Characterization of Fer1L6 in the Mouse C2C12 Cell Line.

by

Jacob T. Huegel

A PROJECT

Submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for
the degree of

Honors Baccalaureate of Science in Biochemistry & Biophysics

(Honors Associate)

Presented May 7th, 2015

Commencement June 13th, 2015

AN ABSTRACT OF THE THESIS OF

Jacob T. Huegel for the degree of Honors Baccalaureate of Science in Biochemistry & Biophysics presented on May 7th, 2015. Title: Characterization of Fer1L6 in the Mouse C2C12 Cell Line.

Abstract Approved: _____

Colin P. Johnson

Fer1L6 is an uncharacterized member of the ferlin family of proteins, a group of large, calcium sensitive proteins with emerging roles in vesicle trafficking and various disease states. The presence of Fer1L6 has been identified within the C2C12 cell line of mouse skeletal muscle. To determine the protein's subcellular location within the cell, we used immunofluorescence. Results indicated Fer1L6 localized to the plasma membrane and perinuclear region, specifically at lamellopodia and adhesion junctions. Co-immunofluoresence, proximity ligation, and co-immunoprecipitation experiments were then performed to identify any Fer1L6 binding partners so as to aid in elucidating the protein's function. The proteins syntaxin-4, SNAP-23, vimentin, and actin were confirmed to bind Fer1L6. Additionally, the focal adhesion proteins vinculin and talin-1, were observed to be in close proximity to Fer1L6. This suggests that Fer1L6 may play a role in vesicle trafficking and membrane fusion at focal adhesions. Thus, Fer1L6 may function as a regulator in the recycling of the focal adhesion complex.

Key words: Fer1L6, focal adhesion, ferlin, vesicle trafficking, C2C12
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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Jacob T. Huegel, Author

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TABLE OF CONTENTS

LIST OF FIGURES

Figure Page

DEDICATION

This thesis is dedicated to my parents for their support throughout both the good times and the bad, and to my friends Jeff, Steve, and Alex, to show them what is possible with a lot of hard work spread out over long periods of time.

INTRODUCTION

The ferlins are a family of large (200-240 kDa) calcium sensitive proteins with functions in vesicle trafficking and membrane fusion events (Lek *et al.*, 2011). To date, six mammalian ferlin proteins are known; however, only three of these proteins have been extensively characterized. Fer1L1 (dysferlin) has been shown to function in calcium mediated muscle repair. Mutations in dysferlin have been linked to muscular dystrophy, miyoshi myopathy, cardiomyopathy, and more recently linked to abnormal phagocytosis in white blood cells and leaky blood vessels in multiple sclerosis patients (Lek *et al.*, 2011; Abdullah *et al.*, 2014). Fer1L2 (otoferlin) plays a role in synaptic fusion events involving neurotransmitter release within the inner hair cells of the ear. As such, mutations in Fer1L2 have been linked to non-sydromic profound hearing-loss (Roux *et al*., 2006; Lek *et al*., 2011; Padmanarayana *et al.*, 2014). Fer1L3 (myoferlin) is involved in myoblast fusion and tyrosine kinase recycling, and its mutation has been associated with abnormal muscle development and breast cancer, and abnormal angiogenesis (Posey *et al*., 2011; Lek *et al*., 2011). These three proteins thus establish a trend of disease states amongst the mammalian ferlins, and motivate studies on the three uncharacterized family members. It is likely that the uncharacterized ferlin proteins, Fer1L4, Fer1L5, and Fer1L6 may also have similar roles in disease and thus their characterization remains a subject of interest.

