACTG	Human	Q RT-PCR
Wipi1-Forward	TATATCGTGGAGCGCCTCTT	Mouse	Q RT-PCR
Wipi1-Reverse	CGGTTGAGCCGAATAGACA	Mouse	Q RT-PCR
Wipi2-Forward	GTTCTCCAGCAGCCTAGTGG	Human	Q RT-PCR
Wipi2-Reverse	ACAGCCAGAATCGTGTTGGA	Human	Q RT-PCR
Wipi2-Forward	AGATGACCTGGGTGCTGTG	Mouse	Q RT-PCR
Wipi2-Reverse	GGAGGATGTTCGCTGTCTTC	Mouse	Q RT-PCR
GAPDH-Forward	CCAACGTGTCAGTGGTGGA	Human	Q RT-PCR
GAPDH-Reverse	CGTCAAAGGTGGAGGAGTGG	Human	Q RT-PCR
β Actin-Forward	CGTCAAAGGTGGAGGAGTGG	Human	Q RT-PCR
β Actin-Reverse	AAGGAAGGCTGGAAGAGTGC	Human	Q RT-PCR
HPRT-Forward	GCGATGATGAACCAGGTTATGA	Mouse	Q RT-PCR
HPRT-Reverse	TCCAAATCCTCGGCATAATGA	Mouse	Q RT-PCR

Conclusions, Current and Future Directions

Chapter 3

Marc C. Liggins and Anand K. Ganesan

3.1 Elucidating the Role of PIKfyve in Melanogenesis *In Vivo*

The studies presented in chapter 2 demonstrate that the PIKfyve complex regulates melanogenesis. Our studies showed that lineage-dependent PIKfyve-knockout mice exhibit progressive whitening of the mouse coat (Figure 2.1). Similarly, previous studies have noted that *VAC14 ingls* (1) and *FIG4* pale tremor mice (2), both exhibited similar lightning of coat color, however this phenotype was not characterized. Interestingly, partial loss of function mutations in *FIG4* had a milder phenotype when compared to *FIG4* knockout mice (3), further suggesting that the phenotype was not a result of loss of melanocyte stem cells but instead a direct impact on melanogenesis. When coupled with previously published studies, it is clear that the PIKfyve complex plays a critical role in melanogenesis. While these studies have directly implicated a role for PIKfyve in melanogenesis, the mechanism has not been thoroughly elucidated.

The phenotype presented by our lineage-specific knockout mice would is characteristic of melanocyte stem cell loss, however, as indicated by TYRP2 staining, there were no significant differences in the total melanocyte population (Figure S2.1). The progressive loss of pigmentation in the mouse coat does however exhibit phenotypic similarity to our *in vitro* inhibitor studies (Figures 2.2 and 2.3) which would suggest that melanosome trafficking is being disrupted as a result. To determine how PIKfyve controls melanosome maturation *in vivo*, skin biopsies were accessed at the ultrastructural level by DOPA histochemistry electron microscopy (EM). Preliminary analysis of anagen hair follicles from control and flox mice were compared by EM (Figure 3.1A). Melanocytes from knockout mice exhibit three morphological phenotypes

(Figure 3.1B). About 40% of melanocytes appear normal with all stages of melanosomes present, while another 40% exhibit extensive vacuolization with few melanosomes, and the remaining 20% present an intermediate phenotype (Figure 3.1C). Similarly, after DOPA incubation, morphologically normal cells contain melanin reaction product, while uncharacteristic deposition is observed in intermediate cells and abnormal cells lack reaction product completely (Figure 3.1C). As a result, anagen hair follicles from knockout mice exhibit minimal or no melanin in intermediate and abnormal melanocytes respectively (Figure 3.1). The lack of DOPA staining would further suggest the loss of TYR protein. These results complement our Nanostring findings that indicated the reduced accumulation of tyrosinase mRNAs (Figure 2.1). Taken together, these studies indicate that PIKfyve regulates melanosome maturation *in vivo* and would also suggests that PIKfyve regulates the accumulation of tyrosinase mRNA. The PIKfyve complex functions as a critical phosphoinositol kinase in mammalian cells as it is responsible for generating the majority of PI(5)P and all $PI(3,5)P_2$ (4-6). To further determine how PIKfyve regulates melanogenesis *in vivo* it is necessary to assess the contributions of its product phosphoinositides.

Previous studies have implicated that $PI(3,5)P_2$ regulates membrane trafficking as loss of the phospholipid or PIKfyve machinery results in the accumulation of large intracellular vacuoles both *in vitro* and *in vivo* (1, 3, 4, 6-8). Furthermore, $PI(3,5)P_2$ has also been implicated in melanogenesis, specifically mutations in two PIKfyve effectors have been identified that result in pigment abnormalities (9-13). To elaborate upon the differing roles of PI(5)P and all $PI(3,5)P_2$ *in vivo*, we are currently utilizing established protocols (14) to isolate and culture primary melanocytes from *PIKfyve* flox and control

mice and inducing PIKfyve deletion with tamoxifen *in vitro*. The isolated melanocytes will be treated with PI(5)P or $PI(3,5)P_2$ and phenotypically characterized. Given prior studies and our *in vitro* findings (Figure 2.6), we hypothesize that $PI(3,5)P_2$ treatment will result in rescue of all defects while PI(5)P may induce the transcription of tyrosinase. Future characterization of the effects of these lipids would require identification of specific phosphoinositide effectors.

Recent *in vitro* studies have identified PIKfyve point mutations in the kinase domain which bias PIKfyve production towards $PI(3,5)P_2$ or PI(5)P (5, 6). $PIKfyve^{K1999E}$ exhibits significantly reduced levels of $PI(3,5)P_2$ and profound vacuolization, while $PIKfyve^{K2000E}$ only perturbed PI(5)P accumulation but no aberrant morphology was observed (5, 6). To determine the *in vivo* roles of $PI(3,5)P_2$ and PI(5)P in melanogenesis, we will employ lineage-specific, inducible, PIKfyve point mutations. Isolating and culturing melanocytes from these models will serve to further complement our studies through in depth molecular and biochemical analysis as previously conducted. We hypothesize that $PIKfyve^{K1999E}$ will exhibit phenotypic similarity to our PIKfyve flox mice, characterized by significant vacuolization, trafficking defects, and progressive pigment loss. In support of our reasoning, only $PIKfyve^{K1999E}$ cells exhibited vacuolization which is indicative of trafficking defects (5, 6). As $PI(3,5)P_2$ has been shown to be essential for multivesicular body (MVB) (7, 8) and lysosomal trafficking (15-17), it stands to reason that loss of the phospholipid would similarly effect LRO trafficking. Complimentary to this, our *in vitro* studies have shown that PIKfyve inhibition resulted in the accumulation of tyrosinase within multivesicular bodies (Figure 2.3). In contrast, if PI(5)P does significantly contribute to melanogenesis, we would expect

PIKfyve^{K2000E} mutant mice to exhibit an intermediate pigment phenotype. Partly, due to only partial loss of function (5, 6) and unclear contributions from MTMRs, residual levels of PI(5)P would remain. Furthermore, the contributions of PI(5)P to melanosomal gene expression are less defined. Given that neither PIKfyve knockdown or knockout completely depleted TYR mRNA (Figures 2.1 and 2.4) and that studies have shown that incomplete depletion of TYR does not profoundly affect pigment accumulation (18), we expect only a mild pigment phenotype would be observed if at all. Given that our established model results in deletion of the kinase domain (19), a double mutant should be phenotypically identical to our established mouse model. Furthermore, another potential caveat in our analyses is that these models exhibited incomplete depletion of the specific lipids *in vitro* (5, 6). To overcome similar limitations, previous studies have transfected reporter plasmids or overexpressed PtdIns5P 4-kinases in order to further deplete PI(5)P levels (20). Utilizing similar methodology, we can deplete PI(5)P in isolated melanocytes using melanocyte-optimized protocols (14). While our *in* vivo studies have clearly demonstrated the PIKfyve is required for melanogenesis *in vivo,* these additional studies will provide significant insights into the regulatory mechanism and will help elucidate the individual roles of PI(5)P and PI(3,5)P_2.

3.2 PI(3,5)P_2 coordinates trafficking to melanosome and melanosome acidification

Given our findings, it's likely that PIKfyve coordinates different melanogenic processes through synthesis of different phosphoinositides. While several challenges

are presented in determining the trafficking dynamics of PIKfyve, the challenges posed in understanding transcriptional regulation are more easily overcome. Determining the functional roles of the individual phosphoinositide products presents several challenges as they have not been well characterized. Furthermore, as PIKfyve is responsible for generating both $PI(3,5)P_2$ and PI(5)P (4-6), it is difficult to ascertain specific functions to either phosphoinositide unless the specific effectors are known. Very few effector proteins have been identified and even fewer that only bind $PI(3,5)P_2$ or PI(5)P. However, it is plausible that subcellular localization of different phosphoinositides may confer different function to these factors.

During melanosome biogenesis, PMEL and MART-1 are transported together to early endosomes via AP-2 vesicles (21-23). Endosome acidification is necessary to induce proteolytic cleave of PMEL prior to progression into stage II melanosomes (21-23). Our studies have shown that upon PIKfyve inhibition, there are increased numbers of stage I melanosomes as verified by immunofluorescence staining with MART-1 (Figure 2.3). As the melanosome matures, MART-1 is believed to be recycled back to the trans-Golgi network (24). As $PI(3,5)P_2$ has been implicated in retrograde trafficking to the TGN (8, 25, 26), the melanosomal accumulation of MART-1 would suggest dysfunction in the pathway. However, this would also suggest that that AP-2 trafficking has not been disrupted as a result. Even though this would suggest that PMEL is being trafficked to the developing melanosomes despite PIKfyve inhibition, our results indicate that proteolytic cleavage of PMEL is not occurring (Figure 2.2) as further evidenced by our EM studies (Figure 2.3). Recent studies have identified TPC2 as a melanosomal $PI(3,5)P_2$ effector (12, 13). $PI(3,5)P_2$ activates the TPC2 cation channel resulting in

acidification of the melanosome (12, 13). Low pH is necessary for PMEL cleavage given that experiments have shown that the primary PMEL protease, beta-secretase 2, exhibits optimal proteolytic activity at pH 4.5 (27-29). Complimentary to our own studies, this would suggest that TPC2 is required for melanosomal acidification to induce proteolytic cleavage of PMEL. This may seem contradictory given that at low pH negatively effects tyrosinase activity (30) and decreases pigmentation (12, 13). Cellularly, OCA2 functions antagonistically of TPC2 in regulating melanosomal pH and pigmentation (31, 32). OCA2 is trafficked along with TYR and TYRP1 using AP-1/AP-3 vesicles to the developing melanosome. Given that TYR and TYRP1 trafficking is disrupted as a result of PIKfyve inhibition (Figure 2.3), OCA2 trafficking is likely also disrupted. Taken together, defects in acidification and deacidification, coupled with lower enzymatic trafficking, could explain the pronounced pigment phenotypes associated with PIKfyve dysfunction *in vitro* (Figure 2.1) and *in vivo* [Figure 2.3 and (1, 2)]. It would also stand to reason why an intermediate phenotype is exhibited as a result of partial loss function (3). Overall, these studies provide evidence directly implicating $PI(3,5)P_2$ in both melanosomal function and trafficking. Future studies will focus on confirming these hypotheses and elucidating the role of $PI(3,5)P_2$ in AP-1/AP-3 trafficking and confirming the role of TPC2 in PMEL processing.

Elucidating the regulatory mechanisms and expanding upon the roles of $PI(3,5)P_2$ in melanogenesis presents several caveats that must be overcome. Most prominently is that very few $PI(3,5)P_2$ effectors are known and that many PI binding proteins have affinity for more than one phosphoinositide. We will investigate the role of TPC2 in melanosomal acidification using RNAi knockdown to determine determining if

PMEL proteolytic cleavage is affected by immunoblotting. Additionally, we will measure the effects on melanosomal acidification using pH sensitive fluorophores. Our PIKfyve^{K1999E} studies will provide significant insight into the roles of PI(3,5)P_2 in membrane trafficking and acidification. To compliment these studies, we will conduct immunofluorescence analyses complimented with phosphoinositide rescue experiments to verify our findings. However, even if proposed melanosomal roles of PI(3,5)P_2 are verified through these studies, the appropriate effector proteins must be identified to complete our studies.

Three PI(3,5)P_2 effectors that have been characterized in melanocytes, however only TPC2 and TRPML3 affected pigmentation (9-13). Furthermore, pigment abnormalities associated with TPC2 and TRPML3 do not appear to be related to melanosomal trafficking (9-13). Given that members of TRPML family regulate endolysosomal trafficking (33, 34) and are specifically activated by PI(3,5)P_2, TRPML1 and TRPML2 remain prime candidates in our attempts to identify trafficking effector proteins (35). While TRPML1 has not been characterized in melanocytes, *in vitro* studies have implicated that its role in endolysosomal trafficking (35, 36). Given that there is a significant overlap in both the developmental processes and machinery utilized in both lysosome biogenesis and melanogenesis, it is reasonable to hypothesize that TRPML1 may regulate melanosomal trafficking. Permitting our hypothesis is correct, RNAi knockdown of the different TRPMLs combined with immunofluorescence analysis will allow us to determine if TRPML1 does indeed regulate melanosomal trafficking.

3.3 PI(5)P regulates gene expression in melanocytes

In our studies we sought to elucidate the role of PI(5)P in melanogenesis. Studies have found that $PI(3,5)P_2$ and PI(5)P both function in autophagy and lysosome biogenesis, but in functionally distinct and even antagonistic manners (20, 37-40). Coupling published studies with our own work would suggest that PIKfyve regulates melanosomal trafficking and development via $PI(3,5)P_2$. The contributions of PI(5)P are to melanogenesis are much less defined. PIKfyve knockdown resulted in the downregulation of several melanosomal proteins (Figure 2.4) exhibiting remarkable similarity to previous studies that had implicated WIPI1 in melanosomal regulation (41). Given these findings, it was not surprising that PIKfyve knockdown also resulted in decreased accumulation of WIPI1 protein, thus suggesting a functional relationship (Figure 2.5). WIPI1 is highly expressed melanocytes and melanoma cells (42, 43) and expression can be further up upregulated via keratinocyte signaling (44), thereby further highlighting its melanosomal significance. Given that WIPI1 can bind to PI(3)P, PI(3,5)P2, and PI(5)P (Figure 2.6A), it is plausible that recruitment by different phosphoinositides mediates differential function.

