University of Glasgow | College of Medical, Veterinary & Life Sciences

Head of College Scholars List Scheme

Summer Studentship

Report Form

This report should be completed by the student with his/her project supervisor. It should summarise the work undertaken during the project and, once completed, should be sent by email to: jill.morrison@glasgow.ac.uk within four weeks of the end of the studentship.

1. Student

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2. Supervisor:

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3. Research Project Report

3.1 **Project Title** (maximum 20 words):

Characterisation of a novel trafficking pathway leading to GLUT4 compartment formation.

3.2 **Project Lay Summary** (copied from application):

A major action of insulin is to increase the rate of glucose transport into fat and muscle. This is achieved through the release of the facilitative glucose transporter GLUT4 from intracellular storage compartments to the plasma membrane and is defective in individuals suffering from insulin-resistance and Type-2 diabetes. Understanding the molecular mechanisms which underlie this process therefore represents an important step in the development of rational therapies for these disease states. Endocytic pathways targeting GLUT4 to the intracellular stores after insulin-mediated release have been defined extensively in rodent models. Recently, a novel biosynthetic trafficking pathway leading to initial store formation has been postulated, which is less characterised, and differs between humans and rodents. The student will use siRNA transfection approaches to further characterize this new pathway with the aim to identify SNARE proteins that underpin its organization.

3.3 **Start Date:** 19/06/2017 Finish Date: 31/07/2017

3.4 **Original project aims and objectives** (100 words max):

To further characterize a novel biosynthetic trafficking pathway that leads to the formation of the intracellular GLUT4 storage compartment (GSC).

To identify SNARE proteins that underpin GSC formation.

To further characterize the role of the ER-to-Golgi intermediate compartment (ERGIC) in GSC formation.

To optimize experimental design to further improve accuracy and reliability of data.

3.5 **Methodology: Summarise and include reference to training received in research methods etc.** (250 words max):

Cell culture

HA-GLUT4-GFP tagged HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% L-glutamine at 37°C in 5% CO_2, and passaged at ~70% confluence. Training was given for sterile cell culture techniques including thawing, splitting and feeding, cell counting and cell seeding.

Transient siRNA transfection

The day prior to transfection approx. 9000 HeLa cells were plated onto glass bottomed 96-well plates. Cells were transfected with 3µM siRNA using the transfection reagent Lipofectamine 2000 and Opti-MEM reduced serum medium. Prior to transfection, cells were washed once with DMEM lacking serum and antibiotics. 28µl of transfection medium was added into each well and incubated for 4 h. Lastly, 65µl of 30% FBS/DMEM was added to each well, cells were incubated for 48 h and stained as described below. DNA-mediated gene transfer is a powerful widely used technique; training in this area is therefore useful.

Immunofluorescence labelling and image analysis

For immunofluorescence labelling cultured cells were fixed using 4% (w/v) p-formaldehyde for 20 minutes at room temperature. Coverslips were then quenched in 50mM NH_4Cl and permeabilized

using 0.1% (v/v) Triton in PBS. Cells were blocked in immunofluorescence (IF) buffer containing 0.1% (v/v) goat serum for 30 minutes. Coverslips were then incubated in primary antibody solution for 1 h followed by an incubation in secondary antibody solution for 1 h at room temperature. Lastly, for nuclear staining cells were incubated for 5 minutes in DAPI and evaluated by confocal microscopy. If grown in 12-well plates coverslips were mounted onto glass slides using a drop of Immunomount prior to evaluation.

Training was given for operating a Zeiss Confocal Microscope and LSM software. Image analysis was performed using ImageJ and co-localization was determined with the JACoP plugin.

Immunoblotting

Samples were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The samples were blocked and incubated in monoclonal antibody specific to the relevant knockdown target overnight. After incubation in secondary antibody specific signals were visualized using Odyssey Sa.

3.6 **Results: Summarise key findings** (300 words max). Please include any relevant tables or images as an appendix to this report:

Optimization of experimental design

The most suitable antibody concentration for IF labelling was determined empirically by incubating cells with dilutions of 1:1000, 1:500, 1:200 and 1:50 of the primary antibodies against the ERGIC markers p115 and ERGIC-53 (Figure 1). Based on a comparison of images shown in Figure 1 the appropriate dilutions were found to be 1:50 and 1:500 for p115 and ERGIC-53, respectively.

Transient siRNA transfection and co-localization analysis

Results of the GLUT4-ERGIC co-localization analysis for the first siRNA transfection are summarized in Figure 2. The SNARE proteins knocked down during this experiment were selected based on published data which suggested their implication in ERGIC trafficking. STX6 and STX16 were included as positive controls as they are known to have a role in GLUT4 sorting (Bryant and

Gould, 2011). Hence it was anticipated to observe a disruption in GLUT4 distribution in cells treated with these siRNAs. Cells treated with the other four siRNAs were expected to show a disruption in the pattern of ERGIC staining. Interestingly, silencing of STX6, STX16 and SEC22C led to an increase in co-localization between GLUT4 and the ERGIC. Silencing of SEC22B, on the other hand, resulted in a major decrease in co-localisation between GLUT4 and ERGIC-53. Furthermore, silencing of STX5 and YKT6 did not seem to have any impact on GLUT4-ERGIC co-localisation.

During the second siRNA transfection cells were stained for both, p115 and ERGIC-53. It targeted a different set of SNARE proteins but no significant impact on GLUT4-ERGIC co-localization could be detected (Figure 3).