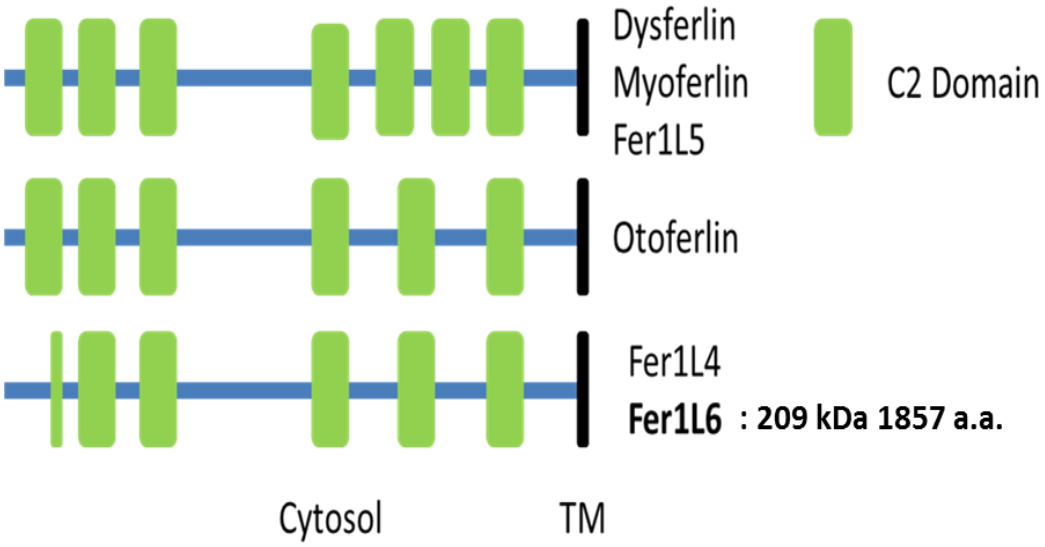

Figure 1. Topography of human ferlins. The ferlins are C-terminal anchored transmembrane proteins containing between 5 and 7 C2 domains. The C2 motif is believed to be involved in calcium-dependent phospholipid binding and membrane targeting, hence the observed roles of the characterized ferlins in vesicle trafficking and membrane fusion events.

In situ hybridization studies performed by our lab had identified Fer1L6 within the muscle of zebrafish. In addition, PCR screening identified the expression of Fer1L6 within the C2C12 line of undifferentiated mouse skeletal muscle, providing a convenient model in which to study the function of Fer1L6. The goal of this study was to locate where Fer1L6 was expressed within undifferentiated C2C12 cells and identify any proteins that interact with it. By uncovering where Fer1L6 is located and what proteins act as its binding-partners while it is located there, it becomes possible to elucidate the protein interactions of Fer1L6 and the potential function for an otherwise uncharacterized protein.

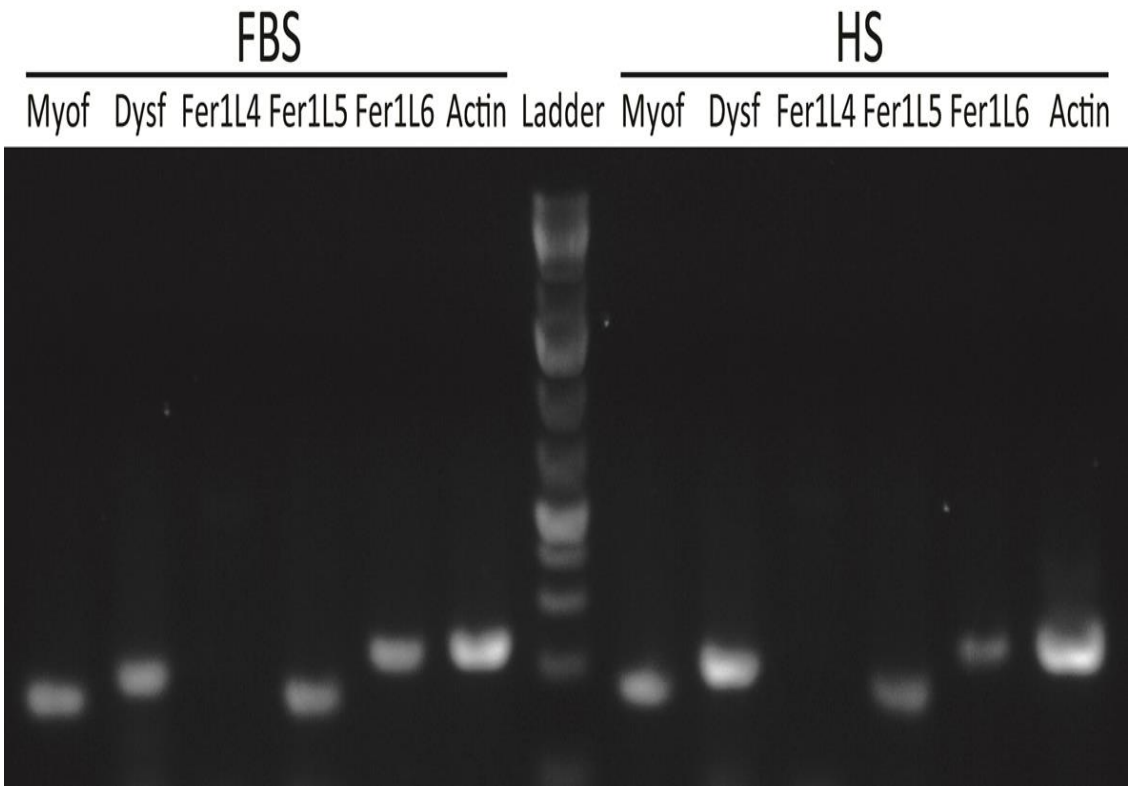

Figure 2. C2C12 PCR screen for Fer1L6 Expression. A PCR screen for expression of Fer1L6 in the C2C12 mouse skeletal muscle line revealed that Fer1L6 is present in undifferentiated (FBS) and differentiated (HS) cells. Fer1L6 expression appears to decrease with differentiation while dysferlin is upregulated and Fer1L5 is down regulated compared to an actin standard. Myoferlin, also appear to be expressed in this cell line, though at constant level.

