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1 Introduction

The incidence of type 2 diabetes and diet-induced obesity is increasing rapidly and reaching epidemic proportions throughout the world populations. In 2011, the global prevalence of diabetes was 366 million, an increase of 82 million from the previous year. Projections for the year 2030 estimate that the prevalence will be about 552 million (Whitling, 2011). Although the cause of type 2 diabetes remains unclear, it is known that insulin resistance is closely related to the development of the disease. Defective glucose uptake in skeletal muscle and adipose tissues plays a major role in causing insulin resistance and glucose intolerance symptoms associated with type 2 diabetes. In type 2 diabetes, there is a failure to increase glucose disposal into peripheral tissues in response to insulin, leading to chronically elevated levels of glucose (hyperglycemia) in the circulation followed by a compensatory rise in insulin (hyperinsulinemia). The elevated glucose and insulin levels, in turn exacerbate insulin resistance, contributing significantly to the pathogenesis of the disease (Huang et al., 2002). In humans, skeletal muscle accounts for nearly 40% of the body’s mass and serves as the main tissue involved in glucose uptake during insulin stimulation; adipose tissue accounts for only 5-20% of glucose disposal. Insulin-induced glucose uptake into muscle and adipose tissue involves a series of intracellular signaling cascades, culminating in glucose disposal and metabolism.

Insulin binds to the extracellular β subunits of its transmembrane receptor protein (IR) and initiates the signaling cascade through the recruitment and activation of a series of kinases culminating in glucose clearance. The activated IR kinase phosphorylates the most insulin responsive substrate proteins, IRS1 and IRS2. Numerous studies using transgenic knockout mouse models have shown that the deletion of IRS-1 resulted in growth retardation and insulin resistance, but not diabetes. However, when IRS-2 was deleted, the knockout mice displayed peripheral insulin resistance resulting in hyperglycemia, diabetes, and premature death. As such, these results demonstrated a crucial role for IRS-2 in insulin sensitivity of skeletal muscle. Insulin-receptor signaling involves the mitogen-activated protein kinase (MAPK) pathway mediating the mitogenic effects of insulin, and the phosphatidylinositol 3-kinase (PI3K) pathway mediating the metabolic effects of insulin. The MAPK pathway can exert its mitogenic effect without any GLUT4 translocation. However, recently there are indications to suggest otherwise. PI3K pathway leads to the activation of a cascade of PI3K-dependent kinases, the most prominent of which is protein kinase B (Akt). Akt has been implicated in the insulin stimulation of GLUT4 translocation. Akt exists in three isoforms, Akt1, Akt2 and Akt3. Akt2 is of particular interest in insulin signaling as it plays a key role in insulin-stimulated glucose uptake. This has been reported by isoform-targeted knockdown of Akt2. Moreover, Akt2-null mice demonstrated impaired glucose tolerance and reduced insulin-stimulated glucose uptake, while Akt1-null mice had normal glucose regulation. Most importantly, reduced Akt2 expression and impaired insulin-stimulated Akt2 activation were reported for diabetic and insulin-resistant human skeletal muscle (McCurdy & Caretee, 2005). In summary, both the MAPK and PI3K pathways are important for the action of insulin and ultimately affect glucose clearance to maintain homeostasis.

Processes downstream to the above pathways are mediated primarily by two facilitative glucose transporters, 1 and 4 (GLUT1 and GLUT4). Both GLUT1 and GLUT4 are mainly expressed in insulin responsive peripheral tissues, the skeletal muscle, cardiac muscle, and adipose tissues. GLUT1 is responsible for glucose uptake in the basal state whereas, GLUT4 is regulated by insulin. Insulin-mediated activation of the insulin receptor and/or its downstream molecules, ultimately effect the expression and translocation of GLUT4 (Katz et al., 1996; Watson et al., 2004; Shulman, 2000; Watson & Pessin, 2001; Shepherd & Kahn, 1999; Wallberg-Henniksson & Zierath, 2001). Under normal conditions, in response
to insulin, GLUT4 translocates from the cytoplasm to the cell membrane and mediates the transport of glucose into the cell (Halaby et al., 2008; Dhar et al., 2006; Larance et al., 2008; Baus et al., 2008). In normal cells, GLUT4 is distributed among the plasma membrane and various intracellular membrane compartments, referred to as the glucose storage vesicles (GSVs). Insulin stimulation significantly increases the exocytic rate of glucose from the GSVs while inhibiting GLUT4 endocytosis (Bryant et al., 2002; Huang et al., 2002; Lopez-Marque et al., 2010; Muretta et al., 2008). This action strongly promotes GLUT4 incorporation into the cell surface.

Insulin resistant conditions arise when insufficient insulin is synthesized by the β cells in the pancreas, or due to an impaired insulin action or signaling. Insulin action as described above, is complex and can be affected either due to the upstream or downstream elements including the MAPK and PI3K pathway effect or proteins or due to alterations in the synthesis and sequestration of GLUT4 itself. Under these conditions there is a shift away from GLUT4 as the main player in glucose clearance to GLUT1 as the responsible transporter (Brozinick et al., 2007). Studies of this process of GLUT4 translocation have been carried out using two approaches. The first is an “outside–inside” approach that focuses on the PI3K pathway, in skeletal muscle and fat cells with the view to identify downstream targets that directly control GLUT4 translocation. Conversely, the second approach is an “inside–outside” which is used to map the intracellular transport itinerary of GLUT4 with the aim of identifying insulin-regulated steps (Foster & Klip, 2000) which may or may not be regulated by MAPK pathway. Moreover, several researchers have established that glucose consumption in skeletal muscle decreases with type 2 diabetes. This reduced glucose consumption can be the result of a combination of impaired activities of PI3K and MAPK, transduction of insulin signals, such as insulin receptor substrate (IRS) phosphorylation; PI3K activity; synthesis, exocytosis or endocytosis of the insulin-responsive GLUT4; and/or other insulin-independent mechanisms (Hayata, et al., 2008).