The results of the third siRNA transfection are summarized in Figure 4. All knockdowns showed decreased co-localization with ERGIC-53 whereas no significant changes could be observed regarding the co-localization with p115. In particular, knockdown of SNAP29 and STX17 had a large impact on co-localisation with ERGIC-53 and also led to a dispersion of the ERGIC-53 staining as shown in Figure 5.

Care must be taken when interpreting this co-localization data due to large standard errors in most samples and the low cell count (5-20 cells per image) used during this analysis. However, the data are a useful and worthwhile first-screen of the role of the different SNAREs in Glut4/ERGIC localisation.

3.7 **Discussion** (500 words max):

Endocytic pathways targeting GLUT4 to the intracellular stores after insulin-mediated release have been defined extensively in rodent models (Figure S1). Recently, a novel biosynthetic trafficking pathway leading to initial store formation has been postulated, which is less characterised, and differs between humans and rodents (Brodsky et al., n.d., Figure S2). Brodsky et al. report that in human muscle and adipocytes, GSC formation involves the non-canonical isoform of clathrin,

CHC22. It is hypothesized that CHC22 is recruited to act as a membrane coat that combines the players of GSC formation (including sortilin, IRAP and p115) and stabilizes their interaction. The results reported here suggest that the SNARE proteins SEC22B, SNAP29 and STX17 may play a role in this novel pathway (Figure 2 and 4).

As shown in Figure 2, silencing of R-SNARE protein SEC22B significantly decreased co-localisation between GLUT4 and ERGIC-53. SEC22B is known to be involved in trafficking between the ER and cis-Golgi (Hay et al., 1997). Furthermore, there is evidence that suggests that CHC22 in fact recruits SEC22B for membrane traffic emerging from the ER (Brodsky et al., n.d.). Hence, we hypothesize that sorting of newly synthesized GLUT4 from the ER into the ERGIC is dependent on SEC22B because the knockdown showed decreased co-localisation and possibly impaired GSC formation.

Similar to SEC22B, SEC22C is thought to be involved in traffic between the ER and Golgi. Hence it would be sensible to expect a similar effect on co-localization upon silencing, however, this was not the case. Due to the small sample size and manual analysis of images we suspect this result to simply be inaccurate.

The second siRNA transfection summarized in Figure 3 did not yield any significant results and should be repeated. The accuracy of these results might have been compromised by disturbances during the capture of images which affected the focus of the microscope.

Silencing of both SNAP29 and STX17 led to a significant decrease in co-localization between GLUT4 and ERGIC-53 but did not have any impact on co-localization with p115 (Figure 4). STX17 is thought to cycle between the ER and ERGIC and, more importantly, has also been shown to interact with SNAP29 during fusion of autophagosomes with endosomes/lysosomes (Itakura et al., 2012). This combined with our results suggests a similar interaction of STX17 and SNAP29 during transport from the ER to the ERGIC and a possible involvement in GSC formation.

Furthermore, STX17 is required for structural maintenance of the ERGIC and Golgi (Muppirala et al., 2011). Our results are consistent with this and indicate a dispersal of the ERGIC as shown in Figure 5. This could be an additional (or even the primary) factor that led to a decrease in co-localization between GLUT4 and ERGIC-53. Interestingly, dispersion of the marker p115 was affected to a lesser extent or not at all. This could be linked to the fact that STX17 silencing leads to complete ERGIC disintegration (where the majority of ERGIC-53 is found) but only to a fragmentation of the Golgi where the majority of p115 is localized minimizing its dispersion (Seemann et al., 2000).

Further experiments are needed to investigate whether knockdown of STX17 and SNAP29 in fact decreases the level of GLUT4 expressed in these cells or if the decrease of co-localization was simply due to disintegration of the ERGIC.

4. **Reflection by the student on the experience and value of the studentship** (300 words max):

The studentship allowed me to get insight and experience of the kind of work which I might find myself doing after my undergraduate course is over. I learned a lot about team work, planning of experiments, critical self-analysis and, most importantly, skills that are mandatory to know before going “out-there” into the real world.

After receiving my training during the first week my supervisor left for a 2-week holiday. At first I was worried that I might not be able to cope with the workload and doing all my experiments independently. However, looking back, it actually turned out to be the most rewarding time of my project as I was forced to do everything on my own without a supervisor “holding me by the hand”. Ultimately, I enjoyed the freedom of planning my days and thinking about the next steps on my own initiative. That being said, my co-workers and supervisor were always very helpful whenever I got stuck or had a question.

In addition to gaining practical skills, I have come to realize that the planning and design of experiments is a continuous process with many potential set-backs along the way. This showed

me that, in order to become a good researcher, it is essential to be passionate enough about the topic of interest to cope with these set-backs and to have the necessary determination to continue.

Another valuable experience I gained from my studentship was to give a presentation of my results to the entire lab group. It took me quite a bit of courage to give a talk in front of scientists that have a lot more experience in the field than I do. However, I am now really glad that I did it and the feedback was very encouraging.

Overall, I am very grateful to have been given this opportunity and I'd like to stress that without the studentship provided by the Head of Scholar's none of this would have been possible.

5. **Dissemination**: (note any presentations/publications submitted/planned from the work):

   We expect to use this data as part of a larger study which we hope to publish over the next 18-24 months.

6. Signatures: Supervisor  Date 22/08/17 Student  Date 22/08/17

References

Brodsky, F., Camus, S., Camus, M., Sadacca, L., Esk, C., Gould, G., Kioumourtzoglou, D., Bryant, N. and Mukherjee, S. (n.d.). GLUT4 Compartment Formation by CHC22 Clathrin Bypasses the Golgi.

