Regulating GLUT4 Sorting into the Insulin-Responsive Compartment in Muscle Cells

Kevin Patrick Foley

Doctorate of Philosophy in Biochemistry

Department of Biochemistry
University of Toronto
2014

Abstract

Skeletal muscle and adipose tissue serve as the major storage sites for glucose, and insulin is the major signal for glucose uptake into these tissues. Glucose transporter 4 (GLUT4) is responsible for the uptake of glucose into muscle and adipose tissues. This protein constitutively recycles between the plasma membrane and intracellular depots. Under resting conditions, most GLUT4 molecules are maintained in intracellular compartments. Insulin shifts this dynamic equilibrium towards the plasma membrane by recruiting a fraction of GLUT4 to the plasma membrane from insulin responsive vesicles. However, steady-state measurements of GLUT4 localization have failed to reveal the subcellular localization of these vesicles or how GLUT4 is sorted to them.

By analyzing the sorting of GLUT4 as it internalizes from the cell surface, advances may be made in revealing how GLUT4 acquires insulin-responsiveness and the intracellular location in which this occurs. In L6 myoblasts stably expressing myc-tagged GLUT4, surface-labelled GLUT4myc that internalizes for 30 min accumulates in a Syntaxin-6 (Stx6)-positive perinuclear compartment and displays insulin-responsive exocytosis. Although Stx6 knockdown does not alter the perinuclear accumulation of internalized GLUT4myc, it does inhibit by ~50% the ability of internalized GLUT4myc to undergo insulin-responsive exocytosis. These results suggest that the Stx6-positive perinuclear compartment consists of at least two sub-compartments – one that serves as a dynamic retention compartment and one that is insulin-responsive. Microtubule
disruption with nocodazole prevents internalized GLUT4myc from reaching the Stx6-positive perinuclear compartment and undergoing insulin-responsive exocytosis. Removing nocodazole allows internalized GLUT4myc to re-acquire insulin-responsive exocytosis in correlation with recovering its accumulation in the Stx6-positive perinuclear compartment. C2-ceramide, which induces insulin resistance, inhibits GLUT4 sorting into the Stx6-positive perinuclear compartment and insulin-responsive exocytosis independently of affecting insulin-stimulated Akt. I propose that internalized GLUT4 must sort through a Stx6-positive compartment that is normally perinuclear as a preamble to acquiring insulin-responsiveness.
Acknowledgments

I can honestly say that pursuing a PhD has been the most challenging undertaking of my life. I also know that, without a doubt, I would not have gotten this far without the tutelage and support of a number of people. First and foremost I would like to thank my supervisor, Dr. Amira Klip. Her guidance has been instrumental in shaping my growth into the scientist I have become. Equally important has been her unwavering support, particularly during my most trying times, which motivated me to push forward and to use those experiences as opportunities to learn instead of as moments to despair. I would also like to thank my supervisory committee members, Dr. David Williams and Dr. Daniela Rotin. Their input and encouragement, and not least of all tremendous patience, has helped guide me to where I am today.

I have had the tremendous pleasure of working with some very intelligent, kind, and all around wonderful people during my 6 years in Amira’s lab. Everyone with whom I worked was positive and supportive and provided a great environment in which to pursue science – which was great since I spent a lot of time with them in the lab. I’d like to thank everyone, past and present, from the Klip lab for their friendship and support during my time in the lab. It truly was a better experience because of you all. In particular, Phil was always available for helpful discussions – whether it be to help solve my problem of the day or to distract me from my problem of the day with “hockey talk”. It was especially fun when “hockey talk” proceeded to the pub to watch a game. Zhi was there for help with any technical procedures I needed to learn in the lab and to make sure everything ran smoothly. It was also a great comfort to have my boy Timmy with me to share in this roller coaster of a journey. If it was a rainy day I could always count on him to have an umbrella….in his beverage. Nico later joined the lab and provided a great friendship and somehow always had a ridiculous scientific paper to show me. He also provided one thing that Timmy couldn’t – long blonde hair. Special thanks go to Yi who made sure I slept and stayed healthy and who made me delicious Chinese dumplings to cheer me up when I was working late on seemingly futile experiments. I’d also like to thank all of the other people I was able to work with outside of the Klip Lab. In particular, the Grinstein Lab was always good for a laugh or social outing, not to mention reagents and tremendous scientific advice. Special thanks goes to Jay and Ron who helped me work out some experimental issues that took over a year to decipher.
Finally, I would like to thank my friends and family who supported me during my PhD studies, even when I neglected to call or visit while I hibernated in the lab. My aunt put it best when she said that when I finish each of them will feel their part in this accomplishment, as they have invested much time and effort in supporting me through this process…and this is an understatement as to the encouragement and moral uplifting they were able to provide to me. This is especially true of my “brother from another mother” Rock (Andrew), my Aunt Julie, and lastly (and left to the end on purpose) my parents, Pat and Julie. The love and support my parents have given me during this time and during all of my previous years have been immeasurable and much appreciated.
Preface

The work presented in this Ph.D. thesis is the research conducted from January 2008 to August 2013 under the supervision of Dr. Amira Klip in the Program in Cell Biology, The Hospital for Sick Children, Toronto, ON, Canada. Financial support was provided by grants from the Canadian Institutes of Health Research and Canadian Diabetes Association. Financial stipend to Kevin Patrick Foley was provided by the National Sciences and Engineering Research Council (NSERC), the Banting and Best Diabetes Centre (BBDC), and the Canadian Diabetes Association (CDA). Results previously published in journals are reprinted in this thesis with the copyright permission from the respective journals.
# Table of Contents

**Contents**

Acknowledgments........................................................................................................ iv
Preface.......................................................................................................................... vi
Table of Contents......................................................................................................... vii
List of Tables ................................................................................................................. xii
List of Figures ............................................................................................................... xiii
List of Appendices ........................................................................................................ xvii
List of Abbreviations ................................................................................................... xviii

Chapter 1....................................................................................................................... 1

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

1.1 Regulated intracellular protein transport ......................................................... 1

1.1.1 Compartmentalization of cellular functions............................................... 1

1.1.2 Proteins involved in regulating inter-endomembrane traffic ....................... 3

1.1.3 Retrograde transport pathways ..................................................................... 9

1.2 Glucose Transporter 4 (GLUT4) ...................................................................... 13

1.2.1 Distribution of GLUT4 in intracellular compartments .......................... 14

1.2.2 GLUT4 retention ....................................................................................... 18

1.2.3 GLUT4 mobilization in response to insulin ............................................. 21

1.2.4 Dynamics of GLUT4 sorting .................................................................... 24

1.2.5 GLUT4 traffic in models of insulin resistance ........................................ 50

1.3 Rationale and hypotheses................................................................................... 51

Chapter 2....................................................................................................................... 54

2 Materials and Methods ......................................................................................... 54
2.1 Cell culture reagents .............................................................................................................. 54
2.2 Antibodies, reagents, cDNA, and siRNA .............................................................................. 54
   2.2.1 Antibodies ...................................................................................................................... 54
   2.2.2 Reagents ....................................................................................................................... 54
   2.2.3 cDNA constructs and siRNA .......................................................................................... 55
2.3 Cell culture and transfections ............................................................................................... 55
2.4 Detection of total GLUT4myc by immunofluorescence ....................................................... 56
2.5 Imaging GLUT4 internalization in single cells ...................................................................... 56
2.6 Insulin-responsive GLUT4 re-exocytosis ............................................................................ 57
2.7 Detection of cell surface GLUT4 .......................................................................................... 58
2.8 GLUT4 endocytosis .............................................................................................................. 58
2.9 GLUT4 Immunoprecipitation ............................................................................................... 59
   2.9.1 Myc immunoprecipitation .............................................................................................. 59
   2.9.2 GLUT4 immunoprecipitation ........................................................................................ 59
2.10 Immunoblotting .................................................................................................................... 60
2.11 Fluorescence microscopy and image analysis ....................................................................... 60
2.12 Statistical analysis .............................................................................................................. 60
Chapter 3 ..................................................................................................................................... 61
3 Dynamics of GLUT4 traffic following internalization from the plasma membrane .............. 61
   3.1 Steady-state localization of GLUT4myc in L6 muscle cells ............................................. 62
   3.2 Tracking the internalization of GLUT4myc in L6 muscle cells ....................................... 68
      3.2.1 Antibody Validation ..................................................................................................... 68
      3.2.2 Sorting of GLUT4 upon its internalization from the plasma membrane, vis-à-vis the localization of endogenous markers .............................................................. 71
      3.2.3 Sorting of GLUT4 following internalization from the plasma membrane relative to Rab-GFP markers ................................................................................................. 79
3.2.4 Sorting of GLUT4 following internalization from the plasma membrane relative to Phosphatidylserine ................................................................. 81

3.3 Conclusions ............................................................................................................. 83

Chapter 4 ......................................................................................................................... 84

4 GLUT4 sorting in response to insulin: Syntaxin-6 regulates the acquisition of insulin-responsive GLUT4 exocytosis ................................................................. 84

4.1 The steady-state localization of intracellular GLUT4 does not suffice to reveal the importance of Stx6 to GLUT4 traffic ...................................................... 84

4.2 Syntaxin-6 regulates GLUT4 sorting into the IRC/IRV but not GLUT4 accumulation in the perinuclear compartment ......................................................... 90

4.3 GLUT4 accumulation in the Syntaxin-6-positive perinuclear compartment does not depend on canonical sorting mechanisms ......................................................... 94

4.3.1 Molecules involved in retrograde transport ......................................................... 94

4.3.2 Molecules linked to GLUT4 availability and fusion at the plasma membrane ... 98

4.4 Conclusions ............................................................................................................. 100

Chapter 5 ......................................................................................................................... 102

5 Alterations in GLUT4 sorting caused by Nocodazole ................................................ 102

5.1 Nocodazole inhibits insulin-stimulated GLUT4 translocation but does not alter the steady-state localization of intracellular GLUT4 ......................................................... 102

5.2 Microtubule disruption inhibits both GLUT4 sorting into the Syntaxin-6-positive perinuclear region and insulin-responsive GLUT4 re-exocytosis ..................... 108

5.3 Conclusions ............................................................................................................. 112

Chapter 6 ......................................................................................................................... 113

6 Insulin resistance modulates GLUT4 traffic ............................................................. 113

6.1 C2-ceramide disrupts the intracellular localization of GLUT4 and insulin-stimulated GLUT4 translocation ................................................................. 113

6.2 C2-ceramide inhibits insulin-responsive GLUT4 re-exocytosis independent of insulin signalling ................................................................. 117

6.3 Conclusions ............................................................................................................. 122

Chapter 7 ......................................................................................................................... 123
7 Summary ........................................................................................................................................ 123

7.1 GLUT4 accumulation in the Syntaxin-6-positive perinuclear compartment correlates with insulin-responsive GLUT4 re-exocytosis ......................................................... 123

Chapter 8 ......................................................................................................................................... 126

8 Discussion .................................................................................................................................... 126

8.1 Characterizing GLUT4 distribution .......................................................................................... 126

8.2 Characterization of GLUT4 sorting dynamics .......................................................................... 127

8.2.1 Perinuclearly localized Stx6 defines GLUT4 sorting into the IRC/IRV in muscle cells .......... 128

8.2.2 GLUT4 sorting at the Stx6-positive perinuclear compartment .............................................. 129

8.2.3 GLUT4 sorting into the Stx6-positive compartment .............................................................. 130

8.2.4 GLUT4 sorting into the IRC/IRV ......................................................................................... 133

8.3 GLUT4 sorting in insulin resistant muscle cells ........................................................................ 134

8.4 Conclusions .............................................................................................................................. 136

8.5 Future Directions ...................................................................................................................... 136

8.5.1 Defining the organelle identity of the perinuclear GLUT4 compartment ................. 137

8.5.2 Characterizing GLUT4 sorting into the IRC/IRV ............................................................... 139

Chapter 9 ......................................................................................................................................... 141

9 Appendix I: Secondary authorship publications ........................................................................ 141

9.1 Contraction-related stimuli regulate GLUT4 traffic in C2C12-GLUT4myc skeletal muscle cells .......................................................................................................................... 141

9.2 NOD2 Activation Induces Muscle Cell-Autonomous Innate Immune Responses and Insulin Resistance .............................................................................................................. 141

9.3 Myo1c binding to submembrane actin mediates insulin-induced tethering of GLUT4 vesicles .............................................................................................................................. 142

9.4 Endocytosis, Recycling, and regulated exocytosis of Glucose Transporter 4 ................. 143

Chapter 10 ...................................................................................................................................... 144

10 References ................................................................................................................................. 144
List of Tables

Table 1.1: Key regulatory proteins in the retrograde transport pathway

Table 7.1: Summary of GLUT4 re-exocytosis parameters under each treatment condition
List of Figures

Figure 1.1: Endomembrane compartments and retrograde transport pathways

Figure 1.2: Rab GTPase cycle

Figure 1.3: Rab protein localization and generalized routes of function

Figure 1.4A: Intracellular membranes in which GLUT4 resides

Figure 1.4B: Differentiating proteins involved in GLUT4 retention from proteins involved in sorting GLUT4 into the IRC/IRV

Figure 1.5A: Insulin signalling cascade to AS160 that releases the retention of GLUT4-containing IRC/IRV

Figure 1.5B: Regulation of insulin-stimulated GLUT4 translocation

Figure 1.6: Cartoon schematics of GLUT4 linear sequence and 2-dimensional folded structure showing localization of key sorting motifs

Figure 1.7: Dynamic retention model

Figure 1.8: Static retention model

Figure 1.9: Model of GLUT4 dynamics

Figure 1.10A: Regulation of GLUT4 sorting through the constitutively recycling pathway

Figure 1.10B: Regulation of GLUT4 sorting from sorting/recycling endosomes into the IRC/IRV

Figure 1.10C: Regulation of GLUT4 sorting from sorting/recycling endosomes to the TGN en route to the IRC/IRV

Figure 3.1: GLUT4myc localization in a resting L6 muscle cell

Figure 3.2: GLUT4myc co-localizes with Stx6 and Stx16, but not with furin, in the perinuclear region

Figure 3.3: Tfn-labelled endosomes co-localize with GFP-GLUT4myc in cytosolic puncta

Figure 3.4: GLUT4myc partially co-localizes with EEA1 and Tfn

Figure 3.5: VAMP2-HA expression in L6 muscle cells

Figure 3.6: GLUT4myc co-localizes with VAMP2-HA in the perinuclear region

Figure 3.7: Quantification of GLUT4myc co-localization with markers of endomembrane compartments
Figure 3.8: GLUT4 interacts with Stx6, but not Stx16

Figure 3.9: GLUT4 does not interact with sortilin

Figure 3.10: Within 20 min, GLUT4myc internalized from the plasma membrane begins to accumulate in the perinuclear region

Figure 3.11: The 9E10 antibody labels cell surface GLUT4myc in both fixed and non-fixed cells

Figure 3.12: Labelling cell surface GLUT4myc with both primary and secondary antibodies before allowing GLUT4 internalization inhibits GLUT4 accumulation in the perinuclear region

Figure 3.13: GLUT4myc accumulates in the perinuclear region between 20 and 30 min following internalization from the plasma membrane

Figure 3.14: Internalized GLUT4 sorts into a Syntaxin-6-positive perinuclear compartment

Figure 3.15: Internalized GLUT4 segregates away from Transferrin

Figure 3.16: Internalized GLUT4 sorts into a Syntaxin-6-positive perinuclear compartment independent of insulin

Figure 3.17: Internalized GLUT4 localizes to EEA1-positive cytosolic vesicles

Figure 3.18: Internalized GLUT4 co-localizes with VAMP2-HA in cytosolic vesicles and in the perinuclear region

Figure 3.19: Internalized GLUT4myc exhibits insulin-responsive re-exocytosis by 30 min

Figure 3.20: Internalized GLUT4myc co-localizes with Rab5-GFP in cytosolic vesicles and in the perinuclear region

Figure 3.21: Internalized GLUT4myc partly co-localizes with Rab11-GFP in the perinuclear region

Figure 3.22: Internalized GLUT4myc co-localizes with Rab7- and Rab9-GFP in the perinuclear region

Figure 3.23: Internalized GLUT4myc does not localize to PS-containing membranes

Figure 4.1: Stx6 depletion does not alter the localization of GLUT4 to the Stx16-positive perinuclear compartment

Figure 4.2: Stx6 depletion does not alter the segregation of GLUT4 from furin or EEA1 compartments

Figure 4.3: Stx6 depletion does not alter GLUT4 localization relative to Tfn or VAMP2-HA

Figure 4.4: Stx6 depletion does not alter GLUT4 localization. GLUT4myc co-localization with markers of endomembrane compartments was quantified using Pearson correlations
Figure 4.5: Stx6 depletion increases cell surface GLUT4 and inhibits insulin-stimulated GLUT4 translocation

Figure 4.6: Stx6 depletion does not inhibit internalized GLUT4 from accumulating in the perinuclear region

Figure 4.7: Stx6 depletion does not inhibit internalized GLUT4 sorting to the VAMP2-HA compartment

Figure 4.8: Stx6 depletion inhibits insulin-responsive GLUT4 re-exocytosis

Figure 4.9: Stx6 depletion does not inhibit GLUT4 endocytosis

Figure 4.10: DN-Dynein, but not DN-Rab5, perturbs GLUT4 localization in L6 muscle cells

Figure 4.11: DN-Dynein, but not DN-Rab5 or DN-Rab22a, inhibits GLUT4 sorting to the perinuclear region

Figure 4.12: Retromer does not regulate GLUT4 sorting into the perinuclear region

Figure 4.13: Rab11a, Rab14, and Rab8a do not regulate GLUT4 sorting into the Stx6-positive perinuclear compartment

Figure 4.14: VAMP2 does not regulate GLUT4 sorting into the Stx6-positive perinuclear compartment

Figure 5.1: 3 µM nocodazole does not disrupt GLUT4 co-localization with Stx6 or Stx16

Figure 5.2: 3 µM nocodazole does not disrupt GLUT4 segregation from furin or Tfn

Figure 5.3: 3 µM nocodazole does not disrupt GLUT4 co-localization with VAMP2-HA

Figure 5.4: Nocodazole does not alter GLUT4 localization. GLUT4myc co-localization with markers of endomembrane compartments was quantified using Pearson correlations

Figure 5.5: Nocodazole inhibits insulin-stimulated GLUT4 translocation but not GLUT4 endocytosis

Figure 5.6: Nocodazole treatment for 5 min disrupts microtubules

Figure 5.7: Nocodazole prevents GLUT4 sorting into the Stx6-positive perinuclear compartment

Figure 5.8: Nocodazole inhibits insulin-responsive GLUT4 re-exocytosis

Figure 6.1: C2-ceramide disrupts intracellular membrane compartments and disperses intracellular GLUT4

Figure 6.2: C2-ceramide inhibits insulin-stimulated GLUT4 translocation

Figure 6.3: C2-ceramide prevents GLUT4 sorting into the Stx6-positive perinuclear compartment
Figure 6.4: Quantification of internalized GLUT4myc co-localization with Stx6 with and without C2-ceramide treatment

Figure 6.5: C2-ceramide inhibits insulin-responsive GLUT4 re-exocytosis and insulin signalling

Figure 6.6: C2-ceramide inhibits insulin-responsive GLUT4 re-exocytosis independent of insulin signalling

Figure 7.1: GLUT4 co-localization with Stx6 correlates with insulin-responsive GLUT4 re-exocytosis

Figure 7.2: Model of GLUT4 sorting in L6 muscle cells
List of Appendices

Appendix I: Secondary Authorship Publications
List of Abbreviations

Adenosine diphosphate: ADP
ADP-ribosylation factor 6: Arf6
Akt Substrate of 160 kDa: AS160
Bovine Serum Albumin: BSA
C2-ceramide: C2-cer
Clathrin Heavy Chain: CHC
Conserved Oligomeric Golgi Complex: COG
Constitutively Active: CA
Dimethyl Sulfoxide: DMSO
Dominant Negative: DN
Early Endosome Antigen 1: EEA1
Endocytic Recycling Compartment: ERC
Endoplasmic Reticulum: ER
Endosomal Sorting Complex Required for Transport: ESCRT
Fluorescence Recovery After Photobleaching: FRAP
Golgi-localized Gamma Adaptin Ear-containing Arf-binding Protein: GGA
Glucose Transporter 1: GLUT1
Glucose Transporter 4: GLUT4
GLUT4 Storage Vesicles: GSV
Golgi-associated Retrograde Protein Complex: GARP
Green Fluorescent Protein: GFP
GTPase Activating Protein: GAP
Guanine Nucleotide Dissociation Inhibitor: GDI
Guanosine Diphosphate: GDP
Guanine Nucleotide Exchange Factor: GEF
Guanosine Triphosphate: GTP
Guanosine Triphosphatase: GTPase
Insulin Receptor Substrate 1: IRS-1
Insulin-Responsive Aminopeptidase: IRAP
Insulin Responsive Compartment: IRC
Insulin Responsive Motif: IRM
Insulin Responsive Vesicles: IRV
Lactadherin-C2 domain: Lact-C2
Lipoprotein Receptor Related Protein 1: LRP1
Mannose-6-Phosphate Receptor: MPR
Multi-Vesicular Bodies: MVB
N-ethylmaleimide sensitive fusion protein: NSF
Paraformaldehyde: PFA
Phosphate Buffered Saline: PBS
PBS with 0.49 mM MgCl₂ and 0.68 mM CaCl₂: PBS+
Phosphatidylinositol-3-kinase: PI3K
Phosphatidylinositol-3-phosphate: PI3P
Phosphatidylinositol-3,4,5-triphosphate: PI(3,4,5)P₃
Phosphatidylserine: PS
Room Temperature: RT
Shiga Toxin Subunit B: STxB
Soluble NSF attachment protein receptor: SNARE
Sorting Nexin: Snx
Syntaxin-16: Stx16
Syntaxin-6: Stx6
Tether containing UBX domain for GLUT4: TUG
Total Internal Reflection Fluorescence: TIRF
Transferrin: Tfn
Transferrin Receptor: TfnR
Trans-Golgi Network: TGN
Vesicle-associated membrane protein: VAMP
Wild Type: WT
Chapter 1

1 Introduction

1.1 Regulated intracellular protein transport

1.1.1 Compartmentalization of cellular functions

Mammalian cells have evolved to separate cellular functions into spatially discrete compartments. One major mechanism through which cells accomplish compartmentalization is to physically separate distinct areas of the cell using membranes. Organelles separate major divisions in function (for example, replication, biosynthesis, degradation, energy production) while vesicular intermediates carry cargo between compartments (Diekmann and Pereira-Leal, 2013). Together, these membrane-bound structures form the endomembrane system, which can be functionally organized into two main pathways (Diekmann and Pereira-Leal, 2013). First, the anterograde pathway is responsible for protein biosynthesis/folding and for protein transport to organelles/cytosol, to the plasma membrane, or to secretory vesicles (Barlowe and Miller, 2013; Bonifacino and Glick, 2004). Second is the retrograde pathway, which is responsible for protein endocytosis, for extracellular material uptake, and for sorting endocytosed cargoes to be recycled or degraded (Bonifacino and Rojas, 2006; Johannes and Popoff, 2008). Both anterograde and retrograde transport are highly regulated and selective to maintain organelle composition and function despite bidirectional membrane transport between organelles of both pathways (Mizuno-Yamasaki et al., 2012). Here I will focus on these pathways with regards to the transport of transmembrane proteins.

The anterograde pathway consists of the Endoplasmic Reticulum (ER), Golgi cisternae, secretory vesicles, and target membranes (e.g. plasma membrane) (Bonifacino and Glick, 2004). The Golgi is organized into 3 distinct regions: the cis-Golgi, the medial-Golgi, and the Trans Golgi Network (TGN). Newly synthesized proteins fold in the ER and are transported to the cis-Golgi. Biosynthetic cargo must pass through the Golgi cisternae and be sorted into appropriate vesicular carriers at the TGN (Bonifacino and Glick, 2004). Thus, the TGN plays a central role in protein sorting, as it is here that biosynthetic cargo proteins diverge to alternative routes and sort into secretory vesicles, to the plasma membrane, or to endomembranes.
The retrograde pathway consists of the plasma membrane and an intricate system of endosomal membranes that sort endocytosed proteins for degradation or recycling (Diekmann and Pereira-Leal, 2013). These endomembrane organelles have been classically defined based on morphology and resident membrane proteins (regulatory or cargo proteins). They include early endosomes, sorting endosomes, recycling endosomes, the Endocytic Recycling Compartment (ERC), late endosomes (multi-vesicular bodies, MVB), lysosomes, the TGN, and specialized storage vesicles (Figure 1.1). However, defining these endomembranes has been confusing due to redundant nomenclature and difficulty in differentiating some of these compartments. First, to simplify nomenclature, early endosomes are comprised of sorting endosomes and recycling endosomes (Hsu et al., 2012; Maxfield and McGraw, 2004). Also, recycling endosomes and ERC have been used to describe the same organelle (Hsu et al., 2012; Johannes and Popoff, 2008; Maxfield and McGraw, 2004). Second, these endomembranes are dynamic in that many ‘resident’ membrane proteins are in fact transported into and out of a compartment such that the average distribution concentrates the ‘resident’ membrane protein in a particular organelle (Maxfield and McGraw, 2004). Further, many organelles actually mature into subsequent compartments and so are themselves transient in nature (Maxfield and McGraw, 2004). Thus, defining an organelle by a single resident protein is problematic. Organelles are defined by more than one characteristic, such as morphology, relative enrichment in multiple proteins, or kinetics of cargo sorting (Maxfield and McGraw, 2004).

**Figure 1.1:** Endomembrane compartments and retrograde transport pathways. Endocytosed membrane proteins enter sorting endosomes where they are sorted to the plasma membrane, recycling endosomes, the TGN, or late endosomes. From recycling endosomes cargo proteins may sort to the plasma membrane, the TGN, or to specialized storage vesicles. From the TGN cargo proteins may sort to specialized storage vesicles, the plasma membrane, or back into sorting endosomes. Late endosomes deliver cargo proteins to lysosomes or the TGN.
Sorting endosomes are the first discernible organelle encountered by cargo proteins endocytosed from the plasma membrane (Maxfield and McGraw, 2004). Here, cargo proteins are sorted for one of four destinations: the plasma membrane, recycling endosomes, the TGN, or late endosomes (Johannes and Popoff, 2008; Maxfield and McGraw, 2004) (Figure 1.1). Sorting endosomes mature into late endosomes (MVB), which then acidify to form lysosomes. Alternatively, tubule networks form on sorting endosomes for cargo sorting into recycling endosomes or to the TGN. Recycling endosomes are often perceived as differentiated subdomains of these tubular networks or as extensions of sorting endosomes (Bonifacino and Rojas, 2006; Chia et al., 2013; Sönnichsen et al., 2000); as such, sorting endosomes and recycling endosomes are the most difficult organelles between which to distinguish. Cargo may be sorted from recycling endosomes to the plasma membrane, to the TGN, or to specialized vesicles. Recycling to the TGN allows endocytosed cargo proteins access to the secretory pathway such that membrane proteins can be recycled back to the plasma membrane, to endomembranes, or into specialized storage vesicles for regulated exocytosis. Thus, the TGN acts as a central hub at the intersection of anterograde and retrograde transport pathways. The regulatory molecules that define the organelles of the retrograde pathway and the endosomal sorting pathways encompassed under the retrograde transport pathway are outlined below.

1.1.2 Proteins involved in regulating inter-endomembrane traffic

The transport of cargo proteins within the endomembrane system requires a complex network of regulatory proteins to mediate selective and directional traffic of cargo. Cargo proteins carry sorting motifs that supply the destination address; however, how these sorting motifs are identified and processed for cargo protein delivery is an ongoing area of investigation. Furthermore, the sorting motif is not always known for a given cargo protein. Here I outline key regulatory proteins in the transport of cargo through the retrograde pathway (see Table 1.1 for summary).
1.1.2.1 Rab small G proteins

The Rab family of small G proteins (Rab GTPases) play an important role in defining organelle function and identity (Mizuno-Yamasaki et al., 2012). These proteins act as molecular switches to control vesicular traffic and fusion (Stenmark, 2009). They cycle between GDP-bound ‘inactive’ and GTP-bound ‘active’ states and reversibly associate with membranes by prenyl groups attached to the C-terminus Cys residues [reviewed in (Stenmark, 2009)] (Figure 1.2).
When bound to GDP, a Rab protein binds to GDP dissociation inhibitor (GDI), which masks the prenyl tail and causes the Rab protein to dissociate from membrane. GDI dissociation factor (GDF) competes with GDI to localize a GDP-bound Rab protein to membrane. In the membrane, a Guanine nucleotide exchange factor (GEF) catalyzes exchange of the GDP for GTP. The GTP-bound Rab protein associates with membrane through the freed prenyl groups and binds to effector proteins with functions directly linked to vesicle transport and delivery. Effectors include motor proteins, tethers, and regulators of SNARE complex assembly (Mizuno-Yamasaki et al., 2012). A GTPase activating protein (GAP) catalyzes hydrolysis of GTP to GDP, converting the Rab protein back to the ‘inactive’ state. Each Rab protein consists of a hypervariable region which plays a key role in targeting of Rabs to specific membranes (Hutagalung and Novick, 2011). Also, Rab-specific GEF and GAP proteins ensure the fidelity of Rab protein activation and inactivation (Mizuno-Yamasaki et al., 2012).

**Figure 1.2:** Rab GTPase cycle. See text for details.

The Rab cycle is critical for regulating inter-endosomal traffic and for defining membrane identity (Hutagalung and Novick, 2011). Multiple Rab proteins work cooperatively within the same pathway through a mechanism termed ‘Rab cascades’ (Mizuno-Yamasaki et al., 2012). A particular Rab localized to an endosome recruits a specific set of effectors that direct vesicle transport. One of these effectors acts as the GEF for a subsequent Rab protein required to
continue the flow of membrane traffic. Likewise, one effector of the second Rab proteins acts as the GAP to the first Rab protein, completing the transition of membrane identity to that dictated by the second Rab protein.

**Figure 1.3**: Rab protein localization and generalized routes of function. See text for details.

In the retrograde transport pathway, key Rab proteins have been identified that confer membrane identity and that regulate the sorting of cargo proteins through endomembranes (Mizuno-Yamasaki et al., 2012) (Figure 1.3). The Rabs 4, 5, 14, and 22 localize to sorting endosomes and regulate specific steps in cargo protein sorting; however, Rab5 is the major regulator of retrograde cargo sorting in sorting endosomes (Hutagalung and Novick, 2011; Stenmark, 2009). The generalized roles of Rab14 and Rab22 are defined as regulating cargo transport between sorting endosomes and the TGN, while Rab4 regulates sorting endosome to plasma membrane transport (Galvez et al., 2012). Rab11 concentrates in recycling endosomes and the TGN and functions in transport of cargo proteins to the plasma membrane (Maxfield and McGraw, 2004; Mizuno-Yamasaki et al., 2012). Late endosomes are marked by Rab7 and Rab9 while lysosomes accumulate Rab7 only (Hutagalung and Novick, 2011). Rab7 is involved in late endosome maturation while Rab9 regulates transport from late endosomes to the TGN (Galvez et al., 2012). Many Rab proteins are found in the Golgi, including Rab6, Rab8, Rab11, and Rab14 (Hutagalung and Novick, 2011).
1.1.2.2 Retromer

The retromer is a sorting complex located within sorting and recycling endosomes that directs protein traffic to the TGN (Bonifacino and Rojas, 2006). It is comprised of 2 sub-complexes: a trimer that mediates cargo selection (Vps35-29-26) and an accessory dimer that may mediate tubule formation or membrane recruitment of the cargo selection complex (Seaman, 2012). The accessory complex consists of 2 sorting nexins (Snx, Snx1 or Snx2 with Snx5 or Snx6) that contain Bin-Amphipysin-Rvs (BAR) and Phox homology (PX) domains. Vps34 (a PI3K) activity is required to generate phosphatidylinositol-3-phosphate (PI3P) to which the Snx dimer binds through its PX domains (Seaman, 2012). The Snx dimer interacts with dynein and may also mediate tubule formation by invoking membrane curvature through the BAR domains (Johannes and Popoff, 2008). The cargo selection complex requires Rab7a and Snx3 for membrane recruitment, although the exact mechanism remains undefined (Seaman, 2012). Once on the membrane, the retromer associates with both cargo proteins and a number of effector proteins to drive vesicle formation and mobilization (Seaman, 2012). Retromer engages Rab7a for recruitment of the cargo selection complex, which is a Rab protein associated with late endosomes and not sorting endosomes. Therefore, it is hypothesized that retromer functions late in sorting endosome maturation at the interface of the conversion of the sorting endosome to late endosome (Seaman, 2012).

1.1.2.3 Motors and tracks

Rab proteins and retromer not only regulate cargo protein sorting, but also the recruitment of motor proteins to direct vesicle traffic (Akhmanova and Hammer, 2010). For sorting through the retrograde pathway, the cargo protein-containing vesicles require a mechanism to undergo long range directional transport. Microtubules function as bi-directional highways for vesicle movement – they form a radial array of tracks extending from a centrosome with their plus ends pointing outwards (Dogterom and Surrey, 2013). Motor complexes link cargo vesicles to the microtubule tracks and confer directionality of movement (Hunt and Stephens, 2011). While the kinesin family of motor proteins directs anterograde traffic (towards plus end), retrograde traffic (towards minus end) is directed by the motor complex dynein (Hunt and Stephens, 2011). Rab proteins mediate directionality of vesicle transport by recruiting motor proteins. Rab5, Rab7, and Rab11 all recruit dynein through interactions between dynein and Rab effector molecules (Hunt...
and Stephens, 2011). The retromer also links vesicles to microtubules through Snx1 and Snx4, which interact with components of the dynein complex (Hunt and Stephens, 2011).

### 1.1.2.4 Tethers

Upon arriving at an endomembrane destination, tethers are required to capture the target vesicle and its cargo proteins to ensure efficient fusion (Chia and Gleeson, 2011). Many tethering factors localize to the TGN, some of which also act to maintain the structural integrity and dynamic organization of the organelle (Chia and Gleeson, 2011). The homodimeric golgins (p230/golgin-245, golgin-97, GCC185, and GCC88) are long coiled-coil proteins that act as tethers for endosomal retrograde transport (Chia and Gleeson, 2011). Golgins are ideal for long range interactions with incoming vesicles and serve as Rab protein effectors that regulate vesicle recruitment (Chia and Gleeson, 2011; Hutagalung and Novick, 2011). The multi-subunit tethering complexes COG (conserved oligomeric Golgi complex), and GARP (Golgi-associated retrograde protein complex) are also required for efficient transport of several cargo proteins (Chia and Gleeson, 2011; Hutagalung and Novick, 2011). The COG complex interacts with Rab proteins as well as TGN resident SNAREs and golgins to mediate vesicle tethering just before fusion (Hutagalung and Novick, 2011; Miller and Ungar, 2012). Similarly, the GARP complex interacts with both Rab and SNARE proteins to regulate the tethering of incoming vesicles (Chia and Gleeson, 2011; Hutagalung and Novick, 2011). Multiple tethers at the TGN may distinguish between alternate routes of retrograde sorting, between different cargo proteins that use the same route, or act in concert to promote efficient vesicle recruitment.