The etiology of human obesity and type 2 diabetes is complex with both genetic predisposition and environmental factors playing a role. These polygenic diseases involve a complex interplay of multiple genes and their interactions with each other, hence can be relatively more easily studied in murine models and then translated into human homologs and phenotypes (Milagro et al., 2011). Heterozygous Atp10c mice represent a novel mouse model of diet-induced obesity and type 2 diabetes. These mice present with the disease states of insulin resistance associated with diet-induced obesity, along with hyperlipidemia and hyperinsulinemia (Dhar et al., 2002, 2004). Phenotypic analysis of these mice supports that Atp10c gene is a strong candidate gene for diet-induced obesity and type 2 diabetes (Dhar et al., 2004). Atp10c is a putative phospholipid translocase or “flippase,” which encodes for a type 4 P-type ATPase. P-type ATPases in multicellular organisms show distinct tissue distribution and specific roles in development. In addition, P-type ATPases reside in multiple, often very specific, cellular compartments, from the plasma membrane to specialized secretory vesicles suggesting high spatial regulation of P-type ATPase function. As such, dysfunctional P-type ATPases can potentially influence the spatial and/or temporal availability of phospholipids, and thus the distribution and activity of proteins (Hua et al., 2002). The type 4 P-type ATPases assumed role involves the translocation of phospholipid molecules across biological membranes rather than cations (Paulusma & Elferink, 2005; Zhou & Graham, 2009). The highly organized and maintained distribution of phospholipids is crucial for many physiological processes including signal transduction, cell morphology, cell movement, activity of membrane proteins, and vesicle biogenesis. One such ATPase is Atp10c which maps to the p-locus on mouse chromosome 7, to a region of a quantitative trait locus associated with body weight, body fat and diabetic phenotypes (Dhar et al., 2000). Although flippases, like Atp10c, have been studied for many years, their exact character and
function remain unclear. It has been shown however that deficiencies in these proteins cause defects in lipid metabolism and have been implicated in the disease states of diet-induced obesity, type 2 diabetes, and non-alcoholic fatty liver disease (NAFLD) (Dhar et al., 2004). Not much is known about the role of Atp10c/ATP10C in regulating insulin resistance in skeletal muscle, if any, and its possible molecular and cellular targets have not been investigated.

In view of the above data, we hypothesized that the type 4 P-type ATPase, Atp10c/ATP10C has an important role in glucose metabolism. Since ATP10C is a putative transmembrane protein it should localize in or around the plasma membrane and as such might exert its effect at the plasma membrane and/or downstream via multiple signaling processes. For our investigations, we used three in vitro cell culture models, HEK293T, mouse C2C12 and modified rat L6 cells, all of which allow the manipulation of target genes, helping investigators in recognizing biological functions without having to deal with the complexities involving whole animals or human subjects. HEK293 cells have been grown in tissue culture for many years and are very widely used as they are relatively easy to grow and transfect. An important variant of this cell line is the HEK293T cell line that contains the SV40 large T antigen, which allows for replication of transfected plasmids containing the SV40 origin of replication. This benefit allows for amplification of transfected plasmids and extended expression of the desired gene products (Baldi et al., 2005; Durocher et al., 2002). Two skeletal muscle models, murine C2C12 and rat L6 cells overexpressing a GLUT4-myc tag are widely used to study insulin-induced glucose transport systems. These cells are available as myoblasts and under permissive conditions can differentiate into myotubes mimicking the in vivo status. To prove our hypothesis, we first assessed the subcellular localization and the size of ATP10C protein in HEK293T cells. Next we altered the endogenous level of Atp10c expression by siRNA technology in mouse C2C12 and rat L6-GLUT4-myc cells, and tested whether the key protein factors of the MAPK and PI3K pathways as well as GLUT4 were potential targets of ATP10C under acute insulin stimulated conditions. Finally, we measured GLUT4 translocation using the GLUT4-myc exofacial tag in L6 cells.

2 Materials and Methods

HEK293T, a commercially-available cell line, was purchased from Open Biosystems (Thermo Fisher Scientific, Waltham, MA). Mouse skeletal muscle cell line C2C12, a commercially-available cell line, was kindly provided by Dr. Seung Baek, College of Veterinary Medicine, the University of Tennessee, Knoxville, TN, USA. Rat L6 muscle cells stably expressing GLUT4 with an exofacial myc-epitope (L6-G4myc) were commercially obtained from Dr. Amira Klip (The Hospital for Sick Children, Toronto, Canada). Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 mg/L glucose and 4.5 mM/L L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin, heat-inactivated fetal bovine serum (FBS), bovine calf serum (BCS), radioimmunoprecipitation assay (RIPA) buffer, and Hank’s balanced salt solution (HBSS) were purchased from Thermo Fisher Scientific (Waltham, MA). DMEM with 1% antibiotics and 10% FBS is furthermore referred to as the HEK293T complete growth media. DMEM with 1% antibiotics and 10% BCS is furthermore referred to as the C2C12 complete growth media. Horse serum, protease inhibitor (pi) cocktail in DMSO solution, and human insulin solution (10 mg/mL in HEPES, pH 8.2) were from Sigma Aldrich (St. Louis, MO). α-Minimum essential medium (α-MEM) containing 5.5 mM glucose was obtained from Gibco (Grand Island, NY). α-MEM with 10% FBS is furthermore referred to as the L6 complete growth media. A Bicinchoninic Acid Kit (BCA) and an En-
hanced Chemiluminescence (ECL) Western Blotting Detection Kit were purchased from Pierce Biotech Inc. (Rockford, IL) and used in protein experiments. HiPerfect transfection reagent and Atp10c-specific siRNA constructs were obtained from Qiagen (Valencia, CA). The ATP10C-GFP plasmid and TurboFectin along with anti-GFP monoclonal antibody were purchased from OriGene Technologies, Inc. (Rockville, MD). The GLUT4-expression plasmid containing GLUT4 open reading frame fused to a red fluorescent protein (RFP) tag at the C-terminal (GLUT4-RFP) was obtained from Dr. Jeffrey Pessin (University of Iowa, Iowa City, IA). Primary antibodies (β-tubulin, Calnexin, Rab5, p38, phospho-p38, JNK, phospho-JNK, ERK1/2, phospho-ERK1/2, phospho-Akt2 (Ser473) and phospho-Akt2 (Ser/Thr) referred hereafter as AS160) as well as the secondary antibody, HRP-conjugated anti-rabbit IgG, were obtained from Cell Signaling Technology (Danvers, MA). PY20 and its specific secondary antibody (goat anti-mouse) were purchased from BD BioSciences, Sparks, MD). Caveolin-1 was used as an immunoblot control and was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Primary antibodies for PI3K, Akt2, myc, GLUT4 and the secondary antibody, HRP-conjugated anti-goat IgG, were also obtained from Santa Cruz Biotechnologies. All immunofluorescence materials (protein blocks [normal rabbit], [normal goat], negative control [normal rabbit], [normal goat] and antibody diluent) were purchased from BioGenex (San Ramon, CA). Secondary antibodies specific for immunofluorescence application, Alexa Fluor 568 donkey anti-rabbit and Alexa Fluor 568 donkey anti-goat as well as Prolong Gold Anti-fade reagent and wheat-germ-agglutinin (WGA) were purchased from Invitrogen (Carlsbad, CA). All immunocytochemistry materials (Odyssey Blocking Buffer and infrared-conjugated secondary antibody) were purchased from LI-COR Biosciences (Lincoln NE). Syto60 was purchased from Molecular Probes (Eugene, OR). Glass bottom culture dishes used for immunofluorescence experiments were purchased from MatTek Corporation (Ashland, MA).