Bryant, N. and Gould, G. (2011). SNARE Proteins Underpin Insulin-Regulated GLUT4 Traffic. Traffic, 12(6), pp.657-664.

Chen, Y. and Scheller, R. (2001). SNARE-mediated membrane fusion. Nature Reviews Molecular Cell Biology, 2(2), pp.98-106.

Hay, J., Chao, D., Kuo, C. and Scheller, R. (1997). Protein Interactions Regulating Vesicle Transport between the Endoplasmic Reticulum and Golgi Apparatus in Mammalian Cells. Cell, 89(1), pp.149-158.

Itakura, E., Kishi-Itakura, C. and Mizushima, N. (2012). The Hairpin-type Tail-Anchored SNARE Syntaxin 17 Targets to Autophagosomes for Fusion with Endosomes/Lysosomes. Cell, 151(6), pp.1256-1269.

Muppirala, M., Gupta, V. and Swarup, G. (2011). Syntaxin 17 cycles between the ER and ERGIC and is required to maintain the architecture of ERGIC and Golgi. Biology of the Cell, 103(7), pp.333-350.

Seemann, J., Jokitalo, E. J., & Warren, G. (2000). The Role of the Tethering Proteins p115 and GM130 in Transport through the Golgi Apparatus In Vivo. Molecular Biology of the Cell, 11(2), 635–645.

Appendix

Figure 1:

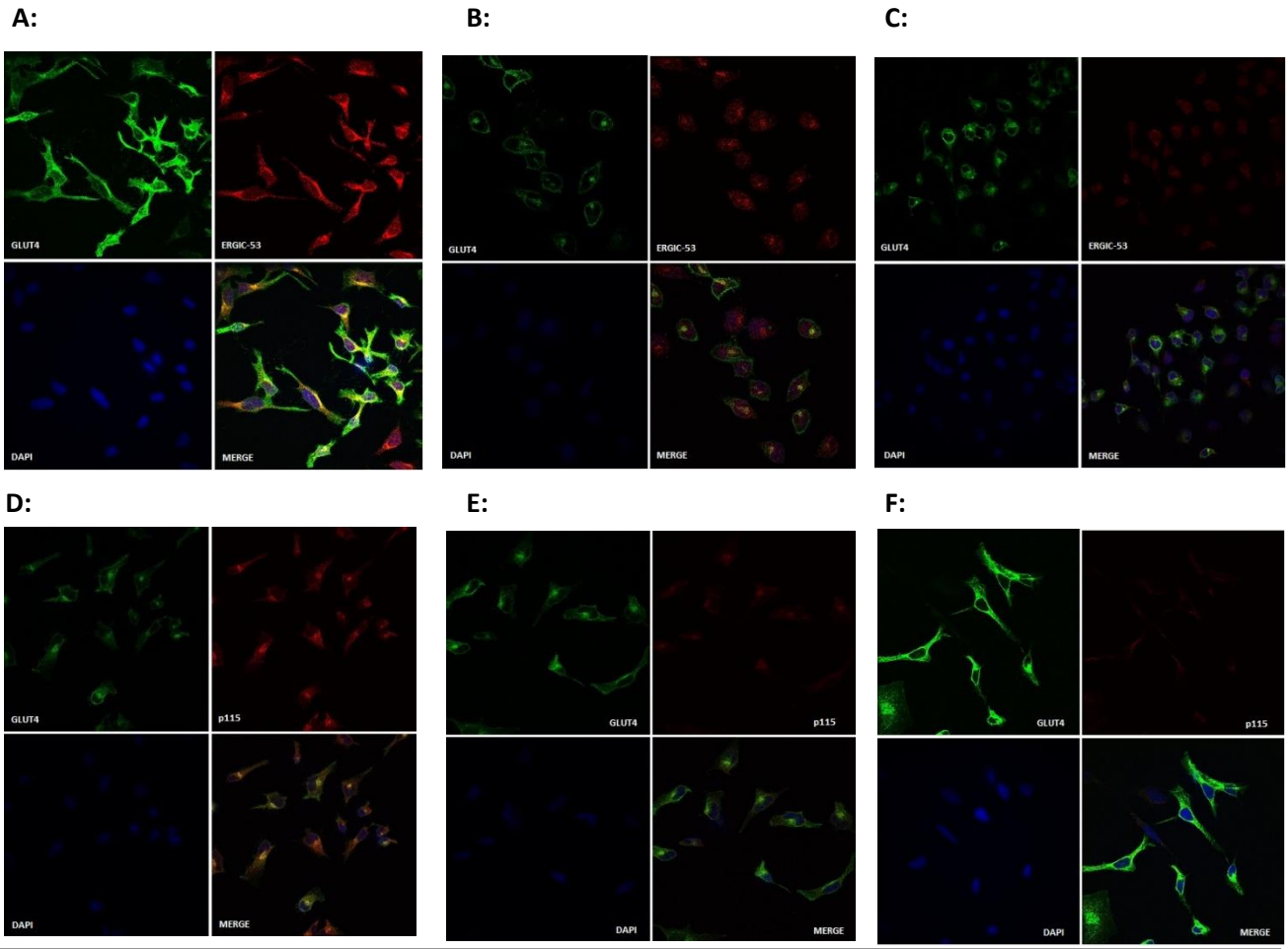


Figure 1, *HeLa-GLUT4 cells stained with antibodies against ERGIC markers ERGIC-53 and p115 at different dilutions.*

(**A-C**) Representative images of GLUT4 (exofacial HA-tag, internal GFP tag) in HeLa-GLUT4 cells labelled with ERGIC-53 antibody (red) at dilutions of 1:200, 1:500 and 1:1000.