Previous studies within our lab demonstrated that WIPI1 modulates transcription via inhibition of mTORC1, thus resulting in activation of the mTORC2-AKT pathway (41). In turn this leads to β-Catenin stabilization which leads MITF-M transcription (41). Consistent with WIPI1 knockdown studies (41), PIKfyve knockdown decreased accumulation of β-Catenin and phosphorylated AKT (Figure 3.2A). Interestingly, addition of exogenous $PI(3,5)P_2$ or PI(5)P to untreated cells demonstrated that the

phospholipids oppositely effect AKT phosphorylation (Figure 3.2B) and TYR protein accumulation (Figure 2.6). These results would suggest that PI(5)P activates the AKT signaling pathway to increase melanosomal gene expression. Additionally, these findings compliment previous studies that have shown that PI(5)P upregulates AKT phosphorylation (5, 40, 45, 46). It is somewhat surprising that phosphorylated S6K increased as a result of PIKfyve knockdown (Figure 3.2A) given that $PI(3,5)P_2$ has been suggested to activate mTORC1 (37). Furthermore, decreased levels of $PI(3,5)P_2$ should increase activation of the mTORC2-AKT pathway, yet the opposite was observed. Taken together, this may suggest that WIPI1 functions downstream of mTOR and imply that WIPI1 may function as a PI(5)P effector. Overall, these studies would suggest that PI(5)P may modulate melanosomal transcription via AKT and WIPI1, however much work must be completed to flesh out these studies and formulate a solid hypothesis. Future studies will elaborate upon how these factors may work together in concert to regulate melanosomal processes.

Our findings suggest that PIKfyve may be modulating melanosomal transcription through PI(5)P synthesis. As nuclear PI(5)P is synthesized in a PIKfyve-independent manner, our findings would suggest that cytoplasmic PI(5)P is predominantly regulating melanogenic transcription. To confirm that PIKfyve is modulating melanosomal transcription, chromatin immunoprecipitation (ChIP) assays with RNA Polymerase II must be conducted. This will allow us to determine how transcription of MITF-M and TYR are being effected under different conditions and how $PI(3,5)P_2$ and PI(5)P are affecting promoter occupancy. Interestingly, WIPI1 can also localize to the nucleus (39). Permitting availability of ChIP-grade antibodies for WIPI1, ChIP-seq analysis in the

presence or absence of phosphoinositides will allow us to determine if WIPI1 is modulating melanosomal transcription cytoplasmically or nuclearly.

3.4 Conclusions

Previous studies have identified aberrant pigmentation associated with PIKfyve kinase complex mutations and in $PI(3,5)P_2$ effector proteins, however, with one very recent exception, PIKfyve has not been implicated in regulating melanosome biogenesis. The thesis work presented here not only defines novel roles of PIKfyve in regulating melanogenesis, but expands upon and bridges findings from previous studies. We demonstrate that PIKfyve coordinates both melanogenic trafficking and transcription through the synthesis of $PI(3,5)P_2$ and PI(5)P. Overall, our findings support the hypothesis that $PI(3,5)P_2$ regulates melanosomal pH and trafficking and that PI(5)P and WIPI1 coordinate melanosomal transcription. Future studies will elaborate upon the individual roles of the phosphoinositides themselves in melanogenesis and define their regulatory mechanisms. Nevertheless, this work expands upon the pool of knowledge and provides new insights into both melanocyte and phosphoinositide biology.

3.5 References

1. Jin N, Chow CY, Liu L, Zolov SN, Bronson R, Davisson M, et al. VAC14 nucleates a protein complex essential for the acute interconversion of PI3P and PI(3,5)P(2) in yeast and mouse. EMBO J. 2008;27(24):3221-34.

2. Chow CY, Zhang Y, Dowling JJ, Jin N, Adamska M, Shiga K, et al. Mutation of FIG4 causes neurodegeneration in the pale tremor mouse and patients with CMT4J. Nature. 2007;448(7149):68-72.

3. Lenk GM, Ferguson CJ, Chow CY, Jin N, Jones JM, Grant AE, et al. Pathogenic mechanism of the FIG4 mutation responsible for Charcot-Marie-Tooth disease CMT4J. PLoS Genet. 2011;7(6):e1002104.

4. Zolov SN, Bridges D, Zhang Y, Lee WW, Riehle E, Verma R, et al. In vivo, Pikfyve generates PI(3,5)P2, which serves as both a signaling lipid and the major precursor for PI5P. Proc Natl Acad Sci U S A. 2012;109(43):17472-7.

5. Shisheva A. PtdIns5P: news and views of its appearance, disappearance and deeds. Arch Biochem Biophys. 2013;538(2):171-80.

6. Shisheva A, Sbrissa D, Ikonomov O. Plentiful PtdIns5P from scanty PtdIns(3,5)P2 or from ample PtdIns? PIKfyve-dependent models: Evidence and speculation (response to: DOI 10.1002/bies.201300012). Bioessays. 2015;37(3):267-77.

7. Whitley P, Reaves BJ, Hashimoto M, Riley AM, Potter BV, Holman GD. Identification of mammalian Vps24p as an effector of phosphatidylinositol 3,5-bisphosphate-dependent endosome compartmentalization. J Biol Chem. 2003;278(40):38786-95.

8. Jin N, Lang MJ, Weisman LS. Phosphatidylinositol 3,5-bisphosphate: regulation of cellular events in space and time. Biochem Soc Trans. 2016;44(1):177-84.

9. Xu H, Delling M, Li L, Dong X, Clapham DE. Activating mutation in a mucolipin transient receptor potential channel leads to melanocyte loss in varitint-waddler mice. Proc Natl Acad Sci U S A. 2007;104(46):18321-6.

10. Sulem P, Gudbjartsson DF, Stacey SN, Helgason A, Rafnar T, Jakobsdottir M, et al. Two newly identified genetic determinants of pigmentation in Europeans. Nat Genet. 2008;40(7):835-7.

11. Lin-Moshier Y, Keebler MV, Hooper R, Boulware MJ, Liu X, Churamani D, et al. The Two-pore channel (TPC) interactome unmasks isoform-specific roles for TPCs in endolysosomal morphology and cell pigmentation. Proc Natl Acad Sci U S A. 2014;111(36):13087-92.

12. Ambrosio AL, Boyle JA, Aradi AE, Christian KA, Di Pietro SM. TPC2 controls pigmentation by regulating melanosome pH and size. Proc Natl Acad Sci U S A. 2016;113(20):5622-7.

13. Bellono NW, Escobar IE, Oancea E. A melanosomal two-pore sodium channel regulates pigmentation. Sci Rep. 2016;6:26570.

14. Godwin LS, Castle JT, Kohli JS, Goff PS, Cairney CJ, Keith WN, et al. Isolation, culture, and transfection of melanocytes. Curr Protoc Cell Biol. 2014;63:1 8 1-20.

15. Kim GH, Dayam RM, Prashar A, Terebiznik M, Botelho RJ. PIKfyve inhibition interferes with phagosome and endosome maturation in macrophages. Traffic. 2014;15(10):1143-63.

16. Min SH, Suzuki A, Stalker TJ, Zhao L, Wang Y, McKennan C, et al. Loss of PIKfyve in platelets causes a lysosomal disease leading to inflammation and thrombosis in mice. Nat Commun. 2014;5:4691.

17. Kerr MC, Wang JT, Castro NA, Hamilton NA, Town L, Brown DL, et al. Inhibition of the PtdIns(5) kinase PIKfyve disrupts intracellular replication of Salmonella. EMBO J. 2010;29(8):1331-47.

18. Paterson EK, Fielder TJ, MacGregor GR, Ito S, Wakamatsu K, Gillen DL, et al. Tyrosinase Depletion Prevents the Maturation of Melanosomes in the Mouse Hair Follicle. PLoS One. 2015;10(11):e0143702.

19. Takasuga S, Horie Y, Sasaki J, Sun-Wada GH, Kawamura N, Iizuka R, et al. Critical roles of type III phosphatidylinositol phosphate kinase in murine embryonic visceral endoderm and adult intestine. Proc Natl Acad Sci U S A. 2013;110(5):1726-31.

20. Vicinanza M, Korolchuk VI, Ashkenazi A, Puri C, Menzies FM, Clarke JH, et al. PI(5)P regulates autophagosome biogenesis. Mol Cell. 2015;57(2):219-34.

21. Watt B, van Niel G, Raposo G, Marks MS. PMEL: a pigment cell-specific model for functional amyloid formation. Pigment Cell Melanoma Res. 2013;26(3):300-15.

22. Hoashi T, Watabe H, Muller J, Yamaguchi Y, Vieira WD, Hearing VJ. MART-1 is required for the function of the melanosomal matrix protein PMEL17/GP100 and the maturation of melanosomes. J Biol Chem. 2005;280(14):14006-16.

23. Sitaram A, Marks MS. Mechanisms of protein delivery to melanosomes in pigment cells. Physiology (Bethesda). 2012;27(2):85-99.

24. De Maziere AM, Muehlethaler K, van Donselaar E, Salvi S, Davoust J, Cerottini JC, et al. The melanocytic protein Melan-A/MART-1 has a subcellular localization distinct from typical melanosomal proteins. Traffic. 2002;3(9):678-93.

25. Odorizzi G, Babst M, Emr SD. Fab1p PtdIns(3)P 5-kinase function essential for protein sorting in the multivesicular body. Cell. 1998;95(6):847-58.

26. Rutherford AC, Traer C, Wassmer T, Pattni K, Bujny MV, Carlton JG, et al. The mammalian phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) regulates endosome-to-TGN retrograde transport. J Cell Sci. 2006;119(Pt 19):3944-57.

27. Shimshek DR, Jacobson LH, Kolly C, Zamurovic N, Balavenkatraman KK, Morawiec L, et al. Pharmacological BACE1 and BACE2 inhibition induces hair depigmentation by inhibiting PMEL17 processing in mice. Sci Rep. 2016;6:21917.

28. Rochin L, Hurbain I, Serneels L, Fort C, Watt B, Leblanc P, et al. BACE2 processes PMEL to form the melanosome amyloid matrix in pigment cells. Proc Natl Acad Sci U S A. 2013;110(26):10658-63.

29. Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendiaz EA, Denis P, et al. Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science. 1999;286(5440):735-41.

30. Slominski A, Tobin DJ, Shibahara S, Wortsman J. Melanin pigmentation in mammalian skin and its hormonal regulation. Physiol Rev. 2004;84(4):1155-228.

31. Bellono NW, Escobar IE, Lefkovith AJ, Marks MS, Oancea E. An intracellular anion channel critical for pigmentation. Elife. 2014;3:e04543.

32. Bellono NW, Oancea EV. Ion transport in pigmentation. Arch Biochem Biophys. 2014;563:35-41.

33. Cheng X, Shen D, Samie M, Xu H. Mucolipins: Intracellular TRPML1-3 channels. FEBS Lett. 2010;584(10):2013-21.

34. Puertollano R, Kiselyov K. TRPMLs: in sickness and in health. Am J Physiol Renal Physiol. 2009;296(6):F1245-54.

35. Dong XP, Shen D, Wang X, Dawson T, Li X, Zhang Q, et al. PI(3,5)P(2) controls membrane trafficking by direct activation of mucolipin Ca(2+) release channels in the endolysosome. Nat Commun. 2010;1:38.

36. Li X, Saitoh S, Shibata T, Tanimura N, Fukui R, Miyake K. Mucolipin 1 positively regulates TLR7 responses in dendritic cells by facilitating RNA transportation to lysosomes. Int Immunol. 2015;27(2):83-94.

37. Bridges D, Ma JT, Park S, Inoki K, Weisman LS, Saltiel AR. Phosphatidylinositol 3,5-bisphosphate plays a role in the activation and subcellular localization of mechanistic target of rapamycin 1. Mol Biol Cell. 2012;23(15):2955-62.

38. Jin N, Mao K, Jin Y, Tevzadze G, Kauffman EJ, Park S, et al. Roles for PI(3,5)P2 in nutrient sensing through TORC1. Mol Biol Cell. 2014;25(7):1171-85.

39. Polson HE, de Lartigue J, Rigden DJ, Reedijk M, Urbe S, Clague MJ, et al. Mammalian Atg18 (WIPI2) localizes to omegasome-anchored phagophores and positively regulates LC3 lipidation. Autophagy. 2010;6(4):506-22.

40. Boal F, Mansour R, Gayral M, Saland E, Chicanne G, Xuereb JM, et al. TOM1 is a PI5P effector involved in the regulation of endosomal maturation. J Cell Sci. 2015;128(4):815-27.

41. Ho H, Kapadia R, Al-Tahan S, Ahmad S, Ganesan AK. WIPI1 coordinates melanogenic gene transcription and melanosome formation via TORC1 inhibition. J Biol Chem. 2011;286(14):12509-23.

42. Proikas-Cezanne T, Waddell S, Gaugel A, Frickey T, Lupas A, Nordheim A. WIPI-1alpha (WIPI49), a member of the novel 7-bladed WIPI protein family, is aberrantly expressed in human cancer and is linked to starvation-induced autophagy. Oncogene. 2004;23(58):9314-25.

43. Uhlen M, Fagerberg L, Hallstrom BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Proteomics. Tissue-based map of the human proteome. Science. 2015;347(6220):1260419.