2.1 Cell Culture

HEK293T cells were cultured as described elsewhere (Kummer & Klocker, 2012; Baldi et al., 2005; Durocher et al., 2002). Roughly $1.0 \times 10^3$ cells were seeded in either a 60-mm dish or a T-75 culture flask. They were then maintained at 37°C and 5% CO2 in complete growth media. At 70 – 80% confluency, cells were harvested and used in subsequent experiments.

C2C12 myoblasts were cultured as described elsewhere (Hurst et al., 2012). Roughly $2.0 \times 10^5$ cells were seeded in a 60-mm dish or a T-75 culture flask. They were maintained at 37°C and 5% CO2 in complete growth media. Cells at 70% confluency were differentiated in the presence of 2% horse serum-enriched media for 3–5 days. Completely differentiated myotubes (days 3–5) were subjected to various treatments described in the relevant sections and harvested for use in subsequent experiments. L6-G4myc cells were cultured as previously described (McCarthy et al., 2006).

L6-G4 myoblasts were maintained at 37°C and 5% CO2 in complete growth media. Cells at 70% confluency were differentiated into multinucleated myotubes with the addition of 2% FBS. Completely differentiated myotubes (days 4-6) were subjected to various treatments described in the relevant sections and harvested for use in subsequent experiments. Cells were serum starved for 30 min before all experiments. Subsequently, cells were left in either the basal state or were acutely stimulated with 100 nM insulin.

2.2 Transfection of ATP10C plasmid

A custom-generated expression plasmid, containing a fusion of ATP10C open reading frame to a C-terminal green fluorescent protein (GFP) tag (ATP10C-GFP) and TurboFectin, a transfection reagent
optimized for nucleic acid delivery into eukaryotic cells were obtained from OriGene Technologies (Rockville, MD). Transfection of HEK293T cells with ATP10C-GFP and GLUT4-RFP was as per the standard protocol provided by the manufacturer. Briefly, two days before transfection, HEK293T cells were plated at a density of 1.0 x 10^5 cells/60 mm dish in complete growth medium and grown to obtain 50-70% confluency. To form the transfection complex, 100 uL of serum free medium plus the appropriate amount of TurboFectin (2-6 uL per 1 ug DNA) were mixed by gentle pipetting and, this solution was incubated at room temperature for 5 min. The appropriate plasmid DNA (1-3 ug per well) was then added to the TurboFectin-containing media prepared above. The two solutions were gently mixed and allowed to incubate at room temperature for 30 min. During this incubation period, the cells were removed from the incubator and the spent medium replaced with 2 mL of fresh complete medium per dish. Upon complex formation, the solution was added to the seeded cells drop-wise and distributed evenly throughout the dish. Cells were cultured for 24 and 48 hours before undergoing immunofluorescence analysis.

2.3 siRNA Transfection

Three different siRNA oligonucleotides against Atp10c were commercially obtained (Qiagen) and transfection protocols were optimized in each cell line before use. Only one siRNA, SI00906220 resulting in roughly 88% and 98% knockdown in C2C12 and L6-G4myc myotubes, respectively was selected for all further experiments. Transient transfection of C2C12 and L6-G4myc myotubes using HiPerfect and quantitation of Atp10c knockdown by qPCR were carried out according to methods described earlier (Hurst et al., 2012). C2C12 and L6-G4myc myotubes demonstrating efficient Atp10c knockdown were designated as C210c/- and L6-G4myc10c/-, respectively. Mock transfected (i.e. transfected with HiPerfect only) C2C12 and L6-G4myc myotubes designated as C2wt and L6-G4mycwt, respectively were used as corresponding controls.