(**D-F**) Representative images of GLUT4 (exofacial HA-tag, internal GFP tag) in HeLa-GLUT4 cells labelled with p115 antibody (red) at dilutions of 1:50, 1:200 and 1:1000.

The ideal dilutions for labelling the ERGIC after transient siRNA transfection were found to be 1:500 and 1:50 for ERGIC-53 and p115, respectively.

Figure 2:

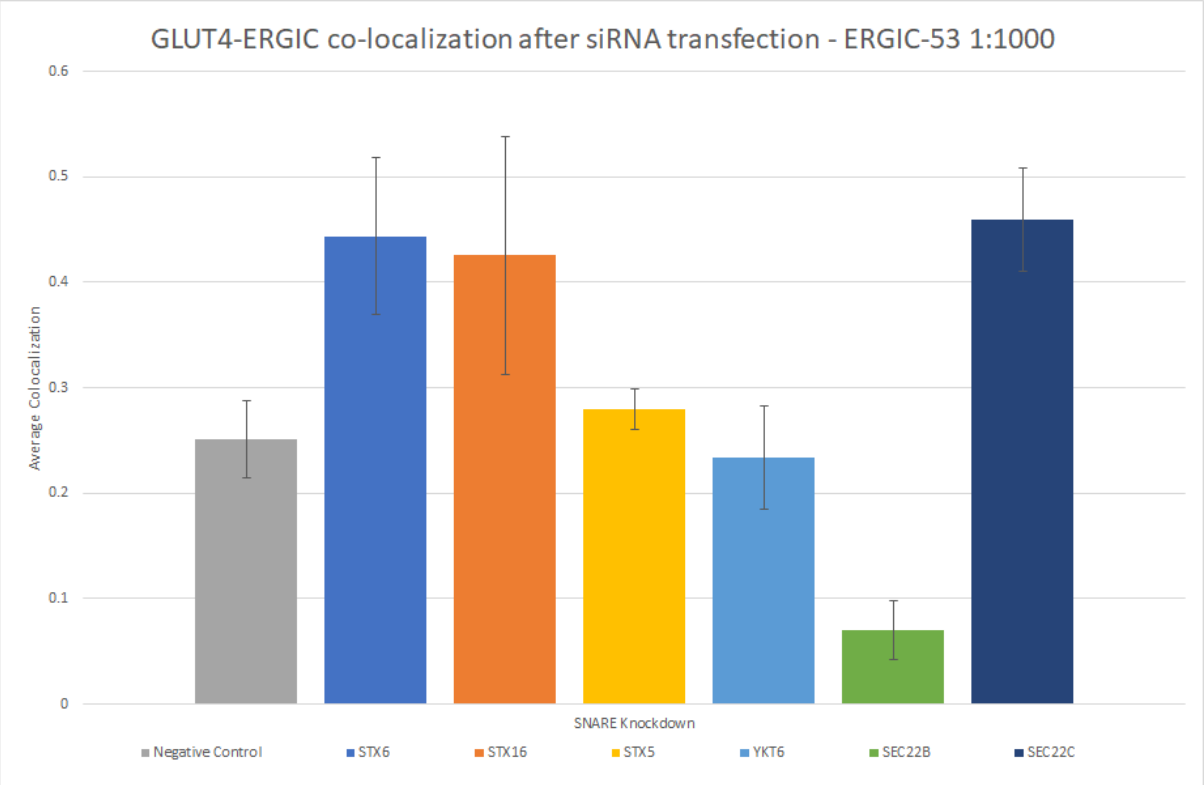


Figure 2, *Results of GLUT4-ERGIC co-localization analysis after knockdown of SNARE proteins as indicated.*

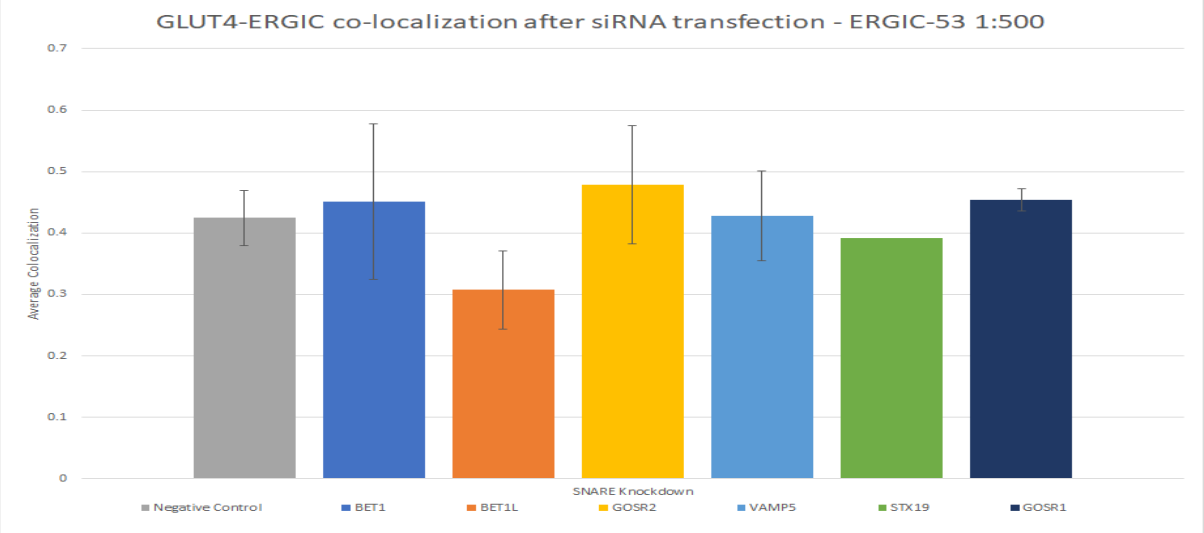
Images were captured using a Zeiss LSM confocal microscope and analysed using the JAcoP plugin on ImageJ. Each bar shows the mean Pearson's correlation coefficient above the Costes auto threshold for the green (GLUT4) and red (ERGIC-53) channels of the respective knockdown. Cells were labelled with an antibody against the ERGIC marker ERGIC-53 at a 1:1000 dilution.