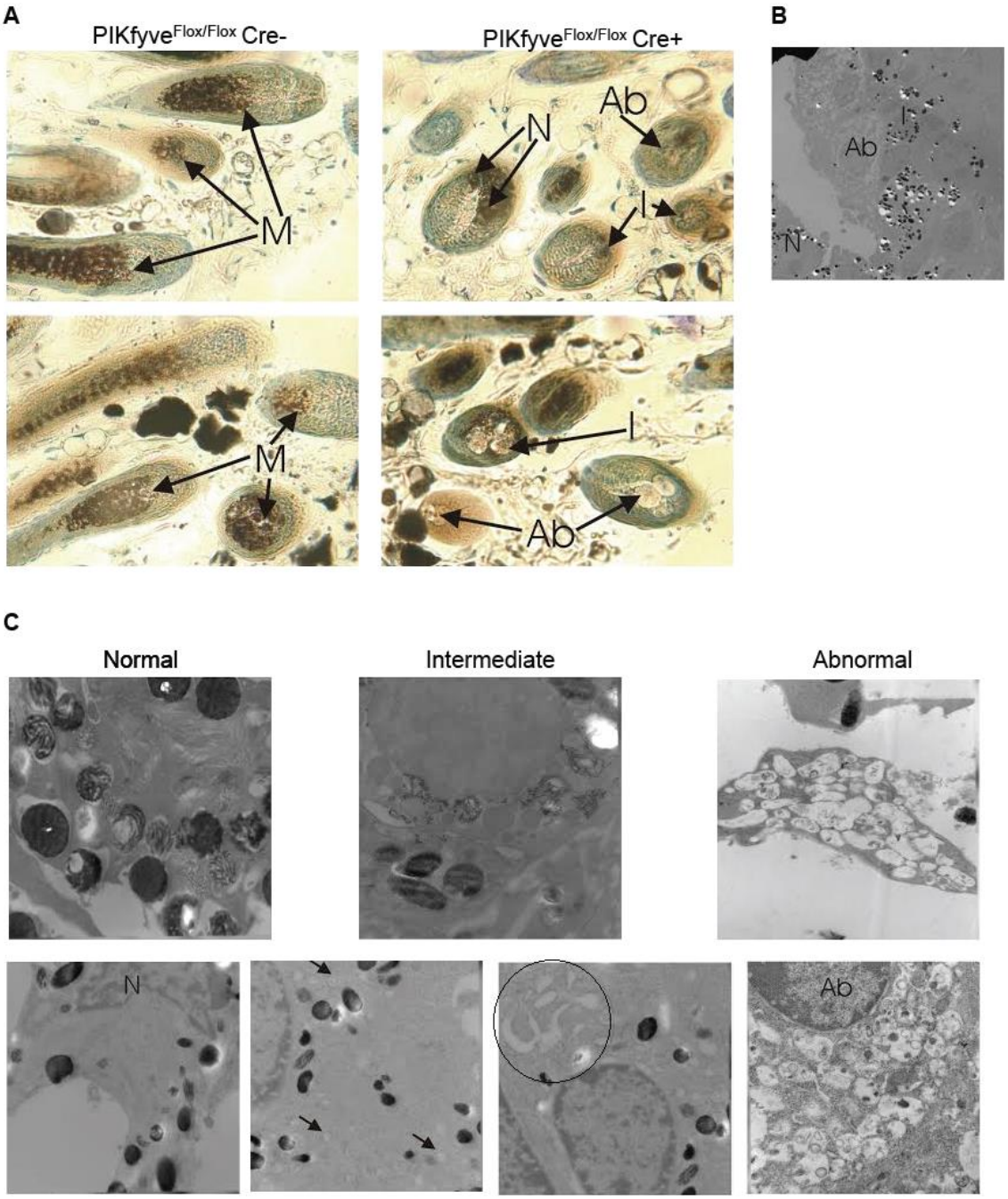
44. Murase D, Hachiya A, Kikuchi-Onoe M, Fullenkamp R, Ohuchi A, Kitahara T, et al. Cooperation of endothelin-1 signaling with melanosomes plays a role in developing and/or maintaining human skin hyperpigmentation. Biol Open. 2015;4(10):1213-21.

45. Ramel D, Lagarrigue F, Pons V, Mounier J, Dupuis-Coronas S, Chicanne G, et al. Shigella flexneri infection generates the lipid PI5P to alter endocytosis and prevent termination of EGFR signaling. Sci Signal. 2011;4(191):ra61.

46. Pendaries C, Tronchere H, Arbibe L, Mounier J, Gozani O, Cantley L, et al. PtdIns5P activates the host cell PI3-kinase/Akt pathway during Shigella flexneri infection. EMBO J. 2006;25(5):1024-34.

Figure 3.1: Melanocytes from PIKfyve knockout mice exhibit lack pigment and exhibit profound vacuolization. All mice were shave depilated at p35 and subsequently fed normal chow beginning at p50 for the subsequent days. A) 4mm skin biopsies were obtained from experimental mice in early anagen at P60 observed by microscopy. Melanocytes (M) from anagen hair follicles from control mice were observed by light microscopy at the epidermal/dermal papilla interface from control life (left panels) and knockout mice (right panels). In experimental mice, arrows refer melanocytes that appear morphologically normal (N), abnormal without melanin (Ab) and intermediate with minimal melanin (I). B) Different morphological melanocyte variants as observed by electronmicroscopy. C) Increased magnification with with DOPA histochemistry illustrating melanocyte morphological variants. Normal (leftmost panels), Intermediate (center panels), and abnormal (rightmost panels)

Figure 3.1


A
PIKfyve Flox/Flox Cre-
PIKfyve Flox/Flox Cre+
M
N
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B
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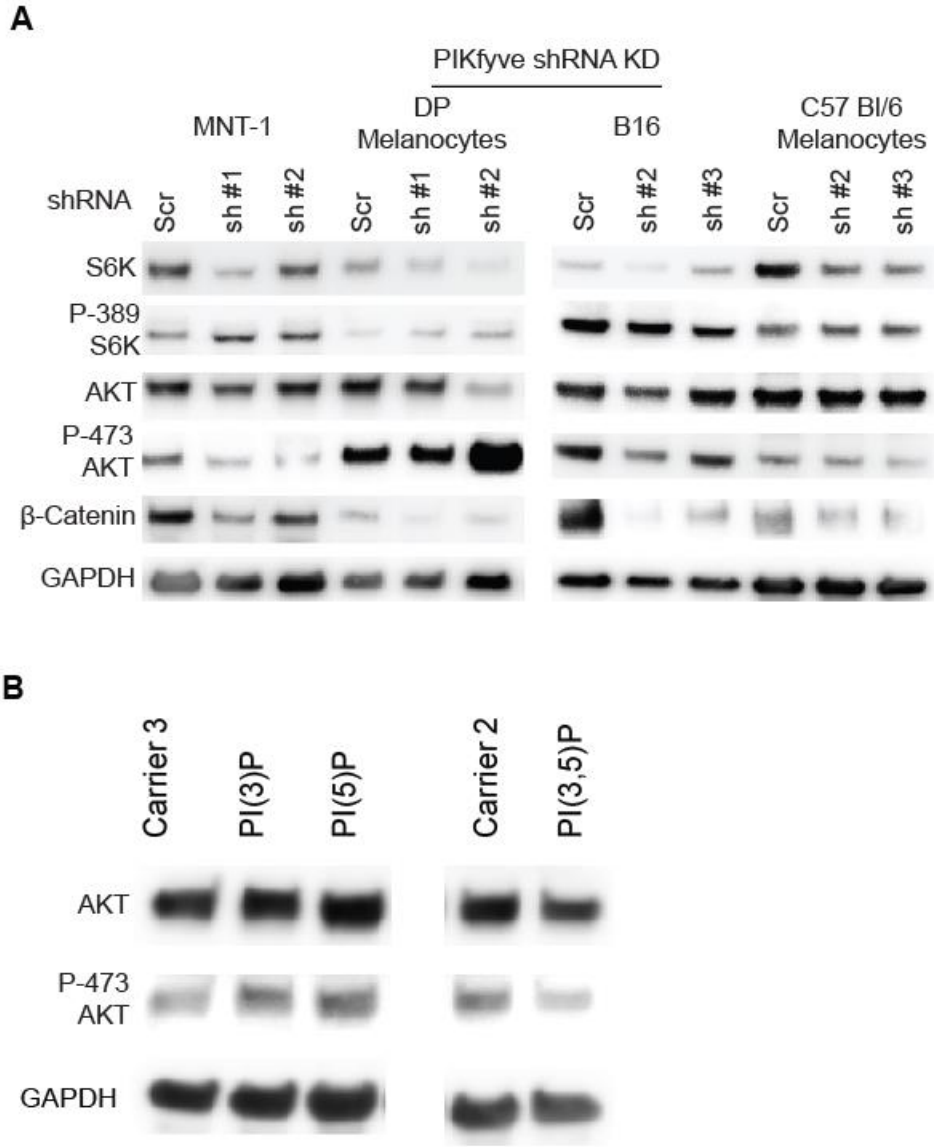
C
Normal
Intermediate
Abnormal
N
Ab

Figure 3.2: PI(5)P activates AKT signaling pathways. A) MNT-1 cells, darkly pigmented (DP) melanocytes, B16 mouse melanoma cells, and C57/B6 mouse epidermal melanocytes expressing shRNA's against PIKfyve or non-targeting scramble control were analyzed. The amount of accumulated proteins was assessed relative to GAPDH using immunoblotting. B) The amount of TYR and WIPI1 protein that accumulated in lipid treated cells was measured by immunoblotting. Each experiment was performed with three biological replicates and three technical replicates.

Figure 3.2


A
PIKfyve shRNA KD
MNT-1
DP Melanocytes
B16
C57 Bl/6 Melanocytes
shRNA
Scr
sh #1
sh #2
sh #3
S6K
P-389 S6K
AKT
P-473 AKT
β-Catenin
GAPDH
B
Carrier 3
PI(3)P
PI(5)P
Carrier 2
PI(3,5)P
AKT
P-473 AKT
GAPDH

Bibliography

UniProt: a hub for protein information. Nucleic Acids Res 43, Database issue (Jan 2015), D204-212.

ALGHAMDI, T. A., HO, C. Y., MRAKOVIC, A., TAYLOR, D., MAO, D. and BOTELHO, R. J. Vac14 protein multimerization is a prerequisite step for Fab1 protein complex assembly and function. J Biol Chem 288, 13 (Mar 29 2013), 9363-9372.

AMBROSIO, A. L., BOYLE, J. A., ARADI, A. E., CHRISTIAN, K. A. and DI PIETRO, S. M. TPC2 controls pigmentation by regulating melanosome pH and size. Proc Natl Acad Sci U S A 113, 20 (May 17 2016), 5622-5627.

ANTONSSON, B. Phosphatidylinositol synthase from mammalian tissues. Bba-Lipid Lipid Met 1348, 1-2 (Sep 4 1997), 179-186.

BAJAJ PAHUJA, K., WANG, J., BLAGOVESHCHENSKAYA, A., LIM, L., MADHUSUDHAN, M. S., MAYINGER, P. and SCHEKMAN, R. Phosphoregulatory protein 14-3-3 facilitates SAC1 transport from the endoplasmic reticulum. Proc Natl Acad Sci U S A 112, 25 (Jun 23 2015), E3199-3206.

BARDEN, H. and LEVINE, S. Histochemical observations on rodent brain melanin. Brain Res Bull 10, 6 (Jun 1983), 847-851.

BASKARAN, S., RAGUSA, M. J., BOURA, E. and HURLEY, J. H. Two-site recognition of phosphatidylinositol 3-phosphate by PROPPINs in autophagy. Mol Cell 47, 3 (Aug 10 2012), 339-348.

BAULAC, S., LENK, G. M., DUFRESNOIS, B., OULED AMAR BENCHEIKH, B., COUARCH, P., RENARD, J., LARSON, P. A., FERGUSON, C. J., NOE, E., POIRIER, K., HUBANS, C., FERREIRA, S., GUERRINI, R., OUAZZANI, R., EL HACHIMI, K. H., MEISLER, M. H. and LEGUERN, E. Role of the phosphoinositide phosphatase FIG4 gene in familial epilepsy with polymicrogyria. Neurology 82, 12 (Mar 25 2014), 1068-1075.

BELLONO, N. W., ESCOBAR, I. E., LEFKOVITH, A. J., MARKS, M. S. and OANCEA, E. An intracellular anion channel critical for pigmentation. Elife 3 (2014), e04543.

BELLONO, N. W., ESCOBAR, I. E. and OANCEA, E. A melanosomal two-pore sodium channel regulates pigmentation. Sci Rep 6 (2016), 26570.

BELLONO, N. W. and OANCEA, E. V. Ion transport in pigmentation. Arch Biochem Biophys 563 (Dec 1 2014), 35-41.

BENTLEY, N. J., EISEN, T. and GODING, C. R. Melanocyte-specific expression of the human tyrosinase promoter: activation by the microphthalmia gene product and role of the initiator. Mol Cell Biol 14, 12 (Dec 1994), 7996-8006.

BERGER, P., SCHAFFITZEL, C., BERGER, I., BAN, N. and SUTER, U. Membrane association of myotubularin-related protein 2 is mediated by a pleckstrin homology-GRAM domain and a coiled-coil dimerization module. Proc Natl Acad Sci U S A 100, 21 (Oct 14 2003), 12177-12182.

BERGER, P., TERSAR, K., BALLMER-HOFER, K. and SUTER, U. The CMT4B disease-causing proteins MTMR2 and MTMR13/SBF2 regulate AKT signalling. J Cell Mol Med 15, 2 (Feb 2011), 307-315.

BLONDEAU, F., LAPORTE, J., BODIN, S., SUPERTI-FURGA, G., PAYRASTRE, B. and MANDEL, J. L. Myotubularin, a phosphatase deficient in myotubular myopathy, acts on phosphatidylinositol 3-kinase and phosphatidylinositol 3-phosphate pathway. Hum Mol Genet 9, 15 (Sep 22 2000), 2223-2229.

BOAL, F., MANSOUR, R., GAYRAL, M., SALAND, E., CHICANNE, G., XUEREB, J. M., MARCELLIN, M., BURLET-SCHILTZ, O., SANSONETTI, P. J., PAYRASTRE, B. and TRONCHERE, H. TOM1 is a PI5P effector involved in the regulation of endosomal maturation. J Cell Sci 128, 4 (Feb 15 2015), 815-827.

BOCCACCIO, A., SCHOLZ-STARKE, J., HAMAMOTO, S., LARISCH, N., FESTA, M., GUTLA, P. V., COSTA, A., DIETRICH, P., UOZUMI, N. and CARPANETO, A. The phosphoinositide PI(3,5)P(2) mediates activation of mammalian but not plant TPC proteins: functional expression of endolysosomal channels in yeast and plant cells. Cell Mol Life Sci 71, 21 (Nov 2014), 4275-4283.

BOISSY, R. E. Melanosome transfer to and translocation in the keratinocyte. Exp Dermatol 12 Suppl 2 (2003), 5-12.

BOLINO, A., BOLIS, A., PREVITALI, S. C., DINA, G., BUSSINI, S., DATI, G., AMADIO, S., DEL CARRO, U., MRUK, D. D., FELTRI, M. L., CHENG, C. Y., QUATTRINI, A. and WRABETZ, L. Disruption of Mtmr2 produces CMT4B1-like neuropathy with myelin outfolding and impaired spermatogenesis. J Cell Biol 167, 4 (Nov 22 2004), 711-721.

