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The Zinc Transporter, *Slc39a7 (Zip7)* Is Implicated in Glycaemic Control in Skeletal Muscle Cells

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Abstract

Dysfunctional zinc signaling is implicated in disease processes including cardiovascular disease, Alzheimer’s disease and diabetes. Of the twenty-four mammalian zinc transporters, Zip7 has been identified as an important mediator of the ‘zinc wave’ and in cellular signaling. Utilising siRNA targeting Zip7 mRNA we have identified that Zip7 regulates glucose metabolism in skeletal muscle cells. An siRNA targeting Zip7 mRNA down regulated Zip7 mRNA 4.6-fold (p = 0.0006) when compared to a scramble control. This was concomitant with a reduction in the expression of genes involved in glucose metabolism including Akl, Dlst, Galm, Gbe1, Idh3g, Pck2, Pgam2, Pgm2, Phkb, Pygm, Tpi1, Gusb and Glut4. Glut4 protein expression was also reduced and insulin-stimulated glycogen synthesis was decreased. This was associated with a reduction in the mRNA expression of *Insr*, *Irs1* and *Irs2*, and the phosphorylation of Akt. These studies provide a novel role for Zip7 in glucose metabolism in skeletal muscle and highlight the importance of this transporter in contributing to glycaemic control in this tissue.

Introduction

Cellular zinc storage, release and distribution are controlled by a family of zinc transporters and metallothioneins. In mammals two families of zinc transporters exist: the zinc efflux (*Slc30/ZnT*) and the zinc influx (*Slc39/ZIP*) proteins [1]. ZnT proteins transport zinc out of the cell or into subcellular compartments in the presence of high cytoplasmic zinc. In contrast, ZIP proteins transport zinc into the cell or out of subcellular compartments when cytosolic zinc is low or depleted [2].

There is increasing interest in the importance of zinc transporters in diseases associated with dysfunctional cellular signaling. In particular, a significant role for these transporters in maintaining essential glucose and lipid metabolism has been identified. For example, in myocytes isolated from the femoral muscle of ZnT7 knockout mice, a reduction in insulin signaling pathway activity was observed [3]. The ZnT7 null mice were susceptible to diet-induced glucose intolerance and insulin resistance and this was associated with a decrease in the expression of the insulin receptor, insulin receptor substrate 2 and Akt1 [3]. ZnT3, ZnT5 and ZnT8 gene expression are differentially regulated by glucose in INS-IE cells, and streptozotocin-treated ZnT3 null mice have decreased insulin gene expression and insulin secretion that resulted in hyperglycemia [4]. Moreover, ZnT3 plays a critical role in the synthesis and secretion of insulin and therefore represents a pharmacological target for treating disorders of insulin secretion including diabetes [5].

Zinc mediates its effects through two mechanisms; early zinc signaling (EZW) and late zinc signaling (LZW) [6]. LZW occurs several hours after an extracellular signaling event and depends on changes in the expression of zinc-related molecules such as zinc transporters and metallothioneins [6,7]. In contrast, EZW occurs minutes after an extracellular stimulus and does not involve transcriptional-dependent changes [6,7]. Zinc signaling mechanisms are involved in eliciting an increase in intracellular zinc concentrations—the ‘zinc wave’ phenomenon [8]. Thus, in this situation zinc acts as a second messenger that activates pathways associated with cellular signaling. In fact, zinc has been categorized as an insulin-mimetic with several groups examining the role of its mimetic activity on glucose [9–13] and lipid [13,14] metabolism. In this context ZIP7 is predominately localized to the Golgi apparatus [15], the ER [16], or both [17] and has been implicated in breast cancer progression [8,17,18]. Studies in tamoxifen-resistant MCF-7 breast cancer cells identified that ZIP7 was responsible for activation of multiple tyrosine kinases that are implicated in the aggressive phenotype of tamoxifen-resistant breast cancer [8,19,20]. Recent evidence in MCF7 cells suggests that ZIP7 is phosphorylated by CK2 and is associated with the regulated release of zinc from intracellular stores to phosphorylate kinases implicated in cell proliferation and migration [8].

Given the role of ZIP7 in modulating zinc flux, and the role of zinc as an insulin mimetic in cellular processes, we propose that...
ZIP7 may also be implicated in metabolic processes associated with glycaemic control. Here we report evidence for a novel role for Zip7 in modulating glycaemic control in skeletal muscle cells. We find that the attenuation of Zip7/mRNA in C2C12 skeletal muscle cells modulates genes involved in carbohydrate metabolism and glycolysis in vitro. These studies demonstrate a previously unprecedented role for Zip7 in regulating glycemic control in skeletal muscle and provide a platform to further explore the potential of this transporter in skeletal muscle insulin resistance.

Materials and Methods

Cell culture

Proliferating mouse C2C12 myoblasts in all experiments were cultured and maintained in DMEM supplemented with 10% Fetal Bovine Serum and physiological zinc concentrations (20 μM ZnSO4), (Life Technologies, Mulgrave, Victoria, Australia). Differentiation of myoblasts into post-mitotic, multi-nucleated myotubes was induced by mitogen withdrawal (i.e. DMEM supplemented with 20 μM ZnSO4 and 2% horse serum for three days). Assessment of the muscle-specific, contractile and metabolic C2C12 muscle phenotype was assessed by measuring the expression of markers of differentiation and metabolic processes as previously described [21]. The time course experiments on differentiated C2C12 skeletal muscle cells were performed over 60 minutes in the presence of 10 nM insulin, 20 μM ZnSO4 and 10 μM pyridoxine (see Figures S1 and S2).