2.4 Preparation of Cellular Extracts and Immunoblotting

Total cell lysates were isolated using RIPA buffer according to standard methods (Roshwalb et al., 2010; Peterich et al., 2009; Hance et al., 2008). Briefly, cells were washed twice with 1X HBSS and lysed in RIPA buffer containing pi cocktail at 4°C for 30 min. Lysates were centrifuged at 16,000g for 10 min at 4°C and protein was recovered in the supernatant. Protein estimation was performed using the BCA kit (Pierce Biotech) with bovine serum albumin (BSA) as the standard, according to the manufacturer’s instructions. Immunoblot analysis was carried out according to procedures described earlier (Roshwalb et al., 2010; Peterich et al., 2009; Hance et al., 2008). Equal concentrations (25–100 µg) of proteins were resolved on 10% SDS-PAGE, using 5X Laemmli sample buffer containing Tris-HCL (375 mM, pH 6.8), glycerol (48%), SDS (6%), β-mercaptoethanol (6%), and bromophenol (0.03%). Cell lysates were denatured by heating before being applied to SDS-PAGE gel. Non-denaturing conditions, i.e., proteins were not denatured by heating when they were used in the analysis of GLUT4 protein. After electrophoresis, proteins were transferred to nitrocellulose membranes, blocked for 1 h in blocking solution (1-5% BSA in 0.05% Tween-20 in western blot washing buffer (TBST), and incubated with specific primary antibodies overnight at 4°C. Primary antibodies were detected with HRP-conjugated secondary antibodies, and antibody-protein complexes were visualized using ECL (Pierce Biotech). Results are expressed as the ratio of target protein expression to that of an internal loading control, caveolin-1.
2.5 Immunofluorescence and Immunocytochemistry

For immunofluorescence, 2.0 × 10^4 cells were seeded onto glass bottom culture dishes (Mat Tek) and subjected to the appropriate treatments as described in the relevant sections. Immunofluorescence assays were carried out according to standard methods as described by others (Bisht & Dey, 2008; Bisht et al., 2007). Briefly, cells were fixed with 1% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.3 for 10 min at room temperature. Cells were washed in 1X HBSS, permeabilized by incubating with 0.01% Tween-20/HBSS for 10 min, and then washed again with 1X HBSS. After the last wash, cells were blocked using blocking buffer (1% BSA, 2% normal serum, 0.1% Tween-20 in HBSS) for 30 min. Blocking solution contained normal goat or normal rabbit serum, the animal in which the primary antibody was generated. Once blocking was complete, the cells were incubated with specific primary antibodies overnight at 4°C. Bound antibody was visualized by incubating for 1 h with secondary antibody labeled with Alexa Fluor 568 [Tetramethyl Rhodamine Iso-Thiocyanate (TRITC)]. To visualize the nucleus, cells were exposed for 5 min at room temperature to 300 nM DAPI in HBSS. DAPI was prepared and diluted based on manufacturer’s instructions (Invitrogen). After washing, cells were mounted using ProLong Gold Antifade reagent (Invitrogen). Dishes were sealed and allowed to dry overnight before imaging. Appropriate positive and negative controls were prepared and imaged alongside the samples to correct for any background fluorescence for quantitative analysis. Images were captured using confocal microscopy (Leica SP2 confocal laser-scanning microscope, Leica Microsystems, Wetzlar, Germany) with a 63× oil objective lens (NA 1.32) and an automated stage.

For immunocytochemistry, GLUT4myc myotube labeling was performed as previously described (McCarthy et al., 2006). Briefly, myotubes were fixed with 2% paraformaldehyde/HBSS. After fixation, cells were left un-permeabilized and blocked in Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE). The samples were then incubated in primary myc antibody (Santa Cruz Biotechnology) overnight, washed and incubated with an infrared-conjugated secondary antibody (LI-COR) for 1 h. Images were then taken and quantified for further analysis.

2.6 Immunofluorescence and Immunocytochemistry Quantitation

Immunofluorescence images were analyzed using NIS-Elements software (AR v. 3.1) (Nikon Instruments). For each sample, image stacks (z-series) were acquired for 3 fields with a step size of 0.5 µm. The percentage of co-localization was calculated using Pearson’s correlation with a perfect correlation of 1 as compared to the internal control.

For immunocytochemistry, images were collected and quantified with the Odyssey system as previously described (McCarthy et al., 2006). Immunofluorescent intensity was normalized to intensity from Syto60, a fluorescent nucleic acid stain (Molecular Probes).

2.7 Densitometry Analysis

Relative densitometry analyses of the immunoblots were determined using Image J (http://rsb.info.nih.gov/ij/index.html) analysis software. By giving an arbitrary value of 1.0 to the respective control sample (caveolin-1) of each experiment, a ratio of relative density was calculated for each protein of interest.
2.8 Statistical Analysis

The data are expressed as mean ± SE. For comparison of two groups, \( P \) values were calculated using the two-tailed paired Student t test. In all cases \( P < 0.05 \) was considered statistically significant and \( P < 0.10 \) was indicative of a trend.

3 Results and Discussion

HEK293T cells are frequently used to exogenously express target proteins for analyses of protein-protein interactions, and their subcellular localization. Transfection protocols using commercially-available transfection reagents are routinely validated by the biotech companies in HEK293T cells and are thus used extensively by many research laboratories for these experiments (Kummer & Klocker, 2012; Baldi et al., 2005; Durocher et al., 2002). Since, numerous attempts to transfect ATP10C-GFP expression plasmid into C2C12 and L6-G4myc cells failed (data not shown), we used HEK293T cells to detect the subcellular localization and assess the size of the ATP10C protein. ATP10C-GFP was transfected into HEK293T cells using TurboFectin as the transfection reagent (Origene), and total cellular lysates were collected at 24 and 48 hours post-transfection. Without a good antibody available for ATP10C, an anti-GFP antibody was used to detect the ATP10C-GFP fusion protein (Figure 1). We have previously demonstrated that \( Atp10c \) cDNA is 4.5kb (Dhar et al., 2000), and since the molecular weight of GFP protein is 30kD, western blot analysis confirmed that the molecular weight of ATP10C protein is 165kD.

