The Clathrin Adaptor AP-1 and Type II Phosphatidylinositol 4-kinase are Required for Glue Granule Biogenesis in *Drosophila*

by

Jason Anthony Burgess

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Department of Molecular Genetics

University of Toronto

© Copyright by Jason Anthony Burgess 2011
The clathrin adaptor AP-1 and Type II Phosphatidylinositol 4-Kinase are Required for Glue Granule Biogenesis in *Drosophila*

Jason Anthony Burgess

Doctor of Philosophy

Department of Molecular Genetics
University of Toronto

2011

Abstract

Regulated secretion of hormones, digestive enzymes and other biologically active molecules requires formation of secretory granules. However, the molecular machinery required for secretory granule biogenesis is incompletely understood. I used powerful genetic approaches available in the fruit fly *Drosophila melanogaster* to investigate the factors required for biogenesis of mucin-containing ‘glue granules,’ which form within epithelial cells of the third-instar larval salivary gland. I discovered that clathrin and the clathrin adaptor protein complex (AP-1), as well the enzyme type II phosphatidylinositol 4-kinase (PI4KII), are indispensable for glue granule biogenesis.

Clathrin and AP-1 are necessary for maturation of exocrine, endocrine and neuroendocrine secretory granules in mammalian cells. I found that *Drosophila* clathrin and AP-1 colocalize at the TGN and that clathrin recruitment requires AP-1. I further showed that clathrin and AP-1 colocalize with secretory cargo at the TGN and on glue granules. Finally, I demonstrated that loss of clathrin or AP-1 leads to a profound block in secretory granule biogenesis. These findings establish a novel role for AP-1/clathrin-dependent trafficking in the formation of mucin-containing secretory granules.
Type II phosphatidylinositol 4-kinase (PI4KII) generates the membrane lipid phosphatidylinositol 4-phosphate (PI4P) at the trans-Golgi network and is required to recruit cargo to endosomes in mammalian cells. I generated null mutations in the sole Drosophila PI4KII and demonstrated a role for PI4KII in both glue granule and pigment granule biogenesis. PI4KII mutant salivary gland cells exhibit small glue granules and mislocalize glue protein to abnormally large late endosomes. Additionally, PI4KII mutants exhibit altered distribution of the granule specific SNARE, SNAP-24. These data point to a crucial role for PI4KII in sorting of regulated secretory products during granule biogenesis. Together, my results indicate that the larval salivary gland is a valuable system for investigating molecular mechanisms involved in secretory granule biogenesis, and provide a framework for future studies using this system.
Acknowledgments

I would like to thank my supervisor Julie Brill for being such a fantastic mentor. Julie has been tremendously supportive and exceptionally generous with her time during my PhD. She has made me a better public speaker, a more concise and clear writer and hopefully a better thinker. She also taught me the value of good citizenry in the scientific community by sending me to many conferences, encouraging me to give talks and share my data, and to embrace scientific collaborations. I have no doubt that the values of sharing and cooperation will serve me well in future endeavors.

I would also like to thank the members of my supervisory committee, Gabrielle Boulianne and Sean Egan, for excellent guidance and advice. They consistently kept me on track and showed genuine interest in my project, as well as my development as a graduate student. I am very grateful for their time and effort.

During my eight years at SickKids, I have had the opportunity to meet many wonderful, funny, warm and inspiring people. I would like to thank all members of the Brill lab past and present for their help, advice and time. Thank you Gordon, Julie, Phil, Ho-Chun, Ray, Lala and Anya for being a great source of moral support. You have became like a family to me and made it a pleasure to come into the lab everyday. Thanks to the various undergraduate students Marta, Ling, Ramtin, Preethy and Milu who have been a tremendous help with genetic screens. Thanks to Farzad, Wael and Lara for all the good times, as well as all the other MaRS and MoGen friends. You all helped make graduate school a very memorable experience.

I am thankful for the chance to have collaborated with many talented and experienced scientists. I would like to thank Helmut Kramer for getting me interested in the salivary gland and providing direction over the years. He generated many useful reagents and really helped to frame my research project. I would also like to thank Janet Rollins for making the trek up to Toronto and generously offering her time to help out with electron microscopy. I am also thankful to Joseph Albanesi and David Hipfner for conducting the PI4KII kinase assays and PI4KII RNAi tissue culture experiments, respectively. I am very appreciative of Roland Le Borgne, Henry Chang and Peter Leventis for generously sharing unpublished reagents. I would
also like to thank Paul Paroutis and Michael Woodside of the SickKids Imaging Facility for training and assistance on confocal microscopy, and Robert Temkin of the Mount Sinai Advanced Bioimaging Centre for help with electron microscopy. I would also like to thank NSERC and OGS for generous scholarships during my PhD studies.

Finally, I would like to thank my friends and family for their support throughout graduate school. I am grateful to Laura Berazadi for her friendship and joie de vivre, and to her family for their kindness and warmth. Thanks to Nick and Martin for providing a place to stay when taking holidays in beautiful Montreal, and to my parents Ray and Thérèse for their unwavering support (including financial!) during my PhD. Thank you!
Table of Contents

Acknowledgments........................................................................................................................................iv
Table of Contents........................................................................................................................................vi
List of Figures.............................................................................................................................................xi
List of Abbreviations.................................................................................................................................xiii
Chapter 1 ....................................................................................................................................................1

1 Introduction..............................................................................................................................................1

1.1 The regulated secretory pathway........................................................................................................1

1.1.1 Regulated secretion..........................................................................................................................1

1.1.2 Sorting mechanisms at the trans-Golgi network..............................................................................4

1.2 Sorting for entry .......................................................................................................................................8

1.2.1 Heterologous expression of secretory proteins.................................................................................8

1.2.2 Selective aggregation .........................................................................................................................9

1.2.3 RSP sorting domains.........................................................................................................................10

1.2.4 Membrane lipids ............................................................................................................................12

1.2.5 Linking luminal proteins with the TGN membrane...........................................................................14

1.2.6 A role for cytosolic coat proteins in granule formation .....................................................................16

1.3 Sorting by Retention ...........................................................................................................................18

1.3.1 Sorting by retention..........................................................................................................................18

1.3.2 Characteristics of immature secretory granules...............................................................................19

1.3.3 Clathrin coats and the removal of non-RSP cargo ..........................................................................19

1.3.4 Retention by processing ..................................................................................................................20

1.3.5 Homotypic fusion............................................................................................................................21

1.4 Summary and outstanding questions ...................................................................................................21

1.5 Drosophila Glue Granules ..................................................................................................................22
1.5.1 Glue granule production is developmentally regulated ........................................22
1.5.2 SNAREs implicated in glue granule biogenesis ..................................................23
1.5.3 Sgs glue genes .....................................................................................................24
1.5.4 Modeling granule biogenesis using *Drosophila* salivary glands .......................25
1.6 Thesis Overview .....................................................................................................26
Chapter 2 ....................................................................................................................27

2 AP-1 and clathrin are essential for secretory granule formation in *Drosophila* ........27

2.1 Summary ................................................................................................................28
2.2 Introduction .............................................................................................................28
2.3 Experimental Procedures .....................................................................................31
  2.3.1 Fly Stocks and genetic crosses ........................................................................31
  2.3.2 Molecular biology ............................................................................................32
  2.3.3 RNA isolation and RT-PCR of salivary glands .............................................32
  2.3.4 Fluorescence microscopy and imaging .........................................................33
  2.3.5 Electron microscopy .......................................................................................34
2.4 Results .....................................................................................................................35
  2.4.1 Glue granule biogenesis is developmentally regulated ...................................35
  2.4.2 Clathrin heavy chain and AP-1 localize to the *trans*-Golgi network ............39
  2.4.3 AP-1 recruits clathrin to the *trans*-Golgi network .........................................42
  2.4.4 Newly synthesized glue proteins pass through an AP-1 and clathrin positive
       compartment at the TGN ....................................................................................45
  2.4.5 AP-1 and clathrin are required for glue granule formation ............................50
  2.4.6 Glue transcripts are expressed in AP-1γ-depleted salivary glands ..........53
  2.4.7 Glue protein accumulates at the *trans*-Golgi network and in aberrant
       vacuolated organelles in AP-1γ-depleted cells ..............................................56
2.5 Discussion ..............................................................................................................59
  2.5.1 Glue granules and WPBs require AP-1 for formation ...................................59
2.5.2 A potential role for AP-1 in granule maturation ........................................... 59
2.5.3 Possible roles of AP-1 during granule formation ............................................. 60
2.5.4 AP-1 and epithelial polarity .............................................................................. 60
2.5.5 Depletion of AP-1 down regulates glue protein synthesis ............................. 61
2.5.6 Additional clathrin binding coat proteins in *Drosophila* ............................... 61

Chapter 3 ................................................................................................................ 63

3 Type II phosphatidylinositol 4-kinase is required for glue granule biogenesis in *Drosophila* ........................................................................................................ 63

3.1 Summary ........................................................................................................... 64
3.2 Introduction ....................................................................................................... 64

3.3 Experimental Procedures .................................................................................. 67

3.3.1 Fly stocks and genetic crosses ...................................................................... 67
3.3.2 Molecular biology ......................................................................................... 68
3.3.3 *Drosophila* S2 cell culture and RNAi ............................................................ 69
3.3.4 RT-PCR to detect PI4KII transcripts .............................................................. 70
3.3.5 Fluorescence microscopy and imaging of salivary glands ............................. 71
3.3.6 Transmission electron microscopy .................................................................. 71

3.4 Results ................................................................................................................ 73

3.4.1 *PI4KII* null mutant flies are viable ............................................................... 73
3.4.2 PI4KII localizes to the TGN and late endosomes in larval salivary gland cells ... 77
3.4.3 PI4KII localization is highly dynamic in live cells and colocalizes with microtubules ................................................................. 82
3.4.4 PI4KII colocalizes with glue cargo at the TGN and is required for proper granule biogenesis ................................................................. 85
3.4.5 PI4KII does not obviously affect recruitment of Golgi adaptor proteins ....... 89
3.4.6 PI4KII is required for sorting of the granule SNARE SNAP-24 ................. 92
3.4.7 Glue protein accumulates in enlarged late endosomes in *PI4KII* mutants .... 95
3.4.8 PI4KII and Fwd are redundant for an essential process in *Drosophila* ..........98

3.5 Discussion .................................................................................................................101

3.5.1 In which compartment does PI4KII exert its effect on granule biogenesis? ......101

3.5.2 Are glue granules lysosome-related organelles? ..................................................103

3.5.3 Role of tubular endosomes in salivary gland cells ............................................104

3.5.4 General functions of PI4KII in *Drosophila* .......................................................105

3.5.5 Genetic redundancy between *PI4KII* and *fwd* ................................................105

Chapter 4 ..........................................................................................................................107

4 Discussion and Future Directions .............................................................................107

4.1 Summary of results ....................................................................................................107

4.2 Role of AP-1 and clathrin in granule biogenesis .....................................................109

4.2.1 Clarifying the localization of coat proteins during glue granule biogenesis ......109

4.2.2 Biochemical approach to defining granule coat composition .........................110

4.2.3 Identifying AP-1 cargo and accessory factors required for granule formation ...111

4.3 Analysis of the role of PI4KII in granule biogenesis .............................................111

4.3.1 Does PI4KII regulate granule biogenesis by producing PI4P? .........................111

4.3.2 Role of endocytosis in granule formation ..........................................................112

4.4 General questions pertaining to glue granule biogenesis ....................................115

4.4.1 Do glue granules form by an aggregation-based mechanism? .........................115

4.4.2 Are glue proteins packaged together or sequentially? .......................................116

4.4.3 Sorting for entry versus sorting by retention .......................................................117

4.4.4 Identifying machinery required for homotypic fusion and exocytosis ..........119

4.4.5 Assessing glue granule secretion .......................................................................121

4.4.6 A genetic screen to identify *PI4KII* interacting genes .....................................122

4.4.7 Summary Model ....................................................................................................123

Appendix 1: PI4KII is required for pigment granule biogenesis .................................126

ix
Appendix 2: PI4KII is required for male fertility in *Drosophila*.......................... 132

Bibliography ........................................................................................................... 137
List of Figures

Chapter 1 - Introduction

Figure 1 - Constitutive versus regulated secretion.........................................................3
Figure 2 - Key steps involved in secretory granule biogenesis.........................................7

Chapter 2 – AP-1 and Clathrin are Essential for Secretory Granule Formation in
Drosophila

Figure 3 - Glue granule biogenesis is developmentally regulated.................................38
Figure 4 - Clathrin heavy chain and AP-1 localize to the trans-Golgi network...............41
Figure 5 - AP-1 is required to recruit clathrin to the trans-Golgi network.....................44
Figure 6 - Sgs3-DsRed co-localizes with AP-1 and clathrin at the trans-Golgi network.....47
Figure 7 - Glue protein and AP-3 localize to distinct cellular compartments...............49
Figure 8 - AP-1 is essential for granule biogenesis.......................................................52
Figure 9 - Glue transcripts are expressed in AP-1γ-depleted salivary glands...............55
Figure 10 - Glue protein accumulates at the trans-Golgi network and in aberrant vacuolated
organelles in AP-1γ-depleted cells.................................................................58

Chapter 3 – Type II phosphatidylinositol 4-kinase is required for glue granule biogenesis in
Drosophila

Figure 11 - Deletions in PI4KII are protein null............................................................76
Figure 12 - PI4KII localizes to the TGN and endosomes……………………………………79
Figure 13 - PI4KII localization in live cells………………………………………………………81
Figure 14 - PI4KII localizes to highly dynamic tubular endosomes………………………84
Figure 15 - PI4KII is required for proper glue granule biogenesis………………………..88
Figure 16 - Mutations in PI4KII do not disrupt AP-1 and EpsinR localization………………91
Figure 17 - The SNARE SNAP-24 is mislocalized in PI4KII mutants………………………94
Figure 18 - Glue protein accumulates in late endosomes of PI4KII mutants……………….97
Figure 19 - PI4KII and Fwd double mutant phenotype……………………………………..100

Chapter 4 – Discussion and Future Directions

Figure 20 - Possible trafficking steps in granule biogenesis…………………………………125

Appendix 1 – PI4KII is required for pigment granule biogenesis

Figure 21 - PI4KII regulates eye pigmentation……………………………………………131

Appendix 2 – PI4KII is required for male fertility in Drosophila

Figure 22 - PI4KII is essential for spermatogenesis…………………………………………136
List of Abbreviations

General abbreviations

ACTH  
adrenocorticotropic hormone, secreted by the anterior pituitary gland
CCV  
clathrin coated vesicle
DTT  
dithiothreitol
GPI  
glycophosphatidylinositol
EM  
electron microscopy
ER  
endoplasmic reticulum
ISG  
immature secretory granule
L3  
third-instar larval stage
LE  
late endosome
LRO  
lysosome-related organelle
MSG  
mature secretory granule
PAO  
phenylarsine oxide
PI  
phosphatidylinositol
PIP  
phosphatidylinositol phosphate
PI4P  
phosphatidylinositol 4-phosphate
PI(4,5)P2  
phosphatidylinositol 4,5-bisphosphate
PBS  
phosphate buffered saline
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PM</td>
<td>plasma membrane</td>
</tr>
<tr>
<td>PNA</td>
<td>peanut agglutinin</td>
</tr>
<tr>
<td>POMC</td>
<td>proopiomelanocortin</td>
</tr>
<tr>
<td>RSP</td>
<td>regulated secretory protein</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse-transcription polymerase chain reaction</td>
</tr>
<tr>
<td>rER</td>
<td>rough endoplasmic reticulum</td>
</tr>
<tr>
<td>SG</td>
<td>secretory granule</td>
</tr>
<tr>
<td>t-SNARE</td>
<td>target SNARE</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TGN</td>
<td>trans-Golgi network</td>
</tr>
<tr>
<td>v-SNARE</td>
<td>vesicle SNARE</td>
</tr>
<tr>
<td>VWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>WPB</td>
<td>Weibel-Palade body</td>
</tr>
</tbody>
</table>
List of Protein and Genetic Abbreviations

AP-1  clathrin adaptor protein complex 1
AP-3  clathrin adaptor protein complex 3
AP-47 gene encoding Drosophila AP-1μ
APs  clathrin adaptor protein complexes
BLOC biogenesis of lysosome-related organelles complex
CERT ceramide transfer protein
Chc  clathrin heavy chain
CgA  chromogranin A
CgB  chromogranin B

cm carmine; gene encoding Drosophila AP-3μ
CPE  carboxypeptidase E
DsRed Discosoma red fluorescent protein
dor deep orange; gene encoding Drosophila HOPS complex subunit Vps18
FAPP four-phosphate adaptor protein
fwd four wheel drive; gene encoding Drosophila type III phosphatidylinositol 4-kinase β

g garnet; gene encoding Drosophila AP-3δ
GFP  green fluorescent protein
GGA  Golgi-localized, gamma adaptin ear-containing, ARF-binding protein
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOPS</td>
<td>homotypic fusion and vacuole protein sorting complex</td>
</tr>
<tr>
<td>LAMP</td>
<td>lysosome-associated membrane protein</td>
</tr>
<tr>
<td>LERP</td>
<td>lysosomal enzyme receptor protein</td>
</tr>
<tr>
<td>Lva</td>
<td>Lava lamp; <em>Drosophila</em> golgin</td>
</tr>
<tr>
<td>lqfr/epsinR</td>
<td><em>liquid facets related</em>; gene encoding <em>Drosophila</em> homolog of epsinR</td>
</tr>
<tr>
<td>M6PR</td>
<td>mannose-6-phosphate receptor</td>
</tr>
<tr>
<td>mCherry</td>
<td>monomeric cherry fluorescent protein</td>
</tr>
<tr>
<td>meGFP</td>
<td>monomeric enhanced green fluorescent protein</td>
</tr>
<tr>
<td>mRFP</td>
<td>monomeric red fluorescent protein</td>
</tr>
<tr>
<td>NSF</td>
<td>N-ethylmaleimide-sensitive fusion protein</td>
</tr>
<tr>
<td>OSBP</td>
<td>oxysterol binding protein</td>
</tr>
<tr>
<td>PAM</td>
<td>peptidylglycine alpha-amidating monooxygenase enzyme</td>
</tr>
<tr>
<td>PC1/3</td>
<td>prohormone convertase 1 (also known as PC3)</td>
</tr>
<tr>
<td>PC2</td>
<td>prohormone convertase 2</td>
</tr>
<tr>
<td>PI4KII</td>
<td>gene encoding <em>Drosophila</em> type II phosphatidylinositol 4-kinase</td>
</tr>
<tr>
<td>PI4KII&lt;sup&gt;KD&lt;/sup&gt;</td>
<td>mutated PI4KII containing a D465A substitution that renders it kinase dead</td>
</tr>
<tr>
<td>PI4KIIα</td>
<td>type II phosphatidylinositol 4-kinase α</td>
</tr>
<tr>
<td>PI4KIIβ</td>
<td>type II phosphatidylinositol 4-kinase β</td>
</tr>
<tr>
<td>PI4KIIIα</td>
<td>type III phosphatidylinositol 4-kinase α</td>
</tr>
<tr>
<td>PI4KIIIβ</td>
<td>type III phosphatidylinositol 4-kinase β</td>
</tr>
</tbody>
</table>
sGFP  secreted green fluorescent protein
SgII  secretogranin II
SgIII  secretogranin III
SgIII  secretogranin IV
sgs  genes encoding *Drosophila* salivary gland secretion proteins
SNARE  Soluble NSF attachment protein receptor
VFP  Venus fluorescent protein
Vps29  vacuolar protein sorting 29, retromer subunit
List of cell lines

A35C    rat PC12 variant cell line, lacks regulated secretory organelles
AR42J   rat pancreatic acinar cell line with exocrine properties
AtT-20  mouse pituitary epithelial-like cell line
β-cell  pancreatic β-cell that produces the hormone insulin
chromaffin    neuroendocrine cells of the adrenal medulla
COS-1   African green monkey kidney fibroblast cell line
COS-7   African green monkey kidney fibroblast cell line
GH4     rat pituitary cell line
GH4C1   rat pituitary cell line
HEK 293 human embryonic kidney cell line
HUVEC   human umbilical vein endothelial cells
HeLa    human epithelial cell line from Henrietta Lacks
INS-1   rat insulinoma cell line
Neuro-2a mouse neural crest-derived cell line
NRK     normal rat kidney cells
PC12    rat adrenal medulla cells
RBL-2H3 rat mucosal mast cell line
S2      *Drosophila* hemocyte-like Schneider cells
Chapter 1

1 Introduction

1.1 The regulated secretory pathway

1.1.1 Regulated secretion

Constitutive secretion of proteins and lipids from the trans-Golgi network (TGN) towards the cell surface is believed to operate in all cells. Constitutive secretion is characterized by the rapid deployment of newly synthesized cargo towards its final cellular destination. In specialized secretory cells such as endocrine, neuroendocrine and exocrine cells, an additional pathway exists termed the regulated secretory pathway. The hallmark of this pathway is the storage of regulated secretory proteins (RSPs) at high concentration in dense-core secretory granules (Fig. 1). In contrast to vesicles involved in constitutive secretion, secretory granules fuse with the cell surface only in response to an appropriate external signal. This allows a cell to release large quantities of cargo, such as hormones, digestive enzymes or mucous, into the extracellular space. Dysregulation of the regulated secretory pathway results in abnormal secretion of these crucial biologically active molecules, and can profoundly affect physiology, causing disease. For example, changes in release of the hormone insulin can lead to diabetes; mucus hypersecretion is associated with asthma and cystic fibrosis; and defective secretion of von Willebrand factor can affect blood coagulation. A mechanistic understanding of secretory granule biogenesis could aid in therapeutic design for many of these frequently occurring and often devastating diseases.
**Figure 1 – Constitutive versus regulated secretion.**

**Schematic diagram of the secretory pathway.** Secreted cargo is translocated across the endoplasmic reticulum and then transported to the Golgi complex after proper folding. Secreted cargo can then undergo post-translation modification in the Golgi, followed by sorting into transport vesicles at the *trans*-Golgi network. All cells are believed to exhibit constitutive secretion (1), whereas specialized secretory cells also exhibit regulated secretion (2). Note that although the constitutive and regulated secretory pathways are shown to diverge at the Golgi, this might occur in post-Golgi immature secretory granules in some cells. (1) During constitutive secretion, newly synthesized proteins (red filled circles) are rapidly transported from the Golgi to the plasma membrane. Due to their short half-life, transport vesicles are difficult to find by electron microscopy. When detected, they lack the electron opaque ‘dense core’ found in conventional secretory granules. In addition, constitutively-secreted vesicles do not require an external stimulus to fuse with their target membrane. (2) Professional secretory cells can store regulated secretory proteins (green open circles) in secretory granules for long periods of time. Secretory granules are readily observed by electron microscopy and occupy most of the cytoplasm. Additionally, secretory granules undergo a process of condensation and maturation whereby their contents become greatly concentrated, giving rise to an electron dense core. Furthermore, secretory granules require an external signal and generation of a second messenger such as calcium to fuse with the plasma membrane (lightning bolt). Consequently, secretory cells are able to release large quantities of RSPs in a brief period of time. (adapted from Regis B. Kelly, *Science*, (1985) 230:25-32, Figure 1). Plasma membrane (PM).
1.1.2 Sorting mechanisms at the trans-Golgi network

The trans-Golgi network (TGN) is a major site of protein sorting in the secretory pathway (De Matteis and Luini, 2008). Constitutively trafficked proteins destined towards the plasma membrane or the endosomal system must be correctly sorted and packaged into transport vesicles. Diverse sorting mechanisms help direct cargo towards their unique destinations in the cell. Transmembrane proteins targeted towards endosomes utilize specific sorting sequences found on their cytoplasmic tails that are recognized by clathrin adaptor proteins, such as the heterotetrameric adaptor protein complex-1 (AP-1) and Golgi-localized, gamma adaptin ear-containing, ARF-binding protein (GGA) (Bonifacino and Traub, 2003). For example, the mannose 6-phosphate receptor (M6PR) contains a dileucine motif (DXXLL) that is recognized by GGAs, as well as a tyrosine motif (YxxΦ) that binds to AP-1 (Puertollano et al., 2001; Takatsu et al., 2001; Ghosh and Kornfeld, 2004). In addition to defining cargo content, clathrin and associated adaptor coat proteins also provide a mechanical scaffold during vesicle formation (Edeling et al., 2006). Post-translational modifications can also be used to partition cargo at the TGN. For example, ubiquitin can be recognized by GGA coat proteins to direct cargo towards endosomes (Scott et al., 2004). In polarized epithelial cells, O- and N-glycosylation can act as a luminal signal for apical targeting (Scheiffele et al., 1995; Yeaman et al., 1997). Lipid tags such as glycosylphosphatidylinositol (GPI) anchors can also confer localization to the apical membrane (Lisanti et al., 1989).

By analogy to sorting mechanisms used for targeting to other compartments in the cell, it has long been assumed that RSP cargo must contain information for entry into the regulated secretory pathway. Several studies have focused on identifying RSP sorting signals, as well as membrane associated RSP receptors (see below) (Dikeakos and Reudelhuber, 2007). Additionally, researchers have tried to determine whether RSP cargo is segregated from constitutively secreted proteins at the TGN or in a post-Golgi compartment (Arvan and Castle, 1998). Although no consensus sorting mechanism has emerged, several general properties of granule formation have been elucidated from studies on endocrine pancreatic β-cells and neuroendocrine adrenal PC12 cells. Two major, non-exclusive models for granule biogenesis have emerged. These are termed ‘sorting for entry’ and ‘sorting by retention’ (Arvan and Castle, 1998; Tooze, 1998). For the purpose of this thesis, sorting for entry will cover sorting
mechanisms believed to operate at the TGN, such as active segregation of RSP cargo by aggregation, binding of RSP cargo by membrane associated receptors and the role of cytosolic sorting machinery. Sorting by retention will cover mechanisms operating in post-Golgi immature secretory granules, including proteolytic processing of core proteins, and refinement of granule contents by removal of non-RSP cargo via budding of coated vesicles (Figure 2).
Figure 2 – Key steps involved in secretory granule biogenesis.

Summary of key mechanisms involved in secretory granule biogenesis. Note that not all features necessarily operate in a given cell type. (1) Selective aggregation of RSP cargo (green open circles) occurs in response to the low pH and high Ca$^{2+}$ concentration in the TGN lumen. Association between luminal cargo and granule membrane proteins (red line) allows the forming aggregate to be anchored to a luminal membrane. Although not shown, membrane proteins may preferentially localize to cholesterol-rich lipid rafts. (2) Membrane-tethered RSP aggregates are sorted into nascent secretory granules. Note that some non-secreted luminal proteins such as lysosomal hydrolases (purple square), as well as transmembrane receptors (not shown), can also enter the forming secretory granule. In endothelial cells, coat proteins such clathrin and the clathrin adaptor complex AP-1 (blue lines) facilitate WPB formation. (3) SNAREs (pink loops) mediate homotypic fusion of immature secretory granules. (4) RSPs undergo proteolytic cleavage in immature secretory granules. RSP cargo becomes highly multimeric and organized, increasing the efficiency of retention in secretory granules. Non-RSP cargo is selectively removed from immature secretory granules via formation of CCVs (blue lines). Non-RSP cargo might be transported to late endosomes and then undergo retrograde transport to the Golgi for future rounds of granule formation. (5) Granules fuse with the plasma membrane in response to an external signal. (6) Granule membrane proteins can then be recycled via endocytosis and delivered to endosomes before undergoing retrograde transport to the Golgi (adapted from Tooze, Gerard and Huttner, *Trends Cell Biol*, (2001) 11:116-22, Figure 1).
Figure 2

1. Secreted protein
2. Lysosomal hydrolase
3. Transmembrane protein
4. SNARE
5. AP-1/clathrin

endosome
trans-Golgi network
1.2 Sorting for entry

1.2.1 Heterologous expression of secretory proteins

To test whether RSP proteins carry sufficient information for sorting into secretory granules, early studies used heterologous expression to ask if a given RSP could be sorted into the regulated secretory pathway in a non-endogenous cell. For example, the pancreatic β-cell prohormone proinsulin expressed in the pituitary cell line AtT-20 can be packaged into secretory granules along with endogenously produced ACTH hormone (Moore et al., 1983). Strikingly, proinsulin is proteolytically cleaved to an apparently mature form and can be released upon stimulation. Similarly, the parathyroid hormone preproparathyroid, when expressed in pituitary GH4C1 cells, can be processed and released in response to secretagogue stimulation, suggesting that prohormones share a common mechanism of sorting in endocrine cells (Hellerman et al., 1984). Furthermore, heterologous expression of exocrine RSPs in endocrine and neuroendocrine cell lines indicates that at least some exocrine RSPs can be correctly sorted. For example, the pancreatic exocrine protein trypsinogen expressed in pituitary AtT-20 cells is packaged with endogenous ACTH hormone in secretory granules (Burgess et al., 1985).