### 1.1.2.5 SNARE proteins

Soluble NSF (N-ethylmaleimide sensitive fusion protein) attachment protein receptor (SNARE) proteins are necessary for vesicle fusion (Jung et al., 2012). For successful vesicle fusion with a target membrane, a SNARE complex consisting of 4 α-helices must be formed in which 1 helix is provided by a SNARE protein on the incoming vesicle (v-SNARE) and 3 helices are provided by SNARE proteins on the target membrane (t-SNAREs) (Wendler and Tooze, 2001). There are two main classes of SNAREs based on the presence of a conserve glutamine or arginine residue within the conserved central coiled-coil SNARE motif that mediates SNARE-SNARE protein interactions: R-SNAREs and Q-SNAREs (Jung et al., 2012). R-SNAREs are mostly located on the vesicle membrane (termed vesicle-associated membrane proteins, VAMPs) while Q-
SNAREs are mostly found in the target membrane. Q-SNAREs can be further divided into three classes: Qa (syntaxins), Qb (SNAP-25 N-terminal), and Qc (SNAP-25 C-terminal) (Wendler and Tooze, 2001). After fusion, the SNARE complex can be disassembled and SNARE proteins recycled for another round of fusion via NSF and α-SNAP (Wendler and Tooze, 2001).

There are 38 distinct SNAREs, each interacting with specific SNARE partners to form productive vesicle fusion events (Jung et al., 2012). In this way, expressing different combinations of SNARE family members and selectively distributing them on organelles allows for the delivery of cargo to specific destinations (Jung et al., 2012). In the retrograde pathway, VAMP3 and VAMP4 are localized to the TGN, but are proposed to function in vesicle fusion on the incoming vesicles (Johannes and Popoff, 2008). Syntaxins (Stx) 5, 6, 10, and 16 and the Qb-SNARE Vti1a are proposed to function in vesicle fusion at the TGN (Johannes and Popoff, 2008).

Stx6 is of particular interest due to its unique structure and many SNARE binding partners. This protein shares homology with both Qa and Qc SNAREs and has been classified as both (Jung et al., 2012). Stx6 has been reported to bind VAMPs 2, 3, 4, 7, and 8; Qa SNAREs Stx4, 7, and 16; Qb SNAREs Vti1a and Vti1b; and Qab SNAREs SNAP23, 25, and 29 (Wendler and Tooze, 2001). It localizes to the TGN and sorting endosomes and contains a Tyr-based sorting motif that regulates its retrograde transport from the plasma membrane to the TGN (Jung et al., 2012). Stx6 also binds to the Rab5 effector EEA1 and has been implicated in homotypic endosome fusion and endosomal recycling (Jung et al., 2012). In the TGN, Stx6 binds to the COG complex, which regulates Stx6 expression as well as the expression of the Stx6 SNARE partners Stx16, Vti1a, and VAMP4 (Jung et al., 2012). However, the role of Stx6 in regulating intracellular transport and the binding partners with which it interacts are cell type-specific (Jung et al., 2012).

### 1.1.3 Retrograde transport pathways

Membrane proteins endocytosed from the plasma membrane first pass through sorting endosomes marked by the regulatory proteins Rab5 and EEA1. From here, cargo proteins are sorted for degradation, for recycling, or for specialized storage compartments. These different sorting pathways have been characterized based on the traffic of model cargo proteins and resident regulatory molecules associated with each organelle. Here, I outline these retrograde sorting pathways and the regulatory molecules associated with each pathway.
1.1.3.1 Degradation

The degradative pathway is defined by cargo protein disposal in the lysosome (Figure 1.1). Sorting endosomes form from endocytosed membrane and continue to fuse with incoming vesicles as they mature through a process regulated by Rab5. Sorting endosomes mature into late endosomes as they lose Rab5 and acquire Rab7 through a process regulated by the HOPS (homotypic fusion and vacuole protein sorting) complex (Luzio et al., 2009). This maturation process occurs within about 20 min of formation of the sorting endosome (Maxfield and McGraw, 2004). The ESCRT (endosomal sorting complex required for transport) proteins are recruited to sorting endosomes and regulate the formation of MVBs as the endosomes mature into late endosomes (Luzio et al., 2009). Late endosomes acquire a Rab9 subdomain that directs traffic of proteins away from lysosome degradation (Stenmark, 2009). As they mature, late endosomes undergo homotypic fusion through the SNARE proteins VAMP8, Stx7, Vti1b, and Stx8 (Luzio et al., 2009). Mature late endosomes then fuse with lysosomes through the SNARE complex VAMP7, Stx7, Vti1b, and Stx8 (Luzio et al., 2009). Late endosomes that fuse with lysosomes are depleted of the cargo cation-dependent mannose-6-phosphate receptor (MPR), but it is unclear what identifies a late endosome as being ready for fusion (Luzio et al., 2009).

1.1.3.2 Constitutive recycling

Constitutive recycling is defined as the sorting of internalized cargo proteins and their traffic back to the plasma membrane. Depending on the cargo protein, different pathways are possible. First, cargo may be shuttled back to the plasma membrane directly from sorting endosomes through a fast recycling pathway dependent on Rab4 (Mizuno-Yamasaki et al., 2012) (i.e. sorting endosomes-to-plasma membrane transport). Alternatively, cargo proteins may traverse the canonical recycling pathway consisting of transport from sorting endosomes to recycling endosomes, and back to the plasma membrane (Figure 1.1) (i.e. recycling endosome-to-plasma membrane transport). Recycling endosomes are most often defined as being Rab11-positive and Rab5-negative, and are distinguished from sorting endosomes based on the kinetics of cargo protein traffic (Maxfield and McGraw, 2004). However, no single protein is present on recycling endosomes but absent from sorting endosomes. Moreover, in different cell types, recycling endosomes have been defined as subdomains of sorting endosomes (either vacuolar or tubular extensions) that form during endosome maturation as sorting centres (Bonifacino and Rojas, 2006; Chia et al., 2013; Sönnichsen et al., 2000). Transferrin Receptor (TfR) is the best
characterized cargo protein that undergoes constitutive recycling through recycling endosomes. TfnR internalized from the plasma membrane transits through sorting endosomes with a $t_{1/2}$ of approximately 2 min, and is sorted into recycling endosomes and back to the plasma membrane with a $t_{1/2}$ of 10 min (Maxfield and McGraw, 2004). Regulated transport of TfnR from recycling endosomes to the plasma membrane requires Rab11 (Maxfield and McGraw, 2004).

Some cargo proteins sort to the TGN in a retromer-dependent process through sorting, recycling, or late endosomes (Johannes and Wunder, 2011). From the TGN, cargo proteins may sort back to sorting endosomes or to the plasma membrane. Sorting endosome-to-TGN traffic has been characterized using the cargo protein TGN38/46 and the toxin Shiga toxin subunit B (STxB); this pathway is retromer-dependent and requires the SNARE complex Stx6, Stx16, Vti1a, and VAMP3/4 at the TGN (Johannes and Popoff, 2008; Lieu and Gleeson, 2010). The requirements for Golgi tethers depend on the cargo, with GCC88 being required for TGN38/46 and GCC185 for STxB (Johannes and Popoff, 2008). A role for the GARP complex has been suggested based on observations that this complex is recruited to sorting endosomes and then functions at the TGN to promote SNARE complex formation between Stx6, Stx16, and VAMP4 (Johannes and Wunder, 2011). Although not implicated in the sorting of STxB or TGN38/46, Rab22 is reported to regulate transport from sorting endosomes to the TGN while Rab14 regulates the reverse event (Galvez et al., 2012).

Recycling endosome-to-TGN transport has been described for both STxB (Lieu and Gleeson, 2010; McKenzie et al., 2012) and TGN38/46 (Chia et al., 2013). While the role of recycling endosomes in sorting TGN38/46 has been questioned, data demonstrating that STxB sorts through Rab11-positive recycling endosomes in a retromer-dependent fashion suggest that STxB may access the TGN through both sorting and recycling endosomes (Chia et al., 2013; Lieu and Gleeson, 2010; McKenzie et al., 2012). STxB and TGN38/46 retrograde transport depend on different Snx proteins in association with retromer (Lieu and Gleeson, 2010) and STxB has also been shown to require the Golgi tethers golgin-97 and golgin-245 (Johannes and Popoff, 2008). The utilization of independent transport pathways provides a possible explanation for the requirements of multiple Snx and tether proteins. However, some question remains about whether recycling endosomes represent an independent route of transport to the TGN based on the difficulty in defining recycling from sorting endosomes.
Late endosome-to-TGN transport has been described for the cargo proteins MPR and furin. Transport from late endosomes to the TGN is Rab9 dependent and requires the Golgi tether GCC185 (Chia et al., 2013). The SNARE complex Stx16/Stx10/Vti1a/VAMP3 is required for vesicle fusion – Stx10 is a human paralog of Stx6 (Chia et al., 2013). MPR traffic to late endosomes requires retromer (Johannes and Popoff, 2008).

1.1.3.3 Specialized storage compartments

In addition to the above traffic arms that are rather generalized in all cell types, there are specific instances of more cell type-specific and cargo-specific transport to and from ‘specialized’ storage compartments. Sorting into specialized storage compartments may proceed from recycling endosomes or the TGN, although this pathway is the least understood. By recycling through the TGN, cargo proteins can be re-packaged for subsequent transport to target membranes. Some cargo proteins are recycled from the TGN back into secretory vesicles, such as the Menkes proteins (Johannes and Popoff, 2008). Menkes proteins are components of the mammalian copper transport pathway which recycle between the plasma membrane and Golgi – but the rate of exit from the TGN is regulated by extracellular copper concentrations (Johannes and Popoff, 2008). In yeast, iron transporters and Atg9 (autophagy protein) cycle through retrograde transport but remain sequestered at the TGN until stimulated to translocate to the plasma membrane or autophagosomes, respectively (Johannes and Popoff, 2008). In some cell types, polarized cell secretion from storage vesicles and recycling back to such vesicles through the TGN maintains a dynamic pool of enzymes for angiogenesis (metalloproteases) or for cell division (yeast chitin synthase 3) (Johannes and Popoff, 2008). One protein of particular interest is the mammalian Glucose Transporter 4 (GLUT4), which is specifically sorted into storage compartments in muscle and adipose tissues. In myocytes and adipocytes, GLUT4 continuously recycles between the plasma membrane and intracellular stores that concentrate in a ‘specialized’ storage compartment. This compartment defines the ability of GLUT4 to respond to insulin and has therefore also been termed the insulin-responsive compartment (IRC). The IRC generates vesicles that translocate to the plasma membrane selectively in response to insulin (Foley et al., 2011). How GLUT4 sorts through the retrograde transport pathway into the insulin-responsive compartment/vesicles in an ongoing area of research and is the research topic of this Thesis.
1.2 Glucose Transporter 4 (GLUT4)

GLUT4 is a member of the SLC2 family of membrane transporters that encompasses 14 family members (Mueckler and Thorens, 2013). The SLC2 proteins are all predicted to be constituted by 12-transmembrane domains and to mediate facilitative sugar transport across the plasma membrane (Mueckler and Thorens, 2013). GLUT4 is one of two major glucose transporters expressed in skeletal muscle, cardiac muscle, and adipose tissue – the other being GLUT1. Whereas GLUT1 is ubiquitously expressed, GLUT4 expression is vastly restricted to muscle and adipose tissue (Mueckler and Thorens, 2013). Unique to GLUT4 is its intracellular distribution and regulation by insulin. Under resting conditions most GLUT4 molecules are maintained in intracellular compartments. Some of these compartments are in dynamic equilibrium with the plasma membrane such that GLUT4 continuously recycles. The IRC acts as a retention compartment from which GLUT4 can be mobilized to the cell surface in response to insulin [reviewed in (Foley et al., 2011)]. This recruitment of GLUT4 from the IRC to the cell surface in response to insulin is termed GLUT4 translocation. Insulin induces a 2 fold increase in cell surface GLUT4 in muscle cells and about a 10 fold increase in adipocytes (Govers et al., 2004; Karylowski et al., 2004; Ueyama et al., 1999). Upon insulin removal, GLUT4 must sort back into the IRC. GLUT4 is a long-lived protein (48 h half-life) such that a single GLUT4 molecule will undergo multiple cycles of translocation (Sargeant and Pâquet, 1993). As such, efficient mechanisms are required to sort GLUT4 into its intracellular compartments and to maintain its intracellular distribution.

Maintaining glucose homeostasis is essential for life. Skeletal muscle and adipose tissue serve as the major storage sites for glucose, and insulin is the major signal for glucose uptake into these tissues. GLUT4 translocation in response to insulin clears dietary blood glucose and is critical for the maintenance of glucose homeostasis. However, physiologically muscle is the major contributor to the clearance of blood glucose based on the enormous size of the muscle pool when compared to that of adipose tissue. In fat and muscle cells of obese or type 2 diabetic individuals or animal models, insulin resistance is characterized by impairment in the ability of insulin to induce GLUT4 translocation (Björnholm et al., 2002; Bogan, 2012; Foley et al., 2011; JeBailey et al., 2007; Karlsson et al., 2005; Summers et al., 1998). In the whole body this results in a reduced ability to regulate glucose homeostasis. Thus, understanding the regulation of
GLUT4 traffic is crucial to understanding how conditions of insulin resistance lead to defects in insulin-stimulated GLUT4 translocation.

Two main cellular models have been used to study the regulation of GLUT4: mouse 3T3-L1 adipocytes and rodent L6 skeletal muscle cells. These cell culture models allow for transient or stable transfection of tagged GLUT4, which can then be tracked throughout the cells. However, one must consider idiosyncratic differences inherent to the species, cell type, or mechanism of study. Although the regulation of GLUT4 translocation has been studied extensively in both muscle and adipose cells, the mechanism with which GLUT4 sorts into the IRC is mostly restricted to work in adipocytes. Importantly, 3T3-L1 preadipocytes do not contain specialized GLUT4 storage compartments – the sorting of GLUT4 into an insulin-regulated storage compartment is dependent on differentiation into 3T3-L1 adipocytes. Our current understanding of how GLUT4 maintains its intracellular distribution, how GLUT4 is sorted into the insulin-responsive compartment/vesicles, and how GLUT4 traffic is perturbed in models of insulin resistance are detailed in Chapter 1.2.

1.2.1 Distribution of GLUT4 in intracellular compartments

In skeletal muscle and adipose cells, GLUT4 localizes to cytosolic vesicles and as an accumulated pool in the perinuclear region – note that the term cytosolic is used throughout this thesis to describe cytoplasmic structures and not molecules soluble in the cytosol (Boguslavsky et al., 2012; Fujita et al., 2010; Larance et al., 2005; Randhawa et al., 2008). Through biochemical methods and immunofluorescence imaging, GLUT4 is found to populate multiple cellular compartments – these include the plasma membrane, sorting and recycling endosomes, the TGN, and a specialized insulin-responsive compartment (IRC), often termed insulin-responsive vesicles (IRV) or GLUT4-storage vesicles (GSV) (Hashiramoto and James, 2000; Kupriyanova et al., 2002; Larance et al., 2005; Perera et al., 2003; Shewan et al., 2003; Zaid et al., 2008; Zeigerer et al., 2002) (Figure 1.4A). It is important to note that the intracellular localization of the IRC is undefined. Also, although the terms IRC, IRV, and GSV, have been used interchangeably in the literature, it is unknown if an insulin-responsive storage compartment exists from which insulin-responsive vesicles bud or if the entire IRC is composed of pre-formed insulin-responsive vesicles. This confusion in nomenclature arises from methodology. IRC/IRV sometimes refers to the biochemically isolated endomembranes that
contain GLUT4, sometimes to actual GLUT4-containing vesicles detected by immunofluorescence and total internal reflection fluorescence (TIRF) microscopy, and sometimes to GLUT4 enriched regions within the cell. Functionally, any of these may contain both the storage compartment and the emanating vesicles destined for the plasma membrane. For the purpose of this thesis, I will use the terminology IRC to refer to the compartment where GLUT4 is destined to acquire insulin responsiveness and hence has been retrieved from the constitutively recycling pathway. IRV will be used to describe those vesicles in the cell periphery whose characteristics match those of the IRC and whose behaviours are regulated by insulin.

**Figure 1.4A**: Intracellular membranes in which GLUT4 resides. See text for details. Resident proteins reported to accumulate in each GLUT4-containing compartment are labelled.

In the resting adipocyte, 1-5% of the total cellular GLUT4 localizes to the plasma membrane (Blot and McGraw, 2008). In muscle cells this is about 10% (Li et al., 2001). The remaining GLUT4 undergoes both constitutive recycling and retention, resulting in the presence of GLUT4 in a number of intracellular organelles at steady-state. In particular, GLUT4 is found to distribute between sorting/recycling endosomes and the TGN, but from a functional perspective these or other locales must contain the IRC/IRV.

At steady-state, about 40-50% of GLUT4 resides in sorting/recycling endosomes based on GLUT4 localization to TfnR-containing membranes (Aledo et al., 1997; Martin et al., 2006; Shewan et al., 2000; Zaid et al., 2008; Zeigerer et al., 2002). GLUT4 also localizes to the TGN region in adipocytes and co-localizes with proteins known to traffic between the TGN and endosomes, including AP-1 and MPR (Kandror and Pilch, 1996; Martin et al., 2000a; Martin et al., 2000b; Slot et al., 1991). In particular, GLUT4 localization to the TGN is suggested from its
co-localization with Stx6 and Stx16 in perinuclear membranes in adipocytes (Larance et al., 2005; Li et al., 2009; Perera et al., 2003; Shewan et al., 2003). The TGN tether golgin-97 also co-localizes with GLUT4 in the perinuclear region and golgin-97 depletion dispersed Stx6 (Hatakeyama and Kanzaki, 2011). These data support the assertion that Stx6 and Stx16 localize in the TGN. However, GLUT4 does not localize to furin or TGN38/46 positive endomembranes, and these two proteins are bona fide resident markers of the TGN (Karylowski et al., 2004; Martin et al., 1994; Shewan et al., 2003). GLUT4 may occupy a sub-domain of the TGN enriched in Stx6 and Stx16, or perhaps Stx6 and Stx16 also localize to a unique compartment distinct from the TGN.

The existence of a physically separate compartment that houses GLUT4, is responsible for its detraction from constitutive recycling, and affords the transporter its susceptibility to be regulated by insulin, has been supported by a number of biochemical observations. Membrane fractionation analysis demonstrates that GLUT4 is enriched in TfnR-negative membrane fractions that are depleted of GLUT4 in response to insulin (Aledo et al., 1997; Hashiramoto and James, 2000; Livingstone et al., 1996). Furthermore, quantitative immuno-electron microscopy demonstrates that although GLUT4 partially co-localizes with MPR, GLUT4 vesicles that translocate to the plasma membrane in response to insulin do not contain MPR (Martin et al., 2000b).

Proteomics analysis of GLUT4 immuno-purified membranes from 3T3-L1 adipocytes identified VAMP2 and Insulin-Responsive Aminopeptidase (IRAP) as being enriched in GLUT4-containing membranes (Larance et al., 2005). VAMP2 is the R-SNARE required for IRV fusion with the plasma membrane in response to insulin (Ramm et al., 2000; Randhawa et al., 2000; Williams and Pessin, 2008). IRAP is a recycling receptor under near identical regulation as GLUT4 and has been reported to be essential for GLUT4 localization (Jordens et al., 2010; Keller, 2003). VAMP2 and IRAP co-localize with GLUT4 in both perinuclear and peripheral vesicles and translocate to the plasma membrane in response to insulin (Larance et al., 2005; Martin et al., 1996). However, because GLUT4 localizes to multiple endomembrane compartments, purifying GLUT4-containing vesicles alone cannot distinguish between the IRC/IRV and other organelles. In this respect, cellugyrin was identified as a component of GLUT4-containing vesicles that does not re-distribute in response to insulin (Kupriyanova and Kandror, 2000). Cellugyrin is a 4-transmembrane containing homologue of synaptogyrin (major
constituent of synaptic vesicles) with unknown function (Kupriyanova et al., 2002). Approximately 50% of GLUT4 is located in cellugyrin-negative membranes which are also depleted of TfnR (Kupriyanova et al., 2002). Because cellugyrin and TfnR accumulate in GLUT4-containing compartments that do not respond to insulin, the IRC/IRV are represented in the remaining 50% of GLUT4. Performing proteomics on GLUT4 immuno-purified vesicles that were first depleted of cellugyrin-containing endomembranes allowed for analysis of GLUT4 vesicles that were enriched for the IRC/IRV (Jedrychowski et al., 2010). IRAP, VAMP2, sortilin, and low density lipoprotein receptor related protein 1 (LRP1) were abundant on the GLUT4-containing membranes enriched for IRC/IRV and translocated to the plasma membrane in response to insulin (Jedrychowski et al., 2010). Sortilin and LRP1 are required for GLUT4 entry into the IRC/IRV, as discussed in Chapter 1.2.4.3. Thus, insulin-responsive GLUT4 endomembranes that are enriched with selective proteins supports the existence of a specialized compartment.

A key impasse in the understanding of GLUT4 traffic is that the intracellular localization of the IRC/IRV is unknown. Although IRAP, VAMP2, sortilin, and LRP1 are enriched in the IRC/IRV, no protein has been identified that can serve as an independent marker of the IRC/IRV. Interestingly, the TGN-resident proteins Stx6 and Stx16 have received special attention as possible components of the IRC. As mentioned above, Stx6 and Stx16 co-localize with GLUT4 in the perinuclear region, presumably in the TGN (Hatakeyama and Kanzaki, 2011; Larance et al., 2005; Li et al., 2009; Shewan et al., 2003). However, perinuclearly localized GLUT4 mobilizes to the cell periphery in response to insulin (Boguslavsky et al., 2012; Fujita et al., 2010; Randhawa et al., 2008; Yu et al., 2007). Although these experiments could not determine if the GLUT4 mobilized from the perinuclear region reaches the plasma membrane, these results suggest that a component of the Stx6- and Stx16-positive perinuclear compartment may consist of the IRC. This hypothesis is supported by two observations. First, GLUT4 partially co-localizes with sortilin in the perinuclear region (Shi and Kandror, 2007). Second, Stx6 (Perera et al., 2003; Shewan et al., 2003) and Stx16 (Shewan et al., 2003) translocate to the plasma membrane in response to insulin. Thus, it may be that Stx6 and Stx16 concentrate in the TGN but also partially localize to the IRC in the perinuclear region.

The IRC is also proposed to include IRV which localize in the cell periphery and respond to insulin. In extracts from fat and muscle cells, GLUT4 mainly localized to small membrane
vesicles (Xu and Kandror, 2002). In response to insulin, GLUT4 vesicles in the cell periphery are preferentially depleted over perinuclearly localized GLUT4 (Larance et al., 2005). These observations suggest that IRV are a major regulated pool of GLUT4. Using TIRF microscopy, highly mobile GLUT4 vesicles are observed within 100 nm of the plasma membrane that rarely fuse with the plasma membrane in resting 3T3-L1 adipocytes (Bai et al., 2007; Lizunov et al., 2005). In response to insulin, however, these vesicles slow in their movements and become much more prone to fusion with the plasma membrane (Bai et al., 2007; Foley et al., 2011; Huang et al., 2007). Based on this insulin-dependent change in vesicle behaviour, it is assumed that these vesicles are bona fide IRV destined for fusion with the plasma membrane. Further studies have examined GLUT4 vesicle behaviour in the TIRF zone using VAMP2 or IRAP as markers of the IRV. These studies confirm that insulin promotes the fusion of these vesicles with the plasma membrane but also that insulin treatment recruits additional IRV to the TIRF zone (Chen et al., 2012; Xiong et al., 2010; Xu et al., 2011). In particular, it was estimated that IRV are of smaller diameter than recycling endosomes in the TIRF zone (Xu et al., 2011). IRV may be in dynamic equilibrium with a perinuclear IRC such that the IRC functions to retain GLUT4 in intracellular membranes and to replenish IRV in response to insulin. However, IRV may also bud directly from endosomal or TGN membranes such that the IRV are the functional IRC. Identifying how GLUT4 acquires insulin-responsiveness and the endomembrane compartments in which this occurs will be key in defining the IRC and IRV beyond functional characterization.

1.2.2 GLUT4 retention

A key aspect of GLUT4 regulation is that in resting cells GLUT4 is retained in intracellular membranes. Current models suggest that GLUT4 is retained in the IRC or in IRV such that GLUT4 contained in these compartments is not accessible to the plasma membrane (Brewer et al., 2011; Foley et al., 2011; Jordens et al., 2010; Stöckli et al., 2011). Localization to retention compartments also stabilizes GLUT4 expression, as GLUT4 localization to constitutively recycling endosomes promotes GLUT4 degradation (Shi and Kandror, 2005). Multiple proteins have been identified that regulate the retention of GLUT4, including Akt substrate of 160 kDa (AS160), tether containing a UBX domain for GLUT4 (TUG), the small ubiquitin-related modifier conjugating enzyme Ubc9, Golgi-localized gamma adaptin ear-containing Arf-binding protein (GGA), sortilin, LRP1, golgin-160, p115, and IRAP [reviewed in (Foley et al., 2011)]. Perturbing GLUT4 retention in the IRC/IRV should shift GLUT4 localization from the IRC/IRV
to constitutively recycling endosomes. As a result, an increase in cell surface GLUT4 and an increase in GLUT4 degradation should be observed. Interestingly, TUG is the only regulator of GLUT4 retention whose transient depletion increases cell surface GLUT4 and reduces GLUT4 expression (Yu et al., 2007). Depleting AS160 or IRAP increases cell surface GLUT4 in resting cells and shifts GLUT4 into recycling endosomes without affecting GLUT4 expression (Eguez et al., 2005; Jordens et al., 2010). However, both AS160 knockout mice (Lansey et al., 2012; Wang et al., 2013) and IRAP knockout mice (Keller et al., 2002) have reduced GLUT4 expression in adipose and muscle tissues. Perhaps the effects of AS160 or IRAP knockdown on GLUT4 expression take longer to manifest than those for TUG. On the other hand, Ubc9, sortilin, or LRP1 knockdown reduces GLUT4 expression without increasing cell surface GLUT4 in resting cells (Jedrychowski et al., 2010; Kim and Kandror, 2012; Liu et al., 2007; Shi and Kandror, 2005). These results suggest that the mechanism of GLUT4 retention is complex and cannot be deciphered through simple examination of cell surface GLUT4 and GLUT4 stability. As outlined below, AS160 and TUG (in conjunction with golgin-160 and possibly p115) appear to be the major regulators of GLUT4 retention while Ubc9, GGA, sortilin, LRP1, and IRAP affect GLUT4 retention by regulating GLUT4 sorting (Figure 1.4B).

![Figure 1.4B](image)

**Figure 1.4B:** Differentiating proteins involved in GLUT4 retention from proteins involved in sorting GLUT4 into the IRC/IRV. See text for details.

In both muscle cells and adipocytes, AS160 (aka TBC1D4) is a major regulator of GLUT4 retention. AS160 is a Rab GAP with activity against Rabs 2, 8A, 10, and 14 *in vitro* (Miine et al., 2005). AS160 retains GLUT4 intracellularly by preventing the activation of IRC/IRV resident Rab proteins necessary for GLUT4 mobilization to the plasma membrane (Eguez et al.,
Rab10 in adipocytes (Sano et al., 2007; Sano et al., 2008) and Rabs 8A and 13, and possibly 14, in muscle cells (Ishikura and Klip, 2008; Ishikura et al., 2007; Sun et al., 2010) appear to be the relevant AS160 targets for insulin-responsive GLUT4 translocation. Insulin causes the phosphorylation of AS160 on 6 residues which inactivates its GAP activity to release GLUT4 retention (Sano et al., 2003). Expression of an AS160 mutant in which 4 of these phosphorylation sites are mutated to alanine (AS160-4A) blocks insulin-stimulated GLUT4 translocation (Sano et al., 2003; Thong et al., 2007) whereas knockdown of AS160 increases cell surface GLUT4 in resting muscle cells and adipocytes (Eguez et al., 2005; Sano et al., 2007; Thong et al., 2007). Based on these observations, the model that has developed characterizes AS160 as a “brake” for GLUT4 recruitment to the plasma membrane (Zaid et al., 2008). AS160 keeps GLUT4 sequestered in the IRC/IRV until insulin acts to release the “brake” by phosphorylating AS160 to inhibit its GAP activity. Thus, although AS160 functions in GLUT4 retention, it acts not as a physical retainer of GLUT4 but as a signal for the availability of GLUT4 to respond to insulin.

In adipocytes, the protein TUG was discovered as an additional regulator of GLUT4 retention (Bogan et al., 2003; Yu et al., 2007). TUG binds to GLUT4 through N-terminal and central domains and acts to tether GLUT4 through its C-terminal domain (Bogan et al., 2003; Yu et al., 2007). Thus, unlike AS160, TUG physically retains GLUT4. TUG co-localizes with GLUT4 in the perinuclear region and upon expressing the C-terminal fragment of TUG (TUG UBX-Cterm) GLUT4 and Stx6 are dispersed (Bogan et al., 2003; Bogan et al., 2012; Yu et al., 2007). Insulin stimulates endoproteolytic cleavage of TUG to release GLUT4 retention and allow GLUT4-containing vesicle recruitment to the plasma membrane (Bogan et al., 2012). Interfering with TUG using siRNA or by over-expressing TUG UBX-Cterm increases cell surface GLUT4 in resting cells, depletes GLUT4 from IRC/IRV-containing membrane fractions, and inhibits insulin-stimulated GLUT4 translocation (Bogan et al., 2003; Yu et al., 2007). Furthermore, TUG disruption promotes GLUT4 degradation (Yu et al., 2007). Together, these data support a model in which TUG sequesters GLUT4 in the IRC/IRV, which at least partially localizes to the perinuclear region. Insulin releases GLUT4 retention by disrupting TUG-GLUT4 binding. IRV then move towards and fuse with the plasma membrane as directed by Rab effectors that are activated upon AS160 inhibition.
Recently, the function of TUG in GLUT4 retention has been linked to the proteins PIST, golgin-160, and the GTPase TC10α (Bogan et al., 2012). TC10α is activated by insulin (Chiang et al., 2001; Okada et al., 2008). PIST, an effector of TC10α, interacts with GLUT4 through TUG and with the Golgi matrix through golgin-160 (Bogan et al., 2012). It is proposed that PIST anchors TUG/GLUT4 membranes to the Golgi matrix through golgin-160 and that insulin signalling through TC10α promotes TUG cleavage to release IRV from Golgi anchoring (Bogan et al., 2012). p115 shares a similar distribution as golgin-160 and regulates GLUT4 distribution and translocation (Hosaka et al., 2005). Over-expressing the N-terminal fragment of p115 disperses GLUT4 and blocks insulin-stimulated GLUT4 translocation (Hosaka et al., 2005). It is possible that p115 functions in tethering GLUT4 at the Golgi matrix in conjunction with golgin-160. A similar mechanism is proposed to exist in muscle (Loffler et al., 2013). As in adipocytes, TUG binds to GLUT4 isolated from skeletal muscle and insulin promotes their dissociation (Schertzer et al., 2009). Over-expression of TUG UBX-Cterm in mouse muscle also inhibits the ability of endogenous TUG to sequester GLUT4; TUG UBX-Cterm expression promotes the endoproteolytic cleavage of endogenous TUG and increases cell surface GLUT4 in resting muscle (Loffler et al., 2013).

### 1.2.3 GLUT4 mobilization in response to insulin

#### 1.2.3.1 Insulin signalling cascade

AS160 and TUG are positioned to maintain GLUT4 in the IRC/IRV. Insulin acts on these proteins to release GLUT4 retention, but how does insulin binding to its receptor at the plasma membrane trigger IRV release? Insulin-derived signals must impinge on one or more traffic steps that regulate GLUT4 distribution (Figure 1.5A). Upon binding, insulin elicits tyrosine autophosphorylation of the Insulin Receptor, which generates binding sites for IRS-1 (Insulin Receptor Substrate -1) (Myers and White, 1993; Myers et al., 1995). The tyrosine kinase activity of the receptor catalyzes IRS-1 tyrosine phosphorylation at multiple sites (Myers and White, 1993; Myers et al., 1995). At the plasma membrane, phosphorylated IRS-1 binds class I phosphatidylinositol-3-kinase (PI3K), which rapidly phosphorylates PI(3,4)P₂ to generate PI(3,4,5)P₃ [reviewed in (Asano et al., 2007)]. The kinase Akt2 is recruited to the plasma membrane through interaction of its PH domain with PI(3,4,5)P₃, where it is phosphorylated on two residues (Asano et al., 2007). The kinase PDK (PI3K dependent kinase) phosphorylates T308 and mTORC2 phosphorylates S473 to fully activate Akt2 (Asano et al., 2007; Humphrey et
Akt2 then localizes to a number of endomembranes in addition to the plasma membrane, notably GLUT4-containing endomembranes (Calera et al., 1998; Gonzalez and McGraw, 2009). Phosphorylated Akt2 acts on a number of substrates, and of key importance in GLUT4 translocation is AS160 (Humphrey et al., 2013; Zaid et al., 2008). Akt2 phosphorylation of AS160 inhibits its GAP activity and allows Rab proteins associated with the IRC/IRV to become activated [reviewed in (Zaid et al., 2008)].

Each of the above signals are required for GLUT4 translocation to the plasma membrane in response to insulin, as evinced by use of selective inhibitors (of the receptor tyrosine kinase activity, PI3K, and Akt2), dominant negative mutants (of all the proteins in the above signalling cascade), or gene expression knockdown or gene knockout of IRS-1, PI3K, Akt2, and selective Rab GTPases (Cho et al., 2001; Eguez et al., 2005; Gonzalez and McGraw, 2006; Hausdorff et al., 1999; Ishikura et al., 2007; Jiang et al., 2003; Kido et al., 2000; Lansey et al., 2012; Okada et al., 1994; Sanchez-Margálet et al., 1994; Sano et al., 2003; Sano et al., 2007; Sun et al., 2010; Terauchi et al., 1999; Wang et al., 1998b; Zeigerer et al., 2004). However, the above pathway is likely incomplete, and indeed based on the incomplete accounting of all of Akt2 signalling through AS160, it is apparent that additional Akt targets must contribute to relating the signal to GLUT4 and are still awaiting identification (Bai et al., 2007; Brewer et al., 2011; Chen et al., 2011; Humphrey et al., 2013).

**Figure 1.5A:** Insulin signalling cascade to AS160 that releases the retention of GLUT4-containing IRC/IRV. See text for details.

In addition, insulin regulates the actin cytoskeleton to support or synergize with the above signalling cascade. This cytoskeletal contribution operates in both muscle and adipose cells but
its regulation is more amply characterized in muscle cells and mature muscle [reviewed in (Chiu et al., 2011)]. Downstream of PI(3,4,5)P_3 there is activation of the Rho GTPase Rac1 which initiates a dynamic cycle of actin branching and depolymerisation. All elements of this actin remodelling response are found necessary for insulin-stimulated GLUT4 translocation (Chiu et al., 2010; Chiu et al., 2011). The Rac1 signalling pathway has also been validated in the regulation of insulin dependent glucose uptake into mature skeletal muscle (Sylow et al., 2013; Ueda et al., 2010).

1.2.3.2 Recruitment of GLUT4 to the plasma membrane from IRC/IRV

As outlined above, insulin signals inhibit AS160, leading to the activation of Rab10 in adipocytes and Rab8A and Rab13 in muscle cells. Rab protein activation leads to the recruitment of, as of yet, unknown effectors that are necessary for IRC/IRV mobilization to the plasma membrane. In addition, insulin signals additional mechanisms to increase the efficiency of IRV tethering and fusion with the plasma membrane. The mechanisms through which IRV are mobilized to, and fuse with, the plasma membrane are active areas of research, with numerous proteins being implicated in these processes (Figure 1.5B).