RNA Extraction and cDNA Synthesis

Mouse quadriceps muscle was a kind gift from Dr. Paul Lewandowski, Deakin University, Australia with approval from the Deakin University Animal Welfare Committee (A37/2007). Total RNA was extracted from C12C2 cells and C57Bl/6J mouse quadriceps using TRI-Reagent (Sigma-Aldrich, Castle Hill, NSW, Australia) according to the manufacturer’s protocol. Total RNA was then treated with 2 U of DNase1 for 30 min at 37°C followed by purification of the RNA through an RNaseasy purification column system (Qiagen, Chadstone, Victoria, Australia). RNA quantity and quality was measured using a Nanodrop spectrophotometer (Thermo Scientific, Scoresby, Victoria, Australia). A High Capacity cDNA Synthesis kit was used to synthesize cDNA from 2 μg of total RNA using random hexamers according to the manufacturer’s instructions (Life Technologies). The cDNA was diluted to 400 μl in nuclease-free water and stored at −20°C.

Mouse glucose metabolism and zinc transporter arrays

The Mouse Glucose Metabolism RT2 Profiler PCR Array was purchased from SA Biosciences, Qiagen; Catalogue No. 330321. This array profiles the expression of 84 genes involved in the regulation and enzymatic pathways of glucose and glycogen metabolism (Table S1). The Zinc Transporter RT2 Profiler Custom PCR Array (Qiagen) contained the genes for the two zinc transporter families, Slc30a1-10 and Slc39a1-14.

cDNA synthesis of RT2 mouse glucose metabolism and zinc transporter PCR array

cDNA synthesis using the RT2 First Strand Kit was performed as described by the manufacturer (Qiagen). Briefly, potential genomic DNA was eliminated from 500 ng of total RNA using the manufacturer’s recommended protocol. Excess RNA binding buffer was then removed by heating the sample at 95°C for 5 min.

Quantitative Real-time PCR

Quantitative PCR (qPCR) was performed on a RealPlex PCR detection system (Eppendorf, North Ryde, New South Wales, Australia) in triplicate on at least three independent RNA preparations. Target cDNA levels were analyzed in 10 μl reactions with SensiMix SYBR No-ROX (Bioline, Alexandria, New South Wales, Australia). Primers (GeneWorks, South Australia, Australia) for markers of skeletal muscle cell differentiation and metabolism, Myogenin, Tnn1, Tnn2, Abca1, Fabp3 and Sbeb-1c (Table S2) have been previously described [21–23]. Other primers (Table S2) for the amplification of target gene sequences were designed using the NCBI Primer Blast Tool http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi, with the exception of Insr (PrimerBank ID: 29825829a1), Irs2 (PrimerBank ID: 3661525a1) and Irs (PrimerBank ID: 6754560a1) which were obtained from the PrimerBank Database http://pga.mgh.harvard.edu/primerbank/index.html [24–26]. Note: all primers were rigorously analyzed by BLAST as described in the results section and associated errors were calculated using the guidelines described by Bookout and Mangelsdorf [27].

Primer design to detect endogenous and exogenous pCMV-Zip7

Primers were designed to specifically target endogenous and exogenous Zip7 mRNA. For the pCMV-Zip7 overexpression plasmid, we placed the forward primer on the Zip7 mRNA sequence and the reverse primer on the plasmid C-myc tag (see Table S2). For the specific amplification of endogenous Zip7 we designed primers on the 5’UTR. This region is omitted on the pCMV-Zip7 expression plasmid.

Quantitative Real-time PCR of RT2 mouse glucose metabolism and zinc transporter PCR array

The qPCR for the RT2 glucose metabolism and zinc transporter arrays were performed as outlined in the guidelines supplied by the manufacturers (Qiagen). Briefly, cDNA (102 μl) from the RT2 First Strand Kit was added to 1350 μl of 2 x RT2 SYBR Green and 1248 μl of H2O. To each well of the glucose metabolism assay 96-well plate, 25 μl of sample was added. qPCR was performed on a RealPlex PCR detection system (Eppendorf) using 45 cycles of amplification consisting 95°C for 15 seconds and 60°C for 1 minute.

Transient transfections of siRNA molecules

The transient transfection of siRNA molecules Zip7 (Catalogue No: AM16708), Zfp1 (Catalogue No: AM16708), Gapkdh (Catalogue No: 4390771) and the scramble control (Catalogue No: AM4635) (Life Technologies) were performed using RNAiMAX reagent as instructed by the manufacturer (Life Technologies). Briefly, C2C12 cells were transfected in 6-well dishes with 10 nM of siRNA molecular or the scramble control in RNAiMAX reagent. The cells were subsequently maintained in 2% horse serum and differentiated over three days and collected in 1 ml of TRI-Reagent per three wells for RNA extraction or 1 ml of RIPA Buffer (Thermo Scientific) (containing Halt Protease and Phosphatase Inhibitor Cocktail; Thermo Scientific) for protein analysis.
Zip7 overexpression plasmid and transient transfection in C2C12 skeletal muscle cells

A full-length mouse cDNA Zip7 expression plasmid was obtained from Origene Technologies, Inc (Clone ID MR216331; Rockville, MD). The pCMV control plasmid was created by excision of the full-length Zip7 gene by restriction digest at enzyme sites Sgfl and MluI followed by end-filling and blunt-end ligation. Briefly, 1 μg of pCMV-Zip7 plasmid was digested in the presence of 10X Fast Digest Green Buffer and 1 unit of Sgfl and MluI restriction enzymes (Fermentas, Thermo Scientific) for 5 min at 37°C. For the end-filling, approximately 1 μg of pCMV plasmid DNA was incubated with 0.5 mM dNTPs and 1 unit of Klenow fragment and incubated at 30°C for 15 minutes. The pCMV control plasmid was circularized in the presence of 2 μl of T4 DNA ligase, 100 ng of plasmid vector and 1 μl of T4 DNA ligase and incubated at 4°C for approximately 16 h.