BOLIS, A., COVIELLO, S., VISIGALLI, I., TAVEGGIA, C., BACHI, A., CHISHTI, A. H., HANADA, T., QUATTRINI, A., PREVITALI, S. C., BIFFI, A. and BOLINO, A. Dlg1, Sec8, and Mtmr2 regulate membrane homeostasis in Schwann cell myelination. J Neurosci 29, 27 (Jul 8 2009), 8858-8870.

BONG, S. M., SON, K. B., YANG, S. W., PARK, J. W., CHO, J. W., KIM, K. T., KIM, H., KIM, S. J., KIM, Y. J. and LEE, B. I. Crystal Structure of Human Myotubularin-Related

Protein 1 Provides Insight into the Structural Basis of Substrate Specificity. PLoS One 11, 3 (2016), e0152611.

BOSENBERG, M., MUTHUSAMY, V., CURLEY, D. P., WANG, Z., HOBBS, C., NELSON, B., NOGUEIRA, C., HORNER, J. W., 2ND, DEPINHO, R. and CHIN, L. Characterization of melanocyte-specific inducible Cre recombinase transgenic mice. Genesis 44, 5 (May 2006), 262-267.

BOTELHO, R. J., EFE, J. A., TEIS, D. and EMR, S. D. Assembly of a Fab1 phosphoinositide kinase signaling complex requires the Fig4 phosphoinositide phosphatase. Mol Biol Cell 19, 10 (Oct 2008), 4273-4286.

BRIDGES, D., MA, J. T., PARK, S., INOKI, K., WEISMAN, L. S. and SALTIEL, A. R. Phosphatidylinositol 3,5-bisphosphate plays a role in the activation and subcellular localization of mechanistic target of rapamycin 1. Mol Biol Cell 23, 15 (Aug 2012), 2955-2962.

BUA, D. J., MARTIN, G. M., BINDA, O. and GOZANI, O. Nuclear phosphatidylinositol-5-phosphate regulates ING2 stability at discrete chromatin targets in response to DNA damage. Sci Rep 3 (2013), 2137.

BULTEMA, J. J., AMBROSIO, A. L., BUREK, C. L. and DI PIETRO, S. M. BLOC-2, AP-3, and AP-1 proteins function in concert with Rab38 and Rab32 proteins to mediate protein trafficking to lysosome-related organelles. J Biol Chem 287, 23 (Jun 1 2012), 19550-19563.

BULTEMA, J. J. and DI PIETRO, S. M. Cell type-specific Rab32 and Rab38 cooperate with the ubiquitous lysosome biogenesis machinery to synthesize specialized lysosome-related organelles. Small GTPases 4, 1 (Jan-Mar 2013), 16-21.

BUSCA, R., BERTOLOTTO, C., ORTONNE, J. P. and BALLOTTI, R. Inhibition of the phosphatidylinositol 3-kinase/p70(S6)-kinase pathway induces B16 melanoma cell differentiation. J Biol Chem 271, 50 (Dec 13 1996), 31824-31830.

CABEZAS, A., PATTNI, K. and STENMARK, H. Cloning and subcellular localization of a human phosphatidylinositol 3-phosphate 5-kinase, PIKfyve/Fab1. Gene 371, 1 (Apr 12 2006), 34-41.

CAI, X., XU, Y., CHEUNG, A. K., TOMLINSON, R. C., ALCAZAR-ROMAN, A., MURPHY, L., BILLICH, A., ZHANG, B., FENG, Y., KLUMPP, M., RONDEAU, J. M., FAZAL, A. N., WILSON, C. J., MYER, V., JOBERTY, G., BOUWMEESTER, T., LABOW, M. A., FINAN, P. M., PORTER, J. A., PLOEGH, H. L., BAIRD, D., DE CAMILLI, P., TALLARICO, J. A. and HUANG, Q. PIKfyve, a class III PI kinase, is the target of the small molecular IL-12/IL-23 inhibitor apilimod and a player in Toll-like receptor signaling. Chem Biol 20, 7 (Jul 25 2013), 912-921.

CALVO, P. A., FRANK, D. W., BIELER, B. M., BERSON, J. F. and MARKS, M. S. A cytoplasmic sequence in human tyrosinase defines a second class of di-leucine-based sorting signals for late endosomal and lysosomal delivery. J Biol Chem 274, 18 (Apr 30 1999), 12780-12789.

CAMPEAU, P. M., LENK, G. M., LU, J. T., BAE, Y., BURRAGE, L., TURNPENNY, P., ROMAN CORONA-RIVERA, J., MORANDI, L., MORA, M., REUTTER, H., VULTO-VAN SILFHOUT, A. T., FAIVRE, L., HAAN, E., GIBBS, R. A., MEISLER, M. H. and LEE, B. H. Yunis-Varon syndrome is caused by mutations in FIG4, encoding a phosphoinositide phosphatase. Am J Hum Genet 92, 5 (May 2 2013), 781-791.

CARLTON, J., BUJNY, M., PETER, B. J., OORSCHOT, V. M., RUTHERFORD, A., MELLOR, H., KLUMPERMAN, J., MCMAHON, H. T. and CULLEN, P. J. Sorting nexin-1 mediates tubular endosome-to-TGN transport through coincidence sensing of high-curvature membranes and 3-phosphoinositides. Curr Biol 14, 20 (Oct 26 2004), 1791-1800.

CHENG, X., SHEN, D., SAMIE, M. and XU, H. Mucolipins: Intracellular TRPML1-3 channels. FEBS Lett 584, 10 (May 17 2010), 2013-2021.

CHINTALA, S., LI, W., LAMOREUX, M. L., ITO, S., WAKAMATSU, K., SVIDERSKAYA, E. V., BENNETT, D. C., PARK, Y. M., GAHL, W. A., HUIZING, M., SPRITZ, R. A., BEN, S., NOVAK, E. K., TAN, J. and SWANK, R. T. Slc7a11 gene controls production of pheomelanin pigment and proliferation of cultured cells. Proc Natl Acad Sci U S A 102, 31 (Aug 2 2005), 10964-10969.

CHOW, C. Y., LANDERS, J. E., BERGREN, S. K., SAPP, P. C., GRANT, A. E., JONES, J. M., EVERETT, L., LENK, G. M., MCKENNA-YASEK, D. M., WEISMAN, L. S., FIGLEWICZ, D., BROWN, R. H. and MEISLER, M. H. Deleterious variants of FIG4, a phosphoinositide phosphatase, in patients with ALS. Am J Hum Genet 84, 1 (Jan 2009), 85-88.

CHOW, C. Y., ZHANG, Y., DOWLING, J. J., JIN, N., ADAMSKA, M., SHIGA, K., SZIGETI, K., SHY, M. E., LI, J., ZHANG, X., LUPSKI, J. R., WEISMAN, L. S. and MEISLER, M. H. Mutation of FIG4 causes neurodegeneration in the pale tremor mouse and patients with CMT4J. Nature 448, 7149 (Jul 5 2007), 68-72.

CHOW, C. Y., ZHANG, Y., DOWLING, J. J., JIN, N., ADAMSKA, M., SHIGA, K., SZIGETI, K., SHY, M. E., LI, J., ZHANG, X., LUPSKI, J. R., WEISMAN, L. S. and MEISLER, M. H. Mutation of FIG4 causes neurodegeneration in the pale tremor mouse and patients with CMT4J. Nature 448, 7149 (Jul 5 2007), 68-72.

CICHOREK, M., WACHULSKA, M., STASIEWICZ, A. and TYMINSKA, A. Skin melanocytes: biology and development. Postepy Dermatol Alergol 30, 1 (Feb 2013), 30-41.

CLARKE, J. H., GIUDICI, M. L., BURKE, J. E., WILLIAMS, R. L., MALONEY, D. J., MARUGAN, J. and IRVINE, R. F. The function of phosphatidylinositol 5-phosphate 4-kinase gamma (PI5P4K gamma) explored using a specific inhibitor that targets the PI5P-binding site. Biochemical Journal 466 (Mar 1 2015), 359-367.

CLARKE, J. H., GIUDICI, M. L., BURKE, J. E., WILLIAMS, R. L., MALONEY, D. J., MARUGAN, J. and IRVINE, R. F. The function of phosphatidylinositol 5-phosphate 4-kinase gamma (PI5P4K gamma) explored using a specific inhibitor that targets the PI5P-binding site. Biochemical Journal 466 (Mar 1 2015), 359-367.

CLARKE, J. H., GIUDICI, M. L., BURKE, J. E., WILLIAMS, R. L., MALONEY, D. J., MARUGAN, J. and IRVINE, R. F. The function of phosphatidylinositol 5-phosphate 4-kinase gamma (PI5P4Kgamma) explored using a specific inhibitor that targets the PI5P-binding site. Biochem J 466, 2 (Mar 1 2015), 359-367.

CLARKE, J. H. and IRVINE, R. F. Evolutionarily conserved structural changes in phosphatidylinositol 5-phosphate 4-kinase (PI5P4K) isoforms are responsible for differences in enzyme activity and localization. Biochemical Journal 454 (Aug 15 2013), 49-57.

COSTIN, G. E. and HEARING, V. J. Human skin pigmentation: melanocytes modulate skin color in response to stress. FASEB J 21, 4 (Apr 2007), 976-994.

CURRINN, H., GUSCOTT, B., BALKLAVA, Z., ROTHNIE, A. and WASSMER, T. APP controls the formation of PI(3,5)P(2) vesicles through its binding of the PIKfyve complex. Cell Mol Life Sci 73, 2 (Jan 2016), 393-408.

CURRINN, H. and WASSMER, T. The amyloid precursor protein (APP) binds the PIKfyve complex and modulates its function. Biochem Soc Trans 44, 1 (Feb 15 2016), 185-190.

DALL'ARMI, C., DEVEREAUX, K. A. and DI PAOLO, G. The role of lipids in the control of autophagy. Curr Biol 23, 1 (Jan 7 2013), R33-45.

DE LARTIGUE, J., POLSON, H., FELDMAN, M., SHOKAT, K., TOOZE, S. A., URBE, S. and CLAGUE, M. J. PIKfyve regulation of endosome-linked pathways. Traffic 10, 7 (Jul 2009), 883-893.

DE MAZIERE, A. M., MUEHLETHALER, K., VAN DONSELAAR, E., SALVI, S., DAVOUST, J., CEROTTINI, J. C., LEVY, F., SLOT, J. W. and RIMOLDI, D. The melanocytic protein Melan-A/MART-1 has a subcellular localization distinct from typical melanosomal proteins. Traffic 3, 9 (Sep 2002), 678-693.

DELL'ANGELICA, E. C. AP-3-dependent trafficking and disease: the first decade. Curr Opin Cell Biol 21, 4 (Aug 2009), 552-559.

DELL'ANGELICA, E. C., MULLINS, C., CAPLAN, S. and BONIFACINO, J. S. Lysosome-related organelles. FASEB J 14, 10 (Jul 2000), 1265-1278.

DI PAOLO, G. and DE CAMILLI, P. Phosphoinositides in cell regulation and membrane dynamics. Nature 443, 7112 (Oct 12 2006), 651-657.

DONG, X. P., SHEN, D., WANG, X., DAWSON, T., LI, X., ZHANG, Q., CHENG, X., ZHANG, Y., WEISMAN, L. S., DELLING, M. and XU, H. PI(3,5)P(2) controls membrane trafficking by direct activation of mucolipin Ca(2+) release channels in the endolysosome. Nat Commun 1 (2010), 38.

DOOLEY, H. C., RAZI, M., POLSON, H. E., GIRARDIN, S. E., WILSON, M. I. and TOOZE, S. A. WIPI2 links LC3 conjugation with PI3P, autophagosome formation, and pathogen clearance by recruiting Atg12-5-16L1. Mol Cell 55, 2 (Jul 17 2014), 238-252.

DOVE, S. K., MCEWEN, R. K., MAYES, A., HUGHES, D. C., BEGGS, J. D. and MICHELL, R. H. Vac14 controls PtdIns(3,5)P(2) synthesis and Fab1-dependent protein trafficking to the multivesicular body. Curr Biol 12, 11 (Jun 4 2002), 885-893.

DROUBI, A., BULLEY, S. J., CLARKE, J. H. and IRVINE, R. F. Nuclear localizations of phosphatidylinositol 5-phosphate 4-kinases alpha and beta are dynamic and independently regulated during starvation-induced stress. Biochem J 473, 14 (Jul 15 2016), 2155-2163.

DUEX, J. E., NAU, J. J., KAUFFMAN, E. J. and WEISMAN, L. S. Phosphoinositide 5-phosphatase Fig 4p is required for both acute rise and subsequent fall in stress-induced phosphatidylinositol 3,5-bisphosphate levels. Eukaryot Cell 5, 4 (Apr 2006), 723-731.

EFE, J. A., BOTELHO, R. J. and EMR, S. D. Atg18 regulates organelle morphology and Fab1 kinase activity independent of its membrane recruitment by phosphatidylinositol 3,5-bisphosphate. Mol Biol Cell 18, 11 (Nov 2007), 4232-4244.

ELIAS, P. M. The skin barrier as an innate immune element. Semin Immunopathol 29, 1 (Apr 2007), 3-14.

ER, E. E., MENDOZA, M. C., MACKEY, A. M., RAMEH, L. E. and BLENIS, J. AKT facilitates EGFR trafficking and degradation by phosphorylating and activating PIKfyve. Sci Signal 6, 279 (Jun 11 2013), ra45.

FENG, X., HUANG, Y., LU, Y., XIONG, J., WONG, C. O., YANG, P., XIA, J., CHEN, D., DU, G., VENKATACHALAM, K., XIA, X. and ZHU, M. X. Drosophila TRPML forms PI(3,5)P2-activated cation channels in both endolysosomes and plasma membrane. J Biol Chem 289, 7 (Feb 14 2014), 4262-4272.

FERGUSON, C. J., LENK, G. M. and MEISLER, M. H. Defective autophagy in neurons and astrocytes from mice deficient in PI(3,5)P2. Hum Mol Genet 18, 24 (Dec 15 2009), 4868-4878.

FULLGRABE, J., KLIONSKY, D. J. and JOSEPH, B. The return of the nucleus: transcriptional and epigenetic control of autophagy. Nat Rev Mol Cell Biol 15, 1 (Jan 2014), 65-74.

GALVAN, I., GHANEM, G. and MOLLER, A. P. Has removal of excess cysteine led to the evolution of pheomelanin? Pheomelanogenesis as an excretory mechanism for cysteine. Bioessays 34, 7 (Jul 2012), 565-568.