Whether an active mechanism exists to exclude constitutively secreted proteins from entering regulated secretory granules is unclear. Examples of exclusion include the viral stomatitis virus G protein, which is not stored in secretory granules when expressed in pituitary AtT-20 cells (Moore and Kelly, 1985). Similarly, the Golgi resident protein Cab45 (missing the retention signal to allow for secretion) behaves as a constitutively secreted protein and is not sorted into secretory granules when expressed in pancreatic INS-1 cells (Lara-Lemus et al., 2006). However, a truncated form of alkaline phosphatase lacking a GPI anchor can efficiently enter secretory granules in INS-1 cells. Discrepancies in the behavior of different constitutively secreted proteins are likely due to two key issues. Firstly, the intrinsic biochemical properties of a protein, such as a propensity to aggregate or associate with membranes, can influence entry into forming secretory granules. Consequently, constitutively secreted proteins expressed at high levels might fortuitously enter the regulated secretory pathway due to aggregation. Secondly, some constitutively trafficked proteins, such as lysosomal hydrolases, are normally sorted into
immature secretory granules and then removed as the granules mature (Klumperman et al., 1998). Consequently, to formally demonstrate that constitutively secreted proteins are excluded from entering the regulated secretory pathway, the ability to detect transient residence in ISGs is crucial. Despite evidence that some constitutively secreted proteins can enter granules, the numerous examples of constitutively secreted proteins that do not enter secretory granules suggest active mechanisms exist to prevent entry of cargo into forming granules.

Heterologous expression experiments have explored the ability of RSPs to induce secretory granule formation in cells lacking regulated secretion. Expression of prohormones in COS-1 cells results in formation of granule-like structures (Beuret et al., 2004). However, the prohormones remain unprocessed and are not efficiently retained in the cell. More striking evidence has come from expression in COS-1 cells of the acidic gramin secretogranin II, which is stored in granules with higher efficiency than prohormones and shows some degree of stimulation-induced release (Beuret et al., 2004). Additionally, expression of the hemostatic protein VWF in human embryonic kidney (HEK 293) cells is sufficient to induce formation of secretory granules capable of stimulus-induced secretion (Michaux et al., 2003). Taken together, these results suggest that granule formation is driven, in part, by cargo content.

1.2.2 Selective aggregation

Formation of a secretory granule requires that certain types of cargo be packaged together. For instance, prohormones need to be packaged with prohormone convertases in the forming granule. Selective aggregation of RSPs has been proposed as a potential sorting mechanism. A key principle of this mechanism is that certain RSPs have a propensity to aggregate in response to the mildly acidic pH (pH 5-6) and high Ca$^{2+}$ concentrations found in the TGN lumen. This property has been documented for a wide range of RSPs, including prohormones, prohormone convertases and granins. Many of these RSPs can self-aggregate, as well as co-aggregate, allowing for multiple stable associations. In addition, some RSPs have membrane-associated domains, which could allow the luminal aggregate to be tethered to membranes.
Acidic granins localize to the peptide- and amine-containing secretory granules of endocrine, neuroendocrine and neuronal cells. Granins, which include chromogranins A (CgA) and B (CgB) and secretogranins II-IV (SgII-IV), have received considerable attention due to their ability to promote aggregation (Helle, 2004). Granins exhibit several characteristics that fulfill the role of a granule promoting or ‘granulogenic’ protein. For instance, CgA, CgB and SgII aggregate in response to low pH and high calcium concentration, conditions that mimic the TGN (Gerdes et al., 1989; Gorr et al., 1989; Chanat and Huttner, 1991). Formation of aggregates also facilitates condensation of other RSPs, including prohormones, but not constitutively secreted proteins (Colomer et al., 1996). Additionally, depletion of CgA, CgB or SgII in neuroendocrine PC12 cells results in a decrease in dense-core secretory granules and a reduction in levels of other RSPs (Kim et al., 2001; Huh et al., 2003; Courel et al., 2010). Finally, overexpression of CgA, CgB or SgII in non-endocrine fibroblasts is sufficient to induce formation of secretory granule-like structures that contain a dense core (Kim et al., 2001; Huh et al., 2003; Beuret et al., 2004). Notably, no single granin appears to function as a master regulator of secretory granule biogenesis, as genetic ablation of CgA, CgB or SgIII in mice does not abolish SG formation or cause lethality (Kingsley et al., 1990; Mahapatra et al., 2005; Hendy et al., 2006; Diaz-Vera et al., 2010).

The ability of exocrine RSPs to aggregate under conditions mimicking the TGN has also been investigated. In pancreatic zymogen granules, which contain digestive enzymes, there is extensive evidence that a wide range of serine-endoproteinases and metallo-exoproteinases can aggregate under low pH and high calcium conditions (Freedman and Scheele, 1993). In addition, zymogen aggregates are able to bind more efficiently to membrane phospholipids at lower pH (Laine and Lebel, 1999). However, the parotid granule matrix proteins amylase and parotid secretory protein do not appear to aggregate in vitro, suggesting that other mechanisms for segregation of RSPs might exist (Venkatesh et al., 2004).

1.2.3 RSP sorting domains

Several studies have focused on identifying sorting domains in RSPs that allow entry into the regulated secretory pathway. However, experiments designed to test for sorting domains in truncated proteins suffer from the complication that even truncated RSPs are prone to form
aggregates. Thus, overexpressed protein fragments might be fortuitously sorted into secretory granules due to low affinity interactions with endogenous aggregating proteins. With that caveat in mind, one signal that has received considerable attention is an N-terminal disulfide loop present in the granins CgA, CgB, as well as numerous prohormones including proopiomelanocortin (POMC). Early studies noted that disruption of the disulfide bond of CgB using reducing agents causes the protein to be missorted in adrenal PC12 cells (Chanat et al., 1993). However, this effect appears to be cell-type specific, as DTT treatment in pituitary GH4C1 cells does not affect routing of CgB to secretory granules (Gorr et al., 1999). These results suggest that the disulfide loop is crucial for sorting in only some cellular contexts, indicating that possible RSP-RSP interactions might also contribute to entry into the regulated secretory pathway.

A more direct test for the importance of the CgB disulfide loop has come from studies in which the disulfide loop was deleted, or added to a constitutively secreted protein. Deletion of the CgB disulfide loop in PC12 cells impairs sorting only slightly (Kromer et al., 1998). However, when endogenous granin production was shut off by infection with vaccinia virus, the truncated form of CgB was missorted to the constitutive secretory pathway. This result highlights the contribution of endogenous RSPs to sorting of CgB, and suggests that multiple redundant sorting mechanisms likely operate. Strikingly, addition of the CgB disulfide loop to a constitutively secreted protein, α1-antitrypsin, was sufficient to direct it into secretory granules (Glombik et al., 1999). Thus, the CgB disulfide loop contributes directly to sorting into the regulated secretory pathway.

The importance of the CgA disulfide loop is less clear. The N-terminal domain, which contains the disulfide loop, is necessary and sufficient for sorting of CgA into the regulated secretory pathway in adrenal PC12 cells (Taupenot et al., 2002). However, point mutations specifically disrupting the disulfide bridge of CgA did not affect sorting, nor was the disulfide loop sufficient to direct a chimeric GFP protein into the regulated secretory pathway, indicating that another sorting determinant is present in the N-terminal domain. Intriguingly, the N-terminal domain of CgA was also shown to induce granule-like structures in COS-1 cells (Stettler et al., 2009). The CgA N-terminal domain could also rescue regulated secretion in A35C cells, which are a PC12 cell variant lacking regulated secretory organelles (Courel et al., 2006). In contrast, another group found that the N-terminal domain of CgA was entirely dispensable for sorting into
secretory granules in GH4C1 cells, and that instead the C-terminal domain was required (Cowley et al., 2000). Taken together, these results suggest that multiple sorting signals reside on granins and that the relative importance of a given signal depends on the cellular context, including the presence of other RSPs participating in aggregate formation.

1.2.4 Membrane lipids

The precise composition of the secretory granule membrane, including phospholipid and cholesterol content, is crucial for proper secretory granule formation. Early studies noted that cholesterol is greatly enriched in the TGN and in secretory granule membranes of both exocrine acinar cells and endocrine pancreatic β-cells, as revealed by filipin labeling (Orci et al., 1981). Furthermore, cholesterol depletion using lovastatin in the pituitary cell line AtT-20 blocks secretory granule biogenesis and causes the prohormone POMC to accumulate at the Golgi (Wang et al., 2000). Addition of cholesterol to lovastatin-treated cells is sufficient to restore granule biogenesis, indicating that cholesterol is specifically required. Similarly, mice lacking enzymes in the cholesterol biosynthesis pathway exhibit defects in granule formation in both exocrine and endocrine cells (Gondre-Lewis et al., 2006). More recent studies indicate that cholesterol might participate in granule formation by localizing peripheral and integral membrane proteins into cholesterol-rich membrane domains referred to as lipid rafts. For instance, SgIII can bind directly to cholesterol through its N-terminal domain, and its association with secretory granule membrane is severely affected when cholesterol is depleted (Hosaka et al., 2004).

Phosphatidylinositol (PI) is a crucial lipid involved in multiple aspects of cell trafficking (De Matteis and Godi, 2004). A unique feature of PI is that it can undergo rapid phosphorylation and dephosphorylation at positions 3, 4 and 5 of the inositol head group to generate seven differentially phosphorylated PIs. The unique subcellular distribution of different PI species is largely determined by the organelle-specific localization of different PI-kinases and PI-phosphatases (De Matteis and Godi, 2004). The lipid phosphatidylinositol 4-phosphate (PI4P), which is generated by the action of phosphatidylinositol 4-kinases (PI4Ks), is enriched at the TGN and plays a crucial role in post-Golgi secretion (Balla and Balla, 2006; D'Angelo et al., 2008). PI4Ks have been characterized biochemically and fall into two classes. Type II PI4Ks are
inhibited by low concentrations of adenosine, whereas type III PI4Ks are inhibited by high concentrations of adenosine and the drug wortmannin. In yeast, mutations in PI4KIIIβ/Pik1p disrupt anterograde secretion and PI4P itself has been shown to be required for secretion (Hama et al., 1999; Walch-Solimena and Novick, 1999; Audhya et al., 2000). In mammalian cells, both PI4KIIIβ and PI4KIIα localize to the Golgi and contribute to distinct pools of PI4P on this organelle (Wong et al., 1997; Wang et al., 2003; Weixel et al., 2005). Consistent with this, manipulating PI4KIIIβ, either by overexpression or using dominant-negative constructs, or depleting PI4KIIα affects post-Golgi trafficking (Godi et al., 1999; Wang et al., 2003; Hausser et al., 2005). PI4P has been shown to recruit a broad range of effector proteins to Golgi membranes. For example, PI4P can recruit the coat proteins AP-1, GGA and EpsinR, which function as clathrin adaptors (Hirst et al., 2003; Wang et al., 2003; Wang et al., 2007). Additionally, FAPP, OSBP and CERT all contain a PI4P-binding pleckstrin homology domain and function in non-vesicular transport of glucosylceramide, cholesterol and ceramide, respectively (Hanada et al., 2003; Godi et al., 2004; Im et al., 2005; D'Angelo et al., 2007). More recently, GOLPH3 was identified as a PI4P specific effector that is required for Golgi morphology, as well as for retention of Golgi-resident enzymes (Dippold et al., 2009; Wood et al., 2009). Given the crucial role PI4P plays in regulating coat proteins, lipid content and Golgi function, which are all processes that impinge on granule formation, it is likely that phosphoinositides also participate in secretory granule biogenesis.

Intriguingly, PI4K activity has been detected on a broad range of secretory granule membranes, including those of pancreatic zymogen granules, mast cell granules, adrenal chromaffin granules and adrenal PC12 ISGs (Kurosawa and Parker, 1986; Husebye et al., 1990; Conway et al., 1993; Barylko et al., 2001; Panaretou and Tooze, 2002; Ishihara et al., 2006). In addition, PI4K activity has also been detected on a subset of glucose transporter 4 containing vesicles from fat and skeletal muscle cells (Del Vecchio and Pilch, 1991; Kristiansen et al., 1998; Xu et al., 2006). In most cases, the biochemical activity associated with granule membranes is consistent with a type II phosphatidylinositol 4-kinase (PI4KII), which is palmitoylated and behaves as an integral membrane protein (Barylko et al., 2001; Barylko et al., 2009). In support of this, fluorescently tagged PI4KII expressed in RBL-2H3 mast cells localizes to granule membranes (Ishihara et al., 2006).
Although the role of PI4KII on granule membranes remains relatively unexplored, inhibition of type III PI4K activity using phenylarsine oxide (PAO) decreases stimulus-dependent secretion in both pancreatic β-cells and adrenal chromaffin cells, indicating a role for phosphoinositides in granule exocytosis (Wiedemann et al., 1996; Olsen et al., 2003). Interestingly, PI4P 5-kinase and PI transfer protein are required for ATP-dependent calcium activated secretion in PC12 cells, implicating the lipid PI(4,5)P₂ in granule priming and exocytosis (Hay and Martin, 1993; Hay et al., 1995). Recent studies have identified a range of PI(4,5)P₂ binding proteins involved in granule fusion events (Osborne et al., 2007). Based on these observations, it is tempting to speculate that granule membrane PI4P acts as a precursor for PI(4,5)P₂ synthesis during exocytosis. However, studies using various PI(4,5)P₂ probes failed to detect this lipid on chromaffin granule membranes, and instead revealed that PI(4,5)P₂ is enriched on the plasma membrane (Holz et al., 2000). An alternative possibility is that PI4P is required to recruit coat proteins and lipid transport proteins to secretory granule membranes, similar to its function at the TGN. For example, coat proteins could be required to remove factors from granule membranes that inhibit exocytosis.

1.2.5 Linking luminal proteins with the TGN membrane

In addition to aggregation, which allows luminal RSPs to be sorted together, the aggregate must be tethered to the membrane to ensure efficient packaging into forming secretory granules. Several granule membrane proteins contain transmembrane domains, including phogrin, peptidyl-α-amidating monooxygenase (PAM), P-selectin and muclin (Koedam et al., 1992; De Lisle and Ziemer, 2000; Bell-Parikh et al., 2001; Wosmeier et al., 2002). In addition, several RSPs can peripherally associate with the luminal membrane, including SgIII, carboxypeptidase E (CPE) and the prohormone convertases PC1/3 and PC2 (Jutras et al., 2000; Dhanvantari et al., 2002; Arnaoutova et al., 2003; Assadi et al., 2004; Hosaka et al., 2004). In principle, any integral or peripheral membrane protein capable of binding to luminal RSPs could contribute to sorting of aggregates into forming granules. Additionally, the cytoplasmic tails of granule membrane proteins could act as potential links between granule cargo and cytosolic sorting machinery.
CPE, which was initially identified as a prohormone carboxypeptidase, has been proposed to function as a sorting receptor (Cool et al., 1997). CPE associates with lipid rafts through a C-terminal α-helix, and this domain is sufficient to direct a chimeric interleukin-2 receptor into secretory granules (Dhanvantari et al., 2002; Zhang et al., 2003). In addition, CPE can bind to the N-terminal loop of the prohormone POMC at a site distinct from its substrate-binding site (Zhang et al., 1999). Consistent with its role as a sorting receptor, depletion of CPE in Neuro-2a cells prevents accumulation of ACTH, the processed form of POMC, in secretory granules (Cool et al., 1997). Additionally, POMC is constitutively secreted in an unregulated manner, shows no response to stimulation, and is only partially processed into mature ACTH in pituitary cells of Cpe^fat^ mutant mice (Cool et al., 1997; Shen and Loh, 1997). However, the role of CPE as a prohormone sorting receptor has been challenged (Thiele et al., 1997). It was proposed that loss of CPE, either directly or indirectly, leads to inefficient processing of prohormones, consequently affecting retention in the regulated secretory pathway. In support of this alternate view, pancreatic islet cells isolated from Cpe^fat^ mutant mice exhibit slower processing of proinsulin (Irminger et al., 1997). More importantly, this work indicates that CPE cannot be the crucial sorting receptor, as the prohormone form of proinsulin is still efficiently transported into regulated secretory granules in Cpe^fat^ mutant mice.

The granin SgIII has also been proposed to function as an RSP receptor (Hosaka and Watanabe, 2010). SgIII can bind to cholesterol and also to CPE and the granin CgA, suggesting it might function to link the aggregate core with the cholesterol-rich secretory granule membrane (Hosaka et al., 2002; Hosaka et al., 2004; Hosaka et al., 2005). Consistent with a putative role for SgIII as a receptor, CgA association with secretory granule membranes is SgIII dependent (Hosaka et al., 2004). Additionally, depletion of endogenous SgIII in PC12 cells impairs intracellular retention of both CgA and the hormone adrenomedullin (Han et al., 2008). Thus, SgIII might function as a multimodal receptor capable of binding to cholesterol rich domains as well as RSP cargo.

Granule membrane proteins contain numerous sorting domains that mediate transport into secretory granules, as well as retrieval from the plasma membrane after granule fusion. One such example is phogrin (IA-2β), a transmembrane protein-tyrosine phosphatase-like protein found in dense core granules of neuronal and endocrine cells. Phogrin has been shown to play a role in glucose-stimulated insulin secretion (Kubosaki et al., 2004; Doi et al., 2006), although this has
been contested (Torii et al., 2009). Phogrin contains a dileucine sorting motif in its cytoplasmic tail that can bind to the clathrin adaptor complexes AP-1 and AP-2 and is required for sorting into granules, as well as for endocytosis at the plasma membrane (Torii et al., 2005). Phogrin also contains a tyrosine-based sorting motif that can bind to AP-2 and, when mutated, causes phogrin to accumulate at the cell surface (Wasmeier et al., 2005). The tyrosine-sorting motif is sufficient to direct an interleukin-2 receptor chimeric protein into secretory granules, whereas mutation of the motif caused the chimeric protein to accumulate in a perinuclear region, as well as on the cell surface. The luminal domain of phogrin also contains targeting information, as a soluble luminal form can be sorted into secretory granules, probably through interactions with RSPs (Wasmeier et al., 2002). Intriguingly, the cytoplasmic domain of phogrin has been shown to possess phosphatidylinositol phosphatase activity and can deplete plasma membrane PI(4,5)P₂ when overexpressed in INS-1 cells (Caromile et al., 2010). Similarly, PI(4,5)P₂ levels are elevated when phogrin is depleted. By modifying lipid content, phogrin could influence granule priming and exocytosis at the cell surface. Consequently, phogrin is uniquely positioned to influence membrane dynamics via its PIP phosphatase activity, as well as acting as a potential link between cytoplasmic clathrin adaptors and luminal cargo.

Muclin is a sulfated glycoprotein that has been proposed to function as a sorting receptor in exocrine cells. Muclin has been assigned a role as a putative receptor as the unprocessed form contains a transmembrane domain and can aggregate with zymogen granule secretory proteins at mildly acidic pH (De Lisle and Ziemer, 2000; Boulatnikov and De Lisle, 2004). Moreover, muclin deficient mice exhibit decreased release of amylase in response to stimulation and are impaired in producing a newly made stimulus-releasable pool of amylase (De Lisle et al., 2008). In addition, expression of promuclin in the poorly differentiated rat pancreatic exocrine cell line AR42J, which lacks a regulated secretory pathway, is able to induce regulated secretion (De Lisle et al., 2005). This indicates promuclin acts as a sorting receptor and can promote formation of regulated secretory granules.

1.2.6 A role for cytosolic coat proteins in granule formation

Coat proteins selectively sort cargo into forming vesicles, and also provide a scaffold for vesicle formation. Clathrin and its associated heterotetrameric adaptor proteins (APs) form
vesicular coats (Edeling et al., 2006). APs can bind to sorting signals found in the cytoplasmic tails of transmembrane cargo and also to the clathrin lattice (Bonifacino and Traub, 2003). There are four different AP complexes (AP-1 to 4) that have distinct roles in the cell (Boehm and Bonifacino, 2001). Of these, AP-1 has been implicated in transport of cargo between the TGN and endosomes (Doray et al., 2002; Hinners and Tooze, 2003; Waguri et al., 2003).

Additionally, AP-1 has been implicated in two aspects of granule biogenesis. The first is in formation of rod-shaped secretory granules known as Weibel-Palade bodies (WPBs) (Lui-Roberts et al., 2005). The second is in maturation of ISGs by selective removal of non-RSP cargo (Dittie et al., 1996; Dittie et al., 1997; Klumperman et al., 1998; Dittie et al., 1999).

WPBs, which are produced in endothelial cells, require clathrin and AP-1 for their formation (Lui-Roberts et al., 2005). WPBs contain the hemostatic protein von Willebrand factor (VWF), as well as the integral membrane protein P-selectin. VWF is a large multimeric glycoprotein that can bind to platelet receptors and is involved in hemostasis (Metcalf et al., 2008). Similar to other RSPs, aggregation of VWF is believed to play an important role in WPB formation. For example, expression of VWF in HEK 293 cells, which lack regulated secretion, is sufficient to drive formation of WPB-like structures (Michaux et al., 2003). Furthermore, the membrane proteins P-selectin and CD63 can be incorporated into these structures when co-expressed with VWF. These pseudo-WPBs can even respond to secretagogue, indicating that they are regulated secretory granules.

Analysis of human umbilical vein endothelial cells (HUVECs) prepared by high-pressure freezing, followed by transmission electron microscopy (TEM), revealed that VWF-containing rods first form at the TGN as finger-like projections that are coated in clathrin (Zenner et al., 2007). This suggested that coat proteins might provide a stabilizing scaffold during VWF tubulation. Additional evidence came from the observation that clathrin and AP-1 colocalize with VWF at the TGN (Lui-Roberts et al., 2005). Indeed, interfering with clathrin function or depleting AP-1 prevents the formation of normal WPBs, leading to accumulation of small VWF-containing organelles. These structures are not simply small WPBs, as they lack granule membrane markers such as P-selectin and Rab27a. Additionally, depletion of AP-1 causes increased constitutive secretion of VWF and a profound loss of regulated secretion. AP-1 depletion does not generally disrupt Golgi function, as AP-1 depleted cells exhibit normal Golgi morphology and can secrete a constitutive secretory marker (a signal sequence fused to
horseradish peroxidase) at normal levels. However, AP-1 is required for the maintaining the steady state distribution of several proteins that traffic between the TGN and endosomes, such as the protein convertase furin (Folsch et al., 2001). Consequently, it is also conceivable that the WPB defect observed in AP-1 depleted cells is primarily caused by a failure to retrieve cargo from endosomes required for future cycles of WPB formation.

Further studies investigated the role of AP-1 effector proteins aftiphilin and γ-synergin on WPB formation (Lui-Roberts et al., 2008). In contrast to AP-1 depletion, RNAi directed against aftiphilin or γ-synergin did not obviously affect WPB formation. In addition, WPBs still recruited the membrane marker P-selectin and VWF was processed into highly multimeric forms. However, depletion of either aftiphilin or γ-synergin resulted in a striking decrease in stimulus-releasable VWF, as well as an increase in constitutive VWF secretion. This result indicates that WPB formation can be uncoupled from the ability of WPBs to undergo regulated secretion. Although aftiphilin is not predicted to regulate exocytosis directly, it is thought to function by binding and recruiting proteins necessary for conferring regulated exocytosis. In this manner, AP-1 and its associated effector proteins might couple recruitment of the clathrin scaffold to incorporation of cargo proteins required for regulated secretion.

1.3 Sorting by Retention

1.3.1 Sorting by retention

The mechanisms described so far have focused on the importance of aggregate formation at the TGN to ensure that RSP cargo is concentrated into nascent ISGs. Additionally, coaggregation of RSPs provides an explanation of how luminal cargo can be coordinated with membrane content. A second model, termed sorting by retention, considers ISGs as a functional extension of the Golgi, in which additional protein sorting and post-translational modification can occur (Arvan and Castle, 1998; Morvan and Tooze, 2008). This model is not mutually exclusive with sorting for entry and has the following tenets. Firstly, that non-aggregated luminal proteins are free to enter forming ISGs through the fluid-phase and that no active exclusion mechanism exists. Secondly, that processing and multimerization of RSP cargo in ISGs increases
efficiency of retention in maturing granules. Lastly, that clathrin-coated vesicles (CCVs) remove proteins lacking significant retention properties. In this sense, sorting by retention can be thought of as a process that leads to refinement of secretory granule content. Sorting by retention has been most extensively studied in endocrine β-cells and neuroendocrine PC12 cells, but has also been documented in exocrine pancreatic acinar cells and salivary gland parotid cells.

1.3.2 Characteristics of immature secretory granules

ISGs are the intermediate compartment between the TGN and mature granules. ISGs are usually proximal to the TGN, although not physically connected, and often possess the ability to undergo stimulation-induced secretion. In endocrine and neuroendocrine cells, ISGs are typically mildly acidic and contain prohormones, whereas mature granules are more acidic and contain mature, processed hormones (Orci et al., 1986). Indeed, there is considerable evidence that processing of prohormones, for example pro-insulin, occurs in ISGs (Orci et al., 1985; Huang and Arvan, 1994). ISGs are also partially coated with clathrin and budded profiles observed by TEM are believed to represent sites of protein removal. Mature secretory granules, on the other hand, exhibit highly organized luminal cargo that appears electron dense and is sometimes found in crystalline arrays.

1.3.3 Clathrin coats and the removal of non-RSP cargo

Early studies noted the existence of clathrin patches on ISGs that were absent from mature granules leading to the proposal that CCVs might function to remove missorted proteins from ISGs (Tooze and Tooze, 1986; Kuliawat et al., 1997). Using pancreatic β-cells as a model, the lysosomal enzyme procathepsin B was shown by immuno-EM to be present in ISGs, but mostly absent from mature β-granules, demonstrating this soluble cargo protein is removed during granule maturation (Kuliawat et al., 1997). Similar results were obtained by pulse-chase labeling, in which the enzyme could be stimulated for exocytosis at early time points as it moved through the ISG compartment, but was refractory to regulated secretion at later time points as it exited maturing granules. Furthermore, the cation-dependent M6PR, which binds to lysosomal hydrolases, is required for removal of procathepsin B from mature β-granules. Further studies
revealed that M6PR and clathrin adaptor AP-1 could be detected on ISGs, in particular on budded profiles, but only rarely on mature granules (Klumperman et al., 1998). Taken together, it was proposed that clathrin/AP-1-containing coats remove M6PRs, together with bound lysosomal enzymes.

A role for clathrin and AP-1 in removal of proteins from ISGs was further substantiated in PC12 cells using a subcellular fractionation protocol that allowed ISGs to be distinguished from MSGs (Tooze and Huttner, 1990; Tooze et al., 1991). ISGs were shown to contain AP-1, M6PR and furin, and these components were depleted from mature granules (Dittie et al., 1996; Dittie et al., 1997; Dittie et al., 1999). AP-1 can bind directly to furin and the cation-independent M6PR, indicating that AP-1 participates directly in removal of these proteins from ISGS. More recently, the clathrin adaptor GGA was also shown to function in removal of VAMP4 from ISGs in PC12 cells (Kakhlon et al., 2006). Taken together, these results indicate that clathrin and its associated coat proteins mediate removal of non-RSP cargo during granule maturation.

1.3.4 Retention by processing

Processing of RSP cargo can facilitate multimerization and increase the efficiency with which RSPs are stored in immature secretory granules. Prohormone convertases (PC) are endopeptidases that cleave prohormones and other RSPs that contain a dibasic amino acid cleavage site. For example, proinsulin is processed by PC1/3, PC2 and CPE in immature secretory granules (reviewed in Davidson, 2004). Interestingly, processing of insulin correlates with its ability to multimerize. For instance, proinsulin cannot multimerize beyond hexamers, whereas mature, processed insulin can form highly multimeric complexes. In addition, processing of insulin correlates with organization of insulin into crystalline arrays (Michael et al., 1987). To directly test whether prohormone conversion increases storage in secretory granules, proinsulin was ectopically expressed in GH4C1 cells, which lack endogenous PC1 (Kulawat et al., 2000). Proinsulin was able to enter immature secretory granules in GH4C1 cells, indicating processing is not required for entry into the regulated secretory pathway. However, proinsulin was only poorly retained in these cells. Strikingly, GH4C1 cells that stably express PC1 exhibit increased storage of processed insulin. This suggests that processing of RSP cargo increases retention.
1.3.5 Homotypic fusion

Several morphological studies have noted that granules increase in size as they mature, leading to the proposal that immature secretory granules might undergo homotypic fusion (Smith and Farquhar, 1966; Tooze et al., 1991; Farkas and Suakova, 1999). One of the first direct demonstrations of ISG homotypic fusion made use of a cell-free fusion assay in which PC2 was present in one population of ISGs and radiolabeled SgII substrate was present in another population (Urbe et al., 1998). Mixing of the two populations resulted in cleavage of SgII, which could be detected with an antibody that specifically recognizes the cleaved product. Interestingly, granule fusion occurred only between ISGs, but was not detected when ISGs were mixed with a population of MSGs expressing PC2, indicating specificity of fusion. Further studies investigating the molecular machinery required for ISG fusion in PC12 cells identified requirements for N-ethylmaleimide-sensitive fusion protein (NSF), the SNARE syntaxin 6 and synaptotagmin IV (Urbe et al., 1998; Wendler et al., 2001; Ahras et al., 2006).