The pCMV-Zip7 and pCMV control plasmid were transiently transfected into C2C12 skeletal muscle cells using Lipofectamine 2000 (Life Technologies) as instructed by the supplier. Briefly, C2C12 skeletal muscle cells were grown to 80% confluence and 2000 (Life Technologies) as instructed by the supplier. Briefly, C2C12 skeletal muscle cells were grown to 80% confluence and 2000 (Life Technologies) as instructed by the supplier. C2C12 cells were transfected with siRNA-

Protein Extraction and Western Blot

Total cellular protein from the scramble control and the siRNA-Zip7 transfected C2C12 cells was isolated by scraping cells with RIPA buffer (contains protease and phosphatase inhibitor cocktail) then the samples were place on ice for 1 hr with constant vortexing every 10 mins. The cells were then sonicated for 5 seconds pulses for 30 seconds at 50% duty followed by boiling for 10 min. The protein samples were centrifuged at 13,000 rpm for 5 min and the supernatant was collected. Total protein concentration was measured using a BCA kit (BIORAD, Gladesville, New South Wales) as outlined by the manufacturer’s instructions.

Total soluble protein (100 μg) from the scramble control and siRNA-Zip7 transfected C2C12 cell lines was resolved on a 4–15% SDS-PAGE gradient gel (BIORAD) and transferred to a nitrocellulose membrane. The membranes were blocked overnight in 5% skim milk in TBS-Tween 20 followed by an overnight incubation with either Glut4 (Cell Signaling, 1:2000; Catalog No: 66513); Gapsdh (Santa Cruz Biotechnology, Santa Cruz, CA), Akt (Cell Signaling 1:5000; Catalog No: 9272), pAkt (Cell Signaling 1:5000; Catalog No: 4058) antibodies.

Glycogen synthesis assay

The glycogen synthesis assay was performed as described by the manufacturer’s (BioVision, Life Research, Scoresby Victoria, Australia). Briefly, C2C12 cells were transfected with siRNA-Zip7 and the scramble control as described above. Following differentiation, C2C12 cells were treated with 10 nM insulin for 60 minutes. Cell lysates were collected in 200 μl of dH2O on ice and homogenates were boiled for 5 min to inactivate enzymes. Samples were then centrifuged at 13000 rpm for 5 min and the supernatant was collected. Samples were then prepared by performing hydrolysis of glycogen to glucose and then mixed with OxiRed probe to generate color (λmax = 570 nm). Note: a glucose control was also performed in the absence of glucose oxidase to determine background glucose levels. These were subsequently subtracted from the glycogen readings. The glycogen concentration in the samples was calculated by C = Ay/Sv where Ay is the amount of glycogen (μg) in the sample as determined from a standard curve and Sv is the sample volume (μl).

Statistics

Data obtained from individual qPCR was assessed by a Student’s unpaired t-test on at least three independent biological replicates. Statistical significance was denoted as the average ± standard deviation of the mean. Data was considered statistically significant when the P-value was ≤0.05, *P<0.05; **P<0.01 and ***P<0.001. The analysis of the gene arrays was performed with the RT² Profiler PCR Array Data Analysis Software v3.5 (SA Biosciences).

Results

The Slc39a (Zip) zinc transporters are differentially expressed in C2C12 skeletal muscle cells and mouse quadriceps

To determine the expression levels of the Slc39a zinc transporter family in mouse C2C12 skeletal muscle cells and mouse quadriceps we utilized a custom zinc gene array (SABiosciences, Qiagen) with primer sequences that are specific for the Slc39a (Zip) mouse zinc transporter genes (i.e. Slc39a1-14). Quantitative real-time PCR (qPCR) was performed and the expression of each zinc transporter was measured relative to the ‘housekeeping gene’, Gapdh.

The zinc transporters Slc39a1 and Slc39a7 were highly expressed in C2C12 skeletal muscle cells (Figure 1A). Lower levels of expression were observed for Slc39a3, 6, 9, 10, 11, 13 and 14. Minimal or no expression was observed in Slc39a2, 4, 5, 8, and 12 (Figure 1A). In mouse quadriceps we observed high levels of expression for all of the Slc39a transporters with the exception of Slc39a5 (Figure 1B).

Slc39a7 (Zip7) is expressed during C2C12 skeletal muscle differentiation

We were most interested in Slc39a7 (Zip7) as this transporter is predominately localized to the ER and the Golgi apparatus and is suggested to be involved in the ‘zinc wave’ and associated cellular signaling [8]. Accordingly, to elucidate the role of Zip7 in skeletal muscle we initially investigated the expression profile of this zinc transporter relative to E47 in the mouse C2C12 myoblast cell line. Proliferating myoblasts can be induced to biochemically and morphologically differentiate into post-mitotic multinucleated myotubes by mitogen withdrawal. This transition from a non-muscle phenotype to a contractile phenotype is associated with the activation and repression of a structurally diverse group of genes.
responsible for contraction and the extreme metabolic demands placed on this tissue [21]. During this period of differentiation, we observed that Zip7 mRNA is highly expressed in proliferating myoblasts and was constitutively expressed during skeletal muscle cell differentiation when normalized to 

In order to assess the differentiation status of the C2C12 cells and to demonstrate that they had acquired a differentiated, contractile and metabolic phenotype, qPCR was performed on the marker genes myogenin (MynoG), a gene that encodes the hierarchical basic helix loop regulator and is specifically required for differentiation [28], the slow twitch (type I) and the fast twitch (type II) isoforms of the contractile protein troponin I (Tnni1 and TnniII), and the metabolic genes Abca1 (ATP-binding cassette proteins), Fabp3 (fatty acid binding protein 3) and Srebp1c (sterol regulatory binding element protein). Expression of both MynoG and the contractile protein genes (type I and II, Tnni1 and Tnni2, respectively) were dramatically increased and confirmed the differentiation of the myoblast C2C12 skeletal cell line to the myotube phenotype (Figure 2B–D). Additionally, genes involved in lipid metabolism (Abca1 and Srebp1c) (Figure 2E and 2G) were also induced while Fabp3 was downregulated during muscle differentiation (Figure 2F) which is consistent with previous studies [22,29–31] and confirms that the muscle cells had acquired the appropriate contractile and metabolic phenotype.