GANESAN, A. K., HO, H., BODEMANN, B., PETERSEN, S., ARURI, J., KOSHY, S., RICHARDSON, Z., LE, L. Q., KRASIEVA, T., ROTH, M. G., FARMER, P. and WHITE, M. A. Genome-wide siRNA-based functional genomics of pigmentation identifies novel genes and pathways that impact melanogenesis in human cells. PLoS Genet 4, 12 (Dec 2008), e1000298.

GAUGEL, A., BAKULA, D., HOFFMANN, A. and PROIKAS-CEZANNE, T. Defining regulatory and phosphoinositide-binding sites in the human WIPI-1 beta-propeller responsible for autophagosomal membrane localization downstream of mTORC1 inhibition. J Mol Signal 7, 1 (2012), 16.

GEE, J. A., FRAUSTO, R. F., CHUNG, D. W., TANGMONKONGVORAGUL, C., LE, D. J., WANG, C., HAN, J. and ALDAVE, A. J. Identification of novel PIKFYVE gene mutations associated with Fleck corneal dystrophy. Mol Vis 21 (2015), 1093-1100.

GERONDOPOULOS, A., LANGEMEYER, L., LIANG, J. R., LINFORD, A. and BARR, F. A. BLOC-3 mutated in Hermansky-Pudlak syndrome is a Rab32/38 guanine nucleotide exchange factor. Curr Biol 22, 22 (Nov 20 2012), 2135-2139.

GIORDANO, F., BONETTI, C., SURACE, E. M., MARIGO, V. and RAPOSO, G. The ocular albinism type 1 (OA1) G-protein-coupled receptor functions with MART-1 at early stages of melanogenesis to control melanosome identity and composition. Hum Mol Genet 18, 23 (Dec 1 2009), 4530-4545.

GIUDICI, M. L., CLARKE, J. H. and IRVINE, R. F. Phosphatidylinositol 5-phosphate 4-kinase gamma (PI5P4Kgamma), a lipid signalling enigma. Adv Biol Regul 61 (May 2016), 47-50.

GODWIN, L. S., CASTLE, J. T., KOHLI, J. S., GOFF, P. S., CAIRNEY, C. J., KEITH, W. N., SVIDERSKAYA, E. V. and BENNETT, D. C. Isolation, culture, and transfection of melanocytes. Curr Protoc Cell Biol 63 (2014), 1 8 1-20.

HAN, B. K. and EMR, S. D. Phosphoinositide [PI(3,5)P2] lipid-dependent regulation of the general transcriptional regulator Tup1. Genes Dev 25, 9 (May 1 2011), 984-995.

HAUCKE, V. Phosphoinositide regulation of clathrin-mediated endocytosis. Biochem Soc T 33 (Dec 2005), 1285-1289.

HE, S. S., NI, D. J., MA, B. Y., LEE, J. H., ZHANG, T., GHOZALLI, I., PIROOZ, S. D., ZHAO, Z., BHARATHAM, N., LI, B. H., OH, S., LEE, W. H., TAKAHASHI, Y., WANG, H. G., MINASSIAN, A., FENG, P. H., DERETIC, V., PEPPERKOK, R., TAGAYA, M., YOON, H. S. and LIANG, C. Y. PtdIns(3)P-bound UVRAG coordinates Golgi-ER retrograde and Atg9 transport by differential interactions with the ER tether and the beclin 1 complex. Nature Cell Biology 15, 10 (Oct 2013), 1206-U1192.

HENNESSY, A., OH, C., DIFFEY, B., WAKAMATSU, K., ITO, S. and REES, J. Eumelanin and pheomelanin concentrations in human epidermis before and after UVB irradiation. Pigment Cell Res 18, 3 (Jun 2005), 220-223.

HIDA, T., SOHMA, H., KOKAI, Y., KAWAKAMI, A., HIROSAKI, K., OKURA, M., TOSA, N., YAMASHITA, T. and JIMBOW, K. Rab7 is a critical mediator in vesicular transport of tyrosinase-related protein 1 in melanocytes. J Dermatol 38, 5 (May 2011), 432-441.

HILL, E. V., HUDSON, C. A., VERTOMMEN, D., RIDER, M. H. and TAVARE, J. M. Regulation of PIKfyve phosphorylation by insulin and osmotic stress. Biochem Biophys Res Commun 397, 4 (Jul 9 2010), 650-655.

HIROBE, T. Origin of melanosome structures and cytochemical localizations of tyrosinase activity in differentiating epidermal melanocytes of newborn mouse skin. J Exp Zool 224, 3 (Dec 30 1982), 355-363.

HNIA, K., VACCARI, I., BOLINO, A. and LAPORTE, J. Myotubularin phosphoinositide phosphatases: cellular functions and disease pathophysiology. Trends Mol Med 18, 6 (Jun 2012), 317-327.

HO, H., ARURI, J., KAPADIA, R., MEHR, H., WHITE, M. A. and GANESAN, A. K. RhoJ regulates melanoma chemoresistance by suppressing pathways that sense DNA damage. Cancer Res 72, 21 (Nov 1 2012), 5516-5528.

HO, H. and GANESAN, A. K. The pleiotropic roles of autophagy regulators in melanogenesis. Pigment Cell Melanoma Res 24, 4 (Aug 2011), 595-604.

HO, H., KAPADIA, R., AL-TAHAN, S., AHMAD, S. and GANESAN, A. K. WIPI1 coordinates melanogenic gene transcription and melanosome formation via TORC1 inhibition. J Biol Chem 286, 14 (Apr 8 2011), 12509-12523.

HOASHI, T., WATABE, H., MULLER, J., YAMAGUCHI, Y., VIEIRA, W. D. and HEARING, V. J. MART-1 is required for the function of the melanosomal matrix protein PMEL17/GP100 and the maturation of melanosomes. J Biol Chem 280, 14 (Apr 8 2005), 14006-14016.

HOEK, K., RIMM, D. L., WILLIAMS, K. R., ZHAO, H., ARIYAN, S., LIN, A., KLUGER, H. M., BERGER, A. J., CHENG, E., TROMBETTA, E. S., WU, T., NIINOBE, M., YOSHIKAWA, K., HANNIGAN, G. E. and HALABAN, R. Expression profiling reveals novel pathways in the transformation of melanocytes to melanomas. Cancer Res 64, 15 (Aug 1 2004), 5270-5282.

HOEK, K. S., SCHLEGEL, N. C., EICHHOFF, O. M., WIDMER, D. S., PRAETORIUS, C., EINARSSON, S. O., VALGEIRSDOTTIR, S., BERGSTEINSDOTTIR, K., SCHEPSKY, A., DUMMER, R. and STEINGRIMSSON, E. Novel MITF targets identified using a two-step DNA microarray strategy. Pigment Cell Melanoma Res 21, 6 (Dec 2008), 665-676.

HORNBECK, P. V., ZHANG, B., MURRAY, B., KORNHAUSER, J. M., LATHAM, V. and SKRZYPEK, E. PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. Nucleic Acids Res 43, Database issue (Jan 2015), D512-520.

HSU, F., HU, F. and MAO, Y. Spatiotemporal control of phosphatidylinositol 4-phosphate by Sac2 regulates endocytic recycling. J Cell Biol 209, 1 (Apr 13 2015), 97-110.

HU, Y., CARRARO-LACROIX, L. R., WANG, A., OWEN, C., BAJENOVA, E., COREY, P. N., BRUMELL, J. H. and VORONOV, I. Lysosomal pH Plays a Key Role in Regulation of mTOR Activity in Osteoclasts. J Cell Biochem 117, 2 (Feb 2016), 413-425.

HUIZING, M., HELIP-WOOLEY, A., WESTBROEK, W., GUNAY-AYGUN, M. and GAHL, W. A. Disorders of lysosome-related organelle biogenesis: clinical and molecular genetics. Annu Rev Genomics Hum Genet 9 (2008), 359-386.

HUME, A. N., COLLINSON, L. M., RAPAK, A., GOMES, A. Q., HOPKINS, C. R. and SEABRA, M. C. Rab27a regulates the peripheral distribution of melanosomes in melanocytes. Journal of Cell Biology 152, 4 (Feb 19 2001), 795-808.

IKONOMOV, O. C., FLIGGER, J., SBRISSA, D., DONDAPATI, R., MLAK, K., DEEB, R. and SHISHEVA, A. Kinesin adapter JLP links PIKfyve to microtubule-based endosome-to-trans-Golgi network traffic of furin. J Biol Chem 284, 6 (Feb 6 2009), 3750-3761.

IKONOMOV, O. C., SBRISSA, D., DELVECCHIO, K., XIE, Y., JIN, J. P., RAPPOLEE, D. and SHISHEVA, A. The phosphoinositide kinase PIKfyve is vital in early embryonic

development: preimplantation lethality of PIKfyve-/- embryos but normality of PIKfyve+/- mice. J Biol Chem 286, 15 (Apr 15 2011), 13404-13413.

IKONOMOV, O. C., SBRISSA, D., FENNER, H. and SHISHEVA, A. PIKfyve-ArPIKfyve-Sac3 core complex: contact sites and their consequence for Sac3 phosphatase activity and endocytic membrane homeostasis. J Biol Chem 284, 51 (Dec 18 2009), 35794-35806.

IKONOMOV, O. C., SBRISSA, D., FENNER, H. and SHISHEVA, A. PIKfyve-ArPIKfyve-Sac3 core complex: contact sites and their consequence for Sac3 phosphatase activity and endocytic membrane homeostasis. J Biol Chem 284, 51 (Dec 18 2009), 35794-35806.

IKONOMOV, O. C., SBRISSA, D., IJUIN, T., TAKENAWA, T. and SHISHEVA, A. Sac3 is an insulin-regulated phosphatidylinositol 3,5-bisphosphate phosphatase: gain in insulin responsiveness through Sac3 down-regulation in adipocytes. J Biol Chem 284, 36 (Sep 4 2009), 23961-23971.

IKONOMOV, O. C., SBRISSA, D., MLAK, K., DEEB, R., FLIGGER, J., SOANS, A., FINLEY, R. L., JR. and SHISHEVA, A. Active PIKfyve associates with and promotes the membrane attachment of the late endosome-to-trans-Golgi network transport factor Rab9 effector p40. J Biol Chem 278, 51 (Dec 19 2003), 50863-50871.

IKONOMOV, O. C., SBRISSA, D., MLAK, K., KANZAKI, M., PESSIN, J. and SHISHEVA, A. Functional dissection of lipid and protein kinase signals of PIKfyve reveals the role of PtdIns 3,5-P2 production for endomembrane integrity. J Biol Chem 277, 11 (Mar 15 2002), 9206-9211.

IKONOMOV, O. C., SBRISSA, D., VENKATAREDDY, M., TISDALE, E., GARG, P. and SHISHEVA, A. Class III PI 3-kinase is the main source of PtdIns3P substrate and membrane recruitment signal for PIKfyve constitutive function in podocyte endomembrane homeostasis. Biochim Biophys Acta 1853, 5 (May 2015), 1240-1250.

JENKINS, N. C. and GROSSMAN, D. Role of melanin in melanocyte dysregulation of reactive oxygen species. Biomed Res Int 2013 (2013), 908797.

JEWELL, J. L., RUSSELL, R. C. and GUAN, K. L. Amino acid signalling upstream of mTOR. Nat Rev Mol Cell Biol 14, 3 (Mar 2013), 133-139.

JHA, A., AHUJA, M., PATEL, S., BRAILOIU, E. and MUALLEM, S. Convergent regulation of the lysosomal two-pore channel-2 by Mg(2)(+), NAADP, PI(3,5)P(2) and multiple protein kinases. EMBO J 33, 5 (Mar 3 2014), 501-511.

JIN, N., CHOW, C. Y., LIU, L., ZOLOV, S. N., BRONSON, R., DAVISSON, M., PETERSEN, J. L., ZHANG, Y., PARK, S., DUEX, J. E., GOLDOWITZ, D., MEISLER, M.

H. and WEISMAN, L. S. VAC14 nucleates a protein complex essential for the acute interconversion of PI3P and PI(3,5)P(2) in yeast and mouse. EMBO J 27, 24 (Dec 17 2008), 3221-3234.

JIN, N., CHOW, C. Y., LIU, L., ZOLOV, S. N., BRONSON, R., DAVISSON, M., PETERSEN, J. L., ZHANG, Y., PARK, S., DUEX, J. E., GOLDOWITZ, D., MEISLER, M. H. and WEISMAN, L. S. VAC14 nucleates a protein complex essential for the acute interconversion of PI3P and PI(3,5)P(2) in yeast and mouse. EMBO J 27, 24 (Dec 17 2008), 3221-3234.

JIN, N., LANG, M. J. and WEISMAN, L. S. Phosphatidylinositol 3,5-bisphosphate: regulation of cellular events in space and time. Biochem Soc Trans 44, 1 (Feb 15 2016), 177-184.

JIN, N., MAO, K., JIN, Y., TEVZADZE, G., KAUFFMAN, E. J., PARK, S., BRIDGES, D., LOEWITH, R., SALTIEL, A. R., KLIONSKY, D. J. and WEISMAN, L. S. Roles for PI(3,5)P2 in nutrient sensing through TORC1. Mol Biol Cell 25, 7 (Apr 2014), 1171-1185.

JO, I. S., SOHN, K.-C., KIM, C. D. and LEE, Y. H. Effect of MITF-M and MITF-A Overexpression on the Dendrtic Formation in Melanocytes. Korean J Phys Anthropol 29, 1 (2016), 27-34.

JONES, D. R., BULTSMA, Y., KEUNE, W. J., HALSTEAD, J. R., ELOUARRAT, D., MOHAMMED, S., HECK, A. J., D'SANTOS, C. S. and DIVECHA, N. Nuclear PtdIns5P as a transducer of stress signaling: an in vivo role for PIP4Kbeta. Mol Cell 23, 5 (Sep 1 2006), 685-695.

KALIE, E., RAZI, M. and TOOZE, S. A. ULK1 regulates melanin levels in MNT-1 cells independently of mTORC1. PLoS One 8, 9 (2013), e75313.

KAMARAJ, B. and PUROHIT, R. Mutational analysis of oculocutaneous albinism: a compact review. Biomed Res Int 2014 (2014), 905472.

KANITAKIS, J. Anatomy, histology and immunohistochemistry of normal human skin. Eur J Dermatol 12, 4 (Jul-Aug 2002), 390-399; quiz 400-391.