Evidence suggests that IRV move along microtubules towards the cell periphery, at least in adipocytes [as reviewed in (Zaid et al., 2008)] – microtubule integrity is required for insulin-stimulated GLUT4 translocation (Karylowski et al., 2004) and the kinesin motors KIF3 and KIF5B have been reported to mediate GLUT4 translocation (Imamura et al., 2003; Semiz et al., 2003). In muscle, the requirement of microtubules has been questioned; microtubule disrupting agents did not inhibit insulin-stimulated glucose uptake in isolated muscles (Ai et al., 2003). Once in the periphery, IRV may transition onto actin filaments. This hypothesis is based on reports that dynamic actin filaments (regulated by Rac1) are required for GLUT4 translocation but not for IRV accumulation in the cell periphery (Lopez et al., 2009; Randhawa et al., 2008). Myosin Va, a processive actin based motor protein, is required for GLUT4 translocation (Chen et al., 2012; Yoshizaki et al., 2007). In addition, myosin 1c, which is single headed and non-processive, also regulates GLUT4 translocation in adipocytes and muscle cells through an undefined mechanism (Boguslavsky et al., 2012; Bose et al., 2002; Bose et al., 2004). Together, these results support a mechanism through which regulated actin dynamics act to transport the IRV along actin filaments to position them for tethering to the plasma membrane.
Figure 1.5B: Regulation of insulin-stimulated GLUT4 translocation. The roles for regulatory proteins in mediating GLUT4 translocation are outlined. Akt and AS160 regulate a pre-fusion step at the plasma membrane, but their exact roles are unknown. See text for details.

At the plasma membrane, Akt2 and AS160 regulate pre-fusion steps in GLUT4 translocation, although the exact mechanisms are unknown (Bai et al., 2007; Brewer et al., 2011; Ng et al., 2010; Tan et al., 2012). Several proteins have been reported to regulate IRV tethering to the plasma membrane, but there is still a lack of agreement on their precise participation [reviewed in (Foley et al., 2011)]. Manipulation of phosphoinositides may recruit tethering factors such as actinin-4 and the exocyst complex to facilitate IRV tethering [reviewed in (Foley et al., 2011; Zaid et al., 2008)]. Fusion of IRV at the plasma membrane requires the SNARE complex of VAMP2, SNAP23, and Stx4 (Klip, 2009). The formation of this SNARE complex is regulated by the protein Munc18c (Jewell et al., 2011). Thus, the recruitment of GLUT4 to the plasma membrane in response to insulin is a complex process about which our understanding is continually evolving.

Complementing the intricacies of GLUT4 traffic to the plasma membrane, the transporter must be retrieved back to the IRC/IRV in order to re-acquire insulin responsiveness. Strikingly, the mechanism whereby internalized GLUT4 is sorted back to an insulin-sensitive storage compartment is very scantily analyzed, and is the research subject of this Thesis.

1.2.4 Dynamics of GLUT4 sorting

1.2.4.1 GLUT4 sorting motifs

GLUT4 contains several sorting motifs that dictate its intracellular sorting and distribution (Figure 1.6). There is an N-terminal motif (F5QQI), the cytoplasmic loop region between trans-
membrane helices six and seven (TM6-7 loop), and three C-terminal motifs (TE^{499}LE^{501}Y, LL^{490} and the insulin-responsive motif – IRM L^{500}XXLXPDEX(D/E)^{509}). The IRM and TELEY motifs partially overlap, although the essential residues for each motif are distinct. Mutations in these motifs alter GLUT4 dynamics, although some discrepancies have been reported with respect to the contributions of each sorting motif to regulating GLUT4 traffic in basal versus insulin-stimulated cells, as outlined below.

The FQQI motif is a member of the aromatic-based internalization motif family that has been implicated in regulating endocytosis and targeted intracellular traffic (Blot and McGraw, 2006; Bonifacino and Traub, 2003). It is similar to the tyrosine-based sorting motif (YXXØ) found in TfnR, but is less efficient in directing clathrin-mediated endocytosis (Blot and McGraw, 2006). In adipocytes, the N-terminal phenylalanine F^5QQI motif is required for efficient endocytosis, for intracellular sorting, and for basal retention of GLUT4 (Al-Hasani et al., 2002; Blot and McGraw, 2006; Blot and McGraw, 2008; Capilla et al., 2007). Mutating the FQQI motif to AQQI (FA mutant) reduces GLUT4 endocytosis and results in more (~4x) GLUT4 being directed to the plasma membrane in resting cells (Al-Hasani et al., 2002; Blot and McGraw, 2008; Capilla et al., 2007; Govers et al., 2004). Interestingly, the FA mutant does not display altered intracellular distribution by immunofluorescence inspection (Blot and McGraw, 2008; Song et al., 2008). This is in spite of evidence that the FA mutant shifts its localization from the IRC/IRV to constitutively recycling endosomes (Blot and McGraw, 2008; Xiong et al., 2010). In adipocytes the TfnR-positive recycling endosomes concentrate in the perinuclear region. Therefore, it may be that the shift in GLUT4 localization is not readily detectable by visual inspection. In addition, the degree to which the FA mutant inhibits insulin-stimulated GLUT4 translocation is unclear. Insulin stimulates at least the same level of cell surface GLUT4 in

**Figure 1.6:** Cartoon schematics of GLUT4 linear sequence and 2-dimentional folded structure showing localization of key sorting motifs. FQQI, TM6-7 loop, LL, and TELEY sorting motifs are highlighted by red boxes. The IRM is outlined by the black box. See text for details.
adipocytes expressing the FA mutant as in those expressing WT GLUT4 (Al-Hasani et al., 2002; Blot and McGraw, 2008; Capilla et al., 2007; Govers et al., 2004). But because the FA mutant increases cell surface GLUT4 in resting cells, the fold response in insulin-stimulated GLUT4 translocation has been reported to be inhibited (Al-Hasani et al., 2002; Capilla et al., 2007) and unaffected (Blot and McGraw, 2008; Govers et al., 2004). One report examined the ability of internalized GLUT4 to respond to a second insulin stimulus 4 h after being internalized; the FA mutant failed to display insulin-stimulated translocation (Capilla et al., 2007). Together, these data show that the FQQI motif directs GLUT4 sorting away from constitutively recycling endosomes such that GLUT4 is retained intracellularly. Some evidence indicates that the FQQI motif directs GLUT4 into the IRC/IRV and is necessary for insulin-stimulated GLUT4 translocation. However, the role of the FQQI motif in regulating GLUT4 dynamics may be cell type specific, as this motif was reported to be disposable for GLUT4 sorting and retention in L6 muscle cells (Haney et al., 1995). Given the more recent findings in 3T3-L1 adipocytes, a role for the FQQI motif in muscle cells may warrant re-visiting.

The large cytoplasmic loop of GLUT4, located between trans-membrane helices 6-7, is required for biosynthetic sorting of GLUT4 into the IRC/IRV, for endocytosis of GLUT4, and for AS160 dependent regulation of insulin-stimulated GLUT4 translocation in adipocytes (Capilla et al., 2007; Khan et al., 2004). These conclusions are based on studies in which GLUT1-GLUT4 chimeras were expressed in 3T3-L1 adipocytes. A chimera in which the TM6-7 loop of GLUT1 was replaced with that of GLUT4 could gain GLUT4-like retention and insulin-responsiveness (Capilla et al., 2007; Khan et al., 2004). Likewise, replacing the TM6-7 loop of GLUT4 with that of GLUT1 resulted in a loss of GLUT4 retention and insulin-responsiveness (Capilla et al., 2007; Khan et al., 2004). The exact sorting motif(s) located in this loop is unknown, and it may be that multiple GLUT4-interacting proteins bind GLUT4 in this region. For example, both TUG and the protein ACAP1 bind GLUT4 at the cytoplasmic loop (Li et al., 2007; Yu et al., 2007). How the interactions between this GLUT4 cytoplasmic loop and regulatory proteins mediate the functions attributed to the cytoplasmic loop remain to be identified.

In adipocytes, the TELEY motif has defined roles in GLUT4 sorting and retention (Blot and McGraw, 2008; Shewan et al., 2003). Mutating the TELEY motif to TALAY (EEAA mutant) results in a small increase (~2x) in cell surface GLUT4 and reduced insulin-stimulated GLUT4 translocation, without altering the intracellular distribution of GLUT4 by immunofluorescence
detection (Blot and McGraw, 2008). However, using a fluorescence quenching assay, 84% of GLUT4 localized to TfnR-containing membranes in cells expressing the EEAA mutant of GLUT4, compared to 44% of WT GLUT4 (Shewan et al., 2000). As with the FA mutant, the localization of the TfnR-positive recycling endosomes to the perinuclear region may explain why the EEAA mutant does not appear dispersed upon visual inspection. The increase in cell surface GLUT4 and reduced insulin-responsiveness supports a model in which the TELEY motif regulates GLUT4 sorting into the IRC/IRV and therefore GLUT4 retention. However, such a large shift in GLUT4 distribution to TfnR-containing membranes would predict a larger increase in cell surface GLUT4 unless other mechanisms function to maintain GLUT4 retention.

Mutating both FQQI and TELEY motifs has an additive effect on increasing cell surface GLUT4 (Blot and McGraw, 2008). Thus, multiple sorting motifs may act independently to ensure GLUT4 sequestration. As GLUT4 retention is functionally linked to insulin-responsiveness, these sorting motifs act to ensure insulin-responsive GLUT4 translocation.

The LL motif has been linked to GLUT4 sorting from constitutive recycling endosomes into retention compartments immediately following insulin removal (Blot and McGraw, 2006; Blot and McGraw, 2008). However, mutating the LL motif (LA mutant) does not alter cell surface GLUT4 under steady-state basal or insulin conditions (Blot and McGraw, 2008; Capilla et al., 2007). Of the four identified GLUT4 sorting motifs, the LL motif has thus far been the least important as assessed by mutational analysis.

The IRM is essential for maintaining GLUT4 distribution and insulin-stimulated GLUT4 translocation (Song et al., 2008). Unlike mutations to the FQQI or TELEY motifs, mutating the IRM not only abrogates insulin-stimulated GLUT4 translocation but drastically alters the localization of GLUT4 to an undefined compartment that is distinct from the IRC/IRV or constitutively recycling endosomes (Song et al., 2008; Song et al., 2013). Furthermore, less GLUT4 is present in the plasma membrane (Song et al., 2008). Recent characterization of the IRM mutant compartment suggests that GLUT4 is diverted to a “dead end” pathway where it is unable to reach the IRC/IRV (Song et al., 2013). This drastic phenotype may point to a role for the IRM in biosynthetic sorting or overall GLUT4 stability and not necessarily in GLUT4 sorting through the endomembrane system. Interestingly, the observed phenotype of the IRM mutant is more drastic than that observed after replacing the last 12-30 amino acids of GLUT4 with that of GLUT3 or cellugiyn – these chimeras mis-localize and display higher cell surface expression in
resting adipocytes but maintain insulin-responsive translocation (Govers et al., 2004; Li et al., 2009). Thus, the C-terminus of GLUT4 plays a role in targeting and retention, but in what capacity each sorting motif acts is uncertain. In L6 muscle cells the C-terminal 25 amino acids (484-509), which encompass all three C-terminal sorting motifs, were sufficient for GLUT4 retention and insulin-responsiveness (Haney et al., 1995). Whether muscle cells and adipocytes utilize these sorting motifs in the same manner is unknown.

1.2.4.2 Mechanisms of GLUT4 endocytosis

Membrane endocytosis is a fundamental process to maintain a balance with exocytic functions. However, this is not a generalized membrane phenomenon, as retrieval of membrane proteins from the plasma membrane is selective and regulated. In particular, membrane proteins are retrieved from the membrane in the form of vesicular cargo, and this occurs through two major types of processes: clathrin-mediated endocytosis or one of many clathrin-independent pathways.

As a constitutively recycling protein, the permanence of GLUT4 at the plasma membrane must be limited, even in the presence of insulin. Surprisingly, the routes of GLUT4 endocytosis are different in adipose and muscle cells. In resting adipocytes, GLUT4 endocytosis occurs mainly through a cholesterol-dependent pathway that requires caveolae and VAMP8 (Blot and McGraw, 2006; Shigematsu et al., 2003; Williams and Pessin, 2008). GLUT4 also enters the cell via clathrin-mediated endocytosis, mediated at least in part by the FQQI sorting motif (Bernhardt et al., 2009; Blot and McGraw, 2006). Insulin inhibits GLUT4 endocytosis through the cholesterol-dependent pathway such that GLUT4 internalization shifts to the clathrin-mediated endocytosis pathway (Blot and McGraw, 2006). Clathrin-mediated endocytosis of GLUT4 is slow due to the sub-optimal nature of the FQQI motif, which contributes to an apparent decrease in the rate of GLUT4 endocytosis in the presence of insulin (Blot and McGraw, 2006).

In L6 muscle cells, GLUT4 is endocytosed via clathrin-mediated and cholesterol-dependent pathways, although the cholesterol pathway is caveolae independent (Antonescu et al., 2008). Evidence from VAMP8-null mice suggests that VAMP8 may also regulate GLUT4 endocytosis in muscle, as these mice display elevated cell surface GLUT4 (Zong et al., 2011). Unlike in resting adipocytes, GLUT4 does not display a preference for its route of internalization in resting L6 cells (Antonescu et al., 2008). In the presence of insulin, neither the rate nor mode of GLUT4 endocytosis appears to be altered (Antonescu et al., 2008).
1.2.4.3 Sorting of GLUT4 into constitutive recycling and insulin-responsive compartments

Upon internalization, GLUT4 must be differentially sorted between constitutively recycling endosomes and retention compartments. Here, I will summarize current findings with regards to GLUT4 sorting and offer a model from which to build our understanding of the mechanisms regulating GLUT4 sorting into the IRC/IRV.

1.2.4.3.1 Models of GLUT4 retention

Two mechanisms have been proposed to model how the intracellular distribution of GLUT4 is maintained by inter-endomembrane transport. Although not mutually exclusive, elements exist in each model that are incompatible with the other. The most important of these elements is the question regarding the degree of membrane exchange between the IRC/IRV and constitutively recycling endosomes in a resting cell. Addressing this question is essential, as the interpretation of data examining GLUT4 sorting into the IRC/IRV is dependent on the accessibility of GLUT4 to each endomembrane compartment.

1.2.4.3.1.1 Dynamic Retention model

First, in the dynamic retention model, the total population of GLUT4 is accessible to the plasma membrane in resting cells (Figure 1.7). This model is experimentally supported by measurements showing that the entire complement of cellular GLUT4 gains exposure to the cell surface over time in 3T3-L1 adipocytes (Karylowski et al., 2004) and L6 muscle cells (Foster et al., 2001). A key observation was that GLUT4 internalized from the plasma membrane equilibrated with constitutively recycling (Tfn-positive) and retention (Tfn-negative) compartments more quickly than predicted by the kinetics of GLUT4 exposure to the cell surface (Karylowski et al., 2004). Thus, it was proposed that GLUT4 retention compartments undergo bidirectional membrane exchange with recycling endosomes but very rarely fuse with the plasma membrane (Karylowski et al., 2004). Through this mechanism, GLUT4 is retained in intracellular stores yet is able to recycle to the plasma membrane through slow membrane exchange between the IRC/IRV and the plasma membrane. Insulin increases the rate of GLUT4 exocytosis from the IRC/IRV to the plasma membrane.
Figure 1.7: Dynamic retention model. See text for details. TELEY is a GLUT4 C-terminal sorting motif. FQQI is a GLUT4 N-terminal sorting motif. Proteins labelled in boxes define intracellular compartments. Stx6, Stx16, and IRAP are positive regulators of GLUT4 sorting into the IRC/IRV. AS160 retains GLUT4 in the IRC/IRV, regulating its availability to respond to insulin. TfnR, Transferrin Receptor; Stx6, Syntaxin-6; Stx16, Syntaxin-16; IRAP, Insulin-Responsive Aminopeptidase; AS160, Akt-substrate of 160 kDa (TBC1D4).

That the GLUT4 retention compartment consists of TGN and IRC/IRV components stems from work examining GLUT4 sorting mutants. As mentioned above, both the FA and EEAA GLUT4 mutants localize to a greater extent with Tfn-containing endosomes than WT GLUT4 and these mutations are additive in increasing cell surface GLUT4 in resting adipocytes (Blot and McGraw, 2008; Shewan et al., 2000). In addition, the FA mutant, but not the EEAA mutant, is additive to AS160 knockdown in increasing cell surface GLUT4 in adipocytes (Blot and McGraw, 2008). It was proposed that the TELEY motif regulates GLUT4 sorting into the IRC/IRV (since the EEAA mutant is no longer under control of AS160) and the FQQI motif regulates GLUT4 retention in a non-TfnR, non-IRC/IRV compartment that may consist of the TGN. Upon defects in sorting, GLUT4 defaults to the donor compartment for IRC/IRV formation, in this case recycling endosomes. Since approximately 80% of all GLUT4-containing membranes also contain IRAP, IRAP likely localizes to both the TGN and IRC/IRV retention compartments (Blot and McGraw, 2008; Zeigerer et al., 2002).

1.2.4.3.1.2 Static retention model

In the static retention model, the GLUT4 retention compartments do not exchange membrane with the constitutively recycling endosomes – GLUT4 is maintained in 2 distinct cycles (Figure 1.8). Evidence for this model originated from work showing that less than 30% of GLUT4 reaches the cell surface in basal 3T3-L1 adipocytes, but yet greater that 70% of GLUT4 reaches the cell surface over the same time course in insulin treated cells (Govers et al., 2004). It was concluded that insulin increased the amount of GLUT4 in the recycling pathway. GLUT4 was
released from retention and into the recycling pool in quantile amounts that was dependent on insulin concentration (Govers et al., 2004). A second study confirmed that insulin acts to increase the amount of GLUT4 in the actively recycling pool, but also to increase the rate of exocytosis of the recycling pool (Coster et al., 2004). Thus, in the static retention model insulin has two functions: increasing the size of the recycling GLUT4 pool and increasing the rate of exocytosis of the recycling GLUT4 pool.

**Figure 1.8:** Static retention model. See text for details. Sorting from the constitutive recycling pathway into the retention compartments occurs transiently (blue arrows) after insulin removal to sequester GLUT4 back into the IRC/IRV such that basal GLUT4 distribution can be restored. Proteins labelled in boxes define intracellular compartments. As in the dynamic retention model, Stx6, Stx16, and IRAP are positive regulators of GLUT4 sorting into the IRC/IRV. AS160 retains GLUT4 in the IRC/IRV, regulating its availability to respond to insulin. TfnR, Transferrin Receptor; Stx6, Syntaxin-6; Stx16, Syntaxin-16; IRAP, Insulin-Responsive Aminopeptidase; AS160, Akt-substrate of 160 kDa (TBC1D4).

Originally, it was not known if GLUT4 release into the recycling pathway occurred between the IRC/IRV and recycling endosomes or between the IRC/IRV and the plasma membrane. However, considerable evidence now demonstrates that insulin releases GLUT4 retention by recruiting GLUT4 from the IRC/IRV to the plasma membrane directly (Chen et al., 2012; Fujita et al., 2010; Habtemichael et al., 2011; Sano et al., 2007; Xiong et al., 2010). Together, these studies show that GLUT4-containing vesicles that demonstrate IRV behaviour are TfnR-negative and Rab10- and IRAP-positive. IRV fusion with the plasma membrane in response to insulin is Rab10-dependent. Importantly, insulin does not increase the localization of GLUT4 in TfnR-positive endosomes, indicating that it is unlikely that GLUT4 moves from the IRC to recycling endosomes en route to the plasma membrane. GLUT4 is also transported with different kinetics than TfnR, suggesting that these two cargo proteins are transported on distinct vesicles.
In the continued presence of insulin, the static retention model dictates that GLUT4 recycles through the constitutive pathway. In one report, the size of GLUT4-containing vesicles were estimated based on fluorescence intensity during docking and fusion (Xu et al., 2011). Prolonged insulin stimulation caused GLUT4-containing vesicle fusion events to switch from small IRV to larger exocytic vesicles characteristic of recycling endosomes (Xu et al., 2011). This observation fits well with an earlier finding that fluorescence quenching of recycling endosomes with HRP-Tfn decreases insulin-stimulated GLUT4 translocation by ~50% (Zeigerer et al., 2002). However, if insulin does not increase the localization of GLUT4 in Tfn-containing vesicles and these two cargo proteins traffic with unique kinetics, how can GLUT4 recycle through the constitutive pathway? Two studies in Chinese Hamster Ovary (CHO) cells, which do not form the IRC/IRV, demonstrate that although GLUT4 and TfnR populate the same recycling endosomes, they are transported to and from the plasma membrane on distinct vesicles (Lampson et al., 2001; Wei et al., 1998). These data fit well with later evidence that GLUT4 and TfnR recycle with different kinetics in adipocytes under basal and insulin-stimulated conditions (Habtemichael et al., 2011). It is plausible that GLUT4 recycles through TfnR-positive recycling endosomes in the continued presence of insulin, but because GLUT4 transport to and from recycling endosomes occurs on separate vesicles from TfnR the apparent co-localization between GLUT4 and TfnR remains consistent.

Upon insulin removal, GLUT4 must sort from recycling endosomes into the static retention compartment comprised of the TGN and IRC/IRV. It is presumed that transport from recycling endosomes to retention compartments is unidirectional and transient based on basal GLUT4 recycling kinetics (Govers et al., 2004; Muretta et al., 2008). This transition is slow, as it takes more than 3 h to recover full GLUT4 retention (Muretta et al., 2008). Independent roles for the TGN and IRC/IRV stems from evidence that the C-terminus of GLUT4 targets GLUT4 to the TGN but that disruption of this targeting reduces, but does not abolish, insulin-responsive GLUT4 translocation (Govers et al., 2004; Li et al., 2009; Shewan et al., 2003). Thus, GLUT4 must be able to sort into the IRC/IRV retention compartment through multiple routes – supposedly through recycling endosomes and through the TGN.
1.2.4.3.1.3 Consolidating dynamic and static retention models

In both models, assumptions are made that do not fit with observations reported in the literature. It is likely that components of both models function in regulating GLUT4 dynamics. In the dynamic retention model, that the FQQI motif directs GLUT4 traffic to the perinuclear region has never been reported. The role of the FQQI motif is implied based on the assertion that GLUT4 sorting into the IRC/IRV occurs at the recycling endosomes and is dependent on the TELEY motif, but not the FQQI motif (Blot and McGraw, 2008). However, multiple reports have claimed that the C-terminus of GLUT4 is required for perinuclear localization (Li et al., 2009; Shewan et al., 2003; Song et al., 2008). In particular, the TELEY motif was required for internalized GLUT4 to sort into the Stx6/16-positive perinuclear compartment (Shewan et al., 2003). Thus, it is unlikely that the FQQI motif, but not the TELEY motif, targets GLUT4 to the perinuclear retention compartment. Furthermore, the FQQI motif does not regulate GLUT4 transit into the IRC/IRV in the dynamic retention model. However, using a “gain-of-function” approach, it was shown that only the N-terminus and cytoplasmic loop of GLUT4 were required to recapitulate normal GLUT4 dynamic behaviour, including AS160-dependent regulation of GLUT4 retention and insulin-responsiveness (Capilla et al., 2007). Over-expressing the AS160-4A mutant had no effect on reducing cell surface GLUT4 in resting adipocytes expressing the FA mutant (Capilla et al., 2007). If the TELEY motif alone controlled GLUT4 sorting into the IRC/IRV, it would be expected that over-expressing AS160-4A would increase the retention of the FA mutant. This is because the TELEY motif would sort GLUT4 into the IRC/IRV which would then act as a sink to improve GLUT4 retention. Also, the FA mutant failed to undergo insulin-responsive re-exocytosis after being internalized from the plasma membrane for 4 h, suggesting that the FA mutant did not sort into the IRC/IRV (Capilla et al., 2007). Thus, a model in which the FQQI motif directs GLUT4 to a Stx6-positive perinuclear compartment and the TELEY motif directs GLUT4 to the AS160 regulated IRC/IRV is incompatible with previous findings. It may be that the FQQI and TELEY motifs act through independent mechanisms to sort GLUT4 into the same compartment. As such, the sorting of the FA or EEAA GLUT4 mutant would be less efficient than that of WT GLUT4 and result in more GLUT4 in the constitutively recycling pathway; however, GLUT4 would maintain its steady-state distribution by relying on the remaining sorting pathway to reach retention compartments.
In terms of GLUT4 retention, two independent groups, using different methods, have shown that GLUT4 is maintained in two different populations – one recycling and the other statically retained (Fujita et al., 2010; Muretta et al., 2008). Insulin acts to release GLUT4 retention such that more GLUT4 is available in the recycling pool (Fujita et al., 2010; Muretta et al., 2008). However, contrary to the static retention model, evidence suggests that these two pools are not completely segregated in resting cells. In resting adipocytes or muscle cells in which AS160 has been depleted, GLUT4 accumulates at the cell surface but still retains insulin-responsiveness (Eguez et al., 2005; Ishikura et al., 2007; Sano et al., 2007). With AS160 depletion, GLUT4 may be expected to be slightly increased in the plasma membrane due to an increased number of IRV in the periphery; however, these IRV show poor fusion competence in the absence of insulin (Bai et al., 2007; Huang et al., 2007; Lizunov et al., 2005). If the GLUT4-containing vesicles in the IRC/IRV cannot fuse with either the plasma membrane or recycling endosomes (as in the static retention model), then releasing AS160-dependent GLUT4 retention should not increase cell surface GLUT4. Rab10 knockdown can partially restore basal GLUT4 retention in AS160 depleted adipocytes, providing evidence that IRV can fuse with the plasma membrane in the absence of insulin (Sano et al., 2007). The FA and EEAA GLUT4 mutants also show increased cell surface GLUT4, as well as increased localization to Tfns-containing membranes (Blot and McGraw, 2008; Shewan et al., 2000). Given the roles of the FQQI and TELEY motifs in sorting GLUT4 into retention compartments, these mutants should not accumulate in recycling compartments in resting cells unless bidirectional traffic between the IRC/IRV and recycling endosomes is possible. Furthermore, if 50% of GLUT4 resides in Tfns-positive endosomes in a resting adipocyte, how can only 30% of GLUT4 be accessible to the plasma membrane unless these recycling endosomes engaged in recycling with GLUT4 retention compartments?

Taking into consideration the above data, a general model for GLUT4 recycling can be developed in which GLUT4 resides in constitutively recycling and retention compartments that slowly exchange membrane (Figure 1.9). GLUT4 recycles to the plasma membrane through the constitutively recycling pathway in resting cells. The retention compartment consists of the TGN and IRC/IRV, although these compartments are likely in communication. GLUT4 may enter the IRC through both recycling endosomes and the TGN. This mechanism is similar to that proposed for STxB sorting to the TGN through both sorting and recycling endosomes. IRV may fuse with the plasma membrane in resting cells, although these vesicles have poor fusion competence and
are mostly retained at intracellular sites in the absence of insulin. Current evidence cannot conclusively assign the GLUT4 sorting motifs to one pathway or another and GLUT4 localization to the Stx6-positive perinuclear compartment has not been linked conclusively to either the TGN or IRC. Insulin releases GLUT4 retention from the IRC/IRV such that IRV are recruited to the plasma membrane.

**Figure 1.9:** Model of GLUT4 dynamics. GLUT4 is maintained in constitutively recycling and retention compartments that slowly recycle with each other. The retention compartments consist of the TGN and IRC/IRV, which likely are in equilibrium. GLUT4 internalized from the plasma membrane is transported from recycling endosomes to either the TGN or IRC, while some GLUT4 recycles back to the plasma membrane. IRV are maintained in intracellular stores such that only a fraction of the GLUT4 retention compartment is in the cell periphery near the plasma membrane. Insulin releases GLUT4 retention such that IRV translocate to the plasma membrane. Insulin also increases the rate of recycling in the constitutive pathway.

This model necessitates a complex mechanism through which to regulate GLUT4 traffic. Several proteins have been identified that regulate GLUT4 sorting through the endomembrane system. However, how they function together to regulate GLUT4 sorting into the IRC/IRV remains unresolved. Based on the model in Figure 1.9, the roles of these proteins in sorting GLUT4 into insulin-responsive compartments will be addressed.

### 1.2.4.3.2 GLUT4 sorting through the constitutive recycling pathway

To date, no studies have examined whether GLUT4 endocytosed from the plasma membrane is processed differently depending on the mode of internalization. It is assumed that GLUT4, once internalized, accumulates in sorting endosomes along with other cargo proteins internalized via independent mechanisms. Given that TfnR recycling requires transit through sorting endosomes, it is presumed that GLUT4 also transits this compartment. In 3T3-L1 adipocytes, Rabs 4, 11, and 14, but not Rab5, associate with GLUT4-containing membranes (Cormont et al., 1996a; Cormont
et al., 1996b; Larance et al., 2005). All four of these Rab proteins localize to sorting endosomes, although Rab11 is mostly associated with recycling endosomes. Sorting endosomes are enriched with Rab5 and its effector EEA1, which are critical for homotypic endosome fusion and endosome maturation (Stenmark, 2009). Interestingly, internalized GLUT4 co-localizes with EEA1 within 2 min in L6 muscle cells (Foster et al., 2001) and inhibiting Rab5 increases cell surface GLUT4 without altering insulin-stimulated GLUT4 translocation in adipocytes (Huang et al., 2001). Inhibiting Rab5 also reduces GLUT4 internalization after insulin removal (Huang et al., 2001). Together, these observations suggest that internalized GLUT4 transits sorting endosomes and that Rab5 is required for early GLUT4 sorting steps.

From sorting endosomes, the path of GLUT4 sorting through constitutively recycling or retention pathways is poorly understood (Figure 1.10A). Rab4 has a defined role in regulating protein transport from sorting endosomes to the plasma membrane through a fast recycling pathway (Galvez et al., 2012). Insulin increases active Rab4, which has been linked to insulin-stimulated GLUT4 translocation and kinesin binding to microtubules (Imamura et al., 2003). Rab4-positive GLUT4-containing vesicles are observed in the TIRF zone and undergo frequent docking behaviour after insulin stimulation (Chen et al., 2012). Thus, Rab4 may direct GLUT4 from sorting endosomes to the plasma membrane. However, Rab4 has also been reported to regulate GLUT4 sorting into the IRC/IRV (Cormont et al., 1996b; Mari et al., 2006; Reed et al., 2013) (see next section). Whether this transport is direct or via an intermediate endomembrane compartment is unknown. However, TfnR accumulation in recycling endosomes and the presence of TfnR and Rab11 in GLUT4-containing membranes support the conclusion that GLUT4 transits from sorting endosomes into recycling endosomes.

**Figure 1.10A:** Regulation of GLUT4 sorting through the constitutively recycling pathway. See text for details.
Once in recycling endosomes, GLUT4 may be recycled to the plasma membrane or sorted into a retention compartment. However, just because GLUT4 localizes to recycling endosomes does not mean that constitutively recycling GLUT4 recycles through the same mechanism as TfnR [discussed above, (Habtemichael et al., 2011; Lampson et al., 2001; Wei et al., 1998)]. In 3T3-L1 adipocytes, GLUT4 recycles to the plasma membrane 5-15 times slower than TfnR and GLUT4 endocytosis is 5x slower than that for TfnR in resting cells (Habtemichael et al., 2011). VAMP7 regulates GLUT4 (Randhawa et al., 2004; Williams and Pessin, 2008) and VAMP3 regulates TfnR (Galli et al., 1994) recycling to the plasma membrane, respectively. Also, while Rab11 is required for Tfn recycling from recycling endosomes to the plasma membrane, the requirement for Rab11 in GLUT4 recycling is less clear. Current evidence suggests that Rab11 regulates GLUT4 sorting from recycling endosomes to the IRC/IRV (Schwenk et al., 2007; Uhlig et al., 2005; Zeigerer et al., 2002), although a role in regulating GLUT4 exocytosis cannot be totally discounted, as DN-Rab11 decreases insulin-stimulated GLUT4 translocation in cardiomyocytes (Uhlig et al., 2005) and inhibiting Rab11 function reduces basal IRAP recycling in adipocytes (Zeigerer et al., 2002). Rab14 has also been suggested to mediate GLUT4 traffic from recycling endosomes to the plasma membrane (Chen et al., 2012). In this case, Rab11-positive GLUT4 vesicles were not observed in the TIRF zone whereas Rab14-positive GLUT4 vesicles that contained TfnR were apparent (Chen et al., 2012). Rab14 knockdown partially inhibited insulin stimulated GLUT4 translocation, consistent with observations that insulin increases the rate of GLUT4 exocytosis in the recycling pool (Chen et al., 2012). Rab14 knockdown also inhibits insulin-stimulated GLUT4 translocation in L6 muscle cells, although the exact role of Rab14 remains unknown (Ishikura et al., 2007). Thus, although current evidence supports a model in which GLUT4 recycles to the plasma membrane from recycling endosomes, the defined roles of Rab11 and Rab14 in mediating this process requires further investigation. Given the efficiency of GLUT4 retention, the model of GLUT4 dynamics presumes that most GLUT4 is sorted from recycling endosomes to retention compartments. These retention compartments have been defined as the TGN and IRC/IRV, although the exact identity of these compartments and their level of overlap are uncharacterized.
1.2.4.3.3 GLUT4 sorting from sorting/recycling endosomes to the IRC/IRV

A model in which GLUT4 sorts from recycling endosomes to the IRC/IRV is based on the accumulation of GLUT4 in Tfn-positive endosomes upon perturbations to GLUT4 sorting. The assertion is that, upon defective sorting, GLUT4 regresses to the donor compartment from which the IRC/IRV was formed. If the IRC/IRV was formed from the TGN, GLUT4 should accumulate in this compartment and not in recycling endosomes. However, whether these Tfn-positive endosomes are sorting or recycling endosomes cannot be determined based on steady-state co-localization with Tfn alone. Based on the accumulation of TfnR in recycling endosomes and the considerable localization of GLUT4 in Tfn-positive membranes it is presumed that GLUT4 sorting away from Tfn occurs at recycling endosomes. This may not be the case, as Rab4 regulation of GLUT4 sorting appears to occur in a sub-domain of sorting endosomes that does not contain Tfn (Mari et al., 2006). Furthermore, many of the proteins described to regulate GLUT4 sorting, as outlined in subsequent paragraphs, have been characterized to function in sorting endosomes. Part of this discrepancy may stem from observations that key sorting events occur as sorting endosomes mature, making it difficult to differentiate between sorting events in sorting versus recycling endosomes. As such, these compartments will not be distinguished further when considering evidence for GLUT4 sorting from these compartments to the IRC/IRV.