### siRNA-Zip7 Expression Represses Endogenous Zip7 mRNA in Skeletal Muscle Cells

To elucidate the biological role of Zip7 in the context of glucose metabolism we selectively ablated the expression of this transporter in C2C12 skeletal muscle cells utilizing a siRNA-Zip7 molecule. An siRNA targeting mouse Gapdh and a scramble sequence that contains no known homology to the mouse, rat or human genome were utilized as controls. The siRNA-Gapdh was used to determine the robustness of the transfection and the ability to successfully attenuate a specific target gene that is constitutively expressed. Accordingly, C2C12 cells were transfected with the scramble control, siRNA-Gapdh or the siRNA-Zip7 and subsequently differentiated for three days.

Initially, we aimed to validate the specificity and robustness of the siRNA transfection in C2C12 cells by transfecting an siRNA-Gapdh to determine transfection efficacy and siRNA specificity. We identified a significant reduction in Gapdh mRNA (4-fold, p = 0.0023) in the siRNA-Gapdh transfected cells compared to the scramble control (Figure 3A). We then transfected C2C12 skeletal muscle cells with an siRNA targeting Zip7 mRNA. Quantitative PCR was then performed to measure the expression levels of endogenous Zip7 relative to Eef2 in RNA isolated from the scramble control and Zip7 transfected cell lines. We observed a significant reduction in the mRNA levels of Zip7 (4.6-fold, p = 0.0006) when compared to the scramble control (Figure 3B).

To determine that the attenuation of Zip7 was not due to differential Eef2 mRNA expression, qPCR was also performed on Eef2 normalized to Gapdh. No change in the level of Eef2 in the Zip7-siRNA cell lines were observed when normalized to Gapdh mRNA (Figure 3C). We also tested the relative expression of Zip7 in siRNA-Gapdh C2C12 cells. There was no change in Zip7 mRNA expression in the Gapdh reduced C2C12 cell lines (Figure 4D).

Since Zip7 was also highly expressed in C2C12 skeletal muscle cells (Figure 1) we decided to selectively reduce the expression of this transporter with an siRNA-Zip1 to determine if there were any compensatory changes in Zip7 expression. C2C12 cells were transfected with the scramble control and siRNA-Zip1 and endogenous Zip1 and Zip7 mRNA was measured. We successfully attenuated endogenous levels of Zip1 mRNA (approximately 3-fold, p = 0.0025) in the C2C12 cell lines (Figure 3E). No change in endogenous expression of Zip7 mRNA (p = 0.1040) was observed in the siRNA-Zip1 cell lines (Figure 3F).

### The attenuation of Zip7 resulted in no change in other zinc transporters

To determine the expression status of the other zinc transporter family members in the presence of the Zip7 reduced C2C12 cell lines we utilized a custom gene array that contains the primer sequences for the Slc39a1-Zip17 (1–10) and Slc39a1-Zip7 (1–14) family members. cDNA from the scramble control and the siRNA-Zip7 C2C12 cells were assayed to assess for compensatory changes in the other family members due to reduced Zip7 mRNA. We identified that the reduction of Zip7 had no effect on the expression of the Slc39a1-Zip17 family members (Figure 4A). In the Slc39a1-Zip arrays, reduced expression of Zip7 resulted in a significant attenuation of Zip7 mRNA as expected. We also
Figure 2. Relative expression of Slc39a7 (Zip7) and markers of skeletal muscle differentiation in C2C12 cell lines. A). Slc39a7 (Zip7) expression relative to Eef2. B–D). Markers of skeletal muscle differentiation: myogenin (MioG) and the troponins 1 and 2 (Tnni1 and Tnni2), respectively. E–G). Markers of metabolism: ATP-binding cassette transporter protein 1 (Abca1), fatty-acid binding protein 3 (Fabp3) and sterol regulatory element binding protein 1c (Srebp-1c), respectively. PMB = proliferating myoblasts; D1–3 = day 1 to day 3 of differentiation of myotubes, respectively. Error bars indicated the ± SD from three independent biological samples. doi:10.1371/journal.pone.0079316.g002

Figure 3. Zip7 mRNA is attenuated by si-RNA-Zip7. Relative expression of Gapdh, Zip7, Zip1 and Eef2 in the scramble control and corresponding siRNA cells, respectively. A). Gapdh relative to Eef2 in siRNA-Gapdh cells. B). Zip7 relative to Eef2 in siRNA-Zip7 cells. C). Eef2 relative to Gapdh in siRNA-Zip7 cells. D). Zip7 relative to Eef2 in siRNA-Gapdh cells. E). Zip1 relative to Eef2 in siRNA-Zip1 cells, and F). Zip7 relative to Eef2 in siRNA-Zip1 cells. Error bars indicated the ± SD from three independent biological samples. **P<0.01, ***P<0.001. doi:10.1371/journal.pone.0079316.g003
observed a small, but significant reduction in the expression of Zip13 and Zip14 to independently test the validity of this observation. We performed qPCR on Zip13 and Zip14 expression in the scramble control and siRNA-Zip7 C2C12 cells. No significant changes in the level of expression for these zinc transporters were observed (Figure 4C).