KAWAKAMI, A., SAKANE, F., IMAI, S., YASUDA, S., KAI, M., KANOH, H., JIN, H. Y., HIROSAKI, K., YAMASHITA, T., FISHER, D. E. and JIMBOW, K. Rab7 regulates maturation of melanosomal matrix protein gp100/Pmel17/Silv. J Invest Dermatol 128, 1 (Jan 2008), 143-150.

KERR, M. C., WANG, J. T., CASTRO, N. A., HAMILTON, N. A., TOWN, L., BROWN, D. L., MEUNIER, F. A., BROWN, N. F., STOW, J. L. and TEASDALE, R. D. Inhibition of

the PtdIns(5) kinase PIKfyve disrupts intracellular replication of Salmonella. EMBO J 29, 8 (Apr 21 2010), 1331-1347.

KIM, E. S., CHANG, H., CHOI, H., SHIN, J. H., PARK, S. J., JO, Y. K., CHOI, E. S., BAEK, S. Y., KIM, B. G., CHANG, J. W., KIM, J. C. and CHO, D. H. Autophagy induced by resveratrol suppresses alpha-MSH-induced melanogenesis. Exp Dermatol 23, 3 (Mar 2014), 204-206.

KIM, G. H., DAYAM, R. M., PRASHAR, A., TEREBIZNIK, M. and BOTELHO, R. J. PIKfyve inhibition interferes with phagosome and endosome maturation in macrophages. Traffic 15, 10 (Oct 2014), 1143-1163.

KIM, S. A., TAYLOR, G. S., TORGERSEN, K. M. and DIXON, J. E. Myotubularin and MTMR2, phosphatidylinositol 3-phosphatases mutated in myotubular myopathy and type 4B Charcot-Marie-Tooth disease. J Biol Chem 277, 6 (Feb 8 2002), 4526-4531.

KIOSCHIS, P., WIEMANN, S., HEISS, N. S., FRANCIS, F., GOTZ, C., POUSTKA, A., TAUDIEN, S., PLATZER, M., WIEHE, T., BECKMANN, G., WEBER, J., NORDSIEK, G. and ROSENTHAL, A. Genomic organization of a 225-kb region in Xq28 containing the gene for X-linked myotubular myopathy (MTM1) and a related gene (MTMR1). Genomics 54, 2 (Dec 1 1998), 256-266.

KIPPENBERGER, S., BERND, A., BEREITER-HAHN, J., RAMIREZ-BOSCA, A. and KAUFMANN, R. The mechanism of melanocyte dendrite formation: the impact of differentiating keratinocytes. Pigment Cell Res 11, 1 (Feb 1998), 34-37.

KONDO, T. and HEARING, V. J. Update on the regulation of mammalian melanocyte function and skin pigmentation. Expert Rev Dermatol 6, 1 (Feb 1 2011), 97-108.

KOTOULAS, A., KOKOTAS, H., KOPSIDAS, K., DROUTSAS, K., GRIGORIADOU, M., BAJRAMI, H., SCHORDERET, D. F. and PETERSEN, M. B. A novel PIKFYVE mutation in fleck corneal dystrophy. Mol Vis 17 (2011), 2776-2781.

KRICK, R., BUSSE, R. A., SCACIOC, A., STEPHAN, M., JANSHOFF, A., THUMM, M. and KUHNEL, K. Structural and functional characterization of the two phosphoinositide binding sites of PROPPINs, a beta-propeller protein family. Proc Natl Acad Sci U S A 109, 30 (Jul 24 2012), E2042-2049.

KUSHIMOTO, T., BASRUR, V., VALENCIA, J., MATSUNAGA, J., VIEIRA, W. D., FERRANS, V. J., MULLER, J., APPELLA, E. and HEARING, V. J. A model for melanosome biogenesis based on the purification and analysis of early melanosomes. Proc Natl Acad Sci U S A 98, 19 (Sep 11 2001), 10698-10703.

LAMOREUX, M. L., WAKAMATSU, K. and ITO, S. Interaction of major coat color gene functions in mice as studied by chemical analysis of eumelanin and pheomelanin. Pigment Cell Res 14, 1 (Feb 2001), 23-31.

LE PAPE, E., WAKAMATSU, K., ITO, S., WOLBER, R. and HEARING, V. J. Regulation of eumelanin/pheomelanin synthesis and visible pigmentation in melanocytes by ligands of the melanocortin 1 receptor. Pigment Cell Melanoma Res 21, 4 (Aug 2008), 477-486.

LEE, S. T., NICHOLLS, R. D., JONG, M. T., FUKAI, K. and SPRITZ, R. A. Organization and sequence of the human P gene and identification of a new family of transport proteins. Genomics 26, 2 (Mar 20 1995), 354-363.

LENK, G. M., FERGUSON, C. J., CHOW, C. Y., JIN, N., JONES, J. M., GRANT, A. E., ZOLOV, S. N., WINTERS, J. J., GIGER, R. J., DOWLING, J. J., WEISMAN, L. S. and MEISLER, M. H. Pathogenic mechanism of the FIG4 mutation responsible for Charcot-Marie-Tooth disease CMT4J. PLoS Genet 7, 6 (Jun 2011), e1002104.

LENK, G. M., FERGUSON, C. J., CHOW, C. Y., JIN, N., JONES, J. M., GRANT, A. E., ZOLOV, S. N., WINTERS, J. J., GIGER, R. J., DOWLING, J. J., WEISMAN, L. S. and MEISLER, M. H. Pathogenic mechanism of the FIG4 mutation responsible for Charcot-Marie-Tooth disease CMT4J. PLoS Genet 7, 6 (Jun 2011), e1002104.

LENK, G. M., FREI, C. M., MILLER, A. C., WALLEN, R. C., MIRONOVA, Y. A., GIGER, R. J. and MEISLER, M. H. Rescue of neurodegeneration in the Fig4 null mouse by a catalytically inactive FIG4 transgene. Hum Mol Genet 25, 2 (Jan 15 2016), 340-347.

LI, S., TIAB, L., JIAO, X., MUNIER, F. L., ZOGRAFOS, L., FRUEH, B. E., SERGEEV, Y., SMITH, J., RUBIN, B., MEALLET, M. A., FORSTER, R. K., HEJTMANCIK, J. F. and SCHORDERET, D. F. Mutations in PIP5K3 are associated with Francois-Neetens mouchetee fleck corneal dystrophy. Am J Hum Genet 77, 1 (Jul 2005), 54-63.

LI, X., SAITOH, S., SHIBATA, T., TANIMURA, N., FUKUI, R. and MIYAKE, K. Mucolipin 1 positively regulates TLR7 responses in dendritic cells by facilitating RNA transportation to lysosomes. Int Immunol 27, 2 (Feb 2015), 83-94.

LI, X., WANG, X., ZHANG, X., ZHAO, M., TSANG, W. L., ZHANG, Y., YAU, R. G., WEISMAN, L. S. and XU, H. Genetically encoded fluorescent probe to visualize intracellular phosphatidylinositol 3,5-bisphosphate localization and dynamics. Proc Natl Acad Sci U S A 110, 52 (Dec 24 2013), 21165-21170.

LINDSKOG, C. The potential clinical impact of the tissue-based map of the human proteome. Expert Rev Proteomics 12, 3 (Jun 2015), 213-215.

LIN-MOSHIER, Y., KEEBLER, M. V., HOOPER, R., BOULWARE, M. J., LIU, X., CHURAMANI, D., ABOOD, M. E., WALSETH, T. F., BRAILOIU, E., PATEL, S. and

MARCHANT, J. S. The Two-pore channel (TPC) interactome unmasks isoform-specific roles for TPCs in endolysosomal morphology and cell pigmentation. Proc Natl Acad Sci U S A 111, 36 (Sep 9 2014), 13087-13092.

LIPATOVA, Z., TOKAREV, A. A., JIN, Y., MULHOLLAND, J., WEISMAN, L. S. and SEGEV, N. Direct interaction between a myosin V motor and the Rab GTPases Ypt31/32 is required for polarized secretion. Mol Biol Cell 19, 10 (Oct 2008), 4177-4187.

LIU, Y. and BANKAITIS, V. A. Phosphoinositide phosphatases in cell biology and disease. Progress in Lipid Research 49, 3 (Jul 2010), 201-217.

LIU, Y., LAI, Y. C., HILL, E. V., TYTECA, D., CARPENTIER, S., INGVALDSEN, A., VERTOMMEN, D., LANTIER, L., FORETZ, M., DEQUIEDT, F., COURTOY, P. J., ERNEUX, C., VIOLLET, B., SHEPHERD, P. R., TAVARE, J. M., JENSEN, J. and RIDER, M. H. Phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) is an AMPK target participating in contraction-stimulated glucose uptake in skeletal muscle. Biochem J 455, 2 (Oct 15 2013), 195-206.

MAJERUS, P. W. and YORK, J. D. Phosphoinositide phosphatases and disease. J Lipid Res 50 (Apr 2009), S249-S254.

MARKERT, C. L. and SILVERS, W. K. The Effects of Genotype and Cell Environment on Melanoblast Differentiation in the House Mouse. Genetics 41, 3 (May 1956), 429-450.

MARKS, M. S. Organelle biogenesis: en BLOC exchange for RAB32 and RAB38. Curr Biol 22, 22 (Nov 20 2012), R963-965.

MARKS, M. S., HEIJNEN, H. F. and RAPOSO, G. Lysosome-related organelles: unusual compartments become mainstream. Curr Opin Cell Biol 25, 4 (Aug 2013), 495-505.

MARTIN, S., HARPER, C. B., MAY, L. M., COULSON, E. J., MEUNIER, F. A. and OSBORNE, S. L. Inhibition of PIKfyve by YM-201636 dysregulates autophagy and leads to apoptosis-independent neuronal cell death. PLoS One 8, 3 (2013), e60152.

MAUTHE, M., JACOB, A., FREIBERGER, S., HENTSCHEL, K., STIERHOF, Y. D., CODOGNO, P. and PROIKAS-CEZANNE, T. Resveratrol-mediated autophagy requires WIPI-1-regulated LC3 lipidation in the absence of induced phagophore formation. Autophagy 7, 12 (Dec 2011), 1448-1461.

MCCARTNEY, A. J., ZHANG, Y. and WEISMAN, L. S. Phosphatidylinositol 3,5-bisphosphate: low abundance, high significance. Bioessays 36, 1 (Jan 2014), 52-64.

MCMAHON, H. T. and BOUCROT, E. Molecular mechanism and physiological functions of clathrin-mediated endocytosis. Nat Rev Mol Cell Bio 12, 8 (Aug 2011), 517-533.

MESMIN, B., BIGAY, J., MOSER VON FILSECK, J., LACAS-GERVAIS, S., DRIN, G. and ANTONNY, B. A four-step cycle driven by PI(4)P hydrolysis directs sterol/PI(4)P exchange by the ER-Golgi tether OSBP. Cell 155, 4 (Nov 7 2013), 830-843.

MIN, S. H., SUZUKI, A., STALKER, T. J., ZHAO, L., WANG, Y., MCKENNAN, C., RIESE, M. J., GUZMAN, J. F., ZHANG, S., LIAN, L., JOSHI, R., MENG, R., SEEHOLZER, S. H., CHOI, J. K., KORETZKY, G., MARKS, M. S. and ABRAMS, C. S. Loss of PIKfyve in platelets causes a lysosomal disease leading to inflammation and thrombosis in mice. Nat Commun 5 (2014), 4691.

MIYAMURA, Y., COELHO, S. G., SCHLENZ, K., BATZER, J., SMUDA, C., CHOI, W., BRENNER, M., PASSERON, T., ZHANG, G., KOLBE, L., WOLBER, R. and HEARING, V. J. The deceptive nature of UVA tanning versus the modest protective effects of UVB tanning on human skin. Pigment Cell Melanoma Res 24, 1 (Feb 2011), 136-147.

MONTELL, C. The TRP superfamily of cation channels. Sci STKE 2005, 272 (Feb 22 2005), re3.

MORT, R. L., JACKSON, I. J. and PATTON, E. E. The melanocyte lineage in development and disease. Development 142, 7 (Apr 1 2015), 1387.

MORYA, V. K., DUNG, N. H., SINGH, B. K., LEE, H. B. and KIM, E. K. Homology modelling and virtual screening of P-protein in a quest for novel antimelanogenic agent and in vitro assessments. Exp Dermatol 23, 11 (Nov 2014), 838-842.

MURASE, D., HACHIYA, A., KIKUCHI-ONOE, M., FULLENKAMP, R., OHUCHI, A., KITAHARA, T., MORIWAKI, S., HASE, T. and TAKEMA, Y. Cooperation of endothelin-1 signaling with melanosomes plays a role in developing and/or maintaining human skin hyperpigmentation. Biol Open 4, 10 (2015), 1213-1221.

NAKAJIMA, J., OKAMOTO, N., SHIRAISHI, J., NISHIMURA, G., NAKASHIMA, M., TSURUSAKI, Y., SAITSU, H., KAWASHIMA, H., MATSUMOTO, N. and MIYAKE, N. Novel FIG4 mutations in Yunis-Varon syndrome. J Hum Genet 58, 12 (Dec 2013), 822-824.

NICHOLS, S. E., JR. and REAMS, W. M., JR. The occurrence and morphogenesis of melanocytes in the connective tissues of the PET/MCV mouse strain. J Embryol Exp Morphol 8 (Mar 1960), 24-32.

NICOT, A. S., FARES, H., PAYRASTRE, B., CHISHOLM, A. D., LABOUESSE, M. and LAPORTE, J. The phosphoinositide kinase PIKfyve/Fab1p regulates terminal lysosome maturation in Caenorhabditis elegans. Mol Biol Cell 17, 7 (Jul 2006), 3062-3074.

NISHIMURA, E. K., JORDAN, S. A., OSHIMA, H., YOSHIDA, H., OSAWA, M., MORIYAMA, M., JACKSON, I. J., BARRANDON, Y., MIYACHI, Y. and NISHIKAWA, S. Dominant role of the niche in melanocyte stem-cell fate determination. Nature 416, 6883 (Apr 25 2002), 854-860.

OBARA, K., SEKITO, T., NIIMI, K. and OHSUMI, Y. The Atg18-Atg2 complex is recruited to autophagic membranes via phosphatidylinositol 3-phosphate and exerts an essential function. J Biol Chem 283, 35 (Aug 29 2008), 23972-23980.