1.2.4.3.3.1 GLUT4 sorting directed by Rab4, Rab11, and Rab14

Rab4, Rab11, and Rab14, all of which reside in the constitutively recycling pathway and localize to GLUT4-containing membranes, have been implicated in regulating GLUT4 sorting into insulin-responsive compartments (Cormont et al., 1996b; Mari et al., 2006; Reed et al., 2013; Sadacca et al., 2013; Zeigerer et al., 2002). Moderately over-expressing Rab4 reduces cell surface GLUT4 in resting adipocytes without affecting insulin-stimulated GLUT4 translocation (Cormont et al., 1996b). In contrast, expressing a Rab4 mutant unable to bind to membranes decreases insulin-stimulated GLUT4 translocation (Cormont et al., 1996b). These results suggest that Rab4 promotes the accumulation of GLUT4 in the IRC/IRV such that inhibiting this function depletes insulin-responsive stores. In particular, the Rab4 effector Rabip4, which does not co-localize with GLUT4 at steady-state in resting cells, co-localizes with internalized GLUT4 in membranes that do not contain EEA1 or TfnR, but that are accessible to internalized Tfn (Mari et al., 2006). Interfering with the Rabip4-Rab4 interaction increases both cell surface
GLUT4 and GLUT4 co-localization with TfnR in resting adipocytes (Mari et al., 2006). This observation supports a role for Rab4 in mediating GLUT4 sorting from constitutive recycling endosomes into the IRC/IRV.

Likewise, interfering with Rab11 function redistributes GLUT4 to Tfn-containing membranes in resting adipocytes and inhibits insulin-stimulated GLUT4 translocation (Zeigerer et al., 2002). Work in cardiomyocytes also supports a function for Rab11 in mediating GLUT4 sorting – Rab11 knockdown increases while Rab11 over-expression decreases cell surface GLUT4 (Schwenk et al., 2007; Uhlig et al., 2005). DN-Rab11 was also reported to inhibit insulin-stimulated GLUT4 translocation in cardiomyocytes and primary skeletal muscle cells (Uhlig et al., 2005). These results are consistent with those for Rab4 in which inhibiting Rab11 function depletes, and promoting Rab11 function enhances, GLUT4 retention in the IRC/IRV.

Rab14 localizes to GLUT4-containing perinuclear endosomes in adipocytes, but does not alter its localization in response to insulin (Larance et al., 2005). Two recent reports suggest that Rab14 functions in GLUT4 sorting into the IRC/IRV (Reed et al., 2013; Sadacca et al., 2013). Rab14 knockdown inhibited the insulin-stimulated translocation of WT GLUT4 and of the FA mutant, but not of the EEAA mutant (Sadacca et al., 2013). Since the EEAA mutant is allegedly responsible for sorting GLUT4 into the IRC/IRV, Rab14 was proposed to also function in this step. Furthermore, Rab14 knockdown was not additive with that of Rab10, suggesting that they regulate the same pool of GLUT4 – the IRC/IRV (Sadacca et al., 2013). However, unlike with Rab4 or Rab11 knockdown, Rab14 knockdown did not increase cell surface GLUT4 in resting cells (Sadacca et al., 2013), which would be expected if GLUT4 sorting into the IRC/IRV was perturbed. Perhaps Rab14 depletion inhibited both GLUT4 sorting into the IRC/IRV and GLUT4 recycling from recycling endosomes to the plasma membrane. This hypothesis fits with previous work describing Rab14 as a mediatior of GLUT4 traffic from recycling endosomes to the plasma membrane (Chen et al., 2012). However, Chen et al. reported that Rab14 and Rab10 knockdowns were additive in decreasing insulin-stimulated GLUT4 translocation (Chen et al., 2012), an observation not recapitulated by Sadacca et al. (Sadacca et al., 2013). Also, Chen et al reported that 40% of Rab14 co-localizes with GLUT4-containing vesicles (marked by IRAP) beneath the plasma membrane while a more recent report suggests that only 3.3% of Rab14 co-localizes with IRAP-positive vesicles (Chen et al., 2012; Reed et al., 2013). Thus, the function of Rab14 in mediating recycling endosome to plasma membrane transport is unclear. In support of a Rab14
role in GLUT4 sorting, CA-Rab14 over-expression caused GLUT4 to accumulate in enlarged vesicular structures with Tfn and Rab14, but with very little Rab5 or Rab11 (Reed et al., 2013). As with Rab4 or Rab11 over-expression, cell surface GLUT4 was reduced and the fold response in insulin-stimulated GLUT4 translocation was maintained (Reed et al., 2013). However, the reduction in cell surface GLUT4 observed in cells over-expressing CA-Rab14 appeared to be due to defective sorting rather than increased sorting into the IRC/IRV. Interestingly, unlike with CA-Rab14, no defects in GLUT4 sorting could be ascertained by immunofluorescence detection in Rab14-depleted cells. However, electron microscopy of Rab14-depleted cells showed that internalized GLUT4 accumulated more in early endosome structures (Reed et al., 2013). The effect of Rab14 knockdown on cell surface GLUT4 and insulin-stimulated GLUT4 translocation were not reported. It was concluded that Rab14 functions in GLUT4 sorting into the IRC/IRV. It is difficult to reconcile that both CA-Rab14 and Rab14 knockdown show similar phenotypes. Perhaps Rab14 functions early in GLUT4 sorting such that CA-Rab14 inhibits endosome maturation that is required for GLUT4 sorting into the IRC/IRV.

Together, these data suggest that Rab4, Rab11 and Rab14 function to sort GLUT4 from the constitutively recycling pathway into the IRC/IRV (Figure 1.10B). It is unknown if these proteins act sequentially or through independent mechanisms. Interestingly, Rab14 and Rab4 share a common effector in Rabip4 (Yamamoto et al., 2010). Rab14 acts upstream of Rabip4 to recruit Rab4 to endosomal membranes and down regulation of any one of these proteins inhibits TfnR recycling (Yamamoto et al., 2010). Given that Rabip4 is implicated in directing GLUT4 sorting and that CA-Rab14 causes GLUT4 to accumulate in enlarged structures with Tfn but not Rab11, it is possible that Rab14 and Rab4 act upstream of Rab11 in directing GLUT4 into the IRC/IRV. Rab14/Rab4 and Rab11 could also act through two independent mechanisms to sort GLUT4. Although these Rab proteins localize to sorting/recycling endosomes, it is important to note that current evidence cannot discern if Rab4, Rab11, and Rab14 sort GLUT4 into the IRC/IRV directly or indirectly.
Figure 1.10B: Regulation of GLUT4 sorting from sorting/recycling endosomes into the IRC/IRV. See text for details.

The accumulation of GLUT4 in enlarged endosomal structures upon CA-Rab14 over-expression is similar to that observed when the ESCRT-III complex function is disrupted. Expression of DN-ESCRT-III constructs in rat adipose cells traps GLUT4 in a swollen compartment with EEA1, TfnR, and Stx6 (Koumanov et al., 2012). This construct also inhibits insulin-stimulated GLUT4 translocation (Koumanov et al., 2012). Do these findings suggest that ESCRT-III is required for GLUT4 sorting or perhaps disrupting endosomal maturation blocks general protein traffic out of sorting endosomes? Of note, this compartment is also enriched with ubiquitinated proteins, which is interesting considering findings that GLUT4 ubiquitination is required for its proper sorting (Lamb et al., 2010). However, the ubiquitination of GLUT4 is proposed to act in GLUT4 sorting through the TGN (Lamb et al., 2010), which suggests that if there is a role for ESCRT-III in directing GLUT4 traffic, it cannot yet be ascribed to GLUT4 sorting from sorting/recycling endosomes directly into the IRC/IRV.

1.2.4.3.3.2 GLUT4 sorting directed by IRAP

IRAP not only accumulates with GLUT4 in the IRC/IRV, but it also regulates GLUT4 stability and traffic. However, the exact role of IRAP has been clouded by inconsistent phenotypes upon IRAP depletion. IRAP knockout mice have reduced GLUT4 expression and reduced glucose uptake, yet no impairment in the relative GLUT4 distribution in subcellular fractions (Keller et al., 2002). By membrane fractionation, transient IRAP depletion in adipocytes was reported to decrease insulin-stimulated GLUT4 translocation without affecting basal levels of plasma membrane GLUT4 or GLUT4 expression (Yeh et al., 2007). In contrast, IRAP depletion increases cell surface GLUT4 in resting adipocytes but does not inhibit insulin-stimulated
GLUT4 translocation (although the fold response appears reduced) when measured by tagged-
GLUT4 insertion into the plasma membrane (Jordens et al., 2010). This latter phenotype was
compared with those of GLUT4 mutants. The EEAA mutant did not increase cell surface
GLUT4 more than IRAP knockdown alone, suggesting that these two proteins operate in the
same pathway for sorting GLUT4 from sorting/recycling endosomes to the IRC/IRV (Jordens et
al., 2010). However, the EEAA mutant alone increased cell surface GLUT4 less than IRAP
knockdown or double knockdown. Furthermore, IRAP knockdown was additive to that of
AS160, whereas the EEAA mutant is not (Blot and McGraw, 2008; Jordens et al., 2010). The
meaning of these data is unclear, but may indicate that IRAP functions in sorting GLUT4 into
the IRC/IRV through multiple routes. In this way IRAP could function in concert with multiple
regulatory partners and seem to function additively with any one partner. IRAP has also been
linked to GLUT4 sorting in conjunction with sortilin and LRP1 (Jedrychowski et al., 2010; Shi
and Kandror, 2007; Shi et al., 2008), which are proposed to function in sorting GLUT4 through
the TGN (see next section). Thus, although it is evident that IRAP is required for GLUT4 sorting
into the IRC/IRV, it is unclear whether this sorting can occur from sorting/recycling endosomes
to the IRC/IRV directly.

1.2.4.3.3 GLUT4 sorting directed by Grp1, Arf6, ACAP1, and CHC

The proteins ACAP1, Grp1, and Arf6 have all been implicated in mediating GLUT4 sorting into
the IRC/IRV. Arf6 is a small GTPase that localizes to the plasma membrane and endosomal
compartments, but not the TGN (Schweitzer et al., 2011). It mediates the recycling of multiple
membrane proteins involved in cell motility, cytokinesis, cell adhesion, and cell migration
[reviewed in (Schweitzer et al., 2011)]. ACAP1 is an Arf6-GAP, but it also acts as a clathrin coat
sorting adaptor (Li et al., 2007). It localizes to recycling endosomes and functions in TfnR and
integrin transport from recycling endosomes to the plasma membrane (Dai et al., 2004; Li et al.,
2007). Grp1 is an Arf6-GEF important in growth factor signalling (D’Souza-Schorey and
Chavrier, 2006). Interestingly, insulin induces Akt-dependent phosphorylation of both ACAP1
and Grp1 (Li et al., 2005; Li et al., 2012).

ACAP1 is a GLUT4-interacting protein through which clathrin heavy chain (CHC) associates
with GLUT4 (Li et al., 2007). ACAP1 and CHC co-localize with GLUT4, sortilin, and Stx6 in
the perinuclear region of adipocytes (Li et al., 2007). Likewise, Arf6 co-localizes with ACAP1,
CHC, and GLUT4. Knockdown of Arf6, ACAP1, or CHC inhibits insulin-stimulated GLUT4 translocation without altering the perinuclear accumulation of GLUT4 (Li et al., 2007). Interestingly, Arf6 knockdown reduced GLUT4 co-localization with CHC and ACAP1 although CHC or ACAP1 knockdown did not affect GLUT4 co-localization with Arf6 (Li et al., 2007). However, knockdown of CHC reduced the co-localization of GLUT4 with ACAP1 and vice versa (Li et al., 2007). It was concluded that Arf6 acts upstream in recruiting ACAP1 to a clathrin-coat complex that functions in GLUT4 recycling. ACAP1 mediates the recruitment of GLUT4 into the clathrin coat complex that couples vesicle formation with cargo sorting (Figure 1.10B).

An interesting observation was that ACAP1 GAP activity was required for insulin-stimulated glucose uptake (Li et al., 2007). If ACAP1 is functioning as a clathrin coat adaptor downstream of Arf6 activation, how is ACAP1 GAP activity required for glucose uptake? It is possible that ACAP1 functions as both an adaptor and as the signal to dissociate the coat complex for GLUT4 vesicle delivery to the IRC/IRV. However, some reports have questioned the requirement of Arf6 GTPase activity in regulating GLUT4 traffic – DN-Arf6 has no effect on insulin-stimulated GLUT4 translocation (Bose et al., 2001; Lawrence and Birnbaum, 2001; Yang and Mueckler, 1999). A more recent study shows that Grp1 acts upstream of Arf6, ACAP1, and CHC in forming GLUT4 vesicles by an in vitro budding assay (Li et al., 2012). Knockdown of Grp1 disrupts GLUT4 co-localization with Arf6, ACAP1, and CHC, but knockdown of any of these proteins does not disrupt GLUT4 co-localization with Grp1. Knockdown of Grp1, as with that of Arf6, ACAP1, or CHC, inhibits insulin-stimulated GLUT4 translocation (Li et al., 2012). Based on these data, activation of Arf6 by Grp1 may recruit ACAP1 and CHC to sorting/recycling endosomes to form a coat complex necessary for GLUT4 sorting. ACAP1 binding to GLUT4 may act to recruit GLUT4 into coated vesicles that will then shed the coat complex via ACAP1 GAP activity and transit to the IRC/IRV. However, Grp1 was shown to promote GLUT4 vesicle budding in an insulin-dependent manner and CA-Grp1 bypasses the requirement of Rab10 for stimulating GLUT4 translocation through the insulin-regulated pathway (Li et al., 2012). It is possible that this mechanism functions in resting cells to regulate GLUT4 sorting and that insulin upregulates the formation of GLUT4 vesicles to match the increase in GLUT4 recycling; however, it seems odd that knockdown of Grp1, Arf6, ACAP1, or CHC does not increase cell surface GLUT4 in resting adipocytes. How do proteins involved in GLUT4 sorting into the
IRC/IRV inhibit insulin-stimulated GLUT4 translocation without increasing cell surface GLUT4 in resting cells? Does this mechanism rely on Rab protein effectors or IRAP? As ACAP1 is also required for TfnR recycling, is it that unique accessory proteins dictate constitutive recycling versus specialized storage? Thus, the exact mechanism through which these proteins act remains to be determined.

In summary, although it is evident that GLUT4 transits sorting/recycling endosomes en route to the IRC/IRV, convincing evidence that GLUT4 sorts from sorting/recycling endosomes directly into the IRC/IRV is lacking. As such, the described roles of the above mentioned regulatory proteins in diverting GLUT4 from constitutively recycling endosomes into the IRC/IRV cannot be conclusively mapped. Although these proteins localize to sorting/recycling endosomes, GLUT4 sorting from sorting/recycling endosomes to the TGN or from sorting/recycling endosomes to the IRC/IRV are equally probable scenarios.

1.2.4.3.4 GLUT4 transit through the TGN en route to the IRC/IRV

A model in which GLUT4 sorts through the TGN en route to the IRC/IRV is supported by the involvement of regulatory proteins in sorting GLUT4 that have defined functions in endosome to TGN transport. The accumulation of GLUT4 in Tfn-positive endosomes upon perturbations in GLUT4 sorting may indicate that GLUT4 cannot traffic from sorting/recycling endosomes into the TGN. Alternatively, GLUT4 sorting from the TGN to the IRC/IRV may be inhibited, and through mass action, GLUT4 that accumulates in the TGN is cycled back into sorting/recycling endosomes due to reduced retention capabilities.

1.2.4.3.4.1 GLUT4 sorting directed by SNAREs

The SNARE complex Stx6, Stx16, Vti1a, and VAMP3/4 localizes at the TGN and functions in endosome to TGN transport (Johannes and Popoff, 2008). In 3T3-L1 adipocytes, internalized GLUT4 is targeted to the Stx6/16-positive perinuclear region by the TELEY motif (Shewan et al., 2003). As such, Stx6 and Stx16 are proposed to regulate GLUT4 sorting at the TGN. But do Stx6 and Stx16 function in sorting/recycling endosome to TGN transport as described for STxB or could these proteins function in TGN to IRC/IRV transport?

In addition to Stx6 and Stx16, Vti1a localizes in GLUT4-containing membranes which become depleted in response to insulin treatment (Bose et al., 2005). GLUT4 and Vti1a co-localize in the
perinuclear region and Vti1a depletion inhibits insulin-stimulated glucose uptake (Bose et al., 2005). Treatment of adipocytes with brefeldin A, which disrupts Golgi and TGN marker proteins, does not alter Vti1a or GLUT4 localization. These data suggest that this SNARE complex may localize to the IRC/IRV. The R-SNARE that functions in this SNARE complex is unknown. Neither VAMP3 nor VAMP4 depletion in adipocytes inhibits the ability of GLUT4 to respond to insulin after being labelled at the cell surface and allowed to internalize for 6 h (Williams and Pessin, 2008).

Stx16 knockdown in 3T3-L1 adipocytes inhibits insulin-stimulated GLUT4 accumulation in plasma membrane fractions, inhibits insulin-stimulated glucose uptake, and delays the return-to-basal kinetics of glucose transport following insulin removal (Proctor et al., 2006). However, Stx16 depletion does not alter basal glucose transport. One caveat is that Stx16 depletion also reduces GLUT4 expression by 30%, which could account at least partially for the reduced insulin-stimulated glucose transport (Proctor et al., 2006). It was hypothesized that depleting adipocytes of Stx16 reduces the amount of GLUT4 in the IRC/IRV.

Interfering with Stx6 function in 3T3-L1 adipocytes by over-expressing a cytosolic domain of Stx6 (Stx6-cyto) delays the disappearance of GLUT4 from the plasma membrane following insulin removal and prevents GLUT4 from accumulating in the IRC/IRV membrane fractions (Perera et al., 2003). However, Stx6-cyto does not inhibit insulin-stimulated glucose transport, although it does increase basal glucose transport (Perera et al., 2003). Interestingly, Stx6 knockdown inhibits IRAP sorting into the IRC/IRV but does not inhibit IRAP transport from the IRC/IRV to the plasma membrane (Watson et al., 2008). However, IRAP labelled at the cell surface and allowed to internalize for 6 h cannot respond to insulin in Stx6 depleted cells. Although this suggests that GLUT4 also requires Stx6 to enter the IRC/IRV, this effect of Stx6 knockdown on GLUT4 traffic has yet to be tested. Interestingly, DN-GGA, which inhibits GLUT4 sorting into the IRC/IRV (Li and Kandror, 2005), was not required to sort IRAP into the IRC/IRV (Watson et al., 2008).

Recently, the Sec1/Munc18 protein Vps45 was reported to mediate IRC/IRV formation using an in vitro GLUT4-vesicle budding assay derived from 3T3-L1 adipocytes (Roccisana et al., 2013). Sec1-Munc18 proteins facilitate vesicle SNARE complex formation and function. Vps45 binds Stx16 and its depletion reduces the expression of GLUT4 (by 76%), Stx16, IRAP, and VAMP4.
but not that of Stx6 or Vti1a (Roccisana et al., 2013). Vps45 knockdown also inhibits insulin-stimulated GLUT4 translocation and reduces GLUT4 content in IRC/IRV-containing fractions. It was concluded that Vps45 functions with Stx16 to regulate GLUT4 sorting into the IRC/IRV. It is interesting to note that of the canonical SNARE complex in endosome to TGN sorting only Stx16 and VAMP4 expression are affected by Vps45 knockdown but not Vti1a or Stx6.

Together, these data support a role for the Stx6-Stx16-Vti1a SNARE complex in regulating GLUT4 sorting in adipocytes, although it is difficult to explain how Stx16 knockdown provides a different phenotype than that of Stx6-cyto if these proteins regulate the same step of sorting GLUT4 into the IRC/IRV. In which compartment Stx6 and Stx16 act is also debatable. Some fractionation data from Stx6-cyto treated adipocytes suggests that endosome to TGN traffic is impaired; however, an increase in GLUT4 localization to recycling endosomes has never been reported after Stx6 or Stx16 depletion. Furthermore, localization of GLUT4 to the Stx6/16-positive perinuclear region does not dictate insulin-responsiveness (Li et al., 2009). As such, the perturbation in GLUT4 sorting that occurs upon Stx6 or Stx16 depletion has never been linked to GLUT4 sorting through the Stx6/16-positive perinuclear compartment and sorting through this compartment has never been linked to insulin-responsiveness.

1.2.4.3.4.2 GLUT4 sorting directed by sortilin, IRAP, and LRP1

Sortilin is a member of the Vps10 family of sorting receptors that regulate endosomal sorting. It is a type I transmembrane protein with a ligand binding site in its luminal N-terminus and internalization and sorting motifs within its cytoplasmic tail [reviewed in (Lane et al., 2012)]. In adipocytes, sortilin expression induces the formation of the IRC/IRV and subsequent insulin-responsive glucose uptake (Shi and Kandror, 2005). Knockdown of sortilin reduces GLUT4 vesicle budding and inhibits insulin-stimulated GLUT4 translocation (Shi and Kandror, 2005). In addition, sortilin depletion increases GLUT4 degradation, leading to a 35% reduction in cellular GLUT4 content (Shi and Kandror, 2005). The luminal domain of sortilin binds GLUT4 to direct GLUT4 sorting into the IRC/IRV (Shi and Kandror, 2007). Interestingly, expression of a cellugyrin-GLUT4 chimera containing the luminal loop of GLUT4 was sufficient to target cellugyrin to the IRC/IRV through a sortilin dependent mechanism (Kim and Kandror, 2012). How this observation fits with previous work showing the importance of cytosolic GLUT4 sorting motifs is unclear. IRAP interacts with sortilin indirectly by binding GLUT4 in its luminal
loop (Shi et al., 2008). LRP1 also interacts with the sortilin-GLUT4-IRAP complex and LRP1 depletion decreases the expression of all three proteins (Jedrychowski et al., 2010). Thus, it is hypothesized that sortilin binds GLUT4 in sorting/recycling endosomes, which then recruits IRAP. Since both sortilin and IRAP are critical for targeting GLUT4 to the IRC/IRV, it is likely that these proteins bind effectors necessary for targeting GLUT4 to the IRC/IRV. LRP1 is recruited to stabilize the luminal interactions of these proteins, which are then sorted into the IRC/IRV as a complex.

Evidence suggests that sortilin directs GLUT4 sorting into the IRC/IRV through the TGN. Sortilin interacts with retromer, which functions in sorting/recycling endosome to TGN transport (Johannes and Popoff, 2008; Lane et al., 2012). Indeed, sortilin requires retromer for its retrograde transport from endosomes to the TGN (Canuel et al., 2008). In adipocytes, GLUT4 exists in mobile and immobile populations when examined by single-particle tracking microscopy, with the immobile population being concentrated in the perinuclear region (Hatakeyama and Kanzaki, 2011). Sortilin and retromer are required for internalized GLUT4 to acquire localization to the immobile population (Hatakeyama and Kanzaki, 2011). Mutating C-terminal sorting motifs in sortilin prevents the ability of sortilin to sort GLUT4 into the immobile population (Hatakeyama and Kanzaki, 2011). Interestingly, expressing sortilin in 3T3-L1 preadipocytes causes GLUT4 to localize to the perinuclear region (Hatakeyama and Kanzaki, 2011). Internalized GLUT4 accumulates in the perinuclear region with golgin-97, Stx6, and Vti1a in sortilin expressing 3T3-L1 preadipocytes (Hatakeyama and Kanzaki, 2011). Golgin-97, but not golgin-245, is required for internalized GLUT4 to enter the immobile population (Hatakeyama and Kanzaki, 2011). Golgin-97 depletion also disperses perinuclear Stx6 (Hatakeyama and Kanzaki, 2011). Thus, it is reasoned that sortilin targets GLUT4 for retromer dependent sorting into the TGN where it is captured by the Golgi tether golgin-97 and the t-SNARE complex of Stx6-Stx16-Vti1a (Figure 1.10C).

1.2.4.3.4.3 GLUT4 sorting directed by GGA, ubiquitination, and Ubc9

The sorting adaptor GGA (Golgi-localized, gamma adaptin ear-containing, ARF-binding protein) is required for GLUT4 localization to the IRC/IRV in adipocytes (Li and Kandror, 2005; Watson et al., 2004). Specifically, GGA2 co-localizes with GLUT4 and Stx6 in the perinuclear region (Li and Kandror, 2005). GLUT4 immunoprecipitation pulls down both GGA adaptors and sortilin
(Li and Kandror, 2005). DN-GGA not only inhibits insulin-stimulated GLUT4 translocation and IRC/IRV formation \textit{(in vitro} GLUT4 vesicle budding), but also reduces glucose uptake in cells re-stimulated with insulin after 30-120 min of recovery (Li and Kandror, 2005). Sortilin is known to bind effector molecules through its cytoplasmic C-terminus, one of which is GGA (Takatsu et al., 2001). It is hypothesized that GGA regulates GLUT4 sorting into the IRC/IRV at the TGN through its interaction with sortilin.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.10c.png}
\caption{Regulation of GLUT4 sorting from sorting/recycling endosomes to the TGN en route to the IRC/IRV. See text for details.}
\end{figure}

In addition, ubiquitination of GLUT4 may be critical for GGA dependent sorting of GLUT4 into the IRC/IRV (Lamb et al., 2010). GGA proteins facilitate TGN to endosomes delivery of cargo proteins by recognizing attached ubiquitin moieties (Lamb et al., 2010). Some evidence suggests that GLUT4 must be transiently ubiquitinated to sort into the IRC/IRV. Less than 0.1% of GLUT4 is ubiquitinated in adipocytes and insulin does not alter this; however, ubiquitin resistant GLUT4 fails to sort into the IRC/IRV and shows reduced insulin-stimulated translocation (Lamb et al., 2010). GGA2 has also been linked to CHC22 in human muscle cells. CHC22 binds to GGA2, AP-1, VAMP2, and GLUT4 (Vassilopoulos et al., 2009). Depletion of CHC22 disperses cellular GLUT4, increases glucose uptake in resting cells, and prevents any further increase in glucose uptake upon insulin stimulation (Vassilopoulos et al., 2009). Through CHC22 deletion studies, CHC22 was characterized to regulate endosome to TGN transport of STxB and CI-MPR in HeLa cells, supporting a general role for CHC22 in retrograde transport (Esk et al., 2010). Stx10 was found to regulate CI-MPR traffic and GLUT4 localization downstream of CHC22 (Esk et al., 2010). Interestingly, Stx10 is a paralog of Stx6 that, like CHC22, is found in humans but not mice (Esk et al., 2010).
The small ubiquitin-related modifier (SUMO)-conjugating enzyme Ubc9 directly binds GLUT4 and is required for GLUT4 accumulation in the IRC/IRV in both adipocytes and muscle cells (Giorgino et al., 2000; Liu et al., 2007). Over-expressing Ubc9 increases GLUT4 expression by delaying its degradation, supposedly by increasing GLUT4 targeting to the IRC/IRV (Giorgino et al., 2000; Liu et al., 2007). Interestingly, Ubc9 activity was not required for its function in sorting GLUT4 in adipocytes, but was reported as necessary in L6 muscle cells (Giorgino et al., 2000; Liu et al., 2007). In adipocytes, Ubc9 depletion inhibits insulin-stimulated glucose transport and reduces the expression of GLUT4, IRAP, sortilin, and Stx6 (Liu et al., 2007). Ubc9 localizes to the TGN, which suggests that it may function in concert with sortilin, GGA, or TUG in regulating GLUT4 accumulation in the IRC/IRV (Liu et al., 2007).

In summary, a number of regulatory proteins function in sorting GLUT4 into the IRC/IRV. Based on the general localization and functions of these regulatory proteins, evidence suggests that GLUT4 sorts from sorting/recycling endosomes, through the TGN, and into the IRC/IRV. However, GLUT4 accumulation in the perinuclear region alone does not define GLUT4 localization to the TGN. Proteins that co-localize with GLUT4 in this region also localize to the IRC/IRV, some of which translocate to the cell surface in response to insulin. The mechanism(s) through which these regulatory proteins function to direct GLUT4 into the IRC/IRV is not yet understood.

1.2.4.3.5 Summary of GLUT4 sorting into constitutively recycling and retention compartments

Current understanding suggests that GLUT4 recycles between the plasma membrane and intracellular stores (Figure 1.9). Internalized GLUT4 sorts into sorting/recycling endosomes where most GLUT4 is efficiently sorted into the IRC/IRV. GLUT4 may enter the IRC/IRV from recycling endosomes directly or first be sorted through the TGN. In this way, GLUT4 traffic may be similar to that of STxB in that it may reach its destination organelle through multiple pathways. IRV can fuse with the plasma membrane, although the fusion competence of these vesicles is poor. The IRC/IRV is more likely to engage in idle cycling with recycling endosomes or TGN membranes. GLUT4 is maintained in perinuclear membranes that consist of TGN and/or the IRC/IRV by the proteins TUG and AS160, possibly by anchoring to the Golgi matrix through golgin-160 and p115. Transit through the TGN could act to localize GLUT4 to the Golgi matrix where interactions with TUG and golgin-160 stabilize perinuclear IRC/IRV retention. However,
it is unknown whether this Golgi compartment consists of the IRC/IRV or if it is a retention compartment that may replenish IRV upon insulin stimulation.

1.2.5 GLUT4 traffic in models of insulin resistance

Obesity and Type 2 Diabetes are characterized by insulin resistance. A prevailing hypothesis is that the accumulation of lipids or lipid by-products (diacylglycerol – DAG, ceramides) in muscle and adipose tissues can cause inflammation and insulin resistance [reviewed in (Chavez and Summers, 2012; Samuel et al., 2010; Shoelson et al., 2007)]. The role of ceramides in causing insulin resistance is of particular interest. A large body of literature has associated elevated intramuscular and circulating levels of ceramides to increased inflammation and insulin resistance [reviewed in (Chavez and Summers, 2012)]. Cellular studies have revealed that a cell permeable ceramide analog, C2-ceramide (C2-cer), inhibits Akt activation and GLUT4 translocation in response to insulin without affecting upstream IRS-1 or PI3K activation (Hajduch et al., 2001; JeBailey et al., 2007; Summers et al., 1998; Wang et al., 1998a).

In most models of insulin resistance defects in insulin signalling events (typically at the levels of IRS-1 or Akt) have been described [reviewed in (Boura-Halton and Zick, 2009; Chavez and Summers, 2012; Copps and White, 2012)]. However, insulin resistance may potentially also arise from defects in GLUT4 translocation or GLUT4 sorting. In adipocytes, overnight incubation with 17 nM insulin inhibits GLUT4 translocation when cells are re-challenged with insulin (Xiong et al., 2010). During this insulin challenge, GLUT4 accumulation beneath the plasma membrane is also impaired despite normal phosphorylation of AS160 (Xiong et al., 2010). These data suggest that insulin resistance can affect the molecular machinery involved in GLUT4 translocation. Indeed, changes have been reported in the expression and localization of sortilin, Myo1c, and the SNARE complex components SNAP-23, VAMP2, syntaxin-4, and their regulator Munc18c under conditions that provoke insulin resistance [reviewed in (Foley et al., 2011)]; however, most of these changes are so far only correlative to defective GLUT4 translocation and glucose uptake.

Can conditions that cause insulin resistance also impair GLUT4 sorting? Treatment of 3T3-L1 adipocytes with endothelin-1 reduces the amount of GLUT4 present in the immobile (retention) population and prevents insulin from further influencing GLUT4 movements (Fujita et al., 2010). Given that the immobile population seems to represent the IRC/IRV, this result suggests
that insulin resistance can impair GLUT4 sorting. However, whether the lack of insulin action was due to defective insulin signalling or GLUT4 mis-localization is not clear. In support of the latter, insulin resistance mis-localizes intracellular GLUT4 in humans, suggesting that GLUT4 sorting may be disrupted (Klip et al., 1990; Maianu et al., 2001; Vassilopoulos et al., 2009). Also, models of insulin resistance can impair GLUT4 translocation independent of IRS-1 and Akt in cells in culture (Hoehn et al., 2008). These studies highlight the complexity with which different models of insulin resistance may affect insulin-stimulated GLUT4 translocation. Importantly, to date the mechanism of ceramide induced insulin resistance has been attributed to defects in insulin signalling at the level of Akt. The possibility that ceramide also inhibits GLUT4 sorting has been neglected. Given that the mechanism through which C2-ceramide inhibits insulin signalling has been established in adipocytes and myocytes in culture, this model provides an excellent platform to investigate the dependence of ceramide-induced insulin resistance on GLUT4 traffic versus insulin signalling.

1.3 Rationale and hypotheses

The model of GLUT4 dynamics outlined in Figure 1.9 has been built largely from studies in adipocytes examining the intracellular localization of GLUT4 at steady-state, cell surface localization of GLUT4, or distribution of GLUT4 in membrane fractions. Overlap between GLUT4 and Tfn recycling pathways seems minimal, as the kinetics of GLUT4 recycling have been probed at the plasma membrane and found to be slower than that for Tfn in adipocytes. Some studies have examined the localization of internalized GLUT4 upon its equilibration with intracellular stores; however, very little is known about the acute dynamics of GLUT4 sorting through intracellular membranes or how regulatory proteins control this process. Importantly, understanding GLUT4 sorting dynamics in both adipocytes and muscle cells is important, as differences exist in their regulation of GLUT4 traffic. For example, muscle and adipose cells utilize different Rab proteins to mediate insulin-stimulated GLUT4 translocation (Rab8a and Rab10, respectively) and regulate GLUT4 endocytosis through different mechanisms. Thus, building a generic model of GLUT4 dynamics based solely on studies performed in adipocytes may not be valid.

The overall process of insulin-stimulated GLUT4 translocation has been extensively studied in muscle and adipose cells. However, the mechanism through which GLUT4 sorts into the
IRC/IRV is largely unexplored in either cell type, and in this Thesis I focus on this process in muscle cells. I propose to study the kinetics of GLUT4 sorting such that GLUT4 intracellular localization can be linked to insulin responsiveness. Like in adipocytes, a major roadblock in characterizing GLUT4 sorting is that the intracellular localization of the IRC/IRV is unknown. Understanding how and where GLUT4 acquires insulin-responsiveness are key initiatives for characterizing the IRC/IRV. Answering these questions will also provide tools to investigate the role, if any, of defective GLUT4 sorting in models of insulin resistance.