MATERIALS AND METHODS

Cell Culture

C2C12 cells were acquired from ATCC (ATCC® CRL-1772™) and grown on manufacturer treated plastic t-75 culture flasks (Denville Scientific) at 37 °C with 5% CO_2 humidity. Cells were grown using DMEM (Invitrogen, 10% Fetal Bovine Serum, 0.2% Penicillin Streptomycin). To prevent differentiation, cells were only grown to 80% confluence prior to harvesting or passaging.

Immunofluorescence

Cells were plated on glass coverslips and incubated overnight. Cells were fixed in 4% paraformaldehyde in PBS and permeabilized for ten minutes using 10% Triton X-100 in PBS. Fixed samples were blocked by rocking in 2.5% BSA in PBS for one hour at room temperature. Fixed samples were rocked in primary antibody overnight at 4 °C at 1/250 (Goat anti-Fer1L6, Santa Cruz Biotech) or 1/500 (all others, Sigma-Aldrich) dilution. All coverslips were treated with secondary antibody (Rabbit anti-Goat HRP Conjugate or Donkey anti-Rabbit HRP Conjugate) for one hour at room temperature. Coverslips were then mounted to glass slides using Fluoromount G. All wash steps were performed using PBS. Slides were imaged using a Zeiss LSM 780 NLO Confocal Microscope System.

Proximity Ligation Assay

Cells used for proximity ligation assays were prepared according to manufacturer guidelines (Olink Bioscience). Samples were prepared with a 1/250 (Goat anti-Fer1L6, Santa Cruz Biotech) or 1/500 (all others, Sigma-Aldrich) dilution.

Co-immunoprecipitation

All cell samples used for co-immunoprecipitation were lysed in PBS with 0.5% Tween and protease inhibitors. Co-immunoprecipitation samples were prepared using IgG magnetic beads according to manufacturer guidelines using 20µg Goat anti-Fer1L6, (Santa Cruz Biotech) (GenScript).

Western Blot

Proteins were separated using a 10% SDS gel. All western blots were transferred according to manufacturer guidelines (Thermo Scientific) using PVDF membranes. Membranes were blocked for one hour at room temperature using PBS (2% milk, 0.05% Tween). Membranes were treated with primary antibody overnight at 4 $^{\circ}$C at 1/1000 antibody dilution. Membranes were treated with secondary antibody at room temperature at 1/5000 antibody dilution. Membranes were developed using horseradish peroxidase chemiluminescence according to manufacturer instructions (Thermo Scientific).

RNA Extraction

Cells prepared for RNA extraction were grown using DMEM (10% Fetal Bovine Serum, 0.2% Penicillin Streptomycin) until reaching 40% confluence, after which cells were switched to DMEM (10% Horse Serum, 0.2% Penicillin Streptomycin) and allowed to differentiate at 37 ^{o}C with 5% CO2 humidity. RNA samples were extracted at 0 and 7 days of differentiation using the Qiagen RNeasy kit.

Quantitative RT-PCR

cDNA was prepared from 1 µg of total RNA using the Bio-Rad iScript Reverse Transcriptase reagents and protocol. qPCR was conducted using the 2X SYBR® 6 Green master mix (Life Technologies) in a 20µL reaction with gene-specific primers (Sigma-Aldritch). Amplification of desired gene products was performed using the following cycle conditions: 95ºC for 10 min and 15 sec during the holding stage, 40 cycles at alternating 95ºC for 15 sec and 60ºC for 1 min, followed by a melt curve. Results were analyzed using Applied Biosystems 7500 Software. Three to five biological replicates were used for all samples.