1.4 Summary and outstanding questions

Existing data are consistent with a model whereby homophilic and heterophilic interactions among RSPs, some of which are peripherally associated with membrane patches or rafts, allow for RSPs to be sorted into immature secretory granules at the TGN. The demonstration that CgA, CgB, CPE and SgIII knockout mice are all viable strongly supports the notion that no single RSP is crucial for granule biogenesis (Kingsley et al., 1990; Cawley et al., 2004; Mahapatra et al., 2005; Hendy et al., 2006; Diaz-Vera et al., 2010). Instead, RSPs appear to be partially redundant in their ability to form aggregates and can be sorted into the regulated secretory pathway, albeit less efficiently, in the absence of other RPSs. Although current evidence argues against a universal sorting receptor, there are several examples of cell type-specific granule membrane proteins and membrane-associated RSPs that can interact with luminal cargo proteins and potentially function as receptors.

A role for coat proteins in granule formation is currently limited to WPBs (Lui-Roberts et al., 2005). However, coat proteins are likely to play a broader role in granule formation, as
condensing secretory products at the TGN are associated with clathrin coats in both pituitary AtT20 cells and pancreatic β-cells (Orci et al., 1984; Tooze and Tooze, 1986). Also, despite extensive biochemical and EM analysis of ISG and mature granules, there are few studies involving functional characterization of molecular components believed to participate in granule maturation. For example, there are no loss-of-function studies assessing contributions of clathrin and AP-1 to ISG maturation or removal of non-RSP cargo. This highlights the importance of using a genetically tractable system to address these and other questions relating to secretory granule biogenesis.

1.5  *Drosophila* Glue Granules

1.5.1  Glue granule production is developmentally regulated

Polarized epithelial cells of the *Drosophila* larval salivary gland produce regulated secretory granules termed ‘glue granules’. Glue granules contain highly glycosylated ‘glue’ proteins that adhere the pupal case to a solid substrate during metamorphosis. Glue production is developmentally regulated, with glue gene transcription beginning midway through the third larval stage (L3) in response to a low-titre pulse of the steroid hormone 20-hydroxyecdysone (henceforth referred to as ecdysone) (Lehmann, 1996; Costantino et al., 2008). From mid-L3 until late-L3, glue granules accumulate in the cytoplasm of salivary gland cells. During this time, granules increase dramatically in size (Farkas and Suakova, 1999). At the end of L3, granules are secreted into the salivary gland lumen in response to a high-titre pulse of ecdysone. Glue granules are released by compound exocytosis, which involves granule-granule fusion in addition to granule-plasma membrane fusion. The multi-granular fusion complexes reach diameters exceeding 10 μm (Biyasheva et al., 2001). Similar to regulated exocytosis in other cell types, the ecdysone stimulus leads to elevated levels of calcium. The salivary gland protein E63-1, which is a member of the EF-hand family of Ca^{2+} binding proteins, is induced by ecdysone, suggesting it might play a role in granule secretion (Vaskova et al., 2000). Consistent with this prediction, ectopic expression of E63-1, together with the addition of a calcium ionophore to raise intracellular calcium levels, is sufficient to trigger secretion (Biyasheva et al., 2001).
Glue granule biogenesis has been extensively characterized morphologically. Granule biogenesis is first initiated in the distal most cells of the salivary gland at the mid-third instar larval stage, with more proximal cells initiating granule biogenesis at later time points. Quantitative studies revealed that glue granules slowly increase in size as their overall number decreases, consistent with maturation through homotypic fusion (Farkas and Suakova, 1999). Morphologically, fine filamentous strands can be observed that connect glue granules undergoing homotypic fusion (Thomopoulos and Kastritis, 1979). At maturity, glue granules reach an average diameter of 3 µm, and contain three types of material (elements I-III) recognizable by electron microscopy. Element I has a highly electron dense granular appearance, is found near the granule periphery, and is enriched in polysaccharides, as determined by the Rambourg technique. Element II exhibits medium electron density and has a filamentous appearance. Element III exhibits low electron density and is found between elements I and II (Thomopoulos and Kastritis, 1979). Based on morphological evidence, element I appears to be produced separately from the other two elements. Element I can be seen in small vesicles near the Golgi and in the vicinity of large secretory granule bodies (Thomopoulos and Kastritis, 1979). It is unclear if only some Golgi bodies can produce element I, or whether this component is only produced for a brief period. During secretion, the distinct electron dense elements are lost and granule contents appear more homogenous.

1.5.2 SNAREs implicated in glue granule biogenesis

Relatively little is known about the molecular machinery required for glue granule homotypic fusion or exocytosis. Vesicle fusion typically requires bringing together vesicle-localized SNAREs (v-SNAREs), such as syntaptobrevin, with target-membrane snares (t-SNAREs), such as syntaxin and SNAP-25. For example, this machinery is required for fusion of synaptic vesicles with the presynaptic membrane. The Drosophila genome encodes SNAP-25, which is expressed primarily in neuronal cells, as well as a highly homologous gene termed SNAP-24 (Risinger et al., 1997; Niemeyer and Schwarz, 2000). Intriguingly, SNAP-24 localizes to the limiting membrane of glue granules throughout granule maturation. Just like SNAP-25, SNAP-24 can form a stable complex in vitro with both syntaxin 1A and either a ubiquitous or neuron-specific form of syntaptobrevin. However, whereas syntaxin 1 and SNAP-25 are found on
the same target membrane in synapses, syntaxin 1 and SNAP-24 localize to opposing membranes in salivary gland cells. Syntaxin 1 is strongly concentrated at the apical membrane of salivary gland cells, in contrast to SNAP-24 found on granule membranes. Consequently, SNAP-24 might associate with another SNARE on granule membranes in vivo. Functional analysis of *Drosophila* SNAP-24 has yet to be reported, and it is currently unknown whether SNAP-24 is required for homotypic granule fusion and/or exocytosis. Although SNAP-24 has slightly higher sequence similarity to mammalian SNAP-25, it might be more functionally related to mammalian SNAP-23. SNAP-23 can interact with multiple syntaxins and regulates compound exocytosis of mast cell granules (Ravichandran *et al*., 1996; Guo *et al*., 1998).

1.5.3  **Sgs glue genes**

The major components of glue granules were first identified as chromosomal puffs, or regions of high transcription, that are easily visualized on the giant polytene chromosomes of salivary gland cells. Different titres of the hormone ecdysone regulate appearance and regression of chromosomal puffs (Ashburner, 1973). For example, during the mid-third instar larval stage, a low-titre pulse of ecdysone induces intermolt puffs, which contain genes that encode the glue mix (Lehmann, 1996; Costantino *et al*., 2008). At the onset of purparation, a high-titre pulse of ecdysone causes intermolt puffs to regress, and initiates induction of a small number of ‘early’ puffs, followed several hours later by a larger number of ‘late’ puffs (Ashburner, 1972). This led to the proposal that early puffs contain genes encoding transcription factors that can induce late puff genes (Ashburner *et al*., 1974). In addition to transcription factors, some of these early puffs also encode genes required for granule secretion (Biyasheva *et al*., 2001). Thus, distinct pulses of ecdysone can induce intermolt puffs that encode the glue mix, as well as early/late puffs that encode proteins required for granule secretion. The distinct physiological responses appear to be due to different levels of ecdysone acting through different ecdysone receptor complexes (Costantino *et al*., 2008).

The *Sgs* (Salivary Gland Secretion) genes were initially mapped to chromosomes as intermolt puffs. In parallel, biochemical analysis of the glue mix by gel electrophoresis identified six major proteins, four of which are glycosylated (Beckendorf and Kafatos, 1976; Korge, 1977). By identifying allelic variants whose products exhibit different electrophoretic mobility, *Sgs*
genes were genetically mapped to regions corresponding to chromosomal puffs (Korge, 1975). The six cloned Sgs genes show a salivary gland specific expression pattern, and are expressed only during the latter half of third-instar larval development (Lehmann, 1996). This tissue specific expression pattern of Sgs genes has been independently confirmed using gene expression arrays (Chintapalli et al., 2007). Sgs1 contains extended repeats rich in threonine and serine, whereas Sgs3 and Sgs4 contain extended repeat containing threonine and proline (Muskavitch and Hogness, 1982; Garfinkel et al., 1983; Roth et al., 1999). The threonine and serine sites represent potential sites for O-linked glycosylation (Syed et al., 2008). Sgs5, 7 and 8 are small proteins of 163, 74 and 75 amino acids, respectively, with no identifiable domains (Garfinkel et al., 1983; Guild, 1984). Sgs5, 7 and 8 appear to be unique to Drosophila, with no identifiable homologs in other species. Although the function of these smaller glue proteins is not known, they might allow for efficient packaging of the mucin-like glue proteins into granules.

A fluorescently tagged version of Sgs3, expressed under control of its endogenous promoter, localizes to the lumen of glue granules and can be efficiently secreted (Biyasheva et al., 2001). This Sgs3 transgene has been used to investigate the ecdysone receptor complex that leads to glue gene transcription and the ecdysone-responsive genes required for secretion (Biyasheva et al., 2001; Costantino et al., 2008). However, this marker has not been used to dissect the cellular machinery required for granule biogenesis.

### 1.5.4 Modeling granule biogenesis using Drosophila salivary glands

I sought to establish a genetically tractable system to analyze regulated secretion. Previous studies using the unicellular ciliate Tetrahymena thermophila have demonstrated the power of reverse genetic analysis to study granule formation (Turkewitz, 2004). Drosophila larval salivary glands exhibit several properties that make them an attractive system to investigate granule biogenesis in a multicellular organism. The Drosophila genome has been entirely sequenced, revealing numerous evolutionarily conserved genes that function in protein sorting at the Golgi (Adams et al., 2000; Kametaka et al., 2010). In addition, several large-scale consortia have generated genome-wide RNAi transgenes, which can be used for genetic screens (Dietzl et al., 2007). These transgenes utilize the Gal4/UAS system, which allows for expression to be directed in specific tissues, including the larval salivary gland (Brand and Perrimon, 1993).
Furthermore, salivary glands are dispensable for viability under laboratory conditions, allowing for analysis of genes with essential functions in the context of an otherwise healthy organism (Costantino et al., 2008). Salivary glands can be dissected and cultured in vitro, and fluorescently tagged granule proteins allow granules to be imaged using confocal microscopy (Biyasheva et al., 2001; Costantino et al., 2008). Moreover, both granule formation and secretion can be stimulated in vitro by addition of different concentrations of exogenous ecdysone (Farkas and Sutakova, 1998; Costantino et al., 2008). Hence, this system provides a powerful model in which to study secretory granule formation, maturation and regulated secretion.

1.6 Thesis Overview

In this thesis, I present data demonstrating that the Drosophila larval salivary gland can be used effectively to explore granule biogenesis in a highly genetically tractable model organism. Chapter 2 describes a role for clathrin and the clathrin adaptor AP-1 in formation of mucin-containing glue granules in salivary gland cells. I show that AP-1 and clathrin colocalize at the TGN and that AP-1 is required to recruit clathrin. I also show that AP-1 and clathrin colocalize with secretory cargo at the TGN and on granules. Finally, I demonstrate that a null mutation in AP-1µ leads to a profound block in granule biogenesis. Chapter 3 describes a role for type II phosphatidylinositol 4-kinase in the biogenesis of glue granules in salivary gland cells. I show that PI4KII colocalizes with secretory cargo at the TGN, but not at mature granules. In addition, fluorescently tagged PI4KII localizes to a highly dynamic endosomal network. I demonstrate that a null mutation in PI4KII results in strikingly small glue granules and accumulation of glue protein in endosomes. Additionally, I present evidence that PI4KII regulates pigment granule biogenesis (Appendix 1), as well as male fertility (Appendix 2). In Chapter 4, I relate my observations to what was previously known about secretory granule biogenesis.
Chapter 2

2 AP-1 and clathrin are essential for secretory granule formation in Drosophila

Jason Burgess, Miluska Jauregui, Julie Tan, Janet Rollins, Henry Chang, Sylvie Lallet, Roland
Le Borgne, Helmut Krämer and Julie Brill

Statement of Contributions:

Henry Chang generated fluorescently tagged Che constructs and transgenic flies. Roland Le
Borgne and Sylvie Lallet recombined $AP-4^{she11}$ onto an FRT chromosome and generated anti-
AP-1γ antibodies. Julie Tan analyzed Sgs transcripts in AP-1γ RNAi experiments. Miluska
Jauregui aided in preparing salivary gland samples for immunofluorescence. Janet Rollins aided
in preparing salivary gland samples for electron microscopy. Helmut Krämer contributed to
experimental design. I performed all other experiments and analysis. Additional reagents or
antibodies that were provided have been indicated in the materials and methods section. All of
my research was performed in the laboratory of Julie Brill.
2.1 Summary

Regulated secretion of hormones, digestive enzymes and other biologically active molecules requires formation of secretory granules. Clathrin and the clathrin adaptor protein complex 1 (AP-1) are necessary for maturation of exocrine, endocrine and neuroendocrine secretory granules. However, the initial steps of secretory granule biogenesis are only minimally understood. We used powerful genetic approaches available in the fruit fly Drosophila melanogaster to investigate the molecular pathway for biogenesis of mucin-containing ‘glue granules’ that form within epithelial cells of the third-instar larval salivary gland. We show that clathrin and AP-1 colocalize at the TGN and that clathrin recruitment requires AP-1. We further show that clathrin and AP-1 colocalize with secretory cargo at the TGN and on granules. Finally, we show that loss of clathrin or AP-1 leads to a profound block in secretory granule biogenesis. These findings establish a novel role for AP-1/clathrin-dependent trafficking in the formation of mucin-containing secretory granules.

2.2 Introduction

How secreted proteins enter the regulated secretory pathway is a source of debate and may prove to be cargo and cell type specific (Dikeakos and Reudelhuber, 2007). In the case of endocrine and neuroendocrine cells, sorting of secreted cargo is believed to be content driven, with selective aggregation of regulated secretory proteins at the trans-Golgi network (TGN) playing a major role in secretory granule biogenesis (Borgonovo et al., 2006). However, little is known about the coat proteins that might be required on the cytoplasmic face to promote budding of lumenal secretory cargo from the TGN. Initial studies in the AtT20 pituitary cell line noted that condensing secretory products accumulate in dilated regions of the TGN and are coated with clathrin (Tooze and Tooze, 1986). Similarly, in β-cells treated with monensin to perturb intracellular trafficking, proinsulin accumulates in a clathrin-coated compartment related to the TGN (Orci et al., 1984). These observations raise the possibility that trafficking of regulated secretory cargo might require passage through a clathrin-coated compartment at the TGN. In addition to sorting events that occur at the TGN, clathrin has also been detected on post-Golgi immature secretory granules in endocrine and neuroendocrine cells, where this coat
protein functions during granule condensation to refine granule content and remove proteins not destined to be part of the mature granule (Arvan and Castle, 1998; Morvan and Tooze, 2008).

Coat proteins selectively incorporate cargo into vesicles and provide a scaffold for vesicle formation. Clathrin and its associated heterotetrameric adaptor proteins (APs) make up a major class of vesicular coats. APs bind to sorting motifs found in the cytoplasmic tails of membrane cargo and function as links between vesicular cargo and the clathrin lattice (Bonifacino and Traub, 2003; Edeling et al., 2006). The four different AP complexes (AP-1 to 4) have distinct sites of action in the cell (Boehm and Bonifacino, 2001; Robinson, 2004). Of these, the AP-1 complex has perhaps the most diverse roles, acting at the TGN to promote constitutive secretion (Chi et al., 2008); at the TGN and endosomes to sort mannose 6-phosphate receptors (Doray et al., 2002; Hiners and Tooze, 2003; Waguri et al., 2003); at recycling endosomes of polarized cells to sort basolateral proteins (Cancino et al., 2007; Gravotta et al., 2007; Deborde et al., 2008); and at immature secretory granules of specialized secretory cells to retrieve missorted proteins (Arvan and Castle, 1998; Klumperman et al., 1998; Dittie et al., 1999; Molinete et al., 2000; Morvan and Tooze, 2008). Indeed, a coat composed of clathrin and AP-1 is required for maturation and condensation of regulated secretory granules (Dittie et al., 1997; Klumperman et al., 1998; Dittie et al., 1999). In contrast to maturation, the roles of AP-1 and clathrin in initial stages of secretory granule formation are unclear. AP-1 and clathrin were shown to be required for formation of Weibel-Palade bodies (WPBs), secretory organelles that store the hemostatic protein von Willebrand factor (Lui-Roberts et al., 2005). However, a dominant-negative clathrin construct did not interfere with insulin granule production in neuroendocrine cells, suggesting these granules form through a clathrin-independent mechanism (Molinete et al., 2001). Thus, it is not clear how general a role AP-1 and clathrin play in granule formation.

The larval salivary gland in the fruit fly Drosophila melanogaster provides an excellent system for molecular genetic analysis of membrane trafficking pathways (Tojo et al., 1987; Xu et al., 2002; Abrams and Andrew, 2005; Wendler et al., 2010). During the last half of third-instar larval development, prior to pupariation, salivary gland cells initiate production of mucin-type secretory granules termed ‘glue’ granules (Beckendorf and Kafatos, 1976; Korge, 1977; Lehmann, 1996). These granules contain highly glycosylated glue proteins that are required to adhere the pupal case to a solid substrate during metamorphosis (Fraenkel, 1952; Fraenkel and Brookes, 1953). Of the six known glue proteins (also called Salivary gland secretion or Sgs
proteins), Sgs1, 3 and 4 contain extended amino acid repeats that are likely sites of oligosaccharide linkage (Muskavitch and Hogness, 1982; Garfinkel et al., 1983; Roth et al., 1999; our unpublished observations). These proteins, which are synthesized in response to a low-titer pulse of the steroid hormone ecdysone at the mid-third instar larval stage, are stored until an additional high-titer pulse of ecdysone promotes their release at the onset of pupariation (Boyd and Ashburner, 1977; Berendes and Ashburner, 1978; Biyasheva et al., 2001).

Secreted mucin-type glycoproteins are ubiquitous in metazoans and serve important roles in animal physiology. Mucins are typically stored in specialized granules that form within dedicated secretory cells, yet the cellular mechanisms required for their formation remain unknown (Davis and Dickey, 2008). Here, we analyze the mechanism of mucin-type glue granule biogenesis in third-instar larval salivary gland cells. We show that AP-1 and clathrin localize to the TGN prior to glue production; colocalize at the TGN with newly synthesized glue proteins during early stages of granule formation; and are found at later stages on maturing glue granules. Using molecular genetic approaches, we provide evidence that AP-1 and clathrin are required for glue granule formation; in the absence of AP-1, glue granules do not form, and reducing AP-1 levels causes glue cargo to accumulate at the TGN and in small post-Golgi vesicles. Our results reveal a requirement for AP-1 and clathrin in the initial formation of mucin-type secretory granules.
2.3 Experimental Procedures

2.3.1 Fly Stocks and genetic crosses

Flies were raised on standard cornmeal molasses agar at 25°C (Ashburner, 1990). Visible markers and balancer chromosomes are as described (Lindsley and Zimm, 1992). $AP^{47^{SHE-11}}$, a deletion of amino acids 146-158 followed by a frameshift in the gene encoding the AP-1 subunit mu-adaptin, was isolated in a screen for modifiers of Presenilin-dependent Notch phenotypes (Mahoney et al., 2006). $AP^{47^{SHE-11}}$ was recombined onto $P\{\text{neoFRT}\}82B$ to generate mosaic clones and to remove the second site $Psn^{143}$ mutation (Xu and Rubin, 1993). $P\{\text{GawB}\}AB1$-GAL4 and $P\{w^{+}, Sgs3\text{-GFP}\}$, were obtained from the Bloomington Drosophila stock center. Transgenic fly stocks carrying $P\{w^{+}, UAS\text{-mRFP-Chc}\}$, $P\{w^{+}, UAS\text{-EGFP-Chc}\}$, $P\{w^{+}, UAS\text{-VFP-AP-47}\}$, $P\{w^{+}, c\text{tauB-mCherry-AP3}\}$ or $P\{w^{+}, c\text{tauB-AP3}\delta\text{-mEGFP}\}$ (see below) were generated by injection of Drosophila embryos using standard techniques. $P\{w^{+}, Sgs3\text{-DsRed}\}$ flies were from A. Andres (Costantino et al., 2008). RNAi stocks expressing double-stranded RNAs under control of GAL4 upstream activating sequences (UAS) (Vienna Drosophila RNAi Center; Dietzl et al., 2007); were as follows: #3275 (AP1γ), #24017 (AP1µ) and #23666 (Chc).

To examine salivary glands depleted for AP-1 or clathrin heavy chain, a stock carrying Sgs3-DsRed; AB1-GAL4 was crossed to flies carrying the corresponding UAS-RNAi insertion.

Mosaic clones were generated using the FLP/FRT system (Golic and Lindquist, 1989) by crossing $y^{1}, w^{118}, P\{70FLP\}3F$ ; $P\{\text{neoFRT}\}82B, P\{\text{Ub}\text{-GFP.D}\}83 / TM6B, Hu, Tb$ to flies containing Sgs3-DsRed ; $P\{\text{neoFRT}\}82B, AP-47^{SHE-11} / TM6B, Hu, Tb$. To examine clathrin in $AP-47^{SHE-11}$ clones, the heat shock FLP stock was crossed to $UAS\text{-mRFP-Chc} / +$ ; $P\{\text{neoFRT}\}82B, AP-47^{SHE-11}, AB1\text{-GAL4} / TM6B, Hu, Tb$. To generate mosaic clones in the salivary gland, adult flies were allowed to lay embryos in a vial during a 60 min collection window. Flies were then removed and embryos were aged for 2.5 hours at room temperature. Embryos were then heat-shocked for 90 min at 37°C by placing the vial in a water bath. Embryos were then incubated at 25°C to allow for further development. Salivary gland cells (approximately 100 per lobe) are specified early in embryonic development and differentiate without dividing (Campos-Ortega and Hartenstein, 1985). Consequently, mosaic clones generated in early embryos typically give rise to single cell mutant clones.
2.3.2 Molecular biology

For Chc constructs, mRFP and EGFP were inserted in frame at the N-terminus of *Drosophila* Chc. For EGFP-Chc, an EcoRI-NotI fragment containing EGFP (without the stop codon) was first cloned into pUAST. pOT2-Chc, a plasmid containing the entire Chc ORF, was obtained from the *Drosophila* Genomics Research Center. This plasmid contained a point mutation in Chc, which was repaired using the QuikChange Site-Directed Mutagenesis Kit (Strategene). QuikChange was also used to introduce a NotI site before the ATG and a KpnI site after the stop codon of Chc. After being verified by sequencing, the Chc ORF was cloned as a NotI-KpnI fragment into pUAST-EGFP, resulting in pUAST-EGFP-Chc (GFP-Chc). pUAST-mRFP-Chc (RFP-Chc) was made in a similar fashion.

To generate mCherry-AP3δ and AP3δ-mEGFP (AP3δ-GFP), we used pCaSpeR-tub::mCherry, which contains an N-terminal mCherry (gift of R. Tsien; Shaner *et al.*, 2004) followed by an XbaI site, or pCaSpeR-tub::GFP, which contains a C-terminal mEGFP (gift of E. Snapp; Zacharias *et al.*, 2002) preceded by a KpnI site. Both vectors are derived from a version of pCaSpeR4 (Pirrotta, 1988) containing the αTub84B promoter (gift of S. Eaton; Marois *et al.*, 2006). Restriction sites were introduced into a full-length *garnet* (RE06749) cDNA (Berkeley Drosophila Genome Project; obtained from the Canadian *Drosophila* Microarray Centre) by PCR and orientation after subcloning was confirmed by DNA sequencing (The Centre for Applied Genomics, SickKids, Toronto). AP3δ-GFP was fully functional, in that it rescued the eye pigmentation defect of *garnet* mutant flies (J. B. and J. A. B., not shown). Construction of an in-frame insertion of Venus fluorescent protein (VFP) between amino acids 232 and 233 of AP-1µ (VFP-AP-47) will be described elsewhere (S. L. and R. L. B., not shown).

2.3.3 RNA isolation and RT-PCR of salivary glands

To obtain salivary glands for RNA extraction, larvae were generated by crossing *w*; *Sgs3-DsRed*; *AB1-Gal4* virgin females to *UAS-AP1γ* RNAi / *TM6B, Hu, Tb* males. AP-1γ-depleted salivary glands were dissected from non-Tb larvae, and salivary glands from Tb siblings were used as controls. 10 pairs of salivary glands for each genotype were dissected and pooled in
a microfuge tube containing 0.7% NaCl buffer. 600 µL of TRIzol (Invitrogen) was added, and tubes were centrifuged at 14,000 rpm for 1 minute. Following addition of 120 µL of chloroform, the mixture was shaken vigorously by hand, incubated at room temperature for 3 minutes, and centrifuged at 14,000 rpm for 15 minutes at 4°C. The upper aqueous phase was transferred to a new microfuge tube, to which 0.7 volumes of isopropanol was added. RNA was left to precipitate overnight at -20°C, pelleted by centrifugation at 14,000 rpm for 30 minutes at 4°C, washed with 500 µL cold 70% ethanol, and dried by centrifugation in a SpeedVac (Eppendorf) for 5 minutes. The dry pellet was resuspended in 15 µL of DEPC water and incubated for 15 minutes at 37°C.

CDNA libraries were generated from 1.7 µg of total salivary gland RNA using random decamers and the RETRO-SCRIPT reverse transcription kit (Ambion) following manufacturer’s recommendations for the two-step procedure. The linear range of PCR amplification was determined empirically, and PCR products of salivary gland cDNA were evaluated after 20 cycles. PCR primers for each transcript were designed to span one or more introns.

2.3.4 Fluorescence microscopy and imaging

Salivary glands from third instar larvae were dissected in PBS (pH 7.4), and were either mounted and imaged directly in dissection buffer or fixed for 20 min on ice in PLP (4% paraformaldehyde, 0.01M sodium meta-periodate, 0.075 lysine, 0.035 phosphate buffer, pH 7.4). Fixed salivary glands were then washed once in PBS (pH 7.4), and permeabilized in PBST (PBS + 0.1% Triton X-100). Primary antibody incubation was performed overnight at 4°C in PBST with 5% normal goat serum. Salivary glands were mounted in PPD (0.1x PBS, 90% glycerol, 1 mg/ml p-phenylenediamine). Antibodies were used as follows: 1:1000 rabbit anti-Lva (gift of John Sisson; Sisson et al., 2000), 1:500 mouse anti-GFP monoclonal 3E6 (Molecular Probes), 1:50 rabbit anti-Rab5 (gift of Marcos González-Gaitán; Wucherpfennig et al., 2003), and 1:500 mouse anti-AP-1γ (S. Lallet and R. Le Borgne, unpublished). Anti-rabbit and anti-mouse secondary antibodies conjugated to Alexa-488, Alexa-568 or Alexa-633 were purchased from Molecular Probes (Invitrogen) and used as recommended by the manufacturer.
Fluorescence micrographs were acquired on a Zeiss LSM510 inverted laser scanning confocal microscope equipped with LSM objectives (20x – FLUAR NA 0.75, 40x – Plan-APOCHROMA NA 1.3, 63x – Plan-APOCHROMAT NA 1.4 or 100x – Plan-APOCHROMAT NA 1.4) and LSM510 software, or on a Quorum spinning disk confocal microscope equipped with an SD 63x - LCI Plan-NEOFLUAR 1.3 DIC Imm Kor (water) objective and Volocity acquisition software (SickKids Imaging Facility). Spinning disk confocal images were deconvolved using the Iterative Restoration function of Volocity 4. 3D reconstructions (Fig. 4, C-C’’) were created using the 3D opacity renderings of Volocity 4. All images were further processed for brightness and contrast levels using Adobe Photoshop CS2.

2.3.5 Electron microscopy

Salivary gland samples were prepared for transmission electron microscopy (TEM) as previously described (Bazinet and Rollins, 2003). Salivary gland samples were staged using Sgs3-DsRed, which is expressed from endogenous promoter/enhancer elements, properly sorted into glue granules and secreted into the lumen in response to ecdysone (Biyasheva et al., 2001). The presence of Sgs3-DsRed did not noticeably alter the appearance of mature granules by electron microscopy (not shown). Sections were viewed with a JEOL JTE 141011 microscope (SickKids/Mt. Sinai Advanced Centre for Bioimaging). Images were obtained using AmtV542 acquisition software, and brightness/contrast adjusted using Adobe Photoshop CS2.
2.4 Results

2.4.1 Glue granule biogenesis is developmentally regulated

To characterize the process of glue granule formation and maturation in third-instar larval salivary glands, we used a fluorescently tagged glue protein (Sgs3-DsRed) expressed under control of its own promoter (see Materials and methods). Glue expression was first visible in distal-most cells of the salivary gland, and proceeded proximally over time (Fig. 3, a-c’), with the salivary gland increasing in size as glue production progressed (Biyasheva et al., 2001). We defined the stages as 0 (no expression), 1 (small granules/expression in distal cells), and 2 (fully mature granules/expression in distal and proximal cells). Unlike glue protein, gamma-adaptin (AP-1γ), a large subunit of the AP-1 complex, was expressed in all cells of the salivary gland throughout third-instar larval development (Fig. 3, a’-c’).