In adipocytes, Stx6 and Stx16 appear to define the perinuclear compartment to which GLUT4 sorts; however, how this compartment relates to the acquisition of insulin-responsiveness is unknown. Previous studies examined glucose transport and GLUT4 localization in membrane fractions of 3T3-L1 adipocytes over-expressing Stx6-cyto or depleted of Stx16 (Perera et al., 2003; Proctor et al., 2006). Although it was concluded that both Stx6 and Stx16 are required to sort GLUT4 from the plasma membrane to the IRC/IRV following insulin removal, the perturbation in GLUT4 sorting attributed to Stx6-cyto or Stx16 knockdown has never been linked to insulin-responsive GLUT4 translocation. Such a role is suggested by the inhibition of IRAP re-exocytosis following insulin-stimulated translocation and re-internalization for 6 h in 3T3-L1 adipocytes depleted of Stx6 (Watson et al., 2008). However, a definite role for Stx6 in mediating GLUT4 sorting into the IRC/IRV is unproven and GLUT4 sorting has yet to be examined in either adipocytes or muscle cells depleted of Stx6. Therefore, Stx6 is a good candidate protein to begin to explore GLUT4 sorting in muscle cells. In this Thesis I address two main questions:

1. Does Stx6 regulate GLUT4 sorting into insulin-responsive compartments? If so, in what compartment does Stx6 function?

2. Do conditions of insulin resistance perturb GLUT4 sorting and subsequent insulin-responsiveness?

To address these questions I use the L6 rodent skeletal muscle cell line stably expressing GLUT4 with an exofacial myc epitope tag (L6GLUT4myc). In these cells, GLUT4myc has been shown to translocate to the cell surface in response to insulin (Ueyama et al., 1999). The increase in cell surface GLUT4myc is approximately two fold, matching the increase in insulin stimulated glucose uptake observed in isolated skeletal muscle and the increase in insulin stimulated
GLUT4 translocation observed by membrane fractionation of skeletal muscle (Aledo et al., 1997; Ueyama et al., 1999; Zierath et al., 1996). These similarities suggest that GLUT4myc behaves in a manner representative of endogenous GLUT4; however, exactly how endogenous GLUT4 behaves cannot be studied, as the strategies used to study endogenous GLUT4 (glucose uptake and membrane fractionation) do not allow for the study of GLUT4 traffic. L6 cells do have limitations: these cells do not contain contractile apparatus and cannot mimic the complexity of mature muscle. Nonetheless, they are the best available cellular model for the study of insulin-dependent GLUT4 traffic in muscle. The major advantage of this model system is that L6GLUT4myc cells provide a means to exocytically label cell surface GLUT4, which overcomes limitations in the study of endogenous GLUT4 traffic. GLUT4 regulation in this cell line is virtually unchanged during the progression from myoblasts to myotubes (Ueyama et al., 1999). In this Thesis I use exclusively L6 myoblasts, since they are easier to manipulate through molecular strategies of gene gain and loss of function. By tracking the intracellular sorting of GLUT4 after pulse-labelling at the cell surface, I characterize the itinerary of GLUT4 transit en route to acquiring insulin-responsiveness. Tracking the acute dynamics of GLUT4 sorting into insulin-responsive compartments allowed us not only to address the role of Stx6 in regulating GLUT4 sorting, but also to identify the time course with which GLUT4 acquires insulin-responsiveness, the step at which Stx6 functions, and the role of C2-ceramide in perturbing this process.

I hypothesized that:

1. Internalized GLUT4 sorts into a Stx6-positive perinuclear compartment that preludes the acquisition of insulin-responsiveness.

2. Stx6 directs GLUT4 sorting into the insulin-responsiveness compartment

3. C2-ceramide inhibits GLUT4 sorting into insulin-responsive compartments. Defective GLUT4 sorting contributes to the insulin resistance observed with ceramide treatment.
2 Materials and Methods

2.1 Cell culture reagents

\(\alpha\)-minimum essential medium (\(\alpha\)MEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), trypsin, and antibiotics solution were from Wisent. Cell growth media was composed of \(\alpha\)MEM supplemented with 10\% (vol/vol) FBS and 1\% (vol/vol) antibiotics solution.

2.2 Antibodies, reagents, cDNA, and siRNA

2.2.1 Antibodies

Rabbit polyclonal anti-\textit{myc}, mouse monoclonal anti-actinin-1, and rabbit anti-Rab11 antibodies were from Sigma-Aldrich. Mouse anti-\textit{HA} antibody (HA.11 Clone 16B12) and \textit{HA}-A594 conjugated antibody were from Covance. Mouse monoclonal anti-Stx6, mouse anti-EEA1, mouse anti-sortilin and rabbit anti-Rab8 antibodies were from BD Transduction Laboratories. Rabbit polyclonal anti-Stx6 and rabbit anti-Stx16 antibodies were from Synaptic Systems. Mouse monoclonal anti-Tubulin and rabbit anti-Rab14 antibodies were from Abcam. Mouse anti-\textit{myc} (c-Myc 9E10) and rabbit anti-furin (H-220) were from Santa-Cruz Biotechnology. Mouse anti-GLUT4 (1F8) and Polyclonal anti-P-Akt(308) and P-Akt(473) were obtained from Cell Signaling Technology. A549-, Cy3- and A488-conjugated donkey anti-rabbit and donkey anti-mouse secondary antibodies (full length and Fab) were purchased from Jackson ImmunoResearch Laboratories. Rabbit anti-\textit{myc}-A549-conjugated antibody was from Rockland Immunochemicals. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody was from Jackson ImmunoResearch Laboratories.

2.2.2 Reagents

DMSO, protease inhibitor cocktail (PIC), NaF, and Na\textsubscript{3}VO\textsubscript{4}, were from Sigma-Aldrich. Nocodazole was purchased from EMD Biosciences Inc. (10mM stock in DMSO) and C2-ceramide was purchased from Enzo Life Sciences (50mM stock in DMSO). Human insulin was purchased from Eli Lilly. Human holo-transferrin conjugated to A488 was from Invitrogen. Hygromycin was purchased from InvivoGen. Tween-20 and NP-40 were from Calbiochem.
2.2.3 cDNA constructs and siRNA

GFP-GLUT4myc subcloned into pcDNA3 was provided by Dr. Jeff Pessin (Albert Einstein College of Medicine, New York, USA). VAMP2-HA was generated by amplification of VAMP2 fragment from VAMP2-GFP (Randhawa et al., 2008) and subcloning into pQCXIH using AgeI-PacI restriction sites. VAMP2-HA was stably expressed in L6GLUT4myc cells using Hygromycin selection. GFP-tagged constructs for Rab5, Rab7, Rab9, Rab11 and DN-Rab22a were kind gifts from Dr. John Brumell (The Hospital for Sick Children, Toronto, Canada). GFP-tagged constructs for 2xFYVE, DN-Rab5, lactadherin-C2, and Dynamitin (DN-Dynein) were provided by Dr. Sergio Grinstein (The Hospital for Sick Children, Toronto, Canada).

Pre-designed siRNA for Syntaxin-6 (siStx6: 5’-CCGAGTCATCAGAAGAACTAA-3’), non-related (siNR: 5’-AATAAGGCTATGAAGAGATAC-3’), Rab11 (siRab11: 5’-GACAATTCTAACAGAGATATA-3’), Rab14 (siRab14: 5’-AAGGAACCTCACCACCCAAA-3’), VAMP2 (siVAMP2: 5’-TCCCTGTGGGTACAATGATA-3’), and Rab8a (siRab8a: 5’-CAGCGCAAGGCCAACATCAA-3’) were from Qiagen.

2.3 Cell culture and transfections

The rat L6 muscle cell line stably expressing GLUT4 with an exofacial myc epitope tag (L6GLUT4myc) was cultured as described previously (Ueyama et al., 1999). Cells were cultured in growth media in a humidified incubator with 5% CO2 at 37C. Myoblasts were split every 48 h to prevent aggregation of the cells.

Transfections of cDNA constructs were performed using Lipofectamine 2000 reagent according to the manufacturer’s protocol (Life Technologies). Cells were seeded onto glass coverslips 16 h before transfection such that cell density was ~50% on the day of transfection. Cells were transfected with 0.5 µg cDNA (1:2 ratio with reagent), incubated in serum-free media for 6 h, and then placed in growth media overnight. Experiments were performed 24 h after transfection. Maximal transfection efficiency in L6 myoblasts is ~20%.

Transfection of siRNA was performed using jetPRIME reagent according to the manufacturer’s protocol (Polyplus transfection). Cells were transfected with 200nM siRNA for 24 h and then cultured for 48 h. Experiments were performed 72 h after transfection. For steady-state detection of cell surface GLUT4myc and insulin-responsive GLUT4myc re-exocytosis experiments, cells
were grown in 24-well plates, transfected at ~70% confluence, and grown to confluence by the
day of the experiment. For immunofluorescence experiments, cells were re-seeded onto glass
coverslips at ~50% confluence 24-48 h before experiments.

2.4 Detection of total GLUT4\textit{myc} by immunofluorescence

The protocol for detection of total GLUT4\textit{myc} in single cells was adapted from previously
established protocols (Ishikura et al., 2010). L6GLUT4\textit{myc} cells were serum starved 2 h before
being washed twice in PBS+ (PBS with 0.49 mM MgCl$_2$ and 0.68 mM CaCl$_2$) and fixed in 3%
PFA for 30 min (10 min on ice and 20 min at RT). Cells were washed in 0.1 M glycine for 10
min, permeabilized with 0.1% Triton for 15 min, placed in blocking buffer (5% goat serum in
PBS+) for 20 min, and labelled with primary antibodies for 1 h at RT. GLUT4\textit{myc} was labelled
with mouse anti-\textit{myc} (9E10) antibody (1:100). Endogenous proteins were labelled as follows:
rabbit anti-Stx6 (1:750), rabbit anti Stx16 (1:750), and rabbit anti-furin (1:500). For VAMP2-HA
and EEA1 labelled with mouse anti-HA (1:1000) and mouse anti-EEA1 (1:1000) antibodies,
GLUT4\textit{myc} was labelled with rabbit anti-\textit{myc} (1:300). Recycling TfnR was labelled with Tfn-
A488 (or A555) conjugates. Cells were incubated with Tfn-A488 for 30 min prior to fixation (50
µg/mL in serum free media supplemented with 1% BSA). Fixation for 1 h at RT with 4% PFA
was required for Tfn labelling. Appropriate fluorophore conjugated secondary antibodies were
then used to label GLUT4\textit{myc} and markers for 1 h at RT before coverslips were mounted for
imaging.

For nocodazole experiments, 3 µM nocodazole was added for the last 30 min during serum
starvation. For C2-ceramide experiments, C2-ceramide was present during the entire 2 h serum
starvation.

2.5 Imaging GLUT4 internalization in single cells

The GLUT4\textit{myc} internalization protocol was adapted from previously established protocols
(Ishikura et al., 2010). L6GLUT4\textit{myc} cells were serum starved for 2 h before being washed twice
in PBS+ and placed in blocking buffer (5% goat serum in PBS+) for 20 min on ice. Cell surface
GLUT4\textit{myc} was pulse-labelled with rabbit anti-\textit{myc} antibody (1:250) at 4C for 1 h before cells
were washed 5x in PBS+ and re-warmed in serum free media at 37C for indicated times. Cells
were then fixed in 3% PFA for 20 min on ice, washed with 0.1 M glycine for 10 min on ice, and
permeabilized with 0.1% Triton for 7 min at RT. Internalized GLUT4\textit{myc} was detected by secondary antibody conjugated to fluorophore (1:400, 1 h at RT). In some preliminary experiments (Chapter 3.2.1), modifications were made to the detection of internalized GLUT4\textit{myc}. In one case, internalized GLUT4\textit{myc} was detected by Fab secondary antibody. Alternatively, GLUT4\textit{myc} was labelled with secondary antibody at 4\textdegree{}C prior to cell re-warm (full length or Fab). When cell surface GLUT4\textit{myc} was detected by immunofluorescence no re-warm was performed and fixation was tested with GLUT4\textit{myc} pulse-labelling before and after fixation.

In experiments where markers of endomembranes were required, antibody against the appropriate marker was used after permeabilization. Endogenous Stx6 was detected by mouse anti-Stx6 antibody (1:100) or rabbit anti-Stx6 (1:750) depending on the species of the matched antibody to label GLUT4. EEA1 and VAMP2-\textit{HA} were labelled was stated above.

For Tfn-A488 experiments, Tfn-A488 (50 \(\mu\)g/mL) in serum free media supplemented with 1% BSA was added to cells for 30 min prior to cell surface GLUT4\textit{myc} detection. Tfn-A488 was kept present during cell re-warm after GLUT4\textit{myc} pulse-labelling. Cells were fixed for 1 h in 4% PFA at room temperature.

For nocodazole experiments, 3 \(\mu\)M nocodazole was added during the 30 min cell re-warm after cell surface GLUT4\textit{myc} pulse-labelling. During nocodazole recovery, cells were washed once with PBS and placed in serum free media after 25 min nocodazole treatment. For C2-ceramide treatment, C2-ceramide was added during the initial 2 h serum starvation and kept present during re-warming. During 50 \(\mu\)M C2-ceramide recovery, cells were washed once with PBS and placed in serum free media for 15 min after the 2 h C2-ceramide treatment during serum starvation. There was no C2-ceramide present during the 30 min re-warming (total 45 min recovery). During 25 \(\mu\)M C2-ceramide recovery, C2-ceramide was removed during the 30 min re-warming (total 30 min recovery).

2.6 Insulin-responsive GLUT4 re-exocytosis

Cell surface GLUT4\textit{myc} was detected as described previously, with slight modifications (Ishikura et al., 2010). Cells were serum starved for 2 h prior to 15 min stimulation with 100 nM insulin. Cell surface GLUT4\textit{myc} was pulse-labelled at 4\textdegree{}C before cells were washed and re-warmed to 37\textdegree{}C in serum free media for indicated times (in most assays 30 min). Cells were then
treated with or without 100 nM insulin for 5 or 10 min, to stimulate GLUT4<em>myc</em> re-exocytosis. Cells were placed on ice, fixed in 4% PFA for 10 min, treated with 0.1 M glycine for 10 min, and surface GLUT4<em>myc</em> detected by horse-radish peroxidase conjugated secondary antibody (1 h at 4C) as previously described (Ishikura et al., 2010).

For nocodazole experiments in which nocodazole was used to disrupt GLUT4 sorting, 3 µM nocodazole was added during the 30 min cell re-warm after cell surface GLUT4<em>myc</em> pulse-labelling, but removed during insulin-stimulated GLUT4<em>myc</em> re-exocytosis. During nocodazole recovery, cells were washed once with PBS and placed in serum free media after 25 min nocodazole treatment. Nocodazole remained absent during insulin-stimulated GLUT4<em>myc</em> re-exocytosis. For nocodazole experiments in which nocodazole was used to inhibit the GLUT4 exocytosis step, 3 µM nocodazole was added 5 min prior to insulin-stimulated GLUT4<em>myc</em> re-exocytosis and remained present during re-exocytosis.

For C2-ceramide experiments, C2-ceramide was added during the initial 2 h serum starvation and kept present during re-warming and re-exocytosis. During 50 µM C2-ceramide recovery, cells were washed once with PBS and placed in serum free media for 15 min after the 2 h C2-ceramide treatment during serum starvation. There was no C2-ceramide present during the 30 min re-warming (total 45 min recovery) or re-exocytosis. During 25 µM C2-ceramide recovery, C2-ceramide was removed for the 30 min re-warming (total 30 min recovery) and re-exocytosis.

2.7 Detection of cell surface GLUT4

Cell surface GLUT4<em>myc</em> was detected as described previously, with slight modifications (Ishikura et al., 2010). Cells were serum starved for 2 h prior to 15 min stimulation with 100 nM insulin. Cells were then fixed in 3% PFA for 30 min (10 min on ice and 20 min at RT), washed with 0.1 M glycine for 10 min, and blocked in 5% goat serum for 20 min. Cell surface GLUT4<em>myc</em> was labelled at RT (rabbit anti-<em>myc</em> 1:500) and surface GLUT4<em>myc</em> detected by horse-radish peroxidase conjugated secondary antibody (1 h at RT, 1:1000) as previously described (Ishikura et al., 2010).

2.8 GLUT4 endocytosis

The disappearance of GLUT4<em>myc</em> from the plasma membrane was measured as previously described (Antonescu et al., 2008). Briefly, cells were serum starved for 2 h before being treated
with or without 100 nM insulin for 15 min. Cell surface GLUT4myc was pulse-labeled at 4C. Cells were washed and re-warmed to 37C for 10 min to induce GLUT4 endocytosis. Insulin was present during to re-warm for the condition labelled ‘insulin’. The ‘return to basal’ condition required cells to be treated with insulin for 15 min after serum starvation, but re-warmed in the absence of insulin. After the GLUT4 endocytosis re-warm step, cells were fixed on ice and GLUT4myc remaining at the cell surface was detected by horseradish peroxidase-conjugated secondary antibody.

2.9 GLUT4 Immunoprecipitation

2.9.1 Myc immunoprecipitation
L6GLUT4myc cells were grown to confluence in 10 cm dishes. Cells were serum starved for 2.5 h and then treated with or without insulin for 15 min. After being washed in PBS, cells were lysed in 800 µL 0.2% C12E8 lysis buffer (1 mM NaF, 10 nM okadaic acid, 1 mM Na3VO4, 1:500 PIC) in PBS. Lysates were passed through a 29-gauge syringe needle 10 times, and supernatants were collected by a 10-min spin at 12,000 × g. Lysates were pre-cleared with Sepharose beads for 30 min at 4C on a bench-top rotator. Supernatants were recovered and 30 µL of each sample was kept for total lysate measurements. The remaining supernatants were incubated with anti-myc conjugated Sepharose beads for 16 h at 4C on a bench-top rotator. Beads were centrifuged at 950 g for 30 s, supernatants were aspirated, and beads were washed (twice with lysis buffer and twice with PBS). Beads were then re-suspended in 50 µL 2x Laemmlli sample buffer for Western blotting analysis.

2.9.2 GLUT4 immunoprecipitation
L6GLUT4myc cells were grown to confluence in 10 cm dishes. Cells were serum starved for 2.5 h and then treated with or without insulin for 15 min. After being washed in PBS, cells were lysed in 600 µL 1% NP-40 lysis buffer (1 mM NaF, 10 nM okadaic acid, 1 mM Na3VO4, 1:500 PIC, 5% glycerol) in 1xTBS. Lysates were passed through a 29-gauge syringe needle 10 times, and supernatants were collected by a 10-min spin at 12,000 × g. Supernatants were recovered and 15 µL of each sample was kept for total lysate measurements. The remaining supernatants were incubated with Protein-G beads conjugated to either anti-GLUT4 1F8 antibody or in house rabbit anti-GLUT4 serum. Samples were incubated for 16 h at 4C on a bench-top rotator. Beads were then centrifuged at 950 g for 30 s and supernatants were aspirated. Beads were washed
three times with wash buffer (0.1% NP-40 in 1xTBS) and then once with 1xTBS. Beads were then re-suspended in 30 µL 2x Laemmli sample buffer for Western blotting analysis.

2.10 Immunoblotting

For siRNA, replicate wells from each experiment were lysed with 1% NP-40. Lysates were passed through a 29-gauge syringe needle 10 times, and supernatants were collected by a 10-min spin at 12,000 x g. Protein samples were mixed with 5x Laemmli sample buffer, resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Bio-Rad), and immunoblotted with the appropriate primary antibodies. Actinin-1 was blotted as a loading control (1:10000). Primary antibodies were detected with the appropriate HRP-conjugated species-specific IgG secondary antibodies. Immunoblotting was completed with Western Lightning Chemiluminescence Reagent Plus and HyBlot CL autoradiography film from Denville Scientific.

For detection of Akt phosphorylation, cells were grown to confluence in 12-well plates and treated with nocodazole or C2-ceramide exactly as described for insulin-responsive GLUT4 re-exocytosis. Cells were lysed in 1% NP-40 as above and blotted for P-Akt S473 (1:1000) and P-Akt T308 (1:1000).

2.11 Fluorescence microscopy and image analysis

Fluorescent images were acquired with an Olympus IX81 inverted fluorescence microscope equipped with a 60x objective (1.35 NA), Hamamatsu C9100-13 back-thinned EM-CCD camera, and Yokogawa CSU X1 spinning disk confocal scan head (with Spectral Aurora Borealis upgrade). Images were acquired of multiple z-slices (0.3 µm) and collapsed xy projections presented. Image analysis was performed using Perkin Elmer Volocity software. Single cells were selected and the Pearson’s correlation calculated for the whole cell using Volocity software.

2.12 Statistical analysis

Statistical analyses were carried out using Prism 4.0 software (GraphPad Software, San Diego, CA). Groups were compared using one-way analysis of variance Newman–Keuls post hoc analysis or Student T-Test. p < 0.05 was considered statistically significant.
3 Dynamics of GLUT4 traffic following internalization from the plasma membrane

According to the model of GLUT4 dynamics, GLUT4 localizes to three endomembrane compartments, namely sorting/recycling endosomes, the TGN, and the IRC/IRV (Figure 1.9). However, much of this model was developed in adipocytes. Given differences in the regulation of GLUT4 traffic in muscle and adipose cells, it is important to test this model in both cell systems. In muscle, GLUT4 occupies both cytosolic and perinuclear membranes and perinuclear GLUT4 disperses with insulin treatment (Boguslavsky et al., 2012; Fecchi et al., 2006; Lauritzen et al., 2008; Randhawa et al., 2008). Approximately 50% of GLUT4 occupies TfnR-positive endosomes at steady-state (Aledo et al., 1997; Zaid et al., 2008). The identity of the perinuclear pool of GLUT4 has not been well characterized.

In L6 muscle cells GLUT4 co-localizes with over-expressed VAMP2, Rab8a and Rab13 in the perinuclear region (Ishikura and Klip, 2008; Randhawa et al., 2000; Sun et al., 2010), but the localization of GLUT4 relative to endogenous proteins is unknown. GLUT4 vesicles in the periphery co-localize with Myo1c and, in response to insulin, over-expressed Rab13 (Boguslavsky et al., 2012; Sun et al., 2010). Localization of the IRC/IRV has not been identified, which may be due in part to limitations in studying steady-state GLUT4 localization.

In this chapter I characterize the steady-state localization of GLUT4 relative to protein markers of different intracellular compartments, focusing on the identity of the perinuclear GLUT4 pool. Then, to overcome limitations of previous studies, I label and track the transit of cell surface pulse-labelled GLUT4. This method allows me to characterize the localization of a sub-population of GLUT4 as it is sorted into insulin-responsive compartments. This GLUT4 labelling technique has been used previously in adipocytes to show that GLUT4 accumulates in a Stx6/Stx16-positive perinuclear compartment 1 hour after being internalized from the plasma membrane (Shewan et al., 2003). However, the acute kinetics with which GLUT4 sorts into this
compartment or how GLUT4 sorting into the Stx6-positive perinuclear compartment relates to insulin responsiveness have never been reported.

I find that GLUT4 is concentrated in the perinuclear region where it co-localizes with Stx6, Stx16, and over-expressed VAMP2. GLUT4 that has been pulse-labelled at the cell surface and allowed to internalize accumulates in a Stx6-positive perinuclear region by 30 min, which correlates with the acquisition of insulin-responsive GLUT4 re-exocytosis. These data invite the hypothesis that GLUT4 sorts from the plasma membrane to the Stx6-positive perinuclear compartment in order to acquire insulin responsiveness.

3.1 Steady-state localization of GLUT4<sub>myc</sub> in L6 muscle cells

In resting L6 muscle cells, GLUT4<sub>myc</sub> localizes in both cytosolic puncta and in the perinuclear region (Figure 3.1). The directionality of GLUT4 vesicle movement cannot be determined from studying steady-state GLUT4 localization; the cytosolic puncta represent both retrograde and anterograde vesicles. Likewise, the population of perinuclear GLUT4 is not necessarily homogeneous and is maintained in dynamic equilibrium.

**Figure 3.1:** GLUT4<sub>myc</sub> localization in a resting L6 muscle cell. Cells were fixed, permeabilized, and labelled with mouse anti-<i>myc</i> antibody. Dashed lines represent the outlines of individual cells. The red oval highlights the nucleus of the centred cell.

Despite these limitations, defining the intracellular localization of GLUT4 is critical for understanding the regulation of GLUT4 dynamics. Here, I characterize the localization of total GLUT4<sub>myc</sub> in L6 muscle cells under resting conditions. This characterization will serve as a reference for internalization studies. As shown in Figure 3.2, the perinuclear pool of GLUT4<sub>myc</sub>
co-localizes with Stx6 and Stx16, but shows little overlap with the TGN-resident protein furin. Stx6 and Stx16 also show considerable overlap.

Figure 3.2: GLUT4myc co-localizes with Stx6 and Stx16, but not with furin, in the perinuclear region. A) Cells were fixed, permeabilized, and labelled with mouse anti-myc antibody (red) and rabbit anti-Stx6, Stx16, or furin antibodies (green), scale = 6 µm. Inset images show single perinuclear slices of the boxed regions. B) Stx6 (red) and Stx16 (green) co-localize. Cells were treated as in A, scale = 17 µm. Inset image shows single perinuclear slice of the boxed region.

Sorting/recycling endosomes are labelled with internalized Tfn conjugated to a fluorophore (note: throughout this Thesis, when Tfn is used as a marker it is always internalized Tfn).

Preliminary experiments were conducted in L6 cells transiently expressing GFP-GLUT4myc in which Tfn uptake progressed for 10, 20, 30, or 60 min (Figure 3.3). Tfn failed to accumulate in the perinuclear GLUT4 compartment. However, many cytosolic puncta were positive for both Tfn and GFP-GLUT4myc at all time points of Tfn uptake.
Figure 3.3: Tfn-labelled endosomes co-localize with GFP-GLUT4myc in cytosolic puncta. Cells were incubated with Tfn conjugated to A555 fluorophore (red) for indicated times. Cells were then fixed and imaged (scale = 6 µm). Enlarged images show collapsed images of boxed regions.

These data demonstrate that internalized Tfn populates sorting/recycling endosomes that are also positive for GLUT4. From visual inspection it appears that all sorting/recycling endosomes are labelled with Tfn by 30 min of Tfn uptake. Thus, L6GLUT4myc cells were treated with Tfn-A488 for 30 min and examined for GLUT4myc co-localization with Tfn. One experiment also examined GLUT4myc co-localization with the early endosome marker EEA1. GLUT4myc shows some overlap with EEA1 and Tfn in cytosolic puncta, indicative that GLUT4 populates early/recycling endosomes (Figure 3.4). Perinuclear GLUT4myc was devoid of Tfn and EEA1.
Figure 3.4: GLUT4myc partially co-localizes with EEA1 and Tf. TOP panel: Cells were fixed, permeabilized, and labelled with rabbit anti-myc antibody (red) and mouse anti-EEA1 antibody (green), scale = 6 µm. BOTTOM panel: Cells were incubated with Tf conjugated to A488 fluorophore (green) for 30 min prior to being fixed, permeabilized, and labelled for GLUT4myc (red). Inset images show single perinuclear slices of the boxed regions. Far right column shows enlarged regions of collapsed images as outlined by blue lines.

These data suggest that GLUT4 accumulates in a specialized compartment that is distinct from sorting/recycling endosomes or the general TGN. However, none of these data allude to the localization of the IRC/IRV. The protein VAMP2, which is required for GLUT4-containing vesicle fusion with the plasma membrane in response to insulin, is enriched in the perinuclear region and translocates to the plasma membrane in response to insulin when expressed exogenously as a GFP fusion protein (Dugani et al., 2008; Randhawa et al., 2008). We have thus far been unable to visualize endogenous VAMP2 by immunofluorescence in L6 cells; however, stable expression of VAMP2-HA in L6GLUT4myc cells allows for the localization of GLUT4myc relative to VAMP2-HA to be examined. Stably expressed VAMP2-HA localizes to the perinuclear region and in cytosolic puncta in a similar manner as GLUT4myc (Figure 3.5).

VAMP2-HA stably transfected in L6GLUT4myc cells

Figure 3.5: VAMP2-HA expression in L6 muscle cells. VAMP2-HA was stably expressed in L6 muscle cells. Cells were fixed, permeabilized, and labelled with mouse anti-HA antibody (scale = 17 µm).
Thus, it is not surprising that GLUT4myc co-localizes with VAMP2-HA in the perinuclear region (Figure 3.6). Such an observation invites the hypothesis that the perinuclear compartment containing GLUT4 may be partially comprised of the IRC/IRV. However, VAMP2 is not exclusive to the IRC/IRV.

![Image of GLUT4myc co-localization with VAMP2-HA](image)

**Figure 3.6:** GLUT4myc co-localizes with VAMP2-HA in the perinuclear region. Cells were fixed, permeabilized, and labelled with rabbit anti-myc antibody (red) and mouse anti-HA antibody (green), scale = 15 μm. Inset image shows a single perinuclear slice of the boxed region.

Pearson correlations were used to quantify GLUT4myc co-localization with each of these protein markers (Figure 3.7). The best markers of GLUT4 localization also localize to the perinuclear region; however, not all proteins in the perinuclear region co-localize with GLUT4, as demonstrated by furin. The Pearson correlation for Stx6 co-localization with Stx16 was similar to that for each of these markers with GLUT4 (0.58, N=2; 7 cells quantified in each experiment).

![Graph of Pearson Correlations](image)

**Figure 3.7:** Quantification of GLUT4myc co-localization with markers of endomembrane compartments. Single cells were selected and Pearson correlations calculated using Volocity software. Tfn uptake was for 30 min prior to GLUT4 labelling, as described in Figure 3.4 (Stx6, N=4; Stx16, N=3; Furin, N=3; EEA1, N=1; Tfn, N=3; VAMP2-HA, N=2). 8-15 cells were quantified in each experiment.

To further explore the relationship between GLUT4 and the syntaxin proteins, GLUT4 was immunoprecipitated and probed for interactions with Stx6 or Stx16 (Figure 3.8).
Interestingly, GLUT4 interacts with Stx6, but not Stx16, and insulin does not affect this interaction. In adipocytes, GLUT4 interacts with both Stx6 and Stx16 (Perera et al., 2003). This difference may represent tissue-specific differences in the regulation of GLUT4 dynamics. Similarly, unlike in adipocytes, an interaction between GLUT4 and sortilin could not be detected (Figure 3.9).
Figure 3.9: GLUT4 does not interact with sortilin. GLUT4 was immunoprecipitated using anti-GLUT4 (in house rabbit anti-serum) antibody. Samples were then probed for sortilin (1:1000) by Western blot analysis. In one sample, the IgG control was spiked with lysate to confirm the sortilin band in IP samples. Whole lysate equivalents were blotted as controls.

In summary, GLUT4\text{myc} localization in L6 muscle cells can be best described as exhibiting accumulation in a Stx6- and Stx16-positive perinuclear compartment that is devoid of TfnR (internalized Tfn) and furin. However, Stx6 may be the more relevant marker, as GLUT4\text{myc} interacts with Stx6 but not Stx16. GLUT4\text{myc} also localizes to cytosolic vesicles that partially consist of sorting/recycling endosomes. Based on these data, the sorting of GLUT4\text{myc} was explored in order to characterize the dynamics of GLUT4 accumulation in these compartments.

3.2 Tracking the internalization of GLUT4 from the plasma membrane

3.2.1 Antibody Validation

In order to track the sub-population of GLUT4 being actively sorted into intracellular compartments, strategies require that cell surface GLUT4\text{myc} be labelled at 4\text{C}. As a first step, I sought to confirm that antibodies against the \text{myc} tag faithfully labelled cell surface GLUT4\text{myc} at 4\text{C} in non-fixed cells and that GLUT4\text{myc} proceeded to internalize. Using a rabbit polyclonal antibody against \text{myc}, GLUT4\text{myc} internalized and began to accumulate in the perinuclear region by 20 min; alternatively, GLUT4\text{myc} labelled using a mouse monoclonal antibody (9E10) remained mostly in cytosolic puncta (Figure 3.10).
Within 20 min, GLUT4\textsubscript{myc} internalized from the plasma membrane begins to accumulate in the perinuclear region. Cell surface GLUT4\textsubscript{myc} was labelled at 4C with rabbit anti-\textit{myc} or mouse anti-\textit{myc} (9E10) antibody. Cells were then re-warmed to 37C for 20 min before being fixed, permeabilized, and treated with fluorophore conjugated secondary antibody to visualize internalized GLUT4\textsubscript{myc} (scale = 3 µm).

To determine if the 9E10 antibody bound to cell surface GLUT4\textsubscript{myc} I conducted an experiment in which cell surface GLUT4\textsubscript{myc} was labelled with 9E10 antibody either before or after fixation (Figure 3.11). Cell surface GLUT4\textsubscript{myc} appeared to be labelled regardless of fixation timing. However, during image acquisition it was noted that the signal intensity while using 9E10 was much lower and was in many cases difficult to detect. Preliminary experiments that examined steady-state cell surface GLUT4\textsubscript{myc} levels with or without insulin stimulation suggested that the 9E10 antibody was inefficient at binding to the \textit{myc} epitope in non-fixed cells.

The 9E10 antibody labels cell surface GLUT4\textsubscript{myc} in both fixed and non-fixed cells. Cells were incubated with mouse anti-\textit{myc} (9E10) antibody for 1 h at 4C either before or after fixation. Surface GLUT4\textsubscript{myc} was detected with fluorophore conjugated secondary antibody (scale = 3 µm). In one sample, cells were fixed and permeabilized before labelling with mouse anti-\textit{myc} (9E10) antibody.
Next, I tested whether surface GLUT4myc could be labelled with secondary antibody and still faithfully sorted into the perinuclear region. As shown in Figure 3.12, labelling cell surface GLUT4myc with both primary and secondary antibodies before internalization prevented GLUT4myc from accumulating in the perinuclear region. Similarly, replacing the full length secondary antibody with a Fab fragment secondary antibody also inhibited proper GLUT4myc sorting. In one experiment, cell surface GLUT4myc was labelled with Fab secondary antibody followed by fixation and labelling with A647 full length secondary antibody to determine if free binding sites not occupied by the Fab secondary antibody were available on the rabbit anti-myc antibody. As no A647 signal was detected, it was presumed that the Fab fragment secondary antibody bound efficiently to the rabbit anti-myc antibody. Experiments were also conducted in which primary antibody was directly conjugated to a fluorophore: this antibody failed to detect cell surface GLUT4myc, total GLUT4myc, or GLUT4myc after 30 min internalization.

Figure 3.12: Labelling cell surface GLUT4myc with both primary and secondary antibodies before allowing GLUT4 internalization inhibits GLUT4 accumulation in the perinuclear region. Cells were incubated with rabbit anti-myc antibody for 1 h at 4C. In control experiments (columns 1 and 3), cells were rewarmed to 37C for 20 min before being fixed, permeabilized, and treated with fluorophore conjugated secondary antibody for GLUT4myc detection (scale = 3 µm). In test experiments (columns 2 and 4), cells were treated with fluorophore conjugated secondary antibody for 1 h at 4C. Cells were then rewarmed for 20 min and fixed.

In summary, the 9E10 antibody did not reliably label GLUT4myc in non-fixed cells. As such, the 9E10 antibody was not used in GLUT4myc internalization experiments. The use of antibody strategies that fluorescently labelled cell surface GLUT4myc either prevented normal
accumulation of GLUT4myc into the perinuclear compartment or could not be detected by fluorescence microscopy. As such, tracking GLUT4 sorting in live cells was not pursued. Instead, the rabbit anti-myc antibody was used for the detection of GLUT4myc in all experiments in which cell surface GLUT4myc was labelled in non-fixed cells. In some cases, mouse anti-HA antibody was used to label cell surface GLUT4-HA in L6GLUT4-HA cells. These studies examined GLUT4myc sorting in fixed cells.

3.2.2 Sorting of GLUT4 upon its internalization from the plasma membrane, vis-à-vis the localization of endogenous markers

The transit of GLUT4myc through intracellular compartments was tracked by single cell immunofluorescence imaging. First, the time course of GLUT4myc internalization was tracked over time (Figure 3.13). Surface labelled GLUT4myc that had internalized for 2-10 min localized to cytosolic puncta. By 20 min GLUT4myc began to accumulate in the perinuclear region; however, not all cells showed perinuclear accumulation. Perinuclear accumulation of GLUT4myc was homogenous by 30 min such that the internalized GLUT4myc showed similar distribution to that of total GLUT4myc. In some cells GLUT4myc also showed accumulation in a superficial compartment distinct from the perinuclear compartment (compare 30 min images, red arrow). Insulin treatment did not visibly alter the accumulation of internalized GLUT4myc (data not shown).