RESULTS

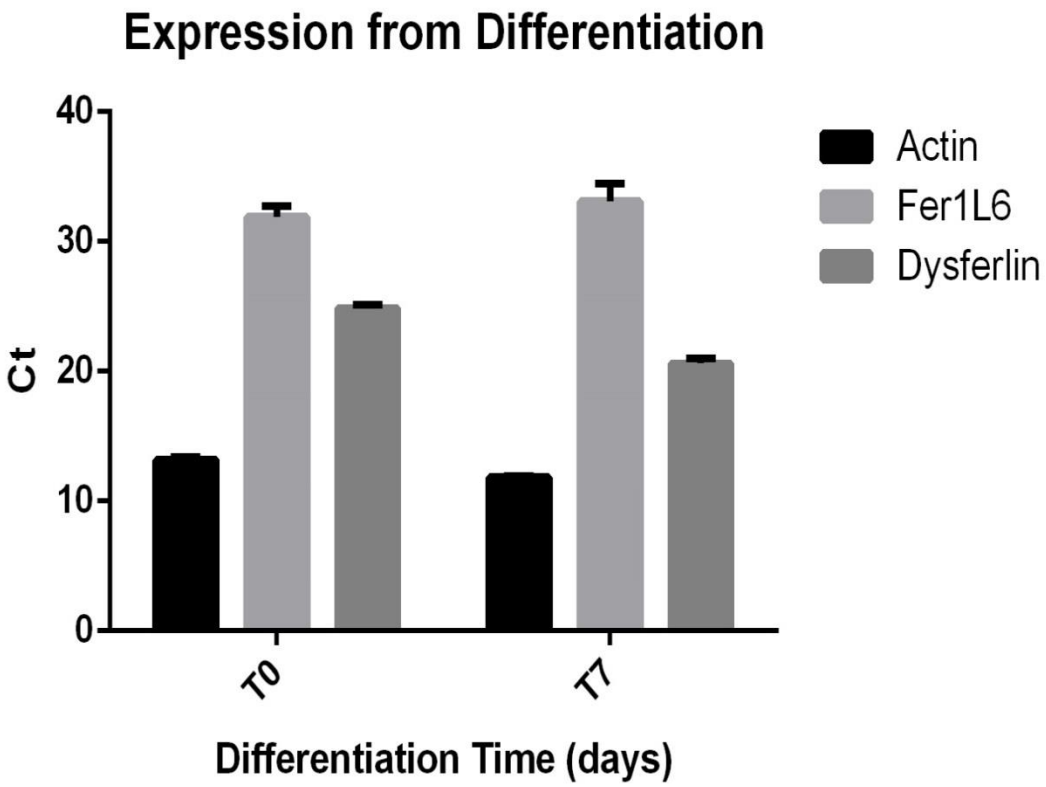

Figure 3. Fer1L6 Expression from Differentiation. cDNA samples prepared from RNA extracted from undifferentiated and differentiated C2C12 cells (t = 7 days) were used to run RT-qPCR. Comparison of the Ct values between time points suggest little difference in Fer1L6 expression as C2C12 differentiate.

Comparison of the Ct values between undifferentiated C2C12 cells (t = 0 days) and differentiated C2C12 cells (t=7 days) shown in Fig 3 indicates little variance of the levels of Fer1L6 expression between these two developmental time points. Little variance is observed in actin expression levels between these time points as well. Levels of dysferlin, however, increase between the time points.

Confirmation of the presence of Fer1L6 with in the undifferentiated C2C12 cell line was probed by western blot on whole C2C12 cell lysate. A band at the correct molecular weight (209 kDa) indicated Fer1L6's presence within the undifferentiated cells. (Figure 4)

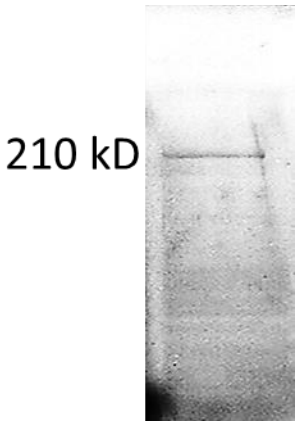

Figure 4. Western Blot of Fer1L6 Fer1L6 was probed by western blotting C2C12 lysate. A band at 209 kDa, the correct molecular weight for Fer1L6, was detected.

Subsequently**,** the location of Fer1L6 in the C2C12 cell line was probed independently by using two different goat anti-Fer1L6 antibodies, one raised against the Fer1L6 N terminal, the other raised against the Fer1L6 C terminal. Fer1L6 labeling in this manner revealed the protein localized both within the perinuclear region and at the plasma membrane, specifically along the leading edge of the cell and at lamellipodia. The fact that this labeling was similar for both antibodies suggested specificity. Fer1L6's position at the lamellipodia suggested a potential role of Fer1L6 at focal adhesions, and leads us to probe for co-localization with other structural and membrane proteins including vimentin, actin, syntaxin-4, talin-1, and vinculin via immunofluorescence.