ODORIZZI, G., BABST, M. and EMR, S. D. Fab1p PtdIns(3)P 5-kinase function essential for protein sorting in the multivesicular body. Cell 95, 6 (Dec 11 1998), 847-858.

OHGUCHI, K., BANNO, Y., NAKAGAWA, Y., AKAO, Y. and NOZAWA, Y. Negative regulation of melanogenesis by phospholipase D1 through mTOR/p70 S6 kinase 1 signaling in mouse B16 melanoma cells. J Cell Physiol 205, 3 (Dec 2005), 444-451.

OHNO, H., AGUILAR, R. C., YEH, D., TAURA, D., SAITO, T. and BONIFACINO, J. S. The medium subunits of adaptor complexes recognize distinct but overlapping sets of tyrosine-based sorting signals. J Biol Chem 273, 40 (Oct 2 1998), 25915-25921.

OKIMOTO, D. K., BLAUS, A., SCHMIDT, M. V., GORDON, M. K., DENT, G. W. and LEVINE, S. Differential expression of c-fos and tyrosine hydroxylase mRNA in the adrenal gland of the infant rat: evidence for an adrenal hyporesponsive period. Endocrinology 143, 5 (May 2002), 1717-1725.

OZEKI, H., ITO, S., WAKAMATSU, K. and HIROBE, T. Chemical characterization of hair melanins in various coat-color mutants of mice. J Invest Dermatol 105, 3 (Sep 1995), 361-366.

OZEKI, H., ITO, S., WAKAMATSU, K. and THODY, A. J. Spectrophotometric characterization of eumelanin and pheomelanin in hair. Pigment Cell Res 9, 5 (Oct 1996), 265-270.

PARK, H. Y., KOSMADAKI, M., YAAR, M. and GILCHREST, B. A. Cellular mechanisms regulating human melanogenesis. Cell Mol Life Sci 66, 9 (May 2009), 1493-1506.

PATERSON, E. K., FIELDER, T. J., MACGREGOR, G. R., ITO, S., WAKAMATSU, K., GILLEN, D. L., EBY, V., BOISSY, R. E. and GANESAN, A. K. Tyrosinase Depletion Prevents the Maturation of Melanosomes in the Mouse Hair Follicle. PLoS One 10, 11 (2015), e0143702.

PENG, Y. and WEISMAN, L. S. The cyclin-dependent kinase Cdk1 directly regulates vacuole inheritance. Dev Cell 15, 3 (Sep 2008), 478-485.

POCCIA, D. and LARIJANI, B. Phosphatidylinositol metabolism and membrane fusion. Biochem J 418, 2 (Mar 1 2009), 233-246.

POLSON, H. E., DE LARTIGUE, J., RIGDEN, D. J., REEDIJK, M., URBE, S., CLAGUE, M. J. and TOOZE, S. A. Mammalian Atg18 (WIPI2) localizes to omegasome-anchored phagophores and positively regulates LC3 lipidation. Autophagy 6, 4 (May 2010), 506-522.

PROIKAS-CEZANNE, T., TAKACS, Z., DONNES, P. and KOHLBACHER, O. WIPI proteins: essential PtdIns3P effectors at the nascent autophagosome. J Cell Sci 128, 2 (Jan 15 2015), 207-217.

PROIKAS-CEZANNE, T., WADDELL, S., GAUGEL, A., FRICKEY, T., LUPAS, A. and NORDHEIM, A. WIPI-1alpha (WIPI49), a member of the novel 7-bladed WIPI protein family, is aberrantly expressed in human cancer and is linked to starvation-induced autophagy. Oncogene 23, 58 (Dec 16 2004), 9314-9325.

PROKSCH, E., BRANDNER, J. M. and JENSEN, J. M. The skin: an indispensable barrier. Exp Dermatol 17, 12 (Dec 2008), 1063-1072.

PUERTOLLANO, R. and KISELYOV, K. TRPMLs: in sickness and in health. Am J Physiol Renal Physiol 296, 6 (Jun 2009), F1245-1254.

RAPOSO, G. and MARKS, M. S. Melanosomes--dark organelles enlighten endosomal membrane transport. Nat Rev Mol Cell Biol 8, 10 (Oct 2007), 786-797.

RAPOSO, G., MARKS, M. S. and CUTLER, D. F. Lysosome-related organelles: driving post-Golgi compartments into specialisation. Curr Opin Cell Biol 19, 4 (Aug 2007), 394-401.

RICHMOND, B., HUIZING, M., KNAPP, J., KOSHOFFER, A., ZHAO, Y., GAHL, W. A. and BOISSY, R. E. Melanocytes derived from patients with Hermansky-Pudlak Syndrome types 1, 2, and 3 have distinct defects in cargo trafficking. J Invest Dermatol 124, 2 (Feb 2005), 420-427.

ROBINSON, F. L. and DIXON, J. E. The phosphoinositide-3-phosphatase MTMR2 associates with MTMR13, a membrane-associated pseudophosphatase also mutated in type 4B Charcot-Marie-Tooth disease. J Biol Chem 280, 36 (Sep 9 2005), 31699-31707.

ROCHIN, L., HURBAIN, I., SERNEELS, L., FORT, C., WATT, B., LEBLANC, P., MARKS, M. S., DE STROOPER, B., RAPOSO, G. and VAN NIEL, G. BACE2 processes PMEL to form the melanosome amyloid matrix in pigment cells. Proc Natl Acad Sci U S A 110, 26 (Jun 25 2013), 10658-10663.

RUTHERFORD, A. C., TRAER, C., WASSMER, T., PATTNI, K., BUJNY, M. V., CARLTON, J. G., STENMARK, H. and CULLEN, P. J. The mammalian phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) regulates endosome-to-TGN retrograde transport. J Cell Sci 119, Pt 19 (Oct 1 2006), 3944-3957.

SAKURAI, Y., KOLOKOLTSOV, A. A., CHEN, C. C., TIDWELL, M. W., BAUTA, W. E., KLUGBAUER, N., GRIMM, C., WAHL-SCHOTT, C., BIEL, M. and DAVEY, R. A. Ebola virus. Two-pore channels control Ebola virus host cell entry and are drug targets for disease treatment. Science 347, 6225 (Feb 27 2015), 995-998.

SASAKI, T., TAKASUGA, S., SASAKI, J., KOFUJI, S., EGUCHI, S., YAMAZAKI, M. and SUZUKI, A. Mammalian phosphoinositide kinases and phosphatases. Prog Lipid Res 48, 6 (Nov 2009), 307-343.

SASAKI, T., TAKASUGA, S., SASAKI, J., KOFUJI, S., EGUCHI, S., YAMAZAKI, M. and SUZUKI, A. Mammalian phosphoinositide kinases and phosphatases. Progress in Lipid Research 48, 6 (Nov 2009), 307-343.

SBRISSA, D., IKONOMOV, O. C., FENNER, H. and SHISHEVA, A. ArPIKfyve homomeric and heteromeric interactions scaffold PIKfyve and Sac3 in a complex to promote PIKfyve activity and functionality. J Mol Biol 384, 4 (Dec 26 2008), 766-779.

SBRISSA, D., IKONOMOV, O. C., FENNER, H. and SHISHEVA, A. ArPIKfyve homomeric and heteromeric interactions scaffold PIKfyve and Sac3 in a complex to promote PIKfyve activity and functionality. J Mol Biol 384, 4 (Dec 26 2008), 766-779.

SBRISSA, D., IKONOMOV, O. C., FU, Z., IJUIN, T., GRUENBERG, J., TAKENAWA, T. and SHISHEVA, A. Core protein machinery for mammalian phosphatidylinositol 3,5-bisphosphate synthesis and turnover that regulates the progression of endosomal transport. Novel Sac phosphatase joins the ArPIKfyve-PIKfyve complex. J Biol Chem 282, 33 (Aug 17 2007), 23878-23891.

SBRISSA, D., IKONOMOV, O. C. and SHISHEVA, A. PIKfyve, a mammalian ortholog of yeast Fab1p lipid kinase, synthesizes 5-phosphoinositides. Effect of insulin. J Biol Chem 274, 31 (Jul 30 1999), 21589-21597.

SBRISSA, D., IKONOMOV, O. C. and SHISHEVA, A. Phosphatidylinositol 3-phosphate-interacting domains in PIKfyve. Binding specificity and role in PIKfyve. Endomenbrane localization. J Biol Chem 277, 8 (Feb 22 2002), 6073-6079.

SCHALETZKY, J., DOVE, S. K., SHORT, B., LORENZO, O., CLAGUE, M. J. and BARR, F. A. Phosphatidylinositol-5-phosphate activation and conserved substrate specificity of the myotubularin phosphatidylinositol 3-phosphatases. Curr Biol 13, 6 (Mar 18 2003), 504-509.

SCHULZE, U., VOLLENBROKER, B., BRAUN, D. A., VAN LE, T., GRANADO, D., KREMERSKOTHEN, J., FRANZEL, B., KLOSOWSKI, R., BARTH, J., FUFEZAN, C., WOLTERS, D. A., PAVENSTADT, H. and WEIDE, T. The Vac14-interaction network is linked to regulators of the endolysosomal and autophagic pathway. Mol Cell Proteomics 13, 6 (Jun 2014), 1397-1411.

SCOTT, G. Rac and rho: the story behind melanocyte dendrite formation. Pigment Cell Res 15, 5 (Oct 2002), 322-330.

SETTY, S. R., TENZA, D., SVIDERSKAYA, E. V., BENNETT, D. C., RAPOSO, G. and MARKS, M. S. Cell-specific ATP7A transport sustains copper-dependent tyrosinase activity in melanosomes. Nature 454, 7208 (Aug 28 2008), 1142-1146.

SHAH, Z. H., JONES, D. R., SOMMER, L., FOULGER, R., BULTSMA, Y., D'SANTOS, C. and DIVECHA, N. Nuclear phosphoinositides and their impact on nuclear functions. FEBS J 280, 24 (Dec 2013), 6295-6310.

SHEN, J., YU, W. M., BROTTO, M., SCHERMAN, J. A., GUO, C., STODDARD, C., NOSEK, T. M., VALDIVIA, H. H. and QU, C. K. Deficiency of MIP/MTMR14 phosphatase induces a muscle disorder by disrupting Ca(2+) homeostasis. Nat Cell Biol 11, 6 (Jun 2009), 769-776.

SHIM, H., WU, C., RAMSAMOOJ, S., BOSCH, K. N., CHEN, Z., EMERLING, B. M., YUN, J., LIU, H., CHOO-WING, R., YANG, Z., WULF, G. M., KUCHROO, V. K. and CANTLEY, L. C. Deletion of the gene Pip4k2c, a novel phosphatidylinositol kinase, results in hyperactivation of the immune system. Proc Natl Acad Sci U S A 113, 27 (Jul 5 2016), 7596-7601.

SHIMSHEK, D. R., JACOBSON, L. H., KOLLY, C., ZAMUROVIC, N., BALAVENKATRAMAN, K. K., MORAWIEC, L., KREUTZER, R., SCHELLE, J., JUCKER, M., BERTSCHI, B., THEIL, D., HEIER, A., BIGOT, K., BELTZ, K., MACHAUER, R., BRZAK, I., PERROT, L. and NEUMANN, U. Pharmacological BACE1 and BACE2 inhibition induces hair depigmentation by inhibiting PMEL17 processing in mice. Sci Rep 6 (2016), 21917.

SHISHEVA, A. PIKfyve: Partners, significance, debates and paradoxes. Cell Biol Int 32, 6 (Jun 2008), 591-604.

SHISHEVA, A. PtdIns5P: news and views of its appearance, disappearance and deeds. Arch Biochem Biophys 538, 2 (Oct 15 2013), 171-180.

SHISHEVA, A., SBRISSA, D. and IKONOMOV, O. Cloning, characterization, and expression of a novel Zn2+-binding FYVE finger-containing phosphoinositide kinase in insulin-sensitive cells. Mol Cell Biol 19, 1 (Jan 1999), 623-634.

SHISHEVA, A., SBRISSA, D. and IKONOMOV, O. Plentiful PtdIns5P from scanty PtdIns(3,5)P2 or from ample PtdIns? PIKfyve-dependent models: Evidence and speculation (response to: DOI 10.1002/bies.201300012). Bioessays 37, 3 (Mar 2015), 267-277.

SHISHEVA, A., SBRISSA, D. and IKONOMOV, O. Plentiful PtdIns5P from scanty PtdIns(3,5)P2 or from ample PtdIns? PIKfyve-dependent models: Evidence and speculation (response to: DOI 10.1002/bies.201300012). Bioessays 37, 3 (Mar 2015), 267-277.

SIMONSEN, A., WURMSER, A. E., EMR, S. D. and STENMARK, H. The role of phosphoinositides in membrane transport. Current Opinion in Cell Biology 13, 4 (Aug 2001), 485-492.

SITARAM, A. and MARKS, M. S. Mechanisms of protein delivery to melanosomes in pigment cells. Physiology (Bethesda) 27, 2 (Apr 2012), 85-99.

SLOMINSKI, A., TOBIN, D. J., SHIBAHARA, S. and WORTSMAN, J. Melanin pigmentation in mammalian skin and its hormonal regulation. Physiol Rev 84, 4 (Oct 2004), 1155-1228.

SMITH, J. W., KOSHOFFER, A., MORRIS, R. E. and BOISSY, R. E. Membranous complexes characteristic of melanocytes derived from patients with Hermansky-Pudlak syndrome type 1 are macroautophagosomal entities of the lysosomal compartment. Pigment Cell Res 18, 6 (Dec 2005), 417-426.

STELZER, G., ROSEN, N., PLASCHKES, I., ZIMMERMAN, S., TWIK, M., FISHILEVICH, S., STEIN, T. I., NUDEL, R., LIEDER, I., MAZOR, Y., KAPLAN, S., DAHARY, D., WARSHAWSKY, D., GUAN-GOLAN, Y., KOHN, A., RAPPAPORT, N., SAFRAN, M. and LANCET, D. The GeneCards Suite: From Gene Data Mining to Disease Genome Sequence Analyses. Curr Protoc Bioinformatics 54 (2016), 1 30 31-31 30 33.

STIJF-BULTSMA, Y., SOMMER, L., TAUBER, M., BAALBAKI, M., GIARDOGLOU, P., JONES, D. R., GELATO, K. A., VAN PELT, J., SHAH, Z., RAHNAMOUN, H., TOMA, C., ANDERSON, K. E., HAWKINS, P., LAUBERTH, S. M., HARAMIS, A. P., HART, D., FISCHLE, W. and DIVECHA, N. The basal transcription complex component TAF3 transduces changes in nuclear phosphoinositides into transcriptional output. Mol Cell 58, 3 (May 7 2015), 453-467.