Glue granules exhibit several hallmarks of regulated secretory granules, including post-Golgi maturation and storage in the cytoplasm until an external stimulus triggers release. Sgs3-DsRed expression was undetectable prior to mid-third instar (compare Fig. 3, d and e). Accordingly, at stage 0, glue granules were not detected by transmission electron microscopy (TEM) (Fig. 3 g). At the onset of glue production (stage 1), small electron-dense glue granules were visible in the cytoplasm with an average diameter of 1.0 µm (Fig. 3, e and h). Occasional L-shaped granules were also visible (Fig. 3 h), consistent with previous data suggesting growth by accretion (Farkas and Suakova, 1999). Between stages 1 and 2, glue granules increased in size (Fig. 3 i, j) and electron dense material became more prominent near the granule membrane (Fig. 3 i, arrowhead). During stage 2, fully mature glue granules reached an average diameter of 3.5 µm (Fig. 3 j). Following stage 2, glue granules fused with the apical membrane and glue cargo was secreted into the salivary gland lumen (Thomopoulos and Kastritis, 1979; Farkas and Sutakova, 1998; Biyasheva et al., 2001; not shown).

To investigate the origin of glue granules, we used electron microscopy to analyze stage 1 salivary glands in which glue granule production was just starting. At the beginning of glue granule synthesis (stage 1), Golgi units composed of clusters of vesicles and tubules were present in close proximity to the rough endoplasmic reticulum (Thomopoulos et al., 1992; Farkas and Sutakova, 1998; Kondylis et al., 2001; Fig. 3 k). Small glue granules were visible in the
cytoplasm adjacent to the Golgi complex. We also observed coated vesicles in the vicinity of the Golgi and early glue granules (Fig. 3 l). These ranged from 60-90 nm in diameter and were bristle-like in appearance, reminiscent of clathrin coats. The presence of small coated vesicles near the Golgi and developing glue granules suggested that granule formation might proceed via formation of coated intermediate vesicles, and consequently require coat proteins such as clathrin and its adaptors.
Figure 3 - Glue granule biogenesis is developmentally regulated.

(a-c') Confocal micrographs of whole third-instar larval (L3) salivary glands expressing Sgs3-DsRed (red) and stained for AP-1 γ (green), show developmental timing of Sgs3-DsRed expression from stage 0 (no granules) through stage 1 (initiation of granule production) to stage 2 (fully mature granules/glands). AP-1γ is expressed in all cells of the salivary gland throughout development, whereas Sgs3-DsRed is first detected in distal (d) mid-L3 salivary gland cells (b, b'), and is expressed in more proximal (p) cells as development proceeds (c, c'). (d-f) Confocal micrographs of individual salivary gland cells showing developmental expression of Sgs3-DsRed. Sgs3-DsRed is not expressed in stage 0 (d). In stage 1, granules surround the nucleus (n) and appear uniformly small (e). In stage 2, granules are larger and occupy most of the cytoplasmic space (f). (g-i) Transmission electron micrographs (TEM) of L3 salivary glands staged using the Sgs3-DsRed marker. No granules were detected in stage 0 (g). Glue granule (Gr) maturation observed by TEM (h, i) parallels that seen by Sgs3-DsRed, validating this marker for following glue granule biogenesis (e, f). (j) Granules increase in size over time, from an average length of 1.0 μm ± 0.3 (n=91) at stage 1 (red bar) to a maximum length of 3.5 μm ± 1.0 (n=54) at stage 2 (green bar). (k, l) TEM of stage 1 salivary gland cells. Rough ER, transitional ER (tER), Golgi and trans-Golgi network (TGN) (defined morphologically as in Thomopoulos et al., 1992; Kondylis and Rabouille, 2009) are present near small glue granules (Gr) (k). Coated vesicles (CV) were also observed near glue granules (Gr) (l).
Figure 3

Stage 0
Early L3

Stage 1
Mid L3

Stage 2
Late L3

Sgs3

AP-1γ

Sgs3

TEM

Granule size (μm)

0 1 2 3 4 5

Stage

Gr
TCN
Golgi
tER

ER

CV

Gr

CV

500nm

100nm
2.4.2 Clathrin heavy chain and AP-1 localize to the *trans*-Golgi network

To identify coats that might function in granule biogenesis, we investigated the subcellular distribution of clathrin heavy chain, as well as subunits of the clathrin adaptor protein complexes AP-1 and AP-3, which reside on intracellular organelles (note that *Drosophila* lacks AP-4; Boehm and Bonifacino, 2001). We first examined clathrin, AP-1 and AP-3 in salivary gland cells at stage 0, just prior to glue production. At this stage, Golgi bodies are easily visualized using antibodies directed against the golgin Lava lamp (Lva), which localizes to the cup-shaped *cis*-Golgi (Fig. 4, a-d’’). A red fluorescent protein fusion to clathrin heavy chain (RFP-Chc) predominantly localized to large puncta adjacent to the concave face of the *cis*-Golgi (Fig. 4, a-a’’). Endogenous AP-1γ showed a similar distribution (Fig. 4, b-b’’). A projection constructed from serial confocal sections revealed numerous Golgi units scattered throughout the cytoplasm (Fig. 4 c). There was a one-to-one correspondence between AP-1γ- and Lva-positive structures, with the *cis*-Golgi cups surrounding AP-1γ in a manner consistent with AP-1 localizing to the TGN (Fig. 4, c-c’’’). Indeed, AP-1γ and RFP-Chc colocalized with the *trans*-Golgi protein EpsinR (also called Liquid facets-Related or LqfR; Lee *et al.*, 2009; not shown). AP-1γ and RFP-Chc colocalized at the TGN (Fig. 4, d-d’’’), although AP-1γ distribution appeared slightly more diffuse in salivary gland cells expressing RFP-Chc than in non-expressing cells (compare Fig. 4, b and d). Localization of AP-1 to the TGN is adaptor-protein specific, as a functional mCherry fusion to AP-3δ (called Garnet in *Drosophila*), showed no overlap with a Venus fluorescent protein (VFP) fusion to AP-1µ (called AP-47 in *Drosophila*) (Fig. 4, e-e’’’), but rather colocalized with the late endosome marker Rab7 (not shown). Given the high degree of colocalization of clathrin and AP-1, we wondered whether AP-1 might be required to recruit clathrin to the TGN.
Figure 4 - Clathrin heavy chain and AP-1 localize to the trans-Golgi network.

Confocal micrographs of stage 0 salivary gland cells. (a-a’’) RFP-Chc (green) localizes adjacent to, but does not overlap with, the cis-Golgi marker Lva (red). (b-b’’) Endogenous AP-1γ (green) localizes adjacent to Lva (red). (c-c’’’) Projection of a series of spinning disc confocal images of salivary gland cells stained for AP-1γ (green), Lva (red) and DNA (stained with DAPI; blue) reveals numerous Golgi bodies scattered throughout the cytoplasm (c). A 3D rotation of a single Golgi body shows AP-1γ (green) adjacent to the cup-shaped Lva-positive cis-Golgi (red) (c’-c’’’). Images were generated from Z-stacks of 28 (c) or 5 (c’-c’’’) optical sections acquired at a distance of 0.3 μm (see Materials and methods). (d-d’’’) AP-1γ (green) and RFP-Chc (red) colocalize adjacent to Lva (blue). Colocalization of AP-1γ and RFP-Chc appears yellow in the merged image. (e-e’’) Spinning disc confocal images reveal that VFP/AP-47 (green) does not colocalize with mCherry-AP3δ (red). Boxed region is shown at 2x higher magnification in the insets.
Figure 4

Stage 0
2.4.3 AP-1 recruits clathrin to the \textit{trans}-Golgi network

To test whether AP-1 recruits clathrin to the TGN, we made use of a \( \mu 1 \)-adaptin null allele, \( AP-47\text{SHE-11} \) (see Materials and methods). To bypass late embryonic lethality caused by this allele, we generated mosaic clones in the salivary gland using FLP/FRT based recombination (see Materials and methods). \( AP-47\text{SHE-11} \) clones were generated during embryogenesis and analyzed in third-instar larval salivary glands at stage 0, just prior to glue production. To determine whether other AP-1 subunits can localize to the TGN in the absence of AP-47, we examined the distribution of AP-1\( \gamma \), and found that its localization was entirely lost in \( AP-47\text{SHE-11} \) mutant cells (Fig. 5, a-a’’). Hence, AP-47 is required for efficient recruitment or stability of AP-1\( \gamma \), similar to what was previously observed in \( \mu 1 \)-adaptin deficient mouse embryonic fibroblasts (Meyer et al., 2000). Not all trafficking markers were affected by the loss of AP-47, as the early endosome marker Rab5 was unperturbed (Fig. 5, b-b’’).

Strikingly, in \( AP-47\text{SHE-11} \) mutant cells, RFP-Chc localization to the Golgi was dramatically reduced (Fig. 5, c-c’’’). The effect on RFP-Chc distribution was also observed in salivary gland cells in which expression of a double-stranded RNA was used to knock down expression of AP-1\( \gamma \) by RNA interference (RNAi) (Fig. 9). In cells depleted of AP-1\( \gamma \), RFP-Chc was strongly delocalized (compare Fig. 5, d-d’’’ with e-e’’’), indicating that the TGN is the major site of clathrin recruitment in these cells. Importantly, Golgi integrity \textit{per se} (as assessed by distribution of Lva) was not affected by disruption of AP-1 (Fig. 5, c’’ and e’’).
Figure 5 - AP-1 is required to recruit clathrin to the trans-Golgi network.

(a-c”’) Confocal micrographs of stage 0 salivary glands showing mutant clones (cells) marked by absence of GFP (green) and outlined in yellow (a-c”’). (a-a”) AP-1γ (red) localization is lost in an AP-1µ (AP-47SHE-11) mutant cell. (b-b”) Rab5-positive early endosomes (red) are unaffected in AP-47SHE-11 mutant cells. (c-c”’) RFP-Chc (red) becomes largely cytoplasmic in an AP-47SHE-11 mutant cell, whereas the distribution of the cis-Golgi marker Lva (blue) is unaltered. Note that Lva shows a gradient of signal intensity due to incomplete antibody penetration of tissue. (d-d”’) Control salivary gland cells expressing the ABI-GAL4 driver alone show colocalization of AP-1γ (green) and RFP-Chc (red) adjacent to Lva (blue). (e-e”’) Salivary gland cells expressing both the ABI-GAL4 driver and a UAS-AP-1γ RNAi transgene are depleted of AP-1γ (green) and show cytosolic distribution of RFP-Chc (red), whereas Lva (blue) is largely unaffected.
Figure 5

AP-47 (RHE 11)

GFP

AP-1γ

Merge

10 μm

AP-47 (RHE 11)

GFP

Rab5

Merge

10 μm

AP-47 (RHE 11)

GFP

Chc-RFP

Merge

10 μm

Control

AP-1γ

Chc-RFP

Merge

10 μm

AP-1γ-RNAi

AP-1γ

Chc-RFP

Merge

10 μm
2.4.4 Newly synthesized glue proteins pass through an AP-1 and clathrin positive compartment at the TGN

Glue granule production is believed to follow the classical model of secretion, as outlined by Palade and co-workers, with secretory cargo being transported through the ER and Golgi prior to incorporation into secretory granules (Jamieson and Palade, 1967a, 1967b; Thomopoulos and Kastritis, 1979). To determine if glue cargo passes through a clathrin/AP-1 positive compartment, we analyzed Sgs3-DsRed localization in cells that had just switched on glue protein expression. In early stage 1, only a subset of distal most salivary gland cells had initiated glue production (Fig. 3 b and Fig. 6, a-a’’ and b-b’’’) and Sgs3-DsRed and AP-1γ partially colocalized adjacent to Lva in a subset of Golgi bodies (Fig. 6, a-a’’). This partial colocalization likely reflects glue protein passing through an AP-1γ positive compartment, as some of the glue was also present in AP-1-negative puncta outside the Golgi (Fig. 6, a-a’’, yellow arrows). Importantly, Sgs3-DsRed colocalized at the TGN with GFP-Chc as well as AP-1γ (Fig. 6, b-b’’’).

Later in stage 1, both GFP-Chc (Fig. 6, c-c’’’), and AP-1γ (Fig. 6, d-d’’’) could be observed partially coating a subset of granules. At stage 2, when granule biogenesis is nearly complete, GFP-Chc could still be seen coating large granules, as well as numerous smaller organelles (Fig. 6, e-e’’). In contrast to AP-1 and clathrin, AP3δ-GFP did not coat stage 1 or stage 2 glue granules. Instead, AP3δ-GFP and Rab7 positive late endosomes were located adjacent to the granules and became more prominent as granule biogenesis proceeded (compare Fig. 7, a-a’’ with b-b’’; Rab7, not shown). The finding that AP-1 and clathrin are associated with the TGN and with post-Golgi glue granules suggested that these coat proteins might play a role in either glue granule biogenesis or maturation (or both).
Figure 6 - Sgs3-DsRed co-localizes with AP-1 and clathrin at the trans-Golgi network.

Confocal fluorescence micrographs of third instar salivary glands at the onset (early stage 1) (a-b’’’), stage 1 (c-d’’’), and stage 2 (e-e’’) of glue production. (a-a’’) Projections of a series of spinning disc confocal images showing cells initiating Sgs3-DsRed (red) expression, stained with AP-1γ (green) and Lva (blue). In an early stage 1 cell, Sgs3-DsRed and AP-1γ partially colocalize (yellow) adjacent to the cis-Golgi marker Lva in a subset of Golgi bodies (a’’’). Boxed region is shown at 2x higher magnification in the insets. Note that a subset of the Sgs3-DsRed puncta do not colocalize with AP-1γ (yellow arrows). Images were generated from a Z-stack of 5 optical sections acquired at a distance of 0.3 µm. (b-b’’’’) Sgs3-DsRed (red) partially co-localizes with both GFP-Chc (green) and AP-1γ (blue). Colocalization of Sgs3-DsRed with GFP-Chc and AP-1γ appears white in the merged image (b’’’’). (c-c’’’’) Low magnification view of a portion of a salivary gland expressing GFP-Chc (green) and Sgs3-DsRed (red), showing a distal cell with a large number of stage 1 glue granules (c, boxed region; shown at higher magnification in c’-c’’’’). GFP-Chc partially coats a subset of Sgs3-DsRed-containing stage 1 glue granules (c’-c’’’’, yellow arrows). (d-d’’’’) Low magnification view of a portion of a salivary gland stained for AP-1γ (green) and expressing Sgs3-DsRed (red) reveals numerous cells with stage 1 granules (d; boxed region is shown at higher magnification in d’-d’’’’). AP-1γ partially coats a subset of Sgs3-DsRed-containing granules (d’-d’’’’, yellow arrows). (e-e’’’’) Spinning disc confocal micrographs of cells from a mature stage 2 salivary gland expressing GFP-Chc (green) and Sgs3-DsRed (red). GFP-Chc coats a broad range of Sgs3-DsRed containing structures, including large stage 2 granules, as well as smaller vesicles (e’’). Boxed regions 1-3 in e’’ are shown at 2x higher magnification in the images on the right.
**Figure 7 - Glue protein and AP-3 localize to distinct cellular compartments.**

Confocal fluorescence micrographs of third-instar salivary glands expressing AP3δ-GFP (green) and Sgs3-DsRed (red). *(a-a’’)* AP3δ-GFP does not colocalize with Sgs3-DsRed in early stage 1 glue granules (a’’, yellow arrows). Boxed region in a’’ is shown at 2x higher magnification in the images on the right. *(b-b’’)* AP3δ-GFP does not colocalize with Sgs3-DsRed in more mature stage 2 granules. (b’’, yellow arrows). Boxed region in b’’ is shown at 2x higher magnification in the images on the right. Note that AP3δ-GFP-containing puncta become more prominent at later stages of granule biogenesis.
Figure 7

Stage 1

Stage 2
2.4.5 AP-1 and clathrin are required for glue granule formation

To determine if AP-1 is required for glue granule formation, we examined AP-47\textsuperscript{SHE-11} homozygous mutant cells in late third-instar larvae, when glue granules are fully mature (stage 2). AP-47\textsuperscript{SHE-11} homozygous mutant cells either lacked detectible Sgs3-DsRed containing glue granules (8/13 cells) (Fig. 8, a-a’’) or accumulated small granules in the cytoplasm (5/13 cells) (Fig. 8, b-b’’). This difference is likely due to variations in perdurance of AP-1\textmu protein in mutant cells. Remarkably, AP-1\textmu showed dosage dependence, in that cells with only one wild-type copy of AP-47 (marked by one copy of GFP) had intermediate-sized glue granules, whereas cells with two functional copies of AP-47 (marked by two copies of GFP) had granules of normal size (Fig. 8, a-a’’). In support of the idea that AP-1 is limiting for granule biogenesis, third-instar larvae heterozygous for AP-47\textsuperscript{SHE-11} and the hypomorphic allele AP-47\textsuperscript{EP1112} were viable and exhibited glue granules of intermediate size (compare Fig. 8, e and f).

Salivary gland cells in which AP-1\textgreekgamma was knocked down using RNAi were morphologically similar to AP-47\textsuperscript{SHE-11} mutant cells; they either lacked detectible glue granules, or accumulated very small granules (compare Fig. 8, c and d). Since AP-1 is required to recruit clathrin to the TGN (Fig. 5, c-c’’’ and e-e’’’), we asked if clathrin might also be required for glue granule formation. The effect of depleting clathrin heavy chain by RNAi was even more dramatic than for AP-1, resulting in a complete block in glue granule formation in most cells, with only rare cells exhibiting small granules (Fig. 8 g). These effects were specific to loss of AP-1 and clathrin, as mutations in carmine (cm\textsuperscript{1}) and garnet (g\textsuperscript{50c}), which encode AP-3 subunits, AP-3\textdelta and AP-3\mup, exhibited normal-sized glue granules (Fig. 8, h and i).
Figure 8 - AP-1 is essential for granule biogenesis.

Confocal fluorescence micrographs of late-third instar (stage 2) larval salivary glands. (a-b'') AP-1µ (AP-47\(^{SHE-11}\)) mutant clones (cells marked by the absence of GFP (green) and outlined in yellow) exhibit a complete block in production of Sgs3-DsRed -containing glue granules (red) (a’ and a’’), or strikingly small glue granules (b’ and b’’). Note that cells with two copies of wild-type AP-47 (marked by two copies of GFP) have larger granules than heterozygous cells (marked by one copy of GFP). (c-i) Confocal fluorescence micrographs of stage 2 larval salivary gland cells expressing Sgs3-DsRed. (c) Control salivary gland cells expressing the AB1-GAL4 driver alone have granules of normal size (c, boxed region; shown at 2x higher magnification in inset). (d) Salivary gland cells expressing both the AB1-GAL4 and a UAS-AP-1γ RNAi transgene completely lack glue granules (outlined cell) or have strikingly small glue granules (d, boxed region; shown at 2x higher magnification in inset). (e-i) Spinning disc confocal micrographs of salivary gland cells expressing Sgs3-DsRed. (e) Control wild-type cells showing normal-sized glue granules. (f) Larvae bearing the heteroallelic genotype AP-47\(^{SHE-11/EP1112}\) exhibited intermediate-sized granules. (g) Depletion of clathrin heavy chain by RNAi in cells expressing AB1-GAL4 and a UAS-Chc causes a complete block in glue production in most cells, whereas a minority of cells produced small amounts of glue. (h,i) Strong loss-of-function mutations in AP-3µ (carmine\(^{l}\) [cm\(^{l}\)]) (h) or AP-3δ (garnet\(^{50e}\) [g\(^{50e}\)]) (i) have no effect on glue granule biogenesis.
Figure 8

AP-47

GFP
Sgs3-DsRed
Merge
10μm

wild type

AP-1γ RNAi

Sgs3-DsRed
10μm

Chc RNAi

Sgs3-DsRed
10μm

cm (AP-3μ)

Sgs3-DsRed
10μm

g506 (AP-3δ)

Sgs3-DsRed
10μm
2.4.6 Glue transcripts are expressed in AP-1γ depleted salivary glands

To rule out an indirect effect of AP-1 on glue granule biogenesis, we tested whether glue gene transcripts were expressed at normal levels. Glue granule cargo consists of at least eight different proteins, all of which are expressed specifically in the salivary gland during the third-instar larval stage. Reverse transcriptase-coupled PCR (RT-PCR) confirmed that AP-1γ transcripts were depleted in salivary glands in which AP-1γ had been knocked down by RNAi, whereas transcript abundance of a control gene, α-tubulin84B, was unaffected (Fig. 9a,b). Interestingly, depletion of AP-1 had a moderate effect on glue gene expression. Levels of glue gene transcripts, including Sgs3-DsRed, were reduced by approximately 50% in AP-1γ knockdown glands as compared to controls. Importantly, glue gene transcription was still robustly initiated in salivary glands depleted of AP-1γ.
Figure 9 - Glue transcripts are expressed in AP-1γ-depleted salivary glands.

(a) RT-PCR analysis of transcripts performed on mRNA extracted from wild-type control and AP-1γ-depleted salivary glands of late-third instar larvae (stage 2). Expression of *ABI-GAL4* and *UAS-AP-1γ* to induce RNAi directed against AP-1γ is indicated. *AP-1γ* transcripts were robustly knocked down by RNAi (lanes 1 and 2), whereas transcripts of the control α-tubulin gene *catub84B* were not altered in abundance (lanes 3 and 4). Abundance of transcripts encoding the glue proteins Sgs1, Sgs5, Sgs7, Sgs8 and the Sgs3-DeRed fusion protein, was reduced by 30-60% in AP-1γ-depleted cells, although all glue genes were still transcribed. (b) Quantification of relative transcript levels in AP-1γ-depleted salivary glands (yellow) versus control salivary glands (red). Transcript abundance in controls was arbitrarily set at 100%. Average intensity and standard deviation are shown for samples from three independent experiments, with the exception of Sgs3-DsRed, which was assayed only once.
Figure 9

(a) Gel electrophoresis showing the effect of AP-1γ RNAi on AP-1γ and αtub84B expression. The lanes are labeled with AP-1γ RNAi: - or +, and the samples are: AP-1γ, αtub84B, Sgs1, Sgs5, Sgs7, Sgs8, Sgs3-DsRed. The gel shows bands at 1000 bp and 500 bp.

(b) Bar graph representing the total intensity of each transcript as a percentage of the control. The x-axis labels are AP-1γ, αtub84B, Sgs1, Sgs5, Sgs7, Sgs8, Sgs3-DsRed, while the y-axis shows the total intensity ranging from 0 to 120.
2.4.7 Glue protein accumulates at the trans-Golgi network and in aberrant vacuolated organelles in AP-1γ-depleted cells

If AP-1 is required to sort glue protein into vesicles at the TGN, we reasoned that disruption of this complex should result in increased association of glue protein with Golgi bodies. In wild-type stage 2 salivary gland cells, Lva-containing Golgi bodies appeared scattered throughout the cytoplasm, but were not obviously associated with Sgs3-DsRed containing mature glue granules (Fig. 10, a-a’’). In contrast, in AP-1γ depleted salivary gland cells, glue protein was associated with numerous Lva containing Golgi bodies (Fig. 10, b-b’’), and these appeared swollen relative to those in control cells (compare Fig. 10, a and b). Indeed, the Golgi in AP-1γ knockdown cells more closely resembled those of cells prior to onset of glue production (compare Fig. 4, a’, b’ and d’ with Fig. 10 b). A similar increase in TGN volume was observed when post-Golgi secretion was blocked by incubation of cells at moderately low temperatures (20°C) (Griffiths et al., 1989; Ladinsky et al., 2002). In addition to its localization at the TGN, Sgs3-DsRed could also be seen in small granules (Fig. 8, d and d’) and in larger cytoplasmic structures (Fig. 10, b’ and b’’).

To determine the morphology of these structures in AP-1γ depleted cells, we examined salivary glands by TEM. Numerous enlarged, vacuolated structures containing fibrillar cargo were visible throughout the cytoplasm, likely representing highly abnormal post-Golgi glue-containing organelles (compare Fig. 10, c and d). Notably, these structures did not resemble early secretory granules observed in wild-type cells, clearly indicating an essential role for AP-1 in normal granule formation.
Figure 10 - Glue protein accumulates at the *trans*-Golgi network and in aberrant vacuolated organelles in AP-1γ-depleted cells.

(a-b’’) Confocal fluorescence micrographs of late-third instar (stage 2) salivary glands expressing Sgs3-DsRed (red) and stained for the *cis*-Golgi marker Lva (green). Lva is distributed throughout the cytoplasm but is not associated with mature Sgs3-DsRed containing granules in control cells expressing *AB1-GAL4* alone (a-a’’; boxed regions 1-3 in a’’ are shown at 2x higher magnification in the images on the right). In cells expressing both the *AB1-GAL4* and a *UAS-AP-1γ* RNAi transgene, Sgs3-DsRed is associated with Lva containing Golgi bodies, and can also be seen in larger organelles (b-b’’; boxed regions 1-3 in b’’ are shown at 2x higher magnification in the images on the right). (c and d) TEM of stage 1 salivary glands. Rough ER, mitochondria (m), nascent granules (Gr) and coated vesicles (CV) are visible in a wild-type cell (c). AP-1γ-depleted cells expressing both the *AB1-GAL4* and a *UAS-AP-1γ* RNAi transgene exhibit rough ER and mitochondria (m), as well as a large number of aberrant vacuolated organelles (d, white arrow). Micrograph is from a single experiment.
2.5 Discussion

We provide compelling evidence of a previously unknown function for clathrin and AP-1 in formation of mucin-type secretory granules. We show that clathrin and AP-1 localize to the TGN prior to synthesis of secretory cargo; colocalize with newly synthesized secretory cargo as it passes through the TGN; and both are required for secretory granule formation. Hence, the primary role of AP-1 and clathrin in these cells is in anterograde trafficking of regulated secretory cargo.

2.5.1 Glue granules and WPBs require AP-1 for formation

Our results show that formation of mucin-containing glue granules and WPBs is similar. WPBs have an unusual cigar shaped appearance and it was proposed that AP-1 and clathrin might participate in their formation at the TGN by allowing for luminal cargo to properly fold and aggregate or by preventing premature scission (Lui-Roberts et al., 2005; Metcalf et al., 2008). Indeed, depletion of AP-1 in endothelial cells results in formation of small, round von Willebrand factor-containing vesicles lacking other WPB markers. Our data demonstrate that the requirement for clathrin and AP-1 is not restricted to one specific type of granule, as depletion of clathrin or AP-1 in Drosophila salivary glands similarly resulted in accumulation of glue protein both at the TGN and in vesicles of aberrant morphology. Furthermore, some cells were completely devoid of granules, clearly demonstrating AP-1’s role in their formation. This extends the role of AP-1 and clathrin to the formation of granules containing mucoprotein cargo, and suggests a broader requirement for this coat complex in granule production.

2.5.2 A potential role for AP-1 in granule maturation

Development of Drosophila glue granules is characterized by an overall increase in size and decrease in number, consistent with homotypic fusion of smaller granules over time (Farkas and Suakova, 1999). Whether small and large granules are equally capable of fusing, and whether fusion events are temporally regulated is not known. Remarkably, reduced levels of AP-1 resulted in intermediate sized granules, suggesting AP-1 is limiting for some aspect of granule
maturation. AP-1 might regulate granule maturation at the TGN by properly sorting into nascent granules the full complement of membrane proteins required for homotypic fusion and eventual exocytosis. Additionally, AP-1 might function directly on maturing granules to promote their maturation and condensation by refining their content, similar to what has been reported for other types of secretory granules (Arvan and Castle, 1998; Morvan and Tooze, 2008). For example, AP-1 might be required for retrieval and recycling of SNAREs or other factors implicated in homotypic fusion and granule growth.

2.5.3 Possible roles of AP-1 during granule formation

How AP-1, a cytosolic coat protein, interacts with luminal cargo at the TGN remains to be determined. As none of the identified glue proteins contains a predicted transmembrane domain, a yet-unidentified transmembrane receptor might mediate this interaction. A putative AP-1 interacting receptor need not be abundant, so long as it is capable of linking condensing cargo proteins at the TGN to AP-1 in the cytoplasm. In mammalian cells, several transmembrane proteins that are targeted to regulated secretory granules have been identified including peptidyl-α-amidating monooxygenase, muclin and phogrin (Bell-Parikh et al., 2001; Wasmeier et al., 2002; Boulatnikov and De Lisle, 2004; Dikeakos and Reudelhuber, 2007). Indeed, phogrin has been shown to bind to AP-1 and AP-2 through well-conserved tyrosine and dileucine sorting motifs present in the cytosolic tail (Torii et al., 2005; Wasmeier et al., 2005). Alternatively, aggregation at the TGN might be sufficient to concentrate the glue proteins, which might then deform the lipid bilayer in such a way that AP-1 and clathrin can promote budding.

2.5.4 AP-1 and epithelial polarity

In mammalian cells, AP-1A is ubiquitously expressed and is required for trafficking between TGN and endosomes, whereas AP-1B is present only in polarized epithelial cells and is required for basolateral sorting from recycling endosomes (Folsch et al., 1999; Folsch et al., 2001; Cancino et al., 2007; Gravotta et al., 2007; Deborde et al., 2008). The sole AP-1 complex in Drosophila might mediate both functions in a single cell type. Interestingly, depletion of AP-1γ in salivary glands after granule formation caused the basolateral protein discs large to
redistribute to the apical surface (Peng et al., 2009), suggesting that AP-1 is required for targeting of proteins to the basolateral membrane in this epithelial tissue. However, an independent analysis of AP-1µ null cells in the dorsal thorax epithelium failed to reveal a polarity defect (R. Le Borgne; pers. comm.). This discrepancy might be due to cell type specific requirements for AP-1, or due to differences in RNAi versus mosaic clone analysis. Since depleting AP-1 in salivary cells clearly disrupts polarity, it will be interesting to test if disrupting polarity affects secretory granule exocytosis.