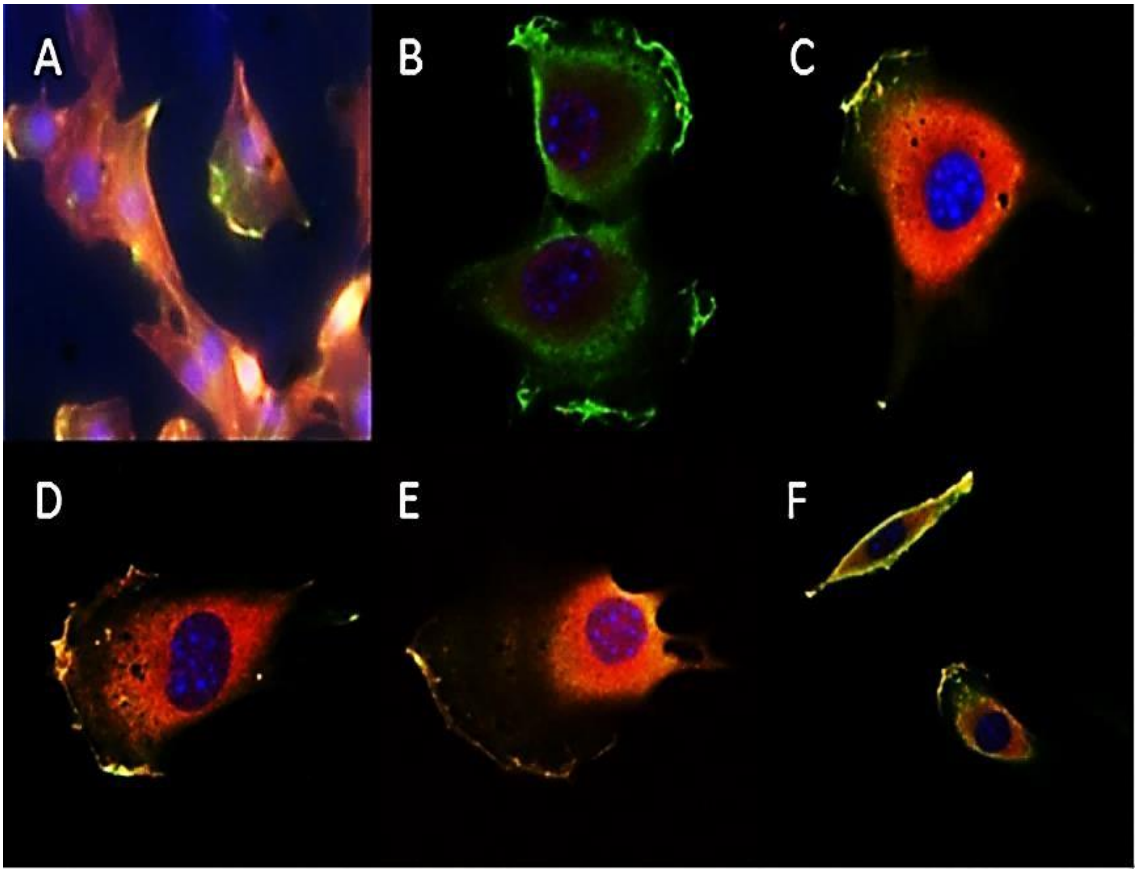

Figure 5. Co-localization studies of Fer1L6. Fer1L6 was labeled with a FITC secondary antibody (green), the nucleus was stained using Dapi (blue), and all other proteins were labeled with a TRITC secondary antibody (red). Images show localization of Fer1L6 and a) actin, b) H3, c) syntaxin -4, d) vimentin, e) vinculin, and f) talin-1. Co-localization is observed between Fer1L6 and syntaxin-4, vimentin, and actin. Co-localization is seen between Fer1L6, talin-1, and Vinculin to a lesser extent. H3 and Fer1L6 do not appear to co-localize.

Immunofluorescence images shown in Fig. 5 confirmed Fer1L6 localization both around the C2C12 cell nucleus and at the plasma membrane, specifically along the leading edge of the cell and at lamellipodia. Little Fer1L6 expression is observed between the perinuclear region and the plasma membrane. Additionally, Fer1L6 demonstrated strong co-localization with the proteins syntaxin-4, actin and vimentin, with minor co-localization with vinculin and talin-1. (Fig 5a, c, d, e, f) No co-localization was observed between Fer1L6 and the nuclear histone marker, H3. (Fig 5b)