Attenuation of Zip7 mRNA in C2C12 cells is associated with changes in several genes implicated in glucose metabolism

We utilized a Mouse Glucose Metabolism RT2 Profiler PCR Array (SA Biosciences, Qiagen) that contains profiles for the expression of 84 key genes implicated in the regulation of enzymatic pathways of glucose and glycogen metabolism to assess potential pathways that are modulated by Zip7 (Table S1). We observed that the attenuation of Zip7 mRNA in C2C12 skeletal muscle cells resulted in changes in several genes implicated in glucose metabolism. These include Agl (Amylo-1,6-glucosidase, 4 alpha-glucanotransferase, p = 0.002997), Dlat (Dihydrolipoamide S-acetyltransferase, p = 0.035894), Galm (Galactose mutarotase, p = 0.001714), Ghe1 (Glucan-1,4-alpha branching enzyme 1, p = 0.003227), Idh3g (Isocitrate dehydrogenase 3 NAD+ gamma, p = 0.015324), Pkm2 (Phosphoglyceraldehyde mutase 2, p = 0.031514), Pim2 (Phosphoglucomutase 2, p = 0.027981), Pkhb (Phosphorylase kinase beta, p = 0.032247), Pym (Muscle glycogen phosphorylase, p = 0.004097), Tps1 (Triosephosphate isomerase 1, p = 0.021080) and Gusb (Glucuronidase beta, p = 0.013637) (Table 1 and Table S1).

We further validated several of these genes with a focus on glycogen metabolism (Pgm2, Phkhb, Pym and Ghe1) by designing new primer pairs and performing qPCR on the scramble control versus the siRNA-Zip7 cDNA. We observed significant downregulation in these genes in concordance with the PCR array data (Figure 5A–D).

We speculated that given genes implicated in glycogen metabolism were affected by reduced Zip7 mRNA levels, that perhaps the glucose transporter, Glut4 might be downregulated in the siRNA-Zip7 cells. Glut4 predominately transports glucose across the plasma membrane which is further processed by oxidative (glycolysis) or non-oxidative (glycogenesis) pathways [32]. Accordingly, qPCR was performed for Glut4 mRNA expression in the scramble control and the siRNA-Zip7 C2C12 cells. We observed a significant downregulation of Glut4 in the siRNA-Zip7 cells (p = 0.0096) (Figure 6A). We also tested for Glut4 immunoreactive protein in the scramble control and siRNA-Zip7 C2C12 cells. Accordingly we observed a significant reduction in immunoreactive Glut4 in the siRNA-Zip7 C2C12 cells compared to the scramble control (Figure 6B). Gapdh was used as a protein loading control and showed that similar amounts of total soluble protein were resolved (Figure 6B).

Reduced Zip7 compromises insulin-induced glycogen synthesis and phosphorylation of AKT in C2C12 skeletal muscle cells

Cellular glucose utilization by Glut4 is responsible for glycogenesis in muscle [33] and with increasing plasma insulin concentration, glycogen synthase is activated by insulin and glycogen synthesis predominates [34]. Moreover, a core component of glycogen synthesis is the insulin-induced phosphorylation of AKT in a process that leads to the activation of glycogen synthase [34]. To test the efficacy of insulin to induce phosphorylation of Akt and thus confirm the robustness of the C2C12 skeletal muscle cell line to respond to insulin, skeletal muscle cells
were treated with 10 nM insulin over 60 min and subsequent protein was extracted as described in Material and Methods. We observed that 10 nM of insulin activated pAkt after 5 min followed by a robust phosphorylation of Akt over the 60 min time course (Figure S1), and thus confirmed the validity of our cell culture system in the presence of 20 μM of pyrithione (Figure S2). Accordingly, 10 nM of ZnSO4 alone and thus confirmed that maintaining our cell culture) affected the phosphorylation status of AKT we treated with 10 nM insulin over 60 mins and performed glycogen synthesis. We observed a significant reduction in glycogen synthesis in the siRNA-Zip7 when compared to the scramble control (Figure 6C). As expected, we observed a significant induction of glycogen synthesis on exposure to insulin in the scramble control cells, however this effect was blunted in the Zip7-siRNA C2C12 (Figure 6C).

To determine a potential mechanism of action for the reduced glycogen synthesis in the presence of reduced Zip7 mRNA we performed qPCR on the insulin receptor (Insr) and the most predominant isoforms of the insulin receptor substrate molecules that are expressed in skeletal muscle, insulin receptor substrate 1 (Irs1), and insulin receptor substrate 2 (Irs2) [32]. These substrates serve as docking molecules for several SH2-containing proteins and the subsequent activation of downstream signaling molecules that result in the activation of AKT, which mediates many of insulin’s metabolic effects by modulating gluconeogenesis, protein synthesis and glycogen synthesis [33]. Accordingly, the reduced expression of Zip7 in the C2C12 skeletal muscle cells resulted in a significant reduction in the expression of the Insr, Irs1 and Irs2 (Figure 7A–C). In order to confirm that the reduction of these key genes was associated with a reduction in signaling we performed immunoblot analysis on phosphorylated Akt (pAkt). We observed a significant reduction in pAkt in the Zip7-siRNA compared to the scramble control (Figure 7D).