Interestingly, knockdown of STX6, STX16 and SEC22C led to an increase in co-localization. Knockdown of SEC22B, on the other hand, decreased co-localization between GLUT4 and ERGIC-53.

The large standard errors are most likely the result of two complications: Firstly, double images at the same depth and settings were required for analysis. Due to an unknown disturbance the focus of the microscope changed slightly between the capture of the two images affecting the analysis. Secondly, each image only contained approx. 5-20 cells which clearly affects the reliability of the results.

Figure 3:

A:



B:

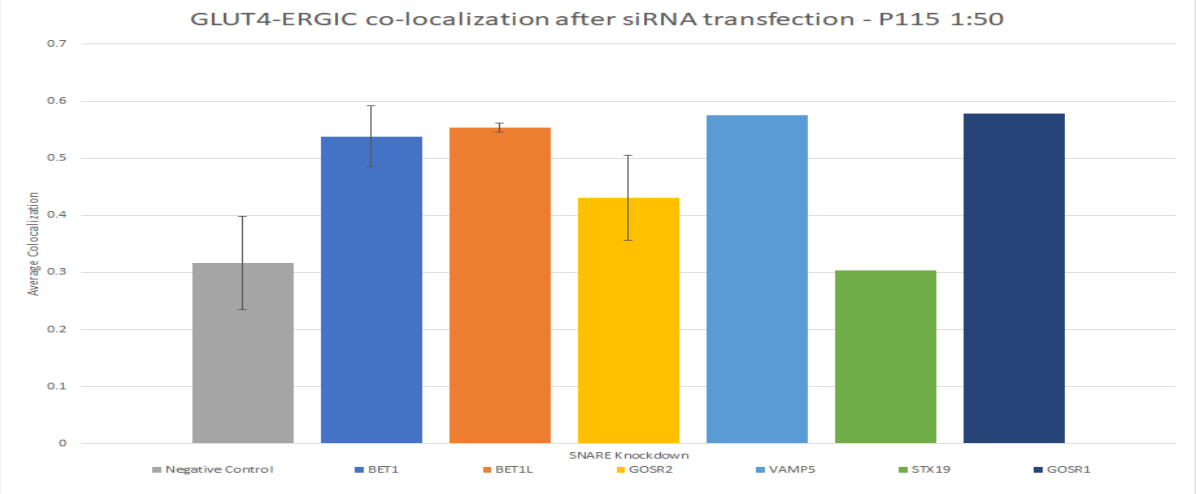


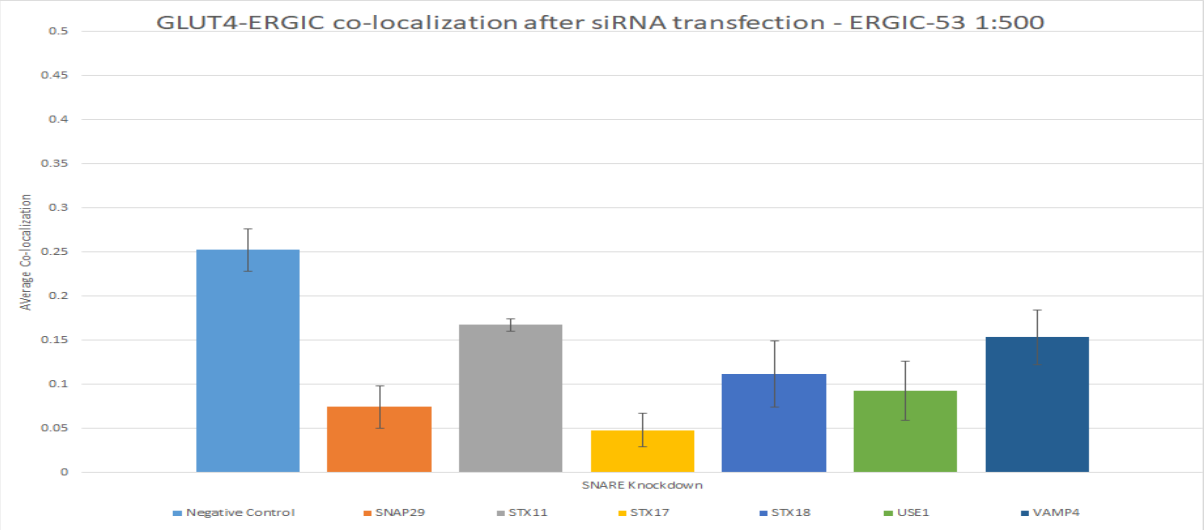
Figure 3, *Results of GLUT4-ERGIC co-localization analysis after knockdown of SNARE proteins as indicated.*

Images were captured using a Zeiss LSM confocal microscope and analysed using the JAcoP plugin on ImageJ. Each bar shows the mean Pearson's correlation coefficient above the Costes auto threshold for the green (GLUT4) and red ((**A**) ERGIC-53 and (**B**) P115) channels of the respective knockdown. Cells were labelled with an antibody against the ERGIC markers ERGIC-53 and p115 at a 1:500 and 1:50 dilution, respectively.