STIJF-BULTSMA, Y., SOMMER, L., TAUBER, M., BAALBAKI, M., GIARDOGLOU, P., JONES, D. R., GELATO, K. A., VAN PELT, J., SHAH, Z., RAHNAMOUN, H., TOMA, C., ANDERSON, K. E., HAWKINS, P., LAUBERTH, S. M., HARAMIS, A. P., HART, D., FISCHLE, W. and DIVECHA, N. The basal transcription complex component TAF3

transduces changes in nuclear phosphoinositides into transcriptional output. Mol Cell 58, 3 (May 7 2015), 453-467.

SULEM, P., GUDBJARTSSON, D. F., STACEY, S. N., HELGASON, A., RAFNAR, T., JAKOBSDOTTIR, M., STEINBERG, S., GUDJONSSON, S. A., PALSSON, A., THORLEIFSSON, G., PALSSON, S., SIGURGEIRSSON, B., THORISDOTTIR, K., RAGNARSSON, R., BENEDIKTSDOTTIR, K. R., ABEN, K. K., VERMEULEN, S. H., GOLDSTEIN, A. M., TUCKER, M. A., KIEMENEY, L. A., OLAFSSON, J. H., GULCHER, J., KONG, A., THORSTEINSDOTTIR, U. and STEFANSSON, K. Two newly identified genetic determinants of pigmentation in Europeans. Nat Genet 40, 7 (Jul 2008), 835-837.

TAKASUGA, S., HORIE, Y., SASAKI, J., SUN-WADA, G. H., KAWAMURA, N., IIZUKA, R., MIZUNO, K., EGUCHI, S., KOFUJI, S., KIMURA, H., YAMAZAKI, M., HORIE, C., ODANAGA, E., SATO, Y., CHIDA, S., KONTANI, K., HARADA, A., KATADA, T., SUZUKI, A., WADA, Y., OHNISHI, H. and SASAKI, T. Critical roles of type III phosphatidylinositol phosphate kinase in murine embryonic visceral endoderm and adult intestine. Proc Natl Acad Sci U S A 110, 5 (Jan 29 2013), 1726-1731.

TAKASUGA, S. and SASAKI, T. Phosphatidylinositol-3,5-bisphosphate: metabolism and physiological functions. J Biochem 154, 3 (Sep 2013), 211-218.

THEOS, A. C., TENZA, D., MARTINA, J. A., HURBAIN, I., PEDEN, A. A., SVIDERSKAYA, E. V., STEWART, A., ROBINSON, M. S., BENNETT, D. C., CUTLER, D. F., BONIFACINO, J. S., MARKS, M. S. and RAPOSO, G. Functions of adaptor protein (AP)-3 and AP-1 in tyrosinase sorting from endosomes to melanosomes. Mol Biol Cell 16, 11 (Nov 2005), 5356-5372.

THERIAULT, L. L. and HURLEY, L. S. Ultrastructure of developing melanosomes in C57 black and pallid mice. Dev Biol 23, 2 (Oct 1970), 261-275.

THINGNES, J., LAVELLE, T. J., HOVIG, E. and OMHOLT, S. W. Understanding the melanocyte distribution in human epidermis: an agent-based computational model approach. PLoS One 7, 7 (2012), e40377.

THODY, A. J., HIGGINS, E. M., WAKAMATSU, K., ITO, S., BURCHILL, S. A. and MARKS, J. M. Pheomelanin as well as eumelanin is present in human epidermis. J Invest Dermatol 97, 2 (Aug 1991), 340-344.

THOST, A. K., DONNES, P., KOHLBACHER, O. and PROIKAS-CEZANNE, T. Fluorescence-based imaging of autophagy progression by human WIPI protein detection. Methods 75 (Mar 15 2015), 69-78.

TOBIN, D. J. Biochemistry of human skin--our brain on the outside. Chem Soc Rev 35, 1 (Jan 2006), 52-67.

TOBIN, D. J. Aging of the hair follicle pigmentation system. Int J Trichology 1, 2 (Jul 2009), 83-93.

TSATMALI, M., ANCANS, J. and THODY, A. J. Melanocyte function and its control by melanocortin peptides. J Histochem Cytochem 50, 2 (Feb 2002), 125-133.

TSUJITA, K., ITOH, T., IJUIN, T., YAMAMOTO, A., SHISHEVA, A., LAPORTE, J. and TAKENAWA, T. Myotubularin regulates the function of the late endosome through the gram domain-phosphatidylinositol 3,5-bisphosphate interaction. J Biol Chem 279, 14 (Apr 2 2004), 13817-13824.

UHLEN, M., FAGERBERG, L., HALLSTROM, B. M., LINDSKOG, C., OKSVOLD, P., MARDINOGLU, A., SIVERTSSON, A., KAMPF, C., SJOSTEDT, E., ASPLUND, A., OLSSON, I., EDLUND, K., LUNDBERG, E., NAVANI, S., SZIGYARTO, C. A., ODEBERG, J., DJUREINOVIC, D., TAKANEN, J. O., HOBER, S., ALM, T., EDQVIST, P. H., BERLING, H., TEGEL, H., MULDER, J., ROCKBERG, J., NILSSON, P., SCHWENK, J. M., HAMSTEN, M., VON FEILITZEN, K., FORSBERG, M., PERSSON, L., JOHANSSON, F., ZWAHLEN, M., VON HEIJNE, G., NIELSEN, J. and PONTEN, F. Proteomics. Tissue-based map of the human proteome. Science 347, 6220 (Jan 23 2015), 1260419.

URABE, K., AROCA, P., TSUKAMOTO, K., MASCAGNA, D., PALUMBO, A., PROTA, G. and HEARING, V. J. The inherent cytotoxicity of melanin precursors: a revision. Biochim Biophys Acta 1221, 3 (Apr 28 1994), 272-278.

VACCARI, I., DINA, G., TRONCHERE, H., KAUFMAN, E., CHICANNE, G., CERRI, F., WRABETZ, L., PAYRASTRE, B., QUATTRINI, A., WEISMAN, L. S., MEISLER, M. H. and BOLINO, A. Genetic interaction between MTMR2 and FIG4 phospholipid phosphatases involved in Charcot-Marie-Tooth neuropathies. PLoS Genet 7, 10 (Oct 2011), e1002319.

VASSAR, R., BENNETT, B. D., BABU-KHAN, S., KAHN, S., MENDIAZ, E. A., DENIS, P., TEPLOW, D. B., ROSS, S., AMARANTE, P., LOELOFF, R., LUO, Y., FISHER, S., FULLER, J., EDENSON, S., LILE, J., JAROSINSKI, M. A., BIERE, A. L., CURRAN, E., BURGESS, T., LOUIS, J. C., COLLINS, F., TREANOR, J., ROGERS, G. and CITRON, M. Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science 286, 5440 (Oct 22 1999), 735-741.

VERGNE, I., DELGADO-VARGAS, M., PONPUAK, M. and DERETIC, V. Regulation of autophagy by PI3P phosphatases. Autophagy 5, 6 (Aug 16 2009), 906-906.

VERGNE, I. and DERETIC, V. The role of PI3P phosphatases in the regulation of autophagy. Febs Letters 584, 7 (Apr 2 2010), 1313-1318.

VICINANZA, M., KOROLCHUK, V. I., ASHKENAZI, A., PURI, C., MENZIES, F. M., CLARKE, J. H. and RUBINSZTEIN, D. C. PI(5)P regulates autophagosome biogenesis. Mol Cell 57, 2 (Jan 22 2015), 219-234.

VICINANZA, M. and RUBINSZTEIN, D. C. Mirror image phosphoinositides regulate autophagy. Mol Cell Oncol 3, 2 (Mar 2016), e1019974.

VIJAYASARADHI, S., XU, Y., BOUCHARD, B. and HOUGHTON, A. N. Intracellular sorting and targeting of melanosomal membrane proteins: identification of signals for sorting of the human brown locus protein, gp75. J Cell Biol 130, 4 (Aug 1995), 807-820.

WALKER, D. M., URBE, S., DOVE, S. K., TENZA, D., RAPOSO, G. and CLAGUE, M. J. Characterization of MTMR3. an inositol lipid 3-phosphatase with novel substrate specificity. Curr Biol 11, 20 (Oct 16 2001), 1600-1605.

WANG, X., ZHANG, X., DONG, X. P., SAMIE, M., LI, X., CHENG, X., GOSCHKA, A., SHEN, D., ZHOU, Y., HARLOW, J., ZHU, M. X., CLAPHAM, D. E., REN, D. and XU, H. TPC proteins are phosphoinositide- activated sodium-selective ion channels in endosomes and lysosomes. Cell 151, 2 (Oct 12 2012), 372-383.

WASMEIER, C., HUME, A. N., BOLASCO, G. and SEABRA, M. C. Melanosomes at a glance. J Cell Sci 121, Pt 24 (Dec 15 2008), 3995-3999.

WATANABE, Y., KOBAYASHI, T., YAMAMOTO, H., HOSHIDA, H., AKADA, R., INAGAKI, F., OHSUMI, Y. and NODA, N. N. Structure-based analyses reveal distinct binding sites for Atg2 and phosphoinositides in Atg18. J Biol Chem 287, 38 (Sep 14 2012), 31681-31690.

WATT, B., VAN NIEL, G., RAPOSO, G. and MARKS, M. S. PMEL: a pigment cell-specific model for functional amyloid formation. Pigment Cell Melanoma Res 26, 3 (May 2013), 300-315.

WEI, A. H. and LI, W. Hermansky-Pudlak syndrome: pigmentary and non-pigmentary defects and their pathogenesis. Pigment Cell Melanoma Res 26, 2 (Mar 2013), 176-192.

WEISMAN, L. S. Organelles on the move: insights from yeast vacuole inheritance. Nat Rev Mol Cell Biol 7, 4 (Apr 2006), 243-252.

WHITLEY, P., REAVES, B. J., HASHIMOTO, M., RILEY, A. M., POTTER, B. V. and HOLMAN, G. D. Identification of mammalian Vps24p as an effector of phosphatidylinositol 3,5-bisphosphate-dependent endosome compartmentalization. J Biol Chem 278, 40 (Oct 3 2003), 38786-38795.

XU, H., DELLING, M., LI, L., DONG, X. and CLAPHAM, D. E. Activating mutation in a mucolipin transient receptor potential channel leads to melanocyte loss in varitint-waddler mice. Proc Natl Acad Sci U S A 104, 46 (Nov 13 2007), 18321-18326.

YAJIMA, I., SATO, S., KIMURA, T., YASUMOTO, K., SHIBAHARA, S., GODING, C. R. and YAMAMOTO, H. An L1 element intronic insertion in the black-eyed white (Mitf[mi-bw]) gene: the loss of a single Mitf isoform responsible for the pigmentary defect and inner ear deafness. Hum Mol Genet 8, 8 (Aug 1999), 1431-1441.

YAMAGUCHI, Y. and HEARING, V. J. Physiological factors that regulate skin pigmentation. Biofactors 35, 2 (Mar-Apr 2009), 193-199.

YOO, K. Y., SON, J. Y., LEE, J. U., SHIN, W., IM, D. W., KIM, S. J., RYU, S. E. and HEO, Y. S. Structure of the catalytic phosphatase domain of MTMR8: implications for dimerization, membrane association and reversible oxidation. Acta Crystallogr D Biol Crystallogr 71, Pt 7 (Jul 2015), 1528-1539.

ZECCA, L., TAMPELLINI, D., GERLACH, M., RIEDERER, P., FARIELLO, R. G. and SULZER, D. Substantia nigra neuromelanin: structure, synthesis, and molecular behaviour. Mol Pathol 54, 6 (Dec 2001), 414-418.

ZECCA, L., ZUCCA, F. A., COSTI, P., TAMPELLINI, D., GATTI, A., GERLACH, M., RIEDERER, P., FARIELLO, R. G., ITO, S., GALLORINI, M. and SULZER, D. The neuromelanin of human substantia nigra: structure, synthesis and molecular behaviour. J Neural Transm Suppl, 65 (2003), 145-155.

ZHANG, C. F., GRUBER, F., NI, C., MILDNER, M., KOENIG, U., KARNER, S., BARRESI, C., ROSSITER, H., NARZT, M. S., NAGELREITER, I. M., LARUE, L., TOBIN, D. J., ECKHART, L. and TSCHACHLER, E. Suppression of autophagy dysregulates the antioxidant response and causes premature senescence of melanocytes. J Invest Dermatol 135, 5 (May 2015), 1348-1357.

ZHANG, X., CHOW, C. Y., SAHENK, Z., SHY, M. E., MEISLER, M. H. and LI, J. Mutation of FIG4 causes a rapidly progressive, asymmetric neuronal degeneration. Brain 131, Pt 8 (Aug 2008), 1990-2001.

ZHANG, Y., ZOLOV, S. N., CHOW, C. Y., SLUTSKY, S. G., RICHARDSON, S. C., PIPER, R. C., YANG, B., NAU, J. J., WESTRICK, R. J., MORRISON, S. J., MEISLER, M. H. and WEISMAN, L. S. Loss of Vac14, a regulator of the signaling lipid phosphatidylinositol 3,5-bisphosphate, results in neurodegeneration in mice. Proc Natl Acad Sci U S A 104, 44 (Oct 30 2007), 17518-17523.

ZHAO, Y. G., SUN, L., MIAO, G. Y., JI, C. C., ZHAO, H. Y., SUN, H. Y., MIAO, L., YOSHII, S. R., MIZUSHIMA, N., WANG, X. Q. and ZHANG, H. The autophagy gene

Wdr45/Wipi4 regulates learning and memory function and axonal homeostasis. Autophagy 11, 6 (Jun 2015), 881-890.

ZOLOV, S. N., BRIDGES, D., ZHANG, Y., LEE, W. W., RIEHLE, E., VERMA, R., LENK, G. M., CONVERSO-BARAN, K., WEIDE, T., ALBIN, R. L., SALTIEL, A. R., MEISLER, M. H., RUSSELL, M. W. and WEISMAN, L. S. In vivo, Pikfyve generates PI(3,5)P2, which serves as both a signaling lipid and the major precursor for PI5P. Proc Natl Acad Sci U S A 109, 43 (Oct 23 2012), 17472-17477.