Table 1. Fold changes in expression of glucose metabolic genes in the siRNA-Zip7 compared to the scramble control.

| PATHWAY: GLUCOSE METABOLISM | T-TEST | Fold Up- or Down-Regulation |
|----------------------------|--------|---------------------------|
| **GLYCOLYSIS**             |        |                           |
| Gene Symbol                | Description | Gene Name | p value* | siRNA-Zip7/Scramble |
| NM_176963                  | Galm    | Galactose mutarotase      | 0.00171  | -1.71               |
| NM_010358                  | Gusb    | Glucuronidase, beta       | 0.01363  | -1.15               |
| NM_018870                  | Pgam2   | Phosphoglycerate mutase 2 | 0.03151  | -1.6                |
| NM_028132                  | Pgam2   | Phosphoglucomutase 2     | 0.02798  | -1.36               |
| NM_009415                  | Tpi1    | Triosephosphate isomerase 1 | 0.02108 | -1.24              |
| **GLUCONEOGENESIS**        |        |                           |
| Gene Symbol                | Description | Gene Name | p value* | siRNA-Zip7/Scramble |
| NM_028994                  | Pck2    | Phosphoenolpyruvate carboxykinase 2 (mitochondrial) | 0.00219  | 1.82               |
| **TCA CYCLE**             |        |                           |
| Gene Symbol                | Description | Gene Name | p value* | siRNA-Zip7/Scramble |
| NM_030225                  | Dist    | Dihydrolipoamide S-succinyltransferase | 0.03589  | -1.14              |
| NM_008323                  | Idh3g   | Isocitrate dehydrogenase 3 (NAD+), gamma | 0.01532  | -1.31              |
| **PATHWAY: GLYCOGEN METABOLISM** |        |                           |
| **SYNTHESIS**             |        |                           |
| Gene Symbol                | Description | Gene Name | p value* | siRNA-Zip7/Scramble |
| NM_028803                  | Gbe1    | Glucan (1,4-alpha-), branching enzyme 1 | 0.00322  | -1.98              |
| **DEGRADATION**           |        |                           |
| Gene Symbol                | Description | Gene Name | p value* | siRNA-Zip7/Scramble |
| NM_001081326               | Agl     | Amylo-1,6-glucosidase, 4-alpha-glucanotransferase | 0.00299  | -1.41              |
| NM_011224                  | Pgm2    | Muscle glycogen phosphorylase | 0.00409  | -1.75              |
| **REGULATION**            |        |                           |
| Gene Symbol                | Description | Gene Name | p value* | siRNA-Zip7/Scramble |
| NM_009446                  | Phkb    | Phosphorylase kinase beta | 0.03224  | -1.43              |

*p values < 0.05

A Mouse Glucose Metabolism RT2 Profiler PCR Array was utilized to profile the expression of 84 genes involved in the regulation and enzymatic pathways of glucose and glycogen metabolism. Three independent biological samples were utilized and the data was considered statistically significance when the P value was ≤0.05. doi:10.1371/journal.pone.0079316.t001
Overexpression of Zip7 in C2C12 cells induces genes associated with glucose metabolism

We observed that reduced Zip7 mRNA in C2C12 skeletal muscle cells was associated with changes in genes implicated in glucose metabolism. For example, a significant reduction in the expression of Pgm2, Phkb, Pygm, Gbe1, Glut4, Insr, Irs1 and Irs2 was observed in the Zip7-siRNA C2C12 cells compared to the scramble control (see Figures 5, 6, 7). Accordingly, to determine if by overexpressing Zip7 we could observe the converse effect on gene expression, we transiently transfected an overexpression Zip7 plasmid (pCMV-Zip7) into C2C12 skeletal muscle cells and after 72 hours collected RNA for subsequent qPCR analysis. We observed a significant induction in the expression of exogenous Zip7 in the pCMV-Zip7 expressing C2C12 cells compared to the pCMV control (Figure 8A and B). To confirm that the major Zip7 mRNA transcript observed was from the overexpression of the pCMV-Zip7 plasmid we performed PCR using primers that were specific for the endogenous form of Zip7 mRNA. We observed that Zip7 mRNA was expressed at relatively much lower levels in both the pCMV and pCMV-Zip7 transfected cells (Figure 8C).

**Figure 5. Reduced Zip7 expression alters gene expression of key glucose metabolic genes. A–E).** Relative expression of Pgm2, Phkb, Pygm, and Gbe1 mRNA to Eef2 in the scramble control and the siRNA-Zip7, respectively. Error bars indicated the ± SD from three independent biological samples. *P<0.05, **P<0.01. doi:10.1371/journal.pone.0079316.g005
confirming that the major \textit{Zip7} transcript resulted from the overexpression system. Moreover, we found that the overexpression of \textit{Zip7} mRNA induced the expression of the insulin receptor (\textit{Insr}); insulin receptor substrate 1 (\textit{Isr1}) and insulin receptor substrate 2 (\textit{Isr2}) (see Figure 8D–F). This was in contrast to Figure 7 where a reduction in the expression of \textit{Zip7} mRNA resulted in reduced expression of \textit{Insr}, \textit{Isr1} and \textit{Isr2}. We also observed an increase in \textit{Glut4} mRNA in the pCMV-\textit{Zip7} overexpression system, however this result did not attain significance (p = 0.0590). Similarly, \textit{Glut4} protein levels were not significantly changed in the pCMV-\textit{Zip7} overexpression system when compared to the pCMV control (data not shown).

Discussion

Intracellular zinc homeostasis is largely regulated by two families of zinc transporters (ZnTs and ZIPs) that traffic zinc across biological membranes [35,36]. Dysregulation of zinc signaling leads to a number of disease states including cancer [19,37], autoimmune disease [38,39], cardiovascular disease [40,41] and diabetes [42–45]. Of this family, ZIP7 is important in maintaining physiological and cellular zinc homeostasis through its ability to initiate the ‘zinc wave’ and provide cytosolic zinc ions that are involved in cellular signaling processes. Although many zinc transporters respond to fluctuating zinc levels and alter their subcellular localization, ZIP7 is an exception and is restricted constitutively to the membrane of the Golgi apparatus and/or the endoplasmic reticulum [15–17]. Furthermore, \textit{Zip7} gene expression and intracellular location are not altered in response to changes in intracellular zinc status [3]. Studies in breast cancer cells have elucidated a role for this transporter in cell signaling events [8,20]; however, the role of ZIP7 with respect to the control of the genetic programs associated with carbohydrate metabolism in skeletal muscle has not been addressed. Here we provide the first evidence for a metabolic role for \textit{Zip7} in modulating glycaemic control in skeletal muscle and provide support for further studies in processes associated with insulin resistance in this tissue.