None of the knockdowns caused any significant changes to the co-localization between GLUT4 and the ERGIC. This is quite possibly due to the complications described in Figure 2.

Figure 4:

A:



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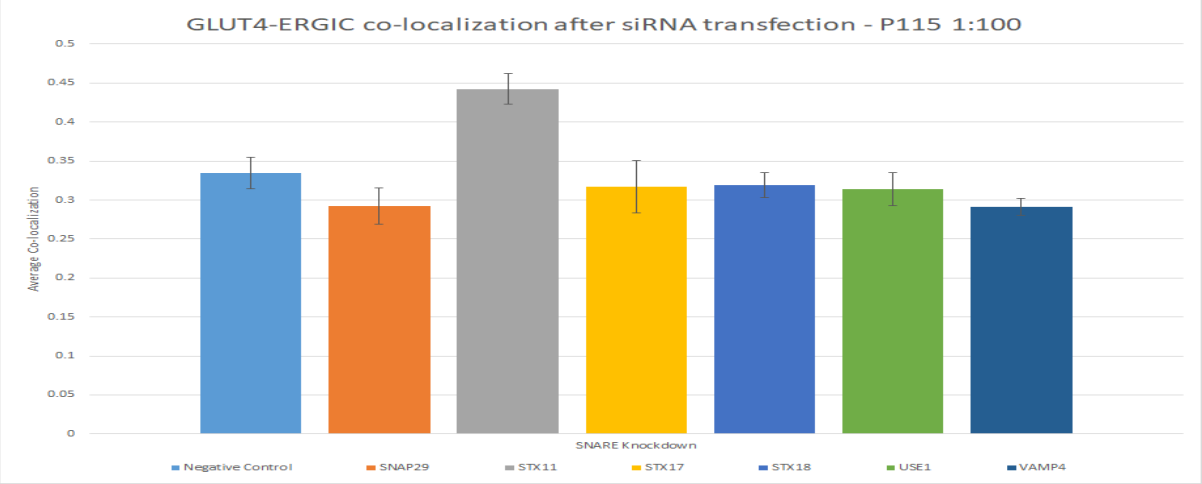


Figure 4, *Results of GLUT4-ERGIC co-localization analysis after knockdown of SNARE proteins as indicated.*

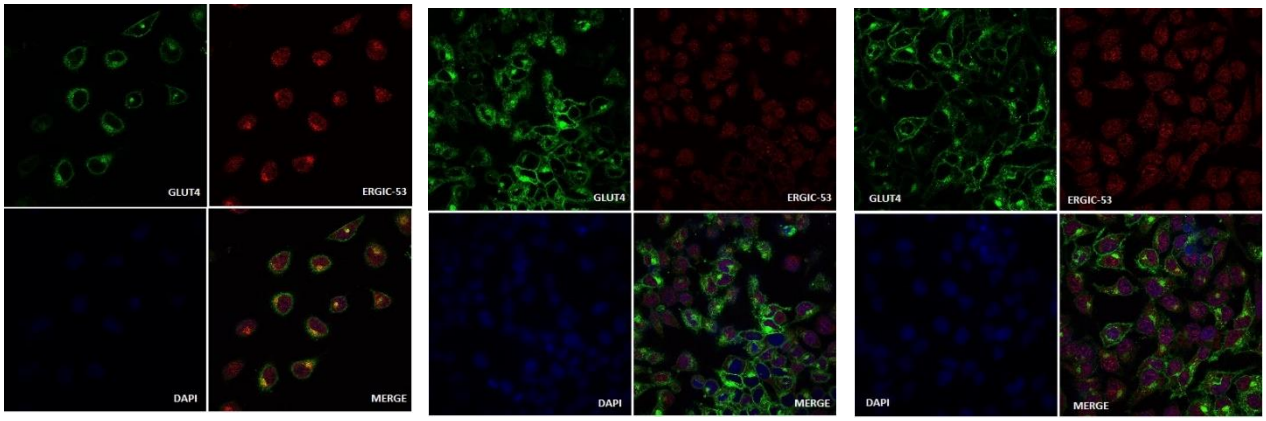
Images were captured using a Zeiss LSM confocal microscope and analysed using the JAcoP plugin on ImageJ. Each bar shows the mean Pearson's correlation coefficient above the Costes auto threshold for the green (GLUT4) and red ((**A**)ERGIC-53 and (**B**) P115) channels of the respective knockdown. Cells were labelled with an antibody against the ERGIC markers ERGIC-53 and p115 at a 1:500 and 1:100 dilution, respectively.

In **Panel A,** which shows co-localization between GLUT4 and ERGIC-53, a general decrease in co-localization could be observed for all knockdowns. The most significant decrease was caused by silencing of SNAP29 and STX17.

Interestingly, as shown in **Panel B**, the exact same knockdowns had no apparent effect on co-localization with p115.