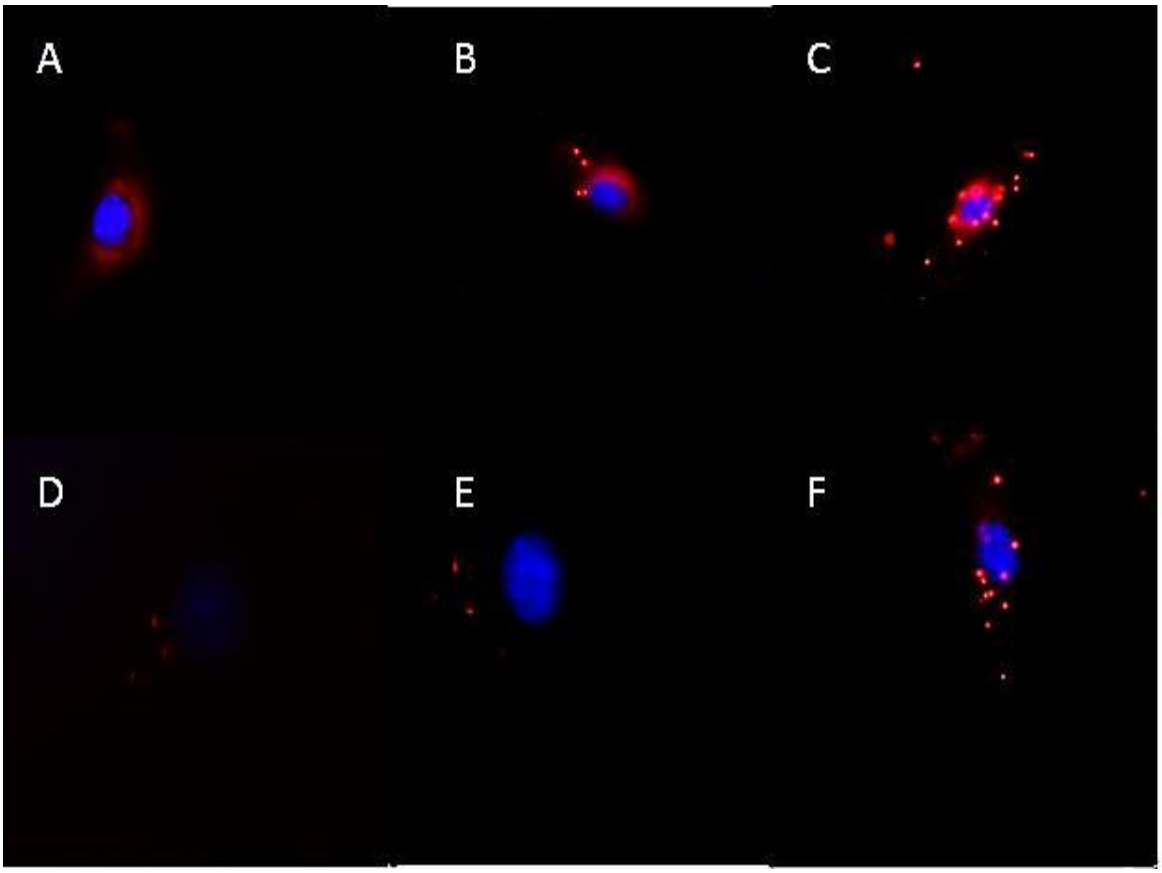

Figure 6. Proximity ligation studies of Fer1L6. Proximity ligation assays were performed between Fer1L6 and a) H3, b) SNAP-23, c) syntaxin-4, d) talin-1, e) vimentin, and f) vinculin. Puncta could be seen in all cases with the exception of H3.

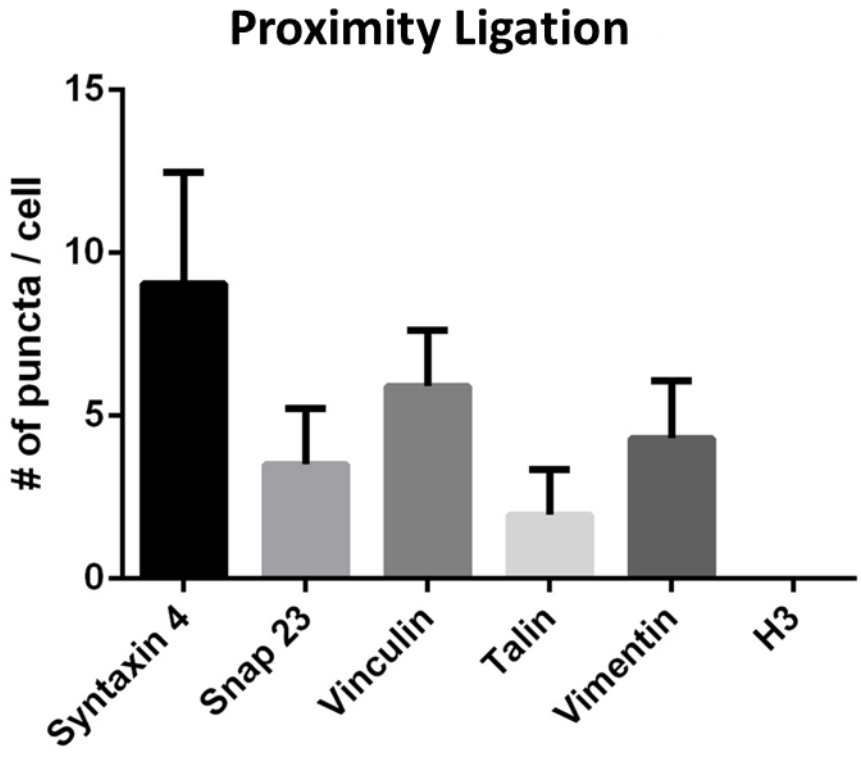

Figure 7. Proximity ligation puncta/cell between Fer1L6 and denoted proteins. A statistically significant number of puncta per cell were observed between Fer1L6 and syntaxin 4, snap-23, vimentin, and vinculin, but not H3.