Figure 3.13: GLUT4myc accumulates in the perinuclear region between 20 and 30 min following internalization from the plasma membrane. Cell surface GLUT4myc was labelled at 4C with rabbit anti-myc antibody. Cells were then re-warmed to 37C for indicated times before being fixed and permeabilized. Internalized GLUT4myc was visualized with fluorophore conjugated secondary antibody (scale = 8 µm).
Time points of 10 and 30 min were selected to examine GLUT4\textsubscript{myc} co-localization with Stx6 and internalized Tfn. GLUT4\textsubscript{myc} internalized for 10 min showed little overlap with Stx6. However, at 30 min, GLUT4\textsubscript{myc} had accumulated in the Stx6-positive perinuclear compartment (Figure 3.14A). The Pearson correlation for the co-localization of internalized GLUT4\textsubscript{myc} and Stx6 was statistically significant after 30 min (Figure 3.14B).

![Figure 3.14: Internalized GLUT4 sorts into a Syntaxin-6-positive perinuclear compartment. A) Cell surface GLUT4 was labeled at 4C before cells were re-warmed to 37C for 0, 10, or 30 min. Fixed cells were then stained for endogenous Stx6 (green), scale = 6 μm. Inset = single slice of perinuclear region. B) Quantification of the co-localization between GLUT4 and Stx6 using Pearson’s Correlation coefficient (N=3, 10-15 cells per experiment), *p<0.01; # p<0.001.](image)

Conversely, GLUT4\textsubscript{myc} that had co-internalized with Tfn localized to Tfn-positive cytosolic puncta but sorted into a segregated perinuclear compartment by 30 min (Figure 3.15A). Tfn also
accumulated in a superficial endosome compartment, similar to that described for GLUT4myc in Figure 3.13. Similar results were obtained when Tfn-positive endosomes were pre-labelled for 30 min prior to cell surface GLUT4myc labelling and internalization (Figure 3.15B). Thus, GLUT4myc that had been previously labelled at the cell surface localized to the Stx6-positive perinuclear compartment distinct from sorting/recycling endosomes by 30 min.

**Figure 3.15:** Internalized GLUT4 segregates away from Transferrin. A) Cells were incubated with rabbit anti-myc antibody and A488-conjugated Tfn for 10 or 30 min. Cells were then fixed, permeabilized, and stained for internalized GLUT4myc (red), scale = 6 µm. Inset = single slice of perinuclear region. Enlarged images show collapsed area outlined by blue lines. B) Cells were incubated with A488-conjugated Tfn for 30 min. Cell surface GLUT4myc was then labelled at 4C before cells were re-warmed to 37C for 30 min, fixed, and permeabilized for staining of internalized GLUT4myc (red), scale = 6 µm. Inset = single slice of perinuclear region.

Although insulin did not appear to alter the accumulation of GLUT4myc in the perinuclear region (data not shown), this result was readdressed by quantitative means. The accumulation of GLUT4myc in the Stx6-positive perinuclear compartment was measured in the presence and absence of insulin (Figure 3.16). Insulin did not alter the co-localization of internalized GLUT4myc with Stx6 at 10 or 30 min.
Figure 3.16: Internalized GLUT4 sorts into a Syntaxin-6-positive perinuclear compartment independent of insulin. A) Cell surface GLUT4myc was labeled at 4C before cells were re-warmed to 37C for 0, 10, or 30 min. Fixed cells were then stained for endogenous Stx6 (green), scale = 15 μm. Inset = single slice of perinuclear region. B) Same protocol as in A, but cells were treated with insulin for 20 min prior to cell surface labelling of GLUT4myc and insulin was present during cell re-warm. C) Pearson correlations were used to quantify the co-localization between GLUT4 and Stx6 (N=3, 10 cells per experiment).

GLUT4myc co-localization with EEA1 was also examined. GLUT4myc localized to EEA1-positive endosomes upon internalization, but accumulated in the perinuclear compartment that was devoid of EEA1 (Figure 3.17).
Figure 3.17: Internalized GLUT4 localizes to EEA1-positive cytosolic vesicles. Cell surface GLUT4\textit{myc} was labelled at 4\(^\circ\)C before cells were re-warmed to 37\(^\circ\)C for 2, 5, 10, or 30 min. Fixed cells were then stained for endogenous EEA1 (green), scale = 6 \(\mu\)m. Inset = single slices of peripheral (2 and 5 min) or perinuclear (10 and 30 min) regions. Enlarged images show collapsed area outlined by blue lines.

GLUT4\textit{myc} accumulation with VAMP2-HA was examined as a potential marker of the IRC/IRV. Surprisingly, internalized GLUT4\textit{myc} co-localized with VAMP2-HA-positive puncta at 2 min and accumulated in these endosomes at 5 and 10 min (Figure 3.18). By 30 min, internalized GLUT4\textit{myc} had accumulated in the VAMP2-HA-positive perinuclear compartment. Insulin did not appear to alter the sorting of GLUT4\textit{myc} into the VAMP2-positive compartment.
Figure 3.18: Internalized GLUT4 co-localizes with VAMP2-HA in cytosolic vesicles and in the perinuclear region.
A) Cell surface GLUT4myc was labelled at 4°C before cells were re-warmed to 37°C for 0, 2, 5, 10, or 30 min. Fixed cells were then stained for VAMP2-HA (green), scale = 5 μm. Inset = single slices of peripheral (2, 5, and 10 min) or perinuclear (30 min) regions. B) Same protocol as in A, but cells were treated with insulin for 20 min prior to cell surface labelling of GLUT4myc and insulin was present during cell re-warm.

The co-localization of internalized GLUT4myc with VAMP2-HA in cytosolic puncta suggests that perhaps GLUT4 sorts through specialized endosomes that acquire insulin-sensitivity early upon internalization. However, it is also possible that VAMP2 normally localizes to recycling endosomes or that the over-expression of VAMP2-HA caused VAMP2 to localize to all endosomes. Tagged-VAMP2 has been used as a surrogate of the IRC/IRV in vesicle fusion assays in response to insulin (Xu et al., 2011). I examined the recycling and insulin-responsive exocytosis of VAMP2-HA-positive endosomes by monitoring cell surface VAMP2-HA. VAMP2-HA could not be detected at the cell surface of L6GLUT4myc cells in either resting cells or in response to insulin. Furthermore, recycling VAMP2-HA could not be detected by
fluorescence microscopy. Thus, it may be that the stably expressed VAMP2-HA does not faithfully represent the IRC/IRV as suggested in adipocytes.

Internalized GLUT4 transits cytosolic vesicles most likely representing sorting/recycling endosomes en route to a Stx6-positive perinuclear compartment. GLUT4 reaches the Stx6-positive perinuclear compartment by 30 min and appears distributed very similarly to that of total GLUT4 in steady-state resting muscle cells. However, how does this localization of GLUT4 relate to insulin-responsiveness? Previous work in adipocytes reports that internalized GLUT4 regains insulin-responsiveness at 4 h without investigating earlier time points (Capilla et al., 2007). I performed a time-course for GLUT4 re-acquisition of insulin-responsiveness in L6 cells. Cells were treated with insulin for 15 min followed by cell surface GLUT4myc labelling at 4C. Cells were then re-warmed to 37C to allow GLUT4 to equilibrate with intracellular membranes for 15 min to 2 h. The previously internalized GLUT4myc displayed insulin-responsive re-exocytosis 30 min following internalization (Figure 3.19). By 90 min the insulin-responsive re-exocytosis was even greater, demonstrating that more of the previously internalized GLUT4myc had equilibrated with insulin-responsive vesicles (IRC/IRV).

The acquisition of insulin-responsive GLUT4myc re-exocytosis most closely correlates with the accumulation of GLUT4myc in the Stx6-positive perinuclear compartment. As such, Stx6 is a candidate for proteins that regulate GLUT4 sorting into the IRC/IRV in the perinuclear region in muscle cells.

![Figure 3.19: Internalized GLUT4myc exhibits insulin-responsive re-exocytosis by 30 min (N=2), *p<0.05; # p<0.01. Cells were treated with insulin for 15 min before being placed at 4C and cell surface GLUT4myc labelled. Cells were re-warmed to 37C for indicated times, treated with insulin for 10 min, and then fixed at 4C for detection of cell surface GLUT4myc.](image)
3.2.3 Sorting of GLUT4 following internalization from the plasma membrane relative to Rab-GFP markers

Rab proteins act as critical regulators of intracellular transport. Rab cascade models have emerged in which the sequential recruitment and activation of Rab proteins recruits effectors necessary for the retrograde and anterograde transport of cargo proteins (Mizuno-Yamasaki et al., 2012). Several Rab proteins have been implicated in different steps of GLUT4 traffic. For GLUT4 retrograde traffic, Rab5 has been suggested to regulate GLUT4 sorting through early endosomes while Rab11 has been suggested to regulate GLUT4 sorting from recycling endosomes to the IRC/IRV (Huang et al., 2001; Zeigerer et al., 2002). I transiently transfected Rab5- and Rab11-GFP constructs in L6GLUT4myc cells and examined GLUT4myc sorting relative to these markers. Rab5-GFP localized to cytosolic puncta and in the perinuclear region. Internalized GLUT4myc co-localized with Rab5-GFP in cytosolic puncta at 2, 5, 10, and 20 min (Figure 3.20). By 20 min GLUT4myc began to co-localize with Rab5-GFP in the perinuclear region.
Figure 3.20: Internalized GLUT4myc co-localizes with Rab5-GFP in cytosolic vesicles and in the perinuclear region. Cell surface GLUT4myc was labeled at 4C before cells were re-warmed to 37C for 2, 5, 10, or 20 min. Fixed cells were then stained for internalized GLUT4myc (red), scale = 6 μm. Enlarged images show collapsed areas outlined by boxed regions.

In contrast, Rab11-GFP displayed mostly perinuclear localization. Internalized GLUT4myc showed little co-localization with Rab11-GFP at 10 min, but these proteins began to show some co-localization in the perinuclear region at 20 min (Figure 3.21). The strongest co-localization between internalized GLUT4myc and Rab11-GFP was observed in the superficial compartment above the nucleus (marked by Tfn in Figure 3.15).

Figure 3.21: Internalized GLUT4myc partly co-localizes with Rab11-GFP in the perinuclear region. Cell surface GLUT4myc was labeled at 4C before cells were re-warmed to 37C for 0, 10, or 20 min. Fixed cells were then stained for internalized GLUT4myc (red), scale = 6 μm. Inset = single slices of perinuclear region.

It was not expected that Rab5 would localize to the perinuclear region with GLUT4. It is possible that over-expression of Rab proteins does not allow for interpretation of co-localization with GLUT4 in the perinuclear region. To address this concern, GLUT4myc co-localization with Rab proteins not previously linked to regulating GLUT4 traffic was examined. Both Rab7- and Rab9-GFP co-localized with internalized GLUT4myc in the perinuclear region between 20 and
30 min (Figure 3.2). These data suggest that Rab protein over-expression is not useful for characterizing the localization of GLUT4\textit{myc} in the perinuclear compartment.

![GLUT4\textit{myc} and Rab proteins](image)

**Figure 3.2**: Internalized GLUT4\textit{myc} co-localizes with Rab7- and Rab9-GFP in the perinuclear region. Cell surface GLUT4\textit{myc} was labeled at 4°C before cells were re-warmed to 37°C for 20 or 30 min. Fixed cells were then stained for internalized GLUT4\textit{myc} (red), scale = 5 μm. Inset = single slices of perinuclear region.

In summary, only Rab5-GFP co-localizes with GLUT4\textit{myc} in cytosolic puncta and only Rab11-GFP co-localizes with GLUT4\textit{myc} in the superficial endosome compartment. These data suggest that internalized GLUT4\textit{myc} transits sorting/recycling endosomes but then accumulates in the Stx6-positive perinuclear compartment that is distinct from general recycling compartments. However, because GLUT4\textit{myc} co-localizes with each Rab-GFP protein in the perinuclear region, the over-expression of Rab proteins is not a useful tool for characterizing the perinuclear accumulation of internalized GLUT4\textit{myc} or how this relates to the acquisition of GLUT4 insulin-responsiveness.

### 3.2.4 Sorting of GLUT4 following internalization from the plasma membrane relative to Phosphatidylserine

Phosphatidylserine (PS) is a negatively charged phospholipid that concentrates in the plasma membrane; however, PS is also distributed to a lesser degree in endomembrane compartments (Uchida et al., 2011; Yeung et al., 2008). Recent work attributes a regulatory role for PS in retrograde transport at recycling endosomes (Lee et al., 2012; Uchida et al., 2011). Specifically,
PS depletion impairs TGN38/46 sorting from endosomes to the TGN (Lee et al., 2012). The lactadherin-C2 domain (Lact-C2) has previously been used as a probe that binds specifically to PS (Yeung et al., 2008). Here, the co-localization of internalized GLUT4myc with the Lact-C2-GFP probe was examined. Internalized GLUT4myc did not concentrate in Lact-C2-GFP-positive cytosolic puncta (Figure 3.23). Likewise, GLUT4myc did not appear to accumulate with Lact-C2-GFP in the perinuclear compartment – although overlap between GLUT4myc and Lact-C2-GFP is apparent at 20 min in the merged image, examining the perinuclear concentration of GLUT4myc versus the perinuclear signal of Lact-C2-GFP in the single channel 20 min images highlights that GLUT4myc is not accumulating in a PS-positive compartment. Given this lack of co-localization, a role for PS in regulating the sorting of GLUT4 was not pursued.

**Figure 3.23:** Internalized GLUT4myc does not localize to PS-containing membranes. Cell surface GLUT4myc was labeled at 4C before cells were re-warmed to 37C for 5, 10 or 20 min. Fixed cells were then stained for internalized GLUT4myc (red), scale = 30 μm. Enlarged images show collapsed areas outlined by boxed regions.
3.3 Conclusions

In L6 muscle cells, GLUT4 localizes to both cytosolic vesicles and in a Stx6- and Stx16-positive perinuclear compartment that is segregated from sorting/recycling endosomes (marked by internalized Tf). Perinuclear GLUT4 also shows little overlap with the TGN-resident protein furin. The cytosolic vesicles are partially comprised of sorting/recycling endosomes labelled with EEA1 and Tf. These endosomes are also marked by Rab5-GFP and VAMP2-HA. GLUT4 that has been labelled at the cell surface and allowed to internalize localizes to sorting/recycling endosomes before being sorted into the Stx6-positive perinuclear compartment that is devoid of Tf. Internalized GLUT4\textit{myc} accumulates in the Stx6-positive perinuclear compartment by 30 min. Interestingly, GLUT4 that has been recruited to the cell surface by insulin treatment, labelled at the cell surface, and allowed to re-internalize displays insulin-responsive re-exocytosis 30 min after internalization. Thus, GLUT4 accumulation in the Stx6-positive perinuclear compartment correlates with GLUT4 acquisition of insulin-responsive exocytosis. These data suggest that the Stx6-positive perinuclear compartment may be comprised of, or at least give rise to, the IRC/IRV. How GLUT4 sorts into the Stx6-positive perinuclear compartment and possible regulation by VAMP2, Rab5, and Rab11 will be addressed in subsequent chapters.
GLUT4 sorting in response to insulin: Syntaxin-6 regulates the acquisition of insulin-responsive GLUT4 exocytosis

GLUT4 localization to a Stx6-positive, but furin negative, perinuclear compartment suggests that this GLUT4-containing compartment may represent a sub-compartment of the TGN or even the IRC/IRV. Although internalized GLUT4 sorts into the Stx6-positive perinuclear compartment, the functional significance of this localization is unknown. The participation of Stx6 in GLUT4 traffic in muscle cells remains unexplored, yet physiologically muscle is the major tissue responsible for insulin-dependent glucose disposal, and the cell biology of GLUT4 traffic in muscle and adipose cells shows similarities but also differences (Foley et al., 2011). In this chapter I report that Stx6 regulates, at least in part, GLUT4 acquisition of insulin-responsive exocytosis but not GLUT4 sorting into its perinuclear compartment.

4.1 The steady-state localization of intracellular GLUT4 does not suffice to reveal the importance of Stx6 to GLUT4 traffic

To test if Stx6 is required to maintain normal GLUT4 localization in L6 muscle cells, Stx6 was knocked down via cognate siRNA and total cellular GLUT4 distribution examined relative to endogenous protein markers and VAMP2-HA. Stx6 knockdown was observed by immunofluorescence (Figure 4.1A). Since Stx16 localizes to the same compartment as Stx6, GLUT4myc co-localization with Stx16 was used to measure any deviation in GLUT4myc distribution upon Stx6 knockdown. GLUT4myc displayed strong perinuclear localization with Stx6 knockdown and remained co-localized with Stx16 (Figure 4.1B).
Figure 4.1: Stx6 depletion does not alter the localization of GLUT4 to the Stx16-positive perinuclear compartment. Cells were fixed, permeabilized, and labelled for A) GLUT4myc (red) and Stx6 (green) or B) GLUT4myc (red) and Stx16 (green), scale = 6 μm. Inset = single slices of perinuclear regions.

Likewise, Stx6 knockdown did not alter GLUT4myc co-localization with either furin or EEA1 (Figure 4.2).
Figure 4.2: Stx6 depletion does not alter the segregation of GLUT4 from furin or EEA1 compartments. Cells were fixed, permeabilized, and labelled for A) GLUT4myc (red) and furin (green) or B) GLUT4myc (red) and EEA1 (green), scale = 6 μm. Inset = single slices of perinuclear regions.

In non-related treated cells GLUT4myc appeared segregated from Tfn in the perinuclear region and Stx6 knockdown did not alter this localization (Figure 4.3A). Furthermore, GLUT4myc remained co-localized with VAMP2-HA in Stx6 knockdown cells (Figure 4.3B).
Figure 4.3: Stx6 depletion does not alter GLUT4 localization relative to Tfnn or VAMP2-HA. A) Cells were loaded with Tfnn-A488 for 30 min before being fixed, permeabilized, and labelled for GLUT4myc (red) B) Cells were fixed, permeabilized, and labelled for GLUT4myc (red) and VAMP2-HA (green), scale = 15 μm. Inset = single slices of perinuclear regions.

The co-localization of GLUT4myc with each of these markers is quantified in Figure 4.4. Stx6 is not required to maintain GLUT4 localization to the perinuclear compartment or to keep GLUT4 segregated from Tfnn or furin. However, it is possible that Stx6 functions within the perinuclear compartment to sort GLUT4 into insulin-responsive compartments.
Figure 4.4: Stx6 depletion does not alter GLUT4 localization. GLUT4myc co-localization with markers of endomembrane compartments was quantified using Pearson correlations. TfN uptake was for 30 min prior to GLUT4 labelling. Single cells were selected and Pearson correlations calculated using Volocity software (N=2; EEA1 and VAMP2-HA, N=1). 8-15 cells were quantified in each experiment.

To test if Stx6 is functionally required for insulin-stimulated GLUT4 translocation, cell surface GLUT4myc was measured before and after acute insulin stimulation, with and without Stx6 depletion. Stx6 knockdown caused a 1.4-fold increase in cell surface GLUT4myc in resting cells (Figure 4.5A). Addition of insulin caused a 2-fold increase in cell surface GLUT4myc in non-related cells but only a 1.7-fold increase in Stx6 knockdown cells. This reduction is reflected as an 18% reduction in the delta % change in cell surface GLUT4myc in Stx6 knockdown cells (Figure 4.5B).
Figure 4.5: Stx6 depletion increases cell surface GLUT4 and inhibits insulin-stimulated GLUT4 translocation. A) Cells were treated with or without insulin for 15 min before being fixed for the detection of cell surface GLUT4myc. * p<0.01 (N=7). Representative Western blot of Stx6 knockdown is shown. B) Data from A, expressed as delta % change [(Insulin-Basal/Insulin)] in cell surface GLUT4myc in response to insulin stimulation, * p<0.01.

It is interesting to note that, in Stx6 knockdown cells, the absolute increase in cell surface GLUT4myc in the basal condition (0.4 units relative to siNR basal) is of the same magnitude as that observed in the insulin stimulated condition (0.4 units relative to siNR insulin). These data support a role for Stx6 in maintaining GLUT4 retention, perhaps through regulating GLUT4 sorting away from constitutively recycling compartments. By depleting Stx6, GLUT4 may not accumulate in the IRC/IRV as efficiently. This would reduce insulin-responsive GLUT4 translocation, as observed by the 18% reduction in Figure 4.5B. However, GLUT4 still displays a 1.7 fold increase in insulin-responsive translocation, indicating that GLUT4 is still recruited to the plasma membrane in response to insulin. It is possible that GLUT4 was not sufficiently depleted from the IRC/IRV upon Stx6 knockdown. If sufficient GLUT4 remained in the IRC/IRV for a single response to insulin and Stx6 does not regulate the recruitment of GLUT4 from the IRC/IRV to the plasma membrane, then Stx6 knockdown would have minimal effect on insulin stimulated translocation. In this case, GLUT4 sorting defects upon Stx6 knockdown
would not be easily observed by measuring steady-state changes in cell surface GLUT4. It may be that these measures underestimate the role of Stx6 in regulating GLUT4 traffic.

4.2 Syntaxin-6 regulates GLUT4 sorting into the IRC/IRV but not GLUT4 accumulation in the perinuclear compartment

To address the role of Stx6 in GLUT4 sorting, the sub-population of GLUT4 being actively sorted along its retrograde pathway needs to be examined. Here, Stx6 was knocked down via cognate siRNA and the perinuclear accumulation of internalized GLUT4myc previously labelled at the cell surface was measured. After 30 min of internalization, GLUT4myc accumulation in the perinuclear region was indistinguishable from that in control cells (Figure 4.6).

![Figure 4.6](image)

**Figure 4.6:** Stx6 depletion does not inhibit internalized GLUT4 from accumulating in the perinuclear region. A) Cell surface GLUT4myc was labelled at 4C. Cells were re-warmed to 37C for 30 min before being fixed, permeabilized, and stained for Stx6 (green), scale = 3 µm. B) Quantification of perinuclear GLUT4myc accumulation, expressed as the perinuclear GLUT4myc fluorescence intensity / total GLUT4myc fluorescence intensity (N=3). An average of 10 cells was quantified per experiment.

As confirmation that GLUT4myc accumulation in the perinuclear compartment was not altered by Stx6 knockdown, GLUT4myc co-localization with VAMP2-HA was measured 10 min and 30
min after GLUT4myc internalization (Figure 4.7). Stx6 knockdown did not alter GLUT4myc sorting to VAMP2-HA-positive membranes.

These data indicate that Stx6 does not regulate GLUT4 sorting into the perinuclear compartment. However, is internalized GLUT4 insulin-responsive in Stx6 knockdown cells? To test this, L6GLUT4myc cells were treated with insulin, cell surface GLUT4myc was labelled at 4C, and cells were re-warmed to 37C for 30 min. This allowed GLUT4myc to sort into the perinuclear compartment as previously defined. Next, cells were treated with insulin for 10 min to stimulate GLUT4 re-exocytosis and cell surface GLUT4myc was measured. In this way, only GLUT4myc that had been recruited to the cell surface and re-internalized into insulin-responsive vesicles would be detected. After 30 min of internalization, Stx6-depleted cells had 2.6-fold more GLUT4myc at the plasma membrane than control cells (Figure 4.8A). Insulin induced an
increase in cell surface GLUT4\textit{myc}, but the insulin-responsive GLUT4\textit{myc} re-exocytosis was reduced by 40% in cells depleted of Stx6 (Figure 4.8B). Even though the level of cell surface GLUT4\textit{myc} is higher in Stx6-depleted cells, that a 2-fold increase in insulin-responsive GLUT4 re-exocytosis was not observed highlights the importance of Stx6 to GLUT4 sorting. This is because approximately 50% of GLUT4 is contained in the IRC/IRV, yet only one fifth of that is required to double cell surface GLUT4. Thus, despite the increase in cell surface GLUT4 in the resting state, a sufficiently large pool of GLUT4 would remain in the IRC/IRV to elicit a 2-fold increase in GLUT4 re-exocytosis in Stx6-depleted cells if Stx6 did not play a role in GLUT4 sorting.

\textbf{Figure 4.8:} Stx6 depletion inhibits insulin-responsive GLUT4 re-exocytosis. A) All cells were treated with insulin for 15 min and then cell surface GLUT4\textit{myc} was labelled at 4C. Cells were re-warmed to 37C for 30 min, treated with or without insulin for 10 min, and then fixed for the detection of cell surface GLUT4\textit{myc} (N=5). B) Data from A, expressed as delta % change [(Insulin-Basal/Insulin)] in cell surface GLUT4\textit{myc} in response to insulin stimulation, * p<0.01.

This behaviour is consistent with defective GLUT4 sorting into the insulin-responsive compartment and suggests that only by measuring GLUT4 internalization and re-exocytosis can
one determine the impact of Stx6 on GLUT4 sorting. It is reasoned that inefficient GLUT4 sorting into the IRC/IRV leads to its accumulation in a readily recycling compartment that shuttle GLUT4 back to the plasma membrane. Although insulin stimulates a partial GLUT4 translocation response, this can be due to accelerated general recycling of GLUT4 accumulated in pre-IRC/IRV compartments or to recruitment of a diminished pool of labelled GLUT4 in the IRC/IRV.

Alternatively, Stx6 knockdown may inhibit the rate of GLUT4myc endocytosis. By membrane fractionation, inhibiting Stx6 function (Stx6-cyto) delays the disappearance of GLUT4 from the plasma membrane following insulin removal in adipocytes (Perera et al., 2003). However, such a role for Stx6 in L6 muscle cells was ruled out by direct measurements of the effect of Stx6 knockdown on GLUT4myc disappearance from the plasma membrane (Figure 4.9A). Furthermore, the rate of GLUT4myc disappearance from the plasma membrane was not altered by “return to basal” conditions in L6 muscle cells (Figure 4.9B).
Figure 4.9: Stx6 depletion does not inhibit GLUT4 endocytosis. A) Cells were treated with or without insulin for 15 min and then cell surface GLUT4myc was labelled at 4°C. Cells were re-warmed to 37°C for 10 min in the absence or presence of insulin and then fixed for the detection of cell surface GLUT4myc (N=6). B) Cells were treated as in A, but the “return to basal” condition was added (Rtn). For Rtn, cells were treated with insulin for 15 min, cell surface GLUT4myc labelled, and then cells re-warmed to 37°C for 10 min in the absence of insulin (N=2).

In summary, although Stx6 is not necessary for GLUT4 sorting into the perinuclear region, these data suggest that Stx6 regulates in part the dynamic re-entry of GLUT4 into the IRC/IRV. GLUT4 may also be more accessible to constitutive recycling in Stx6-depleted cells. Given that internalized GLUT4 accumulates in a Stx6-positive perinuclear compartment, it is plausible that this compartment contains both slowly recycling vesicles and the IRC/IRV.

4.3 GLUT4 accumulation in the Syntaxin-6-positive perinuclear compartment does not depend on canonical sorting mechanisms

Although Stx6 regulates GLUT4 sorting into the IRC/IRV, it is unknown how GLUT4 sorts into the Stx6-positive perinuclear compartment. To this end, a number of molecules were investigated with roles related to retrograde transport, GLUT4 availability, and GLUT4 fusion at the plasma membrane. Except for dynein, none of these proteins were found to regulate GLUT4 sorting into the perinuclear region.

4.3.1 Molecules involved in retrograde transport

Rab5, Rab22a, and dynein were investigated with regards to GLUT4 sorting into the perinuclear region. Rab5, which regulates GLUT4 sorting in adipocytes (Huang et al., 2001), co-localizes with internalized GLUT4myc in cytosolic puncta when over-expressed in L6 muscle cells (Chapter 3 above). Rab22a is localized to endosomes and is involved in retrograde transport between sorting endosomes and the TGN (Galvez et al., 2012). Dynein is required for minus end directed microtubule transport of vesicles (Hunt and Stephens, 2011). Regulation of dynein function requires a multi-subunit complex called dynactin. Over-expressing one subunit of the dynactin complex, dynamitin, acts in a dominant negative fashion to dynein function (Burkhardt et al., 1997), here referred to as DN-Dynein.

DN-Rab5-GFP and DN-Dynein-GFP were transiently transfected into L6GLUT4myc cells and total GLUT4myc localization was examined. Whereas DN-Rab5-GFP had no effect on GLUT4myc distribution, DN-Dynein-GFP dispersed the perinuclear pool of GLUT4myc (Figure
One hundred percent of GFP control and DN-Rab5-GFP cells had normal GLUT4\textit{myc} localization. One hundred percent of DN-Dynein-GFP cells had disrupted GLUT4\textit{myc} localization.

![Image](image_url)

**Figure 4.10**: DN-Dynein, but not DN-Rab5, perturbs GLUT4 localization in L6 muscle cells. DN-Rab5-GFP or DN-Dynein-GFP was transiently transfected into L6 muscle cells for 18 h. Cells were then fixed and permeabilized for the detection of total GLUT4\textit{myc} (red), scale = 6 µm (N=1). 15-21 cells were counted for each condition. 100% of GFP control and DN-Rab5-GFP cells had normal GLUT4\textit{myc} localization. 100% of DN-Dynein-GFP cells had disrupted GLUT4\textit{myc} localization.

Next, GLUT4\textit{myc} distribution was examined after being internalized for 30 min. Neither DN-Rab5-GFP nor DN-Rab22a-GFP altered the perinuclear accumulation of GLUT4\textit{myc}. However, DN-Dynein-GFP prevented GLUT4\textit{myc} from accumulating in the perinuclear region, instead maintaining GLUT4\textit{myc} in cytosolic puncta (Figure 4.11). GLUT4\textit{myc} accumulated in the perinuclear region in: 100% of GFP control cells, 100% of DN-Rab5-GFP cells, 92% of DN-Rab22a-GFP cells, and 19% of DN-Dynein-GFP cells.
These data suggest that microtubule minus end directed movement is required to sort GLUT4 into the perinuclear region but that Rab5 and Rab22a may be dispensable for this action. Retromer may serve to link GLUT4 sorting to microtubule minus end directed movement.

Retromer not only interacts with dynein, but is required for sortilin sorting to the TGN (Canuel et al., 2008; Hunt and Stephens, 2011). Furthermore, sortilin cannot direct GLUT4 sorting to its immobile population (retention compartments) in adipocytes depleted of retromer (Hatakeyama and Kanzaki, 2011). As a measure of retromer function, the compound LY294002 was used to inhibit the class III PI3K Vps34. Vps34 is required to form PI3P, which in turn is required to recruit sorting nexins to the retromer complex (Seaman, 2012). Thus, inhibiting Vps34 using
LY294002 should inhibit retromer function. LY294002 fails to inhibit GLUT4\textit{myc} from accumulating in the perinuclear region (Figure 4.12A). GLUT4\textit{myc} accumulated in the perinuclear region in 100% of control cells and 83% of LY294002 cells. As a positive control for LY294002 activity, tandem FYVE domain-GFP (2xFYVE-GFP) was transiently transfected into L6 cells and its localization monitored. 2xFYVE-GFP binds to PI3P to form distinct puncta on PI3P-positive endosomes. After 10 min of GLUT4\textit{myc} internalization 2xFYVE-GFP was observed on cytosolic puncta in control cells but not in LY294002 treated cells (Figure 4.12B). Based on these data, a role for retromer in regulating GLUT4 sorting was not pursued further. However, examining GLUT4 sorting in L6 cells depleted of retromer is required to directly test retromer involvement in GLUT4 regulation.
Figure 4.12: Retromer does not regulate GLUT4 sorting into the perinuclear region. A) 2xFYVE-GFP was transiently transfected into L6 muscle cells for 18 h. Cells were incubated with or without LY294002 for 30 min. Cell surface GLUT4\(_{\text{myc}}\) was then labelled at 4°C. Cells were re-warmed to 37°C for 30 min, with or without LY294002, before being fixed and permeabilized for the detection of internalized GLUT4\(_{\text{myc}}\) (red), scale = 6 µm (N=1). 12 cells were counted for each condition. GLUT4\(_{\text{myc}}\) accumulated in the perinuclear region in 100% of control cells and 83% of LY294002 cells. B) Cells were treated as in A, but re-warmed to 37°C for 10 min only.

In summary, dynein function is required to maintain normal GLUT4 distribution and to sort GLUT4 into the perinuclear region. Preliminary evidence failed to support a role for Rab5, Rab22a, or retromer in the regulation of GLUT4 sorting.

4.3.2 Molecules linked to GLUT4 availability and fusion at the plasma membrane

Multiple Rab proteins have been linked to cell surface GLUT4 availability. Rab11 and Rab14 are implicated in regulating GLUT4 sorting into the IRC/IRV in adipocytes (Reed et al., 2013; Sadacca et al., 2013; Zeigerer et al., 2002). Rab8a, and possibly Rab14, is required for insulin-stimulated GLUT4 exocytosis in muscle cells (Ishikura and Klip, 2008; Ishikura et al., 2007). Using siRNA-mediated knockdown, the accumulation of internalized GLUT4\(_{\text{myc}}\) in the Stx6-positive perinuclear compartment was measured in cells depleted of each of these Rab proteins. The co-localization of internalized GLUT4\(_{\text{myc}}\) with Stx6 was not altered by depletion of Rab11a, Rab14, or Rab8a (Figure 4.13).
Figure 4.13: Rab11a, Rab14, and Rab8a do not regulate GLUT4 sorting into the Stx6-positive perinuclear compartment. Rab proteins were depleted by siRNA for 60 h. A) Cell surface GLUT4myc was labelled at 4C. Cells were re-warmed to 37C for 30 min before being fixed and permeabilized for the detection of internalized GLUT4myc (red), scale = 15 µm (N=1). 10-12 cells were counted for each condition. B) Quantification of experiment in A. Pearson correlations were calculated for GLUT4myc co-localization with Stx6 for each condition. Representative Western blots are shown.
VAMP2 is required for GLUT4 vesicle fusion with the plasma membrane in response to insulin (Ramm et al., 2000; Randhawa et al., 2000; Williams and Pessin, 2008). VAMP2 has also been proposed to regulate GLUT4 sorting into the IRC/IRV (Williams and Pessin, 2008). However, VAMP2 depletion via cognate siRNA in L6 muscle cells does not alter GLUT4myc accumulation in the perinuclear region (Figure 4.14).

![Figure 4.14: VAMP2 does not regulate GLUT4 sorting into the Stx6-positive perinuclear compartment. VAMP2 was depleted by siRNA for 60 h. Cell surface GLUT4myc was labelled at 4C. Cells were re-warmed to 37C for 30 min before being fixed and permeabilized for the detection of internalized GLUT4myc, scale = 6 µm (N=1). 28-32 cells were counted for each condition. GLUT4myc accumulated in the perinuclear region in 93% of siNR and 94% of siVAMP2 cells. A representative Western blot is shown.](image)

In summary, evidence based on siRNA-mediated depletion of Rab11a, Rab14, Rab8a, and VAMP2 suggests that GLUT4 accumulates in the perinuclear region independently of these proteins.