![ATP10C-GFP expressed in HEK293T cells. ATP10C expression was examined by western immunoblotting using HEK293T-ATP10C-GFP total proteins collected in RIPA buffer at 24 h and 48 h post-transfection. The 30kD molecular weight corresponds to the GFP fragment of the plasmid, while the 165 kD is the expected size of ATP10C protein.](image)

Since, immunoblot analysis demonstrated efficient transfection of ATP10C-GFP plasmid in HEK293T cells, using immunofluorescence and confocal microscopy, we were able to show that the ATP10C fusion protein localized to the plasma membrane of HEK293T cells (Figure 2). These data are consistent with similar studies previously reported for three other members of the type 4 P-type ATPase family, ATP8B1, ATP8B2 and ATP8B4. Additionally, ATP8A1 displayed a peri-nuclear staining and co-localized with the Golgi-marker form immunofluorescence cyclodeaminase indicating expression in the Golgi complex. When expressed at very high levels, these type 4 ATPases also showed nuclear-envelope and reticular staining that coincided with the endoplasmic reticulum (ER) marker protein, disulfide isomerase (Bryde et al., 2010).
Figure 2: Localization of ATP10C-GFP in HEK293T cells. ATP10C localization was determined by immunofluorescence followed by confocal microscopy using HEK293T cells. HEK293T cells were transfected with an ATP10C-GFP expression plasmid (indicated with white arrows). A control-GFP plasmid (pMax) was transfected into HEK293T cells and served as a positive control. All samples were fixed at a designated time point (48 h). Confocal images were captured on a Leica SP2 laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) with a 63x oil objective lens (NA 1.32). For each sample, image stacks (z-series) were acquired for 3 fields with a step size of 0.5 μm. Images courtesy of J. Dunlap, Advanced Microscopy and Imaging Center, UTK.

Since, we were able to show the subcellular localization of ATP10C using a GFP reporter gene, we next sought to co-localize ATP10C with known subcellular markers using antibodies against subcellular proteins (Cell Signaling). Since, ATP10C is tagged to GFP as the reporter gene; it is visualized as a green fluorescent molecule with absorption at 488nm. For co-localization studies, all subcellular marker proteins are visualized using TRITC labeled secondary antibodies with an absorption at 647nm. Hence, their expression is assessed by the red fluorescence. Thus, if ATP10C co-localizes with any of these proteins the region of overlap should appear as a merge of green and red resulting in yellow fluorescence. Currently, none of the antibodies tested co-localized with ATP10C. Representative data suggesting that ATP10C does not localize to the microtubules (β-tubulin), the ER (Calnexin), or the endosome (Rab5) is shown (Figure 3A). Interestingly, when ATP10C-GFP was co-transfected with a GLUT4-RFP expression plasmid, some overlap in their localization was observed (Figure 3B). By measuring a particular region of interest (ROI) within a given field, and using Pearson’s correlation of the two proteins there was 81% co-localization of ATP10C and GLUT4 in HEK293T cells. This value was obtained by comparing the image with significant overlap with an image that had none (0.1% co-localization).

Since, HEK293T cells are non-responsive to insulin they do not contain the machinery necessary to form GSVs and hence do not express the glucose transporter proteins, thus, cannot be used to assess insulin signaling and glucose metabolism when Atp10c is overexpressed or silenced. Hence, future experiments to co-localize ATP10C and GLUT4 and assess changes in basal and insulin stimulated states in insulin responsive cells should be initiated. Future experiments to co-localize ATP10C with any of the known Golgi, VAMP and SNARE markers should also be conducted.
Co-localization of ATP10C-GFP with known protein markers. ATP10C localization was determined by immunofluorescence microscopy using HEK293T cells transfected with ATP10C-GFP (green). For co-localization, transfected cells were immunostained with primary polyclonal antibodies and detected using TRITC-conjugated secondary antibodies (red). Confocal images were captured on a Leica SP2 laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) with a 63x oil objective lens (NA 1.32). For each sample, image stacks (z-series) were acquired for 3 fields with a step size of 0.5 μm. Scale bars are 15 μm. Images courtesy of J. Dunlap, Advanced Microscopy and Imaging Center, UTK.

One of the limitations of global gene-targeting and whole-animal approach is that adaptations over time might occur, possibly producing secondary phenotypes that are not directly linked to the mutation. In this case, the role of Atp10c could be shown either by generating knockout mice or using specific inhibitors against ATP10C. Since there are no known inhibitors of ATP10C, and to avoid whole-animal complexities in transgenic models, we modulated Atp10c expression in vitro in C2C12 and L6-G4myc cells. These two cell lines grow as myoblasts and under permissive conditions undergo differentiation to form insulin-responsive myotubes (Nedachi & Kanzaki, 2006; Nedachi et al., 2008; Niu et al., 2010; Kobayashi et al., 2010; Lui et al., 2010). Hence, to investigate the biological role, if any, of ATP10C in insulin signaling and glucose metabolism, Atp10c mRNA was transiently knocked down in both C2C12 (Hurst et al., 2012) and L6-G4myc cells (Figure 4) by Atp10c-specific siRNA (Qiagen). Since these transfections are transient, Atp10c mRNA knockdown was confirmed in each and every experiment by qPCR.
Figure 3B: Co-localization of ATP10C-GFP with GLUT4-RFP. ATP10C localization was determined by immunofluorescence microscopy using HEK293T cells co-transfected with both plasmids, ATP10C-GFP and GLUT4-RFP. Confocal images were captured on a Leica SP2 laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) with a 63x oil objective lens (NA 1.32). For each sample, image stacks (z-series) were acquired for 3 fields with a step size of 0.5 μm. Overlap is indicated with white arrows showing yellow areas, which is the merge of green and red (TRITC) fluorescence. Scale bar in co-transfection cells is 15 μm. Images courtesy of J. Dunlap, Advanced Microscopy and Imaging Center, UTK.