Figure 5:

A - Control: **B – SNAP29:** **C – STX17:**



D - Control: **E – SNAP29:** **F – STX17:**

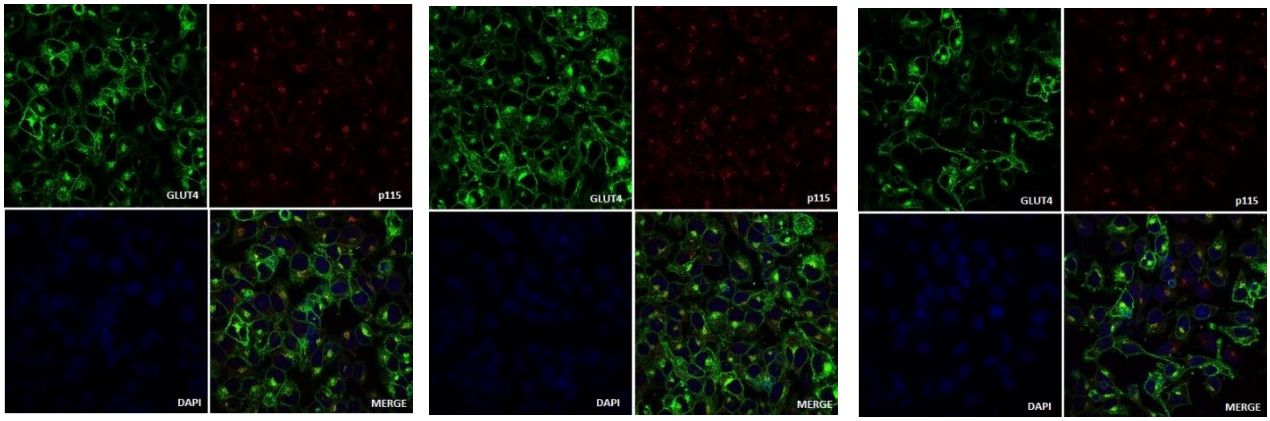


Figure 5, *HeLa-GLUT4 cells silenced for SNAP29 (B and E) and STX17 (C and F) and stained with antibodies against ERGIC markers ERGIC-53 (A-C) and p115 (D-F).*

(**A-C**) Representative images of GLUT4 (exofacial HA-tag, internal GFP tag) in HeLa-GLUT4 cells labelled with ERGIC-53 antibody (red). **Panel C** shows how STX17 silencing led to a dispersion of the ERGIC when compared to the negative control in **Panel A**. Silencing of SNAP29 also led to ERGIC disintegration as shown in **Panel B**.

(**D-F**) Representative images of GLUT4 (exofacial HA-tag, internal GFP tag) in HeLa-GLUT4 cells labelled with p115 antibody (red). **Panels D-F** show that localisation and dispersion of the marker p115 was seemingly unaffected by silencing of SNAP29 (**Panel E**) and STX17 (**Panel F**).

Supplementary material:

Figure S1:

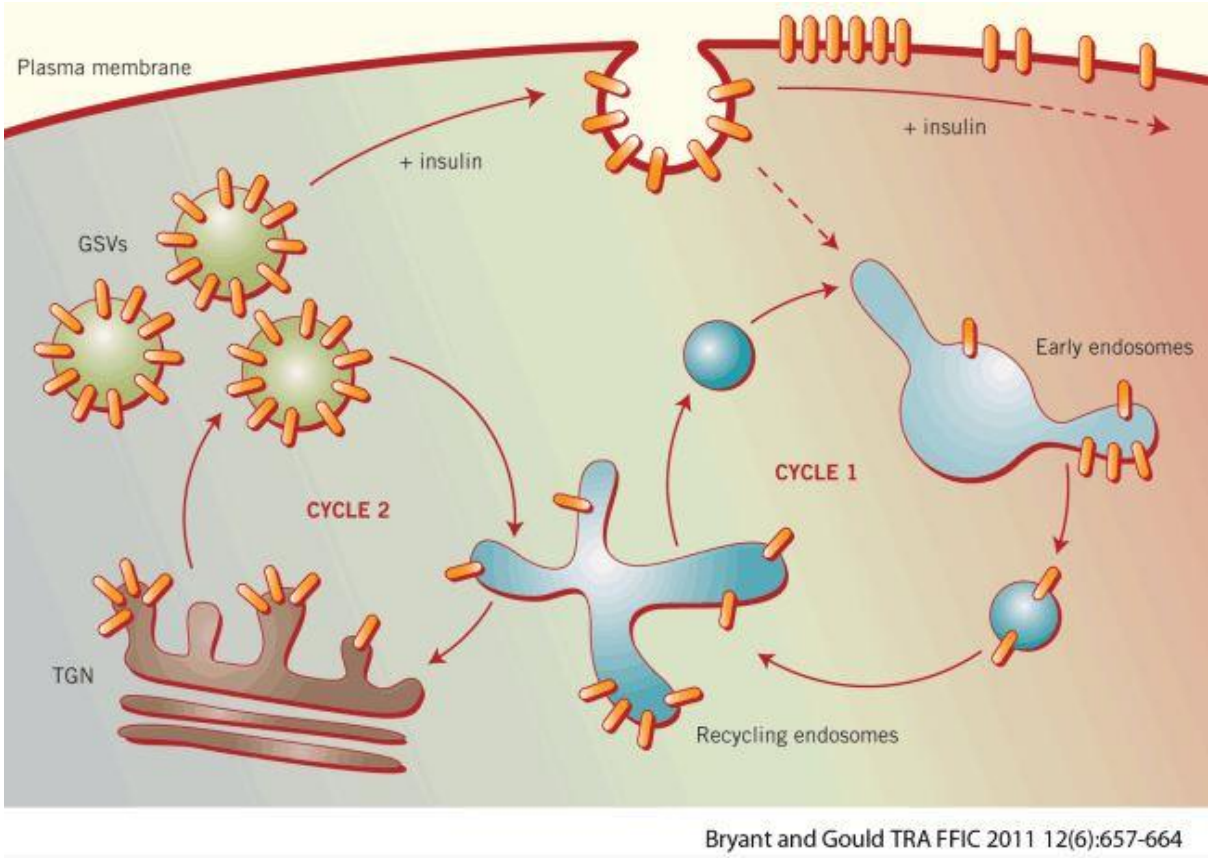


Figure S1, *A diagram of the GLUT4 trafficking itinerary and a summary of the "outdated" model of human GSC formation.*