## 4.4 Conclusions

Stx6 knockdown does not impact sufficiently to be noted when analyzing the overall steady-state localization of GLUT4. It also does not affect sorting of internalized GLUT4 into the perinuclear region. However, Stx6 is involved in GLUT4 sorting into the functionally defined IRC/IRV. Also, Stx6 may function in the retention of GLUT4 in intracellular membranes, as suggested by the elevated cell surface GLUT4 in L6 cells depleted of Stx6 (Figures 4.5 and 4.8). These results are consistent with the hypothesis that the Stx6-positive perinuclear compartment is comprised of two distinct populations: one that consists of a slowly recycling population and one that consists of the IRC/IRV. In this model, GLUT4 maintains its perinuclear localization independent of Stx6
by sorting into the slowly recycling population. Stx6 would then function in sorting GLUT4 from the slowly recycling population to the IRC/IRV. GLUT4 sorting into Stx6-positive perinuclear compartment is dependent on dynein, but may not require retromer, Rab5, Rab8a, Rab11, Rab14, or VAMP2. Thus, although microtubule minus-end directed movement is essential for GLUT4 sorting, the regulatory molecules mediating GLUT4 sorting into the Stx6-positive perinuclear region remain elusive.
5  Alterations in GLUT4 sorting caused by Nocodazole

Although I hypothesize that GLUT4 sorts into the IRC/IRV within the Stx6-positive perinuclear compartment, this has not been experimentally demonstrated. Stx6 localizes to both TGN membranes and recycling endosomes (Jung et al., 2012). As such, the Stx6-positive perinuclear compartment may not be in direct contact with the IRC/IRV. GLUT4 may sort from recycling endosomes into the IRC/IRV. To further examine the relationship between Stx6 and GLUT4 sorting into insulin-responsive compartments, I searched for strategies that would preclude GLUT4 from reaching the Stx6-positive perinuclear compartment. Nocodazole is a microtubule disrupting agent that inhibits insulin-stimulated GLUT4 translocation in adipocytes (Karylowski et al., 2004). Nocodazole prevents microtubule re-assembly which effectively promotes depolymerization due to continuous turn over. Given that DN-Dynein inhibits GLUT4 sorting, nocodazole was a strong candidate for such an approach. However, the use of nocodazole to investigate GLUT4 traffic has been controversial, as high concentrations of nocodazole inhibit glucose transport independent of microtubule disruption and treatments with nocodazole greater than 1 h cause GLUT4 to become dispersed (Molero et al., 2001; Shigematsu et al., 2002). Thus, interpretation of data using nocodazole may prove difficult due to confounding effects. Here, I report that nocodazole inhibits GLUT4 sorting into the Stx6-positive perinuclear region and insulin-responsive re-exocytosis. Importantly, I use a low dose of nocodazole for short duration that does not perturb steady-state GLUT4 distribution. Based on these data, I conclude that GLUT4 must pass through the Stx6-positive perinuclear compartment to acquire insulin-responsiveness.

5.1  Nocodazole inhibits insulin-stimulated GLUT4 translocation but does not alter the steady-state localization of intracellular GLUT4

Treatment of L6 muscle cells with 3 µM nocodazole for 30 min does not disperse perinuclear GLUT4myc and does not alter GLUT4myc co-localization with Stx6 or Stx16 (Figure 5.1).
Figure 5.1: 3 µM nocodazole does not disrupt GLUT4 co-localization with Stx6 or Stx16. Cells were treated with 3 µM nocodazole for 30 min before being fixed, permeabilized, and labelled for A) GLUT4myc (red) and Stx6 (green) or B) GLUT4myc (red) and Stx16 (green), scale = 15 µm. Inset = single slices of perinuclear regions.

Furthermore, GLUT4myc maintains its segregation from furin and Tfn after treatment with 3 µM nocodazole for 30 min (Figure 5.2).
Figure 5.2: 3 µM nocodazole does not disrupt GLUT4 segregation from furin or Tfn. A) Cells were treated with 3 µM nocodazole for 30 min before being fixed, permeabilized, and labelled for GLUT4myc (red) and furin (green). B) Cells were loaded with Tfn-A488 for 30 min during nocodazole treatment. Cells were then fixed, permeabilized, and labelled for GLUT4myc (red), scale = 15 μm. Inset = single slices of perinuclear regions.

GLUT4myc co-localization with VAMP2-HA is also unchanged by nocodazole treatment (Figure 5.3).
Figure 5.3: 3 µM nocodazole does not disrupt GLUT4 co-localization with VAMP2-HA. Cells were treated with 3 µM nocodazole for 30 min before being fixed, permeabilized, and labelled for GLUT4myc (red) and VAMP2-HA (green), scale = 15 μm. Inset = single slices of perinuclear regions.

The co-localization of GLUT4myc with each of these markers is quantified in Figure 5.4. Treatment of L6 muscle cells with 3 µM nocodazole for 30 min does not disrupt the steady-state distribution of GLUT4 as defined by its perinuclear accumulation and co-localization with the markers examined.

Figure 5.4: Nocodazole does not alter GLUT4 localization. GLUT4myc co-localization with markers of endomembrane compartments was quantified using Pearson correlations. Tfn uptake was for 30 min prior to GLUT4 labelling. Single cells were selected and Pearson correlations calculated using Volocity software (N=2; VAMP2-HA, N=1). 8-10 cells were quantified in each experiment.

With a treatment protocol established that does not alter GLUT4 distribution, the effects of nocodazole on insulin-stimulated GLUT4 translocation and GLUT4 sorting could be determined without confounding factors. Nocodazole did not affect the levels of cell surface GLUT4myc in resting cells; however, it inhibited the insulin-stimulated gain in cell surface GLUT4 by 48%.
To determine if nocodazole inhibited GLUT4 exocytosis directly, GLUT4\textit{myc} was labelled at the cell surface (at 4°C) and allowed to internalize for 30 min (at 37°C) before insulin-responsive re-exocytosis was measured. Cooling L6 cells to 4°C for cell surface labelling of GLUT4\textit{myc} disrupts microtubules; however, microtubules re-form within 1-2 min (Klip lab, unpublished findings). Thus, microtubule disruption by the GLUT4\textit{myc} labelling procedure has a negligible impact on GLUT4 endocytosis and sorting. Nocodazole was added after 25 min of GLUT4\textit{myc} internalization and remained present during re-exocytosis. Nocodazole inhibited insulin-responsive GLUT4\textit{myc} re-exocytosis by 70% (Figure 5.5B). In contrast, nocodazole was without effect on the disappearance of GLUT4\textit{myc} from the plasma membrane (Figure 5.5C). These data suggest that microtubules are required for GLUT4 delivery from the IRC/IRV to the plasma membrane but not for GLUT4 endocytosis.
Figure 5.5: Nocodazole inhibits insulin-stimulated GLUT4 translocation but not GLUT4 endocytosis. A) Insulin-stimulated GLUT4 translocation. Cells were treated with or without 3 µM nocodazole for 30 min. During the last 15 min of nocodazole treatment, cells were treated with or without 100nM insulin. Cells were then fixed for the detection of cell surface GLUT4myc (N=5), p<0.05. B) Insulin-responsive GLUT4 re-exocytosis. All cells were treated with insulin for 15 min. Cell surface GLUT4myc was labelled at 4C before cells were re-warmed to 37C for 30 min and then treated with or without insulin for 10 min. Nocodazole was added during the last 15 min of incubation. Cells were then fixed for detection of cell surface GLUT4myc (N=5), p<0.01. C) GLUT4 endocytosis. Cells were treated with or without insulin for 15 min before cell surface GLUT4myc was labelled at 4C. Cells were re-warmed to 37C for 10 min in the absence or presence of nocodazole and insulin. GLUT4myc remaining at the cell surface was expressed relative to 0 min control in which cells were not re-warmed (N=4).

In Figure 5.5B, nocodazole was only present for 5 min before insulin stimulation. Treatment of L6 muscle cells with nocodazole for 5 min was sufficient to disrupt microtubules (Figure 5.6; compare with Figure 5.7A).

Figure 5.6: Nocodazole treatment for 5 min disrupts microtubules. Cell surface GLUT4myc was labelled at 4C before cells were re-warmed at 37C for 25 min before 3 µM nocodazole was added for 5 min. Cells were then fixed, permeabilized, and stained for internalized GLUT4myc and tubulin, scale = 15 µm.

In summary, treatment of L6 muscle cells with 3 µM nocodazole for 30 min is sufficient to inhibit insulin-stimulated GLUT4 translocation without altering GLUT4 endocytosis. Because nocodazole does not disrupt the intracellular distribution of GLUT4, its effects are due to defective GLUT4 traffic and not due to defects in steady-state GLUT4 localization.
5.2 Microtubule disruption inhibits both GLUT4 sorting into the Syntaxin-6-positive perinuclear region and insulin-responsive GLUT4 re-exocytosis

Although nocodazole inhibits GLUT4 exocytosis, it is unknown if nocodazole also disrupts GLUT4 sorting. To address this question, cell surface pulse-labelled GLUT4myc was allowed to internalize for 30 min in the presence of a 3 µM nocodazole. Nocodazole prevented GLUT4myc from accumulating in the Stx6-positive perinuclear compartment (Figure 5.7A). Microtubule disruption was confirmed by the staining of tubulin. This result confirms that observed with DN-Dynein – that transport along microtubules is required for GLUT4 sorting to the Stx6-positive perinuclear compartment.
Figure 5.7: Nocodazole prevents GLUT4 sorting into the Stx6-positive perinuclear compartment. A-D) Cell surface GLUT4myc was labelled at 4°C before cells were re-warmed at 37°C for 30 min with or without 3 µM nocodazole. For recovery, nocodazole was washed out after 25 min and replaced with media for indicated recovery times. Fixed cells were stained for Stx6 (green), scale = 5 µm. Inset = single slices of perinuclear regions. Representative tubulin staining is shown for each condition (far right). E) Quantification of GLUT4myc co-localization with Stx6. Single cells were selected and Pearson correlations calculated using Volocity software (N=2-4), * p<0.05; # p<0.01. 10-15 cells were quantified per experiment.

Next, I determined if the nocodazole effect on GLUT4 sorting could be reversed. Cell surface pulse-labelled GLUT4myc was allowed to internalize for 25 min in the presence of 3 µM nocodazole before the nocodazole was removed. Removing nocodazole for 5 min allowed for microtubules to reform but not for GLUT4 to re-acquire localization to the Stx6-positive perinuclear compartment (Figure 5.7B). Nocodazole removal for 10 min allowed GLUT4 to partially recover its perinuclear localization; however, a population of GLUT4myc was still cytosolic (Figure 5.7C). By 15 min following nocodazole removal GLUT4myc re-acquired its localization to the Stx6-positive perinuclear compartment (Figure 5.7D). The quantification of GLUT4myc co-localization with Stx6 is summarized in Figure 5.7E.
Nocodazole inhibits GLUT4 sorting to the Stx6-positive perinuclear compartment, but does this perturbed sorting translate into defective insulin-stimulated GLUT4 translocation? To answer this question, cell surface pulse-labelled GLUT4\textit{myc} was allowed to internalize for 30 min in the presence of nocodazole and insulin-responsive GLUT4 re-exocytosis was measured. Because nocodazole inhibits GLUT4 re-exocytosis (see Figure 5.5B), nocodazole was removed during the re-exocytosis step in order to assess the effects of GLUT4 mis-sorting independently from microtubule requirements for GLUT4 exocytosis. Removing nocodazole during the re-exocytosis step allows microtubule recovery but maintains GLUT4\textit{myc} dispersion (see Figure 5.7, B and E). In this condition, nocodazole inhibited insulin-responsive GLUT4\textit{myc} re-exocytosis by 56% (Figure 5.8A). By 10 min after nocodazole removal GLUT4\textit{myc} was still not insulin-responsive (Figure 5.8B). It did not matter if nocodazole was washed out before insulin stimulation (Figure 5.8B, left, 55% inhibition) or during insulin stimulation (Figure 5.8B, right, 52% inhibition).

However, by 15 min, when GLUT4\textit{myc} had re-acquired its perinuclear localization in the Stx6-positive compartment, insulin-responsive GLUT4\textit{myc} re-exocytosis was also restored (Figure 5.8C). Importantly, insulin signalling to Akt was not affected under any of these conditions (Figure 5.8D), illustrating an uncoupling between insulin signalling and loss of GLUT4 insulin responsiveness.
Figure 5.8: Nocodazole inhibits insulin-responsive GLUT4 re-exocytosis. A-C) All cells were treated with insulin for 15 min before cell surface GLUT4myc was labelled at 4°C. Cells were then re-warmed at 37°C for 30 min with or without 3 μM nocodazole and treated with or without insulin for 10 min. For recovery, nocodazole was washed out after 25 min and replaced with media (with or without insulin) for indicated recovery times (5-15 min). Cells were then fixed for detection of cell surface GLUT4myc (N=7-8), * p<0.01. White bars = DMSO and colour bars = Nocodazole. D) Nocodazole does not inhibit the insulin-induced phosphorylation of Akt. Cells were treated exactly as in A-C. Noco I = nocodazole for 30 min prior to insulin stimulation; Noco R+I = nocodazole for 25 min with 5 min recovery prior to insulin stimulation.
In summary, nocodazole inhibits both GLUT4 sorting into the Stx6-positive perinuclear compartment and insulin-responsive GLUT4 re-exocytosis. These effects are reversible, with GLUT4 sorting and insulin-responsive re-exocytosis recovering in identical time frames. Collectively, these results suggest that GLUT4 sorting to the Stx6-positive perinuclear compartment is required for GLUT4 to acquire insulin-responsiveness.

5.3 Conclusions

Treatment of L6 cells with 3 µM nocodazole for 5-30 min disrupts microtubule formation. This acute disruption of the microtubule network does not alter the intracellular distribution of GLUT4 or increase cell surface GLUT4 levels; however, GLUT4 traffic is perturbed. Microtubule disruption inhibits GLUT4 sorting into the Stx6-positive perinuclear compartment in a manner that is reversible. GLUT4 arrival at the Stx6-positive perinuclear compartment correlates with the acquisition of GLUT4 insulin responsiveness. These data suggest that it is the perinuclearly localized Stx6 that directs GLUT4 sorting into the IRC/IRV.
6 Insulin resistance modulates GLUT4 traffic

Lipid overload in vivo leads to insulin resistance, and this effect has been linked to the accumulation of intracellular ceramides (Chavez and Summers, 2012). Treatment with C2-ceramide is an acknowledged strategy to confer insulin resistance to both L6 muscle cells (JeBailey et al., 2007) and 3T3-L1 adipocytes (Summers et al., 1998). The conferred insulin resistance is so far ascribed to interference with insulin signalling (JeBailey et al., 2007; Powell et al., 2003; Stratford et al., 2001), and the possibility that C2-ceramide also inhibits GLUT4 sorting has been neglected. Here, I use two different C2-ceramide concentrations (25 and 50 µM) to show that these treatments inhibit proper GLUT4 sorting, disrupt normal Stx6 distribution, and inhibit insulin signalling. Upon removal of 50 µM C2-ceramide, defects in GLUT4 sorting persist despite recovery of insulin signalling. These results demonstrate that ceramides perturb both GLUT4 sorting and insulin signalling and that defective GLUT4 sorting alone can confer insulin resistance.

6.1 C2-ceramide disrupts the intracellular localization of GLUT4 and insulin-stimulated GLUT4 translocation

L6 muscle cells were treated with 25 or 50 µM C2-ceramide and examined for total GLUT4myc localization relative to endogenous markers of intracellular compartments. GLUT4myc was dispersed, but so were Stx6, Stx16, and furin (Figure 6.1, A-C). However, GLUT4myc remained co-localized with both Stx6 and Stx16 in the dispersed structures. Interestingly, GLUT4myc did not disperse into recycling endosomes, as GLUT4myc co-localization with Tfn did not increase in C2-ceramide treated cells (Figure 6.1D).
Figure 6.1: C2-ceramide disrupts intracellular membrane compartments and disperses intracellular GLUT4. A-C) Cells were treated with 25 or 50 µM C2-ceramide for 2 h before being fixed, permeabilized, and labelled for A) GLUT4myc (red) and Stx6 (green) or B) GLUT4myc (red) and Stx16 (green) or C) GLUT4myc (red) and furin (green), scale = 15 μm. Inset = single slices of perinuclear regions. D) As performed in A-C, but cells were loaded with Tfn-A488 for the last 30 min of C2-ceramide treatment. E) GLUT4myc co-localization with markers of endomembrane compartments was quantified using Pearson correlations. Single cells were selected and Pearson correlations calculated using Volocity software (50 µM, N=2; 25 µM, N=1). 8-12 cells were quantified in each experiment.

The co-localization of GLUT4myc with each of these markers is summarized in Figure 6.1E. Cells treated with 50 µM C2-ceramide show a greater dispersion of GLUT4myc than those treated with 25 µM C2-ceramide. Stx6 and Stx16 display a similar phenotype as that for GLUT4myc, with both Stx6 and Stx16 showing a more dramatic dispersion with 50 µM C2-ceramide and co-localizing with GLUT4myc in a superficial compartment. On the contrary, furin is dispersed but does not redistribute to the superficial compartment; both 25 and 50 µM C2-ceramide appear to have equal effects.

Next, C2-ceramide effects on insulin-stimulated GLUT4 translocation were examined. 50 µM C2-ceramide increased cell surface GLUT4myc in resting cells and inhibited the insulin-stimulated increase in cell surface GLUT4myc (Figure 6.2A). Although 25 µM C2-ceramide appeared to alter cell surface GLUT4myc in a similar manner, it failed to significantly alter cell surface GLUT4myc (Figure 6.2B). However, when the % change (i.e., the delta caused by insulin expressed as 100% insulin response) in cell surface GLUT4myc was examined, both 25 and 50 µM C2-ceramide inhibited the insulin-stimulated increase in cell surface GLUT4myc (Figure 6.2C).
Figure 6.2: C2-ceramide inhibits insulin-stimulated GLUT4 translocation. Cells were treated with or without 50 (A) or 25 (B) µM C2-ceramide for 2 h before being stimulated with or without insulin for 15 min. Cells were then fixed for the detection of cell surface GLUT4myc (50 µM, N=5; 25 µM, N=3), * p<0.01. C) Data from A and B, expressed as delta % change [(Insulin-Basal/Insulin)] in cell surface GLUT4myc in response to insulin stimulation, * p<0.01; # p<0.05.

In summary, C2-ceramide alters the intracellular localization of GLUT4 and markers of endomembrane compartments. C2-ceramide also inhibits insulin-stimulated GLUT4 translocation. However, these data do not link altered GLUT4myc localization to inhibition of GLUT4 translocation. Impaired insulin signalling may be responsible for the reduced insulin-responsiveness of GLUT4, with the IRC/IRV being dispersed but still functional.

6.2 C2-ceramide inhibits insulin-responsive GLUT4 re-exocytosis independent of insulin signalling

To investigate the relationship between GLUT4 localization and its insulin-responsiveness, the sorting of cell surface pulse-labelled GLUT4myc was tracked in C2-ceramide treated L6 muscle cells. Cells were treated with C2-ceramide for 2 h prior to pulse-labelling cell surface
GLUT4myc for subsequent internalization. Both 50 and 25 µM C2-ceramide treatments inhibited the co-localization of internalized GLUT4myc with Stx6 (Figure 6.3; compare 1st, 2nd and 4th rows). As observed above, these treatments also dispersed Stx6 from its perinuclear localization.

**Figure 6.3:** C2-ceramide prevents GLUT4 sorting into the Stx6-positive perinuclear compartment. Cells were treated for 2 h with or without C2-ceramide before cell surface GLUT4myc was labelled at 4C. Cells were then re-warmed at 37C for 30 min with or without C2-ceramide. For 50 µM C2-ceramide recovery, C2-ceramide was removed for 15 min after the 2 h treatment and was absent during the 30 min re-warm. For 25 µM C2-ceramide recovery, C2-ceramide was absent during the 30 min re-warm. Fixed cells were stained for Stx6 (green), scale = 5 µm (50 µM) or 15 µm (25 µM). Inset = single slices of perinuclear regions.
Conditions were also tested in which C2-ceramide was washed out during GLUT4myc internalization. 50 μM C2-ceramide was administered for 2 h and then washed out for a total of 45 min (50 μM C2-cer + recovery). Cells were incubated in ceramide-free media for 15 min prior to pulse-labelling the surface GLUT4myc and then allowing it to internalize for 30 min. In spite of the ceramide washout, internalized GLUT4myc did not recover co-localization with Stx6 and remained cytosolically dispersed (Figure 6.3, 3rd row). On the contrary, 25 μM C2-ceramide washed out for a total of 30 min (25 μM C2-cer + recovery) allowed GLUT4myc to recover co-localization with Stx6 in the perinuclear region (Figure 6.3 5th row). These results are quantified in Figure 6.4.

**Figure 6.4:** Quantification of internalized GLUT4myc co-localization with Stx6 with and without C2-ceramide treatment (from Figure 6.3). Single cells were selected and Pearson correlations calculated using Volocity software (50 μM, N=3; 25 μM, N=2), * p<0.01. 10-15 cells were quantified per experiment.

Next, insulin-responsive GLUT4 re-exocytosis was measured in C2-ceramide treated cells. Treatments with 50 and 25 μM C2-ceramide inhibited insulin-responsive GLUT4myc re-exocytosis (by 55% and 63%, respectively, Figure 6.5, A and B). Defects in insulin signalling could have contributed to these effects as well, as these treatments also impaired Akt phosphorylation (Figure 6.5C, 3rd and 6th columns; Figure 6.5D, 3rd column). Similar to that observed for GLUT4myc co-localization with Stx6, 25 μM C2-cer + recovery allowed insulin-dependent phosphorylation of Akt to recover (Figure 6.5C, 7th column). However, 50 μM C2-cer + recovery allowed insulin-dependent phosphorylation of Akt to recover (Figure 6.5D, 4th column) despite GLUT4 sorting remaining defective (see Figure 6.3 and 6.4). Thus, treatment of
L6GLUT4myc cells with 50 µM C2-ceramide followed by 45 min washout allows the contribution of defective GLUT4 sorting to be tested independently from defects in insulin signalling.

**Figure 6.5:** C2-ceramide inhibits insulin-responsive GLUT4 re-exocytosis and insulin signalling. A-B) Cells were treated with or without C2-ceramide for 2 h. All cells were then treated with insulin for 15 min before cell surface GLUT4myc was labelled at 4°C. Cells were re-warmed at 37°C for 30 min with or without C2-ceramide and treated with or without insulin for 10 min. Cells were then fixed for detection of cell surface GLUT4myc (N=6), * p<0.01. C) Cells were treated as in A and B. For recovery, C2-ceramide was absent during cell re-warm and subsequent insulin stimulation. D) Cells were treated as in C, but for recovery C2-ceramide was removed after the 2 h treatment and remained absent for all subsequent steps. I=insulin; R+I = recovery prior to insulin stimulation.
Next, I examined whether the effects of C2-ceramide on insulin-responsive GLUT4 re-exocytosis could be reversed. In spite of the recovery of insulin signalling, 50 µM C2-ceramide + recovery did not restore GLUT4 insulin responsiveness. Insulin-induced GLUT4myc re-exocytosis remained inhibited by 46% after the C2-ceramide washout (Figure 6.6A). However, 25 µM C2-cer + recovery restored insulin-responsive GLUT4myc re-exocytosis (Figure 6.6B). This result was expected, as both GLUT4 sorting and insulin-dependent Akt phosphorylation were restored.

**Figure 6.6:** C2-ceramide inhibits insulin-responsive GLUT4 re-exocytosis independent of insulin signalling. A) Cells were treated with or without 50 µM C2-ceramide for 2 h. All cells were then treated with insulin for 15 min before cell surface GLUT4myc was labelled at 4°C. Cells were re-warmed at 37°C for 30 min with or without C2-ceramide and treated with or without insulin for 10 min. For recovery, C2-ceramide was removed after the 2 h treatment and remained absent for all subsequent steps. Cells were then fixed for detection of cell surface GLUT4myc (N=3), * p<0.01. B) Exactly as performed in A, except with 25 µM C2-ceramide. For recovery, C2-ceramide was removed during the 30 min re-warm (N=7).

In summary, both 50 and 25 µM C2-ceramide inhibit GLUT4 sorting into the Stx6-positive perinuclear compartment. These treatments also reduce insulin-dependent signalling at the level of Akt phosphorylation and inhibit insulin-responsive GLUT4 re-exocytosis. However, removing 25 µM C2-ceramide for 30 min during cell re-warm allows cells to recover insulin-dependent
Akt phosphorylation, GLUT4 sorting, and insulin-responsive GLUT4 re-exocytosis. On the contrary, removing 50 µM C2-ceramide for 45 min allows insulin-dependent Akt phosphorylation to recover but not GLUT4 sorting or insulin-responsive GLUT4 re-exocytosis.

6.3 Conclusions

As our group has previously shown, C2-ceramide reduces insulin-dependent phosphorylation of Akt and inhibits insulin stimulated GLUT4 translocation in L6 muscle cells. However, C2-ceramide also inhibits GLUT4 sorting into the Stx6-positive perinuclear compartment. Defective GLUT4 sorting occurs in parallel with defective insulin-responsive GLUT4 re-exocytosis. GLUT4 sorting is more susceptible to perturbation by C2-ceramide than insulin signalling through Akt, as removing C2-ceramide allows insulin signalling to recover despite no recovery in GLUT4 sorting or insulin-responsive re-exocytosis. These results reveal that impairing GLUT4 sorting per se can contribute to insulin resistance. They also endorse the concept that failure of GLUT4 sorting to a perinuclearly-located, Stx6-positive compartment causes insulin resistance, irrespective of normal insulin signalling.
Chapter 7

7 Summary

7.1 GLUT4 accumulation in the Syntaxin-6-positive perinuclear compartment correlates with insulin-responsive GLUT4 re-exocytosis

In Chapters 5 and 6, I conclude that GLUT4 sorting to the Stx6-positive perinuclear compartment correlates with the acquisition of insulin-responsive GLUT4 re-exocytosis. To quantify this relationship I examined the correlation of these two parameters across the diverse conditions described in this Thesis. Figure 7.1 presents the Pearson correlations of internalized GLUT4\textit{myc} and Stx6 co-localization \textit{versus} GLUT4\textit{myc} insulin-responsive re-exocytosis at matched time points. A strong correlation (r=0.8731) is found between internalized GLUT4\textit{myc} co-localization with Stx6 and insulin-responsive GLUT4\textit{myc} re-exocytosis.

Furthermore, insulin-responsive GLUT4 re-exocytosis is observed only when Stx6 is expressed and GLUT4/Stx6 are located in the perinuclear region (Table 7.1).
Table 7.1: Summary of GLUT4 re-exocytosis parameters under each treatment condition. Noco = nocodazole; C2-cer = C2-ceramide; R = recovery.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stx6 KD</th>
<th>Noco</th>
<th>Noco+R</th>
<th>50 µM C2-cer</th>
<th>50 µM C2-cer+R</th>
<th>25 µM C2-cer</th>
<th>25 µM C2-cer+R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internalized GLUT4</td>
<td>perinuclear</td>
<td>cytosolic</td>
<td>perinuclear</td>
<td>cytosolic</td>
<td>cytosolic</td>
<td>cytosolic</td>
<td>perinuclear</td>
</tr>
<tr>
<td>Insulin-responsive re-exo</td>
<td>inhibited</td>
<td>inhibited</td>
<td>normal</td>
<td>inhibited</td>
<td>inhibited</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>pAkt</td>
<td>N/A</td>
<td>normal</td>
<td>normal</td>
<td>inhibited</td>
<td>normal</td>
<td>inhibited</td>
<td>normal</td>
</tr>
<tr>
<td>Stx6 localization</td>
<td>N/A</td>
<td>perinuclear</td>
<td>perinuclear</td>
<td>cytosolic</td>
<td>cytosolic</td>
<td>cytosolic</td>
<td>perinuclear</td>
</tr>
</tbody>
</table>

These data support a model in which GLUT4 internalized from the plasma membrane is sorted into a perinuclear compartment that is positive for Stx6 but distinct from the recycling endosomes containing TfnR (Figure 7.2). Arrival at this compartment correlates with the acquisition of insulin responsiveness.

![Figure 7.2: Model of GLUT4 sorting in L6 muscle cells. GLUT4 internalized from the plasma membrane transits recycling endosomes marked by Transferrin Receptor en route to a Stx6-positive perinuclear compartment. This Stx6-positive compartment is comprised of a dynamic retention compartment and the IRC/IRV. Stx6 is required to sort GLUT4 from the retention compartment to IRC/IRV. Both nocodazole and C2-ceramide prevent GLUT4 from accumulating in the Stx6-positive perinuclear compartment.](image)

In summary, GLUT4 internalized from the plasma membrane accumulates in a Stx6-positive perinuclear compartment by 30 min. However, GLUT4 accumulation in the perinuclear region is not sufficient to gain insulin responsiveness; Stx6 knockdown inhibits insulin-responsive GLUT4 re-exocytosis without affecting GLUT4 accumulation in the perinuclear region. Sorting into the insulin-responsive compartment facilitates GLUT4 retention, as Stx6 depletion increases cell surface GLUT4 expression in resting cells. Experiments using nocodazole provide evidence
that the perinuclear region is where Stx6 functions to mediate GLUT4 sorting into the insulin-responsive compartment. Nocodazole prevents GLUT4 accumulation in the Stx6-positive perinuclear region in a reversible manner that correlates with GLUT4 acquisition of insulin-responsive re-exocytosis. Treatment with C2-ceramide, which causes insulin resistance, inhibits GLUT4 sorting into the Stx6-positive compartment, insulin-dependent phosphorylation of Akt, and insulin-responsive GLUT4 re-exocytosis. Interestingly, GLUT4 sorting is more sensitive than Akt phosphorylation to perturbation by C2-ceramide such that this condition of insulin resistance can inhibit GLUT4 sorting into the Stx6-positive compartment and insulin-responsive GLUT4 re-exocytosis independent of insulin signals. Together, these data support the hypothesis that GLUT4 sorting through a Stx6-positive perinuclear compartment significantly contributes to GLUT4 acquisition of full insulin responsiveness. The Stx6-positive perinuclear compartment is likely comprised of two sub-compartments: one involved in GLUT4 dynamic retention and one that is insulin-responsive.
Chapter 8

8 Discussion

In contrast to extensive studies on the insulin-dependent signals regulating GLUT4 exocytosis, there is little consensus on the defining mechanisms of GLUT4 sorting into the IRC/IRV. From this lapse it is clear that studying the steady-state distribution of GLUT4 as the only endpoint does not allow scrutiny of the intracellular steps required for GLUT4 to attain insulin responsiveness. As an alternative approach, I have followed the internalization of GLUT4 pulse-labelled at the surface in L6 muscle cells to examine its sorting into intracellular compartments and to match sorting events to the acquisition of insulin responsiveness. I provide evidence that GLUT4 internalized from the plasma membrane enters a segregated perinuclear compartment marked by Stx6 from where it acquires insulin-responsiveness. I further show that C2-ceramide treatment, which confers insulin resistance, impairs GLUT4 sorting into the Stx6-positive perinuclear compartment and inhibits insulin-responsive GLUT4 re-exocytosis in the absence of defects in insulin signalling to Akt. These findings suggest that certain conditions conferring insulin resistance may act by disrupting GLUT4 sorting in the absence of insulin and independently of any change in insulin signalling.

8.1 Characterizing GLUT4 distribution

Not only has much of the model of GLUT4 sorting been developed from studying the steady-state distribution or the cell surface localization of GLUT4, but these studies were also mostly performed in adipocytes. The steady-state localization of GLUT4 has not been characterized in muscle cells. It is unknown if myocytes and adipocytes regulate GLUT4 distribution in the same manner.

I report that, in L6 muscle cells, GLUT4 co-localizes with Stx6 and Stx16 but to a much lesser extent with furin. Stx6, Stx16, and furin all concentrate in the perinuclear region. GLUT4 co-localization with Tfn is limited to cytosolic puncta, indicative of sorting/recycling endosomes. These data are consistent with that observed in adipocytes where GLUT4 localizes to perinuclear membranes positive for Stx6 and Stx16 but does not accumulate in furin-positive membranes (Karylowski et al., 2004; Li et al., 2009; Perera et al., 2003). Unlike in adipocytes, Tfn-positive
endosomes do not accumulate in the perinuclear region, which actually makes it easier to distinguish recycling endosomes from other perinuclear compartments in L6 muscle cells.

Although GLUT4 accumulates in a perinuclear compartment, the organelle identity of this compartment is unknown. Due to their predominant localization to the TGN, the Stx6- and Stx16-positive perinuclear membranes are presumed to consist of the TGN. However, I cannot determine if GLUT4 is localized to the TGN based on co-localization with Stx6 and Stx16 alone. In adipocytes, perinuclear GLUT4 co-localizes with multiple proteins known to localize to distinct membrane compartments. GLUT4 co-localizes with the TGN-resident proteins golgin-97, sortilin, GGA, and MPR but not with TGN-residents furin or TGN38/46 (Hatakeyama and Kanzaki, 2011; Karylowski et al., 2004; Li and Kandror, 2005; Martin et al., 2000b; Shewan et al., 2003; Shi and Kandror, 2007). Sortilin, and possibly Stx6 and Stx16, also localizes to the IRC/IRV (Jedrychowski et al., 2010; Perera et al., 2003; Proctor et al., 2006). Furthermore, perinuclear GLUT4 co-localizes with Arf6, a protein that localizes to the plasma membrane and endosomes, but not the TGN (D’Souza-Schorey and Chavrier, 2006; Li et al., 2012). Apart from MPR, all of these proteins have been reported to regulate insulin-responsive GLUT4 translocation. These data suggest that the perinuclear GLUT4 compartment may actually consist of two or more sub-compartments. In line with this hypothesis, I show that GLUT4 remains localized to the Stx16-positive perinuclear compartment in cells depleted of Stx6. GLUT4 sorts into the perinuclear region in the absence of Stx6, yet Stx6 is involved in GLUT4 acquiring insulin-responsiveness. Technically, Stx6 could be regulating GLUT4 insulin-responsiveness at a single homogeneous perinuclear compartment, perhaps through insulin-dependent vesicle budding. However, Stx6 depletion increases cell surface GLUT4 in resting cells, an effect that would not be expected if Stx6 was regulating insulin-dependent GLUT4 exit from a single perinuclear compartment. It is more likely that Stx6 regulates a GLUT4 sorting step upstream of the IRC/IRV. Thus, the Stx6-positive perinuclear GLUT4 pool likely consists of a pre-IRC/IRV compartment and the IRC/IRV. The pre-IRC/IRV compartment may consist of, or at least bud from, the TGN.

8.2 Characterization of GLUT4 sorting dynamics

GLUT4 may localize to a Stx6 and Stx16-positive perinuclear compartment, but the functional significance of this localization is unknown. I report that GLUT4 sorts into the Stx6-positive
perinuclear compartment within 30 min and that internalized GLUT4 acquires insulin responsiveness by this same time. This observation led me to question if perinuclearly localized Stx6 regulates GLUT4 sorting into the IRC/IRV.

8.2.1 Perinuclearly localized Stx6 defines GLUT4 sorting into the IRC/IRV in muscle cells

Previous work in adipocytes has examined the relationship between GLUT4 and Stx6. Although inhibiting Stx6 function perturbs GLUT4 sorting following insulin removal (Perera et al., 2003), how this relates to the acquisition of insulin-responsiveness is not resolved. Furthermore, no study has examined where in the cell Stx6 functions in regulating GLUT4 traffic.

The observation that Stx6 knockdown inhibits insulin-responsive GLUT4 re-exocytosis but not the accumulation of internalized GLUT4 in the perinuclear region suggests that Stx6 functions after GLUT4 reaches this location. GLUT4 endocytosis is not inhibited in Stx6 depleted cells, yet 30 min after being internalized more GLUT4 is detected at the cell surface. These data suggest that internalized GLUT4 recycles back to the plasma membrane within 30 min; thus, even though GLUT4 sorts to the perinuclear region it is likely more accessible to constitutive recycling than in control cells.