Figure 4: Optimization of siRNA in L6-G4myc cells. L6-G4myc myoblasts (MB) and myotubes (MT) were transfected at each concentration of siRNA (SI00906220) (0 and 50 nM), and collected at the above time points (24 and 48 h). Gapdh (housekeeping) and Atp10c (target) mRNA expression was analyzed using qPCR. The percentage of knockdown was calculated at each concentration and time point, and is based on the expression of the mock-transfected (0 nM) samples (*P < 0.7 criteria determined by the manufacturer). Data represents three independent experiments with each sample repeated in triplicate.
The glucose transporter GLUT4 is essential for insulin-stimulated glucose uptake, and is expressed primarily in both adipose and skeletal muscle tissues. Ideally, in response to insulin, intracellular GLUT4 vesicles move near the cell surface, fuse with the plasma membrane, and begin the process of glucose uptake. Defective uptake of glucose is a central feature of type 2 diabetes, and may involve the alteration of the insulin signaling to GLUT4 vesicles, the trafficking of GLUT4 vesicles to the plasma membrane, and/or the docking and fusion of GLUT4 vesicles with the plasma membrane. We have recently demonstrated that transient transfection of C2C12 myotubes with 50nM of *Atp10c*-specific siRNA, SI00906220 for 24h resulted in a 88% knockdown of *Atp10c*mRNA along with a 2.54 fold significant decrease in glucose uptake. Interestingly there was no change in GLUT4 protein expression, suggesting that alterations, if any must be related to GLUT4 translocation and sequestration (Hurst *et al.*, 2012). Hence, we next assessed whether this decrease in glucose uptake is due to any alteration in GLUT4 translocation. L6 cells derived from rat skeletal muscle and modified to stably overexpress GLUT4 were transiently transfected with *Atp10c*-specific siRNA, SI00906220, and a 98% knockdown of *Atp10c*mRNA was observed (Figure 4). Fold stimulation of GLUT4 in insulin stimulated state was measured using the exofacial myc tag attached to GLUT4. Immunocytochemistry experiments, which are cell-population based assays wherein a sheet of cells in a 6-well tissue culture-treated plate is analyzed using a plate reader rather than microscopic imaging of a single cell are used. Figure 5 shows a decrease in insulin-stimulated GLUT4 translocation to the plasma membrane after 48 h post-transfection in L6-G4myc/10c-myotubes. These results suggest that silencing of *Atp10c* is altering normal GLUT4 regulation at the endocytosis stage and potentially, causing a decrease in insulin-stimulated glucose uptake in these cells.

Being a transmembrane protein, ATP10C may play a role in the retention of GLUT4 storage vesicles away from the plasma membrane or may act as a protein regulatory barrier to GLUT4 fusion with the plasma membrane. Therefore, when ATP10Cexpression is altered, the cells are able to release the GLUT4 storage vesicles by inhibiting the level of vesicle retention or inhibiting the level of the "protein barrier" at the plasma membrane. In turn, ATP10C may serve a scaffold role with other proteins at or near the plasma membrane to regulate the distal regulatory events of GLUT4 translocation which involve docking, tethering, and fusion of these vesicles with the plasma membrane. Although GLUT4 exocytosis has been studied extensively, GLUT4 endocytosis and its possible regulation is less scrutinized. Recent studies have begun to shed light on GLUT4 endocytic mechanisms and their metabolic regulation (Ishikira *et al.*, 2010). Endocytosis involves a “ready-set-go” mechanism, where GLUT4 molecules get ‘ready’ by concentrating within a small region of the plasma membrane. Secondly, they get ‘set’ by interacting with specific membrane proteins and/or lipids, resulting in membrane bending and invagination. Finally, the bud is formed occluding GLUT4 from the extracellular milieu and allowing them to ‘go’ into the cell interior to intracellular compartments. It has been reported that the internalization of GLUT4 occurs by clathrin-mediated endocytosis (Huang *et al.*, 2006). Moreover, it has been argued that GSVs are formed from endosomes and that GLUT4 may be retained intracellularly by an interaction between GSVs and retention machinery, or by restricting the access of GSVs to the vesicle tethering/docking/fusion apparatus at the cell surface (Foster & Klip, 2000; Huang *et al.*, 2006). Co-localization of ATP10C and GLUT4 (Figure 3B) and a decreased GLUT4 translocation to the cytoplasm in L6-G4myc/10c-myotubes (Figure 5) suggest that *Atp10c*/ATP10C is involved in any one of these steps in addition to its other effects on the metabolic pathways described below.
GLUT4 translocation in \textit{Atp10c}-silenced L6-G4myc myotubes is decreased 48 h post-transfection. L6-G4myc myotubes were transfected at each concentration of siRNA (SI00906220) (0nM and 50 nM) at the designated time point (48 h). Cells were then stimulated with insulin (100 nM), and an immunocytochemistry assay was performed (*$P<0.05$). Data is reported as the fold stimulation rather than the absolute GLUT4 translocation value. Results are expressed as the ratio of GLUT4myc intensity under basal and stimulated conditions and indicated as arbitrary units (A.U.). Data is normalized with respect to 0nM basal and stimulated controls.

Previous work by our laboratory has demonstrated a direct correlation between \textit{Atp10c}/\textit{ATP10C} and glucose metabolism, at least in part via the MAPK pathway under basal glucose uptake conditions (Hurst et al., 2012). As demonstrated above, it is possible that \textit{Atp10c}/\textit{ATP10C} affects glucose metabolism either by insulin-dependent pathways or directly at the level of GLUT4. Since the process of glucose metabolism is a complex interplay of a variety of signaling pathways and because changes in protein expression have not been studied in \textit{Atp10c}-silenced cells, we sought to identify changes in the key proteins of relevant signaling cascades, the PI3K and the MAPK pathway in the presence of any acute insulin stimulation in C2/10c- and L6-G4myc/10c- myotubes.