2.5.5 Depletion of AP-1 down regulates glue protein synthesis

The observation that abundance of Sgs3-DsRed cargo protein and several Sgs mRNAs is reduced upon AP-1 knockdown suggests the existence of a negative feedback loop, whereby a block in anterograde secretory trafficking results in downregulation of secretory genes. A block in secretion at the TGN could potentially induce the unfolded protein response, analogous to what happens upon depletion of the Arf1 GEF GBF1 (Citterio et al., 2008). However, GBF1 functions early in the secretory pathway, and knockdown of two Arf-GEFs that act on the TGN did not elicit a similar response (Citterio et al., 2008). Alternatively, a block in anterograde trafficking might repress transcriptional activation of secretory genes by Drosophila CrebA and Forkhead (Fkh) (Abrams and Andrew, 2005; Abrams et al., 2006).

2.5.6 Additional clathrin binding coat proteins in Drosophila

In addition to the AP-1 complex, the Drosophila genome encodes two other clathrin adaptor proteins, EpsinR/LqfR and a single Golgi-localized, gamma-ear-containing, ADP-ribosylation factor-binding (GGA) protein (Hirst et al., 2009; Lee et al., 2009; Kametaka et al., 2010). LqfR partially colocalizes with AP-1 at the TGN in salivary gland cells and lqfR mutants exhibit small salivary glands, suggesting defects in granule biogenesis (Lee et al., 2009). It will be interesting to determine if GGA and LqfR participate in glue granule biogenesis, especially since these clathrin adaptors might facilitate sorting of other types of cargo. For example, EpsinR has been shown to bind SNARE proteins and could function to provide vesicle identity to nascent glue-containing granules (Miller et al., 2007; Chidambaram et al., 2008). SNAP-24 was
previously identified as a glue granule specific SNARE, although whether this SNARE mediates homotypic fusion of granules or functions during the exocytosis of granules at the plasma membrane is unclear (Niemeyer and Schwarz, 2000). Given the apparent similarities between glue granule and WPB biogenesis, as well as the high degree of conservation of TGN sorting machinery in *Drosophila*, our findings suggest that *Drosophila* salivary glands will be of great utility to further elucidate the mechanisms of biogenesis of regulated secretory granules.
Chapter 3

3 Type II phosphatidylinositol 4-kinase is required for glue granule biogenesis in *Drosophila*

Jason Burgess, Miluska Jauregui, David Hipfner, Janet Rollins, Joseph P. Albanesi, Helmut Krämer and Julie A. Brill

**Statement of Contributions:**

David Hipfner carried out the S2 cell RNAi experiments. Joseph Albanesi generated the PI4KII antibody. Miluska Jauregui assisted in preparing salivary gland samples for immunofluoresence. Janet Rollins assisted in preparing salivary gland samples for electron microscopy. Helmut Krämer made the initial observation that salivary gland cells were affected in PI4KII mutants and assisted with experimental design. Additional reagents or antibodies that were provided are indicated in the materials and methods section. I generated all other reagents and performed all other experiments. My research was performed in the laboratory of Julie Brill.
3.1 Summary

Type II phosphatidylinositol 4-kinase (PI4KII) is a lipid modifying enzyme conserved from yeast to man. PI4KII produces the lipid phosphatidylinositol 4-phosphate (PI4P), a key regulator of trafficking events at the Golgi. Additionally, PI4KII is required for proper endosomal function. To gain further insight into the function of PI4KII during metazoan development, I generated null mutations in the sole Drosophila PI4KII gene. PI4KII null mutants are viable, but exhibit defects in the formation of mucin-containing ‘glue granules’ in the larval salivary gland. PI4KII localizes to late endosomes and the Golgi, where it colocalizes with glue proteins. Loss of PI4KII results in formation of small granules, and accumulation of glue protein in late endosomes. Additionally, PI4KII mutants mislocalize the granule associated SNARE SNAP-24. These data demonstrate an important role for PI4KII in ensuring proper sorting of both luminal secretory cargo and SNARE proteins during glue granule biogenesis.

3.2 Introduction

How cargo is sorted at the trans-Golgi network (TGN) into distinct vesicles with unique cellular destinations remains an ongoing source of investigation (De Matteis and Luini, 2008). Cargo can be sorted on the basis of signal motifs, post-translational modifications or even by self-aggregation (Bonifacino and Traub, 2003; Borgonovo et al., 2006). Additionally, cytoplasmic coat proteins play a key role in defining the contents of forming vesicles. For example, adaptor proteins can recognize specific signal sequences on the cytoplasmic domain of transmembrane proteins, while also binding to the clathrin lattice (Edeling et al., 2006). Furthermore, membrane components, including sphingolipids, cholesterol and phosphatidylinositol phosphates (PIPs), have been recognized to play an important role in sorting of cargo proteins. PIPs in particular have emerged as key regulators, due to their ability to recruit specific sorting machinery, such as coat proteins, as well as by altering physical properties of membranes (De Matteis and Godi, 2004).

The unique subcellular distribution of different phosphoinositide species is largely determined by the activity of organelle-specific PI-kinases and phosphatases (De Matteis and Godi, 2004). PI 4-kinases and phosphatidylinositol 4-phosphate (PI4P) are enriched at the Golgi,
where they play crucial roles in the function of this organelle (D'Angelo et al., 2008). In yeast, mutations in PI4KIIIβ/Pik1p perturb secretion and PI4P itself is required for secretion (Hama et al., 1999; Walch-Solimena and Novick, 1999; Audhya et al., 2000). In mammalian cells, PI4KIIIβ and PI4KIIα both localize to the Golgi and contribute to distinct pools of PI4P on this organelle (Wong et al., 1997; Wang et al., 2003; Weixel et al., 2005). Consistent with this, manipulating PI4KIIIβ by overexpression, or by using dominant-negative constructs, affects post-Golgi trafficking (Godi et al., 1999; Hausser et al., 2005). Similarly, depleting PI4KIIα decreases the rate of transport of constitutively secreted protein hemaglutinin from the TGN to the plasma membrane (Wang et al., 2003).

The identification of PI4P-binding effector proteins has helped to define functions for this lipid. These include AP-1, GGA and EpsinR, which function as clathrin adaptor proteins (Hirst et al., 2003; Wang et al., 2003; Wang et al., 2007); FAPP, OSBP and CERT, which function in non-vesicular transport of glucosylceramide, cholesterol and ceramide, respectively (Hanada et al., 2003; Godi et al., 2004; Im et al., 2005; D'Angelo et al., 2007); and GOLPH3, which is required for proper Golgi morphology and retention of Golgi-resident enzymes (Dippold et al., 2009; Wood et al., 2009).

In addition to its role at the Golgi, PI4KIIα localizes to endosomal membranes and is required for proper endosomal function (Balla et al., 2002; Minogue et al., 2006; Craige et al., 2008). For instance, PI4KIIα binds to the AP-3 complex via a dileucine cargo-sorting motif present in PI4KIIα (Salazar et al., 2005; Craige et al., 2008). Depleting PI4KII causes AP-3 to redistribute from endosomes to the cytoplasm. Furthermore, the AP-3 cargo LAMP-1, as well as the SNAREs syntaxin 8 and Vti1b, accumulate on enlarged late endosomes in PI4KII depleted cells. Conversely, in AP-3 mutant mocha mice, PI4KII accumulates in a perinuclear region, indicating AP-3 is required for PI4KII transport. Thus, localization of PI4KII and AP-3 is interdependent. PI4KII is also required for efficient endosomal trafficking and degradation of the EGF receptor (Minogue et al., 2006). In PI4KII depleted cells, lysosomal degradation of activated EGF receptor is dramatically impaired. PI4KII has been associated with synaptic vesicles, and mice lacking PI4KIIα activity exhibit late onset neurodegeneration (Guo et al., 2003; Simons et al., 2009). PI4KII activity has also been detected on immature secretory granules, as well as on a subset of glucose transporter 4 containing vesicles (Del Vecchio and
Pilch, 1991; Kristiansen et al., 1998; Panaretou and Tooze, 2002; Xu et al., 2006). In fact, PI4KIIα was originally cloned following partial purification of this enzyme from chromaffin granule membranes, indicating that PI4KII might play a role in granule biogenesis (Barylko et al., 2001). The surprising range of organelles and cell types that exhibit type II PI4KII activity suggest that this enzyme is involved in many processes within cells of a complex animal.

Here, we investigate the function of PI4KII in the fruit fly Drosophila melanogaster. Drosophila has a single type II PI4K that accounts for the majority of PI4K activity in S2 cells (Barylko et al., 2002). Flies bearing null mutations in PI4KII are fully viable, but exhibit defects in glue granule formation in the specialized secretory cells of the salivary gland. During glue granule production, PI4KII colocalizes with glue protein cargo as it passes through the TGN, but does not localize to mature granules. In addition, PI4KII localizes to highly dynamic and interconnected tubular endosomes in salivary gland cells. Mutations in PI4KII affect sorting of the granule associated SNARE SNAP-24, but do not affect recruitment of the clathrin adaptor proteins AP-1 and EpsinR. Additionally, PI4KII mutants exhibit enlarged late endosomes that aberrantly accumulate glue protein. We propose that PI4KII is required for proper sorting at both the TGN and in endosomes, and that loss of PI4KII leads to missorting of a crucial factor, possibly SNAP-24, required for granule maturation.
3.3 Experimental Procedures

3.3.1 Fly stocks and genetic crosses

Flies were raised on standard cornmeal molasses agar at 25°C (Ashburner, 1990). Visible markers and balancer chromosomes are as described (Lindsley and Zimm, 1992). Deletions in PI4KII/CG2929 were generated by imprecise excision of P\{EP\}CG12746\textsuperscript{GE28807} (obtained from GenExel Inc.) as described previously (Timakov et al., 2002). Briefly, flies carrying the transposable P element GE28807 were crossed to a stock containing the P\{Δ2-3\}99B transposase source to mobilize the transposon. An additional cross was performed to replace the Δ2-3 transposase chromosome with a TM6B balancer chromosome. Individual fly lines exhibiting eye color that varied from the original GE28807 stock (indicating mobilization of the P element), were then screened by PCR to look for deletions in PI4KII. Briefly, 5 flies were ground in a 1.5 ml eppendorf tube and DNA extracted using standard procedures. Deletions were detected using two primers outside of the region spanned by GE28807 and PI4KII. Using this strategy, we isolated Df(3R)730, which removes the entire coding region of PI4KII and CG14671. To generate mosaic clones, we recombined Df(3R)730 onto a chromosome containing P\{neoFRT\}82B (Xu and Rubin, 1993). To rescue lethality associated with loss of CG14671, we further recombined the genomic rescue transgene P\{w\+, CG14671\} onto the deficiency containing chromosome to generate P\{w\+, CG14671\}, P\{neoFRT\}82B, Df(3R)730 which is referred to henceforth as PI4KII\textDelta. This stock is maintained over a third chromosome balancer since males are sterile. Transgenic markers or mutations were introduced into the PI4KII\textDelta mutant stock using standard genetic techniques. Mosaic clones were generated using the FLP/FRT system (Golic and Lindquist, 1989) by crossing y\textsuperscript{l}, w\textsuperscript{1118}, P\{70FLP\}3F \;; P\{neoFRT\}82B, P\{Ubi-GFP.D\}83 / TM6B, Hu, Tb to flies containing w\textsuperscript{1118} ; PI4KII\textDelta / TM6B, Hu, Tb. To examine glue granules in PI4KII\textDelta clones, the heat shock FLP stock was crossed to Sgs3-DsRed ; PI4KII\textDelta / TM6B, Hu, Tb. The heat shock protocol used to generate mosaic clones is described in Chapter 2.

Transgenic flies were generated by injection of w\textsuperscript{1118} embryos with a mix containing the transgenic construct and the helper Δ2-3 transposase-expressing plasmid, pTurbo (Mullins et al., 1989). Transgenic stocks carrying P\{w\+, atub-mCherry-PI4KII\}, P\{w\+, atub-mCherry-
Additional stocks were generously provided as follows: P\{w\+\, αtub\-mCherry\-PI4KII\ATP\}, P\{w\+, αtub\-mCherry\-Vps29\}, P\{w\+, αtub\-Vps29-GFP\} or P\{w\+, αtub\-garnet-GFP\} (see below) were mapped and balanced using w\1118\; Sco/CyO ; TM3/TM6B.

The epsinR\D66\ mutant was created by imprecise excision of the P-element P\{PZ\}lqfr\03685, which is located in this first exon of epsinR (P. Leventis and G. Boulianne, unpublished data). The deletion removes 1102 bp from the insertion point of the P-element in the 3’ direction into the first intron, including the start-ATG.

### 3.3.2 Molecular biology

Genomic rescue constructs for PI4KII and CG14671 were generated by PCR amplification from clone BACR24024 (BACPAC resource) using Phusion DNA Polymerase (Finnzymes). Restriction sites were introduced using primers containing SmaI and NotI cut sites and PCR products subcloned into pCaSpeR4 (Pirrotta, 1988). The CG14671 genomic rescue construct begins just before the first exon of CG12746 (which is antiparallel to CG14671) and ends before the first predicted ATG of PI4KII. The PI4KII genomic rescue construct begins in exon 2 of CG14671 and ends 2 Kb downstream of the last exon of PI4KII.

For low-level ubiquitous expression of fluorescently tagged proteins, cDNAs were subcloned into a modified pCaSpeR4 expression vector containing the αTub84B promoter (Marois et al., 2006). This vector contained either mCherry or mEGFP, allowing proteins to be fluorescently tagged at their N-terminal or C-terminal ends, respectively (Zacharias et al., 2002; Shaner et al., 2004). cDNAs placed after αTub84B-mCherry were inserted into an XbaI site. cDNAs placed between αTub84B and GFP were inserted into a KpnI site. The only exception to this strategy was GFP-PI4KII, in which GFP was placed at the N-terminus, and PI4KII was
inserted into a unique XbaI site. Restriction sites were introduced into cDNAs by PCR, and orientation after subcloning confirmed by DNA sequencing (The Centre for Applied Genomics, Hospital for Sick Children, Toronto). PI4KII kinase dead (D465A) and ATP-binding mutations (K311A) (Barylko et al., 2002), were generated by site directed mutagenesis (Invitrogen). These constructs were tested in vitro and verified to be kinase dead (J. Burgess, G. Polevoy, B. Barylko, J. Albanesi and J. A. Brill, unpublished observations). Full-length cDNAs (Berkeley Drosophila Genome Project) for PI4KII (LD24833), garnet (RE06749), Vps29/CG4764 (GH25884) were obtained from the Canadian Drosophila Microarray Centre.

3.3.3 Drosophila S2 cell culture and RNAi

fwd and PI4KII dsRNA templates were prepared by PCR amplification of genomic DNA from w1118 flies. As a negative control, a β-galactosidase (β-gal) dsRNA template was prepared by PCR amplification of genomic DNA from dpp10638 flies containing a β-gal-encoding enhancer trap insertion. Top strand oligonucleotides included a 5′ T3 promoter sequence (5´-AATTAACCCTCACTAAAGGGAGA-3´), bottom strand oligonucleotides a 5′ T7 promoter sequence (5´-TTAATACGACTCACTATAGGGAGA-3´). Gene specific oligonucleotide sequences were: fwd (5´-CCAAAGAATGCCATATTTCGC-3´ and 5´-GGAGCACATCAGACACAGG-3´); PI4KII (5´-TTCGTGGAGGGTTACAAGG-3´ and 5´-AAGGGAAAAGCGAGACCAT-3´); β-gal (5´-CACCAGCGAAATGGATTTTT-3´ and 5´-AGTAAGGCGGTCCGGATAGT-3´). Double stranded RNA was prepared from these templates using MegaScript T7 and T3 in vitro transcription kits (Ambion). Equal amounts of the T3 and T7 transcription products were mixed, heated to 95°C for 10 minutes, and cooled slowly to room temperature to anneal.

To make pMTpuro/FAPP-PH-YFP, an EcoRI-BamHI fragment from pEYFP-c1/FAPP-PH (Dowler et al., 2000), was cloned into a modified version of pMT vector containing a puromycin selection cassette (pMTpuro) (Denef et al., 2000).

S2 cells were cultured in serum-free insect medium (Sigma) supplemented with 16.5 nM L-glutamine and 1x Penicillin/Streptomycin (Invitrogen). To establish cells stably transfected with the pMTpuro/FAPP-PH-YFP vector, 1.5x10^6 S2 cells were plated in one well of a 6-well
plate and transfected using Cellfectin transfection reagent (Invitrogen) according to the manufacturer’s protocol. Starting two days later, stably transfected cells were selected and maintained by inclusion of 10 µg/ml puromycin in the culture medium.

For FAPP-PH localization experiments, 1.25 x 10^5 S2 cells stably transfected with pMT^puro/FAPP-PH-YFP were plated in 325 µl puromycin-containing serum-free medium in 4-well Lab-Tek Permanox chamber slides (Thermo Scientific). Double-stranded RNA was immediately added (2 µg per well) and mixed. FAPP-PH-YFP expression was induced 24 h later by addition of CuSO_4 to 0.5 mM final concentration. Three days after induction, bright field and fluorescence microscopy images of live cells were acquired with Volocity 4.1 (Perkin Elmer) using a DM4000 microscope (Leica) equipped with a Retiga EXi camera (QImaging). For immunofluorescence experiments, normal S2 cells were treated with dsRNA as above. After four days in dsRNA-containing medium, cells were rinsed once with PBS and fixed for 10 minutes in PBS containing 0.2% Triton X-100 (PBX) and 4% formaldehyde. After washing with PBX and blocking for 1 h in PBX containing 0.1% BSA, slides were processed according to standard immunofluorescence protocols, using rabbit anti-Lva (1:250) or mouse anti-AP-1 (1:300) as primary antibodies. Samples were mounted in 90% glycerol, 10% PBS, 100 mM N-propyl gallate.

3.3.4 RT-PCR to detect PI4KII transcripts

To extract RNA, 5 adults were placed in a microfuge tube and crushed with a pestle. RNA was extracted using TRIzol (Invitrogen) followed by chloroform separation and isopropanol precipitation. cDNA libraries were generated from 2 µg of total adult RNA using random decamers and the RETRO-SCRIPT reverse transcription kit (Ambion). PCR was then performed using primers specific to either the somatic or testis cDNA. To detect the PI4KII somatic transcript, primer sequences were selected from exon 1 and 3. To detect the testis specific transcript, primers were selected from the first predicted exon of the testis isoform and exon 4.
3.3.5 Fluorescence microscopy and imaging of salivary glands

Salivary glands from third instar larvae were dissected and prepared for immunostaining as previously described (Chapter 2). Antibodies were used as follows: 1:1000 rabbit anti-Lva (gift of J. Sisson, Sisson et al., 2000), 1:500 mouse anti-AP1γ (gift of R. Le Borgne, unpublished), 1:350 rabbit anti-PI4KII (gift of J. Albanesi, unpublished), 1:100 rabbit anti-Lqfr (gift of P. Leventis and G. Boulianne, unpublished), 1:100 rabbit anti-SNAP-24 (gift of T. Schwarz, Niemeyer and Schwarz, 2000) and 1:500 mouse anti-GFP monoclonal 3E6 (Molecular Probes). Anti-rabbit and anti-mouse secondary antibodies conjugated to Alexa-488, Alexa-568 or Alexa-633 were purchased from Molecular Probes (Invitrogen) and used as recommended by the manufacturer. Lectin peanut agglutinin conjugated to Alexa Fluor 568 was purchased from Molecular Probes (Catalog # L-32458) and used at a dilution of 1:10,000.

For live imaging of salivary glands, samples were dissected and transferred to a 25 µl drop of PBS (pH 7.4) on a glass slide. We used inert silicone (placed on all four edges of the cover slip) as a bridge between the glass slide and cover slip to seal each sample and prevent it from being crushed. For lysotracker staining, live salivary glands were incubated in PBS contained lysotracker (Molecular Probes) at a dilution 1:1,000 for 1 min, and then mounted in PBS for imaging. Live salivary glands were imaged for no longer than 10 min after being mounted.

All live sample images, and some fixed sample images, were acquired on a Quorum spinning disk confocal microscope using Volocity acquisition software (SickKids Imaging Facility). Images of fixed samples were obtained using a Zeiss LSM510 inverted laser scanning confocal microscope with LSM510 software (SickKids Imaging Facility). Images were exported using Volocity, and further processed for brightness and contrast using Adobe Photoshop CS2.

3.3.6 Transmission electron microscopy

Salivary gland samples were prepared for transmission electron microscopy (TEM) as previously described (Bazinet and Rollins, 2003). Sections were viewed with a JEOL JTE 141011 microscope (SickKids/Mt. Sinai Advanced Centre for Bioimaging). Images were
obtained using AmtV542 acquisition software, and brightness and contrast adjusted using Adobe Photoshop CS2.
3.4 Results

3.4.1 *PI4KII* null mutant flies are viable

*Drosophila melanogaster* possesses a single *PI4KII* gene, CG2929. Two alternative transcripts are predicted for *PI4KII* based on cDNAs sequenced by the Berkeley *Drosophila* Genome Project, with a longer transcript found in numerous somatic tissues, and a shorter transcript detected only in the male germline (Fig. 11a) (Stapleton *et al.*, 2002a; Stapleton *et al.*, 2002b). The two transcripts encode PI4KII isoforms with unique N-terminal domains and identical kinase domains (Fig. 11b). The N-terminal regions of both *Drosophila* PI4KII isoforms are unrelated to either human PI4KIIα or PI4KIIβ. A high degree of conservation can be seen in the C-terminal kinase domain, although *Drosophila* contains a 99 amino acid insertion in this region not found in mammals (Barylko *et al.*, 2002). We confirmed the existence of both *PI4KII* transcripts in *Drosophila* by reverse transcriptase-coupled polymerase chain reaction (RT-PCR). The longer somatic transcript was detected in both male and female fruit flies, whereas the shorter testis transcript was detected exclusively in males (Fig. 11c).

To investigate the function of *PI4KII*, we generated a *PI4KII* deletion by imprecise excision of a nearby transposable P-element GE28807 (Fig. 11a). We isolated deletion Df(3R)730, which removes the entire coding region of *PI4KII*, as well as the uncharacterized upstream gene CG14671 (Fig. 11a). Flies homozygous for Df(3R)730 were larval lethal and could be rescued by a genomic transgene containing both CG14671 and *PI4KII*. To determine which gene is responsible for lethality, we generated separate genomic transgenes for CG14671 and *PI4KII* (Fig. 11a). Df(3R)730 flies carrying the CG14671 genomic transgene were fully viable, indicating that this gene is responsible for the observed lethality. To further characterize *PI4KII* function, the CG14671 genomic rescue transgene was recombined onto the Df(3R)730 chromosome (this chromosome is henceforth referred to as *PI4KIIΔ*). Phenotypes caused by *PI4KIIΔ* (see below) can be fully rescued by a *PI4KII* genomic rescue construct.

*PI4KIIΔ* mutants are protein null, as determined by immunoblotting and immunofluorescence using polyclonal antiserum directed against the N-terminal portion of the somatic isoform of PI4KII. The antiserum recognized a single protein of ~90kDa that could be detected in protein extracts prepared from flies at different developmental stages (Fig. 11d). The
antiserum is specific, as PI4KII was not detected in extracts prepared from \textit{PI4KIIΔ} mutant flies. Moreover, examination of larval salivary glands containing \textit{PI4KIIΔ} mutant cells revealed that PI4KII localized to intracellular puncta in cells with a wild-type copy of the \textit{PI4KII} gene (Fig. 12a), whereas no signal was detected in cells that lacked \textit{PI4KII} (Fig. 12a, dashed line).
Figure 11 - Deletions in \( \text{PI4KII} \) are protein null.

(a) Physical map of \( \text{PI4KII} \) (CG2929). \( \text{PI4KII} \) maps to cytological region 83B2 on the right arm of chromosome 3. Predicted genes (blue bars) mapping to this region are shown. Pointed ends indicate the 3’ end of each gene. The bottom half of this panel shows a magnified view of the \( \text{PI4KII} \) locus. \( \text{PI4KII} \) spans 6Kb and encodes two alternative transcripts with unique start sites. The predicted exons (dark blue, coding regions; light blue, untranslated regions; red, stop codon) for somatic and testis specific isoforms of \( \text{PI4KII} \) are shown. The P element GE28807 (triangle) was excised to generate the deletion Df(3R)730 (red bar), which removes both \( \text{PI4KII} \) and \( \text{CG14671} \). The Df(3R)730 deletion is homozygous lethal and can be rescued by introducing the \( \text{CG14671} \) genomic rescue transgene (light grey bar), but not the \( \text{PI4KII} \) genomic rescue transgene (dark grey bar). This indicates that \( \text{CG14671} \) is essential for viability and that \( \text{PI4KII} \) is a non-essential gene. (b) The C-terminal kinase domain (dark grey bars) of \( \text{Drosophila} \) \( \text{PI4KII} \) is homologous to human \( \text{PI4KII}\alpha \) and \( \text{PI4KII}\beta \), whereas the N-terminal regions (white bars) are not conserved. \( \text{Drosophila} \) \( \text{PI4KII} \) also contains a 99 amino acid insertion in the kinase domain (light grey bars). For sequence comparisons, the kinase domain was split into two regions to exclude a non-conserved insertion. Percentage sequence identity and similarity (in brackets) are shown for both homologous portions of the kinase domain, and all comparisons were made relative to the \( \text{Drosophila} \) \( \text{PI4KII} \) somatic isoform. (c) RT-PCR performed on whole adult male or female flies. The somatic transcript can be detected in both males and females (left panel), whereas the testis specific transcript is detected only in males. (d) Western blot demonstrating that \( \text{PI4KII}\Delta \) mutants are protein null. \( \text{PI4KII} \) is expressed in embryos, third instar larvae (L3), male adults (M adult) and female adults (F adult) (lanes 1-4 respectively). Male and female adult flies homozygous for \( \text{PI4KII}\Delta \) are protein null for \( \text{PI4KII} \) (lanes 5 and 6).
Figure 11

a

Cytological Band
Chromosome Position
Predicted Genes

b

N-terminal domain  C-terminal kinase domain

D. melanogaster PI4KII Somatic Isoform
D. melanogaster PI4KII Testis Isoform
H. sapiens PI4KIIα
H. sapiens PI4KIIβ

N-step transcript  Testis transcript

M Adult  F Adult  M Adult  F Adult

D

wild type  PI4KII

embryo L3  M Adult  F Adult  M Adult  F Adult

PI4KII

tubulin
3.4.2  PI4KII localizes to the TGN and late endosomes in larval salivary gland cells

The enzyme PI4KII, as well as the membrane lipid PI4P, is enriched in chromaffin granule and immature secretory granule membranes of PC12 cells. Intriguingly, *Drosophila* PI4KII is most abundantly expressed in larval salivary glands (5.5x enrichment), a tissue that produces a large number of secretory ‘glue granules’ (Chintapalli *et al.*, 2007). To determine if PI4KII might participate in glue granule formation, we examined localization of this enzyme in salivary gland cells. Endogenous PI4KII is found to colocalize with markers for the Golgi and late endosomes (LEs) in third instar larval salivary gland cells. PI4KII localized adjacent to a functional *Drosophila* PI4KIIβ GFP fusion (GFP-Fwd), suggesting these two enzymes are concentrated in non-overlapping compartments of the Golgi (Fig. 12b). Similarly, PI4KII showed partial colocalization with the TGN-localized clathrin adaptor subunit AP-1γ (Fig. 12c). PI4KII showed strong colocalization with a LAMP GFP fusion protein (GFP-LAMP) on LEs near the cell periphery (Fig. 12d). Likewise, PI4KII showed extensive colocalization on LEs with a functional GFP fusion to the clathrin adaptor subunit AP-3δ (Fig. 12e).

Similar to endogenous PI4KII, fluorescently tagged PI4KII localized to both Golgi and endosomal compartments. In late third instar salivary gland cells, a functional fluorescent PI4KII fusion protein (mCherry-PI4KII) localized adjacent to, but did not overlap with, a fluorescently tagged Golgi marker (Fig. 13a). mCherry-PI4KII also localized to LEs containing YFP-Rab7 (Fig. 13b). Strikingly, mCherry-PI4KII was found on numerous tubular structures emanating from LEs (Fig. 13b, inset). Notably, YFP-Rab7 was excluded from the tubular portion. A projection constructed from serial confocal sections revealed that PI4KII localizes to round endosomes connected by smaller tubules (Fig. 13c). This tubular endosomal network was most dense near the cell periphery, with tubules extending both parallel and perpendicular to the cell cortex (Fig. 13c). Examination of mCherry-PI4KII and LAMP-GFP revealed that LEs appear enriched near the cell cortex (Fig. 13d). PI4KII containing tubular endosomes were not observed in salivary cells prior to glue production, despite localization of PI4KII to LEs. (Fig. 13e, f). Similarly, tubular endosomes were not observed in non-granule producing duct cells at the proximal end of the salivary gland, nor in imaginal disc cells or spermatocytes (not shown).
Figure 12 - PI4KII localizes to the TGN and endosomes.