That perinuclearly localized Stx6 directs GLUT4 sorting into the IRC/IRV is consistent with the finding that nocodazole prevents GLUT4 co-localization with Stx6 and inhibits GLUT4 insulin-responsiveness. The recovery of GLUT4 insulin-responsiveness upon nocodazole removal correlates with the re-acquisition of GLUT4 co-localization with perinuclearly localized Stx6. Previous studies in adipocytes had found that nocodazole treatment (3 µM) for 1-2 h did not inhibit GLUT4 translocation, despite preventing GLUT4 accumulation at the perinuclear region (Molero et al., 2001; Shigematsu et al., 2002). However, in those conditions nocodazole dispersed the entire GLUT4 pool, not only the internalizing fraction, and moreover the localization of Stx6 was not investigated. Here we used a shorter treatment with nocodazole that did not disrupt the steady-state perinuclear pool of GLUT4, and, under these conditions the perinuclear localization of Stx6 was also unaffected. Ostensibly, in the adipocytes study described above, nocodazole failed to inhibit GLUT4 translocation because the bona-fide insulin-responsive pool (i.e., containing Stx6) may have been pre-mobilized to the cytosol. In
line with this hypothesis, a later study found that the shorter (30 min) treatment with nocodazole did preclude insulin-dependent GLUT4 exocytosis in adipocytes (Karylowski et al., 2004).

8.2.2 GLUT4 sorting at the Stx6-positive perinuclear compartment

As described above, these observations support the hypothesis that the Stx6-positive perinuclear compartment consists of at least two sub-compartments. According to the model of GLUT4 sorting (Figure 1.9), these data indicate that GLUT4 follows a path to the IRC/IRV that traverses the TGN. The perinuclear GLUT4/Stx6 compartment is likely devoid of recycling endosomes, in so far as Tfn recycling from the membrane does not reach this compartment. Because GLUT4 is mobilized from the perinuclear location to the cytosol and periphery in response to insulin (Boguslavsky et al., 2012; Fujita et al., 2010; Randhawa et al., 2008), the perinuclear locale is linked to insulin responsiveness. The pre-IRC/IRV sub-compartment may serve a storage function where decisions are made to progress to insulin responsiveness or to slowly recycle back to the plasma membrane (Figure 7.2). Whether the insulin-responsive sub-compartment truly consists of the functional IRC/IRV as characterized by membrane fractionation or is a compartment from which the IRC/IRV forms is unknown. GLUT4 co-localization with sortilin and TUG in the perinuclear region in adipocytes (Bogan et al., 2003; Hatakeyama and Kanzaki, 2011) is consistent with the former; however, further work is required to fully characterize this compartment, especially in muscle cells.

In adipocytes, mis-targeting GLUT4 from the Stx6-positive perinuclear region does not prevent insulin-stimulated GLUT4 translocation (Li et al., 2009). It was proposed that mis-targeted GLUT4 traverses the perinuclear region but that it cannot accumulate there. As such, localization to the perinuclear region increases the efficiency of GLUT4 transport to the IRC/IRV but is not itself comprised of the IRC/IRV. However, upon close examination the average fold translocation of mis-targeted GLUT4 was 50% of that of WT; a large error in the translocation response of WT GLUT4 may have reduced the statistical power. Interestingly, Stx6 knockdown in L6 muscle cells reduces insulin-responsive GLUT4 re-exocytosis by 40%. Considering the above study in the context of Stx6 depletion in muscle cells, two hypotheses may explain why half of the insulin-responsive GLUT4 re-exocytosis response was preserved in Stx6-depleted muscle cells. First is that the remaining insulin-responsiveness in Stx6 knockdown cells is due to an increased pool of GLUT4 in the constitutive recycling pathway. Insulin induced acceleration
of GLUT4 constitutive recycling could play a bigger role in the total GLUT4 translocation response if the constitutively recycling pool of GLUT4 is larger. Second is that GLUT4 can gain access to the IRC/IRV through two independent pathways: one that requires perinuclearly localized Stx6 and another that functions upstream of Stx6. In this case, the Stx6 independent route of GLUT4 sorting could preserve partial insulin-responsiveness. These two possibilities can be tested by knocking down regulatory proteins that mediate GLUT4 recruitment from the IRC/IRV to the plasma membrane; the remaining insulin-responsive GLUT4 re-exocytosis in cells depleted of Stx6 should be inhibited only if the second hypothesis is valid.

8.2.3 GLUT4 sorting into the Stx6-positive compartment

The ability of GLUT4 to sort into the IRC/IRV through two independent routes fits with the model presented in Figure 1.9. This may also explain why insulin-stimulated GLUT4 translocation was only inhibited by 18% in Stx6-depleted cells. If only one route through which GLUT4 sorts into the IRC/IRV is functioning, GLUT4 sorting is less efficient; however, over time sufficient GLUT4 will accumulate in the IRC/IRV to support insulin-responsive GLUT4 exocytosis. By studying the dynamics of GLUT4 sorting, the system cannot re-establish equilibrium and the impairment in GLUT4 sorting can be observed. But how does GLUT4 sort into the Stx6-positive perinuclear compartment? If the pre-IRC/IRV Stx6-positive sub-compartment consists of the TGN, perhaps insight can be gained from examining the sorting routes of other cargoes destined for the TGN.

8.2.3.1 Roles of Sortilin and Stx16

A major sorting mechanism proposed in adipocytes centres on GLUT4 binding to Stx6, Stx16, and sortilin (Perera et al., 2003; Shi and Kandror, 2007) (Figure 1.10C). Sortilin links GLUT4 sorting to the retromer in sorting/recycling endosomes and to GGA in the TGN. Stx6 and Stx16 in the TGN direct GLUT4 sorting into the IRC/IRV. I show that this may not be the case in muscle cells. GLUT4 interacts with Stx6, but not with Stx16 or sortilin. It is important to note that detection of sortilin binding to GLUT4 in adipocytes required the use of a cross-linker (Shi and Kandror, 2005); thus, this interaction may have been too weak to detect by the methods used in this Thesis. Interestingly, sortilin is not required for insulin-stimulated GLUT4 translocation in L6 cells (Ishikura and Klip, unpublished finding) and indirectly inhibiting retromer function fails to prevent GLUT4 sorting to the perinuclear region (Figure 4.12). These data raise questions
regarding the requirement of sortilin for GLUT4 sorting in muscle cells. However, sortilin appears to be required for the development of the IRC/IRV and for insulin-stimulated GLUT4 translocation in the mouse C2C12 muscle cell line (Ariga et al., 2008; Tsuchiya et al., 2010). A major difference between L6 and C2C12 muscle cells is that the IRC/IRV is functional in L6 myocytes while this compartment forms upon differentiation in C2C12 cells. Myogenic differentiation requires sortilin expression in C2C12 cells (Ariga et al., 2008), suggesting that sortilin may regulate the formation of the IRC/IRV indirectly. A role for sortilin in directing GLUT4 sorting requires further investigation. First, sortilin binding to GLUT4 has not been demonstrated in C2C12 cells. Second, GLUT4 re-exocytosis in cells depleted of sortilin has never been investigated. It may be that, like Stx6, sortilin-dependent sorting of GLUT4 can best be identified by measuring acute GLUT4 sorting dynamics.

That GLUT4 does not interact with Stx16 suggests that either Stx16 is not required to regulate GLUT4 sorting or that it binds transiently and was just not captured by GLUT4 immunoprecipitation. Since Stx6 was captured in the GLUT4 pull-down and Stx6 and Stx16 are known to function as part of the same SNARE complex, the latter would imply that Stx6 functions in some additional capacity to its SNARE function for regulating GLUT4 sorting. However, if Stx16 is not required for GLUT4 sorting, how does Stx6 function as a SNARE to regulate GLUT4 sorting? SNAP25, which partially resides in a perinuclear TGN compartment in neuroendocrine cells, can form a SNARE complex with Stx6 (Aikawa et al., 2006, 25; Wendler and Tooze, 2001). SNAP25 supplies two helices to its SNARE complex, and so could replace Stx16 and Vti1a as the SNARE partner for Stx6 to regulate GLUT4 sorting in muscle cells.

Although GLUT4 localizes to similar compartments in both adipocytes and L6 muscle cells, the reliance of GLUT4 sorting on sortilin and Stx16 in muscle cells is questionable. Is there a possible explanation as to why muscle and adipose cells would regulate GLUT4 sorting differently? As outlined in Figures 1.10 A-C, many proteins have been reported to regulate GLUT4 sorting into the IRC/IRV. Sortilin and Stx16 may function in adipocytes to increase the efficiency of GLUT4 retention. Muscle imports glucose for both energy utilization and storage purposes; thus, it may not be advantageous for muscle to maintain the same level of GLUT4 retention as in adipocytes. Like insulin, contraction stimulates GLUT4 translocation in muscle. As such, muscle may require a larger mobilized pool of GLUT4. Indeed, the rate of GLUT4 recycling is faster in L6 muscle cells than in 3T3-L1 adipocytes (Foster et al., 2001; Govers et
al., 2004; Karylowski et al., 2004), suggesting that GLUT4 retention is more dynamic. Perhaps by regulating GLUT4 sorting independent of sortilin and Stx16 GLUT4 is more readily available for recycling to the plasma membrane.

8.2.3.2 Roles of Rab5, Rab11, Rab14, Rab22, and VAMP2

Work in adipocytes has proposed multiple Rab proteins to be involved in regulating GLUT4 sorting; in particular, Rab11 and Rab14 regulate GLUT4 sorting into the IRC/IRV (Reed et al., 2013; Sadacca et al., 2013; Zeigerer et al., 2002). However, it is unknown where in the pathway of GLUT4 sorting these proteins function or whether they act in parallel or in series. Some evidence suggests that Rab14 functions in GLUT4 exit from recycling endosomes into the IRC/IRV – CA-Rab14 traps GLUT4 in enlarged vesicular structures with Tfn and Rab14, but with very little Rab5 or Rab11 (Reed et al., 2013). I find that depletion or neither Rab14 nor Rab11 inhibits internalized GLUT4 from accumulating in the Stx6-positive perinuclear compartment. Likewise, over-expressing DN-Rab5 or DN-Rab22a does not prevent GLUT4 sorting to the perinuclear region. Thus, if any of these proteins mediates GLUT4 sorting from sorting/recycling endosomes to the IRC/IRV, it may be through a direct pathway that does not traverse the perinuclear GLUT4 compartment.

VAMP2 has been proposed to regulate GLUT4 sorting from recycling endosomes into the IRC/IRV in adipocytes (Williams and Pessin, 2008). Insulin-responsive GLUT4 re-exocytosis after 6 h of endocytosis was not inhibited by depletion of VAMP3, VAMP4, VAMP5, VAMP7, or VAMP8. VAMP2 depletion inhibited GLUT4 re-exocytosis, but since VAMP2 is required for insulin-dependent GLUT4 translocation, this was expected. Because knockdown of no other VAMP impaired insulin-responsive GLUT4 re-exocytosis, it was concluded that VAMP2 mediates GLUT4 sorting and GLUT4 exocytosis. Interestingly, there was no increase in cell surface GLUT4 in resting cells after the 6 h endocytosis as would be expected if GLUT4 could not sort from recycling endosomes into the IRC/IRV. I report that VAMP2 depletion does not impair GLUT4 sorting into the Stx6-positive perinuclear compartment. This observation supports a hypothesis in which VAMP2 does not regulate retrograde GLUT4 traffic. Furthermore, it suggests that the co-localization between GLUT4 and VAMP2-HA observed upon GLUT4 internalization (Figure 3.18) does not serve a regulatory function in sorting GLUT4 into the Stx6-positive perinuclear compartment. An obvious candidate R-SNARE for
directing GLUT4 sorting into the Stx6-positive perinuclear compartment is VAMP4; VAMP4 interacts with Stx6 and is known to function in retrograde transport between sorting/recycling endosomes and the TGN (Johannes and Popoff, 2008; Wendler and Tooze, 2001). Although VAMP4 depletion does not inhibit insulin-responsive GLUT4 re-exocytosis in adipocytes, this was after 6 h endocytosis. Examining the dynamic sorting of GLUT4 in cells depleted of VAMP4 may identify changes in GLUT4 distribution.

8.2.3.3 Potential regulators of GLUT4 sorting into the Stx6-positive perinuclear compartment

It remains unresolved how GLUT4 sorts into the Stx6-positive perinuclear compartment. Perhaps Arf6 regulates GLUT4 sorting through a mechanism similar to that proposed by Li et al., 2012 (Figure 1.10B) (Li et al., 2012). Also, IRAP may regulate GLUT4 sorting through a mechanism independent of its interaction with the sortilin-GLUT4-LRP1 complex (Figure 1.10C). One avenue of particular interest is that of Golgi tethers. Golgin-97 is required for internalized GLUT4 to display static retention (Hatakeyama and Kanzaki, 2011). This tether is also required for STxB sorting to the TGN. Examining Golgi tethers that influence GLUT4 sorting and distribution may uncover similarities between the sorting of GLUT4 and other more defined cargoes. Tracking the co-internalization of GLUT4 and one of these cargoes may assist in deciphering the mechanism through which GLUT4 sorts into its retention compartments.

8.2.4 GLUT4 sorting into the IRC/IRV

According to the model outlined in Figure 7.2, Stx6 is hypothesized to regulate GLUT4 sorting in the perinuclear region from a dynamic retention compartment to an insulin-responsive compartment. This fits with the model outlined in Figure 1.9 in which GLUT4 sorts from the TGN to the IRC/IRV. The motor protein dynein directs GLUT4 vesicles along microtubules into the Stx6-positive perinuclear compartment. Although the potential regulators of GLUT4 sorting examined in this Thesis did not inhibit GLUT4 sorting into the Stx6-positive perinuclear compartment, it is possible that these molecules regulate GLUT4 sorting into the IRC/IRV through an alternative pathway, possibly from sorting/recycling endosomes directly into the IRC/IRV. Examining insulin-responsive GLUT4 re-exocytosis in muscle cells depleted of these regulatory proteins and whether their depletion is additive to that of Stx6 in inhibiting insulin-
responsive GLUT4 re-exocytosis will uncover the contributions of multiple sorting pathways to establishing GLUT4 insulin-responsiveness.

8.3 GLUT4 sorting in insulin resistant muscle cells

Obesity and lipid overload are associated with elevated intramuscular and circulating levels of ceramides that contribute to insulin resistance (Chavez and Summers, 2012). While an underlying mechanism is inhibition of Akt activation (Stratford et al., 2001), effects alternative to inhibition of signalling had not been considered. Here, I provide evidence that C2-ceramide inhibits GLUT4 sorting into the Stx6-positive perinuclear compartment and that this occurs even when insulin stimulated phosphorylation of Akt is allowed to recover. C2-ceramide inhibits insulin-responsive GLUT4 re-exocytosis even after Akt signalling has recovered, showing that defects in GLUT4 sorting alone can inhibit an ensuing insulin-responsive GLUT4 exocytosis event.

Unlike with Stx6 depletion or nocodazole treatment, C2-ceramide disrupts the steady-state localization of GLUT4. This is most reminiscent of the phenotype observed in adipocytes expressing a GLUT4 C-terminal mutant in which the last 30 amino acids of GLUT4 are replaced with the C-terminus of cellugyrin (Li et al., 2009). The GLUT4-cellugyrin chimera mis-localizes to the periphery, losing perinuclear co-localization with Stx6. In C2-ceramide treated myocytes, GLUT4 still co-localizes with Stx6, Stx16, and Tfn; however, because Stx6, Stx16 and furin localizations are also altered by C2-ceramide, either these endomembrane compartments are disrupted or select resident-proteins of these compartments are more sensitive to mis-localization than others. C2-ceramide disruption of endomembrane compartments could inhibit multiple processes such that its major effect on the cell may not be disrupting insulin-responsive GLUT4 translocation. Future work will need to define if C2-ceramide disperses multiple markers of defined organelles or if this effect is specific to the Stx6/16 compartment. Nonetheless, these observations indicate that defects in insulin signalling may not be solely responsible for defects in GLUT4 translocation in insulin resistant cells.

The dispersion of GLUT4 in C2-ceramide treated cells may not be related to insulin-responsiveness. In adipocytes, because the GLUT4-cellugyrin chimera was insulin-responsive it was suggested that GLUT4 transits the perinuclear compartment but that it cannot accumulate there (mis-targeted GLUT4 discussed in Section 8.2.2) (Li et al., 2009). I not only show that C2-
Ceramide prevents the perinuclear accumulation of GLUT4 and that this correlates with inhibition of insulin-responsive GLUT4 re-exocytosis, but that defective GLUT4 sorting appears sufficient to inhibit insulin-responsive GLUT4 re-exocytosis in C2-ceramide treated myocytes. It is important to note that even under resting conditions GLUT4 did not localize to the perinuclear region in C2-ceramide treated cells. Thus, it is unlikely that GLUT4 would traverse the perinuclear region upon internalization. However, that insulin-responsive GLUT4 re-exocytosis was inhibited by C2-ceramide and that recovery of this response was dependent on GLUT4 and Stx6 re-acquiring perinuclear localization supports the hypotheses that GLUT4 sorting into the Stx6-positive perinuclear compartment is necessary for the acquisition of normal insulin-responsiveness, that C2-ceramide disrupts this sorting, and that C2-ceramide disruption of GLUT4 localization likely contributes to insulin resistance.

Interestingly, 50 µM C2-ceramide (Figure 7.1, closed triangle) and 3 µM nocodazole with 5 min recovery (Figure 7.1, open triangle) inhibit insulin-responsive GLUT4 re-exocytosis to a similar degree, yet the co-localization between internalized GLUT4 and Stx6 is much higher in 50 µM C2-ceramide treated cells. This observation supports the conclusion that Stx6 functions in the perinuclear compartment to sort GLUT4. In C2-ceramide treated cells Stx6 is dispersed. It is reasoned that although GLUT4 partially co-localizes with Stx6, these proteins are not in the correct location; the endomembrane compartment in which these proteins co-localize is important in determining Stx6-mediated sorting of GLUT4 into the IRC/IRV.

Although these results highlight the importance of proper GLUT4 sorting, they also suggest that defective insulin signalling also plays a role in C2-ceramide mediated insulin resistance. Comparing 50 µM C2-ceramide (Figure 7.1, closed triangle) with 50 µM C2-ceramide + recovery (Figure 7.1, closed upside down triangle) shows that recovery increases insulin-responsive GLUT4 re-exocytosis without changing GLUT4 co-localization with Stx6. The difference between these two conditions is not in GLUT4 traffic, but in the recovery of insulin signalling to Akt. Thus, although insulin-responsive GLUT4 re-exocytosis remains inhibited in the 50 µM C2-ceramide + recovery condition, it is less inhibited than in the 50 µM C2-ceramide condition. These results indicate that the recovery of insulin signalling likely improved insulin-responsive GLUT4 re-exocytosis independent of defective GLUT4 sorting.
8.4 Conclusions

The mechanism whereby GLUT4 is sorted into insulin-responsive vesicles is poorly understood. Current models, developed mostly in adipocytes, rely largely on the study of steady-state GLUT4 localization to infer modes of GLUT4 sorting. Here I report that, in muscle cells, GLUT4 internalized from the plasma membrane sorts into a Stx6-positive perinuclear compartment and provide evidence that GLUT4 sorting through this compartment is a prerequisite for acquisition of full insulin responsiveness. This compartment is likely comprised of two sub-compartments: a pre-IRC/IRV dynamic retention compartment and an insulin-responsive compartment. Furthermore, I show that C2-ceramide inhibits GLUT4 sorting into this compartment thereby inhibiting insulin-responsive GLUT4 exocytosis independent from effects on insulin signalling. These data further our understanding of how GLUT4 sorts from the plasma membrane back into the IRC/IRV (how and where GLUT4 acquires insulin-responsiveness) and how insulin resistance leads to defects in GLUT4 translocation. Also, leads are provided into the characterization of the perinuclear GLUT4 compartment(s) and the intracellular location of the IRC/IRV.

8.5 Future Directions

It remains unknown what directs GLUT4 into the Stx6-positive perinuclear compartment and whether this compartment is comprised of the functional IRC/IRV. Previous studies, performed mostly in adipocytes, have relied heavily on steady-state measurements of GLUT4 localization to interpret GLUT4 sorting events. Using kinetic approaches to study GLUT4 intracellular traffic can provide novel insights into the mechanisms through which GLUT4 acquires and maintains insulin-responsiveness. Furthermore, it is imperative to examine GLUT4 sorting events in both adipose and muscle cells, as differences in the regulation of GLUT4 translocation and endocytosis in these cell types suggests that simply extrapolating findings from one cell type into the other is not necessarily valid. Labelling cell surface GLUT4myc and tracking its internalization relative to Stx6 provides an assay with easily distinguishable phenotypes that is relatable to insulin-responsive GLUT4 re-exocytosis. By systematically depleting potential regulatory proteins and examining 1) GLUT4 steady-state distribution; 2) GLUT4 sorting; and 3) insulin-responsive GLUT4 re-exocytosis; the roles of these proteins in mediating GLUT4 sorting
8.5.1 Defining the organelle identity of the perinuclear GLUT4 compartment

To address the organelle identity of the perinuclear GLUT4 compartment, further investigation is required into its make-up beyond that of containing Stx6 and Stx16. Examining GLUT4 co-localization relative to those proteins reported to be enriched in the IRC/IRV will characterize whether the perinuclear compartment may consist of the functional IRC/IRV. These proteins include sortilin, IRAP, and LRP1. Those proteins found to co-localize with GLUT4 exclusively in the perinuclear region can be immunoprecipitated and probed for GLUT4 interactions. Similar experiments can be performed examining proteins known to regulate GLUT4 recruitment from the IRC/IRV to the plasma membrane, such as TUG, AS160, VAMP2, and Rab8a.

Although GLUT4 may co-localize in the perinuclear region with proteins known to regulate its accumulation in the IRC/IRV, it remains to be shown that this pool of GLUT4 consists of the functional IRC/IRV. In adipocytes, disrupting TUG function causes GLUT4 to become dispersed (Yu et al., 2007). Likewise, AS160 knockdown increases to pool of recycling GLUT4 (Brewer et al., 2011). Both of these perturbations reduce insulin-stimulated GLUT4 translocation; however, it is unknown if AS160 depletion causes perinuclear GLUT4 to become dispersed. AS160 may not play a predominant role in maintaining perinuclear GLUT4, as AS160 depletion minimally affects the static behaviour of GLUT4 in adipocytes (Hatakeyama and Kanzaki, 2011). TUG co-localization with, and regulation of, perinuclearly localized GLUT4 confirms that perinuclearly localized GLUT4 is linked to insulin-responsiveness, but it does not solidify this compartment as containing the functional IRC/IRV. Similarly to how GLUT4 sorting into the Stx6-positive perinuclear compartment correlates with acquisition of insulin-responsive re-exocytosis, GLUT4 exit from the perinuclear compartment can be correlated with insulin-responsive exocytosis. The maximum insulin-stimulated increase in cell surface GLUT4 is observed by 5 min of insulin treatment (Somwar et al., 2001). Using short exposures to insulin can minimize the recruitment of GLUT4 from non-IRC/IRV compartments that may serve to replenish IRV. Using fluorescence recovery after photobleaching (FRAP), one can quantify GLUT4 exit from the perinuclear compartment over time. In these experiments, GFP-GLUT4myc is transfected into L6 myocytes. The cytosolic GLUT4 signal is photobleached such that only the perinuclear pool.
of GLUT4 is visible. By tracking the dispersion of the perinuclear GLUT4 signal in response to insulin, the mobilization of the perinuclear GLUT4 compartment can be correlated to the increase in cell surface GLUT4 observed in paired samples at matched time points. In the reverse experiment, the perinuclear GLUT4 compartment can be photobleached and the accumulation of GLUT4 at the plasma membrane can be detected. If the perinuclear GLUT4 compartment does not contribute to the initial increase in cell surface GLUT4 upon insulin stimulation then the fluorescence intensity of GLUT4 at the cell surface should not be reduced upon photobleaching the perinuclear GLUT4 pool. These FRAP techniques can then be used to test the reliance of insulin-stimulated GLUT4 translocation on Stx6, TUG, AS160, or other regulatory proteins hypothesized to function in the perinuclear GLUT4 compartment. These experiments will test how GLUT4 insulin-responsiveness is linked to the perinuclear region and provide insight into whether this compartment consists of the functional IRC/IRV.

Additionally, the Stx6-positive perinuclear GLUT4 compartment is hypothesized to partially consist of the TGN. In particular, the pre-IRC/IRV dynamic retention sub-compartment may be composed of TGN membranes. To further investigate this hypothesis, the TGN-resident golgin tethers and the tether complexes GARP and COG can be examined for co-localization with GLUT4. Perturbation of GLUT4 localization in cells depleted of any of these tethers would provide more conclusive evidence that GLUT4 partially resides in the TGN. Furthermore, to better quantitate whether GLUT4 localizes to furin-positive membranes in the perinuclear region, the strategy employed by Karylowski et al. can be used in which Tac-furin is expressed in adipocytes and labelled with HRP-anti-Tac antibody (Karylowski et al., 2004). By labelling Tac-furin with HRP and examining fluorescence quenching of internalized GLUT4myc the degree of overlap between furin and GLUT4 membranes can be determined in L6 muscle cells. As a positive control for cargo sorting into the TGN, GLUT4 co-localization with STxB can be examined relative to TGN-resident proteins. The accumulation of internalized GLUT4 and STxB in the perinuclear region can be used as an indicator of the overlap between the retrograde pathways of these two cargoes. Together, these experiments will aid in characterizing the pre-IRC/IRV sub-compartment in the Stx6-positive perinuclear region.
8.5.2 Characterizing GLUT4 sorting into the IRC/IRV

To address the mechanism through which GLUT4 sorts into the IRC/IRV, GLUT4 sorting into the Stx6-positive perinuclear compartment and insulin-responsive GLUT4 re-exocytosis assays can be used to test the effects of siRNA knockdown of suspected regulatory proteins. In this Thesis I reported some inroads into this approach. Although Rab5, Rab11, Rab14, and retromer did not appear necessary for GLUT4 sorting into the Stx6-positive perinuclear compartment, it is unknown if these proteins regulate GLUT4 sorting into the IRC/IRV through an alternative pathway. If knockdown of any of these proteins inhibits GLUT4 re-exocytosis then the additive nature of this effect with that of Stx6 knockdown will be tested. These experiments will provide insight into the number of pathways through which GLUT4 can reach the IRC/IRV. Similarly, GLUT4 sorting and re-exocytosis should be tested in L6 myocytes depleted of sortilin or Golgi tethers. It is hypothesized that Golgi tethers are required to capture GLUT4 at the Stx6-positive pre-IRC/IRV sub-compartment in the perinuclear region. If sortilin knockdown proves inconsequential for GLUT4 sorting, similar experiments can be performed using the C2C12 mouse muscle cell line. These experiments could define the requirement, if any, for sortilin in mediating GLUT4 sorting in muscle cells.

It is possible that GLUT4 must sort through the Stx6-positive perinuclear compartment to acquire localization in the IRC/IRV. In this scenario, the insulin-responsive GLUT4 re-exocytosis observed in cells depleted of Stx6 occurs through the constitutive recycling pathway due to an increased pool of recycling GLUT4. To test this hypothesis, insulin-responsive GLUT4 re-exocytosis can be measured in cells depleted of Stx6 and a regulatory protein known to function in GLUT4 recruitment from the IRC/IRV to the plasma membrane (ex. Rab8a, VAMP2, or Myo1c). In addition, knockdown of proteins that function in constitutive recycling can be tested for additivity with Stx6 knockdown (ex. VAMP7). These experiments will characterize the mechanism of insulin-responsive GLUT4 re-exocytosis in Stx6-depleted cells and provide insight into the routes of GLUT4 sorting into the IRC/IRV.

In addition to knockdown strategies, expression of GLUT4 mutants in L6 cells can be used to correlate phenotypes in the sorting of GLUT4 mutants to those of regulatory proteins. In this way, insight can be gained into the retrograde sorting pathways mediated by the different sorting motifs of GLUT4. For example, TUG binds to the large cytoplasmic loop of GLUT4. By
studying the sorting of a GLUT4-GLUT1 chimera in which this loop of GLUT4 is replaced by that of GLUT1, the role for this region in directing GLUT4 to the perinuclear insulin-responsive compartment can be determined. It can also be investigated why the FA and EEAA GLUT4 mutants do not display drastic changes in localization despite shifting GLUT4 localization to sorting/recycling endosomes. Examining the dynamic sorting of internalized GLUT4 mutants may uncover which sorting motifs in GLUT4 direct GLUT4 to the insulin-responsive perinuclear compartment in muscle cells.

Together, these future studies will build on the framework established in this Thesis to characterize the organelle identity of the Stx6-positive perinuclear compartment, the mechanisms of GLUT4 sorting into the IRC/IRV, and the intracellular location of the IRC/IRV.
Appendix I: Secondary authorship publications

Over the course of my Ph.D. studies I participated in three projects as a secondary author in peer reviewed publications. I also co-authored one peer-reviewed review article on GLUT4 traffic.

9.1 Contraction-related stimuli regulate GLUT4 traffic in C2C12-GLUT4myc skeletal muscle cells

Wenyan Niu,1,2 Philip J. Bilan,1 Shuwei Ishikura,1 Jonathan D. Schertzer,1 Ariel Contreras-Ferrat,1,3 Zhengxiang Fu,2 Jie Liu,2 Shlomit Boguslavsky,1 Kevin P. Foley,1 Zhi Liu,1 Jinru Li,2 Guilan Chu,2 Thomas Panakkezhum,5 Gary D. Lopaschuk,5 Sergio Lavandero,3,4 Zhi Yao,2 and Amira Klip1

1Program in Cell Biology, The Hospital for Sick Children, Toronto, Ontario, Canada; 2Department of Immunology, Key Laboratory of Immuno Microenvironment and Disease of the Educational Ministry of China, Tianjin Medical University, Tianjin, China; 3Centro Estudios Moleculares de la Célula, Facultad Ciencias Quimicas y Farmaceuticas; 4Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile; and 5Cardiovascular Research Group, Mazankowski Alberta Heart Institute, University of Alberta, Edmonton, Alberta, Canada

This paper was published in the American Journal of Physiology Endocrinology and Metabolism, 2010; 298(5): E1058-E1071. My contributions to this work were in the experimental design and writing. I was involved in the discussions about the conceptual framework of the project and the building of this concept into a testable hypothesis. This report characterizes carbachol-stimulated GLUT4 translocation in C2C12 myotubes. Carbachol is a stable analog of acetylcholine and can be used to induce muscle contraction. This cellular model allows one to study the mechanisms through which physiological stimulation of muscle contraction signals the recruitment of GLUT4 to the cell surface. We found that carbachol activates AMPK (AMP Kinase) through force generation and that inhibiting contraction via the myosin II ATPase inhibitor BTS blocks a significant proportion of the carbachol-stimulated GLUT4myc translocation response. Inhibiting AMPK directly using the inhibitor Compound C or by siRNA knockdown also prevents the rise in cell surface GLUT4myc mediated by carbachol. We conclude that muscle contraction stimulates an AMPK dependent signalling pathway in the mobilization of GLUT4 in response to carbachol.

9.2 NOD2 Activation Induces Muscle Cell-Autonomous Innate Immune Responses and Insulin Resistance

Akhilesh K. Tamrakar, Jonathan D. Schertzer, Tim T. Chiu, Kevin P. Foley, Philip J. Bilan, Dana J. Philpott, and Amira Klip

Program in Cell Biology (A.K.T., J.D.S., T.T.C., K.P.F., P.J.B., A.K.), The Hospital for Sick Children,
This paper was published in *Endocrinology, 2010; 151(12): 5624-5637*. My contributions to this work were in conducting revision experiments, preparing all of the figures, and revisions in writing. This report characterizes how activation of NOD2 in muscle cells induces inflammation and inhibits insulin-stimulated GLUT4 translocation. In the context of obesity, immune cell infiltration into adipose tissue causes inflammation and cytokine production that contribute to insulin resistance in adipocytes. Furthermore, inflammatory cytokines cause insulin resistance in muscle and muscle cells in culture. NOD2 is an intracellular pathogen sensor that functions as part of the innate immune system. It activates proinflammatory signalling pathways upon recognizing intracellular bacterial components containing peptidoglycan muramyl dipeptide (MDP). Although typically associated with immune cell responses, NOD2 is ubiquitously expressed. We show that activation of NOD2 with MDP in L6 myotubes inhibits insulin-stimulated glucose uptake and GLUT4 translocation. Knockdown of NOD2 partially recovers the insulin-stimulated increase in cell surface GLUT4. MDP also induces an inflammatory response in L6 myotubes; MDP provokes increases in the phosphorylation of the stress kinases JNK, ERK1/2, and p38 MAPK, the degradation of IKBα, and expression of the proinflammatory cytokines TNFα and KC. JNK and ERK1/2 are known to inhibit insulin signalling by phosphorylating serine and threonine residues on IRS-1. We show that MDP inhibits insulin signalling at the level of IRS-1 and that inhibitors of JNK or ERK1/2 significantly reverse this effect. Thus, activation of NOD2 in muscle cells causes insulin resistance independent of immune cell participation.

### 9.3 Myo1c binding to submembrane actin mediates insulin-induced tethering of GLUT4 vesicles

Shlomit Boguslavsky¹, Tim Chiu¹, Kevin P. Foley¹, Cesar Osorio-Fuentealba²³, Costin N. Antonescu⁵, K. Ulrich Bayer⁴, Philip J. Bilan⁶, and Amira Klip⁶

¹Cell Biology Program, Hospital for Sick Children, Toronto, ON M5G 1X8, Canada; ²FONDAP-CEMC Instituto de Ciencias Biomédicas, University of Chile, Santiago 6530499, Chile; ³Department of Chemistry and Biology, Ryerson University, Toronto, ON M5B 2K3, Canada; ⁴Department of Pharmacology, University of Colorado Denver–School of Medicine, Aurora, CO 80045-0508

This paper was published in *Molecular Biology of the Cell, 2012; 23(20): 4065-4078*. My contributions to this work were in conducting revision experiments. In particular, I developed the protocol used for the detection of recycling transferrin by immunofluorescence and I created the L6GLUT4myc cells that stably express VAMP2-HA used for co-localization experiments. This
report characterizes the insulin-induced tethering of GLUT4-containing vesicles in response to insulin. Actin remodelling is critical for insulin-stimulated GLUT4 translocation. We show that the non-processive myosin motor Myo1c links the actin cytoskeleton to GLUT4-containing vesicles and that this function is required for insulin-stimulated GLUT4 translocation. Specifically, Myo1c is required for GLUT4-vesicle docking beneath the plasma membrane. This action of Myo1c is specific to the exocytosis of GLUT4 vesicles, as GLUT4 endocytosis is not altered by Myo1c knockdown. Furthermore, Myo1c knockdown does not inhibit Tfn recycling. Thus, insulin reduces the mobility of GLUT4 vesicles beneath the plasma membrane through the action of Myo1c, which functions to facilitate GLUT4 translocation.

9.4 Endocytosis, Recycling, and regulated exocytosis of Glucose Transporter 4

Kevin Foley,†,‡ Shlomit Boguslavsky,† and Amira Klip*,†,‡

†Cell Biology Program, The Hospital for Sick Children, Toronto, Ontario M4G 1X8, Canada
‡Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada

This review was published in Biochemistry, 2011; 50(15):3048-61. My contributions to this work were in writing sections of the review on GLUT4 endocytosis and recycling and in revising the entire manuscript.
10 References