\textit{Zip7} mRNA is highly expressed in differentiated C2C12 cells and mouse quadriceps. Although \textit{Slc39a1} was also highly expressed in C2C12 skeletal muscle cells, homozygous knockout of \textit{Slc39a1} in mice produces no phenotype when dietary zinc intake is normal [46] suggesting compensatory actions from other family members. To explore compensatory mechanisms from other zinc transporters we performed qPCR on all of the family members in the scramble control and the siRNA-\textit{Zip7} C2C12 skeletal muscle cells. We did not observe major changes in expression of the other members of the zinc transporters which suggest that the attenuation of \textit{Zip7} has no other effect on these genes. Of the zinc transporters, it should be emphasized that, in addition to ZIP13, [47] ZIP7 is the only other zinc transporter localized to the Golgi apparatus and not the plasma membrane [15] and compensation is therefore unlikely. Given that ZIP7 is localized exclusively on the Golgi apparatus and not the plasma membrane [15] and compensation is therefore unlikely. Given that ZIP7 is localized exclusively on the Golgi apparatus and not the plasma membrane [15] and compensation is therefore unlikely. Given that ZIP7 is localized exclusively on the Golgi apparatus and not the endoplasmic reticulum [15–17], \textit{Zip7} gene expression and intracellular location are not altered in response to changes in intracellular zinc status [3]. Studies in breast cancer cells have elucidated a role for this transporter in cell signaling events [8,20]; however, the role of ZIP7 with respect to the control of the genetic programs associated with carbohydrate metabolism in skeletal muscle has not been addressed. Here we provide the first evidence for a metabolic role for \textit{Zip7} in modulating glycaemic control in skeletal muscle and provide support for further studies in processes associated with insulin resistance in this tissue.

In contrast to the \textit{Zip} expression profile in C2C12 cells, we also observed moderate levels of expression for all of the \textit{Zip} transporters (except for \textit{Zip3}) in mouse quadriceps. \textit{Zip7} mRNA was more highly expressed in C2C12 cells (approximately 15-fold) when compared to the expression found in quadriceps. It should be noted that quadriceps contain a mix of muscle fibre-types (oxidative type I and glycolytic type II) [48]. Similar studies on
Figure 7. Reduced Zip7 expression reduces the mRNA expression of the insulin receptor (Insr), insulin receptor substrate 1 (Irs1), insulin receptor substrate 2 (Irs2) and the phosphorylation of AKT. A–C. Relative expression of Insr, Irs1 and Irs2 mRNA to Eef2 in the scramble control and the siRNA-Zip7 C2C12 skeletal muscle cells. D. Western blot for immunoreactive pAkt and Akt in protein lysates isolated from
scramble control and siRNA-Zip7 transfected C2C12 skeletal muscle cells. Error bars indicate the ± SD from three independent biological samples for the mRNA analysis of Inrs, Irs1 and Irs2. **P<0.01, ***P<0.001. Western blot analysis for pAkt and Akt was performed three times on six independent and pooled transient transfections of the scramble control and siRNA-Zip7.

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Figure 8. The exogenous overexpression of Zip7 mRNA induces the expression of the insulin receptor (Insr), insulin receptor substrate 1 (Irs1), insulin receptor substrate 2 (Irs2) and Glut4 mRNA. A. Relative expression of exogenous Zip7 mRNA. B. Relative expression of endogenous and exogenous Zip7 mRNA. C. Relative expression of endogenous expression of Zip7 mRNA. D-F. Relative expression of Insr, Irs1 and Irs2 mRNA. G. Relative expression of Glut4 mRNA (not significant P = 0.0590). Error bars indicate the ± SD from two independent biological samples for the mRNA analysis that consisted of at least three independent transfections. **P<0.01, ***P<0.001.

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other muscle fibre types; soleus (type I), plantaris (type II) and anterior tibialis (type II) also demonstrated differences in the level of expression for the orphan nuclear receptor, Coup-tfII in these tissues in comparison to C2C12 cells [49]. Moreover, studies on protein arginine methyltransferase 3, 4 and 5 (PRMT3–5) in mouse skeletal muscle tissue and C2C12 cells found high expression of PRMT3–5 in gastrocnemius in comparison to only high expression of PRMT4 (with no or minimal expression of PRMT3 and 5, respectively) [50]. Although these relative expression discrepancies exist between in vitro and in vivo model systems, the C2C12 cell culture model is a well-established and validated system to study the effects of metabolic processes [21,51,52]. For example, data derived from this in vitro model with liver X receptor (LXR) and peroxisome proliferating activated receptor (PPAR) agonists and their role in metabolism (e.g. energy expenditure, running endurance, lipid metabolism and cholesterol efflux) has been validated and reproduced in mice [51–53].