Confocal fluorescence micrographs of third instar salivary gland cells. (a) Late third instar salivary gland showing a $PI4KII\Delta$ mutant clone cell. Endogenous PI4KII exhibits a punctate distribution in cells containing a wild-type copy of $PI4KII$ (marked by the presence of GFP), and cannot be detected in a $PI4KII\Delta$ mutant cell (marked by the absence of GFP and outlined in white). (b) Colocalization of GFP-Fwd and PI4KII in a mid third instar salivary gland. Endogenous PI4KII partially overlaps with GFP-Fwd at the Golgi (arrows), although the two enzymes largely localize to distinct domains (inset). (c) Colocalization of PI4KII and AP-1γ in mid third instar salivary glands. Endogenous PI4KII exhibits partial co-localization with the TGN marker AP-1γ (arrows). (d) Colocalization of LAMP-GFP and PI4KII in late third instar salivary glands. Endogenous PI4KII colocalizes with the lysosomal marker LAMP-GFP (arrows). Confocal sections were obtained near the cortex where LAMP-GFP puncta were enriched. (e) Colocalization of AP-3δ-GFP and PI4KII in late third instar salivary glands. Endogenous PI4KII colocalizes with AP-3δ-GFP near the cortex.
Figure 12
Figure 13 - PI4KII localization in live cells.

Confocal fluorescence micrographs of live third instar salivary gland cells. (a) mCherry-PI4KII localizes adjacent to the Golgi marker YFP-Golgi in late third instar salivary gland cells (arrows). PI4KII also localizes to larger tubulated structures that lack YFP-Golgi (arrowhead). Since mCherry-PI4KII and YFP-Golgi are not coincident, these two proteins localize to distinct compartments of the Golgi (inset). (b) mCherry-PI4KII colocalizes with YFP-Rab7 at late endosomes in late third instar salivary gland cells (arrows). mCherry-PI4KII also localizes to long tubules (arrowhead) from which YFP-Rab7 is excluded (inset). (c) A projection constructed from serial confocal sections of a late third instar salivary gland cell expressing mCherry-PI4KII. (XY) Image generated from a Z-stack of 7 optical sections acquired at a distance of 0.3 µm each starting from the cell surface. Note the presence of tubules (arrowheads) linking round endosomes (arrows). (XZ) Image generated from a Z-stack of 60 optical sections acquired at a distance of 0.3 µm each. The Y-axis was cropped and this slice turned 90° to get a side view of the cell. Note that mCherry-PI4KII is enriched at the cell cortex and localizes to tubules (arrowheads) that extend perpendicular to the cell surface. (d) A projection constructed from serial confocal sections of a late third instar salivary gland cell expressing mCherry-PI4KII and GFP-LAMP. This image was generated from a Z-stack of 52 optical sections acquired at a distance of 0.3 µm each. The Y-axis was cropped and the resulting slice turned 90°. mCherry-PI4KII and LAMP-GFP are enriched near the cell cortex, and colocalize on late endosomes (arrowheads). mCherry-PI4KII can also be seen deeper in the cell. (e) GFP-LAMP and mCherry-PI4KII expression in early third instar salivary cells. mCherry-PI4KII shows partial colocalization with GFP-LAMP on late endosomes, although no mCherry-PI4KII containing tubules are present. (f) GFP-LAMP and mCherry-PI4KII expression in late third instar salivary cells. mCherry-PI4KII localizes to LAMP-GFP containing puncta, and numerous mCherry-PI4KII containing tubules emanate from these structures.
3.4.3 PI4KII localization is highly dynamic in live cells and colocalizes with microtubules

To further characterize the dynamics of tubular endosomes, we performed live imaging experiments of mCherry-PI4KII in salivary gland cells. Long endosomal tubules containing mCherry-PI4KII showed extensive colocalization with microtubules labeled with tubulin-GFP (Fig. 14a). Indeed, time-lapse fluorescence microscopy revealed that these tubules emanate from round endosomes and extend and retract along microtubules (Fig. 14b). Additionally, tubules formed transient connections with each other, temporarily linking distinct round endosomes (Fig. 14c). Strikingly, these tubule links occasionally retracted resulting in displacement of one endosome towards another (Fig. 14c). In addition to the dynamic movement of the tubular endosomal network, small mCherry-PI4KII-containing vesicles could be seen moving rapidly along microtubules (Fig. 14d).

The retromer complex, which mediates retrograde transport of cargo from endosomes to the TGN, was recently shown to localize to tubular endosomal structures (Rojas et al., 2008). To determine if PI4KII and the retromer complex localize to the same tubular compartment on endosomes, we generated transgenic flies expressing a fluorescent fusion to the retromer subunit Vps29. As previously described, mCherry-Vps29 localized to highly dynamic foci on LEs, where it colocalized with YFP-Rab7 (not shown). Intriguingly, mCherry-Vps29 was restricted to the round endosome domain and was not found on endosomal tubules in late third instar salivary cells. Consistent with this, co-expression experiments revealed that GFP-PI4KII and mCherry-Vps29 colocalized on round endosomes, but not on the highly elongated tubules that contain PI4KII (Fig. 14e). This indicates that PI4KII-containing endosomal tubules are distinct from previously described retromer-containing tubules. We conclude that the localization of PI4KII is highly dynamic in live cells and that PI4KII resides on the TGN, a highly tubulated endosomal network and rapidly moving microtubule-associated vesicles.
Figure 14 - PI4KII localizes to highly dynamic tubular endosomes.

Confocal fluorescence micrographs of live third instar salivary gland cells. (a) mCherry-PI4KII tubules colocalize with β-tubulin (Tubulin-GFP) (arrowheads). Shown is a projection of 9 optical sections acquired at a distance of 0.3 μm each. (b-d) Time-lapse fluorescence micrographs of late third instar salivary glands. Time after the start of imaging (in seconds) is shown above each frame. (b) mCherry-PI4KII containing tubules (arrowheads) extend and retract from a round endosome (arrow). Tubules extend on microtubules marked by Tubulin-GFP. (c) mCherry-PI4KII containing tubules rapidly form (frames 2 and 3, green arrowhead) and break (frame 4, red arrowhead). Additionally, retracting tubules can exert a pulling force on endosomes and displace them (frames 4-6, arrows). Each frame is a projection of 5 optical sections acquired at a distance of 0.3 μm each. (d) Small mCherry-PI4KII containing vesicles move rapidly along microtubules marked by Tubulin-GFP (arrowhead). Each frame is a projection of 3 optical sections acquired at a distance of 0.3 μm each. (e) GFP-PI4KII colocalizes with mCherry-Vps29 at round endosomes (arrows). However, mCherry-Vps29 is excluded from mCherry-PI4KII containing tubules (arrowheads).
Figure 14
PI4KII colocalizes with glue cargo at the TGN and is required for proper granule biogenesis

To determine if PI4KII participates in glue granule biogenesis, we compared the localization of PI4KII to that of a fluorescently tagged glue protein (Sgs3-GFP) under control of its endogenous promoter. At the earliest stages of glue production, typically only a few of the distal-most salivary gland cells had initiated glue protein expression (Fig. 15a). At this timepoint, Sgs-GFP was detected predominantly in Golgi bodies and localized adjacent to the cis-Golgi marker Lva. Similarly, mCherry-PI4KII<sup>KD</sup> also localized adjacent to Lva foci (note that mCherry-PI4KII<sup>KD</sup> exhibits identical localization to mCherry-PI4KII in wild-type cells). Importantly, in cells that had initiated glue protein expression, mCherry-PI4KII<sup>KD</sup> showed partial colocalization with Sgs3-GFP at the Golgi (Fig. 15a). To establish whether PI4KII localizes to the limiting membrane of mature glue granules, we examined localization of this enzyme in late third instar larval salivary glands. At this stage, Sgs3-GFP was concentrated in mature glue granules that filled most of the cytoplasm (Fig. 15b). In contrast, mCherry-PI4KII localized to the TGN and LEs, but was not detected on mature granules (Fig. 15b and Fig. 13a,b).

To directly test whether PI4KII participates in glue granule biogenesis, we examined late third instar larval salivary glands, a stage when wild-type granules are fully mature. Sgs3-DsRed-containing granules from wild-type animals had an average diameter of 3.8 ± 1.0 µm (n=222), whereas PI4KII<sup>Δ</sup> mutant glue granules were much smaller, with an average diameter of 1.7 ± 0.3 µm (n=292) (Fig. 15d). Glue granule size was fully rescued to normal diameter, 4.0 ± 0.9 µm (n=249), by introducing a PI4KII genomic rescue construct into a PI4KII<sup>Δ</sup> mutant background (Fig. 15e). To rule out the possibility that PI4KII<sup>Δ</sup> mutants exhibited small granules due to a global developmental delay or defective ecdysone signaling, we generated PI4KII<sup>Δ</sup> single cell mutant clones in the salivary gland. PI4KII<sup>Δ</sup> mutant cells exhibited small granules, demonstrating that PI4KII functions cell autonomously in granule biogenesis (Fig. 15f). To further characterize this phenotype at the ultrastructural level, we prepared late-third instar salivary gland samples for analysis using transmission EM (Fig. 15g,h). PI4KII<sup>Δ</sup> mutants exhibited small glue granules with grossly normal morphology. In particular, mutant glue granules contained the three types of structural elements (granular, filamentous and electron lucent) previously described (Thomopoulos and Kastritis, 1979). Intriguingly, PI4KII<sup>Δ</sup> mutant cells also contained large vacuolar structures in the cytoplasm that were not apparent in wild-
type samples (Fig. 15h, arrowheads). Notably, these vacuoles contained filamentous material that was much less dense than that found in granules.
Figure 15 - PI4KII is required for proper glue granule biogenesis.

(a-f) Confocal fluorescence micrographs of third instar salivary gland cells. (a) Mid-third instar salivary gland cells expressing Sgs3-GFP and mCherry-PI4KII<sup>KD</sup>, and stained for Lva (blue). The cell in the lower left corner has initiated expression of Sgs3-GFP. mCherry-PI4KII<sup>KD</sup> localizes adjacent to the cis-Golgi marker Lva (arrowhead) and colocalizes with Sgs3-GFP (arrow). (b) Projection generated from serial confocal sections of a late third instar salivary gland cell expressing Sgs3-GFP and mCherry-PI4KII. Images were generated from a Z-stack of 16 optical sections acquired at a distance of 0.3 µm. mCherry-PI4KII does not localize to the surface of mature glue granules. (c-e) Late third instar salivary gland cells expressing the glue granule marker Sgs3-DsRed. Wild-type animals exhibit large granules (c), whereas PI4KII<sup>Δ</sup> mutants exhibit strikingly small glue granules (d). This phenotype is rescued by introducing a PI4KII genomic transgene into PI4KII<sup>Δ</sup> mutants (e). (f) PI4KII<sup>Δ</sup> mutant cells (marked by the absence of GFP) exhibit strikingly small glue granules, as visualized using Sgs3-DsRed. (g-h) Transmission electron micrographs of late third instar salivary glands. Wild type animals have large, well-organized glue granules (g, left panel). Note that electron dense material is found near the granule membrane, and filamentous electron intermediate material is found near the centre (g, middle and right panel. PI4KII<sup>Δ</sup> mutant salivary glands exhibit strikingly small granules (g, left panel, arrow). In addition, vacuolated structures are visible in the cytoplasm (yellow arrows). The vacuoles also contain filamentous material (g, middle and right panel, arrow).
3.4.5 PI4KII does not obviously affect recruitment of Golgi adaptor proteins

AP-1 is required for glue granule formation (Chapter 2). To test if other clathrin adaptors are required for glue granule biogenesis, we examined granule formation in late third instar larval salivary glands of epsinR mutants. The strong loss-of-function mutation epsinR<sup>D66</sup> (P. Leventis and G. Boulianne, unpublished reagent), gave rise to small glue granules, similar to PI4KII mutants (Fig 16a,b). Furthermore, both AP-1 and EpsinR colocalize with Chc, supporting a role for these proteins as clathrin adaptor proteins (Fig. 16c). Since PI4KII is required to recruit AP-1, and possibly EpsinR, to the TGN by producing PI4P (Hirst <i>et al.</i>, 2003; Wang <i>et al.</i>, 2003), we asked whether localization of AP-1 and EpsinR is disrupted in PI4KII mutants. PI4KII<sub>A</sub> single cell mutant clones generated in the salivary gland exhibited normal recruitment of endogenous AP-1 (Fig. 16d). Similarly, the clathrin adaptor EpsinR was not affected in PI4KII<sub>A</sub> single cell mutant clones (Fig. 16e). Since the PI4P binding proteins AP-1 and EpsinR are not obviously affected in PI4KII mutants, this indicated PI4KII might regulate other factors.
**Figure 16 - Mutations in PI4KII do not disrupt AP-1 and EpsinR localization.**

(a-e) Confocal fluorescence micrographs of third instar salivary gland cells. (a) Wild-type late third instar larval salivary gland stained with the lectin peanut agglutinin (PNA) to detect glue granules. (b) epsinR<sup>D66</sup> mutants exhibit small glue granules, as detected by PNA staining. (c) Mid-third instar salivary gland expressing Chc-RFP and stained for AP-1γ (green) and epsinR (blue). Note that the clathrin adaptors AP-1 and EpsinR colocalize with Chc (arrows). (d) PI4KIIΔ mutant cells (marked by the absence of GFP and outlined in yellow) exhibit normal AP-1γ localization. (e) PI4KIIΔ mutant cells exhibit normal EpsinR localization.
3.4.6 PI4KII is required for sorting of the granule SNARE SNAP-24

*PI4KII* mutants have small glue granules that otherwise appear morphologically normal, suggesting the granules may fail to undergo homotypic fusion. The SNARE SNAP-24 was previously identified as being abundantly enriched on granule membranes (Niemeyer and Schwarz, 2000). Indeed, SNAP-24 colocalizes with the glue protein Sgs3-DsRed as cells initiate glue biogenesis (Fig. 17a). SNAP-24 also localizes to the limiting membrane of mature granules (Niemeyer and Schwarz, 2000). To test whether PI4KII might be required to localize SNAP-24, we analyzed the distribution of SNAP-24 in *PI4KII* mutant clone cells. Whereas SNAP-24 localized uniformly in control cells, SNAP-24 accumulated on the limiting membrane of structures lacking Sgs3-DsRed in *PI4KII* mutant cells (Fig. 17b). This indicates that PI4KII is required for proper localization of SNAP-24.
Figure 17 - The SNARE SNAP-24 is mislocalized in PI4KII mutants.

(a-b) Confocal fluorescence micrographs of third instar salivary gland cells. (a) Endogenous SNAP-24 colocalizes with the glue protein Sgs3-DsRed in cells initiating granule biogenesis. (b) PI4KIIΔ mutant clone cells (marked by the absence of GFP and outlined in yellow) exhibit altered distribution of the SNARE SNAP-24. Note that SNAP-24 is barely detectable in adjacent control cells, but strongly accumulates in organelles lacking Sgs3-DsRed glue protein in PI4KIIΔ mutant cells.
3.4.7 Glue protein accumulates in enlarged late endosomes in \textit{PI4KII} mutants

The presence of large vacuolated structures in \textit{PI4KII}\textsubscript{Δ} mutant cells, as well as the observation that PI4KII localizes to LEs, suggested that this compartment might be altered. To test this possibility, we performed live cell imaging of fluorescently-tagged endosomal markers. LEs labeled by YFP-Rab7 were much larger in \textit{PI4KII}\textsubscript{Δ} mutants, as compared to wild type cells (Fig. 18a,b). Additionally, YFP-Rab7 labeled endosomes formed clusters in wild type cells, but appeared as individual units in \textit{PI4KII}\textsubscript{Δ} mutants. LEs marked by AP-3δ-GFP were also larger and did not form clusters in \textit{PI4KII}\textsubscript{Δ} mutants (Fig. 18c,d). \textit{PI4KII}\textsubscript{Δ} mutants also appeared to lose LE microdomains, as Vps29-GFP became more uniformly distributed around LE membranes, whereas Vps29-GFP localized tightly to foci on wild type LEs (Fig. 18e,f and i,j). This suggests that partitioning of the endosome into dynamic microdomains is lost in the absence of PI4KII. Acidic LEs or lysosomes were also enlarged in \textit{PI4KII}\textsubscript{Δ} mutants, as compared to wild type cells (Fig. 18g,h). Notably, lysotracker dye appeared restricted to the surface of the acidic compartment and was largely excluded from the centre, suggesting this compartment might contain a large aggregate or occlusion in the lumen. Endosomes appeared normal in \textit{PI4KII}\textsubscript{Δ} mutant salivary gland cells prior to granule biogenesis (data not shown), suggesting that glue granule production might be linked to the enlarged LE phenotype in \textit{PI4KII}\textsubscript{Δ} mutants.

Since PI4KII colocalizes with glue protein as it passes through the TGN, we reasoned that PI4KII might function to direct secreted cargo into the regulated secretory pathway, or alternatively to prevent entry into vesicles destined for late endosomes. Strikingly, \textit{PI4KII}\textsubscript{Δ} mutants aberrantly accumulate glue protein in LEs, as revealed by colocalization of Sgs3-DsRed with YFP-Rab7. Sgs3-DsRed was never detected in YFP-Rab7 containing LEs of wild-type animals (Fig. 18k). However in \textit{PI4KII}\textsubscript{Δ} mutants, Sgs3-DsRed could clearly be observed in the lumen of YFP-Rab7 contained LEs (Fig. 18l). Additionally, LEs were visible throughout the cytoplasm of \textit{PI4KII}\textsubscript{Δ} mutants, whereas LEs were concentrated near the cell periphery in wild-type cells. This indicates that PI4KII is required for proper sorting of secreted glue proteins.
**Figure 18 - Glue protein accumulates in late endosomes of PI4KII mutants.**

Confocal fluorescence micrographs of late third instar salivary gland cells. (a-i) Images were acquired in live salivary glands. (a,b) YFP-Rab7 labeled endosomes increase dramatically in size in PI4KIΔ mutant cells (b), compared to wild-type cells (a). Panel (a) is a projection of 32 slices and panel (b) is a projection of 101 slices. For both images, slices were acquired at a distance of 0.3 µm each. (c,d) AP-3δ-GFP labeled endosomes increased dramatically in size in PI4KIΔ mutant cells (d), compared to wild-type cells (c). Both panels are projections of 13 optical slices acquired at a distance of 0.3 µm each. (e,f) Vps29-GFP becomes uniformly distributed around enlarged endosomes in PI4KIΔ mutant cells (f), relative to wild-type cells (e). Both panels are projections of 22 optical slices acquired at a distance of 0.3 µm each. (g,h) Acidic late endosomes or lysosomes increased dramatically in size in PI4KIΔ mutant cells (h), compared to wild-type cells (g). Both panels are projections of 19 optical slices acquired at a distance of 0.3 µm each. (i,j) Time-lapse fluorescence micrographs of late third instar salivary glands. Vps29-GFP localizes to dynamic foci on the limiting membrane of late endosomes of wild-type cells (i), whereas Vps29-GFP is more uniformly and stably distributed around the membrane of enlarged LEs in PI4KIΔ mutants (j). (k,l) Late third instar salivary glands expressing YFP-Rab7 and Sgs3-DsRed. (k) YFP-Rab7 positive late endosomes are found near the cell cortex in wild-type cells. Sgs3-DsRed is absent from the lumen of these LEs (inset). (l) Enlarge late endosomes appear scattered throughout the cell in PI4KIΔ mutants. These endosomes accumulate luminal Sgs3-DsRed (inset).
Figure 18

Wild type

- YFP-Rab7
- AP-3Δ-GFP
- Vps29-GFP
- lysotracker

PI4K1Δ

- YFP-Rab7
- AP-3Δ-GFP
- Vps29-GFP
- lysotracker

Vps29-GFP

0.0s  3.1s  7.7s  17.0s  20.0s  30.8s

Wild type

PI4K1Δ

0.0s  5.5s  11.1s  15.2s  22.8s  30.4s

Wild type

- YFP-Rab7
- Sgs3-DsRed
- Merge

PI4K1Δ

- YFP-Rab7
- Sgs3-DsRed
- Merge
3.4.8 PI4KII and Fwd are redundant for an essential process in *Drosophila*

Dual localization of PI4KII and GFP-Fwd to the Golgi (albeit to different subcompartments), suggested that these enzymes might function redundantly for synthesis of PI4P at this organelle (Fig. 12b). To test this prediction, we used yellow fluorescent protein (YFP) fused to the pleckstrin homology (PH) domain of FAPP (YFP-PH-FAPP) to monitor Golgi-associated PI4P in S2 cells (Dowler *et al.*, 2000; Godi *et al.*, 2004) (Fig. 19a). Depletion of Fwd caused YFP-PH-FAPP to become less concentrated to the Golgi, while depletion of PI4KII had no observable effect (Fig. 19b, c). Strikingly, depletion of both enzymes caused YFP-PH-FAPP to become strongly cytoplasmic, indicating that these enzymes are partially redundant for synthesis of PI4P at the Golgi (Fig. 19d)

To test if PI4KII and Fwd function redundantly *in vivo*, we recombined a *fwd* mutation on to a *PI4KIIΔ* chromosome. Indeed, *fwd PI4KIIΔ* double mutants were larval lethal, with development arresting at the third instar larval stage. In wild-type salivary glands, the *cis*-Golgi marker Lva localized adjacent to the TGN marker AP-1 with a one-to-one correspondence (Fig. 19e). In *fwd PI4KII* double mutants, Lva staining was reduced and the *cis*-Golgi appeared smaller (Fig. 19f). Importantly, localization of the PI4P-binding protein AP-1 to individual Golgi bodies was dramatically reduced in *fwd PI4KII* double mutants. Single mutants exhibited normal looking Golgi as assessed by Lva and AP-1 staining (data not shown).
Figure 19 - PI4KII and Fwd double mutant phenotype.

(a-d) Confocal fluorescence micrographs of *Drosophila* S2 cells stably transfected with YFP-PH-FAPP. (a) Control cells treated with dsRNA directed against β-gal exhibit normal YFP-PH-FAPP localization. (b) RNAi directed against *fwd* causes YFP-PH-FAPP to become more cytoplasmic. (c) RNAi directed against *PI4KII* did not obviously affect YFP-PH-FAPP localization. (d) *fwd PI4KII* double RNAi caused YFP-PH-FAPP to become strongly cytoplasmic. (e-f') Confocal fluorescence micrographs of early third instar salivary glands. The cis-Golgi marker Lva localizes adjacent to AP-1γ in wild-type cells (e). In *fwd PI4KII* mutant cells, Lva staining is reduced and AP-1γ localization is absent from some of the Golgi bodies. Images show in (e) and (f) were obtained using identical confocal microscope settings. AP-1 appears to be absent from Golgi bodies even when (f) is adjusted for brightness and contrast (f').
Figure 19

YFP-PH-FAPP

wild type

AP-1γ
Lva
Merge

fwd PI4KIIΔ

AP-1γ
Lva
Merge

fwd PI4KIIΔ

AP-1γ
Lva
Merge
3.5 Discussion

We report the first analysis of PI4KII null mutants in the multicellular organism Drosophila melanogaster. PI4KII mutants are fully viable and do not exhibit any obvious developmental defects. This is in agreement with a recent report showing that PI4KIIα deficient mice are viable, although they exhibit late onset degeneration of axons in the spinal cord (Simons et al., 2009). Using salivary glands as a model for granule biogenesis, we find that PI4KII mutants exhibit small glue granules and enlarged late endosomes that accumulate glue protein. Additionally, distribution of the granule specific SNARE SNAP-24 is dramatically altered in PI4KII mutant cells. We propose that PI4KII is necessary for proper sorting of secretory granule proteins.

3.5.1 In which compartment does PI4KII exert its effect on granule biogenesis?

Mammalian PI4KIIα has long been suspected to participate in regulated secretion, based on biochemical purification from immature secretory granules, chromaffin granules, glucose-transporter containing vesicles and synaptic vesicles (Del Vecchio and Pilch, 1991; Kristiansen et al., 1998; Barylko et al., 2001; Panaretou and Tooze, 2002; Guo et al., 2003; Xu et al., 2006). Contrary to expectation, Drosophila PI4KII does not localize to the limiting membrane of glue granules, but instead localizes to the TGN and to endosomes. Depending on whether PI4KII regulates granule formation at the Golgi or at endosomes, several scenarios are possible.

Possible roles for PI4KII at the Golgi

Since PI4KII and glue protein colocalize at the TGN, it is tempting to conclude that the TGN is the site where PI4KII acts during glue granule formation. For instance, PI4KII may be required to generate a TGN-specific pool of PI4P, which in turn recruits one or more factors required for granule biogenesis. Several PI4P binding proteins predicted to function in post-Golgi vesicular trafficking are conserved in Drosophila, including AP-1, GGA, EpsinR and GOLPH3 (Dippold et al., 2009; Hirst et al., 2009; Lee et al., 2009; Kametaka et al.).
Importantly, the AP-1 complex is essential for formation of *Drosophila* glue granules and mammalian Weibel-Palade bodies, and GGA function is required for maturation of neuroendocrine secretory granules (Chapter 2 and Lui-Roberts *et al.*, 2005; Kakhlon *et al.*, 2006). AP-1 might participate in granule formation by binding to a crucial secretory cargo receptor or by recruiting the scaffold protein clathrin. Additionally, EpsinR is required for sorting the endosomal SNAREs syntaxin 7, syntaxin 8 and Vti1b in mammalian and yeast cells (Hirst *et al.*, 2004; Miller *et al.*, 2007; Chidambaram *et al.*, 2008). Consequently, a failure to recruit this adaptor protein could result in missorting of either endosomal or granule specific SNAREs.

Localization of AP-1 or EpsinR is not obviously affected in *PI4KIIΔ* mutant cells. This is in contrast to the observation that AP-1 becomes strongly cytoplasmic when PI4KIIα is depleted in HeLa cells, but consistent with a recent study demonstrating PI4KIIα is not required for AP-1 localization in HEK 293 cells (Wang *et al.*, 2003; Craige *et al.*, 2008). Nonetheless, PI4KII may exert a subtle influence on AP-1 or EpsinR localization in a manner not detected by confocal microscopy. For example, changes in the levels or distribution of PI4P might influence either the kinetics of AP-1 or EpsinR recruitment or their association with particular subdomains of the TGN. Alternatively, PI4KII might affect recruitment of GGA, GOLPH3 or other unidentified proteins. It is also possible that PI4KII is not required to recruit PI4P binding proteins or may be redundant with Fwd (see below). Systematic analysis of PI4P-binding proteins should help define how PI4P participates in glue granule formation, and may help identify novel proteins involved in granule biogenesis.

Sorting of proteins at the TGN is generally thought to direct cargo to secretory versus endosomal pathways. Hence, PI4KII might function at the TGN to ensure that specific cargo proteins are properly sorted into their cognate vesicles. For example, failure to properly segregate SNARE proteins could result in glue granule SNAREs being missorted to endosomes. Similarly, a failure to properly segregate cargo receptors could lead to mixing of glue proteins with lysosomal hydrolases destined for late endosomes.

**Possible roles of PI4KII at endosomes**
In *Drosophila* salivary gland cells, loss of PI4KII results in enlarged late endosomes that accumulate secretory protein. This raises the intriguing possibility that PI4KII might contribute to granule formation by regulating retrograde transport of cargo from endosomes to the TGN. Disrupting this process might lead to a decreased steady state level of secretory granule proteins at the TGN, and their accumulation at endosomes. Consistent with this possibility, SNAP-24 accumulates on aberrant structures in *PI4KII* mutants. It will be of interest to determine if SNAP-24 accumulates on endosomes. Indeed, knockdown of mammalian PI4KIIα results in enlarged endosomes that aberrantly accumulate SNAREs (Syntaxin 8 and Vti1b) (Craige *et al*., 2008). In addition, retromer complex dynamics are altered in *PI4KII* mutants, suggesting defective retrograde transport from endosomes to the TGN. Assuming granule membranes and proteins need to be recycled during successive cycles of granule formation, PI4KII might be required to ensure cargo is efficiently transported from endosomes back to the TGN. A null mutation in the retromer subunit Vps35 could be tested for the ability to phenocopy mutations in *PI4KII* (Franch-Marro *et al*., 2008; Port *et al*., 2008). Additionally, a comprehensive set of endocytic mutants that blocks transport of cargo at distinct steps is available (Vaccari *et al*., 2008). These mutants could be used to systematically test which steps of endocytosis are required for glue granule formation.

Another possibility is that PI4KII contributes to proper lysosomal function. For instance, if glue proteins are missorted to the lysosomal compartment at low levels in normal cells, compromised lysosomal function might lead to accumulation of cargo in LEs/lysosomes. To test this possibility, glue protein localization could be examined in mutants with impaired lysosomal function. If glue cargo accumulates in LEs in these mutants, this would suggest that *PI4KII* mutants have compromised lysosomal function.

### 3.5.2 Are glue granules lysosome-related organelles?

Cargo proteins destined for LROs are transported through endosomes en route to their final destination. However, despite the observation that glue proteins accumulate in endosomes in *PI4KII* mutants, it is unlikely that glue proteins normally follow this intracellular route. First, glue cargo is not usually observed in Rab7-positive late endosomes, even during early stages of granule biogenesis. Second, glue granules exhibit none of the hallmarks of LROs, as they are not
acidic and do not contain lysosomal markers such as LAMP. Finally, mutations in genes encoding subunits of the AP-3 complex, which severely disrupt LRO formation, do not obviously affect glue granules (Chapter 2).