In the present study, the effect of \textit{Atp10c}-silencing was considered on native and activated forms of three essential MAPKs; p38, JNK, and ERK1/2, in addition to PI3K, native Akt2, phospho-Akt2 (Ser473), phospho-Akt2 (Ser473/Thr608), and AS160. Additionally, proteins upstream of MAPK and PI3K, IR-β and IRS-2 were examined. Even though caveolin-1 expression can be a characteristic of differentiated cells and therefore is a gene inducible by muscle differentiation processes, we did not see any change in its expression under any of the experimental conditions and hence used it as an internal control for all immunoblot analyses.

Immunoblot analysis indicates significant up-regulation of native p38 ($P = 0.02$) whereas a significant down-regulation of phospho-p38 ($P = 0.01$), JNK ($P = 0.0003$), and phospho-ERK1/2 ($P = 0.015$ and $P = 0.017$, respectively) is observed (Figures 6A–C).
Figure 6A: MAPK proteins, p38 and phospho-38 are significantly altered after Atp10c-silencing and acute insulin stimulation in C2C12 myotubes. C2C12 myoblasts were differentiated into myotubes. Myotubes were transfected at each concentration of siRNA (SI00906220) (0 and 50 nM), stimulated with insulin (100 nM, 30 min) and collected at the designated time point (24 h). Proteins were collected from these samples and subjected to immunoblot analysis. Data shown are representative of multiple independent experiments (n = 2 to 4), all analyzed in triplicate. The expression of p38 and phospho-p38 is denoted as arbitrary units (A.U.) and represented as the fold change normalized to caveolin-1; *P < 0.05.

The exact mechanism by which the MAPKs mediate glucose uptake is debatable. MAPKs, specifically, p38 and ERK1/2 proteins regulate glucose uptake via both insulin-dependent and insulin-independent pathways (Fujishiro et al., 2001; Kumar & Dey, 2002; Wijesekara et al., 2006; Koistinaho & Koistinaho, 2002). Most importantly, there are many reports which show the involvement of the MAPK pathway in glucose uptake without an effect on GLUT4 translocation. Our results show that when myotubes become insulin resistant by Atp10c-silencing, even though there is a significant increase in the expression of native MAPK proteins, there is a significant down-regulation of their active phosphorylated forms, clearly suggesting that both p38 and ERK1/2 are responsive to changes in Atp10c and thus, MAPK pathway is affected. The significant increase of p38 and ERK1/2, under both basal (Hurst et al., 2012) and acute insulin-stimulated conditions suggests that the MAPK proteins are molecular targets of Atp10c and they increase in expression to combat the insulin resistant state however, their corresponding activated forms are unable to do so, which might result in an altered MAPK signaling. The current data does support our hypothesis that Atp10c-silencing affects glucose metabolism via both insulin-independent and insulin-dependent manners, the exact mechanism however cannot be deduced. With the experiments
Figure 6B: A MAPK protein, phospho-ERK1/2, is significantly down-regulated after Atp10c-silencing and acute insulin stimulation in C2C12 myotubes. C2C12 myoblasts were differentiated into myotubes. Myotubes were transfected at each concentration of siRNA (SI00906220) (0 and 50 nM), stimulated with insulin (100 nM, 30 min) and collected at the designated time point (24 h). Proteins were collected from these samples and subjected to immunoblot analysis. Data shown are representative of multiple independent experiments (n = 2 to 4), all analyzed in triplicate. The expression of ERK1/2 and phospho-ERK1/2 is denoted as arbitrary units (A.U.) and represented as the fold change normalized to caveolin-1; *P < 0.05.

demonstrated in this study, it is not possible to assess whether a decreased GLUT4 translocation is correlated with the alterations in the expression/activation of MAPK proteins, or whether these two effects are independent of each other. These experiments are important however, beyond the scope of this study. We attribute the decrease in native JNK with an obvious, but not significant increase in activated JNK to the stress mechanism initiated by the entire process of transfection in addition to insulin stimulation.

Results from PI3K pathway proteins, indicate a significant decrease in PI3K (P = 0.02), Akt2 (P = 0.03), phospho-Akt (Ser473) (P = 0.01), and a significant increase in AS160 (P = 0.008) (Figures 7A-7B). The upstream elements, IR-β (P = 0.02), and IRS-2 (P = 0.004) are all up-regulated significantly under these conditions (Figure 7A).

While not significant, there was also an up-regulation of Actin (P = 0.1), hence, it was not used as an internal control. Interestingly, and of importance to this study, GLUT4 protein expression is significantly up-regulated under these conditions in L6-G4myc/10c- cells (P = 0.01) (Figure 7C). These data further strengthen the notion that alteration in Atp10c/ATP10C expression renders the L6 cells insulin resistant as a result; there is a significant increase in GLUT4 protein expression, which does not improve
Figure 6C: A MAPK protein, JNK, is significantly down-regulated after Atp10c-silencing and acute insulin stimulation in C2C12 myotubes. C2C12 myoblasts were differentiated into myotubes. Myotubes were transfected at each concentration of siRNA (SI00906220) (0 and 50 nM), stimulated with insulin (100 nM, 30 min) and collected at the designated time point (24 h). Proteins were collected from these samples and subjected to immunoblot analysis. Data shown are representative of multiple independent experiments (n = 2 to 4), all analyzed in triplicate. The expression of JNK and phospho-JNK is denoted as arbitrary units (A.U.) and represented as the fold change normalized to caveolin-1; *P < 0.05.

the sensitivity of cells since its translocation is down-regulated. Transfection of ATP10C in L6-GLUT4myc/wt and changes in GLUT4 translocation in basal and insulin-stimulated conditions should be analyzed to confirm this mechanism.