GLUT4 is constantly cycled between the plasma membrane and intracellular membrane-bound compartments. After internalization, GLUT4 populates two interrelated endosomal cycles. The first is the classic endosomal system, trafficking between the plasma membrane and early endosomes (cycle 1). GLUT4 is further sorted into a slowly recycling pathway, operating between recycling endosomes, the *trans*-Golgi network (TGN) and a population of vesicles termed GSVs. GSVs bud from recycling endosomes and/or TGN. GSVs only cycle to the plasma membrane in the presence of insulin. This is thought to be achieved by an intracellular retention mechanisms that may involve futile cycling with recycling endosomes or the TGN, or physical anchoring to an intracellular structure.

This diagram also summarizes the "outdated" model of GSC formation in humans. Previously it was thought that, instead of going straight to the GSVs, GLUT4 traffics first to the cell surface and then gets sorted into GSVs from recycling endosomes. The new model proposed by Brodsky et al. is shown in Figure S2.

Figure S2:

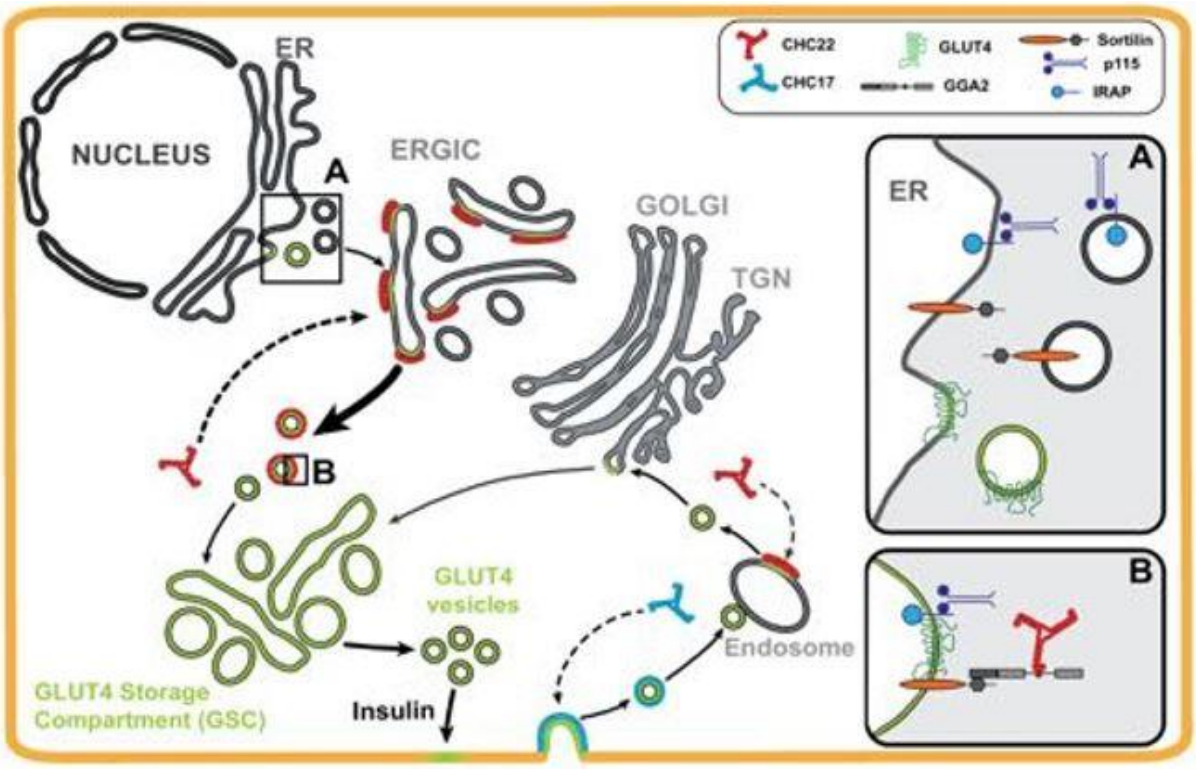


Figure S2, adapted from Brodsky et al. (n.d.) *Summary of the proposed biosynthetic trafficking pathway leading to initial GSC formation in humans.*

Newly synthesized GLUT4, IRAP (insulin-responsive aminopeptidase) and sortilin traffic from the endoplasmic reticulum to the ER-to-Golgi Intermediate Compartment (ERGIC). At the ERGIC, a complex forms between sortilin, IRAP-p115 and GLUT4 that recruits the adaptor protein GGA2. It is thought that CHC22 is recruited as a membrane coat that combines these players for GSC formation and stabilizes their interaction for forming a specialised compartment. The initial sorting to the GSC is thought to be triggered by the meeting of the p115-IRAP complex with GLUT4 and sortilin. GLUT4 storage vesicles then leave the GSC by translocation to the plasma membrane in response to insulin. After plasma membrane translocation, GLUT4 undergoes CHC17 clathrin dependent endocytosis, and is recycled back to the GSC via the retrograde pathway from late endosomes to the TGN, which could involve CHC22, CHC17 or both clathrins.