3.5.3 Role of tubular endosomes in salivary gland cells

PI4KII localizes to an extensive network of highly interconnected tubular endosomes. The emergence of tubular endosomes coincides with the onset of glue biogenesis. However, it is unclear if the function of tubular endosomes in this context is related to the process of granule biogenesis. A key feature of tubular endosomes is that they have a very large surface to volume ratio, in contrast to ‘rounded up’ endosomes, which have a low surface to volume ratio. This might serve two purposes. The first is to increase the amount of membrane available for recycling back to the TGN. This would be important if granule membrane is not newly synthesized but constantly reutilized in cycles. A second function is to increase the number of endosomal domains where membrane proteins can be sorted for exit from the endosomal system. This would be crucial if granule membrane proteins such as SNAREs and receptors need to be recycled back to the TGN for future cycles of granule formation. Alternatively, salivary gland cells may also require the uptake of exogenous sources of proteins and lipids. Indeed, salivary glands are physically attached to fat cells, which function as a lipid reservoir. TEM analysis revealed that membrane invaginations originating from the basal lamina of the fat body protrude into salivary gland cells during early third instar (Thomopoulos, 1988). Consequently, a highly dynamic tubular endosomal network could function to deliver proteins and lipid membrane to the TGN using a retrograde transport pathway.

A second possibility is that emergence of tubular endosomes might be related to the process of autophagy that is induced in salivary glands approximately 10 hours after pupariation formation (Jiang et al., 1997; Berry and Baehrecke, 2007). Autophagy relies on fusion of autophagosomes with lysosomes to form autophagolysosomes, leading to degradation by acidic lysosomal hydrolases. Establishing an extensive endosomal network may be a prerequisite to the autophagic process that occurs several hours later.
3.5.4 General functions of PI4KII in Drosophila

PI4KII mutants do not exhibit a general defect in endocytic function. In fact, endosomes appear normal in size in non-granule producing cells such as epithelial imaginal disc cells. Consistent with this observation, PI4KII mutants do not exhibit obvious developmental defects, indicating that cell signaling pathways are not disrupted. Consequently, PI4KII is distinct from previously isolated trafficking mutants such as lethal giant discs (lgd), which result in enlarged endosomes in epithelial cells and disrupt the Notch cell-signaling pathway (Jaekel and Klein, 2006). In contrast, the most obvious PI4KII mutant phenotypes occur in cell types that require an enormous biosynthetic effort during a short time period. For instance, salivary cells produce glue granules during the last 20 hours of third-instar larval development; eye pigment cells produce pigment granules during the last 48 hours of pupal development (Appendix 1); and sperm cells complete growth in less than 24 hours (Appendix 2). Consequently, PI4KII function may be largely dispensable in most cells types, perhaps due to redundancy with Fwd (see below), and may become crucial only during periods of high biosynthetic effort when sorting fidelity may be most stringently required. Neuronal cells likely also fit into this category, as synaptic vesicle pools must be rapidly replenished and secreted cargo efficiently sorted at synapses. Indeed, PI4KIIα is known to localize to synaptic vesicles and PI4KIIα deficient mice exhibit late onset neurodegeneration (Guo et al., 2003; Simons et al., 2009). My preliminary analysis indicates that PI4KII mutant flies exhibit slower geotactic movement after being banged down to the bottom of a vial, suggesting a potential neurological defect. A more thorough analysis of neuronal function using electrophysiology might reveal a role for PI4KII in the Drosophila nervous system.

3.5.5 Genetic redundancy between PI4KII and fwd

Both PI4KII and Fwd localize to the Golgi, albeit to partially non-overlapping compartments. This is consistent with the observation that mammalian PI4KIIβ localizes predominantly to early Golgi compartments, whereas PI4KIIα is found at the TGN (Weixel et al., 2005). Indeed, PI4KII and Fwd carry out specialized roles. PI4KII is required for glue granule formation, whereas fwd mutants have normal granule morphology. Fwd is essential for male germ cell cytokinesis, and is required to recruit Rab11 to Golgi membranes and vesicles during cytokinesis (Brill et al., 2000; Polevoy et al., 2009), whereas PI4KII is dispensable for
male germ cell cytokinesis and has a role much later in sperm development (Appendix 2). Strikingly, despite these differences in function, simultaneous depletion of PI4KII and Fwd results in redistribution of the PI4P reporter YFP-PH-FAPP from Golgi membranes to the cytoplasm in S2 cells, similar to what was observed in COS-7 cells (Balla et al., 2005). Thus PI4KII and Fwd are partially redundant for synthesizing PI4P at the Golgi, an idea further supported by the observation that PI4KII and fwd single mutants are fully viable, whereas the double mutant is inviable. Indeed, fwd PI4KII double mutants exhibit decreased recruitment of AP-1 to the TGN. Lethality associated with PI4KII and fwd supports the observation that PI4P plays a crucial role in Golgi morphology and function (Hama et al., 1999; Dippold et al., 2009). However, unlike yeast, where deletion of the essential gene PIK1 (PI4KIIIβ) inhibits secretion and LSB6 (PI4KII) cannot compensate for this function, even when overexpressed (Walch-Solimena and Novick, 1999; Han et al., 2002), PI4KII appears to have a more prominent role in metazoans.
Chapter 4

4 Discussion and Future Directions

4.1 Summary of results

The results presented in this thesis significantly advance our understanding of the biogenesis of secretory granules, and establish the Drosophila larval salivary gland as a model for studying granule biogenesis. Moreover, I present the first analysis of a null mutation in the Drosophila lipid kinase PI4KII. In particular, I show that clathrin and the clathrin adaptor AP-1 have conserved, essential roles in formation of secretory granules (Chapter 2); that PI4KII has roles in both glue granule and pigment granule biogenesis (Chapter 3 and Appendix 1); and that PI4KII plays an essential role in spermatogenesis (Appendix 2). My observations can be summarized as follows:

1. **Clathrin and AP-1 are required for glue granule formation.** Prior to glue production, AP-1 and clathrin localize predominantly to the TGN, and recruitment of clathrin to the TGN is AP-1 dependent. At the onset of glue granule formation, both clathrin and AP-1 colocalize with glue protein at the TGN. At later time points, clathrin and AP-1 localize to the limiting membrane of glue granules. Null mutations in AP-1, or depletion of clathrin heavy chain, profoundly block granule biogenesis. Mutant or depleted cells either completely lack glue granules, or exhibit small cytoplasmic glue-containing vesicles. These data point to a crucial role for clathrin and AP-1 in the formation of mucin-containing granules, and suggest that these proteins may also play roles at a later stage in glue granule maturation (Chapter 2).

2. **PI4KII is required for formation of glue granules of normal size.** In larval salivary gland cells, type II phosphatidylinositol 4-kinase localizes to the TGN and to highly dynamic, tubular late endosomes. At the onset of glue formation, PI4KII colocalizes with the glue protein Sgs3 at the TGN. Null mutations in PI4KII result in accumulation of small glue granules. PI4KII
is dispensable for recruitment of AP-1 or EpsinR to the Golgi. However, *PI4KII* mutants aberrantly accumulate glue protein in enlarged late endosomes and mislocalize the granule associated SNARE SNAP-24. These data demonstrate an important role for PI4KII by ensuring proper sorting of both luminal secretory cargo and SNARE proteins during glue granule biogenesis (Chapter 3).

3. **PI4KII is required during formation of pigment granules.** Null mutations in *PI4KII* mildly affect formation of eye pigment granules, which are lysosome-related organelles. Surprisingly, rescue of this mild defect does not require catalytic activity of PI4KII. A hypomorphic mutation in the Sac1 phosphatase, which dephosphorylates PI4P, strongly disrupts pigment granule formation. Mutations in PI4KII suppress the Sac1 phenotype, restoring pigment granule formation back to near wild type levels. These genetic interactions indicate that levels of PI4P must be precisely regulated to ensure proper pigment granule formation and that PI4KII has an additional non-catalytic role in LRO biogenesis (Appendix 1).

4. **PI4KII is required for male fertility.** In wild-type testes, sperm nuclei remain tightly associated at one end of each group of clonally-related spermatids during elongation, individualization and coiling. In *PI4KII* mutants, spermatid nuclei become scattered along the length of the cyst as the spermatids begin to coil. These abnormal cysts accumulate at the base of the testes where they degenerate. This suggests that PI4KII might be required to anchor sperm cells to the somatic cyst cells that envelop spermatids throughout their development (Appendix 2).

While these studies add to our knowledge of granule biogenesis, they also raise important questions relating to the function of PI4KII and AP-1 during glue granule formation that could be addressed in future research. Possible strategies to approach these questions, as well as implications for the field of secretory granule biogenesis are considered below.
4.2 Role of AP-1 and clathrin in granule biogenesis

4.2.1 Clarifying the localization of coat proteins during glue granule biogenesis

Defining the precise localization of clathrin and AP-1 during granule biogenesis has proved crucial in defining their roles in this process (Dittie et al., 1996; Klumperman et al., 1998; Lui-Roberts et al., 2005). For example, the presence of clathrin-coated structures on forming VWF tubules at the TGN suggested a role for coat proteins in formation of these unique granules. Additionally, the presence of coat proteins on ISGs, but not MSGs, suggested a role for coat proteins in refining granule contents. In Chapter 2, I present evidence that fluorescently tagged clathrin and AP-1 colocalize with glue protein at the TGN, as well as on early and late stage granules. Two predictions follow from these observations. The first is that coat proteins might participate in formation of glue granules at the TGN. However, since confocal microscopy cannot distinguish objects less than 200 nm apart, it is unclear if coat proteins are present at the TGN or found on nearby post-Golgi glue containing vesicles or nascent granules. The second is that the coat proteins might be required to refine the contents of both early and late glue granules. This would be consistent with the known role of coat proteins in removing cargo from maturing granules. Indeed, the content of glue granules may continue to be modified until secretion is triggered by ecdysone. To better understand how coat proteins function during glue granule biogenesis, their precise localization needs to be more clearly defined at the ultrastructural level.

To determine if clathrin coated structures are required for granule formation at the TGN, immuno-EM could be used to compare the localization of tagged clathrin or AP-1 relative to tagged Sgs3 containing vesicles. Alternatively, new techniques for preparing EM samples such as high-pressure freezing allow for remarkable preservation of both secreted cargo and clathrin-coated structures (Zenner et al., 2007). Indeed, this technique was used to demonstrate that VWF is packaged into CCVs at the TGN. To test whether coat proteins localize to the limiting membrane of maturing granules, immuno-EM could be used to compare the localization of clathrin/AP-1 to that of the granule specific SNARE SNAP-24. In particular, localization of coat
proteins to bud sites on granules would be a strong indicator that they might participate in vesicle budding (Klumperman et al., 1998).

4.2.2  Biochemical approach to defining granule coat composition

A complementary strategy to define the coat composition of glue granules would be to use a biochemical approach. Late-third instar larval salivary glands are relatively large organs that are easy to dissect, making it feasible to collect significant quantities of tissue. Additionally, clathrin heavy and light chains, as well as subunits of the AP-1 complex are highly expressed in this tissue (FlyAtlas) (Chintapalli et al., 2007). To demonstrate that glue proteins pass through a clathrin coated vesicle compartment, CCVs could be purified from salivary glands and the presence of tagged glue protein analyzed by immunoblotting. This approach was recently used in *Drosophila* S2 cells to demonstrate that lysosomal enzyme receptor protein (LERP) is enriched in CCVs (Hirst et al., 2009). It might also be possible to directly purify glue granules using sucrose gradient centrifugation. Glue granule fractions could then be biochemically distinguished from Golgi and endosomal fractions by enrichment of the granule specific marker SNAP-24. The presence of coat proteins could then be examined by immunoblotting. In PC12 cells, immature granules, which are relatively small, can be further separated from larger mature granules using a combination of velocity and equilibrium sucrose gradient centrifugation (Dittie et al., 1996). This approach was used to demonstrate that clathrin and AP-1, as well as specific cargo proteins, could be detected only on ISGs. This strategy could be applied to glue granules to more precisely define the coat composition during various stages of maturation. Finally, a highly purified granule fraction could be analyzed by mass spectrometry to identify novel glue granule components. Indeed, proteomic analysis of insulin secretory granules has helped give a more complete picture of the machinery required for biogenesis and trafficking of this secretory granule (Brunner et al., 2007). For instance, the association of Rab37 and VAMP8 with ISGs was shown for the first time. Additionally, this approach identified numerous lysosomal hydrolases in ISGs, providing support for the sorting by retention hypothesis.
4.2.3 Identifying AP-1 cargo and accessory factors required for granule formation

Identification of AP-1 cargo proteins, as well as accessory factors, would provide mechanistic insight into how this adaptor protein complex participates in granule biogenesis. The AP-1 complex can sort cargo by binding to canonical tyrosine and dileucine sorting motifs (Bonifacino and Traub, 2003). In addition, the gamma-adaptin ear domain of AP-1γ can bind other adaptor proteins such as EpsinR and GGA, as well as accessory factors required for CCV formation (Hirst et al., 2003; Bai et al., 2004). The identification of a core tetrapeptide motif found in accessory proteins has been used to identify novel AP-1 accessory factors such as aftiphilin (Mattera et al., 2004). Aftiphilin forms a complex containing p200 and gamma-synergin, and depletion of individual components resembles loss of AP-1 (Hirst et al., 2005). Importantly, depletion of aftiphilin and gamma-synergin revealed these proteins to be dispensible for WPB formation, but necessary for WPBs to acquire the property of regulated secretion (Lui-Roberts et al., 2008). These results raise the intriguing possibility that AP-1 accessory proteins might coordinate recruitment of proteins required for distinct aspects of granule biogenesis. Although there are no clear homologs of aftiphilin and gamma-synergin in Drosophila, a computational search could be used to identify proteins containing accessory protein motifs. Similarly, cargo proteins containing dileucine or tyrosine sorting motifs could be identified computationally. This search could be further refined using FlyAtlas to identify proteins that are abundantly expressed in salivary glands. Candidate AP-1 cargo proteins, as well as accessory proteins, could then be rapidly tested using tissue-specific RNAi. Proteins required for glue granule biogenesis could then be characterized in more depth by performing colocalization and physical interaction studies with AP-1.

4.3 Analysis of the role of PI4KII in granule biogenesis

4.3.1 Does PI4KII regulate granule biogenesis by producing PI4P?

In Chapter 3, I present evidence that loss of PI4KII results in small glue granules. The most straightforward interpretation of this phenotype is that immature secretory glue granules
fail to undergo homotypic fusion. Thus, the primary cause of this PI4KII mutant phenotype could be a defect in sorting of proteins required for fusion. Indeed, distribution of the granule specific SNARE SNAP-24 is altered in PI4KII mutant salivary gland cells. However, it is as yet unclear whether SNAP-24 participates in homotypic fusion. Potentially, loss of PI4KII could result in a failure to recruit a crucial PI4P binding protein required for SNARE sorting. For example, the clathrin adaptors EpsinR and AP-1 bind directly to SNAREs (Peden et al., 2001; Hinners et al., 2003; Miller et al., 2007; Chidambaram et al., 2008). Alternatively, PI4KII itself could bind and directly recruit a crucial factor required for granule fusion. In support of this idea, mammalian PI4KIIα associates with the SNAREs VAMP3 and VAMP7 (G. Polevoy, M.J. Kean, J.A. Brill and A.C. Gingras; unpublished data). The identification of VAMP7 as a PI4KIIα associated protein is particularly intriguing, as it is implicated in granule exocytosis (Logan et al., 2006). In summary, PI4KII might regulate the sorting of SNAREs either indirectly, by recruiting PI4P-binding proteins, or through a direct physical interaction.

To determine if the catalytic activity of PI4KII is required for granule biogenesis, I generated transgenic lines that express wild-type and kinase-dead versions of PI4KII in a PI4KIIΔ mutant background (Chapter 3). If PI4KII activity is required for granule biogenesis, then the role of known PI4P binding proteins could be systematically tested. However, if the kinase dead isoform can rescue the granule phenotype, this would imply that PI4KII has a non-catalytic role in binding one or more additional proteins involved in glue granule biogenesis. In this scenario, a proteomic screen to identify novel PI4KII interacting proteins might prove more fruitful. For example, affinity purification coupled with mass spectrometry could be used to identify fly-specific PI4KII interactors (Gingras et al., 2007). PI4KII binding partners could then be verified by co-immunoprecipitation, and a role for interactors in granule maturation could be tested in vivo using tissue specific RNAi.

4.3.2 Role of endocytosis in granule formation

PI4KII localizes to an extensive and highly dynamic tubular endosomal system in salivary gland cells. Intriguingly, the emergence of this tubular endosomal system coincides with initiation of glue granule biogenesis. Based on numerous studies, tubules emanating from endosomes are believed to represent sites where cargo can be sorted for exit from the endosomal
compartment (Bonifacino and Rojas, 2006). Tubular endosomal networks typically consist of multiple domains, with specific sorting complexes localizing to distinct sites in the network. These complexes function to sort cargo to different locations in the cell. For example, the retromer complex transports M6PRs from endosomes to the TGN, whereas the AP-3 complex transports the lysosomal membrane glycoprotein LAMP-1 from endosomes to lysosomes (Arighi et al., 2004; Peden et al., 2004). In Drosophila salivary cells, it is currently unclear if the tubular endocytic pathway is linked to the secretory pathway during glue granule biogenesis. However, evidence that endosomal sorting complexes can direct cargo to the TGN, as well as to specialized post-Golgi organelles, suggests that endosomal trafficking might impinge on secretory granule biogenesis in several ways.

AP-3 and the BLOC complex

The AP-3 complex, biogenesis of lysosome-related organelles (BLOC-1, -2 and -3) complexes and homotypic fusion and vacuole protein (HOPS) complex mainly localize to tubular endosomes (Oh et al., 2000; Theos et al., 2005; Di Pietro et al., 2006). These complexes mediate transport of cargo to lysosomes and to specialized lysosome-related organelles (LROs) such as melanosomes and platelet dense granules. Mutations in subunits of AP-3, BLOC1-3 and HOPS give rise to Hermansky-Pudlak Syndrome, a genetic disease associated with albinism and excessive bleeding.

These complexes are required not only for formation of specialized organelles, but also have more general roles in endocytic trafficking (Wilkin et al., 2008). AP-3 and BLOC-1 regulate sorting of SNARE proteins, and deficiencies in either of these complexes lead to a selective reduction in both vesicular and total VAMP7 levels (Salazar et al., 2006). In the case of AP-3, binding was shown to occur between the AP-3δ subunit and the amino terminal longin domain of VAMP7 (Martinez-Arca et al., 2003). Strikingly, this interaction is conserved in Drosophila, as a yeast two-hybrid screen using VAMP7 as bait isolated the delta subunit of AP-3, as well syntaxin 1 and SNAP-29. Similarly the BLOC-1 complex subunits pallidin and snapin can interact with syntaxin 13 and SNAP23/25, respectively (Huang et al., 1999; Ilardi et al., 1999; Buxton et al., 2003). Since AP-3 and BLOC-1 can bind to many of the SNAREs implicated in granule fusion or exocytosis, disruption of these complexes could directly or
indirectly affect the sorting of SNAREs to granule membranes. Although AP-3 is not required for glue granule formation, I did not test for a role of this complex in granule secretion. Indeed, if AP-3 is required for granule function, it is likely to be in trafficking cargo to mature granules.

Classical regulated secretory granules are believed to be distinct from LROs. RSP cargo is transported directly from the TGN to ISGs, whereas LRO cargo is usually transported to endosomes and then sorted into LROs. However, there are several examples in which secretory granule content is derived from both the TGN and endosomes. For instance, AP-3 is required to transport the tetraspanin CD63 from endosomes to prebudded WPBs (Kobayashi et al., 2000; Harrison-Lavoie et al., 2006). CD63 is ubiquitously expressed and commonly used as a late endosomal marker (Rous et al., 2002). Thus, although WPBs form at the TGN like classical secretory granules, addition of the endosomal marker CD63 after granule formation also confers LRO-like properties to these granules (Metcalf et al., 2008). This reveals that machinery required to transport cargo from tubular endosomes to LROs can also target cargo to regulated secretory granules. Since mutations affecting AP-3, BLOC-1, -2 and -3, as well as HOPS complex subunits have been identified in Drosophila, these mutants could be used to test whether trafficking from endosomes to glue granules is important during granule biogenesis.

The retromer complex

Retromer is a pentameric complex that transports cargo from tubular endosomes to the TGN (Bonifacino and Hurley, 2008). The retromer complex is required for retrieval of the cation-independent M6PR from endosomes to the TGN. Additionally, retromer is required for retrieval of the endosomal SNARE Pep12p to the Golgi (Hettema et al., 2003). Pep12p is equally homologous to the mammalian endosomal SNAREs syntaxin 7 and 12. Interestingly, Pep12p appears to cycle continuously between Golgi and endosomal compartments. It is possible that SNAREs such as syntaxin 6, which are implicated in the homotypic fusion of both ISGs and endosomes, might undergo retrograde trafficking to the TGN. Mutations that disrupt recycling of SNAREs to the TGN would likely decrease their availability for incorporation into forming granules. Additionally, RSP processing enzymes might also need to be recycled back to the TGN for future rounds of granule formation. Since processing of RSP cargo is crucial for proper granule maturation, failure to incorporate processing enzymes would profoundly affect granule
biogenesis. The retromer complex is conserved in *Drosophila*, and is required to recycle the Wingless receptor Wntless to the TGN (Franch-Marro *et al.*, 2008; Port *et al.*, 2008). Mutant cells lacking retromer could be generated in the larval salivary gland to test for the contribution of retromer in glue granule biogenesis.

4.4 General questions pertaining to glue granule biogenesis

A mechanistic understanding of granule biogenesis could aid in treatment of a variety of human diseases, including relatively common disorders such as cystic fibrosis, asthma and diabetes. In the following section, I will explore how the *Drosophila* larval salivary gland could be used to investigate broader biological questions relating to granule biogenesis. I will also highlight areas in which our knowledge of glue granule formation is lacking. By understanding how glue granule biogenesis resembles (and diverges) from mammalian secretory granule biogenesis, findings in *Drosophila* could be more easily translated to mammalian secretory disorders.

4.4.1 Do glue granules form by an aggregation-based mechanism?

Relatively little is known about how glue granules form in salivary gland cells. In mammalian cells, aggregation of RSP cargo at the TGN in response to low pH and high calcium concentration is a key mechanism in granule formation (Tooze and Stinchcombe, 1992). A related property is the ability of certain RSPs to induce formation of dense core granules when ectopically expressed in cells lacking regulated secretion. A relatively small number of glue-encoding genes have been cloned (<10), and these encode major constituents of the glue mix. Thus, it should be feasible to systematically address the contribution of each glue protein to granule formation. To test if individual glue proteins can drive granule formation, tagged proteins could be expressed in *Drosophila* Schneider (S2) cells, which lack regulated secretion. The localization of glue proteins could then be examined using confocal microscopy. The appearance of granule-like structures could be assessed by demonstrating lack of colocalization
with well-established markers for the Golgi, endosomes, and lysosomes. Additionally, S2 cells could be used to purify large quantities of Sgs proteins to test if these proteins aggregate at mildly acidic pH and high calcium concentration, properties commonly exhibited by mammalian RSPs.

Proteins that drive aggregation can bind to other RSPs allowing them to be incorporated into forming granules. For example, in endocrine cells, granins are known to promote incorporation of other RSPs into secretory granules. Individual glue proteins could be depleted using tissue specific RNAi, and the effect on granule formation could be assessed morphologically, using either confocal or electron microscopy. Similarly, glue proteins might be required for the sorting or stability of other RSPs in salivary gland cells. To test this possibility, individual Sgs proteins could be depleted and the effect on the remaining O-glycosylated glue proteins quantified by gel electrophoresis and blotting, using antibodies or lectins that recognize O-glycosylated epitopes (Beckendorf and Kafatos, 1976; Korge, 1977). Individual cargo proteins that robustly disrupt granule formation when depleted might function as aggregation driving proteins. If aggregation-driven granule formation occurs in *Drosophila*, it would highlight the similarity to mammalian granules and further underline the relevance of this system for studying granule formation. Alternatively, these studies could reveal that glue granules form by a novel mechanism. Since glue granules are similar to mucin-containing granules, the general relevance of such a mechanism could be tested in human epithelial cell culture models of mucin granule formation.

4.4.2 Are glue proteins packaged together or sequentially?

Morphological analysis of forming glue granules indicates that some glycoproteins are transported separately to preformed granules (Thomopoulos and Kastritis, 1979). This observation raises several interesting questions relating to the temporal and/or spatial order of glue protein production. For instance, some glue proteins might be produced for only a brief period during glue granule biogenesis. Alternatively, some glue proteins might be coexpressed, but physically segregated at the TGN into distinct ISGs and then later mixed by homotypic fusion. A last possibility is that distinct Golgi bodies might produce separate glue proteins. In
support of this, Golgi bodies isolated from *Drosophila* imaginal disc cells were shown to be functionally diverse and to possess distinct sets of glycosylation enzymes (Yano *et al.*, 2005).

To assess whether glue proteins are either temporally or spatially segregated, transgenic larvae expressing fluorescently tagged glue proteins could be generated. Fluorescently tagged Sgs3, under the control of its endogenous promoter, has proved to be an indispensable tool for monitoring the localization of this glue protein (Biyasheva *et al.*, 2001; this thesis). Using a similar approach, the remaining Sgs proteins could be tagged with distinct fluorescent proteins to visualize multiple glue proteins at various stages of granule biogenesis (Shaner *et al.*, 2005). The distribution of glue proteins could be compared at early time points when they localize to Golgi bodies, as well as later in development when localization to ISGs becomes prominent. Glue proteins that exhibit colocalization only in mature granules would provide strong evidence for granule growth via homotypic fusion.

To identify SNAREs required for homotypic fusion, it would be useful to develop a cell-free assay to measure fusion between distinctly labeled granules (Urbe *et al.*, 1998). For example, a transgenic line could be generated with a glue protein tagged with a genetically encoded enzyme substrate, and a separate line encoding a glue protein tagged with an enzyme that specifically cleaves this substrate. ISGs could then be purified from the two transgenic lines and homotypic fusion of the ISG populations measured as the amount of product generated from the enzyme-substrate reaction. Using this *in vitro* approach, granule fusion could then be quantified in the presence of inhibitors or antibodies directed towards specific SNAREs. In summary, transgenic lines expressing uniquely tagged cargo proteins could be used to study secretory protein synthesis, transport and mixing.

### 4.4.3 Sorting for entry versus sorting by retention

The precise cellular location at which constitutive secretory proteins are segregated from regulated secretory proteins is currently unclear (Arvan and Castle, 1998; Dikeakos and Reudelhuber, 2007). Proponents of sorting for entry argue that cargo is sorted at the TGN, whereas those in favor of sorting by retention posit that cells lack an active mechanism to block entry of non-specific cargo into forming secretory granules. *Drosophila* is an excellent system to
explore this question, as a range of fluorescently tagged secretory proteins can be analyzed and expression levels can be ‘tuned’ using the Gal4/UAS system (Brand and Perrimon, 1993; Elliott and Brand, 2008). This is particularly useful when determining the ability of a protein to enter secretory granules, as aggregation induced by overexpression can lead to inappropriate sorting into forming granules (Tooze et al., 2001).

To test whether constitutively secreted luminal proteins can enter ISGs, the localization of secreted GFP (sGFP) could be compared to the granule marker Sgs3-DsRed (Pfeiffer et al., 2000). sGFP contains GFP fused to the signal peptide from the secreted protein Wingless (Wg) and localizes to the lumen of secretory vesicles. If sGFP accumulates in the lumen of secretory granules, this would indicate that entry into forming granules is not restricted. To determine if constitutively trafficked transmembrane proteins pass through ISGs, localization of mCD8-GFP could be analyzed (Lee and Luo, 1999). mCD8-GFP contains the murine lymphocyte receptor mCD8 fused to GFP. The chimeric mCD8-GFP protein is known to localize to the apical membrane of polarized epithelial salivary gland cells, although its localization relative to glue granules has not been described (Xu et al., 2002; Wendler et al., 2010). If this protein accumulates on granule membranes, this would indicate that membrane domains are not restricted during granule formation. From my initial observations, the lysosomal transmembrane protein GFP-LAMP does not localize to secretory granules at any stage, indicating that at least this particular transmembrane protein is segregated from regulated secretory cargo at the TGN.