When Atp10c mRNA is knocked down; a significant up-regulation of both the IR-β and IRS-2 is observed, suggesting that their expression increases to combat this state of insulin resistance. IRS-2 plays a key role in transmitting signals to PI3K pathway. The increase however is not able to rescue the decrease in the downstream proteins, PI3K, Akt2, and phospho-Akt2 (Ser473). This is consistent with their usual pattern under conditions of acute insulin resistance (Huang et al., 2002). Activation of Akt2 plays a critical role in the PI3K pathway by further activating AS160 (phospho-Akt Ser473/Thr308). It is important to note that only 10–20% of Akt2 needs to be phosphorylated to achieve full glucose transport activation (Somwar et al., 2002; Somwar et al., 2001). Hence, an unexpected finding in our study is the up-regulation of phospho-Akt Ser473/Thr308 or AS160. While it is known that Akt activation requires phosphorylation at both Ser473 and Thr308. Additionally, Ser473 phosphorylation has been shown to precede the phosphorylation of Thr308 and is in fact a prerequisite for Thr308 phosphorylation (Halaby et al., 2008). It is possible that AS160 is not fully expressed and less activated, and thus, is trying to
compensate for the decrease in Akt2 expression. This can help explain the up-regulation of AS160. Importantly, Akt2, which regulates GLUT4 exocytosis, is not required for insulin regulation of GLUT4 endocytosis (Ishikura et al., 2010).

Similarly, IRS-2 also plays a key role in transmitting signals to MAPK pathway, and it appears that the major effect of altering Atp10c expression is via p38 potentially through insulin dependent and independent processes (Hurst et al., 2012). Finally, we cannot disregard any cross-talk between all these signal transduction processes. It appears as though the cells are trying to augment signaling to compensate for the down-regulation (of PI3K proteins) or over expression (as in MAPK proteins) in these pathways. In the case of the PI3K pathway, Atp10c appears to be halting the expression of PI3K (p85) and phospho-Akt2. As a result, there is an up-regulation both up-stream (IR-β and IRS2) and downstream (AS160). In turn, IRS-2 may be sending these compensatory signals to MAPK proteins (Dhar et al., 2004). As such, the up-regulation of p38 must be in response to shift the glucose uptake from GLUT4 to GLUT1, since; p38 has been directly implicated in the regulation of these glucose transporters. As p38
Figure 7B: PI3K proteins, Akt2, phospho-Akt, and AS160 are significantly altered after Atp10c-silencing and acute insulin stimulation in L6-G4myc myotubes. L6-G4myc myoblasts were differentiated into myotubes. Myotubes were transfected at each concentration of siRNA (SI00906220) (0 and 50 nM), stimulated with insulin (100 nM, 30 min) and collected at designated time point (48 h). Proteins were collected from these samples and subjected to immunoblot analysis. Data shown are representative of multiple independent experiments (n = 2 to 4), all analyzed in triplicate (*P < 0.05). The expression of Akt2, phospho-Akt, and AS160 is denoted as arbitrary units (A.U.) and represented as the fold change normalized to caveolin-1.

up-regulation resulted in an increase in GLUT1 and an assumed decrease in GLUT4 (Hurst et al., 2012), it is highly likely that p38 is central in the regulation of glucose metabolism. This is supported by data from other laboratories as well as ours (Bazuine et al., 2005; Koistinaho & Koistinaho, 2002; Wang et al., 2008; Hurst et al., 2012).

4 Conclusion

Atp10c/ATP10C is a putative phospholipid translocase that plays a role in the maintenance of the phospholipid asymmetry and fluidity of the plasma membrane. As such, loss of Atp10c/ATP10C function upsets the normal membrane environment and perturbs glucose and lipid metabolism. To understand the role of type 4 P-type ATPases, it is important not only to study the cellular consequence when they are non-functional, but also to elucidate the specific conditions required for their activation as well as their
Figure 7C: GLUT4 is significantly up-regulated after Atp10c-silencing and acute insulin stimulation in L6-G4myc myotubes. L6-G4myc myoblasts were differentiated into myotubes. Myotubes were transfected at each concentration of siRNA (SI00906220) (0 and 50 nM), stimulated with insulin (100 nM, 30 min) and collected at designated time point (48 h). Proteins were collected from these samples and subjected to immunoblot analysis. Data shown are representative of multiple independent experiments (n = 2 to 4), all analyzed in triplicate (*P < 0.05). The expressions of Actin and GLUT4 are denoted as arbitrary units (A.U.) and represented as the fold change normalized to caveolin-1.

(subcellular) localization (Daleke, 2007). In the present study, we used metabolic pathways of insulin signaling and glucose uptake to identify its putative molecular and cellular targets which will help us delineate the biological role of ATP10C in glucose homeostasis. Our results demonstrated that ATP10C, a 165 kD protein, localizes in or around the plasma membrane and co-localizes with GLUT4 in HEK293T cells. Based on these results, Figure 8 represents putative Atp10c/ATP10C mechanisms of action and possible protein/pathway targets. Results indicate that alteration of ATP10C expression potentially affects the expression of the upstream and downstream elements of insulin signaling. ATP10C may function by affecting the expression of the key proteins in the PI3K pathway which can subsequently influence glucose uptake via GLUT4 translocation (i), or directly by its action on the GLUT4 protein (ii). Additionally, it may exert its affect via the MAPK pathway in insulin dependent and/or independent manner (iii). Finally, ATP10C may function directly either/or on the endocytosis and exocytosis of GLUT4. ATP10C can maintain lipid bilayer asymmetry and cell homeostasis (iv), aid in GSV formation, retain the GSVs inside the cell (v), or stimulate the endocytosis of GLUT4 back inside the cell (vi). Future experiments to analyze the mechanisms of each of these actions should be initiated.
Figure 8: Disruptions in insulin-stimulated and un-stimulated glucose uptake when Atp10c/ATP10C is silenced and/or missing completely. Possible effects include the expression of the key proteins in the PI3K pathway (i), the expression of the GLUT4 protein (ii), or the expression of MAPK pathway proteins (iii). Furthermore, the maintenance of the lipid bilayer asymmetry and cell homeostasis (iv), the retention of the GSVs inside the cell (v), and/or the stimulation the endocytosis of GLUT4 back inside the cell (vi) may be affected by this silencing.

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