Proximity ligation is a technique which highlights proteins that reside within 40 nm of one another. In this technique two proteins of interests can be tagged via specific primary antibodies and specialized secondary antibodies carrying fluorophore labeled oligonucleotide sequences. Provided these two secondary antibodies are within 40 nm of each other their oligonucleotides will ligate together and undergo circular a circular polymerase reaction amplifying their fluorophore signal. Each of these signals is seen as individual puncta underneath the microscope, and are a good indication not only of close proximity between two proteins, but also potential binding. As shown in Fig. 6, puncta formed between Fer1L6, syntaxin-4, SNAP-23, talin-1, vimentin and vinculin, with no

puncta forming between Fer1L6 and H3. Counts of puncta per cell as seen in Figure 7 strongly support co-localization and potential binding between Fer1L6 syntaxin-4, vinculin, SNAP-23 and vimentin. Lower puncta per cell were observed between Fer1L6 and talin-1, suggesting limited co-localization between these two proteins. No puncta were observed between Fer1L6 and H3, thus puncta per cell remained zero. This supports observations seen during the immunofluorescence experiments and further suggests that these proteins maybe Fer1L6 binding partners.

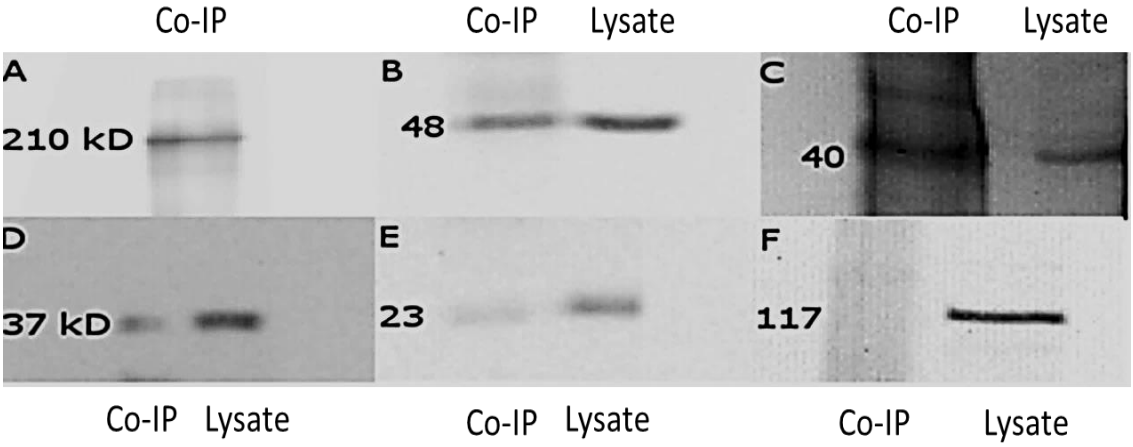

Figure 8. Western blot analysis of Fer1L6 co-immunoprecipitation samples. The left column represents co-immunopercipitation samples probed for a) Fer1L6 b) actin, c) vimentin, d) syntaxin-4, e) snap-23, and f) vinculin. The right column represents C2C12 lysate. Results suggest Fer1L6 co-immunoprecipitates with actin, vimentin, syntaxin-4, and SNAP-23. No pull down is observed for vinculin.

Western blot analysis of co-immunopercipitation samples prepared using undifferentiated C2C12 lysate demonstrated in Figure 8 indicates interaction between Fer1L6, actin, vimentin, syntaxin-4, and SNAP-23, with no observed pulldown of vinculin or talin-1. These results identify that Fer1L6 may either directly or indirectly bind these proteins.

DISCUSSION

Analysis of the Fer1L6 Ct values generated from the RT-qPCR experiments indicate that Fer1L6 expression is relatively consistent as the C2C12 cells differentiated. This suggests that the role Fer1L6 might play in the C2C12 cells is constant and not dependent on the differentiation stage of the cell. Therefore, Fer1L6 is more likely to serve a function that is needed continuously throughout the cell's development.

Based upon the immunofluorescence images, it is apparent that due to the lack of expression within the C2C12 cytosol, and abundant expression at the perinuclear region and plasma membrane, Fer1L6 is most likely synthesized at the endoplasmic reticulum, and directly transported to the plasma membrane where the protein serves its function. The fact that Fer1L6 binds vimentin and actin, both of which are structural proteins, suggests that these filaments may serve as the path on which Fer1L6 is trafficked within vesicles from the ER to the plasma membrane.

Immunofluorescence studies have also shown that Fer1L6 localizes at numerous points on the plasma membrane, especially at lamellipodia, actin cytoskeletal structures at the cell's leading edge responsible for cell adhesion and migration. Such localization suggests Fer1L6 functions at the focal adhesions complex. Co-localization of Fer1L6 with several proteins located within the focal adhesion complex, namely vinculin, and talin-1, supports this hypothesis. Proximity ligation assays further support co-localization between Fer1L6 and the focal adhesion proteins, talin-1 and vinculin. Together, the demonstrated proximity of Fer1L6 with the focal adhesion complex, but absence of binding to the complex itself, suggests that Fer1L6 does not directly associate with the complex. Instead, it may function in the recycling of the focal adhesion complex proteins,

similar to the role of myoferlin in the recycling of tyrosine kinase receptors at the cell membrane of endothelial cells (Yu *et al*., 2011).