Our study revealed that subsets of genes involved in glucose metabolism (Agl, Dist, Galn, Gbe1, Idh3g, Pck2, Pgm2, Pygm, Pkh2, Pygm, Tpi1, Gash and Glut4) are altered when Zip7 expression was reduced. This is further highlighted by the fact that related genes in similar and other pathways (see Table S1) were refractory to the attenuation of Zip7 expression. These data are noteworthy for several reasons. For example, in skeletal muscle, GLUT4 predominately transports glucose across the plasma membrane which is further processed by oxidative (glycolysis) or non-oxidative (glycogenesis) pathways [52]. Thus, the decline in Glut4 protein in the Zip7-reduced C2C12 cells would suggest a reduction in glucose transport and subsequent genes associated with oxidative and non-oxidative pathways. Similarly, this is supported by the observation that several genes implicated in glycolysis (Galm, Gash, Pgm2, Pogm2 and Tpi1) and glycogen synthesis (Gbe1, Agl, Pgm2, Pogm and Pkh2) were reduced in the Zip7 attenuated cells. In skeletal muscle, these genes play critical roles in the oxidative and non-oxidative pathways, respectively. This is further supported by the reduction in the mRNA of the insulin receptor (Insr), and the insulin receptor substrates 1 and 2 (Irs1 and Irs2) and the subsequent reduction of basal and insulin-mediated glycogen storage in C2C12 myotubes when Zip7 expression was attenuated (see Figure 6).

Skeletal muscle is particularly important in maintaining glucose homeostasis because approximately 70-90% of whole body insulin-mediated induction of glucose uptake occurs in muscle where it is incorporated into glycogen for storage [56]. Moreover, in insulin-resistant states, insulin-induced glucose uptake and glycogen synthesis is markedly reduced in skeletal muscle [32,57]. Accordingly, in association with a reduction in genes involved in glycogen metabolism and the fact that there was a reduction in glycogen synthesis, we also observed a significant decrease in the phosphorylation status of AKT in the reduced Zip7 expressing C2C12 cells. This is consistent with recent studies where a siRNA targeting Zip7 significantly decreased zinc-induced pAKT after 5 minutes of 20 μM zinc treatment in MCF-7 tamoxifen-resistant breast cancer cells [8]. Moreover, in a recent study in a Zip7 gene knockout chicken DT40 cell model, the levels of phosphorylation of Akt and Erk were significantly reduced [58]. Given the role of Zip7 in facilitating zinc flux into the cytosol [8], and the fact that previous studies have shown that zinc can activate pAKT [8,59], it will be important to determine whether Zip7 in skeletal muscle plays a similar role in mediating zinc flux and signaling events that lead to phosphorylation of AKT and the mobilization of glucose transporters.

Zinc is a well-known inhibitor of protein tyrosine phosphatases (PTPs) [60] with a reported inhibition constant in the nanomolar range [61]. Zinc inhibits PTP1B, a cytoplasmic phosphatase that interacts with the insulin receptor and catalyzes its dephosphorylation resulting in the attenuation of insulin signaling [62]. Based on these results, and the fact that the insulin signaling pathway depends on the status of tyrosine phosphatases and the release of zinc into the cytosol, we hypothesize that reduced expression of Zip7 could lead to a reduction in the cytosolic zinc pool that is available for cellular signaling. For example, in the testes of diabetic mice treated with the zinc chelator, TPEN, a significant down-regulation of Akt-mediated glucose metabolism signaling was observed that was reflected by reduced phosphorylation of Akt and Gsk-3β [63]. Moreover, treatment of 3T3-L1 adipocytes with ZnCl2 increased tyrosine phosphorylation of the insulin receptor beta subunit and enhanced the transport of glucose in the absence of insulin through the PI3-kinase/Akt pathway [53]. Furthermore, in myocytes isolated from the femoral muscle of mice with a ZnT7 knock-out (these mice display low zinc status) there was reduced insulin signaling pathway activity and these mice were insulin resistant. This was also congruent with a reduction in the mRNA expression of Irs, Irs2 and Akt [3].

Based on the observations that Zip7 plays a crucial role in facilitating cytosolic zinc flux [8], and the given role of zinc as a second messenger that activates pathways associated with cellular signaling, these studies now show a new role for Zip7 in regulating the critical gene programs involved in glucose uptake and glycogen storage in skeletal muscle. In particular, the mRNA down-regulation of Insr, Irs1 and Irs2, in association with reduced phosphorylation of Akt and reduced Glut4 expression, suggests that Zip7 activity may be amenable to manipulation as a novel approach for the treatment of insulin resistance in skeletal muscle.

Supporting Information

Figure S1 A. Western blot analysis for insulin-induced phosphorylation of AKT in C2C12 skeletal muscle cells. C2C12 skeletal muscle cells were differentiated in 2% horse serum for 3 days and then treated in the absence or presence of 10 nM of insulin for 60 minutes. Total cellular protein was collected and the presence for immunoreactive pAkt and Akt was assessed. This immunoblot is a representation of three independent biologically insulin-treated C2C12 cell preparations. B. Average densitometry quantification of pAkt/Akt. pAkt quantified by densitometry on immunoblots from three independent experiments normalized to total Akt and displayed as the mean ± SD with significant (P ≤ 0.001) changes over time 0.

Figure S2 Western blot analysis for zinc induced phosphorylation of AKT in the absence and presence of 10 μM pyrithione in C2C12 skeletal muscle cells. C2C12 skeletal muscle cells were differentiated in 2% horse serum for 3 days and then treated in the presence (+) or absence (-) of 10 μM of pyrithione over 60 minutes. Total cellular protein was extracted and the presence for immunoreactive pAKT and AKT was performed by western blot analysis. This immunoblot represents at least three independent biological replicates.

Table S1 Fold changes in expression of glucose metabolic genes in the siRNA-Zip7 compared to the scramble control.

Table S2 Primer sequences for the amplification of target genes.
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Author Contributions
Conceived and designed the experiments: SM AN GC MAM. Performed the experiments: SM AN GC. Analyzed the data: SM AN GC MAM. Contributed reagents/materials/analysis tools: SM MAM. Wrote the paper: SM.

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