An important prediction of the sorting by retention hypothesis is that additional protein sorting can occur in immature secretory granules, particularly the removal of non-RSP cargo. The identification of a cargo protein that is present in early glue granules, but absent at later stages would provide strong evidence for sorting by retention in Drosophila salivary cells. Lysosomal hydrolases, as well as M6PRs, are known to transiently enter immature secretory granules in a range of cell types (Kuliawat et al., 1997; Klumperman et al., 1998). M6PRs are selectively removed from ISGs by a coat consisting of clathrin and the adaptor proteins AP-1 or GGA (Klumperman et al., 1998; Dittie et al., 1999; Kakhlon et al., 2006). Drosophila contains a functional homolog to the cation-independent M6PR, termed lysosomal enzyme receptor protein (LERP) (Dennes et al., 2005). LERP can rescue the missorting of lysosomal hydrolases in mammalian cells lacking M6PRs, suggesting LERP functions like mammalian M6PRs. GGA and AP-1 are required for sorting of LERP in Drosophila S2 cells, similar to the role these
clathrin adaptors play in sorting M6PRs in mammalian cells (Hirst et al., 2009; Kametaka et al., 2010). In the case of GGA, binding is mediated by a dileucine signal found in the cytoplasmic domain of LERP (Kametaka et al., 2010). Consequently, the similarity of the LERP receptor, as well as of AP-1 and GGA sorting machinery, indicates that LERP could be used to test for sorting by retention during Drosophila glue granule biogenesis. GFP-LERP, which is efficiently incorporated into CCVs (Hirst et al., 2009), could be expressed in salivary cells, and its localization could then be compared to the granule specific marker Sgs3-DsRed at various stages of granule maturation. If GFP-LERP localizes to the membrane of ISGs, but is depleted from larger, more mature granules, this would provide compelling evidence that sorting by retention occurs in Drosophila. Subsequently, GFP-LERP could be analyzed in PI4KII and AP-1 mutants to determine if sorting by retention is disrupted.

4.4.4 Identifying machinery required for homotypic fusion and exocytosis

The formation of SNARE complexes is a key step in fusion of vesicles with target membranes. A SNARE complex consists of four alpha helices, which are contributed by both vesicle and target membrane localized SNARE proteins. Analysis of the SNARE superfamily in Drosophila reveals 11 syntaxin genes, three SNAP genes and three synaptobrevin/VAMP related genes (Lloyd et al., 2000). Several of these SNAREs exhibit homology to SNARE proteins implicated in homotypic fusion or exocytosis of granules in mammalian cells.

SNAREs required for homotypic fusion

The SNARE protein syntaxin 6 has been implicated in homotypic fusion of ISGs in PC12 cells (Wendler et al., 2001). Interestingly, syntaxin 6 is also implicated in the homotypic fusion of early endosomes, where it forms a complex with syntaxin 13, vti1a and VAMP4 (Brandhorst et al., 2006). Indeed, it is thought that syntaxin 6 only transiently passes through immature secretory granules and is ultimately delivered to the endo-/lysosomal system (Klumperman et al., 1998). Since PI4KII mutants exhibit small granules and large endosomes, missorting of syntaxin 6 might underlie both phenotypes. Drosophila encodes homologs of each SNARE, including syntaxin 6 (CG7736), syntaxin 13 (CG11278) and vti1 (CG3279), as well as three possible
VAMP4 homologs. Gene expression arrays indicate that all of these components are expressed in late third instar salivary glands (Chintapalli et al., 2007). To test whether this SNARE complex is required for homotypic fusion of glue granules, individual SNARE proteins could be depleted using tissue specific RNAi. Any effects on homotypic fusion could then be assessed in late third instar larvae, a time point at which mature glue granules reach an average diameter of 3 µm in wild type animals.

**SNAREs required for exocytosis**

_Drosophila_ SNAP-24, which shows high sequence similarity to mammalian SNAP-25 and SNAP-23, localizes to glue granule membranes (Niemeyer and Schwarz, 2000). SNAP-24 can form a complex with syntaxin 1 and synaptobrevin _in vitro_. Since syntaxin 1 is concentrated on the apical membrane of salivary gland cells, it might act as a cognate SNARE for SNAP-24 during exocytosis. Intriguingly, both mammalian SNAP-25 and SNAP-23 belong to SNARE complexes implicated in regulated exocytosis in mammalian cells. For example, a SNARE complex containing SNAP-25, syntaxin 1 and VAMP1/2 mediates exocytosis of chromaffin granules in chromaffin cells and secretory granules in PC12 cells (Banerjee et al., 1996; Glenn and Burgoyne, 1996). There is also evidence that a portion of SNAP-25 is found on chromaffin granule membranes, similar to the localization of _Drosophila_ SNAP-24 on glue granule membranes (Tagaya et al., 1996). SNAP-23 can form a SNARE complex with syntaxin 4 and VAMP7, and is required for compound exocytosis in mast cells (Guo et al., 1998; Sander et al., 2008). Interestingly, SNAP-23 translocates from the plasma membrane to mast cell secretory granules in response to stimulation. The SNAP-23/syntaxin 4/VAMP7 SNARE complex has also been implicated in regulated exocytosis of lysosomes in NRK cells (Rao et al., 2004). Direct experimental testing will be necessary to determine which candidate SNARE complexes are required for glue granule exocytosis.

Finally, the SNARE accessory protein NSF has been implicated in immature secretory granule homotypic fusion (Urbe et al., 1998). NSF is a hexameric ATPase that is believed to function in disassembly of the four-alpha-helix SNARE bundle. There are two NSF genes in _Drosophila_ encoded by NSF-1/comatose and NSF-2 (Ordway et al., 1994; Boulianne and
Trimble, 1995). NSF-1 is abundantly enriched in neuronal tissue, whereas NSF-2 is more broadly expressed and enriched in larval salivary glands (FlyAtlas; Chintapalli et al., 2007). Of particular note is the observation that NSF-2 is expressed sporadically in salivary gland cells, suggesting it might play a stage specific role during granule maturation (Boulianne and Trimble, 1995).

4.4.5 Assessing glue granule secretion

The primary function of the regulated secretory pathway is to secrete proteins into the extracellular space. In the case of Drosophila salivary cells, glue protein is secreted apically into the salivary gland lumen in response to a high titre pulse of ecdysone. After secretion, the glue mix is then expectorated, allowing pupae to stick to a solid substrate. Defective secretion might result in pupae that are easily washed into the soil, or food source, preventing the adult flies from eclosing from their pupal cases. At the molecular level, defective secretion might indicate that granules lack the fusogenic machinery necessary for exocytosis.

A bioassay could be easily developed to measure the ability of pupae to stick to a solid substrate, and this could be used as readout of how efficiently glue proteins are being secreted. For example, mutant larvae could be transferred to a Petri dish with a small source of food and a lid. Larvae will typically migrate away from the food source to the top of the lid to begin pupariation. A jet of pressurized air could then be applied to the pupae to test how adherent it is. This assay would be particularly useful to test whether the formation of abnormally small granules in PI4KII mutants results in a physiologically relevant phenotype.

A complementary approach would be to monitor granule secretion by confocal microscopy using the granule marker Sgs3-DsRed. Salivary glands could be dissected from early white pupae, a stage when most glue protein has been secreted into the lumen, but not yet expectorated. In secretion defective mutants, numerous glue granules should still be visible in the cytoplasm of salivary gland cells. Alternatively, salivary glands from late third instar larvae could be dissected and incubated in a high-titre ecdysone solution to trigger secretion. Granule secretion could then be monitored live using a spinning disc confocal.
A genetic screen to identify \textit{PI4KII} interacting genes

The high degree of conservation in several core components of the Golgi sorting machinery demonstrates that \textit{Drosophila} is particularly well suited for investigation of the secretory pathway. Genome-wide screens performed in \textit{Drosophila} have uncovered novel components of the metazoan secretory pathway (Wendler et al., 2010). More recently, several studies have highlighted a conserved function for \textit{Drosophila} homologs of the Golgi-localized clathrin adaptors AP-1, GGA and EpsinR in post-Golgi trafficking (Hirst et al., 2009; Lee et al., 2009; Kametaka et al., 2010). Moreover, \textit{Drosophila} Golgi shares numerous morphological and functional similarities with mammalian Golgi (Kondylis and Rabouille, 2009). Hence, \textit{Drosophila} provides an ideal system for identifying and studying conserved aspects of secretory trafficking.

Mutations in \textit{PI4KII} result in small glue granules and enlarged late endosomes that accumulate glue protein. It is unclear whether these granule and endosome phenotypes are linked. To further understand how PI4KII regulates glue granule biogenesis, a suppressor/enhancer screen could be performed in larval salivary gland. Genetic interactors would provide information about how PI4KII regulates granule formation, and possibly allow the granule and endosomal phenotypes to be dissected. In addition, such a screen would provide a rich source of new genes involved in granule biogenesis. Briefly, a stock carrying a PI4KII RNAi transgene, as well as Sgs3-DsRed to monitor glue granule biogenesis and YFP-Rab7 to assess late endosomes, could be crossed to a collection of stocks containing defined chromosomal deletions (Ryder et al., 2004). Salivary glands of appropriate progeny could be tested for enhancement or suppression of the PI4KII small granule phenotype. This screen could identify several different classes of mutants. For instance, mutations that suppress/enhance only the small granule phenotype, or mutations that suppress/enhance only the large LE phenotype, would identify genes specifically involved in one of these processes. Such a result would indicate that the granule and endosomal mutant phenotypes are not connected. The identity of these genes would provide useful information about how PI4KII mediates its effects in these two compartments. Alternatively, mutations that suppress/enhance both phenotypes likely participate in both granule and endosome function. If only mutations in this class are identified, this would indicate that the small granule and LE phenotypes are linked.
4.4.7 Summary Model

Glue granule biogenesis is likely to involve multiple trafficking steps utilizing several types of transport complexes (Fig. 20a). It is currently unclear which of these pathways operate during glue granule biogenesis, and which processes are regulated by PI4KII and AP-1. Careful analysis of mutants that disrupt specific transport steps could be used to resolve this issue. My analysis indicates that PI4KII acts redundantly with Fwd at the TGN to recruit AP-1, clathrin and possibly EpsinR. PI4KII, AP-1 and EpsinR are required for granule biogenesis. AP-1 and EpsinR also function in transport of cargo between the TGN and endosomes, and possibly in retrieval of missorted cargo from ISGs. PI4KII is required at late endosomes to transport cargo to lysosomes, or for recycling back to the TGN (Fig. 20b). Further studies are required to determine the precise role, if any, for retromer in recycling proteins to the TGN.
Figure 20 – Possible trafficking steps in granule biogenesis.

Model depicting putative trafficking steps required for glue granule biogenesis. Note that endosomes might act as a convergence point for cargo received from several sites in the cell, including the Golgi, ISGs and the cell surface (not shown). Only step 1 has been confirmed in glue granule biogenesis. (a) In wild-type cells, PI4KII acts redundantly with Fwd at the TGN to produce PI4P and recruit AP-1, clathrin and possibly EpsinR (1). ISGs fuse to form MSGs (2). Non-RSP cargo might be removed from ISGs and transported to LEs via AP-1 and clathrin (3). PI4KII is required at LEs to transport cargo to lysosomes (4) or to recycle cargo and/or membrane back to the TGN (5). Retromer complex might be required to recycle cargo to the TGN (6 and 5). AP-1 and EpsinR are required to transport cargo to endosomes (7). (b) In PI4KII mutants, Fwd generates PI4P and recruits AP-1 and clathrin to form small granules (1), but these granules do not fuse (2). Cargo transported to LEs from ISGs (3), early endosomes (6) or the TGN (7) is not recycled to the TGN (5), but rather accumulates in LEs. Lysosomal trafficking may also be affected (4).
Figure 20

A

Golgi

Immature Granules

Mature Granules

1

AP-1, clathrin

EpsinR?

P14KII

Fwd

2

AP-1 clathrin

Rab5

Early Endosome

Glue cargo

Retromer?

P14KII

Rab7

Late Endosome

MPR

Lysosome

wild type

B

Golgi

Small Granules

1

AP-1, clathrin

Glue cargo

Rab7

Late Endosome

2

AP-1 clathrin

Rab5

Early Endosome

Glue cargo

Retromer?

P14KII mutant

4

Lysosome
Appendix 1: PI4KII is required for pigment granule biogenesis

Introduction

A unique reciprocal interaction between PI4KII and the endosomal adaptor complex AP-3 has been described in mammalian cells. PI4KII contains a canonical dileucine-sorting motif in its N-terminal domain that is necessary for binding and colocalizing with the AP-3 complex (Craigie et al., 2008). Since PI4KII associates integrally with the membrane due to palmitoylation (Barylko et al., 2001), PI4KII can be thought of as an AP-3 specific membrane cargo protein. Consistent with this view, PI4KII redistributes from endosomes to a perinuclear compartment in AP-3 deficient mocha mutant mice (Salazar et al., 2005). Interestingly, depletion of PI4KII also disrupts the localization of AP-3, causing AP-3 to redistribute from endosomes to the cytoplasm (Craigie et al., 2008). Furthermore, PI4KII depletion results in mis-sorting of AP-3 specific cargo and gives rise to enlarged late endosomes. As PI4KII kinase activity is required to rescue this late endosomal sorting phenotype, this has led to a model in which (1) PI4KII is recruited to AP-3 containing membranes via a dileucine-sorting motif, and (2) PI4KII catalytic activity is further required to maintain AP-3 on membranes. Since AP-3 plays a crucial role in formation of lysosome-related organelles (LROs), PI4KIIα is also likely to be involved in this process via regulation of AP-3. However, such a role for PI4KIIα has never previously been demonstrated.

In Drosophila, AP-3 is required for biogenesis of eye pigment granules, a commonly used invertebrate model for LRO formation (Ooi et al., 1997; Lloyd et al., 1999). Mutations in genes encoding subunits of AP-3 result in fruit flies with decreased eye pigmentation (Mullins et al., 1999; Kretzschmar et al., 2000; Mullins et al., 2000). Additionally, several other complexes required for pigment granule formation are conserved in Drosophila, including HOPS, BLOC-1, -2 and -3 (Sevrioukov et al., 1999; Falcon-Perez et al., 2007; Cheli et al., 2010). Typically, mutations that affect one of these complexes show strong genetic interactions, including synthetic lethality, with mutations affecting other complexes (Lloyd et al., 1998). Since both the palmitoylation motif and a putative AP-3 dileucine sorting signal (EEAVLL) are conserved in Drosophila PI4KII, this raises the possibility that PI4KII might interact with AP-3 to promote pigment granule biogenesis.
Materials and Methods

The Garnet-GFP transgenic stock is described in Chapter 3. Eye color mutants were obtained as follows: garnet<sup>50e</sup> (g<sup>50e</sup>) (Kyoto Drosophila Stock Center), garnet<sup>l</sup> (g<sup>l</sup>) and deep orange<sup>l</sup> (dor<sup>l</sup>) (Bloomington Drosophila Stock Center). The sac<sup>L4R3</sup> (sac<sup>ts</sup>) allele, which exhibits temperature-sensitive lethality at 25°C, was isolated in a genetic screen for lethal mutations in polytene interval 61D-61F (Wei et al., 2003). sac<sup>L4R3</sup> contains a missense mutation (G382R) in a residue that lies immediately upstream of the conserved phosphatase domain. The PI4KIIΔ mutation is described in Chapter 3. Double mutant stocks were generated using standard genetic crosses.

Results

To determine if PI4KII is required for pigment granule formation, I compared the eye color of PI4KIIΔ mutants to a wild type reference stock (Oregon-R). PI4KIIΔ mutants exhibited a mild eye pigmentation defect, with a less prominent pseudopupil (a dark spot visible on the compound eye when pigments are normal) (Fig. 21a, b). If PI4KII regulates pigmentation by acting through the AP-3 complex, then PI4KII mutations should not enhance strong loss-of-function mutations in AP-3. To test this, I looked for genetic interactions between PI4KII and garnet (g), which encodes the AP-3δ subunit. Interestingly, genetic interactions appeared to be allele specific. For example, PI4KIIΔ did not enhance the g<sup>l</sup> mutant phenotype, but mildly enhanced the stronger g<sup>50e</sup> allele (data not shown; quantified by R. Wilk). This ambiguity might be due to the fact that these garnet alleles are not null (see Discussion).

Mutations in LRO trafficking complex subunits typically show strong genetic interactions with mutations in subunits of other complexes. If PI4KII acts through AP-3, then mutations in PI4KII should give rise to strong genetic interactions with mutations affecting HOPS, BLOC-1, -2 and -3. To test this possibility, I examined genetic interactions between PI4KIIΔ and the HOPS complex gene deep orange (dor<sup>l</sup>). Double mutants exhibited synthetic lethality when raised at 25°C and dramatically decreased pigmentation when raised at 21°C (Fig. 21c, d). This suggests
that PI4KII might participate in pigment granule formation by regulating AP-3. To test whether PI4KII is required for localization of AP-3, I generated transgenic flies expressing either GFP or mCherry tagged Garnet fusion proteins. I confirmed that the Garnet-GFP fusion construct was functional and fully rescues the eye color of garnet mutants (Fig. 21e,f). This transgenic stock should prove useful for testing whether PI4KII regulates pigmentation via AP-3 (see Discussion). Moreover, this is the first example of a functional GFP fusion to an AP-3 subunit, and should prove generally useful for studying AP-3 dynamics and regulation during LRO formation.

To determine if the lipid PI4P is generally required for pigment granule formation, I studied a previously isolated temperature-sensitive allele in the gene coding for the PI 4-phosphatase Sac1 (H.C. Wei; Wei et al., 2003, unpublished data). Mutations in this gene are predicted to increase levels of PI4P, and sac1ts mutants exhibit a robust eye pigmentation defect (Fig. 21g). Remarkably, the PI4KIIΔ mutation fully suppressed the sac1ts mutant phenotype, restoring eye color to that of PI4KIIΔ single mutants (Fig. 21h). This indicates that at least some of the excess PI4P produced in sac1ts mutants is generated by PI4KII (Fig. 21i). Taken together, these results suggest that PI4P levels must be precisely regulated for proper pigment granule formation.

Discussion

PI4KIIα is predicted to regulate LRO formation due to its ability to recruit AP-3 to endosomal membranes in mammalian cells (Craige et al., 2008). I provide the first evidence that PI4KII is required to form pigment granules, which are Drosophila LROs. Based on this observation, human PI4KIIα gene is a candidate susceptibility factor for Hermansky-Pudlak syndrome, a disorder associated with defective LRO function. Preliminary genetic analysis indicates that PI4KII might regulate eye pigmentation via the AP-3 complex. In particular, PI4KII shows no (or mild) genetic interactions with mutations disrupting AP-3, but exhibits robust interactions with a mutation in the HOPS complex. It is curious that PI4KII exhibits allele specific interactions with garnet. This might be due to the fact that g1 and g50e are not null alleles (Lloyd et al., 1999). Alternatively, PI4KII might act through another complex that regulates
pigment granule formation. For example, PI4KII can form a tripartite complex containing both AP-3 and BLOC-1 (Salazar et al., 2009). Systematic testing of genetic interactions between PI4KII and representative mutations in AP-3, HOPS, BLOC-1, -2 and -3 should allow PI4KII to be placed in a genetic pathway.

Since PI4KII can physically bind to AP-3 in mammalian cells, this raises the possibility that PI4KII has a non-catalytic role in eye pigmentation. Remarkably, kinase-dead PI4KII was able to fully rescue the mild eye pigmentation defect of PI4KII mutants (Wilk et al.; unpublished data). This suggests that PI4KII regulates pigmentation through a physical interaction, perhaps by binding to AP-3. It will be interesting to test whether Drosophila PI4KII can bind to AP-3 using co-immunoprecipitation. Additionally, localization of Garnet-GFP could be examined in PI4KII mutants during pigment granule formation. If Garnet localization is altered in PI4KII mutants, the ability of kinase-dead PI4KII to rescue Garnet localization could be tested. Alternatively, if AP-3 localization is not altered in PI4KII mutants, then localization of HOPS, BLOC-1, -2 and -3 complex subunits could be examined to determine which of these conserved LRO trafficking complexes is regulated by PI4KII. Hence, the fly is likely to be a useful model for defining how PI4KII participates in formation of LROs.
Figure 21 - PI4KII regulates eye pigmentation.

(a-h) Photomicrographs of eyes from three-day old adult w+ males raised at 25°C (unless otherwise indicated). The genotypes are as follows: (a) wild type (Oregon-R), (b) PI4KIIΔ, (c) dorΔ, (d) dorΔ;PI4KIIΔ, (e) g50e (f) g50e, p{w+, garnet}, (g) sac1ts, (h) sac1ts, PI4KIIΔ. (a,b) PI4KIIΔ mutants have a less prominent pseudopupil than wild type flies. (c,d) PI4KIIΔ enhances the eye pigmentation defect of dorΔ mutants. (e,f) GFP-Garnet transgene rescues the pigmentation defect of g50e mutants. (g,h) PI4KIIΔ suppresses the pigmentation defect of sac1ts flies.
Appendix Figure 21

wild type  \( PI4KII\Delta \)  \( dor^1 \)  \( dor^1; PI4KII\Delta \)

\( g^{so} \)  \( g^{so} + \text{rescue} \)  \( sac1^{ts} \)  \( sac1^{ts}, PI4KII\Delta \)

\( PI \xrightarrow{\text{PI4KII}} \text{PI4P} \)

\( \text{Sac1} \)
Appendix 2: PI4KII is required for male fertility in *Drosophila*

**Introduction**

The growth and differentiation of sperm cells into highly elongated spermatids (over 2 mm in length) requires a tremendous biosynthetic effort coupled with a high degree of coordination in trafficking of intracellular cargo. Several Golgi localized proteins required for germ cell cytokinesis have been identified, including the type III phosphatidylinositol 4-kinase β (Fwd) and the SNARE syntaxin 5 (Brill *et al*., 2000; Xu *et al*., 2002). Additionally, the endosomal-sorting complex required for transport (ESCRT-I) subunit dVps28, is required for later stages in spermiogenesis (Sevrioukov *et al*., 2005). Furthermore, sperm cells contain highly specialized structures such as the acrosome, a membrane-bound vesicle located at the apical tip of the sperm head, which is required for fertilization. Acrosomes are secretory lysosomes (LROs) and contain a mixture of unique proteins and enzymes commonly found in lysosomes (Cattaneo *et al*., 2006; Moreno and Alvarado, 2006). Another specialized structure is the basal body, which anchors the axoneme tail to the nucleus. Importantly, depletion of PI(4,5)P₂ affects basal body organization and anchoring of the basal body to the nucleus (Wei *et al*., 2008). Clearly, multiple aspects of trafficking and phosphatidylinositol metabolism must be precisely regulated for proper spermatid formation.

*Drosophila* spermatogenesis occurs in a syncytium in which two somatic cyst cells encapsulate each clonally-related group of germ line cells (Fuller, 1993; White-Cooper, 2010). The testis can be thought of as a blind-ended tube in which both the germline spermatogonial cells and the somatic cyst cells originate from a population of stem cells at the apical tip (White-Cooper, 2010). Cysts progressively differentiate as they move towards the basal end of the testis. After four mitotic and two meiotic divisions, the 64 haploid spermatids grow sperm tails towards the apical end of the testis, while the nuclei remain attached to the head cyst cell, which becomes embedded in the epithelium at the basal end of the testis (Fig. 22b). A process of spermatid individualization then occurs, in which the sperm are invested with their own membranes. This is followed by coiling of the cysts at the basal end of the testis, somatic cyst cell degeneration and transfer of mature sperm to the seminal vesicle. Since PI4KII encodes a testis specific transcript
and is required for intracellular trafficking in other cell types, I asked whether PI4KII might function in spermiogenesis.

**Results**

To test if PI4KII is required for spermiogenesis, I tested the fertility of *PI4KII*Δ mutants, which lack both the somatic and testis isoforms of PI4KII (see Chapter 3 for details). *PI4KII*Δ mutant males were completely sterile as determined by test-crosses with wild-type virgin female flies. The fertility of *PI4KII*Δ mutant females was not obviously affected. The male sterility of *PI4KII*Δ mutants was rescued by introducing a transgene containing *PI4KII* genomic DNA, confirming that sterility is caused by a mutation in the *PI4KII* gene. Interestingly, the ubiquitously expressed mCherry-PI4KII transgene, which encodes the somatic isoform of PI4KII, was able to partially rescue *PI4KII*Δ male sterility. This suggests that the unique amino terminus of the PI4KII testis isoform is dispensible for male fertility.

Since mCherry-PI4KII is functional in the male germline, I analyzed the localization of mCherry-PI4KII during spermatogenesis. mCherry-PI4KII colocalized with the lysosomal marker LAMP-GFP throughout spermiogenesis (data not shown). In particular, PI4KII and LAMP colocalized adjacent to spermatid nuclei (Fig. 22a). The striking concentration of mCherry-PI4KII to the head of elongated sperm suggested that PI4KII might be required later in spermiogenesis. To test this possibility, I analyzed live squashed preparations of *PI4KII*Δ male germ cells. Early stages of spermatogenesis appeared normal in *PI4KII* mutants. PI4KII was not required for cytokinesis, in contrast to Fwd (PI4KIIIβ), which is essential for germ cell division (Brill et al., 2000; Polevoy et al., 2009). *PI4KII* mutants also exhibited normal spermatid elongation and individualization (not shown). Importantly, during coiling, *PI4KII* mutant cysts exhibited scattered nuclei, whereas, in wild-type cysts, the germ cell nuclei remained tightly bundled (Fig. 22b-e). *PI4KII* mutant cysts accumulated at the basal end of the testis and no mature sperm were detected in the seminal vesicles.
Discussion

PI4KII mutants exhibit scattered nuclei in late stage cysts, suggesting that sperm cell heads are not properly anchored to the somatic cyst cell. Consistent with this possibility, mCherry-PI4KII is concentrated at the sperm head adjacent to the nucleus. However, without further marker analysis, it is difficult to conclude if PI4KII localizes to the basal body or the apical acrosome, which are found on opposite sides of the nucleus. If PI4KII localizes to the basal body, it might be required to anchor the nucleus to the axoneme tail. Since PI(4,5)P₂ provides a crucial signal to organize the basal body, PI4KII might regulate this process by generating the precursor lipid PI4P. To examine whether PI4KII regulates basal body organization, the localization of the basal body marker Unc-GFP could be examined relative to Hoescht stained nuclei in PI4KII mutants (Baker et al., 2004). In wild type cells, the Unc-GFP and Hoescht signals are always adjacent. Consequently, if Unc-GFP is not associated with nuclei in PI4KII mutants, this would indicate that the basal body is detached.

If PI4KII localizes to the acrosome, it might be required for formation of this lysosome-related organelle. This would be consistent with a requirement for PI4KII in formation of pigment granules, as well as the observed colocalization between PI4KII and LAMP-GFP in spermatids. To test if the acrosome forms normally in PI4KII mutants, the acrosomal marker Sneaky-GFP could be examined (Wilson et al., 2006). If Sneaky-GFP localization is altered, this might indicate a novel role for the acrosome in anchoring spermatids to the cyst cell.
Figure 22 – PI4KII is essential for spermatogenesis.

(a) Epifluorescence micrograph of spermatids. LAMP-GFP (green) and mCherry-PI4KII (red) colocalize adjacent to spermatid nuclei stained with Hoechst (blue). Note that PI4KII and LAMP are primarily localized at the end of the nucleus near the basal body. This image was acquired by L. Fabian (b, c) Schematic diagram of a Drosophila testis. 64 haploid nuclei remain tightly associated in elongated wild type cysts (b), whereas the nuclei are scattered along the length of the cyst in PI4KII mutants (c). (d, e) Phase (left) and epifluorescence (right) micrographs of live testes stained with the vital dye Hoechst to visualize nuclei. PI4KII mutants exhibit disorganized spermatid nuclei in late-stage coiling cysts (e), whereas nuclei appear tightly bundled in wild type cysts (d).
Appendix Figure 22

(a) LAMP-GFP, mCherry-PI4KII, Hoechst, Merge

(b) wild type

Apical

Basal

Sperm tails

Bundle of 64 nuclei

(c) PI4KIIΔ

Scattered nuclei

d) wild type Phase, Hoechst

e) PI4KIIΔ Phase, Hoechst
Bibliography


Costantino, B.F., Bricker, D.K., Alexandre, K., Shen, K., Merriam, J.R., Antoniewski, C.,
receptor mediates steroid-regulated developmental events during the mid-third instar of

Courel, M., Rodemer, C., Nguyen, S.T., Pance, A., Jackson, A.P., O'Connor D, T., and
Taupenot, L. (2006). Secretory granule biogenesis in sympathoadrenal cells:
identification of a granulogenic determinant in the secretory prohormone chromogranin

Courel, M., Soler-Jover, A., Rodriguez-Flores, J.L., Mahata, S.K., Elias, S., Mon
secretogranin II regulates dense core secretory granule biogenesis in catecholaminergic

domains direct cell type-specific sorting of chromogranin A to secretory granules. J Biol
Chem 275, 7743-7748.

contains an AP-3-sorting motif and a kinase domain that are both required for endosome

D'Angelo, G., Polishchuk, E., Di Tullio, G., Santoro, M., Di Campli, A., Godi, A., West, G.,
Bielawski, J., Chuang, C.C., van der Spoel, A.C., Platt, F.M., Hannun, Y.A., Polishchuk,
R., Mattjus, P., and De Matteis, M.A. (2007). Glycosphingolipid synthesis requires


40, 143-158.

Physiol 70, 487-512.

Cell Physiol 289, C1169-1178.

on the gastrointestinal system. Am J Physiol Gastrointest Liver Physiol 294, G717-727.

products to zymogen granules and the apical plasma membrane of pancreatic acinar cells.


273-284.


Fraenkel, G., and Brookes, V.J. (1953). The process by which the puparia of many species of flies become fixed to a substrate. Biol Bull 105, 442-449.


Hypertension from targeted ablation of chromogranin A can be rescued by the human ortholog. J Clin Invest 115, 1942-1952.