Immunofluorescence and proximity ligation data also indicated co-localization of Fer1L6 with SNAP-23 and syntaxin-4, two SNARE proteins that function in the docking and fusion of transport vesicles. Syntaxin-4 and SNAP-23 also co-IP with Fer1L6, suggesting a potential role for Fer1L6 in vesicle fusion. This is especially interesting due to Fer1L6's localization to the lamellipodia, where the cell would require a greater flux of vesicles containing plasma membrane components. As such, a possible function of Fer1L6 maybe in facilitating vesicle trafficking to and from the leading edge of the cell. Given Fer1L6's co-localization with several components of the focal adhesion complex, it is likely that these vesicles contain focal adhesion proteins.

Future studies of Fer1L6 would likely include the identification of additional binding partners to further elucidate Fer1L6's function. Other potential binding partners may include membrane recycling proteins, other structural proteins, including actinin and tubulin, or known calcium sensitive proteins at focal adhesions, such as calpain-2. Identification of these binding partners would help further narrow down Fer1L6's role within the cell, and potentially further identify its function. Additionally, the knockdown of Fer1L6 and determination of any change in phenotype, including loss of adhesion or migration, would further establish the protein's role. Future studies may also include repletion of immunofluorescence experiments following cycloheximide treatment of the cells. This procedure would prevent protein synthesis and allow determination of whether all Fer1L6 traffics to the plasma membrane or if some remains resident at the perinuclear region, which would suggest possible function in this subcellular area.

In conclusion, based upon the binding of Fer1L6 to syntaxin-4 and SNAP-23, we hypothesize that Fer1L6 plays a role in facilitating the trafficking of vesicles to the plasma membrane. These vesicles likely contain focal adhesion proteins, as Fer1L6 may function as a regulator of the recycling of the focal adhesion complex.

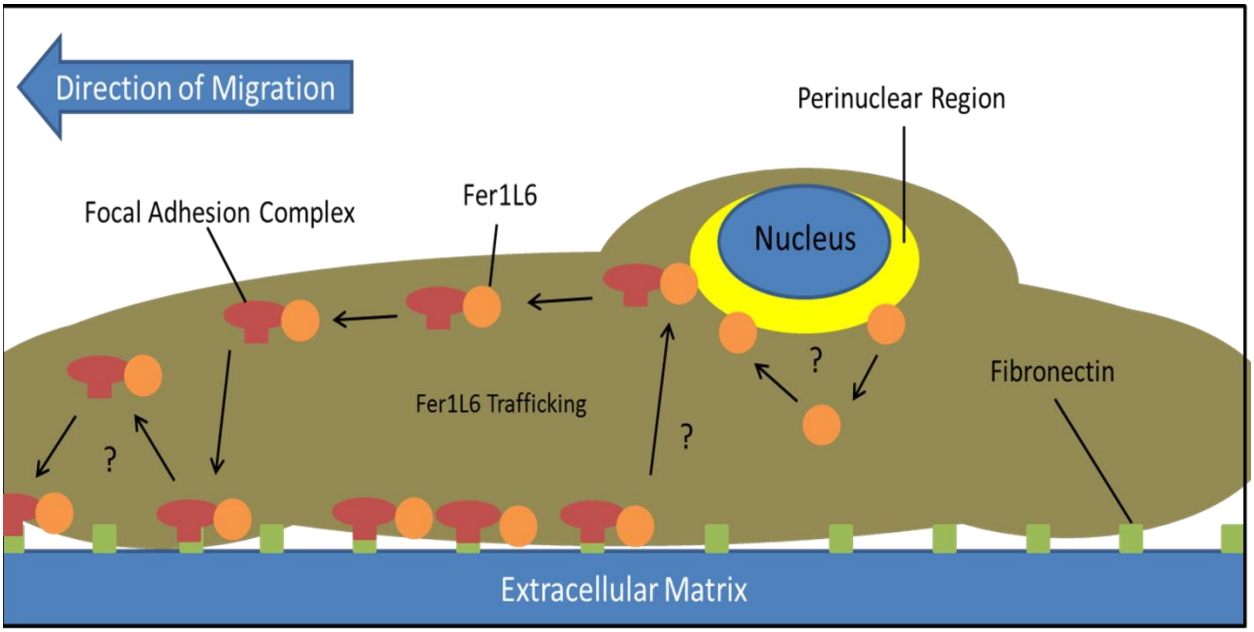

Figure 9. Proposed model of Fer1L6 trafficking. As a cell migrates along the extracellular matrix, Fer1L6 is transported to the cell's leading edge. Fer1L6 may also be directly recycled at the leading edge, at the perinuclear region, and may be trafficked back to the perinuclear region as the cell migrates along the extracellular matrix. Fer1L6 likely signals the trafficking of the focal adhesion complex.

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